Combining Different Genetics-Based TSNA Reduction Strategies in Burley Tobaccos

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Philip Morris International, Neuchâtel, Switzerland

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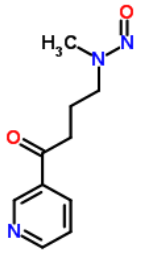
Reducing Tobacco-Specific Nitrosamines (TSNA) via Genetic Modification

Nicotine Nornicotine Anatabine Anabasine

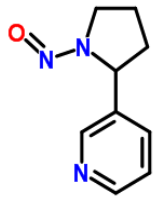
Target the alkaloid precursor

Nitrous oxides, such as NO, NO_2, N_2O_3, and N_2O_4

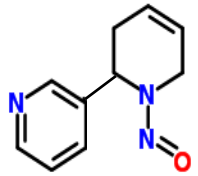
Target the nitrosating agent



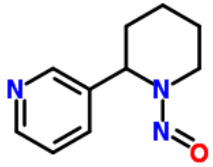
N-Nitrosonornicotine Ketone (NNK)



N-Nitrosonornicotine (NNN)



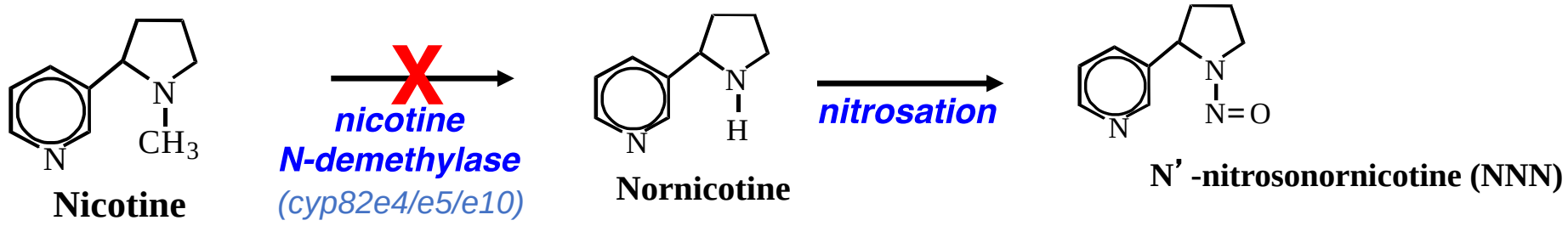
N′-nitrosoanatabine (NAT)



N-nitrosoanabasine (NAB)

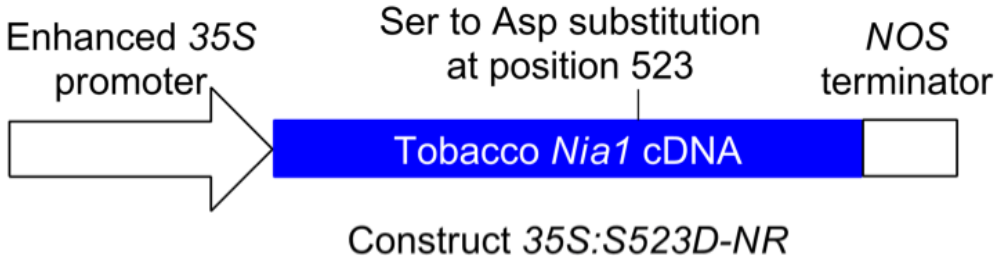
Effective Genetic-Based Strategies to Reduce TSNAs

A) Inactivation of Nicotine Demethylase Genes (Zyvert™)

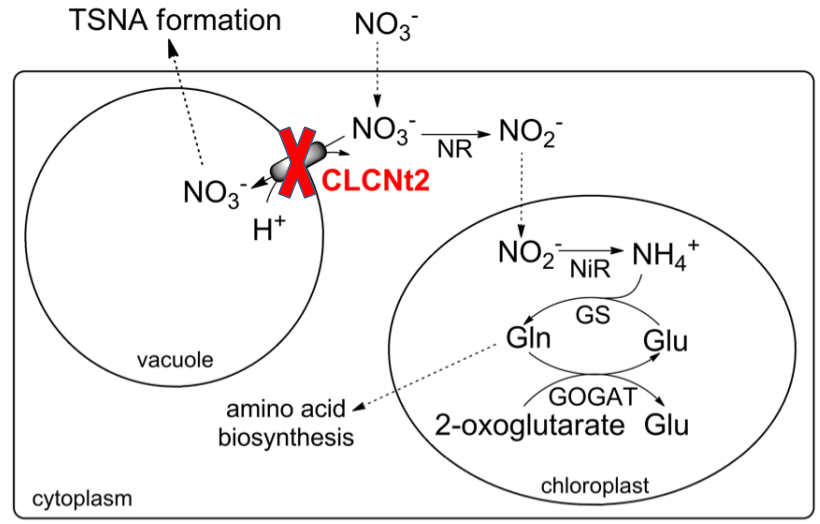


B) Reducing the Levels of Free Nitrate

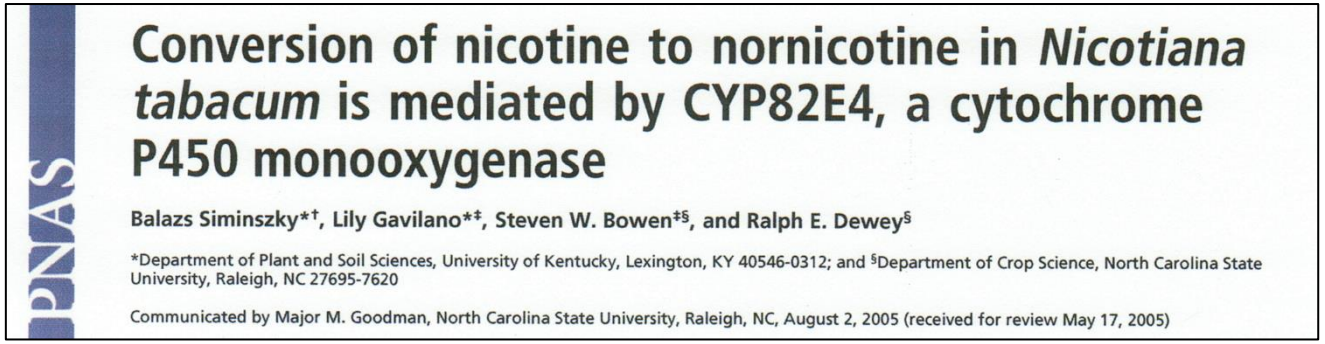
1. Constitutive activation of a deregulated nitrate reductase gene



2. Inactivation of putative nitrate transporters (*CLC-Nt2* genes)



Inactivation of Nicotine Demethylase Genes (*Zyvert*™)



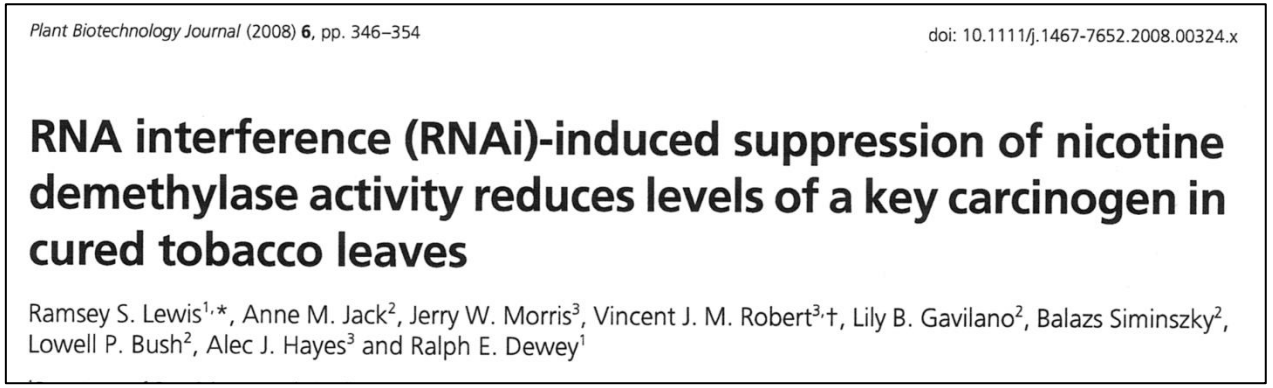
PNAS

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

Balazs Siminszky*†, Lily Gavilano*‡, Steven W. Bowen‡§, and Ralph E. Dewey§

*Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546-0312; and §Department of Crop Science, North Carolina State University, Raleigh, NC 27695-7620

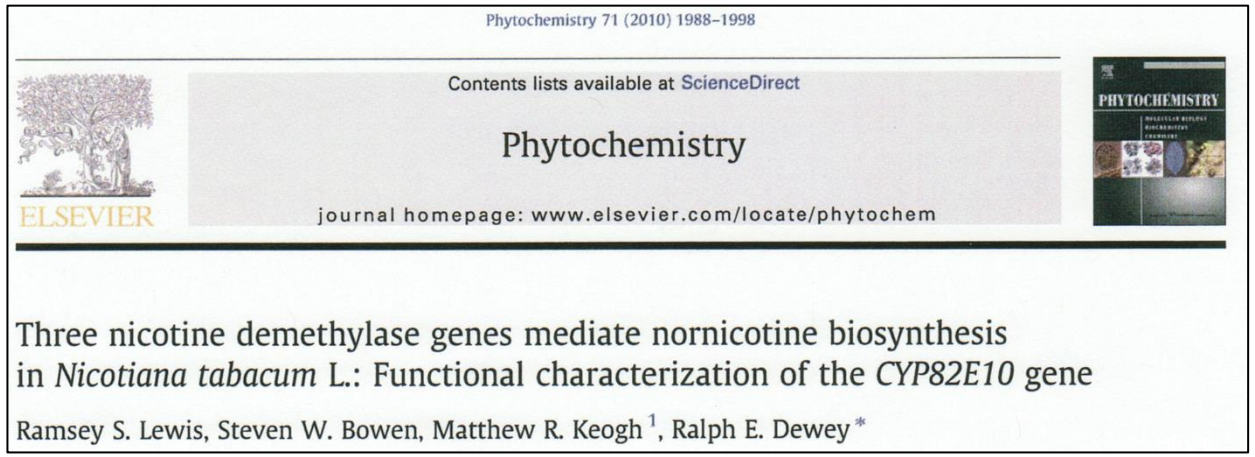
Communicated by Major M. Goodman, North Carolina State University, Raleigh, NC, August 2, 2005 (received for review May 17, 2005)



Plant Biotechnology Journal (2008) **6**, pp. 346–354 doi: 10.1111/j.1467-7652.2008.00324.x

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]



Phytochemistry 71 (2010) 1988–1998

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Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

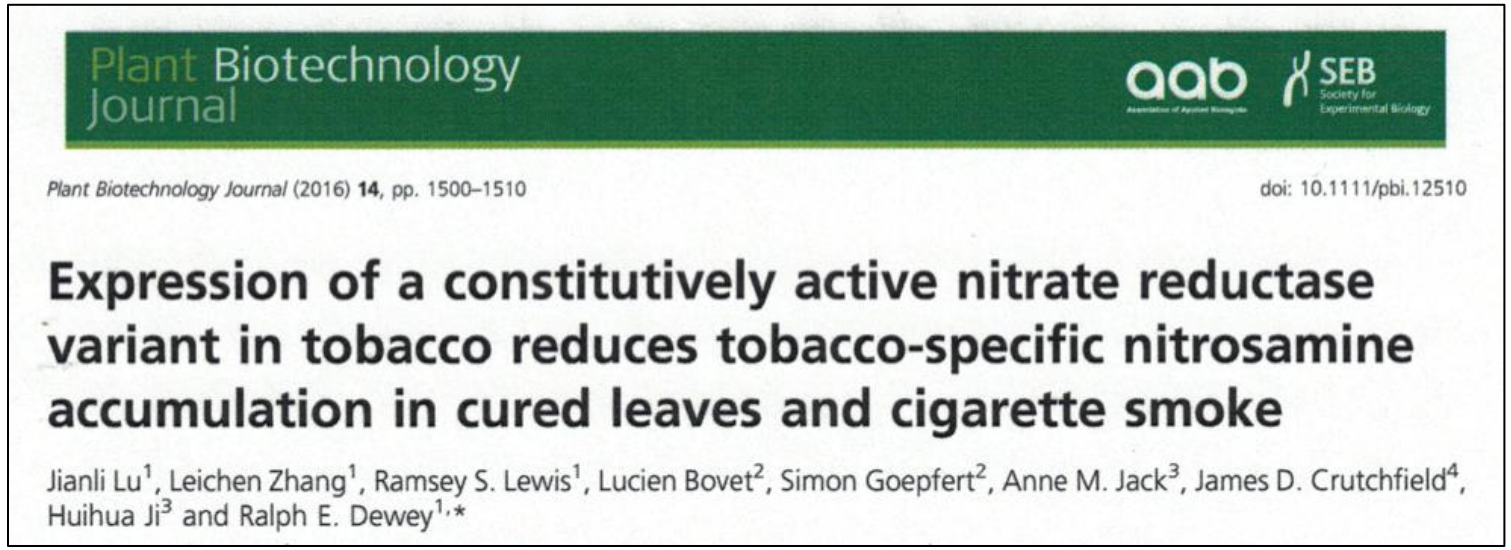
Three nicotine demethylase genes mediate nornicotine biosynthesis in *Nicotiana tabacum* L.: Functional characterization of the *CYP82E10* gene

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

Summary of Results

- **Can reduce both nornicotine and NNN accumulation by ~80%**
- **Appears to have no significant impact on yield or flowering time**

Constitutive Expression of a Deregulated Nitrate Reductase Gene



Plant Biotechnology Journal

aab SEB Society for Experimental Biology

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,*]

scientific reports

Scientific Reports | (2021) 11:4222 | https://doi.org/10.1038/s41598-021-83797-7 nature portfolio

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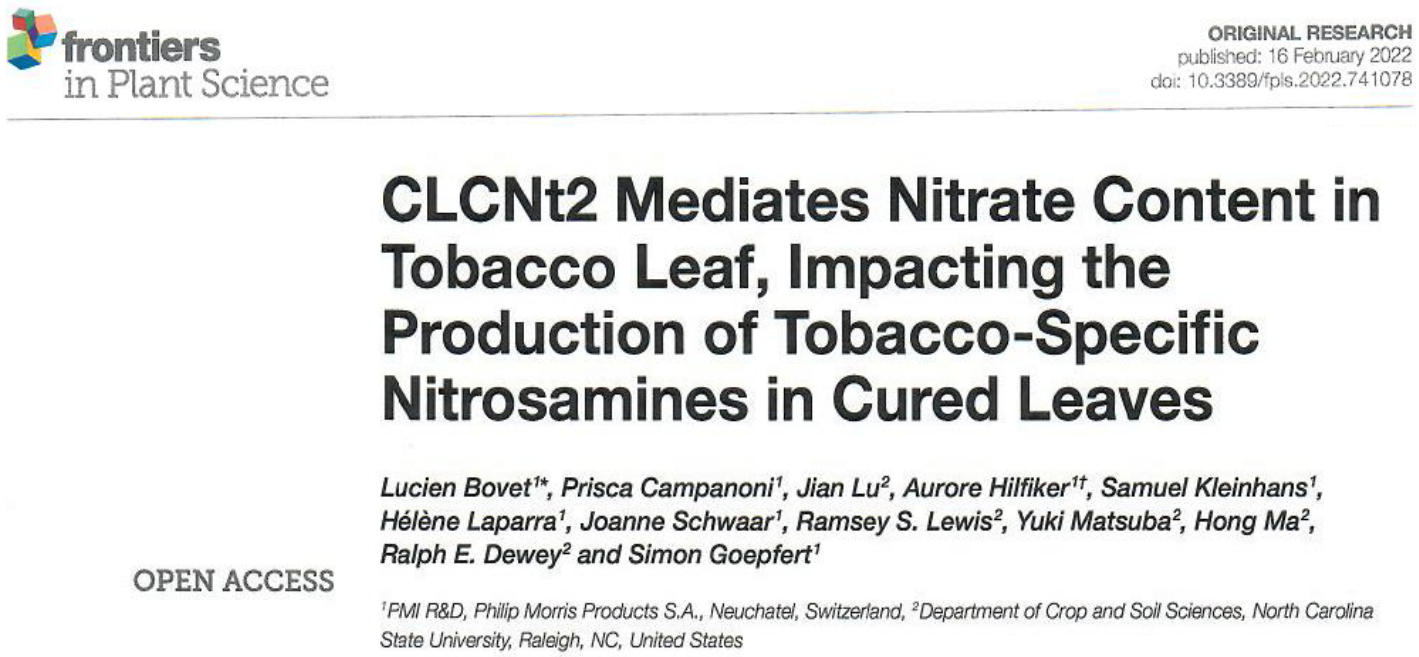
OPEN **Constitutive activation of nitrate reductase in tobacco alters flowering time and plant biomass**

Jianli Lu[1,3], Niharika N. Chandrakanth[1], Ramsey S. Lewis[1], Karen Andres[1], Lucien Bovet[2], Simon Goepfert[2] & Ralph E. Dewey[1]

Summary of Results

- **Can reduce free nitrates levels by ~95% in the cured leaf/cut filler**
- **Can reduce total TSNAs (including NNK) by ~52% in the cut filler and ~76% in the cigarette smoke**
- **Can accelerate flowering by 7 to 10 days**
- **Can lead to reductions in biomass by 30 to 50%**
- ***The reductions in biomass can be overcome while retaining the low TSNA trait by crossing 35S:S523D-NR plants with late flowering burley cultivars***

Inactivation of Putative Nitrate Transporter Genes (*CLC-Nt2*)



frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 February 2022
doi: 10.3389/fpls.2022.741078

CLCNt2 Mediates Nitrate Content in Tobacco Leaf, Impacting the Production of Tobacco-Specific Nitrosamines in Cured Leaves

Lucien Bovet[1], Prisca Campanoni[1], Jian Lu[2], Aurore Hilfiker[1†], Samuel Kleinhans[1], Hélène Laparra[1], Joanne Schwaar[1], Ramsey S. Lewis[2], Yuki Matsuba[2], Hong Ma[2], Ralph E. Dewey[2] and Simon Goepfert[1]*

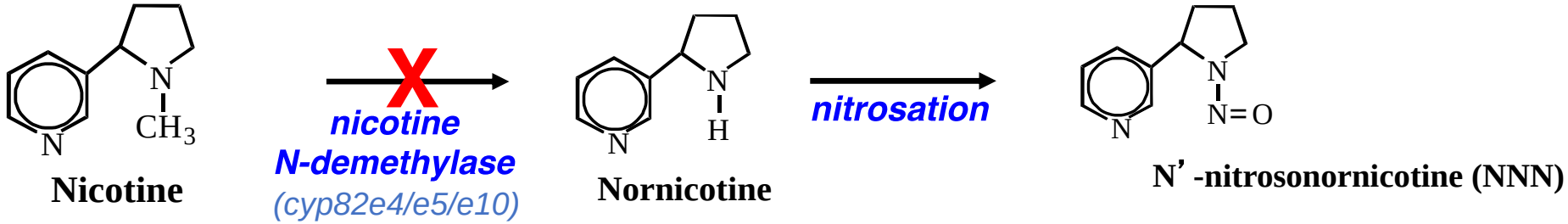
[1]PMI R&D, Philip Morris Products S.A., Neuchatel, Switzerland, [2]Department of Crop and Soil Sciences, North Carolina State University, Raleigh, NC, United States

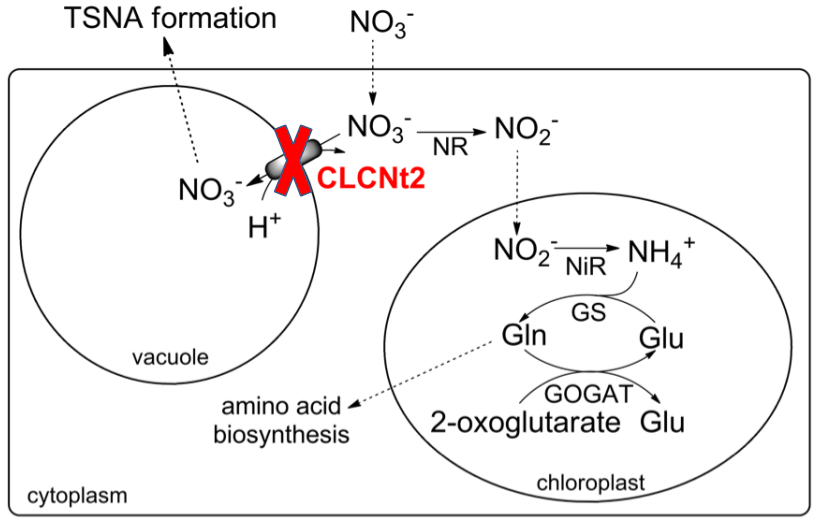
OPEN ACCESS

Summary of Results

- **Nitrate concentration reduced by ~65%**
- **Total TSNAs in cured leaf lamina reduced by ~40% and in mainstream cigarette smoke by ~52%**
- **Inactivation of *CLC-Nt2* genes was not associated with reductions in biomass but was associated with an approximate 10-day delay in average flowering time**

Combining Zyvert™ Trait with *CLC-Nt2* Knockout Mutations





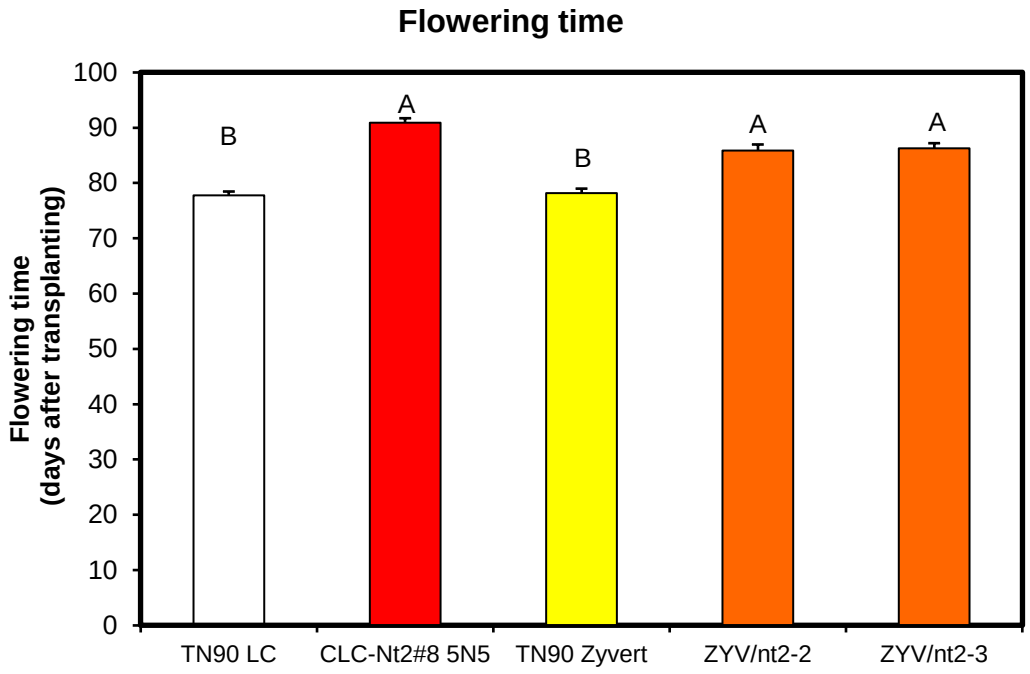
2022 Field Study Conducted in Clayton, NC

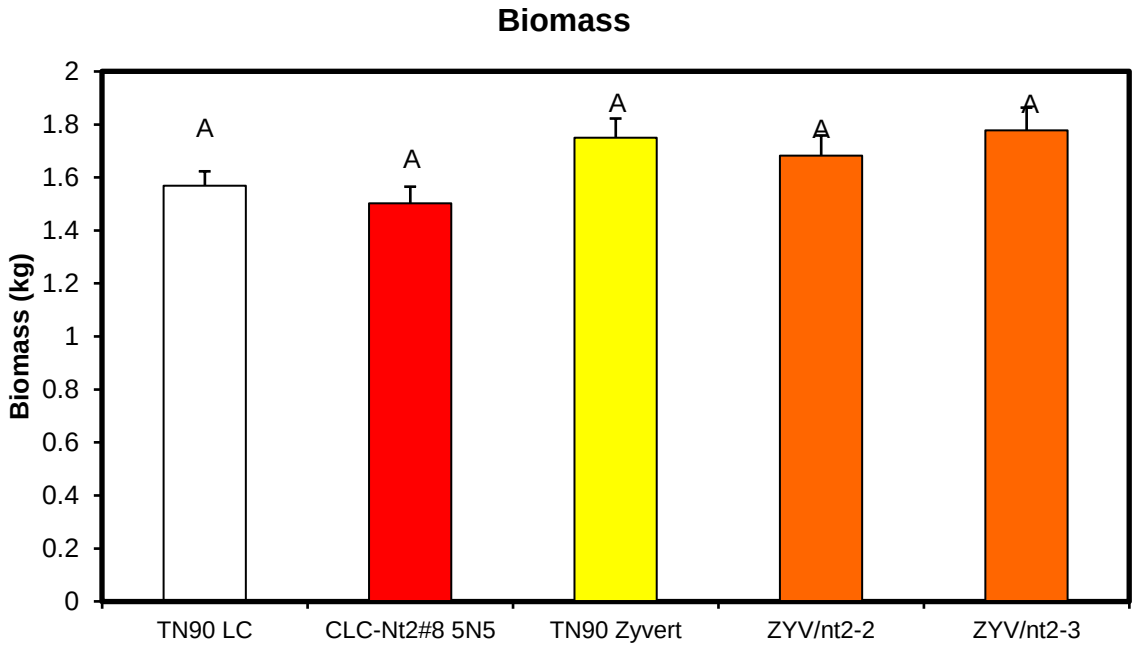
Line	Genotype
TN90 LC	Control
CLC-Nt2#8 5N5	clc-nt2 mutations alone
TN90 Zyvert	cyp82e4/e5/e10 mutations alone
ZYV/nt2-2	clc-nt2 + cyp82e4/e5/e10
ZYV/nt2-3	clc-nt2 + cyp82e4/e5/e10

Traits Measured

- Flowering Time
- Aerial Biomass at Harvest
- Nitrate (after air-cure)
- Alkaloids (after air-cure)
- TSNAs (after air-cure)

Flowering Time and Biomass Results

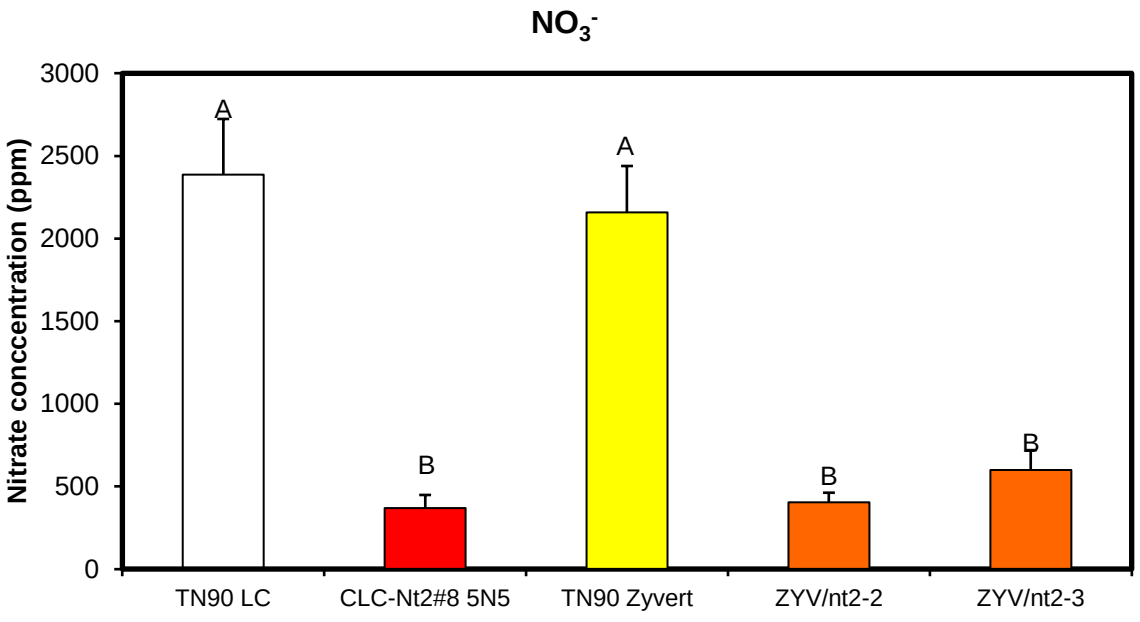


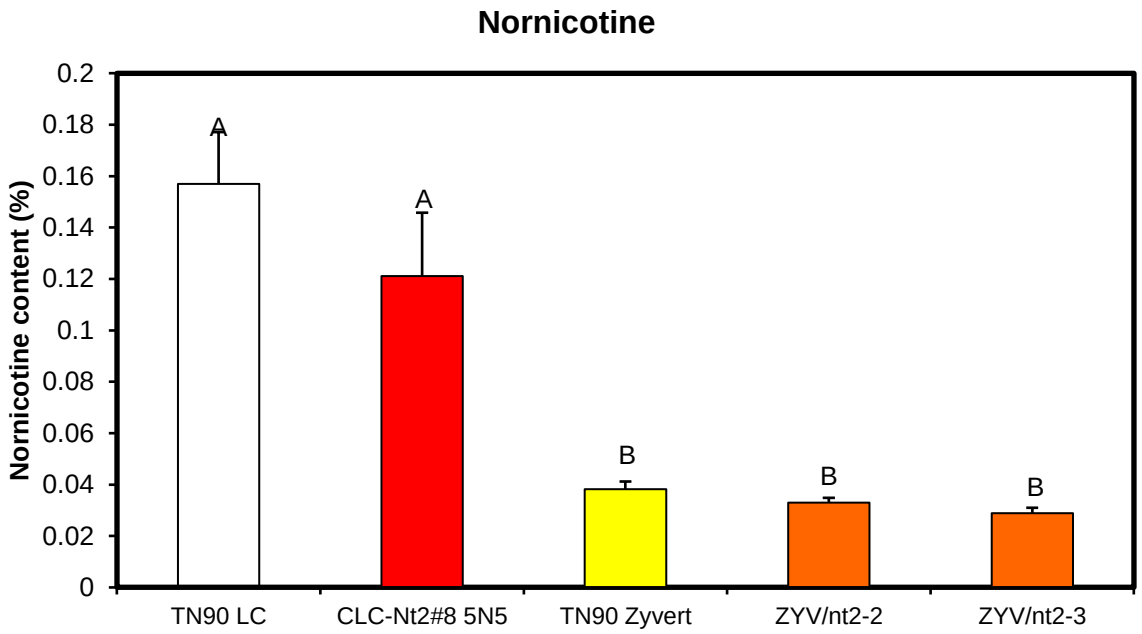


On average, lines possessing the clc-nt2 mutations flowered 8 – 12 days later than the TN90 LC and TN90 Zyvert lines

There were no significant differences in plant biomass observed among the lines

Nitrate and Nornicotine Results

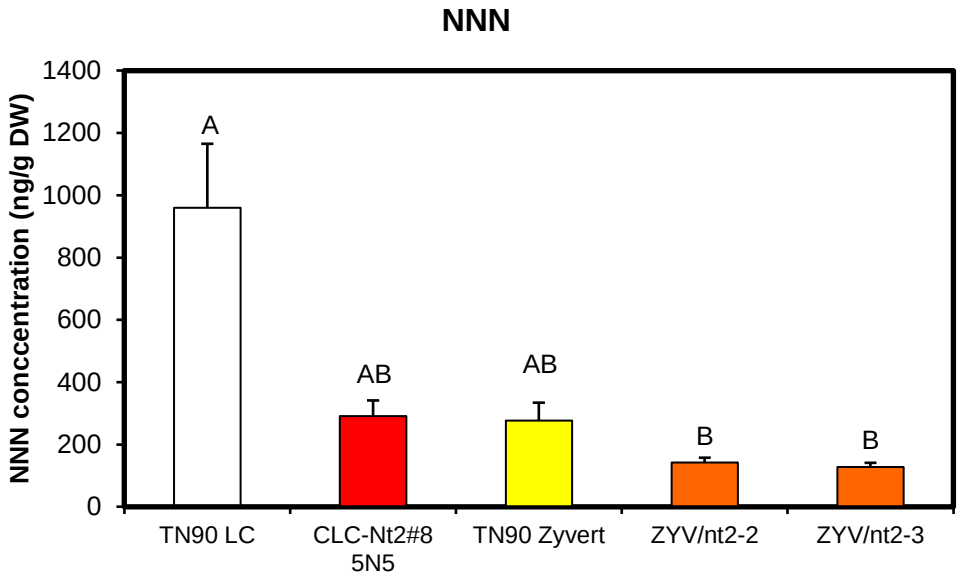


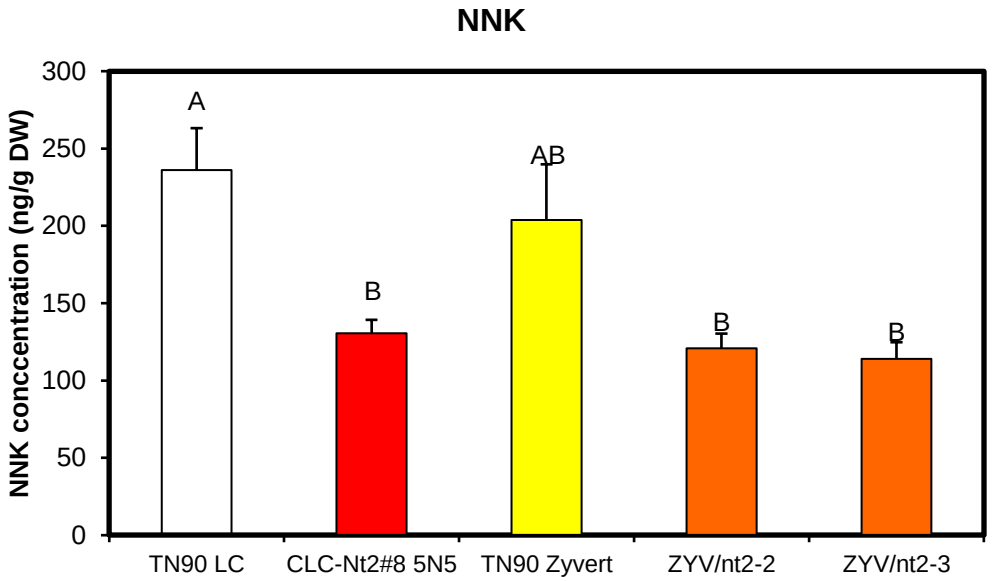


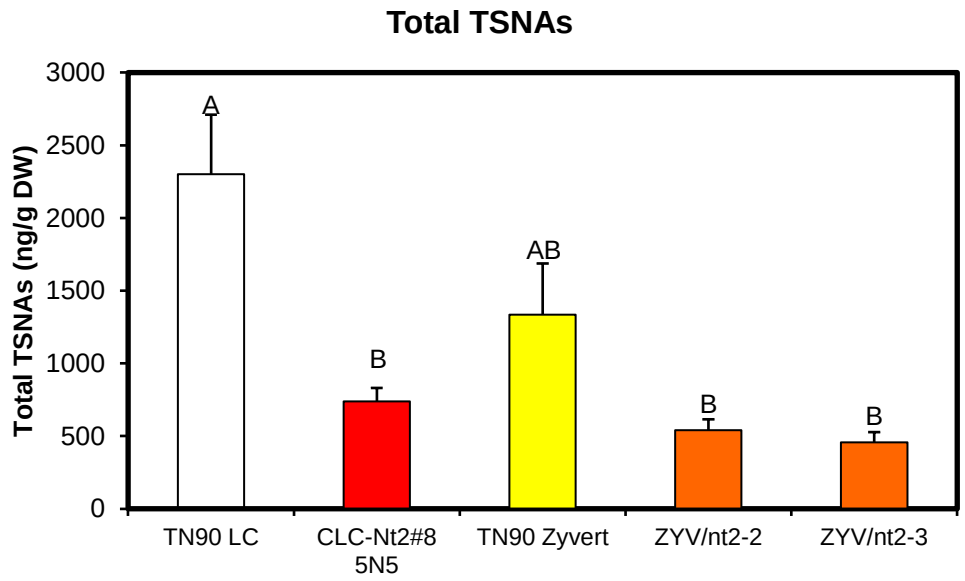
Lines possessing the clc-nt2 mutations accumulated 72 – 84% less nitrate in the cured leaf than the lines TN90 LC or TN90 Zyvert

Lines possessing the Zyvert mutations accumulated 69 – 82% less nornicotine than the lines without these mutations

TSNAs

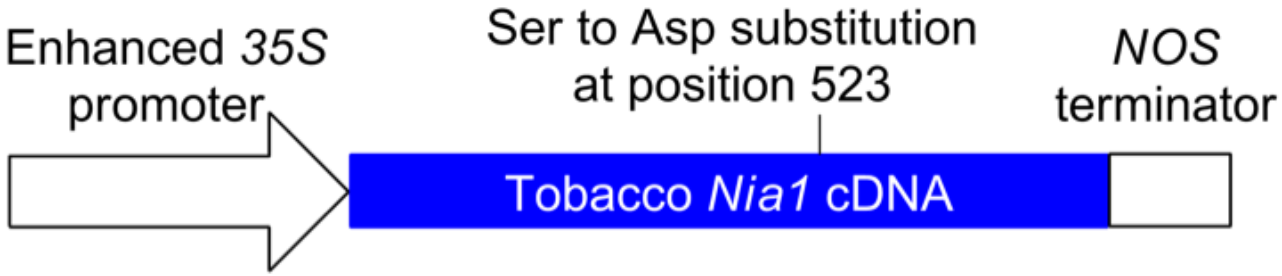




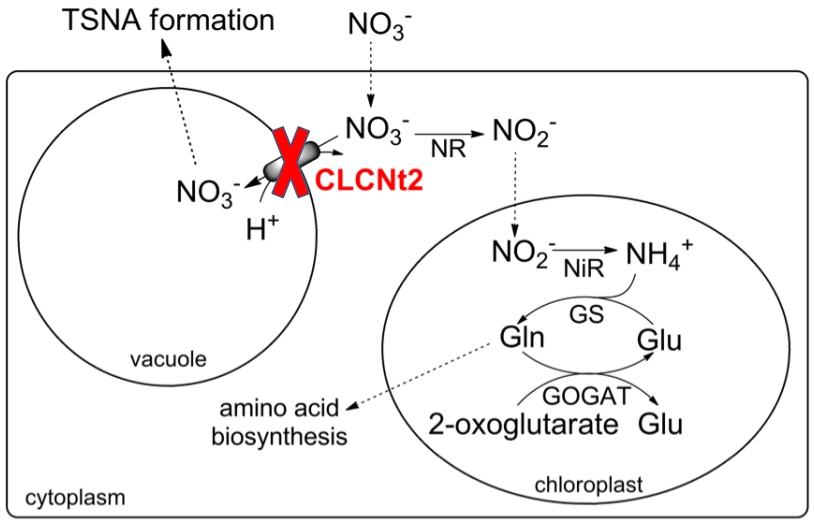


Due to the high costs of TSNA assays, only 10 plants per genotype were analyzed. The low sample size, combined with high inherent variability in TSNA formation, prevented some observations from achieving statistical significance, but the trends strongly suggest that combining the two technologies can reduce NNN levels below that achieved separately

Combining 35S:S523D-NR Transgene with *CLC-Nt2* Knockout Mutations



Construct *35S:S523D-NR*



2022 Field Study Conducted in Clayton, NC

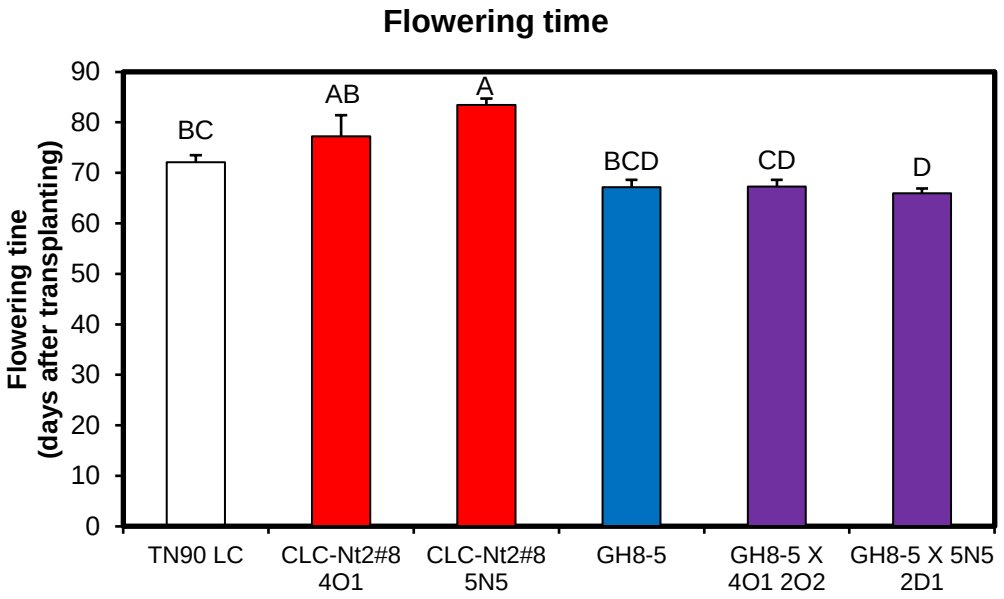
Line	Genotype
TN90 LC	Control
CLC-Nt2#8 4O1	clc-nt2 mutations alone
CLC-Nt2#8 5N5	clc-nt2 mutations alone
GH8-5	35S:S523D-NR alone
GH8-5 4O1 2O2	35S:S523D-NR + clc-nt2
GH8-5 5N5 2D1	35S:S523D-NR + clc-nt2
GH8-5 5N5 2H3*	35S:S523D-NR + clc-nt2

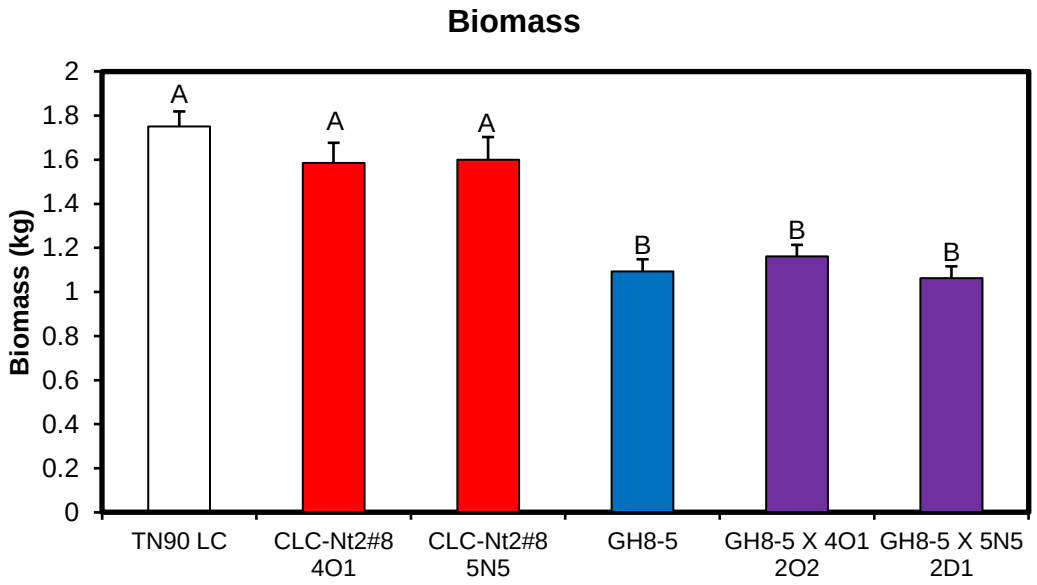
*This line converted (40% nornicotine) so data from this line will be omitted

Traits Measured

- Flowering Time
- Aerial Biomass at Harvest
- Nitrate (after air-cure)
- Alkaloids (after air-cure)
- TSNAs (after air-cure)

Flowering Time and Biomass Results

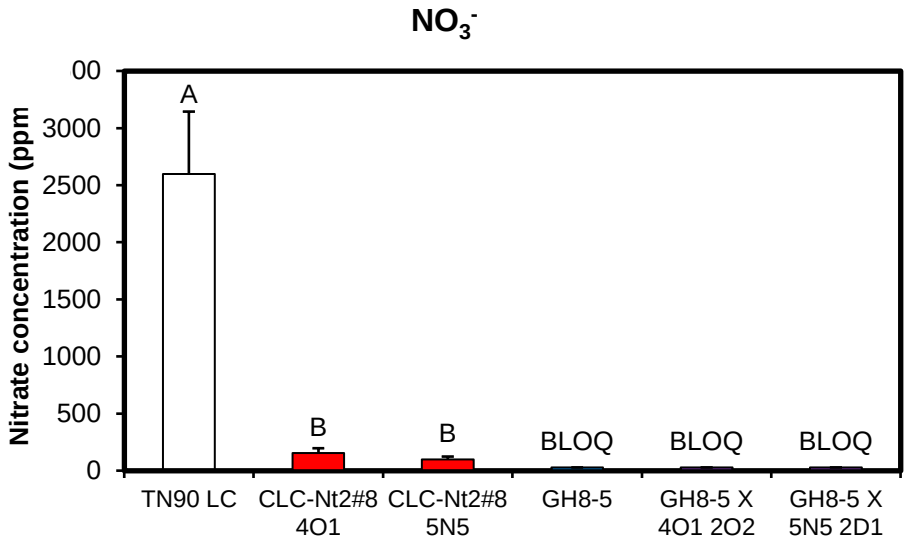


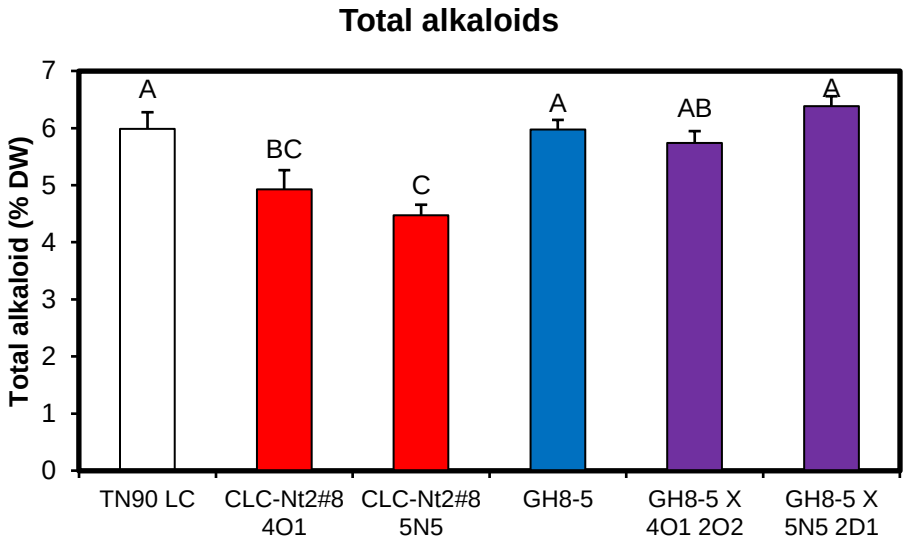


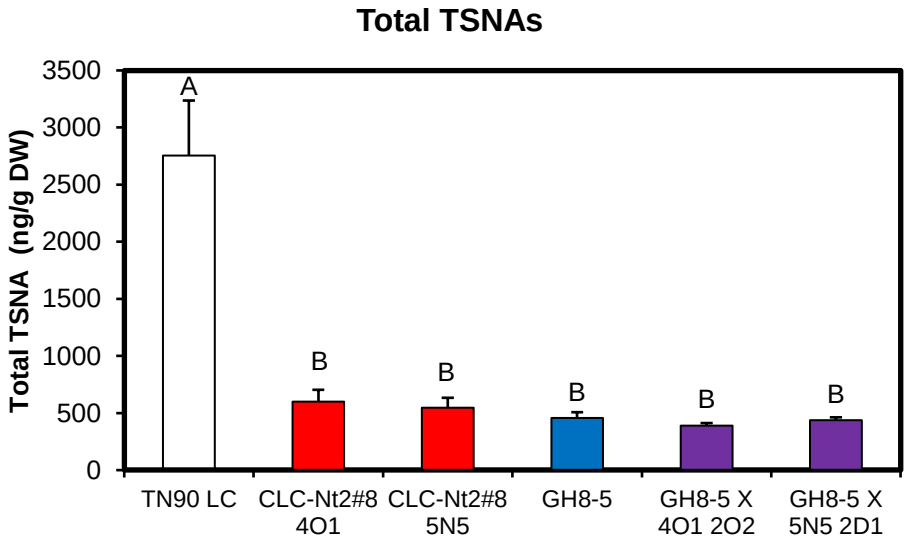
Lines with only clc-nt2 mutations tended to flower later than the rest (only one had statistical significance across all other lines), and all lines with the 35S:S523D-NR construct tended to flower earlier (though not all comparisons were statistically significant)

All lines possessing the 35S:S523D-NR transgene showed major reductions in aerial biomass (34 – 39% decrease)

Nitrate, Alkaloids, and TSNAs







- *Both nitrate-reducing technologies greatly lowered the nitrate content*
- *The clc-nt2 mutations alone were associated with a modest reduction in alkaloids (possibly attributed to later topping dates)*
- *In 2022, both technologies led to similar overall TSNA reductions (79 – 86% decrease)*

Conclusions

- Of the two nitrate-lowering traits tested, the 35S:S523D-NR transgene was dominant to the CLC-Nt2 mutations with respect to flowering time and biomass.
- Although the CLC-Nt2 mutations alone can mediate substantial reductions in TSNA content, they would need to be deployed in combination with Zyvert mutations or LC screening because of the propensity of burley varieties to convert.
- Combining CLC-Nt2 knockout mutations with Zyvert mutations represents a promising strategy for maximally lowering TSNAs without compromising yield.