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A mitogen-activated protein kinase phosphatase influences grain size and weight in rice

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SUMMARY

Grain size and weight are directly associated with grain yield in crops. However, the molecular mechanisms that set final grain size and weight remain largely unknown. Here, we characterize two large grain mutants, *large grain8-1* (*large8-1*) and *large grain8-2* (*large8-2*). *LARGE8* encodes the mitogen-activated protein kinase phosphatase1 (OsMKP1). Loss of function mutations in *OsMKP1* results in large grains, while overexpression of *OsMKP1* leads to small grains. OsMKP1 determines grain size by restricting cell proliferation in grain hulls. OsMKP1 directly interacts with and deactivates the mitogen-activated protein kinase 6 (OsMAPK6). Taken together, we identify OsMKP1 as a crucial factor that influences grain size by deactivating OsMAPK6, indicating that the reversible phosphorylation of OsMAPK6 plays important roles in determining grain size in rice.

Keywords: OsMKP1, OsMAPK6, cell proliferation, grain size, *Oryza sativa*.

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INTRODUCTION

Rice grain size and weight are directly associated with the yield. In recent years, a number of factors associated with grain size have been characterized in rice. These factors are involved in multiple pathways, including phytohormones, G-proteins, mitogen-activated protein kinases, ubiquitin-proteasome-related factors and transcriptional factors (Li and Li, 2016). Several grain size- and weight-related genes have been artificially selected during modern rice breeding (Fan *et al.*, 2006; Mao *et al.*, 2010; Li *et al.*, 2011; Wang *et al.*, 2012, 2015b,c; Ishimaru *et al.*, 2013; Xu *et al.*, 2015; Zhou *et al.*, 2015; Si *et al.*, 2016; Duan *et al.*, 2017; Liu *et al.*, 2017). However, the detailed mechanisms setting final grain size and weight remain largely elusive, despite their potentially fundamental importance to grain yield improvement.

The mitogen-activated protein kinases (MAPKs) play important roles in mediating multiple developmental and defensive signals in plants (Group, 2002; Xu and Zhang, 2015; Bi and Zhou, 2017). Dual phosphorylation of the Thr-X-Tyr activation loop of MAPK by its upstream MAPK kinase (MAPKK) is required for MAPK activity (Colcombet and Hirt, 2008), while dual-specific mitogen-activated protein kinase phosphatases (MKPs) negatively regulate the MAPK signaling by specifically dephosphorylating the phosphorylated activation loop of MAPKs (Kerk *et al.*, 2002; Luan, 2003; Andreasson and Ellis, 2010; Bartels *et al.*, 2010). The Arabidopsis genome encodes five MKPs, including AtMKP1, AtMKP2, PROPYZAMIDE HYPERSENSITIVE 1 (AtPHS1), INDOLE-3-BUTYRIC ACID RESPONSE 5 (AtIBR5) and DUAL-SPECIFICITY PROTEIN TYROSINE PHOSPHATASE 1 (AtDsPTP1; Kerk *et al.*, 2002; Ulm *et al.*, 2002; Bartels *et al.*, 2010). These Arabidopsis MKPs play roles in stress responses and hormone signals (Ulm *et al.*, 2001, 2002; Monroe-Augustus *et al.*, 2003; Naoi and Hashimoto, 2004; Yoo *et al.*, 2004; Quettier *et al.*, 2006; Lee and Ellis, 2007; Strader *et al.*, 2008a,b; Bartels *et al.*, 2009; Lee *et al.*,

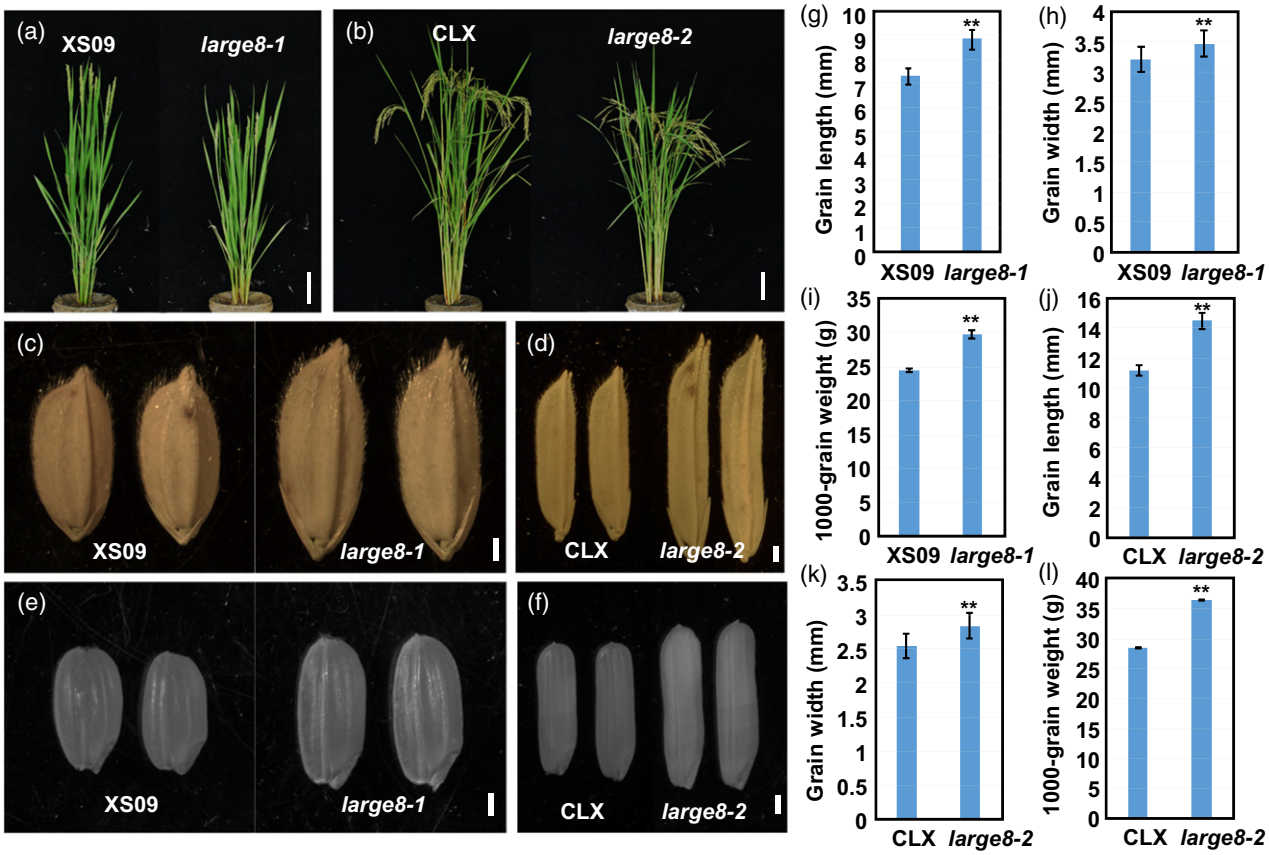

Figure 1. *large8-1* and *large8-2* form large grains.
(a) Plants of Xiushui09 (XS09) and *large8-1*.
(b) Plants of Changlixian (CLX) and *large8-2*.
(c) Mature paddy rice grains of XS09 and *large8-1*.
(d) Mature paddy rice grains of CLX and *large8-2*.
(e) Brown rice grains of XS09 and *large8-1*.
(f) Brown rice grains of CLX and *large8-2*.
(g–i) Grain length (g), grain width (h) and 1000-grain weight (i) of XS09 and *large8-1*.
(j–l) Grain length (j), grain width (k) and 1000-grain weight (l) of CLX and *large8-2*.
Values (g–l) are given as mean ± SD. $^{**}P < 0.01$ compared with the wild-type by Student's *t*-test. Scale bars: 10 cm (a and b); 1 mm (c–f). [Colour figure can be viewed at wileyonlinelibrary.com].

2009; Andreasson and Ellis, 2010). Phylogenetic analysis shows that rice MKPs share similarity with MKPs in Arabidopsis (Katou *et al.*, 2007). However, the functions of MKPs in rice are largely unknown, except that OsMKP1 and OsIBR5 have been reported to be involved in wound and drought stress responses, respectively (Katou *et al.*, 2007; Li *et al.*, 2012).

To identify new potentially beneficial genes for rice grain yield and understand the mechanisms that set final grain size and weight, we have tried to screen for mutants with large grains. In this study, we characterized two large grain mutants, *large grain8-1* (*large8-1*) and *large grain8-2* (*large8-2*). *LARGE8* encodes OsMKP1, which influences grain size by restricting cell proliferation in the spikelet hulls. OsMKP1 directly interacts with and deactivates OsMAPK6. These findings indicate that OsMKP1 is a factor affecting grain size and weight, suggesting the critical role of the reversible phosphorylation of OsMAPK6 in grain size and weight determination.

RESULTS

The *large8-1* and *large8-2* mutants form large grains

To identify potentially beneficial genes for grain yield and investigate the molecular mechanisms setting the final size and weight of rice grains, we have identified several large-grain mutants. The *large8-1* and *large8-2* mutants were isolated from the ethyl methane sulfonate (EMS)-treated *japonica* variety Xiushui09 and *indica* variety Changlixian, respectively. The *large8-1* and *large8-2* mutants produced obviously large grains compared with their respective wild-type plants (Figure 1a–f). The grain lengths of *large8-1* and

large8-2 were dramatically increased compared with that of Xiushui09 and Changlixian, respectively (Figure 1g and j), while the grain widths of *large8-1* and *large8-2* were only slightly increased (Figure 1h and k). Moreover, grains of the *large8-1* and *large8-2* mutants were significantly heavier than those of Xiushui09 and Changlixian, respectively (Figure 1i and l). The 1000-grain weights of Xiushui09 and *large8-1* were 24.5 and 29.8 g, respectively (Figure 1i). The 1000-grain weights of Changlixian and *large8-2* were 28.5 and 36.4 g, respectively (Figure 1l). Taken together, these results indicate that the *large8-1* and *large8-2* mutations affect grain size and weight mainly by increasing grain length.

Increased grain size generally corresponds to decreased grain number. We also counted the number of grains per panicle of the *large8-1* and *large8-2* mutants. The panicle length of *large8-1* was similar to that of XS09, while the numbers of grains, primary branches and secondary branches of *large8-1* panicles were significantly reduced (Figure S1). Similarly, the numbers of grains, primary branches and secondary branches of *large8-2* panicles were significantly reduced compared with those of Changlixian (Figure S2). Thus, the *large8-1* and *large8-2* mutations not only affect grain size and weight, but also influence panicle size.

Identification of the *LARGE8* gene

To identify the causal genes of *large8-1* and *larg8-2*, a Mut-map method was applied (Abe *et al.*, 2012), which was based on genome resequencing of pooled DNA from a segregating population. We crossed *large8-1* with Xiushui09, and *large8-2* with Changlixian to generate F1 plants. The grain size of F1 plants was similar to that of the corresponding wild-type plants, suggesting *large8-1* and *larg8-2* are recessive mutants. The F1 plants were selfed to produce F2 populations. The large-grain phenotypes of both *large8-1* and *larg8-2* were caused by single recessive mutations. Then the genomic DNAs from segregated large-grain F2 plants were pooled and applied for whole-genome resequencing. The genomic DNAs from Xiushui09 and Changlixian were also resequenced as control. The candidate causal genes were identified as described before (Fang *et al.*, 2016). Six and three candidate causal SNPs for *large8-1* and *large8-2* were identified, respectively (Tables S1 and S2). Interestingly, *LOC_Os05g02500* is a candidate causal gene for both *large8-1* and *large8-2*, although the mutation sites are different in the *large8-1* and *large8-2* mutants (Figure 2a; Tables S1 and S2). The mutations of *LOC_Os05g02500* in the *large8-1* and *large8-2* mutants were further confirmed by a derived cleaved amplified polymorphic sequences (dCAPS) marker (Figure 2b) and a cleaved amplified polymorphic sequences (CAPS) marker, respectively (Figure 2c), which were designed according to the corresponding SNPs. Considering that *large8-1* and *large8-2* mutants had similar large-grain phenotypes, these analyses suggest that *LOC_Os05g02500* is a potential causal gene for both the *large8-1* and *large8-2* mutants.

To confirm that *LOC_Os05g02500* corresponds to the *LARGE8* gene, the coding sequence (CDS) of *LOC_Os05g02500* driven by its native promoter (*pLARGE8:LARGE8*) was transformed into the *large8-1* mutant. The *pLARGE8:LARGE8* construct complemented the large-grain phenotype of *large8-1* (Figure 3), demonstrating that *LOC_Os05g02500* corresponds to the *LARGE8* gene.

LARGE8 encodes mitogen-activated protein kinase phosphatase1

The *LARGE8* gene (*LOC_Os05g02500*) encodes mitogen-activated protein kinase phosphatase1 (OsMKP1), which is known to regulate wound response in rice (Katou *et al.*, 2007). OsMKP1 contains a dual specificity phosphatase (DSP) catalytic domain, a Gelsolin-homology domain and a calmodulin-binding domain (Figure 2d; Katou *et al.*, 2007). The *large8-1* mutation results in a premature stop codon, producing premature translational products without the C-terminal 38 amino acids (Figure 2d). The *large8-2* mutation causes an arginine changed to threonine in the DSP catalytic domain (Figure 2d).

To further study the roles of *OsMKP1* in the regulation of grain size and weight, we generated another mutant of *OsMKP1* (*large8-3*) in the *japonica* variety Zhonghua11 background by employing the CRISPR-CAS9 method (Figure S3). The *large-3* mutation leads to a single nucleotide insertion near the 5′-end of the CDS of *OsMKP1*, which is predicted to result in a frame-shift and generate a premature stop codon (Figure 2a). Thus, *large-3* may be a null mutant allele. Like *large8-1* and *large8-2*, the *large8-3* mutant produces larger grains than Zhonghua11 (Figure S3). Taken together, these results further support the conclusion that *LARGE8* encodes OsMKP1, and reveal that loss function of *OsMKP1* results in large grains. As OsMKP1 regulates grain size and panicle size, the expression of *OsMKP1* in panicles was examined by employing the real-time reverse transcriptase-polymerase chain reaction (RT-PCR) method. The mRNA of *OsMKP1* could be detected in developing panicles (Figure S4), which is consistent with its roles in influencing grain and panicle size.

Overexpression of *OsMKP1* results in small grains

As loss of function of *OsMKP1* causes large and heavy grains, we asked if overexpression of *OsMKP1* could lead to small grains. To address this question, the CDS of *OsMKP1* driven by the *Actin* promoter (*pActin:OsMKP1*) was introduced into the Zhonghua11 variety. Transgenic plants overexpressing *OsMKP1* formed obviously small grains compared with Zhonghua11 (Figure 4). The grain length of plants overexpressing *OsMKP1* was dramatically reduced compared with that of Zhonghua11 (Figure 4c), while the grain width of plants overexpressing OsMKP1 was

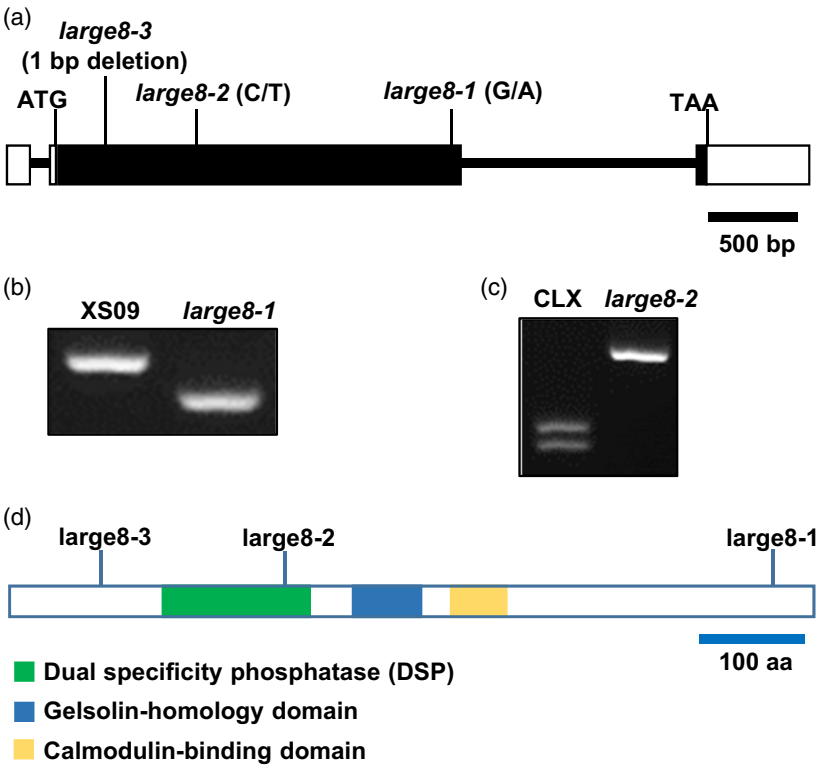

Figure 2. Cloning of the *LARGE8* gene.
(a) The *LARGE8* gene structure. White boxes indicate the 5′- and 3′-untranslated regions, black boxes indicate the coding sequence (CDS), and lines between boxes represent introns. The mutation sites of *large8-1*, *large8-2* and *large8-3* are shown.
(b) The derived cleaved amplified polymorphic sequence (dCAPS)
marker is developed according to the *large8-1* mutation. The restriction enzyme *Alu* I was used to digest the polymerase chain reaction (PCR) products.
(c) The cleaved amplified polymorphic sequence (CAPS) marker is developed according to the *large8-2* mutation. The restriction enzyme *Sma* I was used to digest the PCR products. CLX represents Changlixian.
(d) Schematic diagram of the OsMKP1 protein. The OsMKP1 protein contains a dual specificity phosphatase (DSP) domain, a Gelsolin-homology domain and a calmodulin-binding domain. The mutation sites of large8-1, large8-2 and large8-3 are shown. [Colour figure can be viewed at wileyonlinelibrary.com].

indistinguishable from that of Zhonghua11 (Figure 4d). As a result, the grains of plants overexpressing *OsMKP1* are significantly lighter than those of Zhonghua11 (Figure 4e). Taken together, OsMKP1 regulates grain size and grain weight mainly by repressing grain growth in the longitudinal direction.

OsMKP1 influences grain size by restricting cell proliferation

The spikelet hulls limit grain growth and determine the final grain size in rice (Li and Li, 2016). The final size of a spikelet hull is coordinately determined by cell number and cell size (Li and Li, 2016). To understand how OsMKP1 regulates grain size in cellular level, cells in spikelet hulls were measured. As shown in Figure 5, the average length of outer epidermal cells in *large8-1* lemmas was indistinguishable from that of Xiushui09 lemmas (Figure 5a–c). However, cell number in *large8-1* lemmas was dramatically increased in the longitudinal direction compared with that in Xiushui09 lemmas (Figure 5d). These results reveal that OsMKP1 controls grain size by restricting cell proliferation.

OsMKP1 physically interacts with and dephosphorylates OsMAPK6

In Arabidopsis, the homolog of OsMKP1, AtMKP1, interacts with and deactivates AtMAPK6 (Bartels *et al.*, 2009). In addition, OsMAPK6 plays an important role in influencing grain size (Liu *et al.*, 2015). Thus, OsMKP1 might control grain size by regulating the activity of OsMAPK6. To validate this hypothesis, we sought to test the interaction between OsMKP1 and OsMAPK6. We first tested the interaction of OsMKP1 and OsMAPK6 by employing the yeast two-hybrid. As we expected, OsMKP1 interacted with OsMAPK6 in yeast cells (Figure 6a).

The interaction between OsMKP1 and OsMAPK6 was further confirmed by *in vivo* bimolecular fluorescence complementation (BiFC) analyses. OsMKP1 and OsMAPK6 were fused with the C-terminal and N-terminal fragments of yellow fluorescent protein (YFP), respectively. Co-

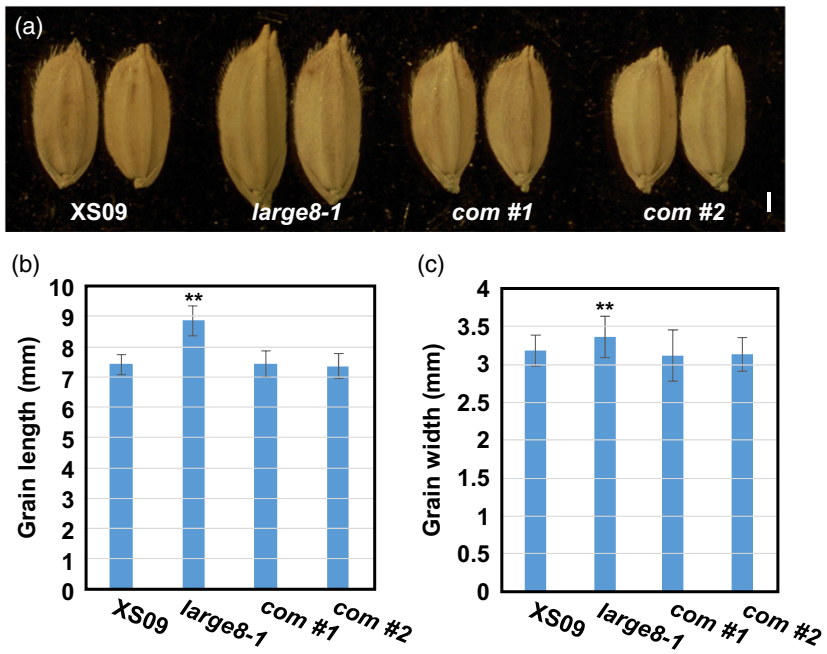

Figure 3. Expression of wild-type OsMKP1 could complement the large-grain phenotype of *large8-1*.
(a) Mature paddy rice grains of Xiushui09 (XS09), *large8-1*, *com #1* and *com #2*. *com* is *large8-1* transformed with *pLARGE8:LARGE8* plasmid. Scale bar: 1 mm.
(b and c) Grain length (b) and grain width (c) of XS09, *large8-1*, *com #1* and *com #2*. Values are given as mean ± SD. $^{**}P < 0.01$ compared with XS09. [Colour figure can be viewed at wileyonlinelibrary.com].

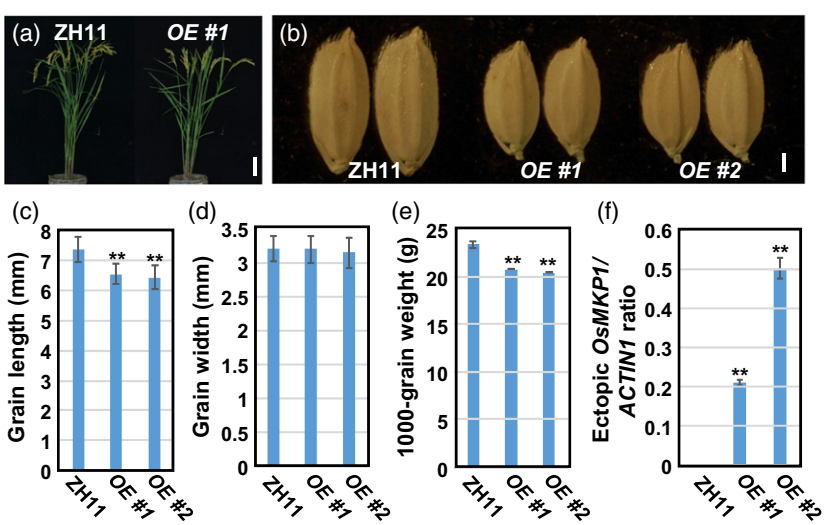

Figure 4. Overexpression of *OsMKP1* leads to small grains.
(a) Plants of Zhonghua11 (ZH11) and *OE #1*. *OE* represents a plant overexpressing *OsMKP1*, which is ZH11 transformed with the *pACTIN:OsMKP1* plasmid. Scale bar: 10 cm.
(b) Mature paddy rice grains of ZH11, *OE #1* and *OE #2*. Scale bar: 1 mm.
(c–e) Grain length (c), grain width (d) and 1000-grain weight (e) of ZH11, *OE #1* and *OE #2*.
(f) Expression levels of ectopic *OsMKP1* in ZH11, *OE #1* and *OE #2*.
Values are given as mean ± SD. $^{**}P < 0.01$ compared with ZH11. [Colour figure can be viewed at wileyonlinelibrary.com].

expression of cYFP-OsMKP1 with nYFP-OsMAPK6 in leaves of *Nicotiana benthamiana* resulted in strong YFP fluorescence, while the negative controls did not, suggesting that OsMKP1 interacts with OsMAPK6 *in planta* (Figure 6b).

We also confirmed the interaction by co-immunoprecipitation (Co-IP) analyses. OsMKP1 and OsMAPK6 were tagged by green fluorescent protein (GFP) and Myc, respectively, and then were co-expressed in leaves of *N. benthamiana*.

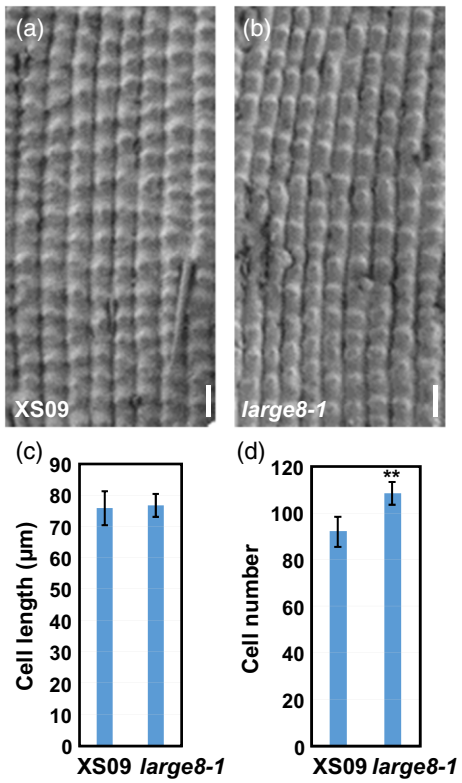

Figure 5. OsMKP1 regulates grain size by affecting cell proliferation but not cell expansion.
(a and b) Scanning electron microscope images of Xiushui09 (XS09) (a) and *large8-1* (b) lemmas. Scale bars: 100 μm.
(c and d) The average length (c) and number (d) of outer epidermal cells in the longitudinal direction in XS09 and *large8-1* lemmas. Values are given as mean ± SD. $^{**}P < 0.01$ compared with XS09. [Colour figure can be viewed at wileyonlinelibrary.com].

Anti-GFP beads were used for immunoprecipitation, and anti-Myc antibody was used to detect the immunoprecipitated proteins by Western blot. As shown in Figure 6c, Myc-OsMAPK6 were co-immunoprecipitated with GFP-OsMKP1, further supporting that OsMKP1 interacts with OsMAPK6 *in vivo*.

To test whether OsMKP1 could directly interact with OsMAPK6, *in vitro* pull-down experiments were performed. OsMKP1 and OsMAPK6 were expressed as glutathione *S*-transferase (GST)-tagged and His-tagged proteins in *Escherichia coli*, respectively. His-OsMAPK6 bound to GST-OsMKP1, but not to the negative control (Figure 6d). Thus, OsMKP1 directly interacts with OsMAPK6.

As OsMKP1 physically interacts with OsMAPK6, we sought to test if OsMKP1 could dephosphorylate the activated OsMAPK6. A previous study showed that MAPK kinase4 (OsMKK4) could phosphorylate and activate OsMAPK6 (Kishi-Kaboshi *et al.*, 2010). Consistently, when we co-expressed a constitutive active OsMKK4 (OsMKK4-DD) with OsMAPK6 in leaves of *N. benthamiana*, the amount of the activated OsMAPK6 was enhanced (Figure 7), while co-expression of OsMKP1 with OsMKK4DD and OsMAPK6 reduced the amount of the activated OsMAPK6 (Figure 7), revealing that OsMKP1 could dephosphorylate the OsMKK4-activated OsMAPK6. Thus, OsMKP1 physically interacts with and dephosphorylates OsMAPK6, suggesting that OsMKP1 may influence grain size by deactivating OsMAPK6.

DISCUSSION

Grain size and weight are critical determinants of grain yield. Several factors associated with grain size and weight have been identified in rice (Li and Li, 2016). Although some of these factors have been selected during breeding processes, the mechanisms setting final size and weight of grains are remain largely unknown (Zuo and Li, 2014). In this study, we identify OsMKP1 as a factor affecting grain size and weight, which may influence grain size by deactivating OsMAPK6, suggesting the critical role of the reversible phosphorylation of OsMAPK6 in grain size and weight determination.

Mitogen-activated protein kinases play important functions in transduction of multiple developmental and defense signals (Widmann *et al.*, 1999; Xu and Zhang, 2015). MKPs negatively regulate MAPK signaling through specifically inactivating the activated MAPKs (Kerk *et al.*, 2002; Luan, 2003; Andreasson and Ellis, 2010; Bartels *et al.*, 2010). The functions of MKPs in stress responses and hormone signals have been characterized in rice and Arabidopsis (Bartels *et al.*, 2010). In this study, we describe the roles of OsMKP1 in regulating grain size. Loss of function of OsMKP1 leads to large and heavy grains (Figures 1 and S3), while overexpression of OsMKP1 results in small grains (Figure 4), revealing that OsMKP1 negatively regulates grain size, and could be used for improving grain size during rice breeding. Spikelet hull, the final size of which is coordinately regulated by cell proliferation and cell expansion, limits grain growth and determines grain size (Li and Li, 2016). Cellular analyses show that the increased grain size of *large8-1* is mainly due to increased cell number (Figure 5). Thus, these findings indicate that OsMKP1 negatively regulates grain size by restricting cell proliferation.

Previous studies have shown that loss of function of OsMKK4 or OsMAPK6 results in small grains (Duan *et al.*, 2014; Liu *et al.*, 2015). OsMAPK6 could be phosphorylated and activated by OsMKK4 (Kishi-Kaboshi *et al.*, 2010), suggesting that the OsMKK4-OsMAPK6 module is involved in regulation of grain size. The activity of OsMAPK6 was increased in an *osmkp1* mutant under both standard growth and wound-treated conditions (Katou *et al.*, 2007). In this study, our biochemical analyses revealed that OsMKP1 physically interacts with and deactivates OsMAPK6 (Figures 6 and 7). Thus, OsMKP1 should control grain size by regulating the activity of OsMAPK6. However,

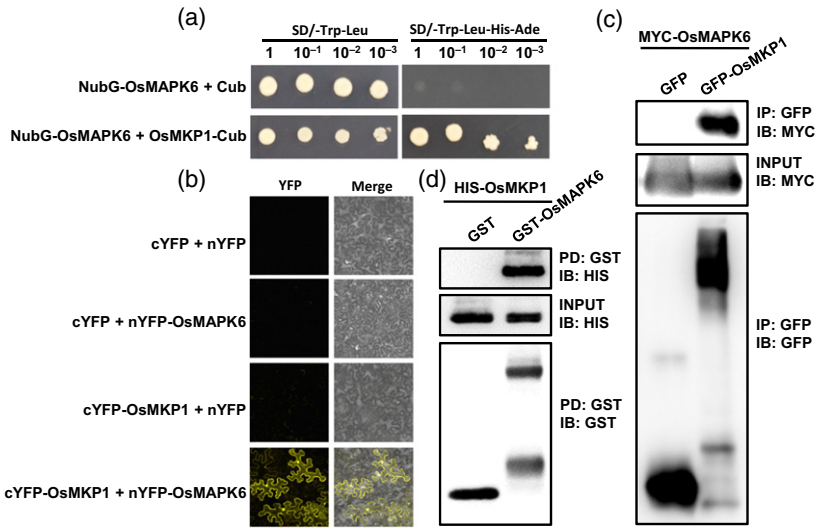

Figure 6. OsMKP1 physically interacts with OsMAPK6.
(a) OsMKP1 interacts with OsMAPK6 in yeast cells. Yeast cells were cultured on SD/-Trp-Leu or SD/-Trp-Leu-His-Ade media.
(b) Bimolecular fluorescence complementation (BiFC) assays indicate that OsMKP1 interacts with OsMAPK6 in *Nicotiana benthamiana* leaves. OsMAPK6 was fused to the N-terminal fragment of YFP (nYFP), and OsMKP1 was fused to the C-terminal fragment of YFP (cYFP), respectively. Then cYFP-OsMKP1 and nYFP-OsMAPK6 were co-expressed in leaves of *N. benthamiana*.
(c) Co-immunoprecipitation (Co-IP) assays indicate that OsMKP1 associates with OsMAPK6 in *N. benthamiana* leaves. GFP-OsMKP1 and MYC-OsMAPK6 were co-expressed in *N. benthamiana* leaves. Proteins were immunoprecipitated (IP) with GFP beads, and then analyzed by immunoblot (IB) using anti-GFP and anti-MYC antibodies, respectively.
(d) OsMKP1 binds OsMAPK6 *in vitro*. HIS-OsMKP1 was incubated with GST-OsMAPK6 and pulled down by Glutathione-Sepharose beads. The precipitate was analyzed by IB using anti-GST and anti-HIS antibodies, respectively. [Colour figure can be viewed at wileyonlinelibrary.com].

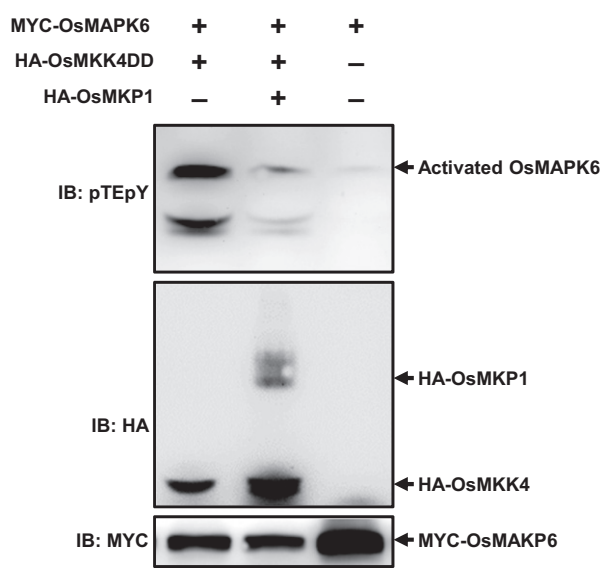

Figure 7. OsMKP1 dephosphorylates the activated OsMAPK6.
The indicated combinations of MYC-OsMAPK6, HA-OsMKP1 and HA-OsMKK4DD were transiently expressed in *Nicotiana benthamiana* leaves. Levels of MYC-OsMAPK6, HA-OsMKP1 and HA-OsMKK4DD were determined by immunoblot (IB) using anti-MYC and anti-HA antibodies, respectively. The activated OsMAPK6 was detected by antiphospho-p44/42 MAPK (Thr202/Tyr204) (anti-pTEpY) antibody.

it is unknown whether OsMKP1 could target other MAPKs to regulate grain size. OsMAPK3 plays a similar role as OsMAPK6 in defense responses (Kim *et al.*, 2012; Hu *et al.*, 2015). It would be interesting to investigate whether OsMKP1 could target OsMAPK3 to regulate grain size in future studies. In addition, OsMKP1 not only influences grain size but also grain number (Figures 1, S1 and S2), suggesting that OsMKP1 might play roles in balancing grain number per panicle and grain size by affecting the developmental process of inflorescence meristem.

According to these results, a model of how OsMKP1 and OsMKK4 control grain size and weight by regulating the activity of OsMAPK6 was suggested (Figure 8). The growth signals may activate OsMKK4 through its upstream MAPK kinase kinase (MKKK). The activated OsMKK4 then phosphorylates and activates OsMAPK6. The activated OsMAPK6 could promote the growth of spikelet hulls by promoting cell proliferation, thereby increasing grain size and weight, while the signaling restricting grain growth promotes OsMKP1 to dephosphorylate and deactivate OsMAPK6, thereby reducing grain size and weight. Therefore, the precise control of the activity of OsMAPK6 by reversible phosphorylation should play a critical role in final grain size and weight determination.

EXPERIMENTAL PROCEDURES

Plant materials

The *japonica* variety Xiushui09 (XS09) and *indica* variety Changlixian (CLX) were used for screening large-grain mutants using EMS mutagenesis. Rice plants were grown under natural growth

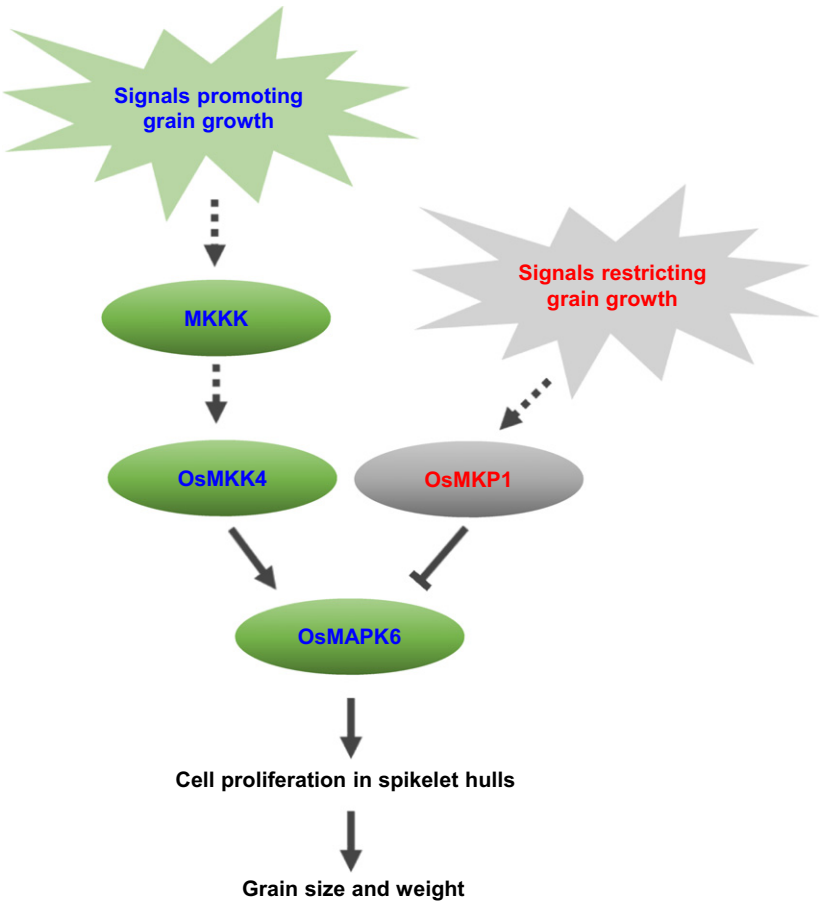

Figure 8. Dual regulation of OsMAPK6 activity by reversible phosphorylation is critical for final grain size and weight determination.
The signaling promoting grain growth may activate OsMKK4 through its upstream mitogen-activated protein kinase (MAPK) kinase kinase (MKKK). The activated OsMKK4 then phosphorylates and activates OsMAPK6. The activated OsMAPK6 could promote cell proliferation in spikelet hulls, thereby increasing grain size and weight, while the signaling restricting grain growth promotes dephosphorylation and deactivation of OsMAPK6 by OsMKP1, and thereby reducing grain size and weight. [Colour figure can be viewed at wileyonlinelibrary.com].

conditions in Lingshui (110° 03′E, 18° 51′N), Hangzhou (119° 95′E, 30° 07′N) and Beijing (116° 20′ E, 40° 22′N).

Grain size and cellular analysis

Grains were scanned to produce digital images, and then grain size was measured by software named SC-G (Wseen, China). At least 50 grains were measured for each sample. For cellular analyses, grains were scanned by a scanning electron microscope. Image J software was used for measuring cell size, and cell numbers were calculated according to grain size and cell size.

Molecular cloning of *LARGE8/OsMKP1*

MutMap approach was used to identifying the candidate genes for the *large8-1* and *large8-2* mutants (Abe *et al.*, 2012; Fang *et al.*, 2016).

Construction and transformation

Primers used for this study were listed in Table S3. A Seaming Cloning Kit (Genebank Biosciences, GBI) was used for constructing plasmid.

The 3.4-kb promoter sequence of *LARGE8* was amplified from wild-type Zhonghua11 using primers *LARGE8-PcomF* and *LARGE8-PcomR* to generate the promoter fragment. The CDS of *LAEGR8* was amplified from Zhonghua11 variety using *LARGE8-comF* and *LARGE8-comR* primers. The *pMDC164* vector was digested by *Pac* I and *Sac* I restriction enzymes to remove the GUS expression cascattes. Then the promoter and CDS fragments were ligated into the digested *pMDC164* to get the *pLARGE8:LARGE8* construct.

pACTIN:OsMKP1 was generated by subcloning *OsMKP1* CDS to the *pIPKb003* vector. *OsMKP1* CDS was amplified using *OsMKP1-F* and *OsMKP1-R* primers.

The *pC1300-cas9-OsMKP1cri* plasmid was constructed as described before (Wang *et al.*, 2015a). The 20-bp target sequence was GAGCCTCGACGAGTGGCCGC. The used primers were *OsMKP1-CRI-F* and *OsMKP1-CRI-R*.

Agrobacterium-mediated genetic transformation was used to transform the *pLARGE8:LARGE8*, *pACTIN:OsMKP1* and *pC1300-cas9-OsMKP1*cri plasmids to rice (Hiei *et al.*, 1994).

Real-time PCR

The TIANGEN RNA extraction kit (DP432) was used for extracting total RNA. The SuperScript III (Invitrogen) was used for generating cDNA. Real-time PCR was performed using a CFX96 PCR machine (Bio-Rad).

Yeast two-hybrid assay

OsMKP1 CDS was cloned into the *pPR3-N* vector and the *OsMAPK6* CDS was cloned into the *pDHB1* vector, respectively. The DUAL yeast two-hybrid system was used for conducting yeast two-hybrid.

BiFC assay

The expression plasmid of nYFP-OsMAPK6 was generated by subcloning the nYFP and OsMAPK6 into the expression vector, *pGWB414*. Similarly, the expression plasmid of cYFP-OsMKP1 was generated by subcloning the cYFP and OsMKP1 into *pGWB414*. The BiFC experiments were conducted as described before (Duan *et al.*, 2015).

Co-IP assay

The *35S:GFP-OsMKP1* was generated by subcloning *OsMKP1* CDS into the *pMDC43* vector. The *35S:Myc-OsMAPK6* plasmid was generated by subcloning *OsMAPK6* CDS into *pCAMBIA1300-221-Myc*. Co-IP assays were conducted as described before (Wang *et al.*, 2016).

Pull-down assay

OsMKP1 CDS was subcloned into the *pET-28a*(+) vector. *OsMAPK6* CDS was subcloned into the *pGEX-4T-1* vector. Pull-down assay was conducted as described before (Xia *et al.*, 2013), and the precipitates were analyzed by Western blot using GST and His antibodies (Abmart).

Dephosphorylation analyses

The HA-tagged *OsMKP1* and HA-tagged OsMKK4DD were subcloned into *pGWB414* vector to generate *35S:HA-OsMKP1* and *35S:HA-OsMKK4DD* plasmids, respectively. Different combinations of Myc-OsMAPK6, HA-OsMKP1 and HA-OsMKK4DD were transiently expressed in *N. benthamiana* leaves, and total proteins were extracted as described before (Wang *et al.*, 2016). Then, the activated OsMAPK6 was detected by Western blot with antiphospho-p44/42 MAPK antibody (Cell Signaling Technology). Protein inputs were, respectively, detected by Western blot with HA and Myc antibodies (Abmart).

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AUTHOR CONTRIBUTIONS

R.X. and H.Y. conducted most of the experiments. J.W. and P.D. isolated the *large8-1* and *large8-2* mutants, respectively. B.Z. performed rice transformation. Jing L., Yu L., J.X., Jia L., N.L. and T.C. helped to do phenotypic and biochemical assays. R.X. and Y.L. analyzed data and wrote the article.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The panicle size of *large8-1* is reduced.
Figure S2. The panicle size of *large8-2* is reduced.
Figure S3. The *large8-3* mutant forms large and heavy grains.
Figure S4. Expression of *OsMKP1* in developing panicles.
Table S1. Identification of the *large8-1* mutation.
Table S2. Identification of the *large8-2* mutation.
Table S3. Primers used in this study.

REFERENCES

Abe, A., Kosugi, S., Yoshida, K. *et al.* (2012) Genome sequencing reveals agronomically important loci in rice using MutMap. *Nat. Biotechnol.* **30**, 174–178.

Andreasson, E. and Ellis, B. (2010) Convergence and specificity in the Arabidopsis MAPK nexus. *Trends Plant Sci.* **15**, 106–113.

Bartels, S., Anderson, J.C., Gonzalez Besteiro, M.A., Carreri, A., Hirt, H., Buchala, A., Metraux, J.P., Peck, S.C. and Ulm, R. (2009) MAP kinase phosphatase1 and protein tyrosine phosphatase1 are repressors of salicylic acid synthesis and SNC1-mediated responses in Arabidopsis. *Plant Cell*, **21**, 2884–2897.

Bartels, S., Gonzalez Besteiro, M.A., Lang, D. and Ulm, R. (2010) Emerging functions for plant MAP kinase phosphatases. *Trends Plant Sci.* **15**, 322–329.

Bi, G. and Zhou, J.M. (2017) MAP kinase signaling pathways: a hub of plant-microbe interactions. *Cell Host Microbe*, **21**, 270–273.

Colcombet, J. and Hirt, H. (2008) Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. *Biochem. J.* **413**, 217–226.

Duan, P., Rao, Y., Zeng, D., Yang, Y., Xu, R., Zhang, B., Dong, G., Qian, Q. and Li, Y. (2014) SMALL GRAIN 1, which encodes a mitogen-activated protein kinase kinase 4, influences grain size in rice. *Plant J.* **77**, 547–557.

Duan, P., Ni, S., Wang, J., Zhang, B., Xu, R., Wang, Y., Chen, H., Zhu, X. and Li, Y. (2015) Regulation of OsGRF4 by OsmiR396 controls grain size and yield in rice. *Nat. Plants*, **2**, 15203.

Duan, P., Xu, J., Zeng, D. *et al.* (2017) Natural variation in the promoter of GSE5 contributes to grain size diversity in rice. *Mol. Plant*, **10**, 685–694.

Fan, C., Xing, Y., Mao, H., Lu, T., Han, B., Xu, C., Li, X. and Zhang, Q. (2006) GS3, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. *Theor. Appl. Genet.* **112**, 1164–1171.

Fang, N., Xu, R., Huang, L., Zhang, B., Duan, P., Li, N., Luo, Y. and Li, Y. (2016) SMALL GRAIN 11 controls grain size, grain number and grain yield in rice. *Rice (N Y)*, **9**, 64.

Group, M. (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci.* **7**, 301–308.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.

Hu, L., Ye, M., Li, R., Zhang, T., Zhou, G., Wang, Q., Lu, J. and Lou, Y. (2015) The rice transcription factor WRKY53 suppresses herbivore-induced defenses by acting as a negative feedback modulator of mitogen-activated protein kinase activity. *Plant Physiol.* **169**, 2907–2921.

Ishimaru, K., Hirotsu, N., Madoka, Y. *et al.* (2013) Loss of function of the IAA-glucose hydrolase gene TGW6 enhances rice grain weight and increases yield. *Nat. Genet.* **45**, 707–711.

Katou, S., Kuroda, K., Seo, S., Yanagawa, Y., Tsuge, T., Yamazaki, M., Miyao, A., Hirochika, H. and Ohashi, Y. (2007) A calmodulin-binding mitogen-activated protein kinase phosphatase is induced by wounding and regulates the activities of stress-related mitogen-activated protein kinases in rice. *Plant Cell Physiol.* **48**, 332–344.

Kerk, D., Bulgrien, J., Smith, D.W., Barsam, B., Veretnik, S. and Gribskov, M. (2002) The complement of protein phosphatase catalytic subunits encoded in the genome of Arabidopsis. *Plant Physiol.* **129**, 908–925.

Kim, S.H., Oikawa, T., Kyozuka, J., Wong, H.L., Umemura, K., Kishi-Kaboshi, M., Takahashi, A., Kawano, Y., Kawasaki, T. and Shimamoto, K. (2012)

The bHLH Rac Immunity1 (RAI1) is activated by OsRac1 via OsMAPK3 and OsMAPK6 in rice immunity. *Plant Cell Physiol.* **53**, 740–754.

Kishi-Kaboshi, M., Okada, K., Kurimoto, L. *et al.* (2010) A rice fungal MAMP-responsive MAPK cascade regulates metabolic flow to antimicrobial metabolite synthesis. *Plant J.* **63**, 599–612.

Lee, J.S. and Ellis, B.E. (2007) Arabidopsis MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. *J. Biol. Chem.* **282**, 25020–25029.

Lee, J.S., Wang, S., Sritubtim, S., Chen, J.G. and Ellis, B.E. (2009) Arabidopsis mitogen-activated protein kinase MPK12 interacts with the MAPK phosphatase IBR5 and regulates auxin signaling. *Plant J.* **57**, 975–985.

Li, N. and Li, Y. (2016) Signaling pathways of seed size control in plants. *Curr. Opin. Plant Biol.* **33**, 23–32.

Li, Y., Fan, C., Xing, Y. *et al.* (2011) Natural variation in GS5 plays an important role in regulating grain size and yield in rice. *Nat. Genet.* **43**, 1266–1269.

Li, Y., Feng, D., Zhang, D., Su, J., Zhang, Y., Li, Z., Mu, P., Liu, B., Wang, H. and Wang, J. (2012) Rice MAPK phosphatase IBR5 negatively regulates drought stress tolerance in transgenic Nicotiana tabacum. *Plant Sci.* **188–189**, 10–18.

Liu, S., Hua, L., Dong, S., Chen, H., Zhu, X., Jiang, J., Zhang, F., Li, Y., Fang, X. and Chen, F. (2015) OsMAPK6, a mitogen-activated protein kinase, influences rice grain size and biomass production. *Plant J.* **84**, 672–681.

Liu, J., Chen, J., Zheng, X. *et al.* (2017) GW5 acts in the brassinosteroid signalling pathway to regulate grain width and weight in rice. *Nat. Plants*, **3**, 17043.

Luan, S. (2003) Protein phosphatases in plants. *Annu. Rev. Plant Biol.* **54**, 63–92.

Mao, H., Sun, S., Yao, J., Wang, C., Yu, S., Xu, C., Li, X. and Zhang, Q. (2010) Linking differential domain functions of the GS3 protein to natural variation of grain size in rice. *Proc. Natl Acad. Sci. USA*, **107**, 19579–19584.

Monroe-Augustus, M., Zolman, B.K. and Bartel, B. (2003) IBR5, a dual-specificity phosphatase-like protein modulating auxin and abscisic acid responsiveness in Arabidopsis. *Plant Cell*, **15**, 2979–2991.

Naoi, K. and Hashimoto, T. (2004) A semidominant mutation in an Arabidopsis mitogen-activated protein kinase phosphatase-like gene compromises cortical microtubule organization. *Plant Cell*, **16**, 1841–1853.

Quettier, A.L., Bertrand, C., Habricot, Y., Miginiac, E., Agnes, C., Jeannette, E. and Maldiney, R. (2006) The phs1-3 mutation in a putative dual-specificity protein tyrosine phosphatase gene provokes hypersensitive responses to abscisic acid in Arabidopsis thaliana. *Plant J.* **47**, 711–719.

Si, L., Chen, J., Huang, X. *et al.* (2016) OsSPL13 controls grain size in cultivated rice. *Nat. Genet.* **48**, 447–456.

Strader, L.C., Monroe-Augustus, M. and Bartel, B. (2008a) The IBR5 phosphatase promotes Arabidopsis auxin responses through a novel mechanism distinct from TIR1-mediated repressor degradation. *BMC Plant Biol.* **8**, 41.

Strader, L.C., Monroe-Augustus, M., Rogers, K.C., Lin, G.L. and Bartel, B. (2008b) Arabidopsis iba response5 suppressors separate responses to various hormones. *Genetics*, **180**, 2019–2031.

Ulm, R., Revenkova, E., di Sansebastiano, G.P., Bechtold, N. and Paszkowski, J. (2001) Mitogen-activated protein kinase phosphatase is required for genotoxic stress relief in Arabidopsis. *Genes Dev.* **15**, 699–709.

Ulm, R., Ichimura, K., Mizoguchi, T., Peck, S.C., Zhu, T., Wang, X., Shinozaki, K. and Paszkowski, J. (2002) Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. *EMBO J.* **21**, 6483–6493.

Wang, S., Wu, K., Yuan, Q. *et al.* (2012) Control of grain size, shape and quality by OsSPL16 in rice. *Nat. Genet.* **44**, 950–954.

Wang, C., Shen, L., Fu, Y., Yan, C. and Wang, K. (2015a) A simple CRISPR/Cas9 system for multiplex genome editing in rice. *J. Genet. Genomics*, **42**, 703–706.

Wang, S., Li, S., Liu, Q. *et al.* (2015b) The OsSPL16-GW7 regulatory module determines grain shape and simultaneously improves rice yield and grain quality. *Nat. Genet.* **47**, 949–954.

Wang, Y., Xiong, G., Hu, J. *et al.* (2015c) Copy number variation at the GL7 locus contributes to grain size diversity in rice. *Nat. Genet.* **47**, 944–948.

Wang, Z., Li, N., Jiang, S., Gonzalez, N., Huang, X., Wang, Y., Inze, D. and Li, Y. (2016) SCF(SAP) controls organ size by targeting PPD proteins for degradation in Arabidopsis thaliana. *Nat. Commun.* **7**, 11192.

Widmann, C., Gibson, S., Jarpe, M.B. and Johnson, G.L. (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* **79**, 143–180.

Xia, T., Li, N., Dumenil, J., Li, J., Kamenski, A., Bevan, M.W., Gao, F. and Li, Y. (2013) The ubiquitin receptor DA1 interacts with the E3 ubiquitin ligase DA2 to regulate seed and organ size in Arabidopsis. *Plant Cell* **25**, 3347–3359.

Xu, J. and Zhang, S. (2015) Mitogen-activated protein kinase cascades in signaling plant growth and development. *Trends Plant Sci.* **20**, 56–64.

Xu, C., Liu, Y., Li, Y., Xu, X., Xu, C., Li, X., Xiao, J. and Zhang, Q. (2015) Differential expression of GS5 regulates grain size in rice. *J. Exp. Bot.* **66**, 2611–2623.

Yoo, J.H., Cheong, M.S., Park, C.Y. *et al.* (2004) Regulation of the dual specificity protein phosphatase, DsPTP1, through interactions with calmodulin. *J. Biol. Chem.* **279**, 848–858.

Zhou, Y., Miao, J., Gu, H. *et al.* (2015) Natural variations in SLG7 regulate grain shape in rice. *Genetics*, **201**, 1591–1599.

Zuo, J. and Li, J. (2014) Molecular genetic dissection of quantitative trait loci regulating rice grain size. *Ann. Rev. Genet.* **48**, 99–118.