CRISPR/SpCas9-mediated double knockout of barley Microrchidia MORC1 and MORC6a reveals their strong involvement in plant immunity, transcriptional gene silencing and plant growth

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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 the MORCs and their potential interactors in regulating plant immunity via analysis of CRISPR/SpCas9-mediated single and double knockout (dKO) mutants, hvmore1 (previously generated and characterized by our group), hvmore6a, and hvmore1/6a. For generation of hvmore1/6a, we utilized two different strategies: (i) successive Agrobacterium-mediated transformation of homozygous single mutants, hvmore1 and hvmore6a CRISPR/SpCas9 constructs. Total mutation efficiency in transformed homozygous single mutants ranged from 80 to 90%, while upon simultaneous transformation, SpCas9-induced mutation in both HvMORC1 and HvMORC6a genes was observed in 58% of T0 plants. Subsequent infection assays showed that HvMORC6a covers a key role in resistance to biotrophic (Blumeria graminis) and necrotrophic (Fusarium graminearum) plant pathogenic fungi, where the dKO hvmore1/6a 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 HvMORC1 and HvMORC6a form distinct nucleocyttoplasmic homo- and heteromers with other HvMORCs 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.

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

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). AtMORC proteins, especially AtMORC1, AtMORC2, and AtMORC6 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 as acting as positive modulators of immunity (Bordiye et al., 2016; Harris et al., 2016; Kang et al., 2008, 2012). Furthermore, in response to microbial pathogens or their microbe-associated patterns (MAMPs), AtMORC1 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., HvMORC1 and HvMORC2), potato (Solanum
tuberosum viz. StMORC1), tomato (Solanum lycopersicum, viz., SMORC1), and tobacco (Nicotiana benthamiana, viz., NbMORC1) 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 AtMORC proteins and two to human HsMORC1 to HsMORC4. The Arabidopsis-like group comprises of HvMORC1 [HORVU7Hr1G083280.15], HvMORC2 [HORVU1Hr1G006770.1], HvMORC6a [HORVU3Hr1G046280.3], HvMORC6b [HORVU3Hr1G078330.4], and HvMORC7 [HORVU2Hr1G066650.2], while HvMORCCW1 [HORVU1Hr1G080470.1] and HvMORCCW2 [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/SpCas9) to generate hvmorc6a KO mutants and hvmorc6a/hvmorc1 KO dKO mutants to further explore the role of HvMORC1 and HvMORC6a in plant immunity. HvMORC6a shares 58.2% aa similarity with AtMORC6 and 55.0% with AtMORC1. AtMORC6 was reported to function in the condensation of pericentromeric heterochromatin, thereby facilitating transcriptional silencing. Furthermore, it has been hypothesized that AtMORC1 and AtMORC2 form small nuclear heterodimers with AtMORC6, 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 HvMORC6a, like HvMORC1, is involved in disease resistance against biotrophic and necrotrophic pathogens. We show that HvMORC1 and HvMORC6a form nucleocytoplasmic homo-/heteromers, interact with components of the epigenetic gene silencing machinery, and function as repressors of transposable elements (TE).

**Results**

CRISPR/SpCas9-mediated generation of KO hvmorc6a and dKO hvmorc1/6a mutants

In barley, HvMORC1 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 HvMORC6a in modulating plant immunity and genome stabilization is inadequately understood. To assess the function of the HvMORC6a protein, we generated hvmorc6a and hvmorc1/6a mutants using CRISPR/SpCas9. 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 HvMORC6a 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). SpCas9 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 SpCas9, we generated dKO barley mutants, 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/SpCas9 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 SpCas9-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, SpCas9-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/SpCas9-generated hvmorc6a and hvmorc1/6a mutants display no off-target effects in other barley MORCs

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): Δhvmorc6a-L9 and L16 carrying a 1bp insertion and 25bp deletion respectively in HVMORC6a; and Δhvmorc1/6a-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 90 Matteo Galli et al.
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 mediated mRNA decay pathway which is involved in degradation [45x320]ª immunity in a species-specific manner (for details see Koch [45x254]et al [45x753], 2021).

HvMORC6a 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 assayed the resistance of hvmorC6a and hvmorC1/6a plants to the biotrophic powdewild 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 (Δhmorc1-L3), hvmorC6a (Δhmorc6a-L9 and Δhmorc1/6a-L16), and hvmorC1/6a (Δhmorc1/6a-L4 and Δhmorc1/6a-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 (Δhmorc1/6a-L4: 55% and Δhmorc1/6a-L5: 50%), while single mutant lines retain a more moderate resistance (Δhmorc1-L3: 77%, Δhmorc6a-L9: 79%, Δhmorc6a-L16: 72%) (Figure 2a).

Defence pathways involved in resistance to biotrophic and necrotrophic pathogens often function antagonistically (Glazebrook, 2005; Jaroch 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 × 10⁶ 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 (Δhmorc6a-L9: 84%, Δhmorc6a-L16: 82%, and Δhmorc1/6a-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: AM403313.1) 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,
and non-LTR retrotransposons. Analysing transcription profiles of long terminal repeat (LTR) elements in Hv, we assessed the effect of HvMORC1 hypothesis that HvMORC6a mutant, Moissiard et al., 2012). As observed in Arabidopsis ATMORC1 mutant, HvMORC1 plants showed derepression of TEs, raising the hypothesis that HvMORC1 contributes to genome stabilization (Kumar et al., 2018; Langen et al., 2014). To prove this further, we assessed the effect of HvMORC6a 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 hvmorc6a-L9 (fold increase, HvInga: 1.7, hvmorc6a-L9: 2.1, hvmorc6a-L16: 2.1, hvmorc6a-L4: 3.2, hvmorc6a-L5: 3.5; HvRLG-S: hvmorc6a-L3: 3.7, hvmorc6a-L4: 6.4, hvmorc6a-L9: 6.5, hvmorc6a-L7: 10.8, hvmorc6a-L5: 11.5; HvVagabond: hvmorc6a-L3: 1.5, hvmorc6a-L9: 1.6, hvmorc6a-L16: 1.3, hvmorc6a-L4: 21, hvmorc6a-L5: 18.4; HvBianca: hvmorc6a-L3: 3.6, hvmorc6a-L9: 3.8, hvmorc6a-L4: 3.2, hvmorc6a-L5: 5.1; HvVagabond: hvmorc6a-L3: 1.1, hvmorc6a-L9: 1.8, hvmorc6a-L16: 2, hvmorc6a-L4: 3.2, hvmorc6a-L5: 2; Figure 4).

HvMORC6a is involved in TGS-mediated transposable element silencing

AtMORC1 and AtMORC6 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 HvMORC1 contributes to genome stabilization (Kumar et al., 2018; Langen et al., 2014). To prove this further, we assessed the effect of HvMORC6a 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 hvmorc6a-L9 (fold increase, HvInga: 1.7, hvmorc6a-L9: 2.1, hvmorc6a-L16: 2.1, hvmorc6a-L4: 3.2, hvmorc6a-L5: 3.5; HvRLG-S: hvmorc6a-L3: 3.7, hvmorc6a-L4: 6.4, hvmorc6a-L9: 6.5, hvmorc6a-L7: 10.8, hvmorc6a-L5: 11.5; HvVagabond: hvmorc6a-L3: 1.5, hvmorc6a-L9: 1.6, hvmorc6a-L16: 1.3, hvmorc6a-L4: 21, hvmorc6a-L5: 18.4; HvBianca: hvmorc6a-L3: 3.6, hvmorc6a-L9: 3.8, hvmorc6a-L4: 3.2, hvmorc6a-L5: 5.1; HvVagabond: hvmorc6a-L3: 1.1, hvmorc6a-L9: 1.8, hvmorc6a-L16: 2, hvmorc6a-L4: 3.2, hvmorc6a-L5: 2; Figure 4).

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Moreover, HvMORC2 and HvMORC6a did not form homomeric complexes in our Y2H assays, but HvMORC2 interacted with HvMORC1 and HvMORC6a (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 HvMORC1, HvMORC2, and HvMORC6a 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 HvMORC2 (Figure 5b, right panel). Surprisingly, and in contrast to AtMORC6, HvMORC6a did not show any homomorphic interaction in either the Y2H assay or the BiFC assay (Figure 5a-b). Furthermore, since it has been hypothesized that AtMORC1 and AtMORC2 form small nuclear heterodimers with AtMORC6, we also transiently expressed chimeric GFP::HvMORC1 and GFP::HvMORC6a 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-Vird2NL5, serving as a nuclear marker to allow the observation of the nucleus in protoplasts (Citovsky et al., 2006; Lin et al., 2018). Both HvMORC1 and HvMORC6a showed nucleocytoplasmic localization in the barley WT background (Figure 5b, left panel). Interestingly, in the hvmorc6a background, HvMORC1 localized almost exclusively in the cytoplasm while HvMORC6a remained nucleocytoplasmic.

HvMORCs interact with components of the RdDM machinery in vivo

AtMORC1, AtMORC2, and AtMORC6 were identified as components of the RdDM pathway by their interaction with SUPPRESSOR OF VARIEGATION 3-9 (SUVVAR) 3-9 homologs SUVH2 and/or SUVH9. These two proteins are canonical components of RdDM that interact directly with DEFECTIVE IN MERISTEM...
Therefore, we identified orthologs of AtMORC interactors in barley to further investigate the involvement of HvMORCs in RdDM (Table S2). HvDMS3, HvSUVH9, the double-stranded RNA-binding protein INVOLVED IN DE NOVO 2 (HvIDN2) and the SWITCH SUBUNIT 3C (HvSWI3C) component of the chromatin-remodelling complex SWITCH/SUCROSE NON-FERMENTABLE (SW/SNF) were cloned and tested in a Y2H assay against HvMORC1, HvMORC2, and HvMORC6a. We found that HvMORC1 interacts with HvDN2 (Figure 6a), in contrast to previous results on AtMORCs, where Y2H showed no interaction between IDN2 and AtMORC1 or AtMORC2 (Liu et al., 2016). Consistent with previous Y2H results, revealing an interaction of AtSUVH9 with AtMORC1, AtMORC2, and AtMORC6 (Liu et al., 2014), all three tested HvMORCs interacted with HvSUVH9 (Figure 6a). Surprisingly, none of the tested HvMORCs showed any interaction with HvSWI3C (Figure 6a), which is inconsistent with what was found in Arabidopsis (Jing et al., 2016). In addition, we did not detect interactions between HvDMS3 with any of the tested HvMORCs (Figure 6a). However, this might be consistent with what was reported for AtMORCs (Jing et al., 2016; Liu et al., 2014, 2016; Moissiard et al., 2014), since AtMORC6 interaction with AtDMS3 was only shown once in an in vitro pull-down experiment (Lorković et al., 2012) and in vivo SILENCING 3 (DMS3; Liu et al., 2014, 2016; Jing et al., 2016).
Figure 6 Localization of some barley orthologs of the RdDM pathway and their interactions with barley MORCs. (a) Y2H screen for possible interactions between HvMORCs and HvRdDM 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 HvRdDM 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-HvRdDMS were C-terminally fused to GFP, and pBiFP2-HvMORCs and pBiFP3-HvRdDM 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).
immunoprecipitation experiments failed to detect the interaction of AtDM53 with AtMORC6, 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 HvMORC 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 HvSUVH9 with HvMORC1, HvMORC2, and HvMORC6a, 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, AtMORC6 was shown to interact with AtIDN2 (Jing et al., 2016). We detected an interaction between HvMORC6a and HvIDN2 in the nucleus of N. benthamiana cells (Figure 6b right panel, Figure S5a). The interaction between either HvMORC1 or HvMORC2 with HvIDN2 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 HvMORC1, HvMORC2 or HvMORC6a with HvSWI3C 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 HvMORCs with HvDM53 in planta (Figure S5b). Since AtMORC6 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 HvSWI3C, HvIDN2, HvSUVH9, and HvDM53 in barley WT and hvMORC6a background (Figure 6b, left panel; Figure S5b). CLSM of barley WT mesophyll protoplasts revealed that HvSWI3C and HvSUVH9 were exclusively localized to the nucleus, HvIDN2 and HvDM53 showed a cytoplasmic and nuclear-cytoplasmic localization, respectively (Figure 6b left panel, Figure S5b left panel). In hvMORC6a background, HvSWI3C, HvSUVH9, and HvIDN2 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 HvDM53 (Figure S5b left panel).

HvMORC6a 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 HvMORC1 and HvMORC6a 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 Δhvmorc1-L3 single mutants did show aberrant growth compared with WT (13% reduction of shoots and roots), Δhvmorc6a-L9 and Δhvmorc1/6a-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/SpCas9-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; Lavrenson 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 (Po) III-dependent U3 small nuclear RNA promoter (GenBank: CAJX01195286.1) for efficient sgRNA expression and KO of HvMORC1 (Kumar et al., 2018). Here we show that a HvU3-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 HvU3 promoter is suitable to drive sgRNA expression in the type II CRISPR/SpCas9 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, SpCas9 induced a mutation in both HvMORC1 and HvMORC6a in 58% of T0 generation plants. Expression analyses of MORC genes confirmed that the CRISPR/SpCas9 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/SpCas9 genome editing for analysing plant gene function in a multigene family such as that of barley MORCs.

HvMORC6a 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/SpCas9-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). AtMORC6 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
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 AtMORC1 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 AtMORC1 and AtMORC6 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 AtMORC1 and/or AtMORC6 are involved in both gene silencing and gene induction. It is likely that in barley, interaction of HvMORC1 and HvMORC6a 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 hvmorcr1/6a 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 HvMORC1 in hvmorcr6a 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 homo- and in addition, heteromers with MORC6 (Liu et al., 2014; Moissiard et al., 2014). Using Y2H and BiFC assays, we could detect heteromerization of HvMORC6a with HvMORC1 and with
HMORC2; and we confirmed complex formation of HMORC1 with HMORC2 as found in Arabidopsis. Unlike in Arabidopsis, we could not find homomerization of HMORC6a, though we could confirm homomerization of HMORC1 and heteromerization of HMORC1 with HMORC2. HMORC1 and HMORC2 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 HMORC1/6a and HMORC2/6a was mainly found in the nucleus of N. benthamiana plants (Figure 5b right panel, Figure S4). HMORC1 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 HMORC1 and its homolog HMORC2 also reside in the cytoplasm of barley cells. On the other hand, our data suggest that HMORC1 and HMORC6a 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 HMORC proteins, we tested the interactions of barley MORC family members with selected barley orthologs of the RdDM pathway. In Arabidopsis, AtSUVH9 together with AtMORC6 and AtSUVH2 regulates silencing of some TEs (Liu et al., 2016). In addition, the SWISNF chromatin remodelling complex components SWI3B, SWI3C, and SWI3D, together with IDN2, interact with AtMORC6 to mediate TGS at some AtMORC6-specific loci (Liu et al., 2016). It was suggested that AtMORC proteins act as adaptors to recruit RNA Polymerase V, in conjunction with AtSUVH2 and AtSUVH9 to facilitate the production of long non-coding RNAs (IncRNAs) to promote DNA methylation (Jing et al., 2016; Liu et al., 2014, 2016). Furthermore, it was proposed that AtMORC1, AtMORC2 and/or AtMORC6 act together with the SWISNF 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 HvMORC1, HvMORC2, and HvMORC6a with HvIDN2, HvSUVH9, and HvSWI3C 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 HvMORC-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 (Alzhohairy 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 HMORC6a 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 mm² 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 cm² 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 × 10⁵ 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 (HvUbiquitin), respectively (Table S1).
Generation of CRISPR/SpCas9 constructs and plant transformation

Twenty nucleotides (nt) target sequence present immediately adjacent to a Protoscaler Adjacent Motif (PAM) was selected using CRISPR sgRNA design online tool (https://fatur.bio/eCommerce/ca-s9Input) 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. GTACGCGTTCAGATCCTGCCGCGGGGG was selected, and sgRNA was assembled and cloned into CRISPR/SpCas9 binary destination vector, as described (Kumar et al., 2018). The CRISPR/SpCas9 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 (hvMorC6a/hvMorC16a), co-knockout of the hvMorC6a and hvMorC16a genes was obtained using a mixture of two Agrobacterium cultures which contained hvMorC6a- and hvMorC16a-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 5′ and 3′ 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 pBb 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 fluorescence 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 (MATa), with genotype: leu2-3,112 trp1-901 his3-200 ura3-52 galδΔ gal180δ GAL2-2ADE2 lys2:: GAL1-HIS3 MET2::GAL7-lacZ cyh2 (Dreze et al., 2010) were transformed with CDS-containing pAD and pBb (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, pBb 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-stere 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-VE (empty vector) and DB-VE 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 soya, 5 g sucrose, 0.5 g MgCl₂ and 20 g agar) complemented 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 (OD₆₀₀) of 0.5, while culture harbouring the silencing suppressor p19 protein of tomato bushy stunt virus (Chen et al., 2011) construct was set to OD₆₀₀ 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 mM 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 mM mannitol, 15 mM MgCl₂) to a final of 5 × 10⁵ protoplasts/ml. 200 µL (1 × 10⁵ 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–CaCl₂. 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 mM 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,
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 a 1.0 unit for both protoplasts and leaf cells. Images for CLSM with nuclear marker were taken in sequential mode, while BFC 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.

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**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. and J.K. 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**

Abrohany, 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., Drewseker, S., Camilleri, C., Amiour, N., Parcy, F. et al. (2008) Arabidopsis TONNELAUI 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., Chugurankar, S., Grego, T., Kandasamy, S., Mitchell, A., Naka, 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., Yam, J.C., Bonnard, A.C., Cho, 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., Lennens, I., Yu, H., Sahulé, J.M. et al. (2009) An experimentally derived confidence score for protein–protein interactions. Nat. Methods. 6, 91–97.

Chen, Q., He, J., Phoolcharoen, W. and Mason, H.S. (2011) Geminiviral vectors for transformation of rice, maize and potato. Plant Methods. 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 interaction mapping. Methods Enzymol. 470, 281–315.

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

Gasparis, S., Kała, M., Przyborowski, M., Jżycki, Ł., 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 and Genome Research. 110, 229–241.

Harris, C.J., Husmann, D., Liu, W., Kasmie, F.E., Wang, H., Papkian, 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-Humanej, J., Deulearan, L.C., Starker, C.G., Voytas, D.F. and Brinch-Pedersen, H. (2017) Evaluation of the mature grain phylase candidate HvPAPhy1 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.
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., Abhiraman, 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 trichodene synthase gene disrupted *Fusarium graminearum*. *Proc. Natl Acad. Sci. USA*, 102, 16892–16897.

Jarosch, B., Kogel, K.H. and Safffrath, 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, I.C., Kachroo, P. and Klessig, D.F. (2008) CR1, an Arabidopsis APATase 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) Endosomase-associated CR1T 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., Buva, S.X. et al. (2012) CR1T is a nuclear-translocated MORC endonuclease that participates in multiple levels of plant immunity. *Nat. Commun.* 3, 1–11.

Kapusi, E., Corcueria-Gómez, M., Melnik, S. and Stoger, E. (2017) Heritable genomic fragment deletions and small indels in the putative ENGIase 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, E., 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., Steibnerbner, 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., Martin, N., Szabados, L., Hrouda, M., Bachmair, A. and Schell, J. (1994) Specialized vectors for gene tagging and expression studies. *Plant Molec. 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., Manosalva, M., Ehlers, K. et al. (2014) The compromised recognition of turnip crinkle virus1 subfamily of microchordia ATPases regulates disease resistance in barley to biotrophic and necrotrophic pathogens. *Plant physiology* 164(2), 866–878.

Lawrenson, T., Shorrinola, O., Stacey, N., Li, C., Østgaard, L., Patron, N., Uauy, C. et al. (2015) Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nucleases. *Genome Biol.* 16, 1–13.
Zhu, H., Li, C. and Gao, C. (2020) Applications of CRISPR-Cas9-enabled genome editing. *Ann. Bot.* **126**, 929–942.

Zhu, H., Li, C. and Gao, C. (2020) Applications of CRISPR-Cas9 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.

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**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, SpCas9-based *HvMORC6a* gene editing. pCMV35S, CaMV 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 *hvmorc6a*-sgRNA and *hvmorc6a*-sgRNA (20 nt, underlined) with PAM sequence (grey highlighted) in *HvMORC1* and *HvMORC6a* protein architecture, respectively; hallmark domains (HATPase_C, S5, and CC) are highlighted in bounding boxes; thunder indicates precise location of SpCas9 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/SpCas9 efficiency and cleavage sites in *hvmorc6a* barley mutant lines. (a) Schematic summary of the transformation efficiency in SpCas9-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** SpCas9-induced frame-shift mutations in *HvMORC1* and *HvMORC6a*. (a) Homozygous mutated lines used in this study: *hvmorc1* (∆*hvmorc1-L3*), *hvmorc6a* (∆*hvmorc6a-L9* and L16), and *hvmorc1/6a* (∆*hvmorc1/6a-L4* and L5) T3 homozygous mutants. (b) CRISPR/SpCas9 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 *HvMORCs*. *HvMORC* combinations were detected in lower epidermal cells of tobacco after 48 h (left panel). pBiFP2-*HvMORCs* and pBiFP3-*HvMORCs* 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::*HvDMS3* localization and YFP signals of homo-/heteromerization of different *HvMORCs* 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 *HvDMS3* 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.