Julius Kühn-Institut
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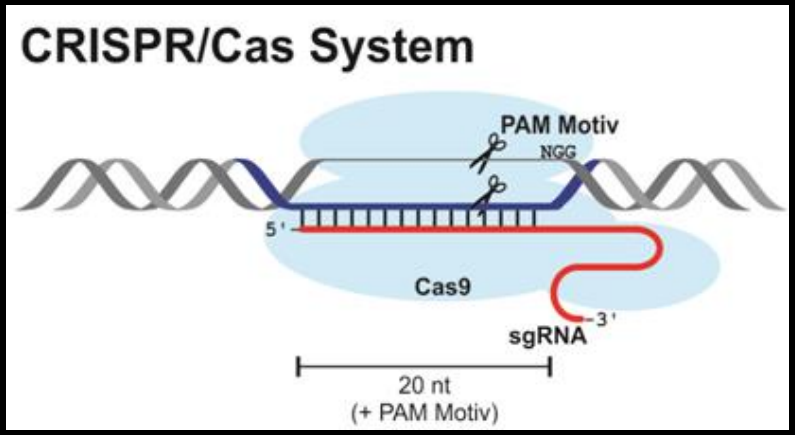


Fauser & Puchta, 2014

Application of CRISPR/Cas in Grapevine – Potentials and Problems

04.09.2018

Oliver Trapp

Institute for Grapevine Breeding Geilweilerhof

Origin of the main grapevine pests

Powdery Mildew

Phylloxera

Downy Mildew

Viticulture on around 100.000 ha in Germany (equals ~1% of agriculture area)

Production of 9-10 mio. hl wine per year (5,8 in Rhineland-Palatinate)

Export of 1 million hl with a value of around 300 mio. €

Portion of fungicides used in the EU for viticulture: 60-70%

→ Breeding for resistance

Breeding for resistance in grapevine

Vitis vinifera

no resistance

X

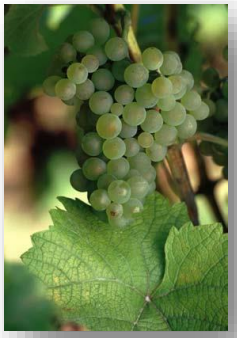
Wild Vitis species

high resistance

F1

pBC1

New variety



Problems with new varieties

Since 1992 35 new fungus tolerant varieties were protected/registered in Germany

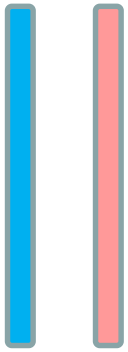
Acreage total: ~3.000 ha (3% of viticultural area)

Consumers buy according to the variety name

	Variety	Acreage in 2017 in ha
1.	Riesling, Weißer	23.809
2.	Müller-Thurgau	12.397
3.	Spätburgunder, Blauer	11.767
4.	Dornfelder	7.649
5.	Ruländer	6.402
6.	Burgunder, Weißer	5.334
7.	Silvaner, Grüner	4.853
8.	Portugieser, Blauer	2.956
9.	Kerner	2.591
10.	Trollinger, Blauer	2.194
(…)		
14.	Regent	1.811
(…)		
36.	Solaris	147

Limitations of resistance breeding

Grapevine is **diploid** and highly **heterozygous**:



A genetic locus is **heterozygous** if there are different **alleles** present in an organism

Heterozygous advantage is the base of the heterosis effect:

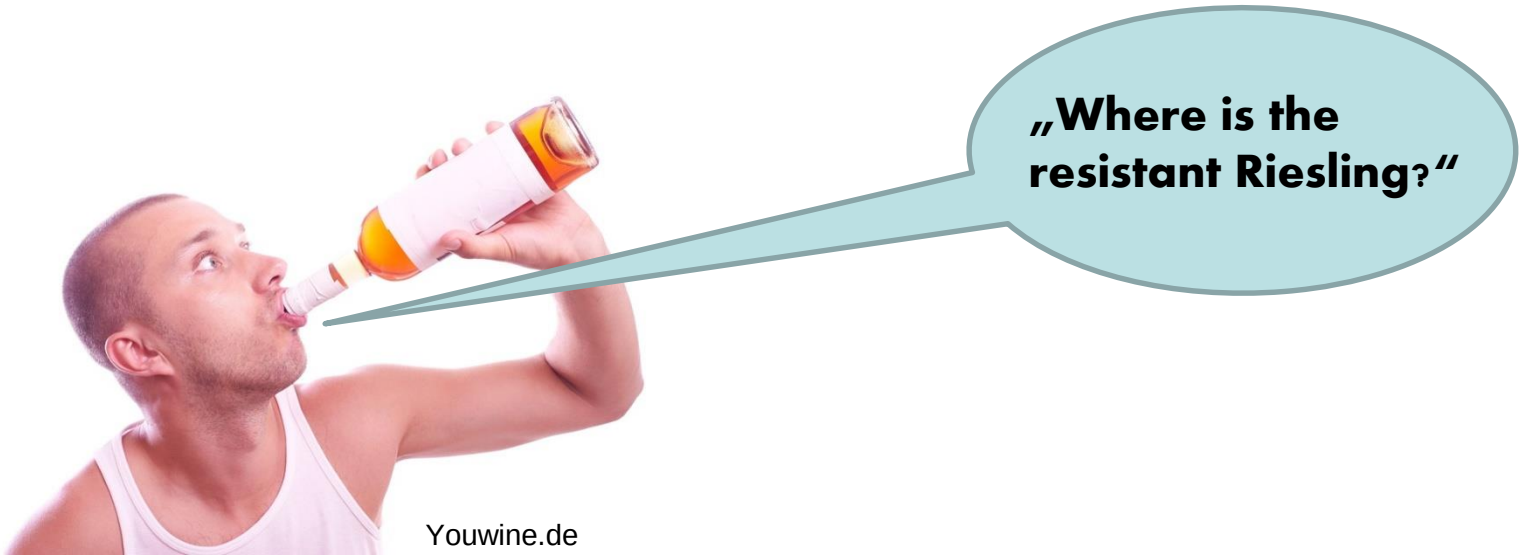
E.g. hybrid maize:

Limitations of resistance breeding

The combination of alleles defines a variety

Every crossing mixes the alleles of the parents in a new way
(Cross with Riesling will not results in new „Rieslings“)

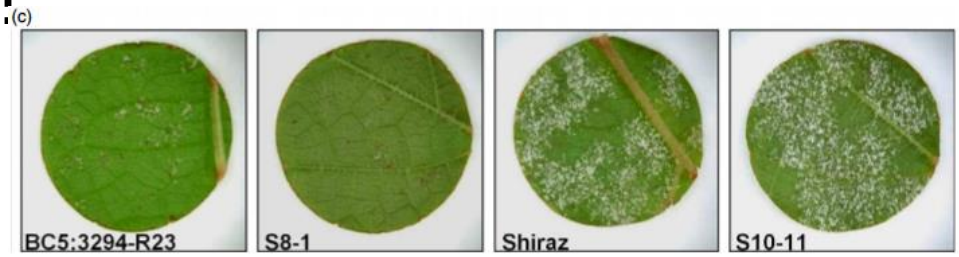
(Vegetative propagation of grapevines)



Youwine.de

The Solution: Biotechnology

1) Introduction of resistance genes:



Feechan *et al.,* 2013

Drawbacks: Not enough knowledge about most genes responsible for resistances
Transgenic plants

2) Mutagenesis:

Via chemicals, radiation or Site-Directed Nucleases (*CRISPR/Cas9*)

Drawbacks: Not enough knowledge about genes responsible for **susceptibility**
Transgenic plants (*CRISPR/Cas9*)

Decision of the European Court of Justice

- Plants created by CRISPR/Cas9 have to be treated as GMOs (other mutagenesis methods: no GMOs)

  → Lengthy and expensive approval process

- No acceptance for a GMO CRISPR/Cas9-created variety in society

- “I can’t see (how) CRISPR–Cas9 and all these new technologies will be profitable in the European Union. I can’t see this happening. I think this research will move somewhere else.” (Kai Purnhagen, Nature **560**, 16 (2018))

- Severly reduced funding for research?

Genome Editing by CRISPR/Cas9

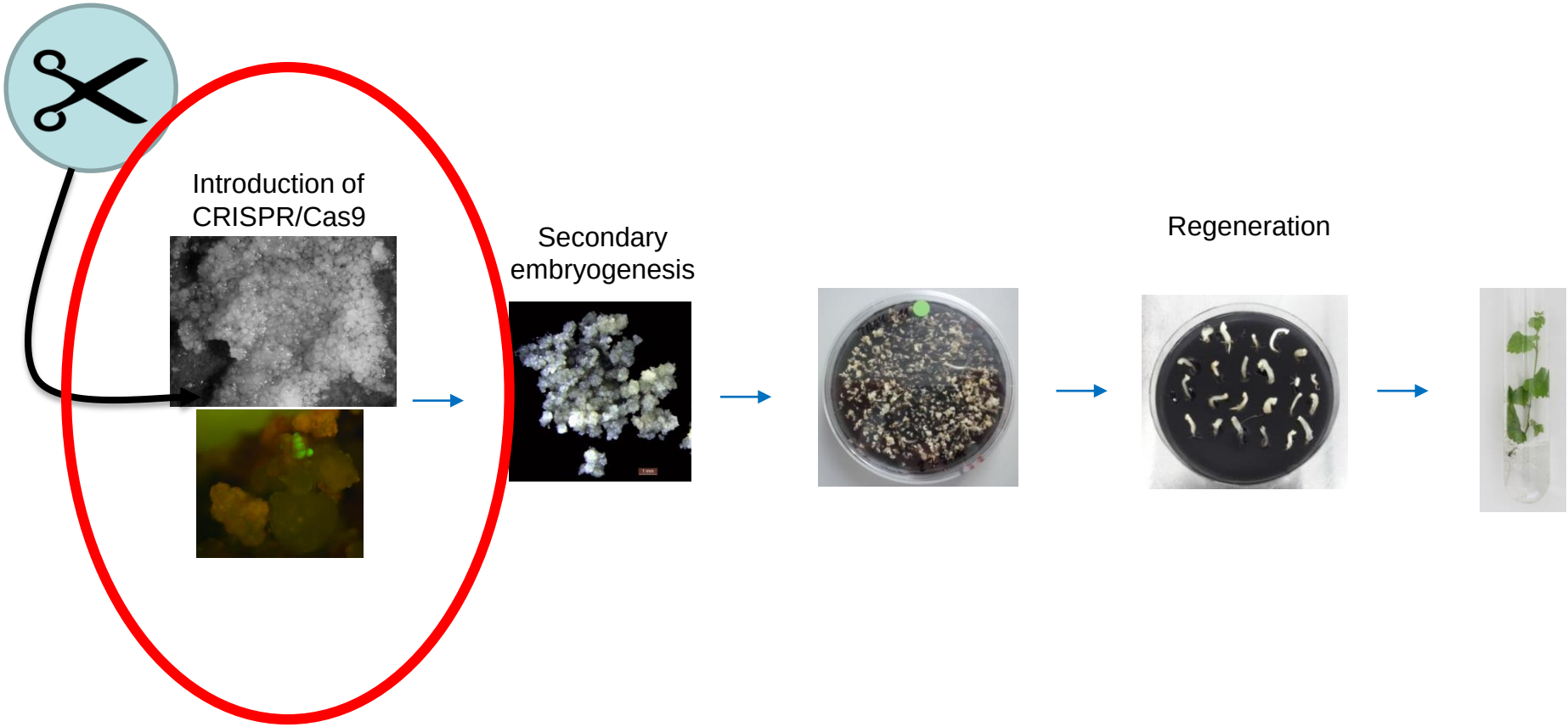
- Method relies on the cell´s DNA repair mechanisms
- Changes are not distinguishable from natural mutations
- In grapevine: clonal selections harbor more mutations than CRISPR would introduce

Three clones of Pinot Noir:

Photo: Antes

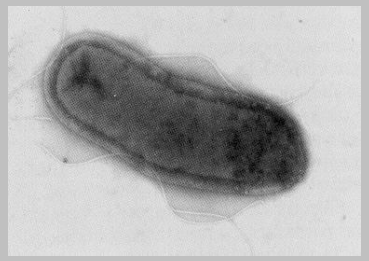
Photos: Robert Richter

Workflow for grapevine



Options for the application of CRISPR/Cas9

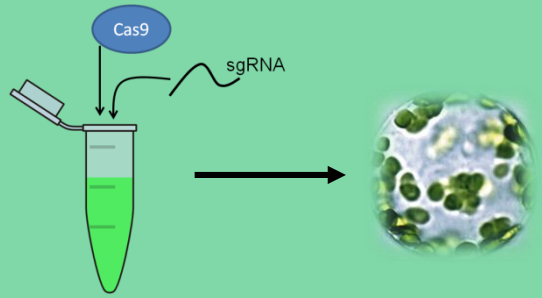
- **Stable transformation** of the CRISPR/Cas9 genes via Agrobacterium



- *Offtarget*-activity
- High efficiency

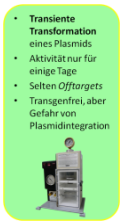
- **Transient transformation** of a plasmid
- Activity for a few days
- Rare *Offtargets*
- Usually transgene-free

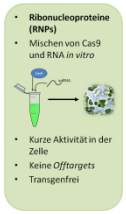
- **Ribonucleo-proteins (RNPs)**
- Mixing Cas9 and RNA *in vitro*



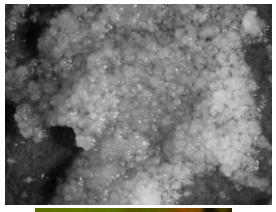
- Short activity in the cell
- No *Offtargets*
- Transgene-free

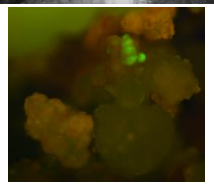
Limitations in grapevine – Transformation & Regeneration





Introduction of CRISPR/Cas9





Secondary embryogenesis

Regeneration

Efficiency of introduction: ?? (<1%?)

Mutagenesis efficiency*: 0,1 – 10%

1 cell cluster → 10 seedlings

Healthy seedlings: 20%

Time: ~12 Months

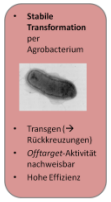
→ *Genome Editing* efficiencies will be very low
(~1 edited plant per 5000 cell masses via RNPs)

*: transient transformation or RNPs

What was done with CRISPR/Cas9 in grapevine?

„CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay „ (Ren et al, 2016)

- Stable transformation
- L-Idonat Dehydrogenase (IdnDH) as target → change in tartaric acid



Vector	No. of obtained CMs	No. of CMs with T-DNA	No. of examined CMs	No. of CMs with mutation	Mutation rate in CMs (%)	No. of regenerated plants	No. of plants with T-DNA	No. of plants with mutation	Mutation rate in plants (%)
AtU6-(None)-CaMV35S-Cas9	62	27	10	0	0.0	4	1	0	0.0
AtU6-sgRNA1-CaMV35S-Cas9	58	21	10	10	100.0	6	3	3	100.0
AtU6-sgRNA2-CaMV35S-Cas9	15	3	3	1	33.3	0	0	0	0.0

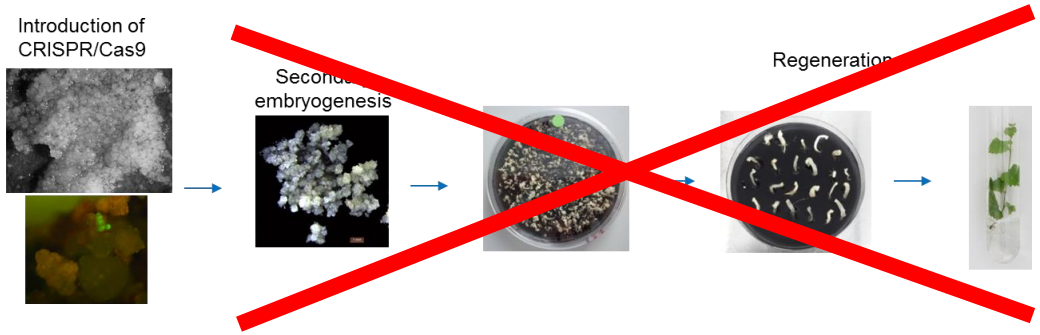
Outcome: 3 edited plants (GMOs) out of 58 cell masses

What was done with CRISPR/Cas9 in grapevine?

„DNA-free genetically edited grapevine (…) protoplasts using CRISPR/Cas9 RNPs“ (Malnoy et al, 2017)

- Introduction of RNPs into Chardonnay
- MLO-7 as target (putative susceptibility gene)

Target gene	Sample name	Number of Reads (more than minimum frequency)			Number of insertion mutations			Number of deletion mutations			Indel ratio (average, %)
		1	2	3	1	2	3	1	2	3	
MLO-7 (RG4, grape)	sgRNA only	56302	52455	54565	0	0	0	0	0	0	0.00
	Cas9 only	9924	10123	10001	0	0	0	0	0	0	0.00
	Cas9: sgRNA, 1:1	51558	52015	52206	0	0	0	49	55	64	0.10
	Cas9; sgRNA, 1:3	56546	55432	56421	2	4	6	71	74	69	0.10
	Cas9; sgRNA, 3:1	67286	64532	66876	42	57	68	10	12	9	0.10



What was done with CRISPR/Cas9 in grapevine?

CRISPR/Cas9-mediated targeted mutagenesis in grape (Nakajima et al., 2017)

- Stable transformation into cultivar Neo Muscat
- Target was Phytoene Desaturase (PDS) leading to loss of chlorophyll

- **Stable transformation** of the CRISPR/Cas9 genes via Agrobacterium

- *Offtarget*-activity
- High efficiency

Table 3. Emergence rate of mutated plants via embryo induction method.

Target	Agrobacterium infected calli	Regenerated plants	Regenerated plants with chlorophyll deficiency	Ratio of plants with chlorophyll deficiency (%)
PDS-t2 (Exp.2)	21	29	8	27.6
PDS-t2 (Exp.3)	58	31	1	3.2
PDS-t3 (Exp.3)	72	18	2	11.1

→ Efficiency was quite high, however…

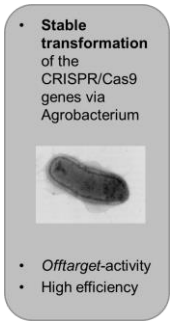
…however: Only chimeric plants were regenerated:

What was done with CRISPR/Cas9 in grapevine?

Scienza Biotechnologies work in progress (Giacomelli et al., 2018):

Targeting different susceptibility genes with a stable transformation approach:

- DMR6_1, DMR6_2
- DLO1, DLO2, DLO3
- MLO7



Most candidates found by their homology to Arabidopsis

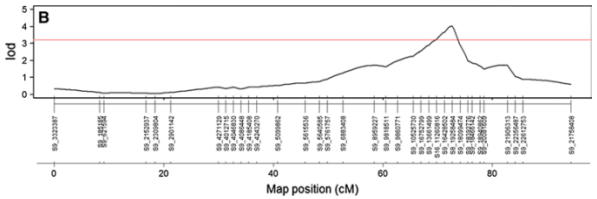
DMR6_1: 480 regenerated plants of which 29 are completely edited

currently being screened for resistance

Limitations of grapevine - genomic targets?

Susceptibility loci:

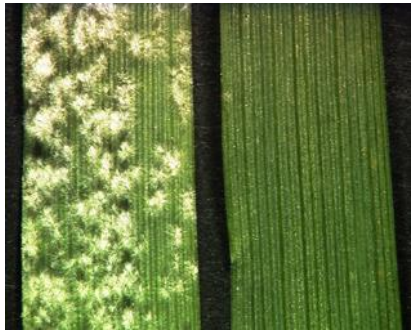
Only one known so far:
Sen1 (Barba et al., 2014)
Size approx. 1Mb



Susceptibility gene *MLO*:

1997 first characterized in barley.
Mutation → Powdery Mildew resistance

But: In grapevine there seem to be around 19 MLO genes present



→ Lack of knowledge for good susceptibility genes

Comparison of biotechnological approaches:

Transformation of resistance genes	Mutagenesis by CRISPR/Cas9 (transient or RNPs)
GMO	GMO
Insertion of DNA	No cis-/transgene insertion
Insertion not defined	Well defined change in the genome (No offtargets with RNPs)
Some knowledge about resistance genes	Almost no knowledge about susceptibility genes
Higher efficiency	Lower efficiency

- Need for better introduction/transformation methods and better regeneration protocols

Low efficiencies in the introduction of CRISPR/Cas9 and the regeneration of plants together with the **lack of knowledge** for good susceptibility genes hinder CRISPR/Cas9-based mutagenesis in grapevine, although it is the **„cleaner“** method.
Especially when the resulting plants will be GMOs.

New breeding technologies vs classical breeding

State of the art in classical breeding:

- 3 resistance genes against each: downy and powdery mildew in current seedlings
- Creation of locus specific homozygous lines for higher throughput when searching for new varieties of a certain type
- New varieties open new possibilities to deal with climate change

Calardis blanc

Thank you for your attention!

Combining resistances