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Research

Natural variation in a molybdate transporter controls grain molybdenum concentration in rice

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Summary

- Molybdenum (Mo) is an essential micronutrient for most living organisms, including humans. Cereals such as rice (*Oryza sativa*) are the major dietary source of Mo. However, little is known about the genetic basis of the variation in Mo content in rice grain.
- We mapped a quantitative trait locus (QTL) *qGMo8* that controls Mo accumulation in rice grain by using a recombinant inbred line population and a backcross introgression line population.
- We identified a molybdate transporter, *OsMOT1;1*, as the causal gene for this QTL. OsMOT1;1 exhibits transport activity for molybdate, but not sulfate, when heterogeneously expressed in yeast cells. *OsMOT1;1* is mainly expressed in roots and is involved in the uptake and translocation of molybdate under molybdate-limited condition. Knockdown of *OsMOT1;1* results in less Mo being translocated to shoots, lower Mo concentration in grains and higher sensitivity to Mo deficiency. We reveal that the natural variation of Mo concentration in rice grains is attributed to the variable expression of *OsMOT1;1* due to sequence variation in its promoter.
- Identification of natural allelic variation in *OsMOT1;1* may facilitate the development of rice varieties with Mo-enriched grain for dietary needs and improve Mo nutrition of rice on Mo-deficient soils.

Introduction

As one of the most important staple crops, rice (*Oryza sativa*) not only provides more than one-fifth of daily calories for half of the world's human population but is also a major source of mineral nutrients such as molybdenum (Mo), an essential micronutrient for almost all living organisms. In humans, Mo is required for the biosynthesis of Mo cofactor (Moco), which forms the active site of Mo-requiring enzymes (molybdoenzymes), including aldehyde oxidase, xanthine dehydrogenase, sulfite oxidase and amidoxime reducing component (Schwarz & Mendel, 2006). These enzymes participate in crucial processes such as purine metabolism and sulfite detoxification and play a vital role in maintaining human health (Schwarz & Mendel, 2006). Deficiency of Moco biosynthesis in humans results in the decrease of molybdoenzyme activity, which leads to inheritable progressive neurological damage and even early childhood death (Johnson *et al.*, 1980; Schwarz, 2005). Although Mo deficiency in humans is extremely rare, food crops grown on soils that are freely drained, acidic and rich in iron oxides are known to have lower Mo concentrations (Marschner & Rengel, 2012).

Mo is also an essential micronutrient for plants. Plants take up Mo mainly as molybdate. Molybdate itself is biologically inactive and must be incorporated into tricyclic pterin to form Moco. Moco serves as electron donors and/or acceptors in molybdoenzymes and plays key roles in the assimilation and biogeochemical cycles of carbon, nitrogen (N), and sulfur (S) (Schwarz & Mendel, 2006; Bittner, 2014). Although Mo deficiency in humans is rare, Mo deficiency in crops is becoming an agricultural problem, especially for crops grown on acid soils. In acid soils, the high levels of reactive iron oxides/hydroxides are known to have a strong adsorption of molybdate which deceases the bioavailability of Mo (Marschner & Rengel, 2012). It is estimated that up to 70% of the world's arable land is characterized as acidic; Mo deficiency is thus a widespread agricultural concern (von Uexküll & Mutert, 1995). The deficiency of Mo in soils has been shown to inhibit plant growth and agricultural productivity (Kaiser *et al.*, 2005). Plants suffering from Mo deficiency develop

the typical 'whiptail' phenotypes, which includes mottled lesions on the leaves, rolling of leaves and wilting of leaf edges (Arnon & Stout, 1939).

Living organisms that require Mo to synthesize molybdoenzymes take up Mo from the environment in an energy-dependent process. In *Escherichia coli*, Mo is taken up by a high-affinity ABC-type transport system. This system is encoded by *modABC* genes and composed of a periplasmic molybdate-binding protein (ModA), a membrane channel protein (ModB), and an energy-transducing ATPase protein (ModC) (Grunden & Shanmugam, 1997; Hollenstein *et al.*, 2007). A large number of ABC transporter genes are present in the genome of eukaryotes; however, none of the ABC-type Mo-specific transporters have been identified in eukaryotes (Kaiser *et al.*, 2005). The first eukaryotic high-affinity molybdate transporter encoded by *MoT1* (*CrMOT1*) was identified in the green alga *Chlamydomonas reinhardtii* (Tejada-Jiménez *et al.*, 2007). Knockdown of *CrMOT1* by an antisense RNA strategy inhibited the molybdate transport activity and the activity of the Mo-containing enzyme nitrate reductase, indicating a function of CrMOT1 in molybdate transport (Tejada-Jiménez *et al.*, 2007). A high-affinity molybdate transporter AtMOT1;1 (also named as MOT1) that shows sequence similarity to CrMOT1 was also identified in *Arabidopsis thaliana* (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008). *AtMOT1;1* belongs to group V of the sulfate transporter superfamily and was previously named as *Sultr5;2* (Tejada-Jiménez *et al.*, 2013). Knockout of *AtMOT1;1* led to decreased accumulation of Mo in both roots and shoots, and the *atmot1;1* mutant showed Mo-deficiency symptoms when grown under limited Mo supply conditions, suggesting an essential role of *AtMOT1;1* in uptake of Mo from soil in *A. thaliana* (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008). Another member of group V of the sulfate transporter superfamily, *AtMOT1;2* (*Sultr5;1*; previously named *MOT2*), was also identified in *A. thaliana*, which localizes to the tonoplast and functioned in vacuolar molybdate export (Gasber *et al.*, 2011). Recently, *LjMOT1* was isolated from *Lotus japonicus*, which is essential for *L. japonicus* to take up Mo from the soil (Gao *et al.*, 2016; Duan *et al.*, 2017). Very recently, *MtMOT1.3* was identified in the model legume *Medicago truncatula*, which is required for nitrogenase activity in root nodules (Tejada-Jiménez *et al.*, 2017). In *C. reinhardtii*, there is another molybdate transporter, CrMOT2, which shows no sequence similarity to CrMOT1 and thus is not related to the MOT1 family (Tejada-Jiménez *et al.*, 2011). CrMOT2 is also a high-affinity molybdate transporter and mainly functions under the molybdate-deficient conditions (Tejada-Jiménez *et al.*, 2011). To date, molybdate transporters that control Mo concentration have not been characterized in staple food crops.

In this study, we identify the quantitative trait locus (QTL) *qGMo8* that controls the variation in grain Mo concentration in rice. We show that *qGMo8* encodes a molybdate transporter OsMOT1;1. *OsMOT1;1* is mainly expressed in roots and exhibits molybdate transport activity when heterogeneously expressed in yeast cells. Loss of function of *OsMOT1;1* results in decreased Mo translocation from roots to shoots, lower Mo concentration in grains and higher sensitivity to Mo deficiency. We further reveal that the natural variation of Mo concentration in rice grains is attributed to the variable expression of *OsMOT1;1* in roots.

Materials and Methods

Plant materials and growth conditions

The rice (*O. sativa* L.) recombinant inbred lines derived from a cross between 'Lemont' (LM, *japonica*) and 'TeQing' (TQ, *indica*) (LT-RILs), and 'TeQing'-into-'Lemont' backcross introgression lines (TILs) were described previously (Tabien *et al.*, 2000; Pinson *et al.*, 2012; Huang *et al.*, 2016b). The heterogeneous inbred families (HIFs) of *OsMOT1;1* locus were generated as previously described (Tuinstra *et al.*, 1997; Loudet *et al.*, 2005). A PCR marker was developed based on the 222 bp deletion in the promoter of *OsMOT1;1* in TQ and was used for genotyping the TILs. The line TIL669.4 was determined to be heterozygous at the *OsMOT1;1* locus. Plants fixed with TQ allele (HIF669.4-TQ) and plants fixed with LM allele (HIF669.4-LM) were identified in the next generation of TIL669.4, resulting in HIFs for comparing phenotypic effects of the *OsMOT1;1* alleles from TQ and LM. The transfer DNA (T-DNA) insertion mutant for *OsMOT1;1* which is in 'Zhonghua 11' background was obtained from Huazhong Agricultural University, China (http://rmd.ncpgr.cn). The segregated plants without T-DNA insertion in *OsMOT1;1* gene were used as a wild-type (WT) control. The WT and T-DNA seeds were planted into soil in a glasshouse at the University of Aberdeen, UK, or hydroponically grown in Nanjing Agricultural University, China. The *A. thaliana* T-DNA insertion mutant for *AtMOT1;1* (SALK_118311) was obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc/).

The growth of LT-RIL and TIL populations in the field in Texas, USA, and the growth of the TIL population in a glasshouse in Purdue University were described previously (Zhang *et al.*, 2014; Huang *et al.*, 2016b). For analysis of the grains and different tissues of the WT and *osmot1;1*, plants were grown in soil in a glasshouse at the University of Aberdeen, UK, as described previously (Huang *et al.*, 2016b). For the hydroponic experiment, WT and *osmot1;1* plants were grown in 96-well plates with the bottom removed. The plates were put in tip boxes containing half-strength Kimura B solution with different concentrations of Mo. The growth condition was described previously (Huang *et al.*, 2016b). *A. thaliana* transgenic plants were grown on MGRL agar media in a growth chamber at the University of Aberdeen, UK, as described (Huang *et al.*, 2016a).

QTL analysis and fine mapping of *qGMo8*

The QTL mapping has been performed previously by using both multiple interval mapping and Bayesian information criterion methods based on the least-squares means of the 5 yr replications for the grain Mo of LT-RIL and three replications of TIL (Zhang *et al.*, 2014). We performed new QTL analyses based on the individual year data under flooded or unflooded field conditions

using Windows QTL Cartographer v.2.5 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) with a composite interval mapping method according to previous studies (Huang *et al.*, 2016b). Seven markers were developed in the *qGMo8* mapping interval and used to genotype the entire population of 123 TILs. Three representative TILs with different genotypes in the *qGMo8* mapping interval and two control TILs were chosen for further replicated phenotypic analyses and fine mapping. By integration of the genotypes and grain Mo concentrations of these lines, the *qGMo8* was fine mapped to a 522 kb region on the top of chromosome 8. The primer sequences are listed in Supporting Information Table S1.

Tissue elemental analysis

The elemental concentrations in grains of LT-RILs, TILs, WT and *osmot1;1* were determined using an Elan DRCe or or NexION 300D inductively coupled plasma mass spectrometer (ICP-MS) (PerkinElmer Corp., Norwalk, CT, USA) according to previous studies (Zhang *et al.*, 2014; Huang *et al.*, 2016b). For determination of elemental concentrations in different organs of WT and *osmot1;1* grown in soil, tissues were excised and washed with Milli-Q water and dried at 88°C overnight. For analysis of roots of WT and *osmot1;1* grown hydroponically, roots were excised and washed with 0.5 mM calcium chloride solution three times and rinsed with Milli-Q water once and then dried at 88°C overnight. Samples were digested with concentrated nitric acid (HNO_3) at 118°C for 4 h. The elemental concentrations in the digested samples were determined using an ICP-MS (Huang *et al.*, 2016b). The Mo concentrations in shoots and roots of transgenic *A. thaliana* plants were determined according to previous studies (Huang *et al.*, 2016a).

Genetic and transgenic complementation test

For genetic complementation, *osmot1;1* and WT T-DNA progeny were crossed with HIF669.4-TQ and HIF669.4-LM, respectively. The concentrations of Mo in grains of F1 plants from each cross were then determined. For transgenic complementation, the full-length coding sequence of *OsMOT1;1* was PCR amplified using the complementary DNA of TQ or LM as templates. The correct PCR fragments confirmed by sequencing were ligated into the *Sal*I-*Spe*I site of p1301GFP vector (Huang *et al.*, 2009) to generate the *35S:OsMOT1;1(TQ)-GFP* and *35S:OsMOT1;1(LM)-GFP* constructs. The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain HA105 and introduced into rice *japonica* cv Zhonghua 11 as described previously (Hiei *et al.*, 1994). *osmot1;1* was crossed with two transgenic *OsMOT1;1* overexpression lines: *35S:OsMOT1;1(TQ)-GFP* and *35S:OsMOT1;1(LM)-GFP*. Two independent transgenic lines of *35S:OsMOT1;1(TQ)-GFP* and *35S:OsMOT1;1(LM)-GFP* were used for crossing. In subsequent F_2 populations, plants containing the transgene in homozygous *osmot1;1* mutant background were identified by genotyping. Plants without the transgene in WT or homozygous *osmot1;1* mutant background were used as controls. The grain Mo concentrations in grains from these F_2 plants were determined using an ICP-MS. The primer sequences using for genotyping are listed in Table S1.

Expressing *OsMOT1;1* in an *A. thaliana atmot1;1* mutant

OsMOT1;1 was expressed in an *A. thaliana atmot1;1* T-DNA mutant using the 35S promoter or *AtMOT1;1* native promoter. The construction of *35S:OsMOT1;1(TQ)-GFP* and *35S:OsMOT1;1(LM)-GFP* vectors was as described earlier. To generate the *AtMOT1;1* promoter-driven *OsMOT1;1* expression vectors, the 1777 bp promoter sequence of *AtMOT1;1* was PCR amplified from the genomic DNA of Col-0 using the primers listed in Table S1. The PCR fragment confirmed by sequencing was used to substitute the 35S promoters in the *35S:OsMOT1;1(TQ)-GFP* and *35S:OsMOT1;1(LM)-GFP* vectors to generate *MOT1pro:OsMOT1;1(TQ)-GFP* and *MOT1pro:OsMOT1;1(LM)-GFP* vectors, respectively. These plasmids were also transformed into *A. tumefaciens* strain GV3101 and introduced into *A. thaliana mot1* mutant (SALK_118311) using the floral dip method (Clough & Bent, 1998). To assay low pH sensitivity, homozygous T3 transgenic plants were grown on MGRL medium with Mo omitted. The low-pH media were prepared as described earlier by adding 35 µl 6 M hydrochloric acid (HCl) into 100 ml media after autoclaving. Plants were grown on plates horizontally in a climate-controlled room with temperature of 19–22°C, photoperiod of 10 h : 14 h, light (100 ± 10 µmol m^{-2} s^{-1}) : dark and humidity of 60%. The shoots were weighed after growing for 20 d.

Functional analysis of *OsMOT1;1* in yeast

The molybdate transporting activity of OsMOT1;1 in yeast was determined according to previous studies with modifications (Tomatsu *et al.*, 2007). To generate the plasmids for expression of *OsMOT1;1* in yeast (*Saccharomyces cerevisiae*), the full-length coding sequence of *OsMOT1;1* was PCR amplified from the complementary DNA of TQ or LM using the primers listed in Table S1. The correct PCR fragments confirmed by sequencing were subcloned into the *Eco*RI-*Xho*I site of the yeast expression vector pYX222x (Tomatsu *et al.*, 2007). The expression of *OsMOT1;1* in this plasmid was driven by a constitutive triose phosphate isomerase promoter. The resultant plasmids and empty vector were transformed into yeast strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) using a Frozen-EZ Yeast Transformation II Kit (ZYMO Research, Irvine, CA, USA). For molybdate transporting assay, the BY4741 strains transformed with the empty vector pYX222x, *pYX222x-OsMOT1;1(TQ)* or *pYX222x-OsMOT1;1(LM)* were inoculated at 30°C overnight in 3 ml of Mo-free synthetic defined (SD)/−His media (6.7 g l^{-1} yeast N base without amino acids and without molybdenum (Formedium Ltd, Norfolk, UK), 1.92 g l^{-1} dropout mix without histidine (His), 2% (w/v) glucose). A 100 µl sample of overnight yeast cells was then transferred to 10 ml Mo-free SD/−His media and incubated at 30°C until the optical density at 600 nm (OD_{600}) reached *c.* 1. Hexaammonium heptamolybdate was then added to the media to a final concentration of 0.5 µM. After

shaking at 30°C for 30 min, yeast cells were harvested by centrifugation and then washed three times with 1 mM EDTA disodium salt and once with Milli-Q water. Cells were dried at 80°C overnight, then digested with concentrated HNO_3. The Mo concentration in digested samples was determined using an ICP-MS. The amount of yeast was converted by 1 ml of $OD_{600} = 1$ culture containing 3×10^7 cells (Baxter *et al.*, 2008).

To test the sulfate transporting activity of OsMOT1;1, the empty vector pYX222x, *pYX222x-OsMOT1;1(TQ)* and *pYX222x-OsMOT1;1(LM)* were transformed into a yeast mutant CP154-7B (MATa, his3, leu2, ura3, ade2, trp1, sul1::LEU2, sul2::URA3) as earlier (Tomatsu *et al.*, 2007). An *A. thaliana* high-affinity sulfate transporter gene, *SULTR1;2*, was used as positive control. The overnight yeast cultures were collected by centrifugation and washed once with Milli-Q water. After adjusting the OD_{600} of yeast cultures to 0.5, 10 µl yeast suspensions were spotted on synthetic medium (0.5 mM sulfate, 20 g l^{-1} glucose, 10 g l^{-1} agarose, 20 mg l^{-1} adenine, 30 mg l^{-1} leucine, 20 mg l^{-1} uracil, and 20 mg l^{-1} trypsin) with or without 20 mg l^{-1} methionine (Met). The plates were incubated at 30°C for 6 d.

Other methods can be found in Methods S1.

Results

Map-based cloning of *qGMo8*

We previously used two synthetic rice mapping populations composed of LT-RILs and TILs to identify QTLs that control the variation in concentration of 16 elements in unmilled rice grain (Zhang *et al.*, 2014). A total of 134 QTLs were identified in either one or both mapping populations grown under flooded and/or unflooded field conditions (Zhang *et al.*, 2014). Among eight QTLs that control the variation in grain Mo concentration, one was detected on the top of chromosome 8 (designated as *qGMo8*) in both mapping populations in field trials over multiple years and in both flooded and unflooded fields. This QTL explains up to 35% of the variation in grain Mo (Fig. 1a,b). The

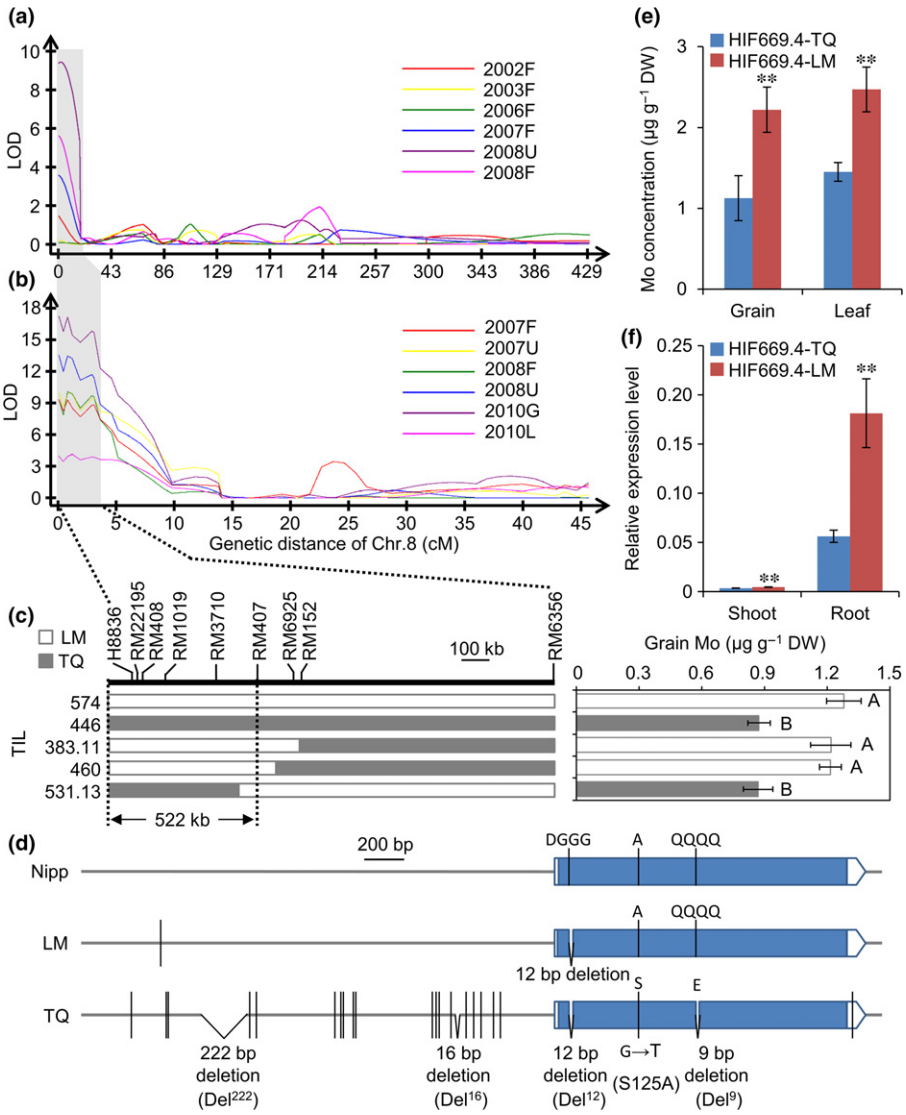

Fig. 1 Quantitative trait locus (QTL) analysis and cloning of *qGMo8* in rice. (a, b) The logarithm of the odds (LOD) profiling of *qGMo8* on chromosome 8 in the 'TeQing'–'Lemont' recombinant inbred line (LT-RIL) population (a) and 'TeQing'-into-'Lemont' backcross introgression line (TIL) population (b) grown in multiple years under different conditions. F, flooded; U, unflooded; G, grains of TILs grown in glasshouse; L, leaves of glasshouse-grown TILs. (c) Grain molybdenum (Mo) concentration and genotype at QTL interval of selected TILs. Data are presented as mean ± SD ($n = 3$). Columns with different letters indicate significant difference at $P \leq 0.01$ (Tukey's honestly significant difference test). (d) Gene structure and sequence variation of *OsMOT1;1* among Nipponbare (Nipp), 'TeQing' (TQ) and 'Lemont' (LM). Blue bars, exons; white bars, untranslated regions; vertical lines, single nucleotide polymorphisms. (e) The Mo concentration in the grain and leaf of HIF669.4-TQ and HIF669.4-LM. (f) Expression level of *OsMOT1;1* in shoots and roots of HIF669.4-TQ and HIF669.4-LM. Data in (e, f) are presented as mean ± SD with (e) $n = 6$ and (f) $n = 3$. Significant differences are indicated (Student's *t*-test): **, $P \leq 0.01$.

qGMo8 first observed in grain from field-grown materials was also detected in both grain and leaf tissues from glasshouse-grown TILs, which allows us to fine map the QTL using plants cultivated in the glasshouse (Fig. 1b).

To narrow down the *qGMo8* mapping interval, we developed additional markers to genotype the TIL population. Integration of grain Mo concentration and genotype data of three TILs narrowed the QTL interval down to a 522 kb region on the top of chromosome 8 (Fig. 1c). In this region, a gene (LOC_Os08g01120) annotated as sulfate transporter encodes a protein that shows 57.8% sequence similarity to *A. thaliana* high-affinity molybdate transporter AtMOT1;1 (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008), which makes it a strong candidate gene for *qGMo8* (Fig. S1a). We thus designated LOC_Os08g01120 as *OsMOT1;1*. Sequence analysis revealed a 9 bp deletion (Del^{9}) and a single nucleotide polymorphism (SNP) in the coding sequence of *OsMOT1;1* in TQ (Figs 1d, S1b). The 9 bp deletion alters the amino acid sequence from four glutamines in LM to a glutamic acid in TQ (Figs 1d, S1b). The SNP in the coding sequence also alters the amino acid sequence, with an alanine in LM and a serine in TQ at the position of 125 (S125A) (Figs 1d, S1b). A 222 bp deletion (Del^{222}) and a 16 bp deletion (Del^{16}) were found in the promoter sequence of *OsMOT1;1* in TQ. Meanwhile, there are 19 SNPs in the promoter sequence of *OsMOT1;1* in TQ. Comparison of *OsMOT1;1* sequences of TQ and LM with Nipponbare reference sequence identified a 12 bp deletion (Del^{12}) in the coding region of *OsMOT1;1* in both TQ and LM, and only an SNP in the promoter sequence of *OsMOT1;1* between LM and Nipponbare (Fig. 1d). Sequence alignment showed that none of the variable amino acids between TQ and LM are conserved in the MOT1 protein family (Fig. S1a), suggesting that these amino acids might not alter the function of OsMOT1;1.

The existence of residual heterozygosity in TILs is useful for developing appropriate nearly isogenic lines by generating HIFs (Tuinstra *et al.*, 1997; Loudet *et al.*, 2005; Huang *et al.*, 2016b). We identified TIL669.4, which is heterozygous at the *OsMOT1;1* locus, and isolated HIF669.4-TQ and HIF669.4-LM in the next generation (Fig. S2). These two HIFs have similar genomic backgrounds and only differ in a small genomic region containing homozygous *OsMOT1;1* alleles from TQ or LM, respectively. Both grain and leaf Mo concentrations of HIF669.4-TQ are significantly lower than that of HIF669.4-LM, suggesting that the TQ allele is a weak allele (Fig. 1e). Quantitative reverse transcription PCR analysis showed that the expression levels of *OsMOT1;1* in both shoots and roots of HIF669.4-TQ were significantly lower than that of HIF669.4-LM, suggesting that the low Mo in HIF669.4-TQ might be due to the lower expression level of *OsMOT1;1* (Fig. 1f).

OsMOT1;1 is the causal gene for *qGMo8*

To investigate the function of *OsMOT1;1*, we isolated a T-DNA insertion mutant of *OsMOT1;1*. The T-DNA insertion at the promoter of *OsMOT1;1* significantly decreases its expression (Fig. S3a–c). The *osmot1;1* mutant has no obvious phenotype difference from the WT when grown in soil in a glasshouse (Fig. 2a). Elemental profile analysis showed that the Mo concentration in the grain of *osmot1;1* is *c.* 58% lower than that of the WT ($P < 0.001$, Student's *t*-test, $n = 10$; Fig. 2b,c). The low-Mo phenotype was also observed in the blades and sheaths of *osmot1;1* (Fig. 2b). Of the 22 elements determined, Mo is the only element that is significantly changed in the grain, leaves and sheaths of *osmot1;1*, suggesting a specific effect of *OsMOT1;1* mutation on Mo concentrations (Fig. 2c). To test whether *OsMOT1;1* is the causal gene for *qGMo8*, we performed an allelic complementation by crossing *osmot1;1* and WT with HIF669.4-TQ and HIF669.4-LM, respectively. The expression level of *OsMOT1;1* in the roots of both HIF669.4-TQ and HIF669.4-LM was higher than in that of *osmot1;1* (Fig. S3e). The Mo concentrations in both leaves and grain of *osmot1;1* × HIF669.4-TQ and *osmot1;1* × HIF669.4-LM F_1 plants grown in soil in a glasshouse were significantly higher than that of *osmot1;1* (Fig. 2d,e), similar to the levels in HIF669.4-TQ and HIF669.4-LM, respectively, suggesting the complementation of *OsMOT1;1* from both TQ and LM to the knockout allele. However, significant differences of both leaf and grain Mo concentrations between *osmot1;1* × HIF669.4-TQ F_1 and *osmot1;1* × HIF669.4-LM F_1 were observed (Fig. 2d,e), indicating differential functional activity of OsMOT1;1 between TQ and LM. To further confirm *OsMOT1;1* as the causal gene, we crossed *osmot1;1* with *OsMOT1;1* overexpression lines in which *OsMOT1;1* from either TQ or LM was expressed from a cauliflower mosaic virus (CaMV) 35S promoter in the cv Zhonghua 11 background. When grown in soil in a glasshouse, the Mo concentrations in grains of F_2 plants containing the transgene in homozygous *osmot1;1* mutant background were significantly higher than those homozygous *osmot1;1* plants without the transgene (Fig. 2e). These results demonstrate that overexpression of *OsMOT1;1* is able to complement the low-Mo phenotype of the *osmot1;1* mutant.

Low-Mo phenotype of *osmot1;1* knockout mutant

At grain maturity stage, the *osmot1;1* mutant grown in soil in the glasshouse has a lower concentration of Mo than WT does not only in the grain, but also in the blade and sheath of the flag leaf, and most of the nodes and internodes of the main tiller (Fig. 3a). Analysis of the seedlings grown hydroponically in the nutrient solution containing 1 nM Mo showed that *osmot1;1* accumulated significantly lower Mo in both roots and shoots (Fig. 3b,c). However, the difference in the root Mo concentration between *osmot1;1* and WT disappeared when plants were grown in the nutrient solution containing 10 nM or higher concentration of Mo (Fig. 3b). Similarly, significant differences in the shoot Mo concentration were observed only at the low levels of Mo supply (1 and 10 nM), but not at the high levels of Mo supply (100 nM or 1 μM) (Fig. 3c). These results suggest that *OsMOT1;1* might function mainly at low Mo concentration. Consistent with lower Mo level in shoots, the Mo concentration in the xylem sap of *osmot1;1* was significantly decreased compared with the WT (Fig. 3d), suggesting that Mo translocation from roots to shoots

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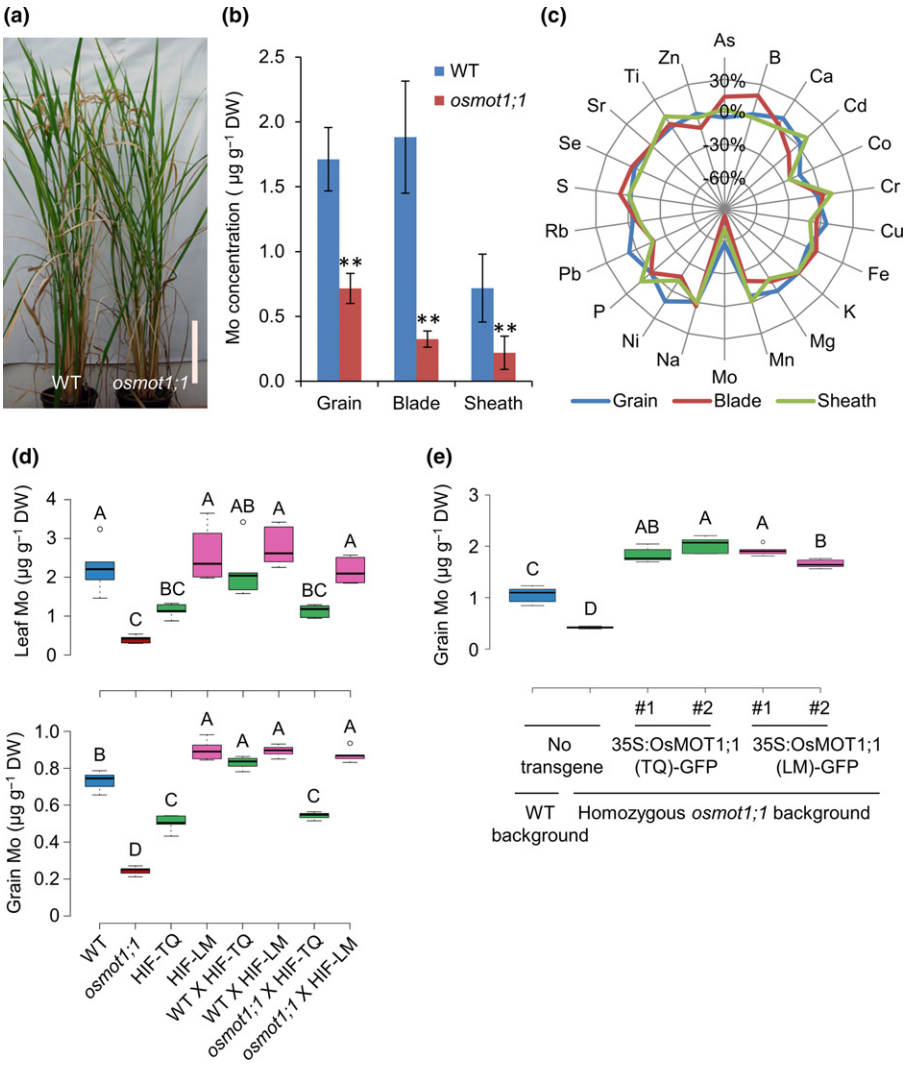

Fig. 2 Characterization of the rice *osmot1;1* mutant and complementation test. (a) Glasshouse-grown wild-type (WT) and *osmot1;1* plants grown at harvesting stage. Bar, 15 cm. (b) Molybdenum (Mo) concentrations in the grain, blade and sheath of WT and *osmot1;1*. Data are presented as means ± SD ($n = 8$). Significant differences between WT and *osmot1;1* are indicated (Student's *t*-test): **, $P \leq 0.01$. (c) Percentage difference of 22 elements in the grain, blade and sheath of *osmot1;1* compared with the WT. Data are visualized in the radar chart. (d) Genetic complementation of *osmot1;1* by crossing with HIF669.4-TQ or HIF669.4-LM. The Mo concentrations in the grain of F_1 plants were determined. (e) Transgenic complementation of *osmot1;1* by crossing with *OsMOT1;1* overexpression lines. The grain Mo concentrations of F_2 plants in a homozygous mutant background containing *35S:OsMOT1;1(TQ)-GFP* or *35S:OsMOT1;1 (LM)-GFP* constructs were determined. Two independent complemented lines were used for crossing. Data in (d, e) are presented as boxplots ($n = 6$) with center lines for medians, box limits for the 25th and 75th percentiles, whiskers for 1.5 times the interquartile range from the 25th and 75th percentiles, and outliers are represented by dots. Boxes with different letters indicate significant difference at $P \leq 0.01$ (Tukey's honestly significant difference test). TQ, 'TeQing'; LM, 'Lemont'; HIF, heterogeneous inbred family.

was affected in *osmot1;1*. Further analysis showed that the Mo concentrations in all tissues except the leaf sheaths of the fifth and sixth leaves of *osmot1;1* were significantly lower than that of WT (Fig. 3e).

The *osmot1;1* mutant is sensitive to limited Mo supply

Given that *osmot1;1* accumulates less Mo in shoots, we investigated the sensitivity of *osmot1;1* to Mo deficiency. The bioavailability of Mo is strongly dependent on the soil pH, with Mo becoming much less bioavailable in acid soils (Marschner & Rengel, 2012). When grown in acidified solid media without supplementation of Mo, most of the seeds of *osmot1;1* did not germinate, and the growth of the plants from seeds that did germinate was strongly inhibited (Fig. 4a,b). By contrast, the WT plants were able to germinate and grow, even though the root growth was also inhibited by low pH (Fig. 4a,b). These results establish that, without Mo added into the media, *osmot1;1* is more sensitive to low pH than the WT is. Supplementation of 1 μM Mo to the acidified solid media was able to restore the growth of *osmot1;1* to the level of WT (Fig. 4a,b), indicating that the sensitivity of *osmot1;1* to low pH is due to the deficiency of Mo in the media. We further showed that the HIF669.4-TQ, which accumulated less Mo (Fig. 1e), was more sensitive to Mo deficiency at low pH condition compared with the HIF669.4-LM (Fig. S4a,b).

Expression pattern and subcellular localization of OsMOT1;1

OsMOT1;1 was expressed in most of the plant tissues throughout the growth period of LM except the panicles at the reproductive stage (Fig. 5a). However, the expression of *OsMOT1;1* was much stronger in the roots than in the other tissues. In roots of 2-wk-old seedlings, *OsMOT1;1* was strongly expressed in the lateral roots as investigated by *OsMOT1;1* promoter–β-glucuronidase (GUS) transgenic rice plants (Fig. 5b). Low levels of GUS signals were detected in other tissues. To test whether the expression of *OsMOT1;1* was affected by Mo supply, LM plants were grown in nutrient solution containing 1 μM Mo for 1 wk and transferred to nutrient solution with Mo omitted for a further week. The expression of *OsMOT1;1* in roots was strongly suppressed by Mo

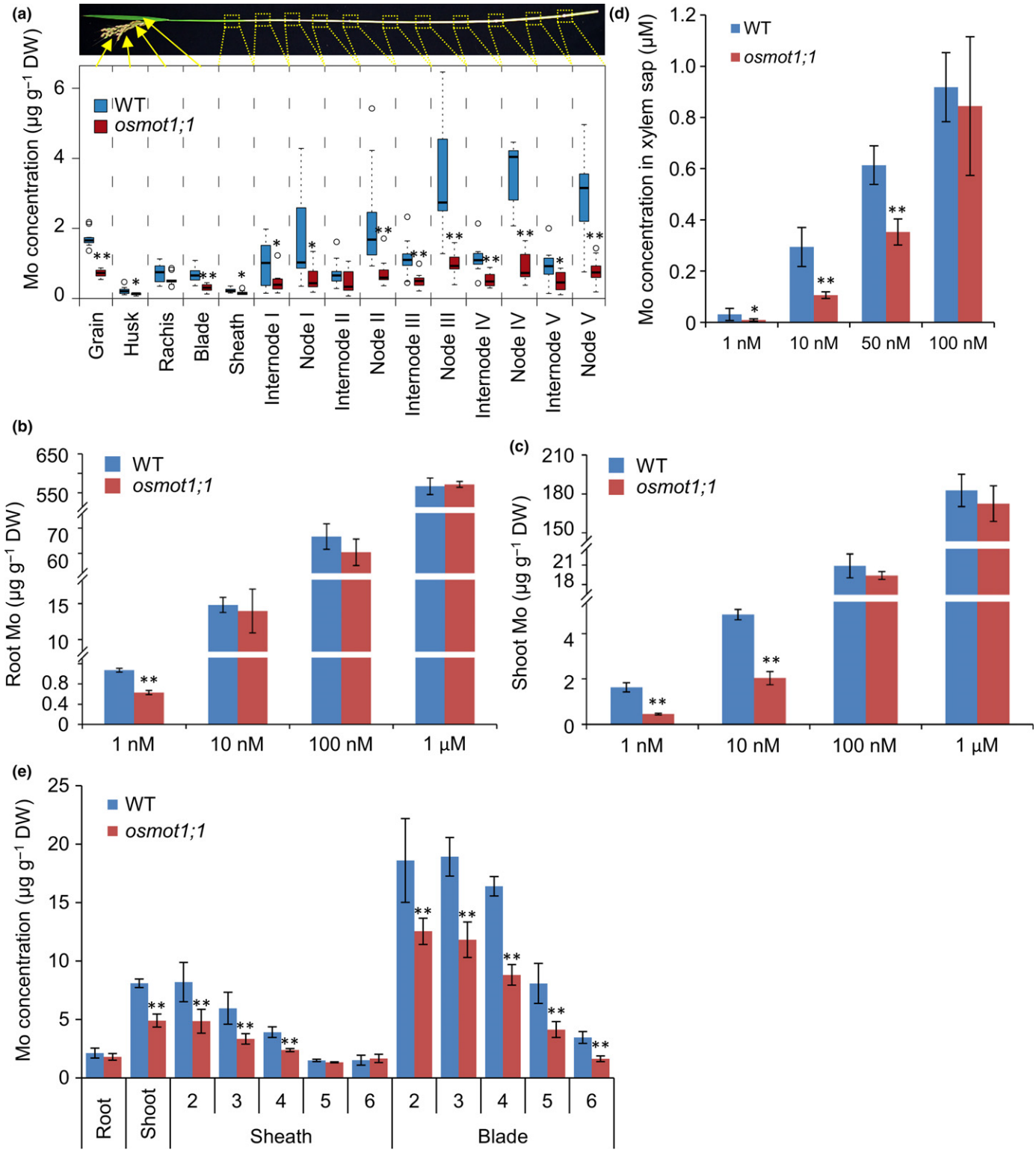

Fig. 3 Low-molybdenum (Mo) phenotype of the rice *osmot1;1* mutant. (a) The Mo concentrations in different tissues of glasshouse-grown wild-type (WT) and *osmot1;1* plants at harvesting stage. (b, c) The Mo concentrations in (b) roots and (c) shoots of WT and *osmot1;1*. Plants were hydroponically grown in nutrient solution containing various concentrations of Mo for 2 wk. (d) The Mo concentration in the xylem sap of WT and *osmot1;1*. Plants were hydroponically grown in Mo-free nutrient solution for 1 wk and treated with various concentrations of Mo for another week. (e) The Mo concentrations in different tissues of WT and *osmot1;1* seedlings. Plants were hydroponically grown in nutrient solution containing 10 nM of Mo for 2 wk. Data in (a) are presented as boxplots (WT, $n = 11$; *osmot1;1*, $n = 10$) with center lines for medians, box limits for the 25th and 75th percentiles, whiskers for 1.5 times the interquartile range from the 25th and 75th percentiles, and outliers represented by dots. Data in (b–e) are presented as means ± SD with (b, c) $n = 4$, (d, e) $n = 8$. Significant differences between WT and *osmot1;1* are indicated (Student's *t*-test): *, $P \leq 0.05$; **, $P \leq 0.01$.

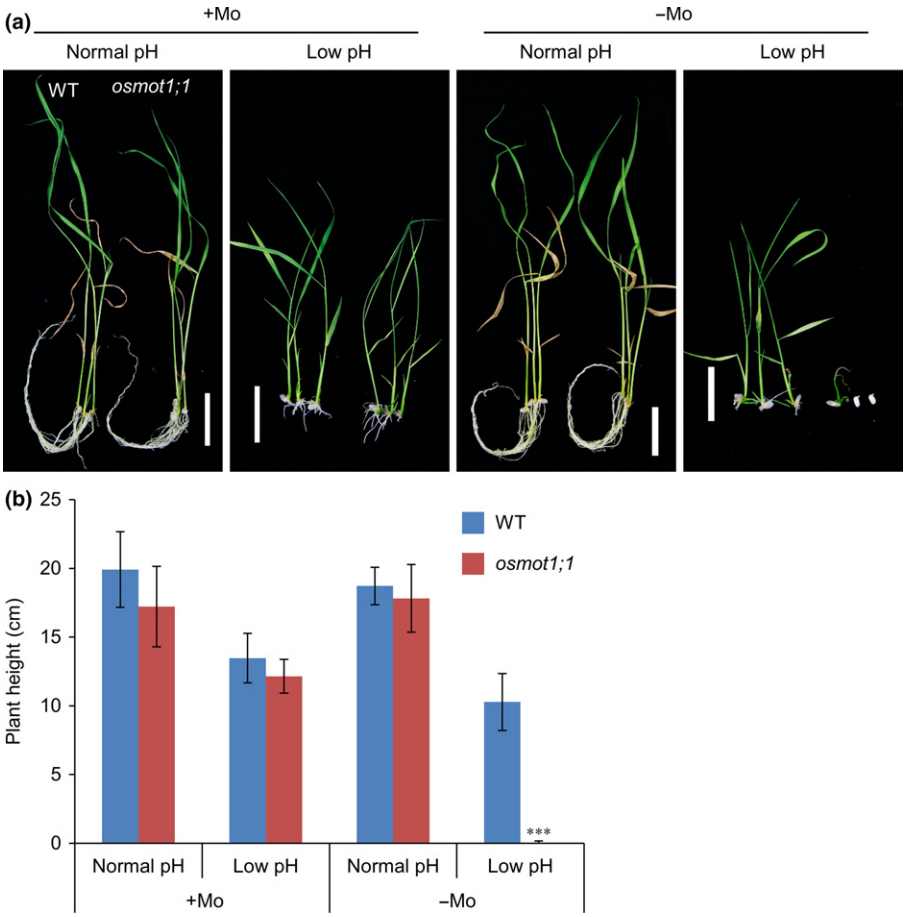

Fig. 4 The rice *osmot1;1* mutant is sensitive to molybdenum (Mo) deficiency at low pH condition. (a) The phenotype of wild-type (WT) and *osmot1;1* grown under normal or low pH media with or without 1 μM Mo added. Low-pH medium was made by adding 50 μl 6 M hydrochloric acid to 100 ml agar medium after autoclaving. Plants were grown for 20 d. Bars, 3 cm. (b) The plant height of WT and *osmot1;1* grown as in (a). Data are presented as means ± SD with $n = 6$. Significant differences between WT and *osmot1;1* are indicated (Student's *t*-test): ***, $P \leq 0.001$.

depletion as determined by quantitative reverse transcription PCR. However, such suppression was not found in shoots (Fig. 5c).

To investigate the subcellular localization of OsMOT1;1, the OsMOT1;1 from TQ or LM was fused with green fluorescent protein (GFP) to the C-terminus and expressed under the control of 35S promoter in *japonica* variety 'Zhonghua 11' (Fig. S3d). The GFP fluorescence was co-localized with the signal of the mitochondrial dye MitoTracker™, suggesting OsMOT1;1 localizes to the mitochondria (Fig. 5d). We observed that OsMOT1;1 from both TQ and LM was localized to the mitochondria, indicating that the amino acid variation of OsMOT1;1 between TQ and LM has no effect on the subcellular localization (Fig. 5d). Furthermore, the Mo concentrations in roots and shoots of transgenic lines were higher than the nontransgenic control line, suggesting the mitochondria-localized OsMOT1;1 is functional (Fig. S5).

Functional analysis of *OsMOT1;1* in *A. thaliana*

OsMOT1;1 shares 57.8% sequence similarity to AtMOT1;1 (Fig. S1a), a high-affinity molybdate transporter in *A. thaliana*. The *atmot1;1* mutant accumulates lower levels of Mo in leaves than WT does (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008). To investigate whether expression of *OsMOT1;1* in *atmot1;1* could suppress its low-Mo phenotype, we heterologously expressed *OsMOT1;1-GFP* in *atmot1;1* using 35S promoter (Fig. S6). In the root cells of the transgenic plants, the GFP signals were observed to co-localize with the mitochondria-specific dye MitoTracker (Fig. S7), further confirming the mitochondria localization of OsMOT1;1. The Mo concentrations in both roots and shoots of transgenic plants expressing *OsMOT1;1* from either TQ or LM were significantly higher than that of *atmot1;1*, even higher than the WT Col-0 (Fig. 6a). These results suggest that expression of *OsMOT1;1* from either TQ or LM could enhance Mo accumulation in *atmot1;1*. To rule out the ectopic effect of overexpression of *OsMOT1;1* driven by 35S promoter, we also expressed *OsMOT1;1* in *atmot1;1* using *AtMOT1;1* native promoter (Fig. S6). The Mo concentrations in both roots and shoots of transgenic plants were significantly higher than that of *atmot1;1*, similar to the level in WT Col-0 (Fig. 6b), suggesting that expression of *OsMOT1;1* using *AtMOT1;1* native promoter was able to complement the *AtMOT1;1* knockout mutant. Notably, there was no significant difference in either root or shoot Mo concentration between transgenic plants expressing *OsMOT1;1* from TQ or LM (Fig. 6b), indicating no functional difference between the *OsMOT1;1* allele of TQ and LM. Previous studies have shown that the *atmot1;1* mutant is sensitive to low-Mo stress (Tomatsu *et al.*, 2007) and shows defective growth in acidic soil, in which the bioavailability of Mo is low (Poormohammad Kiani *et al.*, 2012). Similar to the sensitivity to acidic soil,

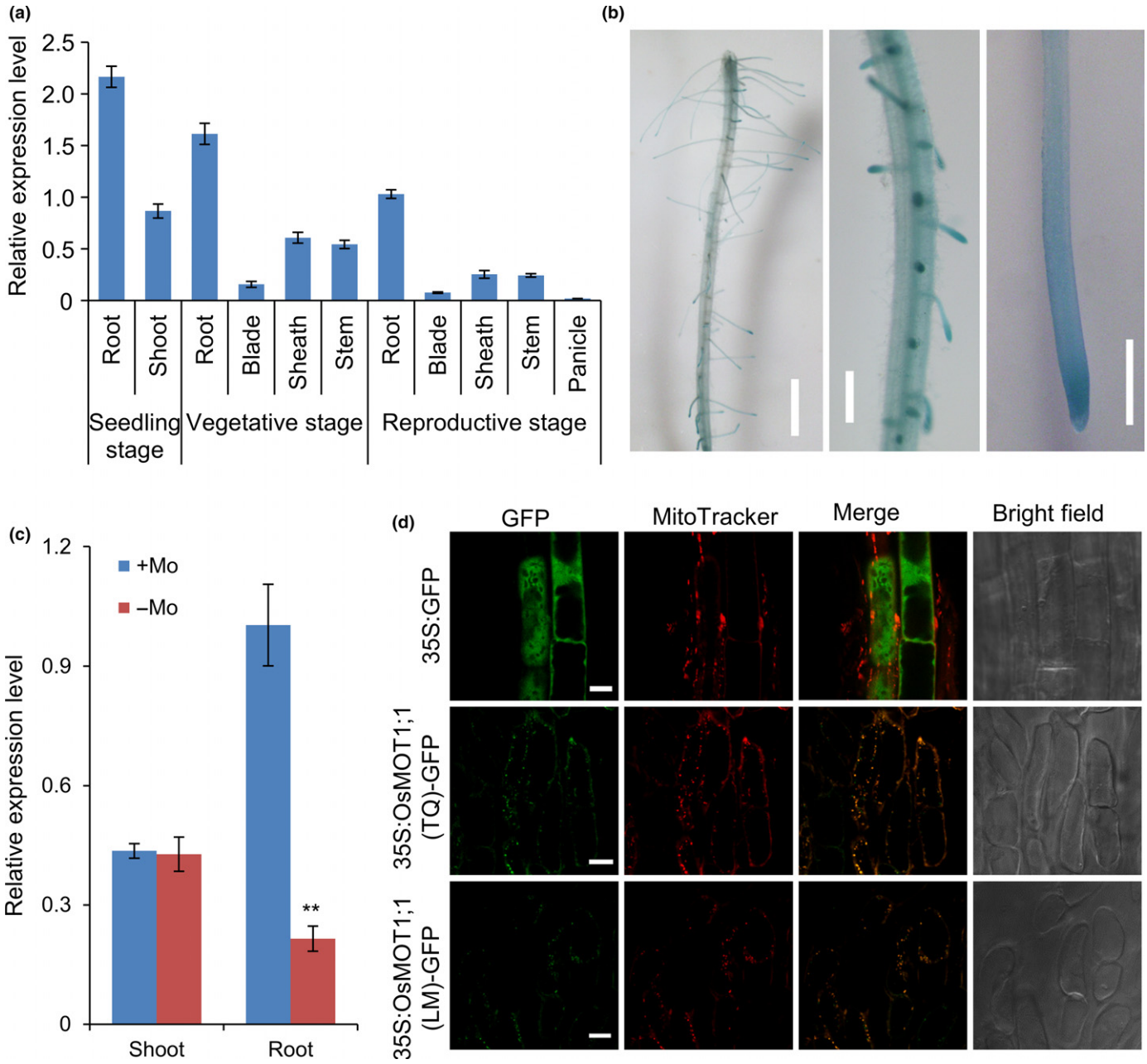

Fig. 5 Expression pattern and subcellular localization of *OsMOT1;1* in rice. (a) Expression level of *OsMOT1;1* in different organs of 'Lemont' (LM) at different growth stages. (b) Histochemical GUS (β-glucuronidase) staining of roots of transgenic rice plants transformed with *OsMOT1;1* promoter-driven GUS constructs. The mature zone (left), elongation zone (middle) and root tip (right) of roots of 2-wk-old plants are showed. (c) Expression of *OsMOT1;1* was suppressed in roots under molybdenum (Mo) deficiency. LM plants were grown hydroponically with 1 μM Mo for 1 wk and then treated with 1 μM Mo (+Mo) or without Mo (−Mo) for another week. Relative expression level was determined by quantitative reverse transcription PCR with three biological replicates. (d) Subcellular localization of OsMOT1;1 in stable transgenic rice plants. OsMOT1;1 from 'TeQing' (TQ) or LM were fused with green fluorescent protein (GFP) at N-terminus and overexpressed under the control of cauliflower mosaic virus 35S promoter. Mitochondria were stained with the specific dye MitoTracker™. Data in (a, c) are presented as means ± SD with $n = 3$. Significant differences are indicated (Student's *t*-test): **, $P \leq 0.01$. Bars: (b, left) 2 mm; (b, middle) 0.5 mm; (b, right) 2 mm; (d) 10 μm.

atmot1;1 also showed growth inhibition on agar media at low pH without added Mo (Fig. 6c–e). The transgenic plants expressing *OsMOT1;1* from TQ or LM using 35S promoter or *MOT1* native promoter completely restore the growth of *atmot1;1* on agar media at low pH (Fig. 6c–e). The results further confirm that *OsMOT1;1* is able to complement the mutation of *AtMOT1;1* in *A. thaliana*.

Molybdate transport activity of OsMOT1;1

The molybdate transport activity of OsMOT1;1 was tested by heterologous expression of *OsMOT1*;1 in yeast (*S. cerevisiae*) strain BY4741. Yeast strain BY4741 transformed with empty vector or *OsMOT1;1* from TQ or LM was cultured in Mo-free media to the mid-log phase and then transferred to the media

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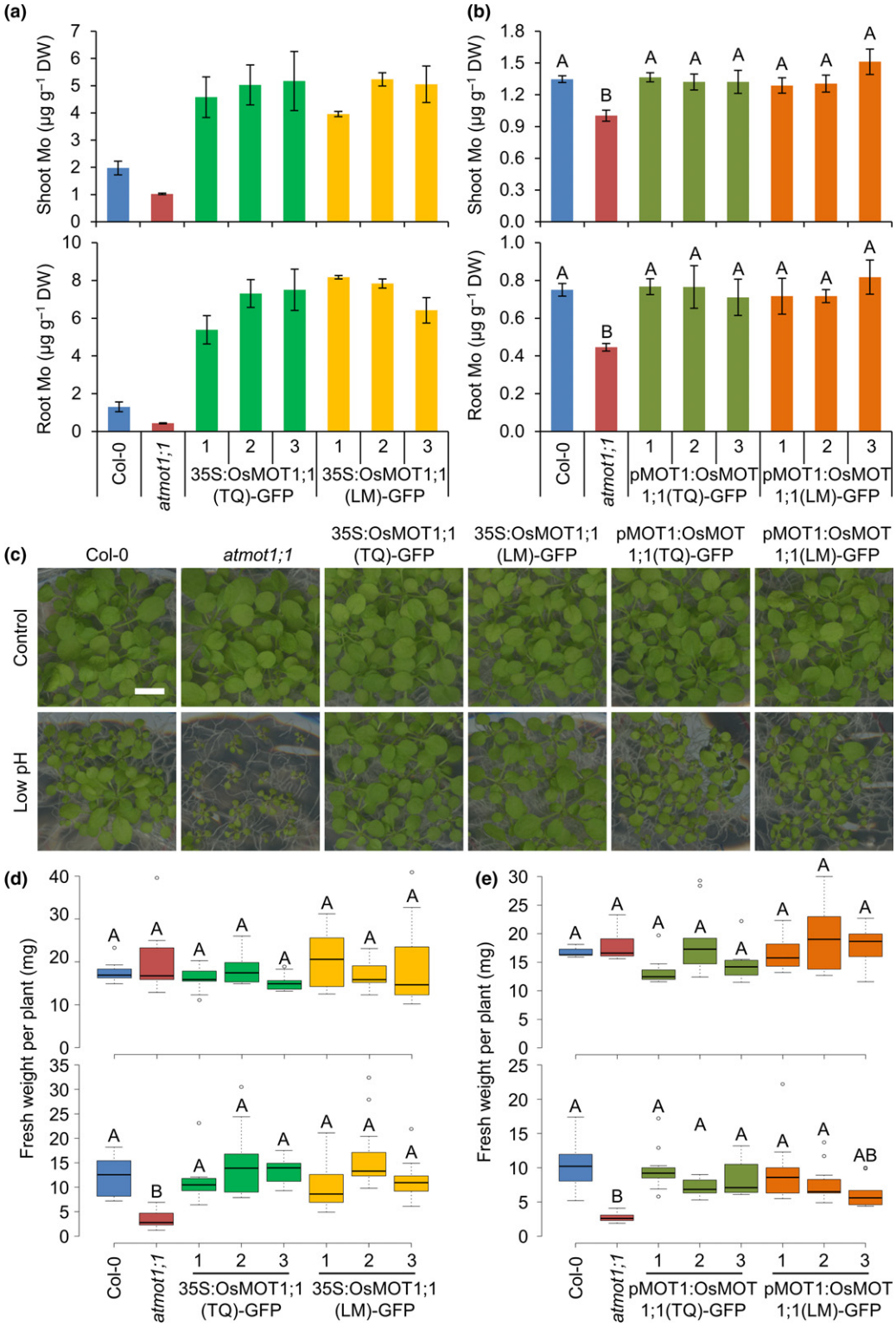

Fig. 6 Functional analysis of *OsMOT1;1* in *Arabidopsis atmot1;1* mutant. (a, b) Molybdenum (Mo) concentrations in roots and shoots of the *atmot1;1* mutant transformed with *OsMOT1;1-GFP* from 'TeQing' (TQ) or 'Lemont' (LM) driven by (a) cauliflower mosaic virus (CaMV) 35S promoter or (b) *AtMOT1;1* native promoter. Plants were grown on MGRL media containing 24 nM Mo for 2 wk. Three independent transgenic lines are shown. (c) Phenotype of *OsMOT1;1-GFP* transgenic lines in *atmot1;1* background. Plants were grown on MGRL media without added Mo at control or low pH condition for 20 d. Bar, 1 cm. (d, e) Fresh weight of *atmot1;1* transformed with *OsMOT1;1-GFP* from TQ or LM driven by (d) CaMV 35S promoter or (e) *AtMOT1;1* native promoter. Plants were grown as in (c). Three independent transgenic lines are shown. Data in (a, b) are shown as means ± SD with three biological replicates. Six plants were combined in each replicate. Data in (d, e) are shown as boxplots ($n = 7$–12) with center lines for medians, box limits for the 25th and 75th percentiles, whiskers for 1.5 times the interquartile range from the 25th and 75th percentiles, and outliers are represented by dots. Columns or boxplots with different capital letters in (b, d and e) indicate significant difference at $P \leq 0.01$ (Tukey's honestly significant difference test).

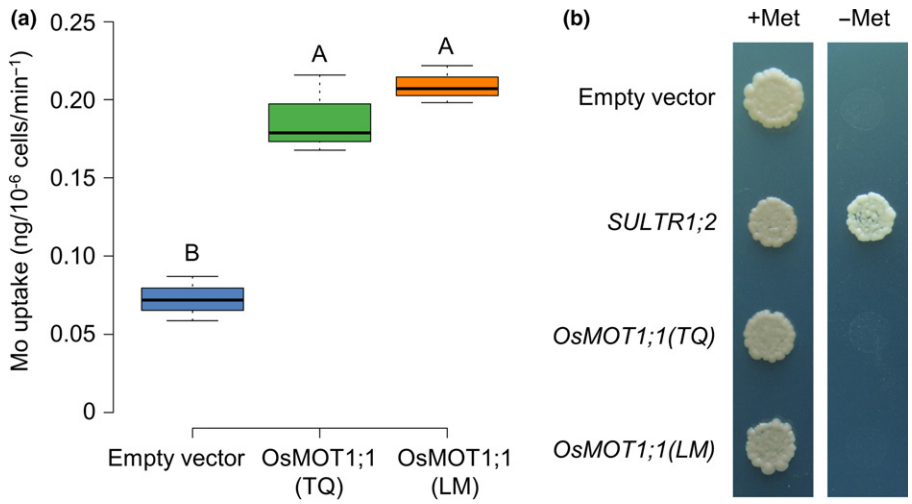

Fig. 7 Functional analysis of *OsMOT1;1* in yeast. (a) Molybdate transport activity of OsMOT1;1. Yeast strains transformed with empty vector or *OsMOT1;1* from 'TeQing' (TQ) or 'Lemont' (LM) were incubated in media containing 0.5 μM molybdenum (Mo) for 30 min. Mo concentrations in yeast cells were determined. Data are shown as boxplot ($n = 3$) with center lines for medians, box limits for the 25th and 75th percentiles, whiskers for 1.5 times the interquartile range from the 25th and 75th percentiles, and outliers are represented by dots. Boxplots with different letters indicate significant difference at $P \leq 0.01$ (Tukey's honestly significant difference test). (b) Complementation analysis of a yeast mutant defective in sulfate uptake. The yeast mutant strains transformed with empty vector, *SULTR1;2*, or *OsMOT1;1* from TQ or LM were incubated on media containing 0.5 mM sulfate with or without added methionine (Met) for 4 d.

containing 0.5 μM Mo and incubated for 30 min. The Mo concentration in yeast cells transformed with *OsMOT1;1* was significantly higher than the control strain transformed with an empty vector (Fig. 7a). These results support the conclusion that OsMOT1;1 is able to transport molybdate. Further comparison revealed no difference of Mo concentrations in the strains transformed with *OsMOT1;1* from TQ or LM (Fig. 7a), indicating that OsMOT1;1 from TQ and LM exhibited similar molybdate transporting activity.

OsMOT1;1 shares sequence similarity to sulfate transporter genes in rice, and thus was previously annotated as a member of the group V sulfate transporter family (Kumar *et al.*, 2011). To determine whether OsMOT1;1 exhibits a sulfate transport activity, we performed complementation analysis of a yeast mutant CP154-7B, which is defective in two high-affinity sulfate transporters and is unable to grow on media containing < 1 mM sulfate as the sole S source (Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002; Tomatsu *et al.*, 2007). The yeast mutant transformed with *SULTR1;2*, an *A. thaliana* high-affinity sulfate transporter, was able to grow on the −Met media. However, expression of *OsMOT1;1* from either TQ or LM was unable to complement the growth defect of the mutant strain on −Met media (Fig. 7b). These results suggest that OsMOT1;1 likely does not exhibit sulfate transport activity.

Analysis of natural variation of *OsMOT1;1*

To investigate the contribution of natural variation at *OsMOT1;1* to the variation of grain Mo in rice, we analyzed the coding sequences of 1479 rice accessions that have been resequenced (Zhao *et al.*, 2015). Five nonsynonymous polymorphisms in the coding sequence of *OsMOT1;1* were identified, including the two polymorphic sites between TQ and LM (S125A and Del^{9}), and the 12 bp deletion (Del^{12}) in both TQ and LM (Table S2; Fig. 1d). To determine the contribution of these three polymorphic sites on the variation of grain Mo, we genotyped 1097 accessions of the United States Department of Agriculture (USDA) rice core collection for which we have previously reported the grain ionomic profile (Pinson *et al.*, 2015). We found no significant difference of grain Mo between the two alleles on any of these three polymorphic sites (Figs 8a, S8a). We further compared the grain Mo concentrations of eight haplotypes derived from the combination of these three polymorphic sites. We observed no significant differences of grain Mo among the eight haplotypes (Figs 8b, S8b). These results suggested that the variation in the coding region of *OsMOT1;1* might not contribute to the variation of grain Mo in the rice population.

There are 19 SNPs and two sequence deletions (Del^{222} and Del^{16}) in the promoter sequence of *OsMOT1;1* in TQ (Fig. 1d). We determined the contribution of the two major sequence variances, Del^{222} and Del^{16}, on the variation of grain Mo. We genotyped the 1097 accessions of the USDA core collection and compared the grain Mo concentrations of accessions with or without the deletions. We found that there was no significant difference between the accessions with or without the Del^{222} or Del^{16} (Figs 8c, S8c). Furthermore, the grain Mo concentrations among the accessions with combination of Del^{222} and Del^{16} were fairly similar (Figs 8d, S8d). These results suggested that Del^{222} and Del^{16} in the promoter of *OsMOT1;1* might not contribute to the variation of grain Mo. Thus, the different promoter activity of *OsMOT1;1* between TQ and LM was likely due to the 19 SNPs, which might cause different expression levels of *OsMOT1;1* between TQ and LM.

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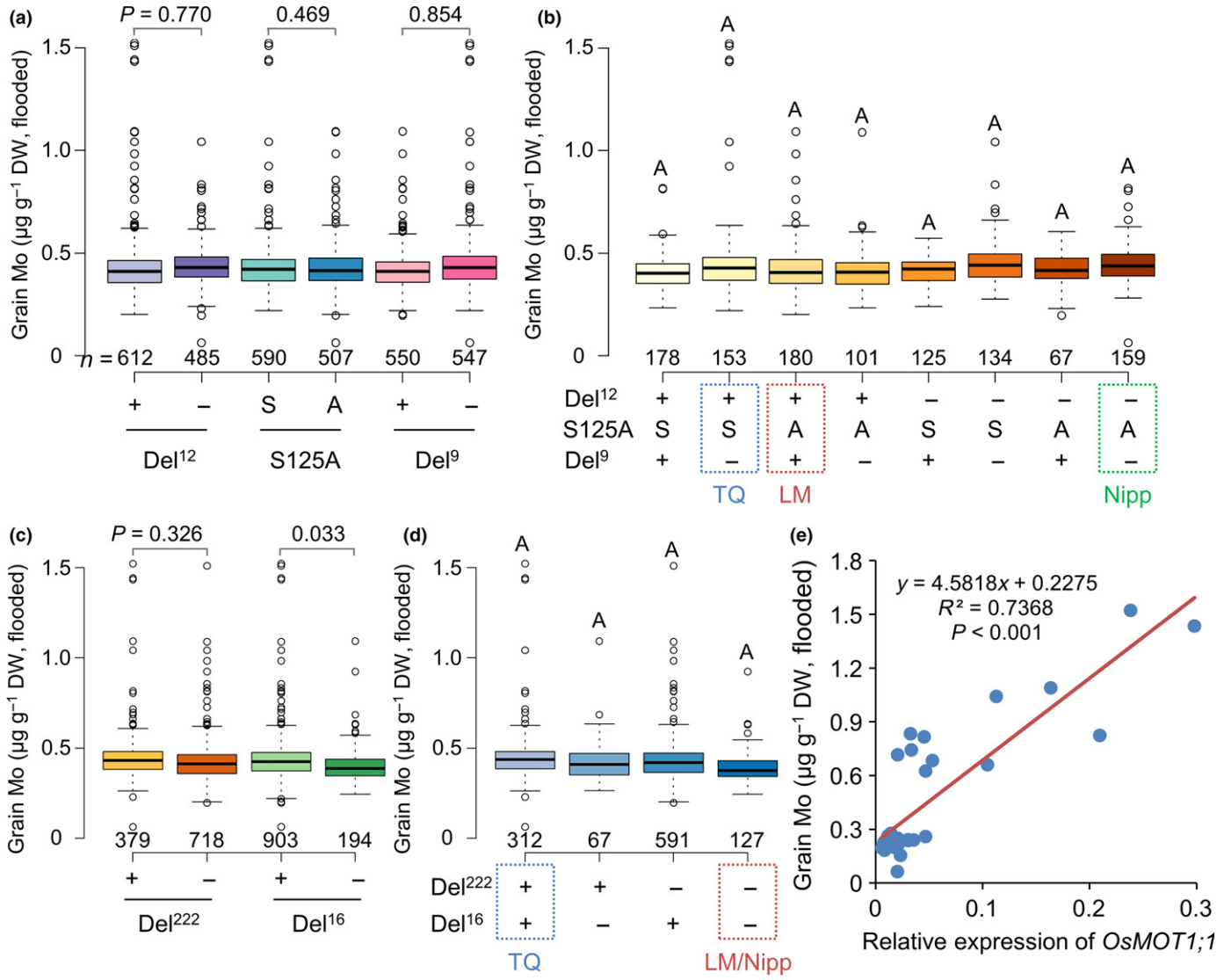

Fig. 8 Contribution of allelic variation of *OsMOT1;1* on the variation of grain molybdenum (Mo) in the United States Department of Agriculture rice core collection grown in flooded condition. (a) The grain Mo in rice accessions with different alleles at three polymorphic sites in the coding region of *OsMOT1;1*. (b) The grain Mo in rice accessions with different haplotypes derived from the combination of three polymorphic sites in the coding region of *OsMOT1;1*. (c) The grain Mo in rice accessions with or without the deletions in the promoter region of *OsMOT1;1*. (d) The grain Mo in rice accessions with different promoter haplotypes derived from the combination of two deletions in the coding region of *OsMOT1;1*. Data in (a–d) are shown as boxplots with center lines for medians, box limits for the 25th and 75th percentiles, whiskers for 1.5 times the interquartile range from the 25th and 75th percentiles, and outliers are represented by dots. Numbers under the boxes are the accession number; numbers above the boxes are the *P*-values; the same letters above the boxes indicate no significant difference at $P \leq 0.01$ (Tukey's honestly significant difference test). + and – represent the presence and absence of the deletion, respectively. The dotted color boxes in (b, d) mark the haplotypes in 'TeQing' (TQ), 'Lemont' (LM) and Nipponbare (Nipp). (e) The correlation of the relative expression of *OsMOT1;1* in roots with the grain Mo concentration in 35 rice accessions grown in flooded condition. The expression of *OsMOT1;1* in the roots of 3-wk-old plants was determined by quantitative reverse transcription PCR. The relative expression level of *OsMOT1;1* was normalized to the rice actin gene and is presented as the mean of $2^{\Delta\Delta Ct}$ with three biological replicates.

Correlation of grain Mo with the expression of *OsMOT1;1*

To determine whether the variation of grain Mo was due to the variation in the *OsMOT1;1* expression level, we selected 35 rice accessions from the USDA core collection with grain Mo concentrations in the range 0.063–1.52 μg g^{-1} under flooded growth condition and 0.084–1.23 μg g^{-1} under unflooded condition. The expression levels of *OsMOT1;1* were determined in the roots of 3-wk-old plants grown hydroponically with 1 μM Mo. We found highly significant correlations between the expression level of *OsMOT1;1* in roots from hydroponically grown plants with the Mo concentrations in grains from plants grown in the field under both flooded (Pearson's $R^2 = 0.7368$, $P < 0.001$; Fig. 8e) and unflooded (Pearson's $R^2 = 0.6207$, $P < 0.001$; Fig. S8e) conditions. These results suggest the natural variation of Mo concentration in rice grains is attributed to the variable expression of *OsMOT1;1* in roots.

Discussion

As one of the essential mineral nutrients required by plants, Mo plays important roles in nitrate assimilation, abscisic acid biosynthesis, purine degradation and sulfite detoxification (Schwarz & Mendel, 2006; Bittner, 2014). However, the mechanisms of Mo uptake and transport and the regulation of these processes are largely unknown in plants, especially in crops. In this study, we identified a QTL, *qGMo8*, that controls the variation of Mo concentration in rice shoots and grains. We determined the causal gene for this QTL to be *OsMOT1;1* by genetic and transgenic complementation (Fig. 2d,e). OsMOT1;1 was previously annotated as a member of the group V sulfate transporter superfamily (Tejada-Jiménez *et al.*, 2013). We provided evidence that OsMOT1;1 is a molybdate transporter, including the fact that OsMOT1;1 enhances molybdate but not sulfate uptake when heterogeneously expressed in yeast (Figs 7a, 2b). We found that the difference in grain Mo concentrations between rice cultivars TQ and LM was not due to altered molybdate transporting activity but most likely to allelic variation at the gene expression level of *OsMOT1;1*. This conclusion is supported by several lines of evidence. First, the molybdate transporting activity of OsMOT1;1 from TQ and LM was similar when heterogeneously expressed in yeast (Fig. 7a). Second, the *OsMOT1;1* from either TQ or LM was able to complement the low-Mo phenotype of *atmot1;1* to a similar level when expressed by the *AtMOT1;1* native promoter (Fig. 6b,c,e). Third, the HIFs line HIF669.4-LM with higher expression level of *OsMOT1;1* accumulates more Mo in the leaves and grains than that of HIF669.4-TQ does. Knockout of *OsMOT1;1* resulted in decreased Mo concentration in the shoots and grains (Fig. 2b,c). We thus conclude that OsMOT1;1 is a molybdate transporter in rice and that the natural variation of Mo concentration in rice grains is attributed to allelic variation in *OsMOT1;1* at the gene expression level.

Several molybdate transporters have been identified, including CrMOT1 and CrMOT2 from *C. reinhardtii* (Tejada-Jiménez *et al.*, 2007, 2011), AtMOT1;1 and AtMOT1;2 from *A. thaliana* (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008; Gasber *et al.*, 2011), LjMOT1 from *L. japonicus* (Gao *et al.*, 2016; Duan *et al.*, 2017) and MtMOT1.3 from *M. truncatula* (Tejada-Jiménez *et al.*, 2017). *AtMOT1;1* has been shown to control the natural variation in leaf Mo concentration in *A. thaliana* (Baxter *et al.*, 2008). A 53 bp deletion in the promoter of *AtMOT1;1*, which is located 13 bp upstream from the transcription start site of *AtMOT1;1*, was identified as the functional polymorphism contributing to decreased leaf Mo concentration (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008). Further analysis revealed a total of six noncoding structural polymorphisms in the *AtMOT1;1* promoter, including the 53 bp deletion originally identified in L*er*-0 and a duplicated 330 bp insertion that has undergone a 4 bp deletion (Forsberg *et al.*, 2015). The 53 bp deletion is associated with decreased leaf Mo, whereas the duplicated 330 bp insertion is associated with elevated leaf Mo. These two polymorphisms control Mo concentration in leaves by either decreasing or increasing, respectively, the expression level of *AtMOT1;1*.

In this study, we identified 19 SNPs and two deletions, a 222 bp deletion (Del^{222}) and a 16 bp deletion (Del^{16}), in the promoter of *OsMOT1;1* between TQ and LM (Fig. 1d). We showed that the difference in grain Mo concentration between TQ and LM was due to the different expression level of *OsMOT1;1* (Fig. 1e,f). Further analysis of 35 rice accessions revealed a significant positive correlation between the expression level of *OsMOT1;1* in roots and Mo concentration in grains (Figs 8e, S8e). However, the variation in grain Mo appears unrelated to the two main noncoding structural polymorphisms, Del^{222} and Del^{16}, as grain Mo concentrations of rice accessions with or without these two deletions were similar (Figs 8c,d, S8c, d). Therefore, the difference in grain Mo concentration between TQ and LM may be due to the SNPs in the promoter of *OsMOT1;1* that lead to different expression levels. At least two SNPs that are significantly associated with the variation of leaf Mo were also identified on the *AtMOT1;1* locus, even though the effect of these SNPs on its expression is not clear (Forsberg *et al.*, 2015). Thus, the variation of Mo in both rice and *A. thaliana* is attributed to the variable expression of the *MOT1;1* gene. Such conserved genetic variation structure across plant species suggests a role of *MOT1;1* gene in adaptation to the environment. Indeed, *A. thaliana* accessions with the weak allele of *AtMOT1;1* from West Asia appear to adapt to their native habitats, where the water-extractable Mo content is high in soils (Poormohammad Kiani *et al.*, 2012). We found that the *osmot1;1* mutant is sensitive to limited Mo supply condition (Fig. 4a,b), similar to the sensitivity of *atmot1;1* to Mo deficiency (Tomatsu *et al.*, 2007) and to acidic soils in which the bioavailability of Mo is low (Poormohammad Kiani *et al.*, 2012). Therefore, the *MOT1;1* gene may play an important role in adaptation to variable molybdate availability in soils caused by environmental changes.

The polymorphisms in the coding region of *AtMOT1;1* also affect its function. A single amino acid variation on AtMOT1;1 in L*er*-0 accession alters its molybdate transporting activity (Tomatsu *et al.*, 2007). The hypofunction of AtMOT1;1 in Sha accession was also proved to be caused by a single amino acid change (Poormohammad Kiani *et al.*, 2012). In the present study, we found two amino acid polymorphisms in OsMOT1;1 between TQ and LM (Fig. 1d). However, neither appears to alter the function of OsMOT1;1, as these two protein versions showed similar molybdate transporting activity and were able to complement the low-Mo phenotype of the *atmot1;1* mutant to a similar level (Figs 6b,c,e, 7a). A 12 bp deletion (Del^{12}) in the coding region of *OsMOT1;1* of both TQ and LM, compared with Nipponbare, seems not to change its function, because the presence/absence of Del^{12} is not associated with the variation of grain Mo (Figs 8a,b, S8a,b). Thus, the large sequence diversity of the *MOT1;1* locus explains well the identification of *AtMOT1;1* in controlling the variation of leaf Mo by genome-wide association analysis based on either the mean or variance of leaf Mo (Shen *et al.*, 2012; Forsberg *et al.*, 2015), and *OsMOT1;1* as the potential locus responsible for the variation of rice grain Mo concentration (Norton *et al.*, 2014).

The expression of *OsMOT1;1* is stronger in roots than in shoots (Fig. 5a). This is similar to the expression pattern of *AtMOT1;1* in *A. thaliana* (Tomatsu *et al.*, 2007), which is consistent with the fact that *AtMOT1;1* mainly functions in roots (Baxter *et al.*, 2008). Under Mo-limited conditions, *OsMOT1;1* is downregulated in roots but not in shoots (Fig. 5a). However, such downregulation was not observed for *AtMOT1;1* in roots, though it was in shoots (Tomatsu *et al.*, 2007), suggesting the different behavior of *MOT1;1* genes in rice and *A. thaliana* under Mo-scarce conditions. *OsMOT1;1* may mainly function under low Mo conditions, as we only observed the difference of Mo concentrations between WT and *osmot1;1* in the hydroponic growth system containing 1 or 10 nM Mo but not at the relatively higher Mo condition (100 nM or 1 μM) (Fig. 3b,c). The Mo concentration in the xylem sap of *osmot1;1* is lower than that of WT (Fig. 3d), suggesting *OsMOT1;1* is also involved in the translocation of Mo from roots to shoots. Meanwhile, the Mo concentrations in the grain, blade and sheath of *osmot1;1* only decrease by 58–82% compared with the WT (Fig. 2d), suggesting the existence of additional transporters that control the accumulation of Mo in rice. Further studies are required to elucidate the detailed function of *OsMOT1;1* and to identify other transporters in controlling the Mo homeostasis in rice.

In summary, we have identified *OsMOT1;1* as the causal gene underlying the QTL for Mo accumulation in rice shoots and grains. OsMOT1;1 exhibits molybdate transport activity. The identification of *OsMOT1;1* provides an important insight into the regulation of Mo homeostasis in rice and a useful gene to breed rice varieties resistant to Mo deficiency in soils. Given the importance of cereals as a source of Mo in the human diet, the identification of natural variation at the *OsMOT1;1* locus provides an efficient way to breed rice varieties with Mo enrichment in the grain, which could improve the nutrient quality of grains.

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Author contributions

X-YH and DES designed the research; X-YH, HL, Y-FZ, SRMP, H-XL and MLG performed the experiments. X-YH and DES analyzed the data. X-YH, F-JZ and DES wrote the paper with contributions from SRMP and MLG.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Fig. S1 Sequence alignment of MOT1 proteins.

Fig. S2 Schematic diagram of development of heterogeneous inbred families (HIF).

Fig. S3 Molecular characterization of OsMOT1;1 T-DNA insertion and OsMOT1;1 overexpression lines.

Fig. S4 The sensitivity of HIF669.4-TQ and HIF669.4-LM to Mo deficiency at low pH condition.

Fig. S5 Overexpression of *OsMOT1;1* increases Mo concentrations in both roots and shoots.

Fig. S6 Expression level of OsMOT1;1 in Arabidopsis transgenic lines.

Fig. S7 Subcellular localization of OsMOT1;1 in Arabidopsis.

Fig. S8 Contribution of allelic variation of OsMOT1;1 on the variation of grain Mo in USDA rice core collection grown in unflooded condition.

Methods S1 Supporting information for Materials and Methods.

Table S1 The primers used in this study.

Table S2 Sequence variation and allele frequency of OsMOT1;1.

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