Expression characteristics of *GFP* driven by *NAC1* promoter and its responses to auxin and gibberellin*

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Abstract A 1050 bp fragment upstream transcription start site of a transcription factor gene *NAC1* in *Arabidopsis thaliana* was amplified and cloned into plasmid pRD420 to construct a green fluorescent protein(GFP) fusion system under the control of *NAC1* promoter. Plasmids were introduced into tobacco by *Agrobacterium*-mediated method to regenerate plants with *NAC1-GFP* gene, and expression pattern of *NAC1-GFP* and its responses to auxin and gibberellin (GA) were observed. GFP was found to accumulate specifically in root, and was detected after treatment of auxin, N-1-Naphthylphthalamic acid (NPA, an auxin antagonist) or GA_3. It was indicated that the expression of *GFP* driven by *NAC1* promoter was induced not only by auxin but also by GAs, suggesting that *NAC1* mediated both the auxin signaling and the GAs signaling involved in lateral roots development.

Keywords: auxin, gibberellin, *NAC1*, GFP.

Initiation of lateral root primordium (LRP) and formation of lateral roots has been one of the hot areas in plant developmental biology, but its mechanism is unclear by now[1,2]. In *Arabidopsis thaliana*, *NAC1* has been proved to be a gene of the important transcription factor mediating the auxin signaling to promote lateral roots initiation, and its expression showed high correlation with both the auxin concentration and the number of LRP[3,4]. Three gibberellin-responsive-elements (GARE) besides auxin-responsive-elements(AuxRE) were found in the 1 kb upstream region of *NAC1* by sequencing[5] and bioinformatics analysis, which suggested that *NAC1* could be induced by gibberellins (GA). The study on the responses of *NAC1* to GA will be helpful to understanding the mechanism of development of LRP and the functions of GA in the process.

The functional enantiotropy of phytohormones has been proved in the development of upground organs and roots in the studies on acting mechanism of auxins, cytokinins and other phytohormones. Based on the fact that GAs play important roles in the formation of lateral tissues such as leaf primordium and floral primordium[6,7], GA is deduced to mediate the initiation of LRP. The previous studies in morphology and physiology have revealed that the functions of GA during roots development are mainly on promoting root elongation, but no molecular evidence supports that GA is involved in formation of LRP[6,8]. In this research, expression of *GFP* driven by the *NAC1* promoter was studied in tobacco to explore its expression characteristics in different tissues of tobacco under the induction of auxins and gibberellins, and to probe into the functions of gibberellins during the development of lateral root primordium.

1 Materials and methods

1.1 Materials

Seeds of tobacco (*Nicotiana tabaccum*) variety SR1 were kindly provided by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Seeds of *Arabidopsis thaliana* variety Columbia c-type were provided by the State Key Laboratory of Plant Physiology and Biochemistry, China Agricultural University. Vector pGEM-T (Promega Ltd.) and plasmid PBI121 were supplied by the Forestry Institute of China Forestry Academy.

1.2 Cloning of *NAC1* promoter and adding of *Bam*HI/*Hin*dⅢ sites

Genomic DNA was extracted from *Arabidopsis*

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thaliana. Primers were designed as 5′-acgacactggctttgttt-3′, 5′-ccaggatttgatcctttt-3′ based on the upstream sequence of *NAC1*[5] so as to amplify the upstream fragment of 1050 bp from the *NAC1* transcription start site. The PCR mixture contained 1 ng template DNA, 1 μmol of each primer, 200 μmol/L dNTPs, 2.5 μL PCR buffer and 1 unit *Taq* DNA polymerase in a volume of 20 μL. Template DNA was initially denatured at 94 ℃ for 3 min followed by 30 cycles of PCR amplification with the following parameters: 1 min of denaturation at 94 ℃, 30 s of primer annealing at 52 ℃ and 40 s of primer extension at 72 ℃. The 1050 bp fragment upstream the *NAC1* transcription start site was amplified and cloned into vector pGEM-T for sequencing to confirm the accuracy of the sequence. Then using the above plasmid DNA as template, PCR was run with upstream primer with a restriction *Hin*d Ⅲ site (mNAC1-F: 5′-aggaagcttacgacactggctttgttt-3′) and downstream primer with an *Xba* Ⅰ site (mNAC1-R: 5′-tgctctagaccaggatttgatcctttt-3′). The program for PCR was: 1 min at 94 ℃ for template denaturing, followed by 8 cycles at 94 ℃ for 30 s, 30 s annealing at 52 ℃, and a 40 s elongation at 72 ℃, and by another 25 cycles at 94 ℃ for 30 s, 30 s annealing at 68 ℃, and a 40 s elongation at 68 ℃, with a final 7 min incubation at 72 ℃ for completion of primer extension. The obtained fragment was cloned into the pGEM-T vector and confirmed by sequencing. A new plasmid with the confirmed sequence was obtained and named GEM-mNAC1pro.

1.3 Construction of the plant expression vector

Plasmids pGEM-mNAC1pro and pRD420 were respectively digested by both *Hin*d Ⅲ and *Xba* Ⅰ, and then fragments were separated by electrophoresis. Recovered target fragments were ligated with T4 ligase and then linked products were transformed into prepared competent cells of *E. coli* stain DH5α. The transformed cells were screened in medium with kanamycin; the positive colonies were checked by PCR to confirm that the inserted fragment and the assembly were correct. The selected positive plasmid was named pRD-mNAC1pro.

1.4 *Agrobacterium*-mediated tobacco transformation

The plasmid pRD-mNAC1pro was introduced into an *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Agrobacterium*-mediated tobacco transformation was performed according to the leaf-disc-infection method described as follows. The infected leaf discs were grown on differentiation medium MS (0.5 mg/L 6-BA, 0.1 mg/L NAA, 0.25% agar) without any antibiotics for 4 days in the dark, then were transferred to select the regenerate sprout with resistance to kanamycin on another differentiation medium with 50 mg/L kanamycin and 50 mg/L carbenicillin in continual light. Explants were subcultured every 20 days till the differentiated sprout grew to 2 cm, then transferred to rooting medium (1/2 MS, with 0.5 mg/L IBA, 0.5 mg/L NAA, 100 mg/L kanamycin, 50 mg/L carbenicillin, 0.25% agar).

1.5 PCR identification for transformed plants

As a large gene family, *NAC* is highly conserved in different plant species[3]. To avoid the false positive, in this study, the transformants were identified by PCR using the specific primers for *GFP* (GFP-F: 5′-cacaagttcagcgtgtccg-3′, GFP-R: 5′-gttcacttgatgccgttc-3′). The program for PCR was: 5 min at 94 ℃ for template denaturing, followed by 30 cycles at 95 ℃ for 40 s, 35 s annealing at 55 ℃, and a 40 s elongation at 72 ℃, with a final 7 min incubation at 72 ℃ for completion of primer extension.

1.6 Determination of *GFP* transcripts in different organs using semi-quantitative reverse transcription PCR (RT-PCR)

Fresh tissues of root, stem, and leaf were first frozen in liquid nitrogen, then homogenized to extract total RNA with the Qiagen Total RNA Extract Kit. The total RNA was treated with DNAase, affirmed by 0.8% agarose gel electrophoresis, and then RNA concentration was quantified. 1μg total RNA extracted from each tissue sample was respectively mixed with 25 ng oligo-dT, primer, and dissolved in distilled water pretreated by diethypyrocarbonate (DEPC), then incubated at 65 ℃ for 5 min and cooled to room temperature. Afterwards 2 μL 0.1 mol/L DTT, 1 μL 20 mmol/L dNTP, 2 μL 10 × reverse transcription buffer(0.1 mol/L, 1 μL RNase, 0.5 μL(19 U) AMV reverse transcriptase) were added and the mixture was kept at 42 ℃ for 1 h, then at 92 ℃ for 4 min to inactivate the reverse transcriptase. The above cDNA was used as the template for RT-PCR amplification. PCR program and primers were described in 1.5. PCR products were separated by 1% agarose gel electrophoresis, then scanned and analyzed for semi-quantification.

1.7 Treatments with auxins, gibberellins and auxin antagonist

Individuals of transformed tobacco plants were cultured in sand. The roots were washed using flash water to remove soil granules, then split, fixated and pretreated for 24 h in the tube with 1/4 MS liquid culture medium while the height of individuals was 5 cm. Then GA_3, 3-indolebutyric acid (IBA), auxin antagonist N-1-naphthylphthalamic acid (NPA) solutions were respectively added into culture medium to certain concentrations, and the treatment continued for 8 h, using dH_2O added as control. GFP fluorescence observed in roots under a fluorescence microscope was evaluated based on the method described by Aspuria[9]. For all the treatments of GA_3, IBA and NPA, two concentrations of 0.1 mg/L and 1.0 mg/L were tested.

1.8 Observation of GFP fluorescence in the tobacco plants

The roots of transformed tobacco plants were sampled and prepared by pellet method. Then GFP fluorescence in these samples was observed under a Nikon Eclipse TE300 fluorescence microscope.

2 Results

2.1 Bioinformatics analysis of upstream regulatory region of *NAC1* sequence

Based on the bioinformatics analysis on upstream regulatory region of the reported *NAC1* sequence[5], three gibberellin responsive elements (GARE, 'TATCCAC', 'C/TCTTTC/T')[10] at − 996/ − 1001 bp, − 827/ − 832 bp and − 57/ − 62 bp respectively were found besides an auxin responsive element (AuxRE, 'TGTCTC')[10] at − 503/ − 508 bp (Fig. 1).

Fig. 1. Diagrams of the GARE and AuxRE in the upstream region of *NAC1*. GARE, gibberellin responsive element; AuxRE, auxin responsive element.

An obvious upstream element 'CCAAT' box was found at − 88— − 84 bp upstream the start code 'ATG'. Most bases from − 20 bp to − 10 bp upstream of this element were 'A', which flanked with one or two pyrimidine nucleotides. These demonstrated typical characteristics of a transcription start site. Based on the above analysis and the fact that most promoters are of 100 bp upstream the transcription start site and generally do not exceed 1 kb in length, the upstream regulatory region (promoter region) of *NAC1* should be included within the 1 kb region upstream the coding region.

2.2 Cloning of *NAC1* promoter and construction of the expression vector

NAC1pro, a 1050 bp fragment of upstream regulatory region of *NAC1*, was amplified from *Arabidopsis thaliana*. Sequencing result showed that its sequence was the same as the reported *NAC1* sequence in GenBank.

The expression vector pRD-mNAC1pro constructed by the method described in Section 1.3 is showed in Fig. 2. After the inserted fragments integrated into the genome of tobacco, the *GFP* expression in transformed tobacco was controlled by *NAC1pro*, and the expression patterns of *GFP* could be used to illustrate the functional features of *NAC1pro*. After the plasmid pRD-mNAC1pro was digested with restriction enzymes, the pattern shown on an agrose gel proved that *NAC1pro* had been inserted into plasmid pRD420 in a correct direction (Fig. 3).

Fig. 2. Diagram of the schematic structure of plasmid pRD-mNAC1pro.

2.3 Identification of the transformed tobacco plants by PCR

A total of 205 transformed tobacco plants were obtained through leaf-disk transformation, differentiation, rooting and plant regeneratation. The PCR identification of a part of the transformed tobacco plants is shown in Fig. 4. In this group, *GFP* specific band of 420 bp was amplificated from 5 tested transformants, while the transformed plants with the plasmid without *NAC1-GFP* showed no such a band. This indicates that the target gene has inte-

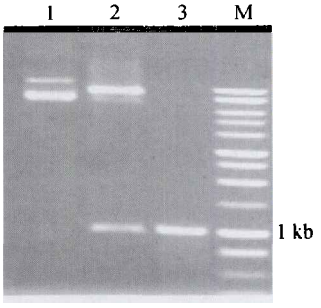

Fig. 3. The restriction digestion of pRD-mNAC1pro plasmid resolved on agarose gel. 1, pRD-mNAClpro plasmid; 2, *Hin*d Ⅲ/*Xba* Ⅰ digestion; 3, PCR product of *mNAC1pro*; M, 1 kb DNA ladder.

grated into genome of tobacco in these transformed individuals.

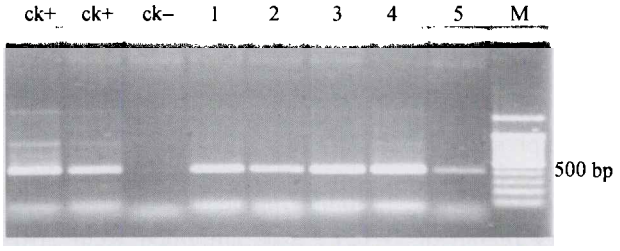

Fig. 4. Identification of the transformed tobacco plant by PCR. ck +, positive plasmid; ck −, transgenic plants with the plasmid without *NAC1-GFP*; Lanes 1—5, PCR products with the transformed tobacco DNA as template; M, DNA molecular marker.

2.4 Expression of *GFP* in different tissues of transformed plants

The *GFP* expression was determined by the method of semi-quantitive-RT-PCR (Fig. 5), using the total RNAs extracted from root, stem and leaf of T1 generation of transformed plants. The expression of *GFP* driven by *NAC1-pro* was strong in root, trace in stem and none in mature leaves, indicating that the *NAC1pro* expression is root-specific.

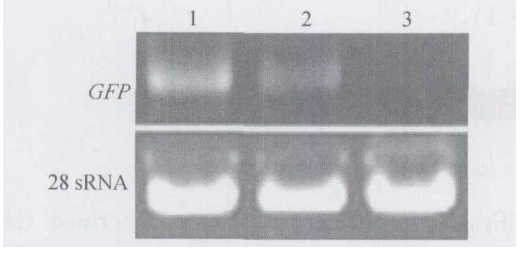

Fig. 5. *GFP* expression in different tissues of transformed tobacco plants, checked by RT-PCR. 1, Root; 2, stem; 3, leaf.

2.5 Expression of *GFP* in roots of transformed tobacco plants

GFP driven by *NAC1pro* was predominantly expressed in the meristem area of root apical (Fig. 6), which was in accordance with the enrichment position of *NAC1* transcripts[3]. The quantitative analysis of GFP supported that the cloned *NAC1pro* has an integrated promoter region of *NAC1* gene, which is located at the 1050 kb fragment upstream the transcription start site.

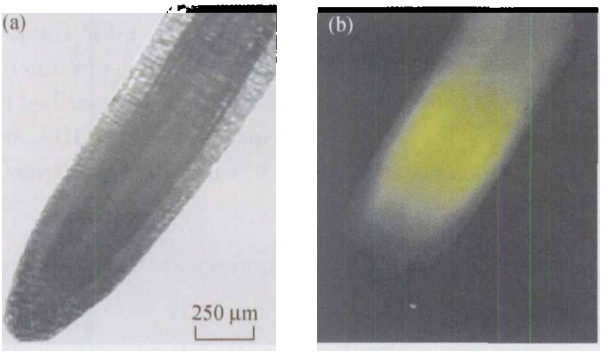

Fig. 6. GFP expression in pRD-mNAClpro transgenic tobacco root. (a) Tobacco root in visual light; (b) GFP fluorescence in UV-light.

2.6 Effects of gibberellin and auxin on *GFP* expression in transgenic tobacco plants

GFP fluorescence in root apical of all treated transgenic tobacco plants was observed after 8 h continual treatment by GA, IBA and NPA. GA_3 and IBA at concentrations of 1.0 mg/L and 0.1 mg/L induced *GFP* expression obviously, whereas auxin antagonist NPA at 1.0 mg/L inhibited *GFP* expression (Table 1). Fig. 7 shows a strengthened GFP fluorescence in root induced by 1 mg/LGA_3. The *GFP* expression was enriched mainly in the meristem area of root apical, especially in stele cells. A similar trend was found for the treatments of 0.1 mg/L IBA, 1.0 mg/L IBA and 0.1 mg/L GA_3.

Table 1. GFP fluorescence in root apical of transgenic tobacco plants in response to IBA, GA_3 and NPA

Treatment	GFP fluorescence[a)]
0.1 mg/L IBA	+ + +
1.0 mg/L IBA	+ + +
0.1 mg/L GA_3	+ + +
1.0 mg/L GA_3	+ + +
dH_2O	+ +
1 mg/L NPA	+

a) GFP fluorescence was observed with a fluorescence microscope; + + +, strong; + +, moderate; +, weak.

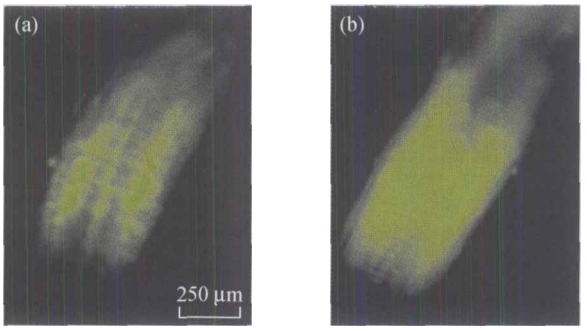

Fig. 7. GFP fluorescence after 0.1 mg/L GA_3 treatment for 6 hours. (a) Before treatment; (b) after treatment.

3 Discussion

The specific expression of genes driven by root-specific promoters in root tissues is a prerequisite to successfully improve the root traits by molecular manipulation. *NAC1* is an auxin-responsive gene specifically expressed in root tissues. To investigate its expression characteristics, a 1050 bp promoter fragment upstream the coding region of *NAC1* was cloned and fused with *GFP*. Just like the expression of *NAC1* in *Arabidopsis*, the expression of *GFP* driven by *NAC1*-promoter in tobacco was mainly observed in the meristem area of root apical, but not in the whole roots. Furthermore, it was found that *NAC1-GFP* was strongly expressed in LRP initiation area, which was identical to a previous observation by Xie et al. who used *GUS* as reporter gene in *Arabidopsis*[3].

It is well known that phyto-hormones often functionally interact to regulate plant growth and development. Auxin was reported to control the elongation of lateral root with an interaction with gibberellin signaling[8,10]. An AuxRE together with three GAREs can be found in *NAC1* promoter, implying that *NAC1* could be possibly regulated by gibberellins. As expected, the expression of *NAC1-GFP* was found to be responsive to GA treatment, suggesting that the expression of *NAC1* can be indeed regulated by GA. In view of the importance of *NAC1* in LRP initiation, the present results strongly suggest that GA plays crucial roles in LRP development.

With progressing in investigation of molecular mechanisms for plant growth and development, it is very important to rapidly and sensitively monitor the expression of the genes in response to phytohormone signaling. A method for rapidly testing the gene expression can be achieved by constructing transient expression vectors carrying some reporter genes such as GFP and RFP driven by hormone responsive promoters. Here, we have developed a transient expression system by transforming *NAC1-GFP* into tobacco plants, which was proved to be successful. Hence, with the presently developed technique, it might be possible to screen the active factors controlling root development. Furthermore, in combination with bioinformatics methods, this technique can be applied to find the hormone responsive elements, therefore deeply revealing the molecular mechanisms for the development of lateral roots or organs, which has already been proved to be powerful for the investigation of the auxin-responsive promoter DR5[9,10].

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