Functional Role of CAAT Box Element of the Nopaline Synthase (*nos*) Promoter

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The nopaline synthase (*nos*) promoter is active in a wide range of plant tissues and regulated by various environmental stimuli. It was previously found that the CAAT box region is important for *nos* promoter activity. In the present study, the location of the CAAT box element was determined by site specific mutation analysis. Point mutations within the conserved CAAT box element significantly reduced the promoter response in transgenic tobacco plants and calli to wounding, H_2O_2, methyl jasmonate, and 2,4-D, but not to salicylic acid. However, mutations immediately upstream from the CAAT box did not affect these responses. These results suggest that the CAAT box element is important in responding to certain stimuli.

Key words: auxin, CAAT box element, 2,4-D, hydrogen peroxide, methyl jasmonate, salicylic acid

The *nos* gene is located in the transferred DNA (T-DNA) of the Ti plasmid in *Agrobacterium tumefasciens*. The gene is transferred along with the T-DNA to plant chromosomes following *Agrobacterium*-mediated plant transformation and is expressed in many plant cells. Initially it was considered to be constitutively expressed in different plant tissues. It was later found, however, that the *nos* promoter activity was organ specific and developmentally regulated (An et al., 1988; Yu et al., 1998).

Extensive deletion mutational analysis of the *nos* promoter indicated that the promoter activity is controlled primarily by three regions of DNA sequences: the TATA box, CAAT box, and an upstream region (An et al., 1986; Ebert et al., 1987; Ha and An, 1989; Mitra and An, 1989; Kim et al., 1993; Dai and An, 1995). Deletion of the TATA box region resulted in about a 10-fold reduction in promoter activity and deletion of the CAAT box region caused a much stronger loss in activity. However, the role of the CAAT box region in plant promoters is not well understood. It was postulated that the CAAT box sequence acted cooperatively with heat shock elements to increase the activity of a soybean heat shock promoter (Rieping and Kosuge, 1992).

We have observed that the CAAT box region is important for wounding, methyl jasmonate (MJ), and H_2O_2 inducibility (Kim et al., 1993; Dai and An, 1995). The wild type *nos* promoter (263 nucleotides from the transcription start site) is inducible by 1 mM salicylic acid (SA), 0.05 μM MJ, and 5 mM H_2O_2 to an almost identical level. Deletion mutants lacking the TATA box region (3′ deletion −25) or the far upstream region (5′ deletion −155, or 5′ deletion −130) responded to H_2O_2, MJ and SA almost equally though the promoter strength was decreased. However, deletion of the CAAT box region (internal deletion between −81 and −63) dramatically reduced inducibility by H_2O_2, or MJ compared to that of SA. These results indicate that the *nos* CAAT box plays an important role in controlling the MJ- or H_2O_2-inducibility. To further understand the functional role and organization of the *nos* CAAT box region, we have generated a series of deletions surrounding the CAAT box region and tested these in transgenic tobacco plants (Kim et al., 1993). The deletion between −97 and −83 did not affect the inducibility by SA or MJ (Kim et al., 1993). However, deletion between −97 and −79 did significantly reduce the MJ response compared to SA. Further deletions from −97 to −75 or −68 showed similar responses, indicating that the regulatory element involved in the MJ or H_2O_2 responses is located between −83 and −63.

In this study, the importance of the CAAT box region was further evaluated by site specific mutations. In order to facilitate creation of mutations, a restriction enzyme site, BstB I (TTCGAA), was generated in the upstream of the conserved CAAT box region by changing the nucleotide A at position −91

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to G. It was previously shown by the deletion analysis that the sequences at this region are not important for the *nos* promoter activity (Kim et al., 1993). To confirm this observation, a mutant promoter was fused to the chloramphenicol acetyltransferase (CAT) reporter gene, resulting in generation of pGA1337. This plasmid was introduced by electroporation into protoplasts isolated from a NT-1 suspension tobacco cell (Ebert et al., 1987). Transient analysis of the gene expression showed that the point mutation did not alter the promoter activity (data not shown).

Using plasmid pGA1337, four additional mutant promoters were generated by substituting the CAAT box region of the plasmid pGA1337 with synthetic oligomers. There is a restriction enzyme site, NheI at –67/–62. Both strands of the DNA sequence between the BstBI and NheI sites were synthesized with some changes in the sequence. The first mutant promoter contains the four nucleotide substitutions at –82/–79 to GCGC, generating a BssHII site. These alterations occurred at the immediate upstream sequence of the CAAT box element. Three mutant promoters were generated within the CAAT box sequence: changes from GGTCAC at –78/–73 to GCATGC (SphI), and TATCAG at –72/–67 to either CATATG (NdeI) or TCGCGA (NruI). The mutant promoter-CAT fusion molecules were subcloned into the binary vector pGA628 and transferred to a tobacco suspension cell line, NT1, via *Agrobacterium* mediated cocultivation method. Transformants were selected on a MS agar medium containing 200 μg mL^{-1} kanamycin. Fifty independent calli were pooled

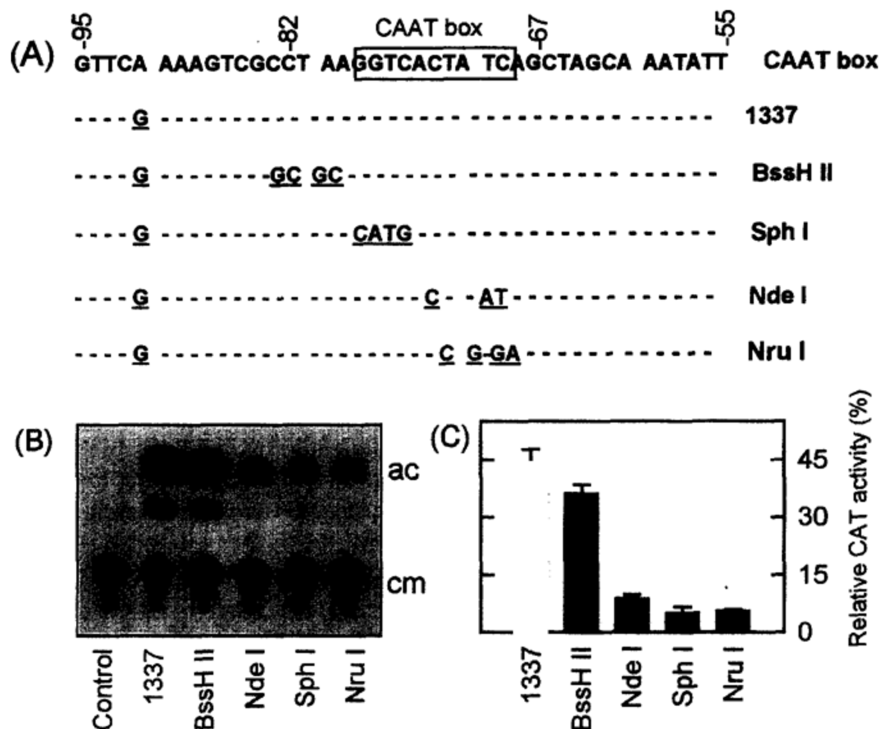

Figure 1. The CAT activity of transgenic tobacco calli carrying different point mutations in the CAAT box region of *nos* promoter. **A.** Nucleotide sequence of the CAAT box region and the positions of site-specific mutations. The 9 nucleotide sequence between –78 and –70 has a homology to the consensus CAAT box sequence element, GGCCAATCT. Both strands of the 31 oligonucleotide sequence between –94 and –63, which carried different site-specific mutations at the CAAT box region, were prepared using an Applied Biosystems DNA synthesizer. In order to facilitate subcloning, the BstBI and NheI sites were included at the ends of the oligomers. Equal amounts of the oligomers were mixed and purified with an OPC cartridge (Applied Biosystems). The oligomers were used to replace the BstBI-NheI fragment of pGA1337. **B.** The TLC autoradiograph showing representative samples. The *nos* mutant promoters carrying various synthetic oligomers were subcloned into the binary Ti-plasmid vector pGA 628 that contains the *cat* reporter gene. *A. tumefaciens* LBA4404 carrying the binary vector was cocultivated with actively growing suspension cultured cells. Fifty independently transformed calli were selected on MS agar medium containing 200 μg mL^{-1} kanamycin. These calli were pooled and soluble protein was extracted for chloramphenicol acetyltransferase (CAT) assay. Control sample is nontransformed NT1 calli. **C.** Relative CAT activity of site specific mutations. The amount of total protein used for each CAT assay was 1 μg. Each bar is the mean of three independent samples of transformed tobacco calli.

together and soluble protein was extracted for assaying CAT activity (An, 1987). The results in Figure 1 showed that the mutation at −82/−79 reduced the promoter strength by approximately 20% whereas the three mutations within the CAAT box element caused a dramatic reduction of promoter activity. These results indicate that the CAAT box element sequence is important for the *nos* promoter activity.

The roles of the CAAT box element in response to various stimuli were studied using transgenic tobacco plants. At least five plants were obtained for each construct. We have observed previously that, although there is a significant difference in the expression level among independently transformed plants, there is a little variation of the relative induction levels of the *nos* promoter by various stimuli (An et al., 1986, 1990; Kim et al., 1993, 1994; Dai and An 1995). Therefore, five plants should have been sufficient to generate reliable data. Fully expanded leaves were wounded by cutting into small pieces (about 1 cm^2) and incubating on liquid MS medium for 20 h at room temperature and 30-50 μmol quantum m^{-2} s^{-1} light intensity. The effect of various chemicals was evaluated by adding 1 mM SA, 5 mM H_2O_2, 50 μM MJ, or 1 μM 2,4-D to the incubation medium. The results in Figure 2 show that one nucleotide sequence substitution at −91 (BstBI) did not alter the *nos* promoter characteristics. The plants were inducible by wounding and further enhanced by the four chemicals. The activity levels induced by SA, H_2O_2, and MJ were almost identical, whereas the level induced by 2,4-D was highest. This induction pattern is identical to that of the wild-type *nos* promoter (Kim et al., 1993; Dai and An, 1995), indicating that the single nucleotide substitution did not alter the inducibility of the *nos* promoter by the stimuli.

Tobacco plants transformed with the BssH II mutant promoter carrying alterations at the sequence immediately upstream of the CAAT box element were also inducible by various stimuli and the induced levels were similar to that of the wild type promoter (Fig. 2). However, the characteristics of the other three mutant promoters (SphI, NdeI, and NruI) carrying the mutations in the CAAT box elements were different. They were inducible by SA but not by H_2O_2 or MJ. In addition, the 2,4-D-induced level of the NruI mutant promoter was lower compared with wild type *nos* promoter. These results suggest that the CAAT box element is important in responding to H_2O_2, MJ, and 2,4-D. They also support the early observations that the induction mechanisms between SA and other stimuli are different from each other (Kim et al., 1993; Dai and An, 1995).

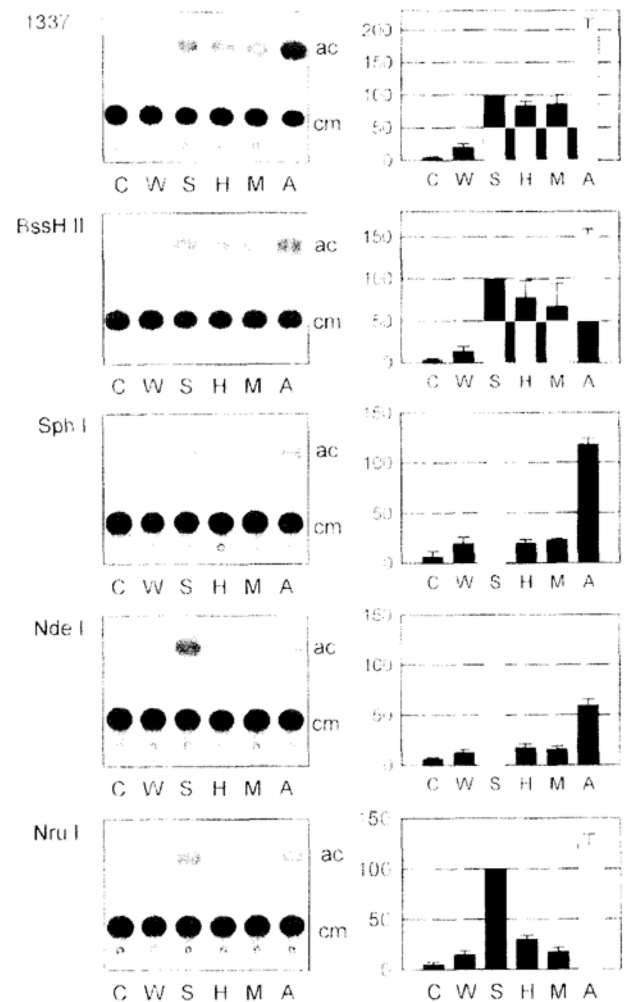

Figure 2. The induction of *nos* promoter activity by wound, SA, H_2O_2, MJ, and auxin. Transgenic tobacco plants were obtained by cocultivation of young leaf segments with *A. tumefaciens* (1). The T1 plants were the first generation obtained by selfing individual transgenic plants. The transgenic plants were maintained in greenhouse conditions. The CAT activity was measured using the crude extracts standardized at 1 to 100 μg of the total soluble protein. Fully expanded leaves of transgenic tobacco plants carrying point mutation: BstBI, BssHII, SphI, NdeI, or NruI, which were replaced with the nucleotide sequence between −91/−63, were sampled before (C) or after 22 h induction by wounding (W), 1 mM SA (S), 5 mM H_2O_2 (H), 50 μM MJ (M), or 1 μM 2,4-D (A) treatment. Representative samples are shown in the TLC autoradiographs on the left. Relative CAT activity of site specific mutations in response to various treatments, normalized to 100% base level induced by SA (S), are shown in bar graphs on the right. The amount of total soluble protein used for CAT assay was 5 μg for BstB I and BssHII, and 100 μg for SphI, NdeI. NruI. Each bar is the mean of five independent transgenic tobacco plants ± SD.

It was reported earlier that the CAAT box region

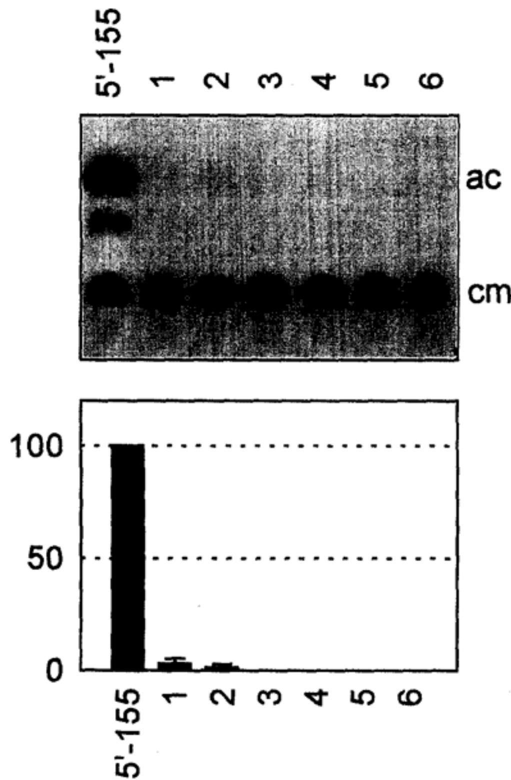

Figure 3. Transient analysis using NT-1 tobacco protoplasts electroporated *nos* promoter containing multimers of the CAAT box region. The nineteen nucleotide *nos* CAAT box element between −81 and −63 was self ligated and inserted into the minimal *nos* promoter 5′-63 with the upstream region (samples −155/1, 2, 3) or without the upstream region (samples 4, 5, 6). The upper panel is the TLC autoradiograph of samples. The lower panel shows the relative CAT activity. Sample 5′-155 is the 5′ deletion mutant to −155; sample 1 contained two copies of the CAAT box region plus the upstream region (−155/−63); sample 2 contained three copies of the CAAT box region plus the upstream region (−155/−63); sample 3 contained four copies of the CAAT box region plus the upstream region (−155/−63); sample 4 contained two copies of the CAAT box region inserted into the minimal *nos* promoter 5′-63; sample 5 contained three copies of the CAAT box region inserted into the minimal *nos* promoter 5′-63; sample 6 contained four copies of the CAAT box region inserted into the minimal *nos* promoter 5′-63. Data from samples 1, 2, 3, 4, 5, and 6 were normalized to the 100% base level of 1 copy of the CAAT box region (wild type). Each bar the means of four independent replicates ± SD.

alone was insufficient in activating the nos promoter and that the upstream 20 nucleotide sequence is necessary (Kim et al., 1994). We therefore tested whether multimerization of the CAAT box element would activate the *nos* promoter in the absence of the upstream element. Both strands of the nineteen nucleotide sequence region between −81 and-63 that contain the CAAT box region were synthesized and made into multimers by self-ligation. Insertion of two to four copies into the 5′-deletion mutant −63 did not activate the nonfunctional promoter (Fig. 3). Likewise, placing the upstream sequence between −63 and −155 in front of the multicopy CAAT box sequences did not activate the *nos* promoter either. This suggests that the CAAT box region alone is not sufficient for promoter activity and that there is in addition to the CAAT box region, an optimal distance between the upstream and downstream elements.

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