

# Downregulation of a putative nitrate transporter (CLCNt2) substantially reduces the accumulation of TSNA's in air-cured tobacco

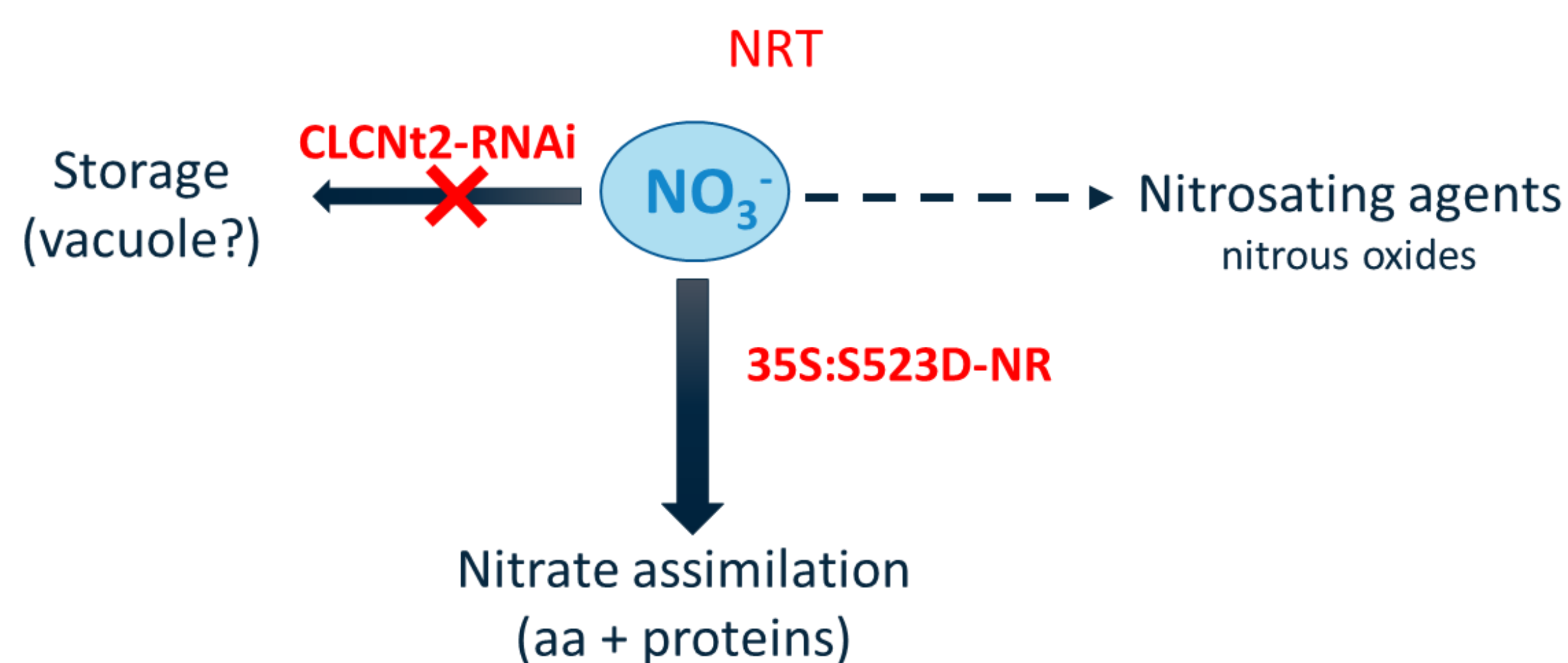
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## Background

- Nicotine-derived nitrosamine ketone (NNK) and N'-nitrosonornicotine (NNN) belong to the family of tobacco-specific nitrosamines (TSNAs). Classified as carcinogenic, they are naturally synthesized in tobacco leaves and during tobacco smoke.
- Leaf nitrate serves as a source of nitrosating agents contributing to the generation of NNN and NNK; therefore, we developed a strategy to reduce nitrate storage in leaf in order to limit the formation of NNN and NNK in tobacco products.

## Nitrate assimilation targets



### Strategies:

Reduce storage: *CLCNt2-RNAi* = chloride/proton antiporters (this study)

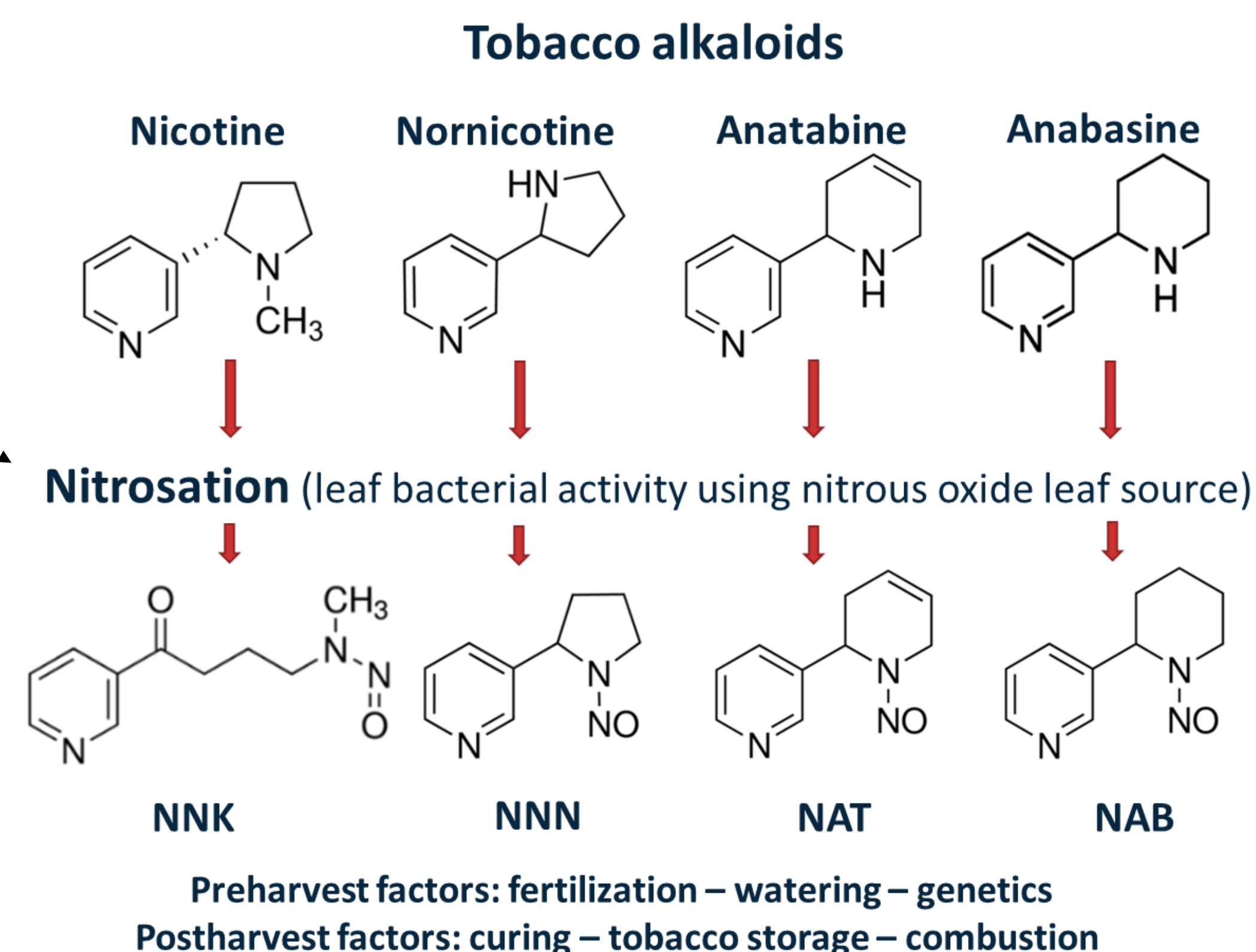
Increase assimilation: NR = nitrate reductase (Lu et al., Plant Biotech Journal, 2016)

Reduce uptake: *NRT-RNAi* = nitrate transporter (in progress)

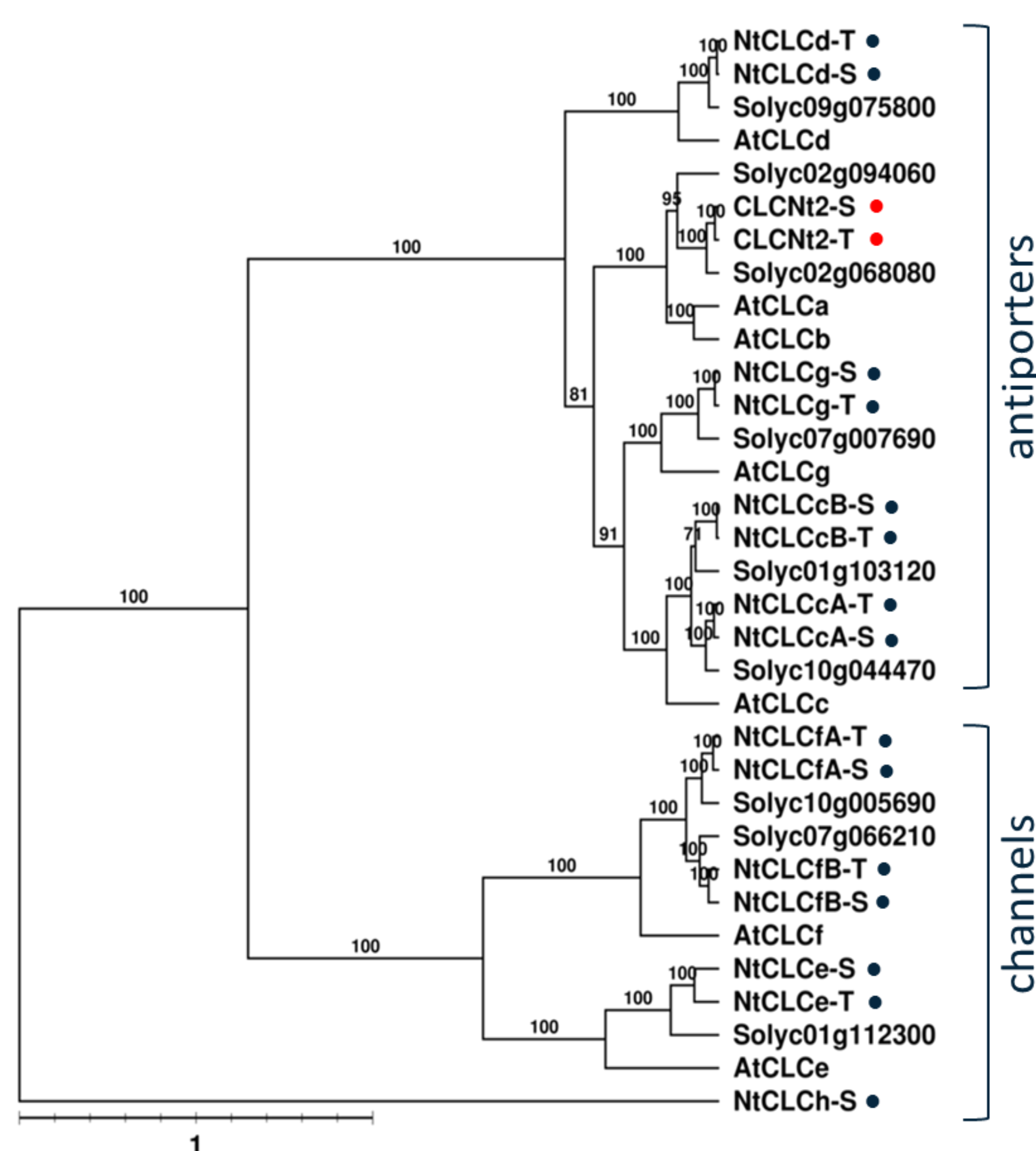
## Approach

- Identify plants with lower nitrate content in leaf to measure the impact in TSA formation.
- Use reverse genetics to identify candidate genes.
- Work with Burley tobacco types high in leaf nitrate and TSNAs.
- Experimental:
  - Field trial in U.S. (North Carolina, 2 locations, 2 years)
  - 3 *CLCNt2-RNAi* lines in Burley TN90e4e5 background
  - 50 plants per line
  - Analysis performed with middle leaf material

## TSNAs



## CLC gene family identification in tobacco



Phylogenetic tree of *Arabidopsis thaliana* AtCLCs, *Solanum lycopersicum* (Solyc...) and *Nicotiana tabacum* (NtCLCs, bullet points) based on gene product sequence homologies.

*CLC* protein family includes both chloride channels (Cl<sup>-</sup>) and chloride/proton antiporters (Cl<sup>-</sup>/H<sup>+</sup>) (1, 2, 3).

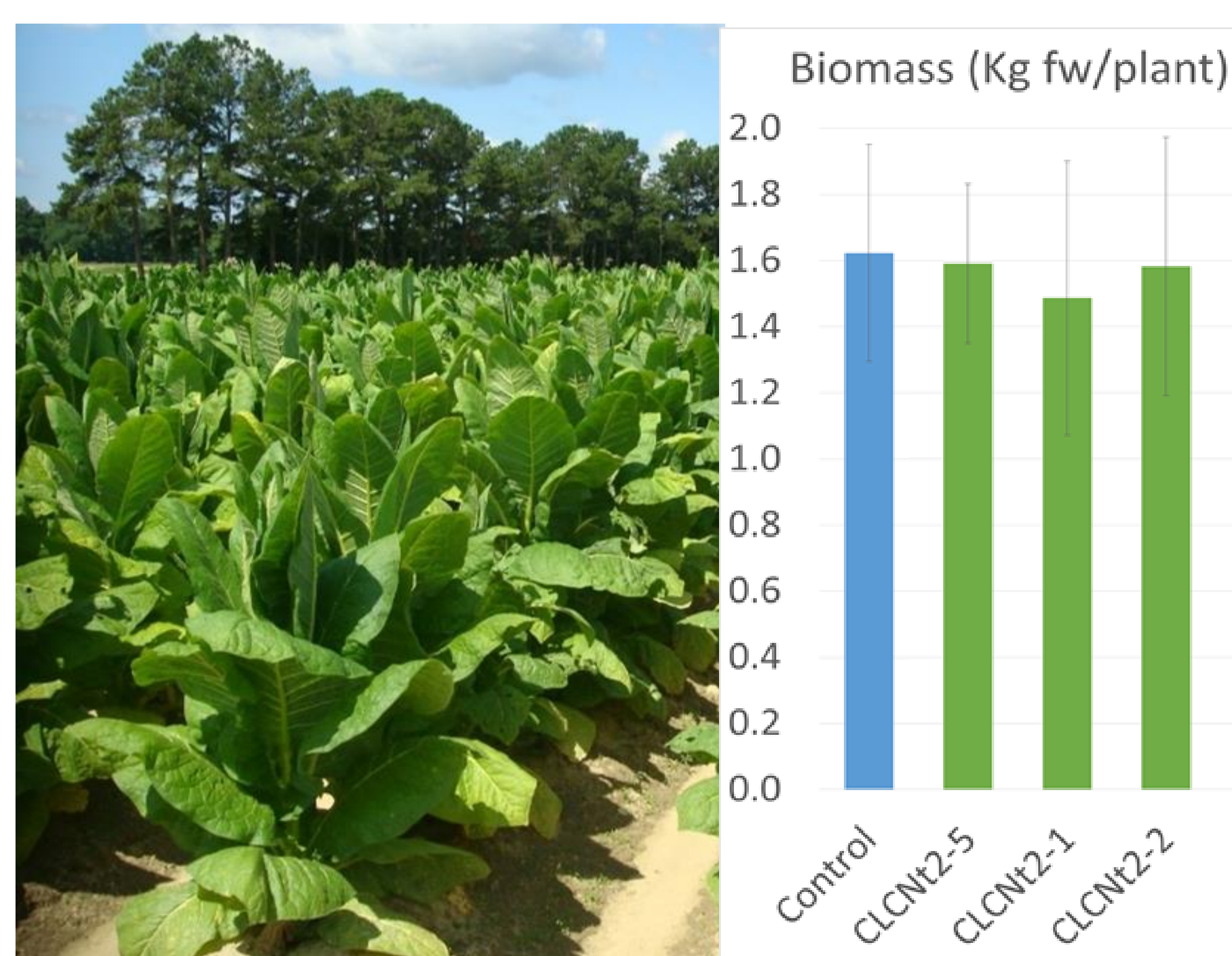
7 and 9 *CLC* genes are identified in *Arabidopsis thaliana* and *Solanum lycopersicum*, respectively. 17 *CLC* genes have been identified in *Nicotiana tabacum* (4).

*AtCLCa* gene product is a Cl<sup>-</sup>/H<sup>+</sup> antiporter localized in the tonoplast membrane mediating the sequestration of NO<sub>3</sub><sup>-</sup> in vacuole (2)

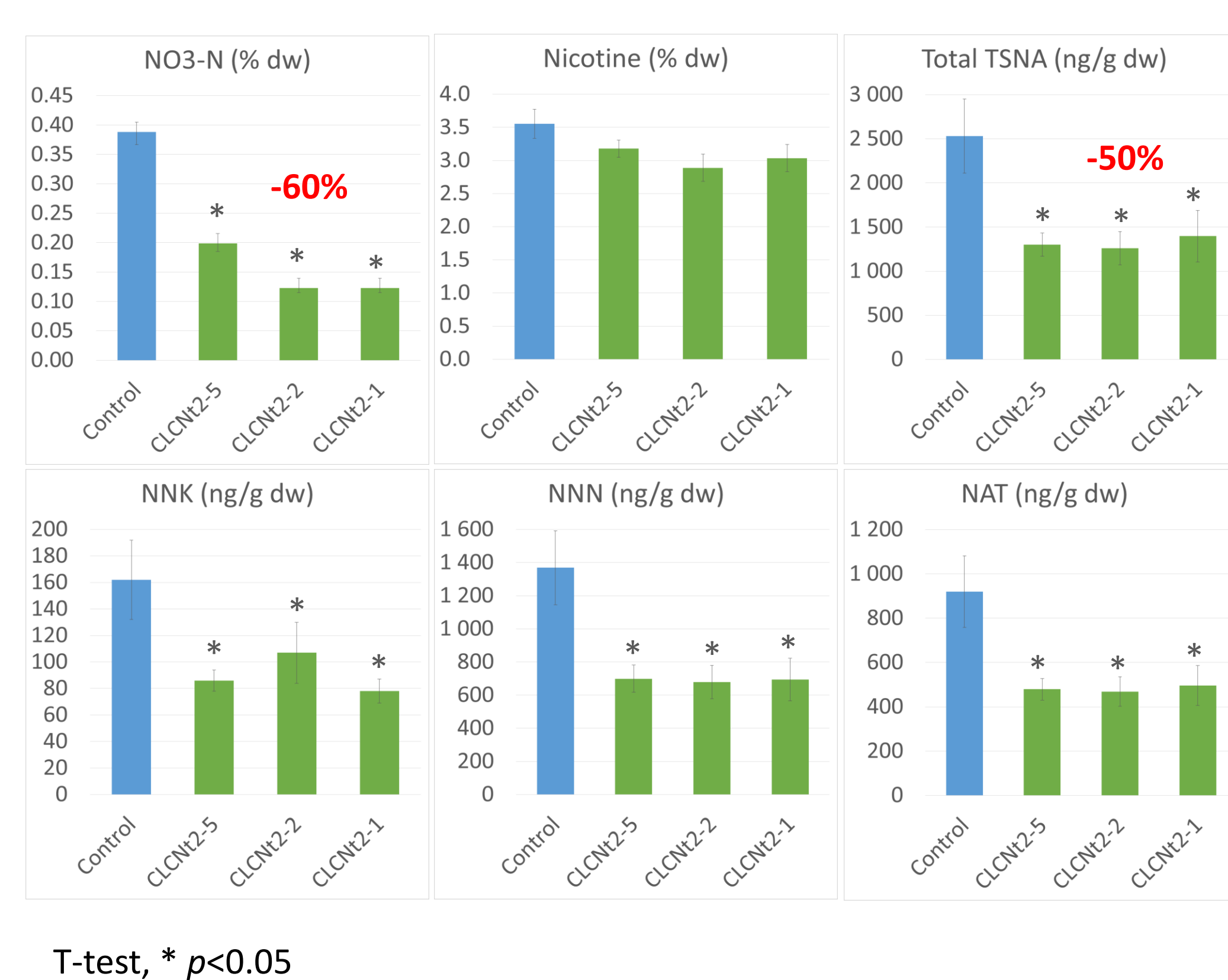
*CLCNt2*, which are the best tobacco orthologues of *AtCLCa* (5), are present in two copies: one originating from *N. sylvestris* (*CLCNt2-S*) and the other one from *N. tomentosiformis* (*CLCNt2-T*).

References : (1) Lv et al., Plant Science, 2009, 176(5), p650-66 (2) De Angeli et al., Nature, 2006, 442, p939-42 (3) De Angeli et al., Biological Science, 2009, 364, p195-201 (4) Zang et al., Gene, 2018, 676, p56-64 (5) Sandré Lurin, theses.fr, 1998

## Impact of *CLCNt2-RNAi* on plant biomass (field 2015)



## Chemistry – cured leaf (field 2015)



T-test, \* *p*<0.05

## Chemistry – cigarette smoke (field 2015-2016)



6 prototype cigarettes, pure grade, 3 cigarettes with field material per year.

## Conclusion

The storage of nitrate (NO<sub>3</sub><sup>-</sup>) in tobacco leaf is reduced under field conditions via the silencing of *CLCNt2*.

- Comparison between *CLCNt2-RNAi* and control plants indicates:
  - No impact on plant biomass
  - 65% lower nitrate levels in leaf
  - 50% lower TSA levels in leaf
  - 45% lower TSA levels in cigarette smoke

A breeding strategy based on loss-of-function of *CLCNt2* can generate plants with conserved agronomic potential and lower generation of total TSNAs.