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CRISPR/Cas9-targeted mutagenesis of a representative member of a novel PR10/Bet v1-like protein subfamily significantly reduces rice plant height and defense against *Meloidogyne graminicola*

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Abstract

Plant PR10/Bet v1-like proteins are involved in defense against microbial pathogens, however, whether they participate in host defense against plant-parasitic animals including nematodes remains unknown. Here, according to the phylogeny, sequence-similarity, and secondary structure analyses, we found that the rice OsBet v1 protein and its homologs constitute a new subfamily of PR10/Bet v1-like protein. Reverse transcription-quantitative PCR assay showed that *OsBet v1* was highly expressed in rice roots and induced by *Meloidogyne graminicola* infection. Using a transient expression assay in rice protoplasts, we demonstrated that OsBet v1 was localized in the cytoplasm and nucleus. Furthermore, CRISPR/Cas9-targeted mutagenesis of *OsBet v1* significantly increased rice susceptibility to *M. graminicola*, but reduced rice plant height. In addition, the expression levels of two peroxidase genes (*peroxidase 5* and *peroxidase 56*) were significantly down-regulated in *OsBet v1* knockout mutants compared with those in wild-type rice plants. Taken together, this study identified a new PR10/Bet v1-like protein subfamily in plant and revealed the involvement of OsBet v1, a representative member of the subfamily, in rice growth and defense against *M. graminicola*. This provides a new insight into the role of plant PR10/Bet v1-like proteins in plant–nematode interactions.

Keywords: *Meloidogyne graminicola*, *OsBet v1*, CRISPR/Cas9, PR10/Bet v1-like protein, Novel protein subfamily

Background

In nature, a broad spectrum of pathogens including fungi, bacteria, viruses, and nematodes attack plants and cause major yield reduction. To fight multiple biotic stresses, plants have developed varied defense mechanisms including activation of genes involved in different defense-related signaling pathways, of which the induction and accumulation of pathogenesis-related (PR) proteins in plants are significantly important (Fritig et al. 1998; Ji et al. 2015). The first plant PR protein was identified in tobacco plants infected by tobacco mosaic virus (van Loon and van Kammen 1970). Later, PR proteins were discovered in all kinds of plants. They comprise a huge superfamily, which is divided into 17 families based on protein structure, genetic relationship, and biological activity (van Loon and van Strien 1999; van Loon et al. 2006). Among them, PR10/Bet v1-like proteins are

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of particular interest due to their anti-pathogen activity. Most PR10/Bet v1-like proteins are small and acidic proteins of 15–18 kDa, with no nuclear localization signal sequences and signal peptides. The open reading frame (ORF) generally consists of two exons, approximately 456–489 bp in length, separated by one intron. The isoelectric point (pI) usually ranges from 4.75 to 6.65 (Hoffmann-Sommergruber et al. 1997; Hashimoto et al. 2004; Liu and Ekramoddoullah 2006). On the basis of sequence-similarity and phylogenetic analyses, the plant PR10/Bet v1-like protein family is usually grouped into 9 subfamilies, i.e., dicot PR-10, monocot PR-10 type I, conifer PR-10, monocot PR-10 type II, cytokinin-specific binding protein (CSBP), (S)-norcoclaurine synthase (NCS), moss PR-10-like protein, major latex protein/ripening-related protein (MLP/RRP), and polyketide cyclase-like protein (Radauer et al. 2008).

PR10/Bet v1-like proteins are ubiquitously present in the plant kingdom and play a variety of roles in plant growth, biosynthesis, metabolism, and stress resistance (Morris et al. 2021). Proteins in the CSBP subfamily have cytokinin, gibberellic acid, and trans-zeatin binding activities (Fujimoto et al. 1998; Ruszkowski et al. 2014). Members of the NCS subfamily participate in the biosynthesis of benzylisoquinoline alkaloids (Samanani et al. 2004; Luk et al, 2007; Berkner et al, 2008). Most members of the MLP/RRP subfamily are expressed in fruits and upregulated during fruit ripening (Nessler et al. 1985; Radauer et al. 2008). In addition, MLP/RRP subfamily proteins are also involved in biotic or abiotic stress responses, like the cotton protein GhMLP28, zucchini protein MLP-PG1, and sugar beet proteins BvMLP1 and BvMLP3, which enhance plant resistance to fungi (Yang et al. 2015; Fujita et al. 2021; Holmquist et al. 2021); whereas the tobacco protein NtMLP423 and *Arabidopsis thaliana* protein MLP43 improve plant tolerance to drought stress (Wang et al. 2016; Liu et al. 2020).

In rice, PR10/Bet v1-like proteins have been found to be linked to antifungal responses. For example, *OsPR10a/PBZ1*, *OsPR10b*, *JIOsPR10*, and *RSOsPR10* are reported to be induced by the invasion of *Magnaporthe grisea* (Jwa et al. 2001; McGee et al. 2001; Hashimoto et al. 2004). *Meloidogyne graminicola*, commonly known as rice root-knot nematode (RKN), is one of the most damaging nematodes on rice, causing production losses of 17–32% (Kyndt et al. 2014; Mantelin et al. 2017). Recent studies have demonstrated that rice RKN-derived effectors suppress plant defense responses via various mechanisms (Chen et al. 2017, 2018; Naalden et al. 2018; Zhuo et al. 2019; Song et al. 2021). Of these effectors, MgMO237 inhibits host defense responses by interacting with three host defense-related proteins, one of which is OsBet v1 (Chen et al. 2018). Bet v1 refers to the main allergen of birch pollen (Breiteneder et al. 1989), which is one of the best known PR10/Bet v1-like proteins and ubiquitously present in plants. However, whether Bet v1 participates in plant defense against RKN is still unknown.

In recent years, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 derived from the adaptive immunity system of *Streptococcus pyogenes* has become a more reliable gene editing tool. The Cas9 protein functions as a nuclease and is directed to a target site by an engineered sequence-specific single guide RNA (sgRNA), and thus precise gene editing is achieved (Ma et al. 2015). Recently, an increasing number of plant genes including *PR* genes have been functionally characterized by the CRISPR/Cas technology. For example, CRISPR/Cas9-mediated mutagenesis of a *PR4* gene *VvPR4b* in grapevine reduces the accumulation of reactive oxygen species around stomata and increases host susceptibility to downy mildew caused by *Plasmopara viticola*, demonstrating that *VvPR4b* functions in grapevine defense against downy mildew (Li et al. 2020a). Here, we characterized *OsBet v1*, a rice Bet v1-coding gene, and generated *OsBet v1* knockout rice mutants using CRISPR/Cas9 technology. Our results demonstrate that OsBet v1 and its homologs constitute a novel subfamily of PR10/Bet v1-like proteins; and OsBet v1 plays important roles in rice growth and defense against nematodes.

Results

OsBet v1 and its homologs form a novel subfamily of PR10/Bet v1-like proteins

The *OsBet v1* gene contains an ORF of 456 bp, separated by an intron of 145 bp. The intron/exon boundary has a conserved 5'-GT-AG-3' intron splice-site junction. The ORF encodes a 151-amino-acid polypeptide with a predicted molecular size of 16.5 kDa and pI value of 5.13. No signal peptide was found based on SignalP analysis. OsBet v1 possesses a Bet v1 domain (residues 3–143; Pfam: PF00407.19) and shares the highest amino acid identity (95.30%) with a strawberry allergen Fra a 1-E-like protein (XP_006659351) from *Oryza brachyantha*. The other 98 homologs of OsBet v1 from various plant species were also revealed through a protein BLAST search against the National Center for Biotechnology Information (NCBI) database. Alignment of the amino acid sequence of OsBet v1 and its homologs showed that all the proteins contain a conserved motif of GD/NGXPG (Fig. 1).

A neighbor-joining (NJ) tree was constructed to examine the phylogenetic relationships among these PR10/Bet v1-like proteins, with a bacterial PR10 sequence (UniprotKB accession number Q98K03) as an outgroup taxon. The resulting NJ tree contains ten major clades, nine of which belong to the known plant PR10/

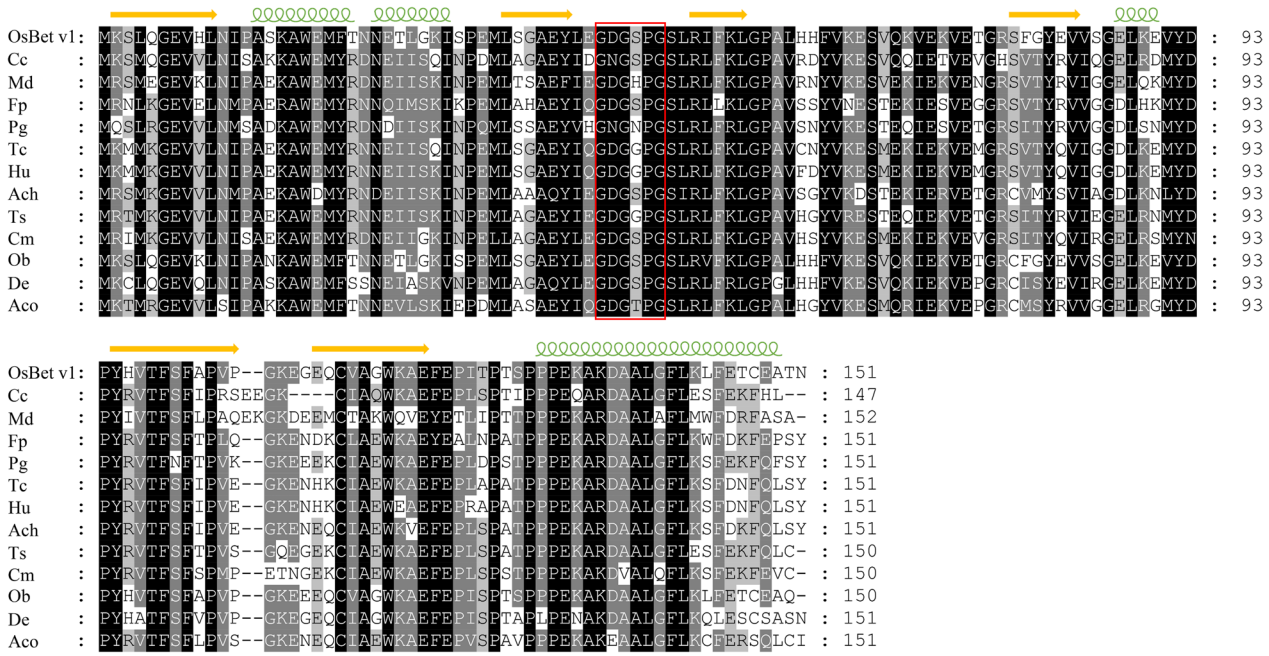

Fig. 1 Multiple sequence alignment of the predicted OsBet v1 protein with some other plant homologous PR10/Bet v1-like proteins. OsBet v1, *Oryza sativa* (LOC_Os08g28670.1); Fp, *Ficus pumila* (Q71HN3); De, *Digitaria exilis* (CAB3482801); Ts, *Tetracentron sinense* (KAF8409242); Ach, *Actinidia chinensis* (PSS05718); Cm, *Cinnamomum micranthum* (RWR89143); Ob, *Oryza brachyantha* (XP_006659351); Tc, *Theobroma cacao* (XP_007043599); Aco, *Ananas comosus* (XP_020105256); Hu, *Herrania umbratical* (XP_ 021293588); Pg, *Punica granatum* (OWM78653); Cc, *Coptis chinensis* (KAF9588434); Md, *Malus domestica* (RXH90448). The red box indicates the P-loop motif. Green wavy lines and yellow arrows indicate alpha helices and beta strands, respectively

Bet v1-like protein subfamilies described by Radauer et al. (2008), while OsBet v1 and its 99 homologs form a phylogenetically-distinct cluster that is closely related to the moss PR-10-like subfamily (Fig. 2). The amino acid identity among proteins of this cluster is greater than 56%, however it is less than 32% between members of this cluster and other PR10/Bet v1-like proteins (Additional file 1: Figure S1). In addition, the proteins of this cluster contain 4 conserved regions, i.e., region_1, region_4, region_6, and region_7 (Additional file 1: Figure S2). Unlike other PR10/Bet v1 subfamily proteins, there are four α-helical segments and six β strands in OsBet v1 (Fig. 1).

Taken together, the results suggest that OsBet v1 and its 99 homologs constitute a novel PR10/Bet v1-like protein subfamily, and we named it monocot & dicot PR-10 (Fig. 2).

OsBet v1 is expressed in all rice tissues and up-regulated upon *M. graminicola* invasion

Reverse transcription-quantitative PCR (RT-qPCR) was performed to investigate the expression levels of *OsBet v1* in different tissues of rice plants. The results showed that *OsBet v1* transcripts were detected in all examined tissues including roots. The transcript level of *OsBet v1* in shoots was set as 1, which was used to calculate the relative expression level of the gene in other tissues. Among all tested tissues, the transcript accumulation of *OsBet v1* in dry seeds was the highest, with an approximately 381-fold increase compared with that in shoots. The expression of *OsBet v1* remained at a higher level in germinating seeds and roots, with approximately 81- and 56-fold increase, respectively, compared with that in shoots (Fig. 3a).

Furthermore, the expression levels of *OsBet v1* in roots were analyzed at 2 and 7 days post-inoculation (dpi) with *M. graminicola* via RT-qPCR. As a result, the transcript levels of *OsBet v1* were significantly increased by approximately 3- and eightfold at 2 and 7 dpi, respectively, compared with that in uninfected roots (Fig. 3b).

OsBet v1 is localized in the cytoplasm and nucleus of rice cells

Transient expression assay was performed to test the subcellular localization of OsBet v1 in plant cells. Enhanced green fluorescent protein (eGFP) and OsBet v1:eGFP were transiently expressed in rice protoplast cells. After 18 h, the fluorescent signal generated by OsBet v1:eGFP was found in the cytoplasm and nucleus of transformed cells. In the control cells transformed with eGFP alone,

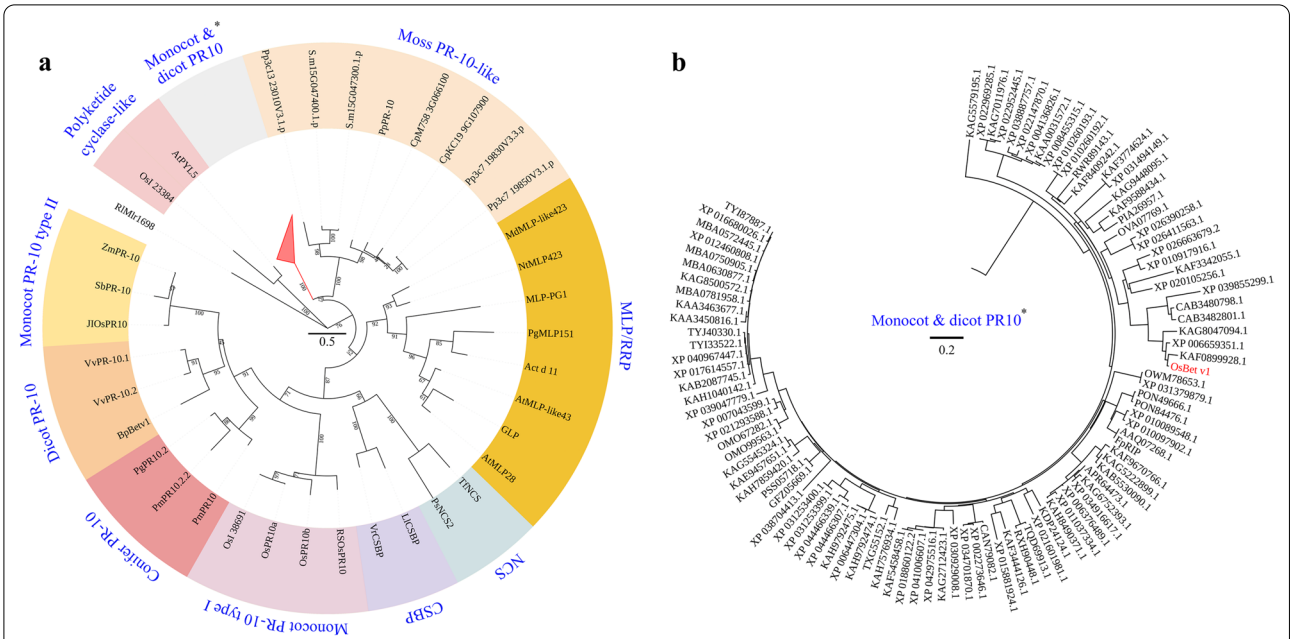

Fig. 2 The phylogenetic tree of plant PR10/Bet v1-like protein homologs. **a** Distance-based phylogenetic tree of members from all plant PR10/Bet v1-like protein subfamilies. The tree was rooted with *Burkholderia pseudomallei*. **b** Distance-based phylogenetic tree of members from the newly discovered subfamily 'Monocot & dicot PR-10' in this study. Blue letters indicate the name of each subfamily. Accession numbers of all genes are provided in Additional file 3: Table S2

the fluorescence signal was also observed in the cytoplasm and nucleus (Fig. 3c). Further Western blot using an anti-GFP antibody detected bands of approximately 44 and 27 kDa for OsBet v1:eGFP and eGFP, respectively (Fig. 3d), showing that the OsBet v1:eGFP fusion protein was intact.

CRISPR/Cas9-targeted mutagenesis of *OsBet v1*

For targeted disruption of *OsBet v1* by CRISPR/Cas9, two sgRNAs targeting the first and second exon of *OsBet v1* were designed based on the online tool CRISPR-GE. These two sgRNAs and their protospacer adjacent motif (PAM) sequences are shown in Fig. 4a, with Target1's PAM sequence (CGG) located at 63 bp away from the 5' splicing site and Target2's PAM sequence (CCT) at 60 bp away from the 3' splicing site of the *OsBet v1*'s intron. An edit vector pYLC-OsBet v1 (Fig. 4b) expressing CRISPR/Cas9, two gRNAs (guide RNA, gRNA1 for Target1 and gRNA2 for Target2), and hygromycin B phosphotransferase that confers hygromycin B resistance selection marker was constructed and delivered into rice cells through *Agrobacterium*-mediated transformation.

In T_0 generation of transgenic rice plants, two homozygous mutations, *OsBet v1#1* and *OsBet v1#4*, were obtained. For *OsBet v1#1*, there is a 1-bp insertion (+A) in Target2; for *OsBet v1#4*, a 4-bp deletion (-TGTC) and a 1-bp insertion (+G) occurred in Target1 and Target2, respectively (Fig. 4c, d). Analysis of changes in amino acid sequence of these rice mutants showed that the translation of *OsBet v1* was terminated at the 81st amino acid in *OsBet v1#1* and at the 53rd amino acid in *OsBet v1#4* (Fig. 4e), indicating that the OsBet v1 protein was successfully mutated.

Possible off-target sites of the two sgRNAs were predicted using the CRISPR-P web tool. Genomic DNA was extracted from T_0 generation rice plants, and subjected to PCR and sequencing analysis. As a result, no mutations were detected in potential off-target sites of the two mutant lines (Table 1).

Identification of 'transgene-free' homozygous *OsBet v1* mutants obtained by CRISPR/Cas9 editing

To identify 'transgene-free' homozygous *OsBet v1* knockout mutants, the presence or absence of *Cas9* and sgRNA in transgenic rice plants of T_1 generation was determined by PCR. In T_1 generation plants obtained through self-pollination, two mutant plants inherited from the *OsBet v1#1* line, and seven mutant plants inherited from the *OsBet v1#4* line did not generate sgRNA- and *Cas9*-specific amplicons (Fig. 5). The results suggested that these mutants have no any

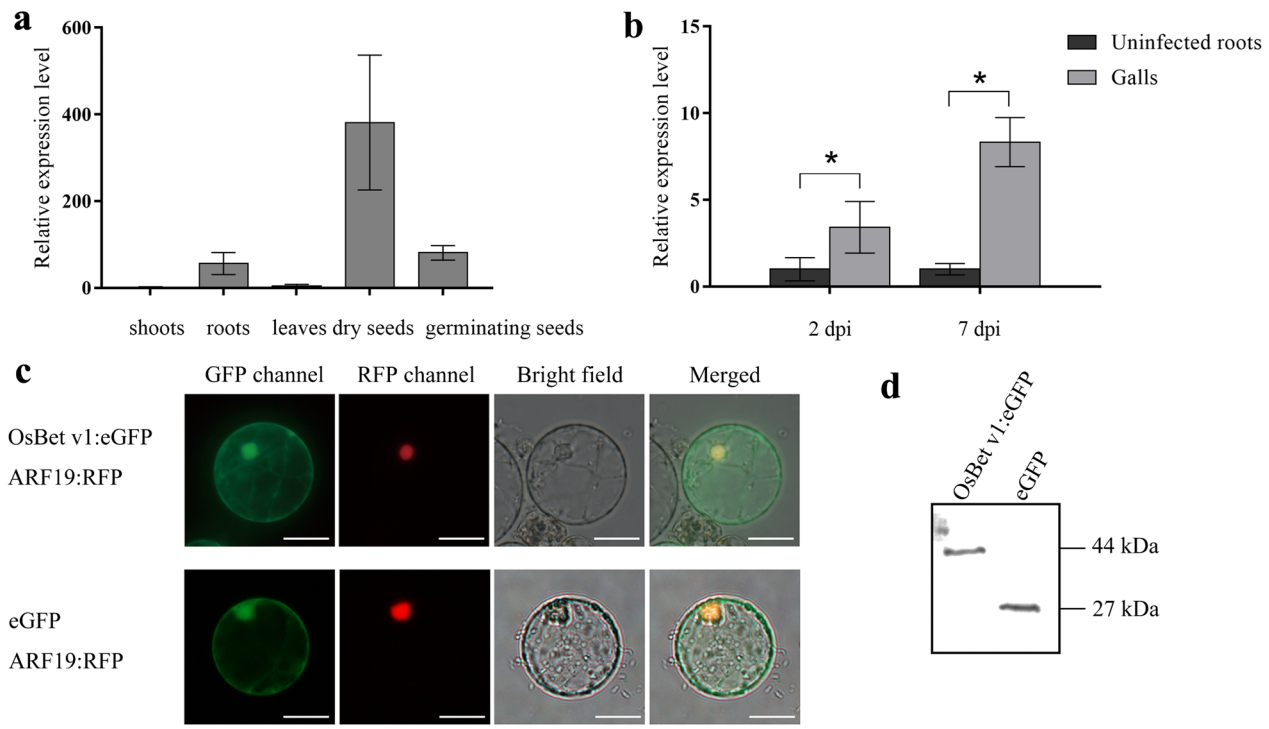

Fig. 3 Expression of *OsBet v1* in rice tissues and in response to *Meloidogyne graminicola* invasion and subcellular localization of OsBet v1. **a** Expression of *OsBet v1* in different rice organs. The level of *OsBet v1* transcript at the shoots was set as 1 and used to calculate the relative expression level of this gene in other tissues. **b** Expression of *OsBet v1* in rice roots infected with *M. graminicola* at 2 and 7 days post-inoculation (dpi). **c** Subcellular localization of OsBet v1. OsBet v1:eGFP was transformed into rice root protoplasts, with transformation of eGFP alone as the control. ARF19:RFP was used as a nuclear-localized marker. Scale bars: 20 μm. **d** Western blotting detection of the target proteins extracted from transformed cells. Protein bands with expected size of approximately 44 kDa for OsBet v1:eGFP and 27 kDa for eGFP were observed. eGFP, enhanced green fluorescent protein; RFP, red fluorescent protein. Data are presented as the mean ± SD. *$P < 0.05$, Student's *t*-test

transgenic elements of the *OsBet v1*-sgRNA/Cas9 vectors, showing that they are 'transgene-free' homozygous rice.

Disruption of *OsBet v1* slows the growth and promotes infection of rice plants by *M. graminicola*

By further self-pollinating 'transgene-free' homozygous *OsBet v1#1* and *OsBet v1#4* lines, T_2 generation were obtained and used for growth phenotype analysis and nematode inoculation. Growth phenotypes were observed at 14 days after germination. It was found that the plant heights of the mutants are obviously shorter than those of the wild-type controls (WT), as the average plant height of *OsBet v1#1* and *OsBet v1#4* was 28% and 37% shorter, respectively, than that of WT, whereas no significant difference in root length was observed between mutants and WT (Fig. 6a, b).

To assess the role of *OsBet v1* in plant susceptibility to nematode infection, T_2 generation of 'transgene-free' *OsBet v1* knockout rice plants were inoculated with *M. graminicola*. Compared with WT, *OsBet v1#1* and *OsBet v1#4* mutant lines exhibited a significantly higher susceptibility to *M. graminicola*, and the average numbers of mature females of *M. graminicola* per root in these two lines were increased by 116% and 109%, respectively (Fig. 6c).

Furthermore, the expression levels of some common defense genes were analyzed in the two mutant lines by RT-qPCR. Compared with that in WT, the expression of two peroxidase genes, *peroxidase 5* (LOC4337732) and *peroxidase 56* (LOC4327052), in these mutants were significantly down-regulated; the average expression levels of *peroxidase 5* in *OsBet v1#1* and *OsBet v1#4* were both decreased by 76%, and those of *peroxidase 56* in *OsBet v1#1* and *OsBet v1#4* were decreased by 54% and 41%, respectively (Fig. 7).

Discussion

The rice OsBet v1 protein was previously shown to interact with the *M. graminicola* effector MgMO237 (Chen et al. 2018). In this study, we analyzed the *OsBet v1* gene that encodes a protein of 16.5 kDa. The protein contains a typical Bet v1 domain with a conserved sequence motif of 'GD/NGXPG', consistent

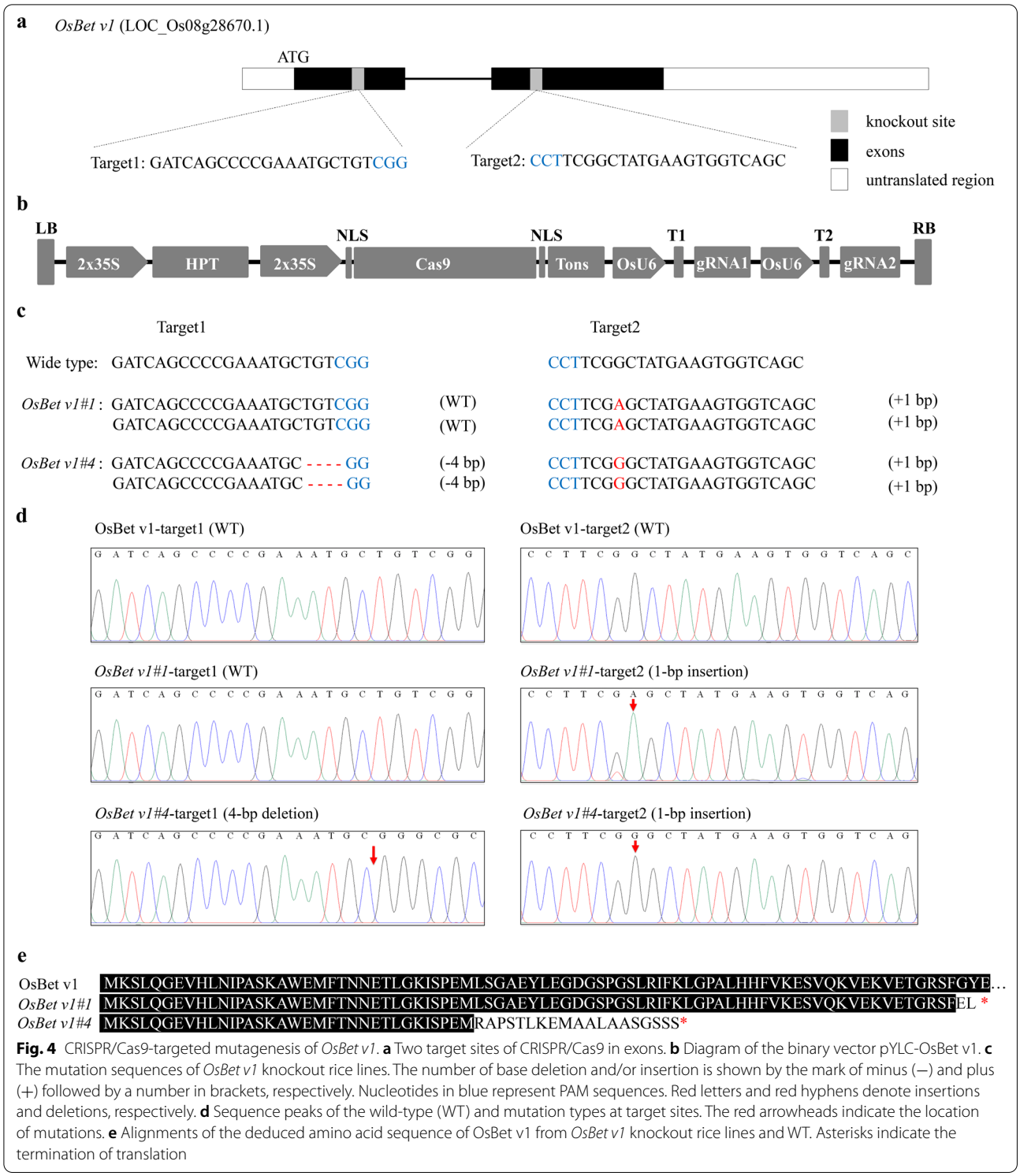

Fig. 4 CRISPR/Cas9-targeted mutagenesis of *OsBet v1*. **a** Two target sites of CRISPR/Cas9 in exons. **b** Diagram of the binary vector pYLC-OsBet v1. **c** The mutation sequences of *OsBet v1* knockout rice lines. The number of base deletion and/or insertion is shown by the mark of minus (—) and plus (+) followed by a number in brackets, respectively. Nucleotides in blue represent PAM sequences. Red letters and red hyphens denote insertions and deletions, respectively. **d** Sequence peaks of the wild-type (WT) and mutation types at target sites. The red arrowheads indicate the location of mutations. **e** Alignments of the deduced amino acid sequence of OsBet v1 from *OsBet v1* knockout rice lines and WT. Asterisks indicate the termination of translation

with the characteristics of PR10/Bet v1-like proteins (Morris et al. 2021). The PR10/Bet v1-like protein family consists of 9 plant protein subfamilies and 2 bacterial protein subfamilies (Radauer et al. 2008). We thus constructed a phylogenetic tree to examine the relationship between OsBet v1 and other plant PR10/Bet v1-like proteins. The dendrogram is generally similar to the phylogenetic tree reported by Radauer et al.

Table 1 Detection of the putative off-target effect in potential off-target sites of the rice mutants

gRNA	Putative off-target gene	Off-target sequence	No. of mismatches*	Mutation
Target1	OS11G0162700	G**C**TCAG**T**C**A**CGAAATGCT**C**T*TGG*	4	Not detected
	OS01G0748200	G**T**TCAGCCCCG**GG**AT**C**CTGT*CGG*	4	Not detected
	OS03G0828800	GAT**G**AGCCCC**C**AA**T**TGCTG**G***TGG*	4	Not detected
Target2	OS04G0409100	GCTGA**T**CAC**GA**CATAGCCGA*AAG*	3	Not detected
	OS01G0717700	**C**CT**C**A**T**CAC**G**TCATAGCCGA*AGG*	4	Not detected
	OS06G0137700	GCTG**T**C**TT**CTTCATAGCCG**G***CGG*	4	Not detected
	OS12G0570400	GCTG**T**C**TT**CTTCATAGCCG**G***CGG*	4	Not detected
	OS01G0717400	GCT**C**A**T**CAC**G**TC**G**TAGCCGA*AGG*	4	Not detected
	OS01G0215000	GC**CT**ACCACTTCAT**T**GC**T**GA*TGG*	4	Not detected
	OS09G0298500	GCTGA**T**CACTTCA**A**AGCC**CC***AAG*	4	Not detected

* Mismatching bases are in bold. Nucleotides in italics represent PAM sequences

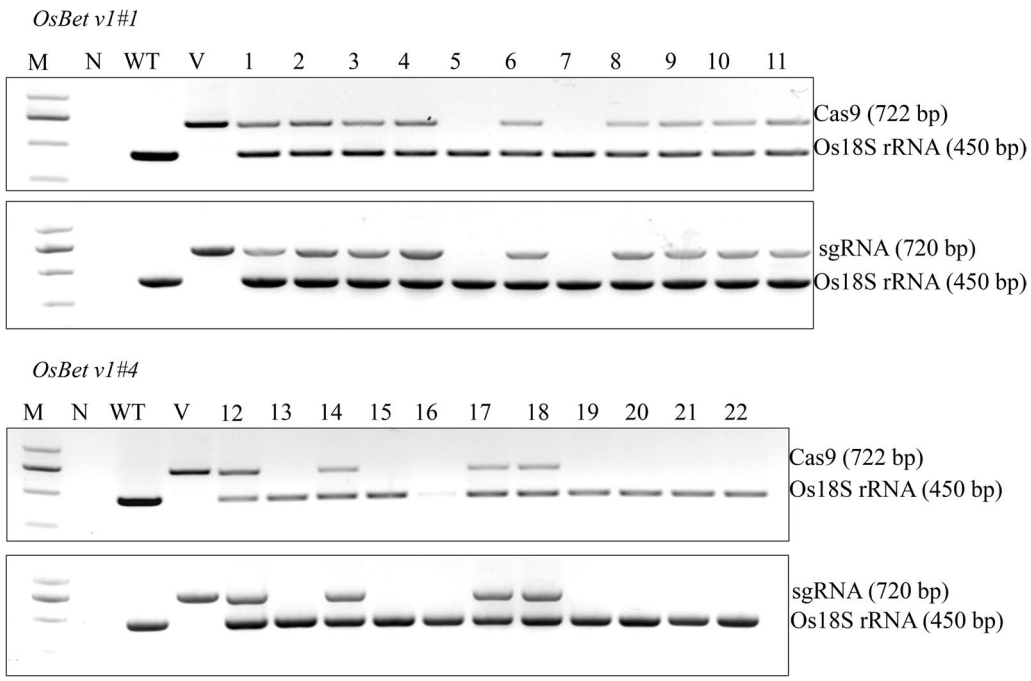

Fig. 5 Detection of 'transgene-free' homozygous *OsBet v1* knockout mutants. Cas9 (722 bp), part of the Cas9-coding sequence; sgRNA (720 bp), a region spanning from the sgRNA to the downstream vector; Os18S rRNA (450 bp) was used as a normalization control; M, DL2000 DNA ladder; N, negative control with sterilized ddH_2O as template; V, the plasmid vector; WT, wild-type plant. Lanes 1–11, 11 mutant individuals of *OsBet v1#1*. Lanes 12–22, 11 mutant individuals of *OsBet v1#4*

(2008), but OsBet v1 and its homologous proteins were grouped in a separate clade that differs from the known 9 plant PR10/Bet v1-like protein subfamilies. Amino acid sequence similarity analysis also showed that OsBet v1 and its homologs have a low similarity with the members of the known 9 plant PR10/Bet v1-like subfamilies (< 32%). Further bioinformatic analyses indicated that OsBet v1 has 4 α-helical segments and 6 β strands, and this differs from the classical Bet v1 structure which contains 3 α-helical segments and 7 β strands (Liu and Ekramoddoullah 2006). Thus, OsBet v1 and its homologs form a novel PR10/Bet v1-like protein subfamily, representing the tenth subfamily of plant PR10/Bet v1-like proteins.

In this study, we observed that OsBet v1 was localized in the cytoplasm and nucleus of rice cells. To our knowledge, PR10/Bet v1-like protein is considered an intracellular PR protein because of its specificity to be free in the cytoplasm (Lebel et al. 2010). Most PR10/Bet v1-like proteins have cytosolic localization (Liu and

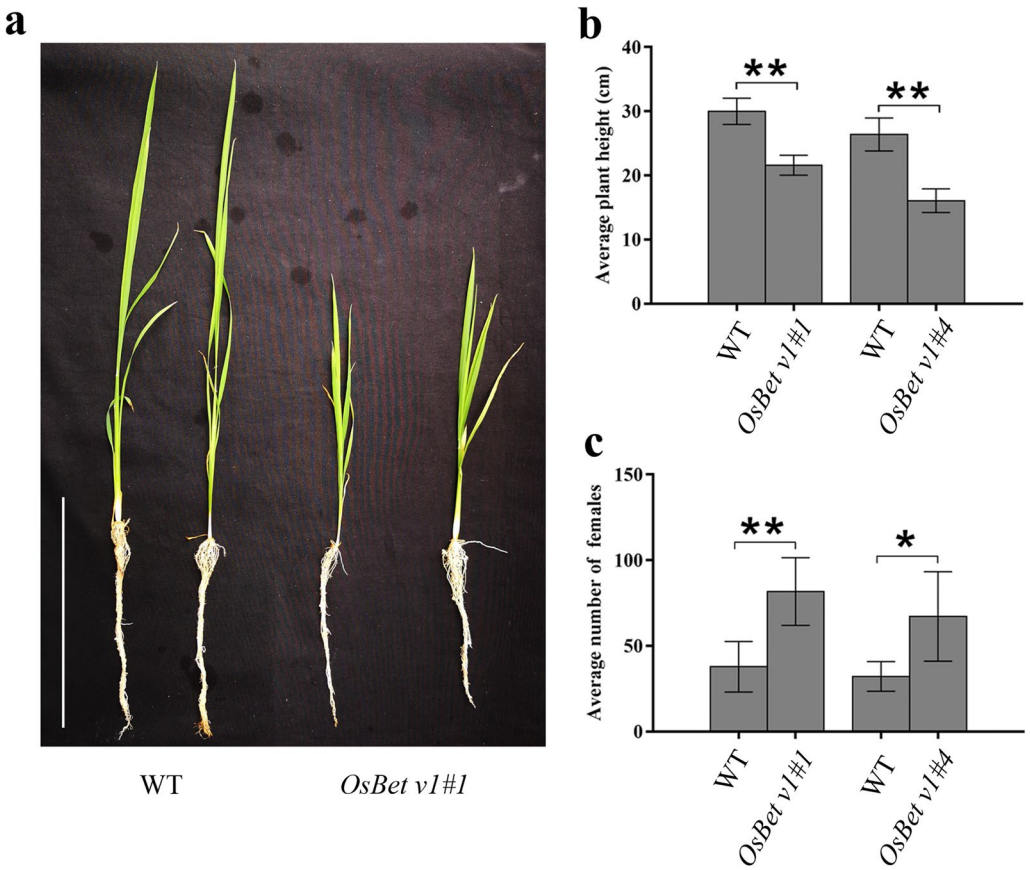

Fig. 6 Growth phenotypes of 'transgene-free' *OsBet v1* knockout mutants and their susceptibility to *Meloidogyne graminicola*. **a**, **b** Plant height of the wild-type (WT) and *OsBet v1* knockout mutants in potting experiment at 14 days after germination. Scale bar: 20 cm. **c** The average number of females in indicated rice lines at 14 days post-inoculation (dpi) with *M. graminicola*. Each experiment was performed two times. Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, Student's *t*-test

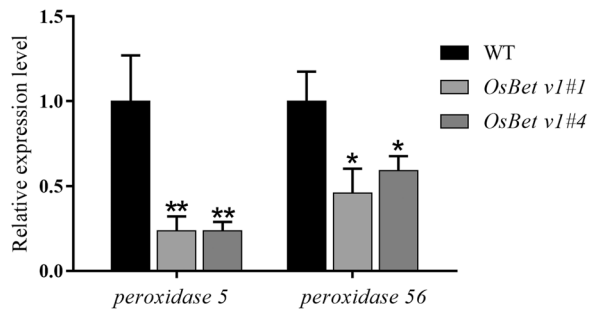

Fig. 7 RT-qPCR analysis of the expression levels of *peroxidase 5* and *peroxidase 56* in *OsBet v1* knockout mutants and wild-type (WT) plants. Data are presented as the mean ± SD. The experiments were performed three times with similar results, and three technical replicates for each reaction. **P* < 0.05, ***P* < 0.01, Student's *t*-test

Ekramoddoullah 2006). However, a few PR10/Bet v1-like proteins, such as cotton GhMLP28 and *A. thaliana* MLP43, have been demonstrated to be localized in the cytoplasm and nucleus, although they have no nuclear localization signal sequences (Yang et al. 2015; Wang et al. 2016). Interestingly, it was found that GhMLP28 interacted with cotton ethylene response factor 6 (GhERF6), and the accumulation of GhMLP28 in nucleus relied on the presence of GhERF6, which contributed to plant resistance against *Verticillium dahliae* (Yang et al. 2015). Therefore, besides cytosolic localization, nucleus localization may be important for certain PR10/Bet v1-like proteins to function normally.

In plants, PR10/Bet v1-like proteins are usually considered to be involved in defense against microbial pathogens (Liu and Ekramoddoullah 2006). For example, sugar beet plants overexpressing BvMLP1 and BvMLP3, two members of the major latex protein subfamily, exhibited a reduction in severity of *Rhizoctonia* root rot (Holmquist et al. 2021); the moss PR10-like subfamily protein PpPR-10 is active in the defense against *Pythium irregulare* in both *A. thaliana* and *Physcomitrella patens*, two evolutionary distant plants (Castro et al. 2016);

Arabidopsis overexpressing a corn monocot PR-10 type I gene *ZmPR10.1* displayed significantly less necrosis and chlorosis when challenged by *Pseudomonas syringae* DC3000, compared with wild-type plants (Xie et al. 2010). Although *OsTHI7*, a rice gene belonging to the PR13 family, was shown to play a role in defense against *M. graminicola* (Ji et al. 2015), whether PR10/Bet v1-like proteins are also involved in the defense against RKN is unknown. Here, we demonstrated that *OsBet v1* was highly expressed in root tissues and its expression could be further induced by *M. graminicola* infection, suggesting that *OsBet v1* may be involved in plant defense against *M. graminicola*. We therefore produced *OsBet v1* knockout rice mutants using the CRISPR/cas9 technology and found that CRISPR/Cas9-targeted mutagenesis of *OsBet v1* significantly increased rice susceptibility to *M. graminicola*, illustrating that *OsBet v1* is involved in rice defense against *M. graminicola*. After further investigation, we found that two rice peroxidase genes, *peroxidase 5* and *peroxidase 56*, were obviously down-regulated in the *OsBet v1* knockout rice mutants compared with WT. Plant peroxidases are involved in a wide range of physiological processes, including cellular growth, cell wall modification, lignin synthesis, and resistance to various stresses (Passardi et al. 2005). One recent example is the *Citrus sinensis* class III peroxidase CsPrx25, which could alter reactive oxygen species homeostasis accompanied by enhanced H_2O_2 levels, leading to enhanced citrus resistance to *Xanthomonas citri* subsp. *citri* (Li et al. 2020b). Accordingly, the resistance to *M. graminicola* mediated by *OsBet v1* in rice plants may be closely related to rice peroxidases.

Besides their roles in defense, PR10/Bet v1-like proteins, especially those in the major latex protein subfamily, are also crucially important for plant growth and development (Liu and Ekramoddoullah 2006). For example, the down-regulation of the *MLP423* gene resulted in mild alterations in *Arabidopsis* leaf curvature (Litholdo et al. 2016). Similarly, knockdown of *MLP28* in *Arabidopsis* also led to alterations in leaf curvature, but the mutant exhibited elongated petioles simultaneously (Litholdo et al. 2016); *A. thaliana* overexpressing three *BvMLP* genes of sugar beet developed faster and formed larger rosettes than wild-type plants (Holmquist et al. 2021). In addition, a rice monocot PR-10 type I subfamily gene *OsPR10A* was reported to be involved in rice growth and reproduction. When the *OsPR10A* was overexpressed in rice, the plant height, panicle length, and seed setting rate were lower than those in wild-type plants (Zhang et al. 2019). It has been suggested that PR10/Bet v1-like proteins participate in the production of plant phenolics, which contribute to plant structure and growth regulation (Morris et al. 2021). In this study, we found that *OsBet v1* can affect rice growth, as the plant height of the mutant rice lines *OsBet v1#1* and *OsBet v1#4* was significantly lower than that of WT, although it remains unknown how *OsBet v1* regulates rice growth. Additionally, given that the high expression of *OsBet v1* also occurs in rice seeds, it remains unclear whether *OsBet v1* can affect rice reproduction. Further research is needed to clarify the answers to these questions and better understand the roles of *OsBet v1* in plant growth, development, and defense response.

Conclusions

In this study, we identified a new PR10/Bet v1-like protein subfamily named monocot & dicot PR-10, which consists of the rice OsBet v1 and its 99 homologs. OsBet v1 was shown to play important roles in rice growth and defense against *M. graminicola* infection.

Methods

Nematode and plant materials

M. graminicola was isolated from rice in Hainan, China, and then purified and propagated on rice (*Oryza sativa* 'Nipponbare') in a greenhouse. Egg masses, preparasitic second stage juveniles (pre-J2s), and parasitic stage nematodes were collected as described previously (Huang et al. 2020). Rice seeds were germinated on wet filter paper at 30 °C for 3 days and then transferred and cultivated in sand and soil (3:1) at 27 °C under a 16 h light /8 h dark photoperiod in a greenhouse (Zhuo et al. 2019).

Gene amplification and sequence analysis of *OsBet v1*

Total RNA was extracted using the RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China) from two-week-old rice roots. The cDNA was synthesized with the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The full-length coding sequence of *OsBet v1* was amplified with the primer pair OsBetv1_F and OsBetv1_R. All primers used in this study are shown in Additional file 2: Table S1. PCR reaction was performed in a total volume of 50 μL containing 1.0 U KOD-FX (Toyobo, Osaka, Japan), 25 μL 2 × PCR Buffer, 10 μL 2 mM dNTPs, 1.5 μL of each of the 10 pM primers, 200 ng cDNA, and sterilized distilled water up to 50 μL. The PCR procedure was as follows: 95 °C for 5 min, followed by 30 cycles of 98 °C for 10 s, 55 °C annealing for 30 s, 68 °C elongation for 30 s, and final elongation at 68 °C for 5 min.

The sequence of the predicted OsBet v1 protein was used to identify homologous sequences by searching in NCBI database. The alignment of OsBet v1 and its homologous sequences from different plants was performed using ClustalW (https://www.genome.jp/

tools-bin/clustalw) and visualized by the GeneDoc software. The pairwise sequence identity matrix of PR10/Bet v1-like protein sequences was generated with DNAstar software, and the heatmap was produced with GraphPad Prism 7 software. The signal peptide was predicted using SignalP v. 4.0. Molecular mass was analyzed using ProtParam. The phylogeny tree was constructed using NJ method based on MEGA-X (Kumar et al. 2018) and visualized by iTOL v6 (https://itol.embl.de/). Bootstrap values were obtained from 1000 replicates. Conserved regions in the predicted OsBet v1 subfamily protein sequences were identified using MEME Version 5.4.1 (https://meme-suite.org/meme/tools/meme) as described previously (Zhang et al. 2018). The parameters of MEME were used as follows: maximum number of motifs are 10 and the optimum width of each motif is between 6 and 50 residues. The secondary structure elements of the complete amino acid sequence of OsBet v1 were predicted by trRosetta web server (Yang et al. 2020).

Reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR was performed using Green qPCR SuperMix (TransGen Biotech, Beijing, China) on a Thermal Cycler Dice Real Time System (Takara, Beijing, China). Total RNA isolation and the first-strand cDNA synthesis were performed as described above, and samples were prepared from three independent experiments. The primer pairs OsBetv1_qpcr_F/OsBetv1_qpcr_R, peroxidase5_F/peroxidase5_R, peroxidase56_F/peroxidase56_R, and OsUBQ_F/OsUBQ_R (Chen et al. 2018) were used to amplify *OsBet v1*, *peroxidase 5*, *peroxidase 56*, and the reference gene *OsUBQ* (Os03g13170), respectively. The qPCR reaction system was as follows: 1.0 μL cDNA, 0.4 μL of each of the 10 pM primers, 10 μL 2 × Green qPCR SuperMix, and 8.2 μL RNase-free ddH_2O. The relative changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Subcellular localization

eGFP was fused to the C-terminus of OsBet v1 to generate the plasmids pUbi:OsBet v1:eGFP. pUbi:eGFP alone was used as a control. Red fluorescent protein (RFP) fused to the C-terminus of the auxin response factor 19 (ARF19) was used as a nuclear marker. All constructs were purified using a High Pure Maxi Plasmid Kit (Tiangen Biotech, Beijing, China) and transformed into rice protoplasts through polyethylene glycol (PEG). The rice protoplast isolation and PEG medium transformation were performed as described previously (Chen et al. 2018). Finally, the fused proteins were observed and photographed using a Nikon ECLIPSE Ni microscope (Nikon, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed as described previously (Zhuo et al. 2017). Briefly, total protein was extracted from rice protoplasts using Plant Protein Extraction Kit (CoWin Biosciences, Beijing, China) and boiled for 10 min by adding 6 × Protein Loading Buffer (TransGen Biotech, Beijing, China). Approximately 10 μg of total protein was loaded onto a 12% SDS-PAGE gel, the primary antibody was Anti-GFP Tag Mouse Monoclonal Antibody (Abbkine Scientific, Wuhan, China), and the secondary antibody was HRP-conjugated Goat Anti-Mouse IgG (H + L) (Abbkine Scientific, Wuhan, China). Protein bands were visualized using an Immobilon Western Chemiluminescence System (Thermo Fisher Scientific, San Jose, CA, USA) with Pierce ECL Western Blotting Substrate.

Construction of CRISPR/Cas9 vectors and plant transformation

The two sgRNA-targeted sequences were designed using the online tool CRISPR-GE (http://skl.scau.edu.cn/; Xie et al. 2017), which displayed all optional sgRNA sequences (20 bp) immediately followed by 5'-NGG (protospacer adjacent motif, PAM) in the forward or reverse strand. Two selected sgRNA sequences targeting *OsBet v1* were cloned into the pYLC vector to produce the vector pYLC-OsBet v1, which could express Cas9 and sgRNA simultaneously (Ma et al. 2015). The pYLC-OsBet v1 vector was then transformed into *E. coli* DH5α and purified using the TIANpure Midi Plasmid Kit (Tiangen Biotech, Beijing, China) for the subsequent experiments.

The purified pYLC-OsBet v1 vectors were transformed into *Agrobacterium tumefaciens* (EHA105) by electroporation. The transgenic rice plants were generated by *Agrobacterium*-mediated transformation of rice calli as described previously (Zhou et al. 2005).

Identification of positive transgenic rice

To confirm that *OsBet v1* was edited successfully, genomic DNA was extracted using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) from the leaves of each individual plant in T_0 generation, and then the regions of the target sites were amplified by PCR with the primers gDNA_OsBetv1_F and gDNA_OsBetv1_R. Then the amplified products were sequenced and decoded through DSDecodeM (http://skl.scau.edu.cn/dsdecode/).

Off-target analysis and identification of 'transgene-free' CRISPR-edited plants

To investigate whether off-target effects occurred, CRISPR-P 1.0 (http://crispr.hzau.edu.cn/CRISPR/) was firstly used to predict potential off-target sites for each

gRNA. Subsequently, fragments of ten potential off-target sites were amplified with their respective specific primers that listed in Additional file 2: Table S1. Finally, the amplified fragments were sequenced and analyzed.

To identify 'transgene-free' T_1 generation plants, genomic DNA of T_1 mutant plants was extracted using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China). And the primer pairs Cas9p-F/Cas9p-R and sgRNA-F/sgRNA-R were used to amplify the transgenic elements, the *Cas9* gene, and the OsU6 promoter, respectively, by a multiple PCR.

Infection assay

The rice plants were cultivated in PVC tubes filled with a mixture of white sand and superabsorbent polymers (SAP) (Song et al. 2021). Fourteen-day-old rice plants, including WT and mutant lines of T_2 generation, were inoculated with 250 *M. graminicola* pre-J2s. At 14 dpi, roots were collected, washed, and stained with acid fuchsin, and the number of females was counted (Naalden et al. 2018). Each experiment was performed two times with counting on six plants of each line in each replicate. Statistically significant differences between treatments and controls were determined by a Student's *t*-test.

Abbreviations

Bet v1: The main allergen of birch pollen; CRISPR: Clustered regularly interspaced short palindromic repeats; dpi: Days post-inoculation; eGFP: Enhanced green fluorescent protein; ETI: Effector-triggered immunity; gRNA: Guide RNA; NJ: Neighbor-joining; pI: Isoelectric point; ORF: Open reading frame; PAM: Protospacer adjacent motif; PAMP: Pathogen-associated molecular pattern; PEG: Polyethylene glycol; PR10: Plant pathogenesis-related protein family 10; PTI: PAMP-triggered immunity; RKN: Root-knot nematode; RT-qPCR: Reverse transcription-quantitative PCR.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00143-z.

Additional file 1: Figure S1. Pairwise sequence identify matrix for plant PR10/Bet v1-like protein sequences. Red and green boxes indicate high and low pairwise identity. **Figure S2**. Architecture of conserved regions in plant PR10/Bet v1-like proteins.

Additional file 2: Table S1. Primers used in this study.

Additional file 3: Table S2. Accession numbers, names, description, and sources of proteins in Fig. 2.

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Author contributions

KZ designed the research; ZWL, QLH, JW, and CHH conducted the experiments; KZ, ZWL, and QLH wrote the manuscript; ZWL, KZ, BRL, QLH, and BG analyzed the data; JLL and KZ revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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