Efficiency of *Agrobacterium tumefaciens*-mediated transformation of tobacco (*Nicotiana tabacum* L.) with rice *OsNAC6* gene

A Rachmat*, Chairunisa and B S Maulana

Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Jalan Raya Bogor KM 46, Cibinong, West Java, Indonesia

*Email: guess_btk@yahoo.com

**Abstract.** Crop improvement by genetic engineering requires efficient plant regeneration derived from cultured cells or tissues. The success of cell or tissue culture regeneration depends on reliable explant source. In this study, three different types of explant (callus, stem, and leaf) of tobacco (*Nicotiana tabacum* L.) were evaluated to determine the most effective explant source for *Osna6* gene transformation. *OsNAC6* encodes a transcription factor family in rice (*Oryza sativa* L.). OsNAC6 transcription factor has been reported in some studies to confer tolerance to drought stress. *Agrobacterium tumefaciens* strain EHA 105 harboring pC1305 plasmid containing the *OsNAC6* gene driven by CaMV 35S promoter (pARNAC6) was used for transformation. The T-DNA contains hygromycin phosphotransferase (*hpt*) gene as a selectable marker. PCR confirmed the presence of the *hpt* gene in the T0 generation of the transformed tobacco plants. The PCR result showed that a total of 50 tobacco plants were positive for *hpt* gene which 18, 20, and 12 of them derived from callus, stem, and leaf explants respectively. The highest transformation efficiency was obtained by using the stem as explant material, i.e. 17%. Meanwhile, the obtained transformation efficiency by using callus and leaf was 15% and 10% respectively.

1. **Introduction**

Tobacco (*Nicotiana tabacum* L.) is grown worldwide in about 120 countries and it is an important industrial non-food crop [1]. In Indonesia, tobacco is cultivated in 15 provinces with a total production of around 200,000 tons in 2019 [2]. The leaf of tobacco is the main component in the cigarette industry supporting the life of Indonesian tobacco farmers for a long time. Other than the cigarette industry, tobacco is highly potential to be used for medicinal purposes such as the production of recombinant plant-based drugs and vaccines [3-6]. Indonesia has a big chance to become one of the world’s leading tobacco-producing countries. The abundance of natural resources and the diversity of tobacco germplasms are two crucial assets for developing tobacco commodity in Indonesia. However, there are still many environmental challenges that can reduce the quality and the yield of Indonesian tobacco.

Drought is among abiotic stresses which have the most devastating effects to plant growth and development. Unpredictable rainfall and gradual raising in the earth’s temperature are predicted to increase the severity of drought events. Drought is always challenging for any agricultural practice, including tobacco. Although tobacco is relatively drought-tolerant, a decrease in tobacco leaf quality and yield due to water stress has been reported in some studies [7-9]. Therefore, it is important to...
develop drought-tolerant tobacco plants to maintain or improve their quality and yield. This can be done, for example, by conventional crossing followed by screening or by marker-assisted breeding. Another approach is genetic engineering by Agrobacterium-mediated transformation which enables direct insert of a specific target gene to alter and improve the plant trait.

Agrobacterium tumefaciens was firstly reported as the cause of crown gall disease [10]. Along with the advancement in plant biotechnology, scientists invented a genetic transfer method utilizing the bacteria. For decades, Agrobacterium-mediated transformation is a common method to modify or improve plant genetic because of several benefits, mainly its stable genetic modification and lower copy number [11]. Agrobacterium-mediated transformation in tobacco plant has been widely practiced not only to study gene functions or biosynthesis of recombinant drugs but also to improve the plant genetic traits [12,13]. We employed Agrobacterium-mediated transformation to deliver OsNAC6 gene into the tobacco genome. NAC (NAM, ATAF1/2, and CUC2) is one of the largest family of plant-specific transcription factors [14,15]. NAC proteins have important roles in plant development [14,16,17] and the expression of NAC genes is induced by various abiotic and biotic stresses such as drought, high salinity, and wounding [18-21]. In rice, 151 NAC genes have been identified [22], including OsNAC6- which is used in this study. OsNAC6 gene has been reported to confer tolerance to drought and salinity in various plants [15,21,23-26]. This study employed Agrobacterium-mediated transformation to introduce OsNAC6 gene into the tobacco genome and investigated the transformation efficiency among three different types of explants (callus, stem, leaf disc).

2. Material and methods

2.1. Gene construct
The pARNAC6 binary vector was constructed by inserting a full coding sequence of OsNAC6 gene (912bp) into pCAMBIA 1305 plasmid (CAMBIA-Australia). The T-DNA of the vector contains hygromycin phosphotransferase (hpt) gene under the control of CaMV 35S constitutive promoter [26] as shown in figure 1.

![Figure 1. Schematic illustration of the T-DNA structure of pARNAC6 binary vector. 35S: 35S CaMV constitutive promoter, OsNAC6: OsNAC6 gene sequence, hpt: hygromycin-phosphotransferase gene sequence, nos: nopaline synthase terminator, LB: left border of the T-DNA structure, RB: right border of the T-DNA structure.](image)

2.2. Explant sterilization and preparation
Three different type of tobacco explants were used i.e. callus, stem, and leaf disc, 120 explants for each type. Surface sterilization of the explants was performed in two steps as per Hendaryono and Wijayani [27]. The first step was done in the preparation room where tobacco leaf and stem taken from greenhouse were washed under running tap water for a few minutes. The second step was done in laminar air flow under sterile condition. The tobacco leaf and stem were immersed in 70% ethanol for 30 seconds and rinsed with sterile double-distilled water (ddH2O). The leaf and stem were subsequently soaked in 1% sodium hypochlorite for 10 minutes and then rinsed thoroughly with sterile ddH2O, three or four times. The explant materials were then air-dried on sterile filter papers. Sterile stems or leaf discs of tobacco were grown on regeneration medium to regenerate calli explant.
2.3. Tobacco transformation and plantlet regeneration
Callus induction and transformation protocol by Hiei and Komari [28] which is the standard routine protocol in our laboratory, is also applicable for tobacco and was used in this study to perform tobacco transformation with OsNac6 gene. Agrobacterium tumefaciens strain EHA 105 harboring pARNAC6 binary vector was used to deliver the gene of interest into the tobacco genome. Tobacco callus, stem, and leaf were co-cultivated with Agrobacterium harboring the pARNAC6 binary vector. Viable plantlets were then selected. Greenish plantlets with reasonable size were transferred onto regeneration medium (MS medium with 1 mg/L NAA, 3 mg/L BA, 4 g/L agarose type-1) containing 50 mg/L hygromycin. Survived plants were subsequently transferred into acclimatization soil in pots and grown for seed set.

2.4. Selection of transgenic plants and gene integration analysis by PCR
PCR analysis targeting hpt gene was done to indirectly confirm the presence of OsNAC6 gene in the first generation (T₀) of the putative transgenic plants. Genomic DNA was extracted from leaf of control and transgenic plants using CTAB (hexadecyl trimethyl ammonium bromide) method by Sambrook et al. [29]. A pair of gene-specific primers were used to amplify the hpt gene. The sequence of forward primer was 5’-GATGCCCTCCGCTCGAAGTAGCG-3’ and 5’-GCATCTCCCGCGTGCCAC-3’ for reverse primer. DNA amplification was done using thermal cycler (Biometra) with the following temperature setting; initial denaturation (95°C, 3 minutes), [denaturation (95°C, 1 minute), annealing (65°C, 1 minute), extension (72°C, 1 minute)] × 35 cycles, and final extension (95°C, 3 minutes). PCR products were subsequently analyzed by electrophoresis on 1% agarose gel and visualized under UV light.

3. Results and discussion
Agrobacterium tumefaciens-mediated transformation has several advantages over direct gene transfer methods such as particle bombardament or electroporation. This method requires minimal equipment, results in lower copy number; higher stability of transgene integration, less somaclonal variation, and enables the introduction of large DNA fragments [30,31]. Although some plants are recalcitrant to A. tumefaciens-mediated transformation, many important crops and other economic plants have been genetically modified and enhanced by using this method [30,32,33].

3.1 Plantlet regeneration from different explant sources
Tobacco callus, stem, and leaf disc (120 each) were co-cultivated with Agrobacterium harboring pARNAC6 binary vector by immersing the explants with Agrobacterium culture solution followed by incubation. About 40% of the transformed explants grew on selection medium, showing resistance to 50 mg/L hygromycin. The number of regenerated plantlets was 29, 37, and 16 each derived from callus, stem, and leaf disc respectively (figure 2). These plantlets were subsequently tested by PCR to confirm the insertion of the transgene.

3.2 Selection of transgenic plants and gene integration analysis by PCR
A total of 82 in vitro plantlets (T₀) were transferred to acclimatization soil in plastic pots. PCR analysis was conducted on all the plants to confirm the presence of OsNAC6 gene using hpt-specific primers with a product size of 492 bp. Since the hpt and the OsNAC6 genes were both at the T-DNA region of the vector and were delivered together in one cassette, positive result for hpt indicates the insertion of OsNAC6 gene into the plant genome. The PCR results showed that 50 out of 82 plants were positive for hpt gene by the presence of amplicon nearly aligned with 500 bp fragment size of the DNA marker (figure 3). The number of hpt-positive plants was 18, 20, 12 each derived from the callus, stem, and leaf disc respectively (table 1).
Figure 2. The process of *Agrobacterium*-mediated transformation using three different explant materials. A: Tobacco calli (regenerated from tobacco stem or leaf), B: Tobacco stem, C: Tobacco leaf disc, D: Tobacco plantlets regenerated after being co-cultivated, E: tobacco plantlets grew on regeneration medium containing 50 mg/L hygromycin.

Figure 3. The result of PCR analysis using a pair of *hpt*-specific primers on putative tobacco transgenic plants. M: 100 bp marker, 1: *hpt* positive control, 2: negative plant control, 3: negative PCR reaction control, 4-28: plant samples.

Transformation efficiency was calculated as the number of *hpt*-positive plants divided by the number of initial explants used in the transformation. The highest transformation efficiency was 17% obtained by using the stem as explant followed by callus (15%), and leaf disc (10%) as the lowest (table 1).

Table 1. *Agrobacterium*-mediated transformation efficiency in tobacco using three different types of explants.

| Explant   | Σ Transformed calli | Σ Hygromycin resistant calli | Σ Regenerated calli | Σ + hpt plants | Regeneration efficiency (%) | Transformation efficiency (%) |
|-----------|---------------------|------------------------------|---------------------|----------------|-----------------------------|-------------------------------|
| Callus    | 120                 | 40                           | 29                  | 18             | 24                          | 15                            |
| Stem      | 120                 | 70                           | 37                  | 20             | 31                          | 17                            |
| Leaf disc | 120                 | 40                           | 16                  | 12             | 13                          | 10                            |
The choice of explant affects transformation efficiency [34]. Excised leaf disc is commonly used as explant in tobacco transformation [34-40]. Although the use of callus could result in undesirable somaclonal variations [39], we also included tobacco callus in addition to leaf disc and tobacco stem to determine the best explant material which gives the highest transformation efficiency.

Transformation efficiency (in percent) is the ratio of the number of \( hpt \)-positive plants with the number of initial inoculated explants. We found that the highest transformation efficiency was 17% by using stem as explant, followed by 15% by using callus, and the lowest was 10% by using leaf disc. These values were significantly lower compared to similar tobacco transformation studies, for example by Pathi et al. [39] and Mubeen et al. [41], which transformation efficiency was around 80%-90%.

Pathi et al. developed an optimized transformation method for \( Nicotiana \) spp by using leaf disc as explant with transformation efficiency of more than 95% [39]. In contrast to our experiment, the optimized method used \( Agrobacterium \) \( tumefaciens \) strain LBA4404 harboring pCAMBIA1301 vector and a different combination of nutrients and hormones for the regeneration medium. Mubeen et al. reported an average transformation efficiency of 79.5% using \( Agrobacterium \) strain LBA4404 harboring pGA482 vector to transform callus regenerated from tobacco seed [41].

However, there are also some reports with low transformation efficiency. Bakhsh et al. [11] evaluated the transformation efficiency of tobacco leaf (\( Nicotiana tabacum \) cv. Samsun) using five different \( Agrobacterium \) strains. The highest transformation efficiency was only 20% obtained by using \( Agrobacterium \) strain LBA4404 [11]. A wide range of transformation efficiency was reported by Duan et al. which was between 2% to 75% by using \( A. \) \( tumefaciens \) strain EHA105 to transform three different wild tobacco genotypes [42]. The last report clearly demonstrates the effect of plant genotype on transformation efficiency. In this study, the low transformation efficiency may be because a transformation protocol for rice was used instead of for tobacco. The results may be different, and the transformation efficiency may be higher if a specific transformation protocol for tobacco was used.

\( Agrobacterium \)-mediated transformation is a complex process, and multiple biological and physical factors affect transformation efficiency. Other than the strain of the \( Agrobacterium \)/the vector, the genotype of the host plant, and the type of explant, transformation efficiency is also affected by the co-cultivation conditions, the regeneration and selection method, the mode of plant regeneration, the inducers of \( vir \) genes, and bacterial density and its growth phase [13,39,43]. In our study, a major improvement could be done by choosing a suitable transformation protocol for tobacco instead of for rice. Nevertheless, the \( hpt \)-positive transgenic plants obtained from this study are enough as early material for further development of drought-tolerant tobacco plant.

4. Conclusion

\( Agrobacterium \)-mediated transformation of tobacco with OsNAC6 gene has been successfully performed. A total of 50 \( hpt \)-positive tobacco plants were obtained. The highest transformation efficiency was 17% obtained by using the stem as explant, followed by 15% and 10% by using callus and leaf respectively. Transformation efficiency could be improved by choosing a suitable transformation protocol for tobacco.

Acknowledgement

Authors would like to thank Dr. Satya Nugroho for access to the facilities at the Laboratory of Genetics and Crop Improvement, LIPI.

References

[1] Kari B 2019 Tobacco Research 43 56
[2] Direktorat Jenderal Perkebunan Indonesia 2020
[3] Tregoning J S, Nixon P, Kuroda H, Svab Z, Clare S, Bowe F, Fairweather N, Ytterberg J, Wijk K Jv, Dougan G and Maliga P 2003 Nucleic Acids Res. 31 1174
[4] Molina A, Hervás-Stubbs S, Daniell H, Mingo-Castel A M and Veramendi J 2004 Plant Biotechnol. J. 2 141
[5] Fernández-San Millán A, Ortizosa S M, Hervás-Stubbs S, Corral-Martínez P, Seguí-Simarro J M, Gaëtan J, Coursaget P and Veramendi J 2008 *Plant Biotechnol. J.* 6 427
[6] Gottschamel J, Lössl A, Ruf S, Wang Y, Skaugen M, Bock R and Clarke J L 2016 *Plant Mol. Biol.* 91 497
[7] Biglouei M H, Assimi M H and Akbarzadeh A 2010 *Plant, Soil, and Environment* 56 67
[8] Çakir R and Cebi U 2010 Irrigation and Drainage 59 453
[9] Celik O and Atak C 2012 *Turkish Journal of Biology* 36 339
[10] Smith E F and Towsend C O 1907 *Science* 25 671
[11] Bakhsh A, Anayol E and Ozcan S 2017 *Emirates Journal of Food and Agriculture* 26 259
[12] Japelaghi R H, Haddad R, Valizadeh M, Uliaie E D and Javaran M J 2018 *Journal of Plant Molecular Breeding* 6 38

Kumar N, Gopalakrishnan V A, Reddy M, Singh A and Narayanan S 2009 *Journal of Forest and Environmental Science* (USA) 25 195
[14] Jensen M K, Hagedorn P H, De Torres-Zabala M, Grant M R, Rung J H, Collinge D B and Lyngkjaer M F 2008 *Plant Journal* 56 867-880
[15] Tang G, Shao F, Xu P, Shan L and Liu Z 2017 *Russian Journal of Plant Physiology* 64 525
[16] Kim Y S, Kim S G, Park J E, Park H Y, Lim M H, Chua N H and Park C M 2006 *The Plant Cell* 18 3132
[17] Mitsuda N and Ohme-Takagi M 2008 *Plant J.* 56 768
[18] Collinge M and Boller T 2001 *Plant Mol. Biol.* 46 521

[19] Hegedus D, Yu M, Baldwin D, Gruber M, Sharpe A, Parkin I, Whitwill S and Lydiate D 2003 *Plant Mol. Biol.* 53 383
[20] Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran L S, Yamaguchi-Shinozaki K and Shinozaki K 2004 *Plant J.* 39 863
[21] Nakashima K, Tran L S, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K and Yamaguchi-Shinozaki K 2007 *Plant J.* 51 617
[22] Nuruzzaman M, Manimekalai R, Sharoni A M, Satoh K, Kondoh H, Ooka H and Kikuchi S 2010 *Gene* 465 30

[23] Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q and Xiong L 2006 *Proc. of the Natl. Academy of Sciences* vol 103 (USA) 12987
[24] Liu Q L, Xu K, Zhao L J, Pan Y Z, Jiang B B, Zhang H Q and Liu G L 2011 *Biotechnol. lett.* 33 2073
[25] Liu X, Liu S, Wu J, Li B, Xian Y and Li L 2013 *Plant Physiol. Biochem.* 70 354
[26] Rachmat A, Nugroho S, Sukma D, Aswidinnoor H and Sudarsono S 2014 *Emirates Journal of Food and Agriculture* 26 519
[27] Hendaryono DPS and Wijayani A 1994 Teknik kultur jaringan pengenalan dan petunjuk perbanyakan tanaman secara vegetatif-modern (Yogyakarta: Kanisius)

[28] Hiei Y and Komari T 2006 *Plant Cell, Tiss. Org. Cult.* 85 271
[29] Sambrook J, Fritsh E F and Maniatis T 1989 *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press)
[30] Hau-Hsuan H, Manda Y and Erh-Min L 2017 *The Arabidopsis Book* 15 e0186
[31] Jefferson R A 1987 *Plant Molecular Biology Reporter* 5 387
[32] Murai N 2013 *American Journal of Plant Sciences* 4 932
[33] Lopez S J, Kumar R R, Pius P K and Muraleedharan N 2004 *Mol. Biol. Rep.* 22 201
[34] Rai G K, Rai N P, Kumar S, Yadav A, Rathaur S and Singh M 2012 *In Vitro Cell. Dev. Biol. - Plant* 48 565
[35] Kwon H B, Park S C, Peng H P, Goodman H M, Dewdney J and Shih M C 1994 *Plant physiol.* 105 357
[36] Dhaliwal H S, Yeung E C and Thorpe T A 2004 *In Vitro Cell. Dev. Biol. - Plant* 40 235
[37] Han S E, Park S R, Kwon H B, Yi B Y, Lee G B and Byun M O 2005 *Plant Cell, Tiss. Org. Cult.* 82 151
[38] Gubis J, Vaňková R, Červená V, Dragúňová M, Hudcovicova M, Lichtnerová H, Dokupil T and Jureková Z 2007 *S. Afr. J. Bot.* **73** 505

[39] Pathi K M, Tula S and Tuteja N 2013 *Plant Signaling & Behavior* **8** e24354

[40] Li W, Li X, Chao J, Zhang Z, Wang W and Guo Y 2018 *Frontiers in Plant Science* **9**

[41] Mubeen H, Bashir A, Ameen A, Masood A and Raza S 2017 *African Journal of Biotechnology* **16** 945

[42] Duan W, Wang L and Song G Q 2016 *American Journal of Plant Sciences* **7** 1

[43] Opabode J T 2006 *Biotechnology and Molecular Biology Reviews* **1** 12