Rice plastidial NAD-dependent malate dehydrogenase 1 negatively regulates salt stress response by reducing the vitamin B6 content

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Summary
Salinity is an important environmental factor that adversely impacts crop growth and productivity. Malate dehydrogenases (MDHs) catalyse the reversible interconversion of malate and oxaloacetate using NAD(H)/NADP(H) as a cofactor and regulate plant development and abiotic stress tolerance. Vitamin B6 functions as an essential cofactor in enzymatic reactions involved in numerous cellular processes. However, the role of plastidial MDH in rice (Oryza sativa) in salt stress response by altering vitamin B6 content remains unknown. In this study, we identified a new loss-of-function osmdh1 mutant displaying salt stress-tolerant phenotype. The OsMDH1 was expressed in different tissues of rice plants including leaf, leaf sheath, panicle, glume, bud, root and stem and was induced in the presence of NaCl. Transient expression of OsMDH1-GFP in rice protoplasts showed that OsMDH1 localizes to chloroplast. Transgenic rice plants overexpressing OsMDH1 (OsMDH1OX) displayed a salt stress-sensitive phenotype. Liquid chromatography–mass spectrometry (LC-MS) metabolic profiling revealed that the amount of pyridoxine was significantly reduced in OsMDH1OX lines compared with the NIP plants. Moreover, the pyridoxine content was higher in the osmdh1 mutant and lower in OsMDH1OX plants than in the NIP plants under the salt stress, indicating that OsMDH1 negatively regulates salt stress-induced pyridoxine accumulation. Furthermore, genome-wide RNA-sequencing (RNA-seq) analysis indicated that ectopic expression of OsMDH1 altered the expression level of genes encoding key enzymes of the vitamin B6 biosynthesis pathway, possibly reducing the level of pyridoxine. Together, our results establish a novel, negative regulatory role of OsMDH1 in salt stress tolerance by affecting vitamin B6 content of rice tissues.

Introduction
Salt stress is a major environmental factor affecting plant growth and development. Efforts to increase salt stress tolerance of crop plants would enable sustainable agriculture on marginal lands and improve crop yields. Salinity causes ionic stress (mainly because of sodium [Na⁺], chloride [Cl⁻] and sulphate [SO₄²⁻] ions), osmotic stress and secondary stresses including nutritional imbalances and oxidative stress (Zhu, 2002). Salinity-mediated oxidative stress reduces the availability of CO₂ and consumption of NADPH by the Calvin cycle. When the level of ferredoxin is reduced during photosynthetic electron transfer, electrons are transferred from photosystem I (PS I) to oxygen to form superoxide radicals (O²⁻) via the Mehler reaction, which initiates chain reactions that produce more oxygen radicals (Hsu and Kao, 2003). Reactive oxygen species (ROS) are continuously generated during normal metabolic processes in peroxisomes, mitochondria and cytoplasm, which destroy normal metabolism by damaging proteins, nucleic acids and lipids (McCord, 2000). Plants have evolved efficient ROS removal systems including ROS-scavenging antioxidative enzymes and small nonenzymatic molecules such as polyphenolic compounds, carotenoids, anthocyanin, flavonoids, glutathione, ascorbate and α-tocopherol.

Vitamin B6, which comprises pyridoxal, pyridoxine, pyridoxamine and their phosphorylated derivatives, is an essential cofactor of numerous metabolic enzymes involved in amino acid metabolism and antibiotic biosynthesis (Tambasco-Studart et al., 2005). Moreover, vitamin B6 is a potent antioxidant, with particular ability to quench ROS, and plays a key role in biotic and abiotic stress responses (Chen and Xiong, 2005; Denslow et al., 2007; González et al., 2007; Tambasco-Studart et al., 2005). Recently, de novo biosynthesis of vitamin B6 has been unravelled in plants (Burns et al., 2005; Ehrenshaft et al., 1999; Osmani et al., 1999; Raschle et al., 2005, 2007; Tambasco-Studart et al., 2005). Pyridoxal phosphate synthase protein 1 (PDX1) and PDX2, which are essential for the biosynthesis of vitamin B6, form a complex that directly synthesizes the cofactor form of the vitamin, pyridoxal S'-phosphate, from ribose-5-phosphate, glyceraldehyde-3-phosphate and glutamine (Burns et al., 2005; Raschle et al., 2005). The Arabidopsis thaliana genome encodes three functional homologs of PDX1, AtPDX1.1, AtPDX1.2 and AtPDX1.3, and a single homolog of PDX2 (Raschke et al., 2011; Tambasco-Studart et al., 2005). Arabidopsis loss-of-function pdx1.1 and pdx1.3 mutants are sensitive to photoinhibition and salt and osmotic stresses (Titiz et al., 2006). Moreover, vitamin B6 deficiency caused by loss-of-function mutation of PDX1.3 reduces the antioxidant capacity of pdx1.3 mutant plants...
Results

Loss-of-function OsMDH1 mutants exhibit salt stress-tolerant phenotypes

In a forward genetic screening of a genome-scale mutagenesis library of rice (Oryza sativa L. var. Nipponbare) CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-associated nuclease 9) mutant pool RGGKO-ALL (a genome-scale mutagenesis library of rice) (Lu et al., 2017), we identified a mutant line 7 (L7) showing increased tolerance to 100 mM NaCl treatment compared with the wild type (NIP) (Figure 1a,b). Information obtained from the barcoded next-generation sequencing (NGS) data and Sanger sequencing analysis (Lu et al., 2017) was combined, which revealed a 4 bp deletion located 84 bp downstream of the ATG start codon of the gene encoding MDH1 (LOC_Os01g61380), resulting in the generation of a premature stop codon in L7 mutant (osmdh1-1) (Figure 1c). The rice genome encodes approximately 10 MDHs, of which OsMDH1 contains highly conserved NAD\(^+\)-binding and proton acceptor sites, which are required for its activity (Figure S1). To confirm whether the loss-of-function mutation of OsMDH1 increased salt tolerance, we generated two independent mutants, osmdh1-2 and osmdh1-3, using the CRISPR/Cas9 system. Specific guide RNA (gRNA) target sites for OsMDH1 were cloned into the CRISPR/Cas9 vector, in which Cas9 was driven by the U8Q10 promoter (Ma et al., 2015). The vectors were then transformed into NIP plants, and homozygous osmdh1-2 and osmdh1-3 mutant lines were identified via Sanger sequencing (Figure S2a). In the osmdh1-2 mutant, 1 bp deletion was found 647 bp downstream of the ATG, resulting in a frame shift mutation and consequently a premature stop codon before the core catalytic domain (Figure S2a). In the osmdh1-3 mutant, 1 bp insertion was detected 742 bp downstream of the ATG, causing a frame shift (Figure S2a). To exclude the potential confounding effect of the Cas9 gene per se, we isolated osmdh1-1, osmdh1-2 and osmdh1-3 mutants by screening for nonhygromycin resistance (Figure S2b). Cas9-free osmdh1-1, osmdh1-2 and osmdh1-3 mutants were used in this study. The osmdh1-2 and osmdh1-3 mutants displayed salt stress-tolerant phenotypes similar to osmdh1-1 (Figure 1d,e). To further confirm whether the loss-of-function mutation of OsMDH1 was responsible for salt stress tolerance, we generated complementation lines by transfecting the osmdh1-1 mutant with a construct expressing OsMDH1 cDNA fused to GFP under the control of the OsMDH1 promoter (OsMDH1\(_{pro}\) OsMDH1-GFP). Two independent complementation lines (Com#1 and Com#2) were chosen for further analysis, and the level of OsMDH1-GFP protein in these lines was detected using Western blot analysis (Figure S2c). Both Com#1 and Com#2 lines showed similar survival rates compared with NIP plants under salt stress (Figure 1d,e). The reduced rate of photosynthesis under salt stress increases the production of ROS including hydrogen peroxide (H\(_2\)O\(_2\)) and O\(_2^-\) (Sharma et al., 2012). Staining of leaves with diaminobenzidine (DAB) and nitrotetrazolium blue chloride (NBT) revealed that osmdh1 mutants accumulated less ROS than NIP plants under normal conditions and under salt stress (Figure 1f). Of notes, osmdh1 mutants displayed a late flowering phenotype compared with NIP plants under normal conditions (Figure S2d,e). Taken together, these results indicate that OsMDH1 participates in salt stress tolerance.

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OsMDH1OX plants exhibit salt stress-sensitive phenotypes

To further investigate the role of OsMDH1 in salt stress tolerance, we generated three independent OsMDH1OX lines (OsMDH1OX-1, OsMDH1OX-2 and OsMDH1OX-3). Real-time quantitative PCR (RT-qPCR) analysis revealed that transcript levels of OsMDH1 were dramatically increased in all three OsMDH1OX lines (Figure S3a). The OsMDH1OX lines displayed an early flowering phenotype compared with NIP plants under normal conditions (Figure S3b,c). Under the salt stress, OsMDH1OX lines showed salt stress-sensitive phenotypes compared with NIP plants, and the survival rate of OsMDH1OX lines was lower than that of NIP plants (Figure 2a,b). Additionally, we detected higher levels of ROS in OsMDH1OX lines than in NIP plants (Figure 2c).

Tissue-specific expression patterns of OsMDH1 and subcellular localization of OsMDH1

To examine the spatial and temporal expression patterns of OsMDH1, we generated transgenic plants expressing the β-glucuronidase (GUS) gene under the control of the OsMDH1
promoter (OsMDH1pro::GUS) and examined the promoter activity of OsMDH1 in different tissues at distinct developmental stages. GUS signals were detected in leaf, leaf sheath, panicle, glume, bud, root and stem tissues (Figure 3a). We also confirmed these results using RT-qPCR (Figure 3b). Additionally, OsMDH1 transcripts were rapidly induced under 100 mM NaCl treatment (Figure 3c); the GUS signals were detected in leaf, leaf sheath, panicle, glume, bud, root and stem tissues (Figure 3a). We also confirmed these results using RT-qPCR (Figure 3b). Additionally, OsMDH1 transcripts were rapidly induced under 100 mM NaCl treatment (Figure 3c); the gene (LOC_Os01g66120), a NaCl-responsive marker, was used as a positive control in this experiment (Figure 3c). To examine the subcellular localization of OsMDH1, we cloned the GFP gene at the 3' end of the OsMDH1 coding sequence to generate the OsMDH1-GFP fusion. The OsMDH1-GFP construct was transfected into rice protoplasts. GFP signals colocalized with chloroplast autofluorescence, indicating that OsMDH1 localizes to the chloroplast (Figure 3d). Western blot analysis using anti-GFP antibody revealed two bands (Figure 3e). We speculated that the higher molecular weight band represented a premature form of OsMDH1-GFP harbouring a transit peptide, while the lower molecular weight band represented the mature OsMDH1-GFP protein localized to chloroplast after transit peptide cleavage. We also confirmed the existence of the transit peptide using the ChloroP 1.1 Server bioinformatics tool (Table S1) (Emanuelsson et al., 1999).

Analysis of OsMDH1 biochemical activity

To examine the biochemical activity of OsMDH1 in vitro, we expressed an N-terminal fusion of OsMDH1 with glutathione S-transferase (GST) in Escherichia coli. We also tested the GST fusion of the catalytically inactive form of OsMDH1[M], in which histidine located at the core catalytic domain was substituted with alanine (Figure 4a). The recombinant proteins were purified (Figure 4b) and incubated with OAA and NADH. We detected that the activity of NAD-MDH was dramatically increased after introducing OAA and NADH in GST-OsMDH1, whereas the activities of GST and GST-OsMDH1[M] showed no changes (Figure 4c). To test the biochemical activity of OsMDH1 in vivo, we purified chloroplast proteins from NIP plants and OsMDH1OX lines and incubated chloroplast proteins with OAA and NADH. The activity of NAD-MDH was dramatically increased in OsMDH1OX lines compared with NIP plants (Figure 4d). Taken together, these results indicate that OsMDH1 is a plastid-localized, NAD-dependent enzyme. Since salt stress rapidly induces the expression of OsMDH1, we further examined the NAD-MDH activity under salt stress using chloroplast proteins purified from NIP, osmdh1-1 and OsMDH1OX-1 plants. The results showed that plastidial NAD-MDH activity was dramatically increased in OsMDH1OX lines compared with NIP plants under salt stress but only marginally altered in osmdh1-1 mutants (Figure 4e). Additionally, plastidial NAD-MDH activity increased more quickly in the OsMDH1OX-1 line than in NIP plants under salt stress (Figure 4e). These results indicate that salt stress up-regulates the expression of OsMDH1, thus inducing plastidial OsMDH1 activity.

Ectopic expression of OsMDH1 alters metabolic profiles

To determine the metabolic consequences of the ectopic expression of OsMDH1, we compared the metabolic profiles of NIP and OsMDH1OX-1 plants using LC-MS under normal conditions (Przyborowska et al., 2004). The metabolome was analysed using three methods: orthogonal projections to latent structures discriminant analysis (OPLS-DA), partial least square discriminant analysis (PLS-DA) and principal components analysis (PCA). A clear separation was observed between NIP and OsMDH1OX-1 plants (Figure S4). Compared with NIP plants, levels of 144...
metabolites were higher and 41 metabolites were lower in OsMDH1OX-1 plants (Table S2). The differentially accumulated metabolites were mapped to KEGG (Kyoto Encyclopedia of Genes and Genomes) ID using MetaboAnalyst online software (Xia and Wishart, 2016). The results showed that ‘flavone and flavonol biosynthesis’ and ‘tyrosine metabolism’ pathways were significantly enriched ($P < 0.05$; Table S3). Additionally, malic acid content was dramatically increased in OsMDH1OX-1 plants compared with NIP plants, which is consistent with the sharp increase in NAD-MDH activity in OsMDH1OX-1 plants compared with NIP plants (Figure 5a). We further confirmed this result in three independent OsMDH1OX lines and three osmdh1 mutant lines. Compared with NIP plants, malate contents of OsMDH1OX lines were significantly increased, while those of osmdh1 mutants were dramatically reduced (Figure 5b). Intriguingly, we found that the amount of pyridoxine was lower in OsMDH1OX lines and significantly higher in osmdh1 mutants than in NIP plants (Figure 5a,c). We further examined the effect of salt stress on malate and pyridoxine contents of plants. Under the salt stress, both malate and pyridoxine contents were increased in NIP plants, whereas osmdh1 mutants showed a marginal change in malate level and a dramatic increase in pyridoxine content (Figure 5d,e). Additionally, OsMDH1OX-1 plants contained more malate and less pyridoxine than NIP plants under salt stress (Figure 5d,e). These results indicate that ectopic expression of OsMDH1 induces metabolic alterations in different pathways.

Pyridoxine improves salt tolerance of OsMDH1OX plants

To determine whether the salt stress-sensitive phenotype of OsMDH1OX was caused by the reduction in the amount of pyridoxine, we examined whether the addition of pyridoxine to the culture solution could rescue the salt stress-sensitive phenotype of OsMDH1OX lines. Under normal conditions, the addition of pyridoxine to the culture solution caused no phenotypic alterations in OsMDH1OX lines compared with NIP plants. However, in the presence of NaCl, addition of $10 \mu M$ pyridoxine to the culture solution rescued the salt stress-sensitive phenotype of OsMDH1OX lines (Figure 6a,b). We also examined the effect of $10 \mu M$ pyridoxine on loss-of-function osmdh1 mutant lines in the presence or absence of $100 \mu M$ NaCl. Under normal conditions, we observed no differences between NIP and osmdh1 plants upon the addition of pyridoxine. By contrast, in the presence of $100 \mu M$ NaCl and no pyridoxine, the osmdh1 mutant showed salt stress-tolerant phenotypes, whereas NIP plants were sensitive to salt stress. However, after the addition of pyridoxine to the culture solution, NIP plants and osmdh1 mutant lines showed...
similar phenotypes (Figure 6c,d). Taken together, these results indicate that plant pyridoxine levels determined by OsMDH1 play an important role in the salt stress response.

**Ectopic expression of OsMDH1 alters gene expression**

To determine the effect of OsMDH1 overexpression on the transcriptome, we performed RNA-seq analysis under normal conditions (Table S4). A total of 1059 genes were up-regulated and 2516 genes were down-regulated in OsMDH1OX plants compared with NIP plants (Table S5). Gene enrichment analysis revealed that gene ontology (GO) terms including ‘response to salt stress’, ‘response to cold stress’, ‘response to water deprivation’, ‘response to abscisic acid’ and ‘cellular water homeostasis’ were significantly enriched among the down-regulated genes, whereas GO terms including ‘defense response to fungus’, ‘response to chitin’ and ‘response to ethylene’ were enriched among the up-regulated genes (Figure 7a; Table S6). Using RT-qPCR, we further examined the expression levels of genes encoding key enzymes involved in vitamin B6 biosynthesis including OsPDX1.1, OsPDX1.2, OsPDX1.3, OsPDX2, OsPDX3, OsSOS4 and OsPLR1. Consistent with RNA-seq results (Figure 7b), we found that the expression of OsPDX1.1, OsPDX1.2, OsPDX2 and OsPLR1 was substantially reduced, while expression of OsPDX1.3 and OsPDX3 was increased in OsMDH1OX lines compared with NIP plants (Figure 7c). Notably, the expression of OsSOS4 did not show noticeable alterations between OsMDH1OX lines and NIP plants (Figure 7c). These results indicate that ectopic expression of OsMDH1 alters the expression of genes encoding key regulators of vitamin B6 biosynthesis, which possibly reduces the pyridoxine content. Thus, we conclude that, under salt stress, overexpression of OsMDH1 indirectly impacts the expression of genes, such as OsPLR1, OsPDX1.1, OsPDX1.2 and OsPDX2, which reduces the pyridoxine content (Figure 7d).

**Discussion**

In this study, we screened a genome-wide mutagenesis library of rice CRISPR/Cas9 mutant pool (Lu et al., 2017) and identified a salt stress-tolerant loss-of-function mutant, osmdh1. We showed that OsMDH1 is expressed in leaf, leaf sheath, panicle, glume, bud, root and stem tissues of rice plants and is rapidly induced by NaCl treatment. The activity of OsMDH1 was dramatically higher in OsMDH1OX plants and significantly impaired in the osmdh1 mutant. In plants, it has been reported that MDH is important in providing malate for C4 metabolism, pH balance, stomatal and pulvinal movement, respiration, β-oxidation of fatty acids and legume root nodule functioning (Miller et al., 1998). Since ectopic expression of OsMDH1 leads to changes of metabolic profiles, we guess the alteration of metabolome in
overexpression lines might cause the genes expression change. RNA-seq analysis of OsMDH1OX transgenic plants revealed that GO terms including ‘response to salt stress’, ‘response to cold stress’, ‘response to water deprivation’, ‘response to abscisic acid’ and ‘cellular water homeostasis’ were significantly enriched among the down-regulated genes, while ‘defense response to fungus’, ‘response to chitin’ and ‘response to ethylene’ were enriched among the up-regulated genes. Thus, it is likely that OsMDH1 impacts the expression of genes in abiotic and biotic stress responses. In apple (Malus domestica), the cytosolic MDH gene (MdcyMDH) is induced by mild cold and salt stresses, and transgenic apple plants overexpressing MdcyMDH exhibit improved cold and salt tolerance compared with wild-type plants (Wang et al., 2016). Under salt and cold stresses, the reductive activity of MdcyMDH overexpression lines was significantly higher than that of wild-type plants (Wang et al., 2016). Although the reductive activities of OsMDH1 and MdcyMDH are increased under salt stress, plants overexpressing the

Figure 5 Ectopic expression of OsMDH1 causes metabolic alterations in different pathways. (a) Metabolites including vitamins, organic acids and amino acids were selected from differential metabolites identified between OsMDH1OX-1 and NIP plants under normal conditions. Error bars indicate ± SD (n = 10). (b, c) Malate and pyridoxine contents in NIP, osmdh1 and OsMDH1OX plants were measured under normal conditions. Error bars indicate ± SD (n = 10). Statistical analyses were performed by comparing osmdh1 mutants and OsMDH1OX lines with NIP plants, respectively. **, P-value < 0.01 (Student’s t-test). PN, pyridoxine. (d) Malate contents in NIP, osmdh1-1 and OsMDH1OX-1 plants were measured under 100 mM NaCl treatment for 0 and 24 h. Relative malate content of osmdh1-1 and OsMDH1OX-1 plants was normalized by malate content in NIP plants. Error bars indicate ± SD (n = 10). Statistical analyses were performed by comparing osmdh1-1 and OsMDH1OX-1 with NIP plants, respectively. (Student’s t-test, a = 4.82*10^-3, b = 3.56*10^-5, c = 7.42*10^-5, and d = 7.52*10^-5). (e) Pyridoxine contents in NIP, osmdh1-1 and OsMDH1OX-1 plants were measured under 100 mM NaCl treatment for 0 and 24 h. Relative pyridoxine content of osmdh1-1 and OsMDH1OX-1 plants was normalized by pyridoxine content in NIP plants. Error bars indicate ± SD (n = 10). Statistical analyses were performed by comparing osmdh1-1 and OsMDH1OX-1 with NIP plants, respectively. (Student’s t-test, a = 6.82*10^-4, b = 7.92*10^-3, c = 5.83*10^-6, and d = 9.16*10^-7). PN, pyridoxine.
corresponding genes display opposite phenotypes; lines overexpressing OsMDH1 are sensitive to salt stress, whereas those overexpressing MydcyMDH lines are tolerant to salt stress. One of the possible explanations for this difference is that MDHs located in different subcellular compartments have different contributions to salt stress tolerance. It has been reported that plastidial malate dehydrogenases mostly contribute to generate malate and NAD by catalysing oxaloacetate using NADH as a cofactor (Scheibe, 2004). After exportation from the chloroplast, malate can work as a source for NADH in the cytosol and feeds mitochondrial ATP production (Scheibe, 2004). Previously, Arabidopsis pdNAD-MDH protein has been shown to play an important role in plastid development during embryogenesis (Ferro et al., 2010). The pdNAD-MDH protein interacts with members of a proposed large AAA protease complex, comprising FtsH12 and FtsHi subunits and Ycf2, localized to the inner chloroplast membrane (Schreier et al., 2018). Intriguingly, NAD(H) and NADP(H) levels did not change in pdNAD-MDH knock-down mutant (mir-mdh-1) compared with wild-type plants (Beeler et al., 2014). It is possible that OsMDH1 also participates in chloroplast development; however, whether and how salt stress-responsive phenotype of OsMDH1 connected to chloroplast biogenesis remains to be investigated. Further studies should be performed by introducing the catalytically inactive form of OsMDH1 (OsMDH1[Δ]) into the osmdh1 mutant background.

Figure 6 Pyridoxine improves the salt stress tolerance of OsMDH1OX plants. Phenotypes of OsMDH1OX lines (a, b) and osmdh1 mutants (c, d) grown on the culture solution with 10 μM PN, 100 mM NaCl or 10 μM PN and 100 mM NaCl. PN, pyridoxine. Images were taken, and the survival rates were measured before the treatment and after the recovery from the treatment. Bars, 4 cm. Error bars indicate ± SD (n = 3). Statistical analyses were performed by comparing OsMDH1OX lines and osmdh1 mutants with NIP plants, respectively. **, P-value <0.01 (Student’s t-test).
to observe whether OsMDH1[M] is able to complement the salt stress-tolerant phenotype of osmdh1.

In this study, OsMDH1OX transgenic lines showed an early flowering phenotype, indicating that OsMDH1 is involved in the regulation of flowering time in rice. Previously, it has been reported that salt stress delays flowering in Arabidopsis by reducing the transcript levels for CO and FT (Li et al., 2007). Exogenous application of gibberellin (GA) causes delayed or inhibited transition from vegetative growth to reproductive development (Li et al., 2007). In addition, the CONSTANS (CO)/FLOWERING LOCUS T (FT) module may also play a role in mediating the effects of salt on flowering (Li et al., 2007). In our RNA-seq analysis, we detected that the expression of OsCO3, a negative regulator in flowering, was decreased in OsMDH1OX-1 compared with NIP (Table S5). Since the expression of OsMDH1 was increased under the treatment of NaCl, we deduce that increased OsMDH1 transcript level might lead to reduced expression of OsCO3, thereby causing early flowering in OsMDH1OX lines. Recently, a floury endosperm mutant in rice, f16, was characterized and found to display defective starch grain formation (Teng et al., 2019). FLO16 encodes a NAD-dependent cytosolic MDH. The ATP content was reduced in the f16 mutant, leading to significant reduction in the activity of enzymes involved in starch biosynthesis (Teng et al., 2019). Thus, it is likely that OsMDHs located in different subcellular compartments play different physiological roles during plant development and growth. Similar functions of MDHs in plant growth and development have been investigated in other plant species. In Arabidopsis, mitochondrial MDH (mMDH) and peroxisomal MDHs (PMDH1 and PMDH2) have function in plant growth (Pracharoenwattana et al., 2007). In tomato (Solanum lycopersicum), MDH regulates starch biosynthesis in the amyloplast (Centeno et al., 2011).

Vitamin B6 comprises six related compounds including pyridoxal, pyridoxine, pyridoxamine and the corresponding 5'-phosphorylated esters. Vitamin B6 is also thought to function as an antioxidant because the level of vitamin B6 is positively correlated with oxidative stress responses in both animals and plants.
AtSOS4 encodes a pyridoxal kinase, which is involved in pyridoxal impacts the homeostasis of vitamin B6 in et al. increases the level of vitamin B6 in shoots and desiccated was significantly reduced in AtPUP1 and, subsequently, were washed three times with rite solution and, subsequently, were washed three times with chamber with the temperature at 28 °C/25 °C (day/night) under approximately 200 μm photons m⁻² s⁻¹. Four-week-old seedlings were grown hydroponically in Yoshida’s culture solution (Yoshida, 1976) and cultured in a growth for 3 days. Seedlings were grown hydroponically in Yoshida’s growth solution for the recovery. Survival rates were measured after 3 days recovery, and survival rates were shown as percentages of alive seedlings. Plants not showing green shoots were regarded as dead seedlings. For testing the flowering phenotype, NIP, osmdh1 and OsMDH1OX lines were grown in the soil with at least 10 plants in each genotype.

Plasmid construction and generation of transgenic plants
Gene-specific primers, OsMDH1-F/R, were used to isolate OsMDH1 cDNA from a cDNA library by PCR. To generate the pCSV1300-OsMDH1 construct, full-length OsMDH1 was amplified and cloned into the pCSV1300 vector using the XbaI and BamHI sites (Xu et al., 2012). To generate OsMDH1-GFP construct for the rice protoplast transfection, OsMDH1 cDNA was inserted into 326-GFP using XbaI and BamHI sites (jin et al., 2001). To generate OsMDH1pro::GUS construct, 2.98-kb fragment upstream of OsMDH1 was amplified by PCR using primer sets, OsMDH1p-F/R, and was inserted into the binary vector pCAM-BIA3301 using EcoRI and Ncol sites (Kim et al., 2013). To generate GST-OsMDH1 construct, full-length OsMDH1 cDNA fragment was amplified by PCR using primer sets, GST-OsMDH1-F/R as well as OsMDH1[M]-F/R, and were inserted into pGEX-4T-1 vector (Ahmad et al., 2019) using BamHI and EcoRI sites, respectively. To knockout OsMDH1 gene, two OsMDH1 CRISPR/Cas9 constructs were designed using the pYLSgRNA-OsU6a and pYLCRISPR/Cas9Pubi-H plasmids according to the method described previously (Ma et al., 2015). CRISPR/Cas9 targeted sequences were designed in the following website (http://skl.sca.edu.cn/). Constructs were transformed into NIP plants by the Agrobacterium-mediated transformation method (Hiei et al., 1994). PCR and Sanger sequencing were used to examine the mutation sequences. T2 seeds were screened with hygromycin (Liu et al., 2018). All primers are listed in Table S7.

Tissue-specific expression test using GUS staining and examination of subcellular localization
GUS staining experiment was performed using transgenic plants expressing the β-glucuronidase (GUS) gene under the control of the OsMDH1 promoter (OsMDH1pro::GUS) as previously described (Jefferson et al., 1987). Rice protoplasts obtained from three-week-old seedlings (grown under 12-h light and 12-h dark conditions) were used for examining the subcellular localization of OsMDH1-GFP (Zhang et al., 2011). The fluorescence microscope (Olympus BX53, Japan) was used to detect GFP and chloroplast autofluorescence signals. Western blot analysis was performed using a monoclonal mouse anti-GFP antibody (Transgen Biotech, Cat. HTB01-01).

NBT and DAB staining methods
The nitroterazolium blue chloride (NBT) and diaminobenzidine (DAB) staining assays were performed according to the method as previously described with a slight modification (Wu et al., 2017). Four-week-old seedlings were treated without or with 100 mM NaCl for 12 h. To detect O₂⁻, the leaves of plants were vacuum infiltrated for 30 min and then stained for 12 h with 0.05% NBT (w/v) and 10 mM NaNO₃ in 10 mM potassium phosphate buffer (pH = 7.8). To detect H₂O₂, the leaves of...
plants were vacuum infiltrated for 1 h and then stained with 0.1% DAB for 24 h (pH = 5.8). Subsequently, leaves were incubated in the destaining buffer (ethanol:lactic acid:glyceraldehyde 3:1:3) (Zhang et al., 2018).

**Protein purification**

Different constructs expressing GST, GST-OsMDH1 or GST-OsMDH1[M] were transformed to E. coli BL21 cell line. Protein purification was performed as previously described (Xu et al., 2013).

**Chloroplast isolation**

Chloroplasts were isolated from 1 g of leaves of four-week-old seedlings as described previously (van Wijk et al., 2007). The intactness of the purified chloroplast fraction was over 80%–90% as judged by the ferricyanide test. Protein amounts were quantified using the Protein Assay Dye Reagent Concentrate (Bio-Rad, Cat. 5000006).

**Analysis of OsMDH1 biochemical activity**

Malate dehydrogenase activity of purified proteins including GST, GST-OsMDH1, GST-OsMDH1[M] and proteins extracted from chloroplasts was measured using Malate Dehydrogenase Assay Kit (Sigma, Cat. MAK196). Briefly, after initiating the reaction by adding MDH substrate, spectrophotometric change at 340 nm was monitored automatically at 40-s intervals for 5 min. Activities were calculated according to the NADH standard curve after each measurement (Berkemeyer et al., 1998).

**Profiling of metabolites by liquid chromatography–mass spectrometry (LC-MS)**

Nearly 50 mg of leaves of four-week-old seedlings from Nip and OsMDH1OX-1 plants was incubated with 500 µl methanol with ten biological replications. The mixture was homogenized for 1 min at 70 Hz and mixed by vortex mixer for 30 s. Then, the mixture was centrifuged at 18 000 g 4 °C for 15 min. Two hundred micro litre supernatant was transferred to sampler vials and detected. An in-house quality control (QC) was prepared by mixing equal amount of each sample. Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6545 UHD and Accurate-Mass Q-TOF/MS was used for LC-MS analysis (Ebert et al., 2010; Gabay et al., 2019).

**Metabolome analyses**

The acquired MS data from GC-MS were converted into the common data format (.mzdata) by Agilent Mass Hunter Qualitative Analysis version B.08.00 software (Agilent Technologies, Palo Alto, California, USA). Using the R software platform (https://www.r-project.org/, Lucent Technologies, New Providence, NJ), the XCMS was applied for data pretreatment such as nonlinear retention time alignment, peak discrimination, filtering, alignment, matching and identification. Subsequently, visualization matrices containing m/z and RT pair, peak area as well as sample names were obtained. Then, multivariate analyses including PCA, PLS-DA and OPLS-DA were conducted. The differential metabolites were screened out by combining VIP (Variable Importance in the Projection) value of OPLS-DA model (VIP = 1) with P-value lower than 0.05 (Student’s t-test). Then, the differential metabolites were mapped to KEGG ID using MetaboAnalyst online software (https://www.metaboanalyst.ca/). Pathway analysis was conducted, and the model organism selected was Oryza sativa L. ssp. japonica. Pathways that have P-value lower than 0.05 were picked out (Fiehn et al., 2000).

**Determination of pyridoxine and malate contents**

The leaves samples of four-week-old seedlings were taken and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Extraction was performed by rapid grinding of tissue in liquid nitrogen and immediate addition of the appropriate extraction buffer. Malate was measured by enzymatic analysis (Nunes-Nesi et al., 2007). Pyridoxine was measured by HPLC as described previously (Szylowski et al., 2013).

**RNA extraction and RT-qPCR analysis**

Total RNA was extracted with Trizol (Invitrogen, Cat. AM1912). Two microgram RNA were used to prepare first-strand cDNA using Transcript One-Step qDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech, Cat. ALU311-02), RT-qPCR analysis was performed using the THUNDERBIRD SYBR qPCR Mix Without Rox reagent (TOYOBO, Cat. QPS-20V). Each sample was normalized against OsACT1 control, and fold change relative to wild type was calculated according to the 2^ΔΔCt method (Livak and Schmittgen, 2001). All primers are listed in Table S7.

**Bioinformatics analyses of RNA-seq data**

Total RNAs were extracted from NIP and OsMDH1OX plants and were subsequently used for transcriptome analysis using the Illumina HiSeq2500 platform (Illumina, San Diego, CA) with three biological replications. The Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) was used to determine the quality and concentration of RNA. Sequencing was performed in paired-end mode with a read length of 150 nucleotides. Next, low-quality (< Q20) reads were excluded from raw data using FASTX-Tookit v.0.0.13 (http://hannonlab.cshl.edu/fastx_tool-kit/).

The clean reads were mapped to rice reference genome MSU7.0 using HISAT2 v.2.1.0 (https://ccb.jhu.edu/software/hsisat2/index.shtml) with default parameters (Kim et al., 2015). Gene quantification was performed using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/) with genomic annotation. The differentially expressed genes were filtered according to the fold change (|log2 FC| > 1) and an adjusted P-value (<0.05), calculated with Cuffdiff (a subpackage of Cufflinks) (Yu et al., 2016, 2018). The gene ontology (GO) grouping of differentially expressed genes (DEGs) was performed by hypergeometric distribution in R in v.3.1.0 (https://www.r-project.org/, Lucent Technologies), with an adjusted P-value <0.05 as a cut-off to determine significantly enriched GO terms.

**Accession number**

Data generated in this study are deposited in NCBI Sequence Read Archive (accession number PRJNA528686).

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**Author contributions**

Z.-Y.X. and N.N. devised and supervised the project. N.N. performed most of experiments and analysed data. J.W. and Y.J.S. generated transgenic plants. Y.W.Q. provided mutant lines.
Conflict of interest

The authors have no conflict of interest to declare.

References

Ahmad, R., Liu, Y., Wang, T.-J., Meng, Q., Yin, H., Wang, X., Wu, Y. et al. (2019) GOLDEN2-LIKE transcription factors regulate WRKY40 expression in response to abscisic acid. Plant Physiol. 179, 1844–1860.

Beeler, S., Liu, H.C., Schreier, T., Eicke, S., Lue, W.L., Truernit, E. et al. (2014) Plastidal NAD-dependent malate dehydrogenase is critical for embryo development and heterotrophic metabolism in Arabidopsis. Plant Physiol. 164, 1175–1190.

Berkemer, M., Scheibe, R. and Ocheretina, O. (1998) A novel, non-redox-regulated NAD-dependent malate dehydrogenase from chloroplasts of Arabidopsis thaliana L. J. Biol. Chem. 273, 27927–27933.

Burns, K.E., Xiang, Y., Kinsland, C.L., McLafferty, F.W. and Begley, T.P. (2005) Reconstitution and biochemical characterization of a new pyridoxal-5'-phosphate biosynthetic pathway. J. Am. Chem. Soc. 127, 3682–3683.

Centeno, D.C., Osorio, S., Nunes-Nesi, A., Bertolo, A.L.F., Carneiro, R.T., Araújo, W.L., Steinhauser, M.-C. et al. (2011) Malate plays a crucial role in starch metabolism, ripening, and soluble solute content of tomato fruit and affects postharvest softening. Plant Cell, 23, 162–184.

Chen, H. and Xiong, L. (2005) Pyridoxine is required for post-embryonic root function in abscisic acid and dehydration stress responses. Plant J. 40, 396–408.

Christine, G. (1992) Malate dehydrogenase isoenzymes: cellular locations and role in the flow of metabolites between the cytoplasm and cell organelles. Biochim. Biophys. Acta, 1100, 217–234.

Denslow, S.A., Rueschoff, E.E. and Daub, M.E. (2007) Regulation of the Arabidopsis thaliana vitamin B6 biosynthesis genes by abiotic stress. Plant Physiol. Biochem. 45, 152–161.

Ebert, B., Zoller, D., Erban, A., Fehrle, I., Hartmann, J., Niehl, A., Kopka, J. et al. (2010) Metabolic profiling of Arabidopsis thaliana epidermal cells. J. Exp. Bot. 61, 1321–1335.

Ehrensreich, M., Bilski, P., Li, M.Y., Chignell, C.F. and Daub, M.E. (1999) A highly conserved sequence is a novel gene involved in de novo vitamin B6 biosynthesis. Proc. Natl Acad. Sci. USA, 96, 9374–9378.

Emanuelsson, O., Nielsen, H. and Von Heijne, G. (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci. 8, 978–984.

Ferro, M., Brugière, S., Salvi, D., Seigneurin-Berny, D., Court, M., Moyet, L. and Ramus, C. et al. (2010) AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. Mol. Cell. Proteomics, 9, 1063–1084.

Fiehn, O., Kopka, J., Dörmann, P., Altman, T., Trethewey, R.N. and Willmitzer, L. (2000) Metabolite profiling for plant functional genomics. Nat. Biotechnol. 18, 1157–1161.

Gaby, B., Faigenboim, A., Dahan, Y., Izhaki, Y., Itkin, M., Malitsky, S., Elkind, Y. et al. (2019) Transcriptome analysis and metabolic profiling reveal the key role of α-linolenic acid in dormancy regulation of European pear. J. Exp. Bot. 70, 1017–1031.

González, E., Danehower, D. and Daub, M.E. (2007) Vitamin levels, stress response, enzyme activity, and gene regulation of Arabidopsis lines mutant in the pyridoxine/pyridoxamine 5'-phosphate oxidase (PDX3) and the pyridoxal kinase (SOS5) genes involved in the vitamin B6 salvage pathway. Plant Physiol. 145, 985–996.

Halket, J.M., Waterman, D., Przyborowska, A.M., Patel, Raj K.P., Fraser, P.D. and Bramley, P.M. (2004) Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. J. Exp. Bot. 56, 219–243.

Havaux, M., Kias, B., Szewczyk, A., Rumeau, D., Franck, F., Caffarri, S. and Triantaphylides, C. (2009) Vitamin B6 deficient plants display increased sensitivity to high light and photo-oxidative stress. BMC Plant Biol. 9, 130.

Hellmann, H. and Mooney, S. (2010) Vitamin B6: a molecule for human health? Molecules, 15, 442–459.

Herrero, S., González, E., Gillikin, J.W., Vélez, H. and Daub, M.E. (2011) Identification and characterization of a pyridoxal reductase involved in the vitamin B6 salvage pathway in Arabidopsis. Plant Mol. Biol. 76, 157–169.

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

Hsu, S.-Y. and Kao, C.H. (2003) Differential effect of sorbitol and polyethylene glycol on antioxidant enzymes in rice leaves. Plant Growth Regul. 39, 83–90.

Huang, S., Zhang, J., Wang, L. and Huang, L. (2013) Effect of abiotic stress on the abundance of different vitamin B6 vitamer in tobacco plants. Plant Physiol. Biochem. 66, 63–67.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907.

Jin, J.B., Kim, Y.A., Kim, S.J., Lee, S.H., Kim, D.H., Cheong, G.-W. and Hwang, I. (2001) A new dynamin-like protein, AD6L, is involved in trafficking from the trans-golgi network to the central vacuole in arabidopsis. Plant Cell, 13, 1511–1526.

Kim, D., Abbaei, N. and Choi, S.-B. (2013) Bruno-like proteins modulate flowering time via 3' UTR-dependent decay of SOCI mRNA. New Phytol. 198, 747–756.

Kim, D., Langmead, B. and Salzberg, S.L. (2015) HISAT: a fast spliced aligner with low memory requirements. Nat. Methods, 12, 357–360.

Li, K.-T. (2014) Metabolic engineering of vitamin B1 and vitamin B6 in staple crops. Li, K., Wang, Y., Han, C., Zhang, W., Jia, H. and U, X. (2007) GA signaling and COFT regulatory module mediate salt-induced late flowering in Arabidopsis thaliana. Plant Growth Regul. 53, 199–206.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^–ΔΔCT method. Methods, 25, 402–408.

Lu, Y., Ye, X., Guo, R., Huang, J., Wang, W., Tang, J., Tan, L. et al. (2017) Genome-wide targeted mutagenesis in rice using the CRISPR/Cas9 system. Mol. Plant, 10, 1242–1245.

Lunn, H.K., Kwok, F. and Lo, S.C. (2002) Cloning and characterization of Arabidopsis thaliana pyridoxal kinase. Planta, 215, 870–879.

McCord, J.M. (2000) The evolution of free radicals and oxidative stress. Am. J. Med. 108, 652–659.

Miller, S.S., Driscoll, B.T., Gregerson, R.G., Garrt, J.S. and Vance, C.P. (1998) Alfalfa malate dehydrogenase (MDH): molecular cloning and characterization of five different forms reveals a unique nodule-enhanced MDH. Plant J. 15, 173–184.

Nunes-Nesi, A., Carrari, F., Gibon, Y., Sulèpcz, L., Lytochenko, A., Fishan, J., Graham, J. et al. (2007) Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. Plant J. 50, 1093–1106.

Ocheretina, O. and Scheibe, R. (1997) Cloning and sequence analysis of cDNAs encoding plant cytosolic malate dehydrogenase. Gene, 199, 145–148.

Osmani, A.H., May, G.S. and Osmani, S.A. (1999) The extremely conserved pyroGlu gene of Aspergillus nidulans is required for pyriodoxine synthesis and is required indirectly for resistance to photosensitizers. J. Biol. Chem. 274, 23365–23369.

Pracharoenwattana, I., Cornah, J.E. and Smith, S.M. (2007) Arabidopsis peroxisomal malate dehydrogenase functions in β-oxidation but not in the glyoxylate cycle. Plant J. 50, 381–390.

Raschke, M., Boycheva, S., Crevencoceur, M., Nunes-Nesi, A., Witt, S., Fermie, A.R., Amrhein, N. et al. (2011) Enhanced levels of vitamin B6 increase aerial organ size and positively affect stress tolerance in Arabidopsis. Plant J. 66, 414–432.

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Tambasco-Studart, M., Arigoni, D., Brunisholz, R., Rechsteiner, H., Amrhein, N. and Szydlowski, N., Shi, H., Xiong, L., Stevenson, B., Lu, T. and Zhu, J.-K. (2002) The Arabidopsis Shreier, T.B., Cl, Raschle, T., Arigoni, D., Brunisholz, R., Rechsteiner, H., Amrhein, N. and Raschle, T., Amrhein, N. and Fitzpatrick, T.B. (2005) On the two components of Nan Nan et al. Acad. Sci. USA Nan Nan et al. 102, 13687–13692.

Xia, J. and Wishart, D.S. (2016) Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. Curr. Protoc. Bioinformatics, 55, 14.10.11-14.10.91.

Xu, Z.-Y., Lee, K.H., Dong, T., Jeong, J.C., Jin, J.B., Kanno, Y., Kim, D.H. et al. (2007) A vacuolar β-glucosidase homolog that possesses glucose-conjugated abscisic acid hydrolyzing activity plays an important role in osmotic stress responses in Arabidopsis. Plant Cell, 24, 2184-2199.

Xu, Z.-Y., Kim, S.Y., Hyeon, D.Y., Kim, D.H., Dong, T., Park, Y., Jin, J.B. et al. (2013) The Arabidopsis NAC transcription factor ANAC096 cooperates with bZIP-type transcription factors in dehydration and osmotic stress responses. Plant Cell, 25, 4708-4724.

Yoshida, S. (1976) Routine procedure for growing rice plants in culture solution. In Laboratory Manual for Physiological Studies of Rice (Yoshida, S., Forno, D.A., Cock, J.H. and Gomez, K.A., ed), pp. 61–66. Philippines: International Rice Research Institute.

Yu, X., Jiang, L., Wu, R., Meng, X., Zhang, A., Li, N., Xia, Q. et al. (2016) The core subunit of a chromatin-remodeling complex, ZmCHB101, plays essential roles in maize growth and development. Sci. Rep. 6, 38504.

Yu, X., Meng, X., Liu, Y., Li, N., Zhang, A., Wang, T.-J., Jiang, L. et al. (2018) The chromatin remodeler ZmCHB101 impacts expression of osmotic stress-responsive genes in maize. Plant Mol. Biol. 97, 451–465.

Zhang, Y., Su, J., Duan, S., Ao, Y., Dai, J., Liu, J., Wang, P. et al. (2011) A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. Plant Methods, 7, 30.

Zhang, Z., Liu, H., Sun, C., Ma, Q., Bu, H., Chong, K. and Xu, Y. (2018) H. C2H2 zinc-finger protein OsZFP213 interacts with OsMAPK3 to enhance salt tolerance in rice. J. Plant Physiol. 229, 100–110.

Zhu, J.-K. (2002) Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53, 247–273.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure 51 Phylogenetic relationship of plastidial NAD-MDH in monocot plants and catalytic sites prediction of OsMDH1.

Figure 52 Phenotypes of the osmdh1 mutants.

Figure 53 Phenotypes of the OsMDH1OX lines.

Figure 54 OPLS-DA, PLSDA and PCA loading plots for the discrimination between NIP vs OsMDH1OX-1 plants under normal conditions.

Table 51 Chloroplast transit peptide cleavage site prediction of OsMDH1 using ChloroP.

Table 52 Differential metabolites identified comparing OsMDH1OX-1 with NIP plants under normal conditions.

Table 53 Metabolic pathways of differential metabolites identified comparing OsMDH1OX-1 with NIP plants under normal conditions.

Table 54 Reads numbers and data size of RNA-Seq data.

Table 55 Differentially expressed genes comparing OsMDH1OX with NIP plants under normal conditions.

Table 56 Enriched GO terms of differentially expressed genes comparing OsMDH1OX with NIP plants under normal conditions.

Table 57 Primer sequences used in different experiments.