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Systematic characterization of the ubiquitin-specific proteases in Magnaporthe oryzae

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Abstract

Ubiquitin-specific protease (UBP) family is the largest group of deubiquitinases, which plays important roles in eukaryotic organisms. Comprehensive analysis of UBP genes has not been conducted in the plant pathogenic fungi. In this study, 11 putative UBP genes were identified and characterized in the rice blast fungus Magnaporthe oryzae. Expression profile analysis showed that UBP3, UBP6, UBP12 and UBP14 were highly expressed in different tissues of M. oryzae. In all ubp mutants, especially Δubp3, Δubp12 and previously reported Δubp14, the ubiquitination levels were evidently elevated, which is consistent with their molecular roles in de-ubiquitination. The Δubp1, Δubp3, Δubp4, Δubp8 and Δubp14 mutants were reduced in colony growth. Most of the ubp mutants were severely reduced in conidia production capacity, indicating important roles of the UBPs in conidia formation. Except for Δubp2 and Δubp16, all of the other mutants were decreased in virulence to host plants and defective in invasive growth. These ubp mutants also induced massive ROS accumulation in host cells. We also found that the UBP may function as both positive and negative regulators in stress response and nutrient utilization of M. oryzae. Collectively, UBP are important for development, stress response, nutrient utilization and infection of M. oryzae.

Keywords: Ubiquitin, Deubiquitinases, Ubiquitin-specific proteases, Infection process, Gene family, Magnaporthe oryzae

Background

Ubiquitination is one of the most important posttranslational modifications, which plays central roles in diverse cellular pathways such as cell-cycle progression, signal transduction, DNA repair and endocytosis and apoptosis (Hershko and Ciechanover 1998; Pickart and Eddins 2004; Dikic et al. 2009). The ubiquitin molecules are catalyzed by a cascade of three enzymes: the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3) (Pickart and Eddins 2004). The fate of ubiquitinated substrate proteins mainly depends on the number of conjugated ubiquitin and the type of linkage. Proteins linked by Lys48 bearing four or more ubiquitin chains are mostly recognized by the 26S proteasome complex system for degradation. Attachment of ubiquitin to cellular proteins as different linkage types are involved in distinct cellular processes (Pickart and Eddins 2004).

As a reversible event, ubiquitins can be removed from proteins, which is also important for regulation of different cellular processes. Cellular ubiquitin monomers are kept in homeostasis by processing from ubiquitin precursors or deubiquitinating from protein substrates (Amerik and Hochstrasser 2004; Nijman et al. 2005). These processes are regulated by the de-ubiquitinating enzymes (DUBs), which can specifically remove ubiquitin moiety. The cleavage of ubiquitin from proteins by DUBs can affect the activity and fate of substrates (Wilkinson 1997;
Amerik and Hochstrasser 2004; Crosas et al. 2006). Among several types of DUBs, the ubiquitin-specific proteases (UBPs) are highly conserved and the largest subfamily among eukaryotic organisms (Wilkinson 1997; Amerik and Hochstrasser 2004; Nijman et al. 2005), which commonly possess a triad of catalytic residues in conserved cysteine and histidine boxes (Hu et al. 2002). Ubiquitin C-terminal hydrolases (UCHs) contain a similar triad of catalytic residues in two conserved cysteine and histidine boxes (Johnston et al. 1999; Amerik and Hochstrasser 2004). Other DUBs include the ovarian tumor proteases (OTUs), Machado–Joseph disease protein domain proteases (MJDs), etc. (Balakirev et al. 2003; Burnett et al. 2003; Scheel et al. 2003; Nanao et al. 2004).

There are 16 UBP genes in Saccharomyces cerevisiae (Wilkinson 1997) and 27 UBP genes in Arabidopsis thaliana (Liu et al. 2008). Studies on S. cerevisiae revealed UBP proteins are involved in nutrient utilization, energy metabolism, sexual reproduction, and stress responses (Kahana 2001; Enyenihi and Saunders 2003; Dudley et al. 2005; Aeusukaree et al. 2009). In plants, UBP genes have been proved to play roles in cell proliferation, root hair elongation, mitochondria morphology, pollen development and transmission, and abscisic acid (ABA)-mediated resistance to salt and drought stress, etc. (Doelling et al. 2001; Liu et al. 2008; Du et al. 2014; Xu et al. 2016; Zhao et al. 2016). However, few studies have addressed systematic analysis on the roles of all members of the UBP gene family, although roles of single UBP gene was reported (Liu et al. 2008; Wu et al. 2019).

Magnaporthe oryzae is a fungus threatening rice production worldwide, and also a model fungal plant pathogen (Wilson and Talbot 2009; Yan and Talbot 2016). This fungus forms a specialized infection structure appressorium on the host surface, and penetrates into host cells (de Jong et al. 1997; Dixon et al. 1999; Thines et al. 2000). In the host cells, the fungus forms invasive hyphae to establish biotrophic colonization and eventually converts into necrotrophic growth for spreading (Kankanala et al. 2007). Previous study has found that both M. oryzae UBP14 and UBP4 play key roles in growth, conidiation, stress response, and carbon sources utilization, and meanwhile, are required for virulence of the pathogen (Wang et al. 2018; Que et al. 2019). In order to systematically determine functions of UBP genes, we identified and characterized roles of all M. oryzae UBP genes in development, stress response, carbon source utilization, as well as the virulence.

**Results**

**Identification of the UBP family proteins in M. oryzae**

To carry out a genome-wide identification of the UBP protein family in M. oryzae, S. cerevisiae UBP protein sequences were used as queries to search against the protein database available in the Magnaporthe genome (http://fungi.ensembl.org/Magnaporthe_oryzae/Info/Index). We identified 11 MoUBP candidate proteins, which were used to analyze the conserved domain using the Pfam database (http://pfam.xfam.org). MoUBP proteins exhibited a complete UCH domain with two short but well-conserved motifs specific to UBP proteins (Cys and His) (Amerik and Hochstrasser 2004). They were named UBP1 (MGG_04031T0), UBP2 (MGG_00877T0), UBP3 (MGG_05996T0), UBP4 (MGG_04957T0), UBP6 (MGG_02970T0), UBP8 (MGG_03527T0), UBP12 (MGG_05666T0), UBP13 (MGG_09907T0), UBP14 (MGG_08270T0), UBP15 (MGG_05002T0) and UBP16 (MGG_04494T0), respectively. These UBP genes also had some non-UBP domains, including RHOD (UBP4), UBQ (UBP6), ZF-UBP (UBP8 and UBP14), DUSP (UBP12), UIM (UBP13), DUF3517 (UBP15) and UBA (UBP14) domains (Fig. 1a).

Expression patterns of UBP genes at different developmental stages of M. oryzae

The gene expression pattern can provide vital clues for gene’s function. To characterize the expression patterns of UBP genes, we analyzed their transcription levels in different tissues, including mycelia, conidia, appressoria (at 3 hours post inoculation [hpi] or 12 hpi), and invasive hyphae (at 18, 24 or 42 hpi) according to an unpublished RNAseq data. As shown in Fig. 2, UBP3, UBP6, UBP12 and UBP14 showed high expression levels in nearly all tested tissues, while UBP1, UBP13 and UBP15 showed low expression levels in these tissues. In particular, four genes (UBP3, UBP6, UBP12 and UBP14) in mycelia, seven genes (UBP3, UBP4, UBP6, UBP8, UBP12, UBP14 and UBP16) in conidia, most of these genes in appressoria, and five genes (UBP3, UBP6, UBP12, UBP14 and UBP16) in invasive hyphae showed high expression levels. Taken together, M. oryzae UBP genes exhibited different expression profiles in different tissues, indicating diverse biological functions of UBP genes in development and infection processes.

**Single deletions of the UBP genes increase ubiquitination levels in M. oryzae**

To characterize the functions of UBP genes in M. oryzae, single gene deletion mutant for each UBP gene was
generated by using a split-PCR strategy (Goswami 2012). At least two independent null mutants with identical phenotypes were obtained for each UBP gene, and all deletion mutants were confirmed by polymerase chain reaction (PCR) and gene expression level detection (Additional file 1: Figure S1 and Additional file 2: Figure S2).

In order to test whether deletion of single UBP gene affects total ubiquitination level in M. oryzae, all of the single ubp mutants (except for Δubp14) were tested for

![Fig. 1](image1.png)

**Fig. 1** The UBP family in *M. oryzae*. 

a) Structures and domains of the UBP proteins in *M. oryzae*. UCH, ubiquitin C-terminal hydrolases; RHOD, rhodanese homology domain; UBO, ubiquitin homologues; ZF-UBP, Zn-finger in ubiquitin-hydrolases; DUSP, domain present in ubiquitin-specific protease; UIM, ubiquitin-interacting motif; DUF, domains of unknown function; UBA, ubiquitin-associated domain.

b) Phylogenetic analyses of putative UBP proteins in fungi. The phylogenetic tree was constructed using MEGA7.0 by neighbour-joining method. The Bootstrap replicates were 1000. The sequences were collected from organisms as follows: Sc, Saccharomyces cerevisiae; An, Aspergillus nidulans; Nc, Neurospora crassa; Mo, Magnaporthe oryzae; Fg, Fusarium graminearum; Bc, Botrytis cinerea. The proteins IDs of MoUBPs are shown as below: MoUBP1 (MGG_04031), MoUBP2 (MGG_00877), MoUBP3 (MGG_05996), MoUBP4 (MGG_04957), MoUBP6 (MGG_02970), MoUBP8 (MGG_03527), MoUBP12 (MGG_05666), MoUBP13 (MGG_09907), MoUBP14 (MGG_08270), MoUBP15 (MGG_05002) and MoUBP16 (MGG_04494)

![Fig. 2](image2.png)

**Fig. 2** The heat map shows expression patterns of UBP genes at different developmental stages. The expression level of each gene relative to the mean FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value across all the experimental stages on the log scale has been represented. The color (from green to red) indicates gene expression intensity from low to high. HY: Mycelial hyphae; CO: Conidia; 3H AP: appressoria at 3 hpi; 12H AP: appressoria at 12 hpi; 18H: invasive hyphae at 18 hpi; 24H: invasive hyphae at 24 hpi; 42H: invasive hyphae at 42 hpi
changes of the ubiquitination level (Fig. 3). As expected, all of the mutants displayed increased ubiquitination levels. Especially, the Δubp3 and Δubp12 mutants, as well as our previously reported Δubp14 (Wang et al. 2018), each of them showed significant increase of the ubiquitination level compared to the wild type. The Δubp4, Δubp8, Δubp13, Δubp15 and Δubp16 mutants showed a moderate increase in ubiquitination level. These data reflected that all of the UBP genes are indeed involved in deubiquitinating process in M. oryzae.

Characterization of vegetative growth of the ubp mutants
All of the ubp mutants were examined for phenotypic changes in colony growth, mycelium formation, conidium formation, appressorium formation, and invasive growth. As summarized in Table 1, the deletion mutants of each UBP gene displayed distinctive effects on colony growth, conidiation, appressorium formation and infection-related morphogenesis. Among 11 mutants, Δubp1, Δubp3, Δubp4, Δubp8 and Δubp14 mutants displayed respectively 22.0%, 9.4%, 6.1%, 12.7% and 25.3% reductions of colony diameter on oatmeal tomato agar (OTA) plates (Fig. 4a, b and Table 1). Correspondingly, significant reductions of mycelial cell length were observed in these mutants (Fig. 4c, d). These results indicated that UBP1, UBP3, UBP4, UBP8 and UBP14 are involved in the regulation of vegetative growth in M. oryzae.

Characterization of conidium production of the ubp mutants
Conidia play important roles in the disease cycle of the rice blast fungus. Most UBP genes were found here to be involved in conidiation of M. oryzae. The Δubp1, Δubp3, Δubp4, Δubp6, Δubp8, Δubp12, Δubp13, Δubp14 and Δubp16 mutants were markedly reduced in conidiation compared with the wild type PI31 strain (Fig. 5a and Table 1). For the Δubp8 and Δubp14 mutants, conidia were very rare with only few visible spores following examination of more than ten 10-day-old culture plates. However, the Δubp2 and Δubp15 mutants showed normal conidium production (Fig. 5a and Table 1). Accordingly, conidiophore formation of the Δubp1, Δubp3, Δubp4, Δubp6, Δubp8, Δubp12, Δubp13, Δubp14 and Δubp16 mutants were severely reduced (Fig. 5b). These results showed that most members of the UBP genes regulate conidiation in M. oryzae.

Except for Δubp8, no other ubp mutants were blocked in conidium germination, and most of them did not show significant changes in appressorium formation. The Δubp8 mutant displayed a lower percentage of appressorium formation compared with the wild-type strain and other ubp mutants (Table 1).

Virulence and infection process of the ubp mutants
To further test whether these M. oryzae UBP genes are involved in virulence, conidia of each of the ubp mutants were sprayed onto susceptible rice seedlings. As shown in Fig. 6a, very few lesions were formed on the rice
seedlings inoculated with Δubp1, Δubp3, Δubp4, Δubp6, Δubp8 and Δubp14 mutants. In contrast, rice seedlings sprayed with Δubp2 and Δubp16 mutants developed numerous typical rice blast lesions similar to that inoculated with the wild-type M. oryzae strain (Fig. 6a). When inoculating the mycelial agar plugs onto the wounded rice leaves, all of the virulence-decreased mutants produced evidently restricted spreading of the lesions (Fig. 6b). This result indicated that UBP1, UBP3, UBP4, UBP6, UBP8 and UBP14 play key roles in virulence to host. Since the Δubp1, Δubp3, Δubp4, Δubp6, Δubp8 and Δubp14 mutants exhibited normal appressorium formation, we examined the ability of the appressoria in the penetration of the barley epidermal cells. At 24 hpi, only Δubp14 showed severe reduction of penetration, while no evident changes were found for the other ubp mutants, with majority of the appressoria in these mutants forming invasive hyphae (Fig. 6c). However, at both 24 hpi and 30 hpi, the formation of infection hyphae in the Δubp1, Δubp3, Δubp4, Δubp6, Δubp8 and Δubp14 mutants was significantly retarded, compared with the wild-type strain (Fig. 6c). These results indicate that UBP1, UBP3, UBP4, UBP6, UBP8 and UBP14 regulate virulence of M. oryzae through affecting infection hyphal growth.

### Table 1 Comparison of biological phenotypic characteristics among strains

| Strain     | Growth (cm) | Conidiation (×10⁴/mL) | Appressorium formation (%) |
|------------|-------------|-----------------------|----------------------------|
| p131       | 3.95 ± 0.06A| 239.7 ± 8.2A          | 97 ± 1.2A                  |
| Δubp1      | 3.08 ± 0.05C| 105.3 ± 4.4D          | 88 ± 2.1B                  |
| Δubp2      | 3.95 ± 0.05A| 244.0 ± 7.0B          | 96 ± 1.5A                  |
| Δubp3      | 3.58 ± 0.05D| 49 ± 5.8E             | 88 ± 4.5B                  |
| Δubp4      | 3.71 ± 0.08C| 146.7 ± 9.2C          | 80 ± 2.0C                  |
| Δubp6      | 3.89 ± 0.06AB| 148.3 ± 2.8C       | 88 ± 4.0B                  |
| Δubp8      | 3.45 ± 0.05F| 3.7 ± 0.5G            | 62 ± 4.0F                  |
| Δubp12     | 3.85 ± 0.05AB| 104.7 ± 3.7D         | 87 ± 2.3D                  |
| Δubp13     | 3.90 ± 0.06A| 169.3 ± 3.3B          | 95 ± 1.2A                  |
| Δubp14     | 2.95 ± 0.05G| 267 ± 0.7F            | 80 ± 2.8C                  |
| Δubp15     | 3.95 ± 0.05A| 243.3 ± 6.6A          | 91 ± 3.1AB                 |
| Δubp16     | 3.95 ± 0.05A| 144.3 ± 5.8C          | 81 ± 1.2C                  |

**Fig. 4** Vegetative growth of the ubp mutants. a The wild-type strain and the ubp mutants were inoculated on OTA plate and cultured at 28 °C for 5 days and then photographed. b Colony diameter. Significant differences compared with the wild type are indicated by asterisks (P < 0.05). c Calcofluor White staining of hyphal tips shows the distance of septa. White arrows indicate the cell septa. Bar, 20 μm. d Average cell length of the hyphal tips. Significant differences compared with the wild type are indicated by asterisks (P < 0.05).
The ubp mutants led to accumulation of host reactive oxygen species (ROS)

Because some ubp mutants were blocked in host invasive growth, we explored whether each of these UBP gene deletion mutants can result in host ROS accumulation. ROS accumulation in the barley epidermis cells infected by each strain at 30 hpi was detected by staining with 3,3′-diaminobenzidine (DAB). The result demonstrated that, except for Δubp2 and Δubp16, the barley epidermis cells infected by all other ubp mutants can be easily stained by DAB, which were detected with abundant reddish-brown precipitate in the infected host cells (Fig. 7a, b). At the same time, host cells infected by the wild-type strain were not well stained by DAB (Fig. 7a, b). These results showed that UBP genes are important for M. oryzae to respond to host cellular oxidative stress, and therefore required for invasive growth.

Members of UBP genes are involved in stress response in M. oryzae

We evaluated the effect of UBP genes disruption on stress tolerance of M. oryzae. The wild-type and ubp mutant stains were inoculated onto the CM plates each containing different stresses, including the cell wall stresses (0.1 mg/mL Calcofluor White [CFW], 0.2 mg/mL Congo Red [CR] or 0.005% sodium dodecyl sulfate [SDS]), osmotic stress (0.5 M NaCl or 1 M sorbitol), or oxidative stress (10 mM H2O2). The results showed that, compared with the wild-type strain, the Δubp1, Δubp3, Δubp8, Δubp12 and Δubp14 mutants were more sensitive to cell wall perturbing reagents; the Δubp8, Δubp12 and Δubp14 mutants were more sensitive to osmotic stress; and the Δubp3, Δubp12 and Δubp14 mutants were more sensitive to oxidative stress (Fig. 8). Interestingly, we also noticed some mutants were more resistant to different stresses. For example, the Δubp2 and Δubp4 mutants were more resistant to cell wall stresses; the Δubp1, Δubp2 and Δubp4 mutants were more resistant
to oxidative stress (Fig. 8). These data indicated that UBP genes positively or negatively regulate response to different stresses in *M. oryzae*.

**Nutrient utilization was positively or negatively regulated by different UBP genes**

We also evaluated whether the disruption of *UBP* genes can affect carbon source utilization of *M. oryzae*. The wild-type and *ubp* mutant strains were cultured on MM plates supplemented with each of glucose, NaAc, ethanol and glycerol as the sole carbon source. After 5 days culture at 28 °C, colony diameters of the Δ*ubp*4 and Δ*ubp*14 mutants were significantly reduced when grown on all of these conditions compared with those grown on the CM plates (Fig. 9). These demonstrated that the carbon sources utilization ability of the Δ*ubp*4 and Δ*ubp*14 mutants were significantly reduced when grown on all of these conditions compared with those grown on the CM plates (Fig. 9). These demonstrated that the carbon sources utilization ability of the Δ*ubp*4 and Δ*ubp*14 mutants were significantly reduced, suggesting *UBP*4 and *UBP*14 are positive regulators of carbon source utilization. In contrast, the Δ*ubp*1 was significantly increased in growth in the NaAc and ethanol conditions, and the Δ*ubp*8 mutant was increased in growth in all tested conditions (Fig. 9), suggesting *UBP*1 and *UBP*8 are negative regulators of carbon source utilization. Therefore, nutrient utilization of *M. oryzae* can be either positively or negatively regulated by different *UBP* genes.

**Discussion**

As one of the largest families of DUBs, the eukaryotic-specific UBP family protein plays important roles in fungal growth and development. Detailed characteristics and functions of *UBP* genes have been reported in *S. cerevisae* (Amerik et al. 2000), but not been systematically described in plant pathogenic fungi. Here, we identified and characterized 11 putative *UBP* genes in the rice blast fungus *M. oryzae* using a genome-wide analysis.

The expression profiles of the *UBP* genes at different developmental stages of *M. oryzae* have been evaluated. According to the gene expression patterns, different UBP proteins may have diverse functions. The *UBP*3, *UBP*6, *UBP*12 and *UBP*14 were highly expressed at different stages, suggesting they might play important roles at these stages. Moreover, some genes showed similar expression patterns in different tissues, suggesting that they might have retained redundant functions in regulating the same biological functions.

In fungi, functions of the *UBP* genes have been revealed in yeast and other fungi including *M. oryzae*.
Fig. 7 Detection of the host cell ROS accumulation. a DAB-staining assays of the wild type and ubp mutants-infected barley epidermis cells. b Percentage of DAB-stained barley epidermis cells infected by the wild-type and ubp mutant strains. Means and standard errors were calculated from three independent replicates. Significant differences were indicated by asterisks ($P < 0.05$).

Fig. 8 Statistical analysis of growth reduction rates of colony growth of the ubp mutants under different stresses. The growth reduction was calculated through dividing growth diameter of each strain grown under normal condition by the disparity between one grown under normal condition and stress conditions. Means and standard errors were calculated from three independent replicates. Significant differences were indicated by asterisks ($P < 0.05$).
In many fungal pathogens, including *M. oryzae*, conidiogenesis is a key step in the infection process. Our result showed the Δubp1, Δubp3, Δubp4, Δubp6, Δubp8, Δubp12, Δubp13, and Δubp14 were significantly reduced in conidiation, suggesting that *UBP* gene family plays key roles in conidium formation. We also found that the *UBP* genes may function as both positive and negative regulators in stress responses and nutrient utilization of *M. oryzae*. For example, *UBP1*, *UBP3*, *UBP8*, *UBP12* and *UBP14* could be positive regulators in different stress responses of *M. oryzae*, *UBP2* and *UBP4* were negative regulators in cell wall stress response, and *UBP1*, *UBP2* and *UBP4* were negative regulators in oxidative stress response (Fig. 8). Similar situation was also found in nutrient utilization. *UBP4* and *UBP14* were positive regulators of nutrient utilization, while *UBP1* and *UBP8* were negative regulators. These data suggest that de-ubiquitination system function in both positive and negative manners in different cellular processes.

**Conclusions**

In this study, we reported that 11 members of the UBP family are involved in the growth, development, stress responses, nutrient utilization, and pathogenicity of *M. oryzae*. Given the importance of these proteins, further investigation of the *UBP* genes and their respective regulatory networks are required, and they may also serve as putative fungicide targets for disease control.

**Methods**

**Strains and culture conditions**

The wild-type strain P131 and mutant strains of *M. oryzae* were grown at 28 °C on Oatmeal Tomato Agar (OTA) plates. Mycelia cultured in liquid CM at 28 °C were used for extracting genomic DNA, RNA, protein, and isolating protoplasts. Colony growth and conidiation were performed as described previously (Chen et al. 2012).
Conidia harvested from 7-day-old OTA cultures were used for testing virulence and observing infection process. Stress sensitivity of different strains were tested at 5 days after incubation on CM plates containing 0.1 mg/mL CFW, 0.2 mg/mL CR, 0.005% SDS, 0.5 M NaCl, or 10 mM H_2O_2. Colony diameters were recorded to calculate the growth reduction rates. Carbon source utilization was tested by recording colony diameters of different strains incubated on MM agar plates containing 1% glucose, 5 mM sodium acetate, 2% ethanol, or 2% glycerol as the sole carbon source. Colony diameters were recorded to calculate the growth reduction rates.

Gene deletion transformation
A split-PCR gene deletion strategy was used to delete the UBP genes (Goswami 2012). Gene replacement construct was generated by respectively fusing 1.5-kb upstream and 1.5-kb downstream flanking sequences with part of the hygromycin segment, and then transformed into protoplasts of the wild type (Chen et al. 2014) (Additional file 3: Table S1). The deletion transformants were selected by using 250 μg/mL hygromycin B (Roche, USA), and verified by PCR-mediated methods (Liu et al. 2018) (Additional file 3: Table S1).

Virulence test and infection process observation
Conidia suspensions (5 × 10^4 conidia/mL in ddH_2O containing 0.025% Tween 20) of different strains were sprayed onto one-month-old rice seedlings (Oryza sativa cv. LTH). The inoculated rice seedlings were incubated at 28 °C with full humidity, and the disease lesions were observed and photographed at 5 days post inoculation [dpi]. For infection process observation, conidia suspensions (1 × 10^5 conidia/mL) were dropped onto the barley leaves, and the leaves were then put into a dark chamber with full humidity at 28 °C. Infection processes were observed under a microscope (Nikon Ni90, Japan) at different time points (24 hpi and 30 hpi).

Expression level analysis of UBP genes
To examine UBP gene transcript levels at different tissues of M. oryzae, samples of mycelia, appressoria and infection hyphae were harvested as previously described (Hendy et al. 2019), and were used to extract total RNA for the RNaseq experiment, from which the expression levels of UBP genes were determined by FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values.

Phenotypic characterization of the ubp mutants
Mycelial growth of the different strains was measured on OTA plates after 120 h of incubation at 28 °C. Conidiation were calculated from conidia produced on OTA plates as previously reported (Chen et al. 2014).

Appressoria formation was observed by dropping conidia suspension (1 × 10^5 conidia/mL) onto a hydrophobic coverslip, then incubating in a moistened chamber at 28 °C. Appressoria formation percentage was calculated under a microscopy with at least 100 conidia per replicate and three replicates per experiment.

Staining assays
For CFW staining assay, mycelia were harvested from CM medium and stained with 10 μg/mL CFW (Sigma-Aldrich, USA) for 10 min in the dark. Before observation under a fluorescence microscope (Nikon Ni90 microscope, Japan), the samples were rinsed twice with PBS buffer. For DAB staining assay, the barley leaves were inoculated with different strains by dropping with conidia suspension (1 × 10^5 conidia/mL), then leave samples were stained with DAB solution (1 mg/mL, pH 3.8, Sigma-Aldrich, USA) at 36 hpi. After staining for 8 h, the leave samples were de-stained with an ethanol/acetic acid solution (ethanol/acetic acid; 94:4) for 1 h and observed under an epifluorescence microscope (Nikon Ni90 microscope, Japan).

Ubiquitination level detection
Total proteins of different strains were extracted as previously reported (Wang et al. 2018), which were subsequently assayed by Western blot analysis with an anti-ubiquitin as the primary antibody (1:5000, Sigma, USA) and an anti-mouse horseradish peroxidase as the secondary antibody (1:10000, Sigma, USA). Results were visualized with the ECL detection system (Amersham Biosciences, Piscataway, NJ).

Statistical analyses
All the data analysis was performed using SPSS (Statistical Program for Social Sciences) Version 10.0.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s42483-020-00050-1.

Additional file 1 Figure S1. Replacement strategy for M. oryzae UBP family genes through a split-marker approach. White bars represent genomic regions upstream and downstream of the UBP coding sequence that were amplified and fused to segments of the hygromycin phosphotransferase (HYG) cassette.

Additional file 2 Figure S2. Verification of the putative deletion mutants of M. oryzae UBP family genes. a PCR verification of the flanking sequences beside the replacement fragment by using primer pairs of LCK/HCK-up and RCK/HCK-down. b PCR verification by amplifying the UBP genes in the transformants and the wild-type strain (WT).

Additional file 3 Table S1. Primers used in this study.

Abbreviations
CFW: Calcofluor White; CR: Congo Red; DAB: 3,3′-diaminobenzidine; DUB: De-ubiquitinating enzyme; OTA: Oatmeal tomato agar; ROS: Reactive oxygen species; CM: complete medium; H2O2: hydrogen peroxide; dpi: days post inoculation; M. oryzae: Magnaporthe oryzae; H2O: water; CR: Congo red; CFW: Calcofluor white; DAB: 3,3′-diaminobenzidine; DUB: De-ubiquitinating enzyme; OTA: Oatmeal tomato agar; ROS: Reactive oxygen species.
species; SDS: Sodium dodecyl sulfate; UBP: Ubiquitin-specific protease; UCH: Ubiquitin C-terminal hydrolase

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Not applicable.

Authors’ contributions
X-LC conceived and designed the experiments. XC, ZW, YH, CL and HA conducted the experiments. JX provided technical support and edited the manuscript. X-LC and XC wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Rice (Hunan Hybrid Rice Research Center) (2019KF04). proved the final manuscript. X-LC and XC wrote the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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