International Journal of Horticultural Science and Technology
Journal homepage: https://ijhst.ut.ac.ir

Potential of Pre-treatment in Enhancing of Transformation Efficiency In-planta Agrobacterium-mediated Genetic Transformation on Soybean (*Glycine max* L.) Seeds

Muhammad Burhanuddin Irsyadi[1]*, Supriyatin Kartika Sari[2], Erlinda Oktiastuti[2], Innaka Ageng Rineksane[2], Etty Handayani[2], Siti Nur Aisyah[2], Setiyono[1]

1 Study Program of Agricultural Science, Faculty of Agriculture, Universitas Jember, Jember-68121, Indonesia

2 Department of Agrotechnology, Faculty of Agriculture, Universitas Muhammadiyah Yogyakarta, Yogyakarta-55183, Indonesia

ARTICLE INFO

Article history:

Received: 9 August 2024,
Received in revised form: 14 January 2025,
Accepted: 28 January 2025,

Article type:

Research paper

Keywords:

Efficacy
Pre-treatment
Soaking
Soybean
Transgenic
Warm water

*Corresponding author's email: burhanuddin@unej.ac.id

ABSTRACT

The genetic modification of soybean continues to be developed to meet market and consumer demands. However, transformation efficacy has been inconsistent. To enhance this efficacy, various pre-treatment methods can be applied. This study aimed to evaluate the potential of the soaking method as a pre-treatment to improve the in-planta transformation efficiency of soybean seeds. Soybean seeds of the Dega I variety were used in this study, which involved four infection methods. In the first and second methods, seeds were directly infected by soaking in a bacterial suspension for 30 and 60 minutes, respectively. In the third and fourth methods, seeds were first pre-treated by soaking in warm water at 35 °C and 45 °C for 10 min before being soaked in the bacterial suspension for 30 min. Transformation efficiency was confirmed through PCR detection of the CaMV-35S promoter and *npt*II genes, with successful transformation indicated by the presence of 235 bp and 550 bp DNA bands, respectively. The results showed that transformation efficiency increased to 30% when seeds were pre-treated in warm water at 35 °C before inoculation for 30 min. In contrast, direct infection without pre-treatment resulted in an efficacy ranging from 4% to 21%. These findings highlight that seed soaking can serve as an effective heat pre-treatment method to enhance the efficiency of in-planta genetic transformation in soybean seeds.

Abbreviation: Base pairs (bp), Cauliflower Mosaic Virus (CaMV), Neomycin phosphotransferase II (*npt*II), Polymerase chain reaction (PCR).

Introduction

Soybean (*Glycine max* L.) is one of the world's major agricultural commodities, alongside rice and wheat, due to its high demand. It is a rich source of protein and essential nutrients, including carbohydrates, minerals, isoflavones, and vitamins, all of which contribute to human health. Soybean seeds are widely used as food ingredients in products such as tofu, soymilk, tempeh, and natto (Carneiro et al., 2019; Gallino et al., 2022; Li et al., 2017). Continuous improvements in soybean cultivars are essential to meet global market demands. The development of superior varieties aims to enhance resistance to pests and diseases, improve production quality and aesthetics, and increase nutritional value (Bezerra et al., 2023). Genetic improvement can be achieved through conventional breeding and biotechnological approaches. Hybridization, a traditional method of developing superior varieties, often requires a long time and results in genetic heterogeneity (Hada et al., 2018).

Among biotechnological approaches, *Agrobacterium*-mediated genetic transformation is

one of the most widely used methods for developing genetically modified soybean. This method continues to be refined to enhance gene stability and improve transformation efficiency (Yang et al., 2019). Genetic transformation involves inserting foreign genes into the plant genome, with *Agrobacterium*-mediated transformation being preferred due to its simplicity and cost-effectiveness. *Agrobacterium tumefaciens*, a soil bacterium that causes crown gall disease, carries a tumor-inducing (Ti) plasmid, along with virulence and chromosomal virulence genes. These features make *A. tumefaciens* an effective tool for transferring foreign genes into plants (Mayavan et al., 2015). Traditional plant genetic transformation methods typically rely on *in vitro* culture, which requires aseptic conditions to prevent contamination from fungi or bacteria (Chen et al., 2018; Kumari et al., 2016; Souza et al., 2017). However, *in vitro* culture demands specialized expertise, costly equipment, and controlled environments, making it an expensive and complex approach (Handayani et al., 2022). Furthermore, soybean is considered a difficult species to genetically transform, and transformation efficiency remains relatively low (Jia et al., 2015; Zhang et al., 2016). *Agrobacterium*-mediated transformation often necessitates *in vitro* plant regeneration, further complicating the process and limiting success rates.

To address these challenges, alternative methods such as *in-planta* genetic transformation have been developed. This approach offers a simpler and more efficient alternative by eliminating the need for *in vitro* culture (Purwantoro et al., 2023). *In-planta* transformation enables the direct infection of target plant organs using an *Agrobacterium* suspension, making it a practical method for obtaining a high number of transformant plants within a shorter timeframe (Jan et al., 2016; Karthik et al., 2018; Mangena, 2019; Suputri et al., 2019; Tarafdar et al., 2019). Studies on *in-planta* genetic transformation in soybean seeds have reported transformation efficiencies of 3.8% (Paz et al., 2006), 6.71% (Jia et al., 2015), 10% (Li et al., 2017), and 14.51% (Hada et al., 2018). Similar methods have been applied to other species, with transformation efficiencies of 16.7% in *Citrus nobilis* (Suputri et al., 2019) and 8.1% in *Macrotyloma uniflorum* Lam. Verdc. (Amal et al., 2020). Despite its practicality and cost-effectiveness, *in-planta* transformation still presents challenges in achieving high efficiency (Jia et al., 2015). Therefore, this study aimed to enhance transformation efficiency through specific pre-treatment strategies.

Pre-treatment methods are commonly used to address seed characteristics that may hinder imbibition, a crucial process during germination (Amusa, 2011; Pérez-García and González-Benito, 2005). Several factors can affect seed permeability during imbibition, including hard seed coats, seed dormancy, and restricted water uptake (Alvarado et al., 2015). Various pre-treatment techniques have been developed to overcome these issues, such as scarification, mechanical disruption, seed priming, and seed soaking (Fredrick et al., 2017). Among these methods, seed soaking in hot water has been shown to enhance imbibition and germination, particularly in dormant and hard-coated seeds. High-temperature water softens the seed coat, thereby improving water and oxygen permeability. Additionally, water uptake during imbibition increases seed size compared to dry conditions (Amusa, 2011; Aydın and Uzun, 2001; Finch-Savage et al., 2004; Pérez-García and González-Benito, 2005).

Since genetic transformation involves *A.grobacterium tumefaciens* infection to facilitate gene transfer, ensuring high seed permeability may contribute to improved transformation efficiency. Studies have shown that heat treatment enhances genome editing efficiency in *Arabidopsis thaliana* and *Nicotiana tabacum* (Blomme et al., 2022) as well as *Citrus sinensis* (LeBlanc et al., 2019). Wang et al. (2016) reported that this method can also induce intergenerational high-temperature stress tolerance in *Triticum aestivum.* Furthermore, heat treatment has been found to improve transformation efficiency in *A. thaliana* (Malzahn et al., 2019) and *T. aestivum* (Milner et al., 2020). However, the effect of heat pre-treatment on transformation efficiency remains underreported in other plant species, particularly soybean. Therefore, this study aimed to investigate the potential of heat pre-treatment applied to soybean seeds to enhance transformation efficiency in *in-planta Agrobacterium*-mediated genetic transformation. The findings of this study provide a practical reference for genetic transformation methods using plant seeds and commonly available laboratory equipment, which may be applicable to other plant species.

Material and methods

Preparation of A. tumefaciens

The *A. tumefaciens* used in this study was strain GV3101 containing the binary vector pRI101AN-SoSPS1 (Fig. 1). This strain was collected from the Center for Development of Advanced Science and Technology, Universitas Jember. *A. tumefaciens* was cultured on Luria Bertani Agar (supplemented with 100 ppm Rifampicin, 50 ppm Kanamycin, and 12.5 ppm Gentamicin) for 48 h at 28 °C in the dark. Once the bacteria grew, quality control was carried out by PCR to detect the presence of the 35S-CaMV promotor gene.

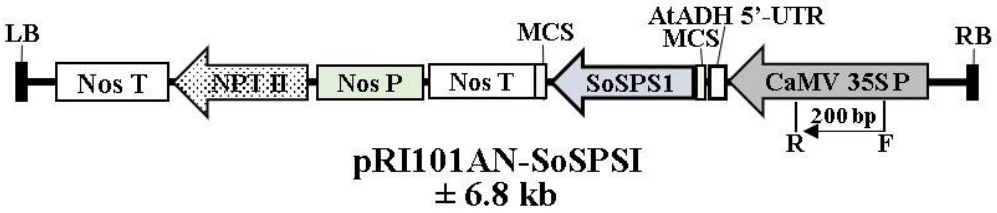

Fig. 1. T-DNA map of binary vector strain pRI101AN-SoSPS1.

Seed soaking-incorporated in-planta Agrobacterium-mediated genetic transformation

Soybean used in this study was *Dega I* variety collected from the Agency of Rice Seeds and Secondary Crops (Yogyakarta, Indonesia). The *A. tumefaciens* suspension was prepared in 50 mL LB broth contained 100 ppm Rifampicin, 50 ppm Kanamycin, and 12.5 ppm Gentamicin. The suspension was agitated at 150 rpm and 28 °C for 17 h in dark condition until it reached OD_{600}= 1.0. This research consisted of 4 infection methods. First and second methods, seeds were infected directly by soaking them in bacterial suspension for 30 and 60 min, respectively. For Third and fourth methods, the soybean seeds were soaked for 10 min as pre-treated in lukewarm distilled water adjusted in two different temperatures (30 and 45 °C), respectively (Fig. 2A). After being soaked, seeds undergo imbibition and enlarge in size (Fig. 2B). Afterwards, the pre-treated seeds were soaked with the bacterial suspension for 30 min (Fig. 2C).

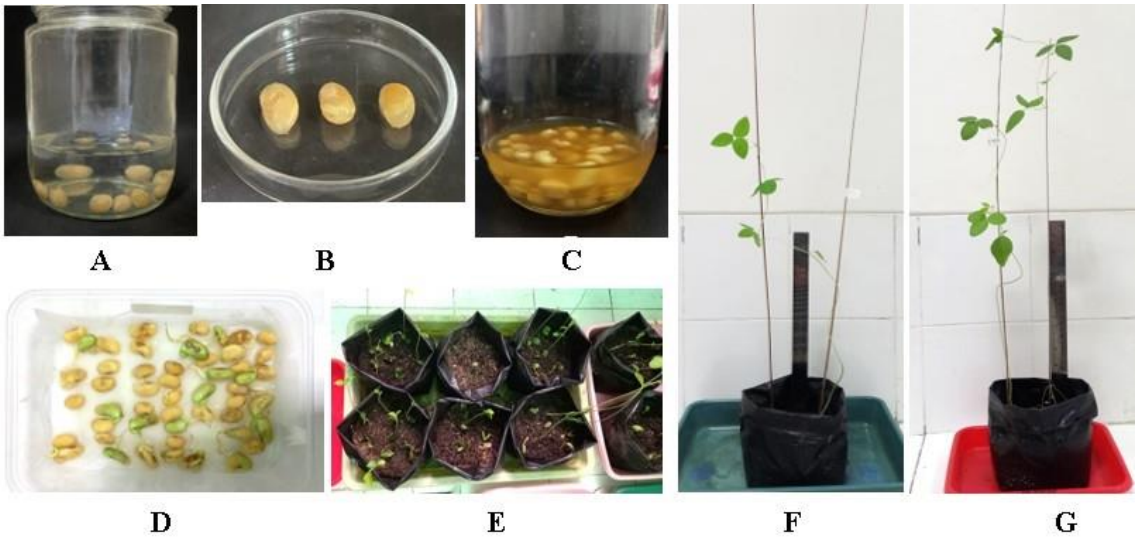

Fig. 2. Procedures *Agrobacterium*-mediated transformation in planta soybean seeds, **A**) Pre-heating seeds soaked in warm water at 35 and 40 °C for 10 min, **B**) Imbibition seeds after treatment, **C**) *Agrobacterium* infection seeds, **D**) Selection of seedling resistance to kanamycin, **E**) Seedlings on medium, **F**) wild-type plants, **G**) transgenic plants.

Screening of putative transformant seedlings

After infection with *A. tumefaciens*, seeds were rinsed with sterile distilled water. The seeds were then placed on filter paper infused with 50 ppm Kanamycin to induce germination. The seeds were incubated for 7 d at room temperature. Normal seedlings were assumed to be the putative transformants and transplanted into planting medium mixture of soil and fertilizer in a greenhouse for the enlargement process.

Conformation of transgenic plants

DNA extraction was performed based on Irsyadi et al. (2024) using soybean leaves collected from 1 month-old, transplanted seedlings as well as wild-type plants. Genomic DNA was subjected to PCR to detect the 35S-CaMV promoter and *npt*II genes. 35S-CaMV PCR was performed using forward primer P35S-S (5'-GATAGTGGGATTGTGCGTCA-3') and reverse primer P35S-R (5'-GCTCCTACAAAATGCCATCA-3') which produced a PCR product measuring 235 bp. The *npt*II PCR was performed using *npt*II-F1 (5′-

GTCATCTCACCTTGCTCCTGCC −3′) and *npt*II-R1 (5′- GTCCGTTGGTCGGTCATTTCG −3′) which a PCR product 550 bp. About 1 µL soybean DNA (200 ng μL^{-1}) was mixed with 6 µL GoTaq® Green master mix (Promega, USA), 0.5 µL for each primer (10 mM), and 2 µL Nuclease-free water. DNA samples were amplified using PCR SensoQuest® (LabCycler, Germany). DNA amplification was carried out using the following setting, namely pre-denaturation at 95 °C for 3 min and followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, elongation at 72 °C for 60 s, and final elongation at 72 °C for 5 min. The PCR products were confirmed through electrophoresis (Mupid-EXu®, Japan) on 1.5% agarose gel based on the presence a single band 235 and 550 bp, respectively. The DNA bands were visualized on blue light trans-illuminator BluPAD (Bio-Helix®, Taiwan).

Data analysis

Data on plant resistance and transformation efficiency were analyzed descriptively. Comparison of transformation efficiency between direct infection and pre-treatment methods was analyzed using T-test with α: 0.05 and standard deviation using R-studio software.

Results

Preliminary detection of recombinant vector in A. tumefaciens colonies

Based on Figure 3, bacterial colonies from Plates 1 and 3 were confirmed to carry the binary vector pRI101AN-SoSPS1, as indicated by the presence of a 235 bp DNA amplicon. This confirmation is essential to ensure that the bacterial strains used contain the targeted vector and gene of interest. Additionally, culturing bacteria on fresh plates serves to rejuvenate the colonies, increase *Agrobacterium* stock carrying the binary vector, and allow for longer storage under optimal conditions.

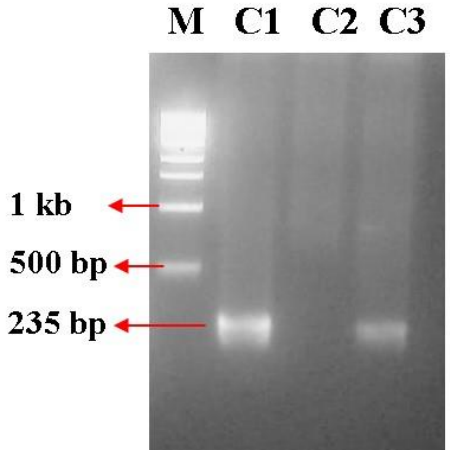

Fig. 3. Detection of *Agrobacterium* colonies containing 35S-CaMV gene with band size 235 bp on binary vector pRI101AN-SoSPS1.

Selection of kanamycin-resistant seedlings

Table 1 shows that seed soaking–incorporated genetic transformation resulted in higher germination rates (42–50%) compared to direct infection, which yielded lower germination rates ranging from 2% to 26%. These findings suggest that seed soaking may enhance seed imbibition during *A. tumefaciens* inoculation, leading to improved resistance to kanamycin. In contrast, wild-type and non-transformant seeds failed to germinate under kanamycin exposure (Fig. 2D). The putative transformed seeds were transplanted into the growing medium and observed for one week (Fig. 2E). After seven weeks, putative transformant plants exhibited growth, as confirmed by DNA analysis (Figs. 2F and G).

Table 1. Screening of resistant to kanamycin and transformation efficiency of transgenic soybean.

Agrobacterium infection treatments	No. of seeds	No. of resistant to kanamycin	Percentage of resistant to kanamycin (%)	No. of transformant	Transformation efficiency (%)
Soaking in suspension for 30 min	50	13	26	4	8
Soaking in suspension for 60 min	50	1	2	0	0
Pre-treatment soaking water at 35 °C for 10 min and suspension for 30 min	50	25	50	15	30
Pre-treatment soaking water at 45 °C for 10 min and suspension for 30 min	50	21	42	6	12

Transformation efficiency of soybean

Molecular analysis of putative transformed and wild-type plants was conducted to amplify specific genes. PCR analysis confirmed the presence of 35S-CaMV and *npt*II gene amplicons, measuring 235 bp and 550 bp, respectively, in the genomic DNA of transgenic

soybean plants (Fig. 4). This indicates the successful insertion of T-DNA, whereas no corresponding DNA bands were detected in wild-type plants. As shown in Table 1, optimizing *in-planta* genetic transformation by pre-soaking seeds in warm distilled water at 35 °C and 45 °C resulted in transformation efficiencies of 30% and 12%, respectively. In contrast, direct infection for 30 minutes yielded an 8% efficiency, while a 60-minute direct infection did not produce any transformant plants. The overall transformation efficiency achieved in this study was 25%. These results highlight that pre-soaking seeds at 35 °C is an effective pre-treatment for enhancing transformation efficiency.

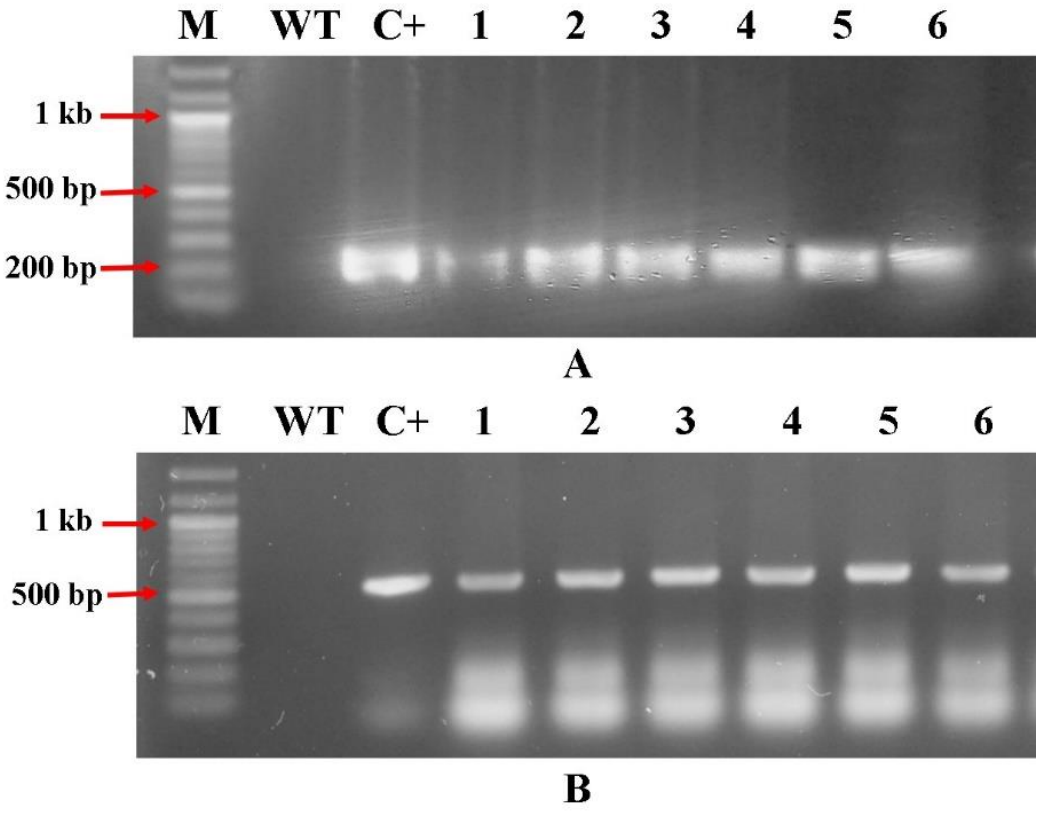

Fig. 4. Amplicon gene from PCR product on 1.5% agarose gel, **A)** 35S-CaMV with a band size 235 bp, **B)** *npt*II with a band size 550 bp. WT: Wild-type, M: ladder 1kb, C+: *Agrobacterium* colony, Lines 1-6: DNA samples of transgenic soybean plants.

Comparison of transformation efficiency between direct infection and warm water pre-treatment of soybean seeds

As shown in Figure 5, transformation efficiency was significantly higher when seeds were pre-soaked in warm distilled water (35–45 °C), achieving 21±9%, compared to direct seed infection, which resulted in only 4±4%, based on the t-test. This pre-treatment demonstrated that heating seeds before infection effectively optimized T-DNA transfer to the plant genome. Additionally, seeds subjected to warm water pre-treatment increased in size and became softer compared to non-soaked seeds (Fig. 2B).

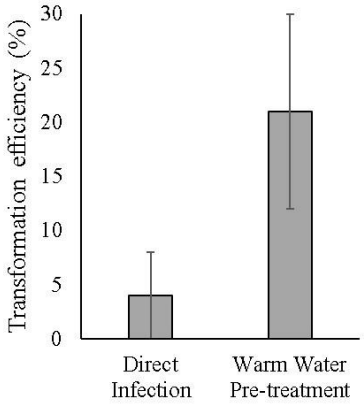

Fig. 5. Comparation of transformation efficiency between direct infection and warm water pre-treatment soaking soybean seeds before infection using t-test with *p*-value: 0.048* and α: 0.05.

Discussion

The *in-planta* method is a simple, effective, and efficient genetic transformation technique that does not require *in vitro* culture. It serves as an alternative approach for achieving high transformation yields. In this method, the target organ is directly infected with an *Agrobacterium* suspension (Jan et al., 2016; Karthik et al., 2018; Mangena, 2019; Suputri et al., 2019; Tarafdar et al., 2019). Zhang et al. (2016) reported that soybean is one of the most challenging species for genetic transformation. Consequently, research efforts continue to focus on enhancing transformation efficiency in soybeans (Yang et al., 2019). Seeds are used as target organs for transformation because their rapidly dividing cells during germination facilitate the integration of target genes into the plant genome (Amal et al., 2020). The density of the *Agrobacterium* suspension significantly affects transformation efficiency (Li et al., 2017). An optical density (OD_{600}) of 0.8–1.0 with a 30-minute infection period has been found effective for soybean genetic transformation (Amal et al., 2020; Jia et al., 2015; Yang et al., 2019). *A. tumefaciens* strain GV3101, which has a C5b chromosomal background with nopaline opine and carries selectable marker genes conferring resistance to gentamicin and rifampicin, is commonly used in transformation studies (Yadav et al., 2014). The pRI101AN binary vector enhances gene expression and transformation efficiency in transgenic plants, while its low DNA copy number minimizes disruption to the plant genome (Bahramnejad et al., 2019; Chetty et al., 2013).

Screening is a crucial step in genetic transformation. The selection of putative transformant plants typically involves antibiotic or herbicide resistance markers. However, a common challenge in this process is the presence of false-positive transgenic plants (Li et al., 2017). Successfully transformed plants exhibit normal growth without signs of damage such as chlorosis, necrosis, or organ deformities (Mangena, 2019). The *neomycin phosphotransferase II* (*npt*II) gene serves as a selectable marker, providing resistance to kanamycin in transgenic plant selection. Kanamycin inhibits protein synthesis and ribosome binding in wild-type plants, whereas the presence of the *npt*II gene allows transgenic plants to survive (Davey et al., 2010). However, natural resistance characteristics can also be used for selection. High antibiotic concentrations cause etiolation and death in non-transformant plants while effectively eliminating escape mutants and increasing selection pressure on transformed plants (Chen et al., 2020). Kanamycin concentrations of 50 ppm have been successfully used for selecting transgenic *Cosmos sulphureus* Cav. (Irsyadi et al., 2022; Purwantoro et al., 2023), *G. max* (Isda, 2012), and *Saccharum officinarum* (Fibriani et al., 2019). Hada et al. (2018) also reported that 35 ppm kanamycin was effective for shoot selection in half-seed soybean transformation.

Low concentrations of antibiotic agents during the screening stage led to an increased number of escapees from non-transformed plants. This is because different plant species exhibit varying levels of tolerance to kanamycin at different concentrations (Dalton et al., 1995). Chen et al. (2020) reported that *A. thaliana* seedlings tolerated kanamycin concentrations up to 1,400 ppm, while *Lycopersicum esculentum* was tolerant at levels below 100 ppm (Subaila and Saleh, 2010). In contrast, 100 ppm kanamycin successfully screened 70% of putative transformed plants in transgenic *M. uniflorum* Lam. Verdc (Amal et al., 2020). Warm water pre-treatment by soaking seeds accelerates seed coat loosening, tissue softening, and imbibition (Amusa, 2011; Pérez-García and González-Benito, 2005). Imbibition refers to the water absorption process in seeds, which follows a triphasic pattern. In the initial stage, seeds rapidly absorb water due to low water potential (Alvarado et al., 2015; Aydın and Uzun, 2001; Qadir et al., 2012). The second stage is characterized by reduced water absorption as the embryo prepares for germination. The final stage sees a rapid increase in water uptake, resulting in seed coat rupture and radicle emergence (Doria et al., 2019; Hada et al., 2018; Siddique and Kumar, 2018). Additionally, soaking seeds removes dirt and chemicals from the seed coat (Mwase and Mvula, 2011) and accelerates seed dormancy breakdown while enhancing seed permeability. Shine et al. (2011) reported that soaking soybean seeds in water for 40 minutes resulted in a 33% increase in water uptake. An impermeable seed coat is a major challenge in legume germination, contributing to dormancy and hardness (Tadros et al., 2011). Hot water treatment has been widely used in legumes to reduce seed coat impermeability (Muhammad and Amusa, 2003). In legumes, water permeability is influenced by weak tissue structures in the strophiole, which facilitate water absorption. Furthermore, water transport carries phenolic compounds from the embryo to the aleurone layer of the endosperm, enhancing seed metabolism and germination (Amri, 2010). These conditions improve the effectiveness of *Agrobacterium* infection and facilitate foreign DNA transfer into the plant genome. Moreover, soaking seeds increases their water content and size compared to non-soaked seeds (Finch-Savage et al., 2004). Heat treatment has been shown to improve genome editing efficiency and increase indel rates in *A. thaliana* and *N. tabacum* (Blomme et al., 2022) as well as *C. sinensis* (LeBlanc et al., 2019). Additionally, heat priming enhances the induction of intergenerational high-temperature stress tolerance in *T. aestivum* (Wang et al., 2016).

In this study, we successfully increased *in-planta* genetic transformation efficiency in soybean to 21% using heat pre-treatment. Previous *in-planta* transformation studies in soybeans have reported lower efficiencies, such as 3.8% (Paz et al., 2006) and 16% (Yang et al., 2019) using half-seed methods. Other transformation approaches, such as chlorine gas treatment, achieved efficiencies of only 10% (Li et al., 2017), 6.71% (Jia et al., 2015), and 4.6–6.1% in *M. uniflorum* Lam. Verdc (Amal et al., 2020). Furthermore, heat treatment at 37 °C has been reported to enhance transformation efficiency in *T. aestivum* (Milner et al., 2020), while temperatures ranging from 28 to 37 °C have been found effective for *A. thaliana* (Malzahn et al., 2019).

Conclusions

Soaking soybean seeds at 35 °C for 30 minutes prior to infection, followed by *Agrobacterium* infection for 30 minutes, resulted in a transformation efficiency of 30%. Additionally, the warm water soaking pre-treatment method significantly enhanced transformation efficiency compared to direct infection, achieving an average efficiency of 21±9% based on the T-test. The successful insertion of the 35S-CaMV and *npt*II genes into the plant genome was confirmed through PCR analysis, which detected DNA bands of 235 bp and 550 bp, respectively. This research provides a simple and efficient approach for genetic transformation using seeds as target organs.

Acknowledgments

We would like to thank Professor Bambang Sugiharto from Universitas Jember and Dr. Widhi Dyah Sawitri from Universitas Gadjah Mada, Indonesia, for providing the *A. tumefaciens* strain and recombinant vector. Our sincere gratitude goes to the Study Program of Agrotechnology, Faculty of Agriculture, Universitas Muhammadiyah Yogyakarta, for providing the research facilities. We also extend our thanks to Rizqi Fauzi and Raninda D. Khairunnisa for their assistance throughout the research stages.

Author Contributions

Conceptualization, MBI and EO; methodology, MBI, EO, and SKS; validation, EH, IAR, SNA, and MBI; formal analysis, MBI, EO; resources, IAR, EH, and SNA; data curation, MBI, and SKS; writing—original draft preparation, MBI, and SKS; writing—review and editing, MBI, SKS, S and SNA; visualization, MBI and S; supervision, IAR. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors indicate no conflict of interest in this work.

References

Alvarado ST, Buisson E, Rabarison H, Rajeriarison C, Birkinshaw C, Ii PPL. 2015. Effects of heat on the germination of sclerophyllous forest species in the highlands of Madagascar. Austral Ecology 40, 601–610. https://doi.org/10.1111/aec.12227.

Amal TC, Karthika P, Dhandapani G, Selvakumar S, Vasanth K. 2020. A simple and efficient Agrobacterium-mediated in planta transformation protocol for horse gram (*Macrotyloma uniflorum* Lam. Verdc.). Journal of Genetic Engineering and Biotechnology 18(9), 1–9. https://doi.org/10.1186/s43141-020-00023-z.

Amri E. 2010. The effects of pre-sowing seed treatments on germination of snake bean (*Swartzia madagascariensis*), a reported medicinal plant. Research Journal of Agricultural and Biological Sciences 6(255), 557–561.

Amusa TO. 2011. Effects of three pre-treatment techniques on dormancy and germination of seeds of *Afzelia africana* (Sm. Ex Pers.). Journal of Horticulture and Forestry 3(4), 96–103.

Aydın I, Uzun F. 2001. The effects of some applications on germination rate of Gelemen Clover seeds gathered from natural vegetation in Samsun. Pakistan Journal of Biological Sciences 4, 181–183.

Bahramnejad B, Naji M, Bose R, Jha S. 2019. A critical review on the use of *Agrobacterium rhizogenes* and their associated binary vectors for plant transformation. Biotechnology Advances 37(7), 107405. https://doi.org/10.1016/j.biotechadv.2019.06.004.

Bezerra ARG, Resende MDV, de-Silva FL, da-Sediyama T, Cruz CD, Silva FC, dos-S., Silva AFda. 2023. Combined BLUP selection indexes with parents and F2 populations in soybean (*Glycine max*) breeding. Crop Breeding and Applied Biotechnology 23(4), 1–10. https://doi.org/10.1590/198470332023v23n4a41.

Blomme J, Develtere W, Köse A, Arraiza-Ribera J, Brugmans C, Jaraba-Wallace J, Decaestecker W, Rombaut D, Baekelandt A, Daniel Fernández Fernández Á, Van Breusegem F, Inzé D, Jacobs T. 2022. The heat is on: a simple method to increase genome editing efficiency in plants. BMC Plant Biology 22(1), 1–14. https://doi.org/10.1186/s12870-022-03519-7.

Carneiro AK, Bruzi AT, Pereira JL de AR, Zambiazzi EV. 2019. Stability analysis of pure lines and a multiline of soybean in different locations.

Crop Breeding and Applied Biotechnology 19(4), 395–401. https://doi.org/10.1590/198470332019v19n4a56.

Chen C, Fu X, Peng R, Tian Y, Yao Q. 2020. Detoxifying processes during kanamycin-induced stress to *Arabidopsis thaliana* seedling growth. Biotechnology & Biotechnological Equipment 34(1), 673–679. https://doi.org/10.1080/13102818.2020.1798811.

Chen L, Cai Y, Liu X, Yao W, Guo C, Sun S, Wu C, Jiang B, Han T, Hou W. 2018. Improvement of soybean *Agrobacterium*-mediated transformation efficiency by adding glutamine and asparagine into the culture media. International Journal of Molecular Sciences 19(10), 1–17. https://doi.org/10.3390/ijms19103039.

Chetty VJ, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas ML. 2013. Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom. Plant Cell Reports 32(2), 239–247. https://doi.org/10.1007/s00299-012-1358-1.

Dalton SJ, Bettany AJE, Timms E, Morris P. 1995. The effect of selection pressure on transformation frequency and copy number in transgenic plants of tall fescue (*Festuca arundinacea* Schreb.). Plant Science 108(1), 63–70. https://doi.org/10.1016/0168-9452(95)04127-G.

Davey MR, Sonej JR, Rao MN, Kourmpetti S, Bhattacharya A. 2010. Generation of development of transgenic crop plants: an overview. Transgenic Crop Plants 1(1), 1–30.

Doria E, Pagano A, Ferreri C, Larocca AV, Macovei A, Araújo S de S, Alma B. 2019. How does the seed pre-germinative metabolism fight against imbibition damage? Emerging roles of fatty acid cohort and antioxidant defense. Frontiers in Plant Science 10(11), 1–13. https://doi.org/10.3389/fpls.2019.01505.

Fibriani S, Agustien I, Sawitri WD, Sugiharto B. 2019. Genetic Transformation and Expression of Sucrose Phosphate Synthase Mutant in Tomato Plant. Journal of Biotechnology and Biosciences of Indonesia 6(1), 134–143.

Finch-Savage, WE, Dent KC, Clark LJ. 2004. Soak conditions and temperature following sowing influence the response of maize (*Zea mays* L.) seeds to on-farm priming (pre-sowing seed soak). Field Crops Research 90(2–3), 361–374. https://doi.org/10.1016/j.fcr.2004.04.006.

Fredrick C, Muthuri C, Ngamau K, Sinclair F. 2017. Provenance and pretreatment effect on seed germination of six provenances of *Faidherbia albida* (Delile) A. Chev. Agroforestry Systems 91(6), 1007–1017. https://doi.org/10.1007/s10457-016-9974-3.

Gallino, JP, Castillo A, Ceretta S, Esteves P, Bonnecarrere V. 2022. A simple and inexpensive procedure to more quickly obtain new varieties in soybean. CROP AGRO, Journal of Agricultural Science 22(1), 1–7. https://doi.org/10.1590/198470332022v22n1a06.

Hada A, Krishnan V, Mohamed-Jaabir MS, Kumari A, Jolly M, Praveen S, Sachdev A. 2018. Improved *Agrobacterium tumefaciens*-mediated transformation of soybean [*Glycine max* (L.) Merr.] following optimization of culture conditions and mechanical techniques. In Vitro Cellular and Developmental Biology - Plant 54(6), 672–688. https://doi.org/10.1007/s11627-018-9944-8.

Handayani E, Irsyadi MB, Alawiyah RLMN, Aris I. 2022. Effect of explants sterilization and plant growth regulators on embryo culture of kepel (*Stelechocarpus burahol*). IOP Conference Series: Earth and Environmental Science 985(012016). https://doi.org/10.1088/1755-1315/985/1/012016.

Irsyadi MB, Sari SK, Oktiastuti E, Rineksane IA. 2024. Rapid genomic DNA extraction for Soybean (*Glycine max* L. Merr) using modified CTAB protocol to obtain high-quality DNA. Indian Journal of Biochemistry and Biophysics 61(2), 97–104. https://doi.org/10.56042/ijbb.v61i2.7603.

Irsyadi MB, Sawitri WD, Purwantoro A. 2022. Molecular and phenotypic characteristics of T1 transgenic yellow cosmos (*Cosmos sulphureus*) carrying neomycin phosphotransferase II. Biodiversitas 23(12), 6097–6105. https://doi.org/10.13057/biodiv/d231202.

Isda MN. 2012. Optimization of concentration kanamycin in the soybean explants (*Glycine max* L.) to transformation TcPIN gene. Semirata PTN Barat.

Jan SA, Shinwari ZK, Shah SH, Shahzad A, Zia MA, Ahmad N. 2016. In-planta transformation: Recent advances. Romanian Biotechnological Letters 21(1), 11085–11091.

Jia Y, Yao X, Zhao M, Zhao Q, Du Y, Yu C, Xie F. 2015. Comparison of soybean transformation efficiency and plant factors affecting transformation during the agrobacterium infection process. International Journal of Molecular Sciences 16(8), 18522–18543. https://doi.org/10.3390/ijms160818522.

Karthik S, Pavan G, Sathish S, Siva R, Kumar PS, Manickavasagam M. 2018. Genotype-independent and enhanced in planta *Agrobacterium tumefaciens*-mediated genetic transformation of peanut [Arachis hypogaea (L)]. 3 Biotech, 8, 202.

https://doi.org/10.1007/s13205-018-1231-1.

Kumari S, Krishnan V, Dahuja A, Vinutha T, Jolly M, Sachdev A. 2016. A rapid method for optimization of Agrobacterium-mediated transformation of Indian soybean genotypes. Indian Journal of Biochemistry and Biophysics, 53(6), 218–226.

LeBlanc C, Zhang F, Mendez J, Lozano Y, Chatpar K, Irish VF, Jacob Y. 2019. Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. The Plant Journal, 93(2), 377–386. https://doi.org/10.1111/tpj.13782.

Li SX, Cong YH, Liu YP, Wang TT, Shuai Q, Chen NN, Gai JY. 2017. Optimization of Agrobacterium-mediated transformation in soybean. Frontiers in Plant Science, 8(246). https://doi.org/10.3389/fpls.2017.00246.

Malzahn AA, Tang X, Lee K, Ren Q, Sretenovic S, Zhang Y, Chen H, Kang M, Bao Y, Zheng X, Deng K, Zhang T, Salcedo V, Wang K, Zhang Y, Qi Y. 2019. Application of CRISPR-Cas12a temperature sensitivity for improved genome editing in rice, maize, and Arabidopsis. BMC Biology 17(9), 1–14. https://doi.org/10.1186/s12915-019-0629-5.

Mangena P. 2019. A simplified in-planta genetic transformation in soybean. Research Journal of Biotechnology 14(9), 117–125.

Mayavan S, Subramanyam K, Jaganath B, Sathish D, Manickavasagam M, Ganapathi A. 2015. Agrobacterium-mediated in planta genetic transformation of sugarcane setts. Plant Cell Reports 34(10), 1835–1848. https://doi.org/10.1007/s00299-015-1831-8.

Milner MJ, Craze M, Hope MS, Wallington EJ. 2020. Turning Up the Temperature on CRISPR: Increased Temperature Can Improve the Editing Efficiency of Wheat Using CRISPR/Cas9. Frontiers in Plant Science 11, 583374. https://doi.org/10.3389/fpls.2020.583374.

Muhammad S, Amusa NA. 2003. The effects of sulphuric acid and hot water treatments on seed germination of tamarind (*Tamarindus indica* L.). African Journal of Biotechnology 2, 267–279.

Mwase WF, Mvula T. 2011. Effect of seed size and pre-treatment methods of *Bauhinia thonningii* Schum. on germination and seedling growth. African Journal of Biotechnology 10(13), 5143–5148. https://doi.org/10.5897/AJB10.2135.

Paz MM, Martinez JC, Kalvig AB, Fonger TM, Wang K. 2006. Improved cotyledonary node method using an alternative explant derived from mature seed for efficient Agrobacterium-mediated soybean transformation. Plant Cell Reports 25(3), 206–213. https://doi.org/10.1007/s00299-005-0048-7.

Pérez-García F, González-Benito ME. 2005. Effects of temperature and different pre-treatments on seed germination of four Halimium species. Seed Science and Technology 33(2), 505–509. https://doi.org/10.15258/sst.2005.33.2.24.

Purwantoro A, Irsyadi MB, Sawitri WD, Fatumi NC, Fajriana, SN. 2023. Efficient floral dip transformation method using A*grobacterium tumefaciens* on *Cosmos sulphureus* Cav. Saudi Journal of Biological Sciences 30(7), 103702. https://doi.org/10.1016/j.sjbs.2023.103702.

Qadir M, Khan F, Khan SS. 2012. Effect of pre-treatment on the germination of *Abrus precatorius* Linn. by soaking in water. Indian Journal of Applied and Pure Biology 27(1), 105–108.

Shine MB, Guruprasad KN, Anand A. 2011. Enhancement of germination, growth, and photosynthesis in soybean by pre-treatment of seeds with magnetic field. Bioelectromagnetics 32, 474–484. https://doi.org/10.1002/bem.20656.

Siddique A, Kumar P. 2018. Physiological and biochemical basis of pre-sowing soaking seed treatment-an overview. Plant Archives 18(2), 1933–1937.

Souza RAV, Alves MDC, Carneiro NP, Barros BDA, Borem A, Carneiro AA. 2017. Agrobacterium -mediated genetic transformation of a tropical elite maize line. Crop Breeding and Applied Biotechnology 17, 133–140. https://doi.org/10.1590/1984- 70332017v17n2a2.

Subaila S, Saleh. 2010. Inhibitory effect of kanamycin on *in vitro* culture of *Lycopersicon esculantum* Mill cv. Mt11. Journal of Agrobiotechnology 1, 79–86.

Suputri NPAEO, Dwiyani R, Darmawanti IAP, Sugiharto B. 2019. *Agrobacterium tumefaciens*-mediated in planta transformation method for the sosps1 gene in citrus plants (*Citrus nobilis* L.). International Journal Of Biosciences And Biotechnology 7(1), 31–44. https://doi.org/10.24843/IJBB.2019.v07.i01.p04.

Tadros MJ, Samarah NH, Alqudah AM. 2011. Effect of different pre-sowing seed treatments on the germination of *Leucaena leucocephala* (Lam.) and *Acacia farnesiana* (L.). New Forests 42(3), 397–407. https://doi.org/10.1007/s11056-011-9260-1.

Tarafdar A, Vishwakarma H, Gothandapani S, Bhati M, Biswas K, Prakash A. 2019. A quick, easy and cost effective in planta method to develop direct transformants in wheat. 3 Biotechnology 9, 180. https://doi.org/10.1007/s13205-019-1708-6.

Wang X, Xin C, Cai J, Zhou Q, Dai T, Cao W, Jiang

D. 2016. Heat priming induces trans-generational tolerance to high temperature stress in wheat. Frontiers in Plant Science 7(4), 1–12. https://doi.org/10.3389/fpls.2016.00501.

Yadav S, Sharma P, Trivastava A, Desai P, Shrivastava N. 2014. Strain specific Agrobacterium-mediated genetic transformation of *Bacopa monnieri*. Journal of Genetic Engineering and Biotechnology 12, 89–94. https://doi.org/10.1016/j.jgeb.2014.11.003.

Yang S, Hu Y, Cheng Z, Rice JH, Miao L, Ma J, Hewezi T, Li Y, Gai J. 2019. An efficient Agrobacterium-mediated soybean transformation method using green fluorescent protein as a selectable marker. Plant Signaling and Behavior 14(7), 1–7. https://doi.org/10.1080/15592324.2019.1612682.

Zhang YM, Liu ZH, Yang RJ, Li GL, Guo XL, Zhang HN, Zhang HM, Di R, Zhao QS, Zhang MC. 2016. Improvement of soybean transformation via *Agrobacterium tumefaciens* methods involving alpha-aminooxyacetic acid and sonication treatments enlightened by gene expression profile analysis. Plant Cell Reproduction 35, 1259–1271. https://doi.org/10.1007/s00299-016-1958-2.