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Rice Yellow Mottle Virus resistance by genome editing of the *Oryza sativa* L. ssp. *japonica* nucleoporin gene *OsCPR5.1* but not *OsCPR5.2*

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Summary

Rice yellow mottle virus (RYMV) causes one of the most devastating rice diseases in Africa. Management of RYMV is challenging. Genetic resistance provides the most effective and environment-friendly control. The recessive resistance locus *rymv2* (*OsCPR5.1*) had been identified in African rice (*Oryza glaberrima*), however, introgression into *Oryza sativa* ssp. *japonica* and *indica* remains challenging due to crossing barriers. Here, we evaluated whether CRISPR/Cas9 genome editing of the two rice nucleoporin paralogs *OsCPR5.1* (*RYMV2*) and *OsCPR5.2* can be used to introduce RYMV resistance into the *japonica* variety Kitaake. Both paralogs had been shown to complement the defects of the Arabidopsis *atcpr5* mutant, indicating partial redundancy. Despite striking sequence and structural similarities between the two paralogs, only o*scpr5.1* loss-of-function mutants were fully resistant, while loss-of-function *oscpr5.2* mutants remained susceptible, intimating that *OsCPR5.1* plays a specific role in RYMV susceptibility. Notably, edited lines with short in-frame deletions or replacements in the N-terminal domain (predicted to be unstructured) of *OsCPR5.1* were hypersusceptible to RYMV. In contrast to mutations in the single Arabidopsis *AtCPR5* gene, which caused severely dwarfed plants, *oscpr5.1* and *oscpr5.2* single and double *knockout* mutants showed neither substantial growth defects nor symptoms indicative lesion mimic phenotypes, possibly reflecting functional differentiation. The specific editing of *OsCPR5.1*, while maintaining *OsCPR5.2* activity, provides a promising strategy for generating RYMV-resistance in elite *Oryza sativa* lines as well as for effective stacking with other RYMV resistance genes or other traits.

Introduction

Rice is one of the main staple food crops in developing countries, especially in sub-Saharan Africa (sSA). About 60% of the rice consumed is produced by sSA. Biotic and abiotic constraints impact rice yield in both irrigated and rainfed rice cultivation environments (Diagne *et al.*, 2013). In sSA, rice yellow mottle virus (RYMV, genus *Sobemovirus*, family *Solemoviridae*) causes substantial losses and is often considered as the dominant rice disease in irrigated and lowland ecologies (Ochola *et al.*, 2015; Suvi *et al.*, 2020; Traoré *et al.*, 2015). RYMV disease was first identified in East Africa in 1966 (Bakker, 1970) and has since been observed in almost all rice producing African countries. Typical symptoms include severe leaf mottling, yellow-green streaking, stunting at the vegetative stage, and reduced panicles emergence and sterility at the reproductive stage (Bakker, 1970). Depending on the environmental conditions and the susceptibility of the particular rice cultivar, in severe cases yield loss can exceed 50%. RYMV is transmitted *via* animal vectors – mammals and insects, as well as by inadequate agricultural practices, such as transplanting (Traoré *et al.*, 2010). Prophylactic practices can be implemented to eliminate reservoirs, reduce infection at seedling stages and improve plant health, but their effect is limited and the most effective way to sustainably reduce the impact of the disease is the use of resistant varieties.

Varietal resistance sources were found in cultivated species. High resistance has been observed in about 7% of accessions of *Oryza glaberrima*, a domesticated African rice species, but is very rare in *Oryza sativa*, which is by far the most cultivated rice species around the world and in Africa (Pidon *et al.*, 2020; Thiémélé *et al.*, 2010). Three major resistance genes have been identified to date: *RYMV1 (eIF(iso)4G1)*, *RYMV2* (*OsCPR5.1*), and *RYMV3* (*NB-LRR*) (Albar *et al.*, 2006; Bonnamy *et al.*, 2023; Odongo *et al.*, 2021; Orjuela *et al.*, 2013; Pidon *et al.*, 2017). Interspecific sterility barriers hamper the introgression of interesting traits from *O. glaberrima* to *O. sativa* (Garavito *et al.*, 2010). Thus, breeding for RYMV resistance had focused primarily on *rymv1-2*, the only resistance allele originating from *O. sativa*

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(Bouet *et al.*, 2013; Jaw *et al.*, 2012). *RYMV2*-mediated resistance is inherited recessively; six alleles have been described in *O. glaberrima* (Pidon *et al.*, 2020). Notably, the resistance in all these alleles is caused either by frameshift mutations or premature stop codons, leading to truncated proteins (7–83%), and thus loss of function.

RYMV2 (gene name *OsCPR5.1* in *O. sativa*) is a close homologue of Arabidopsis *AtCPR5* (constitutive expresser of Pathogenesis Related genes 5) (Orjuela *et al.*, 2013; Pidon *et al.*, 2020). AtCPR5 functions as an integral membrane nucleoporin in the transport of cargo across the nuclear pore complex (Gu *et al.*, 2016; Xu *et al.*, 2021). *atcpr5* mutants showed broad spectrum resistance against bacterial, fungal and oomycete pathogens, however, they had severe growth defects, consistent with a central role as part of the nucleopore complex. The exportin *XPO4* was identified as a genetic interactor of *CPR5* (Xu *et al.*, 2021). Moreover, ROS levels and/or signalling (Jing and Dijkwel, 2008), unfolded protein response (UPR) (Meng *et al.*, 2017), endoreduplication, cell division, cell expansion and spontaneous cell death were affected in *atcpr5* mutants (Kirik *et al.*, 2001; Perazza *et al.*, 2011). Overall, the phenotype of *atcpr5* mutants was pleiotropic, including glucose insensitivity and defects in trichome development (Aki *et al.*, 2007; Brininstool *et al.*, 2008). Due to the severe defects such as dwarfing, the gene was not considered suitable for biotechnological applications (Bowling *et al.*, 1997). The mechanism by which loss-of-function in *oscpr5.1* (*rymv2*) causes RYMV resistance has remained unknown. While the *Arabidopsis* genome contains only a single *AtCPR5* copy, two paralogs were found in other species, including rice (*OsCPR5.1; OsCPR5.2*), possibly reflecting functional differentiation.

Marker assisted backcross breeding (MABB) is widely used to introgress major resistance genes into susceptible varieties (Yugander *et al.*, 2018). However, in both conventional or MABB, introgression of single or multiple resistance genes into an elite variety is time-consuming and may not allow removal of unwanted linked loci and traits, even after multiple backcrosses, due to linkage drag. Introgression of resistance genes from *O. glaberrima* into *O. sativa* varieties is hampered by the crossing barrier (Heuer and Miézan, 2003). To overcome these constraints, innovative technologies, particularly the clustered regularly interspaced short palindromic repeats (CRISPR) associated (Cas9) system have been used successfully in field crops including rice (Endo *et al.*, 2016; Jiang *et al.*, 2013; Oliva *et al.*, 2019; Zhang *et al.*, 2014). To evaluate whether editing can be used to effectively generate new *RYMV2* resistance alleles in *O. sativa*, and to analyse the role of the two *OsCPR5* paralogs in resistance, CRISPR/Cas9 was used to edit the two genes in *Oryza sativa* ssp. *japonica* cv. Kitaake. Loss-of-function mutants in both genes and mutants with short in frame mutations in *OsCPR5.1* were evaluated. We observed effective resistance only in *oscpr5.1* loss-of-function mutants, but not in *oscpr5.2* loss-of-function mutants. By contrast to the *Arabidopsis atcpr5* mutants, *oscpr5.1* and *oscpr5.2* knockout mutants and double-mutants did not show detectable defects under greenhouse conditions. We conclude that *OsCPR5.1* and *OsCPR5.2* have distinct roles in virus resistance. Our data indicate that editing provides an option to stack resistance genes against RYMV to better protect against virus strains that overcome resistance, and to stack with other disease resistance genes (Bonnamy *et al.*, 2023; Hébrard *et al.*, 2018; Pinel-Galzi *et al.*, 2016).

Results

Bioinformatic analysis of *OsCPR5.1* and *OsCPR5.2*

OsCPR5.1 and OsCPR5.2 share 76% similarity and 62% identity at the protein level. To evaluate whether *OsCPR5.1* and *OsCPR5.2* might be redundant, or have distinct functions, we analysed transcript levels in public RNAseq databases (Figures S1–S3). Both genes showed broad expression across different tissues and developmental stages, with substantial differences between different tissues, indicative of a possible functional differentiation (Figures S1–S3). No major effects on mRNA levels by carious stresses were found (Rice RNA-seq database, Zhailab@SUSTech, not shown). Analyses of public databases revealed differences in mRNA levels for the two genes, possibly indicating at least partial differentiation rather than redundancy.

Structure predictions indicated that OsCPR5.1 and OsCPR5.2 have similar structures with a helix bundle from which a long helix extended, as well as possibly unstructured domains at the N-terminus (Figure 1a). Prediction of homo- and heterodimer structures is shown in Figure S4; however, the confidence level for the dimer interface is low. For all three CPR dimers, similar dimeric structures were found among the highest ranked models, only the heterodimer of OsCPR5.1 and OsCPR5.2 appeared different; this can however not be construed as indicating evidence for preferential homomer formation.

Generation of *cpr5.1* mutants by genome editing

In Africa, *O. glaberrima*, the source of most RYMV resistance genes, has been increasingly replaced by higher yielding *O. sativa* varieties that do not have the full spectrum of resistance (R) genes in their gene pool (Linares, 2002). Introgression of suitable R genes for RYMV from *O. glaberrima* into *O. sativa* remains challenging due to crossing barriers (Garavito *et al.*, 2010). It may therefore be valuable to test whether loss-of-function mutations in *OsCPR5.1* in a *japonica* variety would confer resistance. Genome editing with CRISPR/Cas9 can efficiently be used for targeted induction of mutations, e.g., loss of function as a consequence of frameshift mutations. Since *CPR5.1*-based recessive RYMV resistance is associated with naturally occurring loss of function mutations, *OsCPR5.1* was chosen as a target for guide RNAs. To generate loss of function mutations in *OsCPR5.1* through CRISPR/Cas9 editing, gRNAs targeting the first exon of *OsCPR5.1* were designed. Plasmid p-OsCPR5.1A carries gRNA-a1 and -a2, which target two sequences located 35 and 267 bp downstream the ATG to create out-of-frame deletions that would generate premature stop codons (Figure 1b; Table S1). Plasmid p-OsCPR5.1B was designed to express gRNA-b1 and -b2, which target two overlapping sequences 175 bp downstream of the ATG (based on CRISPR efficiency scores and protospacer motif (PAM) sequences; Figure 1b; Table S1). The RYMV-susceptible *O. sativa* L. ssp. *japonica* cv. Kitaake variety was transformed, and T0 events were selected for hygromycin resistance and tested by PCR amplification to check for the presence of T-DNA. Positive T0 transformants were further subjected to sequencing for confirmation of mutations in *OsCPR5.1* with gene specific primers (Table S2). Bi-allelic mutations were identied, demonstrating high efficiency of the editing process. About 50% of the mutations carried single nucleotide insertions or deletions, yet larger deletions were also observed, including a deletion of 232 nucleotides between

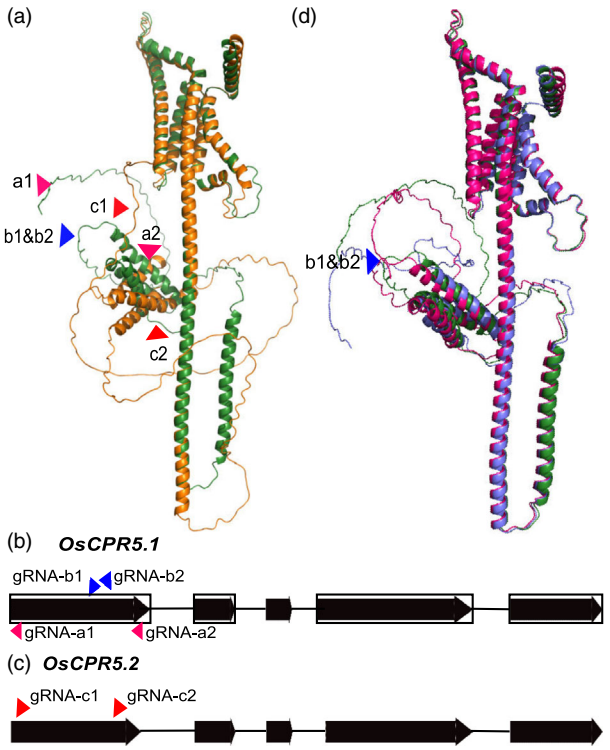

Figure 1 Schematic representation of *OsCPR5.1* and *OsCPR5.2* genes and of the target sites. (a) Prediction of protein structures of rice CPR5. Protein structures of OsCPR5.1 and OsCPR5.2 are represented in green and orange, respectively. (b) The guide RNAs (gRNAs) gRNA-a1, a2, b1 and b2 were designed to target the first exon of *OsCPR5.1* and to induce mutations in the coding sequence. (c) The guide RNAs (gRNAs) gRNA-c1 and c2 were designed to target the first exon of *OsCPR5.2* to induce mutations in the coding sequence. Target editing sites are indicated by pink and blue (OsCPR5.1), or red (OsCPR5.2) triangles. (d) Prediction of protein structures of rice OsCPR5 and in frame deletion mutants (green: wildtype OsCPR5.1, hot pink: OsCPR5.1-B8 and blue: OsCPR5.1-B9). CPR5 dimer predictions are shown in Figure S4.

gRNA-a1 and gRNA-a2. A subset of 13 transformants with frameshift or in-frame mutations, in homo- or heterozygous states, were selfed. Progenies from 15 lines with homozygous *oscpr5.1* mutations and an absence of Cas9 and the *hygromycin resistance* gene, as determined by PCR, were obtained and used for further phenotypic characterization (Figure 2a; Table S3). Ten lines (*cpr5.1-A1*, *A2*, *A4*, *B1*, *B2*, *B3*, *B4*, *B5*, *B6* and *B7*) carried mutations that cause frameshifts and/or early stop codons (Data S1). Three lines (*cpr5.1-B8*, *B10* and *B12*) had mutations that lead to amino-acid substitutions, and two lines (*cpr5.1-B9* and *cpr5.1-B11*) carried 42 bp in-frame deletions in the region corresponding to the N-terminus in a domain that was predicted to be unstructured, yet able to encode an otherwise intact protein (Data S1). The predicted structure of the mutated OsCPR5.1 variants was compared to the wildtype protein for two mutants in edited lines *cpr5.1-B8* and *cpr5.1-B9*, chosen to represent two different in frame mutations, a stretch of amino-acid change and a deletion, respectively. The *cpr5.1-B8* line is characterized by a substitution of eight sequential amino-acids; *cpr5.1-B9* by a 14-amino acids deletion. The predicted structures of the CPR5.1 variants from *cpr5.1-B8* and *cpr5.1-B9* were similar to that of wildtype (Figure 1d).

RYMV resistance of *OsCPR5.1* edited lines

Ten homozygous lines carrying either frameshift mutations or premature stop codons, and five lines with in-frame mutations were evaluated for RYMV resistance. Plants were mechanically inoculated with RYMV (BF1 isolate) and disease symptoms were recorded (Table S4). Wildtype Kitaake and IR64 served as susceptible controls, and two independent *oscpr5.1* T-DNA insertions lines as resistant controls (Pidon *et al.*, 2020). Three independent experiments were performed on 3–17 plants per line, and for a specific line, responses were comparable (Figure 3; Figures S5, S6). Virus load was determined using double antibody sandwich ELISA assays in two independent experiments. ELISA tests agreed with symptom observations 2 weeks after inoculation (Tables S4, S5). The virus was not detected by ELISA in 4 of the 153 plants noted as symptomatic, indicating that in these plants, mottling and yellowing was possibly not due to the disease. Conversely, virus was detected in 16 out of 276 symptomless plants.

Lines with frameshift mutations or premature stop codons showed full resistance. Only 8 out of 564 plants showed mottling and chlorosis two weeks after inoculation (Figure 3a,b; Figures S5, S6). Loss-of-function of *OsCPR5.1* is thus sufficient to confer RYMV resistance in a *japonica* variety (Figure 3a,b). By comparison, symptoms were observed in 265 out of 267 plants with in frame mutations in *OsCPR5.1* indicating that lines with in frame mutations were susceptible (Figure 1d). Most likely, the possibly unstructured domain at the N-terminus is not relevant for virulence of RYMV (Figure 1d). Interestingly, *oscpr5.1-B8* and *oscpr5.1-B11* showed more severe symptoms compared to Kitaake wild-type in all the four experiments, while a comparable result was observed in two experiments for *oscpr5.1-B9*, *-B10* and *-B12*. This result may indicate a hyper-susceptibility associated with the in-frame deletions. Taken together, loss-of-function of *OsCPR5.1* is sufficient to confer RYMV resistance to a *O. sativa* ssp. *japonica* variety.

Sheath blight disease resistance evaluation

To test whether the resistance was due to constitutive activation of defence responses in *oscpr5.1* mutants, we tested for susceptibility to another pathogen, the basidiomycete *R. solani*. Seven homozygous frameshift mutants, one in frame mutant of *oscpr5.1* and Kitaake wildtype were evaluated for sheath blight disease resistance. Plants were inoculated with the virulent *R. solani* strain RSY-04 and observations were recorded. Based on lesion length produced on rice leaves, all tested *oscpr5.1* mutants were susceptible, and lesions were similar as in Kitaake wildtype (Figure S7). We surmise that the resistance of the *oscpr5.1* mutants is possibly specific to the RNA virus RYMV.

Agro-morphological characters of *oscpr5.1* mutants

Arabidopsis *atcpr5* mutants showed increased resistance against diverse pathogens, viz., *Pseudomonas syringae* pv *Pst DC3000*, *Pseudomonas syringae* pv *maculicola ES4326*, *Erisyphe cruciferarum*, and *Hyaloperonospora arabidopsidis*, as well as compromised growth, indicating that *AtCPR5* plays important roles in broad spectrum pathogen susceptibility and in the physiology of uninfected plants (Höwing *et al.*, 2017; Wang, *et al.*, 2017). In contrast to *atcpr5*, uninfected *oscpr5.1* loss of function mutants did not display lesion mimic phenotypes. To test whether *oscpr5.1*-mediated RYMV resistance is associated to a reduction of plant growth, we evaluated key agro-morphological characters

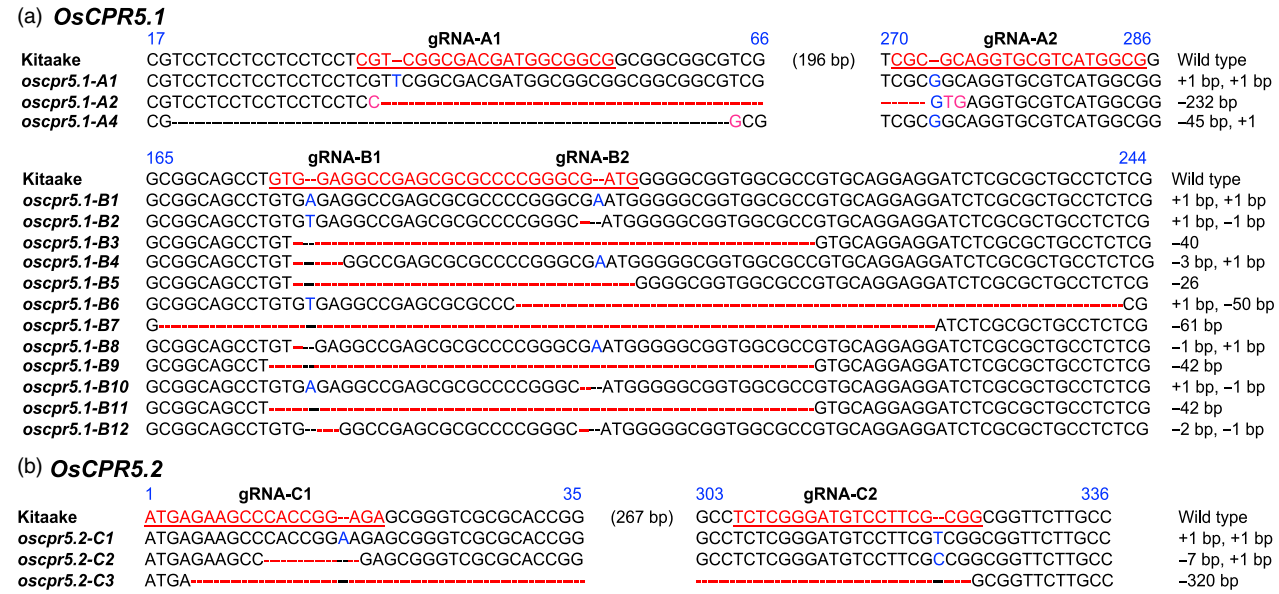

Figure 2 CRISPR/Cas9-induced indels in *OsCPR5.1* and *OsCPR5.2* in transgenic lines. gRNAs are underlined in the wildtype sequence. (a) Homozygous mutations in *OsCPR5.1*. (b) Homozygous mutations in *OsCPR5.2*. Mutations are indicated in red; "-" indicates a single base deletion. Numbering starts from ATG. The number of nucleotides inserted or deleted are indicated on the right.

of the mutants in the absence of the virus in three independent experiments at HHU (Table S6). Plant height, panicle length, reproductive tillers/plant and grain weight were evaluated in seven frameshift mutants and five in-frame mutants and no significant differences from wildtype Kitaake were found (Figure 4; Figures S8, S9). Only *oscpr5.1-B4* showed defects, possibly due to second site mutations that may impact panicle length. Three frameshift mutant lines *oscpr5.1-B5*, *oscpr5.1-B6* and *oscpr5.1-B7* showed increased reproductive tiller/plant and grain weight/plant (Figure 4). In summary, *oscpr5.1* edited frameshift and in frame mutants were generally not significantly different from Kitaake wildtype regarding various growth parameters. Since *atcpr5* mutants showed defects in trichome development (Brininstool *et al*., 2008), we also analysed the *oscpr5.1 knock out* mutants for trichome phenotypes using scanning electron microcopy (SEM). We did however not detect apparent differences in trichome density of structure (Figure 5).

The role of *OsCPR5.2* in RYMV resistance

OsCPR5.2 shares high sequence and predicted structural similarity with OsCPR5.1 (60% identity, 74% similarity; Figure 1a; Figure S10). Although natural *oscpr5.2* alleles of have not been described, in particular not in the context of RYMV resistance, it is conceivable that *oscpr5.2* knockout mutants could also confer RYMV resistance. To explore the role of *OsCPR5.2*, specifically for RYMV resistance, two sgRNAs were designed to target sequences in the first exon of *OsCPR5.2* (Figure 1c; Table S1). Seventeen independent T0 transformants of the *O. sativa L*. ssp. *japonica cv*. Kitaake variety were selected based on hygromycin resistance tests and positive PCR amplification of *Cas9*. Mutations in *OsCPR5.2* were confirmed by DNA sequencing using gene specific primers (Table S2). Most lines carried bi-allelic mutations in *OsCPR5.2*. Three transformants with frameshift mutations or premature stop codons, leading to loss-of-function alleles, were advanced to T2 homozygous progenies (Figure 2b; Table S7). Three lines with frameshift mutations (*oscpr5.2-C1*, *oscpr5.2-C2* and *oscpr5.2-C3*) were evaluated for RYMV resistance (Data S2). All three mutants were susceptible to RYMV and not significantly different from the wildtype Kitaake control regarding symptoms and virus load (Figure 3a,b; Figures S5, S6). The agro-morphological characters of *OsCPR5.2* frameshift mutants were analysed, as described above for *OsCPR5.1* frameshift mutant lines (Figure S11, Table S8). Plant height and grain weight of *oscpr5.2-C3* were reduced compared to the Kitaake wildtype, but *oscpr5.2-C1* and *oscpr5.2-C2* mutants were not significantly different to Kitaake wildtype for any of the three traits evaluated. The results obtained of *oscpr5.2-C1* and *oscpr5.2-C2* indicate that loss of function of *OsCPR5.2* does not affect growth under the conditions tested (Figure S11). Defects observed in *oscpr5.2-C3* were likely due to second site mutations.

Phenotype of *oscpr5.1*, *oscpr5.2* double mutants

Surprisingly, and in contrast to *atcpr5* mutants, the single *oscpr5.1* and *oscpr5.2* loss of function mutants did not show obvious lesion mimic phenotypes and no major defect in growth. This absence of strong growth and yield defect in the single knockout mutants of Kitaake, and in *rymv-2* mutants in various *O. glaberrima* genetic backgrounds, could have suggested compensatory effects and overlapping functions for *OsCPR5.1* and *OsCPR5.2* (Orjuela *et al*., 2013; Pinel-Galzi *et al*., 2016). One might thus hypothesize that *oscpr5.1*, *oscpr5.2* double mutants would be as severely impacted similar as the *atcpr5* mutant in Arabidopsis. To evaluate this hypothesis, a series of *oscpr5.1*, *oscpr5.2* double mutants was generated by crossing and phenotypes were analyzed. Surprizingly, however, the double mutants showed neither apparent lesion mimic phenotypes nor severe stunting (Figure 6).

Discussion

To evaluate whether genome editing of the two rice *CPR5* homologues can be used to confer resistance to RYMV, a major

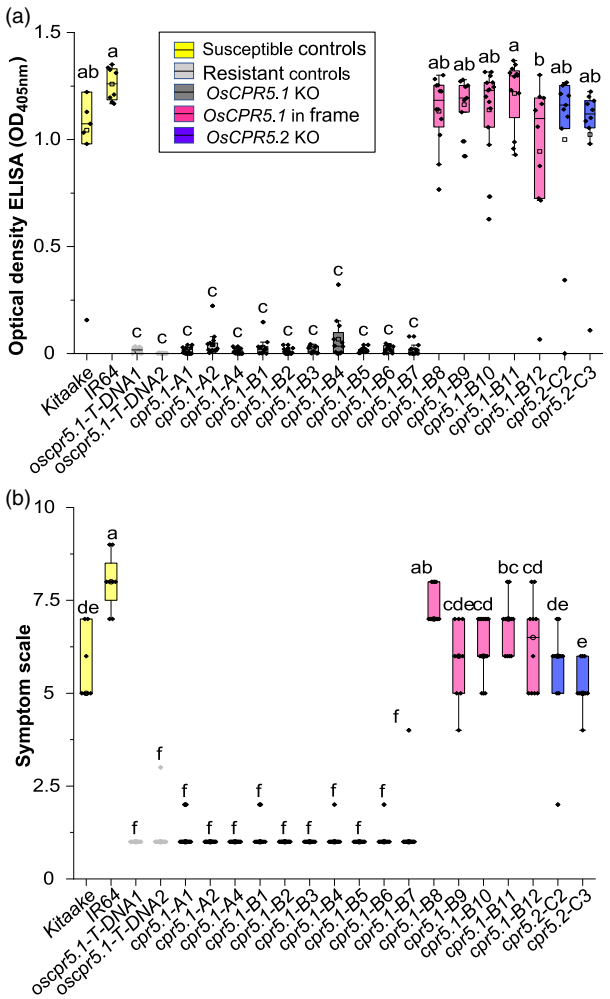

Figure 3 RYMV resistance evaluation in *oscpr5.1* and *oscpr5.2* mutant lines after mechanical inoculation with the BF1 isolate of RYMV. (a) Virus load in RYMV-infected rice leaves collected 2 weeks after inoculation quantified by DAS-ELISA. (b) Bonification of disease symptoms on infected rice plants on a scale from 1 to 9, 2 weeks after inoculation. Boxes extend from 25th to 75th percentiles and display median values as centre lines. Whiskers plot minimum and maximum values, asterisks indicate individual data points; significance evaluated by one-way ANOVA followed by Tukey's test, different letters indicate significant differences ($P < 0.05$). Analyses were performed four times independently with comparable results. Experiments were performed in greenhouse conditions at PHIM and only results from Experiment 2 are reported here.

biotic constraint affecting rice production in sSA (Odongo *et al.*, 2021), the two nucleoporin paralogs were edited in the japonica rice variety Kitaake (Figure S10). Homozygous loss of function alleles carrying premature stop codons in *OsCPR5.1* led to full resistance to the RYMV isolate BF1, while loss of function mutations in *OsCPR5.2* did not show a detectable increase in resistance. Interestingly, deletions in the N-terminal part of *OsCPR5.1* appeared to trigger increased susceptibility. Thus, despite the predicted structural similarity and the ability of the two rice paralogs to complement the mutant phenotype of the Arabidopsis *atcpr5* mutant (Mei *et al.*, 2022), *OsCPR5.1* and *OsCPR5.2* seem to have distinct functions with respect to susceptibility to RYMV (Figure 3; Table 1). The *oscpr5.1* mutant did not show obvious signs of constitutive defence activation, since susceptibility to other pathogens was retained. The mutants also appeared to have normal trichomes, in contrast to the Arabidopsis *atcpr5* mutant (Brininstool *et al.*, 2008). Notably, neither *oscpr5.1* nor *oscpr5.2* single *knockout* mutants showed apparent penalties under greenhouse conditions. The observation that *oscpr5.1* mutants have no obvious defects is consistent with analyses of naturally occurring *rymv2* variants. The lack of an apparent phenotype of the single mutants could be due to compensation by the respective other paralog, which could be evaluated, e.g., by determining mRNA levels of the genes in the respective mutants. We may speculate that compensation of *oscpr5.1* defects by OsCPR5.2 is insufficient at least in the context of RYMV resistance, since *oscpr5.1* single mutants show resistance, while *oscpr5.2* mutants do not. Moreover, in the case of functional compensation, one might have expected that *oscpr5.1*, *oscpr5.2* double mutants would show more severe defects relative to the single mutants, similar to those seen in the case of mutants in the sole *CPR5* gene in the Arabidopsis genome. Since the *oscpr5.1*, *oscpr5.2* double mutants did not appear substantially different from wild type and the single *oscpr5.1* and *oscpr5.2* mutants, compensation does not appear to be a major factor. The absence of a phenotype in the double mutants and the OsCPR5.1-specific resistance may indicate differences in the roles of the paralogs in rice as well as differences regarding the role of the CPR5 orthologues in rice and Arabidopsis. Overall, the role of the nucleoporins AtCPR5, OsCPR5.1 and OsCPR5.2 appears to be distinct and the signalling networks appear to be wired differently in Arabidopsis and rice. Our data indicate that genome editing events that cause loss of function in *OsCPR5.1* provide an effective approach to introduce resistance to RYMV into elite varieties of rice, which otherwise cannot be introduced easily by conventional breeding or marker assisted selection due to sterility barriers between *O. glaberrima* and *O. sativa* L. (Garavito *et al.*, 2010). Since some RYMV isolates are able to overcome individual R-genes against RYMV in experimental conditions, editing may provide an effective tool to stack multiple R genes and obtain more robust resistance (Bonnamy *et al.*, 2023; Hébrard *et al.*, 2018; Pinel-Galzi *et al.*, 2016). Other groups recently showed that genome editing can be used to generate resistance against other viruses by targeting translation initiation factors, known as major susceptibility factors in plant/virus interactions (Atarashi *et al.*, 2020; Kuroiwa *et al.*, 2022; Macovei *et al.*, 2018; Pechar *et al.*, 2022).

Potential role of *OsCPR5.1* in RYMV susceptibility

Since loss of the function leads to resistance, *OsCPR5.1* enables the virus to become virulent. Thus, *OsCPR5.1* serves as a recessive susceptibility gene. All CPR5 proteins appear to encode integral membrane proteins with five predicted transmembrane helices and a large soluble domain and likely function as membrane-anchored nucleoporins. AtCPR5 had been shown to interact genetically and physically with at least three other nucleoporins (Nup85, Nup96, NUP160) as well as the karyopherin Exportin-4 (Gu *et al.*, 2016; Xu *et al.*, 2021). AtCPR5 can form homodimers, which are disrupted when effector-triggered immunity (ETI) is activated, resulting in conformational changes that trigger the release of cyclin-dependent kinase inhibitors and other factors involved in defence ultimately leading to programmed cell death and hypersensitive responses, as well as other defences that block the reproduction/replication and limit the spread of the pathogen (Xu *et al.*, 2021). This fundamental pathway therefore is relevant to broad spectrum resistance/susceptibility to bacterial, fungal

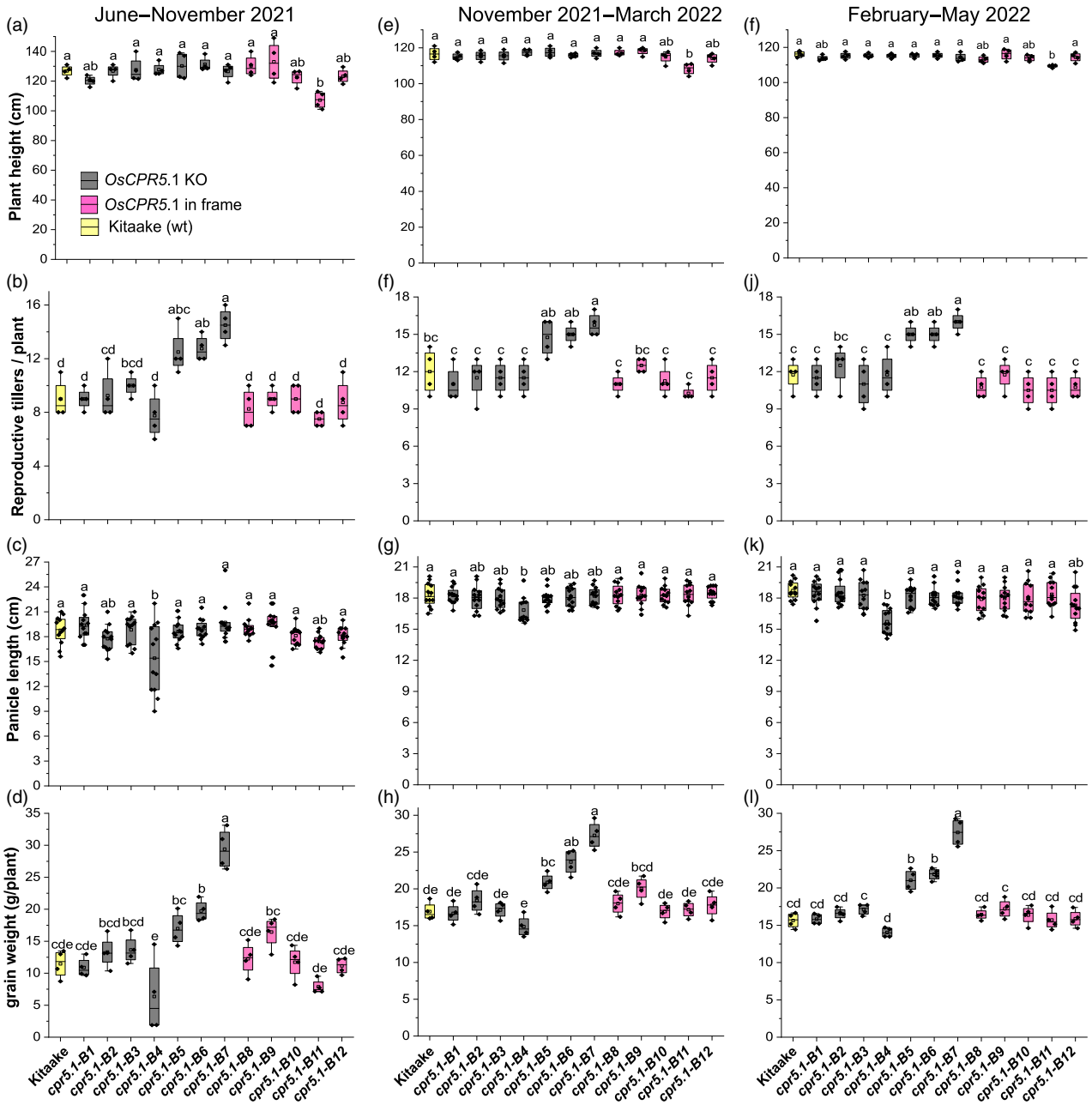

Figure 4 Agro-morphological characters of *oscpr5.1* mutants. Plant height, reproductive tiller number and grain weight were recorded in three independent experiments for the wildtype control Kitaake and the *knockout* mutant lines *oscpr5.1-B1*, *2*, *3*, *5*, *6* and *7*. Boxes extend from 25th to 75th percentiles and display median values as centre lines. Whiskers mark minimum and maximum values, asterisks indicate data points. Significance was evaluated by one-way ANOVA followed by Tukey's test, different letters indicate significant differences ($P < 0.05$). Data from three independent experiments in HHU greenhouses (dates indicated on top).

and viral diseases. Besides, since CPR5, at least in Arabidopsis, appears to be a component of the nuclear pore, one may speculate that the nucleus is required for effective virus replication. One may propose two alternative hypotheses: (i) Resistance against RYMV in *OsCPR5.1* loss of function mutants could possibly result from the constitutive activation of defence, including the salicylic acid (SA) signalling pathway (Bowling *et al*., 1997). Consistent with this hypothesis, *atcpr5* mutants are resistant to the bacterial pathogen *P. syringae* and the oomycete *H. arabidopsidis* (Bowling *et al*., 1997). CPR5 was found to bind to the N-terminus of the ethylene receptor ETR1, and is thought to regulate the export of ethylene-related mRNAs from the nucleus (Chen *et al*., 2022). In the case of virus, SA is known to induce effective resistance to a wide range of plant virus by inhibiting viral replication, blocking intercellular transport and systemic spread (Chivasa *et al*., 1997; Métraux *et al*., 1990; Murphy *et al*., 2020; Yalpani *et al*., 1993). For instance, cauliflower mosaic virus failed to systemically spread in *atcpr5* mutants (Love *et al*., 2007). It is generally assumed that constitutive defence activation brings trade-offs; consistent with

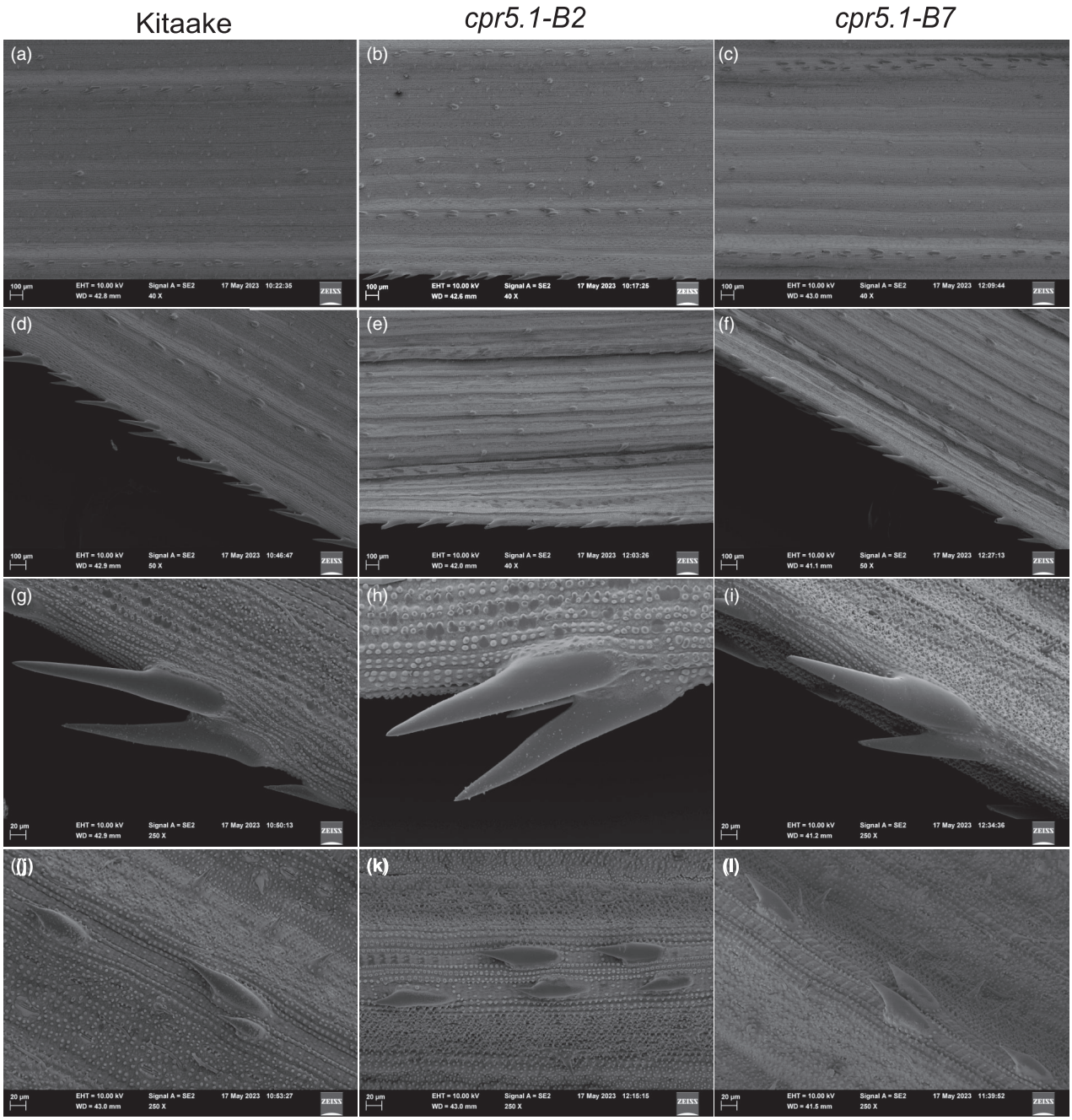

Figure 5 Trichome phenotypes of *oscpr5.1* mutants. SEM of the various types of trichomes on leaves of wild type Kitaake and on two loss-of-function *oscpr5.1* mutants. (a–c) Top view of leaves of wildtype and mutant rice leaves; (d–f) higher magnification with leaf margin; (g–i) higher magnification with large trichomes at margins; (j–l) higher magnification with large trichomes on leaf. Scale bar 100 μm (a–f), 20 μm (i–l).

the massive penalty observed in *atcpr5* mutants in Arabidopsis. Since in *O. glaberrima* and *O. sativa oscpr5.1* mutants did not show apparent penalties, it is unlikely that the *rymv2* resistance is due to defence priming. Moreover, *rymv2* would be expected to confer broad disease resistance, which was however not been observed in preliminary tests using *Rhizoctonia solani* (sheath blight) *Magnaporthe grisea* (blast), and *Xanthomonas oryzae* pv. *oryzae* (bacterial leaf blight) (Figure S7 and unpublished results). (ii) Alternatively, CPR5.1 could play a specific role that is required for RYMV virulence. RNA virus replication does not strictly depend on import into the nucleus, but various viruses, including RNA viruses, require proteins from the nucleus and disrupt the nucleocytoplasmic transport or target the nucleolus to promote their own replication (Flather and Semler, 2015; Walker and Ghildyal, 2017). Opalka *et al*. (1998) showed that RYMV virions can be detected in the nuclei of mesophyll cells (Opalka *et al*., 1998). However, at present, it is not known whether RYMV RNA enters the nucleus and whether virus replication is impaired in the *cpr5.1* mutants.

No obvious role of *OsCPR5.2* in RYMV susceptibility

By contrast to *oscpr5.1* loss of function mutants, *oscpr5.2* mutants did not show detectable RYMV resistance, implicating that *OsCPR5.2* is not directly involved in the interaction with the

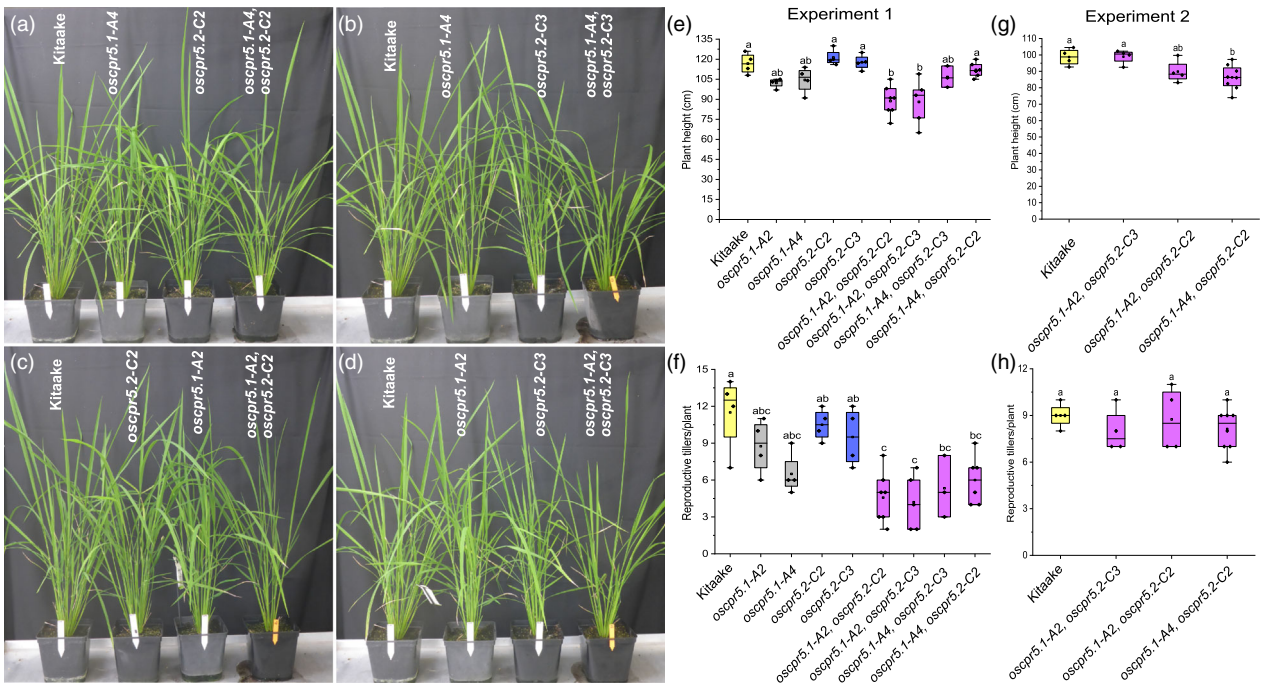

Figure 6 Phenotypic characterization of *oscpr5.1*, *oscpr5.2* double mutants. (a–d) Plant phenotype of *oscpr5.1*, *oscpr5.2* double mutants; (e–h) plant height and number of reproductive tillers of *oscpr5.1*, *oscpr5.2* double mutants. Boxes extend from 25th to 75th percentiles and display median values as centre lines. Whiskers mark minimum and maximum values, asterisks indicate individual data points. Significance calculated by one-way ANOVA followed by Tukey's test, different letters indicate significant differences ($P < 0.05$). Data obtained from two independent experiments conducted in PHIM and HHU greenhouses under controlled conditions (a–f: from Experiment 1; g, h: from independent Experiment 2).

virus, at least when *OsCPR5.1* is functional. However, the functional redundancy between paralogs of virus susceptibility genes can affect the resistance durability. Resistance-breakdown can result from the restoration of the interaction of the virus with the susceptibility factor when resistance alleles are characterized by substitutions or small deletions (Charron *et al.*, 2008; Hébrard *et al.*, 2010), which is not possible when resistance is conferred by knockout mutations. In that case, some viruses can recruit paralogs or isoforms of the missing susceptibility factor, as documented in the potyvirus/eIF4E and eIF(iso)4E interactions (Bastet *et al.*, 2018; Takakura *et al.*, 2018). Pinel-Galzi *et al.* (2016) reported that some RYMV isolates can overcome *RYMV2*-mediated resistance by acquiring mutations in the membrane anchor domain of the P2a polyprotein (Pinel-Galzi *et al.*, 2016). Such mutations could possibly result from an adaptation of the virus to *OsCPR5.2* when *OsCPR5.1* is defective. Besides, *OsCPR5.1* and *OsCPR5.2* genes are broadly expressed in all plant tissues (Figure S1); one, thus, may hypothesize that they can form heterodimers. However, if heterodimerization occurs, it does not appear to be relevant for RYMV susceptibility, since *oscpr5.2* mutants did not show a substantial increase in resistance. To dissect the resistance mechanism, it will be important to determine the subcellular localization of the two isoforms, the ability to dimerize, the effect of the virus on localization and dimerization as well as the effect of loss of CPR5.1 function on viral RNA entry into the nucleus and viral replication.

Summary and outlook

Our results confirmed that the disruption of *OsCPR5.1* in the *O. sativa L.* ssp. *japonica cv.* Kitaake confers resistance to the RYMV isolate BF1 without obvious yield penalty, at least under greenhouse conditions. Novel mutations can effectively be introduced by CRISPR/Cas9 into rice cultivars to obtain resistance against the RYMV and might help in accelerated breeding applications to develop resistance in popular cultivars for sSA. The next step will be to edit *OsCPR5.1* in elite varieties that are better adapted to rice culture in rainfed lowland and irrigated areas of Africa, where rice production is particularly affected by RYMV disease. Suitable transformation protocols have been established (Luu *et al.*, 2020). To obtain more robust resistance, it may be useful to stack the three known RYMV resistance genes to generate a more robust broad-spectrum resistance and to protect against new RYMV variants that could break the resistance genes if deployed individually. It may however be worthwhile to explore whether simultaneous deployment of all three known R-genes may lead to the evolution of new virus strains that can overcome all three resistance genes, and thus reduce our ability to control the disease. The resistance-breaking of *RYMV2* is associated with mutations in the membrane anchor domain of the P2a polyproteine (Pinel-Galzi *et al.*, 2016). *RYMV1* encodes the translation initiation factor eIF(iso)4G1 that is recruited by the virus and directly interacts with the viral protein genome-linked (VPg) covalently fused to the 5′ ends of the positive strand viral RNA (Albar *et al.*, 2006; Hébrard *et al.*, 2010). In incompatible interactions, non-synonymous mutations in eIF(iso)4G1 impair the interaction with the VPg causing a resistant phenotype and mutations in the VPg allow virus adaptation and resistance-breaking (Hébrard *et al.*, 2006, 2010). *RYMV3* is a classical NB-LRR that recognizes the coat protein of the virus and likely acts upstream of CPR5.1 (Bonnamy *et al.*, 2023). Independent viral determinants are thus involved in the resistance-

Table 1 Summary of features of *oscpr5.1* and *oscpr5.2* edited lines

Genotype	Mutation	Symptoms	ELISA	Plant height %	Tillers/plant %	Panicle length %	Grain weight %
Kitaake	Wildtype	+	S	100	100	100	100
IR64	Wildtype	+	S	NA	NA	NA	NA
oscpr5.1-A1	Stop codon after aa 33	–	R	NA	NA	NA	NA
oscpr5.1-A2	Stop codon after aa 15	–	R	NA	NA	NA	NA
oscpr5.1-A4	Stop codon after aa 90	–	R	NA	NA	NA	NA
oscpr5.1-B1	Stop codon after aa 93	–	R	96.9	96.2	97.2	98.8
oscpr5.1-B2	Stop codon after aa 59	–	R	98.0	101.5	96.0	109.7
oscpr5.1-B3	Stop codon after aa 79	–	R	100.5	103.1	93.3	108.8
oscpr5.1-B4	Stop codon after aa 104	–	R	98.8	93.1	90.2	80.1
oscpr5.1-B5	Stop codon after aa 96	–	R	101.0	122.9	95.2	133.8
oscpr5.1-B6	Stop codon after aa 59	–	R	100.2	129.8	96.0	148.2
oscpr5.1-B7	Stop codon after aa 72	–	R	98.8	138.2	103.8	191.1
oscpr5.1-B8	Substitution 60–67 aa	+	S	99.8	106.9	95.5	106.1
oscpr5.1-B9	Deletion 59–73 aa	+	S	102.2	96.2	103.5	122.0
oscpr5.1-B10	Substitution 60–67 aa	+	S	95.7	96.2	92.9	102.2
oscpr5.1-B11	Deletion 59–73 aa	+	S	92.2	88.5	98.3	92.6
oscpr5.1-B12	Substitution 60–67 aa	+	S	96.3	93.9	94.8	101.8
Kitaake (IRD)	Wildtype	+	S	100	100	NA	100
oscpr5.2-C1 (IRD)	Stop codon after 138 aa	+	S	98.2	101.0	NA	101.1
oscpr5.2-C2 (IRD)	Stop codon after 19 aa	+	S	99.7	96.2	NA	102.7
oscpr5.2-C3 (IRD)	Stop codon after 8 aa	+	S	91.3	93.5	NA	80.4

Values are from average of all experiments; +, positive; –, negative; R, resistant (blue); S, susceptible; NA, not analysed. Yellow and green indicate significant deviation from controls.

breaking of the different resistance genes. Stacking of suitable alleles of all three S/R genes will likely help to increase robustness. The highly efficient prime editing tools that have been developed will enable precise editing of RYMV1 and 3 (Gupta *et al*., 2023). Further clarification of the susceptibility and resistance mechanisms might help to design robust resistance. Subsequently, field trials will be required to validate that resistance is maintained under the local conditions in Africa, and to evaluate possible penalties in the relevant agro-ecosystems.

Experimental procedures

Plasmid construct and generation of transgenic rice lines

Mutations in the target genes *OsCPR5.1* (*LOC_Os01g68970.1* gene model on the reference Nipponbare sequence) and *OsCPR5.2* (*LOC_Os02g53070.1*) were generated through CRISPR/Cas9 editing in *Oryza sativa* cv. Kitaake, which is susceptible to RYMV. Two pairs of gRNAs (gRNA-a1/gRNA-a2 and gRNA-b1/gRNA-b2 (Figures S12, S13)) were designed on the first exon of *OsCPR5.1* and one pair of gRNAs (gRNA-c1/gRNA-c2) on the first exon of *OsCPR5.2* gene using online tools (https://cctop.cos.uni-heidelberg.de/; https://www.benchling.com/; Figure 1a,b; Table S1).

For the gRNA-b1/gRNA-b2 pair, single-stranded oligonucleotides were synthesized with adapter sequences for forward (5′-GCAG-3′) and reverse primers (5′-AAAC-3′) (IDT). The single-stranded DNA oligos were annealed to produce double-stranded oligonucleotides. Two double-stranded oligonucleotides were individually inserted into the *BsmBI*-digested pTLNtgRNA-1 to T2 to generate two tRNA-gRNA units. Two tRNA–gRNA units were transferred into *BsmBI*-digested pENTR4-U6.1PccdB using the Golden Gate ligation method. The cassettes containing two tRNA-gRNA units under control of U6.1P were finally mobilized to a binary vector pBY02-ZmUbiP:SpCas9 (Char *et al*., 2017; Zhou *et al*., 2014) using Gateway LR Clonase (Thermo Fisher Scientific, Dreieich, Germany) and designated as p-OsCPR5.1-B (Figure S13).

For the two other gRNA pairs, two cassettes containing the two gRNAs under the control of the rice promoters U6.1p and U6.2p, the sgRNA1 and attL1 and attL2 motives, were synthesized. These cassettes were sub-cloned into a modified binary cas9 vector pH-Ubi-cas9-7 (Fayos *et al*., 2020; Miao *et al*., 2013) in a Gateway LR reaction (Invitrogen, Thermo Fisher, Dreieich, Germany) to generate p-OsCPR5.1-A and p-OsCPR5.1-C constructs (Figures S12, S14).

Agrobacterium tumefaciens strain EHA105 was transformed by electroporation with Cas9/gRNA-expressing binary vectors. Rice transformation was performed by *A. tumefaciens* as previously described (Blanvillain-Baufumé *et al*., 2017; Sallaud *et al*., 2003; Wang, Karki, *et al*., 2017). Transgenic seedlings at 5-leaf stage were transferred to greenhouse.

Genotyping and sequencing of edited lines

Genomic DNA was extracted using the peqGOLD Plant DNA Mini Kit (PeqGold; VWR International GmbH, Darmstadt, Germany) for lines transformed with pOsCPR5.1-B construct while extraction was done according to (Edwards *et al*., 1991) for lines transformed with p-OsCPR5.1-A and p-OsCPR5.1-C constructs. Presence of T-DNA insertion was detected by specific amplification of SpCas9 and hygromycin resistance gene fragments. Primer pairs OsCas9-F/OsCas9-R and Hygro-IIF/Hygro-IIR were used for

lines transformed with pOsCPR5.1-B construct, and primer pairs Cas9-F(IRD)/cas9-R(IRD) and Hygro-F(IRD)/Hygro-R(IRD) for lines transformed with p-OsCPR5.1-A and p-OsCPR5.1-C constructs (Table S2). Amplifications were performed using the High-Fidelity Phusion PCR Master Mix (NEB, Frankfurt am Main, Germany) or the GoTaq G2 polymerase (Promega, Madison) following the manufacturer's instructions. Genome edition was analysed by sequencing of amplification products containing the target loci. Lines obtained with constructs p-OsCPR5.1-A, p-OsCPR5.1-B, p-OsCPR5.1-C were amplified with gene specific primers pairs (Table S2), respectively. Sequencing was subcontracted to Genewiz or Microsynth. Superimposed sequencing chromatograms were analysed with DSDecodeM to decode heterozygous profiles and validated manually.

RYMV resistance evaluation

Rice plants were grown at IRD in plastic trays in greenhouse, at a 28 °C/22 °C day/night temperature, 70% humidity and a 14 h light/24 h photoperiod. The wildtype *O. sativa L*. ssp. *japonica* cv. Kitaake and *O. sativa indica* IR64 were used as susceptible controls; T-DNA insertion lines in *O. sativa L*. ssp. *japonica* cv. Hwayoung and Dongjin (Pidon *et al*., 2020) were used as resistant controls. Four independent experiments were performed. Plants were mechanically inoculated with RYMV about 2 weeks after sowing as described in (Pinel-Galzi *et al*., 2018) with the BF1 isolate of RYMV. Resistance reaction was assessed based on the presence/absence of symptoms, completed with double-antibody sandwich (DAS-) ELISA for experiments 1 and 2. Symptoms were observed on the leaves emerged after inoculation 2 weeks after inoculation. Samples from the most recently emerged leaf were collected 1 or 2 weeks after inoculation for experiments 2 and 1, respectively, and virus load was estimated by direct double-antibody sandwich (DAS-) ELISA, as described by (Pinel-Galzi *et al*., 2018), using a polyclonal antiserum directed against an RYMV isolate from Madagascar (N'Guessan *et al*., 2000).

Sheath blight disease resistance evaluation

To explore resistance of *oscpr5.1* mutants to *R. solani*, seven *oscpr5.1* frameshift mutants (*oscpr5.1*, *B1-B7*), one in frame mutant (*oscpr5.1-B11*) and wildtype Kitaake were grown at HHU. 30-day-old plants were used for inoculation with *R. solani* (RSY-04). Individual sclerotia from three-day old fresh *R. solani* grown on Potato Dextrose Agar (PDA) were inoculated onto individual rice leaves and incubated at 27 °C. Phenotypic observations (8 plants/mutant) were recorded 5 days post inoculation. Notably, this experiment was performed only twice (Figure S7).

CPR5 structure predictions

Protein structures for AtCPR5 (UniProt ID: Q9LV85), OsCPR5.1 (UniProt ID: Q5JLB2), OsCPR5.2 (UniProt ID: Q6ZH55) selected mutants and possible dimers were generated using the AlphaFold Colab notebook interface (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb) based on the AlphaFold v2 structure prediction program (Jumper *et al*., 2021; Mirdita *et al*., 2022) using a selected portion of the BFD database (https://bfd.mmseqs.com). Accuracy in predicted structures might, therefore, differ slightly from those obtained from the full AlphaFold system. We note that these are predictions that may not reflect the actual in vivo structures.

Plant growth and analysis of agro-morphological characters

Plants obtained with pOsCPR5.1-B construct were grown at HHU in greenhouse conditions at 30 °C during the day time and 25 °C during the night time with 60–70% relative humidity. Light conditions in the greenhouse are determined by natural daylight and additional lamplight (8/16 day/night photoperiod). Plants obtained with pOsCPR5.1-A and -C constructs were grown at IRD in greenhouse conditions with a 28 °C/22 °C day/night temperature, 70% humidity and a 14 h light/24 h photoperiod. To determine the agro-morphology of *oscpr5* lines, four to five plants of each line were grown in greenhouses and measured for different morphological characters viz., plant height, number of reproductive tillers, panicle length and grain weight. Three independent experiments were performed for each set of lines.

SEM analysis of trichomes on rice leaves

Scanning electron microscopy (SEM)was prepared and performed in the following steps. The samples were first fixed in glutaraldehyde (2.5%) for one hour, then washed three times with phosphate buffer (PBS) and subsequently dehydrated in an ascending alcohol series (50% – 70% – 80% – 90% – 96% – 100%) for 10 min each. Subsequently, the samples were dried in a critical point dryer (Baltec CPD 030). The dried samples were mounted on SEM sample plates using conductive tabs (Plano) and sputtered with gold for 120 s (Agar Sputter Coater Model: 108). Observations were performed on the scanning electron microscope ZEISS Supra 55VP at 10 kV.

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Conflict of interest

The authors declare no conflict of interest. The project aims at supporting the food security of small scale producers in sSA.

Author contributions

YA, SC, WBF and LA developed the concept. YA, FA, MS, SC, and LA performed experiments. YA, FA, LA, MMW and VSL have performed in silico analyses. YA, LA, MM and WBF have written

the manuscript. All authors have given approval to the final version of the manuscript.

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Supporting information

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

Data S1 Predicted amino acid sequence of OsCPR5.1 in wildtype and in mutants.

Data S2 Predicted amino acid sequence of OsCPR5.2 in wildtype and mutants.

Figure S1 Tissue specific and developmental stages expression levels of *OsCPR5.1* and *OsCPR5.2*.

Figure S2 Analysis of leaf region-specific mRNA levels for *OsCPR5.1* and *OsCPR5.2* using RiceXPro3.0.

Figure S3 Analysis of organ-specific mRNA levels for *OsCPR5.1* and *OsCPR5.2* using RiceXPro3.0.

Figure S4 Alphafold prediction of AtCPR5, OsCPR5.1 and OsCPR5.2 dimer conformations.

Figure S5 Disease resistance phenotypic reaction of *oscpr5.1* and *oscpr5.2* mutants.

Figure S6 Bonification of RYMV-induced disease symptoms on *oscpr5.1* and *oscpr5.2* mutant lines 2 weeks after inoculation with the BF1 isolate of RYMV.

Figure S7 Disease phenotypic reaction of *oscpr5.1* mutants with *R. solani* AG1-1A.

Figure S8 Phenotypic characters of *oscpr5.1* mutants.

Figure S9 Panicle characters of *oscpr5.1* mutant plants.

Figure S10 Alignment of CPR5 homologues from rice and *Arabidopsis*.

Figure S11 Morphological characters of *oscpr5.2* frameshift mutant plants.

Figure S12 Map of the binary CRISPR/Cas9 vector p-OsCPR5.1-A.

Figure S13 Map of the binary CRISPR/Cas9 vector p-OsCPR5.1-B.

Figure S14 Map of the binary CRISPR/Cas9 vector p-OsCPR5.2-C.

Table S1 List of gRNAs used to develop CRISPR/Cas9 mediated mutations of *OsCPR5.1* and *OsCPR5.2* in Kitaake.
Table S2 List of primers used in the present study.
Table S3 List of CRISPR/Cas9-induced insertions or deletions in T2 homozygous plants of *OsCPR5.1*.
Table S4 RYMV disease resistance phenotypic reaction of *oscpr5.1* and *oscpr5.2* (additional file).
Table S5 Virus detection using ELISA in symptomatic and symptomless plants at two weeks after inoculation with BF1 isolate of RYMV.
Table S6 Agro-morphological characters of *oscpr5.1* (additional file).
Table S7 List of CRISPR/Cas9-induced deletions in T2 homozygous plants of *oscpr5.2*.
Table S8 Agro-morphological characters of *oscpr5.2* (additional file).