

CRISPR/Cas9 with ribonucleoprotein complexes allows highly efficient marker-free editing approaches in *Botrytis cinerea* and other fungi

Matthias Hahn

The grey mold fungus *Botrytis cinerea*

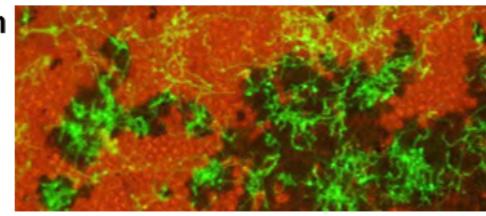


- Major cause of fruit, vegetable and flower rots
- Wide host range
- Abundant sporulation
- 'High risk' pathogen for fungicide resistance

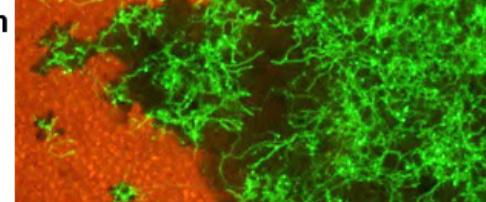


B. cinerea on tomato leaf

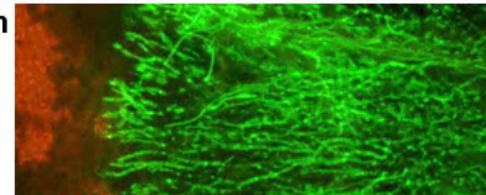
18 h



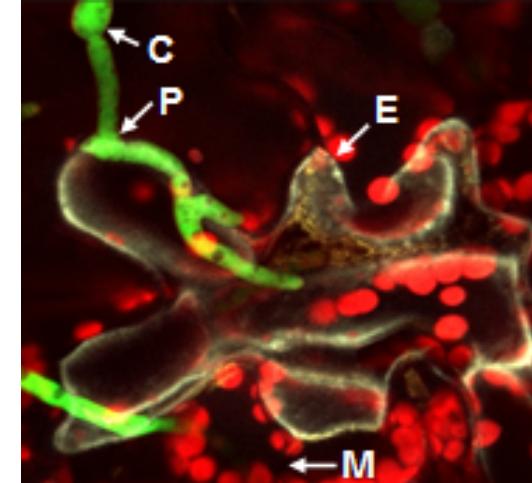
24 h



36 h



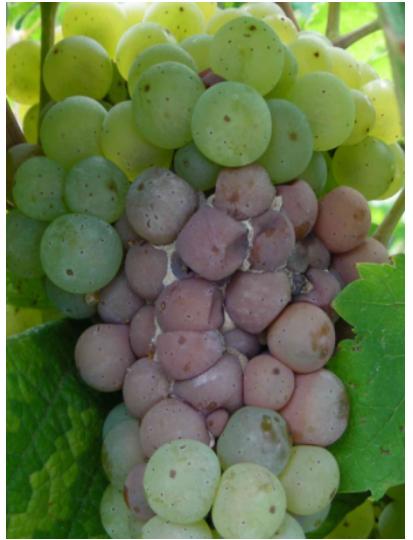
... inducing HR in Arabidopsis



Fungicides against *Botrytis*

Compound	Target	Resistance risk
Fenhexamid	Sterole biosynthesis (3-ketoreductase)	medium
Boscalid, Fluopyram (SDHI)	Respiration (complex II)	high to medium
Cyprodinil, Pyrimethanil (AP)	Mitochondrial function	medium*
Fludioxonil	Osmoregulation	low*

* Risk for efflux-based partial resistance (MDR)



Grapevine

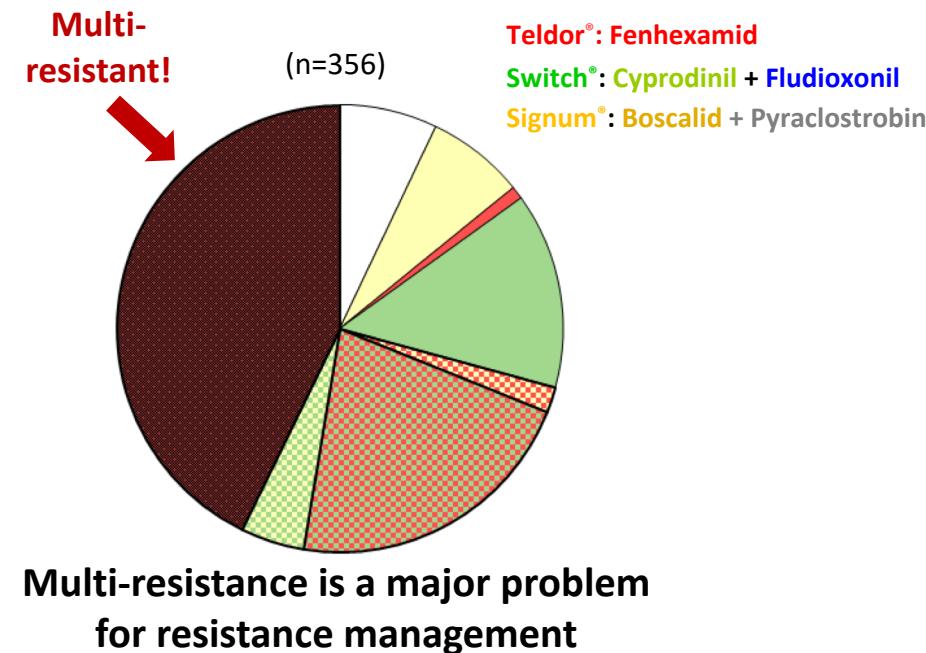
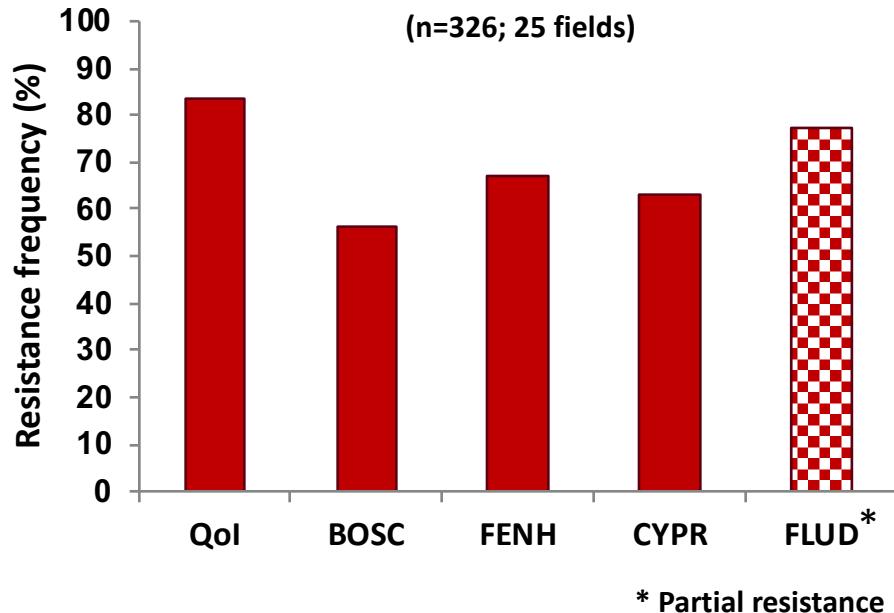
- ca. 1 treatment/ season
- Low to intermediate resistance frequencies

Strawberries

- 4 to >6 treatments
- High resistance frequencies



Grey mould on strawberries: Resistance situation in Southern Germany 2013



Culture	Resistance frequencies
Small fruits	(Very) High
Grapevine	Low to intermediate
Stone fruits	Intermediate to high
Ornamentals	Low to high

Resistance development in Botrytis in the last 30 years

Early 1990ies



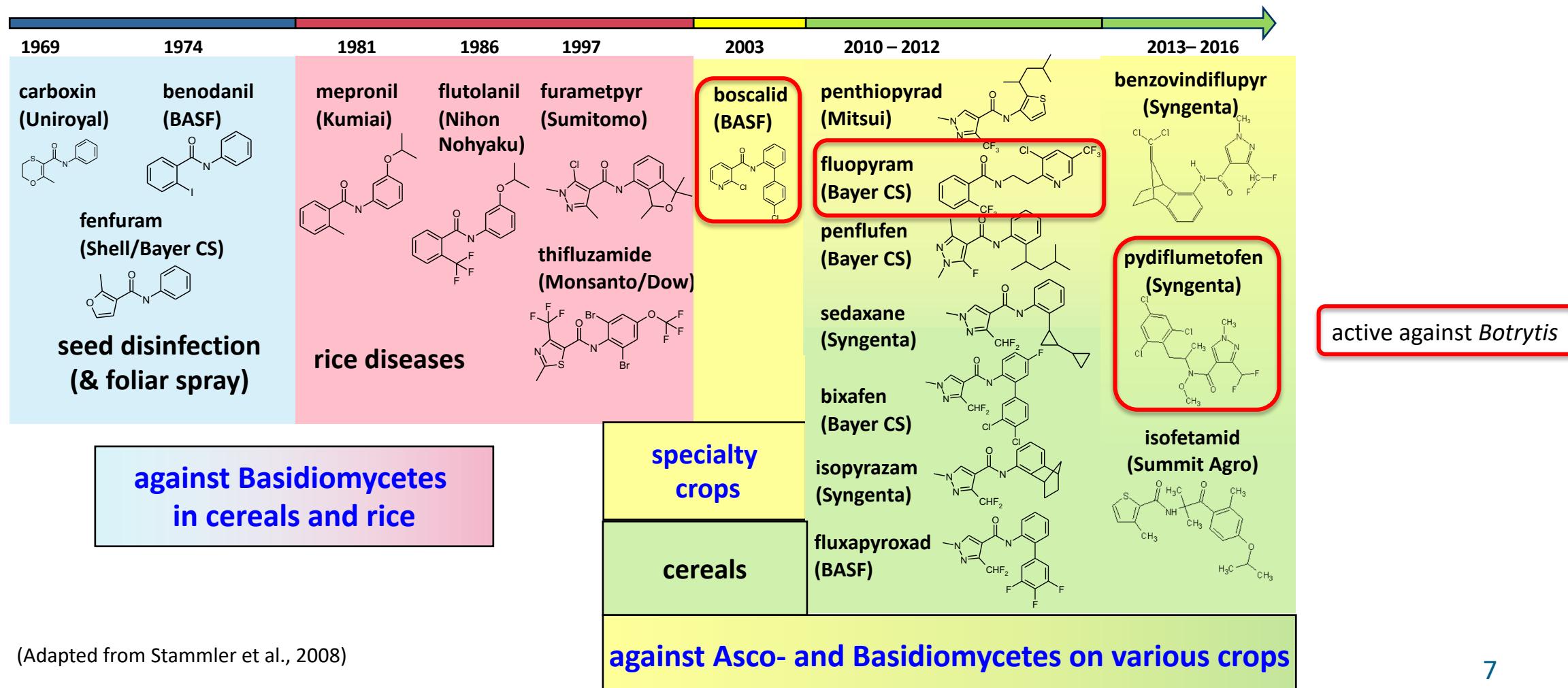
Today



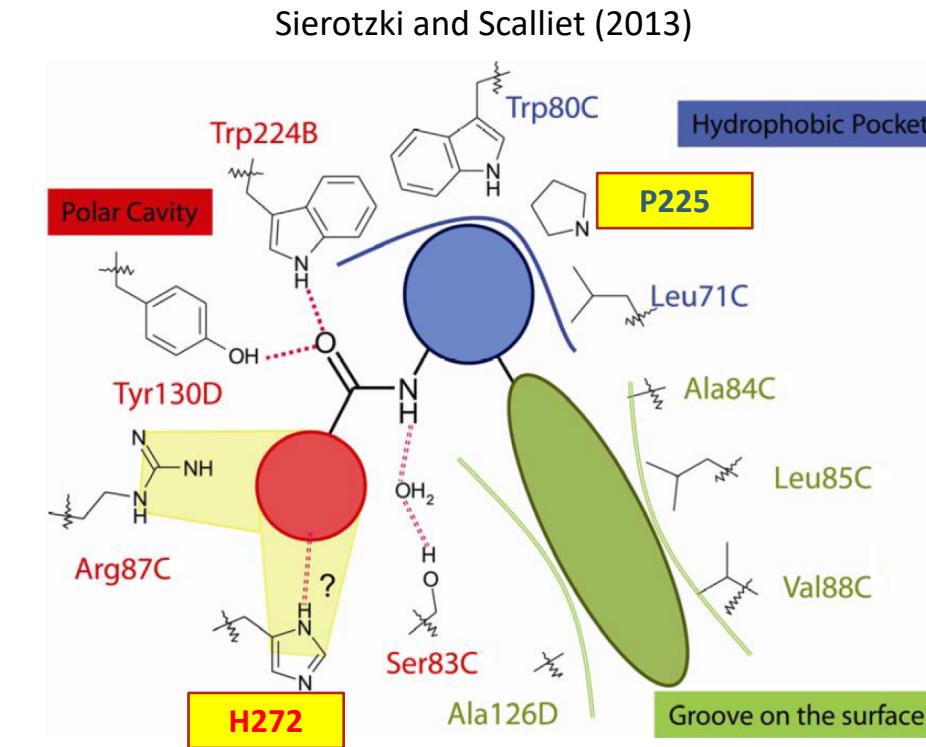
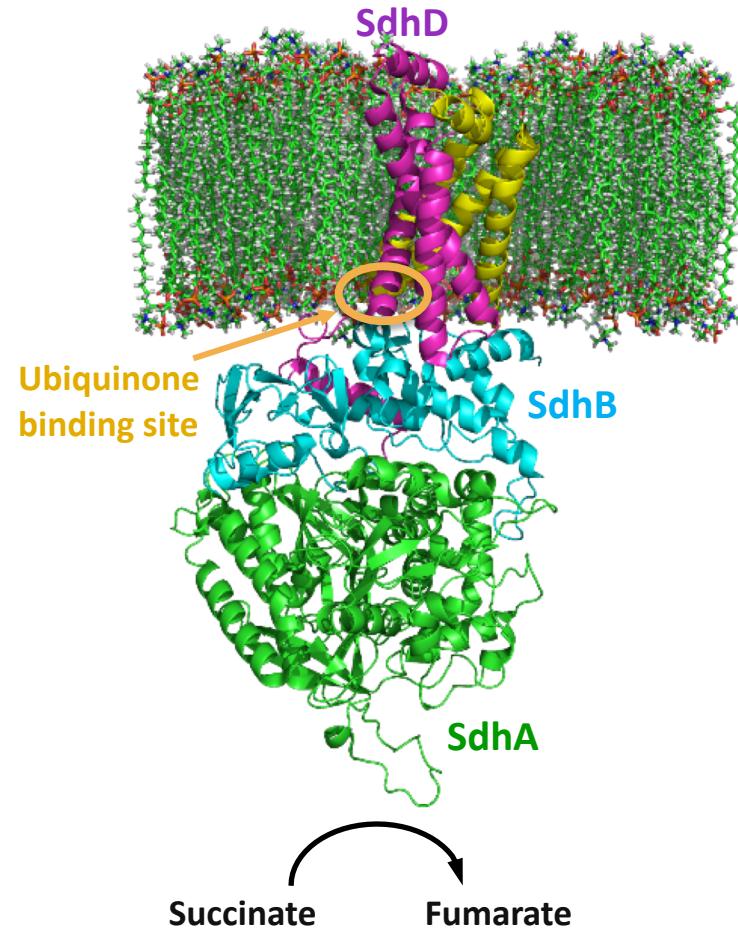
Courtesy G. Stammler (BASF)

Succinate dehydrogenase inhibitors (SDHIs)

Market entry of selected SDHIs & their main targets



Succinate dehydrogenase, target of SDHI fungicides



SDHIs bind to ubiquinone-binding pocket
(interface of *sdhB/C/D*)

Major SDHI resistance mutations in *B. cinerea* SdhB

SdhB**P225 N230**

...ECILCACCS**T**SCP**S**YWW**N**SEEYLGPAILLQS**R**WLADSRD**Q**KKEERKAALDN**S**MSLYR**C****H**T**I**LNC**S**RTCP...

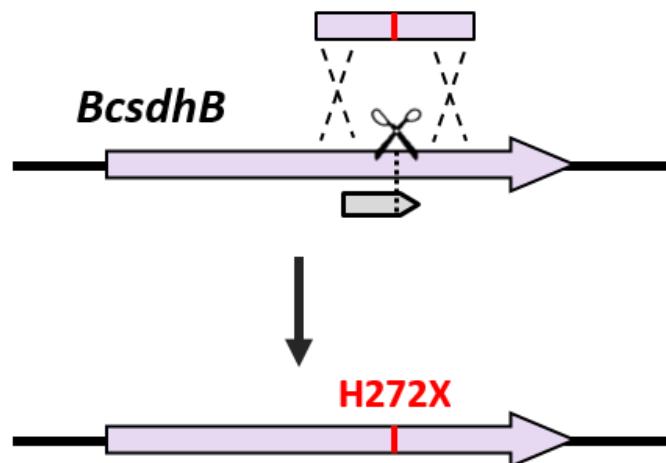
↓ ↓

P225L N230I
P225T
P225F

H272

Resistance to	Exchange	R272 Y272	L272 V272
Boscalid	yes	yes	yes
Fluopyram	no	yes	
Pydiflumetofen	?	?	

Goal: Replace H272 with
all 20 amino acids

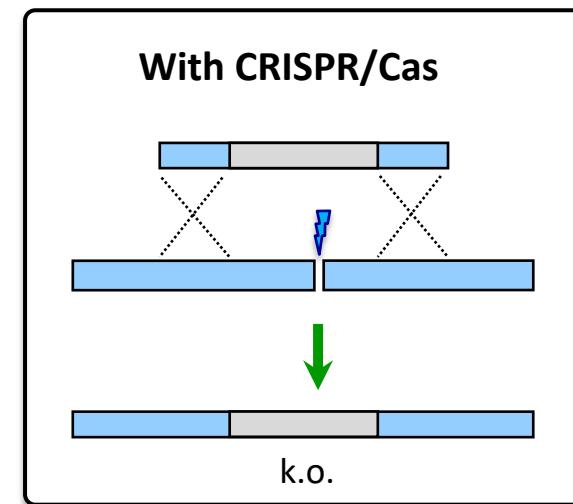
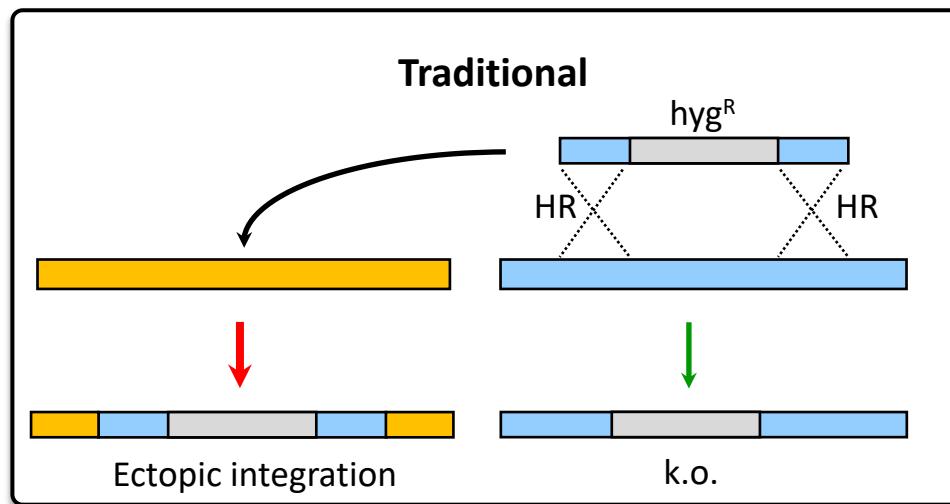


Targeted mutagenesis in fungi

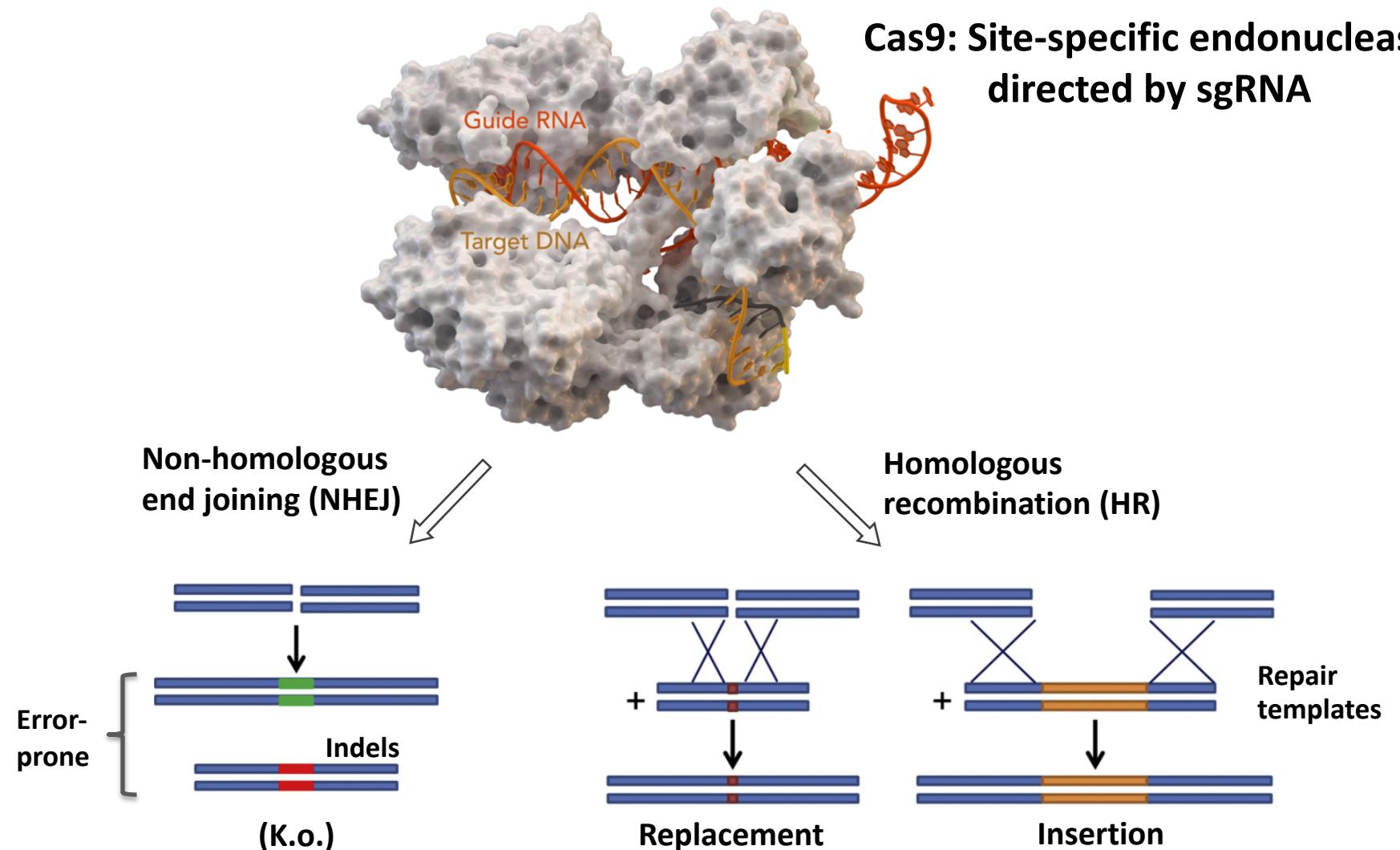
Delivery method:

Protoplasts

Agrobacterium



CRISPR/Cas

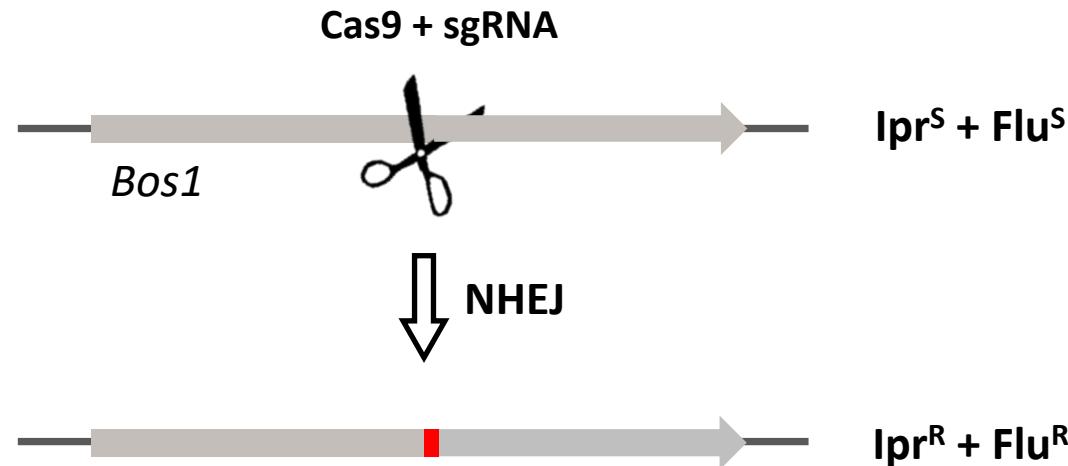


CRISPR/Cas already used in >40 fungi/oomycetes (Schuster and Kahmann, 2019)

Establishing of CRISPR/Cas in *B. cinerea*: Positive selection for loss-of-function mutations

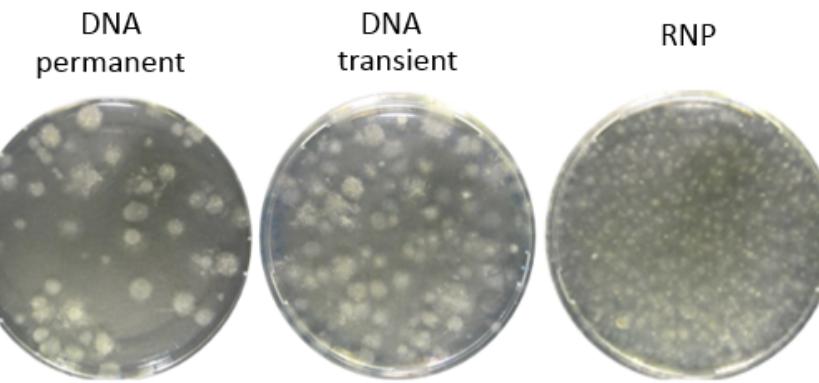
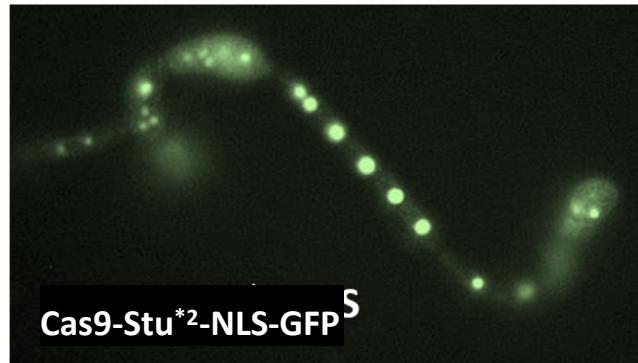
Bos1: Sensor histidine kinase in the HOG MAPK pathway

Bos1 k.o. leads to iprodione & fludioxonil resistance (Viaud et al., 2006)

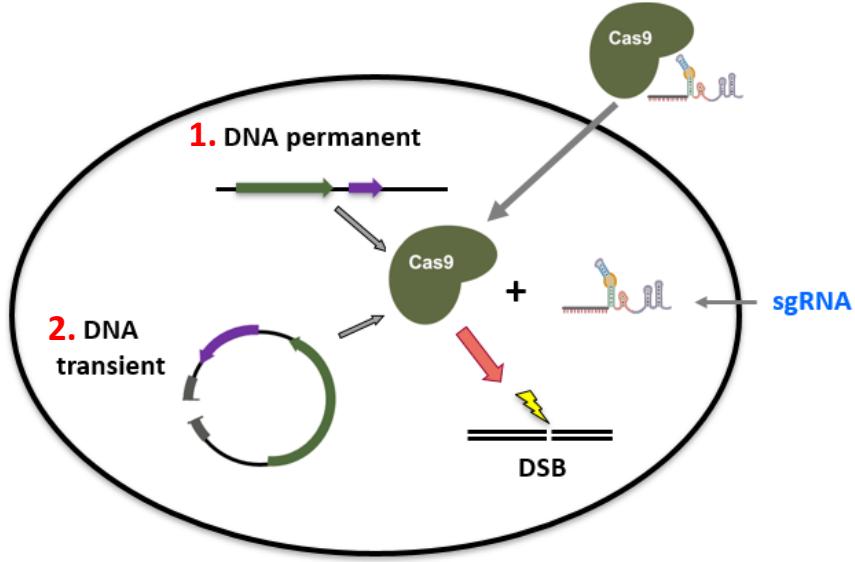
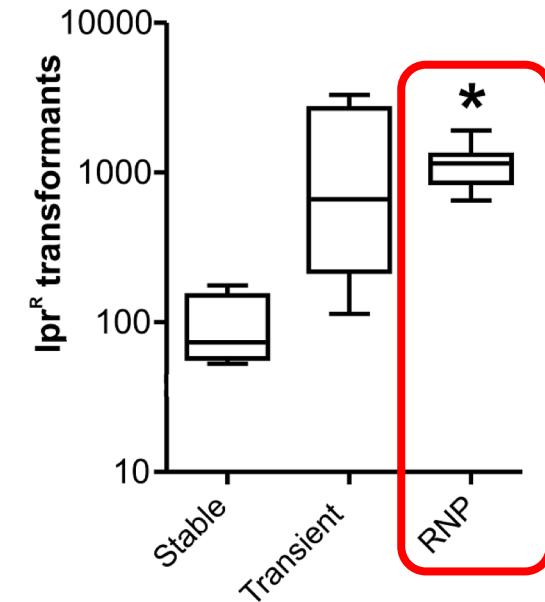


- Positive selection for CRISPR/Cas-induced NHEJ events

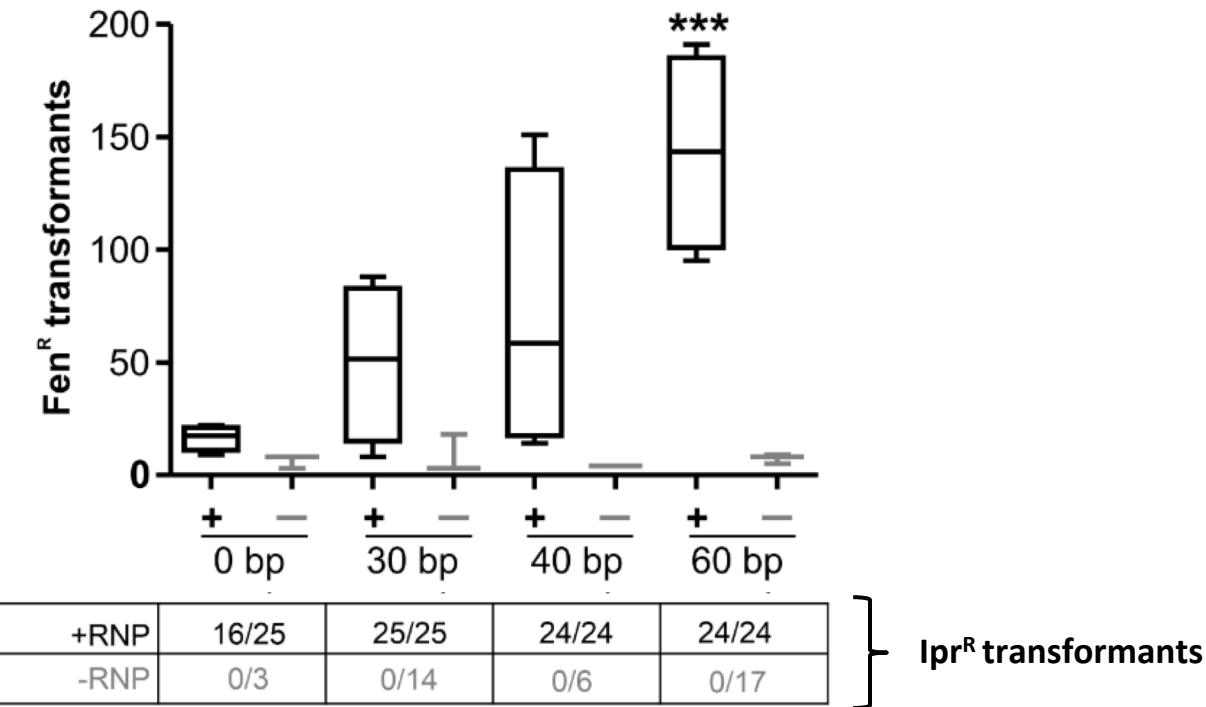
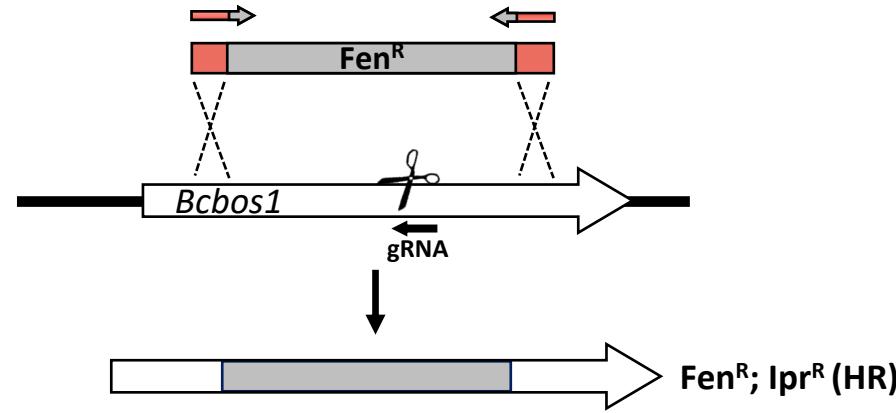
Cas9 with optimized NLS



3. RNP: Ribonucleoprotein complex

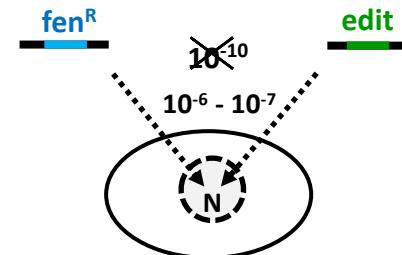
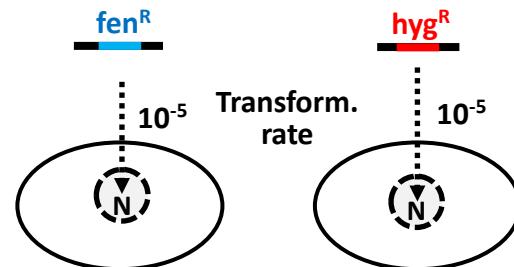
Ipr^R transformants / 10⁶ protoplasts

CRISPR/Cas editing with short homology flanks



- Simplified k.o. mutagenesis using flanking primers overlapping with resistance cassette

1. Cotransformation efficiency

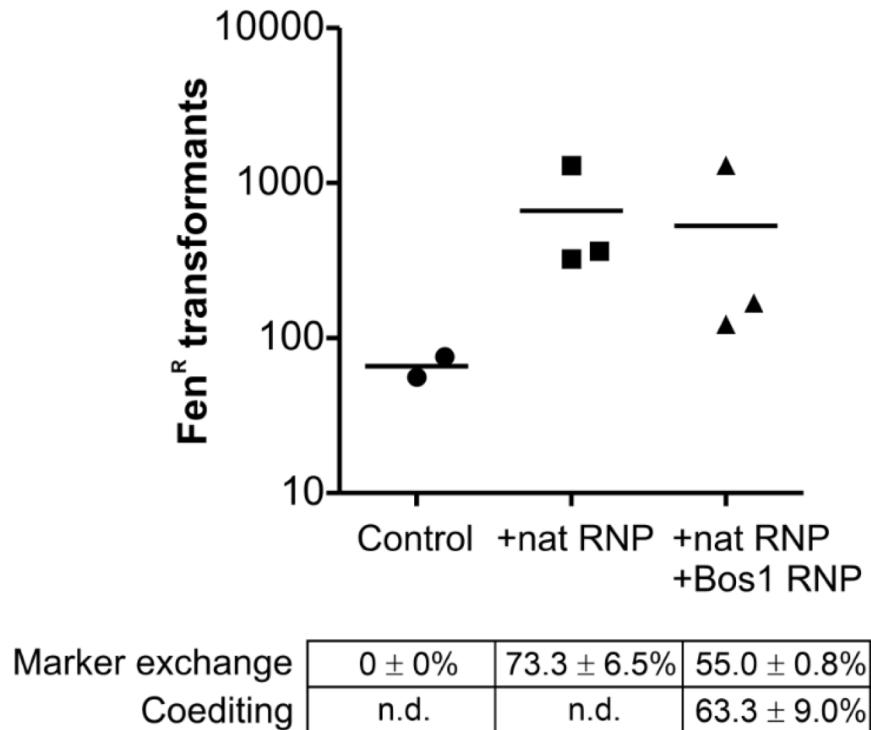
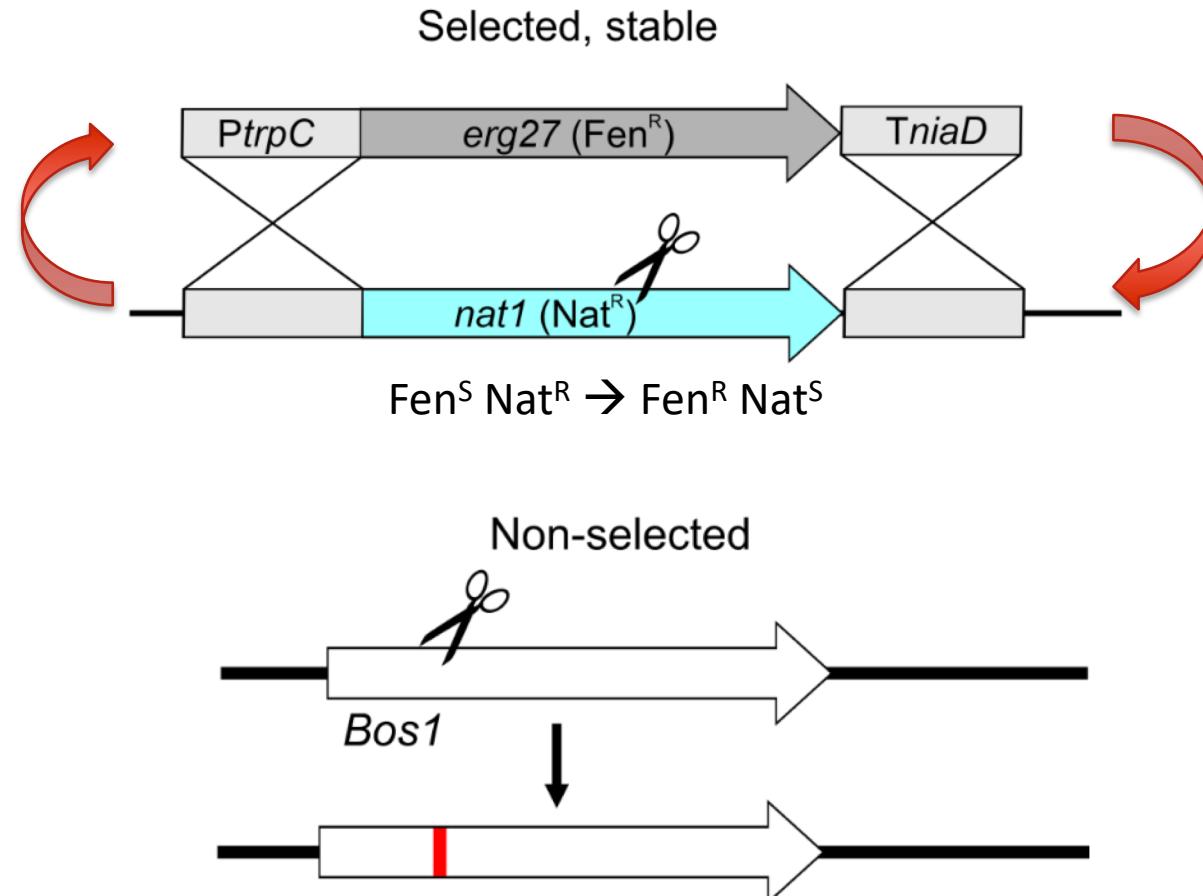


- Selection of only one event, followed by screening for the other event

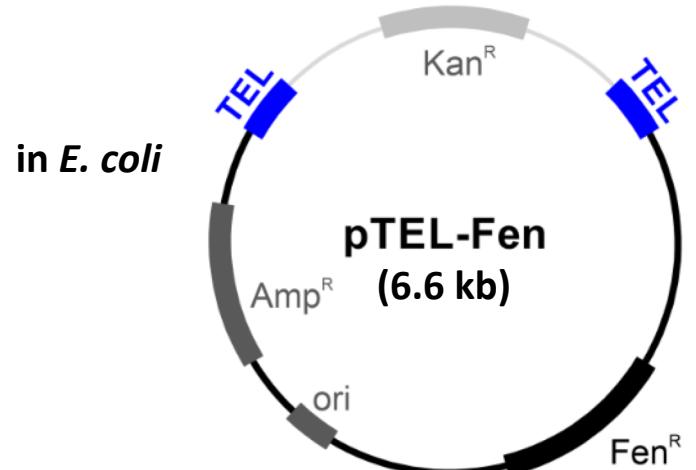
2. Transient selection marker

- a) Replacing a resident marker (marker switching)
- b) Delivery by unstable vector

Marker shuttling



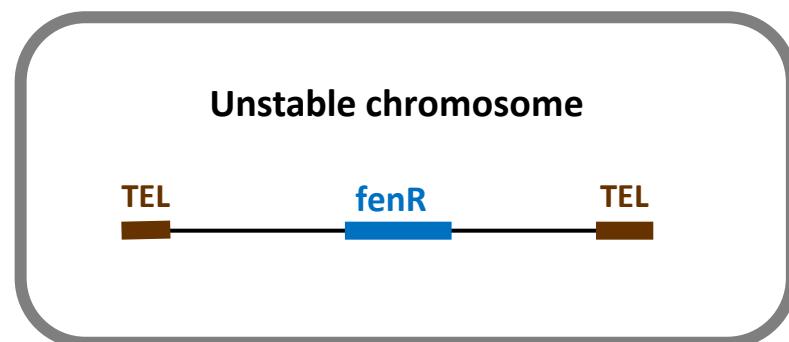
Telomere vectors



Barreau et al. (1998); S. Fillinger (upubl.)

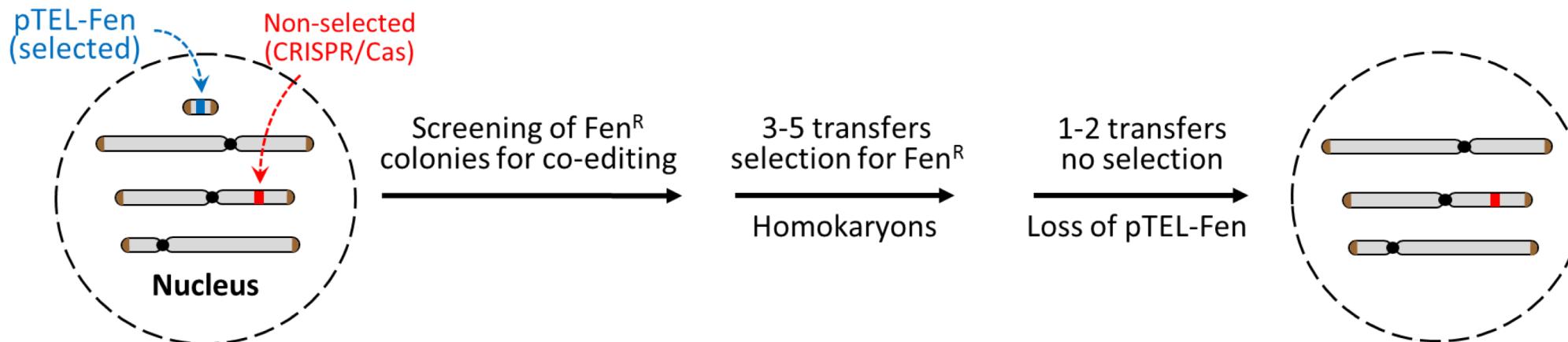


Transformation
into fungus

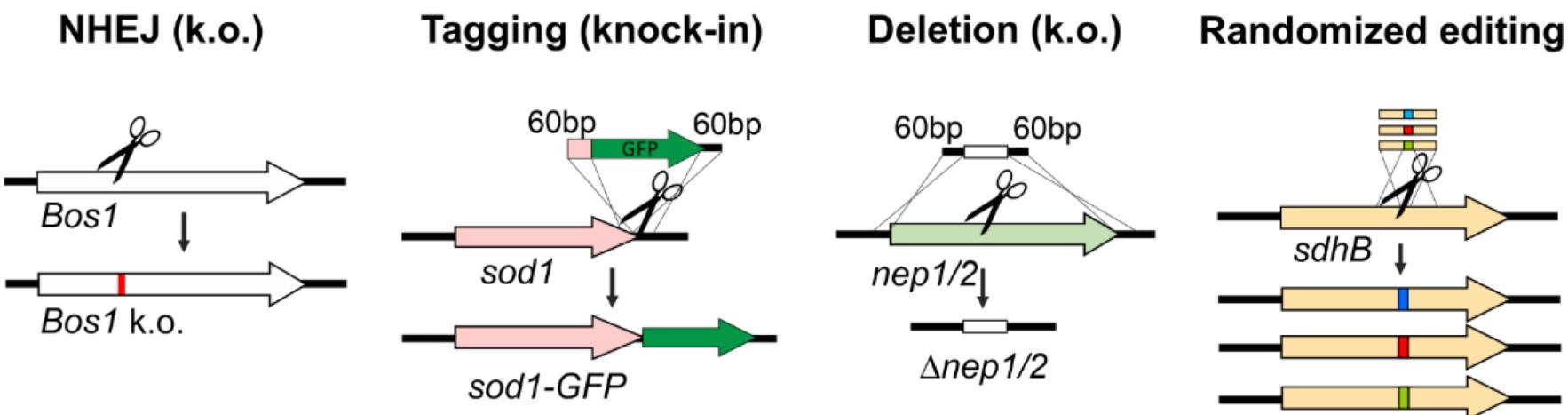


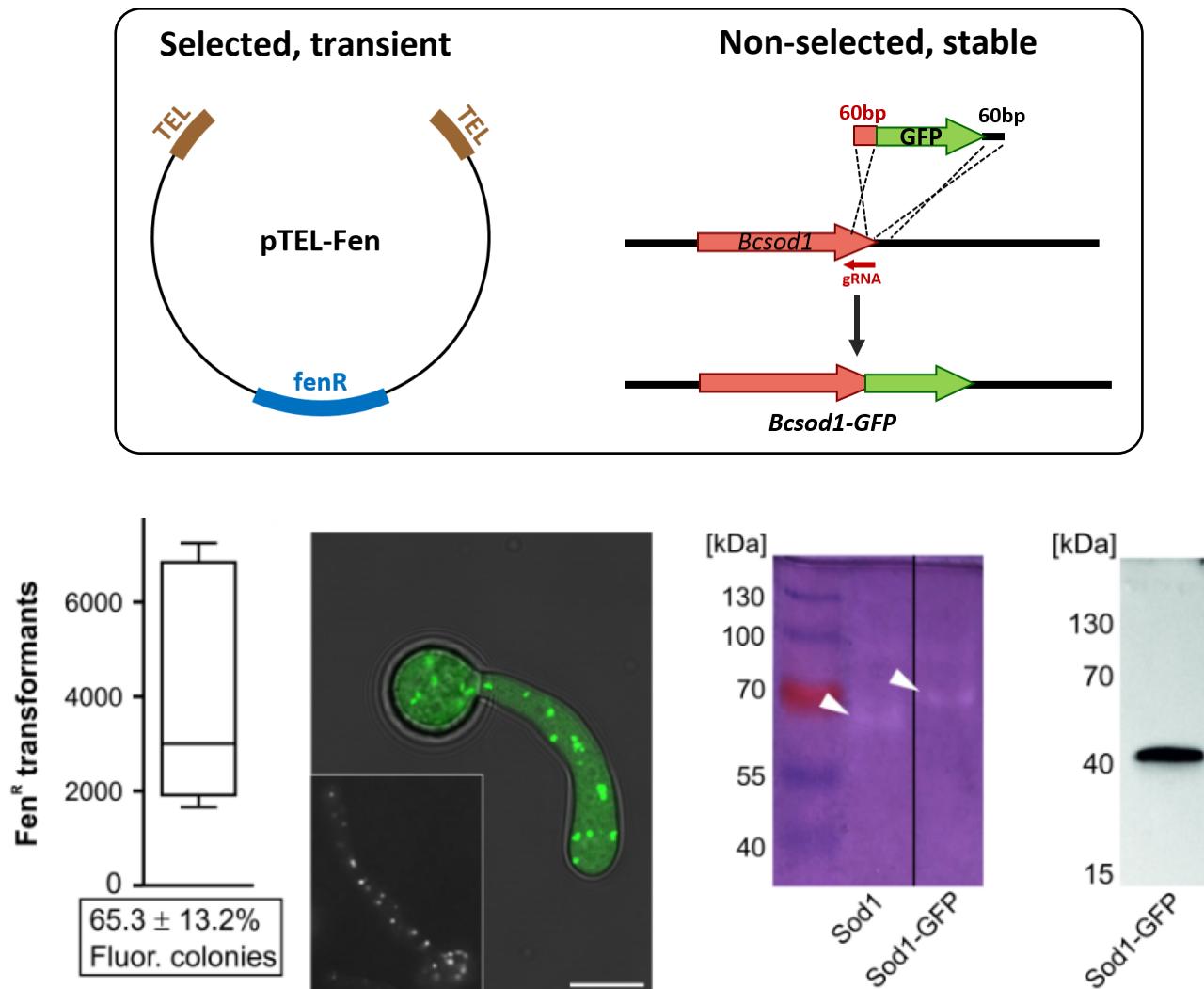
- High transformation rates
- Rapid loss without selection

Telomere vector mediated co-editing



Non-selected marker-free CRISPR/Cas events



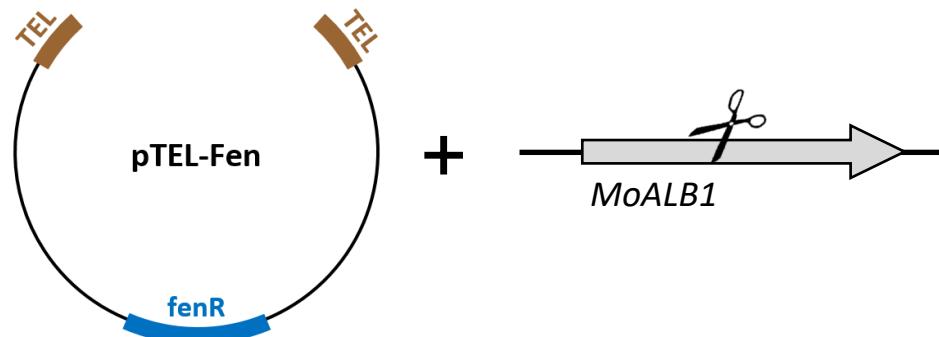


Efficiency of marker-free coediting

Transformants (co-editing rates)

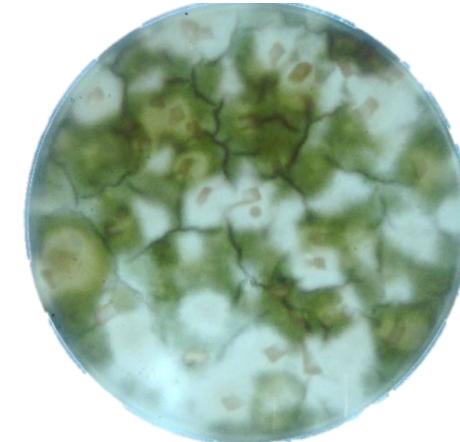
Selected	Non-selected	
	NHEJ	HR
HR	~500 (63%)	n.a.
pTEL uptake	~1000 (58%)	250 to >5000 (10 – 70%)

RNP-mediated transformation of *M. oryzae* described by Foster et al. (2019)

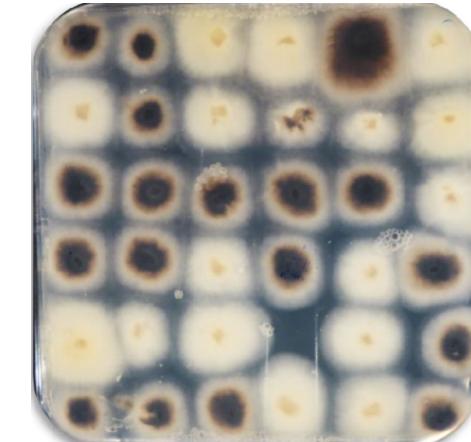


Experiment	Transformants	White colonies (coediting rate)
1	96	17/36 (47%)
2	280	17/35 (49%)
3	120	13/36 (36%)

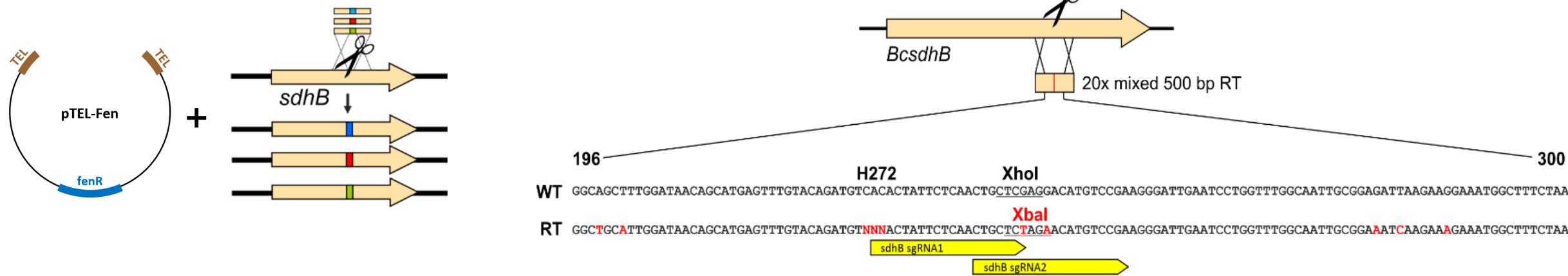
Primary Fen^R transformants



Transformants after transfer



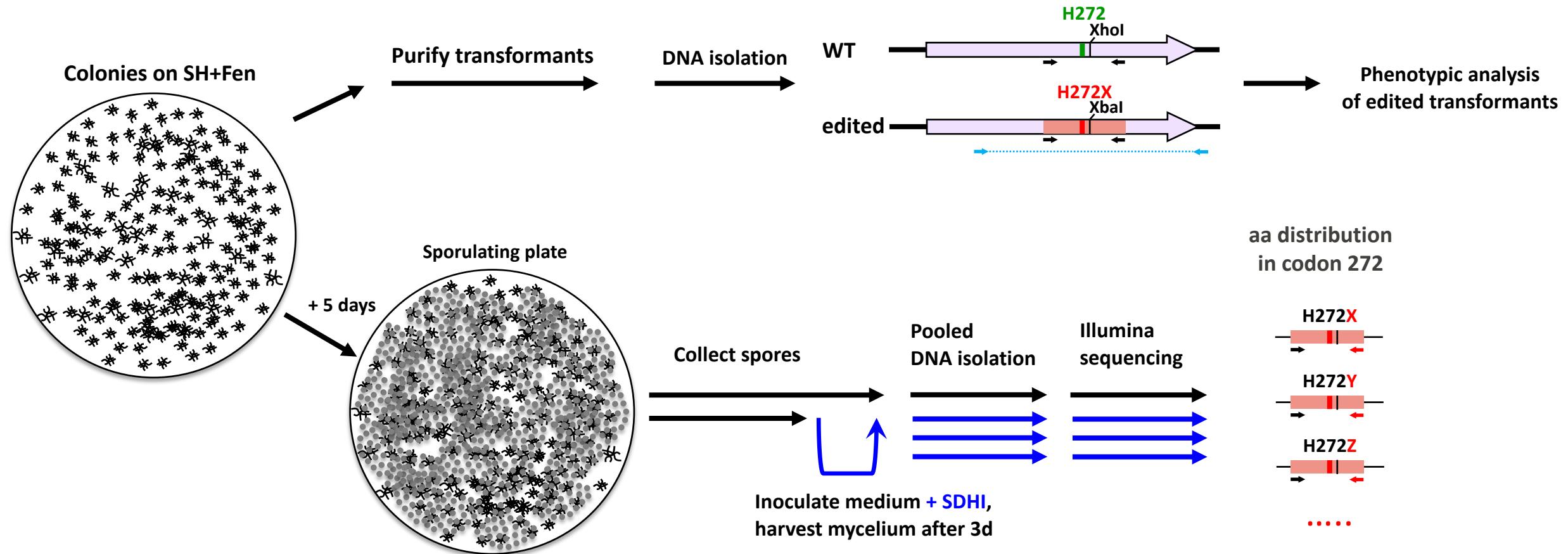
Bulk replacement of codon 272 in *sdhB*



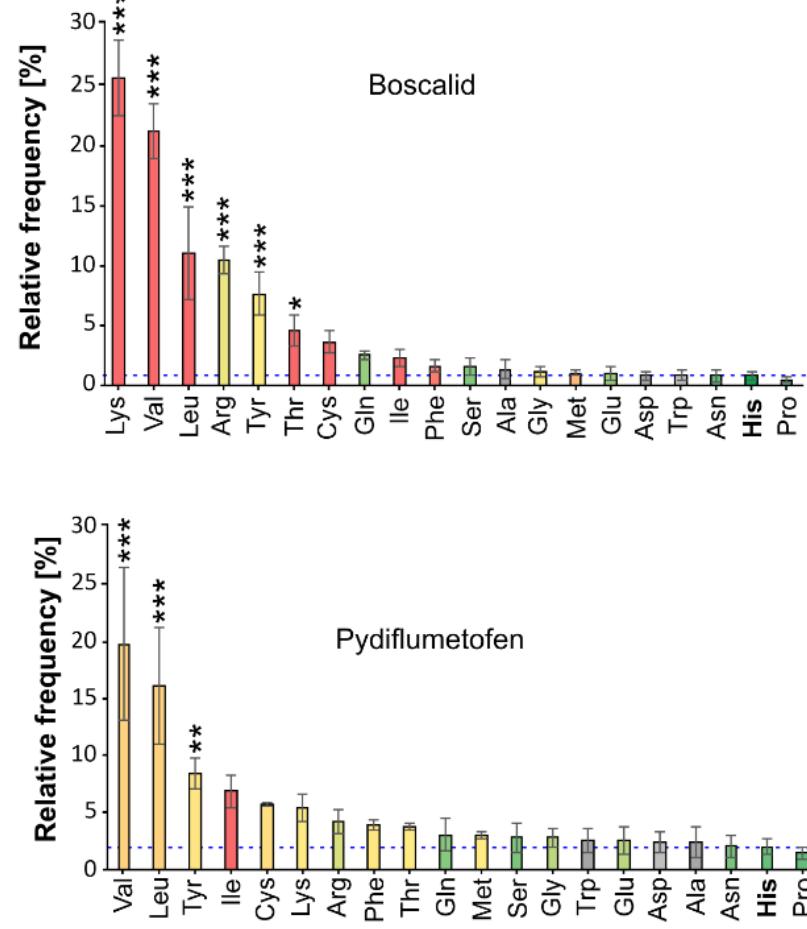
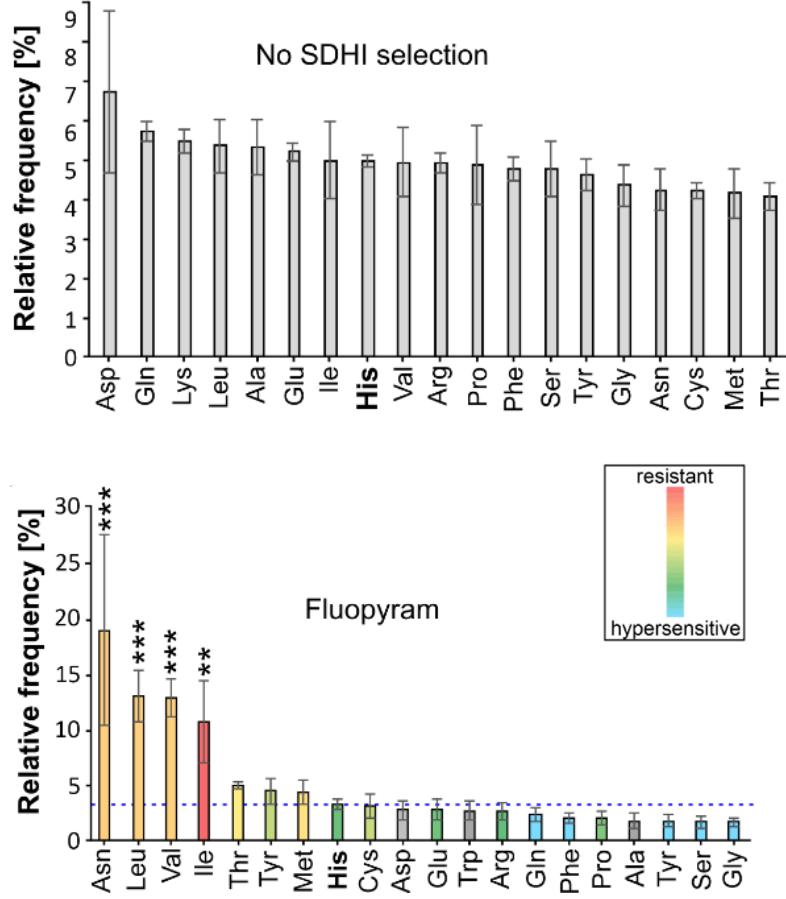
Transformation results

Transformation	sgRNA	Fen ^R colonies/ transformation	Sequenced transformants	Editing frequency	
					Illumina seq.
D	sdhB272-2	4,820	6,100		31.6 %
E	sdhB272-2	6,680	6,200		21.4 %
F	sdhB272-1	7,080	10,400		11.4 %

Bulk replacement of codon 272 in *sdhB*

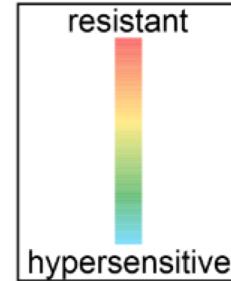


Distribution of amino acids in codon 272 among edited *B. cinerea* transformants



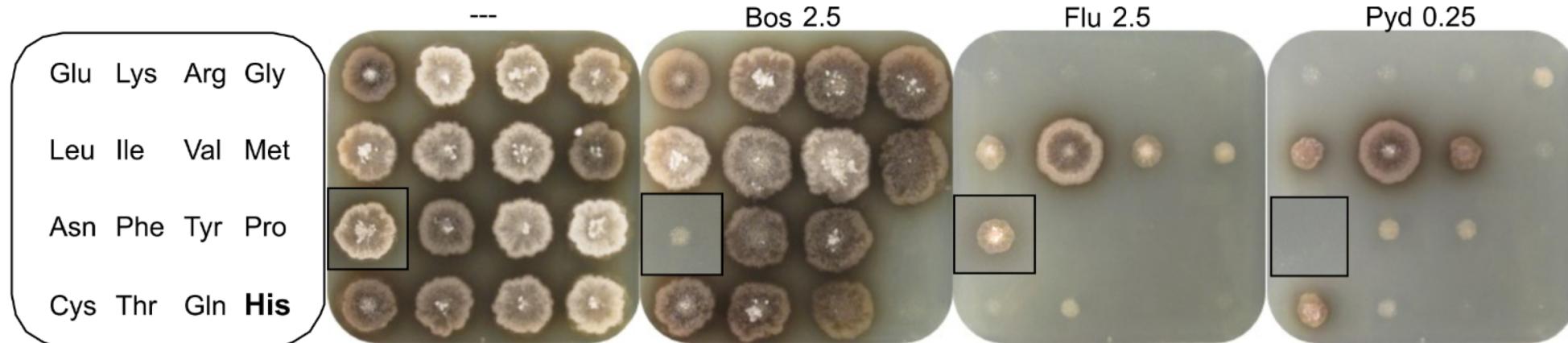
➤ Known and new exchanges are selected differently by SDHIs

SDHI resistance levels of *B. cinerea* transformants with codon 272 amino acid exchanges

EC₅₀ values of purified mutants

	His	Lys	Arg	Gly	Leu	Ile	Val	Met	Phe	Tyr	Pro	Cys	Thr	Ser	Glu	Asn	Gln
Bos	1.23 ±0.04	>10	6.04 ±2.11	6.53 ±1.85	>10	>10	>10	8.07 ±3.18	9.55 ±0.82	6.85 ±2.49	0.25 ±0.02	>10	>10	1.45 ±0.6	2.78 ±0.14	0.26 ±0.03	2.37 ±0.53
Flu	0.24 ±0.02	0.53 ±0.16	0.21 ±0.03	0.030 ±0.01	3.41 ±1.03	>10	2.61 ±0.29	1.55 ±0.57	0.084 ±0.02	0.029 ±0.00	0.31 ±0.03	0.55 ±0.16	0.91 ±0.10	0.013 ±0.004	0.26 ±0.02	2.83 ±0.37	0.099 ±0.00
Pyd	0.012 ±0.01	0.078 ±0.01	0.060 ±0.02	0.048 ±0.06	0.61 ±0.24	2.00 ±0.6	0.49 ±0.27	0.089 ±0.02	0.16 ±0.04	0.18 ±0.06	0.008 ±0.00	0.28 ±0.03	0.092 ±0.01	0.020 ±0.01	0.048 ±0.02	0.018 ±0.01	0.024 ±0.01

Growth on SDHI-containing plates



- Majority of aa lead to boscalid resistance, only few aa to fluopyram and pydiflumethofen resistance
- Amino acids conferring high SDHI resistance (Ile!) not found in field isolates!?

Summary

- CRISPR/Cas with RNPs is a powerful method for gene editing in *Botrytis cinerea*
- Marker switching and telomere vectors allow highly efficient marker-free editing
- Both approaches can be applied with other fungi and maybe beyond
- Bulked amino acid exchanges can be performed in single experiments, allowing ...
 - systematic analysis of fungicide binding sites in the target organism
 - faster and more precise prediction of resistance risk for new fungicides

Acknowledgements



Thomas Leisen

Janine Werner

Sabrina Rupp

Nora Fischbach

Patrick Pattar

David Scheuring



Roland Weber



Gabriel Scalliet

Andreas Mosbach



We create chemistry

Gerd Stammler



Alex Wegner

Ulrich Schaffrath



Sabine Fillinger



Bundesanstalt für
Landwirtschaft und Ernährung