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Specific transporter for iron(III)-phytosiderophore complex involved in iron uptake by barley roots*

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Abstract: Iron (Fe) is an essential element for plant growth. Gramineous plants have generally developed a distinct strategy to efficiently acquire insoluble Fe, which is characterized by the synthesis and secretion of an Fe-chelating substance, phytosiderophore (PS) such as mugineic acid (MA), and by a specific uptake system for Fe(III)-PS complexes. In a previous study, we identified a gene specifically encoding an Fe(III)-PS transporter (HvYS1) in barley. This gene as well as the encoded protein is specifically expressed in the epidermal cells of the roots, and gene expression is greatly enhanced under Fe-deficient conditions. The localization and substrate specificity of HvYS1 indicate that it is a Fe(III)-PS specific transporter in barley roots. In contrast, ZmYS1, which has been reported as an Fe-PS transporter from maize, possesses broad substrate specificity despite a high homology with HvYS1. By assessing the transport activity of a series of HvYS1-ZmYS1 chimeras, we revealed that the outer membrane loop between the 6^{th} and 7^{th} transmembrane regions is essential for the substrate specificity. We also achieved an efficient short-step synthesis of MA and 2'-deoxymugineic acid (DMA). Our new synthetic method enabled us to use them in a large quantity for biological studies.

Keywords: soil; plants; iron; particle synthesis; phytosiderophore.

INTRODUCTION

Iron (Fe) is an essential element for all living organisms. Since animals ultimately depend on plants for their Fe, the primary uptake of Fe by plants from soil is very important for all living beings [1–4]. The availability of Fe often limits plant growth, particularly in alkaline soils, because Fe is present in the forms of insoluble $Fe(OH)_3$ at high pH, which has low bioavailability for plants [5]. Gramineous plants have a distinct Fe uptake system compared to all other plants [6], which is characterized by the synthesis and secretion of Fe-chelating substance phytosiderophore (PS) [7] and by a specific uptake for

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Fe(III)-PS complex (Fig. 1). After the identification of the first PS named mugineic acid (MA) from barley by Takemoto et al. [8], its nine analogs (MAs) have been isolated from various gramineous species and cultivars [9]. In plant tissues, nicotianamine (NA), which is a biosynthetic precursor of 2'-deoxymugineic acid (DMA) and MA, chelates Fe(II) and other divalent metals. The Fe(II)-NA complex plays a major role in the intracellular and intercellular transport of Fe in plants [10,11].

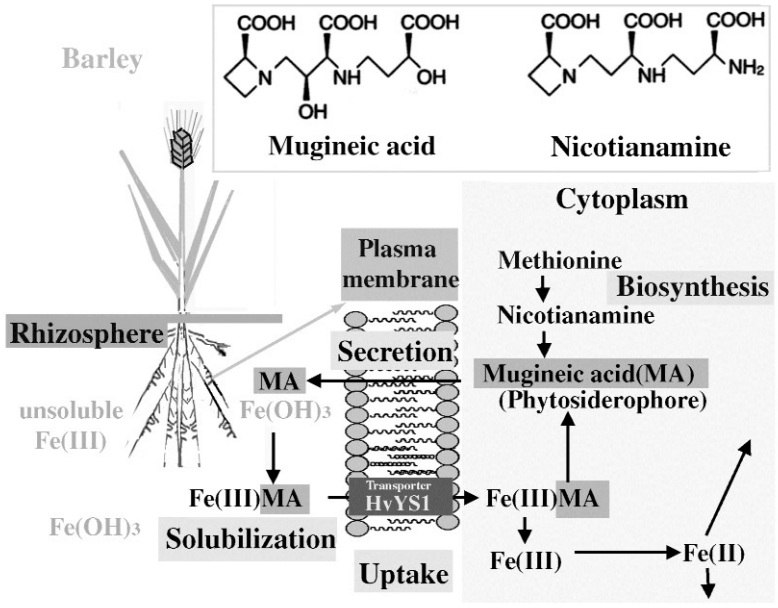


Fig. 1 Model of the specific uptake system for Fe(III)-MA in barley.

In a previous study, we have identified a transporter HvYS1 [12] from barley (*Hordeum vulgare* L.). The cDNA of *HvYS1* is 2430 bp long, and the deduced polypeptide comprises 678 amino acids. The *HvYS1* gene is predicted to encode a plasma membrane protein and a BLAST search shows that HvYS1 belongs to the oligopeptide transporter (OPT) family, which is reported from several organisms, including bacteria, archaea, fungi, and plants [13]. HvYS1 shows high homology to ZmYS1, a first protein identified as an Fe(III)-PS transporter in maize [14], with 72.7 % identity and 95.0 % similarity (Fig. 2). In particular, all predicted transmembrane regions of the two proteins have high similarities. The expression pattern of *HvYS1* gene in barley [12] showed that the *HvYS1* gene was mainly expressed in the roots. Furthermore, the expression was enhanced 50-fold in the Fe-deficient roots compared to the Fe-sufficient roots. These results suggest that HvYS1 is a transporter involved in primary Fe uptake from soil in barley roots.

In the present study, we achieved an efficient short-step synthesis of MAs [16]. We further investigated the substrate specificity of HvYS1 and elucidated the structural element responsible for the specificity by using HvYS1–ZmYS1 chimeric proteins [17].

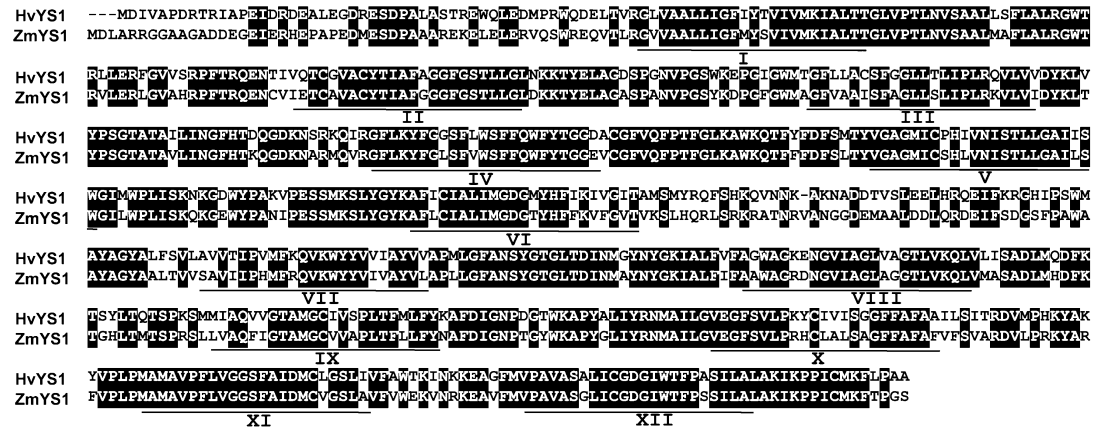


Fig. 2 Comparison of amino acid sequences of HvYS1 (barley) and ZmYS1 (maize). Twelve transmembrane regions of ZmYS1, predicted by the SOSUI program [15], are underlined. The consensus sequences are indicated by black boxes.

Synthesis of MA and DMA

Before we started our study on the function of HvYS1 and its transport mechanism, we had to establish efficient routes to supply sufficient MA **1** and DMA **2** by chemical synthesis: their supply from natural sources was quite limited (Fig. 3). Although several syntheses of DMA and MA also have been reported [18], we needed to develop a more practical synthesis to supply a large quantity of DMA with simple operation. Our efficient synthesis of DMA began with commercially available Boc-L-allylglycine **4** as shown in Scheme 1. After ozonolysis of **4**, the mixture containing **5** was directly treated with $NaBH_3CN$ and L-azetidine-2-carboxylic acid and warmed up to room temperature to give the coupled product **6**. After evaporation of the solvent, the residue was directly treated with dry hydrogen chloride in ethanol. Clean cleavage of the Boc group proceeded in the presence of the remaining excess $NaBH_3CN$ with concurrent conversion of the dicarboxylic acids to the corresponding diethyl ester **7**. The reaction mixture was evaporated to remove the excess hydrogen chloride and the residue treated with a solution of aldehyde **8** [19] and $NaBH_3CN$. The second reductive amination again proceeded smoothly to give protected DMA **9**. All the water-soluble by-products including amino acids and the excess reducing agent and its degradation products can be readily removed by simple extraction with an organic solvent at this stage. Single-flash chromatography on silica gel afforded the product **9** in a pure state. The protected DMA was obtained in a good overall yield from commercially available **4** through one-pot synthesis after just a simple chromatography at the final step. Finally, the deprotection of **9** gave DMA in a quantitative yield. After deionization by ion-exchange resin, recrystallization from EtOH-MeOH-H_2O gave free DMA **2** [16].

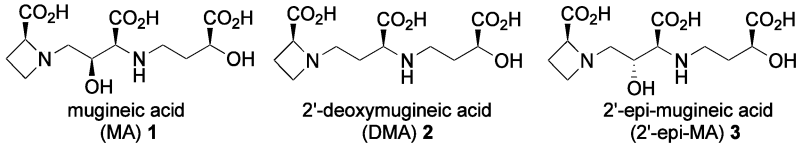
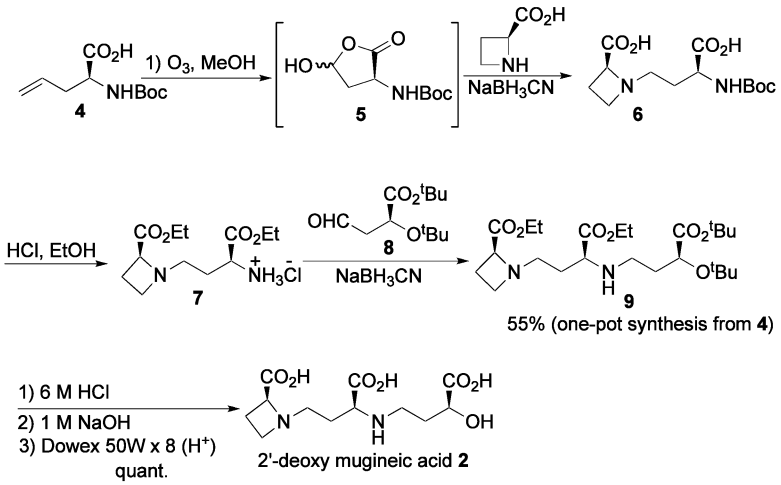


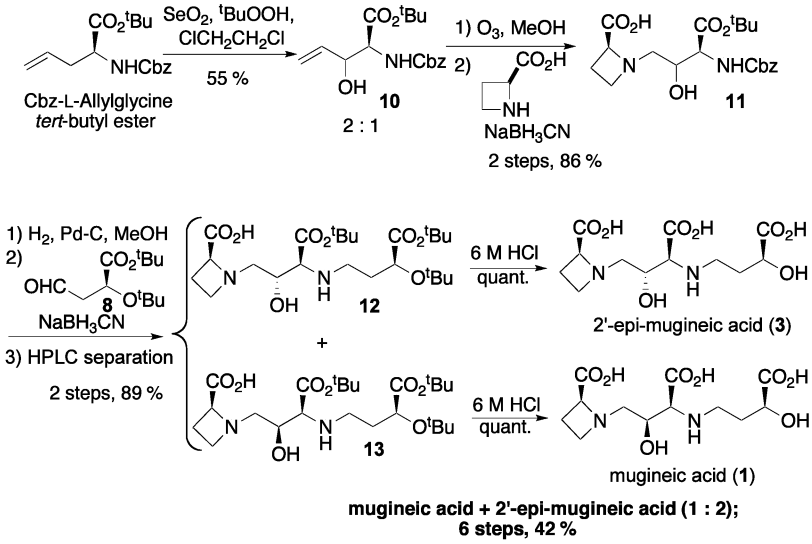
Fig. 3 The structures of MA **1**, DMA **2**, and 2'-epi-MA **3**.



Scheme 1 One-pot synthesis of a protected DMA from commercially available Boc-L-allylglycine **4**.

The same strategy was then applied to the synthesis of MA whose supply has also been required for the functional study. The same Boc-L-allylglycine was expected to be a good starting material in this case, too. 2-Hydroxy-Boc-L-allylglycine obtained by its allylic oxidation is expected to give **1** through the same series of reactions as experienced in the above synthesis of **2**.

Oxidation by selenium oxide of Boc-L-allylglycine ethyl ester proceeded smoothly at the allylic position. This oxidation, however, resulted in the formation of 9:1 diastereomeric mixture in favor of the unnatural α-configuration of the hydroxy group. Extensive investigation led to a better result: oxidation of Cbz-L-allylglycine *tert*-butyl ester by selenium oxide resulted in the formation of ca. 2:1 mixture of 2-hydroxy allylglycine **10** in a modest yield. Though the diastereoselectivity at the 2-position was still in favor of the unnatural configuration, this was the best result obtained and the mixture **10** was thus subjected to the subsequent reactions toward MA derivatives as shown in Scheme 2. Ozonolysis of **10** followed by reductive amination with free L-azetidine-2-carboxylic acid gave the desired **11** in a



Scheme 2 Synthesis of MA and its 2'-epimer (2-epi-MA).

good yield as a mixture of diastereomers. Hydrogenolytic deprotection of **11** and subsequent direct reductive amination with aldehyde **8** gave a mixture of **12** and **13** in high yields. Separation of the diastereomers was carried out by higher-performance liquid chromatography (HPLC) at this stage. The isolated diastereomers **12** and **13** were deprotected separately to give 2'-epi-MA **3** and MA **1**, respectively, in quantitative yields [16].

Substrate specificity of HvYS1 transporter

We examined the substrate specificity of HvYS1 transporter from barley and compared with that of ZmYS1 from maize using expression system in *Xenopus laevis* oocytes (Fig. 4) [12]. Oocytes were voltage-clamped at –60 mV in ND96 buffer solution at pH 7.6 and then superfused with buffer containing various metal-MA and Fe(II)-NA complexes at 50 μM. Currents were absent in water-injected control oocytes (Fig. 4). In oocytes injected with the cRNA encoding *HvYS1*, currents were induced by Fe(III)-MA. However, MA in complexes with other metals than Fe(III), including copper, zinc, nickel, manganese, or cobalt, showed very lower activities compared with that of Fe(III)-MA. Fe(II)-NA complex did not induce a significant current, either. In contrast, in oocytes injected with *ZmYS1* cRNA, currents were induced not only by Fe(III)-MA, but also by the other metal-MA complexes (Fig. 4). Fe(II)-NA also induced similar currents as Fe(III)-MA did [12]. Yeast complementation experiments also showed that ZmYS1 has broad specificity [12,20,21]. The difference in the substrate specificity between HvYS1 and ZmYS1 indicates that HvYS1 is a specific transporter for Fe(III)-MA.

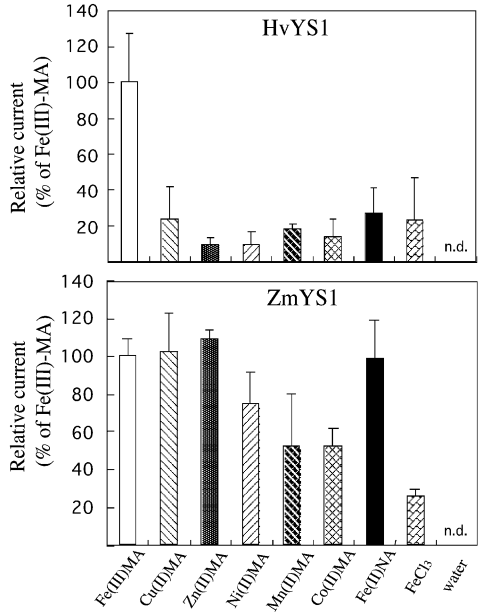


Fig. 4 Transport activity of HvYS1 and ZmYS1 by electrophysiological assay in *X. laevis* oocytes. Currents induced by various metal complexes at 50 μM in oocytes expressing HvYS1 or ZmYS1. Measurement was carried out at –60 mV. Relative currents to Fe(III)-MA are shown. Error bars show (n = 3–6).

We also investigated the effect of the hydroxy group at C2' of MA in the Fe uptake with the sufficient quantities of synthetic MAs, such as MA **1**, DMA **2**, and 2'-epi-MA **3** in hand by the same method using HvYS1 transporter. Although the structure–activity relationship of this family of MAs has been studied by uptake of Fe in barley roots [21], the influence of this secondary alcohol has not been

known. In the oocytes injected with cRNA encoding *HvYS1*, currents induced by Fe(III) complexes were similar among synthetic compounds **1**, **2**, **3**, and natural MA-Fe(III) (Fig. 5) [16]. The above result that the presence or absence of the 2'-hydroxy group has no definite effect on their Fe-transport ability of MAs. We also obtained similar results using oocytes injected with the cRNA of *ZmYS1* [16].

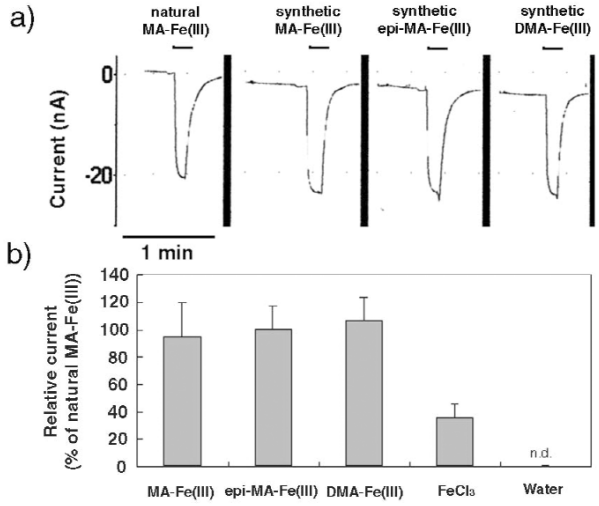


Fig. 5 Transporting activities of HvYS1 for natural MA-, synthetic MA-, 2'-epi-MA-, and DMA-Fe(III). The activity was assayed by two-electrode voltage clamp analysis with *X. laevs* oocytes. (a) Electrogenic Fe(III) complex transport activities by HvYS1. Each compound was added as Fe(III) complex at 50 μM (indicated by the black bars). (b) Currents relative to natural MA-Fe(III) are shown. Error bars: s. d. (n = 5–8).

Structure elements responsible for HvYS1 specificity

To understand the mechanism involved in the difference of substrate specificity between HvYS1 and ZmYS1 transporters, we determined the region essential for Fe(III)-PS specificity of HvYS1 using HvYS1-ZmYS1 chimeric proteins. The proteins were constructed by exchanging low-homology regions; an N-terminal fragment and the outer membrane loop connecting the 6^{th} and 7^{th} transmembrane regions (Fig. 2). Totally, we prepared two HvYS1-ZmYS1 chimeric proteins (Fig. 6) and assayed the transport activity using the expression system with *X. laevis* oocytes. All chimeric constructs as well as the wild-type HvYS1 and ZmYS1 showed similar Fe(III)-DMA transport activities. The result suggests that the Fe(III)-DMA transport activity is not affected by the exchange, either in the N-terminal region or in the particular outer membrane loop. In the next step, the relative currents inducer by Fe(II)-NA/Fe(III)-DMA were measured to investigate the substrate specificity of the chimeras (Fig. 6). The results indicate that residues 314–385 in HvYS1 are responsible for the higher selectivity of Fe(III)-DMA over Fe(II)-NA in HvYS1. We concluded that the variable regions in the middle outer membrane loops are essential and sufficient to define the transport specificity, whereas the N- and C-terminal regions have no influence on the substrate specificity.

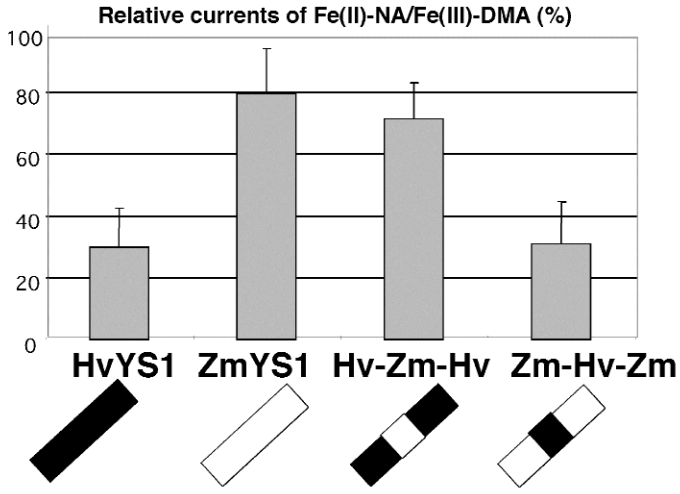


Fig. 6 Substrate-specific transporter activities of HvYS1-ZmYS1 chimeras. Replaced residues 317–389 of ZmYS1 are aligned with residues 314–385 of HvYS1. Transport activities of the wild-type HvYS1 and ZmYS1 and chimeric proteins were measured by two-electrode voltage clamp analysis in *X.* oocytes. Currents induced by 50 μM Fe(III)-DMA or Fe(II)-NA in oocytes. Error bars show standard deviation. The experiment was repeated 4–14 times.

We compared the structural differences in the outer membrane loops between HvYS1 and ZmYS1 proteins, and estimated their helix structures using AGADIR program [23], which was employed to predict the helical propensities of peptides. Higher helical propensity was estimated for the HvYS1 segment than for the ZmYS1 segment (Fig. 7A). Extensive AGADIR predictions suggested that 20 amino acids of HvYS1 (residues 373–392) were the minimal length to maintain the high helical propensity (data not shown). Based on these results, we synthesized the peptides, HvYS1pep (residues 373–392) and ZmYS1pep (residues 377–396) (Fig. 7A) using a peptide synthesizer 433A, and measured their circular dichroism (CD) spectra by a JASCO J-715 spectropolarimeter (Fig. 7B) [17]. The helical contents were estimated to be 40 % for HvYS1pep and 0 % for ZmYS1pep by the SELCON3 algorithm in the CDPro software package [24].

Our present results provide new insights into the mechanism by which Fe(III)-PS and Fe(II)-NA are specifically recognized by HvYS1. The outer membrane loop of HvYS1 is responsible for the distinct specificity for Fe(III)-PS, but the N-terminal region does not contribute to the specificity. The CD spectra indicated that HvYS1pep forms an α-helix structure, which is expected to be involved in substrate recognition. This work provides us with an additional piece of the puzzle, and NMR-based studies are currently underway in our laboratory to obtain further evidence for the interaction between Fe(III)-PS complexes and the particular loop. We are preparing two peptides of 40 amino acid residues corresponding to the outer membrane loop between the 6^{th} and 7^{th} transmembrane domains for HvYS1 and ZmYS1 to identify the amino acid residues that associate with Fe(III)-MAs. We are also planning structure analysis and transport activities by the site-specific mutants of HvYS1 or ZmYS1 transporter.

It is estimated that 2 billion people have Fe-deficiency anemia [25]. The increase in plant Fe content improves nutrient value. The study of PS, its synthesis and transport, not only helps us to understand the mechanism of primary Fe uptake, which has supported major foodstuff for human beings since ancient times, but also to produce crops with enhanced ability for Fe supply.

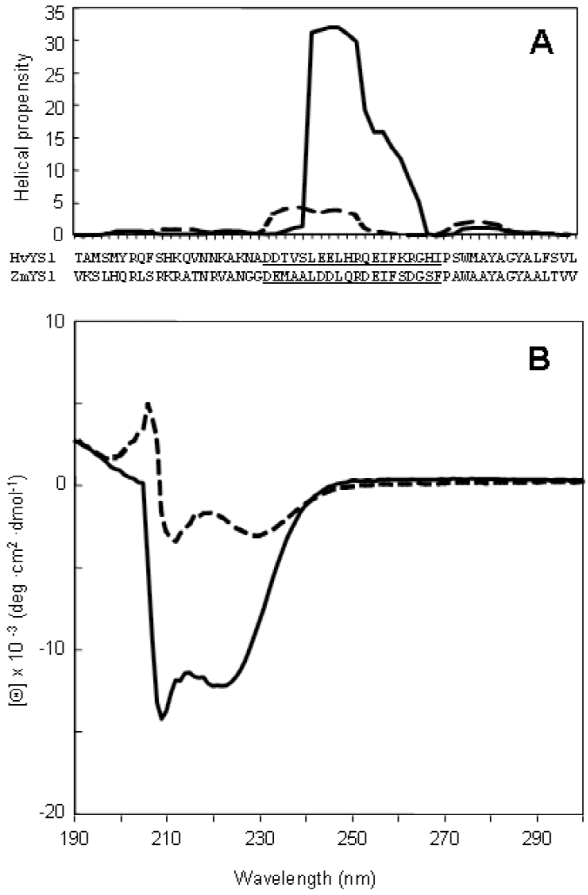


Fig. 7 Helical propensities of the outer membrane regions in HvYS1 (solid line) and ZmYS1 (broken line). (A) Amino acid sequences of HvYS1pep and ZmYS1pep are indicated by lines. (B) CD spectra of HvYS1pep (solid line) and ZmYS1pep (broken line). The buffer spectrum has subtracted from both sample spectra.

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