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Hairy Root-mediated Transgenic-Plant Regeneration in Egyptian Clover (*Trifolium alexandrinum* L.)

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Abstract

Transformed plants of Egyptian clover (berseem clover, *Torifolium alexandrinum* L.) were obtained from hairy roots induced by infection with *Agrobacterium rhizogenes* strain DC-AR2 (harboring mikimopine-type pRi1724). Among established 16 hairy root lines showing growth variations on MS medium containing 0.5 mg l^{-1} NAA, two lines with moderate or slow proliferation regenerating shoots spontaneously. The presence of T-DNA of pRi1724 in the genome of the regenerated shoots was confirmed by the DNA gel blot analysis. The regenerated shoots repeatedly formed shoots but scarcely formed roots on hormone-free MS. Whole plants were obtained from the shoots when they were cultured on Florialite for two months. The regenerated plants displayed the hairy root syndrome such as dwarfing and alterations of leaf shape. We propose here that hairy root-mediated regeneration is one of the effective tools for obtaining transgenic plants of Egyptian clover.

Key words: *Agrobacterium rhizogenes*, regeneration, transformation.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; BA, benzyladenine; MS, Murashige and Skoog's medium; NAA, naphthaleneacetic acid; *rol*, rooting locus.

Transformed roots, called hairy roots, are induced by infection with *Agrobacterium rhizogenes*, which is a soil-borne gram-negative bacterium, causes the hairy root disease. The hairy roots are able to regenerate shoots spontaneously or by addition of plant growth regulators such as auxin and cytokinin (Tepfer, 1984). Generally, in the case of plant species which easily regenerate shoots from the dedifferentiated tissues, hairy roots derived from the plant species also frequently regenerated shoots. However, in some leguminous plants such as Chinese milk betch, which have difficulty in regeneration of shoots from the dedifferentiated tissues, shoots are occasionally regenerated from the hairy roots (Cho *et al.*, 1998).

Egyptian clover (berseem clover, *Torifolium alexandrinum* L.) has been cultivated since ancient times. This clover is an important crop, serving as forage for domestic animals and a material for improvement of soil in a wide range of areas from northern Africa to India. Mokohtarzadeh and Constantin (1978) and Aly *et al.* (1994) succeeded in regeneration of whole plants from the calli on several cultivars of Egyptian clover and from somatic embryos induced from cotyledons by addition of high concentration of 2,4-D. Here we report on the regeneration of plants from hairy root culture of Egyptian clover (*Trifolium alexandrinum* L. cv. Miskawy-Ahaly 1).

Hairy roots from the Egyptian clover were induced by infection with *Agrobacterium rhizogenes* strain DC-AR2 (a kanamycin-sensitive mutant derived from strain MAFF301724) harboring mikimopine-type Ri plasmid pRi1724 (Tanaka and Oka, 1994) and 16 hairy root lines were established (Tanaka *et al.*, 2001). The established hairy root lines showed various proliferations accompanied by dedifferentiation and then differentiation of new adventitious roots on MS medium containing 0.5 mg l^{-1} NAA (0.5NMS). The proliferation pattern shown by each hairy root line appeared to be divided into three types: good (**Fig. 1A**), moderate

(**Fig. 1B**), and poor (**Fig. 1C**). These proliferation patterns were related to the expression levels of *rolB* and *rolC* genes which are involved in hairy root induction in each hairy root line (Tanaka *et al.*, 2001).

To obtain regenerated plants of Egyptian clover transformed by pRi1724 T-DNA, we attempted to culture the hairy roots of the established 16 lines on the MS containing several plant growth regulators and vitamins as reported previously (Mokohtarzadeh and Constantin, 1978; Aly *et al.*, 1994). We used MS containing 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BA (Mokohtarzadeh and Constantin, 1978) or a modified MS containing MS salts, B5 vitamins, 6% (w/v) sucrose, and 2- to 10 mg l^{-1} 2,4-D (Aly *et al.*, 1994) for shoot regeneration. Shoots could not, however, regenerate from any of the hairy root lines, though many shoot primordia-like green calli were induced (**Fig. 1D**).

On the other hand, shoots spontaneously regenerated from the differentiated basal side of hairy roots in lines No. 101 and No. 102, which had shown moderate and poor proliferation, respectively, during culture on 0.5NMS in the dark (**Fig. 1E**). Regenerated shoots were cultured on half-strength MS (1/2 MS) solidified with 1% (w/v) agar in the light. During the culture, the regenerated shoots grew slowly with lateral shoot production (**Fig. 1F**), and showed a rapid aging and rarely produced new roots.

When the regenerated shoots were cultured on Florialite™ (Asahi Techno Glass Co., Tokyo, Japan), an artificial soil composed of vermiculite and fiber, permeated with 1/2 MS without plant hormone for 2 months, roots could differentiate from the shoots, and some of these regenerants had flowers (**Fig. 1G**). The whole plants were transferred onto vermiculite in pots and were cultivated by addition of 1:1000 diluted fertilizer Hyponex (Hyponex Japan, Osaka) at 22℃ in a 16-h photoperiod under a fluorescent daylight lamp (50 μmol m^{-2} s^{-1}) in a climate-controlled room. The regenerated plants cultivated on vermiculite in pots exhibited so-called "hairy root syndrome", with dwarfing by decreased the internode distances, and alterations of leaf shape (**Fig. 1H**), and they have never produced flowers. Except for the established 16 hairy root lines, some of the newly induced hairy root lines showing moderate or poor proliferation also regenerated shoots spontaneously, whereas no lines showing good proliferation regenerated shoots (data not shown).

We confirmed the T-DNA insertion in the hairy root line 101. Genomic DNA was isolated from the shoots regenerated from the hairy root line 101 and leaves of untransformed plants by minipreparation using DNeasy Plant Mini Kit (QIAGEN Inc. Valencia, CA, USA). Genomic DNA (5 μg) digested with *Bam*HI or *Eco*RI was fractionated in 1% agarose gel in Tris-acetate and EDTA (TAE) buffer. After transfer to a Hybond-N^+ membrane (Amersham Pharmacia Biotech, UK), non-RI DNA gel blot analysis using Gene Images™ (Amersham Pharmacia Biotech) was performed. A 9.5-kb *Bam*HI fragment containing pRi1724 core-T-DNA (probe A) or a 5.3-kb *Bam*HI fragment containing a right border of pRi1724 T-DNA and its adjacent sequence (probe B) was used as a probe (**Fig. 2A**). Hybridization signals were detected by X-ray film (RX-U, Fuji Photo Film, Japan). DNA gel blot analysis using the probe A showed that one band of 9.5-kb in *Bam*HI digestion and two bands of 7.6-kb and 1.3-kb in *Eco*RI digestion were detected in the genome of hairy root line 101, while no signal bands were detected in genome of control plants (**Fig. 2B**, left). However, no fragments containing the junction region of the pRi1724 T-DNA/the genome DNA of Egyptian clover could be detected in the *Eco*RI digestion in the hairy root line 101. The fragment might happen to wrap over the 7.6-kb *Eco*RI fragment. When using the probe B, some strong signal bands (3.3 kb and 1.3 kb in *Bam*HI digestion and 4.8 kb in *Eco*RI digestion) were found in the genomes of both the control plant and the regenerant from the hairy root line 101 (**Fig. 2B**, right, indicated by asterisks). The genome of untransformed plants of Egyptian clover might contain the homologous sequence to pRi1724 T-DNA similar to that of *A. reptans* (Tanaka *et al.*, 1998). One band of 6.3 kb in *Bam*HI digestion and three bands of 7.6 kb, 1.4 kb and 0.9 kb in *Eco*RI digestion were detected in the genome of hairy root line 101, while no signal bands originated from the pRi1724 T-DNA were not in the genome of control plants (**Fig. 2B**, right). The band of 6.3 kb in *Bam*HI digestion seemed to be the fragment containing the junction region of the pRi1724 T-DNA/the genome DNA of Egyptian clover. No other bands were found in *Bam*HI digestion, suggesting that the inserted T-DNA of pRi1724 was one copy. The band containing the junction region in *Eco*RI digestion was not detected because the region between the rightmost *Eco*RI site of T-DNA and the right border might be too short to detect the hybridization signal.

In the present study, we demonstrated that the hairy root cultures of Egyptian clover which showed moderate or poor proliferation spontaneously regenerated whole plants. The vigorous growth of hairy roots may inhibit shoot regeneration. The regen-

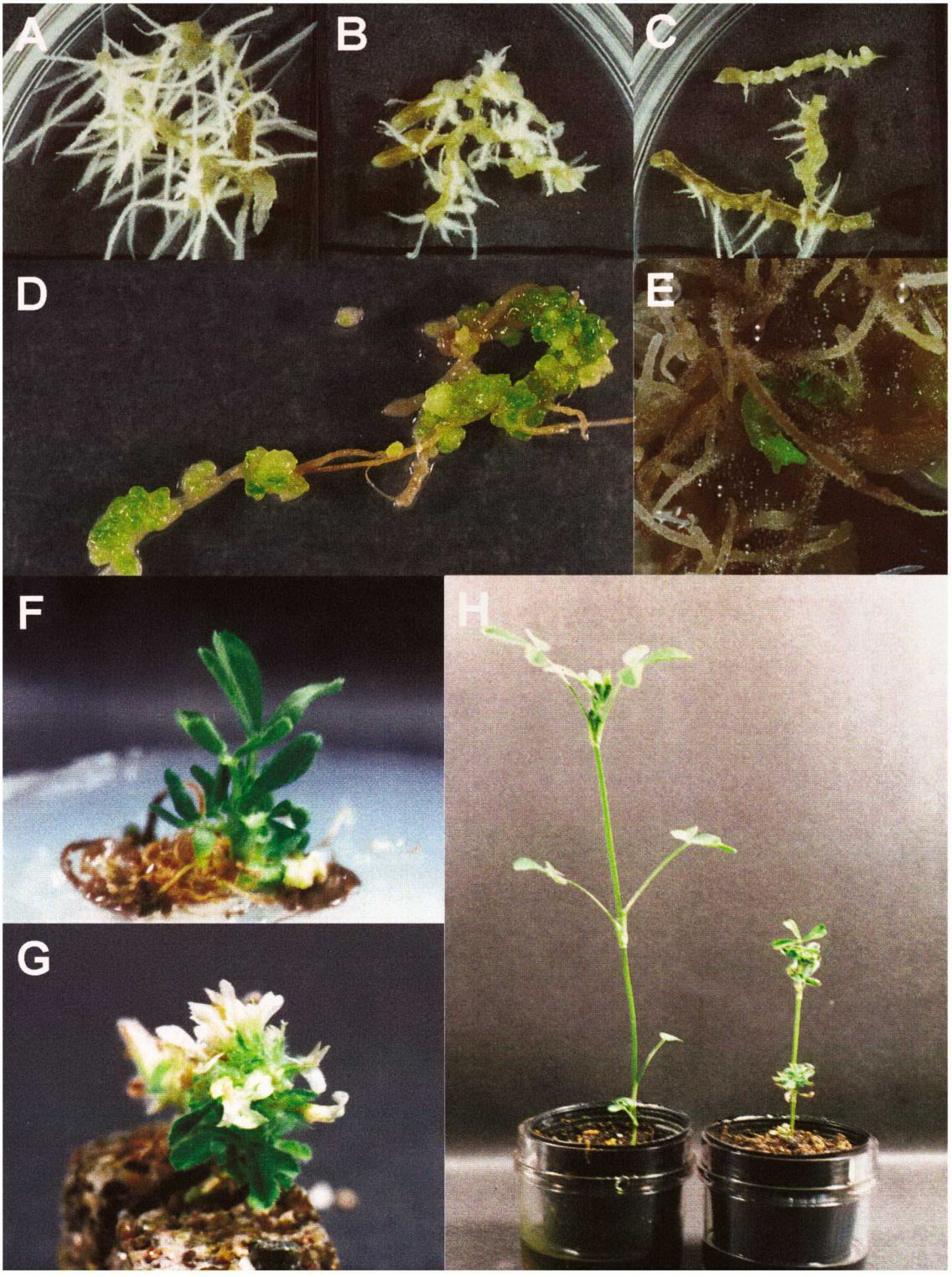

Fig. 1 A–C : Three proliferation types of hairy roots of Egyptian clover on 0.5 NMS. Segments of hairy roots (*ca.* 1.5 cm each in length) cultured on 0.5 NMS medium for 30 days showing good growth (A), moderate growth (B) and poor growth (C). D–H : Shoot regeneration from hairy roots line No. 101. Formation of shoot primordia-like green calli on hairy roots cultured on a modified MS medium containing 2,4-D (D). Spontaneously regenerated shoots from hairy roots (E). Regenerated shoots cultured on 1/2 MS medium for 30 days (F). Flowering on the shoots cultured on Florialite (G). An untransformed plant (left) and a transformed regenerant (right) cultivated in soil for 1 month (H).

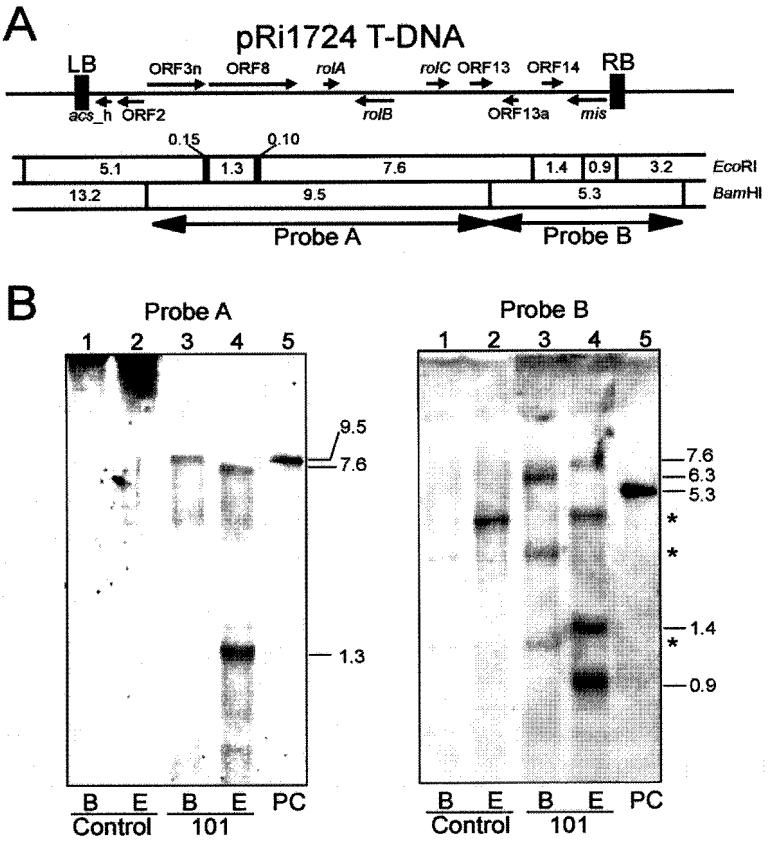

Fig. 2 DNA gel blot analysis for the detection of pRi1724 T-DNA in hairy root line No. 101 of Egyptian clover induced by *A. rhizogenes* strain DC-AR2 infection. A, Schematic illustration of pRi1724 T-DNA. (top) The line wedged between two black rectangles indicates the T-DNA of pRi1724. RB and LB above black rectangles mean the right and left borders of the pRi1724 T-DNA, respectively. The small arrows represent the genes coded on the pRi1724 T-DNA. (middle) The *Bam*HI and *Eco*RI restriction maps of vicinity of pRi1724 T-DNA. (bottom) Lines with arrowhead indicate a 9.5-kb and a 5.5-kb *Bam*HI fragments used as probes of DNA gel blot analysis. B, The result of DNA gel blot analysis. *Bam*HI or *Eco*RI digested DNA (5 μg) of untransformed roots (Control) or the hairy root line 101 was used for non-RI DNA gel blot analysis. (left) A 9.5-kb fragment containing pRi1724 core-T-DNA (Probe A) was used as a probe. (right) A 5.3-kb fragment containing the right border of pRi1724 (Probe B) was used as a probe. Lane 1 and 2, untransformed plant; lane 3 and 4, hairy root line 101; lane 5, 9.5-kb *Bam*HI fragment (left) and 5.3-kb *Bam*HI fragment (right) of pRi1724 T-DNA used as positive controls. Asterisks indicate signal bands detected in the genome of untransformed Egyptian clover.

erated plants showed a slow growth and an altered shape known as "hairy root syndrome" (Tepfer, 1984). Ebinuma *et al.* (1998) developed a useful binary vector called *rol*-type MAT vector which has a transposon combined with *rol* genes of pRi1724 T-DNA. The *rol* genes on the MAT vector induce hairy roots, and then are spontaneously removed by transposon moving during the culture. By using the MAT vector in combination with the present hairy root mediated transformation, transgenic Egyptian clover showing the normal phenotype with healthy growth will be obtained.

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