Genetic engineering of barley to improve *Fusarium* head blight resistance

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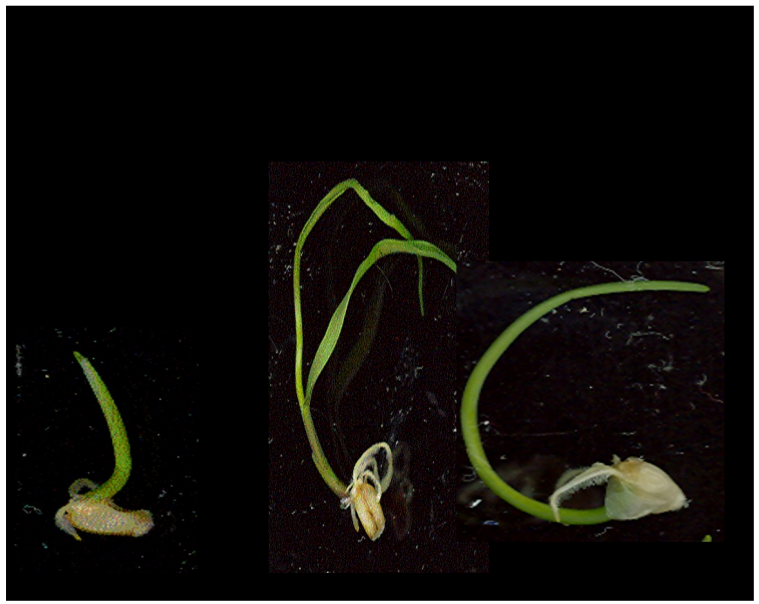
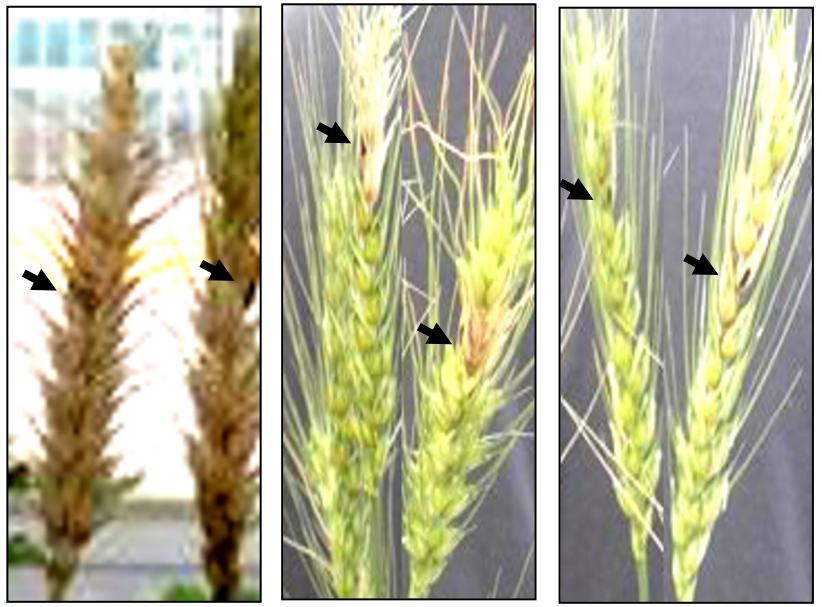
Engineering FHB resistance in wheat and barley

Many transgenic genes have been engineered into mostly wheat to enhance FHB resistance including yeast ribosomal protein L3 (Di *et al*. , 2010) >>> GMO



wt RUT772 RUT8153



wt RUT772 RUT8153

- Di, R., A. Blechl, R. Dill-Macky, A. Tortora, and N. E. Tumer. 2010. Plant Science 178:374-380.
- U.S. Patent #8,026,410 B2. Tumer, N.E. and R. Di. Sept. 27, 2011. Transgenic plants expressing L3 delta proteins are resistant to trichothecene fungal toxins.

CRISPR-gene editing to improve disease resistance

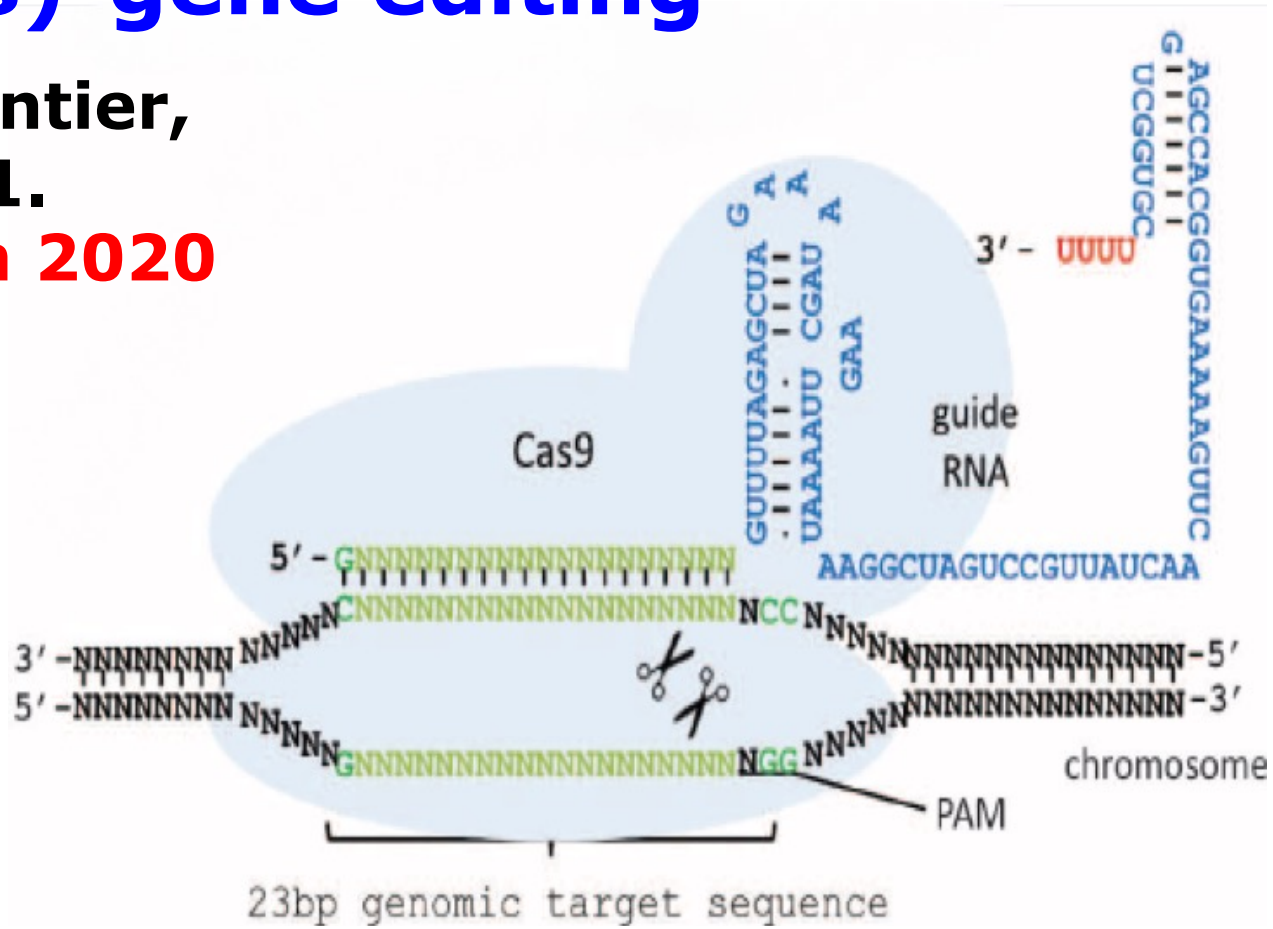
- **Knocking-out host disease susceptibility genes to interrupt plant-pathogen interaction**
- **gRNA and Cas9 transgene cassettes can be segregated from gene-mutated genomes.**
- **Transgene-free, gene-edited mutant plants can be produced in 1-2 generations.**

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-gene editing

Jinek…Doudna and Charpentier, 2012, Science 337:816-821.
Nobel Prize in Chemistry in 2020

Key components:

- **snRNA promoter**
- **gRNA (19-23 nt)**
- **scaffold**
- **Cas9 nuclease**
  -codon optimized
- **Double-stranded breaks (DSB) in gDNA**

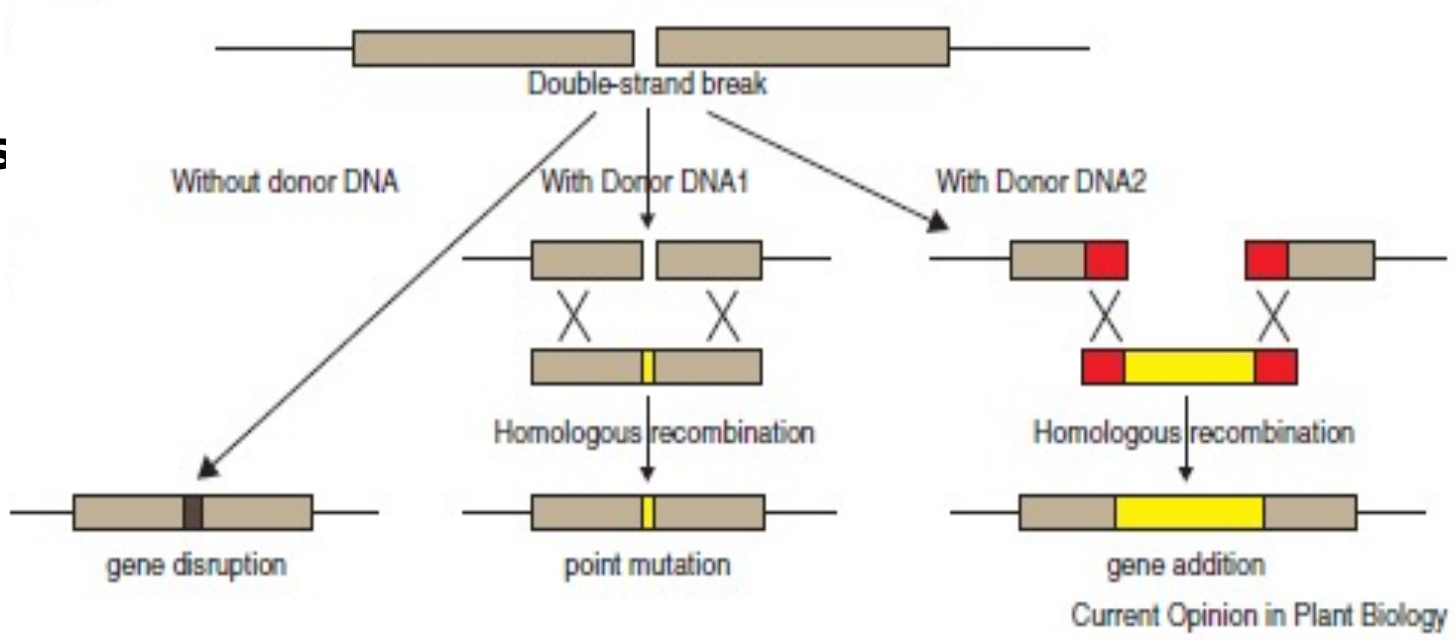


Mali et al., 2013, Science

Mutations or gene editing by CRISPR

DSBs are repaired in nearly all cells by two highly conserved processes:

- **non-homologous end joining (NHEJ), which often results in small insertions or deletions (Indels), leading to gene disruption, gene editing.**
- **homologous recombination (HR), which can be used for point mutation, gene insertion or replacement, gene editing.**



Chen and Lin, 2013

FHB in wheat and barley

FHB in wheat

-- *Fusarium* spp. infects wheat during anthesis
-- colonizes florets first
-- penetrates floral tissues
-- spreads within spikes
-- Two types of resistance:
Type I: resistance to initial infection
Type II: resistance to spread of infection

FHB in barley

-- *Fusarium* spp. infects after anthesis
-- colonizes the brush hairs
-- invades the developing caryopsis
-- Natural Type II resistance;
Symptoms do not spread in spikes

(Huang, Muehlbauer et al. 2016)

Knocking out host factors to prevent initial infection will lead to effective FHB resistance in wheat and barley.

FHB susceptibility gene: *2OGO*

***2-oxoglutarate Fe(II)-dependent oxygenase (2OGO)* gene**

-- Arabidopsis EMS-*At2OGO* mutant showed enhanced plant immunity to *Fg* infection. Knocking-out *At2OGO* gene seems to induce the expression of host defense genes. (Brewer *et al.,* 2014)

-- *At2OGO* gene functions as salicylic acid 5-hydroxylase (S5H) to break down SA into gentisic acid and its impairment leads to SA accumulation. (Zhang *et al*., 2017)

-- We CRISPR-edited *At2OGO* specifically. *At2OGO*-KO plants are resistant to *F. graminearum.* Host defense genes are up-regulated.

-- The *Hv2OGO*-complemented *At2OGO*-KO plants regained susceptibility to *Fg*, indicating *Hv2OGO* might function similarly as *At2OGO.*

Low, Y., M. A. Lawton and R. Di. 2020. Validation of barley *2OGO* gene as a functional orthologue of Arabidopsis *DMR6* gene in *Fusarium* head blight susceptibility. Sci. Reports. 10:9935. DOI:10.1038/s41598-020-67006-5.

FHB susceptibility gene: *EIN2*

***Ethylene insensitive 2* (*EIN2*) gene**

-- ***Fg*** **exploits the ethylene signaling pathway *via* host *EIN2*-regulated signal transduction to promote susceptibility in both Arabidopsis and wheat. (Chen *et al.,* 2009)**

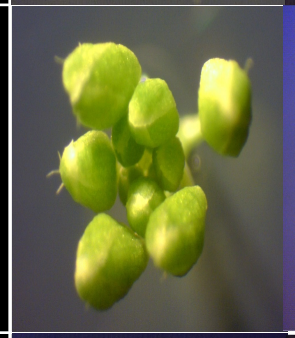
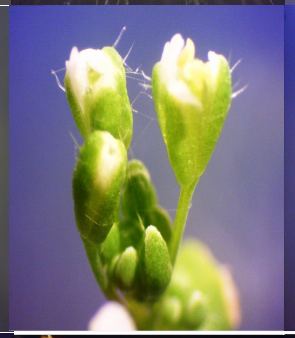
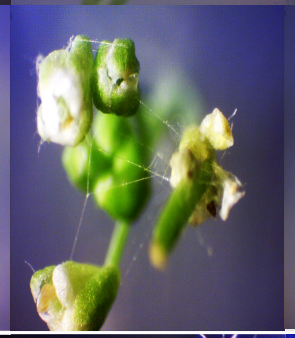
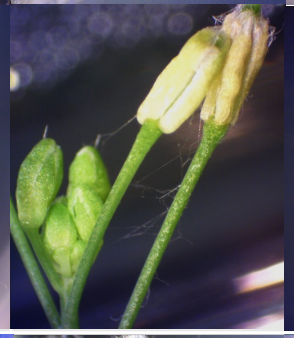
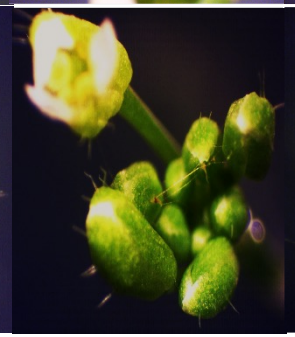
-- **RNAi knocking-down of *EIN2* in wheat enhances FHB resistance. (Travella *et al.*, 2006)**

-- **We CRISPR-edited *AtEIN2* specifically. *AtEIN2*-KO plants are resistant to *F. graminearum.* Ethylene signaling was down-regulated, but the SA and JA signaling pathways were unaffected.**

-- **The *HvEIN2*-complemented *AtEIN2*-KO plants regained susceptibility to *Fg*, indicating *HvEIN2* might also function similarly as *AtEIN2.***

Low, Y. C., M. A. Lawton and R. Di. 2022. *Ethylene insensitive 2* (*EIN2*) as a Potential target gene to enhance *Fusarium* head blight disease resistance. Plant Sci. 322:111361. DOI: 10.1016/j.plantsci.2022.111361.

The *AtEIN2*-KO plants were more resistant to *Fg* infection, compared to *At* WT *and AtEIN2*-KO/*HvEIN2*

Samples	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
At WT						
AtEIN2-KO						
AtEIN2-KO/ *HvEIN2*						

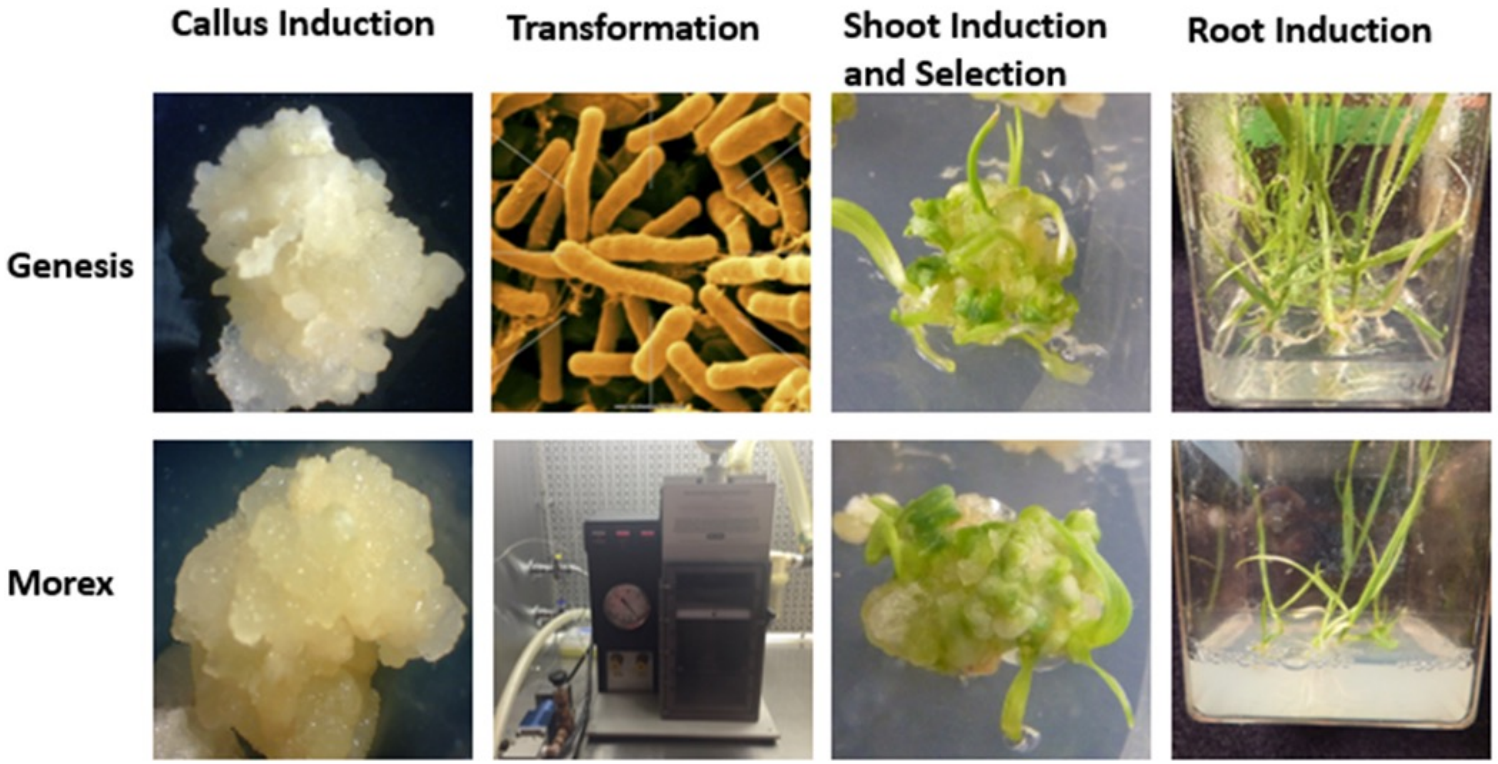
Involvement of *HvUGT* gene in barley FHB resistance

Muehlbauer group (2016) showed that the *HvUGT* (*uridine diphosphate glycosyltranferase*, converting DON to the less toxic D3G) is induced by DON in both the resistant and susceptible barley. So the *HvUGT* gene expression modulation is critical for FHB resistance in barley.

Our barley FHB gene targets:

***Hv2OGO, HvEIN2* and *HvUGT* promoter**

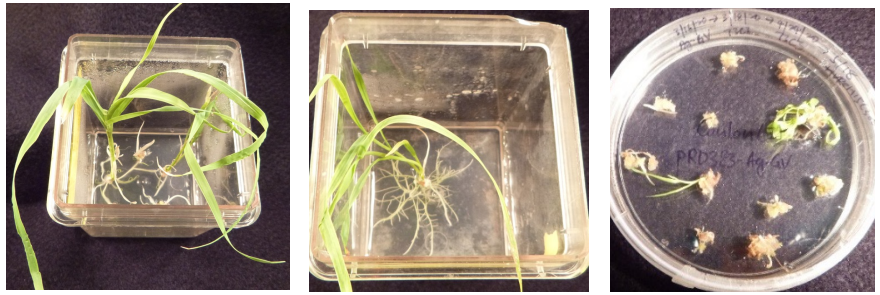
Improved barley tissue culture and transformation system

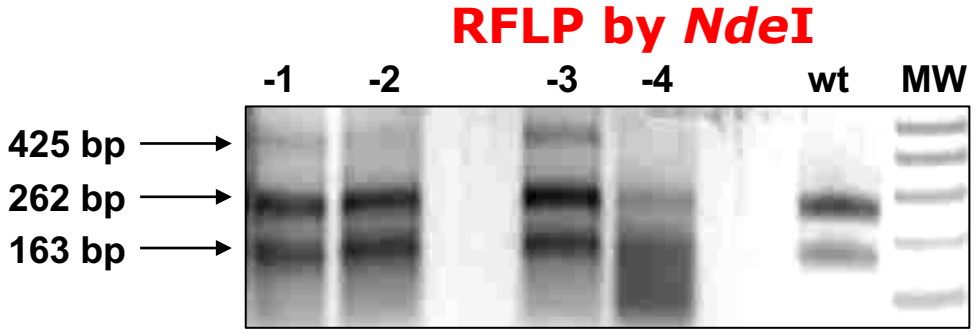


- **Embryogenic calli are induced from immature scutellum.**
- **Calli are transformed by biolistic bombardment or Agrobacterium.**
- **Multiple transgenic shoots are generated from calli initiated from a single immature seed.**

Production of barley *Hv2OGO* mutant plants with pRD383 (POsU3/*Hv2OGO*-gRNA::Cas9) by CRISPR-editing

By Gene gun **By Agro**





PCR-amplifying gDNA, cloning and sequencing

NdeI

```
WT        TCTACCCC AAG TGC CCC TCG CCG GAG CTG ACA TAT GGGCTCCC
383-C1    TCTACCCC AAG TGC CCC TCG CCG GAG CAG ACG TAT GGGCTCCC
                                          L204Q H205R
383-C2    TCTACCCC AAG TGC CCC CCG CCG GAG CTG ACA TAT GGGCTCCC
                                 S201P
383-CA2   TCTACCCC AAG TGC CCC CCG CCG GAG CTG ACA TAT GGGCTCCC
                                 S201P
```

Improvement of barley CRISPR-editing platform

Conventional plant CRISPR-gene editing platform

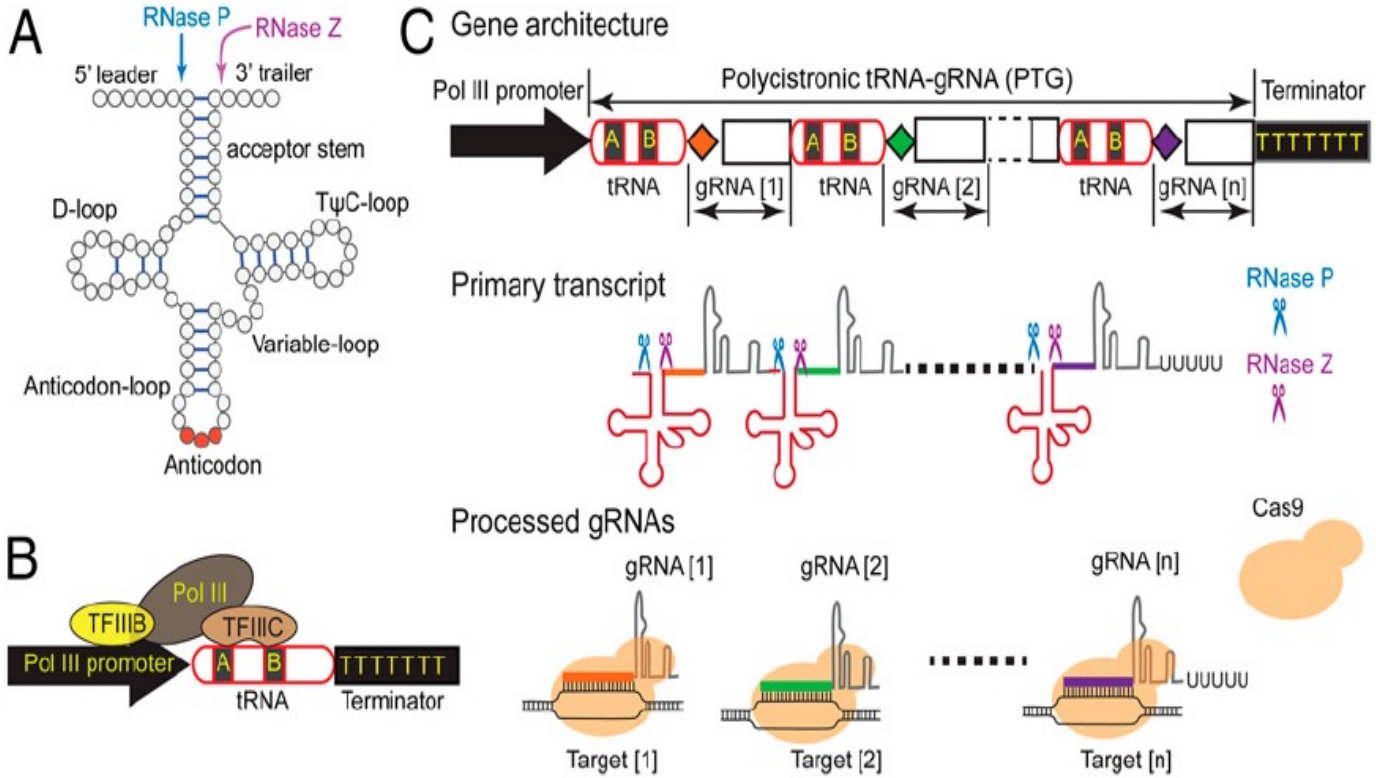
- P_{OsU3}/*Hv* gRNA/P_{ZmUbi}::Cas9-Mo. The gRNA expression is not very efficient.

The polycistronic tRNA-gRNA (PTG) platform

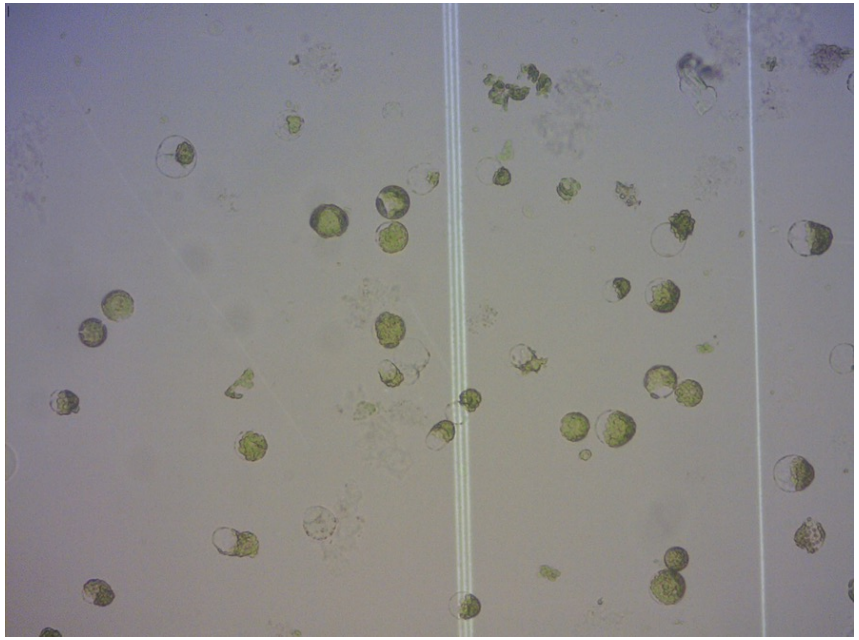
- Developed by the Yang group at Penn State U. (2015)
- tRNA processing is robust.
- No "G" or "A" requirement in the target sequence
- Multiplexing

Dual barley CRISPR-editing vectors:

pRD543 (transient; dual *HvPUGT*)
pRD549 (integrating; dual *HvPUGT*)
pRD550 (transient; dual *HvEIN2*)
pRD554 (integrating; dual *HvEIN2*)



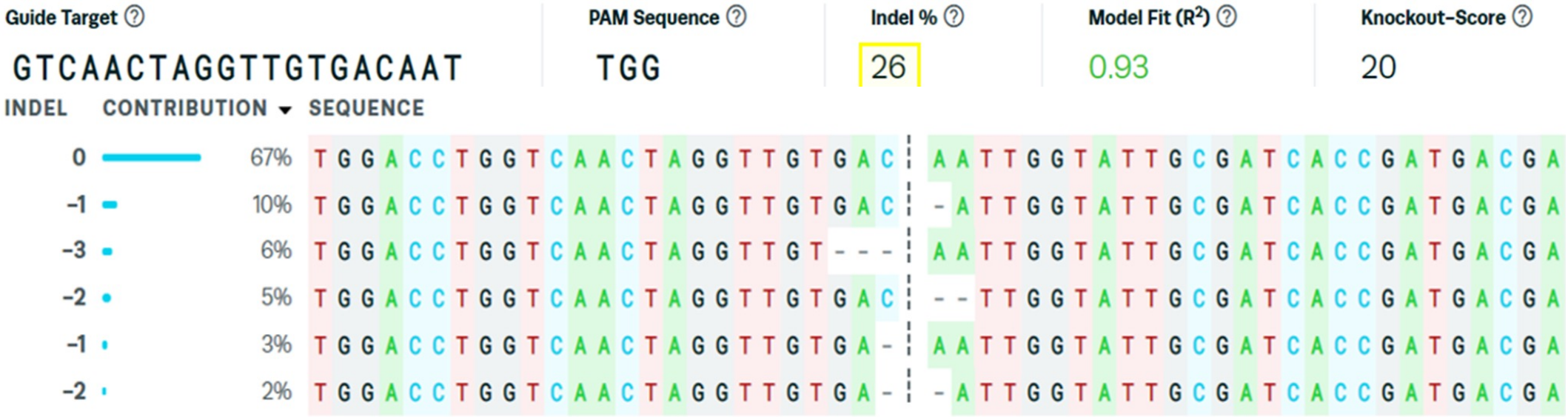
Using barley protoplast to assess the mutagenesis efficiency of CRISPR-editing constructs



- Isolate protoplast
- PEG-Ca2+ mediated-transformation
- Isolate gDNA after 48 hr
- PCR-amplify gDNA spanning the target site
- Analyze for mutations

Using Synthego ICE CRISPR online tool to decipher the Indel frequency in the potential mutant tissues and plants

HvUGT promoter target site

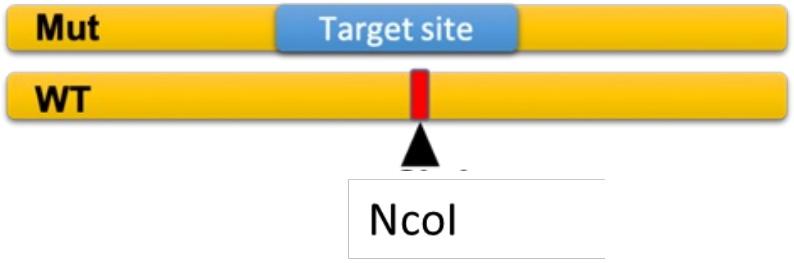


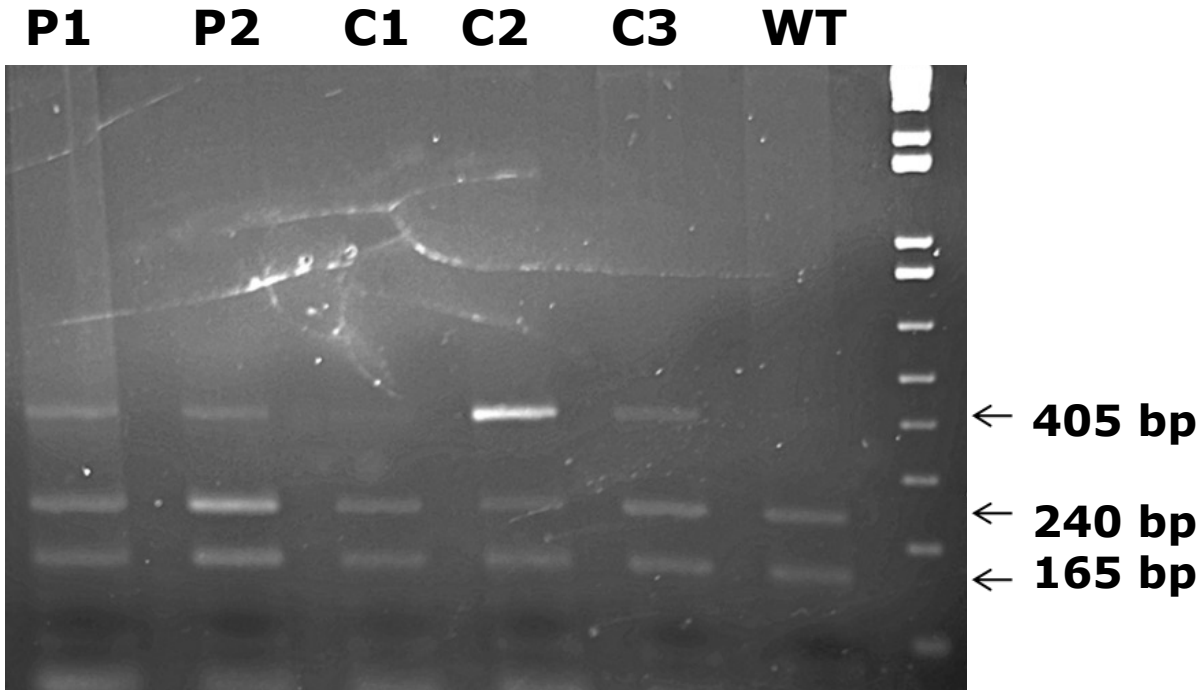
- **Putative mutations were confirmed by Sanger sequencing of individual clones of PCR products.**

Restriction fragment length polymorphism (RFLP) assay to analyze CRISPR-induced mutation

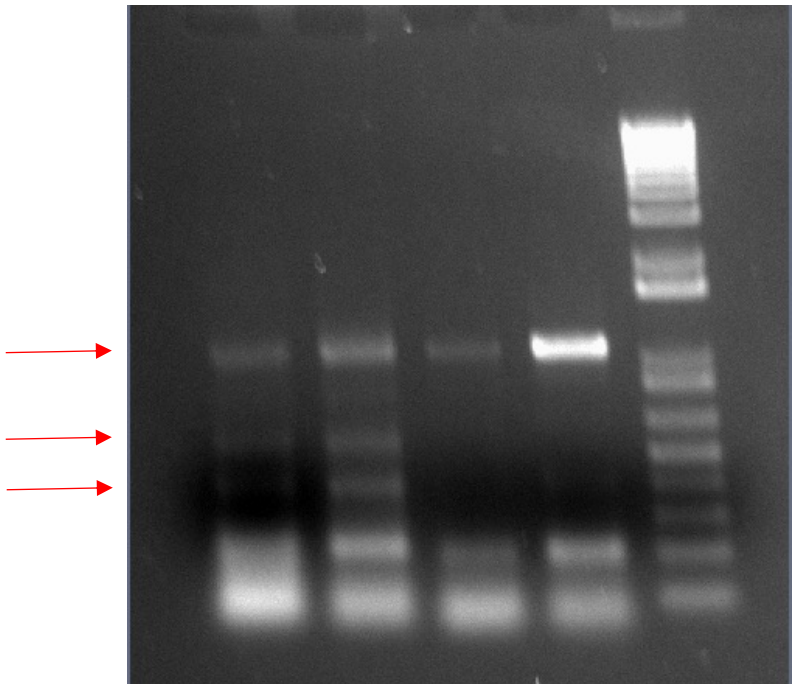
pRD549 (integrating, dual targeting *HvUGT* promoter)-transformed protoplasts (P) or callus (C)

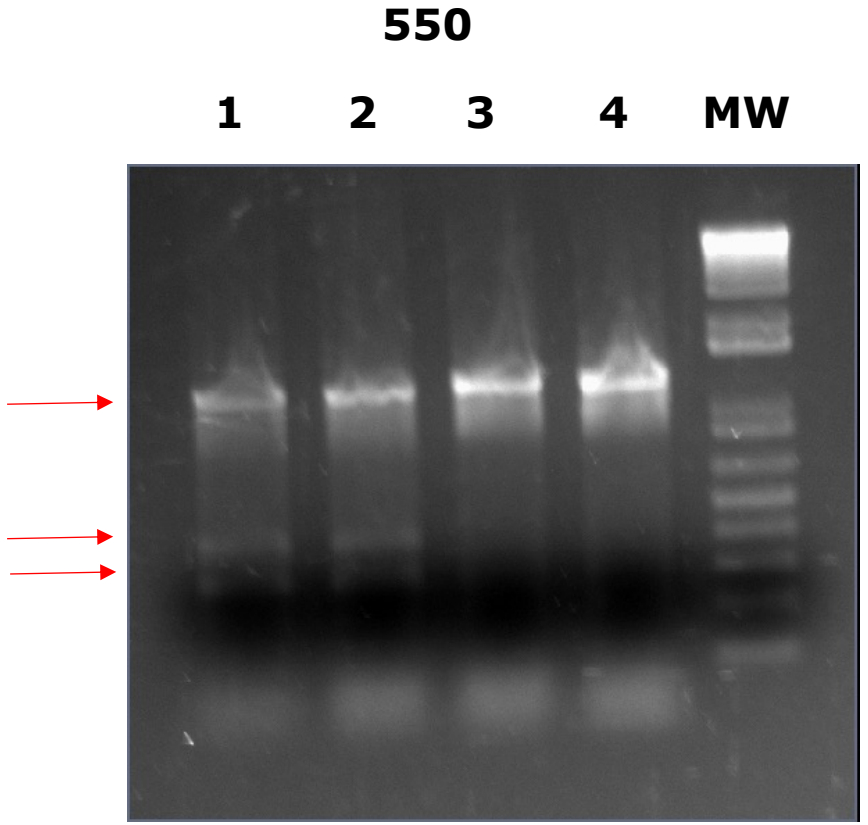
PCR product of *HvUGT* promoter *Nco*I target site





PCR-amplification and sequencing of gDNAs spanning the dual target sites of *HvGUT* promoter and *HvEIN2*





- The full-length products have mostly WT sequences.
- The shorter products have insertions from other barley gDNA parts.

Our gene-edited sweet basil plants produced by the transient CRISPR vector do not contain the transgene and are resistant to downy mildew disease

X. Zhang, Y. C. Low, M. A. Lawton, J. E. Simon and R. Di. 2021. CRISPR-editing of sweet basil (*Ocimum basilicum* L.) homoserine kinase gene for improved downy mildew disease resistance. Frontiers in Genome Editing-Genome Editing in Plants 3:629769. DOI:10.3389/fgeed.2021.629769.

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NATURE | NEWS

Gene-edited CRISPR mushroom escapes US regulation

A fungus engineered with the CRISPR–Cas9 technique can be cultivated and sold without further oversight.

Emily Waltz

14 April 2016

CHEMISTRY WORLD

ACCESS PROVIDED BY RUTGERS STATE UNIVERSITY

By Emma Stove, 26 April 2016

Yinong Yang and colleagues developed the anti-browning mushroom at Pennsylvania State University in the US. 'We used the Crispr-Cas9 technology to create small deletions in a specific gene encoding a polyphenol oxidase (PPO), says Yang.

The team used a bacterial plasmid construct to deliver the guide RNA and Cas9 enzyme into mushroom cells and achieve the necessary deletions. The final product does not contain any DNA from a donor or vector organism, so it is not covered by current regulations that control the import, movement or release of GMOs in the US.

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Genetically Modified Browning-Resistant Apple Reaches U.S. Stores

Success for the "Arctic apple" could herald a new wave of lab-grown foods

By Amy Maxmen, Nature magazine on November 7, 2017

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Availability of Deregulation Extension Request and Preliminary Determination for Apples Developed Using Genetic Engineering

Published: Jun 28, 2021 | Print

Okanagan Specialty Fruits, Inc. (OSF): non-browning apple

-- PG451 Arctic Gala, GD743 Arctic Golden and GS784 Arctic Granny apple

-- RNAi silencing/Agrobacterium transformation to knock-down 4 PPO (polyphenol oxidase) genes to prevent browning

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NJAES

Collaborator: Dr. M. Lawton

Technician: A. Dineen


Q&A