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Immunological characterization of a 36 kDa Fe deficiency specific peptide in barley roots

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In a previous paper we reported that an acidic 36 kDa peptide is the most strongly induced peptide among several peptides induced by Fe deficiency in barley roots. In this paper, polyclonal antibodies were raised against the 36 kDa peptide. This peptide appeared in the roots of all the graminaceous species tested (barley, rye, wheat, oat, maize, sorghum and rice) in response to Fe deficiency. More of the peptide was found in the roots of graminaceous species which secrete higher amounts of mugineic acids (MAs) under Fe deficient nutrition status. Induction of the 36 kDa peptide was first observed on the third day of Fe deficiency, rising to a maximum value on the seventh day. The trend has a positive correlation with secretion of MAs during Fe deficiency. Further, resupply of Fe resulted in a decrease in peptide production on the second day, reaching a control level on the seventh day. The rate of decrease in peptide production was observed to be slower than that of MA secretion. Other nutrient stresses such as B excess, B deficiency, Cu excess, Cu deficiency, Mn excess, Mn deficiency, Zn excess and Zn deficiency induced far less of the peptide. The specific expression of the 36 kDa peptide in roots of graminaceous species under Fe deficiency suggested the positive association of the peptide with a specific Fe deficiency tolerance mechanism in graminaceous plants.

Keywords: barley roots, graminaceous plants, immunoblotting, iron deficiency, 36 kDa peptide, 2D–PAGE

Introduction

Iron is an essential element for plant growth and is involved in various biochemical processes such as photosynthesis, respiration, redox reactions, and nitrogen fixation. Although Fe is the fourth most abundant element in the earth's crust, plants often suffer from Fe deficiency in soils with high pH, mainly calcareous soils where Fe solubility is very low. In order to obtain adequate Fe for plant growth, higher plants have developed at least two different strategies for Fe acquisition (Römheld & Marschner 1986). In response to Fe deficiency, graminaceous plants synthesize and secrete mugineic acid (MA) family phytosiderophores into the rhizosphere. These solubilize sparingly soluble Fe, and the resulting chelate complex (Fe^{3+}–MAs) is absorbed into the root through a specific transporter (Mihashi & Mori 1989). This mechanism is known as a 'Strategy -II' system specific to graminaceous plants. Among graminaceous plants, barley is known to have the strongest induction of this system, showing relatively higher Fe deficiency tolerance (Römheld 1986, Mori *et al.* 1991).

Methionine was the main precursor of MAs (Mori & Nishizawa 1987, Kawai *et al.* 1988, Mori *et al.* 1987) and *S*-adenosyl-L-methionine (SAM) was observed as a direct precursor of MAs in an *in vitro* study of barley roots (Shojima *et al.* 1989). However, this SAM synthetase activity was not induced by Fe deficiency (Takizawa *et al.* 1996). The key enzymes

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for MA biosynthesis in Fe deficiency stress are considered to be nicotianamine synthase (NAS) and nicotianamine aminotransferase (NAAT). The former synthesizes nicotianamine from three molecules of SAM and the latter transfers an amino group of nicotianamine to produce an unstable 3″-oxo intermediate which is rapidly reduced to form deoxymugineic acid (DMA) by NADPH (or NADH) (Shojima *et al.* 1990, Ohata *et al.* 1993). Several genes have been cloned by differential hybridization methods, and the genomic DNA has been sequenced. *Ids1* encodes plant metallothionein, the function of which is thought to be related to heavy metal metabolism (Okumura *et al.* 1991, 1992). Sequences of both *Ids2* (Okumura *et al.* 1994) and *Ids3* (Nakanishi *et al.* 1993) have significant homology with plant dioxygenase (Prescott 1996). It is speculated that these genes are involved in one of the synthetic pathways from DMA to other mugineic acid families (Mori & Nishizawa 1989, 1990, Ma & Nomoto 1993).

Several peptides determined by two-dimensional gel electrophoresis (2D–PAGE) were induced by Fe deficiency in barley roots (Suzuki *et al.* 1995). A 36 kDa peptide was the peptide most strongly induced by Fe deficiency. Its partial amino acid sequences were determined and no highly homologous protein was found according to the homology search. Peptide induction seemed to be specific to Fe deficiency as it was not observed in other nutrient stresses by Coomassie brilliant blue staining.

In the present study, polyclonal antibodies were raised against the 36 kDa peptide. We sought to observe whether the peptide was specifically induced in the roots of all Fe deficient graminaceous plants in order to determine the relationship between the induced 36 kDa peptide and strategy-II mechanisms.

Materials and methods

Plant materials and culture conditions

Barley (*Hordeum vulgare* L. cv. Ehimehadaka no. 1) seeds were germinated for four days at room temperature on paper towels soaked with distilled water. After germination, plants were transferred to a net floating on tap water at pH 5.5 in a greenhouse under natural light conditions. After 10 days, 36 plants (three plants per hole) were transferred to a continuously aerated nutrient solution (20 l) of the following composition: 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.2 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, 0.1 mM Fe–EDTA. Fe deficiency treatment was started on the 19th day after germination. The pH was adjusted to 5.5 daily, with the nutrient solution being changed every three days. The roots were sampled on the 36th day after germination. Other graminaceous plants, rye (*Secale cereale* L. cv. Imperial), wheat (*Triticum aestivum* L. cv. Chinese Spring), oat (*Avena sativa* L. cv. Yakushin), maize (*Zea mays* L. cv. Alice), sorghum (*Sorghum bicor* L. Moench cv. Big Jim) and rice (*Oryza sativa* L. cv. Koshihikari) were also cultured with or without Fe as described previously (Kanazawa *et al.* 1994).

Tomato (*Lycopersicon esculentum* L. cv. Bonner Beste) seeds were germinated and cultured in vermiculite with a tenfold diluted nutrient solution of the composition described above for barley until they reached a height of 5 cm. They were then transplanted to a continuously aerated solution (20 l) of the same composition used for barley with or without Fe. These Fe sufficient (+Fe) and Fe deficient (–Fe) plants were sampled for protein analysis (Higuchi *et al.* 1996a) after two weeks.

Tobacco (*Nicotiana tabacum* L. cv. Xanthi) seeds were germinated and cultured in vermiculite with a tenfold diluted nutrient solution as described for barley until they reached a height of 5 cm. They were then transplanted to a continuously aerated solution (20 l) of the same composition used for barley with or without Fe (Higuchi *et al.* 1995). After two weeks, both Fe sufficient and Fe deficient plants were sampled for protein analysis.

Isolation of the 36 kDa peptide

Fe deficient barley roots (50 g) were homogenized by means of a juice mixer for one minute in 130 ml of extraction buffer (100 mM Tris–HCl, pH 8.6, 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 5% (w/v) polyvinyl-pyrrolidone (insoluble, purchased from Tokyo Kasei Co, Tokyo, Japan), and 2.1 μM leupeptin). The homogenate was filtered through three layers of cheese cloth and centrifuged at 3000 × *g* for 30 min at 4ºC. The supernatant was mixed with 20% trichloroacetic acid (TCA) (1:1). The mixture was centrifuged at 3000 × *g* for 30 min at 4ºC. The precipitate was suspended in cold (–20ºC) acetone using a mortar and pestle, and the resulting suspension was then centrifuged at 3000 x *g* for 30 min at 4ºC. The precipitate was dried under reduced pressure for 2 h and 400 mg was dissolved in 20 ml of the sample buffer for 2D-PAGE (9 M urea, 2% Triton-X 100, 5% β-mercaptoethanol). The resulting solution was then used for 2D–PAGE following the method of O'Farrell (1975).

After 2D–PAGE, the spot corresponding to the 36 kDa peptide was isolated from the gel and stored at –20ºC. About 400 gel pieces were collected, washed twice for 5 min in 50 ml distilled water and equilibrated for 60 min with 15 ml running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS). The peptide was electro-eluted from the gel at 200 V for 2.5 h, dialysed in a cellulose tube (molar cut at 12–14 k) against several changes of distilled water for 72 h at 4ºC, and dried under reduced pressure.

Antibody production and immunoblot analysis

One rabbit was immunized by subcutaneous injection of a total of 500 μg (dry weight) peptide at two week intervals. For the first injection, the immunogen was emulsified in complete Freund's adjuvant. For the second injection and thereafter, incomplete Freund's adjuvant was used. After the 10th injection, whole blood was collected and the antiserum was stored at –80ºC. The 36 kDa peptide was analysed on SDS–PAGE or 2D–PAGE by Western blotting using the above obtained antibody with secondary antibody of goat anti rabbit IgG (H+L) horse radish peroxidase conjugate (BioRad, Tokyo, Japan) stained with diamino benzidine.

Effect of Fe deficiency treatment and Fe resupply on the appearance of the 36 kDa peptide in barley

At the third leaf developmental stage, Fe deficiency treatment was initiated in barley plants. The roots were harvested on days 0, 1, 3, 5 and 7 after Fe deficiency treatment. Protein was extracted from the roots (0.5 g each) and the 36 kDa peptide was detected by Western blot analysis. Barley plants suffering from severe Fe deficiency at fifth leaf stage were used for Fe resupply treatment with Fe–EDTA. The roots were harvested on days 0, 1, 2, 3, 4, 5, 7, 11 and 14 after Fe resupply. In both cases, root washings were collected and MAs were analysed according to the method described by Mori & Nishizawa (1987).

Results

Induction of the 36 kDa peptide by Fe deficiency and suppression by Fe resupply

Only small quantities of the 36 kDa peptide were found in the control plant which had adequate Fe supply. In Fe deficient plants, induction of the 36 kDa peptide was observed on the third day, although no MAs were detected in the root washings. The amount of peptide gradually increased to a maximum value on the seventh day when secreted MAs were almost at a maximum (483 nmol per plant) and continued until the 15th day of Fe deficiency treatment (data not shown). After Fe resupply to the Fe deficient plants (MAs 297 nmol per plant), the peptide production showed a slightly decreasing trend on the second day and thereafter continued to decline gradually until it reached control level on the seventh day when MA secretion had ceased (Figure 1).

The 36 kDa peptide under developmental stages.

The occurrence of the 36 kDa peptide in the root proteins of barley plants at different developmental stages was monitored over 80 days growth (Figure 2). Plants were germinated under distilled water, and cultured under normal culture conditions with Fe. The 36 kDa peptide was not detected in dry seeds (Figure 2, (+)Fe, 0 days). Soon after germination a small amount of the peptide was detected in the roots (Figure 2, (+)Fe, 4 and 7 days). The amount gradually increased with the age of the seedlings (Figure 2, (+)Fe, 9–30 days). In general, the older plants (40 and 80 days), had lower peptide levels than seedlings. After the transplanting stage (12th day after germination), the Fe deficient plants produced the highest amount of the 36 kDa peptide (80 days after germination; Figure 2, (–)Fe, 80 days).

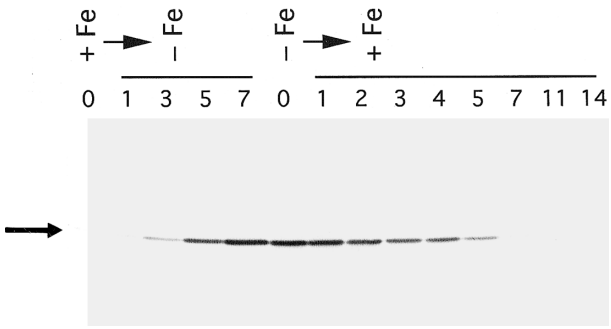

Figure 1. Western blot analysis of the occurrence of the 36 kDa peptide in barley roots as affected by duration of Fe deficiency treatment and its decrease after resupply of Fe to plants which had been cultured without Fe. For the amount of secreted MAs, see text.

Tissue distribution of the 36 kDa peptide

Tissue distribution of the 36 kDa peptide was studied in barley. The peptide was not detected in seeds and leaves (L) under Fe sufficient conditions (Figure 2, (+)Fe, 0 days, and Figure 3, barley, L(+)) or in leaves under Fe deficient conditions (Figure 3, barley, L(–)). The control roots showed low levels of the peptide (Figures 1, 3, 4, 5 and 6). In contrast, roots grown under Fe deficient conditions had higher levels of the peptide (Figure 2, (–)Fe and Figure 3, R(–)).

Induction of the 36kDa peptide under various nutrient stresses

The 36 kDa peptide was induced under several nutrient stresses including deficiency (–) and excess (+) in the following order: (–)Fe >> (–)Zn > (++)B

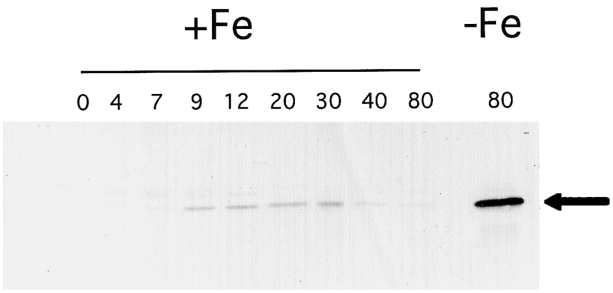

Figure 2. Western blot analysis of the occurrence of the 36 kDa peptide in seeds and in the roots of seedlings after germination. The seeds were germinated without Fe (–Fe) for 12 days, after which the seedlings were transferred to a culture solution with Fe (+Fe) or without Fe (–Fe) for up to 80 days.

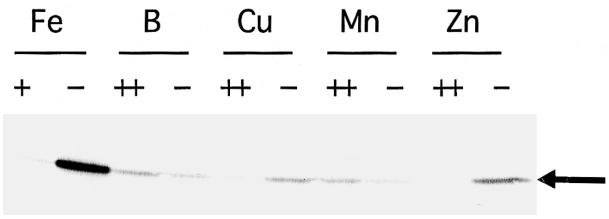

Figure 4. Western blot analysis of the effect of several nutrient stresses on the appearance of the 36 kDa peptide in the roots of barley. Culture conditions were: (–) no supply of the nutrient; (+) adequate supply of the nutrient; and (++) excess supply of the nutrient (see Okumura *et al.* 1991, 1994). For the amount of secreted MAs for each treatment, see text.

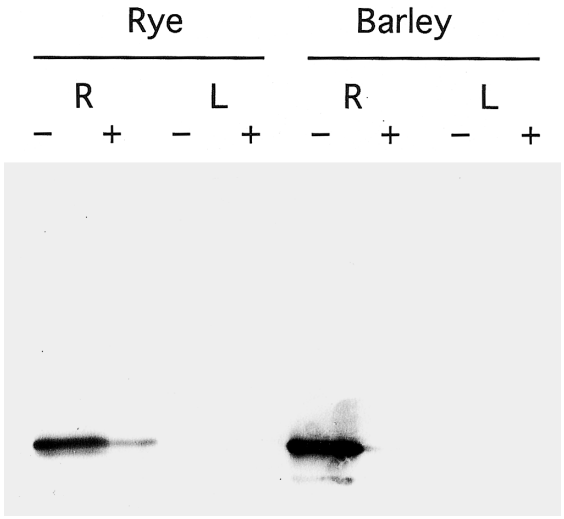

Figure 3. Western blot analysis of the appearance of the 36 kDa peptide in roots (R) and leaves (L) of barley or rye under Fe deficient and Fe sufficient conditions.

> (++)Mn ⩾ (–)Cu. However, Fe deficiency stress showed the most remarkable induction of the peptide (Figure 4). MA secretion as nmol per plant per day under these nutrient stress conditions gave the following values:

control (0.0), –Fe (466), ++B (1.9), –B (2.6), ++Cu (1.5), –Cu (1.0), ++Mn (1.0), ++Zn (1.0), and –Zn (2.3).

These values have previously been reported (Figure 1 of Suzuki *et al.* 1995). Only Fe deficient roots showed a remarkable amount of MA secretion compared to other stresses.

Presence of the 36 kDa peptide in other plant species

The occurrence of the 36 kDa peptide in other graminaceous plants along with two dicot plants was also studied. Peptides were detected by means of immunoblotting in all graminaceous plants under conditions of Fe deficiency (Figures 3 and 5). However, they could not be detected by Coomassie brilliant blue R-250 staining in the control plants of the various graminaceous species. These peptides were strongly induced by Fe deficiency and their apparent molecular weights were similar to 36 kDa with the exception of maize which showed a lower molecular weight (Figure 5). The intensity of the blotting image was in the following order: barley > rye > wheat > oat >> sorghum ⩾ maize ⩾ rice. According to immunoblotting on 2D–PAGE, these peptides have an identical pI of about 5.0 (data not shown). In tomato, the 36 kDa peptide was not detected in either leaves or roots. However, a weak band was observed in Fe deficient tobacco roots (Figure 6).

Discussion

Though the data are not quantitative, the rapid induction of the 36 kDa peptide after Fe deficiency treatment seems to be synchronous with secretion of MAs (Kanazawa *et al.* l995b), nicotianamine synthase activity (Higuchi *et al.* 1996b), nicotianamine aminotransferase activity (Kanazawa *et al.* 1995b), and transcription of *Ids3* (Nakanishi *et al.* 1993). On the other hand, the peptide suppression by Fe resupply was rather slow. On the second day of Fe resupply, the amount of MA secretion was

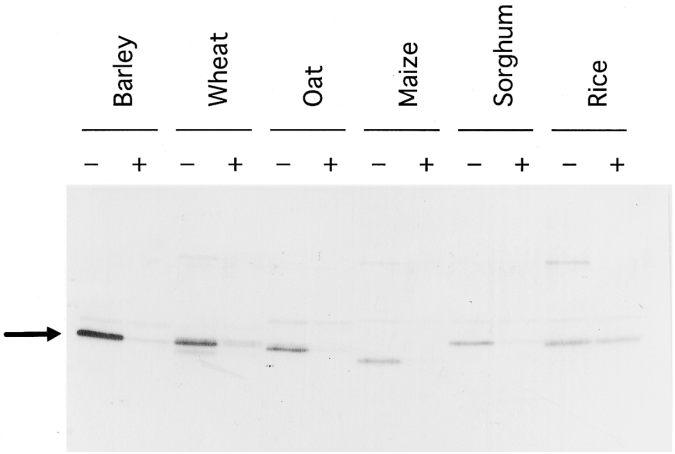

Figure 5. Western blot analysis of the occurrence of the 36 kDa peptide in the roots of various graminaceous plants. Plants were cultured with (+) or without (–) Fe.

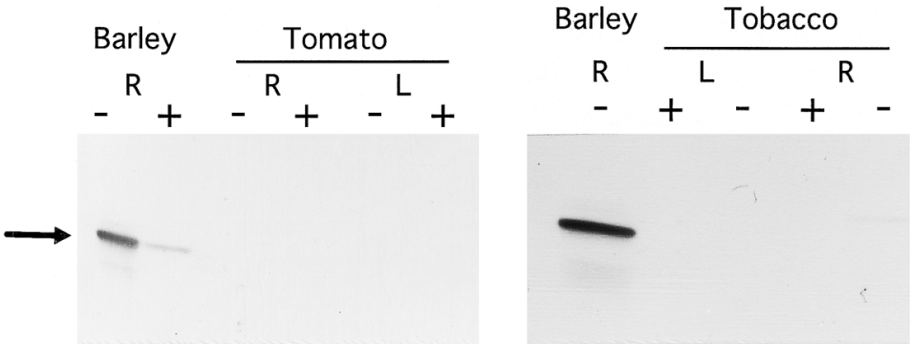

Figure 6. A trace detection of the 36 kDa peptide in the roots (R) of Fe deficient tobacco by Western blot analysis. No 36 kDa peptide was detected in roots (R) and leaves (L) of tomato plants.

reduced to about one-half of its initial value (Mori *et al.* 1988). When Fe was supplied as EpiHMA–Fe, activities of NAS and NAAT were reduced to about one-sixth and one-fourth respectively on the second day, while a decrease in the amount of the 36 kDa peptide was negligible (data not shown). The *Ids3* protein level also decreased more rapidly than the 36 kDa peptide level by Fe resupply according to Western blot analysis of both peptides (data not shown). These data suggest that the 36 kDa peptide might be more stable in roots compared with other indications mentioned above.

In agreement with our previous paper (Suzuki *et al.* 1995), no other nutrient stresses induced this peptide as strongly as conditions of Fe deficiency (Figure 4). Under Zn deficiency conditions, the 36 kDa peptide was greater when compared to other nutrient stresses. In this cultivar, however, Zn deficiency failed to promote an increase in MA release by the roots (Suzuki *et al.* 1995). However, Zn deficiency induces MA secretion only in some genotypes, in particular in Zn efficient cultivars (Zhang *et al.* 1989, Cakmak *et al.* 1994, Walter *et al.* 1994). This Zn deficiency induced MA secretion has been explained by an impaired Fe utilization, mainly Fe translocation, within the Zn efficient genotype (Walter *et al.* 1994). Thus, even a slight increase in the amount of the 36 kDa peptide by Zn deficiency treatment (Figure 4) may support the idea that Fe deficiency is induced by Zn deficiency in the root cells.

The 36 kDa peptide was observed in the roots of all graminaceous plants tested and its expression was strongly induced by Fe deficiency (Figures 3 and 5). The blotting image of the 36 kDa peptide among cultivars *per se* correlates with the order of Fe

deficiency tolerance (Römheld 1987). However, utmost care should be taken as the staining activity of immunoblotting depends on either the amount of the 36 kDa peptide or the specificity of this antibody to the isomeric 36 kDa peptide produced by each species.

Thus, the peptide function appears to be related to the synthesis of MAs in the roots of Fe deficient graminaceous plants, so-called Strategy-II plants (Römheld & Marschner 1986). Possible roles of the 36 kDa peptide may be as a synthetic enzyme of MAs, a transporter of Fe^{3+}–MAs, or a transfactor for an Iron Responsive Element (IRE: although this is not yet known in the plant kingdom) of Fe regulatory genes.

As mentioned in the Introduction, SAM synthetase was not induced by Fe deficiency treatment in the biosynthetic process of MAs (Takizawa *et al.* 1996); in addition, the molecular weights of SAM synthetases calculated from cDNAs of barley obtained by our laboratory were found to be around 28 kDa (accession numbers of *sam1, sam2* and *sam3* are D63835, D85238 and D85237, respectively), and their pI values were approximately 5.3. Therefore, the 36 kDa peptide is not SAM synthetase.

Nicotianamine synthase (NAS) activity is clearly induced in barley roots by Fe deficiency treatment and its molecular weight was speculated to be approximately 40–50 kDa (Higuchi *et al.* 1994). It is possible that the 36 kDa peptide may in fact be a subunit of NAS. Until now this protein has not been suitably purified probably due to its high susceptibility to cysteine protease (Higuchi *et al*. l996b). The existence of a trace amount of the 36 kDa peptide in Fe deficient tobacco roots is also suggestive of this idea. Nicotianamine was first discovered by Noma & Noguchi (1976) in *Nicotiana tobacum*, and NAS activity has also been observed in many varieties of tobacco plants, even though the activity is not induced by Fe deficiency. This is probably because tobacco is a dicot plant and the NAS activity was lower than the control level of Fe sufficient barley roots (Higuchi *et al.* 1995).

Nicotianamine aminotransferase (NAAT) activity was also shown to be strongly induced in barley roots under conditions of Fe deficiency (Kanazawa *et al.* l995b). The molecular weights of the two isomers as determined by gel chromatography were 80 kDa (NAAT-I) and 90 kDa (NAAT-II) as heterodimers (Kanazawa *et al.* 1995). However, during the purification of NAATs by repeated column chromatography, it was observed that as the specific activity of this enzyme increased, the intensity of the Western blotting of the 36 kDa peptide decreased (data not shown). This, therefore, rules out the possibility that one of the heteromers of NAAT is the 36 kDa peptide.

The other possibility is that the 36 kDa peptide may be a transporter of Fe^{3+}–MAs. The transporter activity in barley roots exists even under Fe sufficient conditions and this activity was seen to increase several fold by Fe deficiency treatment (Mihashi & Mori 1989, von Wirén *et al.* 1994). This provides two possibilities: (i) the transporter was activated by Fe deficiency signals; or (ii) the amount of transporter protein was increased *de novo* by Fe deficiency treatment. If the latter is the case, the 36 kDa peptide may be a candidate for the Fe^{3+}–MAs transporter. However, the fact that the transporter may be a transmembrane protein (Frommer *et al.* 1995), very little of which would have been extracted by our sample preparation method (see Materials and methods), makes this theory unlikely.

The possibility of the 36 kDa peptide being *Ids3* is also unlikely in light of the recent identification of the position of IDS3 protein on 2D–PAGE, having a pI value of 5.92 and a molecular weight of 37730 (accession number of *Ids3* is DDBJ 37 796, Nakanishi *et al*. unpublished data). In addition, there was no homologous peptide between *Ids3* protein and the 36 kDa peptide. Homology sequencing has also confirmed that *Ids1* (accession number D50641) and *Ids2* (accession number Dl5051) are not the genes responsible for the 36 kDa peptide.

In future, using the specific antibody, we intend to clone the genes of the 36 kDa peptide from the cDNA library of the expression vector constructed from the mRNA of Fe deficient barley roots.

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