Testing Non-Transgenic CRISPR Technology for Wheat Improvement

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13TH IWGS - TULLN, AUSTRIA

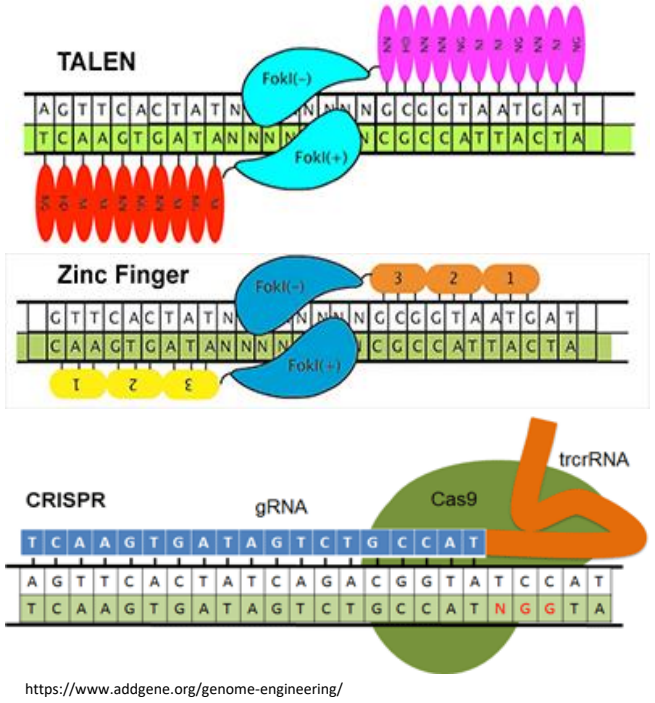
APRIL 28, 2017

Why CRISPR?

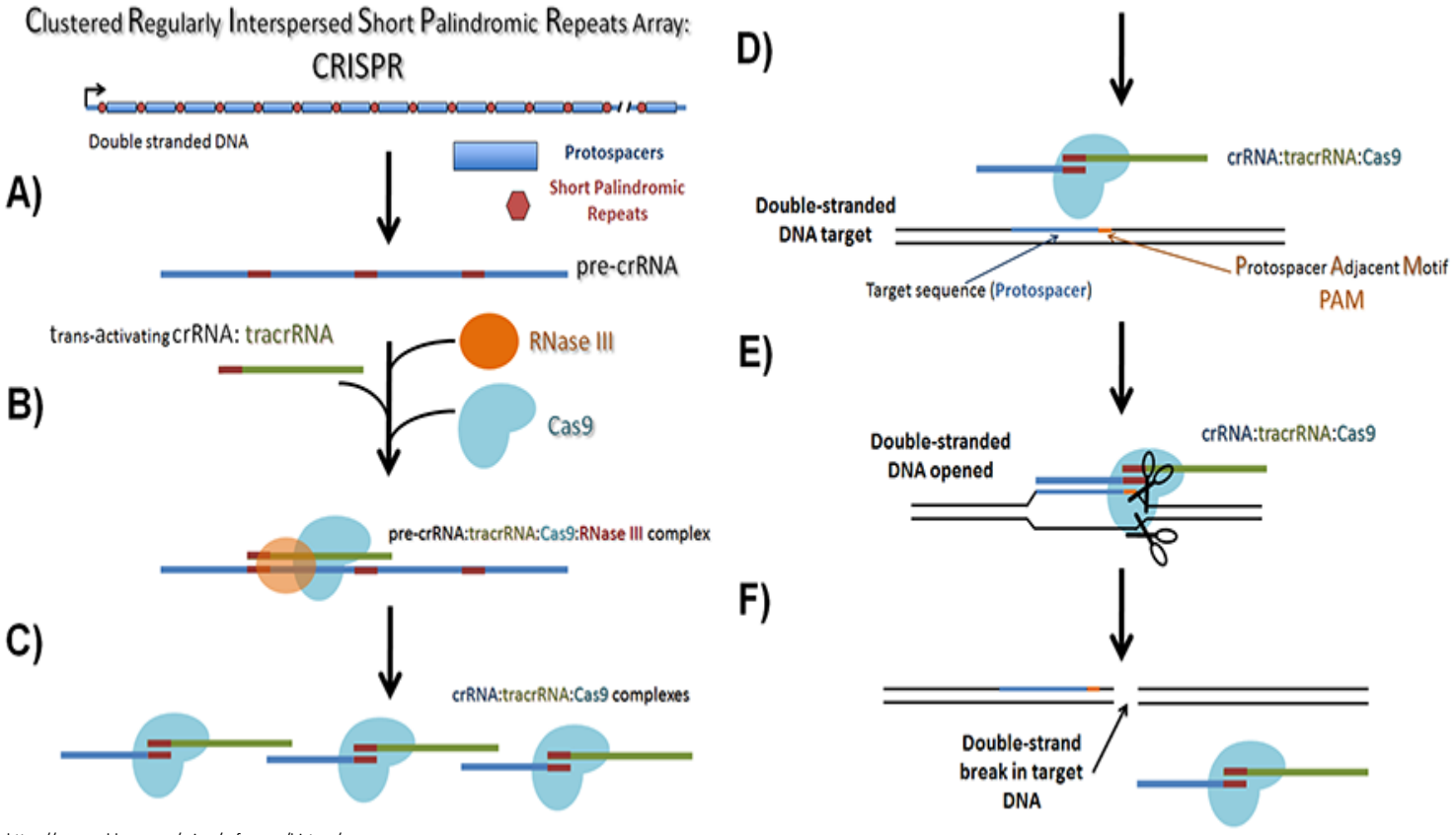
- Easy
- Unlimited Targets
- Cheap
- Non-Transgenic

CRISPR-Cas Bacterial System

- CRISPR
  - Clustered Regularly Interspaced Short Palindromic Repeats
  - Bacterial adaptive immunity system
- Cas
  - CRISPR Associated
- 3 distinct systems: Types I, II, and III
  - Cas9 is Type II
- First eukaryotic use in 2013
  - Previously TALENs and Zinc Fingers for specific targeting



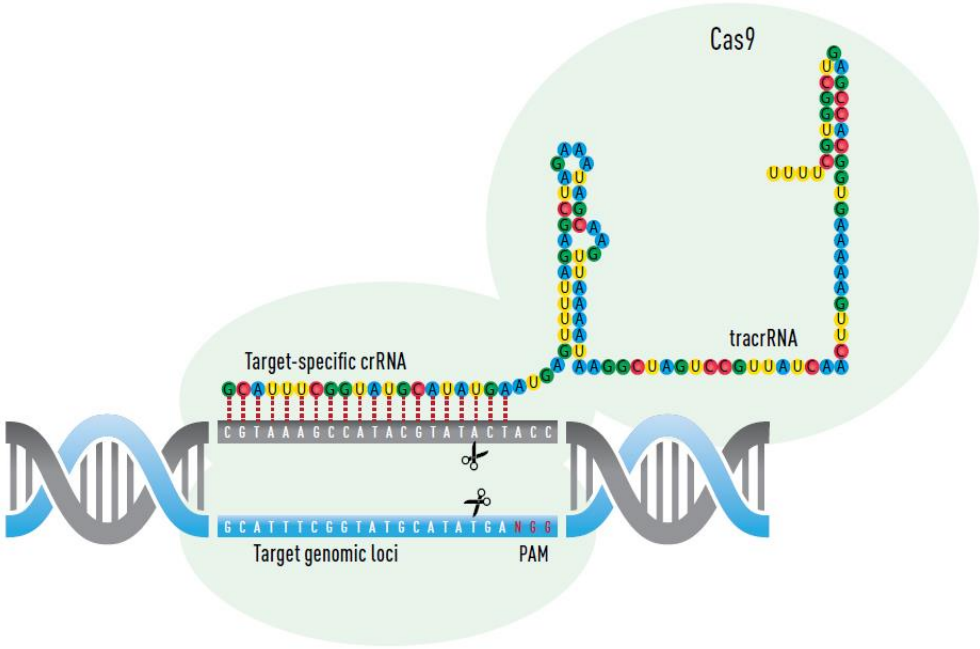
https://www.addgene.org/genome-engineering/



https://www.addgene.org/crispr/reference/history/

How It Works

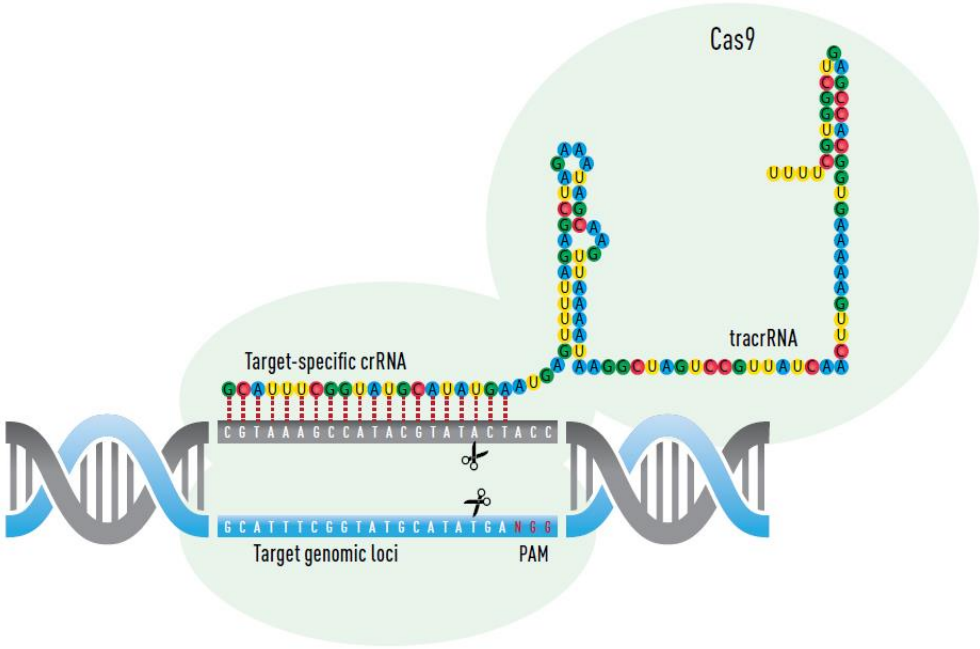
- 2 Components
  - Cas9 endonuclease & Guide RNA (gRNA)
- Can target anywhere that meets 2 conditions:
  - Sequence is unique in the genome
  - Target is 3nt away from a Protospacer Adjacent Motif (PAM)
    - 5’-20nt-NGG and 5’-CCN-20nt



https://www.thermofisher.com/us/en/home/technical-resources/technical-reference-library/genome-editing-support-center/crispr-based-genome-editing-support/crispr-based-genome-editing-support-getting-started.html

How It Works

- Ribonucleoprotein (RNP) complex forms and activates Cas9
  - Successful DNA binding activates RuvC and HNH endonuclease domains
    - DNA strands then cut
- DSB repaired in one of two ways
  - HDR – Homology Directed Repair
  - NHEJ – Non-Homologous End Joining



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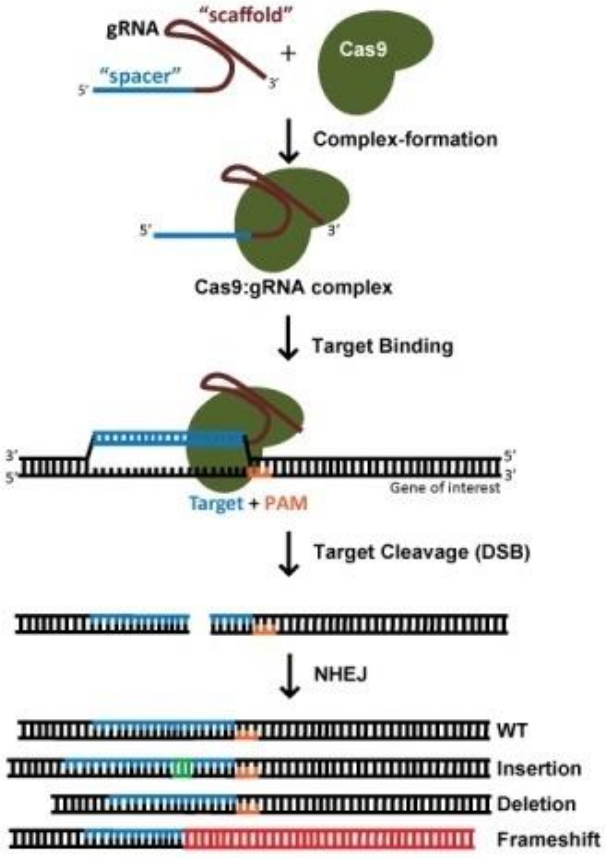
How It's Used

Type of edit:

- Indel
  - NHEJ – most common & most error prone

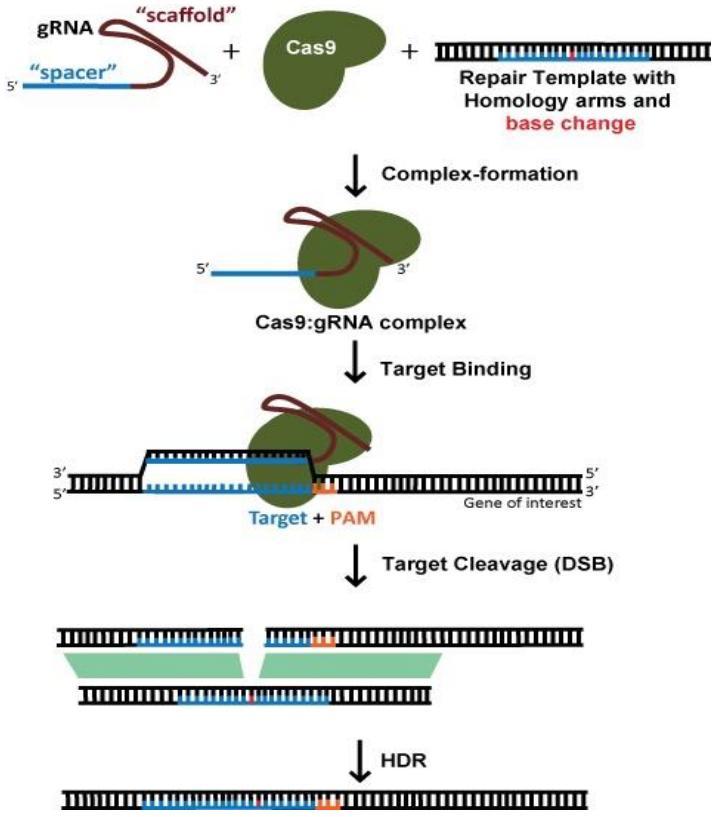


http://blog.addgene.org/how-to-design-your-grna-for-crispr-genome-editing

How It's Used

Type of edit:

- Specific change
  - HDR – less common & more precise
  - Low efficiency
  - Requires single cell cloning and screening



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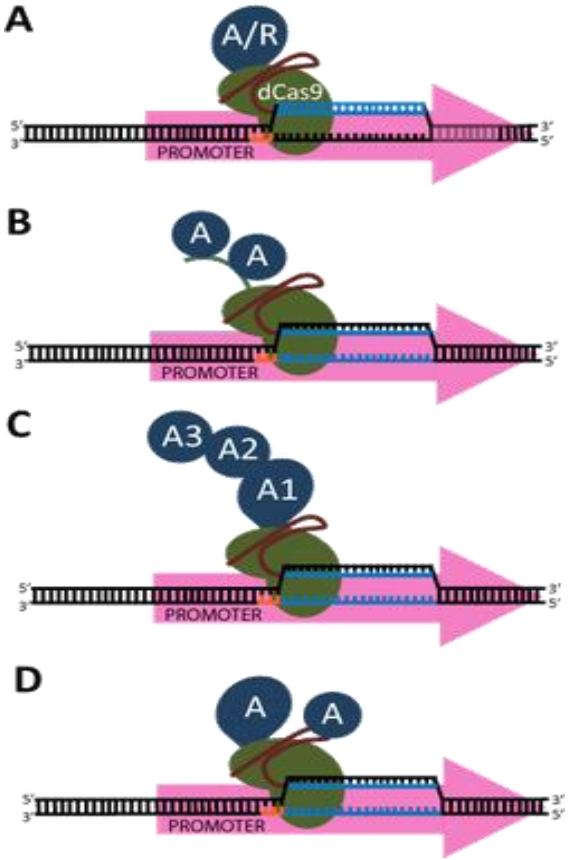
How It's Used

- Activation/Repression
  - Has ability to bind target DNA independent of cleavage
  - Endonuclease domains inactivated → "dead Cas9"
  - Target dCas9 to transcription start sites or promoter regions
    - Add transcription repressors or activators
  - Reversible
- Multiplex Genome Editing
  - Several gRNAs at once



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How It’s Delivered

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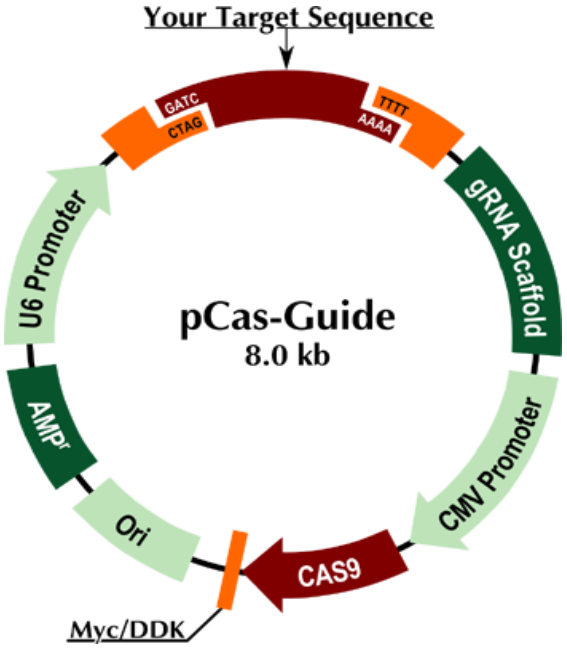
- Plasmid Expression vector
  - Transient or stable, high efficiency



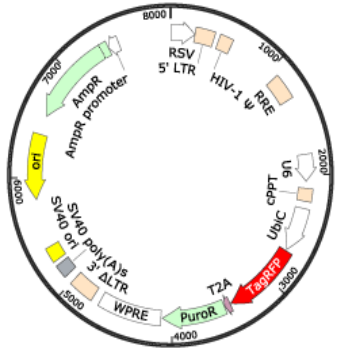
http://www.origene.com/CRISPR-CAS9/GE100001.aspx

How It’s Delivered

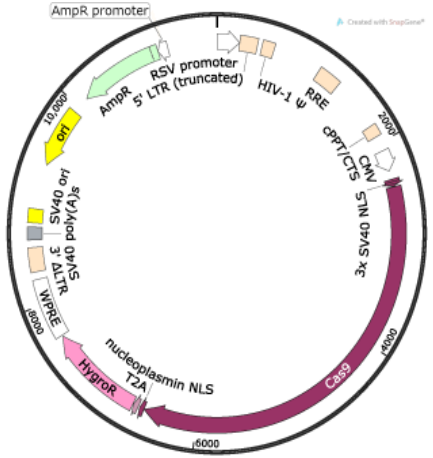
- Plasmid Expression vector
  - Transient or stable, high efficiency

- Integrating virus – eg. lentivirus
  - Stable expression, good for recalcitrant lines, *in vivo*, genome-wide screening

- Transient viral expression – eg. Adenovirus
  - less off-target effects



pRSG16-U6-sg-UbiC-TagRFP-2A-Puro
8.0 kb



pR-CMV-Cas9-2A-Hygro
11.6 kb

https://www.biocat.com/genomics/genome-engineering/crispr-cas9-lentiviral-guide-rna-cloning-vectors-and-control-constructs

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  - stable cell lines
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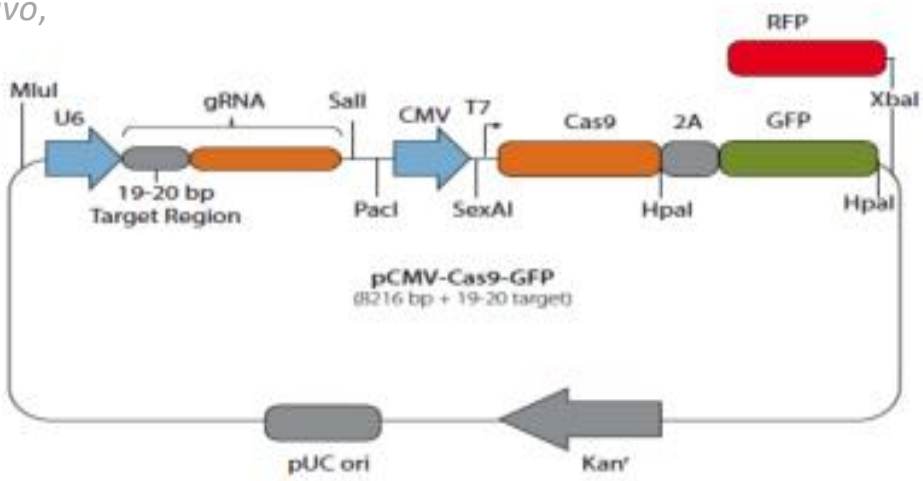
- Cas9 mRNA + gRNA
  - *In vitro* transcription of plasmids
  - Transient (RNA degraded in cell)



https://pluripotentstemcell.wordpress.com/crispr-core-sigma-psc-core/plasmid-based-crispr-mescs-cell-lines/

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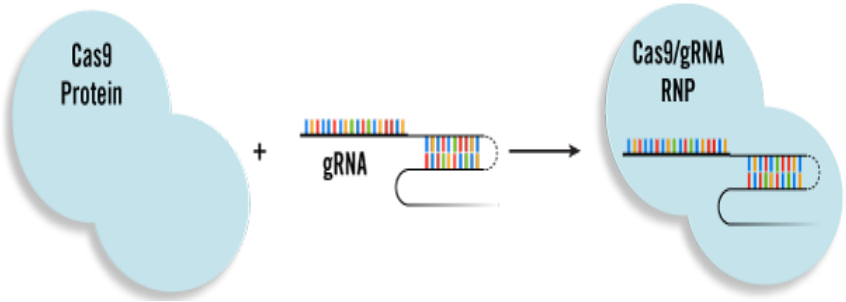

https://www.mirusbio.com/applications/genome-editing-using-crispr-cas/rnp-delivery

- Ribonucleoprotein Complexes (RNP)
  - Cas9 protein + *in vitro* transcribed gRNA form Cas9-gRNA complex
  - Transient

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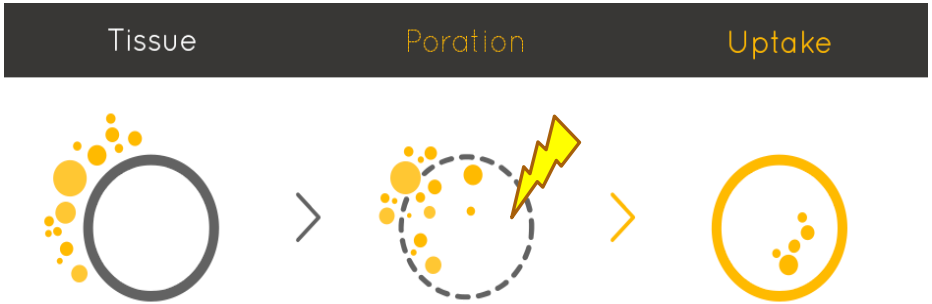

http://oncosec.com/tag/electroporation/

- Electroporation of plasmid DNA, mRNA, Cas9 protein + gRNA, or non-integrating viruses

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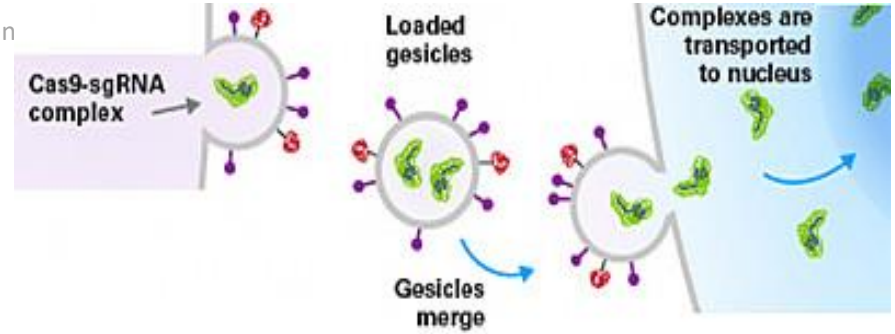


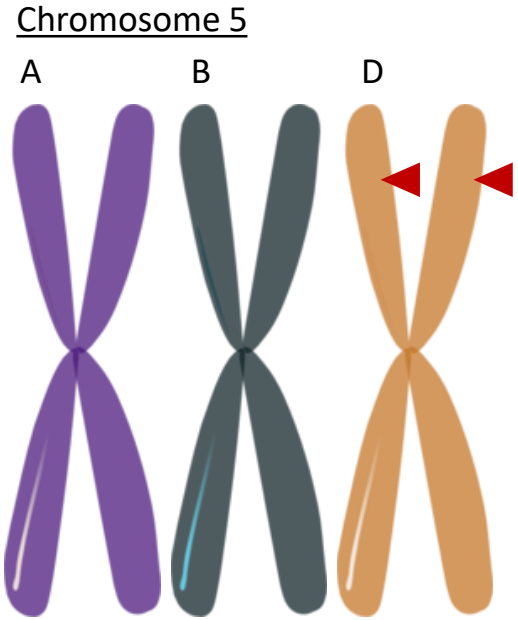
https://www.westburg.eu/blog/crisprcas9-genome-editing-take-it-up-a-notch

- Gesicles (nanovesicles)
  - Cas9-sgRNA RNP complex from producer cell line inside gesicles
  - Gesicles have binding and fusion proteins on surface

Our CRISPR-Cas9 Project

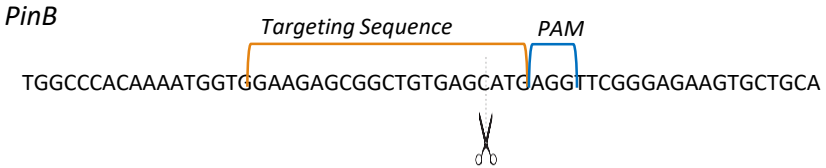
Step 1: Choose Target Gene

- PinB
  - Only on D genome
  - Wt gives soft seeds, knockout gives hard seeds
  - Very specific (as far as wheat genes go)
- We already have a KASP SNP marker
  - So we targeted same base with Cas9



Step 1: Choose Target Gene

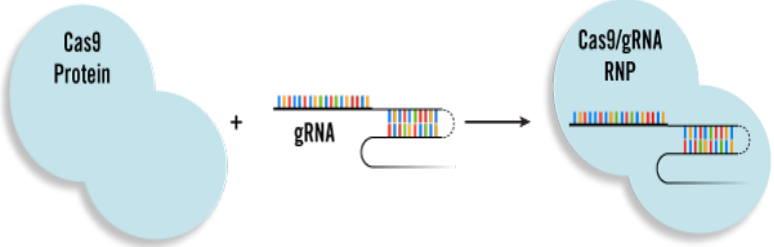
- Variety Bobwhite
  - Wt PinB gene
  - Spring type
  - Goes through tissue culture well



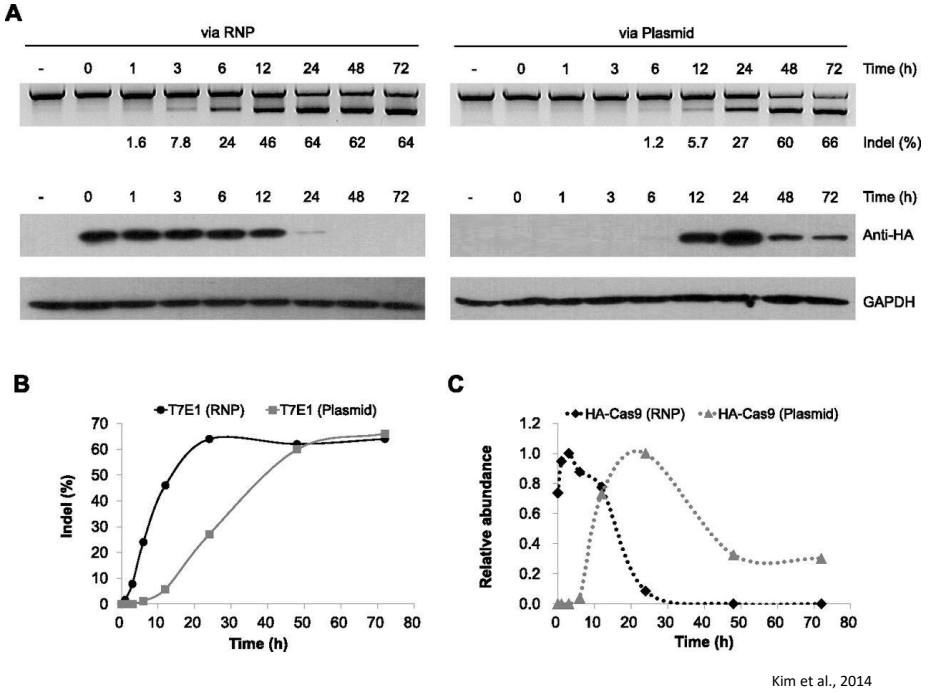
http://plantgrowthfacilities.agsci.colostate.edu

Step 2: Choose Delivery Method

- Ribonucleoprotein Complex (RNPs)
  - Simplest form of delivery
  - Short lifespan in cells
  - Plasmids express for several days
  - "…continuous expression…gives rise to the accumulation of off-target mutations."



https://www.mirusbio.com/applications/genome-editing-using-crispr-cas/rnp-delivery



Kim et al., 2014

Step 3: Choose Transformation Technique

Embryo Culture

- Wheat head cleaned in EtOH → 5% Bleach → rinsed in diH_2O
- Seed removed from primary and secondary florets
- Seed coat cut and peeled back
- Embryo removed and placed scutellum side up on callus induction medium

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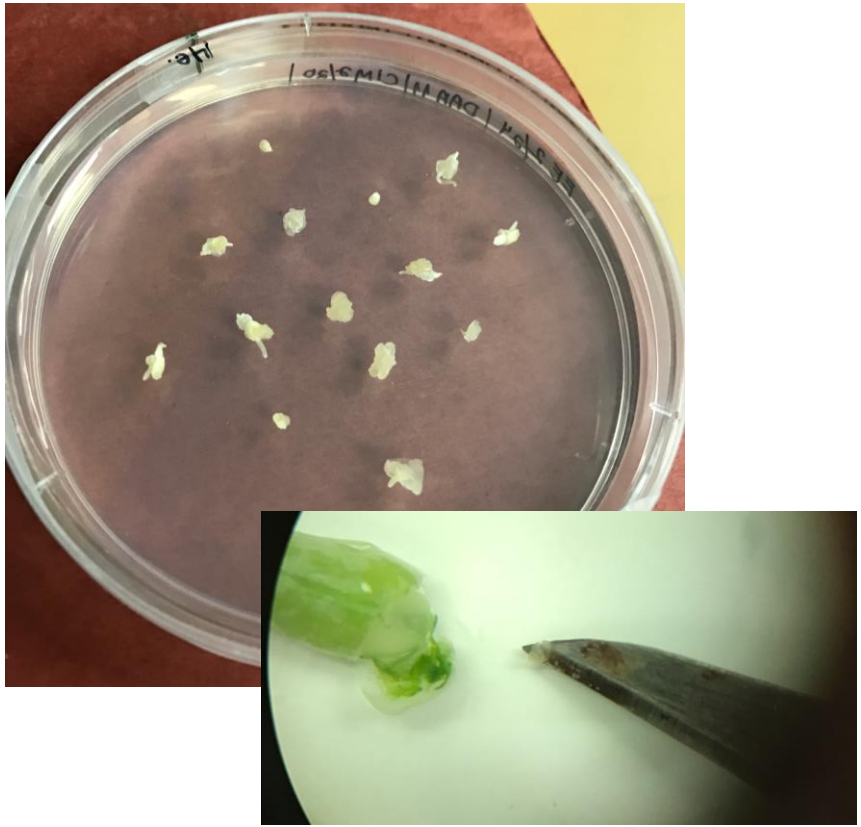
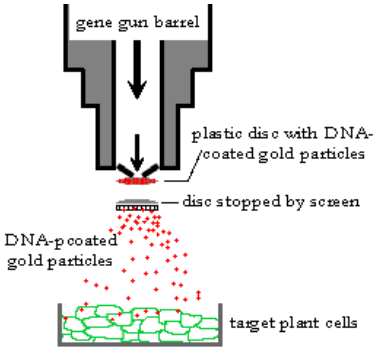
  - 2 weeks later…



Step 3: Choose Transformation Technique

Biolistics/Gene Gun

- No *agrobacterium* required – no transgenics
- Have a protocol in place and all equipment
- Shown to work with RNPs in Wheat
  - Liang et al, 2017, Nature Communications



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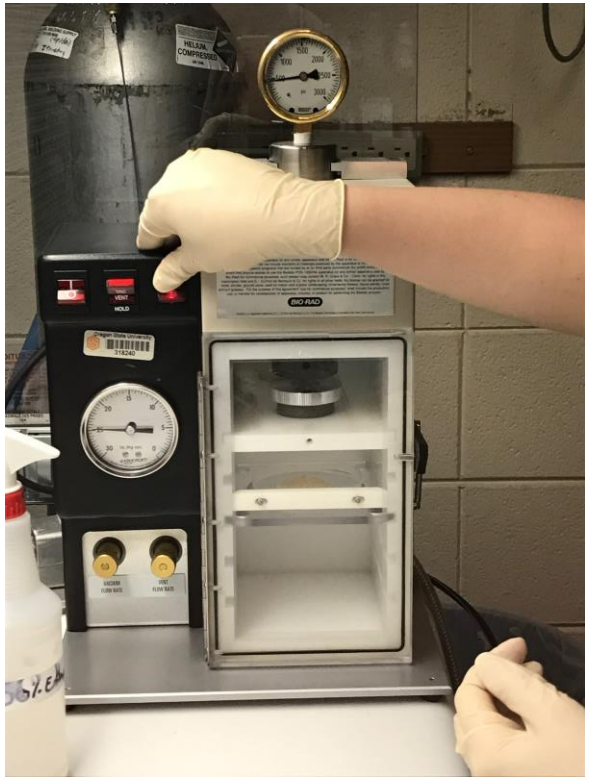
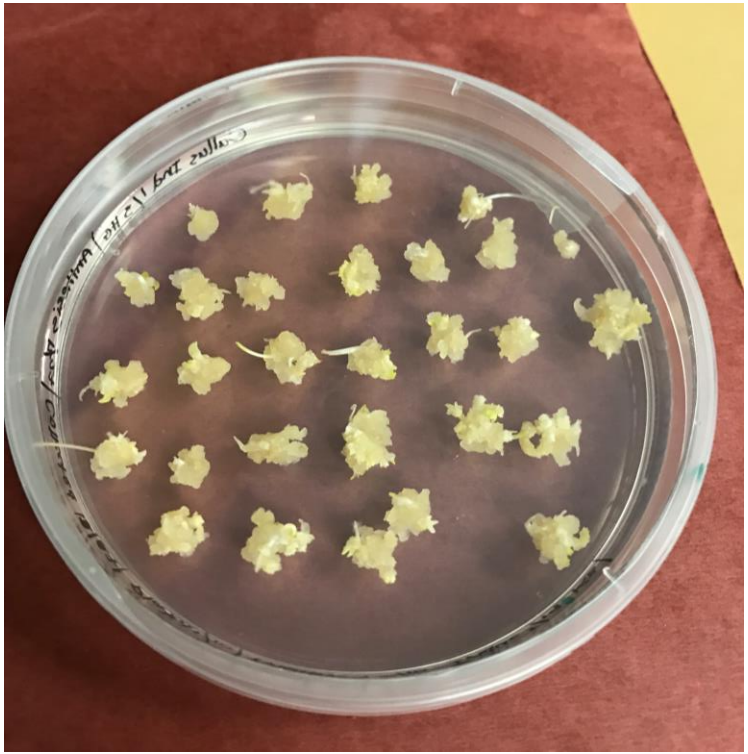


Step 3: Choose Transformation Technique

Tissue Culture/Plantlet Regeneration

- Transform callus
  - Leave on Callus Induction Medium for 2-3 days
- Transfer to Shoot Induction Medium
  - 2-4 weeks



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Tissue Culture/Plantlet Regeneration

- Transform callus
  - Leave on Callus Induction Medium for 2-3 weeks
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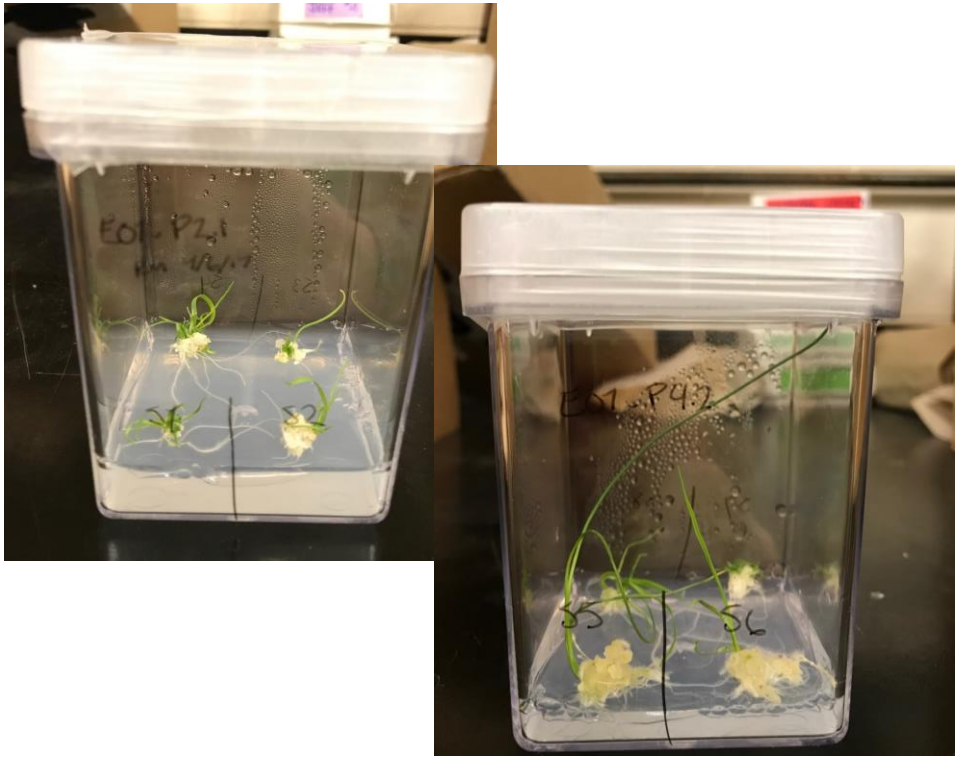
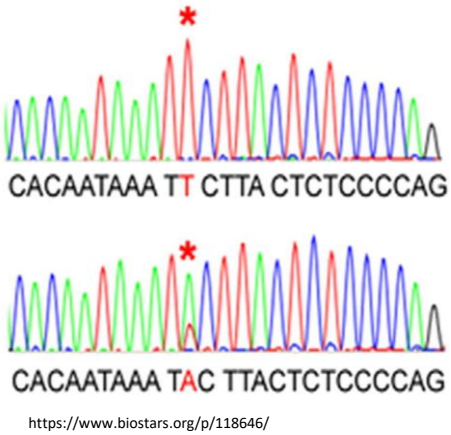
  - 2-4 weeks
- Transfer to Root Induction Medium



Step 4: Transformation Validation

Plantlet Regeneration

- Root Induction Medium
  - Take samples for PinB PCR
  - Sanger Sequence and analyze
- Transformed plantlets moved to soil in greenhouse
  - Phenotype seeds
  - Genotype next generation



https://www.biostars.org/p/118646/

Where We Are Going

- Previous RNP delivery in Wheat and Corn
  - 2.4 – 9.7% transformation efficiency
- Very little to no off-target effects found in literature
  - But still need to confirm
- Lentivirus transformation test
  - *in vivo* → no tissue culture

Conclusions

- Easy ✓ So far…
- (Virtually) Unlimited Targets ✓
- Cheap ✓
- Non-Transgenic ✓

Acknowledgements

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Adam Heesacker

OWC
OREGON WHEAT COMMISSION

ARCS ADVANCING SCIENCE IN AMERICA

Oregon State UNIVERSITY

Questions?

References

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