Rice

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NADP-malic Enzyme OsNADP-ME2 Modulates Plant Height Involving in Gibberellin Signaling in Rice

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Abstract

Plants NADP-malic enzymes (NADP-MEs) act as a class of oxidative decarboxylase to mediate malic acid metabolism in organisms. Despite NADP-MEs have been demonstrated to play pivotal roles in regulating diverse biological processes, the role of NADP-MEs involving in plant growth and development remains rarely known. Here, we characterized the function of rice cytosolic OsNADP-ME2 in regulating plant height. The results showed that RNAi silencing and knock-out of *OsNADP-ME2* in rice results in a dwarf plant structure, associating with significant expression inhibition of genes involving in phytohormone Gibberellin (GA) biosynthesis and signaling transduction, but with up-regulation for the expression of GA signaling suppressor *SLR1*. The accumulation of major bioactive GA_1, GA_4 and GA_7 are evidently altered in RNAi lines, and exogenous GA treatment compromises the dwarf phenotype of *OsNADP-ME2* RNAi lines. RNAi silencing of *OsNADP-ME2* also causes the reduction of NADP-ME activity associating with decreased production of pyruvate. Thus, our data revealed a novel function of plant NADP-MEs in modulation of rice plant height through regulating bioactive GAs accumulation and GA signaling, and provided a valuable gene resource for rice plant architecture improvement.

Keywords Rice (*Oryza sativa* L.), NADP-malic enzyme, OsNADP-ME2, Plant height, Gibberellin signaling

Background

NADP-malic enzymes (NADP-MEs) are a class of oxidative decarboxylase responsible for catalyzing the reversible oxidative decarboxylation of malate to yield pyruvate, carbon dioxide and NADPH in the presence of a divalent metal cation (Edwards and Andreo 1992). NADP-MEs ubiquitously exist in organisms involving in diverse metabolic pathways (Drincovich et al. 2001). In plants, NADP-MEs are divided into photosynthetic and non-photosynthetic subtypes by their physiological function (Drincovich et al. 2001). The photosynthetic NADP-MEs involve in carbon fixation for delivering CO_2 to Rubisco in the bundle sheath chloroplasts of C4 plants and in the cytosol of crassulacean acid metabolism (CAM) plants (Alvarez et al. 2019). While the non-photosynthetic NADP-MEs are generally presented in cytosol or plastid for all C3, C4 and CAM plants. They have been demonstrated to participate in multiple biological processes, including fruit ripening (Edwards and Andreo 1992), cytosolic pH regulation (Edwards and Andreo 1992), stomatal regulation (Laporte et al. 2002), lipogenesis (Shearer et al. 2004), seed germination ability (Arias et al. 2018), abiotic stress responses (Chen et al. 2019; Badia et

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al. 2020) and plant-pathogen interactions (Voll al. 2012; Dangol et al. 2019).

Gibberellins (GAs) are essential phytohormones for multiple growth and developmental processes in controlling root and shoot elongation, seed germination, flowering, fruit patterning and pollen maturation in plants (Yamaguchi 2008; Davière and Achard, 2013). A prominent biological role of GA is to promote stem elongation and plant heightening. Mutations of those genes involving in GA biosynthesis, metabolism, and signaling cascades could influence rice plant height (Davière and Achard, 2013). For example, the mutation of one key gene *SD1*, encoding an enzyme GA20ox-2 for GA synthesis, resulted in a semi-dwarf phenotype in rice, which significantly improved rice yield and lodging resistance during the "Green Revolution" (Sasaki et al. 2002).

GA biosynthesis is originated from the catalyzation of precursor geranylgeranyl pyrophosphate (GGPP) via isopentenyl diphosphate (IPP) into geranylgeranyl diphosphate (GGDP), then GGDP is catalyzed to *ent*-kaurene by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) in plastid. Following with an oxidization reaction catalyzed by kaurene oxidase (KO) and kaurenoic acid oxidase (KAO), entkaurene is transformed to GA_{12} on ER membrane. Finally, GA_{12} is catalyzed by GA3-oxidase (GA3ox) and GA20-oxidase (GA20ox) into gibberellin with higher biological activity in the cytoplasmic matrix (Yamaguchi 2008; Salazar-Cerezo et al. 2018). Although hundreds of GAs are identified, only four GAs including GA_1, GA_3, GA_4 and GA_7 are thought to be major bioactive hormones (Binenbaum et al. 2018). Among them, GA_1 widely functions in various plant species. GA_4 presents in most species, but is thought to be the major bioactive form in *Arabidopsis* and some Cucurbitaceae members (Yamaguchi 2008).

In rice, four NADP-ME genes present in the genome (Chi et al. 2004). Two of them *OsNADP-ME2* (also known as *OscytME1*) and *OsNADP-ME4* (also known as *OscytME3*) have been reported to enhance plant salt and osmotic stress when ectopically expressed in *Arabidopsis* (Liu et al. 2007; Cheng and Long 2007). OsNADP-ME2 was also found to be required for iron- and ROS-dependent ferroptotic cell death and resistance against rice blast fungal pathogen *Magnaporthe oryzae* in rice (Singh et al. 2016; Dangol et al. 2019). However, whether rice OsNADP-MEs function in growth and development processes remains unknown. Here, we characterized that rice cytoplasmic OsNADP-ME2 involves in regulation of plant height through affecting bioactive GAs accumulation and GA signaling transduction, revealing a novel function of plant NADP-MEs in modulation of plant architecture.

Results

OsNADP-ME2 is a Constitutively Expressed Cytosolic NADP-malic Enzyme

Despite rice OsNADP-ME2 was classified into monocot-specific cytosolic group (Chi et al. 2004; Wheeler et al. 2005), its exact subcellular localization remains experimentally inconclusive. To verify OsNADP-ME2 localization, a construct with N-terminal GFP tag fused *GFP:OsNADP-ME2* gene was expressed in rice protoplast, the fluorescence observation showed that the green fluorescence only fills in the cytosol of rice protoplasts, but the control GFP fluorescence presents in the whole cells including cytosol and nucleus (Fig. 1A), confirming that the protein of OsNADP-ME2 indeed localized in cytosol

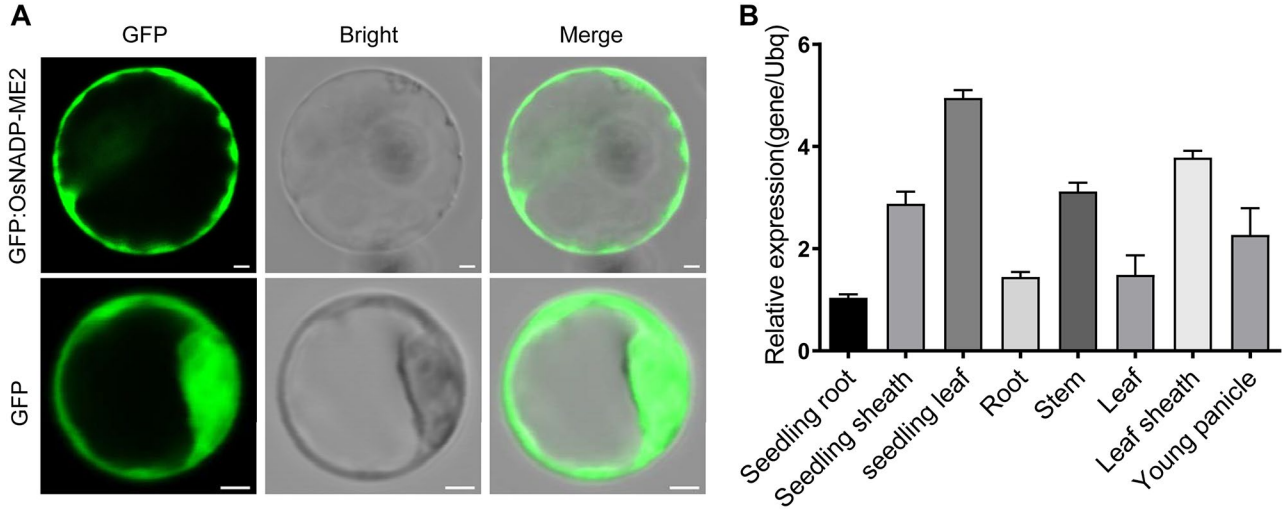

Fig. 1 The subcellular localization of OsNADP-ME2 protein and transcription pattern of *OsNADP-ME2* in rice tissues. (**A**) Subcellular localization of OsNADP-ME2 in rice protoplast. The upper is the fluorescence location of GFP: OsNADP-ME2 protein, below is the fluorescence location of control GFP protein. (**B**) The expression pattern of *OsNADP-ME2* in rice tissues. The data represents the mean of three repeats with SD value as the bar

of rice cells. Further transcripts level analysis by real time RT-qPCR revealed that *OsNADP-ME2* constitutively expressed in all rice tissues with the highest expression level in seedling leaf (Fig. 1B). These results indicated that *OsNADP-ME2* possesses a constitutive expression pattern and is a cytosol localized protein in rice.

Knock-Down of *OsNADP-ME2* Decreases Plant Height in Rice

To understand the biological function of OsNADP-ME2 in rice, the *OsNADP-ME2* RNAi silencing transgenic rice lines were constructed and used for phenotype evaluation (Fig. 2A). The results showed that *OsNADP-ME2* RNAi lines 20−8 and 33−2, with a significant down-regulation of transcripts, display an evidently dwarf phenotype comparing to wild type NPB plants at mature stage (Fig. 2). At mature stage, the total plant height of two RNAi lines was significantly decreased (Fig. 2B, C), and the reduced plant height was caused by the length decreasing of panicle and all the four internodes below the panicle (Fig. 2D, E). The dwarf phenotype was further confirmed that knock-out of *OsNADP-ME2* by the CRIPSR/Cas9-mediated gene editing method in the background of another cultivar ZH11 also exhibited decreased plant height (Fig. 2F; Fig.S2). In *osnadp-me2* mutant, an 1 bp base deletion presented in the 10th exon of *OsNADP-ME2* gene results in a frame shift with a premature termination of protein translation (Fig.S2). These results suggested that OsNADP-ME2 involves in rice plant height modulation.

Furthermore, the evaluation of phenotype for yield traits in *OsNADP-ME2* RNAi lines showed that the number of grains per panicle (Fig. 3A) and seed setting rate (Fig. 3B) were significantly lower than that in wild type NPB. A significant decreasing of 1000-grain weight was presented in RNAi lines (Fig. 3C), and only the significant changes for an increasing of grain length on RNAi line 20−8 and a reduction of grain width for RNAi line 30−3 were observed for grain size traits comparing to NPB (Fig. 3D, E). While the number of effective panicles per plant in RNAi lines was significantly larger than that of NPB (Fig. 3F). Consequently, there is no significant

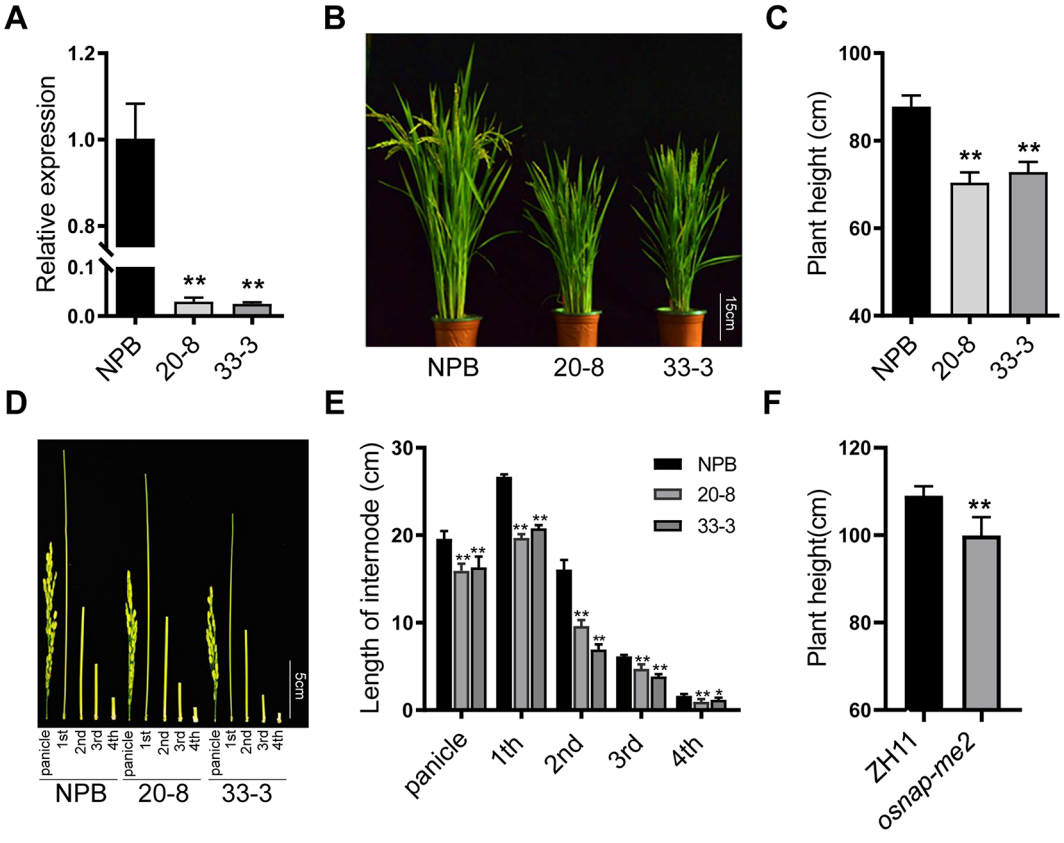

Fig. 2 Phenotype of plant architecture for *OsNADP-ME2* RNAi and CRISPR/Cas9 editing mutant rice lines. (**A**) Transcriptional level of *OsNADP-ME2* in RNAi lines. NPB is the wild type cultivar Nipponbare, 20 − 8 and 33 − 3 are two represented RNAi lines in the background of NPB. (**B**) Phenotype of plant structure for *OsNADP-ME2* RNAi lines at mature stage. (**C**) Plant height of *OsNADP-ME2* RNAi lines at mature stage. (**D**) The phenotype of internode for *OsNADP-ME2* RNAi lines at mature stage. (**E**) The internode length of *OsNADP-ME2* RNAi lines at mature stage. (**F**) Plant height of *OsNADP-ME2* CRISPR/Cas9 gene editing line *osnadp-me2* in the background of japonica cultivar Zhonghua11 (ZH11) at mature stage. Data is the average value of more than 5 replications with a bar of SD value, t-test was used for statistical analysis at $P = 0.05$ and 0.01 respectively with a marker of * and **

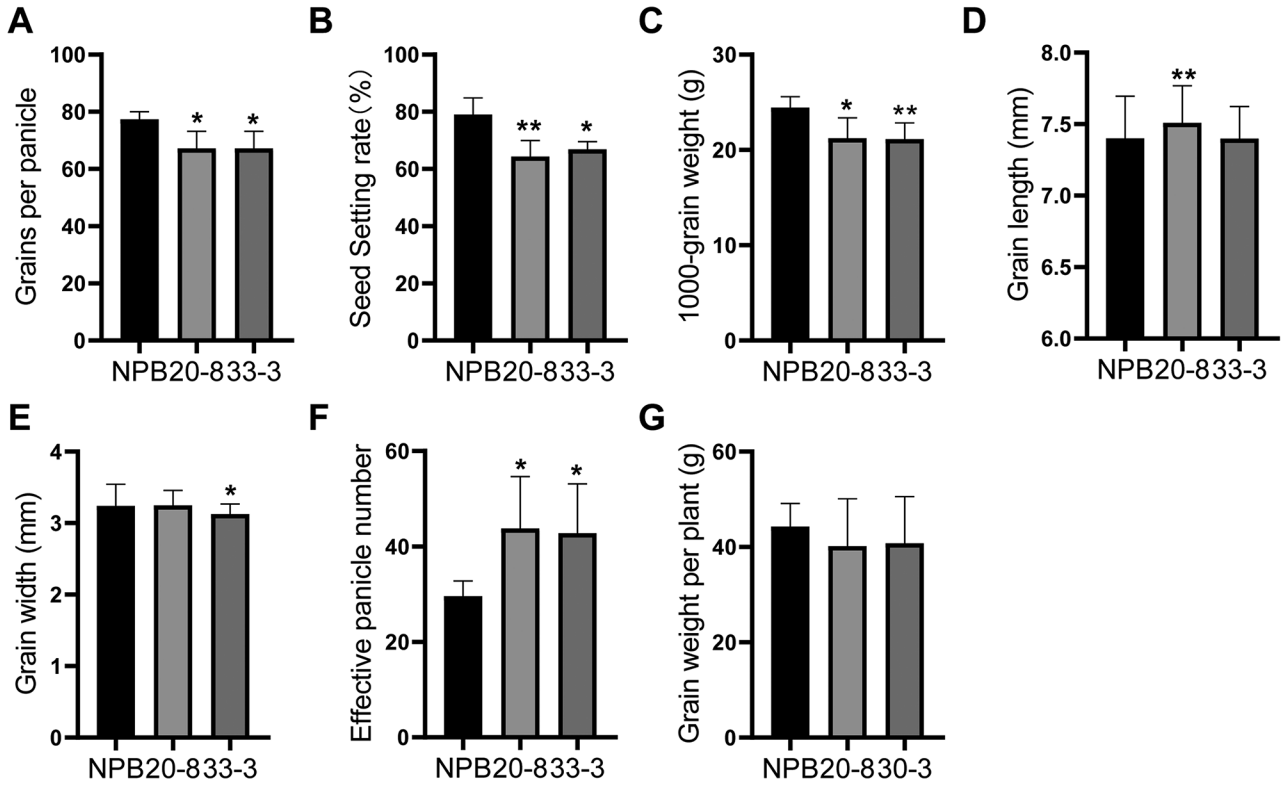

Fig. 3 Phenotype of yield trait for *OsNADP-ME2* RNAi lines. (A-G) Grain number per panicle (**A**), seed setting rate (**B**), 1000-grain weight (**C**), grain length (**D**), grain width (**E**), effective panicle number (**F**), grain weight per plant (**G**) for *OsNADP-ME2* RNAi lines. Data shows the means of more than 5 replications with the bar of SD value, the significance level was indicated as * and ** at the level of $P = 0.05$ and 0.01 respectively under the statistical analysis of t-test

difference for the yield of per plant between RNAi lines and NPB (Fig. 3G).

Knock-down of *OsNADP-ME2* Alters the Expression Pattern of Genes in GA Biosynthesis and Signaling

The phytohormone GA is predominantly associated with plant height modulation (Yamaguchi 2008). To determine whether the GA pathway is affected in *OsNADP-ME2* RNAi lines, the transcription level for the genes involving in GA biosynthesis and signaling pathway was evaluated (Fig. 4). The results showed that the expression level of genes *OsCPS1*, *OsKS1*, *OsKAO*, *OsGA3ox1*, *OsGA3ox2*, *OsGA20ox1*, *OsGA20ox2*, *OsGA20ox3* and *OsGA20ox4*, of which these genes encode metabolic enzymes responsible for GA biosynthesis (Yamaguchi 2008), were significantly down-regulated in both two RNAi lines compared with that in wild type NPB (Fig. 4A, B).

Similarly, the expression level of *OsGID1*, *OsGID2*, *OsPH1* and *OsGRF1/2/8* genes positively regulating GA signaling (Wang and Wang et al., 2022) were also significantly down-regulated in RNAi lines (Fig. 4C, D). However, the transcription level of *SLR1*, the central suppressors of GA signaling pathway (Ikeda et al. 2001), was remarkably up-regulated in RNAi lines (Fig. 4C). These results indicated that OsNADP-ME2 might involve in regulation of GA biosynthesis and signaling to affect plant height in rice.

Knock-down of OsNADP-ME2 Affects Endogenous Bioactive GAs Accumulation

The down-regulated expression of GA biosynthesis genes implicated that GA biosynthesis might be affected. To verify this speculation, the contents of three major bioactive GAs (GA_1, GA_4 and GA_7) (Yamaguchi 2008) in RNAi line 33−3 in the leaf tissues for one week old seedling was firstly measured (Fig. 4E), the results showed that the content of GA_1, GA_4 and GA_7 in RNAi line 33−3 was down-regulated comparing to NPB, although there is no statistical significance for decreased GA_4 content (Fig. 4E, S3). To further confirm that the level of bioactive GAs at late stage of RNAi lines, the quantification of three GAs in the leaf tissues at two months old rice plants revealed that the content of GA_1 and GA_4 was also decreased in both RNAi lines 20−8 and 33−3 comparing to the wild type NPB (Fig. 4F, S4). To be different, the content of GA_7 was increased in the leaf tissues at two months old rice plants (Fig. 4F, S4), that is opposite to the content in the leaf tissues of one week old seedling plants. The similar content pattern for three GAs at two month stage was also observed in *osnadp-me2* gene editing

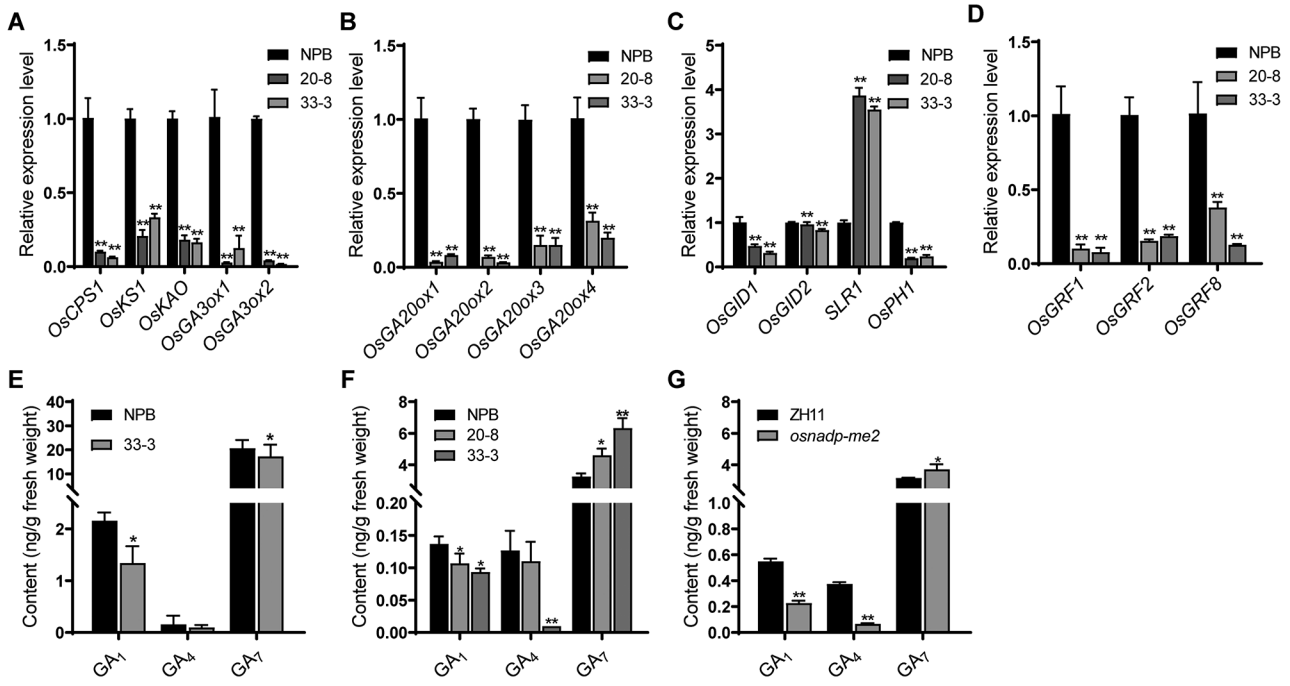

Fig. 4 Transcriptional level of genes for GA biosynthesis and signaling in *OsNADP-ME2* RNAi transgenic rice. (**A**-**B**) Expression level of genes (*OsCPS1*, *OsKS1*, *OsKAO*, *OsGA3ox1*, *OsGA3ox2*, *OsGA20ox1*, *OsGA20ox2*, *OsGA20ox3*, *OsGA20ox4*) for GA biosynthesis in *OsNADP-ME2* RNAi lines. (**C**-**D**) Expression level analysis of genes (*OsGID1*, *OsGID2*, *SLR1*, *OsPH1*, *OsGRF1*, *OsGRF2*, *OsGRF8*) for GA signaling in *OsNADP-ME2* RNAi rice lines. (**E**) The content of endogenous bioactive GA_1, GA_4 and GA_7 in *OsNADP-ME2* RNAi line 33 – 3 and wild type NPB in the leaf tissues of one week old seedling plants. (**F**) The content of endogenous bioactive GA_1, GA_4 and GA_7 in *OsNADP-ME2* RNAi lines 20 – 8 and 33 – 3, and wild type NPB in the leaf tissues of two old months plants. (**G**) The content of endogenous bioactive GA_1, GA_4 and GA_7 in *OsNADP-ME2* gene editing mutant *osnadp-me2* and its wild type ZH11 in the leaf tissues of two old months plants. Data represents of the average of two or three biological repeats with the bar of SD value, t-test was used for difference analysis at the level of $P = 0.05$ and 0.01 marked with * and ** respectively

mutant in another genetic background of ZH11(Fig. 4G, S5). Thus, of these results indicated that OsNADP-ME2 alters endogenous bioactive GAs accumulation pattern in rice.

Exogenous GA Treatment Eliminates the Inhibition of Plant Height in *OsNADP-ME2* RNAi Lines

Because the bioactive GAs level was evidently changed in *OsNADP-ME2* RNAi lines (Fig. 4), we reasoned that whether the dwarf phenotype of *OsNADP-ME2* RNAi lines is caused by GA deficiency. To verify this hypothesis, the seeds of RNAi lines and NPB were germinated with a treatment of 100 μM exogenous GA_3 (Fig. 5). The results revealed that, after one week treatment, the shoot length of RNAi lines was obviously elongated with GA treatment compared to that in RNAi lines without GA treatment (Fig. 5A), so that there was no significant difference for the elongated shoot length between RNAi lines and NPB (Fig. 5B), indicating that exogenous GA treatment rescued the dwarf phenotype of *OsNADP-ME2* RNAi lines.

Knock-down of *OsNADP-ME2* Reduces the NADP-ME Activity and Pyruvate Production

The major role of NADP-ME is to mediate oxidative decarboxylation for catalyzing malate to generate pyruvate, CO_2, and NADPH, thus, the NADP-ME enzyme activity was checked in both two OsNADP-ME2 RNAi lines. The results showed that the NADP-ME activity in the root, sheath, and leaf tissues for two RNAi lines were significantly lower than that in wild type (Fig. 6A). However, the malate content was increased in RNAi lines (Fig. 6B). Furthermore, the content of the reaction product pyruvate was obviously decreased in the root, sheath, and leaf tissues of RNAi lines (Fig. 6C). These results suggested that RNAi silencing of *OsNADP-ME2* decreases the NADP-ME activity resulting in the reduction of oxidative decarboxylation for malate to generate pyruvate.

Discussion

Despite the rice genome only includes four *OsNADP-ME* genes, the biological function for these NADP-MEs remains rarely known. Only OsNADP-ME2 is confirmed to involve in the disease resistance regulation for rice blast (Singh et al. 2016; Dangol et al. 2019). In addition, the ectopic expression of *OsNADP-ME2*

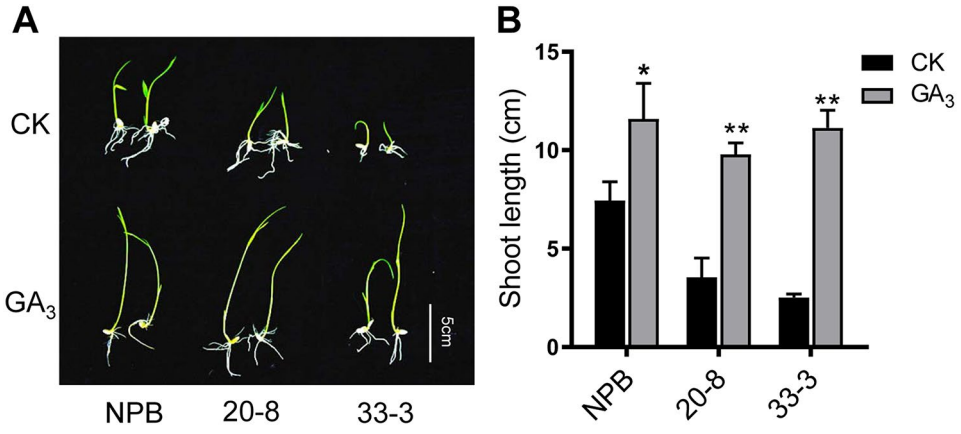

Fig. 5 Phenotype of plant height for *OsNADP-ME2* RNAi lines after exogenous GA treatment. (**A**) Dwarf phenotype of *OsNADP-ME2* RNAi lines was recovered after treatment with 100μM GA_3 for 7 days. (**B**) Shoot length of seedlings for *OsNADP-ME2* RNAi lines with and without 100μM GA_3 treatment for 7 days. Data is the mean of more than 3 repeats with a bar of SD value, t-test was used for significance analysis, the marked * and ** means the significant level under $P = 0.05$ and 0.01 respectively comparing to the control (CK)

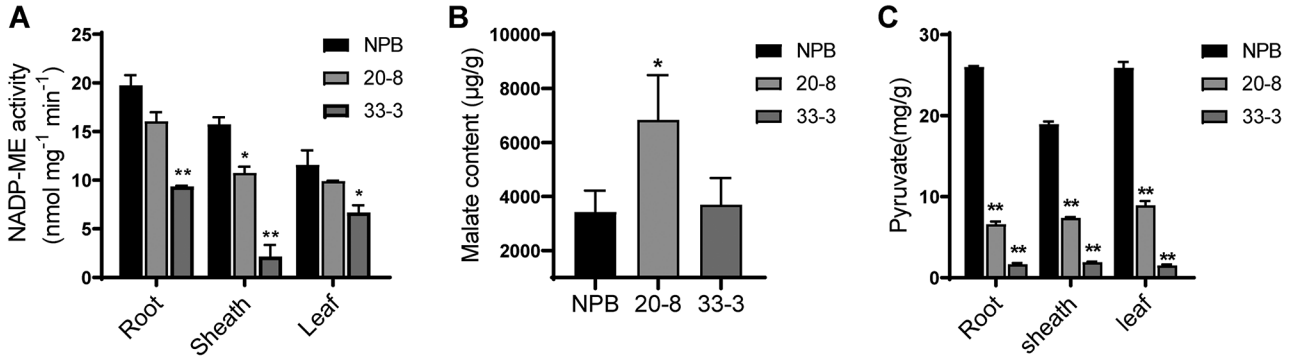

Fig. 6 The NADP-ME enzyme activity and the level of pyruvate and malate content in *OsNADP-ME2* RNAi lines. (**A**) Enzyme activity of NADP-ME in *OsNADP-ME2* RNAi lines. (**B**) Malate content in *OsNADP-ME2* RNAi lines. Data is the average number of two biological replications. (**C**) Pyruvate content in *OsNADP-ME2* RNAi lines. Data represents the mean of three replications with a bar of SD value, t-test was used for significance analysis at $P = 0.05$ and 0.01 level marked with * and ** respectively

and *OsNADP-ME4* in *Arabidopsis* enhanced plant salt and osmic tolerance (Liu et al. 2007; Cheng and Long 2007), but it is still valuable to confirm the function in rice. Here, we found that rice OsNADP-ME2 positively regulates plant height, because knock-down by RNAi silencing and knock-out via CRISPR/Cas9 editing of *OsNADP-ME2* leads a dwarf phenotype (Fig. 2), suggesting a novel function of OsNADP-ME2 in controlling rice plant structure. The previous phylogenetic analysis indicated that OsNADP-ME2 is classified into the group of monocots cytosolic NADP-MEs (Chi et al. 2004), our current experimental results verified the cytosolic subcellular localization of OsNADP -ME2 in rice cells (Fig. 1B). and a constitutive expression pattern for OsNADP-ME2 was detected in various rice tissues (Fig. 1A), implicating a major role of OsNADP-ME2 in plant cytosolic biological processes. It has been well known that plant NADP-MEs participate in diverse physiological processes (Chen et al. 2019), but there was no report for NADP-MEs in controlling plant architecture, our data firstly provides evidence to support the novel function of NADP-MEs in regulating plant structure. However, whether other plant NADP-MEs share similar conserved function is of interest to be investigated.

Plant height is a crucial trait of plant architecture for regulating crop yield performance and lodging resistance (Wang et al. 2017). Despite multiple phytohormones including GA, auxin, brassinosteroids (BRs), ethylene (ET), jasmonates (JAs), and strigolactones (SLs) contribute to plant height controlling, GA is the most predominant regulator with significant roles in agricultural production (Wang and Wang et al., 2022). The semi-dwarf architecture of high yielding crop varieties developed during the "Green Revolution" of 1960s is mainly caused by a significant improvement for GA biosynthesis and signaling pathway (Hedden 2003). Hence, extensive utilization of the *sd1* genes and its alleles in rice breeding has resulted in potential threats of germplasm homogenization for crop production (Gaur et al. 2020). Thus, seeking new beneficial semi-dwarf germplasm or genes

for plant ideal height breeding is urgent for durable rice breeding. Here, our investigation revealed that *OsNADP-ME2* is a new gene conferring rice plant height controlling (Fig. 2). Both RNAi silencing and CRIPSR/Cas9 gene editing of *OsNADP-ME2* gene decreased rice plant height (Fig. 2), indicating that *OsNADP-ME2* is a potential valuable gene for rice breeding application.

The expression level of those genes involving in GA biosynthesis were evidently altered in *OsNADP-ME2* RNAi lines (Fig. 4A, B), implying that OsNADP-ME2 probably contributes to GA biosynthesis. This speculation was confirmed that the content of bioactive GA_1, GA_4 and GA_7 was found to be significantly changed in *OsNADP-ME2* RNAi lines (Fig. 4E and G), in which the content of GA_1 and GA_4 was reduced in both RNAi lines and gene-editing mutant at different growth stages, while the amount of GA_7 level was varied at different growth stages. In rice, it has been demonstrated that GA_1 is the major bioactive form in vegetative tissues of rice (Yamaguchi 2008). Hao et al. showed that the content of GA_1 is majority associated with rice plant height controlling (Hao et al. 2023). Here, our results confirmed that the decreased level of GA_1 is associated with rice dwarf phenotype of *OsNADP-ME2* RNAi lines and knock-out mutant, indicating that the decreased accumulation of GA_1 by knock-down or -out of *OsNADP-ME2* could be one of the causals for the dwarf phenoetype. Interestingly, the decreased level of GA_4 is also correlated with OsNADP-ME2-mediated plant height in rice, however, GA_4 is thought to involve in anther development (Itoh et al. 2001), whether GA_4 is involved in plant height modulation remains inconclusive. Additionally, the amount of GA_7 is the highest one among three bioactive GAs in rice leaf tissues, but the content variation for GA_7 is not correlated with the phenotype changes for *OsNADP-ME2* RNAi lines, that could be partially explained by the reason that GA_7 is mainly involved rice anther development (Kawai et al. 2022). However, why GA_7 possesses so much higher level in rice vegetative tissues remains to be elucidated. Furthermore, the dwarf phenotype of *OsNADP-ME2* RNAi lines was recovered after treatment with exogenous GA (Fig. 5A, B). Thus, of these results suggested that OsNADP-ME2 is a novel regulator for plant height regulation by altering bioactive GAs accumulation pattern.

The plants GA signaling is mainly promoted by GA-GID1-DELLA complex stimulating ubiquitination protein degradation, of which the degradation of DELLA protein is mediated by the F-box protein SLY1/GID2 associated SCF (SKP1, CULLIN, F-BOX) E3 ubiquitin-ligase complexes. GID1 act as a GA receptor, the bound of GA to GID1 promotes the formation of GA-GID1-DELLA complex, and DELLA is a key component for intracellular repressor of GA signaling. The degradation of DELLA releases the suppression of down-stream genes expression for GA response and growth and development regulation (Davière and Achard et al., 2013). Suppression of those genes in GA signaling results in a restrained phenotype of plant growth and development. In our results, consistent with the dwarf phenotype of *OsNADP-ME2* RNAi lines, the expression of genes involving in GA signaling *OsGID1*, *OsGID2*, *OsPH1*, *OsGRF1/2/4* was significantly down-regulated in RNAi lines, that could be partially caused by the reduction of endogenous bioactive GA_1 and GA_4 in RNAi lines (Fig. 4C, D). However, GA_7 displays a higher-level accumulation in RNAi lines (Fig. 4F-G), of which GA_7 has been shown to have a highest binding affinity with GA receptor GID1 (Kawai et al. 2022). Thereby, that is inadequate to explain the suppression of GA signaling activation and dwarf phenotype for RNAi lines. Intriguingly, it is worthy to notice that the expression of GA signaling suppressor *SLR1* (Ikeda et al. 2001) was predominantly up-regulated in RNAi lines (Fig. 4C), that could be one of reasons to explain that the effects of high level of GA_7 on GA signaling activation and plant internode elongation was suppressed by activated SLR1. Nevertheless, how SLR1 was activated in *NADP-ME2* RNAi lines requires further investigation.

Although our data has demonstrated that OsNADP-ME2 involves in the regulation of bioactive GAs accumulation, how OsNADP-ME2 regulates bioactive GAs accumulation remains inconclusive. It is known that the role of NADP-MEs is to catalyzed malic acid into pyruvate, carbon dioxide and NADPH (Edwards and Andreo 1992), the products of pyruvate and NADPH function in multiple metabolic pathways as a source of carbon and reductive coenzyme (Chen et al. 2019). In our results, RNAi silencing of *OsNADP-ME2* decreased the activity of NADP-ME (Fig. 6A) resulting in a reduction of pyruvate (Fig. 6C), that is correlated with the decreased GA synthesis (Fig. 5F), suggesting that the NADP-ME catalyzation product pyruvate might involve in GA biosynthesis. As we know, the GA biosynthesis is started from the catalyzation of GGPP into GGDP (geranylgeranyl diphosphate) (Yamaguchi 2008), while the GGPP is synthesized from the precursor IPP (isopentenyl pyrophosphate), of which IPP biosynthesis is synthesized through the DXP (1-deoxy-D-xylulose-5-phosphate) pathway that requires pyruvate and GA-3P (glyceraldehyde-3-phosphate) as the original rection material (Schwender et al. 2001; Withers and Keasling et al., 2007). Thus, it is possible that the production of pyruvate mediated by NADP-MEs is the causation for GA biosynthesis regulation through restraining the production of GGPP, but it is required for further investigation.

In conclusion, our results revealed that the rice OsNADP-ME2 functions as a novel regulator to modulate plant height involving in regulation of bioactive GAs

accumulation and GA signaling through unknown pathway (Fig. 7), of which shed insights into understanding the novel function of plant NADP-MEs in plant height regulation, and also provided a new gene resource for rice plant architecture improvement.

Materials and Methods

Plant Materials and Growth Conditions

The rice cultivar Nipponbare (*Oryza sativa* L. ssp. japonica) was used for the wild type (NPB) and transformation. For rice planting, seeds were husked and sterilized with 70% ethanol for 2 min, then transferred to 10% NaClO solution for 15 min, after rinsed with sterilized ddH_2O for 3–5 times, the sterilized seeds were finally placed on 1/2 Murashige and Skoog (MS) agar plates for germination. For GA_3 treatment, 100μM GA_3 was included in 1/2 MS agar medium. After germination for 7 days, rice seedling plants were transferred to soil pots and grown in the artificial climate chamber at 28 °C, 70% relative humidity (RH), 12 h light for day time, followed at 22 °C, 70% RH, 12 h dark for night. In the field, rice seedling was transplanted into the field for growth season from May to September with normal managing measurements until mature.

Construct Making and Rice Transformation

The 210 bp sequence fragment of the 3' region for *OsNADP-ME2* was selected for RNAi construct making (Fig.S1), and cloned into the binary vector pANDA with the gateway cloning methods followed the procedure as described in Liu et al. (2015). The constructs were then transformed into cultivar Nipponbare with *Agrobacterium*-mediated transformation method for generating RNAi transgenic plants.

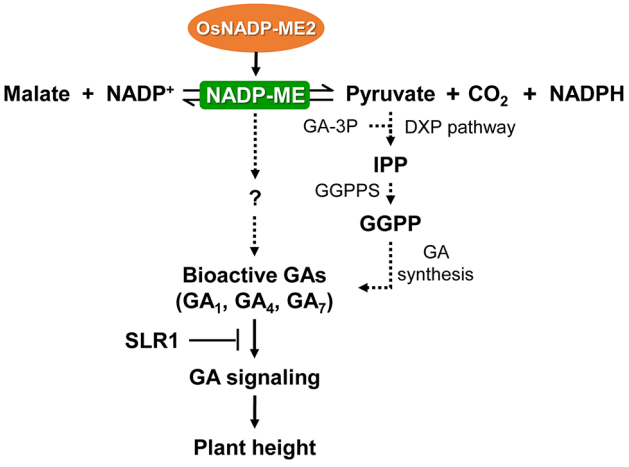

Fig. 7 Proposed model of OsNADP-ME2-mediated plant height regulation. The malic enzyme OsNADP-ME2 functions as a regulator of bioactive GAs accumulation in rice leaf tissues at vegetative stage possibly through regulating GA biosynthesis pathway or unknown pathway. In GA biosynthesis pathway, the product of pyruvate catalyzed by OsNADP-ME2 from malate could be used as a precursor through DXP (1-deoxy-D-xylulose-5-phosphate) pathway to synthesis GGPP, then GGPP is used as an original material to synthesis bioactive GAs (GA_1, GA_4, GA_7), and GAs further activate GA signaling to modulate plant height

Identification of CRISPR/Cas9 Gene Editing Mutant

One CRISPR/Cas9 gene editing mutant for *OsNADP-ME2* in the background of Japonica rice cultivar Zhonghua11 (ZH11) was obtained from a rice CRIPSR/Cas9 mutagenesis library (Lu et al. 2017). For mutant identification, one primer pairs crossing the sgRNA ACAGC TTCCGTGGTCCTTTC site were designed and used for PCR amplification (Fig.S2). The PCR products were sequenced and alignment with the sequence from wild type ZH11 to confirm the mutant type.

Rice Protoplast Isolation and Plasmid Transfection

The procedure of rice protoplast isolation and plasmid transfection was followed by the description in He et al. (2018). A total of 5ug purified plasmid DNA was used for protoplasts transfection via PEG-mediated method. After incubation for approximate 12–20 h, the transfected protoplasts were used for subcellular localization observation.

Subcellular Localization Analysis in Rice Protoplast

The rice protoplasts transfected with the plasmids containing N terminal GFP fused *GFP: OsNADP-ME2* gene and control *GFP* gene were used for fluorescent observation and photo-taking by a confocal microscope (Carl Zeiss LSM T-PMT 880) at excitation wavelength 488 nm and emission wavelength 509 nm.

RNA Extraction and Quantitative RT-PCR

Rice tissues were harvested for total RNA isolation with the Easy Step Super Total RNA Extraction Kit (Promega, Cat# LS1040). After DNA digestion with DNase I, 1 μg total RNA were used for cDNA synthesis with the Go Script™ Reverse Transcription System (Promega, Cat# A5001). 1 μl cDNA with dilution of 10 times was used for real-time quantitative PCR with ChamQ SYBR qPCR Master Mix (Vazyme, Cat#Q311-02). The PCR running program was 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s followed with 40 cycles. Two biological samples with three replications were performed. The relative expression level of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method using *Ubiquitin* gene as reference. The mean of three replications with Standard Deviation (SD) was used for final presented data. The primer pairs was listed in Table S1.

Phenotype Evaluation for Agronomy Traits

The rice plants including NPB, RNAi lines, CRISPR/Cas9 editing line and ZH11 were harvested after mature

for measuring the major agronomy traits of plant height, effective panicles, panicle length, grains per panicle, seed setting rate and thousand-grain weight. The rice seedling plants after treated with (or without) GA_3 for 7 days were used for evaluated the phenotype of plant height, leaf length, sheath length and root length. The means were used for final data with SD with a student's t-test for significance difference analysis.

Quantification Measurement of Endogenous Bioactive GAs

A total of 0.6 g grinded rice seedling tissues frozen in liquid nitrogen with 10 mL acetonitrile solution and 10 µl internal standard solution with corresponding GA_1(1 µg/mL), GA_4 (1 µg/mL) and GA_7 (1 µg/mL) was incubated at 4℃ for overnight. After centrifuge at 12,000 g for 5 min, the supernatant was taken and mixed with 20 mg C18 packing (Octadecylsilyl), then centrifuge at 10,000 g for 5 min, the supernatant was dried in nitrogen flow, and dissolved in 200ul methanol. After filter with 0.22 µm organic phase filter membrane, the filtered solution was used for HPLC-MS/MS detection using the instrument of PE QSight 420 triple quadrupole (PerkinElmer, USA) to quantify the content of bioactive GAs (GA_1, GA_4 and GA_7).

For the recovery rate calculation of GAs measurement, one blank control with 10 ng/mL internal standard GA_1, GA_4 and GA_7 were used for the representative to evaluate the recovery rate, the corresponding recovery rate for GA_1, GA_4 and GA_7 was shown in Table S2.

For the liquid phase, the chromatographic conditions were : the poroshell 120 SB-C18 reverse phase chromatographic column (2.1×150, 2.7 µm); column temperature: 30 ℃; mobile phase: *A: B*=(water/0.02% formic acid): (chromatographic methanol); flow velocity: 0.3 ml/min; and gradient elution mode: 0–1 min, 95% A; 1–9 min, 95−40% A; 9–11 min, 40−5% A; 11–13 min, 5% A; 13–13.2 min, 5–95% A; 13.2–15 min, 95% A; injected sample solution: 10 µL. For mass spectrometry analysis, the major parameters were used as following: Ionization mode: ESI positive and negative ion switching mode; scan type: MRM; air curtain:15psi; spay voltage: +5500/-5000v; atomizing pressure: 65psi; auxiliary pressure:70psi; atomization temperature: 400℃.

The hormone content calculation was used the formula: hormone content (ng/g fresh weight)=detection concentration (ng/mL) × volume coefficient (mL)/mass coefficient (**G**), where volume coefficient is the solution volume used in the final dissolution for tested sample, mass coefficient is the mass of tested sample. Two biological replications for leaf tissues of one week old seedling and three biological replications for leaf tissues of two months old plants were performed for each example. The results represent the means of two or three biological replications.

Pyruvate Content Detection and NADP-ME Activity Assay

The content of pyruvate and NADP-ME activity in rice plants was detected respectively using a pyruvate detection kit (ZC-S0399, ShangHai ZCi BiO) and NADP-ME detection kit (ZC-S0321, ShangHai ZCi BiO) as described procedure. Simply, 0.1 g grinded tissues with 1 ml pyruvate or NADP-ME extraction buffer was mixed and then stood for 30 min at room temperature (RT), after centrifuged at 10,000 g for 8 min at RT, take the supernatant for measurement. For pyruvate, the supernatant was tested at wave length 520 nm. The content of pyruvate was calculated with a standard curve built from the test of labeled sample concentration. For NADP-ME activity, the corresponding extraction supernatant were measured at wave length 340 nm after adding the reaction buffer, the value of preliminary and 1 min after reaction were used for calculating the NADP-ME activity. Two biological replications with three repeats were detected, the means of three repeats for one representative biological replication were used for final data with SD, Student's t-test was used for significance level analysis.

Measurement of Malate Content

The HPLC method was used for malate detection. Briefly, 0.5 g grinded rice leaf tissues within 3 mL ddH_2O was sonicated for 30 min, after centrifuge at 12,000 g for 5 min, the supernatant was transferred and filtered with 0.22 µm aqueous phase filter membrane, and the filtered solution was used for malic acid testing with HPLC method.

Accession Numbers

Genes sequence information used in this investigation was referenced from the Rice Genome Annotation Project version 7 with corresponding accession numbers as following: *OsNADP-ME2* (Os01g52500), *OsCPS1* (Os02g17780), *OsKS1* (Os04g52230), *OsKAO* (Os06g02019), *OsGA3ox1* (Os05g08540), *OsGA3ox2* (Os01g08220), *OsGA20ox1* (Os03g63970), *OsGA20ox2* (Os01g66100), *OsGA20ox3* (Os07g07420), *OsGA20ox4* (Os05g34854), *OsGID1* (Os05g33730), *OsGID2* (Os02g36974), *SLR1* (Os03g49990), *OsPH1* (Os01g65990), *OsGRF1* (Os02g53690), *OsGRF2* (Os06g10310), *OsGRF8* (Os11g35030), *Ubq* (Os03g13170).

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Author Contributions
J.L. Liu and X. Liu designed the project and experiments. B. Li., X. Zhou., W. Yao., J. Lin, X. Ding, Q. Chen, H. Huang, W. Chen, X. Huang, S. Pan performed the experiments and data collection; B. Li, X. Zhou, Y. Xiao, J.F. Liu, X. Liu, J.L. Liu analyzed the data and discussed the results, B. Li, X. Liu and J. Liu wrote and revised the manuscript. All authors reviewed the manuscript.

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Data Availability
No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate
Not applicable.

Consent for Publication
Written informed consent for publication was obtained from all participants.

Competing Interests
The authors declare no competing interests.

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