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A nucleotide-binding site-leucine-rich repeat receptor pair confers broad-spectrum disease resistance through physical association in rice

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Rice blast caused by *Magnaporthe oryzae* is the most destructive fungal disease in crops, greatly threatening rice production and food security worldwide. The identification and utilization of broad-spectrum resistance genes are considered to be the most economic and effective method to control the disease. In the past decade, many blast resistance (*R*) genes have been identified, which mainly encode nucleotide-binding leucine-rich repeat (NLR) receptor family and confer limited race-specific resistance to the fungal pathogen. Resistance genes conferring broad-spectrum blast resistance are still largely lacking. In this study, we carried out a map-based cloning of the new blast *R* locus *Pizh* in variety ZH11. A bacterial artificial chromosome (BAC) clone of 165 kb spanning the *Pizh* locus was sequenced and identified 9 NLR genes, among which only *Pizh-1* and *Pizh-2* were expressed. Genetic complementation experiments indicated that *Pizh-1* but not *Pizh-2* alone could confer blast resistance. Intriguingly, both mutations on *Pizh-1* and *Pizh-2* by CRISPR-Cas9 abolished the *Pizh*-mediated resistance. We also observed that *Pizh-1*-mediated resistance was partially dependent on *Pizh-2*. Pizh-1 and Pizh-2 form a complex of NLRs through direct interaction. This suggests that Pizh-1 may function as the executor NLR and *Pizh-2* as a 'helper' NLR that shares functional redundancy with other NLRs. Our current study provides not only a good tool for rice disease resistance breeding but also deep insight into NLR association and function in plant immunity.

This article is part of the theme issue 'Biotic signalling sheds light on smart pest management'.

1. Introduction

Rice blast, caused by *Magnaporthe oryzae*/*Pyricularia oryzae* (*M. oryzae*) is the most destructive disease in rice, usually causes 10–30% yield loss in epidemic areas [1,2], which greatly threatens rice production and food security worldwide. The identification and utilization of broad-spectrum resistance genes have been thought as the most economic and effective way to control rice blast [3]. To date, over 100 blast resistance (*R*) genes (*Pi*) have been identified, which are distributed on 11 rice chromosomes except chromosome 3. At least 28 *Pi* genes have been cloned. Most of them encode NLR receptors, except *Pi-d2*,

pi21 and *Ptr* [4–24]. *Pi-d2* encodes a receptor-like kinase protein with a predicted extracellular domain of a bulb-type mannose-specific binding lectin (B-lectin) and an intracellular serine-threonine kinase domain [21]. *Pi21* encodes a proline-rich protein that contains a putative heavy metal-binding domain and protein–protein interaction motifs. The resistant allele *pi21* carries deletions in the proline-rich motif and reduces blast infection rate [13]. *Ptr* is an atypical resistance gene encoding a protein with four Armadillo repeats. *Ptr* is required for broad-spectrum blast resistance mediated by the NLR gene *Pi-ta* and by *Pi-ta*^{2} [22]. However, *Pi* genes that confer breeding-approved broad-spectrum blast resistance are still rare and the mechanisms underlying broad-spectrum are still elusive.

Interestingly, several *Pi* loci including *Pik*, *Pikm*, *Pikp*, *Pi5* and *Pia*, which are all present in a head-to-head orientation and separated by short noncoding regions, each require two independent NLR genes for the blast resistance [9,11,16,17,25], which are all present in a head-to-head orientation and separated by short non-coding regions. The Pik pair comprises Pik-1 (the sensor) and Pik-2 (the executor). This Pik pair recognizes the *M. oryzae* effector AVR-Pik that directly binds to an integrated heavy metal-associated (HMA) domain, localized between the coiled-coil (CC) and the nucleotide-binding site (NBS) of Pik-1, releasing Pik-2 to activate immune signalling. Both the AVR-Pik effectors and the Pik NLRs exist as an allelic series in *M. oryzae* and rice, respectively, indicating that they most probably arose through coevolution [26]. Similarly, the *Pia* locus also contains two NLR proteins RGA4 and RGA5 that physically interact with each other; the RATX1 domain of RGA5 acts as a receptor for binding with effectors AVR1-CO39 and AVR-Pia, which disrupt the complex of RGA5 and RGA4, releasing RGA4 to activate downstream of immune signalling [25]. In addition, Pi5-mediated resistance to rice blast is also conferred by a pair of NLRs, Pi5-1 and Pi5-2 [11]. However, these NLRs, when expressed alone, do not confer resistance against *M. oryzae* isolates.

Only a few *R* loci conferring resistance to multiple blast races/isolates have been identified, including *Pigm*, *Pi9*, *Pi2*/*Piz*^{t}, *Pi5*, *Pb1*, *Ptr* [8,11,14,22,24,27]. Among the *Pi* loci, *Pigm*, *Pi9* and *Pi2*/*Piz*^{t} are genetically allelic with different functional NLR genes [27,28]. The *Pigm* locus provides the broadest spectrum for blast resistance discovered so far. Interestingly, the locus contains a cluster of 13 NLR genes. Among the NLRs encoded, a single NLR, PigmR, confers broad-spectrum resistance, whereas another NLR, PigmS, confers susceptibility, which competitively forms a heterodimer with PigmR and suppresses the PigmR-mediated resistance, strongly supporting the notion that NLR self-association is critical to NLR activation, and thereby immune responses; however, the molecular mechanism of the PigmR/PigmS complex in immune activation is obviously different from those of RGA4/RGA5 and Pik-1/Pik-2 [27].

In this study, we identify a new broad-spectrum *Pi* locus through map-based cloning of *Pizh*, from the *Japonica*/*Geng* variety Zhonghua11 (ZH11), which shows good performance in a continuous test of natural blast nursery. We show that *Pizh* contains a pair of NLR proteins that coordinate the broad-spectrum blast resistance through physical association. Introduction of *Pizh* into the elite rice cultivar, Kongyu131, by maker-assisted selection, greatly enhanced blast resistance to different *M. oryzae* isolates, demonstrating its good potential in breeding new varieties with broad-spectrum blast resistance.

2. Methods

(a) Plant materials

Seven rice cultivars, ZH11, Gumei4 (*Pigm*), C101A51(*Pi2*), 75-1-127 (*Pi9*), CO39 and Dongxiang (DX), were used in this study. The F_1 and F_2 populations derived from a cross between ZH11 and highly susceptible cultivar DX were generated for genetic and mapping analysis.

(b) Blast inoculation and disease evaluation

A total of 31 *M. oryzae* isolates were used in the study as listed in electronic supplementary material, table S1. Two-week-old seedlings were spray-inoculated with *M. oryzae* spore suspensions (1.5×10^5 spores ml^{-1}) as previously described [28]. Briefly, inoculated seedlings were kept in darkness at 25°C–27°C and over 90% relative humidity for 24 h, followed by growing under a 12/12 (day/night) photoperiod with the same temperature and relative humidity. To analyse the field resistance response of the same individuals to different isolates, the inject-inoculation method was employed at the tillering stage: each tiller of the same individuals was inoculated with one isolate by injecting 0.1 ml spore suspensions (2.5×10^4 spores ml^{-1}). After 7 days, disease symptoms were scored according to the 0–5 scoring system described by Bonman *et al*. [29]. For punch inoculated with pierced leaves as previously described by Wang *et al*. [30], briefly, leaf segments (5–6 cm long) from the top full-expanded leaf were pierced with a needle, and a droplet of spore suspension (10 µl containing approximately 50 spores) was inoculated on the punctured sites. Inoculated leaves were kept in a growth chamber with 12 h day/12 h night at 26°C. Disease symptoms were recorded at 5 and 7 days after inoculation. Disease symptoms were evaluated at 7 dpi by calculating lesion areas/lengths of fifteen infected leaves using the software ImageJ or a ruler, and measuring relative fungal growth by DNA-based quantitative PCR, as previously reported [27].

(c) Mapping of *Pizh*

A PCR-based mapping approach was performed: briefly, the F_2 population from the cross of ZH11 × DX was inoculated with the *M. oryzae* isolate 85–14, which is avirulent to ZH11 and virulent to DX. Inheritance of blast resistance of ZH11 was analysed based on the separate ratio of resistant and susceptible individuals. A total of 180 simple sequence repeat (SSR) and InDel markers, evenly distributed on 12 chromosomes, were screened for polymorphisms between the parents as previously described [28,31]. The polymorphic markers were then subjected to a modified bulked segregate analysis combined with recessive class analysis [32], using genomic DNAs of the two parents, 20 highly susceptible and 20 resistant individuals for linkage analysis and chromosome location of the *Pizh* locus. An additional 1500 susceptible F_2 individuals were further analysed to finely map the *Pizh* locus.

(d) Screening and sequencing of the candidate bacterial artificial chromosome (BAC) clone of *Pizh*

The genomic BAC library of ZH11 was constructed using the method as previously described [33]. The markers tightly linked to *Pizh* were used for screening of candidate BAC clones. The contig map spanning the *Pizh* locus was constructed based on end-sequencing of candidate BAC clones. The BAC clone 81G2 was selected and further completely sequenced by shot-gun technique using an ABI 3730 sequencer. The genomic sequence of the *Pizh* locus was annotated using the gene prediction program Fgenesh (www.softberry.com) and was manually edited by a homology search against available databases on GenBank (www.ncbi.nlm.nih.gov/genbank).

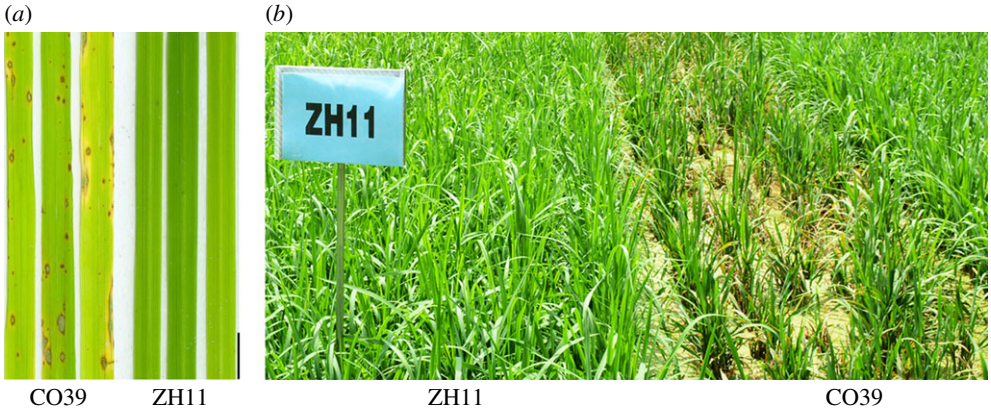

Figure 1. The rice variety ZH11 exhibits broad-spectrum resistance against *M. oryzae*. (*a*) Resistance phenotype of variety ZH11 (*Pizh*) was recognized by spray-inoculation with one representative blast isolate in two-week-old seedlings; CO39 served as a susceptible control. Bar, 1 cm. (*b*) Field disease resistance of ZH11 and CO39 grown in the natural blast nursery, showing that CO39 was almost killed by *M. oryzae*, while ZH11 stood completely heathy.

(e) RNA preparation and quantitative RT-PCR (qPCR) analysis

Total RNAs were extracted from different rice tissues using TRIzol reagent and treated with RNase-free DNase I according to the manufacturer's protocol (Invitrogen). The resulting RNAs were reverse-transcribed using the Superscript III RT kit (Invitrogen). The expression of resistant genes *Pizh-1* and *Pizh-2* was analysed using qRT-PCR, which was performed using SYBR Green (Takara) with the Eppendorf AG 22331 cycler following the manufacturer's instructions. Each qRT-PCR assay was replicated technically three times, and the rice *OsActin1* gene (LOC_Os03g50885) was used as an internal control. The primers for qRT-PCR are listed in electronic supplementary material, table S3.

(f) Plasmid construction and rice transformation

To generate the constructs for complementation test, the entire coding regions (CDS) of *Pizh-1* and *Pizh-2* were amplified using gene-specific primers from cDNA derived from total RNAs of ZH11, and the PCR products were inserted into the binary vector pCAMBIA1300- CaMV35S to generate overexpression plasmids 35S::Pizh-1, 35S::Pizh-2, sequencing to confirm the inserts. The expression constructs were introduced into the susceptible variety Nipponbare (NIPB) via *Agrobacterium*-mediated transformation to generate more than 30 independent transgenic plants for each construct, which were selected by PCR-based gene expression assays and resistance evaluation.

We used the CRISPR/Cas9 system to create *Pizh-1* and *Pizh-2* knockout (KO) mutants. The CRISPR/Cas9 binary vectors were constructed as previously described [34]. The Cas9 plant expression vector (*pYLCRISPR/Cas9Pubi-H*) and sgRNA expression vector (*pYLgRNA*) were kindly provided by the Yao-Guang Liu laboratory (South China Agricultural University). We selected the target of *Pizh-1* (CGACGAGACCAGCCTCCTGC), the target 2 (TGAAACTTAGAGAGCGCCAC) in the exon of *Pizh-2* and the target 3 (CTAGAAATAAACCCAAGCC) in both exons of *Pizh-1* and *Pizh-2* as candidate target sequences. The constructs were introduced into ZH11 to generate more than 30 independent transgenic plants for each construct by the *Agrobacterium*-mediated transformation procedure as described previously [35]. To identify CRISPR KO mutations, regions surrounding the target sites were amplified using gene-specific primers of *Pizh-1* and *Pizh-2* as listed in electronic supplementary material, table S3 and were sequenced to confirm mutations.

(g) Split luciferase complementation assay

For split luciferase complementation assay constructs, the full-length open-reading sequences of *Pizh-1* and *Pizh-2* were inserted into the vector pCAMBIA-35S-NLuc and pCAMBIA-35S-CLuc to generate expression plasmids, CLuc-Pizh-1, CLuc-Pizh-2, Pizh-1-NLuc and Pizh-2-NLuc. Transient expression in *Nicotiana benthamiana* leaves and split luciferase complementation assay were performed as described [36].

(h) Yeast two-hybrid assay

The full-length open-reading sequences of Pizh-1 and Pizh-2 were inserted into the vectors pDEST22 and pDEST32 by the Gateway cloning technology (Invitrogen), respectively, using the gene-specific primers (electronic supplementary material, table S3). The resulting constructs were transformed into yeast strain AH109. Co-transformants were plated on synthetic medium lacking uracil, tryptophan, leucine and histidine, and incubated at 28°C for 3 days. Experimental procedures for Y2H followed the manufacturer's instructions (Invitrogen).

(i) Phylogenetic analysis

We collected protein sequences of relative rice NLR *Pi* genes. The NLR sequences were aligned with the Clustalx program. An unrooted phylogenetic tree was constructed using MEGA5 software with 1000 bootstrap replications [37].

3. Results

(a) ZH11 displays a resistance spectrum different from those conferred by *Pi9*, *Pi2* and *Pigm*

To evaluate the blast response spectrum of ZH11, we used a total of 31 representative *M. oryzae* isolates from different regions (electronic supplementary material, table S1). Three known broad-spectrum resistant cultivars, Gumei4 carrying *Pigm*, 75-1-127 carrying *Pi9* and C101A51 carrying *Pi2*, were used as resistance controls, and the highly susceptible cultivar CO39 was used as a susceptible control. To our surprise, ZH11 was highly resistant to all isolates but one, TH12 (figure 1*b* or electronic supplementary material, table S1). Gumei4 (*Pigm*) was resistant to all the isolates tested, as previously reported [27,28]. By contrast, C101A51 (*Pi2*) was susceptible to eight isolates, and 75-1-127 (*Pi9*) was susceptible to five isolates. We evaluated field resistance performance of ZH11 in the natural blast nursery (Donghui, Zhejiang) from 2013 to 2018. The continuous field trial showed that ZH11 indeed displayed high broad-spectrum resistance to blast (figure 1*b*). Therefore, we conclude that that ZH11 also confers broad-spectrum

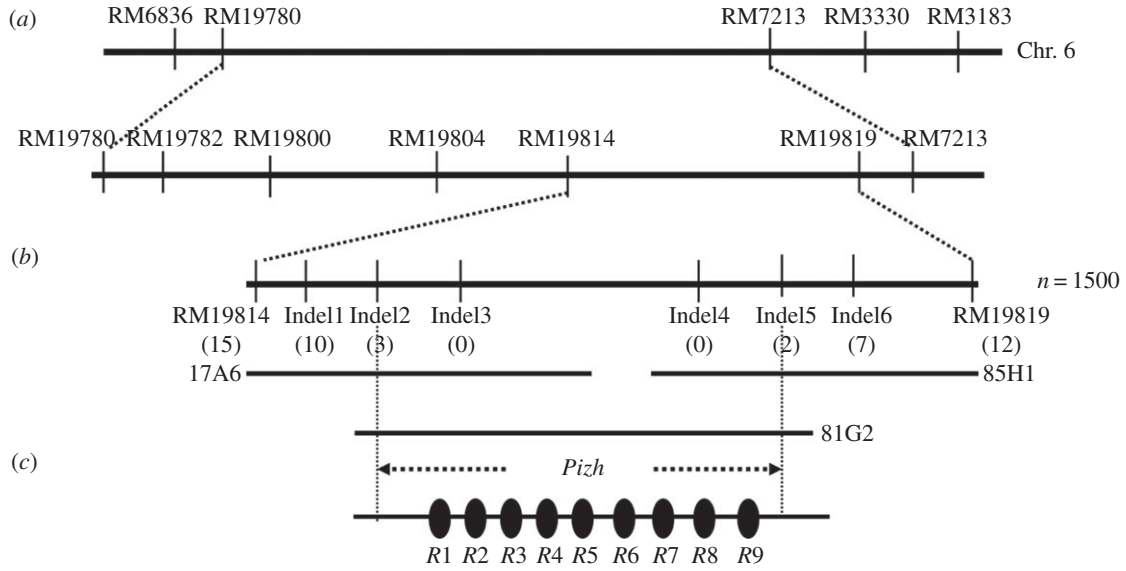

Figure 2. Physical mapping of *Pizh* in a 165 kb region on chrosome 6. (*a*) Preliminary mapping of *Pizh* in the interval between the markers RM19814 and RM19819 on chrosome 6. (*b*) Fine mapping of *Pizh* in a region co-segregating with the markers Indel3 and Indel4, within a contig spanning the *Pizh* locus including three BAC clones that were derived from ZH11 and were end-sequenced. Numbers in parentheses indicate recombinants between markers. (*c*) Sequencing of the BAC clone 81G2 reveals a cluster of 9 NLR genes in the *Pizh* locus.

resistance to *M. oryzae*, with a resistance spectrum different from known *Pi9*/*Pi2*/*Pigm*.

(b) A single dominant locus on chromosome 6 controls broad-spectrum blast resistance in ZH11

Two different *M. oryzae* isolates, 85−14 and 01−19, were used for genetic analysis of the blast resistance in ZH11, using the F_1 and F_2 population derived from a cross between ZH11 and DX. All the F_1 plants were resistant to 85−14 and 01−19, indicating that the dominant inheritance of the *R* gene(s) in ZH11. The segregation of resistant and susceptible individuals in the F_2 population fitted a ratio of 3: 1 (379 resistant: 104 susceptible), suggesting that the resistance to 85−14 and 01−19 is controlled by a single dominant *R* gene in ZH11. We designated this *R* gene in ZH11 as *Pizh*.

We performed map-based cloning to identify *Pizh*. First, a total of 180 SSR and InDel markers distributing evenly on 12 chromosomes were used in bulked segregate analysis (BSA). We found that two polymorphic markers, RM6836 and RM3183 on chromosome 6 (figure 2*a*), were associated with *Pizh*. Further, we confirmed the linkage relationship between RM6836, RM3183 and *Pizh* by analysing the polymorphism of RM6836 and RM3183 in each of 30 resistant and susceptible homozygous individuals confirmed in F_3 progeny, indicating that the RM6836 and RM3183 polymorphism was associated with the *Pizh* locus.

Based on the linkage of *Pizh* with the markers, we further screened the 1500 susceptible/recessive individuals identified from the F_2 population, which were confirmed in F_3 progeny by inoculating with isolate 85−14. The location of *Pizh* was then narrowed down to a small region on chromosome 6 using a set of SSR and InDel markers between RM6836 and RM3183 according to the genomic sequences of Nipponbare (*Japonica*/*Geng*) and the *indica* 9311 (*Indica*/*Xian*) (electronic supplementary material, table S3). The *Pizh* locus was delimited by the flanking markers Indel2 and Indel5, with three and two recombinant events detected, respectively; and co-segregated with the markers Indel3 and Indel4. Therefore, *Pizh* was finally locked within an 80 kb region between the two makers Indel2 and Indel5 on the reference genome of Nipponbare, a region where *Pigm*/*Pi9*/*Pi2* also nest [24,27,28,38]. Therefore, *Pizh* is a novel allele of *Pigm*/*Pi9*/*Pi2*.

(c) Genomic sequence and gene annotation of the *Pizh* locus

To obtain sequence information of the *Pizh* locus, we constructed a genomic BAC library of ZH11 with an average insert size of 140 kb [39]. The co-segregated markers Indel3 and Indel4 were used for PCR screening of the BAC library. Three positive clones were identified and end-sequenced to confirm whether these BAC clones overlapped. A contig map consisting of three overlapping ZH11 BAC clones was constructed that covered the *Pizh* locus (figure 2*b*).

We sequenced the BAC clone 81G2 containing the entire *Pizh* locus using shot-gun strategy, and a total 165 kb region spanning the *Pizh* was assembled. Gene annotation was performed using a BLAST search against the public databases and Fgenesh program (www.softberry.com). There are nine NLR type *R* genes in the *Pizh* locus (figure 2*c*), including two intact genes (*R2*, *R4*), seven pseudogenes or truncated genes (*R1*, *R3* and *R5* to *R9*). We determined the expression and transcripts of the candidate genes by RT-PCR, and found that *R2* (designated as *Pizh-2*) and *R4* (designated as *Pizh-1*) were expressed, with entire CDS encoding 1032 and 1033 amino acids, respectively. Both Pizh-1 and Pizh-2 belong to the family of typical CC-nucleotide-binding leucine-rich repeat receptor proteins (figure 3; electronic supplementary material, figure S1).

Most rice *Pi* genes are constitutively expressed, except for *Pib*, *Pi5-1*, *Pik-m* and *Pik-p*, which exhibit a pathogen-induced expression pattern [4,9,11,17]. To determine whether *Pizh* can be induced during infection by *M. oryzae*, we analysed the induction of *Pizh-1* in infected ZH11 leaves in a time course (0 ~ 72 h post inoculation [hpi]). Expression of *Pizh-1* was not changed by pathogen challenge, indicating that this gene is constitutively expressed. By contrast, *Pizh-2* was

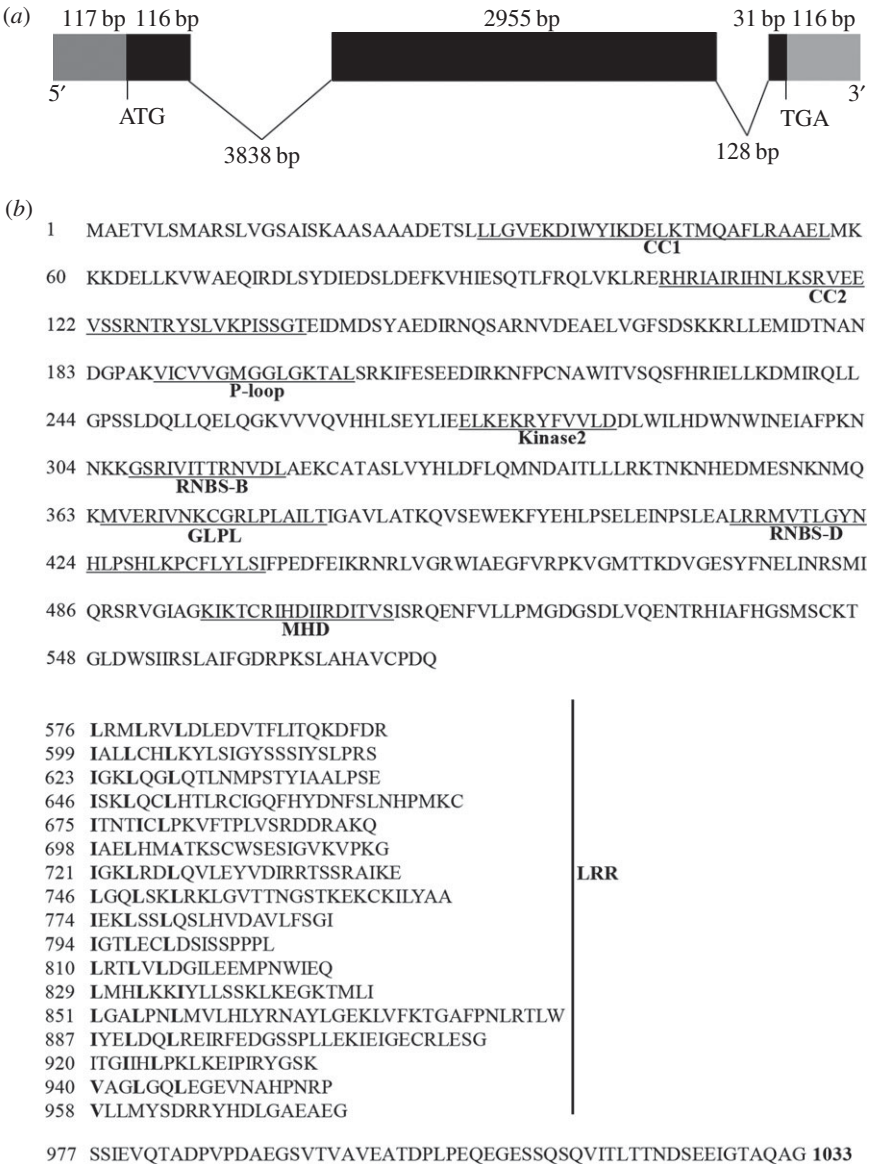

Figure 3. Genomic structure of *Pizh-1* and its amino acid sequence. (*a*) Gene structure of *Pizh-1*. The black box represents exon, lines denote introns, and grey boxes indicate 5′- and 3′-untranslated regions (UTR). (*b*) Amino acid sequence of the *Pizh-1* gene product. The two coiled-coil (CC) motifs are underlined. The conserved motifs (P-loop, Kinase2, RNBS-B, GLPL, RNBS-D, MHD) in the nucleotide-binding site (NBS) region are indicated. The C-terminal leucine-rich repeat (LRR) domain consists of 17 imperfect LRR repeats with the consensus IXX(L)XX(L)XX(L).

slightly induced at 48 hpi, but inhibited at 72 hpi (electronic supplementary material, figure S2). Therefore, *Pizh-1* and *Pizh-2* likely display subtle differences in response to pathogen. Phylogenetic analysis of Pizh and other known NLR proteins in rice revealed that these NLRs could be classified into nine phylogenetic clades (Clades A to I), of which allelic Pizh, Pi2, Piz^{t}, PigmR, Pi50 and Pi9 were grouped in the same sub-group of Clade A (electronic supplementary material, figures S3 and S4). Interestingly, Pizh-1 shares the same protein sequence with Piz^{t}, suggesting their same origin. For consistency in context, we still keep Pizh-1 hereafter. But ZH11 exhibits a broader blast-resistance spectrum than Toride I (*Piz^{t}*) dependent on our previous field resistance evaluation and inoculation result, which indicates that additional resistant genes are also harboured in ZH11, except for *Pizh*.

(d) *Pizh-1* and *Pizh-2* coordinately confer broad-spectrum blast resistance

To determine which one of the candidate genes (*Pizh-1* and *Pizh-2*) is responsible for the *Pizh*-mediated resistance to *M. oryzae*, we developed transgenic plants constitutively expressing *Pizh-1* and *Pizh-2* driven by the 35S promoter in the susceptible Nipponbare (NIPB) background. The transgene plants expressing *Pizh-1* but not *Pizh-2* were resistant to all isolates tested (figure 4*a–e*; electronic supplementary material, table S2).

We also developed KO mutants of *Pizh-1* and *Pizh-2* using the CRISPR/cas9 genome-editing method in the ZH11 background. We only obtained *pizh-1* single and *pizh-1*/*pizh-2* double KO mutants (electronic supplementary material, figure S5), but no *pizh-2* single KO mutant after extensive screening. *Magnaporthe oryzae* inoculation revealed that, as expected, the *pizh-1* KO mutants exhibited susceptible phenotype (figure 4*e–g*). Interestingly, the *pizh-1*/*pizh-2* double mutant plants were shown to be more susceptible than the *pizh-1* single mutants (figure 4*e–g*). Taken together, these results demonstrate that *Pizh-1* confers broad-spectrum blast resistance in the *Pizh* locus, and *Pizh-2* alone does not confer blast function. We suggest that Pizh-2 may act as a 'helper' NLR functioning coordinately with Pizh-1, its loss-of-function impairing the disease resistance mediated by Pizh-1.

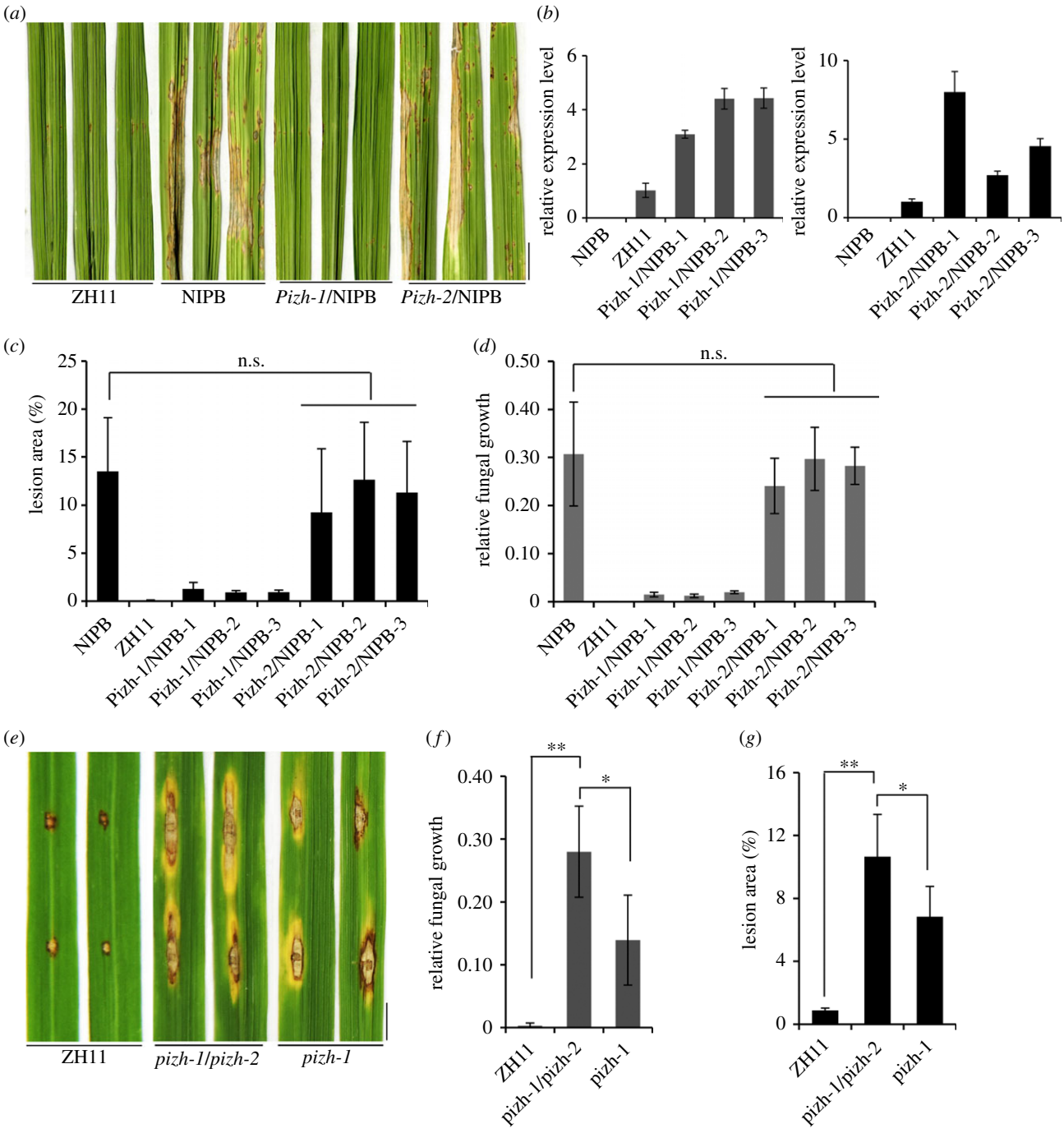

Figure 4. Functional identification of the *Pizh* candidate gene. (*a*) Resistance phenotype of representative transgenic plants expressing Pizh-1 and Pizh-2. Two-month-old adult plants grown in the field were injection-inoculated with isolate 85–14, showing that Pizh-1 but not Pizh-2 confers blast resistance. The gene donor ZH11 and the wild-type NIPB served as resistant and susceptible controls, respectively. (*b*) Gene expression of Pizh-1 and Pizh-2 was detected using qRT-PCR in the transgenic lines and wild-type NIBP. (*c*,*g*) Lesion area of infected leaves was measured at 7 dpi with the ImageJ software. (*d*,*f*) The relative fungal growth was measured at 7 dpi using the calculation MoPot2/OsUbq. (*e*) Resistance phenotype of the transgenic lines gene expression of Pizh-1 and Pizh-2 was detected using qRT-PCR in pizh-1 single and pizh-1/pizh-2 double mutants in the ZH11 background. One-month-old seedlings were punch-inoculated with isolates 85–14, indicating that pizh-1 partially but pizh-1/pizh-2 completely lost blast resistance, compared with the wild-type ZH11. A Student's *t*-test was used to analyse the significance of the difference (*$p < 0.05$, **$p < 0.01$, n.s.: not significant) (*c*,*d*,*g*,*f*).

(e) Pizh-1 interacts physically with Pizh-2 to form hetero-complex

The functionality of Pizh-1 and Pizh-2 described above allowed us to speculate that Pizh-2 may function through direct interaction with Pizh-1 to form an NLR immune complex. To test this hypothesis, we first detected the interaction between Pizh-1 and Pizh-2 in a yeast two-hybrid (Y2H) assay (figure 5*a*). Furthermore, we performed a split luciferase complementation assay to determine the Pizh-1 and Pizh-2 interaction *in planta* (figure 5*b*). Therefore, Pizh-1 and Pizh-2 form a functional NLR pair that confers broad-spectrum blast resistance. Given the fact that the transgenic NIPB plants expressing *Pizh-1* alone developed broad-spectrum blast resistance (figure 4*a*), we consider that an unrecognized NLR(s) likely acts as a 'helper' of Pizh-1 in the NIPB genome, ensuring the Pizh-1-triggered immunity.

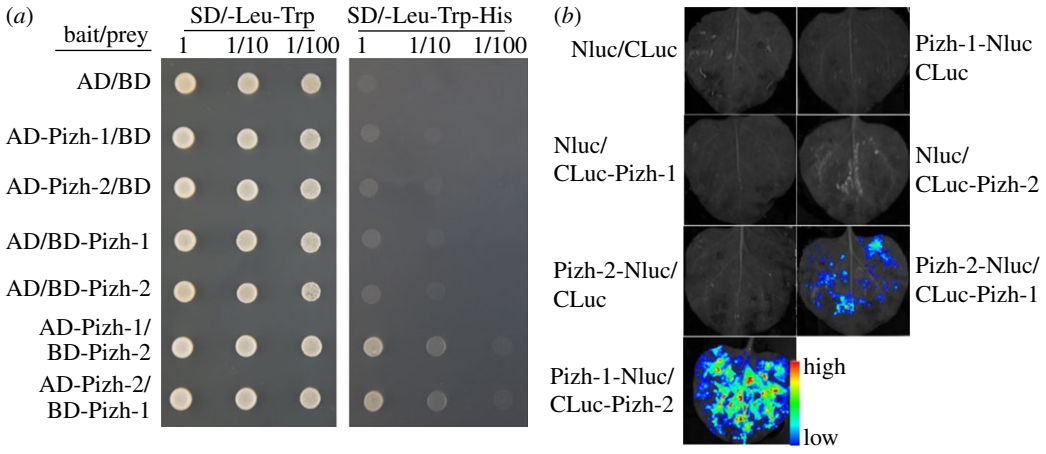

Figure 5. Pizh-1 physically interacts with Pizh-2. (*a*) Pizh-1 interacts with Pizh-2 in a yeast two-hybrid assay. Dilution series of yeast cells expressing GAL4-AD and GAL4-BD fusions of Pizh-1 or Pizh-2 full-length proteins on non-selective synthetic medium lacking Trp and Leu (-Leu-Trp) and selective medium additionally lacking His (-Leu-Trp-His). (*b*) Split luciferase complementation assay indicates in planta interaction between Pizh-1 and Pizh-2, reconstructing the luciferase signal with the pairs of Pizh-1-NLuc and CLuc-Pizh-2, Pizh-2-NLuc and CLuc-Pizh-1. Fluorescence signal intensity is indicated.

(f) Molecular breeding application of *Pizh* elite rice varieties

To develop a practical marker for application of *Pizh* in molecular breeding in rice, we first performed sequence comparison of the *Pizh* alleles between resistant variety ZH11 and the susceptible varieties NIPB, 9311 and CO39, and revealed that the *Pizh-1*(*R4*) is absent in the genome of NIPB, 9311 and CO39. Based on this, we designed a specific marker, M262, in the intron of *Pizh-1*, and used this to genotype the representative resistant and susceptible varieties. We found that this maker could precisely distinguish the *Pizh* locus from different susceptible alleles (figure 6*a*,*b*). Therefore, M262 can be efficiently used as a *Pizh*-specific marker in rice molecular breeding for blast resistance. Using a backcross approach, we have successfully introduced *Pizh* into the high-quality but blast-susceptible variety Kongyu131. The improved new line, KY131-Pizh, exhibited high blast resistance in inoculation assay (figure 6*c*), which will be further tested in natural blast nurseries to determine its field performance.

4. Discussion

The utilization of host *R* genes has long been practised in crop breeding for disease resistance in crops. However, disease resistance breeding rarely succeeds because of the lack of elite broad-spectrum disease resistance genes, particularly in rice breeding for blast resistance because this fungal pathogen prevails in all rice areas worldwide. In this study, we identify a novel blast resistance locus, *Pizh*, from a widely-cultivated rice variety Zhonghua 11, which was bred from resistant varieties Jingfeng5, Tetep and Fukunishiki in 1984, exhibiting a broad-spectrum and durable field resistance against *M.oryzae* for 30 years in Northern China. Interestingly, *Pizh* is allelic to *Pigm*/*Pi2*/*Pi9* but with a different resistance spectrum, providing a new molecular tool for improving rice blast resistance.

Interestingly, at least eight *Pi* genes/loci have been identified in this *Pigm NLR* cluster; each allelic cluster contains different *NLR* gene members and functional NLR(s), resulting in different spectrum resistance against *M. oryzae*. Therefore, this region is a hot spot of *R* gene origin and evolution owing to frequent gene duplication, uneven crossing over or transposon insertion [27]. It is possible that *Pizh* evolved independently of *Pigm*/*Pi9*/*Pi2*, given that *Pizh-1* does not share high sequence similarity to other allelic NLRs (electronic supplementary material, figure S3*a*). Other biological or ecological impacts of these alleles outside their function in disease resistance will be worthy of further investigation.

Recent data suggest a hypothesis that two adjacently located NLRs often work as a pair in the execution of immune action upon pathogen recognition. Among these NLR pairs, a 'sensor' NLR is responsible for pathogen perception and an 'executor' or 'helper' NLR is responsible for activation of downstream signalling. There are two generic models emerging [40]. In the 'sensor–executor model', the two NLRs are encoded by one locus and the NLR proteins physically interact with each other. In this model, the 'sensor' NLR carries an integrated domain that mimics the effector-target and aids pathogen perception. Effector perception by the sensor NLR relieves the suppression of the 'executor' NLR, launching downstream immunity signalling. This is the case for the rice RGA5/RGA4 and *Arabidopsis* RRS1/RPS4 pairs [25,41]. The second model is named 'the sensor–helper model' based on the fact that 'helper' NLRs function downstream of multiple 'sensor' NLRs. In this case, the two NLRs are typically encoded by different loci. Several 'helper' NLRs have been identified in diverse plant species, including to Solanaceae NRC1, tobacco NRG1 and *Arabidopsis* ADR1 [42–44], and the 'helper' NLRs merely require activation by the 'sensor' NLRs, rather than de-repression. In our current study, Pizh-1 itself confers broad-spectrum blast resistance, while Pizh-2 likely acts as a 'helper' NLR contributing to Pizh-1-mediated resistance against *M. oryzae*. Therefore, the Pizh-1/Pizh-2 pair is mechanistically different from the RGA4/RGA5 NLR pair, in which neither NLR can confer resistance on its own [25]. The Pizh-1/Pizh-2 complex is also distinct from the PigmR/PigmS complex functionally and mechanistically [27]. This function mode difference further strengthens the notion that this hot NLR spot could evolve independently and sub-functionalize either in functional NLRs or NLR pairing different alleles. Therefore, our current study also provides new insights into NLR interaction and function in

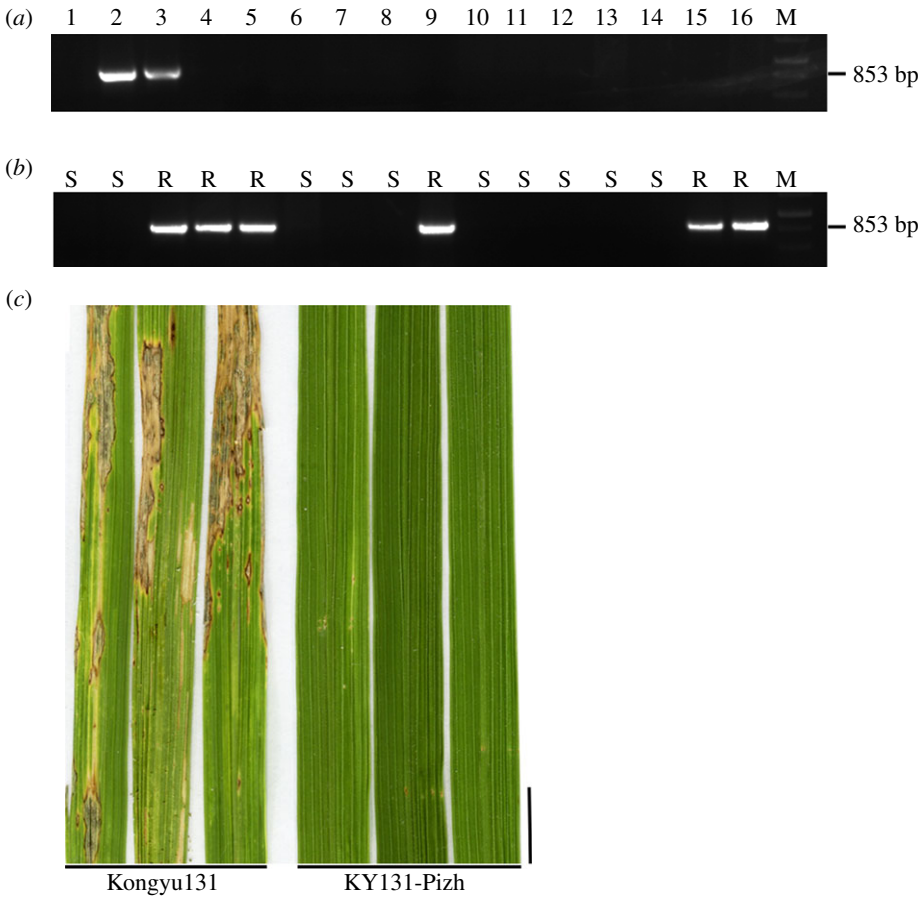

Figure 6. Molecular breeding application of Pizh in elite rice improvement. (*a*) Design of a *Pizh*-specific marker that can efficiently detect the genotype of rice cultivars. 1, susceptible variety Nipponbare; 2, resistant variety ZH11 (*Pizh*); 3, resistant variety Toridel (*Pizt*); 4 to 16, susceptible varieties: 9311, C039, Zhenshan97, Minghui63, Kasalath, Kongyu131, TN1, TP309, Minghui86, Teqing, Longtepu, Wuyujing8, Huanghuazan. (*b*) Genotyping of progeny plants in the breeding population derived from the cross between cultivar ZH11 and Kongyu131 (KY131). R, resistant; S, susceptible. (*c*) Enhance blast resistance of the improved Kongyu131 lines with the Pizh locus (KY131-Pizh) through backcross and maker assistant selection approaches.

plant immunity, in addition to an important new genetic tool for molecular breeding in crop improvement.

5. Conclusion

This study identifies a new broad-spectrum blast resistance locus, *Pizh*, in rice. In the locus, two NLR receptors, Pizh-1 and Pizh-2, coordinately control disease resistance through physical interaction to form a hetero-complex for immune activation. We suggest that Pizh-1 likely functions as the 'executor' NLR and Pizh-2 as a 'helper' NLR. Our study provides a new example of plant NLR pairs performing a resistance function. More importantly, the *Pizh* locus shows good potential in molecular breeding for blast resistance improvement in rice.

Data accessibility. The genomic sequence of the *Pizh* locus was deposited in GenBank under accession no. MH807580.

Authors' contributions. Y.D. and Z.H. designed experiments; Z.X., B.Y., J.S., J.T., X.W., K.Z., J.L., Q.L., M.L., Y.D. and Z.H. performed experiments and data analysis. Y.D. and Z.H. wrote the manuscript. All authors have read, edited and approved the content of the manuscript.

Competing interests. We have no conflict of competing interests.

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