식물조직배양학회지 제22권 제4호
Korean J. Plant Tissue Culture
Vol. 22, No. 4, 201~205(1995)

Synthetic Regulatory Elements of the Nopaline Synthase Promoter in Higher Plants

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고등 식물에서 Nopaline Synthase Promoter의 합성 조절 요소

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The synthetic oligomers called *nos* right palindrome (RP) element and left palindrome (LP) element were inserted into *nos* minimal promoter, *nos* 5'-101 deletion mutant. The activity of *nos* promoter was measured by studying the expression pattern of gene fusion between *nos* promoter and reporter genes such as chloramphenicol acetyltransferase and β-glucuronidase. Analysis of transgenic tobacco plants carrying transgene showed that the activity of *nos* minimal promoter activity was recovered by insertion of synthetic *nos* RP element. *Nos* RP element insertion of *nos* minimal promoter was induced by auxin, dithiothreitol, salicylic acid and methyl jasmonate.

key words: chloramphenicol acetyltransferase, synthetic oligomer, transgenic

Nos gene is one of *Agrobacterium tumefaciens* genes located within the T-DNA of Ti plasmid. It is usually silent in host cells but expressed in plant tissues upon transfer to the plant chromosome. Regulatory regions of *nos* gene was thought to be constitutively active in various plant tissues. However, it was found that *nos* promoter activity is organ specific and developmentally regulated.

The nos promoter consists of the TATA box, CAAT box, and upstream regulatory region. Deletion experiment of the TATA box region resulted in about 10 fold reduction in promoter activity by both stable and transient analyses. In CCAAT box deletion experiment, the promoter activity was reduced by about four fold. In stable assay, CCAAT box is important in *nos* promoter since deletion of this region from the *nos* promoter resulted in a reduction of promoter activity in both vegetative and reproductive organs. 5' deletion analysis showed that the deletion of upstream region above CCAAT box abolished the promoter activity. Internal deletion of this upstream region showed that the sequence located between -130 and -112 is necessary for activity of the promoter[1]. The sequence located at immediate upstream and downstream of this region positively modulate the promoter activity. The analyses had shown that this upstream region is essential for developmental and environmental regulation of the promoter.

DNase I footprint experiments demonstrated that hexamer containing region of the promoter interacts with activation sequence factor (ASF)-1, which also interact with regulatory elements of other plant promoters.

In this study, we show that the synthetic *nos*-RP element containing two hexamer motifs and spacer sequence between them is required to recover the promoter activity of *nos* minimal promoter, 5'-101 deletion mutant and that the hexamer motif containing element is essential for the responses to auxin, dithiothreitol, salicylic acid and methyl jasmonate.

MATERIALS AND METHODS

Bacterial Strains and Plant Materials

Escherichia coli host strains MC 1000 (*ara, leu, lac, gal, str*) was used as a recipient for rutine cloning experiments.

The *Agrobacterium* strain LBA 4404 containing the *Ach*5 chromosomal background and a disarmed helper Ti plasmid PAL4404, was used for transformation of cultured *Nicotiana tabaccum* cell line.

Insertion of Synthetic *nos*-RP Element into *nos* 5′ -101 Minimal Deletion Promoter

Synthetic oligonucleotides including *nos*-RP element were synthesized using an applied Biosystems DNA synthesizer. Both strands were synthesized with GATC at the 5′end of each strands. The oligonucleotides were purified with oligonucleotide purification cartridge (Applied Biosystems), annealed in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer, and ligated into the *Bgl* II site of the pGA 705 which located in the just upstream of the *nos* 5′ -101 deletion promoter. Orientation of the inserts were determined using the dideoxy nucleotide sequencing kit Sequenase Version 2.0 (United States Biochemicals). pGA 705 plasmids are high copy replicons and contain an 5′ -101 *nos* deletion promoter which is fused to the CAT (Chloramphenicol-acetyltransferase) reporter gene and T-DNA gene 6b terminator. The *Eco* RI fragment of new construct which contains an *nos*-RP oligomer on the upstream of the *nos* 5′ -101 promoter was cloned into *Eco* RI site of pGA 891 binary vector and transformed into *Agrobacterium tumefaciens*. pGA 891 plasmids are low copy number replicons and contains a CAT gene and tetracycline and kanamycin resistance genes.

Stable Transformation Analysis

Agrobacterium tumefaciens LBA 4404 carrying the binary vector was cocultivated with wounded leaf disks of 5 weeks old *nicotiana tabaccum* seedlings. 20 independently selected transgenic plants were harvested and proteins were extracted for CAT assay. CATase assays were performed on 5ug of total protein prepared from stably transformed *nicotiana tabaccum* (SR I and Xanthi) leaf tissue, with acetyl Coenzyme A and ^{14}C-chloramphenicol for 60 min at 37°C. Promoter specificity was expressed as the percentage of wild-type activity after subtraction of the background level. For histochemical analysis transgenic tobacco seedlings were incubated in 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid), 50 mM $NaPO_4$, pH 7.0 for 16-20 hrs at 37°C. Chlorophyll was removed by boiling in 95% ethanol for 5 min.

RESULTS

Characteristics of Nopaline Synthase Promoter

The *nos* promoter contains the consensus TATA box and CCAAT box sequences at -26 and -78, respectively (Fig. 1). There are two 11 base-pair repeat elements designed a and two 8-bp repeat elements designed b in alternating tandem array forming two a/b repeat pairs at the upstream region. Also there is a stretch of 10 alternating purine and pyrimidine residues (GCACATACGT) immediately upstream of the downstream b element. This sequence is a potential Z-DNA-forming element similar to those found in a number of other regulatory systems. No other a potential Z-DNA forming sequence was found elsewhere in the promoter.

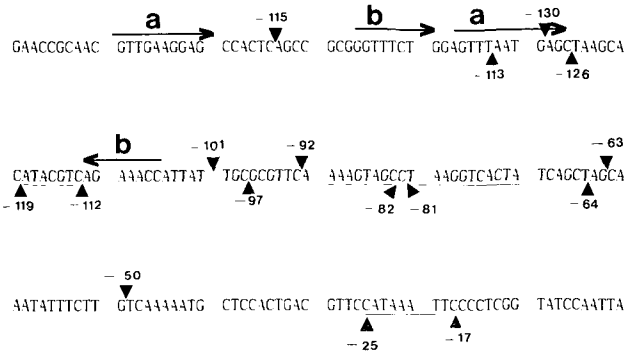

Figure 1. *Nos* promoter sequence showing deletion endpoints. Arrowheads above and below the sequence indicate 5′ and 3′ deletion endpoints, respectively. The arrows above the sequence represents an 11 bp direct repeats (a) and 8 bp inverted repeat (b). The dotted line below the sequence indicates a stretch of 10 alternating purine and pyrimidine residues that constitute a potential Z-DNA forming region (Z). The CCAAT- and TATA-box homologous sequences are indicated by a solid line under the sequence.

Plasmid Construction with Synthetic Oligomers

It was shown that the 5′ deletion mutant -130 of the *nos* promoter retained about one third of the wild type activity while further deletion to -101 resulted in a total loss of promoter activity. This result suggests that the region between -130 and -101 contains an essential sequences for *nos* promoter activity. We studied the regulatory sequences of the *nos* promoter using a 5′ -101 deletion mutant. *Nos*-RP oligomers were inserted into *Bgl* II site of pGA 705 (Table 1). pGA 705 plasmid carries *nos* 5′ -101 deletion mutant

Table 1. Stable transformation of Various synthetic oligomers inserted into 5′ −101 *nos* minimal promoter.

Oligomer	Sequence[a]	Relative strength[b] (mean±SE)
nos wild type element	TGAGCTAAGCACATACGTCA	100.0±16.1
nos RP element	TGACGTATGTACATACGTCA	114.8±30.0
nos LP element	TGAGCTAAGCGCTTAGCTCA	2.7±0.7

[a]Bold type indicates hexamer motifs. The nucleotide sequences which differ from the *nos* wild type element are underlined.

[b]Data are averages of two to six independent transformation experiments.

fused with CAT gene. Multiple cloning sites including *Bgl* II site were located at upstream of the −101 deletion promoter. This construction was moved to binary vector pGA 891. We also constructed a structure containing *nos* RP element fused to GUS reporter gene with pGA 705 plasmid. The mutants having *nos* RP and LP oligomers were selected for the study of expression pattern of *nos* minimal promoter in higher plants. The mutant having *nos* LP oligomer was used as a control sample.

Expression Pattern of *nos*-RP Element at Upstream of *nos* 5′ −101 Minimal Promoter

The *nos* upstream sequence carries two hexamer motifs which showed in other regulatory promoters such as octopine synthase and 35S promoters. The two hexamer motifs are located in the sequence between −112 and −131 of the *nos* promoter (Fig. 1). Downstream hexamer is located in the sequence between −112 and −117 and upstream hexamer is located in the sequence between −126 and −131 of the promoter. The two hexamer motifs are separated by eight nucleotides in the *nos* promoter. Similar spacing was observed in other promoter of the T-DNA and viral promoter. The Z-DNA forming potential sequence is located in the sequence between −114 and −123. Although Z-DNA forming sequence spans through the half of downstream hexamer and 6 nucleotide of spacer sequence, the Z-DNA-forming sequence potential alone is insufficient for regulating promoter activity and the actual sequence at the spacer region is important. The synthetic *nos*-RP element insertion into the upstream of −101 minimal deletion promoter was slightly more active than that of the *nos* wild type element (Table 1). When *nos*-RP element was inserted into the upstream of the −101 *nos* minimal deletion promoter, the activity of *nos* promoter was recovered by treatments such as

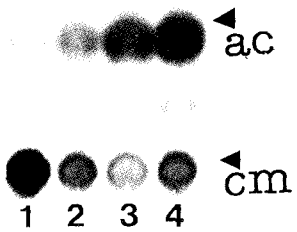

Figure 2. Effect of synthetic *nos*-RP element inserted into the upstream of −101 *nos* minimal promoter. Transgenic tobacco plants carrying synthetic *nos*-RP element fused to cat reporter gene were grown in the green house to the flowering stage. Leaf slices from each transgenic plant were incubated for 20 hrs before wounding (1), after wounding (2), 1 mM salicylic acid (3) and 0.2 ug/mL 2,4-D (4) after wounding in the MS medium. Using 4 ug of total soluble protein to standardize sample, CAT activity was assayed by measuring conversion of chloramphenicol (cm) acetyl-chloramphenicol (ac).

wounding, salicylic acid and 2,4-D (Diphenoxy acetic acid) (Fig. 2). The activity of *nos* promoter was induced by wounding and further enhanced by 2,4-D. However, when *nos*-LP element was inserted into the upstream of the −101 minimal promoter, the activity of *nos* promoter was not recovered by same treatments as we did for the *nos* RP element (data not shown). *Nos*-RP element affected a vertical gradient formation in *nos* minimal promoter through the plant growth. *Nos* promoter is highly active in the lower parts of a plant and gradually decreased in the upper parts of a plant. Overall induction activity of upper parts of leaves was lower with various treatments (Fig. 3).

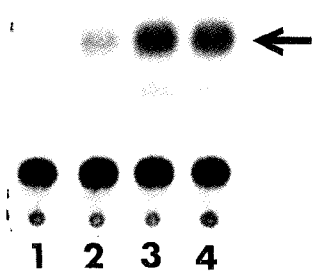

Figure 3. Promoter activity in upper parts of leaves in a transgenic tobacco plant. Upper leaves of transgenic tobacco plants carrying *nos* RP-element, which was inserted into the 5′ −101 *nos* minimal deletion mutant. The upper parts of leaves were incubated in MS medium before wounding (1), after wounding (2), 100 uM methyl jasmonate (3) and 1 mM salicylic acid (4). The data were presented as described for Figure 2.

When two weeks old young seedlings which carried *nos*-RP element in the upstream of −101 minimal deletion promoter were treated with spermidine and ascorbic acids, polyamine spermidine and reducing agent ascorbic acid did not induce the *nos* promoter activity (Fig. 4C and D). However, the reducing agent DTT induced the activity of the *nos* promoter (Fig. 4E). Our study showed that the consistant induction of promoter activity was observed by only GUS assay (Fig. 4E).

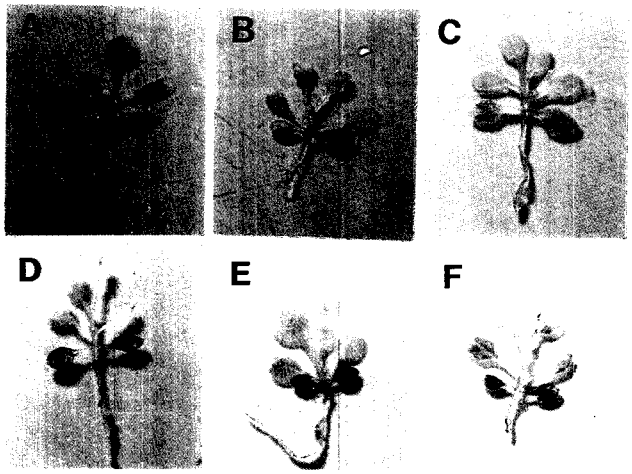

Figure 4. GUS analysis with spermidine, ascorbic acid, dithiothreitol(DTT), and 2,4-D. Leaf slices of randomly selected transgenic tobacco plants carrying *nos*-RP element, which was inserted into 5′ -101 *nos* minimal promoter were incubated for 20 hr before wounding (A), after wounding (B), 2 mM spermidine (C), 1 mM ascorbic acid (D), 1 mM DTT (E) and 2,4-D (F).

DISCUSSION

Nos-RP and *nos*-LP synthetic oligomers were used to study the induction of *nos* upstream regulatory region. Activity of the *nos* promoter is completely destroyed by deletion of *nos* hexamer region. The *nos* upstream region contains an inverted repeat of hexamer motifs. Induction experiment showed that the activity of -101 *nos* minimal deletion promoter was recovered with *nos*-RP element. *Nos*-RP sequence is a palindrome of the right half of spacer sequence and downstream hexamer motif of *nos* promoter. *Nos*-LP sequence is a palindrome sequence of left half of spacer sequence and upstream hexamer motifs of *nos* promoter. *Nos* LP insertion into the upstream of *nos* -101 minimal promoter did not induce the promoter activity with various stimuli. This result indicates that the sequence between -112 and -131 is essential for *nos* promoter activity. However, the hexamer motif which is located in the upstream of the *nos* promoter is not significant as much as that in the downstream. Two hexamer motifs differs only in two nucleotides (Table 1). Recently we have found that when the GC sequence of upstream hexamer was changed to CG like that of downstream hexamer sequence, the promoter activity was significantly increased. The sequence composition of the upstream hexamer element is not a main reason in the weak promoter activity. Therefore it appears that an inversion of the downstream hexamer motif significantly influenced to the function of the promoter element. This result suggests that the hexamer motifs and spacer sequence may function together and that spacer separation of these elements affects promoter activity.

In seedlings the lower parts of *nos* promoter exhibits higher activity compared to upper parts in *Nicotiana tobacco* transgenic plants. Old leaves of transgenic plants was not actively induced with various environmental factors such as salicylic acid and methyl jasmonate. We also observed that When two weeks old young seedlings were treated with spermidine and ascorbic acids, they did not induce the regulatory element of the *nos* promoter. However, the reducing agent DTT induced the RP-element in the upstream of *nos* -101 promoter. Dithiothreitol is a strong reducing agent in biochemical functions. The resulting analysis was observed by GUS assay, but we could not get a consistant data from CAT assay. To quantitate the experimental data, we observed both consistant induction and tissue localization of the wound site by only GUS assay. This study suggests that constitutive *nos* promoter can be altered by insertion of pollen specific promoter fragment into the upstream of 5′ -101 *nos* minimal and wild type promoters. Modified promoters may direct tissue specific gene expression in pollen. Future experiments will focus on the construction of fusion molecules between α-tubulin pollen specific promoter and *nos*-minimal promoter and connect this to anthocyanin genes to analyse the function of flavonoids during gametophyte and pollen developments

적 요

Nopaline synthase promoter의 upstream element region을 본딴 *nos*-RP 요소라고 불리워진 합성 oligomer는 *nos* wild type promoter의 5′ end에서부터 -101까지를 절단한 promoter의 upstream에 삽입하였다. *Nos* promoter의 활성은 *nos* promoter와 연결되어 있는 reporter gene인 Chloramphenicol과 β-glucuronidase유전인자들이 발현되는 현상을 연구함으로써 측정하였다. 형질 전환된 유전인자를 가지고 있는 담배 식물에 대한 분석은 *nos* minimal promoter의 활성이 합성 *nos*-RP 요소가 삽입됨으로써 회복될 수 있음을 보여 주었다. 또한 *Nos* minimal promoter의 upstream에 *nos*-RP 요소의 삽입은 여러 가지 환경적인 요소들인 auxin, dithiothreitol, salicylic acid 그리고 methyl jasmonate에 의해서 활성이 증가됨을 보여 주었다.

ACKNOWLEDGEMENT - This work was supported by a grant

from the Korean Science and Engineering Foundation (KOSEF)

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(Received July 19,1995)