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No Requirement of Vernalization for Flower Formation in Ri-Transformed *Cichorium* Plants

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Hairy roots of *Cichorium intybus* L. cv. Witloof were induced by inoculation with *Agrobacterium rhizogenes* harboring different types of Ri plasmids (pRiA4, pRi15834 and pRi8196). Whole plants were regenerated from the hairy roots by culturing them on a modified Murashige and Skoog's medium containing BA (1 mg/*l*). Transformation was confirmed by the presence of agropine and mannopine in the hairy roots and of internal fragments of the T-DNA of Ri plasmid in the leaves of the regenerated plants. These tansgenic plants flowered without vernalization under a long-day condition, but normal (nontransformed) plants did not show any flower bud formation under the same condition. *Rol*C gene was cloned into T-DNA of a disarmed mini Ti plasmid (pBI101) and transformation was achieved by the leaf disc method. The transgenic plants with *rol*C gene flowered without vernalization under a long-day condition.

Introduction

Infection of dicotyledonous plants by *Agrobacterium rhizogenes* harboring Ri plasmid results in adventitious root formation at the infected sites[1]. The adventitious roots, called hairy roots, can grow well with numerous lateral roots in phytohormone-free medium after the elimination of bacteria[2,3]. Recently, several researchers reported the regeneration of entire plants from hairy roots in some plant species[4,5]. The transgenic plants showed an altered phenotype, called hairy-root syndrome[6,7]. The invariable and characteristic traits of the hairy-root syndrome in tobacco are; (1) rapid growth rate of roots in culture; (2) reduced apical dominance; (3) wrinkled leaves with increased width to length ratio; and (4) plagiotropism in roots. These morphogenic changes are due to the integration of a portion (T-DNA) of the Ri plasmid into plant genomic DNA[2,3,8]. The T-DNA of agropine type Ri plasmid consists of two non-continuous streches of DNA, TL- and TR-DNA[9,10]. The TR-DNA contains two gene homologous to the genes encoding IAA synthesizing enzymes of Ti plasmid, but only plays a conditional and non-essential role in the hairy root formation[11]. On the other hand, 4 loci involved in the hairy root formation have been identified through insertional mutagenesis in the TL-DNA[12]. None of the root loci, known as *rol* A, B, C and D, showed any homology with the T-DNA of Ti plasmid[9].

Recently, it was demonstrated that the integration of each of the root loci (*rol* A, B and C) into tobacco cells resulted in morphogenic alterations of the transgenic plants, such as dwarfism and wrinkled leaves[13-15]. Transgenic plants with entire T-DNA of Ri plasmid or *rol*C gene showed early flowering in a day-neutral cultivar of tobacco[16]. However, transgenic tobacco plants with

chimeric *rol*C gene under the control of cauliflower mosaic virus (CaMV) 35S promoter did not show early flowering[14]. On the other hand, carrot, which requires low temperature treatment for flower differentiation, was transformed with entire T-DNA of Ri plasmid and the transformed plants exhibited flowering without cold treatment[6]. However, nontransformed carrot plants occasionally exhibited precocious flowering during plant regeneration in *in vitro* culture without cold treatment. Thus it remains to be clarified whether Ri-transformed biennial plants could flower without low temperature treatment. *Cichorium intybus* is known to require low temperature treatment for flower formation[17] and in this paper, the flowering response of transgenic *Cichorium intybus* plants with T-DNA of Ri plasmid and *rol*C gene is examined.

Materials and Methods

Induction and culture of hairy roots

Sterilized seeds of *Cichorium intybus* L. cv. Witloof were germinated under a 18 h light (4, 000 lux)/6 h dark condition (18L/6D) at 25°C. A modified Murashige and Skoog's (MS) semisolidified (0. 3 % Gelrite) medium was used for seed germination in which the concentrations of macro-elements and nicotinic acid were reduced to 1/2 and 1/10, respectively, and no phytohormones were added (hereafter referred to as 1/2MS medium)[18]. Three strains of *Agrobacterium rhizogenes* (A4, 15834 and 8196) were used for hairy root induction. The bacteria grown on YEB agar medium[19] were inoculated onto leaves of 4-week-old *Cichorium* seedlings. Two to 5 weeks after the inoculation, tip segments of hairy roots appeared at the inoculated site were excised and cultured on phytohormone -free 1/2MS medium containing Claforan (0. 5 g/*l*). After several transfers to 1/2MS medium with Claforan, growing hairy roots were transferred to phytohormone-free semisolidified (1. 2 % agar) root culture (RC) medium[20] without the addition of antibiotics. The root cultures were maintained under continuous darkness at 25°C and subcultured at monthly intervals.

Plant regeneration

Axenic hairy roots were cultured on 1/2MS (1. 2 % agar) medium containing BA (1 mg/*l*). After 1 to 3 months, adventitious buds differentiated from the roots were excised and transferred to phytohormone-free 1/2MS (1. 2 % agar) medium. In a control experiment, segments of leaves and roots of nontransformed (normal) plants were cultured on 1/2MS (1. 2 % agar) medium with BA (1 mg/*l*). Adventitious buds differentiated from the leaves or roots after 1 to 3 months were excised and transferred to phytohormone-free 1/2MS (1. 2 % agar) medium. All cultures were incubated at 25°C under a 18h light/6h dark condition.

Construction of a plasmid with rolC gene and plant transformation

Construction of a recombinant plasmid was carried out according to the standard methods described earlier[21]. A plasmid (pHIRc) containing only *rol*C gene was obtained by inserting the 4240 base pairs (bp) Hind III fragment of TL-DNA of pRiA4 in the multi-cloning site of the binary vector pBI101[22] (**Fig. 1**). The newly constructed plasmid was transferred from *E. coli* strain to *A.*

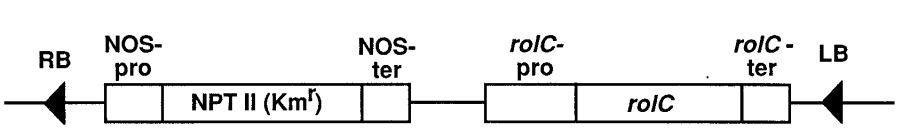

Fig. 1 A schematic drawing of T-DNA of the constructed plasmid (pHIRc) with *rol*C gene.
The plasmid (pHIRc) with *rol*C gene was obtained by inserting the 4240 bp *Hind*III fragment of TL -DNA of pRiA4 in the multi-cloning site of the binary vector pBI101. pro: promoter, ter: terminator, NOS: nopaline synthase gene, RB and LB: right and left border of T-DNA, NPTII: neomycin phosphotransferase II gene.

tumefaciens strain LBA4404 harboring Ti plasmid without T-DNA by the triparental method described earlier[23].

Transformation of *C. intybus* with the newly constructed plasmid containing *rol*C gene was carried out according to the leaf disc method described ealier[24]. Plant regeneration was achieved by culturing the leaf discs on 1/2MS (1.2 % agar) medium containing BA (1 mg/*l*) and Claforan (0.5 g/*l*). Transformants exhibiting kanamycin-resistance were obtained by culturing the adventitious buds on 1/2MS (1.2 % agar) medium containing 100 mg/*l* of kanamycin. The plants were then transplanted to phytohormone-free 1/2MS medium without Claforan and kanamycin.

Detection of opines

Opines were separated by high voltage paper electrophoresis and detected by an alkaline silver nitrate reagent[25].

Southern blot analysis

Total genomic DNA was isolated from leaves of the regenerated plants according to the method described earlier[26]. Purified DNA was digested with *Bam*HI, electrophoresed through a 0.7 % agarose gel and transferred to a nylon membrane (GENE SCREEN, NEN Co., LTD.) according to the supplier's protocol. Southern blot hybridization was carried out with pLJ1 including the entire TL-DNA of pRiA4[10] as a probe. Probe DNA was labelled with [^{32}P]dCTP using Multiprime labelling system (Amersham).

Results

Induction and culture of hairy roots

Two to 5 weeks after inoculating *A. rhizogenes* (A4, 15834 and 8196), numerous adventitious roots appeared at the inoculated sites in the leaves. The axenic root cultures obtained grew well with the production of numerous lateral roots on RC medium (**Fig. 2-A**).

To confirm transformation, the presence of opines in extract of the axenic roots was examined. In the axenic roots obtained by the inoculation with agropine type strains (A4 and 15834), both agropine and mannopine could be detected (data not shown). On the other hand, only mannopine could be detected in the roots induced with a mannopine type strain (8196). Neither mannopine nor

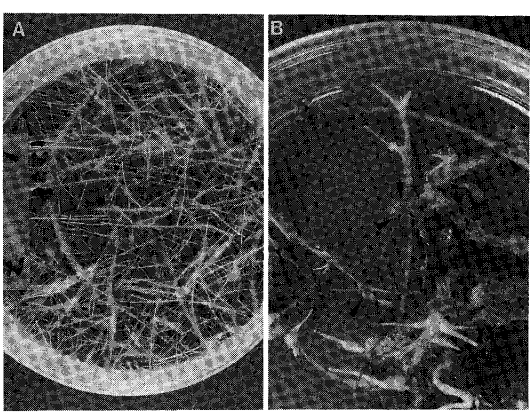

Fig. 2 A: A hairy root transformed with *A. rhizogenes* A4 strain.
The hairy root was cultured for 1 month on hormone-free RC medium at 25°C in continuous darkness.
B: Adventitious buds formed on the hairy roots transformed with *A. rhizogenes* A4 strain.
Hairy roots were cultured for 1 month on 1/2MS medium supplemented with BA (1 mg/*l*) at 25°C under a 18L/6D condition. Arrowheads indicate the differentiated buds.

agropine was detected in roots of nontransformed plants.

Plant regeneration from hairy roots

When axenic hairy roots were cultured on 1/2MS medium containing BA (1 mg/*l*) for 1 to 3

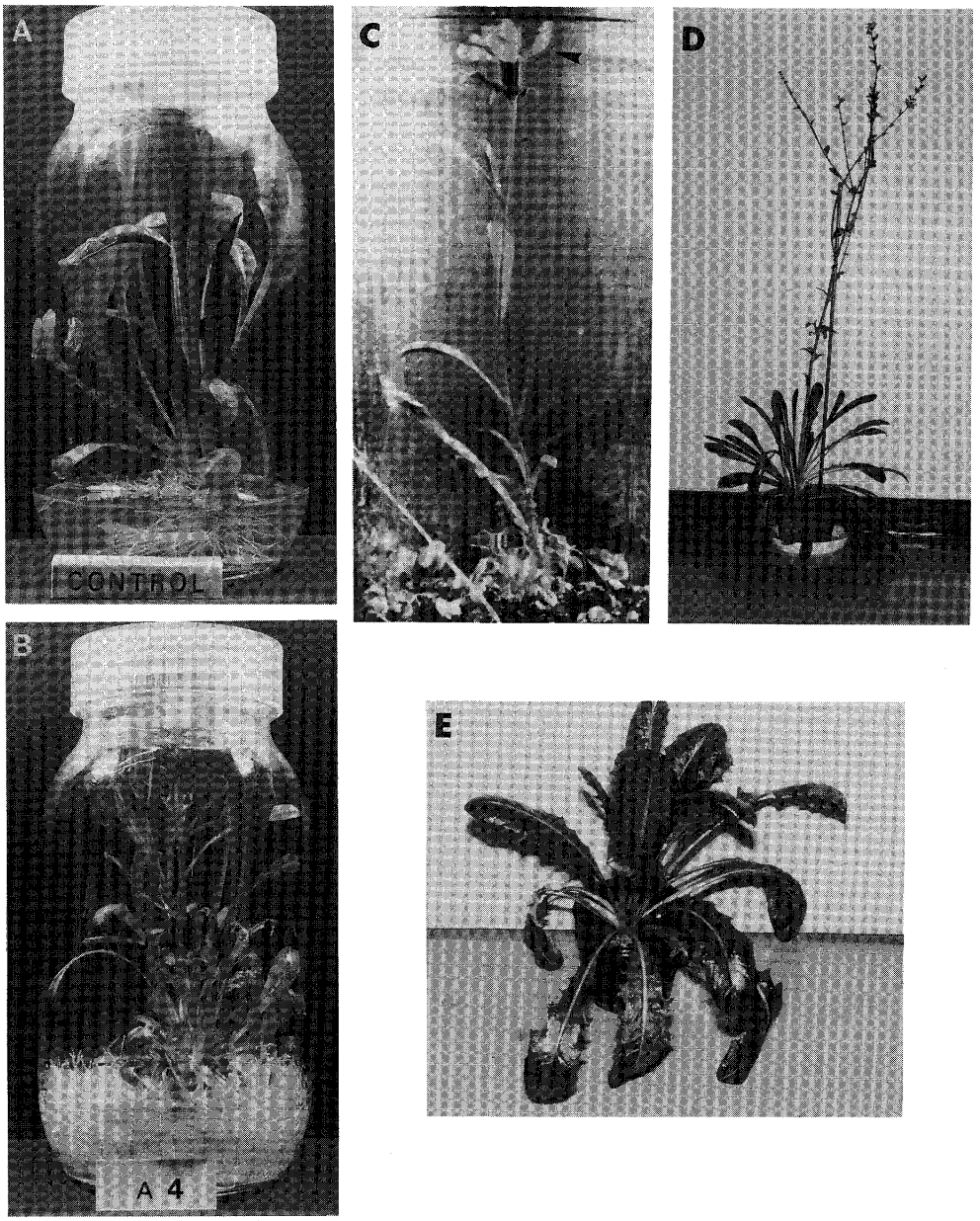

Fig. 3 Regenerated plants from hairy roots.
All plants were cultivated at 25°C under a 18L/6D condition. A: A control plant regenerated from a leaf of nontransformed plant. B: A plant regenerated from a hairy root transformed with *A. rhizogenes* A4 strain showing good root growth and flower bud formation. C: A plant regenerated from a hairy root transformed with *A. rhizogenes* A4 strain showing stalk elongation and flower bud formation. An arrowhead indicates a differentiated flower. D: A regenerated plant from a hairy root transformed with *A. rhizogenes* 15834 strain. The plant was cultivated for 3 months in soil and showed stalk elongation and flower formation. E: A control plant regenerated from a leaf of nontransformed plant. The plant was cultivated for 3 months in soil and showed no flower formation.

months, adventitious buds were differentiated from the roots (**Fig. 2-B**). The buds were excised and transferred to phytohormone-free 1/2MS medium. After 1 to 3 weeks, rooted plants were obtained. The roots grew well, but the leaves did not show any morphological alterations as compared to those of nontransformed plants (**Fig. 3-A, B**). Flower formation was observed in almost all the regenerated plants when they were cultured at 25°C under a 18L/6D condition regardless of the strains of bacteria used (Fig. 3-C). The regenerated plants were then transplanted to soil and cultivated at 25°C under a 18L/6D condition in a culture room. These plants were observed to form flowers continuously under these conditions (**Fig. 3-D**).

Total genomic DNA was extracted from leaves of the plants regenerated from hairy roots, digested with *Bam*HI and analyzed by Southern blot hybridization. When pLJ1 containing entire TL-DNA of agropine type Ri plasmid (pRiA4) was used as a probe, internal fragments including TL-DNA could be detected in the plants transformed with agropine type strains (A4 and 15834) (**Fig. 4**).

Plant regeneration from nontransformed plants

When the segments of roots or leaves of nontransformed plants were cultured on 1/2MS medium containing BA (1 mg/*l*), adventitious buds were differentiated after 1 to 3 months. One to 3 weeks after the transfer of the buds to phytohormone-free 1/2MS medium, rooted plants were obtained. Morphology of the regenerated plants was similar to that of *Cichorium* plants derived from seeds. More than 100 nontransformed plants were tested and none of them showed flower formation at 25°C under a 18L/6D condition (**Fig. 3-A**). The regenerated nontransformed plants were transferred to soil and grew under similar conditions; none of them developed elongated stalk or showed any flower formation (**Fig. 3-E**).

Transformation with rolC gene

A. tumefaciens atrain LBA4404 containing the newly constructed plasmid with *rol*C gene (pHIRc) was co-cultured with leaf discs for 2 days before transfer to a medium containing BA and Claforan as described in Materials and Methods. A large number of adventitious buds were formed after 1

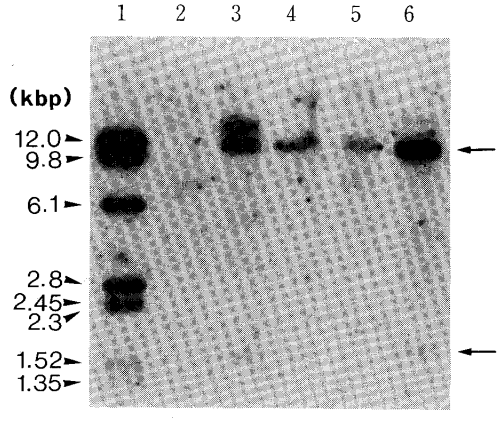

Fig. 4 Southern blot analysis of DNA isolated from transformed and nontransformed plants.
Total genomic DNA isolated from leaves was digested with *Bam*HI, separated electrophoretically on an agarose gel and transferred to a nylon membrane. Hybridization was carried out with pLJ1 (a cosmid clone containing entire TL-DNA of agropine type Ri plasmid) as a probe. Lane 1: pLJ1, Lane 2: nontransformed plant, Lanes 3 and 4: regenerated plants from 2 different clones of hairy roots transformed with *A. rhizogenes* A4 strain, Lanes, 5 and 6: plants individually transformed with *A. rhizogenes* 15834 strain. Arrows indicate internal fragments of TL-DNA of the Ri plasmid and arrowheads indicate the size (kbp) of fragments of pLJ1.

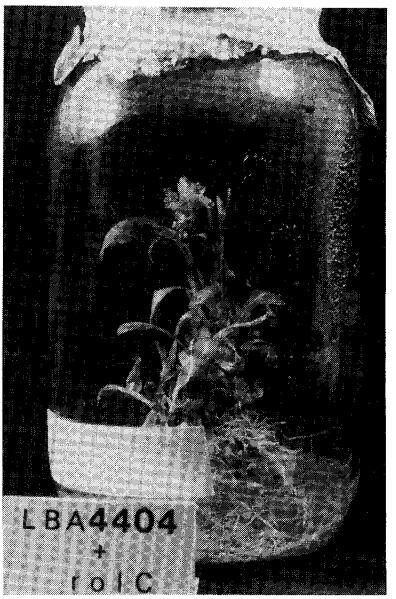

Fig. 5 A plant transformed with pHIRc.
Leaf discs co-cultivated with *A. tumefaciens* LBA4404 harboring pHIRc were cultured on 1/2MS medium containing BA (1 mg/*l*) and Claforan (0. 5 g/*l*). Differentiated buds from the leaf discs were transferred to phytohormone-free 1/2MS medium with Claforan (0. 5 g/*l*) and then elongated shoots were transferred to phytohormone-free 1/2MS medium with kanamycin (100 mg/*l*). The plants exhibiting kanamycin-resistance was cultured at 25°C under a 18L/6D condition. After 1 to 2 months, the plants exhibited stalk elongation and flower bud formation.

month and the plants regenerated from the buds were subcultured on 1/2MS medium with Claforan at 25°C under a 18L/6D condition. After total elimination of bacteria, the regenerated plants were transferred to 1/2MS medium containing 100 mg/*l* of kanamycin for 1 to 2 months. Kanamycin-resistant green plants were then transplanted to phytohormone-free 1/2MS medium without antibiotics; flowering was observed from such plants grown at 25°C under the long-day condition (**Fig. 5**).

Discussion

In this report, we showed that Ri-transformed plants of *Cichorium intybus* could easily be obtained through the culture of hairy roots transformed with various types of Ri plasmids. Recently, it was reported that tobacco cells transformed with Ri plasmid showed higher sensitivity to phytohormones, especially to IAA as compared to nontransformed tobacco cells[27,28]. However, in *Cichorium*, the same concentration of cytokinin (BA, 1 mg/*l*) is effective for regeneration of plants from hairy roots as well as normal roots and leaves. Experience with some other plant species also revealed that required concentrations of phytohormones (auxins and cytokinins) for regeneration of plants from hairy roots and from nontransformed tissues could be similar[4]. Based on these results, the higher sensitivity to IAA observed in Ri transformed tobacco cells[27,28] might be an indirect effect of T-DNA genes or an exceptional phenomenon observed only in tobacco.

Regenerants from hairy roots generally exhibit certain morphological alterations such as shortened-stem, wrinkled leaves with increased width to length ratio and high growth rate of roots in culture[6,7]. These phenotypic traits were induced by the action of *rol* loci in the TL-DNA delivered to the plant genome[6,13,29]. Transgenic *C. intybus* plants had a well growing root system compared

to nontransformed plants, but no morphological alteration was observed in leaves (**Fig. 3**). More interestingly, *Cichorium* plants transformed with entire TL-DNA of agropine type Ri plasmid (pRiA4 and pRi15834) exhibit flower formation without vernalization (**Fig. 3**). A similar phenomenon was observed in the plants transformed with mannopine type Ri plasmid (pRi8196) containing a T-DNA homologous to TL-DNA without the genes for the synthesis of IAA. Field or pot-grown *C. intybus* plants generally require vernalization and long-day condition for flower formation[17]. However, Ri transformed *C. intybus* plants cultivated in pots showed flower formation without vernalization under a long-day condition (**Fig. 3**). Similarly, carrot plants normally require vernalization for flower differentiation, but transgenic plants with entire T-DNA of Ri plasmid exhibited flowering without cold treatment[6]. These results clearly show that vernalization was not required in Ri-transformed plants of some plant species for flower formation. Furthermore, transgenic *Cichorium* plants with *rol*C gene also showed flower formation without vernalization. It had been reported that *rol*C protein acted like cytokinin glucosidase[30]. However, plant regeneration in nontransformed *Cichorium* was achieved by cytokinin treatment in this report, and the regenerated plants did not show flower formation. This result may suggest that *rol*C protein had another physiological function for flower formation. Further detailed studies are required in order to fully explain the results obtained in this work, especially for those concerned with flowering.

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《和文要約》

Riプラスミド形質転換チコリの花芽形成におけるバーナリゼーションの非要求性

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三種の異なった型（pRiA4，pRi15834，pRi8196）のRiプラスミドを含む毛根病菌の感染によってチコリ（*Cichorium intybus*）の毛状根を得た．この毛状根をBA（1 mg/*l*）を含むMS培地で培養したところ植物個体が再分化した．形質転換は，毛状根におけるアグロピンとマンノピンの存在及び再分化植物の葉におけるRiプラスミドT-DNAの内部断片の存在によって確認した．このトランスジェニック植物は長日条件下においてバーナリゼーション無しで花芽分化したが，非形質転換個体では同じ条件下でも花芽分化を示さなかった．一方，Riプラスミドの*rol*C遺伝子のみを遺伝子導入ベクター（pBI101）にクローン化し，リーフディスク法によってチコリに導入したところ，このトランスジェニック植物では，長日条件下においてバーナリゼーション無しで花芽分化した．