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Research Article

Recombinase Based Stacking of Multiple Disease Resistance Genes in Granola Potato

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ABSTRACT

Phytophthora infestans, Potato Virus Y (PVY), and *Potato Leafroll Virus* (PLRV) are major diseases that severely affect potato production and quality worldwide, including Indonesia. These pathogens pose significant threats to crop security, and effective, durable control methods are still limited. This study aimed to establish an efficient transformation system using Gene Assembly in *Agrobacterium* by Nucleic acid Transfer using Recombinase technologY (GAANTRY)-based gene stacking technology to generate multi-pathogen-resistant potato lines, which may reduce reliance on chemical control and support environmentally sustainable cultivation. Two Granola potato clones, Granola Indonesia (Gra I) and Granola MSU (Gra M), were evaluated for their *in vitro* growth and regeneration performance. Gra M exhibited vigorous vegetative growth, while Gra I showed the highest regeneration efficiency using mid-stem node explants cultured on GNZ medium. Transformation using *Agrobacterium rhizogenes* strain ArPORT1 harboring the multigene vector pMSU2DR-02 resulted in 13% transformation efficiency and 100% regeneration with a 10-minute infection period. PCR confirmed stable integration of five resistance genes (*RpiAmr1, RpiAmr3, RpiVnt1, RySto,* and *Rladg*) without vector backbone fragments. These results demonstrate that GAANTRY-based gene stacking is an effective approach for producing potato cultivars with single T-DNA insertions carrying multiple resistance genes, providing broad-spectrum and durable resistance as a sustainable alternative to chemical control for long-term crop protection. The resulting transgenic potato lines have the potential to be utilized to improve disease resistance and productivity among smallholder farmers in Indonesia.

1. Introduction

Potato (*Solanum tuberosum* L.) is the world's fourth most important food crop after rice, wheat, and maize (Poma-Chamana *et al.* 2025). This crop plays a vital role in global food security and nutrition because of its high yield potential (Hopkins *et al.* 2026), broad adaptability to diverse agroecological conditions, and rich nutritional composition (Das Dangol *et al.* 2025), providing carbohydrates, potassium, vitamin C, dietary fiber, and other essential micronutrients required for human health (Gowda *et al.* 2025). Despite its importance, potato productivity remains severely constrained by several devastating pathogens, particularly late blight caused by *Phytophthora infestans* (Nowicki *et al.* 2012; Haverkort *et al.* 2016), which can reduce yields by up to 75% in susceptible cultivars such as Granola and is responsible for annual economic losses exceeding USD 6-10 billion worldwide (Dong and Zhou 2022). In Indonesia, potato production reached approximately 1.27 million tonnes in 2024. Yet, disease management still relies heavily on frequent fungicide applications every 2-3 days,

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increasing production costs while posing environmental and human health risks (Sparks *et al.* 2019; BPS 2025). In addition to late blight, viral diseases such as *Potato virus Y* (PVY) and *Potato leafroll virus* (PLRV) further threaten potato production, reducing marketable yields by 10-80% and 20-50%, respectively, while accelerating seed degeneration and decreasing tuber quality (Quenouille *et al.* 2013; Thomas-Sharma *et al.* 2016).

Conventional breeding efforts have produced several varieties with partial resistance, but limitations such as genetic incompatibility between species, differences in ploidy levels, and limited sources of resistance genes remain obstacles (Douches *et al.* 2001). Most breeding varieties have vertical (race-specific) resistance that is easily broken by pathogen evolution (Vleeshouwers *et al.* 2011). The introgression of resistance genes from wild Solanum species into cultivated potato has proven to be an effective strategy for improving disease resistance while maintaining desirable agronomic characteristics. In Indonesia, this approach led to the development of the genetically engineered Bio Granola cultivar carrying the *Rpi-blb1* (*RB*) gene from *S. bulbocastanum*, which exhibited enhanced resistance to *P. infestans* while retaining the agronomic performance of the Granola cultivar (Hadiarto and Ambarwati 2024). However, monogenic resistance is often overcome by the rapid evolution of pathogen populations, highlighting the need for strategies that combine multiple resistance genes to achieve broader and more durable resistance (Kourelis and Van Der Hoorn 2018; Witek *et al.* 2021).

Thanks to developments in gene stacking technology, it is now possible to combine several resistance genes into a single construct to create more robust and wide-ranging resistance to different pathogens. The successful stacking of three late blight resistance genes—*RB*, *Rpi-blb2* from *S. bulbocastanum*, and *Rpi-vnt1.1* from *S. venturii*—into elite African potato varieties using *Agrobacterium*-mediated transformation demonstrated the potential of combining multiple R genes for durable resistance. The transgenic events were selected using the nptll marker and validated through PCR and Southern blot analyses, resulting in plants with broad and stable resistance to *P. infestans* under field conditions (Ghislain *et al.* 2019).

Similarly, the molecular structure and stability of transgenic potato events carrying three stacked resistance genes—*Rpi-mcq1, Rpi-blb2*, and *Rpi-vnt1.1*—were confirmed through event-specific PCR, Southern blot, and whole-genome sequencing, which verified the integrity and expression of all R genes. These 3-R events exhibited complete resistance to *P. infestans* under both greenhouse and field conditions (Zarka *et al.* 2024). Gene Assembly in *Agrobacterium* by Nucleic acid Transfer using Recombinase technology (GAANTRY) system is a noteworthy innovation that enables precise and effective multigene assembly and transfer in a single transformation vector (Collier *et al.* 2018). This system has been used to combine multiple resistance genes, *Rpi-vnt1, Rpi-amr1,* and *Rpi-amr3* genes (for resistance to *P. infestans*), *RySto* (PVY), and *Rladg* (PLRV), which originate from the wild species *S. venturii, S. americanum, S. stoloniferum,* and *S. andigena*, respectively (Velásquez *et al.* 2007; Grech-Baran *et al.* 2020, 2022; Paluchowska *et al.* 2022, 2024) into a single plant transformation construct pMSU2DR-02. The pMSU2DR-02 construct was newly developed in this study using GAANTRY-based gene stacking to assemble multiple resistance genes.

This study intends to develop methods that allow for the introduction of multiple disease resistance in potato, using different transformation procedures and the pMSU2DR-02 (GAANTRY construct) to introduce the *Rpi-vnt1, Rpi-amr1, Rpi-amr3, RySto,* and *Rladg*. It is anticipated that this strategy will improve food security and lessen reliance on insecticides and fungicides, resulting in a production system that is sustainable, adaptable, and environmentally friendly. Potato T-DNA events created by these methods have the potential to provide a potato biotech product with long-lasting, broad-spectrum late blight and virus resistance.

2. Materials and Methods

2.1. Plant Material and Culture Maintenance

The materials used in the experiment included two potato genotypes (Granola Indonesia/Gra I and Granola MSU/Gra M). Single-node segments were cultivated *in vitro* using vitamin-supplemented Murashige and Skoog (MS) basal medium. Cultures were kept at 20-22°C with a 4,000-5,000 lux light intensity and a photoperiod of 16 hours of light and 8 hours of darkness. The commonly used potato micropropagation and genetic transformation studies served as the model for this culture process (Murashige and Skoog 1962; Badr *et al.* 2015).

2.2. Optimization of *in vitro* Culture

Nodal explants removed from the middle and upper parts of the stem, about 1-2 cm below the shoot apex,

were used to propagate non-transgenic Granola potato genotypes (Gra I and Gra M) *in vitro*. These regions were selected due to their high meristematic activity and regeneration potential. For 3-4 weeks, the explants were cultivated on Murashige and Skoog (MS) medium at a temperature of 20-22°C, a light intensity of 4,000-5,000 lux, and a photoperiod of 16 hours of light and 8 hours of darkness. Each treatment consisted of 5 explants/ bottle with 10 replicates (total 50 explants per clone). Plant height, number of leaves, number of stem nodes, and length of roots were among the parameters noted after four weeks. Stem nodes without buds were used as explants for regeneration optimization (Hajare *et al.* 2021). The explant's origin (A1 = Gra I and A2 = Gra M) and the type of explant (B1 = middle section and B2 = upper section near the tip) were the two factors in this study's 2 × 2 factorial experimental design. Four treatment interactions were produced by the combination of these factors: A1B1 (Gra I - middle section), A1B2 (Gra I - upper section), A2B1 (Gra M - middle section), and A2B2 (Gra M - upper section).

2.3. Optimization of Regeneration

Internode explants from four-week-old cultures were used for regeneration. The medium contained Murashige and Skoog (MS) basal salts supplemented with 1.0 mg/L zeatin, 1.0 mg/L indole-3-acetic acid (IAA), and 1.0 mg/L gibberellic acid (GA_3), hereafter referred to as Zeatin-IAA-GA_3 (ZIG) medium. In contrast, the medium supplemented with 1.0 mg/L GA_3, 1.0 mg/L naphthaleneacetic acid (NAA), and 1.0 mg/L zeatin was referred to as GA_3-NAA-Zeatin (GNZ) medium. Each medium was applied to two clones, Gra I and Gra M. Each dish contained 10 explants, with 10 replicates, for a total of 400 explants (200 GRA I and 200 GRA M). Explants measuring 0.5-1.0 cm were incubated at 22°C, with media replacement every 3 weeks. The percentage of callus formation was one of the observation parameters. The number of calli that gave rise to shoots, the initial shoot emergence time, and the number of shoots in each Petri dish and each explant were also observed (Abeuova *et al.* 2020).

2.4. Bacterial Culture Preparation

The culture medium for *Agrobacterium rhizogenes* strain ArPORT1 carrying the binary plasmid pMSU2DR-02 (nptII marker, GAANTRY system), which harbors five resistance genes (*RpiAmr1, RpiAmr3, RpiVnt1, RySto,* and *Rladg*) (provided by D. Douches, Michigan State University, USA), was prepared by inoculating one PCR-positive colony into 15 mL of Luria-Bertani (LB) medium containing kanamycin (50 mg/L). The culture was maintained in a 28°C shaking incubator at a speed of 200 rpm until it reached a cell density of OD_{600} = 0.3. The suspension was centrifuged for ten minutes at 10,000 rpm, and the pellet obtained was resuspended in a liquid co-culture medium supplemented with acetosyringone (100 mg/L). The suspension was then maintained in a shaking incubator at 28°C for three hours and 200 rpm before being used for the infection process—a physical map of the T-DNA region of pMSU2DR-02 within A. rhizogenes ArPORT1 (Figure 1).

2.5. Transformation Method

The *in vitro* method based on Zarka (2022) was used for transformation. Both Gra I and Gra M were used as explant sources. Stem internodes aged 3-4 weeks from *in vitro* cultures were divided into lengths of 0.5 to 1.0 cm and subcultured on pre-culture medium (pH 5.7-

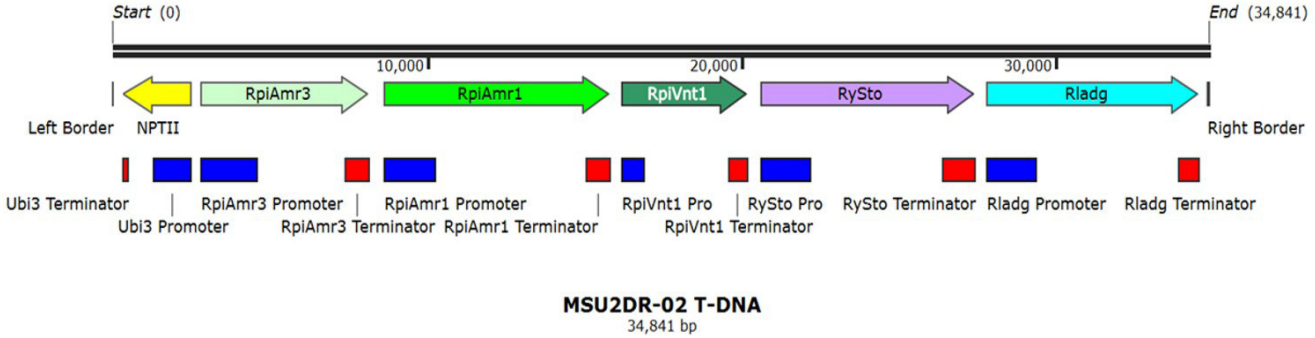

Figure 1. Shows the physical map of T-DNA in the pMSU2DR-02 binary vector for *Agrobacterium*-mediated transformation. The left border (LB) and right border (RB) sequences in the T-DNA region define the transferred DNA segment. The selectable marker nptII (neomycin phosphotransferase II) confers kanamycin resistance. There are five stacked resistance genes in the region: *RySto, Rladg, RpiAmr3, RpiAmr1,* and *RpiVnt1*—each driven by constitutive, native, or endogenous promoters and terminators to confer broad-spectrum resistance in *S. tuberosum* cv. Granola (Douches 2024)

5.8, MS salts 4.4 g/L, 20 g/L sucrose, 3 g/L phytagel, thiamine-HCl (1.0 mg/L), and then autoclaved. After sterilization, zeatin-riboside (1.0 mg/L) and 2,4-D (1.0 mg/L) were incorporated into the medium. They were incubated for two days at 20–22°C. The explants were then infected with an *A. rhizogenes* suspension using the immersion method for 10 minutes or 20 minutes, dried on sterile tissue, then placed on GNZ solid co-culture medium and incubated at 20-22°C for three days in the dark. Each treatment used 100 explants planted in 10 Petri dishes (10 explants per dish). As a control, 60 uninfected explants were prepared, also with 10 explants per Petri dish, bringing the total number of explants used to 260. The explants were placed in a non-selective callus induction medium for three to five days following co-cultivation, then transferred to a selective medium containing timentin, carbenicillin, and kanamycin (100 mg/L). Subcultures were carried out every two weeks until potential transgenic shoots grew. After that, the shoots were cultivated for four weeks in rooting medium (MS0 + kanamycin 50 mg/L) (20-22°C, photoperiod 16/8 hours) until plantlets were formed. All plantlets that developed roots in the selective rooting medium were subjected to PCR analysis to confirm the presence of the transgene. Regeneration and transformation efficiency, the time of the first shoot emergence, the number of selection-resistant shoots, and the number of resistant calli that generated shoots were among the observation parameters. To quantitatively assess the success of *Agrobacterium*-mediated transformation, both transformation (1) and regeneration efficiencies (2) were considered (Sahoo *et al.* 2011). These parameters provide a reliable measure of the responsiveness of potato explants to bacterial infection and subsequent morphogenic recovery under antibiotic selection pressure. Transformation efficiency was determined as the percentage of explants that survived kanamycin selection relative to the total number of transformed events. In contrast, regeneration efficiency represented the proportion of calli that successfully produced shoots among those that survived antibiotic selection. The following formulas were applied:

$$\text{Transformation efficiency (\%)} = \frac{\text{number of kanamycin resistant calli}}{\text{total calli amount}} \times 100 \quad (1)$$

$$\text{Regeneration efficiency (\%)} = \frac{\text{number of regenerating calli}}{\text{number of kanamycin resistant calli}} \times 100 \quad (2)$$

2.6. Isolation of Genomic DNA

Using a commercial plant DNA extraction kit (Geneaid, USA) and the manufacturer's instructions, young leaf tissue was used to extract genomic DNA from kanamycin-resistant Granola potato plantlets for PCR analysis. A NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA) was employed to determine the DNA concentration and purity, and a 1.0% agarose gel electrophoresis stained with RedSafe™ (iNtRON Biotechnology, Korea) was used to evaluate its quality.

2.7. PCR Amplification of Stacked Resistance Genes

The PCR profile consists of one first cycle of denaturation at 95°C for three minutes, 35 cycles of denaturation at 95°C for 30 seconds, and annealing at 50°C for stacked gene fragments (flanking regions of 2 target genes) or 60°C for nptll for 30 seconds, followed by 90-second extension at 72°C, a final 5 minute extension at 72°C followed by cooling at 25°C for 10 minutes marked the end of the cycle. To ensure that the amplified DNA bands came exclusively from the transformed plants, non-transformed plant DNA was used as a negative control. The presence of each gene within the T-DNA was confirmed by PCR analysis of the internal junction regions. The PCR primers used are listed in Table 1. The PCR reaction was carried out in a total volume of 20 µL, consisting of 10 µL of MyTaq Red Master Mix as the source of Taq DNA polymerase, and reaction buffer; 1 µL of forward primer (20 µM) and 1 µL of reverse primer (20 µM); 6 µL of sterile water to adjust the final volume; and 2 µL of DNA template derived from either plant DNA or Agrobacterium DNA containing the pMSU2DR-02 construct. The PCR products were subsequently analyzed by gel electrophoresis. Using 1× TAE buffer stained with RedSafe (iNtRON Biotechnology, Korea), electrophoresis on a 1% (w/v) agarose gel was performed to separate the PCR amplification products. The procedure was conducted at 75 volts for 60 minutes, after which the DNA bands were visualized under a UV transilluminator.

2.8. Screening for Plasmid Backbone Integration

To ensure that only the plant genome contained the T-DNA region, PCR screening was conducted using a primer pair targeting the non-T-DNA backbone region of the binary vector. This test aimed to detect any unintended integration of vector backbone sequences during the transformation process. In addition, primers specific

Table 1. List of primers used for amplification of the stacked resistance genes and marker gene (Douches 2024)

Primer	Sequence	Junction site	Product size (bp)	Annealing temperature (°C)
Stack 1				
1 Stack	GCACGATGTCGCCTTATGTG	left flank border	1,270	50
2b Stack	GCCGAGAAAGTATCCATCAT	NPTII		
Stack 2				
NPT pro out F	GTATTTGATACATACATCTTCATGAC	NPTII	1,032	50
4 Stack	AGTTTAGACTTGGCCATAGC	*RpiAmr3*		
Stack 3				
5 Stack	TGCCCATAAGCCCATTTAT	*RpiAmr3*	942	50
6 Stack	ATCTGTTCTTCCATTAACC	*RpiAmr1*		
Stack 4				
7 Stack	GTCCTTATCACCTTCCGAGA	*RpiAmr1*	1,330	50
8 Stack	CGTTGTCCCACATTTAAGTG	*RpiVnt1*		
Stack 5				
9 Stack	TTTTGTGTGGCAGTAGTTGA	*RpiVnt1*	917	50
10 Stack	TGTGACTGCCGAGATCATTTA	*RySto*		
Stack 6				
11 Stack	CAATGAGTGTAGCAAAGGTC	*RySto*	1,046	50
14 Stack	TGTGAACCAGTTTTGGC	*Rladg*		
Stack 7				
15 Stack	GTCCTCCCATGATAGTTG	*Rladg*	742	50
RB R	ACCCGCCAAATATCCTGTCA	right border		

for the detection of large plasmid backbone sequences were used, as listed in Table 2. PCR amplification was performed under the following conditions: an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute 20 seconds, with a final extension at 72°C for 5 minutes.

3. Results

3.1. Response of Granola Potato Vegetative Growth *in vitro*

The findings showed that the explant source significantly impacted every vegetative growth parameter. Greater mean values for the number of leaves, stem nodes, plant height, and root length showed that the Gra M clone consistently outperformed Gra I in terms of vegetative performance. Another important factor was the type of explant; explants from the middle section (B1) grew better than those from the upper section (B2) (Figure 2 and Table 3). Higher mean values for the number of stem nodes (8.61) and leaves (12.11), plant height (9.65 cm), and root length (6.71 cm) showed that the Gra M clone continuously produced better vegetative growth than Gra I. Similarly, compared to explants from the upper section close to the tip (B2), those from the middle section (B1) typically produced stronger vegetative growth. The A2B1 treatment (Gra M-middle section) produced the highest number of leaves (12.26), maximum number of stem nodes (9.22), and tallest plant height (10.20 cm), indicating the synergistic influence of genotype and explant position on the vegetative performance of Granola potatoes under in vitro conditions.

3.2. Granola Potato Regeneration's Reaction to *in vitro* Growth Media Treatment

The results demonstrated that modifications to the growth media had a major effect on the *in vitro* regeneration of Granola potato plants (Table 4). There were variations between treatments in some parameters, including the percentage of regeneration, the number of explants capable of regeneration, the number of shoots per explant, the number of shoots per Petri dish, and the time required for shoot emergence. The results show that the growth medium composition and the clone source had a significant impact on the Granola potato explants' ability to regenerate *in vitro*. ZIG Gra I showed the lowest regeneration rate (30%), while GNZ Gra I produced the highest regeneration percentage (82%) and the most significant number of shoots per explant (2.04). Furthermore, GNZ Gra I displayed the quickest shoot emergence (8 days), indicating a robust interaction between culture medium and genotype in promoting morphogenic response. Figure 3 shows the regeneration response of granola potato explants under various *in vitro* media treatments. Treatment differences were evident, especially in the

Table 2. Primers used for the detection of large plasmid backbone sequences (Douches 2024)

Primer	Sequence	Product size (bp)	Annealing temperature (°C)
ArPORT1 Backbone1 FWD	CGCACACCGTGGAAACGGATG	500	60
ArPORT1 Backbone1 REV	GAAGGCAGCAAGCGCGATGAA		
ArPORT1 Backbone2 FWD	CAACGCGCTTGGTGCTTATGTG	670	60
ArPORT1 Backbone2 REV	GATCCATGCCCATGACGGCAG		
ArPORT1 Backbone3 FWD	CATGTCGAAGGGAGGGTTGC	650	60
ArPORT1 Backbone3 REV	CTTTGTCCAGGATTGGCCGGG		
ArPORT1 Backbone4 FWD	GGCTGATGTGCGCTGCTTTG	622	60
ArPORT1 Backbone4 REV	GAGGAAGGTCGAGAGTACCG		
ArPORT1 Backbone5 FWD	GGCTCGGCAGAATGTTCACG	595	60
ArPORT1 Backbone5 REV	CAGTCGATTTGGTCGAGCCG		

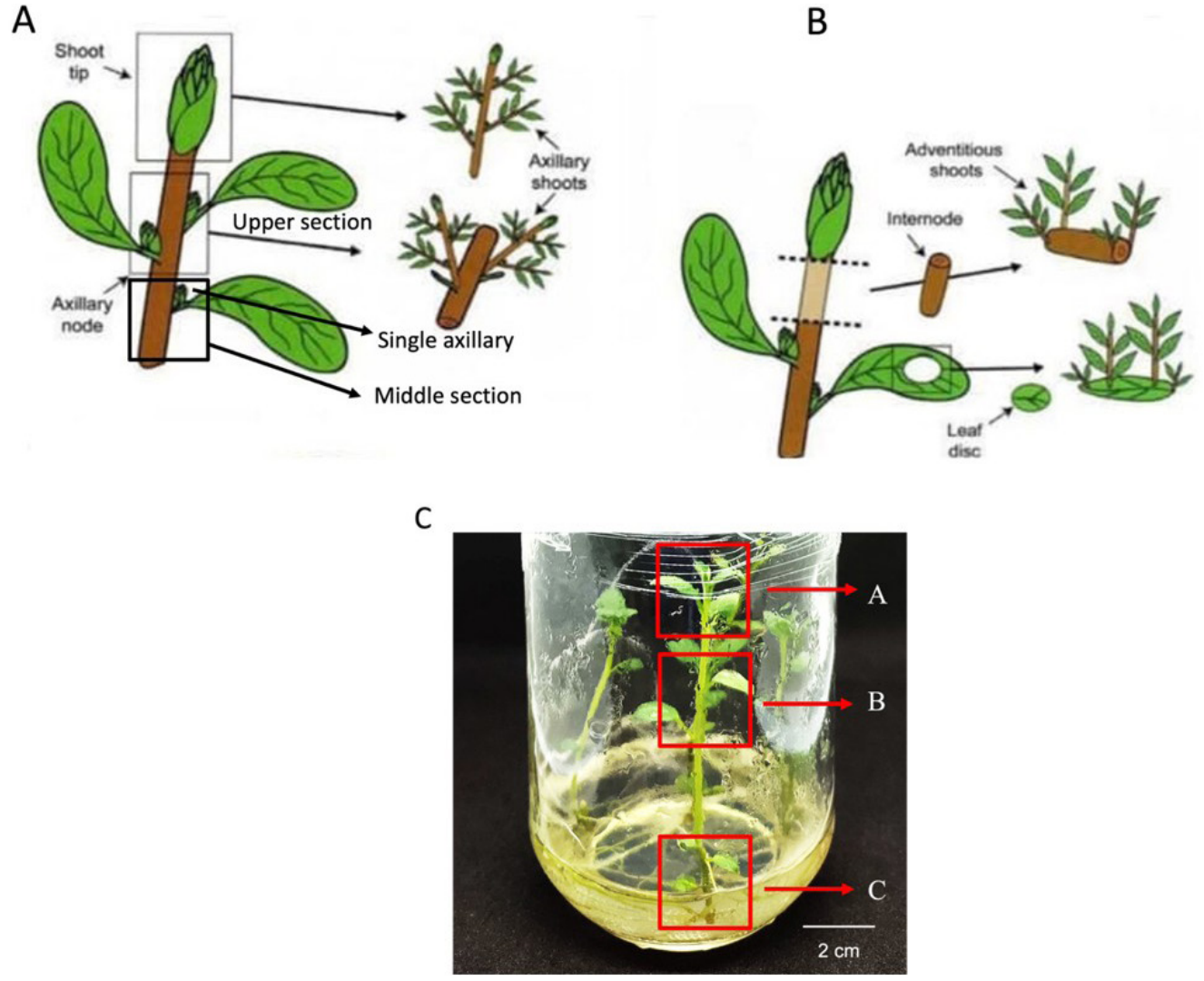

Figure 2. Selection of potato stem explants used for *in vitro* propagation and genetic transformation. (A) Upper stem region containing axillary buds used for clonal propagation. (B) Middle internode region lacking axillary buds, used as explants for regeneration optimization and Agrobacterium-mediated transformation. (C) Lower stem region showing root development. Scale bar = 2 cm

Table 3. The effect of explant source and explant type on the vegetative growth of Granola potatoes in in vitro culture at 4 weeks after planting (WAP)

Factor	Parameter			
	Number of stem nodes	Number of leaves	Plant height (cm)	Root length (cm)
Factor A (Granola)				
A1 (Gra I)	4.45[a]	7.40[a]	7.09[a]	4.08[a]
A2 (Gra M)	8.61[b]	12.11[b]	9.65[b]	6.71[b]
Factor B (Stem Internode)				
B1 (Middle section)	6.95[b]	9.89[b]	8.69[b]	5.47[b]
B2 (Upper section)	6.11[a]	9.62[a]	8.05[a]	5.32[a]
Interaction				
A1B1	4.68[b]	7.52[a]	7.18[a]	4.16[a]
A1B2	4.22[a]	7.28[a]	7.00[a]	4.00[a]
A2B1	9.22[d]	12.26[a]	10.20[c]	6.79[a]
A2B2	8.00[c]	11.96[a]	9.10[b]	6.63[a]

Numbers followed by the same letter in the same column for each treatment indicate no significant difference based on the LSD test at a 5% significance level

Table 4. The effect of growth media on the *in vitro* regeneration of Granola at 8 weeks after planting (WAP)

Treatment	Parameter			
	% Regeneration	Shoots per explant	Shoots per petri dish	Days to shoot emergence
ZIG Gra M	46.00[b]	1.23[b]	5.60[a]	14.60[d]
GNZ Gra M	83.00[c]	1.52[c]	12.60[c]	10.40[b]
ZIG Gra I	30.00[a]	0.87[b]	8.70[b]	11.40[c]
GNZ Gra I	82.00[c]	2.04[d]	20.40[d]	8.00[a]

quantity and vigor of regenerated shoots. Explants cultured on the GNZ medium exhibited a more vigorous regeneration response, producing a greater number of shoots compared to the ZIG medium. The use of the GNZ combination was superior, with a regeneration rate of 82-83% and an average of 8.2-8.3 regenerated explants, compared to ZIG, which only reached 30-46% with an average of 3.0-4.6 explants. This confirms that the use of NAA is more effective than IAA in supporting the regeneration of Granola potato varieties; synthetic auxins are more effective in combination with cytokinins for shoot formation, and the combination of NAA with cytokinins can produce more shoots than IAA. By week 8, both media showed comparable shoot growth, although GNZ had a higher number of regenerated shoots overall. Thus, the success of *in vitro* regeneration is greatly influenced by the type of medium and hormone combination used, so the GNZ Gra I treatment was selected as the most effective medium to support the Granola plant regeneration process and has the potential to be used in genetic transformation.

3.3. Introduction of Stacked Genes into Granola Potato Plants

Transformation tests on 200 explants showed the best efficiency at 10 minutes of infection (13% transformation, 100% regeneration) compared to 20 minutes (7% transformation, 71% regeneration) (Table 5). This suggests that a shorter infection duration is more effective because it minimizes physiological stress and increases the success rate of transgenic shoot regeneration. The regeneration process from these transformed explants successfully produced a total of 25 transformed shoots. Transformed explants refer to explants that successfully integrated the transgene, while regenerated explants refer to transformed tissues that subsequently produced shoots under selective culture conditions. Granola potato explants infected with *A. rhizogenes* ArPoRT1 for 10 minutes, followed by a 3-day co-cultivation period on solid medium, showed higher transformation and regeneration efficiency than those infected for 20 minutes. The shorter infection time likely reduced bacterial stress and tissue damage during transformation, resulting in multiple bright green putative shoots and the emergence of transgenic roots.

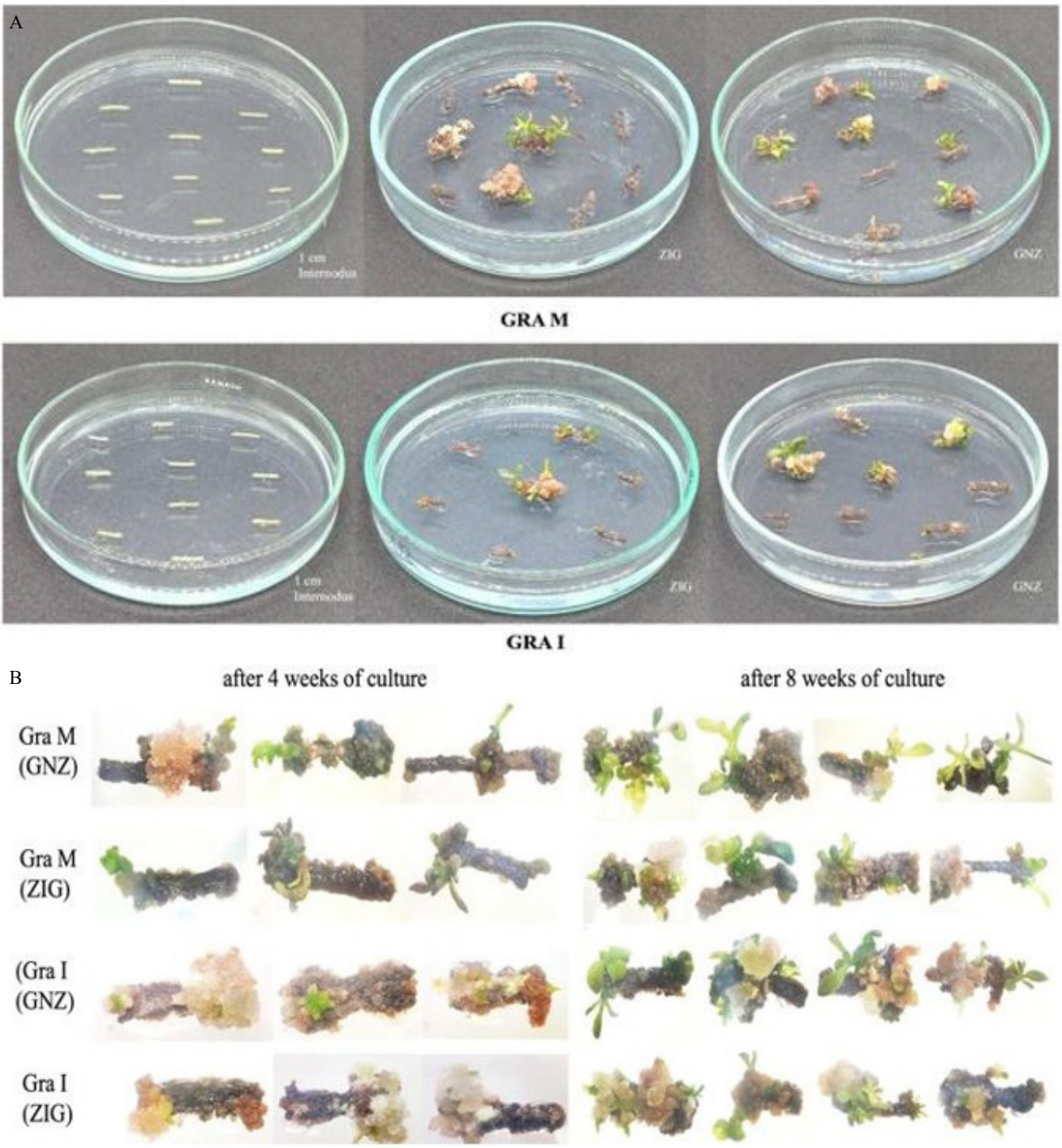

Figure 3. (A) Regeneration response of Granola potato stem explants after *in vitro* treatment using two combinations of growth hormones, and (B) visualization of Gra M and Gra I explant regeneration at 4 and 8 weeks of age using a stereomicroscope. Scale bar = 1 cm

Table 5. Transformation and regeneration efficiency of putative transgenic Granola potatoes

Duration of infection	Total explant	Kanamycin resistant		Transformation efficiency (%)	Regeneration efficiency (%)
		Total resistant callus	Total regenerated callus		
10 minutes	100	13	13	13.0	100.0
20 minutes	100	7	5	7.0	71.4
Controls 10 minutes	30	0	0	0.0	0.0
Controls 20 minutes	30	0	0	0.0	0.0
Total	260	20	18	-	-

These observations were consistent across repeated experiments, supporting the reproducibility of the results. This observation suggests that the tissue maintained a healthy appearance during culture, showing normal shoot differentiation and root initiation. In contrast, explants immersed for 20 minutes exhibited reduced regeneration ability, fewer shoots, and browning of the callus, likely due to excessive stress caused by prolonged bacterial contact. During the pre-culture (Figure 4a), co-cultivation (Figure 4b), and callus induction (Figure 4c) stages, the number of explants placed per Petri dish was not fixed and occasionally exceeded ten explants. This approach was intentionally applied to conserve culture media during the early stages of the experiment. However, in the selection stage, each Petri dish consistently contained 10 explants, as specified in the experimental design.

3.4. Analysis of Stacked Gene Integration in Putative Transgenic Plants

Molecular verification results showed that all 25 transgenic plants successfully integrated the target genes from the pMSU2DR-02 vector, as evidenced by the amplification of DNA fragments of the expected sizes and the absence of plasmid backbone sequences. The molecular verification results were further explained in detail based on the PCR product sizes amplified using each primer stack (1-7). Primer stack 1 produced a DNA fragment of approximately 1270 bp, followed by stack 2 (1,032 bp), stack 3 (942 bp), stack 4 (1,330 bp), stack 5 (917 bp), stack 6 (1,046 bp), and stack 7 (742 bp) (Figure 5 and Table 1).

The organization of the pMSU2DR-02 T-DNA constructs used for Agrobacterium-mediated transformation is shown (Figure 5A). The construct contains five stacked disease resistance genes (*RpiAmr3, RpiAmr1, RpiVnt1, RySto,* and *Rladg*), together with the selectable marker gene (NPTII), assembled sequentially within a single T-DNA region using the GAANTRY gene-stacking system. Seven primer stacks were designed to amplify the junction regions between adjacent genetic elements, enabling verification of the integrity of the entire stacked T-DNA construct before assessing its integration into transgenic Granola plants.

PCR amplification profiles obtained using seven primer stacks confirmed the integrity of the stacked T-DNA construct in transgenic Granola potato plants (Figure 5B). Lane 1 contains the 1 kb DNA ladder used as the molecular size marker. Lanes 2-15 represent genomic DNA from transgenic Granola plants amplified using primer stacks 1-7, with each primer stack analyzed in two independent transgenic lines. Primer stack 1 (lanes 2-3) produced the expected 1,270 bp amplicon corresponding to the junction between the NPTII selectable marker and the *RpiAmr3* gene. Primer stack 2 (lanes 4-5) generated the expected 1,032 bp fragment spanning the *RpiAmr3-RpiAmr1* junction. Primer stack 3 (lanes 6-7) amplified the expected 942 bp fragment corresponding to the *RpiAmr1-RpiVnt1* junction. Primer stack 4 (lanes 8-9) produced the expected 1,330 bp fragment spanning the RpiVnt1-RySto junction. Primer stack 5 (lanes 10-11) generated the expected 917 bp fragment corresponding to the *RySto-Rladg* junction. Primer stack 6 (lanes 12-13) amplified the expected 1,046 bp fragment spanning the *Rladg-KmR* junction, whereas primer stack 7 (lanes 14-15) produced the expected 742 bp fragment covering the terminal region of the stacked T-DNA construct. Lane 16, containing the pMSU2DR-02 plasmid DNA, served as the positive control and produced the expected amplification product, whereas lane 17, containing genomic DNA from non-transgenic Granola plants, showed no amplification. The successful amplification of all expected PCR fragments from the transgenic plants confirmed the integrity of each target region within the pMSU2DR-02 stacked T-DNA construct, demonstrating that the stacked resistance genes were successfully integrated into the Granola potato genome (Table 6).

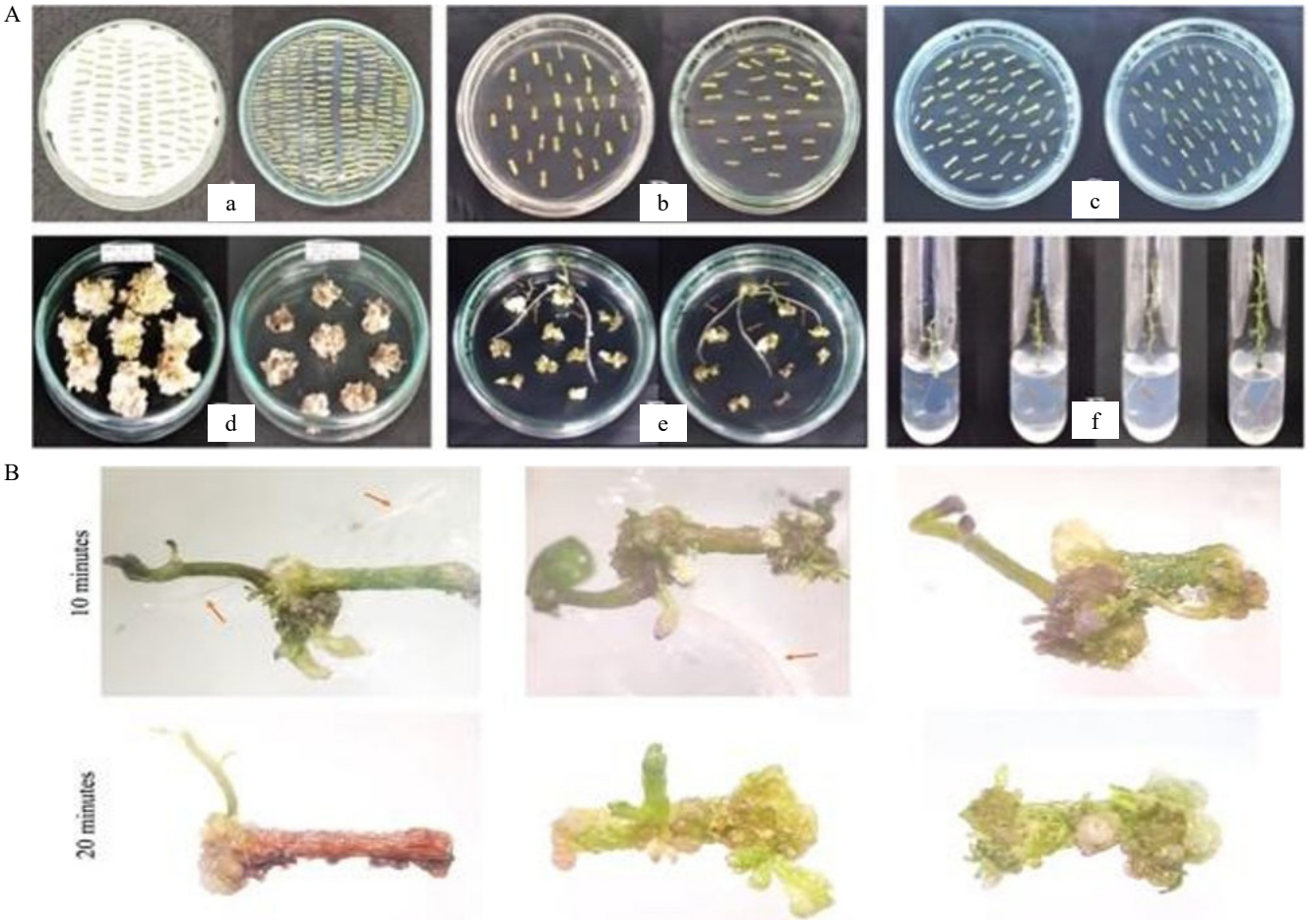

Figure 4. Stages of Granola potato plant transformation using *A. rhizogenes* ArPORT1 at 10- and 20-minute infection durations. (A) Panel A: (a) initial explants before infection, (b) explants after *Agrobacterium* infection, (c) co-culture for 3 days, (d) callus after shoot cutting, (e) shoot regeneration in the 4th week, (f) planlets on MS0 medium in the 1st to 4th weeks. (B) Panel B: Microscopic visualization of transformant regeneration observed at 4 weeks of age

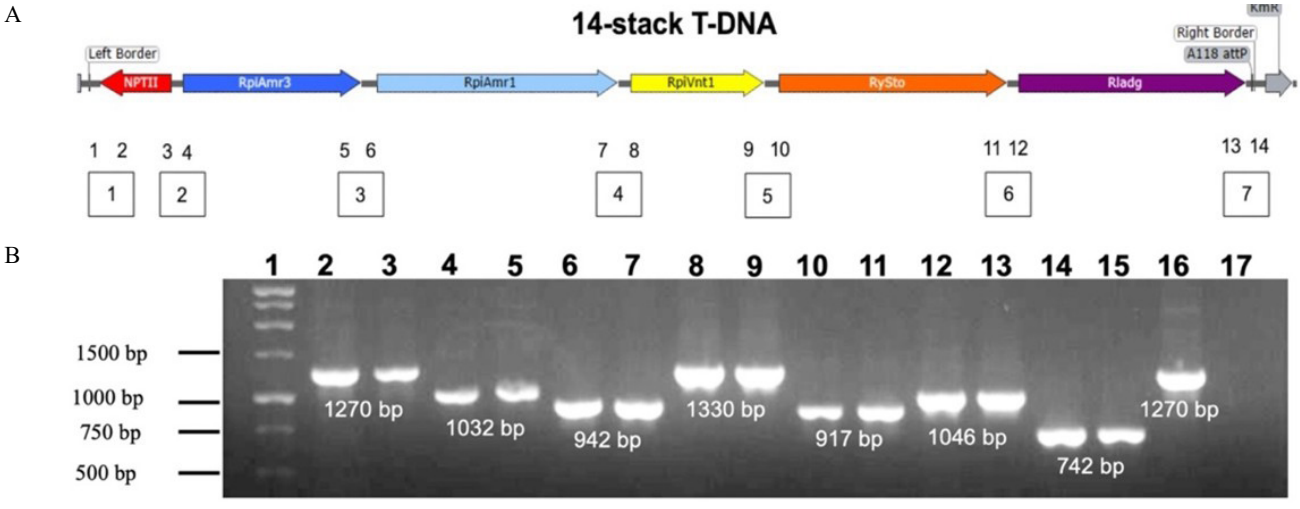

Figure 5. (A) Linear map of stacked gene construction in the pMSU2DR-02 vector showing the position and (B) orientation of primers for each target gene, and PCR results for stacked resistance genes; lane 1: 1 kb DNA ladder marker; lanes 2-15: transgenic granola clones; lane 16: pMSU2DR-02 (positive control); lane 17: non-transgenic granola (negative control)

Table 6. Recapitulation of positive shoots from PCR confirmation results

Duration of infection	Number of shoots tested	Total positive shoots (PCR)
10 minutes	17	17
20 minutes	8	8
Total	25	25

4. Discussion

The comparison of Gra I and Gra M during tissue culture optimization demonstrated that both genotype and explant position influenced regeneration performance and guided the selection of suitable plant material for genetic transformation. Although Gra M exhibited greater vegetative growth, Gra I produced a more consistent regeneration response, particularly from middle internode explants cultured on the optimized regeneration medium. These results indicate that vegetative vigor does not necessarily correspond to regeneration capacity, which is largely determined by genotype-specific responses under *in vitro* conditions. The superior performance of middle internode explants agrees with previous studies showing that explants at an intermediate developmental stage possess greater morphogenic competence and support more efficient shoot organogenesis than younger apical tissues (Schmölling *et al.* 1993). Therefore, Gra I was selected for *Agrobacterium*-mediated transformation because its stable and reproducible regeneration provides a practical advantage for obtaining healthy regenerated shoots after infection and selection. Although the physiological basis of these genotype-dependent responses was not examined in this study, the findings emphasize the importance of regeneration screening before transformation and demonstrate that optimizing regeneration before genetic transformation can improve the reliability and reproducibility of potato transformation protocols.

The composition of plant growth regulators strongly influenced regeneration performance. The GNZ medium, containing NAA, produced the highest regeneration percentages (82-83%) and the fastest shoot emergence. In contrast, the ZIG medium, containing IAA, generated greener but more compact calli with fewer regenerated shoots (Figure 3 and Table 4). These results demonstrate that different auxin formulations significantly affected shoot regeneration under the culture conditions used in this study. Similar responses have been reported in potato and other Solanaceae species, where the interaction between auxin and cytokinin regulates callus proliferation and shoot organogenesis, with Zeatin-NAA combinations generally promoting higher regeneration efficiency than media containing IAA (Pal *et al.* 2012; Hajare *et al.* 2021). Together with the observed genotype and explant effects, these findings indicate that regeneration competence in Granola potato is determined by the combined influence of genotype, explant source, and culture medium composition.

The duration of bacterial exposure influenced both regeneration and transformation efficiency in Granola potato. Explants exposed to *A. rhizogenes* for 10 min produced a higher transformation efficiency (13%) and complete shoot regeneration (100%) than those exposed for 20 min, which showed lower transformation (7%) and regeneration (71.4%) rates (Figure 4 and Table 5). In addition, tissue browning was more frequently observed after the longer exposure period, coinciding with reduced regeneration capacity. Similar observations have been reported in *Agrobacterium*-mediated transformation studies, where prolonged bacterial contact can adversely affect explant viability and reduce recovery of transformed shoots (Bhattacharya *et al.* 2010; Dan *et al.* 2015). Although the physiological basis of this response was not investigated in the present study, these findings indicate that limiting the initial infection period to 10 min provides more favorable conditions for transformation while maintaining regeneration capacity. This result highlights the importance of optimizing infection duration to achieve efficient and reproducible genetic transformation in potato.

The lower regeneration observed after prolonged bacterial exposure indicates that infection duration is an important factor influencing transformation outcomes in potato. Similar findings have been reported in previous *Agrobacterium*-mediated transformation studies, where excessive bacterial exposure reduced explant viability and regeneration efficiency (Joyce *et al.* 2010; Ho-Plágaro *et al.* 2018). Because bacterial concentration, physiological responses, and molecular mechanisms were not evaluated in the present study, the observed reduction in regeneration should be interpreted as an association rather than direct evidence of the underlying cause. Nevertheless, the results demonstrate that optimizing infection duration is essential to maintain regeneration capacity and improve transformation efficiency in potato. Future studies integrating physiological and molecular analyses will be valuable for clarifying the mechanisms responsible for these genotype-dependent responses.

PCR analysis confirmed the presence of all expected resistance gene fragments in the transgenic Granola potato lines, indicating successful transfer of the stacked T-DNA construct. The expected amplification patterns obtained

with all primer sets were consistent with the integrity of the multigene cassette. At the same time, the absence of detectable vector backbone–specific fragments suggested that major backbone sequences were not present in the analyzed transformants. Similar PCR-based validation has been widely used to assess the integrity of GAANTRY-mediated multigene constructs. However, additional analyses such as Southern blot hybridization, sequencing, or long-read genome analysis are required to determine T-DNA copy number, insertion sites, and structural integrity with greater certainty (Zarka *et al.* 2024). Therefore, the molecular evidence obtained in this study provides a solid foundation for subsequent analyses of transgene expression and evaluation of disease resistance in the transformed Granola potato lines.

The findings of this study provide a foundation for further molecular and functional characterization of GAANTRY-mediated transgenic potato lines. Future studies should verify transgene expression and evaluate resistance against *P. infestans*, PVY, and PLRV under greenhouse and field conditions to determine the effectiveness of the stacked resistance genes. In addition, comprehensive molecular analyses, including sequencing or Southern blot hybridization, will be important to confirm T-DNA copy number, insertion sites, and structural integrity beyond PCR-based validation (Zarka *et al.* 2024). Such molecular characterization, together with comprehensive environmental biosafety assessments, will be essential to support the safe deployment and regulatory approval of genetically engineered potato cultivars (Ambarwati *et al.* 2022). These approaches will strengthen the characterization of multigene transgenic events and support the development of durable disease-resistant potato cultivars.

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