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SHORT COMMUNICATION

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

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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

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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

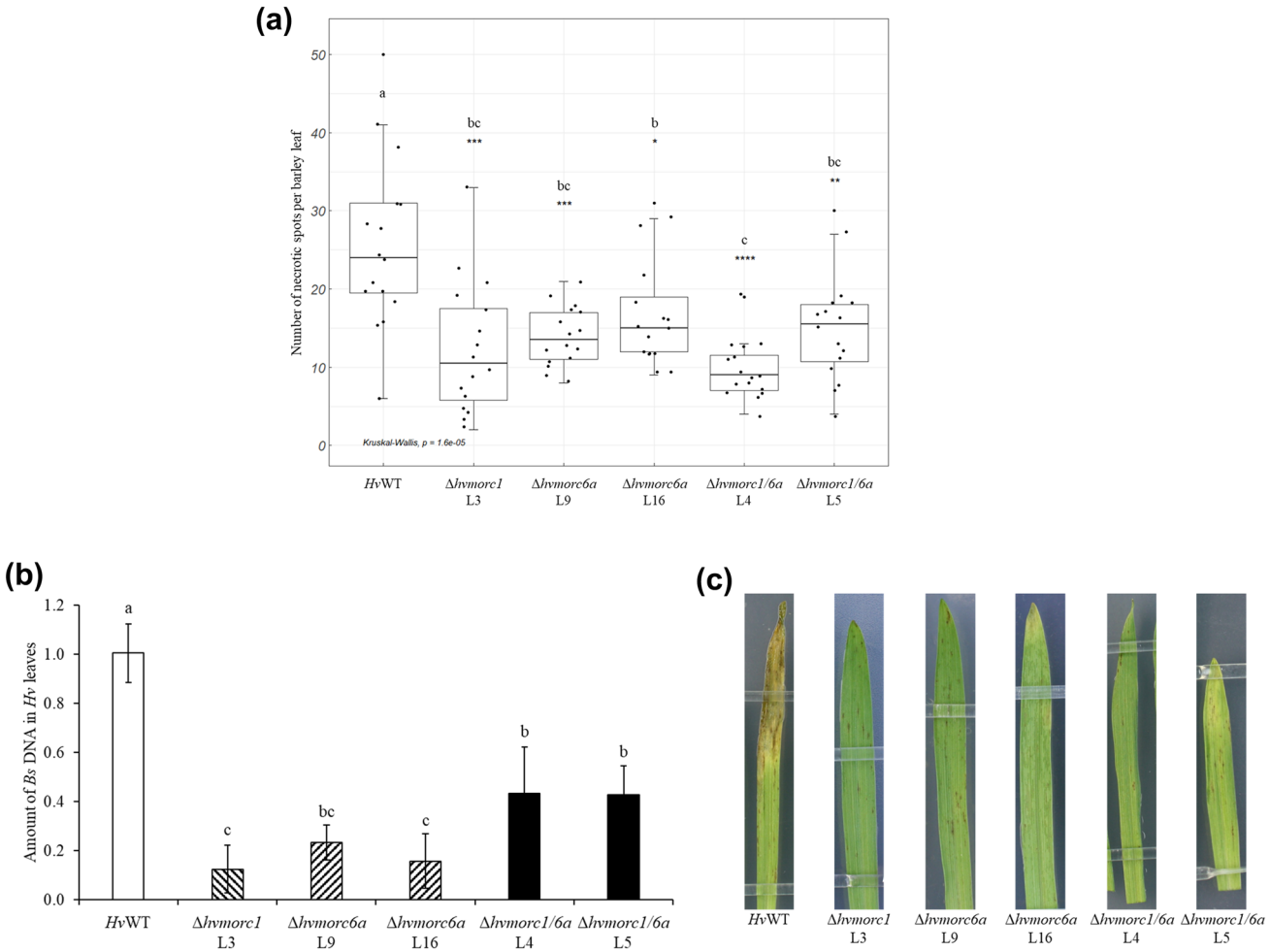

Fig. 1 Response of *Sp*Cas9-induced barley mutants against *Bipolaris sorokiniana* (*Bs*), the causal agent of spot blotch disease. **a** Single T3 mutants Δ*hvmorc1* L3, Δ*hvmorc6a* L9, Δ*hvmorc6a* L16 and T3 double mutants Δ*hvmorc1/6a* L4, Δ*hvmorc1/6a* L5 show increased resistance to the hemibiotrophic 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

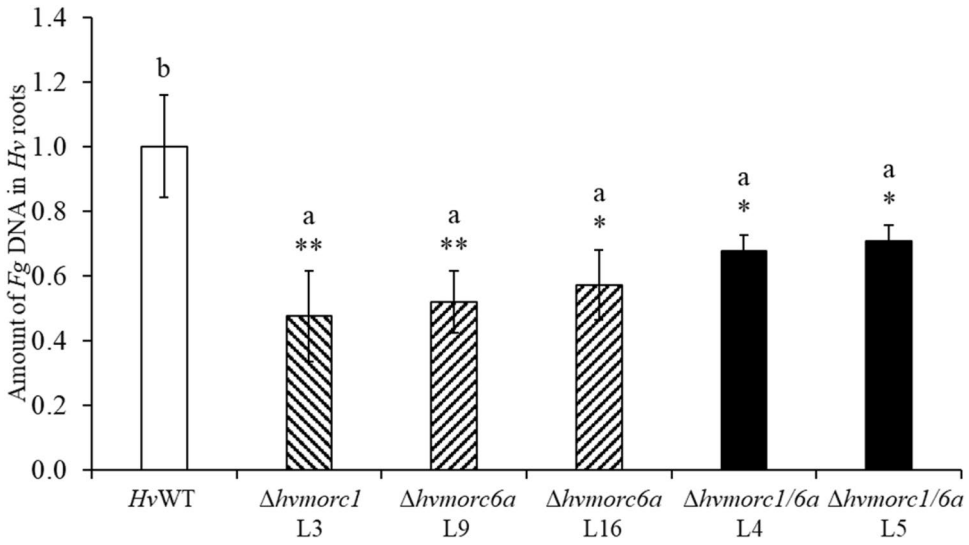

Fig. 2 Response of *Sp*Cas9-induced barley mutants against *Fusarium graminearum* (*Fg*), the causal agent of FRR. Single T3 mutants Δ*hvmorc1* L3, Δ*hvmorc6a* L9, Δ*hvmorc6a* L16 and T3 double mutants Δ*hvmorc1/6a* L4 and Δ*hvmorc1/6a* L5 show increased root resistance to *Fg* as revealed by quantification 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

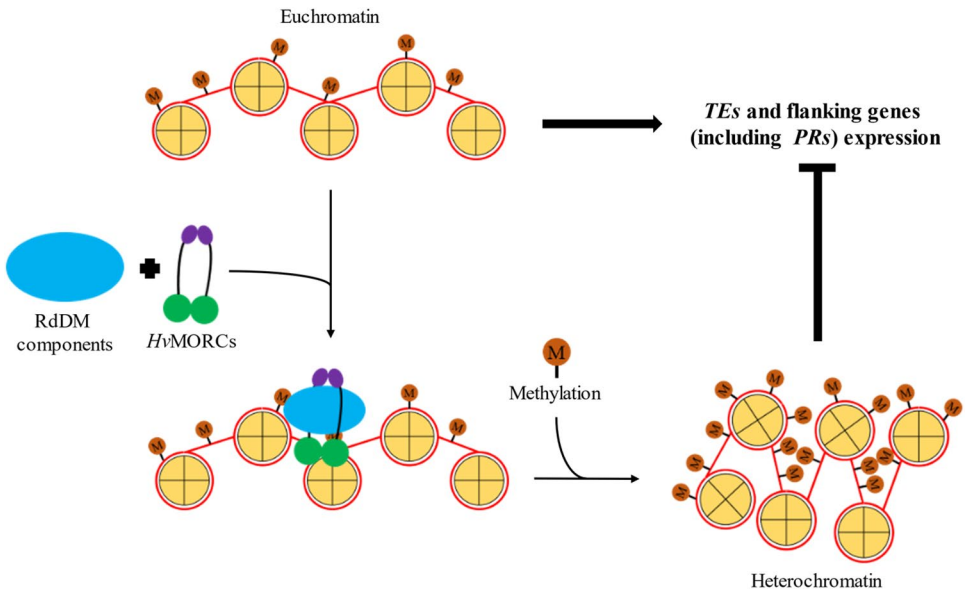

Fig. 3 Simplified working model hypothesis of the role of barley MORC proteins in the regulation of *TEs*, defense genes and disease resistance. MORC protein complexes interact directly with other components of the RdDM pathway to initiate or maintain inhibitory methylation marks, leading to the formation 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.

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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.

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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.

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