OsNBL3, a mitochondrion-localized pentatricopeptide repeat protein, is involved in splicing nad5 intron 4 and its disruption causes lesion mimic phenotype with enhanced resistance to biotic and abiotic stresses

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Summary
Lesion mimic mutants are used to elucidate mechanisms controlling plant responses to pathogen attacks and environmental stresses. Although dozens of genes had been functionally demonstrated to be involved in lesion mimic phenotype in several plant species, the molecular mechanisms underlying the hypersensitive response are largely unknown. Here, a rice (Oryza sativa) lesion mimic mutant natural blight leaf 3 (nbl3) was identified from T-DNA insertion lines. The causative gene, OsNBL3, encodes a mitochondrion-localized pentatricopeptide repeat (PPR) protein. The nbl3 mutant exhibited spontaneous cell death response and H$_2$O$_2$ accumulation, and displayed enhanced resistance to the fungal and bacterial pathogens Magnaporthe oryzae and Xanthomonas oryzae pv. oryzae. This resistance was consistent with the up-regulation of several defence-related genes; thus, defence responses were induced in nbl3. RNA interference lines of OsNBL3 exhibited enhanced disease resistance similar to that of nbl3, while the disease resistance in overexpression lines did not differ from that of the wild type. In addition, nbl3 displayed improved tolerance to salt, accompanied by up-regulation of several salt-associated marker genes. OsNBL3 was found to mainly participate in the splicing of mitochondrial gene nad5 intron 4. Disruption of OsNBL3 leads to the reduction in complex I activity, the elevation of alternative respiratory pathways and the destruction of mitochondrial morphology. Overall, the results demonstrated that the PPR protein-coding gene OsNBL3 is essential for mitochondrial development and functions, and its disruption causes the lesion mimic phenotype and enhances disease resistance and tolerance to salt in rice.

Keywords: OsNBL3, Oryza sativa, disease resistance, salt tolerance, pentatricopeptide repeat (PPR) protein, mitochondria, RNA splicing, nad5, lesion mimic.

Introduction
Programmed cell death (PCD) plays important roles in the embryonic, juvenile and adult phases of plant development. In addition, a type of PCD is often observed during plant response to pathogen attack; this is termed the ‘hypersensitive response’ (HR). HR is a defence mechanism employed by plants to protect themselves from biotic stress and is usually accompanied by the generation of reactive oxygen species (ROS), expression of pathogenesis-related (PR) proteins, accumulation of callose and thickening of cell walls at the infection sites (Jones and Dangl, 1996).

Lesion mimic mutants (LMMs), also called spotted leaf (spl) mutants, spontaneously develop localized cell death lesions resembling those caused by HR in the absence of pathogen infection, abiotic stress or mechanical damage. In many cases, LMMs exhibit significantly enhanced disease resistance; therefore, LMMs are deemed as suitable materials for elucidating the mechanisms underlying plant responses to pathogen attacks and environmental stresses. To date, dozens of genes have been characterized from LMMs and that encode diverse proteins, including porphyrin (Undan et al., 2012), transcription factors (Li et al., 2004), oxidoreductases (Yang et al., 2004), protein kinases (Wang et al., 2015a), ubiquitinations (Zeng et al., 2004), membrane-associated proteins (Noutoshi et al., 2006), zinc finger proteins (Wang et al., 2005), ion channel proteins (Mosher et al., 2010), clathrin-associated adaptor proteins (Qiao et al., 2010), nucleotide-binding site–leucine-rich repeat (NLR) proteins (Tang et al., 2011), mRNA splicing factors (Chen et al., 2012), UDP-N-acetylglucosamine pyrophosphorylase (Wang et al., 2015b), AAA-type ATPase (Fekih et al., 2015), eEF1A-like protein (Wang et al., 2017c) and glycine-rich domain proteins (Zhao et al., 2020). The large range of protein types contributes to the characteristics of LMMs, suggesting the molecular mechanisms regulating defence responses in plants are very complicated.

Mitochondria playing a pivotal role during PCD is well reviewed by Huang et al., (2016). PCD is closely associated with mitochondrial reactive oxygen species (mtROS) production in plants. mtROS production is often increased during stress and PCD, likely due to mitochondrial electron transport chain (mtETC) inhibition (Huang et al., 2016). mtETC enzymes, such as AOX, protect against PCD (Wu et al., 2015). In Arabidopsis, mitochondrial Complex I is responsible for the ROS production that leads to PCD in the mosaic death 1 (mod1) mutant (Wu et al., 2015).
Mitochondria have also been proposed to be linked with PCD via increased ROS production during pathogen defence. In agreement, a wide variety of mutants in mitochondrial functions have been reported to have altered PCD or pathogen defence phenotypes (Huang et al., 2016). Although plant mitochondrial genomes can encode genes, most of these genes are regulated by proteins encoded by nuclear genes (Schmitzlinneweber and Small, 2008). One of the largest families of proteins that modulate mitochondrial gene expression is pentatricopeptide repeat (PPR) proteins, which can be divided into P and PLS subfamilies according to PPR motifs therein. The P-subfamily proteins only consist of canonical 35 amino acid-PPR motifs carrying, whereas the PLS-subfamily proteins contain short motifs (S; 31 amino acids), long motifs (L; 35–36 amino acids) and E, E– or DYW domains at the C terminus (Schmitzlinneweber and Small, 2008). The rice genome is predicted to encode 491 PPR proteins (246 P-subfamily and 245 PLS subfamily). Moreover, 90 E domains and 131 DYW domains have been found in the PLS subfamily (Chen et al., 2018). P-subfamily proteins are mainly involved in mitochondrial RNA splicing, stabilization and translation, while PLS-subfamily proteins mainly play a role in the C-U editing of mitochondrial RNA (Barkan and Small, 2014).

Most of the PPR proteins located in mitochondria are involved in modifying the gene expression of subunits of the mETC, which is composed of respiratory enzyme complexes, such as the nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase system (complex I), succinate-ubiquinone reductase system (complex II), cytochrome b precursor (complex III), cytochrome c oxidase system (complex IV) and ATP synthase system (complex V). Mutations in the PPR genes lead to dysfunction of the mitochondrial ETC, which can cause seed and embryo development defects, growth retardation, pollen abortion, and abiotic stress and ABA sensitivity in plants (Barkan and Small, 2014). In Arabidopsis, the P-subfamily proteins MTL1 (Halli et al., 2016), SLOW GROWTH 3 (Hsieh et al., 2015) and OTP43 (De Longevialle et al., 2007) have been shown to be localized in mitochondria and are involved in the cis- or trans-splicing of the introns of genes encoding subunits of complex I. Recently, it was reported that a rice P-subfamily PPR protein, FLO10, is required for the trans-splicing of the mitochondrial nad1 intron 1 (Wu et al., 2019). Another P-subfamily PPR protein, RL1 of rice, was specifically involved in the splicing of the mitochondrial nad4 intron 1 (Wu et al., 2020). Several mitochondrion-localized P-type PPR proteins have been reported to be involved in the RNA stabilization of genes coding for subunits of complex I. These P-type PPR proteins include Arabidopsis AtMTF51 (Halli et al., 2013), AtMTF52 (Wang et al., 2017a), PPR19 (Lee et al., 2017) and maize PPR78 (Zhang et al., 2017). The mitochondrion-localized PLS-subfamily PPR proteins, including rice OGR1 (Sungryul et al., 2009), MPR25 (Toda et al., 2012), Arabidopsis SLO2 (Zhu et al., 2012), SLO4 (Weissenberger et al., 2017), MEF8 (Diaz et al., 2017), maize DEK36 (Wang et al., 2017b) and DEK39 (Li et al., 2018), are mainly involved in the C-U editing of genes encoding subunits of complexes I and III. Although these studies shed some light on the roles of PPR proteins in plant growth and development via regulation of mitochondrial RNA metabolism, the functions of most PPR proteins remain unclear.

In the incumbent study, we report Oryza sativa NBL3 (OsNBL3) that encodes a mitochondrion-localized P-subfamily PPR protein. The mutation in OsNBL3 leads to a spontaneous lesion mimic phenotype, concomitant with enhanced disease resistance to Magnaporthe oryzae (the causal agent of rice blast disease) and Xanthomonas oryzae pv. oryzae (Xoo; the causative pathogen of rice leaf blight disease) and salt tolerance, and premature leaf senescence. RNA interference (RNAi) lines of OsNBL3 exhibited enhanced disease resistance similar to that of the nbl3 mutant (a T-DNA insertion mutant), while the resistance level in overexpression lines thereof did not differ from that of the wild type. OsNBL3 mainly involves in the cis-splicing of nad5 intron 4 and thus contributes to the precise assembly and construction of mitochondria.

Results

Phenotypic characterization of the nbl3 mutant

The rice mutant nbl3 (natural blight leaf 3) was identified in the paddy field growing T-DNA insertion lines of Geng (japonica) rice cv. Aichiasahi. Under field conditions (Beijing), the lower leaves of nbl3 mutants exhibited irregular, brown necrosis lesions at their tips from approximately 30 days after germination. The lesions then expanded from the tip to the whole leaf, leading to severe leaf withering and premature senescence (Figure 1a). The cell death lesions appeared on the nbl3 plants acropetally (Figure 1b), and by the heading stage, the nbl3 plants exhibited a typical senescence phenotype (Figure 1c). Under greenhouse conditions, the mutant phenotype of nbl3 was delayed and was less severe than its field phenotype. After Trypan blue staining, the nbl3 mutant exhibited dark blue spots, indicating the emergence of cell death or membrane damage on the leaves (Figure 1d). Reactive oxygen species (ROS; e.g. H2O2) accumulation has been reported in many LMMs (Chen et al., 2012; Ma et al., 2019; Qiao et al., 2010; Wang et al., 2017c; Zhao et al., 2020). When tetratniobiole tetrazolium chloride (NBT) solution and 3,3’-diaminobenzidine (DAB) staining were used to assess ROS in the nbl3 mutant, intense brown staining accentuated surrounding lesion sites on the leaves, as opposed to in the wild-type leaves (Figure 1e,f). This indicates that H2O2 accumulation occurred in the nbl3 leaves. In addition to cell death and premature senescence, several agronomic traits including plant height, tiller number and 1000-grain weight were affected in the nbl3 plants (Figure S1).

Enhanced resistance of nbl3 against M. oryzae and Xoo, accompanied by constitutive expression of defence-related genes

The appearance of spontaneous leaf spot is often concomitant with enhanced disease resistance. To determine whether the nbl3 mutation led to enhanced resistance to rice pathogens, one-month-old seedlings of the rice cultivar Aichiasahi (wild type) and the nbl3 mutant were inoculated with M. oryzae (wild type) and the nbl3 mutant were inoculated with M. oryzae. The nbl3 plants growing in greenhouse conditions did not display lesions when inoculated with H535, a virulent isolate of M. oryzae, using the punch inoculation method. The size of lesions on the nbl3 leaves was significantly smaller than that of lesions on the wild-type leaves at 96 h post-inoculation (hpi; Figure 1g,h). Two-month-old seedlings were also inoculated with the bacterial blight pathogen Xoo strain PXO99. It was found that the lesions on the nbl3 leaves were much smaller than those on the wild-type plants two weeks post-inoculation (Figure 1i,j). These results demonstrate that the nbl3 plants display significantly enhanced resistance to both M. oryzae and Xoo.
In many rice LMMs, the constitutive expression of defence-response genes has been found to be accompanied by lesion development (Ma et al., 2019; Zhao et al., 2020). To determine whether the transcription of defence-related genes was affected in the nbl3 mutant, expression analyses were performed using RT-qPCR. Several pathogenesis-related protein genes, including OsPR1b, OsPR2, OsPR3, OsPR5, OsPR8 and OsPR10, and two defence-related genes, OsWRKY45 and OsWRKY62, were significantly up-regulated in nbl3 (Figure 2a). These results are consistent with the enhanced disease resistance of nbl3. Among these up-regulated genes, OsWRKY62 is known to be involved in the jasmonic acid (JA) signalling pathway (Liu et al., 2016), and the other genes are well known to be involved in the salicylic acid (SA) signalling pathway (Tang et al., 2011; Wang et al., 2019). Furthermore, the intensity of jasmonic acid, abscisic acid and salicylic acid are higher in nbl3 than that in the wild type (Figure 2b). These results suggest that the mutation in nbl3 confers enhanced disease resistance and other phenotypes, possibly mediated by integrated signalling pathways.

Cloning of OsNBL3 and its expression patterns

Genetic analysis demonstrated that the nbl3 mutation is a recessive trait that is co-segregated with the T-DNA insertion (Table S1). Thereafter, SiteFinding TAIL-PCR procedures were used to isolate genomic sequences flanking both ends of the T-DNA. The results showed that the T-DNA was integrated into chromosome 3. Mapping the sequences onto the O. sativa cv. Nipponbare genome (The Rice Genome Annotation Project; http://rice.plantbiology.msu.edu/) revealed that the insertion site was located in the 3′-untranslated region of an annotated gene LOC_Os03g06370 (Figure S2a). This insertion event was confirmed by specific PCR using two pairs of primers flanking the insertion site (T-DNA borders; Figure S2b). Using the two pairs of primers to perform the semi-quantitative RT-PCR analysis, the transcript of LOC_Os03g06370 was not detectable in the nbl3 seedlings (Figure S2c). The RT-qPCR analysis also confirmed that the expression level of LOC_Os03g06370 was dramatically lower in nbl3 than in the wild type (Figure S2d). These results indicated

Figure 1  The nbl3 mutant exhibits lesion mimic phenotype companied with reactive oxygen species burst, accelerated leaf senescence and enhanced disease resistance. (a) Whole plants of the wild type (WT) and the nbl3 mutant at flowering stage in the paddy field. Scale bars = 10.0 cm. (b) Lesion mimic phenotypes on different leaves of the nbl3 mutant at the pollination stage, compared with that of wild type. Leaves from lower to upper were arrayed from right to left, respectively. (c) The second leaf of the wild type and the nbl3 mutant. (d–f) Photographs of leaves stained with Trypan blue, NBT and DAB. (g) Lesion length in leaves of (h) indicates significant differences between WT and nbl3 leaves. Data were shown as means ± SD, n = 10 (**P < 0.01; Student’s t-test). (h) The lesions on the WT and nbl3 leaves at 96-hour post-inoculation with isolate HS35 of M. oryzae. (i) Lesion length in leaves of (j) indicates significant differences between the WT and nbl3 leaves. Data were shown as means ± SD, n = 15 (**P < 0.01; Student’s t-test). (i) The lesions on the WT and nbl3 leaves after two-week post-inoculation with Xoo strain PXO99.
that the mutation in LOC_Os03g06370 was responsible for the nbl3 phenotypes. This was further confirmed by overexpression and RNAi analyses of the gene using transgenic procedures (see below). Thus, the LOC_Os03g06370 gene is renamed as OsNBL3 in this study.

Phylogenetic analysis showed that OsNBL3 shared similarity with its homologues in both monocot and dicot plants, and had especially high level of identity with homologues in grasses with its homologues in both monocot and dicot plants, and had OsNBL3 expression in the basal node and flag leaves at the heading stage relatively higher expression in the sheath and roots, and lower seedlings was induced by inoculation with the OsNBL3 was constitutively expressed in all tested tissues, with rice, RT-qPCR analyses were performed. It was revealed that the transcript levels of OsNBL3-RNAi transgenic lines were generated (Figure 3c). In contrast to the RNAi lines, the OE lines were significantly up-regulated in the RNAi lines (Figure S4). Overexpression (OE) lines of the gene were also administered to extract SA, JA and ABA. Log10 intensity values were shown as means ± SD, n = 3 (***P < 0.01; Student’s t-test).

RNAi plants also exhibited enhanced disease resistance

To further confirm that the disruption of OsNBL3 was responsible for the nbl3 phenotypes, we firstly tried to generate knockout lines of OsNBL3 using the CRISPR/Cas9 method (Shan et al., 2013). However, we were unable to obtain transgenic plants. It is possible that the knockout of OsNBL3 is lethal. Therefore, OsNBL3-RNAi transgenic lines were generated (Figure 3c), and the transcript levels of OsNBL3 in each homozygous line were confirmed by RT-qPCR analysis (Figure 3e). Exceeding expectations, the RNAi plants had shorter plant heights similar to those of the nbl3 mutant, while no clear spontaneous cell death was observed on the leaves of the RNAi plants (Figure S3a). The intact mRNA level was detected by RT-PCR analysis using RT-F and RT-R as primers; the result showed that the transcripts of OsNBL3 in the mutant are lower than that in the RNAi plants (Figure S3b), which may be attributed to the phenotypic difference. To determine whether the RNAi plants exhibited enhanced disease resistance, the resistance of three independent RNAi lines to M. oryzae was tested using the punch inoculation method. The results showed that all the tested lines displayed enhanced resistance (Figure 3d,f). In addition, expression analysis showed that two selected defence-related marker genes, OsPR1b and OsPR5, were significantly up-regulated in the RNAi lines (Figure S4). Overexpression (OE) lines of the OsNBL3 gene were also generated (Figure 3c). In contrast to the RNAi lines, the OE lines did not exhibit obvious reduced or enhanced resistance to M. oryzae compared with the wild type (Figure 3d,f). These results further demonstrate that disruption of OsNBL3 is responsible for the enhanced disease resistance.

Both the nbl3 mutant and RNAi plants exhibit enhanced salt tolerance

It has been reported that the mechanism underlying rice LMMs is regulated by hormones and abiotic stresses (Mosher et al., 2010; Wang et al., 2015a; Yamanouchi et al., 2002). To evaluate whether OsNBL3 is associated with abiotic stresses, 10-day-old seedlings, grown in normal conditions, were continuously irrigated with 100 mM and 150 mM NaCl for thirty days, respectively. Compared with the wild-type plants, a greater number of nbl3 and two RNAI plants survived at both salt concentrations, while the two OE lines showed fewer survived plants (Figure 4a), which indicates that nbl3 and RNAI plants are
more tolerant to salt. The germinated seeds were placed on agar plates containing 0, 100, 150 and 200 mM NaCl, and the plant height and fresh weight were measured after seven days. Compared with the wild-type plants, all the nbl3, RNAi and OE plants showed lower inhibition rate of both plant height and fresh weight, at each salt concentration (Figure 4b and Figure S5a,b), further indicating the involvement of OsNBL3 in salt responses. The root length of both the nbl3 and wild-type seedlings grown on agar plates containing 100 mM NaCl were also measured from two to five days post-planting. The inhibition rate of root growth was significantly lower for nbl3 than for the wild type at each sampling point (Figure S5c–e). RT-qPCR analysis was then conducted to determine the expression of OsNBL3 in response to salt treatment. The result showed that OsNBL3 expression was induced by NaCl with a peak at 10 days post-irrigation (Figure 4c). In addition, the expression analysis showed that the potassium transport gene OsHAK1, the late embryogenesis abundant protein-encoding gene OsLEA3 and the transcription factor genes OsNAC22, OsNAP and OsMYB4, which are salt tolerance-related genes (Chen et al., 2015; Chen et al., 2014; Hong et al., 2016; Hu, 2008; Vannini et al., 2006), were significantly up-regulated in the nbl3 plants compared with in the wild-type plants.

Figure 3  Expression patterns of OsNBL3 and enhanced disease resistance of RNA interfere plants. (a) RT-qPCR analysis of OsNBL3 expression patterns in different tissues of wild-type plants. Flag leaf (L1), second leaf (L2), third leaf (L3), fourth leaf (L4), Sheath (Sh), Stem node 1(Sn1), Stem node 2(Sn2), Basal node(Bn), panicle(Pa), Root(Ro). Data were shown as means ± SD, n = 3. Rice OsActin (LOC_Os03g50885) gene was used as an internal control. (b) RT-qPCR analysis of OsNBL3 expression level at different time after inoculation with the compatible isolate H535 of M. oryzae. Two-week-old seedlings were used for inoculation. The seedlings sprayed only with 0.025% Tween 20 were used as negative control (Mock). Rice OsActin (LOC_Os03g50885) gene was used as an internal control. Data were shown as means ± SD, n = 3, (**P < 0.01; Student’s t-test). (c) Schematic diagrams of the OsNBL3-OE and RNAi structures. Ubi, Maize ubiquitin promoter; 3xFlag, three tandem repeat Flag tag; a cDNA fragment from 238 bp to 797 bp downstream of OsNBL3’s ATG was used sense and anti-sense sequence. (d) The lesion on the wild type (WT) and nbl3 leaves at 96-hour post-inoculation with H535. (e) Expression analysis of OsNBL3 in the wild type(WT) and OsNBL3-OE or OsNBL3-RNAi lines by RT-qPCR, respectively. Data were shown as means ± SD, n = 3 (**P < 0.01; Student’s t-test). OsNBL3-RNAi lines include R6, R10, R20; OsNBL3-OE lines include OE-16, OE-21, OE-23. (f) Lesion length in leaves (b) of the wild type, nbl3, OsNBL3-OE and OsNBL3-RNAi lines (**P < 0.01; Student’s t-test). Data were shown as means ± SD, n = 10.
wild type (Figure 4d). These results demonstrate that OsNBL3 contributes in response to salt stress, and disruption of the gene results in enhanced salt tolerance.

**OsNBL3 is a mitochondrion-localized P-type PPR protein**

OsNBL3 encodes a protein with 409 amino acid residues. According to the prediction by https://ppr.plantenergy.uwa.edu.au/ (Cheng et al., 2016), OsNBL3 is a PPR protein that harbours seven canonical P-type PPR repeats (Figure 5a). TargetP prediction analysis showed that the OsNBL3 protein has a mitochondrion-targeting signal at its N terminus. To determine the subcellular location of OsNBL3, the OsNBL3-GFP construct was made in which the full-length cDNA was fused with GFP. When the OsNBL3-GFP construct was transformed into the epidermal cells of the N. benthamiana leaf, no GFP signals were observed. It is possible that the entire OsNBL3-GFP protein was difficult to express or was easily degraded. Therefore, another construct (3N-GFP) was made in which the GFP was fused to the N terminus of OsNBL3, harbouring the putative mitochondrion-targeting signal (Figure 5a). When 3N-GFP was transiently expressed in the epidermal cells of the N. benthamiana leaf, the green fluorescent signals of 3N-GFP overlapped with signals from the mitochondria Mito-Tracker (Figure 5b). When 3N-GFP was transiently expressed in rice protoplasts, similar overlays between GFP and signals from the mitochondria dye, Mito-Tracker Red, were also observed (Figure 5c). These results indicate that OsNBL3 is a mitochondrion-localized protein.

**OsNBL3 participates predominantly in the splicing of nad5 intron 4**

It had previously been implicated that P-type PPR proteins participate in plant organelle RNA metabolism, including 5′ processing, intron splicing and translation (Barkan and Small, 2014). Given that OsNBL3 is a mitochondrion-targeting protein, CR-RT-PCR procedures were firstly used to examine whether the 5′ processing of respiratory complex genes was affected in the nbl3 mutant. Among 17 analysed genes, the amplified products of each gene in the wild-type and nbl3 plants were the same size (Figure S6) suggesting that 5′ processing was not affected by the mutation in nbl3. Next, the mature transcripts of 34 mitochondrial genes were examined using total RNA from 30-day-old wild-type and nbl3 plants with specific primers (Figure 6a). The results showed that only the mature nad5 transcripts were conspicuously reduced in nbl3.

There are four introns in the nad5 gene, including two cis-introns and two trans-introns (Bonen, 2008). To determine whether the reduction in the nad5 transcripts resulted from splicing defects in nbl3, RT-qPCR was conducted to analyse the ratio of spliced to unspliced transcripts of all the 23 introns in mitochondria. The splicing efficiency of nad5 intron 4 was dramatically reduced in nbl3 compared with in the wild type (Figure 6b). To further verify improper splicing of nad5 in nbl3, reverse transcription PCR was performed, allowing amplification across adjacent exons and detection of each splicing event in nad5 (Figure 6c). The amount of cis-spliced transcripts of exons 4–5 (exon 4 + exon 5) was significantly reduced in the nbl3 mutant (Figure 6d). These results suggest that OsNBL3 is required for the cis-splicing of the mitochondrial nad5 intron 4. Interestingly, the amount of trans-spliced transcripts of exons 2–4 (exon 2 + exon 3 + exon 4) was also weakly reduced in the nbl3 mutant, suggesting that OsNBL3 also participates in the trans-splicing of nad5 in mitochondria.

**OsNBL3 mutation affects NADH dehydrogenase activity and mitochondrial morphology**

NAD5 is a subunit of NADH dehydrogenase (complex I) in the mitochondrial respiratory chain. To investigate whether complex I and mitochondrial morphology were affected by the disruption of OsNBL3, the assembly and function of the mitochondrial respiratory chain and complex I in gel activity assays were conducted by Blue Native-PAGE (BN-PAGE) and Coomassie Brilliant Blue (CBB) staining, respectively. No significant difference in the assembly of complex I in nbl3 was observed in BN-PAGE as compared with the wild type, and Western blot using an antibody against NAD9 (a subunit of complex I) showed little reduction in nbl3. However, the activity of complex I was reduced in nbl3 compared with the wild type (Figure 7a). Transmission electron microscopy assays were used to observe the ultrastructure of the mitochondria in mesophyll cells from eight-week-old nbl3 and wild-type plant leaves. The overall number of mitochondria was lower in nbl3 than in the wild type, and the overall size of mitochondria was relatively greater in nbl3. Specifically, swelling cristae with vesicle-like structures and reduced intermembrane content were observed in the nbl3 mesophyll cells (Figure 7b and Figure 7a).

Disruption of ETC in plant mitochondria usually leads to the induction of alternative respiratory pathway genes that are known to be mitochondrial stress markers (Vanlerbergh et al., 2013). RT-qPCR analysis showed that there were significantly more AOX1a and AOX1c transcripts in the leaves of 30-day-old nbl3 seedlings than in the leaves of wild-type seedlings (Figure 7b). Plant mitochondria have internal and external NADH dehydrogenases that function as alternative dehydrogenases (Rasmussen and Wallstrom, 2010). Expression analysis showed that the internal NADH dehydrogenase genes, NDB2 and NDB3, and the external NADH dehydrogenase genes, NDA1 and NDC1, were expressed at a higher level in the leaves of four-week-old nbl3 seedlings than in four-week-old wild-type seedlings (Figure 7b). These results demonstrate that loss of OsNBL3 function affects the assembly of complex I, leading to the induction of the alternative respiratory pathway.

**Discussion**

The nbl3 is a LMM mutant caused by the disruption of a PPR gene

Plant cell death and structural adaptations may protect plants from biotic and abiotic stresses, including pathogens, salt and high temperature but ROS (Ma et al., 2019; Yamanouchi et al., 2002; Zeng et al., 2004). The molecular mechanism underlying plant cell death and defence responses in LMMs mutants have been partially elucidated. LMM mutants show spontaneous HR-like necrotic lesions and bursts of ROS, and activate the expression of defence genes (Wang et al., 2017c; Xu et al., 2014). The nbl3 mutant identified in the study exhibited growth retardation, leaf wilting and premature senescence (Figure 1a–c). Interestingly, unlike most identified LMM mutants that usually display regular-shaped reddish-brown spots, although some of them are regulated developmentally or environmentally. In the case of nbl3, the typical lesion mimic spots of nbl3 only occurred at the seedling stage. From the tilling stage, the nbl3 does not exhibit obvious dark spots, while the leaves of nbl3 gradually withered from the lower to upper leaves. Histochimical staining using Trypan blue, NBT and DAB showed that the nbl3 mutant...
Figure 4  Disruption of OsNBL3 leads to enhanced salt tolerance. (a) Morphology of 40-day-old seedlings of the wild type, nbl3, RNAi-10, -20 and OE-16, -21 that all were continuously irrigated with 100, 150 mM NaCl or water only, for thirty days. Scale Bar = 10 cm. (b) The height inhibition rate of seven-day old seedlings grown on agar plates containing 100, 150 and 200 mM NaCl, respectively. Data were shown as means±SD, n = 5, (Values with same superscript letters are of no significant difference (P > 0.05), those with different letters are of significant or extreme difference (P < 0.05)). (c) RT-qPCR analysis of temporal expression patterns of OsNBL3 in the wild-type seedlings that were continuously irrigated with 100 mM NaCl. The OsActin gene (LOC_Os03g50885) was used as an internal control. Data were shown as means±SD, n = 3, (**P < 0.01; Student’s t-test). (d) RT-qPCR analyses of constitutive expression of several the salt tolerance-related genes in seedlings of the nbl3 and wild type. The OsActin gene (LOC_Os03g50885) was used as an internal control. Data were shown as means±SD, n = 3, (**P < 0.01; Student’s t-test).
displayed ROS accumulation and cell death in the leaves (Figure 1d-f). These symptoms have also been observed in other LMM mutants (Wang et al., 2017c; Zhao et al., 2020). These results indicated that nbl3 is a new LMM mutant.

In addition to ROS bursts and cell death, many LMM mutants exhibit enhanced disease resistance (Chen et al., 2019; Qiao et al., 2010; Yamanouchi et al., 2002). In the present study, it was found that the nbl3 mutant showed enhanced resistance against both fungal and bacterial pathogens (Figure 1g–j). This suggests that OsNBL3 may play roles in regulating PCD and resistance signalling pathways in plants (Figure 2, 3b). Interestingly, the RNAi plants did not show spontaneous cell death in the same way that the nbl3 mutant did (Figure S3a). However, the RNAi plants did display enhanced resistance to M. oryzae (Figure 3d,f). An explanation for this could be that the transcripts were destroyed in the nbl3 mutant by a T-DNA insertion within the 3′-untranslated region of OsNBL3. Meanwhile, the down-regulation of OsNBL3 in the RNAi plants was mediated via a post-transcriptional silencing mechanism, which allowed for a few intact transcripts to be present. Consistent with the explanation of OsNBL3 in the RNAi plants were more than that of mutants, but less than that of wild type (Figure S3b).

OsNBL3 encodes a mitochondrion-localized PPR protein (Figure 5). As RNA binding factors, PPR proteins regulate the RNA expression in organelles, thereby affecting plant growth and development. There are many reports involving PPR mutants that have shown that PPR proteins are involved in regulating the physiological functions of plant growth and development. Such PPR mutants have displayed cytoplasmic male sterility (CMS) (Hu et al., 2012; Huang et al., 2015), defective seed or embryo development (Liu et al., 2020; Wang et al., 2017b), restricted plant growth (Xie et al., 2016; Zhu et al., 2012), organelle development defects (Lin et al., 2015; Wang et al., 2018b), albino leaf phenotype (Su et al., 2012; Tang et al., 2017) and insensitivity to abiotic stress (Yuan and Liu, 2012; Zsigmond et al., 2012). In this study, nbl3 showed almost all of the physiological phenotypes of PPR mutants, such as growth retardation (Figure 1a), reduced seed setting rate (Figure S1) and enhanced salt tolerance (Figure 4). However, unlike the other PPR mutants, disruption of a mitochondrion-localized PPR gene causes the nbl3 phenotype with enhanced disease resistance.

**OsNBL3 is involved in splicing nad5 introns**

PPR proteins are usually encoded by nuclear genes and then transported to organelles to regulate RNA metabolism, including intron splicing, RNA editing, 5′- and 3′-modification, RNA degradation and translation and other post-transcriptional modification processes (Barkan and Small, 2014). It is well known that mitochondria provide energy for cell processes. Group II introns are ubiquitous in the organelle genomes of flowering plants. There are 23 group II introns in rice mitochondria, 19 of which are distributed in the genes encoding subunits of the NADH dehydrogenase complex (complex I) (Bonen, 2008). Complex I is the first enzyme complex in the mitochondrial respiratory chain, the starting point for electrons to enter the respiratory chain, and essential for mitochondrial energy production and electron transfer (Dai et al., 2018; Wu et al., 2019). It has been reported that defects in the intron splicing of nad mRNAs could lead to partial or complete reductions in the activity of ETC complex I, and disturb plant growth and development. Recently, several P-type PPR proteins have been identified as splicing factors of *nad* in *Arabidopsis* and maize (Colas des Francs-Small et al., 2014; Zhang et al., 2017). Defects in *nad* gene intron splicing result in a significant decrease in the assembly and activity of complex I and overexpression of AOX genes in the *misf* and *ppr19* mutants of *Arabidopsis thaliana* (Lee et al., 2017; Wang et al., 2018a). The maize PPR gene mutants *dek37*, *emp12* and *ppr20* displayed compromised splicing efficiencies of *nad2* introns, and mitochondrial morphology and seed development were also affected (Dai et al., 2018; Sun et al., 2019; Yang et al., 2020). Furthermore, the other two PPR proteins DEK41 and EMP602 have been shown to be required for the splicing of *nad4* introns and seed development (Ren et al., 2019; Zhu et al., 2019). The PPR protein
ZmSMK9 affects the development of the kernel and plant architecture by participating in the splicing of nad5 introns in maize (Pan et al., 2019). In rice, two PPR proteins FLO10 and RL1 were reported to be involved in the splicing of nad1 intron 1 and nad4 intron 1, respectively (Wu et al., 2019; Wu et al., 2020). nad5 contains four group II introns, of which introns 1 and 4 are cis-spliced, while introns 2 and 3 are trans-spliced (Bonen, 2008). In the present study, it was found that the disruption of the PPR gene OsNBL3 compromised the splicing of nad5 introns in mitochondria (Figure 6b–d). As mention above, RNA metabolism in organelles is essential for their functions. It was considered that P-type PPR proteins are associated with RNA splicing. In rice genome, there are 246 genes encoding P-type PPR proteins (Chen et al., 2018). Among them, only few were identified involving in several nad genes splicing. It would be needed to deeply determine whether and how the rest of P-type PPR proteins involving in organelles RNA metabolism.

Disruption of the OsNBL3 results in enhanced tolerance to salinity stress

As one of the major abiotic factors limiting crop productivity, high salinity stress induces osmotic stress and ionic stress that inhibit water uptake in roots and photosynthesis in shoots (Deinlein et al., 2014). Salinity stress causes over-reduction in the mitochondrial electron transport chain (mtETC), resulting in electron leakage to O2 and subsequent production of reactive oxygen species (ROS) O2− and H2O2 (Liberatore, et al., 2016). Excess ROS is toxic and leads to metabolic disorders, cell damage and cell death (Liberatore, et al., 2016; Miller, et al., 2010). The complex I and III of the mtETC are major ROS-producing sites under abiotic stress (Miller, et al., 2010). In plants, mitochondria can bypass the oxidative phosphorylation pathway and transport protons without producing ATP, by depending on alternative NAD(P)H dehydrogenases (NDs) and alternative oxidases (AOXs).
tolerance in
Inhibition of mitochondrial complex I enhances high salinity-stress
OsNBL3 (Figure 4 and Figure S5a,b). The mutation of
gene is responsible for the
mechanism against salt stress and triggers HR response, as well
mtROS further destroys the intracellular environment and home-
their leakage into the cytoplasm. Moreover, the leakage of
mitochondrial membrane underlies the production of mtROS and results in
(Figure 7 and Figure S7). The destruction of the inner mitochon-
ditures, and elevated reliance on alternative respiratory pathways
morphology with the collapse of cristae with vesicle-like struc-
tures, and elevated reliance on alternative respiratory pathways
(Figure 7 and Figure S7). The destruction of the inner mitochon-
drial membrane underlies the production of mtROS and results in
their leakage into the cytoplasm. Moreover, the leakage of
mtROS further destroys the intracellular environment and home-
ostasis, which via feedback, activates the plant’s protective
mechanism against salt stress and triggers HR response, as well
as growth retardation and pre-senescence.

Taken together, it is plausible that the mutation of the OsNBL3
gene is responsible for the nbl3 phenotype as the fully function-
ing gene normally participates in the splicing of nad5 introns.
Disruption of OsNBL3 leads to cell death and enhanced disease
resistance and salt tolerance. Our results would provide an insight
to explain the mechanisms underlying plant defence against
biotic and abiotic stresses.

Methods

Plant materials and growth conditions
The nbl3 mutant was identified by screening a T-DNA insertion
population of the rice cultivar Aichiasahi (Oryza sativa ‘Geng’
[japonica]). The nbl3 mutant and wild-type plants were grown in
the experimental field at China Agricultural University in Beijing,
China, or in growth rooms maintained at 28°C during the day,
with a 12-h light/12-h dark photoperiod and 70% humidity.

Pathogen inoculations and salt treatments
Rice seedlings were inoculated with the Magnaporthe oryzae
isolate HS35 using the punch inoculation method (Fang et al.,
2018). In brief, the detached leaves of five-leaf-stage rice
seedlings were wound-inoculated with 10 µL of spore suspension
(2 × 10⁶ spores/mL) supplemented with 0.025% Tween 20.
Moreover, intact leaves were spray-inoculated with HS35 spore
solution (1 × 10⁶ spores/mL) containing 0.025% Tween 20.
The inoculated leaves were then transferred to a chamber at 28°C
under 100% humidity and a 12-h dark/12-h light cycle. The
length of the resulting lesions was measured at 96 h post-
inoculation (hpi). To evaluate rice bacterial blight disease
resistance, the wild type and nbl3 mutant were inoculated with the
Xanthomonas oryzae pv. oryzae (Xoo) strain PXO99 using the
leaf-clipping method at 60 days after sowing (DAS). The over-
night liquid culture of the bacterium was collected and adjusted
to an optical density (OD) = 0.8 using deionized water. The distal
tip (approximately 3 cm) of the flag leaves was removed using
scissors and then dipped into the bacterial suspension. Five
individual plants and three tillers per plant were inoculated with
PXO99, and these plants were grown in a glasshouse. The lesion
length was measured at 14 days post-inoculation (dpi).

For the salt treatments, seeds of the nbl3 and wild-type plants
were treated with sodium hypochlorite solution (1%) to acceler-
ate germination. 10-day-old seedlings grown in normal condi-
tions (28°C and photoperiod of 12-h light/12-h dark) were
continuously irrigated with 100 mM or 150 mM NaCl, while plants
irrigated only with water were used as the control. The whole
plants were photographed after thirty days of irrigation. During
the irrigation with NaCl, wild-type leaves were sampled at
different time points to conduct expression analysis of OsNBL3 by

Figure 7 OsNBL3 mutation affects NADH dehydrogenase activity and mitochondrial morphology. (a) Left, Coomassie Brilliant Blue (CBB) staining of mitochondrial proteins from the wild type (WT) and the nbl3 mutant by Blue native-polyacrylamide gel electrophoresis (BN-PAGE). Middle, in-gel NADH dehydrogenase activity staining for Complex I, C-I: complex I. Right, Western blot analysis with antibody against Nad9 (a subunit of complex I). (b) Transmission electron microscope images of mitochondria in wild-type (WT) and nbl3 leaves. The red arrows indicate mitochondria (Mt). Bar = 0.5 µm. (c) Number of mitochondria per cell in the mutant nbl3 and WT. (d) Average size of mitochondrion of the mutant nbl3 and WT.
real-time quantitative polymerase chain reaction (RT-qPCR). In addition, germinated seeds were placed on agar plates containing 0, 100, 150 or 200 mM NaCl, and grown in a greenhouse (28°C and photoperiod of 12-h light/12-h dark). The seedlings under each treatment were photographed, and plant height and fresh weight were measured at 7 d. Also, roots of the seedlings under 100 mM NaCl were photographed, and the root lengths were measured at 2, 3, 4 and 5 d.

Site finding thermal asymmetric interlaced (TAIL)-PCR cloning for OsNBL3

SiteFinding TAIL-PCR procedures were used to isolate the sequences flanking the T-DNA using a previously described method (Tan et al., 2005). The products of tertiary SiteFinding TAIL-PCR were sequenced and used to search against the rice genome database (http://rice.plantbiology.msu.edu/) to obtain the T-DNA insertion site. Specific primers, L3, R3, JF and JR, were used to reconfirm the T-DNA insertion site. The sequences of all of the primers used in the study are listed in Table S2.

Transmission electron microscopy

Leaf sections of the nbl3 and wild type were sampled from plants at 60 DAS, and immersed in 2.5% glutaraldehyde in a phosphate buffer at room temperature for 48 h and then maintained at 4°C overnight. Subsequently, the samples were washed and incubated in 1% OsO4 at 4°C for 12 h. After dehydration in a gradient ethanol series, the samples were embedded in Spurr’s resin prior to ultrathin sectioning. Sections were stained with uranyl acetate and examined with a Hitachi-H7500 transmission electron microscope.

Histochemical assays

The second leaves of the nbl3 and wild type were used in histochemical assays at 60 DAS. A lactic acid-phenol-trypsin blue solution was used to evaluate cell death, and tetranitroblue tetrazolium chloride (NBT) solution and 3,3’-diaminobenzidine (DAB) solution were used to evaluate H2O2 accumulation. Staining was performed using previously described methods (Wang et al., 2017c). Briefly, leaves of the nbl3 and wild-type plants were immersed in Trypan blue solution (0.25% Trypan blue, 25% lactic acid, 23% water-saturated phenol and 25% glycerol) in a boiling water bath for 20 min, cooled all night and supplemented with chloral hydrate (0.25%) for 30 h. Leaves were also immersed in DAB solution (1 mg/mL DAB and 10 mM Na2HPO4, pH 3.8) or NBT solution (0.5 mg/mL NBT and 10 mM K2HPO4, pH 7.8) in the dark for 16 h at room temperature. The staining leaves were transferred into 95% ethanol to decolourize.

Subcellular localization

To perform transient expression analysis in rice protoplasts and Nicotiana benthamiana leaf epidermal cells, the full-length coding sequence of OsNBL3 without the stop codon and with a total length of 300 bp starting from ATG was amplified using the primer set OsNBL3-GFP-F/R or 3N-GFP-F/R, respectively. The PCR product was fused in frame with green fluorescent protein (GFP) in the pCAMBIA1301 plasmid, to generate 35S:OsNBL3-GFP or 35S:3N-GFP fusion constructs, respectively. The expression vector was introduced into rice protoplasts following previously described methods (Zhao et al., 2020), and the transfected protoplasts were incubated at 28 °C in the dark. The fluorescent GFP signal was examined and photographed using a laser confocal scanning microscope (Leica TCS SP8) after 16 h. The mitochondria dye Mito-Tracker Red (Invitrogen) was incubated at 37°C in a suspension containing the rice protoplasts 15 min before imaging (Shyu et al., 2008). Additionally, pCXSN-COX4-RFP (Chen et al., 2019) as Mito-Marker constructs and 3N-GFP or OsNBL3-GFP fusion constructs were introduced into the Agrobacterium tumefaciens strain EHA105. The constructs were then transformed via A. tumefaciens into Nicotiana benthamiana leaves together using previously described methods (Shyu et al., 2008). Fluorescence was detected 36 h after infiltration. The primers used are listed in Table S2.

RNA isolation and RT-qPCR analysis

For the expression analysis of defence marker genes and AOX or ND genes, the leaves of five-leaf-stage wild-type and nbl3 plants were used. For the expression pattern analysis, the leaves from the top to bottom, the sheath, stem nodes, panicles and roots of the wild type were used. Total RNA was extracted using the KF Fast Plant Total RNA Kit (Beijing Zoman Biotechnology), and cDNA synthesis was performed using the HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) according to manufacturers’ instructions. RT-qPCR was performed using 2x RealStar Green Power Mixtue with ROX II and ABI QuantStudio 6 Flex PCR program, with the rice actin gene (LOC_Os03g50885) used as an internal control. Primers used for the RT-qPCR analyses are listed in Table S2.

Measurement of phytohormones

For measurement, the intensity of JA, ABA and SA, ~1 g (fresh weight) rice seedlings were used to extract phytohormones and their metabolites, followed by Cao et al. (2016). The resulting sample solution was injected into the LC–MS/MS (1260-6520 LC-QTOF, Agilent) for further analysis. Phytohormones and their metabolites were separated on a ZORBAX SB-C18 column (2.1*100 mm, 3.5 μm, Agilent). The mobile phase consisted of water with 0.1% formic acid (solvent A) and ACN with 0.1% formic acid (solvent B). The gradient is started with 5% B and kept for 2 min, then increased to 25% B in 8 min, to 70% in 30 min, to 95% B in 3 min and kept for 7 min. The flow rate was 0.25 mL/min. The parameters for MS are as follows: ESI source, negative mode, capillary voltage = 3000V, gas temperature = 340°C. Data acquisition and analysis were performed using MassHunter software (Agilent). The standard solution of JA, ABA and SA were used for identification. m/z of 209.1182, 138.0317 and 264.1362 were used for identification of JA, SA and ABA, respectively. The intensity of JA, ABA and SA in each sample was log10-transformed and then compared. All the samples were analysed with three biological replicates.

Circularized RNA reverse transcription PCR (CR-RT-PCR)

Following the T4 RNA Ligase I (New England Biolabs) guide, five μg of total RNA from rice leaves at 30 DAS were circularized. First-strand cDNA was synthesized using Prime Script II RTase (TaKaRa) with specific primers (Table S2). The resulting cDNAs were amplified using primers specific to each target gene (Table S2). Each circular RT-PCR fragment was cloned into the pCloneEZ-Blunt TOPO Cloning Kit (Clone Smarter) with ten monoclonals for each target gene sequence.

Vector construction and rice transformation

For the genetic overexpression and RNAi tests, the OsNBL3 coding sequence was amplified using cDNA templates derived from the rice cultivar Aichiasahi. PCR products were cloned into the binary
vector pCAMBIA1301 under the control of the maize ubiquitin promoter to generate fused pUbI:3Flag-OsNBL3 constructs. Bases 238 to 797 of the cDNA fragment of OsNBL3 were selected and used for RNAi vector construction. The 386 bp intron of OsNBL3 was used as a linker. The fragments of OsNBL3 were fused into pUbI:OsNBL3-RNAi constructs. The vectors were introduced into Agrobacterium tumefaciens strain EHA105 and used to infect wild-type calli. Primers used in the study are listed in Table S2.

Blue Native-PAGE electrophoresis and Complex I activity assays

Crude mitochondria were isolated from germinating seedlings of two days, and 120 µg of mitochondria protein samples were run for BN-PAGE according to Zhang et al. (2017). In-gel NADH dehydrogenase activity assay was performed as described by Meyer et al. (2009). Briefly, the gel was washed three times for 5 min with distilled water and incubated in the reaction medium (0.14 mM NADH, 1.22 mM NBT, and 0.1 M Tris-HCl, pH 7.4). When the dark blue stain was strong enough, the reaction was stopped by transferring the gel to 40% methanol/10% acetic acid (v/v).

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

WS Zhao designed the research; TC Qiu, XS Zhao, HI Feng and LL Qi performed the experiments; WS Zhao, TC Qiu, J Yang and YL Peng discussed the results; WS Zhao and TC Qiu wrote the manuscript. The author(s) read and approved the final 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.

**Figure S1** Several agronomic traits of the nbl3 mutant and wild type (WT) at the pollenization stage.

**Figure S2** Molecular cloning of the OsNBL3.

**Figure S3** Phenotypes of the whole plants of the wild type, nbl3 mutant and three representative RNAi lines, and the expression of OsNBL3 in these plants.

**Figure S4** RT-qPCR analyses of two defence-related genes in the seedling of the wild type, nbl3 mutant and three represent RNAi lines.

**Figure S5** Disruption of OsNBL3 results in enhanced salt tolerance in seedlings.

**Figure S6** Circular RT-PCR analysis of seventeen mitochondrial genes.

**Figure S7** Detailed mitochondrial morphology and expression of alternative respiratory pathway genes in the nbl3 and wild type.

**Table S1** Genetic analysis of the nbl3 mutant.

**Table S2** List of primers and their uses.

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