A Putative Function for the Arabidopsis Fe–Phytosiderophore Transporter Homolog AtYSL2 in Fe and Zn Homeostasis

Gabriel Schaaf 1,4, Adam Schikora 2,4, Jennifer Häberle 1, Grégory Vert 2, Uwe Ludewig 3, Jean-François Briat 2, Catherine Curie 2 and Nicolaus von Wirén 1,5

1 Institut für Pflanzenernährung, Universität Hohenheim, D-70593 Stuttgart, Germany
2 Biochimie et Physiologie Moléculaire des Plantes, Centre National de la Recherche Scientifique (Unité Mixte de Recherche 5004)/Institut National de la Recherche Agronomique/Agro-M/Université Montpellier II, 2 place Viala, F-34060 Montpellier cedex 1, France
3 ZMBP-Pflanzenphysiologie, Universität Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany

Although Arabidopsis thaliana does not produce phytosiderophores (PS) under Fe deficiency, it contains eight homologs of the metal–PS/metal–nicotianamine (NA) transporter ZmYS1 from maize. This study aimed to investigate whether one of the closest Arabidopsis homologs to ZmYS1, AtYSL2, is involved in metal–chelate transport. Northern analysis revealed high expression levels of AtYSL2 in Fe-sufficient or Fe-resupplied roots, while under Fe deficiency transcript levels decreased. Quantitative real-time polymerase chain reaction (PCR) and analysis of transgenic plants expressing an AtYSL2 promoter::β-glucuronidase gene further allowed the detection of down-regulated AtYSL2 gene expression under Zn and Fe deficiency. In contrast to ZmYS1, AtYSL2 did not mediate metal–PS or metal–NA transport in yeast mutants defective in Cu or Fe uptake, nor did AtYSL2 mediate Fe(II)–NA–, Fe(III)–NA– or Ni(II)–NA–inducible currents when assayed by two-electrode voltage clamp in Xenopus oocytes. Moreover, truncation of the N-terminus to remove putative phosphorylation sites that might trigger autoinhibition did not confer functionality to AtYSL2. A direct growth comparison of yeast cells transformed with AtYSL2 in two different yeast expression vectors showed that transformation with empty pFL61 repressed growth even under non-limiting Fe supply. We therefore conclude that the yeast complementation assay previously employed does not allow the identification of AtYSL2 as an Fe–NA transporter. Transgenic plants expressing an AtYSL2 promoter::β-glucuronidase gene showed expression in root endodermis and transgenic plants facing the meta-xylem tubes. Taken together, our investigations support an involvement of AtYSL2 in Fe and Zn homeostasis, although functionality or substrate specificity are likely to differ between AtYSL2 and ZmYS1.

Keywords: nicotianamine — iron transport — strategy II — yeast complementation — Xenopus oocytes.

Abbreviations: DMA, 2′-deoxy-mugineic acid; GFP, green fluorescent protein; GUS, β-glucuronidase; NA, nicotianamine; NAAT, nicotianamine amino-transferase; NAS, nicotianamine synthase; ORF, open reading frame; PS, phytosiderophore; TMD, transmembrane-spanning domain.

Introduction

Iron (Fe) is an essential element for all plants and plays a critical role in respiration, photosynthesis and many other cellular functions, including DNA synthesis and hormone production. Although abundant in the earth’s crust, plant availability of Fe is extremely low in oxygenated environments due to the formation of low-soluble ferric hydroxide complexes at neutral or alkaline pH (Guerinot and Yi 1994).

In contrast, under anaerobic conditions, Fe, like other transition metals, can become toxic when hyper-accumulated or maldistributed within cells due to the generation of free radicals by Fenton chemistry (Briat and Lebrun 1999). Therefore, plants need to tightly control Fe uptake, intracellular compartmentation and distribution into the various plant organs (Briat and Lobréaux 1997, Wei and Theil 2000). In addition, due to deleterious effects of Fe(II), control of the Fe redox state and control of the biosynthesis of Fe chelators seem to be of particular importance for the cellular homeostasis of Fe.

Plants have developed two strategies to acquire Fe from soils under Fe-limiting conditions as first proposed by Römheld and Marschner (1986). In strategy I, which is found in dicots and non-graminaceous monocots, Fe has to be reduced prior to uptake. Therefore, proton secretion by plasma membrane H⁺-ATPases and reduction capacity as well as Fe(II) transport are enhanced under Fe-deficient growth conditions (Schmidt 2003). In contrast, in strategy II, as found in graminaceous plant species, Fe is chelated extracellularly by hexadentate chelators, so-called phytosiderophores (PS), and is subsequently taken up as intact Fe(III)–PS complexes. Both PS release and Fe(III)–PS uptake are enhanced under Fe deficiency, whereas the reduction-dependent pathway for Fe uptake remains in most cases constitutively expressed (Zaharieva and Römheld 2001, Bugchio et al. 2002). In the past years, many of the mechanisms involved in Fe acquisition by strategy I and strategy II plants have been described at the molecular level (summarized in Curie and Briat 2003, Schmidt 2003). The genes encoding AtFRO2 and AtIRT1, that are mainly responsible for extracellular Fe reduction and Fe²⁺ uptake into roots in the strategy I plant Arabidopsis, have been isolated and the encoded proteins

4 These authors contributed equally to this work.
5 Corresponding author: E-mail, vonwiren@uni-hohenheim.de; Fax, +49-711-459-3295.

762
characterized (Eide et al. 1996, Robinson et al. 1999). In strategy II, genes encoding nicotianamine synthase (NAS) and nicotianamine amino-transferase (NAAT), both key players in PS biosynthesis, as well as the high-affinity Fe(III)–PS transporter ZmYS1 have been isolated and the encoded proteins have been characterized (Higuchi et al. 1999, Takahashi et al. 1999, Curie et al. 2001, Schaaf et al. 2004). All these proteins are involved in Fe acquisition. In contrast, to date, only a few gene products are known that might play a role in intracellular transport of Fe and other heavy metals. For instance, AtNRAMP3 is localized to the tonoplast of root cells in the vascular bundle and might be involved in mobilizing vacuolar Fe, Zn and Mn to the cytoplasm (Thomine et al. 2003). Due to the presence of highly homologous NRAMP genes in both dicot and monocot plants, and the modulation of Cd and Fe toxicity by different members of the NRAMP family in plants, a more general function in metal homeostasis independent of the strategy for Fe acquisition has been postulated (Belouchi et al. 1997, Curie et al. 2000, Thomine et al. 2000, Mäser et al. 2001, Thomine et al. 2003).

For the chelation of Fe within plants, several potential ligands have been discussed. In the xylem, these are citrate for both strategy I and strategy II plants (Tiffin 1965). Moreover, in strategy II plants, mugineic acid and deoxy-mugineic acid, two PS species, may also chelate Fe in the xylem (Mori et al. 1987, Kawai et al. 2001). One of the most promising candidates for intracellular Fe chelation seems to be nicotianamine (NA). NA, a structural analog of PS and precursor of PS bio-dates for intracellular Fe chelation seems to be nicotianamine (NAAT) (von Wirén et al. 1994, Curie et al. 2000, Thomine et al. 2000, Mäser et al. 2001, Thomine et al. 2003).

NA has been shown to chelate Fe, Cu, Zn and Mn (Benes et al. 1983, Stephan and Scholz 1993, Stephan et al. 1996). The unexpected high kinetic stability of Fe(II)–NA and the low Fenton activity of Fe(II)–NA and Fe(III)–NA suggest that NA possesses an important function in scavenging Fe and protecting cells from oxidative damage (von Wirén et al. 1999). This function of NA has been corroborated by the observation that NA levels correlate with the amount and distribution of Fe in pea and tomato plants (Pich et al. 2001). Furthermore, the maize NAS gene ZmNAS3 is induced under Fe-sufficient conditions and repressed under Fe deficiency (Mizuno et al. 2003), which provides evidence for an involvement of NA in Fe nutrition in grasses other than in PS biosynthesis. A first link between Fe chelation by NA and Fe transport across biological membranes has been made by the observation that the maize ZmYS1 protein mediates transport not only of Fe–PS and other metal–PS complexes (von Wirén et al. 1994, von Wirén et al. 1996, Curie et al. 2001, Schaaf et al. 2004) but also of Fe(II)–, Fe(III)– and Ni(II)–NA complexes as shown by heterologous expression in yeast and Xenopus oocytes (Schaaf et al. 2004). Since eight homologs of ZmYS1, named AtYSL1-8 (for ‘yellow stripe-like’), have been found in the strategy I plant Arabidopsis (Curie et al. 2001), the question arose of whether these homologs might be involved in Fe–NA transport and contribute to Fe homeostasis.

In the present study, we describe several independent approaches to investigate whether AtYSL2, one of the closest homologs of ZmYS1, is involved in Fe nutrition or heavy metal homeostasis. Unlike ZmYS1, we could not observe that AtYSL2 restores growth of the yeast Fe uptake-defective mutant fet3 fet4 in the presence of Fe–NA or Fe–PS chelates. However, expression analyses by Northern gel blots, real-time polymerase chain reaction (PCR) or transgenic plants expressing AtYSL2 promoter::GUS fusions support a function for AtYSL2 in plant Fe and Zn homeostasis.

**Results**

*Isolation of AtYSL2 from an Arabidopsis cDNA library*

The open reading frame (ORF) of AtYSL2 was amplified from an Arabidopsis thaliana Col-0 cDNA library and cloned into the pGEM®-T Easy vector for further subcloning.
Sequence analysis revealed that, in contrast to the database prediction (TAIR, http://www.arabidopsis.org/), the AtYSL2 cDNA contained the additional exon sequences 1,011–1,028 bp and 1,488–1,505 bp (starting from ATG as position 1 in the genomic DNA; GenBank accession number AY648977) which were predicted to represent intron sequences. These additional exon sequences were also present in two cDNA sequences found in the database (accession nos AY139751 and BT005806).

A phylogenetic analysis of the Arabidopsis AtYSL homologs and ZmYS1 suggested that AtYSL proteins cluster into three subgroups, and one of them, containing AtYSL1, AtYSL2 and AtYSL3, is most closely related to ZmYS1 in terms of sequence (Fig. 1). Using the ClustalW software (MegAlign, DNASTAR, Madison, WI, USA), AtYSL2 exhibited the closest homology to ZmYS1 with 59.8% identity at the amino acid level. The AtYSL homologs and ZmYS1 encode proteins of approximately 73–80 kDa that possess typical membrane protein signatures of alternating transmembrane-spanning domains (TMDs) and hydrophilic loops. In general, a longer hydrophilic loop separates the first four TMDs from the following 8–10 TMDs (http://www.cbs.dtu.dk/services/TMHMM-2.0).

Expression of AtYSL2 depends on the Fe and Zn nutritional status

To investigate whether AtYSL2 might be involved in Fe homeostasis, gene expression dependence on the Fe nutritional status was studied by Northern analysis. Therefore, hydroponically grown Fe-sufficient Arabidopsis plants were transferred to Fe-deficient medium for 10 d (–Fe) and then resupplied for 24 h with 50 µM Fe(III)-EDTA (–Fe+RS). As shown in Fig. 2A, AtYSL2 expression could be detected in roots of Fe-sufficient and resupplied plants, but expression in roots was low under Fe starvation. In shoots, a weak expression could be detected only under Fe-sufficient conditions. In addition, we monitored the expression of AtIRT1, an Fe deficiency-induced gene encoding the major high-affinity Fe transporter in the Arabidopsis root epidermis (Vert et al. 2002). In agreement with data obtained by us and others (Connolly et al. 2002, Vert et al. 2003), AtIRT1 expression was strongly enhanced under Fe-limiting conditions and repressed after Fe resupply (Fig. 2A, lower panel), which confirms that –Fe plants had been efficiently starved for Fe. Moreover, interveinal chlorosis in leaves of –Fe plants confirmed their Fe-deficient state. These data demonstrated that AtYSL2 expression in roots and regulation is dependent on the Fe nutritional status in an opposite manner to AtIRT1, thus supporting a different role for AtYSL2 in Fe homeostasis. In an independent approach, gene expression was...
Verification of metal-chelate transport by AtYSL2 investigated by real-time PCR. For this purpose, plants were grown during 5 d either in the presence of 100 µM Fe(III)-EDTA or without Fe in the culture medium. The quantitative analyses revealed that Fe deficiency leads to a 2-fold decrease of the transcript levels in roots as compared with Fe-sufficient roots (Fig. 2B), confirming the Northern analysis. In addition, AtYSL2 mRNAs accumulated four times less in shoots in conditions of Fe deficiency. The amount of transcript in shoots, however, was much lower than in roots (data not shown).

To assess the effect of Zn deficiency on AtYSL2 expression, plants were grown in nutrient solution for 4 weeks and then transferred to Zn-deficient medium for 5 d. As for Fe, Zn starvation resulted in a 2-fold decrease in AtYSL2 mRNA abundance, in both shoots and roots. The data obtained by Northern analysis and real-time PCR were in agreement with a third approach investigating the enzymatic activity of transgenic plants expressing an AtYSL2 promoter::β-glucuronidase (GUS) gene: for this purpose, transgenic plants were grown for 10 d under Fe- and Zn-sufficient conditions and then transferred to Zn- or Fe-depleted medium and analyzed for enzymatic GUS activity. GUS activity of Fe-deficient plants was reduced approximately 2-fold in roots and shoots (Fig. 3I). The effect was even more pronounced in Zn-deficient plants, where the enzymatic activity of the AtYSL2 promoter-driven GUS gene was reduced by >10-fold in roots and >2-fold in leaves. The enzymatic GUS activity was consistent with the histochemical staining of transgenic plants, indicating a reduced GUS expression under Fe and Zn deficiency (Fig. 3C, D, respectively).

Expression of AtYSL2 shows a diurnal rhythm

We then investigated whether AtYSL2 expression depends on the diurnal rhythm. For this purpose, plants were grown under Fe-sufficient conditions and harvested at 9:00 h, 14:00 h, 19:00 h and 0:00 h. The expression level of AtYSL2 in roots was highest at 19:00 h (at the end of the light period) and lowest at 9:00 h (at the end of the dark period, Fig. 2C). We therefore concluded that, similarly to AtIRT1 and AtFRO2, expression of AtYSL2 is subjected to diurnal regulation.

AtYSL2 is expressed in the root central cylinder

Root-specific expression of AtYSL2 was confirmed further by promoter-reporter gene analysis. Twelve transgenic Arabidopsis lines, expressing an AtYSL2 promoter::GUS fusion, were generated and examined with regard to tissue specificity of the AtYSL2 promoter activity. GUS histochemical analyses were performed on plants selected in vitro for kanamycin resistance during 10 d and grown for another 5 d in the absence of kanamycin. In 10 out of 12 lines, the promoter was active in the central cylinder of the root (Fig. 3A). Three lines additionally showed a weak activity in leaves, which was restricted to the veins (Fig. 3B). In cross-sections of young roots, the promoter activity was localized in the pericycle as well as in the parenchyma of the central cylinder.
Verification of metal-chelate transport by AtYSL2 (Fig. 3E). This pattern changed during root development, since mature roots also showed GUS staining in endodermal cells (Fig. 3F). In older parts of the root, the promoter remained active in the pericycle layer as well as in the endodermis, but only in those cells that faced the meta-xylem tubes (Fig. 3G, H).

**Heterologous expression of AtYSL2 in yeast and Xenopus oocytes could not show a function in metal–chelate transport**

ZmYS1 has been shown to complement the growth defect of the fet3 fet4 mutant, which is defective in high- and low-affinity Fe transport (Spizzo et al. 1997) on media containing Fe(III)–DMA (Curie et al. 2001) or Fe(II)–NA and Fe(II)–NA (Schaaf et al. 2004) as a sole Fe source. To investigate whether the selected Arabidopsis homolog was also able to complement the growth defect of the fet3 fet4 mutant, AtYSL2 was subcloned into the yeast expression vector pDR195 under control of the strong PMA1 promoter fragment. Transformants were plated on media supplemented with different concentrations of Fe(II)–NA and Fe(III)–NA or the more easily available structural analog Fe(III)–DMA at different pH values. However, under any tested condition, growth of the yeast fet3 fet4 mutant expressing AtYSL2 was similar to that of yeast transformed with pDR195 alone (Fig. 4A). AtYSL2 in pDR195 was further used for transformation of the Cu uptake-defective yeast mutant ctr1, which is unable to grow on media supplemented with low Cu and a non-fermentable carbon source (Dancis et al. 1994; Fig. 4B). In contrast to ZmYS1, however, AtYSL2 was not able to restore growth of ctr1 on 6 µM Cu(II)–NA.

AtYSL2 was then heterologously expressed in Xenopus oocytes and analyzed by two-electrode voltage clamp. For this purpose, oocytes injected with pOO2-AtYSL2 or pOO2-ZmYS1 cRNA were clamped at –70 mV in choline-based buffer solution at pH 6.0 and then superfused with buffer containing different metal–NA chelates. As expected, in control oocytes injected with cRNA encoding ZmYS1, currents were induced...
Verification of metal-chelate transport by AtYSL2

Fig. 5 (A) Prediction of phosphorylation sites in the N-terminal region of ZmYS1 and the AtYSL homologs. Phosphorylation sites in the 45 N-terminal amino acids of the transporters were predicted by the NetPhos 2.0 program (www.cbs.dtu.dk/services/NetPhos). (B) Construction of the N-terminal truncated versions of AtYSL2. Primers were designed to hybridize to AtYSL2 at positions 61 bp (AtYSL2short1 for1), 103 bp (AtYSL2short2 for1) and 151 bp (AtYSL2short3 for1) relative to position 1 from the initial ATG. Each forward primer contained an additional ATG at the 5’ end (underlined) to allow translation of the truncated AtYSL2 version at this position. (C) Expression of N-terminal truncated versions of AtYSL2 in yeast. Growth of the yeast fet3 fet4 mutant (DEY1453) transformed with pDR196-ZmYS1, pDR195-AtYSL2, pDR195-AtYSL2short1, pDR195-AtYSL2short2, pDR195-AtYSL2short3 or empty pDR195 on media supplemented with 50 µM Fe(II)–NA (left), 35 µM Fe(III)–NA (right) or 30 µM FeCl₃. Similar results were obtained when plating transformants on media supplemented with 7.5 µM Fe(II)–NA or 7.5 µM Fe(III)–NA at pH 7.5 (data not shown). No growth differences could be observed at 10 µM FeCl₃ (data not shown). Plates were incubated at 30°C for 10 d (Fe–NA) or 5 d (FeCl₃).
Verification of metal-chelate transport by AtYSL2

by 24 µM Fe(III)–NA (Fig. 4C), 50 µM Fe(II)–NA or 50 µM Ni(II)–NA (data not shown). Observed currents corresponded to the net influx of positive charge (Schaaf et al. 2004). However, neither 24 µM Fe(III)–NA (Fig. 4C) nor any of the other metal–NA chelates induced currents in oocytes injected with cRNA of AtYSL2, which behaved like water- or non-injected control oocytes (data not shown).

Predicted N-terminal phosphorylation sites suggested post-translational regulation of AtYSL2 and other AtYSL homologs

Since the activity of transport proteins might depend on the phosphorylation status of regulatory N-terminal domains, the N-termini of AtYSL proteins were analyzed for possible phosphorylation sites employing the NetPhos 2.0 programme (www.cbs.dtu.dk/services/NetPhos). As shown in Fig. 5A, all Arabidopsis homologs contain at least one (AtYSL3 and AtYSL8) or several (AtYSL1, AtYSL2, AtYSL4, AtYSL5, AtYSL6 and AtYSL7) putative phosphorylation sites in the N-terminal 45 amino acids, whereas ZmYS1 was not predicted to possess phosphorylation sites in this region. Thus, N-terminal autoinhibition of AtYSL2 might be responsible for its lack of functionality. N-terminally truncated versions of several transporters lacking these phosphorylation sites, such as the Arabidopsis vacuolar Ca2+/H+ antiporter AtCAX1 (Pittman and Hirschi 2001, Pittman et al. 2002) and the vacuolar Mn2+/H+ antiporter AtCAX2 (Schaaf et al. 2002), showed activity in contrast to the corresponding full-length versions. Therefore, a PCR-based approach was set up to generate N-terminally truncated versions of AtYSL2 as illustrated in Fig. 5B. However, none of these versions was able to complement the growth

defect of the yeast fet3 fet4 strain on media supplemented with 50 µM Fe(II)–NA or 35 µM Fe(III)–NA (Fig. 5C) and other Fe(II)–NA and Fe(III)–NA concentrations or on low concentrations of FeCl3 (data not shown).

Co-expression of ZmYS1 with AtYSL2 did not affect growth on Fe–NA or alter uptake and release of Ni–NA in yeast

Since failure of AtYSL2 to complement the growth defect of the yeast fet3 fet4 mutant or to mediate metal–NA-induced currents in oocytes might be due to a function of AtYSL2 in metal–NA release rather than in metal–NA uptake, an experiment was set up that took into account a potential function of AtYSL2 in substrate export. For this purpose, ZmYS1 was co-expressed with pDR195-Leu-ZmYS1 and pDR195-AtYSL2. This approach attempted to load yeast cells with Ni(II)–NA via ZmYS1 to analyze subsequently a potential AtYSL2-mediated release of Ni(II)–NA. In this experiment, NA-chelated Ni was employed, since we observed in previous studies less precipita-
tion and adsorption of the metal to the matrix (e.g. yeast cell walls) when compared with Fe(II)–NA, thus allowing detection of small quantities more reliably (Schaaf et al. 2004 and unpublished results). As a control, yeast cells co-transformed with pDR195Leu-ZmYS1 and the empty vector pDR195 were used. After a 20 min incubation period in Ni(II)–NA, yeast cells were washed and transferred to release buffer. As shown in Fig. 6, no statistically significant differences in uptake or release rates between AtYSL2-expressing cells and control cells were found. This experiment was also designed to investigate a potential intracellular localization of AtYSL2: if this homolog mediated transport of Ni(II)–NA into internal compartments, one might expect that the release of $^{63}$Ni would be decreased or held up after ZmYS1-mediated loading.

The yeast expression vectors pDR195 and pFL61 affect yeast growth differently

In the study of DiDonato et al. (2004), which was published while this manuscript was being reviewed, a growth complementation of Fe and Cu uptake-defective yeast mutants by AtYSL2 on Fe(II)– or Cu(II)–NA was shown, which contrasted with our own observations (Fig. 4). We therefore tested whether this discrepancy with our experiments might be related to the use of different yeast expression vectors and transformed the fet3 fet4 yeast mutant with AtYSL2 either in pDR195 or in pFL61 as used by DiDonato et al. (2004). A direct comparison of yeast growth under non-limiting Fe supply (30 µM Fe-EDTA) showed that yeast colonies were smaller when transformed with empty pFL61 relative to empty pDR195 (Fig. 7A). Impaired growth was not due to Fe deficiency because increasing Fe concentrations did not reverse the growth differences between pFL61 and pDR195 transformants (data not shown) and because pFL61-AtIRT1 transformants did not show better growth relative to cells transformed with pFL61 alone (Fig. 7A). Colony development of AtYSL2-transformed cells, however, was only superior to that of the transformants with empty vector when the pFL61 vector was used but not when pDR195 was used. Moreover, growth of pFL61-AtYSL2 transformants exactly matched that of pDR195 transformants irrespective of whether they carried a cDNA of AtIRT1, ZmYS1 or AtYSL2. The relative growth advantage suggested by the comparison of pFL61-AtYSL2 transformants with empty vector transformants clearly agreed with the data published by DiDonato et al. (2004). However, the apparent AtYSL2-mediated growth advantage was not related to Fe or the presence of Fe(II)–NA, because it appeared under non-limiting Fe supply and in the absence of NA (Fig. 7A). In contrast, under supply of 40 µM Fe(II)–NA, AtYS1 could not improve yeast growth, while expression of ZmYS1 or AtIRT1 clearly overcame Fe-limited growth depression (Fig. 7B). Also under these conditions, pFL61-transformed cells tended to grow poorly compared with pDR195-transformed cells.

Discussion

In non-graminaceous plants such as *Arabidopsis*, the transport of chelated transition metals across the plasma membrane or across intracellular membranes might provide a transport pathway that prevents the cytoplasm from deleterious effects mediated by free metals along the Fenton reaction. Recent studies in the hyperaccumulating plant *Thlaspi caerulescens* provide in addition increasing evidence for the formation of Ni–NA complexes under high Ni supply and transport of Ni–NA in the xylem, most probably for detoxification purposes (Vacchina et al. 2003). Based on the findings that NA is involved in inter- or intracellular trafficking of Fe and other metals (Pich et al. 2001, Takahashi et al. 2003), and that a member of the YS1/YSL transporter family, ZmYS1, also transports NA-chelated metals (Schaaf et al. 2004), we addressed the question of whether AtYSL2, one of the closest *Arabidopsis* homologs to ZmYS1 (Fig. 1), acts as a metal–NA transporter.

Heterologous expression suggests a different function for AtYSL2 and ZmYS1

Neither growth complementation of the yeast mutants fet3 fet4 and ctr1 nor two-electrode voltage clamp analysis allowed the demonstration of the functionality of AtYSL2, although in both assays the positive control, ZmYS1, mediated metal–chelate transport (Fig. 4). A simple explanation for the failure to show the functionality could be that the correct substrate has not yet been found. Indeed, in several cases, sequence homology between transporters did not allow us to come to a conclusion as to the identity of transported substrates. For instance, the OPT family, which includes ZmYS1 and the AtYSL homologs, additionally includes members mediating the transport of tetrpeptides such as the *Schizosaccharomyces pombe* OPT member ISP4 (Lubkowitz et al. 1998) or of the tripeptide glutathione such as ScHGT1 (Bourbouloux et al. 2000). In future experiments, it may thus be essential to conduct a large-scale screening of possible substrates for AtYSL2. The versatile oocyte expression system, which allowed the setting up of an expanded list of substrates transported by ZmYS1, is certainly a suitable approach for this purpose (Schaaf et al. 2004).

An intracellular localization of AtYSL2 might also hamper the functional characterization of transporters in heterologous systems and could explain the failure of yeast complementation and functional analysis in *Xenopus* oocytes in our study. Localization in yeast is in many cases similar to the endogenous localization, as shown for the *Arabidopsis* vacuolar pyrophosphatase and the vacuolar ATP-binding cassette transporter AtMRP2 (Kim et al. 1994, Liu et al. 2001) or as evidenced for the mitochondrial basic amino acid carriers AtmBAC1 and AtmBAC2 (Catoni et al. 2003, Hoyos et al. 2003). However, localization in the plasma membrane in the endogenous system is not in all cases a prerequisite for complementation of yeast mutants defective in nutrient uptake, as shown for AtNRAMP3 which complemented the fet3 fet4 yeast
mutant (Thomine et al. 2000), but for which green fluorescent protein (GFP) fusions localized to tonoplast when expressed in Arabidopsis protoplasts (Thomine et al. 2003). Therefore, cauliflower mosaic virus 35S-driven AtYSL2-GFP fusion proteins were transiently expressed in Arabidopsis protoplasts and analyzed by confocal microscopy. Although in several protoplasts GFP-dependent fluorescence localized to internal compartments (data not shown), fluorescence was too weak to draw firm conclusions. Other methods should therefore describe the native subcellular localization of AtYSL2.

Autoinhibitory domains have been reported to cause post-transcriptional down-regulation of protein activity in various animal, yeast and plant transporters (Pittman and Hirschi 2001). For transporters in intracellular membranes, these domains were mainly localized at the N-terminus, and N-terminal truncation could restore the activity of these proteins as shown for the endoplasmic reticulum-localized Ca$^{2+}$-ATPase ACA2 (Harper et al. 1998), the tonoplast Ca$^{2+}$-ATPases ACA4 (Geisler et al. 2000) and BCA (Malmström et al. 2000), the tonoplast Ca$^{2+}$/H$^+$ exchanger AtCAX1 (Pittman and Hirschi 2001) and the tonoplast Mn$^{2+}$/H$^+$ exchanger AtCAX2 (Schaaf et al. 2002). N-terminal inhibition has also been shown for the plasma membrane-localized Ca$^{2+}$-ATPase SCA1 (Chung et al. 2000). In the case of AtCAX1 and ACA2, the inhibitory effect of these domains depended on the phosphorylation status of serine residues at position 25 and 45, respectively (Hwang et al. 2000, Pittman and Hirschi 2001). Analysis of the encoded AtYSL proteins revealed that in contrast to ZmYS1, all AtYSL2 homologs contained one or several predicted phosphorylation sites within the first 45 amino acids (Fig. 5A). Therefore, truncated versions of AtYSL2 were generated and tested for functionality. However, none of the truncated versions could complement growth of the yeast fet3 fet4 mutant (Fig. 5C). In addition, Ni–NA transport was not affected when N-terminal truncated versions of AtYSL2 were co-expressed with ZmYS1 (data not shown).

The AtYSL2-mediated advantage for yeast growth depended on the yeast expression vector and not on the supply of Fe(II)–NA as an Fe source

In the study of DiDonato et al. (2004), the proof of AtYSL2 functionality as an Fe(II)–NA transporter was based exclusively on a yeast complementation study. A growth comparison between pFL61- and pFL61-AtYSL2-transformed yeast cells on 4 µM Fe in the presence of 5 µM NA yielded better growth of Fe uptake-defective yeast cells, and similarly, yielded better growth of Cu uptake-defective cells when Cu was supplied in the presence of NA (DiDonato et al. 2004). We could reproduce this apparent difference in growth complementation; however, we clearly observed that it occurred independently of the Fe supply and in the absence of NA. Under non-limiting Fe supply of 30 µM Fe-EDTA, pFL61-transformed yeast cells clearly grew more slowly than pDR195 transformants, indicating that the empty pFL61 vector alone confers a relative growth depression (Fig. 7A). Irrespective of the employed vector system, however, AtYSL2 could not complement yeast growth when Fe(II)–NA was supplied at 40 µM (Fig. 7B). We cannot yet explain the reason for the AtYSL2-mediated growth difference in the pFL61 vector system and why it is restricted to pFL61. It is clear, however, that the AtYSL2-mediated growth difference under these conditions did not depend on Fe limitation and on the supply of Fe–NA as an Fe source. We therefore conclude that the yeast complementation assay employed by DiDonato et al. (2004) does not allow identification of AtYSL2 as an Fe–NA transporter. In future, it will thus be important to verify yeast complementation approaches by metal–chelate uptake or electrophysiological studies in heterologous systems or in transgenic plants.

Transcriptional regulation of AtYSL2 supports a role in Fe and Zn homeostasis

As shown by three independent approaches, Northern analysis, real-time PCR and promoter–reporter gene analysis, AtYSL2 is expressed mainly in roots, repressed under Fe and Zn starvation and upregulated upon Fe resupply (Fig. 2A, B, Fig. 3). A decreased expression under Fe starvation clearly argues in favor of a role for AtYSL2 in Fe storage or Fe detoxification rather than in Fe uptake or acquisition as postulated for ZmYS1 (von Wirén et al. 1994). The transcriptional regulation of AtYSL2 resembled that of the NAS genes OsNAS3 and ZmAAS3, for which expression was decreased under Fe deficiency (Inoue et al. 2003, Mizuno et al. 2003). Moreover, AtYSL2 is additionally regulated by Zn. Expression of AtYSL2 was repressed in Zn-deficient plants, supporting the view that Fe and Zn share common pathways in chelation as well as in membrane transport. Several plant Fe transporters with Zn transport capabilities have been described, including AtIRT1, AtIRT2, AtNRAMP3 and ZmYS1 (Eide et al. 1996, Vert et al. 2001, Vert et al. 2002, Thomine et al. 2003, Schaaf et al. 2004). It is noteworthy that both Fe and Zn participate in regulating the Fe uptake machinery in Arabidopsis since accumulation of IRT1 and FRO2 transcripts upon Fe deficiency is repressed by Zn supply (Connolly et al. 2003). However, the Zn control seems to be exerted at the post-transcriptional level for IRT1 and FRO2 genes, while Fe deficiency and Zn deficiency most probably regulate AtYSL2 expression transcriptionally as indicated by the GUS expression analyses.

The diurnal regulation of AtYSL2 was reminiscent of the expression pattern of AtIRT1 and AtFRO2, which showed an increase of transcript levels during the light period (Vert et al. 2003). For AtIRT1 and AtFRO2, this regulation does not seem to be controlled by an endogenous circadian rhythm rather than directly or indirectly by light (Vert et al. 2003). A diurnal rhythm in the transcriptional regulation of plant membrane transporters has also been described for nitrate, ammonium and sulfate transporters (Gazzarrini et al. 1999, Lejay et al. 1999, Lejay et al. 2003). A more detailed study revealed that substrate transport and expression of these transporters was
enhanced by an increase in light intensity and by external sugar supply, indicating that this regulation depends on the stimulation by photoassimilates (Lejay et al. 2003). These authors further postulated that the induction of the nitrate transporter gene AtNRT2;1 during the light period depended on the glycolysis pathway or a metabolite downstream of the reaction catalyzed by hexokinase. Nutrient acquisition is a process that requires free energy. Stimulation of nutrient transporters by light may reflect an adaptation to the huge energy demand of nutrient transport processes and the subsequent metabolic pathways. This seems also to apply for AtYSL2 and thus supports its putative function in Fe and Zn transport.

**Promoter–GUS analysis reveals vascular localization of AtYSL2**

Analyses of transgenic plants expressing the GUS gene under control of a 1.12 kb AtYSL2 promoter fragment confined expression of *AtYSL2* to the vascular tissue (Fig. 3). The localization of *AtYSL2* promoter-driven GUS expression in pericycle and endodermis cells facing the metaxylem tubes resembled the expression pattern of the outwardly rectifying K⁺ channel SKOR (Gaymard et al. 1998), the boron transporter AtBOR1 (Takano et al. 2002) or the phosphate transporter AtPHO1 (Hamburger et al. 2002), which are all involved in xylem loading. The article by DiDonato et al., which was published during the review process of the present study, proposes a function for AtYSL2 in the transport of NA-chelated metals for the lateral movement of NA-chelated metals in the vasculature. This conclusion is based on the localization of *AtYSL2*–promoter–GUS in the vasculature and the subcellular localization of stably transformed AtYSL2–GFP in lateral plasma membranes of elongated cells within the vascular tissue. While our AtYSL2–promoter–GUS studies are fully in agreement with the high promoter activity in the vasculature, transient expression of AtYSL2–GFP in *Arabidopsis* protoplasts did not allow characterization of the subcellular localization (unpublished data), perhaps due to a loss of cell polarity and disturbed AtYSL2 targeting in suspension cultures. Despite its localization in the plasma membrane, however, it remains unclear whether AtYSL2 is indeed involved in xylem loading. If so, AtYSL2 might function as an Fe–chelate exporter even though our co-expression tests with ZmYS1 in yeast cells might argue against such a possibility (Fig. 6).

While the transcriptional regulation and the *AtYSL2* promoter–reporter gene analyses support a function for AtYSL2 in Fe homeostasis, a function for this Fe(III)–PS transporter homolog could not be established in the present study. Since most of the functional assays were conducted in parallel with ZmYS1, which reliably showed functionality in the same assay, we conclude that a different transport mechanism or substrate specificity applies for the in planta function of AtYSL2. However, it is also possible that AtYSL2 is not targeted to the plasma membrane in heterologous expression systems. To elucidate this function in future approaches, the analysis of *AtYSL2*-overexpressing plants and *AtYSL2* T-DNA insertion lines is encouraged.

**Materials and Methods**

**Plasmids, yeast strains and growth conditions**

DNA manipulations were carried out using standard protocols (Sambrook et al. 1989). Yeast media, were prepared using standard recipes (Rose et al. 1990). The ORF of *AtYSL2* was amplified by PCR from an *A. thaliana* Col-0 cDNA library (kindly provided by Karin Schumacher, ZMBP, Tübingen, Germany) using the primers 5’-ATGGGAAAAGGTTGAG-3’ and 5’-TATGAGCCGCGAGTAGTC-3’. PCR products were A-tailed, cloned into the pGEM®-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer’s instructions, sequenced and submitted to GenBank under accession number AY648977.

The *AtYSL2* ORF was subcloned into the yeast expression vector pDR195 (Rentsch et al. 1995) using the NorI restriction sites. AtYSL2 was subcloned from the pGEM®-T Easy vector into the oocyte expression vector pO20 (Ludewig et al. 2002) using the Apal–PstI restriction sites. The construction of pO02-ZmYS1 was described previously (Schaaf et al. 2004). The construct pDR195Leu-ZmYS1 was generated by subcloning ZmYS1 from pDR196-ZmYS1 (Schaaf et al. 2004) into pDR195Leu at the BamHI restriction sites. The pDR195Leu vector is a derivative of the yeast expression vector YEplac181, in which a 1.1 kb HindIII–SpfI fragment of pDR195 was inserted using HindIII–SpfI (Katie Luyten; Sympro GmbH, Tübingen, Germany, personal communication). Yeast transformed with the resulting vector pDR195Leu and its derivatives can be selected on leucine-free medium. The short N-terminal truncated versions of *AtYSL2* were cloned into the pGEM®-T Easy vector and subcloned into pDR195 as illustrated in Fig. 5. The pFL61/AtYSL2 construct kindly provided by Dr. Elisabeth Walker was published earlier in DiDonato et al. (2004).

Yeast strains used for heterologous expression were the *ctr1* mutant (BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YPR124w:: kanMX4, accession No. Y05539 from Euroscarf), its isogenic wild type (BY4741, Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; Euroscarf accession No. Y00000) and the *fet3* *fet4* mutant (DEV1435, *MATa* / *MATα ade2+*/can1/can1 his3/bi3 his4 leu2/try1/try1 ura3/ura3 fet-3/-2/His3/ fet-3/-2/His3/fet-4/-1/Leu2/fet-4/-1/Leu2, Spizyo et al. 1997). Yeast cells were transformed by the LiAc method (Gietz et al. 1992) and transformants were selected on uracil-deficient or uracil- and leucine-deficient medium containing 1% arginine as nitrogen source and the appropriate supplements.

To support growth of the *fet3* *fet4* mutant, solid YNB medium contained additionally 30 µM FeCl₂, while the liquid YPD medium was acidified to pH 5.0 with HCl.

For growth tests, a single colony was washed in 1 ml of 10x TE (pH 7.5) and resuspended in sterile water to obtain an OD (600 nm) of 0.09. Cells were streaked on uracil-deficient or uracil- and leucine-deficient YNB medium, containing 0.1% arginine, 3% glucose, 0.01% tryptophan, histidine and methionine (and leucine when appropriate), and the respective Fe or Cu source. For selective growth of *ctr1* transformants, glucose was substituted by 3% of the non-fermentable carbohydrate glycerol.

Uptake experiments in yeast were performed as described in Schaaf et al. (2004). In a release experiment, yeast cells were incubated for 20 min in uptake solution, washed in release buffer, consisting of 3% glucose, 20 mM NaH₂PO₄, 10 mM EDTA and 50 mM citric acid adjusted to pH 4 with NaOH, and resuspended in release buffer to obtain an OD (600 nm) of 0.6. The release solution was incubated further at 30°C while shaking at 500 rpm A 100 µl aliquot of uptake solu-
tion or 1 ml of release solution were diluted in 10 ml of 10 mM EDTA (pH 5.0, adjusted with NaOH) and filtered (glass microfiber filters, GF/C, Whatman, Maidstone, UK) using a vacuum filtration assembly (Hoeffer Pharmacia Biotech Inc., San Francisco, CA, USA). Filter membranes were analyzed by addition of 3 ml of 1% HCl and 3 ml of scintillation cocktail (QuickSafe A, Zinsser Analytic, Frankfurt, Germany) and liquid scintillation counting.

**Plant cultivation and Northern analysis**

*Arabidopsis thaliana* Col-0 was grown in hydroponic culture under short day conditions at 50% relative humidity with a light intensity of 150 µE s⁻¹ m⁻² and a day/night temperature regimen of 10 h (9:00–19:00) at 22°C and 14 h at 19°C, respectively. For this purpose, 1.5 ml Eppendorf tubes (Hamburg, Germany) were cut at the 1 ml sign and sealed with a nylon net (Ø 400 µm) by heating the Eppendorf cups at the bottom. The lids of the tubes were removed and the Eppendorf cups were filled with quartz sand (Ø 0.6–1.2 mm) that had been washed three times in deionized water and dried sterilized. About 30 of these cups were placed in a perforated insulating mat floating on nutrient solution in 2.5 liters pots and 5–10 seeds were placed on top of one sand-filled Eppendorf tube. In the first week, pots contained 20 mg l⁻¹ CaSO₄ in deionized water and transparent plastic panes covered the seeds. From the second week on, this solution was replaced by nutrient solution consisting of 1.8 mM KNO₃, 1 mM CaSO₄, 1 mM MgSO₄·7H₂O, 0.5 mM KH₂PO₄, 0.22 mM (NH₄)₂SO₄, 50 µM Na-Fe(III)-EDTA unless indicated otherwise, 25 µM H₂BO₃, 2.5 µM MnCl₂, 0.25 µM ZnSO₄, 0.25 µM CuSO₄, and 0.25 µM NH₄NO₃. After 3 weeks, the nutrient solution was aerated and, from the fourth week onwards, the nutrient solution was changed every 3 d. After 4 weeks, the Eppendorf tubes containing the seedlings were transferred to stable PVC trays, staying on top of the 2.5 liters pots to dry out the sand, thereby reducing algal growth. The nutrient solution did not touch the rim of the Eppendorf tubes, but roots remained almost completely immersed in the nutrient solution. Plants were harvested after 7 weeks.

For Northern analysis, 20 µg of total root or shoot RNA were separated by gel electrophoresis and transferred to a Hybond-N membrane (Amersham Biosciences Europe GmbH, Freiburg, Germany) following standard protocols. For *AtIRT1* and *AtYSL2*, fragments obtained by *NdeI* restriction digests of pFL61-IRT1 and pDR195-AtYSL2, respectively, were used as a probe.

**Real-time PCR**

Total RNA was extracted from plants grown for 4 weeks in control hydroponic culture and transferred for another 5 d to Fe- or Zn-depleted media (without added Fe or Zn) for another 4 d before harvesting. For Northern analysis, 20 µg of total root or shoot RNA were treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) for 2 h at 37°C, was used as template for synthesis of the first strand of DNA. A total of 100 U of M-MLV reverse transcriptase (Promega, Madison, WI, USA) and liquid scintillation counting.

### Generation of transgenic plants

**GUS analyses** were performed on 12 transgenic *Arabidopsis* T2 lines expressing a promoter *AtYSL2-uidA* fusion. A 1.12 kb fragment of the *AtYSL2* genomic sequence located upstream of the ATG was amplified by PCR using the primers 5'CCCACCGTTGAAAGATGGTAAG-3' and 5'CGGCCATGGGGAAGAAGACGAACTC-3', thereby introducing a HindIII site in 5' and a NcoI site in 3'. The HindIII–NcoI PCR fragment was cloned in translational fusion with the *uidA* gene in the pBKS-GUS vector (Eyal et al. 1995). The HindIII–XbaI fragment of the resulting plasmid, containing the *AtYSL2* promoter fused to the *uidA* gene, was subsequently inserted into the pBIN19 vector at the respective restriction sites. *Arabidopsis* Col-0 plants were transformed via Agrobacterium-mediated transformation (strain MP90) using the floral dip method (Clough and Bent 1998) and transformed plants were selected on kanamycin.

**GUS expression analyses**

*Histochemical GUS analysis.* Transgenic plants were grown in vitro for 10 d on control medium (containing 100 µM FeEDTA and 3 µM ZnSO₄) and transferred to Fe- or Zn-depleted media (without added Fe or Zn) for another 4 d before harvesting. GUS staining was performed overnight at 37°C in 50 mM PO₄ buffer pH 7.2, using 10 mM X-Gluc (5-bromo-4-chloro-3-indoxyl-β-d-glucuronic acid) (EUROMEDEX, Mundolsheim, France) as a substrate. Stained plants were either photographed or embedded in Technovit 7100 (Heraus-Kulzer, Wehrheim, Germany) and cut in 5 µm sections using a Leica RM 2165 (Wetzlar, Germany) microtome. Prior to the observation using an Olympus BH2 microscope (Tokyo, Japan), sections were counterstained with the Schiff dye.

**Enzymatic GUS activity**

Roots and shoots from plants of eight lines (grown as indicated above) were harvested separately and subsequently homogenized in liquid N₂. Total proteins were extracted in GUS extraction buffer (50 mM PO₄, 5 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, 0.1% Na lauryl sarcosyl). GUS activity was measured fluorometrically using 1 mM MUG (4-methylumbelliferyl-β-D-glucuronide) (EUROMEDEX, Mundolsheim, France) as a substrate (Jefferson et al. 1987). The measured activity was corrected with the total protein content (Bradford 1976).

### Preparation of metal-chelates

DNA was produced as described (von Wirén et al. 1996), and chemically synthesized NA (Miyakoshi et al. 2001) was kindly provided by Professor Takeshi Kitahara, University of Tokyo, Japan. Fe-FeNA and Fe-NA were prepared as described in Schaaf et al. (2004). Cu(II)-NA was prepared like Fe(III)-NA, but NA was supplied in 2-fold excess.

**Electrophysiological studies in Xenopus laevis oocytes**

Capped cRNA was transcribed from pOO2-ZnYS1 and pOO2-AtYSL2 in vitro using the mMessage mMachine kit (Ambion, Austin, TX, USA), after linearization of plasmids with Eco72I and MluI, respectively. Metal-chelate-induced currents in oocytes were detected by two-electrode voltage clamp 3 d after cRNA injection. Oocyte experiments were essentially conducted as described in Schaaf et al. (2004).

**Acknowledgments**

We would like to thank Annegreth Honsbein and Sabine Rauch for their excellent help with plant cultivation as well as Northern analysis,
and we gratefully acknowledge provision of the pFL61-ArYSL2 cDNA by Dr. E.L. Walker, Massachusetts, and of N.A by S. Mori and T. Kitaehara, Tokyo. We would also like to thank the anonymous reviewers for the constructive criticism and their encouragement during the review process of this paper. This study was supported by the Deutsche Forschungsgemeinschaft, Bonn, Germany, with a grant to N.v.W. (WI 1728/1 and 1728/6).

References


Verification of metal-chelate transport by AtYSL2


(Received June 11, 2004; Accepted February 25, 2005)