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Novel resistance to the Bymovirus BaMMV established by targeted mutagenesis of the *PDIL5-1* susceptibility gene in barley

Robert Eric Hoffie[1], Dragan Perovic[2], Antje Habekuß[2], Frank Ordon[2] and Jochen Kumlehn[1,*]

[1]*Plant Reproductive Biology, Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), Seeland, Germany*

[2]*Institute for Resistance Research and Stress Tolerance, Julius Kuehn Institute (JKI), Federal Research Centre for Cultivated Plants, Quedlinburg, Germany*

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**Correspondence* (Tel +49 394825361;
email kumlehn@ipk-gatersleben.de)

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Summary

The Potyviridae are the largest family of plant-pathogenic viruses. Members of this family are the soil-borne bymoviruses *barley yellow mosaic virus* (BaYMV) and *barley mild mosaic virus* (BaMMV), which, upon infection of young winter barley seedlings in autumn, can cause yield losses as high as 50%. Resistance breeding plays a major role in coping with these pathogens. However, some viral strains have overcome the most widely used resistance. Thus, there is a need for novel sources of resistance. In ancient landraces and wild relatives of cultivated barley, alleles of the susceptibility factor *PROTEIN DISULFIDE ISOMERASE LIKE 5–1* (*PDIL5-1)* were identified to confer resistance to all known strains of BaYMV and BaMMV. Although the gene is highly conserved throughout all eukaryotes, barley is thus far the only species for which *PDIL5-1*-based virus resistance has been reported. Whereas introgression by crossing to the European winter barley breeding pool is tedious, time-consuming and additionally associated with unwanted linkage drag, the present study exemplifies an approach to targeted mutagenesis of two barley cultivars employing CRISPR-associated endonuclease technology to induce site-directed mutations similar to those described for *PDIL5-1* alleles that render certain landraces resistant. Homozygous primary mutants were produced in winter barley, and transgene-free homozygous M_2 mutants were produced in spring barley. A variety of mutants carrying novel *PDIL5-1* alleles were mechanically inoculated with BaMMV, by which all frameshift mutations and certain in-frame mutations were demonstrated to confer resistance to this virus. Under greenhouse conditions, virus-resistant mutants showed no adverse effects in terms of growth and yield.

Introduction

Viruses, along with insects and fungi, are among the most important pathogens of crop plants. *Potyviridae* represent the largest group of plant pathogenic viruses. They are enveloped, filamentous single-strand (+) RNA viruses transmitted by vectors, that is insects or soil-borne fungi to various mono- and dicotyledonous plants. From this family, soil-borne representatives of the genus Bymovirus are particularly important in the cultivation of barley and wheat in Europe and Asia (Kühne, 2009). For winter barley grown in the temperate zone, *barley yellow mosaic virus* (BaYMV) and *barley mild mosaic virus* (BaMMV) are of the highest importance. Mainly in autumn, they are transmitted by the plasmodiophorid *Polymyxa graminis* via the roots into young seedlings. The infection initially causes yellow mosaics on the leaves of juvenile plants, reduces winter hardiness and, in spring, impacts plant growth and tillering. As a consequence, as much as 50% yield losses can be caused in infested fields. In Europe, two strains of BaYMV and one strain of BaMMV are common, but additional strains are known (comprehensively reviewed in Jiang *et al*. (2020)).

The *EUKARYOTIC TRANSLATION INITIATION FACTOR 4 E (EIF4E)* and its isoform *EIF(iso)4 E* have been described as interaction factors for *Potyviridae* in a number of di- and monocotyledonous plants (Sanfaçon, 2015). They bind to the viral genome-linked protein (VPg) that mimics the m^7G cap of eukaryotic mRNAs at the 5′ end of the viral RNA. Thus, viral RNA is translated into viral proteins by the translational machinery of the host plant. Barley was the first cereal in which virus resistance-mediating *EIF4E* alleles were identified. In European winter barley breeding, the *EIF4E* alleles *rym4* (resistance to BaYMV-1 and BaMMV) and *rym5* (resistance to BaYMV-1, -2 and BaMMV) are used extensively (Stein *et al*., 2005). For instance, in Germany, the vast majority of current winter barley cultivars is resistant to BaMMV and BaYMV, mostly due to the *rym4* or *rym5* alleles (Bundessortenamt, 2021). Due to single nucleotide polymorphisms that entail alterations in the binding domain of the encoded eIF4E protein, the latter is no longer capable of interacting with the viral genome-linked protein (VPg) cap of the viral RNA. Thus, translation of the viral proteins is prevented. In addition to the early-emerging strain BaYMV-2, which has broken the *rym4* resistance, new pathotypes of BaMMV (BaMMV-Teik, BaMMV-Sil) were identified to break the *rym5*-conferred resistance (Habekuss *et al*., 2007; Kanyuka *et al*., 2004). The viruses have overcome *EIF4E*-based resistance by changing the structure of their VPg RNA cap (Li *et al*., 2016), thus regaining the ability to interact with *rym4* or *rym5* variants of the eIF4E protein that initiates translation of the viral RNA.

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Since only *EIF4E*-mediated resistance is present in current winter barley cultivars, there is an urgent need for new resistance mechanisms in winter barley breeding material. The full knockout of *HvEIF4E* renders barley resistant to bymovirus infection, as described for other plants, but it is associated with a substantial yield penalty (Hoffie *et al*., 2021). In the landrace HOR1363 (formerly PI1963) and the Russian cultivar 'Russia 57', *rym11*, a locus associated with resistance to all known strains of BaYMV and BaMMV, was described (Nissan-Azzouz *et al*., 2005; Sedláček and Mařík, 2010). Later, *rym11* was identified as a knockout allele of the *PROTEIN DISULFIDE ISOMERASE LIKE 5–1* gene (*PDIL5-1*) (Yang *et al*., 2014). Protein disulfide isomerases are highly conserved enzymes in eukaryotes and feature thioredoxin (TRX) domains that act as chaperones catalysing the formation of disulfide bridges between amino acids in the context of protein folding (Houston *et al*., 2005). HvPDIL5-1 carries a KDEL localisation signal for the endoplasmic reticulum. To date, virus resistance based on *PDIL5-1* orthologues has not been described in any plant species other than barley, although it is likely that this highly conserved gene interacts with *Potyviridae* in other plants as well.

By screening diverse barley germplasm, seven resistant haplotypes were found in East Asian landraces of barley, six of which carry deletions or stop codons leading to loss of function of the gene. The seventh haplotype has a single nucleotide polymorphism in exon 3 that results in a substitution of a conserved amino acid within a functional domain of the protein. All of these landraces are resistant to all known strains of BaYMV and BaMMV (Yang *et al*., 2017), while the knockout of *PDIL5-1* does not appear to be associated with yield loss (Yang *et al*., 2014). Thus, *rym11* represents a valuable source of resistance for breeding. However, introgression of resistance-conferring alleles from landraces into current winter barley breeding material will require many years of back-crossing and selection.

RNA-guided Cas endonucleases derived from bacterial CRISPR-Cas immune systems have been established in cereals as effective tools for targeted mutagenesis (Hisano *et al*., 2021). Using this technology, target sequence-specified guide RNAs (gRNAs) are capable of precisely directing Cas endonucleases to predetermined positions in any candidate gene of choice, where the endonucleases induce DNA double-strand breaks whose error-prone repair can cause random mutations at the mended site. In barley, too, Cas endonuclease technology has been applied in some studies to validate gene functions or to introduce advantageous traits without any further modification of the genetic background (Gerasimova *et al*., 2020; Hisano *et al*., 2022; Hoffie *et al*., 2021; Li *et al*., 2019; Steckenborn *et al*., 2022). Since all potential *rym11* donors represent rather exotic germplasm for winter barley breeding, the site-directed generation of *rym11* alleles is considered as being a particularly useful approach towards the development of virus-resistant varieties.

In the present study, a variety of novel resistance-conferring *PDIL5-1* alleles, similar to those identified in some landraces, was generated by *cas9*-mediated site-specific mutagenesis in the winter barley cultivar 'Igri' and the spring barley cultivar 'Golden Promise'. In the M_2 generation, resistance was tested by mechanical inoculation with BaMMV-ASL. In this generation, homozygous mutants lacking the *cas9* transgene were identified and shown to be completely resistant to infection. A greenhouse experiment revealed that *pdil5-1* mutants did not differ in yield components compared with their 'Igri' and 'Golden Promise' wild-type counterparts.

Results

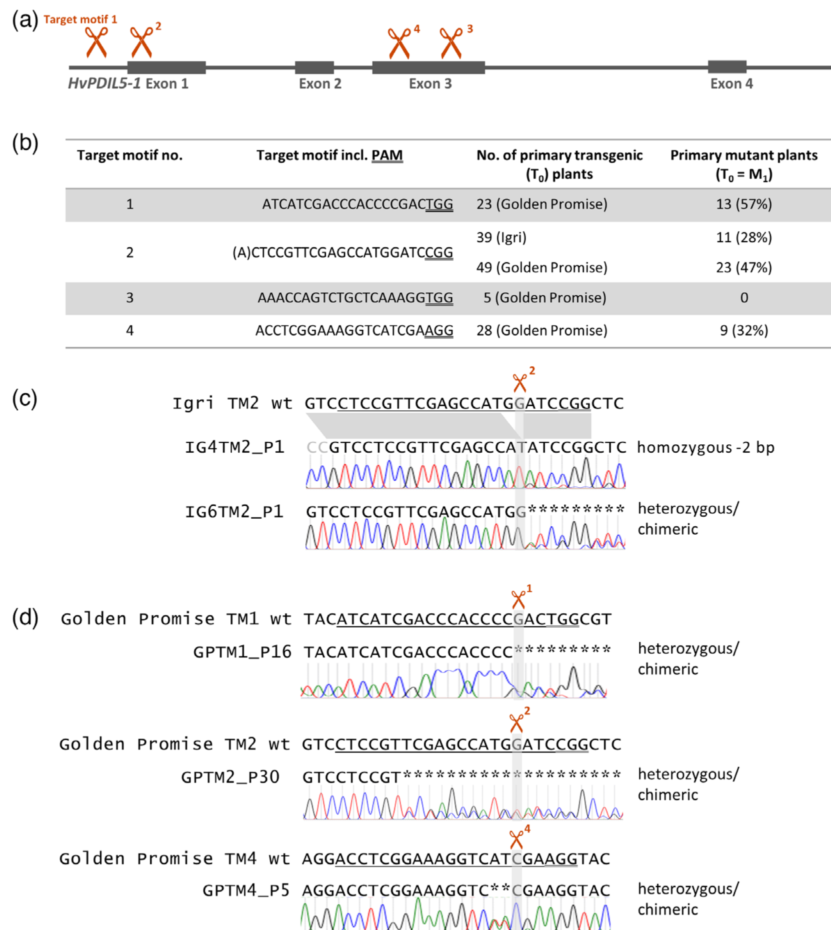
Mutations in the *PDIL5-1* target motif 2 confer resistance of winter barley cv. 'Igri' to BaMMV

Agrobacterium-mediated transformation of winter barley cv. 'Igri' was performed using the vector pBin_PDIL5-1_TM2. In total, 39 plants were obtained from seven transformation experiments. Thirty-three plants were PCR-positive for the *cas9* transgene. Mutation screening by PCR amplification of the target region followed by Sanger sequencing of the amplicons revealed 11 plants (i.e. 28% of the regenerants) to carry mutations at target motif 2 (see Figure 1b for a summary). Two of these mutants (IG4TM2_P1 and IG6TM2_P2) were already homozygous for a 2-bp and a 22-bp deletion, respectively (see, e.g. IG4TM2_P1 in Figure 1c). The others were considered heterozygous or chimeric based on the appearance of double or multiple peaks in the sequencing chromatogram, which indicated that more than one allelic variant was present in the respective plant (see, e.g. IG6TM2_P1 in Figure 1c).

Five selected M_2 progeny were mechanically inoculated with BaMMV-ASL to validate the resistance of individual plants by phenotyping and virus-specific ELISA. In parallel, M_2 plants were genotyped with regard to the presence of the transgene (PCR for *cas9* gene), and for a subset of plants, Sanger sequencing of the target region was performed. In summary, 79 M_2 plants were inoculated with BaMMV-ASL, of which 44 showed resistance according to the results of the ELISA (for detailed results of individual plants, see Table S2). All nine tested M_2 plants originating from the primary mutant IG4TM2_P1, which was homozygous for a 2-bp deletion at target motif 2, were invariably resistant to BaMMV inoculation, and every individual plant carried the same 2-bp deletion as their parent (see Figure 2a). The same features applied to the 19 M_2 plants derived from the primary mutant IG6TM2_P2 that was homozygous for a 22-bp deletion (see Figure 2a). All of these plants were PCR-positive for the *cas9* transgene.

The progeny of the three heterozygous and/or chimeric primary mutants IG4TM2_P6, IG6TM2_P1 and IG7TM2_P1 segregated with regard to transgenicity, mutation of the target gene and phenotype. The results are summarized in Figure 2b, and detailed results per plant are provided in Table S2. Heterozygous or chimeric M_2 plants as well as non-mutated siblings were mainly susceptible to BaMMV inoculation, whereas biallelic mutants were virus-resistant. Of 20 M_2 plants derived from the primary mutant IG4TM2_P6, 10 were ELISA-negative for BaMMV, indicating resistance. Of 16 plants that were Sanger sequenced, five carried a homozygous 58-bp deletion (see Figure 2a) and one was biallelic (10-bp insertion and a 58-bp deletion). Based on the separation of PCR products of different sizes by agarose gel electrophoresis and separate elution, it was possible to individually sequence both alleles. This plant was the only T-DNA free (i.e. *cas9* PCR-negative) mutant with the background of cv. 'Igri'. Among the progeny of IG7TM2_P1, only one M_2 plant was found to be resistant to BaMMV, which was homozygous for a 4-bp deletion in the target motif (see Figure 2a).

Mechanical inoculation of leaves with BaYMV was not successful, BaYMV particles were neither detected in *PDIL5-1* mutants nor in their wild-type counterparts (see Table S2).



Target motif no.	Target motif incl. PAM	No. of primary transgenic (T_0) plants	Primary mutant plants ($T_0 = M_1$)
1	ATCATCGACCCACCCCGACTGG	23 (Golden Promise)	13 (57%)
2	(A)CTCCGTTCGAGCCATGGATCCGG	39 (Igri)	11 (28%)
		49 (Golden Promise)	23 (47%)
3	AAACCAGTCTGCTCAAAGGTGG	5 (Golden Promise)	0
4	ACCTCGGAAAGGTCATCGAAGG	28 (Golden Promise)	9 (32%)

Figure 1 (a) Gene structure of *HvPDIL5-1* (grey line, exons indicated by grey boxes) and location of the target motifs (scissors). (b) Overview of *cas9*/gRNA target motifs, including the respective protospacer-adjacent motifs (PAM), number of primary transgenics with T-DNA carrying *cas9* and the respective gRNA and number of primary mutants screened by Sanger sequencing of target regions. (c) Chromatograms of two primary mutants in the 'Igri' background (target motif 2) aligned to the wild-type sequence of the target region. Homozygous deletion of 2 base pairs in IG4TM2_P1, while double peaks downstream of the cleavage site indicate a heterozygous or chimeric mutation state in IG6TM2_P1. (d) Chromatograms of one primary mutant for each target motif where mutations were successfully induced in the 'Golden Promise' background. Heterozygous InDels are indicated by double peaks downstream of the *cas9* cleavage site. Two double peaks in GPTM4_P5 indicate heterozygous base substitutions.

As indicators for plant performance, the thousand-grain weight and number of grains per plant were assessed, and no significant differences were detected by multiple comparison between all groups (heterozygous mutants, homozygous mutants, non-mutated siblings, 'Igri' wild-type, $P > 0.05$, see Figure 2c,d and Table S2).

InDels as well as single nucleotide polymorphisms in different target motifs confer resistance of spring barley 'Golden promise' to BaMMV

Due to the higher efficiency in generating transgenic plants in spring barley cv. 'Golden Promise' via *Agrobacterium*-mediated DNA transfer to immature embryos, all four target motifs were addressed in this accession using the transformation vectors pBin_PDIL5-1_TM1, pBin_PDIL5-1_TM2, pBin_PDIL5-1_TM3 and pBin_PDIL5-1_TM4. In four independent transformation experiments, 23, 49, 5 and 28 plants were regenerated. All but three of these plants were PCR-positive for the *cas9* transgene. Target regions were sequenced as previously explained. For the individual target motifs, 13, 23, 0 and 9 primary mutants were obtained, which corresponded to 57%, 47% and 32% of the regenerated plants, respectively. All of these mutants were heterozygous/chimeric, as indicated by the double/multiple peaks in the sequencing chromatogram (see Figure 1b,d).

For the target motifs 1, 2 and 4, respectively, five, five and four primary mutants were selected, and their progeny were phenotyped for resistance to BaMMV-ASL and genotyped for transgenicity and target-specific mutation patterns. All of these families showed independent segregation of mutations and the transgene. Heterozygous/chimeric and non-mutated siblings were mainly susceptible to BaMMV-ASL infection, while homozygous frameshift mutations (InDels) as well as in-frame base substitutions caused resistance to BaMMV. A summary of the results is provided in Figure 3b, and detailed data are listed in Table S2. Here, some examples are described in detail. In the progenies of five primary mutants of target motif 1 (5′-UTR), GPTM1_P1, _P7, _P12, _P16 and _P23, two plants were PCR-negative for the *cas9* transgene, carried homozygous deletions of 18 bp (GPTM1_P1_16) and 1 bp (GPTM1_P16_12), respectively, and were resistant to virus infection. Except for plant GPTM1_P1_4, all susceptible individuals

(a)

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GTATCCGCCTTCTCCTCGTCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCGAGCCATGGATCCGGCTCTTCGGCGCC      Igri wild-type
GTATCCGCCTTCTCCTCGTCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCGAGCCAT--ATCCGGCTCTTCGGCGCC      IG4TM2_P1_9
GT----------------------------------------------------------ATCCGGCTCTTCGGCGCC      IG4TM2_P6_1
GTATCCGCCTTCTCCTCGTCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCGA----------------------GCC      IG6TM2_P2_4
GTATCCGCCTTCTCCTCGTCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCGAGCCATGGTCGTATCCGGCTCTTCGGCGCC  IG7TM2_P1_5
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(b)

Plants		Phenotype	Genotype					
Primary mutant (M_1)	Progeny (M_2) tested	BaMMV-free (ELISA)	T-DNA-free plants	Sequenced plants	Non-mutated	Heterozygous mutants	Homozygous mutants	T-DNA-free mutants
Target Motif 2								
IG4TM2_P1	9	9	0	9	0	0	9	0
IG4TM2_P6	20	10	2	16	1	10	5	1
IG6TM2_P1	16	5	0	10	4	6	0	0
IG6TM2_P2	19	19	0	19	0	0	19	0
IG7TM2_P1	15	1	3	5	4	0	1	0
sum	79	44	5	59	9	16	34	1
wild-type								
Igri	10	1	10	6	6	0	0	-

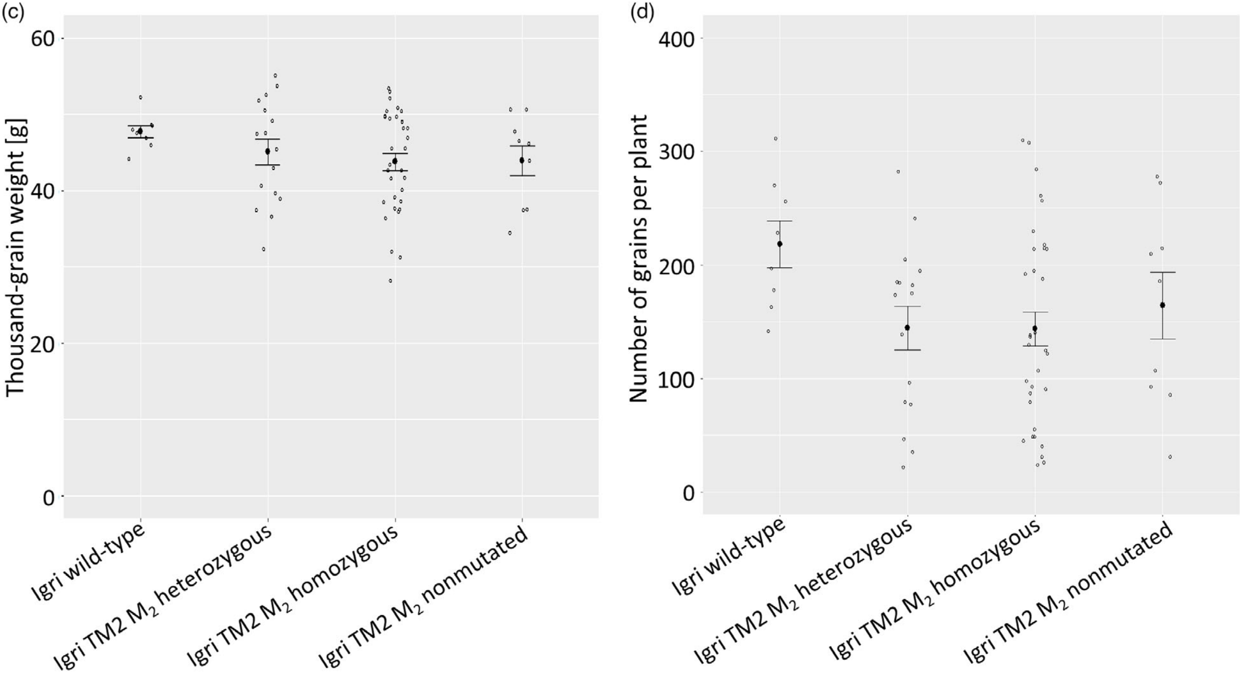

Figure 2 (a) Alignment of sequences from homozygous M_2 mutants in the 'Igri' background (target motif 2) to the wild-type sequence of the target region. Cleavage sites of *cas9* are indicated by scissors, deletions by dashes and insertions in red. Larger deletions of 58 base pairs (IG4TM2_P6_1) and 22 base pairs (IG6TM2_P2_4) were likely to be mediated by microhomologies (bold and dashed underlined). (b) Summary of phenotyping (mechanical inoculation of plantlets with BaMMV-ASL followed by DAS-ELISA for viral particles) and genotyping of M_2 progeny of primary mutants in the 'Igri' background. (c) Thousand-grain weight of heterozygous and homozygous M_2 mutants compared with non-mutated segregants and 'Igri' wild-type plants grown under the same conditions. No significant differences were observed according to the ANOVA and post hoc Tukey's test ($P > 0.05$). (d) Number of grains per plant of heterozygous and homozygous M_2 mutants compared with non-mutated segregants and 'Igri' wild-type plants grown under the same conditions. No significant differences were observed according to the ANOVA and post hoc Tukey's test ($P > 0.05$).

that were sequenced carried either heterozygous/chimeric mutations and/or the non-mutated wild-type allele, while all but one resistant plant showed heterozygous or homozygous InDels between 1 and 21 bp in size.

For target motif 2 (ATG), 95 M_2 plants derived from the primary mutants GPTM2_P9, _P11, _P22, _P30 and _P50 were analysed. Two plants, GPTM2_P11_6 and GPTM2_P22_4, were PCR-negative for *cas9*, resistant to viral infection and had a homozygous 1-bp insertion or 21-bp deletion, respectively. Among the progeny of the primary mutant GPTM2_P11, two plants were identified that carried a homozygous 3-bp insertion. The insertion of 3 bp after base-pair position four of the *PDIL5-1* coding sequence led to the conversion of the second amino acid (aspartate to alanine) and the addition of one amino acid (tyrosine), whereas the rest of the protein sequence remained unaltered (see Data S1). These plants were susceptible to BaMMV

infection, while their siblings with other heterozygous/chimeric mutations or homozygous 1-bp insertions were resistant to the infection. Among the progeny of the primary mutant GPTM2_P30, two plants with biallelic mutations were identified, with one allele carrying a 1-bp deletion and the other allele carrying the same 58-bp deletion that was already found at the same target site in the 'Igri' background. Both plants were resistant to BaMMV infection. Among the seven sequenced progeny of the primary mutant GPTM2P50, none carried any mutation, although five of them were found to be ELISA-negative for BaMMV particles, which was most likely due to incomplete mechanical inoculation.

For target motif 4 (exon 3), four M_2 families derived from primary mutants GPTM4_P5, _P11, _P14 and _P21 with 67 plants in total were analysed. Among 12 M_2 progeny of the primary mutant GPTM4_P5, six individuals exhibited a heterozygous/chimeric mutation state. One plant carried a 2-bp homozygous deletion at the target motif, while another five siblings were homozygous for a substitution of two base pairs, by which the triplet ATC at positions 235–237 of the *PDIL5-1* coding sequence was converted into TAC (see Figure 4a and Data S1). This polymorphism led to an amino acid change from a nonpolar, hydrophobic isoleucine to a polar, aromatic tyrosine within the thioredoxin-like domain at position 79 of the PDIL5-1 protein and is referred to as the I79Y mutant in the following (see Figure 4b). The three-dimensional structures of the wild-type HvPDIL5-1 protein and the I79Y variant were modelled using SWISS-MODEL on the Expasy platform (Waterhouse *et al*., 2018). The protein structure (see Figure 4c) did not seem to be impaired by this specific amino acid exchange, but nonetheless, all I79Y mutants showed resistance to BaMMV infection.

The thousand-grain weight and number of grains per plant were again measured and analysed group-wise per target motif and mutation state (see Figure 3c,d). In the 'Golden Promise' background, ANOVA revealed significant differences in the thousand-grain weight and grain number per plant ($P < 0.05$). Multiple comparison via the post hoc Tukey's test revealed statistically significant differences between non-mutated segregants and homozygous mutants of target motif 2, with the mutants having a higher thousand-grain weight ($P > 0.05$) and more grains ($P > 0.01$). In contrast, no statistically significant differences were found in comparisons between any other groups.

In summary, targeted mutations were induced in three of four target motifs. InDels carrying a loss of the translational reading frame as well as in-frame mutants with base substitutions were identified as rendering winter barley cv. 'Igri' and spring barley cv. 'Golden Promise' resistant to bymovirus infection. T-DNA-free, homozygous mutants with the resistant phenotype were obtained in the M_2 generation.

Discussion

cas9 endonuclease efficiently induces mutations in *PDIL5-1*

Upon *cas9*-induced mutagenesis of the *PDIL5-1* gene of barley, homozygous virus-resistant plants were generated in the M_2 generation. The proportion of mutants among primary transgenic plants was 0%–57%, which is on par with previously published results in targeted mutagenesis with *cas9* in barley (see Koeppel *et al*. (2019), Table 1). The comparatively high proportion of homozygous mutants in M_1 using the cultivar 'Igri' indicated that *cas9* and gRNA could induce mutations prior to spontaneous genome duplication, which is a frequent occurrence during (haploid) microspore-derived plant regeneration (see also Hoffie *et al*. (2021)). However, for the same reason, T-DNA-free individuals occurred rather rarely among Cas endonuclease-induced mutants with an 'Igri' background.

In 'Golden Promise', all primary mutants carried heterozygous or chimeric mutations. Due to independent segregation of integrated T-DNAs and induced mutations, transgene-free (*cas9* PCR-negative), homozygous mutants occur in the M_2 generation as expected.

Microhomologies present in the target region contribute to the diversity of mutation patterns

At three of four targeted positions within the *HvPDIL5-1* sequence, mutations were obtained. Small deletions of 1 or 2 base pairs were mostly observed, corresponding to the typical pattern resulting from the non-homologous end joining (NHEJ) DNA repair pathway. In contrast, larger deletions of up to 58 base pairs were more likely to be triggered by microhomologies within the target region (see Figures 2a and 3a). The principle of microhomology-mediated end joining (MMEJ) is dependent upon sequence repeats of at least 2 bp present on either side of the DNA double-strand break. This condition provides the option that the two 5′-ends of the DNA single strands anneal with one another owing to their complementarity along those repeats (Seol *et al*., 2018). The MMEJ process entails the deletion of the nucleotides residing between the repeats (if there are any) along with one of the repeats. MMEJ is one of the predominant DNA repair pathways in targeted mutagenesis and has been frequently observed for *cas9*-mediated mutagenesis in plants (reviewed in van Vu *et al*. (2021)). Here, it is demonstrated that MMEJ also works very efficiently in barley and thus may be used for the selection of target motifs, by which the predictability of resultant mutations can be increased.

Site-specific induced mutations conferring resistance to BaMMV infection are similar to haplotypes present among barley genetic resources

The progenies of primary mutants were genotyped with regard to mutation patterns and the presence of T-DNA and were phenotyped for BaMMV resistance. For target motifs 1, 2 and 4, mutants resistant to BaMMV inoculation were identified.

At target motif 1, which resides in the 5′-UTR, predominantly small deletions resulted in resistant mutants, while in the progeny of GPTM1_P1, a larger in-frame deletion of 21 bp retained susceptibility in 'Golden Promise'. In the same progeny, a plant with an 18-bp deletion in the 5′-UTR was found to be ELISA-negative for BaMMV particles. Since the latter was just a single case, it was difficult to judge whether the result was due to incomplete inoculation or because of a particular disturbance of *PDIL5-1* translation. Reduction of target gene expression by mutations in UTRs has been described, for instance by Assou *et al*. (2021), where the reduction of a seed storage protein was observed in plants carrying mutations in the 5′- and 3′-UTRs, while frameshift mutations in the coding region led to a complete absence of the encoded protein. The UTRs of genes have many complex functions in mRNA transport, splicing and translation, which are mostly ensured by the appropriate binding sites for several proteins and complexes. In the case of the 5′-UTR, especially the binding of the eukaryotic translation initiation factors and the small subunit of the ribosome, initiating the

(a)

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TACATCATCGACCCACCCCGACTGGCGTATCCGC      GP wild-type
TACATCATC----------------------CGC      GPTM1_P1_4
TACATCATCGACCCACCC-GACTGGCGTATCCGC      GPTM1_P16_12
TACATCATCGACCCACGCTAGTACTGCGCCGTATCCGC  GPTM1_P16_20
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```
AGGACCTCGGAAAGGTCATCGAAGGTAC   GP wild-type
AGGACCTCGGAAAGGTC--CGAAGGTAC   GPTM4_P14_14
AGGACCTCGGAAAGGTCATACGAAGGTAC  GPTM4_P21_10
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GTATCCGCCTTCTCCTCGTCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCGAGCCATGGATCCGGCTCTTCGGCGCC  GP wild-type
GTATCCGCCTTCTCCTCGTCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCG---------------------GCGCC  GPTM2_P22_4
GTATCCGCCTTCTCCCCGCCTCCCCGTCTTCGCCGTGGCCGTCCTCCGTTCGAGCCATGG-TCCGGCTCTTCGGCGCC  GPTM2_P30_10-a1
GT----------------------------------------------------------ATCCGGCTCTTCGGCGCC  GPTM2_P30_10-a2
```

(b)

Plants		Phenotype	Genotype					
Primary mutant (M_1)	Progeny (M_2)	BaMMV-free (ELISA)	T-DNA-free plants	Sequenced plants	Non-mutated	Heterozygous mutants	Homozygous mutants	T-DNA-free mutants
Target Motif 1								
GPTM1_P1	20	4	4	6	1	2	3	1
GPTM1_P7	19	0	3	3	0	3	0	1
GPTM1_P12	20	15	5	13	1	9	3	2
GPTM1_P16	17	15	2	9	0	5	4	2
GPTM1_P23	18	6	6	8	2	4	2	0
Target Motif 2								
GPTM2_P9	20	18	2	19	4	12	3	1
GPTM2_P11	20	6	4	8	0	3	5	3
GPTM2_P22	18	16	2	15	0	9	6	1
GPTM2_P30	18	7	3	5	1	4	0	0
GPTM2_P50	19	5	3	7	7	0	0	0
Target Motif 4								
GPTM4_P5	20	17	7	12	0	6	6	6
GPTM4_P11	19	17	4	9	1	8	0	1
GPTM4_P14	16	13	1	9	0	5	4	1
GPTM4_P21	12	9	1	8	0	5	3	1
sum	256	148	47	131	17	75	39	20
Wild-type								
Golden Promise	11	3	11	5	5	0	0	-

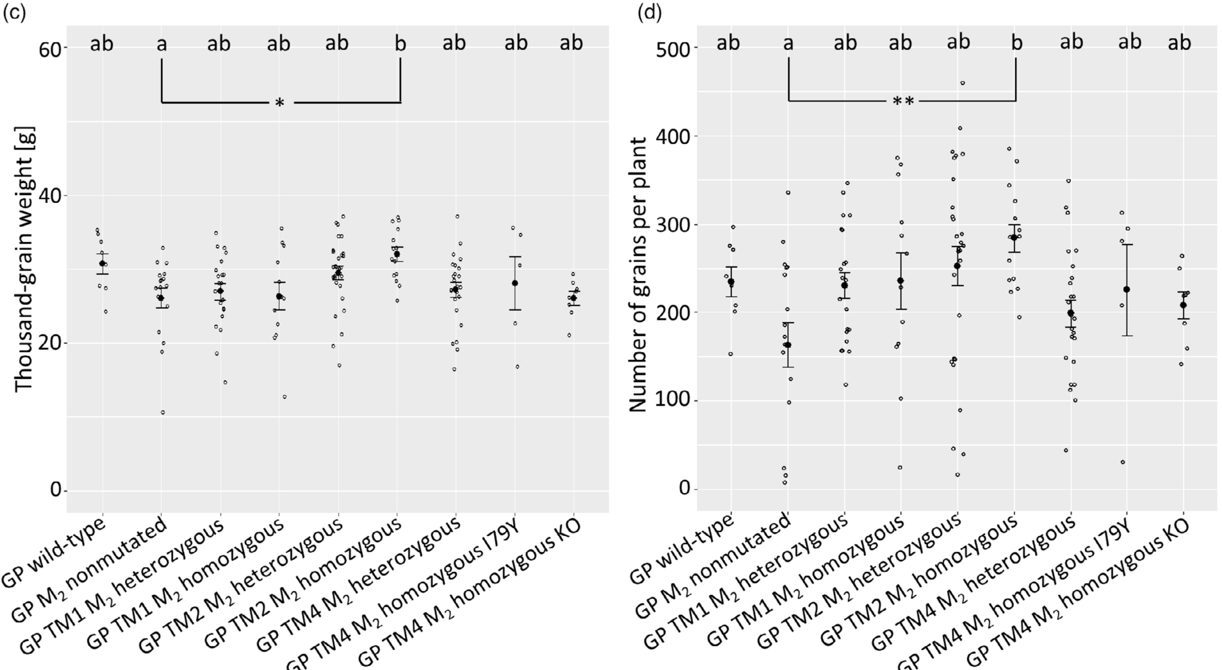

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Figure 3 (a) Alignment of sequences from homozygous M_2 mutants (InDels) in the 'Golden Promise' (GP) background (target motifs 1, 2 and 4) to the wild-type sequence of the respective target region. The cleavage site of *cas9* is indicated by scissors, deletions by dashes and insertions in red. Larger deletions of 21 base pairs (GPTM1_P1_4) up to 58 base pairs (GPTM2_P30_10 allele 2) were likely to be mediated by microhomologies (bold and dashed underlined). (b) Summary of phenotyping (mechanical inoculation of plantlets with BaMMV-ASL followed by DAS-ELISA for viral particles) and genotyping of M_2 progeny of primary mutants in the 'Golden Promise' background. (c) Thousand-grain weight of heterozygous and homozygous M_2 mutants for each target motif compared with non-mutated segregants and 'Golden Promise' wild-type plants grown under the same conditions. Significant differences were only observed between non-mutated segregants and homozygous mutants at target motif 2, according to the ANOVA and post hoc Tukey's test ($P < 0.05$), but not between the other variants ($P > 0.05$). (d) Number of grains per plant of heterozygous and homozygous M_2 mutants for each target motif compared with non-mutated segregants and 'Golden Promise' wild-type plants grown under the same conditions. Significant differences were only observed between non-mutated segregants and homozygous mutants at target motif 2, according to the ANOVA and post hoc Tukey's test ($P < 0.01$), but not between the other variants ($P > 0.05$).

translation of the mRNA into the protein sequence is of particular importance (Leppek *et al*., 2018; Roos and de Boer, 2021). Detection of the start codon AUG is crucial in this process, for which mutations in the 5′-UTR may be deleterious.

Deletions and insertions at target motifs 2 (at the translation start) and 4 (within exon 3) led to frame shifts of the translational reading frame, causing a nonsense protein sequence and stop codons that prevented the formation of functional PDIL5-1 protein. The mutations that lead to the loss of ATG are similar to the haplotype XXVIII (*rym11*-a, Yang *et al*. (2017)), featuring a large deletion of 1375 bp, including ATG and the first exon, and thereby preventing the translation of a functional transcript (see Table 1). Small InDels in exon 3 resulting in shifts of the reading frame are similar to haplotype II, which was described as a 17-bp deletion in exon 3 and was found to be the most abundant BaMMV-resistant haplotype. Barley landraces with the aforementioned haplotypes of the *PDIL5-1* gene showed complete resistance to all known strains of BaYMV and BaMMV (Yang *et al*., 2017), suggesting a corresponding effect for the *cas9*-induced alleles that were generated in the present study. Resistance of these novel mutants to the isolate BaMMV-ASL was already demonstrated under climate chamber conditions within the scope of our study. A similar experiment involving mechanical inoculation of mutant plants with BaYMV did not

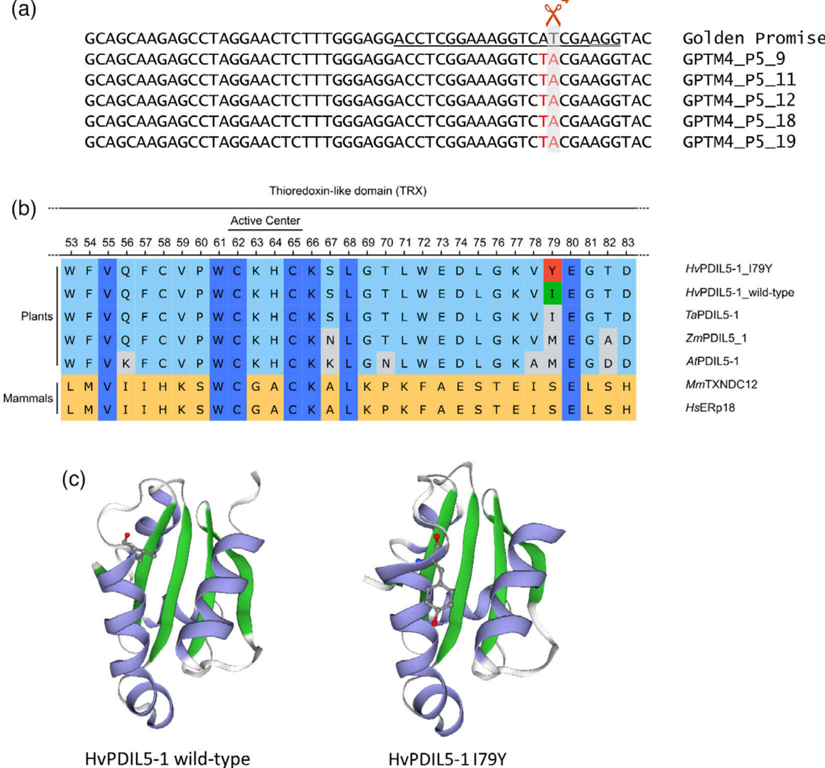

Figure 4 (a) Alignment of sequences from five homozygous M_2 progeny plants of the primary mutant GPTM4_P5 (target motif 4), all carrying the same 2-base-pair substitution causing an amino acid change from isoleucine to tyrosine at position 79 in the resultant protein sequence. The *cas9* cleavage site is indicated by scissors and the substitution in red. (b) Alignment of the thioredoxin-like domain (TRX) protein sequence of *Hv*PDIL5-1 with paralogs from selected plant and mammalian species. Conserved amino acids across all species are shown in dark blue, conserved sequence across plant species in light blue, the isoleucine at position 79 in green and the tyrosine at position 79 of the mutant plants in red. Amino acid positions given for *Hv*PDIL5-1. *Hv*: *Hordeum vulgare*, *Ta: Triticum aestivum, Zm: Zea mays, At: Arabidopsis thaliana, Mm: Mus musculus, Hs: Homo sapiens*. (c) Three-dimensional models based on the SWISS-MODEL of the protein structures of *Hv*PDIL5-1 wild-type protein and the I79Y variant. Structures of isoleucine (wild-type) and tyrosine (I79Y) at position 79 in the protein sequence are highlighted.

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Table 1 Comparison of resistant barley haplotypes of *Hv*PDIL5-1 according to Yang *et al.* (2017) and induced mutations leading to resistance in the present investigation

genomic location	Barley germplasm		Induced mutations	
	Haplotype	Sequence variation	Target	Type of mutation
5′-UTR	–	–	1	Frameshift InDels around pos. -64 bp genomic
5′-UTR/Exon 1	XVIII	1375-bp deletion pos. −567 to 789 bp genomic	2	58-bp deletion pos. −54 to 4 bp genomic 22-bp deletion pos. −3 to 19 bp genomic
Exon 1	-	–	2	Frameshift InDels around pos. 4 bp cds
Exon 3	II	17-bp deletion 315–331 bp cds	4	Frameshift InDels around pos. 236 bp cds
Exon 3	XVII	Base substitution A239G cds, resulting in amino acid substitution E80G	4	Base substitutions A235T + T236A cds, resulting in amino acid substitution I79Y

succeed, not even in the susceptible wild-type control plants virus particles were detected. While mechanical inoculation with BaMMV has been reported in 2007 and used to study new BaMMV pathotypes and resistant barley genotypes since then, mechanical inoculation of barley with a *GFP*-carrying BaYMV strain was published only recently (Tanokami *et al*., 2021). However, the infection rates reported were rather low, being 21%–60% in a susceptible genotype. Of note, also resistant genotypes proved infected by this method with up to 27% efficiency. Taken together, there is no published example yet of an assay based on leaf inoculation providing conclusive data on the resistance of barley to BaYMV. Consequently, growth on *Polymyxa graminis*-infected soil remains the only viable opportunity left to validate the resistance of the *pdil5-1* knockout mutants to BaYMV.

No effect on susceptibility was found in the case of an in-frame mutation at target motif 2 involving a 3-bp (CGT) insertion after position 4 of the coding sequence, which is associated with the addition of a further amino acid after the initial methionine and substitution of the second amino acid. The small modification at the N-terminus of the protein is unlikely to alter its structure or function.

At target motif 4 in exon 3, in addition to small insertions and deletions, a substitution of two base pairs was induced in the heterozygous state in the primary mutant GPTM4_P5 (see Figure 1d). Among its progeny, five homozygous mutants were identified for that substitution (see Figure 4a) that led to a change of the amino acid isoleucine to tyrosine at position 79 in the PDIL5-1 protein (see Figure 4b). At position 80, an amino acid exchange of glutamic acid to glycine has been described for the resistant haplotype hap-XVIII in a highly conserved position of the thioredoxin domain of PDIL5-1 (Yang *et al.*, 2014; Yang *et al.*, 2017). Comparisons with homologous proteins from wheat (TaPDIL5-1), maize (ZmPDIL5-1), Arabidopsis (AtPDIL5-1), mouse (MmTXNDC12) and human (HsERp18, see Figure 4b) suggest that the amino acid at position 79 is more variable than the one at position 80. The substitution of isoleucine, for example methionine or serine, is often well tolerated, whereas tyrosine, the only aromatic amino acid in the comparison of amino acid sequences shown in Figure 4b, is more likely to alter protein function (Barnes and Gray, 2005). Taken together, the tyrosine-for-isoleucine substitution at position 79 has a comparable effect to the nonsynonymous mutation in hap-XVIII that may reduce the functionality of the protein. It is worth mentioning in this context that hap-XVIII is the only resistance-mediating haplotype identified in wild barley (*Hordeum spontaneum*), whereas all others originate from domesticated barley accessions (Yang *et al*., 2017), suggesting a certain advantage of residual PDIL5-1 function over complete knockout under more challenging environmental conditions.

Loss of HvPDIL5-1 function has no apparent adverse effects on plants

In the present investigation, no negative effect on yield-relevant traits was observed under climate chamber and greenhouse conditions, either in the PDIL5-1 I79Y mutants or in the *pdil5-1* knockout lines (see Figures 2c,d and 3c,d). All the studied mutants showed normal development and exhibited no significant decrease in thousand-grain weight or grain number per plant. Surprisingly, there was a significant difference between non-mutated segregants in the 'Golden Promise' background and homozygous mutants for target motif 2, with the mutants having a higher yield than the non-mutated plants. However, this effect was not consistent with the 'Igri' background, where the comparison of mutants for the very same target motif vs. wild-type did not reveal such a difference. Together with the numerous described loss-of-function alleles in landraces, it can be assumed that the full knockout of *PDIL5-1* had no substantial negative effects on the plants. However, further studies, especially in the field and/or under a variety of stress conditions, are necessary to confirm this observation. For this purpose, the perfectly isogenic lines produced in the present study constitute excellent material, whereas linkage drag poses a standard problem in material generated via back-crossing. At present, how the knockout of a highly conserved protein such as *PDIL5-1* incurs no penalty remains an unanswered question. The most likely scenario is that at least one other protein disulfide isomerase (PDI) of barley is capable of compensating for the function of PDIL5-1. Specific data are lacking for barley, whereas 22 PDIs have been identified in maize and nine in wheat (d'Aloisio *et al*., 2010; Houston *et al*., 2005). No interaction of PDIs and plant viruses has yet been described in any other plant species, rendering the utilization of *PDIL5-1* alleles particularly interesting for the breeding of barley and perhaps other cereal crop plants. Intriguingly, in humans, PDIs have been demonstrated to facilitate the uptake of HIV into host cells by decomposing disulfide bridges in the envelope protein of this virus (Barbouche *et al*., 2003). In addition, PDIs have been described as potential targets for influenza and other RNA viruses (Kim and Chang, 2018; Mahmood *et al*., 2021). Since the mechanism of PDIL5-1-based resistance in barley remains elusive, conclusions about the potential stability of resistance are vague. To break

EIF4E-mediated resistance, the viruses adapted the protein structure of their genome-linked viral protein (VPg) cap so that it was recognized again by the modified eIF4E protein, thereby initiating translation of the viral RNA (Li *et al*., 2016). However, the loss of function of *PDIL5-1* makes such comparatively simple viral adaptation unlikely. Pyramiding different resistance-mediating alleles from different genes allows for the combination of diverse resistance mechanisms, thus slowing down the ability of pathogens to overcome resistance. Against BaYMV and BaMMV, marker-assisted selection coupled with doubled haploid production after crossing was used to stack the three resistance loci *rym4*/*rym5*, *rym9* and *rym11* (Werner *et al*., 2005). Despite being a valid approach for classical breeding, such crosses come with off-trades, such as linkage drag and exponential growth of the population size associated with every additional locus to be introgressed. As a more viable and rapid alternative, state-of-the-art genome engineering approaches offer the possibility of generating resistance alleles of a variety of genes in one and the same elite background.

In summary, bymovirus resistance-conferring alleles of *PDIL5-1* were identified in the barley gene pool, as represented by ancient landraces and wild relatives of cultivated barley. The present study provides a proof-of-concept for how those alleles can be generated in barley cultivars by site-directed mutagenesis, circumventing tedious crossings and series of backcrossing, as well as any linkage drag and making the available genetic diversity more accessible to enhance future breeding efforts.

Methods

Target selection, off-target analysis and vector cloning

Target motifs within the *HvPDIL5-1* gene (GenBank: HG793095.1) were individually selected based on their position within the gene sequence, the presence of an NGG protospacer-adjacent motif (PAM) and the 2-dimensional minimum free energy structures of the cognate single-gRNAs (NNNNNNNNNNNNNNNNNNNNGU UUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCG UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU) as modelled by the RNAfold WebServer (Gruber *et al*., 2008). Target motifs were selected to obtain either early shifts of the translational reading frame or to induce single nucleotide polymorphisms in exon 3. The localization of target motifs and their sequences, including the PAM, are shown in Figure 1a,b and Data S1. To the cognate gRNA of target motif 2, an adenosine that does not match with the target motif was added to the 5′-end to include a *U3* promoter-compatible transcription start. For the same reason, the specific 5′-parts of the gRNAs for target motifs 3 and 4 comprised only 19 nucleotides instead of the standard 20 nucleotides to feature an A as the first base.

Off-target analysis was performed by a BLAST search of the target motif, including the PAM, against the barley reference genome 'Morex' v3 and the genomes of 'Golden Promise' and 'Igri' with the GrainGenes BLAST Service (Jayakodi *et al*., 2020; Priyam *et al*., 2019), by which no potential off-targets were detected (see Data S1).

Single-stranded DNA oligonucleotides (see Table S1) were used for every gRNA as forward and reverse strands with overhangs for BsaI-based insertion into the generic vector pSH121 (GenBank-ID: MW145140.1, Gerasimova *et al*. (2018)) containing expression units of a rice *U3* promoter-driven gRNA scaffold and a maize codon-optimized *cas9* from *Streptococcus pyogenes* regulated by the maize *POLYUBIQUITIN 1* promoter.

For *Agrobacterium*-mediated barley transformation, these transgenes were directionally cloned via SfiI digestion and ligation into the binary vector p6i-2x35S-TE9 (DNA cloning Service, Hamburg, Germany) carrying a doubled enhanced *CaMV 35 S* promoter-driven *hpt* as a plant selectable marker gene and left and right border sequences for *Agrobacterium*-mediated gene transfer. The vectors were named pBin_PDIL5-1_TM1, pBin_PDIL5-1_TM2, pBin_PDIL5-1_TM3 and pBin_PDIL5-1_TM4.

Agrobacterium-mediated barley transformation

Winter barley cv. 'Igri' transformation using the vector pBin_PDIL5-1_TM2 was conducted by *Agrobacterium tumefaciens*-mediated DNA transfer into embryogenic pollen cultures according to Kumlehn *et al*. (2006). In brief, highly vacuolated, immature pollen at the microspore stage was isolated from cold-treated spikes and cultivated for 1 week. For DNA transfer via *Agrobacterium* strain LBA4404/pSB1 carrying pBin_PDIL5-1_TM2, cell proliferation-undergoing pollen and agrobacteria were co-cultivated for 48 h, followed by pollen cultivation in the presence of Timentin to remove *Agrobacterium* and in the presence of hygromycin to facilitate the preferential development of transgenic tissue and regenerating plantlets.

Agrobacterium-mediated DNA transfer to immature embryos of the spring barley cv. 'Golden Promise' using pBin_PDIL5-1_TM1, pBin_PDIL5-1_TM2, pBin_PDIL5-1_TM3 and pBin_PDIL5-1_TM4 was performed as previously described by Hensel *et al*. (2009); immature embryos were excised from caryopses 12–16 days after pollination and cocultivated with *Agrobacterium* strain AGL1 carrying the respective binary vector for 48–72 h. Then, the explants were cultivated for callus induction under selective conditions using Timentin and hygromycin followed by plant regeneration.

DNA was extracted from leaf samples using a phenol–chloroform-based protocol as described by Pallotta *et al*. (2000). The presence of T-DNA in regenerated plantlets was confirmed by PCR for *cas9* (for primer sequences, see Table S1).

Amplification and sequencing of target regions of primary mutants

Mutant plants were identified by PCR amplification of the respective target region (for primer sequences, see Table S1) followed by Sanger sequencing. Double peaks in the chromatogram starting upstream of the PAM (see Figure 1c,d) were considered as an indication for chimeric and/or heterozygous mutants. Sequence alignments were performed to characterize the mutation patterns of plants with homozygous insertions or deletions. Mutant plants were grown in a greenhouse until the formation of mature grains.

Mechanical BaMMV and BaYMV inoculation and analysis of M_2 plants

Progenies of primary mutant plants were mechanically inoculated with the BaMMV 'Aschersleben' isolate (BaMMV-ASL) to screen for bymovirus resistance according to Habekuss *et al*. (2007). This procedure is a viable alternative to vector-mediated virus infection by *Polymyxa graminis*.

In material with the 'Igri' background, 20–40 grains each of five M_2 families mutated in target motif 2 were sown in a growth chamber at 12 °C and a 16-h photoperiod, and nine to 20 siblings were obtained. Likewise, material with the 'Golden Promise'

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background was grown, sowing 20 grains each of five M_2 families mutated in target motifs 1 and 2, as well as four M_2 families mutated in target motif 4. Two weeks after germination, mutant and wild-type seedlings were inoculated twice at five- to seven-day intervals with leaf sap from BaMMV-infected barley plants. Six to eight weeks after the first inoculation, virus infection was determined by evaluation of phenotypic symptoms (yellow mosaics) on newly developed leaves. In addition, a double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) was performed with fresh leaves from the inoculated plants. At an extinction of $E405 \leq 0.1$, plants were considered resistant to BaMMV-ASL infection. In parallel, DNA was extracted from leaf samples of selected plants to test for the presence of the *cas9* transgene by PCR. Furthermore, target regions were PCR-amplified, followed by Sanger sequencing of PCR products for genotyping of a subset of mutants.

The same procedure was used to inoculate four selected M_2 families carrying mutations in target motif 2 ('Igri' and 'Golden Promise' background) and target motif 3 ('Golden Promise' background) with BaYMV.

Yield data

Selected M_2 plants were grown to maturity after resistance screening in a greenhouse with 18–20 °C/12–14 °C day/night temperatures with a 16-h photoperiod. The plants were harvested, the ears were threshed, and the total grain number and the total grain weight were recorded for every single plant. The thousand-grain weight (TGW) was calculated as follows:

$$\text{TGW} = \frac{\text{Grain weight}}{\text{Grain number}} * 1000$$

Yield data were collected separately for the two barley genotypes used, as well as for the transformation constructs carrying different target motif-specified gRNA expression units. According to their mutation status, the segregating M_2 plants were then divided into groups for comparison (heterozygous/chimeric mutant, homozygous mutant and non-mutant) to each other and to wild-type plants. One-way analysis of variance (ANOVA) followed by a post hoc Tukey's test (Tukey HSD) was performed using R version 3.6.1 software (R Core Team, 2019). To generate the plots, the R package ggplot2 was used (Wickham, 2016). Significance code: $***P \leq 0.001$; $**P \leq 0.01$; $*P \leq 0.05$.

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Conflict of interests

The authors declare no conflicts of interest.

Author contributions

D.P., J.K., F.O. conceived the project and acquired the funding. J.K., R.E.H., D.P. developed the experimental setup. R.E.H., D.P. and A.H. performed the experiments and analysed the data. R.E.H. drafted the manuscript, and all authors edited and approved 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.

Table S1 Oligonucleotides used as primers and for cloning of gRNAs.

Table S2 M_2 plant yield data and results of BaMMV infection assay Igri, target motif 2.

Data S1 Off-target analysis.

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