Establishment of anthracnose disease resistance of maize by RNA interference and site-directed mutagenesis

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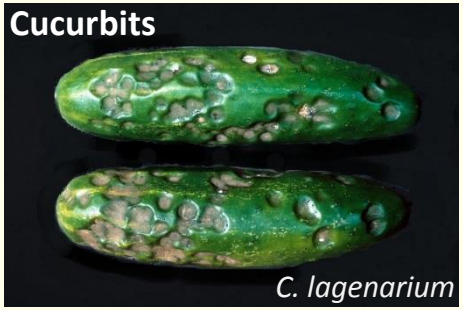
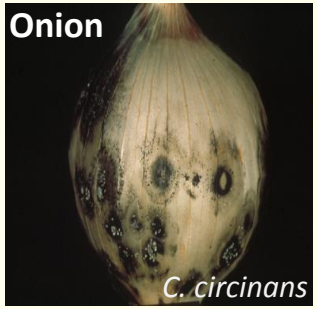
Importance of Maize (*Zea mays*)

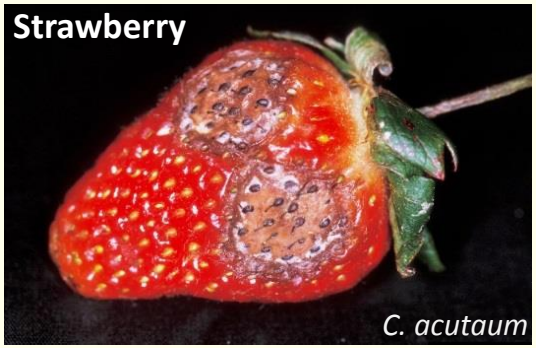
- Maize is one of the four most important crops world-wide
- Queen of cereals to its high genetic yield potential
- Consumed directly as food, feed for livestock
- Industrial raw material
- 75 Million t annual yield loss caused by plant pathogens
- *Colletotrichum graminicola* (Hemibiotrophic fungus)
- Anthracnose disease (yield losses 40% to 80%)

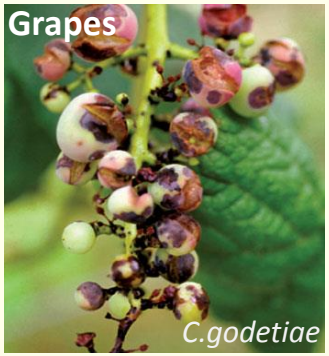
http://www.agroatlas.ru/en/content/diseases/Zeae/Zeae_Colletotrichum_zeae
http://cropwatch.unl.edu/2016/ear-and-stalk-rot-diseases-becoming-more-common-corn-fields

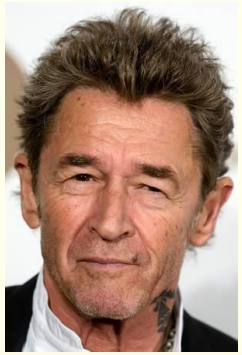
Wide range of anthracnose disease





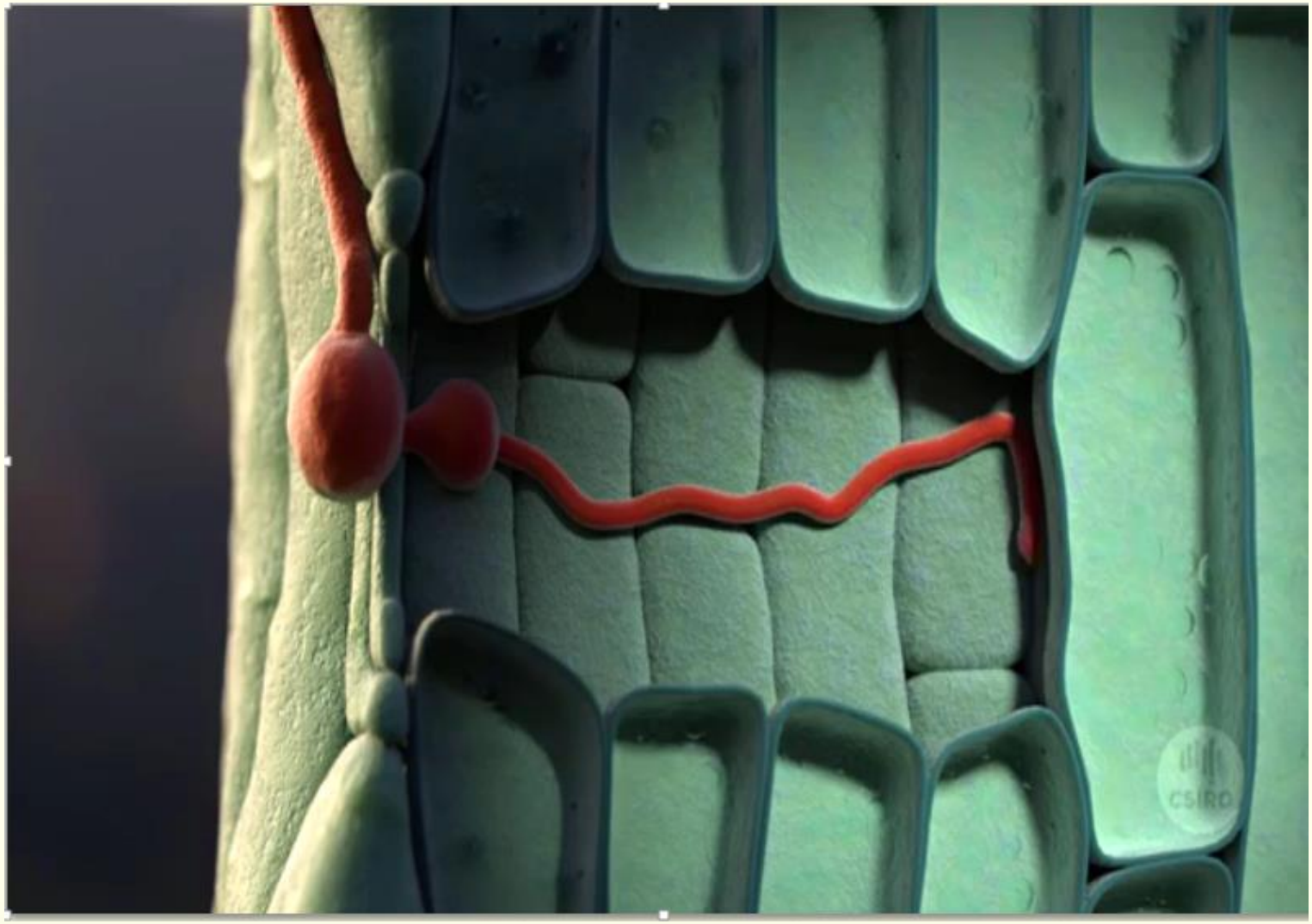






http://www.oisat.org/pests/diseases/fungal/anthracnose.html , https://www.forestryimages.org/browse/detail.cfm?imgnum=1572820 , https://hortnews.extension.iastate.edu/2009/11-11/bitterrot.html

Infection process



https://chrishammang.com.au

Maize anthracnose phenotypes

- **Leaf blight**

  - oval shaped, yellowing, water-soaked lesions on the leaves
  - occurs during early plant development stages

- **Top die-back**

  - necrosis of the top leaves and stalk
  - prominent after tasseling or later during the grain fill period

- **Stalk rot**

  - blackening of the pith tissue in the stalk
  - much delayed generative development

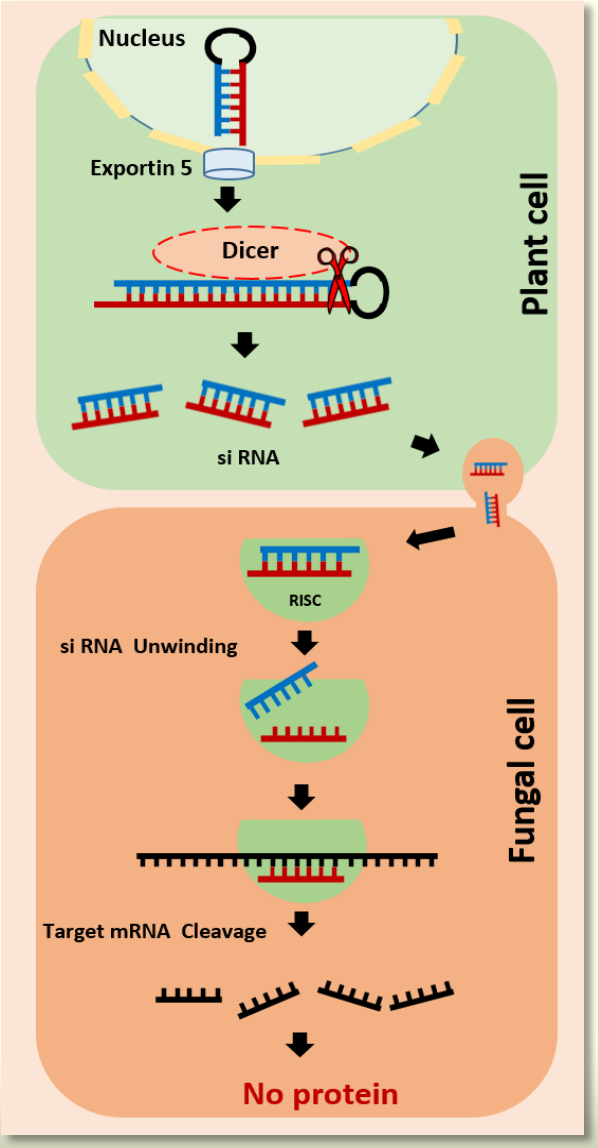
Bergstrom und Nicholson 1999

Disease Management

- Fungal diseases can be controlled by resistance breeding or chemical protection
- Resistance provided by most R-genes as well as administration of fungicides are no durable solutions
- Fungal point mutations can be sufficient to render fungicides ineffective
- Fungal tolerance can also rest upon efflux transporters genetically adapted to fungicidal drugs
- Host-induced gene silencing (HIGS) may confer durable plant resistance
- Knockout of plant susceptibility *(Zm lox-3)* factor with RGEN

Major features of HIGS appoaches

- RNA interference (RNAi)-based process
- Plant-made small RNAs silence gene-specific transcripts after being taken up by a pest or pathogen
- Established principle of plant‘s pathogen control as exemplified for nematodes, insects and fungi
- Acts at the transcriptional level, i.e. no formation or modification of a protein or metabolite required
- Can still address fungicide resistant pathovars

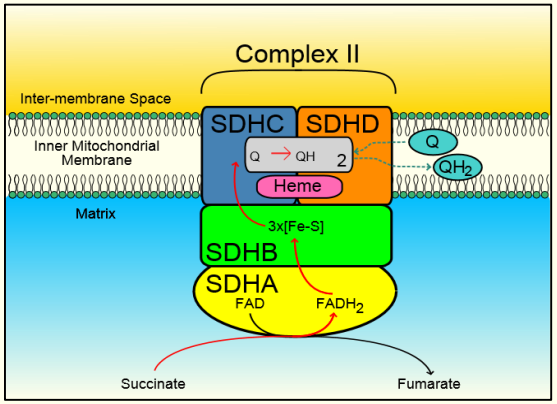


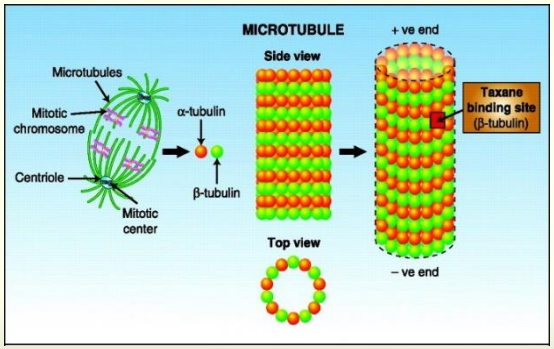
HIGS targets and its functions

- Genes encoding fungicide targets are potential HIGS targets
- Boscalid: succinate dehydrogenase inhibitor
- Benomyl and carbendazim: β-tubulin inhibitors

- Succinate dehydrogenase (SDH)
  - act in citric acid cycle and electron transport chain
  - oxidates succinate to fumarate
  - reduces ubiquinone to ubiquinol

- β-Tubulin
  - essential for cell division, cell shaping
  - multiple further functions

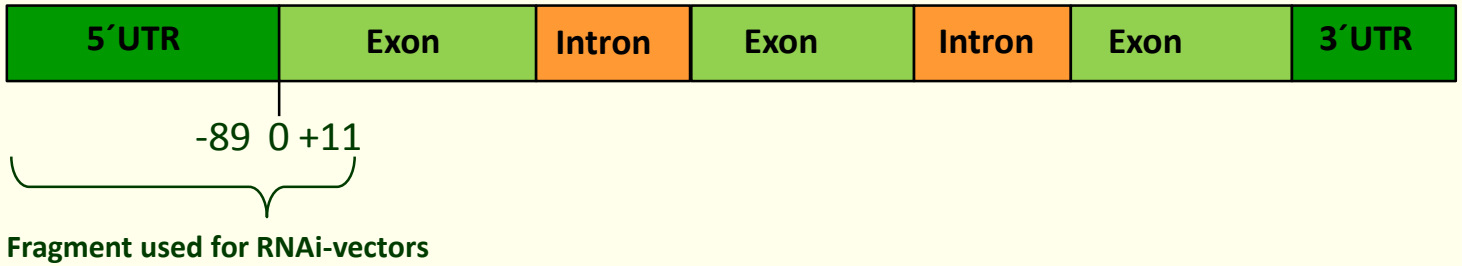




https://en.wikipedia.org/wiki/Succinate_dehydrogenase
https://en.wikipedia.org/wiki/Tubulin

HIGS target sequences and transformation constructs

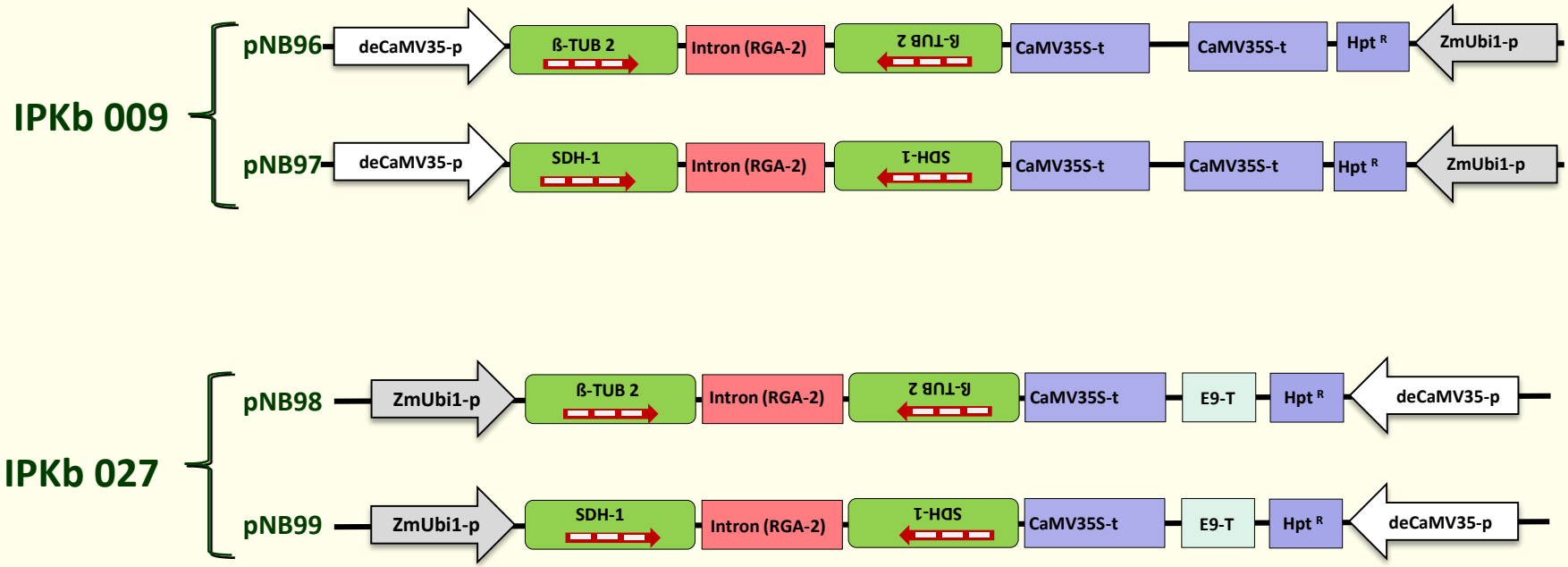
- 5´ UTR regions of both fungal genes showed sufficient sequence diversity from their orthologs of the host genome



- The 5'-untranslated regions of *SDH1* (117 bp) and *ßTUB2* (100 bp) were thus chosen as target sequences
- The generic HairPin (HP) vector contains sense and antisense sequences from a target gene connected by intron
- HP constructs result in formation of dsRNA which triggers post-transcriptional gene silencing

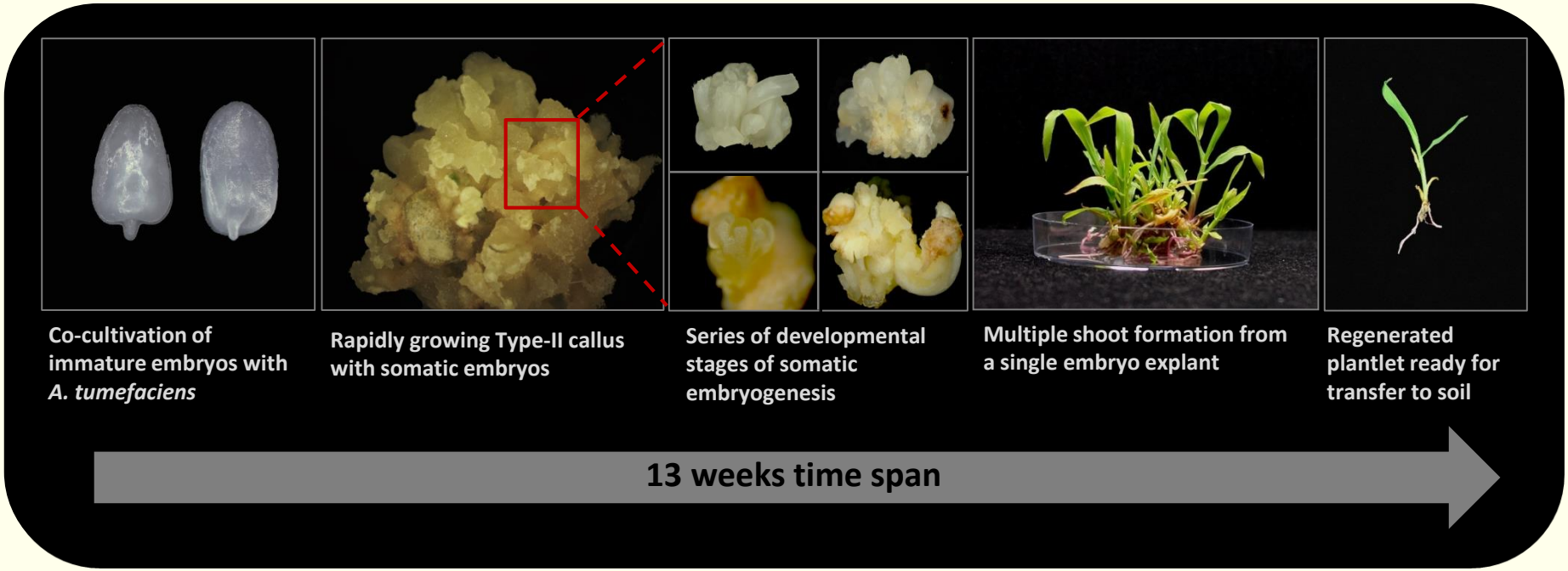
RNAi constructs used for maize transformation

- To produce sufficient amounts of siRNA, three repeats of the 117 bp target sequence were integrated into the modular binary vectors IPKb009 and IPKb027



Maize genetic transformation method

- **The transformable Hi II hybrid was transformed by our standard protocol**



Hensel et al, 2009, Int. Journal of Plant Genomics

Current state of transformation experiments

Vector name	No. of embryos agro-infected	No. of plants produced	Regeneration Efficiency (%)	Transformation efficiency (%) (PCR for selectable marker)	Transformation efficiency (%) (PCR for both inverted repeats)
pNB96	107	5	4.6	4.6	4.6
pNB97	145	34	23.4	22.7	6.8
pNB98	87	28	32.1	32.1	11.4
pNB99	110	0	0	0	0

Summary & Outlook

- *SDH*-1 and *TUB-2* specific hairpin constructs designed and cloned
- Transgenic maize plants generated
- Presence of gene of interest and plant selection marker confirmed
- pNB99 vector used in yet another transformation experiment

- Segregation analysis of T1 plants under progress
- Conduct Colletotrichum infections assays

Knockout of susceptibility factor *ZmLOX-3* using gRNA/Cas9-mediated mutagenesis

Plant lipoxygenases

- 9-Lipoxygenase (*ZmLOX3)* acts as susceptibility factor for *Colletotrichum graminicola*
- ZmLOX3 enzyme produces oxylipins
- Plants produce oxylipins as signal to defend against abiotic stress, pests and pathogens
- In fungi, oxylipins are potent regulators of mycotoxin biosynthesis and sporogenesis
- Pathogens recruit the host (plant) oxylipin pathway to facilitate pathogenesis and reproductive development
- Loss-of-function mutations of *ZmLOX3* reduce susceptibility
- Project aim: knockout of *ZmLOX3* via site-directed mutagenesis

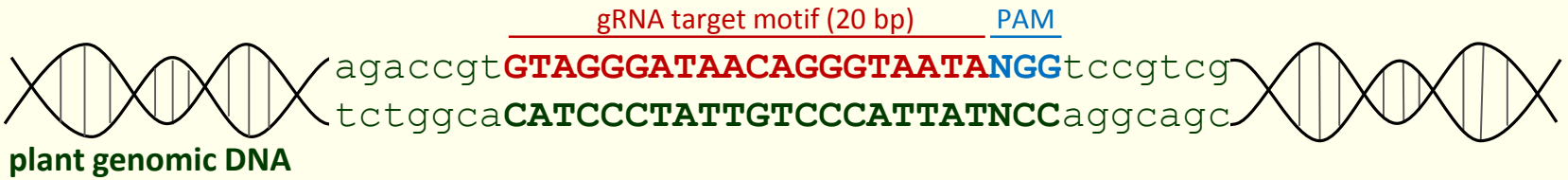
Major features of RGEN appoaches

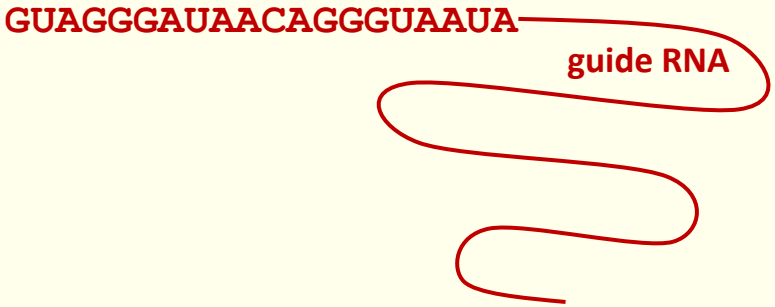
- Microbial defense mechanism, protect against foreign DNA from viruses by cleaving sequence-specific manner

- Emerged as a promising tools for plant genome engineering
  - deletion of detrimental traits or addition of significant characters
  - generate transgene-free mutant plants

- RGEN system is simple, efficient and versatile
  - with multiple gRNAs can edit multiple target genes simultaneously
  - easily reprogrammable by changing the guide RNA sequence

New Molecular Biology Tool: Cas Endonucleases





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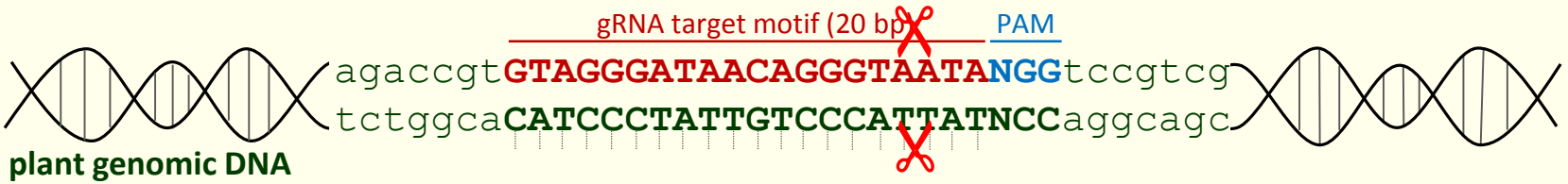
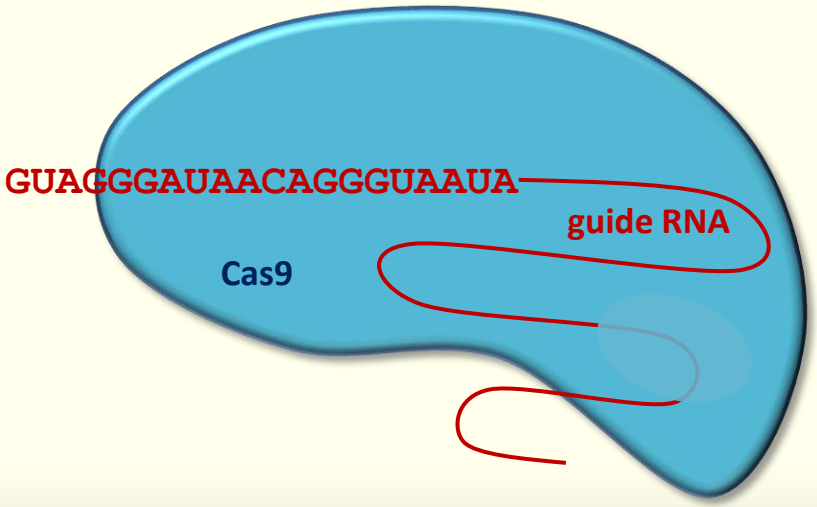
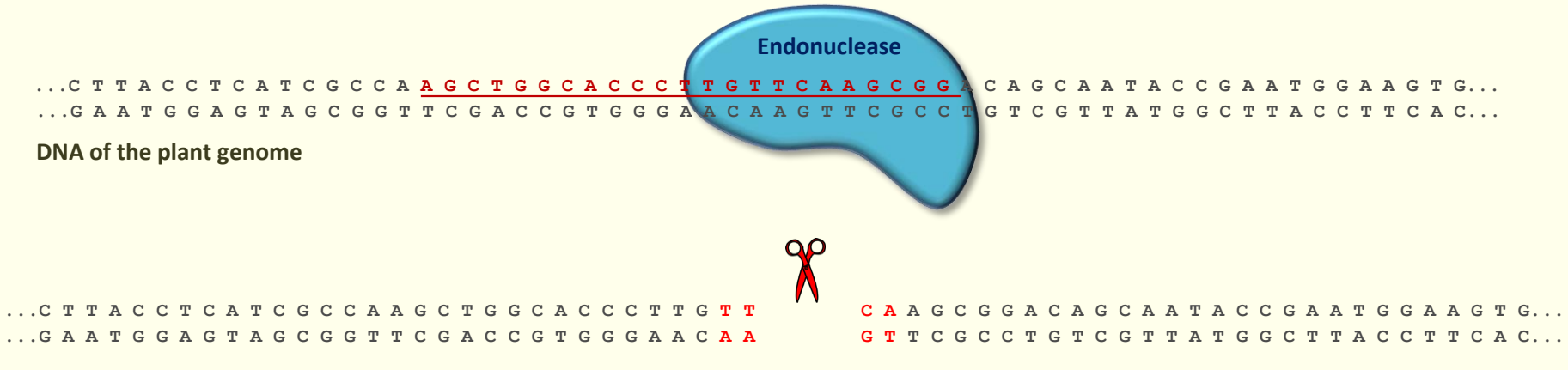




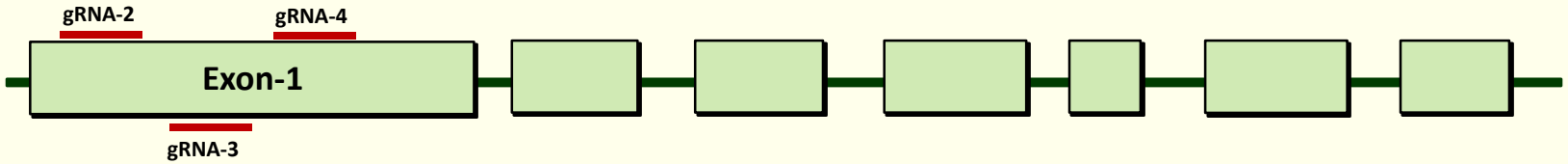
Site-directed mutagenesis using RNA-guided Cas9 endonuclease



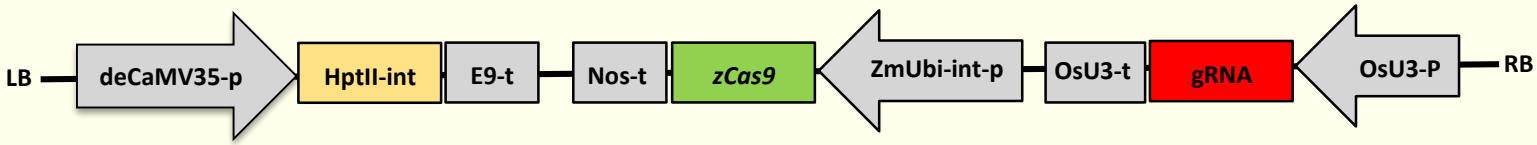
Any genomic sequence of choice can be mutated *in planta.*

Guide RNA design and cloning

- *Zm LOX3* has 7 exons
- Guide RNAs are designed to target the first exon



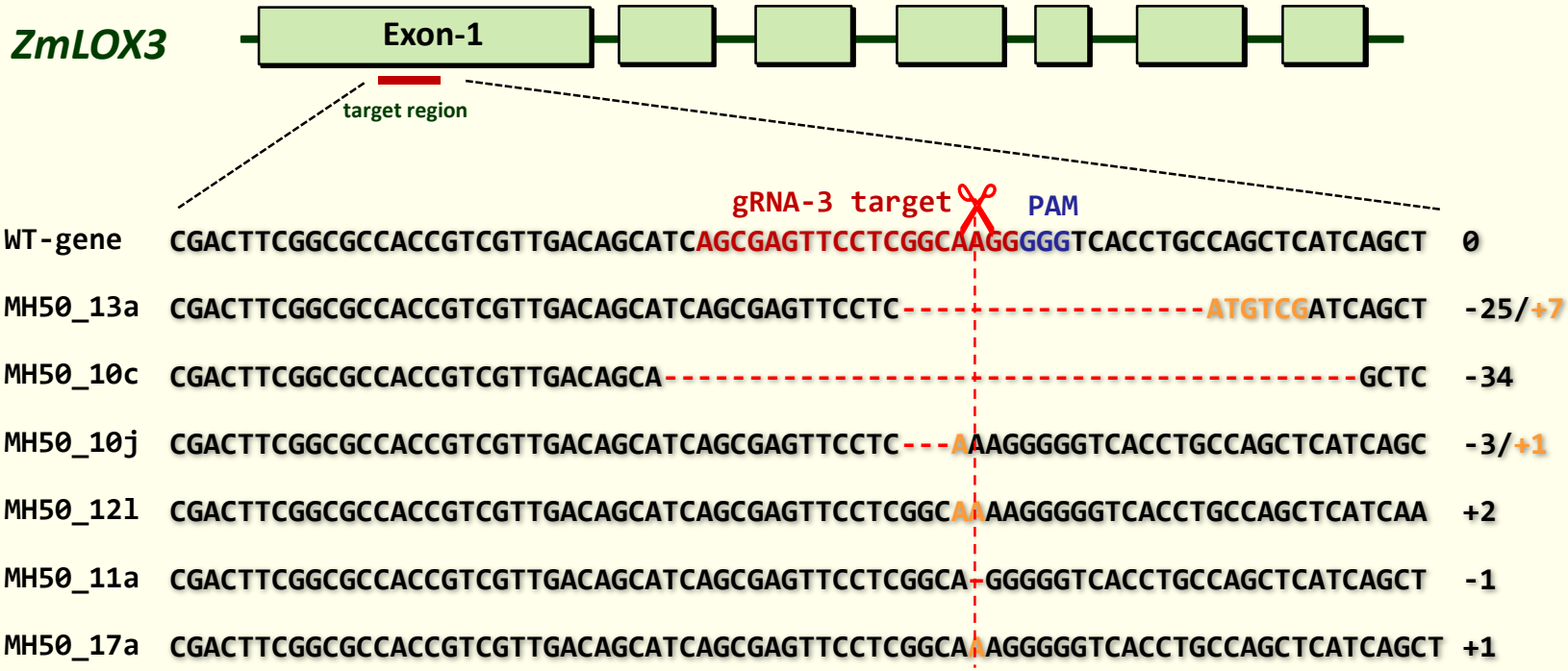
- gRNA/Cas9 constructs for maize transformation



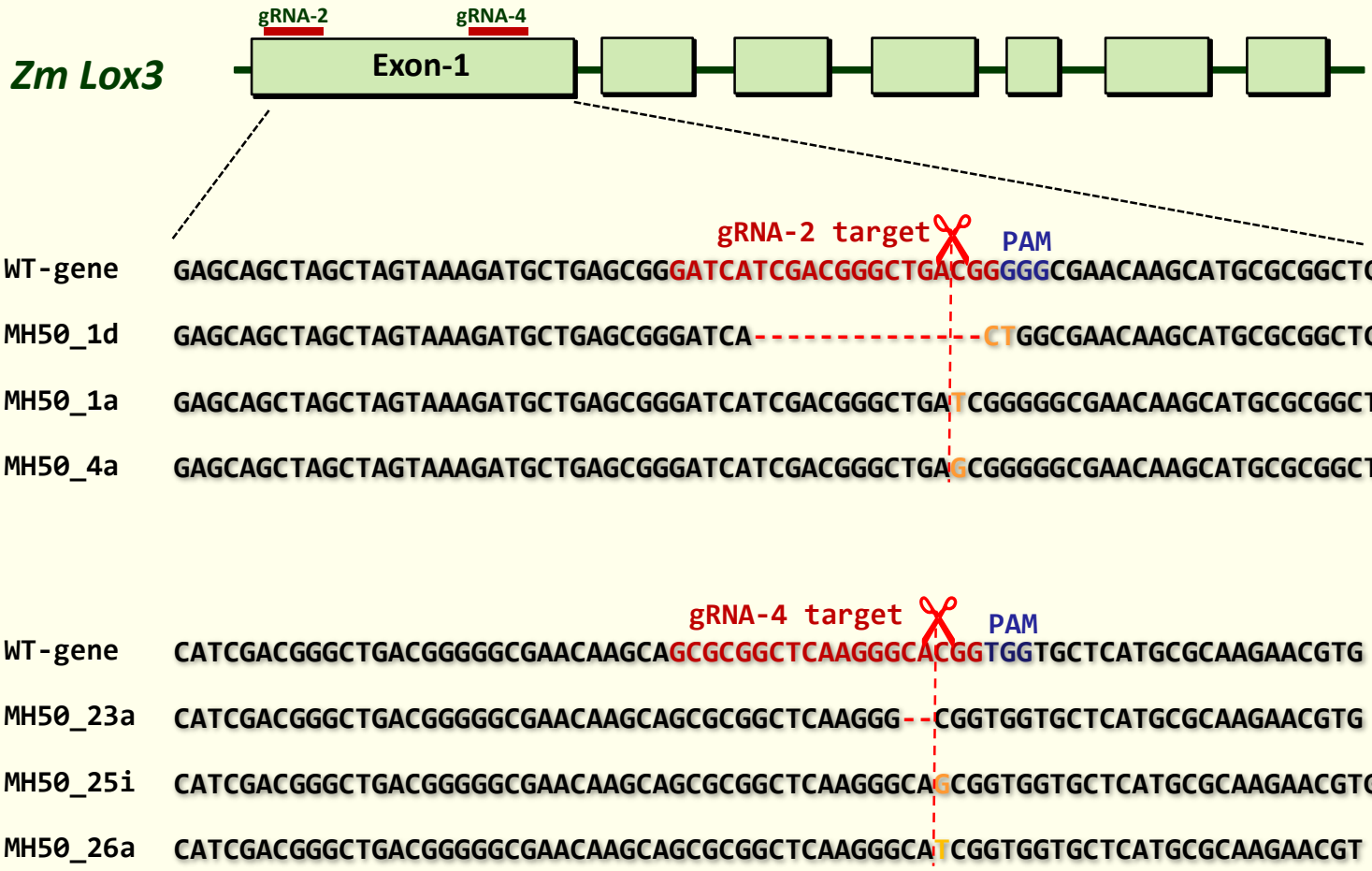
- Maize transformation using Hi II hybrid

Cas9-mediated mutagenesis of maize *ZmLOX3*

- **Screening of primary transgenic (T0) plants**

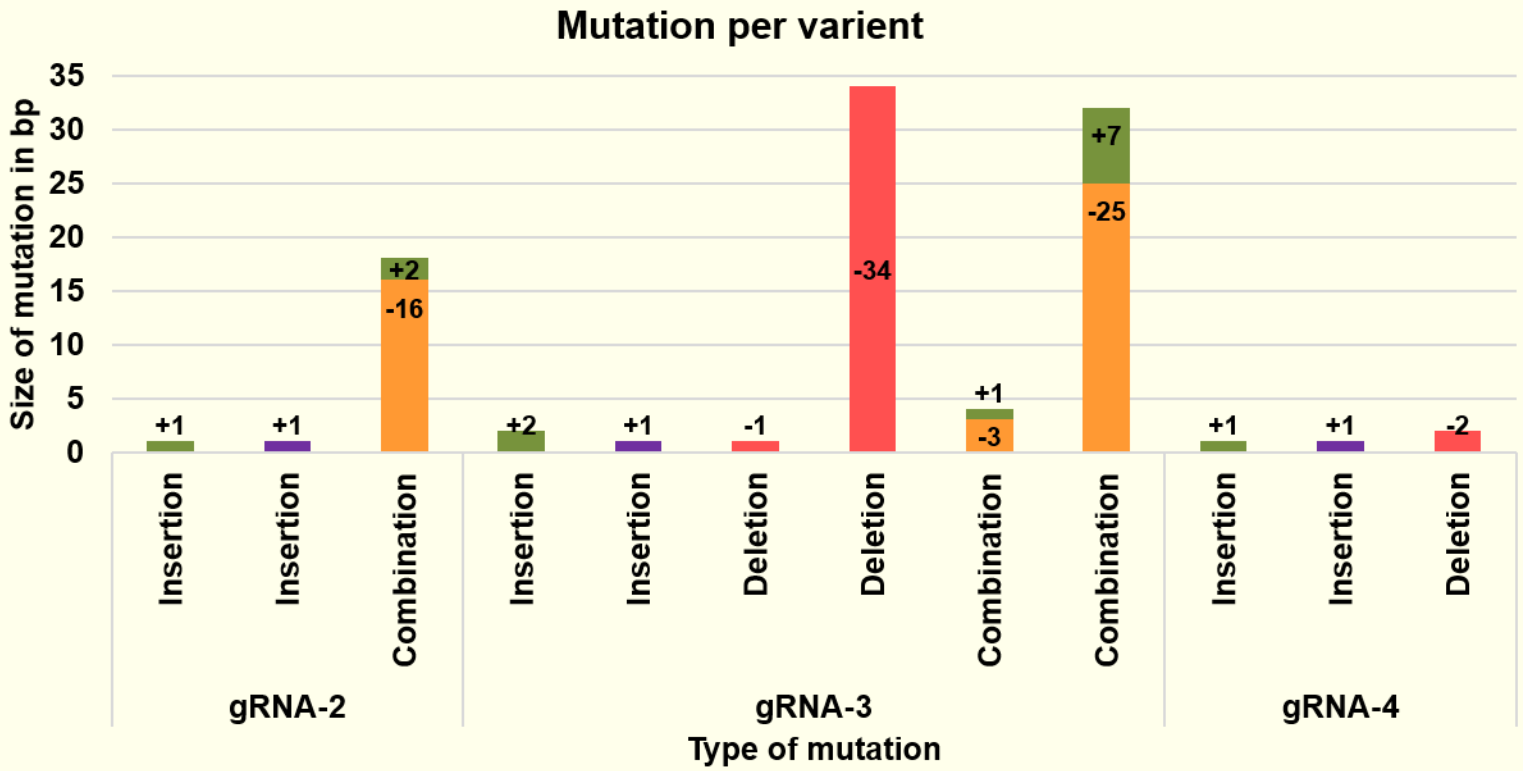


Screening of primary transgenic (T_0) plants



Summary of Cas9 induced mutations

- **Frequency of types and lengths of alterations**



Summary of transformation experiments

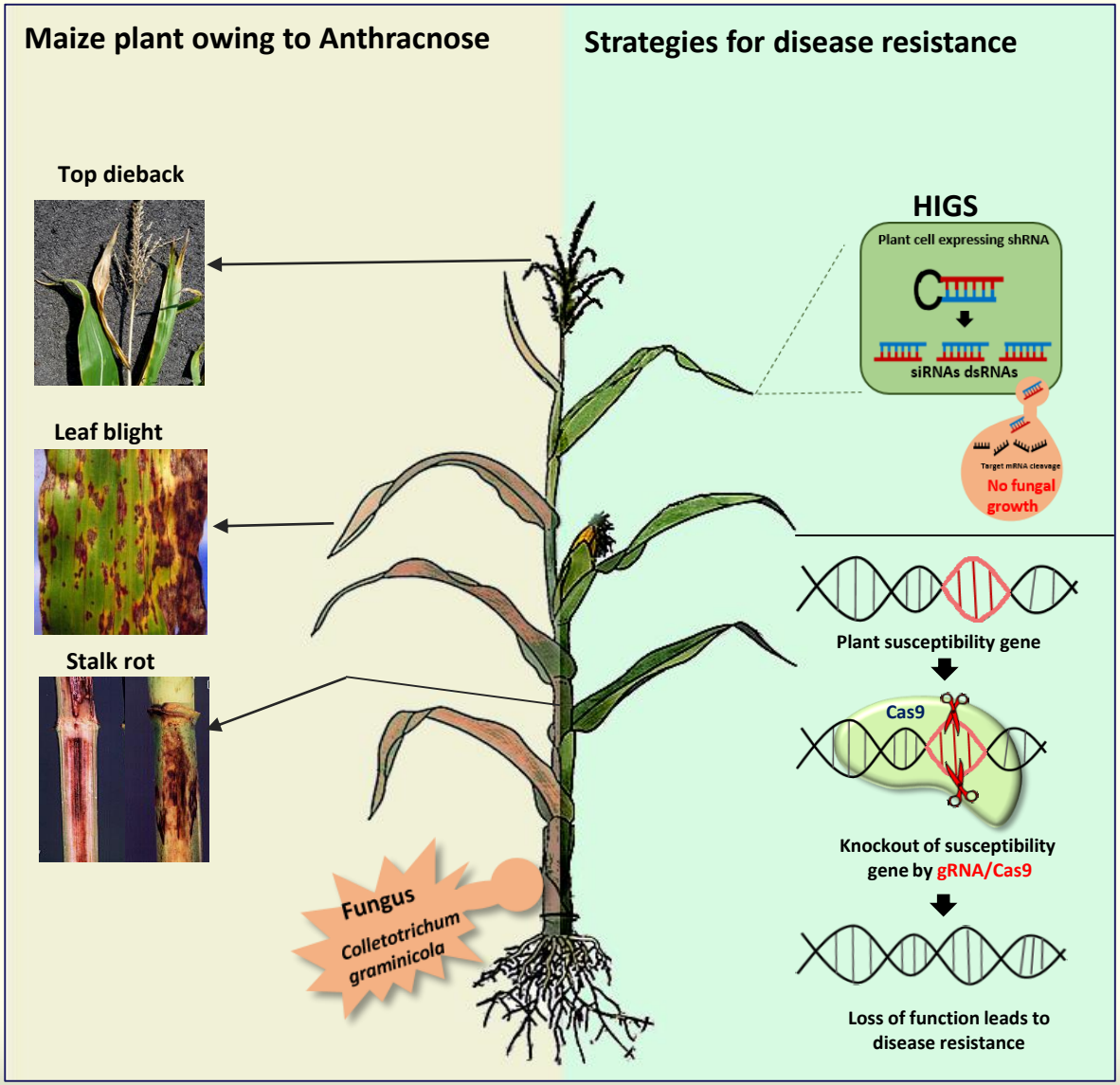
	gRNA-2	gRNA-3	gRNA-4
No. of immature embryo inoculated	118	140	116
Regenerants tested for the presence of T-DNA	6	88	37
PCR-positive plants (Presence of CAS9)	6	88	37
Plants used to sequence the target	6	88	37
Plants with conclusive target sequence	6	85	36
Plants without mutated target	0	0	0
Mutated plants	6	85	37
No. of independent mutational events	3	6	3

Summary & Outlook

- Transgenic maize mutant plants generated
- Detailed mutation analysis in progress

- Production of progeny to achieve homozygosity of mutated alleles
- Validation of homozygous mutant plants vs. WT maize for resistance
  - infection assays using leaf explants and whole plants
  - microscopic validation
  - estimation of fungal development by qPCR

Working Model



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