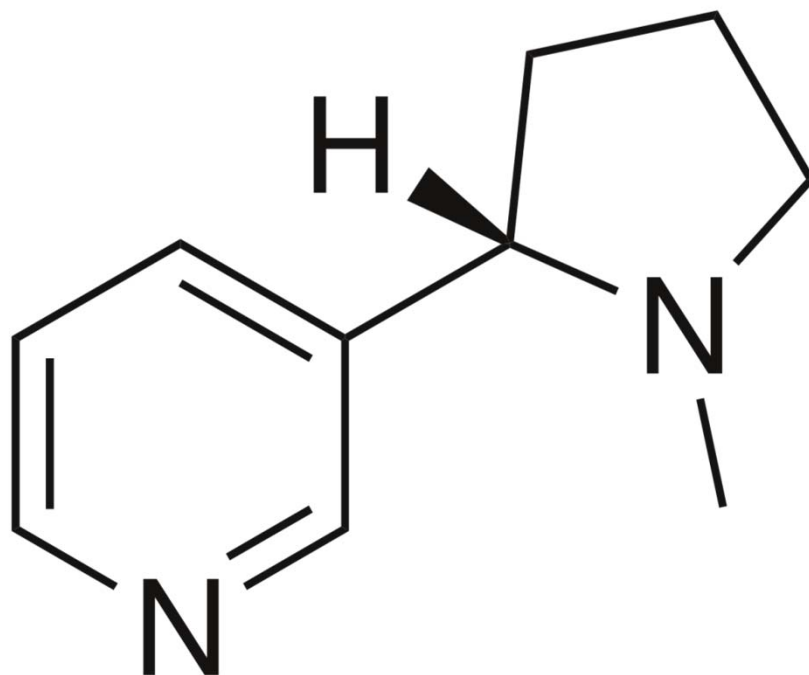
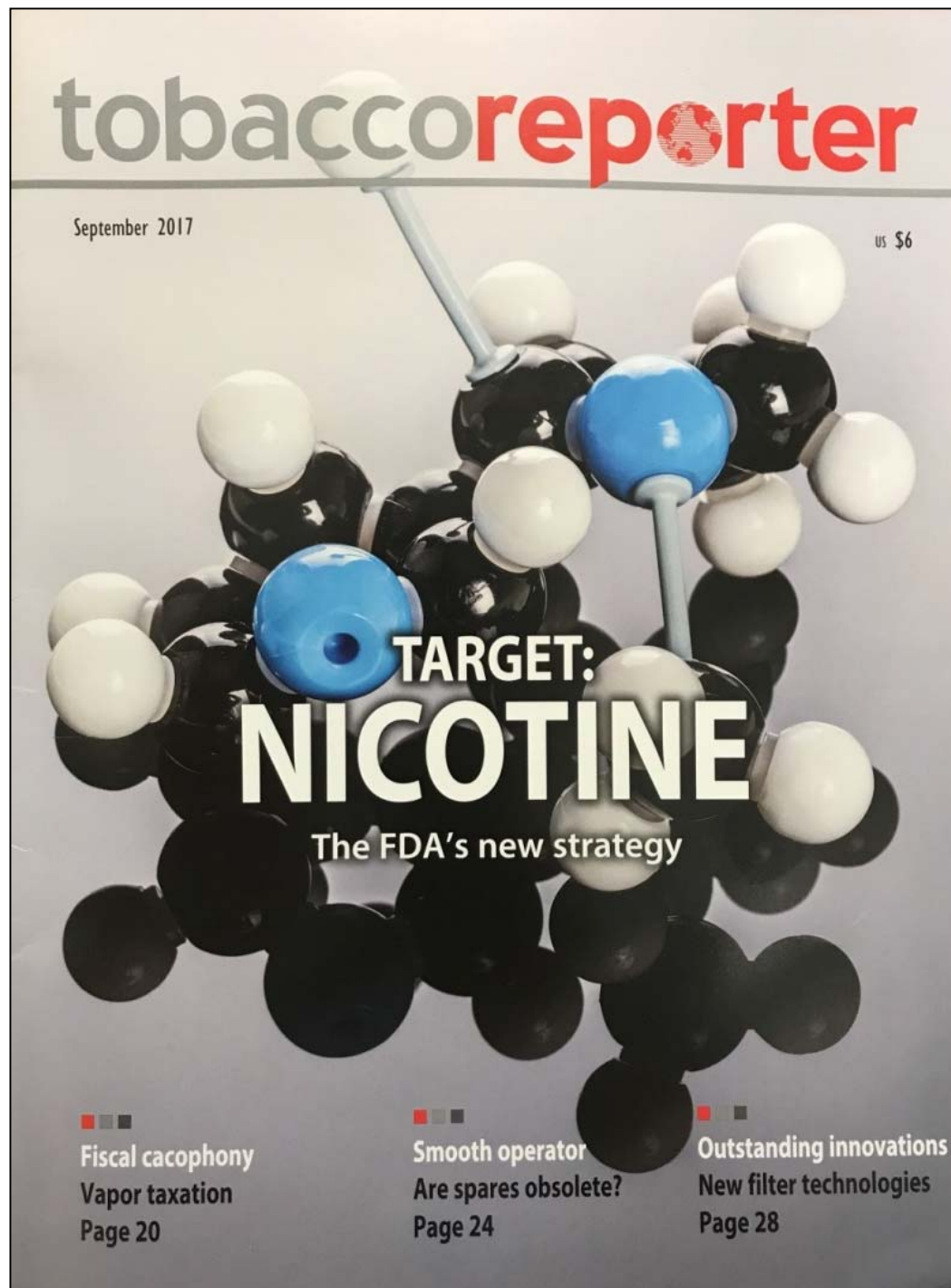


# Evaluation of Burley and Flue Cured Tobacco Lines Carrying Mutations in *BBL* Genes Affecting Nicotine Accumulation



R.S. Lewis, K.E. Drake-Stowe, R.E. Dewey



# Federal Regulation of Tobacco Products

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Public Law 111–31  
111th Congress

An Act

June 22, 2009  
[H.R. 1256]

To protect the public health by providing the Food and Drug Administration with certain authority to regulate tobacco products, to amend title 5, United States Code, to make certain modifications in the Thrift Savings Plan, the Civil Service Retirement System, and the Federal Employees' Retirement System, and for other purposes.

*Be it enacted by the Senate and House of Representatives of  
the United States of America in Congress assembled,*

Family Smoking  
Prevention and  
Tobacco Control  
Act.

## **DIVISION A—FAMILY SMOKING PRE- VENTION AND TOBACCO CONTROL ACT**

# Federal Regulation of Tobacco Products

## “SEC. 907. TOBACCO PRODUCT STANDARDS.

21 USC 387g.

“(4) CONTENT OF TOBACCO PRODUCT STANDARDS.—A tobacco product standard established under this section for a tobacco product—

“(A) shall include provisions that are appropriate for the protection of the public health, including provisions, where appropriate—

“(i) for nicotine yields of the product;

“(ii) for the reduction or elimination of other constituents, including smoke constituents, or harmful components of the product; or

“(3) LIMITATION ON POWER GRANTED TO THE FOOD AND DRUG ADMINISTRATION.—Because of the importance of a decision of the Secretary to issue a regulation—

“(A) banning all cigarettes, all smokeless tobacco products, all little cigars, all cigars other than little cigars, all pipe tobacco, or all roll-your-own tobacco products; or

“(B) requiring the reduction of nicotine yields of a tobacco product to zero,  
the Secretary is prohibited from taking such actions under this Act.

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# Proposed Nicotine Regulation

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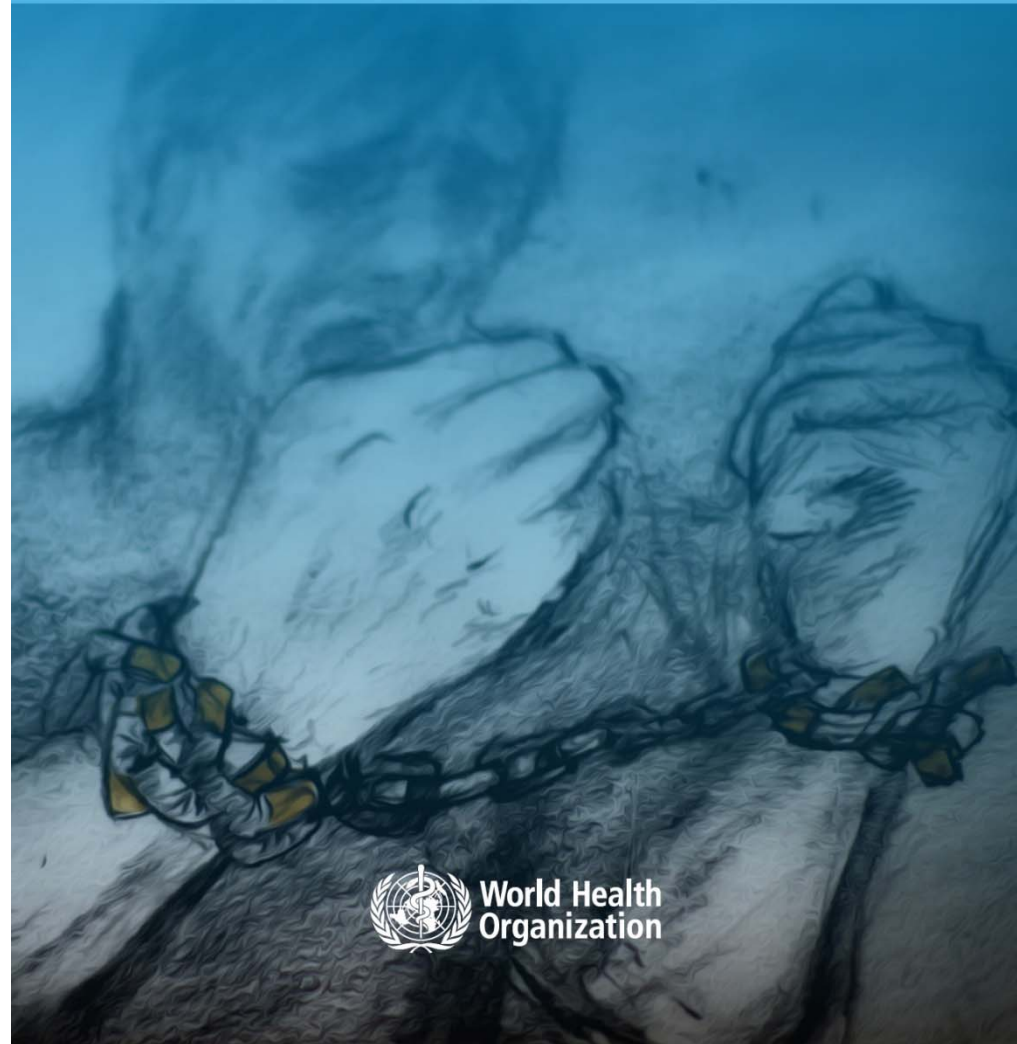
## GOALS

- (1) Reduce overall addiction to cigarettes
- (2) Reduce uptake of smoking by potential new smokers
- (3) Facilitate smoking cessation or transition to alternative nicotine-containing products

ADVISORY NOTE

# Global Nicotine Reduction Strategy

WHO Study Group on Tobacco Product Regulation (TobReg)





# WHO Recommendation

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1-2% Nicotine in Cigarette Filler

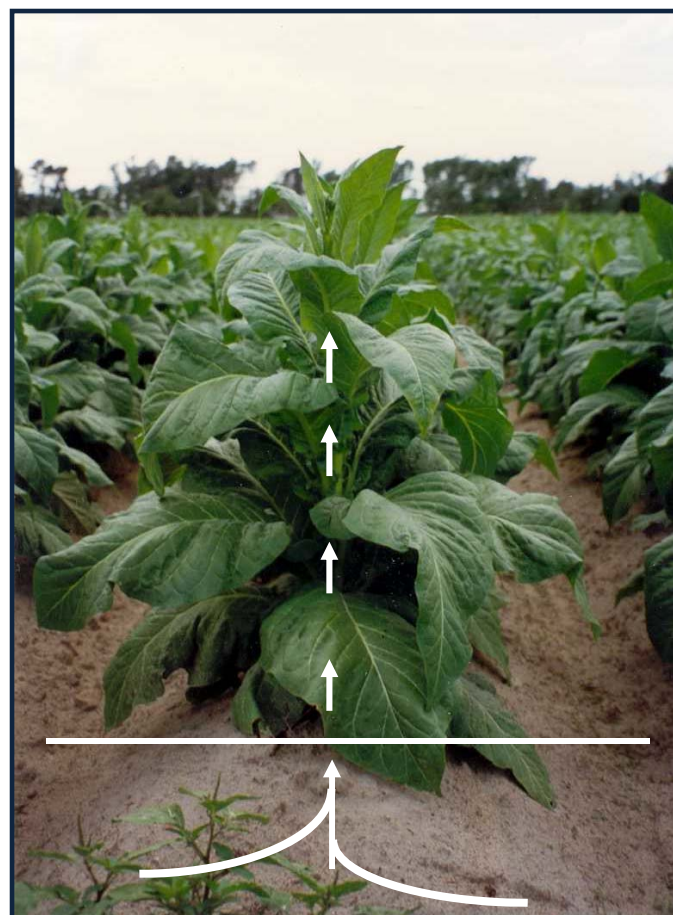
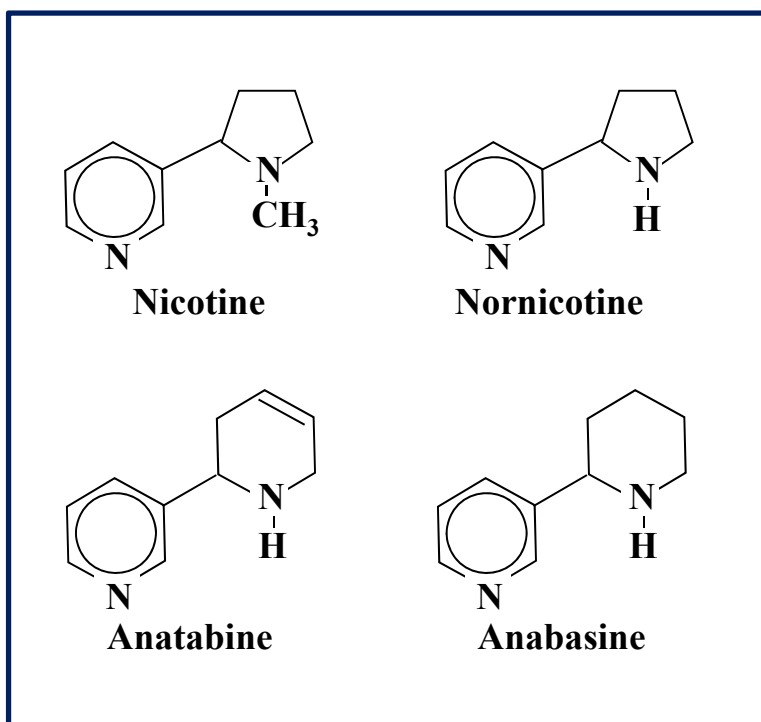


~25-fold Reduction in Nicotine

0.04% Nicotine in Cigarette Filler

# Alkaloids Are Plant Natural Products

Nicotine comprises the major fraction (>90%) of alkaloids in *N. tabacum*. Minor alkaloids are typically *nornicotine*, *anabesine*, & *anatabine*.



# Alkaloids Are Plant Natural Products

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% Nicotine = Genetics + Environment



Naturally Existing Variation

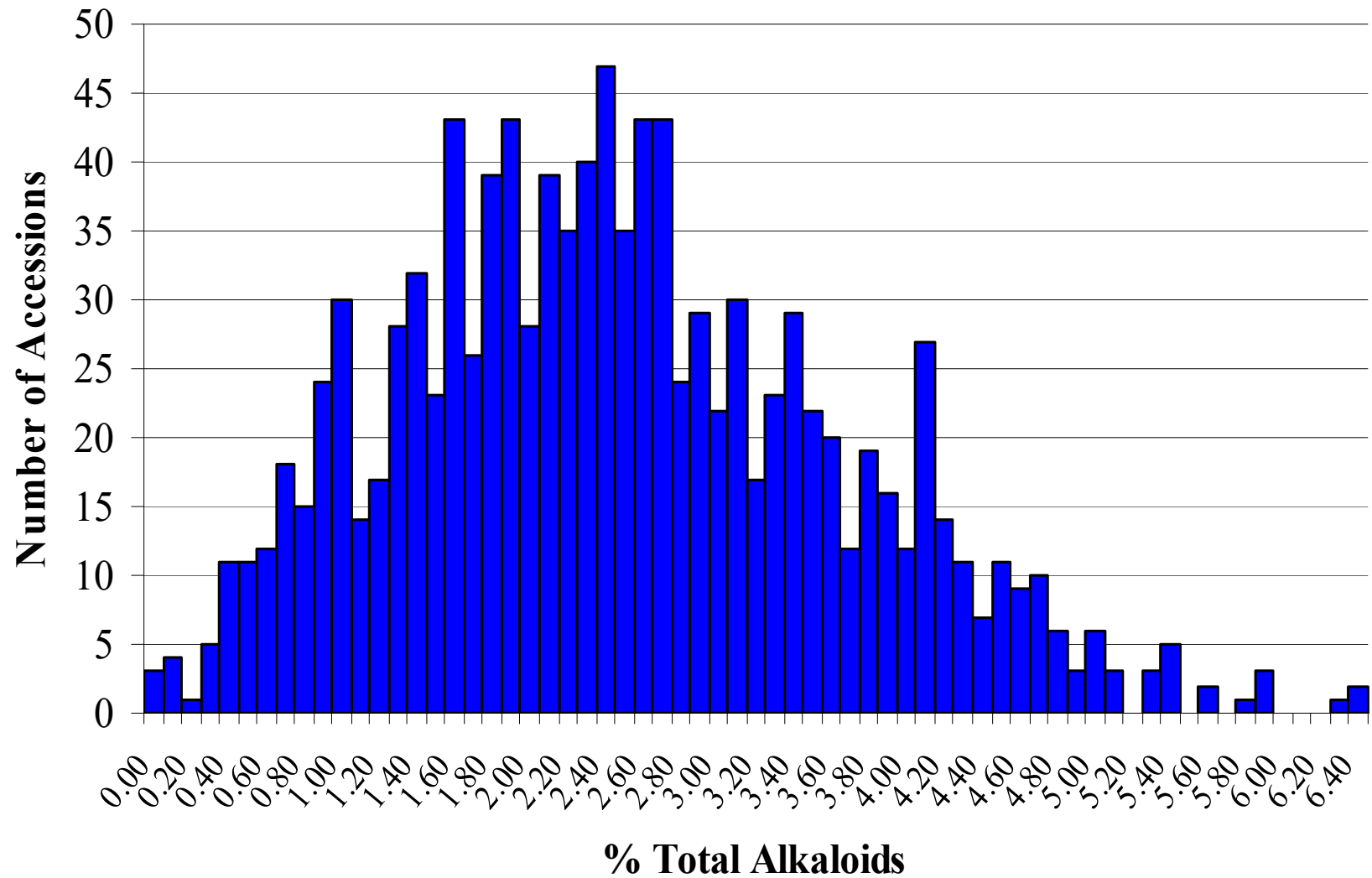
Induced Variation:

Transgenic

Mutation

# Tobacco Alkaloids

## Percent Total Alkaloids in 1108 *N. tabacum* Accessions



Sisson & Saunders, 1982

# Tobacco Alkaloids

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**Known loci with large effects:**

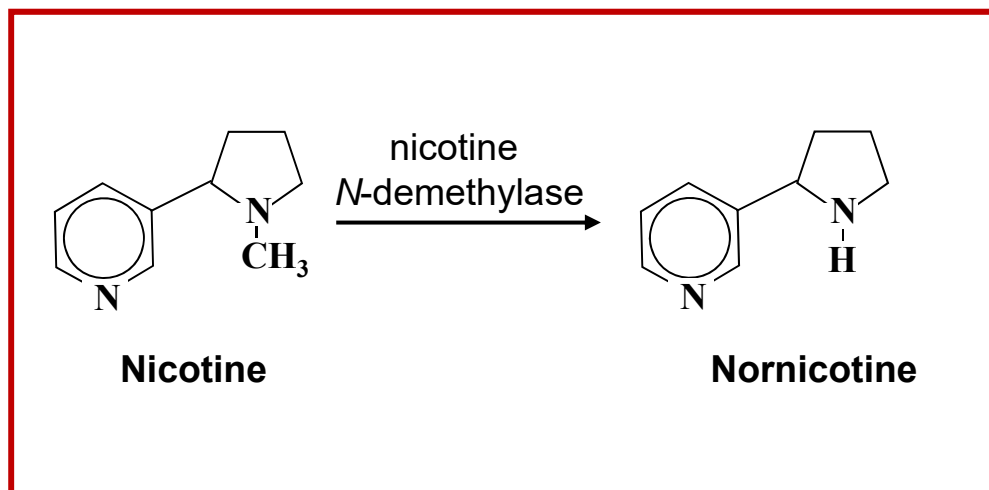
**nicotine converter loci**

***nic1 (A), nic2 (B)***

# Tobacco Alkaloids

Known loci with large effects:

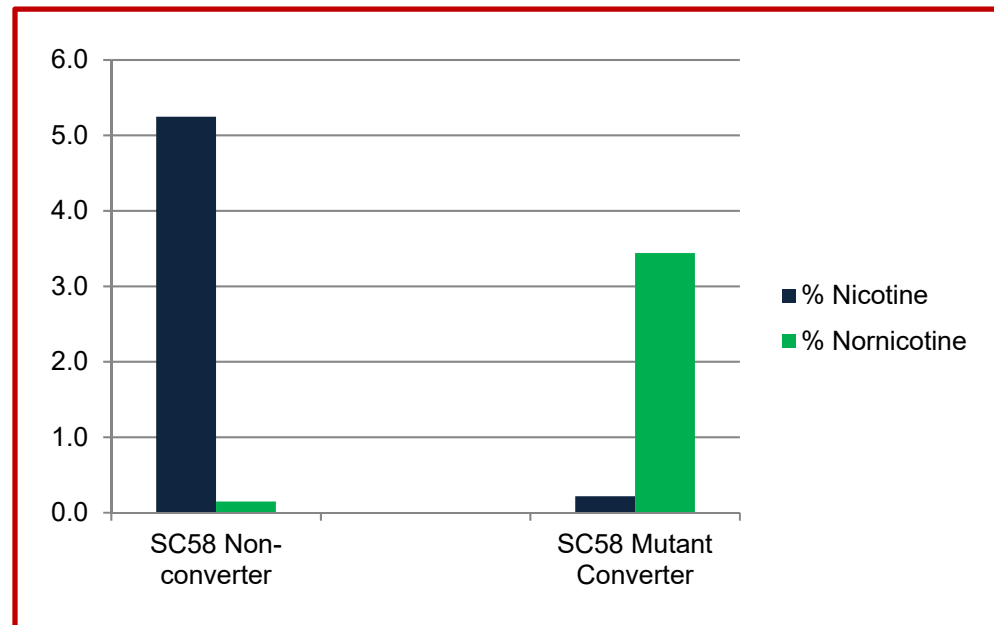
**nicotine converter genes**



# Tobacco Alkaloids

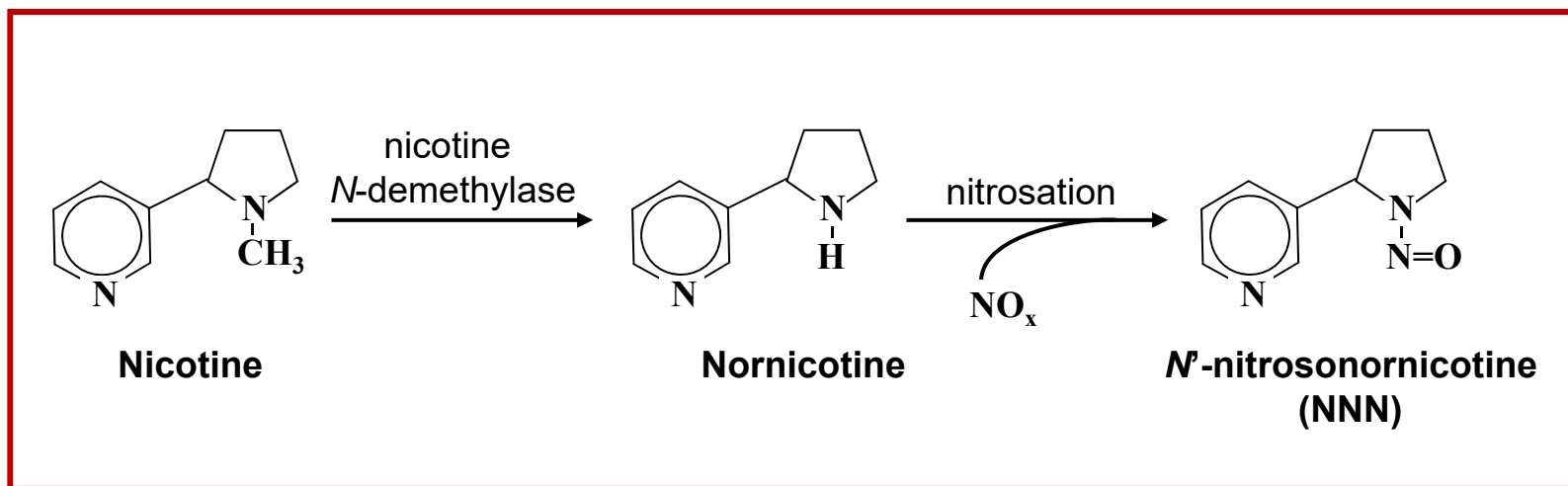
Known loci with large effects:

**nicotine converter genes**



# Increase in Nornicotine

## Potential Increases in NNN, a carcinogenic TSNA

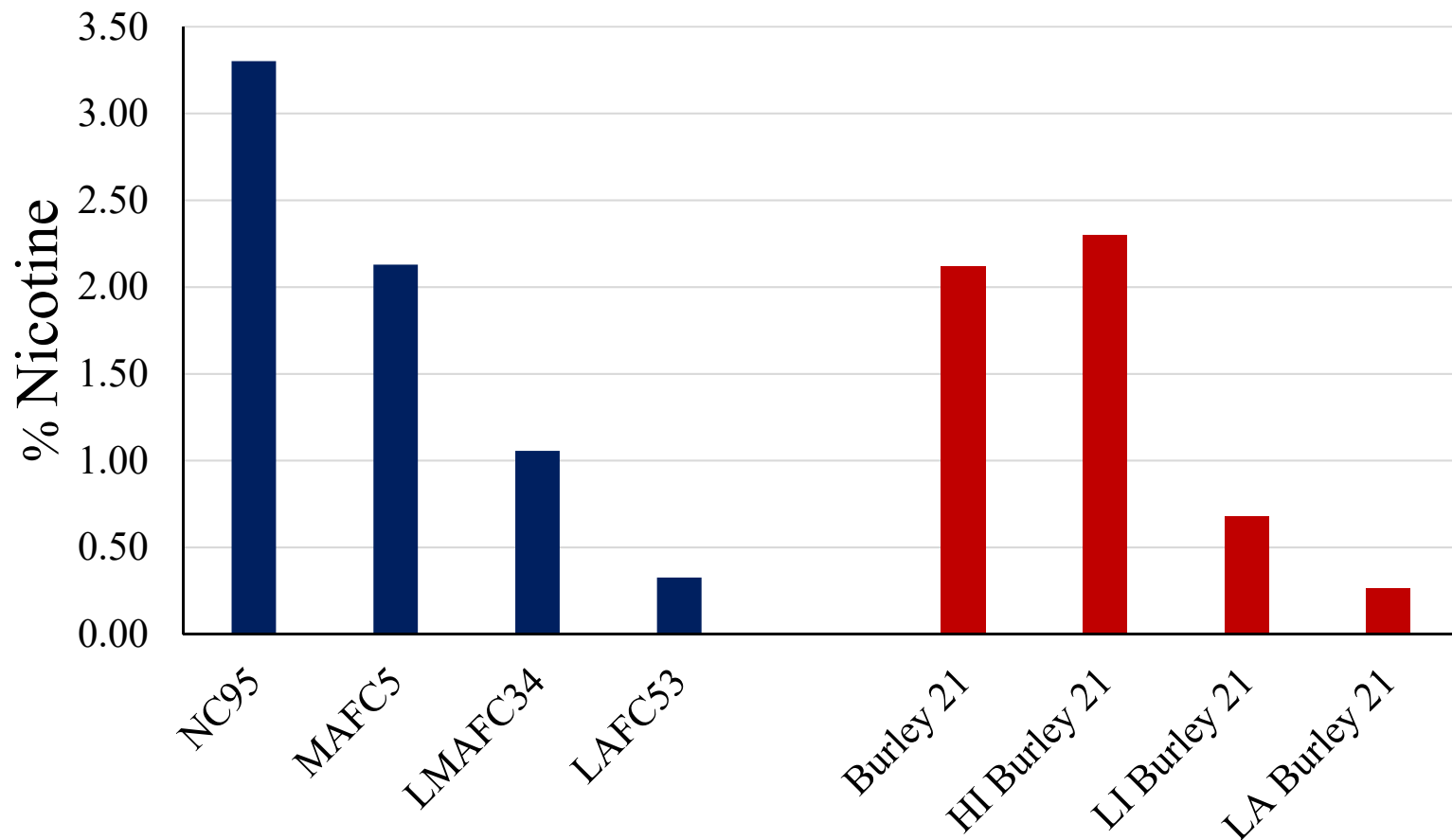




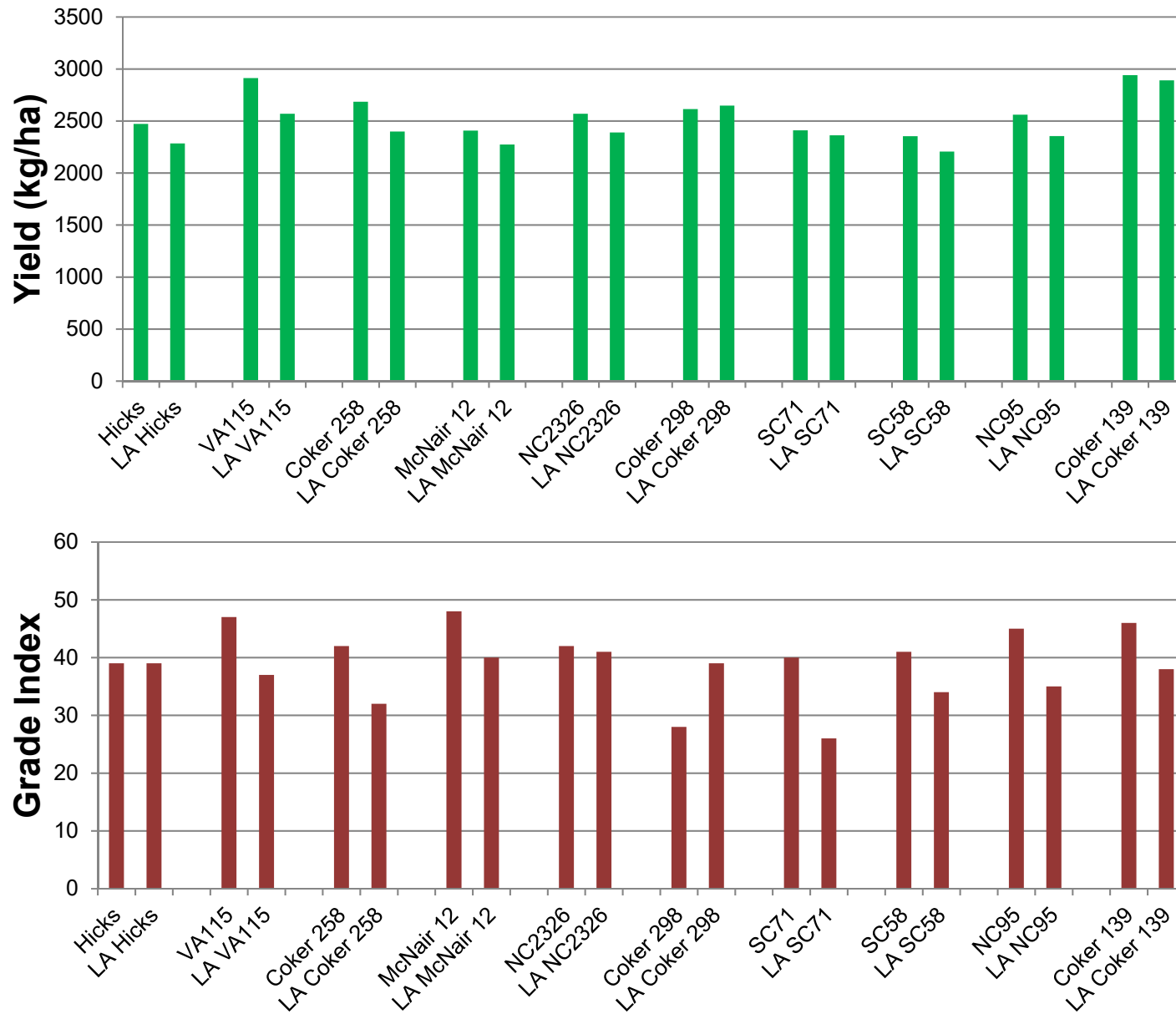
# Tobacco Alkaloids

Known loci with large effects:

*nic1 + nic2*



# *nic1 nic2* Penalty



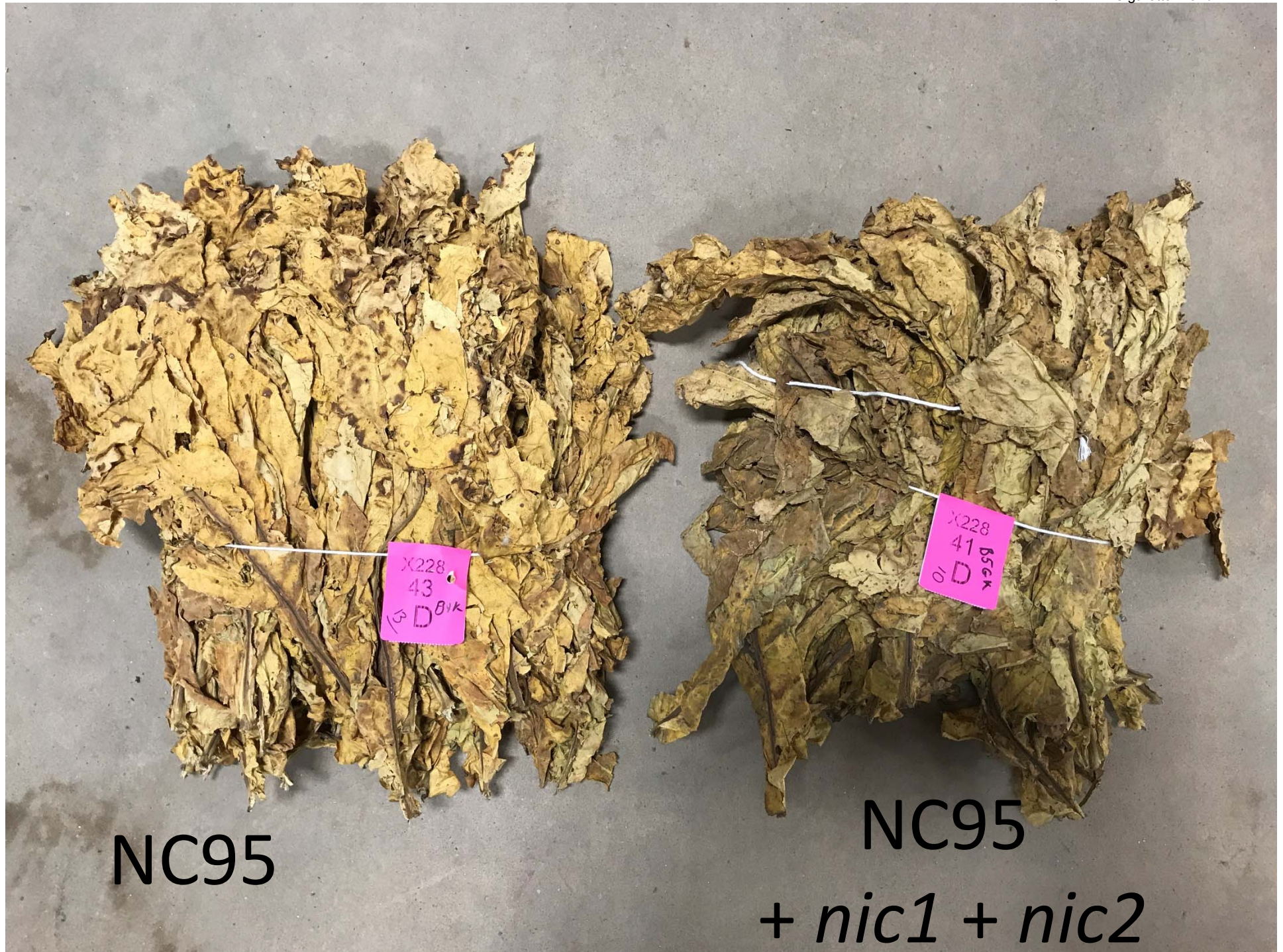
Chaplin and Weeks, 1976



NC95

LAFC53  
(NC95  
+ *nic1* + *nic2*)





# Alkaloids Are Plant Natural Products

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% Nicotine = Genetics + Environment



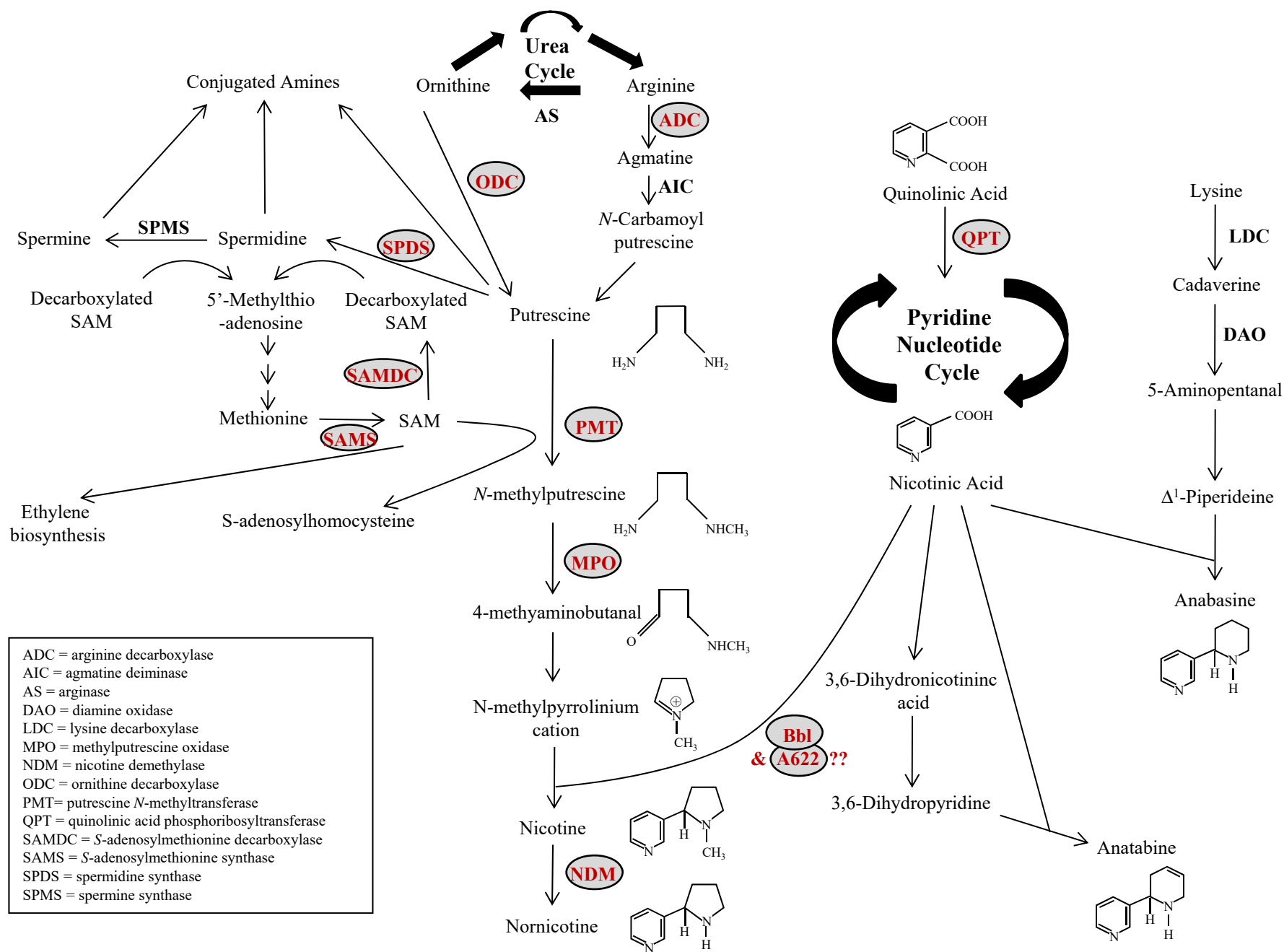
Naturally Existing Variation

Induced Variation:

Transgenic

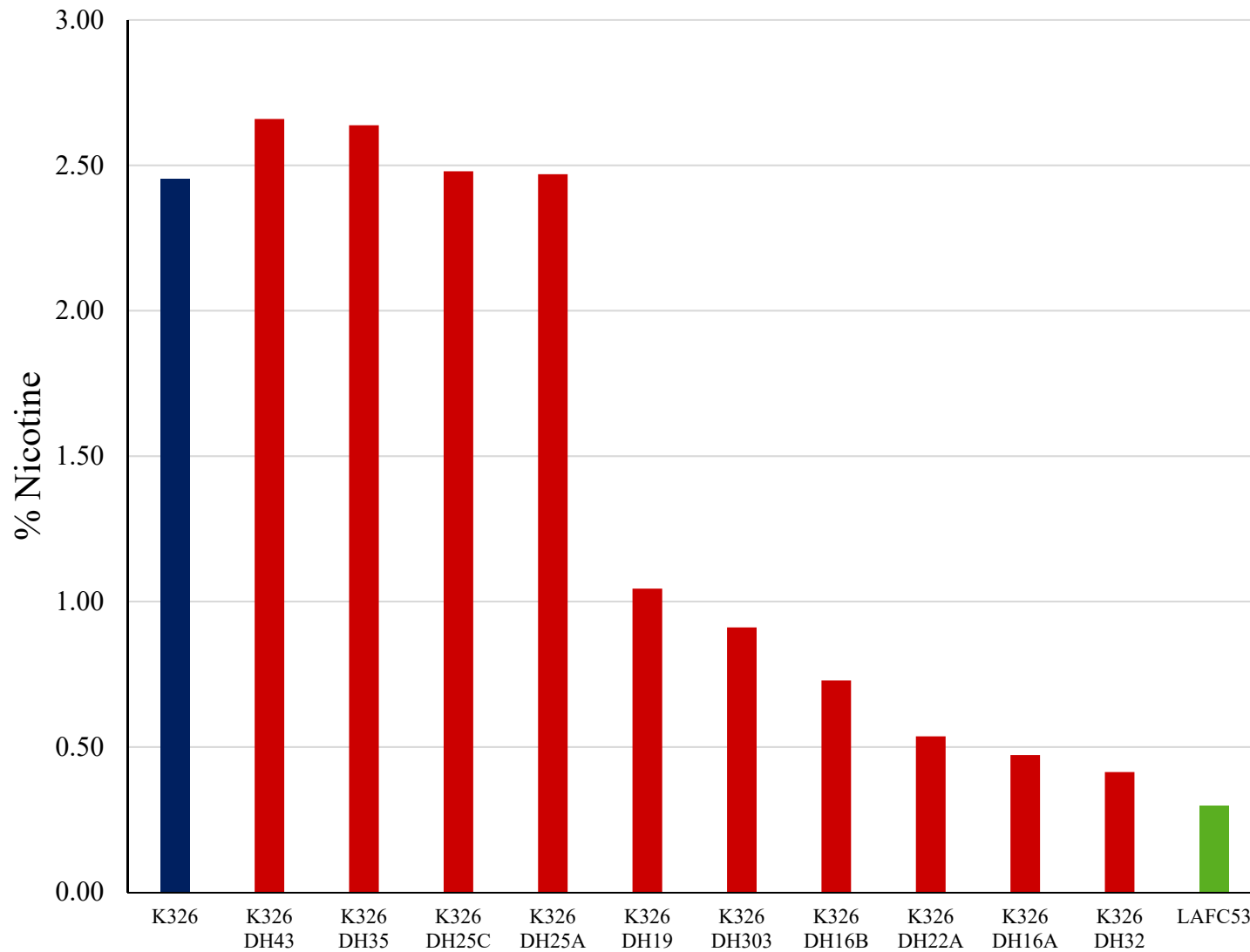
Mutation





Primary biosynthetic pathways for major alkaloids. Enzymes for which genes have been characterized are circled and shaded in grey.

# RNAi of *Bb1* Gene Family in DH Lines of 'K326'





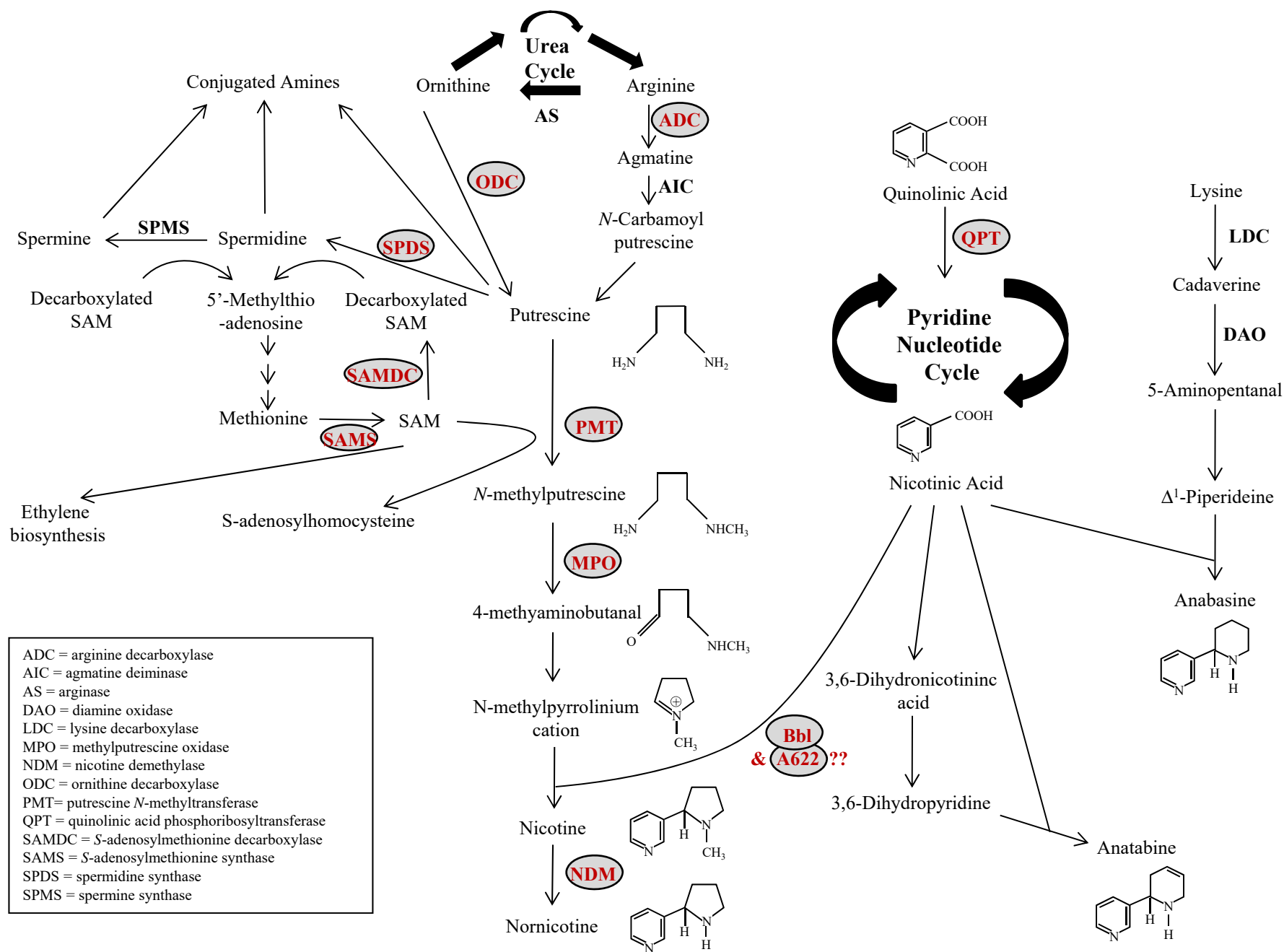


K326 + *BBL*  
silencing

K326







Primary biosynthetic pathways for major alkaloids. Enzymes for which genes have been characterized are circled and shaded in grey.

# Expressed *BBL* Genes

## *In silico* analysis of *BBL* gene expression

<i>BBL</i>	EST Library	Root-Specific
Isoform	Hits <sup>a</sup>	ESTs
<i>BBLa</i>	38	38
<i>BBLb</i>	11	10
<i>BBLc</i>	16	16
<i>BBLd</i>	2	1

<sup>a</sup> In addition to cDNA from libraries generated solely from root tissue, cDNAs isolated from pooled leaf, flower, and root cDNA libraries were also included.

# ***BBL* Gene Mutations**

Position and effect of mutations identified in *BBL* genes in the mutated genetic background of tobacco line DH98-325-6.

Gene	Mutant	Mutation	Position from ATG	Amino Acid Change
<i>BBLa</i>	1435	G/A	681	W227Stop
<i>BBLb</i>	675	G/A	438	W146Stop
<i>BBLc</i>	1771	C/T	448	Q150Stop

# BBL Gene Mutations

Backcross all three mutations into K326,  
 TN90, and TN90 SRC to develop BC<sub>7</sub>F<sub>3</sub>  
 nearly isogenic lines.

<i>BBLa/BBLa</i>	<i>BBLb/BBLb</i>	<i>BBLc/BBLc</i>	(000)
<i>BBLa/BBLa</i>	<i>BBLb/BBLb</i>	<i>bb1c/bb1c</i>	(002)
<i>BBLa/BBLa</i>	<i>bb1b/bb1b</i>	<i>BBLc/BBLc</i>	(020)
<i>bb1a/bb1a</i>	<i>BBLb/BBLb</i>	<i>BBLc/BBLc</i>	(200)
<i>BBLa/BBLa</i>	<i>bb1b/bb1b</i>	<i>bb1c/bb1c</i>	(022)
<i>bb1a/bb1a</i>	<i>BBLb/BBLb</i>	<i>bb1c/bb1c</i>	(202)
<i>bb1a/bb1a</i>	<i>bb1b/bb1b</i>	<i>BBLc/BBLc</i>	(220)
<i>bb1a/bb1a</i>	<i>bb1b/bb1b</i>	<i>bb1c/bb1c</i>	(222)

# ***BBL* Gene Mutations**

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Flue cured NILs evaluated in 6 2016 + 2017  
environments.

Burley NILs evaluated in 4 2016 + 2017  
environments.

# Field Evaluation of *BBL* Mutation Lines

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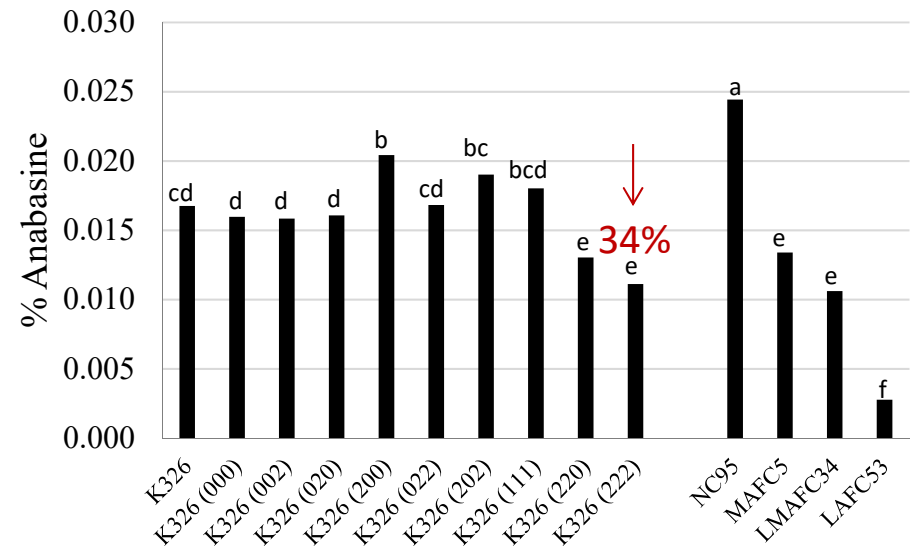
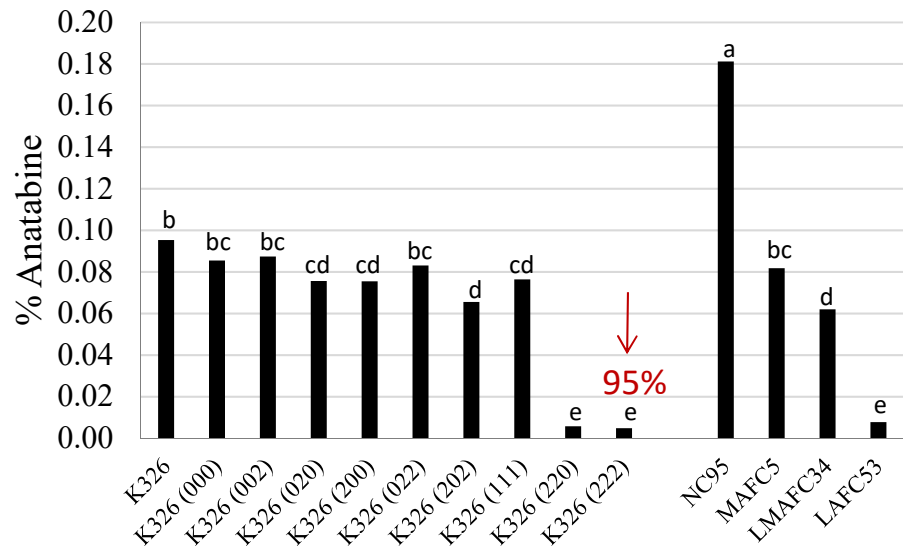
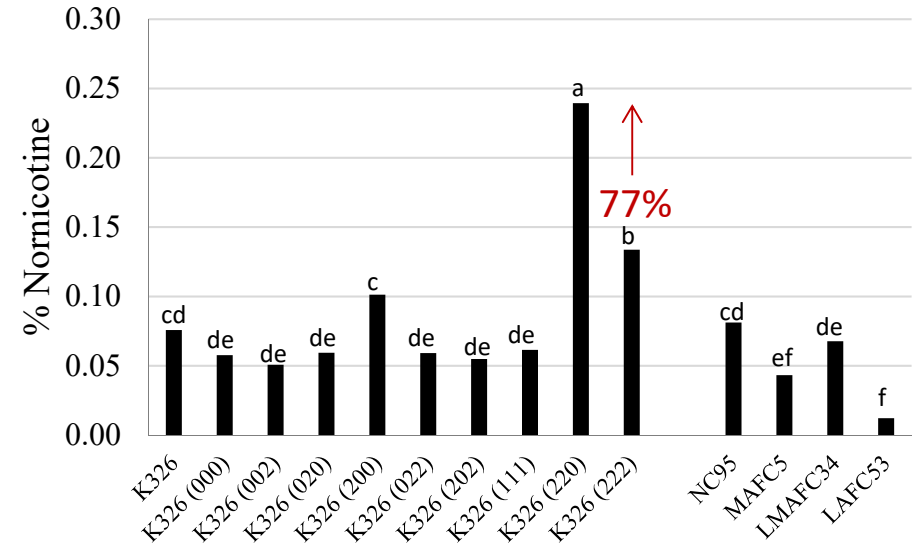
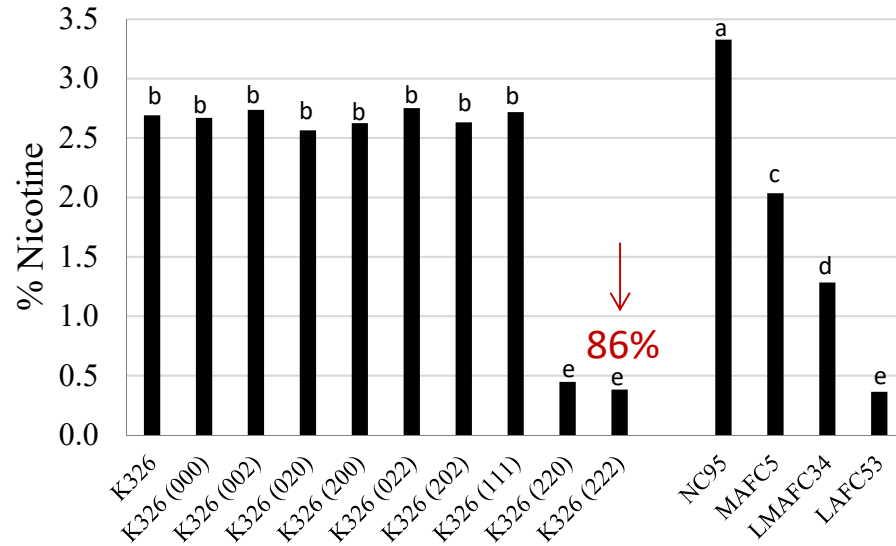
RCBD Design With 4 Replications

*nic1/nic2* isolines included as checks

Yield, quality, and chemistry determined  
for each plot

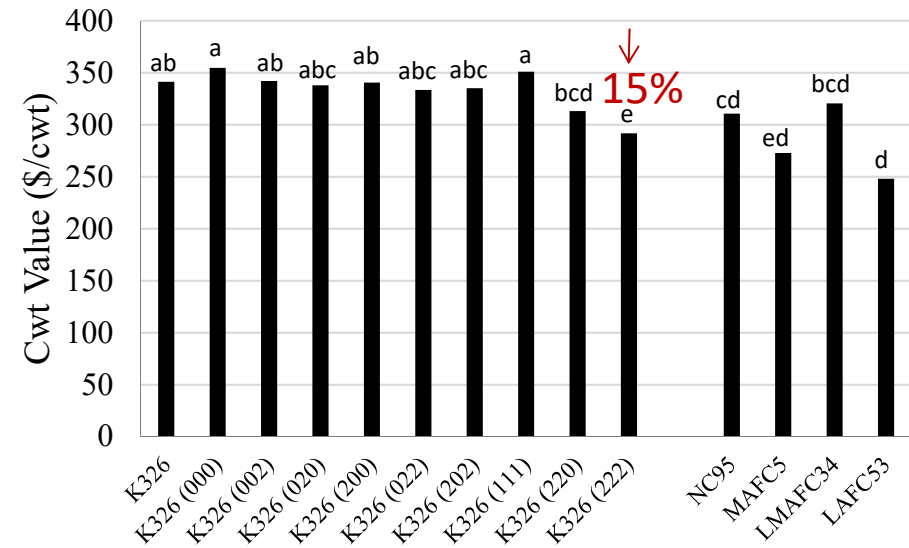
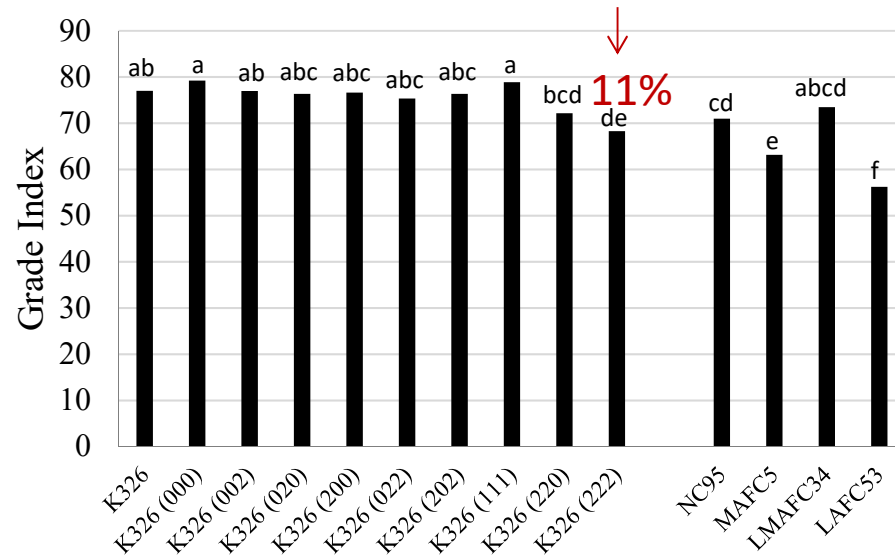
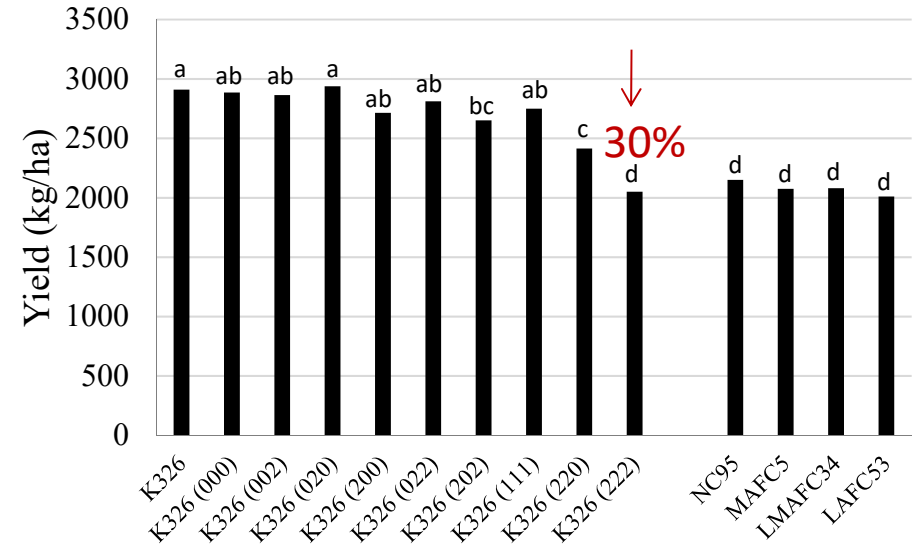
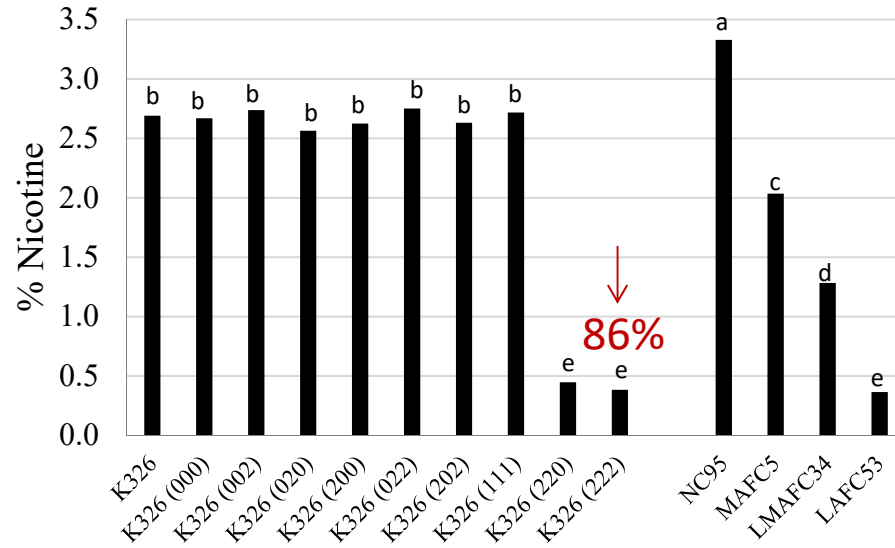
Composite samples used for alkaloid  
quantifications

# BBL Mutant Genetic Series In K326





# BBL Mutant Genetic Series In K326





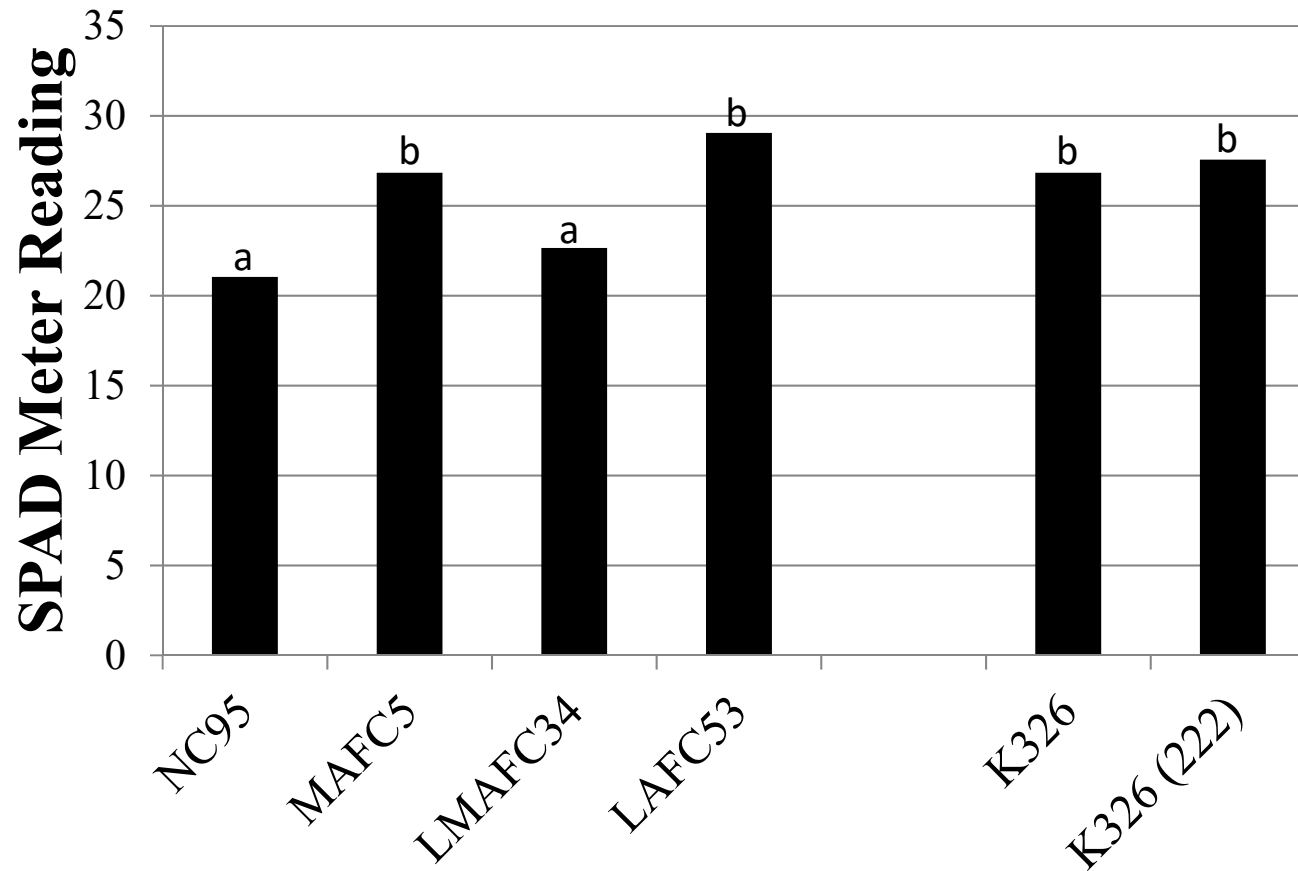


K326

K326 + *BBL*  
Mutant Line



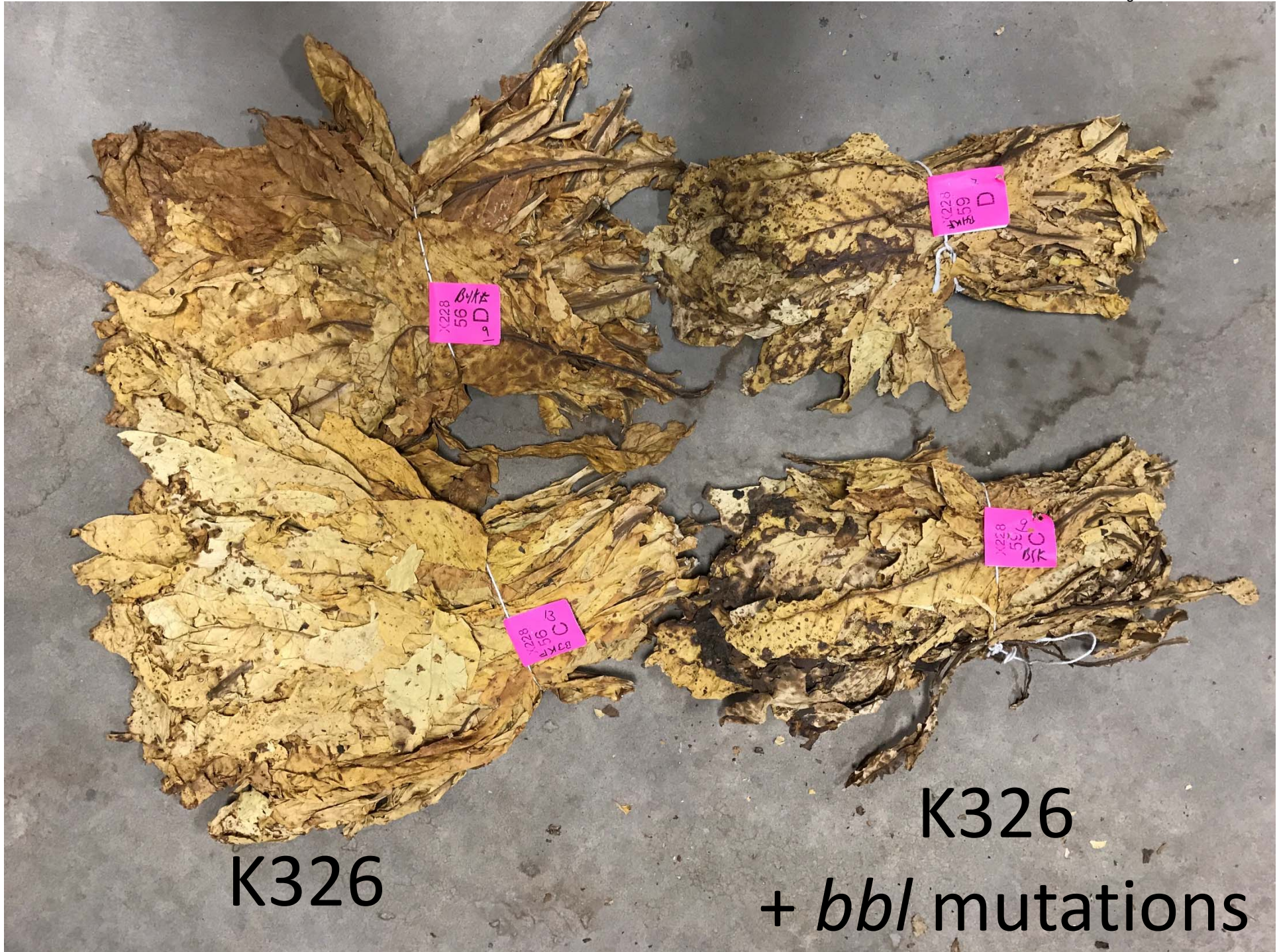
# Chlorophyll Content Before Harvest



# Declines in Leaf Quality

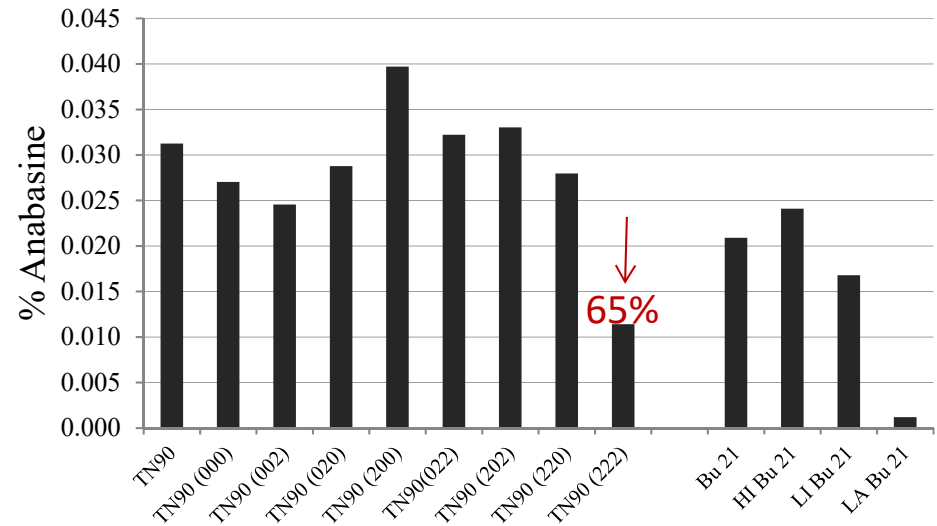
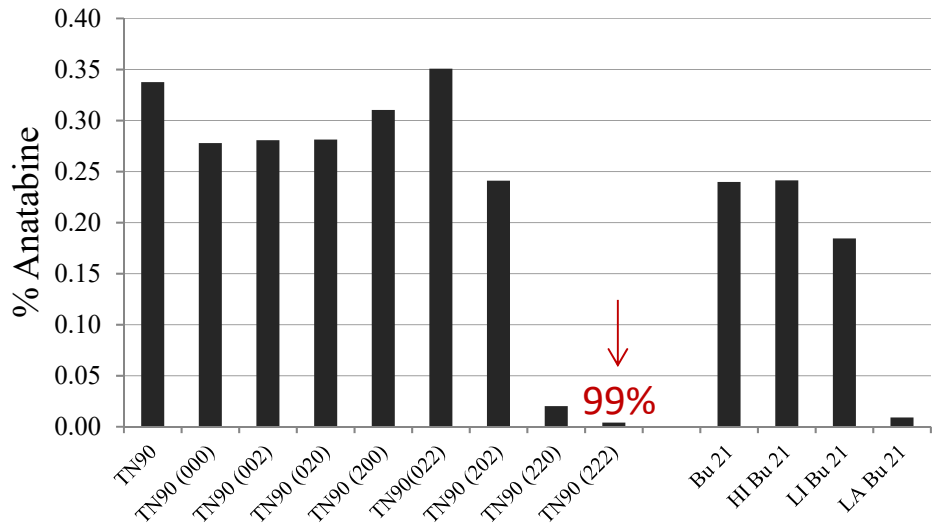
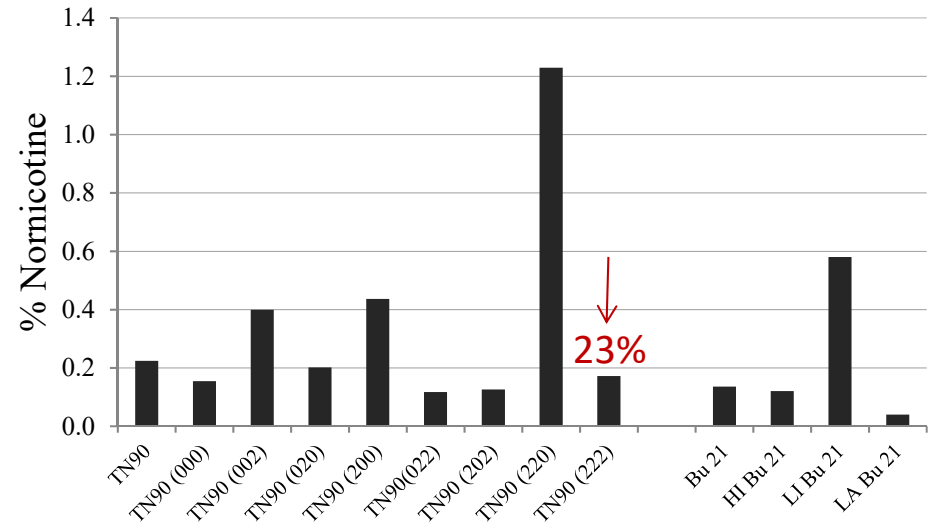
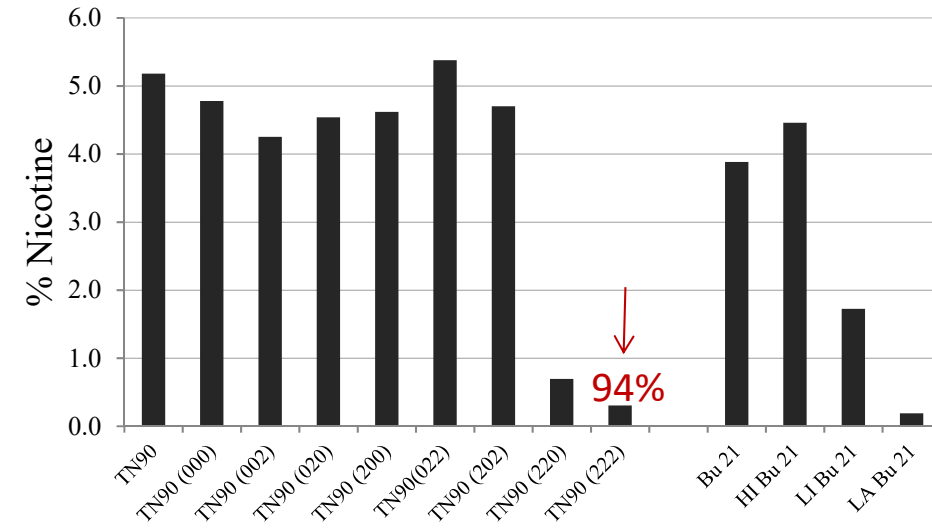
Environment	Rep	Genotype	GovGradeA	GovGradeB	GovGradeC	GovGradeD
Kinston2016	3	K326	X4F	C4KF	B4K	B4K
Kinston2016	3	K326 (222)	X4F	C5F	B5KF	B5K
Kinston2016	4	K326	X3F	C4KM	B4KF	B4K
Kinston2016	4	K326 (222)	X4F	C4KF	B4KF	N1GF
Kinston2017	2	K326	P4F	C5F	B4K	B5KF
Kinston2017	2	K326 (222)	P4F	C4KF	B5KF	B5KF
Oxford2016	1	K326	X4F	C4F	B5K	N1K
Oxford2016	1	K326 (222)	X4F	C4F	N1K	N1K
Oxford2016	2	K326	X5F	X4F	B5K	B5K
Oxford2016	2	K326 (222)	N1XL	X4F	N1K	N1K
Oxford2016	3	K326	X5F	C4F	B5K	B5K
Oxford2016	3	K326 (222)	X4F	X4F	N1K	N1K
Oxford2016	4	K326	C5F	C4F	B5K	B4KF
Oxford2016	4	K326 (222)	X4F	N1XL	B5KF	B5KF
Oxford2017	1	K326	X4F	C4F	B4KF	B4KF
Oxford2017	1	K326 (222)	X5F	C5F	B4KF	B4KF
Oxford2017	3	K326	X4F	C4F	B3KF	B4KF
Oxford2017	3	K326 (222)	X4F	C5F	B5KF	B4KF
Oxford2017	4	K326	X4F	C4F	B4K	B4K
Oxford2017	4	K326 (222)	X4F	C4F	B4KF	B4KF
RockyMount2016	1	K326	X3F	C4F	B5K	B5K
RockyMount2016	1	K326 (222)	X4F	C4F	N1K	N1K
RockyMount2016	2	K326	X3F	C4F	B4F	B4K
RockyMount2016	2	K326 (222)	X4F	C5F	N1K	B6K
RockyMount2017	3	K326	X4F	C4F	B4K	B4KF
RockyMount2017	3	K326 (222)	X4F	X5F	B4K	B5KF
RockyMount2017	4	K326	X4F	C4F	B4K	B4K
RockyMount2017	4	K326 (222)	X4F	C4F	B4KF	B4KF



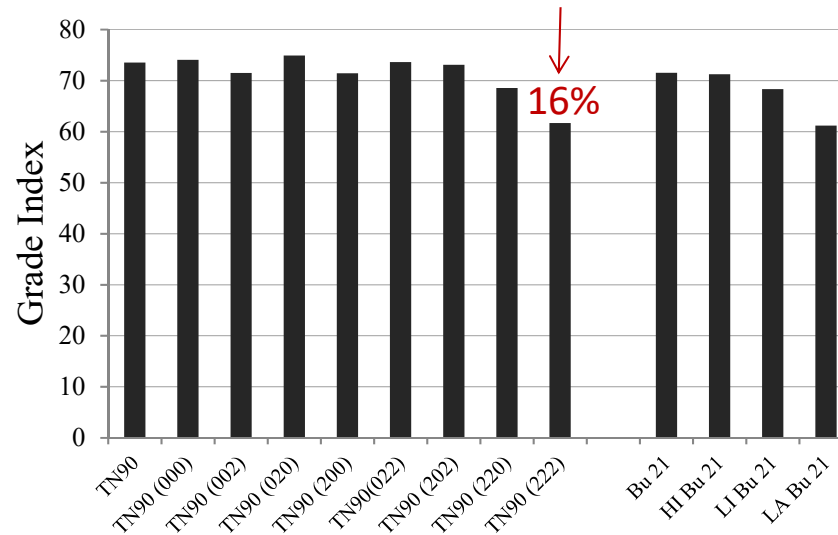
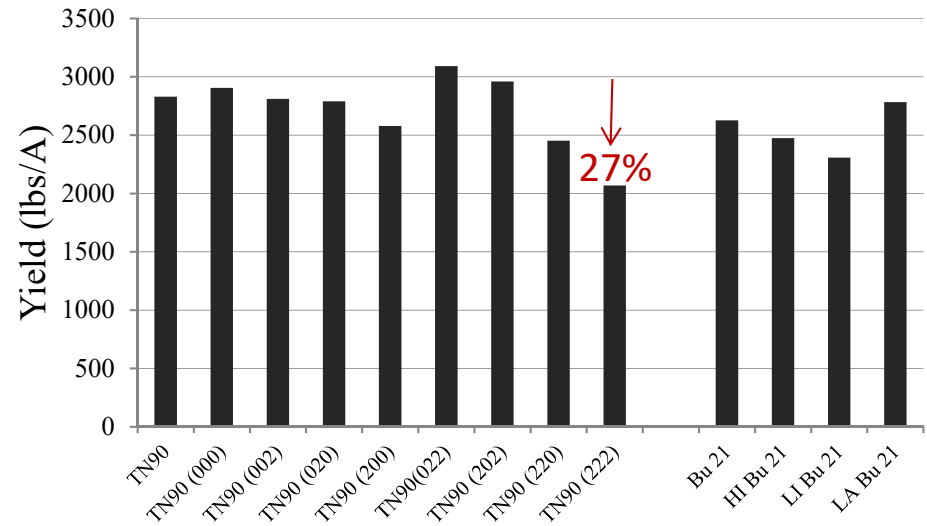
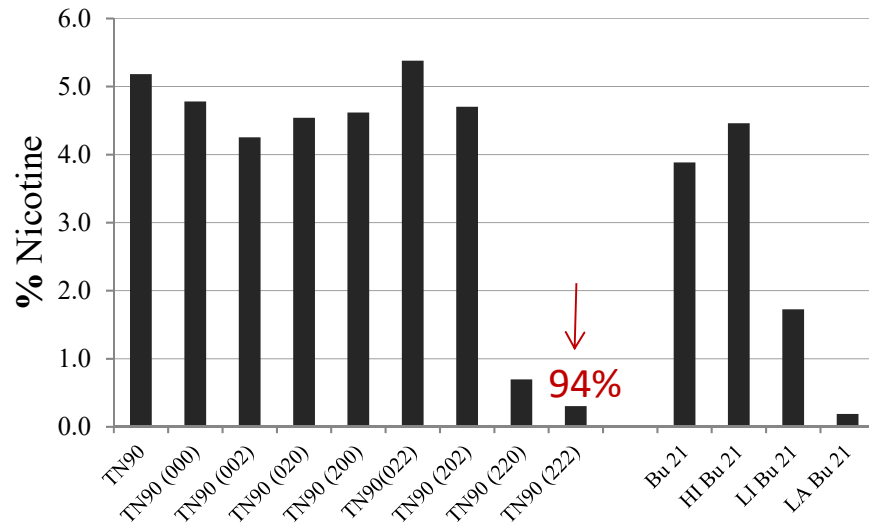




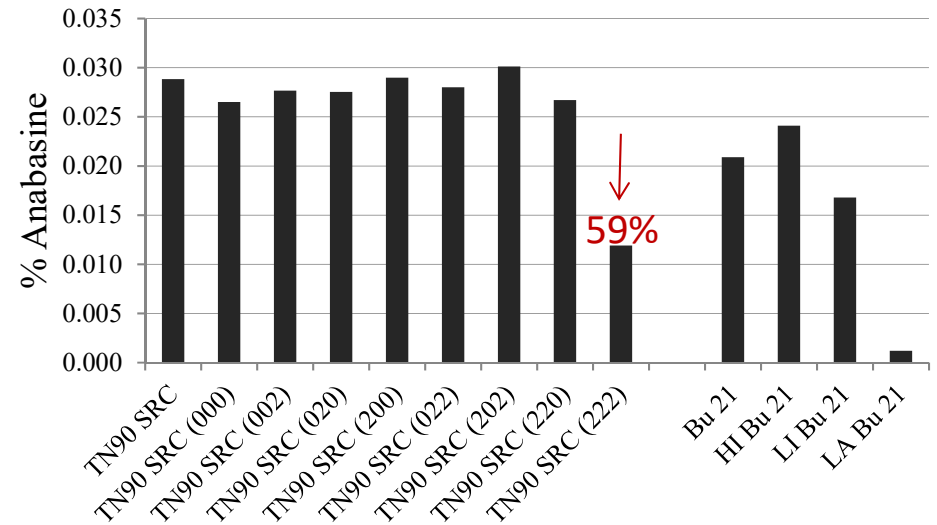
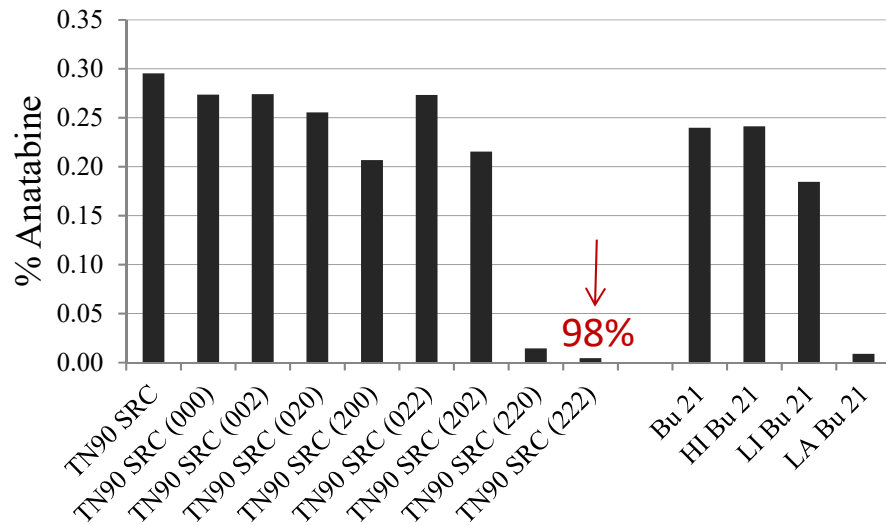
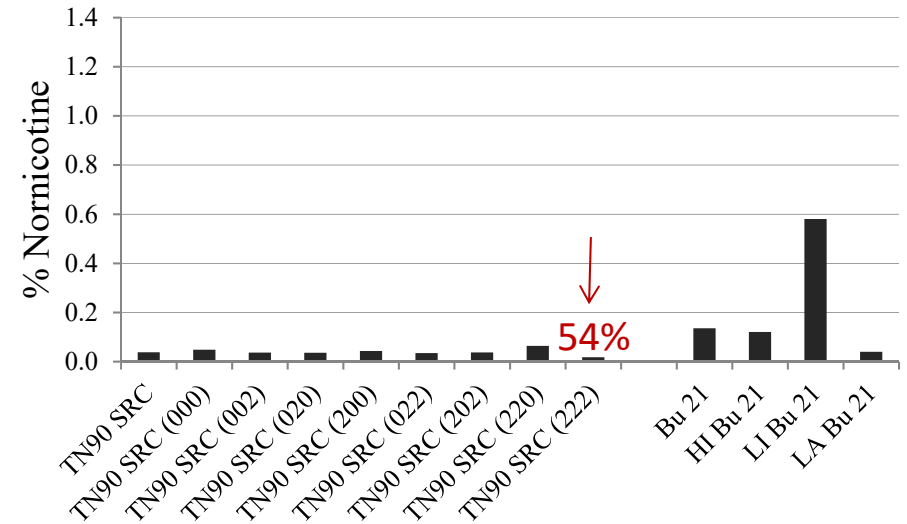
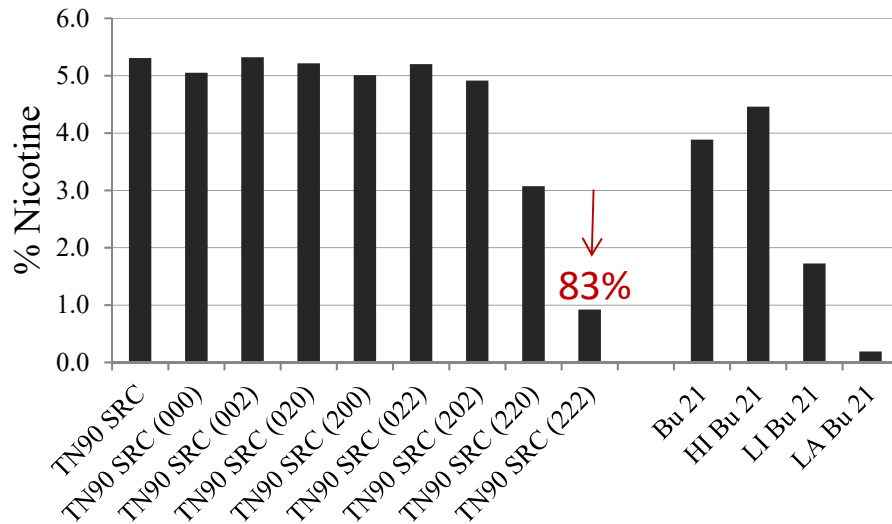
# BBL Mutant Genetic Series In TN90



# BBL Mutant Genetic Series In TN90

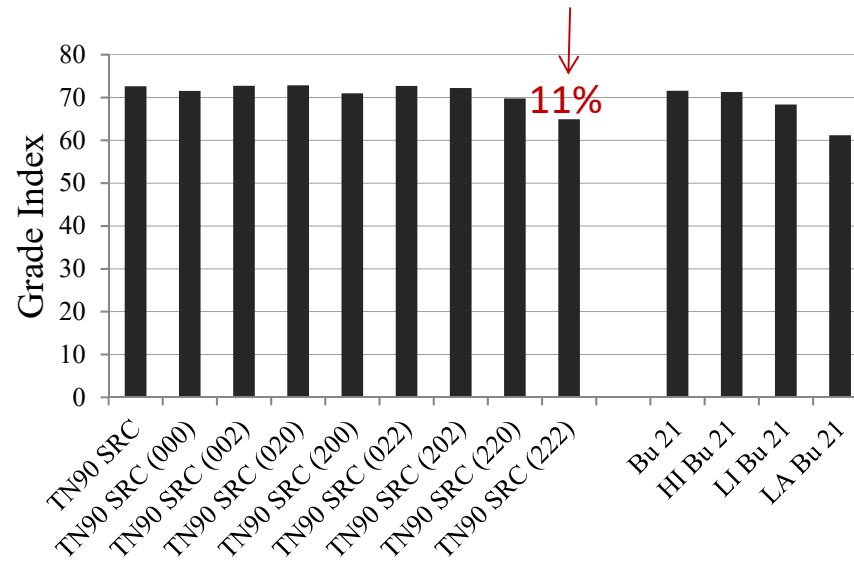
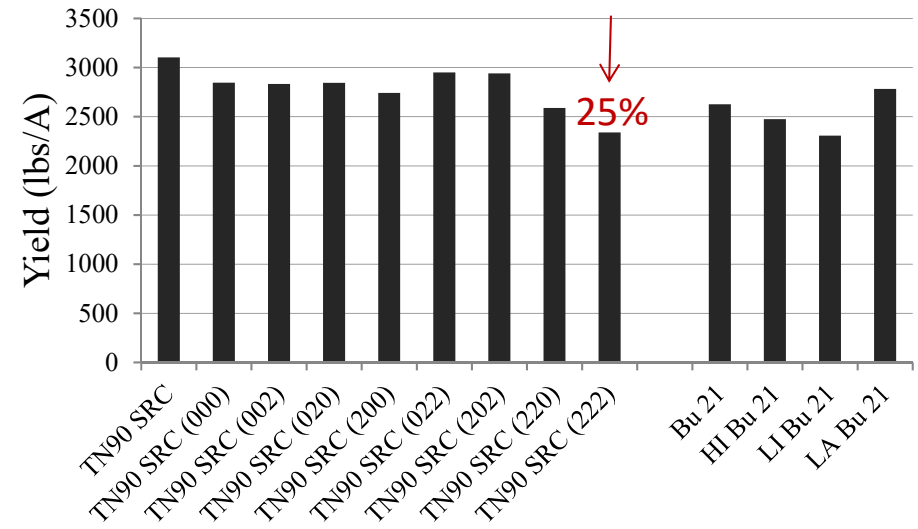
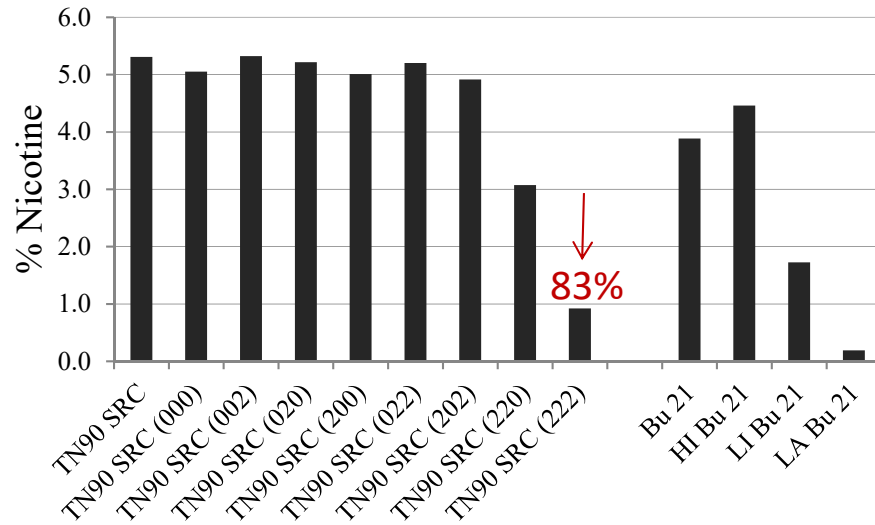


# BBL Mutant Genetic Series In TN90 SRC

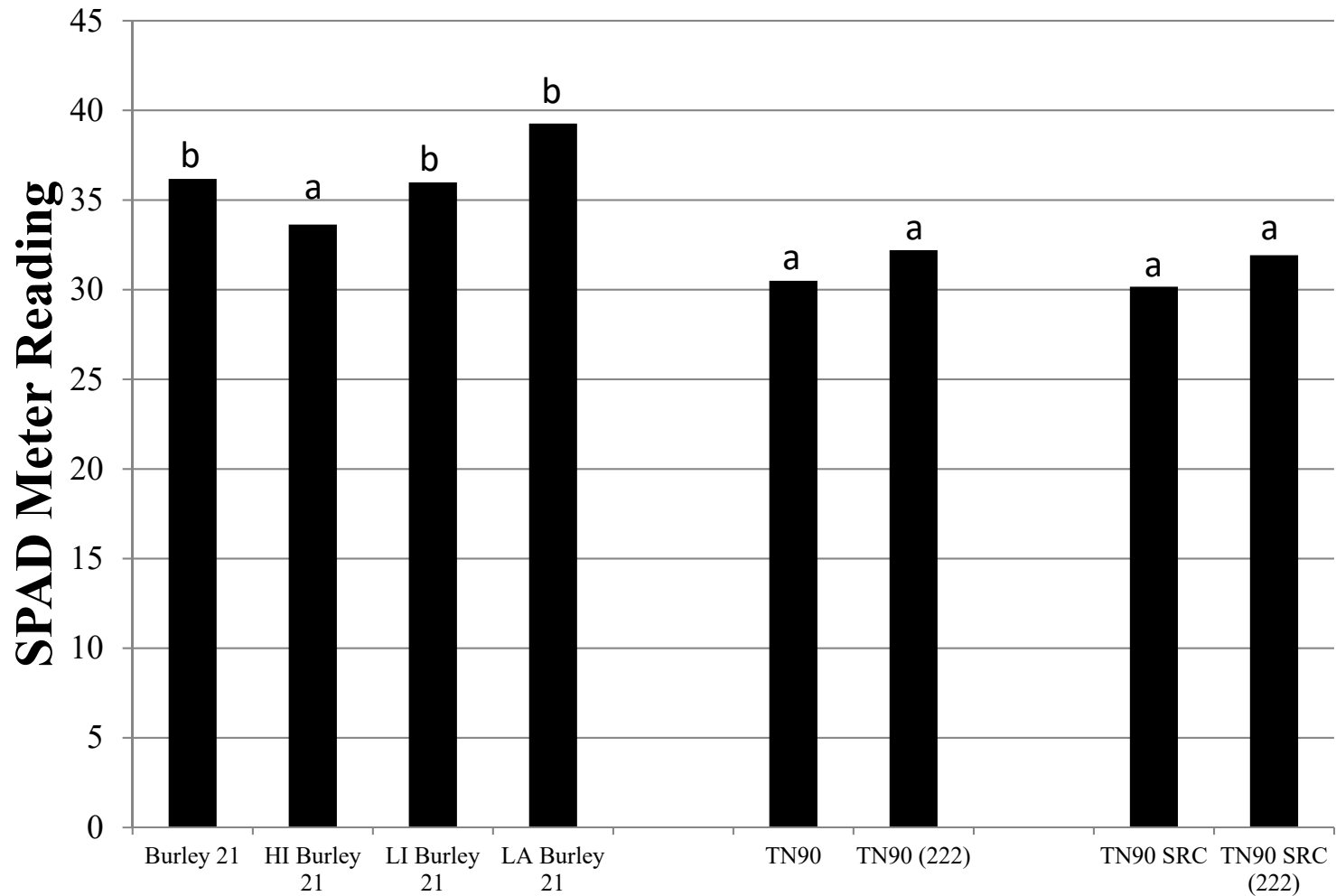




# BBL Mutant Genetic Series In TN90 SRC



# Chlorophyll Content Before Harvest



# Conclusions

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- 1) Mutations in three *BBL* genes reduced nicotine to ~0.20 to 0.40 % in our environments (using a composite sample)
- 2) *BBL* mutations did not provide a lower nicotine level than *nic1* + *nic2* genetic variation
- 3) *BBL* enzymes also likely involved in the biosynthesis of anatabine and anabasine
- 4) *BBL* mutations associated with 25 to 30% reduction in cured leaf yields
- 5) *BBL* mutations associated with reduced cured leaf quality, although possibly not as bad as *nic1* + *nic2*

# Prospects for Reducing Nicotine Content

