Note

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Efficient DNA Minipreparation by Modified Benzyl Chloride Method from Hairy Roots of *Ajuga reptans* to Detect *rolB* Gene from Ri Plasmid

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Ajuga reptans, a member of the family Labiatae, is not only a horticultural plant which is used as ground cover, but also a medicinal herb which has been used for the treatment of jaundice and rheumatism in European countries for centuries. The roots of *A. reptans* contain several types of phytoecdysteroid and mainly 20-hydroxyecdysone (20-HE) known as a principal physiological inducer of molting and metamorphosis in arthropoda. For production of 20-HE, some *A. reptans* hairy root lines with high productivity of 20-HE and their culture methods have been established [see review 1].

Hairy roots are adventitious roots induced by infection with *Agrobacterium rhizogenes*. There are variations in the characteristics, such as morphology, the capacity for growth and the productivity of secondary metabolites, of hairy roots lines and, moreover, the characteristics of each hairy root line are very stable [2]. Since hairy roots of *A. reptans* are relatively thick (1-2 mm in diameter) and firm, the difference of characteristics such as morphology, elongation, branching, and gravity response among the independently isolated root lines can be easily distinguishable [3, 4]. This trait of *A. reptans* hairy roots prompts us to use the hairy roots for analysis of genes, in particular, rooting locus (*rol*) genes, responsible for exhibiting such characteristics [4]. At present, we have isolated more than 15 hairy root lines with distinctive features (unpublished result). During the selection of hairy root lines, we need to develop a small scale DNA isolation method (minipreparation) from the hairy roots to confirm integrated T-DNA of Ri plasmid, in particular, *rol* genes.

In minipreparation of DNA from plant materials, a method using cetyltrimethylammonium bromide (CTAB) is well devised [5], and the freezing of plant materials in liquid nitrogen is also indispensable for disruption of plant materials. The major problem with this method is that part of the disrupted plant materials are lost because the fine powder of the plant materials adheres to the surface of the grinding apparatus. Recently, Kikuchi *et al.* introduced some excellent methods including the benzyl chloride method for the isolation of DNA from plant materials [6]. A plant DNA isolation kit that employed a very strong organic solvent, benzyl chloride, effective to destroy non-grinding plant materials and to release DNA without damage was purchased from Nippongene Ltd. (Tokyo) as ISOPLANT. Following the protocol which accompanied ISOPLANT, we have tried to isolate DNA from hairy roots of *A. reptans* but only a very small amount of DNA could be obtained. The insufficient disruption of plant materials with benzyl chloride seemed to be the cause of the low yield of DNA. Moreover, the isolated DNA was difficult to digest by restriction endonuclease, suggesting that impurities were present in the DNA solution. Thus, to isolate a large amount of DNA of high quality from hairy roots, some modifications of the protocol are necessary.

A hairy root line Ar-24 of *Ajuga reptans* which had been established by infection with *Agrobacterium rhizogenes* strain MAFF301724 harboring mikimopine-type pRi1724 was used as the material for isolation of DNA [3, 4]. For effective grinding of the hairy roots, we employed a small pestle (Pellet mixer, Treff AG, Schweiz, Switzerland) fit to an 1.5 m*l* micro test tube, and quartz sand (powder, Katayama Chemical Industries Co., Ltd). One-month cultured hairy roots (0.1 to 0.5 g) cut to 1 cm in length was quickly and completely ground with a pellet mixer in 300 μl of solution I (Extraction buffer) by addition of 0.01- to 0.02-g quartz sand (sterilized with a dry heated sterilizer at 160°C for 4 hr) in a 1.5 m*l* micro test tube for 1 to 2 min. The plant material must be ground in the solution because the DNase activity must be inhibited in solution I. Five microliters of 10 mg/m*l* RNase was added to solution I for digestion of RNA in the grinding step as described in the ISOPLANT protocol. One-hundred and fifty microliters of solution II (lysis buffer: benzyl chloride) was added to the homogenate, followed by thoroughly mixing with a vortex mixer for 20-30 sec until the mixture became muddy white. After incubation at 50°C for 30 min, 150μl of solution III (sodium acetate, pH5.2) was added to the mixture, followed by brief vortex mixing, and the mixture was incubated on ice for 15 min. By

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centrifugation at 15,000 rpm for 15 min at 4°C, the upper aqueous phase including DNA was recovered and then was precipitated with 2.5 volume of ethanol.

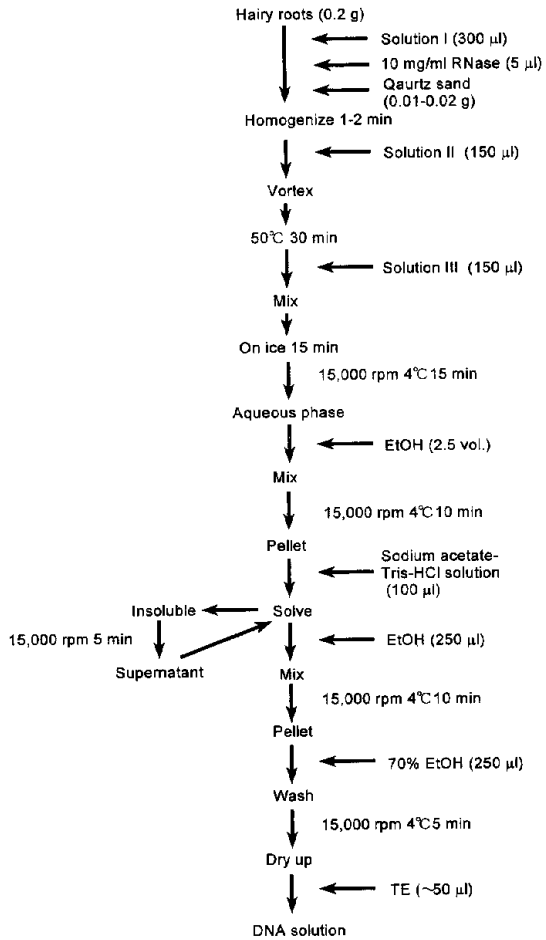

Fig. 1 Scheme of the improved DNA isolation method for *A. reptans* hairy roots.

By centrifugation at 15,000 rpm for 10 min at 4°C, DNA was precipitated. For removing salts and impurities in the precipitant, we added following steps; the precipitant including DNA was solved in 100 μl of sodium acetate-Tris-HCl solution (0.1 M CH_3COONa, 0.01 M Tris-HCl, pH8.0); if insoluble clumps were present, they could be removed with the centrifugation at 15,000 rpm for 5 min at room temperature. These steps gave a good result for digestion of the isolated DNA by restriction endonucleases. DNA was re-precipitated with 2.5 volume of ethanol, followed by centrifugation at 15,000 rpm for 10 min at room temperature. The precipitant was washed with 70% ethanol and was successively dried *in vacuo* for 5 min, and then was resolved in 50 μl of TE (10 mM Tris-HCl, pH8.0, 1 mM EDTA). The improved protocol is shown in **Fig. 1.**

Fig. 2-a shows that an electrophoregram of the total DNA isolated from *A. reptans* hairy roots by the improved protocol described above and the ISOPLANT protocol. Using the improved protocol, a large amount of genome DNA was isolated from the hairy roots, though the band smeared by slight fragmentation of DNA. The destruction of DNA seemed to occur during the grinding of the materials. By contrast, a very small amount of DNA was isolated by the ISOPLANT protocol. The absorbance of isolated DNA could not be measured at 260 nm with spectrophotometer, since impurities showing similar absorbance seemed to exist. To quantify the isolated DNA, 80 μl of 5 $\mu g/\mu l$ Hoechst33342 (Sigma, St. Louis, USA) was mixed with 20 μl of the DNA solu-

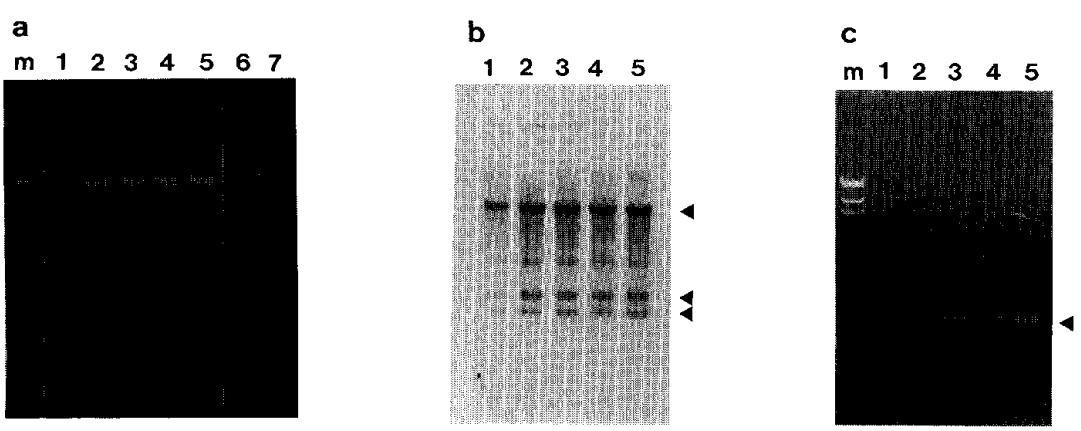

Fig. 2 Characteristics of DNA isolated from *A. reptans* hairy roots by the improved method. (a) Electrophoregram of the isolated DNA. One microliter of each DNA solution was applied in 1% agarose gel. (b) Southern blot analysis using non-RI detection system (Gene Images™). Five micrograms of each DNA was digested with *Bam*HI and *Eco*RI. pRTB9 containing 9.5-kb *Bam*HI fragment of pRi1724 T-DNA was used as a probe. Arrowheads indicate hybridization signals corresponding to 6.4-, 1.8- and 1.3-kb *Bam*HI-*Eco*RI fragments. (c) Electrophoregram of the *rolB* fragments of pRi1724 amplified from the isolated DNA by PCR. An arrowhead indicates 837 bp of amplified *rolB* fragments. Lane m; λ/*Hin*dIII molecular weight marker. Lanes 1, 2, 3, 4 and 5; DNAs isolated from 0.1, 0.2, 0.3, 0.4 and 0.5 g of hairy roots, respectively. Lanes 6 and 7; DNAs isolated from 0.1 and 0.5 g of hairy roots by ISOPLANT protocol.

Table 1. Yeild of DNA from *Ajuga reptans* hairy roots.

Protocol	weight(g)	Yield(μg/g)
Improved	0.1	76.5±30.8*
	0.2	72.4±13.4*
	0.3	52.2±19.7*
	0.4	37.2±14.9*
	0.5	48.8±24.4*
ISOPLANT	0.1	5.5± 1.9*
	0.5	2.6

* The means (±SD) of three samples.

tion and then the solution was measured with Cyto-Flour II (PerSeptive Biosystems, Framingham, USA) at 360 nm for excision and 460 nm for emission. As shown in **Table 1**, 37-76 μg of DNA per g of plant material (μg/g) was isolated by the improved protocol, whereas 5.5μg/g of DNA was isolated by the ISOPLANT protocol. Judging from the yield of DNA and standard deviation (**Table 1**), we considered that the most suitable weight of hairy roots for isolation of DNA was 0.2g.

The DNAs isolated by the improved protocol were enough to use for not only PCR but also Southern blot analysis. Since the hairy root line Ar-24 contains the T-DNA of pRi1724 (probably, single copy) in the genome [4], Southern blot analysis using 9.5-kb *Bam*HI fragment which had contained core-T-DNA of pRi1724 [7] used as a probe was performed. Each 5 μg of DNA samples isolated by the improved protocol could be completely digested with *Bam*HI and *Eco*RI, whereas the same weight of DNA isolated by the ISOPLANT protocol could not (data not shown), suggesting that the DNA samples isolated by ISOPLANT protocol had included considerable impurities. When non-RI Southern blot analysis (Gene Images™, Amersham Pharmacia Biotech, UK) using the DNA samples isolated by the improved protocol was performed, the signals corresponding to 6.4-, 1.8 - and 1.3-kb *Bam*HI-*Eco*RI fragments which had been derived from 9.5-kb *Bam*HI fragment of pRi1724 T-DNA were clearly detected (**Fig. 2-b**). Furthermore, when PCR using KOD dash polymerase (TOYOBO, Osaka, Japan), a set of primers (5′-ATGGCACTGAACTTGCCGTT-3′ and 5′- AGTCGCCGAGGTTTCTTTCT-3′) [4] which are located on both ends of *rolB* gene of pRi1724 and 100ng/m*l* of each DNA sample isolated by the improved protocol used as templates was performed, 837-bp fragments corresponding to *rolB* gene were amplified from all templates (**Fig. 2-c**).

In conclusion, we could improve the protocol of DNA minipreparation suitable for hairy roots of *Ajuga reptans*. Through additions to ISOPLANT protocol, the efficiency of DNA extraction was raised by 1) grinding tissues using quartz sand and 2) removing impurities from precipitated DNA through sodium -acetate/Tris-HCl solution. The DNA isolated from 0.2g of hairy roots by the improved protocol was sufficient to detect single copy *rolB* gene by Southern blot and PCR analysis. The protocol may be applicable for isolation of DNA from wide range of plant materials.

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