Genetic Approaches and Resources for Enabling Compliance to Proposed NNN Standards

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DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

21 CFR Part 1132

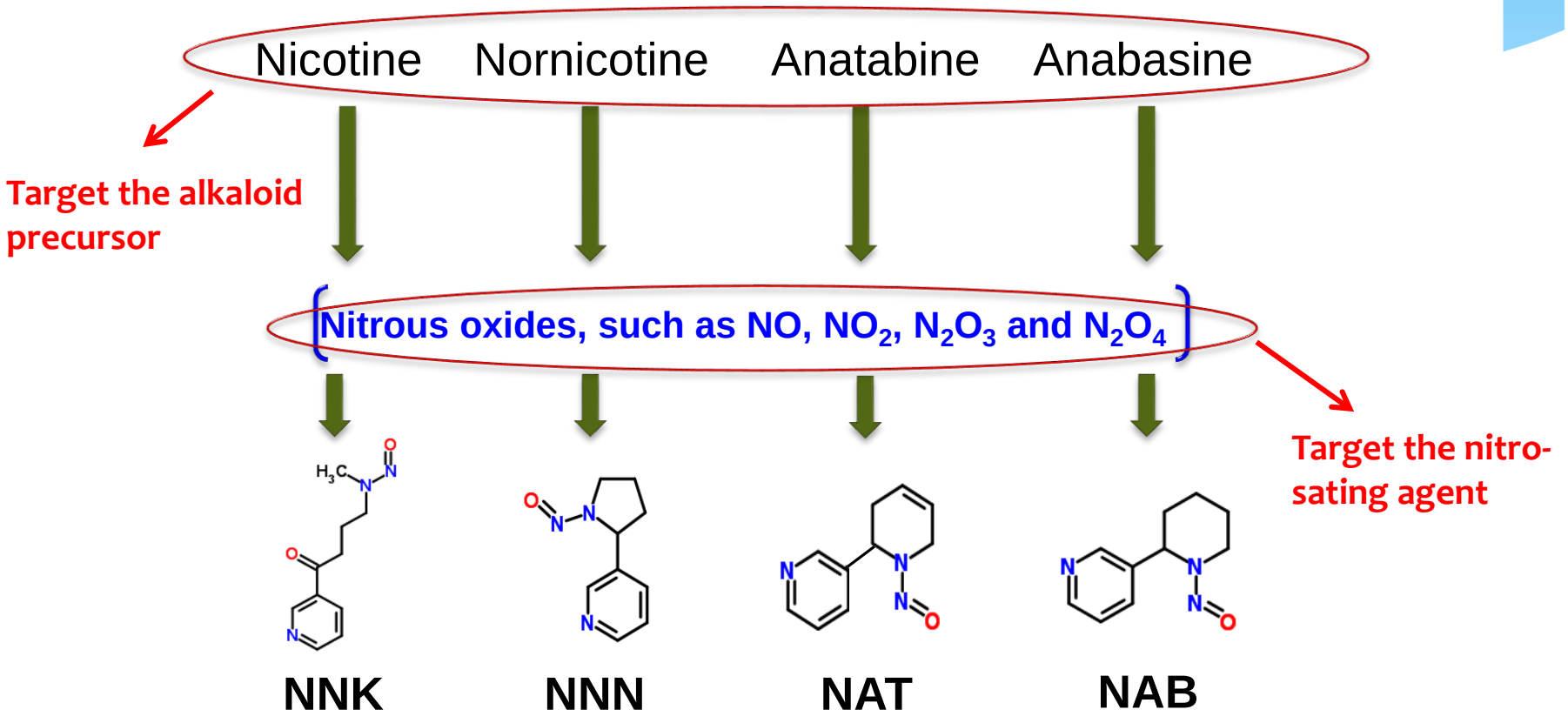
[Docket No. FDA-2016-N-2527]

Tobacco Product Standard for N-nitrosonornicotine Level in Finished Smokeless Tobacco Products

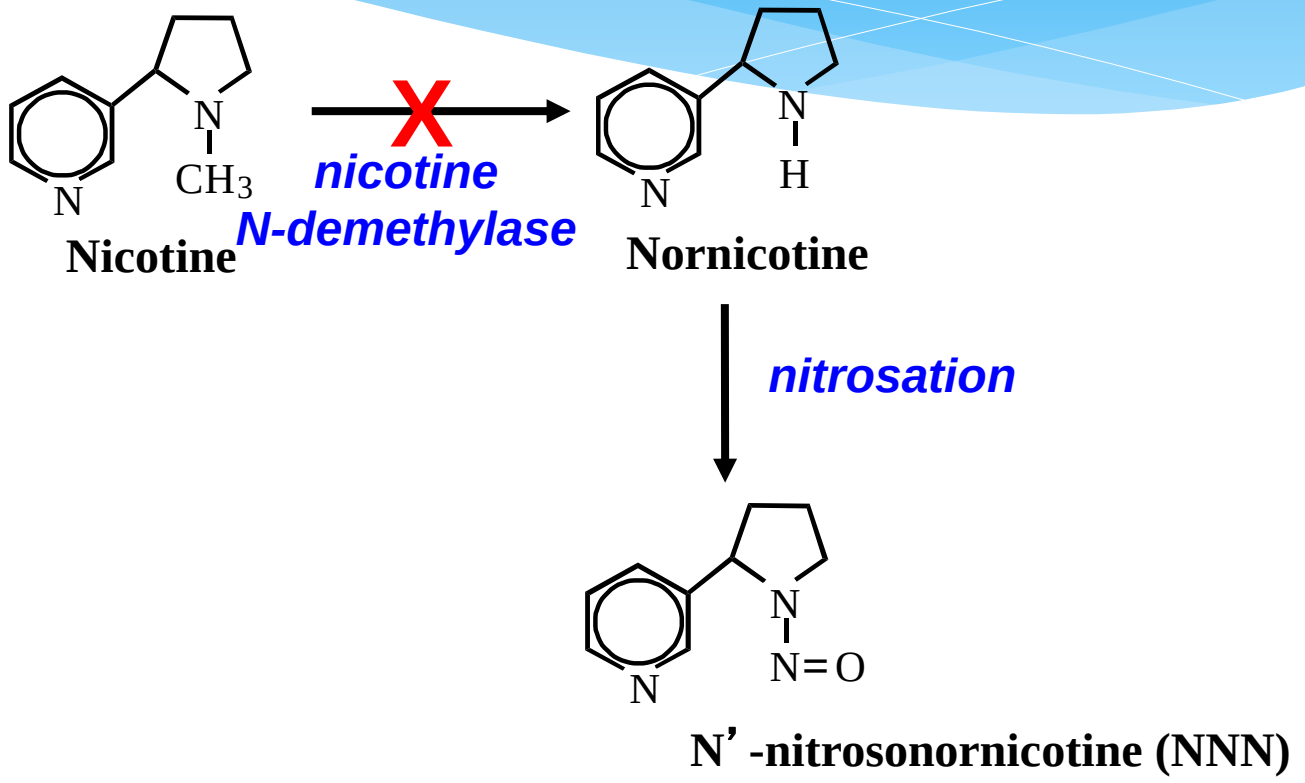
ACTION: Proposed rule.

"The proposed rule would require that the mean level of NNN in any batch of finished smokeless tobacco products **not exceed 1.0 microgram per gram (µg/g)** of tobacco (on a dry weight basis) at any time through the product's labeled expiration date..."

Reducing Tobacco-Specific Nitrosamines via Genetic Modification

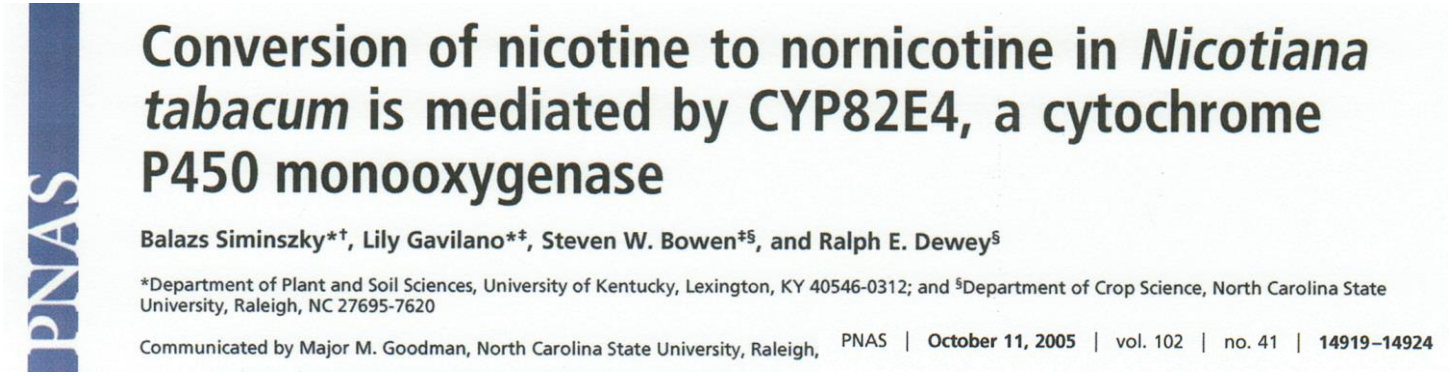


Original Strategy for Reducing NNN = Targeting the Nornicotine Precursor



Phase I: Isolation of *CYP82E4*, the Major Nicotine Demethylase Gene of *Nicotiana tabacum*

- Isolated using a gene expression profiling strategy (microarrays)
- Encodes cytochrome P450 enzyme
- Member of small, closely related gene family unique to *Nicotiana*



PNAS

Conversion of nicotine to nornicotine in *Nicotiana tabacum* is mediated by CYP82E4, a cytochrome P450 monooxygenase

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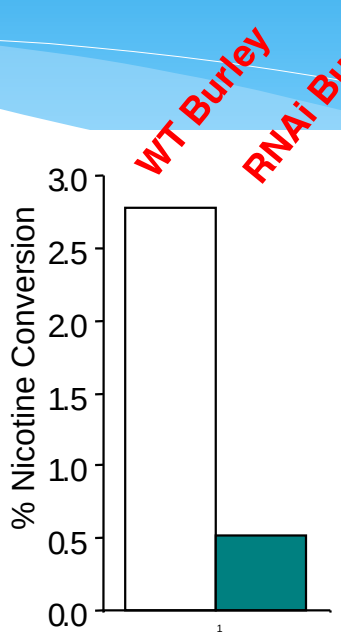
Phase 2: Demonstration that transgenic suppression of *CYP82E4* family can reduce nornicotine and NNN levels



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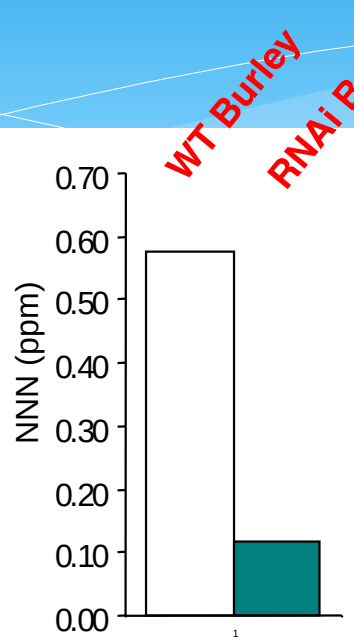

P = <0.0001

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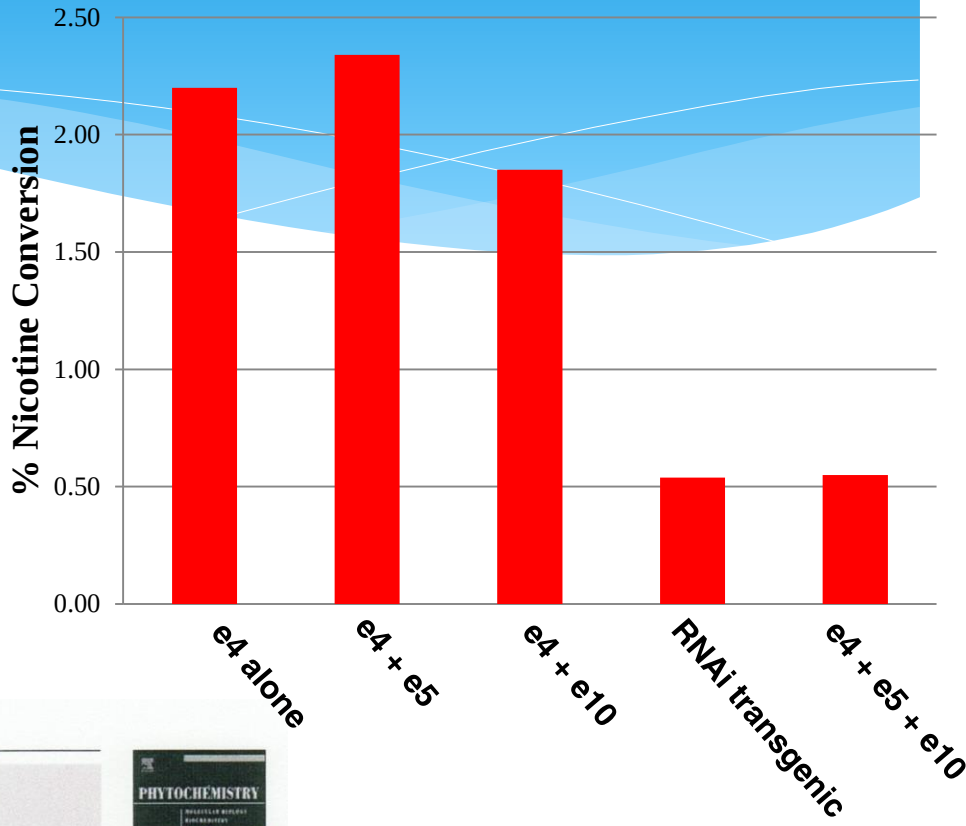
RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves

Ramsey S. Lewis[1,*], Anne M. Jack[2], Jerry W. Morris[3], Vincent J. M. Robert[3,†], Lily B. Gavilano[2], Balazs Siminszky[2], Lowell P. Bush[2], Alec J. Hayes[3] and Ralph E. Dewey[1]

Phase 3: Development of a non-GM strategy for nornicotine (and NNN) reduction

- Generation and selection of knockout mutations (EMS) in all three nicotine demethylase genes (*CYP82E4*, *CYP82E5* and *CYP82E10*)
- Pyramiding the three mutations within the same line



Phytochemistry 71 (2010) 1988–1998

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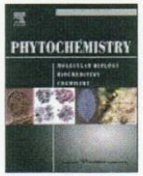


Three nicotine demethylase genes mediate nornicotine biosynthesis in *Nicotiana tabacum* L.: Functional characterization of the *CYP82E10* gene

Ramsey S. Lewis, Steven W. Bowen, Matthew R. Keogh [1], Ralph E. Dewey *

Development of Tobacco Varieties Possessing the Triple Mutant (*Zyvert™*) Low NNN Trait

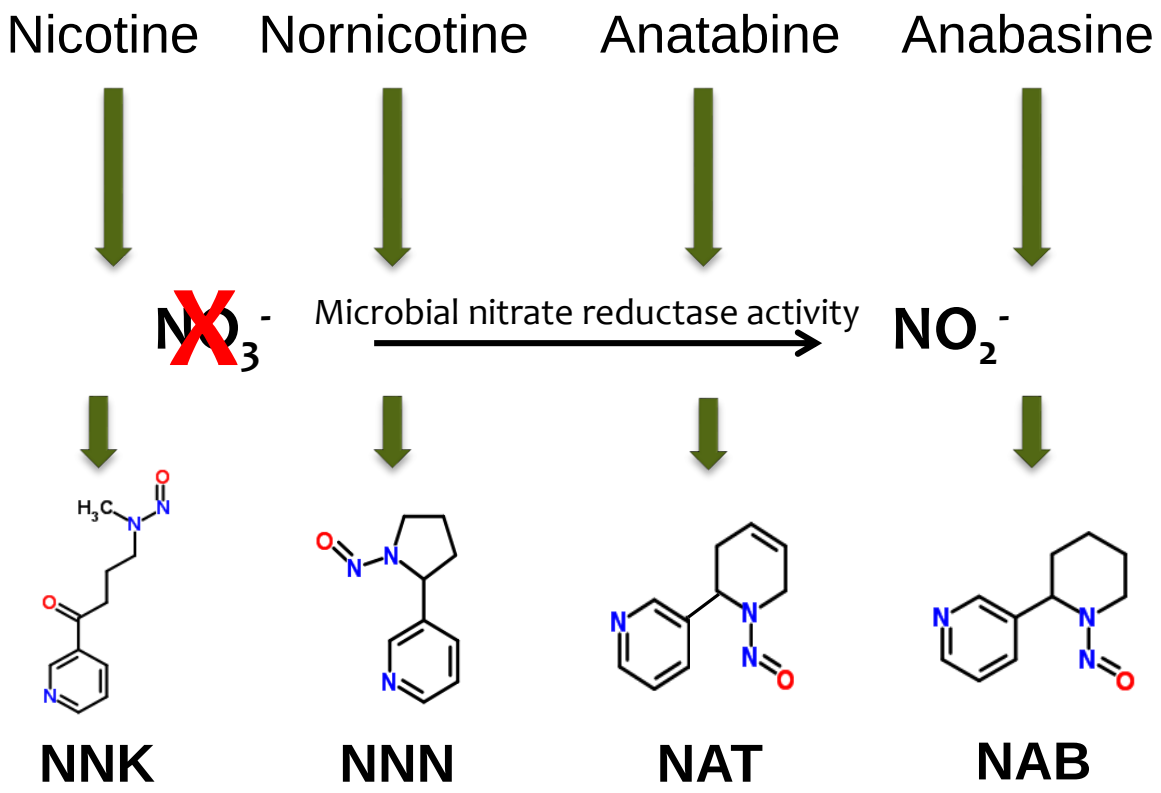
Varieties developed at NCSU and field tested to date:

Burley	Dark	Flue-Cured
TN 86	Ky 171	K 326
NC 7	Ky 160	K 346
Ky 14 x L8	VA 359	NC 196
TN 90	Narrow Leaf Madole	NC 71
NC BH129	Little Crittenden	NC 297
NC 2000	VA 309	Speight 168
NC 2002		NC 55
NC 3		
NC 4		
NC 5		
NC 6		
Banket A1		
Burley 21		

Zyvert™ technology represents a very effective means for greatly reducing the levels of NNN in any air-cured tobacco

Targeting the Nitrosating Agent

In Air-Cured Tobaccos the Predominant Nitrosating Agent is Nitrite (NO_2^-) Produced from Endogenous Leaf Nitrate (NO_3^-) by Leaf Surface Microbes



Targeting the Nitrosating Agent

Strategy #1

- Expression of a deregulated tobacco nitrate reductase (NR) enzyme that continually depletes the endogenous NO_3^- pools of the leaf (S523D-NR mutant)

Strategy #2

- Down-regulation of a putative nitrate transporter gene

Deregulated NR Activity

Both Burley tobaccos and most Dark varieties accumulate large amounts of free nitrate. We speculated that depleting these stores through expression of a mutant, deregulated version of the nitrate reductase gene (S523D-NR) would lead to reductions in all classes of TSNAs

Plant Biotechnology Journal

Plant Biotechnology Journal (2016) **14**, pp. 1500–1510 doi: 10.1111/pbi.12510

Expression of a constitutively active nitrate reductase variant in tobacco reduces tobacco-specific nitrosamine accumulation in cured leaves and cigarette smoke

Jianli Lu[1], Leichen Zhang[1], Ramsey S. Lewis[1], Lucien Bovet[2], Simon Goepfert[2], Anne M. Jack[3], James D. Crutchfield[4], Huihua Ji[3] and Ralph E. Dewey[1,*]

Cured Leaf Alkaloid and TSNA Content (upper leaf position)

A nearly 90% reduction in NNN observed in the cured leaf (upper position)

	WT	35S:GOGAT	35S:S523D-NR
Total TSNA (ng/g)	709	649	162
	A	A	B
NNN (ng/g)	287	288	31
	A	A	B
NAT (ng/g)	293	257	83
	A	A	B
NAB (ng/g)	17	14	0.3
	A	A	B
NNK (ng/g)	111	91	48
	A	A	B
Total alkaloid (%)	2.6	2.8	2.6
	A	A	A
Nicotine (%)	2.5	2.6	2.4
	A	A	A
Nornicotine (%)	0.058	0.063	0.058
	A	A	A
Anabasine (%)	0.011	0.011	0.012
	A	A	A
Anatabine (%)	0.075	0.075	0.091
	A	A	A
Conversion (%)	2.4	2.6	2.5
	A	A	A

Means with the same letter are not significantly different at alpha=0.01. Alkaloid measurements represent % dry weight. Means are grouped according to the REGWG method, N=33.

A 55 – 90% reduction in each individual TSNA was observed in the 35S:S523D-NR plants; total TSNA reduction = 77.5%

No differences were observed in the alkaloid profiles

Down-Regulation of a Putative Nitrate Transporter Gene

Chloride Channel (CLC) Family of H^+-Coupled Antiporters

- Originally believed to function exclusively as Cl^- transporters
- Certain homologues were later shown to serve as NO_3^-/H^+ antiporters that function to transport and sequester nitrate into the vacuole (e.g. AtCLC-a)

Hypothesis: *If one could identify a tobacco gene responsible for transporting nitrate into the vacuole and inhibit its expression, the levels of free nitrate could be reduced, along with TSNA formation*

Down-Regulation of a Putative Nitrate Transporter Gene

Tobacco *CLC-Nt2* Genes

- Two closely related isoforms (*CLC-Nt2_S* and *CLC-Nt2_T*) that encode proteins of 786 aa in size (98% identical)
- Share 77% identity to the Arabidopsis AtCLC-a nitrate transporter
- An RNAi construct was generated to determine the effect of suppressing *CLC-Nt2* expression on leaf nitrate and TSNA formation in Burley tobaccos

Down-Regulating *CLC-Nt2* Lowers Leaf Nitrate Accumulation in Mature Leaves (2015 Field Results)

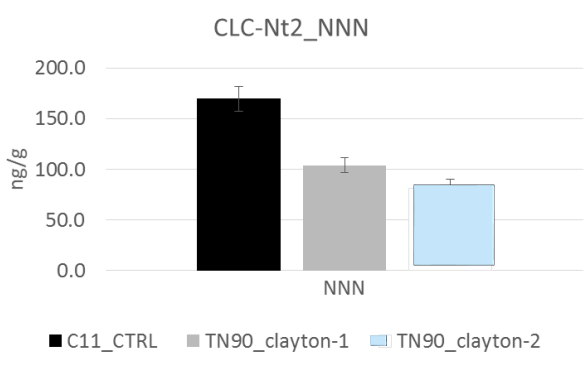
Effects of RNAi lines on NO3N content (ppm) in mature leaves of Burley tobacco line TN90 grown in Clayton, NC

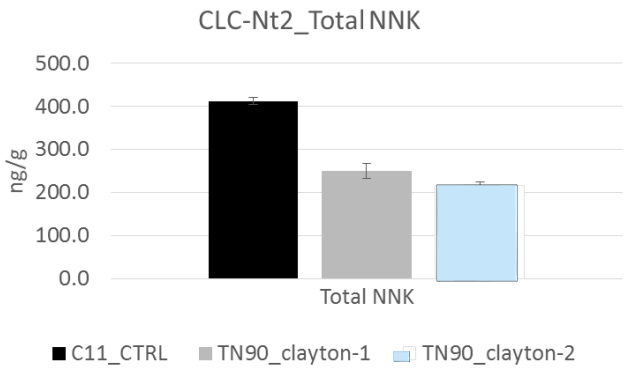
REGWQ Grouping	Means ± Standard Errors	No. of Plants	Line Name
A	1662 ± 197	48	TN90-WT
A	1417 ± 213	49	Anti-CLC-Nt2-4
A	1103 ± 131	48	Anti-CLC-Nt2-3
B	485 ± 55	48	Anti-CLC-Nt2-5
BC	379 ± 54	47	Anti-CLC-Nt2-2
C	281 ± 36	49	Anti-CLC-Nt2-1

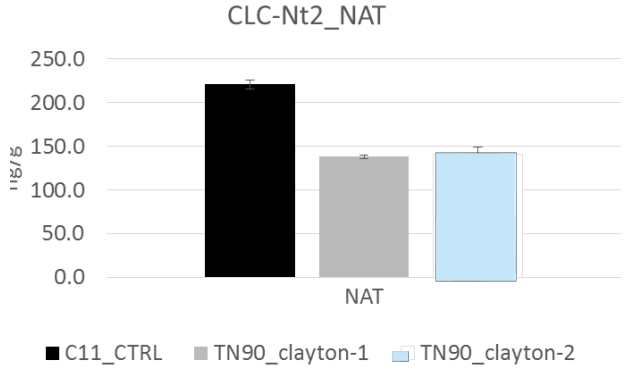
a. Means ± standard errors with the same letter are not significantly different at α=0.05.

Best transgenic lines displayed ~80% reduction in leaf nitrate

Down-Regulating *CLC-Nt2* Lowers TSNA Accumulation in Air-Cured Leaves (2015 Field Results)

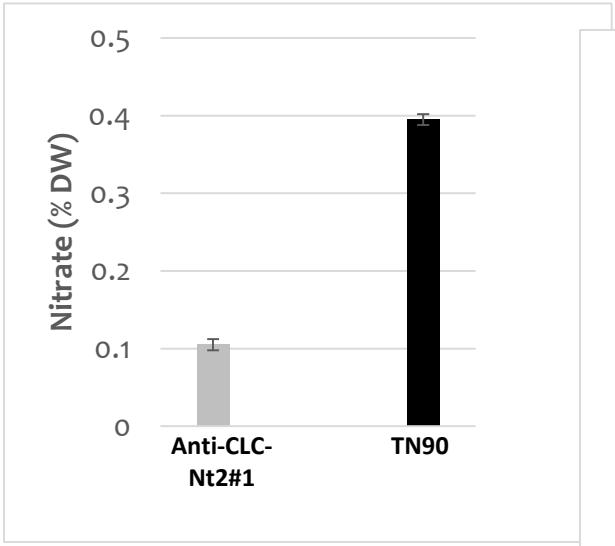




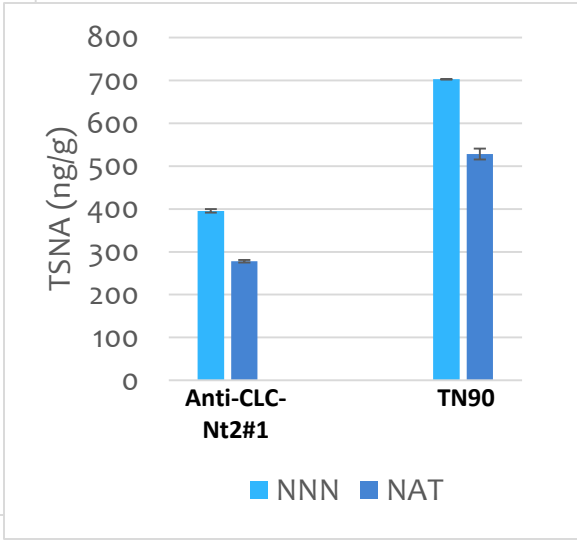


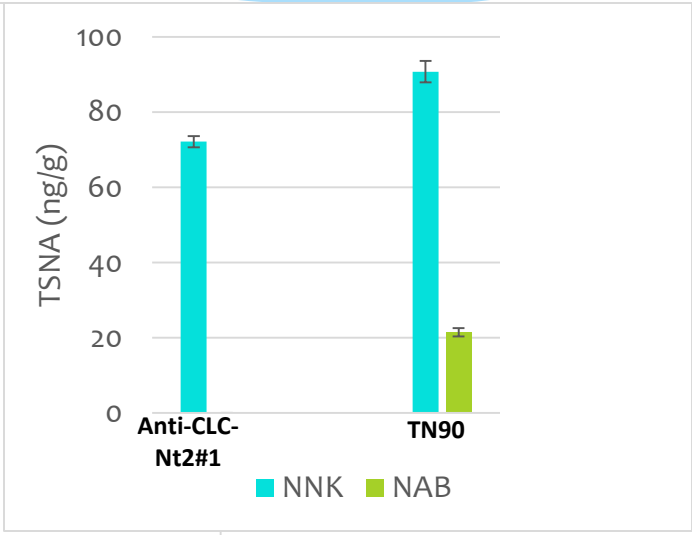
A 40 – 50% reduction was observed in the NNN, NNK and NAT content of the cured leaf

Down-Regulating *CLC-Nt2* Lowers Nitrate and TSNA Accumulation in the Lamina of Air-Cured Leaves (2016 Field Results)



73% reduction in nitrate





TSNA reductions:

- NNN = 44%
- NAT = 48%
- NNK = 21%

Summary of Genetics-Based Technologies for Reducing TSNAs

Mechanism	Harmful TSNA(s) targeted	Estimated timeline for development of efficacious varieties
Nicotine demethylase gene deactivation	NNN	Numerous already developed, including 6 Dark varieties suitable for smokeless products
Deregulated nitrate reductase activity (S523D-NR)	NNN and NNK	4 – 6 years*
Nitrate transporter (*CLC-Nt2*) deactivation	NNN and NNK	3 – 8 years**

*This strategy requires the use of either GM or non-transgenic "new breeding technologies"
**Shorter time estimate based on the use of non-transgenic "new breeding technologies"

The degree to which the use of genetics-based approaches can help in achieving compliance with the proposed 1 μg/g NNN limit in finished smokeless products has yet to be determined