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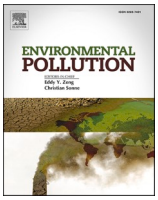

# Malic acid inhibits accumulation of cadmium, lead, nickel and chromium by down-regulation of *OsCESA* and up-regulation of *OsGLR3* in rice plant☆

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ABSTRACT

Malic acid (MA) plays an important role in plant tolerance to toxic metals, but its effect in restricting the transport of harmful metals remains unclear. In this study, *japonica* rice NPB and its fragile-culm mutant fc8 with low cellulose and thin cell wall were used to investigate the influence of MA on the accumulation of 4 toxic elements (Cd, Pb, Ni, and Cr) and 8 essential elements (K, Mg, Ca, Fe, Mn, Zn, Cu and Mo) in rice. The results showed that fc8 accumulated less toxic elements but more Ca and glutamate in grains and vegetative organs than NPB. After foliar application with MA at rice anthesis stage, the content of Cd, Pb, Ni significantly decreased by 27.9–41.0%, while those of Ca and glutamate significantly increased in both NPB and fc8. Therefore, the ratios between Cd and Ca in grains of NPB (3.4‰) and fc8 (1.5‰) were greatly higher than that in grains of NPB + MA (1.1‰) and fc8+MA (0.8‰) treatments. Meanwhile, the expression of *OsCEAS4,7,8,9* for the cellulose synthesis in secondary cell walls were down-regulated and cellulose content in vegetative organs of NPB and fc8 decreased by 16.7–21.1%. However, MA application significantly up-regulated the expression of GLR genes (*OsGLR3.1-3.5*) and raised the activity of glutamic-oxalacetic transaminase for glutamate synthesis in NPB and fc8. These results indicate that hazard risks of toxic elements in foods can be efficiently reduced through regulating cellulose biosynthesis and GLR channels in plant by combining genetic modification *in vivo* and malic acid application *in vitro*.

## 1. Introduction

In the past decades, heavy metals including cadmium (Cd), nickel (Ni), lead (Pb), chromium (Cr) exhibit a fast increase trend in the world, and food products contaminated by heavy metals have been frequently reported (Ahmed et al., 2022; Liao et al., 2019; Peng et al., 2019; Zhang et al., 2020). Most heavy metals absorbed by plants are immobilized in cell walls and the concentrations of toxic elements in grains are much lower than those in vegetative organs (Wang et al., 2021; Liang et al., 2018; Xue et al., 2022). In general, the moving speeds of beneficial elements like Ca, S and P are much higher than that of Cd in plants (Kobayashi et al., 2013; Tao et al., 2022; Toyota et al., 2018). However, the mechanism by which plant cells differentiate between harmful and beneficial elements is still unclear.

Cadmium has serious toxicity to organism and its median lethal concentration ($LC_{50}$) is much lower than other heavy metals like Pb and Zn (Calfee et al., 2014; Mazzei et al., 2013). Therefore, trace amount of Cd in agriculture products has caused great ecological and health risks (Deng et al., 2020; Wang et al., 2021; Xue et al., 2022). A large number of studies have shown that plant cell walls and vacuoles are the preferential sites to detoxify Cd, Pb and As in plants (Deng et al., 2020; Loix et al., 2017; Xue et al., 2021; Yang et al., 2021). Plant cell walls are composed of primary walls, secondary walls and intercellular layers. Their main components are cellulose, hemicellulose and pectin. These macromolecules have high potential to form chelates with Cd in the cell walls (Liu et al., 2013; Ouyang et al., 2022; Ma et al., 2015). Increasing

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the content of pectin and hemicellulose in root cell walls with nitric oxide (NO) and salicylic acid have significantly increased the Cd accumulation in rice root cell walls and improved the tolerance of rice plants to Cd stress (Pan et al., 2021; Xiong et al., 2009). Plants can even enhance the adsorption and fixation of Cd by changing the content and crystal structure of cellulose, hemicellulose and pectin in cell walls (Cheng et al., 2018; Meyer et al., 2015; Zhu et al., 2013). These results indicate that the macromolecular composition and plant cell walls play an important role in Cd transport and stress tolerance of plants (Liu et al., 2021; Rao and Dixon, 2018).

Malic acid (MA) is closely related to amino acid metabolism in plant cells. Malic acid produced from tricarboxylic acid (TCA) or glyoxylate cycle in mitochondria can be converted into oxaloacetic acid (OAA) through oxidation, which is converted into aspartic acid under the catalysis of glutamic transaminase (GOT), and then a series of amino acids (i.e., glutamine, lysine, threonine, etc.) are synthesized (Galili et al., 2016; Zhao et al., 2020; Zamani-Nour et al., 2021). These amino acids can reduce Cd toxicity through chelation and inhibiting Cd transport across plasma membranes in rice cells (Xue et al., 2022; Zhang et al., 2023). In addition, malic acid also has the function of supplementing and balancing other metabolites in TCA cycle to ensure the demand of cells for organic acids in amino acid and sugar metabolism (Lampugnani et al., 2019; Walker et al., 2021). The conversion of organic acids, amino acids, fatty acids, and soluble sugars can be regulated through the storage and release of MA in vacuoles (Eastmond et al., 2015; Walker et al., 2018, 2021). Adding MA can reduce Cd toxicity by enhancing root activity and increasing plant growth (Hawrylak-Nowak et al., 2015; Sebastian et al., 2018). However, whether MA metabolism is related with the development of cell walls and selective permeability of channels in cell membranes to toxic heavy metals in rice has not been reported.

A lot of ions and molecules are transported across plasma membranes by glutamate receptor-like (GLR) proteins to regulate defense and cell regeneration pathways (Grenzi et al., 2021; Hernández-Coronado et al., 2022; Wudick et al., 2018). To determine the effects of cellulose levels in cell walls on the selective permeability of GLR channels to essential and harmful ions, two genotypes with different cellulose content in vegetative organs were planted in Cd-contaminated soil. Meanwhile, the effects of MA treatment on cellulose and ion concentrations, as well as the expression levels of key genes, in different rice organs were examined. The main objectives of this study are revealing the roles of cellulose composition in cell walls on the accumulation of four common toxic elements (i.e., Cd, Ni, Pb and Cr) and essential elements in rice vegetative organs and grains, and explore the physiological mechanism of crosstalk between cell wall cellulose and glutamate-receptor channels in rice plant.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Two rice (*Oryza sativa* L.) genotypes, Nipponbare (*japonica*, normal cellulose content) and a fragile-culm mutant, termed *fragile culm 8* (*fc8*, low cellulose content) were used in this experiment. The seeds of these two genotypes were provided by Biomass and Bioenergy Research Centre, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China. The homozygous mutant *fc8* was developed from a T-DNA mutagenesis pool of Nipponbare (NPB) in 2008 as described in previous report (Wu et al., 2021). The tested seeds were disinfected with 30% $H_2O_2$ for 30 min, and germinated in vermiculite. Seedlings were irrigated with 1/10 of Hoagland nutrient solution every week. After growth for 5 weeks, 12 uniform seedlings were selected for planting in each pot. The soil for pot experiment was collected from 0 to 20 cm topsoil in a Cd-polluted farmland of Hunan province. Its basic physicochemical properties were listed in Table S1.

Each pot was filled with 4.0 kg soil with Cd content of 0.82 mg $kg^{-1}$ and supplemented with 2.0 g of $NH_4NO_3$ (0.7 g N) at tillering stage and elongation stage, respectively. The soil in pots remained flooded (4 cm deep) during the whole growth period of rice. At rice flowering stage, 50 ml of deionized water or 5 mM malic acid was sprayed on the leaves, once in the morning and once in the evening for two consecutive days. The gene expression levels of the developing grains were determined on the 7th day after flowering. Malate dehydrogenase (MDH) and glutamic-oxalacetic transaminease (GOT) activities in developing grains were measured on the 7th, 14th and 21st day after flowering. At mature stage, all plants were divided into grains, rachises, first internodes (panicle neck), flag leaves, second internodes, second leaves, basal stems, roots and other parts. All samples were dried to constant weight at 75 °C.

### 2.2. Cellulose and soluble sugar measurement

Cell wall fractions and cellulose in the rice organs were extracted according to the introduction of assay kits (Solarbio Life Science Co., Ltd., Beijing, China). In brief, 0.3 g of dry samples was extracted twice in 80% ethanol at 90 °C for 20 min and the extract was fixed to 50.0 ml by 80% ethanol for measurement of soluble sugars. The precipitate was washed twice with acetone, then soaked in chloroform-methanol (1:1, v:v) for 15 h, centrifuged at 6000 g and 25 °C for 10 min. After discard the supernatant, the pellet was extracted by acetic-nitric acids-water (8:1:2, v:v:v) at 100 °C for 1 h and dried to get cell wall fractions. 5.0 mg of cell walls was digested with 2.0 ml 60% sulfuric acid solution in ice bath for 30 min. The mixture was centrifuged at 8000 g and 4 °C for 10 min. The supernatant was diluted for the determination of cellulose content. Cellulose content and soluble sugar content were determined by anthrone colorimetry. The OD values of the samples were measured at 620 nm by UV spectrophotometer (UV-2010 type).

### 2.3. Gene expression analysis

Total RNA in fresh panicles was extracted with Trizol reagent with 4 replications. The complementary DNA (cDNA) was synthesized from total RNA with HiScript II Q RT SuperMix for qPCR (Vazyme R223). Relative quantification of gene expression by real-time PCR was performed on a BioRad CFX Manager System (BioRad, USA) with ChamQ Universal SYBR qPCR Master Mix (Vazyme Q711). Primer pairs for *Actin* gene were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primerblast/) based on the corresponding sequences available in the database (http://www.ncbi.nlm.nih.gov/), and used as an internal reference. The primer pairs for *OsMYB103*, *OsCESA* genes, *OsNramp* and GLR genes were designed based on references (Table S2). All gene expression analysis was performed with at least four independent biological replicates. Relative expression levels of genes were calculated according to the $2^{-\Delta\Delta Ct}$ method.

### 2.4. Enzyme activity assay

Fresh panicles were ground into powder in liquid nitrogen. About 0.2 g of powder was weighed and mixed with 1.8 ml of buffer (pH7.4, 0.05 mol $L^{-1}$ Tris-HCl) and left on ice for 5 min. The extract was centrifuged at 16000 g and 4 °C for 10 min. The supernatant was diluted for the determination of enzyme activity. Assay kits (Solarbio Life Science, Beijing, China) were used for measuring the activities of malate dehydrogenase (MDH) and glutamic-oxaloacetic transaminase (GOT). The unit of MDH activity was defined as the amount of enzyme that catalyzes the production of 1 μmol NADH per hour. The unit of GOT activity was defined as the amount of enzyme that catalyzes the production of 1 μmol pyruvic acid per hour.

### 2.5. Determination of Cd content and free amino acids content

The samples were dried and ground into powder for determination of Cd and free amino acids content. The extraction of soluble Cd and

insoluble Cd was carried out as previous report (Xue et al., 2019; 2022). After samples were digested with an instrument DigiBloc ED54 (Lab-Tech, Beijing, China) by concentrated $HNO_3$ and 30% $H_2O_2$ with a volume ratio of 7:1, solutions were diluted to 50 mL with deionized water and filtered through a 0.45 μm filter membrane (Jinteng, Tianjin, China). These solutions were used for analysis of Cd, Ni, Pb, Cr, K, Mg, Ca, Fe, Mn, Zn, Cu and Mo by ICP-MS (iCAP Q, Thermo Fisher Scientific, Waltham, MA, USA). The content of free amino acids in samples was measured by using an Agilent 1200 HPLC systems (Agilent Technologies, Palo Alto, CA) as described in previous article (Xue et al., 2022).

### 2.6. Statistical analysis

The data in the experiment were presented as the mean ± SD (n = 3). The significant differences among treatments were analyzed by one-way analysis of variance (ANOVA) with multiple comparisons by Duncan's test ($p < 0.05$), a mixed model ANOVA ($p < 0.05$) utilizing SPSS 20.0. All tables and figures were generated utilizing Microsoft Excel 2013.

## 3. Results

### 3.1. Effects of MA supply on toxic and essential elements in rice

MYB proteins belong to a super-family of transcription factors and have important roles in plant development and stress responses. The down-regulation of *OsMYB103* in fc8 and MA application significantly reduced the content of Cd, Ni and Pb in grains. Content of Cr in grains was much lower than those of other toxic elements and was not influenced by MA application (Table 1). NPB accumulated much higher Cd (0.9 mg $kg^{-1}$), Ni (0.24 mg $kg^{-1}$), Pb (0.44 mg $kg^{-1}$) and Cr (0.08 mg $kg^{-1}$) in grains than fc8. Grains in fc8+MA treatment displayed much lower Cd, Pb, Ni and Cr content than that in NPB + MA treatment.

The concentrations of essential mineral elements in rice grains were affected by genotypes and MA application at different levels. Mutant fc8 accumulated much more Ca, Fe and Zn than its wild type NPB. MA application increased the content of Ca and Fe by 7.5–12.5% in fc8 and 7.7–8.4% in NPB, respectively, while had little effect on Zn content. On the other hand, the levels of Mn and Cu in fc8 grains were significantly lower than those in NPB grains. MA application decreased the content of Mn and Cu by 8.1–10.9% in fc8 and 6.3–7.0% in NPB, respectively. Concentrations of macro-element K and Mg in rice were in the range of 1.60–1.68 g $kg^{-1}$. NPB had less K and Mg than fc8 but did not reach to the significant level at $p < 0.5$. MA application had little effect on K content and slightly increased Mg content. Micro-element Mo was not significantly influenced by genotype or MA application.

**Table 1**
Effects of malic acid on the content of toxic and essential elements in rice grains.

| Elements | NPB | NPB + MA | fc8 | fc8+MA |
|---|---|---|---|---|
| Toxic elements | | | | |
| Cd(mg $kg^{-1}$) | 0.92 ± 0.06a | 0.32 ± 0.08c | 0.63 ± 0.09b | 0.33 ± 0.03d |
| Ni(mg $kg^{-1}$) | 0.24 ± 0.02a | 0.21 ± 0.03b | 0.19 ± 0.04c | 0.16 ± 0.02d |
| Pb(mg $kg^{-1}$) | 0.44 ± 0.03a | 0.38 ± 0.03b | 0.37 ± 0.07b | 0.34 ± 0.04c |
| Cr(mg $kg^{-1}$) | 0.08 ± 0.03a | 0.08 ± 0.01a | 0.06 ± 0.03b | 0.06 ± 0.01b |
| Essential elements | | | | |
| K(g $kg^{-1}$) | 1.63 ± 0.04a | 1.60 ± 0.14a | 1.68 ± 0.03a | 1.64 ± 0.11a |
| Mg(g $kg^{-1}$) | 0.70 ± 0.05a | 0.73 ± 0.04a | 0.76 ± 0.04a | 0.78 ± 0.05a |
| Ca(mg $kg^{-1}$) | 272.35 ± 12.30d | 295.31 ± 18.12c | 408.67 ± 23.66b | 428.99 ± 18.58a |
| Fe(mg $kg^{-1}$) | 51.82 ± 6.35d | 55.64 ± 3.03c | 58.39 ± 8.05b | 64.87 ± 9.95a |
| Mn(mg $kg^{-1}$) | 62.44 ± 5.83a | 58.08 ± 3.30b | 49.69 ± 3.88c | 42.87 ± 1.18d |
| Zn(mg $kg^{-1}$) | 18.43 ± 1.88b | 18.60 ± 1.89b | 19.65 ± 1.06a | 19.73 ± 1.38a |
| Cu(mg $kg^{-1}$) | 2.86 ± 0.05a | 2.69 ± 0.04b | 2.35 ± 0.04c | 2.12 ± 0.05d |
| Mo(mg $kg^{-1}$) | 0.43 ± 0.05a | 0.44 ± 0.03a | 0.41 ± 0.03a | 0.43 ± 0.06a |

After MA application, the variation scale of Cd content was much higher than that of other elements in grains (Fig. 1A). In comparison with NPB and fc8, NPB + MA and fc8+MA treatments made Cd content in grains decreased by 65.5% and 63.7%, respectively. Under MA treatment, Ni and Pb content decreased by 3.1% and 13.2% in NPB, and 25.4% and 22.7% in fc8, respectively. Cr content showed no significant change among treatments. The total amount of these 4 toxic metals in grains was decreased by 41.0% in NPB and 27.9% in fc8 after spraying MA, respectively. Foliar application with MA generally raised the content of most essential elements in grains, only Mn and Cu content significantly decreased.

Concentrations and species of coexistent cations have pronounce effects on the toxicity of Cd. Genotypes and MA application had significant effects on the ratios between Cd and essential elements in rice grains. After MA application, the Cd:K ratio decreased from 0.6‰ to 0.4‰ to less than 0.2‰ in NBP and fc8 grains, respectively. The Cd:Mg ratio dropped from 1.3‰ to 0.8‰ to below 0.4‰ in NPB and fc8, respectively. The value of Cd:Ca ratio in grains was much higher than that of Cd:K or Cd:Mg, and dropped from 3.4‰ to 1.1‰ in NPB and from 1.5‰ to 0.8‰ in fc8 after MA application (Fig. 1B). Relatively, ratios between Cd and micro-elements were much higher than that of Cd:Ca. The Cd:Fe and Cd:Mn ratios were in the range of 1.5–1.8%, and Cd:Zn ratio was as high as 4.99% in NPB grains (Fig. 1C). After MA application, Cd:Fe and Cd:Mn ratios dropped to 0.5–0.6%, and Cd:Zn ratio fallen to 1.7%, respectively.

*NRAMP* family genes mediate the transport of divalent cations, especially Mn and Cd in rice plants. In comparison with NPB, fragile-culm mutant fc8 had much lower expression levels of *OsNramp1, 2, 3 and 5* in developing panicles. The expression levels of these genes were further inhibited by MA application (Fig. 1D). Foliar application with MA reduced the expression level of *OsNramp1, 2, 3 and* 5 by 55.5–72.4% in NPB and 29.8–51.1% in fc8, respectively.

### 3.2. Effects of MA supply on toxic and essential elements in vegetative organs

When rice plants grew in soil contaminated with heavy metals, NPB accumulated more toxic elements (i.e., Cd, Ni, Pb and Cr) in rachises than fc8. However, the content of these toxic elements displayed litter difference between NPB and fc8 in the first and second internodes. Under foliar application with MA, the content of these toxic elements were significantly decreased in rachises, first and second internodes (Fig. 2A).

Content of essential elements in rachises and internodes displayed different changing models among treatments. Content of Fe and Ca in rachises and internodes significantly increased after MA application, and fc8 generally displayed higher Fe and Ca content than NPB (Fig. 2B). However, MA treatment had little effect on the content of Zn and Cu in rachises and internodes, and fc8 displayed significantly higher Zn and Cu content than NPB (Fig. 2C). Content of Mn in rachises and internodes significantly decreased after MA application, and fc8 displayed lower Mn content than NPB (Fig. 2D). Content of K, Mg, and Mo in rachises and internodes displayed slightly changes among different treatments (Table S3). There was no significant difference in the content of toxic and essential elements in basal stems, roots and other parts among different genotypes and MA treatments.

### 3.3. Effects of OsMYB103 and MA supply on the synthesis of cellulose

Soluble sugars are important precursors for cellulose synthesis. Down-regulation of *OsMYB103* and foliar application with MA had significantly influence on the content of cellulose and soluble sugars in

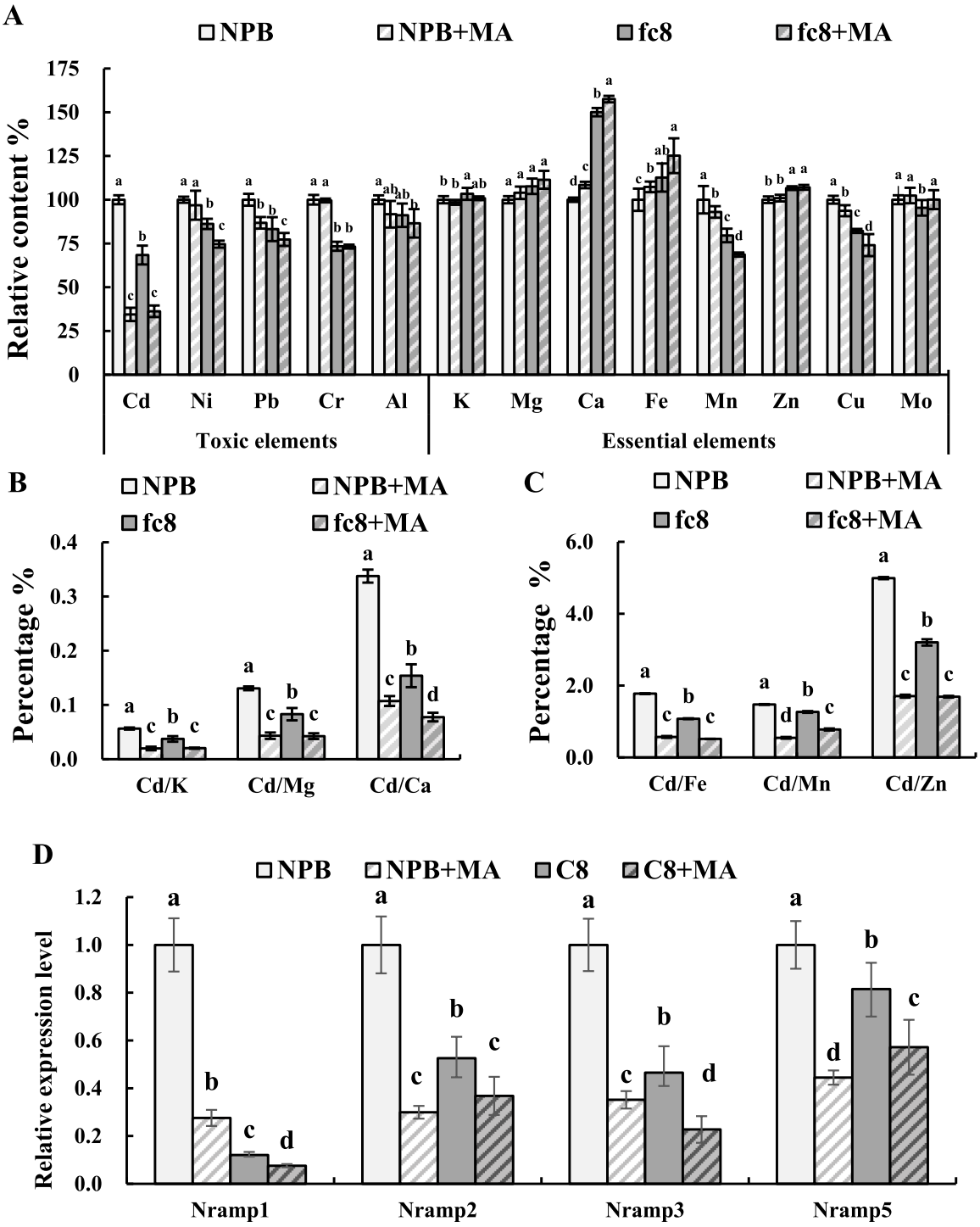


**Fig. 1.** Relative content of toxic and essential elements in grains (A), ratios between Cd and macro-elements (B), and between Cd and micro-elements (C); relative expression levels of genes encoding different proteins in rice panicles (D), which were normalized to the corresponding genes in NPB. Values are the means of four replicates ± SD. Different letters indicate significant difference ($p < 0.05$) among treatments for the same element or the same gene.

vegetative organs of rice plant. In normal *japonica* rice cultivar NPB, cellulose content in the first internodes (305.9 mg $g^{-1}$) and flag leaves (266.9 mg $g^{-1}$) was at the same level. Down-regulation of *OsMYB103* in the mutant fc8 significantly inhibited cellulose synthesis in vegetative organs. The cellulose content in internodes and flag leaves of fc8 was decreased by 10.3–19.4% than that of NPB. Foliar application with MA also significantly inhibited the synthesis of cellulose (Fig. 3A). After MA application, cellulose content was decreased by 16.7–21.1% in internodes, and by 30.1–33.7% in flag leaves of NPB and fc8 at maturity stage, respectively. Content of soluble sugars in flag leaves was significantly higher than that in internodes. Knockdown of *OsMYB103* in mutant fc8 resulted in about 14.0% decrease of soluble sugars in internodes and flag leaves. After foliar application with MA, soluble sugars in internodes and flag leaves were decreased by 23.5% in NPB and by 13.3% in *fc8*, respectively (Fig. 3B).

Cellulose synthase genes (*CESA*) play an important role in the

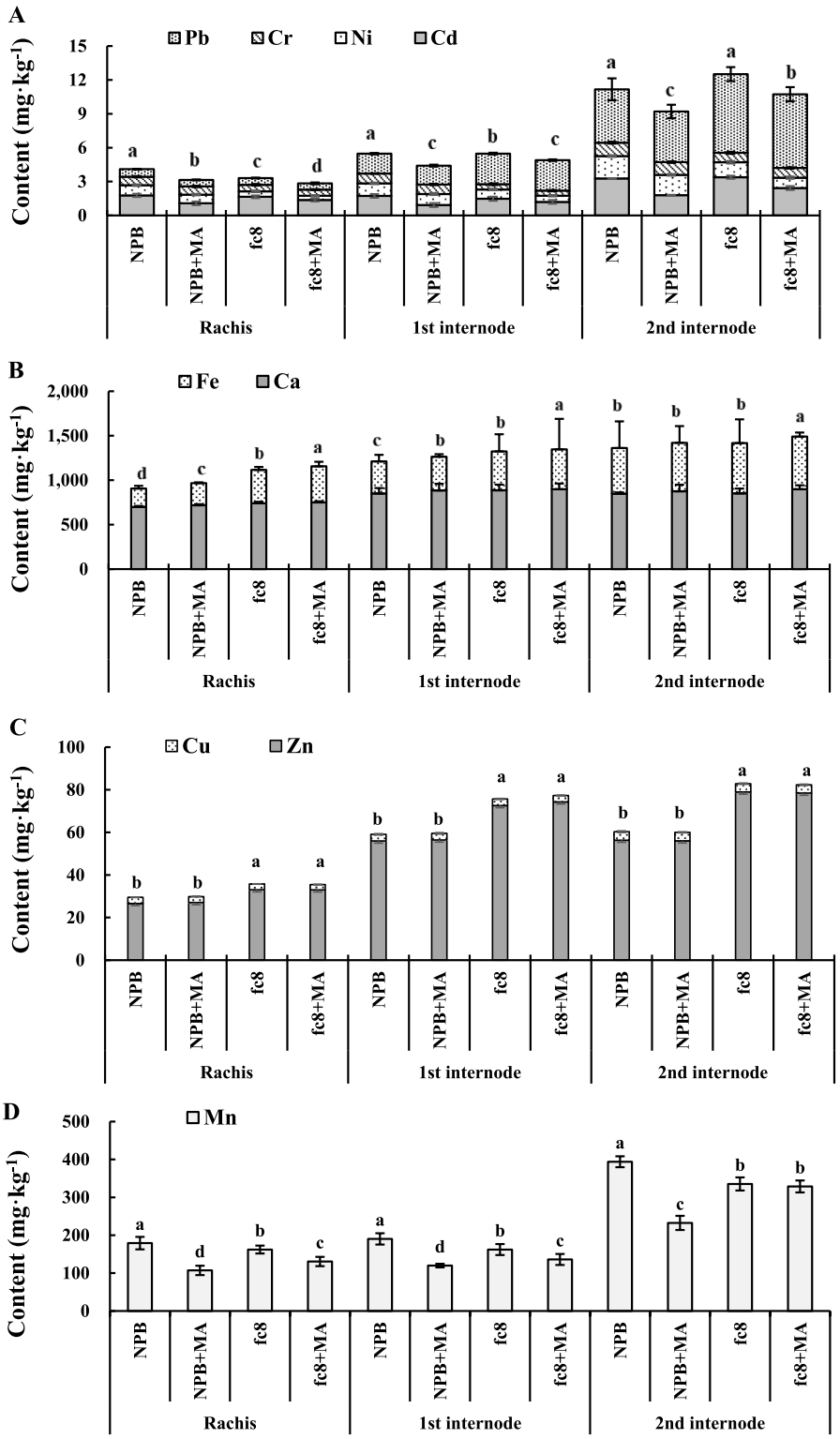


**Fig. 2.** Content of toxic elements (A), Fe and Ca (B), Zn and Cu (C), and Mn (D) in rachises, first and second internodes of rice plant. Values are the means of four replicates ± SD. Different letters indicate significant difference of element content ($p < 0.05$) among treatments in the same organ.

cellulose synthesis of cell walls, and *CESA4, 7, 8, 9* are primarily responsible for the secondary cell wall formation. Comparing with NPB, the mutant fc8 displayed very lower expression level of *OsMYB103* in mutant fc8. MA application had little effect on the expression level of *OsMYB103* in NPB and fc8. However, down-regulation of *OsMYB103* in fc8 or MA application significantly decreased the expression levels of *OsCESA* genes (Fig. 3C). The expression levels of *OsCESA4, 7, 8, 9* in fc8 was decreased by 28.4–62.6% compared to that in NPB. After MA application, the expression levels of *OsCESA4,7,9* was decreased by 46.7–53.5% in NPB, while only decreased by 7.2–18.3% in fc8. In comparison with these genes for the formation of secondary cell walls, gene *OsCESA1* responsible for the formation of primary cell walls was

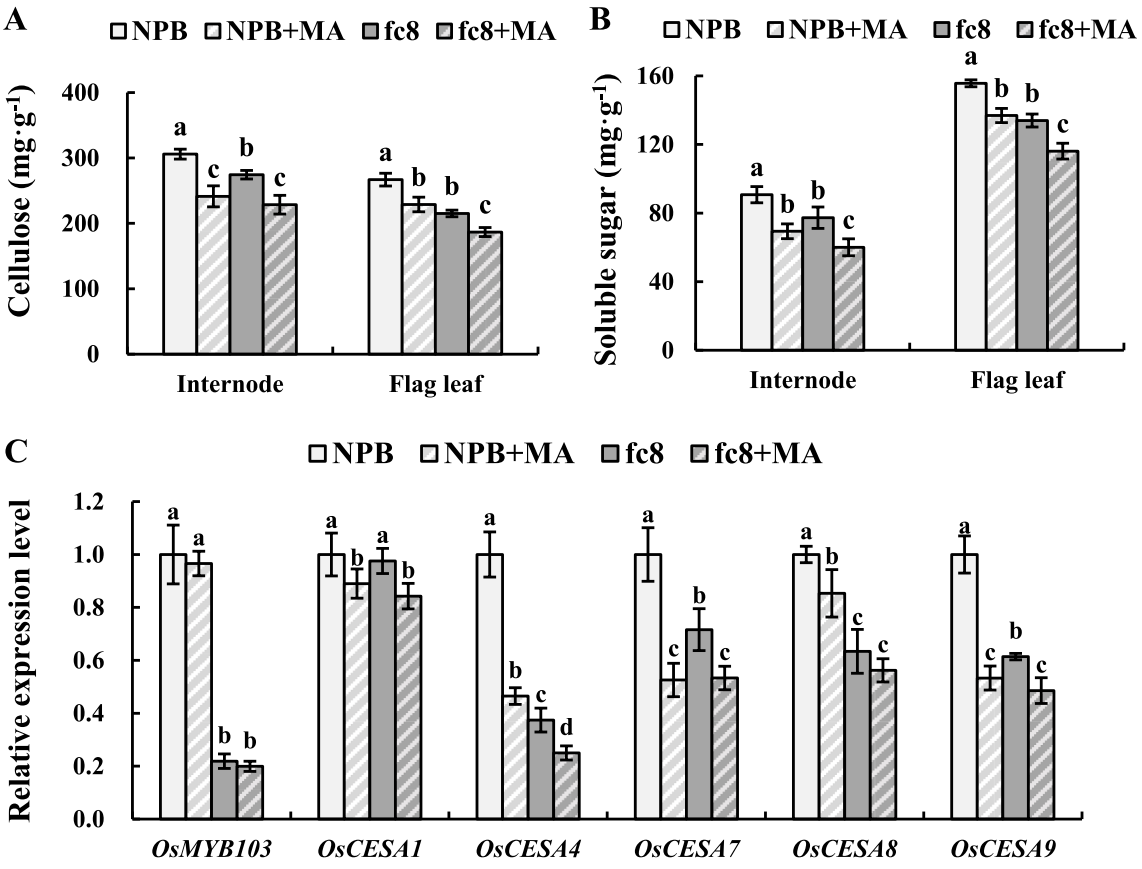


**Fig. 3.** Content of cellulose (A) and soluble sugar (B) in flag leaf and first internode, and relative expression levels of *OsMYB103* and *OsCESA* in NPB and *fc8* (C), which were normalized to the corresponding genes in NPB. Different letters indicate significant difference ($p < 0.05$) among treatments in the same organ or for the same gene.

less influenced by the knockdown of *OsNYB103* and MA application. There was no significant change in the expression level of *OsCESA1* between NPB and fc8 with or without MA treatment.

### 3.4. Effects of MA supply on synthesis of glutamate and glutamate receptor-like channels

Catalyzed by MDH, MA is transformed to oxaloacetic acid which enters into carbohydrate metabolism. GOT is one of the important transaminases involved in amino acid metabolism in plant, and promotes the mutual transformation of amino acids through amino transfer (Fig. 4 A). There was no significant difference in MDH activity between NPB and fc8 during the whole grain filling period. However, MA application significantly increased MDH activity during grain filling period, and the activity of MDH reached to its maximum value on the seventh day after anthesis (Fig. 4B). After MA treatment, MDH activity increased by 33.3% in NPB and by 67.8% in fc8, respectively. The GOT activity in NPB and fc8 showed little change during the whole grain filling period. On the 21st day after anthesis, the GOT activity in fc8 was significantly lower than that in NPB. During grain filling period, MA application increased the activity of GOT by 14.9–16.0% in NPB and by 20.8–53.1% in fc8, respectively (Fig. 4C).

With the increase of GOT activity after MA application, content of free Glu and Asp in grains and first internodes was significantly increased (Fig. 4D). NPB displayed much higher levels of free Glu and Asp in the first internodes (0.38–0.56 g $kg^{-1}$) than that in grains (0.09–0.18 g $kg^{-1}$). Under MA treatment, Glu content increased by 21.9% and 41.0% in the grains and first internodes of NPB, and increased by 29.8% and 15.2% in those of fc8, respectively.

The results of qRT-PCR showed that the expression levels of 5 *OsGLR3* isogenes varied with genotypes and MA treatment in the developing grains. Knockdown of *OsNYB103* in fc8 remarkably down-regulated the expression of *OsGLR3.2-3.5*, while had little effect on *OsGLR3.1*. After MA application, the expression levels of *OsGLR3.1-3.5* were significantly up-regulated (Fig. 4E). Relatively, the expression level of *OsGLR3* in fc8 were more sensitive to MA application than those in NPB. Under MA treatment, the expression levels of *OsGLR3.1-3.5* increased by 42.6–229.1% in fc8 and 30.3–83.5% in NPB, respectively.

### 3.5. Relationship between elements and glutamate in organs

The content of four harmful elements in rice grains was positively correlated with each other as well as with Mn content, but was negatively correlated with the content of Mg, Ca and Fe. Content of 3 toxic elements (Ni, Pb and Cr) except Cd, was negatively correlated with Zn and positively correlated with Cu content (Fig. 5A). Content of Ca was positively correlated with the content of other essential elements like K, Mg, Fe and Zn, but negatively correlated with Mn and Cu content. Relatively, there were very weak correlations between the content of toxic elements in vegetative organs. Only positive correlation coefficient between Ni and Cr content, and negative correlation coefficients between Pb and Cr/Ni content were significant at $p < 0.01$. Similar to the correlative models in grains, content of Ca was negatively correlated with Cd, but Mn content was positively correlated with Cd in vegetative organs (Fig. 5B). Apparently, rice grains had stronger recognition ability to toxic elements than vegetative organs.

In rice grains, the levels of free glutamate (0.11–0.18 g $kg^{-1}$) and Ca (0.26–0.44 g $kg^{-1}$) were at the same level, ~1000 times higher than that

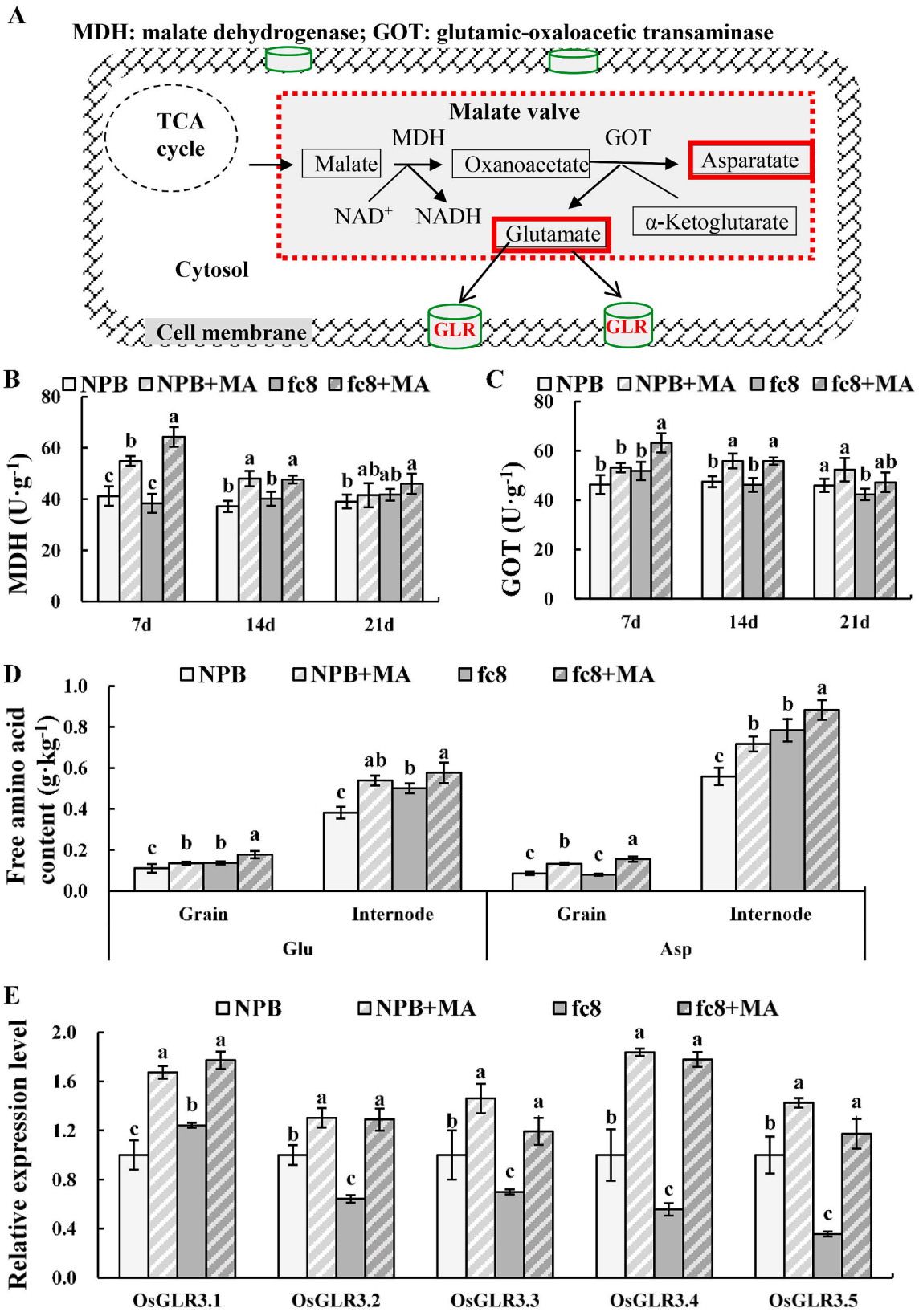


**Fig. 4.** Possible route of glutamate (Glu) and aspartate (Asp) synthesis (A), activities of MDH (B) and GOT (C) in developing grains, free Glu and Asp in organs (D), and relative expression levels of *OsGLR3* isogenes (E) in developing grains of NPB and fc8, which were normalized to the corresponding genes in NPB. Different letters indicate significant difference ($p < 0.05$) among treatments in the same organ for the same enzyme or gene.

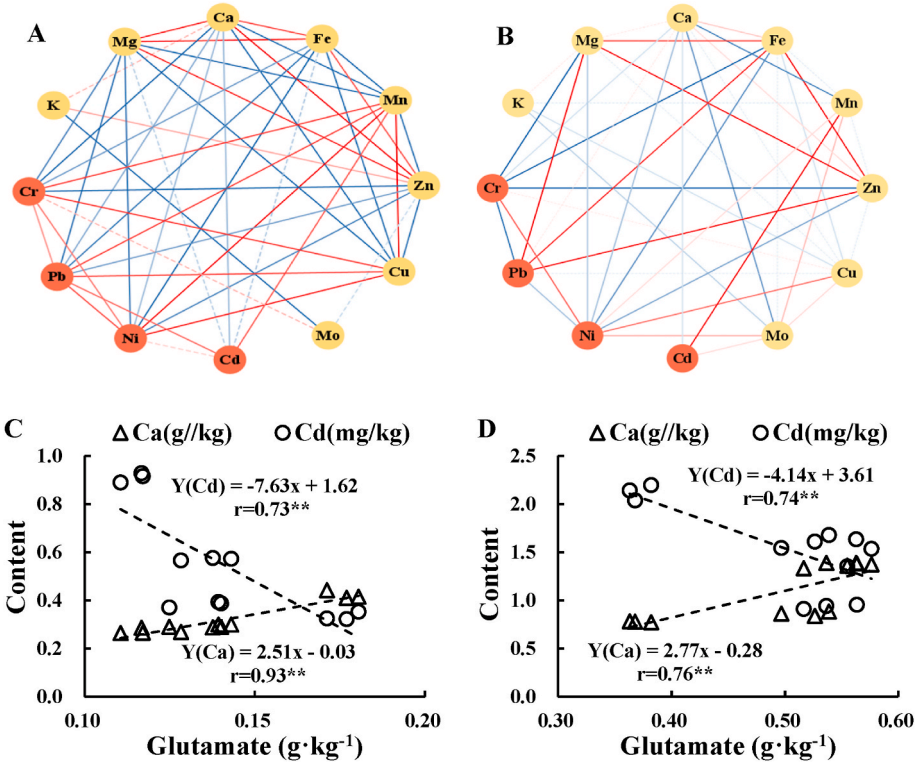


**Fig. 5.** Pearson's correlation analysis of 4 toxic elements (with red background) and 8 essential mineral elements (with yellow background) in grains (A) and first internodes (B), and regression analysis between glutamate content and Cd or Ca content in grains (C) and first internodes (D). Only significant correlations are displayed in the correlations wheels ($P < 0.05$). Solid lines indicate $P < 0.01$, dotted lines indicate $0.01 < P < 0.05$. Positive correlations are denoted by red lines, negative correlations are denoted by blue lines. Darker color of lines mean higher R values ($R > 0.71$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of Cd (0.32–0.92 mg $kg^{-1}$). Glutamate content was positively correlated with Ca, but negatively correlated with Cd content. As the glutamate content increased by 0.1 g $kg^{-1}$ in grains, the Ca content increased by 0.25 g $kg^{-1}$ and Cd content decreased by 0.76 mg $kg^{-1}$ (Fig. 5C). Compared with grains, internodes accumulated much more free glutamate, Ca and Cd. Similarly, the content of free glutamate in the internodes was positively and significantly correlated with Ca content, while negatively correlated with Cd content. With the increase of glutamate content by 0.1 g $kg^{-1}$ in vegetative organs, the Ca content increased by 0.28 g $kg^{-1}$, while Cd content decreased by 0.41 mg $kg^{-1}$ (Fig. 5D).

## 4. Discussion

There are great differences between the transport velocities of essential and toxic elements in plants. For example, some essential major elements like $^{13}N$ and $H_2^{15}O$ move as fast as 110–520 cm $h^{-1}$, while heavy metals like $^{52}Mn$ and $^{107}Cd$ only move at the speed of 2.4–3.6 cm $h^{-1}$ in rice plant (Fujimaki et al., 2010). The distinct uptake patterns among essential and harmful cations in plants are the result of selective transport processes determined by cell walls and membrane transport proteins. Plant cell walls contain elaborate polysaccharide networks and play a key role in regulating nutrient transport (Ogden et al., 2018). Rice organs with thick cell walls (i.e., nodes and roots) concentrate much more Cd ions than those with thin walls like leaves (Xue et al., 2022; Zhang et al., 2023). Inhibition of cellulose synthesis in the secondary wall of rice cells by gene mutation (i.e., *brittle culm13*) can significantly reduce the content of Cd in stems, leaves and xylem (Song et al., 2013). In this study, we found that expressions of *OsCESA* genes responsible for the synthesis of cellulose in the secondary cell walls were significantly suppressed by knockdown of *OsNYB103* in mutant fc8 or by MA application. With the reduction of cellulose content in cell walls, content of K, Mg, Fe, Ca, Zn, Cu and Mo in vegetative organs increased at different levels, while those of toxic elements (Cd, Ni, Pb and Cr) significantly decreased. Therefore, down-regulation of cellulose synthesis may be a feasible strategy to inhibit accumulation of toxic elements in cell walls of rice plant.

It has found that non-selective cation channels (NSCCs) can transport many kinds of ions at the same time (Demidchik and Maathuis, 2007; Han et al., 2019). As one kind of ligand-gated NSCCs, GLR channels play important parts in modulating $Ca^{2+}$ signaling, ion transport and metabolic pathways (Grenzi et al., 2021; Hernández-Coronado et al., 2022; Wudick et al., 2018). Content of essential elements in tissues are usually increased to establish new homeostasis among ions under environments with Cd/Cr stress (Cheng et al., 2016; Li et al., 2019; Xue et al., 2022; Zhang et al., 2023). In this study, we found that MA treatment promoted the expression of *OsGLR3.1-3.5*, accompanied by significant increase of Ca and Fe, while remarkable reduction of Cd, Ni and Pb quantities as well as ratios between Cd and essential elements in rice grains. These results indicate that the up-regulation of *OsGLR3.1-3.*5 has promoted the preferential transport of essential elements through GLR channels and retarded the influx of toxic elements in developing grains.

As an essential microelement for plant development, Mn can enter the cell through transporters and NSCCs (Demidchik and Maathuis, 2010; Han et al., 2019; Yang et al., 2014a,b). The natural resistance associated macrophage proteins (NRAMPs) make a great contribution to Mn transport in rice plants. Knockout of *OsNramp5* gene in *japonica* and *indica* rice has produced mutants with much low concentration of Mn and Cd in rice grains (Ishimaru et al., 2012; Sasaki et al., 2012; Tang et al., 2017). In this study, the content of Mn and Cd in both grains and vegetative organs was significantly decreased after foliar application with MA, accompanied by the reduction of expression levels of *OsNRAMP1, 2, 3, 5* in rice panicles. MA application may inhibit the transport of Cd and Mn through NRAMPs by down-regulation of *OsNRAMP1, 2, 3,*

5 in rice plant.

Glutamate is a very active amino acid and can rapidly induce defense responses in plants (Forde and Roberts, 2014; Jiang et al., 2020; Kan et al., 2017; Ni et al., 2016). Free glutamate can reduce the toxicity of Cd by forming chelates with Cd through α-carboxyl and side chain carboxyl oxygen atoms in rice cells (Jiang et al., 2020; Xue et al., 2023). High levels of Glu are able to activate the GLR channels with a consequent $Ca^{2+}$ influx into the cytosol in the apoplast (Vincent et al., 2017; Toyota et al., 2018; Grenzi et al., 2021). This study found that spraying MA significantly increased the levels of Glu and Asp by raising the activity of glutamic-oxaloacetic transaminase (GOT), while decreased the ratios between Cd and essential elements in rice grains. The elevation of Glu levels may act as sensors to activate the defense action of developing grains and to increase the preferential selectivity of GLR channels for essential ions over toxic ions in rice cells.

Malic acid is an important intermediate in TCA cycle and can freely shuttle among chloroplasts, mitochondria, vacuoles and other organelles in plant cells (Selinski and Scheibe, 2019; Zhao et al., 2020; Zamani-Nour et al., 2021). When glucose produced by photosynthesis is limited, malate is gradually transformed into glucose, glucose uridine diphosphate (UDPG) and other soluble sugars by gluconeogenesis (Walker et al., 2020). These sugars are used for cellulose synthesis at the plasma membrane by CELLULOSE SYNTHASE A (CESA) protein complexes (CSCs) (Allen et al., 2021; Lampugnani et al., 2019). Meanwhile, some malate is converted into amino acids by amino transfer enzymes in the cytosol (Galili et al., 2016; Zamani-Nour et al., 2021). Therefore, malate has been involved in elaborate polysaccharide networks in cell wall rebuild and cell-cell communication for mediation of energy homeostasis (Barnes and Anderson, 2018; Zhao et al., 2020). Based on a fragile-culm rice mutant with low cellulose and MA treatment, we confirmed that the decrease of cellulose content in cell walls is accompanied by the significant decrease of toxic elements. The key roles of malate in suppressing toxic element transport may be attributed to the two aspects: (1) decreasing the mobile toxic ions (Cd, Ni, Pb and Cr) in the secondary cell walls by reducing cellulose content; (2) improving the discrimination and interception of GLR channels to toxic ions in cell membranes by increasing glutamate content in rice cells (Fig. 6). It seems that malate is a key valve to modulate the balance between glutamate and soluble sugars. Malate supplementation can promote the conversion of α -ketoglutaric acid to glutamate by stimulating the activity of transaminase, and may inhibit the synthesis of cellulose in cell walls by restraining the conversion of malate into glucose through gluconeogenesis.

## 5. Conclusion

The concentrations of toxic elements (Cd, Ni, Pb and Cr), cellulose and sugars in internodes of *japonica* genotype NPB were significantly

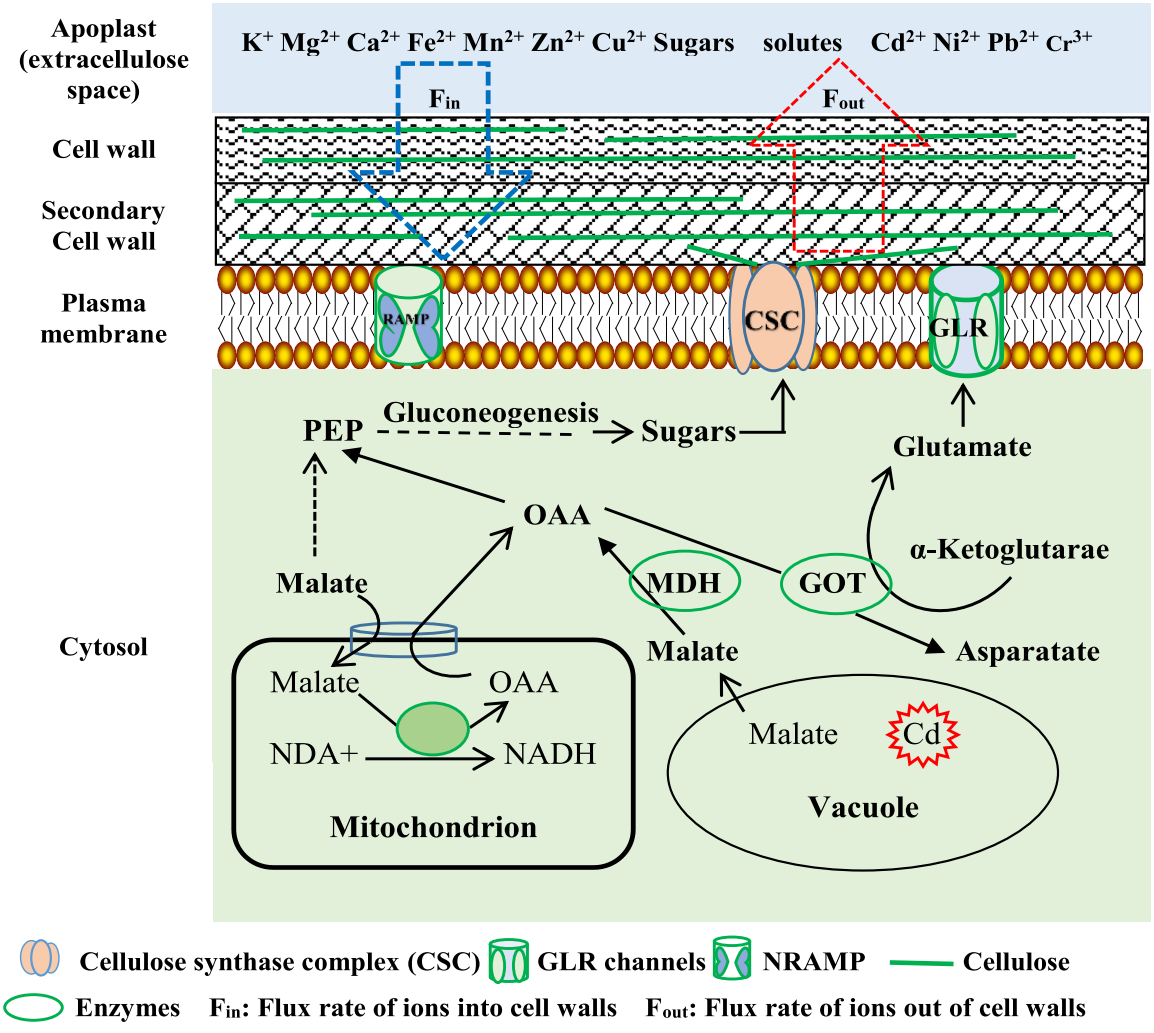


**Fig. 6.** Schematic of cell walls and glutamate receptor-like channels (GLRs) in plasma membrane to selectively transport essential and toxic elements from free space into cell, and effects of malate valve on the synthesis of glutamate and sugars in cell.

higher than those of the fragile-culm mutant fc8 with *OsMYB103* knockdown. Foliar application with malate significantly down-regulated the expression of *OsCEAS4,7,8,9* for cellulose synthesis in secondary cell walls and *NRAMP1,2,3.5* for Mn transport, while up-regulated the expression of GLR genes (*OsGLR3.1-3.5*) for modulating glutamate receptor-like (GLR) channels in both NPB and fc8. Malate supplementation inhibited the accumulation of toxic elements by 27.9–41.0%, while increased the content of Ca and glutamate by 12.5–29.8% in grains of NPB and fc8. As a result, the Cd:Ca ratio dropped from 3.4‰ in NPB to 0.8–1.1‰ in NPB + MA. These results indicate that foliar application with malate inhibits the accumulation of toxic elements by enhancing the permeability of GLR channels to $Ca^{2+}$ and limiting the transport of toxic elements in rice plants. It is a powerful strategy to decrease hazard risks of toxic elements in rice through regulating cellulose biosynthesis and GLR channels in plants by combining genetic modification *in vivo* and malate application *in vitro*.

## CRediT authorship contribution statement

**Xin Zhang:** Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Weijie Xue:** Investigation, Methodology, Resources. **Lin Qi:** Investigation, Methodology, Resource. **Changbo Zhang:** Methodology, Software, Resource. **Changrong Wang:** Conceptualization, Formal analysis, Writing – review & editing. **Yongchun Huang:** Resources, Visualization, Formal analysis. **Yanting Wang:** Investigation, Resources, Visualization. **Liangcai Peng:** Resource, Methodology, Supervision, Project administration, Funding acquisition. **Zhongqi Liu:** Conceptualization, Validation, Formal analysis, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122934.

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