Identification of *rol* Genes on pRi1724 in *Agrobacterium rhizogenes* Strain MAFF 03-01724 Isolated in Japan

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Abstract

Agrobacterium rhizogenes strain MAFF 03-01724 isolated from a diseased melon plant bears pRi1724, a mikimopine-type of hairy-root-inducing plasmid. The 9.5-kb region of pRi1724 showed the root-inducing ability toward leaf disks of *Ajuga reptans* and *Nicotiana tabacum*. This region contained sequences highly homologous to each of the *rolA*, *rolB*, and *rolC* genes of the agropine-type hairy-root-inducing plasmid pRiA4b. The relative position of the three homologs was the same as that of pRiA4b. These results indicate that pRi1724 carries genes both functionally and structurally equivalent to the *rolA*, *rolB* and *rolC* genes of pRiA4b.

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INTRODUCTION

The soil phytopathogenic bacterium *Agrobacterium rhizogenes* causes the hairy root syndrome in many dicotyledonous plants³⁾. The neoplastic roots induced upon infection contain a portion of bacterial plasmid DNA. The causative plasmid is termed hairy-root-inducing plasmid (pRi). The T-DNA includes genes essential for root induction and for synthesis of unique amino acid derivatives called opine¹⁴⁾. In the case of agropine-type pRiA4b, it is known that four genes (*rolA*, *rolB*, *rolC*, and *rolD*) are required for root induction²⁰⁾. The *rolB* gene codes for a β -glucosidase capable of hydrolyzing indole- β -glucosides, leading to the increase of active auxin level by release of auxins from its inactive β -glucosides in transgenic plants⁵⁾. The *rolC* gene encodes another β -glucosidase that hydrolyzes cytokinin- β -glucosides⁴⁾. Other *rol* gene products have not yet been characterized biochemically. Moreover, molecular mechanisms of initiation of root induction by these *rol* gene products have not been elucidated.

A. rhizogenes strains, MAFF 03-01724 to 03-01727 (carrying pRi1724 to 1727, respectively), have been isolated from diseased melon plants in Japan¹⁷⁾, and characterized as a producer of a new opine called mikimopine⁸⁾, a stereoisomer of cucumopine²⁾. We previously constructed pRi1724 gene libraries with the plasmid and cosmid vectors, and characterized its T-DNA region¹⁹⁾. During course of the study, it was found that pRi1724 contains DNA regions highly homologous to the core T-DNA region of pRiA4b. Since the pRiA4b core T-DNA contains the four *rol* genes²⁰⁾, pRi1724 appears to have functionally similar genes.

In this article, we show the precise mapping of the *rol* gene homologs on pRi1724 by Southern-blot hybridization experiments, and demonstrate that DNA fragments carrying these homologs can be independently identified by means of initiation of hairy roots on leaf disks by infection with *A. tumefaciens* harboring binary vectors bearing test DNA fragments.

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MATERIALS AND METHODS

General methods. As described were culture media, chemicals, enzymes, and procedures for transformation of *Escherichia coli*, bacterial conjugation, preparation of plasmid DNA, DNA cleavage with restriction enzymes, purification of DNA fragments, and ligation of DNA fragments^{7,10,16,19}. Electrophoresis of DNA was carried out on 0.7% agarose gel for 4 hr at 80 V in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 7.6).

Bacteria and plasmids. *Agrobacterium rhizogenes* strain MAFF 03-01724 isolated from a diseased melon plant in Japan¹⁷ was used in the present study. *A. tumefaciens* strain LBA4404 (Toyobo Co., Ltd.) was used for infection on plants. The *E. coli* strain JM109²¹ was used for construction of recombinant plasmids. The *E. coli* strain RK2013 (Toyobo) was used as a helper bacterium for plasmid transfer from *E. coli* strain JM109 to *A. tumefaciens* strain LBA4404. Plasmids described below were introduced to these bacterial strains by transformation as needed. Plasmids used were a binary vector pBIN19 (Toyobo) and *E. coli* cloning vectors pUC18 (ampicillin-resistant) and pHSG298 (kanamycin-resistant) (Takara). The recombinant plasmid pRIB106, a clone in plasmid library, carried a portion of the pRi1724 T-DNA on the *Bam*HI site of pBR329 as described in our previous paper¹⁹.

Plasmids carrying a portion of the pRi1724 T-DNA region are shown in Fig. 1. Their vectors were pBIN19 for the three pRTBI plasmids (pRTBI5, 9, and 19), and pHSG298 for pRTB plasmids (pRTB5, 9, and 19) and pRTE7.6. The plasmid pRIB106 carries a portion of the pRi1724 T-DNA consisting of the three *Bam*HI fragments with sizes of 13.5 kb, 9.5 kb, and 5.5 kb¹⁹ (Fig. 1). Its subclones, pRTB5, pRTB9, and pRTB19 contained 13.5-kb, 9.5-kb, and 5.5-kb *Bam*HI fragments, respectively, on pHSG298 (Fig. 1). These plasmids such as pRTBI5, pRTBI9, and pRTBI19 were the same as these subclones except that their vectors were pBIN19. Another pRIB106 subclone was pRTE7.6, which was made by joining pHSG298 with a 7.6-kb *Eco*RI fragment (see Fig. 1).

Both of pB330D and pB216D were deletion derivatives of cosmid clones, pBANK330 and pBANK216, carrying a portion of the pRiA4b T_L-DNA¹³. Recombinant plasmids of pROLA, pROLB, and pROLC were constructed by cloning their subfragments on pUC18, and used for *rolA*, *rolB*, and *rolC* probes, respectively. The DNA regions carried by these clones are indicated in Fig. 2.

Southern-blot hybridization. An ECL gene detection kit (Amersham) was used for DNA labeling, Southern blot, and detection of target sequences according to the supplier's directions. A nylon membrane (Hybond-N⁺, Amersham) was used for blotting. Chemiluminescence signal bands were

Fig. 1. Restriction map of the vicinity of pRi1724 T-DNA and its cloned fragments. The bar, marked to the upper right, represents 5 kb. The numerals in the restriction map are the DNA sizes (kb) of restriction fragments. The shaded rectangle indicates the 6.4-kb *Eco*RI-*Bam*HI region where three *rol* homologs are located.

Fig. 2. Restriction map of the vicinity of pRiA4b T_L-DNA and its cloned fragments. Shaded rectangles represent the position of T_L-DNA of pRi1724 and its *rol* genes. The bar, marked to the upper right, represents 5 kb. The numerals in the restriction map are the DNA sizes (kb) of restriction fragments. Abbreviations: E, *Eco*RI; H, *Hind*III; P, *Pst*I.

Fig. 3. Hairy roots induced on *Ajuga reptans* by strain LBA4404 carrying pRTBI5, pRTBI9 or pRTBI19. CONT indicates the uninoculated leaf disk. These leaf disks were cultured on MS medium at 25°C for 30 days after inoculation in the dark.

visualized by exposure for 30 sec to an X-ray film (Hyperfilm, Amersham).

***Agrobacterium* inoculation and cultivation of roots.** Leaf disks of *Ajuga reptans* var. *atropurpurea* and *Nicotiana tabacum* were inoculated with *A. tumefaciens* strain LBA4404 derivatives harboring various plasmids by briefly immersing in culture medium as described previously¹¹. At 4

weeks after inoculation, induced roots were cut off from the disks and cultured on Murashige-Skoog's (MS) medium¹²⁾ solidified with 0.2% Gellungum (Wako) in the dark condition.

Detection of mikimopine. Mikimopine synthesized in root tissues was assayed with paper electrophoresis as previously described¹⁸⁾.

Fig. 4. Hairy roots induced on *Nicotiana tabacum* by strain LBA4404 carrying pRTBI5, pRTBI9 or pRTBI19. CONT indicates the uninoculated leaf disk. These leaf disks were cultured as in Fig. 3.

Fig. 5. Detection of *rol* genes on pRi1724 T-DNA by Southern hybridization. The *Bam*HI (lane 2) and *Eco*RI (lane 3) digests of the pRiB106-carriers were hybridized to *rolA* (b), *rolB* (c) or *rolC* (d) probe of pRiA4b, respectively. *Bam*HI and *Eco*RI fragments of pRiB106 were visualized by EtBr staining (a). Numerals on the left are DNA sizes in kb referred from λ /*Hind*III digests (lane 1). The figures on right represent *Bam*HI (9.5) and *Eco*RI (7.6) fragments hybridized with the probes.

RESULTS AND DISCUSSION

Ability of root induction by DNA fragments from pRi1724

A 30-kb region of pRi1724 carried by pRIB106 was extremely close to the core T_L-DNA region of pRiA4b¹⁹. For further dissection of this region, the three *Bam*HI fragments, 13.5 kb, 9.5 kb, and 5.5 kb (Fig. 1) on pRIB106 were separately inserted into pBIN19, and the resulting recombinant plasmids were named pRTBI5, pRTBI9, and pRTBI19, respectively. Each of these three plasmids was introduced into *A. tumefaciens* strain LBA4404 by the triparental mating method¹⁰. *A. reptans* and *N. tabacum* were inoculated with the plasmid-carriers. In the case of *A. reptans*, all of the three plasmid-carriers developed adventitious roots though the pRTBI9-carrier gave more intensive results than the pRTBI5- and pRTBI19-carriers (Fig. 3). These induced roots could be cultured on MS medium for so fairly long period as roots made by infection with the parental *A. rhizogenes* strain 1724 (data not shown). However, growth of the former roots were generally less vigorously than that of the latter roots (data not shown). In the case of *N. tabacum*, adventitious root formation occurred only with the pRTBI9-carrier infection (Fig. 4). In addition, the initiated roots were feeble, and could not be maintained on MS medium. The difference of hairy-root-initiation between these plants may depend upon their original rooting-

Fig. 6. Detection of *rol* genes on the 7.6-kb *Eco*RI fragment of pRi1724. pRTE7.6 was digested by *Eco*RI (lane 2) together with *Bam*HI (lane 3), *Apa*I (lane 4), *Bgl*II (lane 5), *Kpn*I (lane 6), *Nae*I (lane 7) or *Sph*I (lane 8). a: EtBr staining; b-d: hybridization with the *rolA* probe (b), *rolB* probe (c), and *rolC* probe (d). Fragment sizes are shown in the left as in Fig. 5.

Table 1. Restriction fragments of pRTE7.6 by digestion with several enzymes and Southern hybridization for detection of *rol* genes in pRi1724

Probe	Enzyme	Fragments (kb) ^{a)}
<i>rolA</i>	<i>EcoRI</i>	<u>7.6</u>
	<i>EcoRI</i> — <i>Bam</i> HI	<u>6.4</u> , 1.2
	<i>EcoRI</i> — <i>Apa</i> I	2.2, <u>1.9</u> , 1.1, <u>1.0</u> , 0.8, 0.6
	<i>EcoRI</i> — <i>Bgl</i> II	<u>3.2</u> , 2.8, 1.4, 0.2
	<i>EcoRI</i> — <i>Kpn</i> I	<u>2.8</u> , 2.5, 1.2, 1.1
	<i>EcoRI</i> — <i>Nae</i> I	4.1, <u>2.0</u> , <u>1.5</u>
	<i>EcoRI</i> — <i>Spl</i> I	5.0, <u>2.6</u>
<i>rolB</i>	<i>EcoRI</i>	<u>7.6</u>
	<i>EcoRI</i> — <i>Bam</i> HI	<u>6.4</u> , 1.2
	<i>EcoRI</i> — <i>Apa</i> I	2.2, <u>1.9</u> , 1.1, 1.0, <u>0.8</u> , 0.6
	<i>EcoRI</i> — <i>Bgl</i> II	<u>3.2</u> , <u>2.8</u> , 1.4, 0.2
	<i>EcoRI</i> — <i>Kpn</i> I	<u>2.8</u> , <u>2.5</u> , 1.2, 1.1
	<i>EcoRI</i> — <i>Nae</i> I	<u>4.1</u> , <u>2.0</u> , 1.5
	<i>EcoRI</i> — <i>Spl</i> I	<u>5.0</u> , 2.6
<i>rolC</i>	<i>EcoRI</i>	<u>7.6</u>
	<i>EcoRI</i> — <i>Bam</i> HI	<u>6.4</u> , 1.2
	<i>EcoRI</i> — <i>Apa</i> I	2.2, 1.9, 1.1, 1.0, 0.8, 0.6
	<i>EcoRI</i> — <i>Bgl</i> II	3.2, <u>2.8</u> , 1.4, 0.2
	<i>EcoRI</i> — <i>Kpn</i> I	2.8, <u>2.5</u> , 1.2, 1.1
	<i>EcoRI</i> — <i>Nae</i> I	<u>4.1</u> , 2.0, 1.5
	<i>EcoRI</i> — <i>Spl</i> I	<u>5.0</u> , 2.6

a) The numerals with underline represent the fragments hybridized with probes.

responses to infection of strain 1724; that is, the response in *A. reptans* was higher than that in *N. tabacum* (unpublished results). Also, it is likely that strain LBA4404 was difficult to infect *N. tabacum* used in this experiment. Anyhow, the results as described above are consistent with the view that the principal gene responsible for root induction on plants is located in the 9.5-kb *Bam*HI fragment although the accessory functions enhancing the root-inducing ability are directed from both of the 13.5-kb and 5.5-kb *Bam*HI fragments. Besides, it is likely that DNA regions outside of this 30-kb region contains genes which magnify the pathogenic activity. Since mikimopine was not synthesized in the roots induced by the pRTBI-carriers, a gene (or genes) directing synthesis of mikimopine appears to be present outside of the 30-kb region.

Similarity between the pRi1724 and pRiA4b T-DNAs

To identify whether the 30-kb region of pRi1724 T-DNA contains homologs of the *rolA*, *rolB*, and *rolC* genes of pRiA4b, Southern-blot hybridization experiments were done using the DNA fragments carrying each of the three *rol* genes of pRiA4b as probes. The results (Fig. 5) indicated that the 9.5-kb *Bam*HI fragment and the 7.6-kb *Eco*RI fragment are hybridized with all of the three probes. We thus concluded that the three *rol* homologs are located within the 6.4-kb *Eco*RI—*Bam*HI region of pRi1724 (see Fig. 1).

Assignment of three *rol* homologs of pRi1724

In order to precisely localize the pRi1724 *rol* homologs, the 7.6-kb *Eco*RI fragment was cleaved by digestion with either each of *Apa*I, *Bam*HI, *Bgl*II, *Kpn*I, *Nae*I, and *Spl*I (Fig. 6a) or their various combinations (Table 1), and a fine restriction map of the 7.6-kb *Eco*RI fragment was constructed (Fig. 7). After separation by gel electrophoresis, the subfragments were blotted and then hybridized to the three *rol* gene probes as in the preceding section (Fig. 6b–d). The *rolA* probe hybridized with 1.9-kb

Fig. 7. A fine restriction map of the 7.6-kb *EcoRI* fragment of pRi1724 and the assignment of *rol* genes. The numerals in the map represent the fragment sizes (kb). Capital letters in the fragments of map represent DNA hybridized to *rolA* (A), *rolB* (B) and *rolC* (C), respectively. Shaded rectangles indicate the position of *rol* homologs of pRi1724.

(*ApaI*), 3.2-kb (*BglII*), 2.8-kb (*EcoRI-KpnI*), 2.0-kb (*NaeI*) and 2.6-kb (*EcoRI-SphI*) fragments showed strong homology, while 1.0-kb (*ApaI*) and 1.5-kb (*EcoRI-NaeI*) ones showed weak homology (Fig. 6b), indicating that the pRi1724 *rolA* homolog is located on the 1.0-kb *NaeI-SphI* region and probably extends to its left side. The *rolB* probe was close to 1.9-kb (*ApaI*), 3.2-kb (*BglII*), 2.5-kb (*KpnI*), 2.0-kb (*NaeI*) and 5.0-kb (*SphI-EcoRI*) fragments showed strong homology, while 0.8-kb (*ApaI*), 2.8-kb (*BglII*), 2.8-kb (*KpnI*) and 4.1-kb (*NaeI-EcoRI*) ones showed weak homology (Fig. 6c), while the *rolC* probe was similar to 2.2-kb (*ApaI*), 2.8-kb (*BglII*), 2.5-kb (*KpnI*), 4.1-kb (*NaeI-EcoRI*) and 5.0-kb (*SphI-EcoRI*) fragments showed strong homology (Fig. 6d). Therefore, the majority of the pRi1724 *rolB* and *rolC* homologs should be located on the 0.7-kb *KpnI-ApaI* fragment and the 1.0-kb *ApaI-KpnI* fragment, respectively. The organization of these *rol* homologs was the same as that of the three *rol* genes of pRiA4b²⁰. These facts together with the root-inducing ability of the 9.5-kb *BamHI* fragment support the view that pRi1724 bears three genes both functionally and structurally equivalent to the *rolA*, *rolB* and *rolC* genes of pRiA4b.

The presence of *rolA*, *rolB* and *rolC* genes on both pRi1724 found in Japan and pRiA4b found in USA is very interesting. Filetici *et al.*⁶ reported that the highly conserved sequences are present on the T-DNAs of various opine-type Ri plasmids (*e.g.* agropine-type pRi1855¹⁵), mannopine-type pRi8196⁹), and cucumopine-type pRi2659⁹). Though the spread of *A. rhizogenes* is not well understood at present for lack of enough reports on wild isolates, similar bacteria may have distributed widely all over the world. DNA sequences analysis of the pRi1724 T-DNA, which is now in progress, might further shed a light on this regard.

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和 文 摘 要

田中伸和・岡 穆宏：日本産 *Agrobacterium rhizogenes* MAFF 03-01724 株(メロン毛根病菌)の pRi1724 上の *rol* 遺伝子の同定

日本産 *Agrobacterium rhizogenes* MAFF 03-01724 株(メロン毛根病菌)のミキモピン型毛状根誘発プラスミド pRi1724 の T-DNA 上に、アジュガおよびタバコのリーフ・ディスクに毛状根を誘発する 9.5 kb の *Bam*HI 断片を見いだした。この断片上にはアグロビン型毛状根誘発プラスミド pRiA4b 上の毛状根誘発に関与する *rolA*, *rolB* および *rolC* 遺伝子と相同性の高い DNA 配列が存在し、その相対的位置も同様であった。以上のことから、pRi1724 上には、pRiA4b の *rolA*, *rolB* および *rolC* 遺伝子と構造的にも機能的にも同様な遺伝子が存在することが示された。