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Physiological and Biochemical Characterization of Metal-Phytosiderophore Transport in Graminaceous Species

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Physiological studies on the uptake of metal-phytosiderophores in strategy II plants indicated that besides iron-phytosiderophores also other phytosiderophore-chelated metals can be taken up. To verify whether this reflects a property of the recently isolated Fe(III)-phytosiderophore transporter ZmYS1, a biochemical study was undertaken using growth complementation of yeast mutants and two-electrode voltage clamp in Xenopus oocytes. These approaches allowed to show that ZmYS1 indeed transports other phytosiderophore-chelated metals, such as Cu, Zn, Co, or Ni, as well as nicotianamine-complexes with Fe(II), Fe(III), and Ni(II). Moreover, it was shown that Fe(III)-DMA is cotransported with protons by ZmYS1, thus allowing substrate transport to be driven by the negative membrane potential. Despite the transport of a broad range of phytosiderophore-chelated heavy metals, neither Zn nor Mn or Cu deficiency led to an upregulation of *ZmYS1* transcript levels in leaves or roots of maize. It is therefore concluded that the transporter ZmYS1 itself does not represent a component of the stress response to Zn-, Mn-, or Cu-limiting growth conditions, but that transport of these metal-phytosiderophores represents rather a side activity of ZmYS1.

Key Words: **iron deficiency, nicotianamine, strategy II, transition metals.**

For most heavy metal micronutrients, and for iron (Fe) in particular, transport in the soil solution as a free ion from the solid phase to the root surface is restricted. On the one hand, heavy metals like Fe, Mn, and Zn can be immobilzed due to oxidation, precipitation, or adsorption to the soil matrix, processes which are favored in well-aerated soils of neutral or alkaline pH (Marschner 1995). Then, diffusion will be the major transport path in the soil solution and thereby often limit plant growth. On the other hand, heavy metals may interact with organic substances, which will increase metal mobility, if the resulting chelate is sufficiently small to allow its transport in the soil solution (Marschner 1995). Since these metal-chelates are neutral or most frequently carry a negative charge, they may reach the root surface primarily by mass flow and thus can potentially provide larger amounts of the metal to the roots.

While chelation by low molecular-weight compounds can improve metal availability and subsequent plant nutrition, if metal solubility is below the concentration required to sustain plant growth, it will decrease metal availability at increasing concentrations of the free metal in solution (Marschner 1995). The latter is the case for Cu in peat soils, where more than 90% of water-soluble Cu can be found in the form of organic complexes (Adriano 1986). As a consequence, plants adapted to different soil environments by employing heavy metal chelation to prevent metal toxicity. For example, with increasing concentrations of soluble Al, several plant species, such as wheat, maize, or lupin, secrete organic acids to form organic Al-chelate species, that are less vulnerable to the roots compared to the free metal (Neumann and Römheld 2000).

In case of Fe, chelation in the rhizosphere solution is required for Fe acquisition in all higher plant species, except if they grow in anaerobic, water-logged soils, where the concentration of free Fe exceeds approx. 10^{-8} M and thus is sufficient to sustain plant growth (Lindsay 1979). So-called strategy I plants respond to Fe deficiency with an enhanced secretion of protons, an enhanced capacity to reduce ferric Fe and an induction of transporters for ferrous Fe. In this case chelation of ferric Fe is supposed to carry solubilized Fe(III) through the rhizosphere to the sites of Fe(III) reduction at the plasma membrane (Neumann and Römheld 2000). These chelates may mainly derive from decomposed organic matter, that provides low molecular-weight chelators, such as fulvic acids, with a considerably high affinity for Fe(III) (Pandeya et al. 1998). Alternatively, organic compounds are liberated from decaying root cells and rhizosphere microorganisms or may even be released as

part of a specific response to Fe-deficient growth conditions. The latter process has been proposed for the release of phenols, such as derivatives of coumaric or caffeic acid, which can complex Fe(III) and might support its reduction (Römheld and Marschner 1983).

In graminaceous plant species, so-called strategy II plants, Fe acquisition from soils with low Fe solubility seems completely dependent on the biosynthesis and release of phytosiderophores (Takagi 1976; Takagi et al. 1984). Phytosiderophores are hexadentate chelators, that coordinatively bind heavy metals by their hydroxy, amino / amide and carboxy residues. Their biosynthesis derives from the amino acid methionine and proceeds through nicotianamine, which is an ubiquitously occurring plant metabolite that also carries the ability to form stable complexes with heavy metals (Beneš et al. 1983; von Wirén et al. 1999). Grasses can further process nicotianamine into phytosiderophores through substitution of the terminal amino group by a hydroxyl residue to form the first phytosiderophore, 2-deoxy mugineic acid (DMA). Most grasses are able to introduce additional hydroxyl groups within the carbon chain or at the N-terminal acetidine ring to form the major hydroxylated phytosiderophores, mugineic acid (MA) or epi- / hydroxy mugineic acid (eHMA and HMA) (Ma and Nomoto 1993). Alternatively, some graminaceous species open the acetidine ring to form other phytosiderophore species like avenic acid (AA) or distichonic acid (DAA) (Ma and Nomoto 1992).

Phytosiderophores possess a relatively high affinity for the chelation of Fe(III), however, other metals, in particular Cu, Zn, and most likely also Ni, form complexes of comparable stability, decreasing in the order Fe(III) > Cu > Ni > Zn (Murakami et al. 1989). Therefore, these heavy metals represent efficient competitors in the rhizosphere that may finally decrease the amount of solubilized Fe(III). As a consequence, roots of graminaceous plants will face a mixture of different phytosiderophore-bound heavy metals according to the endogenous metal and chelate composition of the soil and the competitivity of the metal to form stable phytosiderophore complexes.

Physiological Characterization of Metal-Phytosiderophore Uptake in Roots of Grasses

In their ground-breaking study Römheld and Marschner (1986) directly compared uptake rates of radiolabelled ^{59}Fe from synthetic chelates and from phytosiderophores in strategy I plants with that in grasses, and made two important observations: 1) the uptake of Fe(III)-phytosiderophores by barley roots was not affected by the presence of the Fe(II) chelator BPDS, thus independent of a reduction step, and 2) the capacity for Fe(III)-phytosiderophore uptake in grasses was induced after plant preculture under Fe deficiency. In a subsequent study Römheld and Marschner (1990) showed that various graminaceous plant species took up at similar rates Fe(III) chelates from root exudates of different strategy II plants releasing phytosiderophores of different composition. This was confirmed in a more defined study, in which Ma et al. (1993) used Fe(III)-chelates of isolated phytosiderophore species with different structure to show that the transport system for Fe(III)-phytosiderophore in barley could hardly discriminate among the different phytosiderophore species irrespective of whether they were more or less hydroxylated or lacking the acetidine ring. In contrast, Fe(III)-chelates with synthetic aminocarboxylate ligands were apparently not transported. These observations allowed to conclude that the transport system for Fe(III)-phytosiderophores recognizes a certain backbone structure of the Fe(III)-phytosiderophore complex rather than hydroxy substitutions at the carbon chain or the acetidine ring.

The broad range of soil heavy metals mobilized by phytosiderophores (Treeby et al. 1989) and the finding that phytosiderophores were also released under Zn-deficiency stress (Zhang et al. 1989), prompted Zhang et al. (1991) to conduct uptake studies with ^{65}Zn-labelled phytosiderophores. Indeed, uptake of Zn(II)-phytosiderophores by wheat was induced under Fe deficiency (Zhang et al. 1991). This was confirmed in a subsequent study by Ma et al. (1993), in which ^{65}Zn- and ^{64}Cu-phytosiderophores were also found to be taken up by roots, however, at much lower rates compared to Fe(III)-phytosiderophores. With regard to a characterization of the metal-phytosiderophore transport system, however, these early studies suffered from two major drawbacks. First, determining uptake rates of a phytosiderophore-chelated, labelled metal did not allow to distinguish how much of the metal entered the root in a chelated form or as a free metal, and second, this type of uptake studies integrated over all existing transport proteins in the root and thus could not indicate whether transporters might exist with different metal-chelate transport properties.

It was thus a major advantage to employ the maize mutant yellow-stripe 1 (*ys1*), which showed severe symptoms of Fe deficiency under almost any growth condition due to a defect in Fe-phytosiderophore uptake. By contrast, the amount of phytosiderophores released under Fe deficiency was similar to that in wild type maize (von Wirén et al. 1994). Concentration-dependent uptake of ^{59}Fe-labelled phytosiderophores allowed to determine an almost tenfold lower uptake capacity of *ys1* roots relative to Fe-deficient wild type maize, while

the substrate affinity of the transport system appeared to be unaffected (von Wirén et al. 1995). A comparison of the uptake rates of double-labelled ^{65}Zn-(^{14}C)-phytosiderophores between *ys1* and wild type maize further allowed to show that the defective transport system in *ys1* was also responsible for the uptake of Zn-phytosiderophores (von Wirén et al. 1996).

Isolation of the Fe(III)-Phytosiderophore Transporter ZmYS1

From a transposon-mutagenized maize population a maize line was selected, that was constitutively chlorotic but regreened after excess supply of Fe fertilizers, thus exhibiting the same phenotype as the *ys1* mutant. Cloning and sequencing of the flanking regions of the inserted transposon allowed to identify *ZmYS1* as the disrupted gene in the *ys1* mutant. *ZmYS1* encodes a hydrophobic protein predicted to contain multiple transmembrane-spanning domains (Curie et al. 2001). The *ZmYS1* gene is member of the *YS1/YSL* (YSL for yellow-stripe-like) gene family, that encompasses at least three homologs in maize, 18 homologs in rice, and interestingly, also eight homologs in Arabidopsis, although Arabidopsis represents a non-phytosiderophore-synthesizing species (http://www.aramemnon.de; Curie et al. 2001; Gross et al. 2003). The YS1/YSL protein family further belongs to the superfamily of oligopeptide transporters (OPT), which are present in all organisms and transport a large variety of different substrates. This family was named after several functionally characterized members in yeast that mediate oligopeptide transport including glutathione and enkephalins (Lubkowitz et al. 1997, 1998; Bourbouloux et al. 2000; Yen et al. 2001). Several members of the plant OPT cluster have been characterized by their ability to functionally complement growth of Leu-auxotrophic yeast mutants on media supplemented with Leu-containing tetra- and pentapeptides as a sole Leu source (Koh et al. 2002). Their function in plants has so far mainly been related to long-distance transport of or seed loading with nitrogen- or sulfur-containing oligopeptides (Stacey et al. 2002). One member of this family, AtOPT3, has recently been supposed to mediate Cu transport in yeast (Wintz et al. 2003). The YS1/YSL transporter class represents so far the only transporter family for metal-chelates in the plant kingdom.

Functionality of the ZmYS1 protein has been demonstrated by growth complementation of the yeast *fet3 fet4* mutant that carries a defect in the uptake of Fe (Eide et al. 1996; Curie et al. 2001). Yeast cells expressing ZmYS1 grew on Fe(III)-DMA as a sole Fe source, while mutant cells expressing the empty vector did not (Curie et al. 2001). Since the Arabidopsis Fe(II) transporter AtIRT1 was also able to confer growth complementation on the same medium, it was suggested that small amounts of Fe(II) might be present in the Fe(III)-DMA-containing medium. Indeed, addition of BPDS restricted AtIRT1-transformed cells from growth, while ZmYS1-expressing cells continued to grow (Curie et al. 2001). This observation emphasized that only chelated, but not ionic Fe was a suitable substrate for ZmYS1.

Biochemical Characterization of the Metal-Phytosiderophore Transporter ZmYS1

Relative to dicotyledonous plants graminaceous plant species show a superior tolerance to Fe deficiency-induced chlorosis. This trait has mainly been associated with their capacity to synthesize and release phytosiderophores under Fe-deficient growth conditions (Marschner et al. 1986), while the transport mechanism of Fe(III)-phytosiderophores and its contribution to the strategy II response has so far been poorly investigated.

Using the strong *PMA1*-promoter for constitutive expression in the yeast *fet3 fet4* mutant, Schaaf et al. (2004) first reproduced growth complementation of *fet3 fet4* on Fe(III)-DMA as an Fe substrate (Curie et al. 2001), but further observed that growth complementation depended on substrate pH. To verify the influence of external pH, cRNA of in vitro-transcribed *ZmYS1* was injected into Xenopus oocytes and substrate transport was assayed by two-electrode-voltage clamp. Superfusing ZmYS1-expressing oocytes with Fe(III)-DMA-containing solutions yielded an inward current of net positive charge that increased with substrate concentration and with more negative membrane potential (Schaaf et al. 2004). Michaelis-Menton kinetics of concentration-dependent, substrate-induced currents allowed to calculate a K_m value between 5 and 10 μM for Fe(III)-DMA. Since an inward current of positive net charge was obtained, although Fe(III)-DMA complexes are neutral or even negatively charged at neutral pH (von Wirén et al. 2000), it was supposed that another, positively charged substrate was cotransported. In fact, increasing the external proton concentration strongly increased Fe(III)-DMA-induced currents, while varying external Na^+ concentrations did not. Substrate-induced currents saturated at 0.1 μM H^+ corresponding to pH 7, however, even up to pH 9.5 Fe(III)-DMA-induced currents were detectable. To further verify if Fe-phytosiderophores were transported also in a pH-dependent manner in planta, wild type maize and *ys1* mutant plants were precultured hydroponically under Fe deficiency and subjected to uptake studies at different pH using ^{59}Fe-labelled DMA complexes. Uptake rates of Fe(III)-

DMA in wild-type maize increased by lowering the pH from 7.5 to 4.5 (Schaaf et al. 2004). Furthermore, Fe(III)-DMA uptake was dramatically inhibited in the presence of the proton-uncoupler CCCP, which was in agreement with transport studies in excised barley roots, where CCCP decreased Fe(III)-MA transport by approx. two-thirds (Mihashi and Mori 1990). Taken together, these observations allowed to conclude that neutral or negatively charged Fe-phytosiderophore complexes are cotransported with one or several protons, thereby allowing the negative membrane potential across the root plasma membrane to drive Fe-phytosiderophore transport. Since this transport mechanism allows H^+ / Fe(III)-phytosiderophore transport even at alkaline pH, it is concluded that it is a substantial component of the less pH-sensitive strategy II response to Fe deficiency.

To further characterize metal specificity of ZmYS1-mediated chelate transport, the Zn uptake-defective yeast mutant *zap1* was employed. In fact, expression of ZmYS1 in *zap1* recomplemented growth of *zap1* yeast cells on Zn(II)-DMA as a sole Zn source (Schaaf et al. 2004). Furthermore, wild-type yeast transformed with ZmYS1 also transported ^{63}Ni(II)-DMA in a CCCP-dependent manner. For a broader screening of suitable metal-DMA complexes, chelate-induced currents in ZmYS1-expressing oocytes were determined at two different concentrations. Besides Fe(III)-, Zn(II)-, and Ni(II)-DMA also Co(II)- and Cu(II)-DMA induced large currents, while those of Mn(II)- and Cd(II)-DMA were lower. By contrast, unchelated DMA or free heavy metals did not induce any significant current in ZmYS1- vs. water-injected oocytes. Thus, ZmYS1 was concluded to transport a broader range of phytosiderophore-chelated metals (Schaaf et al. 2004). A partially different conclusion was achieved when transport studies by ZmYS1 were based on growth complementation of yeast mutants (Roberts et al. 2004). While ZmYS1 conferred growth complementation of an Fe or Cu uptake-defective yeast mutant on Fe- or Cu-MA, respectively, ZmYS1 did not complement growth of the yeast mutant *zrt1 zrt2*, which is defective in Zn uptake. It was speculated that the lower substrate concentration used in this study was responsible for the failed complementation on Zn-MA (Roberts et al. 2004). It should be noted, however, that also the preparation of metal-phytosiderophores largely differed, and that in both studies, the formed metal complexes were not documented.

The high structural similarity of phytosiderophores to NA and the ability of NA to form stable chelates with various heavy metals (Beneš et al. 1983) suggested that also these chelates might be suitable substrates for ZmYS1. Indeed, ZmYS1 also allowed growth complementation of the *fet3 fet4* yeast mutant on Fe(II)- and Fe(III)-NA and ZmYS1 mediated Fe(II)- and Fe(III)-NA-induced currents in oocytes (Schaaf et al. 2004). However, in both heterologous systems transport rates of Fe-NA appeared to be considerably lower compared to those of Fe(III)-DMA. Moreover, ZmYS1 also permitted transport of ^{63}Ni(II)-NA into wild-type yeast, supporting the idea that ZmYS1 or related transporter homologs might function in plants for the internal distribution or detoxification of Ni(II)-NA complexes. Such Ni(II)-NA complexes have recently been suggested to be formed in nicotianamine synthase-expressing, Ni-tolerant yeast cells (Vacchina et al. 2003). The repeatedly verified ability for metal-NA transport indicated an extended function of ZmYS1, besides that in Fe acquisition. The verification of a physiologically significant role of ZmYS1 in heavy metal-phytosiderophore and -nicotianamine transport, however, requires urgently a determination of the relevant metal species occurring in planta.

Transcriptional Regulation of ZmYS1 in Dependence of Metal Micronutrient Deficiencies

The observation of Zn(II)-, Mn(II)-, and Cu(II)-DMA transport by ZmYS1 in oocytes raised the question whether this transporter property is also a part of the plant's stress response to deficiencies of these metals. To obtain a first impression whether this might be true, a gene expression analysis was conducted in hydroponically-grown wild-type maize and *ys1* plants that were subjected to micronutrient-deficient growth conditions. In wild-type maize *ZmYS1* transcripts strongly accumulated under Fe deficiency in roots and in leaves (Figs. 1 and 2), clearly confirming the observations made by Curie et al. (2001). In *ys1* mutant plants the same upregulation was observed, although *ZmYS1* transcripts appeared at a lower size due to the insertion of the transposon (Fig. 1; Curie et al. 2001). *ZmYS1* mRNA was also detectable in Zn-deficient 16 d-old plants, however, not in significantly higher amounts compared to Zn-supplied control plants (Fig. 1A). In roots of Zn-stressed *ys1* plants *ZmYS1* transcript levels were even lower than under Zn supply. This could be explained by a better growth of Zn-supplied *ys1* plants that resulted in smaller but greener *ys1* plants (Fig. 1B), as supported by relative chlorophyll concentrations according to SPAD-meter estimations (Fig. 1B). Since Fe-EDTA is an inadequate substrate to suppress completely the Fe deficiency-stress response in strategy II plants (Römheld and Marschner 1990; von Wirén et al. 1995), Zn-deficient *ys1* plants thus were at the same time less Fe deficient than Zn-sufficient plants, resulting in a further repression of *ZmYS1* transcript levels (Fig. 1A).

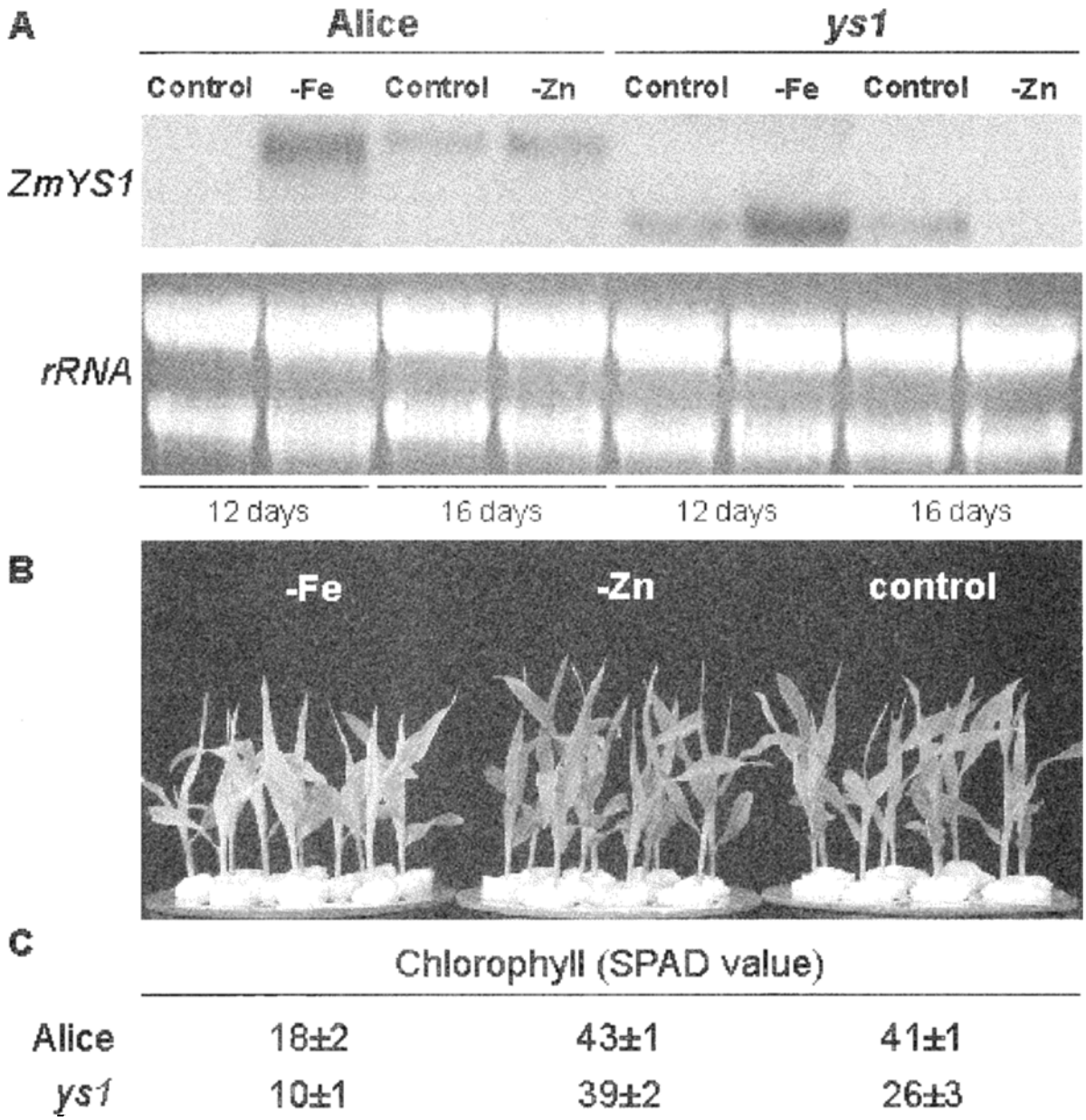


Fig. 1. Expression of *ZmYS1* under Fe and Zn deficiency in roots of maize. (A) Wild-type Alice and *ys1* mutant plants were grown hydroponically for 12 or 16 d in the absence of Fe or Zn, respectively (von Wirén et al. 1996). Twenty μg of root RNA were subjected to RNA gel blot analysis using the *ZmYS1* cDNA as a probe (Sambrook and Russell 2001; the cDNA was kindly provided by C. Curie, Montpellier). Ethidium bromide-stained rRNA is shown as a loading control. (B) Phenotype of *ys1* plants precultured under Fe- and Zn-sufficient conditions (control) or under Zn (−Zn) and Fe (−Fe) deficiency. (C) Relative chlorophyll concentrations as determined by SPAD meter at harvest in leaves of Fe-deficient, Zn-deficient, and control *ys1* plants (from left to right).

Assessing wild-type maize plants after 19 d of growth under Mn or Cu deficiency clearly allowed to observe that neither of these micronutrient deficiencies could upregulate *ZmYS1* transcription. It must be pointed out that in this experiment micronutrient deficiencies were not verified by determining microelement concentrations in the tissue; instead growth conditions were adopted that were verified in previous experiments (e.g. von Wirén et al. 1996). A similar observation was made in a gene expression analysis by Roberts et al. (2004), where *ZmYS1* showed only a 1.3-fold and 1.2-fold increase under Zn and Cu deficiency, respectively. Likewise, *ZmYS1* was not upregulated in leaves when plants were grown under Zn, Mn, or Cu deficiency (Fig. 2; Roberts et al. 2004).

Taken together, the gene expression analyses allowed to conclude that upregulation of *ZmYS1* is restricted to Fe deficiency but does not represent part of a stress response to Zn-, Mn-, or Cu-limiting growth conditions.

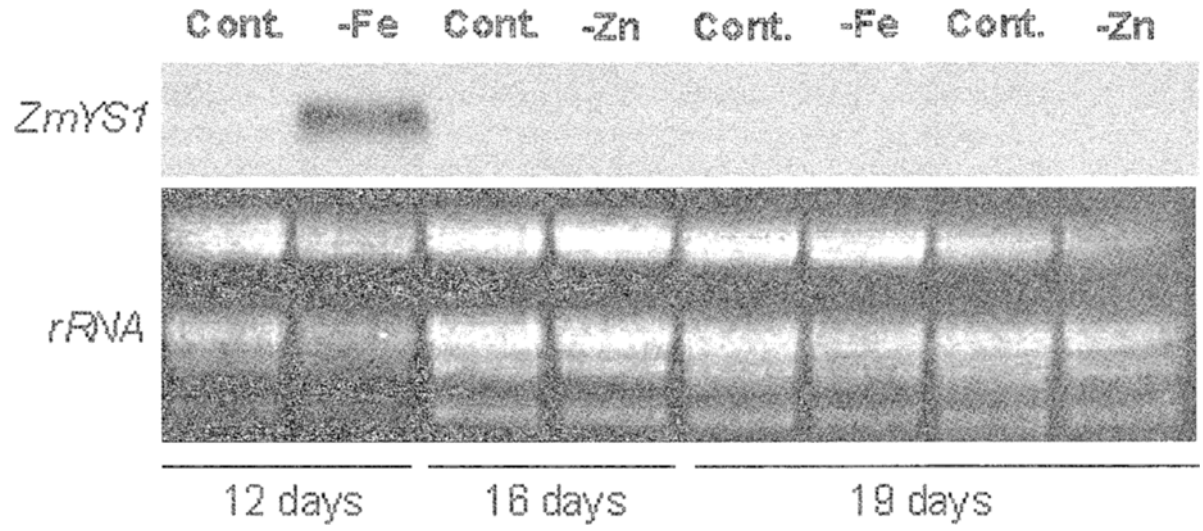


Fig. 2. Expression of *ZmYS1* under Fe, Zn, Mn, and Cu deficiency in shoots of maize. Wild-type Alice plants were grown hydroponically in the absence of Fe for 12 d, of Zn for 16 d, of Mn for 19 d, or of Cu for 19 d (von Wirén et al. 1996). Twenty μg of root RNA were subjected to RNA gel blot analysis using the *ZmYS1* cDNA as a probe (Sambrook and Russell 2001; the cDNA was kindly provided by C. Curie, Montpellier). Ethidium bromide-stained rRNA is shown as a loading control.

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