

Crop Genome Editing and Precision Breeding with CRISPR/Cas9



National Fusarium Head Blight Forum
Dec. 6, 2015



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Conventional Transgenic Approaches

Drawbacks:

- ∅ Random insertion of transgene
- ∅ Not suitable for gene targeting or precise gene mutation
- ∅ Difficult to perform gene replacement or create allelic variation
- ∅ Introduction of undesirable DNA fragments (T-DNA, selection markers)
- ∅ Extensive regulatory requirements
- ∅ Public concerns over transgenic crops

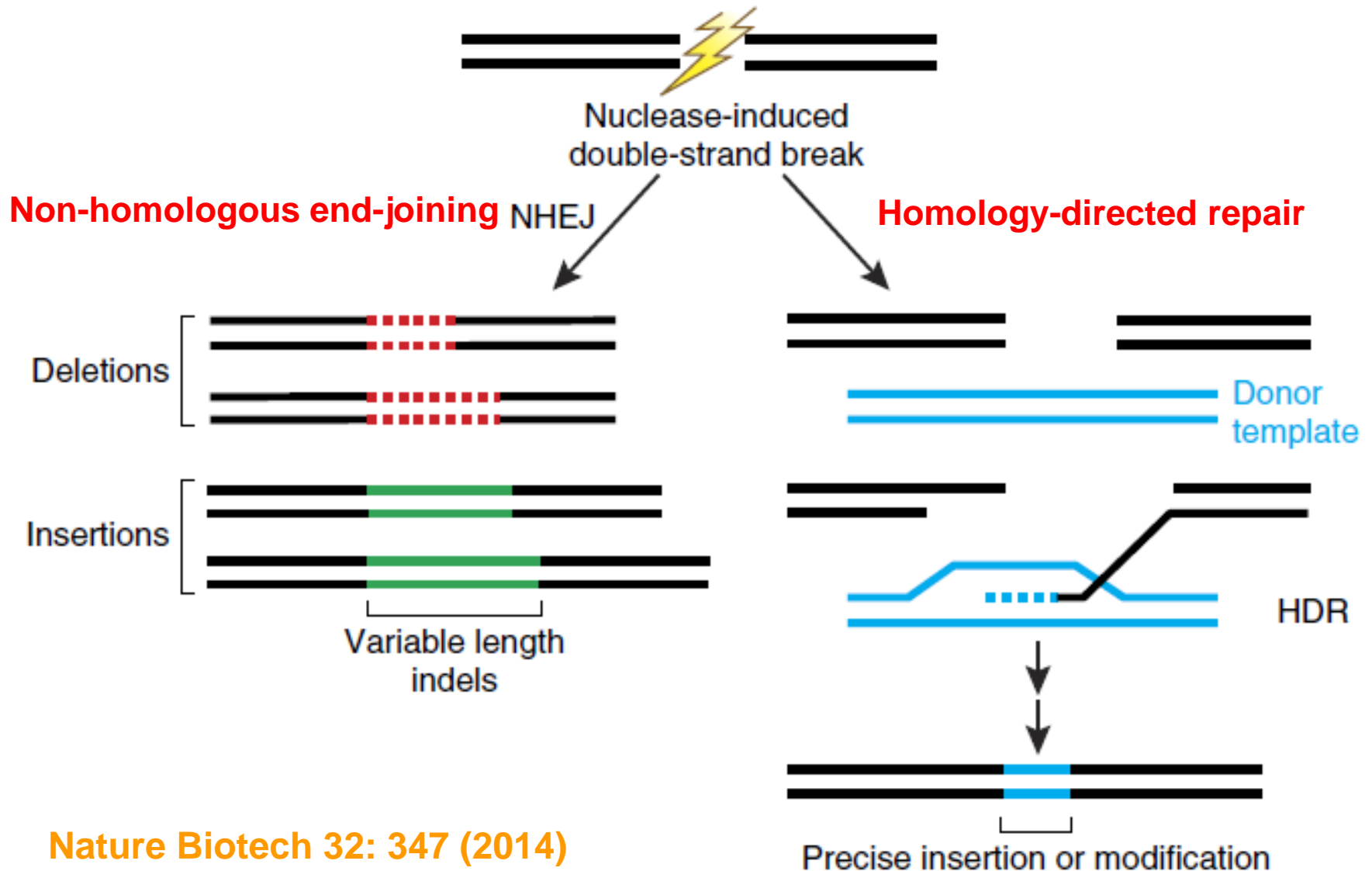
New technology is much needed:

- ∅ To precisely and efficiently manipulate genome for crop improvement
- ∅ To reduce regulatory hurdles and public concerns



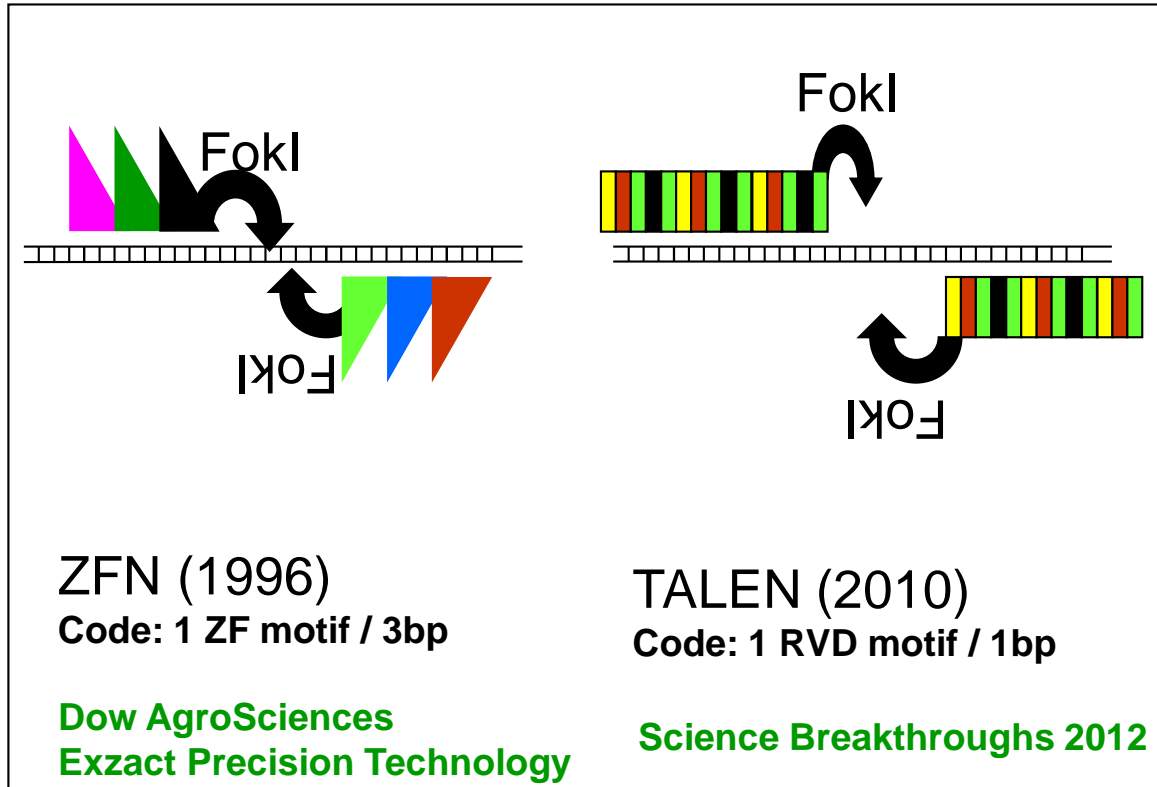
Fig. 2. Hawaiian papaya plot in 2011. Hawaiian papaya plot showing diseased, devastated, non-transformed trees in the foreground and healthy transgenic trees behind. [Photo courtesy of Dennis Gonsalves, Agricultural Research Service, U.S. Department of Agriculture, Hawaii]

Genome Editing: Break and Repair DNA

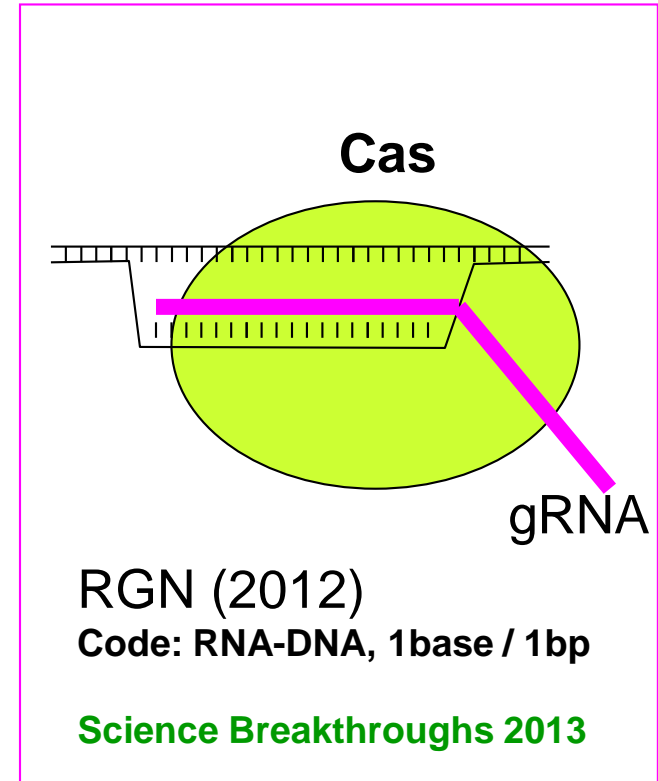


Nature Biotech 32: 347 (2014)

Programmable Nucleases for Genome Editing



Program nuclease based on DNA binding specificity of zinc fingers and TAL effectors

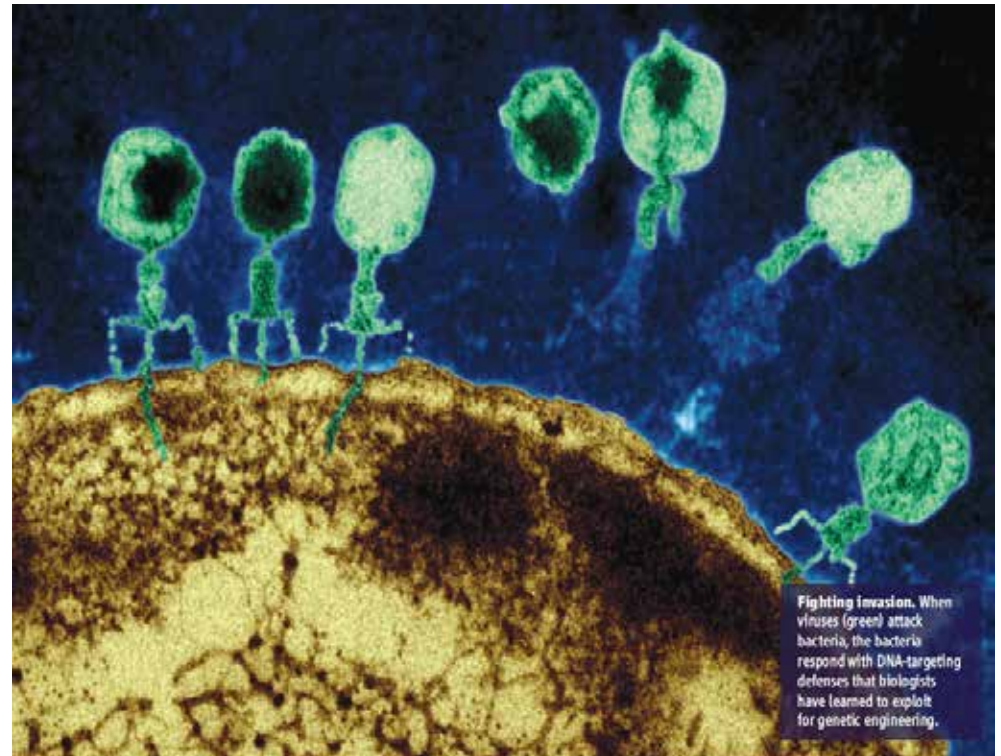
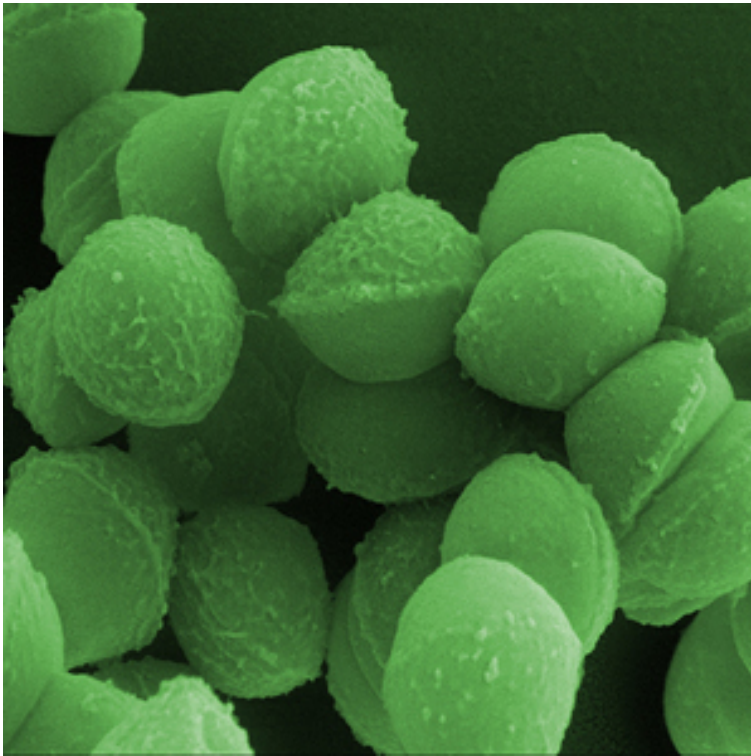


Program nuclease according to RNA:DNA base pairing

CRISPR/Cas: A Bacterial Defense System

CRISPR: clustered regularly interspaced short palindromic repeats

CAS: CRISPR-associated nuclease



These *Streptococcus pyogenes* bacteria use CRISPR/Cas9 system to battle viruses

(Science, Aug. 23, 2013)

2015 Breakthrough Prize



Dick Costolo

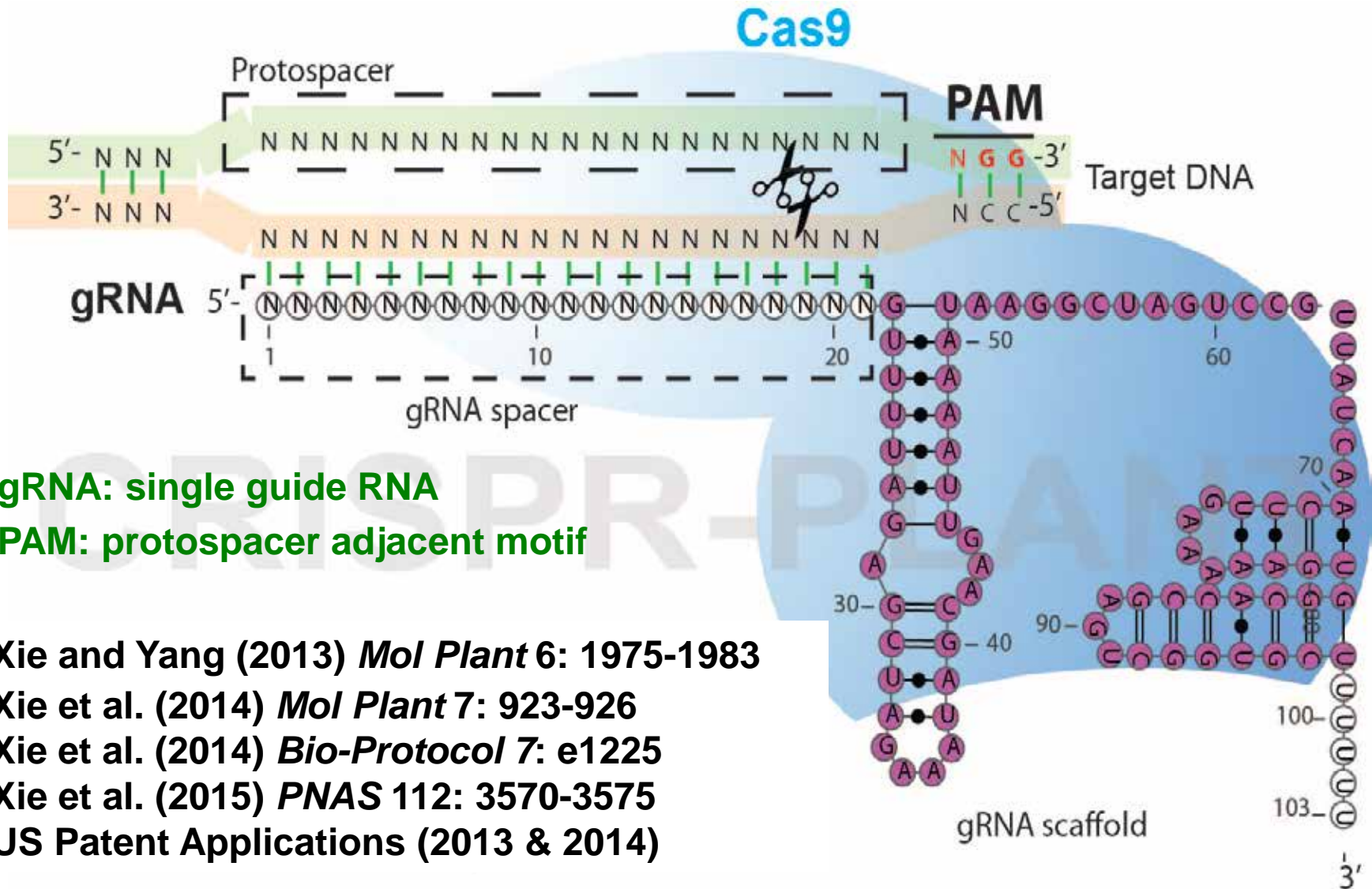
Jennifer Doudna

Emmanuelle Charpentier

Cameron Diaz

2nd Annual Breakthrough Prize Ceremony held at NASA's Ames Research Center in Moffett Field, CA, on November 9, 2014. The event was hosted by Breakthrough Prize founders Sergey Brin and Anne Wojcicki, Jack Ma and Cathy Zhang, Yuri and Julia Milner, Mark Zuckerberg and Priscilla Chan.

Engineering CRISPR/Cas9 for Plant Genome Editing



gRNA: single guide RNA

PAM: protospacer adjacent motif

Xie and Yang (2013) *Mol Plant* 6: 1975-1983

Xie et al. (2014) *Mol Plant* 7: 923-926

Xie et al. (2014) *Bio-Protocol* 7: e1225

Xie et al. (2015) *PNAS* 112: 3570-3575

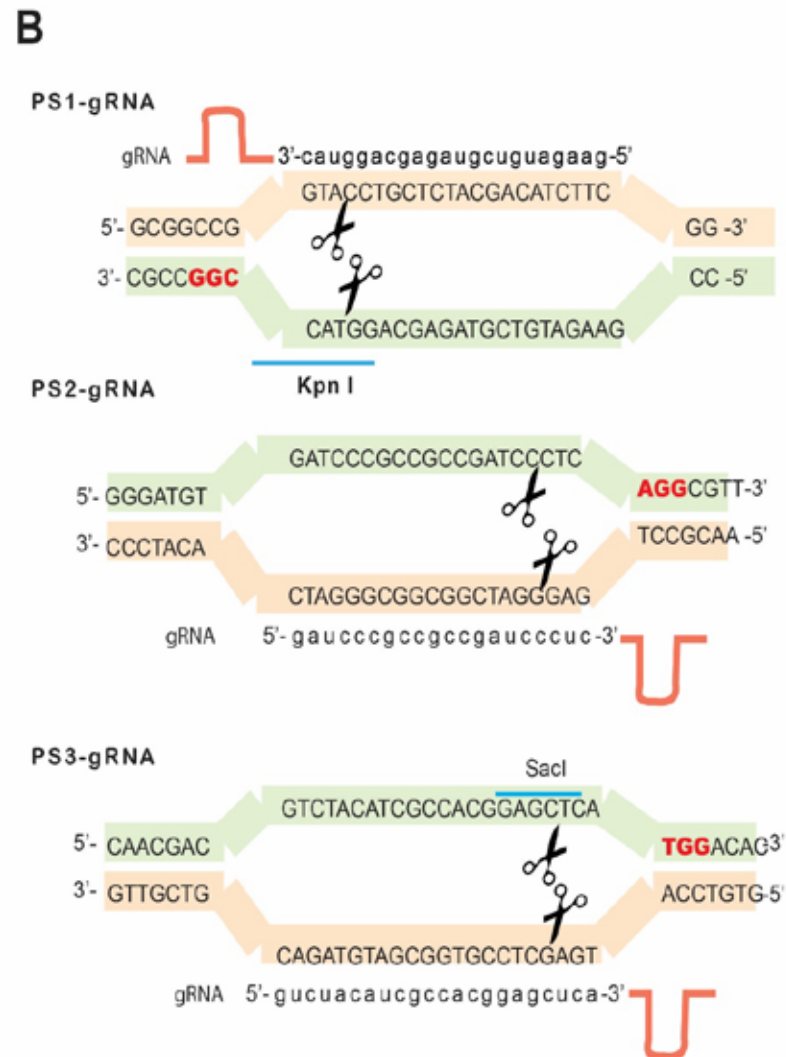
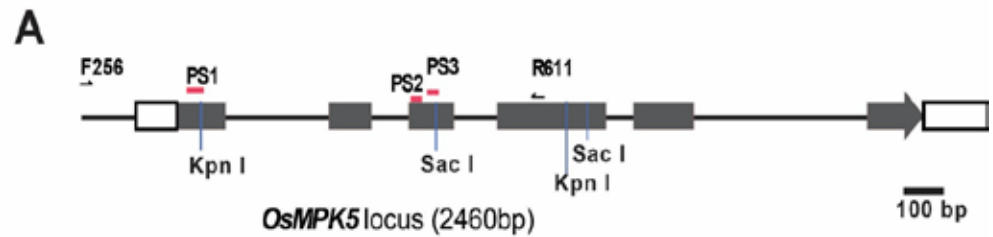
US Patent Applications (2013 & 2014)

Plasmid vectors available via Addgene (www.addgene.org)

Design of gRNAs to Target Three Specific Sites of *OsMPK5*

* *OsMPK5* encodes a stress-inducible MAP kinase which negatively regulates rice disease resistance

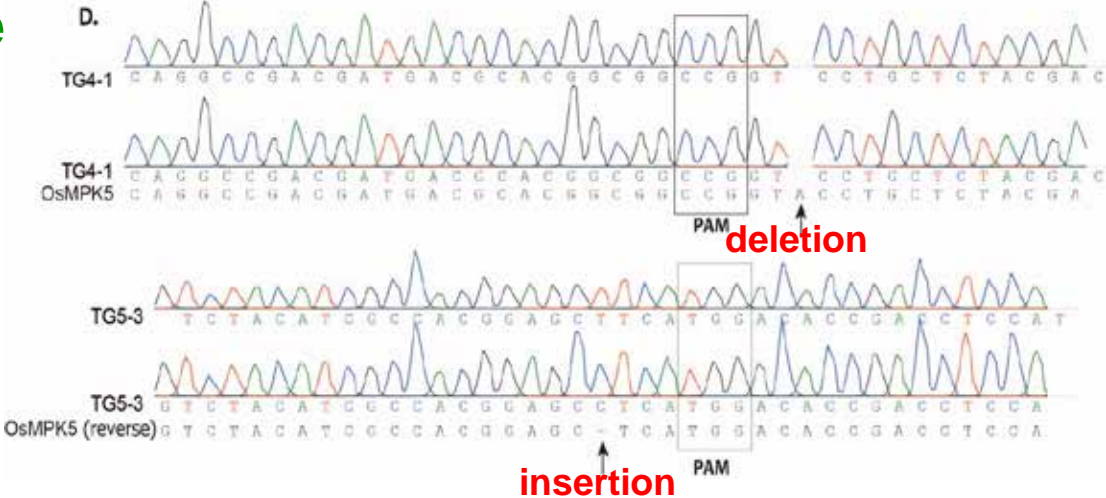
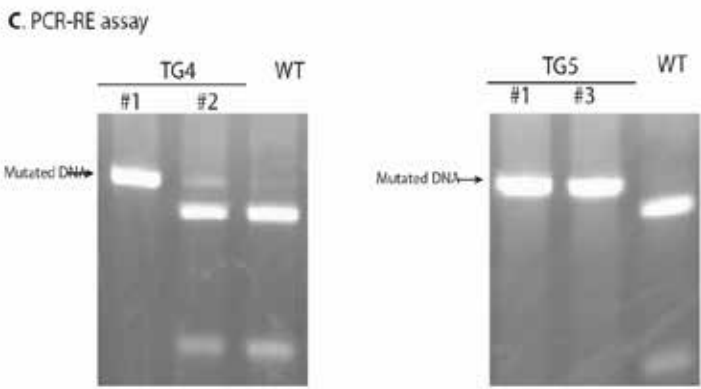
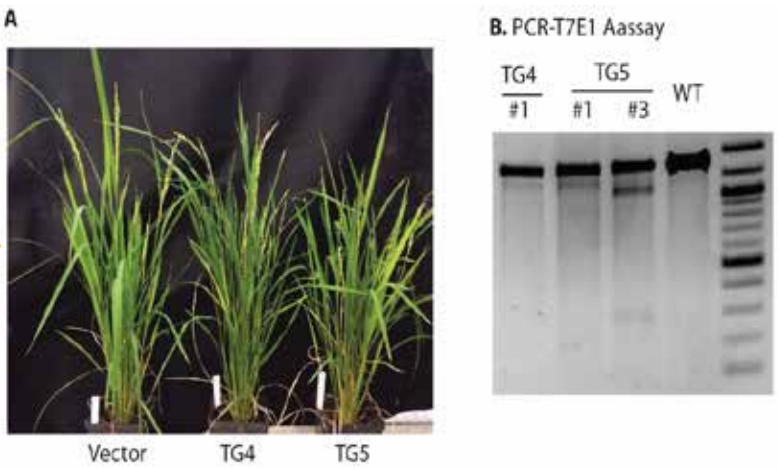
Mol Plant 6: 1975-1983 (2013)



Transgenic Rice Lines with Targeted Mutation of *OsMPK5*

Genome editing resulted in single base indels and frame-shift of *OsMPK5*

gRNA/Cas9 could be readily removed via segregation, resulting in transgene-free mutant lines



CRISPR/Cas9 Editing Specificity

Specificity/off-target determinants:

1. Cas9 protein (some variants are more specific)
2. gRNA/Cas9 concentration in the cell
3. gRNA/Cas9 exposure time/duration (transient vs. stable)
4. DNA sequences of genomic sites

Genome-wide prediction of specific gRNA spacers

CRISPR-PLANT

A Portal of CRISPR-Cas9 Mediated Genome Editing

[Home](#) [Search](#) [Genome Browser](#) [Instruction](#) [Download](#) [More about RGE](#) [About us](#)

Before searching, please read our [instructions](#). Currently, [Class0.0 and Class1.0](#) gRNA spacer sequences, which are expected to be highly specific for the CRISPR-Cas9 mediated genome editing, will be displayed for selected region or gene. All classes of gRNA spacers are available in the [JBrowser](#). Search requests for regions longer than 30kb will not be accepted to maintain server performance. **Searches of bigger genes or longer regions may take more time to download the results**. The search function require your web browsers to support JavaScript (Latest version of Firefox is recommended). Two RGE vectors developed by Yang's Lab are available via Addgene now. (http://www.addgene.org/Yinong_Yang/)

Select species

Chromosome

From

To

Select species

Gene Locus

Select one searching method then **click search**.

CRISPR-PLANT is supported by [Penn State](#) and [AGI](#).

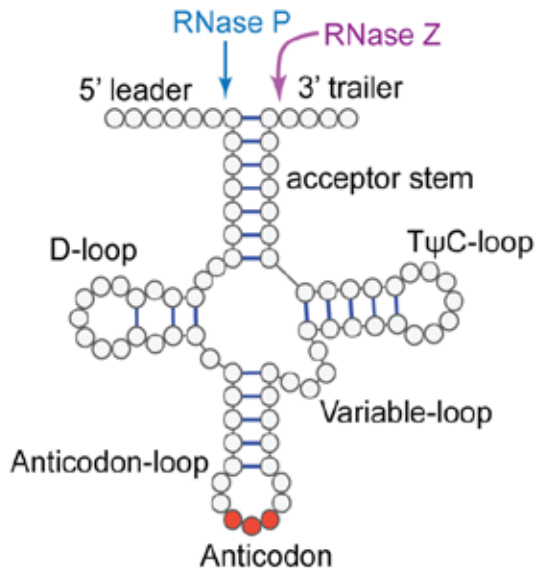
CRISPR-PLANT©, 2014

www.genome.arizona.edu/crispr

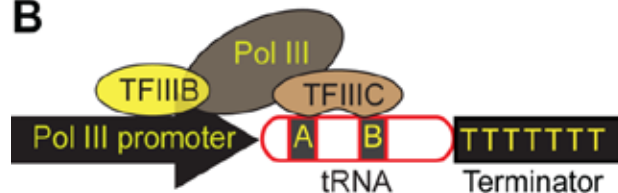
Polycistronic tRNA-gRNA (PTG) Technology

Array gRNAs tandemly in a single polycistronic gene and utilize endogenous tRNA processing system for precise cleavage and production of numerous gRNAs *in vivo*. (PNAS 112: 3570-3575, 2015)

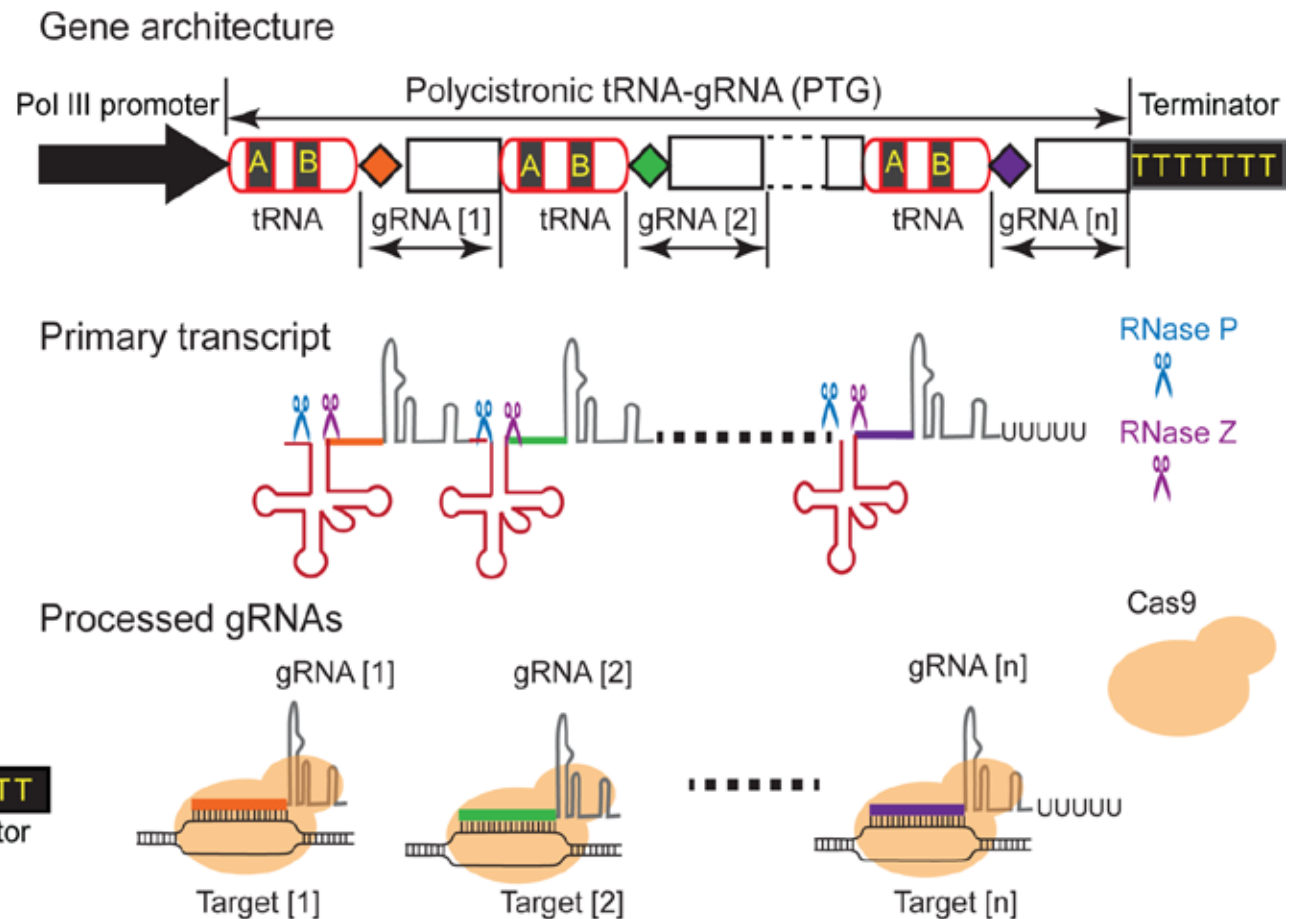
A











B



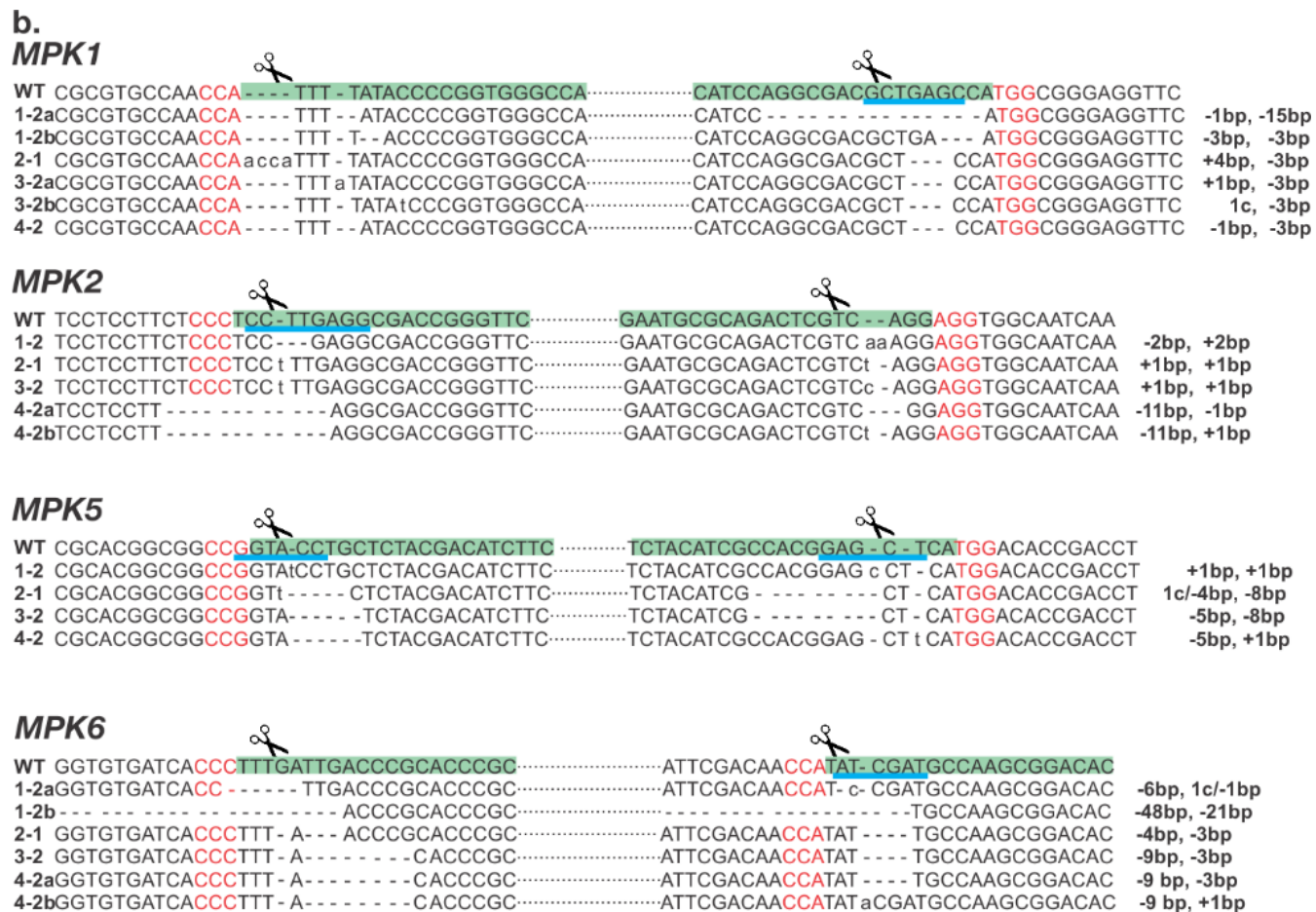
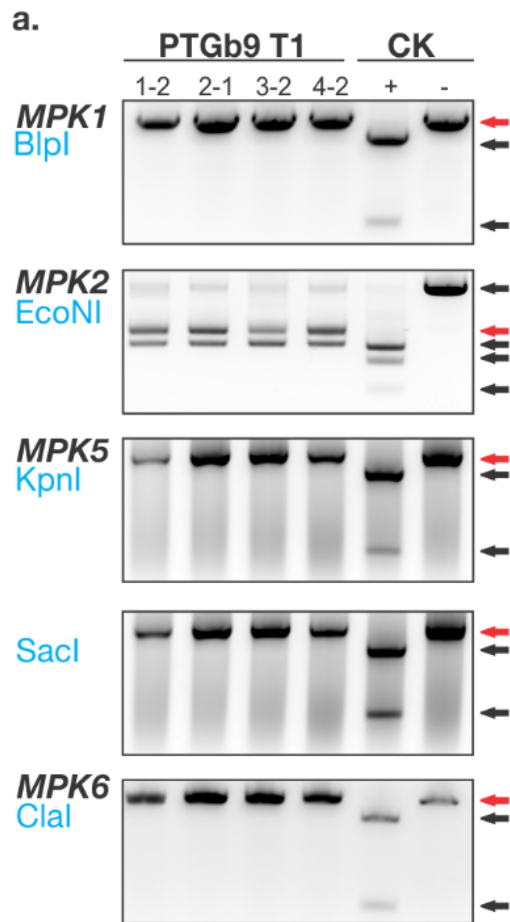
C



Multiplex Targeted Mutagenesis with PTG in Stable Transgenic Rice Plants

MPK1		 gRNA4	 gRNA3	gRNA4 site	gRNA3 site
WT	CGCGTGCCAA CCA TTT TATACCCCGGTGGGCCA.....CATCCAGGCGACGCTGAG CCA TGG CGGGAGGTTC				
#1	CGCGTGCCAA CCA TTT -ATACCCCGGTGGGCCA.....CATCCAGGCGACGCTGA aa CCA TGG CGGGAGGTTC			-1 bp	G->A, +1 bp
#10	CGCGTGCCAA CCA TTT -ATACCCCGGTGGGCCA.....CATCCAGGCGACGCT- -- CCA TGG CGGGAGGTTC			-1 bp	-3 bp
#11	CGCGTGCCAA CCA TTT -ATACCCCGGTGGGCCA.....CATCCAGGCGACGCTGA- CCA TGG CGGGAGGTTC			-1 bp	-1 bp
MPK2		 gRNA5	 gRNA6	gRNA5 site	gRNA6 site
WT	TCCTCCTTCT CCC TCC TTGAGGCGACCGGGTTC.....GAATGCGCAGACTCGTC AGG AGG TGGCAATCAA				
#1	TCCTCCTTCT CCC TCC -TGAGGCGACCGGGTTC.....GAATGCG - -----			-1 bp	-42 bp
#10	TCCTCCTTCT CCC TCCtTTGAGGCGACCGGGTTC.....GAATGCGCAGACTCGTCtAGG AGG TGGCAATCAA			+1 bp	+1 bp
#11	TCCTCCTT-----AGGCGACCGGGTTC.....GAATGCGCAGACTCGTCtAGG AGG TGGCAATCAA			-11 bp	+1 bp
MPK5		 gRNA1	 gRNA2	gRNA1 site	gRNA2 site
WT	CGCACGGCGG CCG GTA CCTGCTCTACGACATCT.....TCTACATCGCCACGGAGC TCAT TGG ACACCGACCT				
#1	CGCACGGCGG CCG GTA ----- TACGACATCT.....TCTACATCGCCACGGAGCaTCAT TGG ACACCGACCT			-7 bp	+1 bp
#10	CGCACGGCGG CCG GTA -----.....TCTACATCGCCACGGAGC tTCAT TGG ACACCGACCT			-23 bp	+1 bp
#11	CGCACGGCGG CCG GTA ----- TCAT TGG ACACCGACCT				delete 727 bp
MPK6		 gRNA8	 gRNA7	gRNA8 site	gRNA7 site
WT	GGTGTGATC ACC TTT GATTGACCCGCACCCGC.....CATTGACAA CCA TAT CGATGCCAAGCGGACAC				
#1	GGTGTGATC ACC TTTTt---- ACCCGCACCCGC.....CATTGACAA CC ATAT ---TGCCAAGCGGACAC			+1bp, -5 bp	-3 bp
#10	GGTGTGATC ACC TTTT -AT -GACCCGCACCCGC.....CATTGACAA CC ATA----- CCAAGCGGACAC			-2 bp	-6 bp
#11	GGTGTGATC ACC TTTT -A- -GACCCGCACCCGC.....CATTGACAA CC ATATaCGATGCCAAGCGGACAC			-3 bp	+1 bp

Inheritance of *MPK1/MPK2/MPK5/MPK6* Quadruple Mutation in T1 Rice Lines



(Yang lab, unpublished)

Broad Application of CRISPR-Cas9 Technology

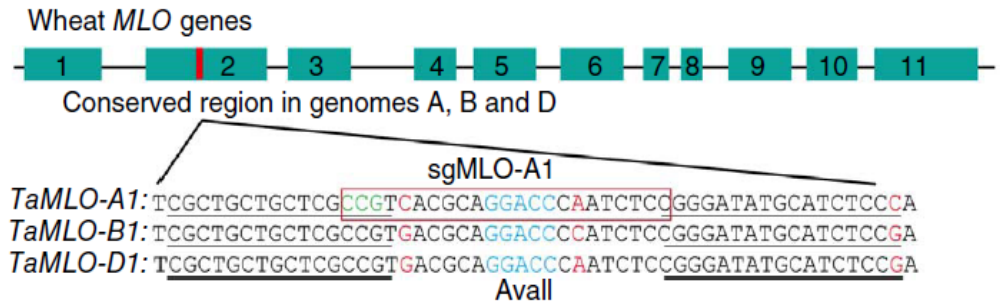
Technical advantages for basic plant biology and crop breeding

- Ø Targeted gene mutation (multiple or redundant genes)
- Ø Site-specific integration and gene stacking
- Ø Gene replacement via homologous recombination
- Ø Site-directed mutagenesis to create allelic variation
- Ø Chromosomal engineering such as deletion or translocation
- Ø Modification and labeling of multiple genomic sites
- Ø Transcriptional modulation of multiple genes and pathways
- Ø Epigenome editing such as methylation and demethylation
- Ø Cisgenesis without introducing undesirable foreign DNA

Economic, regulatory and societal benefits:

- Ø Reduce costs for precise and efficient molecular breeding
- Ø Eliminate or significantly reduce regulatory requirements
- Ø Alleviate public concerns about GM crops

Simultaneous Mutation of Six MLO Alleles Confers Powdery Mildew Resistance



Nature Biotechnology 32: 947–951 (2014)

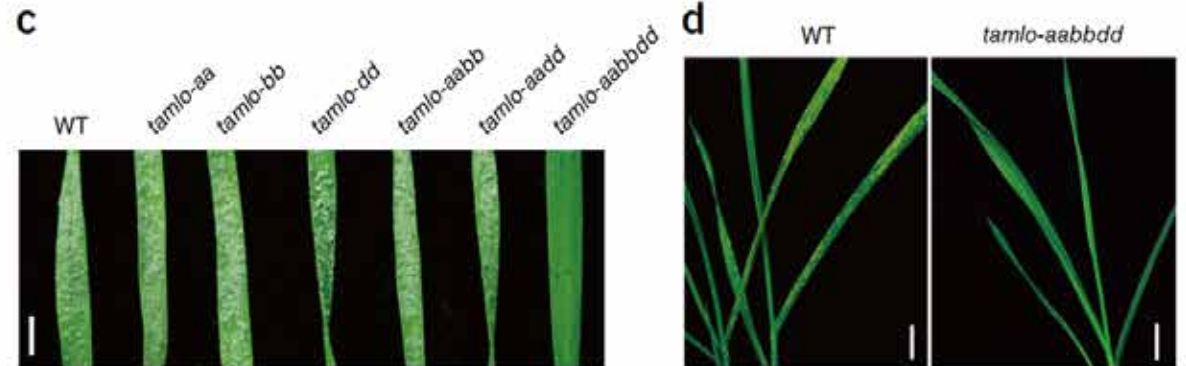
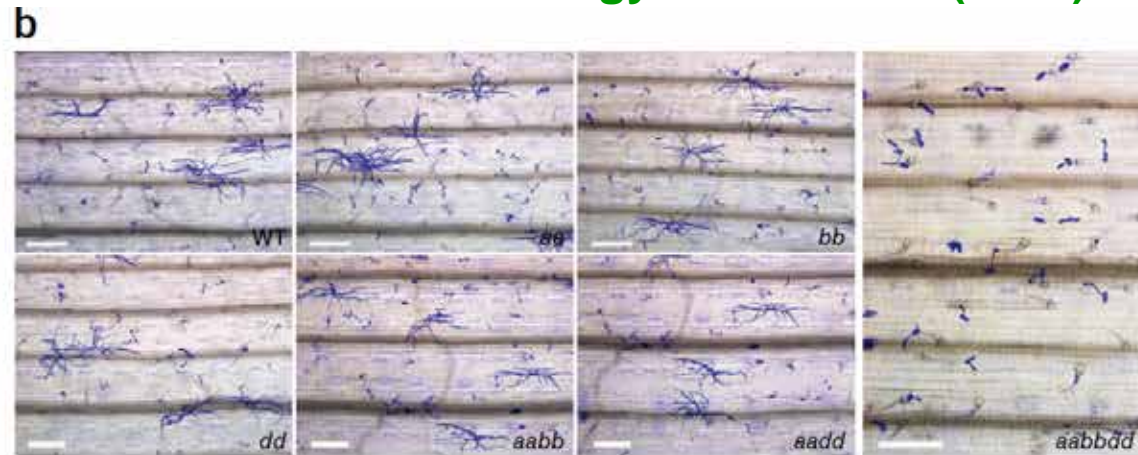
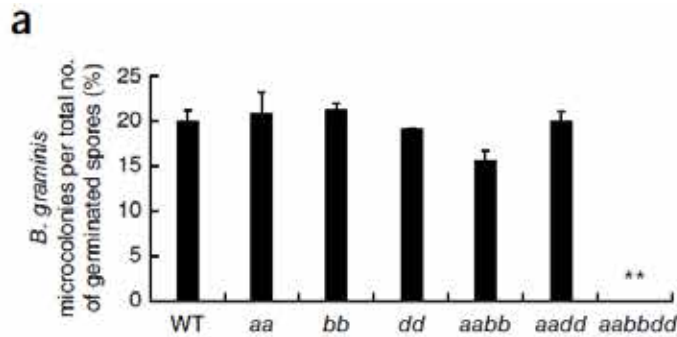
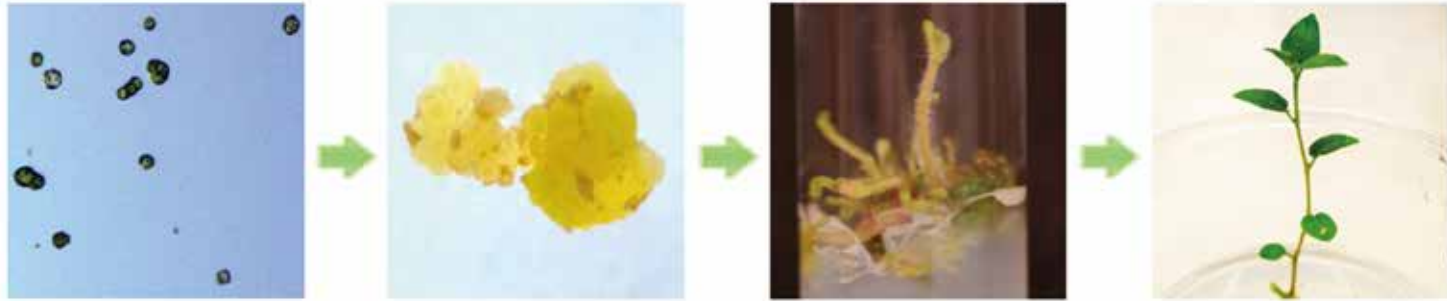


Figure 2 Loss of *TaMLO* function confers resistance of bread wheat to powdery mildew disease. **(a)** Percentage of microcolonies formed from the total number of germinated spores of *Blumeria graminis* f. sp. *tritici* (*Bgt*) inoculated on the leaves of wild-type (WT) and various *tamlo* mutants. At least 2,000 germinated spores per genotype per experiment were examined 72 h after inoculation with virulent *Bgt* isolate E09. Values are the mean \pm s.d. of four independent experiments. ****** $P < 0.01$ (*t*-test). **(b)** Micrographs of microcolony formation of *Bgt* on the surfaces of leaves of the indicated genotypes 3 d postinoculation. Powdery mildew spores and colonies were stained with Coomassie blue. Scale bars, 200 μ m. **(c)** Macroscopic infection phenotypes of representative leaves of WT and the indicated *mlo* mutants 7 d after inoculation of detached leaves with *Bgt*. Scale bar, 1 cm. **(d)** Disease symptoms of wild-type (WT) and *tamlo-aabbdd* mutant plants. The photograph was taken 7 d after inoculation *in planta*. Scale bars, 2 cm.

Transgene-free Mutation of *AS1* for Potential Reduction of Acrylamide Levels in Potato

A

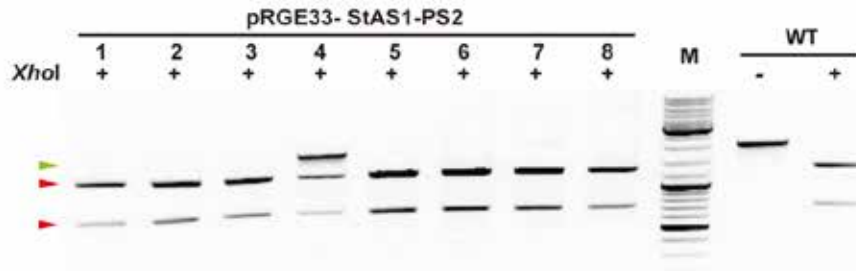


Transient expression

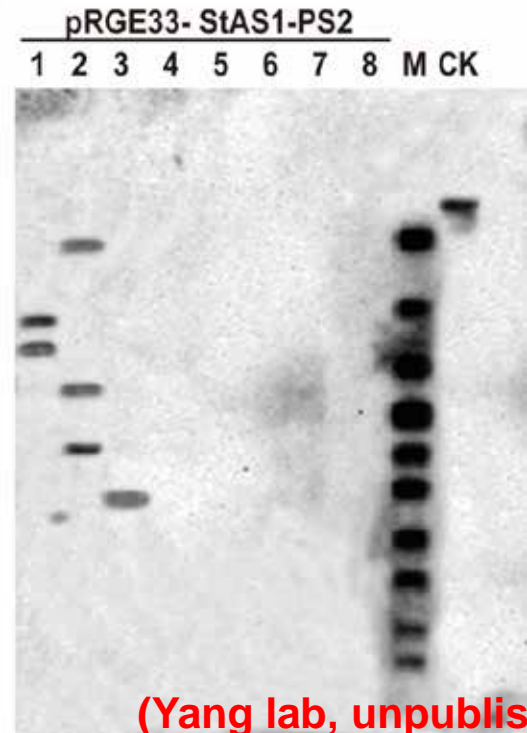
Callus formation w/o antibiotic selection

Regeneration

B



E

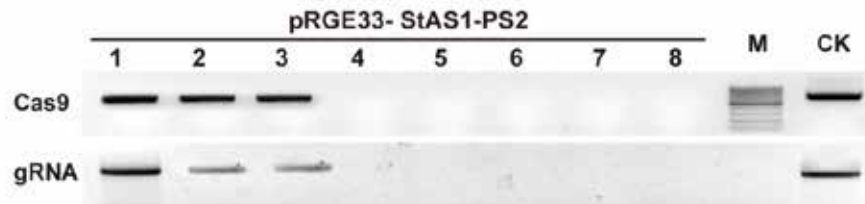


C



AS1 encodes asparagine synthase 1

D

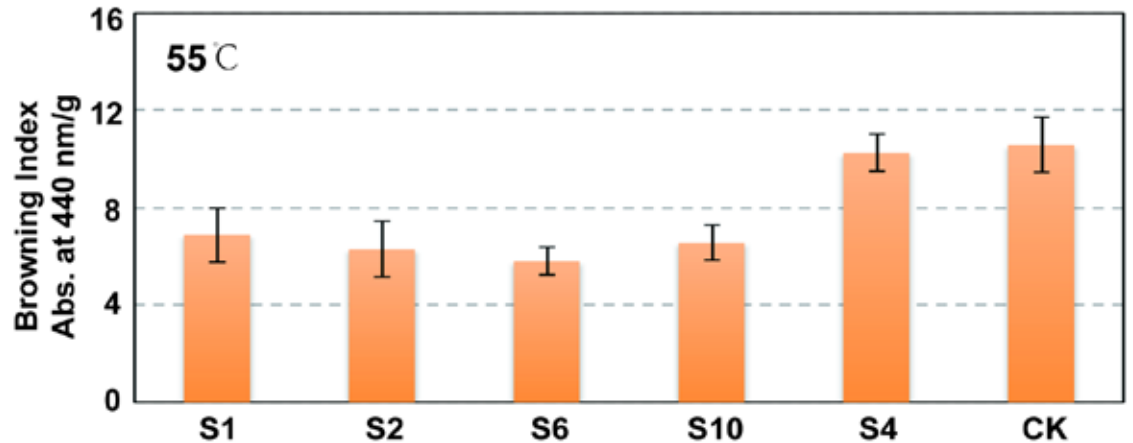
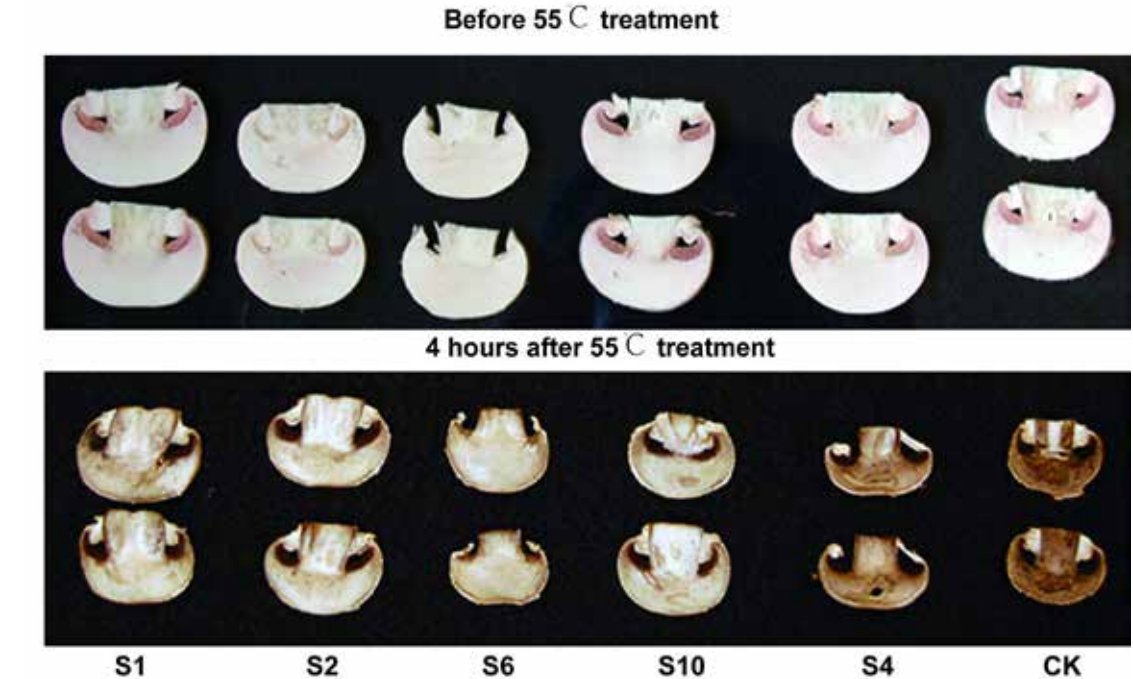


(Yang lab, unpublished)

Transgene-free, Anti-browning Mushroom to Extend Shelf Life and Facilitate Mechanical Harvesting



(Yang lab, unpublished)



Near-term Applications for Crop Breeding

1. Targeted deletion of single or multiple genes for transgene-free, mutational breeding in various crop species.
2. Site-specific integration and precise gene stacking for transgenic or cisgenic breeding.
3. Multiplex editing to create allelic variation at quantitative trait loci to improve multiple agronomic traits (yield, quality, disease resistance and abiotic stress tolerance).

Genome editing in rice for S918A conversion in *Pita*

Rice Variety	Resistant with <i>AVR-Pita</i> Fungus	Rice Type	Amino Acid Position				918
			6	148	158	176	
Yashiro-mochi	Yes	Japonica	I	R	H	D	A
Tetep	Yes	Indica	I	R	H	D	A
C101A51	No	Indica	I	R	H	D	S
Tsuyuake	No	Japonica	S	S	Q	V	S

Table after Bryan et al. (2000), *The Plant Cell*

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Thank you !