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Characterization of the *rolB* promoter on mikimopine-type pRi1724 T-DNA

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Abstract

The promoter activity of the *rolB* gene encoded on mikimopine-type pRi1724 (*1724rolB*) was elucidated. Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that *1724rolB* mRNA was highly accumulated in roots of *1724rolB*-transformed tobacco plants. A β-glucuronidase (GUS) fluorometric assay using a *1724rolB* promoter-GUS construct also showed high promoter activity in the roots. A GUS histochemical assay revealed that *1724rolB* was expressed in the vicinity of root apical meristematic cells of the vascular cylinder in the main roots, in lateral root primordial and meristematic cells, in veins of cotyledons, and faintly in the shoot apex. Since the product of *1724rolB* has a N-terminal stretch 17 amino acid longer than the *rolB* of agropine-type pRi1855 (*1855rolB*), primer extension analysis was used to show that the transcription initiation point of *1724rolB* was located beyond the putative TATA box of *1855rolB*, suggesting that the locations of the minimal promoters of the two *rolB* genes differed from each other. *1724rolB* promoter analysis using deletion constructs indicated that the minimal region necessary for this promoter activity was, at most, −215 bp upstream of the translation initiation point. A predominant auxin-responsive sequence was located at position −313 to −216 upstream of the transcription initiation point, containing the *Nicotiana tabacum rolB* domain B factor 1 (NtBBF1) binding motif ACTTTA.

Keywords: *Agrobacterium rhizogenes*; Auxin; *cis*-element; GUS; Ri plasmid; *rolB* gene

1. Introduction

The soil-borne bacterium *Agrobacterium rhizogenes* is the causal pathogen of hairy root disease in dicotyledonous plants. This disease results from the transfer and integration of a segment, transferred-DNA (T-DNA), of root-inducing (Ri) plasmid to the plant genome [1]. Expression of genes encoded on T-DNA in transformed cells results in the proliferation of highly branched, agravitropic roots at the site of inoculation and synthesis of amino acid derivatives called opines [2]. Partial or complete nucleotide sequences of the T-DNA of pRiA4 [3], pRi8196 [4], pRi1724 [5–7] and pRi2659 [8] have been reported, but the agropine-type pRiA4, which is the same plasmid as pRi1855, has been most extensively investigated. Genetic and molecular analyses of pRiA4 have resulted in identification of T-DNA genes, *rooting locus* (*rol*) *A*, *B* and *C* (corresponding to open reading frame (ORF)10, ORF11 and ORF12, respectively), which are the main determinants of hairy root proliferation [9]. On two other Ri plasmids, the mannopine-type pRi8196 and cucumopine-type pRi2659, ORF10, ORF11 and ORF12 are also involved in hairy root induction [4,10]. In particular, of the three Ri plasmids, the *rolB* gene (ORF11) plays a key role in hairy root induction

Abbreviations: GUS, β-glucuronidase; IAA, indoleacetic acid; NtBBF1, *Nicotiana tabacum rolB* domain B factor 1; NAA, naphthaleneacetic acid; ORF, open reading frame; *rolB*, rooting locus B; T-DNA, transferred-DNA

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[4,9,10]. From its adventitious rooting activity, a relationship between *rolB* function and regulation or perception has been presumed. Tobacco cells transformed with the *rolB* gene show an alteration in auxin-induced hyperpolarization of the plasma membrane [11]. RolB protein has been proposed to function as a glucosidase [12], an auxin-binding protein [13] or a tyrosine phosphatase [14]; however, neither direct evidence nor convincing explanations of the relationships between these functions and adventitious rooting have been provided. Very recently, Moriuchi et al. [15] reported that adventitious root induction by the *rolB* gene correlates with the nuclear localization of RolB protein mediated by its interaction with a 14-3-3 family protein, Nt14-3-3 ωII. Since 14-3-3 family proteins usually have roles, such as modulating enzyme activity, mediating or coordinating the formation of protein complexes, and activating transcription, this result suggested that the RolB protein might intervene in the signal network for adventitious rooting [15].

The phytohormone auxin regulates the activity of the promoter of the *rolB* gene in transgenic plants [16]. The promoters of the *rolB* gene of pRi1855 (also known as pRiA4) T-DNA (*1855rolB*) and cT-DNA of *Nicotiana glauca* (Ng*rolB*) have already been analyzed [17–20], and from the results of these analyses, the regions important for the activation of transcription by auxin have been determined [18,19]. A Dof protein, *Nicotiana tabacum rolB* domain B factor 1 (NtBBF1), has been isolated as a *trans*-element that can bind to a *cis*-element ACTTTA sequence contained in the auxin response domain B in the *1855rolB* promoter [21,22]. However, the regulation mechanism of NtBBF1 protein by auxin compounds is still unknown.

The *A. rhizogenes* strain MAFF301724, originally isolated in Japan [23], harbors the mikimopine-type Ri plasmid pRi1724 [24]. The T-DNA of pRi1724 contains the plant oncogenes *rolABC*, ORF13, and ORF14 [5], and mikimopine synthase (*mis*) genes [25]. pRi1724 is the first Ri plasmid of which the complete nucleotide sequence has been determined [7]. Interestingly, it was reported that T-DNA of the mikimopine-type pRi1724-like plasmid seems to be the origin of cT-DNA in the genome of *N. glauca* [26], since the latter contains the *mis* gene [25]. Regarding the oncogenes on the T-DNA of pRi1724, the *rolABC* genes are also involved in hairy root induction [27], and the status of transcription of these genes in two independent *Ajuga reptans* hairy root lines has been reported [28]. The rooting capacity of the *rolB* gene of pRi1724 (*1724rolB*) has also been reported [15], and the differences in growth among hairy root lines have been shown to be caused by variations in the level of *1724rolB* gene expression caused by endogenous indoleacetic acid (IAA) concentration [29]. It appears that there is a level of *1724rolB* expression required for active growth of hairy roots; growth is poor when the level is either too high or too low [29]. Since exogenous auxin promoted adventitious root formation on hairy roots containing T-DNA of pRi1724, similar to those of other Ri plasmid [29], *1724rolB* expression must also be activated by auxin. However, so far, little is known about the *1724rolB* promoter. The 1724RolB protein has an N-terminal stretch 17 amino acid longer than the 1855RolB protein (and also A4RolB protein) [5], suggesting a difference in the region required for transcription between the two promoters.

In this article, we characterized the *1724rolB* promoter by analyzing the expression pattern of *1724rolB*, the transcription initiation point, and the regions required for basal transcription and activation by auxin. Comparison of auxin-regulatory elements in the *1724rolB* promoter with those in *rolB* and several oncogene promoters in other types of Ri plasmid was discussed.

2. Materials and methods

2.1. Bacterial strains

A. rhizogenes strain MAFF301724 containing a mikimopine-type Ri plasmid (pRi1724) [24] was used as the origin of the *1724rolB* gene. *A. tumefaciens* strain LBA4404 and the kanamycin-sensitive *A. rhizogenes* strain DC-AR2 derived from strain MAFF301724 [30] were used as hosts for binary vectors. *Escherichia coli* strain DH5α was used for cloning.

2.2. Plasmid construction

1724rolB with its own promoter and terminator was constructed as follows: a 2.7-kb fragment isolated from pRTE7.6 [5] by digestion with *Ase*I and *Sal*I was subcloned into pBluescript SK$^+$. The fragment was then isolated from this subclone by digestion with *Bam*HI and *Sal*I, and inserted into the corresponding sites in the binary vector pBIN19 [31], resulting in pB11. This binary vector was introduced into *A. tumefaciens* strain LBA4404.

The *1724rolB* promoter-GUS construct was made as follows: a 1202-bp fragment containing the *1724rolB* promoter (from −1202 to −1 bp upstream from the translation initiation codon of the *1724rolB* gene) was amplified by polymerase chain reaction (PCR) using a forward primer (5′-TATCTAGAAGGCGCTCTGGA-3′, an *Xba*I site is underlined), a reverse primer (5′-CAGGATCCT-TAAAGTGCACC-3′, a *Bam*HI site is underlined) and TaKaRa *Taq* polymerase (Takara Bio Inc., Ohtsu, Japan). The amplified fragment was double-digested with *Xba*I and *Bam*HI and then inserted into the *Xba*I-*Bam*HI site upstream of the *uidA* gene encoding β-glucuronidase (GUS) bound to the nopaline synthase gene (*nos*) terminator in pUC18. The *1724rolB* promoter region was gradually deleted from the *Xba*I site by treatment with Exonuclease III (TOYOBO Co., Osaka, Japan) and mung bean nuclease (TOYOBO Co.) to construct a deletion series from 1076 to 72 bp in length. The full length promoter and each of the deletion series of the *1724rolB* promoter with the *uidA* gene and *nos* terminator