Plant Cell Reports (1985) 4: 74-77

Plant Cell Reports
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Infection of turnip and radish storage roots with *Agrobacterium rhizogenes*

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Received October 1, 1984 / Revised version received February 20, 1985 – Communicated by J. Widholm

ABSTRACT

Within about 10 days after inoculation with Agrobacterium rhizogenes, the vascular bundles of storage root disks of turnip or radish developed small outgrowths with numerous root hairs. Thereafter, adventitious roots (hairy roots) emerged extensively from these outgrowths. The hairy roots which emerged fully supported the growth of host plants, though they lacked geotropism. An excised hairy root could be subcultured as an axenic root culture in the absence of phytohormones. Hairy root cultures with extensive lateral branches grew much more rapidly than those with few lateral branches or ordinary roots. Calli were induced from hairy root cultures in the presence of 2,4-D, and root proliferation from these calli occurred in the absence of 2,4-D. Both the primary hairy roots and the roots which grew from them synthesized agropine and mannopine.

INTRODUCTION

Agrobacterium rhizogenes is the causative agent of the so-called hairy root disease of plants (Elliot 1951). The presence of a root-inducing plasmid (pRi) (Moore et al. 1979; White and Nester 1980) differentiates A. rhizogenes from other gram-negative soil bacteria. Integration of a DNA segment (T-DNA) of pRi into the host genome leads to active proliferation of adventitious roots (hairy roots) at the site of infection (Chilton et al. 1982; White et al. 1982), though the mechanism of integration remains unknown.

An axenic root culture can be established from hairy roots excised from infected plants using a solid or liquid medium without phytohormones (Spanò et al. 1981). Calli derived from hairy roots incite root proliferation in the absence of phytohormones and can be induced to differentiate into complete plants in the presence of suitable phytohormones (Spanò et al. 1981; Spanò and Costantino 1982). Hairy roots as well as in vitro regenerated plants harbour T-DNA within their genomes, and they synthesize T-DNA determined specific compounds, opines (Tepfer and Tempé 1981; Petit et al. 1983; David et al. 1984; Tepfer 1984).

Plant cell transformation by A. rhizogenes has so far been investigated with carrot, tomato and tobacco. The results of the present study indicate that storage roots of turnip and radish are also eligible plant materials for such studies.

MATERIALS AND METHODS

Bacterial strain. Agrobacterium rhizogenes A4 strain (agropine type) (Petit et al. 1983) was obtained from Dr. G. Strobel of Montana State University, U. S. A.. The bacteria were grown in a liquid Difco potato dextrose medium.

Inoculation. All procedures were carried out under aseptic conditions. Fresh storage roots of turnip (Brassica rapa L.) and radish (Raphanus sativus L.) purchased from a local market were soaked in a 2% solution of sodium hypochlorite for 10 min followed by thorough washing with sterile distilled water. They were cut into disks and ca. 0.5 ml of a bacterial suspension (10^8 bacteria/ml) was spread on the upper surface of each root disk. The inoculated root disks were placed on a 1% agar plate with the inoculated surface up and incubated at 25°C in darkness.

Establishment of hairy root culture. After about 2 weeks of incubation, terminal pieces of about 15 mm long were excised from emerging hairy roots and transferred to a 1% agar Murashige-Skoog medium (Murashige and Skoog 1962) containing 3% sucrose and no phytohormones (MS). After 2-3 days of incubation at 25°C in darkness, terminal pieces of actively growing roots without bacterial and fungal contamination were transferred to a liquid MS medium. They were subcultured every 3 or 4 weeks.

Callus formation and root proliferation. A piece of cultured hairy root was transferred onto a 1% agar Linsmaier and Skoog medium (LS) (Linsmaier and Skoog 1965) supplemented with 2% sucrose and 0.5 mg/l 2,4-D and incubated at 25°C. For induction of root proliferation, the calli derived from hairy root cultures were transferred to a solid LS

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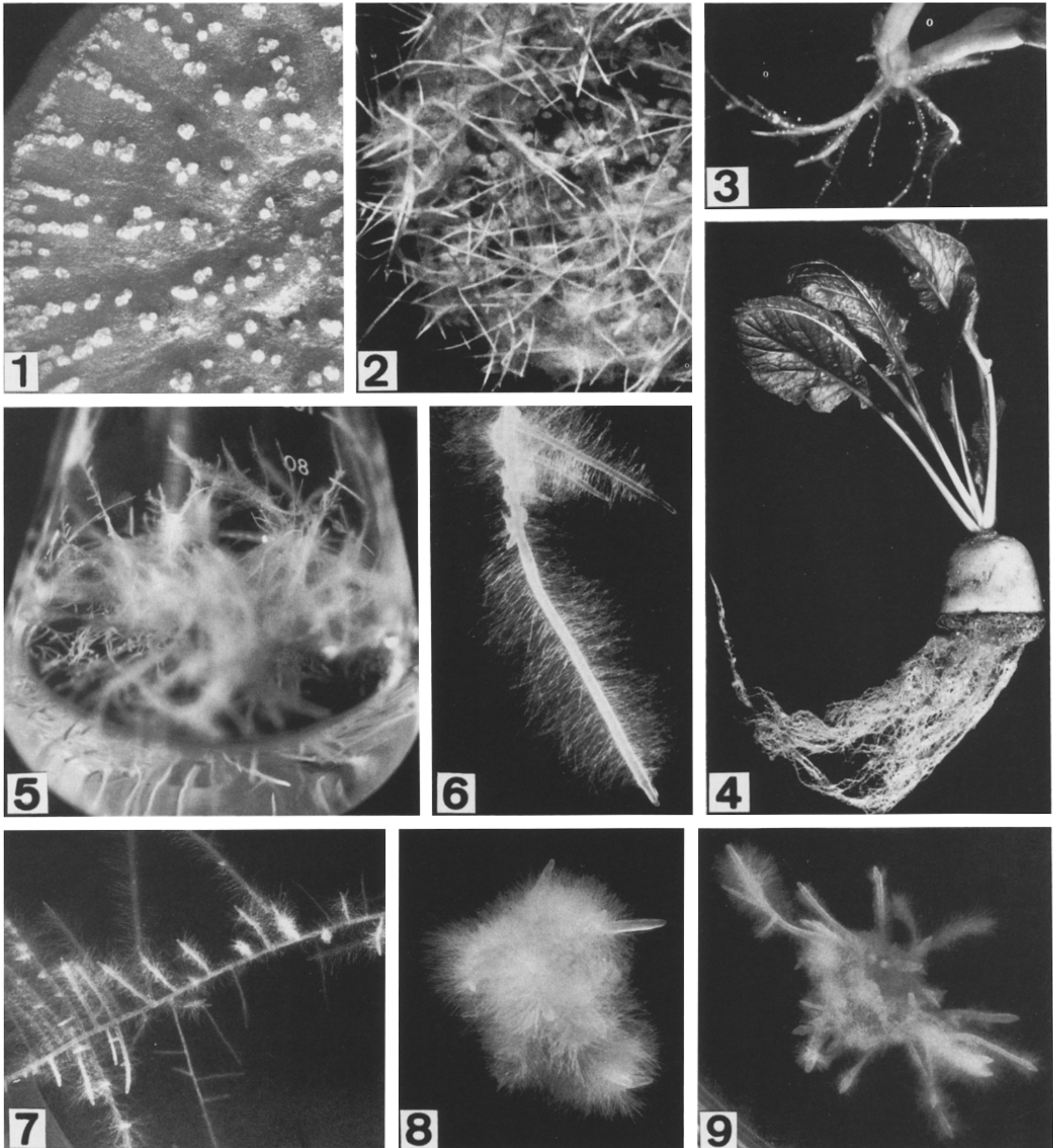

Fig. 1. Outgrowths on radish storage root disk (about 80 mm diam. x 15 mm thick). Photographed 7 days after inoculation with A. rhizogenes.

Fig. 2. Hairy roots emerged from woolly knots on turnip root disk (about 50 mm diam. x 15 mm thick). Photographed 11 days after inoculation.

Fig. 3. Hairy roots emerged from radish hypocotyl. Photographed 14 days after inoculation.

Fig. 4. Turnip root disk with woolly knots and shoot tips cultured for 2 months. Note developed hairy roots of about 24.5 cm long and mature leaves.

Fig. 5. Turnip hairy root culture in a liquid medium. Note aerial root growth.

Fig. 6. Detailed view of turnip hairy root.

Fig. 7. Turnip hairy root with extensive lateral branching. The root has been subcultured for about 2 years.

Fig. 8. Woolly callus derived from turnip hairy root.

Fig. 9. Roots emerged from woolly callus derived from turnip hairy root.

medium without 2,4-D and incubated at 25°C in darkness.

Opine assay. Agropine and mannopine synthesized in hairy roots were analyzed by paper electrophoresis and thin-layer chromatography on cellulose. The standard samples of agropine and mannopine were prepared according to Tate et al. (1982). The structure of both compounds was confirmed by field-desorption mass spectrometry (Hitachi DF/GC/MS M-80) (Tate et al. 1982). Paper electrophoresis was carried out according to Petit et al. (1983). In brief, 1.0 g of the cultured hairy root were homogenized with 50 ml of 70% ethanol and centrifuged at 15,000 x g for 10 min. The supernatant was evaporated, and the residue was dissolved in water (0.1ml/g tissue). The extract and the standard samples were spotted on Whatmann 3MM paper and electrophoresed at 10 V/cm, 150 min in the buffer consisting of formic acid/ acetic acid/water (30/60/910:v/v/v). The dried chromatogram was stained with an alkaline silver nitrate reagent. The root extract and the standard samples were also spotted on a 0.1 mm cellulose HPTLC plate (Merck) and developed with 80% ethanol. The dried plate was stained with an alkaline silver nitrate reagent.

RESULTS AND DISCUSSION

Hairy root proliferation. In both turnip and radish, the vascular bundles of the inoculated surface of the storage root disks began to protrude after 5-7 days of inoculation with *A. rhizogenes*. These protrusions further developed into small tumor-like outgrowths (Fig. 1) with numerous root hairs (woolly knots) within 2-3 days. Thereafter, an adventitious root emerged vigorously from each woolly knot (Fig. 2). Neither tumor-like outgrowths nor adventitious roots appeared on the uninoculated root disks. When the hypocotyl of a turnip or radish seedling was inoculated with the bacteria through wounding with a needle, no woolly knots appeared on the hypocotyl surface. Instead, hairy roots emerged directly from the wound site (Fig. 3). Moore et al. (1979) considered that the pericycle tissue is essential for root proliferation induced by this bacterium.

Characteristics of hairy roots and their calli. When the turnip root disks with woolly knots on one side and only shoot tips on the other were planted in moist vermiculite, the emerging hairy roots fully supported their growth for over 3 months with the shoot tips developing into mature leaves (Fig. 4). No degenerative change was prominent on hairy roots, root disks and leaves. Thus, it is clear that the induced hairy roots are as functional as ordinary roots.

Since the hairy roots completely lacked geotropism (David et al. 1984), active aerial root growth occurred on a liquid as well as on a solid medium (Figs. 5 and 6). Hairy root culture lines each derived from a single piece of explant exhibited extensive or few lateral branching. This was also observed with hairy root cultures of other plants including Leguminosae, Solanaceae and Umbelliferae (unpublished observation). Hairy root culture lines with extensive lateral branching grew more rapidly than those with little lateral branching or than ordinary roots. For example, a turnip hairy root culture line with extensive lateral branching (Fig. 7) showed ca. a 10-fold increase in fresh weight over ordinary roots in 20 days of culture. These phenotypic characters of each root culture line were stably maintained through successive subculturing.

When cultured hairy roots were transferred to a solid LS medium with 2,4-D, callus gradually formed after about 3 weeks. The calli which developed were soft and light brown and could be maintained as undifferentiated masses as long as 2,4-D was present in the medium. However, when callus pieces were transferred to a medium lacking 2,4-D, they began to be covered with root hairs in 7-10 days (Fig. 8) and subsequently roots emerged (Fig. 9). Regeneration of complete plants from hairy roots is currently being carried out.

Opine synthesis by hairy roots. Five lateral roots of a primary hairy root culture of turnip were excised and were cultured independently for 4 weeks. They were designated as R1 roots. R2 roots comprised cultured lateral roots derived from R1 roots and so forth. Extracts from all R1 roots and R6 roots were analyzed for opine synthesis. They all contained the compounds showing the same mobilities by paper electrophoresis and thin-layer chromatography on cellulose as authentic agropine and mannopine, and also reduced the silver nitrate reagent (Figs. 10 and 11). On the other hand, these compounds were absent in uninoculated turnip roots. Since the opine synthesis of *A. rhizogenes* -infected plants is encoded by T-DNA of pRi (Chilton et al. 1982; White et al. 1982), the present results indicate the stable maintenance of T-DNA in these hairy roots.

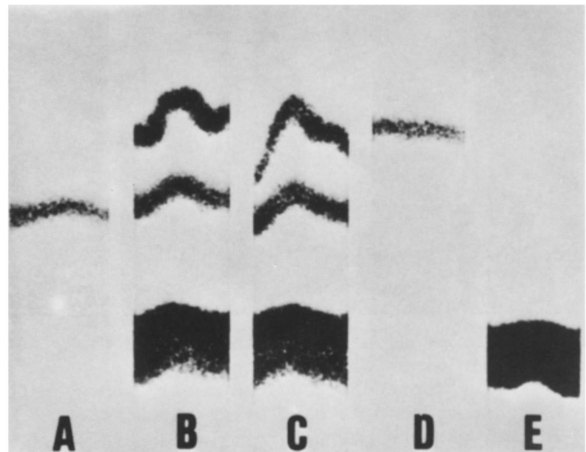

Fig. 10. Paper electrophoretic analysis of extracts from primary and subcultured hairy roots of turnip. Lane A: authentic mannopine; lane B: R1 root (primary hairy root); lane C: R6 root (subcultured hairy root) ; lane D: authentic agropine; lane E: ordinary root.

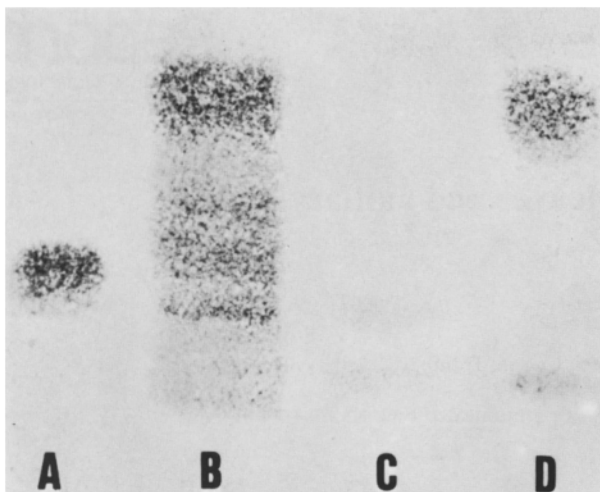

Fig. 11. Thin-layer chromatographic analysis of extracts from hairy and ordinary roots of turnip. Lane A: authentic mannopine; lane B: primary hairy root; lane C: ordinary root; lane D: authentic agropine.

In conclusion, A. rhizogenes can be regarded as a natural engineer capable of endowing plants with rooting potential. In view of the results of the present study, the following new technologies would be envisaged. The fact that hairy roots can fully support the growth of host plants indicates that inoculation of A. rhizogenes may become of practical use to increase the shoot mass of agriculturally important crops. For several solanaceous plants, in fact, inoculation of this bacterium has given rise to plants with about 1.5 times thicker shoots and roots in fresh weight. In addition, these plants revealed drought tolerance as pointed out by Moore et al. (1979) (unpublished observation).

Furthermore, the stable maintenance of phenotypic characters such as vigorous growth and extensive lateral branching in subcultured hairy roots suggests the possibility of replacing conventional callus culture (undifferentiated tissue culture) with hairy root culture (differentiated tissue culture) in production and biotransformation of secondary metabolites. Cultured hairy roots of several solanaceous plants have been found to produce alkaloids in 1-3 times higher amounts than ordinary roots (in preparation).

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