Current progress on developing disease-resistant citrus varieties using the CRISPR technology

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Challenges for breeding disease-resistant citrus varieties via traditional approaches

- The best means of disease control is the use of resistant plant materials.
- The genetic improvement of citrus by using conventional breeding is a lengthy, costly and challenging process due to the complex reproductive biology of citrus, including sexual incompatibility, a highly heterozygous nature, nucellar seedlings, male or female sterility and a long juvenile phase.
- For HLB, all commercial citrus varieties are susceptible. Poncirus trifoliata is known to have resistance/high tolerance to HLB.
- Issues for traditional breeding to keep the features of original elite varieties.
- Biotechnology approaches such as CRISPR-mediated genome editing have the potential to accelerate the citrus improvement process.

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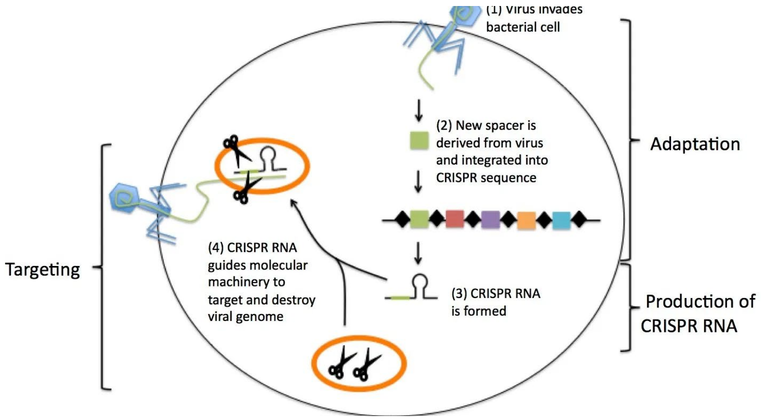
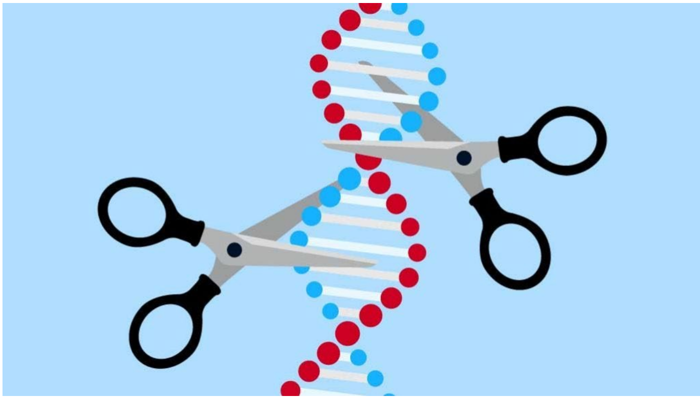
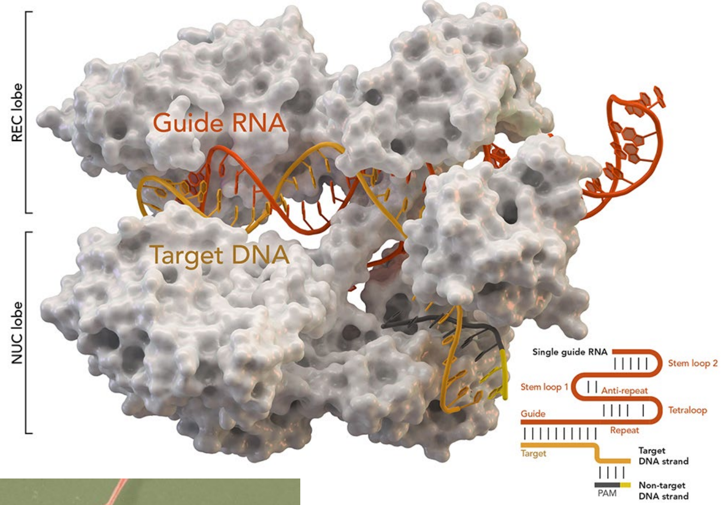
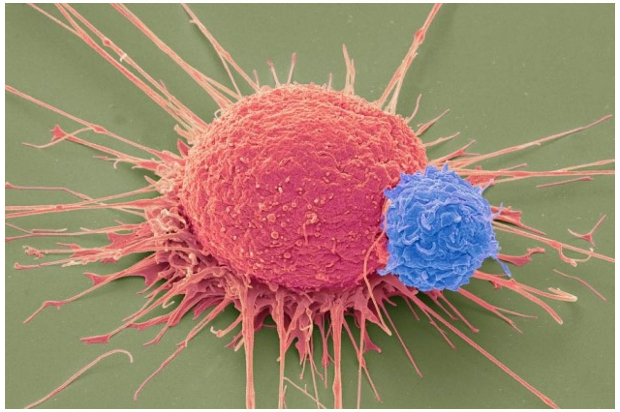
What are CRISPR?





- CRISPR (clustered regularly interspaced short palindromic repeats) and genome editing
- CRISPR: Prokaryotic Adaptive Immune System
- Molecular scissors
- First CRISPR clinical trial gets green light from US panel: 2016, University of Pennsylvania
- Early Results from First-In-U.S. Trial of CRISPR-Edited Immune Cells for Cancer Patients Suggest Safety of Approach 2019





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Regulatory approval of genome-edited crops

The regulatory status of gene-edited organisms under proposed US legislation

Are genome-edited organisms a regulated article?			
	USDA-APHIS	EPA	FDA
YES	If the edit introduces additional nucleic acids	If the edit results in the introduction of a plant-incorporated protectant (PIP)	If it's an animal[1]
NO	1. If the edit is a deletion of any size 2. If the edit is a single base-pair substitution 3. If the edit introduces naturally occurring nucleic acid sequences	If the edit does not constitute a PIP	No, if it's a crop. But a voluntary consultation process

[1]Full risk assessment and market approval as an animal drug.

- On May 18, 2020, APHIS published the Final Rule for its biotechnology regulations 7 CFR part 340. The SECURE rule, which stands for Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient.
- This "gene-editing" approach is both expedient and likely to be free of most regulatory burdens under the new SECURE rule.
- Calyxt became the first to commercially debut a gene-edited food, a soybean oil.
- Successful example of regulatory approval of transgenic 'HoneySweet'(resistant to Plum pox virus (PPV)) in the U.S. (Scorza et al. 2013).

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Application of CRISPR technology for genome editing of citrus

Goal:

- Optimization of the CRISPR scissor for genome editing of citrus
- Disease resistance: HLB and canker
- Identification of right targets
- Non-transgenic, or Non-GMO
- Biallelic or homozygous mutation
- No effects on other horticultural traits
- No off-target

Huanglongbing (HLB, greening) *Candidatus* Liberibacter

Citrus canker (*Xanthomonas citri*)

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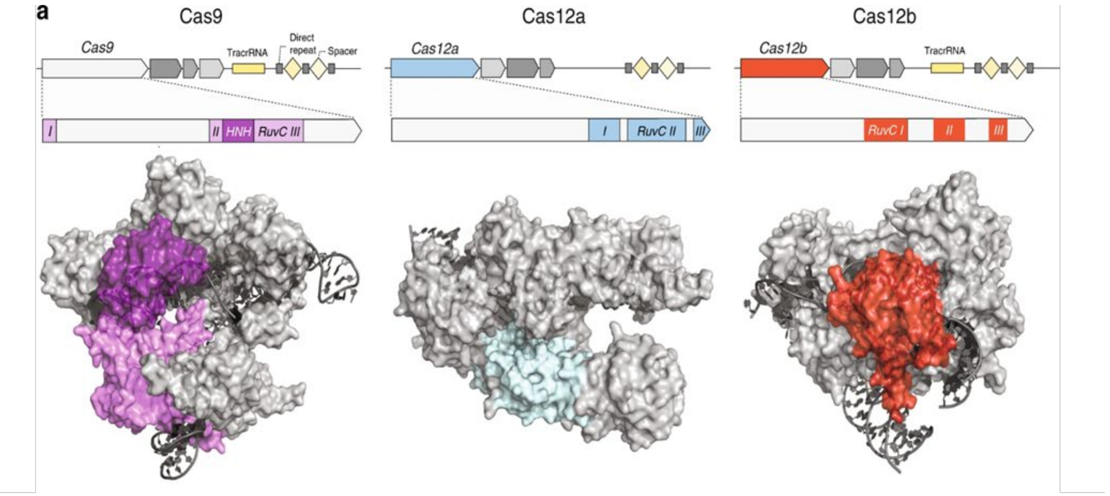
What CRISPR scissors are used on citrus?



Class 2, type II

Class 2, type V

Class 2, type V

Categories	Notes	Protein size (aa)	PAM	Guide RNA	Used on citrus
Cas9	*Streptococcus pyogenes*	1368	5' NGG	crRNA, tracrRNA	v
	Staphylococcus aureus	1053	5'NNGRRT		v
	Streptococcus thermophilus	1388	5'NNGNG		
	Neisseria meningitidis	1081	5'NNNNGATT		
	Campylobacter jejuni	984	5'NNNNACAC		
Cas12a (Cpf1)	*Acidaminococcus sp.*	1307	5' TTTV	crRNA	
	Francisella novicida	1300			
	Lachnospiraceae bacterium	1246			v
Cas12b	*Bacillus hisashii*	1108	T-rich	crRNA, tracrRNA	
Prime editing	a Cas9 H840A nickase fused to a reverse transcriptase, and a prime editing guide RNA (pegRNA)				

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Susceptibility genes/target genes

- Plant genes that facilitate infection and support compatibility are referred to here as susceptibility genes. Specifically, we define HLB susceptibility genes as genes responsible for promoting Las growth in plant or causing HLB symptoms.

- *CsLOB1* is the canker susceptibility gene (Hu et al. 2014; Jia et al. 2016, 2017)

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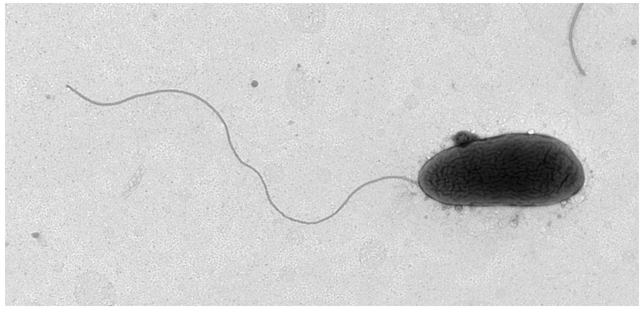
Generating canker resistant citrus varieties

- Resistance can be generated by editing the promoter region or the coding region of the susceptibility gene *CsLOB1*.

CsLOB1



Xanthomonas citri

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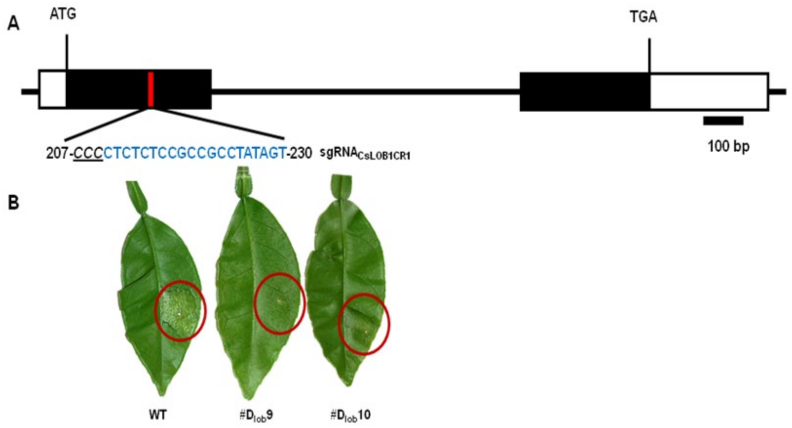
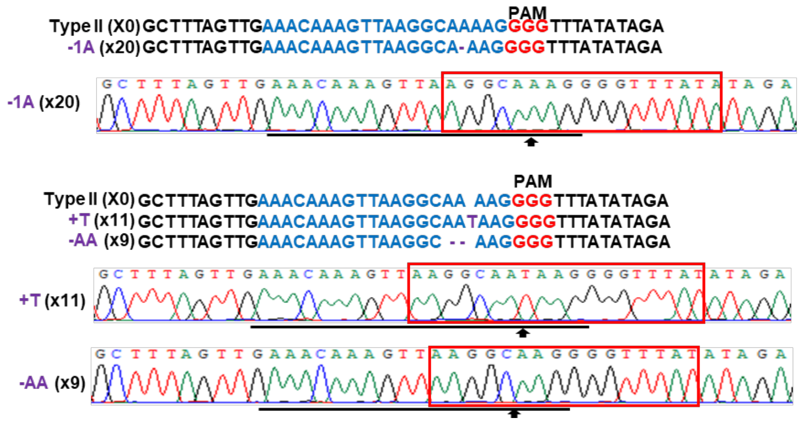
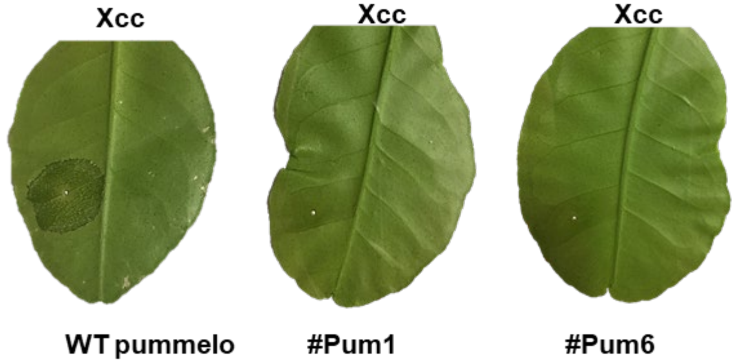
CsLOB1





Homozygous mutation

Biallelic mutation



Duncan grapefruit, 89.36%, coding region

Homozygous canker-resistant citrus, 100%, T0 generation, Pummelo , promoter region

Gene editing the promoter region and coding region of the canker susceptibility gene CsLOB1 generates canker resistant citrus.

Homozygous or biallelic mutants with high efficiency (44%) in the T0 generation

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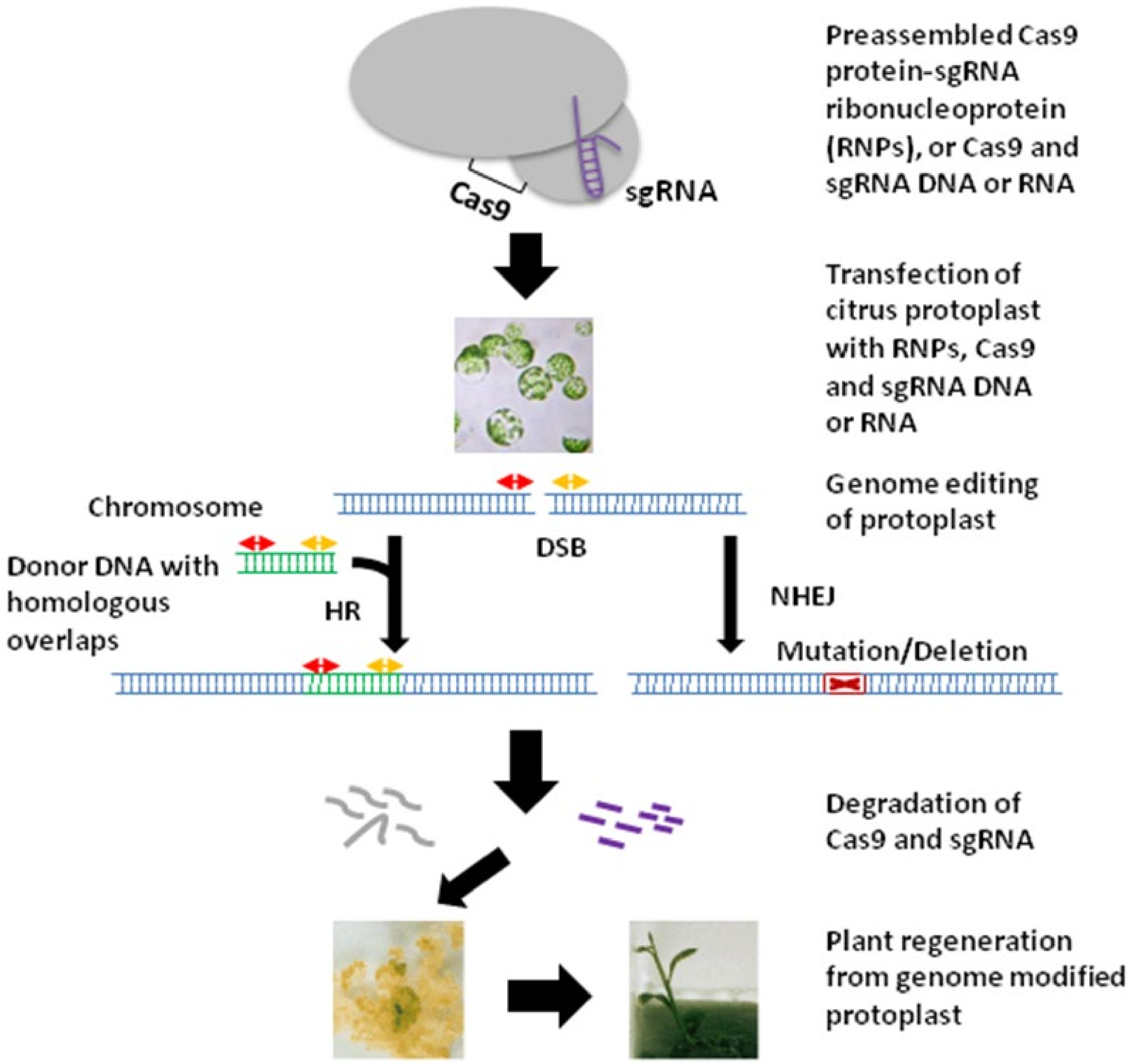
Towards Non-GMO genome editing of citrus



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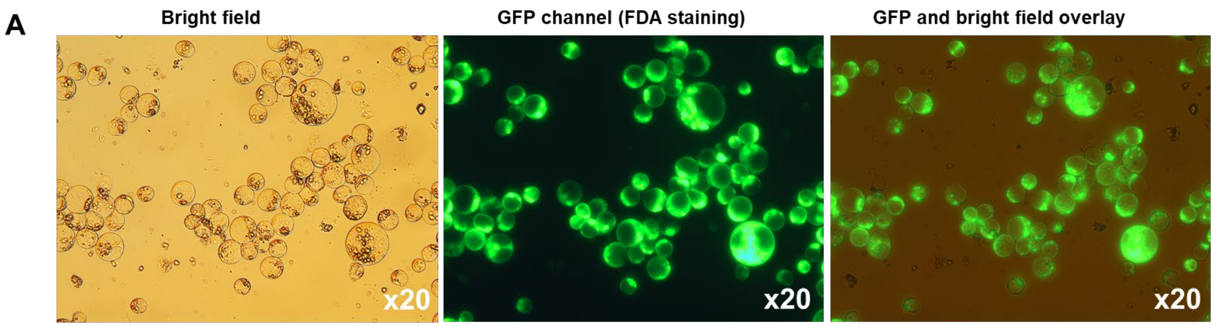
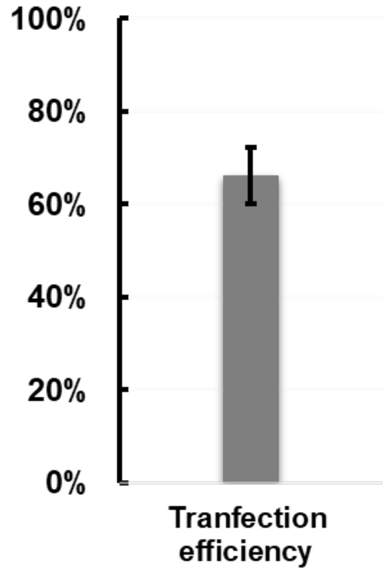
Towards Non-GMO genome editing of citrus

- We optimized citrus protoplast isolation and manipulation. More than 98% of the isolated protoplasts were alive.
- We routinely obtained a transfection efficiency of approximately 66% or above.
- Sweet orange U6 promoters were identified to drive a gRNA, achieved a higher editing efficacy than AtU6.
- tRNA-based multiplex genome editing constructs can not only introduce small InDels, but also create large deletions.





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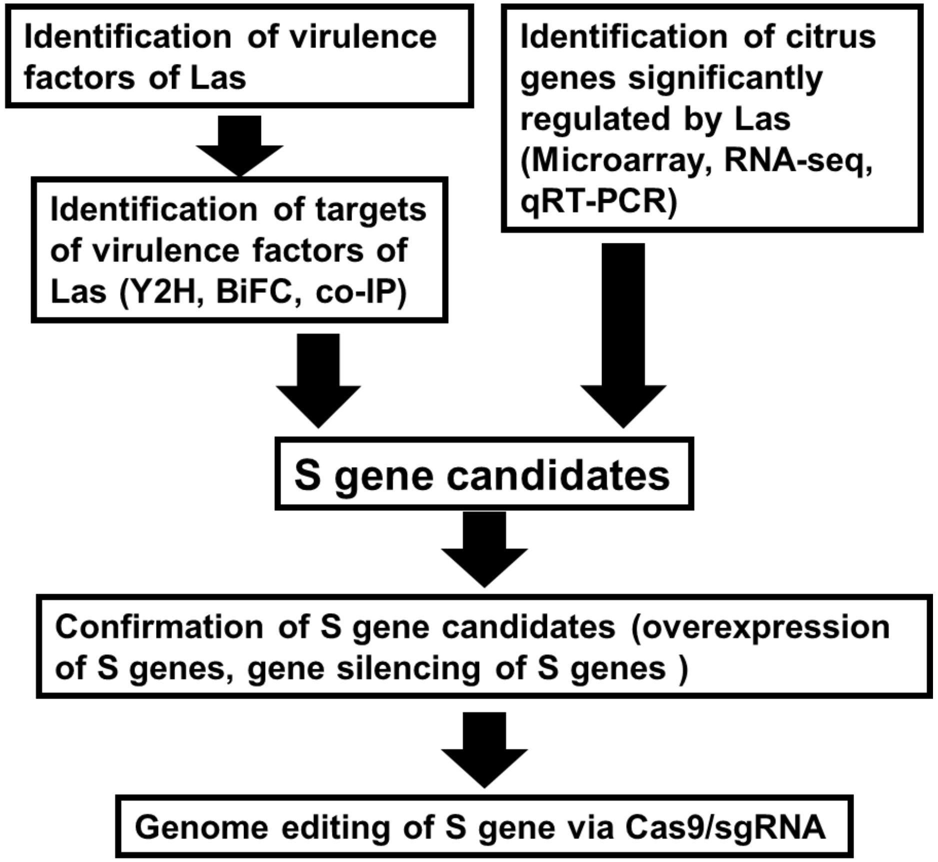
Generating HLB resistant citrus varieties using the CRISPR technology

- Characterization of CLas effector SDE1 found that it directly interacts with and inhibits the activity of members of the papain-like cysteine protease (PLCP) family that likely contribute to HLB resistance.

- Papain-like cysteine protease (PLCP): overexpression using Las inducible promoters.

- ACD2, a negative regulator of plant immunity: mutation of promoter or binding sites with Las effectors.



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To speed up the process…

- Early and frequent consults with APHIS, EPA, FDA
- Last meeting: July 13, 2020

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Summary

- The CRISPR scissors are ready for citrus!
- Canker resistant citrus varieties were successfully generated using the CRISPR technology.
- Ongoing with HLB

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CRDF

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