

1 **Natural variation in a molybdate transporter controls grain molybdenum**
2 **concentration in rice**

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1 **Summary**

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

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21 **Key words: molybdenum, mineral nutrient, natural variation, QTL, rice**

22

1 **Introduction**

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

16 Mo is also an essential micronutrient for plants. Plants take up Mo mainly as molybdate.
17 Molybdate itself is biologically inactive and must be incorporated into tricyclic pterin to
18 form Moco. Moco serves as electron donors and/or acceptors in molybdoenzymes and
19 plays key roles in the assimilation and biogeochemical cycles of carbon, nitrogen, and
20 sulfur (Schwarz & Mendel, 2006; Bittner, 2014). Although Mo deficiency in humans is
21 rare, Mo deficiency in crops is becoming an agricultural problem, especially for crops
22 grown on acid soils. In acid soils, the high levels of reactive iron oxides/hydroxides are
23 known to have a strong adsorption of molybdate which decreases the bioavailability of Mo
24 (Marschner & Rengel, 2012). It is estimated that up to 70% of the world's arable is
25 characterized acidic, Mo deficiency is thus a widespread agricultural concern (von Uexküll
26 & Mutert, 1995). The deficiency of Mo in soils has been shown to inhibit plant growth and
27 agricultural productivity (Kaiser *et al.*, 2005). Plants suffering from Mo deficiency develop
28 the typical "whiptail" phenotypes, which includes mottled lesions on the leaves, rolling of
29 leaves and wilting of leaf edges (Arnon & Stout, 1939).

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

1 conditions (Tejada-Jimenez *et al.*, 2011). To date, molybdate transporters that control Mo
2 concentration have not been characterised in staple food crops.

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

11

12 **Materials and Methods**

13 **Plant materials and growth conditions**

14 The rice (*Oryza sativa* L.) recombinant inbred lines derived from a cross between Lemont
15 (LM, *japonica*) and TeQing (TQ, *indica*) (LT-RILs), and TeQing-into-Lemont backcross
16 introgression lines (TILs) were described previously (Tabien *et al.*, 2000; Pinson *et al.*,
17 2012; Huang *et al.*, 2016b). The heterogeneous inbred families (HIFs) of *OsMOT1;1* locus
18 were generated as previously described (Tuinstra *et al.*, 1997; Loudet *et al.*, 2005). A PCR
19 marker was developed based on the 222 bp deletion in the promoter of *OsMOT1;1* in TQ,
20 and was used for genotyping the TILs. The line TIL669.4 was determined to be
21 heterozygous at the *OsMOT1;1* locus. Plants fixed with TQ allele (HIF669.4-TQ) and
22 plants fixed with Lemont allele (HIF669.4-LM) were identified in the next generation of
23 TIL669.4, resulting in HIFs for comparing phenotypic effects of the *OsMOT1;1* alleles
24 from TQ and LM. The T-DNA insertion mutant for *OsMOT1;1* which is in Zhonghua 11
25 background was obtained from Huazhong Agricultural University, China
26 (<http://rmd.ncpgr.cn>). The segregated plants without T-DNA insertion in *OsMOT1;1* gene
27 were used as a WT control. The WT and T-DNA seeds were planted into soil in a
28 greenhouse at the University of Aberdeen, U.K., or hydroponically grown in Nanjing
29 Agricultural University, China. The *A. thaliana* T-DNA insertion mutant for *AtMOT1;1*

1 (SALK_118311) was obtained from the Arabidopsis Biological Resource Center (ABRC,
2 <http://www.arabidopsis.org/abrc/>).

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

13 **QTL analysis and fine mapping of *qGMo8***

14 The QTL mapping has been performed previously by using both multiple interval mapping
15 (MIM) and Bayesian information criterion (BIC) methods based on the least squares (LS)
16 means of the 5 years-replications for the grain Mo of LT-RIL and three replications of TIL
17 (Zhang *et al.*, 2014). We reperformed QTL analyses based on the individual year data under
18 flooded or unflooded field conditions by using Windows QTL cartographer version 2.5
19 (<http://statgen.ncsu.edu/qtlcart/WQTLCart.htm>) with a composite interval mapping (CIM) method
20 according to previous studies (Huang *et al.*, 2016b). Seven markers were developed in the
21 *qGMo8* mapping interval and used to genotype the entire population of 123 TILs. Three
22 representative TILs with different genotypes in the *qGMo8* mapping interval and two
23 control TILs were chosen for further replicated phenotypic analyses and fine mapping. By
24 integration of the genotypes and grain Mo concentrations of these lines, the *qGMo8* was
25 fine mapped to a 522 kb region on the top of chromosome 8. The primer sequences are
26 listed in Table S2.

27 **Tissue elemental analysis**

28 The elemental concentrations in grains of LT-RILs, TILs, WT and *osmot1;1* were
29 determined using an inductively couple plasma mass spectrometer (ICP-MS, Elan DRCe,
30 PerkinElmer; or NexION 300D, PerkinElmer) according to previous studies (Zhang *et al.*,

1 2014; Huang *et al.*, 2016b). For determination of elemental concentrations in different
2 organs of WT and *osmot1;1* grown in soil, tissues were excised and washed with Milli-Q
3 water and dried at 88°C overnight. For analysis of roots of WT and *osmot1;1* grown
4 hydroponically, roots were excised and washed with 0.5 mM CaCl₂ solution three times
5 and rinsed with Milli-Q water once and then dried at 88°C overnight. Samples were
6 digested with concentrated HNO₃ at 118°C for 4 h. The elemental concentrations in the
7 digested samples were determined by ICP-MS (Huang *et al.*, 2016b). The Mo
8 concentrations in shoots and roots of transgenic *A. thaliana* plants were determined
9 according to previous studies (Huang *et al.*, 2016a).

10 **Genetic and transgenic complementation test**

11 For genetic complementation, *osmot1;1* as well as WT T-DNA progeny were crossed with
12 HIF669.4-TQ and HIF669.4-LM, respectively. The concentrations of Mo in grains of F₁
13 plants from each cross were then determined. For transgenic complementation, the full-
14 length coding sequence of *OsMOT1;1* was PCR amplified using the cDNA of TQ or LM
15 as templates. The correct PCR fragments confirmed by sequencing were ligated into the
16 *SalI-SpeI* site of p1301GFP vector (Huang *et al.*, 2009) to generate the
17 *35S:OsMOT1;1(TQ)-GFP* and *35S:OsMOT1;1(LM)-GFP* constructs. The resulting
18 plasmids were transformed into *Agrobacterium tumefaciens* strain HA105 and introduced
19 into rice *japonica* cv. Zhonghua 11 as described previously (Hiei *et al.*, 1994). *osmot1;1*
20 was crossed with two transgenic *OsMOT1;1* overexpression lines, *35S:OsMOT1;1(TQ)-*
21 *GFP* and *35S:OsMOT1;1(LM)-GFP*. Two independent transgenic lines of
22 *35S:OsMOT1;1(TQ)-GFP* and *35S:OsMOT1;1(LM)-GFP* were used for crossing. In
23 subsequent F₂ populations, plants containing the transgene in homozygous *osmot1;1*
24 mutant background were identified by genotyping. Plants without the transgene in WT or
25 homozygous *osmot1;1* mutant background were used as controls. The grain Mo
26 concentrations in grains from these F₂ plants were determined by ICP-MS. The primer
27 sequences using for genotyping are listed in Table S2.

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

29 *OsMOT1;1* was expressed in an *A. thaliana atmot1;1* T-DNA mutant using the 35S
30 promoter or *AtMOT1;1* native promoter. The construction of *35S:OsMOT1;1(TQ)-GFP*

1 and *35S:OsMOT1;1(LM)-GFP* vectors was as described above. To generate the *AtMOT1;1*
2 promoter driven *OsMOT1;1* expression vectors, the 1,777-bp promoter sequence of
3 *AtMOT1;1* was PCR amplified from the genomic DNA of Col-0 using the primers listed
4 in Table S2. The PCR fragment confirmed by sequencing was used to substitute the 35S
5 promoters in the *35S:OsMOT1;1(TQ)-GFP* and *35S:OsMOT1;1(LM)-GFP* vectors to
6 generate *MOT1pro:OsMOT1;1(TQ)-GFP* and *MOT1pro:OsMOT1;1(LM)-GFP* vectors,
7 respectively. These plasmids were also transformed into *Agrobacterium tumefaciens* strain
8 GV3101 and introduced in *A. thaliana mot1* mutant (SALK_118311) using the floral dip
9 method (Clough & Bent, 1998). To assay low pH sensitivity, homozygous T3 transgenic
10 plants were grown on MGRL medium with Mo omitted. The low pH media was prepared
11 as described above by adding 35 μ l 6 M HCl into 100 ml media after autoclaving. Plants
12 were grown on plates horizontally in a climate-controlled room with temperature of 19-
13 22°C, photoperiod of 10 h light ($100 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$)/14 h dark and humidity of 60%.
14 The shoots were weighed after growing for 20 d.

15 **Functional analysis of *OsMOT1;1* in yeast**

16 The molybdate transporting activity of *OsMOT1;1* in yeast was determined according to
17 previous studies with modifications [12]. To generate the plasmids for expression of
18 *OsMOT1;1* in yeast (*Saccharomyces cerevisiae*), the full-length coding sequence of
19 *OsMOT1;1* was PCR amplified from the cDNA of TQ or LM using the primers listed in
20 Table S2. The correct PCR fragments confirmed by sequencing were subcloned into the
21 *EcoRI-XhoI* site of the yeast expression vector pYX222x (Tomatsu *et al.*, 2007). The
22 expression of *OsMOT1;1* in this plasmid was driven by a constitutive triose phosphate
23 isomerase promoter. The resultant plasmids and empty vector were transformed into yeast
24 strain BY4741 (MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0) using a Frozen-EZ Yeast
25 Transformation II Kit (ZYMO Research). For molybdate transporting assay, the BY4741
26 strains transformed with the empty vector pYX222x, *pYX222x-OsMOT1;1(TQ)* or
27 *pYX222x-OsMOT1;1(LM)* were inoculated at 30°C overnight in 3 mL of Mo-free SD/-His
28 media (6.7 g L⁻¹ yeast nitrogen base without amino acids and without molybdenum
29 (FORMEDIUM), 1.92 g L⁻¹ dropout mix without histidine, 2% (w/v) glucose). A 100 μ L
30 sample of overnight yeast cells was then transferred to 10 ml Mo-free SD/-His media and
31 incubated at 30°C until the optical density at 600 nm (O.D.600) reached to around 1.

1 Hexaammonium heptamolybdate was then added to the media to a final concentration of
2 0.5 μM . After shaking at 30°C for 30 min, yeast cells were harvested by centrifugation and
3 then washed three times with 1mM EDTA-Na and once with Milli-Q water. Cells were
4 dried at 80°C overnight, then digested with concentrated HNO_3 . The Mo concentration in
5 digested samples were determined by ICP-MS. The amount of yeast was converted by 1ml
6 of 1 O.D.600 culture containing 3×10^7 cells (Baxter *et al.*, 2008).

7 To test the sulfate transporting activity of OsMOT1;1, the empty vector pYX222x,
8 *pYX222x-OsMOT1;1(TQ)* and *pYX222x-OsMOT1;1(LM)* were transformed into a yeast
9 mutant CP154-7B (MATa, his3, leu2, ura3, ade2, trp1, sul1::LEU2, sul2::URA3) as above
10 (Tomatsu *et al.*, 2007). An *A. thaliana* high-affinity sulfate transporter gene *SULTR1;2* was
11 used as positive control. The overnight yeast cultures were collected by centrifugation and
12 washed once with Milli-Q water. After adjusting the O.D.600 of yeast cultures to 0.5, 10
13 μL yeast suspensions were spotted on synthetic medium (0.5 mM sulfate, 20 g L^{-1} glucose,
14 10 g L^{-1} agarose, 20 mg L^{-1} Ade, 30 mg L^{-1} L Leu, 20 mg L^{-1} Ura, and 20 mg of L^{-1} Trp)
15 with or without 20 mg L^{-1} methionine. The plates were incubated at 30°C for 6 days.

16 Other methods could be found in Methods S1.

17

18

19 **Results**

20 **Map-based cloning of *qGMo8***

21 We previously used two synthetic rice mapping populations composed of recombinant
22 inbred lines derived from a cross between Lemont (LM, *japonica*) and TeQing (TQ, *indica*)
23 (LT-RILs), and TeQing-into-Lemont backcross introgression lines (TILs), to identify
24 QTLs that control the variation in concentration of 16 elements in unmilled rice grain
25 (Zhang *et al.*, 2014). 134 QTLs were identified in either one or both mapping populations
26 grown under flooded and/or unflooded field conditions (Zhang *et al.*, 2014). Among 8
27 QTLs that control the variation in grain Mo concentration, one was detected on the top of
28 chromosome 8 (designated as *qGMo8*) in both mapping populations in field trials over
29 multiple years and in both flooded and unflooded fields. This QTL explains up to 35% of

1 the variation in grain Mo (Fig. 1a and 1b). The *qGMo8* first observed in grain from field
2 grown materials was also detected in both grain and leaf tissues from greenhouse grown
3 TILs, which allows us to fine map the QTL using plants cultivated in the greenhouse (Fig.
4 1b).

5 To narrow down the *qGMo8* mapping interval, we developed additional markers to
6 genotype the TIL population. Integration of grain Mo concentration and genotype data of
7 three TILs narrowed the QTL interval down to a 522 kb region on the top of chromosome
8 8 (Fig. 1c). In this region, a gene (LOC_Os08g01120) annotated as sulfate transporter
9 encodes a protein that shows 57.8% sequence similarity to *A. thaliana* high-affinity
10 molybdate transporter AtMOT1;1 (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008), which makes
11 it a strong candidate gene for *qGMo8* (Fig. S1a). We thus designated LOC_Os08g01120
12 as *OsMOT1;1*. Sequence analysis revealed a 9-basepair (bp) deletion (Del⁹) and a single
13 nucleotide polymorphism (SNP) in the coding sequence of *OsMOT1;1* in TQ (Fig. 1d, Fig.
14 S1b). The 9-bp deletion alters the amino acid sequence from four glutamines in LM to a
15 glutamic acid in TQ (Fig. 1d, Fig. S1b). The SNP in the coding sequence also alters the
16 amino acid sequence, with an alanine in LM and a serine in TQ at the position of 125
17 (S125A) (Fig. 1d, Fig. S1b). A 222-bp deletion (Del²²²) and a 16-bp deletion (Del¹⁶) were
18 found in the promoter sequence of *OsMOT1;1* in TQ. Meanwhile, there are 19 SNPs in the
19 promoter sequence of *OsMOT1;1* in TQ. Comparison of *OsMOT1;1* sequences of TQ and
20 LM with Nipponbare reference sequence identified a 12-bp deletion (Del¹²) in the coding
21 region of *OsMOT1;1* in both TQ and LM, and only a SNP in the promoter sequence of
22 *OsMOT1;1* between LM and Nipponbare (Fig. 1d). Sequence alignment showed that none
23 of the variable amino acids between TQ and LM are conserved in the MOT1 protein family
24 (Fig. S1a), suggesting that these amino acids might not alter the function of OsMOT1;1.

25 The existence of residual heterozygosity in TILs is useful for developing appropriate nearly
26 isogenic lines (NIL) by generating heterogeneous inbred families (HIFs) (Tuinstra *et al.*,
27 1997; Loudet *et al.*, 2005; Huang *et al.*, 2016b). We identified TIL669.4 which is
28 heterozygous at the *OsMOT1;1* locus, and isolated HIF669.4-TQ and HIF669.4-LM in the
29 next generation (Fig. S2). These two HIFs have similar genomic background and only
30 differ in a small genomic region containing homozygous *OsMOT1;1* alleles from TQ or
31 LM, respectively. Both grain and leaf Mo concentrations of HIF669.4-TQ are significant

1 lower than that of HIF669.4-LM, suggesting that the TQ allele is a weak allele (Fig. 1e).
2 Quantitative RT-PCR analysis showed that the expression levels of *OsMOT1;1* in both
3 shoots and roots of HIF669.4-TQ were significantly lower than that of HIF669.4-LM,
4 suggesting that the low Mo in HIF669.4-TQ might be due to the lower expression level of
5 *OsMOT1;1* (Fig. 1f).

6 ***OsMOT1;1* is the causal gene for *qGMo8***

7 To investigate the function of *OsMOT1;1*, we isolated a T-DNA insertion mutant of
8 *OsMOT1;1*. The T-DNA insertion at the promoter of *OsMOT1;1* significantly decreases
9 its expression (Fig. S3a- S3c). The *osmot1;1* mutant has no obvious phenotype difference
10 from the wild-type (WT) when grown in soil in a greenhouse (Fig. 2a). Elemental profile
11 analysis showed that the Mo concentration in the grain of *osmot1;1* is approximately 58%
12 lower than that of the WT ($P < 0.001$, Student's *t*-test, $n = 10$) (Fig. 2b and 2c). The low
13 Mo phenotype was also observed in the blades and sheaths of *osmot1;1* (Fig. 2b). Of the
14 22 elements determined, Mo is the only element that is significantly changed in the grain,
15 leaves and sheaths of *osmot1;1*, suggesting a specific effect of *OsMOT1;1* mutation on Mo
16 concentrations (Fig. 2c). To test whether *OsMOT1;1* is the causal gene for *qGMo8*, we
17 performed an allelic complementation by crossing *osmot1;1* as well as WT with HIF669.4-
18 TQ and HIF669.4-LM, respectively. The expression level of *OsMOT1;1* in the roots of
19 both HIF669.4-TQ and HIF669.4-LM was higher than in that of *osmot1;1* (Fig. S3e). The
20 Mo concentrations in both leaves and grain of *osmot1;1* × HIF669.4-TQ and *osmot1;1* ×
21 HIF669.4-LM F1 plants grown in soil in a greenhouse were significantly higher than that
22 of *osmot1;1* (Fig. 2d and 2e), similar to the levels in HIF669.4-TQ and HIF669.4-LM,
23 respectively, suggesting the complementation of *OsMOT1;1* from both TQ and LM to the
24 knockout allele. However, significant differences of both leaf and grain Mo concentrations
25 between *osmot1;1* × HIF669.4-TQ F1 and *osmot1;1* × HIF669.4-LM F1 were observed
26 (Fig. 2d and 2e), indicating differential functional activity of *OsMOT1;1* between TQ and
27 LM. To further confirm *OsMOT1;1* as the causal gene, we crossed *osmot1;1* with
28 *OsMOT1;1* overexpression lines in which *OsMOT1;1* from either TQ or LM was expressed
29 from a cauliflower mosaic virus (CaMV) 35S promoter in the cv. Zhonghua 11 background.
30 When grown in soil in a greenhouse, the Mo concentrations in grains of F2 plants
31 containing the transgene in homozygous *osmot1;1* mutant background was significantly

1 higher than those homozygous *osmot1;1* plants without the transgene (Fig. 2e). These
2 results demonstrate that overexpression of *OsMOT1;1* is able to complement the low Mo
3 phenotype of the *osmot1;1* mutant.

4 **Low Mo Phenotype of *osmot1;1* knockout mutant**

5 At grain maturity stage, the *osmot1;1* mutant grown in soil in the greenhouse has a lower
6 concentration of Mo than WT not only in the grain, but also in the blade and sheath of the
7 flag leaf, and most of the nodes and internodes of the main tiller (Fig. 3a). Analysis of the
8 seedlings grown hydroponically in the nutrient solution containing 1 nM Mo showed that
9 *osmot1;1* accumulated significant lower Mo in both roots and shoots (Fig. 3b and 3c).
10 However, the difference in the root Mo concentration between *osmot1;1* and WT
11 disappeared when plants were grown in the nutrient solution containing 10 nM or higher
12 concentration of Mo (Fig. 3b). Similarly, significant differences in the shoot Mo
13 concentration were observed only at the low levels of Mo supply (1 and 10 nM), but not at
14 the high levels of Mo supply (100 nM or 1 μ M) (Fig. 3c). These results suggest that
15 *OsMOT1;1* might function mainly at low Mo concentration. Consistent with lower Mo
16 level in shoots, the Mo concentration in the xylem sap of *osmot1;1* was significantly
17 decreased compared to the WT (Fig. 3d), suggesting that Mo translocation from roots to
18 shoots was affected in *osmot1;1*. Further analysis showed that the Mo concentrations in all
19 tissues except the leaf sheaths of the 5th and 6th leaves of *osmot1;1* were significantly lower
20 than that of WT (Fig. 3e).

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

22 Given that *osmot1;1* accumulates less Mo in shoots, we investigated the sensitivity of
23 *osmot1;1* to Mo deficiency. The bioavailability of Mo is strongly dependent on the soil pH
24 with Mo becoming much less bioavailable in acid soils (Marschner & Rengel, 2012). When
25 grown in acidified solid media without supplementation of Mo, most of the seeds of
26 *osmot1;1* did not germinate, and the growth of the plants from seeds that did germinate
27 was strongly inhibited (Fig. 4a and 4b). In contrast, the WT plants were able to germinate
28 and grow, even though the root growth was also inhibited by low pH (Fig. 4a and 4b).
29 These results establish that, without Mo added into the media, *osmot1;1* is more sensitive
30 to low pH compared to the WT. Supplementation of 1 μ M Mo to the acidified solid media

1 was able to restore the growth of *osmot1;1* to the level of WT (Fig. 4a and 4b), indicating
2 that the sensitivity of *osmot1;1* to low pH is due to the deficiency of Mo in the media. We
3 further showed that the HIF669.4-TQ, which accumulated less Mo (Fig. 1e), was more
4 sensitive to Mo deficiency at low pH condition compared to the HIF669.4-LM (Fig. S4a
5 and S4b).

6 **Expression pattern and subcellular localization of OsMOT1;1**

7 *OsMOT1;1* was expressed in most of the plant tissues throughout the growth period of LM
8 except the panicles at the reproductive stage (Fig. 5a). However, the expression of
9 *OsMOT1;1* was much stronger in the roots than in the other tissues. In roots of 2-week-old
10 seedlings, *OsMOT1;1* was strongly expressed in the lateral roots as investigated by
11 *OsMOT1;1* promoter-GUS transgenic rice plants (Fig. 5b). Low levels of GUS signals
12 were detected in other tissues. To test whether the expression of *OsMOT1;1* was affected
13 by Mo supply, LM plants were grown in nutrient solution containing 1 μ M Mo for one
14 week and transferred to nutrient solution with Mo omitted for a further week. The
15 expression of *OsMOT1;1* in roots was strongly suppressed by Mo depletion as determined
16 by qRT-PCR. However, such suppression was not found in shoots (Fig. 5c).

17 To investigate the subcellular localization of OsMOT1;1, OsMOT1;1 from TQ or LM was
18 fused with GFP to the C-terminal and expressed under the control of 35S promoter in a
19 japonica variety Zhonghua 11 (Fig. S3d). The GFP fluorescence was co-localized with the
20 signal of a mitochondrial dye Mitotacker, suggesting OsMOT1;1 localizes to the
21 mitochondria (Fig. 5d). We observed that OsMOT1;1 from both TQ and LM were localized
22 to the mitochondria, indicating that the amino acid variation of OsMOT1;1 between TQ
23 and LM has no effect on the subcellular localization (Fig. 5d). Furthermore, the Mo
24 concentrations in roots and shoots of transgenic lines were higher than the non-transgenic
25 control line, suggesting the mitochondria-localized OsMOT1;1 is functional (Fig. S5).

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

27 OsMOT1;1 shares 57.8% sequence similarity to AtMOT1;1 (Fig. S1a), a high-affinity
28 molybdate transporter in *A. thaliana*. The *atmot1;1* mutant accumulates lower levels of Mo
29 in leaves than WT (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008). To investigate whether
30 expression of *OsMOT1;1* in *atmot1;1* could suppress its low Mo phenotype, we

1 heterologously expressed *OsMOT1;1-GFP* in *atmot1;1* using 35S promoter (Fig. S6). In
2 the root cells of the transgenic plants, the GFP signals were observed to co-localize with
3 the mitochondria specific dye MitoTracker (Fig. S7), further confirming the mitochondria
4 localization of *OsMOT1;1*. The Mo concentrations in both roots and shoots of transgenic
5 plants expressing *OsMOT1;1* from either TQ or LM were significantly higher than that of
6 *atmot1;1*, even higher than the WT Col-0 (Fig. 6a). These results suggest that expression
7 of *OsMOT1;1* from either TQ or LM could enhance Mo accumulation in *atmot1;1*. To rule
8 out the ectopic effect of overexpression of *OsMOT1;1* driven by 35S promoter, we also
9 expressed *OsMOT1;1* in *atmot1;1* using *AtMOT1;1* native promoter (Fig. S6). The Mo
10 concentrations in both roots and shoots of transgenic plants were significantly higher than
11 that of *atmot1;1*, similar to the level in WT Col-0 (Fig. 6b), suggesting that expression of
12 *OsMOT1;1* using *AtMOT1;1* native promoter was able to complement the *AtMOT1;1*
13 knockout mutant. Notably, there was no significant difference in either root or shoot Mo
14 concentration between transgenic plants expressing *OsMOT1;1* from TQ or LM (Fig. 6b),
15 indicating no functional difference between the *OsMOT1;1* allele of TQ and LM. Previous
16 studies have shown that the *atmot1;1* mutant is sensitive to low Mo stress (Tomatsu *et al.*,
17 2007) and shows defective growth in acidic soil in which the bioavailability of Mo is low
18 (Poormohammad Kiani *et al.*, 2012). Similar to the sensitivity to acidic soil, *atmot1;1* also
19 showed growth inhibition on agar media at low pH without added Mo (Fig. 6c to 6e). The
20 transgenic plants expressing *OsMOT1;1* from TQ or LM using 35S promoter or *MOT1*
21 native promoter completely restore the growth of *atmot1;1* on agar media at low pH (Fig.
22 6c to 6e). The results further confirm that *OsMOT1;1* is able to complement the mutation
23 of *AtMOT1;1* in *A. thaliana*.

24 **Molybdate transport activity of OsMOT1;1**

25 The molybdate transport activity of *OsMOT1;1* was tested by heterologous expression of
26 *OsMOT1;1* in yeast (*Saccharomyces cerevisiae*) strain BY4741. Yeast strain BY4741
27 transformed with empty vector or *OsMOT1;1* from TQ or LM were cultured in Mo-free
28 media to the mid-log phase and then transferred to the media containing 0.5 μ M Mo and
29 incubated for 30 min. The Mo concentration in yeast cells transformed with *OsMOT1;1*
30 was significantly higher than the control strain transformed with an empty vector (Fig. 7a).
31 These results support the conclusion that *OsMOT1;1* is able to transport molybdate. Further

1 comparison revealed no difference of Mo concentrations in the strains transformed with
2 *OsMOT1;1* from TQ or LM (Fig. 7a), indicating that *OsMOT1;1* from TQ and LM
3 exhibited similar molybdate transporting activity.

4 *OsMOT1;1* shares sequence similarity to sulfate transporter genes in rice and thus was
5 previously annotated as a member of the group five sulfate transporter family (Kumar *et al.*,
6 2011). To determine whether *OsMOT1;1* exhibits a sulfate transport activity, we
7 performed complementation analysis of a yeast mutant CP154-7B, which is defective in
8 two high-affinity sulfate transporters and is unable to grow on the media containing lower
9 than 1 mM sulfate as the sole sulfur source (Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002;
10 Tomatsu *et al.*, 2007). The yeast mutant transformed with *SULTR1;2*, an *A. thaliana* high-
11 affinity sulfate transporter, was able to grow on the –Met media. However, expression of
12 *OsMOT1;1* from either TQ or LM was unable to complement the growth defect of the
13 mutant strain on –Met media (Fig. 7b). These results suggest that *OsMOT1;1* likely does
14 not exhibit sulfate transport activity.

15 **Analysis of natural variation of *OsMOT1;1***

16 To investigate the contribution of natural variation at *OsMOT1;1* to the variation of grain
17 Mo in rice, we analyzed the coding sequences of 1,479 rice accessions which have been
18 resequenced (Zhao *et al.*, 2015). Five non-synonymous polymorphisms in the coding
19 sequence of *OsMOT1;1* were identified, including the two polymorphic sites between TQ
20 and LM (S125A and Del⁹), and the 12 bp deletion (Del¹²) in both TQ and LM (Table S1
21 and Fig. 1d). To determine the contribution of these three polymorphic sites on the
22 variation of grain Mo, we genotyped 1,097 accessions of USDA rice core collection for
23 which we have previously reported the grain ionic profile (Pinson *et al.*, 2015). We
24 found no significant difference of grain Mo between the two alleles on any of these three
25 polymorphic sites (Fig. 8a, Fig. S8a). We further compared the grain Mo concentrations of
26 eight haplotypes derived from the combination of these three polymorphic sites. We
27 observed no significant differences of grain Mo among the eight haplotypes (Fig. 8b and
28 Fig. S8b). These results suggested that the variation in the coding region of *OsMOT1;1*
29 might not contribute to the variation of grain Mo in the rice population.

1 There are 19 SNPs and two sequence deletions (Del²²² and Del¹⁶) in the promoter sequence
2 of *OsMOT1;1* in TQ (Fig. 1d). We determined the contribution of the two major sequence
3 variances, Del²²² and Del¹⁶, on the variation of grain Mo. We genotyped the 1,097
4 accessions of USDA core collection and compared the grain Mo concentrations of
5 accessions with or without the deletions. We found that there was no significant difference
6 between the accessions with or without the Del²²² or Del¹⁶ (Fig. 8c and Fig. S8c).
7 Furthermore, the grain Mo concentrations among the accessions with combination of
8 Del²²² and Del¹⁶ were fairly similar (Fig. 8d and Fig. S8d). These results suggested that
9 Del²²² and Del¹⁶ in the promoter of *OsMOT1;1* might not contribute to the variation of
10 grain Mo. Thus, the different promoter activity of *OsMOT1;1* between TQ and LM was
11 likely due to the 19 SNPs, which might cause different expression level of *OsMOT1;1*
12 between TQ and LM.

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

14 To determine whether the variation of grain Mo was due to the variation in the *OsMOT1;1*
15 expression level, we selected 35 rice accessions from the USDA core collection with grain
16 Mo concentrations ranging from 0.063 to 1.52 $\mu\text{g g}^{-1}$ under flooded growth condition and
17 0.084 to 1.23 $\mu\text{g g}^{-1}$ under unflooded condition. The expression levels of *OsMOT1;1* were
18 determined in the roots of three-week-old plants grown hydroponically with 1 μM Mo. We
19 found highly significant correlations between the expression level of *OsMOT1;1* in roots
20 from hydroponically grown plants with the Mo concentrations in grains from plants grown
21 in the field under both flooded (Pearson's $R^2 = 0.7368$, $p < 0.001$) (Fig. 8e) and unflooded
22 (Pearson's $R^2 = 0.6207$, $p < 0.001$) conditions (Fig. S8e). These results suggest the natural
23 variation of Mo concentration in rice grains is attributed to the variable expression of
24 *OsMOT1;1* in roots.

25

26 **Discussion**

27 As one of the essential mineral nutrients required by plants, Mo plays important roles in
28 nitrate assimilation, abscisic acid biosynthesis, purine degradation and sulfite
29 detoxification (Schwarz & Mendel, 2006; Bittner, 2014). However, the mechanisms of Mo
30 uptake and transport and the regulation these processes are largely unknown in plants,

1 especially in crops. In this study, we identified a QTL *qGMo8* that controls the variation
2 of Mo concentration in rice shoots and grains. We determined the causal gene for this QTL
3 to be *OsMOT1;1* by genetic and transgenic complementation (Fig. 2d and 2e). *OsMOT1;1*
4 was previously annotated as a member of the group V sulfate transporter superfamily
5 (Tejada-Jimenez *et al.*, 2013). We provided evidence that *OsMOT1;1* is a molybdate
6 transporter, including the fact that *OsMOT1;1* enhances molybdate but not sulfate uptake
7 when heterogeneously expressed in yeast (Fig. 7a and 2b). We found that the difference in
8 grain Mo concentrations between rice cultivars TQ and LM was not due to altered
9 molybdate transporting activity but most likely to the allelic variation at the gene
10 expression level of *OsMOT1;1*. This conclusion is supported by several lines of evidence.
11 First, the molybdate transporting activity of *OsMOT1;1* from TQ and LM was similar when
12 heterogeneously expressed in yeast (Fig. 7a). Second, the *OsMOT1;1* from either TQ or
13 LM was able to complement the low Mo phenotype of *atmot1;1* to a similar level when
14 expressed by the *AtMOT1;1* native promoter (Fig. 6b, 6c and 6e). Third, the heterogeneous
15 inbred families line HIF669.4-LM with higher expression level of *OsMOT1;1* accumulates
16 more Mo in the leaves and grains than that of HIF669.4-TQ. Knockout of *OsMOT1;1*
17 resulted in decreased Mo concentration in the shoots and grains (Fig. 2b and 2c). We thus
18 conclude that *OsMOT1;1* is a molybdate transporter in rice and the natural variation of Mo
19 concentration in rice grains is attributed to the allelic variation at the gene expression level
20 of *OsMOT1;1*.

21 Several molybdate transporters have been identified, including CrMOT1 and CrMOT2
22 from *C. reinhardtii* (Tejada-Jimenez *et al.*, 2007; Tejada-Jimenez *et al.*, 2011), *AtMOT1;1*
23 and *AtMOT1;2* from *A. thaliana* (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008; Gasber *et al.*,
24 2011), *LjMOT1* from *Lotus japonicus* (Gao *et al.*, 2016; Duan *et al.*, 2017) and *MtMOT1.3*
25 from *Medicago truncatula* (Tejada-Jimenez *et al.*, 2017). *AtMOT1;1* has been shown to
26 control the natural variation in leaf Mo concentration in *A. thaliana* (Baxter *et al.*, 2008).
27 A 53-bp deletion in the promoter of *AtMOT1;1* which is located 13 bp upstream from the
28 transcription start-site of *AtMOT1;1* was identified as the functional polymorphism
29 contributing to decreased leaf Mo concentration (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008).
30 Further analysis revealed a total of six non-coding structural polymorphisms in the
31 *AtMOT1;1* promoter, including the 53-bp deletion originally identified in *Ler-0* and a

1 duplicated 330-bp insertion that has undergone a 4-bp deletion (Forsberg *et al.*, 2015). The
2 53-bp deletion is associated with decreased leaf Mo, while the duplicated 330-bp insertion
3 is associated with elevated leaf Mo. These two polymorphisms control Mo concentration
4 in leaves by either decreasing or increasing, respectively, the expression level of *AtMOT1;1*.
5 In this study, we identified 19 SNPs and two deletions, a 222-bp deletion (Del²²²) and a 16-
6 bp deletion (Del¹⁶), in the promoter of *OsMOT1;1* between TQ and LM (Fig. 1d). We
7 showed that the difference in grain Mo concentration between TQ and LM was due to the
8 different expression level of *OsMOT1;1* (Fig. 1e and 1f). Further analysis of 35 rice
9 accessions revealed a significant positive correlation between the expression level of
10 *OsMOT1;1* in roots and Mo concentration in grains (Fig. 8e and Fig. S8e). However, the
11 variation in grain Mo appears unrelated to the two main non-coding structural
12 polymorphisms, Del²²² and Del¹⁶, as grain Mo concentrations of rice accessions with or
13 without these two deletions were similar (Fig. 8c and 8d, Fig. S8c and S8d). Therefore, the
14 difference in grain Mo concentration between TQ and LM may be due to the SNPs in the
15 promoter of *OsMOT1;1* which lead to different expression level. At least two SNPs which
16 are significantly associated with the variation of leaf Mo were also identified on the
17 *AtMOT1;1* locus, even though the effect of these SNPs on its expression is not clear
18 (Forsberg *et al.*, 2015). Thus, the variation of Mo in both rice and *A. thaliana* is attributed
19 to the variable expression of the *MOT1;1* gene. Such conserved genetic variation structure
20 across plant species suggests a role of *MOT1;1* gene in the adaptation to the environment.
21 Indeed, *A. thaliana* accessions with the weak allele of *AtMOT1;1* from West Asia appear
22 to adapt to their native habitats where the water extractable Mo content is high in soils
23 (Poormohammad Kiani *et al.*, 2012). We found that the *osmot1;1* mutant is sensitive to
24 limited Mo supply condition (Fig. 4a and 4b), similar to the sensitivity of *atmot1;1* to Mo
25 deficiency (Tomatsu *et al.*, 2007) and to acidic soils in which the bioavailability of Mo is
26 low (Poormohammad Kiani *et al.*, 2012). Therefore, the *MOT1;1* gene may play an
27 important role in adaptation to variable molybdate availability in soils caused by
28 environmental changes.

29 The polymorphisms in the coding region of *AtMOT1;1* also affect its function. A single
30 amino acid variation on *AtMOT1;1* in *Ler-0* accession alters its molybdate transporting
31 activity (Tomatsu *et al.*, 2007). The hypofunction of *AtMOT1;1* in *Sha* accession was also

1 proved to be caused by a single amino acid change (Poormohammad Kiani *et al.*, 2012).
2 In the present study, we found two amino acid polymorphisms in *OsMOT1;1* between TQ
3 and LM (Fig. 1d). However, none of them appears to alter the function of *OsMOT1;1* as
4 these two version proteins showed similar molybdate transporting activity and was able to
5 complement the low Mo phenotype of the *atmot1;1* mutant to a similar level (Fig. 6b, 6c
6 and 6e; Fig. 7a). A 12-bp deletion (Del¹²) in the coding region of *OsMOT1;1* of both TQ
7 and LM, compared with Nipponbare, seems not to change its function because the
8 presence/absence of Del¹² is not associated with the variation of grain Mo (Fig. 8a and 8b,
9 Fig. S8a and S8b). Thus, the large sequence diversity of the *MOT1;1* locus explains well
10 the identification of *AtMOT1;1* in controlling the variation of leaf Mo by genome-wide
11 association (GWA) analysis based on either the mean or variance of leaf Mo (Shen *et al.*,
12 2012; Forsberg *et al.*, 2015), and *OsMOT1;1* as the potential locus responsible for the
13 variation of rice grain Mo concentration (Norton *et al.*, 2014).

14 The expression of *OsMOT1;1* is stronger in roots than in shoots (Fig. 5a), similar to the
15 expression pattern of *AtMOT1;1* in *A. thaliana* (Tomatsu *et al.*, 2007), which is consistent
16 with the fact that *AtMOT1;1* mainly functions in roots (Baxter *et al.*, 2008). Under Mo
17 limited condition, *OsMOT1;1* is downregulated in roots but not in shoots (Fig. 5a).
18 However, such downregulation was not observed for *AtMOT1;1* in roots but rather in
19 shoots (Tomatsu *et al.*, 2007), suggesting the different behaviour of *MOT1;1* genes in rice
20 and *A. thaliana* under Mo scarce condition. *OsMOT1;1* may mainly function under low
21 Mo condition, as we only observed the difference of Mo concentrations between WT and
22 *osmot1;1* in the hydroponic growth system containing 1 or 10 nM Mo but not at relative
23 higher Mo condition (100 nM or 1 μ M) (Fig. 3b and 3c). The Mo concentration in the
24 xylem sap of *osmot1;1* is lower than that of WT (Fig. 3d), suggestion *OsMOT1;1* is also
25 involved in the translocation of Mo from roots to shoots. Meanwhile, the Mo
26 concentrations in the grain, blade and sheath of *osmot1;1* only decrease by 58% - 82%
27 compared to the WT (Fig. 2d), suggesting the existence of additional transporters that
28 control the accumulation of Mo in rice. Further studies are required to elucidate the detailed
29 function of *OsMOT1;1* and to identify other transporters in controlling the Mo homeostasis
30 in rice.

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

8

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19 **Author contribution**

20 X.-Y.H. and D.E.S. designed the research; X.-Y.H., H.L., Y.-F.Z., S.R.M.P., H.-X.L., and
21 M.L.G. performed the experiments. X.-Y.H. and D.E.S. analysed the data. X.-Y.H., F.-J.Z.,
22 and D.E.S. wrote the paper with contributions of S.R.M.P. and M.L.G.

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11 12 13 **Figure legends**

14 **Fig. 1. QTL analysis and cloning of *qGMo8*.**

15 (a and b) The LOD profiling of *qGMo8* on chromosome 8 in the LT-RIL population (a)
16 and TIL population (b) grown in multiple years under different conditions. F, flooded; U,
17 unflooded. G, grains of TILs grown in greenhouse; L, leaves of greenhouse grown TILs.
18 (c) Grain Mo concentration and genotype at QTL interval of selected TILs. Data are
19 presented as mean \pm SD ($n = 3$). Columns with different letters indicate significant
20 difference at $P \leq 0.01$ (Tukey's Honestly Significant Difference (HSD) test). DW, dry
21 weight. (d) Gene structure and sequence variation of *OsmOT1;1* among Nipponbare (Nipp),
22 TQ and LM. Blue bars and white bars represent exons and untranslated regions,
23 respectively. Vertical lines represent SNPs. (e) The Mo concentration in the grain and leaf
24 of HIF669.4-TQ and HIF669.4-LM. (f) Expression level of *OsmOT1;1* in shoots and roots
25 of HIF669.4-TQ and HIF669.4-LM. Data in (e) and (f) are presented as mean \pm SD with
26 $n = 6$ in (e) and 3 in (f). ** indicates significant difference at $P \leq 0.01$ (Student's *t* test).

27 **Fig. 2. Characterization of the *osmot1;1* mutant and complementation test.**

28 (a) Greenhouse grown WT and *osmot1;1* plants grown at harvesting stage. (b) Mo
29 concentrations in the grain, blade and sheath of WT and *osmot1;1*. Data are presented as
30 means \pm SD ($n = 8$). ** indicates significant difference between WT and *osmot1;1* at $P \leq$

1 0.01 (Student's *t* test). (c) Percentage difference of 22 elements in the grain, blade and
2 sheath of *osmot1;1* compared with the WT. Data are visualized in the radar chart. (d)
3 Genetic complementation of *osmot1;1* by crossing with HIF669.4-TQ or HIF669.4-LM.
4 The Mo concentrations in the grain of F1 plants were determined. (e) Transgenic
5 complementation of *osmot1;1* by crossing with *OsMOT1;1* overexpression lines. The grain
6 Mo concentrations of F2 plants in a homozygous mutant background containing
7 *35S:OsMOT1;1(TQ)-GFP* or *35S:OsMOT1;1(LM)-GFP* constructs were determined. Two
8 independent complemented lines were used for crossing. Data in (d, e) are presented as
9 boxplots ($n = 6$). Boxes with different letters indicate significant difference at $P \leq 0.01$
10 (Tukey's HSD test). DW, dry weight.

11 **Fig. 3. Low Mo phenotype of the *osmot1;1* mutant.**

12 (a) The Mo concentrations in different tissues of greenhouse grown WT and *osmot1;1*
13 plants at harvesting stage. (b and c) The Mo concentrations in roots (b) and shoots (c) of
14 WT and *osmot1;1*. Plants were hydroponically grown in nutrient solution containing
15 various concentrations of Mo for two weeks. (d) The Mo concentration in the xylem sap
16 of WT and *osmot1;1*. Plants were hydroponically grown in Mo-free nutrient solution for
17 one week and treated with various concentrations of Mo for another week. (e) The Mo
18 concentrations in different tissues of WT and *osmot1;1* seedlings. Plants were
19 hydroponically grown in nutrient solution containing 10 nM of Mo for two weeks. Data (a)
20 are presented as boxplots ($n = 11$ for WT and $n = 10$ for *osmot1;1*). Data in (b-e) are
21 presented as means \pm SD with $n = 4$ in (b, c), 8 in (d, e). * and ** indicate significant
22 difference between WT and *osmot1;1* at $P \leq 0.05$ and 0.01 (Student's *t* test), respectively.
23 DW, dry weight.

24 **Fig. 4. The *osmot1;1* mutant is sensitive to Mo deficiency at low pH condition.**

25 (a) The phenotype of WT and *osmot1;1* grown under normal or low pH media with or
26 without 1 μ M Mo added. Low pH media was made by adding 50 μ L 6 M HCl to 100 mL
27 agar media after autoclaving. Plants were grown for 20 days. Bar, 3 cm. (b) The plant
28 height of WT and *osmot1;1* grown as in (a). Data are presented as means \pm SD with $n = 6$.
29 *** indicated significant difference between WT and *osmot1;1* at $P \leq 0.001$ (Student's *t*
30 test).

1 **Fig. 5. Expression pattern of *OsMOT1;1*.**

2 (a) Expression level of *OsMOT1;1* in different organs of Lemont at different growth stages.
3 (b) Histochemical GUS staining of roots of transgenic rice plants transformed with
4 *OsMOT1;1* promoter driven GUS constructs. The mature zone (left), elongation zone
5 (middle) and root tip (right) of roots of 2-week-old plants are showed. Scale bars from left
6 to right are 2, 0.5 and 2 mm. (c) Expression of *OsMOT1;1* was suppressed in roots under
7 Mo deficiency. Lemont (LM) plants were grown hydroponically with 1 μ M Mo for one
8 week and then treated with 1 μ M Mo (+Mo) or without Mo (-Mo) for another week.
9 Relative expression level of was determined by qRT-PCR with three biological replicates.
10 ** indicates significant difference at $P \leq 0.01$ (Student's *t* test). (d) Subcellular localization
11 of *OsMOT1;1* in stable transgenic rice plants. *OsMOT1;1* from TeQing (TQ) or LM were
12 fused with GFP at N-terminal and overexpressed under the control of CaMV 35S promoter.
13 Mitochondria were stained with the specific dye MitoTracker. Scale bar, 10 μ m.

14 **Fig. 6. Functional analysis of *OsMOT1;1* in *Arabidopsis atmot1;1* mutant.**

15 (a and b) Mo concentrations in roots and shoots of the *mot1* mutant transformed with
16 *OsMOT1;1-GFP* from TeQing (TQ) or Lemont (LM) driven by CaMV 35S promoter (a)
17 or *AtMOT1;1* native promoter (b). Plants were grown on MGRL media containing 24 nM
18 Mo for two weeks. Three independent transgenic lines are shown. (c) Phenotype of
19 *OsMOT1;1-GFP* transgenic lines in *mot1* background. Plants were grown on MGRL media
20 without added Mo at control or low pH condition for 20 days. Bar, 1 cm. (d and e) Fresh
21 weight of *mot1* transformed with *OsMOT1;1-GFP* from TeQing (TQ) or Lemont (LM)
22 driven by CaMV 35S promoter (d) or *AtMOT1;1* native promoter (e). Plants were grown
23 as in (c). Three independent transgenic lines are shown. Data in (a) and (b) are shown as
24 means \pm SD with three biological replicates. Six plants were combined in each replicate.
25 Data in (d) and (e) are shown as boxplots with $n = 7$ to 12. Columns or boxplots with
26 different letters in (a) to (e) indicate significant difference at $P \leq 0.01$ (Tukey's HSD test).
27 DW, dry weight.

28 **Fig. 7. Functional analysis of *OsMOT1;1* in yeast.**

29 (a) Molybdate transport activity of *OsMOT1;1*. Yeast strains transformed with empty
30 vector, or *OsMOT1;1* from TeQing (TQ) or Lemont (LM) were incubated in media

1 containing 0.5 μ M Mo for 30 min. Mo concentrations in yeast cells were determined. Data
2 are shown as boxplot with $n = 3$. Boxplots with different letters indicate significant
3 difference at $P \leq 0.01$ (Tukey's HSD test). (b) Complementation analysis of a yeast mutant
4 defective in sulfate uptake. The yeast mutant strains transformed with empty vector,
5 *SULTR1;2*, or *OsMOT1;1* from TQ or LM were incubated on media containing 0.5 mM
6 sulfate with or without added Met for four days.

7 **Fig. 8. Contribution of allelic variation of *OsMOT1;1* on the variation of grain Mo in**
8 **USDA rice core collection grown in flooded condition.**

9 (a) The grain Mo in rice accessions with different alleles at three polymorphic sites in the
10 coding region of *OsMOT1;1*. (b) The grain Mo in rice accessions with different haplotypes
11 derived from the combination of three polymorphic sites in the coding region of *OsMOT1;1*.
12 (c) The grain Mo in rice accessions with or without the deletions in the promoter region of
13 *OsMOT1;1*. (d) The grain Mo in rice accessions with different promoter haplotypes derived
14 from the combination of two deletions in the coding region of *OsMOT1;1*. Data in (a - d)
15 are shown as boxplots. Numbers under the boxes are the accession number; numbers above
16 the boxes are the P values; the same letters above the boxes indicate no significant
17 difference at $P \leq 0.01$ (Tukey's HSD test). + and - represent the presence and absence of
18 the deletion, respectively. The dash color boxes in (b) and (d) mark the haplotypes in TQ ,
19 LM and Nipp. (e) The correlation of the relative expression of *OsMOT1;1* in roots with the
20 grain Mo concentration in 35 rice accessions grown in flooded condition. The expression
21 of *OsMOT1;1* in the roots of 3-week-old plants were determined by qRT-PCR. The relative
22 expression level of *OsMOT1;1* was normalized the rice actin gene, and presented as the
23 mean of $2^{-(\Delta\Delta Ct)}$ with three biological replicates.

24

25 **Supporting Information**

26 Additional supporting information may be found in the online version of this article.

27 **Fig. S1** Sequence alignment of MOT1 proteins.

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

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

- 1 **Fig. S4** The sensitivity of HIF669.4-TQ and HIF669.4-LM to Mo deficiency at low pH
2 condition.
- 3 **Fig. S5** Overexpression of *OsMOT1;1* increases Mo concentrations in both roots and
4 shoots.
- 5 **Fig. S6** Expression level of *OsMOT1;1* in Arabidopsis transgenic lines.
- 6 **Fig. S7** Subcellular localization of *OsMOT1;1* in Arabidopsis.
- 7 **Fig. S8** Contribution of allelic variation of *OsMOT1;1* on the variation of grain Mo in
8 USDA rice core collection grown in unflooded condition.
- 9 **Table S1.** Sequence variation and allele frequency of *OsMOT1;1*.
- 10 **Table S2.** The primers used in this study.
- 11 **Methods S1** Supporting information for Materials and Methods.
- 12

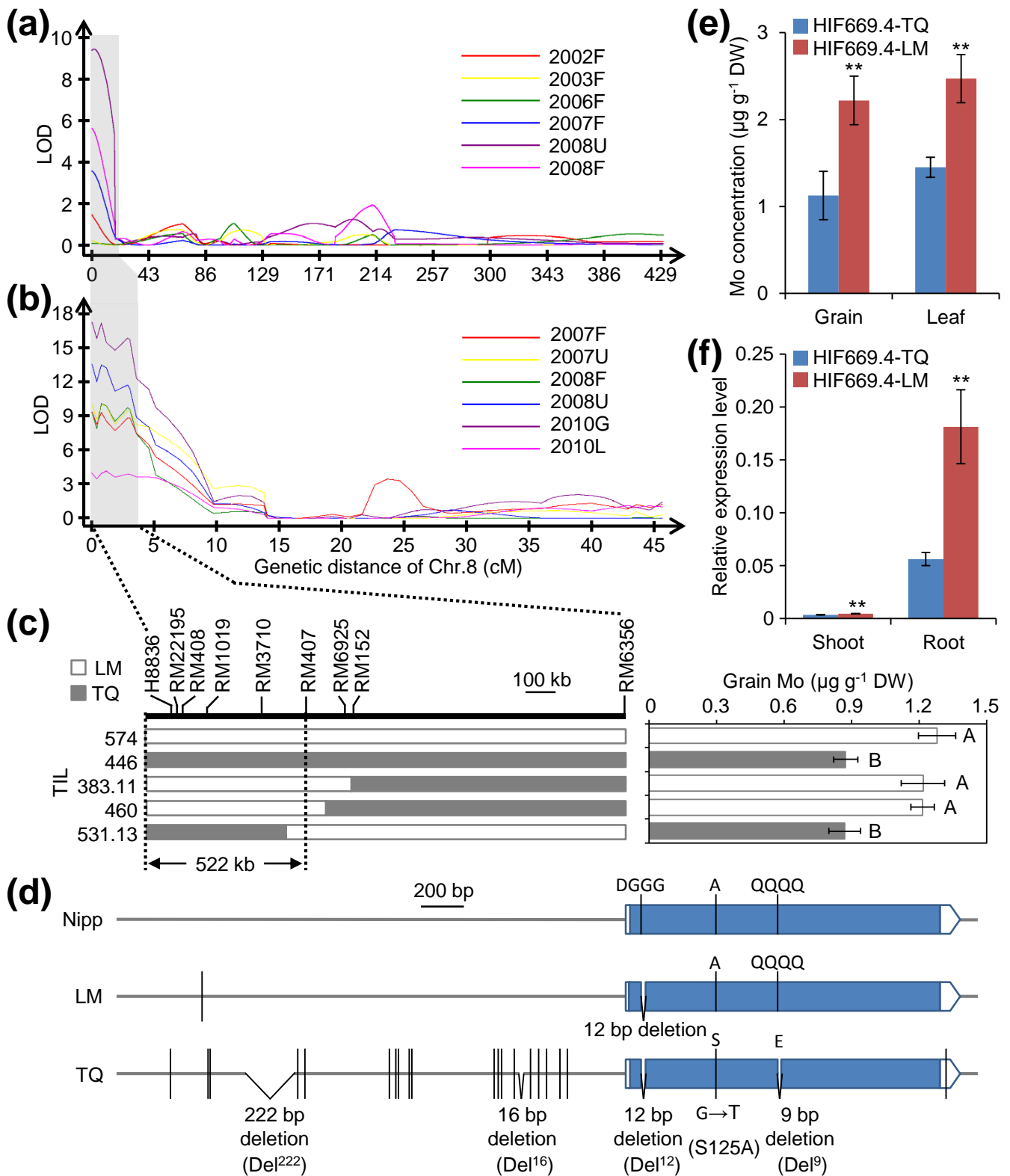


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(a and b) The LOD profiling of *qGMo8* on chromosome 8 in the LT-RIL population (a) and TIL population (b) grown in multiple years under different conditions. F, flooded; U, unflooded. G, grains of TILs grown in greenhouse; L, leaves of greenhouse grown TILs. (c) Grain Mo concentration and genotype at QTL interval of selected TILs. Data are presented as mean \pm SD ($n = 3$). Columns with different letters indicate significant difference at $P \leq 0.01$ (Tukey's Honestly Significant Difference (HSD) test). DW, dry weight. (d) Gene structure and sequence variation of *OsMOT1;1* among Nipponbare (Nipp), TQ and LM. Blue bars and white bars represent exons and untranslated regions, respectively. Vertical lines represent SNPs. (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) and (f) are presented as mean \pm SD with $n = 6$ in (e) and 3 in (f). ** indicates significant difference at $P \leq 0.01$ (Student's *t* test).

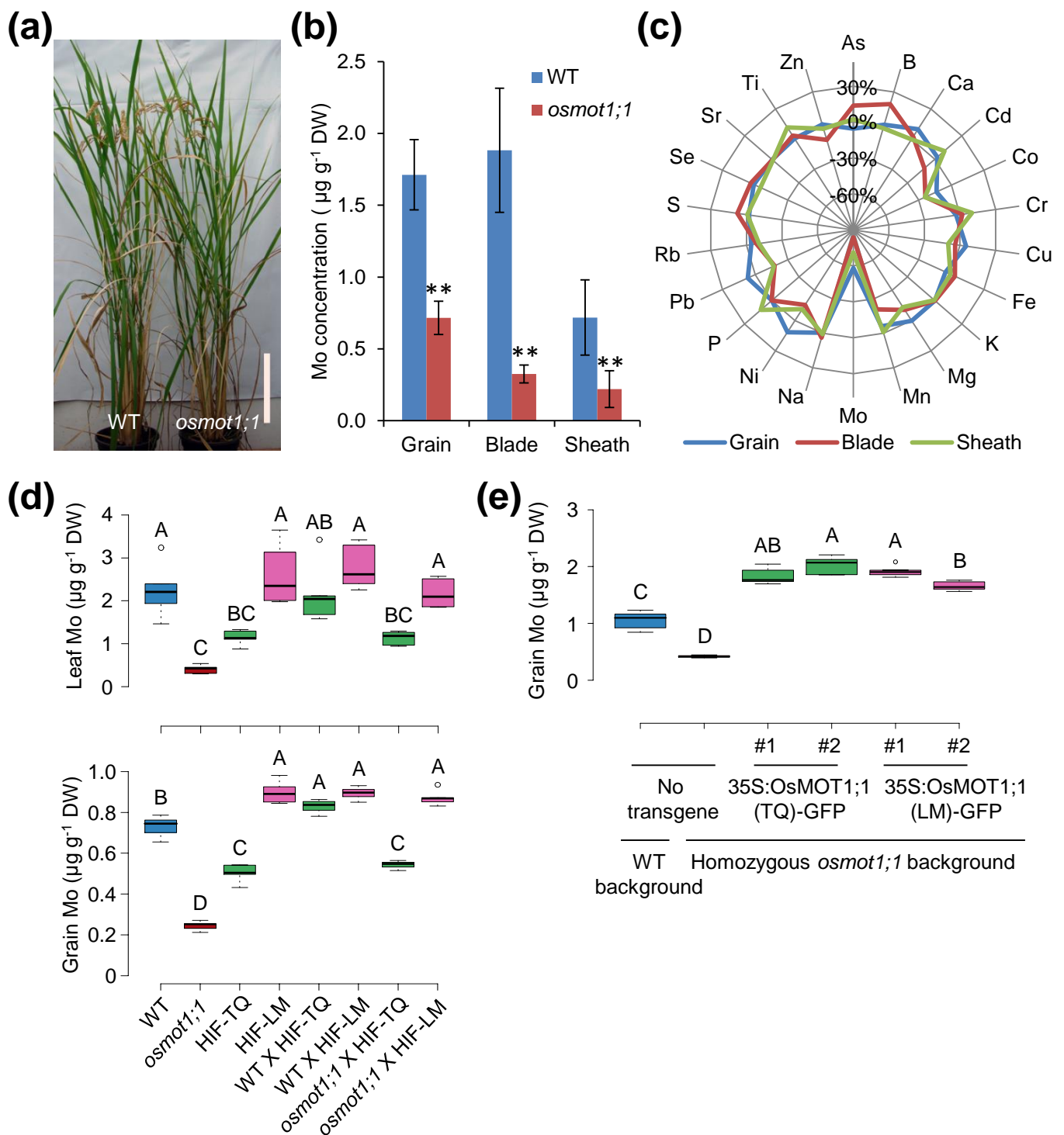


Fig 2. Characterization of the *osmot1;1* mutant and complementation test.

(a) Greenhouse grown WT and *osmot1;1* plants grown at harvesting stage. (b) Mo concentrations in the grain, blade and sheath of WT and *osmot1;1*. Data are presented as means \pm SD ($n = 8$). ** indicates significant difference between WT and *osmot1;1* at $P \leq 0.01$ (Student's *t* test). (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 F1 plants were determined. (e) Transgenic complementation of *osmot1;1* by crossing with *OsMOT1;1* overexpression lines. The grain Mo concentrations of F2 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 ($n = 6$). Boxes with different letters indicate significant difference at $P \leq 0.01$ (Tukey's HSD test). DW, dry weight.

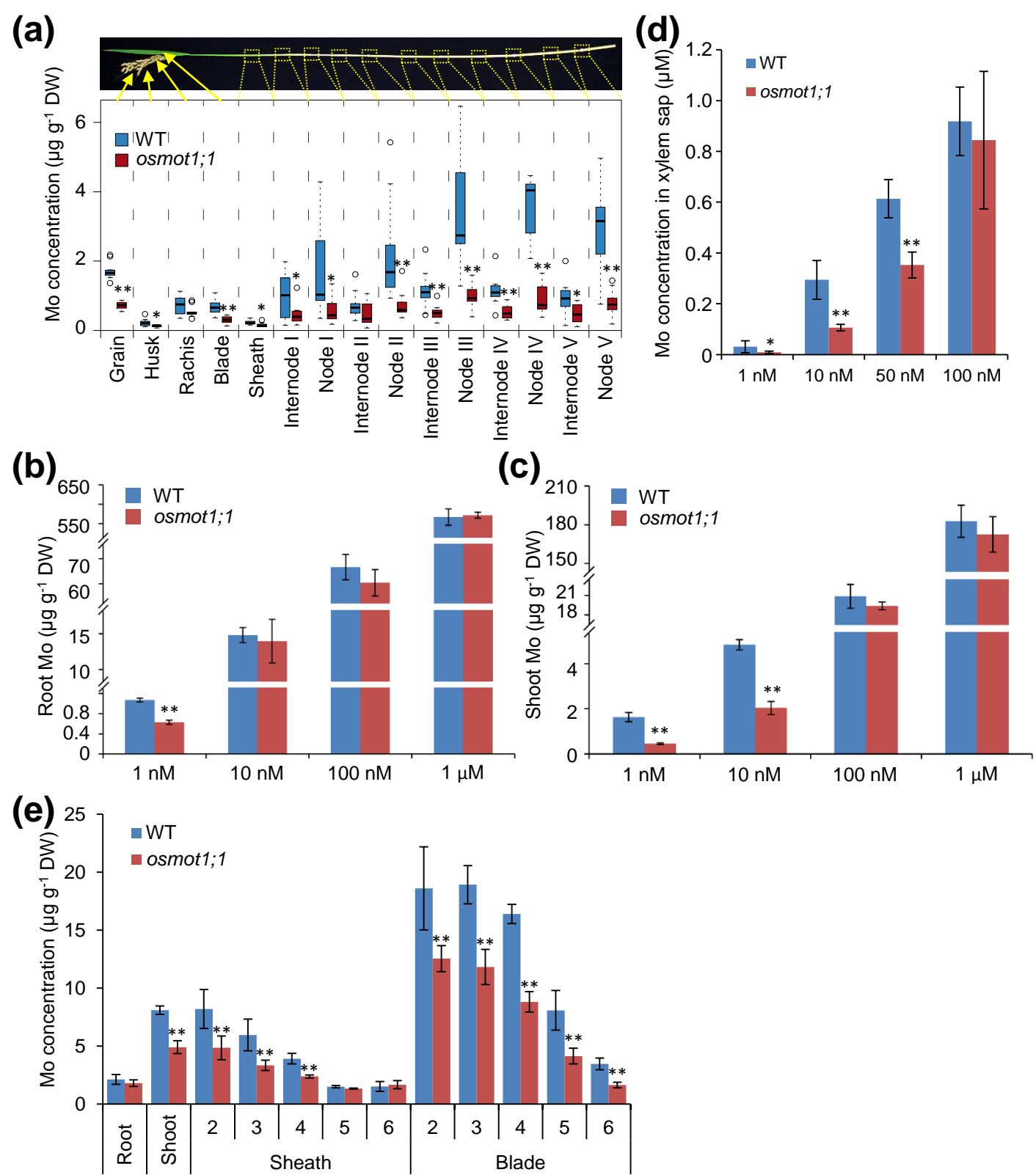


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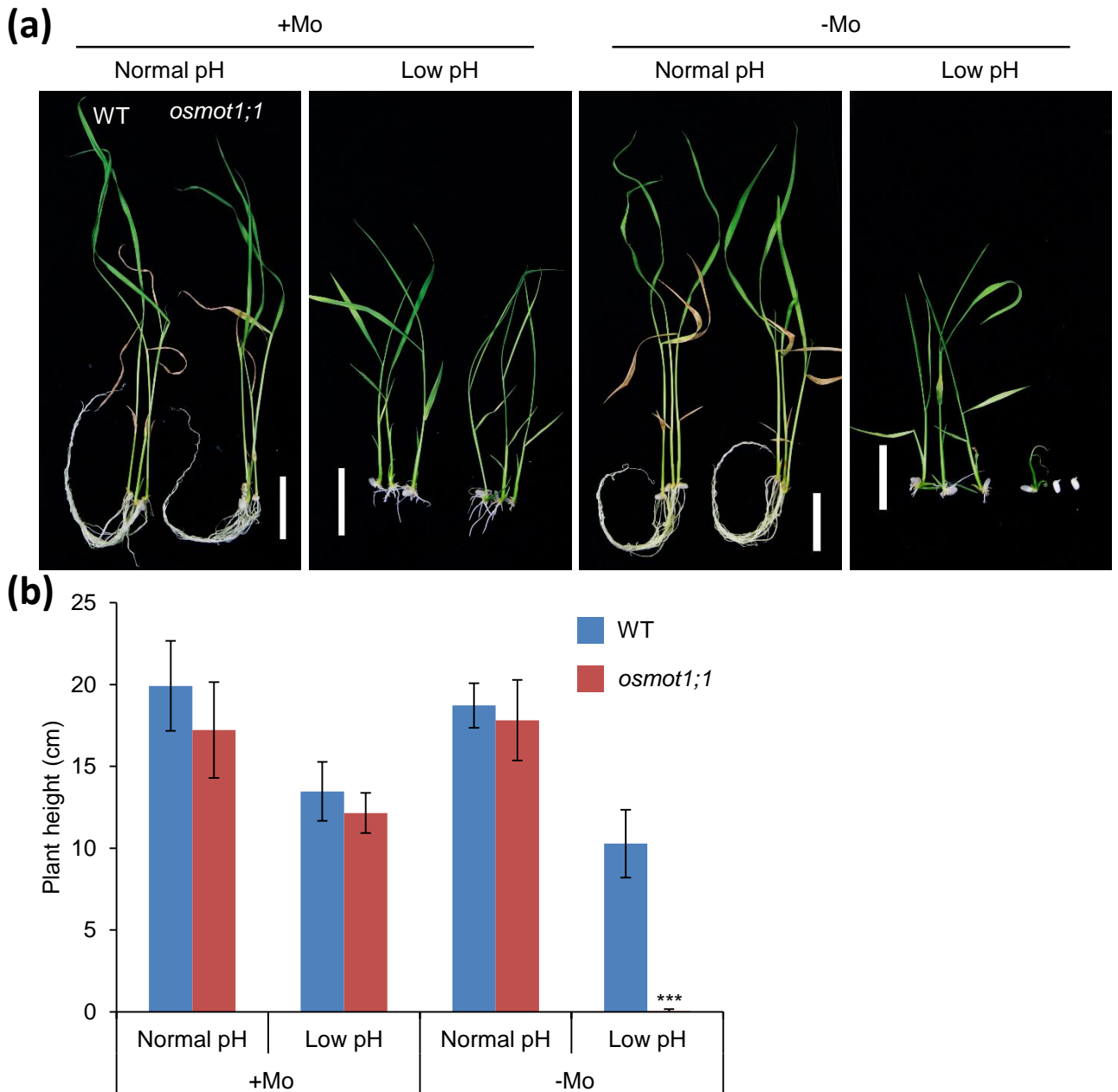


Fig 4. The *osmot1;1* mutant is sensitive to Mo deficiency at low pH condition.

(a) The phenotype of WT and *osmot1;1* grown under normal or low pH media with or without 1 μ M Mo added. Low pH media was made by adding 50 μ L 6 M HCl to 100 mL agar media after autoclaving. Plants were grown for 20 days. Bar, 3 cm. (b) The plant height of WT and *osmot1;1* grown as in (A). Data are presented as means \pm SD with $n = 6$. *** indicated significant difference between WT and *osmot1;1* at $P \leq 0.001$ (Student's t test).

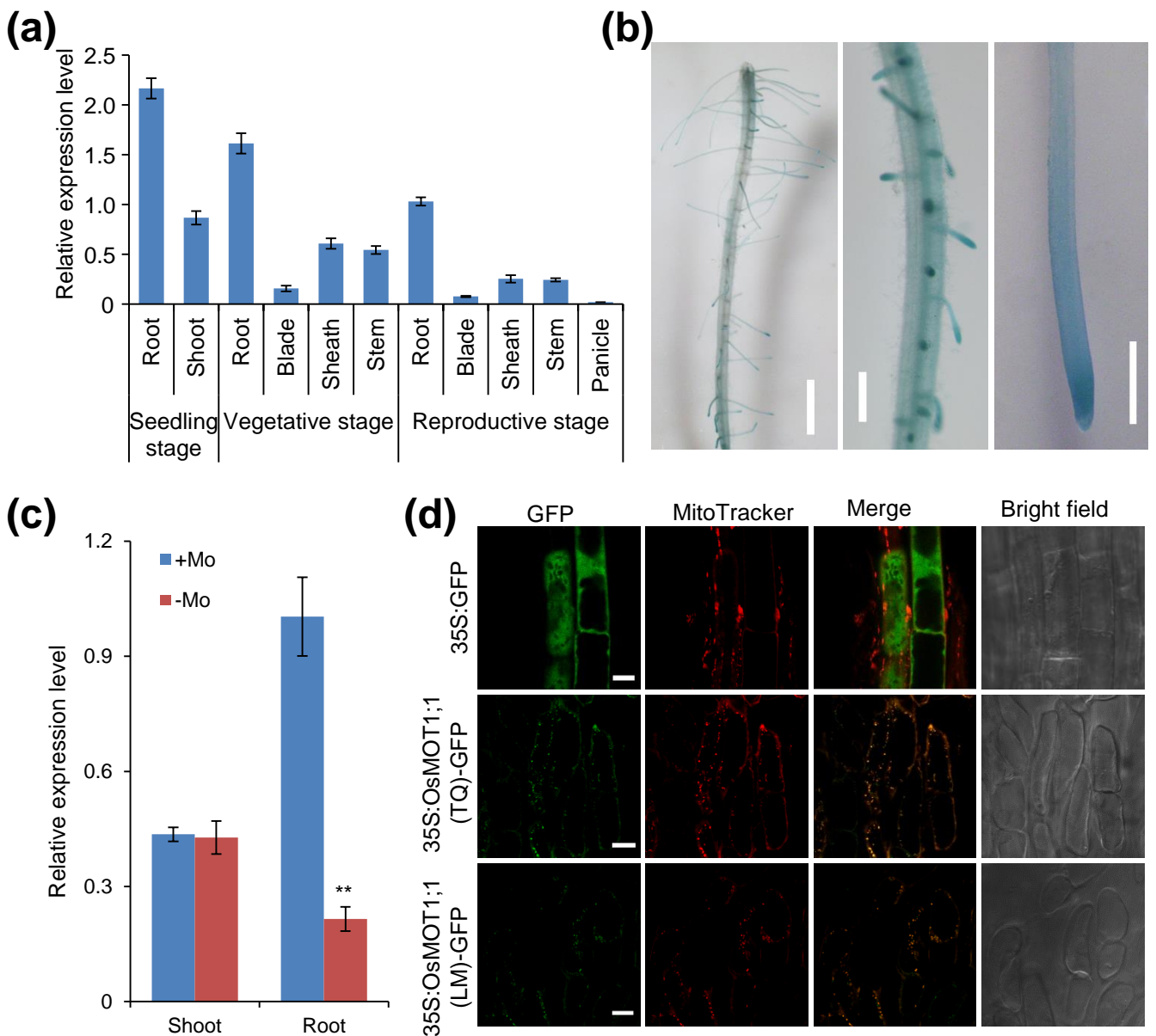


Fig 5. Expression pattern and subcellular localization of *OsMOT1;1*.

(a) Expression level of *OsMOT1;1* in different organs of Lemont at different growth stages. (b) Histochemical GUS 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-week-old plants are showed. Scale bars from left to right are 2, 0.5 and 2 mm. (c) Expression of *OsMOT1;1* was suppressed in roots under Mo deficiency. Lemont (LM) plants were grown hydroponically with 1 μM Mo for one week and then treated with 1 μM Mo (+Mo) or without Mo (-Mo) for another week. Relative expression level of was determined by qRT-PCR with three biological replicates. ** indicates significant difference at $P \leq 0.01$ (Student's *t* test). (d) Subcellular localization of *OsMOT1;1* in stable transgenic rice plants. *OsMOT1;1* from TeQing (TQ) or LM were fused with GFP at N-terminal and overexpressed under the control of CaMV 35S promoter. Mitochondria were stained with the specific dye MitoTracker. Scale bar, 10 μm .

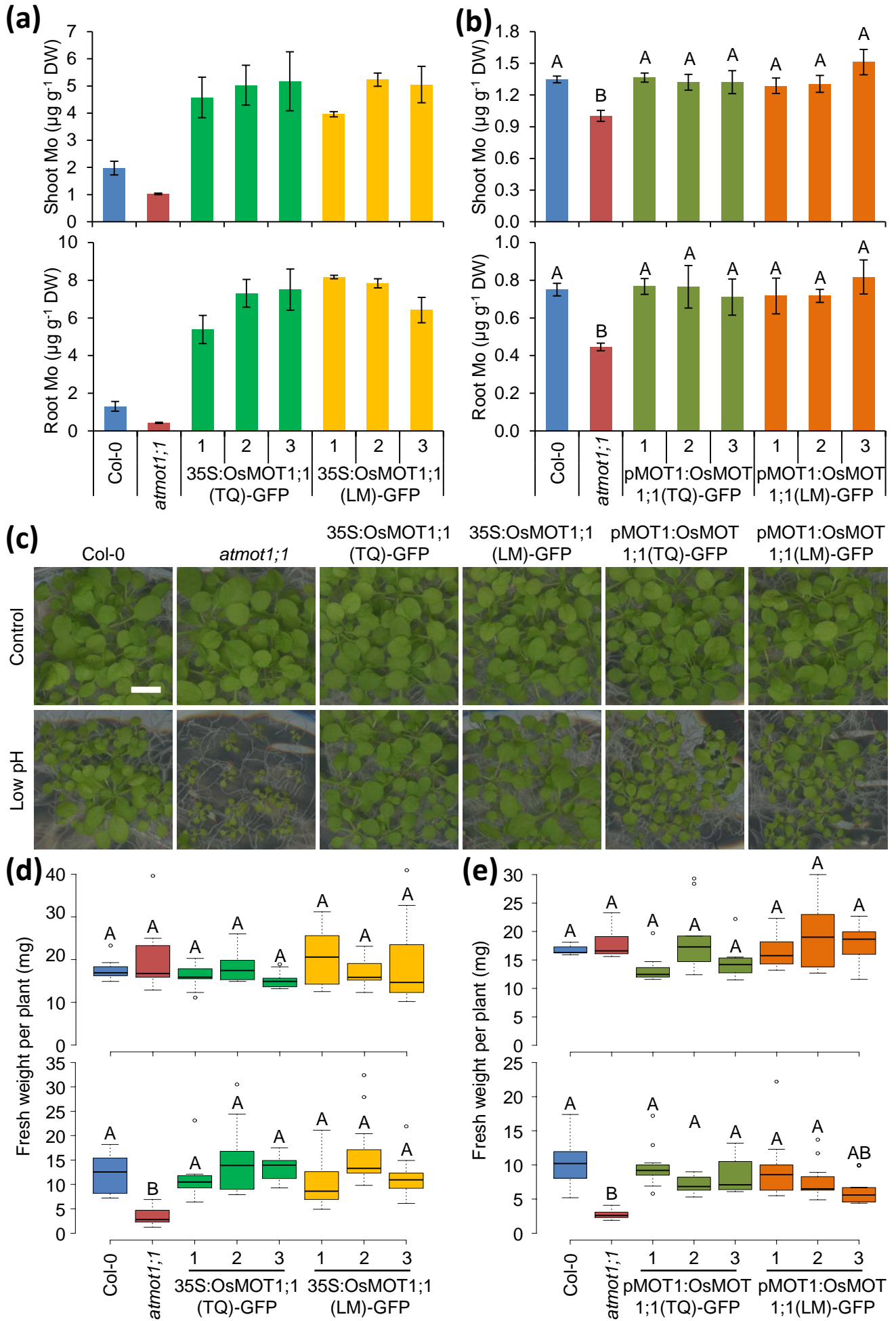
Fig 6

Fig 6 continued

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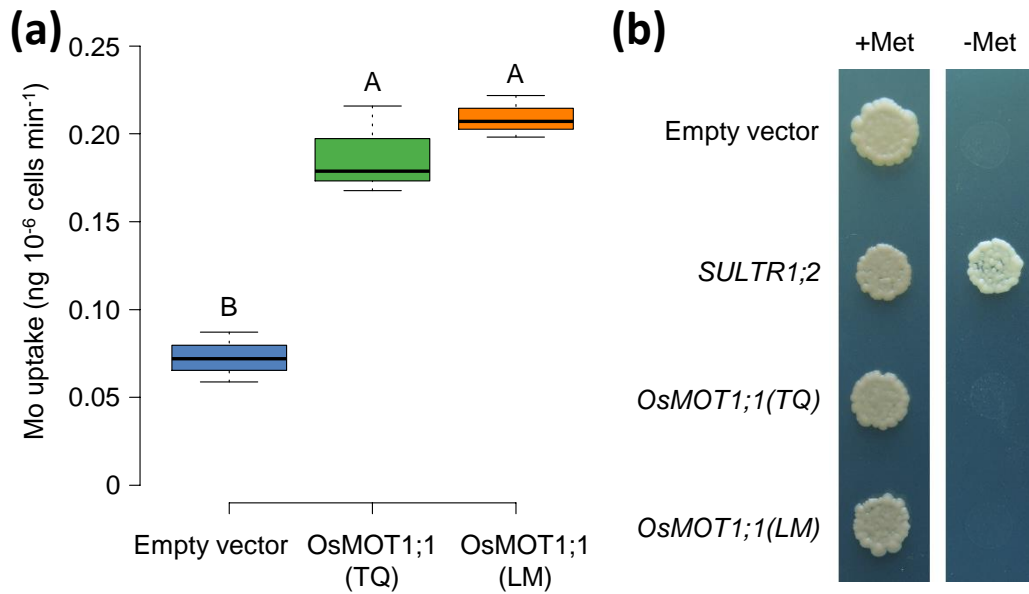


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(a) Molybdate transport activity of *OsMOT1;1*. Yeast strains transformed with empty vector, or *OsMOT1;1* from TeQing (TQ) or Lemont (LM) were incubated in media containing 0.5 μM Mo for 30 min. Mo concentrations in yeast cells were determined. Data are shown as boxplot with $n = 3$. Boxplots with different letters indicate significant difference at $P \leq 0.01$ (Tukey's HSD 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 Met for four days.

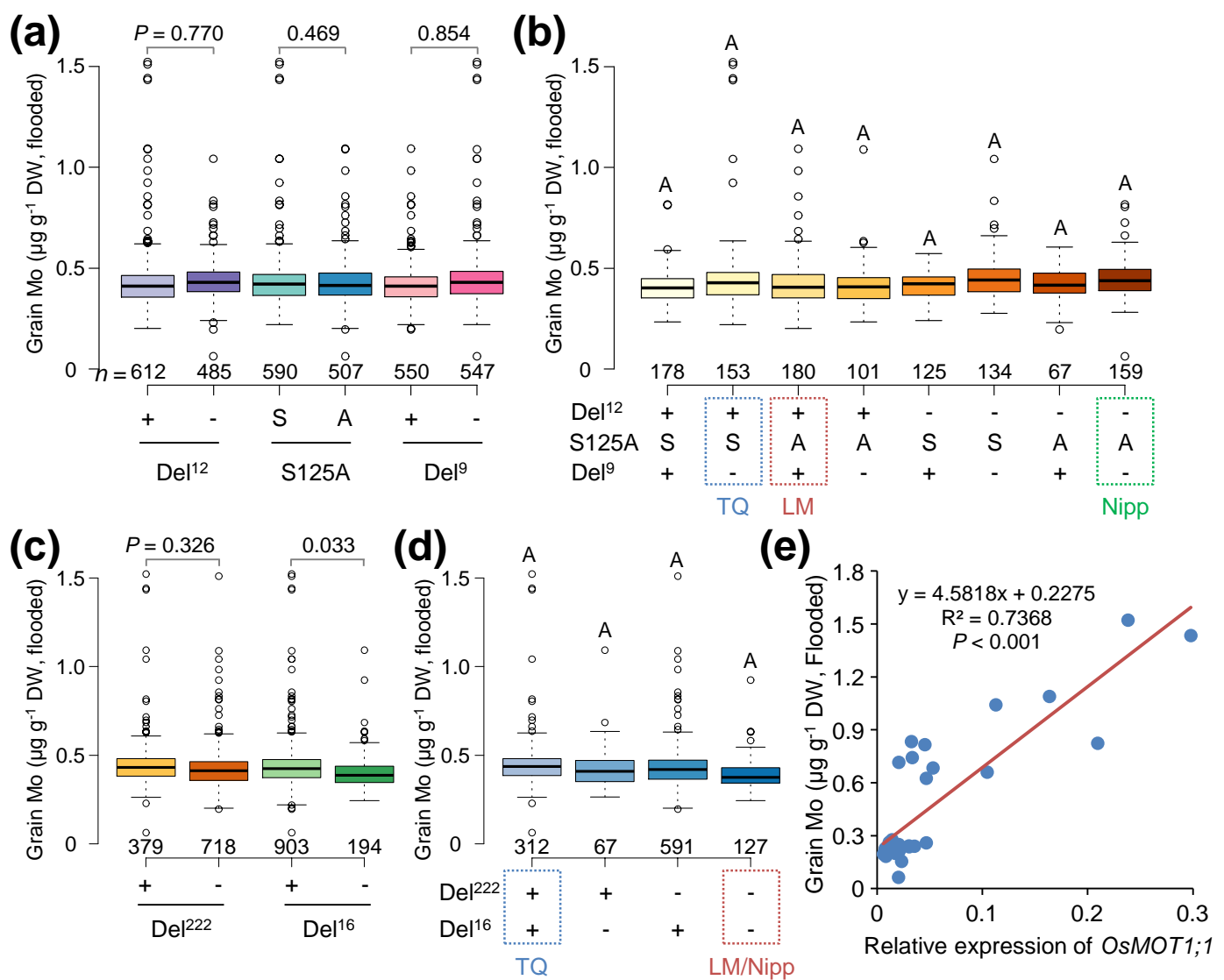


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