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Regeneration of horseradish hairy roots incited by *Agrobacterium rhizogenes* infection

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ABSTRACT

Surface-sterilized leaf disks of horseradish (Armoracia lapathifolia) were immersed in a suspension of Agrobacterium rhizogenes harboring the root-inducing plasmid (pRi) and cultured on a solid medium. Within about 10 days after inoculation, adventitious roots (hairy roots) emerged from the leaf disks. No roots emerged from the uninoculated leaf disks. The excised hairy roots grew vigorously in the dark and exhibited extensive lateral branches in the absence of phytohormones. When the hairy roots were moved into the light, numerous adventitious buds thrust out of the roots within about 10 days, and they developed into complete plants (R0 generation). R0 plants revealed leaf wrinkle. Root masses of cultured R0 plants were of two types. One had fibrous roots only and the other had both fibrous and tuberous roots. Leaf disks of the R0 plants proliferated adventitious roots (R1 generation) on a solid medium after 1-2 weeks of culture. Phenotypical characters of the R1 roots were the same as those observed with the initial hairy roots. The T-DNA sequences of pRi were detected within DNA isolated from the hairy roots and their regenerants.

INTRODUCTION

In contrast to an in vitro grown excised normal roots, hairy roots, which are adventitious roots derived from cells transformed by the root-inducing plasmid (pRi) (Moore et al. 1979; White and Nester 1980a) of Agrobacterium rhizogenes, showed more vigorous growth and extensive lateral branches in the absence of phytohormones (Spanò et al. 1981; Tanaka et al. 1985; Mano et al. 1986). These phenotypical characters of hairy roots provided a new practical technology which allows the production of specific chemicals such as tropane alkaloids by hairy root cultures of Scopolia japonica (Mano et al. 1986). The hairy root harbors a DNA segment (T-DNA) of pRi within its nuclear genomes (Chilton et al. 1982; Spanò et al. 1982; White et al. 1982; Willmitzer et al. 1982; Slightom et al. 1985; Taylor et al. 1985; Boulanger et al. 1986), and this T-DNA codes enzymes which direct the synthesis of novel amino acid derivatives known as opines (Tepfer and Tempè 1981; Petit et al. 1983).

The studies on regeneration of hairy roots indicated that complete plants could be obtained from hairy roots of tobacco (Ackermann 1977; Tepfer 1984; Taylor et al. 1985), carrot (Tepfer 1984), morning glory (Tepfer 1984), potato (Ooms et al. 1985), oilseed rape (Guerche et al. 1987) and N. plumbaginifolia (Jouanin et al. 1987). Since the T-DNA also has morphological consequences, these regenerants showed various phenotypical alterations (Ackermann 1977; Tepfer 1984; Ooms et al. 1985; Taylor et al. 1985; Guerche et al. 1987; Jouanin et al. 1987). Thus, pRi can be regarded as a tool in the study of plant mutation.

In the present paper, we report horseradish hairy roots, their unique regeneration and characteristics of pRi-transformed regenerants.

MATERIALS AND METHODS

Plant. A fresh root tuber of horseradish (Armoracia lapathifolia Gilib.) was purchased from a market and planted in moist vermiculite. Newly developed leaves were used for bacterial inoculation.

Bacteria. Agrobacterium rhizogenes agropine type strain A4 (Tanaka et al. 1985) was grown for 24 hr at 25°C in a liquid LB medium (Maniatis et al. 1982a) with sucrose (10g/l) in place of NaCl.

Inoculation and pre-incubation. Bacterial inoculation was carried out by a modification of the procedure of Horsch et al. (1985). Disks (6 mm in diam.) were punched from the surface-sterilized leaves (Tanaka et al. 1985) and immersed in a bacterial suspension (about 10^9 cells/ml). After gentle shaking for about 10 min, the leaf disks were placed on a sterilized filter paper to remove excess bacterial suspension. The inoculated leaf disks were placed upside-down on a 1% agar plate or a sterilized moist filter paper

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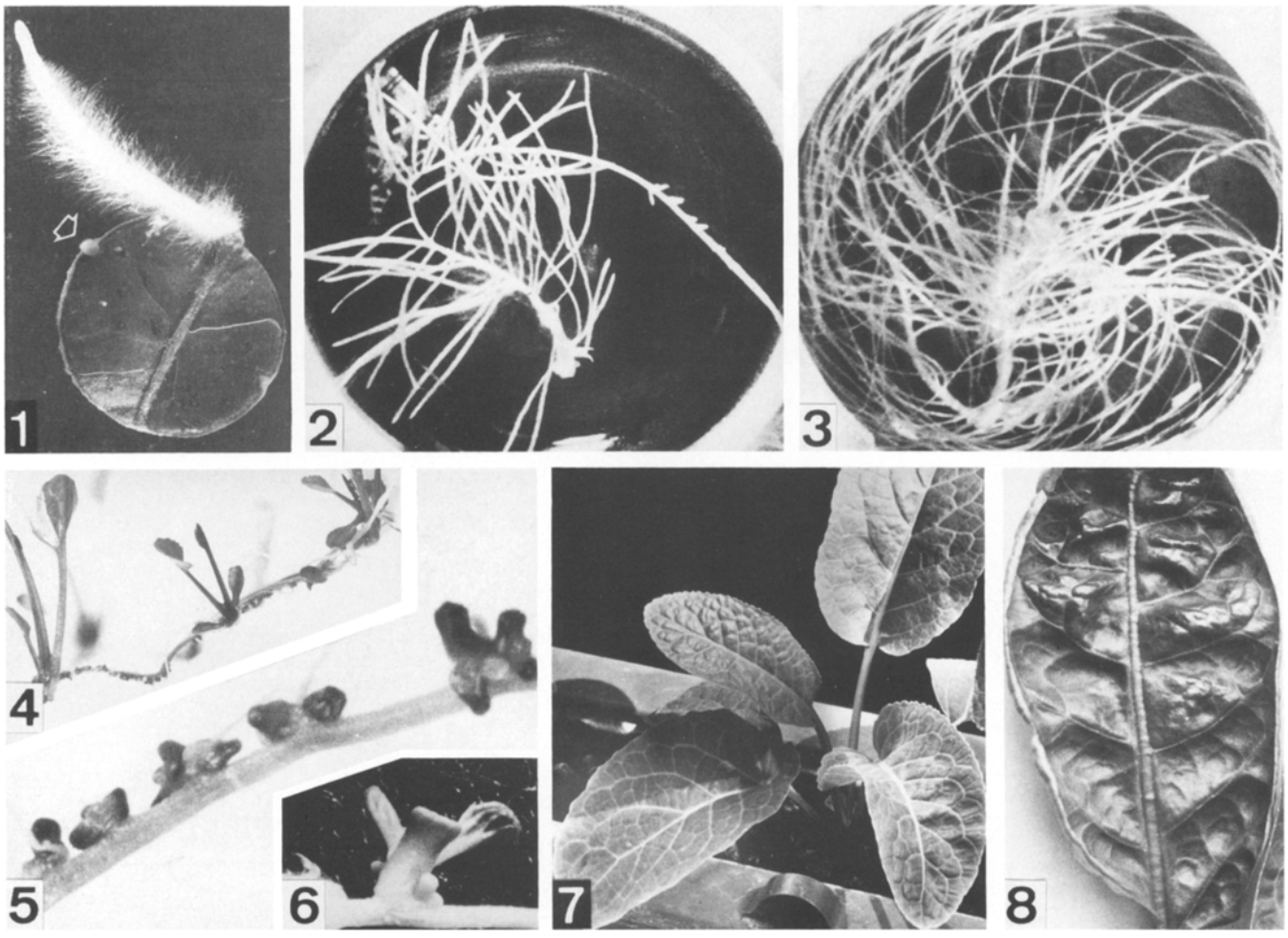


Fig. 1. General view of hairy root emerged from A. rhizogenes-inoculated leaf disks of horseradish. An arrow indicates a fine outgrowth. Photographed 14 days after inoculation.
Figs. 2 and 3. Hairy roots cultured in a flask. The root (Fig. 2) was cultured in MSB medium with 3% sucrose, vitamins and no phytohormones for 10 days (Fig. 3).
Fig. 4. General view of hairy root with plantlets and numerous adventitious buds cultured in MSB medium with 3% sucrose. Photographed 20 days after transfer into the light.
Fig. 5. Detailed view of hairy root with adventitious buds.
Fig. 6. Detailed view of adventitious bud thrust out of the hairy root.
Fig. 7. Regenerant of hairy root.
Fig. 8. Leaf wrinkle of regenerant.

and incubated at 25°C under cool white fluorescent light ca. 2Klx.

Post-incubation. After 3 days of pre-incubation, the inoculated leaf disks were transferred to a 1% agar Murashige-Skoog base medium (MSB medium) (Murashige and Skoog 1962) with 3% sucrose, vitamin mixture, carbenicillin (500 μg/ml), vancomycin (200 μg/ml) and no phytohormones. After about 2 weeks of incubation at 25°C in the light, terminal pieces of 10-15 mm long were excised from actively growing hairy roots without fungal and bacterial contamination. The excised root pieces were transferred to a liquid MSB medium with 3% sucrose, vitamins, no antibiotics and no phytohormones. The root pieces were cultured at 25°C in the complete dark.

Regeneration of hairy roots. The hairy roots cultured in the dark were moved into the light. After several days, root pieces with adventitious buds was excised and placed on a 1% agar MSB medium with 3% sucrose. Plantlets were transferred to soil or water culture.

Cloning of T-DNA probe. E. coli DH1 harboring pBANK210 (Nishiguchi and Oka 1986) was kindly supplied by Dr. A. Oka of Inst. Chem. Res., Kyoto University. The pBANK210 of 41.43Kb was constructed with pHC79 cosmid vector, HindIII fragments 3, 7, 15, 20, 22, 29 and 33 of pRiA4b (Huffman et al. 1984). The fragments 3, 15 and 22 covered the right T-DNA region of pRiA4b. The pBANK210 was digested with HindIII and electrophoresed through 1% agarose gels. The HindIII fragment 22 of 3.7Kb was recovered by electroelution and cloned as T-DNA probe using pUC18 vector (Yanisch-Perron et al. 1985).

Isolation of leaf DNA and Southern blot hybridization. Leaf DNA was isolated from regenerants of hairy roots or intact plants according to White et al. (1982). The leaf DNA was digested with HindIII, electrophoresed through 0.8% agarose gels and transferred to nitrocellulose using 10 X SSC (Maniatis

et al. 1982b). Southern hybridization was carried out using the T-DNA probe, the cloned HindIII fragment 22 which was labeled by nick-translation in the presence of biotin-11-dUTP (Leary et al. 1983).

RESULTS AND DISCUSSION

Hairy root proliferation. During the pre-incubation, it was expected that the transformation of leaf cells was established by pRi of the inoculated bacteria which adhered and grew on the cut ends of leaf disks. No bacterial growth was observed on the agar plate or filter paper on which the inoculated leaf disks were placed. Presumably, lack of nutrients in the agar plate or filter paper inhibited growth of bacteria which adhered to the leaf surface. These bacteria were apparently unavailable for transformation.

The point aimed at during post-incubation was the establishment of axenic hairy root cultures. Within 1 week after bacterial inoculation, fine outgrowths appeared on the cut ends of the leaf disks (Fig. 1). Subsequently, these outgrowths developed into hairy roots with numerous root hairs (Fig. 1). The outgrowths were exclusively detected on the cut ends of lateral veins and veinlets. No roots emerged from the uninoculated leaf disks incubated under the same culture conditions as those of the inoculated ones.

Characteristics of hairy roots. Horseradish hairy roots excised from the leaf disks grew vigorously and exhibited extensive lateral branches when they were cultured in the dark (Figs. 2 and 3). A representative hairy root culture line showed ca. a 100-fold increase in fresh weight in 1 month of culture, and this phenotypical character could be stably maintained through successive cultures. In contrast, a normal root culture line derived from horseradish callus showed ca. a 4-fold increase under the same culture conditions as those of the hairy roots.

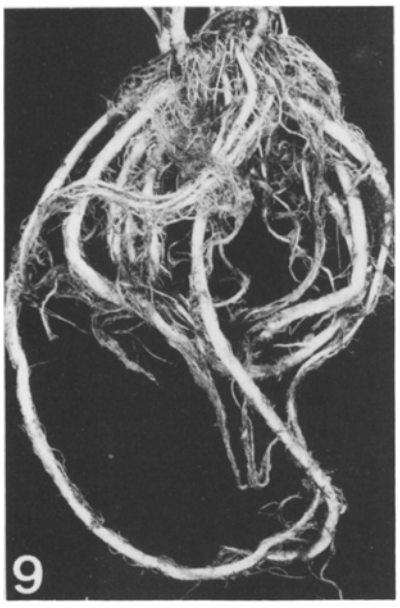
Regeneration of hairy roots. When the hairy roots cultured in the dark were transferred into the light, regeneration occurred throughout the entire root except the apical tips within about 1 week (Fig. 4). In fact, numerous adventitious buds thrust out of the root surface without visible callus formation (Figs. 5 and 6), though the mechanism of light-induced regeneration of hairy roots remains unknown. Five-ten adventitious buds occurred on each hairy root of 10 mm length. Each bud excised from the hairy root developed into a complete plants (R0 generation) (Fig. 7). On the other hand, after about 1 month of transfer into the light, adventitious buds occasionally emerged from normal roots excised from a callus.

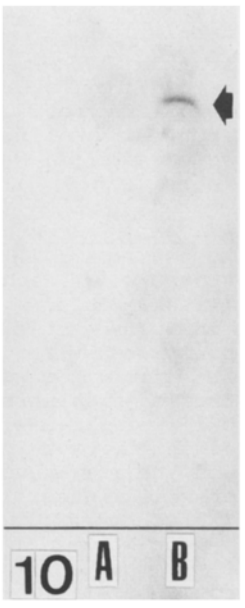
Characteristics of regenerants. Leaf wrinkle associated with pRi-transformed regenerants of tobacco, morning glory and oilseed rape (Ackerman 1977; Tepfer 1984; Slightom et al. 1985; Taylor et al. 1985; Guerche et al. 1987) was also prominent in leaves of some R0 plants (Fig. 8). Taylor et al. (1985) demonstrated that the integration of left T-DNA region of pRi into the plant genomes was responsible for the leaf wrinkle. After about 6 months of soil or water culture, root masses of R0 plants were of two types. One was characterized by an abundant proliferation of fibrous roots only and the other had both fibrous and tuberous roots (Fig.9).

T-DNA sequences of pRi in regenerants. According to the opine assay by paper electrophoresis and thin-layer chromatography (Tanaka et al. 1985), neither agropine nor mannopine could be detected in horseradish hairy roots and their R0 plants. In general, it is apt to be considered that opine synthesis of plants necessarily associated with pRi- or pTi-transformation. However, because of unstable opine synthesis of hairy roots and their regenerants without apparent change of the integrated T-DNA sequences, Tepfer (1984) commented that opine synthesis is a poor marker for pRi-transformation. A similar conclusion was already presented for nopaline synthesis associated with pTi-transformation (Hepburn et al. 1983). Mano et al. (1986) also reported no opine synthesis in some clones of S. japonica hairy roots. Thus, to determine whether T-DNA sequences of pRiA4b were actually integrated within DNA of horseradish hairy roots or their R0 plants in which no opine synthesis was detected, hybridization was examined between the cloned T-DNA sequences and DNA isolated from hairy roots (data not shown) or their R0 plants. As shown in Fig. 10, leaves of R0 plant contained the HindIII fragment 22 derived from right T-DNA region of pRiA4b. This T-DNA sequences are also included in the tms sequences of pTi T-DNA (Huffman et al. 1984). No hybridization was observed between such HindIII fragment and DNA of normal horseradish leaves.

Root proliferation from regenerated leaves. In the previous studies on regeneration of hairy roots (Ackermann 1977; Tepfer 1984; Slightom et al. 1985; Taylor et al. 1985; Guerche et al. 1987; Jouanin et al. 1987), no proliferation of adventitious roots from the regenerated leaves was reported. After 1-2 weeks of incubation on MSB medium with sucrose, vitamins and no phytohormones, adventitious roots emerged from uninoculated leaf disks of R0 plants (Fig. 11). Their phenotypical characters such as vigorous growth with extensive lateral branches, stable maintenance as roots in the dark, easy regeneration in the light and leaf wrinkle of regenerants were the same as those of the initial hairy roots.

In the present study, A. rhizogenes agropine type strain 15834 (Petit et al. 1983), mannopine type strain 8196 (Petit et al. 1983), cucumopine type strain NCPPB2659 (Petit et al. 1986) and 11325 strain were also used as the inocula. The results associated with strain 15834, 8196 or NCPPB2659 inoculation were the same as those of strain A4. In contrast, slender woolly knots (Fig. 12) or calli emerged from leaf disks inoculated with strain 11325. Their growth was slower than the hairy roots and adventitious roots emerging from these outgrowths





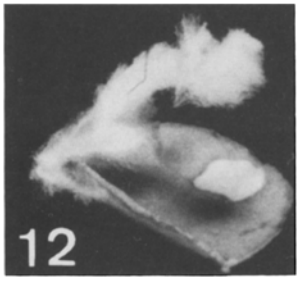


Fig. 9. Root tubers of regenerant.

Fig. 10. Southern blot hybridization between HindIII fragment 22 (an arrow) of pRiA4b T-DNA and leaf DNA of a normal plant(A) or regenerant of hairy root (B).

Fig. 11. Adventitious roots emerging from an uninoculated leaf disk of R0 plant. Photographed after 15 days of incubation.

Fig. 12. Slender woolly knot emerging from a leaf disk inoculated with strain 11325 of A. rhizogenes.

were fragile. These roots revealed poor lateral branches and no plant regeneration under the same conditions as those of hairy roots.

The strain 11325 is regarded as the type strain of A. rhizogenes in Bergey's Manual (Kersters and De Ley 1984). However, White and Nester (1980b) and Petit et al. (1983) demonstrated that the outgrowths incited by strain 11325 infection not only synthesized nopaline as an opine but also this bacteria was able to degrade nopaline. Thus, Petit et al. (1983) commented that A. rhizogenes 11325 strain should be reclassified as a nopaline strain of A. tumefaciens. At any rate, taxonomic descriptions of the species of Agrobacterium in Bergey's Manual (Kersters and De Ley 1984) should be revised on the basis of their native plasmid background, because the species names such as tumefaciens or rhizogenes were originated from their pathogenicity, and both these bacteria do not owe their pathogenicity to their chromosomal background such as biovars but to their plasmids.

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