

# The Demethylase Mutants: Panacea or New Problems?

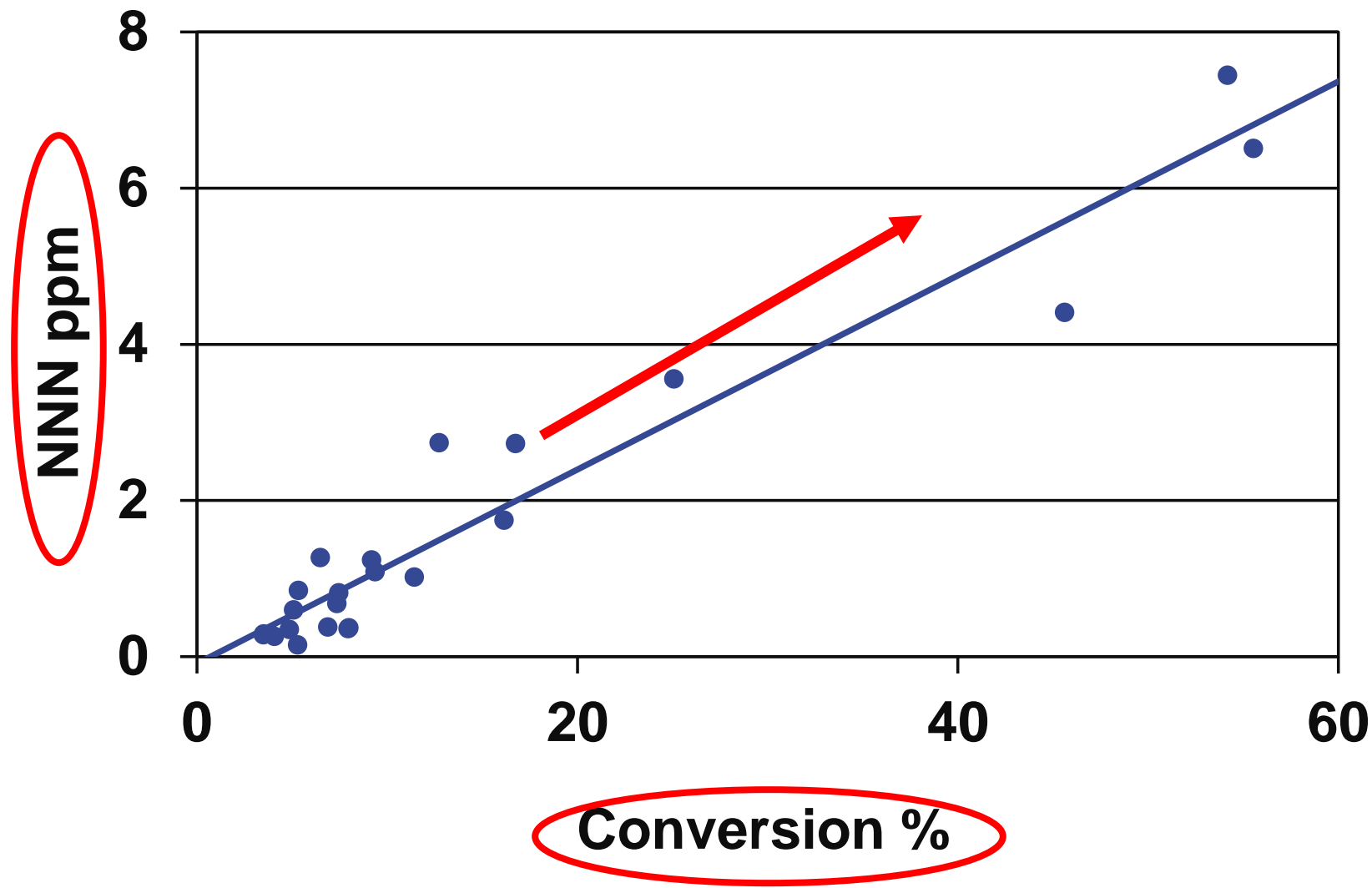
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Angela Schoergendorfer

University of Kentucky



TSRC Sept 2015

# NNN vs Conversion



# Conversion – Anomalies

- **Atypical trait**
- **Variable expression**
  - difficult to measure conversion potential
- **Unpredictable progeny**
- **Unstable, variation between**
  - seedlots
  - varieties
  - locations
- **Screen each seed increase**
- **Never eliminate all converters**



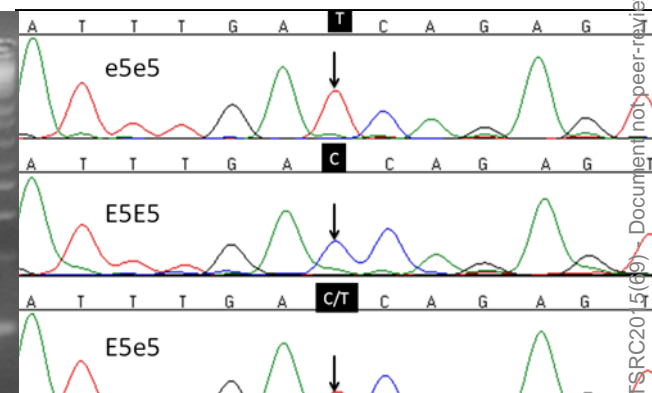
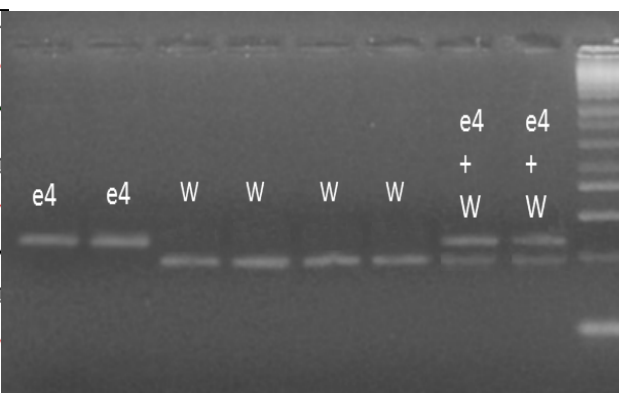
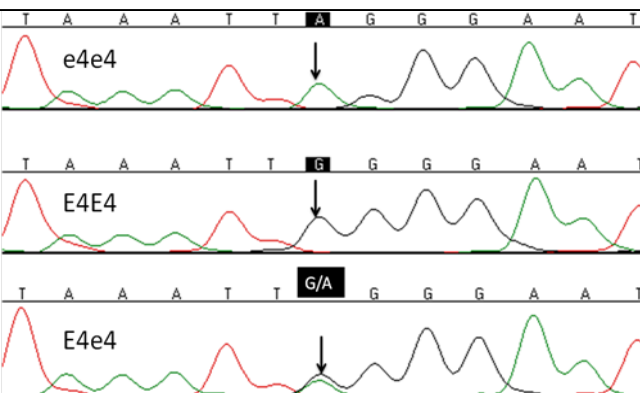
# Seed Screening

- Reducing nornicotine – seed screening
  - Very effective in reducing TSNA
- BUT
  - Expensive
  - Laborious
  - Must be done with each seed increase
  - Not perfect



# $e_4$ , $e_5$ , $e_{10}$ Mutants - Background

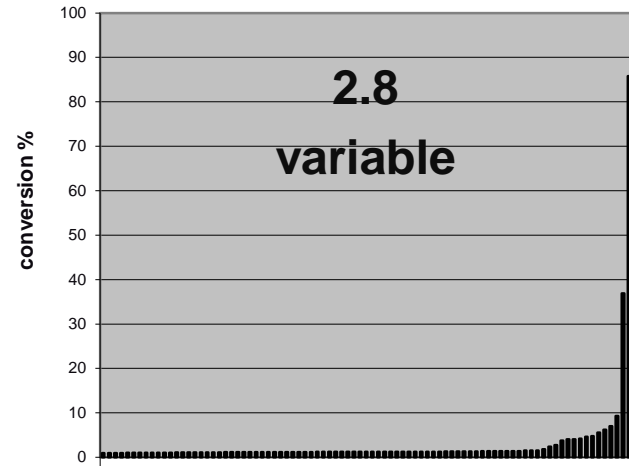
- **Demethylase mutants**
  - **Developed by NCSU**
  - $e_4$ ,  $e_5$ ,  $e_{10}$
  - $e_4/e_4, e_5$  mutants = best LC, but stable
  - **Triple  $e_4e_5e_{10}$  mutant stable, much lower**
    - **Three gene interaction**



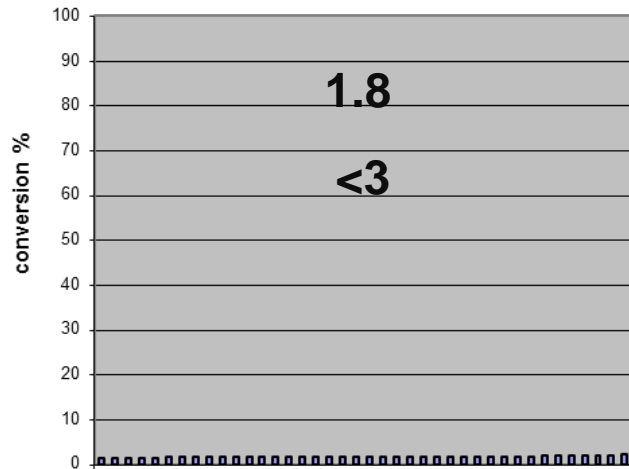
# Nicotine Conversion



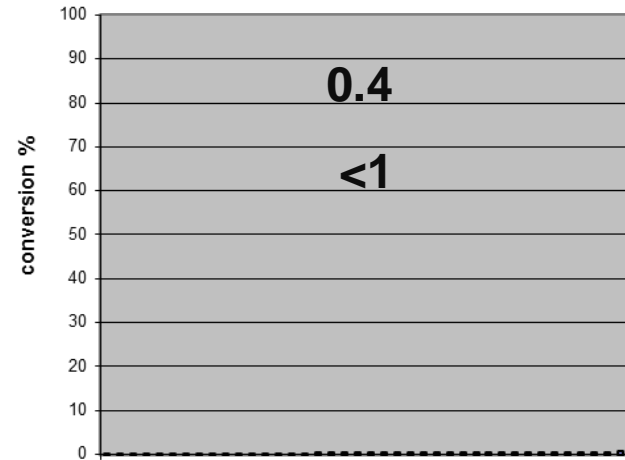
LC



e4

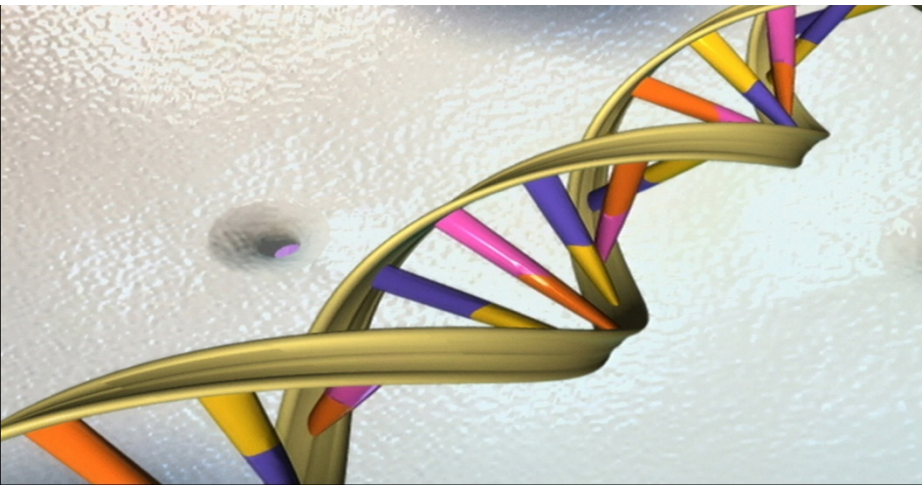


e4e5e10, transgenic



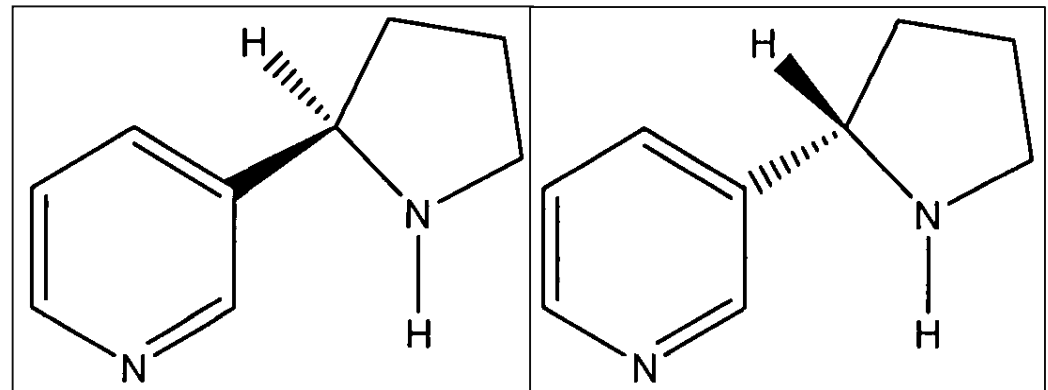
# Benefits of Mutants

- **Conversion reduced to levels not possible with seed screening**
  - NNN reduced ~ tenfold
- **Eliminates seed screening**
- **Cheaper – 7% of screening cost**
- **Do once**



# Possible Problems with Mutants

- **Nicotine & TA  $\uparrow$  as conversion  $\downarrow$** 
  - Nicotine in mutants may be too high
- **Stereo-isomer ratio different**
  - Ratio S-nornic & S-NNN  $\uparrow$  in triple mutant
- **Mutant genes may affect growth**

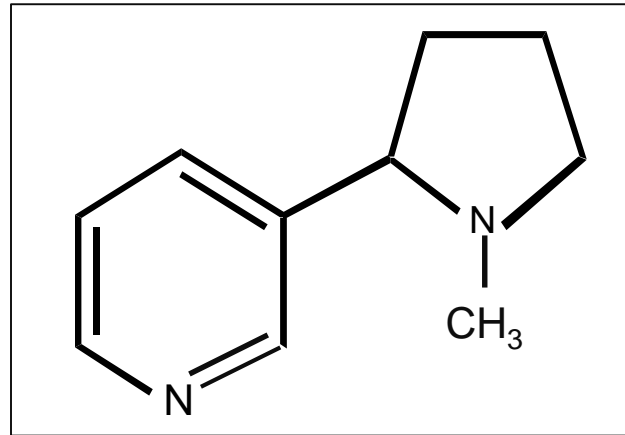




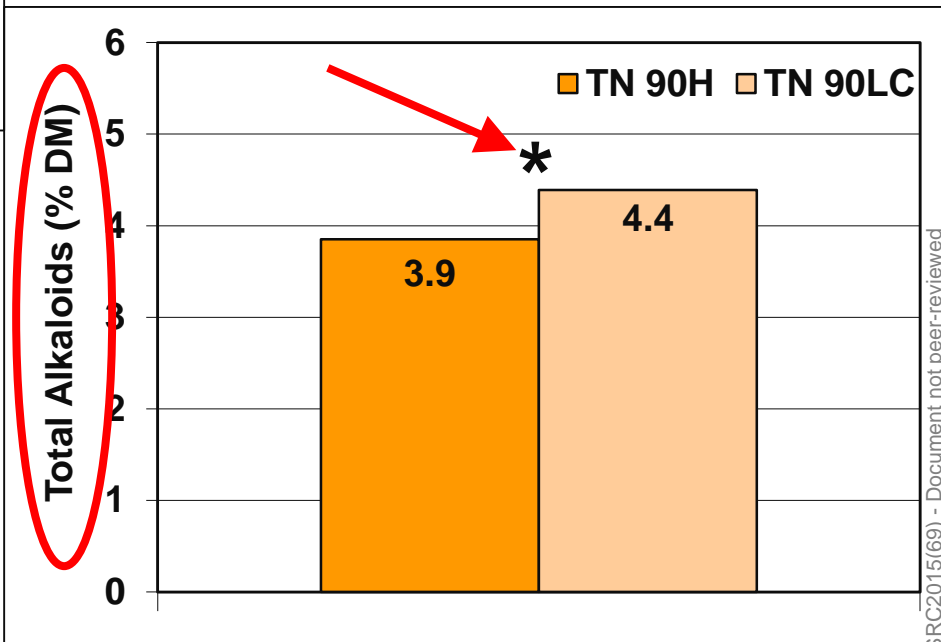
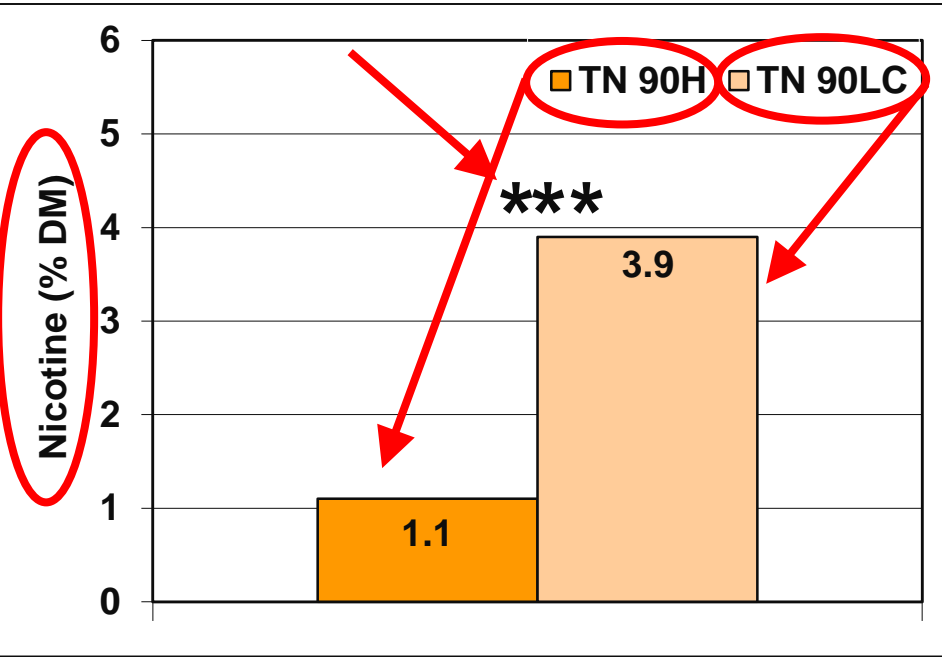
# 2013 Study – Mutants, LC, TG

- **Two varieties**
  - TN 86, TN 90
- **LC ( $E_4E_5E_{10}$ )**
  - Commercial variety
  - Low conversion, unstable
- **$e_4e_5$** 
  - Double mutant; E4 major demethylase
  - Low conversion, stable
- **$e_4e_5e_{10}$** 
  - Triple mutant, 3-gene interaction
  - Ultra low conversion, stable
- **Transgenic**
  - Targets demethylase gene family
  - Ultra-low conversion similar to triple mutant

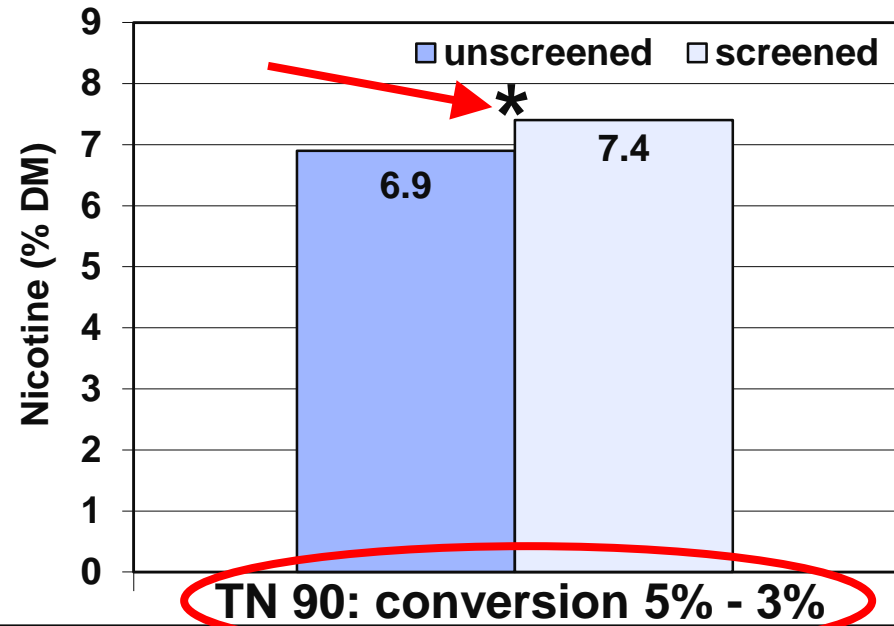
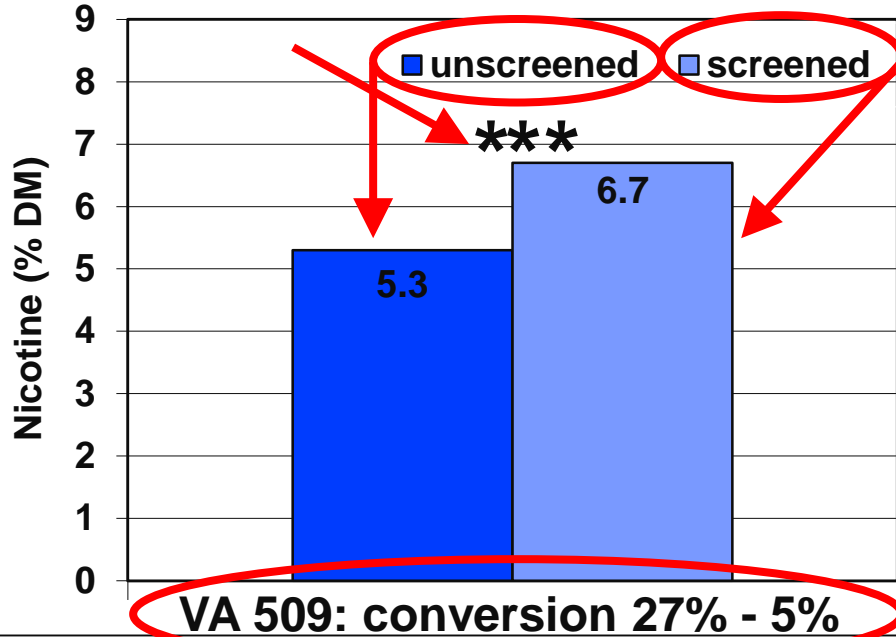
# Nicotine Levels in the Demethylase Mutants



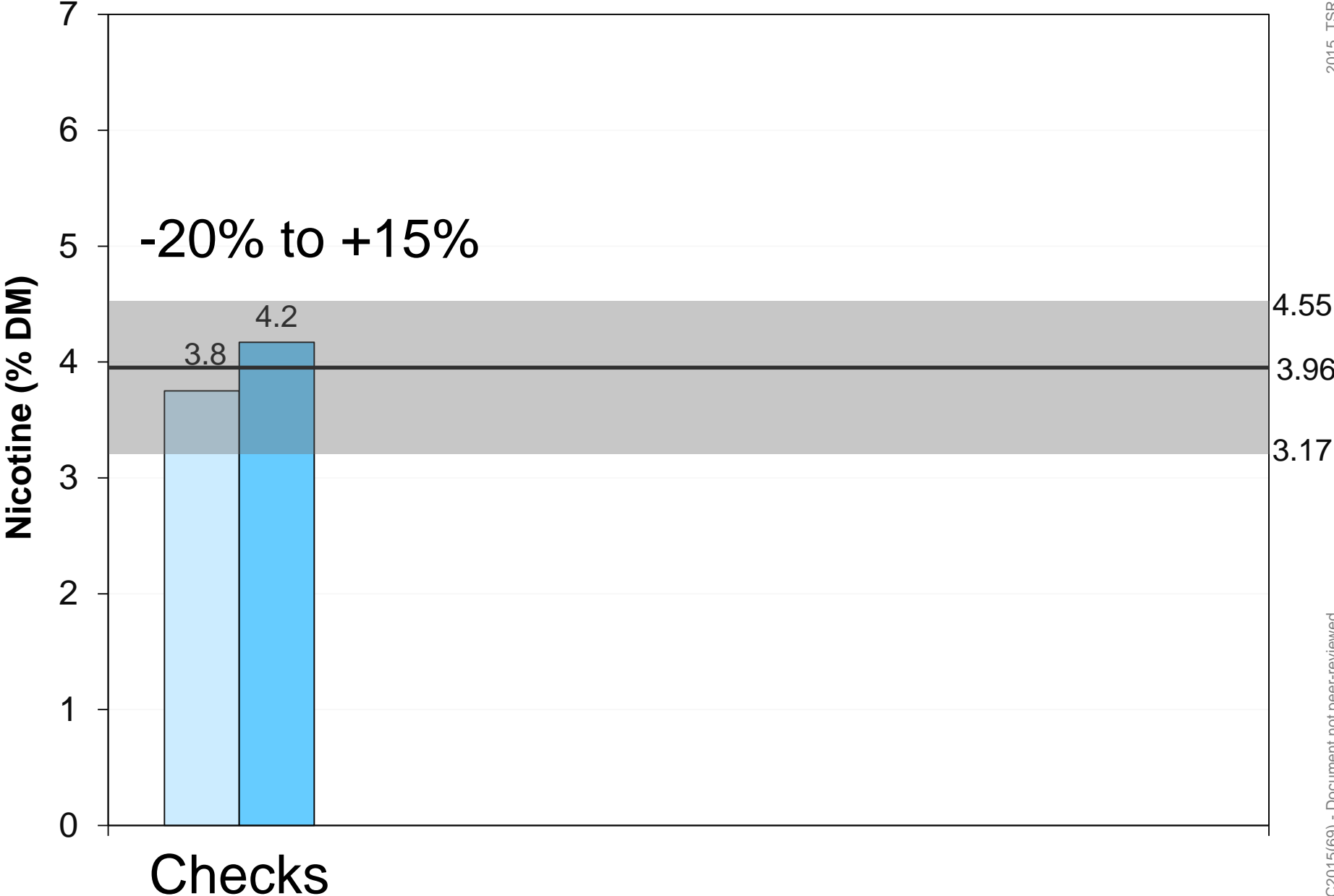
# LC vs. HC: Nicotine, Total Alkaloids



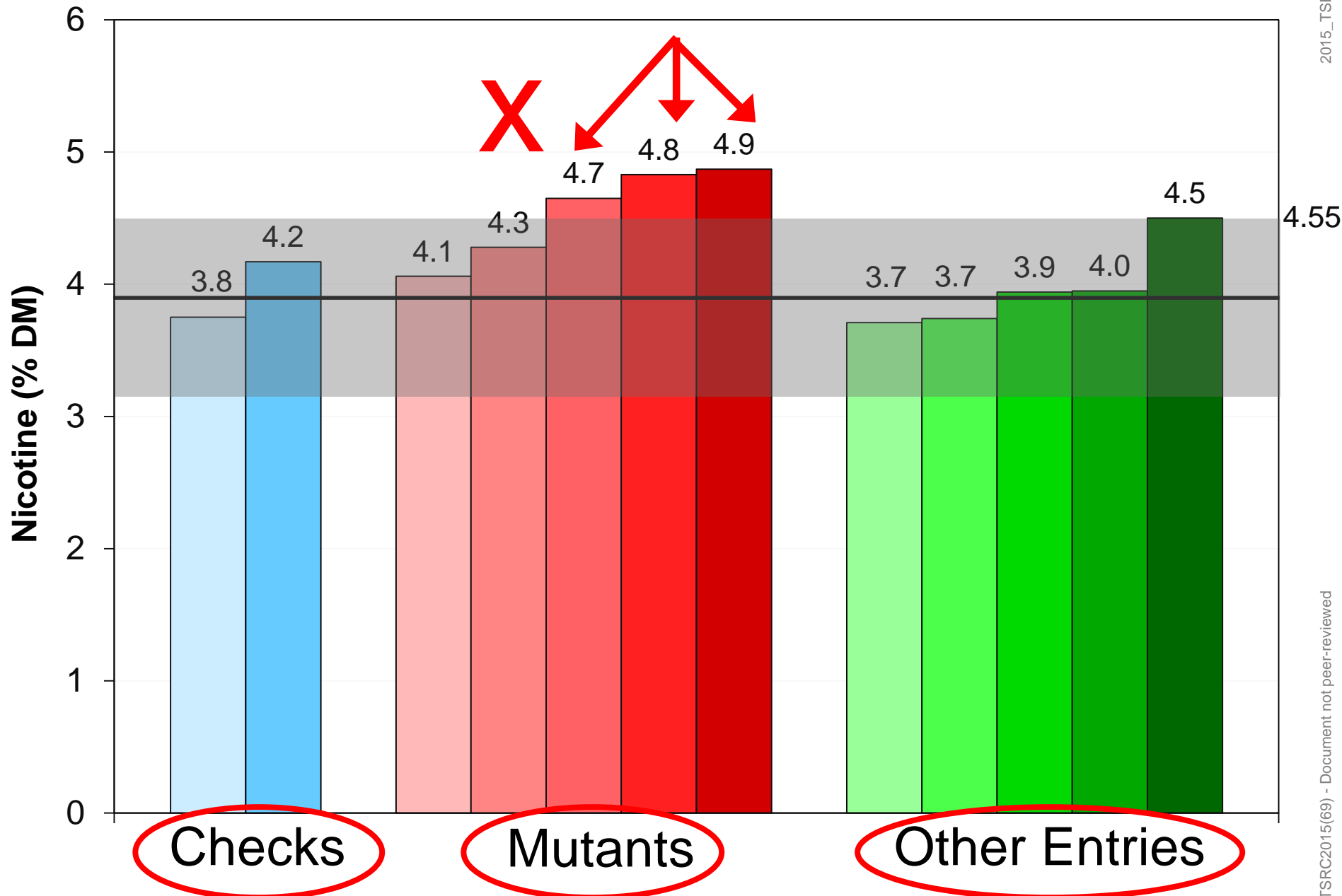
# Unscreened vs. Screened: 2 varieties



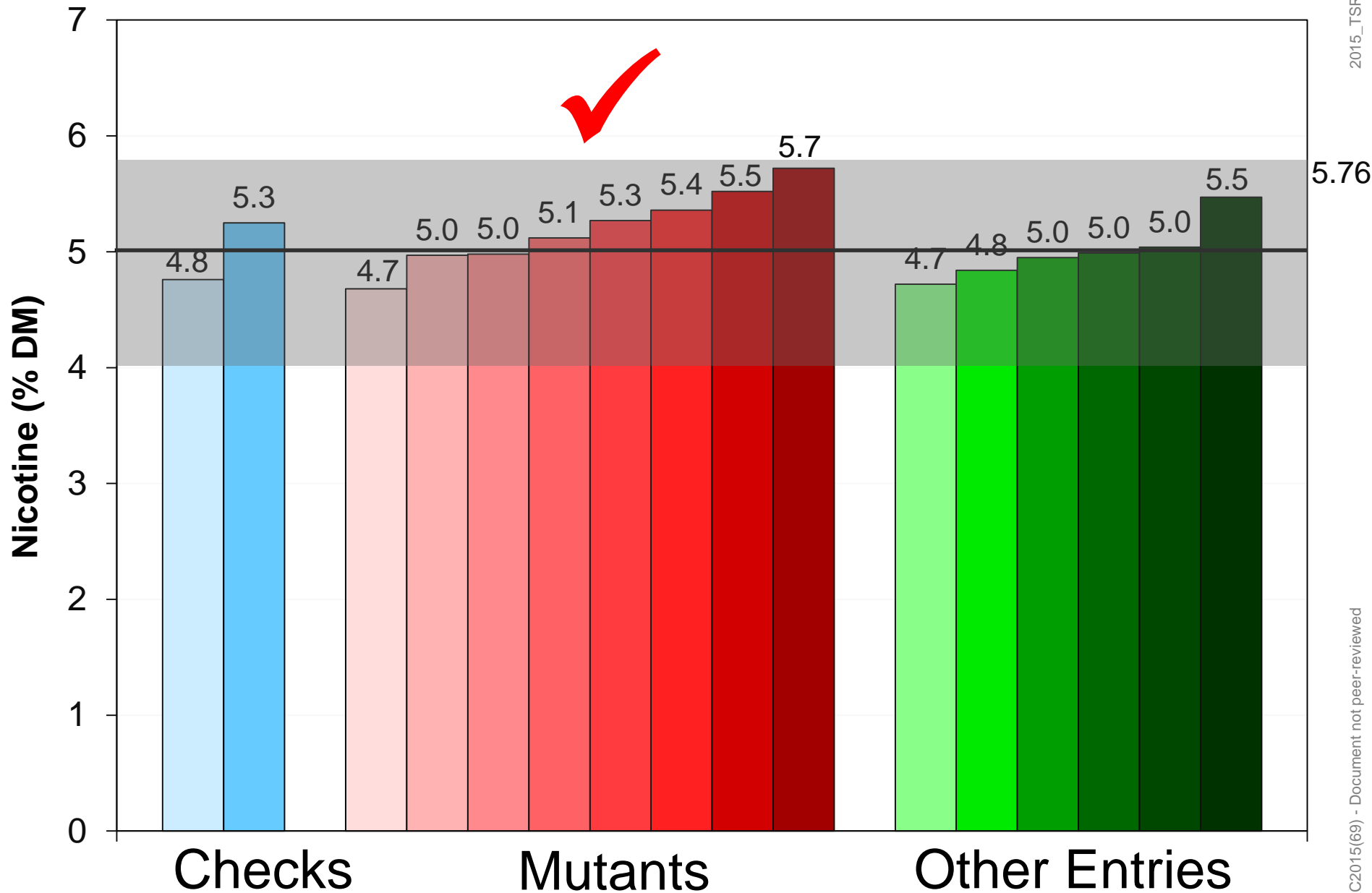
# 2013 RQTs: Nicotine % DM



# 2013 RQTs: Nicotine % DM

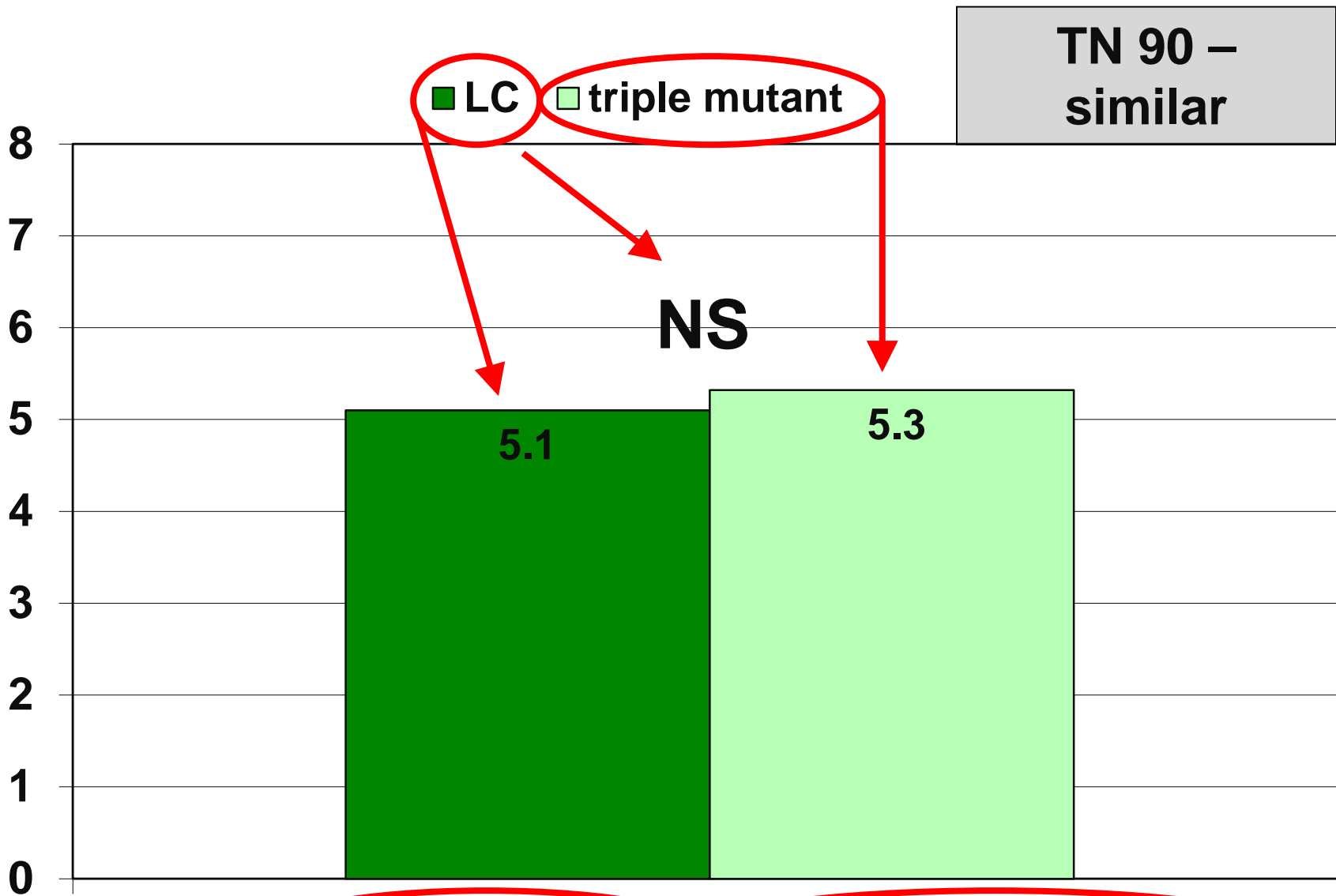


# 2014 RQTs: Nicotine % DM



# LC vs. Triple Mutant: TN 86

Nicotine (% DM)



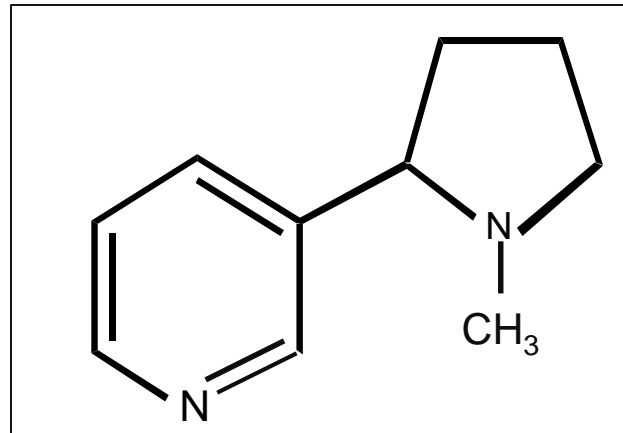
LC: Conversion 2.6%    Triple Mutant: Conversion 0.7%



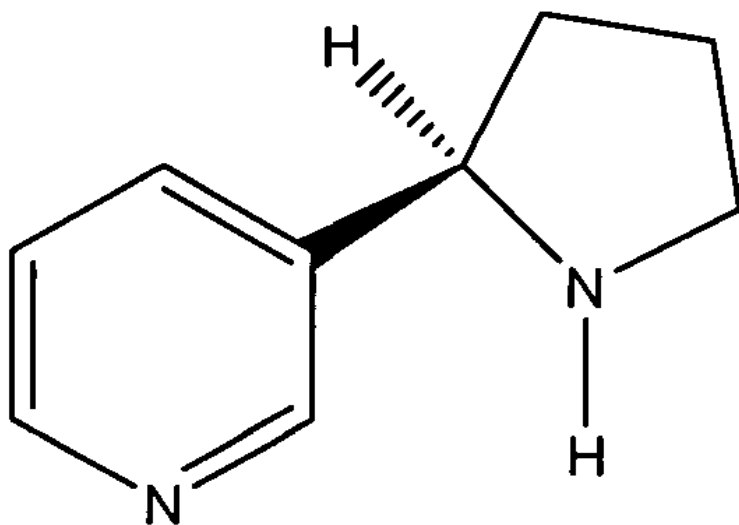
# Nicotine Levels

## Summary

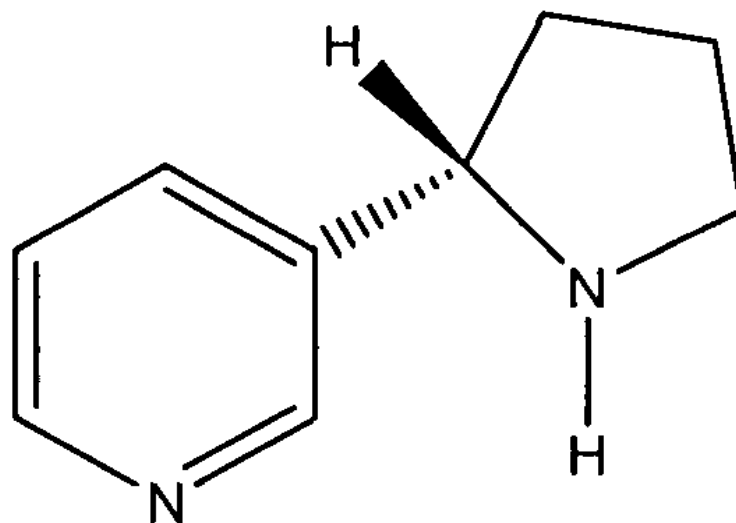
- **LC vs triple mutant – no difference**
  - Trend to  $\uparrow$  nicotine in triple mutant
- **$\uparrow$  Nicotine & TA could be a problem**
  - But not necessarily so
- **Shown by 2013 & 2014 RQT data**



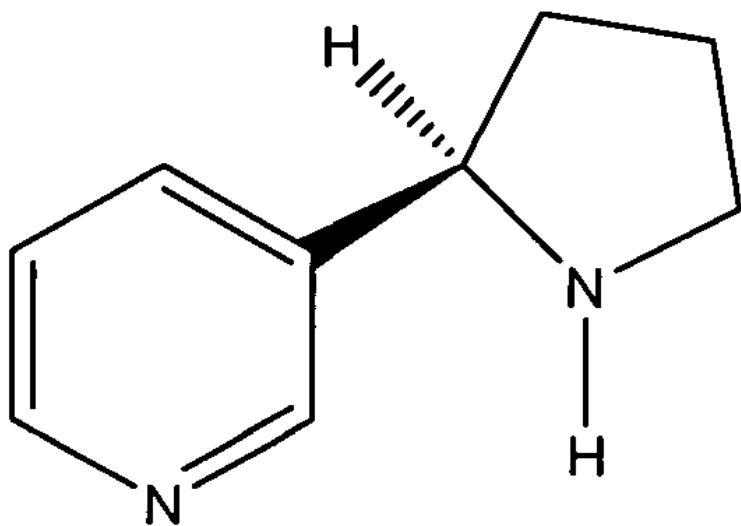
# Alkaloid & TSNA Isomers in the Demethylase Mutants



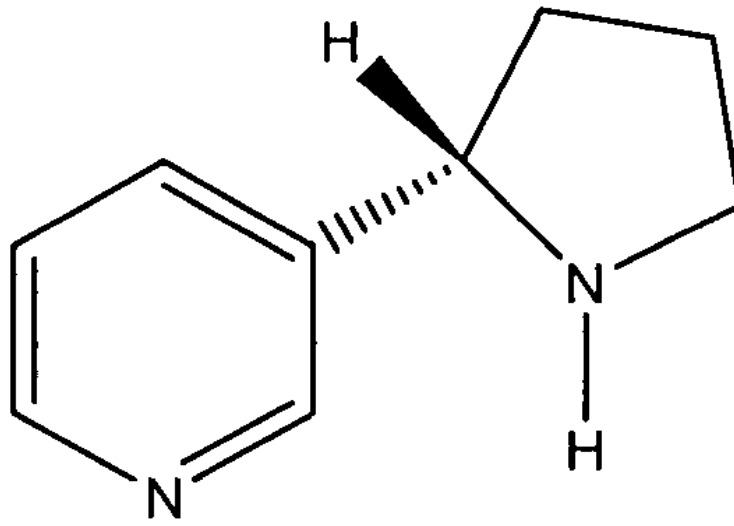
**R-Nornicotine**



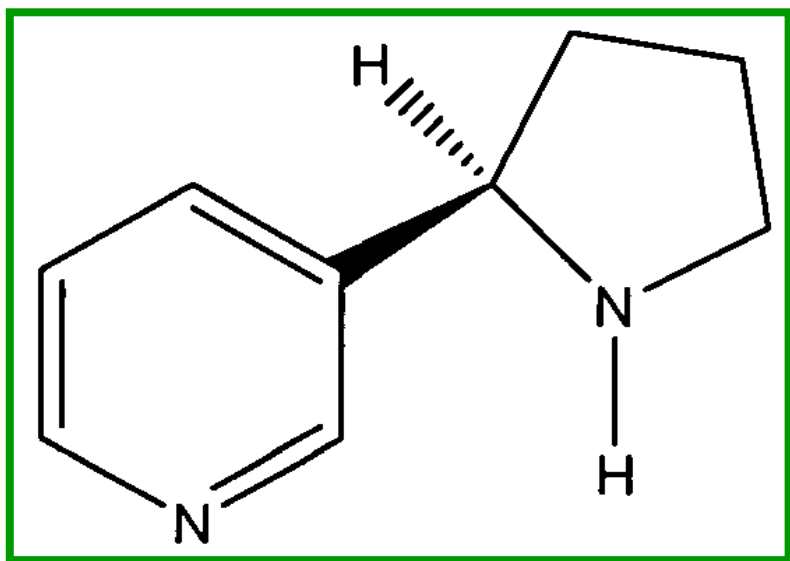
**S-Nornicotine**



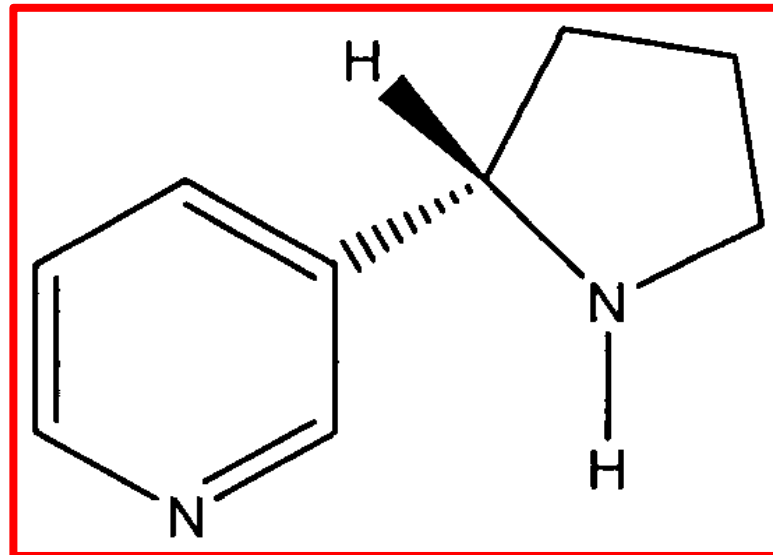
**R-Nornicotine**



**S-Nornicotine**

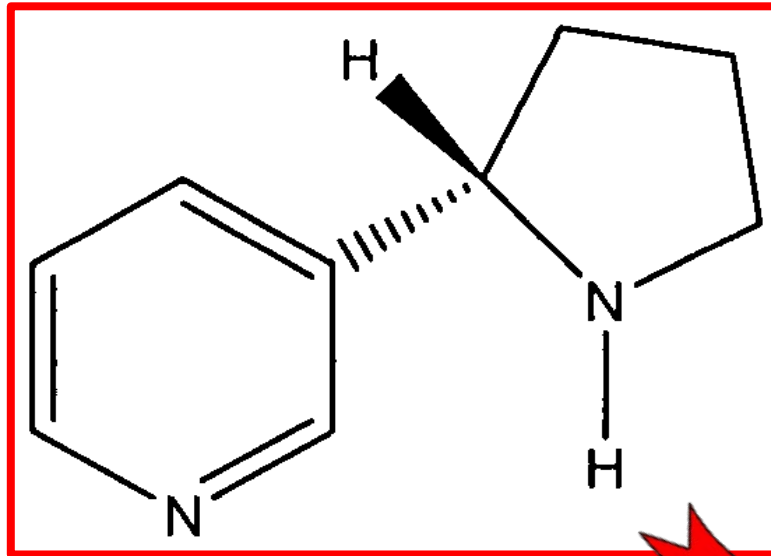


**R-Nornicotine**

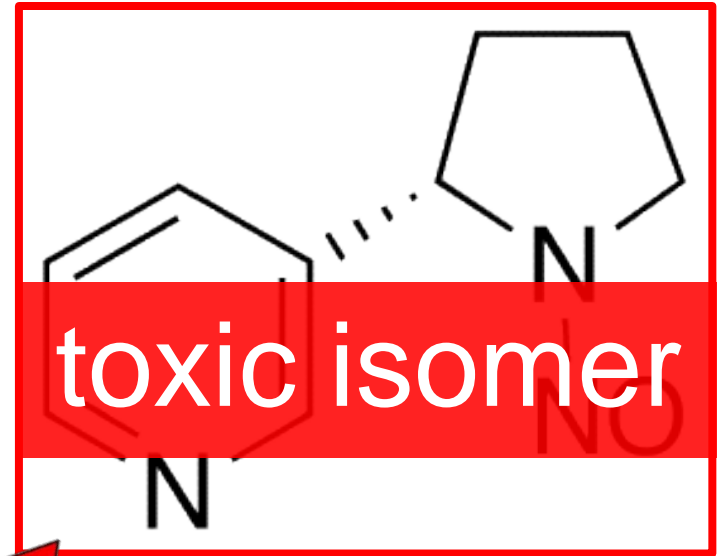


**S-Nornicotine**

# Stereo-Isomers



**S-Nornicotine**



**S-NNN**



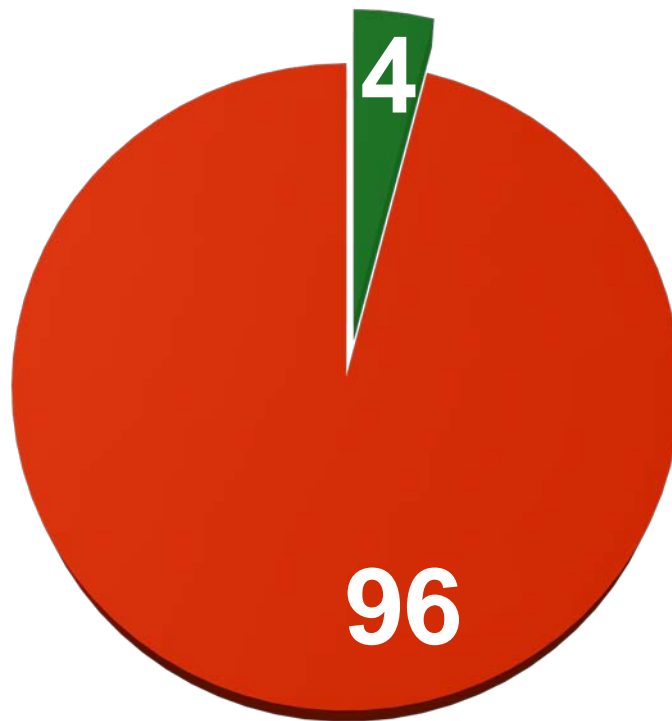
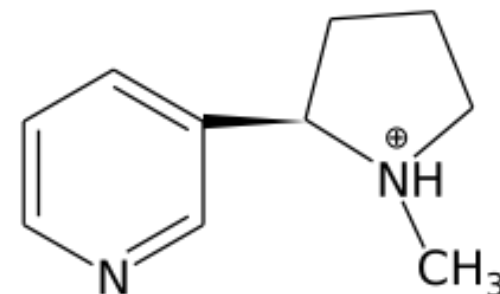
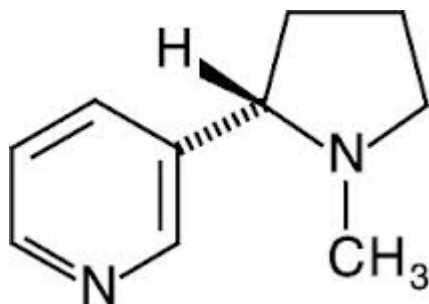
# Nicotine Stereo-Isomers



S-Nicotine



R-Nicotine



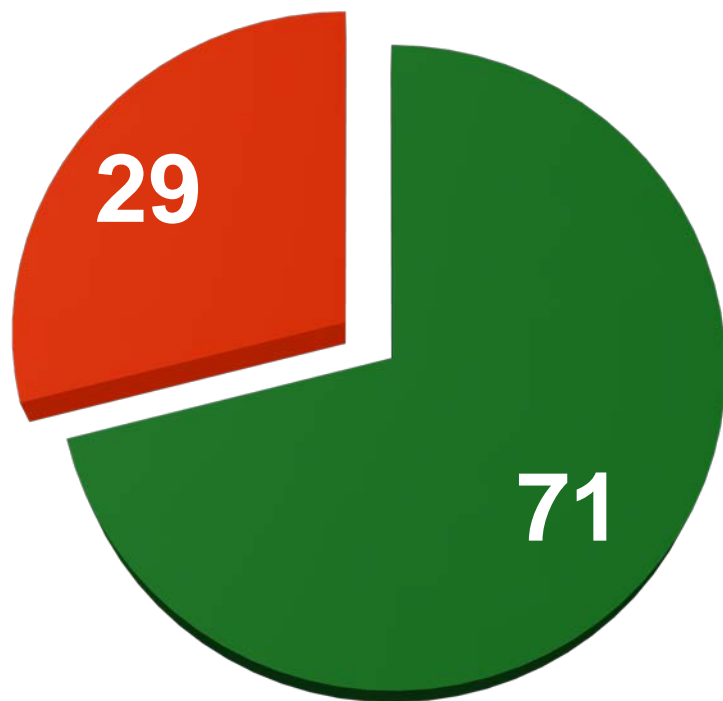
Bin Cai

# Nornicotine Stereo-Isomers

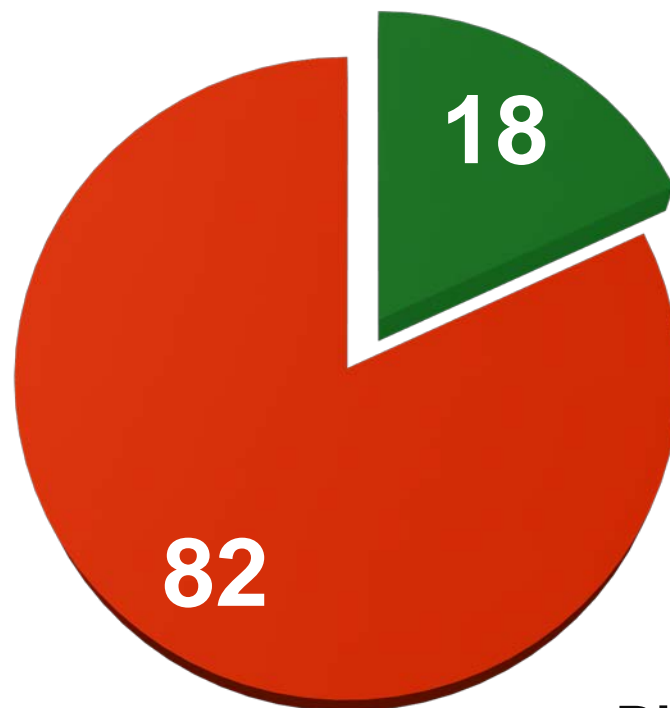
 S-Nornicotine

 R-Nornicotine

LC,  $e_4$



$e_4e_5e_{10}$ , transgenic



Bin Cai

# Why the Difference?

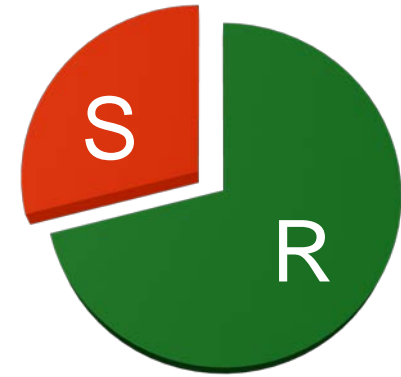
- **Nicotine**

- 4% R, 96% S



- **All three –  $E_4$ ,  $E_5$ ,  $E_{10}$  (LC)**

- Selectively demethylate R-nicotine
- Relatively more R nornicotine where all are active



- **$E_5$ ,  $E_{10}$**

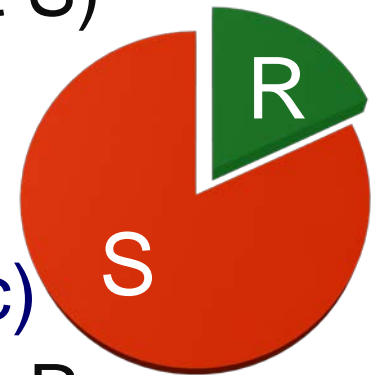
- Exclusively demethylate R-nicotine (not S)

- **$E_4$  (LC)**

- Can demethylate both R & S nicotine

- **Ultra low converters ( $e_4, e_5, e_{10}$ , transgenic)**

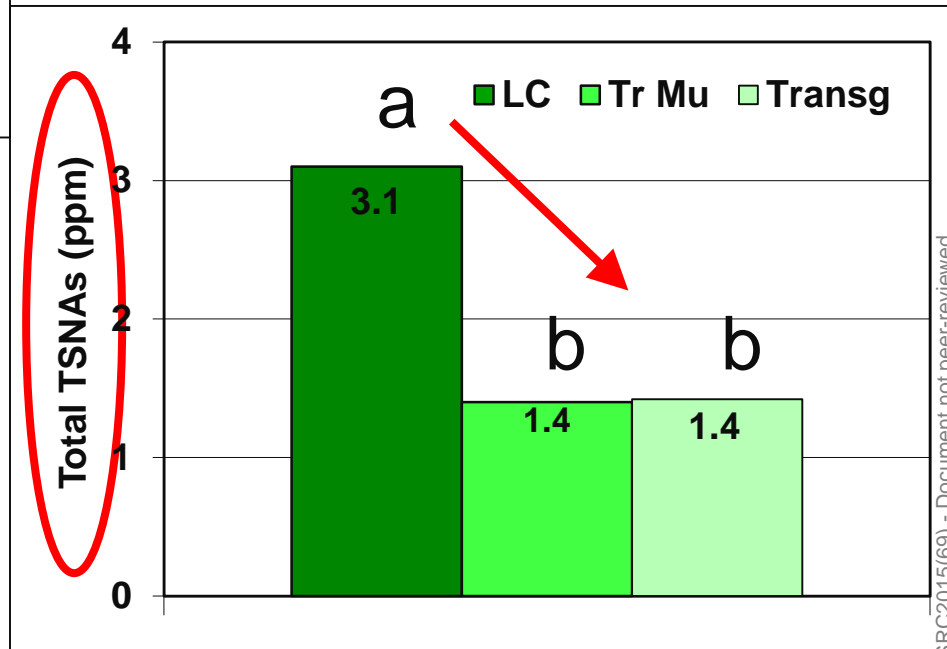
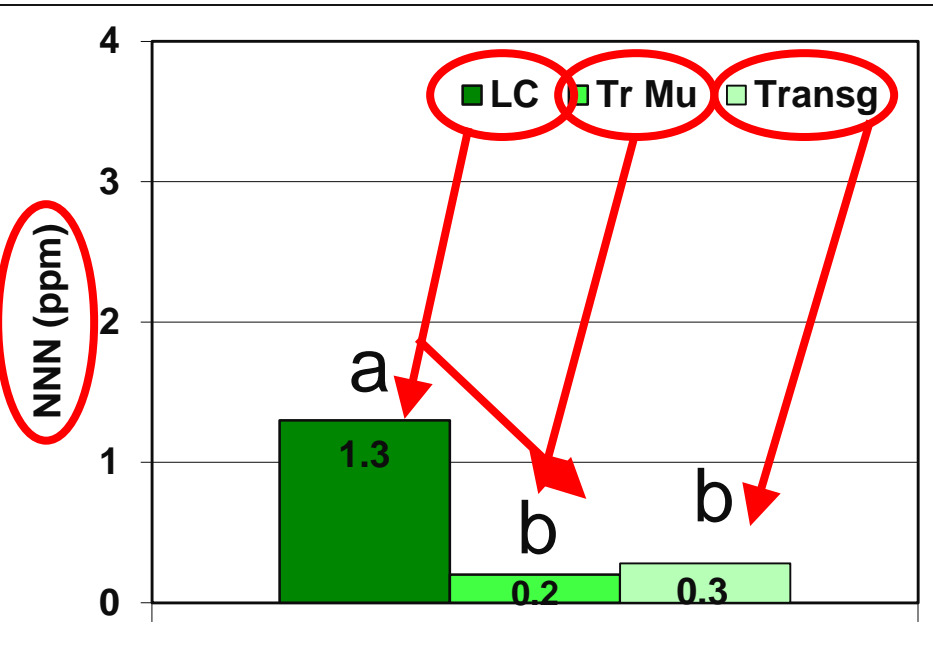
- Reflect nicotine composition in leaf – S>R
- No selective R demethylation





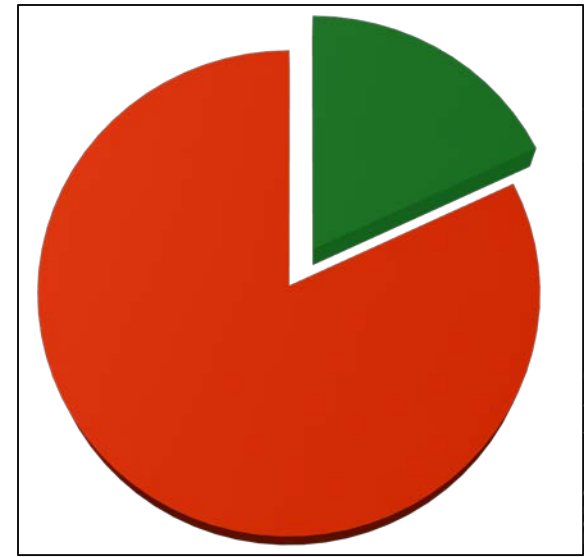
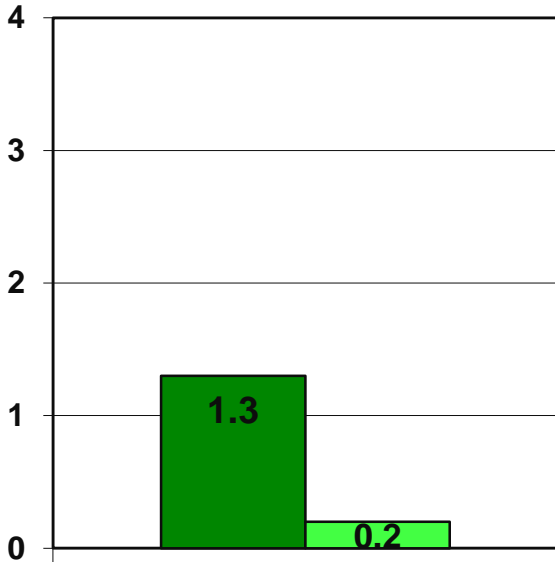
# LC vs. Triple Mutant: NNN, Total TSNAs

## TN 90

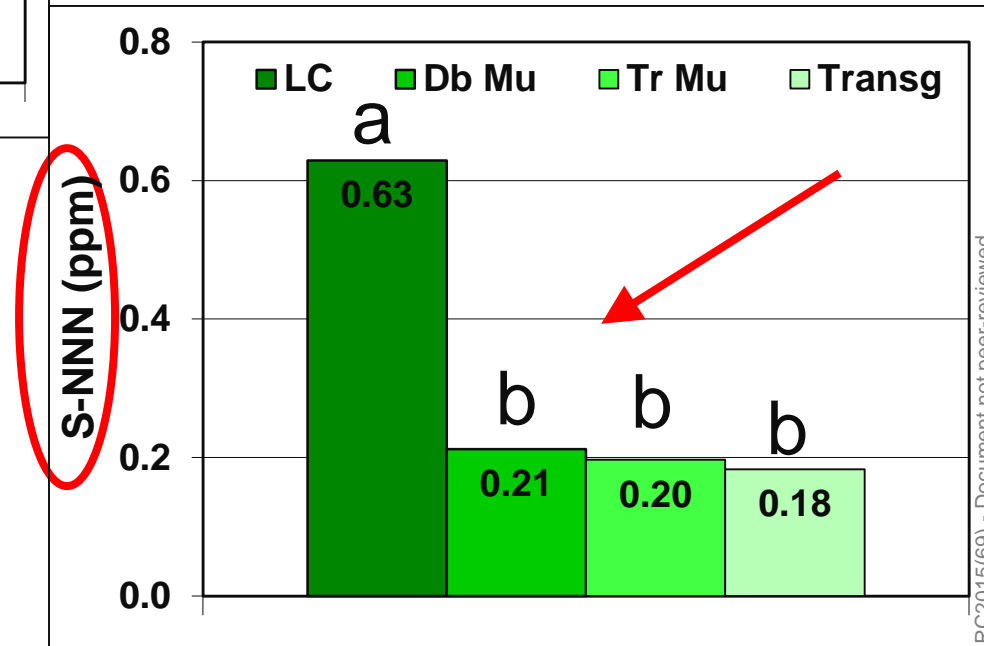
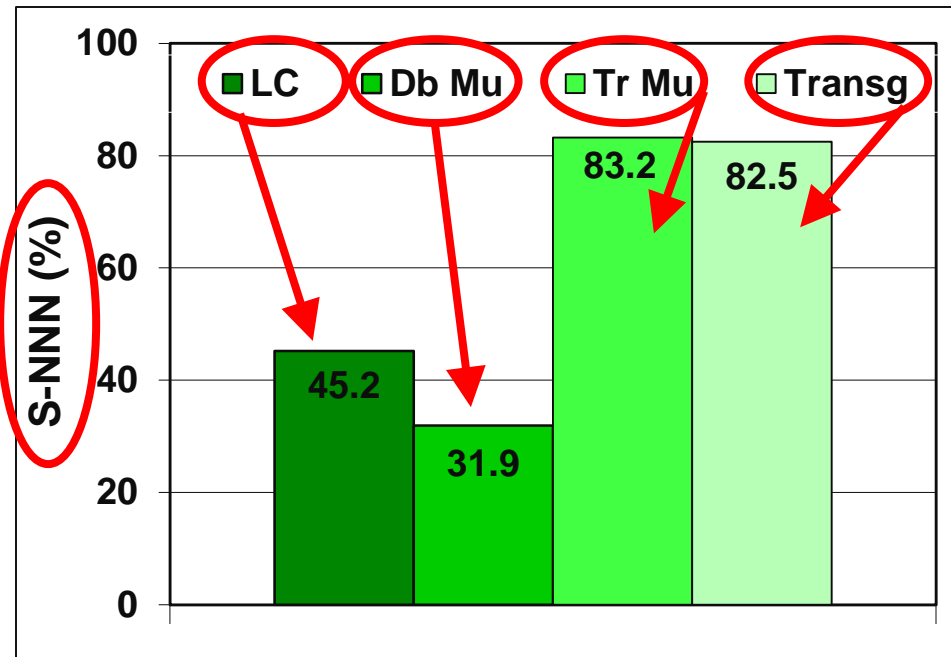


# S-NNN?

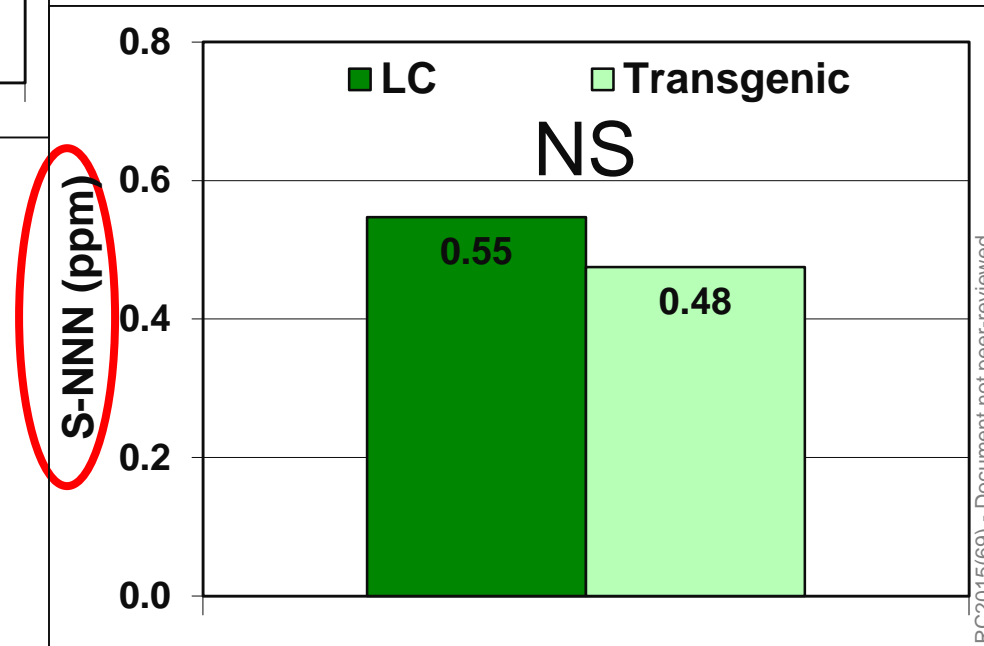
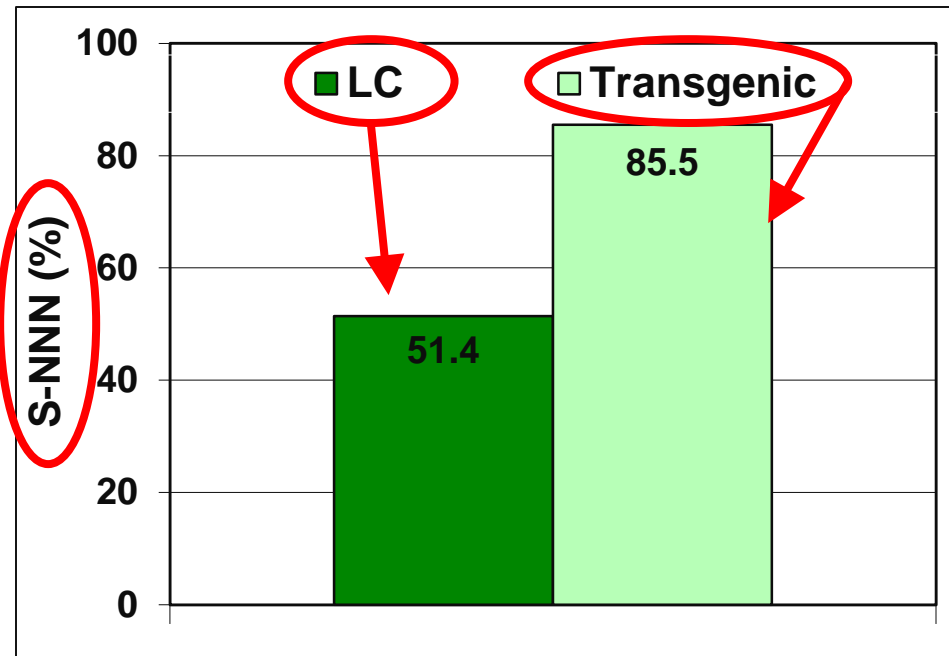
- **Triple mutant vs. LC**
  - Lower conversion
  - Lower absolute amount of NNN
  - Higher ratio of S-NNN
- **Absolute amount of S-NNN**
  - Mutant higher ratio of smaller amount
    - S-NNN less in triple mutant than in LC?



# S-NNN – %, ppm: TN 90



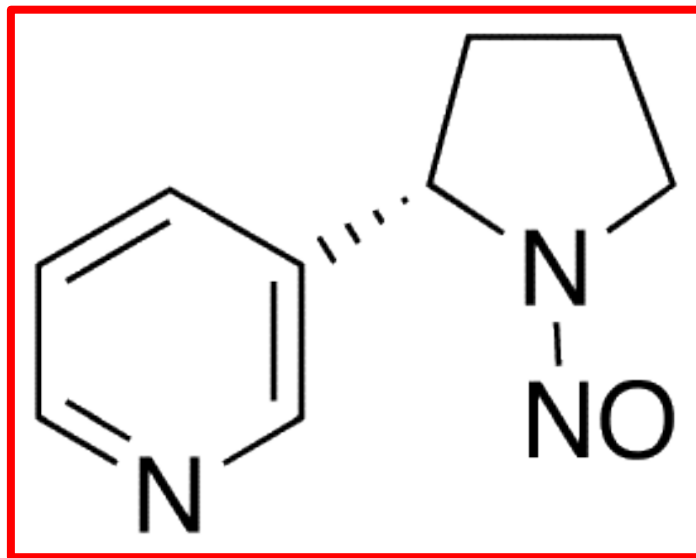
# S-NNN – %, ppm: TN 86



# TSNA Isomers

## Summary

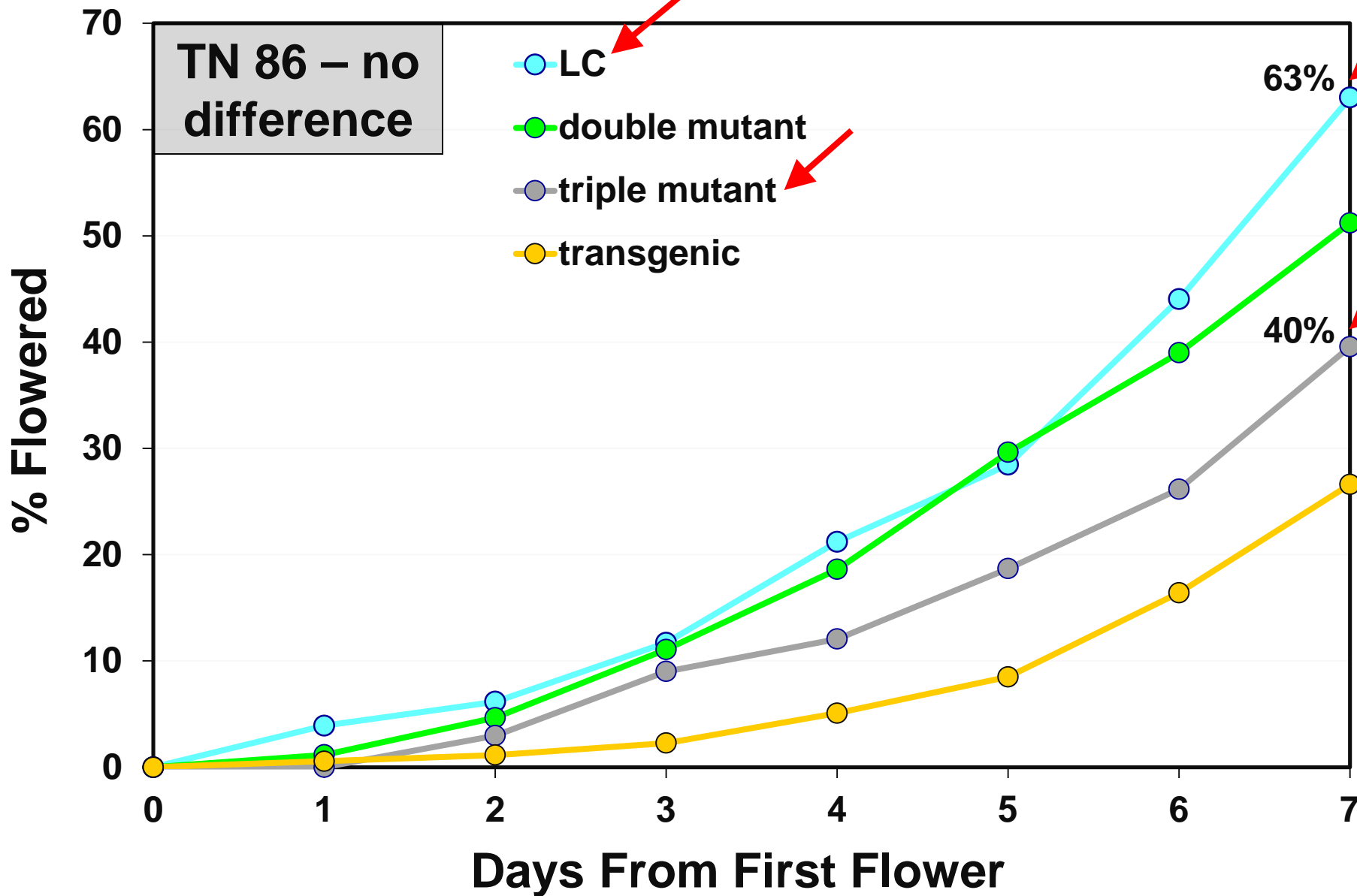
- Mutant S-NNN ppm ↓ or = LC
- No indication ↑ % S-NNN
  - → ↑ S-NNN ppm



# Plant Growth in the Demethylase Mutants



# Days to Flower – TN 90



# Growth Parameters - Summary

- **Flowering time**
  - TN 90 – triple mutant later flowering than LC
  - TN 86 – no difference
- **Leaf shape**
  - Triple mutant longer, narrower leaves
- **Stalk height, leaf number**
  - Triple mutant taller, fewer leaves
- **Internode length**
  - TN 90 – triple mutant longer internodes
  - TN 86 – no difference

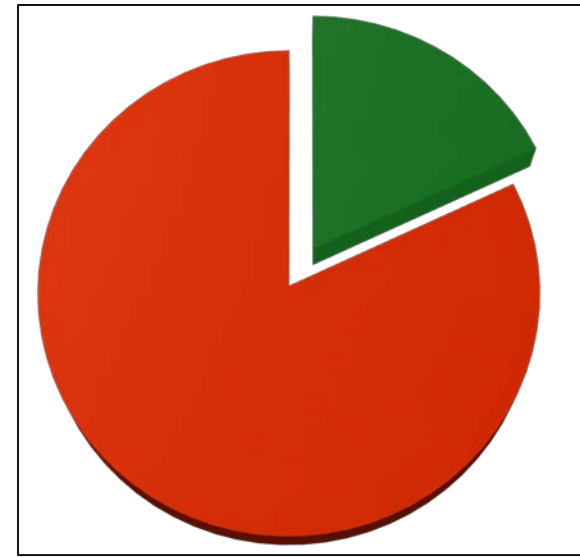
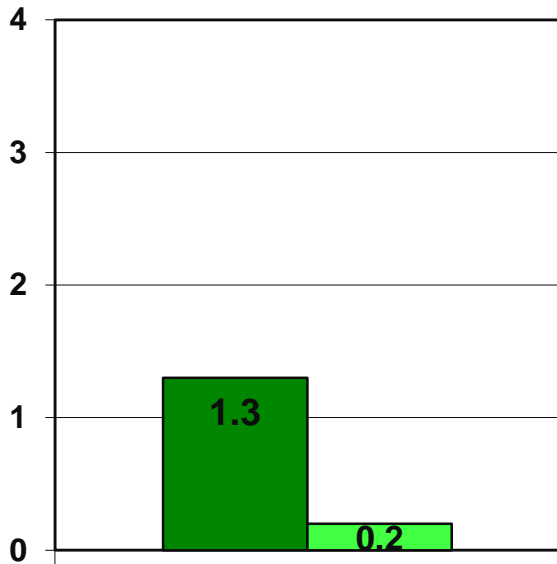
**Unlikely to be major  
problem**





# Conclusion

- On the basis of this (limited) work
- **Triple mutants**
  - Advantages outweigh potential disadvantages



# Acknowledgements

- **Bin Cai**
  - Former grad student
- **Neil Fannin, Huihua Ji**
  - Method development
- **Financial support**
  - PMI
  - Kentucky Research & Development Center

