NC STATE UNIVERSITY

Molecular Biology-Based Approaches for Facilitating Compliance of Future Tobacco Products in an FDA Regulatory Environment

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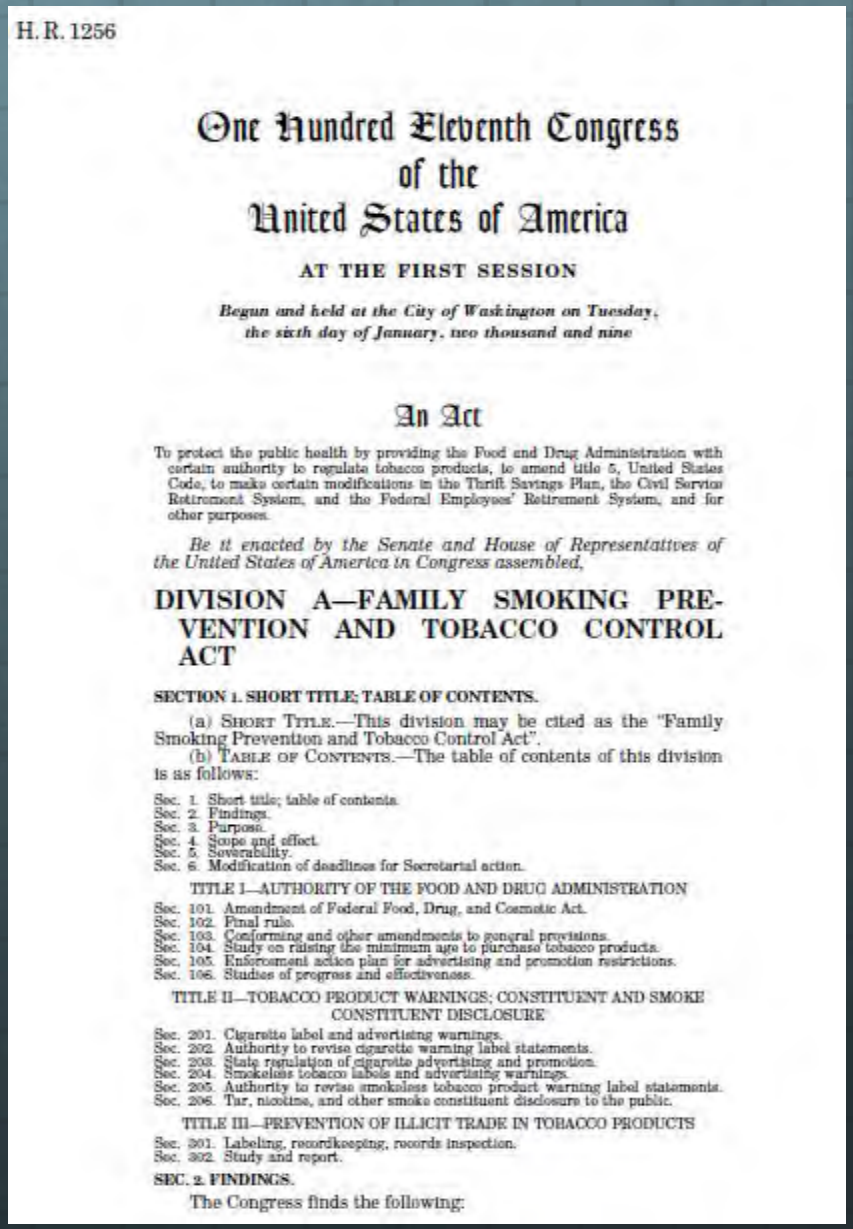
FDA Regulation in the United States



H. R. 1256

One Hundred Eleventh Congress
of the
United States of America

AT THE FIRST SESSION

Begun and held at the City of Washington on Tuesday, the sixth day of January, two thousand and nine

An Act

To protect the public health by providing the Food and Drug Administration with certain authority to regulate tobacco products, to amend title 5, United States Code, to make certain modifications in the Thrift Savings Plan, the Civil Service Retirement System, and the Federal Employees' Retirement System, and for other purposes.

Be it enacted by the Senate and House of Representatives of the United States of America in Congress assembled,

DIVISION A—FAMILY SMOKING PREVENTION AND TOBACCO CONTROL ACT

SECTION 1. SHORT TITLE; TABLE OF CONTENTS.

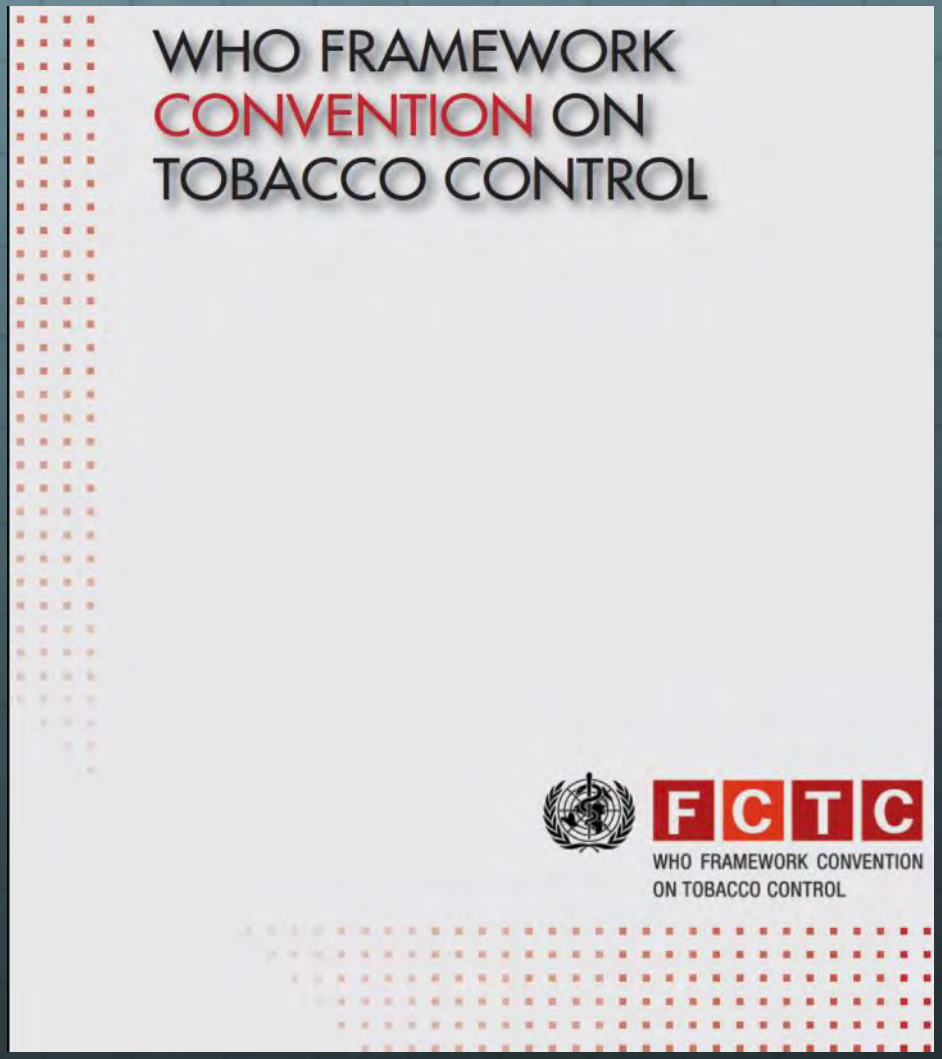
(a) SHORT TITLE.—This division may be cited as the "Family Smoking Prevention and Tobacco Control Act".

(b) TABLE OF CONTENTS.—The table of contents of this division is as follows:

SEC. 2. FINDINGS.

The Congress finds the following:

World Health Organization



Biotechnology (Transgenics) as a Tool for Tobacco Improvement

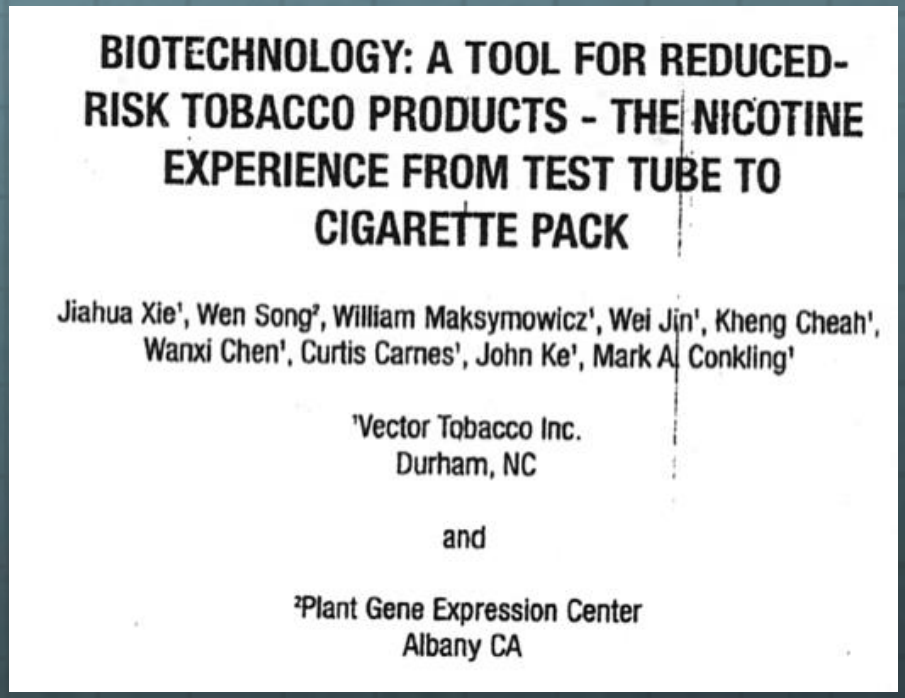
Hundreds if not thousands of manuscripts have been published documenting the ability of transgenics to enhance various aspects of tobacco performance

- Agronomic traits
  - Insect and nematode tolerance
  - Herbicide tolerance
  - Disease resistance (viral, bacterial and fungal)
  - Drought and salinity tolerance
  - Yield, biomass, flowering (maturation)
- Value-added traits
  - Harm reduction traits
  - Altered leaf surface chemistry
  - Flavor components
  - Alkaloid composition

Biotechnology as a Topic for TSRC Symposia

1992 – "Highlights of Current Research on Tobacco and Tobacco Chemisty"
1995 – "Impact of Plant Manipulation and Post Harvest Phenomena on Leaf Composition"
1999 – "Genetics and the Future of Tobacco"
2004 – "Biotechnology: a Tool for Developing Reduced-Risk Products
2007 – "Frontiers in Tobacco Biotechnology"
2010 – "Tobacco Research in the Era of Biotechnology and Genomics"

Biotechnology as a Tool for Producing New Commercial Tobacco Varieties



BIOTECHNOLOGY: A TOOL FOR REDUCED-RISK TOBACCO PRODUCTS - THE NICOTINE EXPERIENCE FROM TEST TUBE TO CIGARETTE PACK

Jiahua Xie[1], Wen Song[2], William Maksymowicz[1], Wei Jin[1], Kheng Cheah[1], Wanxi Chen[1], Curtis Carnes[1], John Ke[1], Mark A. Conkling[1]

[1]Vector Tobacco Inc.
Durham, NC

and

[2]Plant Gene Expression Center
Albany CA

Single Example:
Vector 21-41

Vector 21-41: a transgenic cultivar engineered to contain very low nicotine. Used to produce low nicotine cigarettes designed to help those who want to quit to wean themselves off their nicotine addition.

Grown on minimal acreage.

Why Has Biotechnology Failed to Play a Significant Role in Commercial Tobacco Variety Development?

- Multiple licensing agreements must be negotiated (and paid for) in order to commercialize a GM variety
- Time and costs for deregulation of a transgenic event can be very high
- Fear of market loss by consumers who consider the process unnatural and believe GM crops may lead to unintended negative health and/or environmental consequences

In the Face of these Obstacles, How Can Biotechnology Be of Use to the Tobacco Industry, Particularly with Respect to FDA Regulation?

Current Model: Utilize the techniques of molecular biology to discover genes of interest and validate the effects of their manipulation, then use this information to obtain a similar result using non-GM materials and approaches

Future Model (?): Utilize newly developed genome engineering methodologies to introduce specific changes to the genome in manner that leaves no foreign DNA incorporated

Aspects of FDA Regulation that May be Impacted by Biotechnology

"Harmful and Potentially Harmful Constituents" in Tobacco Products as Used in Section 904(e) of the Federal Food, Drug, and Cosmetic Act

DRAFT GUIDANCE

For the purpose of establishing "a list of harmful and potentially harmful constituents, including smoke constituents, to health in each tobacco product by brand and by quantity in each brand and subbrand," as required under section 904(e) of the Act, FDA believes that the phrase "harmful and potentially harmful constituent" includes any chemical or chemical compound in a tobacco product or in tobacco smoke:

a) that is or potentially is inhaled, ingested, or absorbed into the body; and

b) that causes or has the potential to cause direct or indirect harm to users or non-users of tobacco products. Examples of constituents that have the "potential to cause direct harm" to users or non-users of tobacco products include constituents that are toxicants, carcinogens, and addictive chemicals and chemical compounds. Examples of constituents that have the "potential to cause indirect harm" to users or non-users of tobacco products include constituents that may increase the exposure to the harmful effects of a tobacco product constituent by: 1) potentially facilitating initiation of the use of tobacco products; 2) potentially impeding cessation of the use of tobacco products; or 3) potentially increasing the intensity of tobacco product use (e.g., frequency of use, amount consumed, depth of inhalation). Another example of a constituent that has the "potential to cause indirect harm" is a constituent that may enhance the harmful effects of a tobacco product constituent.

Aspects of FDA Regulation that May be Impacted by Biotechnology

U.S. Food and Drug Administration

Tobacco Products Scientific Advisory Committee

Purpose

The Tobacco Products Scientific Advisory Committee advises the Commissioner or designee in discharging responsibilities as they relate to the regulation of tobacco products.

The Committee reviews and evaluates safety, dependence, and health issues relating to tobacco products and provides appropriate advice, information and recommendations to the Commissioner of Food and Drugs.

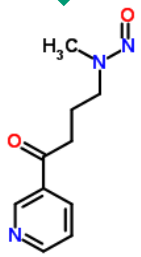
Specifically, the Committee will submit reports or recommendations on tobacco-related topics, including:

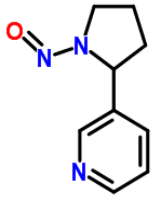
- **The impact of the use of menthol in cigarettes on the public health, including such use among children, African Americans, Hispanics and other racial and ethnic minorities**
- **The nature and impact of the use of dissolvable tobacco products on the public health, including such use on children**
- **The effects of the alteration of nicotine yields from tobacco products and whether there is a threshold level below which nicotine yields do not produce dependence on the tobacco product involved**
- **Any application submitted by a manufacturer**

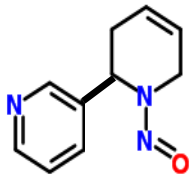
Tobacco-Specific Nitrosamines

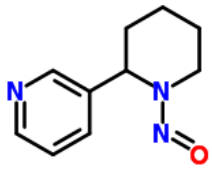
Nicotine **Nornicotine** **Anatabine** **Anabasine**

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









NNK **NNN** **NAT** **NAB**

Highly Carcinogenic **Not Carcinogenic**

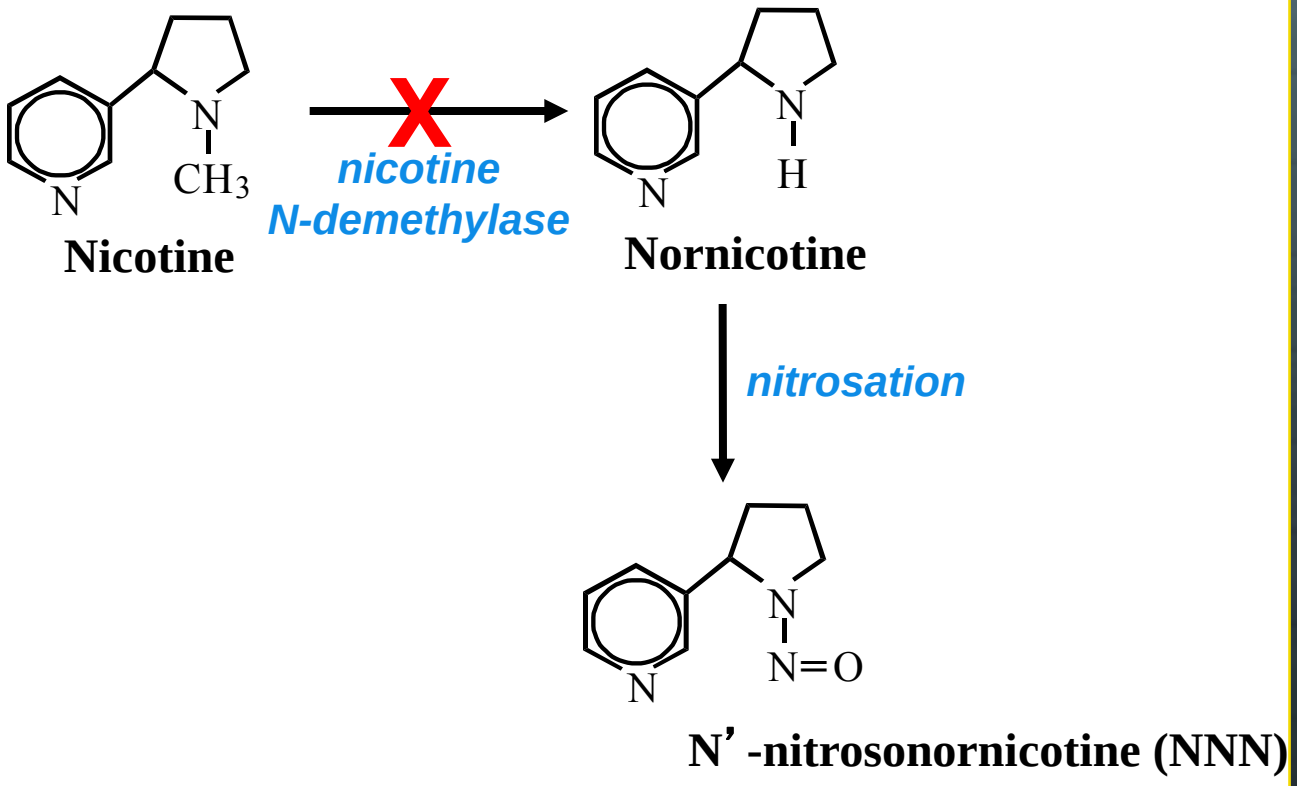
Tobacco-Specific Nitrosamines

Table 1 Toxicants recommended for mandated lowering by WHO

Toxicant	Level in µg/mg nicotine	Criteria for selecting the value
NNK	0.072	Median value of data set
NNN	0.114	Median value of data set
Acetaldehyde	860	125% of the median value of data set
Acrolein	83	125% of the median value of data set
Benzene	48	125% of the median value of data set
Benzo[a]pyrene	0.011	125% of the median value of data set
1,3-Butadiene	67	125% of the median value of data set
Carbon monoxide	18,400	125% of the median value of data set
Formaldehyde	47	125% of the median value of data set

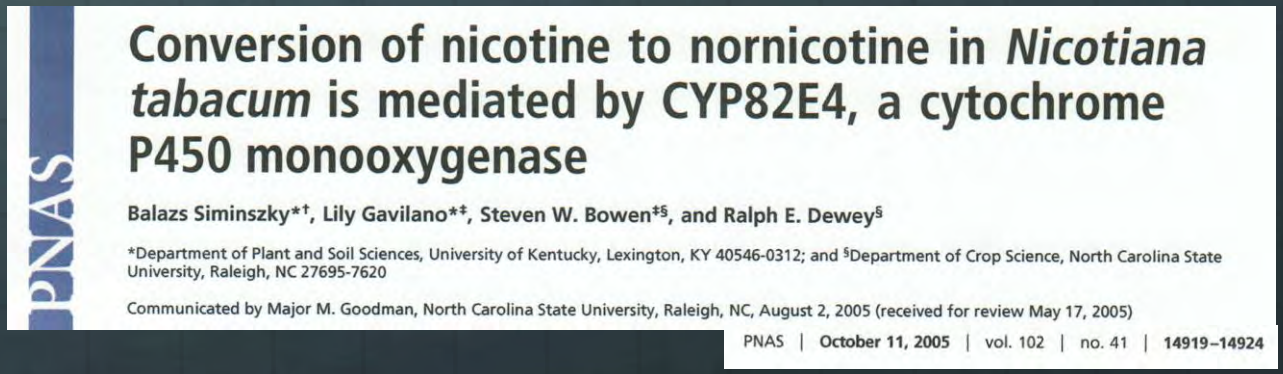
from Burns et al. 2008, Tobacco Control 17: 132-141

Strategy for Reducing NNN



Phase I: Isolation of *CYP82E4*, the Major Nicotine Demethylase Gene of *Nicotiana tabacum*

- Isolated using a gene expression profiling strategy (microarrays)
- Encodes cytochrome P450 enzyme
- Member of small, closely related gene family unique to *Nicotiana*



PNAS

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

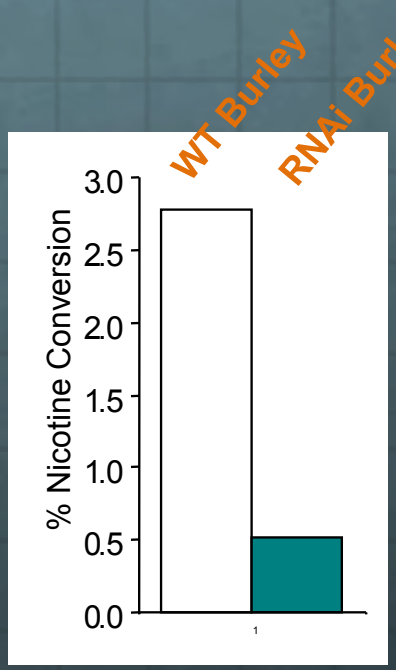
Balazs Siminszky*†, Lily Gavilano*‡, Steven W. Bowen‡§, and Ralph E. Dewey§

*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

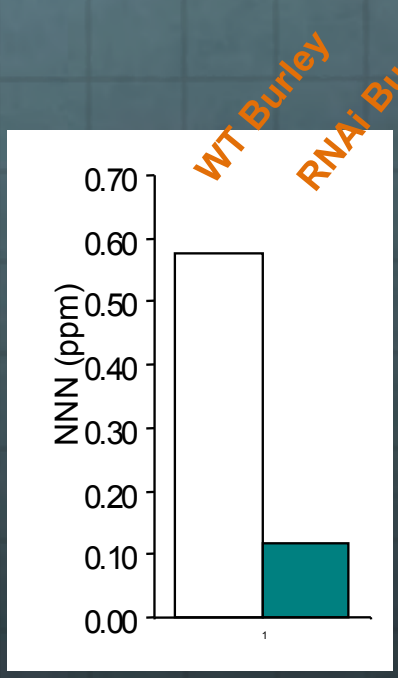
Communicated by Major M. Goodman, North Carolina State University, Raleigh, NC, August 2, 2005 (received for review May 17, 2005)

PNAS | October 11, 2005 | vol. 102 | no. 41 | 14919–14924

Phase 2: Demonstration that Transgenic Suppression of *CYP82E4* Family Can Reduce Nornicotine and NNN Levels



P = <0.0001



P = <0.0001

Plant Biotechnology Journal (2008) **6**, pp. 346–354

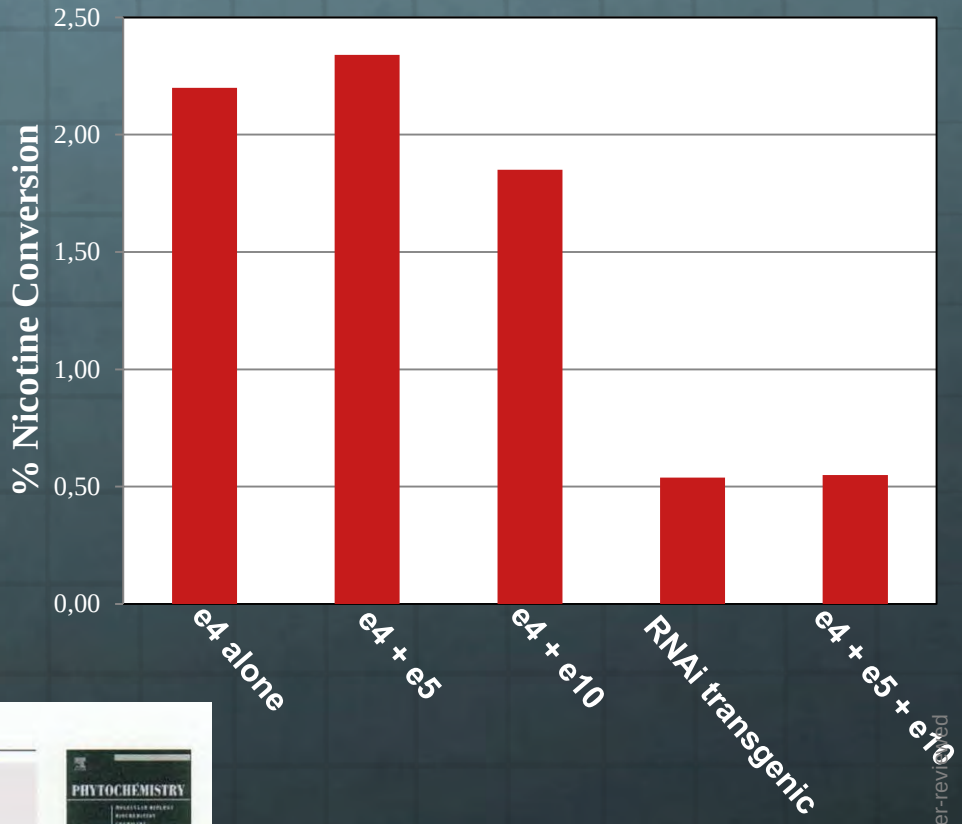
doi: 10.1111/j.1467-7652.2008.00324.x

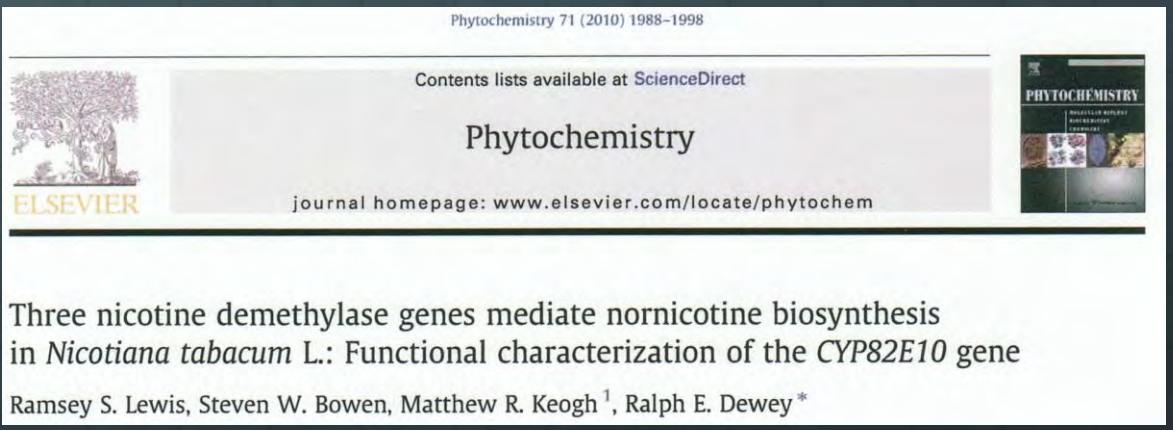
RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves

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]

Phase 3: Development of a non-GM Strategy for Nornicotine and NNN Reduction

- Generation and selection of knockout mutations in all three nicotine demethylase genes (*CYP82E4*, *CYP82E5* and *CYP82E10*)
- Pyramiding the three mutations within the same line





Phytochemistry 71 (2010) 1988–1998

Contents lists available at ScienceDirect

Phytochemistry

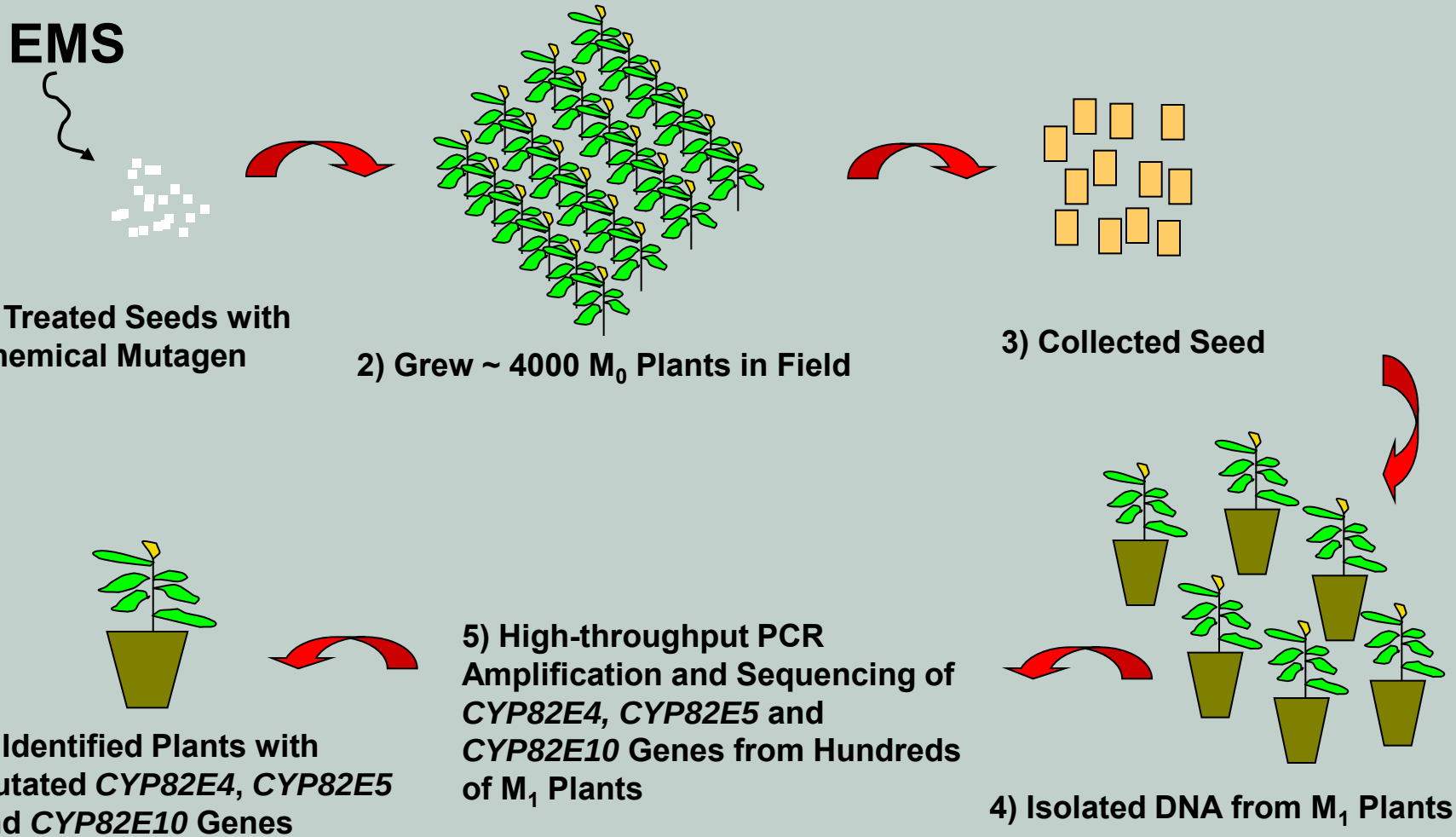
journal homepage: www.elsevier.com/locate/phytochem

ELSEVIER

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 *

Developing Non-GM Varieties with Greatly Reduced Nornicotine



Recovery of *CYP82E* Family Mutant Tobacco Plants

CYP82E4

- # of M_1 Plants Screened: 672
- # of *CYP82E4* Mutations Identified: 11
- Most notable mutant:

Plant	Amino Acid Changed	Effect on Gene Function
#775	Trp (329) to Stop	completely inactive

CYP82E5

- # of M_1 Plants Screened: 768
- # of *CYP82E5* Mutations Identified: 12
- Most notable mutant:

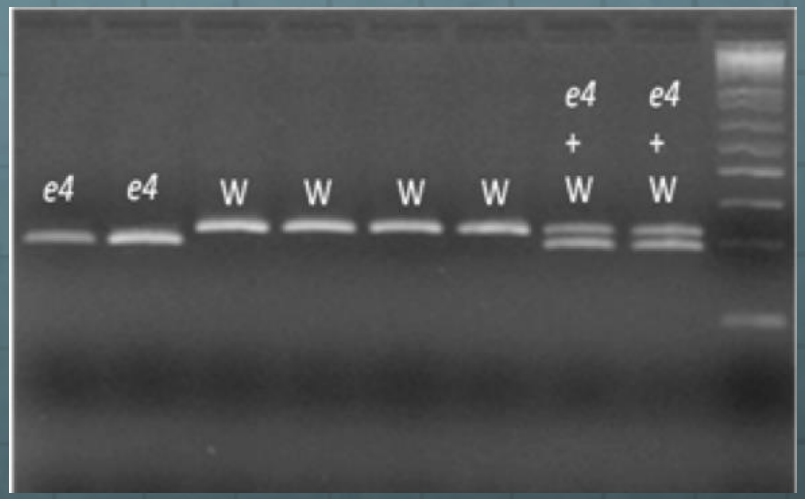
Plant	Amino Acid Changed	Effect on Gene Function
#1013	Trp (422) to Stop	completely inactive

CYP82E10

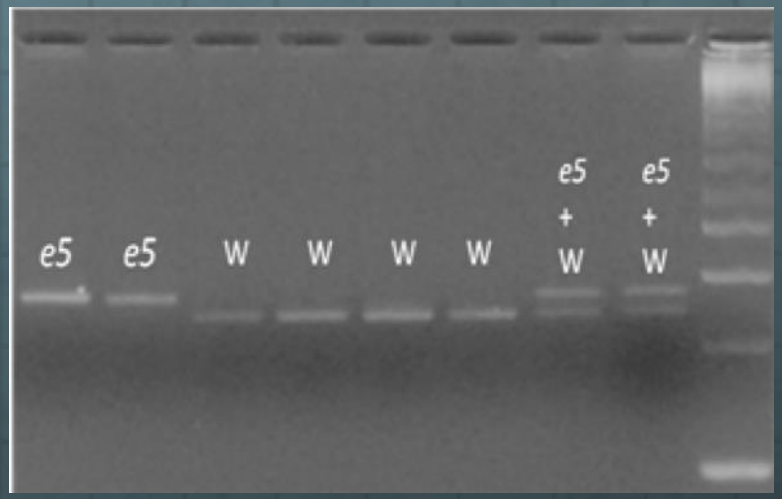
- # of M_1 Plants Screened: >1000
- # of *CYP82E10* Mutations Identified: 15
- Most notable mutant:

Plant	Amino Acid Changed	Effect on Gene Function
#1041	Pro (382) to Ser	completely inactive

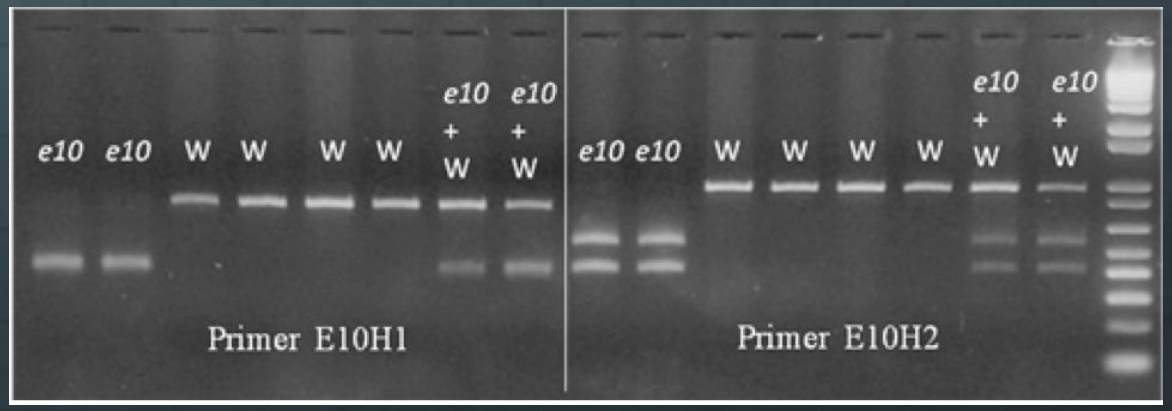
Marker-Assisted Introgression of Mutant *cyp82e* Alleles into Elite Commercial Lines



cyp82e4 marker



cyp82e5 marker



cyp82e10 markers

Deployment of Tobacco Varieties Possessing the Triple Mutant (non-GM) Harm Reduction Trait

New varieties first field tested in 2012:

Burley	Dark	Flue-Cured
TN86	Ky171	K326
NC7	Ky160	K346
Ky14 x L8	VA359	NC196
TN90		
NC BH129		

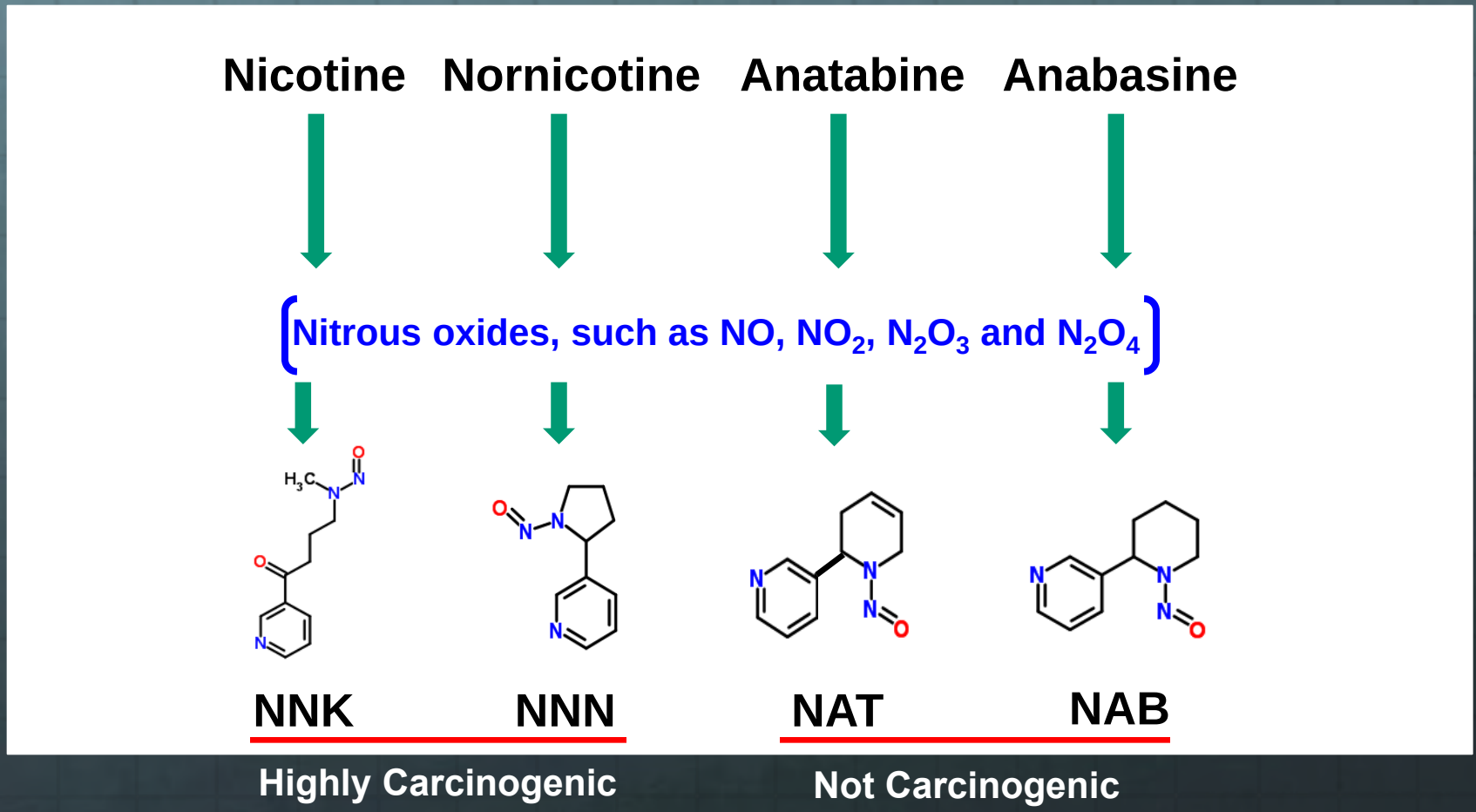
New varieties first field tested in 2013:

Burley	Dark	Flue-Cured
NC 2000	Narrow Leaf Madole	NC 71
NC 2002	VA 309	NC 297
NC 3	Little Crittenden	Speight 168
NC 4		NC 55
NC 5		
NC 6		
Banket A1		
Burley 21		

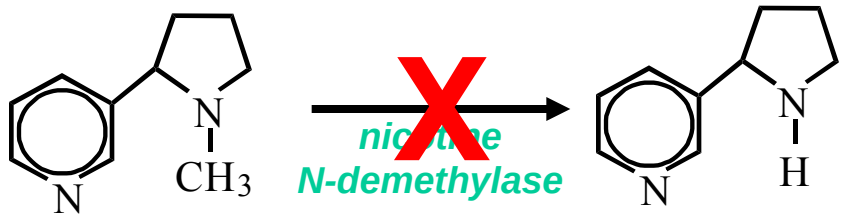
The first commercially released variety is scheduled to be NC7 in 2016

What About NNK?

Of the two carcinogenic TSNAs, NNK is arguably even worse than NNN as it is believed to be one of the major determinants of lung cancer



Strategy for Reducing NNK



Nicotine **Nornicotine**

Nitrosation
(NO+)

4-methylnitrosoamino-1-(3-pyridyl)-1-butanone (NNK)

N'-nitrosonornicotine (NNN)

Cadmium Reduction

- Classified as a Class I carcinogen by the International Agency for Research on Cancer (IARC)
- Associated with the following cancers:
  - Lung
  - Testes
  - Prostate

Because tobacco is a plant known to accumulate relatively high levels of Cd, there is much interest in minimizing its accumulation in the leaf

Cadmium Reduction

Molecular Strategies Designed to Reduce Cd in Tobacco Generally Fall Under the Following Two Categories:

1. Over-expression of heavy metal-binding proteins that chelate Cd and restrict intercellular movement

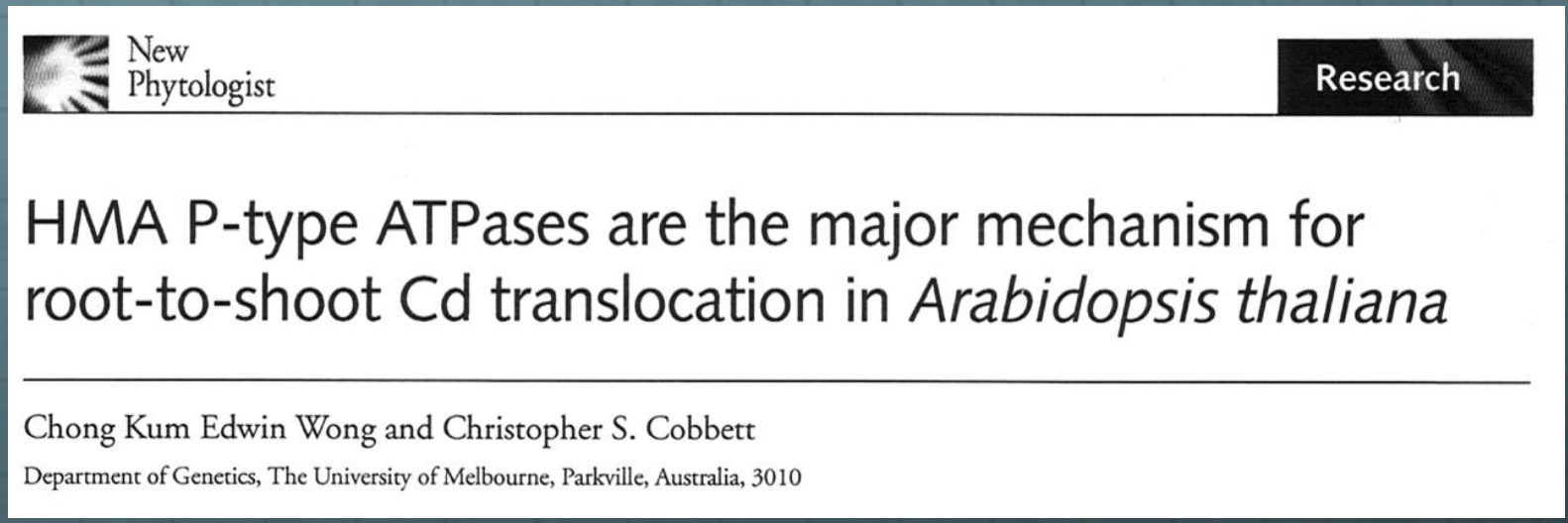
2. Manipulation of genes encoding Cd transporters

Both strategies are based on the concept of sequestering and immobilizing Cd ions within the roots to prevent its transport to leaf tissue

Heavy Metal Transporters

- *CAX* Genes
  - Encode divalent cation/proton antiporters
  - Most function by transporting metal ions into the vacuole while pumping H^+ into the cytosol.
- *HMA* (Heavy Metal ATPase) Genes
  - Drives the transport of heavy metals across cellular membranes, powered by the hydrolysis of ATP
  - Can be localized on either the plasma membrane or the tonoplast (vacuolar membrane)

HMA-Type Heavy Metal Transporters



New Phytologist

Research

HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in *Arabidopsis thaliana*

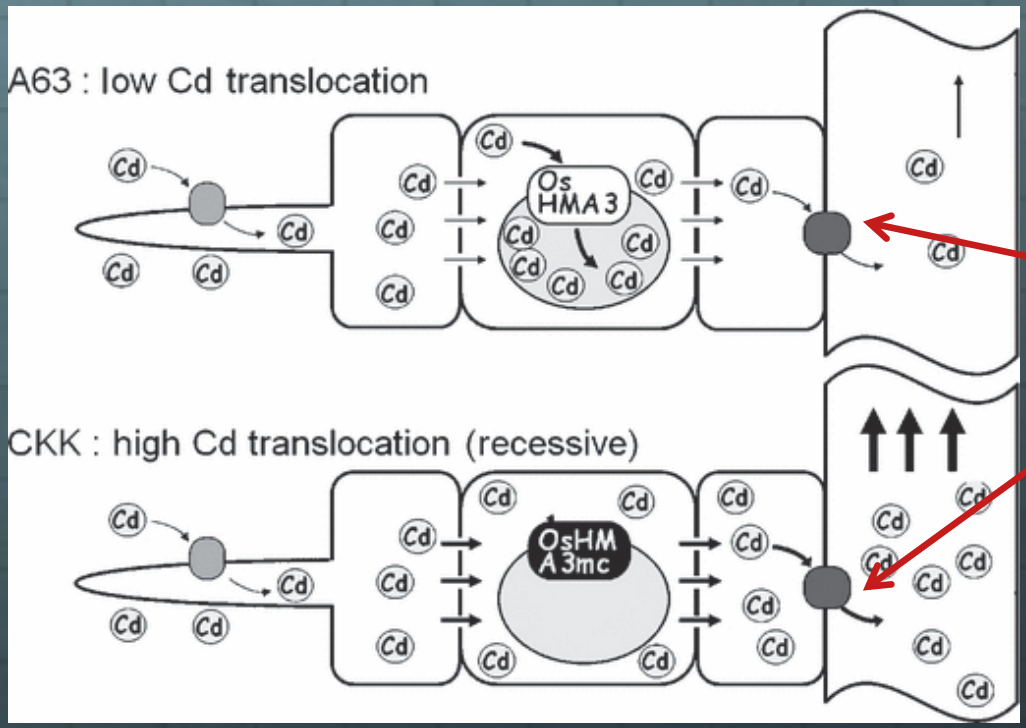
Chong Kum Edwin Wong and Christopher S. Cobbett

Department of Genetics, The University of Melbourne, Parkville, Australia, 3010

Wong et al., 2009. New Phytol. 181:71-78

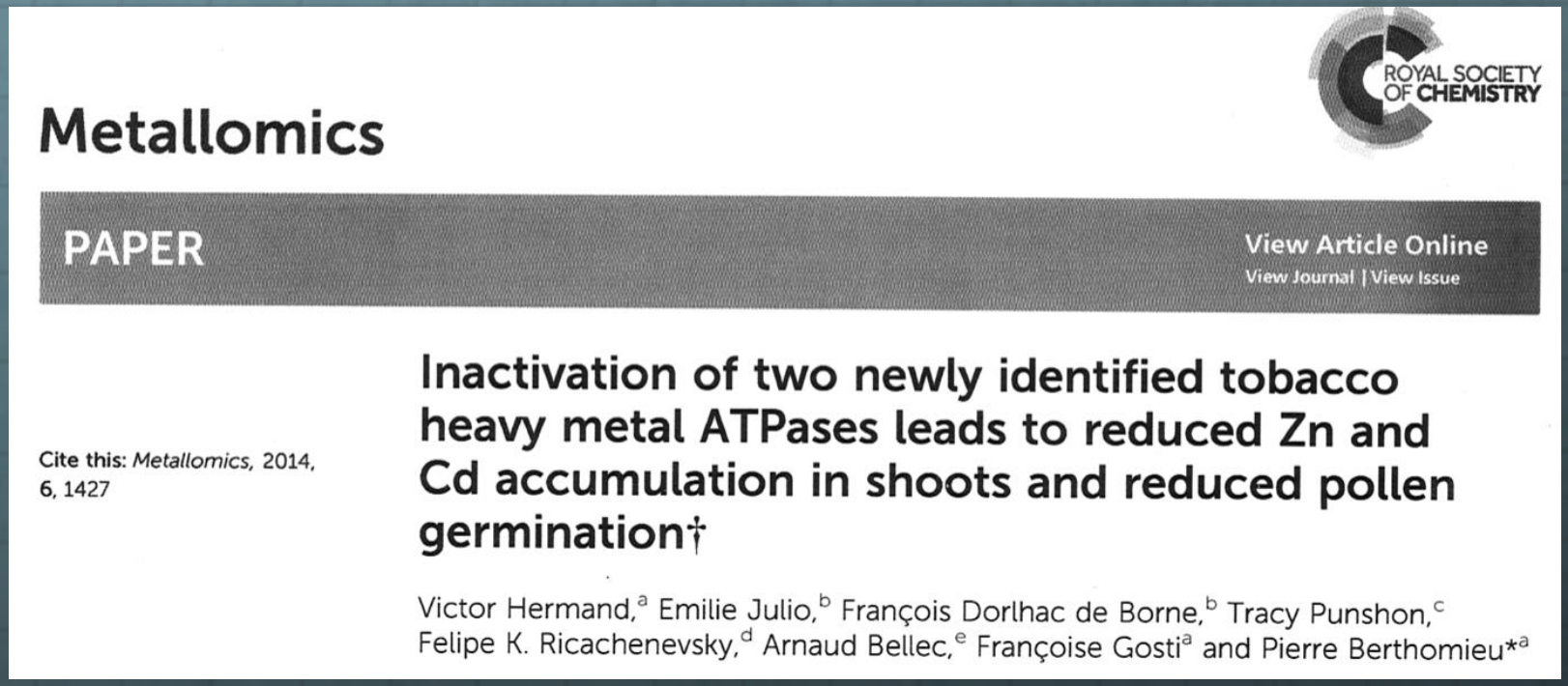
Research conducted in Arabidopsis suggested that certain members of the *HMA* class of metal transporters may be good candidates for reducing leaf Cd levels in plants.

HMA-Type Heavy Metal Transporters



Miyadate et al. 2010. New Phytologist 189: 190-199

HMA-Type Heavy Metal Transporters



Metallomics

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

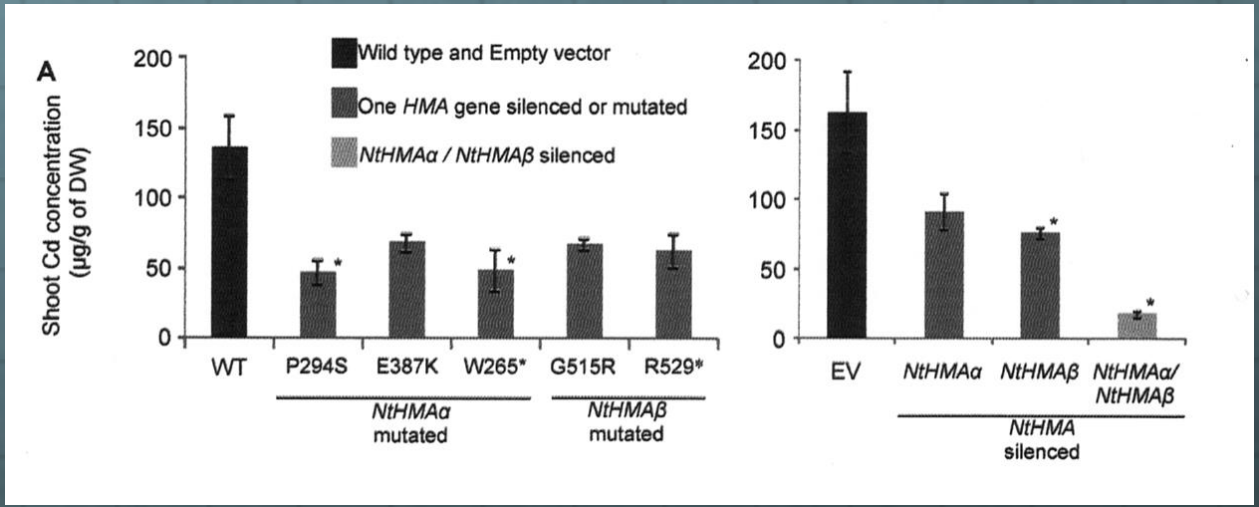
Cite this: *Metallomics*, 2014, 6, 1427

Inactivation of two newly identified tobacco heavy metal ATPases leads to reduced Zn and Cd accumulation in shoots and reduced pollen germination†

Victor Hermand,[a] Emilie Julio,[b] François Dorlhac de Borne,[b] Tracy Punshon,[c] Felipe K. Ricachenevsky,[d] Arnaud Bellec,[e] Françoise Gosti[a] and Pierre Berthomieu*[a]

Researchers from Imperial Tobacco characterized the tobacco homologs of HMA2 and HMA4 to evaluate their potential in reducing cadmium

HMA-Type Heavy Metal Transporters



EMS-induced mutants

Transgenic Suppression

When either *NtHMA*α or *NtHMA*β were down regulated, leaf cadmium was decreased by ~50%. Combining the two mutants, however, was detrimental to normal plant growth and development.

HMA-Type Heavy Metal Transporters

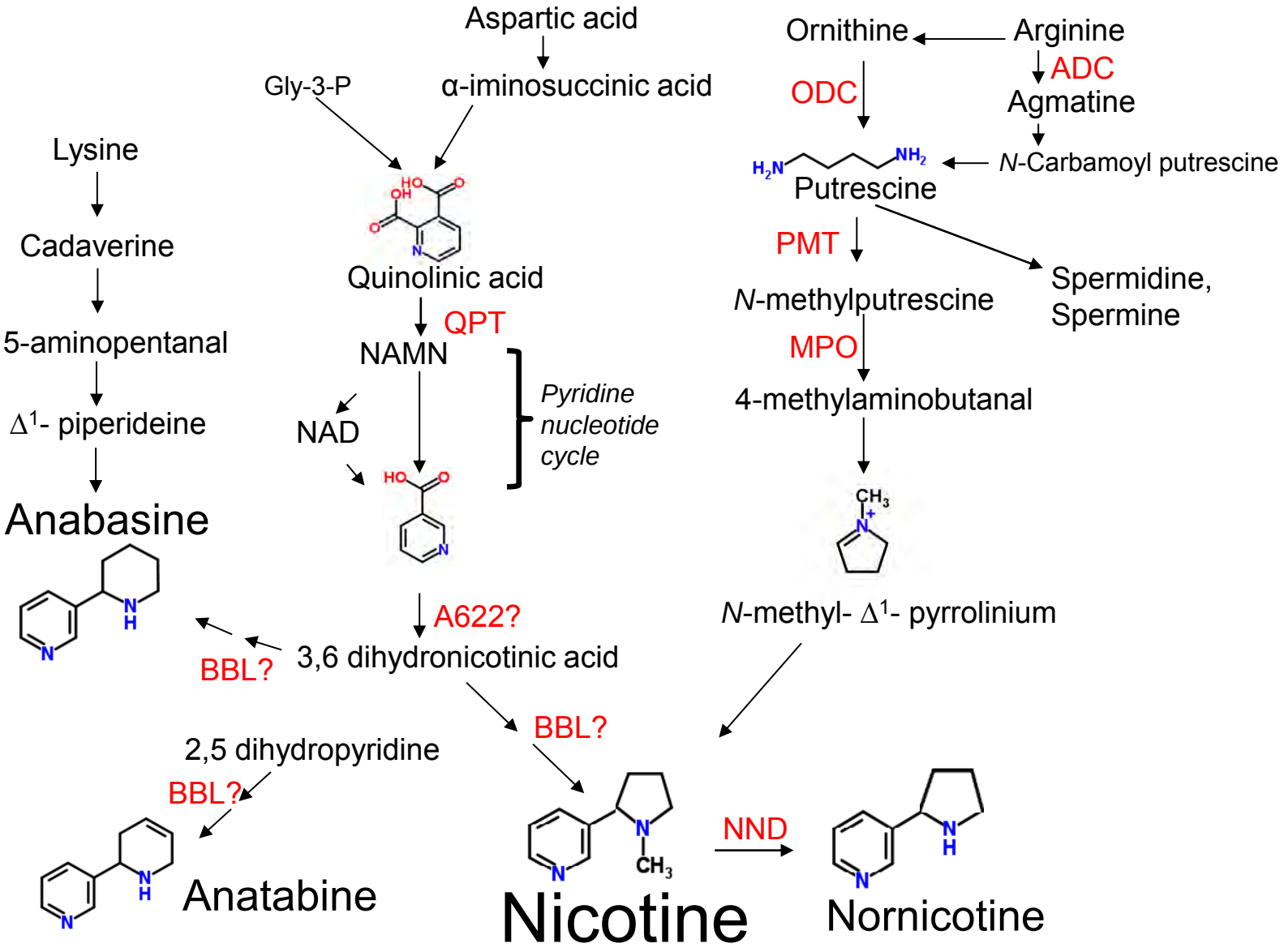
Hermand et al. 2104. Metallomics 6: 1427

Double *NtHMA* mutants likely suffer from zinc deficiencies. However, introducing single *NtHMA* EMS mutants may have potential for developing non-GM tobacco varieties with reduced cadmium.

Reducing Nicotine Content

- There is a large body of literature describing the molecular biology of alkaloid synthesis in tobacco
- The most straightforward and effective way to reduce nicotine is to block an essential step in the nicotine biosynthetic pathway

Tobacco Alkaloid Biosynthetic Pathway



Berberine Bridge Enzyme-Like (*BBL*) Gene Family of Tobacco

- Four identified members
  - *BBLa*
  - *BBLb*
  - *BBLc*
  - *BBLd* (minimal expression observed from this isoform)
- Flavin-containing oxidases
- Enzymatic function unclear, but functions at a final stage of nicotine biosynthesis, after condensation of the pyridine and pyrrolidine rings

Vacuole-Localized Berberine Bridge Enzyme-Like Proteins Are Required for a Late Step of Nicotine Biosynthesis in Tobacco[1][C][W]

Masataka Kajikawa, Tsubasa Shoji, Akira Kato, and Takashi Hashimoto*

Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630–0192, Japan

Plant Physiology®, April 2011, Vol. 155, pp. 2010–2022,

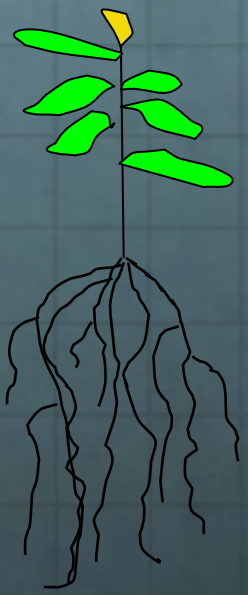
Field Analysis of anti-*BBL* K326 RNAi Doubled Haploid Lines (2012)

Genotype Means (flue-cured leaf, % dry weight)

Genotype	Nic	Nor	Anab	Anat	Total Alk.
K326	2.52	0.043	0.019	0.11	**2.70**
NC95	2.98	0.045	0.028	0.21	**3.26**
LAFC53	0.22	0.011	0.002	0.01	**0.25**
K326 RNAi DH22A	0.40	0.069	0.011	0.007	**0.48**
K326 RNAi DH32	0.43	0.085	0.013	0.007	**0.53**
K326 RNAi DH16A	0.45	0.068	0.011	0.007	**0.54**
K326 RNAi DH19	0.67	0.089	0.016	0.014	**0.79**
K326 RNAi DH303	0.76	0.083	0.015	0.021	**0.88**
K326 RNAi DH16B	0.54	0.069	0.012	0.012	**0.64**
Average of transgenics	0.54	0.077	0.013	0.011	**0.64**

Knockout Lines Have Been Identified in the Three Major *BBL* Genes by High-Throughput DNA Sequencing of a Mutagenized Tobacco Population

Nicotiana tabacum



BBLa
1,248 → **BBLa** knockout line
Plants screened

BBLb
1,344 → **BBLb** knockout line
Plants screened

BBLc
2,112 → **BBLc** knockout line
Plants screened

These mutations have been crossed and combined in all possible combinations to create a series of non-GM tobacco lines with varying levels of nicotine

Mutations in *BBL* Genes Appear to Reduce Alkaloid Content in an Additive Manner

Genotype Means (% dry weight)

Genotype	Nic	Nor	Anab	Anat	Total Alk.
NS BBLa/BBLb/BBLc	0.0907	1.4121	0.0109	0.0984	**1.6121**
SM BBLa/BBLb/bblc	0.1116	1.3256	0.0109	0.1136	**1.5617**
SM BBLa/bblb/BBLc	0.0885	0.9555	0.0090	0.0817	**1.1347**
SM bbla/BBLb/BBLc	0.1086	0.9428	0.0111	0.0583	**1.1208**
DM BBLa/bblb/bblc	0.0471	1.1847	0.0119	0.1042	**1.3478**
DM bbla/BBLb/bblc	0.1054	1.1470	0.0139	0.0916	**1.3579**
DM bbla/bblb/BBLc	0.0056	0.3309	0.0126	0.0073	**0.3564**
TM bbla/bblb/bblc	0.0050	0.1001	0.0080	0.0058	**0.1188**

Relative contribution of each isofrom: *BBLa = BBLb > BBLc*

Reducing Nicotine Content

- The step of alkaloid biosynthesis encoded by *BBL* genes appears to be a particularly good target for the production of high quality, reduced alkaloid tobacco varieties
- We are currently developing a series of elite flue-cured (K326) and burley (TN90) lines that differ in their nicotine contents by pyramiding different combinations of *bbl* mutations
  - Mid-high nicotine (70 – 75% of normal)
  - Low nicotine (20 – 25% of normal)
  - Very low nicotine (5 – 10% of normal)
- Test the agronomic properties of these new low alkaloid, non-GM lines

Precision Genome Editing Technologies

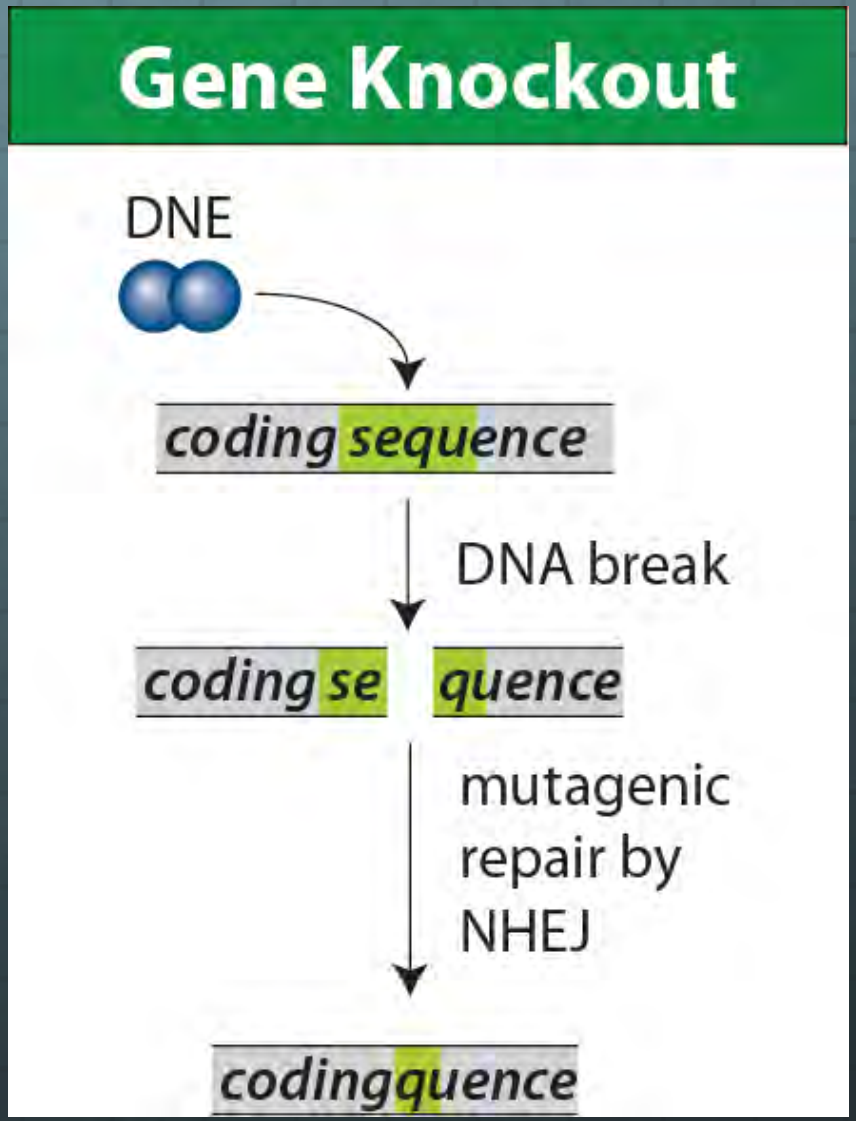
Why All the Excitement?

- Technical Advantages
  - Many desirable traits can be created by the mutagenesis or the subtle modification of endogenous genes as opposed to the introduction of a foreign gene
  - Traditional mutagenic agents (e.g. X-rays, ethyl methane sulfonate) cause genome-wide perturbations that can only be removed by numerous generations of backcrossing
  - Many desired mutations are too complex to recover using non-selective traditional agents
- Regulatory and "Societal" Advantages
  - If plants generated in this manner are not considered "regulated articles" by government agencies, the time and costs associated with commercialization become greatly reduce compared to traditional transgenic crops
  - More likely to gain broader public acceptance since the end product contains no foreign DNA

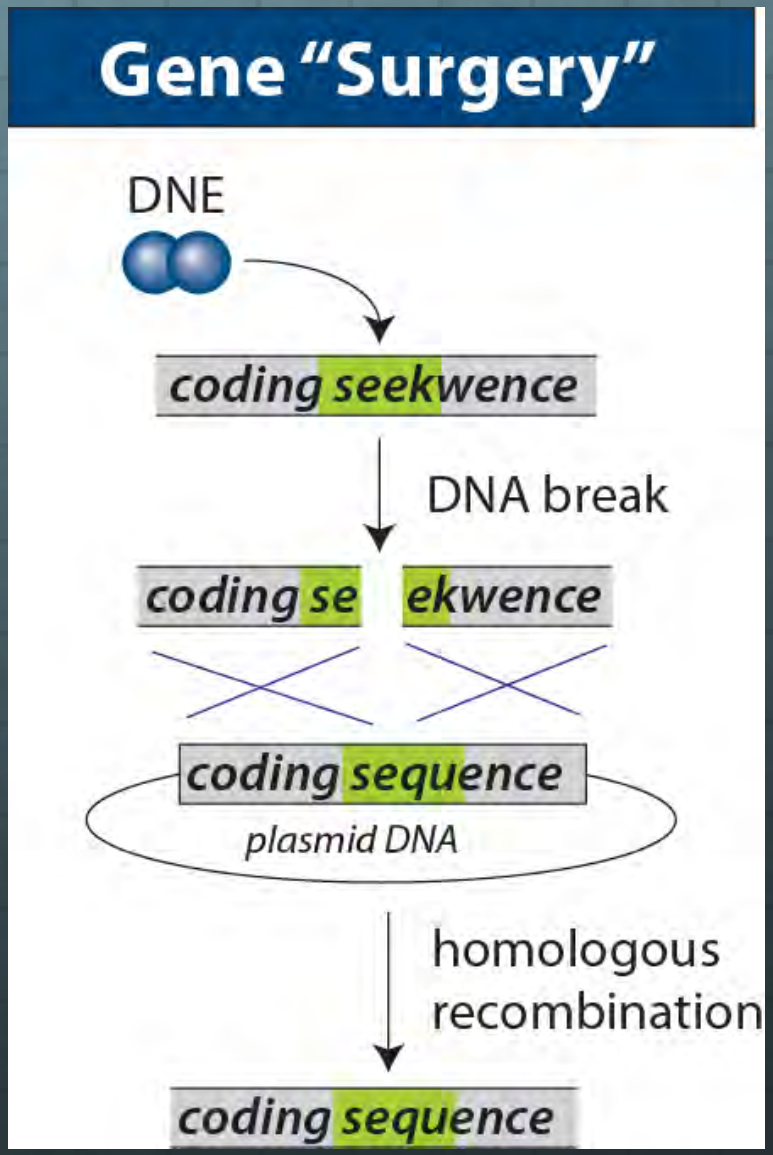
The Key to Precise Genome Modification Relies on the Introduction of Double Strand Breaks (DSBs) into Targeted Genomic Locations

- DSBs are repaired by one of two distinct DNA repair systems
  - Nonhomologous End Joining (NHEJ) = religation of the broken ends
    - Error prone, giving rise to short insertion/deletion events
    - Predominant system in higher eukaryotes, including plants
  - Homologous Recombination (HR) = repair corrected by a homologous template such as a sister chromatid or donor DNA
    - Repair is very accurate
    - Predominant system in bacteria and unicellular eukaryotes

Applications of Targeted DNA breaks



Applications of Targeted DNA breaks



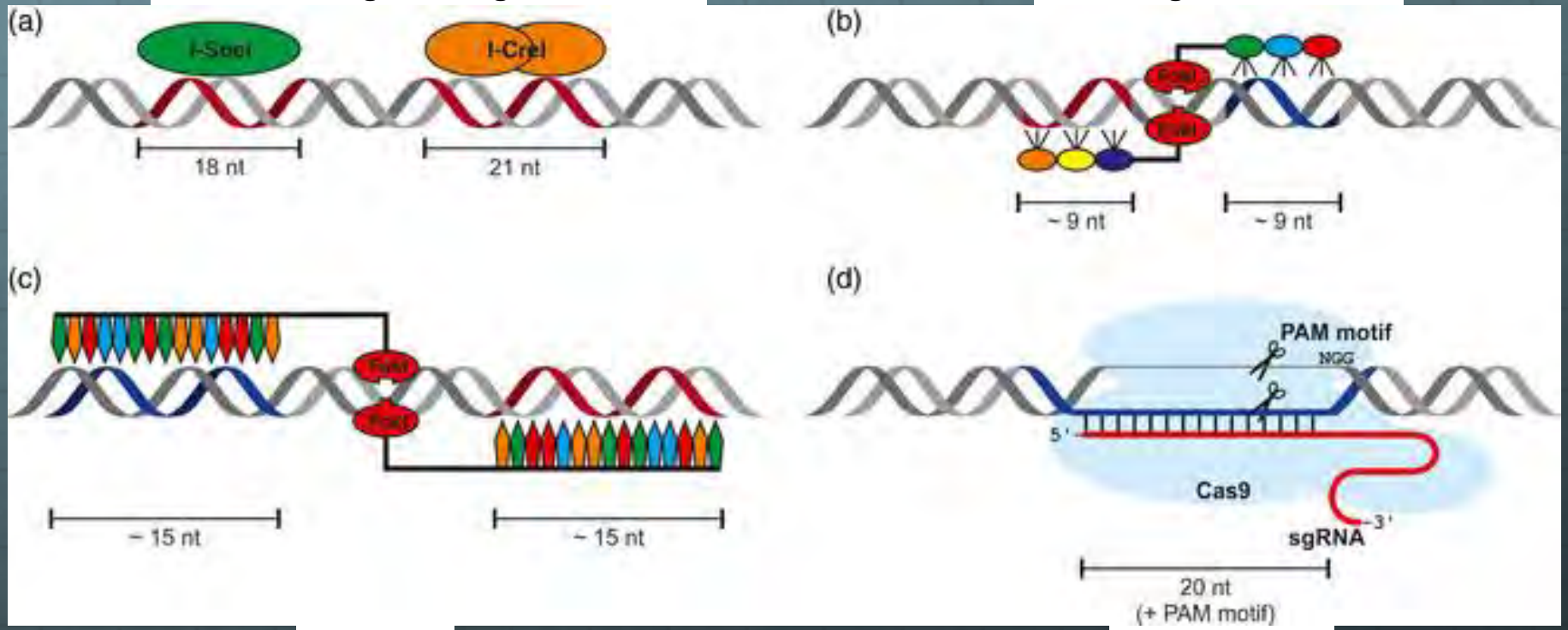
Engineered Nucleases Mediate Targeted Genome Modification

- Zinc finger nucleases (ZFNs)
- Engineered homing enzymes/meganucleases
- Transcription activator-like effector nucleases (TALENs)
- Clustered, regularly interspaced, short palendromic repeats (CRISPR)-Cas system

Engineered Nucleases

Custom-designed meganucleases

Zinc-finger Nucleases

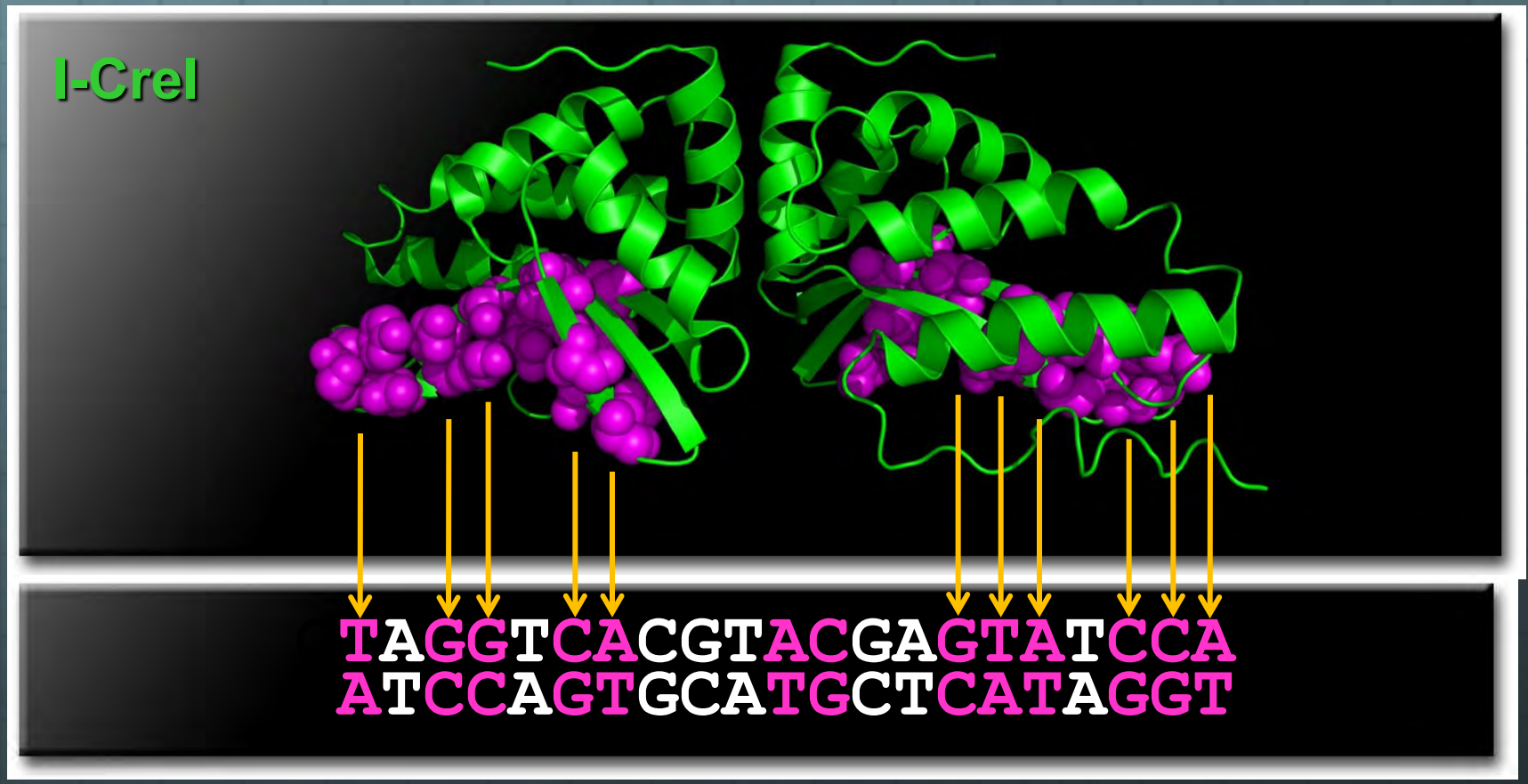


TALENs

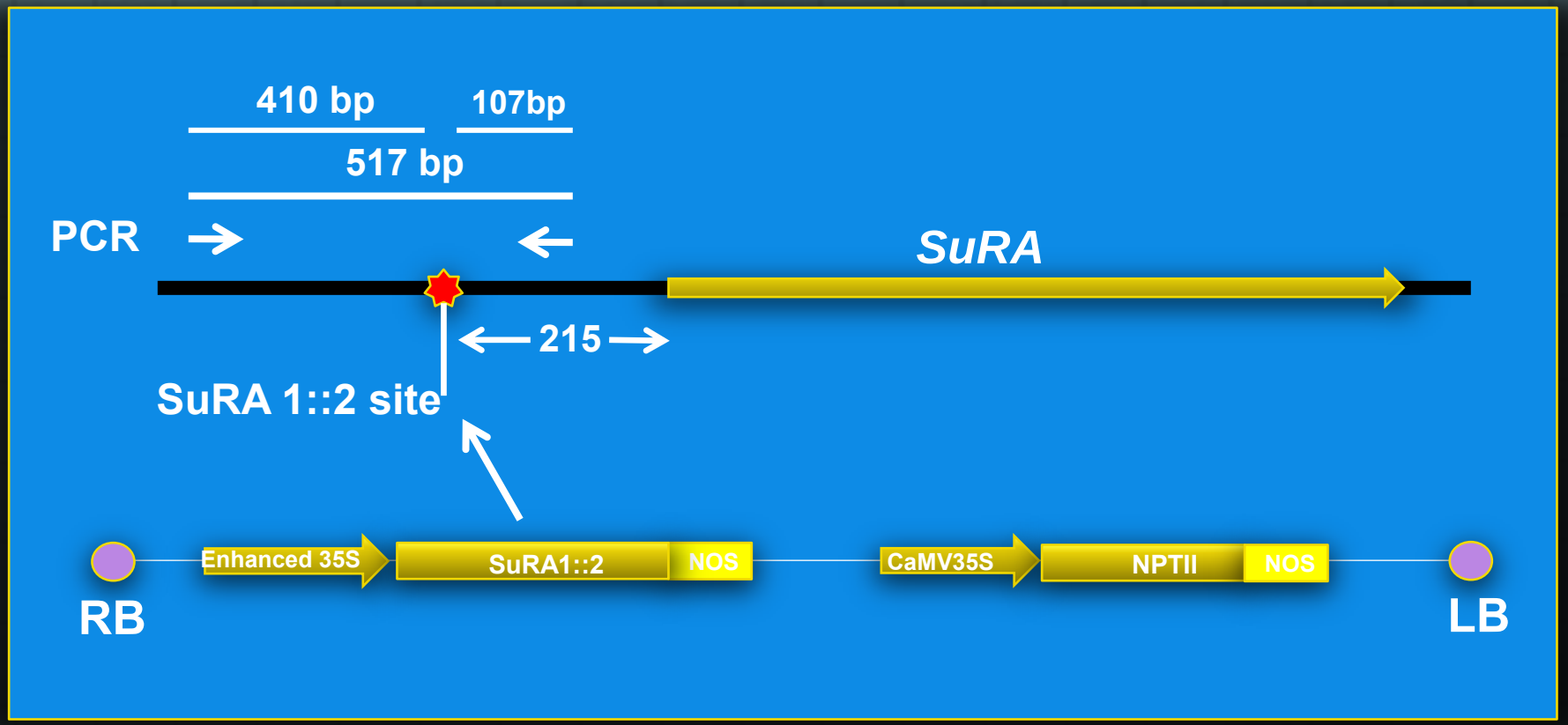
CRISPR-Cas

Puchta and Fauser, 2013. Plant J. doi: 10.1111/tpj.12338

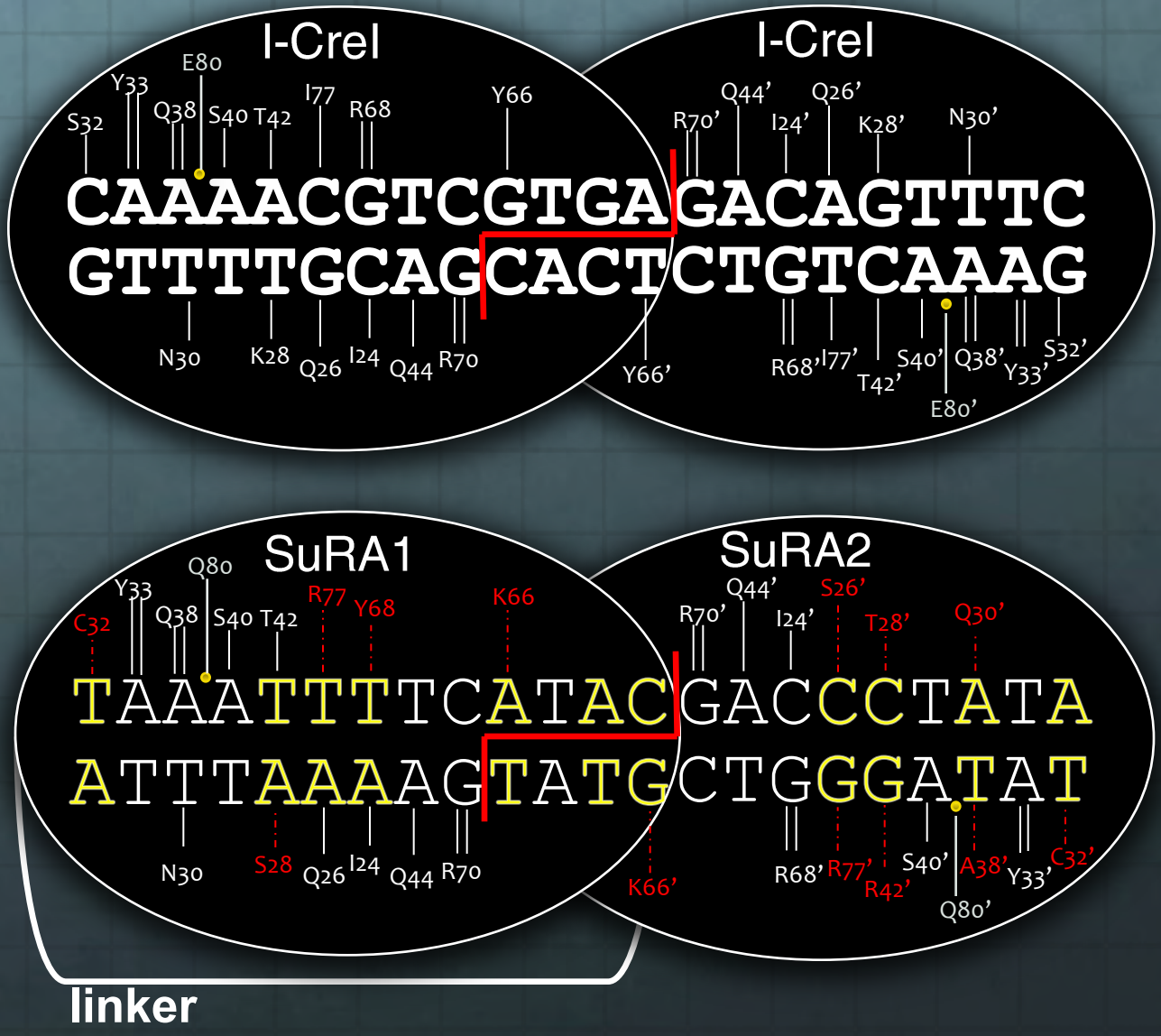
Re-engineering I-CreI Cleavage Specificity



Directed Genome Mutation in Tobacco



Redesign of the I-CreI Enzyme to Recognize The Tobacco SuRA Gene



Characterization of SurA Meganuclease-Induced Deletion Events in Tobacco

```
Plant
 WT    AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTATGAAAATTTAATATTACTTTAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
S143   AAGAAATTTGGTGCCAGCCATAGATATAGGGT GTATGAAAATTTAATATTACTTTAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
S131   AAGAAATTTGGTGCCAGCCATAGATATAGGGT  TATGAAAATTTAATATTACTTTAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
F125   AAGAAATTTGGTGCCAGCCATAGATATAG            ATTTAATATTACTTTAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
S38    AAGAAATTTGGTGCCAGCCATAG     G      ATGAAAATTTAATATTACTTTAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
S81    AAGAAATTTGGTGCCAGCCATAGATAT                          TACTTTAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
F98    AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTA                   CTTTAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
[*]    AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTAT                                                 TTAGTCGCCA
S29    AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTAT T (-167BP)
S131   AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTAT   CCGTCA        TTAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
S24    AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTAT                          AAATTGCACGGGCGCTCTATTTAGTCGCCA
S147   AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTAT                    TAATAGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
S143   AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTAT                       AGTAAAAATTGCACGGGCGCTCTATTTAGTCGCCA
S81    AAGAAATTTGGTGCCAGCCATAGATATAGGGTCGTATG TGAAGAAATTTGGTGCCACC       ATTGCACGGGCGCTCTATTTAGTCGCCA
S111                                                  (-61BP)  AATTGCACGGGCGCTCTATTTAGTCGCCA
```

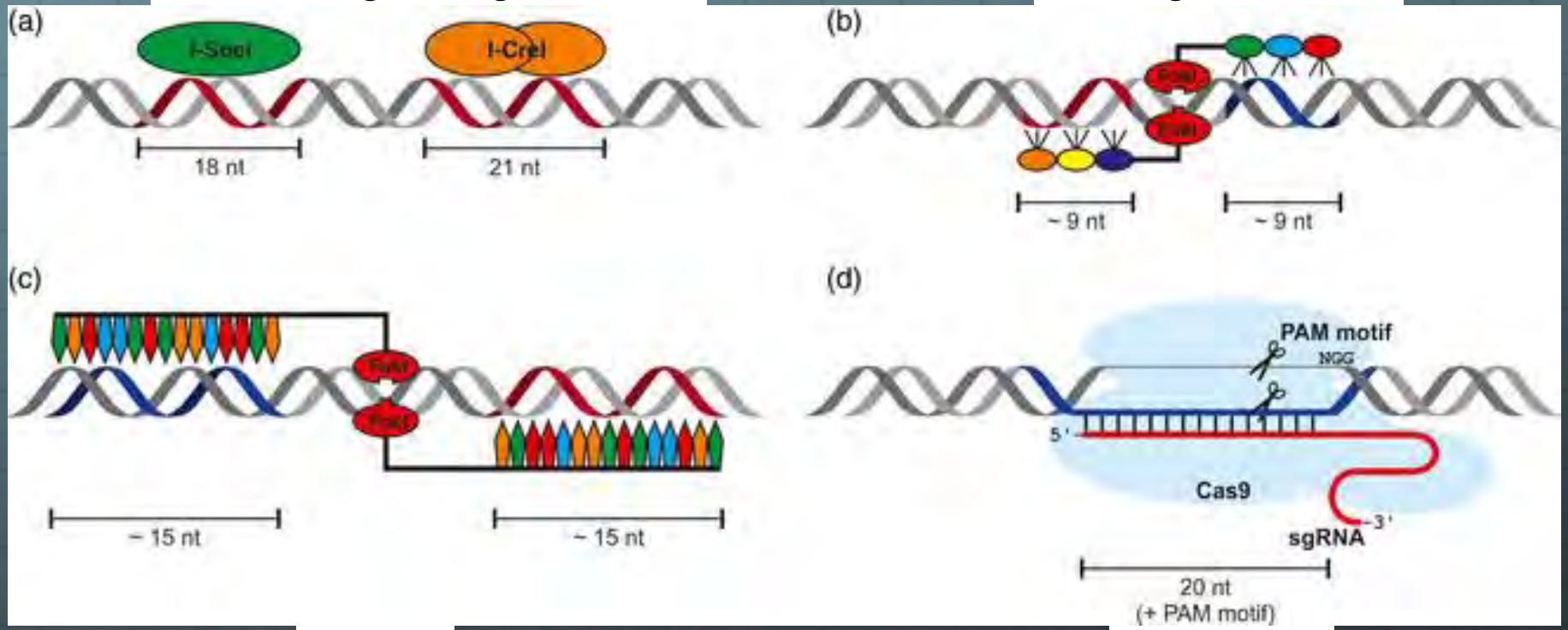
[*]= high frequency event recovered in over 20 independent lines

- Induced mutations found in ~20% of all transgenic plants examined
- The progeny of the transformed plants segregate independently for the introduced transgene and the induce mutations, making it easy to obtain plants with a mutation in the gene of interest and containing no foreign DNA

Engineered Nucleases

Custom-designed meganucleases

Zinc-finger Nucleases

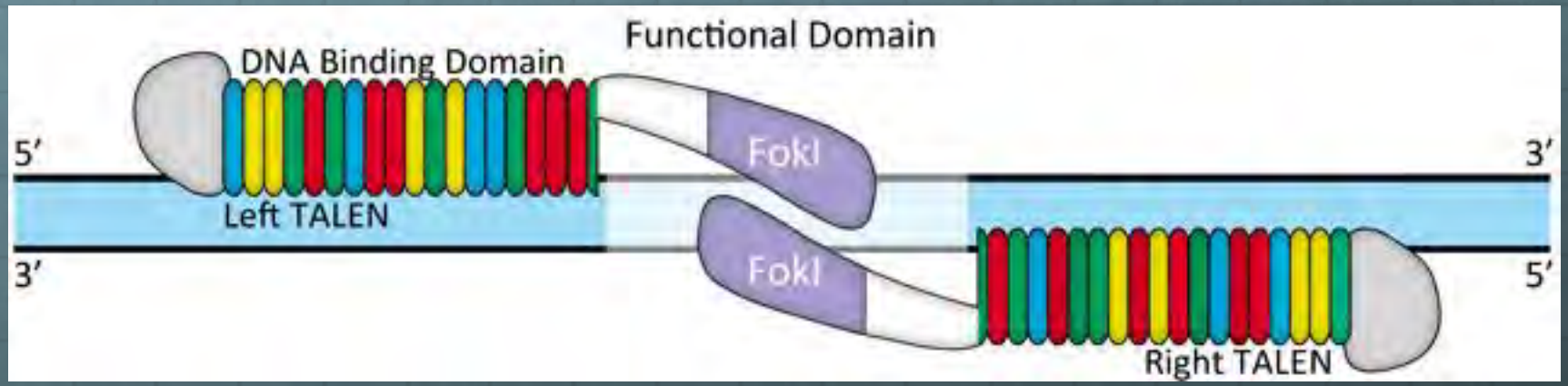


TALENs

CRISPR-Cas

Puchta and Fauser, 2013. Plant J. doi: 10.1111/tpj.12338

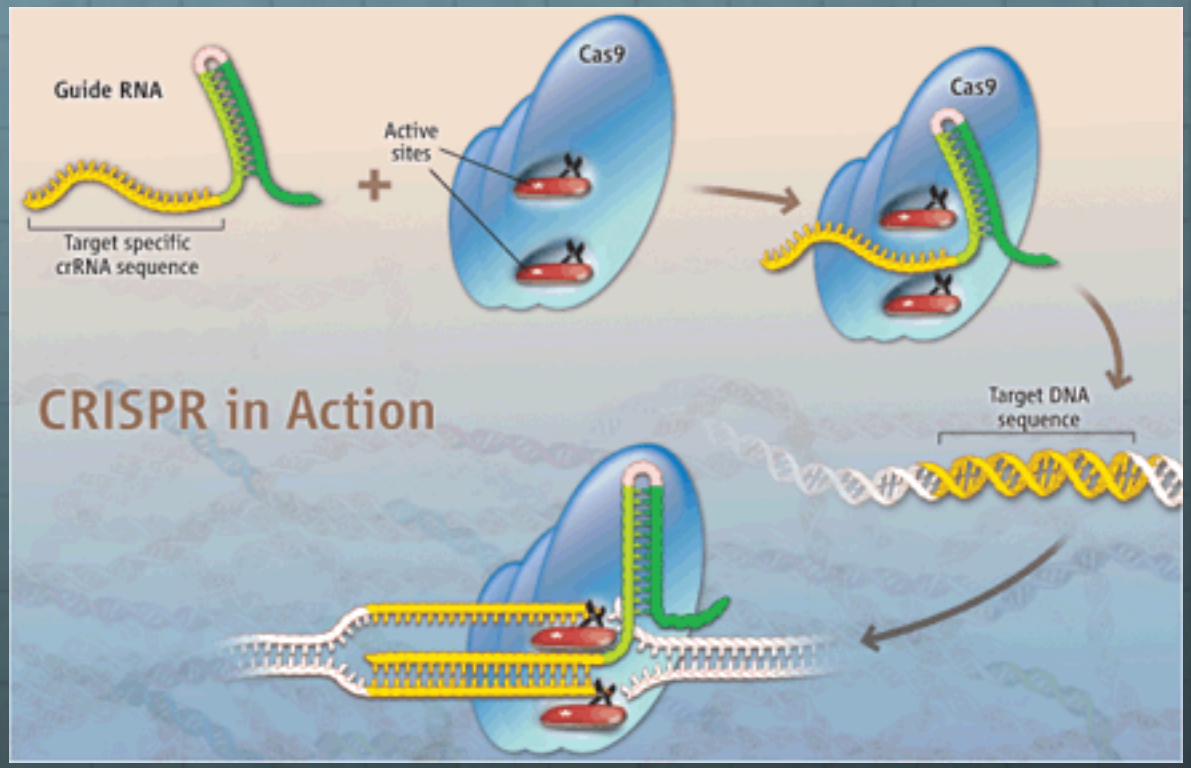
TALENs – Mediated DNA Cleavage



Features:

- The design of TALENs is much simpler than ZFNs or meganucleases. Vectors are available that can enable any molecular biology equipped lab to synthesize a custom TALEN in approximately one week.
- The researcher has great control over the length of specificity (therefore less likely to encounter off-site cleavage
- Very few limitations on where you can effectively direct a TALENs to cut

CRISPR-Cas

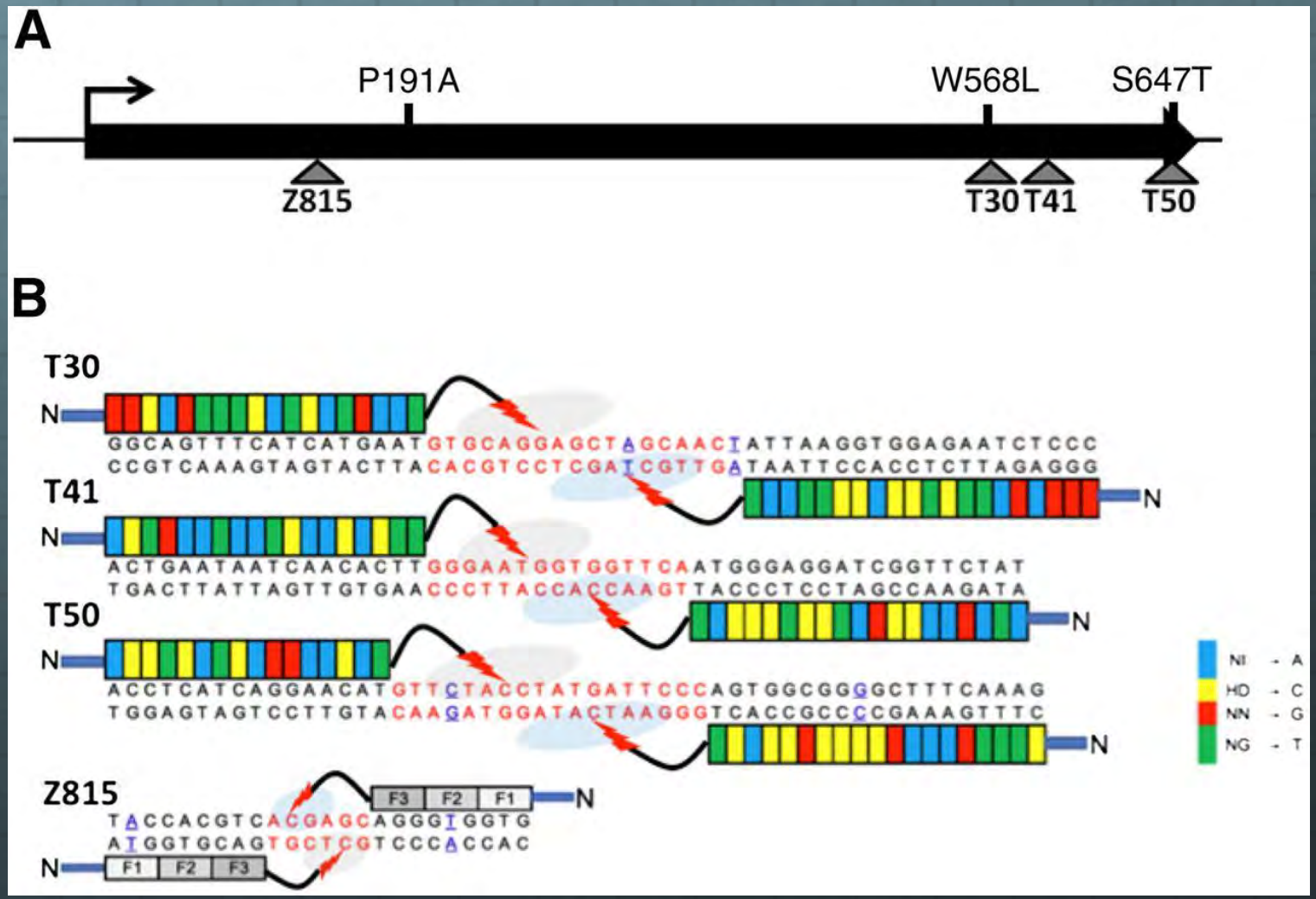


Features:

- The design of CRISPR-Cas nucleases is extremely simple. All you need to do to change target specificity is to change the 20 nt sequence of complementarity in the guide RNA
- Are minimal restrictions for where you can design a CRISP-CAS nuclease
- Multiplexing achieved by simply including additional guide RNA sequences
- Specificity is restricted to 20 nt; therefore can have problems with off-target cutting

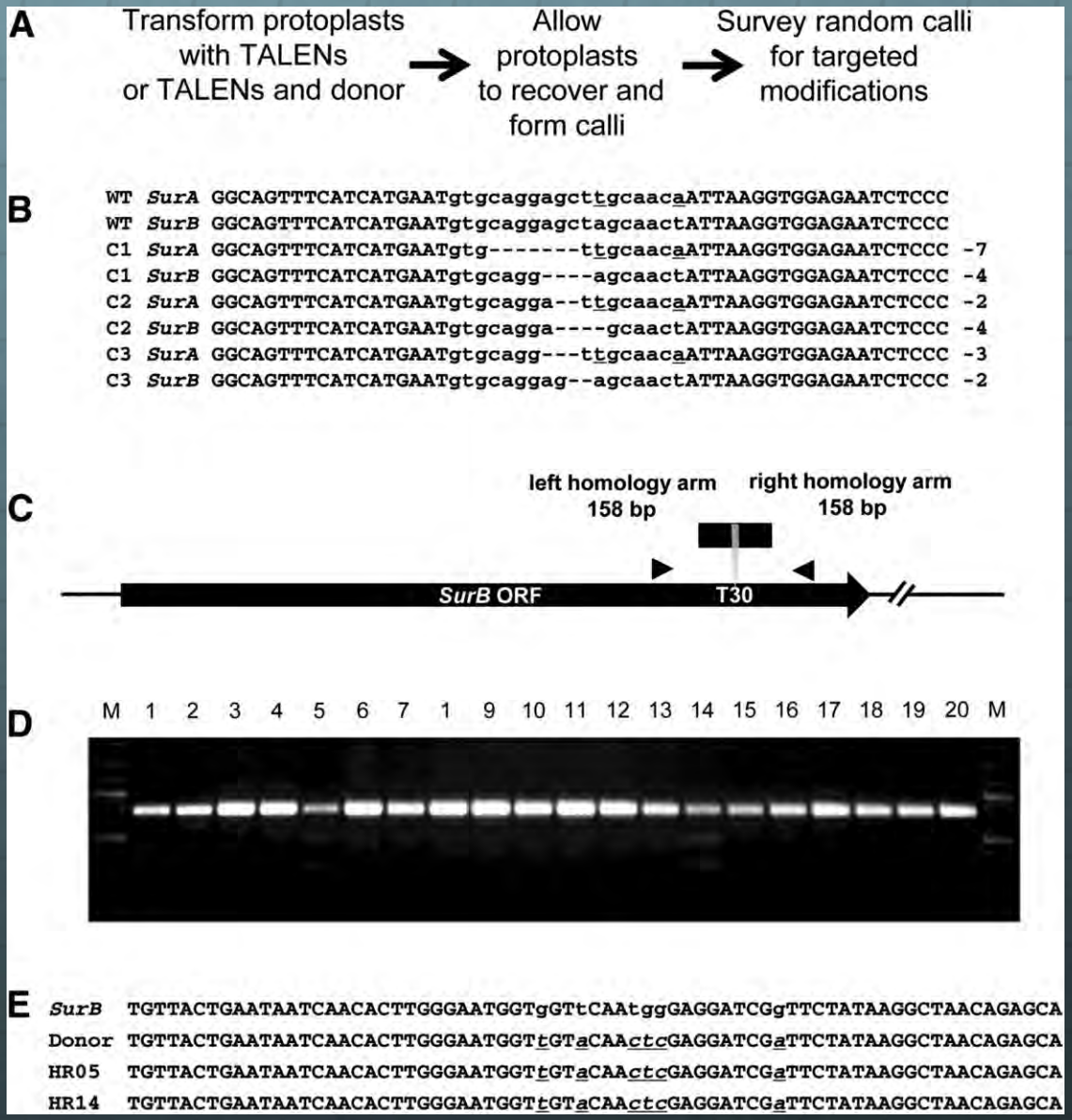
Targeting the tobacco ALS genes with TALENs

(Dan Voytas lab, Univ. of Minnesota)



Zhang Y et al. 2013 Plant Physiol. 161:20-27

Recovery of targeted modifications without selection or enrichment.



Achieved a high frequency of targeted genome modifications *in the absence of selection!!*

- 30% rate of targeted gene mutation
- 4% rate of targeted gene replacement (gene surgery)

Zhang Y et al. 2013 Plant Physiol. 161:20-27

Crops Developed Using Precision Mutagenesis and Gene Editing by Designer Nucleases May be Easier to Commercialize than a "Traditional" Transgenic Plant

Crop	Trait	Developer	Technique
Switchgrass	Easier conversion to biofuels	Ceres	Gene gun
Grapes	Red colour	University of Florida	Gene gun
Turf grasses	Herbicide tolerant	Scotts Miracle-Gro	Gene gun
Maize (corn)	Improved nutrition	Dow AgroSciences	Zinc-finger nuclease
Plums	Faster breeding	Appalachian Fruit Research Station	Non-transgenic offspring of GM parents
Tobacco	Faster breeding	North Carolina State University	Non-transgenic offspring of GM parents
Sorghum grass	Higher yields	University of Nebraska–Lincoln	Epigenetics
Not disclosed	Faster breeding	New Zealand Institute for Plant and Food Research	Non-transgenic offspring of GM parents
Ornamental plants	Not disclosed	BioGlow	Not disclosed
Not disclosed	Not disclosed	Cellectis	Meganuclease-targeted gene deletions

Biotechnological methods/traits that APHIS has stated does not require the resulting crop to be defined as a "regulated article" (i.e. can be grown without any special permission or testing). *From Nature 500: 389-390 (2013)*

Conclusions

- Even within the current "anti-GM" environment, molecular biology-based technologies can still serve to help the tobacco industry address certain issues that are likely to be the subject of future regulation
  - TSNA reduction
  - Cd reduction
  - Alteration of nicotine levels
- Emerging precision genome editing technologies hold the promise of enabling the alteration of genetic traits in a manner that should make them easier to deploy than a conventional transgenic crop: *no foreign DNA in end product; avoid the lengthy time and high costs of deregulation*

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