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Research Article

Transgenic Expression of the SoRIP1 Gene Confers Resistance to *Alternaria alternata* and *Alternaria solani* in Potato

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ABSTRACT

Ribosome-inactivating proteins (RIPs) are glycosylases that specifically inactivate ribosomes by halting the process of translation. *SoRIP1* is a type I RIP isolated from *Spinacia oleracea* (spinach). It is a multifunctional protein with versatile properties and contains the conserved shiga/ricin toxic domain. *In vitro* antifungal assays with purified *SoRIP1* protein demonstrated strong inhibition of pathogenic fungi, including *Alternaria alternata* and *A. solani*. The *SoRIP1* gene was introduced into the potato cultivar 'Desiree' via *Agrobacterium*-mediated transformation under the control of the CaMV 35S promoter. Molecular confirmation of transgenic lines was achieved through PCR and GUS assays, with a transformation efficiency of approximately 57%. Crude protein extracts from transgenic potato plants inhibited the growth of *A. alternata* and *A. solani in vitro*, while non-transgenic plants were highly susceptible in parallel bioassays. In contrast, transgenic plants exhibited resistance when challenged with both pathogens. Quantitative real-time PCR (qRT-PCR) revealed that *SoRIP1* expression increased progressively in transgenic plants following fungal infection, reaching a 5.5-fold induction with *A. alternata* and a 5.9-fold induction with *A. solani* at 72 h post-inoculation compared to control plants. These findings indicate that *SoRIP1* confers antifungal activity and could be an effective candidate for developing disease-resistant potato cultivars. Further validation under greenhouse and field conditions is recommended.

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Introduction

A decline in the production of several important crops has been observed over the years, largely due to pathogen attacks, which contribute to food shortages and significant economic losses (Savary et al., 2019). A survey conducted by the Food and Agriculture Organization (FAO) reported that nearly one billion people worldwide suffer from malnutrition, with pathogenic fungal infections being one of the primary causes (Barrett, 2010). Among staple crops, *Solanum tuberosum* (potato) is recognized as a food security crop and a vital dietary energy source, with its global consumption steadily increasing (Nonhebel, 2012). Although potatoes are affected by a range of pathogens including bacteria, oomycetes, and viruses, fungal pathogens are the predominant threat to its production. Among these, *Alternaria* species are particularly destructive due to their ability to survive under diverse environmental conditions. *Alternaria solani* is the causal agent of early blight, whereas *A. alternata* is responsible for brown spot disease. Both diseases significantly reduce

potato yield (Schmey et al., 2024).

To protect potato from such pathogens, a variety of control measures are employed. Chemical pesticides and fungicides are the most commonly used, alongside agronomic practices such as crop rotation. However, these approaches have limitations, including high costs, environmental toxicity, harm to non-target organisms, and the need for repeated applications (Khan et al., 2017). Conventional breeding offers opportunities to introduce resistance genes, but this approach may unintentionally transfer undesirable traits.

Genetic engineering provides a more precise alternative by enabling the targeted transfer of beneficial genes into crops (Dong and Ronald, 2019). Transgenic crops have been shown to provide both environmental and economic benefits (Kumar et al., 2023). Ribosome-inactivating proteins (RIPs), a group of N-glycosylases, are notable in this context for their ability to enzymatically inactivate ribosomes, thereby inhibiting protein synthesis. In addition, RIPs possess diverse enzymatic activities, including superoxide dismutase, DNase, chitinase, and phosphatase functions. RIPs are classified into three types: Type I (single-chain), Type II (two-chain; an enzymatic A-chain and a lectin B-chain that facilitates cell binding and internalization), and Type III, a less clearly defined class (Kumar et al., 2023). Previous studies have demonstrated that transgenic plants expressing RIPs exhibit enhanced resistance against a wide spectrum of pathogens. For instance, potato, tomato, and tobacco lines transformed via *Agrobacterium*-mediated methods under the CaMV 35S promoter exhibited resistance to fungal, bacterial, and viral pathogens (Kumar et al., 2023).

SoRIP1, a Type I RIP derived from spinach (*Spinacia oleracea*), possesses a ricin-like toxic domain (Ishizaki et al., 2002; Kawade et al., 2008). *In vitro* studies have demonstrated its antifungal and antibacterial activities (Ismail and Sumrin, 2024). In this study, SoRIP1 (BP31) was introduced into potato for the first time. Although other RIPs, such as Pokeweed Antiviral Protein (PAP) and PIP2 from *Phytolacca insularis*, have previously been expressed in potato with antiviral effects, several well-known RIPs, including α-momorcharin from *Momordica charantia*, which confers resistance against viral and fungal pathogens, have not yet been tested in this crop (Zhu et al., 2018).

Given its origin from an edible, non-traditional donor species and its demonstrated *in vitro* activity, SoRIP1 represents a novel candidate gene for engineering disease resistance in potato. In the present study, the antifungal potential of SoRIP1 was evaluated against *A. solani* and *A. alternata* under controlled conditions. Future investigations under greenhouse and field settings are recommended to validate its effectiveness.

Material and Methods

SoRIP1 gene cloning into plant expression vector (pCAMBIA 1301)

In silico cloning of the SoRIP1 gene

The codon-optimized *SoRIP1* gene, with a length of 867 bp, was synthesized by Molecular Biology Products (https://mbps.pk/), XhoI restriction sites were introduced through PCR amplification using the primers listed in Table 1. As a result, the final amplified product had a size of 882 bp. The complete sequence of *SoRIP1*, including the added XhoI sites, is provided below.

(Left primer) →

CTCGAGATGGTGGTTGAGGACGAGGAGATGATAGAGAAT
CTTAGGTATACGACGGTTACCTTCGAGTTGACCAACGGCG
TGACGGGTTATAGCAGCTTTATGACTAGACTTAGGAATGC
AGTTGAGGCTCCTACCAGGGCATGTGGACTGCAGTCCACT
AGATCAACTCCTCTACCTGGTGCTGAGTATATCCATGTCG
ATTTGAAGATTAGTAATACCCAGTGGGTGACATTGGGGAT
TGACGTAAAGGACTTTTACGTCTGGGGATACCAGGATAAC
GTCCGATACAATGGTAACTACAGGGCTAGCTTTCTGAACG
ACGCACCACAGGCAGCAAAGAATTTATTTAGGGGCAGTAC
GATCCGTACAACCCGATTTGGTGGGAATTACAATTCTTTG
GAACCTGCAGCTGGGATCACCAGACGTAATCTAGTTCTGG
GTATACAAAATCTCGATGGAGCCATTCGTACTGTCTATGG
TAAGCAGGAGTCTGAATTGAATAAAGGGAACGCTGAAGCA
CGTTTCTTCTTAATCGCTATTCAGATGGTGGCTGAAGCTG
CTAGATTCAAGTTTATGGAAGAAGCTATAGTAAGAAACGA
CAATACGCCGGACTTTAAGAGAAAGATGGTTGCATTTCAA
AACGACTGGGACCCAATAAGTACAGCTATCCACCGAGCCG
AGGCTGCCACCCCTAAGTGCACAAGAATCACACCTACACT
TGTGATTTCTAATACGGAATATCGACAGGAAGTTAACACC
GTCGCAGAGATTAAGGACGACATGGGGCTGCTGAAATACA
GGTCTACAACTCTATCAAGCATCGCCTCATCAATACTTGA
CGACGACGAGTTCCTAGAGTCAGCCCTTATT**TAGCTCGAG**

(Right primer) ←

Prior to performing *in vitro* cloning, *in silico* cloning of the SoRIP1 gene into the pCAMBIA vector was carried out using SnapGene. The SoRIP1 gene was cloned by replacing the Hygromycin resistance gene in the pCAMBIA1301 vector under the control of the CaMV 35S promoter (Figure 1).

Table 1. Primer sequences used for SoRIP1 gene amplification.

Primer name	Sequence	Source
LEFT PRIMER (forward)	5'-**CTCGAG**ATGGTGGTTGAGGACGAGGA-3'	Self-design
RIGHT PRIMER (reverse)	5'-**CTCGAG**CTAAATAAGGGCTGACTCTAGGAACTCG-3'	Self-design

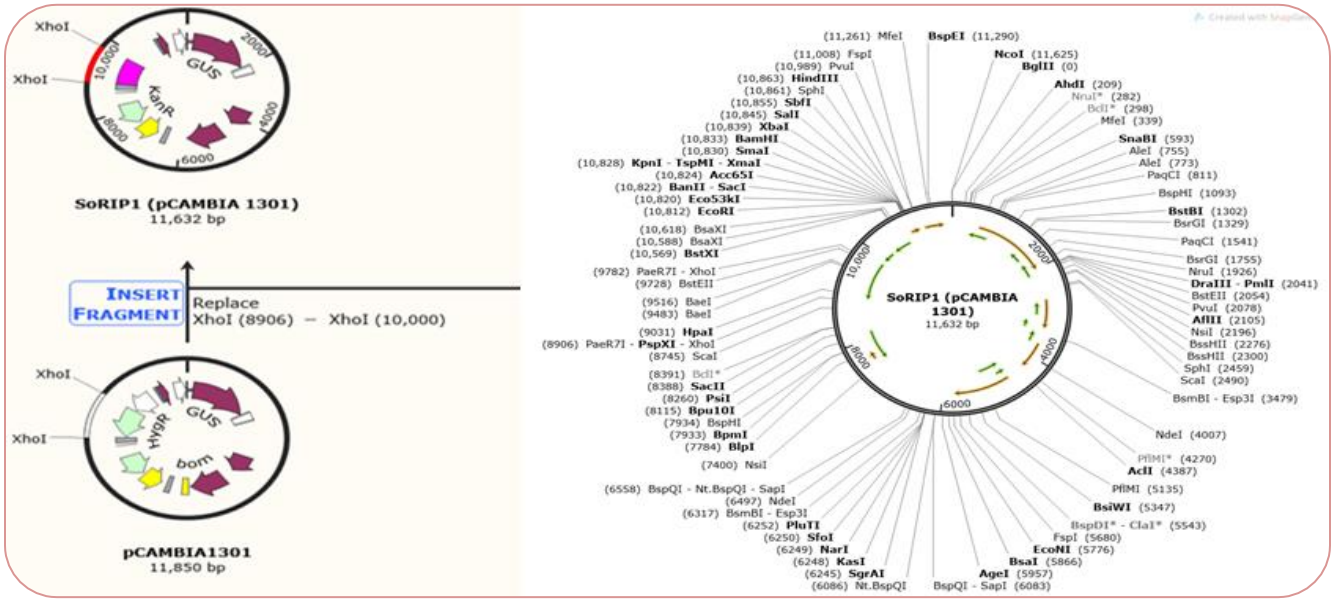


Figure 1. *In silico* cloning of the SoRIP1 gene into the pCAMBIA1301 vector.

***In vitro* cloning of construct pCAMBIA1301_SoRIP1**

To introduce XhoI restriction sites at both ends of the *SoRIP1* gene sequence, specific primers were designed (Table 1). The PCR profile consisted of an initial denaturation at 94°C for 4 min, followed by 30 amplification cycles of denaturation at 95°C for 40 sec, annealing at 60°C for 35 sec, and extension. Each 20 µL PCR reaction mixture contained 1 µl (100 ng) of template DNA, 2 µl (10 pmol) of each primer, 2 µl (25 mM) $MgSO_4$, 2 µl of 10× Taq buffer, 2 µl (200 µM) of dNTPs, 0.5 µl (5 U/µL) Taq DNA polymerase, and 8.5 µl of nuclease-free water. Amplified products were resolved on a 1% agarose gel.

Following gel confirmation, both the PCR-amplified *SoRIP1* gene and the pCAMBIA1301 vector were digested with XhoI, gel-eluted, and purified. The digested insert and vector were then ligated using a commercial ligation kit (Figure 2). The ligation mixture was transformed into *Escherichia coli* Top10 competent cells, and successful recombinants were preliminarily screened by colony PCR.

Confirmation of *SoRIP1* gene orientation in pCAMBIA1301

To verify the orientation of the inserted *SoRIP1* gene, PCR was performed using orientation-specific primers (Figure 3) (Table 2). The PCR profile consisted of an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 95°C for 40 sec and annealing at 57°C for 35 sec, with extension. Each 20 µl reaction mixture contained 1 µl (100 ng) of template DNA, 2 µl (10 pmol) of each primer, 2 µl (25 mM) $MgSO_4$, 2 µl of 10× Taq buffer, 2 µl (200 µM) of dNTPs, 0.5 µl (5 U/µl) Taq DNA polymerase, and 8.5 µl of nuclease-free water. PCR products were resolved on a 1% agarose gel for confirmation.

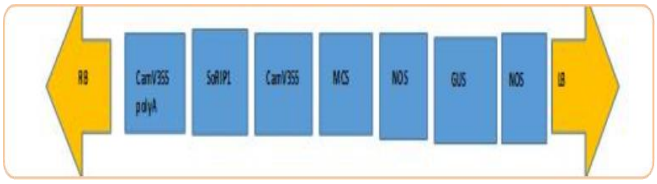


Figure 2. Schematic map of the construct pCAMBIA1301_SoRIP1.

Table 2. Primers used for orientation analysis of SoRIP1 in the pCAMBIA1301 vector.

Primer name	Sequence
Primer1G	5'- GATATACTCAGCACCAGGTAGAGGAGT-3'
Primer2V	5'- CTATCTCTCTCGAGATGGTGGTTGAG-3'

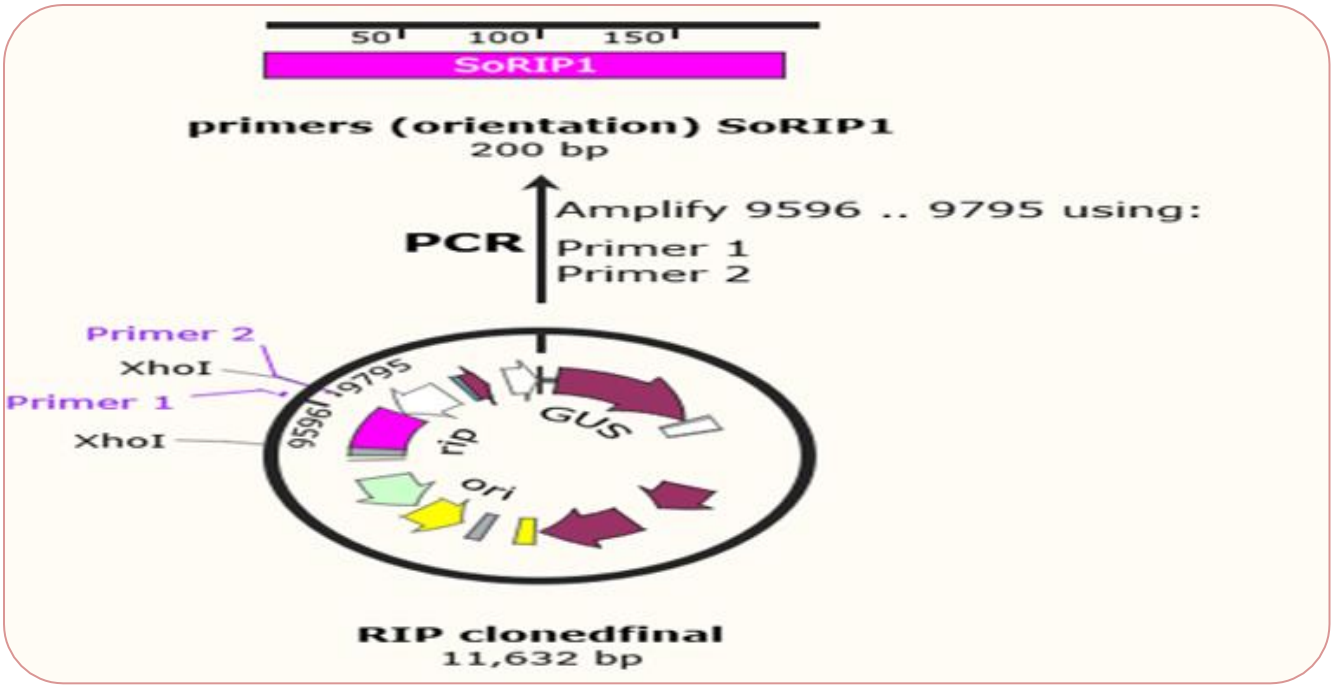


Figure 3. *In silico* primer synthesis for orientation confirmation using SnapGene.

SoRIP1 gene transformation in *S. tuberosum*

Transformation was carried out using the AGL-1 strain of *Agrobacterium tumefaciens*. Freshly prepared competent cells were transformed with approximately 500 ng of the recombinant plasmid (pCAMBIA-SoRIP1). Successful transformation was confirmed by colony PCR and restriction digestion analysis.

For plant transformation, a 24-h-old culture of *Agrobacterium* harboring pCAMBIA-SoRIP1 was co-cultivated with potato nodal explants. The explants were incubated on a shaker for 30 min, followed by drying on blotting paper for approximately 7 min. These explants were then placed on MS basal medium (zero media), sealed with parafilm, and incubated in a growth chamber at 25°C under a 16/8 h light/dark photoperiod. After 48 h, explants were transferred to MS plates containing 250 mg/L cefotaxime for 3 days to eliminate bacterial growth. Subsequently, explants were shifted to MS regeneration medium tubes, and the regenerated shoots were further grown on MS basal medium for two successive two-week periods.

Molecular analyses of transformed potato plants

Transgenic plants were initially screened on MS medium supplemented with kanamycin. Further molecular confirmation was carried out as described below.

GUS Assay for transient expression

Transient expression of the β-glucuronidase (GUS) reporter gene was analyzed using a GUS assay. The GUS staining solution contained 100 mM NaH_2PO_4, 25 mg/L X-gluc, 10 mM EDTA, 0.1% Triton X-100, and 50% methanol, adjusted to pH 8.0. Stem and leaf cuttings from both control (non-transgenic) and putative transgenic plants were immersed in the GUS solution and incubated for 24 h at 37 °C.

SoRIP1 integration analysis in transgenic potato plants

Genomic DNA was isolated from control and transgenic plants using the CTAB method (Allen et al., 2006). PCR amplification was performed using gene-specific primers, and amplicons were analyzed by agarose gel electrophoresis to confirm the integration of SoRIP1.

Transformation efficiency

Regeneration and transformation efficiency were calculated using the standard equations described by Bakhsh (2020), Khan et al. (2017), and Sahoo et al. (2011).

$$\text{Regeneration efficiency} = \frac{\text{Number of plants survived}}{\text{Total number of embryos inoculated}} \times 100$$

$$\text{Transformation efficiency} = \frac{\text{PCR +/GUS positive plants}}{\text{Total number of regenerated plants}} \times 100$$

Dot blot analysis

After confirming transgenic plants through selection media, GUS assay, and PCR, dot blot analysis was performed. A PCR-amplified probe was labeled using the Roche DIG random probe labeling and detection kit. Genomic DNA (~15 µg) was denatured by the heat shock method, spotted onto a nylon membrane, air-dried, and cross-linked under UV light for 3 min. The membrane was pre-hybridized in pre-hybridization buffer for 1.5 h at 60°C, followed by hybridization with the SoRIP1-DIG labeled probe at 60°C for 16 h (the probe was denatured in boiling water before use).

Post-hybridization washes were carried out as follows: (i) 20 min in 0.1% SDS, 2× SSC at room temperature, (ii) 20 min in the same buffer at 60°C, and (iii) rinsing with distilled water. The membrane was then equilibrated in solution I (100 mM Tris, 150 mM NaCl) for 20 min, followed by blocking in solution II (100 mM Tris, 150 mM NaCl, 0.5% blocking reagent) for 20 min with gentle shaking. The membrane was incubated with alkaline phosphatase-conjugated anti-DIG antibody (FAB) for 1 h and 30 min. Signal detection was performed using BCIP/NBT substrate for 20 min in the dark at room temperature. DNA from a non-transformed potato plant served as a negative control, whereas a plasmid containing the SoRIP1 gene was used as a positive control.

Expression analysis of transgenic potato plants

Total RNA was extracted from young leaves of control and transgenic potato plants using the Trizol method with slight modifications (Rio et al., 2010). RNA quality and concentration were assessed using a NanoDrop spectrophotometer and agarose gel electrophoresis. First-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and stored at -20 °C. Expression of the transgene was analyzed by PCR using cDNA from transgenic and control plants with SoRIP1-specific primers and GAPDH primers as an internal control.

Quantification of SoRIP1 gene

Quantitative real-time PCR (qRT-PCR) was conducted to determine the expression levels of the SoRIP1 gene in potato plants. The PCR conditions were as follows: initial denaturation at 94°C for 4 min, denaturation at 95°C for 40 sec, annealing at 57°C for 35 sec, followed by 30 amplification cycles. Gene-specific primers are listed in Table 3. Each 15 µl reaction contained SYBR Green master mix (Thermo Fisher, Lithuania), primers, and cDNA. Threshold cycle (Ct) values were obtained for both SoRIP1 and the reference gene GAPDH. Relative expression levels were calculated using the 2-delta delta method (Livak and Schmittgen, 2001).

Table 3. Primer sequences of the SoRIP1 gene used for real-time PCR analysis.

Primer name	Sequence
PRIMER 1	5'-CTCGAGATGGTGGTTGAGGACGAGGA-3'
PRIMER 2	5'- GATATACTCAGCACCAGGTAGAGGAGT-3'

***In vitro* fungal inhibition activity assay**

For the assay, total crude protein was extracted from both control and transgenic potato plants at the 3-4 leaf stage. Approximately 1 g of leaf tissue was ground in liquid nitrogen and homogenized in 600 µl of protein extraction buffer. The homogenate was centrifuged at 12,000×g for 20 min at 4°C, and the resulting supernatant was collected as the crude protein extract.

For the inhibition assay, 190 µg of crude protein from transgenic plants was used. Crude protein extracted from non-transformed potato plants served as the negative control. Thus, the inhibitory effect of the SoRIP1 protein was evaluated (Mondal et al., 2007).

The percentage inhibition of fungal growth for *A. solani* and *A. alternata* was calculated using the following equation:

$$\% \text{ inhibition} = \frac{\text{Fungal colony diameter in treatment}}{\text{Fungal colony diameter in control}} \times 100$$

Bioassay of transgenic potato plants

For the bioassay, transgenic and control potato plants were grown in jars for approximately 14 days. Thereafter, the plants were challenged with fungal pathogens *A. solani* and *A. alternata*. A PDA block (≈4 mm) containing active fungal growth was carefully placed at the base of each jar. All jars were maintained in a growth chamber at 20°C under a 16/8 h light/dark photoperiod. The morphological responses of both control and transgenic plants were monitored and compared at specific time intervals (Khan et al., 2017).

Differential mRNA (SoRIP1) expression

The best-performing transgenic plants, as identified from the antifungal bioassays, were selected for molecular analysis. A fungal spore suspension (≈2 ml) was used for inoculation. Total RNA was extracted from plant tissues at 0, 24, 48, and 72 h post-inoculation using Trizol reagent. For differential expression analysis, transgenic plants at the 3-4 leaf stage exhibiting maximum antifungal activity in previous assays were chosen.

For quantitative real-time PCR (qRT-PCR), 1 µg of total RNA was used. The thermal cycling profile consisted of an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 95°C for 40 sec, annealing at 57°C for 35 sec, and extension. A 15 µl reaction mixture was prepared, containing SYBR Green Master Mix (Thermo Fisher, Lithuania), gene-specific primers (Table 3), and cDNA. Ct values for *SoRIP1* and the reference gene (*GAPDH*) were recorded. Relative expression levels were calculated using the 2-delta delta method (Livak and Schmittgen, 2001).

Results

Cloning of the *SoRIP1* gene into the plant expression vector pCAMBIA1301

The *SoRIP1* gene (882 bp), flanked by *XhoI* restriction sites at both ends, was cloned into the plant expression vector pCAMBIA1301. For this purpose, both the vector and the *SoRIP1* gene fragment were digested with FastDigest *XhoI* enzyme, followed by ligation. Digestion of the vector released a 1026 bp fragment corresponding to the *hygromycin* resistance gene. The correct orientation of the inserted gene was confirmed using gene-specific primers (Figure 4).

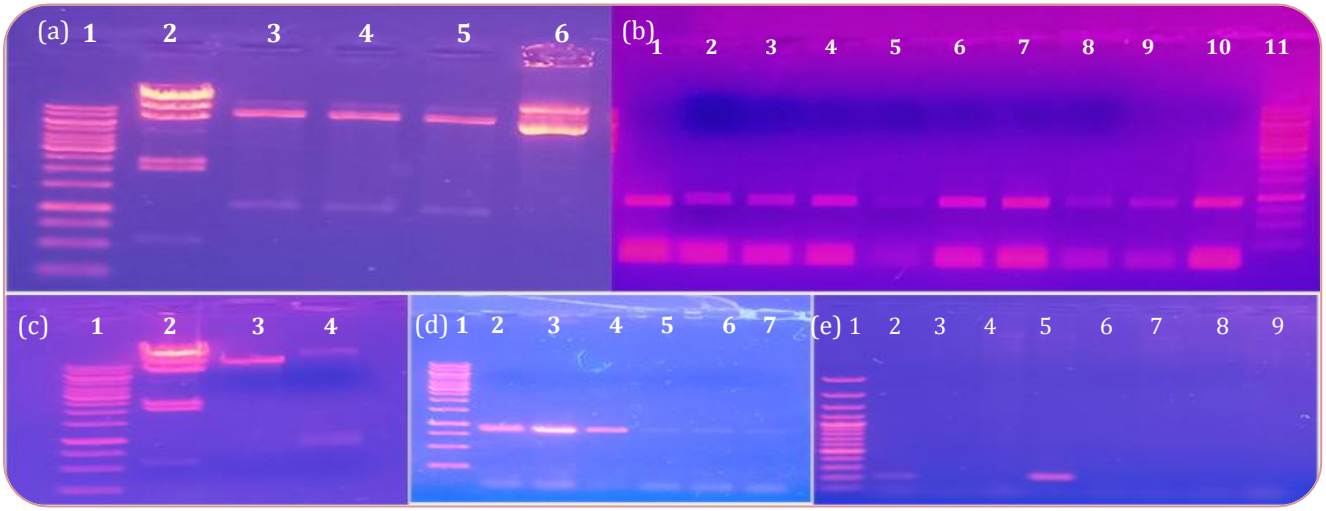


Figure 4. Construction of recombinant plasmid pCAMBIA1301_SoRIP1. (a) Restriction digestion analysis of vector: Lane 1, 1 kb DNA ladder (Thermo, SM0311); Lane 2, λ DNA ladder (Thermo, SM0103); Lanes 3-5, vector digested with FastDigest XhoI; Lane 6, uncut vector. (b) Restriction digestion analysis of SoRIP1 gene: Lane 11, 1 kb DNA ladder; Lanes 1-10, digested gene. (c) Ligation of vector and gene: Lane 1, 1 kb DNA ladder; Lane 2, λ DNA ladder; Lane 3, digested vector; Lane 4, vector ligated with gene. (d) Ligation confirmation by colony PCR: Lane 1, 1 kb DNA ladder; Lanes 2-7, PCR product of gene. (e) Gene orientation confirmation by colony PCR: Lane 1, 100 bp plus DNA ladder (Thermo, SM0323); Lanes 2-9, PCR products of plasmid DNA.

Transformation of the SoRIP1 gene in *S. tuberosum*

After introducing the construct pCAMBIA1301_SoRIP1 into *A. tumefaciens*, successful transfer was confirmed through colony PCR and restriction digestion analysis (Figure 5).

The transformation into *Agrobacterium* strain AGL-1 was followed by transferring the gene construct into the potato variety 'Desiree' (Figure 6). The transformed plantlets were initially grown on MS medium in Petri plates and then transferred to MS medium in glass tubes supplemented with cefotaxime (250 mg/L) to eliminate *Agrobacterium*.

After five weeks, 52 plants (*Agrobacterium*-free) were obtained that were then grown further on regeneration medium for almost one month.

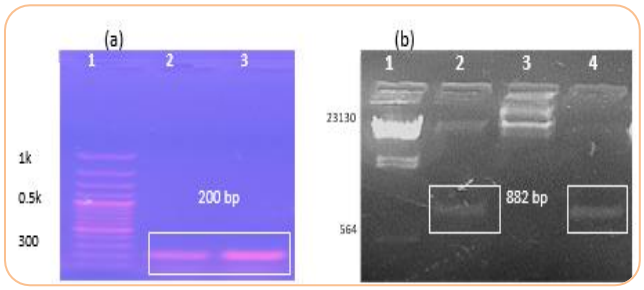


Figure 5. Confirmation of the construct pCAMBIA1301_SoRIP1 in *Agrobacterium tumefaciens*. (a) Colony PCR analysis: Lane 1, 100 bp DNA ladder (Thermo); Lanes 2-3, PCR-amplified product of pCAMBIA1301_SoRIP1. (b) Restriction digestion analysis: Lane 1, λ DNA (Thermo); Lanes 2 and 4, pCAMBIA1301_SoRIP1 digested with FastDigest XhoI enzyme; Lane 3, undigested pCAMBIA1301_SoRIP1.

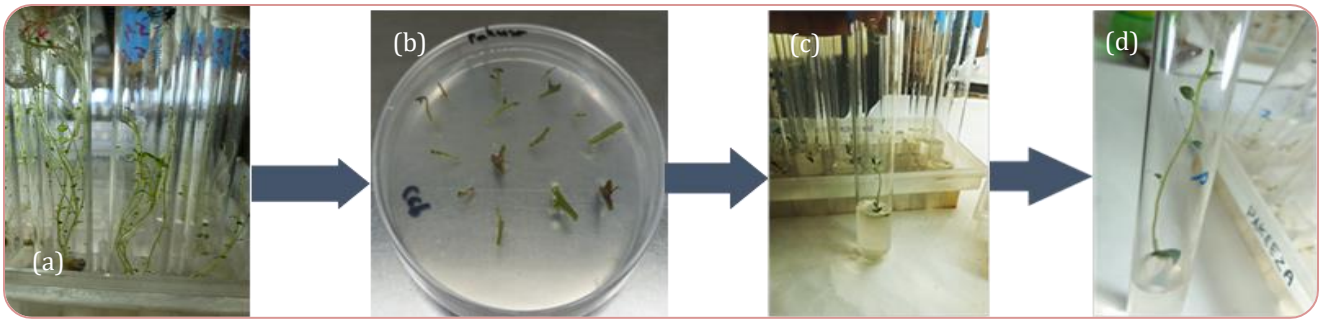


Figure 6. Transformation methodology: a) source potato plant (two-month-old), b) plant cuttings infected with *Agrobacterium* on MS plate, c) incubation of plants on MS medium (with antibiotic), d) incubation of plants on MS basal medium (without antibiotic).

***SoRIP1* integration analyses in transgenic potato plants**

Integration of the *SoRIP1* gene was confirmed through PCR analysis. Genomic DNA was first extracted from the leaves of transgenic and control potato plants. PCR was performed, and the amplified products were separated on a 1.5% agarose gel. A distinct band of 882 bp corresponding to the *SoRIP1* gene was observed in lanes 3, 5, 6, 7, 8, and 10, confirming the presence of transgenic potato plants (Figure 7a). Out of fifty-two plants analyzed, approximately thirty tested positive for the transgene, as indicated by the presence of the PCR band. These PCR-positive plants were further validated using DNA dot blot analysis. In this assay, the probe hybridized to its complementary sequence, resulting in visible color development (Figure 7b).

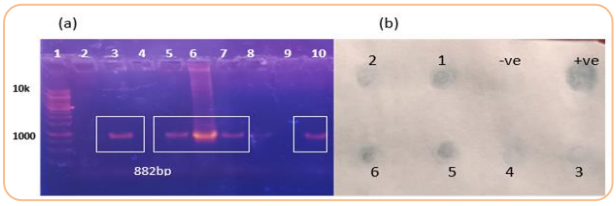


Figure 7. Analysis of SoRIP1 gene integration in potato plants: (a) PCR amplification of the SoRIP1 gene from genomic DNA. Lane 1: DNA ladder (1 kb); Lanes 3, 5, 6, 7, 8, and 10: transformed plants; Lanes 2, 4, and 9: control plants. (b) DNA dot blot analysis of transformed potato plants.

SoRIP1 gene expression analyses

Gene expression was quantitatively analyzed in transgenic potato plants. Initially, conventional PCR was performed to verify cDNA synthesis, followed by real-time PCR using the same cDNA samples. A GUS assay was also conducted, in which the enzyme β-glucuronidase hydrolyzed 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), producing an insoluble blue precipitate in the transgenic plant tissues. The transgenic potato plants developed a distinct blue coloration, whereas no such color was observed in the control plants (Figure 8).

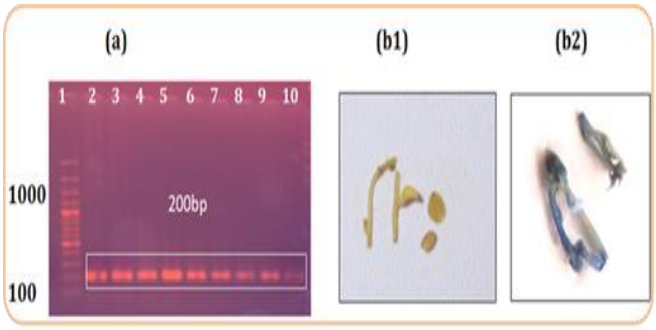


Figure 8. SoRIP1 gene expression analysis. (a) PCR amplification from cDNA: Lane 1, DNA ladder (100 bp plus, Thermo, SM0323); Lanes 2-10, PCR products from cDNA of transformed potato plants. (b1) GUS assay of non-transformed potato plant. (b2) GUS assay of transformed potato plant.

Transformation Efficiency

For the potato cultivar Désirée, a total of 61 explants were inoculated, out of which 52 successfully regenerated on selective medium, resulting in a regeneration efficiency (RE) of 85%. Among the regenerated plants, 30 were confirmed positive through both GUS assay and PCR analysis. Therefore, the overall transformation efficiency (TE) was calculated to be 57% (Table 4).

Table 4. Transformation efficiency of potato cultivar Désirée.

Variety	Total explants inoculated (N)	Regenerated plants (A)	GUS-positive plants (B)	PCR-positive plants (C)	RE = A/N × 100	TE = B/A × 100	TE = C/A × 100
Désirée	61	52	30	30	85%	57%	57%

In the real-time PCR analysis, cDNA from transgenic potato plants (P-01, P-02, P-04, P-08, P-16, P-32, and P-64) was amplified using gene-specific primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal housekeeping control. The results revealed that the transgenic line P-04 exhibited the highest expression level, showing an approximately 4.2-fold increase (Figure 9).

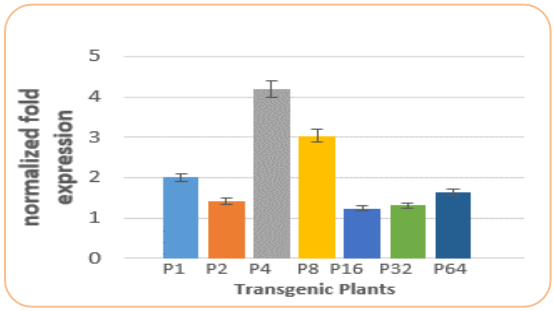


Figure 9. Quantification of SoRIP1 gene expression in transformed potato lines.

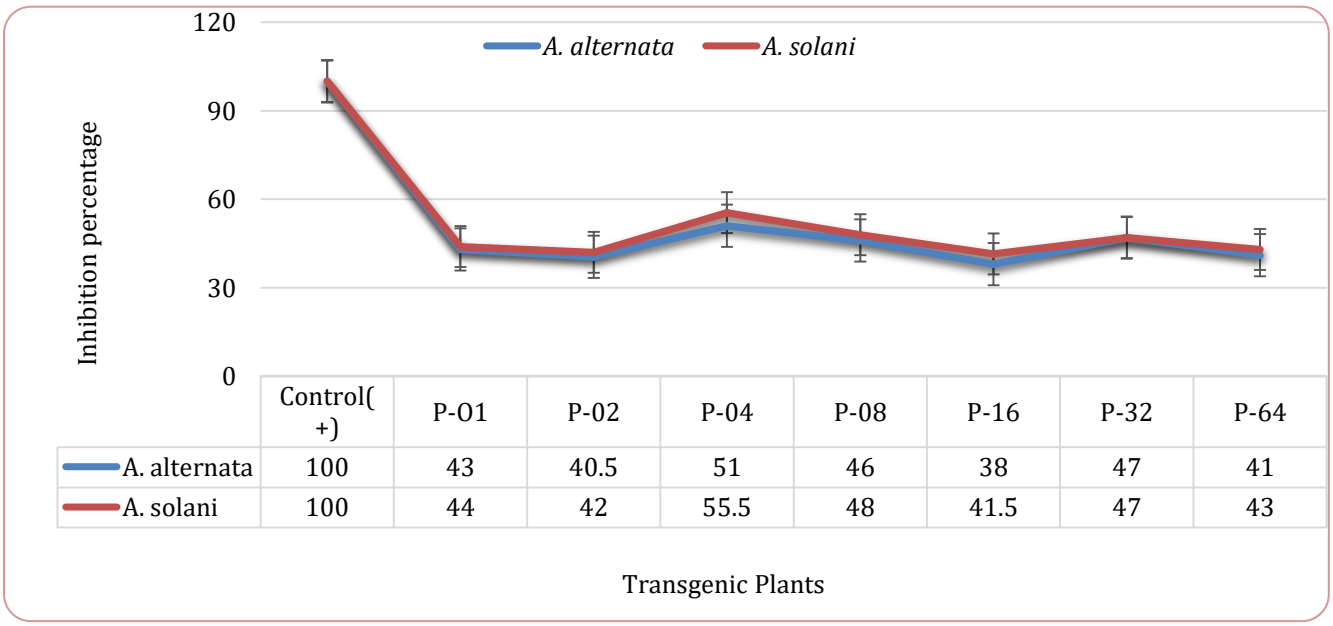
In vitro fungal inhibition activity assay

To evaluate antifungal activity, transgenic potato lines P-01, P-02, P-04, P-08, P-16, P-32, and P-64 were tested. Crude protein extracts from these lines were incorporated into PDA plates inoculated with *A. solani* and A. alternata. Fungal growth inhibition was observed in all transgenic lines. Against *A. solani*, the highest inhibition (55.5%) was recorded for line P-04, while the lowest inhibition (41.5%) was observed for line P-16. Similarly, against A. alternata, line P-04 exhibited the highest inhibition (51.0%), whereas line P-02 showed the lowest inhibition (40.5%) (Figure 10).

In order to calculate the percentage inhibition, crude protein extracted from non-transgenic plants was used as the negative control, while purified SoRIP1 protein was used as the positive control (100%). Against *A. alternata*, the transgenic line P-01 showed 43% inhibition, P-08 showed 46%, P-16 showed 38%, P-32 showed 47%, and P-64 showed 41%. Similarly, against *A. solani*, the transgenic line P-01 showed 44% inhibition, P-02 showed 42%, P-08 showed 48%, P-32 showed 47%, and P-64 showed 43%.

Bioassay of transgenic potato plants

The transgenic line selected for this bioassay was P-04, based on its performance in previous assays. Fourteen days after inoculation, the jars were completely covered with fungal hyphae. During the assay, the morphology of both control and transgenic plants was carefully observed. The control plants turned yellow, and some even collapsed, whereas the transgenic plants remained green and healthy. After three weeks of observation, the control plants completely died, while the transgenic potato plants showed only minor signs of fungal infection (Figure 11).



	Control(+)	P-01	P-02	P-04	P-08	P-16	P-32	P-64
A. alternata	100	43	40.5	51	46	38	47	41
A. solani	100	44	42	55.5	48	41.5	47	43

Figure 10. Fungal inhibition activity assay showing the percentage of inhibition (y-axis) in transgenic potato plants (x-axis).

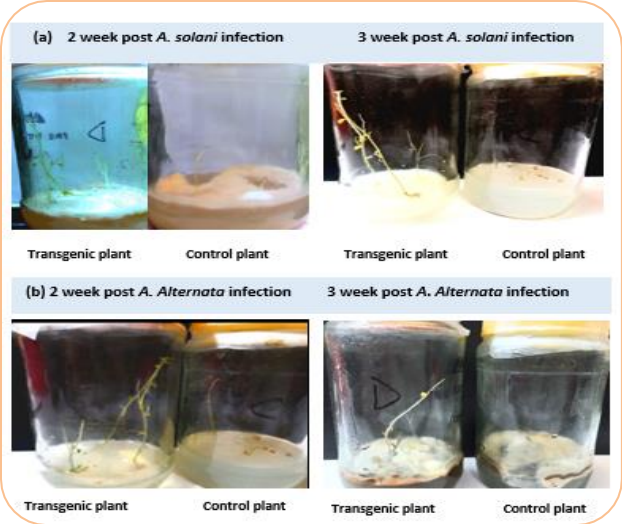


Figure 11. Bioassay of transgenic potato plants: (a) Transgenic and control plants at two and three weeks post-infection with A. solani; (b) Transgenic and control plants at two and three weeks post-infection with *A. alternata*.

Differential mRNA (SoRIP1) expression

Expression analysis was performed using real-time PCR after transgenic potato plants were infected with fungal strains. It was hypothesized that infection by both *A. solani* and *A. alternata* would enhance the mRNA expression of the transgene.

The transgenic line selected for differential mRNA (SoRIP1) expression was P-04. The expression level recorded at 0 h was taken as the control. The results showed that expression increased gradually over time, with higher expression observed at 24 h and 48 h after fungal infection. The maximum expression was recorded at 72 h, reaching a 5.5-fold increase in response to *A. alternata* and a 5.9-fold increase in response to *A. solani*. GAPDH was used as an internal control to normalize the results (Figure 12).

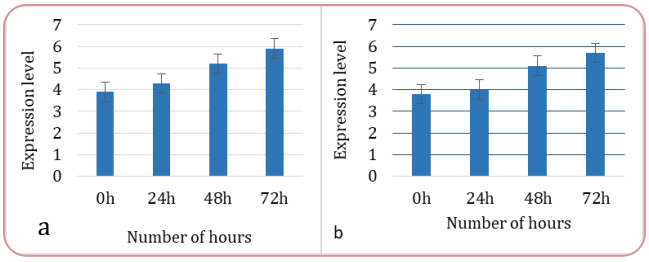


Figure 12. Differential mRNA (SoRIP1) expression analysis of the P-04 transgenic line after fungal infection. (a) Expression in the P-04 transgenic line infected with *A. solani*; (b) Expression in the P-04 transgenic line infected with *A. alternata*.

Discussion

Worldwide, crops including potato are attacked by diverse pathogens such as fungi, viruses, and bacteria, resulting in significant yield losses both in the field and during storage. With the increasing global demand for food, protecting potato production has become crucial. Chemical pesticides have been widely used for crop protection; however, their use raises concerns due to adverse impacts on human health and the environment, as well as the potential development of resistance in pathogens. Consequently, biological control and genetic engineering have emerged as safer and more sustainable alternatives (Khan et al., 2017).

Ribosome-inactivating proteins (RIPs) have been successfully expressed in various transgenic crops, where they demonstrated antifungal, antibacterial, and antiviral properties. Trichosanthin and pokeweed antiviral protein, for example, are natural RIPs that have shown strong protective effects against multiple pathogens in transgenic plants (Fabbrini et al., 2017).

In the present study, the *SoRIP1* gene, encoding a ribosome-inactivating protein, was introduced into the potato cultivar 'Desiree.' To achieve high expression, the gene was codon-optimized for *S. tuberosum*. PCR amplification using primers with engineered XhoI sites (Figure 4) enabled cloning of *SoRIP1* into the plant vector pCAMBIA1301 following *in silico* assembly with SnapGene. The gene orientation was confirmed both *in silico* and *in vitro* using a combination of gene-specific and vector-specific primers (Table 2). Correct integration produced the expected 200 bp fragment during colony PCR. The construct was then introduced into potato via *Agrobacterium*-mediated transformation (Figure 6), a method widely adopted for potato due to its relatively high efficiency compared to other transformation techniques (Ooms et al., 1983; Horsch et al., 1984; Weiland, 2003; Chakravarty et al., 2007). Many other RIPs have also been successfully transferred to plants using this approach (Li et al., 2004; Citores et al., 2021).

Following transformation, shoots were regenerated on MS medium supplemented with cefotaxime to eliminate *Agrobacterium*. Transgenic plants were confirmed by PCR, dot blot analysis, and GUS assays (Figure 7). PCR using gene-specific primers and dot blot hybridization confirmed stable integration of *SoRIP1*. Plants expressing the transgene also exhibited GUS activity due to the CaMV 35S promoter present in the pCAMBIA1301 vector. Transformation efficiency was 57% (Table 4),

consistent with previous reports of variable efficiency ranging from 40% to 92.8% depending on potato cultivar (Badr et al., 2008).

Expression analysis using real-time PCR with cDNA from transgenic plants demonstrated successful transcription of *SoRIP1* (Figure 9). To evaluate antifungal activity, crude protein extracts from transgenic and control plants were tested *in vitro* against *A. solani* and *A. alternata*. Transgenic line P-04 exhibited the highest inhibition, with 55.5% against *A. solani* and 51% against *A. alternata* (Figure 10). A fungal growth inhibition assay performed in jars confirmed these results: control plants developed disease symptoms and eventually died, whereas transgenic plants resisted infection (Figure 11). Further, real-time PCR performed after fungal inoculation revealed that *SoRIP1* expression peaked at 72 h post-infection in P-04 plants (Figure 12).

The observed antifungal activity can be attributed to high-level accumulation of the recombinant SoRIP1 protein, which provided sufficient protection against fungal pathogens. Similar outcomes have been reported with other RIPs and defense-related genes. For example, the barley chitinase and RIP genes conferred resistance against *A. brassicae* in *Brassica juncea* (Chhikara et al., 2012), while transgenic potato expressing RIPs exhibited resistance against *Phytophthora infestans* (Ghosh et al., 2016). Other RIPs such as pokeweed antiviral protein (PAP), PIP2 from *Phytolacca insularis*, and α-MMC in rice have also provided resistance against viral and fungal pathogens (Qian et al., 2014; Zhu et al., 2018). Collectively, these studies highlight the promise of RIPs as safe and effective transgenes in crop protection. The results of this work demonstrate that SoRIP1, derived from spinach, an edible plant, represents a novel and biosafe candidate for improving potato resistance to fungal pathogens.

Conclusion

This study demonstrated the antifungal potential of the spinach-derived ribosome-inactivating protein *SoRIP1* in transgenic potato plants. The *SoRIP1* gene was successfully introduced into *S. tuberosum* via *Agrobacterium*-mediated transformation and confirmed by molecular analyses. Transgenic lines expressed *SoRIP1* at high levels and showed significant inhibition of *A. solani* and *A. alternata* both *in vitro* and *in planta*. As *SoRIP1* originates from an edible plant, it presents minimal biosafety concerns, making it a suitable candidate for deployment in crop improvement strategies. These findings establish *SoRIP1* as a promising gene for the development of disease-resistant potato cultivars and contribute to advancing sustainable agricultural practices.

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Authors' Contributions

Both authors contributed to the study and experimental design; PI prepared the materials, collected data and performed the analysis; AS supervised the work; PI drafted the manuscript; Both authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger
SDG 3: Good Health and Well-being
SDG 13: Climate Action

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