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ORIGINAL PAPER

Overexpression of a *Miscanthus sacchariflorus* yellow stripe-like transporter *MsYSL1* enhances resistance of *Arabidopsis* to cadmium by mediating metal ion reallocation

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Abstract
The yellow stripe-like (YSL) family of transporters mediates the uptake, translocation, and distribution of various mineral elements in vivo by transferring metal ions chelated with phytosiderophore or nicotianamine (NA). However, little is known about the roles of the *YSL* genes against cadmium *in planta*. In this study, we first cloned and characterized a vital member of the *YSL* gene family, *MsYSL1*, from the bioenergy plant *Miscanthus sacchariflorus*. *MsYSL1* localized in the plasma membrane and was widely expressed throughout the whole seedling with the highest expression level in the stem. In addition, its expression in the root was stimulated by excess manganese (Mn), cadmium (Cd), and lead, and a shortage of iron (Fe), zinc (Zn), and copper. Functional complementation in yeast indicated that MsYSL1 showed transport activity for Fe(II)–NA and Zn–NA, but not for Cd–NA. Although they exhibited no significant differences versus the wild type under normal cultivation conditions, *MsYSL1*-overexpressing *Arabidopsis* lines displayed a higher resistance to Cd accompanied by longer root lengths, lower Cd, Zn, and Mn levels in roots, and higher Cd, Fe, and Mn translocation ratios under Cd stress. Moreover, genes related to NA synthesis, metal translocation, long-distance transport, and Cd exclusion were highly induced in transgenic lines under Cd stress. Thus, MsYSL1 may be an essential transporter for diverse metal–NAs to participate in the Cd detoxification by mediating the reallocation of other metal ions.

Keywords *Miscanthus sacchariflorus* · *MsYSL1* · Yeast complementation · Cd tolerance · *Arabidopsis*

Abbreviations

YSL	Yellow stripe-like
PSs	Phytosiderophores
NAs	Nicotianamines
NAS	Nicotianamine synthetase
MAs	Mugineic acids
DMAs	Deoxymugineic acids

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Introduction

Yellow stripe-like (YSL) proteins belong to an oligopeptide superfamily and participate in the uptake and retranslocation of various metal ions in plants (Curie et al. 2009). YSL transporters usually transport metals as metal complexes formed with phytosiderophores (PSs) or nicotianamines (NAs), which are two vital metal ion ligands that are widely distributed in plants. NA is generated from *S*-adenosylmethionine through the catalysis of nicotianamine synthetase (NAS) in plants (Higuchi et al. 1994). PS normally consists of a variety of acids, including mugineic acids (MAs) and deoxymugineic acids (DMAs). MA is derived from NA after a deamination step and a reduction process conducted by NA aminotransferase and DMA synthetase, respectively, especially in graminaceous plants (Takahashi et al. 1999; Bashir et al. 2006). YSL transporters are divided into two groups according to the transport substrates. The metal–PS transporter is mainly responsible for the uptake of metal ions in the grass family, while the metal–NA transporter is closely related to long-distance translocation and redistribution in

monocots and dicots. In recent decades, researches focused primarily on family members of *Zea mays* (ZmYS1), *Oryza sativa* (OsYSL2, 6, 9, 15, 16, and 18), *Hordeum vulgare* (HvYS1, HvYS2, and HvYSL5), and *Arabidopsis thaliana* (AtYSL1, 2, 3, 4, and 6). ZmYS1 was the first YSL member discovered and identified as a typical transporter for the uptake of iron(Fe)(III)–PS from rhizosphere soil (Curie et al. 2001). Further research revealed that ZmYS1 transports several substrates, including Fe(III)–DMA, Fe(III)–MA, Fe(II)–NA, zinc (Zn)–DMA, copper (Cu)–MA, nickel (Ni)–MA, Ni–NA, and cadmium (Cd)–DMA (Schaaf et al. 2004). A homologous gene in rice (*YSL15*) exhibits substrate specificity for Fe(III)–DMA and Fe(II)–NA, and is expressed not only in root epidermis but also in phloem and reproductive organs (Lee et al. 2009), while HvYS1 in barley only transports Fe(III)–DMA and regulates Fe uptake in roots (Murata et al. 2006). Other members are mainly involved in internal metal translocation and redistribution in plants. YSL members in *Arabidopsis* chiefly mediate Fe(II)–NA and Fe(III)–citrate in vivo because PS is not synthesized or secreted. *AtYSL2* is especially expressed in vascular tissues and is capable of transporting Fe(II)–NA and Cu–NA in yeast (DiDonato et al. 2004; Schaaf et al. 2005). AtYSL1 and AtYSL3 are identified as transporters of Fe(II)–NA that mediate Fe, Zn, and Cu redistribution from shoots and leaves to seeds in *Arabidopsis*, and AtYSL3 also has the capacity to transfer Fe(II)–DMA in yeast (Waters et al. 2006; Chu et al. 2010). Additionally, AtYSL4 and AtYSL6 are reported to localize in the vacuole membrane and chloroplast envelope in response to the detoxification of excessive metals in plant cells although there is no transport capacity for Fe(II)–NA in the yeast (Conte et al. 2013; Divol et al. 2013). OsYSL2 was the first characterized transporter of the 18 members in the rice genome. It delivers Fe(II)–NA and manganese (Mn)–NA from roots to shoots and seeds through the phloem (Koike et al. 2004; Ishimaru et al. 2010). OsYSL6 is responsible for transporting Mn–NA and preventing Mn toxicity (Sasaki et al. 2011). Recent research demonstrated that OsYSL9 functions in transferring the Fe(III)–DMA and Fe(II)–NA from endosperm to embryo in developing grains (Senoura et al. 2017). OsYSL16 not only transports Fe(III)–DMA in the vasculature, but also plays an essential role in node phloem for Cu–NA delivery (Kakei et al. 2012; Zheng et al. 2012). A high mRNA abundance of *OsYSL18* with transport activity for Fe(III)–DMA is detected in reproductive tissues (especially in pollen and pollen tubes) and laminar joints in phloem, indicating its function in Fe supply for reproduction (Aoyama et al. 2009). In barley, *HvYS2* expresses in the endodermis and possesses a wide range of transport substrates, including Fe(III)/Zn/Mn/Cu/Cobalt (Co)/Ni–DMA and Fe(II)–NA (Araki et al. 2011). HvYSL5 is a vesicle-located protein involved in the transient storage of Fe, and its mRNA expression follows the rhythm of MA secretion. However, the phenotypes of yeast mutants harboring HvYSL5 expression vector cannot be complemented with Fe(III)–DMA or Fe(II)–NA (Zheng et al. 2011). Some *YSL* genes from cash crops have been cloned in recent years. For example, eight YSL transporters were found in pear (*Pyrus bretschneideri*), and *PbrYSL4* has a high transcript level in all tissues, particularly during pollen tube growth (Yang et al. 2016).

Although most YSL transporter members are induced by Fe deficiency or overload, some respond to other excess nutrient elements or even heavy metals. To explain the high accumulation rate of heavy metals in hyperaccumulators, certain *YSL* homologous genes were isolated. *TcYSL3, TcYSL5*, and *TcYSL7* were characterized in *Thlaspi caerulescens*, and further research on *TcYSL3* implied a role in Ni–NA translocation (Gendre et al. 2007). In total, 27 members were isolated in *Brassica juncea*. Some were induced by heavy metals (Das et al. 2011), and the overexpression of *BjYSL7* in tobacco enhanced the tolerance for Cd and Ni (Wang et al. 2013). A plasma-located transporter, SnYSL3, is capable of delivering multiple metals chelated with NA (Cd–NA was the first reported) in *Solanum nigrum*, and the expression level is upregulated by Cd stress (Feng et al. 2016).

Miscanthus is an excellent candidate for phytoremediation based on its strong ecological adaptability and heavy metal tolerance (Chung and Kim 2012). *Miscanthus* copes with heavy metals by promoting root metabolism (Kayama 2001) and improving photosynthesis or the antioxidant enzyme system (Ezaki et al. 2008; Zhang et al. 2015). However, the molecular mechanisms of heavy metal resistance remain unclear in *Miscanthus* plants. In our previous study, we found that three *Miscanthus* species showed different tolerance levels to Cd, resulting in different growth and physiological responses (Guo et al. 2016a). *Miscanthus sacchariflorus* accumulates less Cd in both roots and leaves by restricting Cd uptake from roots and displays a high Cd tolerance, while *Miscanthus floridulus* not only absorbs more Cd from roots but also transfers more Cd from roots to shoots (Guo et al. 2016a). According to the transcriptomic analysis of *M. sacchariflorus* under Cd stress using high-throughput RNA-sequencing technology (Guo et al. 2016b), we identified one *YSL* gene, *MsYSL1*, which was significantly upregulated by Cd in *M. sacchariflorus*. To explore the molecular mechanism of *MsYSL1* in *M. sacchariflorus*, the full-length coding sequence was cloned, characterized, and overexpressed in the yeast and *Arabidopsis*. The transgenic lines were used to evaluate the functions of *MsYSL1* against Cd stress. In addition, the expression levels of genes related to NA synthesis, metal translocation, and long-distance transport, and Cd exclusion were measured under Cd stress in *Arabidopsis*. MsYSL1 as an essential transporter for diverse metal–NAs participates in Cd detoxification by

mediating the reallocation of other metal ions. This study was aimed to increase knowledge regarding the molecular functions of *YSLs* in plants.

Materials and methods

Plant materials and growth conditions

Mature seeds of *M. sacchariflorus* were collected in the same place as described in our previous study in 2015 and saved at 4 °C until use (Guo et al. 2016a). The seeds were soaked in deionized water in Petri dishes to germinate at 37 °C for 2 days in the darkness. After germination, the seedlings were transferred to several plastic boxes full of vermiculite in a nutrient solution (pH 5.5) recommended by the International Rice Research Institute to ensure vertical growth (Yoshida et al. 1976). The solution contained the following: 1.43 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 0.51 mM K_2SO_4, 1 mM $CaCl_2$, 1.6 mM $MgSO_4$, 9.5 µM $MnCl_2$, 19 µM H_3BO_3, 0.152 µM $ZnSO_4$, 0.155 µM $CuSO_4$, 0.075 µM $(NH_4)_6Mo_7O_{24}$, and 125 µM $FeNa_2$·EDTA. Seven-day-old seedlings were washed with deionized water for hydroponic culture in new boxes, and the nutrient solution (pH 5.5) was changed weekly. Plants were grown in a greenhouse programmed for a 16 h light (white fluorescent light intensity of 1200 µmol photons $m^{-2}·s^{-1}$)/8 h dark cycle with a daytime temperature of 30 °C and a night temperature of 22 ± 2 °C. The relative humidity was maintained at 60%.

Arabidopsis thaliana (Ecotype: Columbia-0) seeds were sown in peat substrate (Pindstrup, Denmark) (for transformation) and 1/5 Hoagland's medium (for screening and growth test). The 1/5 Hoagland's medium (pH 5.5) contained 1 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.4 mM $MgSO_4$, 0.2 mM $(NH_4)H_2PO_4$, 3 µM HBO_3, 0.5 µM $MnCl_2$, 0.2 µM $CuSO_4$, 0.4 µM $ZnSO_4$, 1 µM $(NH_4)_6Mo_7O_{24}$, and 20 µM $FeNa_2$·EDTA. A growth chamber was used for *Arabidopsis* cultivation and maintained a light/dark cycle of 10/14 h at 24/20 °C and 70% relative humidity.

Nicotiana benthamiana seedlings for MsYSL1–green fluorescent protein (GFP) transient expression were cultivated in vermiculite containing 1/5 Hoagland's solution (pH 5.5) in a growth chamber under the same conditions as for *Arabidopsis*.

Gene cloning

The *MsYSL1* gene-coding sequence was cloned from *M. sacchariflorus* cDNA according to the transcriptomic information from our previously published RNA-seq results (Guo et al. 2016b) with specific primers MsYSL1-F and MsYSL1-R (Supplementary Table 1). Total RNA was extracted using a MiniBEST Plant RNA Extraction Kit (Takara, Shiga, Japan) and was then used for cDNA synthesis with a PrimeScript™ 1st-Strand cDNA Synthesis Kit (Takara).

Reverse transcription and quantitative real-time PCR (qRT-PCR) analysis

To investigate the expression patterns in *M. sacchariflorus*, 6-week-old seedlings were used to detect *MsYSL1* expression levels in roots, stems, and leaves. These seedlings were grown in the different nutrient solutions containing 400 µM Fe, 200 µM Zn, 100 µM Mn, 200 µM Cu, 100 µM Cd, or 100 µM lead for 6 h. Solutions deficient in Fe, Zn, Mn, or Cu were also utilized in a 5-day experiment. For related gene expression analyses in transgenic *Arabidopsis*, 6-week-old seedlings were exposed to 50 µM $CdSO_4$ for 24 h. After treatments, *Miscanthus* (roots, stems and leaves) and *Arabidopsis* (roots) were harvested. The harvest tissues were quickly frozen in liquid nitrogen and stored at − 80 °C for subsequent experiments. For the qRT-PCR analysis, three biological replications were performed.

Total RNA was extracted using a MiniBEST Plant RNA Extraction Kit (Takara), and cDNA was synthesized from 1 µg of RNA using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). The qRT-PCR analysis was conducted on a LightCycler® 480II machine (Roche, Penzberg, Bavaria, Germany) with SYBR® Green I Master PCR mix (Roche) in triplicate. The expression data were normalized using *Actin* in *M. sacchariflorus* and *Actin2*/*UBQ10* in *Arabidopsis*. Primers for qRT-PCR were listed in Supplementary Table 1. Data were analyzed by LightCycler® 480 software v1.5.0 and LinRegPCR v2016.1 software according to a previous report (Aglawe et al. 2012).

Plasmid construction

To construct the overexpression vector, the *MsYSL1* coding sequence was amplified without the stop codon using the gene specific primers MsYSL1OE-F and MsYSL1OE-R into the *Bam*HI/*Xba*I sites of pCAMBIA1300–*GFP* (CaMV35s promoter). The vector was also used for the MsYSL1–GFP fusion protein that was transiently expressed in *N. benthamiana*.

For the yeast complementation assay, the *MsYSL1* expression cassette was cloned using primers MsYSL1YE-F and MsYSL1YE-R and introduced into the yeast expression vector pDR196 digested by *Eco*RI/*Xho*I. The pDR196–*OsYSL15* and pDR196–*OsYSL2* expression vectors were constructed by inserting *OsYSL15* and *OsYSL2* coding sequences, respectively, using primers with the same enzyme cutting sites as *MsYSL1* (OsY15-YE-F/OsY15-YE-R and OsY2-YE-F/OsY2-YE-R, respectively). All primers applied for construction of vectors were given in Supplementary Table 1.

Subcellular localization

The CaMV35s::*MsYSL1–GFP* vector and red fluorescent protein (RFP) marker *pm-rbCD3-1008* that localized to the plasma membrane (Nelson et al. 2007) were introduced into *Agrobacterium tumefaciens* GV3101 and transiently expressed in the leaves of *N. benthamiana* as previously described (Xu et al. 2014). After treatment, the leaves were cut into small pieces, and mesophyll cells were observed through a confocal laser scanning microscope (LSM 710, Carl Zeiss, Jena, Germany) with lasers at 488 and 584 nm.

Yeast complementation assay

The yeast complementation assay was adapted to the following *Saccharomyces cerevisiae* mutant strains: Fe uptake-defective mutant DEY1453 (*MATa can1 his3 leu2 trp1 ura3 fet3-2::HIS3 fet4-1::LEU2*), Zn uptake-defective mutant ZHY3 (*MATa ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2; zrt2::HIS3*), and the wild type BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*). The polyethylene glycol/lithium acetate method was used for the yeast transformation (Gietz and Schiestl 1995), and a synthetic dropout medium (SD-Ura, pH 5.8), containing 0.67% yeast nitrogen base (Sigma-Aldrich, St. Louis, MO, USA), 0.2% appropriate amino acid lacking uracil (DO Supplement-Ura, Takara) and 2% glucose, was used to screen transformants harboring expression vectors.

The empty pDR196 vector, pDR196–*MsYSL1*, pDR196–*OsYSL15*, and pDR196–*OsYSL2* were independently transformed into yeast strains DEY1453, ZHY3, and BY4741. Complementation of the yeast mutant phenotype was tested on the solid SD-Ura medium containing 0.67% yeast nitrogen base (Sigma-Aldrich), 0.2% appropriate amino acid lacking uracil (DO Supplement-Ura, Takara), 2% glucose, and 2% agar at pH 7. NA (TRC, Toronto, Ontario, Canada) was purchased from Toronto Research Chemicals. Before solidification, the fresh metal–NA complex was generated as previously reported (Schaaf et al. 2004), and added to the medium. Positive yeast transformants were developed in liquid SD-Ura medium at 30 °C for ~ 16–18 h until the optical density (OD) was over 1.5 at 600 nm. Then, a specified volume of yeast suspension was transferred into fresh SD-Ura liquid medium to grow for ~ 6–8 h from 0.2 to 1.0 at OD_{600}. The fresh yeast suspension was centrifuged at 3000×*g* for 10 min and suspended in autoclave-sterilized water three times, resulting in final serial dilutions (0.1, 0.01, 0.001 and 0.0001 at OD_{600}) of the yeast culture in water. The diluted solutions were spotted on plates with 20 µM Fe–NA, 10 µM Zn–NA, and 20 µM Cd–NA medium for the Fe, Zn, and Cd uptake assays, respectively. After spotting, plates were inverted and incubated at 30 °C for 4 days.

MsYSL1 overexpressing in *Arabidopsis*

The floral dip method incorporating *A. tumefaciens* GV3101 with the CaMV35s::*MsYSL1–GFP* vector was utilized for overexpressing *MsYSL1* in *Arabidopsis* (Clough and Bent 1998). Positive transgenic seedlings were identified at the genome level by PCR and at the transcript level by semi-quantitative PCR using the respective primers listed in Supplementary Table 1.

Transgenic *Arabidopsis* phenotype and metal content analysis

MsYSL1-overexpressing *Arabidopsis* T_3 seeds were surface-sterilized and sown on plates containing 1/5 Hoagland's solid medium (pH 5.5) in the presence of 50 µM $CdSO_4$ or not for 8 days to screen the Cd tolerance by observing the growth. Six-week-old hydroponic seedlings were cultivated for another 5-days period with 10 µM $CdSO_4$ and then washed with 20 mM Na_2·EDTA and deionized water three times. After wiping off the water, fresh samples were weighed and digested in 5 mL HNO_3 and 1 mL H_2O_2 at 160 °C for metal concentration measurement by inductively coupled plasma mass spectrometry (DRC-e, Perkin-Elmer, Norwalk, CT, USA). The ratio of the metal amount in shoots to the amount in the whole plant was considered the translocation ratio. Three biological replicates were applied in these experiments.

Statistical analysis

Experimental data from at least three individual replicates were shown as the means ± SD. Two-tailed *t*-tests were performed, and significance was determined as $*P < 0.05$ or $**P < 0.01$.

Results

Phylogenetic analysis of MsYSL1

The *MsYSL1* gene, which encodes a polypeptide of 678 amino acids, was obtained from RNA-seq results of *M. sacchariflorus*. A phylogenetic analysis revealed that MsYSL1 belonged to the Group I subfamily that includes some typical Fe uptake transporters, such as ZmYS1/HvYS1/OsYSL15 from *Gramineae* and AtYSL1-3 from *Arabidopsis* (Fig. 1). The MsYSL1 protein shared a 94% identity with ZmYS1 and contained 13 predicted transmembrane domains (Fig. S1). The prediction of transmembrane helices also showed that MsYSL1 possessed an N terminus in the cytosol and C terminus outside the membrane (Fig. S2).

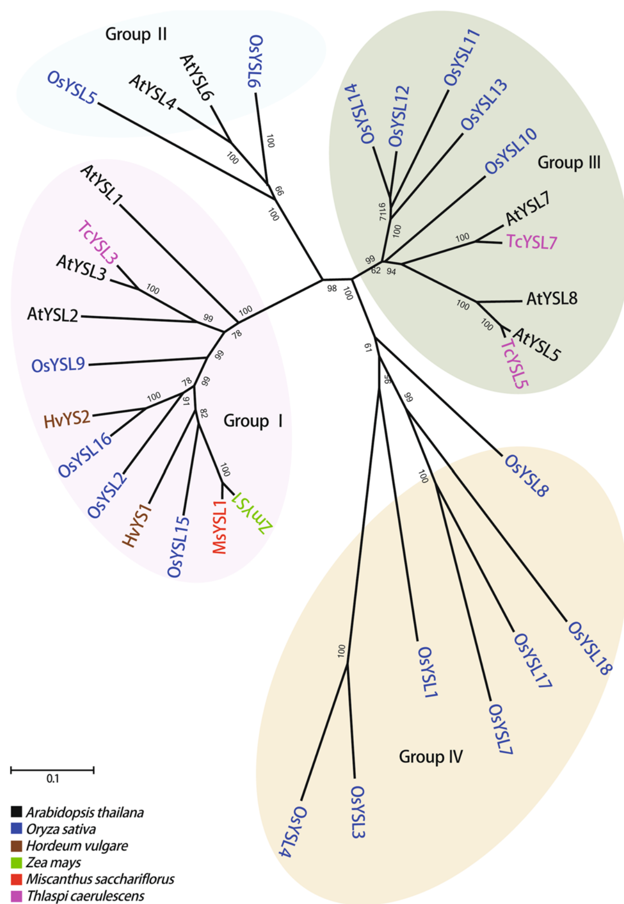

Fig. 1 Polygenetic analysis of MsYSL1. Polygenetic tree of yellow stripe-like proteins. YSL proteins from diverse plants were constructed in MEGA6.0 with neighbor-joining method

Expression patterns and subcellular localization of *MsYSL1*

To understand whether the expression of *MsYSL1* was induced by metals, the transcript levels of the *MsYSL1* gene were detected in the main tissues of *M. sacchariflorus*, suffering from a deficiency or excess of metals, by qRT-PCR with *MsActin* as an internal reference gene for normalization. Under normal conditions, *MsYSL1* had a highest expression level in stems compared with that in roots and leaves (Fig. 2a). In roots, the expression of *MsYSL1* was induced by excess metal treatments (especially by lead, Mn, and Cd) and deficiencies in Fe, Zn, and Cu (Fig. 2b, e). In stems, a deficiency of Fe and an excess of Cu led to an increase of *MsYSL1* at the transcript level (Fig. 2c, f). Either a deficiency or an excess of Fe significantly upregulated the expression of *MsYSL1* in leaves (Fig. 2d, g).

To determine the function, the subcellular localization of MsYSL1 was investigated. The coding sequence, without the stop codon, was introduced into a GFP-fusion vector with a CaMV35S promoter, and then the fusion vector and an RFP marker (*pm-rbCD3-1008*) were transiently expressed in tobacco leaves (Fig. S3a, b, c). The GFP signal was clearly merged with the *pm-rbCD3-1008* marker protein in the plasma and nuclear membranes (Fig. S3d), confirming that MsYSL1 is a plasma membrane protein in *M. sacchariflorus*. Thus, MsYSL1 could be a transporter located in the plasma membrane.

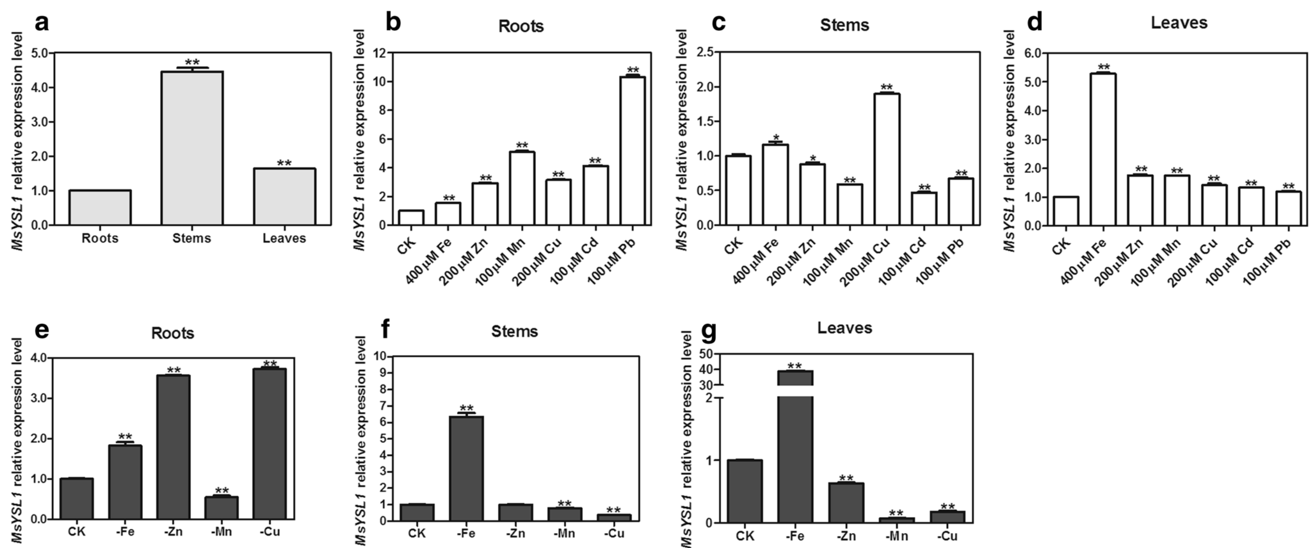

Fig. 2 Expression patterns of *MsYSL1* in *M. sacchariflorus*. **a** Tissue-dependent expression of *MsYSL1*. **b–d** *MsYSL1* expression levels in roots, stems and leaves under excessive metal conditions for 6 h. **e–g** *MsYSL1* expression levels in roots, stems and leaves under various metal deficiency treatments for 5 days. The expression levels were determined by qRT-PCR. *MsActin* was applied as an internal reference and experimental data were indicated as the means ± SD (n = 3)

Yeast transport activity assay of MsYSL1

To investigate its transport activity and substrate specificity, the *MsYSL1* expression vector was introduced into yeast mutants defective in Fe or Zn uptake. The corresponding yeast mutants transformed with pDR196 and pDR196–*OsYSL2*/*15* were used as negative and positive controls, respectively. When exposed to 20 µM $FeSO_4$, all of the Fe uptake-defective mutant DEY1453 yeast lines maintained similar low growth rates. When subjected to 50 µM Fe(III)–citrate, all of the lines grew well and showed no differences. However, when exposed to 20 µM Fe–NA, the lines with *OsYSL2* and *MsYSL1* expression vectors grew better compared with the line containing the empty vector (Fig. 3a). In addition, Zn uptake-defective mutant ZHY3, harboring the *MsYSL1* expression vector, showed better growth versus its counterpart harboring the empty vector in the presence of 10 µM $ZnSO_4$–NA (Fig. 3b). However, there was no difference in growth under the 10 µM $ZnSO_4$ and 100 µM $ZnSO_4$ treatments, suggesting that MsYSL1 only transported chelated $ZnSO_4$–NA. For the wild type yeast BY4741, the lines containing either the pDR196 or *MsYSL1* expression vector showed similar growth in the presence of 20 µM $CdSO_4$–NA and 20 µM $CdSO_4$ (Fig. 3c), indicating that MsYSL1 was not able to transfer Cd–NA or Cd ions.

MsYSL1 improved the tolerance of transgenic *Arabidopsis* to Cd

To identify the physiological and biochemical functions of MsYSL1 in plants, *MsYSL1* was overexpressed using pCAMBIA1300–*GFP* as the overexpression vector under the CaMV35s promoter in *Arabidopsis* (Fig. S4a). A PCR analysis was conducted to ensure that the genetic transformation was successful after obtaining transgenic lines (Fig. S4b). Then, semi-quantitative PCR was used to verify the expression levels in the lines (Fig. S4c). Finally, three lines (Y15, Y21, and Y27) were chosen for further experiments. There was no difference in growth between the wild type and transgenic plants (Fig. 4a, c) under normal cultivation, while the root lengths of the transgenic lines were approximately two-fold longer than those of the wild type after exposure to 50 µM $CdSO_4$ (Fig. 4b, d). Thus, *MsYSL1* played a role in the resistance of *Arabidopsis* to Cd toxicity.

MsYSL1 was involved in metal ion homeostasis

To understand why *MsYSL1* improved the resistance of *Arabidopsis* to Cd toxicity, the concentrations of various metal ions were analyzed and their translocation ratios were calculated. No differences, either in concentrations or in translocation ratios, were found under control conditions (Fig. 5a–c). However the concentrations of Cd, Zn, and Mn

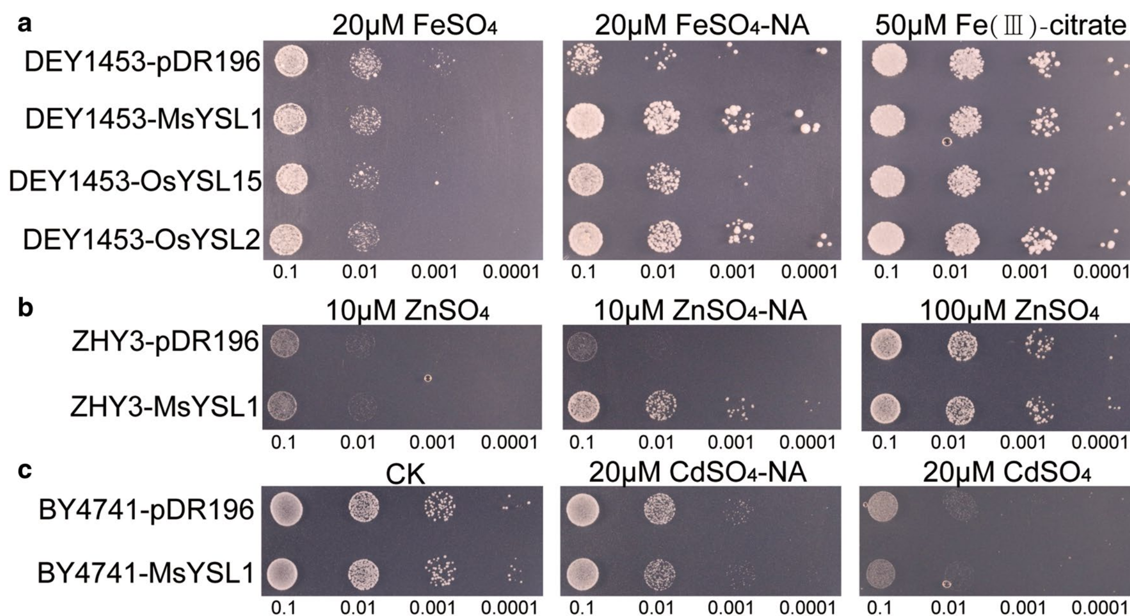

Fig. 3 Functional complementation of mutant yeasts by MsYSL1. **a** DEY1453 yeast expressing empty vector pDR196, vectors with *OsYSL2*/*OsYSL15* (positive control) or *MsYSL1* was grown on SD-Ura (pH 7) containing 20 µM $FeSO_4$, 20 µM $FeSO_4$–NA or 50 µM Fe(III)–citrate. **b** ZHY3 yeast harboring empty vector pDR196 and vector with *MsYSL1* was grown on SD-Ura (pH 7) containing 10 µM $ZnSO_4$, 10 µM $ZnSO_4$–NA or 100 µM $ZnSO_4$. **c** BY4741 yeast with empty vector pDR196 and vector with *MsYSL1* was spotted on SD-Ura (pH 7) containing 0 µM $CdSO_4$, 10 µM $CdSO_4$–NA or 20 µM $CdSO_4$. A serial of culture dilutions (OD_{600} = 0.1, 0.01, 0.001, 0.0001) were spotted on media and been incubated at 30 °C for 4 days

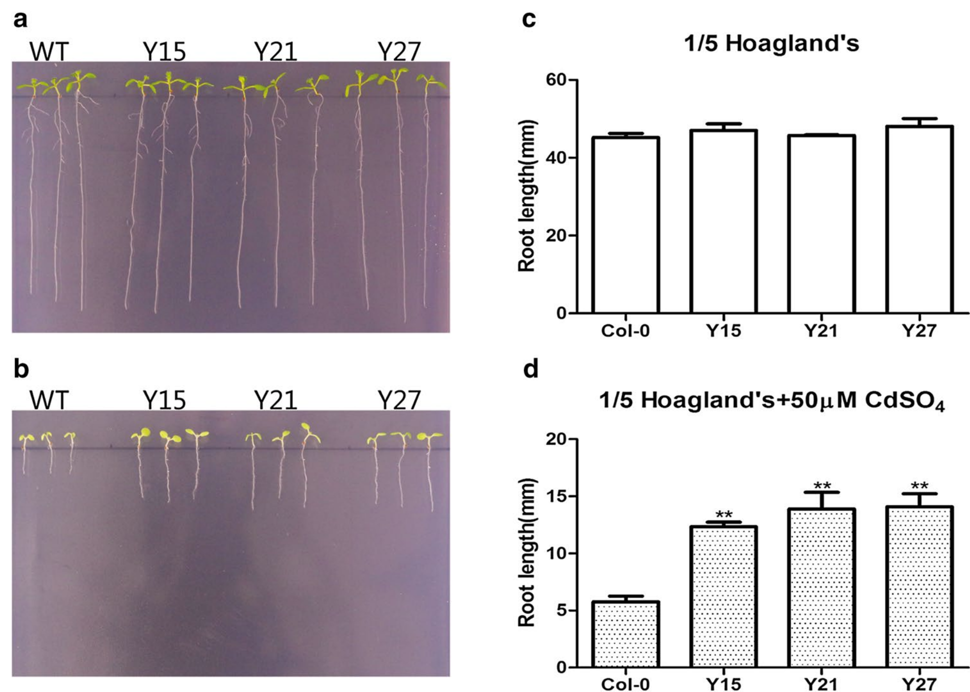

Fig. 4 Phenotype of *MsYSL1*-overexpressing lines under Cd stress. **a** Response of transgenic lines and wild-type line under normal solution. **b** Response of transgenic lines and wild-type line under Cd treatment. **c** Root length under normal condition. **d** Root length under Cd stress. Seedlings were cultivated on plates containing treatment media for 8 days after sowing. Normal solution was 1/5 Hoagland's solution (pH 5.5) and Cd treatment solution was 1/5 Hoagland's solution with 50 µM $CdSO_4$ (pH 5.5). Data were means ± SD (n = 3)

in the roots of *MsYSL1*-overexpressing *Arabidopsis* were lower than those in the roots of wild type (Col-0) under Cd stress, and the concentrations of all the above mentioned metals were not significantly different in the shoots of the lines (Fig. 5d, e). For the translocation ratios, the transgenic lines exhibited obvious transport activities for Cd, Fe, and Mn from roots to shoots (Fig. 5f).

Expression levels of genes involved in Cd uptake and translocation in transgenic *Arabidopsis*

To understand whether the differences of Cd transport activity between transgenic lines and wild type were related to Cd uptake or translocation, we examined the expression levels of genes involved in metal translocation and heavy metal tolerance (Fig. 6). Three homologous *YSL* genes (*YSL1, YSL2*, and *YSL4*) that participated in metal–NA translocation were investigated. Although the expression of *AtYSL1* was not different among the lines, the expression levels of *AtYSL2* and *AtYSL4* in the transgenic lines were higher than those in wild type line. *NAS1* and *NAS2*, which regulate the synthesis of NA, showed a nearly twofold increase in transcript levels in transgenic lines compared to wild type lines. The expression levels of *natural resistance-associated macrophage protein 4* (*NRAMP4*) and *heavy metal transporting ATPase 2/4* (*HMA2/HMA4*), which are related to redistribution from roots to shoots through the vascular bundle, were almost twofold higher than in control lines. The expression level of *AtNRAMP3* was not consistent in the different lines. In addition, two Cd exclusion genes, *detoxification 1* (*DTX1*) and *pleiotropic drug resistance 8* (*PDR8*), were also significantly induced in transgenic plants under Cd stress compared to that in the control.

Discussion

MsYSL1 functioned as a vital metal–NA transporter in vivo

A phylogenetic tree analysis classified the YSL members into four individual subfamilies, and MsYSL1 was categorized as a Group I member along with many other characterized transporters, including primary uptake transporters (ZmYS1, HvYS1, and OsYSL15) and translocation-related transporters (OsYSL2, HvYS2, and AtYSL1/2/3) from different plant species, indicating that MsYSL1 may have similar functions as the abovementioned transporters. Additionally, the subcellular location of MsYSL1 in tobacco was in the plasma membrane (Fig. S3), which was identical to members in the same subfamily (Curie et al. 2009), and also implied a consistent function. MsYSL1 had a high identity with ZmYS1, which has already been identified as a multi-metal transporter for absorbing metal–PS and –NA complexes (Schaaf et al. 2004). Functional complementation assays indicated that MsYSL1 possessed the capacity to transfer Fe(II)–NA and Zn–NA in yeast cells and rescued the mutant yeast phenotype (Fig. 3). MsYSL1 expression rescued the Fe-deficiency phenotype by regulating Fe(II) absorption in DEY1453 yeast. This was similar to its homologous proteins ZmYS1 from maize, OsYSL15 from rice (Lee

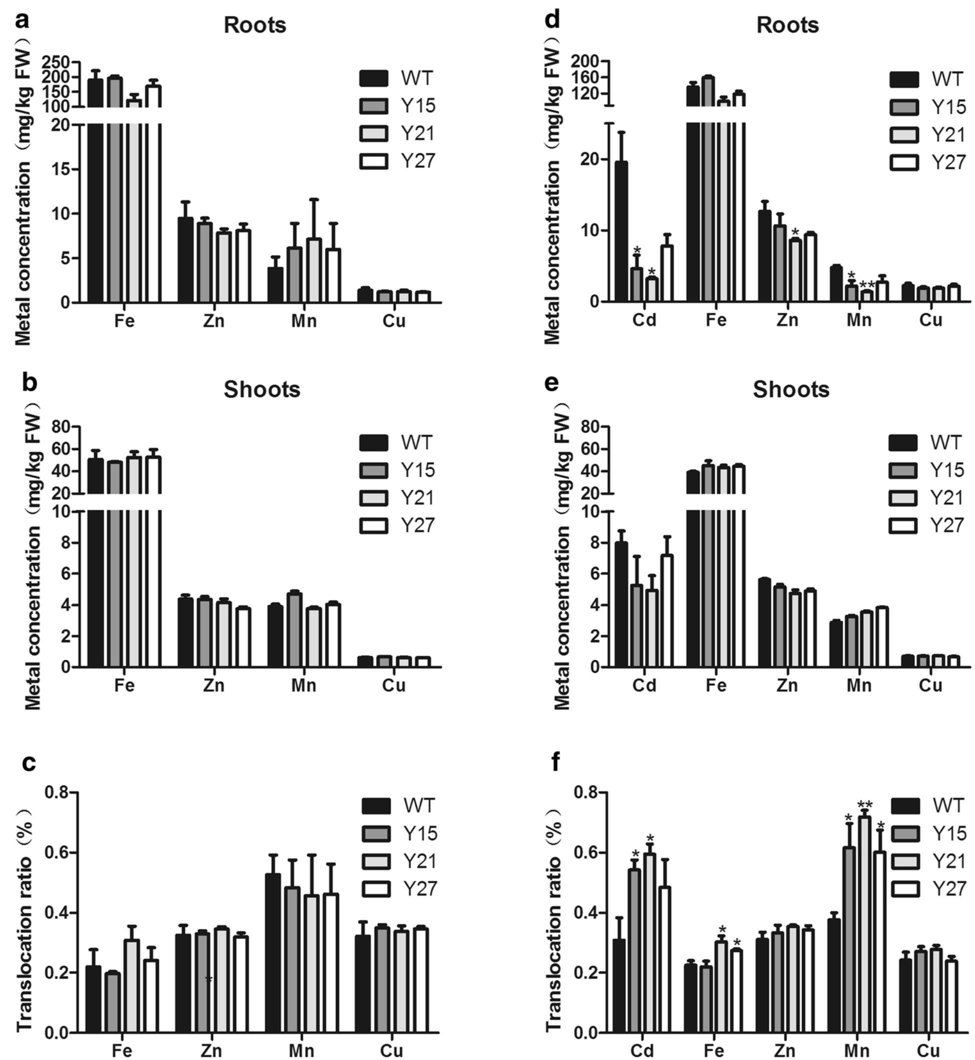

Fig. 5 Metal content determination of *MsYSL1*-overexpressing lines under normal condition and Cd stress. **a** Metal content of roots under normal condition. **b** Metal content of shoots under normal condition. **c** Translocation ratios of multi metal in plants under normal condition. **d** Metal content of roots under Cd stress. **e** Metal content of shoots under Cd stress. **f** Translocation ratios of multiple metals in plants under Cd stress. Hydroponic seedlings were cultivated in 1/5 Hoagland's solution (pH 5.5) for 6 weeks before a 5-day 10 μM $CdSO_4$ treatment. Metal content determination was calculated by fresh weight (FW). Data were means ± SD (n = 3)

et al. 2009), and AtYSL1/2/3 from *Arabidopsis* (DiDonato et al. 2004; Waters et al. 2006). In addition, MsYSL1 was confirmed to possess the ability to transport Zn–NA in the Zn uptake-defective mutant ZHY3, which could also be rescued by an YSL family member from *Solanum nigrum* (SnYSL3) (Feng et al. 2016). Thus, the MsYSL1 transporter may play an important role in regulating the uptake and translocation of Fe and Zn in vivo. Although *MsYSL1* was induced by Cd stress in *M. sacchariflorus*, the overexpression of *MsYSL1* in wild type yeast did not directly mediate the transport of Cd–NA or Cd^{2+}. This implied that *MsYSL1* may indirectly respond to Cd stress by mediating other metal ion homeostasis in *M. sacchariflorus*.

Overexpression of *MsYSL1* enhanced Cd tolerance in *Arabidopsis*

Cd is a non-essential and toxic element to plants, and restricts their normal metabolism, growth, and development (Singh et al. 2016), thus reducing the yield (Cao et al. 2015). Cd is absorbed through some transporters, like divalent cations (Fe, Zn and Mn) or ion channels (Ca and Mg), indicating that a competitive interaction between essential mineral nutrients and Cd occurs in vivo (Lin and Aarts 2012). To further confirm the role of *MsYSL1* in responding to Cd stress, *MsYSL1* was overexpressed in *Arabidopsis*, and the transgenic lines were exposed to a Cd solution. The overexpression of *MsYSL1* in *Arabidopsis* significantly increased its Cd tolerance, resulting in longer root lengths and lower Cd concentrations in roots compared with wild type plants (Figs. 4, 5d). According to the functional complementation experiment of yeast, *MsYSL1* did not transport Cd^{2+} or Cd–NA into yeast cells (Fig. 3c). Therefore, we hypothesized that MsYSL1 mediated Cd tolerance mainly by regulating the redistribution of other metals in transgenic *Arabidopsis* and *M. sacchariflorus*. *OsYSL2* is involved in the reallocation of Fe and Mn from roots to shoots through vascular bundles (Koike et al. 2004). This was in agreement with the effects of Cd stress on the accumulation and

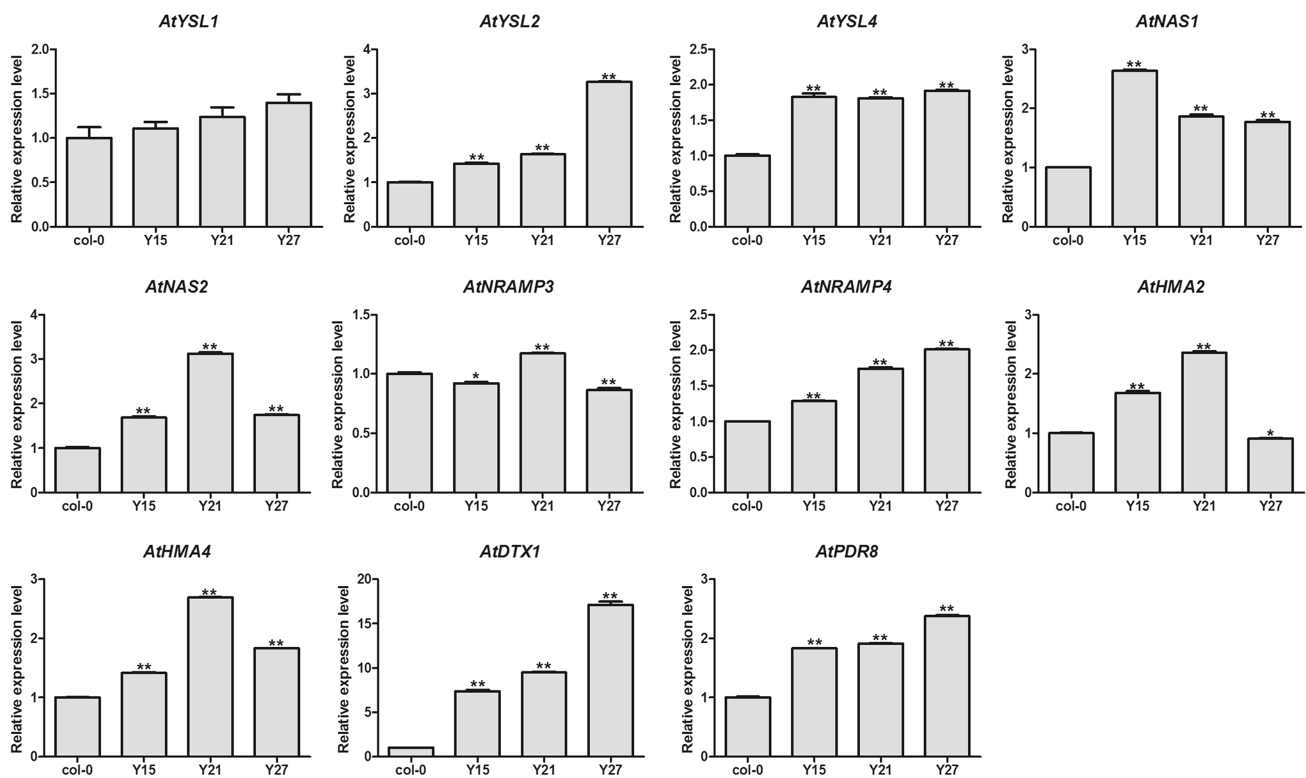

Fig. 6 Expression levels of metal transport and heavy metal tolerance genes in roots of *MsYSL1*-overexpressing lines under Cd stress. Six-week-old hydroponic *Arabidopsis* roots were captured after 24 h Cd stress (50 μM $CdSO_4$). Relative mRNA expression level of each gene was standardized by *AtActin2*/*AtUBQ10*. Data were analyzed according to LinReg method. Data were means ± SD (n = 3)

translocation ratios of other metals, especially Fe and Mn, in the roots of transgenic *Arabidopsis* (Fig. 5d, f). In addition, the highest expression level of *MsYSL1* was found in stems under normal conditions, while its expression was inhibited under Cd stress, further indicating that *MsYSL1* may play an important role in regulating the translocation of metal ions from the root to the shoot in plants.

Cd tolerance was maintained by upregulating metal translocation genes in the *MsYSL1*-overexpressing line

Cd tolerance can be maintained by regulating metal uptake and translocation transporters to mediate metal ion reallocation (Wang et al. 2013; Sasaki et al. 2014; Lin et al. 2016). SnYSL3, which delivers a broad range of metal–NA complexes, increased Cd tolerance in transgenic *Arabidopsis* by decreasing Fe and Mn concentrations in the roots, and increasing the root-to-shoot translocation ratios of Fe and Mn (Feng et al. 2016). *BjYSL7* encodes a plasma-localized metal–NA transporter, and the overexpression of *BjYSL7* in tobacco enhanced Cd and Ni tolerance by increasing their translocation from roots to shoots (Wang et al. 2013). In this study, the overexpression of *MsYSL1* in *Arabidopsis* partly induced the expression of three *AtYSL* genes and two *AtNAS* genes under Cd stress(Fig. 6), indicating that *MsYSL1* participated in the translocation of Fe, Zn, and Mn from roots to shoots in transgenic *Arabidopsis*, thus increasing Cd tolerance (Figs. 3a, b, 5). This was in agreement with that the high expression levels of the *NAS* genes contributed to metal redistribution from roots to shoots (Kim et al. 2005; Klatte et al. 2009), and enhanced Cd resistance (Wu et al. 2012). In addition, in transgenic *Arabidopsis*, the expression profiles of genes involved in Cd uploading in the vasculature, such as *AtHMA2*/*HMA4* and *AtNRAMP4* (Eren and Arguello 2004; Verret et al. 2004; Pottier et al. 2015), were upregulated (Fig. 6). Although the expression level of *AtHMA2* in the Y27 line was lower than that of the wild type, and the mRNA levels of *AtNRAMP3* were not consistent in the transgenic lines, this might be the result of high *AtYSL2* and *AtNRAMP4* expression levels. The overexpression of *AtHMA4*/*AtNRAMP4* decreased the Zn and Cd accumulation in roots (Verret et al. 2004; Pottier et al. 2015). This may also explain why the overexpression of *MsYSL1* in *Arabidopsis* increased Cd, Fe, and Mn translocation ratios under Cd stress (Fig. 5f). In addition, two Cd efflux genes, *AtDTX1* and *AtPDR8* (Li et al. 2002; Kim et al. 2007), were significantly induced by Cd in transgenic *Arabidopsis* (Fig. 6),

indicating that the high Cd tolerance and low Cd accumulation in roots may be partly due to the increase in Cd efflux under Cd stress in transgenic *Arabidopsis*. However, the specific mechanism of the *MsYSL1* overexpression-induced increase in the two Cd efflux gene transcript levels under Cd stress in *Arabidopsis* is not clear and needs to be further investigated in future studies.

Conclusions

In this study, an *YSL1* gene from *M. sacchariflorus* was cloned and characterized. The heterologous expression of *MsYSL1* in yeast indicated that MsYSL1 was a transporter that participated in delivering metal–NA complexes in vivo. The overexpression of *MsYSL1* in *Arabidopsis* enhanced Cd tolerance by decreasing Cd accumulation in roots and increasing Cd translocation from roots to shoots. In addition, the genes related to NA synthesis, metal translocation and long-distance transport, and Cd exclusion were highly induced in transgenic lines under Cd stress, further indicating that *MsYSL1* may play a crucial role in Cd detoxification and internal metal reallocation in *M. sacchariflorus*.

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Author contributions CHM, GHP and JDA conceived and designed the experiments. CHM and ZC performed the experiments. CHM, HYM and HY analyzed the data. CHM, GHP and JDA wrote the paper. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest These authors have declared that no competing interests exist.

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