Efficient Agrobacterium-mediated genetic transformation method using hypocotyl explants of radish (Raphanus sativus L.)

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Received September 1, 2021; accepted October 21, 2021 (Edited by Y. Hoshino)

Abstract To investigate the gene function of radish (Raphanus sativus L.), several attempts have been made to generate genetically transformed radish. However, no efficient and relatively simple method for the genetic transformation of radish has been developed to date. In this study, we established an Agrobacterium-mediated genetic transformation method using the hypocotyl-derived explants of radish cultivar “Pirabikku”. Primarily based on the Brassica transformation procedure, we optimized it for radish transformation. Using this system, the transformation efficiency of radish hypocotyl explants by Agrobacterium tumefaciens strain GV3101 harboring pIG121-Hm was 13.3%. The copy number of transfer DNA integrated into the genome was either one or two in the four independent transgenic plants. Two of the four plants exhibited male sterility and did not produce self-pollinated seeds. Examination of the expression of the β-glucuronidase (GUS) gene in T1 plants from fertile T0 plants showed that the GUS genes were inherited. The improvement in the genetic transformation in this study might pave the way for accelerated molecular breeding and genetic analysis of radish.

Key words: Agrobacterium strain GV3101, molecular breeding, pIG121-Hm vector, Raphanus sativus L., transformation efficiency.

Radish (Raphanus sativus L.) belongs to the family Brassicaceae and is cultivated worldwide. Radish is a popular vegetable in Europe and East Asia. Various radish varieties have been developed, mostly in China, Korea, and Japan, through a long breeding history (Yamagishi 2017). The whole-genome sequences of radish together with the genetic maps were published recently by several groups (Jeong et al. 2016; Kitashiba et al. 2014; Mitsui et al. 2015; Shirasawa et al. 2020; Xiaohui et al. 2015). Although these studies have accelerated the investigation of the correlation between the genes and traits, molecular breeding based on the revealed molecular function of these genes has not been developed because a genetic transformation system with high efficiency and reproducibility has not been constructed in Raphanus. In some previous publications on Raphanus transformation, many different methods such as the floral dip (Curtis and Nam 2001), ultrasonic and vacuum infiltration (Park et al. 2005), and Agrobacterium-mediated genetic transformation using the hypocotyl explants (Cho et al. 2008) have been reported. However, in these methods, the transformation efficiency is insufficient because the number of transgenic plants produced in one experiment is relatively small, which makes the molecular breeding of radish with practical traits unrealistic. In this study, we established a transformation system for radish, based primarily on the transformation of Brassica rapa “Osome” (Takasaki et al. 1997), which is estimated to be a close relative of the radish, but with modifications that can maximize the transformation efficiency.

For transformation using hypocotyl explants, shoot regeneration ability (Sparrow et al. 2004b) and susceptibility of the plant to Agrobacterium tumefaciens infection (Sparrow et al. 2004a) are essential prerequisites. We collected 16 commercially available fixed radish cultivars in Japan and compared their adventitious shoot regeneration frequencies. Radish seeds were sterilized with 99.5% ethanol for 20 min and then with 1%–5% sodium hypochlorite with a small amount of Tween 20 for 20 min. The seeds were then rinsed in sterile distilled water several times.
times until the rinsed water became clear. Excess water was removed and the sterilized seeds were sown in a germination medium (1/2 Murashige and Skoog [MS] medium containing 30 g l\(^{-1}\) sucrose and 4 g l\(^{-1}\) gellan gum (FUJIFILM Wako, Osaka, Japan) with pH 5.8). After 10–14 days of incubation at 25°C in the dark, the etiolated hypocotyls were cut into pieces of size 5–7 mm (hypocotyl explants). The hypocotyl explants were then cultured in a callus induction medium (B5 medium containing 1 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid, 200 mg l\(^{-1}\) carbenicillin, 30 g l\(^{-1}\) sucrose, and 4 g l\(^{-1}\) gellan gum with pH 5.8) at 25°C under long-day conditions (16 h light and 8 h dark) under a light intensity of 60 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) for 7 days. The explants were then transferred to a shoot formation medium (B5 medium containing 3 mg l\(^{-1}\) 6-benzylaminopurine, 1 mg l\(^{-1}\) trans-zeatin, 200 mg l\(^{-1}\) carbenicillin, 10 g l\(^{-1}\) sucrose, and 4 g l\(^{-1}\) gellan gum with pH 5.8) supplemented with 10 mg l\(^{-1}\) AgNO\(_3\), and cultured for 7 days. This was followed by culture in a shoot formation medium for 4 weeks without AgNO\(_3\).

Among the 16 cultivars, “Pirabikku” had the highest shoot regeneration frequency of 83.8% (Supplementary Tables S1, S2).

The next step was the examination of susceptibility of “Pirabikku” hypocotyl explants to Agrobacterium infection. The “Pirabikku” hypocotyl explants were suspended in a liquid infection medium (MS medium containing 200 mg l\(^{-1}\) KH\(_2\)PO\(_4\), 1 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D), 20 mg l\(^{-1}\) acetoxyringone, and 30 g l\(^{-1}\) sucrose with pH 5.2) for 3 h at 25°C (preculture), and then suspended in a liquid infection medium for 30 min with Agrobacterium strains EHA101 harboring pIG121-Hm (Ohta et al. 1990). The binary vector pIG121-Hm contained a GUS gene with an inserted intron as a reporter gene and a hygromycin phosphotransferase gene (hpt) and a neomycin phosphotransferase II gene (nptII) for antibiotic selection. The optical density at 600 nm (OD\(_{600}\)) of bacterial preculture was adjusted to 0.1 before infection. The Agrobacterium plasmid EHA101 was formerly used for the transformation of *B. napus* “Osome” (Takasaki et al. 1997). EHA101 were cultured overnight in YEP medium containing 50 mg l\(^{-1}\) kanamycin and 50 mg l\(^{-1}\) hygromycin at 28°C. The hypocotyl explants were co-cultivated with EHA101 on a solid medium (liquid infection medium supplemented with 4 g l\(^{-1}\) gellan gum) for 3 days in the dark and the \(\beta\)-glucuronidase (GUS) assay was performed. The explants infected by Agrobacterium were soaked in 5-Bromo-4-chloro-3-indolyl-\(\beta\)-D-glucuronic acid solution for 1 h under reduced pressure and then incubated overnight at 37°C (Jefferson et al. 1987). About 64% of the hypocotyl explants were stained blue because the GUS genes were introduced in “Pirabikku”, indicating the susceptibility of “Pirabikku” to EHA101 (Supplementary Figure S1). We did not verify the susceptibility of cultivars to Agrobacterium other than “Pirabikku”. Based on these data, we constructed a transformation method using the hypocotyl explants of “Pirabikku” that belongs to *Raphanus sativus* var. *sativus*.

The procedure of transformation was as follows: The hypocotyl explants infected with *Agrobacterium* harboring pIG121-Hm were transferred to a callus induction medium at 25°C for 7 days. The calluses were induced in all the hypocotyl explants of “Pirabikku”. The explants were then transferred to a shoot formation medium with AgNO\(_3\) and cultured for 7 days, followed by culture in a shoot formation medium without AgNO\(_3\) for 6 weeks (Figure 1A). The shoot formation medium for transformation was supplemented with 10 mg l\(^{-1}\) hygromycin or 50 mg l\(^{-1}\) kanamycin for selection. The explants were subcultured with fresh medium after every 2 weeks. We found that hygromycin (10 mg l\(^{-1}\)) is a more effective antibiotic for the selection of “Pirabikku” transformants than kanamycin. When kanamycin (50 mg l\(^{-1}\)) was used for selection, many GUS-negative kanamycin-resistant plants were obtained, which were inappropriate for the selection. The hygromycin-resistant shoots regenerated were transferred to a shoot maturation medium (B5 medium composed of 10 mg l\(^{-1}\) hygromycin, 200 mg l\(^{-1}\) carbenicillin, and 10 g l\(^{-1}\) sucrose, 4 g l\(^{-1}\) gellan gum with pH 5.8) for 6 weeks (Figure 1B). We compared the transformation efficiency of *Agrobacterium* strains EHA101 and GV3101 because a recent study reported that GV3101 was more efficient in transforming radish (Cho et al. 2008). Hypocotyl explants were infected with an *Agrobacterium* strain GV3101 harboring pIG121-Hm in the same way as EHA101.

To confirm transformation, we checked for the...
presence of the \textit{hpt} gene by polymerase chain reaction (PCR). The PCR primers were 5′-CTCTCGAGGAGCTGATGCTT-3′ and 5′-GATGTTGGGACCTCGTATT-3′, which were designed within the \textit{hpt} gene. Genomic DNA (gDNA) was extracted from the leaves of the candidate plants using an alkaline DNA extraction buffer (100 mM Tris–HCl [pH 9.5], 1 M KCl, and 10 mM EDTA-2Na).

The crude extracts were centrifuged at 20,000\(\times\)g and 1\(\mu\)l of each supernatant was used as a template for PCR. The PCR conditions were as follows: 94°C for 2 min; followed by 30 cycles of 30 s at 94°C; 30 s at 60°C; 30 s at 72°C; and 7 min at 72°C. PCR reactions were performed using the Gene RED PCR Mix Plus Kit (NIPPON GENE, Tokyo, Japan) as per the manufacturer’s instructions. The PCR experiment confirmed the presence of transfer DNA (T-DNA) in all hygromycin-resistant plants with several leaves and detected no escaped plant. Therefore, all hygromycin-resistant plants were transgenic. The result of the comparison between transformation efficiencies is shown in Table 1. The experiments were repeated three times, and in each experiment, the hypocotyl explants were divided in half, and each was used for different \textit{Agrobacterium} infections. The result indicated that in each experiment, the transformation efficiency (No. of transgenic shoots per hypocotyl explants) of GV3101 was higher than that of the EHA101, and the mean value (GV3101: 13.3% and EHA101: 6.2%) also supported the difference. Thus, we could improve the transformation efficiency from 0.26% that was previously described in the publication (Cho et al. 2008) to 13.3% using the “Pirabikku” hypocotyl as the explant source.

Four transgenic shoots transformed by \textit{Agrobacterium} strain GV3101 were transferred to shoot elongation medium (B5 medium containing 1 mg l\(^{-1}\) gibberellic acid, 10 mg l\(^{-1}\) hygromycin, 200 mg l\(^{-1}\) carbenicillin, 10 g l\(^{-1}\) sucrose, and 4 g l\(^{-1}\) gellan gum with pH 5.8)), and these plants were further grown in a root induction medium (B5 medium containing 2 mg l\(^{-1}\) indole-3-butyric acid, 10 mg l\(^{-1}\) hygromycin, 200 mg l\(^{-1}\) carbenicillin, 10 g l\(^{-1}\) sucrose, and 4 g l\(^{-1}\) gellan gum with pH 5.8). The rooted plants were transplanted in the soil and grown for flowering.

Southern blot hybridization and ligation-mediated PCR were performed to confirm the copy number of the inserted T-DNA and independence among the transgenic plants. The gDNA from the leaves of mature plants (T\(_0\)) was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Approximately 5\(\mu\)g of gDNA from each plant was digested with Hind III, electrophoresed on a 0.7% agarose gel, and alkaline transferred to a nylon membrane (Hybond-XL, GE Healthcare, Chicago, USA). The \textit{GUS} genes integrated in the \textit{Raphanus} genome were detected with a 1500-bp probe for the \textit{GUS} gene chemically labelled with the PCR DIG Probe synthesis kit (Roche Diagnostics, Basel, Switzerland). The PCR primers used to amplify the 1500-bp fragment inside the \textit{GUS} gene were 5′-GATCCGGAAAACCTGTTGAAT-3′ and 5′-CGGTTTTTTACCCGAGGTTCA-3′. All the four tested transgenic plants retained either one or two copies of the \textit{GUS} gene in the T-DNA. The different positions of the detected bands indicated that all were independent transformation events (Figure 2A, C).

A ligation-mediated PCR was performed using the Straight Walk® kit (BEX, Tokyo, Japan) following the manufacturer’s instructions (Tsuchiya et al. 2009). The gDNA (1\(\mu\)g) was digested with Bgl II and BamH I. After one base extension, the adaptor DNA was ligated, and the PCR was performed using gene-specific primers and an adaptor-derived primer. The first PCR was performed with the T-DNA-specific primer 5′-GGATACCGGGGAATTTATGGAAC-3′ and the second PCR (nested PCR) was performed with the primer 5′-CTGAGTCCTCTTTTAAGCTGTC-3′. Both primers were designed from the sequence near the right border of the 25-bp repeat of T-DNA. For each plant, one or two distinct band(s) were amplified (Figure 2C). The copy number of the inserted T-DNAs for individual plants was detected by Southern blot hybridization and corresponded to that obtained by ligation-mediated PCR. Also, the electrophoretic band positions from the individual plants were different for different plants, indicating the occurrence of independent transformation events.

The four transgenic T\(_0\) plants (transgenic event No.

### Table 1. Comparison of transformation efficiency with different \textit{Agrobacterium} strains.

| \textit{Agrobacterium} strain | Replicate | No. of hypocotyl explants | No. of transgenic shoots | Transgenic shoots per hypocotyl explants (%) |
|-----------------------------|-----------|---------------------------|--------------------------|--------------------------------------------|
| GV3101                      | 1         | 106                       | 18                       | 17                                         |
|                             | 2         | 127                       | 8                        | 6.3                                        |
|                             | 3         | 120                       | 21                       | 17.5                                       |
|                             | Total     | 353                       | 47                       | 13.3±6.32\(^a\)                             |
| EHA101                      | 1         | 120                       | 2                        | 1.7                                        |
|                             | 2         | 116                       | 5                        | 4.3                                        |
|                             | 3         | 120                       | 15                       | 12.5                                       |
|                             | Total     | 356                       | 22                       | 6.2±5.65\(^a\)                              |

\(^a\) Values represent means±SD of average.
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1, 2, 3, and 4 corresponding the same number in Figure 2A, C) extended growing to a flowering stage; however, two of them (No. 1 and 2) did not produce self-fertilized seeds because of male sterility. The remaining two plants (No. 3 and 4) were self-fertilized and seeds were obtained from them seeds (Figure 1C). We confirmed that almost all “Pirabikku” plants had the male sterile nuclear gene orf138 by PCR (Yamagishi and Terachi 2001). When randomly chosen “Pirabikku” seeds were grown in a greenhouse, 16 of 24 flowers of these plants showed male sterility. We also confirmed that in the four T0 plants analyzed by us, fertile plants can be screened by PCR-restriction fragment length polymorphism (PCR-RFLP) for the presence of fertility restorer gene orf687 (Yasumoto et al. 2008; Supplementary Figure S2).

The transmission of the hpt gene in T1 seedlings was detected by PCR and the data were analyzed by chi-square analysis. The segregation ratio of the hpt gene was consistent with the Mendelian inheritance (Table 2). We detected the inheritance of the GUS gene by the GUS assay (Figure 1D) in the T1 seedlings. The transgenic shoot indicated that the transgenes in T-DNA introduced by Agrobacterium were inherited as a Mendelian factor.

In our screening of cultivars for high regeneration frequency, we found that the regeneration frequency of Raphanus sativus var. longipinnatus L. was lower than that of Raphanus sativus var. sativus L. (Supplementary Table S2). We found the growth condition (especially the hormone concentration and incubation period) used for “Pirabikku” in this study was not optimal for the transformation of Raphanus sativus var. longipinnatus L. In the previous paper, Cho et al. used Raphanus sativus L. cv. Jin Ju Dae Pyong (Korean F1 hybrid radish) with a relatively high frequency of adventitious shoot formation (55%). However, the transformation efficiency was relatively low (0.26%), which was due to their low susceptibility to Agrobacterium infection. Therefore, we investigated the susceptibility of “Pirabikku” to Agrobacterium during the preliminary screening. In the B. rapa “Osome” transformation method, shoot elongation culture was performed in a hormone-free medium. However, in radish, the root tissue near the cut surface in contact with the hormone-free culture medium was enlarged, which might inhibit the transfer of nutrients to the part of the plant above the ground and for root development. Therefore, we used a medium containing 1 mg l−1 gibberellic acid to promote root development.

In conclusion, we established a highly efficient system for the Agrobacterium-mediated genetic transformation of radish. We have recently succeeded in producing gene knockout plants of “Pirabikku” by CRISPR/Cas9 technology (unpublished). The genetic transformation of radish established in this study may contribute to gene function analysis, breeding transgenic plants, and accelerating genome editing in radish, which is one of the most consumed vegetables worldwide.

Acknowledgements

We thank Dr K. Izawa of our laboratory for providing Agrobacterium EHA101, the binary vector pG121-Hm, and technical suggestions for Southern hybridization. This study was supported by grants from the Tokyo NODAI General Director Project of the Tokyo University of Agriculture.
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