

Genetic mapping of a novel, low-anatabine gene mutation in tobacco (*Nicotiana tabacum* L.)

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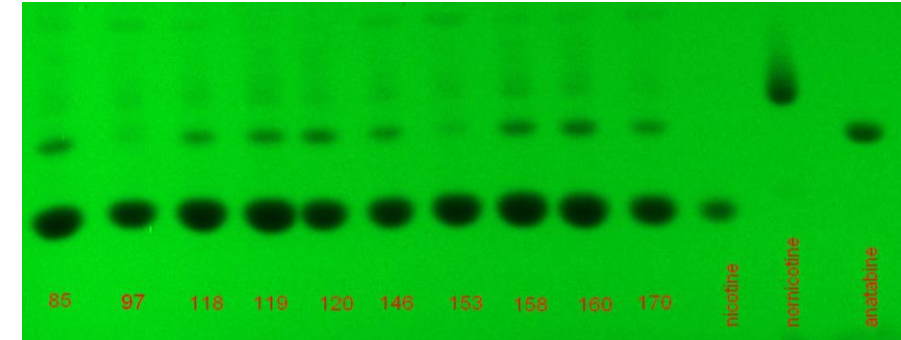
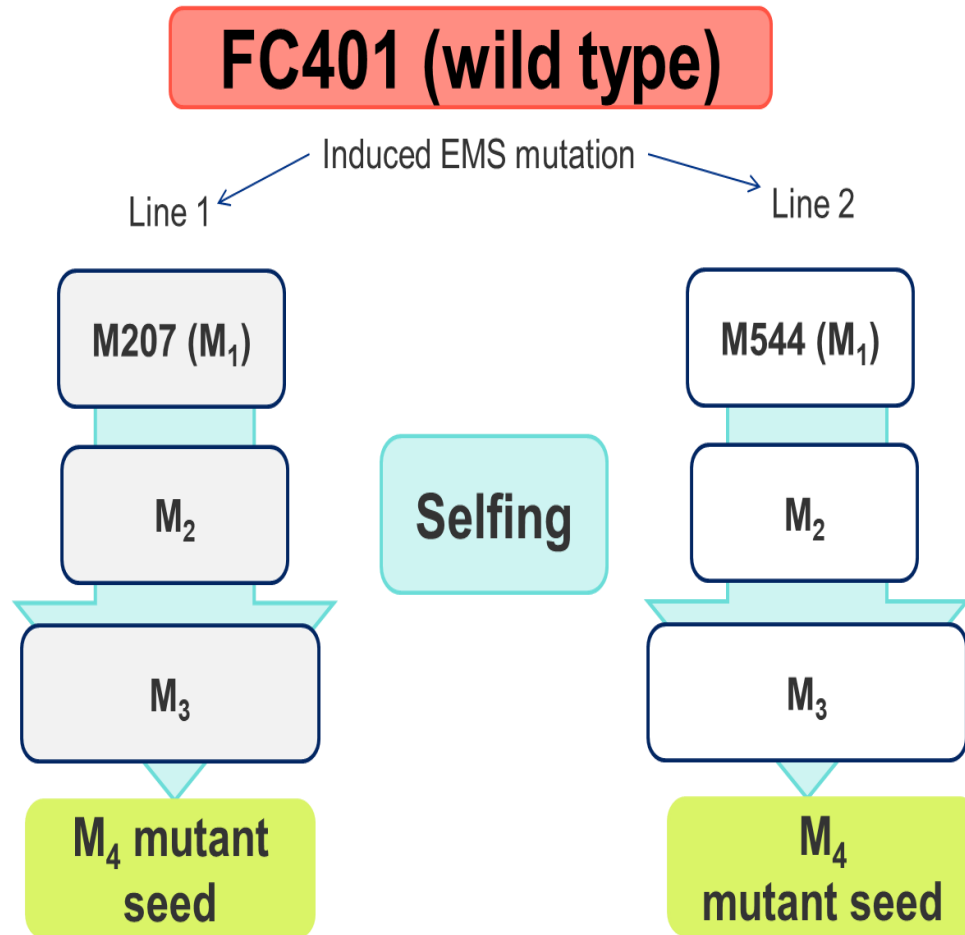
Introduction

- Anatabine, a minor alkaloid with ~3 % of the total alkaloids and precursor for 35 %-50 % of N'-nitrosoanatabine (NAT), is one of four tobacco-specific nitrosamines (TSNAs).
- Reduction or elimination of anatabine content in tobacco could significantly reduce total TSNAs.
- Regulation of anatabine synthesis is not completely understood.
- Identifying molecular markers associated with the anatabine trait will be useful in:
 - Developing tobacco varieties that are low in anatabine and NAT TSNA content in tobacco products.
 - Identifying the locus that regulates anatabine synthesis through map-based cloning.
 - Identifying candidate genes which will help in elucidating the late stages of the alkaloid biosynthetic pathway.

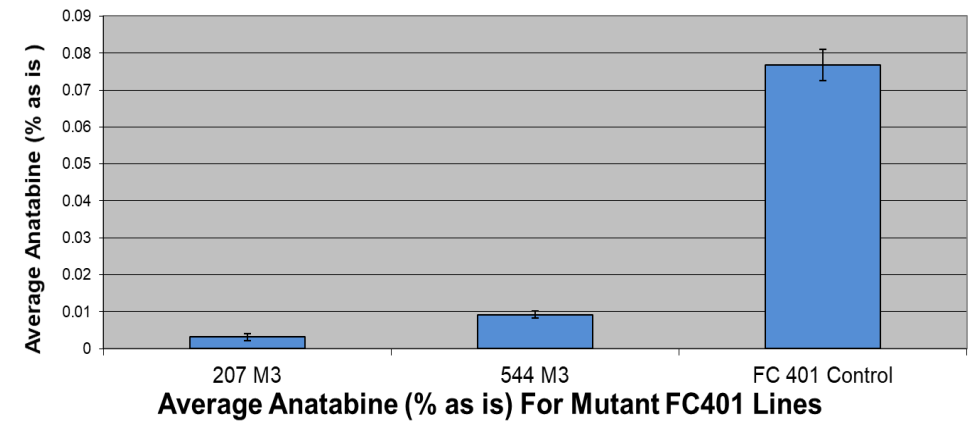
Objectives

- Develop and characterize novel mutant lines that exhibit reduction or elimination of anatabine while maintaining normal levels of other tobacco alkaloids.
- Utilize the high-density linkage map simple sequence repeat (SSR) resources of tobacco to identify genetic loci, molecular markers, and biosynthetic genes linked to the anatabine trait.

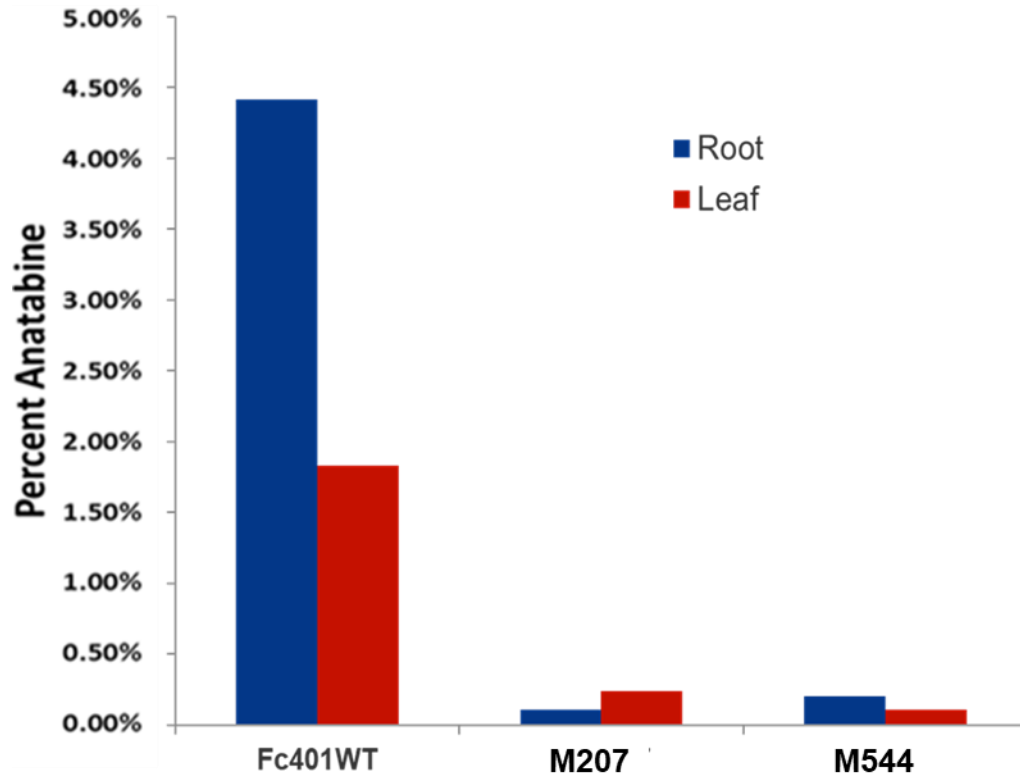
Low anatabine mutant trait development



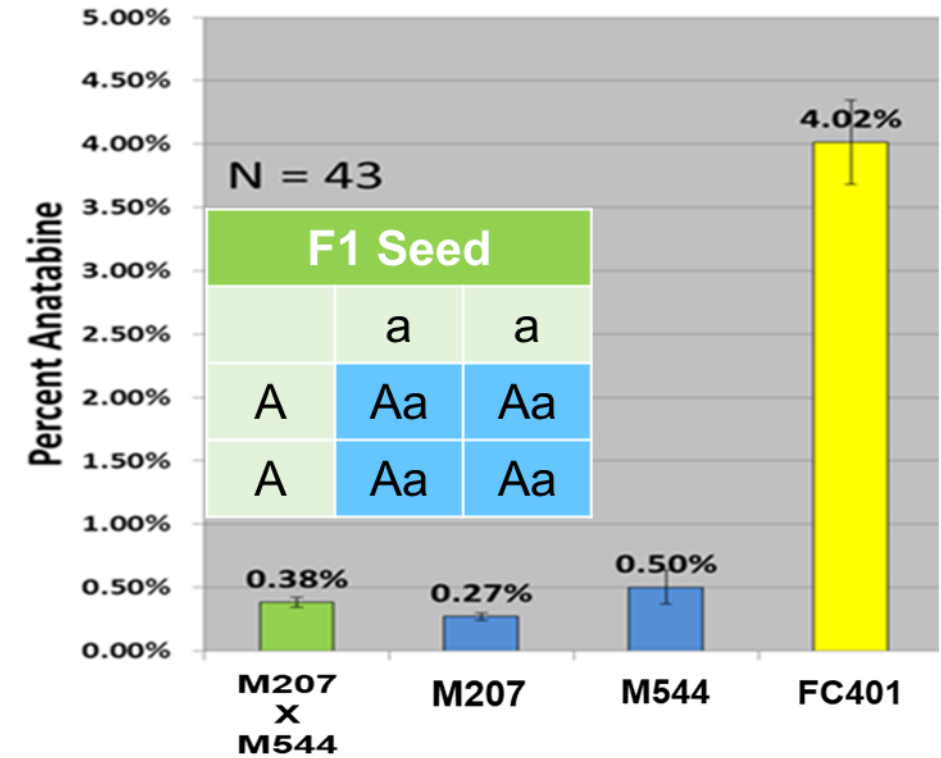
Screening of EMS mutants by High Performance Thin Layer Chromatography (HPTLC) method. Two mutant plants 97 (M207) and 153 (M544) have reduced anatabine content compared to other mutant lines.



Low anatabine mutant lines characterization

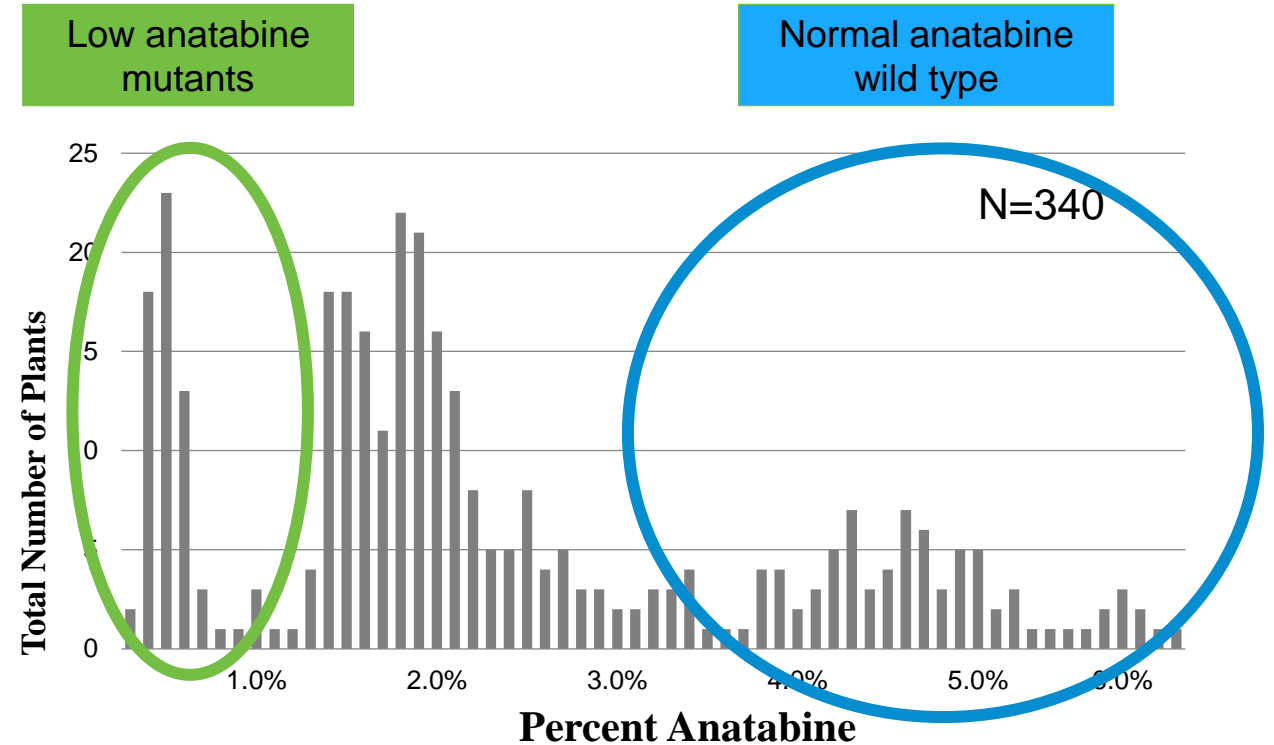
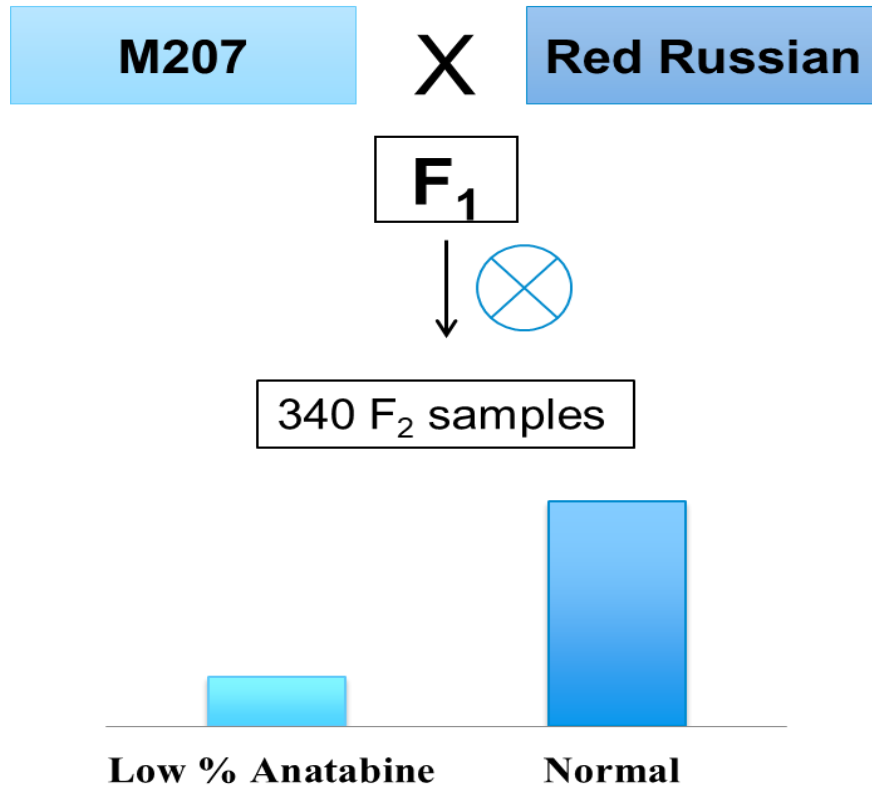


- Anatabine reduced in both roots and leaves of mutant lines.



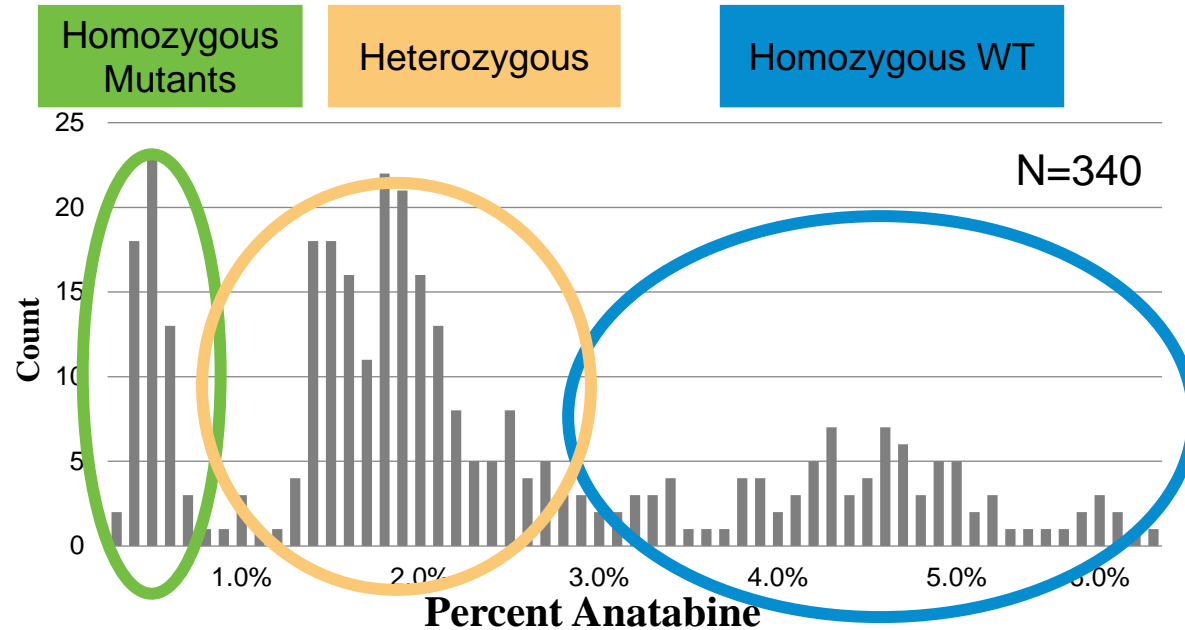
- No segregation when M207 and M544 lines are crossed.
- Crosses indicate a mutation in the same gene.

Low anatabine marker analysis

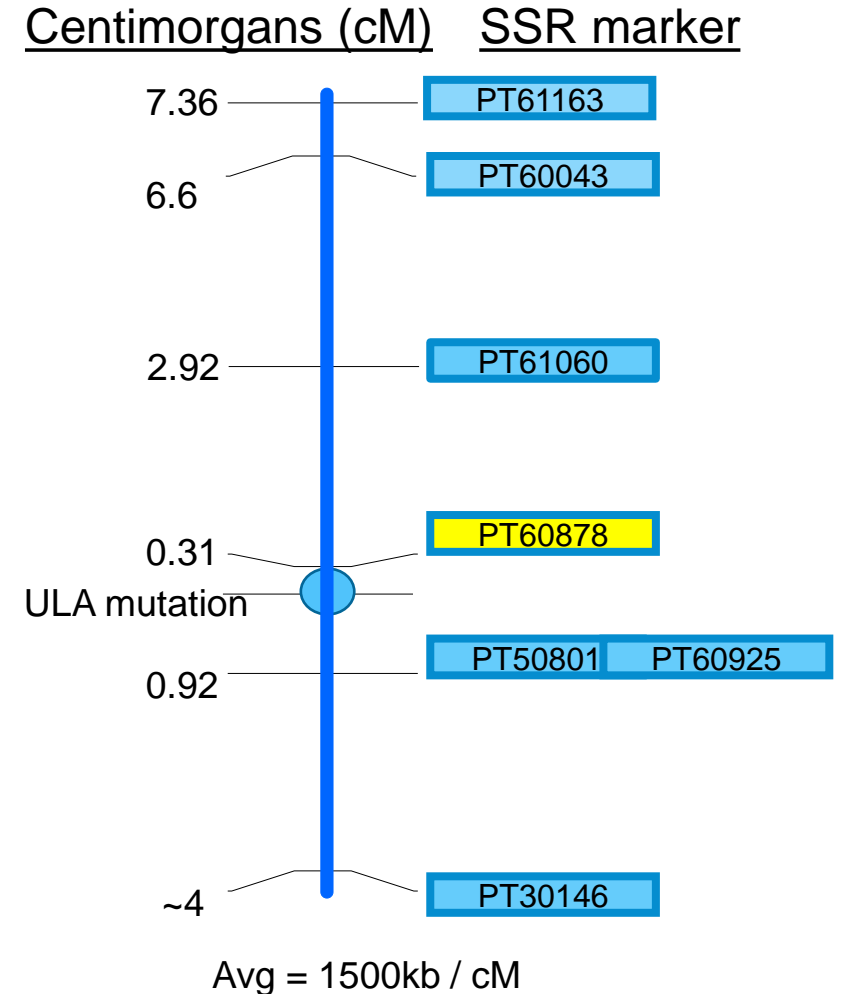


- Mapping population of 340 F₂ plants was generated and 65 M207 and 85 Red Russian plants were included in the alkaloid analysis and downstream genetic analysis.
- Two hundred and forty-six SSR markers were assessed in the parental lines.
- Two hundred and thirty-nine polymorphic markers between the parents were identified and used for screening for the ULA trait in the mapping population in two pools of 23 F₂ ULA and 24 F₂ normal anatabine plants.
- Six markers associated with the low anatabine trait were then genotyped in the mapping population of 340 plants.

Low anatabine marker analysis



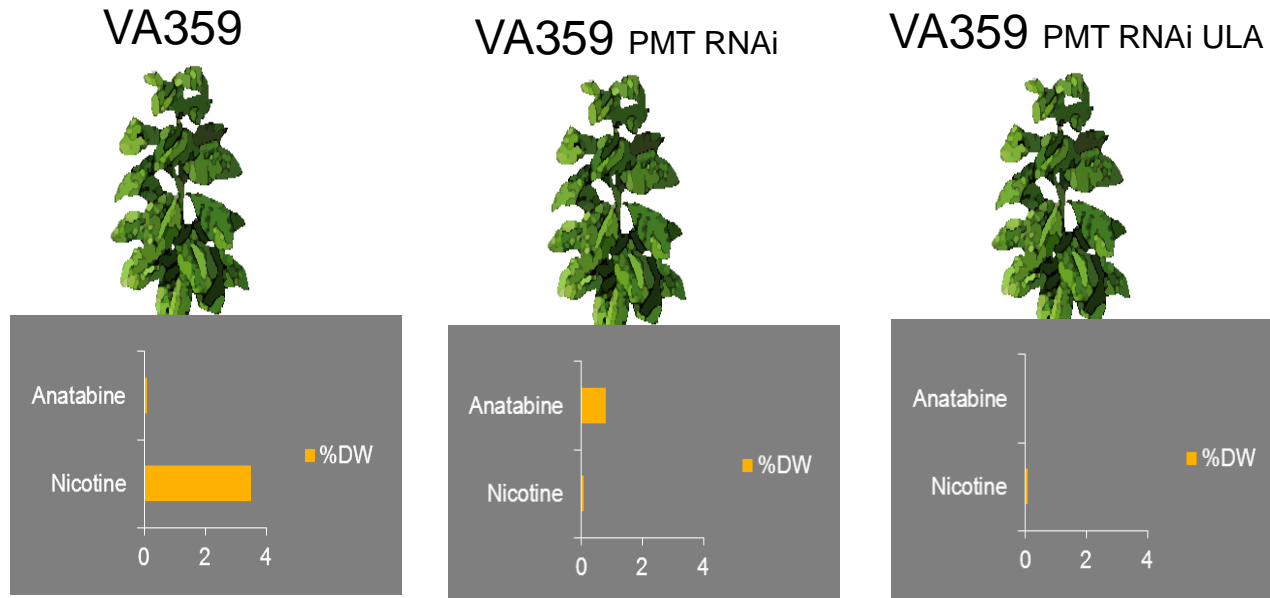
- Anatabine Trait Genetic Mapping Analysis.
- Markers: PT61163, PT60043, PT61060, PT60878, PT50801, PT60925 & PT30146.
- Map using 340 F2 individuals for % anatabine.



Low anatabine gene discovery

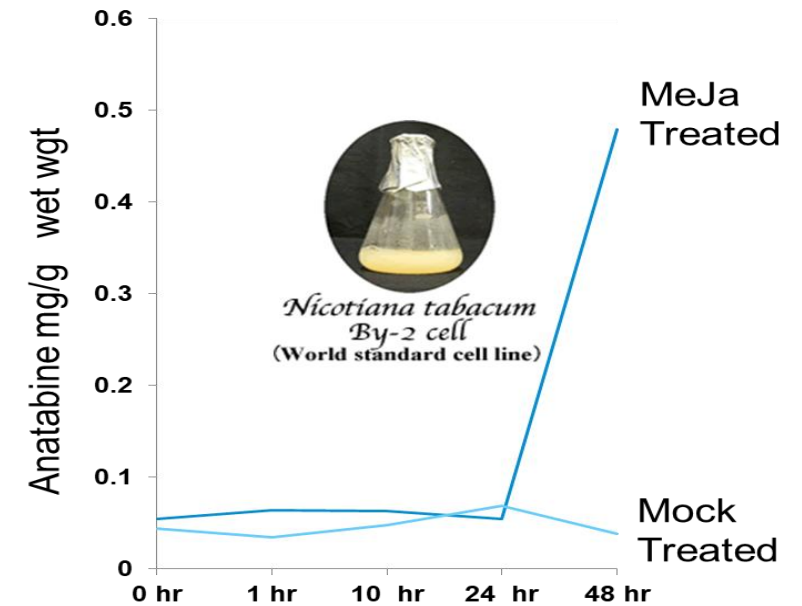
Genetic Materials

- VA 359 wild-type – 0.1 % anatabine
- VA 359 PMT RNAi – 0.8 % anatabine
- VA 359 PMT RNAi ULA – 0.01% anatabine

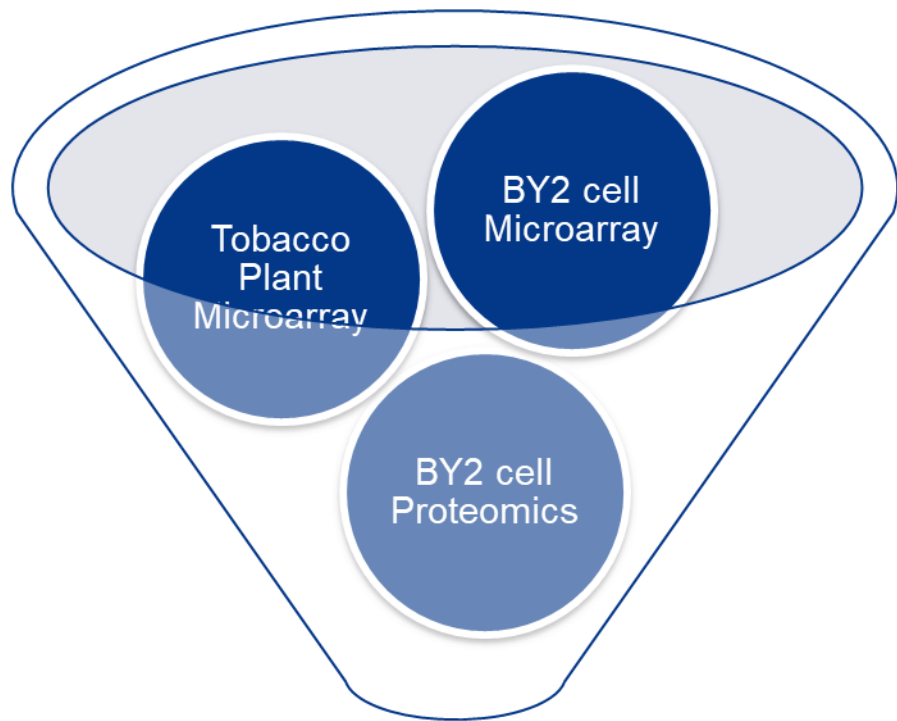


Genetic Materials

- Control BY2 cells grown in liquid medium
- BY2 cells treated with Jasmonic acid

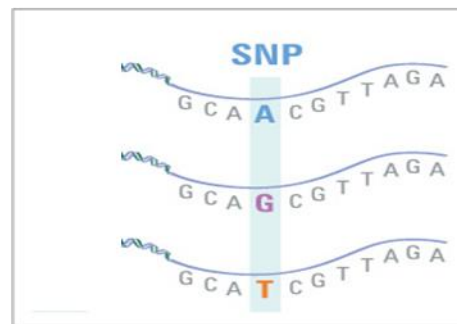


Low anatabine gene discovery



Gene Targets

Sequencing of Target Genes

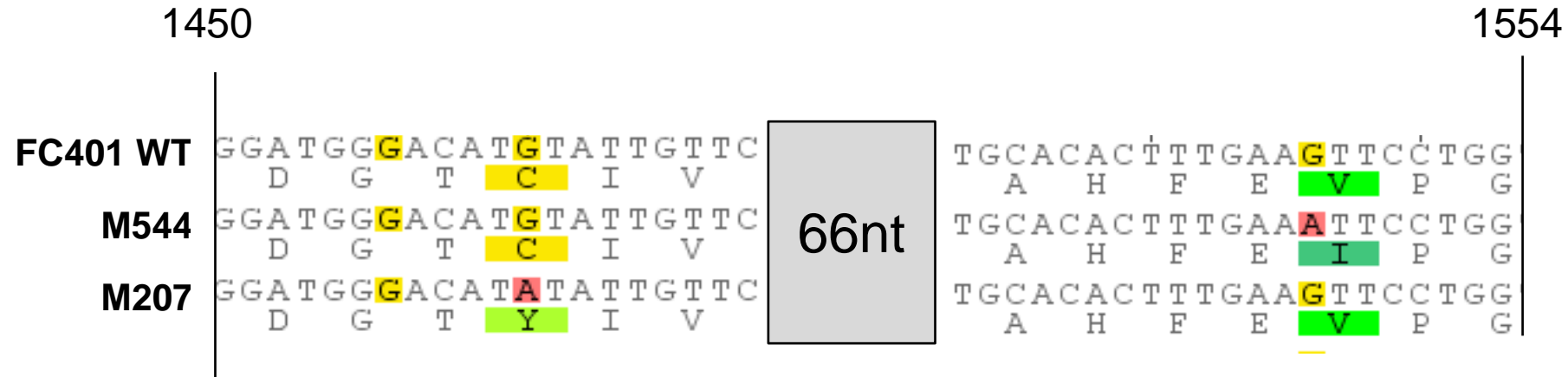


Gene Characterization

- WRKY TFs
- EIG proteins
- MATE transporters
- FAD Oxidoreductase
- 17 Root specific genes
- Universal Stress Protein
- Methylesterase
- Aquaporin
- Quinolate Synthase
- QPT Proteins
- PDR Transporters
- Berberine Bridge Like Enzymes (BBLs)
- Isoflavone reductase (A622)
- Allene Oxide Cyclase
- Allene Oxide Synthase
- Pathogenesis Related Protein 1

Candidate Genes

Quinolinate Synthetase gene sequencing in low anatabine trait



M207

G→A mutation
Cysteine → Tyrosine

M544

G→A mutation
Valine → Isoleucine

Quinolinate Synthetase characterization

Pfam Database

2787 total QS sequences
41 sequences from plants

- Conserved in all sequences
- Null mutation in bacterial system
- Highly conserved in plants
- Not conserved in bacteria

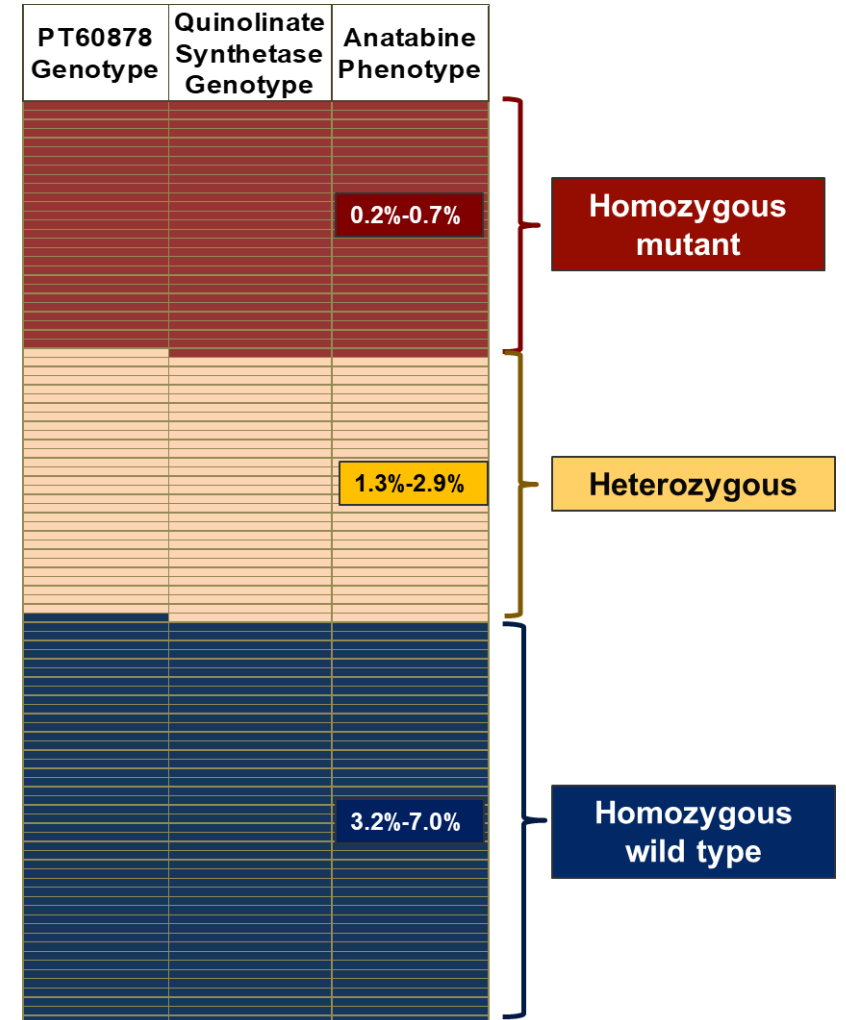
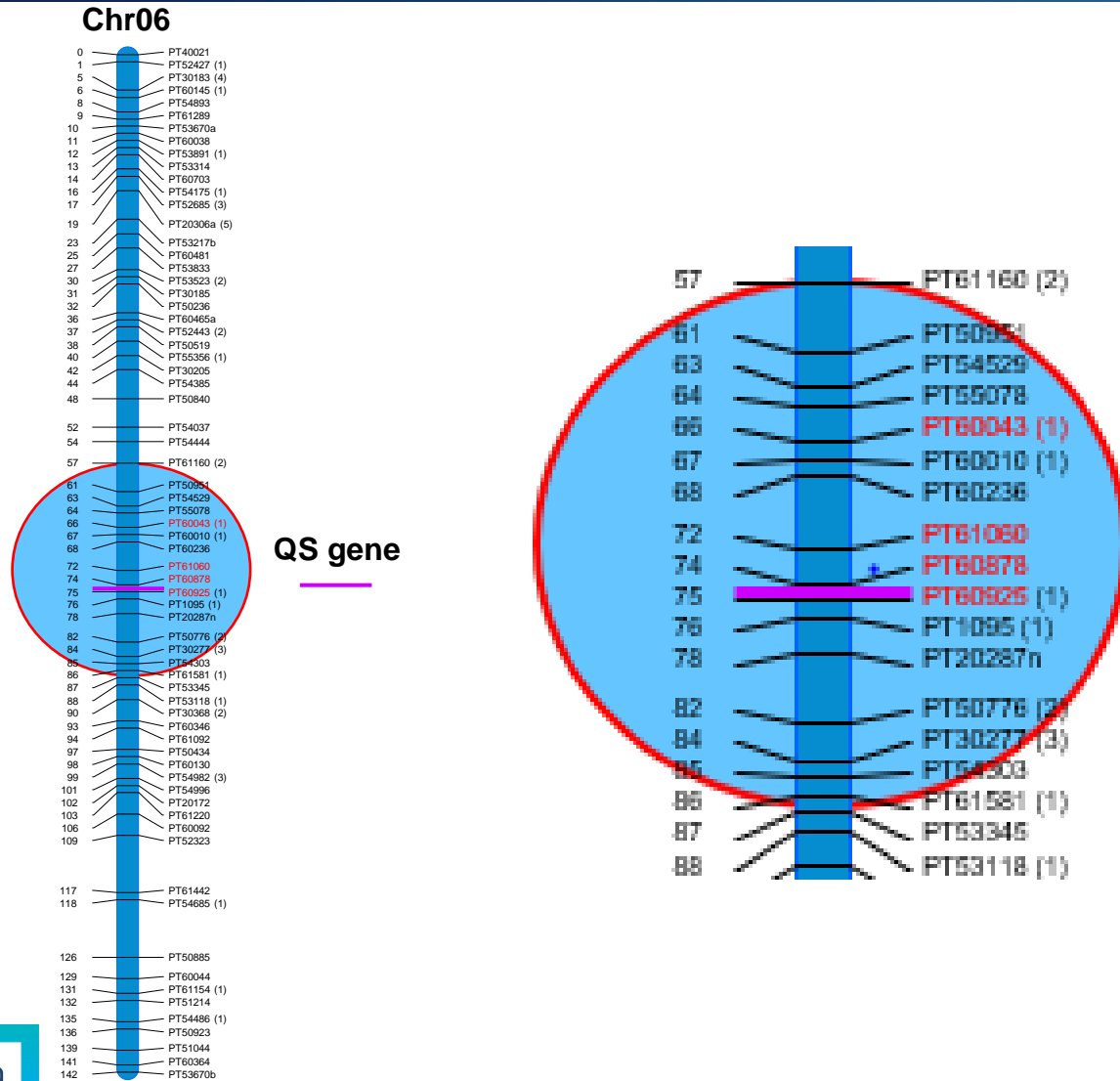
C512Y

V541I

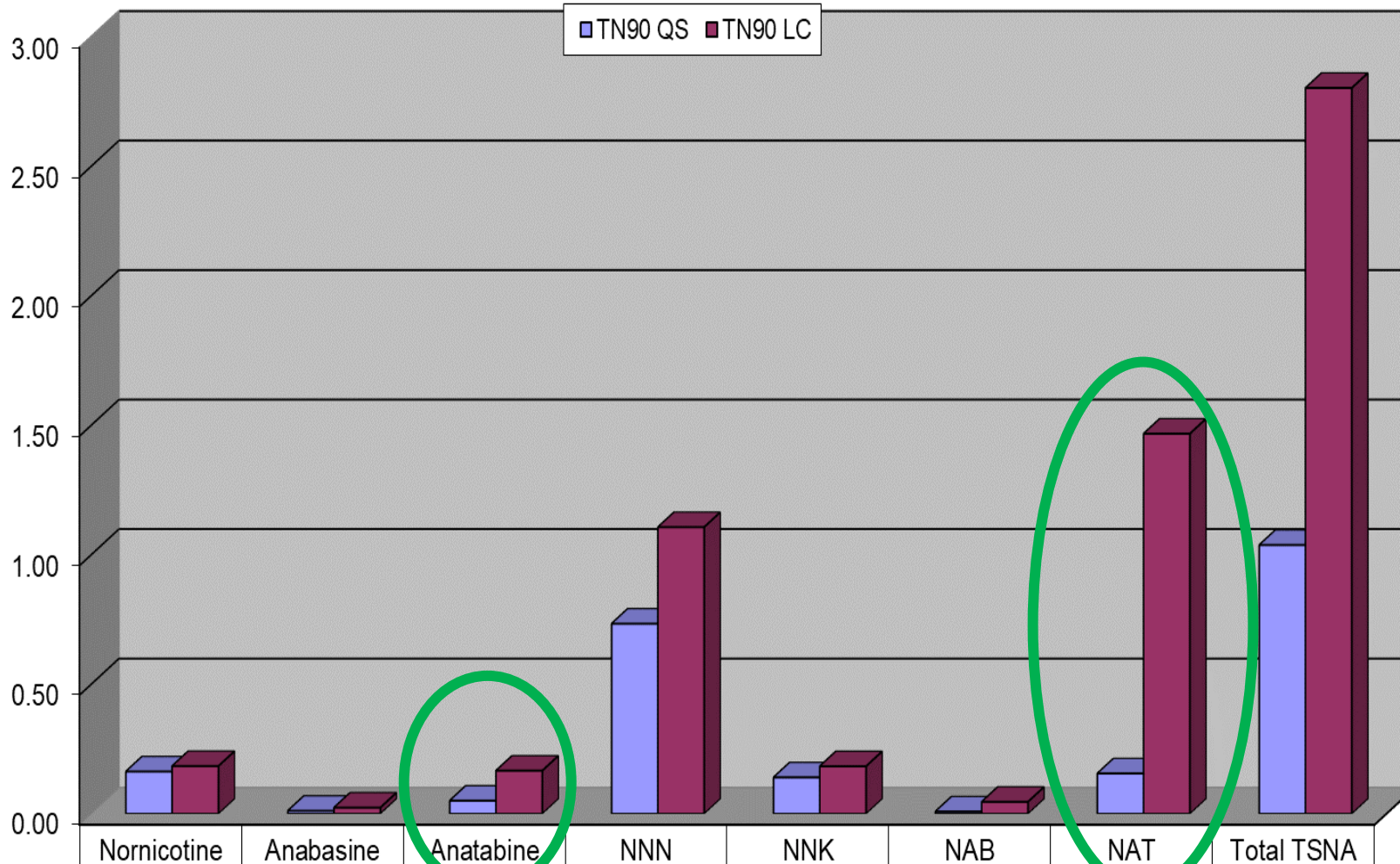
1. Fc401 WT
2. M207
3. M544
4. D5LAT9/169-49
5. D5LAX9/215-54
6. D7FHH3/111-39
7. D7MPK3/260-66
8. D7SHG8/133-45
9. D8QQE7/239-65
10. D8R8I8/237-56
11. D8U8Q3/237-49

G	C	V	H	H	L	F	G	H	E	V	V	E	N	I	N	E	M	Y	G	D	A	F	L	T	A	H	F	E	V	G	E	M	F	S	L	A	M	
G	Y	V	H	H	L	F	G	H	E	V	V	E	N	I	N	E	M	Y	G	D	A	F	L	T	A	H	F	E	V	G	E	M	F	S	L	A	M	
G	C	V	H	H	L	F	G	H	E	V	V	E	N	I	N	E	M	Y	G	D	A	F	L	T	A	H	F	E	V	G	E	M	F	S	L	A	M	
G	C	V	H	H	L	F	G	G	E	V	T	E	L	V	A	A	G	Y	G	D	A	Y	L	A	A	H	F	E	V	G	E	M	F	R	L	A	M	
G	C	V	H	H	L	F	G	G	E	V	T	E	L	V	A	A	G	Y	G	D	A	Y	L	A	A	H	F	E	V	G	E	M	F	R	L	A	M	
G	I	C	V	H	H	M	F	G	D	D	V	V	E	R	V	R	S	N	H	A	D	A	F	H	T	A	H	L	E	V	G	E	M	F	R	L	A	M
G	C	V	H	H	L	F	G	H	E	V	V	E	R	I	K	Y	M	Y	C	D	A	F	L	T	A	H	L	E	V	G	E	M	F	S	L	A	M	
G	C	V	H	H	L	F	G	H	E	V	V	E	K	I	N	E	M	Y	C	D	A	F	L	T	A	H	L	E	V	G	E	M	F	S	L	A	M	
G	S	C	V	H	D	M	F	G	K	D	V	V	S	R	V	R	E	G	Y	G	D	A	Y	L	T	A	H	E	V	G	E	M	F	T	L	A	M	
G	S	C	V	H	D	M	F	G	K	D	V	V	S	R	V	R	E	G	Y	G	D	A	Y	L	T	A	H	E	V	G	E	M	F	T	L	A	M	
G	C	V	H	H	L	F	G	G	E	V	A	E	L	V	R	R	A	Y	G	D	A	Y	L	A	A	H	F	E	V	G	E	M	F	E	G	L	A	M

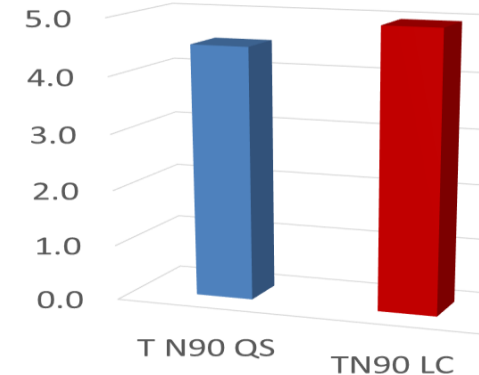
Mapping of SSR markers, QS gene, and their characterization



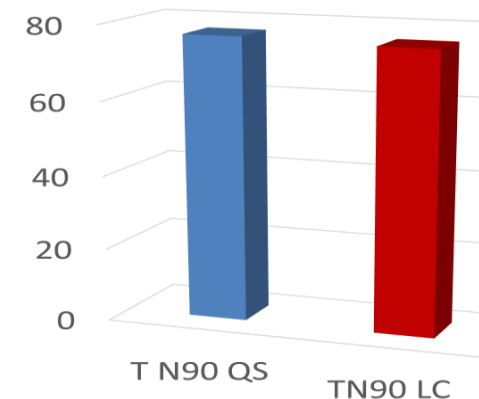
Leaf quality of low anatabine trait lines



TSNA analysis of TN90 anatabine mutant line



% Nicotine in TN90 anatabine mutant line



Grade Index of TN90 anatabine mutant line

Summary

- We developed and characterized a novel group of mutant lines that exhibit 80-95 % reduction of anatabine, while maintaining normal levels of other tobacco alkaloids.
- The low-anatabine trait was mapped to linkage group six, 0.3 cM away from PT60878 on the high-density tobacco map.
- Quinolinate synthetase was directly linked to anatabine biosynthesis and mutation in the gene resulted in 80-95 % reduction of anatabine and NAT.
- The leaf quality of mutant line is similar to the wild type.

Thank you