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Development of molecular mapping populations for tobacco specific nitrosamines

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Abstract
In the present study, an effort was made for the development of mapping population for mapping of Tobacco-specific nitrosamines (TSNA) in tobacco. TSNAs are found only in tobacco products, and are considered to be harmful to tobacco consumers. Molecular mapping of TSNA trait aid in tailoring the tobacco plants to yield lower levels of TSNAs using marker assisted breeding strategies. In order to select the parents for developing mapping populations, 11 burley lines were characterized for their TSNA yielding abilities and molecular polymorphism using 34 RAPD markers. Based on this, three polymorphic lines viz., Banket-A1, By-64 and VA-510 were identified as parents for developing mapping population. Banket-A1 is a high TSNA yielding variety and selected as one of the parent. VA-510, a low TSNA line recording higher molecular diversity with Banket-A1 was crossed with Banket-A1 for developing mapping population. Another line, By-64 recording low TSNA, having desirable morphological characters and least molecular diversity with Banket-A1 was crossed with Banket-A1 for developing another mapping population. Recombinant Inbred Lines (RILs) were developed from both the crosses through single seed descent method. These populations developed using diverse parents are of immense use in mapping of genes governing TSNA content in tobacco.

Keywords: tobacco, tobacco-specific nitrosamines, TSNA, burley, diversity, mapping population

Introduction
Tobacco-Specific Nitrosamines (TSNA) in tobacco products are considered to be harmful to the health of consumers (Lakshminarayana, 1998, Sarala *et al.*, 2012) [1, 2]. Higher the levels TSNA in tobacco, more the associated health risk (Hecht, 2003) [3]. These nitrosamine carcinogens are formed from nicotine and related compounds by a nitrosation reaction that occurs during the curing and processing of tobacco [3]. Essentially the plant's natural alkaloids combine with nitrate forming the nitrosamines. They are called tobacco-specific nitrosamines because they are found only in tobacco products, and possibly in some other nicotine-containing products. Among the tobacco-specific nitrosamines, nicotine-derived nitrosamine ketone (NNK) and *N*-nitrosonornicotine (NNN) are the most carcinogenic.[3] Others include *N′*-nitrosoanatabine (NAT) and *N*-nitrosoanabasine (NAB). The tobacco-specific nitrosamines are present in cigarette smoke and to a lesser degree in "smokeless" tobacco products such as dipping tobacco and chewing tobacco. However, their yields in tobacco products are mainly depending on the TSNA yielding abilities of tobacco used in the preparation of tobacco products. Cigarettes are made by blending flue-cured Virginia (FCV) and burley tobacco in specific proportion with few additives. The TSNA levels in burley tobacco (8-15 ppm) found to be in general high and reducing their levels to 1ppm is considered to lower the health risks associated with the cigarette consumption.
Effort is being made at ICAR-Central tobacco Research Institute (CTRI), Rajahmundry for lowering TSNA levels in burley tobacco through breeding and agronomical approaches. As TSNA formation is genetically controlled, hence, understanding the genetics will helps to bring down the levels of these constituents to safe levels through breeding of low TSNA yielding tobacco varieties. Establishment of mapping populations using selected parents and analyzing them one approach to understand and map the genes involved in various traits of interest. The present paper deals with the development of mapping population for mapping genes involved in TSNA formation. For generation of mapping populations through controlled crosses selection of parents having sufficient variation for the trait of interest at both at DNA sequence level and at phenotypic level is the first step.

The present investigation deals with the selection of burley lines as parents based on their TSNA yielding abilities and molecular diversities and development of mapping population for mapping TSNA trait.

Materials and Methods

Selection of burley lines

Based on the previous studies conducted at ICAR-CTRI, eleven burley lines viz., Banket-A1, Sota-6506, Harrow Velvet, By Resistant, By-64, By Sota-51, Ky-10, T-117, VA-510, BSRB-II and Burley-21 differing in their TSNA abilities were selected for the present study. Banket-A1 and Burley-21 are high TSNA yielding lines and cultivated varieties. Others are low TSNA forming germplasm lines.

Confirmation of tar content of selected burley lines

All the burley lines were raised under field condition during 2005-06, leaf samples were collected from middle picks and air cured. Lamina portion of the air cured samples were analysed for nicotine, nor-nicotine and TSNAs viz., N-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB) and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) using GC -Thermal Energy Analyzer.

Genetic diversity study

DNA was extracted from all the 11 burley lines as per Doyle and Doyle (1990)[4] method with little modification, quality analysed on 0.8% agarose gel and quantified using spectrophotometer (Nanodrop ND-1000, USA). The isolated DNA was amplified in a 25 µl reaction mixture containing 30 ng template DNA, 0.5 units of Taq polymerase, 0.2 mM dNTPs and 30 ng of each primer (Williams *et al.,* 1990) [5]. Thirty four random primers (Operon Technologies, USA) that found to be polymorphic (Xu *et al*., 1998)[6] among tobacco cultivars were used for the amplification of burley lines (Table 1).

Table 1: RAPD (Operon Technologies, USA) used in the present study

S. No	RAPD primer	Sequence
1.	OPAA-04	5'-AGGACTGCTC-3'
2.	OPAA-05	5'-GGCTTTAGCC-3'
3.	OPA-05	5'-AGGGGTCTTG-3'
4.	OPA-08	5-GTGACGTAGG-3
5.	OPAB-12	5-CCTGTACCGA-3
6.	OPAC-10	5-AGGAGCGAGG-3
7.	OPB-01	5-GTTTCGCTCC-3
8.	OPB-13	5-TTCCCCCGCT-3
9.	OPB-14	5-TCCGCTCTGG-3
10.	OPC-11	5-AAAGCTGCGG-3
11.	OPC-15	5-GACGGATCAG-3
12.	OPD-01	5-ACCGCGAAGG-3
13.	OPD-05	5-TGAGCGGACA-3
14.	OPD-09	5-CTCTGGAGAC-3
15.	OPD-12	5-CACCGTATCC-3
16.	OPE-01	5-CCCAAGGTCC-3
17.	OPE-03	5-CCAGATGCAC-3
18.	OPE-06	5-AAGACCCCTC-3
19.	OPE-11	5-GAGTCTCAGG-3
20.	OPF-08	5-GGGATATCGG-3
21.	OPF-16	5-GGAGTACTGG-3
22.	OPH-01	5-GGTCGGAGAA-3
23.	OPH-05	5-AGTCGTCCCC-3
24.	OPJ-15	5-TGTAGCAGGG-3
25.	OPL-08	5-AGCAGGTGGA-3
26.	OPL-09	5-TGCGAGAGTC-3
27.	OPM-02	5-ACAACGCCTC-3
28.	OPM-06	5-CTGGGCAACTC-3
29.	OPM-10	5-TCTGGCGCAC-3
30.	OPM-13	5-GGTGGTCAAG-3
31.	OPN-07	5-CAGCCCAGAG-3
32.	OPN-08	5-ACCTCAGCTC-3
33.	OPN-15	5-TGGCGTCCTT-3
34.	OPN-20	5-ACACACGCTG-3

PCR cycles consisted of one cycle initial denaturation at 95ºC for 5 min followed by 35 cycles of 95ºC for 1 min, 37ºC for 1 min and 72ºC for 2 min with final primer extension cycle of 7 min at 72ºC. Amplified PCR products were separated on 0.8% (w/r) agarose gels having 0.5 µg/ml ethidium bromide in 1xTris-acetate-EDTA (TAE) buffer.

The binary RAPD data was recorded depending on the presence or absence of the bands obtained after the RAPD analysis. Amplified bands were scored as present (1) or absent (0). The genetic variation among the tobacco lines is analyzed using the NTSYS-pc software version 2.02 (Rohlf, 1998) [7] using the RAPD binary data. Using the software Jaccard's (1908) [8] similarity coefficients among 10 cultivars were calculated. Based on UPGMA and SAHN clustering, a dendrogram depicting the genetic relationship among the burley lines was prepared.

Development of mapping populations

Based on TSNA content of burley lines and RAPD genetic

similarities, two crosses were made between selected burley lines. Single seed descent method was followed in handling the segregating generations.

Results and Discussion

In order to select the parents having variability at both TSNA and DNA sequence level for developing mapping population, TSNA yielding abilities and RAPD diversity among the selected lines was studied.

TSNA variation among the selected lines

During TSNA formation, nicotine, a principal alkaloid produced in tobacco is partly converted into nor-nicotine which in turn converted into different types of TSNAs (Hashimoto and Yamada 1994[9]; Bush *et al*. 2001[10]). Hence, the Nicotine and nor-nicotine yields of burley lines were estimated (Table 2). Nicotine content among the eleven burley lines found to range from 0.481 (T-117) to 2.049% (Banket A1) and nor-nicotine from 0.03 (in By Sota-51) to 1.103% (in Banket A1). The total TSNA content recorded from 0.68 (Sota-6506) to 12.82 ppm (Banket A1). Banket A1 has the highest TSNA content (12.82 ppm) followed by BSRB-II (6.75 ppm) and By-21 (6.15 ppm). T-117 recorded medium TSNA (2.31 ppm) and Sota-6506, By-64, Harrow Velvet, Burley resistant, By Sota-51, Ky 10, and VA-510 low TSNA contents ranging between 0.68 to 1.56 ppm. Among the four TSNAs, NNN contents are higher followed by NAT. NAB and NNK are found in either cultivated varieties (Banket A1 and Burley 21) or advanced breeding line BSRB-II). Among the lines tested, Banket-A1, a cultivated burley tobacco variety found to have maximum nicotine, nor-nicotine and TSNAs.

Table 2: Nicotine, Nor-Nicotine and TSNA yielding abilities of burley lines

S. No	Variety	Nicotine (%)	Nor-Nicotine (%)	Tobacco Specific Nitrosamines (ppm)				
				NNN	NAT	NAB	NNK	Total
1	Banket-A1	2.049	1.103	9.18	1.95	0.95	0.74	12.82
2	Sota-6506	1.089	0.114	0.37	0.31	0	0	0.68
3	Harrow Velvet	1.057	0.076	0.56	0.19	0	0	0.75
4	By Resistant	1.281	0.061	0.5	0.44	0	0	0.94
5	By-64	1.312	0.061	0.87	0.25	0	0	1.12
6	By Sota-51	1.057	0.03	1	0.56	0	0	1.56
7	Ky-10	1.441	0.076	0.5	1	0	0	1.5
8	T-117	0.481	0.076	1.88	0.81	0.62	0	2.31
9	VA-510	1.441	0.494	4.4	0.56	0	0	0.96
10	BSRB-II	1.025	0.114	4.5	1.12	0.5	0.62	6.75
11	Burley-21	1.185	0.304	3.53	1.5	0.46	0.66	6.15

Molecular diversity among burley lines

Molecular markers have become useful tools to provide a relatively unbiased method of quantifying genetic diversity in plants (Clegg, 1990) [11]. Del piano *et al.*, (2001) [12] also stated that the molecular marker techniques have become fundamental tool for plant scientist as they can provide a relatively unbiased method of quantifying genetic diversity in plants. Genetic diversity had been studied in many crops using markers like restricted fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites (Aggarwal *et al.* 1999 [13]; Angiolillo *et al.* 1999 [14]). Williams *et al*., (1990) [5] described a procedure for the identification of polymorphism in plants based on PCR. RAPD sequences (or) RAPD markers, are based on the use of single, short (10 mers) synthetic oligonucleotides primers. The primers were generated with attract 60% G+C content to ensure efficient annealing, and with sequence that are not capable of internal pairing that can produce PCR artefacts. RAPD a PCR based method is being preferred in crop plants because of the relative ease with which PCR assays compared to RFLP. Besides, prior knowledge about the genome is also not a pre-requisite, which makes RAPD a common method for such studies in different crops (Nair *et al*. 2002) [15].

In the present study, 34 single arbitrary decanucleotide random primers (Table 1) were tested for their ability to produce polymorphic bands in the burley lines. The RAPD exhibited both high and low intensity bands. Presence of bands indicates the complimentarity between primer and The genome of that particular variety. Absence of bands indicates that there is no complimentarity between the primer and that particular variety. While analyzing the diversity, amp icons with differential migration on agarose gel were of fluorescence. Binary RAPD data was scored depending on the presence or absence of the bands obtained after the RAPD analysis. The binary RAPD data was used for estimating the genetic similarity/diversity among the lines with NTSYS-pc software version 2.02 and the dendrogram was constructed using the UPGMA cluster analysis.

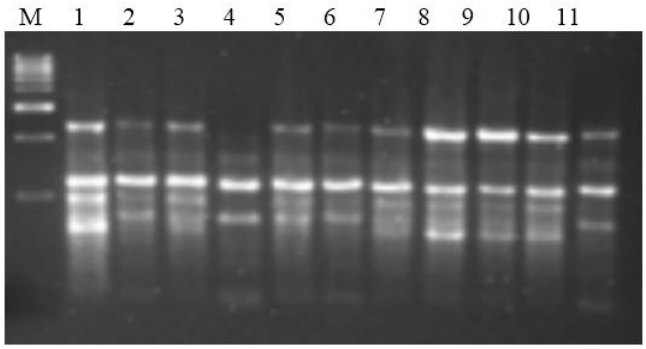

Fig 1: RAPD profile showing the genetic polymorphism among the burley lines detected using primer, OPB-1. Lanes M-Marker, 1. Banket A1, 2. Sota-6506, 3. Harrow Velvet, 4. Burley resistant, 5. By-64, 6. By Sota-51, 7. Ky-10, 8. T-117, 9. VA-510, 10. BSRB-II, 11. By-21

Table 3: Genetic similarity coefficients based on the RAPD data

Lines	Banket -A1	Sota-6506	Harrow velvet	Burley resistant	By-64	By Sota -51	Ky-10	T-117	VA-510	BSRB-II	By-21
Banket-A1	1.00										
Sota-6506	0.62	1.00									
H.velvet	0.78	0.35	1.00								
By.res	0.80	0.46	0.65	1.00							
By-64	0.83	0.44	0.64	0.50	1.00						
By Sota-51	0.64	0.55	0.55	0.59	0.60	1.00					
Ky-10	0.73	0.50	0.65	0.69	0.79	0.74	1.00				
T-117	0.53	0.42	0.59	0.46	0.78	0.46	0.68	1.00			
VA-510	0.43	0.50	0.44	0.46	0.48	0.83	0.61	0.44	1.00		
BSRB-II	0.57	0.46	0.54	0.68	0.55	0.81	0.72	0.47	0.86	1.00	
By-21	0.53	0.54	0.40	0.52	0.59	0.52	0.55	0.50	0.55	0.62	1.00

Based on the genetic similarity values (Table 3), it is observed that the maximum genetic similarity value is found to be 0.86 between VA-510 and BSRB-II showing the maximum genetic relatedness and the minimum genetic similarity value is found to be 0.352 between Sota-6506 and Harrow velvet depicting that they distantly related with greater genetic diversity, followed by 0.417 between T-117 and Sota-6506.

Dendrogram of tobacco lines

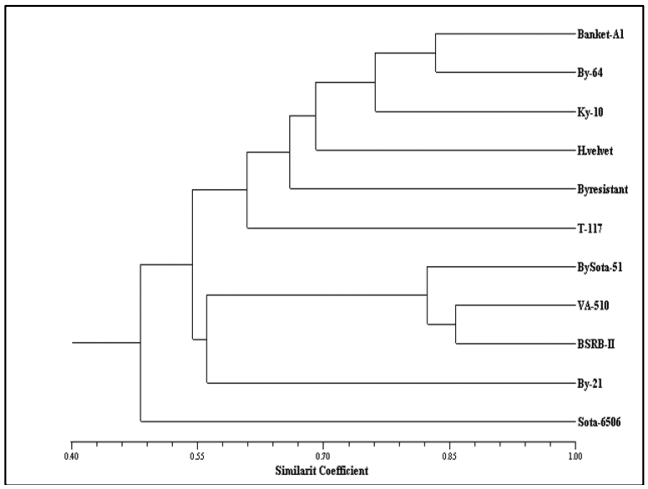

Fig 2: Dendrogram of the tobacco genotypes based on the diversity

The dendrogram constructed (Fig. 2) based on the genetic similarity values showed three major clusters. Cluster one included Banket-A1, By-64, Ky-10, Harrow Velvet, By Resistant and T-117, cluster two included By Sota-51, VA-510, BSRB-II and Burley-21, and cluster three Sota-6506.

In tobacco, the RAPD technique has been mainly used to identify markers linked to genes for resistance to pathogens (Bai *et al.* 1995; Yi *et al.* 1998) [16, 17]. Previously, the genetic diversity in tobacco cultivars have been assessed (Xu *et al.* 1998[6]; Delpiano *et al* 2000 [12]; Zhang *et al.* 2005 [18] using RAPD and found a low degree of polymorphism. Sivaraju *et al.*, (2008) [19] studied the genetic diversity among 46 varieties belonging to 10 different manufacturing tobacco types cultivated under different agro-climatic conditions in India along with two wild species of *Nicotiana* using 40 arbitrary RAPD primers. Sarala and Rao (2008) [20] observed that the RAPD based genetic similarity among eight FCV and two burley varieties ranged between 0.798 and 0.571 with an average of 0.668, indicating close relationship among the cultivars studied. Vicario *et al.*, (1995) [21] used the RAPD technique in their analysis of the genetic relationship among a unique population of *Abies nebrodensis* and *Abies alba populations* along a north-south geographic gradient. This technique was also used by Mulcahy *et al.*, (1993) [22] for distinguishing eight distinct apple cultivars.

In many instances, only a small number of primers are necessary to identify polymorphism within species (Williams *et al.*, 1990) [5]. Indeed, as Mulcahy *et al.*, (1995) [22] had reported, a single primer might often be sufficient to distinguish all of the sampled varieties, which are taxonomically distinct. Williams *et al*. (1990) [5] states that the ease of the RAPD technique could lead to the automation of genetic mapping and to the extension of genetic analysis to cover organisms which lack an ample number of phenotypic markers to completely describe their genome.

Development of mapping population

Banket-A1, being a cultivated tobacco with highest TSNA yield was selected as one of the parent for mapping population. VA-510, having low TSNA and lowest genetic similarity with Banket-A1 was selected as another parent for the development of mapping population. By-64 having desirable morphology for cultivation with reasonably low TSNA level and highest genetic similarity was also selected as another parent for the mapping population development. Crosses were made between By-64 x Banket-A1 and VA-510 x Banket- A1. F_1 and F_2 were raised. Around 250 Individual F_2 plants were selfed and individual plants were advanced to subsequent generations using single seed descent method to develop stable recombinant inbred lines (RILs). RILs are homozygous immortal mapping populations, they can be replicated over various locations and are useful for mapping QTLs and identify tightly linked markers,

Conclusion

Molecular mapping of TSNA trait helps us to tailor the tobacco plants to yield lower levels of TSNAs with desirable traits using marker assisted breeding strategies. In order to develop suitable mapping populations from controlled crosses, parents were selected based on the TSNA yielding abilities and genetic diversities. Using the selected parents, two mapping populations were developed for mapping TSNA trait. These populations developed through diverse parents is of immense use in mapping of genes governing TSNA which in turn helps to breed low TSNA lines. Lowering of TSNA is important for reducing health risks associated with tobacco consumers.

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