The helical propensity of the extracellular loop is responsible for the substrate specificity of Fe(III)-phytosiderophore transporters

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Iron (Fe) is an element essential to life in all organisms from microbes to the animal kingdom. As humans heavily depend on plants for dietary purposes, iron acquisition from plants is important to our nutrition [1,2]. However, Fe forms insoluble ferric (Fe(III)) complexes in neutral and alkaline soils, which generally prevent the effective uptake of Fe into the roots [3,4]. Plants of the Poaceae family efficiently acquire insoluble Fe(III) by secreting iron-chelating phytosiderophores (PS) such as mugineic acids (MAs) [3–7]. The chelated iron is absorbed as soluble complexes by the roots via transporting proteins. In maize (Zea mays), yellow stripe 1 (ZmYS1) was identified as an iron-chelating PS transporter in the roots [8]. We have previously reported that Hordeum vulgare L. yellow stripe 1 (HvYS1) is a selective transporter of Fe (III)-phytosiderophores in barley that is responsible for iron acquisition from the soil. In contrast, maize Zea mays, yellow stripe 1 (ZmYS1) possesses broad substrate specificity. In this study, a quantitative evaluation of the transport activities of HvYS1 and ZmYS1 chimera proteins revealed that the seventh extracellular membrane loop is essential for substrate specificity. The loop peptides of both transporters were prepared and analysed by circular dichroism and NMR. The spectra revealed a higher propensity for α-helical conformation of the HvYS1 loop peptide and a largely disordered structure for that of ZmYS1. These structural differences are potentially responsible for the substrate specificities of the transporters.

Keywords: α-helical content; phytosiderophore; YS1 transporter

Abbreviations
CD, circular dichroism; DMA, 2'-deoxymugineic acid; DPC, dodecylphosphocholine; GB1, protein G B1 domain; MA, mugineic acid; NA, nicotianamine; PS, phytosiderophore; YS1, yellow stripe 1; YSL, yellow stripe 1-like.
considerable substrate promiscuity, transporting metal-MA complexes such as Cd(II)-, Co(II)-, Cu(II)-, Fe(II)-, Mn(II)-, Ni(II)-, Zn(II)- and NA complexed with Fe(II) and Ni(II) in addition to Fe(III)-MAs [15–17]. Membrane trafficking of MA- or NA-metal complexes is mediated by YS1 and yellow stripe 1-like (YSL) transporter proteins, the latter of which are members of the YSL family; both of them play an important role in plant metal homeostasis [11,15]. Despite the high homology among the YS1/YSL family proteins, many of their members, such as HvYS1 and HvYSL5, possess different substrate specificities; HvYSL2 is localized in the endodermis of roots and transports PS complexed with Fe(III), Zn(II), Ni(II), Cu(II), Mn(II) and Co(II) [18], whereas HvYSL5 occurs in all root cells and has an unknown substrate [19]. Eighteen YSL genes have been identified in rice (*Oryza sativa* L.) [15]. OsYSL15 is closely related to ZmYS1 and transports Fe(III)-DMA [20,21]. Recently, the model grass *Brachypodium distachyon* has been shown to express nineteen YS1 orthologues, such as BdYS1A and BdYS1B [22]. However, BdYS1A transports Fe(III)-DMA while BdYS1B lacks this activity [22]. The distinct differences in substrate recognition among the YS1/YSL family transporters motivated us to investigate the mechanism underlying their specificities.

We have previously reported that HvYS1 is the closest homologue to ZmYS1, with a sequence similarity of 95.0% [14], but the N-terminal outer membrane region (residues 1–50 of HvYS1 and 1–53 of ZmYS1) and the seventh extracellular loop (residues 353–392 of HvYS1 and 356–397 of ZmYS1) exhibit relatively low sequence similarities (30.6% and 32.5% respectively). We showed that the loop is responsible for the Fe(III)-PS specificity of HvYS1 based on an extensive assessment of transport activity for the newly constructed chimeras in oocytes, including their transport kinetic parameters, showed that this loop is essential for the substrate specificity of HvYS1. NMR and CD spectra of the peptides corresponding to the loop regions of HvYS1 (41 residues) and ZmYS1 (42 residues) revealed that this loop peptide forms an α-helix in HvYS1, whereas it shows a random coil structure in ZmYS1.

### Materials and methods

#### Electrophysiological studies in *Xenopus laevis* oocytes

HvYS1-ZmYS1 chimeric transporters were constructed by exchanging the extracellular loop regions of HvYS1 (residues 350–392) and ZmYS1 (residues 353–396), designated ‘Hv-Zm-Hv’ and ‘Zm-Hv-Zm’. The Hv-Zm-Hv chimeric construct was prepared by the megaprimer PCR method (first PCR primers: forward, TACCACCTCATAAATTGTTGGTGTACCTGT; reverse, CCATCAGAGGAGGAAAGACCCGTCGCTG; second PCR primers: forward, TACCACACAAAAATGTTGGTGTACCTGTTAAAGAG; reverse, ACAAGGCAATACGCATTGCGTACCCAGCCG) using the QuikChange II site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA) and a HvYS1/pSP64 poly(A) X. laevis oocyte expression vector (Promega, Madison, WI, USA). The Zm-Hv-Zm chimeric construct was prepared using the ZmYS1/pSP64 vector by the megaprimer PCR (first PCR primers: forward, TCTGCATAGCTCATATGGGAGACGGTACATA; reverse, GGGGGA) using the QuikChange II site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA) and a HvYS1/pSP64 poly(A) X. laevis oocyte expression vector (Promega, Madison, WI, USA). The Zm-Hv-Zm chimeric construct was prepared using the ZmYS1/pSP64 vector by the megaprimer PCR (first PCR primers: forward, TCTGCATAGCTCATATGGGAGACGGTACATA; reverse, GGGGGA) using the QuikChange II site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA) and a HvYS1/pSP64 poly(A) X. laevis oocyte expression vector (Promega, Madison, WI, USA).

The PCR products were inserted into ZmYS1/pSP64 poly(A) vector at the HI sites. The Hv-Zm-Hv chimeric construct was prepared by exchanging the extracellular loop regions of HvYS1 (residues 350–392) and ZmYS1 (residues 353–396), designated ‘Hv-Zm-Hv’ and ‘Zm-Hv-Zm’. The Hv-Zm-Hv chimeric construct was prepared by the megaprimer PCR (first PCR primers: forward, TCTGCATAGCTCATATGGGAGACGGTACATA; reverse, GGGGGA) using the QuikChange II site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA) and a HvYS1/pSP64 poly(A) X. laevis oocyte expression vector (Promega, Madison, WI, USA). The Zm-Hv-Zm chimeric construct was prepared using the ZmYS1/pSP64 vector by the megaprimer PCR (first PCR primers: forward, TCTGCATAGCTCATATGGGAGACGGTACATA; reverse, GGGGGA) using the QuikChange II site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA) and a HvYS1/pSP64 poly(A) X. laevis oocyte expression vector (Promega, Madison, WI, USA).
concentrations of 5, 25, 50, 100 and 200 μm respectively). DMA was chemically synthesized as described previously [24] and NA was purchased from T. Hasegawa Co., Ltd. (Tokyo, Japan). Fe(III)-DMA and Fe(II)-NA complexes were prepared as described previously [9,14,17]. To prepare Fe(III)-DMA, 500 mM DMA solution was mixed with 50 mM FeCl₃ in 10 mM MES/Tris Buffer (pH 7.6). To prepare Fe(II)-NA, 200 mM NA (incubated at 65 °C for 10 min) was mixed with 50 mM FeSO₄ in 10 mM MES/Tris Buffer (pH 7.6) containing 1 mM sodium ascorbate (Sigma-Aldrich, St. Louis, MO, USA) for 2 h in the dark. Both Fe(III)-DMA and Fe(II)-NA solutions were filtered using Ultrafree-MC Centrifugal Filter Devices (Merck Millipore, Billerica, MA, USA) by centrifugation at 13 000 g for 10 min at room temperature. Acquisition and all subsequent analyses were performed using p-Clamp 10 (Molecular Devices, Sunnyvale, CA, USA).

**Preparation of HvYS1 and ZmYS1 loop fragments**

The DNA sequence coding for the extracellular loop region of HvYS1 (residues 350–392) or ZmYS1 (residues 353–396) and the gene encoding the protein G B1 domain (GB1) with a hexahistidine tag were inserted into the pET21a vector at the restriction enzyme sites *Arr*II and *Bam*HI [25]. By modifying the *Arr*II site to introduce a factor Xa cleavage site, GB1 fusion HvYS1 (residues 352–392) and ZmYS1 (residues 355–396) constructs were created. All constructs were propagated in the *Escherichia coli* strain BL21 (DE3). GB1 fusion peptides labelled with ¹³C/¹⁵N were prepared by growing cells in minimum M9 medium supplemented with ¹⁵N ammonium chloride, ¹⁵N ammonium sulphate, and ¹³C₆ glucose (as sources of nitrogen and carbon respectively). GB1 fusion peptide expression was induced by adding 0.8–1.0 mM isopropyl β-D-thiogalactopyranoside. The cells were further grown at 287 K for 48–50 h postinduction. After centrifugation, the harvested cells were suspended in 20 mM Tris-HCl (pH 7.6). The GB1 fusion peptides accumulated in the supernatant fraction were purified by C8 reverse-phase high-performance liquid chromatography (HPLC; Waters, Milford, MA, USA) using a water/acetonitrile gradient containing 0.1% trifluoroacetic acid. The purified GB1 fusion peptides were digested with factor Xa (New England Biolabs, Ipswich, MA, USA) in buffer containing 20 mM Tris-HCl (pH 7.4), 200 mM NaCl and 2 mM CaCl₂. The digested peptides were finally purified by HPLC. The molecular weights of the purified peptides were confirmed by Ultraflex III matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS; Bruker Daltonics, Bremen, Germany) and by using an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The peptide concentrations were determined by a standard BCA assay (Thermo Scientific Pierce, Rockford, IL, USA).

**CD spectroscopy**

Circular dichroism experiments were performed using a J-725 spectrophotometer (JASCO, Tokyo, Japan) with a 0.5-mm cell in 10 mM Tris-HCl (pH 7.0) buffer at 298 K. The CD spectra were measured at peptide concentrations of 0.23–0.28 mg·mL⁻¹, with and without membrane-mimicking dodecylphosphocholine (DPC) micelles. The peptide/micelle ratio was 1 : 100. The secondary structures were estimated from the CD spectra using the cdpro program [26,27].

**NMR spectroscopy**

Nuclear magnetic resonance samples contained ~0.5 mM ¹³C/¹⁵N-labelled HvYS1 or ZmYS1 loop fragment in 20 mM Tris-HCl and 50 mM NaCl buffer (pH 7.0) prepared with 95% H₂O/5% D₂O. Standard three-dimensional NMR measurements (HNCO, HNCA, HNCACB, CBCA(CO)NH and CCC(CO)NH) [28–33] were performed using an AVANCE DMX750 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) equipped with a 5-mm cryogenic TXI probe at 298 K. Transverse relaxation-optimized spectroscopy-type three-dimensional measurements [34] were performed for the HvYS1 loop fragment with a 100-fold molar excess of deuterated DPC micelles. NMR data were processed using the nmrpipe program [35], and the backbone chemical shift assignments were performed using the kuhira [36] and magro programs [37]. The secondary structures were predicted from the obtained ¹³C and ¹⁵N chemical shifts using the talos+ program [38].

**Results and Discussion**

**Activities of chimeric transporters**

We have previously assigned the transmembrane regions of HvYS1 in comparison to those of ZmYS1 using the sosui program [39]. However, in a recent review comparing the topology prediction capacity of sosui and the toposcons program [40] toposcons identified fifteen transmembrane regions in ZmYS1 [15]. In this study, the transmembrane topologies of HvYS1 and ZmYS1 including Fe(III)-MAs transporters were re-examined with TMHMM [41] (Table S1 and Fig. S1); in contrast to previous predictions, we predicted that both HvYS1 and ZmYS1 expressed the focused loop fragments on the extracellular side [23]. The transmembrane regions of HvYS1 and ZmYS1 predicted by TMHMM are marked with blue lines in Fig. 1. The HvYS1-ZmYS1 chimeras were prepared by exchanging the predicted extracellular loop regions between the seventh and eighth transmembrane helices of the HvYS1 or ZmYS1 protein (Fig. 1).
Together with HvYS1 and ZmYS1, the chimeric proteins Hv-Zm-Hv and Zm-Hv-Zm (yellow box in Fig. 1) were heterologously expressed in X. laevis oocytes. To measure currents through the expressed transporters, the oocytes were voltage-clamped at \(-60 \text{ mV}\) and superfused with buffer (pH 7.6) containing 0.5, 25, 50, 100 or 200 \(\mu\text{M}\) Fe(III)-DMA (Fig. 2A, B) or Fe(II)-NA (Fig. 2C, D). No currents were detected in water-injected control oocytes (data not shown). As shown in Fig. 2A, B, all proteins exhibited similar transport activities for Fe(III)-DMA, that is, HvYS1 \((K_m = 71.8 \mu\text{M})\), ZmYS1 \((K_m = 65.3 \mu\text{M})\), Hv-Zm-Hv \((K_m = 60.8 \mu\text{M})\) and Zm-Hv-Zm \((K_m = 44.2 \mu\text{M})\). The comparable results for the chimeric and wild-type proteins revealed the stability of their enzymatic activities under the experimental conditions (Fig. S2). The \(K_m\) values of 65.3 \(\mu\text{M}\) for ZmYS1 in this study is higher than that of 5–10 \(\mu\text{M}\) reported before [17]. Small differences in experimental conditions including pH (pH 7.6 in this study and pH 6.0 in the previous one) may influence the difference in \(K_m\) values. We used ND96 buffer at pH 7.6 for oocyte experiments because this buffer and pH are suitable for maintaining the quality of X. laevis oocytes.

For Fe(II)-NA transport, however, the activities of HvYS1 and Zm-Hv-Zm were significantly lower than those of Hv-Zm-Hv and ZmYS1 (Fig. 2C, D), although \(K_m\) values for the Fe(II)-NA complex could not be determined due to a short supply of NA. These results demonstrated that the extracellular loop between the seventh and eighth transmembrane regions of the HvYS1 protein is responsible for its higher selectivity for Fe(III)-DMA than for Fe(II)-NA.

\[\alpha\]-Helix prediction and measurement of CD spectra

Circular dichroism spectra were examined to estimate the secondary structures of the loop fragments in aqueous and membrane-mimicking solutions.
HvYS1 and ZmYS1 transporters exert their effects on substrate export through the membrane. Therefore, the membrane environment is important for maintenance of their native structures. A number of biophysical studies on peptides and proteins corresponding to the natural sequences of loops and cytoplasmic domains such as the G-protein-coupled receptors and viral proteins demonstrate that these protein fragments exhibit different degrees of helicity in aqueous versus membrane-mimicking environments [42,43]. Zwitterionic DPC micelles are frequently used as membrane mimics because the phosphatidylcholine headgroup of DPC is similar to those involved in protein–lipid interactions in eukaryote membranes. The CD spectra of the loop fragments of HvYS1 and ZmYS1 in the absence and presence of membrane-mimicking DPC micelles are shown in Fig. 3. The HvYS1 loop fragment possessed 16.9% helical content, whereas the ZmYS1 loop fragment mostly showed a random coil structure; the intensity of the negative band at 222 nm marked with an asterisk in Fig. 3 indicates α-helical content. The addition of DPC micelles altered the CD spectrum of the HvYS1 loop fragment slightly owing to an increase in the helical content to 31.8%. In contrast, the secondary structure of the ZmYS1 loop fragment remained largely unchanged (from 1.9% to 3.1%).

NMR study of HvYS1 and ZmYS1 loop fragments

The HvYS1 and ZmYS1 loop fragments were analysed by NMR to obtain residue-specific structural information. As seen in the CD spectra, the heteronuclear single quantum coherence (HSQC) spectra of both loop fragments showed narrow chemical shift dispersion in the 1H dimension, corresponding to random coil chemical shifts (Fig. 4A). Compared to the HvYS1 loop fragment, the ZmYS1 loop fragment presented a poorly resolved spectrum (Fig. 4A). 1H and 15N backbone signals were assigned to 35 (out of 41) residues in the HvYS1 loop fragments and 36 (out of 42) residues in the ZmYS1 loop fragments. Residues to
which backbone signals were not assigned were located near the N-terminus of both loop fragments. The secondary structures were predicted by TALOS+ using the obtained chemical shift assignments. We confirmed that residues L378–F387 of the HvYS1 loop fragment form a helical structure, whereas the corresponding loop fragment of ZnYS1 showed a random structure (Fig. 5A,B). Residues E379, E380, H382 and Q384 in the α-helical structural region of HvYS1 were not conserved between HvYS1 and ZnYS1 (Fig. 1), suggesting that these residues are responsible for the formation of helical structures.

Because the CD spectrum of the HvYS1 fragment was changed by the addition of DPC micelles, the HvYS1 loop fragment was analysed by NMR under the addition of the micelles (Fig. 4B,C). Induced chemical shift changes were observed in the regions close to the N- and C-termini of the fragment. In particular, M354–F360 of the N-terminal region, where the unstructured secondary structure was converted to an α-helical structure, showed large chemical shift changes upon the addition of DPC micelles (Fig. 5C). The chemical shift analysis indicated that 24% (10/41) and 41% (17/41) of the amino acid residues in the HvYS1 loop fragment formed an α-helical structure in the absence and presence of DPC micelles respectively. This DPC-elicited increase in helical content of the HvYS1 loop fragment is in agreement with the CD results.

The loop fragment of HvYS1 showed a helical structure at the N-terminus under the membrane-mimicking conditions and the loop region of the transporter was most likely anchored to the membrane surface by neighbouring transmembrane helices. Therefore, the corresponding loop of HvYS1 could be more stable in the membrane environment than in the buffer, which beneficially modulates the transporter function [43,44]. Indeed, the transport activities of the chimeric proteins did not differ from those of wild-type proteins, suggesting that the exchanged loop region mainly contributed to the selectivity of the transporters towards metal-PS complexes. HvYS1 is predicted to possess the highest helical content (29.2% α-helical content) in the homologous loop region among proteins in the YSI/YSL family (Table S1). HvYSL2, an HvYS1 homologue with broad substrate specificity [18], has low α-helix content (2.5%) in the loop region, similar to ZnYS1 (4.1%) and other YSL transporters with 1.9–5.1% α-helices in the corresponding loop as predicted by AGADIR [23]. Therefore, we assumed that the markedly high helical propensity of HvYS1 in the loop region and/or the helical structure induced in the membrane environment acts as a filter for metal-PS complexes to select Fe(III)-DMA complexes (Fig. S3).

Based on these results, we discuss the relationship between Fe(III)-DMA selectivity and structural differences in the loop region. As depicted in Fig. 2D, the chimera Zn-Hv-Zm showed lower Fe(II)-NA transport activity than did ZmYS1. This indicates that the activity of Zm-Hv-Zm, defined as $k_{\text{cat}}/K_m$, is lower than that of ZmYS1. In contrast, at high concentration (200 μM) of the NA complex, Hv-Zm-Hv, which showed a Fe(II)-NA transport activity similar to that of ZmYS1 (Fig. 2C), revealed the significantly higher activity than HvYS1, despite its high overall sequence identity (96%) with Hv-YSL2. Furthermore, Hv-Zm-Hv had little selectivity for the Fe(III)-DMA or Fe(II)-NA complex at the high concentration. These results imply that the $k_{\text{cat}}$ value of HvYS1 is not greatly dependent on the type of PS complex (DMA or NA), and thus, the transport efficiency ($k_{\text{cat}}/K_m$) of HvYS1 is mainly determined by the $K_m$ value. As $K_m$ is defined as $(k_{\text{off}} + k_{\text{cat}})/k_{\text{on}}$ ($k_{\text{on}}$ and $k_{\text{off}}$ are association and dissociation rate constants, respectively, upon binding of a PS complex to a transporter), an increase in the $K_m$ value of HvYS1 for Fe(II)-NA should be caused by a decrease in the $k_{\text{on}}$ value versus the $k_{\text{on}}$ value for Fe(III)-DMA. This means that Fe (II)-NA binds HvYS1 more slowly than Fe(III)-DMA, supporting the hypothesis that the higher helical propensity of the loop region of HvYS1 has a greater preventative effect on the entrance of Fe(II)-NA than on that of Fe(III)-DMA (Fig. S3). Next, we asked why the helical propensity influences the binding of
the PS complexes. The loops possess both positively and negatively charged residues, which can be aligned in a helical structure. On the other hand, the charge distribution and hydration degree differ between Fe(III)-DMA and Fe(II)-NA. Therefore, both or either of these properties are likely important for the interaction between the PS complexes and the loops.

In this study, we focused on the differences in the loop structure between HvYS1 and ZmYS1, and we found that the E379, E380, H382 and Q384 residues in HvYS1 are responsible for forming a helical structure. These residues could possibly be involved in PS recognition, as charged residues are believed to participate in the recognition of trivalent and divalent metal-chelator substrate complexes [15]. YSL proteins belong to oligopeptide transporters (OPT), which are commonly found in bacteria, archaea, fungi and plants [45]. Recent studies have elucidated the crystal structures of several members of the proton-coupled oligopeptide transporter (POT) family of proteins [46–52]. Members of the POT family possess a conserved architecture, consisting of 14-transmembrane \(\alpha\)-helices with N- and C-terminal helix bundles (H1–H6 and H7–H12) and two additional transmembrane \(\alpha\)-helices (HA and HB). Thus, the predicted 14-transmembrane topology of YS1 transporters is in
agreement with the OPT structures. Crystallization of plant YS1/YSL transporters has not been successful. Therefore, the structural features of the loop region shown in this study, which are expected to be responsible for substrate specificity, could facilitate future structure-based investigations into these transporters of plant origin.

Plant physiologists have attempted to produce alkaline-tolerant Poaceae plants by transformation with PS biosynthetic proteins and Fe(III)-PS transporters [53]. The level of tolerance to iron deficiency of barley is significantly higher than that of rice. In addition, transgenic rice that biosynthesizes PS [54,55] and expresses HvYS1 [56] has been shown to acquire alkaline tolerance. We have previously reported that the introduction of HvYS1 into petunia (Petunia hybrida) grants the plant with significantly enhanced tolerance to alkaline hydroponic media in the presence of the Fe(III)-DMA complex [57]. In rice, an entire original transporter gene has been successfully replaced with the whole HvYS1 gene [56]. Based on our data, we propose another strategy for modifying the substrate specificity of metal transporters by altering a small loop portion of the proteins, which could minimize the influence of these transporters on the fate and other functions of intrinsic proteins.

In conclusion, NMR and CD spectra of the loop fragments of HvYS1 and ZmYS1 transporters were determined along with the activities of HvYS1-ZmYS1 chimera transporters, and revealed that structural differences in the loop structure of the YS1/YSL transporter are associated with the substrate specificity of the transporter.

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Author contributions

EH, KS, KN and YM designed and performed the experiments; EH and KS conducted the NMR and CD measurements; YM measured the transport activities; KN performed the synthesis experiments; and EH, KS and YM wrote the paper. All authors contributed to the discussion of the results. The authors declare that they have no competing interests.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Protein sequence alignment of YS1/YSL family transporters.

**Fig. S2.** Transport activities of HvYS1, ZmYS1 and the chimeras.

**Fig. S3.** Schematic illustration of the Fe(III)-DMA selectivity of HvYS1 as compared with ZmYS1 based on the helix propensity of an extracellular loop.

**Table S1.** Transmembrane topology predictions of YS1/YSL transporters and helical propensity predictions of the loop of the transporters.