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RESEARCH ARTICLE

OrMKK3 Influences Morphology and Grain Size in Rice

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Abstract

Although morphology and grain size are important to rice growth and yield, the identity of abundant natural allelic variations that determine agronomically important differences in crops is unknown. Here, we characterized the function of mitogen-activated protein kinase 3 from *Oryza officinalis* Wall. ex Watt encoded by *OrMKK3*. Different alternative splicing variants occurred in *OrMKK3*. Green fluorescent protein (GFP)–*OrMKK3* fusion proteins localized to the cell membrane and nuclei of rice protoplasts. Overexpression of *OrMKK3* influenced the expression levels of the grain size-related genes *SMG1*, *GW8*, *GL3*, *GW2*, and *DEP3*. Phylogenetic analysis showed that *OrMKK3* is well conserved in plants while showing large amounts of variation between *indica*, *japonica*, and wild rice. In addition, *OrMKK3* slightly influenced brassinosteroid (BR) responses and the expression levels of BR-related genes. Our findings thus identify a new gene, *OrMKK3*, influencing morphology and grain size and that represents a possible link between mitogen-activated protein kinase and BR response pathways in grain growth.

Keywords *Oryza officinalis* Wall. ex Watt · *OrMKK3* · Morphology · Grain size

Abbreviation

GFP Green fluorescent protein

Ying Hua Pan, Li Jun Gao and Yun Tao Liang have contributed equally to this work

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Introduction

Rice is the main cereal crop of over 3.5 billion people (Cheng et al. 2013). *Oryza officinalis* Wall. ex Watt (CC, $2n = 2x = 24$), an important and special wild-rice species, represents a significant source of genes influencing important characteristics such as grain size and strong resistance to abiotic and biotic stresses (Zhang et al. 2019). Recent studies have isolated and characterized several genes for disease resistance from *O. officinalis* Wall. ex Watt (Jiang et al. 2019), however, the identities of genes for grain size and plant architecture are unknown.

Plant height and tiller number are two major agronomic traits influencing cereal crop architecture and grain yield (Wang et al. 2018a, b) and are always inversely related in rice (Liao et al. 2019), with increased tillers and decreasing plant height of many mutant and transgenic plants (Chen et al. 2012; Choi et al. 2012; Xu et al. 2012; Zhou et al. 2013). While the specific identities of many trait-influencing genes in rice are unknown, many studies have revealed potential genetic influences on trait regulation. For example, *Grain number, plant height, and heading date7* (*Ghd7*), which encodes a CCT-domain-containing transcription factor and has been reported to have major effects on the

number of grains per panicle, plant height, and flowering time (Gao et al. 2014). In addition, the QTL *DTH8* (QTL for days to heading on chromosome 8) encoding a putative HAP3 subunit of a CCAAT-box-binding transcription factor was shown to regulate heading, plant height, and the number of grains per panicle (Wei et al. 2010). A semi-dwarf and late-flowering mutation altering the function of the WRKY transcription factor Dlf1 caused downregulation of *Ehd2*/*RID1*/*OsId1* in a signal transduction pathway and influenced height in rice (Cai et al. 2014). *OsGA3ox2* encodes a gibberellin (GA) 3β-hydroxylase that catalyzes the conversion of GA20 to GA1FIG. Loss-of-function mutants of *OsGA3ox2* show severe dwarfism due to altered GA processing (Itoh et al. 2002). Overall, these studies indicate that multiple pathways influence rice height.

Tiller number is another critical trait of rice that describes the level of grain yield. A comprehensive understanding of the genetic controls of tiller number will enhance knowledge of basic plant development and improve agriculture. Mutations in one gene, *This1* that encodes a class-III lipase are associated with high tillering, reduced height, and infertile spikelets (Liu et al. 2013). *OsAAP5,* an amino acid permease, regulates tiller, and grain yield by affecting the cytokinin levels (Wang et al. 2019). *Dwarf27* (*D27*) is an iron-containing protein involved in the MORE AXILLARY GROWTH 4 (MAX4)/RAMOSUS 1 (RMS1)/DWARF10 (D10) pathway and participates in strigolactone biosynthesis (Lin et al. 2009). *OsNPF7.2*, a low-affinity nitrate transporter, plays an important role in the control of tiller bud growth and tillering regulation by coordinating the cytokinin and strigolactone pathways (Wang et al. 2018a, b). Tillering is also reported to be inhibited by gibberellin (Duan et al. 2014). Although many studies have been conducted attempting to identify genes that control the tiller development and plant height, only few genes expressed by *O. officinalis* have been identified.

Rice yield depends on three major factors: grain weight, grain number per panicle, and panicle number per plant. Grain weight is related to grain length, width, and thickness (Duan et al. 2014). The QTL grain length and weight on chromosome 7 (q*GLW7*) contains a gene encoding a plant-specific transcription factor that positively regulates cell size in the grain hull, resulting in enhanced rice grain length and yield (Si et al. 2016). In addition, *GSN1* encodes a mitogen-activated protein kinase phosphatase (*OsMKP1*) that directly interacts with and inactivates a mitogen-activated protein kinase (*OsMPK6*) that coordinates the trade-off between grain number and grain size (Tao et al. 2018). Expression levels of GRAIN WIDTH8 (*GW8*) influence grain, shape, and quality (Wang et al. 2012). Another major QTL regulating grain length in rice, *qGL3*, suppresses BR signaling (Gao et al. 2019) while *DWARF2* reduces grain height and size by regulating gibberellin biosynthesis (Chen et al. 2019). Small grain 11 (*SMG11*) controls grain size by promoting cell expansion in grain hulls by regulating BR biosynthesis (Fang et al. 2016). Overexpression of constitutively active versions of *OsMKKK10* and *OsMKK4* causes production of large and heavy grains, since the *OsMKKK10-OsMKK4-OsMitogen-Activated Protein Kinase 6 (MAPK6)* cascade exerts control of grain size and weight in rice (Xu et al. 2018). *SMG1*, encoding MKK4, affects BR responses and thus influences grain size (Duan et al. 2014). Thus, considerable research has revealed that multiple pathways control grain size in rice.

Mitogen-activated protein kinase (Kishi-Kaboshi et al. 2010) cascades transmit exogenous or developmental signals to target molecules through sequential phosphorylation and orchestrate multiple processes related to plant growth, development, and defense response (Andreasson et al. 2010; Pitzschke. 2015; Xu et al. 2015). Mitogen-activated protein kinase 3 (MKK3) mediates resistance to *Nilaparvata lugens* via phytohormone dynamics (Zhou et al. 2019). MKK3 phosphorylates a basic helix–loop–helix transcription factor, activating the MKK3-mitogen-activated protein kinase 6 (MPK6)-Myelocytomatosis module involved in light signal transduction pathways in *Arabidopsis thaliana* (Sethi et al. 2014). In addition, mitogen-activated protein kinase (MKKK62)-MKK3-MAPK7/MAPK14 regulates the transcription of OsMFT in rice to mediate seed dormancy (Mao et al. 2019), with MKK3 also regulating seed dormancy in barley (Nakamura et al. 2016).

We previously have identified genes that affect plant height, tillering, and grain growth in *O. officinalis* Wall. ex Watt. Here, we first describe isolation a gene encoding mitogen-activated protein kinase 3 (*OrMKK3*) in *O. officinalis* Wall. ex Watt by homology-based cloning and its subsequent characterization. *OrMKK3* is an ortholog of *OsMKK3* and appears to influence plant height, tiller, and grain size. Additional results show that *OrMKK3* affects BR responses and the expression levels of BR-related genes. Our findings thus indicate that *OrMKK3* is a factor influencing plant height, tiller, and grain size in *O. officinalis* Wall. ex Watt and indicate a possible connection between the MAPK cascades and BRs in grain growth.

Results

OrMKK3 Cloning and Sequence Analysis

Two pairs of primers (Table S1) were designed based on other available Nipponbare *MKK3* nucleotide sequences (Genbank accessions: *Os06g0473200*) to amplify the *OrMKK3* open reading frame from the Y003 line of *O. officinalis* in Guangxi Province. Five sequences, *OrMKK3.1–3.5*, were obtained (supplementary materials). Comparisons

of nucleotide sequences and gene structures revealed that *OrMKK3.2–3.5* were alternative splicing variants of *OrMKK3.1*. The *OrMKK3* alternative splicing events were classified into four categories: alternative exon 5′-ends (5′-AE), exon skipping (Hamel et al. 2006), mutually exclusive exons, and alternative exon 3′-ends (3′-AE). *OrMKK3.1* and *OrMKK3.5* differed by the presence of a 3′-AE in the 3′-untranslated region (3′-UTR) (Fig. 1a). *OrMKK3.2* possessed a 3′-AE and lacked half of exon 8 (Fig. 1a). *OrMKK3.3* and *OrMKK3.5* both also lacked half of exon 8, with *OrMKK3.3* lacked the first half and *OrMKK3.5* lacking the latter half (Fig. 1a). Multiple exon skip (ES) and AE events distinguish *OrMKK3.4* from *OrMKK3.1–3.3* and *OrMKK3.5*. *OrMKK3.4* has a frameshift mutation in the sixth exon away from the 3′ end of the coding sequence (Fig. 1a). In addition, *OrMKK3.3* and *OrMKK3.5* differed by the presence of a 3′-AE in the 3′-UTR; however, this difference did not cause the production of different proteins. Compared to *OrMKK3.1*, however, *OrMKK3.2*, *OrMKK3.3*, *OrMKK3.4*, and *OrMKK3.5* exhibit a frameshift mutation that is predicted to cause truncated protein production (Fig. 1a).

From NCBI (https://www.ncbi.nlm.nih.gov/), we found five Nipponbare *MKK3* genes named *XM_015788456.2*, *XM_015788457.2*, *XM_015788458.2*, *XM_015788459.2*, and *XM_015788460.2*. *XM_015788458.2* contained 11 exons, as did *OrMKK3.1*. *XM_015788458.2* and *OrMKK3.1* differed by 28 single-nucleotide polymorphisms (SNP) in their open reading frames, although 17 of these SNPs did not affect the encoded proteins while 9 of these SNPs altered the encoded protein sequences. In addition, *XM_015788456.2*, *XM_015788457.2*, *XM_015788459.2*, and *XM_015788460.2* each contained the 17 SNPs described above. *XM_015788456.2* contained an additional exon beyond exon 8 of *OrMKK3.1*. *XM_015788457.2* contained an additional exon beyond exon 8 and additional 3′-AE events compared to *OrMKK3.1*. *XM_015788459.2* contained two additional exons following exons corresponding to exon 1 and exon 8 of *OrMKK3.1*. *XM_015788459.2* contained an additional exon following exon 1 compared to *OrMKK3.1* (Fig. 1b).

OrMKK3 Regulates Height and Tillering

Several experiments were conducted to determine whether the differences in the coding sequences determined *OrMKK3* function. First, four overexpression constructs containing *OrMKK3.1*, *OrMKK3.2*, *OrMKK3.3*, and *OrMKK3.4* from Y003 driven by the 35S promoter from tobacco cauliflower mosaic virus (CaMV35S) were separately introduced into

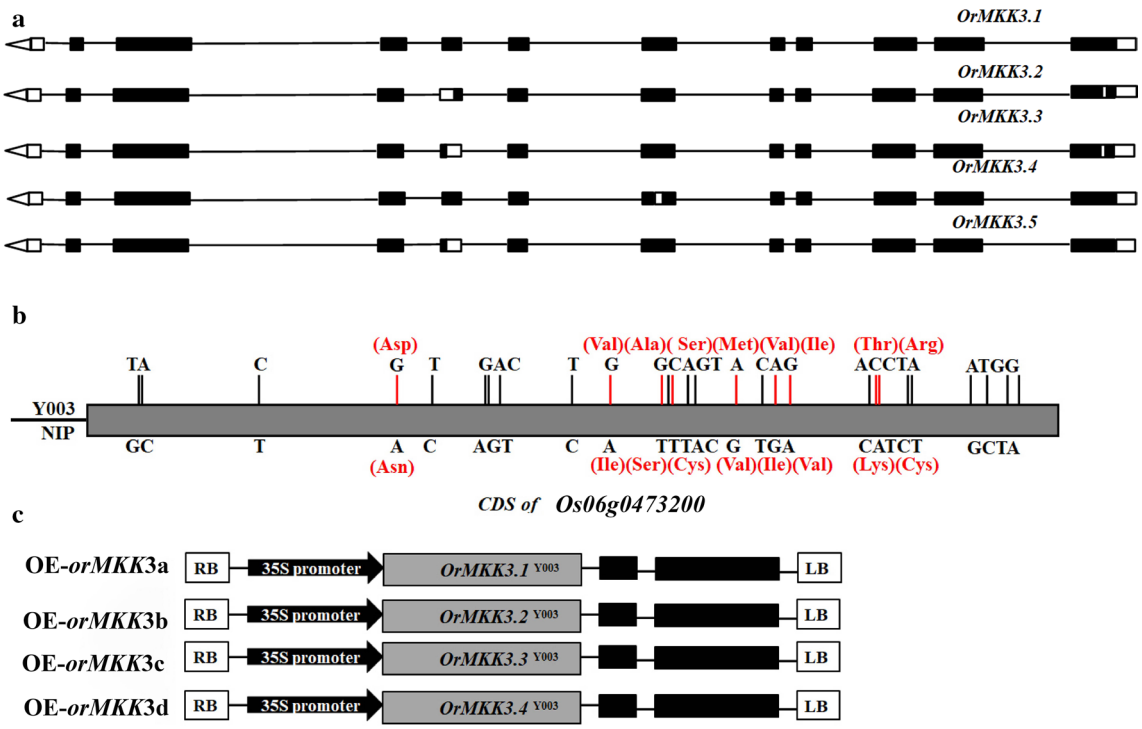


Fig. 1 **a** Five cDNA of alternative splicing in *OrMKK3*. *OrMKK3.1* includes all exons. Alternative 3′ splicing and exon skipping in *OrMKK3.2*. Alternative 5′ splicing in *OrMKK3.3*. Exon skipping in *OrMKK3.4*, Alternative 3′ splicing in *OrMKK3.5*. **b** SNP between Nipponbare and Y003 in *OrMKK3* CDS. **c** Complementation vector: *OrMKK3*a (with CDS of *OrMKK3.1*), *OrMKK3*b (with CDS of *OrMKK3.2*), *OrMKK3*c (with CDS of *OrMKK3.3*), and *OrMKK3*d (with CDS of *OrMKK3*.4) overexpression vector

the Nipponbare cultivar (Fig. 1c). Quantitative real-time polymerase chain reaction (qRT-PCR) results indicated that independent T0 transgenic lines have different expression levels of *OrMKK3.1*, *OrMKK3.2*, *OrMKK3.3*, and *OrMKK3.4*, with expression levels of these genes also determined in T3 lines using qRT-PCR. T3 generations of three overexpression transgenic lines (OE-lines) of each *OrMKK3* splice variant, OE-*OrMKK3a*, OE-*OrMKK3b*, OE-*OrMKK3c*, and OE-*OrMKK3d*, were used for further analysis (Fig. 2a). At the maturation stage, OE-*OrMKK3a*, OE-*OrMKK3b*, OE-*OrMKK3c*, and OE-*OrMKK3d* lines were significantly shorter than wild-type plants (Fig. 2a, c). We performed histocytological analysis of the internal structure of internodes using paraffin sections (Fig. 2d) to reveal the cause of OE-line dwarfism (Fig. 2a, c). Results showed that cell sizes were significantly reduced in OE-line internodes, suggesting that cell size was restrained in the OE-lines. These observations also imply that that *OrMKK3* may negatively affect cell length, thereby limiting rice plant height.

OE-*OrMKK3*a, OE-*OrMKK3*b, OE-*OrMKK3*c, and OE-*OrMKK3*d plants had more tillers than wild-type plants at the full flowering stage (Fig. 2b, c). Tiller number in the OE-lines was significantly more than that in Nipponbare (Fig. 2b, c). In contrast, no significant difference was found between tiller numbers of OE-*OrMKK3*d and Nipponbare lines (Fig. 2b, c). The overexpression of *OrMKK3* thus significantly increased rice tiller number, and the upregulation of *OrMKK3* positively influenced tiller shaping.

OrMKK3 Determines Grain Size and Influences 1000 Seed Weight

We found that *OrMKK3* regulates grain length and width. Grain length and width were reduced in *OrMKK3* overexpression transgenic plants (T3 generation; Fig. 3a, d, e), indicating that altered *OrMKK3* function results in reduced grain length and width. The spikelet hulls of Y003 plants before fertilization were shorter than those of Nipponbare plants. OE-line plants, in which the *OrMKK3* coding sequences were constitutively overexpressed, formed grains that were significantly shorter than those formed by the Nipponbare plants (Fig. 3a, d). We also investigated the 1000-grain weight of Nipponbare and OE-lines. The 1000-grain weight was lower for OE-lines than for Nipponbare lines (Fig. 3c). In addition, histocytological analyses of outer surfaces of husk from OE-lines and Nipponbare revealed that *OrMKK3* likely regulates grain size

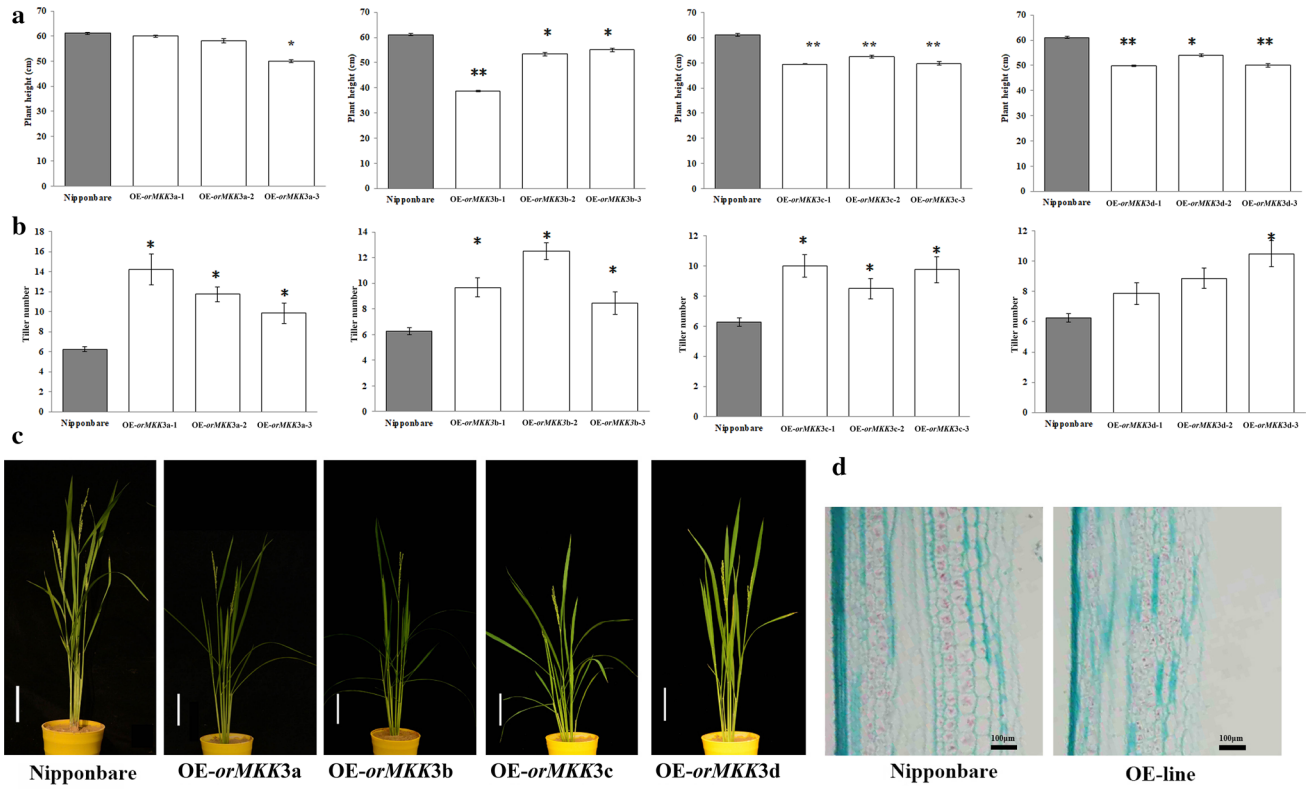


Fig. 2 **a** Plant height of Nipponbare and OE-OrMKK3-lines. **b** Number of tillers of Nipponbare and OE-*OrMKK3*-line. **c** Nipponbare and OE-*OrMKK3*-line plant at the full heading stage. Bar = 10 cm. **d** Histocytological analysis on the internal structure of internodes by paraffin section. Cell growth is inhibited in the OE-OrMKK3-line. Bar = 100 μm

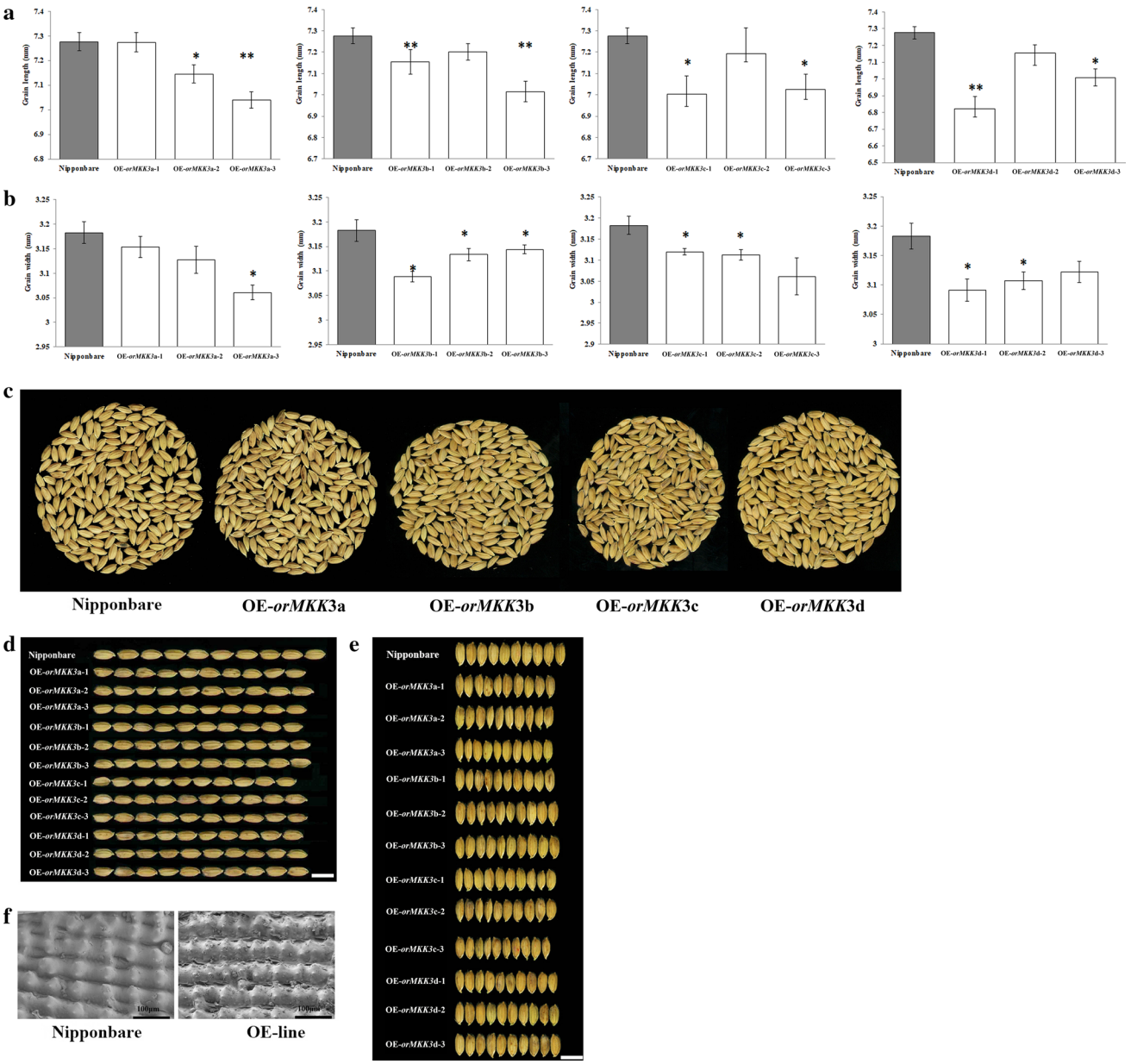


Fig. 3 **a** Grain length of Nipponbare and OE-OrMKK3-lines. **b** Grain width of Nipponbare and OE-OrMKK3-lines. **c** Two hundred seeds of Nipponbare and OE-OrMKK3-lines. **d** Grain length of ten seeds of Nipponbare and OE-OrMKK3-lines. **e** Grain width of ten seeds of Nipponbare and OE-OrMKK3-lines. **f** Scanning electron microscope images of lemmas of Nipponbare and OE-OrMKK3-line

by altering cell numbers. Cell length in either the palea or lemma had little impact on grain size, while total cell numbers of outer and inner epidermal cells in the longitudinal direction in the OE-lines were greater than in Nipponbare (Fig. 3f). These observations suggest that *OrMKK3* may inhibit longitudinal growth by increasing cell proliferation. These results indicate that *OrMKK3* plays a significant role during the full flowering stage by influencing genes regulating grain size and affecting grain production.

OrMKK3 Expression Pattern and Subcellular Localization

OrMKK3 transcripts were detected in various tissues using qRT-PCR with wild-type Y003 plants to determine the spatial expression profile of *OrMKK3*. qRT-PCR analysis showed that *OrMKK3* was expressed ubiquitously in all examined tissues and organs, including the roots, leaves, stems, internodes, and different developmental

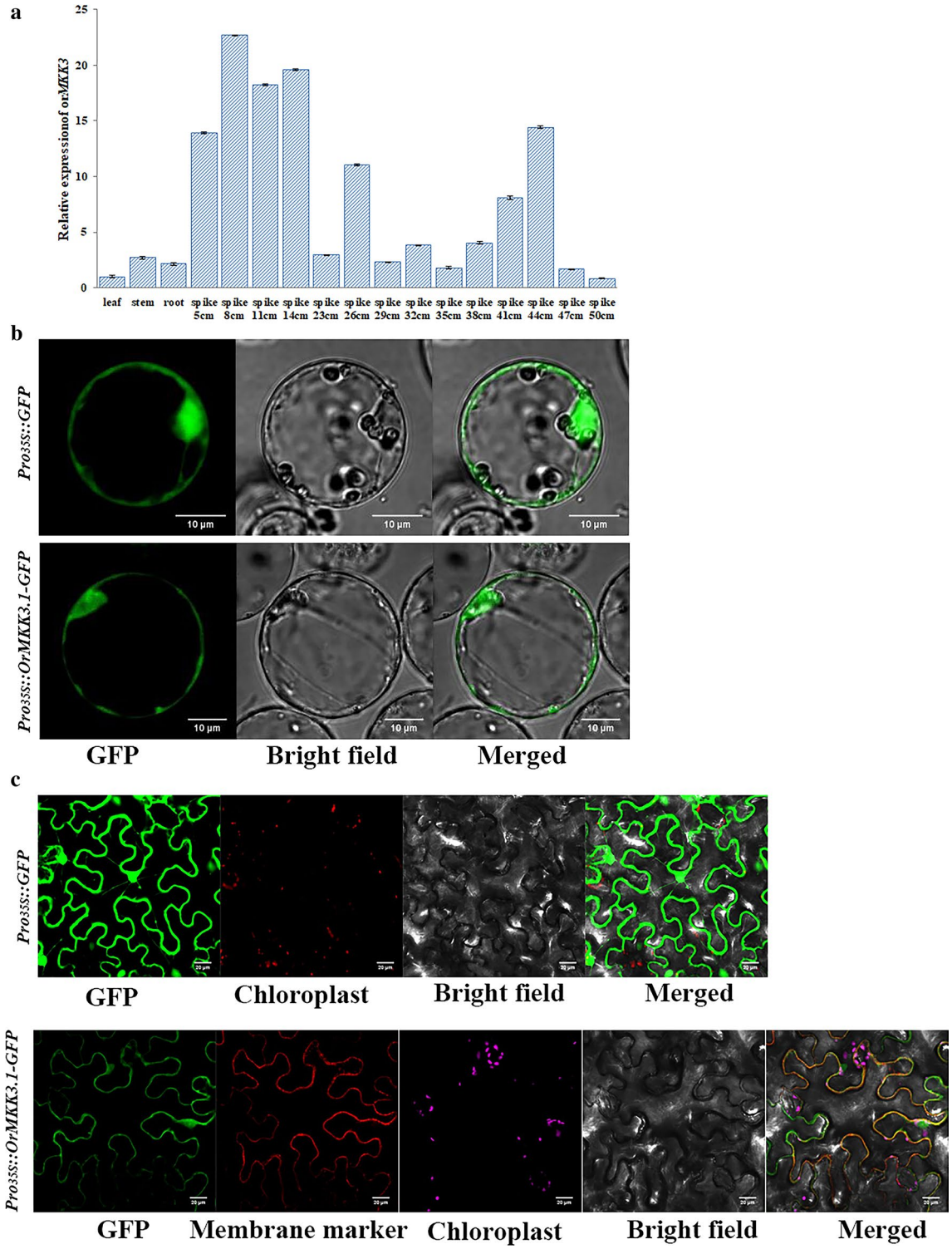


Fig. 4 **a** *OrMKK3* expression in different tissues as analysis by qRT-PCR in Y003 line of *O. officinalis* in Guangxi Province. The *OrMKK3* expression in leaf as 1. **b** Subcellular localization of the *OrMKK3* Protein. Pro35S::GFP as control. Scale bar, 20 mm. **c** Co-localization of 35S: *OrMKK3.1*-GFP with the plasma lemma marker in tobacco epidermal cells. *OrMKK3.1*-GFP overlapped with marker at the plasma membrane. Scale bar, 20 mm

stages of panicles (primarily in the rachillae), lemmas, paleae, lodicules, stamens, and pistils (Fig. 4a). Results also revealed that *OrMKK3* expression is greater in leaves and young panicles than those in other tissues or organs, with *OrMKK3* expression levels gradually increasing during panicle formation.

Since *OrMKK3* contains a kinase domain, we speculated that *OrMKK3* is localized to the plasma membrane. We expressed an *OrMKK3.1*-GFP fusion protein under the control of the 35S promoter in rice leaf protoplasts to determine the subcellular localization of *OrMKK3*. As shown in Fig. 4b, GFP fluorescence from expression of the 35S:*OrMKK3.1*-GFP transgene in rice protoplasts was observed exclusively at the cell membrane and nuclei of rice protoplasts (Fig. 4b) and GFP signal was detected at the cell membrane in tobacco epidermal cells (Fig. 4c). These results indicate that *OrMKK3* encodes a both membrane-localized and nuclear protein.

OrMKK3 Expression Decreases Cell Proliferation

Cell proliferation and expansion significantly influence for organ size (Horiguchi et al. 2006), thus to understand how *OrMKK3* affects grains during panicle growth and height development, we examined cell number and size. Histocytological analysis of stem slices and husks were performed with *OrMKK3* overexpression and Nipponbare lines. The average length of inner stem cells and outer husk cells of the OE-lines and Nipponbare plants were indistinguishable. This result indicates that *OrMKK3* influences cell proliferation rather than cell expansion. Furthermore, we investigated the expression levels of *GL3*, GRAIN WIDTH2 (*GW2*), GRAIN WIDTH8 (*GW8*), *SMALL GRAIN 1* (*SMG1*), and DENSE AND ERECT PANICLE 3 (*DEP3*), which influences grain size by affecting cell proliferation in young panicles (Table S2) (Yan et al. 2011; Qiao et al. 2011; Wang et al. 2012; Chen et al. 2019; Gao et al. 2019). As shown in Fig. 5, *SMG1* expression was not markedly affected by overexpression of *OrMKK3* (Fig. 5a). Compared to Nipponbare plants, expression levels of *GL3* and *DEP3* in 5–10-cm panicles were lower in OE-lines (Fig. 5c, e). Expression levels of *GW2* in OE-lines were initially upregulated in 5–10-cm panicles then decreased in 10–15-cm panicles (Fig. 5d). In young panicles, the expression levels of *GW8* were significantly reduced in OE-lines and Nipponbare in 10-cm panicles than in 5-cm panicles. These decreases in *GL3* and *DEP3* expression levels are probably due to the physiological and biochemical activities involved in rice husk shortening. These results indicate that overexpression of *OrMKK3* likely inhibits the development of plant cells.

MKK3 Phylogenetic Analysis

OrMKK3 encodes mitogen-activated protein kinase kinase 3, with *OrMKK3a* resulting in production of a mutant protein while *OrMKK3*b, *OrMKK3*c, and *OrMKK3*d each contain frameshift mutations. These results thus indicate that *OrMKK3.2*, *OrMKK3.3* and *OrMKK3.4* are loss-of-function alleles. We performed phylogenetic analyses using protein sequences of *OrMKK3* homologs to examine evolutionary relationships. These homolog sequences were obtained in species including *Phoenix dactylifera*, *Elaeis guineensis*, *Ananas comosus*, *Corchorus olitorius*, *Musa acuminata* subsp. *malaccensis*, *Corchorus capsularis*, *Gossypium*, and *Hibiscus syriacus*. The 73 protein homologs were divided into 9 clades. In protein phylogenetic trees, homologs from *O. officinalis*, *O. indica*, *O. japonica*, *Aegilops tauschii*, *Brachypodium distachyon*, *O. brachyantha*, and *O. meyeriana car. granulata* were clustered together with *Triticum aestivum* and *Hordeum vulgare* (Fig. 6a). This indicates a high level of conserved synteny of *MKK3* among multiple plant species. Moreover, these phylogenetic relationships of *MKK3* homologs were based on 446 SNPs from wild rice (*O. rufipogon*) and involved 777 varieties from the world rice core collection. These results indicate that *MKK3* sequences are diverse across the range of *indica*, *japonica*, and wild-rice varieties. Our phylogenetic trees were divided into five clades. OR-I arose from wild-rice varieties in Guangxi, Yunnan, and Guizhou Provinces while both OR-I and OR-II were widely distributed in east and southeast Asia (Fig. 6b).

OrMKK3 OE-Lines Show Altered Responses to BR

OE-lines and Nipponbare plants were treated with homobrassinolide (homo-BL) to identify potential other BR-related *OrMKK3* functions. In germination assays, when treated with 1 μM of homo-BL (a bioactive BR compound), the OE-lines showed larger leaf lamina joint inclination than Nipponbare, indicating enhanced BR sensitivity. In ½ MS medium lacking homo-BL, the OE-line and Nipponbare did not show large leaf lamina joints (Fig. 7a, b). Using qRT-PCR, we compared the expression levels of BR signaling genes including BRASSINOSTEROID INSENSITIVE1 (*OsBRI1*), glycogen synthase kinase 2 (*OsGSK2*), BRASSINAZOLE-RESISTANT1 (*OsBZR1*), BRASSINOSTEROID UPREGULATED1 (*BU1*), and DWARF AND LOW-TILLERING (*DLT*) (Yamamuro et al. 2000; Bai et al. 2007; Tanaka et al. 2009; Zhang et al. 2009; Tong et al. 2009). Expression levels of *OsBZR1* and *BU1* that positively regulate BR signaling were downregulated in OE-lines compared to in Nipponbare (Fig. 7c). These results supported the hypothesis that *OrMKK3* regulates BR biosynthetic genes in rice.

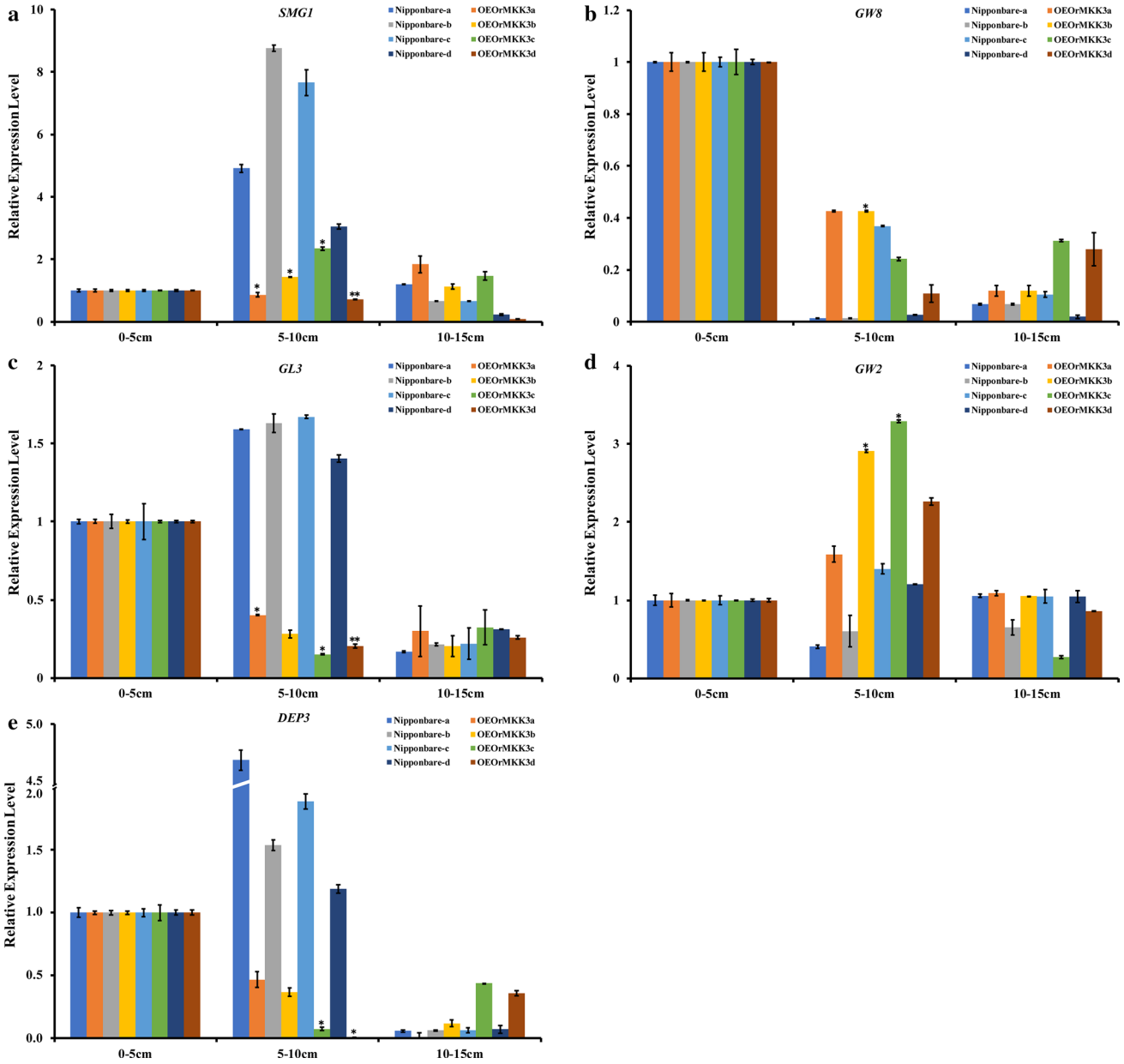


Fig. 5 Relative expression levels of *SMG1*, *GW8*, *GL3*, *GW2* and *DEP3* in Nipponbare and OE-OrMKK3-line young panicles. **a** Relative expression levels of *SMG1* in Nipponbare and OE-OrMKK3-line young panicles. **b** Relative expression levels of *GW8* in Nipponbare and OE-OrMKK3-line young panicles. **c** Relative expression levels of *GL3* in Nipponbare and OE-OrMKK3-line young panicles. **d** Relative expression levels of *GW2* in Nipponbare and OE-OrMKK3-line young panicles. **e** Relative expression levels of *DEP3* in Nipponbare and OE-OrMKK3-line young panicles. * and ** indicate a 5% and 1% significance level, respectively, according to the *t* test. Relative expression levels of genes in 0–5 cm spike as 1, respectively

Discussion

Oryza officinalis Wall. ex Watt is an agriculturally important but seriously endangered species of wild rice. In addition, wild-rice varieties contain abundant genetic diversity and carry many desirable traits, which have proven to be natural genetic reservoirs for the improvement of rice quality and yield. However, comprehensive genetic backgrounds of different *Oryza* species have been a challenge to work with for rice breeders. Fortunately, this has begun to change, and a series of studies utilizing molecular technology have focused on exploiting important genes.

A transformation-competent artificial chromosome clone harboring a NAM-ATAF1/2-CUC (NAC)-positive genomic fragment was selected based on the presence of a conserved sequences of plant stress-related NAC transcription factors

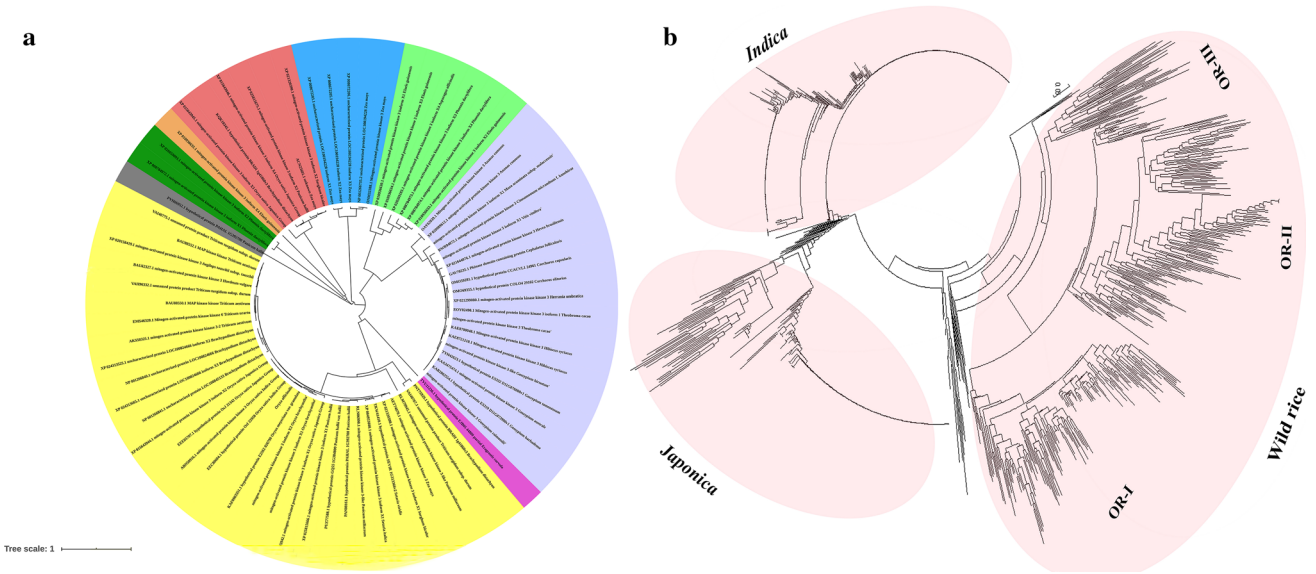
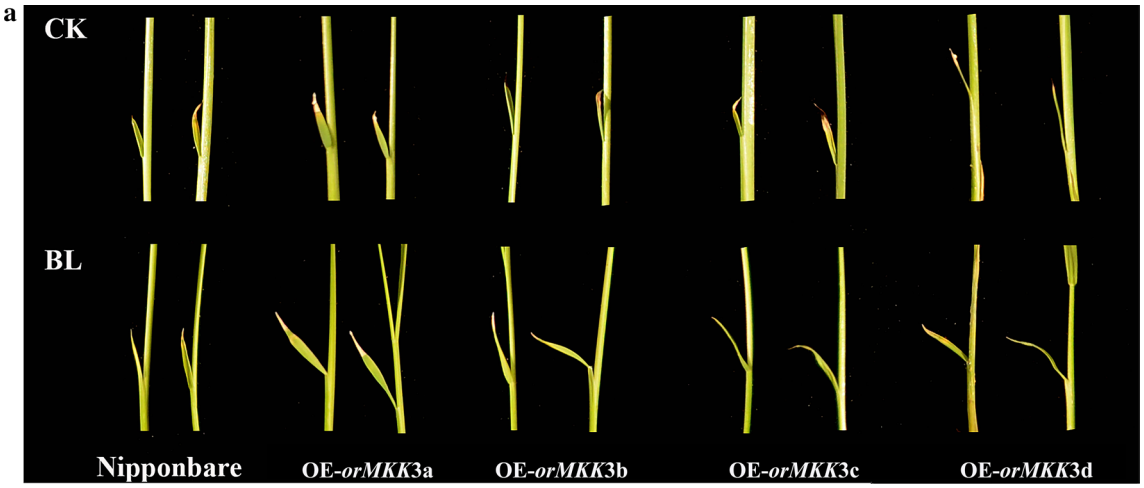


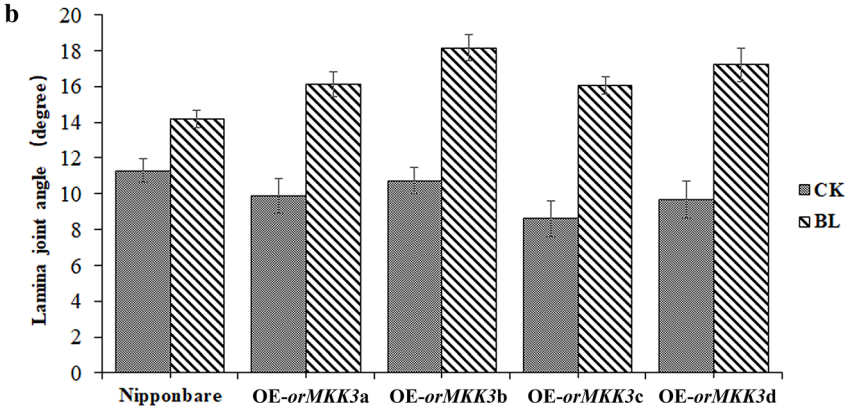
Fig. 6 **a** Phylogenetic tree of the 73 MKK3 proteins in different species. The phylogenetic tree constructed based on different sequences or indels by MAGE 6.0. Different colors represented different clades. **b** The phylogenetic tree of in 446 wild rice (*O. rufipogon*) and 777 varieties from the world rice core collection. The phylogenetic tree constructed based on different functional SNPs or indels by MAGE 6.0. All varieties were categorized by allelic variations in the SNP of *OsMKK3*

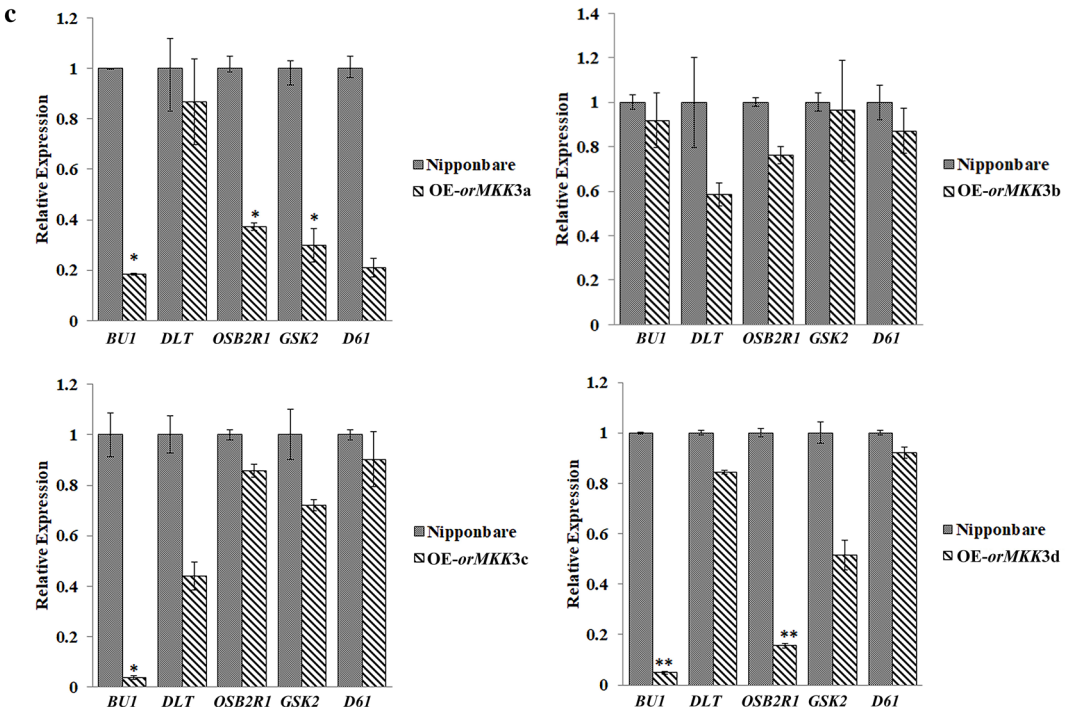
from *O. officinalis* and integrated into the genome of HJX74 (an *indica* rice variety). The resulting transgenic lines exhibited high tolerance to drought stress (Liu et al. 2015). In addition, generation-sequencing methods have been used on *O. officinalis* Wall. ex Watt. A previous study analyzed the leaf transcriptome of *O. officinalis* [CC] using RNA-Seq and de novo assembly of 68,132 unigenes on the basis of approximately 23 million transcriptome reads and identified a total of 476 unigenes related to disease resistance were identified in *O. officinalis* (Bao et al. 2015). In this study, we report that *OrMKK3* in *O. officinalis* Wall. ex Watt exhibits alternative splicing. *OrMKK3* showed multiple types of alternative splicing and amino acid changes compared to Nipponbare.

Height, tiller, and grain size in crops are important agronomic traits. Several factors that influence height and tiller have been described, including small grain and *dwarf 2* (Chen et al. 2019), *DTH8* (Wei et al. 2010), and *DTH7* (Gao et al. 2014). Several genes that affect grain size have been identified, including *GS3*, *GL3*, *GS5*, *GW2*, *GW8*, and *SMG1* (Fan et al. 2006; Song et al. 2007; Mao et al. 2010; Li et al. 2011; Wang et al.2012; Qi et al.2012; Duan et al. 2014). Expression levels of *SMG1*, *GW8*, *GL3*, *GW2*, *and DEP3* were significantly between OE-lines and Nipponbare, suggesting that the effect of *OrMKK3* on grain size may require *SMG1*, *GW8*, *GL3*, *GW2*, and *DEP3*. Xu et al. found that the OsMKKK10-OsMKK4-OsMAPK6 signaling pathway positively regulates grain size and weight in rice (Xu et al. 2018). Sequential phosphorylation activates *OsMAPK6* to promote cell proliferation in spikelet hulls, with *OsMAPK6* potentially also phosphorylating transcription factors thereby regulating grain size. Knock-out mutants confirmed that MKK3-MAPK6 activates blue light responses in Arabidopsis (Sethi et al. 2014). *OsSPL16* (*GW8*) encodes an SBP-domain transcription factor that binds directly to the *GW7* promoter then suppresses *GW7* expression to regulate grain width in rice (Hong et al. 2005; Morinaka et al. 2006; Zhang et al. 2012; Fang et al. 2016; Wu et al. 2016; Gao et al. 2019). It will be important to identify and understand relationships between *OrMKK3* and transcription factors in rice and evaluate their effects on grain characteristics. However, few such underlying genes from *O. officinalis* Wall. ex Watt have been identified, and the molecular mechanisms that influence height, tiller, and grain size, including the function of *OrMKK3*, are still unknown in rice.

Like other MAPKs, MKK3 is involved in various plant physiological–biological activities. Most MKK3 homologs contain a protein kinase domain and NUCLEAR TRANSPORT FACTOR2-like domain in their C-terminal regions (Colcombet et al. 2016). In *A. thaliana*, an MKK3-based MAPK module acts upstream of reactive oxygen species production (Itoh et al. 2002) and signaling (Takahashi et al. 2011). In addition, *MKK3* has been reported to close in response to abscisic acid, ABA activation of MPK7 is blocked in mkk3-1 which expressing a constitutively active version of MKK3 (Huang et al. 2012). Moreover, MKK3 acts in negative regulation in darkness and in light-induced MAPK activation during dark–light transition using knock-out mutants (Lee 2015). MKK3–MPK6 is activated by jasmonic acid and plays a key role in the negative regulation of ATMYC2/JIN1 expression (Takahashi et al. 2007).


a
CK
BL
Nipponbare
OE-orMKK3a
OE-orMKK3b
OE-orMKK3c
OE-orMKK3d


b
Lamina joint angle (degree)
0
2
4
6
8
10
12
14
16
18
20
Nipponbare
OE-orMKK3a
OE-orMKK3b
OE-orMKK3c
OE-orMKK3d
CK
BL


c
Relative Expression
BU1
DLT
OSB2R1
GSK2
D61
Nipponbare
OE-orMKK3a
OE-orMKK3b
OE-orMKK3c
OE-orMKK3d

◂**Fig. 7** OE-*OrMKK3*-lines have response sensitivity to brassinolide (BL). **a** Analysis of brassinolide response by the leaf lamina inclination test. **b** Lamina joint angle show different BR sensitivities. Data were collected from seedlings ($n = 30$) for each concentration and are shown as means.SE. $*P < 0.05$; Student's *t* test. **c** Expression levels of *BU1*, *DLT*, *OSB2R1*, *GSK2*, *D61*. Five plants from each line were used, and three replicates were performed. Relative expression levels of genes in WT as 1, respectively. SE. $*P < 0.05$; $**P < 0.01$; Student's *t* test

MKK3 also plays an important role in the ABA-induced MAPK pathway in plant stress signaling (Danquah et al. 2015). MKK3 interacts with MAP kinase kinase kinase 20 (MKKK20) in two non-complementary signaling cascades involved in root cortical microtubule activities (Matton et al. 2018). Studies of wounding, brown planthopper (*N. lugens*; BPH) infestation (Zhou et al. 2019), and methyl jasmonate or salicylic acid signaling pathways and the expression of MAPK and MAP kinase 3 (*MMK3*) revealed that *OsMKK3*-mediated positive regulation of rice resistance to BPH occurs though the phytohormone system (Zhou et al. 2019).

BRs are steroid hormones that regulate fundamental processes of plant growth and development in plants. In *rice,* BR-deficient mutants (brd2) have small seeds (Hong et al. 2005). This study demonstrated that OE-*OrMKK3* lines have larger leaf lamina joint inclination than Nipponbare after BL treatment. In addition, the expression levels of genes related to BR signaling were reduced significantly in OE-*OrMKK3* lines compared to Nipponbare. These results thus indicate a possible link between MAPK cascades and BR signaling. In *Arabidopsis*, BR inhibits stomatal development by suppressing glycogen synthase kinase 3-like kinase BR-insensitive 2 (BIN2) activity and phosphorylating YDA to inhibit phosphorylation of the YDA substrate MKK4 that mediates MAPK kinase kinase (MAPKKK) signaling (Kim et al. 2012). In addition, *SMG1* may link MAPK pathways and BRs in grain growth, with *OsMKK4*/*SMG1* influencing BR responses and the expression levels of BR-related genes (Duan et al. 2014). In addition, the receptor-like cytoplasmic kinase BRASSINOSTEROID-SIGNALING KINASE1 interacts with and phosphorylates MAPK kinase kinase5 (MAPKKK5); however, MKK3 and MAPKKK5 were not able to form a protein complex in *Arabidopsis* based on detection using an anti-Cluc antibody test (Yan et al. 2018). *GL3.3* encodes OsPPKL that is similar to *Arabidopsis* BSU1, which acts in BR signal transduction to promote cell elongation and cell division (Zhang et al. 2012). Gao revealed that qGL3 improves OsGSK3 via dephosphorylation, and that OsGSK3 regulates OsBZR1 phosphorylation and subcellular distribution (Gao et al. 2019). In addition, in rice, 14–3-3 proteins were shown to interact with OsBZR1 using a yeast two-hybrid screen (Bai et al. 2007). These results suggest that BR regulates grain size by promoting seed growth (Morinaka et al. 2006; Wu et al. 2016). In addition, in mice, protein serine/threonine kinase 38-mediated dissociation of 14–3-3 from the ASK1-14–3-3 complex and the increase of interaction between ASK1 and its substrate MKK3 (Ha et al. 2008). EmMKK2 from the fox tapeworm *Echinococcus multilocularis* closely resembles members of the MKK3/6- and the MEK1/2-MAPKK sub-families that interact with 14–3-3 protein family members within the fox tapeworm (Gelmedin et al. 2010). Collectively, these findings suggest that *OrMKK3* regulates plant height, tiller, grain size, 1000-grain weight, and BR responses, representing a possible link between the MAPK cascades and BRs in cell growth. However, further studies are needed to identify the different roles of *OrMKK3* in BR signaling and seed size control in rice.

Materials and methods

Plant materials, growth conditions, and phenotyping

Wild-type rice plants (*O. officinalis* Wall. ex Watt and *O. sativa* L. spp. *japonica*) and four *OrMKK3* overexpression lines (OE-*OrMKK3*a, OE-*OrMKK3*b, OE-*OrMKK3*c, and OE-*OrMKK3*d) were used in this study. Different tissues, including mature leaves, stems, and roots of *O. officinalis* Wall. ex Watt Y003 and *O. japonica*, were collected and planted in the National Germplasm Nanning Wild Rice Nursery of China. Transgenic lines selected by hygromycin resistance and qRT-PCR after two generations of propagation were cultivated in greenhouses under 16-h light/8-h dark. Three individual overexpression transgenic lines (OE-lines) of OE-*OrMKK3*a, OE-*OrMKK3*b, OE-*OrMKK3*c, and OE-*OrMKK3*d genotypes at the T3 stage were used for further analysis. The overexpression transgenic lines are different transformed cases with ten plants of OE-*OrMKK3*a, OE-*OrMKK3*b, OE-*OrMKK3*c, and OE-*OrMKK3*d.

RNA Extraction and qRT-PCR

Total RNA from the above listed tissues were extracted using a Takara minibest plant RNA extraction kit according to the user's manual (Takara, Catalog no. 9769). Total RNA (2.0 μg) was used for cDNA synthesis with a primeScript™ RT reagent kit with gDNA Eraser (Perfect Real-Time, Takara, Catalog no. RR047B). We performed qRT-PCR using TB Green® Premix Ex Taq™ II and a CFX96 Real-Time system (Bio-RAD, Hercules, CA, USA) following the manufacturer's instructions. qRT-PCR was performed using 10-μL mixtures: 5 μL of 2 × Green qPCR MasterMix, 1 μL of cDNA, 0.25 μL of each primer (10 μM), and 3.5-μL ddH_2O. Amplification steps were 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. 65 °C for 5 s, 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Each experiment

was repeated at least three times. The qRT-PCR analysis was performed using the ΔΔCt method. Details on primers used for detecting the *OrMKK3* and *OsUbiquitin* genes (*LOC_Os03g13170*) (Chen et al. 2019) and probes are provided in Table S1 (*$P < 0.05$; ** $P < 0.01$; Student's *t* test).

Plasmid Construction and Plant Transformation

OrMKK3.1, *OrMKK3.2*, *OrMKK3.3*, *OrMKK3.4*, and *OrMKK3.5* cDNA sequences from *O. officinalis* Wall. ex Watt Y003 were amplified using primers OrMKK3-F and OrMKK3-R (Table S1) and products were subcloned into the pEASY®-Blunt Zero cloning vector (Transgen, Catalog no. CB501-01). Next, this cDNA fragment (*OrMKK3.1–OrMKK3.4*) was cloned into the plant binary vector PMDC32 containing the CaMV 35S promoter. Subsequently, the *OrMKK3.1* cDNA fragment was subcloned into the Gateway binary vector PMDC83 containing the CaMV 35S promoter and GFP and digested with PacI-AscI to generate a 35S::*OrMKK3.1* construct. All constructs were confirmed by sequencing. 35S::*OrMKK3.1*, 35S::*OrMKK3.2*, 35S::*OrMKK3.3*, and 35S::*OrMKK3.4* plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105 by transformation, and the plasmids transformed into Nipponbare in accordance with a previously published protocol (Hiei et al. 1994).

Phylogenetic Analysis

The DNA or deduced amino acid sequences of *OrMKK3* homologs were BLAST searched against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed with MEGA6 using the neighbor-joining method (Tamura et al. 2013). An alignment of rice *MKK3* sequences was constructed using DNAMAN (LynnonBiosoft, V7.0). The SNPs of 777 cultivated rice accessions from 32 countries and 446 wild-rice accessions with publicly available sequencing data from the rice 3 K project (RFGB, http://www.rmbreeding.cn/Index/s) and OryzaGenome (http://viewer.shigen.info/oryzagenome/) were used for phylogenetic analysis (Huang et al. 2012).

BR Treatment

Seeds were surface sterilized by incubation in 20% NaClO for 20 min, washed five times with sterilized water, then germinated in ½ MS medium (hopebio, HB8469-12, China) with 1-μM homo-BL (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) for 10 days in a growth cabinet. Ten-day-old seedlings were used to measure leaf lamina joint inclination.

Subcellular Localization of *OrMKK3*

The plasmids CaMV35S::GFP and CaMV35S::*OrMKK3*-GFP were transformed into rice leaf protoplasts as previously described (Bart et al. 2006). After overnight incubation at 25 °C for 24 h in the dark, GFP was detected using a confocal laser-scanning microscope with excitation at 488 nm (Nikon C2-ER). Excitation of the plasma membrane co-localization red fluorescent protein marker mCherry was done at 580 nm and emission light was collected at 610 nm.

Conclusions

OrMKK3 is a negative regulator of plant height and grain shape, a positive regulator of tiller number, and a possible part of the BR signaling pathways. These findings provide information about a new gene and the crucial role of MAPKs in plant growth.

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Data Availability Statement Relevant data are within the paper including CDS of *OrMKK3*.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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