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# OPTIMIZATION OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION USING DIFFERENT EXPLANTS FOR CRISPR/CAS9 DELIVERY IN POTATO (*SOLANUM TUBEROSUM* L.)

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## SUMMARY

During the dry season, declining soil moisture frequently limits water availability for potatoes (*Solanum tuberosum* L.), exposing plants to drought stress that disrupts normal growth and ultimately reduces tuber yield. Therefore, drought stress-tolerant potato cultivars are essential to address this alarming issue. Stomatal conductance plays a central role in plant responses to drought because it regulates both water losses through transpiration and $CO_2$ uptake for photosynthesis. Hence, optimizing stomatal conductance represents an effective strategy to improve water-use efficiency while sustaining plant growth under water-limited conditions. The *Pain-1* gene with reduced expression promotes the degradation of sucrose into glucose and fructose through genome editing using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9). This study aimed to identify the potato explant type with the highest transformation efficiency to support efficient CRISPR/Cas9-mediated editing of the *Pain-1* gene. As a preliminary study, transformation efficiency analysis ensued through leaf and internode explants of potato clone BR1 plantlets used for genome editing. The transformation took place using the *Agrobacterium tumefaciens* strain EHA101 containing the pGW903 vector plasmid, target sequence, and two sgRNA designs (VIST2 and VIST3). Transformant status received assessment by identifying the presence of the *Cas9* and *hpt* genes. The results showed internode explants exhibited significantly higher transformation efficiency (33.46%) than leaf explants (7.95%).

**Keywords:** Potato (*S. tuberosum* L.), drought tolerance, genome editing, CRISPR/Cas9, explant, sgRNA, tuber yield

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**Key findings:** In potatoes (*S. tuberosum* L.), the efficient genome editing was highly dependent on the explant type. This preliminary study revealed a significant difference between the transformation efficiency of potato leaves and internodes. Internode explants consistently showed higher transformation efficiency across two different sgRNA designs. The results suggested the use of internodes as explants for genome editing in potatoes.

## INTRODUCTION

Potato (*Solanum tuberosum* L.) sustainable production requires low temperature for optimal tuber development. Therefore, potato cultivation in Indonesia largely remains in highland areas because these regions provide optimal growing conditions, including cool temperatures (15 °C–20 °C), high relative humidity, adequate rainfall, and sufficient sunlight. However, in these areas, water supply is insufficient because the farming community mainly relies on rainfall for irrigation (Acquaah, 2012; Ajis and Handoko, 2010). Irrigation water is also increasingly limited due to longer dry seasons and climate change, boosting the risk of drought stress as potatoes require more water than other staple food crops (Byrd *et al.*, 2014; Obidiegwu *et al.*, 2015). Drought stress significantly reduces tuber yield and thereby negatively affects farmers' income (Stark *et al.*, 2013; Gervais *et al.*, 2021; Boguszewska-Mańkowska *et al.*, 2022).

Various agronomic strategies have been applicable to reduce the impact of drought stress, such as improving irrigation efficiency, applying mulches to conserve soil moisture, optimizing nutrient management, and adopting drought-tolerant cultivars. Therefore, the use of drought-tolerant potato cultivars has become the only economic option, leading to ongoing efforts to develop drought-tolerant selection techniques (Nasir and Toth, 2021). These selection strategies may include the use of morphological markers, such as root depth (Steckel and Gray, 1979), physiological traits, viz., stomatal conductance and metabolite levels (Evers *et al.*, 2010). However, these conventional methods are typically labor-intensive, time-consuming, and expensive (Blum, 1989), and the selected genotypes often exhibit limited yield stability under drought stress conditions (Joshi *et al.*, 2020).

Further research extensively focuses on the discovery of molecular markers associated with drought tolerance. Several quantitative trait loci (QTL) associated with drought-tolerant traits have reached discoveries; however, most QTLs have only minor effects, and the procedures are also challenging and expensive (Khan *et al.*, 2016; Joshi *et al.*, 2020). Genetic transformation methods have also been effective in producing drought-tolerant transgenic potatoes (Gazendam *et al.*, 2016). However, regulatory barriers and biosafety concerns pose major challenges to their extensive implementation (Prado *et al.*, 2014).

Genome editing has become an essential tool in modern plant breeding because it allows for precise modifications to target genomic sequences through targeted insertions, deletions, and replacements. Among the various genome-editing technologies available, the CRISPR/Cas9 system has attracted widespread attention due to its simplicity, efficiency, and versatility. The CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) system is a cutting-edge genome-editing technology, facilitating the development of transgene-free mutant plants that are not liable to biosafety regulatory oversight (El-Mounadi *et al.*, 2020; Wada *et al.*, 2020).

Several researchers have obtained transgenic crops with altered stomatal characteristics, making them more efficient at using water without reducing their photosynthetic capacity (Franks *et al.*, 2015). These transgenic genotype plants respond to water-stressed conditions by reducing their stomatal size (Doheny-Adams *et al.*, 2012). Previous studies reported the reduction of stomatal conductance by silencing the *Pain-1*

gene in tobacco, which controls the expression of the vacuolar invertase enzyme in potatoes (Chen *et al.*, 2016). Silencing the *Pain-1* gene using the CRISPR/Cas9 system could improve drought tolerance in potato crops. Nevertheless, the effectiveness of this approach relies on efficient genetic transformation, as successful delivery and stable integration of the CRISPR/Cas9 construct are prerequisites for subsequent genome editing. Therefore, the transformation efficiency is a critical parameter in *Agrobacterium*-mediated CRISPR/Cas9 systems. One of the factors determining transformation efficiency is the explant type in various crop plants (Seçgin *et al.*, 2021; Akter *et al.*, 2024).

However, transformation efficiency largely depends on the biological characteristics of the explant. Leaf and internode tissues were choices because they exhibit distinct cellular and regenerative properties that influence their competence for *Agrobacterium*-mediated transformation. Comparing these explants, therefore, provides valuable insights into the most suitable tissue for efficient genome editing in potatoes. Therefore, this study aimed to identify the potato explant type with the highest transformation efficiency to support an efficient CRISPR/Cas9-mediated editing of the *Pain-1* gene.

## MATERIALS AND METHODS

### Plant materials and plasmid vector

Leaf and internode explants entailed excision from healthy in vitro-grown plantlets of the potato (*S. tuberosum* L.) genotype BR1 plantlets maintained under aseptic culture conditions. The explants' preparation immediately preceded the *Agrobacterium*-mediated transformation according to the respective protocols described by Wang *et al*. (2020) and Bruce and Rupp (2019), with minor modifications. Preparing leaf explants comprised removing the apical and basal portions of the leaf before transformation, whereas internode explants consisted of stem segments approximately 1.5 cm in length. The total number of explants evaluated was 384 and 399 for internode explants transformed with pGW903-VIST2 and pGW903-VIST3, respectively, and 419 and 462 for leaf explants transformed with the corresponding constructs. Each explant treatment continued as an individual experimental unit, with the transformation efficiency calculated based on the total number of explants evaluated for each construct. Transformation succeeded in using *Agrobacterium tumefaciens* carrying the vector plasmid pGW903-ZmUbiP: Cas9-StU6: sgRNA (Figure 1). The potato accession and the vector plasmid came from the collection of PT BISI International Tbk, East Java, Indonesia.

### Target gene and sgRNA

The design of sgRNA sought to target the *Pain-1* (PGSC0003DMG400013856) gene's exon 1, earlier identified using the open-source genome database portal, Ensembl Plants (http://plants.ensembl.org). Using the same portal served to predict off-target sites from the protospacer adjacent motif (PAM) sites found. The two PAM sites appeared in exon 1, i.e., VIST2 (TTAAAATCATCTCCGGCATT) and VIST3 (GATCCTCAACAACCAATCAC). The oligos incurred commercial synthesis for gateway cloning and insertion into a plasmid using the LR clonase recombination method. Confirmation of sgRNA insertion into the plasmid proceeded by the restriction digestion at the HindIII site.


**Figure 1.** Construction of the pGW903 vector plasmid.

***Agrobacterium*-mediated transformation**

The *Agrobacterium tumefaciens* strain EHA101 containing the plasmid, as propagated on selective YEP (yeast extract peptone) media with kanamycin (50 mg $L^{-1}$) and spectinomycin (100 mg $L^{-1}$), sustained incubation at 28 °C for two days and served as the mother culture. Single colonies taken from the mother culture underwent growth in 5 mL of liquid YEP selective medium before incubation for 16 hours. Next, dissolving 1 mL of the culture occurred in 10 mL of the MS10 medium, with the optical density (OD) value measured at a wavelength of 550 nm. When the OD value reached 0.2 ± 0.02, the emerging bacterial culture would be ready for infection. The bacterial suspension adjustment to an $OD_{550}$ of 0.20 ± 0.02 corresponds to a moderate cell density that promotes efficient *Agrobacterium*-mediated infection while minimizing bacterial overgrowth and tissue damage. The use of $OD_{550}$ in this study was according to our laboratory standard protocol and the specifications of the available spectrophotometer.

Transformation using leaf explants continued according to Wang *et al*. (2020), with some modifications. The potato explants placed in *Agrobacterium* suspension media had the supplementation with acetosyringone. The infection process ran for 20 minutes and continued with co-cultivation in callus induction media (CIM) containing acetosyringone. Afterward, the explants entailed transferring to shoot induction media (SIM) containing carbenicillin (500 mg $L^{-1}$) before continuing with the explant selection in the SIM containing hygromycin (2.5 mg $L^{-1}$) until a green callus appeared. The transfer of the green callus proceeded to M13 media containing carbenicillin and hygromycin until shoots appeared and were ready for transfer to the rooting medium.

Transformation with internodal explants followed the method according to Bruce and Rupp (2019), with some modifications. The explants underwent placement in *Agrobacterium* suspension media containing acetosyringone. The infection process ran for 45 minutes and continued with co-cultivation in the CIM containing acetosyringone. The explants then reached their transfer to the SIM 3C5ZR-1 containing ZR (5 mg $L^{-1}$) and carbenicillin (500 mg $L^{-1}$) before transferring to the SIM 3C5ZR-1 containing ZR, carbenicillin, and hygromycin (2.5 mg $L^{-1}$). Next, transferring the explants succeeded in the SIM 3C5ZR-2 containing ZR, carbenicillin, and hygromycin until the shoots were ready for transfer to the rooting medium.

The transformation protocols for leaf and internode explants were adaptations from Wang *et al*. (2020) and Bruce and Rupp (2019), respectively, with minor modifications. The differences in infection duration and regeneration media reflect the explant-specific regeneration requirements reported in these protocols. Therefore, each explant type received evaluation under its respective optimized transformation conditions to ensure maximum regeneration and transformation efficiency.

**Selection of putative transformants**

The shoots formed in the regeneration media, after cutting, incurred transferring to solid MS media containing carbenicillin (500 mg $L^{-1}$) and hygromycin (2.5 mg $L^{-1}$). Allowing the shoots to grow for four weeks had a light intensity of 4000 lux until they formed roots. Shoots that formed roots entailed cutting before transferring back to the rooting media for up to four cycles while discarding those that did not develop and did not form roots. Regenerated shoots sustained four consecutive cycles of hygromycin selection during the rooting stage to minimize the recovery of chimeric transformants. Only shoots consistently surviving hygromycin selection and exhibiting stable root development remained for further analysis. The resulting putative transformants gained subsequent confirmation by PCR amplification using *Cas9* and *hpt* gene-specific primers to verify the presence of the transgene before further evaluation.

**Transformation efficiency observation**

Leaf and internode plantlet samples that were taken had their DNA isolated using GENEzol™

**Table 1**. Specific primer sequences for *hpt* and *Cas9* genes.

| Gene | Code | Sequence (5'-3') |
|---|---|---|
| *hpt* | HPT-F | CTATTGCATCTCCCGCCGTGCACAG |
| | HPT-R | GGCGATTCCATGCCAACAAGGGATGG |
| *Cas*9 | CAS9-F6 | GAATGGATATGCTGGCTATATTGACG |
| | CAS9-R6 | CCTCCGTGACGTACTTGACC |

**Table 2.** Effect of explant sources and sgRNA designs on the transformation efficiency.

| Sources | No. of Explant | No. of Plantlet | *Cas9*/*hpt* Positive | Transformation Efficiency (%) | Transformant Escape Rate (%) |
|---|---|---|---|---|---|
| Effects of explant sources | | | | | |
| Internodes | 783 | 409 | 262 | 33.46 ± 2.71 *a* | 35.94 ± 16.16 |
| Leaves | 881 | 132 | 70 | 7.95 ± 3.12 *b* | 46.97 ± 16.26 |
| p-value | | | | 0.014 * | 0.620 ns |
| Effects of sgRNA designs | | | | | |
| VIST2 | 803 | 318 | 164 | 20.42 ± 15.02 *a* | 48.43 ± 5.33 |
| VIST3 | 861 | 223 | 168 | 19.51 ± 20.86 *a* | 24.66 ± 5.23 |
| p-value | | | | 0.989 ns | 0.049 * |

* = significant at the 5% level of probability, ** = significant at the 1% level of probability, ns = nonsignificant at the 5% level of probability.

DNA Reagent Plant (Geneaid, Cat. No. GR100). The selection of transformant plants continued by amplifying the *Cas9* and *hpt* gene sequences in the isolated plantlet DNA using specific primers via PCR (Table 1). The transformation efficiency calculation proceeded by dividing the number of plantlets found positive for the *Cas9* and *hpt* genes by the number of initial explants, with the results expressed in percentage. Explants with higher transformation efficiency were options for use in subsequent transformation activities.

**Statistical analysis**

The experiment followed a 2 × 2 factorial arrangement with two explant types (leaf and internode) and two CRISPR/Cas9 constructs (pGW903-VIST2 and pGW903-VIST3), resulting in four treatment combinations: VIST2–internode, VIST3–internode, VIST2–leaf, and VIST3–leaf. Transformation efficiency and putative transformant escape rate analysis used a generalized linear model (GLM) with a binomial distribution in R (version 4.4.1), using the number of PCR-confirmed transformants relative to the total number of explants evaluated for each treatment.

**RESULTS AND DISCUSSION**

**Explant type effect**

The results of the study showed the transformation efficiency of potato (*S. tuberosum* L.) internode explants was approximately four times higher (33.46%) than that of leaf explants (7.95%) (Table 2). Reported similar differences in transformation efficiency among explant types were in previous studies, highlighting the importance of explant selection for the success of genetic transformation (Kamrani *et al*., 2015; Dönmez *et al*., 2019; Bakhsh, 2020; Malakhova *et al*., 2021). The higher transformation efficiency in internode explants has a likely relation to greater cellular competence for regeneration and *Agrobacterium*-mediated gene transfer. Inter-node tissues contain actively dividing cells, particularly in the vascular and cambium regions, which are more responsive to dedifferentiation and subsequent shoot organogenesis. Additionally, wounds incurred during explant preparation stimulate the release of phenolic compounds, including acetosyringone-like molecules, which activate *Agrobacterium virulence* genes (*vir*) and

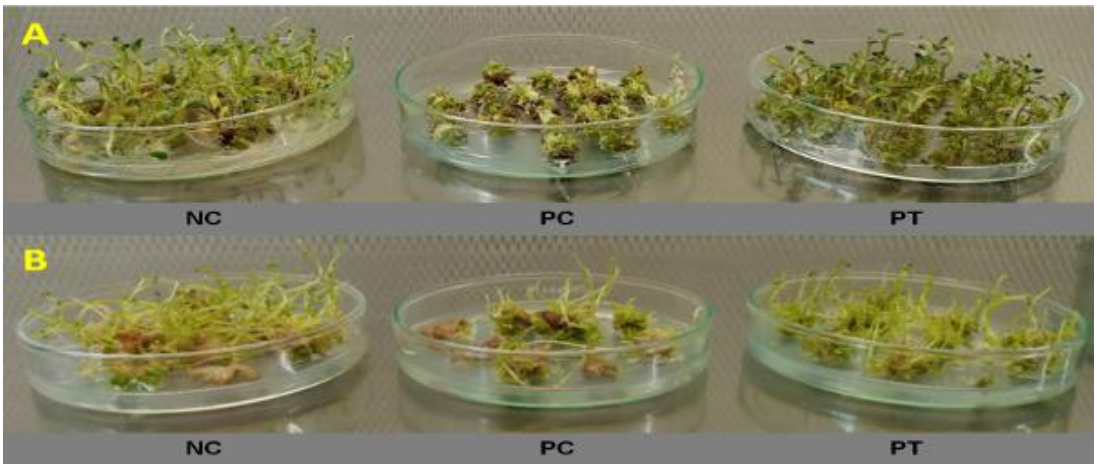


**Figure 2.** Growth of putative transformant plantlets. A) Plantlets derived from internodes' explants, and B) Plantlets derived from leaves' explants. NC = negative control, PC = positive control, and PT = putative transformant.

facilitate T-DNA transfer into plant cells. Successful transformation also depends on the interaction between bacterial virulence factors and the physiological status of the host cell, as competent cells that are actively dividing generally are more receptive to T-DNA integration and subsequent regeneration into transgenic plants (Gelvin, 2003; 2011). Together, these characteristics improve the likelihood of successful transformation and regeneration, thereby contributing to the higher transformation efficiency observed in internode explants.

This observation is consistent with previous reports indicating that internode explants are more tolerant of in vitro culture conditions (Kamrani *et al*., 2015), produce callus more rapidly and in greater quantities (Kumlay and Ercisli, 2015; Bakhsh *et al*., 2020), and exhibit a higher capacity for shoot regeneration (Kaur *et al*., 2020; Mollika *et al*., 2024). Enhanced shoot regeneration has also shown a link to optimized explant preparation, including proper cutting techniques, which further improves the success rate of recovering transformed plants. Conversely, leaf explants are generally more susceptible to mechanical damage during handling (De-Block, 1988) and more prone to somaclonal variation, which can reduce regeneration efficiency and, consequently, lower transformation success (Soto *et al*., 2007).

The comparison of shoot performance between internode and leaf explants is available in Figure 2. In general, the performance of putative transformant (PT) explants, non-transformants on non-selective media (NC), and non-transformants on selective media (PC) showed a similar pattern. The NC and PT explants formed more shoots than PC explants. This also proves the selection pressure of the hygromycin antibiotic as applied to the medium. However, internode explants appeared to form more vigorous and greener shoots than those originating from leaf explants, both on NC and PT explants. The same also had previous reports through the development of efficient, reproducible, and stable *Agrobacterium*-mediated genetic transformation in potato cultivars (Bakhsh *et al.*, 2020).

In contrast, in PC explants, shoot formation appeared to be more prevalent in potato leaf explants than in internodal explants. Internode explants even occurred to experience necrosis and shoot growth inhibition, as reported by Jayakody *et al.* (2023), who also found greater selection pressure by hygromycin on internode explants than on leaf explants in potatoes. The results

confirmed the effectiveness of certain antibiotics as selectable markers as influenced by the explant type. If a particular explant type tends to be less sensitive to the antibiotic, its escape rate will be higher. Although survival rates did not differ significantly between internode explants (35.94%) and leaf explants (46.97%), the two types of explants exhibited different responses under hygromycin selection, particularly for tissue necrosis and shoot regeneration (Table 2). These contrasting responses could reflect inherent differences in tissue physiology and regenerative capacity, rather than merely differences in selection efficiency. Internode explants generally possess a higher capacity for cell division and shoot organogenesis, allowing transformed cells to withstand selection pressure and regenerate more efficiently even when exposed to hygromycin. Conversely, leaf explants are more susceptible to tissue damage and oxidative stress following wounding and antibiotic treatment, which can reduce their regenerative potential even though the transformed cells remain viable. Differences in tissue architecture may also influence the uptake and distribution of hygromycin, leading to variations in cellular sensitivity and the extent of tissue necrosis. Consequently, although their survival rates are comparable, the greater regenerative capacity and tissue tolerance of internodes likely contribute to the higher recovery rates of transformed shoots and the overall transformation efficiency observed in this study (Gelvin, 2003).

The PC explants from internodes (Figure 3) and leaves (Figure 4) both showed tissue damage and necrosis between the resting and one-week selection phases, confirming antibiotic toxicity and the absence of resistance genes to the selectable marker. Meanwhile, the PT explants showed incremental tissue growth, chlorophyll development, robust callus production, and obvious shoot emergence, especially in internode explants, after the 2-week selection phase, indicating successful integration and expression of the selectable marker gene. These results were greatly analogous to previous findings of Miroshnichenko *et al.* (2024), who reported that transgenic explants can maintain vitality and morphogenesis despite the presence of selection agents, owing to the functional expression of selectable marker genes in potatoes.

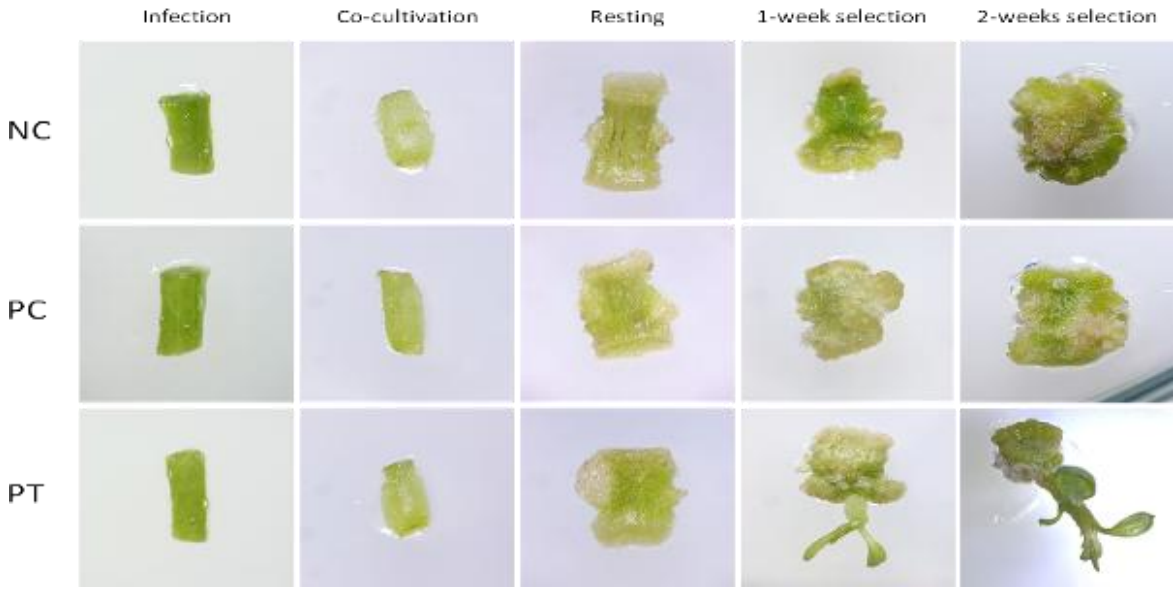


**Figure 3.** Development of internode explants. NC = negative control, PC = positive control, and PT = putative transformant.

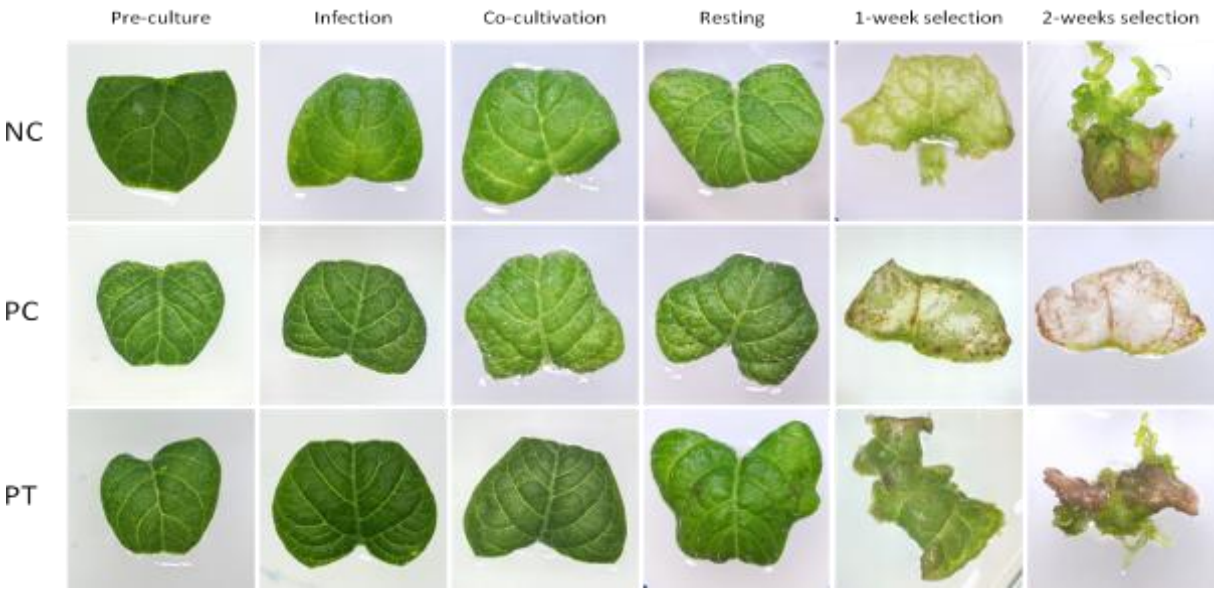


**Figure 4.** Development of leaf explants. NC = negative control, PC = positive control, and PT = putative transformant.

Even so, several past studies have also reported the existence of a genotype-dependent phenomenon, whereby certain potato genotypes exhibited better transformation efficiency by using leaf explants, and vice versa (Dönmez *et al.*, 2019; Bakhsh, 2020). The higher transformation efficiency achieved by using internode explants offers practical advantages for potato transformation by reducing the amount of explant material, culture medium, labor, and time required to obtain a sufficient number of transformants. This improvement is particularly beneficial for CRISPR/Cas9-mediated genome editing, where transformation efficiency directly influences the success of producing edited plants. Future research should evaluate this approach across various potato genotypes to further optimize the transformation protocol. These findings provide a practical basis for developing more efficient and cost-effective transformation systems to support functional genomics studies and precision breeding in potatoes using CRISPR/Cas9 technology.

**sgRNA design effect**

From each targeted PAM, VIST2, and VIST3, the putative transformant plantlets received confirmation by amplifying the *Cas9* and *hpt* genes. The putative transformant plantlets confirmation results showed the *Cas9* and *hpt* gene amplicons were 529 bp and 688 bp in size, respectively (Figure 5). Meanwhile, the transformation efficiency values of VIST2 (20.42%) and VIST3 (19.51%) revealed no differences, and one can conclude that the sgRNA design did not affect the transformation efficiency. The plant genotype, explant type, *Agrobacterium* strain, and vector design emerged to be important factors that influence the success of transformation (Cheng *et al.*, 2004) and the delivery of the CRISPR/Cas9 system (Sapakhova *et al.*, 2025), and not the sgRNA design. Efficient genetic transformation is a critical prerequisite for the success of CRISPR/Cas9-mediated genome editing, as it increases the likelihood of obtaining a sufficient number of transgenic events for molecular characterization and subsequent genome editing analysis. Therefore, the higher transformation efficiency achieved with internode explants in this study provides a practical foundation for the future application of CRISPR/Cas9 in potatoes. Although editing efficiency and mutation yield will ultimately depend on factors such as sgRNA design and target specificity.

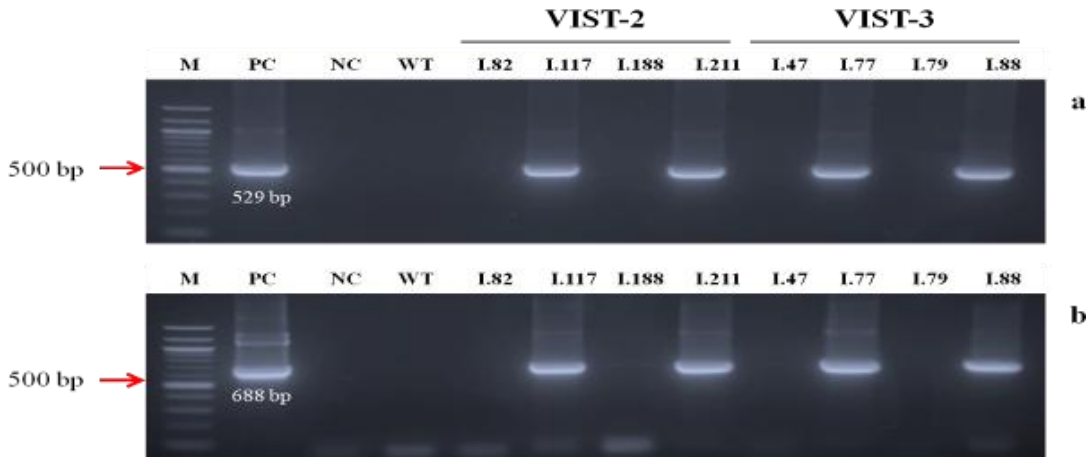


**Figure 5.** Putative transformants confirmation. A) *Cas9* gene amplicons, and B) *hpt* gene amplicons. NC = negative control, PC = positive control, and WT = wild type.

Interestingly, a significant difference in the escape rate of putative transformants was evident between the two sgRNA constructs (Table 2). However, these results require careful interpretation because the sgRNA sequence itself seemed not to directly influence hygromycin selection. Survival under hygromycin selection has its determination primarily by the successful integration and expression of the *hygromycin phosphotransferase* (hpt) selection marker, rather than by the target-specific sgRNA sequence (Gelvin, 2003). Therefore, the observed differences in release rates are more likely due to biological variations during transformation and tissue culture—such as differences in explant physiological status, regeneration response, or stochastic variations in transgene integration and expression—rather than the sgRNA design itself. Reports of similar variability have emerged in *Agrobacterium*-mediated transformation, where transformation outcomes gained influence from various biological and technical factors beyond the gene-editing construct (Gelvin, 2003; 2011). Further experiments using additional independent transformation events and biological replicates are vital to determine whether these differences represent a reproducible biological phenomenon or experimental variation.

## CONCLUSIONS

This study showed internode explants are more suitable than leaf explants for *Agrobacterium*-mediated transformation of the BR1 genotype of potatoes (*Solanum tuberosum* L.), with transformation efficiency approximately four times higher. The higher transformation efficiency achieved with internode explants offers practical advantages by reducing the number of explants, culture media, labor, and time required to obtain sufficient transformants for further applications. These findings established an efficient transformation platform that could facilitate CRISPR/Cas9-mediated genome editing in potatoes in the future. Nevertheless, further optimization of the *Agrobacterium* strain, the selection marker system, and transformation conditions is still essential to improve transformation efficiency and minimize escape rates. Future research should also evaluate genome-editing efficiency, mutation frequency, and drought-related phenotypes, following successful transformation to validate the application of this platform in enhancing drought tolerance.

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