The rice RNase P protein subunit Rpp30 confers broad-spectrum resistance to fungal and bacterial pathogens

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Summary

RNase P functions either as a catalytic ribonucleoprotein (RNP) or as an RNA-free polypeptide to catalyse RNA processing, primarily tRNA 5′ maturation. To the growing evidence of non-canonical roles for RNase P RNP subunits including regulation of chromatin structure and function, we add here a role for the rice RNase P Rpp30 in innate immunity. This protein (encoded by LOC_Os11g01074) was uncovered as the top hit in yeast two-hybrid assays performed with the rice histone deacetylase HDT701 as bait. We showed that HDT701 and OsRpp30 are localized to the rice nucleus, OsRpp30 expression increased post-infection by Pyricularia oryzae (syn. Magnaporthe oryzae), and OsRpp30 deacetylation coincided with HDT701 overexpression in vivo. Overexpression of OsRpp30 in transgenic rice increased expression of defence genes and generation of reactive oxygen species after pathogen-associated molecular pattern elicitor treatment, outcomes that culminated in resistance to a fungal (P. oryzae) and a bacterial (Xanthomonas oryzae pv. oryzae) pathogen. Knockout of OsRpp30 yielded the opposite phenotypes. Moreover, HA-tagged OsRpp30 co-purified with RNase P pre-tRNA cleavage activity. Interestingly, OsRpp30 is conserved in grass crops, including a near-identical C-terminal tail that is essential for HDT701 binding and defence regulation. Overall, our results suggest that OsRpp30 plays an important role in rice immune response to pathogens and provides a new approach to generate broad-spectrum disease-resistant rice cultivars.

Introduction

Acetylation and deacetylation of histones in eukaryotes are two dynamic and reversible post-translational modifications (PTMs) which are regulated by the antagonistic enzymes histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively (Fierz and Poirier, 2019; Shahbazian and Grunstein, 2007). In plants, these enzymes are known to play a crucial role in many cellular processes including plant growth, development, hormone signalling, responses to abiotic stresses and immunity to pathogens (Ma et al., 2013). HATs and HDACs function in plant innate immunity by controlling the expression of defence genes (Ding and Wang, 2015; Song and Walley, 2016).

The rice genome contains eight HATs and 17 HDACs (Ding et al., 2012; Fu et al., 2007; Liu et al., 2012). The HDACs are classified into three families including the plant-specific HD2, which comprises two rice members HDT701 and HDT702 (Fu et al., 2007; Zhao et al., 2015). We previously demonstrated the function of HDT701 in suppressing innate immunity in rice to pathogens (Ding et al., 2012). Overexpression of HDT701 in transgenic rice decreased histone H4 acetylation and enhanced susceptibility to Pyricularia oryzae (syn. Magnaporthe oryzae) and Xanthomonas oryzae pv. oryzae (Xoo). In contrast, silencing of HDT701 in transgenic rice elevated histone H4 acetylation as well as transcription of pattern recognition receptor (PRR) and defence genes, increased generation of reactive oxygen species (ROS) after pathogen-associated molecular pattern (PAMP) elicitor treatment and enhanced resistance to both P. oryzae and Xoo. These results support our working model that HDT701 negatively regulates innate immunity by modulating the level of histone H4 acetylation and expression of PRR and defence genes in rice (Ding et al., 2012). HDT701 has also been reported to negatively regulate plant abiotic stress tolerance (e.g. ABA, salt) and to function in seed germination and flowering (Cho et al., 2018; Zhao et al., 2015). Here, our efforts to identify cellular proteins that may mediate the biological functions of rice HDT701 led to the finding of a surprising partner: OsRpp30, a putative subunit of RNase P.

Keywords: OsRpp30, HDT701, RNase P, Pyricularia oryzae (syn. Magnaporthe oryzae), Xanthomonas oryzae pv. oryzae.
RNase P was initially identified as the ubiquitous endoribonuclease for cleaving 5’ end of precursor tRNAs to make mature tRNAs (Altman, 2007; Gopalani et al., 2018; Lai et al., 2010). Although an RNA-free form of RNase P was discovered later (Daniels et al., 2019; Holzmann et al., 2008), OsRpp30 is a subunit of only the RNP form. The RNase P RNP consists of one catalytic RNA and a varying number of RNase P protein (Rpp) subunits: one in bacteria, up to five in archaea, and 9–10 in eukaryotes (Altman, 2007; Lai et al., 2010; Samanta et al., 2016).

In addition to its principal role in tRNA 5’ biogenesis, RNase P also cleaves long non-coding RNAs (ncRNAs), rRNAs and mRNAs (Hernandez-Cid et al., 2012; Jarrous, 2017; Jarrous and Gopalan, 2010). Moreover, RNase P is required for efficient transcription of various small ncRNAs, including tRNAs, 5S rRNA, SRP RNA and U6 snRNA (Jarrous, 2017; Jarrous and Reiner, 2007). Recent studies show that RNase P is also involved in regulating chromatin structure and function. For instance, the human RNase P subunits Rpp21, Rpp29 and Pop1 act as a repressor of histone H3.3 deacetylation upon HD701 overexpression in vivo and is associated with partially purified rice RNase P that exhibits tRNA 5’-processing. The C-terminal tail of OsRpp30 necessary for binding HD701 is also found in wild rice species and other cereals and is required for inducing expression of defence genes. Our results uncover a link between plant immunity and a protein known to be essential for tRNA processing.

**Results**

**OsRpp30, but not its paralogs, interacts with HD701 in vivo**

To identify HD701-interacting proteins in rice, we screened a yeast two-hybrid (Y2H) rice cDNA library using HD701 as bait. Sequencing of positive clones showed that a gene (LOC_Os11g01074) encoding the putative RNase P protein subunit Rpp30 was the top hit. BLAST searches of the rice genome revealed two paralogs of OsRpp30 (LOC_Os12g07680 and LOC_Os12g07680) that we named OsRpp30-S (shorter) and OsRpp30-L (longer) based on their lengths relative to OsRpp30 (Figures 1a,b). While OsRpp30-S aligns to the N-terminal half of OsRpp30 with 96% identity, OsRpp30-L is identical to OsRpp30 up to amino acid (aa) 699, but its last 31 aa have no homology to the last seven aa in OsRpp30 (Figure 1a,b). It is notable that the C-terminal half of OsRpp30 is a new module specific to plants and missing in other eukaryotes.

Y2H assays revealed that HD701 interacted only with OsRpp30, not with OsRpp30-S or OsRpp30-L (Figure 1c). HD701 also did not interact with just the first 250 aa (OsRpp30(1-250)) or 699 aa (OsRpp30(1-699)) of OsRpp30, regions that are equivalent to OsRpp30-S or OsRpp30-L, respectively. While OsRpp30(607-706) showed robust interaction, further deletions (OsRpp30(607-706) and OsRpp30(607-706)) abrogated HD701 binding. These results point to the importance of the C-terminal half of OsRpp30, particularly its last seven aa that is absent in OsRpp30-L. Remarkably, mutating just the last aa of OsRpp30 (S706A) eliminated all binding with HD701.

We employed multiple approaches to confirm the above findings in planta. First, we authenticated the HD701-OsRpp30 interaction with a luciferase complementation imaging (LCI) assay in Nicotiana benthamiana (Figure 1d). Mirroring the Y2H results, strong luminescence was detected when HD701-cluc and OsRpp30-nLuc were co-expressed under the control of the cauliflower mosaic virus 35S promoter in N. benthamiana leaves. Such luminescence was greatly diminished when (i) OsRpp30-S or OsRpp30-L replaced OsRpp30, (ii) the last seven aa of OsRpp30 was omitted, or (iii) the mutation S706A was introduced in OsRpp30. Second, following co-expression of OsRpp30-HA and HD701-Myc driven by the 35S promoter in rice protoplasts, we performed a co-immunoprecipitation (co-IP) test. The HA antibody successfully pulled down both OsRpp30-HA and HD701-Myc, indicating their interaction in rice (Figure 1e). Finally, because HD701 functions in the rice nucleus (Ding et al., 2012), we postulated likewise for OsRpp30. To test this idea, we first co-transfected rice protoplasts with OsRpp30-green fluorescent protein (GFP) and HD701-red fluorescent protein (RFP) fusion constructs, both under the control of the 35S promoter. Confocal fluorescence microscopy analysis then revealed that both OsRpp30-GFP and HD701-RFP indeed co-localized in the nucleus (Figure 1f). Together, the above results unambiguously demonstrated that HD701 and OsRpp30 interact and are present in the same cellular compartment.

**OsRpp30 positively regulates rice immunity**

To determine whether OsRpp30 is involved in rice immunity akin to HD701 (Ding et al., 2012), we assessed OsRpp30 mRNA levels by quantitative RT-PCR (RT-qPCR) after spray inoculation of wild-type (WT) plants with the compatible fungal pathogen P. oryzae RO1-1. Indeed, OsRpp30 mRNA level increased fourfold at 24 h post-inoculation (h.p.i.), while a modest (~1.5-fold) increase was observed for OsRpp30-L and none for OsRpp30-S (Figure 2a). In a post-inoculation time course analysis, OsRpp30 mRNA level peaked at 48 hpi and remained elevated even at 72 hpi (Figure 2b). To explore the broader significance with a second pathogen, we determined OsRpp30 transcript levels in WT rice plants inoculated with the bacterial blight pathogen Xoo Race 6 POX99 (P6). Likewise, OsRpp30 expression increased and peaked at 48 hpi (Figure 2c). Together with the finding of HD701–OsRpp30 interaction, these results suggest that OsRpp30, but not OsRpp30-L or OsRpp30-S, is involved in rice immunity.

We next examined how overexpression or knockdown of OsRpp30 affect rice immunity. We generated transgenic plants in which HA-OsRpp30 was overexpressed (OX, four- to sevenfold) from a maize ubiquitin promoter (Figure S1a). To evaluate the resistance phenotype of OsRpp30-OX compared to WT plants, we punch-inoculated these plants with the compatible P. oryzae RO1-1. Consistent with the enhanced expression of OsRpp30 post-inoculation playing a role in rice immunity, OsRpp30-OX plants displayed significantly smaller lesions compared to the WT post-inoculation (Figure 2d). Interestingly, while OsRpp30-OX plants showed no developmental or growth defects, their
Figure 1 OsRpp30 interacts with HDT701. (a) Alignment of OsRpp30, OsRpp30-L and OsRpp30-S. (b) Schematic of the three OsRpp30 proteins outlining regions of homology and divergence. Note that OsRpp30 is identical to OsRpp30-L except in the tail end of their C-terminus. The red (a) and yellow (b) bars denote the domain that is present in Rpp30 from other eukaryotes. (c) OsRpp30 interacts with HDT701 but not its paralogs OsRpp30-S and OsRpp30-L. Y2H assays were performed using SD-Leu-Trp-His medium containing 20 mM 3-AT. (d) LCI assay in Nicotiana benthamiana leaves of the interaction between HDT701 and various derivatives of OsRpp30. Each of the eight quadrants was infiltrated with HDT701-cLuc and the indicated construct. Note that quadrants 1 and 6 are repeats. (e) Co-IP analysis of the interaction between OsRpp30 and HDT701 in rice. Protoplasts were transfected with the indicated plasmids, and total protein was used as input for immunoprecipitation with anti-HA beads before immunoblotting with the indicated antibodies. (f) Co-localization of OsRpp30 and HDT701 in the rice nucleus. Confocal fluorescence microscopy of rice protoplasts co-transfected with OsRpp30-GFP and HDT701-RFP plasmids.
OsRpp30 positively regulates plant disease resistance. (a) Expression of OsRpp30, OsRpp30-L, and OsRpp30-S at 0 and 24 h post-inoculation (hpi) with *Pyricularia oryzae* RO1-1. (b) Expression of OsRpp30 induced by *P. oryzae* RO1-1 infection by a spraying inoculation method. (c) Expression of OsRpp30 induced by *Xoo* P6 infection by a leaf-clipping inoculation method. (d) Lesion on WT and OsRpp30-OX (OX) rice leaves at 13 dpi with *P. oryzae* RO1-1. (e) Lesion on WT and OsRpp30-RNAi (Ri) rice leaves at 13 dpi with *P. oryzae* RO1-1. (f) Relative fungal biomass in the necrotic regions of the leaves in panel d. Fungal growth was assessed by qPCR of the fungal Pot2 gene and normalized to rice Ubiquitin gene. (g) Relative fungal biomass in the necrotic regions of the leaves in panel e. Fungal growth was assessed as described in panel f. (a–c, f–g) These data are the mean of at least three biological replicates ± SE. Asterisks denote significant difference based on nested ANOVA (*P* < 0.05, **P** < 0.01). (h) Heatmap showing the differentially expressed genes (DEGs) associated with immune responses and development in OsRpp30-OX plants and WT plants without inoculation. (i) Expression fold change assessed by RT-qPCR of the genes (in panel h) in uninoculated and inoculated (at 24 hpi with *P. oryzae* RO1-1) OsRpp30-OX relative to WT plants. A fold change of 1.0 signifies no difference between OsRpp30-OX and WT plants. The data reported are the mean of three replicates of RT-qPCR ± SE.
seedlings displayed spontaneous cell death under high-humidity growth conditions such as in sealed tissue culture containers, likely on account of stress (Figure S1b). To corroborate findings from the overexpression lines, we also generated transgenic plants in which RNAi was used to knock down OsRpp30 (Figures S1c,d). In direct contrast to OsRpp30-OX plants, OsRpp30-RNAi plants showed larger lesions upon punch inoculation with P. oryzae RO1-1 relative to WT plants (Figure 2e). Correspondingly, the fungal biomass of P. oryzae RO1-1 was much lower post-inoculation in OsRpp30-OX plants and higher in OsRpp30-RNAi plants compared to the WT (Figure 2f,g). To determine the reciprocal regulation of OsRpp30 and HDT701 during rice immunity, we determined HDT701 mRNA levels in OsRpp30-RNAi and OsRpp30-OX plants. We found that HDT701 mRNA levels did not change by more than 25% upon knockdown or overexpression of OsRpp30 (Figure S1e). Likewise, OsRpp30 mRNA levels were only somewhat affected (<25%) by knockout and overexpression of HDT701 (Figure S1f; see below for the description of the HDT701 knockout lines).

To elucidate the OsRpp30-mediated resistance mechanism(s), we examined gene expression differences between OsRpp30-OX and WT plants without any inoculation using RNA sequencing (RNA-seq). Bioinformatic analyses identified 247 differentially expressed genes (DEGs) with at least twofold change: 72 were up- and 75 were down-regulated in the OsRpp30-OX plants (Table S1). Of these, 120 up-regulate d and 46 down-regulate d DEGs are annotated with different gene ontology (GO) terms; the rest are unannotated. Eight of the 120 up-regulate d (~7%) and five of the 46 down-regulate d (~11%) DEGs are annotated as ‘defence response’. Compared to the 445 ‘defence response’ genes of the 22 600 annotated rice genes in total (~2%), our data sets showed that overexpression of OsRpp30 clearly affect expression of defence genes more selectively than by chance (7% (up) and 11% (down) vs. 2%). Consistent with a normal growth and lack of any gross morphological difference in OsRpp30-OX compared to WT plants, we found only a few development-related genes among the DEGs (e.g. flowering promoting factor-like 1 gene, LOC_Os01g15340). The specificity in the altered gene expression profile—as evident from the over-representation of immunity-related genes—suggests that OsRpp30 is mainly involved in plant immunity.

We then substantiated the RNA-seq findings with 11 selected DEGs (Figure 2h): genes encoding immunity factors (PR5, PR10, PR1a, PB21, LOC_Os03g46060), chitinases (CHT-3, CHIT8), phenylalanine ammonia-lyase (PAL1), PAMP receptor (FLS2), lectin-like receptor (LOC_Os07g03810) and development-related protein (LOC_Os01g15340). We performed RT-qPCR using RNA extracted from leaf tissues of OsRpp30-OX and WT plants with or without P. oryzae inoculation. Except FLS2 and LOC_Os07g03810, the eight immunity and the sole development-related (LOC_Os01g15340) genes were induced up to 3.5-fold in the uninoculated OsRpp30-OX compared to WT plants (Figure 2i). Interestingly, 24 hpi with P. oryzae RO1-1, up-regulation of all eight immunity genes was maintained or enhanced even further (PAL1, PR10 and PB21) in OsRpp30-OX compared to WT plants. In contrast, FLS2 and LOC_Os07g03810 were slightly induced, while LOC_Os01g15340 was decreased (Figure 2i). These results suggest that protection afforded by OsRpp30 overexpression was not because of some global alterations but likely mediated by specific (yet deciphered) mechanisms.

As an additional stringency measure, we selected 12 genes for RT-qPCR from different functional categories (immunity, development, metabolism, cytokinesis and abiotic stress) that were not in our list of DEGs. None of these genes was significantly induced or suppressed in the uninoculated OsRpp30-OX compared to WT plants (Figure S2). After inoculation with P. oryzae RO1-1, only the expression of two immune-related genes, RbohB and CERK1, was up-regulated twofold in OsRpp30-OX plants (Figure S2).

OsRpp30 acts genetically and partially upstream of the HDT701-mediated immunity pathway

To further dissect the nexus between OsRpp30 and HDT701, we took a genetic approach and generated osrpp30, hd701 and osrpp30hd701 knockout mutants using the CRISPR/Cas9 strategy. We used single-guide RNA (sgRNA) constructs to generate either an osrpp30 or an hd701 single mutant or both sgRNAs in one construct to obtain the osrpp30hd701 double mutant (Figure S3). The T1-generation mutants used in our functional assays have the following mutations: osrpp30 with a 2-bp deletion, hd701 with a 4-bp deletion and osrpp30hd701 with a 1-bp and a 7-bp deletion, respectively (Figure S3). Because OsRpp30 is identical to OsRpp30-L and highly homologous to OsRpp30-S in the sgRNA-targeted region (nucleotides 440-459 of OsRpp30 coding sequence; Figure S3), we sequenced all three genes in the CRISPR/Cas9 mutants and chose for our phenotypic analysis only those T1 transgenic plants that contained mutations in OsRpp30 but neither in OsRpp30-L nor OsRpp30-S loci. Unlike the Arabidopsis rpp30 mutant (Wang et al., 2012), our osrpp30 mutant did not show defects at the reproductive growth stage, likely because of multiple OsRpp30 homologs in rice compared to the single gene copy in Arabidopsis. Thirteen dpi with P. oryzae RO1-1, hd701 leaves showed enhanced resistance in comparison to the WT (Figure 3a), reminiscent of our finding with HDT701 RNAi lines (Ding et al., 2012); in contrast, osrpp30 leaves were more susceptible mirroring the OsRpp30 RNAi lines in this study (Figures 3a,b vs. 2e,g). Interestingly, osrpp30hd701 leaves displayed a similar level of disease resistance as the WT (Figure 3a). The P. oryzae fungal biomass in WT, hd701, osrpp30 and osrpp30hd701 were consistent with their respective resistance levels (Figure 3b).

Next, we evaluated the resistance of these mutants to Xoo strain P6. While hd701 conferred enhanced resistance to P6, osrpp30 was susceptible to this isolate (Figure 3c). However, osrpp30hd701 was more resistant than osrpp30 but slightly more susceptible than the WT (Figure 3c). The lesion areas correlated with the qualitative trends (Figure 3d). Furthermore, we analysed the accumulation of ROS in these mutants post-PAMP elicitor chitin treatment. Leaf disks from 6- to 7-week-old rice plants were immersed in a chitin solution, and ROS accumulation was measured using the luminol chemiluminescence assay (Schwacke and Hager, 1992). ROS level was highest in hd701 and lowest in osrpp30, with the level in osrpp30hd701 between that in WT and hd701 (Figure 3e).

Our data demonstrate that OsRpp30 expression is linked to resistance towards both fungal and bacterial pathogens of rice. The phenotypic profiles of hd701, osrpp30 and osrpp30hd701 suggest a larger network at play and that OsRpp30 acts genetically and partially upstream of the HDT701-mediated PAMP-triggered immunity pathway in rice.

HDT701 overexpression coincides with decreased acetylation level of OsRpp30

The physical interaction between HDT701 and OsRpp30 prompted us to examine whether OsRpp30 is deacetylated by
HDT701. First, because of the simplicity of performing overexpression studies in bacteria and the existence of HDACs in prokaryote including *E. coli* (Jiang et al., 2017; Zhao et al., 2004), we inquired whether OsRpp30 is a substrate for deacetylation in *E. coli*. Accordingly, we examined the acetylation status of GST-OsRpp30 and His-OsRpp30 in *Escherichia coli* grown with and without trichostatin A (TSA), an HDAC inhibitor (Figure S4). Despite the non-native context, we were encouraged to find that GST-OsRpp30 and His-OsRpp30 were recognized by a pan acetylation monoclonal antibody and that the level of acetylation was enhanced upon treatment with TSA (Figures S4a,b). These results indicate that OsRpp30 is subject to acetylation and deacetylation even in *E. coli*. Second, we then assessed the acetylation level of OsRpp30-Myc in WT rice protoplasts transfected with the construct OsRpp30-Myc alone or together with HDT701-Flag, both driven by the 3SS promoter. After pulling down OsRpp30-Myc with anti-Myc beads, Western blot analysis showed that the acetylation level of OsRpp30-Myc was twofold lower in the presence of HDT701-Flag (Figure S4c, lane 2). To substantiate this finding, we performed the same acetylation assays but with protoplasts isolated from rice plants that overexpress HDT701 under the control of the maize *Ubiquitin* promoter and transfected with the OsRpp30-Myc construct above. Again, we observed a twofold decrease in acetylation of OsRpp30-Myc (Figure S4c, lane 3). While these experiments are preliminary, together with the finding of HDT701 interacting with OsRpp30, these results from experiments in two different contexts suggest that HDT701 could deacetylate OsRpp30 in rice.

OsRpp30 is associated with partially purified rice RNase P

All archaean RNase P protein subunits share homologs in eukaryotic RNase P, and Rpp30 is one of those that are present in both (Evans et al., 2006; Gopalan et al., 2018; Jarrous, 2002; Lai et al., 2010). As the N-terminal half of OsRpp30 shares similarity with archaean and eukaryotic homologs, we investigated the possibility that OsRpp30 is part of the RNase P RNP. We prepared total extracts from WT and HA-OsRpp30-OX rice plants and subjected each to DEAE-Sepharose chromatography. When the eluent fractions were assayed for their ability to cleave a pre-tRNA*Glu*, an identical strong peak of tRNA 5′-processing activity was observed in both preparations (Figure 4a, fractions 6 to 8). The sizes of the products (Figure 4a, tRNA*Glu* and 5′ leader) generated by rice RNase P matched those of the *E. coli* RNase P RNP, which was used as a positive control (Figure 4a, lane +). Strikingly, immunoblot analysis with an HA antibody detected OsRpp30 in these fractions. These results suggest that OsRpp30 is associated with the rice RNase P RNP.
HA-OsRpp30 mainly in fractions 6 to 8, which exhibited the highest RNase P activity among all fractions tested (Figure 4b).

OsRpp30 is present in a number of wild rice species and other cereals

When we searched the database of wild rice genomes for OsRpp30, we found one copy of OsRpp30 in five of the seven well-annotated wild rice species, two copies of OsRpp30-L in one species and one to two copies of OsRpp30-S in five species (Figure 5a, table). All the OsRpp30 orthologs in the five wild rice species share significant overall homology with OsRpp30, and there is almost complete conservation in the last seven aa (Figure 5a, alignment), which is essential for the interaction of OsRpp30 with HDT701 (Figures 1c,d). Moreover, in six Poaceae (grass family) members, OsRpp30 is present in all, OsRpp30-L in four and OsRpp30-S in none (Figure 5b, table). In addition, Sorghum bicolor has two copies of a gene that we termed OsRpp30-like because they code for an OsRpp30 homolog of the same length as OsRpp30 but lacking the 7-aa tail sequence (not shown). Remarkably, the OsRpp30 orthologs in Z. mays (maize), H. vulgare (barley), A. tauschii (aegilops), T. aestivum (wheat) and S. bicolor (sorghum) exhibit high conservation in their last seven aa with those present in OsRpp30, despite a low overall sequence homology (Figure 5b, alignment). To determine the biological function of OsRpp30 C-terminal half and its last seven aa, we overexpressed in rice protoplasts the full-length OsRpp30, the truncated OsRpp30 1-699 lacking the last seven aa and the truncated OsRpp30 407-706 containing only the C-terminus including the 7-aa tail. Expression of full-length OsRpp30 and OsRpp30 407-706, but not OsRpp30 1-699, led to up-regulation of the defence genes PAL1, PR1a and PBZ1 (Figure 5c), thus confirming a pivotal role in immunity for the last seven aa in OsRpp30.

Discussion

We previously reported that rice HDT701 is a histone deacetylase that negatively regulates plant disease resistance (Ding et al., 2012). Here, we show that HDT701 interacts with OsRpp30, a subunit of RNase P. In addition, overexpression of HDT701 in rice, either transiently or stably, coincides with decreased acetylation of OsRpp30; whether this observation is a direct or indirect action of HDT701 will require further confirmation (see below). In contrast to HDT701, OsRpp30 up-regulates the expression of specific immunity genes and promotes resistance to P. oryzae and Xoo. Our overexpression and knockdown studies of HDT701 (Ding et al., 2012) and OsRpp30 (this study) suggest an inverse functional relationship between these two proteins. These findings lead to the conclusion that pathogen infection induces OsRpp30 expression, and OsRpp30 in turn promotes plant immunity by either directly or indirectly regulating expression of defence genes and ROS accumulation.

That OsRpp30 plays a role in rice immunity could be rationalized based on findings elsewhere. First, variants of the human nuclear RNase P RNP complex enhance transcription of rRNA and small ncRNA genes by interacting with RNA polymerase (RNAP) I.
and RNAP III, respectively (Reiner et al., 2006; Reiner et al., 2008; Serruya et al., 2015). Therefore, it is likely that rice RNase P (either in whole or in part) containing OsRpp30 may act similarly to up-regulate transcription of defence genes (e.g. PR1a, PR5). Second, Rpp21, Rpp29 and Pop1—three different subunits of RNase P—repress histone H3.3 chromatin assembly and therefore effect transcriptional silencing in human cells (Newhart et al., 2016). Recently, Rpp29 was also shown to promote heterochromatic PTMs and repress euchromatic PTMs at specific loci (Shastrula et al., 2018). There is evidence from ChIP-seq analysis showing that Lys-/Arg-rich RNA-binding proteins may function together with regulatory RNAs to remodel chromatin and control transcription (Xiao et al., 2019). In light of these precedents, OsRpp30 with its 42 Lys residues could elicit transcriptional changes through epigenetic modifications, perhaps even by acting as a decoy to sequester deacetylases and therefore prolonging the euchromatin status of select loci. Lastly, mutations in Rpp30 cause sterility in Arabidopsis and Drosophila (Molla-Herman et al., 2015; Wang et al., 2012). Defects in tRNA processing were not so pronounced as to warrant attributing the striking infertility phenotype to RNase P dysfunction alone. The Drosophila Rpp30 mutation was postulated to weaken the crosstalk between RNase P and RNAP III, thus accentuating transcription–replication conflicts, which in turn decreases expression of the piRNA genes proximal to tRNA genes (Molla-Herman et al., 2015).

Figure 5 Presence of OsRpp30 in different rice species and other cereals, and its last seven aa are required for inducing defence-related genes expression. (a) OsRpp30 is present in the five wild rice varieties with near-complete sequenced genomes. Each + represents one ortholog copy. Alignment of only OsRpp30 orthologs is shown. The seven-aa tail is boxed. (b) OsRpp30 is present in other cereals. Each + represents one ortholog copy. Alignment of only OsRpp30 orthologs is shown. Despite low similarity elsewhere, high sequence conservation is found in the boxed seven-aa tail of OsRpp30 from these Poaceae members. (c) The last seven aa of OsRpp30 are critical for OsRpp30-mediated expression of defence genes. Rice protoplasts were transfected separately with an empty plasmid or a plasmid harbouring full-length OsRpp30, OsRpp30-699 or OsRpp30-407-706. After a 16-h incubation at room temperature, RNA was extracted and used for RT-qPCR. The data are the mean of three replicates ± SE. Asterisks denote statistically significant differences based on nested ANOVA (*P < 0.05, **P < 0.01), and ns means not significant.
in overexpression of repressed transposable elements, activation of DNA damage checkpoint and arrest of oogenesis. Like this ripple effect in *Drosophila*, modulation of OsRpp30 levels might alter the expression of small RNAs in rice and therefore control immunity, a testable postulate.

Other perspectives stimulated by our findings merit elaboration. First, the cultivated rice species *Oryza sativa* contains OsRpp30-S, OsRpp30-L and OsRpp30, but only expression of OsRpp30-S is up-regulate d significantly post-infection (Figure 2a). This finding is surprising as the promoters of OsRpp30-L and OsRpp30 are identical except for a 2-bp difference in a 2-kb span (no sequence conservation between these promoters and that of OsRpp30-S). These near-identical promoters may underlie the infection-induced expression of OsRpp30-L and OsRpp30; however, the much higher expression of OsRpp30-L suggests the existence of additional regulatory control(s) such as a remote enhancer element and/or mRNA stability. Moreover, the near-identical sequences of OsRpp30 and OsRpp30-L render selective targeting of OsRpp30 by RNAi difficult. However, three lines of evidence suggest that suppression of OsRpp30 and not OsRpp30-L contributes to the susceptibility phenotype of the OsRpp30 RNAi plants. First, only OsRpp30-L is highly induced after *M. oryzae* infection (Figure 2a). Second, we found that OsRpp30-L does not interact with HDT701 unlike OsRpp30. Lastly, OsRpp30-S and OsRpp30-L are intact in osrpp30. Thus, the identical susceptibility phenotypes of the OsRpp30 knockout mutant and RNAi lines confirm that OsRpp30 is the sole player (among the three homologs) in rice immunity.

Second, the evolutionary driving forces that shaped the HDT701-OsRpp30 pairing deserve scrutiny. We recognize that HDT701 may have multiple targets and that OsRpp30 may be a substrate for additional deacetylases (e.g. HDT702) given its multiple Lys residues. The latter notion is consistent with the lack of complete deacetylation of OsRpp30 upon HDT701 overexpression.

Third, despite reports of an RNase P RNP complex in various plants, such as carrot cells, rice and wheat (Franklin et al., 1995; Krehan et al., 2012; Pulukkunat, 2002), only an RNA-free, protein-based form (PRORP) has been characterized in plants (Lechner et al., 2015). However, the lack of lethality upon knockout the single nuclear PRORP gene in the moss *Physcomitrella patens* suggests that a yet unidentified RNFP form might function in the nucleus (Sugita et al., 2014). This conjecture is supported by the presence of different RNase P protein subunits (e.g. Pop1, Pop5, Rpp30, Rpp29) in plant genomes. As there are only reports of a plant RNase MRP RNA (Kiss et al., 1992; Krehan et al., 2012), these proteins were believed to be parts of RNase MRP, the sister enzyme to RNase P that shares several protein subunits with RNase P (Lai et al., 2020). Affinity tagging of OsRpp30, which co-elutes with a RNA-processing activity (Figure 4), could now be exploited as a handle to obtain the elusive plant RNase P RNP.

Last, while there is overwhelming evidence for the HDT701-OsRpp30 interaction, our efforts to identify specific acetylation sites in OsRpp30 that are acted upon by HDT701 in vivo have been hampered by poor yield of purified protein. We are examining newer strategies for isolating rice OsRpp30 in abundant quantities for proteomic studies.

A logical follow-up to this study is to identify the rice acetyltransferase that acetylates OsRpp30 and the sites of acetylation/deacetylation in OsRpp30. An equally important priority is to delineate the effect of acetylation and other PTMs (e.g. succinylation; (He et al., 2016a; Wang et al., 2019) on OsRpp30 function in rice immunity and on RNase P activity. These experiments will help determine if pathogen-sensing modulation of OsRpp30 function by altering the acetylation (or succinylation) status is part of a host stress response. On the other hand, it is likely that pathogens have evolved countermeasures. A recent phospho-proteomic analysis revealed that Xoo infection of rice-triggered dephosphorylation and activation of HDT701 (Hou et al., 2015). Clearly, a suite of PTMs in response to infection dictates the yin-yang balance in terms of resistance and susceptibility: the crop protection afforded by overexpression of OsRpp30, however, suggests that the negative role of HDT701 can be overcome to favour immunity.

Wild and cultivated rice species exhibit both distinct and shared response mechanisms to phytopathogens. Here, we found that the last seven aa are highly conserved in OsRpp30 orthologs in both wild and cultivated rice species as well as in some other cereals of agronomic importance. Somehow co-evolution with pathogens led to a positive selection for this C-terminal tail in a number of Poaceae species, with important payoffs for immunity to pathogens. Dissecting the molecular underpinnings of OsRpp30 function will be key for fine-tuning this innate immunity response. In conclusion, because OsRpp30 overexpression in rice led to enhanced resistance to two key pathogens and the transgenic plants do not show obvious growth defects in the greenhouse and field, this study offers a new direction to generate broad-spectrum disease-resistant cereal crops without yield penalties to feed the growing human population.

## Methods

### Plant growth conditions

Rice (*Oryza sativa* Nipponbare) seeds were sterilized by treatment with 75% (v/v) ethanol for 1 min followed by immersion in 2% (w/v) NaCl for 40 min. After washing the seeds with sterile water, they were germinated on 1/2 Murashige and Skoog (MS) medium for 8–9 days at 26 °C with a 12-h/12-h light/dark photoperiod. Subsequently, the seedlings were transferred to soil and grown in a growth chamber at 26 °C and 80% relative humidity with a 12-h/12-h light/dark photoperiod.

### Yeast two-hybrid assays

The Alkali-cation™ yeast transformation kit (MP Biomedicals) was used in screening for HDT701-interacting proteins and for validating protein–protein interactions. The full-length HDT701 cDNA was cloned into the bait vector pDBLeu, and the construct was used to transform the yeast strain Mav203. An *O. sativa* Nipponbare seedling cDNA library or the candidate interactor cDNAs were cloned into the prey vector pPC86 as described previously (Park et al., 2012), and the resulting clones were used to transform the yeast strain carrying pDBLeu-HDT701. Putative HDT701-interacting clones grown on SD/-Leu-Trp medium were confirmed by growing on SD/-Leu-Trp-His medium containing 20 mM 3-amino-1,2,4-triazole (3-AT), followed by DNA sequencing and bioinformatic analysis.

### Luciferase complementation imaging (LCI) analysis

LCI assay was conducted as described previously with some modifications (Chen et al., 2008; Lai et al., 2010). The *Agrobacterium* strain GV3101, transformed individually with the different constructs, was grown at 28°C for ~18 h with shaking. Subsequently, the cells were harvested by centrifugation at
2500 g for 10 min, then resuspended and incubated in 10 mM MES (pH 5.7) containing 0.2 mM acetosyringone for 3 h at 25–28 °C. The cells were centrifuged again at 5000 g for 10 min and resuspended in the MES solution to yield OD600 ~1. The bacterial suspension was infiltrated into N. benthamiana leaves. The RNAi suppressor P19 construct was included in the infiltration (Lakatos et al., 2004) to dampen the RNA silencing response. Three days later, the treated leaves were sprayed with 5 mM luciferin for fluorescence detection, and imaging was performed using the Universal Hood II Imager (Bio-Rad, Hercules, CA).

**Rice protoplast preparation and transfection**

Rice protoplasts were isolated as previously described (He et al., 2016b). Briefly, 2-week-old etiolated seedlings grown on 1/2 MS medium in the dark at 23 °C in an incubator were cut into 0.5 mm strips and incubated in a solution containing 10.0% (w/v) Cellulase R10 (Yakult, #C8260) and 0.5% (w/v) Macerozyme R10 (Yakult, #MX7351) for 4–6 h in the dark at room temperature with gentle shaking. Protoplasts were collected by centrifugation at 1000 g for 5 min, washed once with W5 solution for 1 h at room temperature and filtered through a Miracloth layer. The Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) was used to analyse the subcellular localization of HDT701-RFP and OsRpp30-GFP. RFP was excited at 584 nm (emission window, 589–620 nm) and GFP at 488 nm (emission window, 498–527 nm). An acousto-optical tunable filter (AOTF) was used to control the intensity of the visible excitation laser light, and an acousto-optical beam splitter (AOBS) was used to split the excitation light and emission light instead of filter blocks. Images were captured digitally at gain value 700 and laser intensity 4% using the Leica TCS HCX PL APO 40x/1.25 NA water-immersion objective.

**Confocal fluorescence microscopy**

The Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) was used to analyse the subcellular localization of HDT701-RFP and OsRpp30-GFP. RFP was excited at 584 nm (emission window, 589–620 nm) and GFP at 488 nm (emission window, 498–527 nm). An acousto-optical tunable filter (AOTF) was used to control the intensity of the visible excitation laser light, and an acousto-optical beam splitter (AOBS) was used to split the excitation light and emission light instead of filter blocks. Images were captured digitally at gain value 700 and laser intensity 4% using the Leica TCS HCX PL APO 40x/1.25 NA water-immersion objective.

**Quantitative RT-PCR (RT-qPCR)**

The Bio-Rad CFX96 Touch Real-Time PCR Thermocycler (Bio-Rad Laboratories, Hercules, CA) and the TransStart Green qPCR SuperMix Kit (TransGen, #AQ101) were used for RT-qPCR analyses. Rice RNA was first transcribed into cDNA with OneStep gDNA Removal and cDNA Synthesis SuperMix (TransGen, #AE311), and the cDNA was then diluted to 20 ng/μL before being used as template for RT-qPCR. Three 25-μL replicates were carried out per sample. The relative quantification of gene expression was calculated using the 2−ΔΔCt method according to the eligible Ct (cycle threshold; 15–35) value of target genes and Ubiquitin, the latter used for normalization. Primers used in these reactions are listed in Table S2.

**Detection of ROS accumulation**

As previously described (Park et al., 2012), leaf disks were punched from 6- to 7-week-old plants and soaked overnight in sterile distilled water. For each test condition, two leaf disks were placed in a 1.5-mL tube containing 98 μL lumenol, 1 μL horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, #112-035-003) and 1 μL PAMP (0.8 μM hexa-N-acetyl-chitoheptaose) or 1 μL water (control). Luminescence was immediately monitored at 1-min intervals for a total of 21 min with a Glomax 20/20 luminometer (Promega). Three technical replicates were performed for each sample and treatment.

**Acetylation assay**

For the experiments in E. coli, GST-OsRpp30 and His-OsRpp30 expressed in E. coli, grown without or with 10 μM TSA, were first isolated by immunoprecipitation with glutathione (Abcam, #ab193267) and nickel (Qiagen, #30210) sepharose beads, respectively. In subsequent immunoblotting, the antibody against (Zhang et al., 2013), and the concentration of Xoo P6 was 2 × 108 colony forming units/mL. At 12–15 dpi when the lesions were obvious and stable, their areas were measured using ImageJ, and the average of three largest lesions was determined.

**RNA-seq analysis**

The leaves of 3-week-old WT and OsRpp30-OX plants were collected, flash-frozen in liquid nitrogen and stored at −80 °C. Three biological replicates were obtained for each sample. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Düsseldorf, Germany, #74904), and the six sequencing libraries were prepared using NEB Next Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Beverly, Massachusetts, #E7530L) following each manufacturer’s recommendations. The libraries were sequenced on an Illumina platform and paired-end reads were generated. Raw FASTQ reads were first processed through in-house Perl scripts, during which clean reads were obtained by removing reads with adapter, ploy-N and low quality sequences. These clean reads were then mapped to the reference rice genome (IRGSP-1.0, http://plantsensembl.org/index.html) using HISAT2 (https://ccb.jhu.edu/software/hisat2/manual.shtml). Only reads with ≤1 mismatch were further analysed and annotated based on the reference genome. Differential expression analysis of the two sample groups (WT and OsRpp30-OX plants) was performed using the DESeq R package, version 1.10.1. A false discovery rate <0.05 and gene expression change of at least twofold by DESeq were set as the threshold for obtaining the inventory of differentially expressed genes. The RNA-seq raw data sets were submitted to NCBI SRA database with the accession number PRIN4664877.
GST (TransGen, #HT601) or the poly-His tag (TransGen, #HT501) was used to assess the amount of pulled down OsRpp30 fusion proteins, and a pan acetylation monoclonal antibody (Proteintech, Rosemont, IL, #66289-1-lg) was used to detect OsRpp30 acetylation. For the experiment in rice protoplasts, the OsRpp30-Myc plasmid alone or together with the HD701-flag plasmid was transfected into protoplasts of the indicated genotypes. Subsequently, OsRpp30-Myc was purified using anti-Myc beads for use in immunoblotting. A Myc antibody (TransGen, #HT101) was used to assess OsRpp30-Myc amount in each lane, and the acetylation antibody was used to determine OsRpp30-Myc acetylation.

Partial purification of rice RNase P

One gram of 4-week-old rice leaves was ground in liquid nitrogen and then transferred to a large glass tube and resuspended with 9 mL of extraction buffer (EB; 20 mM Tris-HCl, 5 mM MgCl₂, pH 8) + 50 mM NaCl (EB50). The resuspension was homogenized with a Polytron and the lysate transferred to a 30-mL Oakridge tube and centrifuged at 21,000 g for 30 min at 4 °C. The cleared lysate was passed through a 0.45-µm syringe filter and loaded onto an EB50-equilibrated 1-mL Hitrap DEAE FF column (GE Healthcare, Uppsala, Sweden). After washing the column with 5 mL EB50, the bound components were eluted with a linear NaCl gradient (50–1000 mM in EB) using the AKTA Purifier FPLC System (GE Healthcare) at a flow rate of 1 mL/min. Sixteen 0.5-mL fractions were collected. Subsequently, 3 µL of each fraction was assayed for RNase P activity in a final volume of 10 µL containing EB, 14 µm 2-mercaptoethanol, 50 nm tobacco chloroplast pre-tRNA Gly (a trace amount of which was internally radiolabeled). The reaction was incubated at 37 °C for 15 min, quenched with 10 µL of stop buffer (7 M urea, 1 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 10% phenol) and separated on an 8% (w/v) polyacrylamide gel containing complex. The resulting gel was then imaged using the Typhoon Phosphorimager (GE Healthcare).

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Author contributions

L.B.L., V.G., G.W. and W. Liu designed research; W. Li, Y.H., L.B.L., K.Z. and Z.L. performed research; W. Li, L.B.L. and H.K. analysed data; L.D., V.G., G.W. and W. Liu supervised and coordinated research; and W. Li, L.B.L., V.G., G.W. and W. Liu wrote the paper.

Conflict of interest

The authors declare no conflicts of interest.

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