Development of Tobacco Lines with Ultra-Low Levels of Nornicotine

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Funding by Philip Morris USA and Philip Morris International

Strategy for Reducing NNN Production

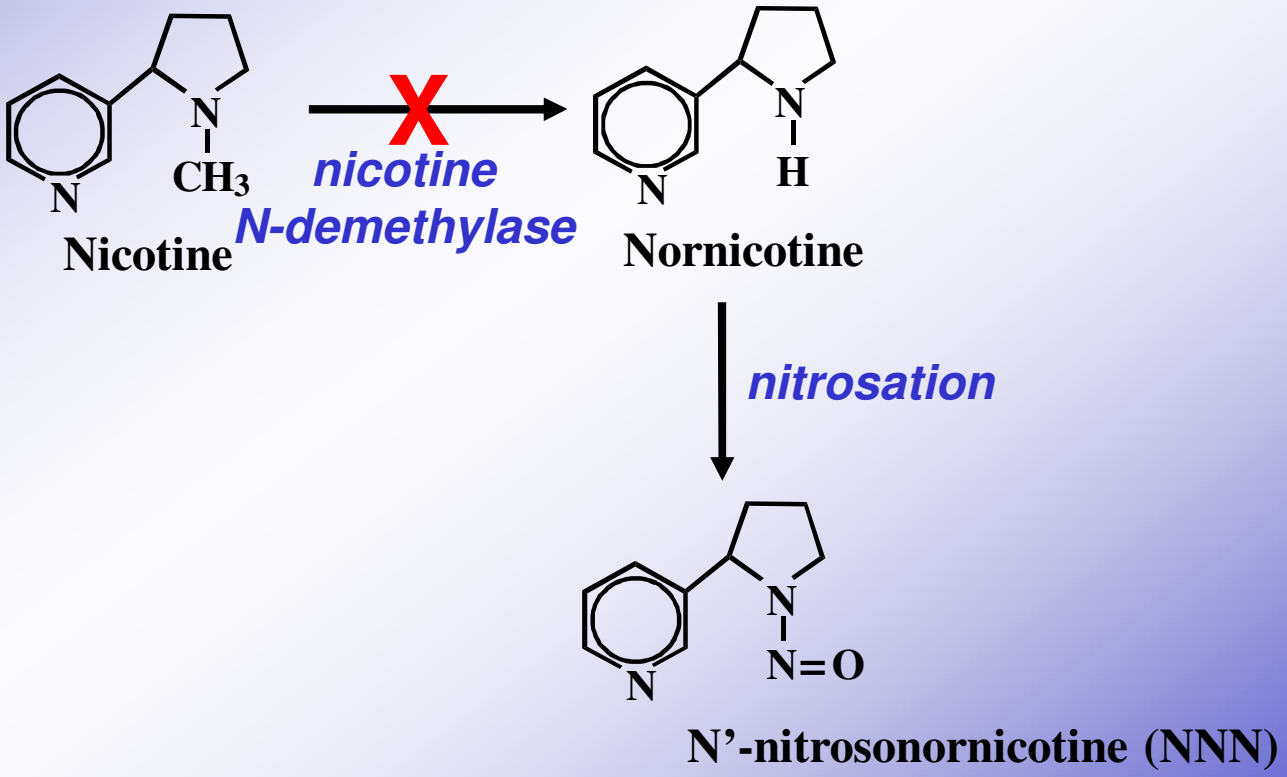


Table 1 Toxicants recommended for mandated lowering by WHO

Toxicant	Level in µg/mg nicotine	Criteria for selecting the value
NNK	0.072	Median value of data set
NNN	0.114	Median value of data set
Acetaldehyde	860	125% of the median value of data set
Acrolein	83	125% of the median value of data set
Benzene	48	125% of the median value of data set
Benzo[a]pyrene	0.011	125% of the median value of data set
1,3-Butadiene	67	125% of the median value of data set
Carbon monoxide	18,400	125% of the median value of data set
Formaldehyde	47	125% of the median value of data set

from Burns et al. 2008, Tobacco Control 17: 132-141

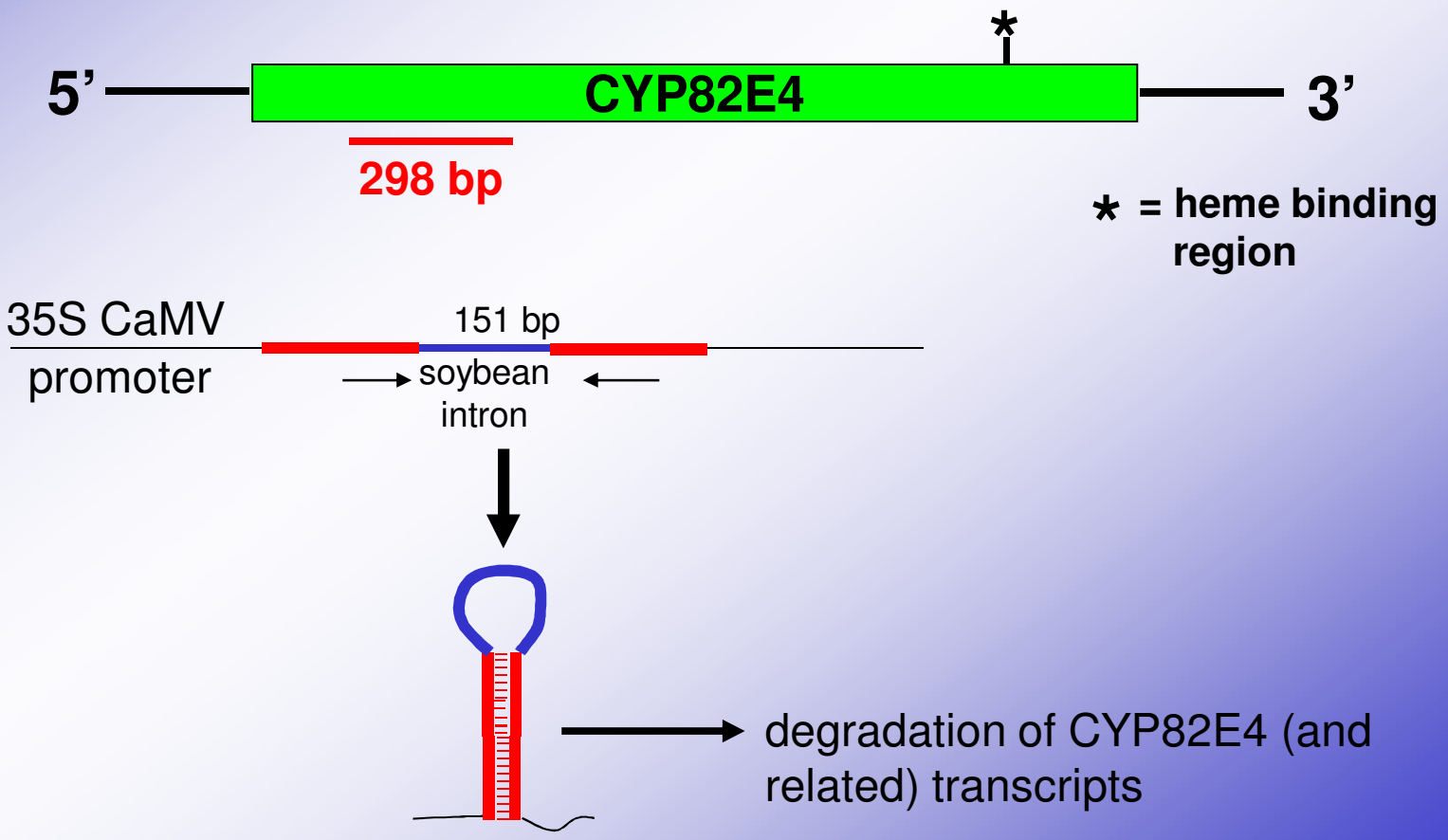
CYP82E4, the Major Nicotine Demethylase Gene of *Nicotiana tabacum*

- **Isolated using large scale gene expression profiling analyses (microarrays) of Converter versus Nonconverter plants**
- **A cytochrome P450 monooxygenase**
- **Member of a closely related gene family found only in *Nicotiana* species**
- **Gene expression is greatly enhanced during senescence, curing and ethylene treatment in Converter plants, but not in Nonconverters**

Siminszky et al., 2005. PNAS 102: 14919-14924
Xu et al. 2007. Physiol. Plantarum 129: 307-319

Transgene-Mediated Silencing of the CYP82E4 Gene (GM approach)

CYP82E4 RNAi Construct



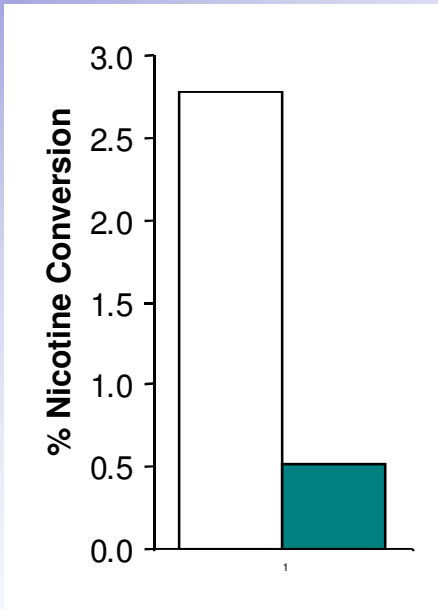
Field Analysis of Transgenic Plants

- Large scale field trail to test the effectiveness of the CYP82E4-RNAi construct was conducted in 2006.
- Plots were located in North Carolina, Kentucky and Virginia

Transgenic Tobacco Plants Produce Significantly Less Nornicotine and NNN than Conventional Cultivars

Three DH98-325-5 control seedlots

Three DH98-325-5 CYP82E4 RNAi lines



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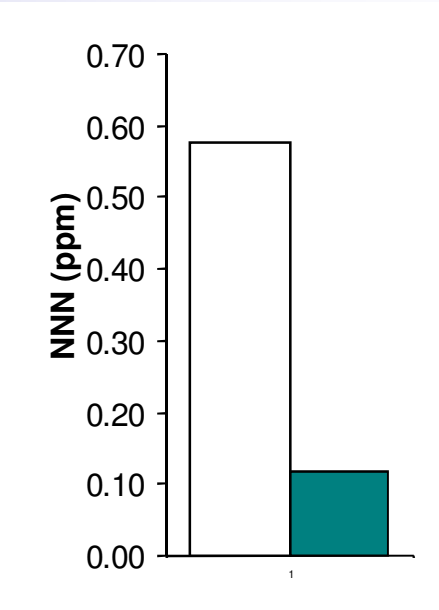

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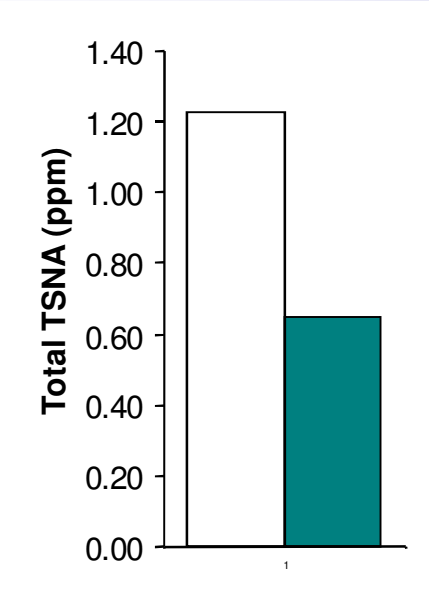


P = 0.0012

The RNAi transgenic plant data showed that reducing conversion from ~2.8% to ~0.5% reduced NNN levels by up to 80% and cut total TSNA levels in half. ***Lewis et al. 2008. Plant Biotech. J. 6: 346-354***

Need for a Non-GM Alternative

- The major tobacco companies refuse to use GM tobacco varieties in their products (potential for rejection by a subset of users philosophically opposed to GMOs)
- Multiple licensing agreements must be negotiated (and paid for) in order to commercialize a GM variety
- Time and costs for deregulation of a transgenic event can be very high

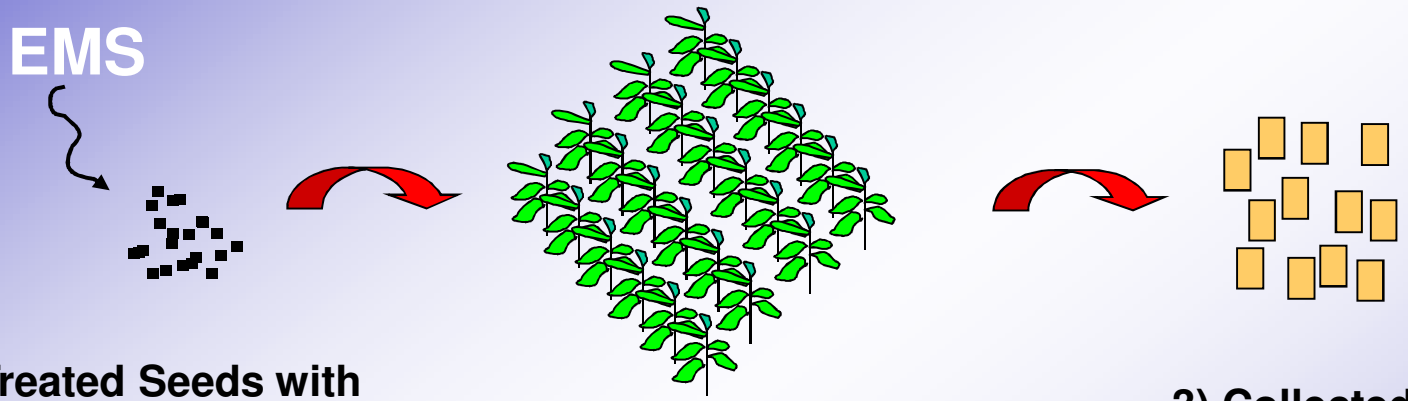
How Many Genes are Involved in Nicotine Demethylation?

Members of CYP82E Gene Family that Display Nicotine Demethylase Activity

- CYP82E2 - **NO** (yeast and plant data)
- CYP82E3 - **NO** (yeast and plant data)
- CYP82E4 - **YES** (yeast and plant data)
- 131A_A02 - **NO** (sequence data = pseudogene)
- 3D_C12-15 - **NO** (sequence data = pseudogene)
- 58-166 - **NO** (yeast data, lack of documented expression)
- CYP82E5 – **YES** (yeast and plant data)

Even more family members exist in the tobacco genome, but are likely to be additional pseudogenes

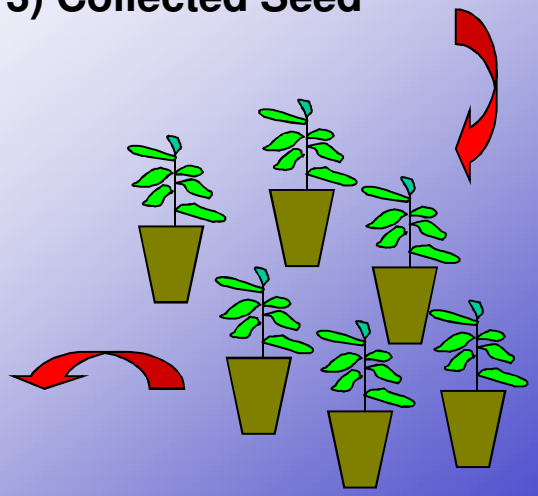
Developing Non-GMO Varieties with Reduced Nornicotine



1) Treated Seeds with Chemical Mutagen

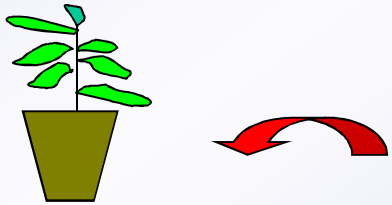
2) Grew ~ 4000 M_1 Plants in Field

3) Collected Seed



4) Isolated DNA from M_2 Plants

5) High-throughput PCR Amplification and Sequencing of *CYP82E4* and *CYP82E5* Genes from Hundreds of M_2 Plants



6) Identified Plants with Mutated *CYP82E4* and *CYP82E5v2* Genes

Recovery of *CYP82E4* and *CYP82E5* Mutant Tobacco Plants

CYP82E4

- # of M_1 Plants Screened: 672
- # of *CYP82E4* Mutations Identified: 11
- Most notable mutant:

Plant	Amino Acid Changed	Effect on Gene Function
#775	Trp (329) to Stop	completely inactive

CYP82E5

- # of M_1 Plants Screened: 768
- # of *CYP82E4* Mutations Identified: 12
- Most notable mutant:

Plant	Amino Acid Changed	Effect on Gene Function
#1013	Trp (422) to Stop	completely inactive

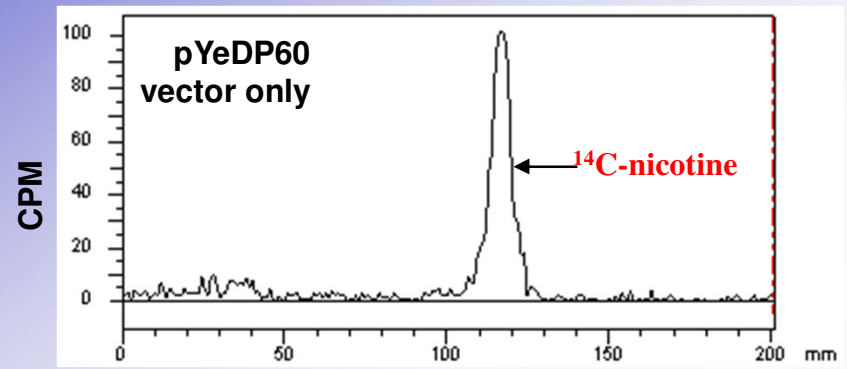
Alkaloid profiles for materials evaluated in 2008 field experiment

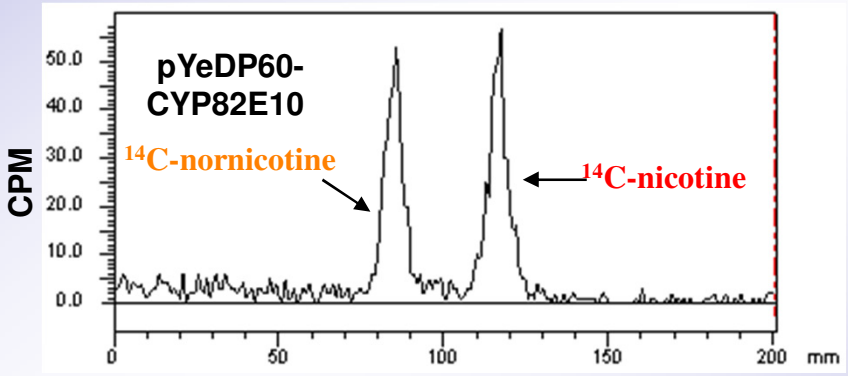
Genotype	Gene targeted	Amino Acid Mutation	Average % Nicotine	Average % Nornicotine	Average % Conversion[d]
DH98-325-6 control (15)[a]	Control		1.228	2.014	**62.4**
TN90LC (14)	Control		4.680	0.157	**3.2**
DH98-325-6 RNAi 300-02 #1 (15)	*CYP82E4 and related*		3.741	0.026	**0.7**
DH98-325-6 #775 Homo. (15)	*CYP82E4*	W329Stop	2.941	0.077	**2.6**
DH98-325-6 #1013 Homo. (14)	*CYP82E5*	W422Stop	1.005	1.876	**65.2**
DH98-325-6 Double Homozygous Mutant (9)	*CYP82E4* and *CYP82E5*	double	3.160	0.076	**2.3**

Conclusion: there must be at least one other nicotine demethylase gene with high sequence homology to *CYP82E4/E5*

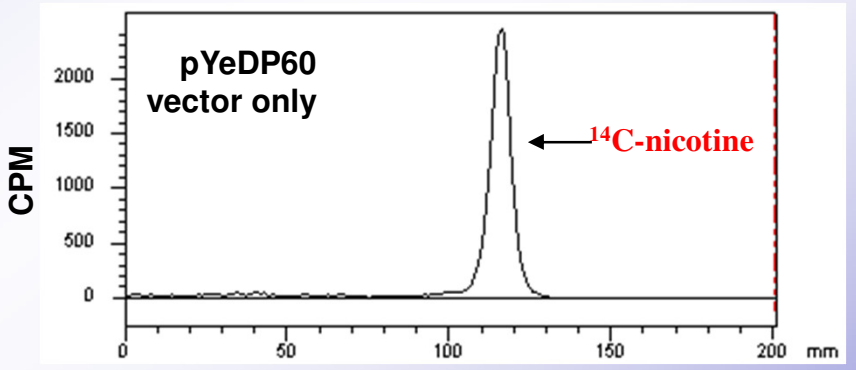
- A GenBank search of tobacco ESTs revealed a previously unidentified *CYP82E*-like gene from libraries generated against root tissue

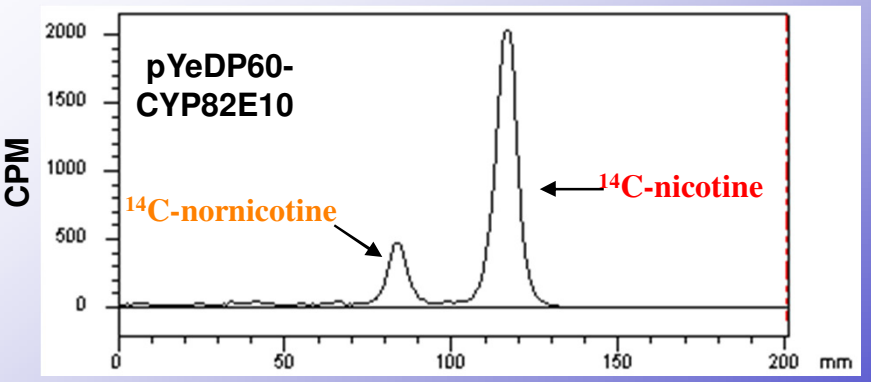
CYP82E10, a root-specific, minor nicotine demethylase gene





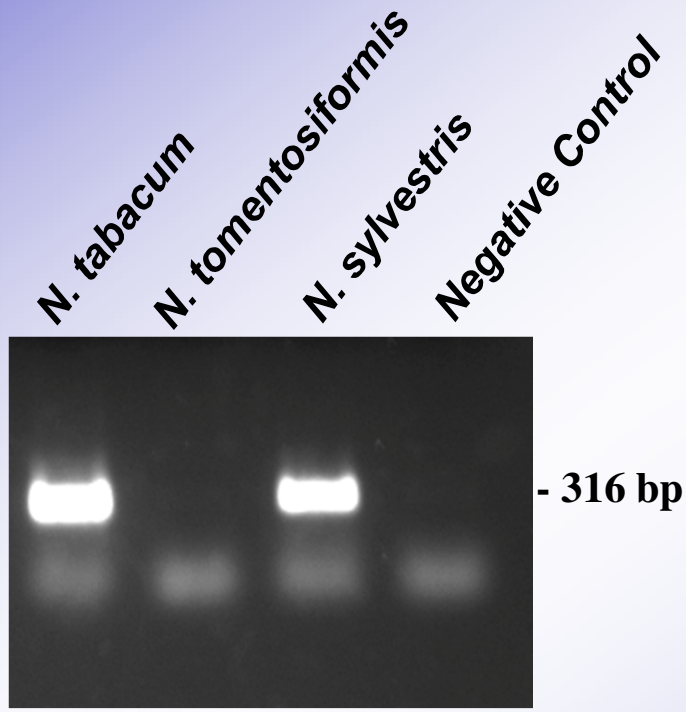
2.45 µM [^{14}C]-nicotine substrate





50 µM [^{14}C]-nicotine substrate

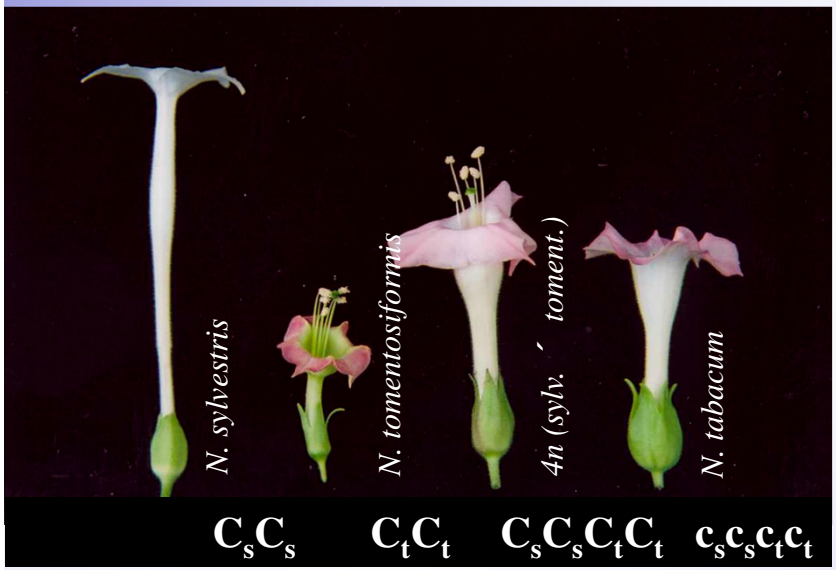
CYP82E10 Originated from the *N. sylvestris* Ancestral Parent of Tobacco

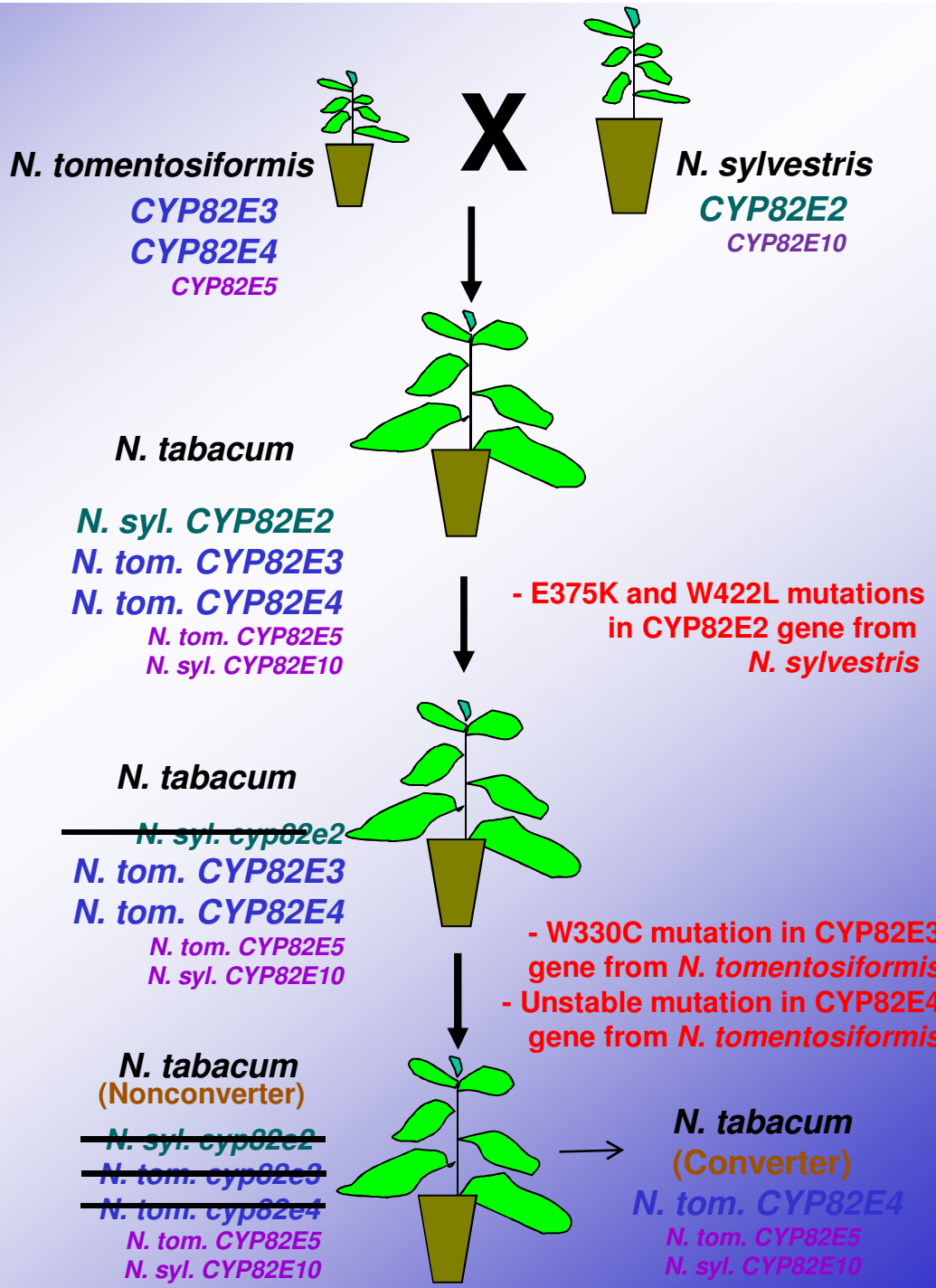


CYP82E10 Gene of *N. sylvestris*

- Exon 1: three polymorphisms, one of which leads to an amino acid substitution (Thr15Ala)
- Intron: 10 polymorphisms (mostly short indels)
- Exon 2: no polymorphisms

Molecular Evolution of the Nicotine Demethylase Gene Family





CYP82E10 Mutation Screen

EMS Treated Lines of DH98-325-6 with Mutations in the *CYP82E10* Gene

Plant Number	Mutation[a]	Amino Acid Change	Activity of Mutant Enzyme[b]
2476	G235A	G79S	not detected
1512	C319T	P107S	not detected
319	C442T	L148F	not tested
634	G514A	G172R	not tested
1035	G1030A	A344T	100%
1041	**C1141T**	**P382S**	**not detected**
817	G1228A	A410T	100%
693	G1250A	R417H	100%
1442	C1255T	P419S	25%

[a]In reference to the start codon of the CYP82E10 cDNA sequence

[b]Relative to the wild type enzyme when expressed in yeast

Nicotine demethylase activity of CYP82E4 and CYP82E10 enzymes possessing the 1041 mutation (Pro382Ser)

Vector	CPM nornicotine at 2.45 µM [^{14}C]-nicotine substrate[a]	CPM nornicotine at 50 µM [^{14}C]-nicotine substrate
pYeDP60-CYP82E4	1,813 ± 623[b]	5,383 ± 505
pYeDP60-CYP82E4/1041	not detected	not detected
pYeDP60-CYP82E10	2,296 ± 99	15,253 ± 465
pYeDP60-CYP82E10/1041	not detected	not detected

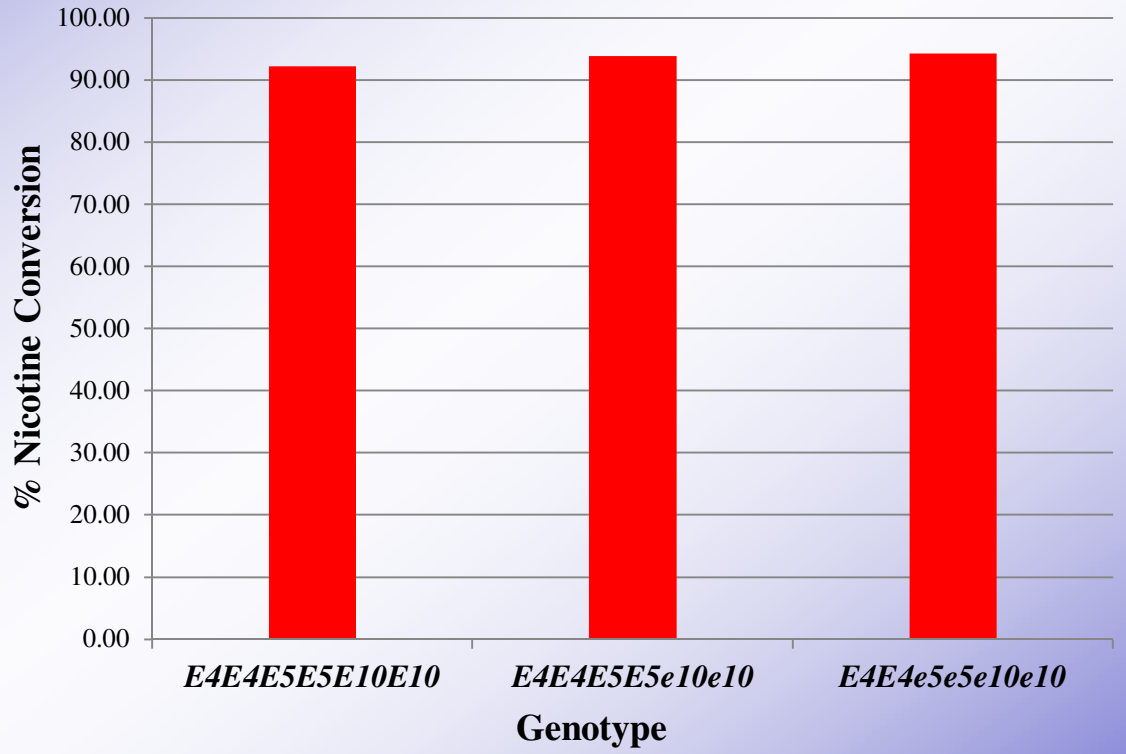
[a]counts per minute of [14C]-nornicotine/mg microsomal protein

[b]standard deviation of two technical replications

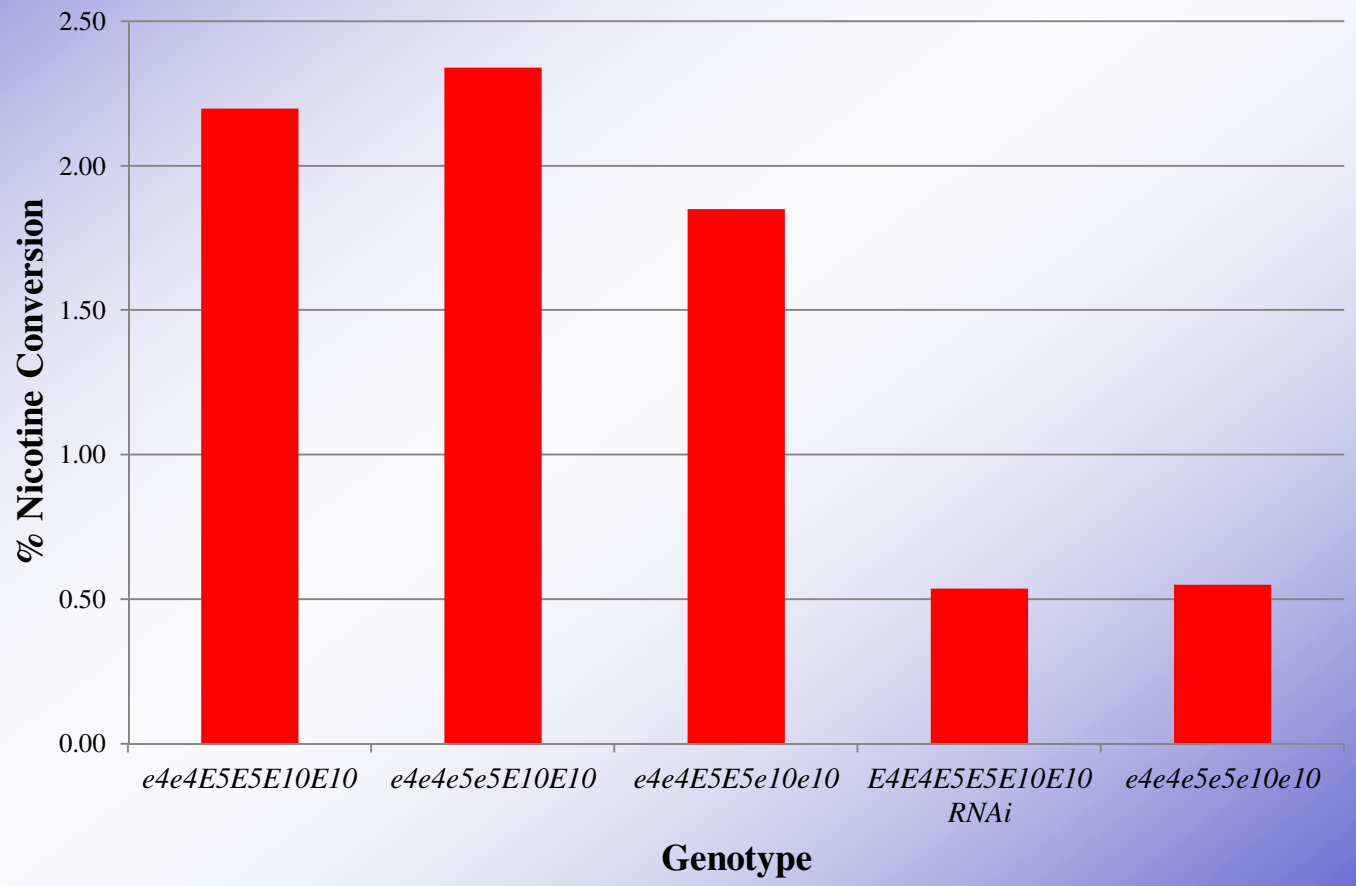
Alkaloid profiles for materials evaluated in 2009 field experiment (35 days after transplant)

Genotype	Gene(s) targeted	Amino Acid Mutation	Average % Nicotine	Average % Nornicotine	Average % Conversion
DH98-325-6 control (8)	Control	-	0.133	1.553	**92.21**
TN90LC (11)	Control	-	1.519	0.104	**7.15**
DH98-325-6 RNAi 300-02 #1 (10)	*CYP82E4* and related	-	1.747	0.009	**0.54**
DH98-325-6 #775 Homo. (10)	*CYP82E4*	W329Stop	1.375	0.030	**2.20**
DH98-325-6 Double Homo. Mutant (11)	*CYP82E4 + CYP82E5*	double	1.524	0.036	**2.34**
DH98-325-6 #1041 Homo. (3)	*CYP82E10*	P382S	0.082	1.302	**93.87**
DH98-325-6 Double Homo. Mutant (5)	*CYP82E5 + CYP82E10*	double	0.081	1.345	**94.31**
DH98-325-6 Double Homo. Mutant (4)	*CYP82E4 + CYP82E10*	double	2.168	0.045	**1.85**
DH98-325-6 Triple Homo. Mutant (5)	*CYP82E4 + CYP82E5 + CYP82E10*	triple	1.793	0.012	**0.55**

Mutations in *CYP82E5* and *CYP82E10* have no effect in a Converter background with an activated *CYP82E4* allele



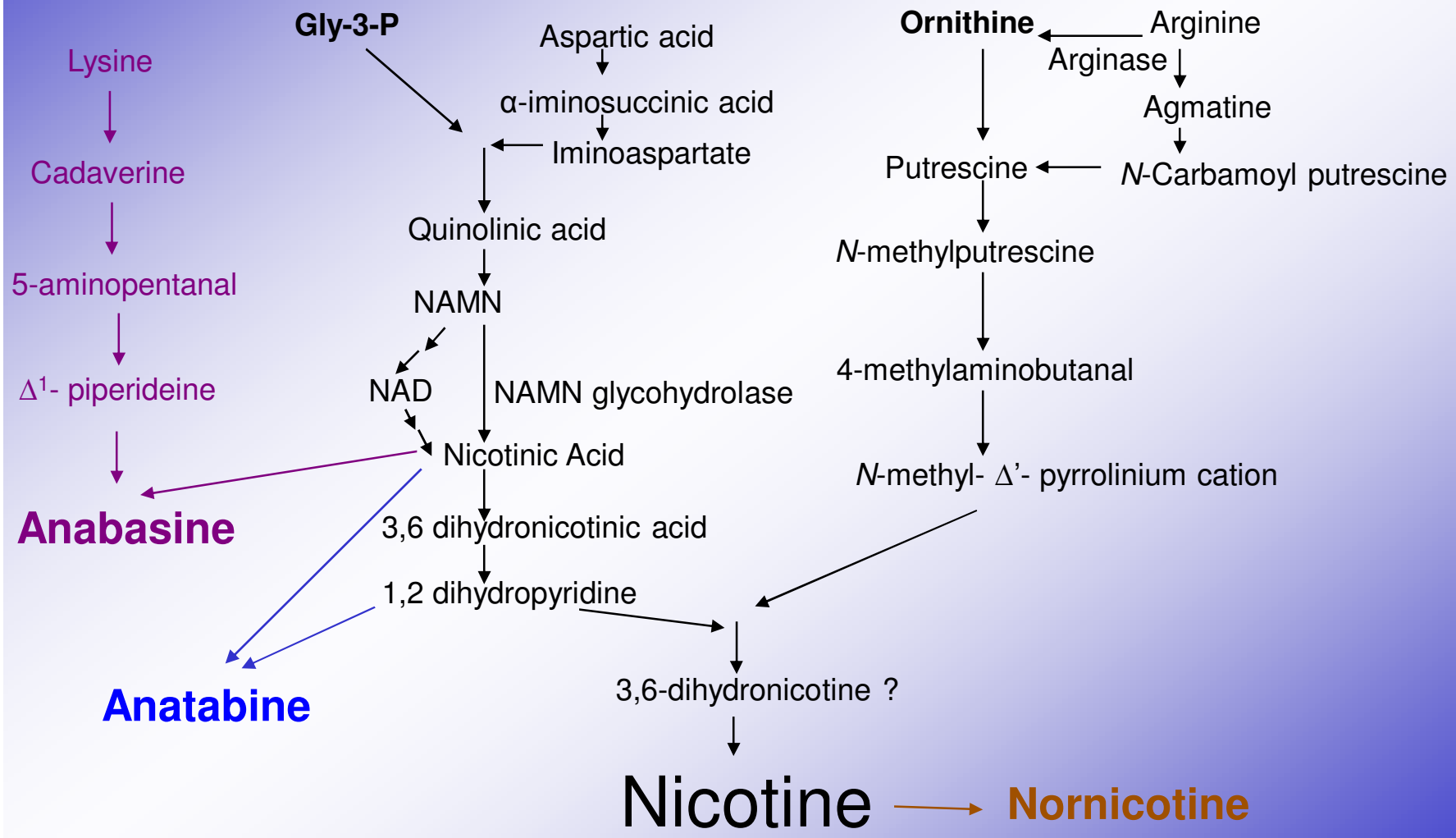
Triple mutant combination reduces nornicotine to the same level as our best RNAi transgenic line



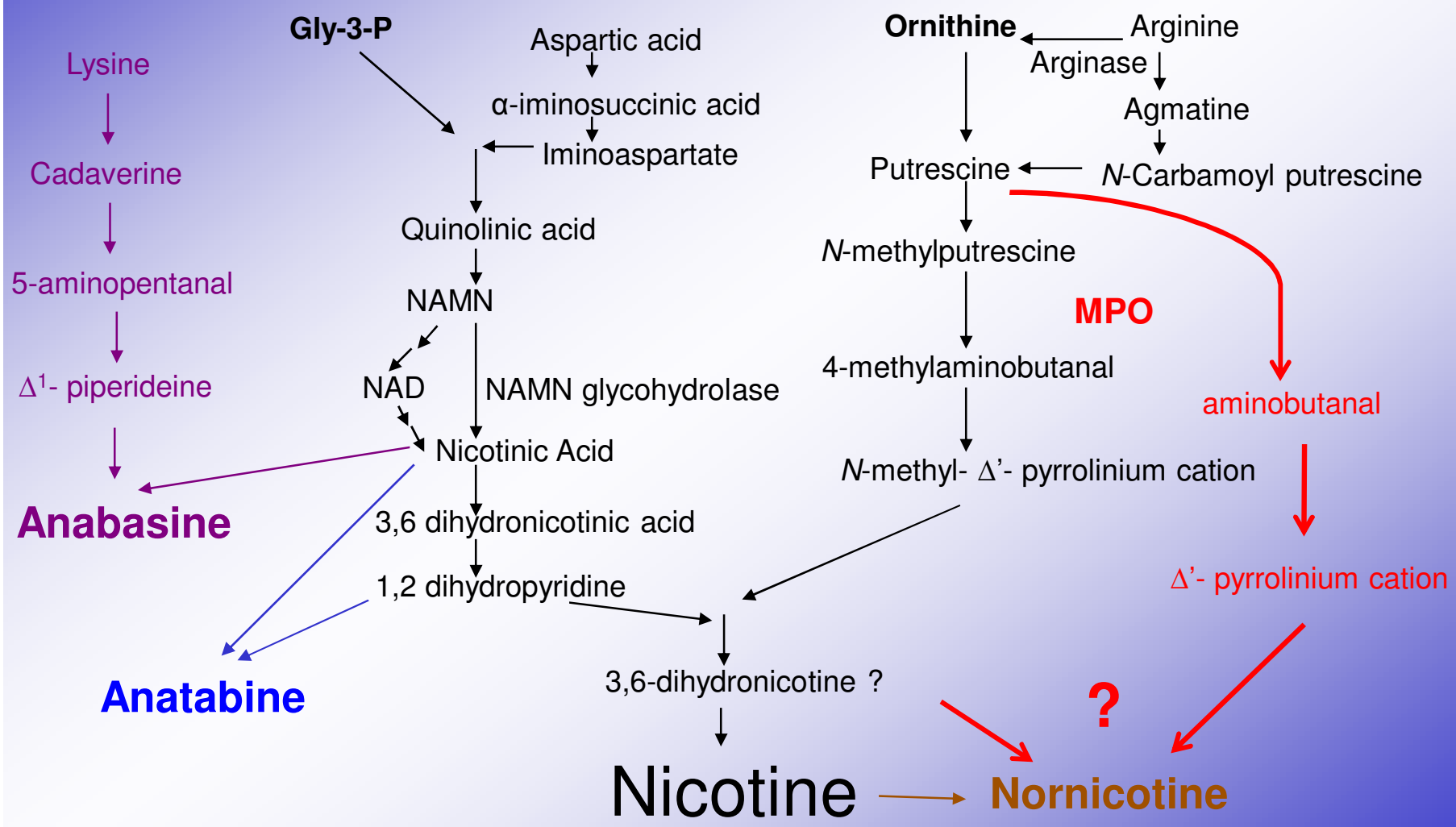
Why is There Any Nornicotine Produced in the Transgenic or Triple Mutant Plants?

- Non-specific oxidation by enzymes with broad substrate specificities (peroxidases, lipoxygenases, etc…)?
- Directly synthesized without going through a nicotine intermediate?

Alkaloid Biosynthesis in Tobacco



Alkaloid Biosynthesis in Tobacco



Conclusions and Directions

- Our biotechnology-based approach is an effective means for substantially reducing the levels of NNN, one of the strongest known carcinogens in tobacco products
- Have developed SNP markers for each mutant *CYP82E* allele and are working to breed the ultra-low nornicotine trait into > 40 of the most popular Burley, Flue-Cured and Dark commercial tobacco varieties
- These new varieties should very useful in helping the industry meet future FDA and WHO imposed TSNA standards