RESEARCH PAPERS

Identification of seed-specific promoter *nap300* and its comparison with *7S* promoter*

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Abstract By fusing seed-specific promoter *nap300* with β-glucuronidase gene, it was found that this about 300bp DNA fragment was sufficient to direct seed-specific gene expression. The substitution mutation in both distB and proxB elements had a little effect on the expression efficiency and almost no effect on the organ-specific expression pattern. In the experiment designed to compare *nap300* with *7S* promoter, the result showed that tissue specificity for *nap300* was higher than that for *7S*, and its expression level was lower than *7S*'s. There was no big difference in their expression pattern, and the maximal activity stage for the two promoters was identical, which indicated they could be used simultaneously for expressing different foreign genes in seeds.

Keywords: ***nap300*** **promoter,** ***7S*** **promoter, seed-specific expression, transgenic tobacco.**

Recent studies on the use of transgenic plants as factories to produce useful materials have revealed that seed, the natural storage organ in plant, is an ideal target for high level accumulation of foreign gene products without deleterious effect on plant growth or phenotype. Various kinds of seed-specific promoters have been isolated and utilized in gene-manipulation and metabolic engineering strategies[1,2]. A number of promoter elements important for seed-specific expression have been identified[2~4]. It is necessary to conduct promoter comparison and select out promoters suitable for organ-specific expression in plants. To realize economic production of transgenic plants, accurate gene expression manipulation is required. Thus, the comparison of promoters, the crucial elements for gene expression, becomes more and more important in genetic engineering. It can not only offer the chance to get a deep insight into their expression mechanism, but also provide valuable information about their relative efficiencies and the temporal, spatial gene expression patterns directed by them. Then, different promoters could be used in a multigene strategy to minimize the possibility of gene silencing caused by promoter homology.

In our laboratory, targeting the products of three poly-3-hydroxybutyrate (PHB)-synthesis genes to rape seed has been achieved via *7S* promoter. However, severe promoter-homology-mediated gene silencing was observed[5]. In order to replace *7S* promoter in some expression cassettes we isolated another seed-specific promoter *nap300*[6]. *Napin* is the promoter of seed storage protein gene in rape. Its function and composition has been extensively investigated. B-box, RY/G complex and some other composite elements in this promoter are found necessary for seed-specific expression[7]. Here we examined the activity of a seed-specific promoter with substitution mutations in B-box. Then, it was compared with another seed-specific promoter *7S* for their similarities and differences. Both of them are promoters for seed storage protein, but *nap300* is for *napinB* gene in rape and *7S* for the α′-gene of β-conglycinin in soybean[8]; neither of them is the full length of the promoter region, but *nap300* with about 300 nucleotides of the 5′ end of *napinB* and *7S* is artificially constructed by inserting the DNA element containing about 300 nucleotides from the 5′ region of the α′-gene into the *35S* promoter. Through comparison detailed information about the characteristics of the two promoters can be obtained, which is necessary for subtly regulated gene expression in plant genetic engi-

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neering.

1 Materials and methods

1.1 Examination of *nap300*'s activity

Seed-specific promoter *nap300* isolated in our laboratory was fused to the reporter gene β-glucuronidase (GUS) to confirm its heterologous expression ability. Transgenic plants obtained through *Agrobacterium*-mediated transformation were analyzed with PCR, Southern blot and finally transferred to the greenhouse[6]. After 3 ~ 5 months, several seed capsules were harvested and frozen in liquid nitrogen every 48 h for each transgenic line from 5 days after flowering (DAF) to 30 DAF for GUS activity analysis. Fluorometric assay was carried out as outlined by Clark[9], protein concentration was determined by the Bradford method[10]. For histochemical assay of GUS, seed capsules at different developmental stages were hand-sectioned and put into staining solution (1 mmol/L X-gluc, 100 mmol/L sodium phosphate buffer, pH7.0, 10 mmol/L EDTA, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide) at 37℃ in dark for 12 h.

1.2 Promoter comparison

1.2.1 Vector construction For promoter comparison, plasmid p7S-A[5] bearing *phbA* gene (the first gene necessary for PHB synthesis, encoding 3-ketothiolase) under the control of seed-specific promoter *7S* and plastid transit peptide *ctp* was modified by replacing *7S* promoter with *nap300* promoter, which resulted in its derivative plasmid pNap-A. The p7S-A and pNap-A differed only in the promoter region.

1.2.2 Plant transformation The two vectors constructed in 1.2.1 were introduced into tobacco (*Nicotiana tabacum* L. cv. Wisconsin38) respectively mediated by *Agrobacterium tumefaciens* (Smith et Townsend) Conn LBA4404 using leaf disc methods[11]. Transformed shoots were selected with 200 mg/L kanamycin.

1.2.3 PCR analysis of transgenic tobacco Genomic DNA was extracted from tobacco leaves as described by Edwards et al.[12]. PCR amplification was carried out using primers for *phbA* gene: PrA1, 5′-CACCATGACTGACGTTGTC-3′, PrA2, 5′-GAAGAGCTCTTCCTTATTT-3′. PCR procedure began with 95℃, 3 min; then 35 cycles of 95℃ for 45 s, 53℃ for 50 s, and 74℃ for 1.5 min; finally an extension at 73℃ for 10 min.

1.2.4 PCR-Southern analysis of transgenic tobacco PCR-Southern analysis of transgenic tobacco with probes for *nap300* and *7S* promoters and *phbA* gene was conducted according to the manual of DIG High Prime Labeling and Detection Starter Kit II (Roche).

1.2.5 Enzyme assay Tobacco seeds harvested every 48h from 14 to 30 DAF were measured for 3-ketothiolase activity in thiolysis direction via spectrophotometric method as described by Senior[13]. Protein concentration was determined by the Bradford method[10].

2 Results

2.1 Identification of *nap300* promoter

Transgenic tobacco expressing GUS gene under the control of *nap300* promoter (Fig. 1) was assayed for GUS activity. As shown in Table 1, there was almost no GUS activity at 5 DAF. However, during 5~10 DAF, a significant increase in GUS activity was detected. During the subsequent developmental stages (10~30 DAF), GUS activity increased steadily and the highest level of GUS expression was found at 30 DAF. Our data showed that there was no obvious decrease in GUS expression directed by *nap300* when compared with the results obtained by Ezcurra et al.[7] and Ellerström et al.[14]. GUS activity was undetectable in leaves and roots of transgenic tobacco, which proved that *nap300* was a seed-specific promoter.

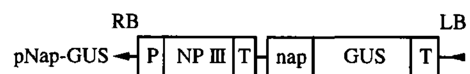

Fig. 1. Plant expression vector pNap-GUS. nap, *nap300* promoter; P and T, the promoter and terminator for nopaline synthase gene; NPTII, plant selective marker gene; GUS, β-glucuronidase gene; LB and RB, left and right border of T-DNA.

Table 1. GUS activity (pmol 4-MU · min^{-1} · mg^{-1} protein) in transgenic tobacco seeds at different DAF

Plant number	5DAF	10DAF	15DAF	20DAF	25DAF	30DAF
8-3	ND	13	35	720	1426	1512
8-7	ND	86	556	1365	2419	2771
8-10	ND	72	495	1098	1783	1807
8-16	9	227	807	1042	3557	3784
8-18	ND	98	463	842	3954	3744
8-27	12	184	1025	2975	4327	5595
Average	4	113	564	1340	2911	3202

ND, no activity has been detected. 4-MU, 4-Methylumbelliferone.

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To further study the spatial expression pattern, histochemical GUS staining was performed for seeds and embryos of transgenic tobacco. From the section of whole seeds, we could see both the embryo and the endosperm were stained. To avoid disturbance from other stained tissues, embryos were stained separately. It was found that the entire embryo or part of it showed GUS activity (Fig. 2).

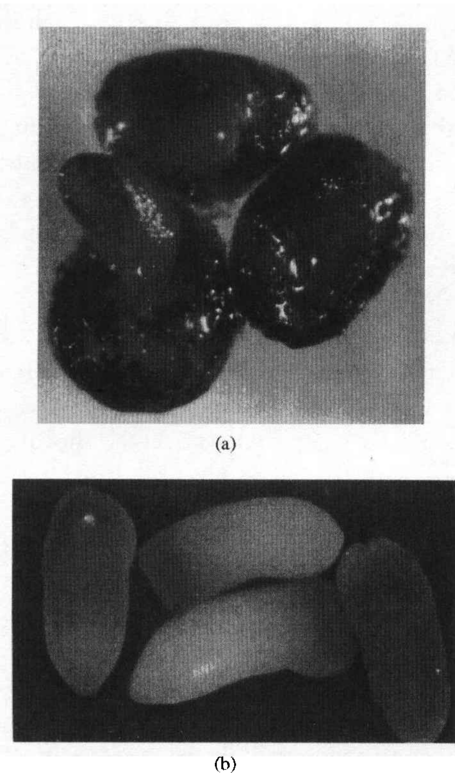

Fig. 2. Histochemical staining of transgenic tobacco seeds. (a), sections of seeds; (b), embryos separated from seeds.

After identifying the function of *nap300* promoter, we deposited this nucleotide sequence in GenBank with the accession number AF403424.

2.2 Promoter comparison

For a direct comparison of seed-specific promoters, *nap300* and *7S* were fused in a similar way to *phbA* gene encoding 3-ketothiolase (Fig. 3). The resulting gene constructs were introduced into tobacco mediated by A. *tumefaciens*. The transformed shoots were selected at 200 mg/L kanamycin and obtained plantlets were screened by PCR using primers for *phbA* gene (Fig. 4). PCR-positive transformants were further analyzed with PCR-Southern, in which *nap300*, *7S* and *phbA* were used as probes for transgenic tobacco (Fig. 5). Twenty-one rooted transgenic tobacco plants harboring pNap-A and twenty harboring p7S-A were transplanted to the greenhouse and grown to maturity.

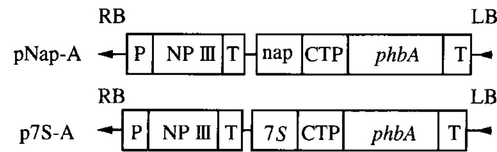

Fig. 3. Plant expression vectors pNap-A and a7S-A. For nap, NPTII, LB and RB, P and T, see Fig. 1; *7S*, *7S* promoter; CTP, chloroplast transit peptide; *phbA*, 3-ketothiolase gene.

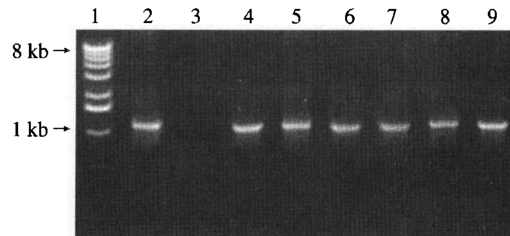

Fig. 4. PCR analysis of transgenic tobacco. 1, 1 kb ladder; 2, positive control, plasmid pNap-A; 3, negative control, untransformed tobacco; 4 ~ 6, the PCR products of *phbA* from genomic DNA of pNap-A transformants; 7 ~ 9, the PCR products of *phbA* from genomic DNA of p7S-A transformants.

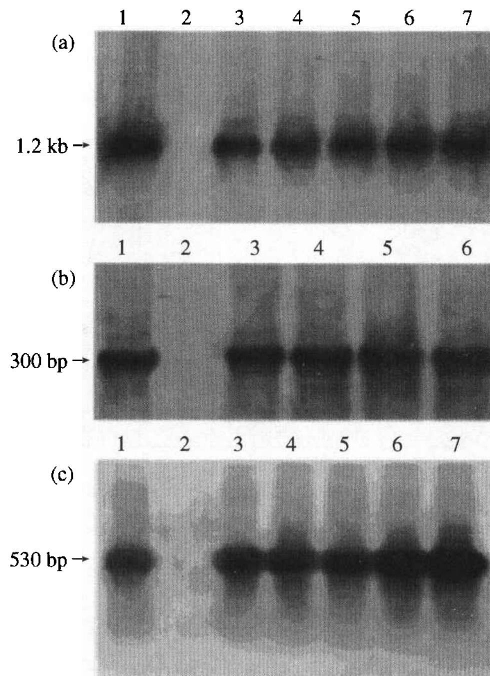

Fig. 5. PCR-Southern analysis of transgenic tobacco for *phbA* gene (a), *nap300* promoter (b) and *7S* promoter (c). Lane 1, plasmid pNap-A, pNap-A, p7S-A (a ~ c) as positive control; lane 2, negative control, untransformed tobacco; (a) the hybridization of transgenic plants harboring pNap-A (3 ~ 5) or p7S-A (6 ~ 7) with *phbA* as probe; (b) the hybridization of transgenic plants harboring pNap-A with *nap300* as probe (3 ~ 6); (c) the hybridization of transgenic plants harboring p7S-A with 7S as probe (3 ~ 7).

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Tobacco seeds were harvested and assayed for 3-ketothiolase. The results indicated that transgenic tobacco harboring either pNap-A or p7S-A showed a maximum of enzyme activity in seeds during 28～30 DAF. It was shown in Fig. 6 that the activity of 3-ketothiolase in transgenic plants harboring p7S-A increased during mid-to-late stages in embryogeny and reached its highest level at seed maturation. The enzyme activity for transgenic plants with pNap-A, which was lower than that for tansgenic plants with p7S-A, showed a similar trend.

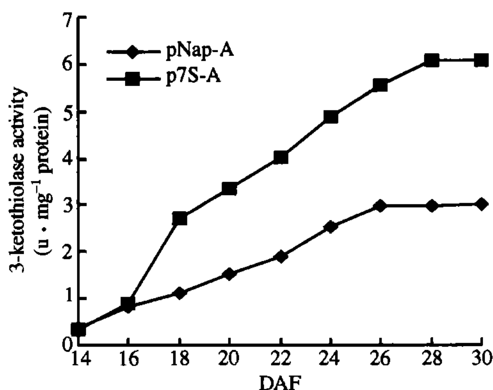

Fig. 6. 3-ketothiolase activity in seeds from transgenic tobacco with pNap-A or p7S-A at different DAF. All the data here are the mean of enzyme activities of six independent transgenic plants.

To investigate the tissue specificity of the two promoters, we analyzed enzyme activity in the leaves and roots of transgenic plants. Although in these tissues enzyme activity was undetectable for transgenic tobacco bearing pNap-A, there was a low activity in the leaves and roots for transgenic tobacco bearing p7S-A, which indicated that the tissue specificity of 7S promoter was not very strict (Table 2).

Table 2. 3-ketothiolase activity ($u \cdot mg^{-1}$ protein) in different organs of transgenic plants harboring pNap-A and p7S-A at 26 DAF

	Seed	Leave	Root
Transgenic plants with pNap-A	3.0	ND	ND
Transgenic plants with p7S-A	5.5	1.9	0.9

All the data here are the mean of the enzyme activities of six independent transgenic plants; ND, no activity has been detected.

3 Discussion

Seed-specific expression of transgenes is particularly attractive either for seed quality improvement or for the production of commercially valuable materials in plant. Now great efforts have been put on isolating and characterizing seed-specific promoters. Some sequence elements crucial for promoter function have been identified. One of them is B-box which includes the ABRE-like element distB (distal B) and the CA-rich element proxB (proximal B). Ezcurra et al. reported that the multi-base substitution mutation of distB element and proxB element simultaneously produced a 44-fold reduction of GUS activity[7]. In this study, there are three substitution mutations in the highly reserved region of *nap300* promoter (Fig. 7). One of them is in TACACAT consensus motif. This motif allows one base mismatch[15], thus we can assume that this mutation is due to the difference between species and will have no influence on *nap300*'s expression. To analyze the effect of the other two mutations in distB and proxB, we fused *nap300* to GUS reporter gene. Our data indicated that this approximate 300bp DNA fragment was sufficient to direct seed-specific gene expression with its activity increasing to the maximum at seed maturation. Compared with the work described by Ezcurra et al.[7], our results showed that the two substitution mutations in B-box led to a slight decrease in promoter's expression efficiency and almost had no effect on its temporal and spatial expression pattern.

There are several seed-specific promoters used in plant genetic engineering. Comparing their activities will provide valuable information about their relative expression efficiency and the difference in their tissue-specific expression patterns. Here the comparison of *nap300* and *7S* promoters indicated that the tissue specificity of *nap300* promoter was higher than that of *7S* promoter, which suggests that *nap300* should

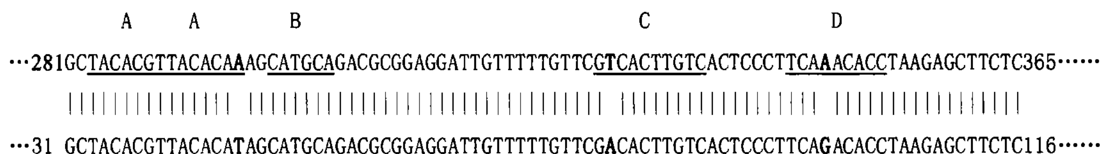

Fig. 7. Substitution mutations in highly reserved region for *nap300* promoter compared with *napinB* promoter. The upper row is a partial sequence of *napinB* promoter, lower row is a partial sequence of *nap300* promoter; A～D underlined stand for the conserved sequences of *napinB* promoter, A: TACACAT consensus motif (allow one base mismatch), B: RY repeats (CATGCA), C: distB, D: proxB; the site for substitution mutation was shown in boldface.

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be used when a ubiquitous expression must be avoided. It was also found that 7S promoter expressed the *phbA* gene more efficiently than *nap300* promoter, which means that 7S should be chosen when gene expression efficiency is a priority. Due to the two promoter's expression efficiency reaching a peak at the same developmental stage of tobacco, they have the advantage of being used simultaneously for expressing different foreign genes in plant. In our previous study, gene silencing is a common phenomenon in seed-specific PHB production because of co-suppression[5]. According to above results, promoter *nap300* will probably help us to solve this problem in plant-mediated PHB production by minimizing promoter-homology-mediated gene silencing. We expect that "green plastics" can compete with traditional plastics in the near future.

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