Agric. Chem. Biotechnol. **44**(2), 92-94 (2001)

Simple Detection of Opines by Paper Electrophoresis for Hairy Roots Transformed with *Agrobacterium rhizogenes* Strains

Hyeon-Je Cho*[1], Hyo-Cheol Ha[2], Jae-Sung Lee[2] and Jack M. Widholm[1]

[1]*Department of Crop Sciences, University of Illinois, 1201 W. Gregory Dr, Urbana, IL 61801, USA*
[2]*Department of Food Science and Technology, Yeungnam University, 214-1, Daedong, Kyongsan, Kyongbuk 712-749, Korea*

Received March 20, 2001

A simple protocol for the detection of opines, cucumopines and mikimopines using a general horizontal or vertical get electrophoresis system for protein or DNA separation in the laboratory are demonstrated. This electrophoresis method can also be applied to other opines as long as correct detection reagent and buffer system are used.

Key words: *electrophoresis, opines,* Agrobacterium rhizogenes.

Bacteria of the *Agrobacterium* genus are Gram-negative, soil microbes, which incite crown gall (*A. tumefaciens*) or hairy root (*A. rhizogenes*) diseases in various of dicotyledonous plants. Both bacteria infect the wound sites and transfer T-DNA from the bacteria to the plant cell. Integration and expression of this DNA in the plant genome lead to the development of the crown gall or hairy root phenotype[1)] and synthesis of novel amino acid derivatives known as opines[2)] that were not present in normal tissues of the same plant. *In vitro* culture of hairy root lines resulting from inoculation with *A. rhizogenes* has been exploited during the last decade for many purposes including fundamental research on the effects of the *rol* genes on root formation,[3)] the production of secondary metabolites,[4)] foreign gene expression,[5)] and studies of root interaction with pathogens.[6)]

Opines are small, water-soluble, generally stable, ionizable molecules, carrying characteristic functional groups (guanido, amino, glycol, acid, etc.). Transformed plant materials from a variety of sources may be screened for the presence of opines. Routine separation of opines from other compounds was achieved through high-voltage (>1000V) paper electrophoresis (HVPE) at 50 to 100 V/cm.[2)] We, here, demonstrate a simple, modified protocol for the detection of opines, cucumopines and mikimopines, using a general horizontal or vertical gel electrophoresis system for protein or DNA separation in the laboratory.

Materials and Methods

A. rhizogenes strains, DC-AR2,[7)] a derivative of strain MAFF301724 (renamed from MAFF03-01724, harboring a mikimopine-type pRi1724) which had been isolated from a diseased melon plant in Japan, and cucumopine strain K599[8)] were used for plant transformation. *A. rhizogenes*-mediated transformations of *Astragalus sinicus* cv. Japan (Yutoubansei Renge; Takayama Seed, Kyoto, Japan)[5,9)] and soybean (*Glycine max*) cultivar Williams 82[6)] for hairy root induction were carried out as described previously. The established hairy root cultures were transferred every 4 to 5 weeks into a fresh MS medium at 26°C in the dark.

Root segments were placed in preweighed Eppendorff tubes. After weighing, the segments (50 - 100 mg) in 6 volumes of distilled water (600 μ*l*/100 mg tissue) were boiled for 10 min, crushed, and centrifuged (1300 × g for 5 min) at room temperature. The supernatant was collected and rotary-evaporated at 40°C under vacuum to yield dried extracts. The extracts were resuspended in 200 μ*l* distilled water per 100 mg of tissues and kept frozen for further studies. Extracts were also prepared from roots of non-transformed seedlings.

The vertical gel electrophoresis system (Model V16, Bethesda Research laboratories, Gaithersburg, MD, USA) and power supply (Model EC600, E-C Apparatus Corporation, St. Petersburg, FL, USA) for general protein or DNA separation were used. The vertical electrophoresis system included a reserve electrophoresis buffer and a tall glassplate against which the filter paper was placed (Fig. 1A), and the electrophoresis tank was filled with an ammonium carbonate (5 g · l^{-1}) buffer adjusted to pH 9.8 with 0.5 N ammonia[2)]. The horizontal electrophoresis system also could be used for the detection of opines (Fig. 1B).

Whatman 3MM filter paper (16 × 26 cm) was prepared (Fig. 2). 3 μ*l* of synthetic cucumopine or mikimopine (1 μg · μl^{-1}) as a standard marker, and 5 μ*l* of 0.1% bromophenol blue as a marker dye were blotted onto the origin of filter paper. Synthetic cucumopine and mikimopine were a kind gifts from Dr. S. K. Farrand (Dept. of Crop Sciences, University of Illinois at Urbana-Champaign) and Dr. A. Isogai (Dept. of Agricultural Chemistry, The University of Tokyo), respectively. 5

*Corresponding author
Phone: 82-53-810-2955; Fax: 82-53-816-7365
E-mail: hjecho@uiuc.edu

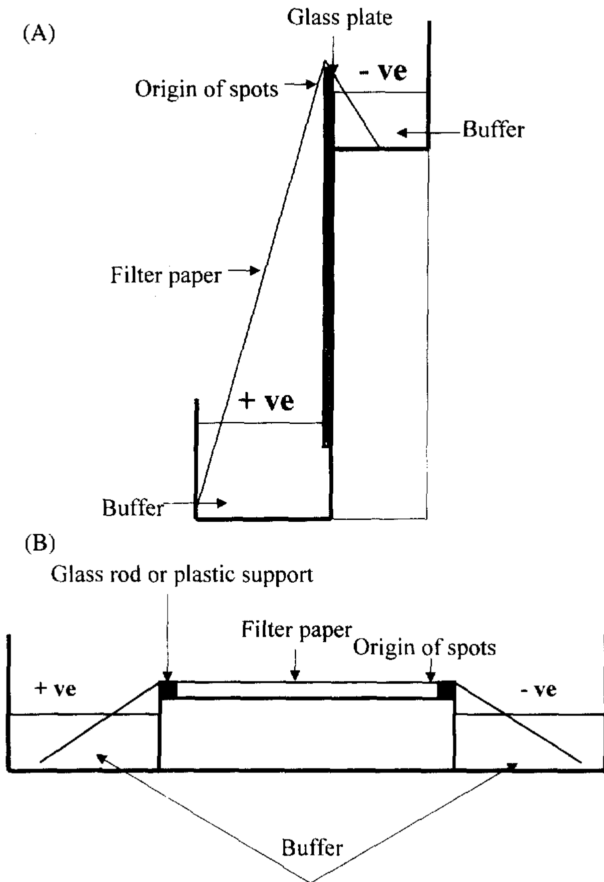

Fig. 1. Layout of vertical (A) or horizontal (B) paper electrophoresis.

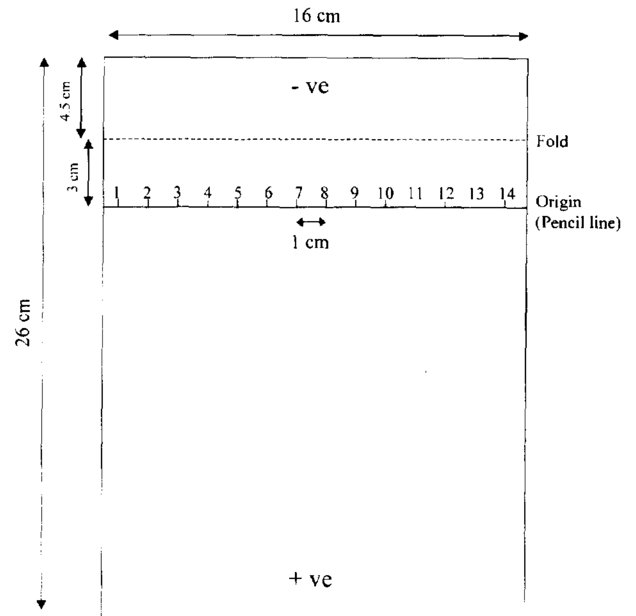

Fig. 2. Layout of an opine paper electropherogram showing the origin where samples are spotted.

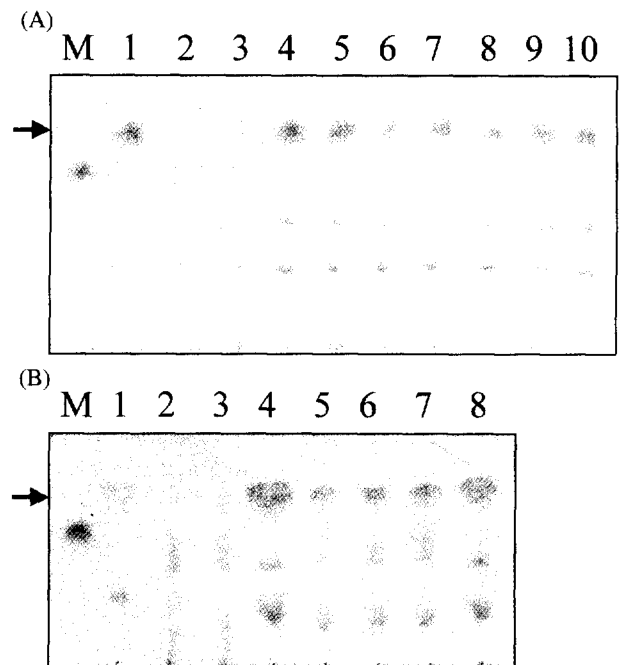

Fig. 3. (A) Detection of cucumopine in extracts of soybean hairy root lines transformed with *A. rhizogenes* K599 following paper electrophoresis. M, Bromophenol blue; 1, authentic cucumopine; 2 and 3, untransformed control roots; 4 to 10, transformed hairy roots. (B) Detection of mikimopine in extracts of *A. sinicus* hairy root lines transformed with *A. rhizogenes* DC-AR2. M, Bromophenol blue; 1, authentic mikimopine; 2 and 3, untransformed control roots; 4 to 8, transformed hairy roots.

μ*l* of the extract was placed onto the origin in spots 1 cm apart and dried using a hot-air dryer. Both sides of the filter paper were immersed in an electrophoresis buffer in separate trays avoiding the 1-cm segment above the origin. Ends of the paper were then placed into the two electrophoresis tanks with the origin at the cathode (-) end, making sure that only the ends of the paper were fully immersed in the buffer. The origin of the filter paper was wet by the diffused buffer within a minute, and the power supply was activated to a constant voltage of 500 V, through it could as well be set at any point within the range of 100 to 500 V depending upon the power supply capacity. Electrophoresis was continued until the bromophenol blue marker moved 3 to 4 cm from the origin, generally for 20 min at 500 V, and the filter paper was thoroughly dried using a hot air dryer.

Visualization of cucumopine and mikimopine was achieved using the sulfanilic acid/sodium nitrite (Pauly's) reagent.[11] Preparation of the reagents is summarized in Table 1. The dried filter paper was lightly sprayed with sulfanilic acid/ sodium nitrite reagent using a Chromist air spray unit (Gelman Instrument Company, Ann Arbor, MI, USA). It was immediately sprayed with the sodium carbonate solution until spots appeared, and allowed to dry in a fume hood. Cucumopine and mikimopine reacted immediately with the Pauly reagent resulting in full development.

Results and Discussion

The results of an electropherogram with cucumopine and mikimopine are shown in Fig. 3. Cucumopine and mikimo-

pine spots gave colors ranging from bright orange to intense pink. The negative control (lanes 2 and 3) did not contain cucumopine and mikimopine. This reaction, though detects the imidazole moiety of the molecule, is not highly specific. Thus, compounds other than histidine, such as tyrosine and indole, could also be detected. An opine standard or extract from known transformed cells should be included with the samples since plant extracts contain various amino acids. Plant tissues also could be smashed with glass rod directly on the origin of filter paper, but the extraction method described above gave better results. This electrophoresis method can also be applied to other opines as long as correct detection reagent and buffer system are used.

Since Sigma-Aldrich Co. supplies only mannopine, cucumopine and mikimopine can be synthesized[10] by dissolving 1 g of histidine and 1.25 g of α-keto glutaric acid in 5 m*l* of 2 M NaOH, and incubating the mixture for 4 h at 80°C. The products, cucumopine and mikimopine, can be purified through HPLC, but the crude mixture can also be used as a standard marker for showing a single spot containing both compounds in a buffer (>pH 2.0) without further purification.

The reporter or selectable markers genes such as β-glucuronidase (*gus*), green fluorescent protein (*gfp*), neomycin phosphotransferase (*nptII*), and hygromycin phosphotransferase (*hpt*) are used routinely for detection or selection of transgenic events in plant cells. However, the simple detection of opines from plant cells or hairy roots transformed by wild type *A. tumefaciens* or *A. rhizogenes* are also useful tools for confirming the transgenic event of T-DNA insertion from the Ri or Ti plasmid.

References

1. Grant, J. E., Dommisse, E. M., Christey, M. C. and Conner, A. J. (1991) Gene transfer to plants using *Agrobacterium. In Advanced Methods in Plant Breeding and Biotechnology,* Murray, D. R. (ed.) pp. 50-73, CAB International, Wallingford.
2. Petit, A., David, C., Dahl, G. A., Ellis, J. G., Guyon, P., Casse-Delbart, F. and Tempé, J. (1983) Further extension of the opine concept: Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Mol. Gen. Genet.* **19**, 204-214.
3. Lemcke, K. and Schmülling, T. (1998) Gain of function assays identify non-*rol* genes for *Agrobacterium rhizogenes* TL-DNA that alter plant morphogenesis or hormone sensitivity. *Plant J.* **15**, 423-433.
4. Nin, S., Bennici, A., Roselli, G., Mariotti, D., Schiff, S. and Magherini, R. (1997) *Agrobacterium*-mediated transformation of *Artemisia absinthium* L. (wormwood) and production of secondary metabolites. *Plant Cell Rep.* **16**, 725-730.
5. Cho, H-J., Brotherton, J. E., Song, H-S. and Widholm, J. M. (2000) Increasing tryptophan synthesis in a forage legume *Astragalus sinicus* by expressing tobacco feedback-insensitive anthranilate synthase (ASA2) gene. *Plant Physiol.* **123**, 1069-1076.
6. Cho, H-J., Farrand, S. K., Noel, G. R. and Widholm, J. M. (2000) High efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. *Planta* **210**, 195-204.
7. Isogai, A., Fukuchi, N., Hayashi, M., Kamada, H., Harada, H. and Suzuki, A. (1990) Mikimopine an opine in hairy roots of tobacco induced by *Agrobacterium rhizogenes. Phytochem.* **29**, 3131-3134.
8. Savka, M. A., Ravillion, B., Noel, G. R. and Farrand, S. K. (1990) Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode. *Phytopathol.* **80**, 503-508.
9. Cho, H-J., Widholm, J. M., Tanaka, N., Nakanishi, Y. and Murooka, Y. (1998) *Agrobacterium rhizogenes*-mediated transformation and regeneration of the legume *Astragalus sinicus* (Chinese milk vetch). *Plant Sci.* **138**, 53-65.
10. Tanaka, N. (1990) Detection of opines by electrophoresis. *Plant Tissue Culture Lett.* **7**, 45-47
11. Dawson, R. M. C., Elliot, D C., Elliot, W H. and Jones, K. M. (1968) In *Data for Biochemical Research*, pp. 530, Clarendon Press, Oxford.