Development of a Simple and Efficient Method for Transformation of Buckwheat Plants (*Fagopyrum esculentum*) Using *Agrobacterium tumefaciens*

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Apical meristems of seedlings of buckwheat (*Fagopyrum esculentum* var. Shinano No. 1) were prickled with a needle and inoculated with *Agrobacterium tumefaciens* (LBA4404, pBI121). The inoculated seedlings were grown to maturation and allowed to pollinate randomly to set the seeds (T1 plants). The transformation efficiency of the T1 plants was estimated by germination in the presence of genetricin (20 μg/ml) and by detection of β-glucuronidase (GUS) gene with PCR, indicating that 36% and 70% of the T1 plants were transformed, respectively. Four plants taking on a mutated morphology were selected from T1 plants which were transformed with the method using *A. tumefaciens* harboring a modified pBI121 for plasmid rescue. Southern blot analysis of plasmids rescued from the 4 T1 plants demonstrated that each plasmid contained a different flanking DNA of the buckwheat genome, an evidence that T-DNA was integrated in different sites of the genomic DNA among the 4 T1 plants.

**Key words:** *Agrobacterium tumefaciens*; apical meristem; *Fagopyrum esculentum*; plasmid rescue; transformation

Transformation has been used to define the functions of plant genes. It, however, is not easy to introduce genes into plants to produce the transgenic plants and obtain their progenies in a short period. Furthermore, somatic mutation or somaclonal variation occurs frequently in plant cells during tissue culture for transformation. To conquer these problems, a simple *in planta* transformation method has been developed with *Arabidopsis*. The method includes infiltration of *Agrobacterium* cells into *Arabidopsis* plants before flowering, and direct selection for rare transformants in the resultant seeds. The small plant size, high germination time, and high seed yield per plant are prerequisites for the methods. Because these features are not shared by other ordinary plants, the usage of this methods is limited to *Arabidopsis*.

Thus, we developed a modified *in planta* transformation method for other plants. In our methods, *A. tumefaciens* was inoculated onto the apical meristems of seedlings. We screened many plants, mainly crop plants, and finally found that buckwheat could be transformed by this method. In addition, buckwheat has several advantages. Buckwheat grows rapidly and produces many seeds within 2 months. Furthermore, buckwheat is an important crop, especially in the district where our university is located. As far as we know, this is the first paper to report the transformation of buckwheat.

Buckwheat seeds (*Fagopyrum esculentum* var. Shinano No. 1) sterilized with sodium hypochlorite were sown on soils in two pots (each 20 seeds) and grown at 25°C and 60% relative humidity under 8 h light (about 4,000 Lux) and 16 h dark in a growth chamber. The seedings of 7–8 cm height with two expanded cotyledons after 4 to 5 days of culture were used for transformation. *A. tumefaciens* (LBA4404 harboring pBI121, Toyobo) was cultured in LB medium containing kanamycin (50 μg/ml), rifampicin (10 μg/ml), and streptomycin (50 μg/ml) at 28°C for 18 h. The cells were harvested by centrifugation, washed with water, suspended in water (1.0 × 10⁶ cell/ml) and used as an inoculum. Two to three points in tops of hypocotyls (3 mm parts) that included apical meristems were prickled with a needle (φ0.71 mm) and then inoculated with a cotton applicator, drenched with either the inoculum or water. The seedlings in one pot were inoculated with water (non-transformed plants, control plants), while the seedlings in another pot were inoculated with *A. tumefaciens* (transformed T0 plants). The inoculated seedlings were kept at 22°C in the dark for 3 days and subsequently grown under the conditions described above. No clear difference was observed in growth between

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seeds in the two pots. The buckwheat plants develop two types of flowers with different length of pistil, about 1.8 and 0.6 mm (heterostylism). Only pollination between different types of flowers results in fertilization. The flowers of plants in the respective pot were allowed to pollinate randomly. About 1,000 seeds were harvested from the plants in each pot. T1 seeds harvested were mixed up well to ensure random sampling.

The inoculated seedlings (T0 plants) were not treated to eliminate the Agrobacteria. Therefore, we examined the presence of the remaining Agrobacteria in T1 plants as follows. The seeds (T1 plants) were sterilized in sodium hypochlorite solution and then germinated aseptically. The resultant seedlings were homogenized in sterile water and the homogenate were incubated on the LB medium containing kanamycin (50 μg/ml), rifampicin (10 μg/ml), and streptomycin (50 μg/ml) on which A. tumefaciens used for transformation could grow. No colonies appeared, confirming that T1 plants were free of Agrobacteria used for transformation.

Because non-transformed buckwheat seeds could germinate in the presence of kanamycin at as high concentration as 500 μg/ml, we tested the germination of T1 and non-transformed seed in the genetin solution (20 μg/ml) to confirm the introduction of npt II gene in pBI121 binary vector (Fig 1). Of 45 T1 seeds, 16 seeds (36%) germinated in the presence of genetin (20 μg/ml), while none of 20 non-transformed seeds germinated.

Histochemical GUS assay were also done to confirm the introduction of β-glucuronidase gene with both non-transformed and T1 seedlings. However, no clear difference was visible between T1 and non-transformed seedlings due to the high background staining with non-transformed plant seedlings. Thus, the presence of β-glucuronidase gene in T1 seedlings were examined by PCR analysis. Genomic DNA was extracted from the seedlings using a Nuclear Phytopure DNA extraction kit (Amersham Pharmacia Biotech) following to the instructions of supplier and further purified using RNase and proteinase K. A portion (1-2 μl, about 500 ng) of the genomic DNA from T1 and non-transformed seedlings was added to a reaction mixture of 50 μl of final volume (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 200 μM each nucleotide, 0.2 μM each primer, and 1.25 units Taq polymerase (Takara)). The PCR was done at 94°C for 30 sec, at 58°C for 1 min, 72°C for 10 min for the first 30 cycles and then 72°C for 10 min. The primers used were: P1, 5’-CGAAGTGATGGACATCAGG-3’, P2, 5’-CACACTGATACCTTCTCAC-3’ which were to amplify the 1.2-kb segment in the GUS gene. Amplified DNAs were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Of 20 T1 plants, 14 (70%) gave the amplified band of 1.2 kb, but none of 3 non-transformed plants gave the amplified band (Fig. 2). The transformation efficiency (70%) estimated by this methods was much higher than that (36%) estimated by genetin resistance test (Fig. 1). Although the reason for the discrepancy is not clear, kanamycin resistance gene in some T1 plants might not be expressed because of its integration in an inactive region of chromosome, e.g., a heterochromatin region, producing T1 plants that are sensitive to genetin in spite of the presence of a kanamycin

Fig. 1. Germination of T1 and Non-transformed Seeds of Fagopyrum esculentum in Presence of Genetin.
Non-transformed seeds (panel A) and T1 seeds (panel B) were germinated at 28°C on cotton pads containing genetin water solution (20 μg/ml) for 5 days.

Fig. 2. Detection of Integrated β-Glucuronidase Gene in Genomic DNAs of T1 Plants by PCR.
PCR was done as described in the text. The 1.2-kb DNA is β-glucuronidase gene segment amplified. M, size marker of DNA; Lane 1, pBI121 binary vector; Lane 2-4, non-transformed plants; Lane 5-24, T1 plants.
resistance gene in their genomes.

A modified pBI121 binary vector was constructed to rescue the plasmids that contained the integrated T-DNA and the flanking host chromosomal DNA. The vector was made by replacing the Smal/EcoRI segment of the β-glucuronidase gene and Nos terminator of pBI121 binary vector with a PvuII/EcoRI segment (2.3 kb) of an ampicillin resistance (β-lactamase) gene and a replication origin (ori) of pBR322 (Nippon Gene) and then introduced into A. tumefaciens (LBA4404). Ten seedlings (T0) were transformed with the A. tumefaciens and about 700 T1 seeds were obtained by random pollination. The randomly selected T1 100 seeds were sown on soil and grown to mature plants. Ninety six T1 plants were normal in their morphology. The remaining four T1 plants took on a clear mutant phenotypes in their morphology; dwarf with smaller leaves (mutant 1), flat stem (mutant 2), inhibited branching (mutant 3), dwarf with thick stem (mutant 4). These 4 plants were presumed to be dominant negative mutants of which some genes involved in their morphology were destroyed by insertion of T-DNA. The genomic DNAs were isolated from those 4 mutant phenotype-T1 plants and 4 non-transformed plants and digested with HindIII. A single HindIII site is included upstream of the segment derived from pBR322 plasmid in T-DNA. The digested DNAs were self-ligated and then used to transform Escherichia coli (HB101), which were then cultured on LB plates containing ampicillin (50 µg/ml). The colonies appeared with all 4 T1 plants, while no colonies appeared with any of 4 non-transformed plants. Plasmids were isolated from the colonies of each sample and digested with HindIII. The digested plasmid DNAs were analyzed by Southern hybridization using a 32P-labeled ampicillin resistance (β-lactamase) gene as a probe (Fig. 3). The plasmids rescued from different T1 plants gave the band in different positions, indicating that each plasmid contained a different flanking genome DNA of buckwheat. The result supported the idea that T-DNA was integrated in a different site of genome DNA in each of the 4 T1 plants.

Taken together with the results described above, the method reported in this paper seems to be a promising transformation method. There are several advantages with this method. First, it does not require sterile conditions, second, it does not involve tissue culture, third, a large number of progeny can be obtained in a short period, and finally, the transformation efficiency is high.

Fig. 3. Southern Hybridization Analysis of Plasmids Rescued from T1 Plants with a Mutant Phenotype in Morphology.
Plasmids were rescued from 4 mutant T1 plants which had been transformed with A. tumefaciens harboring a modified pBI121. The plasmids were digested with HindIII and Southern hybridized using 32P-labeled Amp6 gene as a probe. Lane 1, mutant 1; Lane 2, mutant 2; Lane 3, mutant 3; Lane 4, mutant 4. See text for the phenotypes of the mutants.

References