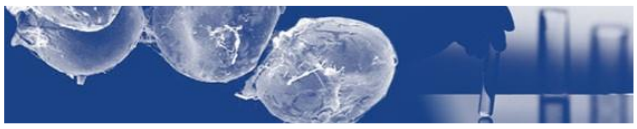
JUSTUS-LIEBIG-UNIVERSITÄT GIESSEN



FB08

USE OF RNA-BASED TECHNOLOGIES FOR TARGETED GENE SILENCING IN CEREALS

Dissertation zur Erlangung des Doktortitels (Dr. rer. nat.) der Naturwissenschaftlichen Fachbereiche der Justus-Liebig-Universität Gießen durchgeführt am Institut für Phytopathologie

vorgelegt von:

M.Sc. Matteo Galli

1. Gutachter:
Prof. Dr. Karl-Heinz Kogel

2. Gutachter:
Prof. Dr. Peter Friedhoff

Justus-Liebig-Universität

Gießen, im Februar 2022

Complete list of publications

Publications relevant to the understanding of this work are marked with an "*****". Contribution to the experimental and writing section is indicated in %.

***** 1. Kumar, N., **Galli, M.**, Ordon, J., Stuttmann, J., Kogel, K.H. and Imani, J., 2018. Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system. *Plant Biotechnology Journal*, 16, pp.1892-1903 – DOI: https://doi.org/10.1111/pbi.12924 (Research paper, experiments 25%, writing 20%).

2. **Galli, M.**, Imani, J. and Kogel, K.H., 2020. Labeling of dsRNA for Fungal Uptake Detection Analysis. *RNA Tagging*, pp.227-238 – DOI: https://doi.org/10.1007/978-1-0716-0712-1_13 (Book chapter, experiments 70%, writing 60%).

***** 3. Kumar, N.†, **Galli, M.**†, Dempsey, D.M., Imani, J., Moebus, A. and Kogel, K.H., 2021. NPR1 is required for root colonization and the establishment of a mutualistic symbiosis between the beneficial bacterium *Rhizobium radiobacter* and barley. *Environmental Microbiology*, 23, pp.2102-2115 – DOI: https://doi.org/10.1111/1462-2920.15356 (Research paper, experiments 40%, writing 30%).
†: these authors contributed equally to the article

4. Zanini, S., Šečić, E., Busche, T., **Galli, M.**, Zheng, Y., Kalinowski, J. and Kogel, K.H., 2021. Comparative analysis of transcriptome and sRNAs expression patterns in the *Brachypodium distachyion – Magnaporthe oryzae* pathosystems. *International Journal of Molecular Sciences*, 22, pp.650 – DOI: https://doi.org/10.3390/ijms22020650 (Research paper, experiments 5%, writing 5%).

***** 5. **Galli, M.**, Martiny, E., Imani, J., Kumar, N., Koch, A., Steinbrenner, J. and Kogel, K.H., 2021. CRISPR/*Sp*Cas9-mediated double knockout of barley Microrchidia MORC1 and MORC6a reveals their strong involvement in plant immunity, transcriptional gene silencing and plant growth. *Plant Biotechnology Journal*, 20, pp.89-102 – DOI: https://doi.org/10.1111/pbi.13697 (Research paper, experiments 60%, writing 60%).

***** 6. **Galli, M.**, Hochstein, S., Iqbal, D., Claar, M., Imani, J. and Kogel, K.H., 2022. CRISPR/*Sp*Cas9-mediated KO of epigenetically active MORC proteins increases barley resistance to *Bipolaris* spot blotch and *Fusarium* root rot. *Journal of Plant Diseases and Protection* – DOI: https://doi.org/10.1007/s41348-022-00574-y (Research paper, experiments 60%, writing 60%).

7. **Galli, M.**†, Jacob, S.†, Zheng, Y., Imani, J., Allasia, V., Keller, H., Coustau, C., Ghezellou, P., Gand, M., Albuquerque, W. W. C., Thines, E., Kogel, K.H., 2022. Chemokine-like MIF1 protein is a key regulator of cellular differentiation and virulence in the fungal pathogen *Magnaporthe oryzae*. *Manuscript under preparation* (Research paper, experiments 40%, writing 60%).

Declaration

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen - Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis - in carrying out the investigations described in the dissertation."

Date Signature of the candidate

Acknowledgement

Throughout my doctoral studies, I have received a lot of support and help from all the staff at the Institute for Phytopathology. I would like to thank you all for your professional and extracurricular assistance during this time.

Abstract

Gene silencing techniques are essential to study gene function and produce crops with desired agronomic traits. Over the past decade, RNA interference (RNAi)-based methods involving experimental modulation of gene expression at the post-transcriptional level have only allowed partial gene silencing. Recently, new biotechnological tools, in particular CRISPR/Cas-based technologies, have become available for precise gene editing. The RNA-directed Cas9 nuclease introduces heritable precise insertions and deletions into the eukaryotic genome that can result both in altered gene function or complete disruption of the codon reading frame.

In this work, an in-depth analysis of the technical aspects and applications of CRISPR/Cas9 and RNAi technologies is performed on the cereal model plant barley (*Hordeum vulgare*). In addition to further evidence for the potential application of RNAi and Cas9-mediated gene silencing, the work also uncovered new roles of Non-expressor of PR1 (NPR1) and the Microrchidia (MORC) protein family in the interaction of barley with microbial pathogens. Specifically, CRISPR/Cas9 was established for precise gene editing in barley and applied to two members of the epigenetically active MORC family. Whereas, the RNAi tool was used to generate barley plants with partial immunodeficiency driven by *NPR1* knockdown. After *Agrobacterium*-mediated transformation, the MORC and NPR1 mutants were functionally characterized and their effects were investigated in plant-microbe interaction and modulation of plant fitness. In CRISPR/Cas9-mediated MORC knockout mutants, we elucidated role of different MORC family members in regulating plant immunity to a broad range of plant pathogens and their role in genome stability. In RNAi-silenced NPR1 plants, its function was investigated during the establishment of the mutualistic symbiosis between barley and the beneficial Alphaproteobacterium *Rhizobium radiobacter* F4 and in induced systemic resistance (ISR). The results presented here suggest that MORC proteins and NPR1 are involved in modulating disease resistance and plant fitness. This reveals potential gene candidates that may contribute to the development of new breeding strategies for higher-yielding and more resistant barley varieties.

Zusammenfassung

Gene Silencing Techniken sind ein wichtiges neues Werkzeug, um die Funktion von Genen zu untersuchen und Nutzpflanzen mit den gewünschten agronomischen Eigenschaften zu erzeugen. In den letzten zehn Jahren haben Methoden auf der Grundlage der RNA-Interferenz (RNAi), mit der eine Veränderung der Genexpression auf posttranskriptioneller Ebene erreicht werden kann, nur eine partielle Abschaltung von Genen ermöglicht. Seit kurzem stehen neue biotechnologische Werkzeuge, insbesondere die CRISPR/Cas-basierten Technologien, für präzises *Gene Editing* zur Verfügung. Die RNA-gesteuerte Cas9 Nuklease führt vererbbare, präzise Insertionen und Deletionen in das eukaryotische Genom ein, die sowohl zu einer veränderten Genfunktion, als auch zu einer vollständigen Unterbrechung des Codon-Leserasters führen können.

In dieser Arbeit wird eine eingehende Analyse der technischen Aspekte und Anwendungen von CRISPR/Cas9- und RNAi-Technologien an der Getreidepflanze Gerste (*Hordeum vulgare*) durchgeführt. Neben weiteren Belegen für die potenzielle Anwendung von RNAi und Cas9-vermitteltem *Gene Silencing* wurden im Rahmen der Arbeit auch neue Funktionen von NPR1 (Non-expressor of PR1) und der Microrchidia (MORC) Proteinfamilie in der Interaktion von Gerste mit mikrobiellen Pathogenen aufgedeckt. Konkret wurde CRISPR/Cas9 für präzises *Gene Editing* in Gerste etabliert und auf zwei Mitglieder der epigenetisch aktiven MORC Familie angewandt. Darüber hinaus wurde das RNAi-Tool zur Erzeugung von Gerstenpflanzen mit partieller Immunschwäche durch Knockdown des *NPR1* Gens eingesetzt. Nach der *Agrobacterium*-vermittelten Transformation wurden die MORC- und NPR1-Mutanten funktionell charakterisiert und die Auswirkungen der Mutationen auf die Interaktion zwischen Pflanzen und Mikroben sowie auf die Modulation der Pflanzenfitness untersucht. Insbesondere haben wir mit Hilfe von CRISPR/Cas9-vermittelten MORC Knockout Mutanten die Rolle verschiedener Mitglieder der MORC Familie bei der Regulierung der pflanzlichen Immunität gegen ein breites Spektrum von Pflanzenpathogenen und ihre Rolle bei der Genomstabilität aufgeklärt. In RNAi-unterdrückten NPR1 Pflanzen wurde seine Funktion während der Etablierung der mutualistischen Symbiose zwischen Gerste und dem nützlichen Alphaproteobakterium *Rhizobium radiobacter* F4 und bei der Induzierten Systemischen Resistenz (ISR) untersucht. Die hier vorgestellten Ergebnisse untermauern die Bedeutung von MORC Proteinen und NPR1 bei der Modulation von Krankheitsresistenz und Pflanzenfitness. Die vorgestellten Arbeiten erweitern unser Verständnis der molekularen Aspekte von Symbiosen und parasitären Interaktionen und weisen auf potenzielle Genkandidaten, die zur

Entwicklung neuer Züchtungsstrategien für ertragreichere und widerstandsfähigere Gerstensorten beitragen könnten.

Abbreviations:

Arabidopsis	*Arabidopsis thaliana*
Avr	Avirulence
Bgh	*Blumeria graminis* f. sp. *hordei*
Bp	Base pair
Bs	*Bipolaris sorokiniana*
Cas	CRISPR-associated protein
cDNA	Complementary DNA
CRISPR	Clustered regularly interspaced short palindromic repeat
crRNA	CRISPR RNA
DAMP	Damage-associated molecular pattern
DCL	DICER-like
dKO	Double knockout
DNA	Deoxyribonucleic acid
DSB	Double-strand breaks
dsRNA	Double stranded RNA
ET	Ethylene
ETI	Effector-triggered immunity
Fg	*Fusarium graminearum*
gRNA	Guide RNA
HR	Hypersensitive response
Hv	*Hordeum vulgare*
hvmorc1	Barley MORC1 KO mutant generated using CRISPR/Cas9
hvmorc1/6a	Barley MORC1/MORC6a dKO mutant generated using CRISPR/Cas9
hvmorc6a	Barley MORC6a KO mutant generated using CRISPR/Cas9
hvnpr1	Barley NPR1 KD mutant generated using RNAi
*Hv*U3	Barley U3 RNA polymerase III-type promoter
ISR	Induced systemic resistance
JA	Jasmonic acid
KD	Knockdown
KO	Knockout
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MORC	Microrchidia

mRNA	Messenger RNA
NB-LRR	Nucleotide-binding leucine-rich repeat
NHEJ	Non-homologous end joining
NHR	Non-host resistance
NPR1	Non-expressor of PR1
nt	Nucleotide
*Os*U3	Rice U3 RNA polymerase III-type promoter
PAM	Protospacer-adjacent motif
PAMP	Pathogen-associated molecular pattern
PRs	*Pathogenesis related*
PRR	Pattern recognition receptor
PTGS	Post-transcriptional gene silencing
PTI	PAMP-triggered immunity
RdDM	RNA-directed DNA methylation
RISC	RNA-induced silencing complex
RITS	RNA-induced transcriptional silencing complex
RNAi	RNA interference
ROS	Reactive oxygen species
*Rr*F4	*Rhizobium radiobacter* F4
SA	Salicylic acid
SAR	Systemic acquired resistance
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Sp	*Streptococcus pyogenes*
TALEN	Transcription activator-like effector nuclease
T-DNA	Transferred DNA
TE	Transposable element
TGS	Transcriptional gene silencing
tracrRNA	Transactivating crRNA
WT	Wild type
ZFN	Zinc finger nuclease

Table of contents

General introduction

The productivity of crops, especially cereals, is under constant threat. Microbial pathogens (fungi, bacteria, viruses), pests (nematodes, insects) and weeds are in constant co-evolutionary competition with crop plants for dominance in the landscape and in the phytobiome (van der Heijden and Hartmann, 2016). According to their lifestyles, plant microbes are generally divided into necrotrophs, biotrophs and mutualists. With the exception of mutualists, where the inter-species collaboration is beneficial to all species partners, all other relationships between plants and microbes are pathogenic. Necrotrophs colonise their host and often kill it through the production of toxins and cell-wall-degrading enzymes to feed on their contents. While biotrophic pathogens invade living plant cells and through specialized feeding structures (*haustorium*) obtain sugars and amino acids from the living host tissues. There is another class of plant pathogens, hemibiotrophs, which use both strategies to feed and thrive on their host.

To breach host barriers, microbes employ offensive physical and chemical weapons and after penetration, parasitism and host resistance run parallel until severe disease develops, the host dies or the microbe is excluded (Dangl and Jones, 2001). This constant race for survival caused plants to develop an extensive and sophisticated array of strategies to perceive attacks and translate this information into an adaptive response. Unlike mammals, plants do not have an adaptive and mobile immune system but rely on the innate immunity of each individual cell and on systemic signals originating from the infection site. It is therefore crucial that the plant reacts promptly to the threat by containing the spread of infection and triggering an immune response. The standard model that summarizes how plants fend themselves from pathogens is the "two-branched [plant] innate immune system" or "zig-zag model" (Jones and Dangl, 2006). Here, the response generated by the plant begins after the recognition of molecules common to many classes of microbes. The triggered signal is masked by virulence effectors of the pathogen that have evolved to suppress it, and these in turn are recognised by another group of plant immune proteins. This hide-and-seek balance regulates the interaction between plant and microbe.

The plant-microbe interaction and the plant immune system

Pathogens use different strategies to invade and multiply in the intercellular plant space (the apoplast). They range from brute-force mechanisms to highly specialised delivery structures. Fungi can form appressoria, dome-like cells that use turgor pressure to puncture the plant cuticle and penetrate the underlying epidermal cell. Several types of microbes can penetrate through stomata, natural openings and wounds present on the plant leaf. Bacteria and viruses

can be transmitted by vectors, most often insects, and many more. Once inside, they self-replicate and secrete effector molecules (virulence factors) to increase microbial fitness and plant susceptibility. Plants, in turn, employ a variety of physical and molecular techniques to repel, contain and fight pathogens. Typical of non-host resistance (NHR), structural elements in the plant body such as the cuticle layer and cork cells, as well as constitutively produced secondary metabolites such as saponins (steroid molecules with antifungal activity), they are the common and passive defence mechanisms employed by plants (Mysore and Ryu, 2004). Cellular transmembrane pattern recognition receptors (PRRs) are the first layer of specialised immune-related proteins. These surface localised receptor-like kinases bind to common microbe-, pathogen- or damage-associated molecular patterns (MAMPs, PAMPs or DAMPs) such as flagellin, chitin or endogenous plant signals and activate the innate immune signalling cascade. Finally, within the cell, polymorphic nucleotide-binding leucine-rich repeat (NB-LRR) proteins form the second layer of resistance proteins of the plant immune system. NB-LRRs recognise pathogen effectors, such as avirulence proteins (Avr) and mediate a strong local defence response (Göhre and Robatzek, 2008; Dodds and Rathjen, 2010).

Independent of the signalling pathways activated in the plant cell, both PAMP-triggered immunity (PTI; recognition of pathogen elicitors) and effector-triggered immunity (ETI; recognition of pathogen effectors) lead to a positive regulation of disease resistance. Following a downstream cascade of mitogen-activated protein kinases (MAPKs), the initial stimulus is carried from the site of infection to the cell nucleus. Here, it is converted into multiple responses, like the up-regulation of the expression of defence-related genes, local cell wall appositions (*papillae*), the increase in cytosolic Ca^{++} levels, the production of reactive oxygen species (ROS), the regulation of plant hormonal networks and, as last resource, the hypersensitive response (HR; localised programmed cell death at the site of infection) (Zhang and Klessig, 2001; Jones and Dangl, 2006). These actions aim to prevent the pathogen from growing and spreading to other cells.

Plant hormones play a central role in regulating developmental process and sustaining the plant immune signalling network. The exact concentration or timing of their induction can determine the susceptibility or resistance of plant tissue to the invading pathogen. The major plant defense hormones are salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Other hormones that also play a role in plant signalling are gibberellins, brassinosteroids, auxins, abscisic acid, cytokinins and nitric oxide (Verhage et al., 2010; Pieterse et al., 2012). SA, JA and ET are involved in plant responses to a variety of biotic and abiotic stresses. With regard to microbes, defence against biotrophic and hemibiotrophic pathogens is largely due to SA-dependent

mechanisms. PTI or ETI induction triggers SA biosynthesis and increased expression of *Pathogenesis Related* (*PRs*) genes. Positive transcription of defence-related antimicrobial genes leads to long-lasting broad-spectrum resistance in both local and distal tissues. This type of defence is called systemic acquired resistance (SAR) and prevents the development of secondary infections into undamaged plant tissues. Finally, high SA accumulation drives HR of the host infected tissue (Vlot et al., 2009; Pieterse et al., 2012). In contrast, necrotrophic pathogens are not limited by HR, but by defence reactions activated by JA and ET signals (Glazebrook, 2005; Pieterse et al., 2012). JA is synthesised from lipids and oxylipin and also plays an important role in the response to mechanical wounding and herbivory. As with the SA pathway, hormonal modulation of JA triggers global changes in gene expression (Howe and Jander, 2008). Last but not least, mutualists and root beneficial microorganisms such as the plant growth-promoting fungus *Piriformospora* (syn. *Serendipita*) *indica* or the Alphaproteobacterium *Rhizobium radiobacter*, also trigger JA hormonal modulation of host immunity. Here, recruitment of the JA pathway is necessary to suppress both early and late defence mechanisms, including SA-mediated defence system (Jacobs et al., 2011; Glaeser et al., 2016). The JA signalling pathway, activated at the site of wounding by herbivores or in root after colonisation by beneficial microbes, triggers a similar JA-dependent response in distal or undamaged aboveground plant parts. It is called induced systemic resistance (ISR). (Conrath et al., 2006; Van Wees et al., 2008; Pieterse et al., 2014).

Gene silencing as a tool for gene discovery

Although plants have developed multiple defence mechanisms to combat microbes and pathogens, systematic losses due to pests and diseases occur annually in agriculture. These range from about 50% in wheat to more than 80% in cotton production (Oerke et al., 2012). Progress in deciphering plant immunity and gene functions is therefore crucial for safeguarding crop protection and increasing agricultural productivity. Since Morgan's discovery of genes on chromosomes and heritable traits, scientists have been working to locate (map) genes in organisms. In the last century, classical genetics linked a particular phenotype to a gene by crossing with individuals carrying the same unusual trait (forward genetics). The advent of sequencing projects such as the Human Genome Project (Lander et al., 2001) opened up new possibilities in loss-of-function genetics. New gene-based approaches aim to discover their function by selectively disrupting a particular gene (and/or its expression) and studying the resulting phenotypes (reverse genetic). This is called gene silencing. Currently, the most

extensively used gene silencing methods are RNA interference (RNAi) and site-directed genome engineering (Gaj et al., 2013; Boettcher and McManus, 2015).

RNA interference

RNAi is a conserved endogenous biological process. Here, small antisense RNA molecules with a length of 21-24 nucleotides (nt) are used to inhibit the transcription of nuclear genes (transcriptional gene silencing, TGS) or to degrade cytoplasmic messenger RNA (mRNA; post-transcriptional gene silencing, PTGS) (Castel and Martienssen, 2013). This self-regulatory mechanism of the cell controls gene expression in response to environmental or metabolic stimuli and helps protect the cell from viral infections and transposable elements (TEs) (Almeida and Allshire, 2005; Umbach and Cullen, 2009). The breakthrough study on the mechanism of RNAi led to the award of the 2006 Nobel Prize in Physiology or Medicine to Andrew Fire and Craig Mello (Fire et al., 1998). The trigger for RNAi is the recognition of an exogenous or endogenous double stranded RNA fragment (dsRNA). This is then processed by a DICER-nuclease mediated cleavage (in plants: DICER-like or DCL) into smaller interfering RNA duplexes (siRNAs). Finally, the siRNAs are loaded onto ARGONAUTE-containing complexes and directed to the silencing mRNA target. If a target is found by nucleotide pairing between the antisense siRNA and the mRNA sequence, ARGONAUTE is activated and cleaves the mRNA or stops its transcription. The multiprotein complexes like the RNA-induced silencing complex (RISC) or the RNA-induced transcriptional silencing complex (RITS) control gene silencing at the translational and transcriptional level, respectively (Fang and Qi, 2016). Recently, it was found that small RNAs also play a crucial role in many interactions between plants and microbes (Weiberg et al., 2013; Wang et al., 2017; Zanini et al., 2021). Small RNAs are exchanged between pathogen and host and vice versa to suppress immune/virulence-related genes. This phenomenon is called "cross-kingdom RNA communication".

RNAi as a reverse genetics technique is instead a transgene-mediated method of gene silencing based on PTGS. Here, the RNAi machinery of the target organism is hijacked by introducing synthetic dsRNA molecules or an RNAi construct to modulate cytoplasmic mRNA expression. The conserved domain of the target gene can be used to make short hairpin RNA (shRNA) constructs for RNAi-mediated silencing. Weaker gene expression thus translates to quantitatively lower protein production (knockdown, KD). Over the past decade, RNAi has been used extensively in several organisms to study gene function. It provides good tissue specificity when the appropriate promoter is used, can silence multiple members of a gene

family simultaneously and is not hindered by changes in chromatin structure (Setten et al., 2019). Importantly, it can also target mRNA transcript independently of ploidy, which is particularly valuable for plant research (McGinnis et al., 2007; Wang et al., 2014). However, RNAi comes with major disadvantages. It is limited only to targets located in the cytoplasm of the cell and is not well suited for targeting RNAi-associated genes. Finally, saturation of the RNAi pathway by overexpression of a transgene can displace the cell's endogenous microRNAs from the RISC, resulting in an off-target phenotype (Unniyampurath et al., 2016). Due to these numerous limitations, novel tools for directly modifying the genetic code of an organism are attracting great interest in the scientific community. These site-specific mutagenesis technologies are slowly displacing RNAi from its dominant position as gene expression modifying technologies in plant research.

Genome engineering using site-directed nucleases

Site-specific genome engineering is the modification of an organism's DNA at a specific, predetermined location. Here, the targeted change in gene expression is a secondary product of the modification and can lead to partial or complete silencing of gene expression (knockout, KO) (Doudna and Charpentier, 2014). Genome engineering utilizes genetically modified nucleases to generate double-strand break (DSB) at a predefined target, which then triggers the cellular DNA repair mechanism. Depending on the DNA repair pathway and the presence of a template, this can lead to gene disruption, insertion, correction and chromosomal rearrangement. In eukaryotic cells, there are two distinct DSB repair pathways: the error-prone non-homologous end joining (NHEJ) and the high fidelity homologous recombination. In NHEJ, the two DNA strands are simply rejoined together. In the presence of overhangs, NHEJ results in the insertion or deletion of random base pairs, which can disrupt the codon reading frame. Homologous recombination instead uses an exogenous or endogenous DNA template to repair the damaged DNA. This method can introduce precise gene modifications at the DSB site (Bortesi and Fischer, 2015).

Until a few years ago, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were the most commonly used programmable site-specific technologies for genome tailing (Kim et al., 1996; Christian et al., 2010). Hybrid restriction enzymes formed by fusion of a DNA cleavage domain (FokI) with multiple customisable DNA binding motifs, zinc fingers and transcription activator-like effectors, respectively. While each ZF motif recognises a 3 bp stretch, TALE motif can recognise a single nucleotide and thus produce a one-to-one match. Because FokI is active only as a dimer, pairs of site-specific molecules are

required, each located on one strand of the DNA sequence to produce the DSB (Bortesi and Fischer, 2015). Once the mutagenesis has been introduced, programmable site-specific activity within the target cell is no longer required. In contrast to the transient nature of RNAi, where loss of siRNA equals loss of the induced phenotype, this allows the generation of new mutant plants in which the transgene is naturally lost via segregation (non-transgenic). Although each of these two platforms has led to important scientific advances and discoveries, the construction of DNA recognition segments remains difficult, time-consuming and far from routine. In 2013, the new CRISPR genome editing technology was used for the first time in plant research (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013). This novel and incredibly powerful site-specific mutagenesis technology, modelled on the adaptive immune system of bacteria, promises to rapidly improve many organisms, including agricultural crops (Chen et al., 2019).

The CRISPR/Cas system

Bacteriophages attack bacteria to insert their genetic code into the bacterial genome and to use them as factories for the production of new phages. When a bacterium survives a viral attack, it captures small fragments of the foreign DNA and stores these sequences in its own genetic code. A saved foreign DNA sequence implicated in the defence system of prokaryotic organisms against viruses is called CRISPR (clustered regularly interspaced short palindromic repeats). In the event of a de novo attack by a similar bacteriophage, the bacterium rapidly generates an RNA copy from the CRISPR archive (crRNA) and loads it into a CRISPR-associated endonuclease protein (Cas). The RNA-loaded Cas then scans the inside of the bacterium looking for complementary DNA or RNA sequences. If it finds a perfect match, it is activated and cuts out the target, rendering it useless and protecting the bacterium (Horvath and Barrangou, 2010). The CRISPR system gained prominence as a genome editing technology when scientists discovered that the crRNA sequence could be programmed and adapted to target any sequence in an organism (Doudna and Charpentier, 2014). In recent years, research into CRISPR diversity has led to a constant evolution of its classification. Current categorization divides CRISPR/Cas systems into two distinct classes. Class 1, where the interference is mediated by several small proteins that form the effector complex, and class 2, where a single, large protein with multiple domains is responsible for generating the DSB. Each class is then further partitioned into several subtypes, with different Cas proteins targeting single- and double-stranded DNA or RNA (Koonin et al., 2017). The class 2 CRISPR/Cas9 type II system from *Streptococcus pyogenes* (*Sp*Cas9) quickly became the most widely used CRISPR tool for genome editing. To achieve interference, only a target-specific crRNA, a

target-independent transactivating crRNA (tracrRNA), and a dual RNA-directed DNA endonuclease Cas enzyme (*Sp*Cas9) are required. It was shown that the dual tracrRNA:crRNA can also be constructed as a single guide RNA chimera (gRNA) to drive sequence-specific Cas9-dsDNA cleavage, further facilitating the genome editing process (Jinek et al., 2012). An important prerequisite for Cas-directed DNA cleavage is the presence of a conserved 2 - 6 base pair protospacer-adjacent motif (PAM) downstream of the target sequence. In *Sp*Cas9, the PAM normally carries the sequence 5'-NGG-3', where "N" stands for any nucleobase and "G" for the nucleobase guanine (Anders et al., 2014). The development and application of the CRISPR/Cas9 method for genome editing led to the award of the 2020 Nobel Prize in Chemistry to Emmanuelle Charpentier and Jennifer Doudna. Meanwhile, it has been successfully used to edit both the human and plant genomes (Demirer et al., 2021). Generation of knockout mutants in higher crop plants such as cereals has never been widely available compared to dicotyledonous plants such as Arabidopsis (*Arabidopsis thaliana*). Immersing thousands of inflorescences in an *Agrobacterium* suspension, followed by rapid antibiotic- or herbicide-selection, is not suitable for higher crop plants with longer growing periods. The efficiency and specificity of CRISPR/Cas9 now allow anyone to generate mutants of many monocotyledonous species, including wheat, rice and barley (Chen et al., 2019; Lawrenson and Harwood, 2019).

Barley as a cereal model for crop improvement

Barley is a member of the Poaceae family and, along with rice and wheat, is one of the oldest domesticated cereals. Cultivated barley (*Hordeum vulgare* L. subsp. *vulgare*), used for both human nutrition and animal feed, is the fourth most widely grown cereal in the world (FAOstat, 2021). It is a self-pollinating crop with a diploid genome, and in 2012 the International Barley Sequencing Consortium (IBSC) made the genetic data of barley cv. "Morex" for the first time publicly available (Mayer et al., 2012).

Barley grows in many temperate regions of the world and is less susceptible to abiotic stresses than many other cereals (Sallam et al., 2019). Due to this widespread distribution and high production volume, barley is a host for a vast variety of pathogens and insect pests that attack the plant at all stages of growth. The main fungal foliar diseases include powdery mildew (*Blumeria graminis*), rusts (*Puccinia* species), barley leaf streak disease (*Pyrenophora graminea*), leaf scald (*Rhynchosporium commune*) and spot blotch (*Bipolaris sorokiniana*) (Walters et al., 2012). Head blight (*Fusarium* species) and covered smut (*Ustilago hordei*) are the main infections affecting ears, heads and seeds of barley (Hollaway et al., 2013; Lanver et

al., 2017). Necrotrophic *Fusarium* species are a particularly important target in barley crop protection research. They not only cause rot in the bulbs and roots but also contaminate the grains with mycotoxins, affecting their quality and availability (Gaffar et al., 2019). In recent years, numerous molecular plant breeding approaches have been built on barley. Robust, simple and reproducible protocols for barley transformation have been developed, making barley an excellent model platform for reverse genetics and functional genomics in cereal research (Lawrenson and Harwood, 2019).

Recently, our group has used both RNAi and CRISPR/*Sp*Cas9 technologies for gene silencing in barley. Each tool targeted a different gene involved in the regulation of the plant immune system. *Non-expressor of PR1* (*HvNPR1*) was the focus of our RNAi construct (Kumar et al., 2020), while two members of the epigenetically active barley *Microrchidia* family (*HvMORC1* and *HvMORC6a*) were the objects of study for the CRISPR/*Sp*Cas9 application (Kumar et al., 2018; Galli et al., 2021; Galli et al., 2022).

Exploring NPR1's role in mutualistic symbiosis, a RNAi approach

As we previously mentioned, plant defense mechanisms can be broadly categorized into two distinct pathways: SA-induced SAR and JA-triggered ISR. SAR is induced in distant uninfected tissues of plants by a prior pathogen challenge and by upregulation of *PRs* expression. ISR on the other hand is primarily stimulated by pests (insects) damage or non-pathogenic root-colonizing microbes and requires components of JA and ET pathways (van Loon et al., 2006). The downstream signalling component NPR1 is a fundamental transcriptional regulatory protein in SAR and *PRs* expression (Cao et al., 1994; Pieterse et al., 2012). Normally NPR1 dimers are sequestrated in the cytoplasm in form of oligomers and when a small amount of NPR1 monomers accidentally moves into the cell nucleus, they are targeted to destruction in the proteasome after ubiquitin tagging (Spoel et al., 2009). Proteasome-mediated turnover of NPR1 is important for preventing autoimmunity in absence of pathogen threat. SA-induced ROS burst increases NPR1 monomerization and nuclear translocation (Tada et al., 2008) and ubiquitin-specific protease stabilize NPR1 by removing ubiquitin chains that signal for its proteasome-mediated degradation (Skelly et al., 2019). In the nucleus, NPR1 acts together with transcriptional activators TGAs which bind to specific 5'-TGA-3' DNA sequences for the transcription of *PRs* and the induction of SAR (Després et al., 2000; Kinkema et al., 2000; Klessig et al., 2018). NPR1 is also an important regulator in the establishment of ISR. Here no defense-related proteins are present in induced leaves before pathogen challenge but upon infection JA-responsive genes activation is faster and stronger –

a phenomenon called priming (Conrath et al., 2002; van Loon et al., 2006). Experimental evidence of the role of NPR1 in priming relays mainly on studies on dicot species, while its function during establishment of mutualistic symbioses between cereals and beneficial microbes is poorly investigated. Furthermore, SAR in cereals was not detected in systemic leaves, but rather in the region adjacent to the initial infection site (Gao et al., 2018). Employing RNAi-mediated barley NPR1 KD plants (Dey et al., 2014) we investigated whether this redox-sensitive protein is involved in establishing mutualistic symbioses between the cereal plant barley and the Alphaproteobacterium *Rhizobium radiobacter* (*Rr*F4). *Rr*F4 was isolated the first time from the sebacinoid basidiomycete fungus *Piriformospora indica* (Weiß et al., 2016). Later, further experiments indicated that the bacterium can also install relationship with plant roots without host preference (Glaeser et al., 2016). *Rr*F4-colonized barley has enhanced biomass and disease resistance to powdery mildew fungus *B. graminis* f.sp. *hordei* (*Bgh*; Glaeser et al., 2016).

Employing NPR1 knockdown (KD-*hvnpr1*) plants we were able to reveal that the NPR1 protein plays a fundamental role in establishing and supporting the mutualistic symbiosis between barley and *Rr*F4. Our results indicated that, i. KD-*hvnpr1* roots, compared to WT, supported substantially fewer bacterial cells and displayed an atypical spatiotemporal colonization pattern; ii. following inoculation, in KD-*hvnpr1* leaves and roots local and systemic expression of SA-induced marker genes, such as *HvPR1b, HvPR2* and *HvPR5* was lowered in colonized plants compared to untreated ones; iii. *Rr*F4-induced root-initiated systemic resistance against *Bgh*A6 was jeopardized in plants with aberrant *NPR1* gene expression, compared to the untreated counterpart; besides these immune-related differences, iv. KD-*hvnpr1* plants produced higher root and shoot biomass, but largely lost the *Rr*F4-mediated growth promotion effects (Kumar et al., 2020). Overall, our findings suggest that *Hv*NPR1 plays important roles in both modulating the tissue-specific capacity for successful *Rr*F4 colonization, as well as transducing the signal for *Rr*F4-induced immune and growth responses in barley.

Functional analysis of the barley MORCs, employing CRISPR/*Sp*Cas9

The nuclear regulator Microrchidia (MORC) protein family compared to the NPR family is involved in regulation of disease resistance as a bioproduct of its main role. Initially discovered to be essential in mice's spermatogenesis and genital development (hence the name, Watson et al., 1998; Inoue et al., 1999), MORC proteins have now been identified in many prokaryotes and eukaryotes (Langen et al., 2014; Dong et al., 2018). Studies of MORC functional analysis

in mammals and plants connected these proteins to the RNA-directed DNA methylation (RdDM) pathway, a branch of the cell TGS machinery (for review see Koch et al., 2017; Kumar et al., 2018; Galli et al., 2021; Xue et al., 2021; Galli et al., 2022). RdDM machinery acts directly on the cell DNA and establishes a repressive form of chromatin (euchromatin). This compaction of the histone-DNA molecules prevents the DNA/RNA polymerases from accessing the DNA, resulting in a very high degree of gene repression (Du et al., 2015; Erdmann and Picard, 2020). Non-coding RNA molecules recruit the RdDM complex at DNA loci complementary to the RNAs. There, the enzyme DNA methyltransferase methylates cytosines in the nearby genetic code. Increased DNA methylation triggers local protein-mediated genome changes in chromatin structure. The eukaryotic RdDM pathway is involved in both de novo and maintenance of DNA methylation loci. This type of epigenetic gene regulation is fundamental for genome integrity, gene expression and the repression of *TEs* (Matzke and Mosher, 2014). In Arabidopsis, MORC proteins have been shown to be required to repress *TEs* expression (Moissiard et al., 2012). Recently, MORCs were also shown to be necessary to establish de novo DNA methylation over newly integrated transgenes (Xue et al., 2021). Initial awareness of the role of MORCs in plants came with the discovery of their involvement in regulating plant immunity (Kang et al., 2008; Kang et al., 2010). More recently, the increased susceptibility has been explained by plant delayed and/or reduced expression of several defense marker genes following plant infection (Kang et al., 2012, Bordiya et al., 2016). Interestingly, MORCs affect plant immunity in species-specific manners. Silencing of MORCs in barley, tomato and *N. benthamiana* enhances ETI or basal resistance, while it has a negative effect on immunity in Arabidopsis and potato (Langen et al., 2014; Manosalva et al., 2015). Consistent with this, transient overexpression of *Hv*MORCs compromised resistance to *Bgh* (Langen et al., 2014).

To overcome the unavailability of respective MORC KO mutants in cereals, we used the *Sp*Cas9-mediated gene-editing approach in barley to target two of the seven *HvMORC* family members. The initial sequence of each target was selected for mutagenesis to disrupt the gene reading frame and characterise their function in plant immunity and epigenetics (Kumar et al., 2018; Galli et al., 2021; Galli et al., 2022). Single and simultaneous *Agrobacterium*-mediated transformation with the two CRISPR constructs achieved KO and double KO (dKO) of barley MORC1 and MORC6a proteins. As expected, barley *morc1*, *morc6a* and *morc1/6a* mutants showed positive resistance to a vast variety of different plant diseases: i. powdery mildew caused by *Bgh* (Kumar et al., 2018; Galli et al., 2021), ii. *Fusarium* leaf spot and *Fusarium* root rot by the necrotrophic *Fusarium graminearum* (Galli et al., 2021; Galli et al., 2022) and iii.

Bipolaris spot blotch caused by the hemibiotrophic pathogen *Bipolaris sorokiniana* (Galli et al., 2022). However, the increased immunity of the plants and the enhanced expression of *PRs* came at a considerable cost to plant fitness. *Hvmorc* mutants displayed overexpression of *TEs*, synonym of disrupted genome stability, and exhibited reduced leaf and root development (Kumar et al., 2018; Galli et al., 2021). Finally, we also presented important advances in MORC protein localisation and interaction with other elements of the RdDM pathway using the mutant background (Galli et al., 2021). It is interesting and worth mentioning that dKO mutants did not show an enhanced additive defence phenotype compared to single KO mutants (Galli et al., 2021; Galli et al., 2022). This is consistent with previous reports in Arabidopsis and barley suggesting that MORC proteins require each other's activity (Moissiard et al., 2014; Galli et al., 2021).

Although a direct comparison between the two studies is not possible due to the different objectives, it still allows us to confront the advantages and limitations of the two technologies.

RNAi *vs.* CRISPR/*Sp*Cas9 to study gene function in barley

Both loss-of-function reverse genetics approaches were successful and delivered novel information defining the target gene function. Nevertheless, these two technologies differ fundamentally in the way they work. Is therefore essential to analyze advantages and limitations of both techniques to chart a predictive picture about their future application for gene manipulations and crop improvements.

Knockdown and knockout

The most important difference between RNAi and CRISPR/*Sp*Cas9 is their impact on the targeted gene. While the first induces modification in the post-transcriptional gene expression machinery, *Sp*Cas9 introduces permanent and heritable changes on the target genome. In our RNAi-targeted barley *NPR1* study, we show results from two different transgenic lines. After gene expression analysis, we confirmed that the line KD-*hvnpr1* E7L2 lost almost 68% of transcript accumulation, whereas KD-*hvnpr1* E11L9 displayed a 53% loss (Fig. 2, Kumar et al., 2020). In the case of slow protein degradation or strong protein activity, a successful decrease in transcript levels may not necessarily lead to a sufficient reduction in protein amounts to trigger an effect. Weaker silencing on barley *NPR1*, as in KD-*hvnpr1* E11L9, produced a weaker impact on plant yield and growth (Fig. S6, Kumar et al., 2020). Similarly, the number of bacteria colonizing KD-*hvnpr1* E11L9 transgenic plants was slightly (not statistically significant) higher compared to KD-*hvnpr1* E7L2. Another example, partial

silencing of MORC1 in barley had little or no effect on the up-regulation of *TEs* (Fig. 10, Langen et al., 2014). Full KO of *Hv*MORC1 instead exhibited robust de-repression of transposable elements (Kumar et al., 2018; Galli et al., 2021). To determine the function of a target gene, CRISPR/*Sp*Cas9 might therefore be more suitable than RNAi. However, the RNAi approach may be a more convenient solution to study essential genes whose depletion would otherwise be lethal to the organism or as a crop improvement technology. Complete KO of *Hv*MORC6a or dKO of *Hv*MORC1 and *Hv*MORC6a had positive effects on disease resistance of barley, but a high price for plant fitness and growth (Fig 7, Galli et al., 2021). From a breeder's point of view, yield is the most important criterion for plant selection. In the case of MORC genes, partial silencing of these proteins may be a better strategy: a positive effect on plant immunity without harmful disruption of genome stability (Langen et al., 2014).

Technical and methodological aspects

Planning and screening of gene expression alteration are also important parameters to consider when choosing one or the other application. While the *Agrobacterium*-mediated transformation approach is similar for both technologies, less genomic information is required for the development of the RNAi construct than for the CRISPR construct (Qi et al., 2013). Accurate protein transcripts are more easily accessible and available than the corresponding gene sequence with its intron and exon distribution. In our case, to generate KD-*hvnpr1* plants described in Dey et al. (2014), the authors used a conserved domain of barley NPR1 with a length of 387 bp derived from the cDNA sequence. To knockout the two barley MORC genes, the designed 20 bp target sequences were obtained from the nucleotide collection of *Hordeum vulgare*. We selected the region immediately after the protein start site of the target genes (Fig. S1b, Galli et al., 2021). Since barley is a fully sequenced organism, we were able to confirm that the 20 bp long target region did not target an intron sequence and that no intron was present in the target region. Therefore, different but appropriate information is needed for the planning and design of the individual target system. After target modification, screening of genome editing activities, especially in early transgenic lines, needs to be evaluated. Amplification of the target region and sequencing of the amplicon is a cost-effective strategy for selecting homozygous KO clones (Fig. 5, Kumar et al., 2018; Fig. 1, Galli et al., 2021). *Sp*Cas9-mediated gene editing can introduce many different types of mutations into each transformed cell. Heterozygous mutations are the most common and are usually indicated by the presence of double (or more) peaks in the sequence chromatogram (Fig. 5c, Kumar et al., 2018; Fig. S2d, Galli et al., 2021). Different mutations on different chromosome strands could still lead to only

partial suppression of mRNA transcript levels. Therefore, several generations are required for segregation of homozygous mutations. In RNAi time-consuming sequencing of the targeted region is not required. Confirmation of transgene presence and silencing effect are the only objectives of the analysis. However, since gene silencing is not a permanent change, but depends on the RNAi construct, the presence of the transgene must be verified in each plant generation previous experiments (Fig. 2, Kumar et al., 2020).

Gene silencing for crop improvement also raises significant legal and health concerns about the presence of transferred DNA (T-DNA) or transgene in genetically modified crops (Jones, 2015). In Europe, several member states have special restrictions on genetically modified organisms (GMO). Just recently, the EU Commission launched a policy action on plants produced by targeted mutagenesis and cisgenesis to propose a new regulatory framework for GMO plants (Legislation for plants produced by certain new genomic techniques, Legislative proposal). In contrast to RNAi tools, genome editing technologies such as the CRISPR system allow the production of mutants without the need for a persistent T-DNA construct (Jones, 2015). Detectability of the T-DNA region in mutant organisms is particularly critical to ensure traceability of GMOs. We were able to identify *Sp*Cas9-edited plants without the T-DNA construct as early as the T1 generation (Kumar et al., 2018; Galli et al., 2021). Conveniently, the loss of T-DNA and thus antibiotic resistance allowed us to re-transform homozygous single mutants devoid of the T-DNA with the second mutagenesis construct. This allowed rapid antibiotic selection of the new mutants and introduction of the second modification in plants only two-generation-old (Galli et al., 2021).

Specificity and off-target effects

It is widely established that both technologies are associated with off-target effects (Castanotto and Rossi, 2009; Hsu et al., 2013). In both approaches, design optimization using computer-aided prediction software can be a valuable strategy to increase efficiency and specificity (Si-fi for RNAi, Galli et al., 2020; CRISPR gRNA Design tool; Kumar et al., 2018 and Galli et al., 2021). Hsu and coworkers (2013) also demonstrated that the specificity of genome editing also depends on the amount of gRNA transcripts and *Sp*Cas9 proteins in the cell (Hsu et al., 2013). To ensure efficient and specific transcription, we drove production of gRNAs in barley cells under the control of the barley and rice U3 RNA polymerase III-type promoter (*HvU3*; *OsU3*) of the small nuclear RNA. The coding *Sp*Cas9 sequence was driven by the maize ubiquitin promoter (*ZmUbi*) (Fig. 3, Kumar et al., 2018; Fig. S1a, Galli et al., 2021). A comparison between expression of gRNA under control of either the *HvU3* or *OsU3* promoter showed

clearly enhanced gRNA accumulation by *HvU3* promoter-driven expression in barley and increase in mutagenesis (Fig. 6, Kumar et al., 2018). In our study, the use of both CRISPR constructs resulted in efficient CRISPR/*Sp*Cas9-mediated multiple gene editing and did not affect the gene activity of other *MORC* paralogs (Fig. 1b, Galli et al., 2021). In Kumar et al. (2020), we also analyzed the specificity of our RNAi construct in KD-*hvnpr1* plants. After the in silico identification of new Arabidopsis *NPRs* paralogs in barley (Fig. 1, Kumar et al., 2020), we investigated possible off-target effects in these genes. As with the CRISPR system, our RNAi approach did not alter the expression of either *HvNPR2*, *HvNPR3*, *HvNPR5*, and *HvNPR6* (Fig. 2b, Kumar et al., 2020). We can only speculate that there were no other off-target effects outside of the target genes that were silenced by both the CRISPR and RNAi tools. Blast alignment analysis and off-target prediction using software revealed no evidence of an inaccurate match between silencing constructs and non-target sequences.

General discussion

Barley is the fourth most important cereal in the world and is grown in more than 100 countries. In recent decades, barley has become a model for research in cereal plant science due to its numerous scientific advantages: i. plant diploidy and relatively low chromosome number (14); ii. time-efficient and routine transformation protocols; iii. multiple high-quality sequence assemblies of the pan-genome infrastructure; and iv. ease of cultivation under a wide range of climatic conditions (Lawrenson and Harwood, 2019). In plants molecular biology, targeted gene silencing using RNA-directed nucleases in agronomically important barley cultivars is playing an increasingly crucial role in discovering useful traits to withstand both biotic and abiotic stresses (Chen et al., 2019). In addition, the advent of faster and higher-throughput sequencing methods, coupled with powerful and reliable bioinformatics tools, offers the opportunity to study gene function and its relationship to disease development in greater detail. In the past, the study of a gene's function started from its mutant phenotype and worked back through crosses to a gene or a gene cluster (forward genetics). Today, the gold standard for deciphering gene function is to disrupt normal gene expression (overexpression, knockdown, and knockout) to study the resulting phenotypes (reverse genetics).

CRISPR/Cas9, the tool for exploring new frontiers in plant science

More than 80% of the barley genome is classified as derived from TEs, while genes cover only 2-3% of it. The remaining 17-18% is still unannotated (Mascher et al., 2017). It comes with no surprise that more and more evidence indicates that TE activity is also important in response

to exogenous environmental and genomic stresses (Dubey and Jeon, 2017, Galindo-González et al., 2017). Epigenetic regulation of plant defense has so far been neglected in breeding because the mechanism is still unexplored and very complex. There is a lack of studies highlighting the role of epigenetics in the modulation of the growth and pathogenicity of fungal plant pathogens. In plants as in mammals, key player in maintenance of DNA stability concerning DNA methylation and TEs regulation is the RdDM pathway. ARGONAUTE-mediated TGS machinery recruits RdDM molecules to ultimately influence chromatin compaction and repression of TEs (Matzke and Mosher, 2014). RNAi tools are not well-suited for silencing genes directly involved with TGS or PTGS. CRISPR on the other hand is not hampered by those proteins and can also target loci where the chromatin is in a compacted state (Hsu et al., 2013). Our research exemplarily demonstrates how this novel tool can be employed to improve and/or explore new modulators of barley genome stability for agronomic purposes (Kumar et al., 2018; Galli et al., 2021; Galli et al., 2022). MORC-mediated epigenetic regulation fine-tunes disease resistance to a very broad range of fungal pathogens with different lifestyles (*Blumeria graminis*, *Fusarium graminearum* and *Bipolaris sorokiniana*). We hypothesized that such "non-hormonal" resistance is due to loose suppression of *TEs* associated with increased basal barley *PRs* expression. An always-active immune system, even as a secondary product of a more general instability in gene expression, upsets the trade-off between plant growth and defense (Huot et al., 2014). In agreement with the results of Kumar et al. (2020), where plants in which *HvNPR1* was knocked down had better growth fitness, here de-repression of MORC-related genes was linked with lower plant biomass of both plant roots and shoots.

RNAi cannot be used to alter genetic elements, such as non-transcribed, non-coding regulatory regions. In contrast to RNAi, CRISPR/Cas9 can be used to alter non-transcribed genomic segments and characterize individual proteins involved in the RdDM pathway and other epigenetics mechanisms. Understanding the molecular basis of these interactions in plants should enable new breeding strategies for developing cereal varieties with an optimal balance between growth and defense to maximize crop yields and meet the growing global demand for food and biofuels.

Conclusion

CRISPR/Cas9 and RNAi are the two best known and most widely used RNA-based molecular tools available to breeders today for loss-of-function experiments. Each tool has its own advantages and drawbacks and can cause a temporary and/or permanent effect on the

expression of the target gene. In the last two decades, RNAi has been considered by many scientists as the best approach to study genes by disrupting their gene expression. The emergence of a new, incredibly powerful mutagenesis tool modeled after the adaptive immune system of bacteria, the CRISPR/Cas system, is displacing RNAi from its dominant position. Undoubtedly, the versatility and programmability of Cas9 have made CRISPR the preferred platform for gene modifying approaches. The routine use of this novel technology for breeding purposes is still a long way off, but there is no evidence-based doubt that this technology will be a fundamental part of any plant breeder's toolbox in the future. New and better CRISPR-based methods are constantly being developed, which will lead to dominate RNAi in the long term.

References

Almeida, R. and Allshire, R.C., 2005. RNA silencing and genome regulation. *Trends in Cell Biology*, 15, pp.251-258.

Anders, C., Niewoehner, O., Duerst, A. and Jinek, M., 2014. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature*, 513, pp.569-573.

Boettcher, M. and McManus, M.T., 2015. Choosing the right tool for the job: RNAi, TALEN, or CRISPR. *Molecular Cell*, 58, pp.575-585.

Bordiya, Y., Zheng, Y., Nam, J.C., Bonnard, A.C., Choi, H.W., Lee, B.K., Kim, J., Klessig, D.F., Fei, Z. and Kang, H.G., 2016. Pathogen infection and MORC proteins affect chromatin accessibility of transposable elements and expression of their proximal genes in Arabidopsis. *Molecular Plant-Microbe Interactions*, 29, pp.674-687.

Bortesi, L. and Fischer, R., 2015. The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnology Advances*, 33, pp.41-52.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X., 1994. Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *The Plant Cell*, 6, pp.1583-1592.

Castanotto, D. and Rossi, J.J., 2009. The promises and pitfalls of RNA-interference-based therapeutics. *Nature*, 457, pp.426-433.

Castel, S.E. and Martienssen, R.A., 2013. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. *Nature Reviews Genetics*, 14, pp.100-112.

Chen, K., Wang, Y., Zhang, R., Zhang, H. and Gao, C., 2019. CRISPR/Cas genome editing and precision plant breeding in agriculture. *Annual Review of Plant Biology*, 70, pp.667-697.

Christian, M., Cermak, T., Doyle, E.L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A.J. and Voytas, D.F., 2010. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics*, 186, pp.757-761.

Conrath, U., Beckers, G.J., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M.A., Pieterse, C.M., Poinssot, B., Pozo, M.J. and Pugin, A., 2006. Priming: getting ready for battle. *Molecular Plant-Microbe Interactions*, 19, pp.1062-1071.

Conrath, U., Pieterse, C.M. and Mauch-Mani, B., 2002. Priming in plant-pathogen interactions. *Trends in Plant Science*, 7, pp.210-216.

Dangl, J.L. and Jones, J.D., 2001. Plant pathogens and integrated defence responses to infection. *Nature*, 411, pp.826-833.

Demirer, G.S., Silva, T.N., Jackson, C.T., Thomas, J.B., Ehrhardt, D.W., Rhee, S.Y., Mortimer, J.C. and Landry, M.P., 2021. Nanotechnology to advance CRISPR-Cas genetic engineering of plants. *Nature Nanotechnology*, 16, pp.243-250.

Després, C., DeLong, C., Glaze, S., Liu, E. and Fobert, P.R., 2000. The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *The Plant Cell*, 12, pp.279-290.

Dey, S., Wenig, M., Langen, G., Sharma, S., Kugler, K.G., Knappe, C., Hause, B., Bichlmeier, M., Babaeizad, V., Imani, J. and Janzik, I., 2014. Bacteria-triggered systemic immunity in barley is associated with WRKY and ETHYLENE RESPONSIVE FACTORs but not with salicylic acid. *Plant Physiology*, 166, pp.2133-2151.

Dodds, P.N. and Rathjen, J.P., 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature Reviews Genetics*, 11, pp.539-548.

Dong, W., Vannozzi, A., Chen, F., Hu, Y., Chen, Z. and Zhang, L., 2018. MORC domain definition and evolutionary analysis of the MORC gene family in green plants. *Genome Biology and Evolution*, 10, pp.1730-1744.

Doudna, J.A. and Charpentier, E., 2014. The new frontier of genome engineering with CRISPR-Cas9. *Science*, 346 pp.1077.

Du, J., Johnson, L.M., Jacobsen, S.E. and Patel, D.J., 2015. DNA methylation pathways and their crosstalk with histone methylation. *Nature Reviews Molecular Cell Biology*, 16, pp.519-532.

Dubey, A. and Jeon, J., 2017. Epigenetic regulation of development and pathogenesis in fungal plant pathogens. *Molecular Plant Pathology*, 18, pp.887-898.

Erdmann, R.M. and Picard, C.L., 2020. RNA-directed DNA methylation. *PLoS Genetics*, 16, pp.1009034.

Fang, X. and Qi, Y., 2016. RNAi in plants: an Argonaute-centered view. *The Plant Cell*, 28, pp.272-285.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391, pp.806-811.

Gaffar, F.Y., Imani, J., Karlovsky, P., Koch, A. and Kogel, K.H., 2019. Different components of the RNA interference machinery are required for conidiation, ascosporogenesis, virulence, deoxynivalenol production, and fungal inhibition by exogenous double-stranded RNA in the head blight pathogen *Fusarium graminearum*. *Frontiers in Microbiology*, 10, pp.1662.

Gaj, T., Gersbach, C.A. and Barbas III, C.F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology*, 31, pp.397-405.

Galindo-González, L., Mhiri, C., Deyholos, M.K. and Grandbastien, M.A., 2017. LTR-retrotransposons in plants: engines of evolution. *Gene*, 626, pp.14-25.

Galli, M., Imani, J. and Kogel, K.H., 2020. Labeling of dsRNA for Fungal Uptake Detection Analysis. *RNA Tagging*, pp.227-238.

Galli, M., Martiny, E., Imani, J., Kumar, N., Koch, A., Steinbrenner, J. and Kogel, K.H., 2021. CRISPR/*Sp*Cas9-mediated double knockout of barley Microrchidia MORC1 and MORC6a reveals their strong involvement in plant immunity, transcriptional gene silencing and plant growth. *Plant Biotechnology Journal*, 20, pp.89-102.

Galli, M., Hochstein, S., Iqbal, D., Claar, M., Imani, J. and Kogel, K.H., 2022. CRISPR/*Sp*Cas9-mediated KO of epigenetically active MORC proteins increases barley resistance to *Bipolaris* spot blotch and *Fusarium* root rot. *Journal of Plant Diseases and Protection*, pp.1-7.

Gao, J., Bi, W., Li, H., Wu, J., Yu, X., Liu, D. and Wang, X., 2018. WRKY transcription factors associated with NPR1-mediated acquired resistance in barley are potential resources to improve wheat resistance to *Puccinia triticina*. *Frontiers in Plant Science*, pp.1486.

Glaeser, S.P., Imani, J., Alabid, I., Guo, H., Kumar, N., Kämpfer, P., Hardt, M., Blom, J., Goesmann, A., Rothballer, M. and Hartmann, A., 2016. Non-pathogenic *Rhizobium radiobacter* F4 deploys plant beneficial activity independent of its host *Piriformospora indica*. *The ISME Journal*, 10, pp.871-884.

Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, 43, pp.205-227.

Göhre, V. and Robatzek, S., 2008. Breaking the barriers: microbial effector molecules subvert plant immunity. *Annual Review of Phytopathology*, 46, pp.189-215.

Hollaway, G.J., Evans, M.L., Wallwork, H., Dyson, C.B. and McKay, A.C., 2013. Yield loss in cereals, caused by *Fusarium culmorum* and *F. pseudograminearum*, is related to fungal DNA in soil prior to planting, rainfall, and cereal type. *Plant Disease*, 97, pp.977-982.

Horvath, P. and Barrangou, R., 2010. CRISPR/Cas, the immune system of bacteria and archaea. *Science*, 327, pp.167-170.

Howe, G.A. and Jander, G., 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology*, 59, pp.41-66.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O. and Cradick, T.J., 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology*, 31, pp.827-832.

Huot, B., Yao, J., Montgomery, B.L. and He, S.Y., 2014. Growth-defense tradeoffs in plants: a balancing act to optimize fitness. *Molecular Plant*, 7, pp.1267-1287.

Inoue, N., Hess, K.D., Moreadith, R.W., Richardson, L.L., Handel, M.A., Watson, M. and Zinn, A.R., 1999. New gene family defined by MORC, a nuclear protein required for mouse spermatogenesis. *Human Molecular Genetics*, 8, pp.1201-1207.

Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Lipka, V., Kogel, K.H. and Schäfer, P., 2011. Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus *Piriformospora indica*. *Plant Physiology*, 156, pp.726-740.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E., 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 337, pp.816-821.

Jones, H.D., 2015. Regulatory uncertainty over genome editing. *Nature Plants*, 1, pp.1-3.

Jones, J.D. and Dangl, J.L., 2006. The plant immune system. *Nature*, 444, pp.323-329.

Kang, H.G., Choi, H.W., Von Einem, S., Manosalva, P., Ehlers, K., Liu, P.P., Buxa, S.V., Moreau, M., Mang, H.G., Kachroo, P. and Kogel, K.H., 2012. CRT1 is a nuclear-translocated MORC endonuclease that participates in multiple levels of plant immunity. *Nature Communications*, 3, pp.1-11.

Kang, H.G., Kuhl, J.C., Kachroo, P. and Klessig, D.F., 2008. CRT1, an Arabidopsis ATPase that interacts with diverse resistance proteins and modulates disease resistance to turnip crinkle virus. *Cell Host and Microbe*, 3, pp.48-57.

Kang, H.G., Oh, C.S., Sato, M., Katagiri, F., Glazebrook, J., Takahashi, H., Kachroo, P., Martin, G.B. and Klessig, D.F., 2010. Endosome-associated CRT1 functions early in resistance gene-mediated defense signaling in Arabidopsis and tobacco. *The Plant Cell*, 22, pp.918-936.

Kim, Y.G., Cha, J. and Chandrasegaran, S., 1996. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proceedings of the National Academy of Sciences*, 93, pp.1156-1160.

Kinkema, M., Fan, W. and Dong, X., 2000. Nuclear localization of NPR1 is required for activation of PR gene expression. *The Plant Cell*, 12, pp.2339-2350.

Klessig, D.F., Choi, H.W. and Dempsey, D.M.A., 2018. Systemic acquired resistance and salicylic acid: past, present, and future. *Molecular Plant-Microbe Interactions*, 31, pp.871-888.

Koch, A., Kang, H.G., Steinbrenner, J., Dempsey, D.M.A., Klessig, D.F. and Kogel, K.H., 2017. MORC proteins: novel players in plant and animal health. *Frontiers in Plant Science*, 8, pp.1720.

Koonin, E.V., Makarova, K.S. and Zhang, F., 2017. Diversity, classification and evolution of CRISPR-Cas systems. *Current Opinion in Microbiology*, 37, pp.67-78.

Kumar, N., Galli, M., Dempsey, D.M., Imani, J., Moebus, A. and Kogel, K.H., 2021. NPR1 is required for root colonization and the establishment of a mutualistic symbiosis between the beneficial bacterium *Rhizobium radiobacter* and barley. *Environmental Microbiology*, 23, pp.2102-2115.

Kumar, N., Galli, M., Ordon, J., Stuttmann, J., Kogel, K.H. and Imani, J., 2018. Further analysis of barley MORC 1 using a highly efficient RNA-guided Cas9 gene-editing system. *Plant Biotechnology Journal*, 16, pp.1892-1903.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. and Funke, R., 2001. Initial sequencing and analysis of the human genome. *Nature*, 409, pp.860-921.

Langen, G., von Einem, S., Koch, A., Imani, J., Pai, S.B., Manohar, M., Ehlers, K., Choi, H.W., Claar, M., Schmidt, R. and Mang, H.G., 2014. The compromised recognition of turnip crinkle virus 1 subfamily of microrchidia ATPases regulates disease resistance in barley to biotrophic and necrotrophic pathogens. *Plant Physiology*, 164, pp.866-878.

Lanver, D., Tollot, M., Schweizer, G., Presti, L.L., Reissmann, S., Ma, L.S., Schuster, M., Tanaka, S., Liang, L., Ludwig, N. and Kahmann, R., 2017. *Ustilago maydis* effectors and their impact on virulence. *Nature Reviews Microbiology*, 15, pp.409-421.

Lawrenson, T. and Harwood, W.A., 2019. Creating targeted gene knockouts in barley using CRISPR/Cas9. *In Barley*, pp.217-232.

Li, J.F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M. and Sheen, J., 2013. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and *Nicotiana benthamiana* using guide RNA and Cas9. *Nature Biotechnology*, 31, pp.688-691.

Lück, S., Kreszies, T., Strickert, M., Schweizer, P., Kuhlmann, M. and Douchkov, D., 2019. siRNA-Finder (si-Fi) software for RNAi-target design and off-target prediction. *Frontiers in Plant Science*, pp.1023.

Manosalva, P., Manohar, M., Kogel, K.H., Kang, H.G. and Klessig, D.F., 2015. The GHKL ATPase MORC1 modulates species-specific plant immunity in Solanaceae. *Molecular Plant-Microbe Interactions*, 28, pp.927-942.

Mascher, M., Gundlach, H., Himmelbach, A., Beier, S., Twardziok, S.O., Wicker, T., Radchuk, V., Dockter, C., Hedley, P.E., Russell, J. and Bayer, M., 2017. A chromosome conformation capture ordered sequence of the barley genome. *Nature*, 544, pp.427-433.

Matzke, M.A. and Mosher, R.A., 2014. RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nature Reviews Genetics*, 15, pp.394-408.

Mayer, K.F., Waugh, R., Langridge, P., Close, T.J., Wise, R.P., Graner, A., Matsumoto, T., Sato, K., Schulman, A., Muehlbauer, G.J. and Stein, N., 2012. A physical, genetic and functional sequence assembly of the barley genome. *Nature*, 491, pp.711-716

McGinnis, K., Murphy, N., Carlson, A.R., Akula, A., Akula, C., Basinger, H., Carlson, M., Hermanson, P., Kovacevic, N., McGill, M.A. and Seshadri, V., 2007. Assessing the efficiency of RNA interference for maize functional genomics. *Plant Physiology*, 143, pp.1441-1451.

Moissiard, G., Bischof, S., Husmann, D., Pastor, W.A., Hale, C.J., Yen, L., Stroud, H., Papikian, A., Vashisht, A.A., Wohlschlegel, J.A. and Jacobsen, S.E., 2014. Transcriptional gene silencing by Arabidopsis microrchidia homologues involves the formation of heteromers. *Proceedings of the National Academy of Sciences*, 111, pp.7474-7479.

Moissiard, G., Cokus, S.J., Cary, J., Feng, S., Billi, A.C., Stroud, H., Husmann, D., Zhan, Y., Lajoie, B.R., McCord, R.P. and Hale, C.J., 2012. MORC family ATPases required for heterochromatin condensation and gene silencing. *Science*, 336, pp.1448-1451.

Mysore, K.S. and Ryu, C.M., 2004. Nonhost resistance: how much do we know?. *Trends in plant Science*, 9, pp.97-104.

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J.D. and Kamoun, S., 2013. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nature Biotechnology*, 31, pp.691-693.

Oerke, E.C., Dehne, H.W., Schönbeck, F. and Weber, A., 2012. Crop production and crop protection: estimated losses in major food and cash crops. *Elsevier*.

Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S.C., 2012. Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology*, 28, pp.489-521.

Pieterse, C.M., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C. and Bakker, P.A., 2014. Induced systemic resistance by beneficial microbes. *Annual review of Phytopathology*, 52, pp.347-375.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P. and Lim, W.A., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*, 152, pp.1173-1183.

Sallam, A., Alqudah, A.M., Dawood, M.F., Baenziger, P.S. and Börner, A., 2019. Drought stress tolerance in wheat and barley: advances in physiology, breeding and genetics research. *International Journal of Molecular Sciences*, 20, pp.3137.

Setten, R.L., Rossi, J.J. and Han, S.P., 2019. The current state and future directions of RNAi-based therapeutics. *Nature Reviews Drug Discovery*, 18, pp.421-446.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., Qiu, J.L. and Gao, C., 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology*, 31, pp.686-688.

Skelly, M.J., Furniss, J.J., Grey, H., Wong, K.W. and Spoel, S.H., 2019. Dynamic ubiquitination determines transcriptional activity of the plant immune coactivator NPR1. *Elife*, 8, pp.47005.

Spoel, S.H., Mou, Z., Tada, Y., Spivey, N.W., Genschik, P. and Dong, X., 2009. Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell*, 137, pp.860-872.

Tada, Y., Spoel, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J. and Dong, X., 2008. Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. *Science*, 321, pp.952-956.

Umbach, J.L. and Cullen, B.R., 2009. The role of RNAi and microRNAs in animal virus replication and antiviral immunity. *Genes and development*, 23, pp.1151-1164.

Unniyampurath, U., Pilankatta, R. and Krishnan, M.N., 2016. RNA interference in the age of CRISPR: will CRISPR interfere with RNAi?. *International Journal of Molecular Sciences*, 17, pp.291.

van der Heijden, M.G. and Hartmann, M., 2016. Networking in the plant microbiome. *PLoS Biology*, 14, pp.1002378.

van Loon, L.C., Rep, M. and Pieterse, C.M., 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology*, 44, pp.135-162.

Van Wees, S.C., Van der Ent, S. and Pieterse, C.M., 2008. Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology*, 11, pp.443-448.

Verhage, A., van Wees, S.C. and Pieterse, C.M., 2010. Plant immunity: it's the hormones talking, but what do they say? *Plant Physiology*, 154, pp.536-540.

Vlot, A.C., Dempsey, D.M.A. and Klessig, D.F., 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology*, 47, pp.177-206.

Walters, D.R., Avrova, A., Bingham, I.J., Burnett, F.J., Fountaine, J., Havis, N.D., Hoad, S.P., Hughes, G., Looseley, M., Oxley, S.J. and Renwick, A., 2012. Control of foliar diseases in barley: towards an integrated approach. *European Journal of Plant Pathology*, 133, pp.33-73.

Wang, M., Thomas, N. and Jin, H., 2017. Cross-kingdom RNA trafficking and environmental RNAi for powerful innovative pre-and post-harvest plant protection. *Current Opinion in Plant Biology*, 38, pp.133-141.

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C. and Qiu, J.L., 2014. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnology*, 32, pp.947-951.

Watson, M.L., Zinn, A.R., Inoue, N., Hess, K.D., Cobb, J., Handel, M.A., Halaban, R., Duchene, C.C., Albright, G.M. and Moreadith, R.W., 1998. Identification of morc (microrchidia), a mutation that results in arrest of spermatogenesis at an early meiotic stage in the mouse. *Proceedings of the National Academy of Sciences*, 95, pp.14361-14366.

Weiberg, A., Wang, M., Lin, F.M., Zhao, H., Zhang, Z., Kaloshian, I., Huang, H.D. and Jin, H., 2013. Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science*, 342, pp.118-123.

Weiß, M., Waller, F., Zuccaro, A. and Selosse, M.A., 2016. Sebacinales-one thousand and one interactions with land plants. *New Phytologist*, 211, pp.20-40.

Xue, Y., Zhong, Z., Harris, C.J., Gallego-Bartolomé, J., Wang, M., Picard, C., Cao, X., Hua, S., Kwok, I., Feng, S. and Jami-Alahmadi, Y., 2021. Arabidopsis MORC proteins function in the efficient establishment of RNA directed DNA methylation. *Nature Communications*, 12, pp.1-13.

Zanini, S., Šečić, E., Busche, T., Galli, M., Zheng, Y., Kalinowski, J. and Kogel, K.H., 2021. Comparative Analysis of Transcriptome and sRNAs Expression Patterns in the *Brachypodium distachyion-Magnaporthe oryzae* Pathosystems. *International Journal of Molecular Sciences*, 22, pp.650.

Zhang, S. and Klessig, D.F., 2001. MAPK cascades in plant defense signaling. *Trends in Plant Science*, 6, pp.520-527.

Publications

Publications are listed in order of acceptance.

1. Kumar, N., **Galli, M.**, Ordon, J., Stuttmann, J., Kogel, K.H. and Imani, J., 2018. Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system. *Plant Biotechnology Journal*, 16, pp.1892-1903.
   DOI: https://doi.org/10.1111/pbi.12924

2. Kumar, N.†, **Galli, M.**†, Dempsey, D.M., Imani, J., Moebus, A. and Kogel, K.H., 2021. NPR1 is required for root colonization and the establishment of a mutualistic symbiosis between the beneficial bacterium *Rhizobium radiobacter* and barley. *Environmental Microbiology*, 23, pp.2102-2115.
   †: authors contributed equally.
   DOI: https://doi.org/10.1111/1462-2920.15356

3. **Galli, M.**, Martiny, E., Imani, J., Kumar, N., Koch, A., Steinbrenner, J. and Kogel, K.H., 2021. CRISPR/*Sp*Cas9-mediated double knockout of barley Microrchidia MORC1 and MORC6a reveals their strong involvement in plant immunity, transcriptional gene silencing and plant growth. *Plant Biotechnology Journal*, 20, pp.89-102.
   DOI: https://doi.org/10.1111/pbi.13697

4. **Galli, M.**, Hochstein, S., Iqbal, D., Claar, M., Imani, J. and Kogel, K.H., 2022. CRISPR/*Sp*Cas9-mediated KO of epigenetically active MORC proteins increases barley resistance to *Bipolaris* spot blotch and *Fusarium* root rot. *Journal of Plant Diseases and Protection*.
   DOI: https://doi.org/10.1007/s41348-022-00574-y

Plant Biotechnology Journal

Plant Biotechnology Journal (2018) **16**, pp. 1892–1903 doi: 10.1111/pbi.12924

Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system

Neelendra Kumar[1], Matteo Galli[1], Jana Ordon[2], Johannes Stuttmann[2], Karl-Heinz Kogel[1,*] and Jafargholi Imani[1,*]

[1]*Research Centre for BioSystems, Land Use and Nutrition, Institute of Phytopathology, Justus-Liebig University Giessen, Giessen, Germany*

[2]*Institute of Genetics, Martin Luther University of Halle-Wittenberg, Halle, Saale, Germany*

Received 30 August 2017; accepted 18 March 2018.
**Correspondence* (Tel +49 641 9937490; fax +49 641 9937499; email Karl-Heinz.Kogel@agrar.uni-giessen.de [K.-H.K.] and Tel +49 641 9937571; fax +49 641 9937499; email Jafargholi.Imani@agrar.uni-giessen.de [J.I.])

Keywords: CRISPR/Cas9, gene-editing, barley, wheat, rice, MORC1, *Blumeria*, *Fusarium*.

Summary

Microrchidia (MORC) proteins comprise a family of proteins that have been identified in prokaryotes and eukaryotes. They are defined by two hallmark domains: a GHKL-type ATPase and an S5-fold. In plants, MORC proteins were first discovered in a genetic screen for *Arabidopsis thaliana* mutants compromised for resistance to a viral pathogen. Subsequent studies expanded their role in plant immunity and revealed their involvement in gene silencing and genome stabilization. Little is known about the role of MORC proteins of cereals, especially because knockout (KO) mutants were not available and assessment of loss of function relied only on RNAi strategies, which were arguable, given that MORC proteins in itself are influencing gene silencing. Here, we used a *Streptococcus pyogenes* Cas9 (*Sp*Cas9)-mediated KO strategy to functionally study *HvMORC1*, one of the current seven *MORC* members of barley. Using a novel barley RNA Pol III-dependent *U3* small nuclear RNA (snRNA) promoter to drive expression of the synthetic single guide RNA (sgRNA), we achieved a very high mutation frequency in *HvMORC1*. High frequencies of mutations were detectable by target sequencing in the callus, the T0 generation (77%) and T1 generation (70%–100%), which constitutes an important improvement of the gene-editing technology in cereals. Corroborating and extending earlier findings, *Sp*Cas9-edited *hvmorc1*-KO barley, in clear contrast to Arabidopsis *atmorc1* mutants, had a distinct phenotype of increased disease resistance to fungal pathogens, while *morc1* mutants of either plant showed de-repressed expression of transposable elements (TEs), substantiating that plant MORC proteins contribute to genome stabilization in monocotyledonous and dicotyledonous plants.

Introduction

Gene-editing methods have arisen as an efficient tool for rapid analysis of gene function. From the agricultural perspective, these new methods can be harnessed to create crop plants with desired traits for agronomic purposes with significantly less undesirable side effects on the plant genome. While traditional plant breeding methods involve chemical and radiation mutagenesis that often create random deleterious and chimeric mutations across genomes, modern gene-editing tools allow precise modification of the genome at a desired position (Lowder *et al.*, 2015; Malzahn *et al.*, 2017; Xiong *et al.*, 2015). Genome modification requires an engineered nuclease to create double-strand breaks (DSBs) at defined targets, which then triggers cellular DNA repair mechanism, depending on the DNA repair pathway and presence of a repair template. There are two known DSB repair pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ in most instances leads to random insertions or deletions (indels) of nucleotides at the repair site. In case DSB generates overhangs, NHEJ can also introduce gene insertions or precise gene modifications with a double-stranded DNA fragment with compatible overhangs (Cristea *et al.*, 2013; Maresca *et al.*, 2013). In the presence of a DNA template with homology to the separated chromosome ends, DSBs can be repaired by HR, although this mechanism is rather exceptional at least in somatic cells. Nevertheless, this process can be used to insert DNA fragments and precisely modify genes (Bortesi and Fischer, 2015).

Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have shown promising results in achieving site-directed DNA breaks. Both enzymes use a dimeric *Fok*1 nuclease for creating DNA breaks (Christian *et al.*, 2010; Smith *et al.*, 2000). In 2013, the type II *clustered regularly interspaced short palindromic repeat* (CRISPR)-associated Cas9 system was discovered in *Streptococcus pyogenes* (*Sp*), which emerged as a powerful tool to induce precise mutations in the human genome (Cong *et al.*, 2013; Mali *et al.*, 2013). It promises high on-target activity and low off-target effects compared to RNAi (Smith *et al.*, 2017). Subsequent implementation of *Sp*Cas9 as RNA-guided, sequence-specific nuclease (SSN) for genome editing in plants led to comparably fast and reliable results (Li *et al.*, 2013). *Sp*Cas9-mediated DNA editing involves introduction of two components, the Cas9 protein and a synthetic single guide RNA (sgRNA), into the target cell (genome) to be mutated. The sgRNA (~80 nucleotide [nt] total length) consists of a ~20 nt sequence with sequence similarity to the target gene and a synthetic RNA sequence that adopts functions of CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) of the original bacterial system (Deltcheva *et al.*, 2011; Jinek *et al.*, 2012; Sorek *et al.*, 2013). *Sp*Cas9 induces DSBs by recruiting the sgRNA. An important requirement for DNA cleavage is the presence of a conserved protospacer adjacent motif (PAM), usually carrying the sequence 5′-NGG-3′ (for *Sp*Cas9) downstream of the target DNA (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012). Since 2013, the *Sp*Cas9 system has successfully been applied for gene-editing in

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.

plants such as *Arabidopsis thaliana*, tobacco and tomato (Brooks *et al.*, 2014; Li *et al.*, 2013; Nekrasov *et al.*, 2013), as well as cereals such as rice, wheat, barley and sorghum (Jiang *et al.*, 2013; Miao *et al.*, 2013; Zhang *et al.*, 2014; reviewed in Ma *et al.*, 2016; Malzahn *et al.*, 2017). Engineering disease resistance in major crops is especially promising because many resistant traits are recessively inherited (Hückelhoven *et al.*, 2003; van Schie and Takken, 2014). A prominent example is powdery mildew resistance in cereals, which is conferred by recessive alleles of the locus *mildew-o* (*mlo*; Acevedo-Garcia *et al.*, 2014). Significantly, *Sp*Cas9-mediated simultaneously editing each of the three *Mlo* homeologs in allohexaploid bread wheat (*Triticum aestivum*) resulted in *mlo*-based disease resistance against the wheat powdery mildew fungus *Blumeria graminis* f. sp. *tritici* (Wang *et al.*, 2014). A limitation of the technology was the low mutation frequencies shown in the above study for wheat (5.6% in the T0 generation). Plant RNA Pol III-dependent promoters from small nuclear RNA (snRNA)-encoding genes (e.g. *U3* snRNA and *U6* snRNA) have been used to express sgRNA that guides the Cas9 protein to its target in the genome (Brooks *et al.*, 2014; Jiang *et al.*, 2013; Li *et al.*, 2013; Miao *et al.*, 2013; Nekrasov *et al.*, 2013; Zhang *et al.*, 2014). Lawrenson *et al.* (2015) exploited the wheat promoter of the *TaU6* snRNA gene for *Sp*Cas9-mediated gene-editing of barley *HvPM19*, which encodes an ABA-inducible plasma membrane protein. Holme *et al.* (2017) edited *HvPAPhy*, a barley phytase gene using a similar construct. Mutation frequencies of 10%–44% were observed in the T0 generation, and induced mutations were transmitted to T1 plants independently of the T-DNA construct. Kapusi *et al.* (2017) used the *Sp*Cas9 system to disrupt a barley *Endo-N-acetyl-β-D-glucosaminidase* (*ENGase*) gene by employing the rice *OsU6* promoter to drive the sgRNA, reaching a *Sp*Cas9-induced mutation frequency of 78%. However, these studies on cereals had some limitations concerning the mutation and/or transformation efficiency, thereby either accessing a mutation enrichment method using restriction enzymes to identify mutated plants in T0 generation (Holme *et al.*, 2017; Lawrenson *et al.*, 2015) or studying a large number of explants (Kapusi *et al.*, 2017) to identify *Sp*Cas9-positive plants (~10%), which reduces the overall efficiency of the *Sp*Cas9 gene-editing system. These results indicate a need to improve the efficiency of *Sp*Cas9-mediated gene-editing in cereals.

In this study, we exemplarily used *HvMORC1* (GenBank: HG316119.1), one of the seven members of the barley *microrchidia* (*MORC*) GHKL (gyrase, Hsp90, histidine kinase, MutL) ATPase subfamily (Koch *et al.*, 2017), to further improve application of the *Sp*Cas9-mediated gene-editing system in the cereal model barley. Plant *MORC* genes were first discovered in a genetic screen for *Arabidopsis* knockout (KO) mutants with compromised resistance against the turnip crinkle virus (TCV), suggesting that they play a role in plant immunity (Kang *et al.*, 2008, 2010, 2012). Subsequent studies in Arabidopsis revealed their involvement in gene silencing and transposable element repression (Lorković *et al.*, 2012; Moissiard *et al.*, 2012, 2014). Unlike Arabidopsis *atmorc* mutants, barley became more resistant to fungal pathogens, such as powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*), when *HvMORC2*, a paralog of *HvMORC1*, was partially silenced by expressing *MORC2*-targeting silencing constructs with inverted promoters in transgenic plants (Langen *et al.*, 2014). Consistent with this, transient overexpression of either of the five at that time-known *HvMORC* paralogs compromised resistance to *Bgh*. Yet, functional analysis of cereal MORC proteins has been hampered by the unavailability of respective KO mutants. Hence, we anticipated that the *MORC* gene family is an excellent model for *Sp*Cas9-mediated gene-editing applications in barley.

Using a novel barley *U3* snRNA promoter to drive the sgRNA, we achieved an unprecedentedly high mutation frequency. Distinct *hvmorc1*-KO mutations were detectable by target sequencing in the transgenic calli, the T0 generation (77%) and T1 (70%–100%) generation, which represents an important improvement of the technology. Extending earlier findings that were based on *hvmorc2*-KD mutants generated by RNAi-mediated knockdown (KD) strategies, *Sp*Cas9-edited *hvmorc1*-KO barley showed increased disease resistance to biotrophic *Bgh* and necrotrophic *Fusarium graminearum*. However, in contrast to barley *hvmorc1*-KD mutants, *hvmorc1*-KO barley, alike *atmorc1* mutants, showed de-repressed expression of transposable elements (TEs), suggesting that barley MORCs also are involved in genome stabilization.

Results

Identification and characterization of a barley RNA Pol III-dependent snRNA promoter

As RNAi-mediated KD may result in low efficiency and thus substantial residual amounts of transcript and protein, we further analysed the function of *MORCs* using stable KO mutant barley lines generated by *Sp*Cas9-based nuclease. To ensure efficient transcription of sgRNAs in barley cells, we first set out to identify suitable regulatory elements by focusing on cereal *U3* snRNA promoters. A *U3* snRNA promoter from rice has been previously characterized (*OsU3*; Qu *et al.*, 1996). This promoter has been used for *Sp*Cas9-mediated gene-editing in rice (e.g. Zhang *et al.*, 2014) and maize (Xing *et al.*, 2014). A suitable *HvU3* regulatory element (GenBank: CAJX011995286.1) was identified by similarity to the wheat *TaU3* promoter (GenBank: X63065.1; Marshallsay *et al.*, 1992) from the database of barley cultivar 'Bowman' (http://webblast.ipk-gatersleben.de/barley/). *U3* snRNA promoter sequences from barley, rice and wheat were compared for the presence of features characteristic for Pol III-dependent promoters in monocotyledonous plants: TATA box, an upstream sequence element (USE), and monocot-specific promoter (MSP) elements (Figure 1a–c). MSPs are conserved G + C-rich sequences with a consensus of RGCCCR in either direction, usually present −30 to −130 bp upstream of the USE (Connelly *et al.*, 1994). Consistent with this, the barley *HvU3* promoter (p*HvU3*) contains a TATA box, located 23 base pairs (bp) upstream of the transcription initiation site, and the USE with the consensus sequence 5′-TCCCACCTCG 25 bp upstream of TATA box, along with five MSPs, thus matching with the characteristics of RNA Pol III-dependent promoters from monocotyledons (Waibel and Filipowicz, 1990).

Assessment of barley and rice *U3* snRNA promoter activities

We first studied the activity of barley and rice *U3* snRNA promoter fragments in the tissue of immature barley embryos; 638 and 380 bp upstream of the predicted transcription start sites of *HvU3* and *OsU3*, respectively, were cloned into pGY1-*35s*:*GFP* (Figure S1). The *U3* regulatory elements replaced the 35S promoter in pGY1 to drive expression of *GFP*. Resulting constructs were delivered to tissues from excised immature embryos of spring barley cultivar (cv.) Golden Promise by particle bombardment. Foci of GFP expression were detected 48 h after bombardment in embryonic cells transformed with either construct

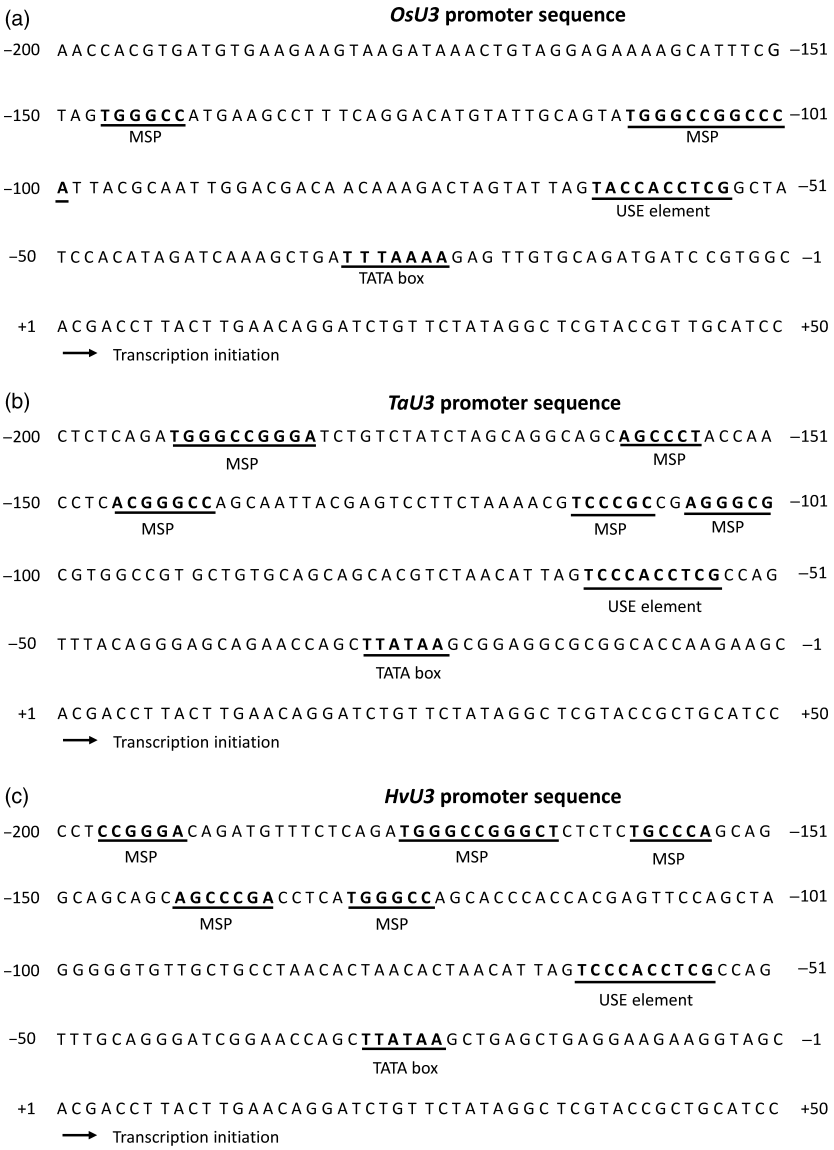


Figure 1 *U3* promoter sequences from rice (a); wheat (b) and barley (c). Sequence motifs are underlined: TATA box, upstream sequence element (USE) and monocot-specific promoter (MSP) element.

(Figure 2a,b). Although foci occurred at rather low frequencies in comparison with our routine observations, when bombarding constructs for Pol-II promoter-driven expression, the results demonstrate activity of both the *HvU3* and *OsU3* promoter fragments in barley. Notably, this also suggests that *U3*-driven transcripts can, at least to some extent, engage the translational machinery in the cytoplasm.

*Sp*Cas9-induced mutation of *HvMORC1*

The barley genome contains seven *MORC* genes, all of which are assumed to act as negative regulators of immunity as deduced from overexpression and RNAi-mediated KD studies (Koch *et al.*, 2017; Langen *et al.*, 2014). To further address MORCs' function in barley, *HvMORC1* was targeted by *Sp*Cas9 to generate loss-of-function alleles. A target site in the 5′ part of the *HvMORC1* gene upstream of the ATPase domain with no potential off-targets in any of the seven barley *MORC*s (Figure S2) or the barley genome (see Experimental procedures) was chosen, and a respective sgRNA was designed (Figure 3a). Two constructs *HvU3:sgRNA* and *OsU3:sgRNA*, containing either p*HvU3* or p*OsU3* driving sgRNA expression and *Sp*Cas9 under control of the maize ubiquitin promoter (*ZmUbi*:*Cas9*), were transformed into immature embryos by agro-transformation (Figure 3b,c; see also Figure S3). Genome editing activity from transformation of the construct with *HvU3*-driven sgRNA was analysed in calli 6 weeks after transformation. Genomic DNA was extracted from randomly chosen embryonic calli grown on hygromycin selective media. The target region was amplified by PCR, and amplicons were analysed by direct sequencing of both strands. From two calli, the wild-type (wt) sequence was obtained (Figure 4a; calli 1 and 6), and chromatograms were not indicative of any nuclease activity at the target site. In contrast, a homozygous deletion of 2 bp was obtained for one callus and also a bi-allelic lesion (Figure 4a, calli 2 and 5). For the remaining five calli, varying degrees of nuclease activity were detected in chromatograms, and precise alleles could not be deduced as multiple sequences were detected (Figure 4a,b), suggesting that samples used for PCR originated from a mixed population of cells containing different molecular lesions at the target site. Thus, nuclease activity was detected in almost 77% (7/9) of analysed calli by direct sequencing, and bi-allelic disruptive mutations affecting both alleles were also readily detected, suggesting occurrence of loss-of-function lines already

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

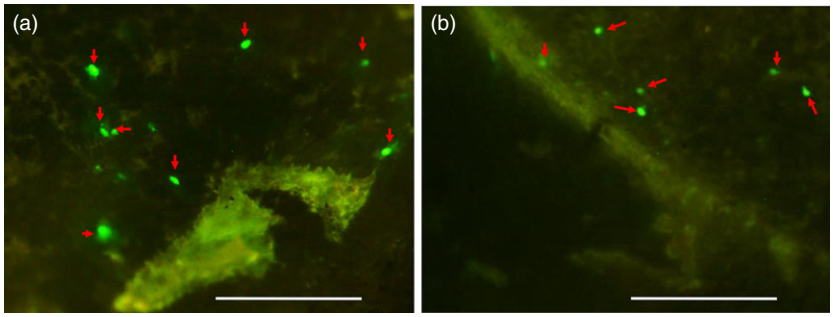


Figure 2 *GFP* expression in immature embryos of barley cv. Golden Promise 48 h after biolistic transformation with (a) pGY1-p*HvU3*:*GFP*, containing 638 bp of the promoter sequence upstream of the coding region of the *U3* snRNA promoter from barley, and (b) pGY1-p*OsU3*:*GFP*, containing 380 bp of the promoter sequence upstream of the coding region of the *U3* snRNA from rice, to drive *GFP* expression. Arrows mark GFP fluorescence. Bar scale 0.5 mm.

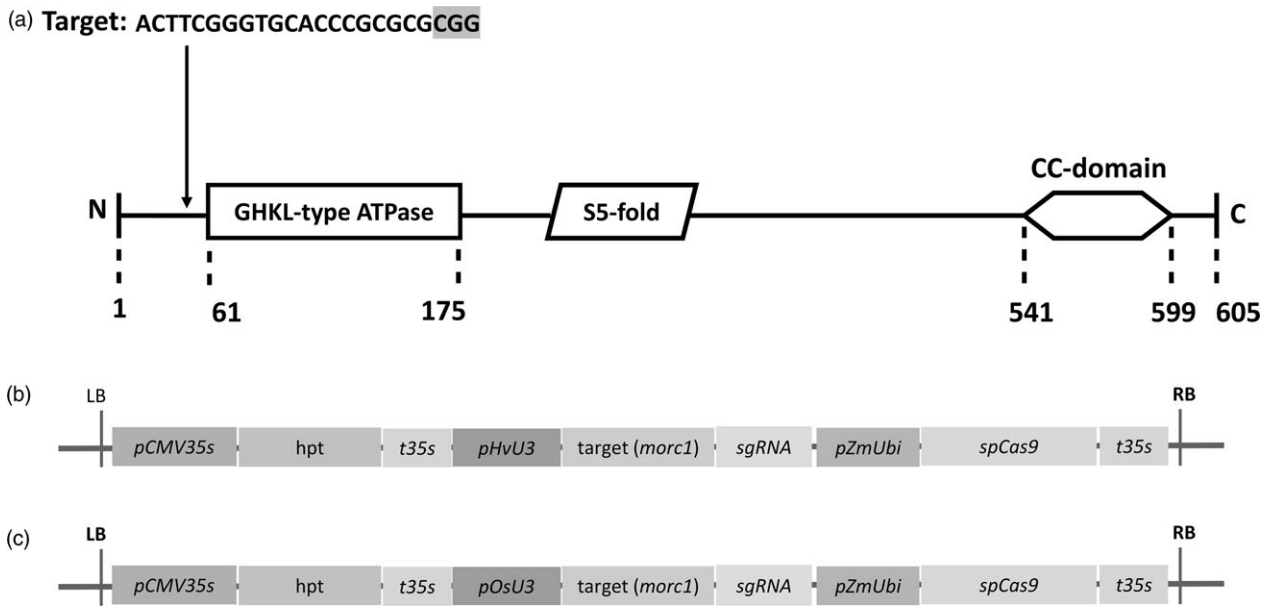


Figure 3 MORC domain structure and constructs used for targeted KO of *HvMORC1* by *Sp*Cas9-mediated gene-editing. (a) Targeted area (20 nt) of *HvMORC1* domain with the PAM sequence in grey shade. The hallmark domains of *HvMORC1*: a GHKL-type ATPase, an S5-fold and a CC-domain, are highlighted. (b, c) Schematic representation of the T-DNA regions containing all components for *Agrobacterium*-mediated, *Sp*Cas9-based *HvMORC1* gene-editing. Construct with barley *U3* promoter (b) and construct with rice *U3* promoter (c). Hygromycin, *hygromycin phosphotransferase gene* [*hpt*]; pZmUbi, *ubiquitin* promoter of *Zea mays*; t35s, *CaMV* 35S terminator; LB, RB, left and right border sequences of the T-DNA; sgRNA, synthetic single guide RNA.

in the T0 generation. We used TIDE (Tracking of Indels by Decomposition; Brinkman *et al.*, 2014) to further access the spectrum and frequency of *Sp*Cas9-induced mutations in calli. Overall, mainly indels of −2, −1 and +1 nt were detected by decomposition of chromatograms, and frequencies were comparable. Deletions of up to 5 nt were also detected, but frequencies were low. Among all calli (including those without detectable mutations), the wt sequence represented in average only 40% (±34%) of all sequence information, suggesting highly efficient genome editing when expressing sgRNAs under control of the *HvU3* promoter.

Selection of homozygous *hvmorc1*-KO barley in the T1 generation

Genome editing activities were further analysed in T1. T0 plantlets regenerating on hygromycin selective medium were randomly selected and propagated in soil to obtain seeds for T1 generation. Notably, the parental T0 lines were not checked for the presence of either T-DNA construct or mutations in *HvMORC1*. In T1 offspring, target sites were PCR-amplified and amplicons were analysed by sequencing. For construct *HvU3*:sgRNA, 71 2-week-old plants from 12 different T1 lines (five to six plants per line) were analysed, and mutations could be detected in all T1 populations (100% efficiency). Similarly, 60 plants from 10 independent T1 lines (six plants per line) carrying *OsU3*:sgRNA were analysed. Mutations were detected in seven of these T1 populations (70% efficiency). For both transformation events, homozygous indel mutations (Figure 5a,b) were identified within the 20 bp target sequence, at a frequency of 38% (*HvU3*) or 42% (*OsU3*). Heterozygous mutations showed the presence of double peaks in the sequencing chromatogram (Figure 5c,d). While homozygous mutations are bi-allelic, heterozygous

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

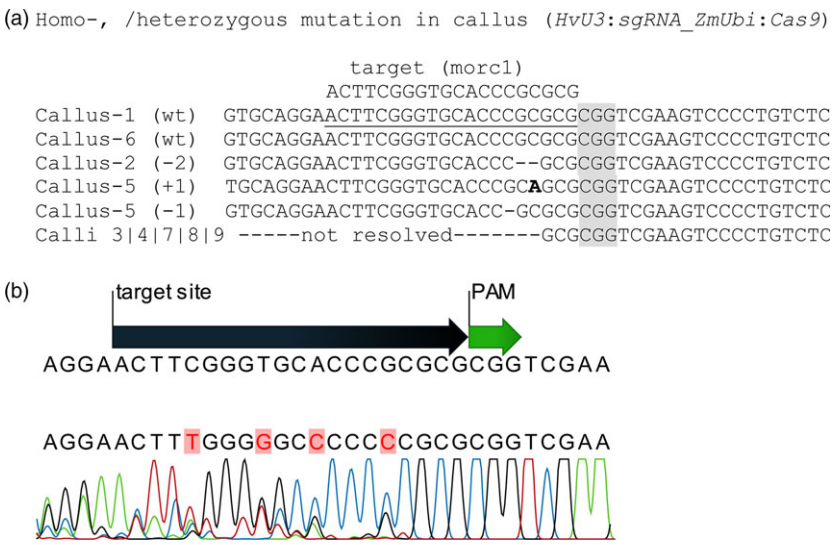


Figure 4 Mutations in the 20 bp target region of *HvMORC1* in 6-week-old T0 calli expressing the construct *HvU3:sgRNA_ZmUbi:Cas9*. (a) Overview of sequences obtained from calli. The PAM (CGG) sequence is highlighted in grey. (b) Example of a typical chromatogram, which could not be resolved into two or less distinct alleles. Note peaks with multiple overlaying signals indicating the presence of at least three different alleles within the sample.

mutations theoretically could be either mono-allelic or bi-allelic (with different mutations on both chromosomes). Further assessment of T1 populations with bi-allelic mutations showed that in each population plants homozygous for each allele could be discovered (Figure 5e). These results confirm that *Sp*Cas9 can induce different mutations on different chromosomal strands of the same T0 plants, resulting in homozygous plants with two different mutation patterns in the T1 generation. Notably, we also identified mutated T1 plants that did not contain a T-DNA construct (nontransgenic): 11 of 73 tested plants (15%) were devoid of the construct, indicating segregation of T-DNA construct and the lesions within *HvMORC1*.

Enhanced sgRNA accumulation by *HvU3 promoter*-driven expression in barley

To further corroborate the suitability of the *HvU3* promoter for genome editing approaches in barley, expression of sgRNA under control of either the *HvU3* or *OsU3* promoter was quantified in T-DNA-positive lines using quantitative RT-PCR (RT-qPCR). To normalize for potential copy number variations and/or transgene insertions at different genome locations, sgRNA expression was normalized to the expression of T-DNA-encoded *Sp*Cas9 (Figure 6) or *hygromycin phosphotransferase* (Figure S4). We observed clearly higher expression of p*HvU3*-driven sgRNA transcripts compared to p*OsU3* under both instances.

hvmorc1-KO mutants show increased resistance to fungal pathogens

Arabidopsis lines deficient in *MORC1* and *MORC2* are severely impaired in resistance to viral, bacterial, oomycete and fungal pathogens (Kang *et al.*, 2008, 2012), while, in contrast, RNAi-mediated reduced transcript levels of *HvMORC2* in barley enhanced resistance (Langen *et al.*, 2014). To substantiate the opposing function of MORCs in barley vs. Arabidopsis, mutated *hvmorc1*-KO T1 plants (consisting of both homozygous and heterozygous bi-allelic mutations) from p*HvU3:sgRNA_*p*ZmUbi:Cas9* construct (*hvmorc1*-L3, *hvmorc1*-L13 and *hvmorc1*-L16; see Figure 5e) were tested for powdery mildew resistance. Detached leaves were inoculated with conidia of *Bgh*A6 (virulent on cv. Golden Promise). Mutant lines developed less fungal colonies 6 days postinoculation (dpi) compared to wt plants (*hvmorc1*-L3: 71.5%; -L13: 71.8%; *hvmorc1*-L16: 76%; Figure 7a). These results were consistent with our expectation that barley MORC paralogs respond similar to *Bgh* (Langen *et al.*, 2014). T1 plants from *hvmorc1*-L10 and *hvmorc1*-L13 that were homozygous for frameshift mutations (*hvmorc1-1* and *hvmorc1-4*; Figure S5) in the 5′ region of *HvMORC1* were propagated for analyses of T2 plants. We further studied *hvmorc1-1* and *hvmorc1-4* T2 homozygous plants for their response to the mycotoxin-producing fungus *Fusarium graminearum*. Detached leaves of *hvmorc1*-KO and wt plants were inoculated with macrospores of *F. graminearum*, and fungal DNA was quantified by quantitative PCR at five dpi. Fungal biomass was significantly reduced in *hvmorc1* KO mutant tissues (Figure 7b).

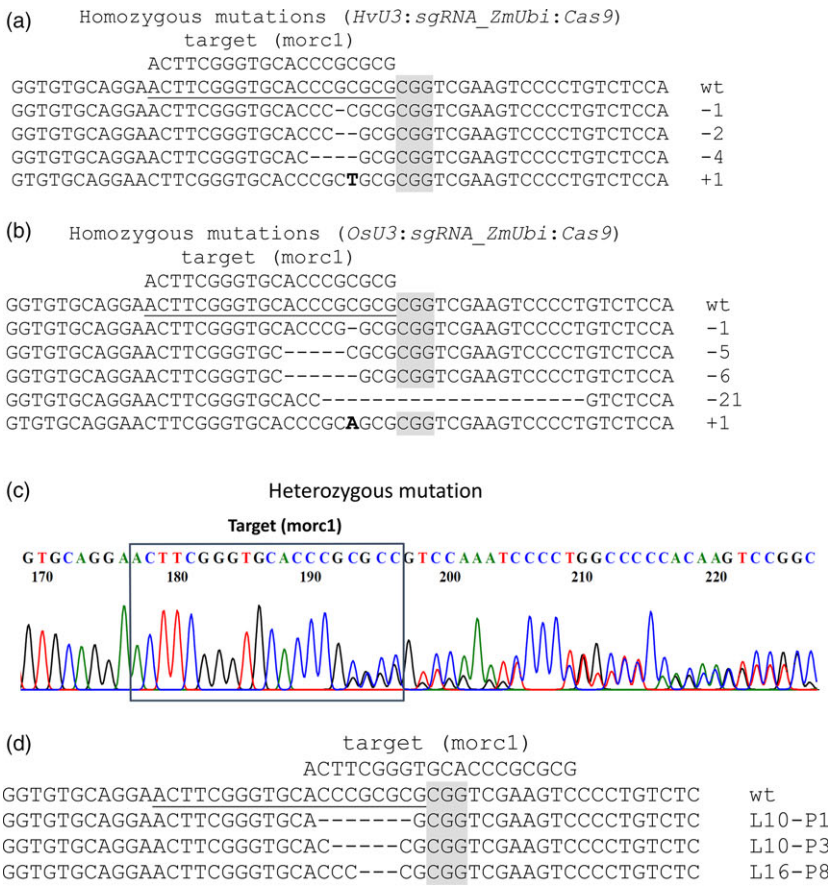
hvmorc1-KO mutants show enhanced expression of *PR* genes

We investigated whether enhanced resistance of *hvmorc1*-KO lines are associated with constitutive activation of defence responses. To this end, we measured expression of defence-related genes. Expression of *HvPR1b* (GenBank: X74940.1), *HvPR2* (GenBank: AF479647.2) and *HvPR5* (GenBank: AM403331.1) in *hvmorc1-1* and *hvmorc1-4* T2 homozygous plants was determined by RT-qPCR at 0, 24, 48 and 72 hpi with *Bgh*. Without pathogen stimulus (0 hpi), all of these genes were expressed to higher levels in *hvmorc1*-KO mutants compared to wt (Figure 8a–c). Upon *Bgh* inoculation, differences in *PR* expression of *hvmorc1*-KO vs. wt were even more pronounced, noticeably at an early infection stage (24 hpi). Most strikingly, expression of *PR1b* was strongly induced in the *hvmorc1*-KO mutant. We concluded that compromised *HvMORC1* functions de-repress at least parts of the plant defence system.

hvmorc1-KO mutants show de-repressed transposable elements

In Arabidopsis *atmorc1* and *atmorc6* mutants, expression of transposable elements (TEs) located around the pericentromeric region is strongly increased (Moissiard *et al.*, 2012), while transposon de-repression has not been observed in barley *hvmorc1*-KD mutants that were only partially silenced for *HvMORC1* (Langen *et al.*, 2014). We refined the analysis of TE expression using the *hvmorc1*-KO lines. To this end, expression of long terminal repeat (LTR) and non-LTR retrotransposons (Long INterspersed Elements; LINE) with sequence similarity to those de-

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,



(e) Mutation patterns in *Hvmorc1* bi-allelic homozygous lines with different constructs

T1 Line	**p*HvU3:sgRNA_pZmUbi:Cas9***	
L3	1 bp deletion	2 bp deletion
L10	1 bp insertion	4 bp deletion
L13	1 bp insertion	4 bp deletion
L14	1 bp deletion	2 bp deletion
L16	1 bp deletion	2 bp deletion
T1 Line	**p*OsU3:sgRNA_pZmUbi:Cas9***	
L1	1 bp Insertion	6 bp deletion
L11	1 bp Insertion	21 bp deletion
L13	1 bp Insertion	21 bp deletion
L15	1 bp insertion	5 bp deletion

Figure 5 *Sp*Cas9-induced mutations in independent barley T1 lines. (a–b) Homozygous mutations in T1 plants containing the *HvU3:sgRNA_ZmUbi:Cas9* and the *OsU3:sgRNA_ZmUbi:Cas9* construct, respectively. (c) Heterozygous mutants have characteristic double peaks in the chromatogram, for example starting 4 bp upstream of the PAM sequence (grey). (d) Mutation patterns of heterozygous mutants determined after sequencing using specific primers (Table S1) from both directions. (e) Examples for mutations in homozygous bi-allelic T1 lines. The PAM (CGG) sequence is highlighted in grey, the 20 bp target region in *HvMORC1* is underlined, and insertions are marked in bold.

repressed in Arabidopsis *atmorc* mutants (Langen *et al.*, 2014; Moissiard *et al.*, 2012) was measured by RT-qPCR in *hvmorc1-1* and *hvmorc1-4* T2 homozygous mutated plants. In contrast to partially silenced *hvmorc1*-KD mutants, *Sp*Cas9-generated *hvmorc1*-KO lines showed significant transposon de-repression as compared to wt (Figure 9), although the degree of TE de-repression was lower than previously reported for Arabidopsis *atmorc* mutants. This suggests that HvMORC1, such as AtMORC1, is involved in genome stabilization.

hvmorc1-KO mutants show increased expression of *HvMORC2* in immature embryos

In Arabidopsis, AtMORC1 and AtMORC2 interact with AtMORC6 to form distinct heteromers to achieve gene silencing. Additionally, the function of AtMORC6 is epistatic to both AtMORC1 and AtMORC2 (Moissiard *et al.*, 2014). We assessed the effect of knocking out *HvMORC1* on the expression of other barley MORC homologs. Expression of *HvMORC2* (GenBank: HG316120) and *HvMORC6a* (GenBank: HG316122) was measured in immature embryos of T1 *hvmorc1*-KO homozygous plants *hvmorc1-1* and *hvmorc1-4* and leaves of their T2 progenies. An increased expression of *HvMORC2* was observed in embryos of *hvmorc1-1* and *hvmorc1-4* compared to wt, while expression of *HvMORC6a* was similar in all genotypes (Figure 10a). This raises the possibility that, in the absence of HvMORC1, there is an increased expression of *HvMORC2* to maintain the cellular concentration of heteromeric complexes involving HvMORC6 for transcriptional repression of TEs, especially in immature embryonic tissue. Notably, *HvMORC2* showed no significant increase in expression in leaves of *hvmorc1*-KO

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

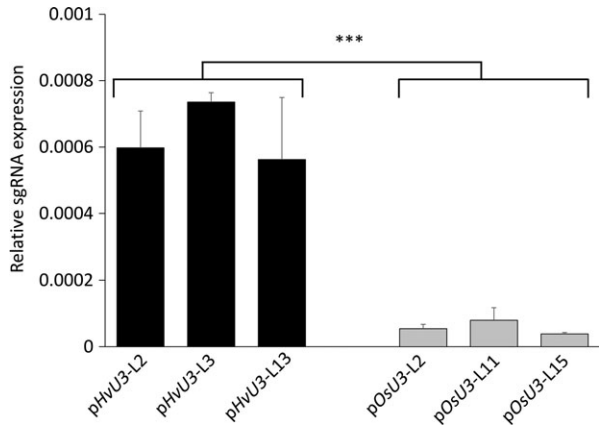


Figure 6 Relative expression of sgRNA under control of barley and rice *U3* promoters (p*HvU3* and p*OsU3*) in leaves of *hvmorc1*-KO T2 homozygous mutants measured by RT-PCR and normalized against *Sp*Cas9. Error bars indicate standard deviation of three repetitions. Asterisks indicate statistical significant difference (Student's *t*-test: ***$P < 0.001$).

mutants (Figure 10b). Both immature embryos and leaves of *hvmorc1*-KO show reduced transcript level of *HvMORC1* (Figure 10a,b), which could be a result of mRNA degradation by non-sense-mediated mRNA decay pathway that identifies and removes mRNA with premature STOP codons (Reviewed in Baker and Parker, 2004).

Discussion

Identification and transient expression of the barley *U3* snRNA promoter

Monocot and dicot RNA Pol III promoters from snRNA genes have been used to express sgRNA for genome editing. Diverse Arabidopsis promoters such as *AtU3b*, *AtU3d*, *AtU6*, *AtU6-1*, *AtU6-26* and *AtU6-29* have been shown to be functional in dicotyledons (Brooks *et al.*, 2014; Fauser *et al.*, 2014; Feng *et al.*, 2014; Gao *et al.*, 2015; Ma *et al.*, 2015; Mao *et al.*, 2013; Nekrasov *et al.*, 2013; Xing *et al.*, 2014). For the expression of sgRNA in cereals, promoter variants *OsU3* and *OsU6* for rice (Ma *et al.*, 2015; Shan *et al.*, 2013), *TaU3* and *ZmU6* for maize (Svitashev *et al.*, 2015; Xing *et al.*, 2014), and *TaU6* for wheat and barley (Lawrenson *et al.*, 2015; Wang *et al.*, 2014) have been used. In the newly isolated barley *U3* promoter, the USE element lies 25 bp upstream of TATA box, which conforms to the consensus sequence of RNA Pol III-dependent promoters (Figure 1c). In maize, deletion or substitution of MSPs decreases the transcription efficiency by 30%–60% (Connelly *et al.*, 1994; Qu *et al.*, 1996). Thus, it was crucial to check the functionality of promoters to be used in our *Sp*Cas9 system. RNA Pol III is able to produce functional mRNA with a low efficiency in human cells (Gunnery and Mathews, 1995), but similar studies have not been carried out in plants. In our study, upon transient transformation both barley and rice (RNA Pol III-dependent) *U3* promoters coupled with *GFP* were expressed in barley immature embryos (Figure 2a,b), confirming their functionality. This finding also suggests that in plants some protein-coding genes might be RNA Pol III-dependent.

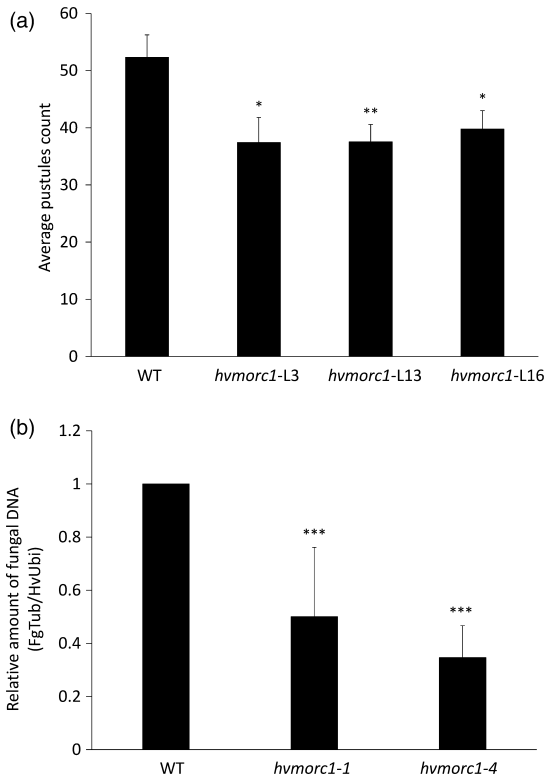


Figure 7 *Sp*Cas9-mediated KO of *HvMORC1* results in enhanced resistance against fungal pathogens. (a) *hvmorc1*-KO T1 barley cv. Golden Promise lines (*hvmorc1*-L3, *hvmorc1*-L13, *hvmorc1*-L16) display enhanced resistance against powdery mildew. Detached second leaves of 14-day-old plants were inoculated with 3–5 conidia per mm^2. *Bgh* colonies were counted at six dpi. Shown is the average number of *Bgh* colonies on 1.5 cm^2 leaf area ($n = 14$). The experiment was repeated twice with similar results. Error bars indicate standard error. Asterisks indicate statistical significant difference (Student's *t*-test *$P < 0.05$, **$P < 0.01$). (b) *hvmorc1*-KO T2 homozygous mutants show enhanced resistance against *Fusarium graminearum* (*Fg*). For inoculation, 20 ul of *Fg* conidia (5×10^4 conidia mL^{-1}) was drop-inoculated on detached third leaves of 21-day-old plants. Quantification of *Fg* on leaves was performed five dpi by quantitative RT-PCR based on the ratio of fungal tubulin (*FgTub*) to plant ubiquitin (*HvUbi*). Significant changes are marked: ***$P < 0.001$ (Student's *t*-test). Presented are mean of 10 leaves. Bars represent standard deviation of three repetitions.

Highly efficient genome editing in barley by *HvU3*-driven sgRNA expression

Several studies have been published that reported the use of *Sp*Cas9 system in barley (Holme *et al.*, 2017; Kapusi *et al.*, 2017; Lawrenson *et al.*, 2015). We used a single T-DNA vector similar to the study by Lawrenson *et al.* (2015), main difference to this previous work being the use of the *U3* promoters of either barley or rice for sgRNA expression (Figures 1 and 3). Using these promoters, we achieved both stringent selection of transgenic plants on hygromycin and highly efficient genome editing with bi-allelic mutations occurring already in T0 (Figure 4a). Although we cannot exclude an extraordinary high efficiency of the sgRNA used in our study, we assume that *U3* promoters are highly suitable for sgRNA expression in barley genome editing applications. Notably, *HvU3*-driven sgRNA

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

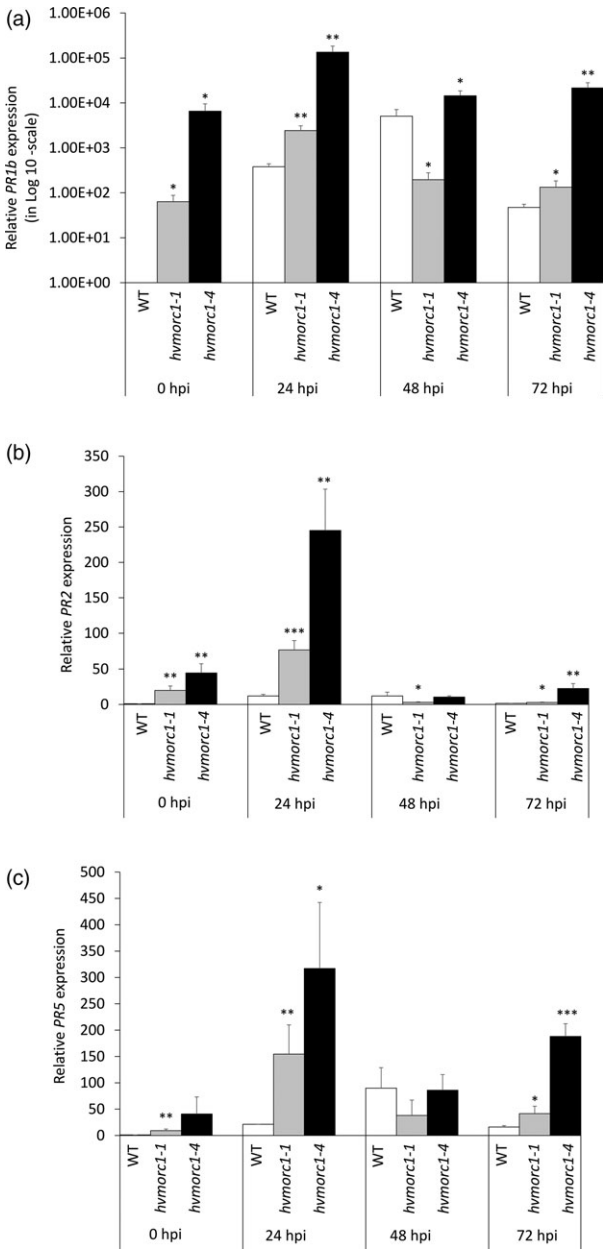


Figure 8 Relative *PR* gene expression in leaves of *Sp*Cas9-generated *hvmorc1*-KO T2 homozygous mutants vs. wt measured by RT-qPCR and normalized to plant *ubiquitin*. Expression of SA pathway marker genes *HvPR1b* (a), *HvPR2* (b) and *HvPR5* (c). Detached second leaves of 14-day-old plants were inoculated with 10 to 15 conidia per mm^2 (n = 5). Error bars indicate standard deviation of three repetitions. Asterisks indicate statistical significant difference (Student's t-test: *P < 0.05, **P < 0.01, ***P < 0.001).

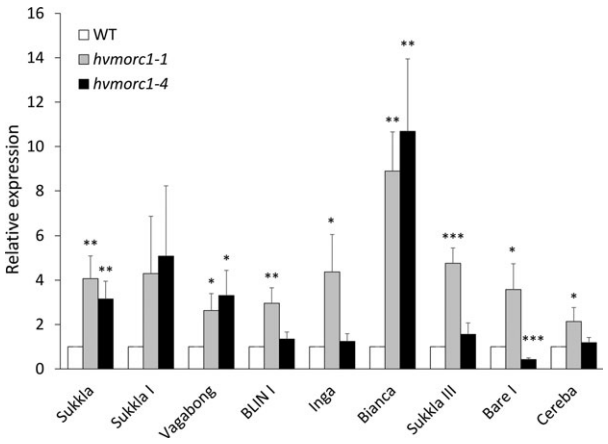


Figure 9 Expression of transposons (TEs) in second leaves of 14-day-old *Sp*Cas9-generated *hvmorc1*-KO T2 homozygous mutants (*hvmorc1-1* and *hvmorc1-4*) vs. wt assayed by RT-qPCR and normalized to plant *ubiquitin* (n = 5). Error bars indicate standard deviation of three repetitions. Asterisks indicate statistical significant difference (Student's t-test: *P < 0.05, **P < 0.01, ***P < 0.001).

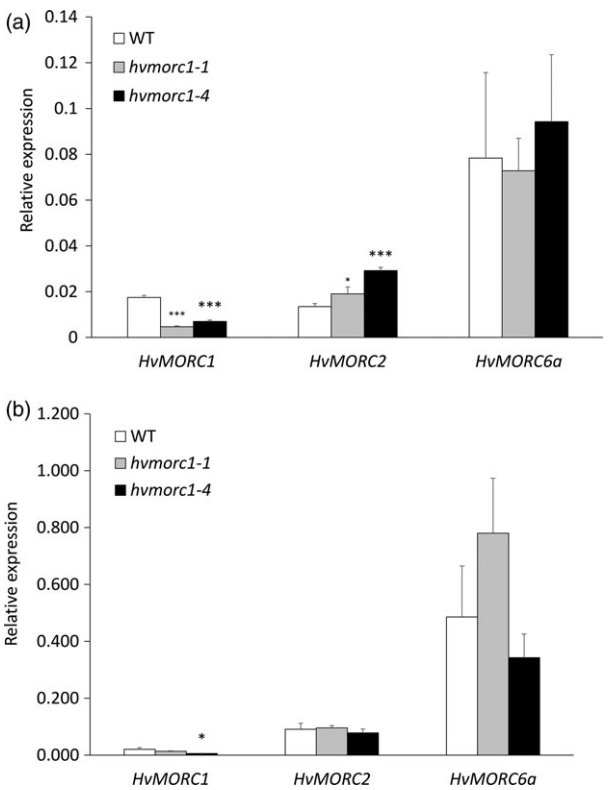


Figure 10 Relative expression of barley *MORC* genes in immature embryos and leaves of *hvmorc1*-KO mutants and wt assayed by RT-qPCR and normalized to plant *ubiquitin*. (a) Expression of *HvMORC1*, *HvMORC2* and *HvMORC6a* in immature embryos of T1 homozygous mutants (*hvmorc1-1* and *hvmorc1-4*). (b) Expression of *HvMORC1*, *HvMORC2* and *HvMORC6a* in leaves of T2 homozygous mutants (*hvmorc1-1* and *hvmorc1-4*). mRNA was extracted from second leaves of 14-day-old plants and immature embryos (n = 5). Error bars indicate standard deviation of three repetitions. Asterisks indicate statistical significant difference (Student's t-test: *P < 0.05, ***P < 0.001).

showed highest transcript accumulation as compared to *OsU3* (Figures 6 and S4). We provide these regulatory elements, and also *TaU6* and *OsU6* promoter fragments used in previous experiments, as part of a convenient toolkit to the plant research community. Our toolkit, which is similar to a previously reported toolkit for genome editing in dicot plants (Ordon *et al.*, 2017), provides simple and rapid (Golden Gate-based) cloning procedures and high multiplexing capacity for expression of four or up to eight sgRNAs. A description of the

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

toolkit with cloning manual (Appendix S1) and vector maps (Appendix S2) is provided.

*Sp*Cas9 nuclease-induced mutations in barley

The aim of our study was to further increase the mutation frequency and select mutated plants growing on hygromycin selective medium using simple PCR and Sanger sequencing. Using *HvU3*, we obtained 77.7% mutation frequency in 6-week-old T0 callus (Figure 4a), which is seemingly high for barley. In separate experiments, T0 plants growing on hygromycin selective medium were selected for seed propagation. Later in T1 offspring of those plants, we obtained mutation frequencies of 100% and 77% in *HvMORC1* using *HvU3* and *OsU3* promoters, respectively. We obtained 38%–41% bi-allelic homozygous plants and 15% plants were T-DNA-free T1 generation. The T-DNA-free homozygous plants do not contain any inserted DNA fragment/gene and carry the same mutation on both chromosomes, thus being ideal for gene function studies. Hence, we show here that it is possible to get a high frequency of mutation in barley using the *Sp*Cas9 technique. No doubt in future this technology would be the first choice of gene modification for plant pathologists, breeders and biochemists.

Higher *HvMORC2* expression compensates for KO of *HvMORC1* in *hvmorc1*-KO embryos

Previous work suggested that AtMORC1 and AtMORC2 do not interact with each other but both interact with AtMORC6, leading to the proposal that AtMORC6 mediates gene silencing by forming mutually exclusive heterodimers with either AtMORC1 or AtMORC2, or as a homodimer (Liu *et al.*, 2014; Moissiard *et al.*, 2014), and the function of AtMORC6 is epistatic to both AtMORC1 and AtMORC2 (Moissiard *et al.*, 2014). Supportive of the former reports, we found an increased expression of *HvMORC2* in immature embryos of *hvmorc1* compared to wt, while expression of *HvMORC6a* was not changed (Figure 10a). These data suggest that in the absence of HvMORC1, there is an increased expression of *HvMORC2* to maintain the cellular concentration of heteromeric complexes involving HvMORC6 for transcriptional repression of TEs, although it is not resolved whether the targets of MORC1-MORC6a and MORC2-MORC6a complexes are identical, different or overlapping. Notably, *HvMORC2* showed no significant increase in expression in leaves of *hvmorc1*-KO (Figure 10b), suggesting that the cell machinery is epigenetically programmed to identify and compensate for defects in DNA methylation during reproductive stage. Finally, constitutive *HvMORC6a* expression is higher compared to *HvMORC1* and *HvMORC2* (Figure 10a,b), further arguing for a prominent cellular requirement of HvMORC6a.

Barley MORC1 modulates plant immunity and regulates TE expression

While in Arabidopsis and potato, *MORC*s positively regulate resistance to microbial pathogens, they are negative regulators in tobacco and tomato (Kang *et al.*, 2008, 2010, 2012; Manosalva *et al.*, 2015). In barley, RNAi-mediated KD of *HvMORC2* also resulted in higher resistance, resembling the situation in tobacco and tomato (Langen *et al.*, 2014). Given MORC proteins are also influencing gene silencing, there remains a technical uncertainty to assess the loss of function using RNAi. In the present study, a complete KO of HvMORC1 also enhances plant immunity against fungal pathogens *Bgh* and *F. graminearum* (Figure 7a,b), confirming similar immune functions of barley paralogs MORC1 and MORC2. Enhanced resistance to fungal pathogens correlated with elevated transcript level of *PR* genes in *hvmorc1*-KO mutants (Figure 8a–c). *PR* expression was further enhanced in response to *Bgh*, particularly during initial phase of fungal colonization, providing *hvmorc1*-KO mutants an early advantage over wt plants. It appears *HvMORC1* controls at least part of the plant's immune system, possibly thereby avoiding autoimmune reactions of an overshooting defence system.

Several lines of evidence suggest that MORC proteins also have nuclear targets. For example, *in vitro* assays demonstrated that AtMORC1 and HvMORC1 bind DNA/RNA, display endonuclease activity and are transferred from cytoplasmic locations to the nucleus in response to PAMP signals such as flagellin (Kang *et al.*, 2012; Langen *et al.*, 2014). Furthermore, MORC proteins from a range of prokaryotes and eukaryotes have been shown to play roles in chromatin modification and/or DNA recombination and repair (Iyer *et al.*, 2008; Pastor *et al.*, 2014; Perry and Zhao, 2003). The identification of AtMORC1 and/or AtMORC6 in three independent forward genetic screens of Arabidopsis mutants defective for transcriptional gene silencing (TGS) provided the first insight into nuclear MORC protein function (Brabbs *et al.*, 2013; Lorković *et al.*, 2012; Moissiard *et al.*, 2012). In plants, TGS plays an important role in repressing TEs, intergenic regions, DNA repeats and some genes; it is mediated by the RNA-directed DNA methylation (RdDM) pathway (Law and Jacobsen, 2010; Matzke *et al.*, 2009, 2015). RdDM utilizes small RNAs to recruit the DNA methylation machinery to targeted sequences. DNA methylation in turn leads to recruitment of histone-modifying enzymes, and the combined effect of these repressive epigenetic marks establishes chromatin in a silenced state. De-repression of silenced reporter genes as well as TEs was observed in most *atmorc* mutants, suggesting that these proteins play a role in epigenetic gene silencing (Bordiya *et al.*, 2016; Brabbs *et al.*, 2013; Harris *et al.*, 2016; Lorković *et al.*, 2012; Moissiard *et al.*, 2012, 2014). In the present study, we found an increased expression of barley TEs in homozygous *hvmorc1*-KO mutants (Figure 9). A huge part (84%) of the barley genome consists of mobile and repeat structures, 76% of which are retrotransposons. Some 99.6% of retrotransposons are long terminal repeat (LTR) transposons, while 0.31% are non-LTR retrotransposons (International Barley Genome Sequencing Consortium (IBSC), 2012). Notably, RNAi-mediated KD of *HvMORC1* did not result in detectable de-repression of barley TEs (Langen *et al.*, 2014), suggesting that the remaining MORC protein activity (degree of gene KD was approx. 50%) was sufficient to repress TEs, which can explain the different phenotypes of RNAi-generated *hvmorc1*-KD vs. *Sp*Cas9-mediated *hvmorc1*-KO plants. Yet, when comparing *morc1* mutants from barley and Arabidopsis, the different degrees of transposon de-repression are conspicuous (barley up to 14-fold [this study] vs. Arabidopsis up to 500-fold [Moissiard *et al.*, 2014;] as compared to the respective wt plants). However, in two subsequent studies, lower expression of transposons (*AtCopia28/RomaniaT5*) was observed in *atmorc1* (Moissiard *et al.*, 2014; Zhang, 2016). Moreover, a previous report showed that barley retrotransposons are responsive to various biotic and abiotic environmental cues (Alzohairy *et al.*, 2012). Consistent with our study, the barley LTRs did not show high transcript level in response to such triggers.

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

While the link between MORC proteins role in immunity and TGS is currently unknown, the discovery that *Pseudomonas syringae* pv. *tomato* (*Pst*) infection alters AtMORC1 binding at genomic regions preferentially associated with TEs provides an important clue (Bordiya *et al.*, 2016). A growing number of studies suggest that TEs are key regulatory elements that control stress-associated gene expression (Dowen *et al.*, 2012). Thus, the finding that *Pst* infection reduces AtMORC1 binding at loci associated with heterochromatic TEs led Bordiya *et al.* (2016) to propose that loss of AtMORC1 binding at these sites disrupts a silencing complex and thus up-regulates heterochromatic TE expression. The de-repressed TEs could serve as enhancers of proximal gene expression in barley. It is tempting to speculate that elevated resistance of *hvmorc1*-KO mutants results from barley MORC role in genome stabilization, which is attenuated in the mutants resulting in higher expression of TEs and concomitantly *PR* gene expression.

Experimental procedures

Plant material and fungal inoculation

Seeds of barley (*Hordeum vulgare*) cv. 'Golden Promise' were germinated for 3 days on filter paper. Seedlings were transferred to soil (Fruhstorfer Erde Typ T) and cultivated in a growth chamber at 22 °C/18 °C (day/night cycle) with 60% relative humidity and a photoperiod of 16 h (240 μmol/m^2/s photon flux density). After complete emergence (12–14 day), the second leaves were detached, laid on 0.5% (w/v) water agar and inoculated with *Bgh*A6 (Langen *et al.*, 2014) at a density of 2 to 5 conidia mm^{-2}. For expression analysis, a high density of 10 to 15 conidia mm^{-2} was used. *F. graminearum* (strain 1003; Jansen *et al.*, 2005) was regularly cultured on SNA (synthetic nutrient-poor agar) plates containing 0.1% KH_2PO_4, 0.1% KNO_3, 0.1% $MgSO_4{\cdot}7H_2O$, 0.05% KCL, 0.02% glucose, 0.02% sucrose and 1.4% agar. Plates were incubated at room temperature under constant illumination from one near-UV tube (Phillips TLD 36 W/08, http://www.philips.de) and one white light tube (Phillips TLD 36 W/830HF, http://www.philips.de). Sterile 0.02% Tween water (v/v) was poured on 2-week-old plates, and conidial suspension was scrubbed using a glass rod and filtered through a miracloth (Calbiochem, http://www.merck-chemicals.de). Conidia concentration was adjusted to 5×10^{-4} spore mL^{-1}; 20 μL of spore suspension was drop-inoculated on detached barley leaves kept on 0.5% water agar plates. Square Petri plates with detached leaves were kept at room temperature under one white tube (Phillips TLD 36 W/830HF, http://www.philips.de). Progression of infection was routinely monitored. For quantification of fungal invasion, leaf samples were harvested at 5 dpi and DNA was extracted (Doyle and Doyle, 1987), which was later used to determine the amount of fungal DNA by quantitative RT-PCR.

Generation of vectors to study barley and rice *U3* promoter activity using *GFP* reporter gene in transient assay system

A 638 bp upstream of barley *U3* coding sequence (GenBank: CAJX011995286.1) and a 380 bp sequence upstream of rice *U3* coding sequence (Miao *et al.*, 2013) were amplified with primers containing restriction sites *XhoI* and *NcoI* (Table S1). Both barley and rice *U3* promoters were coupled with the reporter gene for the green fluorescent protein (*GFP*) by replacing the CMV35s promoter in pGY1-*35s*:*GFP* (Schweizer *et al.*, 1999) using restriction enzyme *XhoI* and *NcoI* to generate plasmid constructs—pGY1p*HvU3*:*GFP* and pGY1p*OsU3*:*GFP*.

Generation of CRISPR/Cas9 constructs

Twenty bp target sequences with NGG (PAM) at 3′ end were selected using CRISPR sgRNA design online tool (https://atum.bio/eCommerce/cas9/input) for *HvMORC1* (GenBank: HG316119.1). The designed 20 bp target sequences was blasted (BlastN) against nucleotide collection of *Hordeum vulgare* (taxid: 4513) at NCBI for putative off-targets, and ACTTCGGGTGCACCCGCGCG was selected. Cloning overhangs (*Hv*U3: agca/aaac; *Os*U3: ggca/aaac) were added and guide sequences cloned as hybridized oligonucleotides. To adapt *OsU3*, *HvU3*, *OsU6* and *TaU6* elements for the multiplexing system, existing *BsaI* and *BpiI* sites were removed, and promoter fragments were cloned together with a ccdB cassette and the sgRNA backbone into a pUC57 derivative as previously described (Ordon *et al.*, 2017). Recipient vectors were assembled by modular cloning as previously described (Engler *et al.*, 2014; Ordon *et al.*, 2017). Details on cloning procedures and primer sequences are available upon request.

Plant transformation

Plasmids were electroporated (Gene Pulser, Biometra) into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991), and the resulting *Agrobacterium* was used to transform spring barley 'Golden Promise' as described (Imani *et al.*, 2011; Tingay *et al.*, 1997). Transient barley transformation was performed as described (Schweizer *et al.*, 1999). Immature barley embryos were shot using a particle inflow gun (PDS-1000/He, BIO-RAD) with DNA-coated on 1-μm gold particles. One microgram of plasmid per shot was used with a rupture disc of 650 psi.

DNA isolation and quantitative PCR analysis

DNA/RNA extraction and quantitative RT-PCR were performed as described (Doyle and Doyle, 1987; Imani *et al.*, 2011). Primer pairs used for expression analysis are listed in Table S1.

Acknowledgements

We thank E. Swidtschenko and C. Dechert for excellent technical assistance. This work was supported by the German Research Council (Deutsche Forschungsgemeinschaft, DFG; Ko 1208/23-1) to K.H.K. and the Deutscher Akademischer Austauschdienst (DAAD) to N.K. JO was supported by grant STU642-1 / 1 from the German Research Council (DFG) to JS, and seed funding from the Collaborative Research Center 648 (Collaborative Research Center 648) to JS. JS and JO acknowledge Ulla Bonas for continous support.

Conflict of interest

The authors declare no conflict of interest.

References

Acevedo-Garcia, J., Kusch, S. and Panstruga, R. (2014) Magical mystery tour: MLO proteins in plant immunity and beyond. 2014. *New Phytol.* **204**, 273–281.

Alzohairy, A.M., Yousef, M.A., Edris, S., Kerti, B., Gyulai, G. and Bahieldin, A. (2012) Detection of LTR retrotransposons reactivation induced by in vitro environmental stresses in barley (*Hordeum vulgare*) via RT-qPCR. *Life Sci. J.* **9**, 1–8.

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

Baker, K.E. and Parker, R. (2004) Nonsense-mediated mRNA decay: terminating erroneous gene expression. *Curr. Opin. Cell Biol.* **16**, 293–299.

Bordiya, Y., Zheng, Y., Nam, J.C., Bonnard, A.C., Choi, H.W., Lee, B.K., Kim, J. *et al.* (2016) Pathogen infection and MORC proteins affect chromatin accessibility of transposable elements and expression of their proximal genes in Arabidopsis. *Mol. Plant Microbe Interact.* **29**, 674–687.

Bortesi, L. and Fischer, R. (2015) The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv.* **33**, 41–52.

Brabbs, T.R., He, Z., Hogg, K., Kamenski, A., Li, Y., Paszkiewicz, K.H., Moore, K.A. *et al.* (2013) The stochastic silencing phenotype of Arabidopsis morc6 mutants reveals a role in efficient RNA-directed DNA methylation. *Plant J.* **75**, 836–846.

Brinkman, E.K., Chen, T., Amendola, M. and van Steensel, B. (2014) Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* **42**, e168.

Brooks, C., Nekrasov, V., Lippman, Z.B. and Eck, J.V. (2014) Efficient gene-editing in tomato in the first generation using the CRISPR/Cas9 system. *Plant Physiol.* **3**, 1292–1297.

Christian, M., Cermak, T., Doyle, E.L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A.J. *et al.* (2010) Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics*, **186**, 757–761.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**, 819–823.

Connelly, S., Marshallsay, C., Leader, D., Brown, J.W.S. and Filipowicz, W. (1994) Small nuclear RNA genes transcribed by either RNA polymerase II or RNA polymerase III in monocot plants share three promoter elements and use a strategy to regulate gene expression different from that used by their dicot plant counterparts. *Mol. Cell. Biol.* **14**, 5910–5919.

Cristea, S., Freyvert, Y., Santiago, Y., Holmes, M.C., Urnov, F.D., Gregory, P.D. and Cost, G.J. (2013) In vivo cleavage of transgene donors promotes nuclease-mediated targeted integration. *Biotechnol. Bioeng.* **110**, 871–880.

Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y., Pirzada, Z.A., Eckert, M.R. *et al.* (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature*, **471**, 602–607.

Dowen, R.H., Pelizzola, M., Schmitz, R.J., Lister, R., Dowen, J.M., Nery, J.R., Dixon, J.E. *et al.* (2012) Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl Acad. Sci. USA*, **109**, E2183–E2191.

Doyle, J.J. and Doyle, J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**, 11–15.

Engler, C., Youles, M., Gruetzner, R., Ehnert, T.M., Werner, S., Jones, J.D., Patron, N.J. *et al.* (2014) A golden gate modular cloning toolbox for plants. *ACS Synth. Boil.* **3**, 839–843.

Fauser, F., Schiml, S. and Puchta, H. (2014) Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant J.* **79**, 348–359.

Feng, Z., Mao, Y., Xu, N., Zhang, B., Wei, P., Yang, D.L., Wang, Z. *et al.* (2014) Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **111**, 4632–4637.

Gao, Y., Zhang, Y., Zhang, D., Dai, X., Estelle, M. and Zhao, Y. (2015) Auxin binding protein 1 (ABP1) is not required for either auxin signaling or Arabidopsis development. *Proc. Natl Acad. Sci. USA*, **112**, 2275–2280.

Gasiunas, G., Barrangou, R., Horvath, P. and Siksnys, V. (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl Acad. Sci. USA*, **109**, E2579–E2586.

Gunnery, S. and Mathews, M.B. (1995) Functional mRNA can be generated by RNA polymerase III. *Mol. Cell. Biol.* **15**, 3597–3607.

Harris, C.J., Husmann, D., Liu, W., El Kasmi, F., Wang, H., Papikian, A., Pastor, W.A. *et al.* (2016) Arabidopsis AtMORC4 and AtMORC7 form nuclear bodies and repress a large number of protein-coding genes. *PLoS Genet.* **12**, e1005998.

Holme, I.B., Wendt, T., Gil-Humanes, J., Deleuran, L.C., Starker, C.G., Voytas, D.F. and Brinch-Pedersen, H. (2017) Evaluation of the mature grain phytase candidate *HvPAPhy_a* gene in barley (*Hordeum vulgare* L.) using CRISPR/Cas9 and TALENs. *Plant Mol. Biol.* **95**, 111–121.

Hückelhoven, R., Dechert, C. and Kogel, K.-H. (2003) Over-expression of barley BAX Inhibitor-1 induces enhanced accessibility to *Blumeria graminis* and breakdown of *mlo*-mediated penetration resistance in barley. *Proc. Nat. Acad. Sci. USA*, **100**, 5555–5560.

Imani, J., Li, L., Schäfer, P. and Kogel, K.-H. (2011) STARTS—a stable root transformation system for rapid functional analyses of proteins of the monocot model plant barley. *Plant J.* **67**, 726–735.

International Barley Genome Sequencing Consortium (IBSC). (2012) A physical, genetic and functional sequence assembly of the barley genome. *Nature*, **491**, 711–716.

Iyer, L.M., Abhiman, S. and Aravind, L. (2008) MutL homologs in restriction modification systems and the origin of eukaryotic MORC ATPases. *Biol. Direct.* **3**, 8.

Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K.-H., Felk, A. and Maier, F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proc. Natl Acad. Sci. USA*, **102**, 16892–16897.

Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B. and Weeks, D.P. (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Res.* **41**, e188.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, **337**, 816–821.

Kang, H.-G., Kuhl, J.C., Kachroo, P. and Klessig, D.F. (2008) *CRT1*, an Arabidopsis ATPase that interacts with diverse resistance proteins and modulates disease resistance to turnip crinkle virus. *Cell Host Microbe*, **3**, 48–57.

Kang, H.-G., Oh, C.S., Sato, M., Katagiri, F., Glazebrook, J., Takahashi, H., Kachroo, P. *et al.* (2010) Endosome associated CRT1 functions early in resistance gene-mediated defense signaling in Arabidopsis and tobacco. *Plant Cell*, **22**, 918–936.

Kang, H.-G., Hyong, W.C., von Einem, S., Manosalva, P., Ehlers, K., Liu, P.P., Buxa, S.V. *et al.* (2012) CRT1 is a nuclear-translocated MORC endonuclease that participates in multiple levels of plant immunity. *Nat. Commun.* **3**, 1297.

Kapusi, E., Corcuera-Gómez, M., Melnik, S. and Stoger, E. (2017) Heritable genomic fragment deletions and small indels in the putative ENGase gene induced by CRISPR/Cas9 in barley. *Front Plant Sci.* **8**, 540.

Koch, A., Kang, H.-G., Steinbrenner, J., Dempsey, D.A., Klessig, D.F. and Kogel, K.-H. (2017) MORC proteins: novel players in plant and animal health. *Front Plant Sci.* **8**, 1720.

Langen, G., von Einem, S., Koch, A., Imani, J., Pai, S.B., Manohar, M., Ehlers, K. *et al.* (2014) The compromised recognition of turnip crinkle virus1 subfamily of microrchidia ATPases regulates disease resistance in barley to biotrophic and necrotrophic pathogens. *Plant Physiol.* **164**, 866–878.

Law, J.A. and Jacobsen, S.E. (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat. Rev. Genet.* **11**, 204–220.

Lawrenson, T., Shorinola, O., Stacey, N., Li, C., Ostergaard, L., Patron, N., Uauy, C. *et al.* (2015) Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. *Genome Biol.* **16**, 258.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. *Nat. Biotechnol.* **9**, 963–967.

Li, J.F., Norville, J.E., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M. *et al.* (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat. Biotechnol.* **31**, 688–691.

Liu, Z.W., Shao, C.R., Zhang, C.J., Zhou, J.X., Zhang, S.W., Li, L., Chen, S. *et al.* (2014) The SET domain proteins SUVH2 and SUVH9 are required for Pol V occupancy at RNA-directed DNA methylation loci. *PLoS Genet.* **10**, e1003948.

Lorković, Z.J., Naumann, U., Matzke, A.J.M. and Matzke, M. (2012) Involvement of a GHKL ATPase in RNA-directed DNA Methylation in *Arabidopsis thaliana*. *Curr. Biol.* **22**, 933–938.

Lowder, L.G., Zhang, D., Baltes, N.J., Paul, J.W. III, Tang, X., Zheng, X., Voytas, D.F. *et al.* (2015) A CRISPR/Cas9 toolbox for multiplexed plant gnome editing and transcriptional regulation. *Plant Physiol.* **169**, 971–985.

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B. *et al.* (2015) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol. Plant*, **8**, 1274–1284.

© 2018 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

Ma, X., Zhu, Q., Chen, Y. and Liu, Y.G. (2016) CRISPR/Cas9 platforms for genome editing in plants: developments and applications. *Mol. Plant*, **9**, 961–974.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E. *et al.* (2013) RNA-guided human genome engineering via Cas9. *Science*, **339**, 823–826.

Malzahn, A., Lowder, L. and Qi, Y. (2017) Plant genome editing with TALEN and CRISPR. *Cell Biosci.* **7**, 21.

Manosalva, P., Manohar, M., Kogel, K.-H., Kang, H.-G. and Klessig, F. (2015) The GHKL ATPase MORC1 modulates species-specific plant immunity in Solanaceae. *Mol. Plant Microbe Interact.* **28**, 927–942.

Mao, Y., Zhang, H., Xu, N., Zhang, B., Gou, F. and Zhu, J.K. (2013) Application of the CRISPR-Cas system for efficient genome engineering in plants. *Mol. Plant*, **6**, 2008–2011.

Maresca, M., Lin, V.G., Guo, N. and Yang, Y. (2013) Obligate ligation-gated recombination (ObLiGaRe): custom-designed nuclease-mediated targeted integration through nonhomologous end joining. *Genome Res.* **23**, 539–546.

Marshallsay, C., Connelly, S. and Filipowicz, W. (1992) Characterization of the U3 and U6 snRNA genes from wheat: U3 snRNA genes in monocot plants are transcribed by RNA polymerase III. *Plant Mol. Biol.* **19**, 973–983.

Matzke, M., Kanno, T., Daxinger, L., Huettel, B. and Matzke, A.J. (2009) RNA-mediated chromatin-based silencing in plants. *Curr. Opin. Cell Biol.* **21**, 367–376.

Matzke, M.A., Kanno, T. and Matzke, A.J. (2015) RNA-directed DNA methylation: the evolution of a complex epigenetic pathway in flowering plants. *Annu. Rev. Plant Biol.* **66**, 243–267.

Miao, J., Guo, D., Zhang, J., Huang, Q., Qin, G., Zhang, X., Wan, J. *et al.* (2013) Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Res.* **23**, 1233–1236.

Moissiard, G., Cokus, S.J., Cary, J., Feng, S.H., Billi, A.C., Stroud, H., Husmann, D. *et al.* (2012) MORC family ATPases required for heterochromatin condensation and gene silencing. *Science*, **336**, 1448–1451.

Moissiard, G., Bischof, S., Husmann, D., Pastor, W.A., Hale, C.J., Yen, L., Stroud, H. *et al.* (2014) Transcriptional gene silencing by Arabidopsis microrchidia homologues involves the formation of heteromers. *Proc. Natl Acad. Sci. USA*, **111**, 7474–7479.

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J.D. and Kamoun, S. (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* **31**, 691–693.

Ordon, J., Gantner, J., Kemna, J., Schwalgun, L., Reschke, M., Streubel, J., Boch, J. *et al.* (2017) Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly genome editing toolkit. *Plant J.* **89**, 155–168.

Pastor, W.A., Stroud, H., Nee, K., Liu, W., Pezic, D., Manakov, S., Lee, S.A. *et al.* (2014) MORC1 represses transposable elements in the mouse male germline. *Nat. Commun.* **5**, 5795.

Perry, J. and Zhao, Y. (2003) The CW domain, a structural module shared amongst vertebrates, vertebrate-infecting parasites and higher plants. *Trends Biochem. Sci.* **28**, 576–580.

Qu, F., Zhai, W., Chen, H., Zhu, L.H. and Morris, T.J. (1996) Cloning, characterization and transient expression of the gene encoding a rice *U3* small nuclear RNA. *Gene*, **172**, 217–220.

van Schie, C.C.N. and Takken, F.L.W. (2014) Susceptibility genes 101: how to be a good host. *Ann. Rev. Phytopath.* **52**, 551–581.

Schweizer, P., Pokorny, J., Abderhalden, O. and Dudler, R. (1999) A transient assay system for the functional assessment of defense-related genes in wheat. *Mol. Plant Microbe Interact.* **12**, 647–654.

Shan, Q.W., Wang, Y.P., Li, J., Zhang, Y., Chen, K.L., Liang, Z., Zhang, K. *et al.* (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 686–688.

Smith, J., Bibikova, M., Whitby, F.G., Reddy, A.R., Chandrasegaran, S. and Carroll, D. (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucleic Acids Res.* **28**, 3361–3369.

Smith, I., Greenside, P.G., Natoli, T., Lahr, D.L., Wadden, D., Tirosh, I., Narayan, R. *et al.* (2017) Evaluation of RNAi and CRISPR technologies by large-scale gene expression profiling in the connectivity map. *PLoS Biol.* **15**, e2003213.

Sorek, R., Lawrence, C.M. and Wiedenheft, B. (2013) CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annu. Rev. Biochem.* **82**, 237–266.

Svitashev, S., Young, J.K., Schwartz, C., Gao, H.R., Falco, S.C. and Cigan, A.M. (2015) Targeted mutagenesis, precise gene-editing, and site-specific gene insertion in maize using Cas9 and guide RNA. *Plant Physiol.* **169**, 931–945.

Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S. and Brettell, R. (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J.* **11**, 1369–1376.

Waibel, F. and Filipowicz, W. (1990) RNA-polymerase specificity of transcription of Arabidopsis U snRNA genes determined by promoter element spacing. *Nature*, **346**, 199–202.

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C. and Qiu, J.L. (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* **32**, 947–951.

Xing, H.L., Dong, L., Wang, Z.P., Zhang, H.Y., Han, C.Y., Liu, B., Wang, X.C. *et al.* (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biol.* **14**, 327.

Xiong, J.S., Ding, J. and Li, Y. (2015) Genome-editing technologies and their potential application in horticultural crop breeding. *Hortic. Res.* **2**, 15019.

Zhang, F. (2016) *Functional analysis of Arabidopsis thaliana* matrix metalloproteinases and MORC in plant immunity (Doctoral dissertation). Retrieved from Giessener Elektronische Bibliothek (http://geb.uni-giessen.de/geb/volltexte/2016/12043/pdf/ZhangFei_2016_04_14.pdf)

Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., Mao, Y. *et al.* (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene-editing in rice in one generation. *Plant Biotechnol. J.* **12**, 797–807.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Maps of constructed plasmid.
Figure S2 Target site conservation in *MORC* genes.
Figure S3 638 bp sequence of barley RNA Pol III promoter (TATA box is underlined) (A); 380 bp sequence of rice RNA Pol III promoter (TATA box is underlined) (B); Sequence of sgRNA is underlined with terminator (C).
Figure S4 Relative expression of sgRNA under control of barley and rice U3 promoter (p*HvU3* and p*OsU3*) in leaves of *hvmorc1*-KO T2 homozygous mutants measured by RT-PCR and normalized against Hygromycin gene.
Figure S5 *Sp*Cas9-induced frame-shift mutations in *HvMORC1* leads to premature STOP codons Predicted *HvMORC*1 open reading frames (in red) with premature stop codons after Cas9 induced mutation (b-d) compared to wt (A) using online tool (http://web.expasy.org/translate/).
Table S1 Oligonucleotide primers used in this study (restriction sites are underlined).
Appendix S1 Cloning manual for pMGE genome editing vectors.
Appendix S2 Annotated sequence files (Genbank) for pMGE vectors.

environmental microbiology

Environmental Microbiology (2021) **23**(4), 2102–2115 doi:10.1111/1462-2920.15356

NPR1 is required for root colonization and the establishment of a mutualistic symbiosis between the beneficial bacterium *Rhizobium radiobacter* and barley

Neelendra Kumar,[1†] Matteo Galli,[1†] D'Maris Dempsey,[1] Jafargholi Imani,[1*] Anna Moebus[2] and Karl-Heinz Kogel[1*]

[1]*Institute of Phytopathology, Research Centre for BioSystems, Land Use and Nutrition, Justus Liebig University Giessen, Giessen, 35392, Germany.*

[2]*Biomedical Research Centre Seltersberg, Justus Liebig University, Giessen, 35392, Germany.*

Summary

Non-expressor of pathogenesis-related genes 1 (NPR1) is a key regulator of plant innate immunity and systemic disease resistance. The model for NPR1 function is based on experimental evidence obtained largely from dicots; however, this model does not fit all aspects of Poaceae family, which includes major crops such as wheat, rice and barley. In addition, there is little scientific data on NPR1's role in mutualistic symbioses. We assessed barley (*Hordeum vulgare*) HvNPR1 requirement during the establishment of mutualistic symbiosis between barley and beneficial Alphaproteobacterium *Rhizobium radiobacter* F4 (*Rr*F4). Upon *Rr*F4 root-inoculation, barley NPR1-knockdown (KD-*hvnpr1*) plants lost the typical spatiotemporal colonization pattern and supported less bacterial multiplication. Following *Rr*F4 colonization, expression of salicylic acid marker genes were strongly enhanced in wild-type roots; whereas in comparison, KD-*hvnpr1* roots exhibited little to no induction. Both basal and *Rr*F4-induced root-initiated systemic resistance against virulent *Blumeria graminis* were impaired in leaves of KD-*hvnpr1*. Besides these immune-related differences, KD-*hvnpr1* plants displayed higher root and shoot biomass than WT. However, *Rr*F4-mediated growth promotion was largely compromised in KD-*hvnpr1*. Our results demonstrate a critical role for HvNPR1 in establishing a mutualistic symbiosis between a beneficial bacterium and a cereal crop.

Received 17 September, 2020; revised 7 December, 2020; accepted 10 December, 2020. *For correspondence. E-mail karl-heinz.kogel@agrar.uni-giessen.de; jafargholi.imani@agrar.uni-giessen.de; Tel. +496419937490; Fax: +496419937499. †These authors contributed equally to the article. [Correction added on 12 January 2021, after first online publication: Jafargholi Imani was designated as co-corresponding author]

Introduction

During co-evolution with pests and microbes, plants have evolved ingenious local and systemic immune pathways. Local immune responses are initiated when highly conserved microbe- or pathogen-associated molecular patterns are detected by cell surface-localized pattern recognition receptors. This recognition triggers pattern-triggered immunity (PTI), which often is sufficient to prevent further pathogen ingress. However, some pathogens are able to suppress PTI. In this situation, the host plant displays a low level of resistance, termed basal resistance. To combat these virulent pathogens, some plants can activate the second layer of local immunity, termed effector-triggered immunity (ETI). Both PTI and ETI are associated with increased synthesis of the phytohormone salicylic acid (SA) and the activation of various defence responses in the infected tissue (Jones and Dangl, 2006; Mishina and Zeiser, 2007; Choi and Klessig, 2016). The systemic immune pathways are broadly categorized into systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Fu and Dong, 2013; Pieterse *et al*., 2014; Klessig *et al*., 2018). SAR is induced in distal uninfected tissues by a prior inoculation of a pathogen (Ross, 1961); like PTI and ETI, it is dependent on the SA signalling pathway. By contrast, ISR is induced primarily by pests (insects) and root-colonizing non-pathogenic microbes. Activation of ISR is mediated by the jasmonic acid (JA) and ethylene (ET) signalling pathways (Van Loon *et al*., 2006.

The discovery that SA is a critical endogenous signal for SAR led to extensive efforts to identify downstream signalling components. Characterization of several Arabidopsis mutants that accumulated endogenous SA but failed to activate SAR after pathogen infection or exogenous SA treatment led to the identification of a single gene, *Non-expressor of pathogenesis-related (PR) genes 1* (*NPR1*)

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

(Cao *et al*., 1994; Delaney *et al*., 1995; Glazebrook *et al*., 1996; Shah *et al*., 1997). Subsequent studies revealed that NPR1 not only plays a critical role in the establishment of SAR but also during ISR (van Loon *et al*., 2006). Structural analyses indicated that NPR1 contains an N-terminal BTB/POZ (broad-complex, tamtrack, bric-a-brac/pox virus, and zinc finger) domain, an ankyrin repeat domain, a C-terminal transactivation domain and nuclear localization sequence (Klessig *et al*., 2018). NPR1 is a redox-sensitive protein that resides in the cytosol as an oligomeric complex formed by intermolecular disulfide bonds (Mou *et al*., 2003). Following microbial infection, SA induces a biphasic change in the cellular redox state. The initial oxidative burst is followed by a more reducing environment that causes the NPR1 oligomer to disassociate (Mou *et al*., 2003; Tada *et al*., 2008). In addition, direct binding of SA promotes NPR1 monomerization (Wu *et al*., 2012). NPR1 monomers are then transported to the nucleus, where they serve as transcriptional coregulators of defence-associated genes, such as *Pathogenesis-related 1*, via their direct interaction with basic leucine zipper transcription factors from the TGA family (Klessig *et al*., 2018).

In comparison to its role in the nucleus, cytosolic NPR1 does not promote SA-induced defence gene expression. Instead, it modulates crosstalk between the SA and JA signalling pathways (Spoel *et al*., 2003). These pathways are thought to form the backbone of plant immunity, with SA generally mediating resistance to biotrophic pathogens and JA regulating resistance to necrotrophic pathogens and herbivorous insects. ET frequently works synergistically with JA to activate resistance to necrotrophs. The relationship between the SA and JA/ET signalling pathways often is mutually antagonistic, although synergistic interactions have been noted (Pieterse *et al*., 2012; De Vleesschauwer *et al*., 2014; Caarls *et al*., 2015; Shigenaga *et al*., 2017). The balance between the SA and JA/ET pathways presumably enables deployment of defences best suited to combat pathogens with different lifestyles.

Although SA's and NPR1's roles in mediating defence signalling have been well documented in many dicot species, their function in monocots is less clear. Studies in rice, which constitutively accumulates elevated levels of SA, as well as other cereals, suggest that SA is involved in immune signalling triggered by at least some pathogens (Klessig *et al*., 2018). In addition, NPR1 is conserved in dicots and monocots (Kogel and Langen, 2005; Balmer *et al*., 2013; Sharma *et al*., 2013). Overexpression of *AtNPR1* either primes or enhances SA-associated disease resistance in wheat (*Triticum aestivum*) and rice (*Oryza sativa*) against various pathogens, including *Xanthomonas oryzae*, *Magnaporthe oryzae* (*Mo*), *Fusarium verticillioides* and *Erwinia chrysanthemi* (Makandar *et al*., 2006; Chern *et al*., 2007; Quilis *et al*., 2008; Xu *et al*., 2017). Similarly, overexpression of wheat *TaNPR1* in barley (*Hordeum vulgare*) conferred enhanced resistance to *Mo*, whereas resistance to *Mo* was suppressed in a barley line with knocked-down (KD) expression of *HvNPR1* (Wang *et al*., 2018). Furthermore, protein interaction between NPR1 and TGAs is critical for NPR1 function in monocots and dicots (Després *et al*., 2003; Chern *et al*., 2007; Cantu *et al*., 2013), including expression of *PR* genes during resistance triggered by *P. syringae* DC3000 (Wang *et al*., 2016).

Despite these findings, the well-established model for NPR1's role in host–microbe interactions is not consistent with some aspects of the family of Poaceae, which includes major crops like wheat, rice and barley. These cereal crops do not develop a canonical SAR in which the activation of *PR* gene expression and broad-spectrum pathogen resistance in the systemic leaves is signalled by increased levels of endogenous SA (Kogel and Langen, 2005; Wang *et al*., 2018). In barley and wheat, inoculation with *Pseudomonas syringae* pv. *tomato* (*Pst*) induces enhanced resistance to secondary infection by other pathogens, a phenomenon termed acquired resistance (AR). Transcriptional profiling of barley tissue adjacent to the primary inoculation revealed similarities with the transcriptional profile of SAR in Arabidopsis, as well as transcripts previously associated with chemically induced AR in cereals (Beßer *et al*., 2000), suggesting that AR in barley and SAR in Arabidopsis may be mediated by analogous pathways. However, AR is not detected in systemic leaves, but rather in the region adjacent to the initial infection site (Colebrook *et al*., 2012; Gao *et al*., 2018). Alternatively, primary leaf infection of barley with *P. syringae* pv. *japonica* (*Psj*) induces systemic resistance in uninfected leaves against a subsequent challenge infection with *X. translucens* pv. *cerealis* (*Xtc*). Unlike SAR in Arabidopsis, however, systemic immunity in barley was not associated with HvNPR1, nor with the local or systemic accumulation of SA (Dey *et al*., 2014). Instead, it was associated with a moderate local, but not systemic, induction of abscisic acid (ABA). Local application of JA methyl ester or ABA, but not SA or BTH, triggered systemic immunity to *Xtc*. The systemic response correlated with the local and systemic induction of two WRKY and two ethylene-responsive factor-like transcription factors.

The role NPR1 and the SA signalling pathway play during establishment of mutualistic symbioses between plants and beneficial microbes also is poorly understood. Legumes are uniquely capable of forming symbiotic interactions with rhizobacteria belonging to the *Rhizobium* genus (Remigi *et al*., 2016). Inoculation of legume roots with symbiotic bacteria, such as *Sinorhizobium meliloti*,

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

or treatment with purified nodulation factors induces various early responses, such as root hair deformation and induction of early and late nodulin genes. Analyses of *Medicago truncatula* with altered levels of NPR1 expression revealed that *S. meliloti*-induced root hair deformation was suppressed in plants that overexpressed *AtNPR1*, while it was accelerated in plants silenced for *NPR1* expression (Peleg-Grossman *et al*., 2009). Interestingly, *S. meliloti*-induced root hair deformation and expression of early nodulin genes also were observed in the non-legume *Arabidopsis*, but only in the *npr1* mutant background rather than WT plants. Thus, NPR1 appears to suppress plant responses to *Rhizobia* (Peleg-Grossman *et al*., 2009). Further implicating the SA signalling pathway as a negative regulator of plant–*Rhizobium* symbiotic interactions, SA treatment of *M. truncatula* inhibits *S. meliloti*-induced root hair deformation, whereas this response is supported in SA-deficient *NahG* but not WT Arabidopsis. Additionally, SA levels are reduced in *M. truncatula* during the first days of *S. meliloti* infection, which may result in reduced NPR1-dependent gene expression (Martinez-Abarca *et al*., 1998). By contrast, the ectomycorrhiza (EM) fungus *Laccaria bicolor* promotes mutualism in *Populus* by expressing the effector protein MiSSP7 (Mycorrhiza-induced small secreted protein 7), which blocks the JA signalling network by binding and protecting the host protein PtJAZ6 (Jasmonate ZIM-domain [JAZ] protein 6) from degradation (Plett *et al*., 2014). Likewise, *Populus* roots colonized with the EM fungus *Paxillus involutus* accumulate elevated levels of the stress-related hormone ABA and SA compared with non-EM colonized roots, whereas JA and auxin levels are reduced (Luo *et al*., 2009). Together, these studies indicate that various plant immune signalling pathways can impact the establishment of mutualistic symbioses between different microbes and their hosts.

In the present work, we investigated whether NPR1 is involved in establishing a mutualistic symbiosis between the Alphaproteobacterium *Rhizobium radiobacter* (*Rr*F4) and the cereal plant barley. The beneficial bacterium *Rr*F4 was originally isolated from the sebacinoid basidiomycete fungus *Serendipita indica* (Weiß *et al*., 2016; syn. *Piriformospora indica*, Verma *et al*., 1998), a host-unspecific root endophyte that colonizes virtually all plants so far tested under greenhouse conditions (Sharma *et al*., 2008). *Rr*F4 shows a high degree of genomic similarity to the plant pathogen *R. radiobacter* (formerly: *Agrobacterium tumefaciens*) C58 (Glaeser *et al*., 2016). Similar to its fungal host *S. indica*, *Rr*F4 colonizes plant roots without host preference and forms aggregates of attached cells and dense biofilms at the root surface of maturation zones. *Rr*F4-colonized plants show increased biomass and systemically enhanced resistance against the powdery mildew fungus *B. graminis* f.sp. *hordei* (*Bgh*) in barley and bacterial leaf pathogens such as *Pst* DC3000 in Arabidopsis and *X. translucens* pv. *translucens* (*Xtt*) in wheat (Sharma *et al*., 2008; Glaeser *et al*., 2016; Alabid *et al.,* 2020).

Here, we examine the influence of *NPR1* on the beneficial bacterium *R. radiobacter* F4 to form mutualistic symbioses with roots of the cereal crop barley and further analyse the signalling pathways modulated during root colonization and induction of systemic resistance. Our results indicate that HvNPR1 plays a critical role in the establishment of a mutualistic symbiosis between a bacterium and a cereal crop. This study hence expands our understanding of the molecular nature of plant–microbe interaction in cereals.

Results and discussion

Phylogenetic analysis and in silico *identification of barley* NPR1-*like genes*

In Arabidopsis, *NPR1* belongs to a gene family that contains five additional members (*AtNPR2-6*). Previous analyses in barley identified *HvNPR1* (GenBank: AM050559.1), which encodes a protein containing the conserved domains identified in other NPR1 homologues such as the BTB/POZ domain, the DUF domain (Domain of Unknown Function), the ankyrin repeat domain and a NPR1/NIM1 like defence protein C terminal domain (Fig. Fig. S1a; Kogel and Langen, 2005). Two additional *HvNPR1*-like genes, *Cul4* (GenBank: AK360734.1) and *Lax-a* (GenBank: AK359086.1) have been published (Tavakol *et al*., 2015; Jost *et al*., 2016; Castelló *et al*., 2018). These genes share high similarity with Arabidopsis *Blade-On-Petiole 1* (*BOP1*; syn. *AtNPR5*) and *BOP2* (syn. *AtNPR6*) respectively (Fig. Fig. S1b). Phylogenetic analyses have divided NPR1-like proteins into three clades: clade I contains AtNPR1 and AtNPR2 homologues, clade II contains AtNPR3 and AtNPR4 homologues and clade III contains AtNPR5 and AtNPR6 homologues (Fig. 1; Backer *et al*., 2019). The clear separation of clade I and II is currently controversial (Toriba *et al*., 2019).

To mine additional barley *NPR1*-like genes, we conducted a genome-wide analysis across several species based on predicted protein data from the National Centre for Biotechnology Information. Using domain prediction analysis (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), we identified two additional genes that encode HvNPR1-like proteins, *HvNPR2* (BAJ86173.1) and *HvNPR3* (BAJ90272.1) (Fig. Fig. S1c,d). Protein sequences corresponding to *HvNPR2* (*HORVU3Hr1G074640.4*) and *HvNPR3* (*HORVU4Hr1G003040.1*) were also found in the barley cv. Morex sequencing database of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK,

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

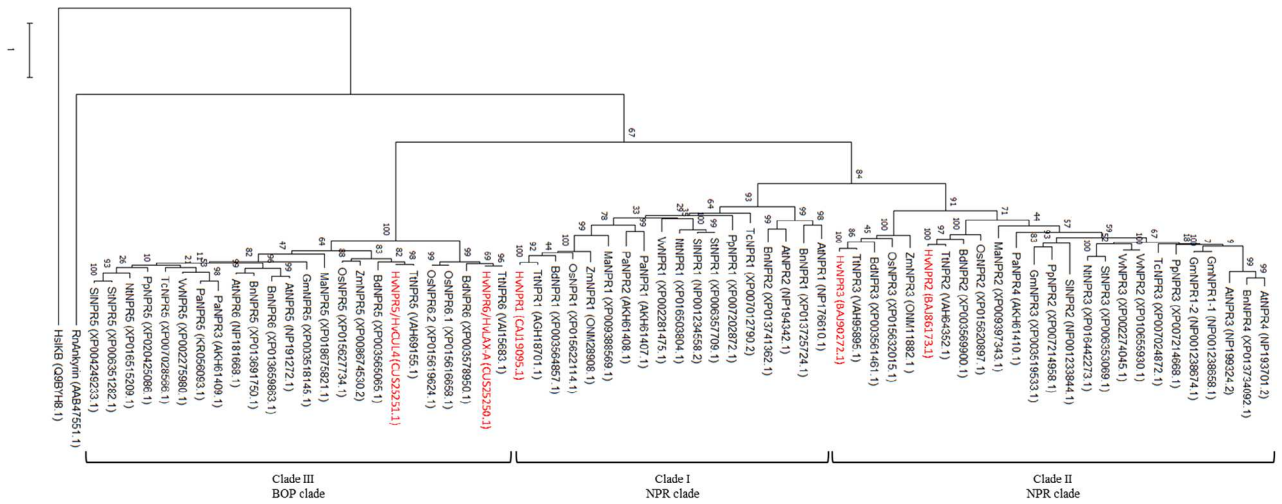


Fig 1. Phylogenetic relationship of barley NPRs with their homologues in other species. Amino acid (aa) sequences of HvNPR1 and its homologues were aligned using the MUSCLE algorithm and the maximum likelihood tree was generated using the MEGA software (MEGA X version 10.0.5, Kumar *et al*., 2018). Numbers in the tree nodes indicate confidence values based on 1000 bootstrap replicates. The following species were included in the analysis: the dicot model *Arabidopsis thaliana* (*At*), the grass model *Brachypodium distachyon* (*Bd*), *Brassica napus* (*Bn*), *Glycine max* (*Gm*), *Hordeum vulgare* (*Hv*, in red), *Musa acuminata* (*Ma*), *Nicotiana tabacum* (*Nt*), *Oryza sativa* (*Os*), *Persea americana* (*Pa*), *Prunum persica* (*Pp*), *Solanum lycopersicum* (*Sl*), *Solanum tuberosum* (*St*), *Theobroma cacao* (*Tc*), *Triticum turgidum* (*Tt*), *Vitis vinifera* (*Vv*) and *Zea mays* (*Zm*). The ankyrin-2 sequence from *Rattus norvegicus* (*Rn*) and human *NF-kappa-B inhibitor zeta* (*Hs*IkB) were used as outgroups. The scale bar at the bottom indicates the evolutionary distance corresponding to one aa substitution per site. [Color figure can be viewed at wileyonlinelibrary.com]

Gatersleben, Germany (https://webblast.ipk-gatersleben.de/barley_ibsc/viroblast.php)). Based on amino acid (aa) sequence, HvNPR2 shares the highest similarity with rice OsNPR2 and the grass model *Brachypodium distachyon* BdNPR2, and HvNPR3 shares the greatest level of similarity with OsNPR3 and BdNPR3, which all cluster in clade II (see Fig. 1). The domain structure of all barley *NPR1*-like genes was also tested by their exon-intron distribution frequency (http://gsds.cbi.pku.edu.cn), further confirming the placement of the barley NPR1-like proteins in the various phylogenetic clades (Fig. Fig. S1e).

HvNPR1 modulates colonization of barley roots by Rr*F4*

To assess the possibility that HvNPR1 plays a role in establishing a mutualistic symbiosis between the beneficial bacterium *Rr*F4 and barley, we monitored root colonization in *Rr*F4-inoculated WT plants and a barley line (cv. Golden Promise) that was partially silenced for *HvNPR1* expression (KD-*hvnpr1* E7L2 and E11L9 lines; Dey *et al*., 2014). As anticipated from a previous study, the relative level of *HvNPR1* transcript in homozygous KD-*hvnpr1* lines was 32% and 47% respectively, compared with wt plants (Fig. 2a), and KD-*hvnpr1* E7L2 plants lost sensitivity to the resistance inducer benzothiadiazole (BTH; Fig. 3; Görlach *et al*., 1996). To confirm that *HvNPR1* silencing was specific, we investigated possible off-target effects on other *HvNPRs*. As expected due to the lack of off-target detection with SiFi software, the KD-hvnpr1 lines E7L2 and E11L9 were silenced for *HvNPR1* expression, while *HvNPR2*, *HvNPR3*, *HvNPR5* and *HvNPR6* expression was not affected (Fig. 2b; Fig. S2).

The roots of 3-day-old WT and KD-*hvnpr1* seedlings were dip-inoculated with a *ß-glucuronidase* (*GUS*)-expressing *Rr*F4 strain (Glaeser *et al*., 2016). Subsequently, seedlings were cultivated in glass jars on half-strength Murashige and Skoog (1/2 MS) medium. Detached roots were treated with the GUS substrate 5-Bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexyl ammonium salt (X-gluc) for visualization of bacterial cells. Starting from 2 days post-inoculation (dpi), WT roots showed a dark blue colour that was spatially restricted to the root hair zone, whereas KD-*hvnpr1* roots showed a fainter colouring that was distributed across the root tips (Fig. 4a–d; Fig. S3). Based on the staining intensity and pattern, bacterial colonization of WT roots appears to be stronger than that of KD-*hvnpr1* roots. This finding raises the possibility that HvNPR1 positively regulates the spatiotemporal colonization pattern of *Rr*F4. To further investigate this possibility, WT and KD-*hvnpr1* plants were inoculated with *Rr*F4 and cultivated in the soil for 3 weeks; DNA was then extracted from roots and quantified by quantitative real-time PCR (qRT-PCR) using bacteria-specific internal transcribed spacer (ITS) primers (Glaeser *et al*., 2016). Based on the relative level of *Rr*F4 ITS, the roots of both KD-*hvnpr1* lines E7L2 and E11L9 supported substantially lower levels of *Rr*F4 than those of WT plants (Fig. 4e). We extended our analysis by transmission electron microscopy (TEM) to understand the

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

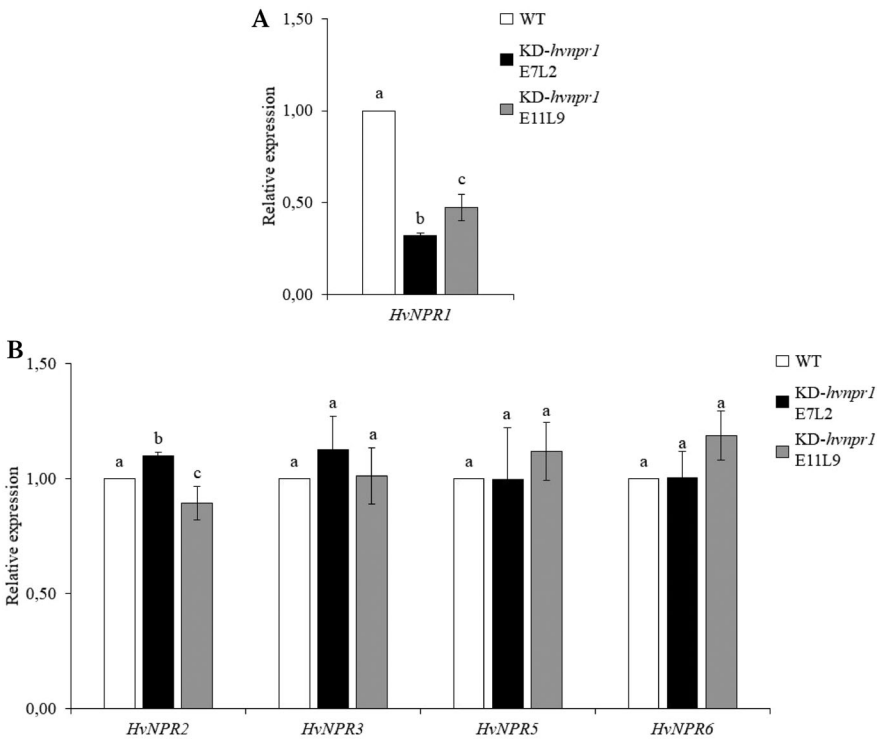


Fig 2. Relative expression of *HvNPR* genes determined by qRT-PCR in wild type (WT) barley cv. Golden Promise (GP) and in two KD-*hvnpr1* lines (Dey *et al*., 2014). The results were obtained using the T3 (E11L9) and T5 (E7L2) generation of transgenic plants. The transcript level of *HvNPR1* (A) and other *HvNPR* family members (B) was normalized to barley *Ubiquitin* (GenBank: M60176.1). Displayed is the mean of six technical repetitions (n = 10 plants). The experiment was conducted two times (n = 10 plants) with similar results. Error bars represent standard deviation (SD). Letters represent the statistical difference among each group means (Tukey's range test, α = 0.05).

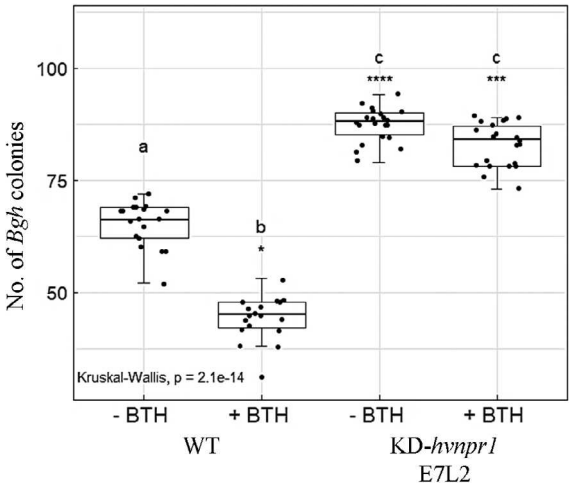


Fig 3. Sensitivity of barley to the resistance-inducing compound benzothiadiazole (BTH). Ten milliliters of 20 ppm BTH in wettable powder (WP) and WP alone as mock control were applied to a 5-day-old cv. Golden Promise WT and KD-*hvnpr1* seedlings as a soil drench. Two days later, detached first leaves were inoculated with *Bgh*A6 and at 6 dpi colonies were counted. *Bgh*A6 colony numbers on BTH-treated WT plants were lower than numbers on mock-treated plants. In contrast, BTH-treated KD-*hvnpr1* plants showed only minor reductions in *BghA6* colony number compared with mock-treated KD-*hvnpr1*, showing that BTH-induced resistance is dependent on HvNPR1. The experiment was conducted two times (n = 20 plants) with similar results. Comparisons between groups were performed via the Kruskal–Wallis test and Dunn's test of multiple comparisons. Asterisks represent the statistical differences of the groups against WT mock (*p < 0.05; ***p < 0.001; ****p < 0.0001). Letters represent statistical difference among all groups (α = 0.05).

rhizodermal colonization pattern of the bacteria. Due to the stronger *HvNPR1* silencing effect, these experiments were done with line KD-*hvnpr1* E7L2. At 5 dpi, *Rr*F4 cells had already penetrated into the WT roots. Significantly, and in accordance with Glaeser *et al*. (2016), *Rr*F4 predominantly colonize the extracellular spaces of the root cortex (Fig. 5A–C). In clear contrast, the roots of KD-*hvnpr1* plants showed a broad layer of bacteria that were located on the outside of the rhizodermis and bacteria could not be found in the extracellular spaces of the cortex (Fig. 5D–F). Thus, HvNPR1 appears to be required, at least in part, for effective colonization of barley roots by the beneficial microbe *Rr*F4.

Rr*F4-induced expression of SA but not JA marker genes is compromised in KD-*hvnpr1 *roots*

Whether *Rr*F4 inoculation impacts the local expression of plant defence genes was then assessed in WT and KD-*hvnpr1* roots over a 6-day time-course. Three-day-old barley seedlings were dip-inoculated with *Rr*F4 or dipped into buffer (mock) and axenically grown roots were harvested for qRT-PCR analysis at the indicated time points (Fig. 6). From 2 dpi onwards, expression of the SA marker genes *HvPR1b* and *HvPR2* was significantly higher in *Rr*F4-colonized WT roots compared with mock-treated roots (Fig. 6A and B). In contrast, *Rr*F4 colonization did not enhance the expression of either *PR* gene in KD-*hvnpr1* roots at 2 or 4 dpi, although a small induction

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

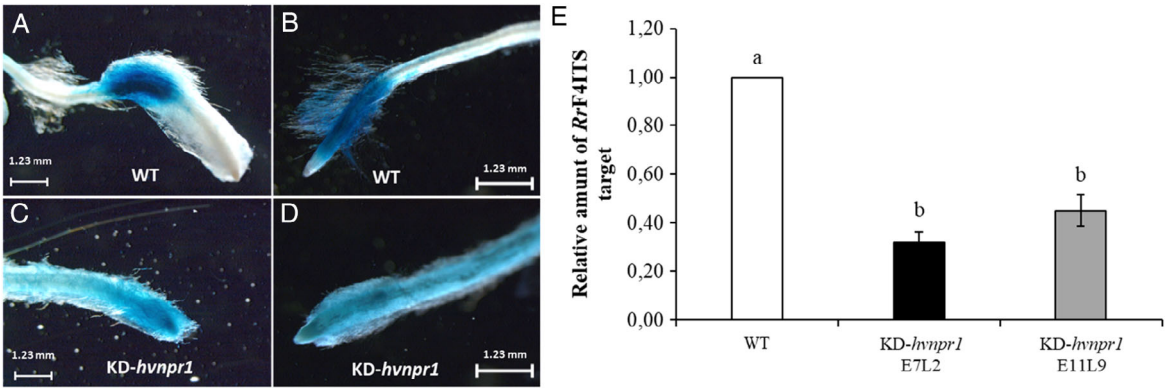


Fig 4. *Rr*F4 colonization pattern and strength in barley cv. Golden Promise WT and KD-*hvnpr1* roots. Root segments colonized by *GUS*-expressing *Rr*F4 at 5 dpi in WT (A, B) and KD-*hvnpr1* E7L2 (C, D) plants. (E) Relative qPCR analysis of the quantity of *Rr*F4 cells in roots of 3-day-old plants at 5 dpi using primers specific for barley *Ubiquitin* and *Rr*F4 *ITS*. The number of bacteria was significantly reduced in both roots of KD-*hvnpr1* mutant lines compared with WT plants. The experiment was conducted two times (n = 10 plants) with similar results. Displayed are means with standard errors of three independent biological experiments. Letters represent the statistical differences among the group means (Tukey's range test, α = 0.05). [Color figure can be viewed at wileyonlinelibrary.com]

was detected at 6 dpi, potentially due to residual NPR1 activity. The JA marker *S-adenosyl-l-methionine: jasmonic acid carboxyl methyltransferase* (*HvJMT*) also was induced by *Rr*F4 colonization of WT roots, although a dramatic increase was not detected until 4 dpi (Fig. 6C). In contrast to either *PR* gene, *HvJMT* expression was strongly enhanced in KD-*hvnpr1* roots after *Rr*F4 inoculation, with transcripts for this gene accumulating to even greater levels than in comparably treated WT plants at 2 and 6 dpi.

Taken together, these data suggest that root inoculation with the mutualistic microbe *Rr*F4 enhances local expression of *HvPR1b* and *HvPR2* via a pathway that is largely dependent on HvNPR1, whereas it induces *HvJMT* expression via a pathway that is largely HvNPR1-independent.

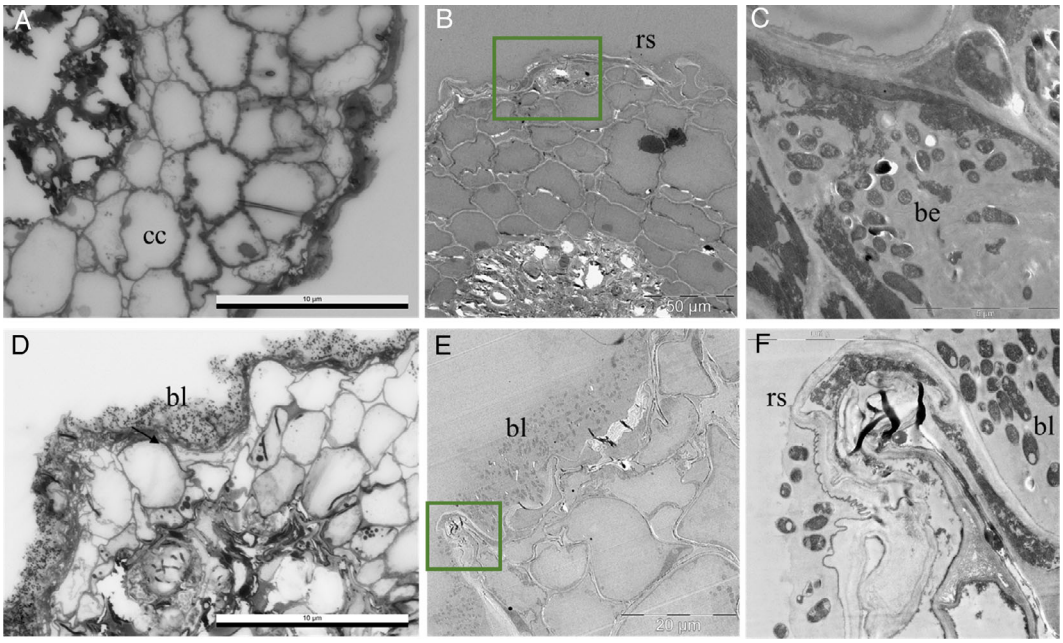


Fig 5. TEM analysis of the colonization pattern of *Rr*F4 in WT vs. KD-*hvnpr1* barley roots. Three-day-old seedlings were dip-inoculated for 30 min into bacterial suspensions (OD_{600} = 1.4–2), the colonization pattern was analysed at 5 dpi. In WT plants, bacterial colonization was located mainly in the root cortex (A, B, C), whereas in KD-*hvnpr1* roots it was located on the outside of the rhizodermis (D, E, F). Cc, root cortex cells; bl, bacterial layer outside on the root surface; be, bacteria in the extracellular space of cortex cells; rs, root surface. [Color figure can be viewed at wileyonlinelibrary.com]

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

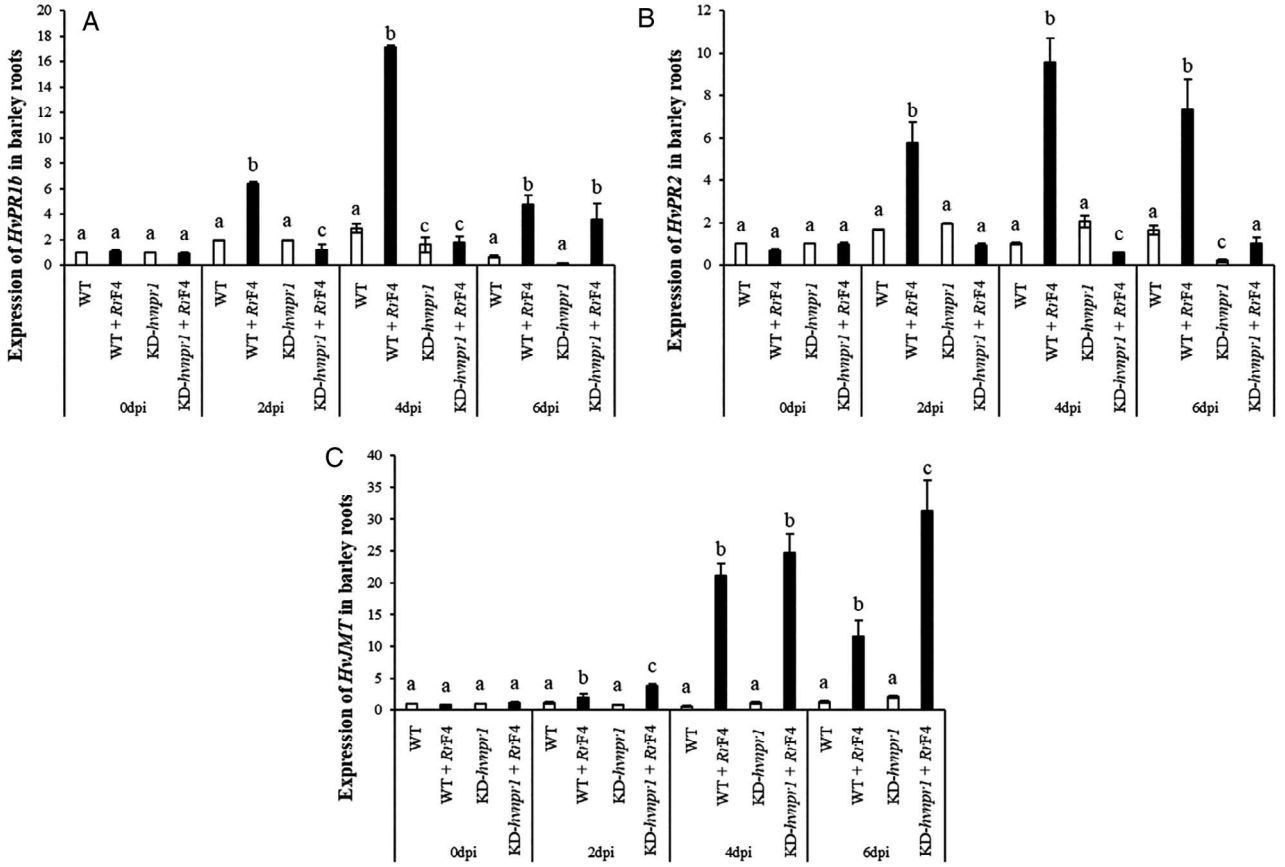


Fig 6. Relative expression of immune-related genes in the roots of KD-*hvnpr1* vs. WT barley in the presence or absence of *Rr*F4. Transcripts of *HvPR1b* (A), *HvPR2* (B), or *HvJMT* (C) were assessed by qRT-PCR and normalized to barley *ubiquitin*. Roots of 3-day-old seedlings were dip-inoculated with *Rr*F4 (OD_{600} = 1.4–2) and harvested at 0, 2, 4 and 6 dpi. The experiment was conducted three times (n = 7 plants) with similar results. Error bars indicate standard deviation. For each gene, the different letters above the bars indicate significant differences in the means determined by one-way ANOVA with post-hoc Tukey HSD test (α = 0.05).

Our data are consistent with a report showing that elevated levels of JA prevent endophytic colonization of rice roots by the nitrogen-fixing *Azoarcus* sp. strain BH72 (Miché *et al*., 2006). Furthermore, our results confirm that an intact immune status of the roots is important for the establishment of a mutualistic interaction, as has been shown for fungal sebacinoid endophytes, such as *S. indica* in Arabidopsis (Lahrmann *et al*., 2015).

HvNPR1 is required for both Rr*F4-induced root-initiated systemic resistance and basal resistance to powdery mildew*

In Arabidopsis, root colonization with *Rr*F4 results in enhanced systemic resistance against *Pst* DC3000 (Glaeser *et al*., 2016). Mutational analysis showed that this systemic resistance does not require NPR1 or SA, but instead is dependent on the JA-induced ISR pathway. The unavailability of similar mutants in cereals has hampered such analyses in these important crops. To assess the requirement of HvNPR1 in root-initiated systemic resistance of a monocotyledonous plant, roots of 3-day-old KD-*hvnpr1* and WT barley seedlings were either dip-inoculated in an *Rr*F4 suspension or mock treated with buffer. The seedlings were grown for 3 weeks in the soil; leaves were then harvested and inoculated with the virulent isolate A6 of *Bgh* (*Bgh*A6). At 6 dpi with *BghA6*, the detached leaves from *Rr*F4-colonized WT plants displayed fewer fungal colonies than the leaves from mock-treated plants (Fig. 7). Thus, root colonization with *Rr*F4 initiated systemic resistance to this virulent fungal pathogen. By contrast, as after BTH treatment (see Fig. 3) comparable numbers of *Bgh*A6 colonies were observed on the detached leaves of KD-*hvnpr1* plants regardless of whether their roots were treated with *Rr*F4 or buffer. It should be noted that the leaves of buffer-treated (as well as *Rr*F4-colonized) KD-*hvnpr1* plants supported a greater number of fungal colonies than the leaves of buffer-treated WT plants.

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

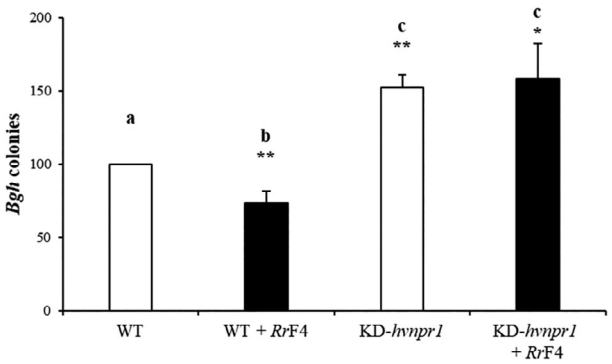


Fig 7. Knock-down (KD) of barley *HvNPR1* results in altered basal and root-initiated systemic disease resistance to the powdery mildew fungus *B. graminis* f.sp. *hordei* (*Bgh*). Number of *Bgh* colonies on detached leaves of WT and KD-hvnpr1 plants whose roots were or were not colonized by *Rr*F4. After dip-inoculating the roots of 3-day-old seedlings in an *Rr*F4 suspension (OD_{600} = 1.4) or 10 mM $MgSO_4$ $7H_2O$ buffer, plants were grown in soil for 3 weeks. Twenty-four-day-old detached third leaves were inoculated with 3–5 *Bgh* conidia/mm^{-2} and fungal colonies were counted 6 days later. The graph shows the percentage in pustules count of three independent experiments (n = 15 plants). Comparisons between groups were performed via Kruskal–Wallis test and Dunn's test of multiple comparisons. Error bars represent standard deviation. Asterisks indicate statistical difference of the group means against WT mock (*p < 0.05; **p < 0.01; ***p < 0.001). Letters represent the statistical differences among all group means (α = 0.05).

Together, these results both confirm our prior demonstration that HvNPR1 is required in the inoculated leaf for basal resistance to *Bgh*A6 (Dey *et al*., 2014), and they reveal a critical role for HvNPR1 in root-initiated systemic resistance. This latter finding extends an earlier report investigating the role of NPR1 in barley during AR (Gao *et al*., 2018). Previously, foliar inoculation of WT barley with *Pst* DC3000 was shown to induce heightened resistance in the adjacent tissue (outside of the initial infection zone) to a secondary infection by *Mo*. This *Pst* DC3000-induced AR was suppressed in the *HvNPR1* knock-down line E7L2 line but enhanced in a barley line overexpressing wheat *wNPR1* (Gao *et al*., 2018). By contrast, a different study indicated that HvNPR1 is not required for systemic immunity triggered by inoculating a lower leaf of barley plants with either *Xtc* or *Psj*. In comparison to plants that received a primary mock inoculation, the systemic leaves of KD-*hvnpr1* (line E7L2) and WT plants that received a primary inoculation with *Xtc* or *Psi* displayed a similar reduction in bacterial growth following challenge inoculation with *Xtc* (Dey *et al*., 2014). Further studies will be required to determine how the location of the primary infection (root vs. leaf) and/or the identity of the pathogen influence activation of systemic resistance via NPR1-dependent or -independent signalling pathways.

Rr*F4-induced systemic defence gene expression is compromised in KD-*hvnpr1 *plants*

Next, we investigated whether the HvNPR1-dependent systemic resistance triggered by *Rr*F4 root colonization is associated with increased defence gene expression in barley leaves. To this end, the roots of WT and KD-*hvnpr1* seedlings were dip-inoculated with *Rr*F4 or dipped into the buffer (mock). After growing the seedlings on soil for 3 weeks, leaves were detached and inoculated with *BghA6* conidia. Relative levels of *HvPR1b*, *HvPR2* and *HvPR5* expression were then determined by qRT-PCR analysis at 0, 18, 36, 48 and 72 h post-inoculation (hpi) (Fig. 8; Fig. S4). At all time points after *Bgh*A6 inoculation, expression levels of *HvPR1b*, *HvPR2* and *HvPR5* were substantially lower in the leaves of *Rr*F4-colonized KD-*hvnpr1* plants compared with comparably treated WT plants. Thus, the ability of *Rr*F4 root colonization to effectively induce systemic *PR* gene expression appears to be largely dependent on HvNPR1.

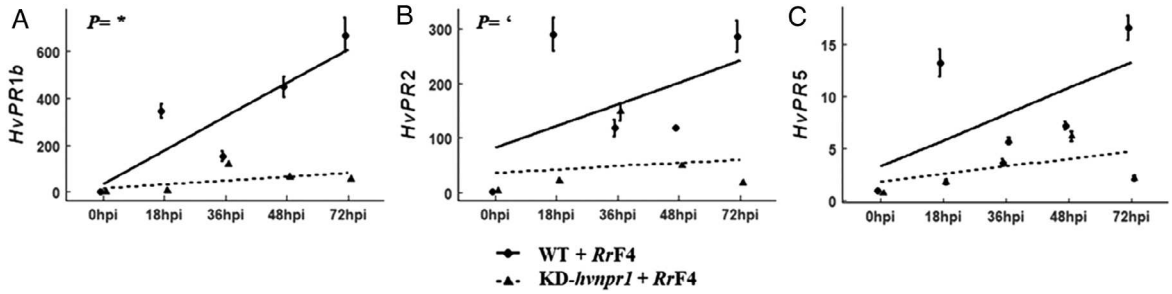


Fig 8. Scatterplots with trendlines of the relative systemic expression of defence-related genes upon *Bgh*A6 challenge inoculation of *Rr*F4-colonized WT or KD-*hvnpr1* plants. Transcripts of *HvPR1b* (A), *HvPR2* (B) and *HvPR5* (C) were assessed by qRT-PCR and normalized to barley *ubiquitin*. Roots of WT and KD-*hvnpr1* seedlings were dip-inoculated with *Rr*F4 (OD_{600} = 2). After growing the seedlings in the soil for 3 weeks, the detached youngest leaves were inoculated with 10–15 *Bgh*A6 conidia/mm^{-2} and harvested 0, 18, 36, 48 and 72 hpi. Displayed are the means of three biological repetitions (n = 4 plants). Error bars indicate standard deviation. Significant differences between the linear regression analyses were determined by one-way ANOVA ('p < 0.1, *p < 0.05).

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

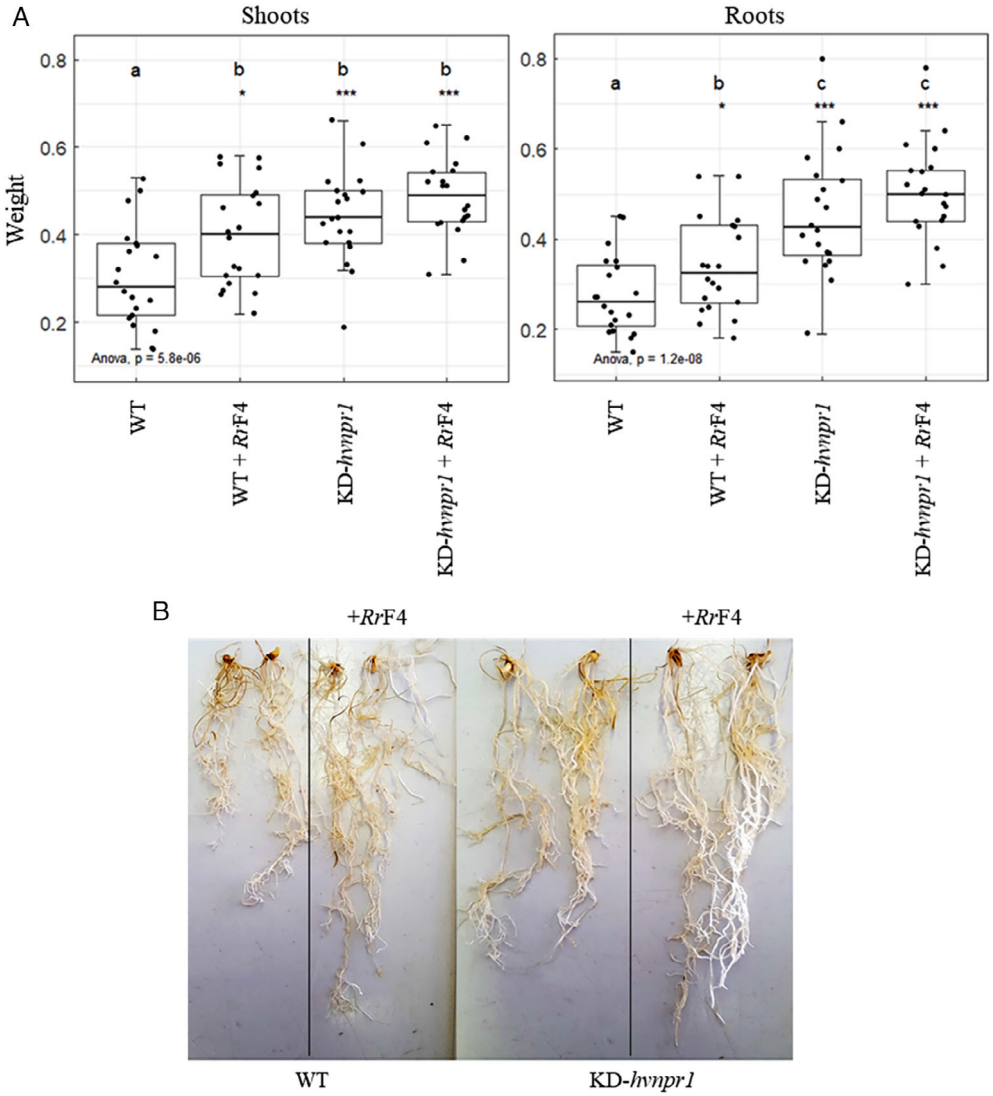


Fig 9. Root and shoot biomass of 3-week-old WT and KD-*hvnpr1* plants after colonization with *Rr*F4 was compared with non-colonized plants. Plants were cultivated in artificial soil containing 2:1 mixture of expanded clay (Seramis) and Oil-Dri in a growth chamber at 22°C/18°C (day/night cycle) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density) (A) Root and shoot fresh weight (FW) and (B) root morphology. The experiment was conducted two times ($n = 20$ plants) with similar results. Comparisons between groups were performed via one-way ANOVA and Tukey's range test. Asterisks represent statistical difference of the group means against WT mock (*$p < 0.05$; ***$p < 0.001$). Letters represent the statistical differences among all group means ($\alpha = 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

KD-hvnpr1 *plants have a higher biomass but are compromised for* Rr*F4-induced growth promotion*

From an agronomic viewpoint, it is critical to determine whether NPR1's function as a key regulator of *PR* gene expression and pathogen defence also has an impact on plant yield (Xu *et al*., 2017). Previous studies have shown that the biomass of Arabidopsis and barley plants is enhanced after root inoculation with *Rr*F4 (Sharma *et al*., 2008; Glaeser *et al*., 2016). To assess whether this response is dependent on HvNPR1, we recorded the biomasses of WT and KD-*hvnpr1* plants whose roots were inoculated with either buffer or *Rr*F4 over a growth period of 3 weeks. *Rr*F4-colonized WT plants showed a strong increase in root and shoot fresh weight (FW) compared with mock-treated WT plants, corroborating the findings of Sharma *et al*. (2008). Strikingly, the root and shoot FWs of mock-inoculated KD-*hvnpr1* plants were significantly higher (Tukey's range test $p < 0.001$) than those of either mock- or *Rr*F4-inoculated WT plants (Fig. 9; Fig. S5). In comparison to WT plants, however, the FW of *Rr*F4-colonized KD-*hvnpr1* plants showed only a slight, statistically insignificant increase over that of mock-treated KD-*hvnpr1* plants. To further substantiate the hypothesis that NPR1 is required for plant fitness and

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

growth, we also recorded the root and shoot biomasses of KD-*hvnpr1* E11L9, which shows a weaker (53%) silencing effect. Both root and shoot FWs were significantly higher compared with WT plants (Fig. S6), suggesting a negative correlation between *HvNPR1* transcript levels and growth promotion. That plants with reduced *HvNPR1* expression display better fitness in terms of root and shoot growth is consistent with the hypothesis that a weakened immune system results in a stronger growth phenotype (Heil and Baldwin, 2002; Abreu and Munné-Bosch, 2009; Yang *et al*., 2012; Huot *et al*., 2014).

Conclusion

The results presented here suggest that *HvNPR1* plays a vital role in the establishment of a mutualistic symbiosis. Following *Rr*F4 inoculation, the roots of KD-*hvnpr1* plants displayed a different spatiotemporal colonization pattern than the roots of WT plants, and they supported substantially fewer bacterial cells. The reduced multiplication of *Rr*F4 in KD-*hvnpr1* roots was associated with reduced local and systemic expression of several SA marker genes, including *HvPR1b*, *HvPR2* and/or *HvPR5*, while local expression of the JA marker *HvJMT* was either comparable to or higher than that detected in *Rr*F4-inoculated WT plants. Based on these findings, we hypothesize that *Rr*F4-mediated activation of the SA signalling pathway may help to downregulate the JA pathway, thereby enhancing the colonization of barley roots. In addition, *KD-hvnpr1* plants were compromised for *Rr*F4-induced root-initiated systemic resistance to *Bgh*A6. Together, these findings suggest that HvNPR1 plays important roles in both modulating the tissue-specific capacity for successful *Rr*F4 colonization, as well as transducing the signal for *Rr*F4-induced immune responses in barley. Finally, HvNPR1 function negatively interferes with the growth of barley roots and shoots, however, reinforces *Rr*F4-induced growth responses.

Experimental procedures

Plant material and inoculation with Bgh

Seeds of spring barley (*H. vulgare*) cv. Golden Promise (GP) and GP-derived KD-*hvnpr1*-E7L2 plants were surface sterilized and grown under sterile conditions for 3 days (Glaeser *et al*., 2016). The generation of KD-*hvnpr1*-E7L2 plants is described in Dey *et al*. (2014). A conserved domain of HvNPR1 (aa 204–333) was used to generate hairpin RNA constructs for RNAi-mediated silencing of *HvNPR1*. Seeds were germinated on sterile filter paper for 3 days at 22°C/18°C (day/night cycle) and roots were dipped in *Rr*F4 suspension buffer (OD_{600} = 1.4–2) or just in suspension buffer (10 mM $MgSO_4$ $7H_2O$) for 2–3 h. Subsequently, the seedlings were transferred, depending on the experiment, to pots (⌀12 cm) containing soil (Fruhstorfer Erde Typ T) or alternatively in 2.5-L glass jars on 1/2 MS medium (150 ml tot. vol.). Plants were cultivated then in a growth chamber at 22°C/18°C (day/night cycle) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). Plants in soil were fertilized weekly with 0.1% WUXAL top N solution (N/P/K: 12/4/6; Aglukon, Düsseldorf, Germany). The detached leaf assay was done with the third leaves of 3-week-old plants. Leaf segments were laid on 1% (wt./vol.) water agar and inoculated with fresh conidia of *Blumeria graminis* f.sp. *hordei* (*Bgh*) race A6 as described in Dey *et al*. (2014). For the root defence-gene analysis, after plants were moved in ½ MS medium, at 0, 2, 4 and 6 dpi roots were harvested, crushed in liquid nitrogen with the help of a mortar and pestle and extracted DNA/RNA analysed via qPCR.

BTH treatment

Barley plants were grown in 200 g capacity pots in soil (Fruhstorfer Erde, Vechta, Germany) under controlled condition 16 h light (240 μmol m^{-2} s^{-1} photon flux density) and 60% relative humidity (22/18°C day/night cycle). Ten milliliters of 20 ppm BTH (CGA245704, Bion®, Novartis, Basel, Switzerland) formulated as 50% active ingredient with wettable powder (WP) in water was applied to 5-day-old seedlings as a soil drench. Control plants were treated with WP. Two days after BTH treatment, first leaf segments were placed on 0.5% (wt./vol.) water agar containing 20 mg L^{-1} benzimidazole (Merck-Schuchardt, Munich, Germany) and inoculated with *Bgh*A6 (5 conidia/mm^2 density) by air current dispersion in an inoculation tower and saved in the same climate chamber for 7 days. *Bgh* colonies were counted using a binocular on a 2.5 cm^2 segment. Comparisons between groups were performed via ANOVA + Tukey with a 95% family-wise confidence level.

Inoculation of roots with Rr*F4, genomic DNA isolation and qRT-PCR*

Bacteria culturing, root inoculation and DNA extraction were performed as described in Glaeser *et al*. (2016). Briefly, the Alphaproteobacterium *R. radiobacter* F4 (*Rr*F4; syn. *Agrobacterium fabrum*, syn. *Agrobacterium tumefaciens*) originally isolated from the beneficial fungus *P. indica* DSM 11827 (Sharma *et al*., 2008; Glaeser *et al*., 2016) was grown overnight in modified LB broth (1% casamino hydrolysate, 0.5% yeast extract and 5% NaCl, pH 7.0, supplemented with 100 μg ml^{-1}

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

gentamicin) at 28°C and 150 r.p.m. GUS-expressing *Rr*F4 was cultured in the presence of 100 μg ml^{-1} spectinomycin. Bacterial cells were collected by centrifugation (3202*g*, 10 min), washed and resuspended in a 10 mM $MgSO_4$ $7H_2O$ solution. Roots of 3-day-old barley seedlings were dip-inoculated for 2–3 h in *Rr*F4 suspensions (OD_{600} = 1.4–2). Control seedlings were dipped into 10 mM $MgSO_4$ $7H_2O$. RNA extraction, qRT-PCR with specific oligonucleotides (Supplemental Table S1) was performed as described (Imani *et al*., 2011). Relative DNA or transcript levels were determined using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Protein structure comparison and phylogenetic analysis

Protein sequences of NPRs from selected crop species were used for the protein structure and phylogenetic analysis. Visualization and comparison of the different NPRs domains were done via the online-tool CDD/SPARCLE (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, Marchler-Bauer *et al*., 2016). Multiple sequence alignments were carried out using the MUSCLE algorithm (Edgar, 2004). The phylogenetic tree was built using the maximum likelihood statistical method based on the WAG protein substitution model (Whelan and Goldman, 2001). Tree nodes accuracy was tested via the bootstrap method with 1000 bootstrap replicates. Phylogenetic and molecular evolutionary analyses were conducted using MEGA software (MEGA X version 10.0.5, Kumar *et al*., 2018). Exon-intron distribution analysis was carried out via the online-tool gene extraction display server ($GSDS_{2.0}$, http://gsds.cbi.pku.edu.cn, Hu *et al*., 2015). Corresponding coding and genomic sequences were obtained from the JGI Phytozome 12.1.6 Plant Comparative Genomics portal (https://phytozome.jgi.doe.gov/pz/portal.html).

Microscopy

Visualization of root colonization by Rr*F4.* The colonization of plant roots was visualized using GUS-expressing *Rr*F4 strains combined with light- and epifluorescence microscopy. Root cross-sections also were analysed by TEM according to methods described in Glaeser *et al*. (2016) (see also Supplementary Materials and Methods).

Acknowledgements

We thank U. Micknass, E. Swidtschenko and C. Dechert for excellent technical assistance. This work was supported by the German Minister of Science (BMBF: PrimedPlant consortium) to K.H.K. and the Deutscher Akademischer Austauschdienst (DAAD) to N.K. and M.G. Open access funding enabled and organized by Projekt DEAL.

References

Abreu, M.E., and Munné-Bosch, S. (2009) Salicylic acid deficiency in *NahG* transgenic lines and *sid2* mutants increases seed yield in the annual plant *Arabidopsis thaliana*. *J Exp Bot* **60**: 1261–1271.

Alabid, I., Hardt, M., Imani, J., Hartmann, A., Rothballer, M., Li, D., Uhl, J., Schmitt-Kopplin, P., Glaeser, S., and Kogel, K.-H. (2020). The N-acyl homoserine-lactone depleted Rhizobium radiobacter mutant RrF4NM13 shows reduced growth-promoting and resistance-inducing activities in mono- and dicotyledonous plants. *Journal of Plant Diseases and Protection*, **127**: 769–781.

Backer, R., Naidoo, S., and van den Berg, N. (2019) The nonexpressor of pathogenesis-related genes 1 (NPR1) and related family: mechanistic insights in plant disease resistance. *Front Plant Sci* **10**: 102.

Balmer, D., Planchamp, C., and Mauch-Mani, B. (2013) On the move: induced resistance in monocots. *J Exp Bot* **64**: 1249–1261.

Beßer, K., Jarosch, B., Langen, G., and Kogel, K.H. (2000) Expression analysis of genes induced in barley after chemical activation reveals distinct disease resistance pathways. *Mol Plant Pathol* **1**: 277–286.

Caarls, L., Pieterse, C.M., and Van Wees, S. (2015) How salicylic acid takes transcriptional control over jasmonic acid signaling. *Front Plant Sci* **6**: 170.

Cantu, D., Yang, B., Ruan, R., Li, K., Menzo, V., Fu, D., *et al*. (2013) Comparative analysis of protein-protein interactions in the defense response of rice and wheat. *BMC Genomics* **14**: 166.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994) Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6**: 1583–1592.

Castelló, M.J., Medina-Puche, L., Lamilla, J., and Tornero, P. (2018) NPR1 paralogs of Arabidopsis and their role in salicylic acid perception. *PLoS One* **13**: 12.

Chern, M.S., Fitzgerald, H.A., Canlas, P.E., Navarre, D.A., and Ronald, P.C. (2007) Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersensitivity to light. *Mol Plant Microbe Interact* **18**: 511–520.

Choi, H.W., and Klessig, D.F. (2016) DAMPs, PAMPs/MAMPs, and NAMPs in plant innate immunity. *BMC Plant Biol* **16**: 232.

Colebrook, E.H., Creissen, G., Mcgrann, G.R., Dreos, R., Lamb, C., and Boyd, L.A. (2012) Broad-spectrum acquired resistance in barley induced by the *Pseudomonas* pathosystem shares transcriptional components with Arabidopsis systemic acquired resistance. *Mol Plant Microbe Interact* **25**: 658–667.

De Vleesschauwer, D., Xu, J., and Höfte, M. (2014) Making sense of hormone-mediated defense networking: from rice to Arabidopsis. *Front Plant Sci* **5**: 611.

Delaney, T.P., Friedrich, L., and Ryals, R.A. (1995) Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc Natl Acad Sci U S A* **92**: 6602–6606.

Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., and Fobert, P.R. (2003) The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

basic domain/leucine zipper transcription factor TGA1. *Plant Cell* **15**: 2181–2191.

Dey, S., Wenig, M., Langen, G., Sharma, S., Kugler, K.G., Knappe, C., *et al*. (2014) Bacteria-triggered systemic immunity in barley is associated with WRKY and ethylene responsive factors but not with salicylic acid. *Plant Physiol* **166**: 2133–2151.

Edgar, R.C. (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**: 113.

Fu, Z.Q., and Dong, X. (2013) Systemic acquired resistance: turning local infection into global defense. *Annu Rev Plant Biol* **64**: 839–863.

Gao, J., Bi, W., Li, H., Wu, J., Yu, X., Liu, D., and Wang, X. (2018) WRKY transcription factors associated with *NPR1*-mediated acquired resistance in barley are potential resources to improve wheat resistance to *Puccinia triticina*. *Front Plant Sci* **9**: 1486.

Glaeser, S.P., Imani, J., Alabid, I., Guo, H., Kumar, N., Kämpfer, P., *et al*. (2016) Non-pathogenic *Rhizobium radiobacter* F4 deploys plant beneficial activity independent of its host *Piriformospora indica*. *ISME J* **10**: 871–884.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1996) Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. *Genetics* **143**: 973–982.

Görlach, J., Volrath, S., Oostendorp, M., Kogel, K.H., Beckhove, U., Staub, T., *et al*. (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates induced systemic resistance in wheat. *Plant Cell* **8**: 629–643.

Heil, M., and Baldwin, I.T. (2002) Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends Plant Sci* **7**: 61–67.

Hu, B., Jin, J., Guo, A.Y., Zhang, H., Luo, J., and Gao, G. (2015) GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics* **31**: 1296–1297.

Huot, B., Yao, J., Montgomery, B.L., and He, S.Y. (2014) Growth–defense tradeoffs in plants: a balancing act to optimize fitness. *Mol Plant* **7**: 1267–1287.

Imani, J., Li, L., Schäfer, P., and Kogel, K.H. (2011) STARTS - a stable root transformation system for rapid functional analyses of proteins of the monocot model plant barley. *Plant J* **67**: 726–735.

Jones, J.D.G., and Dangl, J.L. (2006) The plant immune system. *Nature* **444**: 323–329.

Jost, M., Taketa, S., Mascher, M., Himmelbach, A., Yuo, T., Shahinnia, F., *et al*. (2016) A homolog of blade-on-Petiole 1 and 2 (BOP1/2) controls internode length and homeotic changes of the barley inflorescence. *Plant Physiol* **171**: 1113–1127.

Klessig, D.F., Choi, H.W., and Dempsey, D.M.A. (2018) Systemic acquired resistance and salicylic acid: past, present, and future. *Mol Plant Microbe Interact* **31**: 871–888.

Kogel, K.-H., and Langen, G. (2005) Induced disease resistance and gene expression in cereals. *Cell Microbiol* **7**: 1555–1564.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* **35**: 1547–1549.

Lahrmann, U., Strehmel, N., Langen, G., Frerigmann, H., Leson, L., Ding, Y., *et al*. (2015) Mutualistic root endophytism is not associated with the reduction of saprotrophic traits and requires a noncompromised plant innate immunity. *New Phytol* **207**: 841–857.

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**: 402–408.

Luo, Z.B., Janz, D., Jiang, X., Göbel, C., Wildhagen, H., Tan, Y., *et al*. (2009) Upgrading root physiology for stress tolerance by ectomycorrhizas: insights from metabolite and transcriptional profiling into reprogramming for stress anticipation. *Plant Physiol* **151**: 1902–1917.

Makandar, R., Essig, J.S., Schapaugh, M.A., Trick, H.N., and Shah, J. (2006) Genetically engineered resistance to Fusarium head blight in wheat by expression of Arabidopsis NPR1. *Mol Plant Microbe Interact* **19**: 123–129.

Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C.J., Lu, S., *et al*. (2016) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res* **45**: 200–203.

Martinez-Abarca, F., Herrera-Cervera, J.A., Bueno, P., Sanjuan, J., Bisseling, T., and Olivares, J. (1998) Involvement of salicylic acid in the establishment of the Rhizobium meliloti-alfalfa symbiosis. *Mol Plant Microbe Interact* **11**: 153–155.

Miché, L., Battistoni, F., Gemmer, S., Belghazi, M., and Reinhold-Hurek, B. (2006) Upregulation of jasmonate-inducible defense proteins and differential colonization of roots of *Oryza sativa* cultivars with the endophyte *Azoarcus* sp. *Mol Plant Microbe Interact* **19**: 502–511.

Mishina, T.E., and Zeiser, J. (2007) Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. *Plant J* **50**: 500–513.

Mou, Z., Fan, W., and Dong, X. (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113**: 935–944.

Peleg-Grossman, S., Golani, Y., Kaye, Y., Melamed-Book, N., and Levine, A. (2009) NPR1 protein regulates pathogenic and symbiotic interactions between Rhizobium and legumes and non-legumes. *PLoS One* **4**: e8399.

Pieterse, C.M.J., Does, D.V.D., Zamioudis, C., Leon-Reyes, A., and Wees, C.M.V. (2012) Hormonal modulation of plant immunity. *Annu Rev Cell Dev Biol* **28**: 489–521.

Pieterse, C.M.J., Zamioudis, C., Berendsen, R.L., Weller, D. M., van Wees, S.C.M., and Bakker, P.A. (2014) Induced systemic resistance by beneficial microbes. *Annu Rev Phytopathol* **52**: 347–375.

Plett, J.M., Daguerre, Y., Wittulsky, S., Vayssières, A., Deveau, A., Melton, S.J., *et al*. (2014) Effector MiSSP7 of the mutualistic fungus *Laccaria bicolor* stabilizes the *Populus* JAZ6 protein and represses jasmonic acid (JA) responsive genes. *Proc Natl Acad Sci U S A* **111**: 8299–8304.

Quilis, J., Peñas, G., Messeguer, J., Brugidou, C., and Segundo, B.S. (2008) The Arabidopsis AtNPR1 inversely modulates defense responses against fungal, bacterial, or viral pathogens while conferring hypersensitivity to abiotic

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

stresses in transgenic rice. *Mol Plant Microbe Interact* **21**: 1215–1231.
Remigi, P., Zhu, J., Young, J.P.W., and Masson-Boivin, C. (2016) Symbiosis within symbiosis: evolving nitrogen-fixing legume symbionts. *Trends Microbiol* **24**: 63–75.
Ross, A.F. (1961) Systemic acquired resistance induced by localized virus infections in plants. *Virology* **14**: 340–358.
Shah, J., Tsui, F., and Klessig, D.F. (1997) Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. *Mol Plant Microbe Interact* **10**: 69–78.
Sharma, M., Schmid, M., Rothballer, M., Hause, G., Zuccaro, A., Imani, J., *et al*. (2008) Detection and identification of bacteria intimately associated with fungi of the order *Sebacinales*. *Cell Microbiol* **10**: 2235–2246.
Sharma, R., De Vleesschauwer, D., Sharma, M.K., and Ronald, P.C. (2013) Recent advances in dissecting stress-regulatory crosstalk in rice. *Mol Plant* **6**: 250–260.
Shigenaga, A.M., Berens, M.L., Tsuda, K., and Argueso, C. T. (2017) Towards engineering of hormonal crosstalk in plant immunity. *Curr Opin Plant Biol* **38**: 164–172.
Spoel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., *et al*. (2003) NPR1 modulates cross-talk between salicylate-and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* **15**: 760–770.
Tada, Y., Spoel, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., *et al*. (2008) Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. *Science* **321**: 952–956.
Tavakol, E., Okagaki, R., Verderio, G., Shariati, V., Hussien, A., Bilgic, H., *et al*. (2015) The barley *Uniculme4* gene encodes a BLADE-ON-PETIOLE-like protein that controls tillering and leaf patterning. *Plant Physiol* **168**: 164–174.
Toriba, T., Tokunaga, H., Shiga, T., Nie, F., Naramoto, S., Honda, E., *et al*. (2019) BLADE-ON-PETIOLE genes temporally and developmentally regulate the sheath to blade ratio of rice leaves. *Nat Commun* **10**: 1–13.
Van Loon, L.C., Rep, M., and Pieterse, C.M. (2006) Significance of inducible defense-related proteins in infected plants. *Annu Rev Phytopathol* **44**: 135–162.
Verma, S., Varma, A., Rexer, K.H., Hassel, A., Kost, G., Sarbhoy, A., *et al*. (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia* **90**: 896–903.
Wang, X., Bi, W.S., Gao, J., Yu, X., Wang, H., and Liu, D. (2018) Systemic acquired resistance, NPR1, and pathogenesis-related genes in wheat and barley. *J Integr Agric* **17**: 60345–60347.
Wang, X., Yang, B., Li, K., Kang, Z., Cantu, D., and Dubcovsky, J. (2016) A conserved *Puccinia striiformis* protein interacts with wheat NPR1 and reduces induction of pathogenesis-related genes in response to pathogens. *Mol Plant Microbe Interact* **29**: 977–989.
Weiß, M., Waller, F., Zuccaro, A., and Selosse, M.A. (2016) Sebacinales–one thousand and one interactions with land plants. *New Phytol* **211**: 20–40.
Whelan, S., and Goldman, N. (2001) A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol Biol Evol* **18**: 691–699.
Wu, Y., Zhang, D., Chu, J.Y., Boyle, P., Wang, Y., Brindle, I. D., *et al*. (2012) The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep* **1**: 639–647.
Xu, G., Yuan, M., Ai, C., Liu, L., Zhuang, E., Karapetyan, S., *et al*. (2017) uORF-mediated translation allows engineered plant disease resistance without fitness costs. *Nature* **545**: 491–494.
Yang, D.L., Yao, J., Mei, C.S., Tong, X.H., Zeng, L.J., Li, Q., *et al*. (2012) Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proc Natl Acad Sci U S A* **109**: E1192–E1200.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.

Fig. S1. Domain and genomic analysis of the various HvNPR1-like family members with their homologues in *Oryza sativa*, *Brachypodium distachyon* and *Arabidopsis thaliana*. (a-d) Domain structure comparison via the online-tool CDD/SPARCLE (Marchler-Bauer *et al*., 2016). (e) Comparison of the predicted exon-intron frequency in the genomic sequences. Exons are displayed as yellow boxes while introns as straight black lines.

Fig. S2. Blast alignment of *Hvnpr1*_RNAi (Dey *et al*., 2014) against *HvNPR1* and RNAi off-targets prediction analysis. (a) Blast analysis of the RNAi construct was conducted by EMBOSS Needle (Madeira *et al*., 2019; https://www.ebi.ac.uk/Tools/psa/emboss_needle). (b) Off-targets simulations were run using SiFi software (v1.2.3), program designed for RNAi off-target analysis and silencing efficiency predictions (Lueck, 2017; http://labtools.ipk-gatersleben.de). siRNA hits were found only against *HvNPR1* sequence, while no off-targets hits were found in the other *HvNPR* genes.

Fig. S3. *Rr*F4 colonization pattern and strength in WT and KD-*hvnpr1* roots. Primary root segments colonized by *GUS*-expressing *Rr*F4 at 2 dpi, 4 dpi and 10 dpi. The number of bacteria was reduced in roots of KD-*hvnpr1* as compared to WT plants (methods see Fig. 3).

Fig. S4. Scatterplots with trendlines of the relative systemic expression of defence-related genes upon *Bgh* inoculation in non-colonized WT vs. KD-*hvnpr1* barley. Transcripts of *HvPR1b* (a), *HvPR2* (b), and *HvPR5* (c) were assessed by qRT-PCR and normalized to barley *ubiquitin*. After growing the seedlings in soil for three weeks, the detached youngest leaves were inoculated with 10 to 15 *Bgh* conidia per mm^{-2} and harvested 0, 18, 36, 48, and 72 hpi. Displayed are means of three biological repetitions ($n = 4$ plants). Error bars indicate standard deviation. Significant differences between the linear regression analyses were determined by one-way ANOVA.

Fig. S5. Phenotypic analysis of WT and KD-*hvnpr1* barley cv. Golden Promise seedlings grown for 10 days in artificial soil containing 2:1 mixture of expanded clay and Oil-Dri® in a growth chamber at 22°C/18°C (day/night cycle) with 60%

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). Plants were fertilized one time with 0.1% WUXAL top N solution (N/P/K: 12/4/6; Aglukon, Düsseldorf, Germany).

Fig. S6. Plant root and shoot biomass of three-week-old barley cv. Golden Promise WT and two KD-*hvnpr1* mutant lines (Dey *et al*., 2014). The results were obtained using the T3 (E11L9) and T5 (E7L2) generation of transgenic plants. Plants were cultivated in artificial soil containing 2:1 mixture of expanded clay (Seramis®, Masterfoods, Verden, Germany) and Oil-Dri® (Damolin, Mettmann, Germany) in a growth chamber at 22°C/18°C (day/night cycle) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). The experiment was conducted two times (n = 15 plants) with similar results. Comparisons between groups was performed via One-way Anova and Tukey's Range Test. Asterisks represent statistical difference of the group means against WT mock (**p < 0.01). Letters represent statistical difference among all group means (α = 0.05).

Table S1. List of primers used in the study.

© 2020 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd.,

Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2022) **20**, pp. 89–102 doi: 10.1111/pbi.13697

CRISPR/*Sp*Cas9-mediated double knockout of barley Microrchidia MORC1 and MORC6a reveals their strong involvement in plant immunity, transcriptional gene silencing and plant growth

Matteo Galli[1], Engie Martiny[1], Jafargholi Imani[1], Neelendra Kumar[1], Aline Koch[2], Jens Steinbrenner[1] and Karl-Heinz Kogel[1,*]

[1]*Institute of Phytopathology, Research Centre for BioSystems, Land Use and Nutrition, Justus Liebig University Giessen, Giessen, Germany*
[2]*Institute for Phytomedicine, University of Hohenheim, Stuttgart, Germany*

Received 1 July 2021;
revised 4 August 2021;
accepted 5 August 2021.
**Correspondence* (Tel +49 641/99-37492; fax +49 641/99-37499; email karl-heinz.kogel@agrar.uni-giessen.de)

Keywords: Microrchidia, barley, *Blumeria graminis*, disease resistance, *Fusarium graminearum*, gene editing, RNA interference.

Summary

The Microrchidia (MORC) family proteins are important nuclear regulators in both animals and plants with critical roles in epigenetic gene silencing and genome stabilization. In the crop plant barley (*Hordeum vulgare*), seven *MORC* gene family members have been described. While barley *HvMORC1* has been functionally characterized, very little information is available about other *HvMORC* paralogs. In this study, we elucidate the role of *HvMORC6a* and its potential interactors in regulating plant immunity via analysis of CRISPR/*Sp*Cas9-mediated single and double knockout (dKO) mutants, *hvmorc1* (previously generated and characterized by our group), *hvmorc6a,* and *hvmorc1/6a*. For generation of *hvmorc1/6a*, we utilized two different strategies: (i) successive *Agrobacterium*-mediated transformation of homozygous single mutants, *hvmorc1* and *hvmorc6a,* with the respective second construct, and (ii) simultaneous transformation with both *hvmorc1* and *hvmorc6a* CRISPR/*Sp*Cas9 constructs. Total mutation efficiency in transformed homozygous single mutants ranged from 80 to 90%, while upon simultaneous transformation, *Sp*Cas9-induced mutation in both *HvMORC1* and *HvMORC6a* genes was observed in 58% of T0 plants. Subsequent infection assays showed that *Hv*MORC6a covers a key role in resistance to biotrophic (*Blumeria graminis*) and necrotrophic (*Fusarium graminearum*) plant pathogenic fungi, where the dKO *hvmorc1/6a* showed the strongest resistant phenotype. Consistent with this, the dKO showed highest levels of basal *PR* gene expression and derepression of *TEs*. Finally, we demonstrate that *Hv*MORC1 and *Hv*MORC6a form distinct nucleocytoplasmic homo-/heteromers with other *Hv*MORCs and interact with components of the RNA-directed DNA methylation (RdDM) pathway, further substantiating that MORC proteins are involved in the regulation of TEs in barley.

Introduction

Microrchidia (MORC) proteins have been identified in many prokaryotes and eukaryotes to facilitate DNA structure rearrangement and DNA mismatch repair (Iyer *et al*., 2008). In plants, like in mammals, MORCs are involved in transcriptional gene silencing and maintenance of genome stability (Kang *et al*., 2008, 2010; 2012; Lorković *et al*., 2012; Moissiard *et al*., 2012, 2014; Brabbs *et al*., 2013; Langen *et al*., 2014; Harris *et al*., 2016; Koch *et al*., 2017; Kumar *et al*., 2018; Xue *et al*., 2021). In Arabidopsis, MORC1 was discovered in a forward genetic screen against turnip crinkle virus (TCV), suggesting that MORCs also play a role in plant immunity (Kang *et al*., 2008). Subsequent genome-wide analyses have detected seven *MORC* genes (*AtMORC1-7*) and many orthologs in various monocotyledon and dicotyledon plants (Dong *et al*., 2018; Langen *et al*., 2014). Plant MORC protein architecture is conserved between species, usually consisting of a GHKL (Gyrase, Hsp90, Histidine Kinase, MutL) domain and an ATPase domain at the N-terminus of the protein, followed by an S5-fold domain and a coiled-coil (CC) or zinc-finger CW (named for its conserved cysteine and tryptophan residues) domain at the C-terminus (Iyer *et al*., 2008; Koch *et al*., 2017). *At*MORC proteins, especially *At*MORC1, *At*MORC2, and *At*MORC6 are involved in multiple layers of defence response against a variety of pathogens, such as TCV, the bacterium *Pseudomonas syringae*, and the oomycete *Hyaloperonospora arabidopsidis* by acting as positive modulators of immunity (Bordiya *et al*., 2016; Harris *et al*., 2016; Kang *et al*., 2008, 2012). Furthermore, in response to microbial pathogens or their microbe-associated patterns (MAMPs), *At*MORC1 was shown to translocate to the plant cell nucleus, where it plays a role in DNA recombination and DNA repair (Kang *et al*., 2008, 2010 and 2012). MORCs' action in the nucleus has been linked to the RNA-directed DNA methylation (RdDM) pathway, which is involved in transcriptional gene silencing (TGS) and chromatin remodelling (Lorković *et al*., 2012; Manohar *et al*., 2017; Moissiard *et al*., 2012; Xue *et al*., 2021). MORCs have also been studied in barley (*Hordeum vulgare,* viz., *Hv*MORC1 and *Hv*MORC2), potato (*Solanum*

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.

tuberosum viz. *St*MORC1), tomato (*Solanum lycopersicum*, viz., *Sl*MORC1), and tobacco (*Nicotiana benthamiana,* viz., *Nb*MORC1) and surprisingly, the role of each MORC protein in plant defence is species-specific (Kumar *et al*., 2018; Langen *et al*., 2014; Manosalva *et al*., 2015). While in Arabidopsis and potato MORC proteins are positive regulators in pathogen resistance, in barley, tobacco, and tomato, they negatively affect plant immunity (Kang et al., 2008, 2010, 2012; Kumar *et al*., 2018; Langen *et al*., 2014; Manosalva *et al*., 2015).

As in Arabidopsis, seven members of the MORC family have been identified in barley, with five closely related to *At*MORC proteins and two to human *Hs*MORC1 to *Hs*MORC4. The Arabidopsis-like group comprises of *Hv*MORC1 [HORVU7Hr1G083280.15], *Hv*MORC2 [HORVU1Hr1G006770.1], *Hv*MORC6a [HORVU3Hr1G046280.3], *Hv*MORC6b [HORVU3Hr1G078330.4], and *Hv*MORC7 [HORVU2Hr1G066650.2], while *Hv*MORCCW1 [HORVU1Hr1G080470.1] and *Hv*MORCCW2 [HORVU7Hr1G093640.4], carrying a CW domain at the C-terminal region of the protein instead of the typical CC, belongs to the human-like clade (Koch *et al*., 2017). In marked contrast to corresponding Arabidopsis mutants, barley *hvmorc1* and *hvmorc2* mutants were more resistant to the biotrophic pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*) and the necrotrophic pathogen *Fusarium graminearum* (*Fg*) (Kumar *et al*., 2018; Langen *et al*., 2014). On the other hand, like *atmorc1* mutants, barley *hvmorc1* mutants showed derepression of transposable elements (TEs), further suggesting their engagement in genome stabilization (Kumar *et al*., 2018).

Here, we used the CRISPR-Cas9 systems from *Streptococcus pyogenes* (CRISPR/*Sp*Cas9) to generate *hvmorc6a* KO mutants and *hvmorc1*/*hvmorc6a* dKO mutants to further explore the role of *Hv*MORC1 and *Hv*MORC6a in plant immunity. *Hv*MORC6a shares 58.2% aa similarity with *At*MORC6 and 55.0% with *At*MORC1. *At*MORC6 was reported to function in the condensation of pericentromeric heterochromatin, thereby facilitating transcriptional silencing. Furthermore, it has been hypothesized that *At*MORC1 and *At*MORC2 form small nuclear heterodimers with *At*MORC6, which then act in the nucleus and are required for Pol V occupancy in the RdDM pathway (Liu *et al*., 2016; Moissiard *et al*., 2014). Here, we demonstrate that *Hv*MORC6a, like *Hv*MORC1, is involved in disease resistance against biotrophic and necrotrophic pathogens. We show that *Hv*MORC1 and *Hv*MORC6a form nucleocytoplasmic homo-/heteromers, interact with components of the epigenetic gene silencing machinery, and function as repressors of transposable elements (TE).

Results

CRISPR/*Sp*Cas9-mediated generation of KO *hvmorc6a* and dKO *hvmorc1/6a* mutants

In barley, *Hv*MORC1 has been shown to increase disease resistance to fungal pathogens and to derepress the expression of transposable elements (TEs) (Kumar *et al*., 2018; Langen *et al*., 2014). On the other hand, the role of *Hv*MORC6a in modulating plant immunity and genome stabilization is inadequately understood. To assess the function of the *Hv*MORC6a protein, we generated *hvmorc6a* and *hvmorc1/6a* mutants using CRISPR/*Sp*Cas9. Towards this, we generated *hvmorc6a*-guided RNA, with no potential off-target sites (see Experimental Procedures) in the barley genome or other barley MORC family genes (*HvMORC1, HvMORC2*, *HvMORC6b*, *HvMORC7*, *HvMORCCW1,* and *HvMORCCW2*). To completely disable the *Hv*MORC6a function, sgRNA targeted the 5′ part of *HvMORC6a*, upstream the ATPase domain, and generated plants with *HvMORC6a* loss-of-function alleles (Figure S1a–d). After *Agrobacterium*-mediated transformation and germination of the transformed seedlings, genome editing activity was investigated in 2-week-old first generation (T0) plants. The genomic target region was amplified by PCR and the amplicons were analysed by Sanger sequencing using specific primers (Table S1). Out of 123 candidate *hvmorc6a* plants, 93 plantlets carried Indel mutations within the 20 bp target sequence (76% mutation efficiency), of which 42 contained a bi-allelic homozygous mutation (identical mutation on both alleles) (Figure S2a). *Sp*Cas9 also induced different mutation patterns in T0 plants, including bi-allelic heterozygous mutations (different mutations on the two alleles) (Figure S2b,c). This phenotype was confirmed by the characteristic presence of double peaks in the sequencing chromatogram (Figure S2d).

Next, using *Sp*Cas9, we generated dKO barley plants, mutated in both *HvMORC1* and *HvMORC6a*. To ensure the correct generation of the desired dKO genotype, we utilized two different strategies: (i) simultaneous transformation of wild-type (WT) barley cv. Golden Promise with both *hvmorc1* (Kumar *et al*., 2018) and *hvmorc6a* CRISPR/*Sp*Cas9 constructs, and (ii) transformation of homozygous single mutants with the second construct, where *hvmorc6a* plants were transformed with the *hvmorc1* construct and *hvmorc1* plants were transformed with the *hvmorc6a* construct. For the latter (ii), only single mutants devoid of the T-DNA construct, in which the *hygromycin B* gene could not be detected anymore, were used for transformation. Using both strategies, 55 *morc1* in *morc6a*, 64 *morc6a* in *morc1* and 147 *morc1/6a* T0 266 plants were generated, and *Sp*Cas9-induced mutation efficiencies were compared (Figure 1a). Total mutation efficiency in transformed homozygous single mutants was 89% and 81% with respective *hvmorc1*- and *hvmorc6a*-guided RNA. In the simultaneous transformation, *Sp*Cas9-induced mutations of both *HvMORC1* and *HvMORC6a* genes were observed in 58% of the analysed plants. Thus, we already found in T0 generation plantlets that carried disrupted bi-allelic homozygous mutations in both target genes (17 of 64 in *hvmorc6a* transformed plants, 27%; 13 of 55 plants in *hvmorc1*, 23%; 12 of 147 plants in the simultaneous transformation, 8%).

CRISPR/*Sp*Cas9-generated *hvmorc6a* and *hvmorc1/6a* mutants display no off-target effects in other barley *MORC*s

Homozygous bi-allelic genome-edited T0 *hvmorc6a* and *hvmorc1/6a* plants were selected and propagated in soil to obtain T1 seeds. We further worked only with T1 lines that carried a disruptive mutation in target gene(s): Δ*hvmorc6a*-L9 and L16 carrying a 1bp insertion and 25bp deletion respectively in *HvMORC6a*; and Δ*hvmorc1/6a*-L4 and L5 harbouring both a 2bp deletion in *HvMORC1* and 1 bp insertion and 8 bp deletion respectively in *HvMORC6a* (Figure S3a). First, expression of *HvMORC* homologs was assessed in mutants to confirm the KO phenotype and study possible off-target effects. To this end, *HvMORC1*, *HvMORC2*, *HvMORC6a*, *HvMORC7,* and *HvMORCCW1* transcripts were determined by RT-qPCR in 3-week-old WT, *hvmorc1*, *hvmorc6a*, and *hvmorc1*/6a mutant plants. Notably, transcript levels of *HvMORC1* and *HvMORC6a* were significantly downregulated (~80% reduction) in corresponding mutants, while those of other *HvMORCs* remained unaltered (Figure 1b). As anticipated, further sequencing analysis confirmed that the reduced transcript level of *HvMORC1* and

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

(a)

CRISPR/*Sp*Cas9 construct	p*Hv*U3:***morc1***:sgRNA:p*Zm*Ubi:*Sp*Cas9 in *hvmorc6a*	p*Hv*U3:***morc6a***:sgRNA:p*Zm*Ubi:*Sp*Cas9 in *hvmorc1*	p*Hv*U3:***morc1***:sgRNA:p*Zm*Ubi:*Sp*Cas9 + p*Hv*U3:***morc6a***:sgRNA:p*Zm*Ubi:*Sp*Cas9 in barley wildtype
	Barley CRISPR/*Sp*Cas9 *Agrobacterium*-mediated transformation		
Nr. of plants	55	64	147
Mutation frequency in T0 plants	50 (89%)	52 (81%)	85 (58%)
Bi-allelic homozygous dKO	13 (23%)	17 (27%)	12 (8%)
Pattern of mutation detected	1bp+, 1bp-, 2bp- and 5bp-	1bp+, 2bp-, 5bp-, 9bp- and 25bp-	1bp+, 1bp-, 2bp- and 25bp-

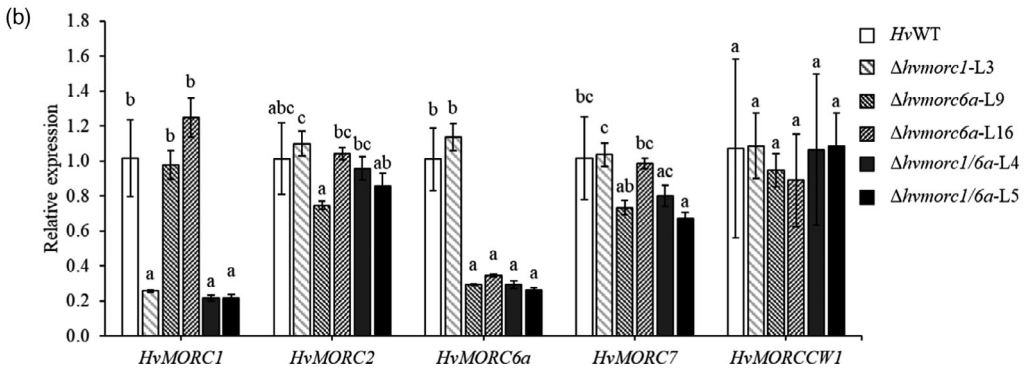


Figure 1 Mutation efficiency and silencing effect in *Sp*Cas9-induced *hvmorc1/6a* dKO mutants. (a) Schematic summary of CRISPR efficacy in the generation of *hvmorc1/6a* dKO in different backgrounds. For transformation with second construct, *hvmorc1* and *hvmorc6a* T2 transgene-free plants were used (Δ*hvmorc1*-L3, Δ*hvmorc6a*-L9). For simultaneous transformation, WT barley plants were transformed with both sgRNAs. (b) Relative *MORC* expression in leaves of barley WT, *hvmorc1* (Δ*hvmorc1*-L3), *hvmorc6a* (Δ*hvmorc6a*-L9 and L16) and *hvmorc1/6a* (Δ*hvmorc1/6a*-L4 and L5) T3 mutants. Transcript amounts of *HvMORC1*, *HvMORC2*, *HvMORC6a, HvMORC7,* and *HvMORCCW1* were measured in the second youngest leaf of 21 days old plants ($n = 8$) via RT-qPCR. Plant ubiquitin (*HvUbiquitin*) was used as the normalization gene. The experiment was repeated twice with similar results. Comparisons between groups were performed via ANOVA and Tukey's range test for multiple comparisons. Letters represent statistical differences among all group means ($\alpha < 0.05$).

HvMORC6a is the result of mRNA degradation by the nonsense-mediated mRNA decay pathway which is involved in degradation of aberrant mRNAs harbouring multiple premature STOP codons (Figure S3b; Reviewed in Yi *et al.*, 2020).

*Hv*MORC6a has a negative regulatory role on barley immunity against biotrophic and necrotrophic fungi

Previous results suggest that MORC proteins are modulators of immunity in a species-specific manner (for details see Koch *et al.*, 2017). To further explore the role of MORC proteins in barley immunity, we assessed the resistance of *hvmorc6a* and *hvmorc1/6a* plants to the biotrophic powdery mildew fungus. Detached leaves of the virulent barley cv. Golden Promise were inoculated with conidia of *Bgh* race A6 and *Bgh* colonies were counted 5 days post-inoculation (dpi). Compared with WT, all mutant lines *hvmorc1* (Δ*hvmorc1*-L3), *hvmorc6a* (Δ*hvmorc1*-L9 and Δ*hvmorc1*-L16), and *hvmorc1/6a* (Δ*hvmorc1/6a*-L4 and Δ*hvmorc1/6a*-L5) showed increased resistance to *Bgh*. These results were consistent with our expectation that barley MORC paralogs respond similarly to *Bgh* (Kumar *et al.*, 2018; Langen *et al.*, 2014). Of note, compared with WT plants, dKO lines displayed the strongest phenotype (Δ*hvmorc1/6a*-L4: 55% and Δ*hvmorc1/6a*-L5: 50%), while single mutant lines retain a more moderate resistance (Δ*hvmorc1*-L3: 77%, Δ*hvmorc6a*-L9: 79%, Δ*hvmorc6a*-L16: 72%) (Figure 2a).

Defence pathways involved in resistance to biotrophic and necrotrophic pathogens often function antagonistically (Glazebrook, 2005; Jarosch *et al.*, 1999; Klessig *et al.*, 2018; Pieterse *et al.*, 2012). With this in mind, we also investigated the resistance of all mutants against the necrotroph *Fusarium graminearum* (*Fg*). Detached leaves were drop-inoculated with 20 μL of a macroconidia suspension (5×10^4 conidia mL^{-1}) and infection was assessed via qPCR at five dpi. A significant reduction in fungal growth was observed in both *hvmorc6a* and *hvmorc1/6a* mutants as compared with WT (Δ*hvmorc6a*-L9: 84%, Δ*hvmorc6a*-L16: 82%, and Δ*hvmorc1/6a*-L4: 70%; Figure 2b).

Basal expression of *pathogenesis-related* (*PR*) genes is enhanced in *hvmorc* mutants

Arabidopsis mutants defective in RdDM show enhanced bacterial resistance, and constitutive expression of *Pathogenesis-related 1* (*PR1*) (Yu *et al.*, 2013). Similarly, depletion of *HvMORC1* in barley resulted in higher expression of canonical markers for disease resistance, such as *PR* genes (Kumar *et al.*, 2018). Based on these findings, we investigated whether KO of *HvMORC6a* also influences expression of *PR* genes and jasmonic acid (JA) marker gene *S-adenosyl-l-methionine: jasmonate O-methyltransferase* (*HvJMT*). The basal expression level of *HvPR1b* (GenBank: X74940.1), *HvPR2* (GenBank: AF479647.2), *HvPR5* (GenBank: AM403331.1) as well as *HvJMT* (GenBank: KAE8819745.1) was determined by RT-qPCR in 3-week-old *hvmorc6a* and *hvmorc1*/6a homozygous plants. Compared with WT, *hvmorc6a* and *hvmorc1*/6a displayed higher *PR* expression levels (fold increase,

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

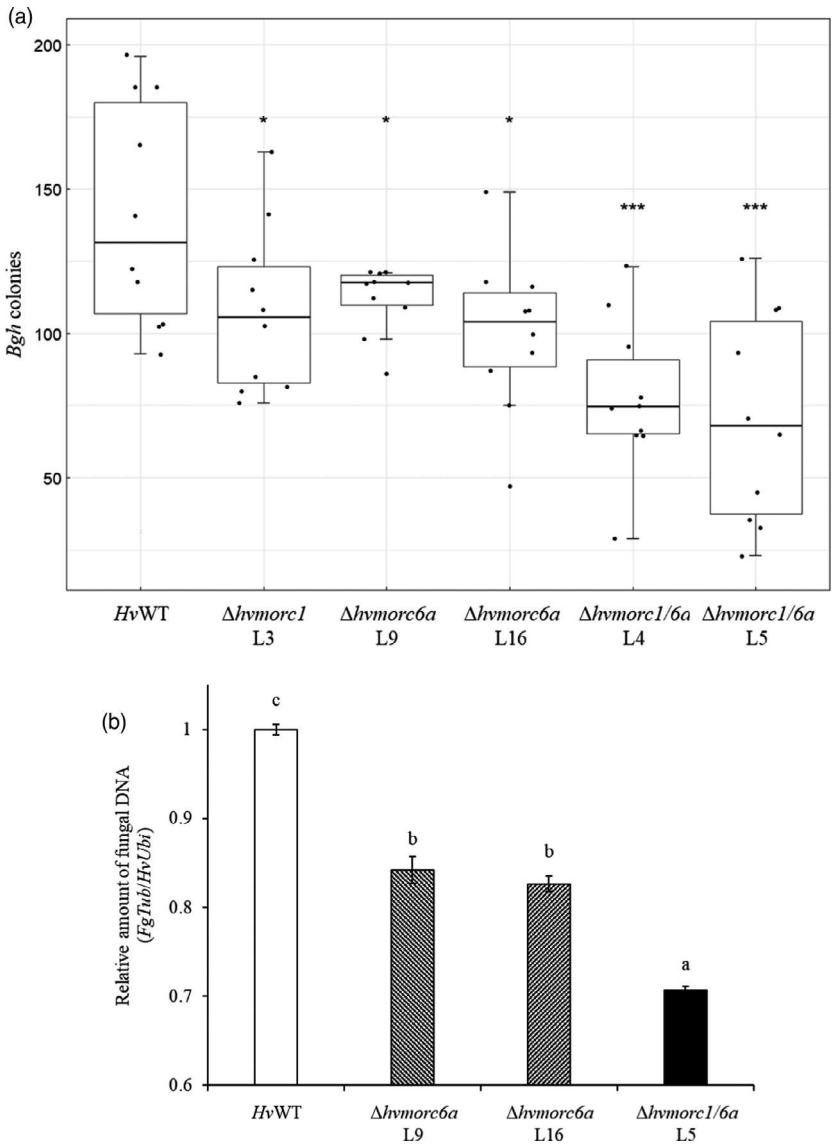


Figure 2 Fitness analysis of *Sp*Cas9-induced mutated lines against fungal pathogens. (a) *hvmorc1* (Δ*hvmorc1*-L3), *hvmorc6a* (Δ*hvmorc6a*-L9 and L16), and *hvmorc1/6a* (Δ*hvmorc1/6a*-L4 and L5) T3 mutants show increased resistance to the biotrophic fungus *Blumeria graminis* f.sp. *hordei* race A6 (*Bgh*). Detached second leaves of 2-week-old plants were inoculated with 3–5 conidia per mm^2 and at 5 dpi, *Bgh* colonies were counted. Shown is the average number of *Bgh* colonies on a 1.5 cm^2 leaf area ($n = 10$). The experiment was repeated twice with similar results. Comparisons between groups were performed via student's t-test between *Hv*WT and mutant lines; asterisks represent statistical difference of the groups against *Hv*WT (*$P < 0.05$; ***$P < 0.001$). (b) *hvmorc6a* (Δ*hvmorc6a*-L9 and L16) and *hvmorc1/6a* (Δ*hvmorc1/6a*-L4 and L5) T3 mutants display enhanced resistance against *Fusarium graminearum* (*Fg*) growth. Detached second leaves of 2-week-old plants were inoculated via drop inoculation assay with 20 µl solution of *Fg* conidia (5×10^4 conidia mL^{-1}). Quantitative PCR was used to measure the *Fg* DNA amount on leaves at 5 dpi (ratio between fungal tubulin to plant ubiquitin; *FgTub/HvUbi*). Bars represent the standard deviation of three technical repetitions; assay was repeated twice with similar results. Comparisons between groups were performed via ANOVA and Tukey's range test for multiple comparisons. Letters represent statistical differences among all group means ($\alpha < 0.05$).

HvPR1b: Δ*hvmorc6a*-L9: 2.1, Δ*hvmorc6a*-L16: 3, Δ*hvmorc1/6a*-L4: 3.6, Δ*hvmorc1/6a*-L5: 4.1; *HvPR2*: Δ*hvmorc6a*-L9: 2.7, Δ*hvmorc6a*-L16: 3, Δ*hvmorc1/6a*-L4: 2.7, Δ*hvmorc1/6a*-L5: 3.9; *HvPR5*: Δ*hvmorc6a*-L9: 2.6, Δ*hvmorc6a*-L16: 2.5, Δ*hvmorc1/6a*-L4: 4.6, Δ*hvmorc1/6a*-L5: 3.4; *HvJMT*: Δ*hvmorc6a*-L9: 2.1, Δ*hvmorc6a*-L16: 1.8, Δ*hvmorc1/6a*-L4: 2.9, Δ*hvmorc1/6a*-L5:3; Figure 3). Most strikingly, expression of all *PR* genes and the *JA* marker gene was strongly induced in the *hvmorc1/6a* mutants.

*Hv*MORC6a is involved in TGS-mediated transposable element silencing

*At*MORC1 and *At*MORC6 have been shown to influence gene silencing downstream of the RdDM pathway, thereby influencing methylation rate and chromatin state (Manohar *et al*., 2017; Moissiard *et al*., 2012). As observed in Arabidopsis *atmorc1* mutant, *hvmorc1* plants showed derepression of TEs, raising the hypothesis that *Hv*MORC1 contributes to genome stabilization (Kumar *et al*., 2018; Langen *et al*., 2014). To prove this further, we assessed the effect of *Hv*MORC6a on TE derepression. Analysing transcription profiles of long terminal repeat (LTR) and non-LTR retrotransposons *HvInga*, *HvRLG-S*, *HvVagabond*, *HvBianca* and *HvCereba* by RT-qPCR, we found increased derepression of TEs in leaves of all *hvmorc* mutants, with significant higher derepression in *hvmorc1/6a* (fold increase, *HvInga*: Δ*hvmorc1*-L3: 1.7, Δ*hvmorc6a*-L9: 2.1, Δ*hvmorc6a*-L16: 2.1, Δ*hvmorc1/6a*-L4: 3.2, Δ*hvmorc1/6a*-L5: 3.5; *HvRLG-S*: Δ*hvmorc1*-L3: 3.7, Δ*hvmorc6a*-L9: 6.4, Δ*hvmorc6a*-L16: 6.5, Δ*hvmorc1/6a*-L4: 10.8, Δ*hvmorc1/6a*-L5: 11.5; *HvVagabond*: Δ*hvmorc1*-L3: 1.5, Δ*hvmorc6a*-L9: 1.6, Δ*hvmorc6a*-L16: 1.3, Δ*hvmorc1/6a*-L4: 21, Δ*hvmorc1/6a*-L5: 18.4; *HvBianca*: Δ*hvmorc1*-L3: 3.6, Δ*hvmorc6a*-L9: 3.8, Δ*hvmorc6a*-L16: 3.2, Δ*hvmorc1/6a*-L4: 6.4, Δ*hvmorc1/6a*-L5: 5.1; *HvCereba*: Δ*hvmorc1*-L3: 1.1, Δ*hvmorc6a*-L9: 1.8, Δ*hvmorc6a*-L16: 2, Δ*hvmorc1/6a*-L4: 3.2, Δ*hvmorc1/6a*-L5: 2; Figure 4).

*Hv*MORC proteins form homomers and heteromers *in vivo*

Because Arabidopsis MORCs form homo-/heteromeric complexes *in vivo* (Harris *et al*., 2016; Liu *et al*., 2014; Moissiard *et al*., 2014), we next performed Y2H assays to determine whether barley MORCs can also interact *in vivo*. We found that *Hv*MORC1 forms both a homomer and heteromers with *Hv*MORC6a, respectively

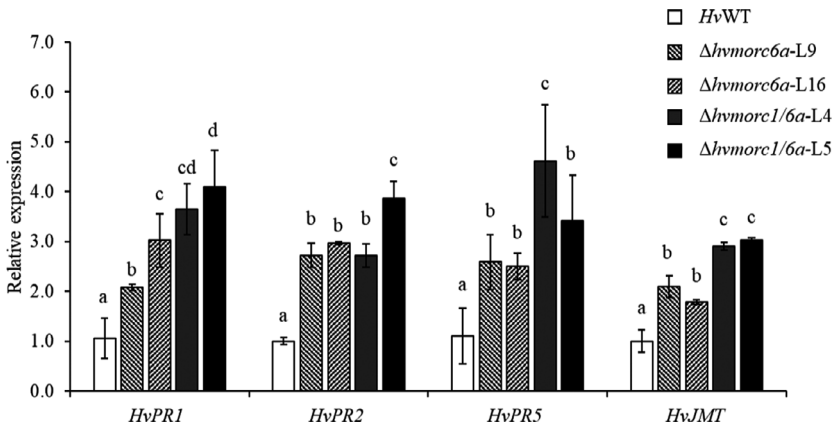


Figure 3 Basal PRs expression in *Sp*Cas9-induced mutated lines. Relative *PR* gene expression in leaves of *Sp*Cas9-generated *hvmorc6a* (Δ*hvmorc6a*-L9 and L16) and *hvmorc1/6a* (Δ*hvmorc1/6a*-L4 and L5) T3 mutants vs. WT. The quantification level of *HvPR1b*, *HvPR2, HvPR5,* and *HvJMT* was measured in the sterile third youngest leaf of 21-day-old plants (n = 8) via RT-qPCR. Plant ubiquitin (*HvUbiquitin*) was used as the normalization gene. The experiment was repeated twice with similar results. Comparisons between groups were performed via ANOVA and Tukey's range test for multiple comparisons. Letters represent statistical differences among all group means ($\alpha < 0.05$).

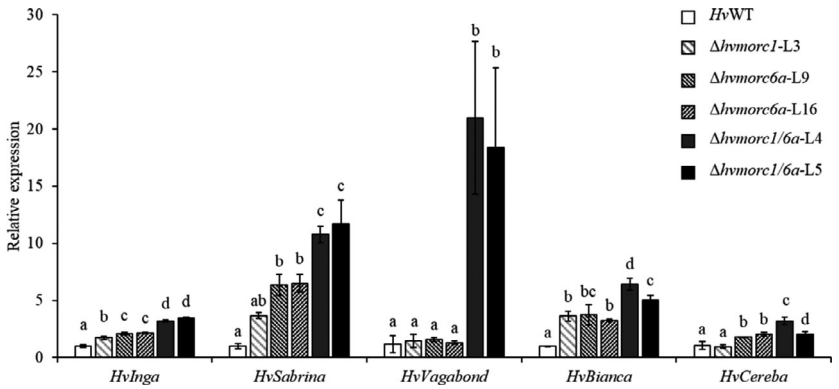


Figure 4 Transposon expression in *Sp*Cas9-induced mutated lines. Relative TEs gene expression in leaves of *Sp*Cas9-generated *hvmorc1* (Δ*hvmorc1*-L3), *hvmorc6a* (Δ*hvmorc6a*-L9 and L16), and *hvmorc1/6a* (Δ*hvmorc1/6a*-L4 and L5) T3 mutants against WT. The quantification level of multiple TEs genes (*HvINGA*, *HvRLG-S*, *HvBianca*, *HvVagabond,* and *HvCereba)* was measured in the second youngest leaf of 21-day-old plants (n = 8) via RT-qPCR. Plant ubiquitin (*HvUbiquitin*) was used as the normalization gene. The experiment was repeated twice with similar results. Comparisons between groups were performed via ANOVA and Tukey's range test for multiple comparisons. Letters represent statistical differences among all group means ($\alpha < 0.05$).

(Figure 5a). Moreover, *Hv*MORC2 and *Hv*MORC6a did not form homomeric complexes in our Y2H assays, but *Hv*MORC2 interacted with *Hv*MORC1 and *Hv*MORC6a (Figure 5a).

To further validate our Y2H results *in planta*, a BiFC assay was conducted using *Nicotiana benthamiana* plants. In this approach, the N- and C-terminal parts of YFP were fused to *Hv*MORC1, *Hv*MORC2, and *Hv*MORC6a and were transiently expressed in *N. benthamiana* leaves (Figure 5b, right panel; Figure S4) confirming our Y2H results. Additionally, contrary to the Y2H, we detected a homodimerization for *Hv*MORC2 (Figure 5b, right panel). Surprisingly, and in contrast to *At*MORC6, *Hv*MORC6a did not show any homomeric interaction in either the Y2H assay or the BiFC assay (Figure 5a-b). Furthermore, since it has been hypothesized that *At*MORC1 and *At*MORC2 form small nuclear heterodimers with *At*MORC6, we also transiently expressed chimeric GFP::*Hv*MORC1 and GFP::*Hv*MORC6a under the control of the cauliflower mosaic virus 35S promoter in barley mesophyll protoplasts. Using confocal laser scanning microscopy (CLSM), we examined the precise protein localization in WT and *hvmorc6* barley background (Figure 5b, left panel). For this, barley protoplasts were simultaneously transformed with pSAT6-mCherry-VirD2NLS, serving as a nuclear marker to allow the observation of the nucleus in protoplasts (Citovsky *et al*., 2006; Lin *et al*., 2018). Both *Hv*MORC1 and *Hv*MORC6a showed nucleocytoplasmic localization in the barley WT background (Figure 5b, left panel). Interestingly, in the *hvmorc6a* background, *Hv*MORC1 localized almost exclusively in the cytoplasm while *Hv*MORC6a remained nucleocytoplasmic.

*Hv*MORCs interact with components of the RdDM machinery *in vivo*

*At*MORC1, *At*MORC2, and *At*MORC6 were identified as components of the RdDM pathway by their interaction with SUPPRESSOR OF VARIEGATION 3- 9- (SUV[VAR] 3-9) homologs SUVH2 and/or SUVH9. These two proteins are canonical components of RdDM that interact directly with DEFECTIVE IN MERISTEM

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

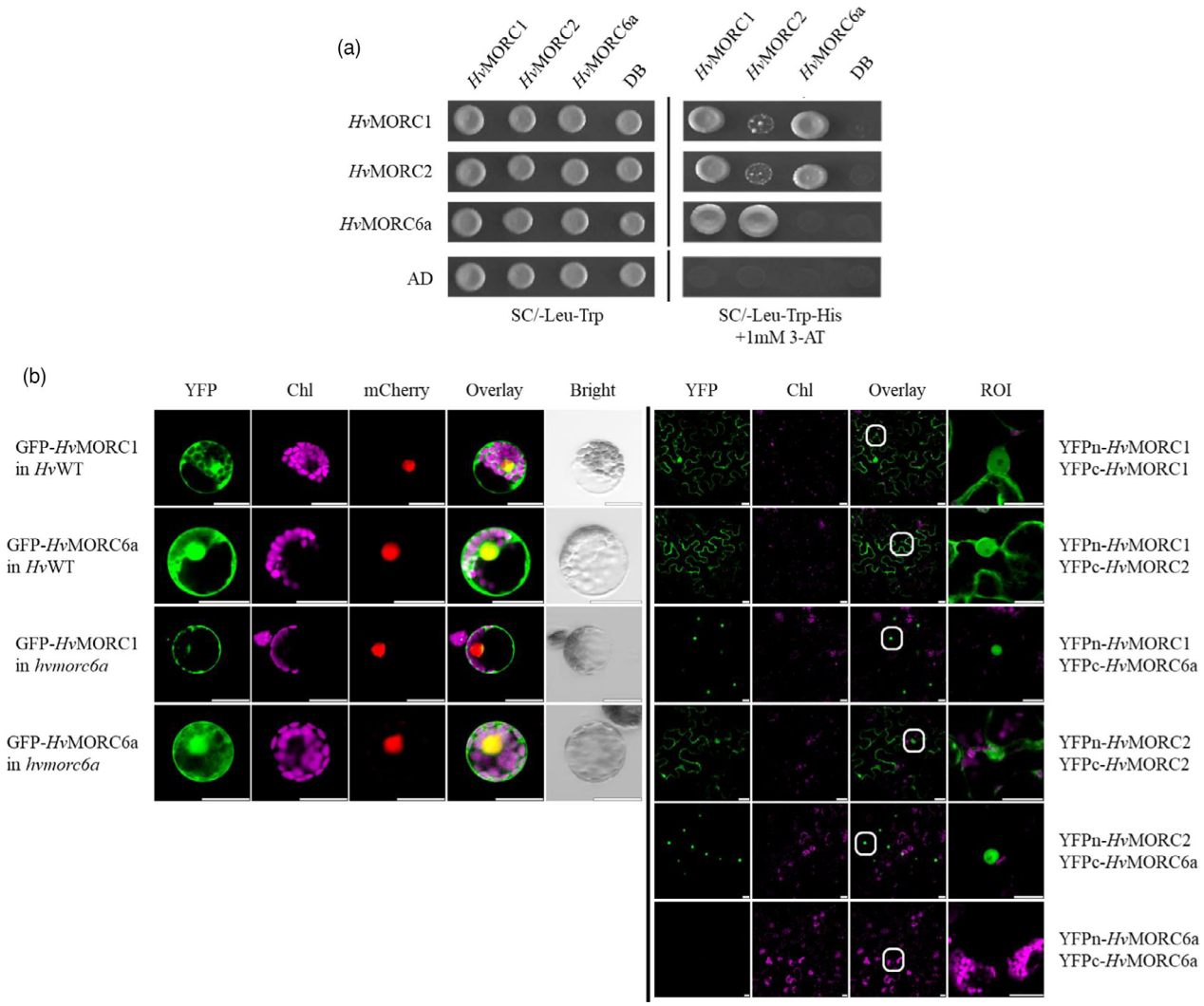


Figure 5 Localization, homomeric and heteromeric interaction of barley MORCs. (a) Y2H screen for possible dimerization between *Hv*MORC proteins. *Hv*MORCs were N-terminally fused to the Gal4-binding domain (DB) and the Gal4 activation domain (AD). Left panel shows growth on SC-Leu–Trp selective media as an indication of successful mating between all combinations. The right panel shows growth on stringent selective media that further lacks histidine and supplemented with 1 mM 3-amino-1,2,4-triazole, a competitive inhibitor of the *HIS3* gene product, indicating interaction between the AD- and DB- constructs and activation of the HIS3 gene. No growth was detected between AD-construct/ DB empty or between DB-construct/AD-empty, indicating that none of the tested constructs is autoactive. (b) GFP signals of barley MORCs detected in barley mesophyll protoplasts after 24 h in barley WT and *hvmorc6a* background (left panel) and YFP signals of homo-/heteromerization of barley MORCs detected in lower epidermal cells of tobacco after 48 h (right panel). p2FGW7-*Hv*MORCs were C-terminally fused to GFP and pBiFP2-*Hv*MORCs and pBiFP3-*Hv*MORCs were C-terminally fused to the N- and C-terminal parts of YFP, respectively. Protoplasts were simultaneously transformed with mCherry-VirD2NLS as a nuclear marker. Images of protoplasts and lower epidermis represent two and three biological replicates, respectively. Scale bar: 20 μm. ROI is a magnification of the bordered region in the overlay column. YFP: yellow fluorescence protein, Chl: chlorophyll autofluorescence, mCherry: nuclear fluorescence, ROI: regions of interest (magnification of the bordered region).

SILENCING 3 (DMS3; Liu *et al*., 2014, 2016; Jing *et al*., 2016). Therefore, we identified orthologs of *At*MORC interactors in barley to further investigate the involvement of *Hv*MORCs in RdDM (Table S2). *Hv*DMS3, *Hv*SUVH9, the double-stranded RNA-binding protein INVOLVED IN DE NOVO 2 (*Hv*IDN2) and the SWITCH SUBUNIT 3C (*Hv*SWI3C) component of the chromatinremodelling complex SWITCH/SUCROSE NON-FERMENTABLE (SWI/SNF) were cloned and tested in a Y2H assay against *Hv*MORC1, *Hv*MORC2, and *Hv*MORC6a. We found that *Hv*MORC1 interacts with *Hv*IDN2 (Figure 6a), in contrast to previous results on *At*MORCs, where Y2H showed no interaction between IDN2 and *At*MORC1 or *At*MORC2 (Liu *et al*., 2016). Consistent with previous Y2H results, revealing an interaction of *At*SUVH9 with *At*MORC1, *At*MORC2, and *At*MORC6 (Liu *et al*., 2014), all three tested *Hv*MORCs interacted with *Hv*SUVH9 (Figure 6a). Surprisingly, none of the tested *Hv*MORCs showed any interaction with *Hv*SWI3C (Figure 6a), which is inconsistent with what was found in *Arabidopsis* (Jing *et al*., 2016). In addition, we did not detect interactions between *Hv*DMS3 with any of the tested *Hv*MORCs (Figure 6a). However, this might be consistent with what was reported for *At*MORCs (Jing *et al*., 2016; Liu *et al*., 2014, 2016; Moissiard *et al*., 2014), since *At*MORC6 interaction with *At*DMS3 was only shown once in an *in vitro* pull-down experiment (Lorković *et al*., 2012) and *in vivo*

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

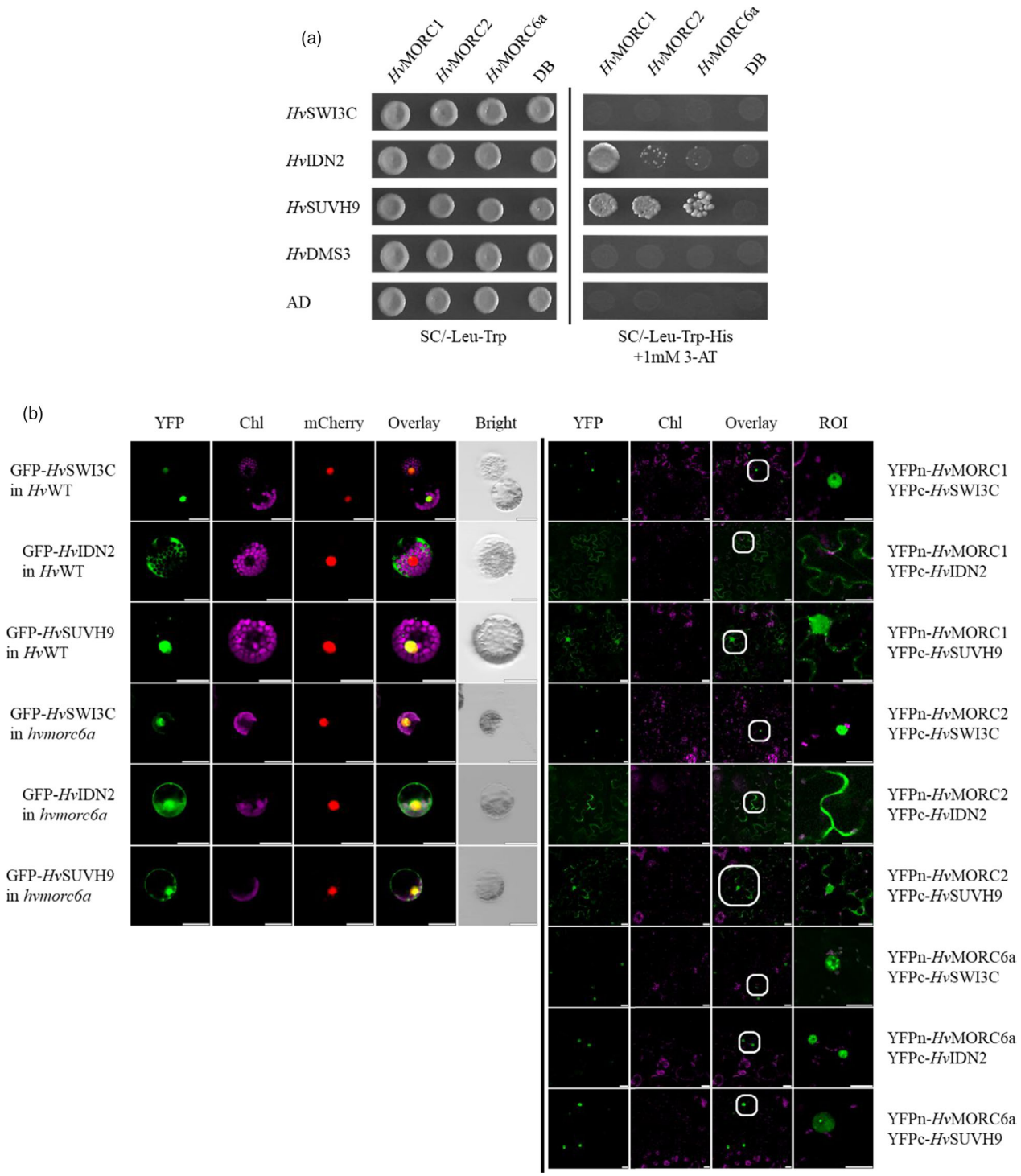


Figure 6 Localization of some barley orthologs of the RdDM pathway and their interactions with barley MORCs. (a) Y2H screen for possible interactions between *Hv*MORCs and *Hv*RdDM components. Barley MORCs and RdDM proteins were N-terminally fused to the Gal4-binding domain (DB) and the Gal4 activation domain (AD). Left panel shows growth on SC-Leu–Trp selective media as an indication of successful mating between all combinations. The right panel shows growth on stringent selective media that further lacks Histidine and supplemented with 1 mM 3-amino-1,2,4-triazole, a competitive inhibitor of the *HIS3* gene product, indicating interaction between the AD- and DB- constructs and activation of the *HIS3* gene. No growth was detected between AD-construct/DB empty or between DB-construct/AD-empty, indicating that none of the tested constructs is autoactive. (b) GFP signals of *Hv*RdDM proteins detected in barley mesophyll protoplasts after 24 h in barley WT and *hvmorc6a* background (left panel) and YFP signals of homo-/heteromerization of barley MORCs detected in lower epidermal cells of tobacco after 48 h (right panel). p2FGW7-*Hv*RdDMs were C-terminally fused to GFP, and pBiFP2-*Hv*MORCs and pBiFP3-*Hv*RdDM were C-terminally fused to the N- and C- terminal parts of YFP, respectively. Protoplasts were transformed with mCherry-VirD2NLS as a nuclear marker. Images of protoplasts and lower epidermis represent two and three biological replicates, respectively. ROI is a magnification of the bordered region in the overlay column. Scale bar: 20 μm. YFP: yellow fluorescence protein, Chl: chlorophyll autofluorescence, mCherry: nuclear fluorescence, ROI: regions of interest (magnification of the bordered region).

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

immunoprecipitation experiments failed to detect the interaction of *At*DMS3 with *At*MORC6, possible due to a weak or ephemeral interaction (Moissiard *et al*., 2014).

Since Y2H only detects approximately 25% of all occurring interactions (Braun *et al*., 2009), we further verified the interactions of *Hv*MORC proteins and the barley RdDM orthologs *in planta* using BiFC in *N. benthamiana* leaf epidermal cells (Figure 6b right panel, Figure S5a–b). BiFC assay revealed an interaction of *Hv*SUVH9 with *Hv*MORC1, *Hv*MORC2, and *Hv*MORC6a, supporting our Y2H results (Figure 6b right panel). Those interactions were predominantly nuclear in epidermal cells of *N. benthamiana* leaves (Figure 6b right panel). Previously, *At*MORC6 was shown to interact with *At*IDN2 (Jing *et al*., 2016). We detected an interaction between *Hv*MORC6a and *Hv*IDN2 in the nucleus of *N. benthamiana* cells (Figure 6b right panel, Figure S5a). The interaction between either *Hv*MORC1 or *Hv*MORC2 with *Hv*IDN2 was entirely cytoplasmic, excluded from the nucleus and was only observed in the nuclear periphery (Figure 6b right panel, Figure S5a). Additionally, we detected interactions between *Hv*MORC1, *Hv*MORC2 or *Hv*MORC6a with *Hv*SWI3C in the nucleus (Figure 6b right panel, Figure S5a), contradictory to our Y2H screens. Finally, and consistent with the Y2H results, we could not detect any interaction of *Hv*MORCs with *Hv*DMS3 *in planta* (Figure S5b). Since *At*MORC6 has been shown to be involved in the regulation of chromatin condensation and we show the interaction in Y2H and BiFC in *N. benthamiana*, we additionally analysed the localization of *Hv*SWI3C, *Hv*IDN2, *Hv*SUVH9, and *Hv*DMS3 in barley WT and *hvmorc6a* background (Figure 6b, left panel; Figure S5b). CLSM of barley WT mesophyll protoplasts revealed that *Hv*SWI3C and *Hv*SUVH9 were exclusively localized to the nucleus, *Hv*IDN2 and *Hv*DMS3 showed a cytoplasmic and nuclear-cytoplasmic localization, respectively (Figure 6b left panel, Figure S5b left panel). In *hvmorc6a* background, *Hv*SWI3C, *Hv*SUVH9, and *Hv*IDN2 could be detected both into the nucleus and in the cytoplasm of the cell (Figure 6b left panel). Lastly, we could not detect any difference in the localization of *Hv*DMS3 (Figure S5b left panel).

*Hv*MORC6a affects plant biomass and growth

High expression of *PR* genes and other defence genes has an impact on plant yield and development (Kumar *et al*., 2021; Xu *et al*., 2017). Based on our findings that *hvmorc* mutants have a high basal level of PRs, we analysed whether KO of *Hv*MORC1 and *Hv*MORC6a affects plant growth and development. For this, we measured the root and shoot biomasses of 3-week-old *hvmorc1*, *hvmorc6a,* and *hvmorc1/6a* plants. While Δ*hvmorc1*-L3 single mutants did show aberrant growth compared with WT (13% reduction of shoots and roots), Δ*hvmorc6a*-L9 and Δ*hvmorc1/6a*-L5 mutants were strongly impaired in growth with root and shoot dry weight lower as compared with WT plants (shoot dry weight 17% and 18% reduction, respectively; root dry weight: 23% and 24% reduction, respectively; Figure 7a–c).

Discussion

Efficient CRISPR/*Sp*Cas9-mediated multiple gene editing in barley

Targeted genome engineering is the modification of the DNA in an organism at a precise, predetermined locus. From an agricultural perspective, gene editing is an important tool to improve yield, grain quality, and resistance/tolerance of crops to biotic and abiotic stress to ensure sustainable, but also effective food production (Fernandez-Cornejo *et al*., 2014; Govindan and Ramalingam, 2016; Kim and Kim, 2014; Zhu *et al*., 2020). Over the last decade, the type II CRISPR-Cas9 editing module has emerged as a powerful tool to induce precise mutations in the genome of many animal and plant species, including barley (Cong *et al*., 2013; Gasparis *et al*., 2018; Holme *et al*., 2017; Jaganathan *et al*., 2018; Kapusi *et al*., 2017; Kis *et al*., 2019; Kumar *et al*., 2018; Lawrenson *et al*., 2015; Lee *et al*., 2021; Li *et al*., 2020; Mali *et al*., 2013; Zeng *et al*., 2020). In a previous study, we already used the barley RNA Polymerase (Pol) III-dependent U3 small nuclear RNA promoter (GenBank: CAJX011995286.1) for efficient sgRNA expression and KO of *HvMORC1* (Kumar *et al*., 2018). Here we show that a *Hv*U3-driven sgRNA construct was equally effective for *HvMORC6a* KO (Figure S1a). After hygromycin selection, using PCR and Sanger sequencing, we detected Indel mutations in 76% of T0 *hvmorc6a* mutants (Figure S2a). The strikingly high mutation frequency supports the technical finding that the *Hv*U3 promoter is suitable to drive sgRNA expression in the type II CRISPR/*Sp*Cas9 system for genome editing in barley. For the generation of the dKO mutant *hvmorc1/6a*, we compared two different strategies: (i) successive *Agrobacterium*-mediated transformation of a homozygous single MORC mutant with the respective second KO construct, and (ii) simultaneous *Agrobacterium*-mediated transformation with two constructs, each targeting one of the two *MORC* genes. Both strategies yielded high mutation rates (Figure 1a). Total mutation efficiency in transformed homozygous single mutants was between 80 to 90%, while in simultaneous transformation, *Sp*Cas9 induced a mutation in both *HvMORC1* and *HvMORC6a* in 58% of T0 generation plants. Expression analyses of *MORC* genes confirmed that the CRISPR/*Sp*Cas9 constructs precisely targeted target *MORC* genes and did not cause off-target effects that resulted in impaired gene activity of other MORC paralogs (Figure 1b). Therefore, our results confirm the effectiveness and usefulness of CRISPR/*Sp*Cas9 genome editing for analysing plant gene function in a multigene family such as that of barley *MORCs*.

*Hv*MORC6a is involved in plant defence and interacts with chromatin remodelling mediator proteins

In mammals, MORC proteins are involved in maintaining genome stability and in consequence, the regulation of cancer and other diseases as well as spermatogenesis (Iyer *et al*., 2008), while in plants, they are involved in maintaining genome stability in addition to their function in immunity to microbial pathogens (Koch *et al*., 2017). MORC proteins in cereals are largely unexplored, because in the past, KO mutants were difficult to produce. Previous studies demonstrated that RNAi-mediated knockdown (KD) of *HvMORC1* and *HvMORC2* rendered barley less susceptible to both biotrophic and necrotrophic fungal pathogen (Langen *et al*., 2014), which was subsequently confirmed with CRISPR/*Sp*Cas9-mediated KO of *HvMORC1* (Kumar *et al*., 2018). These findings agreed with earlier reports showing that the Arabidopsis dKO mutant *atmorc1/2* is compromised in the immune response to inoculation with *Pseudomonas syringae pv. maculicola (Psm)* (Kang *et al*., 2012).

The barley *HvMORC6a* gene shares 58% aa similarity with *AtMORC6* (Koch *et al*., 2017). *At*MORC6 acts as positive regulator of defence against the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) (Harris *et al*., 2016). In agreement with an immune function of MORC6, we show here that

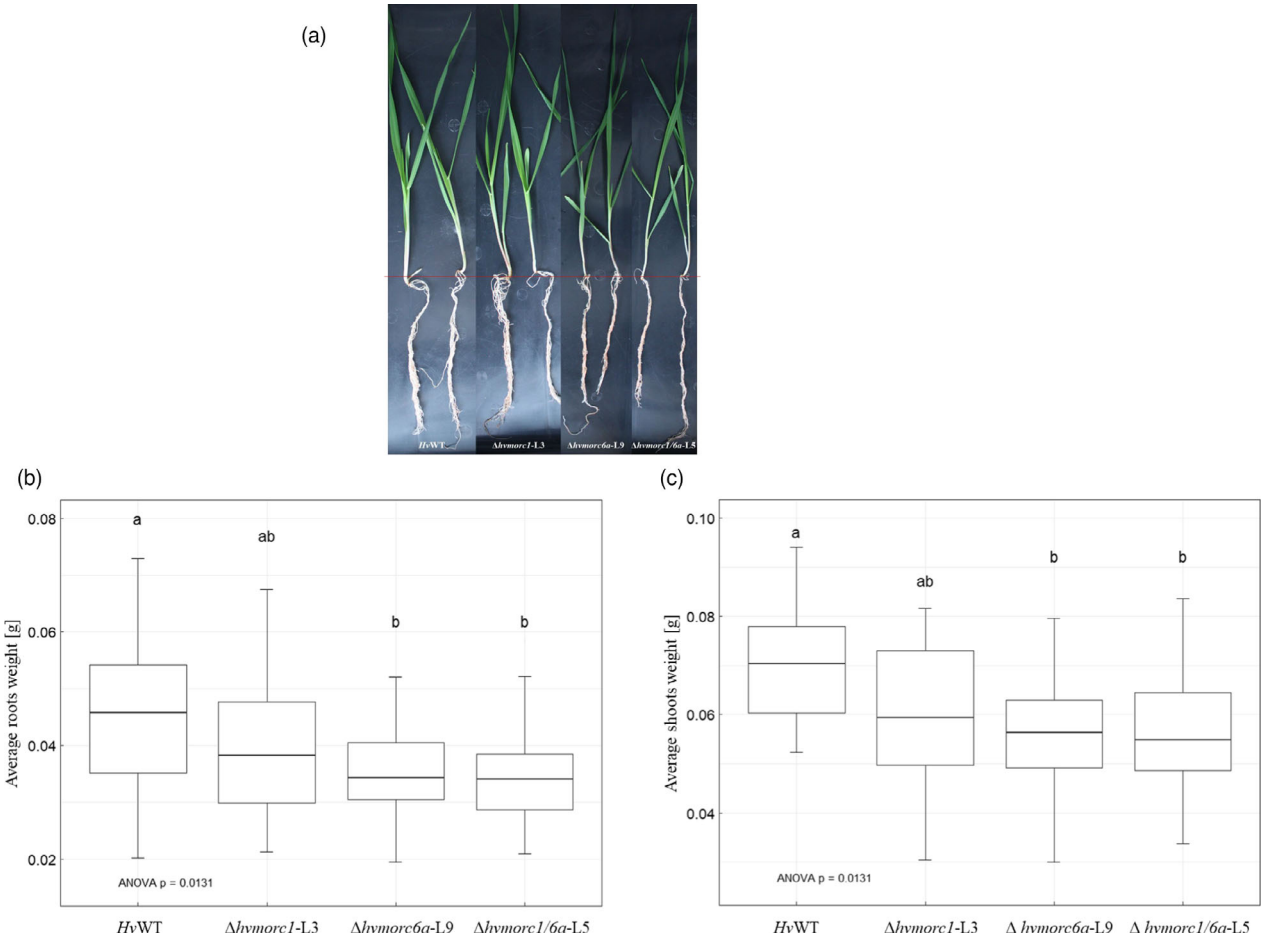


Figure 7 Root and shoot biomass of 3-week-old WT and mutant plants. (a) Plant morphology, (b) roots dry weight, and (c) shoots dry weight of T3 barley plants impaired in the expression of *HvMORC1* (Δ*hvmorc1*-L3), *HvMORC6a* (Δ*hvmorc6a*-L9), and both genes (Δ*hvmorc1/6a*- L5) vs. WT. Plants were cultivated in artificial soil containing a 2:1 mixture of expanded clay (Seramis®, Masterfoods, Verden, Germany) and Oil-Dri® (Damolin, Mettmann, Germany) in a growth chamber at 22 °C/18 °C (day/night cycle) with 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). The experiment was conducted two times (n = 15 plants) with similar results. Comparisons between groups were performed via One-way ANOVA and Tukey's Range Test. Letters represent statistical differences among all group means (α = 0.05).

depletion of *HvMORC6a* enhances the resistance of barley against *Bgh* and *Fg* (Figure 2a,b), and is associated with a higher basal expression of *PR* and JA marker genes (Figure 3). Of note, the highly susceptible Arabidopsis dKO mutant *atmorc1/2* showed attenuated expression of *PR* genes upon infection with *P. syringae* pv. *tomato* (*Pst*; Bordiya *et al*., 2016), which confirms the correlation of MORC-mediated immune phenotypes with defence gene expression.

Despite their contrasting effects on plant immunity, both barley and Arabidopsis MORCs control TE expression in a similar manner (Figure 4; Bordiya *et al*., 2016; Langen *et al*., 2014). We do not yet have a profound explanation for this phenomenon. Bordiya and co-workers suggested that *Pst* infection primarily suppresses binding of *At*MORC1 to DNase I hypersensitive sites (dDHSs), regions of the genome where the chromatin has lost its condensed structure, which are associated with heterochromatic TEs, but enhances its binding at infection-induced dDHSs in genes and TEs. Combined with earlier reports, showing the involvement of *At*MORC1 and *At*MORC6 in upregulation of DNA methylation as well as condensation of compact chromatin (Brabbs *et al*., 2013; Lorković *et al*., 2012; Moissiard *et al*., 2012), the data suggest that *At*MORC1 and/or *At*MORC6 are involved in both gene silencing and gene induction. It is likely that in barley, interaction of *Hv*MORC1 and *Hv*MORC6a with DNA modulating proteins near *PR* loci leads to suppression of *PR* transcription, explaining why barley *MORC* mutants show increased *PR* expression and disease resistance to fungal pathogens.

The dKO mutant *hvmorc1/6a* displays the strongest effect on pathogen defence (Figure 2a, b) and *PR* gene (Figure 3) expression in barley. Therefore, data hint at the possibility that *HvMORC1* and *HvMORC6a* interact with each other and suppress plant defence through epigenetic silencing mechanisms. Microscopic localization showed that *Hv*MORC1 in *hvmorc6a* barley protoplasts was predominantly localized in the cytoplasm of the cell compared with the nuclear-cytoplasmatic localization in barley WT (Figure 5b, left panel). This is consistent with the results in Arabidopsis, where MORC1 and MORC2 form homomers and in addition, heteromers with MORC6 (Liu *et al*., 2014; Moissiard *et al*., 2014). Using Y2H and BiFC assays, we could detect heteromerization of *Hv*MORC6a with *Hv*MORC1 and with

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

*Hv*MORC2; and we confirmed complex formation of *Hv*MORC1 with *Hv*MORC2 as found in Arabidopsis. Unlike in Arabidopsis, we could not find homomerization of *Hv*MORC6a, though we could confirm homomerization of *Hv*MORC1 and heteromerization of *Hv*MORC1 with *Hv*MORC2. *Hv*MORC1and *Hv*MORC2 form homomers and heteromers in the cytoplasm and to a lower extent in the nucleus of *N. benthamiana* cells (Figure 5b right panel, Figure S4). In contrast, the interaction of *Hv*MORC1/6a and *Hv*MORC2/6a was mainly found in the nucleus of *N. benthamiana* plants (Figure 5b right panel, Figure S4). *At*MORC1 was shown to interact with several Resistance (R) proteins, preferable in their inactive state, residing at the plasma membrane (Kang *et al*., 2010). Therefore, it seems plausible that *Hv*MORC1 and its homolog *Hv*MORC2 also reside in the cytoplasm of barley cells. On the other hand, our data suggest that *Hv*MORC1 and *Hv*MORC6a heteromerization likely affects heterochromatin condensation, as reflected by the increased TEs and *PRs* expression and disease resistance in the barley dKO mutants.

To further investigate whether suppression of barley TEs is mediated by *Hv*MORC proteins, we tested the interactions of barley MORC family members with selected barley orthologs of the RdDM pathway. In Arabidopsis, *At*SUVH9 together with *At*MORC6 and *At*SUVH2 regulates silencing of some TEs (Liu *et al*., 2016). In addition, the SWI/SNF chromatin remodelling complex components SWI3B, SWI3C, and SWI3D, together with IDN2, interact with *At*MORC6 to mediate TGS at some *At*MORC6-specific loci (Liu *et al*., 2016). It was suggested that *At*MORC proteins act as adaptors to recruit RNA Polymerase V, in conjunction with *At*SUVH2 and *At*SUVH9 to facilitate the production of long non-coding RNAs (lncRNAs) to promote DNA methylation (Jing *et al*., 2016; Liu *et al*., 2014, 2016). Furthermore, it was proposed that *At*MORC1, *At*MORC2 and/or *At*MORC6 act together with the SWI/SNF chromatin remodelling complex components and IDN2 to alter chromatin structure and therefore reinforce TGS (Jing *et al*., 2016; Koch *et al*., 2017; Liu *et al*., 2016). We observed an interaction of the *Hv*MORC1, *Hv*MORC2, and *Hv*MORC6a with *Hv*IDN2, *Hv*SUVH9, and *Hv*SWI3C with nuclear and cytoplasmatic localization in leaf epidermal cells of *N. benthamiana*, and additionally a shift of localization of these RdDM components in cellular compartments between *hvmorc6a* and barley WT protoplasts (Figure 6b, Figure S5a), further indicating that the barley MORC family members are also involved in RdDM-mediated TEs repression in barley through a *Hv*MORC-dependent pathway.

Derepression of MORC-related genes is linked with lower plant biomass and growth

The barley genome, like most of the plant genomes, consists of a big part of transposable elements or transposons (84%) (International Barley Genome Sequencing Consortium, 2012). Even though these elements are categorized in two classes (retroelement and DNA element transposons, members of the first-class transpose through an RNA intermediate while members of the latter one through a DNA intermediate) their function is nonetheless similar: they move through the genomes to activate and deactivate genes, influence their expression and are fundamental in epigenetic regulation (Bennetzen and Wang, 2014; Galindo-González *et al*., 2017). Notably in plants, TE activity was also detected in response to exogenous environmental and genomic stresses (Alzohairy *et al*., 2012; Galindo-González *et al*., 2017; Grandbastien *et al*., 2005; Salazar *et al*., 2007). Stress has normally a direct effect on the activation of the immune system, which comes always at a great cost for plant development and growth (Huot *et al*., 2014; Kumar *et al*., 2021; Xu *et al*., 2017). To assess whether TEs derepression influences plant fitness and development, we measured the root and shoot biomasses of WT and barley MORC mutants, over a growth period of 3 weeks. Both root and shoot dry weight of all the mutants were lower as compared with WT plants (Figure 7b–c), suggesting a positive correlation between transcript levels and growth promotion. Notably, in *hvmorc6a* and *hvmorc1/6a* mutants, we found strong impairment in growth (Figure 7a) indicating probably a major role of *Hv*MORC6a in nuclear stabilization. Our results underline how important it is to keep the natural chromatin compaction and relaxation for proper plant development and growth.

Our work shows a successful example of how genome editing technologies can be used to introduce desirable agronomic traits into a cereal plant. With CRISPR/Cas, we were able to make plants more resistant to biotic stress, and with significantly fewer undesirable side effects on the plant genome than with chemical and radiation mutagenesis. While conventional breeding produces thousands of random mutations and then requires time-consuming backcrossing to isolate a desired new trait, molecular breeding methods, on the other hand, are easy to use, fast, precise, flexible, and cost-effective. For us, there is no evidence-based doubt that this technology will be a fundamental part of every plant breeder's toolbox in the future.

Experimental procedures

Plant material and fungal inoculation

Seeds of spring barley (*Hordeum vulgare*) cv. 'Golden Promise' were germinated on wet filter paper in large plastic Petri plates. Three days after germination, seedlings were transferred to soil and grown in Typ T soil (Fruhstorfer Erde, Vechta, Germany; 200 g capacity pots) under control condition of 16 h light (240 μmol m^{-2} s^{-1} photon flux density) and 60% relative humidity (22/18 °C day/night cycle). For pathogen assays, the second youngest leaves of 14-day-old plants were cut and laid on 0.7% (w/v) water agar and inoculated with powdery mildew fungus race A6 (*Blumeria graminis* f.sp. *hordei*) at a conidia density of 5 per mm^2 by air current dispersion in an inoculation tower and saved in the same climate chamber for 7 days (Langen *et al*., 2014). *Bgh* colonies were counted using a binocular on a 2.5 cm^2 segment. For *Fusarium graminearum*, strain 1003 (Jansen *et al*., 2005) was selected for inoculation, the fungus was cultured on synthetic nutrient-poor agar medium (SNA) at room temperature under constant illumination as described by Kumar *et al*., 2018. Conidia was isolated from 2-week-old plates, by scrubbing using a Drigalski spatula and filtered through a piece of Miracloth (Calbiochem, http://www.merck-chemicals.de). Conidia was finally resuspended in sterile 0.02% Tween water (w/v) and its concentration was adjusted to 5×10^4 spore mL^{-1}. 20 μL of the suspension was drop-inoculated on detached barley leaves. Progression of infection was routinely monitored and quantification of fungal growth was assessed after 5 days post-inoculation (dpi). Leaf samples were crushed and DNA was extracted via DNA extraction kit (Qiagen, Hilden, Germany). The total fungal/plant DNA ratio was quantified via qPCR normalized with fungal tubulin (*FgTub*) to plant ubiquitin (*HvUbi*), respectively (Table S1).

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

Generation of CRISPR/*Sp*Cas9 constructs and plant transformation

Twenty nucleotides (nt) target sequence present immediately adjacent to a Protospacer Adjacent Motif (PAM) was selected using CRISPR sgRNA design online tool (https://atum.bio/eCommerce/cas9/input) for *HvMORC6a* (GenBank: HORVU3Hr1G046280.3). The designed 20 nt target sequence was blasted (BlastN) against nucleotide collection of *Hordeum vulgare* (taxid: 4513) at NCBI to check for putative off-targets, GTACGGCTTGACATCGCGGGGGG was selected, and sgRNA was assembled and cloned into CRISPR/*Sp*Cas binary destination vector, as described (Kumar *et al*., 2018). The CRISPR/*Sp*Cas9 vector containing *hvmorc6a*-guided RNA was electroporated (Gene Pulser, Bio-Rad) into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*., 1991), and the resulting strain was used to transform spring barley 'Golden Promise' as described (Imani *et al*., 2011). For generation of the double KO line (*hvmorc1/6a*), co-knockout of the *hvmorc6a* and *hvmorc1* genes was obtained using a mixture of two *Agrobacterium* cultures which contained *hvmorc6a*- and *hvmorc1*-guided RNA. The *Agrobacterium* pool was cultured with barley immature embryos as described (Imani *et al*., 2011). All putative single and double knockout barley lines were characterized using PCR followed by Sanger sequencing of the genomic region targeted by respective CRISPR sgRNAs.

DNA isolation and quantitative RT-PCR analysis

DNA/RNA extraction and quantitative RT-qPCR were performed as described in protocol kits (DNA: Qiagen, Hilden, Germany; RNA: Zymo Research, Irvine). Primer pairs used for PCR and expression analysis are listed in Table S1.

Gateway cloning and plasmid DNA preparation

To create Gateway entry, clones of coding sequences (CDS) of barley MORCs (clones obtained from previous work; Langen *et al*., 2014) and the candidate interactors from the barley cultivar Golden Promise were amplified from cDNA using *att*B flanked primer pairs (Table S1) and recombined by Gateway cloning into pDONR™/Zeo vector (Invitrogen, UK) according to the manufacturer's recommendations.

For the yeast two-hybrid (Y2H) assay, entry clones were recombined into pAD and pDB destination vectors (N-terminal fusions of Activation domain AD and DNA-Binding domain DB of the *Saccharomyces cerevisiae* transcriptional activator Gal4, respectively) (Dreze *et al*., 2010). For bimolecular fluorescent complementation (BiFC) assay in *N. benthamiana* plants, entry clones were recombined into pBIFP2 and pBIFP3 destination vectors (N-terminal fusions of the N- and C- parts of yellow fluorescence protein YFP, respectively) (Azimzadeh *et al*., 2008). For CLSM assay in barley protoplasts, entry clones were recombined into p2FGW7 destination vector (Karimi *et al*., 2002). Sanger sequencing was used to validate in-frame cloning and the sequence integrity of all constructs using appropriate primers (Table S1).

Yeast transformation and Y2H assay

Two haploid strains of *S. cerevisiae* of opposite mating types Y8800 (MATa) and Y8930 (MATα), with genotype: *leu2-3,112 trp1-901 his3-200 ura3-52 gal4*Δ *gal80*Δ *GAL2-ADE2 LYS2::GAL1-HIS3 MET2::GAL7-lacZ cyh2*^{R} (Dreze *et al*., 2010) were transformed with CDS-containing pAD and pDB (AD-X and DB-Y) plasmids. Yeast transformation was done using the PEG/Lithium acetate heat-shock method, as previously described (Dreze *et al*., 2010). Four prototrophic markers were used in this screen: TRP1 and LEU2 for the selection of successful transformation of yeast strains with pAD, pDB plasmids on plates lacking Tryptophan or Leucine, respectively. HIS3 and ADE2 were used for the detection of possible AD-X/DB-Y interactions that reconstitute GAL4 transcription factor in the yeast nucleus and initiate transcription of the reporter gene on media lacking Histidine or Adenine, respectively. Y2H screen (or split GAL4 transcription activator) was done in semi-sterile conditions according to the protocol from Dreze *et al*. (2010). Synthetic complete (SC) selective agar plates that lack the amino acids Leucine and Tryptophan (SC-Leu–Trp) were used to assess mating; interaction plates that further lack Histidine were supplemented with 1 mM 3-amino-1,2,4-triazole (a competitive inhibitor of the HIS3 gene product) (Sc-Leu–Trp–His+1 mM *3AT*) were used to detect the interactions. All DB-X constructs and AD-Y were checked for autoactivation by mating with AD-EV (empty vector) and DB-EV on selection media, respectively.

Agrobacterium-mediated transformation of *N. benthamiana* and BiFC assay

pBIFP2 and pBIFP3 harbouring barley MORCs or putative interactors were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Koncz *et al*., 1992, 1994) using a heat-shock method. Positive transformants were selected on YEB plates (for 1 L: 5 g beef extract, 1 g yeast extract, 5 g peptone from soy, 5 g sucrose, 0.5 g $MgCl_2$ and 20 g Agar) complimented with appropriate antibiotics and further confirmed by colony PCR using insert-specific primers (Table S1). Leaves from 4 to 5-week-old *N. benthamiana* plants were used for *Agrobacterium*-mediated transient expression of recombinant proteins. *Agrobacterium* infiltration procedure was performed according to Waadt and Kudla (2008) on the abaxial epidermal leaf layer. The cultures of *Agrobacterium* carrying constructs of interest were set to an optical density (OD_{600}) of 0.5, while culture harbouring the silencing suppressor p19 protein of tomato bushy stunt virus (Chen *et al*., 2011) construct was set to OD_{600} of 0.3. All infiltration combinations for BiFC assay were mixed with p19 before infiltration. Plants were kept at 25 C° for 48 h before visualization under laser scanning confocal microscopy.

Protoplast isolation and transformation

Mesophyll protoplasts were enzymatically released from green leaves of 1–2-week-old barley according to Sheen (1991). After resting on ice for 30 min in WI solution (0.6 M mannitol. 4 mM MES, pH 5.7, 20 mM KCl), the protoplasts were resuspended in MMg solution (4 mM MES, pH 5.7, 0.6 M mannitol, 15 mM $MgC1_2$) to a final of 5×10^5 protoplasts/ml. 200 μL (1×10^5 protoplasts) were used for the PEG-mediated transformation as previously described (Yoo *et al*., 2007). 20–30 μg total plasmid DNA coding for different chimeric N-terminal GFP fusions to the full-length CDS was gently mixed with the protoplasts before slowly adding PEG-Ca^{2+}. Transformation time was set to 13 min. After washing steps as indicated previously (Yoo *et al*., 2007), protoplasts were incubated in modified WI solution (0.6 M mannitol. 4 mM MES, pH 5.7, 4 mM KCl) in the dark at 25 C° for 24 h before visualization using laser scanning confocal microscopy. 10 μg pSAT6-mCherry-VirD2NLS was simultaneously transformed as a nuclear marker.

Confocal laser scanning microscopy

Images were taken using a Leica TCS SP8 confocal laser scanning microscope. GFP::full-length protein samples and BiFC samples were excited using an argon laser at 488 nm and 514 nm,

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

respectively. YFP and GFP fluorescence emission was detected between 519–548 nm. The nuclear marker (mCherry-VirD2NLS) was excited at 561 nm and fluorescence emission was detected between 573 and 626 nm. Chlorophyll autofluorescence was detected between 679 and 789 nm after excitation using a 633 nm Helium–Neon laser. The pinhole was set to 1 airy unit for both protoplasts and leaf cells. Images for CLSM with nuclear marker were taken in sequential mode, while BiFC images without nuclear marker were acquired in standard mode. YFP-, mCherry fluorescence, and chlorophyll autofluorescence are shown in green, red, and purple, respectively. Images were processed using the Leica LAS X software.

Accession numbers

*Hv*MORC1 [HORVU7Hr1G083280.15], *Hv*MORC2 [HORVU1Hr1G006770.1], *Hv*MORC6a [HORVU3Hr1G046280.3], *Hv*MORC6b [HORVU3Hr1G078330.4], *Hv*MORC7 [HORVU2Hr1G066650.2], *Hv*MORCCW1 [HORVU1Hr1G080470.1], and *Hv*MORCCW2 [HORVU7Hr1G093640.4]; *At*IDN2 [NP_001327083.1], *Hv*IDN2 [BAJ90280.1], *At*SWIC3C [NP_173589.1], *Hv*SWIC3C [BAJ93481.1], *At*DMS3 [NP_566916.1], *Hv*DMS3 [BAJ94830.1], *At*SUVH9 [NP_001031625.1], and *Hv*SUVH9 [BAK07491.1].

Acknowledgements

We thank Martina Claar, Dagmar Biedenkopf, Cornelia Dechert and Eugen Swidtschenko for the excellent technical assistance. This work was funded by the Deutsche Forschungsgemeinschaft (DFG) to KHK (RU5116). M.G. and E.M. were supported by German Academic Exchange Service (DAAD). Open Access funding enabled and organized by Projekt DEAL.

Authorship

M.G., J.S. and K-H.K. wrote the manuscript; KHK, M.G., J.S., N.K., A.K. and J.I. designed the study; M.G., E.M., N.K. and J.I. prepared material for the experiments; M.G., E.M. conducted the experiments; M.G., J.I, J.S. and KHK analysed all data and drafted the figures. All authors commented and reviewed the final manuscript.

Consent for publication

All authors declare consent of publication.

Competing financial interests

The authors declare no competing financial interests.

Data availability statement

All data generated or analysed during this study are included in this published article [and its supplementary information files].

References

Alzohairy, A.M., Yousef, M.A., Edris, S., Kerti, B., Gyulai, G. and Bahieldin, A. (2012) Detection of LTR Retrotransposons Reactivation induced by in vitro Environmental Stresses in Barley (*Hordeum vulgare*) via RT-qPCR. *Life Sci. J.* **9**, 5019–5026.

Azimzadeh, J., Nacry, P., Christodoulidou, A., Drevensek, S., Camilleri, C., Amiour, N., Parcy, F. *et al*. (2008) Arabidopsis TONNEAU1 proteins are essential for preprophase band formation and interact with centrin. *Plant Cell*, **20**, 2146–2159.

Bennetzen, J.L. and Wang, H. (2014) The contributions of transposable elements to the structure, function, and evolution of plant genomes. *Annu. Rev. Plant Biol.* **65**, 505–530.

Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., Nuka, G. *et al*. (2021) The InterPro protein families and domains database: 20 years on. *Nucleic Acids Res.* **49**(D1), 344–354.

Bordiya, Y., Zheng, Y., Nam, J.C., Bonnard, A.C., Choi, H.W., Lee, B.K., Kim, J. *et al*. (2016) Pathogen infection and MORC proteins affect chromatin accessibility of transposable elements and expression of their proximal genes in Arabidopsis. *Mol. Plant Microbe Interact.* **29**, 674–687.

Brabbs, T.R., He, Z., Hogg, K., Kamenski, A., Li, Y., Paszkiewicz, K.H., Moore, K.A. *et al*. (2013) The stochastic silencing phenotype of Arabidopsis morc6 mutants reveals a role in efficient RNA-directed DNA methylation. *Plant J.* **75**, 836–846.

Braun, P., Tasan, M., Dreze, M., Barrios-Rodiles, M., Lemmens, I., Yu, H., Sahalie, J.M. *et al*. (2009) An experimentally derived confidence score for binary protein-protein interactions. *Nat. Methods*, **6**, 91–97.

Chen, Q., He, J., Phoolcharoen, W. and Mason, H.S. (2011) Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. *Human Vaccines*, **7**, 331–338.

Citovsky, V., Lee, L.Y., Vyas, S., Glick, E., Chen, M.H., Vainstein, A., Gafni, Y. *et al*. (2006) Subcellular localization of interacting proteins by bimolecular fluorescence complementation *in planta*. *J. Mol. Biol.* **362**, 1120–1131.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D. *et al*. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**, 819–823.

Dong, W., Vannozzi, A., Chen, F., Hu, Y., Chen, Z. and Zhang, L. (2018) MORC domain definition and evolutionary analysis of the MORC gene family in green plants. *Genome Biol. Evol.* **10**, 1730–1744.

Dreze, M., Monachello, D., Lurin, C., Cusick, M.E., Hill, D.E., Vidal, M. and Braun, P. (2010) High-quality binary interactome mapping. *Methods Enzymol.* **470**, 281–315.

Fernandez-Cornejo, J., Wechsler, S., Livingston, M. & Mitchell, L. (2014). Genetically engineered crops in the United States. USDA-ERS Economic Research Report, (162).

Galindo-González, L., Mhiri, C., Deyholos, M.K. and Grandbastien, M.A. (2017) LTR-retrotransposons in plants: Engines of evolution. *Gene*, **626**, 14–25.

Gasparis, S., Kała, M., Przyborowski, M., Łyżnik, L.A., Orczyk, W. and Nadolska-Orczyk, A. (2018) A simple and efficient CRISPR/Cas9 platform for induction of single and multiple, heritable mutations in barley (*Hordeum vulgare* L.). *Plant Methods*, **14**, 1–14.

Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227.

Govindan, G. and Ramalingam, S. (2016) Programmable site-specific nucleases for targeted genome engineering in higher eukaryotes. *J. Cell. Physiol.* **231**, 2380–2392.

Grandbastien, M.-A., Audeon, C., Bonnivard, E., Casacuberta, J.M., Chalhoub, B., Costa, A.-P., Le, Q.H. *et al*. (2005) Stress activation and genomic impact of Tnt1 retrotransposons in *Solanaceae*. *Cytogenetic Genome Res.* **110**, 229–241.

Harris, C.J., Husmann, D., Liu, W., Kasmi, F.E., Wang, H., Papikian, A., Pastor, W.A. *et al*. (2016) Arabidopsis *At*MORC4 and *At*MORC7 form nuclear bodies and repress a large number of protein-coding genes. *PLoS Genet.* **12**, e1005998.

Holme, I.B., Wendt, T., Gil-Humanes, J., Deleuran, L.C., Starker, C.G., Voytas, D.F. and Brinch-Pedersen, H. (2017) Evaluation of the mature grain phytase candidate *HvPAPhy* a gene in barley (*Hordeum vulgare* L.) using CRISPR/Cas9 and TALENs. *Plant Mol. Biol.* **95**, 111–121.

Huot, B., Yao, J., Montgomery, B.L. and He, S.Y. (2014) Growth–defense tradeoffs in plants: a balancing act to optimize fitness. *Molecular Plant*, **7**, 1267–1287.

Imani, J., Li, L., Schaefer, P. and Kogel, K.H.(2011) STARTS–A stable root transformation system for rapid functional analyses of proteins of the monocot model plant barley. *Plant J.* **67**, 726–735.

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

International Barley Genome Sequencing Consortium (IBSC). (2012) A physical, genetic and functional sequence assembly of the barley genome. *Nature*, **491**, 711–716.

Iyer, L.M., Abhiman, S. and Aravind, L. (2008) MutL homologs in restriction-modification systems and the origin of eukaryotic MORC ATPases. *Biology Direct*, **3**, 1–9.

Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S. and Venkataraman, G. (2018) CRISPR for crop improvement: an update review. *Frontiers Plant Sci.* **9**, 985.

Jansen, C., Von Wettstein, D., Schäfer, W., Kogel, K.H., Felk, A. and Maier, F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proc. Natl Acad. Sci. USA*, **102**, 16892–16897.

Jarosch, B., Kogel, K.H. and Schaffrath, U. (1999) The ambivalence of the barley Mlo locus: mutations conferring resistance against powdery mildew (*Blumeria graminis f. sp. hordei*) enhance susceptibility to the rice blast fungus *Magnaporthe grisea*. *Mol. Plant Microbe Interact.* **12**, 508–514.

Jing, Y., Sun, H., Yuan, W., Wang, Y., Li, Q., Liu, Y., Li, Y. *et al*. (2016) SUVH2 and SUVH9 couple two essential steps for transcriptional gene silencing in Arabidopsis. *Molecular Plant*, **9**, 1156–1167.

Kang, H.G., Kuhl, J.C., Kachroo, P. and Klessig, D.F. (2008) CRT1, an Arabidopsis ATPase that interacts with diverse resistance proteins and modulates disease resistance to turnip crinkle virus. *Cell Host Microbe.* **3**, 48–57.

Kang, H.G., Oh, C.S., Sato, M., Katagiri, F., Glazebrook, J., Takahashi, H., Kachroo, P. *et al*. (2010) Endosome-associated CRT1 functions early in resistance gene–mediated defense signaling in Arabidopsis and tobacco. *Plant Cell*, **22**, 918–936.

Kang, H.-G., Woo Choi, H., von Einem, S., Manosalva, P., Ehlers, K., Liu, P.-P., Buxa, S.V. *et al*. (2012) CRT1 is a nuclear-translocated MORC endonuclease that participates in multiple levels of plant immunity. *Nat. Commun.* **3**, 1–11.

Kapusi, E., Corcuera-Gómez, M., Melnik, S. and Stoger, E. (2017) Heritable genomic fragment deletions and small indels in the putative ENGase gene induced by CRISPR/Cas9 in barley. *Front. Plant Sci.* **8**, 540.

Karimi, M., Inzé, D. and Depicker, A. (2002) GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* **7**, 193–195.

Kim, H. and Kim, J.S. (2014) A guide to genome engineering with programmable nucleases. *Nat. Rev. Genet.* **15**, 321–334.

Kis, A., Hamar, É., Tholt, G., Bán, R. and Havelda, Z. (2019) Creating highly efficient resistance against wheat dwarf virus in barley by employing CRISPR/Cas9 system. *Plant Biotechnol. J.* **17**, 1004.

Klessig, D.F., Choi, H.W. and Dempsey, D.M.A. (2018) Systemic acquired resistance and salicylic acid: past, present, and future. *Mol. Plant Microbe Interact.* **31**, 871–888.

Koch, A., Kang, H.G., Steinbrenner, J., Dempsey, D.M.A., Klessig, D.F. and Kogel, K.H. (2017) MORC proteins: novel players in plant and animal health. *Front. Plant Sci.* **8**, 1720.

Koncz, C., Martini, N., Szabados, L., Hrouda, M., Bachmair, A. and Schell, J. (1994) Specialized vectors for gene tagging and expression studies. *Plant Molecul. Biol. Manual*, 53–74.

Koncz, C., Schell, J. and Rédei, G.P. (1992) T-DNA transformation and insertion mutagenesis. *Methods Arabidopsis Res.* 224–273.

Kumar, N., Galli, M., Ordon, J., Stuttmann, J., Kogel, K.H. and Imani, J. (2018) Further analysis of barley MORC 1 using a highly efficient RNA-guided Cas9 gene-editing system. *Plant Biotechnol. J.* **16**, 1892–1903.

Kumar, N., Galli, M., Dempsey, D.M., Imani, J., Moebus, A. and Kogel, K.H. (2021) NPR1 is required for root colonization and the establishment of a mutualistic symbiosis between the beneficial bacterium Rhizobium radiobacter and barley. *Environ. Microbiol.* **23**, 2102–2115.

Langen, G., von Einem, S., Koch, A., Imani, J., Pai, S.B., Manohar, M., Ehlers, K. et al. (2014) The compromised recognition of turnip crinkle virus1 subfamily of microrchidia ATPases regulates disease resistance in barley to biotrophic and necrotrophic pathogens. *Plant physiology* **164**(2), 866–878.

Lawrenson, T., Shorinola, O., Stacey, N., Li, C., Østergaard, L., Patron, N., Uauy, C. *et al*. (2015) Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. *Genome Biol.* **16**, 1–13.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) A DNA transformation–competent Arabidopsis genomic library in *Agrobacterium*. *Bio/Technology*, **9**, 963–967.

Lee, J.H., Won, H.J., Hoang Nguyen Tran, P., Lee, S.-M., Kim, H.-Y. and Jung, J.H. (2021) Improving lignocellulosic biofuel production by CRISPR/Cas9-mediated lignin modification in barley. *GCB Bioenergy*, **13**, 742–752.

Li, Y., Liu, D., Zong, Y., Jiang, L., Xi, X., Cao, D., Shen, Y. *et al*. (2020) New D hordein alleles were created in barley using CRISPR/Cas9 genome editing. *Cereal Res. Commun.* **48**, 131–138.

Lin, H.Y., Chen, J.C. and Fang, S.C.(2018) A protoplast transient expression system to enable molecular, cellular, and functional studies in Phalaenopsis orchids. *Front Plant Sci.* **9**, 843.

Liu, Z.W., Shao, C.R., Zhang, C.J., Zhou, J.X., Zhang, S.W., Li, L., Chen, S. *et al*. (2014) The SET domain proteins SUVH2 and SUVH9 are required for Pol V occupancy at RNA-directed DNA methylation loci. *PLoS Genet.* **10**, e1003948.

Liu, Z.W., Zhou, J.X., Huang, H.W., Li, Y.Q., Shao, C.R., Li, L., Cai, T. *et al*. (2016) Two components of the RNA-directed DNA methylation pathway associate with MORC6 and silence loci targeted by MORC6 in Arabidopsis. *PLoS Genet.* **12**, e1006026.

Lorković, Z.J., Naumann, U., Matzke, A.J. and Matzke, M. (2012) Involvement of a GHKL ATPase in RNA-directed DNA methylation in *Arabidopsis thaliana*. *Curr. Biol.* **22**, 933–938.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E. *et al*. (2013) RNA-guided human genome engineering via Cas9. *Science*, **339**, 823–826.

Manohar, M., Choi, H.W., Manosalva, P., Austin, C.A., Peters, J.E. and Klessig, D.F. (2017) Plant and human MORC proteins have DNA-modifying activities similar to type II topoisomerases, but require one or more additional factors for full activity. *Mol. Plant Microbe Interact.* **30**, 87–100.

Manosalva, P., Manohar, M., Kogel, K.H., Kang, H.G. and Klessig, D.F. (2015) The GHKL ATPase MORC1 modulates species-specific plant immunity in Solanaceae. *Mol. Plant Microbe Interact.* **28**, 927–942.

Moissiard, G., Bischof, S., Husmann, D., Pastor, W.A., Hale, C.J., Yen, L., Stroud, H. *et al*. (2014) Transcriptional gene silencing by Arabidopsis microrchidia homologues involves the formation of heteromers. *Proc. Natl Acad. Sci. USA*, **111**, 7474–7479.

Moissiard, G., Cokus, S.J., Cary, J., Feng, S., Billi, A.C., Stroud, H., Husmann, D. *et al*. (2012) MORC family ATPases required for heterochromatin condensation and gene silencing. *Science*, **336**, 1448–1451.

Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S.C. (2012) Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* **28**, 489–521.

Salazar, M., González, E., Casaretto, J.A., Casacuberta, J.M. and Ruiz-Lara, S. (2007) The promoter of the TLC1. 1 retrotransposon from *Solanum chilense* is activated by multiple stress-related signaling molecules. *Plant Cell Rep.* **26**, 1861–1868.

Sheen, J. (1991) Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. *Plant Cell*, **3**, 225–245.

Waadt, R. and Kudla, J. (2008) In planta visualization of protein interactions using bimolecular fluorescence complementation (BiFC). *Cold Spring Harbor Protocols*, **2008**, 4995.

Xu, G., Yuan, M., Ai, C., Liu, L., Zhuang, E., Karapetyan, S., Wang, S. *et al*. (2017) uORF-mediated translation allows engineered plant disease resistance without fitness costs. *Nature*, **545**, 491–494.

Xue, Y., Zhong, Z., Harris, C.J., Gallego-Bartolomé, J., Wang, M., Picard, C., Cao, X., Hua, S., Kwok, I., Feng, S. and Jami-Alahmadi, Y. (2021) Arabidopsis MORC proteins function in the efficient establishment of RNA directed DNA methylation. *Nature communications*, **12**, 1–13.

Yi, Z., Sanjeev, M. and Singh, G. (2020) The branched nature of the nonsense-mediated mRNA decay pathway. *Trends Genet.* **37**, 143–159.

Yoo, S.D., Cho, Y.H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2**, 1565–1572.

Yu, A., Lepère, G., Jay, F., Wang, J., Bapaume, L., Wang, Y., Abraham, A.L. *et al*. (2013) Dynamics and biological relevance of DNA demethylation in Arabidopsis antibacterial defense. *Proc. Natl Acad. Sci. USA*, **110**, 2389–2394.

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

Zeng, Z., Han, N., Liu, C., Buerte, B., Zhou, C., Chen, J., Wang, M. *et al*. (2020) Functional dissection of HGGT and HPT in barley vitamin E biosynthesis via CRISPR/Cas9-enabled genome editing. *Ann. Bot.* **126**, 929–942.

Zhu, H., Li, C. and Gao, C. (2020) Applications of CRISPR–Cas in agriculture and plant biotechnology. *Nat. Rev. Mol. Cell Biol.* **21**, 661–677.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 *HvMorc6*-sgRNA target location and construct used to generate *hvmorc6a* barley mutants. (a) Schematic representation of the T-DNA region containing all components for *Agrobacterium*-mediated, *Sp*Cas9-based *HvMORC6a* gene editing. *pCMV35s*, *Cauliflower Mosaic Virus* 35S promoter, *hpt*, *hygromycin phosphotransferase gene*; *t35s*, *CaMV* 35S terminator; *pHvU3*, barley *U3* promoter; target *morc6a* sequence; *sgRNA*, synthetic single-guide RNA; pZmUbi, ubiquitin promoter of *Zea mays*; *SpCas9*, *S. pyogenes* Cas9; *LB*, *RB*, left and right border sequences of the T-DNA. (b) Target area of *hvmorc1*-sgRNA and *hvmorc6a*-sgRNA (20 nt, underlined) with PAM sequence (grey highlighted) in *Hv*MORC1 and *Hv*MORC6a protein architecture, respectively; hallmark domains (HATPase_C, S5, and CC) are highlighted in bounding boxes; thunder indicates precise location of *Sp*Cas9 cutting site. Protein domains were drawn after analysis of the protein sequence via the InterPro protein families and domains database (https://www.ebi.ac.uk/interpro/; Blum *et al*., 2021). Note both protein domain structures have been drawn to scale. (c) Target area of *hvmorc6a*-sgRNA in *HvMORC6a* cDNA sequence. (d) Alignment of potential target sites of the *hvmorc6a*-sgRNA in other *HvMORC* paralogs; similar nucleotides to the sgRNA are displayed in red.

Figure S2 CRISPR/*Sp*Cas9 efficiency and cleavage sites in *hvmorc6a* barley mutant lines. (a) Schematic summary of the transformation efficiency in *Sp*Cas9-induced *hvmorc6a* mutants. (b) Homozygous mutations in T0 *hvmorc6a* plants, determined after sequencing using specific primers (Table S1). The PAM (NGG) sequence is highlighted in grey, the 20 bp long target region is underlined, and point mutations are marked in bold. (c) All bi-allelic homozygous mutation patterns found in independent plants. (d) Example of a heterozygous mutant, with the characteristic multiple spikes in the chromatogram.

Figure S3 *Sp*Cas9-induced frame-shift mutations in *HvMORC1* and *HvMORC6a*. (a) Homozygous mutated lines used in this study: *hvmorc1* (Δ*hvmorc1*-L3), *hvmorc6a* (Δ*hvmorc6a*-L9 and L16), and *hvmorc1/6a* (Δ*hvmorc1/6a*-L4 and L5) T3 homozygous mutants. (b) CRISPR/*Sp*Cas9 system inserts STOP codons in *HvMORC1* and *HvMORC6a* open reading frames (in red), leading to the premature termination of the protein. Frame-shift mutations are visualized via the online tool (http://web.expasy.org/translate/).

Figure S4 YFP signals of homo-/heteromerization of different *Hv*MORCs. *Hv*MORCs combinations were detected in lower epidermal cells of tobacco after 48 h (left panel). pBiFP2-*Hv*MORCs and pBiFP3-*Hv*MORCs were N-terminally fused to the N- and C- terminal parts of YFP, respectively. The different combinations show similar interaction results. ROI is a magnification of the bordered region in the overlay column. Scale bar: 20 μm. YFP: yellow fluorescence protein, Chl: chlorophyll autofluorescence, ROI: regions of interest (magnification of the bordered region).

Figure S5 GFP::*Hv*DMS3 localization and YFP signals of homo-/heteromerization of different *Hv*MORCs with orthologs of the RdDM pathway. (a) The interaction between barley MORCs and SWI3C, and IDN2 was detected in both combination directions of the two BiFC vectors in lower epidermal cells of tobacco. Scale bar, 20 μm. (b) Localization of *Hv*DMS3 in barley WT and *hvmorc6a* protoplasts, and interaction between barley MORCs and DMS3 (no signal detected in both directions). Scale bar, 20 μm. YFP: yellow fluorescence protein, Chl: chlorophyll autofluorescence, ROI: regions of interest (magnification of the bordered region).

Table S1 Oligonucleotide primers used in this study.

Table S2 Barley orthologs of the potential Arabidopsis RdDM interactors.

© 2021 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.,

Journal of Plant Diseases and Protection
https://doi.org/10.1007/s41348-022-00574-y

SHORT COMMUNICATION

CRISPR/*Sp*Cas9-mediated KO of epigenetically active MORC proteins increases barley resistance to *Bipolaris* spot blotch and *Fusarium* root rot

Matteo Galli[1] · Selina Hochstein[1] · Danish Iqbal[1] · Martina Claar[1] · Jafargholi Imani[1] · Karl-Heinz Kogel[1]

Received: 30 November 2021 / Accepted: 13 January 2022
© The Author(s) 2022

Abstract
Microrchidia (MORC) proteins are fundamental regulators of genome stabilization, chromatin remodeling and gene expression in both mammals and plants. In Arabidopsis, their activity is linked to the RNA-directed DNA methylation (RdDM) pathway, which utilizes small RNAs (sRNAs) to influence the rate of DNA methylation and chromatin compaction and thus gene expression. In barley, there are a total of seven members of the MORC family, and recent advances showed that *Hv*MORC1 and *Hv*MORC6a also interact with components of the RdDM pathway. CRISPR/*Sp*Cas9-mediated single and double knock-out mutants showed de-repression of transposable elements (*TEs*) and pathogenesis-related (*PR*) genes and interestingly increased resistance to both biotrophic and necrotrophic plant pathogenic fungi. In this study, we further demonstrate the requirement of MORC proteins in the resistance against two devastating cereal diseases, *Bipolaris* spot blotch, caused by *Bipolaris sorokiniana* and *Fusarium* root rot, caused by *Fusarium graminearum*.

Keywords Microrchidia · Barley · CRISPR · *Bipolaris sorokiniana* · *Fusarium graminearum* · Epigenetics

Introduction

In eukaryotes, transcriptional gene silencing (TGS) results in decreased RNA synthesis by establishing and maintaining DNA methylation through the RNA-directed DNA methylation (RdDM) pathway (Law and Jacobsen 2010; Erdmann and Picard 2020). In Arabidopsis, several members of the Microrchidia (MORC) protein family (*At*MORC1 to *At*MORC7) are RdDM downstream players involved in repression of DNA methylated genes as well as transposable elements (*TEs*) by increasing chromatin compaction rate (Lorković et al. 2012; Moissiard et al. 2012, 2014; Brabbs et al. 2013; Liu et al. 2014, 2016; Harris et al. 2016; Jing et al. 2016; Xue et al. 2021). Several studies have shown that MORC proteins play a role in plant defense, but it is highly dependent on the plant species. In Arabidopsis and potato, MORC proteins enhance resistance to pathogens, while in barley, tobacco and tomato, they negatively affect plant immunity (Kang et al. 2008, 2010, 2012; Langen et al. 2014; Manosalva et al. 2015; Kumar et al. 2018). Thus, in clear contrast to Arabidopsis mutants that show reduced expression of pathogenesis-related genes (*PRs*), knock-down (KD) and knock-out (KO) mutants of barley *MORC* genes show enhanced *PRs* expression. Moreover, *Hv*MORC1, *Hv*MORC2 and *Hv*MORC6a proteins also play a crucial role in maintaining genome stability by suppressing *TE*s (Langen et al. 2014; Kumar et al. 2018), and forming nucleocytoplasmic homo-/heteromeric MORC complexes that contain additional components of the RdDM gene silencing machinery (Galli et al. 2021). As a consequence, mutations in *HvMORC1* and *HvMORC6a* resulted in de-repression

✉ Matteo Galli
matteo.galli@agrar.uni-giessen.de

Selina Hochstein
selina.hochstein@ag.uni-giessen.de

Danish Iqbal
danish.iqbal@agrar.uni-giessen.de

Martina Claar
martina.claar@agrar.uni-giessen.de

Jafargholi Imani
jafargholi.imani@agrar.uni-giessen.de

Karl-Heinz Kogel
karl-heinz.kogel@agrar.uni-giessen.de

[1] Institute of Phytopathology, Land Use and Nutrition, Research Centre for BioSystems, Justus Liebig University, 35392 Giessen, Germany

Published online: 25 January 2022

of *TEs* and were associated with increased disease resistance to powdery mildew caused by *Blumeria graminis* f.sp. *hordei* (*Bgh*) and *Fusarium* leaf spot. It is also noteworthy that MORC mutants exhibit reduced leaf and root development in addition to their effects on the plant immune system (Galli et al. 2021).

In the present study, we have extended our analysis on the role of barley MORC proteins in RdDM-mediated epigenetic regulation of disease resistance, using *Bipolaris sorokiniana* (*Bs*) (teleomorph *Cochliobolus sativus*) and *Fusarium* root rot (FRR) caused by *Fg* as study cases as they are two major cereal pathogens of global importance. *Bs* is the causative agent of cereal spot blotch disease, which severely limits grain production in warm, humid South Asian countries, as well as in Canada, the USA, Brazil and Australia, resulting in significant yield losses (Kumar et al. 2002; Singh et al. 2015; Gupta et al. 2018). *Fusarium* fungi, on the other hand, are devastating plant pathogens of wheat and barley that are widespread worldwide causing *Fusarium* head blight (FHB), *Fusarium* crown rot (FCR) and *Fusarium* root rot (FRR) (Hollaway et al. 2013; Balmas et al. 2015). They also contaminate the grain with mycotoxins and thus decrease grain quality and availability (Gaffar et al. 2019). We show here that barley MORC single and double mutants generated with CRISPR/*Sp*Cas9 exhibit increased resistance to *Bipolaris* spot blotch and FRR.

Material and methods

Plant and fungal growth conditions

Seeds of spring barley (*Hordeum vulgare*) cv. 'Golden Promise' and all MORC mutants carrying a homozygous disruptive mutation in target gene(s) (Δ*hvmorc1*-L3: −2 bp; Δ*hvmorc6a*-L9: + 1 bp; Δ*hvmorc6a*-L16: −25 bp; Δ*hvmorc1/6a*-L4: −2 bp/ + 1 bp; Δ*hvmorc1/6a*-L5: −2 bp/-8 bp, Kumar et al. 2018; Galli et al. 2021) were germinated in dark on wet filter paper. Three days after germination, seedlings were transferred to soil and grown in Type T soil (Fruhstorfer Erde, Vechta, Germany; 200 g capacity pots) under control condition of 16 h light (240 µmol m^{-2} s^{-1} photon flux density) and 60% relative humidity (22/18 °C day/night cycle). *Bipolaris sorokiniana* culture KN2 was grown on complete medium at 25 °C and propagated as described in Kumar et al. (2001). *Fusarium graminearum* wild-type strain 1003 (teleomorph: *Gibberella zeae*) was cultured on Haarleen Agar (HA; 8 g malt extract, 3.2 g glucose, 3.2 g yeast extract and 12 g agar per liter) and induction of conidiation as described in Jansen et al. (2005). Both *Bs* and *Fg* conidia were harvested from 10 to 14-day-old axenic cultures with a sterile glass rod and suspended in 0.002% (v/v) Tween-20 after filtering them through a layer of Miracloth (Calbiochem, http://www.merck-chemicals.de).

Plant inoculation

Bs conidia were applied on whole ten-day-old seedlings with a spray brush (20×10^4 conidia ml^{-1}) in 0.002% Tween water (v/v). Mock inoculated control plants were sprayed with Tween water only. After inoculation, the plants were placed in a transparent plastic box with moist paper towels and closed with a lid to ensure > 95% relative humidity. After five days, the first leaf of each plant was cut off, placed on (1% w/v) agar plate, photographed, and the black *Bs* lesions were subsequently counted. For *Fg* root rot analysis, three-day-old barley seedlings were placed in glass jars and roots submerged with 15×10^4 conidia ml^{-1} in Tween water for 90 min. The control group was treated with Tween water only. Subsequently, the plants were wrapped in moist sterile filter paper and placed in 50 mL falcon tubes for seven days under 22 °C, 16/8 h light. Infection was monitored regularly, and fungal growth was assessed seven days post-inoculation (dpi).

Real-time PCR detection for quantification of infection levels

The same plant material was used for quantitative analysis as for symptom assessment. Seven-day-old infected and non-infected leaves with *Bs* were crushed in liquid nitrogen, and DNA was extracted using a DNA extraction kit (Qiagen, Hilden, Germany). Total fungal/plant DNA ratio was quantified by quantitative real-time (qPCR) using fungal *Glyceraldehyde-3-phosphate dehydrogenase* (*BsGPD*) primers (Table 1) normalized to barley *Ubiquitin* (*HvUbi*)

Table 1 Oligonucleotide primers used in this study

Primer name	Sequence 5′ → 3′	Target; use
q*Hv*Ubi_F	TCGCCGACTACAACATCCAG	Barley *Ubiquitin*; qPCR expression
q*Hv*Ubi_R	TGTGCTTGTGCTTTTGCTTC	
q*Fg*EF-1a_F	TGCCAACATGATCATTTCGTGCGTA	*Fg elongation factor-1 α*; qPCR expression
q*Fg*EF-1a_R	CAAGGCCGTCGAGAAGTCCAC	
q*Bs*GPD_F	AACGGCAAGACCATCCGTT	*Bs glyceraldehyde-3-phosphate dehydrogenase*; qPCR expression
q*Bs*GPD_R	GACGACGTAGTAAGCGCCAGT	

as described in Galli et al. (2021). For quantification of *Fg* DNA, root samples were ultrasonically washed three times in a water bath prior to DNA extraction. Quantification by qPCR was performed with fungal *Elongation factor-1 α* (*FgEF-1a*) primers (Table 1) normalized to plant *Ubiquitin* (*HvUbi*) as described in Galli et al. (2021).

Statistical tests used in this study (Student's t test, Kruskal–Wallis test with multiple comparisons and ANOVA with post hoc Tukey HSD test) were selected after analysis of the normal distribution and homogeneity of variances in the different groups. All biological assays were repeated at least twice with similar results.

Results

Barley MORC1 and MORC6a are negative regulators of disease resistance to *B. sorokiniana* and *F. graminearum*

Previous findings suggest that MORC proteins modulate immunity in a species-specific manner (for details see Koch et al. 2017). In barley, MORC1 and MORC6a negatively regulate resistance to biotrophic and necrotrophic fungal leaf pathogens (Langen et al. 2014; Kumar et al. 2018; Galli et al. 2021). To broaden our knowledge on the effect of MORC proteins on fungal pathogens, we first investigated the response of barley MORC mutants to the hemibiotrophic fungus *Bipolaris sorokiniana*. Ten-day-old wild-type barley (*Hv*WT) cultivar 'Golden Promise' (GP) and GP seedlings with knock-out (KO) mutations in genes *HvMORC1* and *HvMORC6a* were spray-inoculated with *Bs* conidia and five days post-inoculation (dpi) the total number of necrotic lesions was recorded. Compared to *Hv*WT, single mutants Δ*hvmorc1* line 3, Δ*hvmorc6* line 9, and Δ*hvmorc6* line 16 as well as double mutants Δ*hvmorc1/6a* line 4 and Δ*hvmorc1/6a* line 5 showed significantly less spot blotch symptoms (Δ*hvmorc1* L3: 2.1-fold decrease; Δ*hvmorc6a* L9: 1.8-fold decrease; Δ*hvmorc6a* L16: 1.5-fold decrease; Δ*hvmorc1/6a* L4: 2.7-fold decrease; Δ*hvmorc1/6a* L5: 1.7-fold decrease; α 0.05, Kruskal–Wallis test with multiple comparisons; Fig. 1a). In line with these results, the amount of fungal DNA extracted from infected leaves (7 dpi) was also significantly lower (Δ*hvmorc1* L3: 8.1-fold decrease; Δ*hvmorc6a* L9: 4.3-fold decrease; Δ*hvmorc6a* L16: 6.4-fold decrease; Δ*hvmorc1/6a* L4: 2.3-fold decrease; Δ*hvmorc1/6a* L5: 2.4-fold decrease; $\alpha = 0.05$, ANOVA with Tukey test; Fig. 1b, c). Consistent with the results of Galli et al. (2021), leaves of *hvmorc1/6a* double mutants were smaller compared to WT and single mutants (not shown), explaining the increased ratio between fungal and plant DNA detected by qPCR.

Compared to leaf-infecting pathogens, fewer studies focus on root pathogens due to the difficulty in observing the infection process. *F. graminearum* is not only a specialized pathogen of the aerial parts but can also cause tremendous damage by root rot (Lanoue et al. 2010; Kazan and Gardiner 2018). We addressed the question of whether KO of MORCs also leads to higher resistance to FRR. To this end, seedlings were dip-inoculated in *Fg* conidia, and infection was assessed by quantifying the fungal DNA by qPCR at 7 dpi. Significant reduction in fungal growth was observed in all mutants as compared to *Hv*WT (Δ*hvmorc1* L3: 2.1-fold decrease; Δ*hvmorc6a* L9: 1.9-fold decrease; Δ*hvmorc6a* L16: 1.7-fold decrease; Δ*hvmorc1/6a* L4: 1.5-fold decrease; Δ*hvmorc1/6a* L5: 1.4-fold decrease; Student's *t* test versus *Hv*WT and $\alpha = 0.05$, ANOVA with Tukey test; Fig. 2).

Discussion

Epigenetic mechanisms in plant defense

MORC proteins were first identified in mice as important regulators of spermatogenesis and genital development (Watson et al. 1998; Inoue et al. 1999). They are also widespread among plants, where they bear highly conserved ATP binding motifs to their counterparts in animal phyla (Langen et al. 2014; Dong et al. 2018; Xue et al. 2021). Accumulating structural and biochemical evidence suggests that both plant and animal MORCs share many similar functions related to disease resistance and epigenetic control (for review see Li et al. 2013; Koch et al. 2017; Xue et al. 2021). Epigenetic gene regulation is fundamental for genome integrity, gene expression and the repression of *TEs*. The plant-specific RdDM pathway achieves stable epigenetic modification via both de novo and maintenance of DNA and chromatin methylation (Lister et al. 2008; Law and Jacobsen 2010; Du et al. 2015; Matzke et al. 2015; Erdmann and Picard 2020). Several studies have shown that epigenetic regulation fine-tunes the trade-off between disease resistance, yield and fitness (Dowen et al. 2012; Deng et al. 2017; Kumar et al. 2021). Here, we show that the CRISPR/*Sp*Cas9-generated KO mutations in *HvMORC1* and *HvMORC6a* have an enhanced immune response to *Bipolaris* leaf spot blotch (Fig. 1a–c) and *Fusarium* root rot (Fig. 2). These results are consistent with previous reports showing that barley *MORC* mutants also have increased resistance to biotrophic (*Blumeria graminis*) and necrotrophic (*Fusarium graminearum*) fungal leaf pathogens (Langen et al. 2014; Kumar et al. 2018; Galli et al. 2021). Interestingly, the enhanced-defense phenotype of single versus double KO mutants was not additive. This finding is consistent with previous reports suggesting that *Hv*MORC1 and *Hv*MORC6a require each other's activity

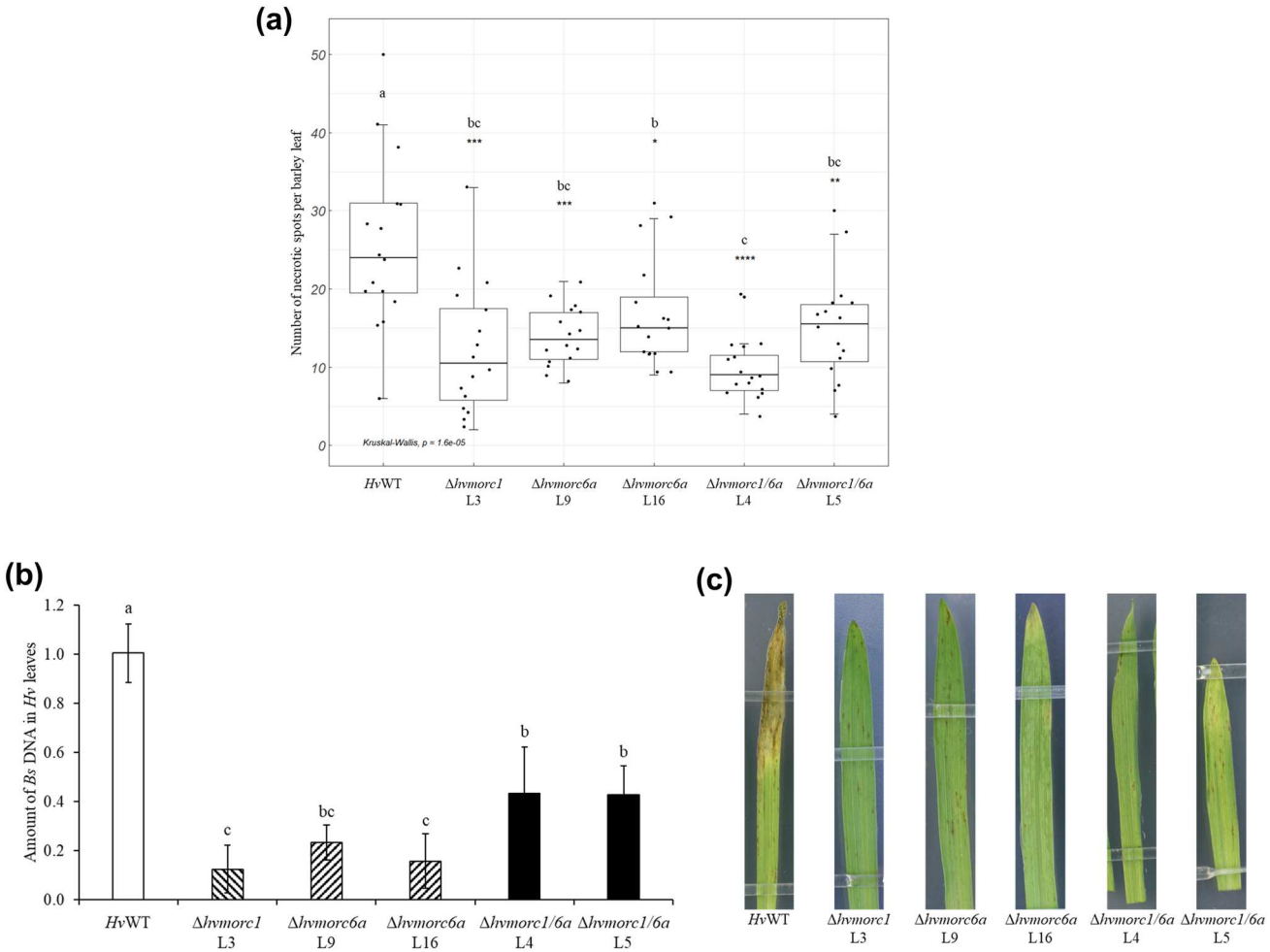


Fig. 1 Response of *Sp*Cas9-induced barley mutants against *Bipolaris sorokiniana* (*Bs*), the causal agent of spot blotch disease. **a** Single T3 mutants Δ*hvmorc1* L3, Δ*hvmorc6a* L9, Δ*hvmorc6a* L16 and T3 double mutants Δ*hvmorc1/6a* L4, Δ*hvmorc1/6a* L5 show increased resistance to the hemibiotrophic fungus *Bs* KN2 as revealed by reduced numbers of necrotic lesions. Shown is the average number of *Bs* necrotic lesions on leaves ($n=8$) at 5 dpi in two biological replicates. Comparisons between groups were performed via student's t test between *Hv*WT and mutants; asterisks represent statistical difference of the groups against *Hv*WT (Student's t test: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$). Comparisons between groups were performed via Kruskal–Wallis test with multiple comparisons. Letters represent statistical differences among all group means ($\alpha<0.05$). **b** Quantitative analysis of fungal infection as calculated as total amount of *Bs* DNA in barley leaves (7 dpi), based on the ratio of fungal *Glyceraldehyde-3-phosphate dehydrogenase* (*BsGPD*) calculated by qPCR. Bars represent the standard deviation (SD) of three technical repetitions; biological assay was repeated twice with similar results. Comparisons between groups were performed by ANOVA and Tukey's range test for multiple comparisons. Letters represent statistical differences among all group means ($\alpha<0.05$). **c** Leaf infection phenotypes after *Bs* spray inoculation (7 dpi)

for immune function because they physically interact as a prerequisite for their action (Galli et al. 2021).

Given that the defense mechanisms involved in resistance to biotrophic, hemibiotrophic and necrotrophic pathogens often function antagonistically due to the antagonism mode of action inherent to the defense pathways associated with the plant hormones salicylic acid and jasmonic acid (Glazebrook 2005; Klessig et al. 2018), our finding is relevant in the plant pathology field: 1. The involvement of MORC proteins in plant defense is independent of the known hormone-driven antagonistic defense mechanisms; 2. epigenetic regulation of plant defense has so far been neglected in breeding because the mechanism is still unexplored and very complex. 3. Breeding strategies with a focus on epigenetics could have the advantage that they may avoid the hormonal antagonism in plant defense and thus work on a broader type of resistance.

In conclusion, we hypothesize that KO of *HvMORC1* and *HvMORC6a* achieves higher immunity by weakening the recruitment of RdDM complexes to their site of action on barley DNA and chromatin. Plant *TEs* are silenced via the RdDM pathway, and methylation often extends to flanking genes (Cui and Cao 2014). *Hv*MORC1 and *Hv*MORC6a form distinct nucleo-cytoplasmic homo-/heteromers with

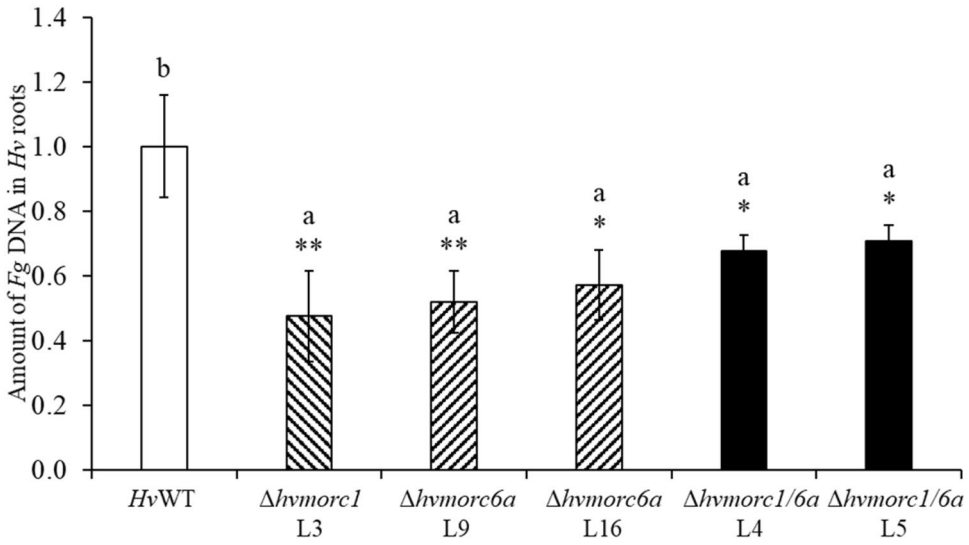


Fig. 2 Response of *Sp*Cas9-induced barley mutants against *Fusarium graminearum* (*Fg*), the causal agent of FRR. Single T3 mutants Δ*hvmorc1* L3, Δ*hvmorc6a* L9, Δ*hvmorc6a* L16 and T3 double mutants Δ*hvmorc1/6a* L4 and Δ*hvmorc1/6a* L5 show increased root resistance to *Fg* as revealed by quantification of total *Fg* DNA. Whole roots of 3-day-old seedlings were dip-inoculated with 30 mL solution of *Fg* conidia (15×10^4 conidia mL^{-1}) for 90 min. qPCR was used to measure the amount of *Fg* DNA at 7 dpi, calculated based on the ratio of fungal *Elongation factor-1 α* (*FgEF-1a*). Bars represent the SD of three technical repetitions; the biological assay was repeated twice with similar results. Asterisks represent statistical difference of the groups against *Hv*WT (Student's *t* test: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$)

other *Hv*MORCs and interact with components of the RdDM pathway (Galli et al. 2021). Our results are also consistent with the idea that in the absence of a pathogen barley MORCs interact with DNA-methylating proteins to repress *TEs* and favorite chromatin compaction of *TEs*` flanking regions, leading to the transcriptional repression of several genes, including *PRs* (Fig. 3). On the contrary, a compromised function of MORC proteins then explains higher disease resistance to various fungal pathogens (Fig. 1a–c and Fig. 2), constitutive higher *TEs* and *PRs* expression (even if no pathogen is present), and atypical plant growth (Kumar et al. 2018; Galli et al. 2021).

Importantly, we show that barley *MORC*-mediated epigenetic regulation fine-tunes disease resistance to a very broad range of fungal pathogens. Previous reports already showed a trade-off between MORC-dependent plant defense and

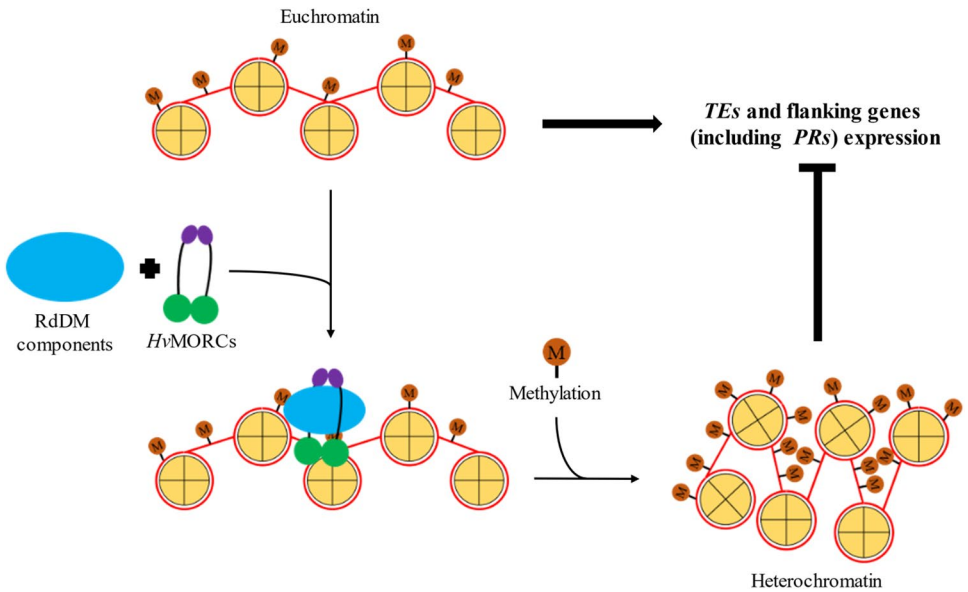


Fig. 3 Simplified working model hypothesis of the role of barley MORC proteins in the regulation of *TEs*, defense genes and disease resistance. MORC protein complexes interact directly with other components of the RdDM pathway to initiate or maintain inhibitory methylation marks, leading to the formation of heterochromatin, silencing of *TEs* and potentially many defense genes in the environment (reduced disease resistance). The absence of MORCs then leads to relaxed chromatin, de-repression of various silenced *TEs* and many flanking genes, including *PRs* (increased disease resistance)

growth (Galli et al. 2021), thus revealing possible gene candidates in a single cluster that have evolved opposing functions in disease resistance and plant fitness. In the future, the discovery of the effects of MORC on these important agronomic traits and the importance of the RdDM pathway in pathological processes may help to develop new breeding strategies for higher yielding and more resistant barley varieties.

Acknowledgements This work was funded by the Deutsche Forschungsgemeinschaft (DFG) to KHK (RU5116). M. G. was supported by German Academic Exchange Service (DAAD).

Authors contribution MG and K-HK wrote the manuscript; K-HK, JI and MG designed the study; SH, DI and MC prepared material for the experiments; MG, SH and MC conducted the experiments; MG, JI and K-HK analyzed all data and drafted the figures. All authors commented and reviewed the final manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL.

Availability of data and material All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare no competing financial interests.

Consent of publication All authors declare consent of publication.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Balmas V, Scherm B, Marcello A, Beyer M, Hoffmann L, Migheli Q, Pasquali M (2015) *Fusarium* species and chemotypes associated with fusarium head blight and fusarium root rot on wheat in Sardinia. Plant Pathol 64(4):972–979

Brabbs TR, He Z, Hogg K, Kamenski A, Li Y, Paszkiewicz KH, Moore KA, O'Toole P, Graham IA, Jones L (2013) The stochastic silencing phenotype of Arabidopsis *morc6* mutants reveals a role in efficient RNA-directed DNA methylation. Plant J 75(5):836–846

Cui X, Cao X (2014) Epigenetic regulation and functional exaptation of transposable elements in higher plants. Curr Opin Plant Biol 21:83–88

Deng Y, Zhai K, Xie Z, Yang D, Zhu X, Liu J, Wang X, Qin P, Yang Y, Zhang G, Li Q (2017) Epigenetic regulation of antagonistic receptors confers rice blast resistance with yield balance. Science 355(6328):962–965

Dong W, Vannozzi A, Chen F, Hu Y, Chen Z, Zhang L (2018) MORC domain definition and evolutionary analysis of the MORC gene family in green plants. Genome Biol Evol 10(7):1730–1744

Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, Dixon JE, Ecker JR (2012) Widespread dynamic DNA methylation in response to biotic stress. Proc Natl Acad Sci 109(32):2183–2191

Du J, Johnson LM, Jacobsen SE, Patel DJ (2015) DNA methylation pathways and their crosstalk with histone methylation. Nat Rev Mol Cell Biol 16(9):519–532

Erdmann RM, Picard CL (2020) RNA-directed DNA methylation. PLoS Genet 16(10):1009034

Gaffar FY, Imani J, Karlovsky P, Koch A, Kogel KH (2019) Different components of the RNA interference machinery are required for conidiation, ascosporogenesis, virulence, deoxynivalenol production, and fungal inhibition by exogenous double-stranded RNA in the head blight pathogen *Fusarium graminearum*. Front Microbiol 10:1662

Galli M, Martiny E, Imani J, Kumar N, Koch A, Steinbrenner J, Kogel KH (2021) CRISPR/*Sp*Cas9-mediated double knockout of barley Microrchidia MORC1 and MORC6a reveals their strong involvement in plant immunity, transcriptional gene silencing and plant growth. Plant Biotechnol J

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43:205–227

Gupta PK, Chand R, Vasistha NK, Pandey SP, Kumar U, Mishra VK, Joshi AK (2018) Spot blotch disease of wheat: the current status of research on genetics and breeding. Plant Pathol 67(3):508–531

Harris CJ, Husmann D, Liu W, Kasmi FE, Wang H, Papikian A, Pastor WA, Moissiard G, Vashisht AA, Dangl JL, Wohlschlegel JA (2016) Arabidopsis AtMORC4 and AtMORC7 form nuclear bodies and repress a large number of protein-coding genes. PLoS Genet 12(5):1005998

Hollaway GJ, Evans ML, Wallwork H, Dyson CB, McKay AC (2013) Yield loss in cereals, caused by *Fusarium culmorum* and *F. pseudograminearum*, is related to fungal DNA in soil prior to planting, rainfall, and cereal type. Plant Disease 97(7):977–982

Inoue N, Hess KD, Moreadith RW, Richardson LL, Handel MA, Watson M, Zinn AR (1999) New gene family defined by MORC, a nuclear protein required for mouse spermatogenesis. Hum Mol Genet 8(7):1201–1207

Jansen C, Von Wettstein D, Schäfer W, Kogel KH, Felk A, Maier FJ (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proc Natl Acad Sci 102(46):16892–16897

Jing Y, Sun H, Yuan W, Wang Y, Li Q, Liu Y, Li Y, Qian W (2016) SUVH2 and SUVH9 couple two essential steps for transcriptional gene silencing in Arabidopsis. Mol Plant 9(8):1156–1167

Kang HG, Choi HW, Von Einem S, Manosalva P, Ehlers K, Liu PP, Buxa SV, Moreau M, Mang HG, Kachroo P, Kogel KH (2012) CRT1 is a nuclear-translocated MORC endonuclease that participates in multiple levels of plant immunity. Nat Commun 3(1):1–11

Kang HG, Kuhl JC, Kachroo P, Klessig DF (2008) CRT1, an Arabidopsis ATPase that interacts with diverse resistance proteins and modulates disease resistance to turnip crinkle virus. Cell Host Microbe 3(1):48–57

Kang HG, Oh CS, Sato M, Katagiri F, Glazebrook J, Takahashi H, Kachroo P, Martin GB, Klessig DF (2010) Endosome-associated CRT1 functions early in resistance gene–mediated defense signaling in Arabidopsis and tobacco. Plant Cell 22(3):918–936

Kazan K, Gardiner DM (2018) Fusarium crown rot caused by *Fusarium pseudograminearum* in cereal crops: recent progress and future prospects. Mol Plant Pathol 19(7):1547–1562

Klessig DF, Choi HW, Dempsey DMA (2018) Systemic acquired resistance and salicylic acid: past, present, and future. Mol Plant Microbe Interact 31(9):871–888

Koch A, Kang HG, Steinbrenner J, Dempsey DMA, Klessig DF, Kogel KH (2017) MORC proteins: novel players in plant and animal health. Front Plant Sci 8:1720

Kumar J, Hückelhoven R, Beckhove U, Nagarajan S, Kogel KH (2001) A compromised Mlo pathway affects the response of barley to the necrotrophic fungus *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) and its toxins. Phytopathology 91(2):127–133

Kumar J, Schäfer P, Hückelhoven R, Langen G, Baltruschat H, Stein E, Nagarajan S, Kogel KH (2002) *Bipolaris sorokiniana*, a cereal pathogen of global concern: cytological and molecular approaches towards better control. Mol Plant Pathol 3(4):185–195

Kumar N, Galli M, Ordon J, Stuttmann J, Kogel K-H, Imani J (2018) Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system. Plant Biotechnol J 16(11):1892–1903. https://doi.org/10.1111/pbi.12924

Kumar N, Galli M, Dempsey DM, Imani J, Moebus A, Kogel KH (2021) NPR1 is required for root colonization and the establishment of a mutualistic symbiosis between the beneficial bacterium *Rhizobium radiobacter* and barley. Environ Microbiol 23(4):2102–2115

Langen G, von Einem S, Koch A, Imani J, Pai SB, Manohar M, Ehlers K, Choi HW, Claar M, Schmidt R, Mang HG (2014) The compromised recognition of turnip crinkle virus1 subfamily of microrchidia ATPases regulates disease resistance in barley to biotrophic and necrotrophic pathogens. Plant Physiol 164(2):866–878

Lanoue A, Burlat V, Henkes GJ, Koch I, Schurr U, Röse US (2010) *De novo* biosynthesis of defense root exudates in response to *Fusarium* attack in barley. New Phytol 185(2):577–588

Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet 11(3):204–220

Li DQ, Nair SS, Kumar R (2013) The MORC family: new epigenetic regulators of transcription and DNA damage response. Epigenetics 8(7):685–693

Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH, Ecker JR (2008) Highly integrated single-base resolution maps of the epigenome in Arabidopsis. Cell 133(3):523–536

Liu ZW, Shao CR, Zhang CJ, Zhou JX, Zhang SW, Li L, Chen S, Huang HW, Cai T, He XJ (2014) The SET domain proteins SUVH2 and SUVH9 are required for Pol V occupancy at RNA-directed DNA methylation loci. PLoS Genet 10(1):1003948

Liu ZW, Zhou JX, Huang HW, Li YQ, Shao CR, Li L, Cai T, Chen S, He XJ (2016) Two components of the RNA-directed DNA methylation pathway associate with MORC6 and silence loci targeted by MORC6 in Arabidopsis. PLoS Genet 12(5):1006026

Lorković ZJ, Naumann U, Matzke AJ, Matzke M (2012) Involvement of a GHKL ATPase in RNA-directed DNA methylation in *Arabidopsis thaliana*. Curr Biol 22(10):933–938

Manosalva P, Manohar M, Kogel KH, Kang HG, Klessig DF (2015) The GHKL ATPase MORC1 modulates species-specific plant immunity in *Solanaceae*. Mol Plant Microbe Interact 28(8):927–942

Matzke MA, Kanno T, Matzke AJ (2015) RNA-directed DNA methylation: the evolution of a complex epigenetic pathway in flowering plants. Annu Rev Plant Biol 66:243–267

Moissiard G, Bischof S, Husmann D, Pastor WA, Hale CJ, Yen L, Stroud H, Papikian A, Vashisht AA, Wohlschlegel JA, Jacobsen SE (2014) Transcriptional gene silencing by Arabidopsis microrchidia homologues involves the formation of heteromers. Proc Natl Acad Sci 111(20):7474–7479

Moissiard G, Cokus SJ, Cary J, Feng S, Billi AC, Stroud H, Husmann D, Zhan Y, Lajoie BR, McCord RP, Hale CJ (2012) MORC family ATPases required for heterochromatin condensation and gene silencing. Science 336(6087):1448–1451

Singh PK, Zhang Y, He X, Singh RP, Chand R, Mishra VK, Malaker PK, Reza MA, Rahman MM, Islam R, Chowdhury AK (2015) Development and characterization of the 4th CSISA-spot blotch nursery of bread wheat. Eur J Plant Pathol 143(3):595–605

Watson ML, Zinn AR, Inoue N, Hess KD, Cobb J, Handel MA, Halaban R, Duchene CC, Albright GM, Moreadith RW (1998) Identification of morc (microrchidia), a mutation that results in arrest of spermatogenesis at an early meiotic stage in the mouse. Proc Natl Acad Sci 95(24):14361–14366

Xue Y, Zhong Z, Harris CJ, Gallego-Bartolomé J, Wang M, Picard C, Cao X, Hua S, Kwok I, Feng S, Jami-Alahmadi Y (2021) Arabidopsis MORC proteins function in the efficient establishment of RNA directed DNA methylation. Nat Commun 12(1):1–13

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.