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Studies on the expression pattern of seed-specific napin promoter (BcNAI) in transgenic (*Nicotiana tabacum* L.) tobacco seeds

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***Abstract*— BcNA1 gene from Brassica napus, is highly expressed in developing seeds. The promoter of this gene, referred to as napin promoter, has been isolated and is shown to confer seed specific expression pattern in heterologous systems and thus has been used in many genetic engineering experiments aimed at expressing transgenes, especially those involved in fatty acid or TAG biosynthesis, in developing seeds. We were interested in studying the expression pattern of seed specific, napin promoter, in the developing seeds of tobacco. Therefore we were interested in isolating the napin promoter and validate the same in tobacco seeds. Promoter specific primers for napin were synthesized using the sequences available in the database and were used for amplifying the promoter using the genomic DNA isolated from Brassica napus. The obtained amplicon of the expected size (1752 bp) was cloned in T/A cloning vector and confirmed by restriction analysis as well as sequencing. The sequence data was subjected to BLAST analysis, which confirmed the cloning of napin gene. The isolated napin promoter was cloned in binary vector pCAMBIA1391Z upstream of uidA gene encoding β-glucuronidase and the confirmed clone was mobilized into Agrobacterium strain LBA4404. Putative transgenic tobacco shoots obtained using this vector were confirmed through PCR and standard GUS assay in the developing seeds of tobacco.**

***Index Terms*— Napin promoter, seed oil, transgenic, Tobacco, X-Gluc, Brassica**

I. INTRODUCTION

Over the last decade plant biotechnology has made a number of rapid advances, resulting in the commercial planting of thousands of hectares of transgenic plants. One area of interest in this rapidly expanding field of plant biotechnology is the modification of the lipid profile of oilseeds (Topfer et al., 1995). The application of the newer techniques of genetic engineering promises to revolutionize plant agriculture. It is envisioned that traditional seed products can be tailored to the end market, as for example, seed oils produced with specific fatty acid profiles. Thus, it has been possible to produce a rapeseed line with 88% oleic acid in the triacylglycerol fraction of the seed oil, by transferring an antisense gene to a fatty acid desaturase, FAD2, to the rapeseed; this desirable characteristic was limited to the seed oils, and therefore did not affect the fatty acids of the membrane lipids of the rest of the plant, by putting the antisense gene under control of the napin seed-specific promoter (Hitz et al., Kader JC and Mazliak P, Kluwer, Dordecht, 1995). It is also envisioned that seeds can be used to produce non-traditional products, such as edible vaccines. However, for these applications as well, it is preferable to utilize seed specific promoters, to limit the presence of such non-traditional products to the seed, and to avoid their presence in other parts of the plant. Using the seed specific promoters from the model systems to regulate, the target gene expression during the seed development has been studied extensively, and many achievements have been made. It is known that napins, which are encoded by a multigene family, are important storage proteins in rapeseed and other *Brassica* species and constitute 20-30% of their total seed proteins. Some of them are NAPI, napA, gNA, BcNAI have been cloned and sequenced. A number of reports are available on seed specific expression pattern of napin genes (Broun et al 1997, Victoria et al 1998, and Vesna katavik et al 2001).

Being seed specific in expression pattern, the promoters of napin genes are expected to be seed specific in their profile. It has been demonstrated that one of the napin genes, BcNAI expresses to high levels in developing seeds and its promoter could also drive the expression of foreign genes efficiently in the embryos of transgenic plants. Based on these reports it is considered that the upstream regulatory region of BcNAI gene can be successfully used in regulation of the deposit of seed in rapeseed or other crops by gene engineering. We have cloned and sequenced the upstream regulatory region of BcNAI gene and to test the tissue specific expression pattern of this promoter, we have cloned in pCAMBIA1391Z binary vector upstream of *gus* gene and the expression pattern of the cloned napin promoter is analysed in the transgenic tobacco plants developed using this construct.

II. MATERIALS AND METHODS

A. Plant, strains and media

Tobacco (*Nicotiana tabacum* L., Var. Xanthi), plants were used in this study. Transformation of tobacco was done with standard leaf-disc method. Leaf-discs were taken from aseptic plants grown in the light at 22°c in Murashige and Skoog medium supplemented with 2% sucrose (Murashige.P and Skoog F., 1962). *Escherichia coli* DH5α strain was used for plasmid cloning and propagation. *Agrobacterium* LBA4404 strain was used for plant transformation. *Agrobacterium* was grown in LB medium at 28°c with

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appropriate antibiotics. *Brassica* plants were grown in greenhouse for isolation of napin gene.

B. DNA isolation

Genomic DNA was isolated from *Brassica napus*.L using CTAB method (Doyle and Doyle., 1987) and was used as template DNA to isolate napin (BcNAI) promoter.

C. Cloning of napin promoter by PCR

Two oligonucleotide primers, napin forward primer 5' gcg tcg acg ata tca cta caa tgt cgg 3' and napin reverse primer 5' cgg atc ctt gtg tat gtt ctg tag tg 3',were synthesized based on the reported napin promoter coding sequence available at national centre for biotechnology information (NCBI) data base (Gene bank accession no. AF302261).

The primers were used to amplify napin promoter from the isolated genomic DNA. After initial denaturating at 94°c for 3 min. amplification was performed in 35 cycles of 20sec. at 92°c, 30 sec. at 55°c and 120sec. at 72°c followed by final extension at 72°c for another 10min. Amplification product was fractionated on 1 % agarose gel, from which the selected DNA band was purified. The purified PCR product was ligated with the InsT/A vector to generate a recombinant plasmid. Plasmid DNA was purified for sequencing using the qiagen QIA prep miniprep kit (Qiagen, United states). Nucleotide sequences were analysed using BLAST analysis and 'gene tool' softwares.

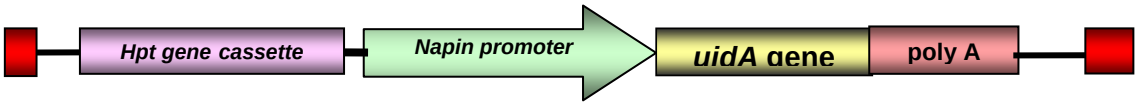

Fig.1: Schematic representation of cloning of napin promoter upstream of *uidA* (*gus)* gene in pCAMBIA 1391Z

D. Plant expression vector construction and tobacco transformation

To develop the required plant transformation vector, pCAMBIA1391Z vector backbone was used. pCAMBIA1391Z vector has a promoter-less *gus* gene cassette so that the promoter to be studied could be cloned upstream of the *uidA* gene and the resultant vector could be used for the promoter analysis. pCAMBIA1391Z vector also hygromycin gene cassette to select primary transformants using hygromycin as the selection agent. Napin promoter was excised out from the pNapin (InsT/A:napin promoter) plasmid using Sal1 and BamHI enzymes and the fragment was cloned into pCAMBIA1391Z vector backbone that was also digested with SalI and BamHI enzymes. This directional cloning ensured cloning of the promoter in right orientation upstream of *uidA* gene present in the vector. The recombinant plasmid was confirmed using restriction analyses and the binary vector was introduced into *Agorbacterium tumifaciens* strain LBA4404 by electroporation. Tobacco was transformed by the standard leaf-disc method (Horsch et al., 1988). Initial transformants were selected on 50µg/ml hygromycin. Plants were maintained in axenic culture under controlled conditions.

E. Molecular confirmation of transgenic plants

a. PCR analysis of transgenic plants

Genomic DNA was isolated from the leaves of untransformed and transformed plants using CTAB method (Doyle & Doyle., 1972). PCR was performed in a total volume of 20 µl containing 10-20µg of template DNA and napin specific primers under identical PCR conditions as described earlier. PCR products were fractionated on 1% agarose gel and documented.

b. GUS histochemical assay

The transgenic plants were assayed histochemically for GUS enzyme activity using X-Gluc (5 bromo-4-chloro-3-indolyl β-D glucuronide) as substrate. *GUS* was tested histochemically according to the procedure of Jefferson *et al*., (1987) in developing seeds of transgenic plants obtained from pBinN construct. Developing seeds of tobacco were collected at 18-20 days after fertilization and incubated overnight at 37˚C in X-Gluc solution (3-5 mg X-Gluc, 0.05 M phosphate buffer, 30 µl dimethyl formamide, 30 µl TritonX100). The seeds were cleared by treating the seeds with 70% alcohol. Finally samples with blue coloration were stored in 70% ethanol at 4˚C and photographs were captured under microscope.

III. Results and Discussion

The PCR product obtained with the primers designed to amplify the napin promoter was of the expected size (1.75 Kb) and so indicated that it could be napin promoter (Fig. 2). This fragment was cloned in InsT/A vector and confirmed by restriction analysis. The analysis indicated the clones (designated pNapin) had the expected size fragment and confirmed cloning (Fig. 3).

The sequence analysis of pNapin indicated that the amplified fragment contained 1.75Kb size and shared a homology of 99.5 % with the available sequence of napin promoter. This promoter region was excised from InsT/A and cloned in pCAMBIA1391Z vector and confirmed. The confirmed vector was designated pBinNGUS. The vector was mobilized into *Agrobacterium* and used for transforming tobacco leaf explants. A total of 17 putative transgenic plantlets were developed and transferred to soil. The transgenicity of these shoots was confirmed using PCRs with napin promoter specific primers. Majority of the shoots tested showed presence of the napin promoter (Fig. 4).

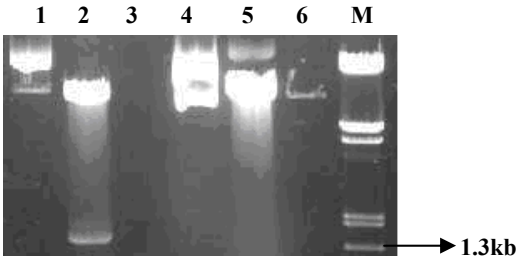

Fig. 3 Restriction analysis of InsT/A clones using *Sal* I+ *Bam*HI
Lane1,4: Uncut Plasmid DNA
Lane 2: Positive Plasmid of expected size 1.7kb
Lane5: Untransformed Plasmid

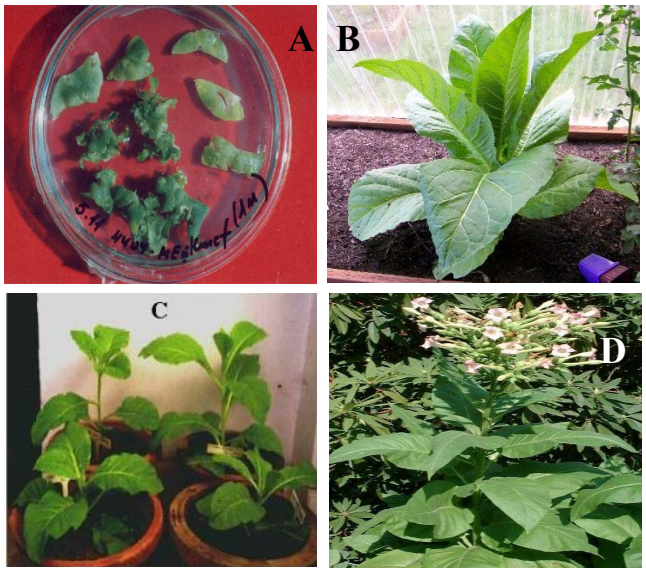

Fig. 5: Development of transgenic tobacco plants
A: Induction of green shoots from tobacco leaf discs, B: Acclimatized putative tobacco plants C: Putative tobacco plants are in vegetative phase in greenhouse D: Putative tobacco plants are at flowering stage in greenhouse

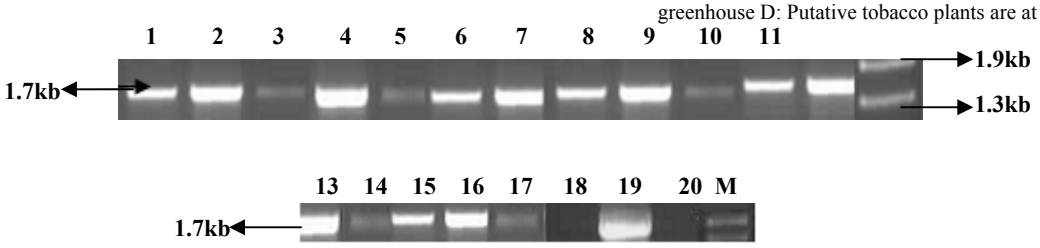

Fig.4: Confirmation of putative transgenic tobacco shoots using napin promoter specific primers (*Sal* I For + *Bam*HI Rev)
Lane 1-18 Transgenic shoots of Tobacco
Lane 19 Negative control (PCR control), Lane 20 Positive Control (plasmid DNA of pBinN*Gus*) Lane 21 PCR blank
Lane M. DNA size marker- λ DNA *Eco*RI + *Hind* III double digest

All the tobacco shoots transferred to half MS medium rooted and they were acclimatized initially in tissue culture lab and then in green house (Fig.5). The plants were normal in morphology and were comparable to the untransformed tobacco plants for all the phenotypic characters. From these confirmed transgenic plants, developing seeds were collected at 18-20 days after fertilization and analysed for the expression of *gus* gene in comparison with control tobacco seeds (Fig.6).Transgenic tobacco seeds showed blue colour while the seeds from untransformed control plant did not take up the stain. This clearly indicated that the isolated napin promoter had a seed specific expression pattern in tobacco.

IV. Conclusion

This might be taken as an indication that seed specific promoters are active in seed tissues and was confirmed by histochemical analysis of reporter gene expression.

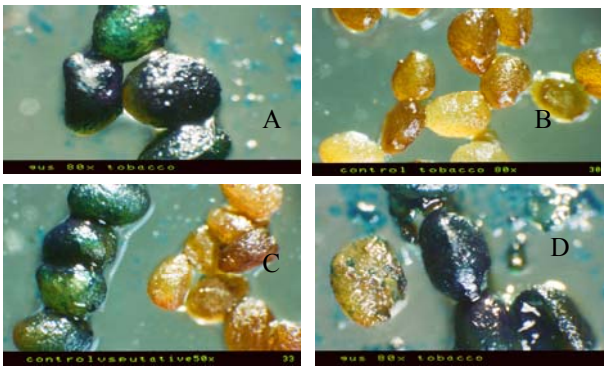

Fig.6: Expression of *gus* in the developing seeds of transgenic tobacco in comparison with control tobacco
A: Transgenic tobacco seeds (50x) B: Control tobacco seeds (50x)
C&D: Comparison of transgenic and control tobacco seeds(50x)

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