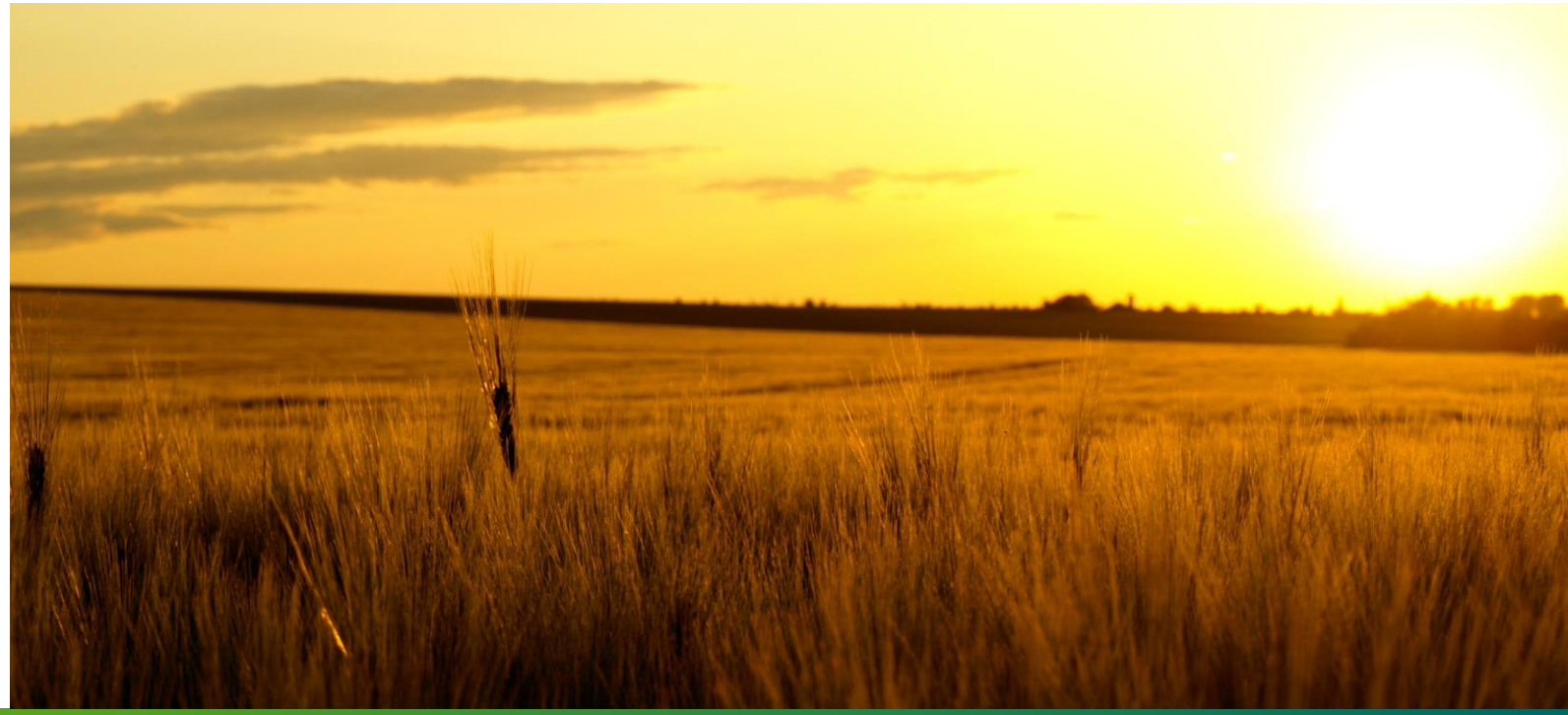
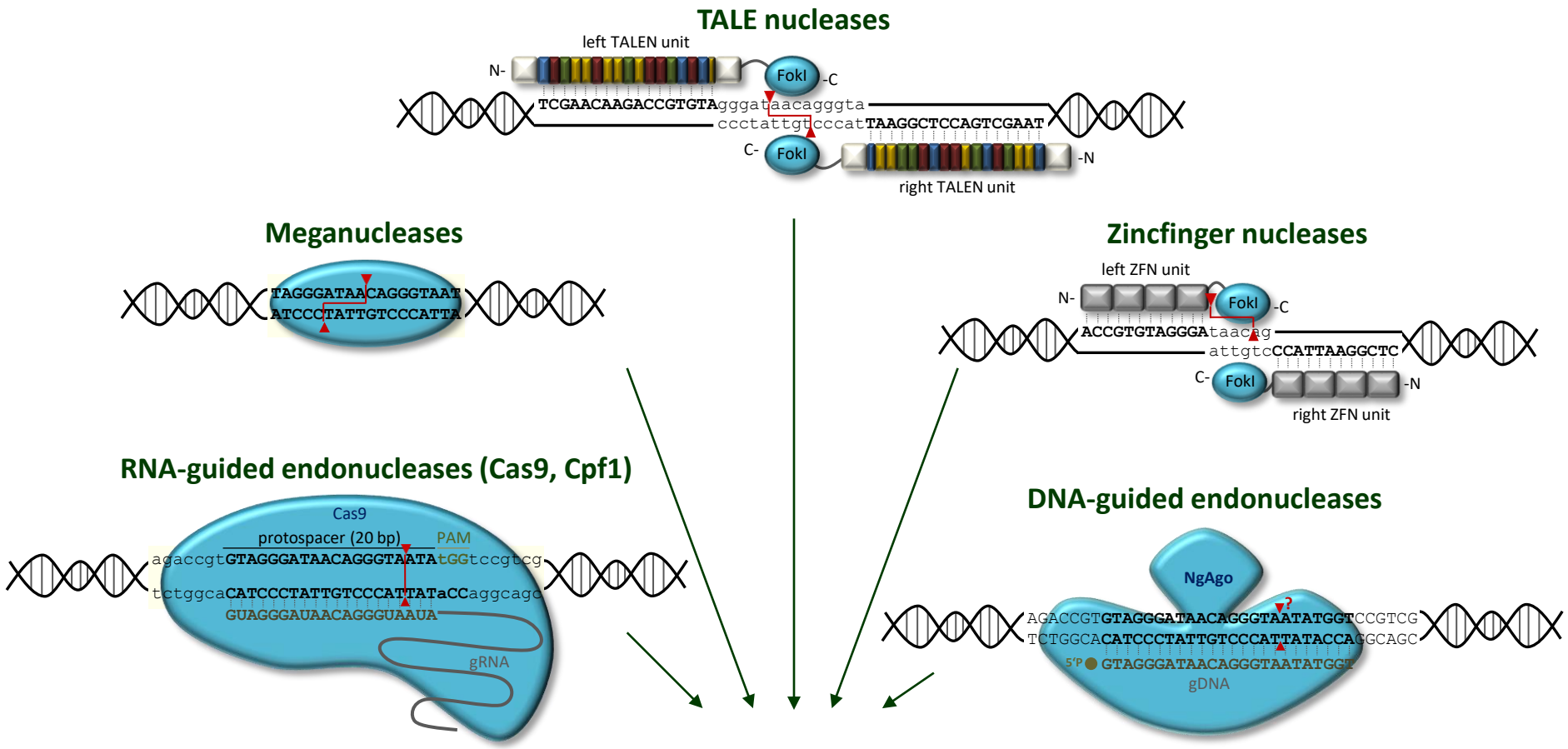
Gene Editing – Gezielte Mutationen für die Resistenzzüchtung



Einleitung

ZNFs, TALEN, CRISPR

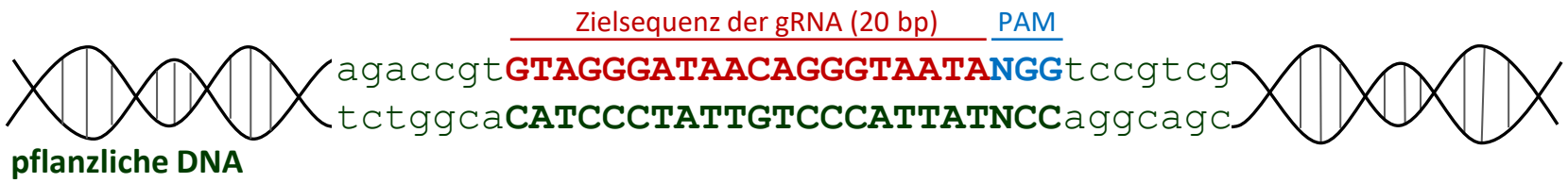
Zielgerichtete Mutagenese

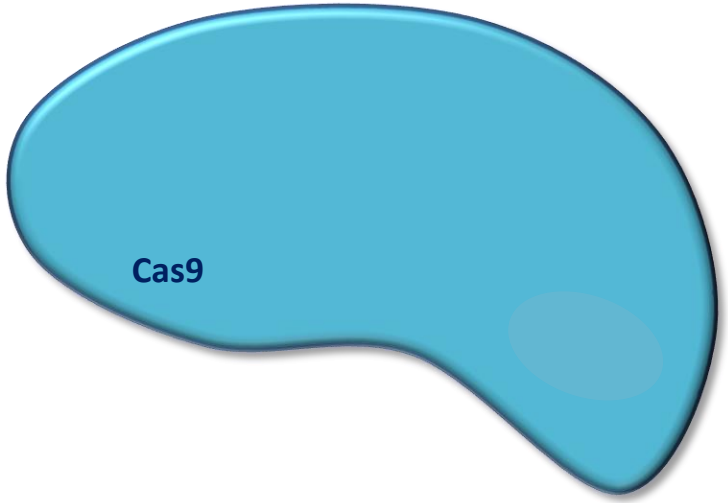


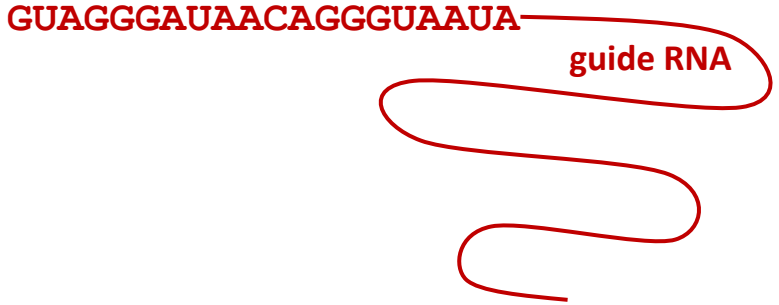
RNA-vermittelte Cas-Endonuklease: “Gen-Schere”

- entstammt einem bakteriellen Immunsystem

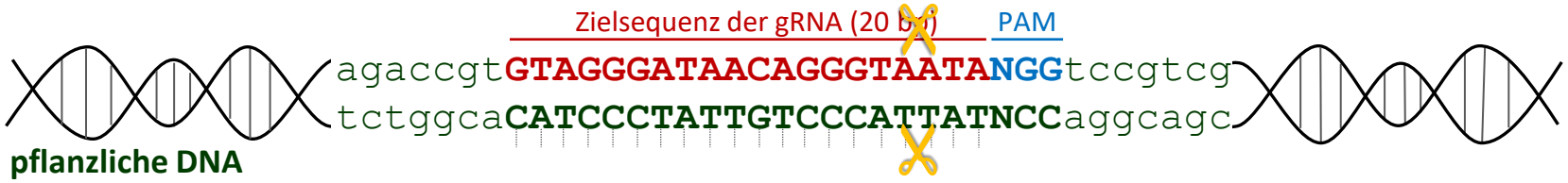
(“CRISPR-Cas”: clustered regularly interspaced short palindromic repeats – CRISPR associated proteins)

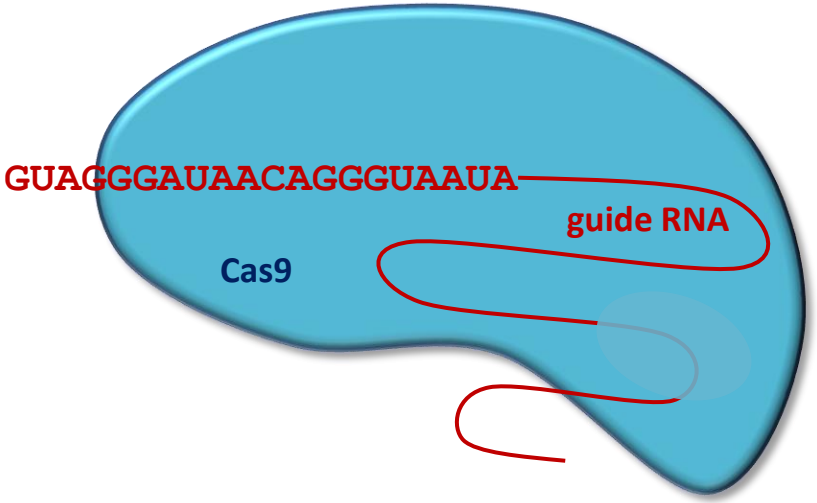




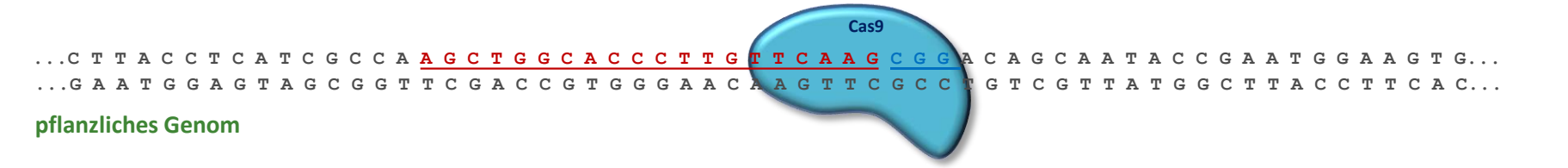


RNA-vermittelte Cas-Endonuklease: “Gen-Schere”





Gezielte Mutagenese



DNA Reparatur

zumeist korrekt

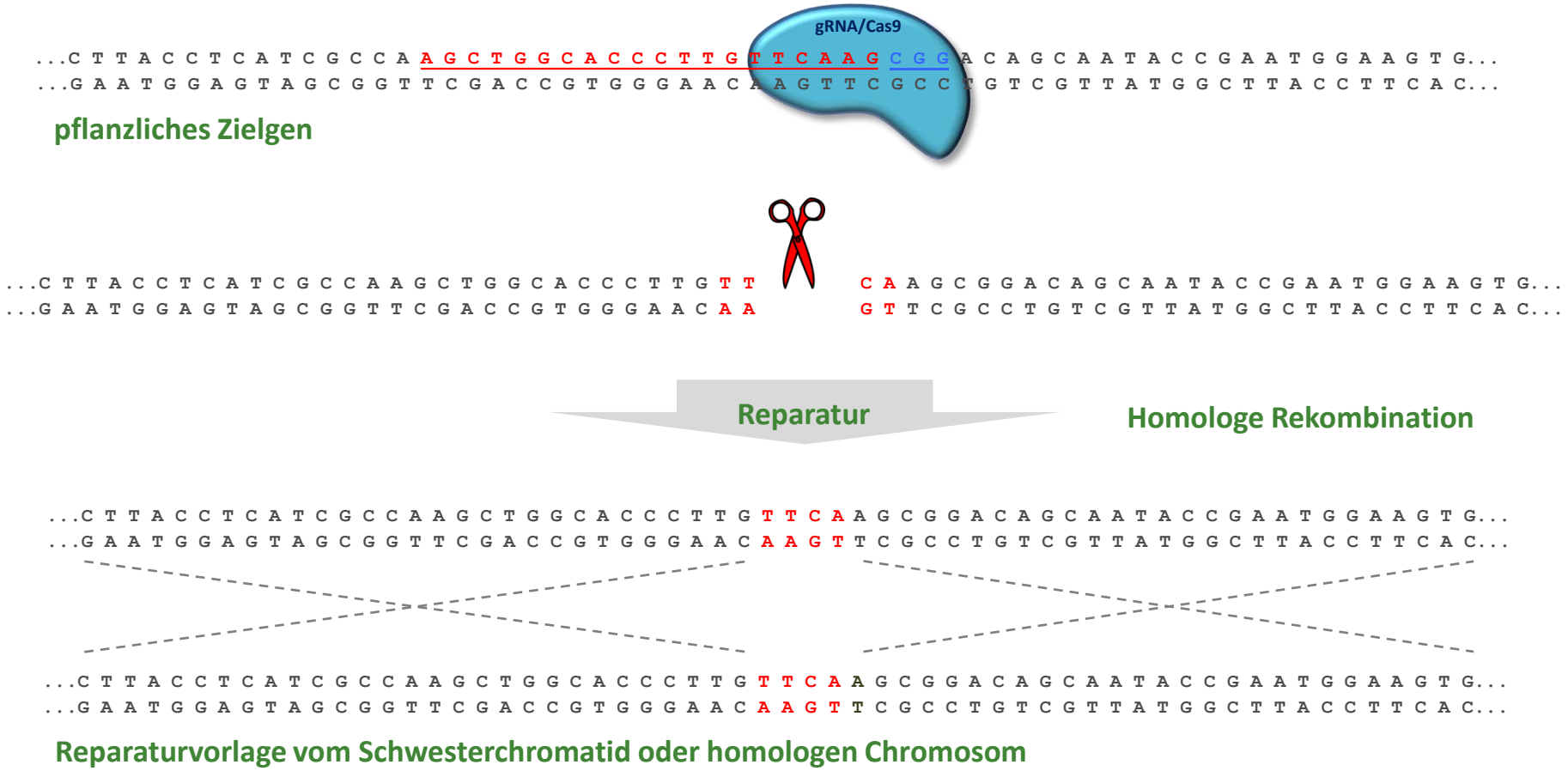
```
...CTTACCTCATCGCCAAGCTGGCACCCTTGTTCAAGCGGACAGCAATACCGAATGGAAGTG...
...GAATGGAGTAGCGGTTCGACCGTGGGAACAAGTTCGCCTGTCGTTATGGCTTACCTTCAC...
```

manchmal fehlerhaft

```
...CTTACCTCATCGCCAAGCTGGCACCCTTGTT-AAGCGGACAGCAATACCGAATGGAAGTG...
...GAATGGAGTAGCGGTTCGACCGTGGGAACAA-TTCGCCTGTCGTTATGGCTTACCTTCAC...
```

➡ Zufällige Veränderung an vorgegebener Position in der DNA

Präzise Editierung: Homologie



Präzise Editierung: Homologie

```
...CTTACCTCATCGCCAAGCTGGCACCCTTGTTCAAGCGGACAGCAATACCGAATGGAAGTG...
...GAATGGAGTAGCGGTTCGACCGTGGGAACAAGTTCGCCTGTCGTTATGGCTTACCTTCAC...
```

pflanzliches Zielgen

```
...CTTACCTCATCGCCAAGCTGGCACCCTTGTT      CAAGCGGACAGCAATACCGAATGGAAGTG...
...GAATGGAGTAGCGGTTCGACCGTGGGAACAA      GTTCGCCTGTCGTTATGGCTTACCTTCAC...
```

Reparatur

homologe Rekombination

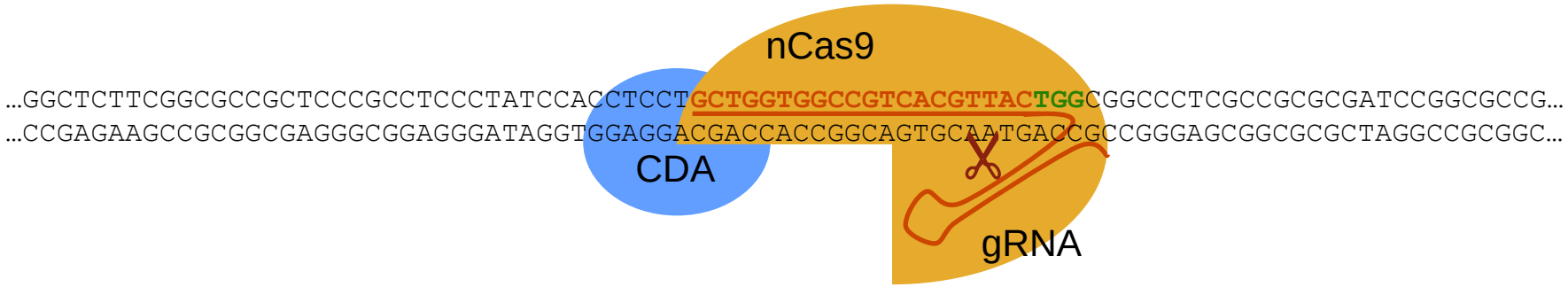
```
...CTTACCTCATCGCCAAGCTGGCACCCTTGGAGAAGCGGACAGCAATACCGAATGGAAGTG...
...GAATGGAGTAGCGGTTCGACCGTGGGAACCTCTTCGCCTGTCGTTATGGCTTACCTTCAC...

...CTTACCTCATCGCCAAGCTGGCACCCTTGGAGAAGCGGACAGCAATACCGAATGGAAGTG...
...GAATGGAGTAGCGGTTCGACCGTGGGAACCTCTTCGCCTGTCGTTATGGCTTACCTTCAC...
```

Reparaturvorlage mit Modifikation der Wahl

➡ **Vorgegebene Veränderung an vorgegebener Position**

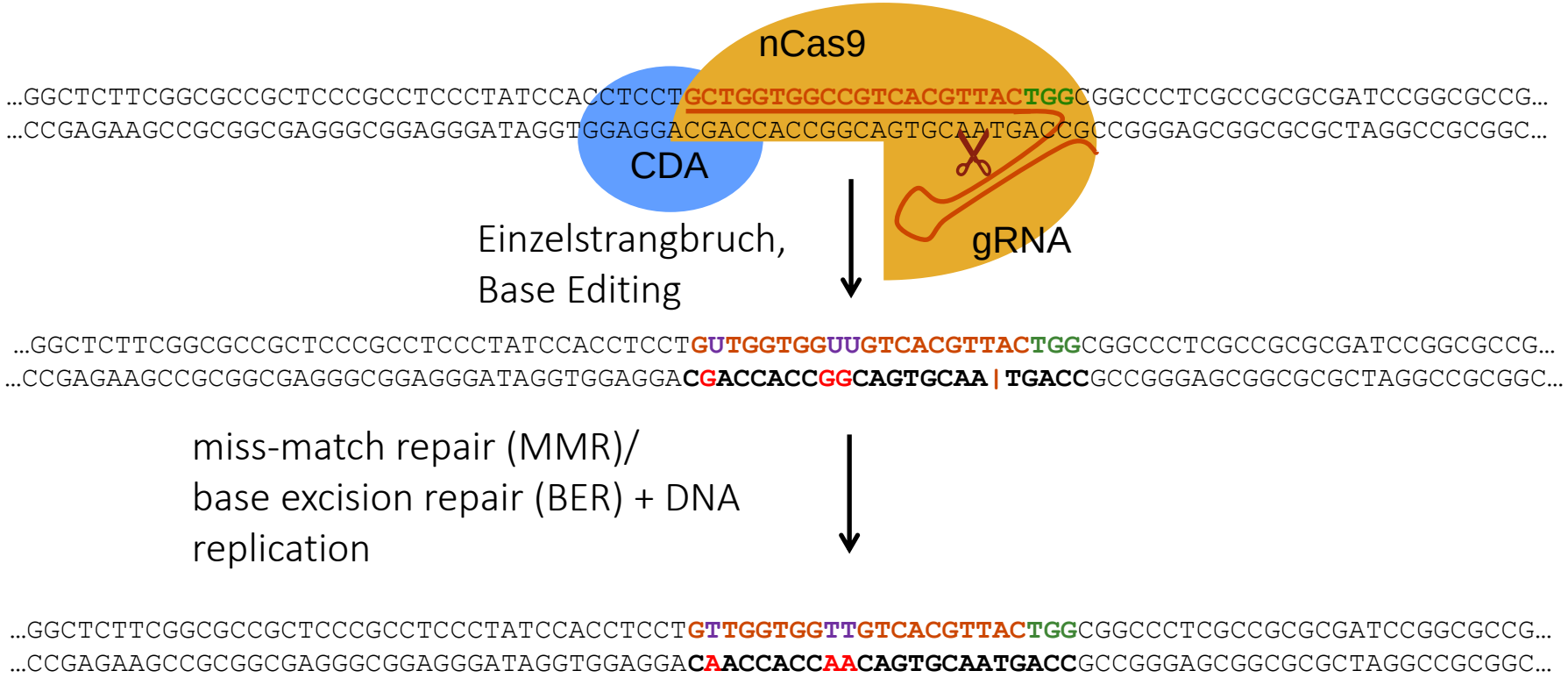
Präzise Editierung: Base Editing



Base editing

- nCas9: Nickase (nur eine funktionale Endonuklease-Domäne)
- CDA: Cytidin Deaminase (C ⇨ T)
- ADA: Adenin Deaminase (A ⇨ G)

Präzise Editierung: Base Editing



➡ **Single Nucleotide Polymorphisms (SNPs) an definierten Positionen**

Mutationen für die Resistenzzüchtung

Beispiel Virusresistenz

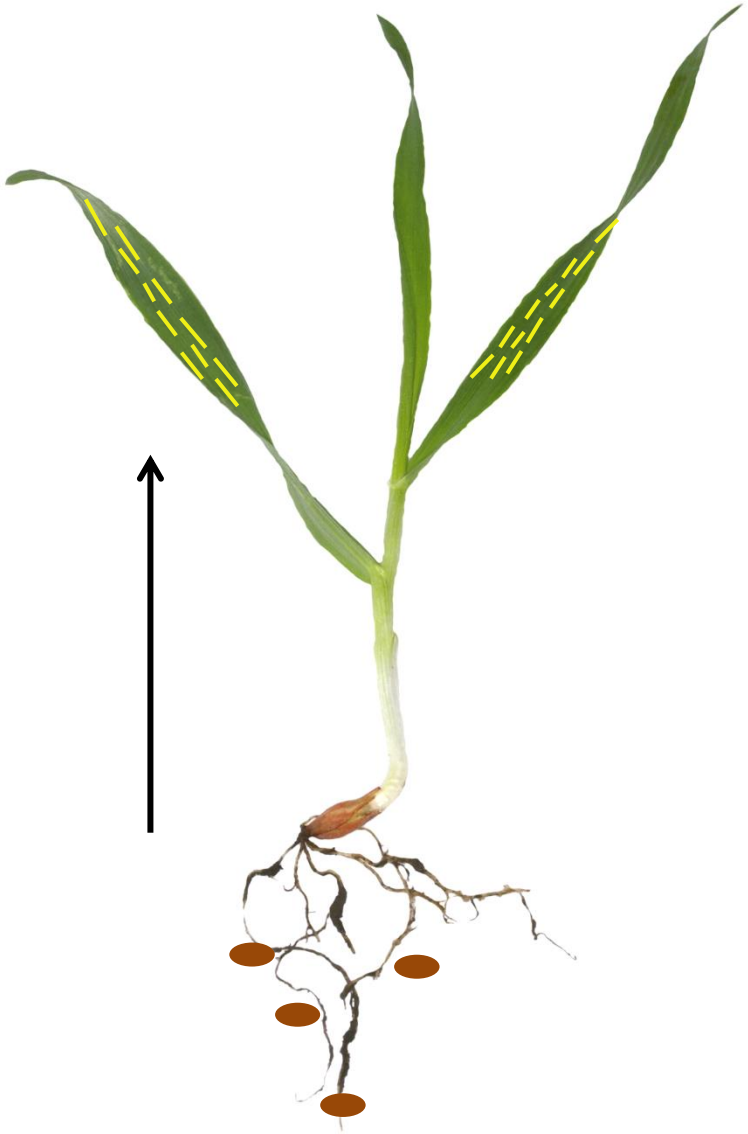
Bymoviren in Gerste

Symptome:
- Gelbe Mosaike auf den Blättern

Später:
- Reduzierte Winterhärte
- Blattnekrosen
- Ertragsverlust bis zu 50 %

Virus verteilt sich in der Pflanze (Zell-zu-Zell, Langstreckentransport)

Polymyxa graminis als Vektor von BaYMV/ BaMMV infiziert die Wurzeln



Barley Yellow Mosaic Virus (BaYMV 1/2)
Barley Mild Mosaic Virus (BaMMV)

- ss(+)RNA-Virus
- Potyviridae, Bymovirus

- Übertragung durch Wurzelinfektion mit Vektor *Polymyxa graminis*

Bekannte Resistenzgene

Gene und QTLs:

rym1-18 (meist rezessiv, Virusstamm-spezifisch)

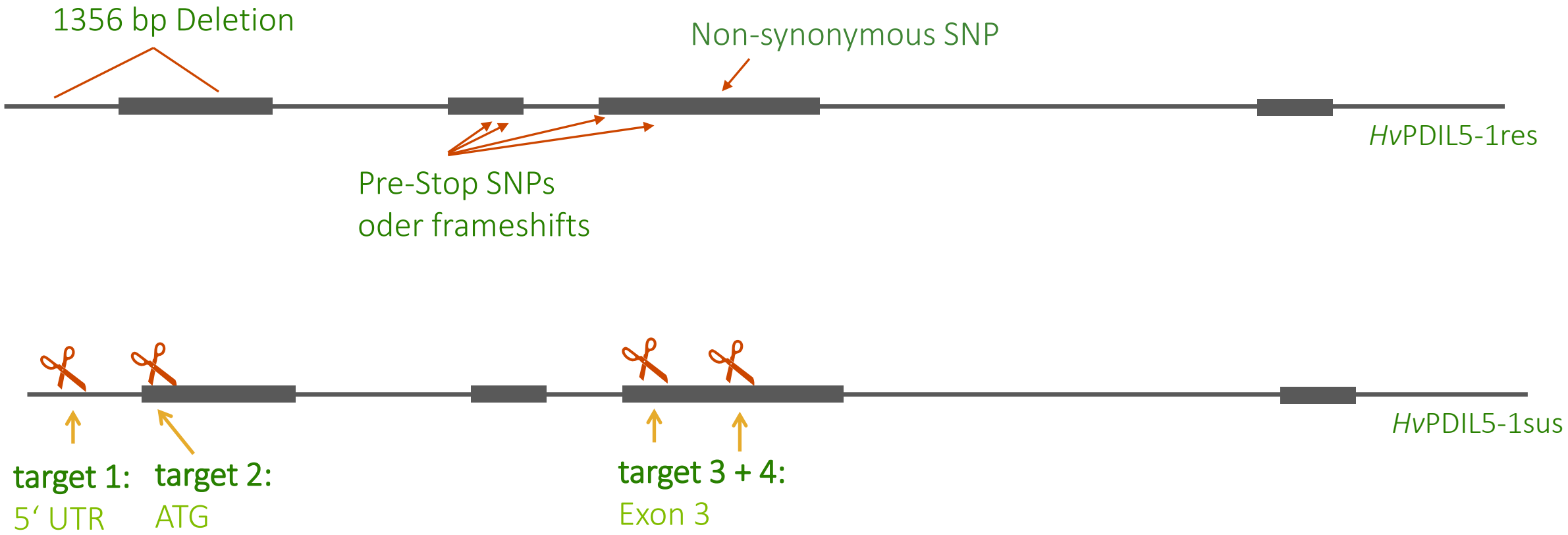
Identifizierte Gene:

rym1, rym11:
PROTEIN DISULFIDE ISOMERASE-LIKE 5-1 (PDIL5-1)

rym4, 5, 6, 10:
EUKARYOTIC TRANSLATION INITIATION FACTOR 4E

Reviewed in Jiang *et al*., 2020 *Theoretical and Applied Genetics*
rym4, 5: Stein *et al*., 2005 *The Plant Journal*
rym11: Yang *et al*., 2014 *PNAS*

PDIL5-1



Yang et al., 2014 (PNAS)

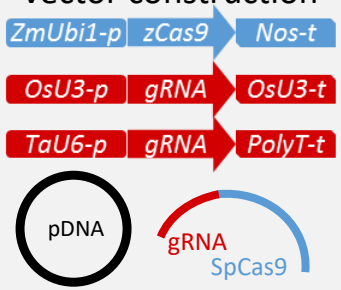
Hoffie et al., in revision

Workflow

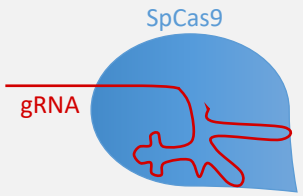
Selection of target motifs

- *Target/off-target selection:* CRISPR-P, WU-CRISPR, DESKGEN, CRISPOR
- *Test of gRNA 2-D structure:* mfold, RNAfold

Vector construction

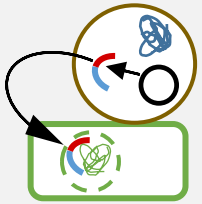


RNP assembly

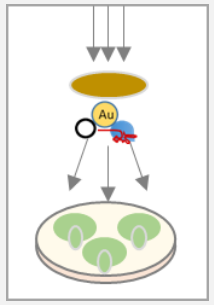


Transfer of endonuclease to embryogenic pollen

- DNA transfer by Agrobacterium



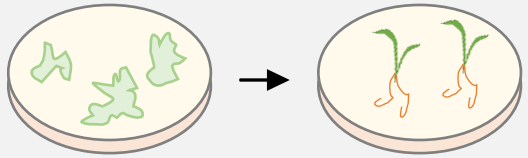
- Ballistic transfer of DNA or RNPs on gold particles



Test of gene expression and gRNA/Cas9 complex activity

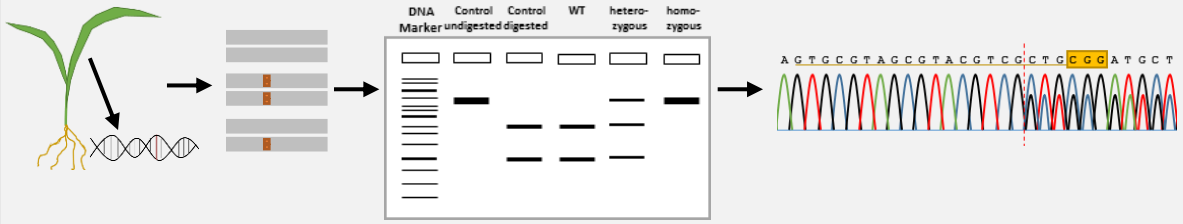
- *In vitro* cleavage assay
- Protoplast transformation and Amplicon sequencing
- Biolistic test of gRNA activity in leaves

Callus and plant formation

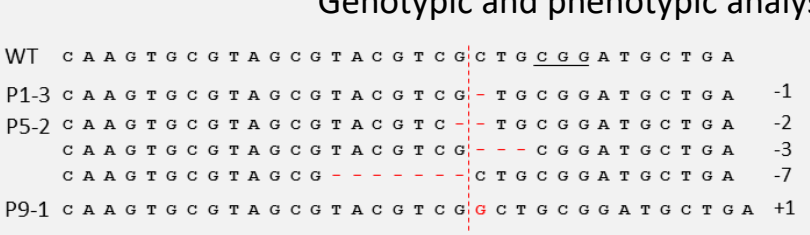


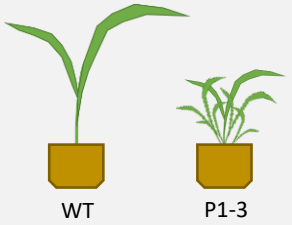
Mutant detection

DNA isolation | Amplification | PCR/RE, T7E1 or PCR/RNP | Sanger sequencing



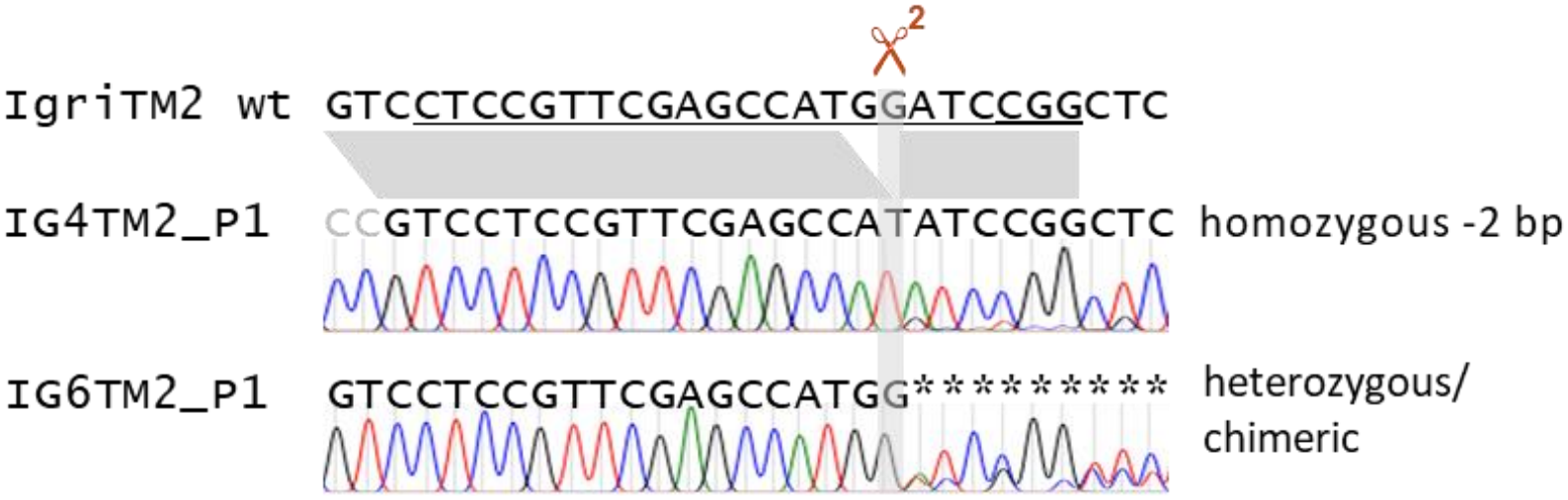
Genotypic and phenotypic analyses





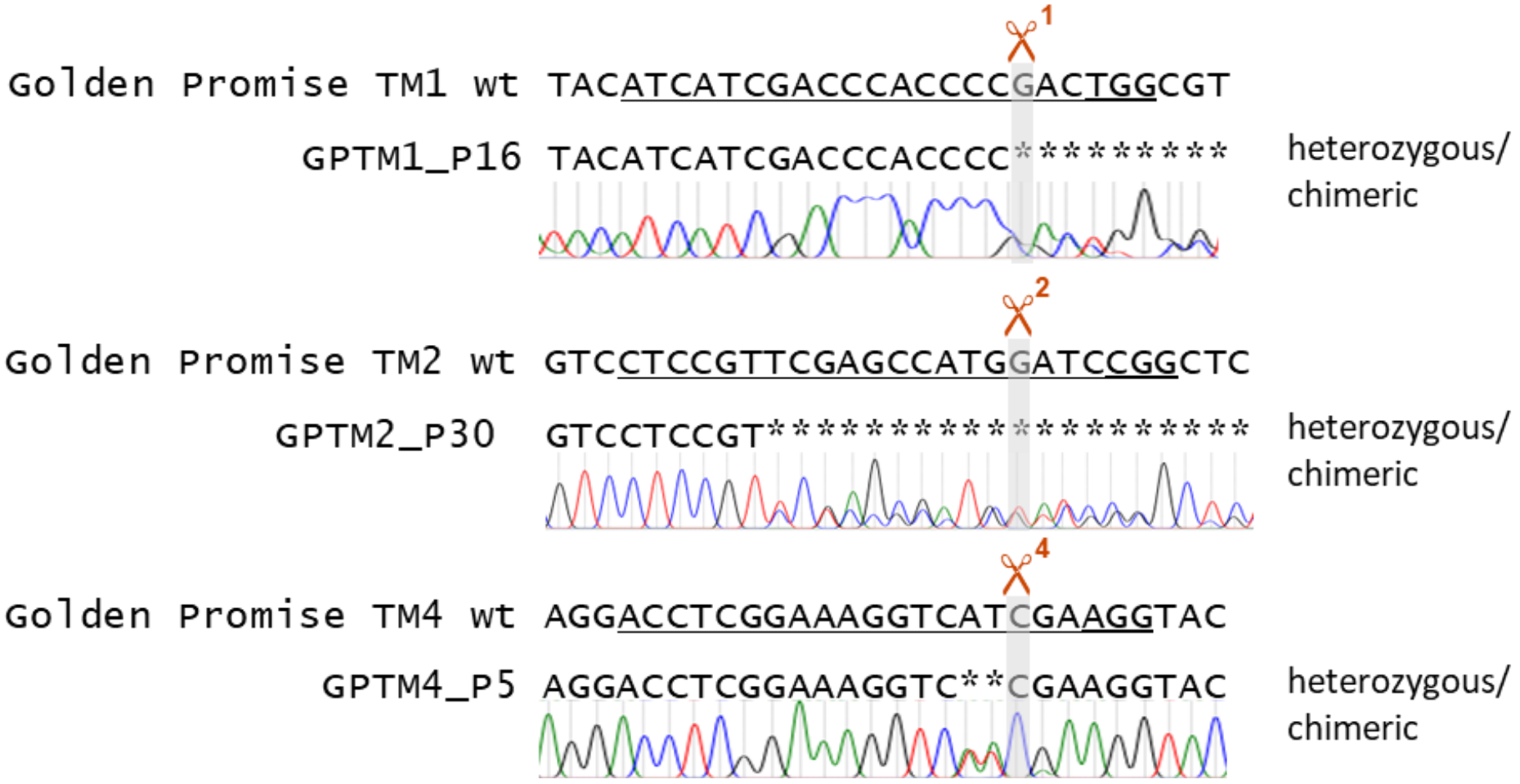
Koeppel *et al.*, 2019 *International Journal of Molecular Sciences*

PDIL5-1 Primärmutanten Igri



Hoffie et al., in revision

PDIL5-1 Primärmutanten Golden Promise



Hoffie et al., in revision

PDIL5-1 M_2 InDels

```
                                   ✂1
TACATCATCGACCCACCCCGACTGGCGTATCCGC     Golden Promise
TACATCATC----------------------CGC     GPTM1_P1_4
TACATCATCGACCCACCC-GACTGGCGTATCCGC     GPTM1_P16_12
TACATCATCGACCCACGCTAGTACTGCGCCGTATCCGC GPTM1_P16_20
```

```
                                                   ✂2
GTATCCGCCTTCTCCTCGTCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCGAGCCATGGATCCGGCTCTTCGGCGCC Golden Promise
GTATCCGCCTTCTCCTCGTCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCG---------------------GCGCC GPTM2_P22_4
GTATCCGCCTTCTCCCCGCCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCGAGCCATGG-TCCGGCTCTTCGGCGCC GPTM2_P30_10 allele1
GT-------------------------------------------------------------ATCCGGCTCTTCGGCGCC GPTM2_P30_10 allele2
```

```
                                  ✂4
AGGACCTCGGAAAGGTCATCGAAGGTAC  Golden Promise
AGGACCTCGGAAAGGTC--CGAAGGTAC  GPTM4_P14_14
AGGACCTCGGAAAGGTCATACGAAGGTAC GPTM4_P21_10
```

Hoffie et al., in revision

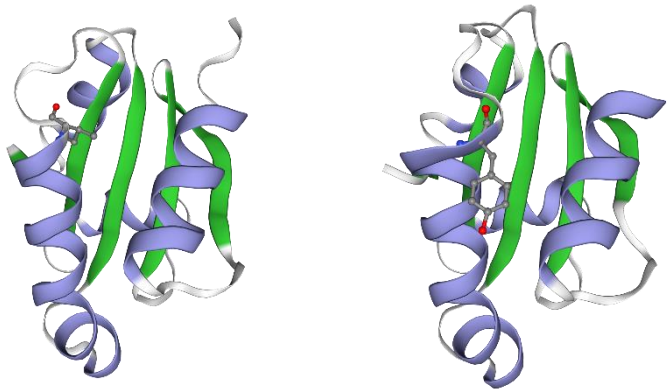
PDIL5-1_I79Y

```
                                                  4
GCAGCAAGAGCCTAGGAACTCTTTGGGAGGACCTCGGAAAGGTCATCGAAGGTAC   Golden Promise
GCAGCAAGAGCCTAGGAACTCTTTGGGAGGACCTCGGAAAGGTCTACGAAGGTAC   GPTM4_P5_9
GCAGCAAGAGCCTAGGAACTCTTTGGGAGGACCTCGGAAAGGTCTACGAAGGTAC   GPTM4_P5_11
GCAGCAAGAGCCTAGGAACTCTTTGGGAGGACCTCGGAAAGGTCTACGAAGGTAC   GPTM4_P5_12
GCAGCAAGAGCCTAGGAACTCTTTGGGAGGACCTCGGAAAGGTCTACGAAGGTAC   GPTM4_P5_18
GCAGCAAGAGCCTAGGAACTCTTTGGGAGGACCTCGGAAAGGTCTACGAAGGTAC   GPTM4_P5_19
```

Thioredoxin-like domain (TRX)

Active Center

	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	
Plants	W	F	V	Q	F	C	V	P	W	C	K	H	C	K	S	L	G	T	L	W	E	D	L	G	K	V	Y	E	G	T	D	*Hv*PDIL5-1_I79Y
	W	F	V	Q	F	C	V	P	W	C	K	H	C	K	S	L	G	T	L	W	E	D	L	G	K	V	I	E	G	T	D	*Hv*PDIL5-1_wild-type
	W	F	V	Q	F	C	V	P	W	C	K	H	C	K	S	L	G	T	L	W	E	D	L	G	K	V	I	E	G	T	D	*Ta*PDIL5-1
	W	F	V	Q	F	C	V	P	W	C	K	H	C	K	N	L	G	T	L	W	E	D	L	G	K	V	M	E	G	A	D	*Zm*PDIL5_1
	W	F	V	K	F	C	V	P	W	C	K	H	C	K	K	L	G	N	L	W	E	D	L	G	K	A	M	E	G	D	D	*At*PDIL5-1
Mammals	L	M	V	I	I	H	K	S	W	C	G	A	C	K	A	L	K	P	K	F	A	E	S	T	E	I	S	E	L	S	H	*Mm*TXNDC12
	L	M	V	I	I	H	K	S	W	C	G	A	C	K	A	L	K	P	K	F	A	E	S	T	E	I	S	E	L	S	H	*Hs*ERp18



HvPDIL5-1 wild-type HvPDIL5-1 I79Y

Hoffie et al., in revision

Resistenztests M_2

Mosaik-Symptome nach mechanischer Inokulation mit BaMMV in Phytokammer
Bild: Antje Habekuß, JKI

Infektion

- Mechanische Inokulierung von M_2 Sämlingen mit BaMMV

Phänotyp

- Bonitur der Symptome, ELISA Test

Genotyp

- Sequenzierung der Zielregion

Resistenztests M_2

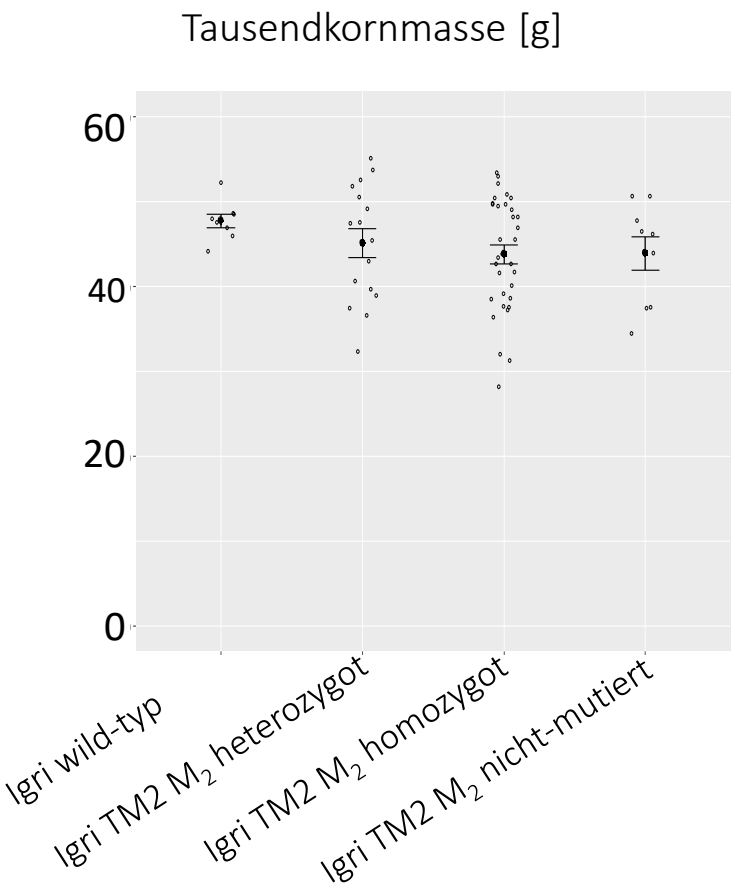
Nicht infiziert vs. infiziert

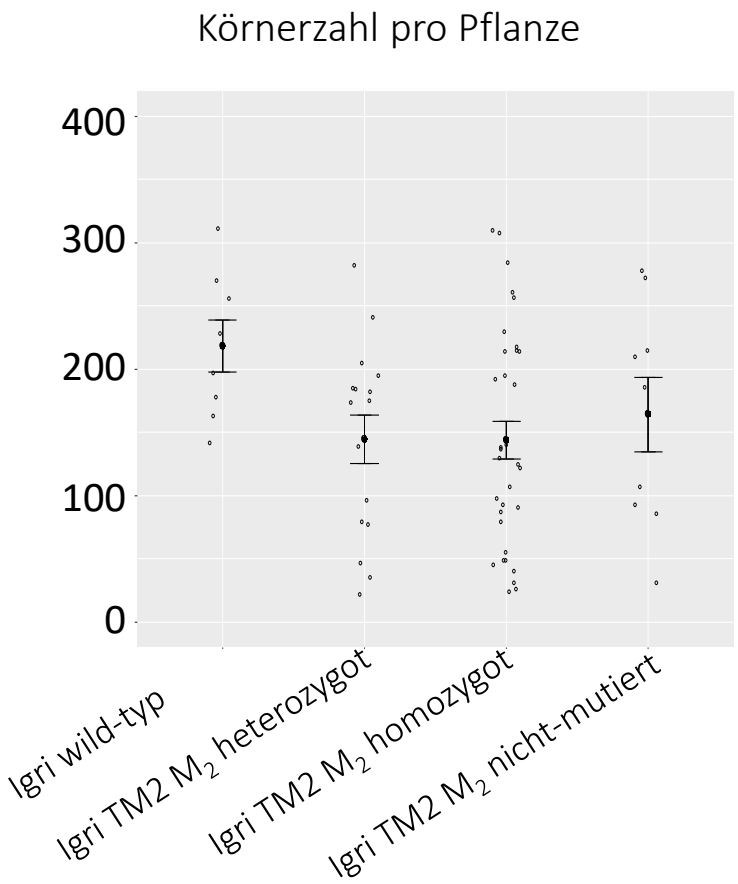
Plants		Phenotype	Genotype					
Primary mutant (M_1)	Progeny (M_2) tested	BaMMV-free (ELISA)	T-DNA-free plants	Sequenced plants	Non-mutated	Heterozygous mutants	Homozygous mutants	T-DNA-free mutants
Target Motif 2								
IG4TM2_P1	9	9	0	9	0	0	9	0
IG4TM2_P6	20	10	2	16	1	10	5	1
IG6TM2_P1	16	5	0	10	4	6	0	0
IG6TM2_P2	19	19	0	19	0	0	19	0
IG7TM2_P1	15	1	3	5	4	0	1	0
sum	79	44	5	59	9	16	34	1
wild-type								
Igri	10	1	10	6	6	0	0	-

Plants		Phenotype	Genotype					
Primary mutant (M_1)	Progeny (M_2)	BaMMV-free (ELISA)	T-DNA- free plants	Sequenced plants	Non-mutated	Heterozygous mutants	Homozygous mutants	T-DNA-free mutants
Target Motif 1								
GPTM1_P1	20	4	4	6	1	2	3	1
GPTM1_P7	19	0	3	3	0	3	0	1
GPTM1_P12	20	15	5	13	1	9	3	2
GPTM1_P16	17	15	2	9	0	5	4	2
GPTM1_P23	18	6	6	8	2	4	2	0
Target Motif 2								
GPTM2_P9	20	18	2	19	4	12	3	1
GPTM2_P11	20	6	4	8	0	3	5	3
GPTM2_P22	18	16	2	15	0	9	6	1
GPTM2_P30	18	7	3	5	1	4	0	0
GPTM2_P50	19	5	3	7	7	0	0	0
Target Motif 4								
GPTM4_P5	20	17	7	12	0	6	6	6
GPTM4_P11	19	17	4	9	1	8	0	1
GPTM4_P14	16	13	1	9	0	5	4	1
GPTM4_P21	12	9	1	8	0	5	3	1
sum	256	148	47	131	17	75	39	20
Wild-type								
Golden Promise	11	3	11	5	5	0	0	-

Hoffie et al., in revision

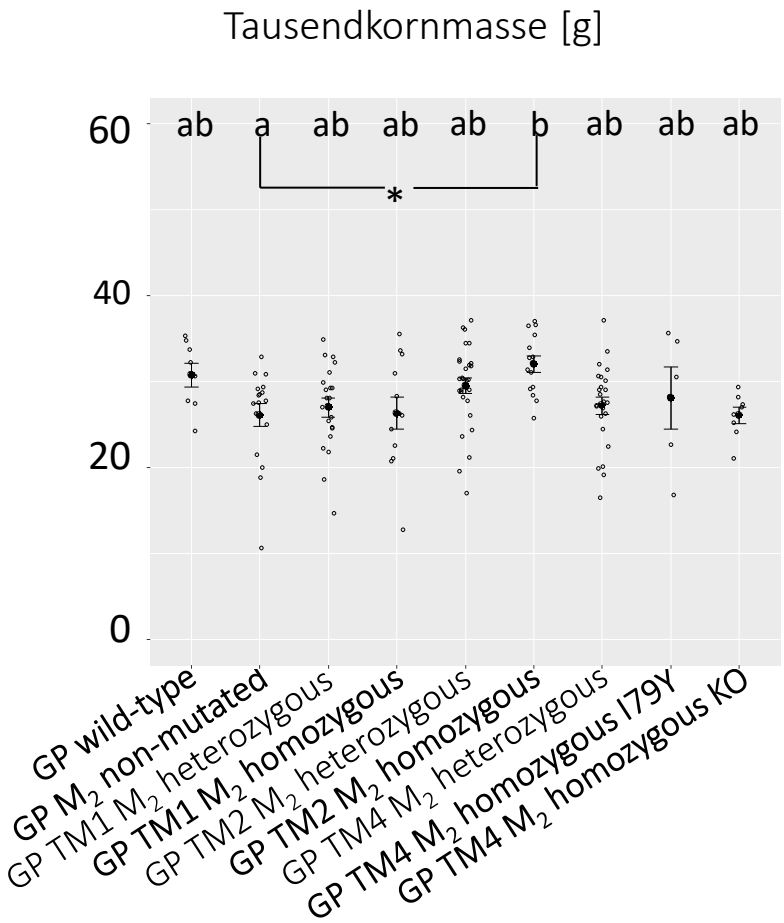
PDIL5-1 Ertragsparameter Igri

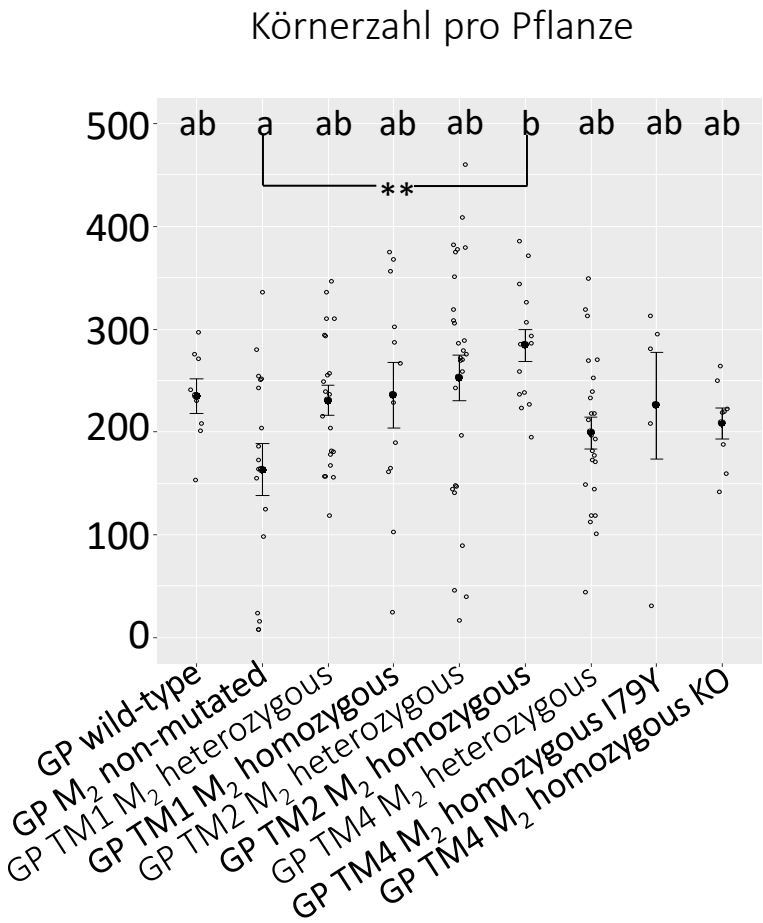




Hoffie et al., in revision

PDIL5-1 yield parameters Golden Promise





Hoffie et al., in revision

Zusammenfassung

Zusammenfassung

- Verschiedene Präzisionsstufen bei genetischen Veränderungen
- Verschiedene PDIL5-1-Allele erzeugt, die zu Virusresistenz führen
- Wissen über Gen(funktionen) erforderlich
- *In-vitro*-Kultur genotypenabhängig
- Monogene Resistenzen, Entkopplung von Eigenschaften, Pyramidisierung
- Besonders geeignet für Allele aus “exotischem” Zuchtmaterial oder Pflanzen mit hoher Heterozygotie oder langer Generationszeit

Acknowledgement

Ag Pflanzliche Reproduktionsbiologie

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Ingrid Otto
Iris Hoffie
Nicole Schäfer
Pooja Satpathy
Pouneh Pouramini
Sabine Sommerfeld
Stefan Hiekel

GIVE **GENES** A CHANCE !

Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen
Federal Research Centre for Cultivated Plants

Dr. Dragan Perovic, Antje Habekuß,
Ingrid Dubsky, Katy Niedung

Bundesministerium für Bildung und Forschung

PLANT 2030

Vielen Dank für Ihre Aufmerksamkeit!