Tobacco Alkaloid Genetics (TAG) Task Force Annual Report 2020

Coordinator: Yongfeng Guo, TRI-CAAS, China

CORESTA Congress Online

October 2020

Objectives

1. To understand the genetics that control alkaloid formation in tobacco plants.
2. To understand the feasibility of conventional and non-conventional breeding techniques to modify alkaloid formation in tobacco plants.
3. To understand the impact of tobacco alkaloid levels on leaf production and quality.

TAG Task Force

❖ Progress

Project No.	Activity	Status	Time
140	Initiation	Completed	March 2017
	Soliciting participants	Completed	January 2018
	Breaking down the report into 9 subtitles	Completed	February 2018
	Assignment of subtitles to participants (9/12)	Completed	April 2018
	Collecting writings	Completed	October 2018
	First draft	Completed	April 2019

TAG Task Force

❖ Progress

Project No.	Activity	Status	Time
140	Publishing the report in a peer-reviewed journal (accepted by the eBook "Studies in Natural Products Chemistry" (Bioactive Natural Products) published by ELSEVIER SCIENCE PUBLISHERS – AMSTERDAM)	Ongoing	October 2020
	Final report	On-going	October 2020

List of participants

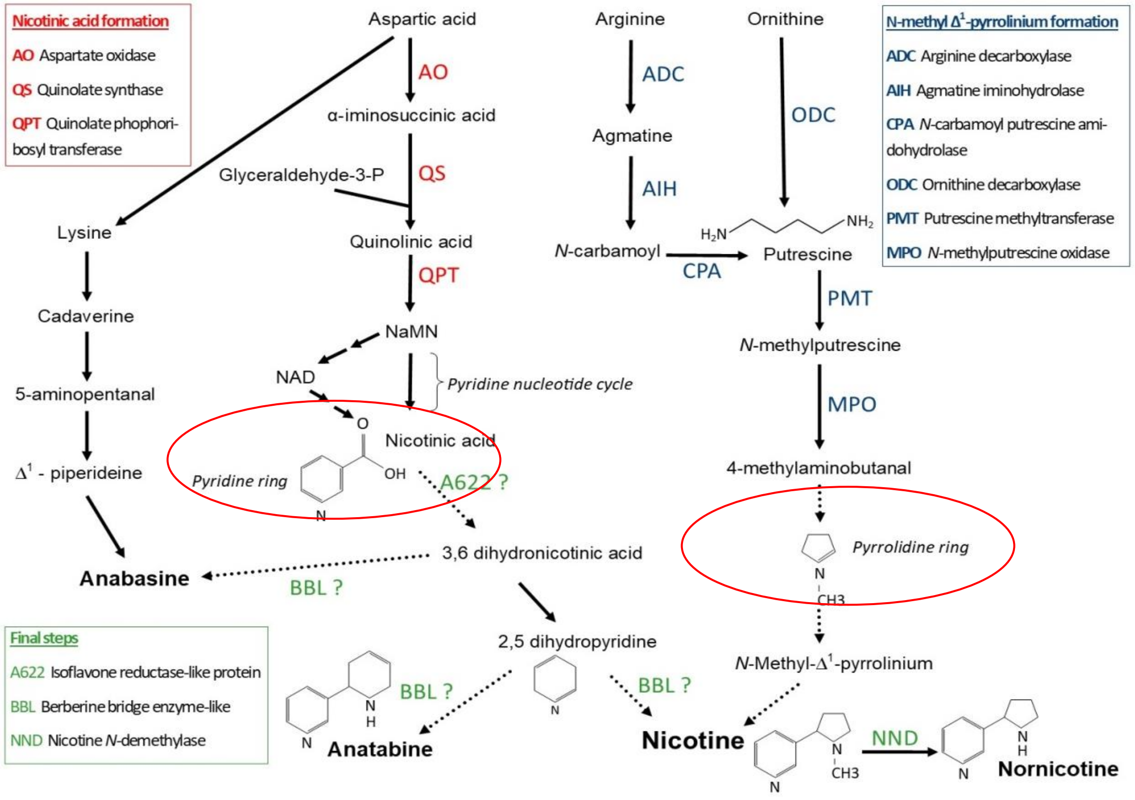
- **Ernie Hiatt** (RJ Reynolds, USA), **Tijs Gilles** (BAT, UK), **Irving J. Berger** (BAT, Brazil), Alkaloid biosynthesis
- **Christelle Bonnet** (JTI, Switzerland), Regulatory mechanisms of alkaloid accumulation
- **Chengalrayan Kudithipudi** (Altria Client Service, USA), Regulatory mechanisms of alkaloid accumulation
- **Ramsey S. Lewis** (NC State University, USA), Traditional breeding for low alkaloid tobacco
- **Hongzhi Shi**, **Mengyue Zhang** (Henan Agricultural University, China), **Marcos Lusso** (Altria Client Services, USA), Leaf production and quality affected by low nicotine tobacco production
- **Barunava Patra**, **Shengming Yang** (University of Kentucky, USA), Transportation of alkaloids
- **Xue Zhao**, **Hongbo Zhang** (Tobacco Research Institute, CAAS, China), Transportation of alkaloids
- **François Dorlhac de Borne** (Imperial Tobacco, France), Introduction
- **Yongfeng Guo**, **Liuying Wen** (Tobacco Research Institute, CAAS, China), **Dongmei Xu** (Altria Client Services, USA), Organization

Structure of the report

- **Title: Molecular regulation and genetic manipulation of alkaloid accumulation in tobacco plants**
- **Abstract**
- **Introduction**
- **Alkaloid biosynthesis in tobacco**
- **Transportation of alkaloids between cells and within the plant**
- **Regulatory mechanisms of alkaloid accumulation in tobacco**
- **Development and use of low alkaloid tobacco**
- **Future prospects for ultra-low alkaloid tobacco**

Alkaloid biosynthesis

1. **Formation of nicotinic acid (the pyridine ring);**
2. **Formation of N-methyl- Δ1-pyrrolinium cation (the pyrrolidine ring);**
3. **Condensation of a pyridine ring and a pyrrolidine ring (A662 and BBLs);**
4. **Nornicotine biosynthesis;**
5. **Anabasine biosynthesis;**
6. **Anatabine biosynthesis;**



Nicotinic acid formation

AO Aspartate oxidase

QS Quinolate synthase

QPT Quinolate phophoribosyl transferase

N-methyl Δ^1-pyrrolinium formation

ADC Arginine decarboxylase

AIH Agmatine iminohydrolase

CPA *N*-carbamoyl putrescine amidohydrolase

ODC Ornithine decarboxylase

PMT Putrescine methyltransferase

MPO *N*-methylputrescine oxidase

Final steps

A622 Isoflavone reductase-like protein

BBL Berberine bridge enzyme-like

NND Nicotine *N*-demethylase

Figure by Christelle Bonnet

Alkaloid transportation

1. **Multidrug and toxic compound extrusion proteins NtMATE 1 and NtMATE2;**
2. **Jasmonate inducible alkaloid transporters JAT1 and JAT2;**
3. **Purine uptake permease-like transporter NUP1;**

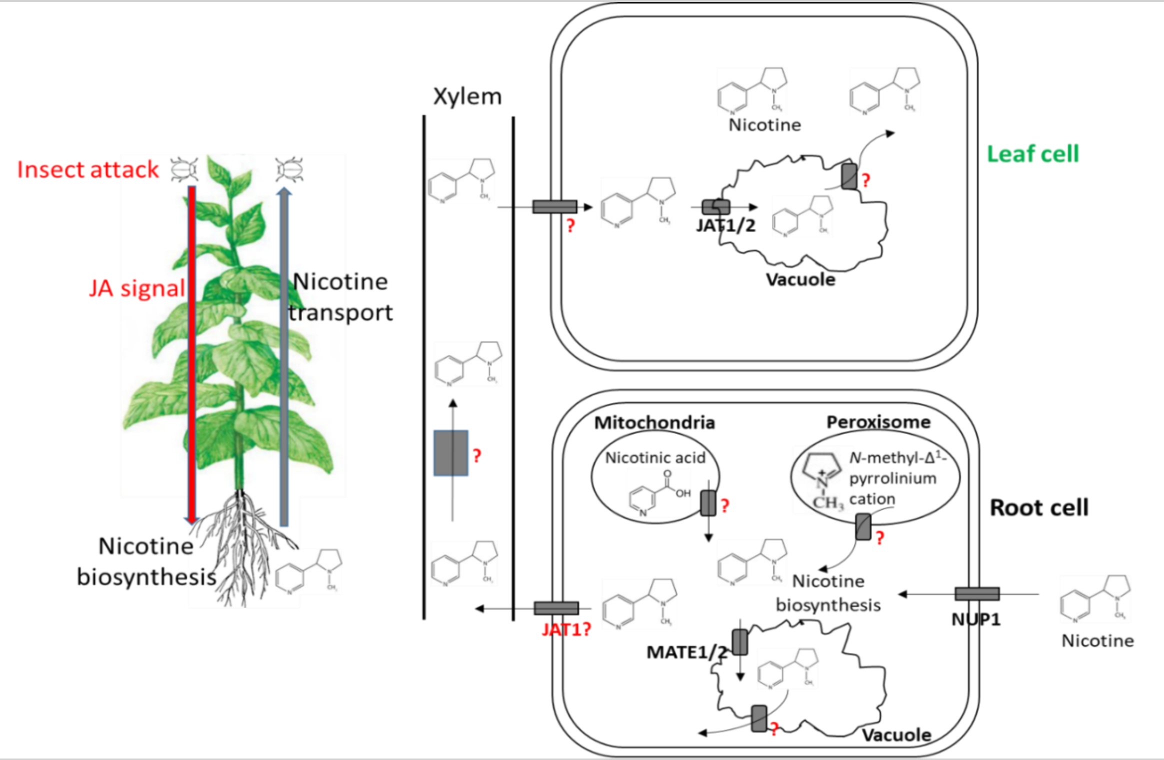


Figure by Shengming Yang

Regulatory mechanisms

1. **The Nic loci and ERF transcription factors;**
2. **The jasmonate pathway and MYC2-like bHLH transcription factors;**
3. **Induction of JA-mediated nicotine accumulation by senescence and abiotic stresses;**
4. **Inhibitory effects of ethylene and auxin on nicotine biosynthesis;**
5. **Small and long non-protein-coding RNAs in nicotine biosynthesis;**

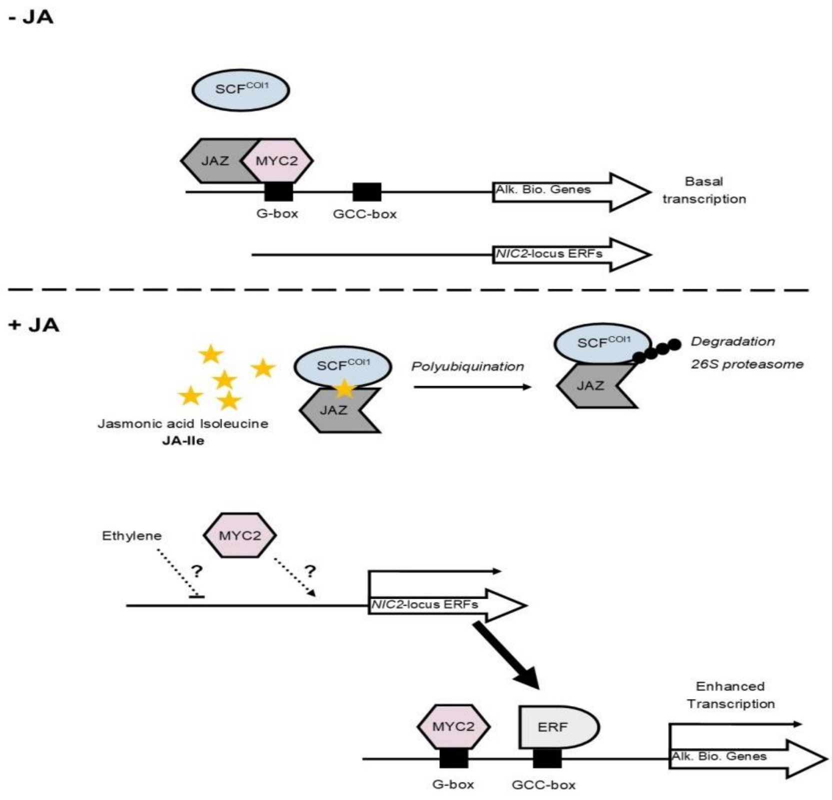


Figure by Christelle Bonnet

Low alkaloid tobacco

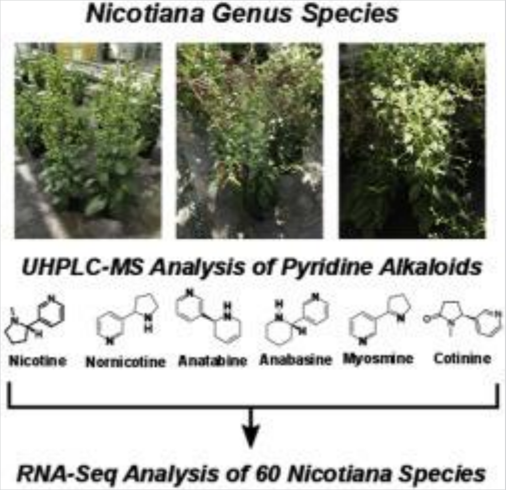
1. **Low alkaloid tobacco developed by traditional breeding;**
2. **Low alkaloid tobacco developed by genetic engineering;**
3. **Reduction of alkaloid accumulation through agronomic practices.**

Table by Ramsey Lewis

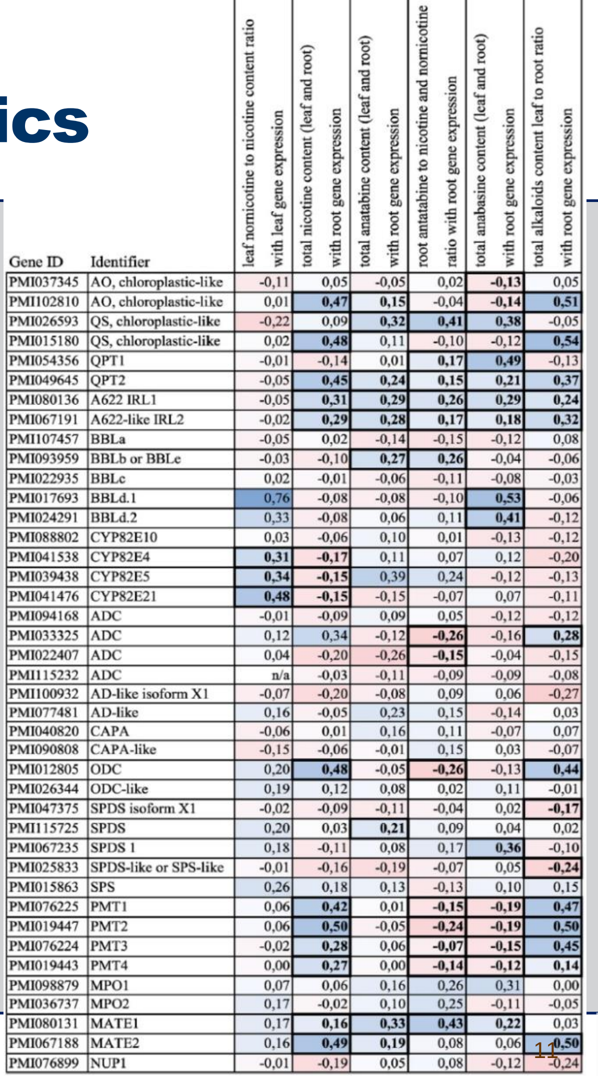
Variability Type	Mechanism	Nicotine (mg/g)	Sample Type	Reference
Wild type	Nic1Nic2	15 – 45	Composite cured leaf sample	Lewis 2018 [121]
Naturally-Occurring	nic1/nic2 (also known as a/b)	2.0 – 2.5[b]	Composite cured leaf sample [c]	Legg and Collins 1971 [118]
		2.99	Composite cured leaf sample	Lewis et al. 2015 [41]
		4.52	Composite cured leaf sample	Lewis 2016 (unpublished data)
Naturally-Occurring	CYP82E4 (nicotine demethylase)	6.45 – 8.33	Fourth leaf from the top	Lewis et al. 2008 [51]
Transgenic	NtQPT1 Antisense	1.44	Composite cured leaf sample	Vector Tobacco Ltd. 2001 [22]
Transgenic	NtPMT Family RNA Interference	0.60	Composite cured leaf sample	Lewis 2014 (unpublished data)
Transgenic	NtPMT Family Co-Suppression	2.20	Composite cured leaf sample	Lewis 2014 (unpublished data)
Transgenic	NtBBL Family RNA Interference	4.14	Composite cured leaf sample	Lewis et al. 2015 [41]
Knockout Mutation	NtBBL Family Inactivation	4.43	Composite cured leaf sample	Lewis 2016 (unpublished data)

Recent progress - omics

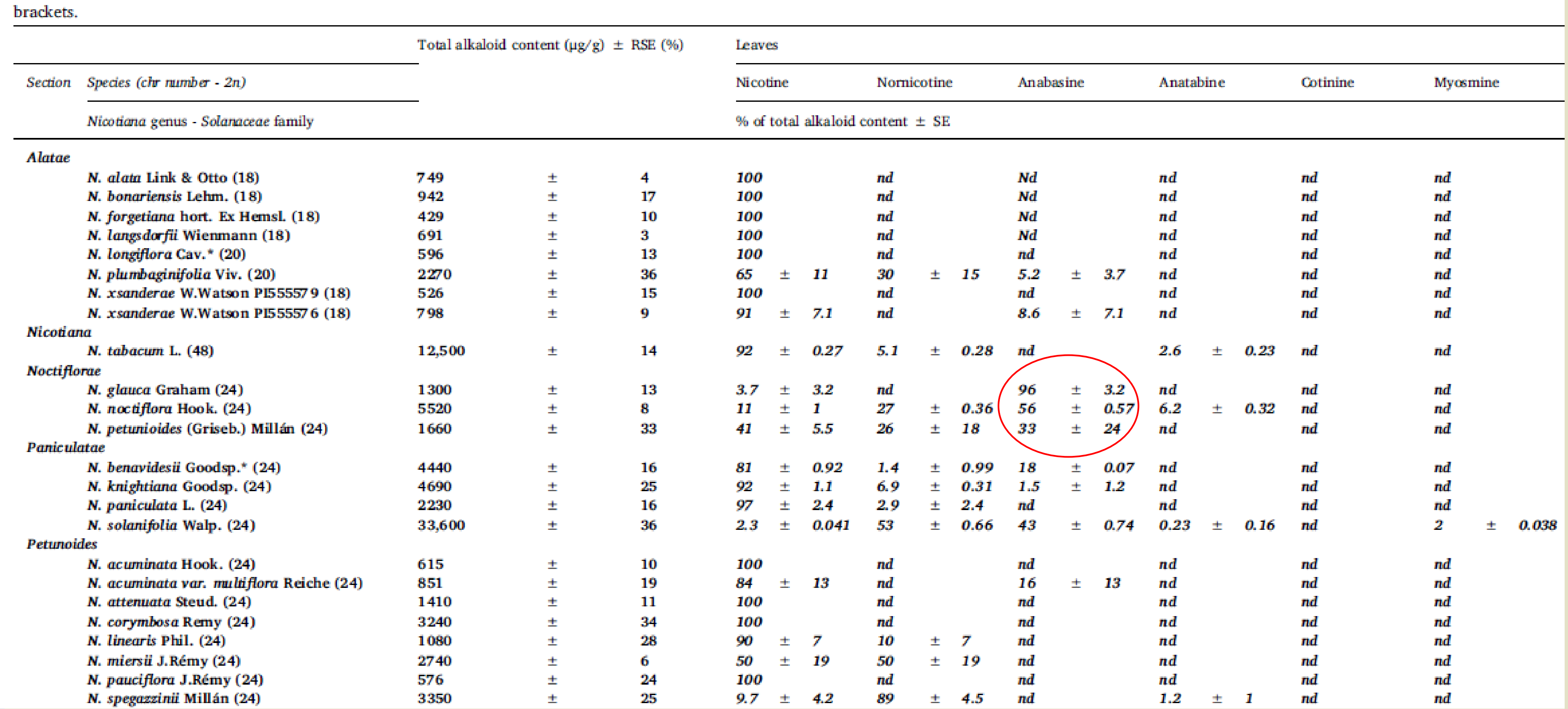
1. **Pyridine alkaloid content in Nicotiana genus correlates with sectional classification.**
2. **Myosmine was detected in 16 *Nicotiana* species.**
3. ***Noctiflorae* and *Suaveolentes* sections accumulated above average levels of anabasine.**
4. **Clustering of *Nicotiana* gene expression reflected sectional classification.**
5. **Correlation of gene expression with alkaloid accumulation was evident.**



K. P. Kaminski et al., Alkaloid chemophenetics and transcriptomics of the Nicotiana genus. Phytochemistry (2020) 177:112424.



Gene ID	Identifier	leaf nornicotine to nicotine content ratio with leaf gene expression	total nicotine content (leaf and root) with root gene expression	total anatabine content (leaf and root) with root gene expression	root antatabine to nicotine and nornicotine ratio with root gene expression	total anabasine content (leaf and root) with root gene expression	total alkaloids content leaf to root ratio with root gene expression
PMI037345	AO, chloroplastic-like	-0,11	0,05	-0,05	0,02	**-0,13**	0,05
PMI102810	AO, chloroplastic-like	0,01	**0,47**	**0,15**	-0,04	**-0,14**	**0,51**
PMI026593	QS, chloroplastic-like	-0,22	0,09	**0,32**	**0,41**	**0,38**	-0,05
PMI015180	QS, chloroplastic-like	0,02	**0,48**	0,11	-0,10	-0,12	**0,54**
PMI054356	QPT1	-0,01	-0,14	0,01	**0,17**	**0,49**	-0,13
PMI049645	QPT2	-0,05	**0,45**	**0,24**	**0,15**	**0,21**	**0,37**
PMI080136	A622 IRL1	-0,05	**0,31**	**0,29**	**0,26**	**0,29**	**0,24**
PMI067191	A622-like IRL2	-0,02	**0,29**	**0,28**	**0,17**	**0,18**	**0,32**
PMI107457	BBLa	-0,05	0,02	-0,14	-0,15	-0,12	0,08
PMI093959	BBLb or BBLe	-0,03	-0,10	**0,27**	**0,26**	-0,04	-0,06
PMI022935	BBLc	0,02	-0,01	-0,06	-0,11	-0,08	-0,03
PMI017693	BBLd.1	0,76	-0,08	-0,08	-0,10	**0,53**	-0,06
PMI024291	BBLd.2	0,33	-0,08	0,06	0,11	**0,41**	-0,12
PMI088802	CYP82E10	0,03	-0,06	0,10	0,01	-0,13	-0,12
PMI041538	CYP82E4	**0,31**	**-0,17**	0,11	0,07	0,12	-0,20
PMI039438	CYP82E5	**0,34**	**-0,15**	0,39	0,24	-0,12	-0,13
PMI041476	CYP82E21	**0,48**	**-0,15**	-0,15	-0,07	0,07	-0,11
PMI094168	ADC	-0,01	-0,09	0,09	0,05	-0,12	-0,12
PMI033325	ADC	0,12	0,34	-0,12	**-0,26**	-0,16	**0,28**
PMI022407	ADC	0,04	-0,20	-0,26	**-0,15**	-0,04	-0,15
PMI115232	ADC	n/a	-0,03	-0,11	-0,09	-0,09	-0,08
PMI100932	AD-like isoform X1	-0,07	-0,20	-0,08	0,09	0,06	-0,27
PMI077481	AD-like	0,16	-0,05	0,23	0,15	-0,14	0,03
PMI040820	CAPA	-0,06	0,01	0,16	0,11	-0,07	0,07
PMI090808	CAPA-like	-0,15	-0,06	-0,01	0,15	0,03	-0,07
PMI012805	ODC	0,20	**0,48**	-0,05	**-0,26**	-0,13	**0,44**
PMI026344	ODC-like	0,19	0,12	0,08	0,02	0,11	-0,01
PMI047375	SPDS isoform X1	-0,02	-0,09	-0,11	-0,04	0,02	**-0,17**
PMI115725	SPDS	0,20	0,03	**0,21**	0,09	0,04	0,02
PMI067235	SPDS 1	0,18	-0,11	0,08	0,17	**0,36**	-0,10
PMI025833	SPDS-like or SPS-like	-0,01	-0,16	-0,19	-0,07	0,05	**-0,24**
PMI015863	SPS	0,26	0,18	0,13	-0,13	0,10	0,15
PMI076225	PMT1	0,06	**0,42**	0,01	**-0,15**	**-0,19**	**0,47**
PMI019447	PMT2	0,06	**0,50**	-0,05	**-0,24**	**-0,19**	**0,50**
PMI076224	PMT3	-0,02	**0,28**	0,06	**-0,07**	**-0,15**	**0,45**
PMI019443	PMT4	0,00	**0,27**	0,00	**-0,14**	**-0,12**	**0,14**
PMI098879	MPO1	0,07	0,06	0,16	0,26	0,31	0,00
PMI036737	MPO2	0,17	-0,02	0,10	0,25	-0,11	-0,05
PMI080131	MATE1	0,17	**0,16**	**0,33**	**0,43**	**0,22**	0,03
PMI067188	MATE2	0,16	**0,49**	**0,19**	0,08	0,06	**0,50**
PMI076899	NUP1	-0,01	-0,19	0,05	0,08	-0,12	-0,24



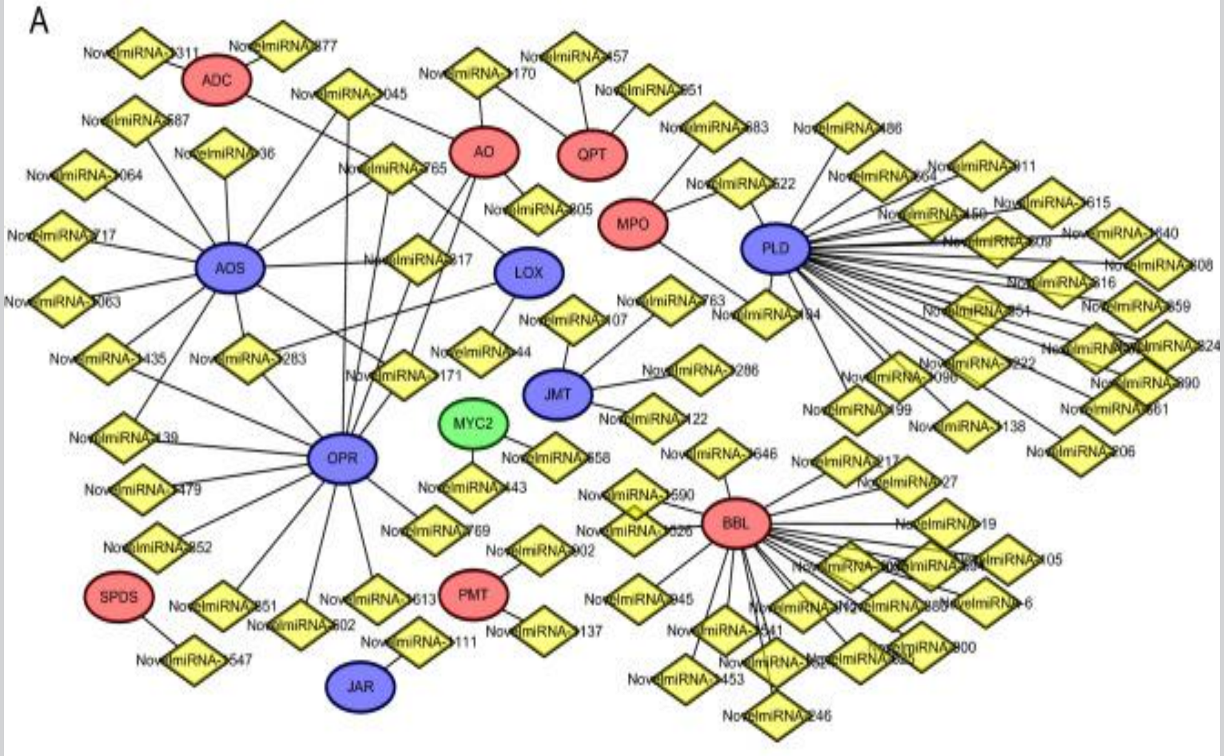
Alkaloid content of *Nicotiana* species in leaves and roots. Total alkaloid content is shown together with relative standard error (RSE), while percentage compositions are shown with simple standard error (SE). Specie name is followed by their botanical authority name abbreviation. A star next to the species name indicates that it did not flower at the time of harvest. The number of chromosomes is shown next to the species name i brackets.

Section	Species (chr number - 2n)	Total alkaloid content (µg/g) ± RSE (%)	Leaves: Nicotine	Nornicotine	Anabasine	Anatabine	Cotinine	Myosmine
	Nicotiana genus - *Solanaceae* family		% of total alkaloid content ± SE					
Alatae								
	N. alata **Link & Otto (18)**	749 ± 4	*100*	*nd*	*Nd*	*nd*	*nd*	*nd*
	N. bonariensis **Lehm. (18)**	942 ± 17	*100*	*nd*	*Nd*	*nd*	*nd*	*nd*
	N. forgetiana **hort. Ex Hemsl. (18)**	429 ± 10	*100*	*nd*	*Nd*	*nd*	*nd*	*nd*
	N. langsdorfii **Wienmann (18)**	691 ± 3	*100*	*nd*	*Nd*	*nd*	*nd*	*nd*
	N. longiflora **Cav.* (20)**	596 ± 13	*100*	*nd*	*nd*	*nd*	*nd*	*nd*
	N. plumbaginifolia **Viv. (20)**	2270 ± 36	*65 ± 11*	*30 ± 15*	*5.2 ± 3.7*	*nd*	*nd*	*nd*
	N. xsanderae **W.Watson PI555579 (18)**	526 ± 15	*100*	*nd*	*nd*	*nd*	*nd*	*nd*
	N. xsanderae **W.Watson PI555576 (18)**	798 ± 9	*91 ± 7.1*	*nd*	*8.6 ± 7.1*	*nd*	*nd*	*nd*
Nicotiana								
	N. tabacum **L. (48)**	12,500 ± 14	*92 ± 0.27*	*5.1 ± 0.28*	*nd*	*2.6 ± 0.23*	*nd*	*nd*
Noctiflorae								
	N. glauca **Graham (24)**	1300 ± 13	*3.7 ± 3.2*	*nd*	*96 ± 3.2*	*nd*	*nd*	*nd*
	N. noctiflora **Hook. (24)**	5520 ± 8	*11 ± 1*	*27 ± 0.36*	*56 ± 0.57*	*6.2 ± 0.32*	*nd*	*nd*
	N. petunioides **(Griseb.) Millán (24)**	1660 ± 33	*41 ± 5.5*	*26 ± 18*	*33 ± 24*	*nd*	*nd*	*nd*
Paniculatae								
	N. benavidesii **Goodsp.* (24)**	4440 ± 16	*81 ± 0.92*	*1.4 ± 0.99*	*18 ± 0.07*	*nd*	*nd*	*nd*
	N. knightiana **Goodsp. (24)**	4690 ± 25	*92 ± 1.1*	*6.9 ± 0.31*	*1.5 ± 1.2*	*nd*	*nd*	*nd*
	N. paniculata **L. (24)**	2230 ± 16	*97 ± 2.4*	*2.9 ± 2.4*	*nd*	*nd*	*nd*	*nd*
	N. solanifolia **Walp. (24)**	33,600 ± 36	*2.3 ± 0.041*	*53 ± 0.66*	*43 ± 0.74*	*0.23 ± 0.16*	*nd*	*2 ± 0.038*
Petunoides								
	N. acuminata **Hook. (24)**	615 ± 10	*100*	*nd*	*nd*	*nd*	*nd*	*nd*
	N. acuminata var. multiflora **Reiche (24)**	851 ± 19	*84 ± 13*	*nd*	*16 ± 13*	*nd*	*nd*	*nd*
	N. attenuata **Steud. (24)**	1410 ± 11	*100*	*nd*	*nd*	*nd*	*nd*	*nd*
	N. corymbosa **Remy (24)**	3240 ± 34	*100*	*nd*	*nd*	*nd*	*nd*	*nd*
	N. linearis **Phil. (24)**	1080 ± 28	*90 ± 7*	*10 ± 7*	*nd*	*nd*	*nd*	*nd*
	N. miersii **J.Rémy (24)**	2740 ± 6	*50 ± 19*	*50 ± 19*	*nd*	*nd*	*nd*	*nd*
	N. pauciflora **J.Rémy (24)**	576 ± 24	*100*	*nd*	*nd*	*nd*	*nd*	*nd*
	N. spegazzinii **Millán (24)**	3350 ± 25	*9.7 ± 4.2*	*89 ± 4.5*	*nd*	*1.2 ± 1*	*nd*	*nd*

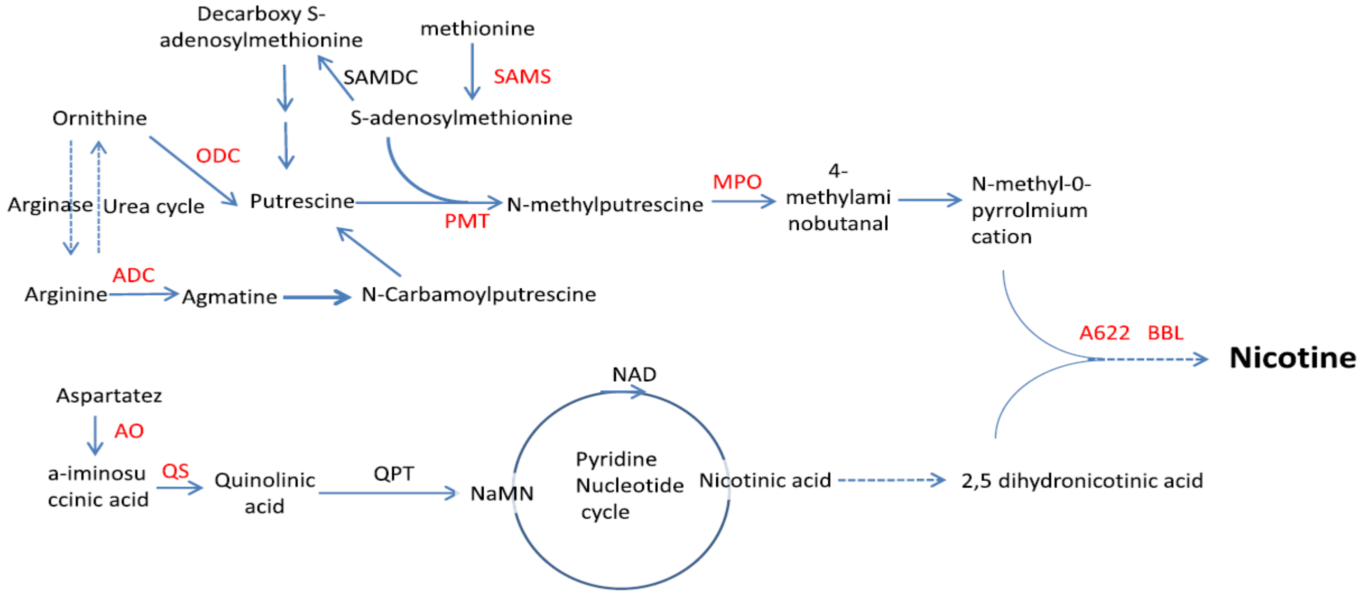
Recent progress - omics

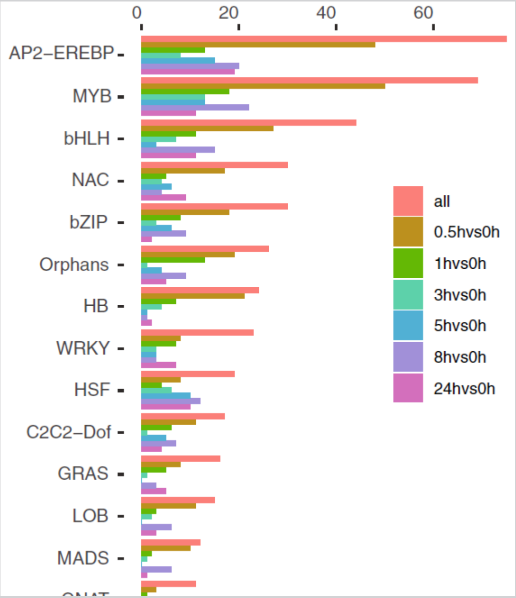
Analysis of high and low nicotine flue-cured tobacco indicated that 6 nicotine biosynthetic genes and 7 jasmonate pathway genes were predicted to be targeted by 77 miRNA loci.

J. Jin et al., Degradome, small RNAs and transcriptome sequencing of a high-nicotine cultivated tobacco uncovers miRNA's function in nicotine biosynthesis. Sci. Rep. (2020), 16;10(1):11751.



Recent progress - omics



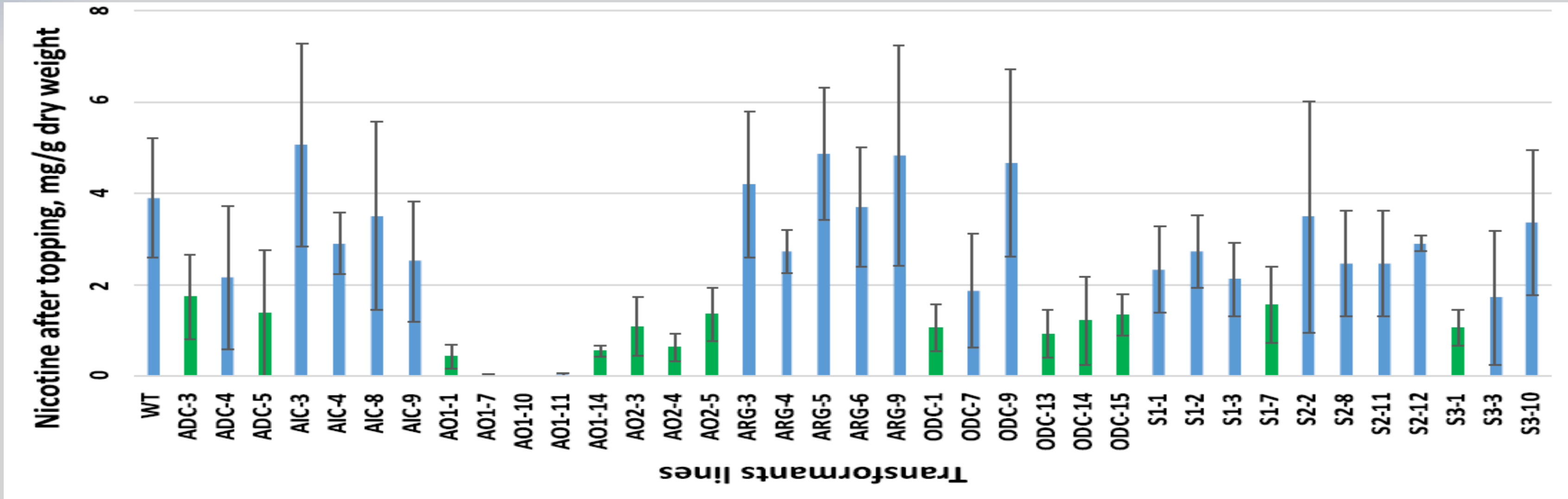


Genes involved in biosynthesis and transport of nicotine are up-regulated within 24 h after topping.

Y. Qin et al., Transcriptome analysis reveals key genes involved in the regulation of nicotine biosynthesis at early time points after topping in tobacco (*Nicotiana tabacum* L.). BMC Plant Biology (2020) 20:30

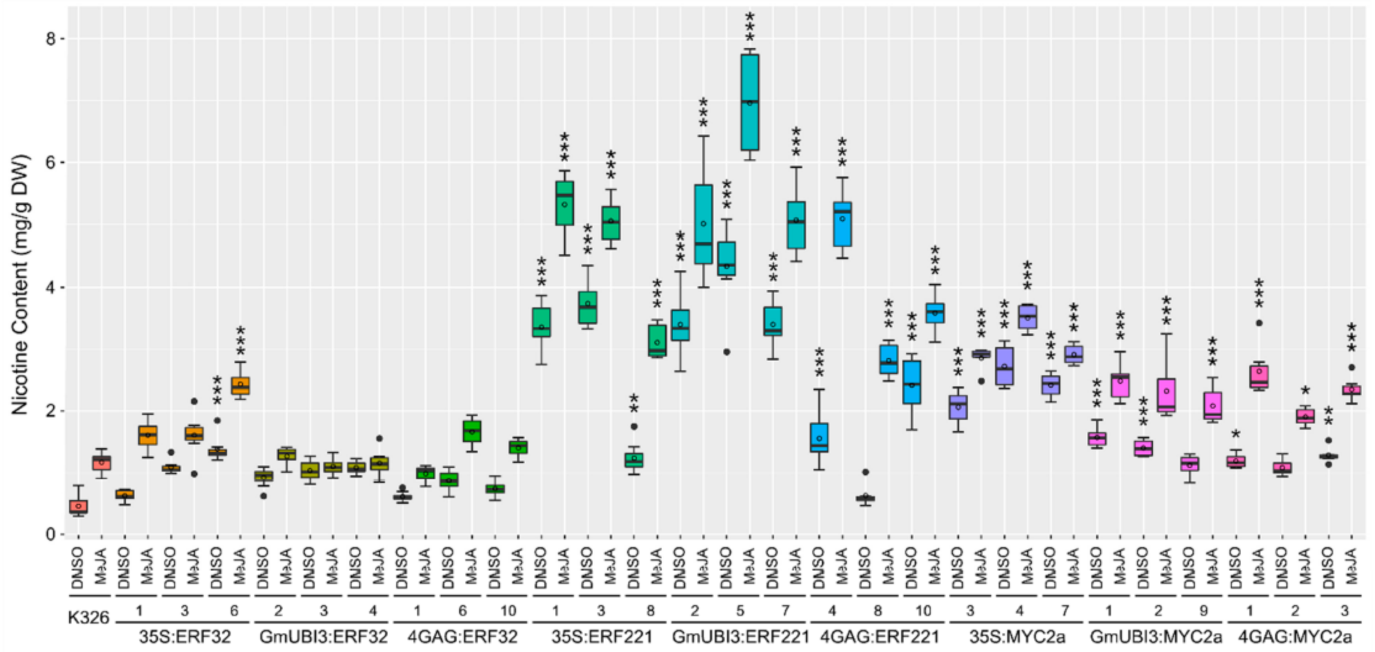
Recent progress – genetic manipulation

Downregulation of alkaloid biosynthesis genes ornithine decarboxylase (ODC), arginine decarboxylase (ADC), and aspartate oxidase (AO) resulted in viable plants with a significantly lower nicotine content.



D. H. Martinez et al., Genetic attenuation of alkaloids and nicotine content in tobacco (*Nicotiana tabacum*). Planta (2020) 251:92 .

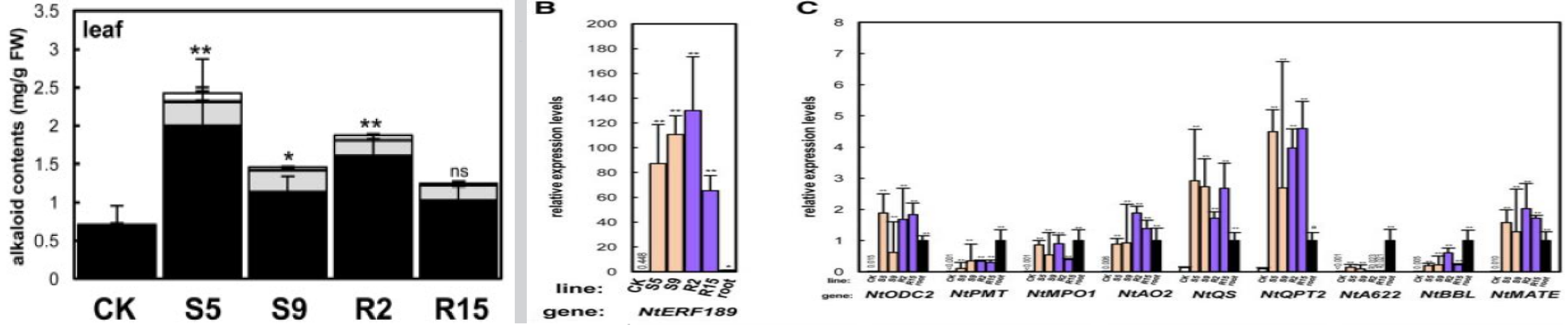
Recent progress – genetic manipulation



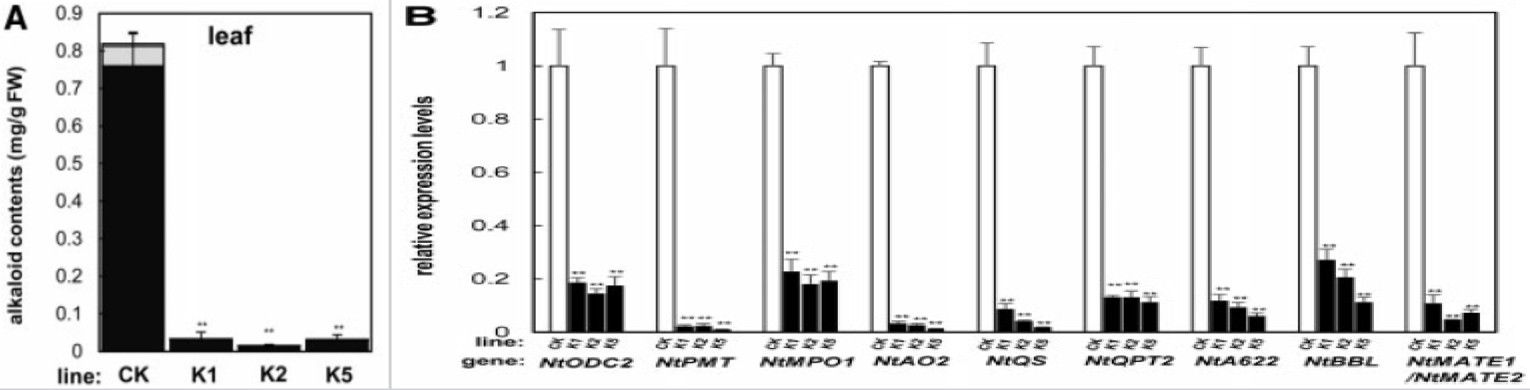
H. Liu et al., Increased Leaf Nicotine Content by Targeting Transcription Factor Gene Expression in Commercial Flue-Cured Tobacco (*Nicotiana tabacum* L.). Genes (Basel) (2019) 10(11): 930.

Overexpression of the tobacco *NtERF32*, *NtERF221/ORC1*, and *NtMYC2a* TFs leads to significant increases in nicotine accumulation.

Recent progress – genetic manipulation



S. Hayashiet al., Genetic Manipulation of Transcriptional Regulators Alters Nicotine Biosynthesis in Tobacco. Plant and Cell Physiology (2020) 61(6) :1041–1053.

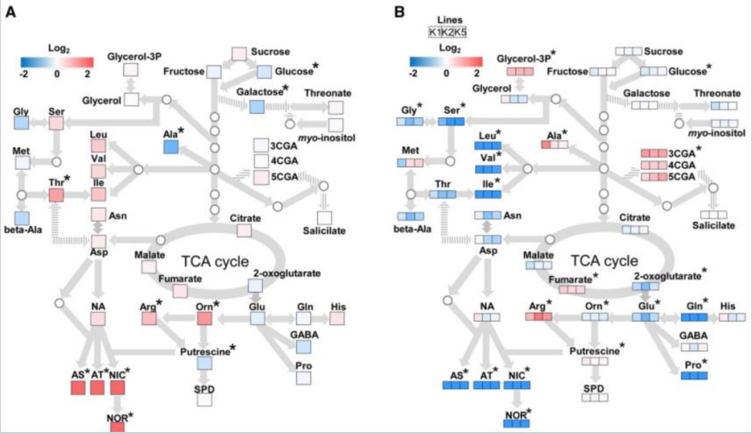


Recent progress – genetic manipulation

1. **Constitutive and leaf-specific overexpression of *NtERF189* increased the accumulation of foliar alkaloids in transgenic tobacco plants but negatively affected plant growth.**

2. **In a knockout mutant of NtERF189 and NtERF199 obtained through CRISPR/Cas9-based genome editing, alkaloid levels were drastically reduced without causing major growth defects.**

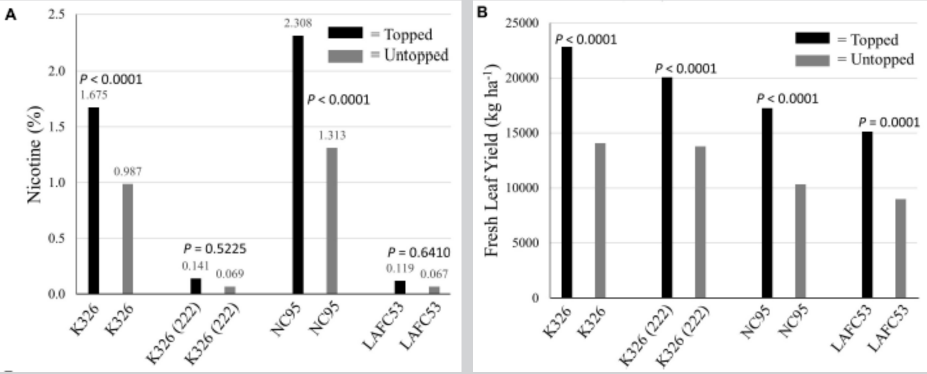
3. **NtERF189 and NtERF199 are primary transcriptional regulators of nicotine biosynthesis**

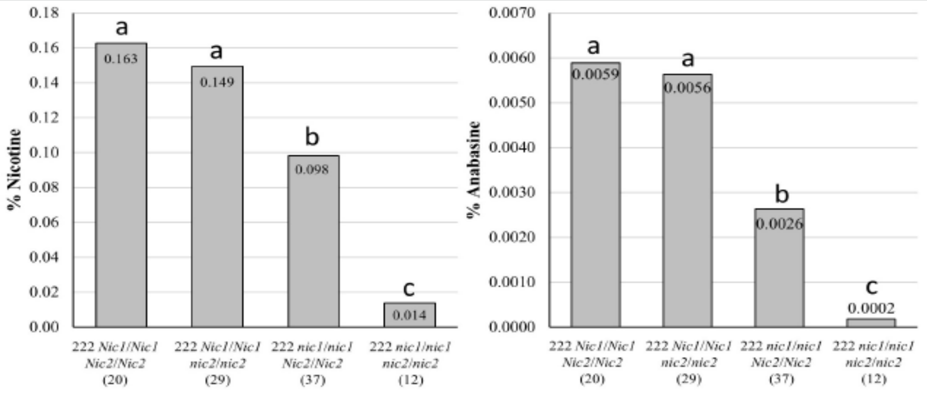


Recent progress – genetic manipulation

1. **Significant reductions (up to 17-fold) in percent leaf nicotine were observed combined mutations in BBL-a, BBL-b, and BBL-c. The addition of mutations in BBL-d1, BBL-d2, and BBL-e had no additional significant.**
2. **Reduced nicotine levels were associated with reductions in cured leaf yields (up to 29 %) and cured leaf quality (up to 15 %).**
3. **BBL mutations combined with Nic1 and Nic2 loci exhibited further reductions in percent nicotine（0.014 %）.**
4. **Existence of a minor, alternative pathway for nicotine biosynthesis in *N. tabacum*.**

R. S. Lewis et al., Genetic and Agronomic Analysis of Tobacco Genotypes Exhibiting Reduced Nicotine Accumulation Due to Induced Mutations in Berberine Bridge Like (BBL) Genes. Front Plant Sci (2020) 11: 368.





Recent progress – other approaches

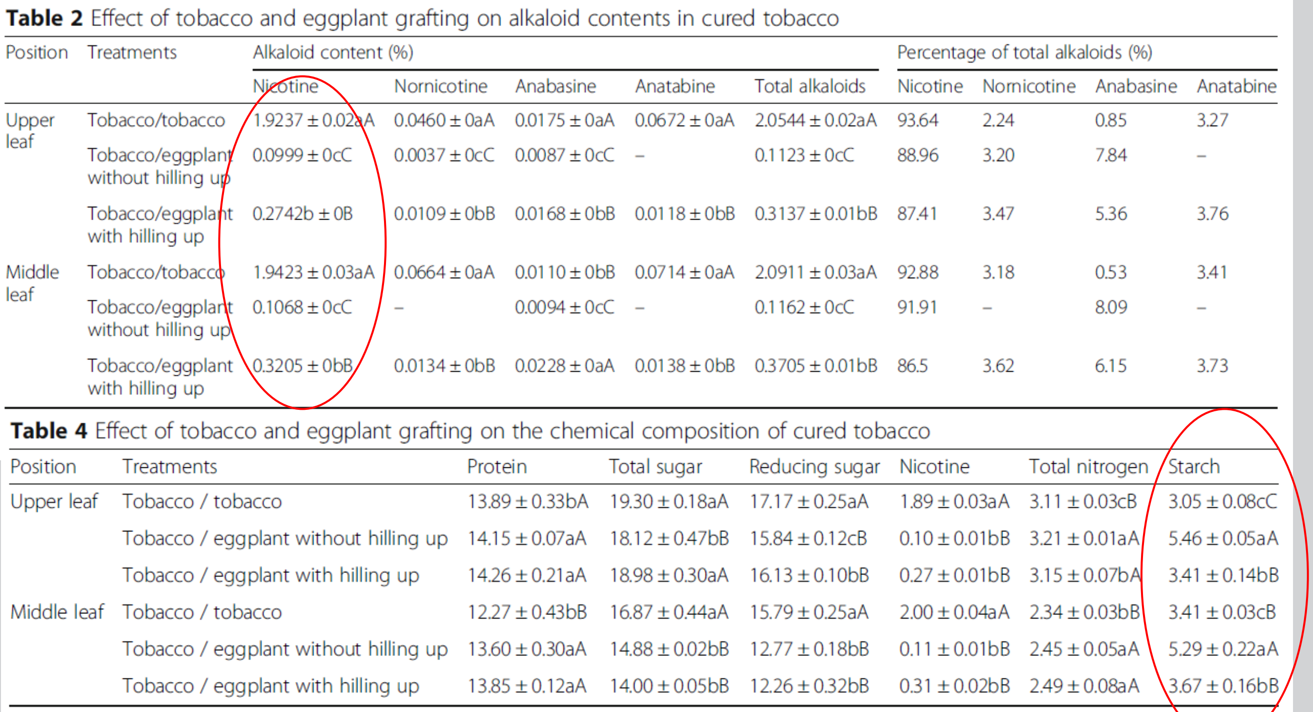


Table 2 Effect of tobacco and eggplant grafting on alkaloid contents in cured tobacco

Position	Treatments	Alkaloid content (%)					Percentage of total alkaloids (%)			
		Nicotine	Nornicotine	Anabasine	Anatabine	Total alkaloids	Nicotine	Nornicotine	Anabasine	Anatabine
Upper leaf	Tobacco/tobacco	1.9237 ± 0.02aA	0.0460 ± 0aA	0.0175 ± 0aA	0.0672 ± 0aA	2.0544 ± 0.02aA	93.64	2.24	0.85	3.27
	Tobacco/eggplant without hilling up	0.0999 ± 0cC	0.0037 ± 0cC	0.0087 ± 0cC	–	0.1123 ± 0cC	88.96	3.20	7.84	–
	Tobacco/eggplant with hilling up	0.2742b ± 0B	0.0109 ± 0bB	0.0168 ± 0bB	0.0118 ± 0bB	0.3137 ± 0.01bB	87.41	3.47	5.36	3.76
Middle leaf	Tobacco/tobacco	1.9423 ± 0.03aA	0.0664 ± 0aA	0.0110 ± 0bB	0.0714 ± 0aA	2.0911 ± 0.03aA	92.88	3.18	0.53	3.41
	Tobacco/eggplant without hilling up	0.1068 ± 0cC	–	0.0094 ± 0cC	–	0.1162 ± 0cC	91.91	–	8.09	–
	Tobacco/eggplant with hilling up	0.3205 ± 0bB	0.0134 ± 0bB	0.0228 ± 0aA	0.0138 ± 0bB	0.3705 ± 0.01bB	86.5	3.62	6.15	3.73

Table 4 Effect of tobacco and eggplant grafting on the chemical composition of cured tobacco

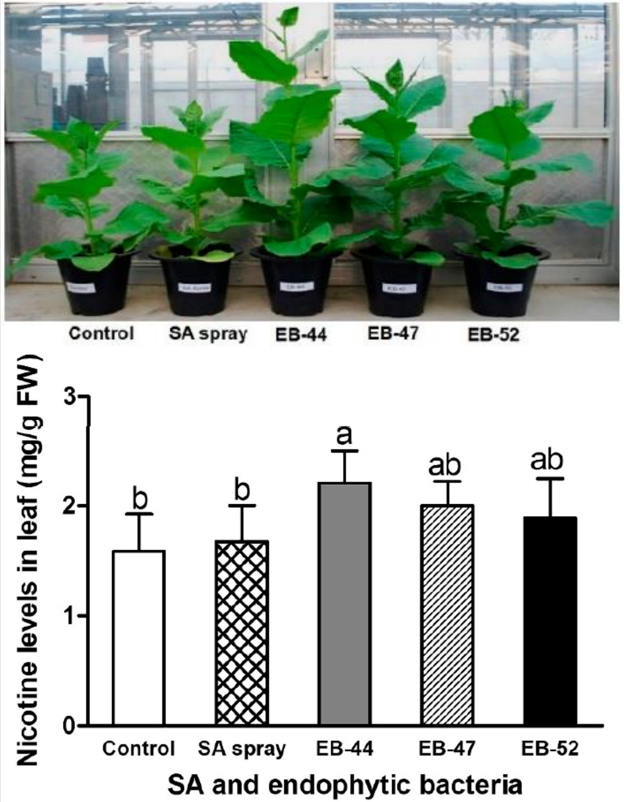
Position	Treatments	Protein	Total sugar	Reducing sugar	Nicotine	Total nitrogen	Starch
Upper leaf	Tobacco / tobacco	13.89 ± 0.33bA	19.30 ± 0.18aA	17.17 ± 0.25aA	1.89 ± 0.03aA	3.11 ± 0.03cB	3.05 ± 0.08cC
	Tobacco / eggplant without hilling up	14.15 ± 0.07aA	18.12 ± 0.47bB	15.84 ± 0.12cB	0.10 ± 0.01bB	3.21 ± 0.01aA	5.46 ± 0.05aA
	Tobacco / eggplant with hilling up	14.26 ± 0.21aA	18.98 ± 0.30aA	16.13 ± 0.10bB	0.27 ± 0.01bB	3.15 ± 0.07bA	3.41 ± 0.14bB
Middle leaf	Tobacco / tobacco	12.27 ± 0.43bB	16.87 ± 0.44aA	15.79 ± 0.25aA	2.00 ± 0.04aA	2.34 ± 0.03bB	3.41 ± 0.03cB
	Tobacco / eggplant without hilling up	13.60 ± 0.30aA	14.88 ± 0.02bB	12.77 ± 0.18bB	0.11 ± 0.01bB	2.45 ± 0.05aA	5.29 ± 0.22aA
	Tobacco / eggplant with hilling up	13.85 ± 0.12aA	14.00 ± 0.05bB	12.26 ± 0.32bB	0.31 ± 0.02bB	2.49 ± 0.08aA	3.67 ± 0.16bB

Ren et al., Reducing the nicotine content of tobacco by grafting with eggplant. BMC Plant Biology (2020) 20:285

1. **Replacing tobacco root with eggplant by grafting produced tobacco leaves with ultra-low nicotine level.**
2. **The contents of nornicotine, anabasine, NNN, NNK, NAT, total TSNAs and nicotine level of mainstream cigarette smoke decreased.**
3. **The contents of amino acids and the precursors of alkaloids increased in grafted tobacco.**

Recent progress – other approaches

SA-prodicing endophytic bacteria (EB) isolated from plants were used in treating tobacco plants. EB-44 can successfully colonize Nicotiana spp., leading to increased endogenous SA production and resistance to tobacco wildfire disease, as well as increased nicotine accumulation.



Md. Nurul Islam et al., Salicylic Acid-Producing Endophytic Bacteria Increase Nicotine Accumulation and Resistance against Wildfire Disease in Tobacco Plants. Microorganisms (2020) 8(1): 31.

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- **Last meeting-Zimbabwe 12 Oct, 2019**
- **Work Completed**
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