

***Medicago truncatula* Yellow Stripe-Like7 encodes a peptide transporter participating in symbiotic nitrogen fixation**

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Running Head: Peptide transporter MtYSL7 participates in SNF

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Abstract

Yellow Stripe-Like (YSL) proteins are a family of plant transporters typically involved in transition metal homeostasis. Three of the four YSL clades (I, II, and IV) transport metal complexes with non-proteinogenic amino acid nicotianamine or its derivatives. No such capability has been shown for any member of clade III, which translocate short peptides across the membranes. The connection between clade III YSLs and metal homeostasis could be masked by the functional redundancy of this family. This was tested by studying Clade III YSLs in root nodules where the evolution of the symbiosis may have provided a new role for a single YSL in providing transition metals for symbiotic nitrogen fixation. *MtYSL7* was mainly expressed in nodules. It encodes a plasma membrane protein located in the pericycle and in the nodule cortex. *MtSYL7* is able to transport short peptides into the yeast cytosol. Reducing *MtYSL7* expression levels resulted in diminished nitrogen fixation rates, as well as reduced plant growth under symbiotic and non-symbiotic conditions. *YSL7* mutant nodules accumulated more copper and iron, the later the likely result of increased expression in roots of iron uptake and delivery genes. The data indicates a role of *MtYSL7* in transition metal homeostasis.

Keywords: *Medicago truncatula*, symbiotic nitrogen fixation, peptide transport, YSL

Introduction

Iron, copper, zinc, and other transition metals are essential nutrients for plants (Marschner, 2011). These elements are structural components or cofactors of proteins involved in almost every physiological process. Therefore, obtaining transition metal nutrients from soil and delivering them to hundreds of metalloproteins in different organelles and tissues is essential for plant development. Several metal transport families participate in transition metal allocation (Kobayashi & Nishizawa, 2012; Olsen & Palmgren, 2014; Pilon, 2011). While most of them are conserved in all domains of life, a few, such as the Yellow Stripe-Like (YSL) proteins, are exclusively present in plants (Curie *et al.*, 2008; Kumar *et al.*, 2017), suggesting selective pressures for specialized metal transport systems. YSLs are evolutionarily related to the Oligopeptide Transporter (OPT) family, sharing the capability of transporting amino acid substrates into the cell cytosol (Lubkowitz, 2011). The amino acid substrates of the biochemically characterized YSLs are typically the non-proteinogenic amino acid nicotianamine or nicotianamine-derived molecules (mugineic acids) in complex with transition elements (Aoyama *et al.*, 2009; Schaaf *et al.*, 2004).

The founding member of the family is *Zea mays* YSL. Mutants of this gene presented interveinal chlorosis in leaves (yellow stripes), caused by a deficiency in iron uptake (Beadle, 1929; Curie *et al.*, 2001). ZmYSL1 and its orthologues like OsYSL15 are the main iron uptake system from soil in grasses, participating in what has been named as Strategy II (in contrast to the Strategy I in non-grasses) (Kobayashi & Nishizawa, 2012). Monocots secrete nicotianamine-derived phytosiderophores to the rhizosphere, where they chelate iron and other transition elements (Nozoye *et al.*, 2011; Roberts, Pierson, Panaviene, & Walker, 2004; Schaaf *et al.*, 2004). Subsequently, YSL proteins mediate the uptake of these complexes into the root epidermal cells (Curie *et al.*, 2001). However, this is not the only role of YSLs. Both in monocots and dicots, YSLs facilitate the transport of metal-nicotianamine complexes as part of a more general role in long-distance metal transport and intracellular transport (DiDonato, Roberts, Sanderson, Easley, & Walker, 2004; Waters *et al.*, 2006). For instance, *Arabidopsis thaliana* YSL1 and YSL3 are responsible for iron uptake from the vascular tissues, redistribution from senescent leaves, and delivery to the seed (Waters *et al.*, 2006). More recently, it has been proposed that YSL proteins also participate in the systemic control of iron homeostasis in plants (Kumar *et al.*, 2017).

Based on their amino acid sequence, YSL transporters can be classified into four different groups (Yordem *et al.*, 2011). Group I includes ZmYS1, AtYSL1, and AtYSL3. Group II is formed by a number of YSL proteins that have intracellular localization. Best characterized AtYSL4 and AtYSL6 are located in the vacuoles and/or in chloroplast membranes, and are involved in the mobilization of metal stores (Conte *et al.*, 2013; Divol *et al.*, 2013). YSL Group IV is formed only by monocot proteins, including metal transporting OsYSL8 (Aoyama *et al.*, 2009). While members from these three groups have been linked to transition metal homeostasis, no physiological function has been attributed to YSLs belonging to Group III.

Group III YSLs AtYSL7 and AtYSL8 are able to transport peptides into the cell (Hofstetter, Dudnik, Widmer, & Dudler, 2013). This is used by plant pathogen *Pseudomonas syringae* pv *syringae* to introduce the virulence factor SylA, a tripeptide involved in proteasome inhibition that dysregulates the plant immune response, into the plant cell (Groll *et al.*, 2008). However, it is unlikely that this is the physiological role of Group III YSLs. Considering that the other three YSL groups are involved in transition metal homeostasis (Conte *et al.*, 2013; Curie *et al.*, 2001), we hypothesize that Group III members would also be participating in the same physiological process. However, no metal-related phenotype for any mutant of the members of this clade has been reported in *Arabidopsis*, perhaps as a consequence of the frequent functional redundancy of the YSL family (Divol *et al.*, 2013; Waters *et al.*, 2006). As an alternative, we have considered the model legume *Medicago truncatula* due to its ability to carry out symbiotic nitrogen fixation, a process that heavily relies on metal transport (González-Guerrero, Escudero, Sáez, & Tejada-Jiménez, 2016). This symbiotic process required the neofunctionalization of many genes (De Mita, Streng, Bisseling, & Geurts, 2014), and could have caused the loss of some of the functional redundancy that characterizes the YSL family.

Transition elements are essential for symbiotic nitrogen fixation as cofactors of many of the enzymes participating in this process, including the enzyme central to the conversion of N₂ into NH₄⁺, nitrogenase (Brear, Day, & Smith, 2013; González-Guerrero, Matthiadis, Sáez, & Long, 2014). Considering the high expression levels of many of the genes encoding these metalloenzymes, a substantial part of the metal incorporated into the plant is directed to the root nodules, oftentimes eliciting metal deficiency responses (Terry, Soerensen, Jolley, & Brown, 1991). Consequently, it is expected that dedicated metal transport systems have been adapted from pre-existing ones to ensure proper metal

supply to the newly developed organs. In those cases where functional redundancy might exist, it could imply that only one of the redundant genes acquire a novel role. Here, we take advantage of this possibility to study *M. truncatula* YSL7 (*Medtr3g063490*), a Group III YSL family member with high expression in nodules important for plant growth in general and nitrogen fixation in particular.

MATERIALS AND METHODS

Plant growth conditions

Medicago truncatula *Tnt1*-insertion mutants *ysl7-1* (NF11536) and *ysl7-2* (NF9504) were identified by PCR-based reverse screening of the *Tnt1* DNA pools as described by Cheng et al. (2014). These mutant and *M. truncatula* R108 (Wild Type) seeds were scarified, sterilized and germinated as indicated by Tejada-Jiménez et al. (2015). Seedlings were planted on sterile perlite pots and inoculated with *Sinorhizobium meliloti* 2011 or the same bacterial strain transformed with pHc60-GFP (Cheng & Walker, 1998). Plants were grown in a greenhouse with the conditions 16 h light / 8 h dark at 25 °C / 20 °C, and watered every two days alternating Jenner's solution with water (Brito, Palacios, Hidalgo, Imperial, & Ruíz-Argüeso, 1994). Nodules were collected at 28 days-post-inoculation (dpi). Non-nodulated plants were grown under the same conditions of light and temperature but were watered every two weeks with solutions supplemented with 2 mM NH₄NO₃.

Hairy-root transformations of *M. truncatula* seedlings with *Agrobacterium rhizogenes* ARqual carrying the appropriate binary vector, were performed following the methodology described by (Boisson-Dernier et al., 2001).

Yeast complementation assays

To analyse MtYSL7 peptide uptake capabilities, *MtYSL7* was amplified using the primers listed in Suppl. Table 1 and cloned in the *PstI* and *XhoI* sites of pDR196. Yeast strains $\Delta opt1$ with full disruption of the *YJL212C* (*OPT1*) gene (Euroscarf) (BY4741; *MATa*; *ura3 Δ 0*; *leu2 Δ 0*; *his3 Δ 1*; *met15 Δ 0*; *YJL212c::kanMX4*) and BY4741 (Winston, Dollard, & Ricupero-Hovasse, 1995) were used. Growth experiments were performed using SD media without nitrogen (N) using the different peptides indicated as a nitrogen

source. SD media supplemented with 5 g/l (NH₄)₂SO₄ was used as a growth control media. Ten-fold serial dilutions were spotted (5 µl) onto SD or YPD plates and incubated at 28 °C for 2-3 days.

Quantitative real-time PCR

Gene expression studies were done using quantitative real-time PCR (RT-qPCR) (StepOne plus, Applied Biosystems) using the Power SyBR Green master mix (Applied Biosystems). Primers used are indicated in Suppl. Table 1. RNA levels were normalized by using the *ubiquitin carboxy-terminal hydrolase* gene as internal standard for *M. truncatula* genes (Kakar *et al.*, 2008). RNA isolation and cDNA synthesis were carried out as previously described (Tejada-Jiménez *et al.*, 2015).

GUS staining

Two kilobases upstream of *MtYSL7* start codon were amplified using the primers indicated in Suppl. Table 1, then cloned in pDONR207 (Invitrogen) and transferred to pGWB3 (Nakagawa *et al.*, 2007) using Gateway Cloning technology (Invitrogen). This led to the fusion of the promoter region of *MtYSL7* with the *gus* gene in pGWB3. An *A. rhizogenes* ARqual derived strain containing pGWB3::*MtYSL7_{prom}* vector was used for root transformation of *M. truncatula*. Transformed plants were transferred to sterilized perlite pots and inoculated with *S. meliloti* 2011. GUS activity was determined in 28 dpi plants as described (Vernoud, Journet, & Barker, 1999).

Immunohistochemistry and confocal microscopy

A DNA fragment of the full length *MtYSL7* genomic region and the 2 kb upstream of its start codon was amplified using the primers indicated in Suppl. Table 1 and cloned into the plasmid pGWB13 (Nakagawa *et al.*, 2007) using the Gateway technology (Invitrogen). This plasmid fuses three C-terminal hemagglutinin (HA) epitopes in-frame. Hairy-root transformation was performed as previously described (Vernoud *et al.*, 1999). Transformed plants were transferred to sterilized perlite pots and inoculated with *S. meliloti* 2011 containing a pHc60 plasmid that constitutively expresses GFP. Roots and nodules collected from 28 dpi plants were fixed by overnight incubation in 4 %

paraformaldehyde, 2.5 % sucrose in PBS at 4 °C. After washing in PBS, nodules were included in 6 % agarose and cut in 100 µm sections with a Vibratome 1000 plus (Vibratome). Sections were dehydrated using methanol series (30, 50, 70, 100 % in PBS) for 5 min and then rehydrated. Cell walls were permeabilized with 4 % cellulase in PBS for 1 h at room temperature and with 0.1 % Tween 20 in PBS for 15 min. Sections were blocked with 5 % bovine serum albumin (BSA) in PBS before their incubation with an anti-HA mouse monoclonal antibody (Sigma) for 2 hours at room temperature. After washing, an Alexa594-conjugated anti-mouse rabbit monoclonal antibody (Sigma) was added to the sections for 1 h at room temperature. DNA was stained with DAPI after washing. Images were acquired with a confocal laser-scanning microscope (Leica SP8) using excitation lights at 488 nm for GFP and at 561 nm for Alexa 594.

Acetylene reduction assay

Nitrogenase activity was measured by the acetylene reduction assay (Hardy, Holsten, Jackson, & Burns, 1968). Nitrogen fixation was assayed in mutants and control plants at 28 dpi in 30 ml vials fitted with rubber stoppers. Each vial contained four or five pooled transformed plants. Three ml of air inside of the vial was replaced with 3 ml of acetylene. Tubes were incubated at room temperature for 30 min. Gas samples (0.5 ml) were analyzed in a Shimadzu GC-8A gas chromatograph fitted with a Porapak N column. The amount of ethylene produced was determined by measuring the height of the ethylene peak relative to background. Each point consists of two vials each. After measurements, nodules were recovered from roots to measure their weight.

Metal content determination

Iron content was determined in shoots, roots, and nodules 28 dpi using Atomic Absorption Spectroscopy (AAS) or Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Plant tissues for AAS were weighted and mineralized in 15.6 M HNO₃ (trace metal grade) for 1 h at 80 °C and overnight at 20 °C. Digestions were completed with 2 M H₂O₂. Samples were diluted in 300 mM HNO₃ prior to measurements. Element analyses were performed in an AAnalyst 800 (Perkin Elmer), equipped with a graphite furnace. All samples were measured in duplicate. ICP-MS analyses were carried out at

the Unit of Metal Analysis in the Scientific and Technology Centre of Universidad de Barcelona (Spain).

Bioinformatics

To identify *M. truncatula* YSL family members, BLASTN and BLASTX searches were carried out in the *M. truncatula* Genome Project site (<http://www.jcvi.org/medicago/index.php>). Protein sequences for tree construction were obtained from Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>), Uniprot (<http://www.uniprot.org/blast>) and NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>): *M. truncatula* MtYSL1 (Medtr1g077840); MtYSL2 (Medtr1g007540); MtYSL3 (Medtr3g092090); MtYSL4 (Medtr1g007580); MtYSL5 (Medtr6g077870); MtYSL6 (Medtr7g028250); MtYSL7 (Medtr3g063490), MtYSL8 (Medtr5g091600) and MtYSL9 (Medtr3g063520); *Arabidopsis thaliana* AtYSL1 (At4g24120), AtYSL2 (At5g24380), AtYSL3 (At5g53550), AtYSL4 (At5g41000), AtYSL5 (At3g17650), AtYSL6 (At3g27020), AtYSL7 (At1g65730), AtYSL8 (At1g48370); *Oryza sativa* OsYSL1 (Os01g0238700), OsYSL3 (Os05g0251900), OsYSL4 (Os05g0252000); OsYSL5 (Os04g0390600), OsYSL6 (Os04g0390500); OsYSL7 (Os02g0116300), OsYSL8 (Os02g0116400), OsYSL10 (Os04g0674600), OsYSL12 (Os04g0524600), OsYSL13 (Os04g0524500), OsYSL14 (Os02g0633300); OsYSL15 (Os02g0650300), OsYSL16 (Os04g0542800); OsYSL17 (Os08g0280300), OsYSL18 (Os01g0829900); *Zea mays* ZmYSL1 (Zm00001d017429), ZmYSL2 (Zm00001d025977), ZmYSL6 (Zm00001d003941), ZmYSL11 (Zm00001d025888), ZmYSL12 (Zm00001d025887), ZmYSL14A (Zm00001d051193), ZmYSL17 (Zm00001d054042); *Brachypodium distachyon* BdYSL1A (BRADI_3g50267), BdYSL1B (BRADI_3g50263), BdYSL2 (BRADI_3g50260), BdYSL3 (BRADI_5g17230), BdYSL9 (BRADI_5g17210); BdYSL10 (BRADI_2g5395), BdYSL11 (BRADI_5g16190), BdYSL12 (BRADI_5g16170), BdYSL13 (BRADI_5g16160) and *Glycine max* GmYSL7 (GLYMA_11G203400), GLYMA_09G164500; GLYMA_16G212900.

Trees were constructed from a ClustalW multiple alignment of the sequences (<http://www.ebi.ac.uk/Tools/msa/clustalw2>), then analyzed by MEGA7 (S. Kumar, Stecher, & Tamura, 2016) using a Neighbour-Joining algorithm with bootstrapping (1,000 iterations). Unrooted trees were visualized with FigTree

(<http://tree.bio.ed.ac.uk/software/figtree>).

Statistical tests

Data were analyzed with Mann-Whitney test to calculate statistical significance of observed differences. Test results with *p*-values lower than 0.05 were considered as statistically significant.

Results

MtYSL7 is expressed in roots and in nodules

The *M. truncatula* genome contains nine *YSL* genes (*MtYSL1-MtYSL9*). Sequence comparison of the encoded proteins with known *YSL* proteins from monocots and dicots, showed that *M. truncatula* *YSLs* are distributed in the same groups as any other dicot (Fig. 1A). Four of them (*MtYSL1-4*) belong to Group I, one (*MtYSL6*) is clustered in group II, and four (*MtYSL5, 7, 8, and 9*) are in Group III. *MtYSL7* is very similar to *AtYSL7* and *GmYSL7*, and likely resulted from a duplication event with *MtYSL8* or *MtYSL9*. Over 89% identity is shared among *MtYSL7*, *MtYSL8*, and *MtYSL9*.

MtYSL7 was the only gene among the four members of the Group III *M. truncatula* *YSLs* to have maximum expression in nodules (Fig. 1B, Suppl. Fig. 1). Transcripts of this gene was specifically detected in roots and nodules, with no significant transcription in shoots. In spite of the high degree of similarity to *MtYSL7*, *MtYSL8* and *MtYSL9* were not expressed in nodules. In fact, no *MtYSL8* or *MtYSL9* transcripts were found in any organs at the time points analysed (Suppl. Fig. 1). In contrast, *MtYSL5* was expressed in all organs tested, with the lowest relative expression detected in nodules.

MtYSL7 is a peptide transporter

YSL transporters have been typically associated with the transport of transition metal complexes with non-proteogenic amino acid nicotianamine or its derivatives (Schaaf *et al.*, 2004). To test whether *MtYSL7* was able to transport transition elements free or complexed to nicotianamine, complementation assays of *S. cerevisiae* metal transport mutants were carried out. For this, the strains *ctr1* (deficient in copper uptake), *zrt1zrt2*

(deficient in zinc uptake), and *fet3fet4* (deficient in iron uptake) were used (Askwith *et al.*, 1994; Dancis *et al.*, 1994; Dix, Bridgham, Broderius, Byersdorfer, & Eide, 1994; Zhao & Eide, 1996). These strains were co-transformed with a plasmid expressing a β -estradiol-dependent transactivator of *Gal* promoters, and a plasmid containing *MtYSL7* coding sequence regulated by a *Gal* promoter. Drop tests were carried out using serial dilutions of the cultures. In the case of iron, both the Fe^{2+} and the Fe^{3+} forms were tested. As shown in Suppl. Fig. 2, there was no restoration of the wild type growth in any of the conditions tested, unlike what has been reported for other metal-nicotianamine transporting YSLs in similar assays (Waters *et al.*, 2006).

Alternatively, it can be hypothesized that the substrate of *MtYSL7* is a peptide and not a metal-NA complex. YSL and OPT proteins are closely related (Lubkowitz, 2011), and the *Arabidopsis* *AtYSL7* protein transports a peptide (Hofstetter *et al.*, 2013). To test this possibility, different peptides were provided as the sole nitrogen source to an *S. cerevisiae* strain lacking an oligopeptide transporter (*opt1*) (Osawa, Stacey, & Gassmann, 2006) transformed with a plasmid containing *MtYSL7* (Fig. 2). *MtYSL7*-expressing yeasts were able to grow when four to six amino acid peptides were provided. Some limited growth was also observed when a 12 amino acid peptide was used as substrate, but not when one of 10 was provided. *MtYSL7* did not appear to transport the tripeptide glutathione when expressed in the yeast glutathione transport mutant *hgt* (Suppl. Fig. 3). In addition, no evidence for transport of iron-regulatory peptide IMA (Grillet, Lan, Li, Mokkaapati, & Schmidt, 2018) was observed (Suppl. Fig. 4).

MtYSL7 is located in the plasma membrane of root pericycle and nodule cortical cells

To determine the tissue localization of the expression of *MtYSL7*, *M. truncatula* seedlings were transformed with a construct fusing the 2 kb region upstream of *MtYSL7* to the β -glucuronidase gene (*gus*). GUS activity was localized in 28 dpi plants based on the blue stain resulting from using X-Gluc as a substrate (Fig. 3). As indicated by the RT-qPCR studies, *MtYSL7* was expressed in roots and nodules, with the signal more intense in nodules (Fig. 3A). In nodules, *MtYSL7* expression was located in the cortical region of the nodule throughout its length (Fig. 3B, C), with no expression observed in the nodule core, regardless of the developmental zone of the nodule. In roots, GUS activity was

detected in the perivascular region, in a zone that contains the pericycle surrounding the vessels (Fig. 3D).

Immunolocalization of a C-terminal (HA)₃-tagged MtYSL7 regulated by its own promoter supported the reporter gene studies (Fig. 4). The signal from the Alexa 594-conjugated antibody used to localize MtYSL7-HA was observed in the periphery of the nodule, in the cortical area, both in the perivascular and intervascular areas (Fig. 4A-F). At a higher magnification, the perivascular distribution of MtYSL7-HA seemed to be confined to the endodermal layer (Fig. 4G-I, Suppl. Fig. 5), mostly to the periphery of the cell. In roots MtYSL7-HA was observed in the pericycle (Fig. 4J-L). The intracellular distribution of MtYSL7-HA was indicative of a plasma membrane association, as also evidenced by its co-localization with a plasma membrane marker when co-transfected into tobacco leaves (Fig. 4M-O). Controls in which no Alexa 594-conjugated antibody was used showed no signal in the measured channels (Suppl. Fig. 6). Furthermore, these antibodies did not exhibit any binding capabilities in nodules not expressing a HA-tagged protein (Castro-Rodríguez et al., 2020)

MtYSL7 mutation affects plant growth and symbiotic nitrogen fixation

To determine the function of MtYSL7, two *M. truncatula Tnt1* insertional mutants were obtained from the Noble Research Institute (Tadege *et al.*, 2008). NF11536 (*ysl7-1*) is a knock-down line with the transposon inserted in its first intron (nucleotide +1118) (Fig. 5A). The *Tnt1* insertion in NF9504 (*ysl7-2*) is in the first exon in nucleotide +315, that reduces *MtYSL7* expression below our detection limit. Regardless of the extent to which expression is reduced, when uninoculated and grown with ammonium nitrate in the medium, both *MtYSL7* mutant alleles had reduced growth compared to wild type plants (Fig. 5B, C). The growth phenotype, particularly in roots, was restored in the knock-out *ysl7-2* mutant allele when a wild type copy of *MtYSL7-HA* expressed under the control of its own promoter was reintroduced (Suppl. Fig. 7). In addition, although it was not evident from visual examination of the plants, both mutant alleles had a slight but significant decrease in chlorophyll content (Fig 5D). In general, no significant differences in iron, copper, or zinc concentrations were observed, the one exception being copper levels in shoots of *ysl7-2* (Fig. E-G).

Similar growth differences were observed when the only source of nitrogen would be provided by *S. meliloti* (Fig. 6). Plant growth was reduced in *MtYSL7* mutants, to a higher degree in the knock-out line than the knock-down line (Fig. 6A, B). These plants developed nodules that had no evident morphological differences from wild type (Fig. 6C). Nitrogenase activity was affected in both *Tnt1* lines, with around 40% of the activity of controls (Fig. 6D). These phenotypes were restored in *ysl7-2* when a wild type copy of *MtYSL7-HA* expressed by its native promoter was reintroduced (Suppl. Fig. 8). In addition, *ysl7-2* nodules had a significantly higher iron and copper content in nodules than wild type (Fig. 6E, F), while no differences were observed for zinc (Fig. 6 G). Increased iron concentrations might be the result of the increased expression in roots of iron uptake and delivery systems, such as ferredoxinase FRO1 and iron transporter MtNRAMP1 (Fig. 7). This induction was much higher in the knock-out mutant *ysl7-2* than in the knock-down *ysl7-1*. However, the expression levels of these genes were not affected under non-symbiotic conditions (Suppl. Fig. 9). The increased iron content in *ysl7-2* nodules did not result in altered iron distribution in nodules (Suppl. Fig. 10). Knock-down *ysl7-1* showed a similar, although non-significant metal accumulation trend, the likely consequence of the remaining *MtYSL7* expression (Fig 6E-G). In spite of the altered iron and copper homeostasis in nodules, reducing or increasing iron or copper content in the nutrient solution did not have any significant effect on plant growth or nitrogenase activity (Suppl. Fig. 11, Suppl. Fig. 12).

Discussion

YSLs play a role in transition metal uptake from soil, its distribution from source to sink tissues, as well as in intracellular metal remobilization (Conte *et al.*, 2013; Curie *et al.*, 2001; Waters *et al.*, 2006). More recently, there is evidence of YSL proteins participating in long-distance signalling of the nutritional status of iron (Kumar *et al.*, 2017). These functions are carried out by members of three of the four known YSL groups (Yordem *et al.*, 2011). The remaining one, Group III, has been associated with the transport of the *P. syringae* pv. *syringae* virulence factor SylA (Hofstetter *et al.*, 2013). Although it is unlikely that this is its physiological role, it showcases the ability of proteins from this family to use peptides instead of non-proteogenic amino acids as their substrate. Further reinforcing this role as peptide transporter, expression of *MtYSL7* in yeast restores the uptake capacity of short peptides. However, there seems to be some

specificity to transport by MtYSL7, since a decapeptide was not transported, as neither were tripeptide glutathione or IMA-related peptides consisting of four, five, or seventeen amino acids.

MtYSL7 is expressed in roots and nodules, with the highest levels in the nodules. Immunolocalization of HA-tagged MtYSL7 indicates that it is associated with the plasma membrane of cells in the root pericycle and in the nodule cortex and vasculature, similar to the subcellular localization of AtYSL7 (Hofstetter *et al.*, 2013). In roots, it could be hypothesized that MtYSL7 could be introducing a yet-to-be determined metal complex into the pericycle cell, either from the root cortex or from the root vessels. In this situation, *ysl7* mutant roots should have altered metal levels, but they do not. Additionally, this hypothetical role of MtYSL7 as a metal-complex transporter would mean that in the nodule cortex cells, it would be introducing metals, possibly iron based in the metal content of the *ysl7-2* mutant, into a cell layer with a specific need for them, with a role in preventing oxygen diffusion into the nodule. This would create a separate iron pool from those elements directed for nitrogen-fixing cells, which very likely is in the form of iron-citrate (Kryvoruchko *et al.*, 2018; Tejada-Jiménez *et al.*, 2015). It would also explain the reduction of nitrogenase activity observed, a result of some oxygen leaking to the interior of the nodule, poisoning nitrogenase. However, if this were the case, we should expect that metal fortification of the nutrient solution would partially complement the phenotype, as reported for other nodule metal transport mutants (Gil-Díez *et al.*, 2019; Tejada-Jiménez *et al.*, 2015; Tejada-Jiménez *et al.*, 2017). Moreover, we should also observe metal accumulation in the apoplast around these cells. The fact that this did not happen could be explained either as the absence of an additional low affinity metal uptake system in those cells or that MtYSL7 is playing a different physiological function.

An alternative hypothesis to the role of *MtYSL7* is that its substrate transmits a peptide signal somehow linked to metal homeostasis. Removing the transport capabilities of the signal would alter metal-dependent processes, among others, and could explain the small reduction of chlorophyll content observed and the altered plant growth. In nodules, sensing low-iron levels would lead to reduced nitrogenase assembly, which would also explain the reduced activity observed. Supporting this theory is the observed increase in iron and copper content of *ysl7-2* nodules, perhaps the consequence of lacking a feedback signal of metal sufficiency in nodules. Since no change in iron distribution was observed, it could be speculated that no defects in overall metal transport would result from *MtYSL7*

loss-of-function. Also consistent with this hypothesis is the upregulation of iron uptake systems in roots of nodulated *ysl7* mutants. *MtFRO1* is a ferredoxinase involved in the iron deficiency response in roots, responsible for the conversion of Fe^{3+} to Fe^{2+} prior to its assimilation (Andaluz, Rodríguez-Celma, Abadía, Abadía, & López-Millán, 2009). *MtFRO1* upregulation in roots should be the result of iron deficiency in the plant. However, we have not observed any significant reduction on iron concentration in the plant, but the contrary, with higher iron concentrations in *ysl7-2* nodules. Similarly, *MtNramp1* expression levels were higher in the roots of *ysl7-2* plants than in the controls, while no statistical differences were observed in nodules. This transporter is responsible for iron uptake by the root endodermis and by rhizobia-infected nodule cells (Tejada-Jiménez *et al.*, 2015). The fact that it is upregulated exclusively in roots would be indicative of a signal controlling whole plant metal iron allocation instead of specifically being targeted to nitrogen-fixing cells. Similarly, close orthologue of *MtYSL7* in soybean can functionally complement the *ysl7* phenotype when driven under the *MtYSL7* promoter (Gavrin *et al.*, 2021). GmYSL7 is a nodule-specific protein, located on the symbiosome membrane, with similar peptide transport characteristics, that might synchronize the metabolism/nutrition between the two symbionts, including metal homeostasis genes. This different subcellular localization of YSL7 in Medicago and soybean could reflect alternative ways of controlling nodule functioning in indeterminate and determinate type nodules.

In any case, regardless of the specific cause of the induction of Fe deficiency responses in the root, these results link Group III YSL function to metal homeostasis, a physiological role shared by all the other three YSL groups (Aoyama *et al.*, 2009; Conte *et al.*, 2013; Curie *et al.*, 2001), that could be suggested but had not been demonstrated previous to the research presented here. However, to conclusively prove any of these alternative theories, future work should focus on the identification of the specific substrate of Group III YSLs, for which a new set of peptides need to be identified.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Fig. 1. *Medicago truncatula* YSL7 is a Group III YSL highly expressed in nodules. A) Unrooted tree of the *M. truncatula* YSL transporters, MtYSL1-MtYSL9 (*Medtr1g077840*, *Medtr1g007540*, *Medtr3g092090*, *Medtr1g007580*, *Medtr6g077870*, *Medtr7g028250*, *Medtr3g063490*, *Medtr5g01600*, and *Medtr3g063520*, respectively), and representative plant homologues. Numbers indicate bootstrapping values. B) MtYSL7 expression relative to internal standard gene *ubiquitin carboxyl-terminal hydrolase*. Data are the mean $\pm$ SE of five independent experiments.

Fig. 2. MtYSL7 transports peptides. The yeast strain *opt1*, mutant in the oligopeptide transporter ScOPT1, was transformed with either the empty pDR196 vector, or with pDR196 containing the coding sequence of MtYSL7 or AtOPT4. Serial dilutions (10x) were grown on SD media supplemented with the nitrogen sources indicated. The selected are representative images from at least three independently carried experiments using each a different yeast transformant.

Fig. 3. MtYSL7 is expressed in the root and nodule vasculature and in the nodule cortex. Histochemical staining of the GUS activity in 28 dpi root and nodules expressing the *gus* gene under the regulation of the MtYSL7 promoter. A) General view of both root and nodule. Bar = 1 mm. B) Longitudinal section of a nodule. Bar = 200 μ m. C) Cross section of nodule. Bar = 200 μ m. D) Cross section of a root. Bar = 100 μ m. The images shown are representative images from four separate *M. truncatula* transformations, each producing at least 15 plants, whose roots and nodules were collected and prepared for GUS expression determination.

Fig. 4. MtYSL7 is located in the periphery of nodule cortical cells, in the nodule endodermis and in the root pericycle. A-C) Longitudinal section of a 28 dpi *M. truncatula* nodule expressing MtYSL7 under its own promoter and fused to three HA epitopes (*MtYSL7-HA*). The HA-tag was detected with the help of a secondary Alexa594-conjugated antibody (red, A). Transformed plants were inoculated with a GFP-expressing *S. meliloti* (green) and DNA was stained with DAPI (blue, B). C shows the overlay of all these channels together with the bright field image. Bars = 200 μ m. D-F) Cross section of a 28 dpi *M. truncatula* nodule expressing MtYSL7-HA and detected with an Alexa594-conjugated antibody (red, D). Transformed plants were inoculated with a GFP-expressing *S. meliloti* (green) and DNA was stained with DAPI (blue, E). Panel F shows the overlay

of all these channels together with the bright field image. Bars = 200 μ m. G-I) Detail of a longitudinal section of a vessel from a 28 dpi *M. truncatula* nodule expressing *MtYSL7-HA* and detected with an Alexa594-conjugated antibody (red, G) and inoculated with GFP-expressing *S. meliloti* (green). DNA was stained with DAPI (blue, H). Panel I shows the overlay of all these channels. The arrowheads indicate the position of the Casparian strip. Bars = 50 μ m. J-L) Cross section of a 28 dpi root expressing *MtYSL7-HA* and detected with an Alexa594-conjugated antibody (red). Lignin autofluorescence was used to identify xylem and Casparian strip lignin (green, J). Panel K shows the brightfield image and all three channels are overlaid in panel L. Endodermis (en) and pericycle (pc) are indicated. Bars = 50 μ m. M-O) Colocalization of *MtYSL7*-GFP and *AtPIP2*-CFP in tobacco leaves. Left panel shows the localization of *MtYSL7* fused to GFP (green, M) transiently expressed in tobacco leaf cells. Middle panel shows the localization of plasma membrane marker *AtPIP2* fused to CFP (cyan, N), transiently expressed in the same cells. Panel O is the overlay of the two previous channels together with the bright field image. Bars = 50 μ m. The images shown are representative images from four separate *M. truncatula* transformations, each producing at least 15 plants, whose roots and nodule were collected and prepared for GUS expression determination.

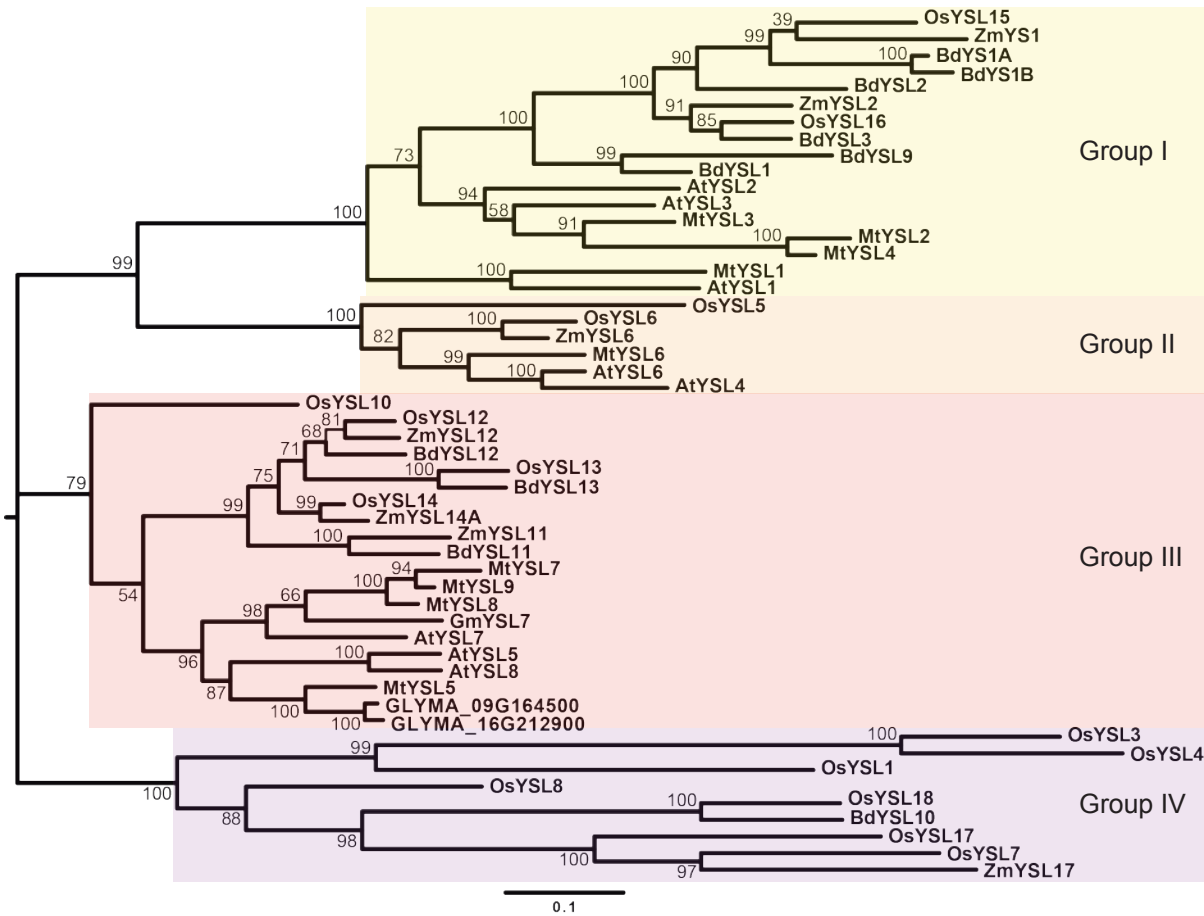
Fig. 5. *MtYSL7* mutation affects plant growth under non-symbiotic conditions. A) Position of the *Tnt1* insertions in *ysl7-1* and *ysl7-2* lines (left side). Right side, *MtYSL7* expression in 28 dpi nodules from WT, *ysl7-1*, and *ysl7-2* plants relative to internal standard gene *ubiquitin carboxyl-terminal hydrolase*. Data are the mean $\pm$ SE of three independent experiments. B). Growth of representative plants. Bar = 3 cm. C) Dry weight of shoots and roots of wild type, *ysl7-1*, and *ysl7-2* plants. Data are the mean $\pm$ SE (n = 5 plants). C) Chlorophyll content of wild type, *ysl7-1*, and *ysl7-2* shoots. Data are the mean $\pm$ SE of three sets of five pooled plants. E) Iron content in roots and shoots of wild type, *ysl7-1*, and *ysl7-2* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. F) Copper content in roots and shoots of wild type, *ysl7-1*, and *ysl7-2* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. G) Zinc content in roots and shoots of wild type, *ysl7-1*, and *ysl7-2* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. * indicates statistical significance ($p < 0.05$).

Fig. 6. *MtYSL7* participates in symbiotic nitrogen fixation. A) Growth of representative wild type, *ysl7-1*, and *ysl7-2* plants. Bar = 3 cm. B) Dry weight of shoots and roots of 28

dpi plants. Data are the mean $\pm$ SE (n = 10-15 plants). C) Detail of representative nodules of 28 dpi wild type, *ysl7-1*, and *ysl7-2* plants. Bars = 1 mm. D) Nitrogenase activity in 28 dpi nodules from wild type, *ysl7-1*, and *ysl7-2* plants. Data are the mean $\pm$ SE measured in duplicate from three sets of five pooled plants. 100 % = 0.28 nmol ethylene h⁻¹ plant⁻¹. E) Iron content in roots, shoots, and nodules of wild type, *ysl7-1*, and *ysl7-2* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. F) Copper content in roots, shoots, and nodules of wild type, *ysl7-1*, and *ysl7-2* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. G) Zinc content in roots, shoots, and nodules of wild type, *ysl7-1*, and *ysl7-2* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. * indicates statistical significance (p < 0.05).

Fig. 7. Mutation of *MtYSL7* alters the expression of iron homeostasis genes in nodulated plants. A) *MtFRO1* expression relative to internal standard gene *ubiquitin carboxyl-terminal hydrolase* in 28 dpi roots and nodules from wild type (WT), *ysl7-1*, and *ysl7-2* plants. Data bars are standardized to wild type values (1) and show the mean $\pm$ SE of three independent experiments. B) *MtNRAMP1* expression relative to internal standard gene *ubiquitin carboxyl-terminal hydrolase* in 28 dpi roots and nodules from wild type (WT) *ysl7-1*, and *ysl7-2* plants. Data bars are standardized to wild type values (1) and show the mean $\pm$ SE of three independent experiments. * indicates statistical significance (p < 0.05).

FIGURE 1
A



B

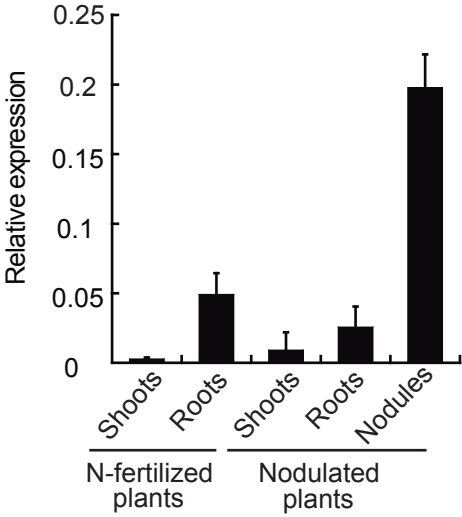


FIGURE 2

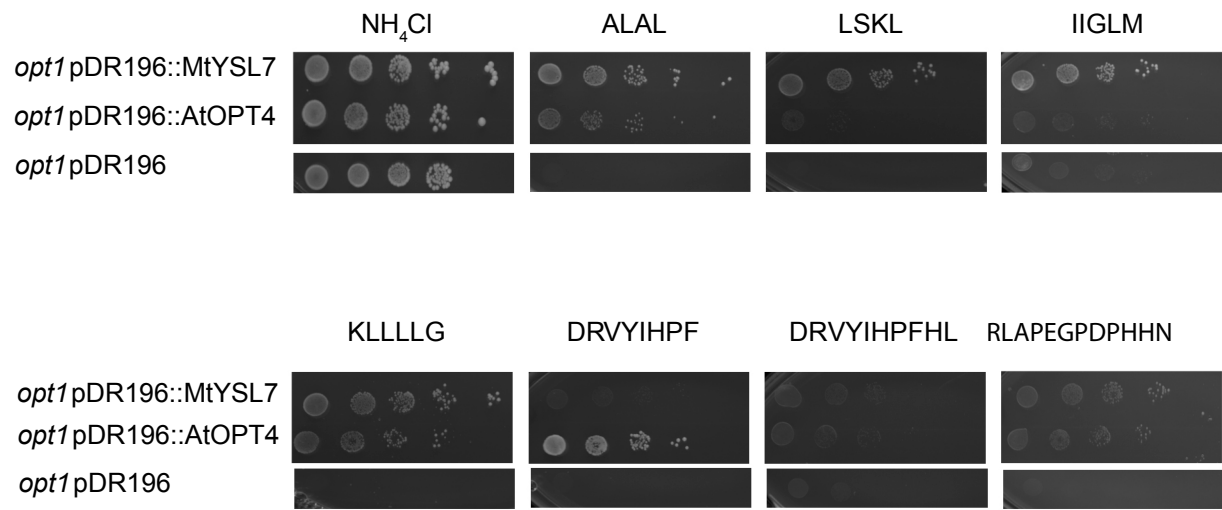


FIGURE 3

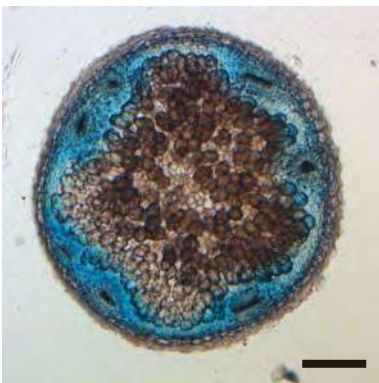
A



B



C



D

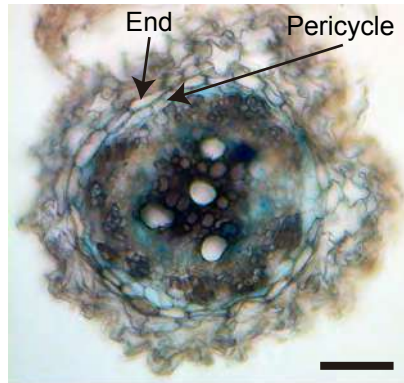


FIGURE 4

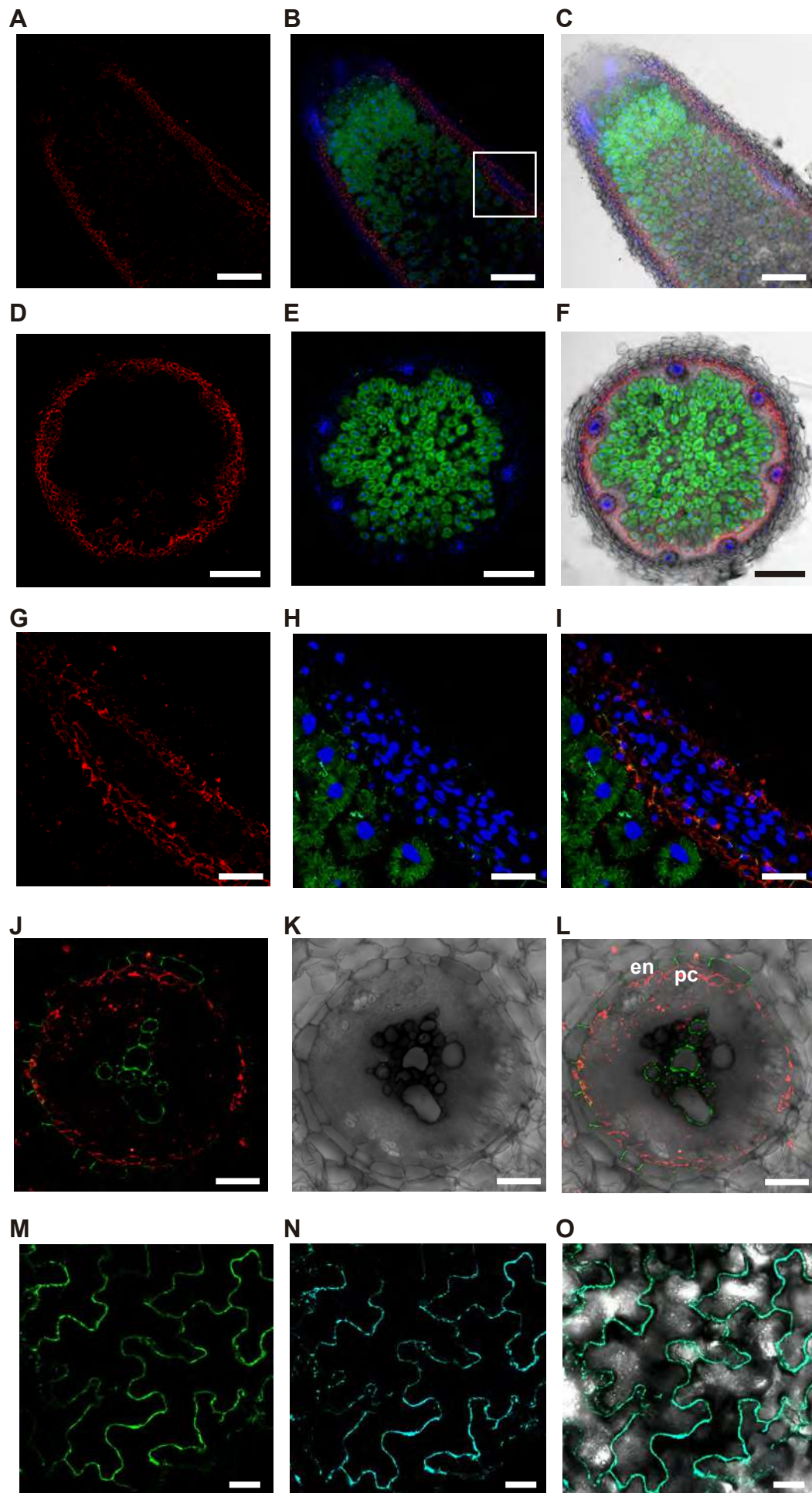


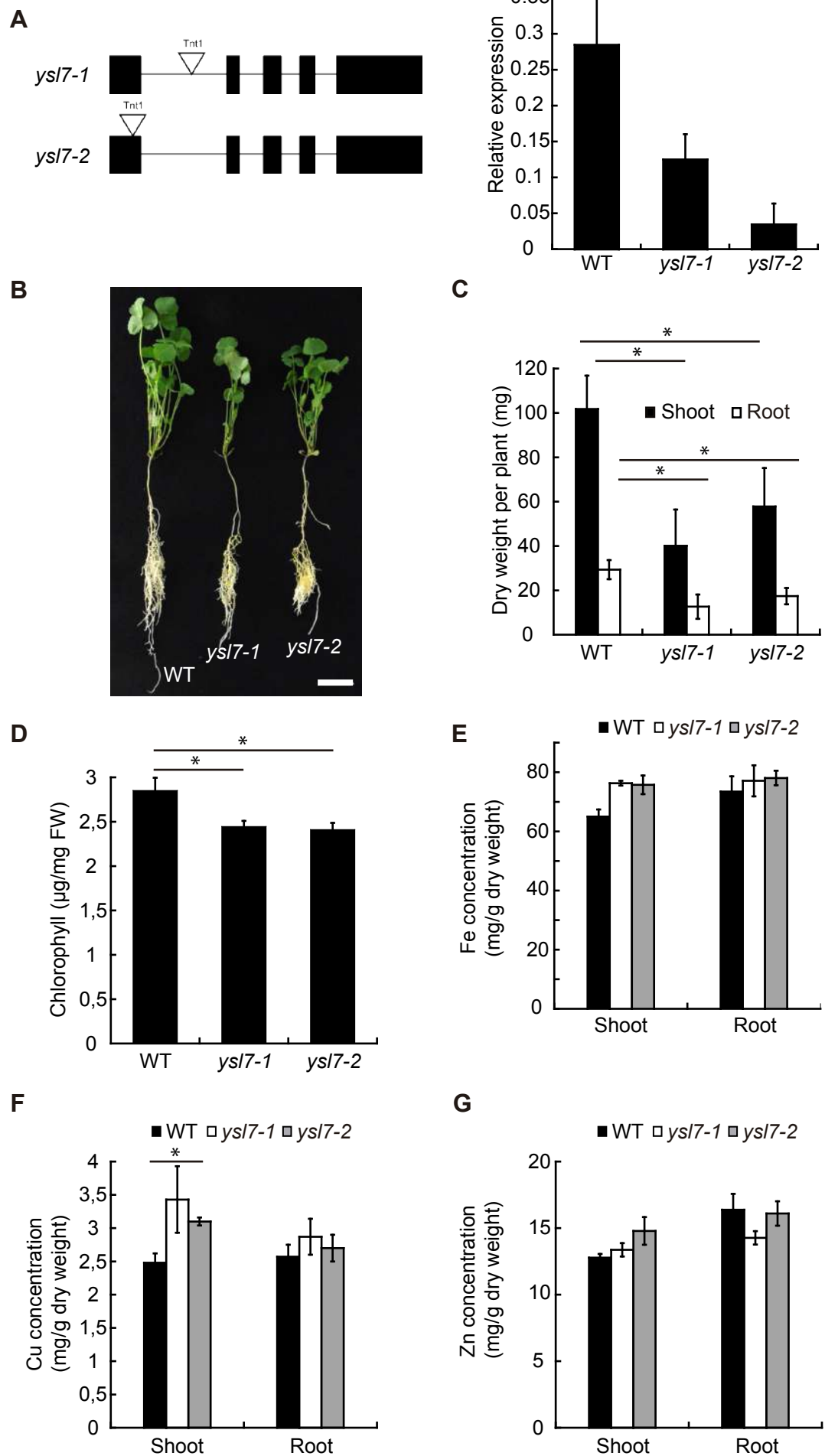
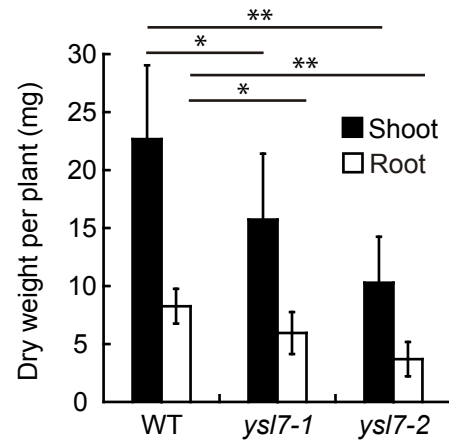
FIGURE 5

FIGURE 6

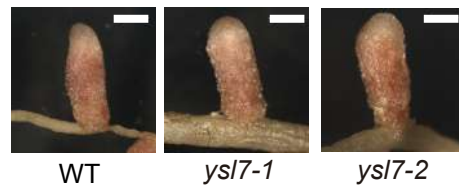
A



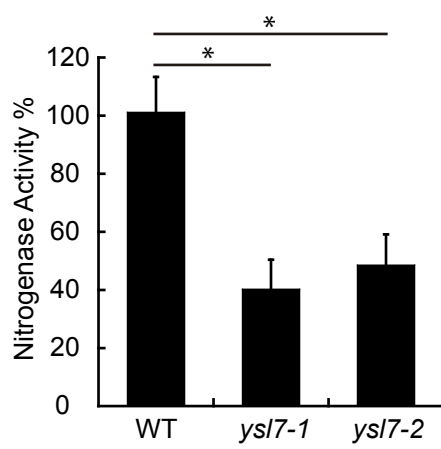
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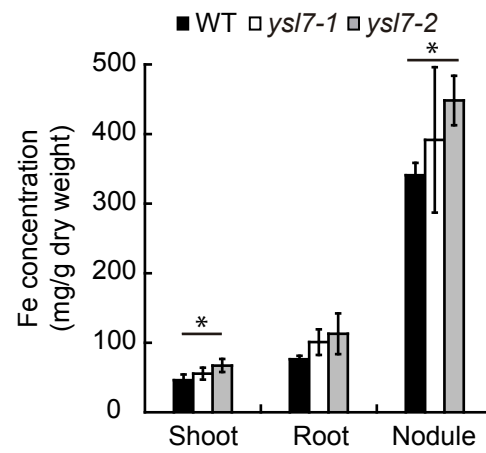
C



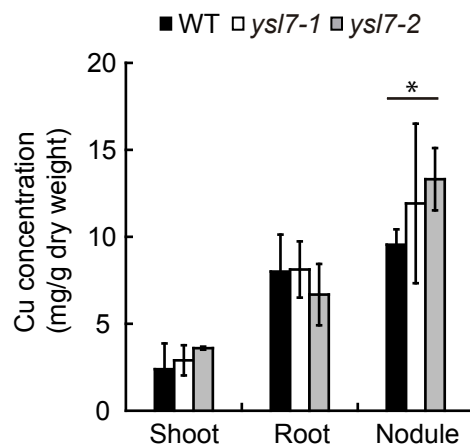
D



E



F



G

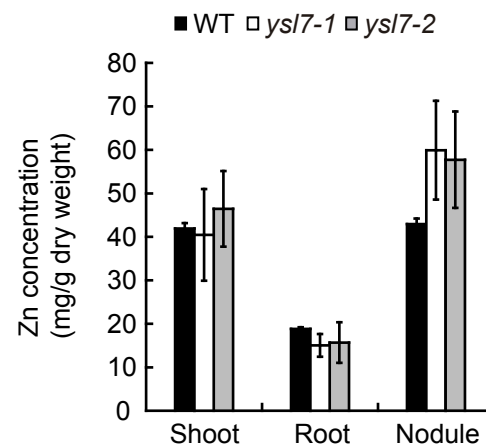
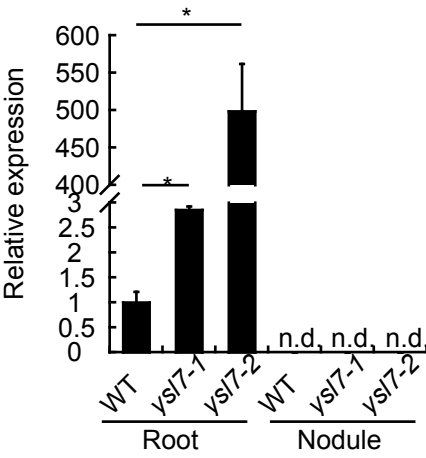
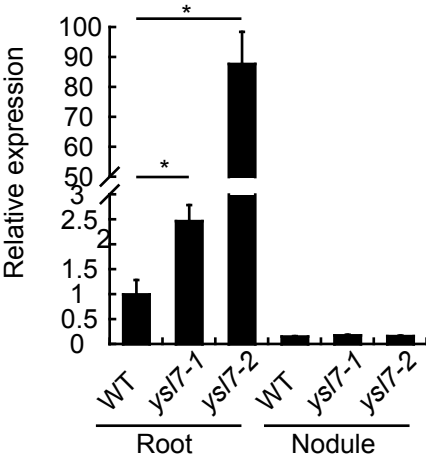


FIGURE 7

A



B



SUPPLEMENTAL MATERIAL AND METHODS

Yeast complementation assays

For metal complementation assays, *MtYSL7*, *MtNramp1*, and *MtZIP6* coding sequences were amplified from cDNA obtained from RNA isolated from 28 dpi *M. truncatula* nodules using the primers listed in Supp. Table 1. Amplicons were cloned in pDONR207 (Invitrogen) and transferred to the destination vector pYES6CT (Invitrogen), using the Gateway Cloning technology (Invitrogen). Empty pYES6CT or pYES6CT::*MtYSL7* were co-transformed with vector pGEV-Trp to drive the expression of the Gal promoter of pYES6CT using β -estradiol (Gao and Pinkham, 2000). Transformations of *Saccharomyces cerevisiae* cells were performed by the lithium acetate method (Schiestl and Gietz, 1989), and selected in SD media by their autotrophy. Iron uptake tests were carried out using the strains DEY1451 (*MATa/MATa ade2/+ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3*; (Dix et al., 1994)), and mutant in iron transport DEY1453 (*ade2/+ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::HIS3/fet3-2::HIS3 fet4-1::LEU2/fet4-1::LEU2*; (Eide et al., 1996)). For DEY1453 strain to grow without the two mutant transporters, the medium has to contain 25 mM sodium citrate (pH 4.2) and 50 μ M FeCl₃. For complementation assays with iron and iron-nicotianamine, plates were made as described by (Chu et al., 2010). To test whether *MtYSL7* was able to restore copper uptake, the parental strain BY4741 (*MATa his3 leu2 met15 ura3*; Yeast Knockout Collection, GE Dharmacon), and mutant in CTR1 copper transporter DY4741 Δ *ctr1* (*MATa his3 leu2 met15 ura3 ctr1::ura3*) were used. For these complementation assays with copper or copper-nicotianamine, glycerol was used as a carbon source in place of dextrose and plates were made following the concentrations indicated by (DiDonato et al., 2004). Finally, the strains DY1457 (*MATa ade6 can1 his3 leu2 lys2 trp1 ura3*) and the mutant in two zinc transporters ZHY3 (*MATa ade6 can1 his3 leu2 lys2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*; (Zhao and Eide, 1996, 1996)) were used to test zinc uptake. For complementation assays with zinc or zinc-nicotianamine, SD-Trp medium was made with zinc-free YNB (yeast nitrogen base) and with 1.4 μ M ZnSO₄ containing or lacking 10 μ M nicotianamine.

To analyse *MtYSL7* IRON-MAN peptide uptake capabilities (Grillet et al., 2018), pDR196 and pDR196::*MtYSL7* were transformed in yeast strains YJL212C (OPT1) (Euroscarf) (BY4741; *MATa*; *ura3 Δ 0*; *leu2 Δ 0*; *his3 Δ 1*; *met15 Δ 0*; *YJL212c::kanMX4*)

and BY4741 (Winston et al., 1995) were used. Growth experiments were performed using SD media without nitrogen (N) using the peptides ENGDDDDDSGYDYAPAA, GDDDD, or APAA, as nitrogen source. SD media supplemented with 5 g/l (NH₄)₂SO₄ was used as a growth control media.

For glutathione transport assays, the parental line ABC733 (BY4741) (*MAT α , his3 leu2 met15 ura3*) (Brachmann et al., 1998) and the mutant in glutathione transport ABC817 (*hgt1*) (*MAT α , his3 leu2 met15 ura3 hgt1::LEU2*) (Bourbouloux et al., 2000) were used. The first of them was transformed with empty pDR196 and the mutant was transformed with empty pDR196 and pDR196::*MtYSL7*. Serial dilutions of each transformant were grown 2-3 days at 30°C in SD media where the unique source of sulfur was 100µM of glutathione (GSH). SD medium with all required amino acids and SD without sulfur and supplemented with methionine were used as growth control media.

Iron localization assays

Roots and nodules from 28 dpi plants were collected, fixed, and included in LR-white resin as previously described (Rodríguez-Haas et al., 2013). Thin sections from these nodules were obtained at Centro Nacional de Microscopía Electrónica, and the Perl-DAB method was used to visualize iron distribution (Roschztardt et al., 2009). Direct observation of sections was performed under a Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany) with an attached digital camera (Leica DFC 420C, Heerburgg, Switzerland). A minimum of three nodules and roots per treatment and three sections per sample were examined.

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SUPPLEMENTAL FIGURE S1

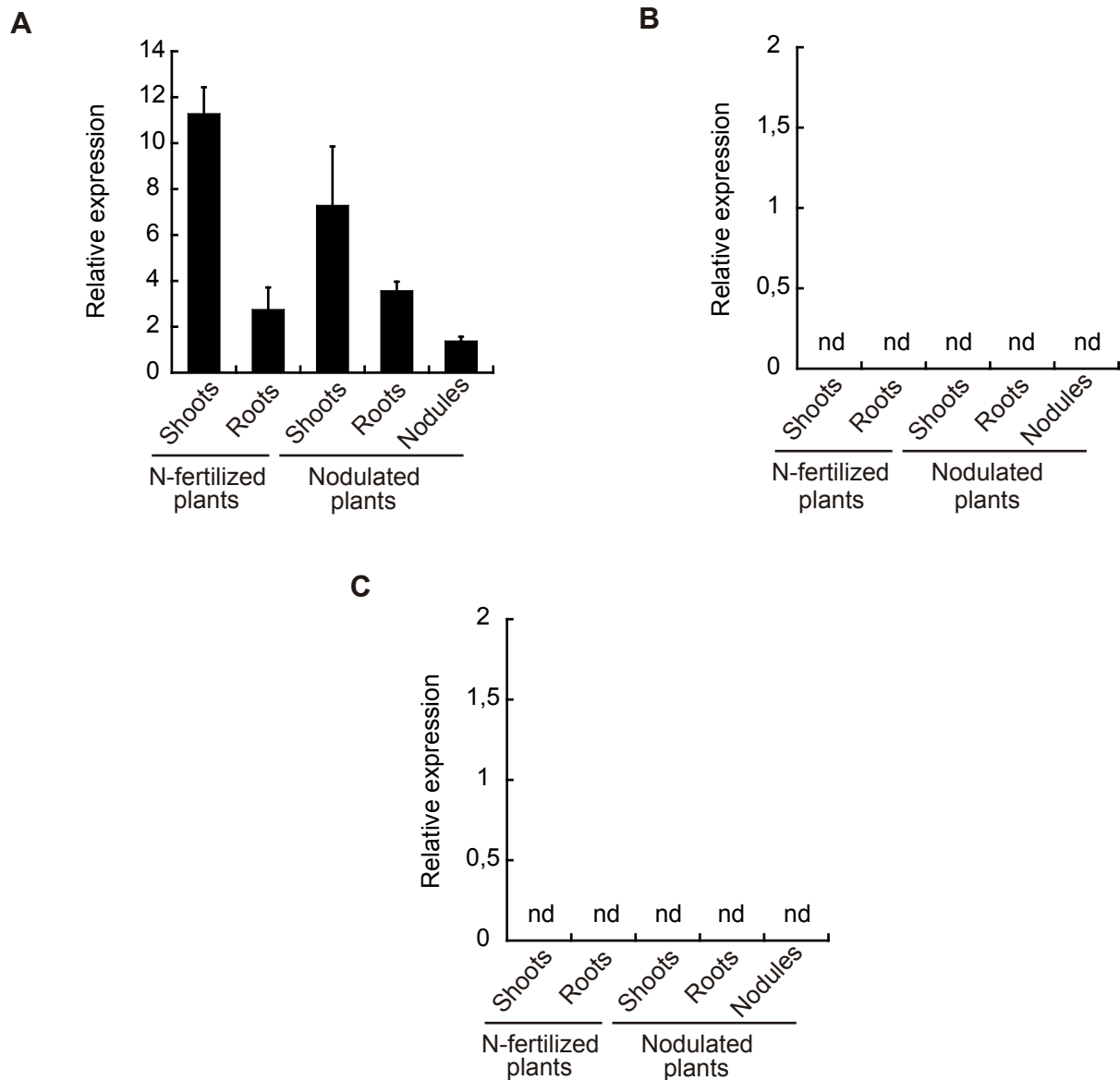


Fig. S1. Expression of *M. truncatula* Group III YSLs. A) *MtYSL5* expression relative to internal standard gene *ubiquitin carboxyl-terminal hydrolase*. Data are the mean $\pm$ SE of five independent experiments. B) *MtYSL8* expression relative to internal standard gene *ubiquitin carboxyl-terminal hydrolase*. Data are the mean $\pm$ SE of five independent experiments. C) *MtYSL9* expression relative to internal standard gene *ubiquitin carboxyl-terminal hydrolase*. Data are the mean $\pm$ SE of five independent experiments. n.d.: not detected.

SUPPLEMENTAL FIGURE S2

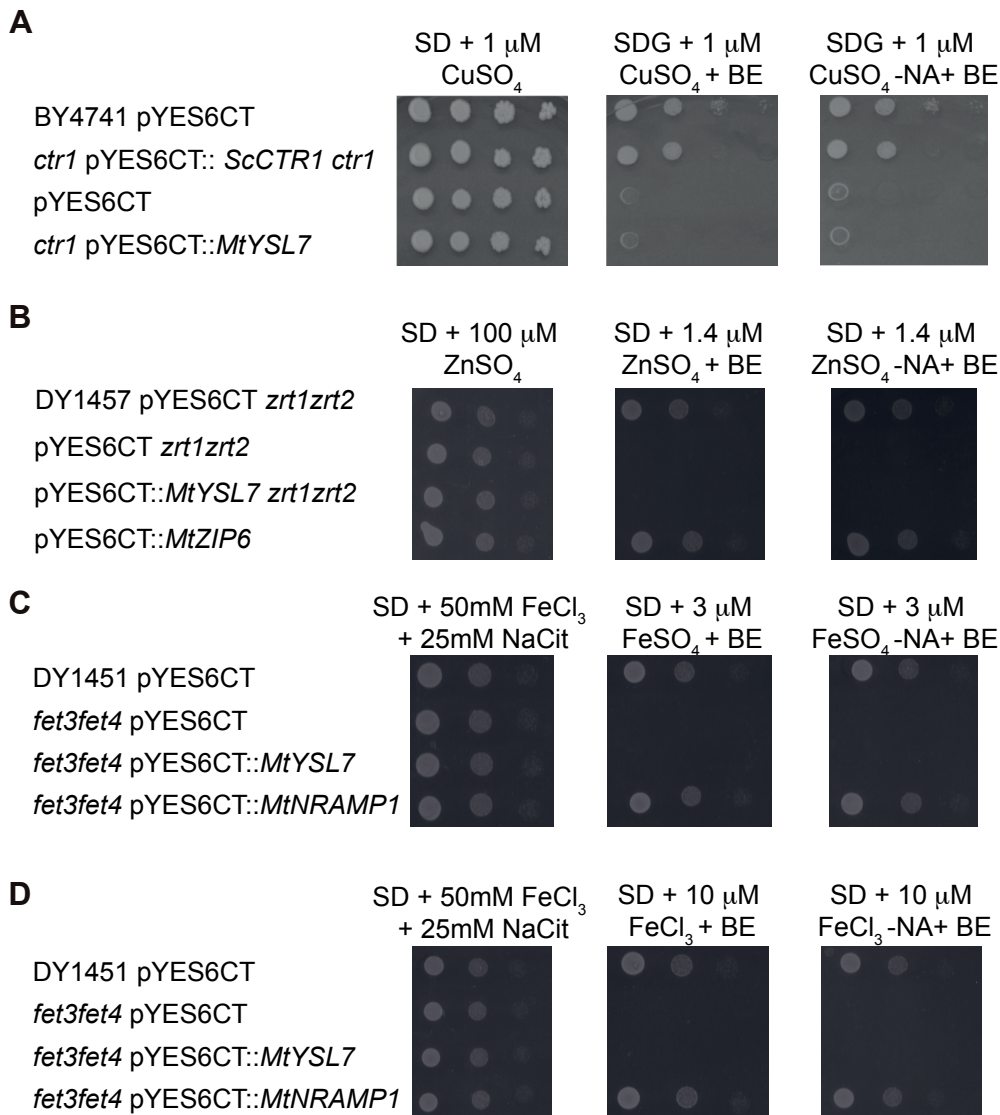


Fig. S2. MtYSL7 metal nicotianamine transport assays in *S. cerevisiae*. Wild type strains were transformed with empty pYES6CT vector, while mutant strains were transformed with empty pYES6CT, pYES6CT containing *MtYSL7* cDNA, or the positive control cDNAs *ScCTR1* (copper), *MtZIP6* (zinc), or *MtNRAMP1* (iron). All of these strains were co-transformed with pGEV vector. A) Copper or copper-nicotianamine (NA) uptake assay in wild type strain BY4741 and *ctr1* copper uptake mutant. Serial dilution were placed in SD or SDG (SD-glycerol) media supplemented with the indicated molecules. *Gal* promoter was induced with the addition of 10 nM of β -estradiol (BE). B) Zinc or zinc-nicotianamine uptake assay in wild type *S. cerevisiae* strain DY14571 and *zrt1/zrt2* zinc uptake double mutant. Serial dilution were placed in SD media supplemented with the indicated molecules. *Gal* promoter was induced with the addition of 10 nM of β -estradiol (BE). C) Iron(II) or Fe²⁺-nicotianamine uptake assay in wild type *S. cerevisiae* strain DY1451 and *fet3/fet4* iron uptake double mutant. Serial dilution were placed in SD media supplemented with the indicated molecules. *Gal* promoter was induced with the addition of 10 nM of β -estradiol (BE). 25 mM sodium citrate (NaCit) was added to the medium to allow growth of *fet3/fet4* mutant strains. D) Iron(III) or Fe³⁺-nicotianamine (NA) uptake assay in wild type *S. cerevisiae* strain DY1451 and *fet3/fet4* iron uptake double mutant. Serial dilution were placed in SD media supplemented with the indicated molecules. *Gal* promoter was induced with the addition of 10 nM of β -estradiol (BE). 25 mM sodium citrate (NaCit) was added to the medium to allow growth of *fet3/fet4* mutant strains. The selected are representative images from at least three independently carried experiments using each a different yeast transformant.

SUPPLEMENTAL FIGURE S3

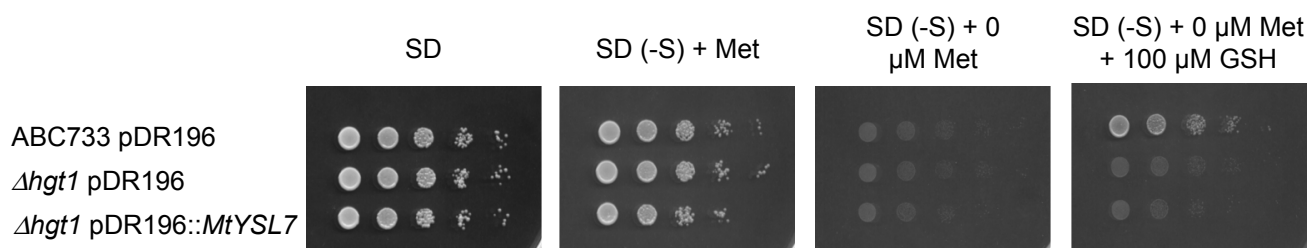


Fig. S3. MtYSL7 glutathione transport assays. *S. cerevisiae* strain ABC733 was transformed with empty pDR196 vector, while $\Delta hgt1$ mutant in the ABC733 strain was transformed with either the empty pDR196 or pDR196 expressing MtYSL7 cDNA. Serial dilutions (10x) of each transformant were placed in SD medium, SD medium using either methionine (Met) or glutathione (GSH) as sole sulfur source. The selected are representative images from at least three independently carried experiments using each a different yeast transformant.

SUPPLEMENTAL FIGURE S4

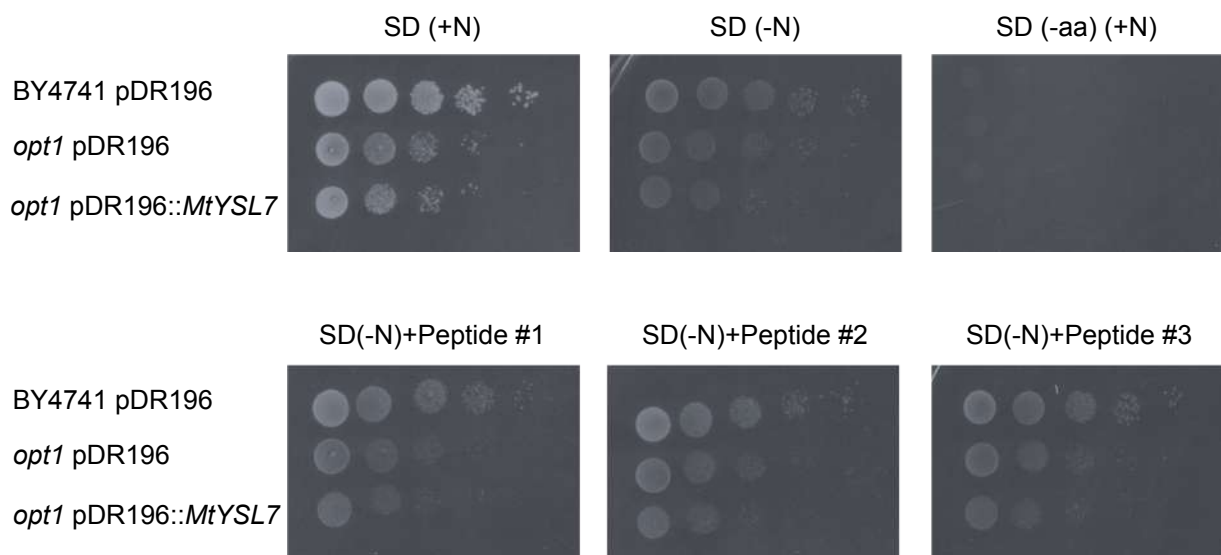


Fig. S4. MtYSL7 IMA peptides transport assays. Parental yeast strain BY4741 was transformed with empty pDR196 vector, while the strain *opt1*, mutant in the oligopeptide transporter ScOPT1, was transformed with either the empty pDR196 vector, or with pDR196 containing the coding sequence of *MtYSL7*. Serial dilutions (10x) were grown on SD media supplemented with one of the following peptides as sole nitrogen source: ENGGDDDDSGYDYAPAA (Peptide 1), GDDDD (Peptide 2), or APAA (Peptide 3). The selected are representative images from at least three independently carried experiments using each a different yeast transformant.

SUPPLEMENTAL FIGURE S5

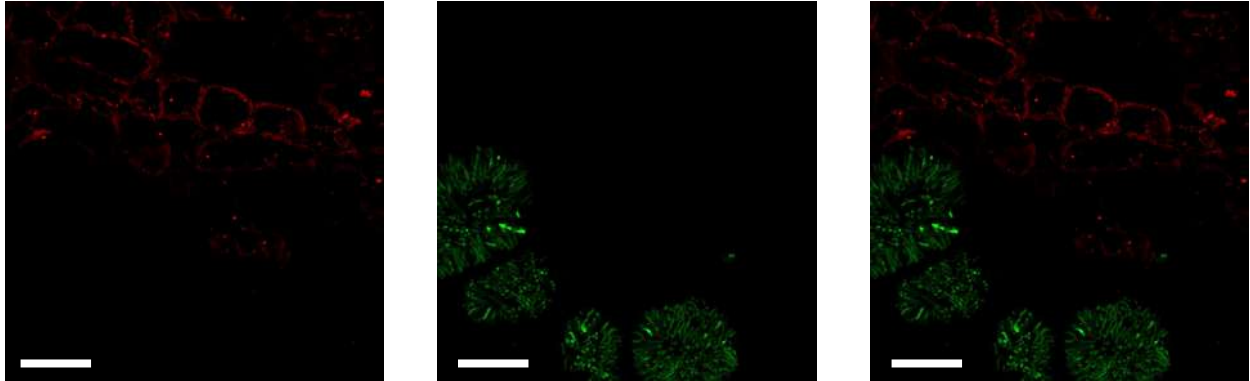


Fig. S5. Detail of MtYSL7-HA localization in nodules. Longitudinal section of a 28 dpi *M. truncatula* nodule expressing *MtYSL7-HA* under its own promoter. HA was visualized with an Alexa594-conjugated antibody (red left panel). Transformed plants were inoculated with a GFP-expressing *S. meliloti* strain (green, centre panel). These two channels are overlaid on the right panel. Bars = 25 μ m. The images shown are representative images from four separate *M. truncatula* transformations, each producing at least 15 plants, whose roots and nodule were collected and prepared for GUS expression determination.

SUPPLEMENTAL FIGURE S6

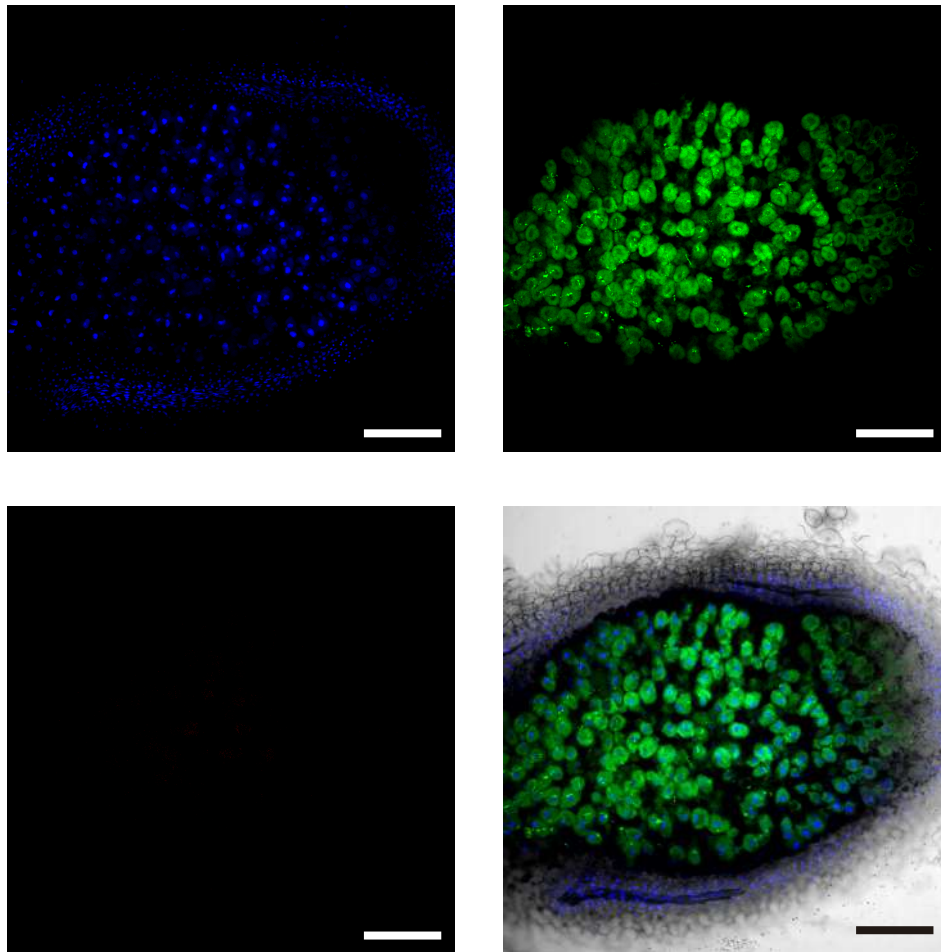


Fig. S6. Autofluorescence control for Alexa594. Longitudinal section of a 28 dpi *M. truncatula* nodule expressing *MtYSL7-HA* under its own promoter. No primary anti-HA antibody was used, but samples were incubated with secondary Alexa594-conjugated antibody and DNA stained with DAPI. Transformed plants were inoculated with a GFP-expressing *S. meliloti* strain. Top left panel corresponds to DAPI signal (blue), top right to the GFP emission (green), and lower left to the Alexa 594 emission with the same settings used in all confocal imaging. These three channels are overlaid with the bright field image in the lower right panel. Bars = 200 μ m. The images shown are representative images from four separate *M. truncatula* transformations, each producing at least 15 plants, whose roots and nodule were collected and prepared for GUS expression determination.

SUPPLEMENTAL FIGURE S7

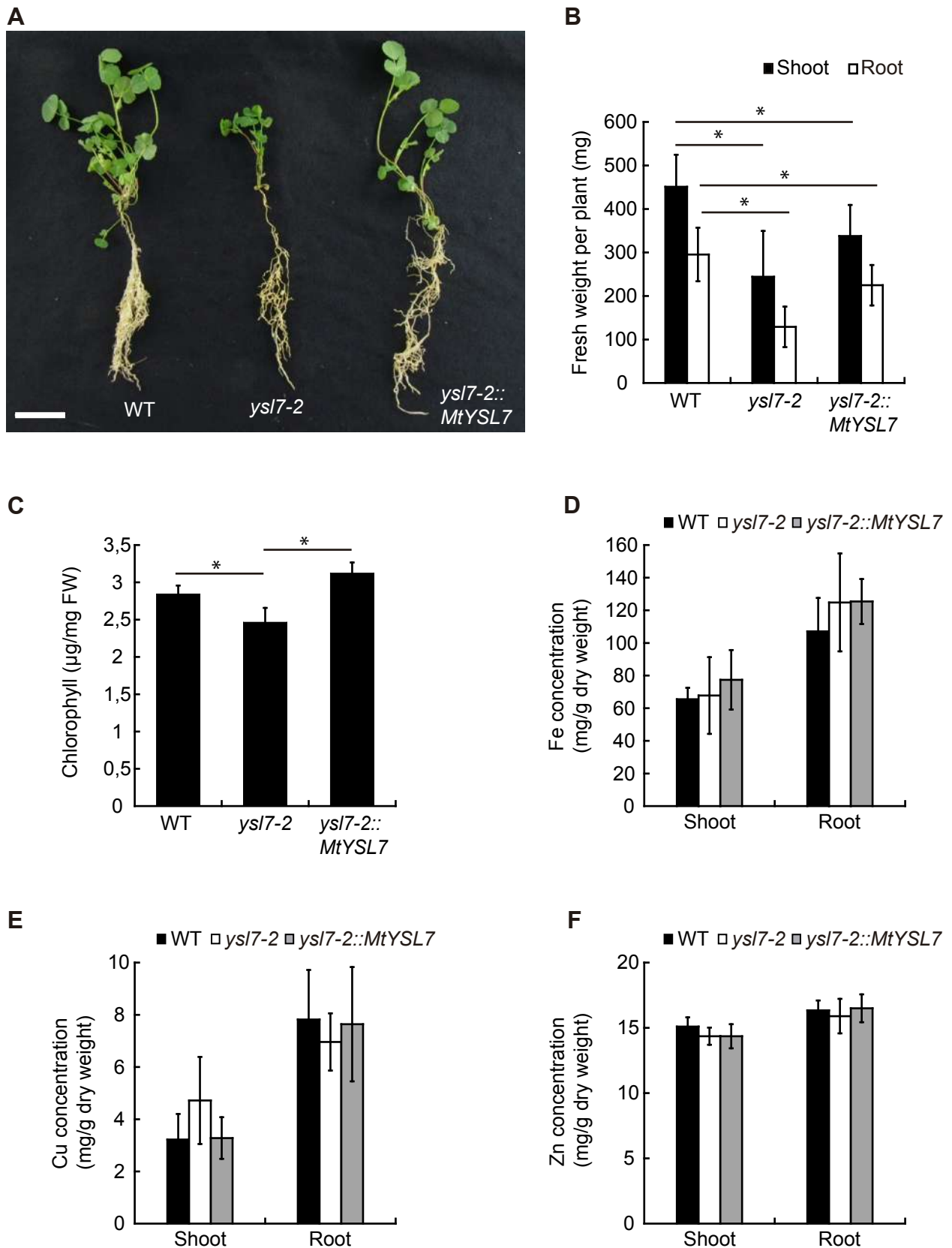


Fig. S7. Genetic complementation of the *ysl7-2* phenotype under non-symbiotic conditions. A). Growth of representative wild type, *ysl7-2*, and *ysl7-2* transformed with a wild type copy of *MtYSL7-HA* regulated by its own promoter (*ysl7-2::MtYSL7*) plants. Bar = 3 cm. B) Fresh weight of shoots and roots of wild type, *ysl7-2*, and *ysl7-2::MtYSL7* plants. Data are the mean $\pm$ SE (n = 6 plants). C) Chlorophyll content of wild type, *ysl7-2*, and *ysl7-2::MtYSL7* shoots. Data are the mean $\pm$ SE of three sets of five pooled plants. D) Iron content in roots and shoots of wild type, *ysl7-2*, and *ysl7-2::MtYSL7* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. E) Copper content in roots and shoots of wild type, *ysl7-2*, and *ysl7-2::MtYSL7* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. F) Zinc content in roots and shoots of wild type, *ysl7-2*, and *ysl7-2::MtYSL7* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. * indicates statistical significance (p < 0.05).

SUPPLEMENTAL FIGURE S8

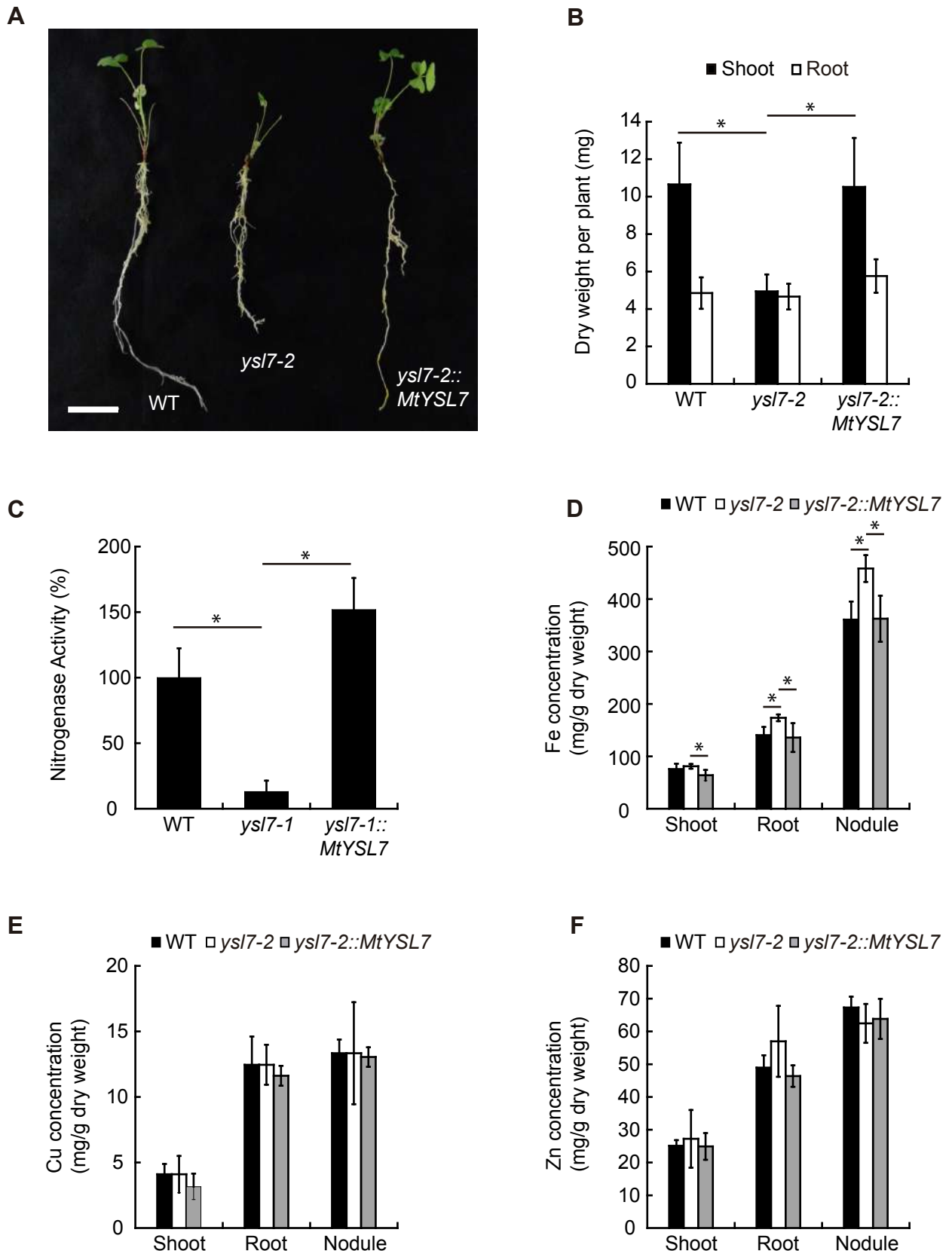
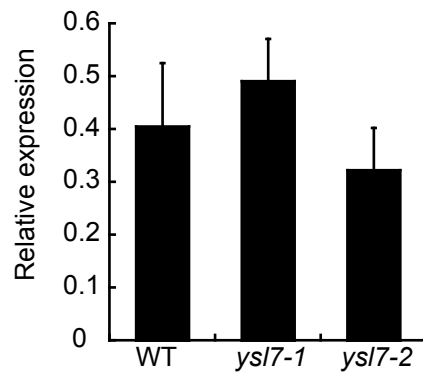


Fig. S8. Genetic complementation of the *ysl7-2* phenotype under symbiotic conditions. A) Growth of representative wild type, *ysl7-2*, and *ysl7-2* transformed with a wild type copy of *MtYSL7-HA* regulated by its own promoter (*ysl7-2::MtYSL7*) plants. Bar = 3 cm. B) Dry weight of shoots and roots of 28 dpi plants. Data are the mean $\pm$ SE ($n = 5$ plants). C) Nitrogenase activity in 28 dpi nodules from wild type, *ysl7-2* and *ysl7-2::MtYSL7* plants. Data are the mean $\pm$ SE measured in duplicate from two sets of five pooled plants. 100 % = 0.115 nmol ethylene h^{-1} plant $^{-1}$. D) Iron content in roots and shoots of wild type, *ysl7-2*, and *ysl7-2::MtYSL7* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. E) Copper content in roots and shoots of wild type, *ysl7-2*, and *ysl7-2::MtYSL7* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. F) Zinc content in roots and shoots of wild type, *ysl7-2*, and *ysl7-2::MtYSL7* plants. Data are the mean $\pm$ SE of five sets of five pooled plants. * indicates statistical significance ($p < 0.05$).

SUPPLEMENTAL FIGURE S9

A



B

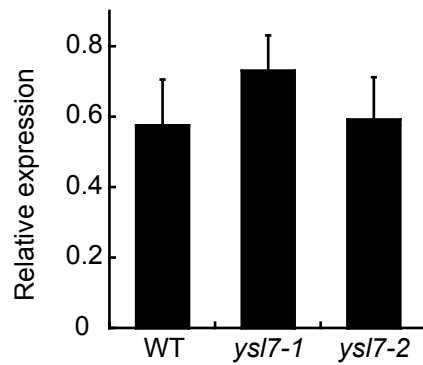


Fig. S9. Mutation of *MtYSL7* does not alter the expression of iron homeostasis genes *MtFRO1* (A) or *MtNRAMP1* (B) in non-symbiotic conditions. Expression of each gene was calculated relative to internal standard gene ubiquitin carboxyl-terminal hydrolase in 28 dpi roots from wild type (WT), *ysl7-1*, and *ysl7-2* plants. Data bars are the mean $\pm$ SE of three independent experiments.

SUPPLEMENTAL FIGURE S10

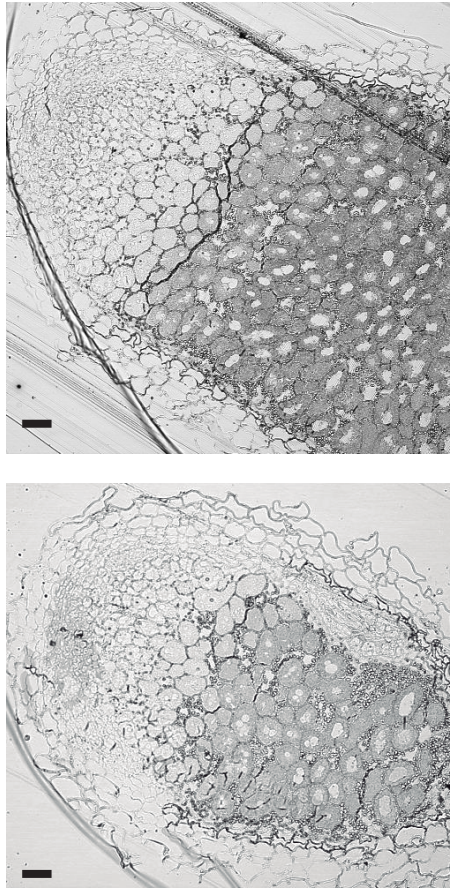


Fig. S10. Iron distribution in 28 dpi wild type (top panel) and *ys/7-2* (bottom panel) nodules. Bars = 50 μ m. The images shown are representative from randomly selected nodules obtained from 10 wild type or 10 *ys/7-2* plants.

SUPPLEMENTAL FIGURE S11

A



B

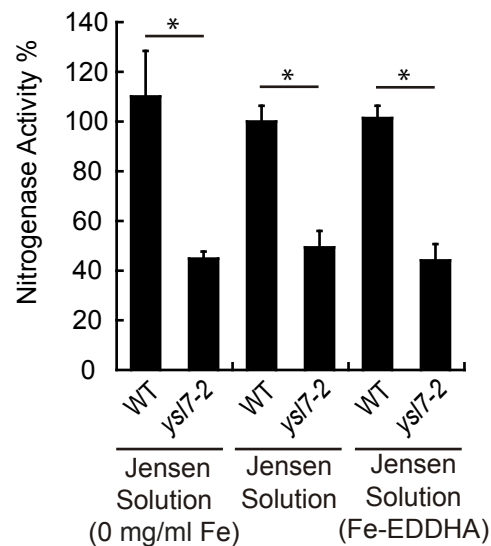


Fig. S11. Effect of changing iron concentrations on the *ysl7-2* phenotype. A) Growth of representative wild type and *ysl7-2* plants watered with standard nutrient solution (Jensen solution), with a modified nutrient solution without adding any iron (0 mg/ml Fe), and one fortified with 0.5 g/l of Fe-EDDHA. Bar = 3 cm. B) Nitrogenase activity in 28 dpi nodules from wild type or *ysl7-2* plants watered with standard nutrient solution (Jensen solution), with a modified nutrient solution without adding any iron (0 mg/ml Fe), and one fortified with 0.5 g/l of Fe-EDDHA. Data are the mean $\pm$ SE measured in duplicate from two sets of five pooled plants. 100 % = 0.645 nmol ethylene h^{-1} plant $^{-1}$. * indicates statistical significance ($p < 0.05$).

SUPPLEMENTAL FIGURE S12

A



B

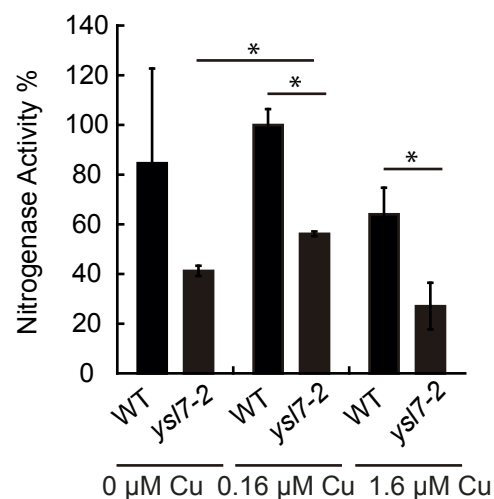


Fig. S12. Effect of changing copper concentrations on the *ysl7-2* phenotype. A) Growth of representative wild type and *ysl7-2* plants watered with standard nutrient solution (Jensen solution), with a modified nutrient solution without adding any copper (0 μM Cu), and one fortified with 1.6 μM Cu. Bar = 3 cm. B) Nitrogenase activity in 28 dpi nodules from wild type or *ysl7-2* plants watered with standard nutrient solution (Jensen solution), with a modified nutrient solution without adding any copper (0 μM Cu), and one fortified with 1.6 μM Cu. Data are the mean $\pm$ SE measured in duplicate from two sets of five pooled plants. 100 % = 0.456 nmol ethylene h^{-1} plant $^{-1}$. * indicates statistical significance ($p < 0.05$).

Supplemental Table 1. Primers used in this study

Primer	Sequence	Use
MtUb v4qF	ATTCTTCACATGCGGCGATTTAC	qPCR of <i>ubiquitin carboxyl-terminal hydrolase</i>
MtUb v4qR	TTTCTCATTTGCTTTTGGTGTGG	qPCR of <i>ubiquitin carboxyl-terminal hydrolase</i>
YSL7qF2	CGTCGAAAGCCATGACAGTGGT	qRT-PCR of <i>MtYSL7</i>
YSL7qR2	ACCGACAACATGAGACTCACGAA	qRT-PCR of <i>MtYSL7</i>
YSL8qF_v2	CGTGACGTCGAAACCTCCGA	qRT-PCR of <i>MtYSL8</i>
YSL8qR_v2	ATGATACTCACTACTATTGCCCTAACC	qRT-PCR of <i>MtYSL8</i>
YSL9qF	CAGTTGCGCCGGTGTGAAA	qRT-PCR of <i>MtYSL9</i>
YSL9qR_v2	TCACCGACAACATGAGACTTACA	qRT-PCR of <i>MtYSL9</i>
YSL5qF	GCTCTTTTGGAGTTAAGCCTCCC	qRT-PCR of <i>MtYSL5</i>
YSL5qR	GGCAGATTTACAATGCATCAACAAGC	qRT-PCR of <i>MtYSL5</i>
5MtNRAMP1-1533	AAGGGGTTTGTAAACGATGGTCAGT	qRT-PCR of <i>MtNramp1</i>
3MtNRAMP1-1659	GCTCTCTAAAATGACTTGAAAGCAA	qRT-PCR of <i>MtNramp1</i>
5MtFRO1qF	GGTGACACGTGGATCATCTG	qRT-PCR of <i>MtFRO1</i>
3MtFRO1qR	TTGCAATCCACAGGAACAAA	qRT-PCR of <i>MtFRO1</i>
5MtYSLpGW	GGGGACAAGTTTGTACAAAAAAGCAG GCTCTTAGGGAGGTTTTTTAGAG	Cloning of <i>MtYSL7</i> promoter and full sequence in pGWB3/13
3MtYSLpGW	GGGGACCACTTTGTACAAGAAAGCTG GGTTATGTTTACAATGAAATTGA	Cloning of <i>MtYSL7</i> promoter in pGWB3
3MtYSLGW	GGGGACCACTTTGTACAAGAAAGCTG GGTTATGTTCTAAGAAGGCATCAAC	Cloning of <i>MtYSL7</i> promoter, full sequence and coding sequence for fused to 3xHA or GFP in C- terminus.
5MtYSL7+1GW-C	GGGGACAAGTTTGTACAAAAAAGCAG GCTATGTCTTCTGAACCCCATCGA	Cloning of <i>MtYSL7</i> coding sequence in Gateway vectors to fuse them to GFP in C-terminus
5MtYSL7+1GW-N	GGGGACAAGTTTGTACAAAAAAGCAG GCTTCATGTCTTCTGAACCCCATCGA	Cloning of <i>MtYSL7</i> coding sequence in Gateway vectors to fuse them to GFP in N-terminus
3MtYSL7+3996GW-N	GGGGACCACTTTGTACAAGAAAGCTG GGTTCAATGTTCTAAGAAGGCATCAAC	Cloning of <i>MtYSL7</i> coding sequence in Gateway vectors to fuse them to GFP in N-terminus
5MtYSL7+1_PstI	AAAACTGCAGATGTCTTCTGAACCCCA TCGA	Cloning of <i>MtYSL7</i> coding sequence in pDR196
3MtYSL7+4002_XhoI	AAAACTCGAGTCAATGTTCTAAGAAG GC	Cloning of <i>MtYSL7</i> coding sequence in pDR196
YSL7.2-Fv.2	CGAAAGCCATGACAGTGGT	Genotyping of Tnt1 mutant lines
YSL7.2-Rv.2	AGGGGGTAATTAGATTAAACCAA	Genotyping of Tnt1 mutant lines.
5MtNramp1 + 1GW	GGGGACAAGTTTGTACAAAAAAGCAG GCTTCATGGCACATCAAGAAGTGAAT	Cloning of <i>MtNramp1</i> coding sequence in Gateway vectors

3GWMtNramp1	GGGGACCACTTTGTACAAGAAAGCTG GGTACAACCTAGGTCCCTCTGGTCG	Cloning of <i>MtNramp1</i> coding sequence in Gateway vectors
5 MtZIP6 1GW	GGGGACAAGTTTGTACAAAAAAGCAG GCTATGGCTTATTCAGTAACTAT	Cloning of <i>MtZIP6</i> coding sequence in Gateway vectors
3' GW Mt ZIP6	GGGGACCACTTTGTACAAGAAAGCTG G GTACTCAATTTGTGTATTGACA	Cloning of <i>MtZIP6</i> coding sequence in Gateway vectors
