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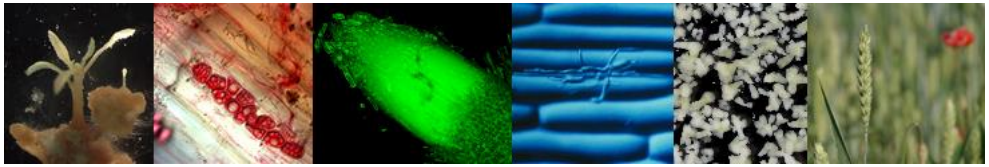


PHYTO Phytopathologie JLU Gießen

Employing the CRISPR/Cas9 system for improvement of plant defense against pathogens

M. Galli, Dr. J. Imani & Prof. Dr. K. H. Kogel

AIPlanta – GfPB Symposium
Neustadt/Mußbach
September 03-04th, 2018

Targeted genome engineering

It's a technological approach which allows **to determine the function of specific genes** via modification of their expression.

Technologies:

I. Gene overexpression
II. Antisense RNA
III. RNA interference (RNAi)

Limitations → incomplete/temporary gene knock-down (KD) and poor specificity

IV. Site-Specific Nucleases (SSNs)

Advantages → complete knock-out (KO), higher sequence fidelity and possibility of transgene-free plants in next generations

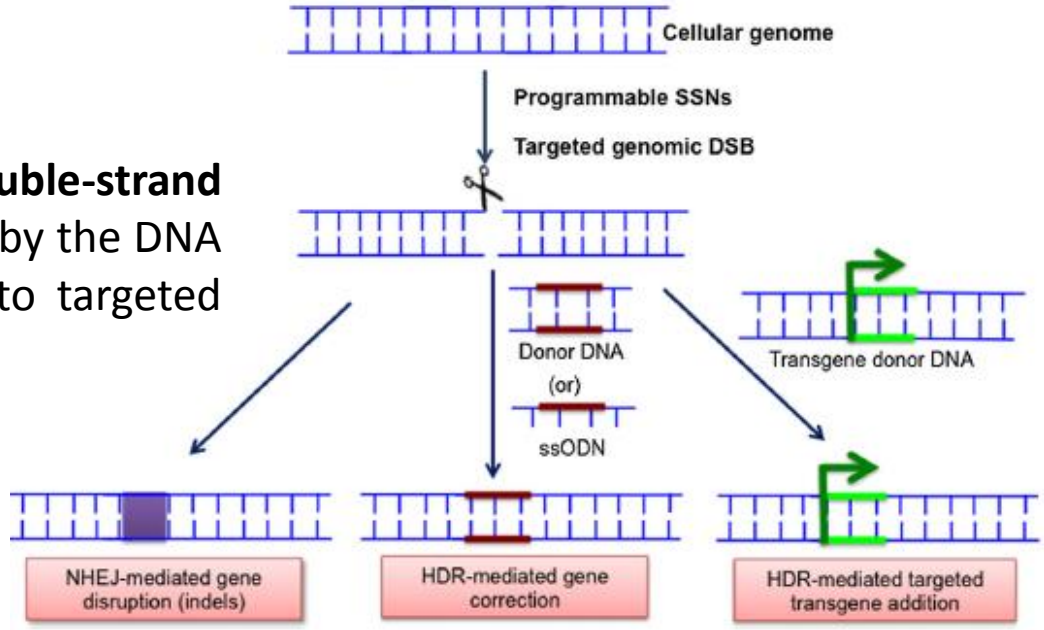
Kim *et al*., 2014; Govindan *et al*., 2016

Site-Specific Nucleases

I. Zinc finger nucleases (Kim *et al*., 1996)
II. Transcription activator-like effector nucleases (Boch *et al*., 2009)
III. RNA-guided engineered nucleases (Sapranauskas *et al*., 2011)

How do they achieve gene KO?

Introducing a site-specific **double-strand break** (DSB), which is **corrected** by the DNA repair cell machinery, leading to targeted **genetic modifications** (Kim *et al*., 2014).



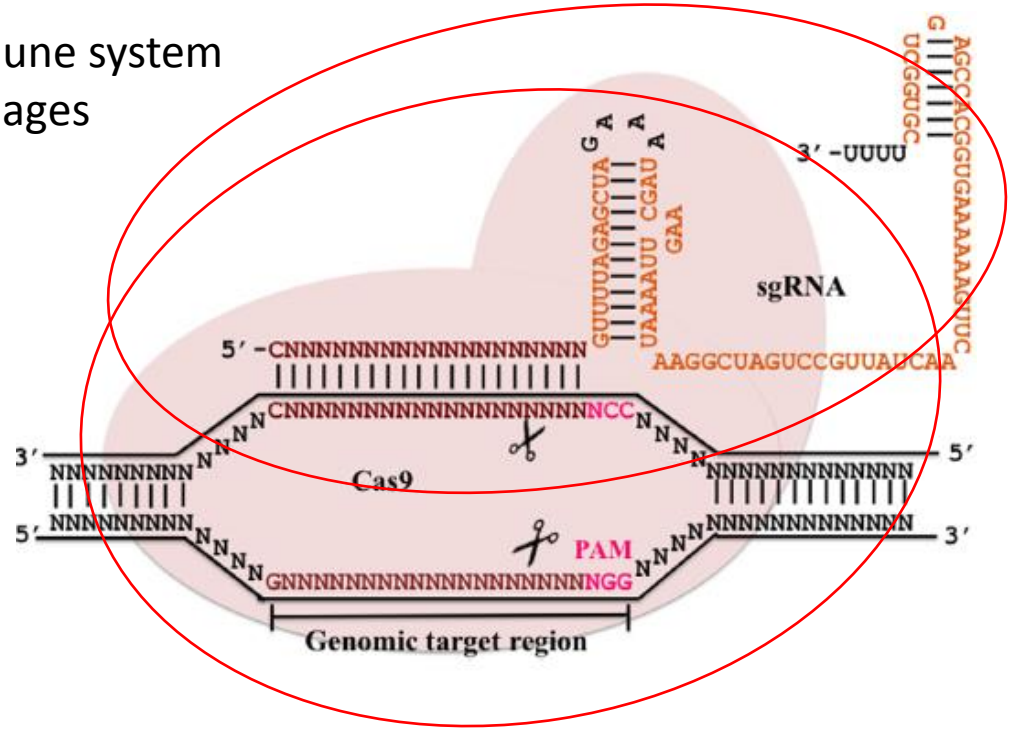
*Sp*Cas9 type II system

Discovered as part of the adaptive immune system of bacteria (*S. pyogenes*) vs. bacteriophages

The CRISPR/Cas type II system requires only two components:

I. single RNA chimera or single guide RNA (sgRNA)
II. CRISPR associated protein (Cas9)

*Sp*Cas9 cleaves in a 20bp DNA sequence targeted, 3-nt upstream of the **PAM** site

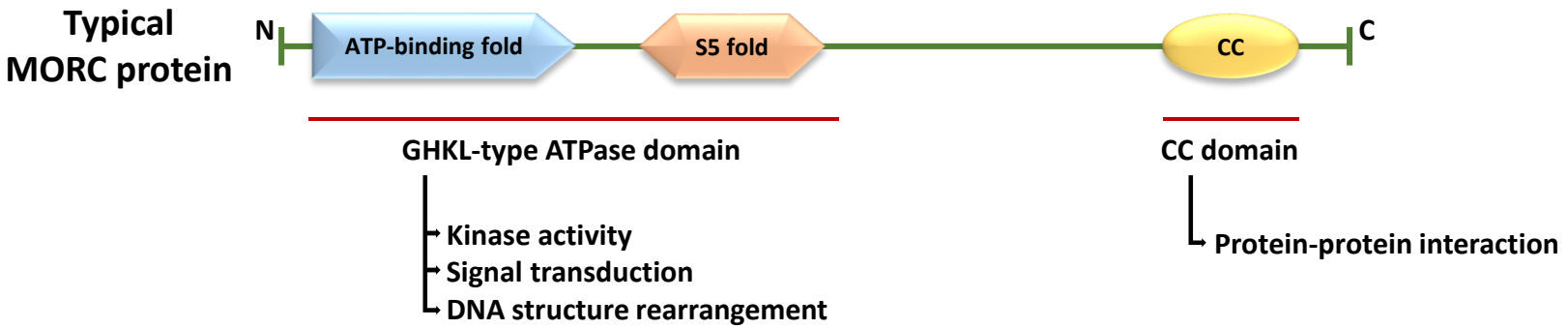


Govindan and Ramalingam, 2016

Microrchidia (MORC) ATPases

- Discovered as critical modulators of mice spermatogenesis (Watson et al., 1998), in both eukaryotes and prokaryotes they have characteristic conserved domains: a GHKL and S5 fold domains and a coiled-coil domain (Dutta and Inouye, 2000). MORC proteins are involved in cell cycle regulation, DNA methylation and repair and transcriptional gene silencing (Moissiard et al., 2012; Kock et al., 2017).

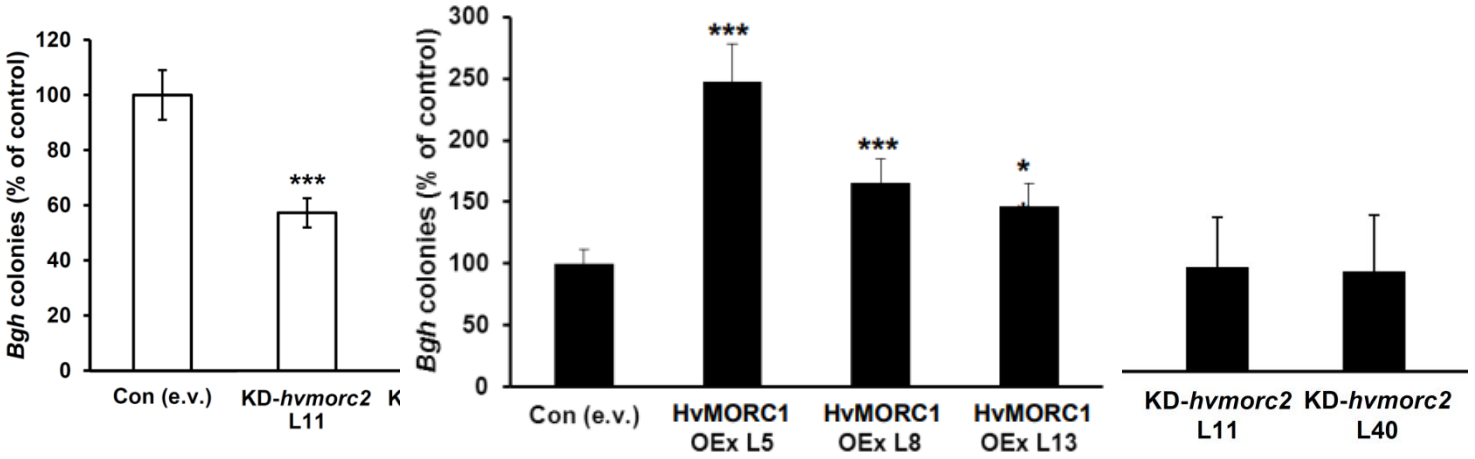
- In plants MORCs participate in multiple layers of plant immunity: in *Arabidopsis* and potatoes, they are positive regulators, whereas in tobacco and tomato they are negative ones (Kang et al., 2008; Manosalva et al. 2015).



Microrchidia (MORC) ATPases in barley

- In the monocot barley, seven *Hv*MORCs are present and they act as positive regulators of plant immunity (Koch et al., 2017).

- RNAi knock-down (KD) of *HvMORC2* resulted in enhanced resistance vs. *Blumeria graminis* f.sp. *Hordei* and *Fusarium graminearum,* while plants constitutively overexpressing *HvMORC1* under control of the *CMV*35S promoter were compromised for resistance (Langen et al., 2014).

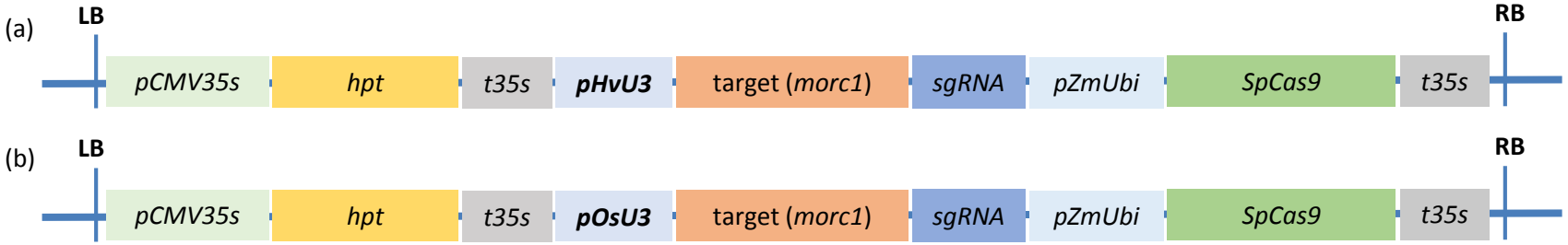


Employing CRISPR/Cas9 system to KO *HvMORC1*

Construction of the T-DNA

- Several monocot and dicot RNA Pol III promoters from snRNA genes have been used to express sgRNA for genome editing.

In this study, barley and rice (RNA Pol III dependent) *U3* promoters were used for active transcription of sgRNA in barley cells.



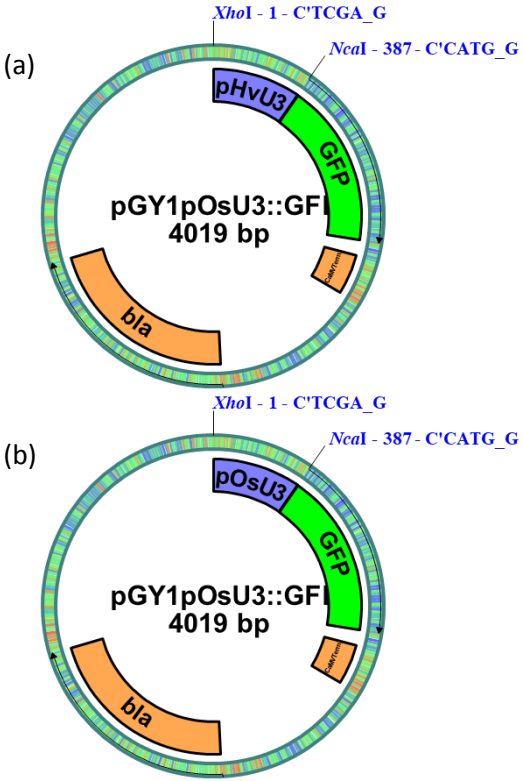
The final *Sp*Cas9 construct, with sgRNA under the control of *HvU3 (a)/OsU3 (b)* promoter and *Cas9* gene under the maize *ubiquitin* promoter, was used for *A. tumefaciens* mediated-transformation of barley embryos.

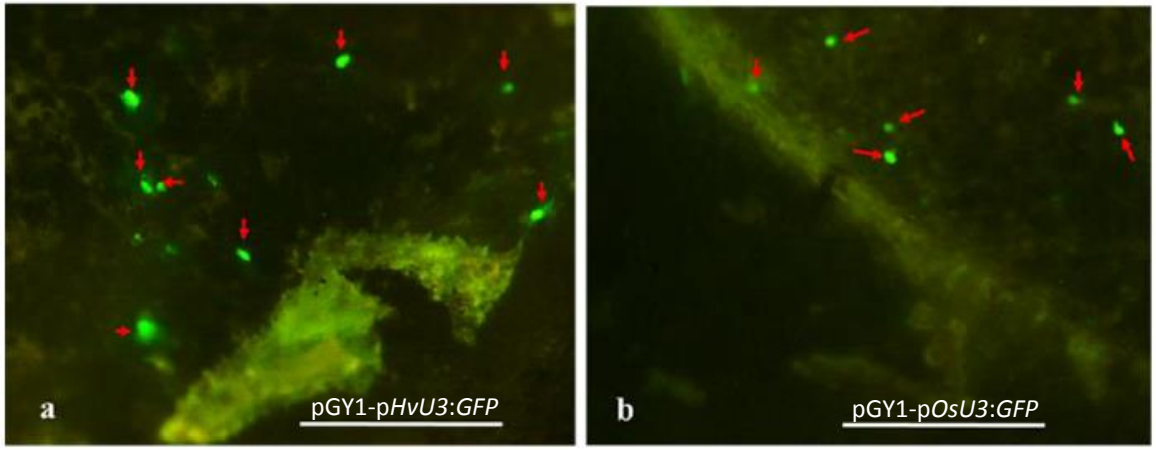
Kumar, Galli, Ordon, Stuttmann, Kogel & Imani, ***Plant biotechnology journal*** **(2018)**

Employing CRISPR/Cas9 system to KO *HvMORC1*

Activity of *Hv*U3 and *Os*U3 promoters in barley

HvU3 and *OsU3* promoters were cloned in pGY1-*35s*::*GFP* (Schweizer et al., 1999) and their activity tested in immature barley embryos via biolistic particle delivery system.



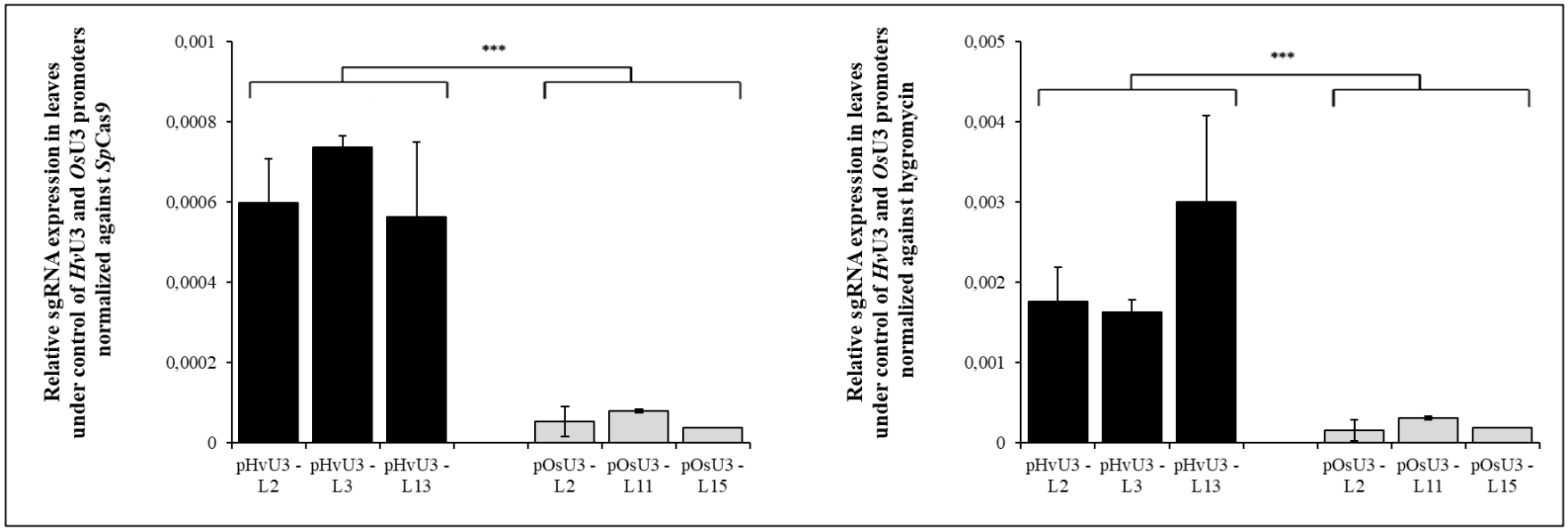


Kumar, Galli, Ordon, Stuttmann, Kogel & Imani, *Plant biotechnology journal* (2018)

Employing CRISPR/Cas9 system to KO *HvMORC1*

Activity of *Hv*U3 and *Os*U3 promoters in barley

Expression of sgRNA under control of either the *Hv*U3 or *Os*U3 promoter.



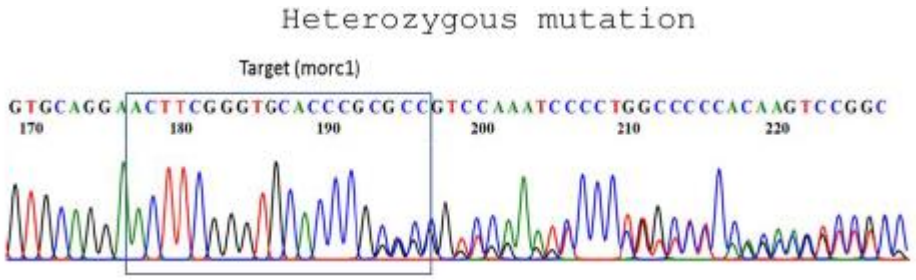
- *Hv*U3 driven sgRNA showed highest transcript accumulation as compared to *Os*U3.

Kumar, Galli, Ordon, Stuttmann, Kogel & Imani, *Plant biotechnology journal* (2018)

Employing CRISPR/Cas9 system to KO *HvMORC1*

_Sp_Cas9 induce mutation in T1 barley plants

Mutation frequency in T0 *calli* for construct *HvU3*:sgRNA was 77%. In T1 plants was 100% and 70% using p*HvU3* and p*OsU3*, respectively.



Homozygous mutations (*HvU3*:*sgRNA_ZmUbi*:*Cas9*)

target (morc1)

ACTTCGGGTGCACCCGCGCG

GGTGTGCAGGAACTTCGGGTGCACCCGCGCGCGGTCGAAGTCCCCTGTCTCCA	wt
GGTGTGCAGGAACTTCGGGTGCACCC-CGCGCGGTCGAAGTCCCCTGTCTCCA	-1
GGTGTGCAGGAACTTCGGGTGCACCC--GCGCGGTCGAAGTCCCCTGTCTCCA	-2
GGTGTGCAGGAACTTCGGGTGCAC----GCGCGGTCGAAGTCCCCTGTCTCCA	-4
GTGTGCAGGAACTTCGGGTGCACCCGCTGCGCGGTCGAAGTCCCCTGTCTCCA	+1

Homozygous mutations (*OsU3*:*sgRNA_ZmUbi*:*Cas9*)

target (morc1)

ACTTCGGGTGCACCCGCGCG

GGTGTGCAGGAACTTCGGGTGCACCCGCGCGCGGTCGAAGTCCCCTGTCTCCA	wt
GGTGTGCAGGAACTTCGGGTGCACCCG-GCGCGGTCGAAGTCCCCTGTCTCCA	-1
GGTGTGCAGGAACTTCGGGTGC-----CGCGCGGTCGAAGTCCCCTGTCTCCA	-5
GGTGTGCAGGAACTTCGGGTGC------GCGCGGTCGAAGTCCCCTGTCTCCA	-6
GGTGTGCAGGAACTTCGGGTGCACC----------GTCTCCA	-21
GTGTGCAGGAACTTCGGGTGCACCCGCAGCGCGGTCGAAGTCCCCTGTCTCCA	+1

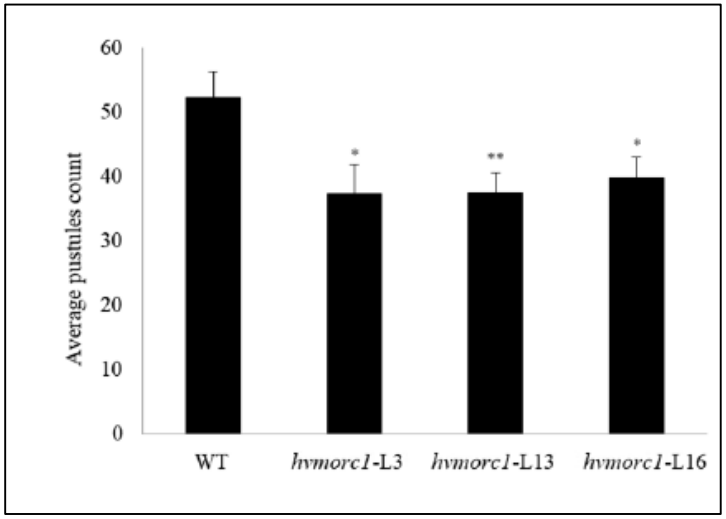
- *Sp*Cas9 technique produces high frequency mutation events in barley.

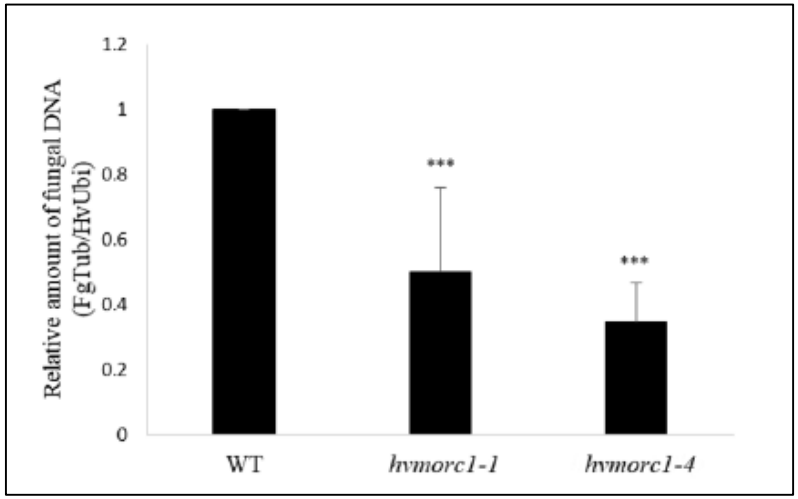
Kumar, Galli, Ordon, Stuttmann, Kogel & Imani, *Plant biotechnology journal* (2018)

Employing CRISPR/Cas9 system to KO *HvMORC1*

Barley MORC1 modulates plant immunity

Mutated *hvmorc1*-KO T1 plants from p*HvU3*:*sgRNA*_p*ZmUbi*:*Cas9* construct were tested for powdery mildew and *F. graminearum* resistance.





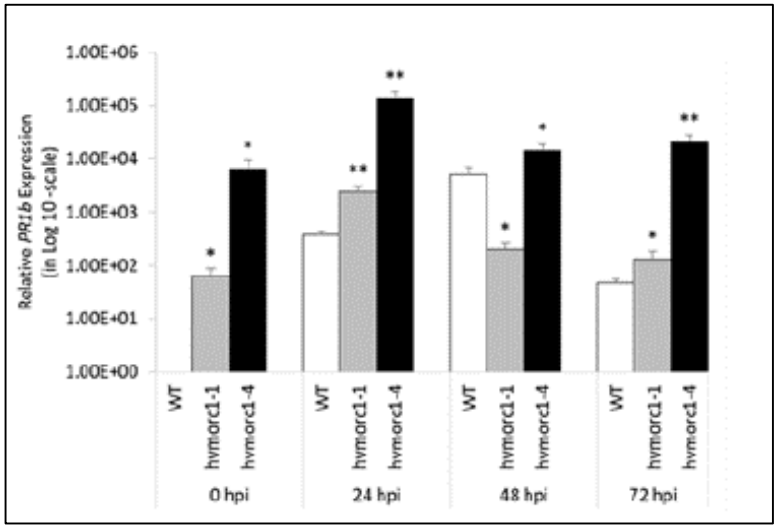
- Complete KO of HvMORC1 enhances plant immunity against fungal pathogens *Blumeria graminis* and *F. graminearum*

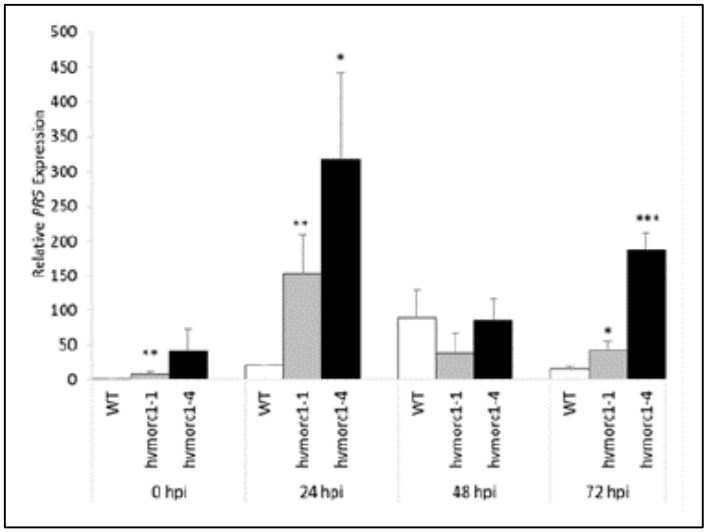
Kumar, Galli, Ordon, Stuttmann, Kogel & Imani, ***Plant biotechnology journal*** **(2018)**

Employing CRISPR/Cas9 system to KO *HvMORC1*

Barley MORC1 modulates plant immunity

Enhanced resistance of *hvmorc1*-KO lines is associated with constitutive activation of defense responses. *HvPR1b* and *HvPR5* were tested by RT-qPCR at 0, 24, 48, and 72 hpi with *Bgh.*





- *PR* expression is enhanced in response to *Bgh*, particularly during initial phase of fungal colonization, providing *hvmorc1*-KO mutants an early advantage.

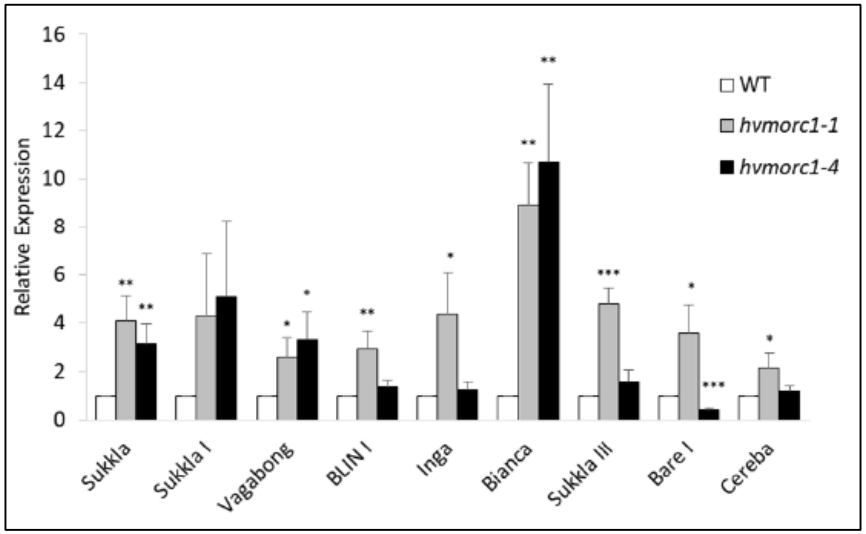
Kumar, Galli, Ordon, Stuttmann, Kogel & Imani, ***Plant biotechnology journal*** **(2018)**

Employing CRISPR/Cas9 system to KO *HvMORC1*

Barley MORC1 regulates TE expression

- Arabidopsis mutants *atmorc1* and *atmorc6* are defective for transcriptional gene silencing (TGS), which plays an important role in repressing TEs (Moissiard et al., 2012).

Barley *morc1*-KO T_1 plants were tested for transposable elements de-repression.



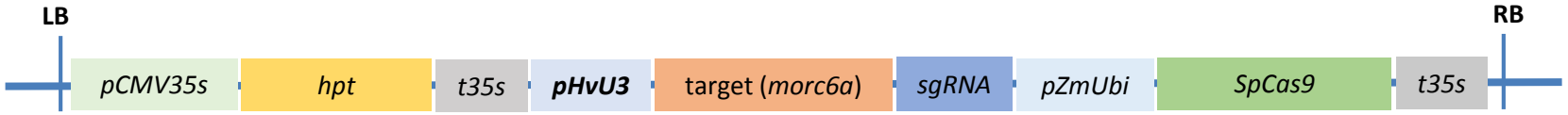
- Barley *MORC1* has a role in genome stabilization, the loss of which results in higher expression of TEs and concomitantly *PR* gene expression.

Kumar, Galli, Ordon, Stuttmann, Kogel & Imani, ***Plant biotechnology journal*** **(2018)**

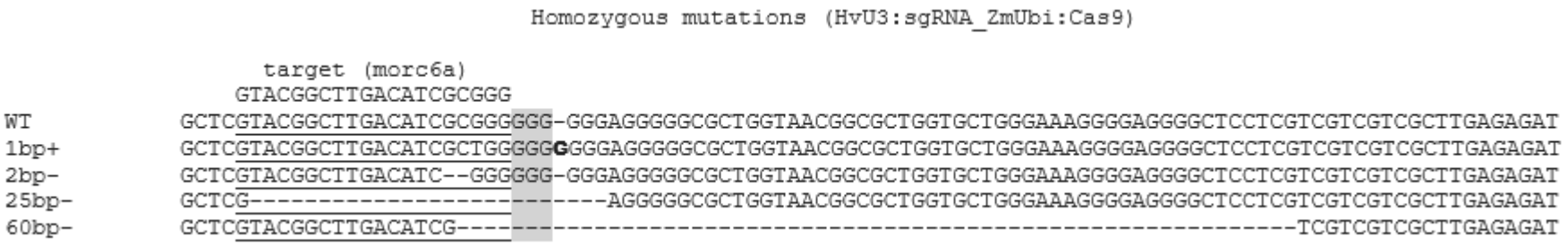
Employing CRISPR/Cas9 system to KO *HvMORC6a*

Preliminary results (I)

Barley (RNA Pol III dependent) *U3* promoters was used for active transcription of sgRNA in barley cells.



Mutation frequency in T0 plants was 73% using p*Hv*U3, with a total of 27% bi-allelic homozygous mutated plants.

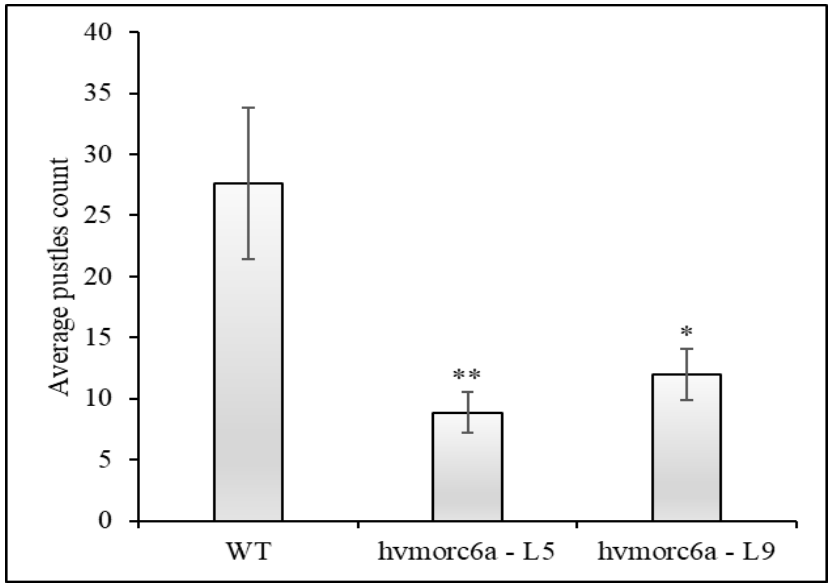


Galli et al., 2019 (unpublished)

Employing CRISPR/Cas9 system to KO *HvMORC6a*

Preliminary results (II)

Mutated *hvmorc6a*-KO T1 plants from p*HvU3*:*sgRNA*_p*ZmUbi*:*Cas9* construct were tested for powdery mildew resistance.



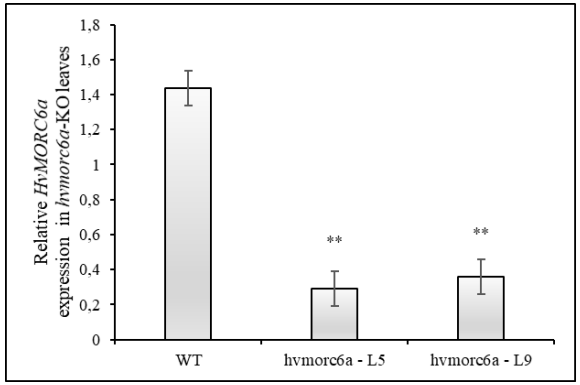
- Complete KO of HvMORC6a enhances plant immunity against bio-trophic fungal pathogens *Blumeria graminis.*

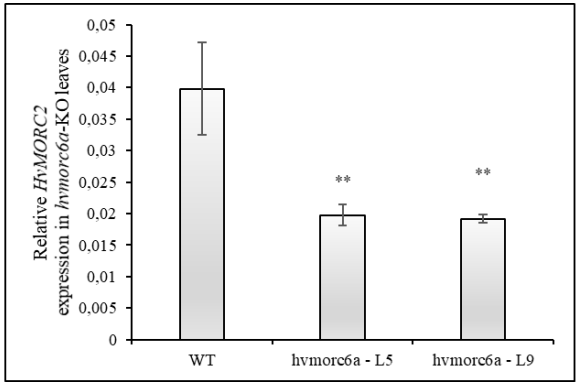
Galli et al., 2019 (unpublished)

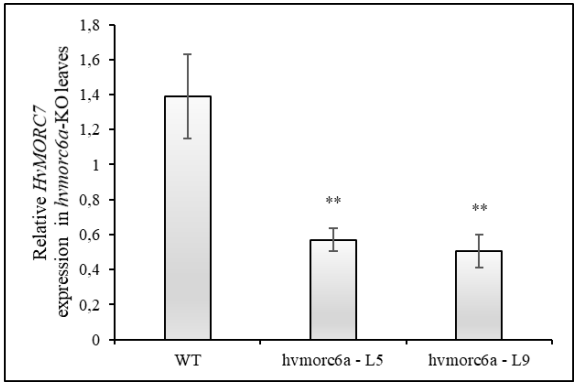
Employing CRISPR/Cas9 system to KO *HvMORC6a*

Preliminary results (III)

Mutated *hvmorc6a*-KO T1 plants show reduced *HvMORC2* and *HvMORC7* expression.







- Possibly HvMORC6a act as a nuclear transcriptional factor.

Galli et al., 2019 (unpublished)

Outlook

Employing CRISPR/Cas9 system to KO both *HvMORC6a* and *HvMORC1*

- Three different strategies will be employed to ensure the achievement of this important step:

  I. conventional crossing of barley homozygous *hvmorc**1*** and *hvmorc**6a*** single mutants

  II. concomitant transformation of “wild-type” barley immature embryos with both CRISPR/Cas9 constructs

  III. transformation of homozygous single mutants with the second construct (*hvmorc**6a*** KO mutants with *hvmorc**1***-construct and vice versa).

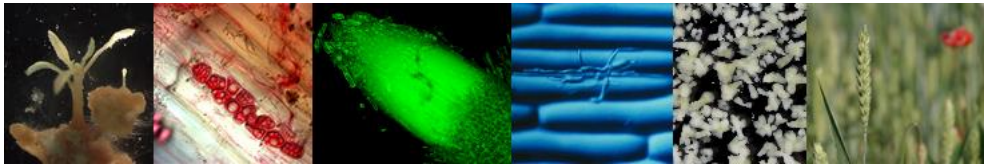
Conclusions

U3 promoters are highly suitable for sgRNA expression in barley genome editing applications, especially barley *U3* promoter.

CRISPR/Cas9 genome editing technology is an efficient tool to modify gene families in cereal crops with potential agronomic applications.

Knock-out *HvMORC1,* as well as *HvMORC6a,* produce a positive impact on fungal pathogen resistance.

Genes expression analysis may indicate that *HvMORC1* and *HvMORC6a*, like *AtMORC1* and *AtMORC6*, are involved in genome stabilization.



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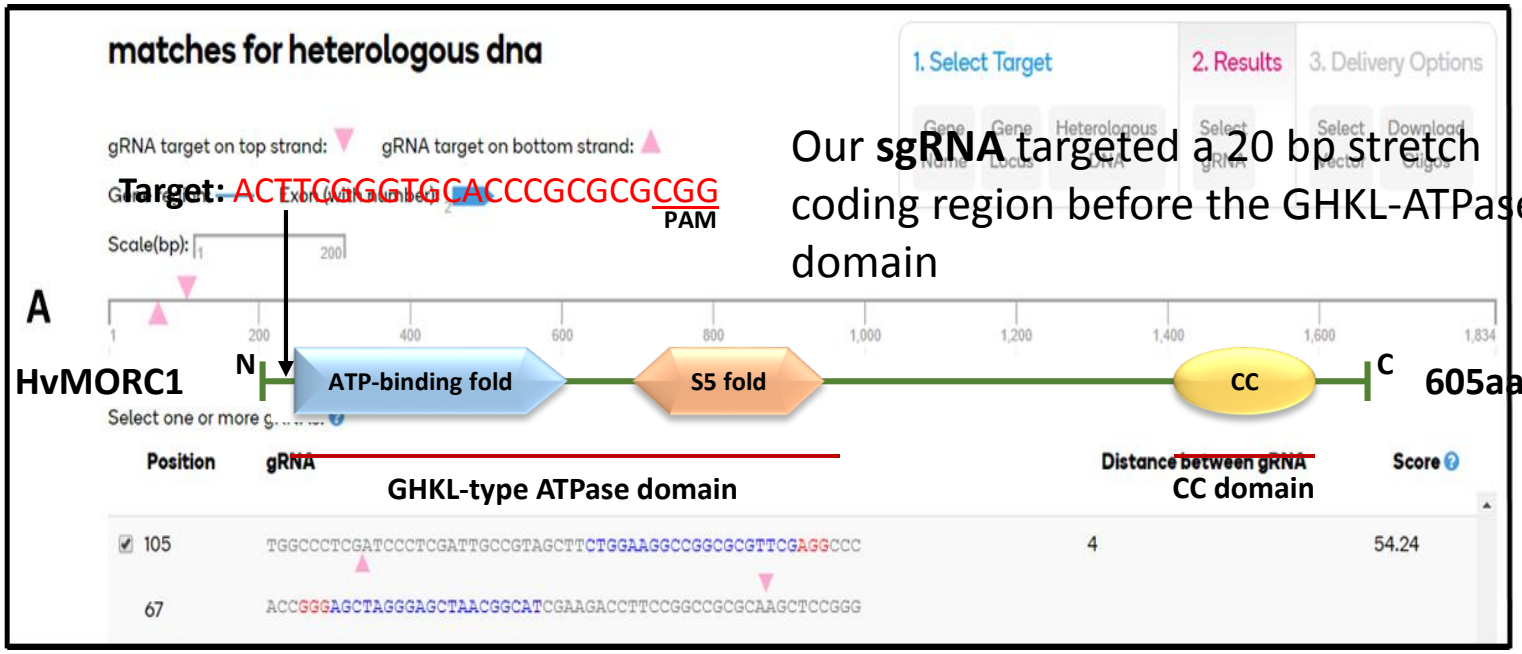
Dr. Neelendra Kumar
B.Sc. Danish Iqbal
and all JLU Phytopatogloy team...

Thank you all for your attention…

Employing CRISPR/Cas9 system to KO *HvMORC1*

Choosing *HvMORC1* 20bp target region

20bp target sequences with NGG (PAM) at 3´end were selected using CRISPR sgRNA design online tool (https://atum.bio/eCommerce/cas9/input)



Kumar, Galli, Ordon, Stuttmann, Kogel & Imani, *Plant biotechnology journal* (2018)