Cytosolic malate dehydrogenase 4 modulates cellular energetics and storage reserve accumulation in maize endosperm

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Introduction

Owing to its high value as animal feed, raw industrial material, food for human consumption and fuel through bioethanol production, maize (Zea mays L.) is widely distributed and cultivated throughout the world. Maize endosperm accounts for over 75% of the kernel dry weight and mainly contains starch and protein. Maize starch endosperm mutants often exhibit changes in the structure and/or accumulation of starch granules and/or protein bodies (PBs), thus affecting grain yield and nutritional quality. Examples include the opaque (o) and floury (fl) mutants. o1, o10, fl1 and fl4 mutants have abnormal PBs and noticeable changes in the levels of zeins and alcohol-soluble prolamins, which are the main components of PBs (Holding et al., 2007; Larkins and Hurkman, 1978; Lending and Larkins, 1989; Wang et al., 2014a; Wang et al., 2012; Yao et al., 2016). Several other opaque/floury mutants, such as o2, o6, o7, o11, fl2, fl3, Mucronate, De*-B30, Mto140 and ocd1, display reduced zeins and a compensatory increase in non-zeins, which result in elevated lysine content (Coleman et al., 1997; Feng et al., 2018; Holding et al., 2010; Kim et al., 2006; Kim et al., 2004; Li et al., 2017; Schmidt et al., 1992; Wang et al., 2011; Wang et al., 2014b; Yang et al., 2018). In case of o5, however, the opaque phenotype is caused by morphological alterations in starch granules rather than changes in protein and/or amino acid composition (Hunter et al., 2002; Myers et al., 2011). These alterations are due to mutations in the structural or regulatory protein genes of zeins. opaque/floury mutants, such as o2, o11, fl2, fl3, Mucronate and De*-B30, involve genes that regulate zein biosynthesis (Coleman et al., 1997; Feng et al., 2018; Kim et al., 2006; Kim et al., 2004; Li et al., 2017; Schmidt et al., 1992), whereas other opaque/floury mutants, such as o1, o10, fl1 and fl4, are controlled by genes that participate in the assembly of zeins into PBs (Holding et al., 2007; Wang et al., 2014a; Wang et al., 2012; Yao et al., 2016). In addition, the o6, o7, Mto140 and ocd1 mutants are impaired in amino acid metabolism or other primary metabolic pathways (Holding et al., 2010; Wang et al., 2011; Yang et al., 2018). For example, O6 (also known as Pro1) and Mto140 encode Δ1-pyrroline-5-carboxylate synthetase and arogenate dehydrogenase 1 that catalyze proline and tyrosine biosynthesis, respectively. Mutations of these two genes result in restricted proline and tyrosine supply for zein biosynthesis (Holding et al., 2010; Wang et al., 2014b). O7 and Ocd1, however, are oxalyl-CoA synthetase and oxalyl-CoA decarboxylase genes that are involved in oxalate degradation. These mutations affect amino acid levels and zein accumulation (Wang et al., 2011; Yang et al., 2018) through an unknown mechanism. Together, these published data suggest a link between amino acid metabolism, zein biosynthesis and endosperm texture. Thus, the cloning and characterization of additional kernel mutants will deepen our understanding of how protein and/or starch biosynthesis are regulated in maize endosperm.

To this end, we isolated a starchy kernel mutant with altered starch, zein and lysine content in the endosperm. The mutant phenotype was found to be the result of a 3-bp deletion in the cytosolic NAD-dependent malate dehydrogenase 4 (Mdh4) gene. MDHs are oxidoreductases that catalyse the reversible interconversion between malate and OAA in the cytosol and other organelles, including glyoxysomes, mitochondria, peroxisomes.
and chloroplasts. In Arabidopsis thaliana, the plastidial NAD-specific MDH shuttles malate and OAA in non-photosynthetic tissues and its null mutant is embryo lethal (Beeler et al., 2014; Selinski et al., 2014). In contrast, NADP-MDH is important for adjusting the ATP/NADPH ratio in light malate valve and loss of NADP-MDH has no or little effect on growth (Hebbelmann et al., 2012; Heyno et al., 2014). Overexpressing maize NADP-dependen MDH in A. thaliana confers salt tolerance (Kandoi et al., 2018). Mitochondrial NAD-MDHs play a critical role in the tricarboxylic acid (TCA) cycle and null mutants of the two NAD-MDH isoforms display viable but abnormal plant development (Sew et al., 2016; Tomaz et al., 2010). Peroxisomal NAD-MDHs mainly generate NAD⁺ for the β-oxidation of fatty acids and double mutant of the two isoforms fail to efficiently mobilize triacylglycerols (Pracharoenwattana et al., 2007, 2010). Although loss-of-function mutants of the plastidial, mitochondrial and peroxisomal MDH isoforms have been characterized, the phenotype expression of cytosolic MDH of A. thaliana mutants is largely unknown. Cytosolic MDH mediates malate biosynthesis in the cytosol and has been reported to participate in plant and cell growth in apple (Wang et al., 2016; Yao et al., 2011a, 2011b). Its overexpression confers abiotic stress tolerance, such as cold and salt exposure, in apple trees by modulating redox homeostasis via malate accumulation (Wang et al., 2016; Yao et al., 2011a, 2011b). Recently, a NAD-dependent cytosolic MDH (flo16) has been reported in rice, revealing a potential role of cytosolic MDHs on reserve storage and seed development in the grass family (Teng et al., 2019). The mutant exhibits reduced ATP production and enhanced oxidation via a reduction in the activities of starch biosynthetic enzymes, leading to decreased starch accumulation in rice endosperm. Conversely, the overexpression of cytosolic MDH significantly improves grain weight (Teng et al., 2019). Taken together, these data point to a role of cytosolic MDHs in regulating starch biosynthesis and seed development in grass species.

The maize genome contains four NAD-dependent MDH isoforms and one NADP-dependent MDH isoform (Goodman et al., 1981). However, data concerning the roles of these isoforms are limited. Of these, ZmMdh1 (Zm00000d009640), ZmMdh2 (Zm00001d039089) and ZmMdh3 (Zm00001d044042) are localized to mitochondria, ZmMdh4 (Zm00001d032695) and ZmMdh5 (Zm00001d014030) to the cytosol, and ZmMdh6 (Zm00001d031899) to plastids (Goodman et al., 1981; Newton and Schwartz, 1980). The cytosolic isoforms ZmMdh4 and ZmMdh5 are a result of gene duplication and reside on different chromosomes (McMillin and Scandalios, 1980). In maize, at least one of the two mitochondrial MDHs needs to be present to ensure normal kernel development (Goodman et al., 1981), suggesting that MDH activity is indispensable for maize kernel and plant development under non-stress conditions. By contrast, the null homozygous mutants of either or both cytosolic MDHs are viable and can grow to maturity (Goodman et al., 1981), suggesting a potential regulatory role in endosperm development and storage reserve accumulation.

In this study, we isolated the maize kernel mutant, mdh4-1, which shows a deformed endosperm, reduced starch and zein content, and enhanced lysine content. Map-based cloning, genetic transformation and allelism analyses confirmed cytosolic ZmMdh4 as the causal gene. A 3 base pairs (bps) deletion in exon 7 of ZmMdh4 was validated as the functional mutational site. This mutation renders the ZmMdh4 enzyme almost inactive for converting OAA to malate, likely by altering the tertiary structure of the enzyme. The mutant shows elevated ZmMdh4 transcript and protein levels, presumably to compensate for the reduction in enzymatic activity. Combined with the transcriptome and metabolome data of immature kernels at 12 days after pollination (DAP), we propose a regulatory model of ZmMdh4 on storage reserve accumulation in maize.

**Results**

A novel naturally occurring mdh4-1 mutant displays small and opaque kernels, reduced starch content and elevated lysine levels

mdh4-1 was originally isolated as a small kernel mutant with a deformed endosperm crown (Figure 1a), which appeared 12 DAP on segregating F₂ ears. These mutants were characterized by the relatively smaller kernel size and delayed development (Figure S1). At maturity, the 100-kernel weight of homoygous mutant kernels was only 55.1% of the wild type (WT), and the length, width and thickness were 10.8%, 16.5% and 29.8%, lower than those of the WT, respectively (P < 0.001; Student’s t-test; Figure 1b and Figure S2a-b). In addition, the longitudinal sections of the kernels showed that the mutant embryo was abnormal and smaller in size compared with that of the WT (Figure 1c). Upon quantification of the storage constituents, a 6.9% and 14.5% reduction in total starch and amylose content, respectively, was observed in mature mutant kernels as compared to the WT (Figure 1d). Furthermore, 12 DAP mdh4-1 endospermis accumulated a much lower level of zein protein than WT (Figure 1e). At maturity, the mutant endosperms had an increased level of non-zein proteins (Figure S2c-d), which was accompanied by an increase in lysine, one of the major components of non-zeins, by approximately 1.4-fold (Figure 1f, Table S2).

In addition to the kernel phenotypes, the mdh4-1 mutant also exhibited various growth defects. For example, it exhibited a 50% lower germination rate than the WT (Figure 1h), and radicle root length of the mutant was much shorter than that of the WT (Figure 1g, i). Moreover, the mutant seedlings produced less and shorter roots than WT seedlings at 13 days after germination (DAG) (Figure 1j-k). Lastly, mutant plant height and ear length were also reduced in comparison to the WT (Figure 1l-m). These observations suggest that the mdh4-1 mutation exerts pleiotropic effects on plant growth and kernel development.

The mdh4-1 mutant exhibits delayed endosperm and embryo development

To further dissect the starchy-like kernel phenotype, sections of mature and developing mdh4-1 and WT kernels were examined. Under a scanning electron microscope (SEM), mature mutant kernels displayed irregularly shaped starch granules with reduced surrounding matrix, whereas the starch granules in mature WT kernels aggregated together and were surrounded by a dense matrix (Figure 2a). The paraffin sections showed that mdh4-1 embryos developed much more slowly than those of the WT. Notably, the shoot and root apical meristems, and leaf primordia were clearly visible in the WT embryos, but were still indistinguishable in mutant embryos at 12 DAP (Figure 2b). Additionally, starchy endosperm development was severely retarded in the mutant as compared with the WT, with the starchy endosperm occupying only 2/3 of the kernel as to 100% occupancy in the WT (Figure 2c). At 16 DAP, the mutant embryo was deformed and the endosperm had developed into three blocks (Figure 2c). These observations support the notion that embryo and
Figure 1  Phenotypic comparison between WT and the *mdh4-1* mutant. (a) Segregation of Zheng58 × *mdh4-1* F₂ seeds. The red arrows indicate *mdh4-1* kernels; scale bar, 1 cm. (b) 100-kernel weight of the WT vs. *mdh4-1*, with five replicates. (c) Comparison between the longitudinal sections of WT and *mdh4-1* kernels. En, endosperm; Em, embryo; scale bar 2 mm. (d) Starch and amylose content in WT vs. *mdh4-1* endosperms with four replicates. (e) Zein proteins in 12 DAP endosperms of WT and *mdh4-1*. (f) Total lysine content in WT vs. *mdh4-1* mature kernels with three replicates. (g) Representative examples of germination of the WT and *mdh4-1* seeds; scale bar, 1 cm. (h) Germination rate of WT vs. *mdh4-1* with three replicates. (i) Radical length of WT vs. *mdh4-1* seedlings with three replicates. (j) Representative examples of WT and *mdh4-1* seedlings 13 days after germination (DAG); scale bar, 5 cm. (k) Height and root length of WT vs. *mdh4-1* seedlings 13 DAG, with ten replicates. (l) Representative examples of mature WT and *mdh4-1* plants; scale bar, 30 cm. (m) Plant height and ear length of mature WT vs. *mdh4-1* plants, with 15 replicates. In all bar graphs, values are represented as means ± SE, **P < 0.01, ***P < 0.001 denote statistically significant differences between WT and *mdh4-1* (Student’s t-test).
endosperm development were delayed in the mdh4-1 mutant. In addition, reduced ingrowth in the cells of the basal endosperm transfer layer (BETL) in mdh4-1 kernels as compared to the WT kernels was observed, suggesting impaired transmission of nutritional constituents from the maternal tissue to endosperm cells (Figure 2b-c).

The starchy endosperm cells were examined by transmission electron microscopy (TEM), and it was found that the shape and number of PBs were similar between 18 DAP WT and mutant endosperms (Figure 2d). However, mdh4-1 kernels exhibited smaller starch granules than the WT (Figure 2e-f), which may be related to the loosely packed starch granules and reduced 100-kernel weight of the mutant kernels (Figures 1b and 2a).

**Map-based cloning of mdh4-1**

For genetic analysis, mdh4-1 was crossed with a widely used Chinese elite inbred line, Zheng58, and the resulting F1 progeny was self-pollinated to generate an F2 population (Figure 1a). The ratio of WT to mutant F2 kernels was roughly 3:1, suggesting the existence of a single recessive mutation. We randomly selected and grew F2 kernels that showed the WT phenotype and collected and analysed 100 F3 ears. The ratio of non-segregating (n = 30, homozygote) to segregating ears (n = 70, heterozygote) was approximately 1:2 ($\chi^2 = 0.5 < \chi^2_{0.05} = 3.84$). In addition, the segregation of normal to mutant F3 kernels obtained from segregating F2 ears followed a 3:1 ratio (normal:mdh4-1 = 5494:1833, $\chi^2 = 0.002 < \chi^2_{0.05} = 3.84$; Table S1). Similar segregation ratios were observed in the other five F2 segregating populations constructed by crossing mdh4-1 with different inbred lines (Table S1 and Figure S2e). Collectively, these results indicate that the mutant phenotype of mdh4-1 is caused by a single recessive locus.

Preliminary genetic mapping using 419 F2 individuals placed the target gene between the simple sequence repeat (SSR) markers umc1245 and umc2181 (Table S3) on bin1.08 of chromosome 1. The interval was further narrowed down to a...
~224-kb region between markers C362 (two recombinants) and Indel-98 (five recombinants) using an F₂ population consisting of 34,080 individuals (Figure 3a). The 224-kb target region contains two predicted open reading frames (ORFs), Zm00001d032695 and Zm00001d032699, in the B73 reference genome (RefGen V4; Figure 3a). The genomic sequences of these two genes were compared between mdh4-1 and Zheng58 and several single nucleotide polymorphisms (SNPs) were identified, as well as insertions and deletions (InDels). However, only the 3-bp deletion in exon 7 of Zm00001d032695 was found exclusively in the mdh4-1 mutant (Figure 3b; Figure S3). Zm00001d032695 is annotated as a cytosolic malate dehydrogenase 4 in Gramene (http://www.gramene.org/Zea_mays/) and was therefore named ZmMdh4. ZmMdh4 is ~5.4-kb in length with seven exons (Figure 3b), and the full-length cDNA of ZmMdh4 is estimated to be 1381-bp. The deduced protein translation contains 332 amino acids, with a molecular mass of ~36 kDa. It has a predicted NAD-dependent cytoplasmic malate dehydrogenase domain (Figure 3c). The polymorphic sites of ZmMdh4 were analysed in an association panel consisting of 540 inbred lines to identify the functional polymorphisms associated with kernel characteristics (http://www.maizego.org/Resources.html; Table S4). Three SNPs in the 5′ UTR were found to associate with lysine content and/or kernel thickness (Figure 3b and Table S5). Based on these results, ZmMdh4 was selected as the candidate gene for further validation and characterization.

To validate whether ZmMdh4 is the causal genetic basis for the observed mdh4-1 kernel phenotype, the ZmMdh4 coding sequence was overexpressed using the constitutive ubiquitin (Ubi) promoter. The resulting transgenic line (Mdh4-OE) was crossed with a homozygous mdh4-1 (—/—) mutant, and F₁ was self-pollinated to generate F₂ seeds (Figure 3d). Twenty-four normal (WT) and 11 mutant F₂ kernels were selected randomly and genotyped with markers detecting the 3-bp deletion in mdh4-1 (—/—) and the Mdh4-OE transgene. Four of the 24 normal kernels had the homozygous mdh4-1 (—/—) genotype with Mdh4-OE (Figure 3e-f). Additionally, they showed phenotypic characteristics analogous to those of the normal kernels at the seedling stage (Figure 3g). These results indicate that Mdh4-OE could rescue the mdh4-1 mutant phenotype. Together, these data confirmed that ZmMdh4 was the causal gene of the mdh4-1 mutant.

**Knocking out ZmMdh4, but not ZmMdh5, phenocopies mdh4-1 phenotypes**

To further confirm ZmMdh4 as the causal gene of the mdh4-1 mutant phenotype, Mu-inserted ZmMdh4 mutants were screened from the Maize Genetics Stock Center. However, the only Mu-mutant identified had the Mu insertion in the non-coding region of ZmMdh4, which exhibited no obvious kernel phenotype. Thus, CRISPR/Cas9 was used to generate ZmMdh4 loss-of-function lines with a specific guide RNA (grRNA) using ZZZ01. Sequencing of the CRISPR/cas9-mediated ZmMdh4-edited transgenic individuals resulted in the identification of two mutants, mdh4-2 and mdh4-3, that carry null mutations in the ZmMdh4 gene (Figure 4a). The T₁ individuals of mdh4-2 and mdh4-3 were self-pollinated for two consecutive generations to produce homozygous T₂ progeny (Figure 4b). Allelism tests were performed by crossing the homozygous mdh4-2 (—/—) and mdh4-3 (—/—) T₁ plants with both the homozygous mdh4-1 (—/—) and heterozygous (+/—) individuals, respectively. The results show that the F₂ kernels of the mdh4-2 (—/—) × mdh4-1 (—/—) cross all phenocopied homozygous mdh4-1 (—/—) kernels (Figure 4c), and the mdh4-2 (—/—) × (+/—) and F₂ kernels exhibited a mutant:WT ratio of 1:1 (Figure 4d and Table S6). Similar results were obtained with mdh4-3 (—/—) derived F₂ kernels. These findings confirmed that the CRISPR/cas9-mediated mutations were allelic to the naturally occurring mdh4-1 mutation. In support of this conclusion, kernel size and 100-kernel weight of the mdh4-2 (—/—) and mdh4-3 (—/—) T₂ individuals were found to be comparable to those of the mdh4-1 mutant (Figure S4a-c). In addition, the germination rate, seedling and adult height, as well as root length of the two cas9 lines, were reduced by more than 50% as compared with the control, similar to that observed for mdh4-1 (Figure S4d-i). These results further supported ZmMdh4 as the target gene.

Due to the high sequence similarity between ZmMdh5 and ZmMdh4 (McMillin and Scandalios, 1980; Gramene; http://ensembler.gramene.org/Zea_mays), both genes were knocked out by CRISPR/cas9 in mdh4-2 and mdh4-3. To test whether the cas9-mediated mutations in ZmMdh5 had contributed to the mutant phenotype, Zmdh5-linked markers were assayed. They did not co-segregate with kernel phenotype in the T₂ generation (Figure 4e-f), suggesting that the ZmMdh5 mutations were not causal of the mutant kernel phenotype in the two cas9 lines. Further, the F₂ kernels derived by crossing mdh4-1 (Mdh5 (+/+)) mdh4-1 (—/—) with mdh4-2 (Mdh5 (+/+)) mdh4-1 (—/—) all exhibited the mutant phenotype (Figure 4g-h). These results suggest that the ZmMdh5 cas9 mutations were unrelated to the mutant kernel phenotype, and ZmMdh4 was the target gene.

**ZmMdh4 is constitutively expressed and its protein is localized to the cytoplasm**

Using qPCR, ZmMdh4 expression was quantified in the root, stem, leaf, embryo, and endosperm of the WT and mdh4-1 mutant. ZmMdh4 was found to be constitutively expressed in all tested tissues, with the highest expression level in the leaf (Figure 5a). During kernel filling, ZmMdh4 expression was mainly detected during the early stages of endosperm development and its transcript level was higher in the mutant than the WT (Figure 5b). Consistent with this result, Western blot analysis with the MDH4 antibody indicated a relatively higher ZmMDH4 protein level in mdh4-1 kernels even during late endosperm development (Figure 5c).

In Gramene (http://ensembl.gramene.org/Zea_mays), ZmMDH4 is predicted to be localized in the cytoplasm. To verify this prediction, the full-length CDS of ZmMDH4 was C-terminally fused to the enhanced green fluorescent protein (eGFP) and transiently expressed under the control of the CaMV 35S promoter in Nicotiana benthamiana epidermal leaf cells and A. thaliana mesophyll protoplasts. In both approaches, the fusion protein was found localized in the cytoplasm (Figure 5d), thus confirming the localization of ZmMDH4 to the cytoplasm. On the contrary, free GFP signal was detected in the nuclei and the cytoplasm.

**ZmMDH4 predominantly catalyzes the conversion from OAA to malate**

The 3-bp deletion in the mdh4-1 mutant results in a Glu deletion at position 322 in the C-terminus of the MDH4 protein, which is distant from the active site (located at 156–168aa; Uniprot database, http://www.Uniprot.org). However, this deletion may alter the overall conformational structure of the ZmMDH4 protein. Specifically, Arg97 and Lys98, the spatial neighbours of Glu322, reside in a loop (88-103aa) associated with substrate binding (SWISS-MODEL algorithm; Waterhouse et al., 2018). The
Figure 3  Mapping and genotyping of ZmMdh4. (a) Fine mapping of ZmMdh4 using the F2 populations derived from Zheng58 and mdh4-1. The ZmMdh4 locus was mapped to a ~224 kb region on chromosome 1. The number of recombinants and the population size are shown on the left and right of each marker, respectively. (b) The gene structure of ZmMdh4. Allelic mutants are indicated by red letters. The black and white boxes indicate exons and introns, respectively. The bold black line indicates the promoter and the gray boxes indicate the untranslated regions (UTRs). (c) A schematic diagram of the ZmMDH4 protein, with conserved domains indicated. (d) Mdh4-OE/Mdh4 × mdh4-1 F2 ear. Mdh4-OE denotes the Mdh4 overexpression line. The red arrow indicates a mdh4-1 mutant kernel. Scale bar, 2 cm. (e-f) Genotyping of randomly selected kernels. Lanes 1–24 and 25–35 show the genotyping results of the normal and mutant kernels, respectively. Lane 36 shows the ZZC01 control. “-” and “+” represent the blank (H2O) and the CUB-ZmMdh4 vector control, respectively. M represents the marker. (g) Differences in the phenotypic expression of Mdh4-OE lines at ten-day after germination (DAG). WT-, non-transgenic lines with ZmMdh4 gene; mdh4+, positive transgenic lines with ZmMdh4 gene; mdh4-, negative transgenic lines with ZmMdh4 gene. The scale bar, 5 cm.
Figure 4 Validation of the ZmMdh4 transgenic lines. (a) Alignment of the genomic and amino acid sequences of the positive Cas9-edited transgenic plants and the control ZZC01. The bold letters in black denote the sgRNA target sequence. (b) Comparison of the mdh4-2 and mdh4-3 ears with ZZC01. Scale bars, 2 cm. (c) Allelism test using homozygous mdh4-1, mdh4-2, and mdh4-3. The scale bars are 2 cm. (d) Allelism test using heterozygous mdh4-1 and homozygous mdh4-2 and mdh4-3 mutants. The scale bars, 2 cm. The red arrows indicate mutant kernels. (e) Representative kernels from T2 Cas9-Mdh4 (+/-) Mdh5 (+/-); scale bars, 1 cm. (f) Linkage analysis of Zmmdh5 with randomly selected normal kernels from the mdh4-2 T2 generation. (g) Representative mdh4-1 × mdh4-2 and mdh4-1 F2 ears; scale bars, 2 cm. (h) Linkage analysis of ZmMdh5 with randomly selected kernels from the mdh4-1 × mdh4-2 F2 ear.
The salt bridge between Arg97 and Glu322 draws the loop and helix 9 together in the WT MDH4 enzyme. In the mutant enzyme, however, the salt bridge is abolished by the change of Lys98, thereby potentially loosening the MDH4 structure by preventing the interaction between the loop and helix 9 (Figure 6a).

To examine the effect of Glu322 deletion on MDH4 function, purified WT and mutant MDH4 protein heterologously expressed in bacteria were used in enzymatic assays to monitor changes in NADH levels (Figure 6b-c). Very little change in NADH levels was observed during the malate-to-OAA conversion for both enzymes but a substantial decrease in NADH with the WT MDH4 enzyme during the OAA-to-malate conversion, suggesting that MDH4 mainly catalyses the reaction from OAA to malate (Figure 6c). Consistent with our hypothesis, the mutant enzyme almost completely lost its activity for catalysing the OAA-to-malate conversion, as no obvious change in NADH was observed (Figure 6c). Thus, 3-bp deletion in exon 7 significantly impacts MDH4 activity presumably by causing a conformational change in MDH4 tertiary structure.

Disruption of ZmMdh4 leads to changes in cellular energetics and impairment of mitochondrial complex I and II function

Because the conversion of OAA to malate is a key step in the TCA cycle, the amount of ATP and metabolites associated with energy metabolism in 12 DAP mdh4-1 and WT kernels were quantified. Specifically, this was undertaken to investigate if the lack in ZmMDH4 activity affected energy production. The levels of lactate, aconitate, 3-phosphoglycerate (3PG), phosphoenolpyruvate (PEP) and cyclic-AMP increased significantly by >1.5-fold in mdh4-1 compared with the WT, whereas those of NAD+, NADH, pyruvate and alpha-ketoglutaric acid (α-KG acid) were reduced by >1.5-fold (Figure 7a-b). These data suggest that both glycolysis and the TCA cycle were altered. By contrast, the ratio of NAD+/NADH, which reflects the redox state, increased by 2.7-fold in the mdh4-1 mutant as compared to the WT (Figure 7c). Taken together, these results suggest that the TCA cycle is impaired in the mdh4-1 mutant. This is further supported by the observation that ATP content decreased ~40% in the 12 DAP mutant kernels compared with the WT (Figure 7d). Consistently, we observed reduced NADH dehydrogenase activity and the disassociation of the mitochondrial ATP-producing I+III super-complex in 15 DAP mutant kernels compared with the WT, as fewer I+III super-complexes were observed in the mutant than WT (Figure 7e). Further supporting evidence came from the substantial decreases in NAD7 and SDH1 proteins, as well as an increase in CYC1 protein in 15 DAP mutant kernels (Figure 7f). These results are consistent with the vacuolization of the mutant mitochondria in 15 DAP mutant kernels (Figure 7g), suggesting that the disruption of ZmMDH4 led to mitochondria dysfunction and reduced ATP production.

The loss of ZmMDH4 activity in mutant kernels also affected the levels of amino acids derived from the intermediates of the TCA cycle (Figure 7h). For example, we detected the accumulation of amino acids derived from the Asp-derived pathway and those from the Glu-derived pathway that competes with Pro, an important component of the zein protein, for the Glu substrate (Figure 7h). These results indicate that the mutant endosperm had developmental defects and alterations in central metabolism.
To further explore the molecular basis of the metabolic changes in the \textit{mdh4-1} mutant, 12 DAP mutant and WT kernels were subjected to RNA-seq analysis. A total of 23,594 transcripts were detected and retrieved, among which 413 were differentially expressed (log2 (fold change) \(>0.78\) or \(<1\) and false discovery rate (FDR) \(<0.01\)) between the WT and the \textit{mdh4-1} mutant. Of the differentially expressed genes (DEGs), 291 were up-regulated and 122 were down-regulated in the \textit{mdh4-1} mutant as compared to WT (Figure 8a). Of those, 309 DEGs could be functionally annotated by BLAST searches against the UniProt database (http://www.uniprot.org) and were analysed by an online Gene Ontology (GO) software (agriGO, Tian et al., 2017). The significantly enriched terms included carbohydrate metabolic process (GO: 0005975), oxidation-reduction process (GO: 0055114) and nutrient reservoir activity (GO: 0045735) (Figure 8b; Table S7).

Several genes were randomly selected and tested with quantitative real-time PCR (qPCR) to validate the RNA-seq results. Consistent with the RNA-seq data, the transcript levels of \textit{Zm00001d018971} (\textit{O2}), \textit{Zm00001d044129} (\textit{Sh2}), \textit{Zm00001d048813} (zein-alfa), \textit{Zm00001d050032} (\textit{Bt2}) and \textit{Zm00001d0502079} (\textit{LKR/SDH}) were lower, and those of \textit{Zm00001d033805}, \textit{Zm00001d039346}, \textit{Zm00001d041671} (\textit{BETL3}) and \textit{Zm00001d047253} (\textit{SuSy}) were higher in \textit{mdh4-1} than in WT (Figure 8c; Table S7). DEGs under carbohydrate metabolic processes (GO: 0005975) and starch biosynthesis and metabolic processes (GO: 0019252 and GO: 0005982) were enriched (Table S7). Key genes involved in starch and amylose biosynthesis, including \textit{Zm00001d044129} (\textit{Sh2}), \textit{Zm00001d050032} (\textit{Bt2}), \textit{Zm00001d033937} (\textit{GBSS}) and \textit{Zm00001d045462} (\textit{wx1}), were significantly down-regulated in the \textit{mdh4-1} mutant. DEGs under nutrient reservoir activity (GO: 0045735) were found to be mainly involved in zein protein biosynthesis (21/24). These DEGs were also significantly down-regulated the \textit{mdh4-1} mutant (Figure 8d; Table S7). The changed expression levels of genes related to starch and protein biosynthesis suggest a role of \textit{ZmMdh4} in storage reserve accumulation.

**Discussion**

\textit{ZmMdh4} is indispensable for kernel development

Gooman and associates have reported that the kernels of homozygous \textit{Zmmdh4} and \textit{Zmmdh5} mutants were viable and could germinate and develop to normal mature plants (Goodman et al., 1981). In this study, both the natural \textit{mdh4-1} mutant and the \textit{cas9 mdh4-2} and \textit{mdh4-3} lines could develop into relatively normal plants but exhibited poor seed germination, retarded vegetative and reproductive growth compared with controls (Figures 1, 4a-b; S4). It is worth noting that \textit{ZmMhd5}, the only paralog of \textit{ZmMdh4}, is unlinked to the mutant kernel phenotype (Figure 4e-h). Our findings point to an indispensable role for \textit{ZmMdh4} in kernel and plant development. Previous studies have reported a positive correlation between the transcript level and enzymatic activity of cytosolic MDHs in apple and cotton (Imran et al., 2017; Yao et al., 2011b). Taken together with the lack of obvious morphological and developmental defects in the \textit{mdh4-1} and \textit{mdh4-cas9} lines, these results indicate the existence of other genetic factors that are functionally redundant to \textit{ZmMdh4}.

\textit{ZmMDH4} is indispensable for ATP production

In non-photosynthetic organs, such as the endosperm, glycolysis in cytoplasm and the TCA cycle in mitochondria are major sources for ATP. The cytosolic MDHs, which catalyse the reversible conversion between malate and OAA, contribute to the partitioning of metabolic flux between glycolysis and the TCA cycle.
ZmMDH4 determines storage reserves by balancing glycolysis and the TCA cycle

(Selinski and Scheibe, 2019), thereby regulate ATP production. Consistent with this function, the loss of ZmMDH4 activity caused substantial changes in the levels of glycolysis and TCA cycle-related metabolites in \( mdh4-1 \) (Figure 7a-b and h). For example, an increase in lactate level was observed, as well as a reduction in ATP content in \( mdh4-1 \) compared with the WT (Figure 7b, d), implying enhanced glycolysis and a role of a malate/OAA shuttle in regulating ATP production (Scheibe, 2004). As a result, the mitochondria of \( mdh4-1 \) were vacuolated, NADH oxidase activity was reduced, and the mitochondrial I + III super-complex was disassociated (Figure 7c-g). These data are in line with a previous report that the rice \( flo16 \) mutant, which was determined to be caused by a mutated cytosolic MDH, had reduced ATP production (Teng et al., 2019). These data suggest that cytosolic ZmMDH maintains mitochondrial complex activity, ATP production and the homeostasis of glycolysis and the TCA cycle.

ZmMDH4 affects starch and zein synthesis in the endosperm

In the cytoplasm, pyruvate phosphate dikinase (PPDK), which reversibly converts PEP to pyruvate, also involved in the malate metabolic pathway. Knockout of maize endosperm PPDK (cytosolic \( pdk2 \)) results in opaque kernel characteristics, elevated glycolysis metabolites, reduced ATP content, but unaffected starch and zein
contents (Lappe et al., 2018). However, mutations in ZmMdh4 cause an elevation in glycolysis metabolites and a reduction in ATP content, with a concomitant decrease in starch and zein contents (Figures 1d-e, 7a-d). These results suggest that different regulators might be involved in cytosolic pdk2 and ZmMdh4 expression to balance glycolysis and the TCA cycle, as indicated that PPK is the direct target of O2 and MDH is the target of thioredoxin-h1 (Hara et al., 2006; Lappe et al., 2018; Manicacci et al., 2009). Loss-function of the cytosolic Mdh leads to increased oxidation, which subsequently results in a reductive ATP-glucose pyrophosphorylase (AGP, the committed enzyme of starch biosynthesis) activation state, thereby reducing starch content in tomato plastids (Centeno et al., 2011) and in rice endosperm (Teng et al., 2019). These findings are consistent with increased NAD+/NADH levels, as well as the reduced expression levels of Bt2 and Sh2, which encode the large and small subunits of AGP observed in the mdh4-1 (Figures 7c and 8c). Thus, it can be inferred that restricted AGP activity causes reduced starch accumulation in mdh4-1 mutants. Another reason could be related to compromised ATP production, which would directly impair the differentiation of BETL cells, or the activity of endosperm cells (Figure 2b-c). This would inhibit the biosynthesis and deposition of storage compounds, resulting in small kernels.

Zeins are the most abundant seed storage proteins (>60%; Wu and Messing, 2014) that determine the nutritional quality of maize grain (Frizzi et al., 2010; Hunter et al., 2002). Published data have demonstrated that mutants with reduced zein contents, especially α-zein, such as the o2, o7, ocd1 and f2 mutants, accumulate lysine, an essential amino acid whose levels in maize grains is not well balanced for human and animal consumption, to compensate (Coleman et al., 1997; Kemper et al., 1999; McIaups et al., 2011; Wang et al., 2011; Yang et al., 2018). Analagous to that observed with these mutants, the biosynthesis of zein (especially 19- and 22-kd α-zein) was restricted in the mdh4-1 mutant and the lysine content was increased in the mdh4-1 mutant; this was further supported by the down-regulation of all DEGs involved in zein biosynthesis and lysine degradation, and enhanced levels of aspartate for lysine and glutamine biosynthesis, which competes with glutamate for zein biosynthesis in the mdh4-1 (Figures 1e-f, 8c-d). Collectively, these results demonstrate that ZmMdh4 influences TCA-derived substrate supply for protein biosynthesis, though the gap between ZmMdh4 activity and the final seed phenotype needs to be comprehensively investigated.

In summary, we cloned the ZmMdh4 gene that encodes cytosolic malate dehydrogenase in maize. The 3-bp deletion in exon 7 eliminates ZmMdh4 enzymatic activity, resulting in reduced ATP supply and an elevated oxidation level, perturbing AGP activity and starch production in the endosperm. Concomitantly, impaired TCA cycle alters the substrate availability for amino acid biosynthesis, which influences the proportions of zein and non-zein protein by modulating the expression levels of related genes in the endosperm as depicted in our proposed model (Figure 9).

### Methods

#### Plant materials

**mdh4-1** is a naturally occurring mutant isolated during maize breeding and was crossed with Zheng58, PH6WC, HCL645, Qi319, Lx9801 and D1798Z to generate F1 hybrids. The resulting F1 plants were self-pollinated to construct six F2 mapping populations. The *mdh4* mutant, Zheng58, PH6WC, HCL645, Qi319, Lx9801, D1798Z and the F2 populations were grown at the research farm of Henan Agricultural University, Zhengzhou, China (113°42’ E, 34°48’ N). The near-isogenic lines (NILs) of *mdh4-1* were produced by self-crossing heterozygous F2 individuals, derived from the by crossing Zheng58 with *mdh4-1*, for eight generations under background and foreground marker-assisted selection. The ears and kernels of the F2 plants and NILs were collected from no less than three individuals at 5, 8, 10, 11, 12, 14, 16, 20, 23, 25, 27, 30 and 35 DAP.

#### Cytological section preparation

To prepare the paraffin sections of kernels, immature seeds were fixed overnight at 4 °C in a formalin-acetic acid-alcohol (FAA) solution containing 50% ethanol, 5% acetic acid and 3.7% formaldehyde. The fixed materials were then dehydrated in an ethanol gradient series (50, 70, 85, 95 and 100% ethanol). Afterwards, the samples were treated with xylene, embedded in paraffin wax via infiltration and cut into 6–10 μm-thick sections under Leica RM2235 (Germany). The sections were stained with toluidine blue (Sinopharm Chemical Reagent Co., Ltd) and examined under the Lecia M165FC stereomicroscope (Germany). The endosperm structures of mature and immature seeds of different developmental stages were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively (Wu and Messing, 2010; Zhang et al., 2016).

#### Map-based cloning of ZmMdh4

A total of 34 080 F2 individuals were used for map-based cloning. The genotypes of key F2 recombinants were verified by examining the corresponding F2 seeds from each F2 ear. Table S3 lists the primers used. Additional polymorphic markers were developed by comparing genomic sequence near the ZmMdh4 locus between *mdh4-1* and Zheng58.

#### Vector construction and gene transformation

To construct the CRISPR/Cas9 vector, the recombinant PBUE411 vector was designed to produce mutations within the coding region of ZmMdh4, using a small guide RNA (sgRNA) alongside the Cas9 endonuclease gene. To obtain ZmMdh4 overexpression lines (Mdh4-OE), the CDS driven by a Ubi promoter was cloned into a CUB skeleton vector. The CRISPR/Cas9 and overexpression constructs were introduced into *agrobacterium* strain EHA105 and used to transform the immature embryos of maize inbred line ZZC01, by co-cultivation at the Life Science and Technology Center of China National Seed Group Co. Ltd (Wuhan, China). Positive transgenic plants were confirmed by amplifying the Bar gene and the knock-out regions by PCR using ZmMdh4-specific primers CUB-f/CUB-R, which span ZmMdh4 and the CUB vector. A co-dominant functional marker for the 3-bp InDel in exon 7, Exon7-L/Exon7-R, was developed to identify the homozygous *mdh4-1* genotype. All primers used for vector construction and gene transformation are listed in Table S3.

#### Starch, protein and total amino acid determination

A minimum of 20 endosperms from mature kernels of the WT and *mdh4-1* mutant were pulverized into fine powder using a pulverizer. For each sample, 50 mg flour was used to measure starch content using the Megazyme kit (K-TSTA; Megazyme). SDS-PAGE was used to analyse the accumulation patterns of zein contents, especially *zein* and non-*zein* protein by modulating the expression levels of related genes in the endosperm as depicted in our proposed model (Figure 9).

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bound amino acids) in the mature kernels were analysed according to the method of Wang et al. (2011) with three replicates.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from immature endosperms, embryos and other tissues using TransZol Plant (Transgen). Five hundred nanogram of total RNA was used for first-strand cDNA synthesis using HiScript C226 QRT SuperMix for qPCR (+gDNA wiper) (Vazyme). All qPCR analyses were carried out in a Bio-Rad iQ5 system (Bio-Rad iQ5 Real Time PCR, ABI 7500) using the SYBR Green I kit (Vazyme). The 2^(-ΔΔCT) method was used to calculate the relative transcript level of the target gene with ZmActin (Zm00001d010159) as the endogenous control. The PCR program was conducted as follows: (1) 5 min at 94 °C; (2) 40 cycles of 10 s at 95 °C, 30 s at 58 °C. The 20-μL reaction volumes contained 2 μL cDNA, 0.4 μL LR primers (10 μM), 10 μL 2 x qPCR SYBR Green I mix and 7.2 μL double-distilled water. Statistically significant differences in gene expression levels were analysed by Student’s t-test. All primers used for qPCR are listed in Table S3.

Subcellular localization of ZmMDH4

The CDS of ZmMdh4 was fused with the enhanced GFP (eGFP) reporter gene and cloned into the pCAMBIA1300 vector (35S::

![](image)
ZmMDH4::GFP). pCAMBIA1300-35S-GFP vector not containing the ZmMdh4 gene was used as the free GFP control (35S::GFP). The 35S::GFP and 35S::ZmMDH4::GFP constructs were transiently expressed in Nicotiana benthamiana (N. benthamiana) leaves and Arabidopsis mesophyll protoplasts as described by Li et al. (2017) and Yang et al. (2018). GFP signal was observed and imaged using a confocal microscope (FV1000, Olympus). All primers used for the subcellular localization analysis are listed in Table S3.

Protein extraction and Western blot

Endosperm proteins were isolated using the method described by Wu and Messing (2012), separated on a 15% SDS PAGE by electrophoresis, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was then incubated with commercial MDH4 (AS153065, Agrisera) and ACTIN (Abclonal, China) antibodies and visualized using the Tanon-5200 system (Tanon Science & Technology Co., Ltd.). The MDH4 and ACTIN antibodies were 1:2000 and 1:5000 diluted, respectively (Yang et al., 2018). The NAD7 (PHY1077S, Phytoab), SDH1 (PHY05585, Phytoab) and CYC1 (PHY05665, Phytoab) antibodies were 1:3,000 diluted, while the α-tubulin antibody (AS10680, Agrisera) was 1:10,000 diluted.

Enzymatic assay of ZmMDH4

The open reading frames (ORFs) of ZmMdh4 and Zmmdh4 were amplified by PCR with gene-specific primers (Table S3) and separately cloned into the pET-28a vector using a One Step Cloning Kit (Vazyme). The resulting ZmMdh4 and Zmmdh4 constructs were each transformed into the Escherichia coli (E. coli) strain BL21 (DE3). 2 mL of the transformed cells was used to inoculate 200 mL LB media in a 500 mL conical flask and cultured at 37 °C until OD600 reached 0.4–0.6, when 0.5 mM isopropyl-d-thiogalactoside (IPTG) was added to induce protein expression. After culturing at 16 °C overnight, the cells were collected by centrifugation and then resuspended in 10 mL lysis buffer [50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5% glycerol, and 5 mM imidazole, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.0037% β-mercaptoethanol]. Next, the cells were disrupted by sonication for 30 min and centrifuged at 5752 g for 50 min. The supernatant was added to the Ni-NTA resin, which was pre-equilibrated with 10 mL lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5% glycerol, and 5 mM imidazole, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.0037% β-mercaptoethanol). Next, the cells were disrupted by sonication for 30 min and centrifuged at 5752 g for 50 min. The supernatant was added to the Ni-NTA resin, which was pre-equilibrated with 10 mL lysis buffer. After centrifugation, the protein solution was eluted by a series of elution buffers containing 10 mM, 50 mM, 100 mM and 200 mM imidazole. The eluted ZmMDH4::GFP).
protein fractions were pooled and dialysed with a 10 kDa ultrafiltration device to remove imidazole and salt, evaluated for purity via SDS-PAGE electrophoresis, and purified by dialysis buffer (50 mM Tris-HCl pH 8.0, 20 mM NaCl, 5% glycerol and 0.037% β-mercaptoethanol). The purified proteins were quantified using a calibration curve based on the absorbance at 280 nm (A280) with BSA as a standard.

The 1 mL oxidative/reductive catalytic reaction included 250 mM HEPES buffer (pH 8.0), 2 mM MgCl2, 0.25 mM NAD+/NADH, 2.5 mM OAA or malate (Solarbio, Beijing, China), and 5 µg (1 µg/µL) of each protein sample. The change in NADH concentration (A260) of each reaction was monitored for 6 min by a spectrophotometer (DU®730, Nucleic Acid/Protein Analyzer, Beckman) at 1 min intervals.

Determination of amino acids and energy metabolites

12-DAP kernels of the WT and mdh4-1 mutant were collected with three biological replications, snap frozen in liquid nitrogen, and stored at −80 °C until use. More than 3 g of kernels were ground and 55 mg fine powder from each sample was mixed with 1 mL of pre-cooled methanol/acetonitrile/H2O solution and vortexed for 30 s. The mixture was then sonicated for 30 min on ice and left at −20 °C for 1 h to allow protein precipitation. Afterwards, the mixture was centrifuged for 15 min at 14,000 g at 4 °C, and the proteins were vacuum dried using a lyophilizer (FD-10-80, BILON, Shanghai). The dried protein extracts were then dissolved in 100 µL 1:1 (v/v) mixture of acetonitrile:H2O and centrifuged at 14,000 g at 4 °C for 15 min. Targeted metabolic analysis was performed using the LC-MS/MS system at Shanghai Applied Protein Technology Co. Ltd. Electrospray ionization was conducted with an Agilent 1290 Infinity chromatography system and AB SCIEX QTRAP 5500 mass spectrometer.

ATP content was determined using an ATP Assay Kit (Beyotime) following the manufacturer’s instructions; three biological replicates were analysed for each sample. Briefly, 100 mg fresh endosperm of WT and mdh4-1 was homogenized in 1 mL pre-cooled lysis buffer and centrifuged at 12,000 g for 5 min at 4 °C. The supernatant was used to determine ATP content. ATP standard solutions of various concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.5, 1 µM) were prepared to generate an ATP calibration curve. The supernatant and ATP standards were separately mixed with the ATP detection solution (working concentration) provided in the Kit in a 1:9 ratio. Luminescence was detected by a Tecan Infinite 200 (BD Infinium, Shanghai) microplate reader (Shanghai), and the ATP content of each sample was calculated based on the calibration curve.

BN-PAGE and the determination of mitochondrial complex activity

The mitochondria were separated from 15 DAP kernels using the Plant mitochondria DNA Extraction Kit (Beijing biolab technology co. LTD) with minor modifications. Briefly, about 400mg kernel was ground in liquid nitrogen and 1.6 mL plant cell lysis buffer (0.5% β-mercaptoethanol) was added to each sample. The samples were mixed and centrifuged at 10,000 g for 5 min at 4 °C. Then, the supernatant was transferred to a new tube and centrifuged at 16,000 g for 10 min at 4 °C. Crude mitochondria were resuspended in cleanout fluid and centrifuged at 1000 g for 5 min at 4 °C. The resulting supernatant was centrifuged at 16,000 g for 10 min at 4 °C to collect highly purified mitochondria and the pellet was resuspended in 100 µL B25G20 solution. BN-PAGE and in-gel activity assay of mitochondrial complexes were performed as described by Chen et al. (2017).

RNA-seq analysis

Total RNA was extracted from 12 DAP endosperms of the mdh4-1 mutant and WT with RNAprep Pure Plant Kit (Tiangen). The VAIHTSTM Stranded mRNA-seq Library Prep Kit for Illumina® (Vazyme) was used to construct the RNA-seq libraries. Clean reads were obtained using the Illumina HiSeq X Ten platform (JiakangBio, Wuhan, China) and mapped to the B73 reference genome (RefGen_v4) using Bowtie2. Gene expression level was converted to Fragments per Kilobase Million (FPKM) for each transcript model. Differentially expressed genes (DEGs) were selected by the following criteria: log2 (fold change) > 0.78 or < -1, false-discovery rate (FDR) < 0.05, as calculated by the DESeq2 software, and P-value < 0.05. GO enrichment analysis of the DEGs was performed using an online version of agrigo (Tian et al., 2017).

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Conflict of interest

This study did not involve human participants and/or animals. All authors declare no financial or commercial conflicts of interest.

Author contributions

Z.F. and J.T. designed and supervised this study. Y.C., H.Z., R.T., H.Y., C.S., L.W. and W.Z. performed the experiments. Z.F., Y.C., Z.G. and X.Z performed the data analysis. Z.F., Y.C. and J.T. prepared the manuscript with inputs from other authors.

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**Supporting information**

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