

# FUNCTIONAL GENOMICS APPROACHES TO HARM REDUCTION IN TOBACCO PRODUCTS



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## **Tobacco use is a leading preventable cause of disease, disability, and death worldwide**

In 2009 the National Survey on Drug Use and Health (NSDUH) reported that 27.7% of the US population age 12 and older (~ 70 million people) used a tobacco product at least once in the month prior to being interviewed.

58.7 million cigarette smokers (23.3 % of the population)

13.3 million smoked cigars (5.3%)

8.6 million used smokeless tobacco (3.5%)

2.1 million smoked tobacco in pipes (0.8%)

<http://oas.samhsa.gov/NSDUH/2k9NSDUH/2k9Results.htm>

The World Health Organization (WHO) estimates that there are ~ 1.1 billion regular smokers in the world today, and ~3 million deaths caused by tobacco use every year.

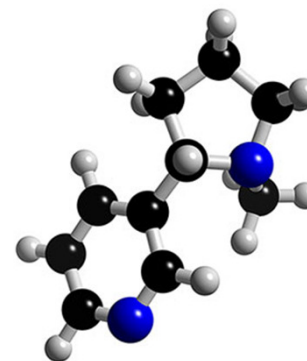
The Centers for Disease Control and Prevention (CDCP) reported that cigarette smoking results in more than 443,000 premature deaths in the US each year — about 1 in 5 deaths — and an additional 8.6 million people suffer with a serious illness caused by smoking.

Most consumers of tobacco products are unwilling or unable to give up their use

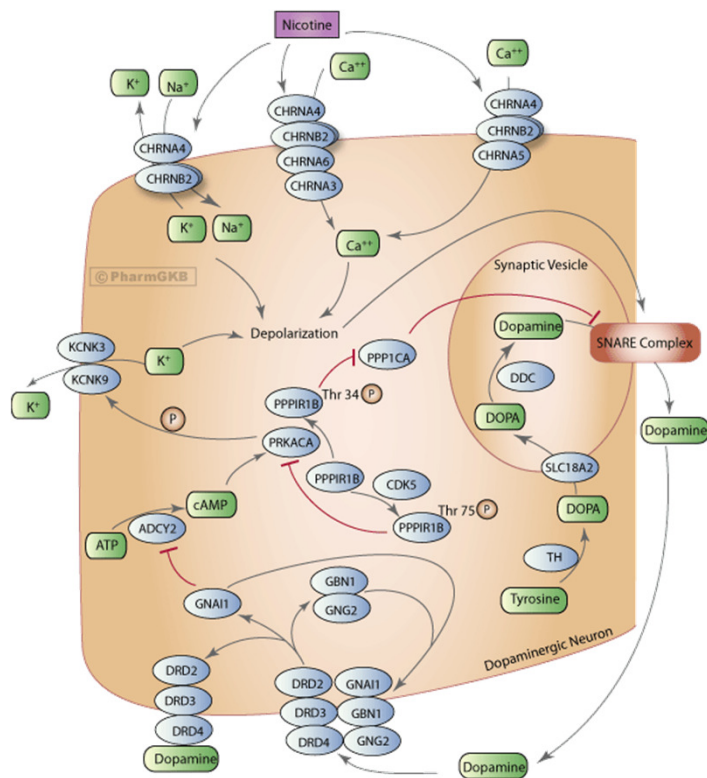
The primary reason people use tobacco products is to obtain **nicotine**.

Accounts for ~ 95% of the total alkaloid content in commercial tobacco

Nornicotine and anatabine are the most abundant minor alkaloids  
~2-3% of the total each; anabasine ~ 0.3%.



(S)-3-(1-methylpyrrolidin-2-yl) pyridine



Source: PharmGKB and Stanford University

Nicotine is readily absorbed into the bloodstream from a chewed, inhaled, or smoked tobacco product.

Nicotine distributes quickly, crosses the blood-brain barrier to enter the central nervous system (CNS) where it binds ganglion and CNS-type nicotinic acetylcholine receptors (nAChRs) triggering epinephrine (adrenaline) release which stimulates the CNS and transiently increases blood pressure, respiration, and heart rate.

Nicotine also increases levels of the neurotransmitter dopamine that affects the brain pathways for reward and pleasure.

Numerous pathological conditions (lung and oral cancers, chronic obstructive pulmonary disease (COPD), cardiovascular (CV) and respiratory disorders) are linked to chronic smoking and use of other forms of tobacco products and exposure to nicotine and its metabolized derivatives in the body.

Tobacco products contain a complex mixture of > 4000 chemical constituents



In the absence of complete cessation of use, the provision of tobacco products to the consumer with reduced levels of "harmful" constituents is an acceptable goal

## **Our Research Goals:**

### **Broadly understand the integrated circuitry that regulates the formation and accumulation of nicotine and related alkaloids in plant cells**

- what genes are responsible for the biosynthesis of the major and minor alkaloid of tobacco
- how are these genes regulated [transcriptional machinery - regulatory elements and protein factors]
- what cellular processes control metabolite flux and alkaloid accumulation

### **Use our acquired knowledge to selectively alter leaf chemistry and composition to reduce levels of "harm components" in tobacco products**

- major (nicotine, nor nicotine) / minor (anatabine, anabasine, anatabine, myosmine) alkaloids
- tobacco specific nitrosamine (TSNA) levels in "cured" and "fermented" tobacco products
- various N-rich substrates, simple and complex sugars (lignins), etc that form particulates and volatiles.

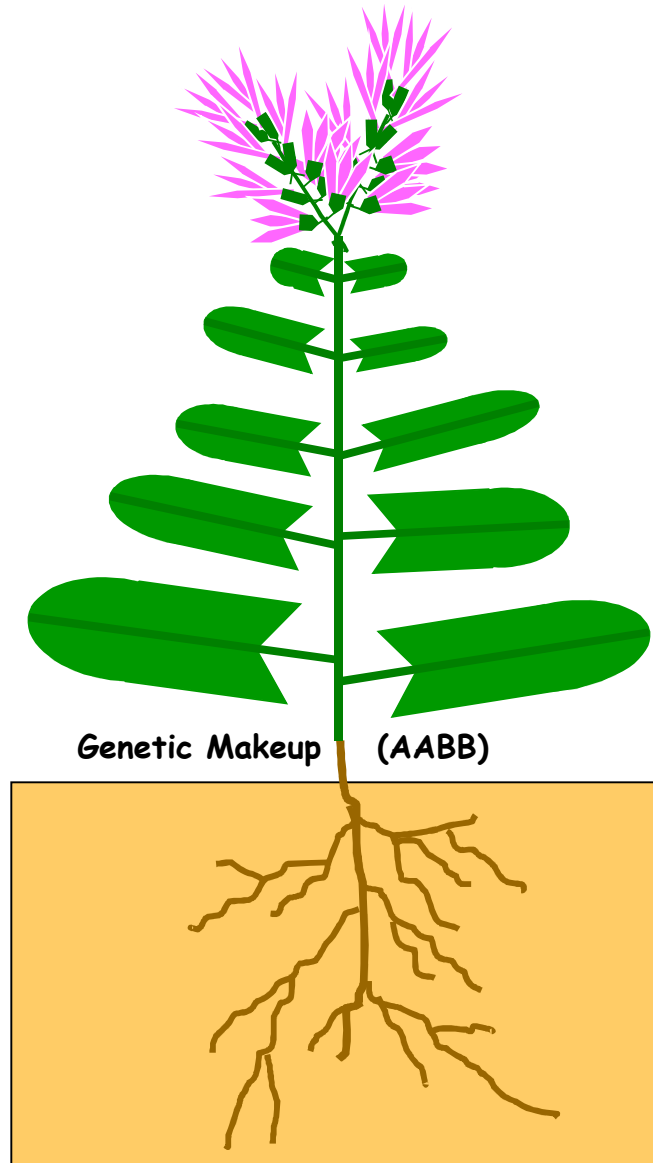
### **Evaluate whether these "Harm Reduced" tobacco products demonstrate a differential ability to influence (potentiate, activate, repress) human cellular process.**

- establish in vitro human lung and oral epithelial cell culture exposure systems
- use transcriptomic, proteomic and metabolomic approaches to describe the effects of whole smoke, smoke condensate (CS), and tobacco soluble extracts (SE) on cellular function

**A large number of biotic and abiotic factors influence tobacco growth and the biosynthesis and accumulation of tobacco alkaloids**

**Abiotic Influences**

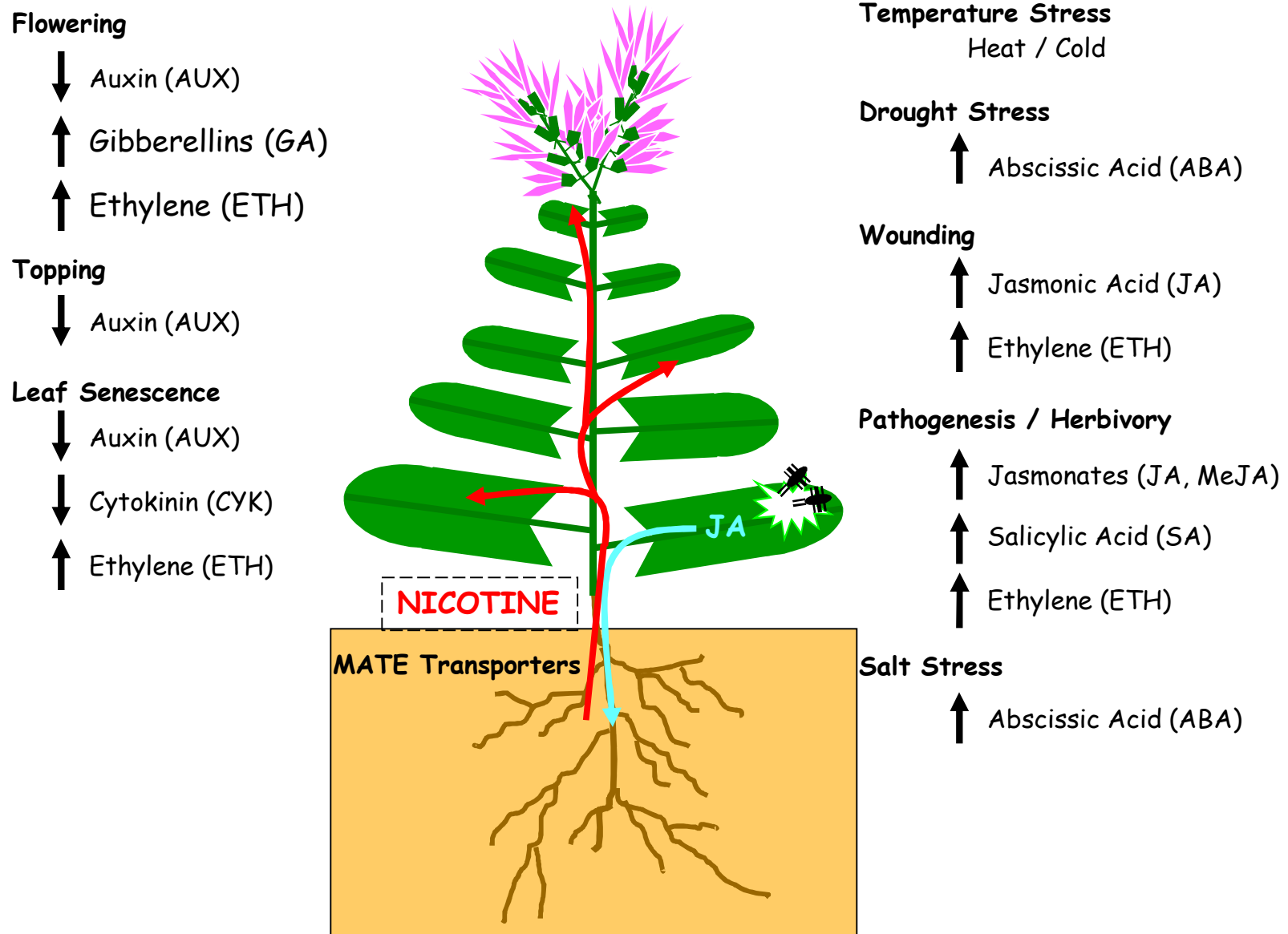
- Light (daylength)
- Temperature
- Humidity
- Water availability  
(yearly/cropping season)
- Mechanical wounding  
(Tilling)
- Pesticide/Herbicide  
Treatment
- Soil Nutrition  
(N, P, K, S, Ca, etc)



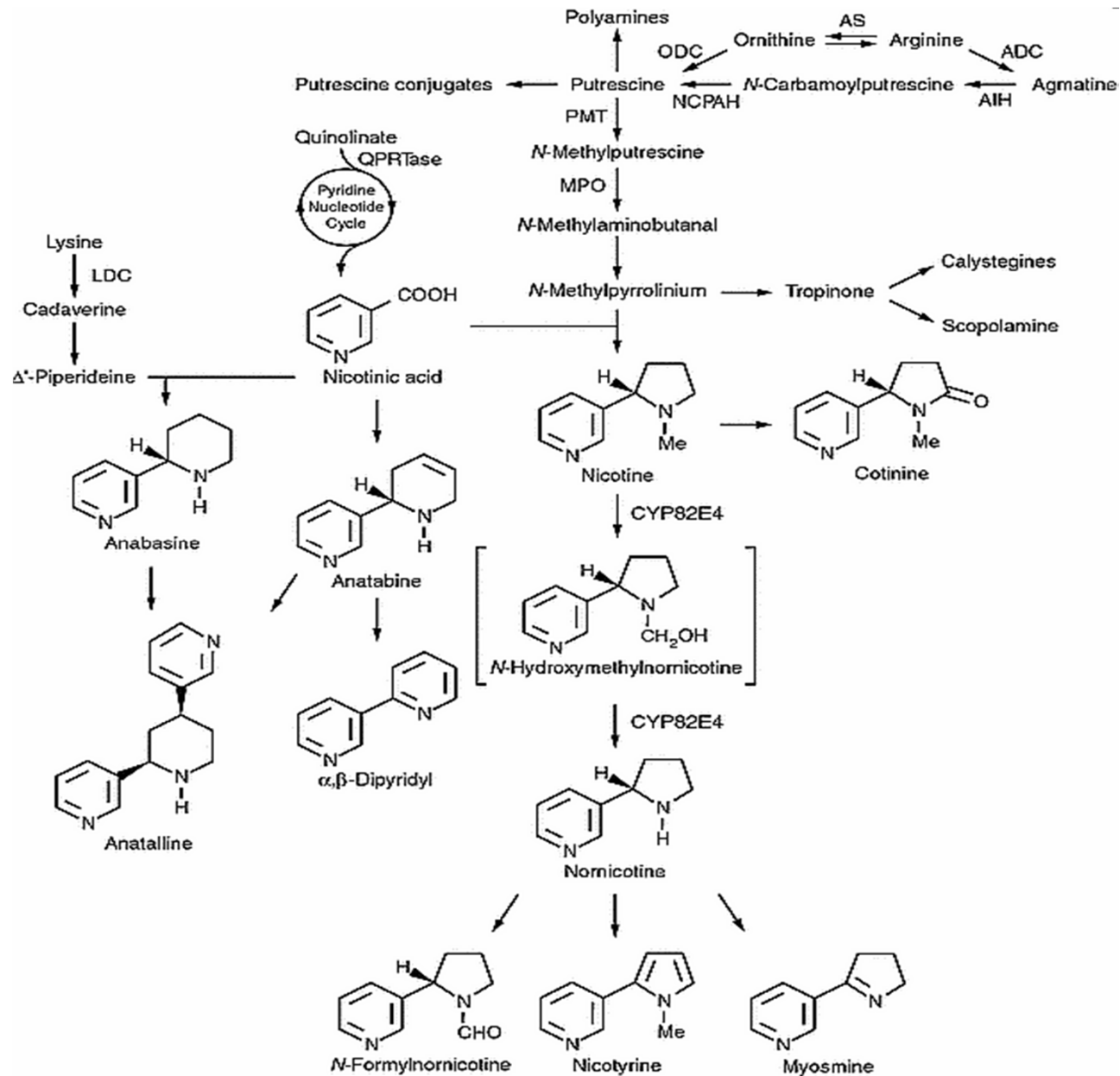
**Biotic Influences**

- Disease Agents
  - Viruses
  - Bacteria
  - Fungi
  - Nematodes
  - Parasitic plants
- Herbivores
- Phytohormonal changes  
(Developmental)  
(Inducible)

Phytohormonal changes underlie the control of nicotine and total leaf alkaloid content

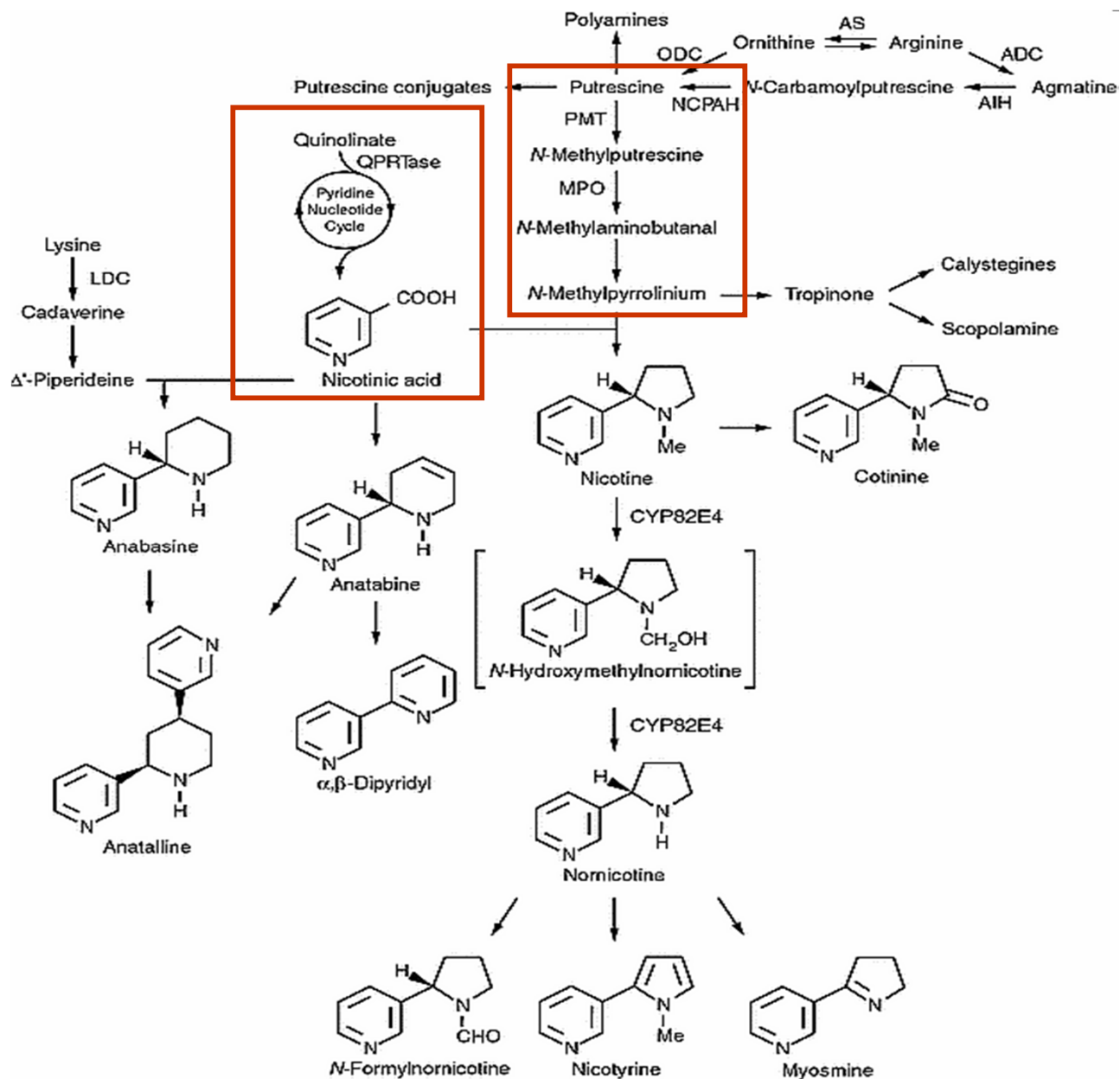


The basic biochemistry of nicotine and minor alkaloid biosynthesis in tobacco is known

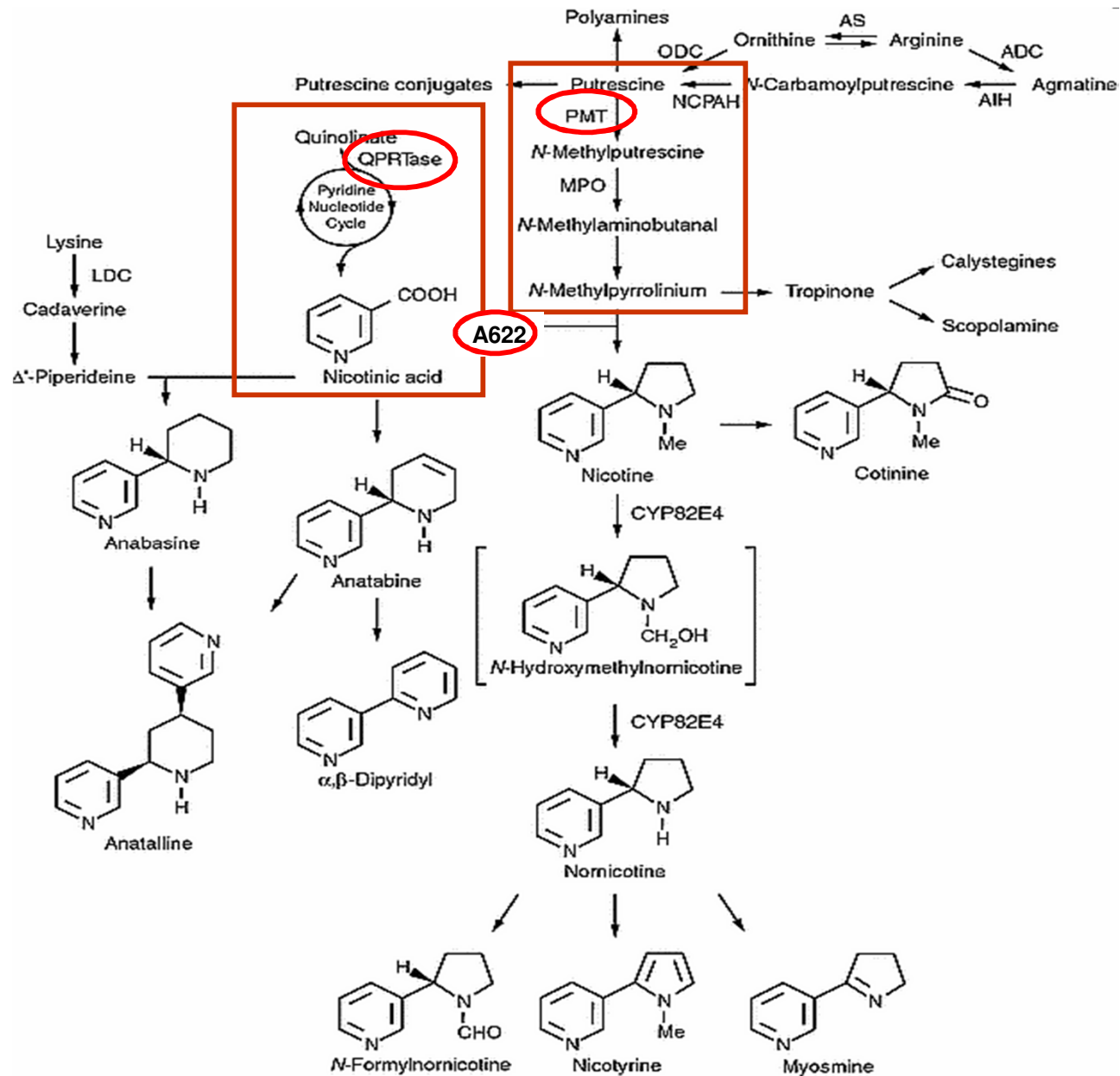




Two main branches contribute to nicotine formation - one leading to a pyridine ring (nicotinic acid) and the other a N-methylpyrrolidine ring



Quinolinic acid phosphoribosyltransferase (QPT) and Putrescine N-methyltransferase (PMT) are branch point regulators, A622 is thought to be a nicotine synthase



## Describing global transcriptome changes during alkaloid formation

**Goal:** To uncover the transcriptional factors and cellular signaling pathway components involved in directing the synthesis of nicotine and minor tobacco alkaloids through a comparative transcriptomics based approach using long oligonucleotide-microarrays.



### Tobacco Custom chip Version 1.2

385,000 feature oligo-based (60-mer) microarray (6 oligos / gene coding region)

Interrogates:

- ~45,000 gene coding regions (BLAST annotated GSRs + ESTs)
- ~ 2,500 transcription factors

Based on curated a dataset of 1.5 million gene-space sequence reads (GSRs) generated by MF technology representing an estimated minimum of 90-95% of tobacco gene space

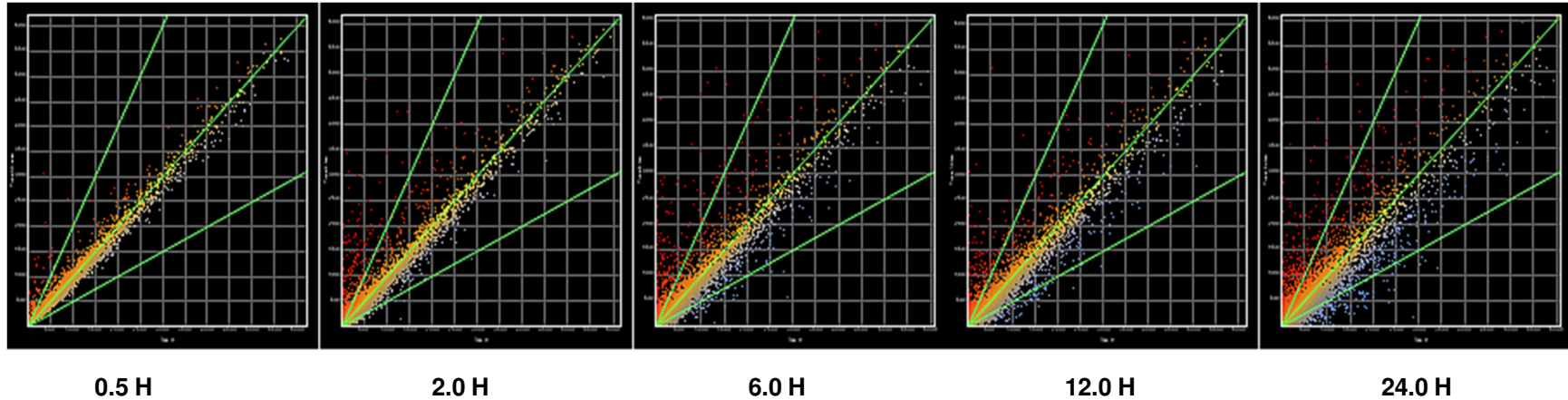
**The Tobacco Genome Initiative (TGI) PM USA - NCSU**

Plus > 83,000 publicly available cDNAs / ESTs

**TOBFAC Database** [ <http://compsysbio.achs.virginia.edu/tobfac/> ] - 64 well-characterized transcription factor (TF) families in tobacco.

Rushton, et al. (2008) Plant Physiology 147: 280-295; Rushton, et al. (2008) BMC Bioinformatics 9:53

## MeJA induced transcriptome changes in tobacco cells



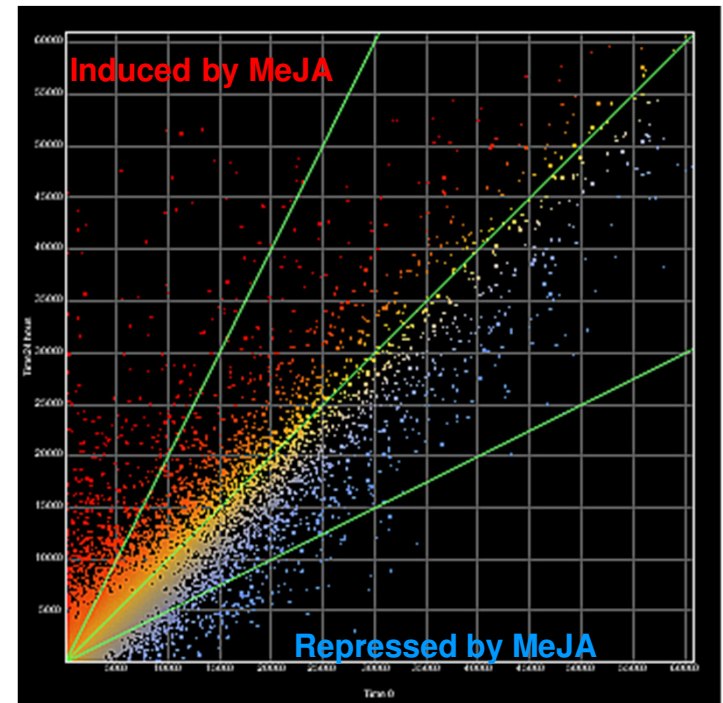
Bright Yellow 2 (BY2) tobacco cells grown in auxin-depleted media treated with 50  $\mu$ M MeJA and then assayed at various times thereafter

Number of genes induced / repressed over time

| post-MeJA (H)     | 0.5 | 2.0  | 6.0  | 12.0 | 24.0  |
|-------------------|-----|------|------|------|-------|
| 2-fold            | 9   | 690  | 979  | 665  | 3167  |
| 4-fold            | 81  | 2730 | 4013 | 3248 | 8311  |
| 8-fold or greater | 172 | 5187 | 6287 | 5340 | 11216 |

Level of confidence

| post- MeJA (H) | 0.5 | 2.0  | 6.0  | 12.0 | 24.0 |
|----------------|-----|------|------|------|------|
| $p = 0.10$     | 562 | 2515 | 3670 | 3593 | 5706 |
| $p = 0.05$     | 189 | 761  | 1093 | 1017 | 1856 |
| $p = 0.01$     | 85  | 352  | 459  | 440  | 790  |



## Major Biosynthetic Processes Significantly Up-Regulated by MeJA Treatment

### Alkaloid biosynthesis:

Lysine decarboxylase, aspartate oxidase, ornithine decarboxylase, quinolinate synthase, quinolinate phosphoribosyl transferase (QPT2), putrescine N-methyltransferase (PMT1, PMT1a, PMT2, PMT3, PMT4), N-methylputrescine oxidase (MPO), nicotine synthase (A622)

### JA biosynthesis:

Lysophospholipase, lipoxygenase, allene oxide synthase, 12-oxophytodienoate reductase, 1-aminocyclopropane-1-carboxylic acid oxidase

### Phenylpropanoid, Flavanoid, and Terpenoid biosynthesis:

caffeoyl-CoA O-methyltransferase, PAL, 4CL, C4H  
quercetin-3-O-glucosidase-6-O-malonyltransferase, vetispiradiene synthase  
wax synthase

### Detoxification and conversion:

Cytochrome P450 monooxygenases (CYP94B1, CYP94C, CYP74C)

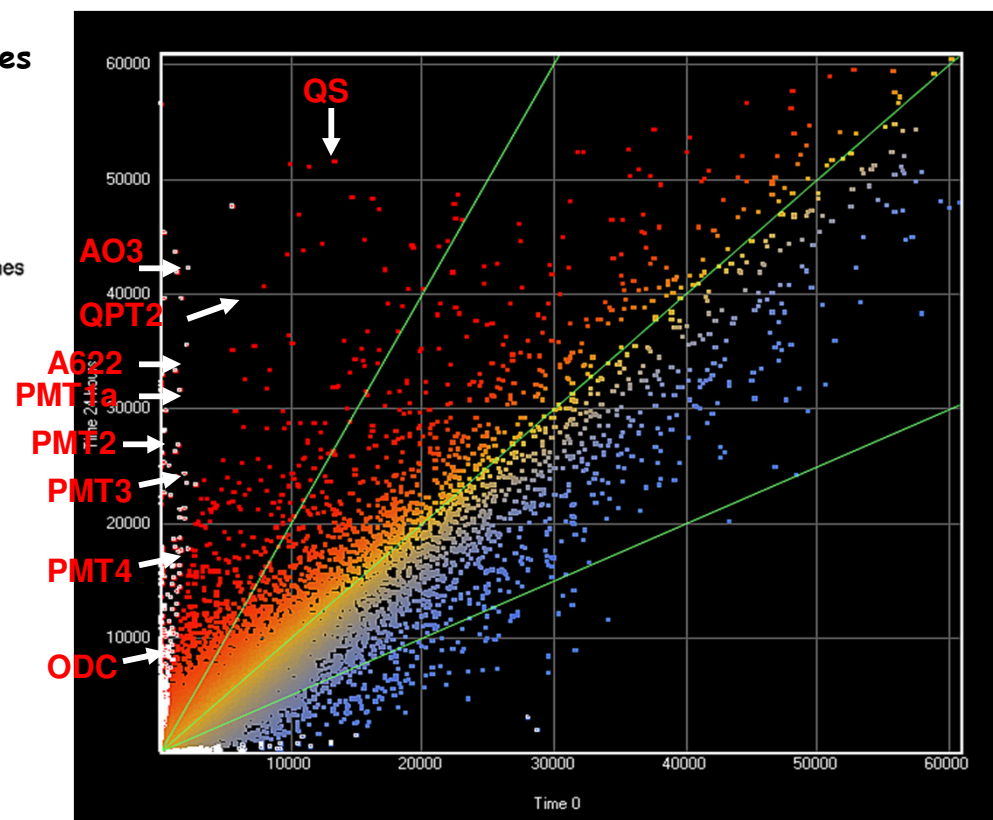
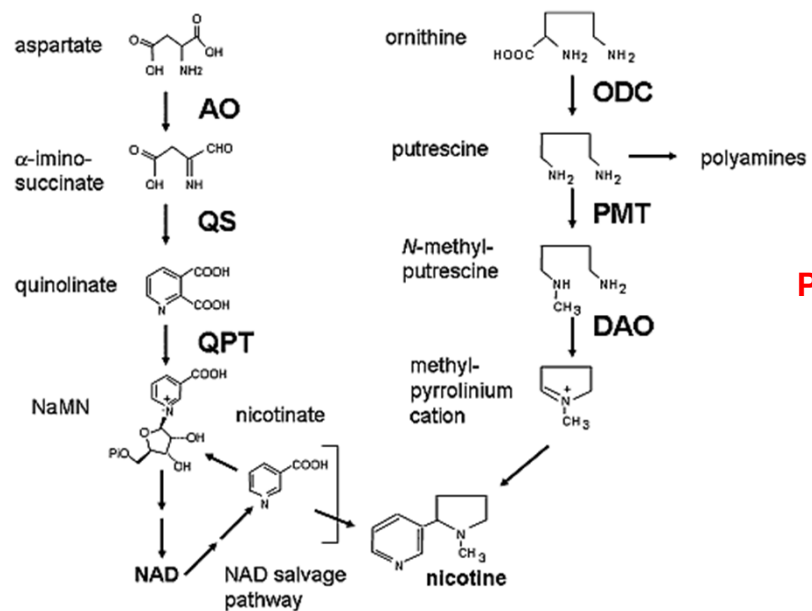
### Ethylene biosynthesis:

ACC synthase, ACC oxidase, ethylene receptor, SAM synthetase

### Hydrolysis of auxin conjugates:

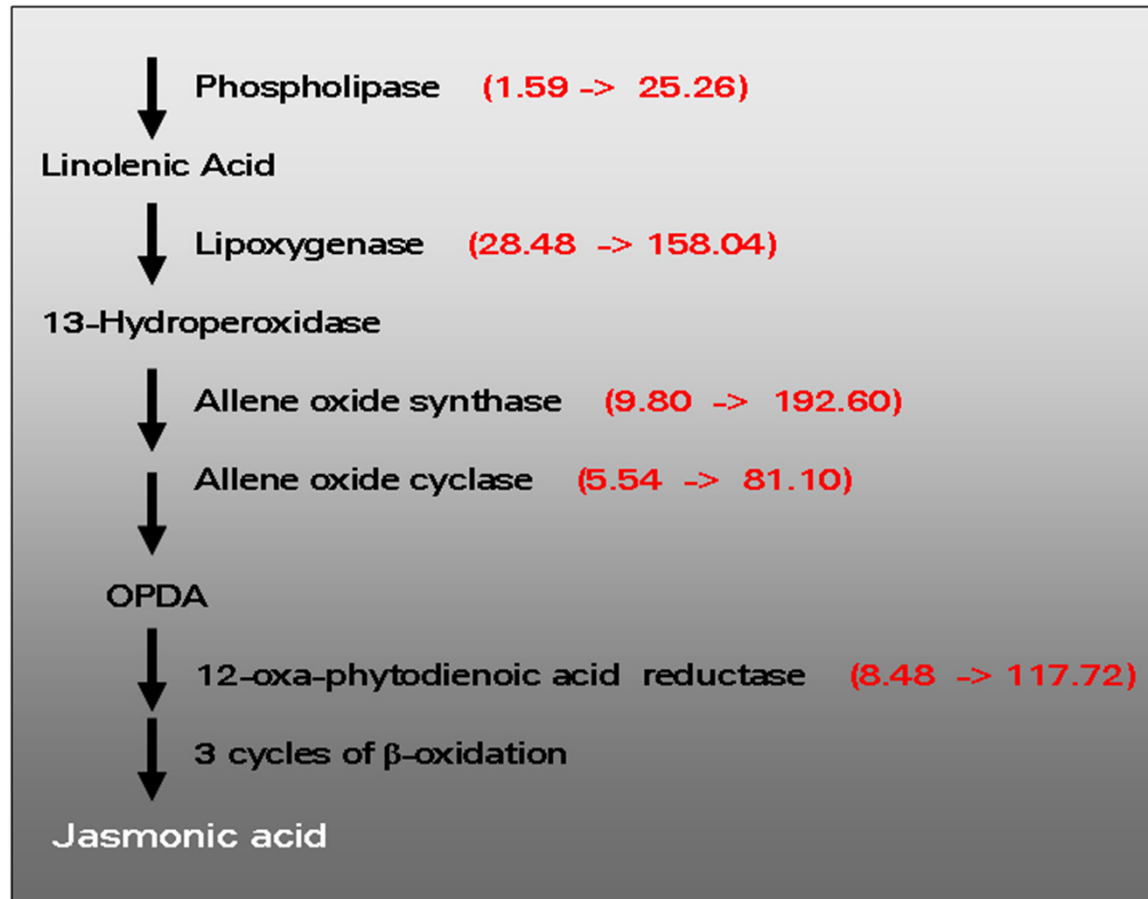
IAA amino acid hydrolase

## MeJA broadly activates alkaloid biosynthetic genes



| Gene Designation | 0.5 H | 2.0 H         | 6.0H   | 12.0 H | 24.0 H        |
|------------------|-------|---------------|--------|--------|---------------|
| ODC              | 1.50  | 11.33         | 17.02  | 13.13  | <b>22.60</b>  |
| AO3              | 0.66  | 61.01         | 91.80  | 101.92 | <b>140.31</b> |
| LDC              | 1.19  | 2.39          | 28.89  | 53.48  | <b>113.65</b> |
| PMT1             | 1.46  | 72.34         | 90.40  | 94.31  | <b>138.86</b> |
| PMT1a            | 1.21  | 65.54         | 85.98  | 76.33  | <b>127.89</b> |
| PMT2             | 1.18  | 47.58         | 64.44  | 59.24  | <b>98.95</b>  |
| PMT3             | 0.97  | 54.39         | 87.34  | 61.22  | <b>180.93</b> |
| PMT4             | 1.40  | 33.00         | 40.00  | 32.03  | <b>57.60</b>  |
| QPT              | 14.8  | <b>159.45</b> | 130.17 | 124.10 | 132.01        |
| A622             | 0.83  | 57.16         | 283.20 | 323.97 | <b>567.84</b> |

MeJA induced increases in the jasmonate biosynthetic pathway



## Regulatory genes induced early and late by MeJA

Early (0.5 H) ~75 genes minimum 8-fold induced- Largely transcription factors, cell signaling components, and oxidation and detoxification

Late (24 H) - Transcription factors, cell signaling components, transporters

| Early Induced (0.5 H)                 |             | Later induced (24 H)                  |               |
|---------------------------------------|-------------|---------------------------------------|---------------|
| Gene                                  | Fold Change | Gene                                  | Fold Change   |
| Cytochrome P450 monooxygenase CYP94B* | 220.51      | <b>MATE Efflux Carrier</b>            | <b>650.31</b> |
| ZIM15 JAZ1                            | 86.76       | Cytochrome P450 CYP74C3*              | 559.20        |
| Cytochrome P450                       | 82.39       | ZIM15 JAZ1/TIFFY10a                   | 354.39        |
| MYB-related transcription factor      | 65.08       | MYB-related transcription factor      | 247.39        |
| Protein kinase                        | 56.14       | ERF91                                 | 230.53        |
| Cytochrome P450 CYP94C1*              | 49.38       | ERF210                                | 171.21        |
| ERF43                                 | 48.97       | Cytochrome P450 monooxygenase CYP94B* | 143.51        |
| JAP1                                  | 46.74       | ERF29                                 | 133.10        |
| MYB137                                | 38.81       | bHLH transcription factor Group N     | 99.34         |
| Protein kinase                        | 36.36       | ERF161                                | 77.81         |
| ERF1                                  | 35.61       | disease resistance protein            | 74.90         |
| NAC137                                | 33.11       | ERF26                                 | 70.89         |
| ERF171                                | 29.64       | ERF16                                 | 69.57         |
| ERF161                                | 28.98       | Cytochrome P450                       | 65.17         |
| NAC165                                | 28.61       | Calcium/calmodulin protein kinase     | 59.44         |
| ERF123/EREBP1                         | 27.17       | MYB25                                 | 59.19         |
| ERF34                                 | 25.00       | ERF104                                | 58.28         |
| ERF142                                | 23.44       | ERF26                                 | 56.71         |
| ERF29                                 | 21.83       | MYB162                                | 51.86         |
| Cytochrome P450                       | 15.92       |                                       |               |
| Cytochrome P450 CYP74C3*              | 11.02       |                                       |               |
| ZIM29                                 | 10.37       |                                       |               |
| ORC                                   | 10.19       |                                       |               |

CYP94 clan members -  $\omega$ -hydroxylation reactions on long chain fatty acids, cutin biosynthesis  
 CYP74A - oxylipin pathway, biosynthesis of jasmonic acid and its methyl ester.  
 CYP74B - C6 volatiles (also known as green leaf volatiles).



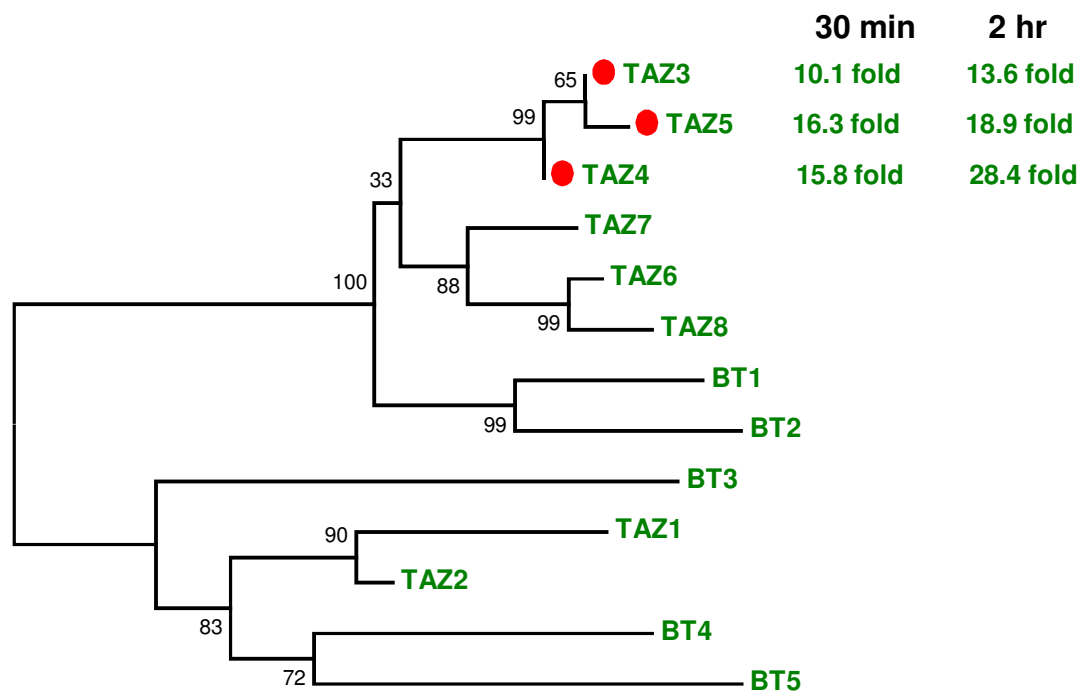
## Major Groups of Transcription Factors Are Rapidly Induced by MeJA Treatment

|  | Gene Name           | 0.5      | 2.0      | 6.0      | 12.0     | 24.0 H   |
|--|---------------------|----------|----------|----------|----------|----------|
| TF families previously implicated in regulation of alkaloid biosynthesis | bHLH80              | 19.26095 | 22.77554 | 20.49593 | 32.87435 | 34.41195 |
|  | ZIM54               | 8.171716 | 15.05944 | 16.41073 | 16.07903 | 18.29839 |
|  | ZIM29               | 10.37856 | 22.38093 | 26.019   | 30.03734 | 31.9194  |
|  | ZIM15 JAZ1/TIFFY10a | 81.4318  | 200.2678 | 207.0056 | 194.1159 | 354.399  |
|  | WRKY94              | 8.783906 | 13.9031  | 10.69285 | 13.12207 | 19.264   |
|  | WRKY86              | 10.28535 | 6.250077 | 3.021051 | 1.684482 | 2.485425 |
|  | WRKY WZZ            | 8.560769 | 8.240814 | 4.354299 | 2.242118 | 2.772749 |
| Novel TF families not previously implicated                              | TAZ5                | 16.26728 | 18.8711  | 11.83132 | 10.08521 | 10.82991 |
|  | TAZ4                | 15.82708 | 28.43111 | 19.70669 | 19.07123 | 19.96179 |
|  | TAZ3                | 10.07997 | 13.6404  | 5.556042 | 7.308234 | 7.814843 |
|  | NAC165              | 28.61289 | 79.4148  | 43.74384 | 21.44671 | 29.22691 |
|  | NAC148              | 8.738688 | 8.565065 | 5.828583 | 5.357513 | 6.32293  |
|  | NAC144              | 25.39449 | 76.45565 | 39.40846 | 20.78165 | 28.16605 |
|  | NAC142              | 7.740277 | 10.15413 | 6.735282 | 5.823688 | 7.02466  |
|  | NAC137              | 33.11684 | 29.07432 | 16.71765 | 15.06963 | 16.92028 |
|  | MYB137              | 38.81437 | 75.48642 | 43.37177 | 13.60557 | 33.26423 |
|  | MYB126              | 15.30421 | 34.33204 | 24.79423 | 12.10359 | 18.67313 |
| MYB-related transcription factor   | 65.08559            | 296.8879 | 232.0247 | 224.3874 | 247.3969 |          |
| TF families previously implicated in regulation of alkaloid biosynthesis | JAP1                | 46.74121 | 96.32362 | 57.80582 | 10.47133 | 27.90388 |
|  | ERF91               | 14.90965 | 138.116  | 117.1518 | 115.3997 | 230.5319 |
|  | ERF43               | 48.97425 | 42.9925  | 12.45338 | 11.32662 | 16.94804 |
|  | ERF34               | 25.00929 | 57.50541 | 37.41603 | 5.373373 | 15.92009 |
|  | ERF29               | 21.83762 | 93.4457  | 75.76626 | 87.87373 | 133.1093 |
|  | ERF221              | 10.19806 | 33.25401 | 24.62997 | 30.17118 | 43.54652 |
|  | ERF210              | 7.772942 | 68.54234 | 53.99681 | 96.38171 | 171.2103 |
|  | ERF200              | 13.13474 | 5.945172 | 2.756405 | 2.200636 | 1.691165 |
|  | ERF171              | 29.64926 | 40.938   | 9.566142 | 9.72084  | 10.66286 |
|  | ERF161              | 28.98894 | 35.11224 | 18.80423 | 29.79476 | 77.81196 |
|  | ERF16               | 9.452124 | 41.04665 | 30.88507 | 27.55179 | 69.57246 |
|  | ERF146              | 10.07655 | 17.6723  | 4.646603 | 1.097782 | 1.201869 |
|  | ERF142              | 22.44951 | 5.630708 | 1.365725 | 0.950111 | 1.146815 |
|  | ERF127              | 16.68055 | 35.45427 | 11.81974 | 4.802914 | 13.89144 |
|  | ERF123 EREBP1       | 27.17103 | 28.46735 | 20.96191 | 8.417083 | 16.88214 |
|  | ERF108              | 7.464297 | 33.72545 | 15.09291 | 3.169867 | 6.702274 |
|  | ERF104              | 8.547192 | 19.11911 | 19.66225 | 22.91799 | 58.28698 |
|  | ERF1                | 35.61383 | 33.42185 | 21.65265 | 12.69124 | 22.26616 |
|  | DOF22               | 8.980808 | 37.00591 | 28.59944 | 16.1694  | 10.9234  |
|  | C2H2 140            | 10.76172 | 13.26983 | 6.740594 | 5.030606 | 9.41924  |
| C2H2 129   | 8.975682            | 8.919238 | 4.348382 | 3.915977 | 7.146894 |          |

## NtTAZ transcription factors - Novel components of jasmonate signaling

**TAZ** (Transcriptional **A**dapter **Z**inc-binding) domains are primarily involved in protein-protein recognition.

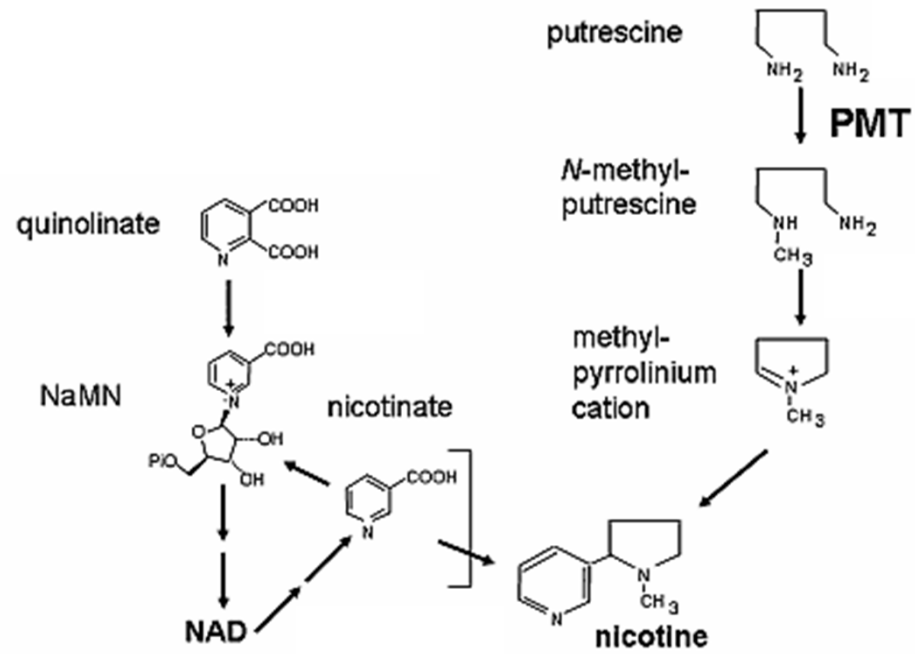
The activation domains of more than 30 TF have been reported to bind to the TAZ domains, and each TAZ domain generally binds a different subset of transcription factors.



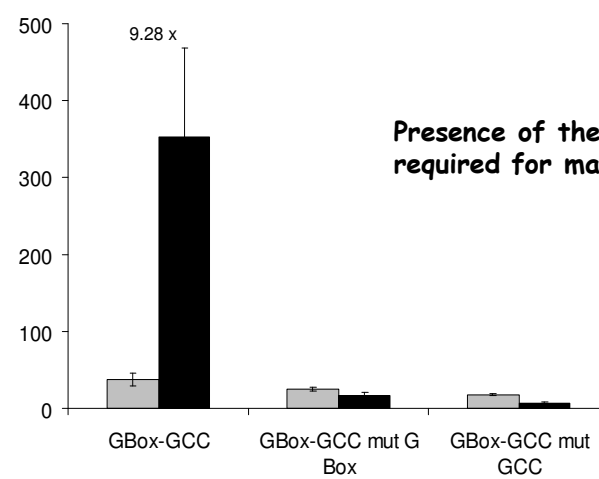
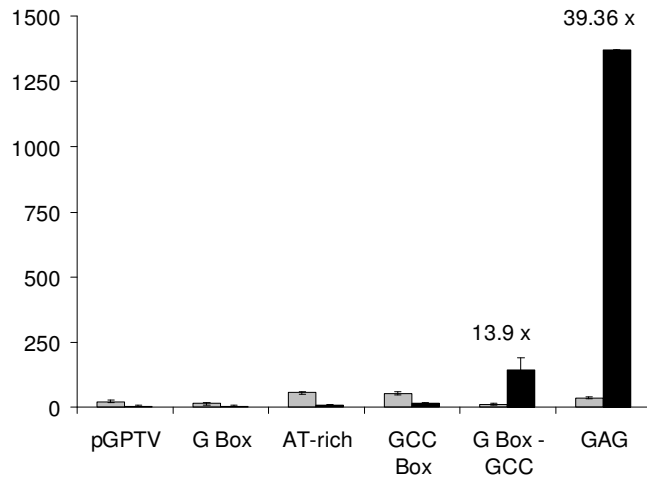
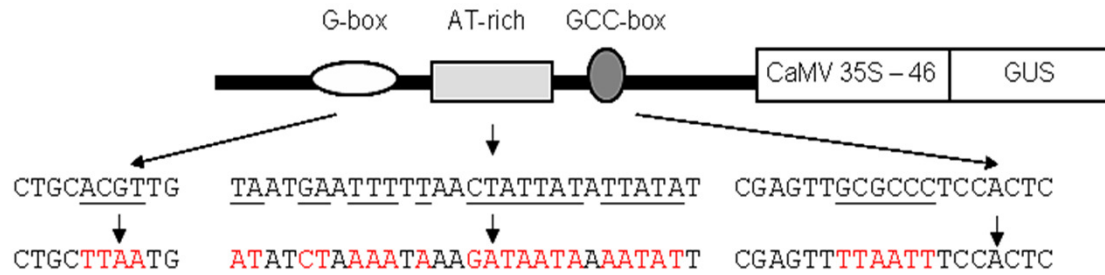
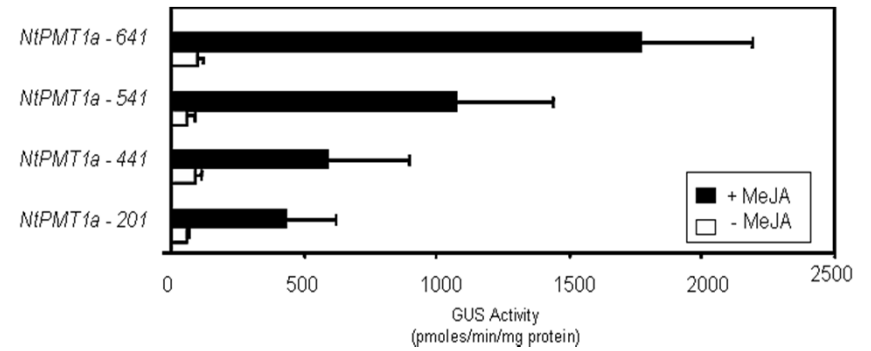
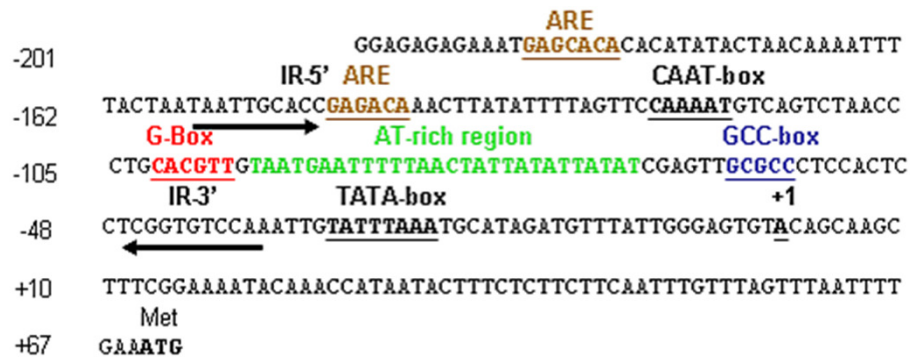
## Jasmonate leads to repression of auxin regulated gene expression

| Gene  | Fold repressed |
|---|----------------|
| Auxin induced protein/small auxin up RNA (SAUR_B) | 77.31          |
| Auxin induced protein                             | 61.14          |
| Endoxyloglucan transferase                        | 52.36          |
| Ripening related protein                          | 39.70          |
| Cytochrome P450                                   | 38.25          |
| Senescence associated protein                     | 36.12          |
| Peroxidase  | 34.42          |
| Invertase   | 33.00          |
| Rhamnosyltransferase                              | 32.15          |
| Unknown   | 29.56          |
| Pectate lyase                                     | 28.75          |
| Stigma-specific Stig1                             | 26.85          |
| Myosin II heavy chain                             | 25.50          |
| Expansin  | 24.84          |
| Accellerated Cell Death-like                      | 24.36          |
| Cyclin  | 24.31          |
| Proline rich protein                              | 24.19          |
| Maturation polypeptide                            | 24.19          |
| Pectinesterase inhibitor                          | 23.58          |
| LRR receptor-like kinase                          | 18.73          |
| Receptor protein kinase                           | 18.38          |
| Homeodomain-leucine zipper protein                | 16.92          |
| Protein kinase                                    | 16.87          |
| AUX1-like permease                                | 16.07          |
| Auxin efflux carrier                              | 14.61          |
| TCP14   | 14.15          |
| AS2 81  | 12.85          |
| MYB-related 26                                    | 12.09          |
| Auxin transporter PIN1-like                       | 9.80           |
| ABI19   | 9.06           |
| R gene like                                       | 8.84           |
| R gene like                                       | 8.83           |
| Homeodomain35                                     | 8.55           |
| Auxin transporter                                 | 8.12           |

Molecular mechanism of jasmonate-regulated *NtPMT1a* gene transcription

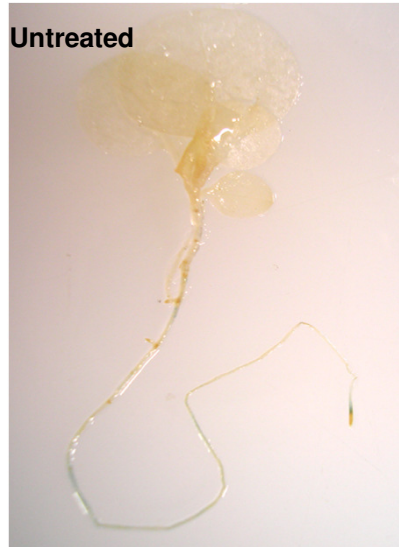


### The novel tripartite "GAG motif" in the NtPMT1a gene mediates MeJA responsiveness



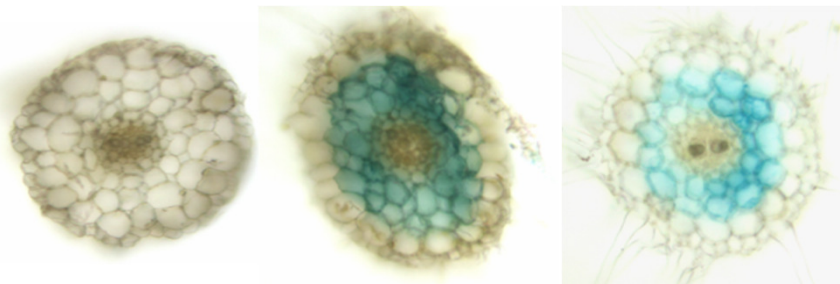
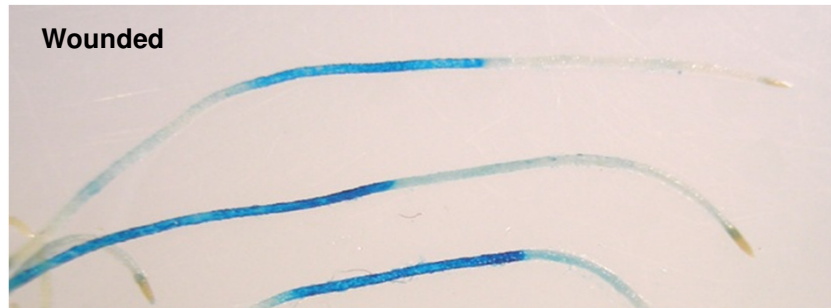
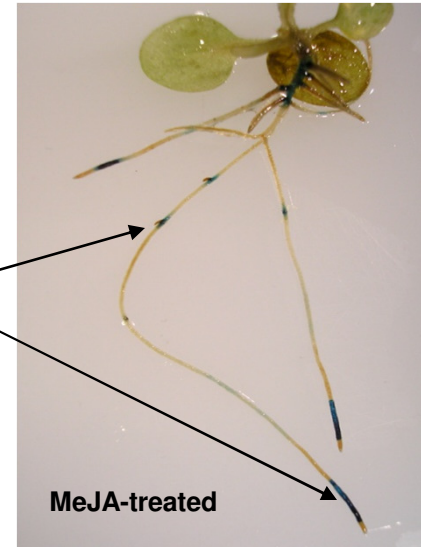
Presence of the G-box and GCC-box are required for maximal MeJA responsiveness

The *GAG* motif differentiates between MeJA and wound inducible signaling in transgenic plants



**Response to wounding** – occurs in a broad zone of the cortex beginning at stem-root transition extending to zone of maturation

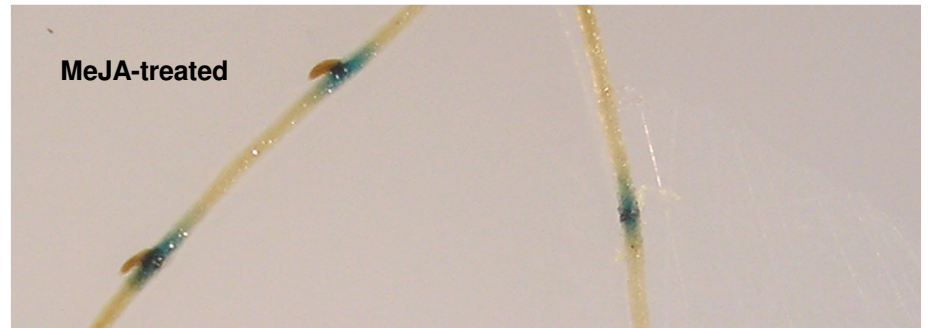
**Jasmonate response** - response is restricted to the zone of elongation and points of lateral root initiation



Untreated

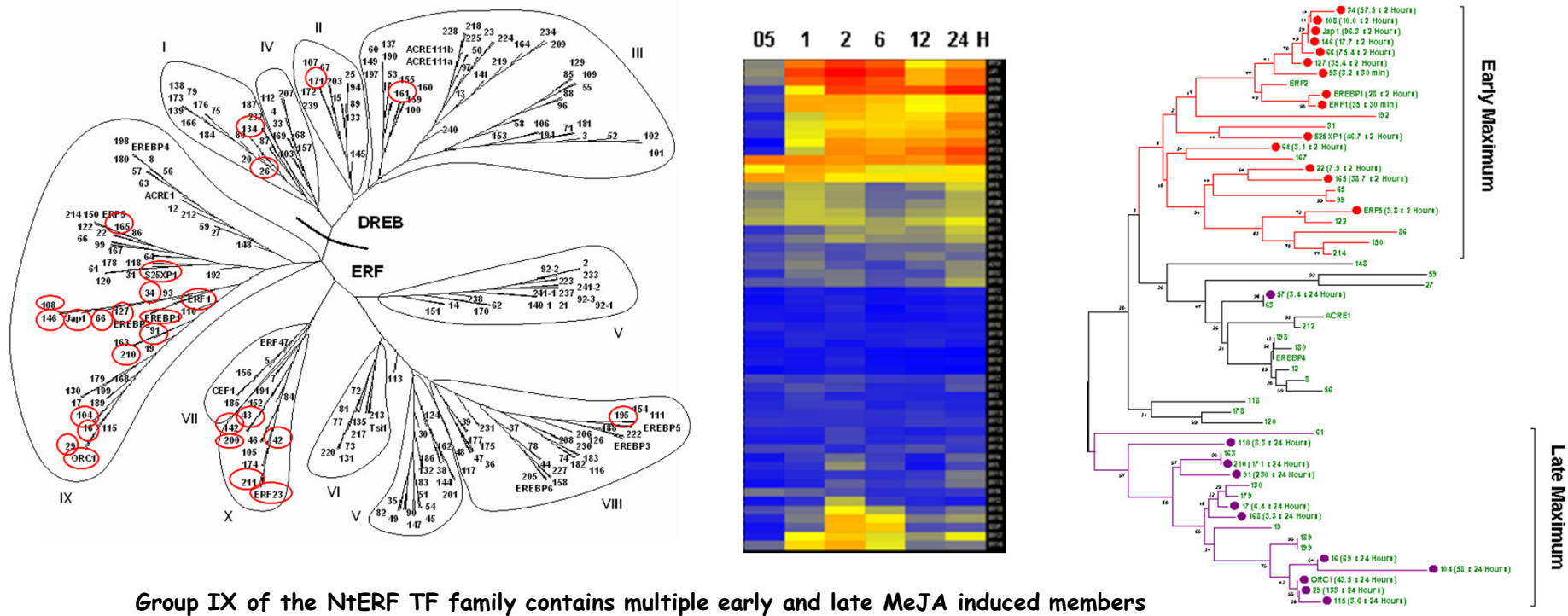
Wounded

MeJA-treated

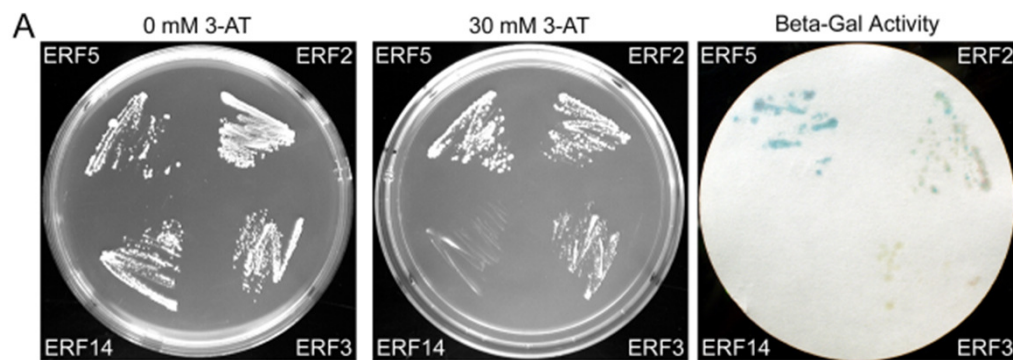


Bokowiec et al (2010) Manuscript in preparation

### Identification of NtERF TFs that bind the GCC-like box in the *NtPMT1a* GAG motif

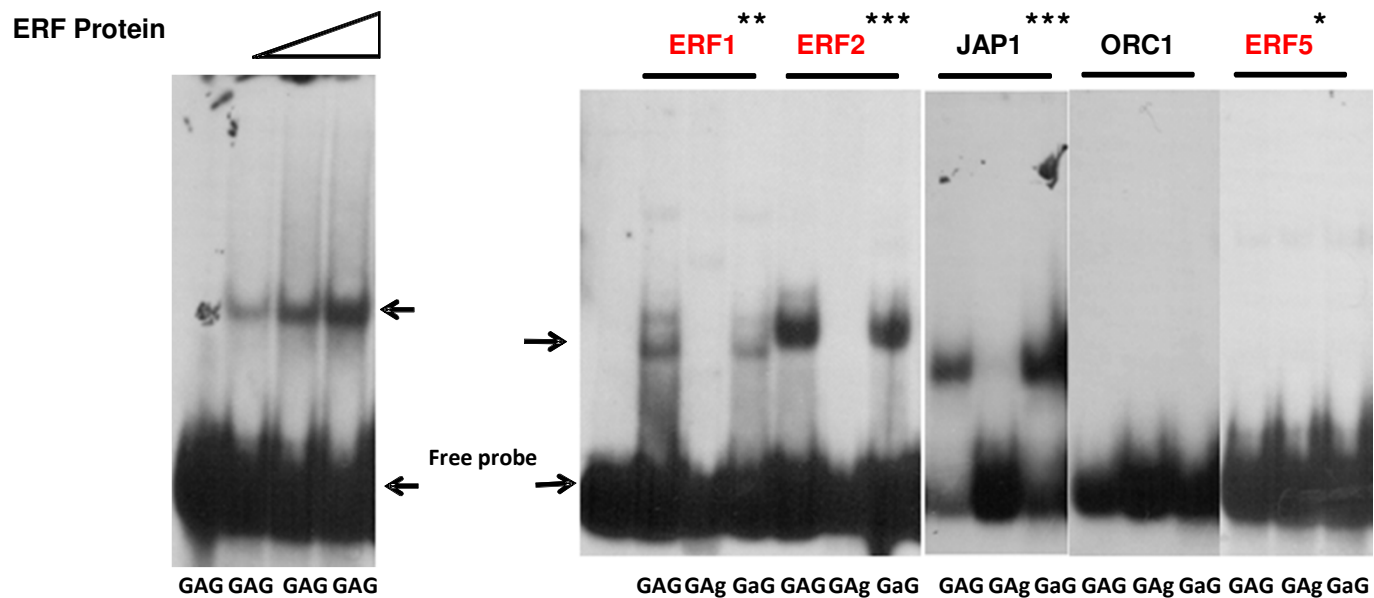


Group IX of the NtERF TF family contains multiple early and late MeJA induced members



Yeast two hybrid screens demonstrate NtERFs can specifically bind the GCC-box in the GAG motif

## NtERF TFs differ in their ability to bind the GCC-like box in the *NtPMT1a* GAG motif



```

GAG  CTGCACGTTG TAATGAATTTTAACTATTATATTATAT CGAGTTGCGCCCTCCACTC
GaG  CTGCACGTTG ATATCTAAAATAAAGATAATAAAATATT CGAGTTGCGCCCTCCACTC
Gag  CTGCACGTTG TAATGAATTTTAACTATTATATTATAT CGATTTTCTCCCTCCACTC
  
```

ORC1 (NtERF221) and JAP1 were previously shown to upregulate nicotine formation in transient expression assays in tobacco protoplasts presumably by activating transcription of the *N. sylvestris* *NsPMT2* promoter (De Sutter et al., 2005).

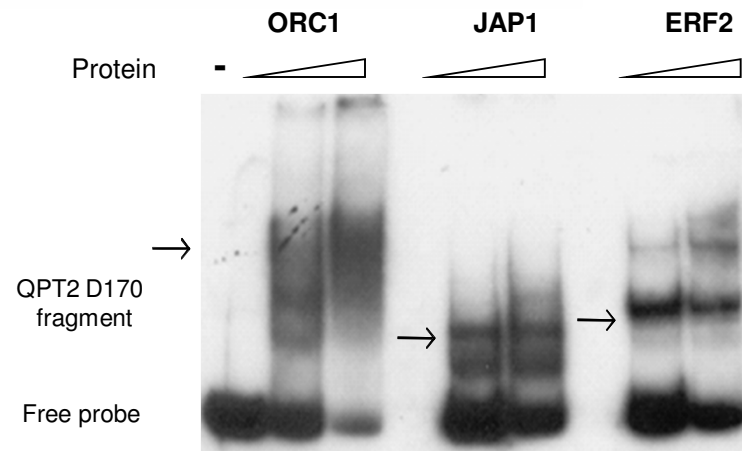
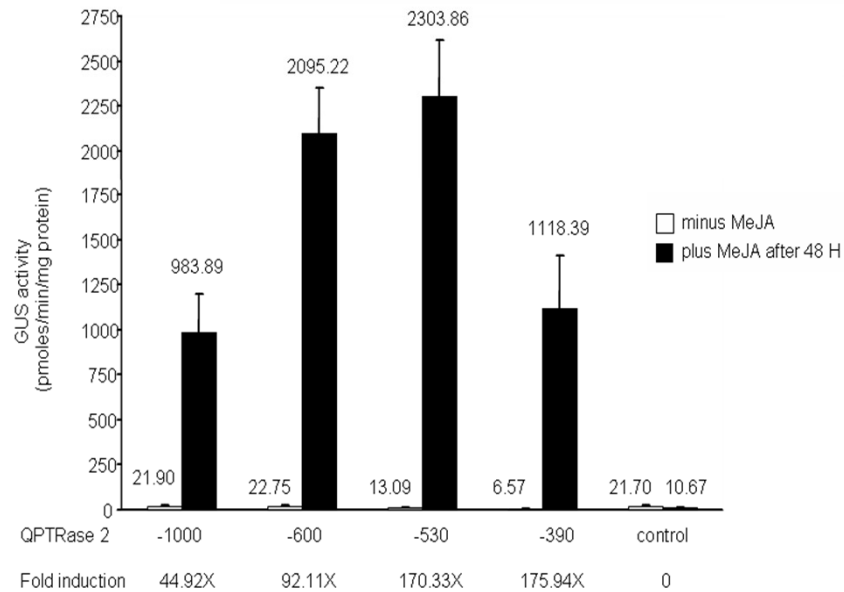


# NtJAP1, NtORC1 and NtERF2 bind the MeJA inducible NtQPT2/RD2 promoter but not in the GAG motif

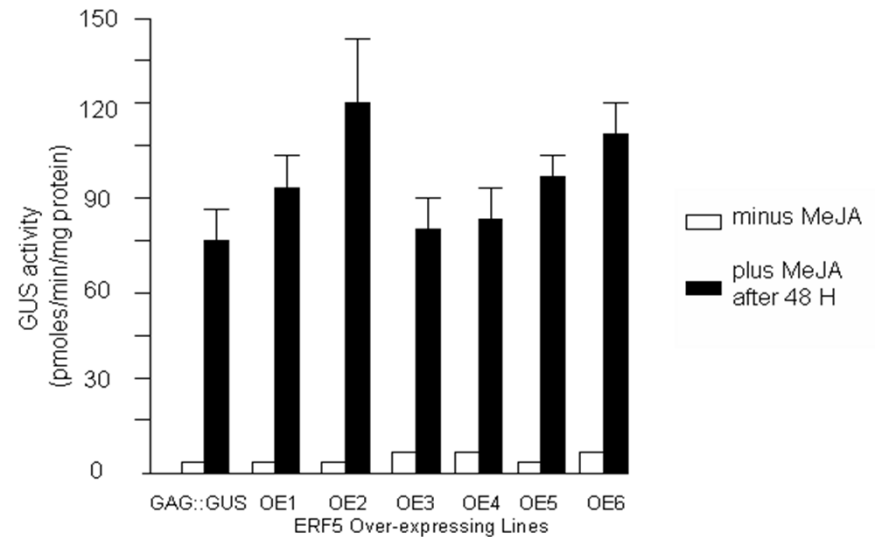
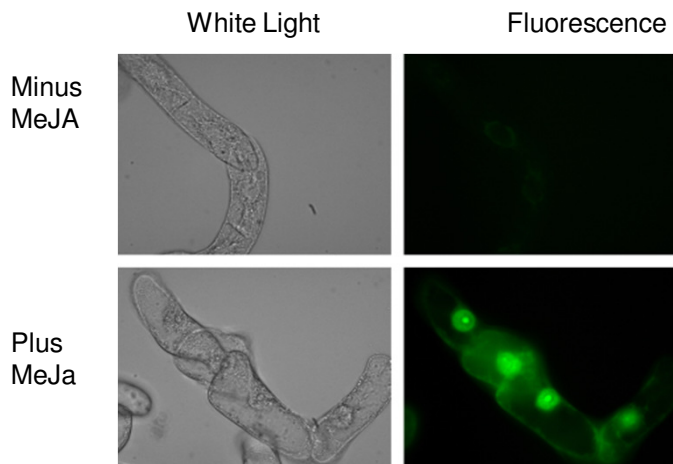
```

1  ctccaggatc taaattgtga gttcaatctc ttcctattg gattgattat cctttctttt ctccaattt ggtttttt ttgctaatt tattgtgta tccccttat cctattttg tctttactt
131  atttatttgc tctatgtct ttgtacaag atttaactc tatggccatc attttaagt tgttagaaaa taaattctt caagattgat gaaagaact ttaattgta gatatttgc agattttat
261  ctctactac caatataacg cttgaattga cgaaaattg tgcocaaata tctagacaaa aggtatccaa tgaaaataa tcatatgta tcttcaatc ttgtgtcta tgaagattg atactttgt
391  caatggaaga gattgtgtgc atattttta aatttttatt agtaataaag attctatata gctgttatag agggataatt ttacaaagaa cactataaat atgattgtg ttgttaggtt gccaatggt
521  cggttcgact gytattttta taaaatttg accataccat ttttttcgat attctatct gtataaccaa aattgactt ttcgaaatcg tcccaatcat gtcggttca cttcggtatc ggtaccgttc
651  gyttaatttt cattttttt taaatgtcat taaaattc acagtaaaat agaatgcaat aacatacgtt cttttatagg acttagcaaa agctctctag acatttttac tgtttaaagy ataatgaatt
781  aaaaaacatg aaagatggct agagtataga tacacaacta ttgcagacga acgtaaaaga aaccaagtaa aagcaagaaa aatataaatc acacgagtgg aaagatatta accaagtgg gattcaagaa
911  taaagtctat attaaatatt caaaaagata aatttaata atagaaaagy aaacatctc aatacattgt agttgtctac tcataatg c tagaatactt tgtgccttgc taataaagat actgaaata
1041  gcttagttta aatataata gcataataga ttttagaat tagtatttt agtttaatta cttattgact tgtaacagtt ttataatc caagcccat gaaaaatta atgctttatt agtttaaac
1171  tctactata aatttttcat atgtaaaatt taatcggat agttcgatc tttttcaatt ttttttata aataaaaaa cttaccctaa ttatcgtac agttatagat ttatataaaa atctacggtt
1301  cttcagaaga aacctaaaaa tcggttcggt gggacgggt cgtacgggtt agtcgattt caaatatca ttgacactc tagttgtgt tataggtaaa aagcagttac agagaggtaa aatataact
1431  aaaaaacag ttctaaggaa aaattgact ttatagtaa tgactgttat ataaggatg ttgtacagag aggtatgagt gtattgtgta aattatgctc ttgacgtgt atgtcacata ttatttata
1561  aaactagaaa aaacagcgtc aaactagca aaaatccaac ggacaaaaaa atcgtgtgaa ttgatttgg ttccaactt taaaaaagt tcagtggaaa agaactcgtg actgtttgat atataaaca
1691  agggcacatt ggtcaatac cataaaaaa tatatgacag ctacagtgg tagcatgtgc tcagctattg aacaaatca aagaaggtac atctgtaac ggaacaccac taaatgact aaattaccct
1821  catcagaag cagatggagt gctacaata acacactatt caacaacct aaataaaagc tgttcagcta ctaaaacaaa tataataaa tctatgtttg taagcactc agccatgta atggagtgtc
1951  attgcctgtt aactctcact tataaaatag tagtagaaaa aatagtaacc aaacacac caaagaagc attaagctc ccaaaaaacta tttccacaa aattcatttc acaaccccc caaaaaaaa
TATA Box >>.....>>
Start of transcription >

```

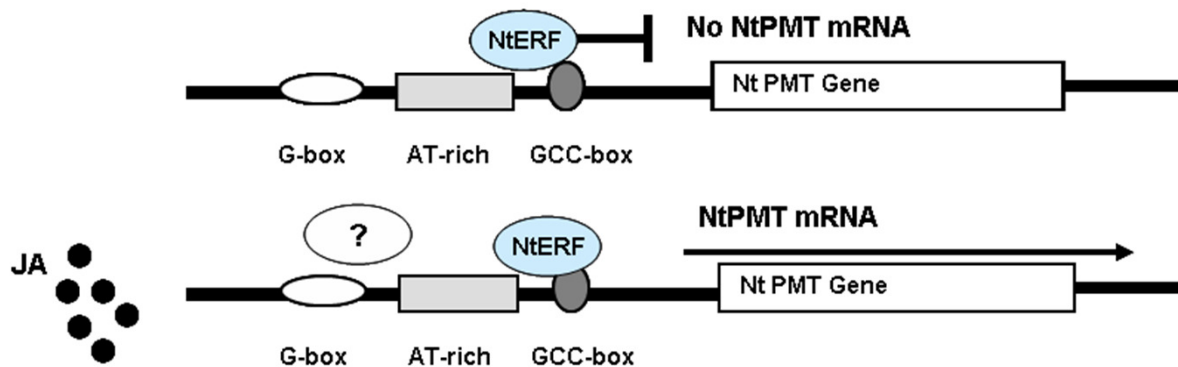


**NtERF binding alone is insufficient to activate MeJA-induced *NtPMT1a* transcription**



Expression of NtERF5-GFP is induced in response to MeJA treatment and the fusion localizes to the nucleus.

Overexpression of NtERF5 TFs does not transactivate pGAG::GUS transgenes in the absence of MeJA



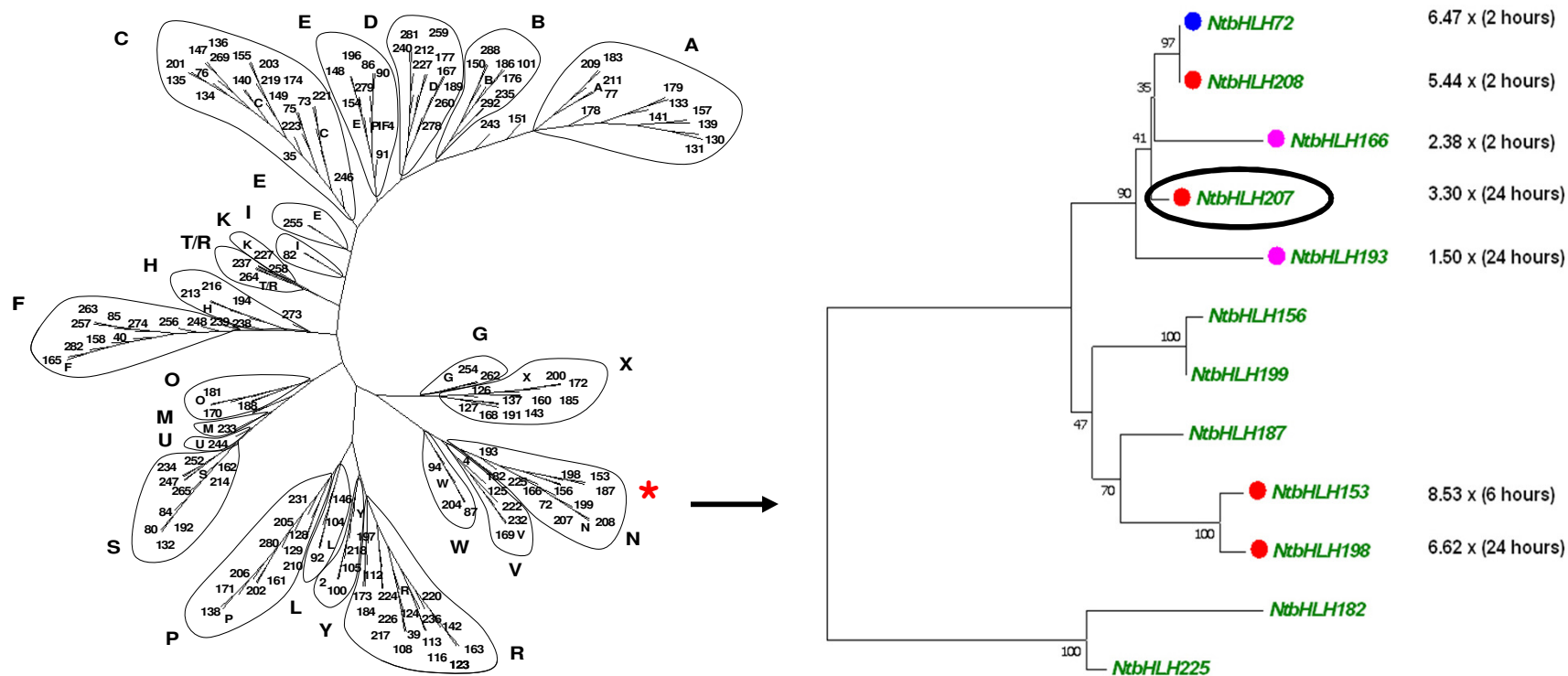
### Mutations in NtERF2 and NtERF5 decrease alkaloid content in tobacco plants

| Gene target | Mutation Nucleotide – AA | Nicotine | Nornicotine | Myosmine | Anabasine | Anatabine | Total alkaloids | % Conversion | % Anatabine |
|-------------|--------------------------|----------|-------------|----------|-----------|-----------|-----------------|--------------|-------------|
| WT T34      |                          | 1.63     | 0.0513      | 0.00137  | 0.010800  | 0.074     | 1.768           | 3.15         | 4.20        |
| NtERF5      | GGT > GAT G > D          | 0.83     | 0.0137      | 0.00128  | 0.0039    | 0.041     | 0.89            | 1.62         | 4.67        |
| NtERF5      | AGA > AAA R > K          | 0.857    | 0.0182      | 0.00125  | 0.0045    | 0.026     | 0.91            | 2.08         | 2.87        |
| NtERF5      | CCA > TCA P > S          | 0.903    | 0.0218      | 0.00131  | 0.0069    | 0.089     | 1.02            | 2.36         | 8.79        |
| NtERF5      | GAG > AAG E > K          | 0.94     | 0.0390      | 0.00130  | 0.0071    | 0.040     | 1.03            | 3.97         | 3.88        |
| NtERF2      | GCT > GTT A > V          | 0.21     | 0.0352      | 0.00135  | 0.0041    | 0.008     | 0.26            | 14.53        | 3.238       |
| NtERF2      | ACC > ATC T > I          | 0.607    | 0.0145      | 0.0013   | 0.0039    | 0.022     | 0.65            | 2.33         | 3.47        |
| NtERF2      | ATA / GTA I > V          | 0.714    | 0.023       | 0.00126  | 0.0039    | 0.036     | 0.78            | 3.12         | 4.63        |
| NtERF2      | AAC > A-C Gap            | 0.994    | 0.0623      | 0.00131  | 0.0079    | 0.056     | 1.12            | 5.90         | 5.06        |
| NtERF2      | GAG > GCG E > A          | 1.12     | 0.0370      | 0.00137  | 0.0042    | 0.044     | 1.21            | 3.20         | 3.67        |
| NtERF2      | TGG > TGA W / stop.      | 1.35     | 0.262       | 0.00293  | 0.0063    | 0.065     | 1.69            | 16.25        | 3.87        |

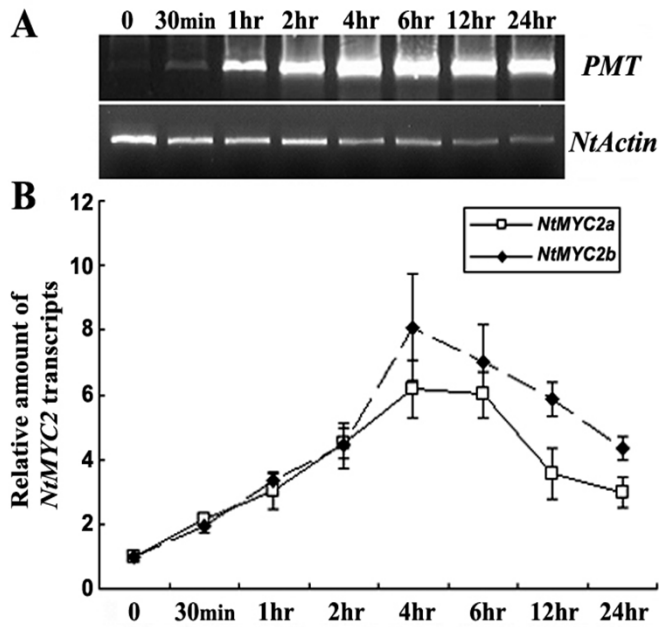
Represent new targets for manipulation of alkaloid content

Bokowiec MT, Kudithipudi C, Hayes A, Timko MT (2010)  
Manuscript in preparation

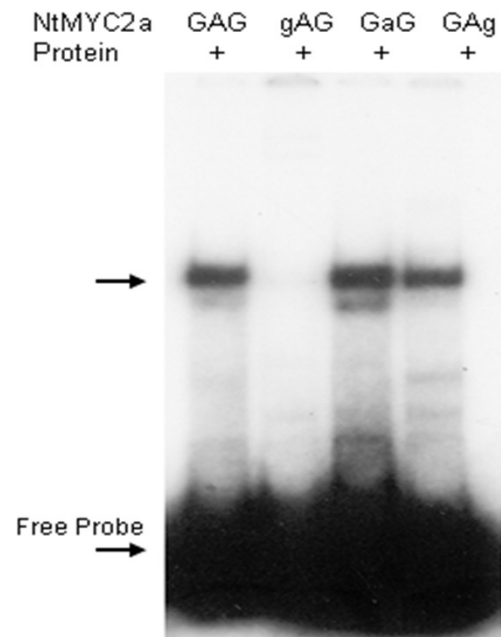
A highly induced NtbHLH (basic Helix-Loop-Helix) TF binds the *G*-box within the *NtPMT1a* GAG motif



Members of Subgroup N of the NtbHLH TF family are MeJA inducible and bHLH207 appear to be the closest homolog of the Arabidopsis jasmonate response regulator AtMYC2.

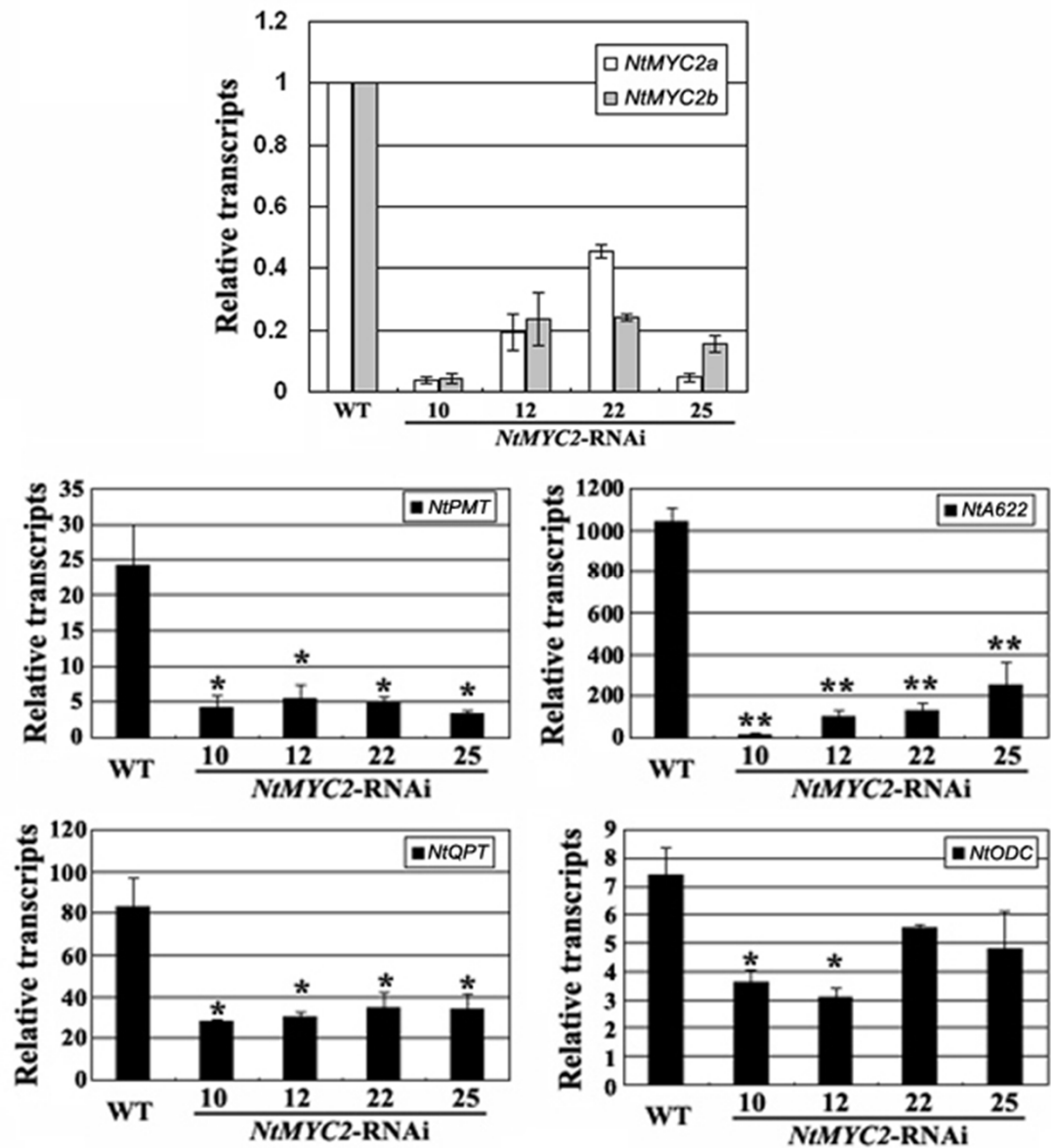


Two expressed protein variants of bHLH207 exist in tobacco, designated NtMYC2a and NtMYC2b

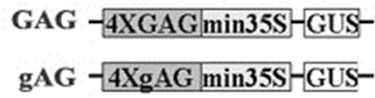


NtMYC2a / NtMYC2b specifically binds the G-box in the GAG motif.

## RNAi knockdown of *NtMYC2* blocks MeJA-induced nicotine biosynthetic gene transcription



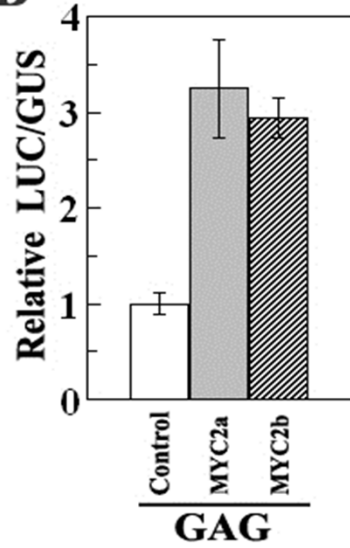
### A Reporter



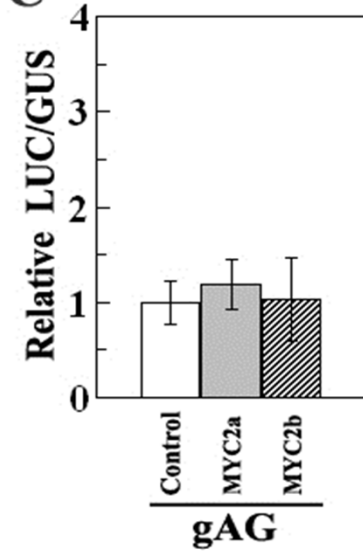
### Effector



### B

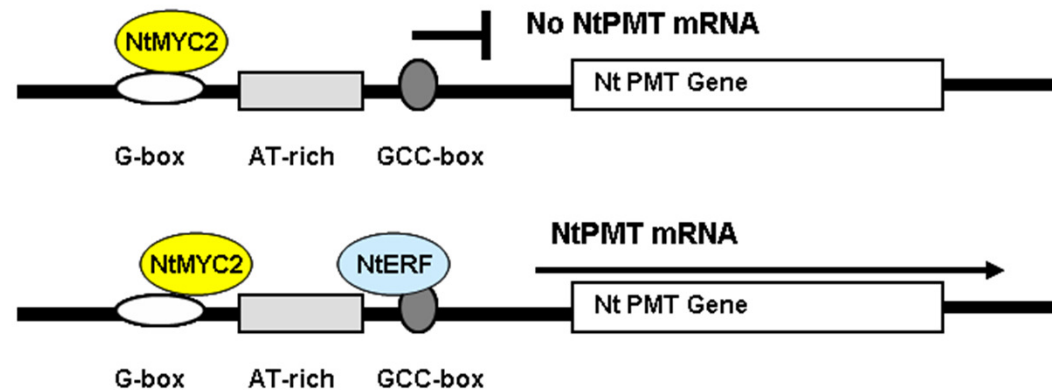


### C

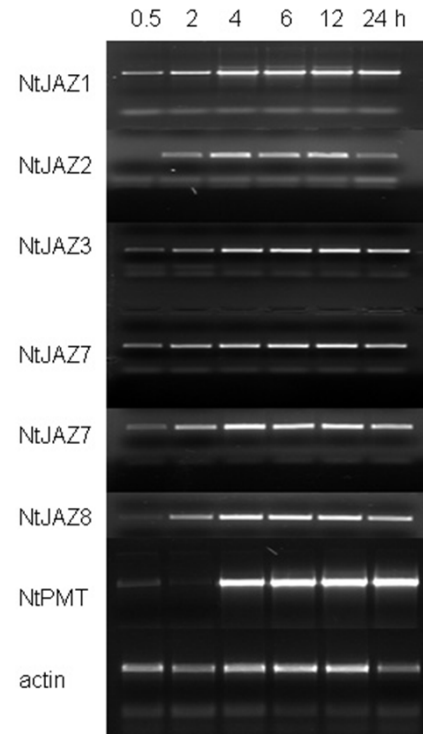
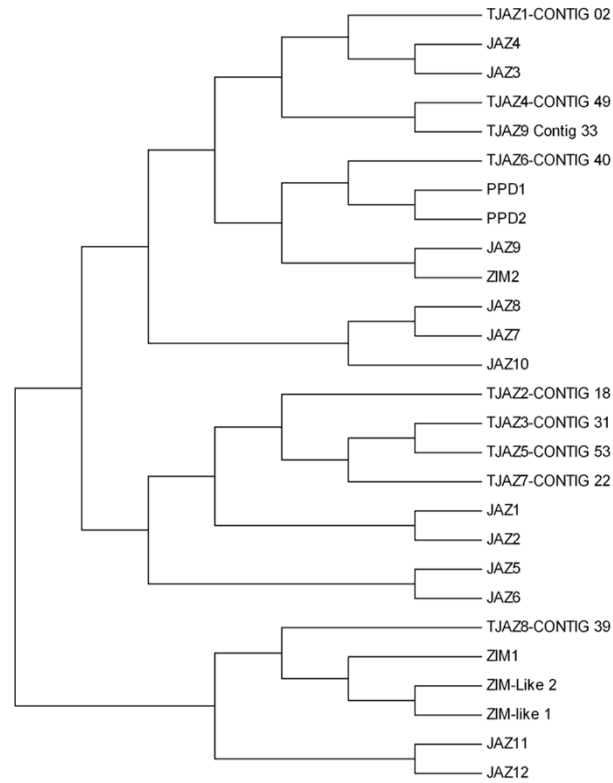


Overexpressed NtMYC2a / NtMYC2b are capable of transactivating pGAG-GUS transgene expression only in the presence of added MeJA

A functional G-box in the GAG motif is required



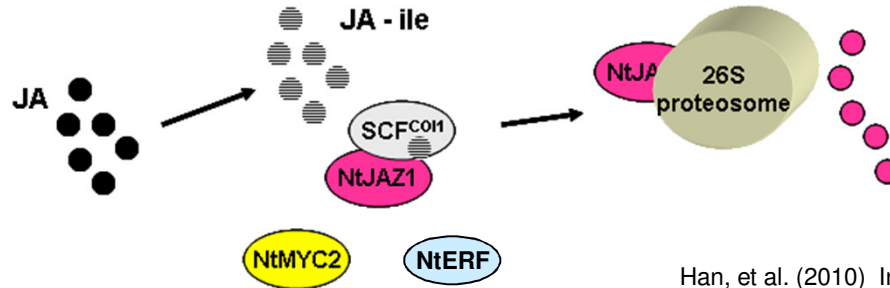
# The *JAZ* family of repressors is the missing link in jasmonate signaling



## Absence of JA



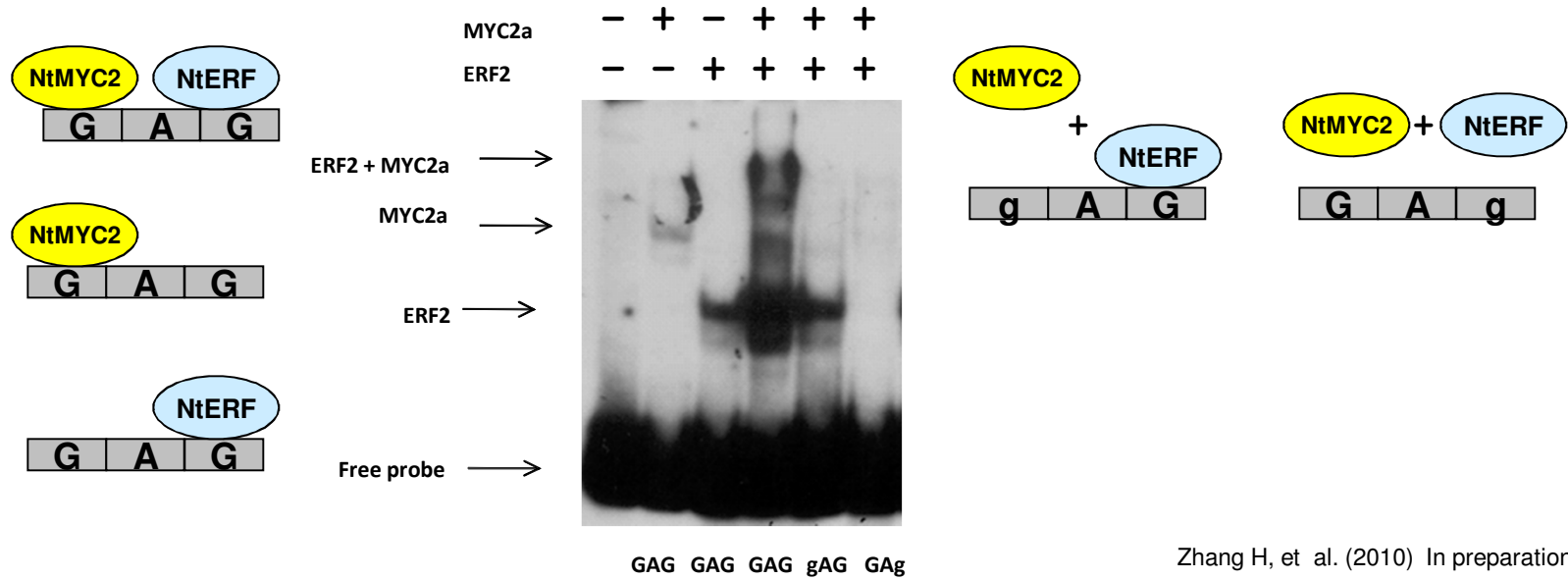
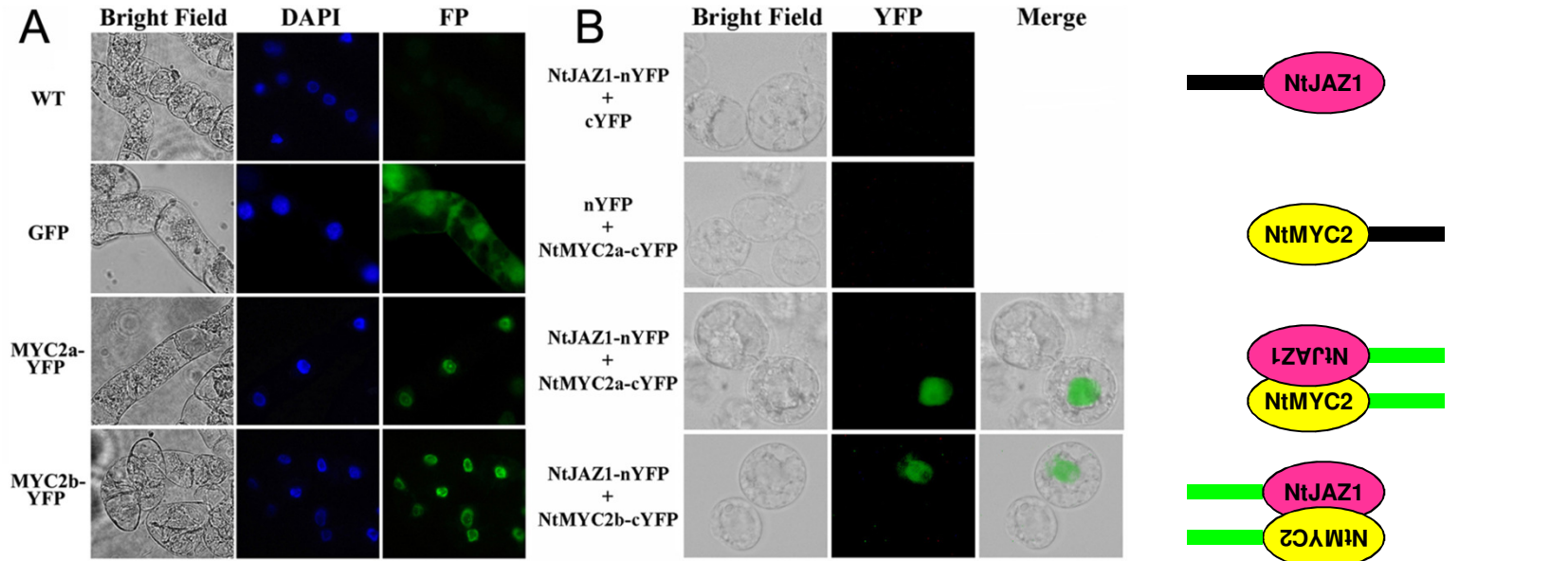
## Presence of JA



Han, et al. (2010) In preparation

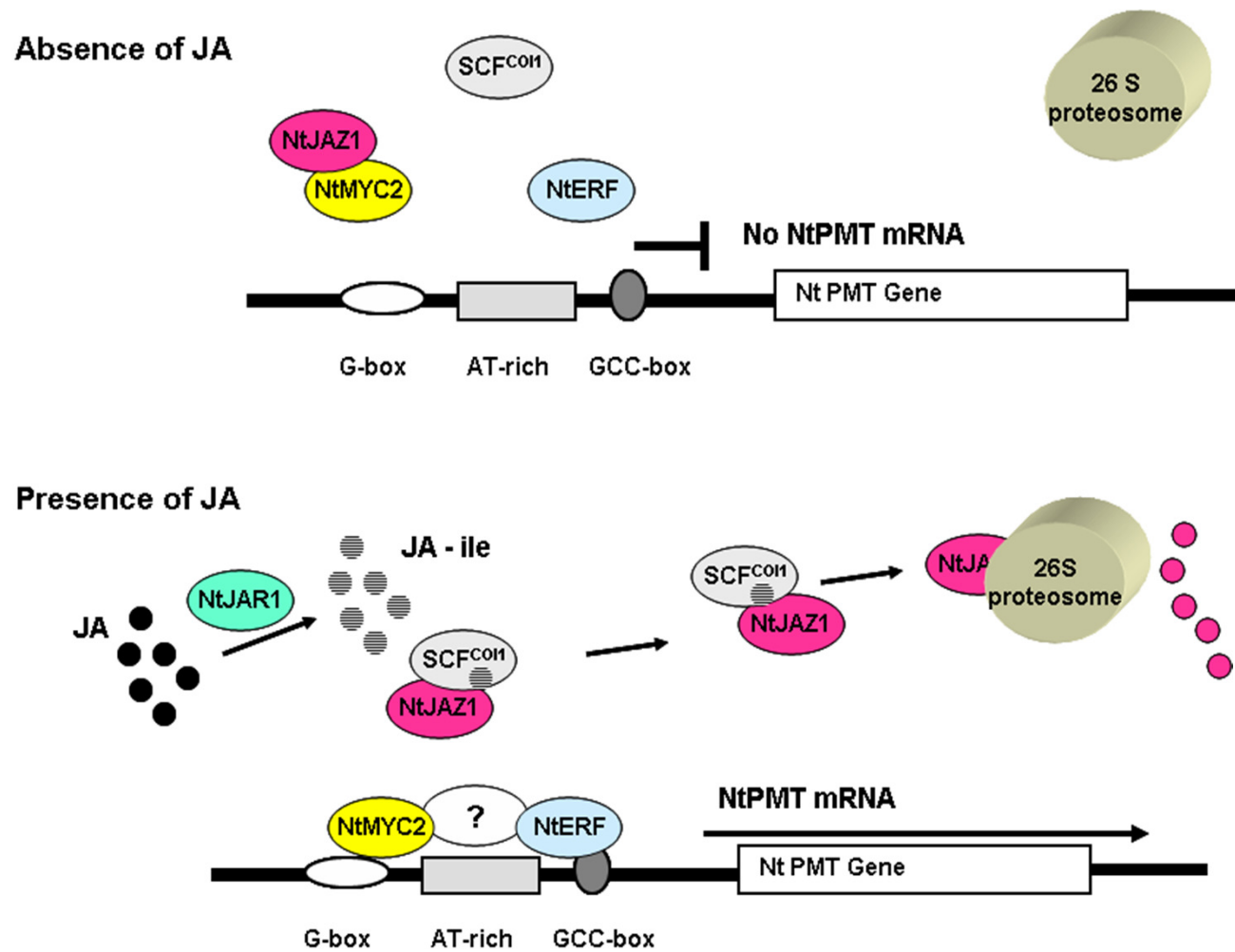


## In vitro and in vivo interaction of NtMYC2, NtJAZ1, and NtERF2



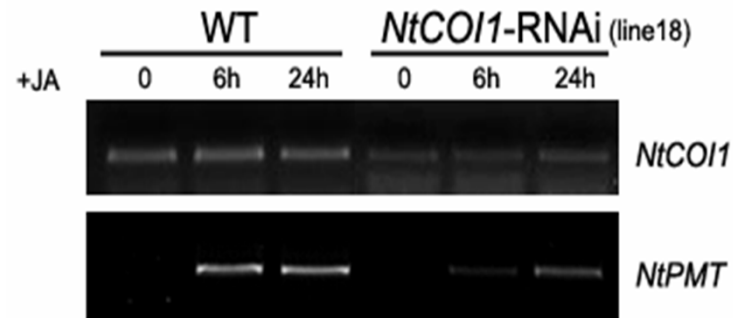
Zhang H, et al. (2010) In preparation

## Current Model for JA inducible *NtPMT* gene expression

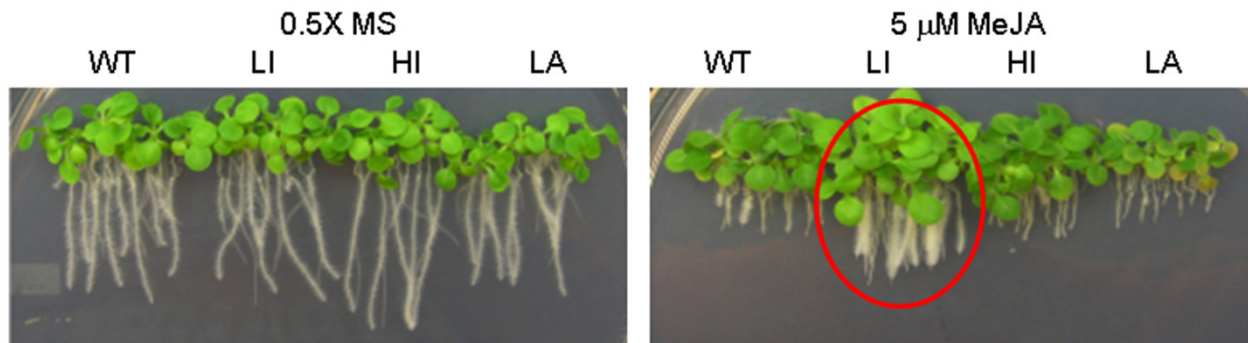


The JAR1 conjugating enzyme forms Jasmonoyl-isoleucine (JA-Ile) in the cytosol. JA-Ile promotes SCF<sup>COI1</sup> interaction with JAZ transcriptional repressors, leading to their ubiquitination and degradation by the 26S proteasome. The MYC2 transcription factor is then free to regulate the expression of genes involved in JA response either alone or in combination with other TFs.

If *COI1* is not available gene expression leading to alkaloid formation can not be activated



LI (*nic2*) mutants of Burley 21 are MeJA insensitive - Is this *NtCOI1*?



Zhang H, et al. (2010) In preparation

## **Our Research Goals:**

**Broadly understand the integrated circuitry that regulates the formation and accumulation of nicotine and related alkaloids in plant cells**

- what genes are responsible for the biosynthesis of the major and minor alkaloid of tobacco
- how are these genes regulated [transcriptional machinery - regulatory elements and protein factors]
- what cellular processes control metabolite flux and alkaloid accumulation

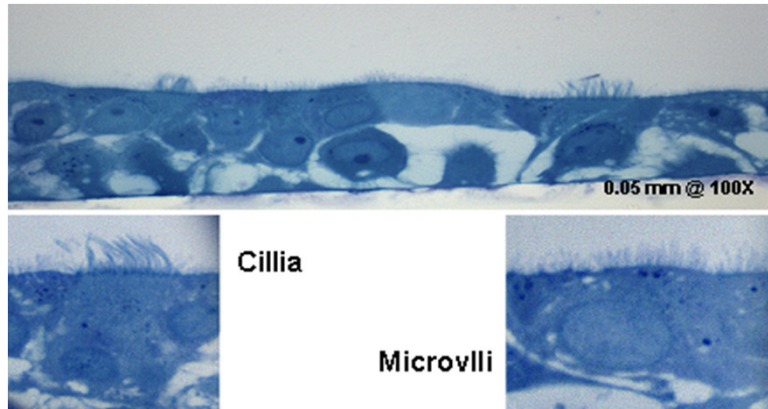
**Use our acquired knowledge to selectively alter leaf chemistry and composition to reduce levels of "harm components" in tobacco products**

- major (nicotine, nor nicotine) / minor (anatabine, anabasine, anatabine, myosmine) alkaloids
- tobacco specific nitrosamine (TSNA) levels in "cured" and "fermented" tobacco products
- various N-rich substrates, simple and complex sugars (lignins), etc that form particulates and volatiles.

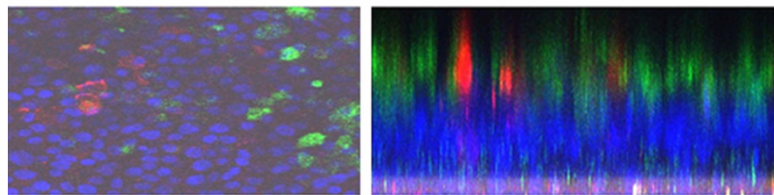
**Evaluate whether these "Harm Reduced" tobacco products demonstrate a differential ability to influence (potentiate, activate, repress) human cellular process.**

- establish in vitro human lung and oral epithelial cell culture exposure systems
- use transcriptomic, proteomic, and metabolomic approaches to describe the effects of whole smoke, smoke condensate (CS), and tobacco soluble extracts (SE) on cellular function

## Mucociliary differentiation of Human Bronchial Epithelial (NHBE) cells for smoke exposure studies

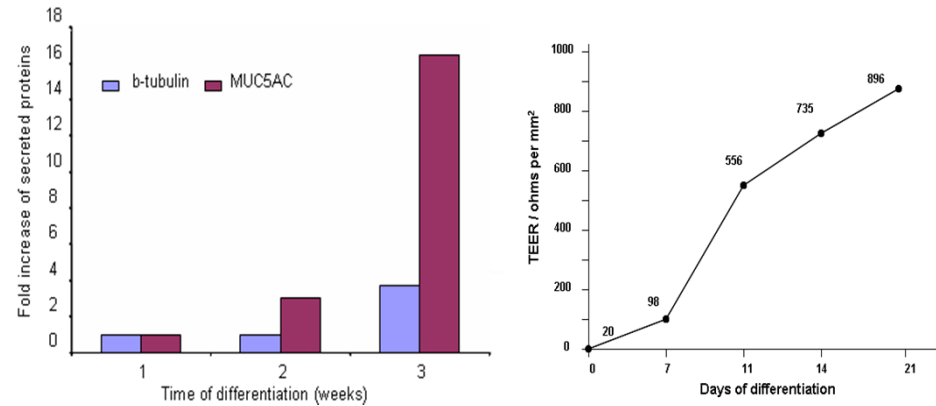


Semithin (0.25  $\mu$ m) plastic embedding of human lung epithelial cells in culture. Photograph by T. Kotova & Dr. MS Forbes (Pediatrics)

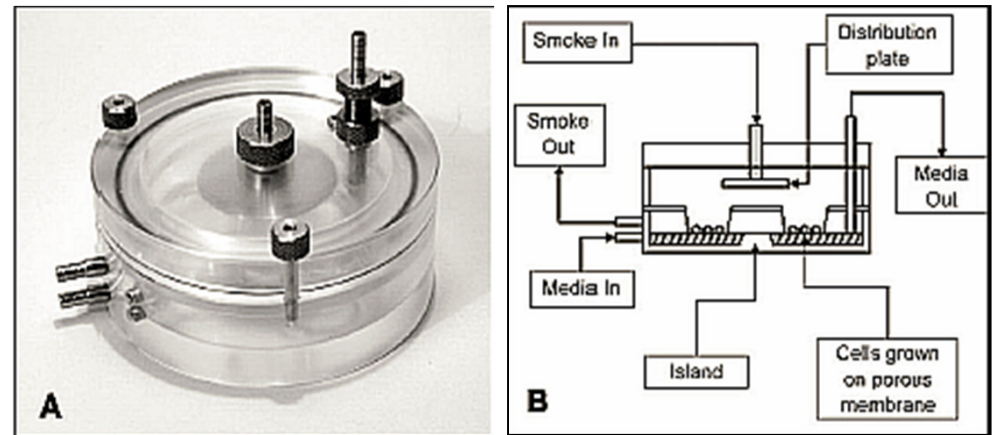


Leica MLL (confocal/spectrum) 25x

**Goblet cells (microvilli)** - ~7.0% Alexa 658 (red) Mouse anti MUC5AC  
**Ciliated cells** - ~21.1% Alexa 488 (green) Rabbit anti  $\beta$ -tubulin  
**Columnar epithelium (basal cells)** - DAPI stained nuclei (blue)



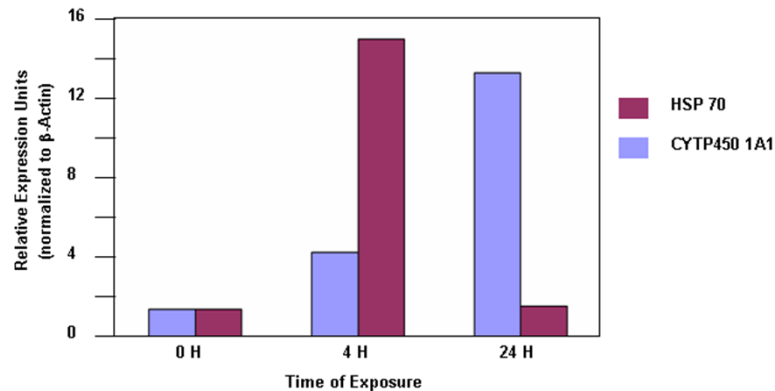
Immunodetection of MUC5AC and  $\beta$ -tubulin secreted by human bronchial epithelial cells during lung cell differentiation (left) and acquisition of Trans Epithelial Electrical Resistance (TEER) during differentiation.



British American Tobacco UK and Curbridge Engineering, Ltd. Southampton, UK  
 Photo from Maunders et al. (2007) *AJP Lung Cell Mol Physiol* 292: L1248–L1256

## Validation of "harm reduction" in selectively modified tobacco products

Transcriptomic analysis of human bronchial epithelial cells (NHBE) to exposure from smoke from tobacco smoke (Kentucky 1R5F). Using qRT-PCR human gene expression arrays and cytokine/inflammatory response assays.

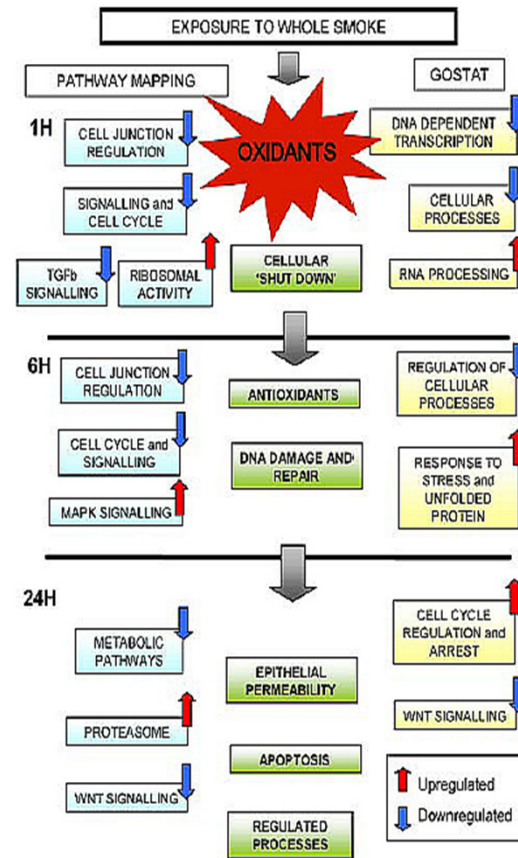


Examination of changes in expression for genes involved in oxidative metabolism, response to stimuli or cell cycle regulation and the response to DNA damage. Controls shown are heat shock protein 70 kDa (HSP70B), cytochrome *P*-450 CYP1A1 (involved in bioactivation of polyaromatic hydrocarbons)

### Future plans

Commercial vs "harm reduced"

Comparison of cigarettes made from wild type leaf and leaf from selectively modified tobacco (e.g., low nicotine/nornicotine/ anatabine; NtERF2/5 mutant lines, NtERF, NtMYC2, NtCOI1 knockdown lines, etc) using *in vitro* cellular assays



Maunder et al. (2007) *AJP Lung Cell Mol Physiol* 292: L1248–L1256

## **Conclusions**

Have begun to unravel the integrated circuitry that regulates the formation and accumulation of nicotine and related alkaloids in plant cells

Identified both key regulatory elements in the promoters of genes controlling key biosynthetic enzymes as well as an array of TFs that work through these elements.

Identified multiple new targets for potential manipulation that could affect cellular processes controlling formation of both major (nicotine, nor nicotine) / minor (anatabine, anabasine, anatabine, myosmine) alkaloids

## **Recommendation**

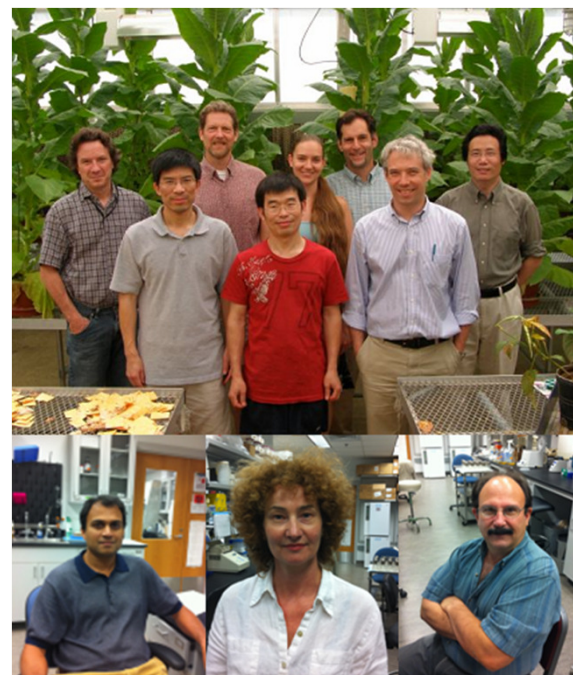
Careful evaluation of the effects of selectively altering leaf chemistry and composition are needed to determine if these products provide "harm reduction" as evidenced by their measured effects on human cellular process

## UVA Tobacco Research Group

|                               |                  |
|-------------------------------|------------------|
| Marta Bokowiec, PhD candidate | NtERFs           |
| Hongbo Zhang, PhD             | NtMYC2           |
| Shengchen Han, PhD            | NtJAZ/ZIM        |
| Tatiana Kotova, Tech          | Cell culture     |
| Rohan Rajapaske, DVM          | Tobacco exposure |
| Michael J. Wolkowicz, PhD     | Tobacco exposure |
| Shuran Li                     | Biostatistician  |
| Jim Roberts UVA HSC           | Bioinformatics   |
| Tom Laudeman UVA HSC          | Web development  |
| Undergraduate assistants:     |                  |
| Gina Kim                      | Sanam Shahid     |
| Cassie Campbell               | Annesha Basu     |

## Collaborators

|                          |                                |
|--------------------------|--------------------------------|
| Paul J. Rushton          | South Dakota State University  |
| John Finer               | Ohio State University          |
| Xianfeng Chen            | US DoD                         |
| Chengalrayan Kudithipudi | PM USA, Altria Client Services |
| Alec Hayes               | PM USA, Altria Client Services |



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