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Senescence specific *nnd* promoter analysis in *Nicotiana tabacum* SNN

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Abstract
Tobacco is a cursed lucrative crop. India being the second largest producer of tobacco (*Nicotiana tabacum*), its most unlikely that due to its high revenue and foreign exchange earnings, government policy would discourage the farmers. The pyridine alkaloids nicotine, nornicotine reported in genus *Nicotiana* (3). The deadly linkage between tobacco and cancers results from a robust combo of two agents - nicotine and carcinogens. Nicotine being present as the single most dominant (>90%) alkaloid is not carcinogenic. Nornicotine constituting around 5% of the entire pyridine alkaloid pool in tobacco, is a N-demethylated derivative of nicotine, concentrated in matured tobacco leaf during senescence and curing. In tobacco, nicotine N-demethylase enzymes (*NND*) belonging to the cytochrome P450 family (7) catalyse the conversion of nicotine to nornicotine, reducing tobacco quality and being detrimental for health. Therefore, the present investigation is envisaged for crop improvement of tobacco through promoter modification as per our research interest.

Keywords: Tobacco, Cancer, Nornicotine, *nnd*, promoter, improvement

Introduction
Plant molecular biology uses an extension of the classical plant breeding technique with important difference which allows for the transfer of a greater variety of genetic information in a high precision and controlled system. Plants accomplish a successful life by assimilating and overcoming a range of environmental stimuli adapting to both inducible and spatio-temporal expression patterns of genes. They are engineered to produce new biological substances which enable them to be used as "production systems" to supply valuable materials such as medicinal drugs or novel food ingredients by expressing a "Gene of Interest" or GOI in the plant. The expression of foreign GOI is controlled by a regulatory element called "Promoter". The expression level of GOI depends on the strength of promoter. The strength of promoter may be enhanced by genetic manipulations and such engineered promoters are very useful in plant biotechnology that has a high potentiality for commercialization. Promoters are cis acting regulatory elements lying upstream of coding sequence where RNA polymerase (RNAP) binds. Nicotine is the principal alkaloid in both commercial and homemade products (e.g., cigarettes, smokeless tobacco, bidis, waterpipes) followed by nornicotine, anabasine, anatabine, and many other basic substances that contain a cyclic nitrogenous nucleus. The functional tobacco nicotine N-demethylase enzymes, affiliated to the cytochrome P450 mono-oxygenase of CYP82E subfamily.The important nicotine N-demethylase enzyme is CYP82E4(two others functional *nnd* enzymes are CYP82E5v2 and CYP82E10) whose CYP82E4-*nnd* gene is developmentally regulated. Its transcript level is high during senescence and air-curing of tobacco as well as under biotic stress [ethylene and tobacco mosaic virus infection] treatments. Expression of CYP82E4 is leaf senescence specific whereas CYP82E5v2 and CYP82E10 [8] are constitutively expressed in green leaves and root tissues respectively. Nicotine is synthesized from aspartate and putrescine through several enzymatic steps, and then de-methylated by N nicotine demethylase enzyme (*NND*) to nornicotine. Tobacco types, leaf position on the plant, agricultural practices, fertilizer treatment, and degree of ripening are among some prominent factors that determine the levels of alkaloids in tobacco leaf. In fact, every step in tobacco production that affects plant metabolism will influence the level of alkaloid content to a certain degree. Depending on blending recipe, type and amount of additives, and product design, all types of tobacco products contain a very wide range of nicotine concentration.

In most tobacco (*Nicotiana tabacum* L.) plants, while nicotine is considered to be the most abundant alkaloid nornicotine is a relatively minor alkaloid of the total pyridine alkaloid pool in the mature leaf. Nornicotine is the demethylation product of nicotine and the precursor of tobacco-specific nitrosamine N'-nitrosonornicotine (NNN) in tobacco *(Nicotiana tabacum* L.). Nornicotine is believed to be a source of many undesirable health effects. Similar to nicotine, nornicotine accumulates in the brain, induces dopamine release and contributes to the pharmacological profile of nicotine.

In tobacco research, nicotine to nornicotine conversion has vital importance. Understanding this conversion phenomenon would help in breeding low nornicotine producing species because nornicotine affects tobacco quality by causing unwanted flavor and decreasing smoking quality. Nicotiana species that convert a large portion of their nicotine content to nornicotine during sensescence and curing are Converters whereas the species that mainly accumulate nicotine in their leaves are Non-Converters. Nicotine to nornicotine conversion in tobacco (*Nicotiana tabacum* L.) is regulated by an unstable converter locus which in its activated state gives rise to a high nornicotine, low nicotine phenotype in the senescing leaves. Cytochromes P450 (CYPs) are proteins of superfamily containing heme as a cofactor and are in general, the terminal oxidase enzymes in electron transfer chains; the term "P450" [7] is derived from the spectrophotometer peak at the wavelength of the absorption maximum of the enzyme (450 nm) when it is in the reduced state. Nicotine N-demethylase (*NND*), the main source for conversion of nicotine to nornicotine, is encoded by cytochrome P450 mono oxygenase genes of the CYP82E subfamily in *N.tabacum*. In plants that carry the high nornicotine trait, nicotine conversion is primarily catalyzed by a cytochrome P450 protein, designated CYP82E4 whose transcription is strongly upregulated during leaf senescence. Changes in gene expression at an unstable locus, however, can give rise to plants that produce high levels of nornicotine, specifically during leaf senescence and curing. Minimizing the nornicotine content in tobacco is highly desirable, because this compound serves as the direct precursor in the synthesis of N'-nitrosonornicotine, a potent carcinogen in laboratory animals. Nornicotine is likely produced almost entirely via the N-demethylation of nicotine, in a process called nicotine conversion that is catalyzed by the enzyme nicotine N-demethylase (*NND*). Previous studies have identified CYP82E4 as the specific *NND* gene responsible for the unstable conversion phenomenon, and CYP82E5v2 as a putative minor *NND* gene. During last decade, numbers of modified promoter with duplicate enhancer were constructed and applied in plant biotechnology-based program. There is an increasing higher public acceptance of the NPPs (novel plant products obtained by cisgenesis, intragenesis and genome engineering using site-specific nucleases and gene-targeting oligonucleotides) devoid of virus sequences and antibiotic genes, and containing only genetic material derived from the species itself or from closely related species. Plant expression vector coupled to promoter with enhanced activity and sequence heterogeneity is one of the prime needs in gene-based approach for plant modification [14]. Exploring CRISPR-Cas9-sgRNA-mediated genome editing technology as an effective nontransgenic approach in dismantling gene structure and subsequent expression of nicotine N-demethylase (*NND*) to reduce the conversion of nicotine to nornicotine in high conversion tobacco lines to nullify tobacco harmful carcinogen tobacco specific nitrosoamines (TSNAs). In a parallel investigation after molecular characterisation of a Flt [12] promoter fragment, cloning of Cas9 with the selection of the best expressing fragment helped in development of binary expression vectors containing M24 (modified full length transcript promoter with duplicated enhancer domains of MMV *Mirabilis mosaic virus*, a member of the Caulimovirus family; that can be efficiently transformed into tobacco plants to deliver and drive the expression of the Cas9/sgRNA genes for optimizing the expression and mutation rates under both promoters). Assays of CRISPR-Cas9-mediated genome editing in plants will support screening for mutations in target sites generated via transient [13] electroporation followed by raising CRISPR-Cas9 edited tobacco plants carrying the test constructs. The edited plants are advanced to subsequent generations for simultaneous analysis of alkaloid content, primarily nicotine and nornicotine levels for stringent evaluation in field trials.

Materials and Methods

Nicotiana tabacum Turkish Samsun NN is the tobacco variety used in this study. A design for modified [6] promoter deletion analysis (Fig.1) was carried out using *nnd*E4 promoter sequence (770-bp with EcoRI-HindIII; co-ordinates 251-1021) and comparison with full-length transcript (FLt) promoter.

Table 1: Experimental materials with their respective source

Name	Source
1. Enzymes and DNA markers T4 DNA ligase, Restriction Endonuclease	NEB, USA
Pfu DNA Polymerase, Klenow DNA polymerase Taq DNA Polymerase, dNTP mix, DNA ladder, Restriction Endonucleases like EcoR1, Hind III.	Promega, USA
2. Bacterial Strains E. *coli* TB01, E. *coli* BL21	Stratgene, Germany
Agrobacterium tumifaciens, strain LBA 4404	NIPGR, India
Plasmids Expression vector, Pklyxgus71, pUCpmagus	Dr. I.B Maiti, Director, Division of Plant Pathology, University of Kentucky, Lexington, USA

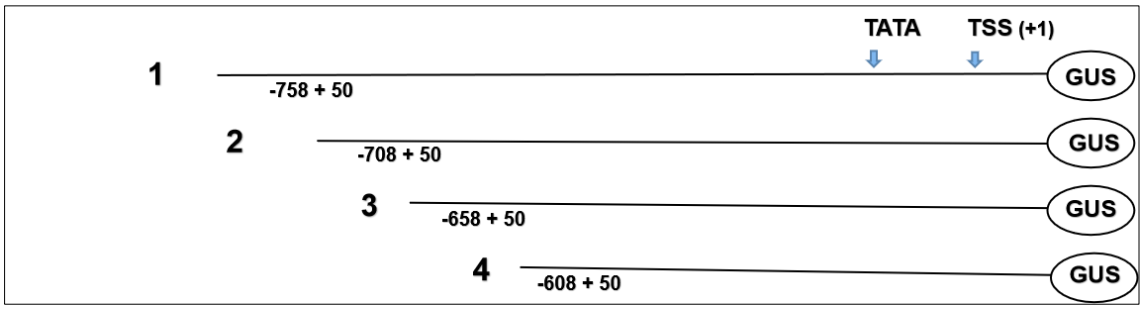

Fig 1: A schematic map of the GUS constructs developed for the leader-deletion analysis of promoter along with the end coordinates of the relative deletion fragments of four representative constructs

Gaattc|GTTAAATCTTAGCCCAACCC

;

TATCGTTGGTTCGACTTTTTTTCT

TTTAATTTTGATTTATTCTGTTCGGTAATTTCGCTTTGTTTGGCTTGAAT

;

AATAACTAGTGCATAAAGTCATATACTCTAATATTTTTAATTGAAGTACT

CACAAATACAAAAATAAAAAACATTCTAAGCTCACATGATAtTTGACAAA

ATCTTTATCCAAAACAAGAGGCGGAGCCAGGATTTGAAACTTATGGGTTC

AGAtTTCTAAATCTCTTAAGTTACTAGGTTCTAAATTAATAATTTATACAT

GTTCAATGAATTTCTTAAGACAAATACATAGTTTGAACGAAAGCTACTGG

GTTCGGCCGAATCCGTAAGTTATACTCTCCCTCCGCCCCGGTCCAAAACC

AGCTAGTATCAATAGAGAGAGAGAGAGAGAGAGAGAGATAATAAATTTGA

CCATTGACAATGGCTTATTACTTGCTTAGAGTTAATTGGTGAACTTAGAG

AATATAATAAGGAATATTTAAACAGATACGTCATCAATCCACGAGTAACG

AAGTAAGAAATACCCTAAAATCGTAGAAACATTACGTTAAATTGCTTGAC

AGCCTATCTAGTAAGAGTCAAAATCTACTATCTATCTTGTTCCGCCATTT

TCTTAAAGAAGTACATGAGCTTTATCATCCACCTCAACATGAATGCAAAA

GAAAATTATTGTGCAACTTAATATGTTATAATCAATGATATGTGTCTTGT

GTAACAAAGTATATATTTCGATACGATATTAATATGTAGGTGTTATATTT

TTAAATATCAAATATCATACTTAACACCGATTTTTTAAAAACTTAGGCCA

ATTACCCTACCAACTAAAATACTGTATATCAAACACTAATGTTTTCTATT

TCGGTACGACAGTTCTCTATTTACCATATTATGGAATTATGCCCATCCTA

CAGTTACCTATAAAAAAGGAAGTTGCCGATAGTTATATTCTCAACTT

CTTATCTAAAAATCCATA aagctt

Fig 2: *nnd* E4 promoter sequence (co-ordinates 251-1021)

Table 2: *nnd* promoter fragments sequence

Promoter Fragment	Forward Primer	Reverse Primer	Construct Size in bp
NND1	NND-FP1	NND-RP	758
NND2	NND-FP2	NND-RP	708
NND3	NND-FP3	NND-RP	658
NND4	NND-FP4	NND-RP	608
NND5	NND-FP5	NND-RP	558
NND6	NND-FP6	NND-RP	508
NND7	NND-FP7	NND-RP	458
NND8	NND-FP8	NND-RP	408
NND9	NND-FP9	NND-RP	358
NND10	NND-FP10	NND-RP	915
NND11	NND-FP11	NND-RP	815

A series of defined Flt promoter fragments (for 3' end and 5'-end deletions) will be amplified by PCR with appropriately designed primers to tailor an EcoRI site at the 5'-end and a Hind III site at the 3'-end of the amplified products. Modified Flt promoter fragments will be cloned in the EcoRI and Hind III sites of pUC119 vector and then sub-cloned into the corresponding sites of pUCPMAGUS vector.

Table 3: Forward and reverse primers for leader-deletion analysis

NND-FLT#1	5' - GGGCGAATTC GTTAAATCTTAGCCCAACCC
NND-F#101	5′- GCGGGCGAATTC AATAACTAGTGCATAAAGTCATA
NND-F#201	5′- GCGGGCGAATTC ATCTTTATCCAAAACAAGAGGCG
NND-F1#251	5′- GCGGGCGAATTC TAAATCTCTTAAGTTACTAGGTT
NND-F2#301	5′- GCGGGCGAATTC TGAATTTCTTAAGACAAATACAT
NND-F3#351	5′- GCGGGCGAATTC CCGAATCCGTAAGTTATACTCTC
NND-F4#401	5′- GCGGGCGAATTC TATCAATAGAGAGAGAGAGAGAG
NND-F5#451	5′- GCGGGCGAATTC ACAATGGCTTATTACTTGCTTAG
NND-F6#501	5′- GCGGGCGAATTC ATAAGGAATATTTAAACAGATAC
NND-F7#551	5′- GCGGGCGAATTC GAAATACCCTAAAATCGTAGAAA
NND-F8#601	5′- GCGGGCGAATTC TCTAGTAAGAGTCAAAATCTACT
NND-F9#651	5′- GCGGGCGAATTC AGAAGTACATGAGCTTTATCATC
NND-R1#1021	5'- ATGCAGAAGCTT TATGGATTTTTAGATAAGAAG

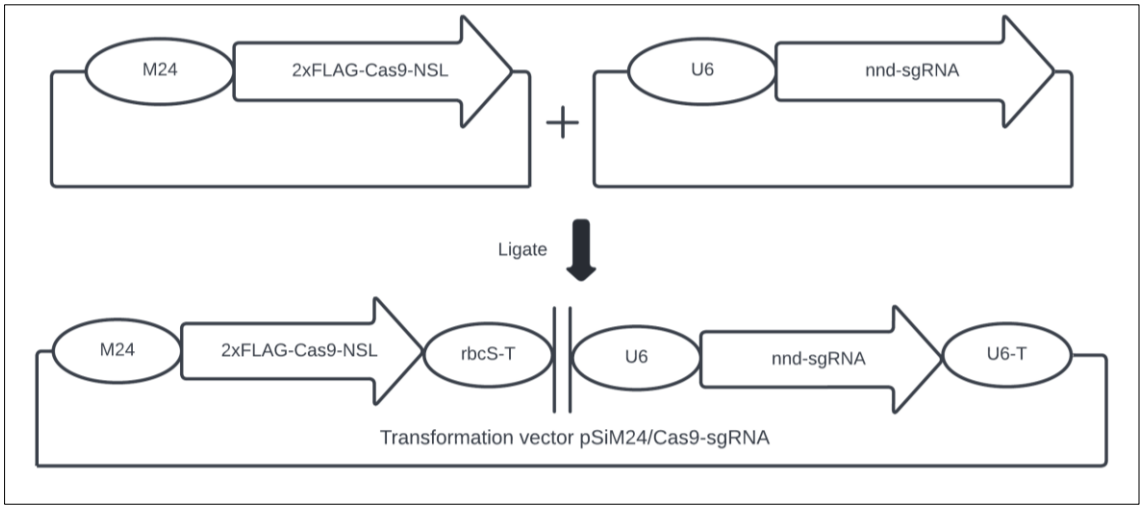

Fig 3: Construction of binary expression vectors for co-expression of Cas9 and two gRNAs for dysfunctioning the nnd gene in protoplasts from tobacco cell suspension culture. The isolated Cas9 gene was cloned into the XhoI and SstI sites of the pSiM24 vector. Cas9 contained a 2X FLAG tag sequence (DYKDDDDKKDYKDDDDKH) at the 5'-end for immuno-detection and a 3'-terminator of ribulose bisphosphate carboxylase small subunit (rbcS-T); g-RNA expressed under RNA-dependent RNA polymerase III promoter (U6 promoter) while its expression is terminated by the U6 terminator.

Methods

It is possible to redesign the promoter architecture employing either site-directed mutagenesis or promoter-DNA shuffling approaches [16]. Such *cis* shuffled/ *cis*-rearranged efficient promoters are certainly 'better' choices for endogenous expression of gene in plant [2]. The present study involved analysis of a senescence specific CYP82E4 Nicotine N-Demethlayse (*nnd*) promoter for targeted metabolic engineering in tobacco. Initially selection of the target i.e. *nnd* CYP82E4 gene and thereby devising a system to induce targeted modifications within its ~350 bp genomic region of the nnd exon sequence for its disruption involves the following:

1. Designing of fragments (promoter-leader deletion analysis) specific to the promoter region of the GOI
A set of twelve 5'-deletion promoter fragments were generated using forward and reverse primers (using the map of *nnd* promoter sequence of 1009 bp). Cloning was done using PCR, agarose gel electrophoresis, gel elution, double digestion using restriction enzymes EcoRI and HindIII in the pucPMA GUS [10] vector (i.e. protoplast expression vector) which was digested and eluted by running through 1% agarose gel electrophoresis. Competent cells were prepared using E.coli TB1 strain in cryovials kept in -80 ºC. After ligation of the insert with the pucPMA*GUS* vector, transformation was carried out in ampicillin (Amp) selection LB medium. These plates are then kept for 12-16 hours in 37 ºC incubator. The colonies on the Amp plates were confirmed by conducting Colony PCR with their respective positive and negative control. The confirmed colonies were inoculated for extracting their plasmids (mini prep and midi prep). The concentration of the purified plasmids is checked and quantified using nanodrop spectrophotometer.
2. Protoplast GUS assay and by sonication, electroporation of ppG constructs (plasmid DNA having the promoter fragment fused with a *beta-glucuronidase (*GUS)-encoding gene using Gene Pulser II apparatus of BioRad) into the previously prepared tobacco protoplasts (*Nicotiana tabacum* L. cv. Xanthi 'Brad'). Relative GUS expression levels will be assayed for all constructs.
3. Methods to evaluate the strength of the various promoter fragments made by 5' leader deletion efficiently in transient expression systems like agroinfiltration assay; assay in tobacco protoplasts using GUS reporter gene to analyse the activity of *nnd* promoter quantitatively and identify the best expressing fragment.
4. Agrobacterium-mediated transformation of tobacco: Functional evaluation of transgenics for future study regarding plant expression after agro-infiltration through protein extraction and purification assays. Cloning of the selected target in the vector followed by sub-cloning in the binary plant expression vector (fig. 2) to integrate the *nnd* promoter fragments stably [9] through plant expression vector pKYLX71GUS. The putative target amplicons were then sequenced to detect the modifications. For visualization of the modifications, the sequences were aligned with the native genomic target sequence and modification percentages were evaluated in earlier studies [11].
5. Raising of transgenic tobacco plants after cloning of best fragments using the plant expression vector *nnd*-pKYLX71GUS [5] alongside transient assays with deletion constructs of promoters in different tobacco plants. Each construct was transfected and assayed [1] in at least two replicates.
6. Selection on the basis of gas chromatography studies of nornicotine levels in foliar analysis of advancing generations of modified plants.

Result and Discussions
In light of this study of functional characterization of the *nnd* promoter which aid in understanding its transcriptional regulation towards development of efficient promoter construct for future genome editing studies to eliminate or minimize the expression of functional *NND* gene by targeting various regions of *NND*-ORF, promoters were constructed and compared for their relative efficacy with the FLt promoter. The promoter M24 was used for gRNA and Cas9 genes respectively, where several types of mutations were registered in target sites. Characterization of promoter by using 5' deletion analysis of fusion construct between the 2.2 kb upstream regulatory region of CYP82E4 and the beta-glucurodinase gene (GUS is the reporter gene cloned downstream of the CYP82E4 promoter for studying the transcriptional regulation and measuring the activity of this promoter). In the leaves, GUS activity was highly correlated with the progression of senescence. Here, we observed that both *NND* 4, *NND* 9 showed stronger activity compared to that obtained from FLt promoter through agroinfiltration-based GUS assays. Both the promoter constructs developed in this study could be useful in engineering putative gene constructs suitable for our GOI and could thus prove to be potential candidate promoters in plant genetic engineering for developing various independent tobacco transgenic lines with reduced nornicotine levels and validation through research subjecting them for molecular and biochemical characterisation of tobacco leaf profiles.

Conclusion
Plant science has been making quantum progress in developing new biotechnology-based plant breeding techniques to alter genetic factors. The work describes the initial work prior to the development of molecular vectors harboring gRNA/Cas9 system for modification of tobacco *nnd* gene. The aforementioned research analysis of *nnd* background provided a novel platform through characterisation of modified Flt and determination of best expressing fragment tailored from promoter-leader deletion analysis (3' and 5'-end deletions) from transient and transgenic assays for the control of target genes in tobacco as a crop plant. This technique will aid in the modification of plant genetic architecture for tobacco crop development. The pSiM24 plasmid [15] can act as a platform for various applications like gene expression studies and different promoter expressional analyses(GenBank: KF032933) vector with a strong constitutive promoter M24 was selected as the source generating material for designing the binary transformation vectors used henceforth. Further research on genetic alteration of tobacco *nnd* gene constructs by the CRISPR-Cas9 system using binary vectors to induce targeted mutations in *NND* gene that could be beneficial to minimize nornicotine formation. RNA guided genome editing in these plants can be done by targeting tobacco *NND* genes (selecting target sites 20 nucleotide of *NND* genes in open reading frame with protospacer adjacent motif, trinucleotide NGG at 3'-end of target region)of "CYP" family. Since it is a senescence specific promoter (4), the basic mechanism that lies behind its stress-specific response/activity needs to be understood. Ultimately reaching a far vision of better marketability of non edible tobacco cultivars that performs well in field trials is the plus point for high revenue but not at the cost of human health.

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