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In vitro clonal propagation of nicotiana tabacum with modified medium and studying its gene alteration through bioinformatic analysis

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Abstract

Tobacco has medicinal uses and was used as an insecticide. Also, tobacco holds social and custom noteworthiness in different social orders so hereditary modifications and change ponders can be effectively analyzed within the test plant due to its simple genome courses of action. In this study, we treated tobacco seeds with EMS chemical and analyzed the changes in the DNA mutation compared with the control. Total nine gene sequences were taken such as AP2, CAD, CHI, PIP1, LTP1, QPT, RBOH, UPL5, PR1 to study the DNA alternations, through sequencing and comparison with mutated genes. Gene mutation was determined using the bioinformatics tool namely Bioedit. From the result obtained in bioedit were higher mutation rate was observed in PR1 followed by AP2 and CHI gene. All gene sequences were mutated except RBOH and UPL5. This preliminary study showed the effects of EMS on treating tobacco plants. This genetic alternation study can be used in constructing highly efficient tobacco lines against future TILLING studies.

Keywords: Tobacco; Gene mutation; Bioedit; EMS; TILLING

1 Introduction

In this study, the effects of EMS on tobacco seeds and its mutational induction potential might be studied to find the effects of mutated protein in tobacco lines. Soufi et al. 2023, explained EMS EMS-treated tobacco yields higher biochemical content in the treated tobacco seeds with EMS. Gichner, 2003, revealed EMS treatment produces DNA damage in the treated seeds after exposure to the chemical and planting. They explained the treatment with EMS at a low concentration (0.1) % led to an increase in the germination rate and speed of tobacco varieties. Thus, it can be suggested to soak seeds at a concentration 0.1% for its role in improving the germination of tobacco varieties (Soufi et al.2022). Hisashi Udagawa et al. 2021, described the development of whole-exome sequencing (WES) procedure in tobacco and its application to characterize a test population of EMS-induced mutations. Xiaoming Gao et al. 2019, they described that the ethyl methane sulfonate (EMS) mutagenesis induced premature leaf senescence mutant yellow leaf 1 (yl1) in common tobacco (Nicotiana tabacum L.) and their study finding the gene controlling the mutation phenotype and revealing the molecular regulation mechanism of tobacco leaf senescence in the next stage. In our study, we like to induce the mutations in tobacco seeds with EMS and analyze the mutations in the genes AP2, CAD, CHI, LTP1, PIPT, PR1, QPT, RBOH, UPL5. These genes were reported that have the functions of modulating the plant architecture, involved in lignin biosynthesis, affecting stem strength and resistance to pathogens, influencing the production of flavonoids, which contribute to plant defense and human health benefits, implicated in plant defense against pathogens and environmental stresses, impacts tobacco plant's response to drought and salinity stresses, marker for plant defense responses against pathogens, influences tobacco's production of secondary metabolites and defense compounds, plays a role in tobacco's response to biotic and abiotic stresses modulates various cellular processes including stress

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responses and hormone signaling in tobacco plants. Altering this gene pattern for the ORF regions or inducing mutations may induce the overexpression of this function.

2 Materials and methods

2.1 Collection of tobacco seeds

Nicotiana tobacco seeds were collected from Indian Tobacco Research Centre, Vedasandur, Tamil Nadu, India. The collected tobacco seeds were used for planting in pots also in direct tissue culture media after the treatment with EMS solution.

2.2 Treatment with EMS

The seeds of tobacco were immersed in either a 0.5% or 1.0%(w/v) EMS solution for 16h, then rigorously washed with water before being grown in a greenhouse. The treated seeds were planted in pots also they were inoculated in tissue culture media simultaneously. Three trials were conducted with seeds each trial contains ten seeds of tobacco. After 15 days of growth in pots and tissue culture bottles, the plantlets were collected and taken for DNA isolation.

2.3 Selection of genes and primers

AP2, CAD, CHI, LTP1, PIP1, PR1, QPT, RBOH, UPL5 were chosen based on their contributions to tobacco growth and defense systems. The wild genome sequences were downloaded from the NCBI database and the Primers were designed based on conserved regions for all isolates. The primer sequence was tabulated.

2.4 PCR amplification of Genes

The PCR setup was assigned based on the primer annealing temperature, and template concentrations. The whole PCR setup was run in 10µl reaction i.e., 10mM primers, 1X PCR buffer, 25mM NaCl, Taq Polymerase, and dNTP mix. The run sequences were directly sent for sequencing.

2.5 Comparison of DNA alterations in treated and non-treated

Bioedit software was used to identify the DNA alterations that occurred with the EMS treated tobacco. The sequenced DNA were retrieved from the Eurofin sequencing service Bangalore, India. Both the EMS-treated and non-treated plantlets DNAs of all nine genes were compared and identified the changes in DNA and also protein sequence. The altered DNA sequences were further analyzed for mutational effects.

2.6 Structure prediction of wild and mutated proteins RASMOL

RasWin, a Windows-based adaptation of the RasMol atomic visualization computer program, is commonly utilized in protein structure prediction. This software was used to draw the protein structure and used to compare alternations that occurred between wild and mutated.

3 Results

3.1 Collection of tobacco seeds

The seeds were planted in both tissue culture and soil pots for the non-treated control. The seeds showed growth on both the media and pots (Figure 1).

Figure 1 Tobacco seeds and planted sterile pot

3.2 EMS (Ethyl Methanesulfonate) treatment

The seeds were EMS treated as per the protocol and the seeds were placed on the tissue culture media (organ culture media) and also in a sterile soil pot (Figure 2). After 15 days of treatment the plantlets were taken for DNA isolation to analyses the mutation in DNA sequences.

Figure 2 A) Tobacco seeds were treated with EMS B) Mutated tobacco lines grown on the tissue culture media C) Mutated lines grown on the soil pots

Table 1 Primer sequences for all nine genes

Gene Names	Forward Primer	Reverse primer	Size of the Amplicon
AP2	CTATCTCCGACTTC	GAACACTGTAAAAA	1012
CAD	GATCTTGGCATGTCCAACTA	TGGAATGGTTCACTATTATA	1045
CHI	GTGAAAATTCCAGAGGGTAT	ATGAATTTGGATGTTGGTGA	1032
LTP1	CTAGCTAGATACTTCATTCC	GTACTTATGATATGTGTCAA	1024
PIP1	TTGCTTGGGCTTTTGGTGGT	GATGCCAAGAGAAATGCTA	1054
PR1	CCGTGAAATCTTCAAGATTT	CAAGAACTTAAATAATTGC	1078
QPT	AAAGCATTAAGCTCCT	GGAGTAAAAGCAGCT	1098
RBOH	CAAAATTCGGAAAATCATCA	TATGAGGTGGTTTTGTTGG	1085
UPL5	ACTCTCTTCAGTCTTCT	ATGACACCAGTGAAT	1043

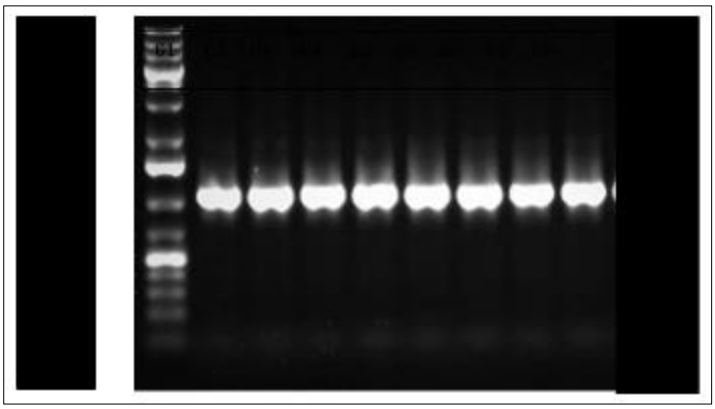

Figure 3 Gene amplification in PCR with designed primers

The genes were amplified in PCR with selected primers all the amplicons sizes were designed at approximately 1kb and the products were amplifying positively in the PCR. The PCR products were sent for sequencing.

3.3 Mutated genes

Table 2 Mutated genes

S. No	Gene Name	Total Number of base pairs	Number of Mutations	Base pair Mutated location	Mutated nucleotide	Name of the Amino acid before mutated	Name of the Amino acid after mutated
1.	AP2	887	6	74,75	AA – TT	_	K (Lysine)
2.	CAD	1397	4	241, 292, 421, 1248	A – T, A – C, A – C, C – A	T(Threonine), M(Methionine), N(Asparagine), S(Serine)	S(Serine), L(Leucine), H(Histidine), S(Serine)
3.	CHI	1008	6	87, 238, 326, 512, 555, 556	A – C, G – A, T – C, G – A, A – C, A – C	Q(Glutamine), *, I(Isoleucine), *, T(Threonine), I(Isoleucine)	H(Histidine), *, T(Threonine), *, T(Threonine), L(Leucine)
4.	LTP1	3112	5	126, 127, 336, 1124, 1318	A – T, G – T, A – C, G – A, T - A	Q((Glutamine), L(Leucine), Q((Glutamine), R(Arginine), F(Phenylalanine)	H(Histidine), L(Leucine), H(Histidine), Q((Glutamine), I(Isoleucine)
5.	PIP1	1093	2	300, 301	A – T, A - T	L(Leucine), T(Threonine)	L(Leucine), S(Serine)
6.	PR1	2038	8	411, 600, 601, 604, 605, 760, 761, 1747	A – T, A – C, T - C, A – T, C – T, T – C, A – C, G - A	L(Leucine), T(Threonine), L(Leucine), T(Threonine), T(Threonine), *, *, E (Glutamic acid),	F(Phenylalanine), T(Threonine), L(Leucine), F(Phenylalanine), F(Phenylalanine), P(Proline), P(Proline), K(Lysine)
7.	QPT	1396	4	137, 138, 251, 325	A – T, A – T, G – A, A - T	E (Glutamic acid), E (Glutamic acid), C(Cysteine), K(Lysine)	V(Valine), V(Valine), Y(Tyrosine), *.

8.	RBOH	3338	-	-	-	-	-
9.	UPL5	3266	-	-	-	-	-

In AP2 gene the Lysine gene is altered, in CAD gene the Serine, Leucine, Histidine, Serine was changed, in CHI gene the Histidine, Threonine, Threonine, Leucine, in LTP1 gene the Histidine, Leucine, Histidine, Glutamine, Isoleucine, PIP1 gene the Leucine, Serine, PR1 gene the Phenylalanine, Threonine, Leucine, Phenylalanine, Phenylalanine, Proline, Proline, Lysine, QPT gene the Valine, Tyrosine, in RBOH and UPL5 genes there were no mutations present in the ORF regions. Totally in gene 1 has 6 AA, in gene 2 has 4 AA, in gene 3 has 6AA, in gene 4 has 5AA, in gene 5 has 2AA, in gene 6 has 8AA, in gene 7 has 4AA.

3.4 Analysis of gene mutation of PR1 gene using bioedit

Figure 4 Analysis of gene mutation of PR1 gene using bioedit

3.5 Comparison of mutated protein and non-mutated protein

In RasWin the protein sequences were compared with the aminoacid changes with 3D model. The open reading frame.

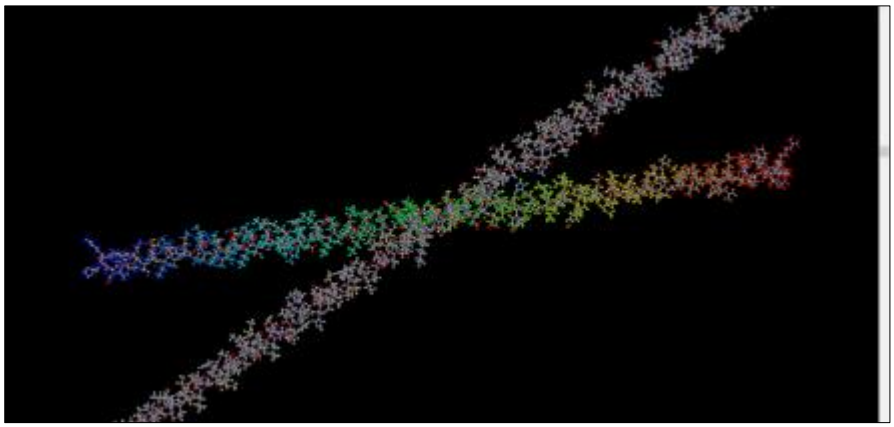

Figure 5 Comparison of mutated protein with non-mutated in RasWin

The sequences of aminoacids with mutated proteins of all genes were analyzed in RasWin in that the side chain changes were observed in the structure prediction.

4 Discussion

EMS (Ethyl Methanesulfonate) treatment is commonly utilized to initiate hereditary changes in living beings, including plants like tobacco, by alkylating DNA bases (Shamshad et al. 2023). In tobacco seeds, EMS treatment regularly comes about in a range of changes, extending from point transformations to huge cancellations, inclusions, or improvements within the genome (Tian et al. 2021). These changes can lead to a variety of phenotypic changes within the coming plants, counting changed development designs, changes in leaf morphology, altered blossom color or structure, and contrasts in regenerative characteristics (Yang et al.2021). Be that as it may, EMS treatment can moreover actuate undesirable impacts such as decreased practicality or sterility in a few seeds, restricting the utility of the mutagenesis approach (Chen et al. 2023). By and large, the impacts of EMS treatment on tobacco seeds can change depending on components such as treatment dose, length, and hereditary foundation, highlighting the significance of cautious optimization and characterization of EMS mutagenesis conventions for particular breeding or investigative purposes (Sonavane, 2016).

The tobacco seeds planted in the earthen pots showed good growth when compared with the Tissue culture media. EMS is a type of non-transgenic chemical mutagen, and EMS mutagenesis is an important way to obtain mutations and discovery of new genes for plants. Special protocols have been established for many plant species (Chen et al. 2023).In our study the tobacco seeds were treated with EMS DNA was isolated from the mutated ones and the DNA was sequenced (Udagawa et al. 2021). The sequenced DNA of mutated genes such as AP2, CAD, CHI, LTP1, PIP1, PR1, QPT, RBOH, UPL5 were shown basepair changes and Aminoacid changes in the sequencing. Alignment with non-treated revealed many SNPs and codon changes in the open reading frame. These changes could be either over-expressive or truncated in protein sequence. It could arrest either the protein function or it may induce the function of the protein. This preliminary study about genetic alterations in tobacco by EMS may provide information regarding gene alteration during chemical mutation and this mutation can be used in constructing highly efficient tobacco lines for disease and quality trait management.

5 Conclusion

In conclusion, our study demonstrated the efficacy of using EMS treatment on tobacco seeds to induce DNA mutations, paving the way for future genetic modification research. The observed higher mutation rates in genes such as PR1, AP2, and CHI highlight the potential for targeted genetic alterations in tobacco plants. Interestingly, RBOH and UPL5 genes remained unaffected by EMS treatment, suggesting potential targets for further investigation into the mechanisms of mutation resistance. These findings contribute to our understanding of tobacco genetics and provide valuable insights for developing highly efficient tobacco lines through future TILLING studies. The medicinal, insecticidal, and socio-cultural significance of tobacco underscores the importance of such genetic research, offering potential applications in agriculture, medicine, and industry. Further exploration of the mutated gene sequences identified in this study could lead to the development of novel tobacco varieties with improved traits and reduced environmental impact.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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