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Regulation of the Iron-Deficiency Responsive Gene, *Ids2*, of Barley in Tobacco

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Abstract

Expression of the *Ids2* (iron deficiency-specific clone 2) gene was compared between native barley and heterogenous tobacco regarding spatial specificity and iron-deficiency responsiveness. The results of Northern blot and *in situ* hybridization analyses demonstrated that the *Ids2* expression in barley was specific to the endodermis and/or pericycle and in the cortex of roots, and responded exactly to iron starvation. Analysis of the GUS activity regulated by eight 5'-deletion clones of the *Ids2* promoter in tobacco exhibited that every clone could promote root specific and iron-deficiency responsive expression. Aspects of the spatial specificity and iron-deficiency responsiveness (e.g. sensitivity, stability and returnability) in tobacco were also similar to those in barley, although a few variations were recognized. It is probable that even the shortest *Ids2* promoter region in this experiment (-272/-47) contains the iron-deficiency-inducible and the root-specific cis-element(s), which is recognized by two different types of iron-acquisition strategy plants, barley and tobacco.

Accession numbers: D10057, D15051.

Key words: *Hordeum vulgare*, *Ids2*, iron-deficiency responsive expression, *Nicotiana tabacum*, promoter analysis.

Abbreviations

DMA, 2'-deoxymugineic acid (DMA); epiHDMA, 3-epihydroxy-2'-deoxymugineic acid; epiHMA, 3-epihydroxymugineic acid; GUS, β-glucuronidase; MA, mugineic acid.

Introduction

Dissolution and/or uptake of iron at the root surface are the first steps for plants to acquire iron. Two strategies, the use of which depends on the plant species, are known to exist for the iron-uptake (Römheld, 1987; Marschner and Römheld, 1995; Moog and Brüggemann, 1995; Welch, 1995). Strategy I is considered to be taken mainly by dicotyledonous and non-graminaceous species, and is characterized by induction of a plasma membrane bound reductase with enhanced net excretion of protons. Reduced iron is taken up into cytoplasm by a ferrous transporter (Kochian, 1991). Strategy II plants, which are confined to graminaceous species, increase biosynthesis and secretion of phytosiderophores designated as mugineic acid family (MAs; Takagi *et al.*, 1984). The rates of MA excretion and MA-Fe^{3+} conjugate re-absorption are clearly enhanced under iron-deficient conditions corresponding with the related enzymatic activities (Bienfait, 1988). Once the MA-Fe^{3+} conjugate has been formed, a specific transporter in the root plasma membrane mediates the uptake of the conjugate. Inside the root cell the conjugate presumably splits after reduction of Fe^{3+} to Fe^{2+} (Marschner and Römheld, 1995).

Various kinds of gene have already been isolated for both strategies. For example, aha1 (H^+-ATPase;

Harper *et al.*, 1989), *irt1* (iron transporter; Eide *et al.*, 1996) and *fro2* (ferric-chelate reductase; Robinson *et al.*, 1999) have been isolated from Arabidopsis and shown to play an important role in Strategy I. For genes of Strategy II, *sams* (*S*-adenosylmethionine synthethase; Takizawa *et al.*, 1996), *nas* (nicotianamine synthase; Herbik *et al.*, 1999; Higuchi *et al.*, 1999), and *naat* (nicotianamine aminotransferase; Takahashi *et al.*, 1999) have been isolated from barley roots. SAMS catalyzes the first step of biosynthesis of MAs from methionine to SAM (Mori and Nishizawa, 1987). NAS and NAAT catalyze the intermediate steps to MAs (Shojima *et al.*, 1990; Ma *et al.*, 1993). Furthermore, even though the cDNA and/or the proteins for catalyzing the last step from [3"-oxo acid] to MAs has not yet been clarified, two genes for putative dioxygenases designated as *Ids2* and *Ids3* (iron-deficiency-specific clones) have also been isolated from barley (Nakanishi *et al.*, 1993; Okumura *et al.*, 1994). Recent studies confirm that *Ids2* and *Ids3* encode hydroxylases that catalyze the hydroxylation of DMA to epiHDMA and/or MA to epiHMA (*Ids2*) and DMA to MA (*Ids3*), respectively (Kobayashi *et al.*, 2000; Nakanishi *et al.*, 2000). Most of these genes have been confirmed to exhibit a root specific and iron-deficiency responsive expression by means of Northern blot and/or RT-PCR experiments, though more precise levels of observation are needed to clarify the detailed regulation mechanism. As the next step, a heterologous transformation system may contribute to reveal conserved regulation mechanisms among species as well as specific mechanisms of the gene expression. Actually, Higuchi *et al.* (2001a, b) have clearly shown differences and similarities of the *HVNAS1* gene expression between native barley and heterogenous rice or tobacco using such a system.

Here, we used the promoter sequence of the *Ids2* gene of barley as a model, no homologue of which can be seen in any dicotyledonous plant species including tobacco. The *Ids2*/GUS chimeric gene was transferred into tobacco (*Nicotiana tabacum* L. cv. petit-Havana SR1), whose iron acquisition strategy is different from that of barley. Then, the *Ids2* gene expression was compared between barley and tobacco regarding the iron-deficiency responsiveness and the spatial specificity.

Materials and Methods

Analysis of the Ids2 gene expression in native barley

Barley (*Hordeum vulgare* L. cv. Ehime-hadaka No.1) seedlings were cultured in modified Kasugai nutrient solution with or without iron for one month as previously described (Nakanishi *et al.*, 1993). The barley roots were separated according to their positions (proximal position, secondary roots, intermediate position, distal position and root cap; **Fig. 1A**) and cut into small pieces. The total RNAs were isolated using a commercial kit based on the guanidium isothiocyanate and phenol system (TRIzol; Invitrogen, USA). Twenty-μg of the total RNAs of each was separated by agarose gel electrophoresis and blotted onto a membrane (Hybond N+; Amersham, USA). The *Ids2* gene expression was detected by the Northern blot hybridization method with a modified buffer system specialized for the digoxigenin (DIG) labeled probe (Engler-Blum *et al.*, 1993). The DIG labeled probe was produced by PCR (PCR DIG probe synthesis kit; Roche, Switzerland) with a set of primers (5'-GAATTC-CAAGCCATCCTCGACT CTGG and 5'-AAGC-TTCGAACGATCGAGGAAGTGAT) for covering most of coding region of the *Ids2* gene. Next, *in situ* hybridization was carried out for more detailed observation. The same barley roots as used for the Northern blot hybridization analysis were fixed, dehydrated and embedded in paraffin wax. The samples were thinly sliced with a microtome (15 μm), hybridized with a DIG-labeled RNA probe (DIG RNA probe synthesis kit, Roche, Switzerland) and the expression of the *Ids2* gene was detected by a modified method of Leitch *et al.* (1995).

Construction of the Ids2/GUS chimeric genes and the transformation of tobacco plant

The cDNA of *Ids2* was isolated from the roots of iron-starved barley (*Hordeum vulgare* L. cv. Ehime-hadaka No.1) using the differential screening method described previously. The 1650 bp of the promoter region was subsequently isolated from the barley genomic libraries using the cDNA as a probe (Okumura *et al.*, 1994, DDBJ accession number: D15051). Eight of the 5'-deletion clones (d1, -1696/-47; d2, -1540/-47; d3, -1292/-47; d4, -1127/-47; d5, -882/-47; d6, -681/-47; d7, -497/-47; d8, -272/-47 from the translation start site) were sequentially synthesized using PCR (**Fig. 4A**). The primers used were:

d1-F: 5'-GGATTGGGGATAAACACCTC,
d2-F: 5'-AGTTTTTCAGGTTTACGTTT,
d3-F: 5'-CAATCTGAATTTTCATGTAC,
d4-F: 5'-CATTTGTAATACTTAGCCGA,
d5-F: 5'-CAGGCTTGTGATTCCCTCCC,
d6-F: 5'-ACAGAGTACTATAATTAGCT,
d7-F: 5'-GGACTATGACTAGCAAAACT,
d8-F: 5'-CGAGGACGATTTGAACGGCA,
R: 5'-GAAGAGTGCGTAGGTGCTTG.

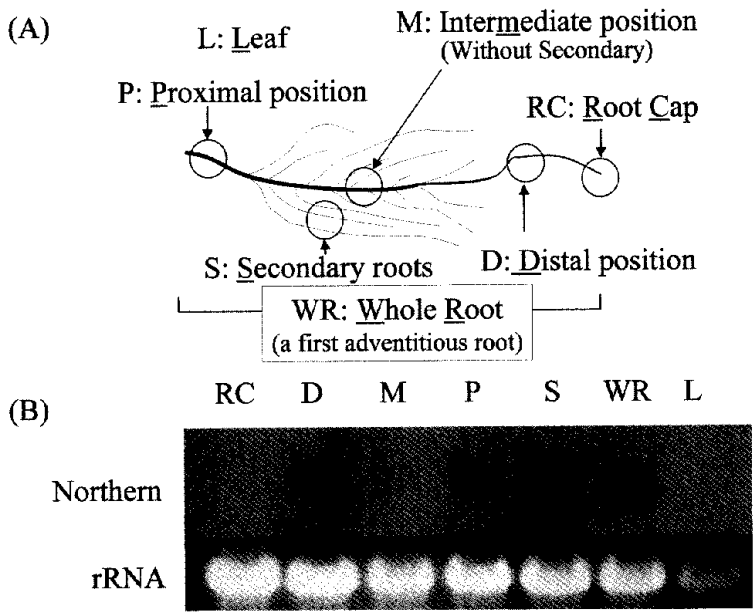

Fig. 1 Northern hybridization analysis for the *Ids2* gene expression in native barley

(A) The figure indicates positions in a root system where the total RNAs were isolated; RC, root cap; D, distal position; M, inter mediate position; P, proximal position; S, secondary roots; WR, a whole root; L, leaf.

(B) Northern hybridization analysis for the *Ids2* gene expression. Barley was grown for about one month under the iron-starved condition. 20 μg of total RNAs from respective positions of root were applied for each lane and probed with almost entire sequence of the *Ids2* gene of barley. Amounts of rRNA were used for a standard of the applied RNA levels.

These were transcriptionally fused to the upstream of the GUS reporter gene in the plasmid vector pBI101, and were introduced into tobacco plants (*Nicotiana tabacum* L. cv. Petit-Havana SR1) via *Agrobacterium* mediated infection. After confirmation of the presence of chimeric genes using PCR (data not shown), eleven to sixteen T0 transformants were subsequently acclimatized for each of the constructs, transplanted to soil and grown in a greenhouse at 30 ± 3℃ under a 12-h light and 12-h dark cycle until the harvesting of seeds (T1).

Growth conditions of the ex-plant for the GUS assay

Seedlings of the T1 generation containing the chimeric constructs were screened and grown on MS agar medium (Murashige and Skoog, 1962), which was supplemented with 3% of sucrose, 0.8% of agar, and 100 mgl^{-1} of Kanamycin under sterilized conditions for about one month. The temperature was kept at 28 ± 1 ℃ under a 16-h light and 8-h dark cycle. Then, four seedlings per line were transplanted onto the raft of a membrane culture system (Life raft, placed in a magenta box, pore size of 25 μm, Life Technologies, Rockville, USA), which was floated individually on the MS liquid medium (the same as the MS agar medium without agar, Kanamycin, and with or without iron). Seedlings were grown for about one month after being transplanted onto the membrane under the same growing conditions as above. Then, the roots and the leaves were harvested after culture, frozen by liquid nitrogen and stored at -80 ℃ until use for the GUS assay. In addition, to examine the time-dependent manner of the regulation of *Ids2* promoter, other seedlings of a typical expression line of the d2 construct were cultured in the same way as described above. To some of the iron-starved seedlings iron was re-supplied with 24 mg ml^{-1} of Fe-EDTA at 10 days after the transplanting to observe the recovery from iron deficiency. Roots and leaves were harvested at 3, 7, 14, 21, 28 days after the transplanting to the iron-deficient condition and used individually for the GUS and chlorophyll content assays.

Fluorometric and histochemical analysis of GUS activity

Assays of GUS activity were performed using the method described by Jefferson (1987). Roots and leaves of each transformant were homogenized with GUS extraction buffer (50 mM sodium phosphate, 10 mM beta-mercaptoethanol, 10 mM Na_2-EDTA, 0.1% sodium lauryl sarcosine and 0.1% (V/V)

Triton X-100), and were centrifuged at 15000 rpm for 5 minutes. The supernatants were mixed with the assay buffer (1 mM 4-methylumbelliferyl-D-glucuronide; 4-MUG in the extraction buffer) and incubated at 37 ℃ for 60 min. The concentration of the reaction product (methylumbelliferone; MU) was determined with excitation at 365 nm and emission at 455 nm by a spectrofluorometer (F2500, Hitachi, Tokyo, Japan). The GUS activity was normalized as pmoles-MU (or nmoles-MU) production per mg-protein per minute. Data were represented as the mean values and the standard errors of eleven to sixteen independent transgenic lines (4 lines for non-transgenic plants and 10 lines for 35S/GUS plants). Significant differences between each construct and the neighboring longer construct were analyzed by the non-parametrical Mann-Whitney test (Ichihara, 1990).

For histochemical observation, a hand-cut section of the root was dipped in staining solution (1 mM 5-bromo-4-indolyl-beta-D-glucuronide (X-gluc) in 100 mM phosphate buffer (pH 7.0), 10 mM Na_2-EDTA, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100) and kept at 37 ℃ overnight. Some of the sections were embedded in paraffin wax after the staining and more thinly sectioned (15 μm) with a microtome. These sections were observed under a light microscope.

Measurement of chlorophyll content

To determine the levels of iron deficiency, the chlorophyll content was measured in the leaves of the plants used for the time-course experiment. An adequate quantity of the leaves without the mid lib was measured for the fresh-weight and then ground with 1.5 ml of cold-buffered 80% aqueous acetone (2.5 mM Na-phosphate buffer, pH 7.8). The homogenates were centrifuged at 2500 rpm for 10 min, and the supernatants were recovered. Absorbance of the supernatants was measured at the wavelength of 663.6 nm and 646.6 nm, and the chlorophyll a+b concentrations in the solution were calculated using the equation described by Porra *et al.* (1989). The content of chlorophyll a+b in each plant was normalized by the fresh-weight.

Results

Ids2 gene expression in native barley

Results from the Northern blot analysis showed that the *Ids2* gene expression in native barley responded exactly to iron starvation and was specific to the root tissues, although the expression was quite variable depending on the position (**Fig. 1**). That is, secondary roots (lateral roots from an adventitious root) exhibited the most vigorous expression in the entire root system. The distal and the proximal position of adventitious roots exhibited the second most vigorous expression. In contrast, the intermediate position of adventitious roots and the root cap showed no or quite low expression. Leaves exhibited no *Ids2* gene expression irrespective of the iron condition in the culture solution. Even in secondary roots, no *Ids2* gene expression could be seen when the plant was cultured with enough iron (data not shown). The *in situ* hybridization analysis clarified the precise expression site of the *Ids2* gene in the iron-starved root (**Fig. 2A, B**). The *Ids2* gene strongly expressed in the endodermis and/or pericycle and in the cortex of the adventitious root. In contrast to these strong expressions, the epidermis and inside of the central vascular cylinder showed no expression of the *Ids2* gene.

Root specific and iron-deficiency responsive GUS expressions in tobacco regulated by the barley Ids2 gene promoter

Histochemical analysis clearly showed that the GUS activities regulated by the barley *Ids2* promoter were specifically observed in roots only when the tobacco was in an iron-deficient condition (**Fig. 2C-G**). No visible increase in the GUS activity could be seen in leaves of the same plantlets (data not shown). The GUS staining was restricted in the cortex, endodermis and/or pericycle of the root tissues. Even when the *Ids2* gene expression was strongly induced in these tissues by the iron-deficient condition, the root cap, epidermis and central part of the vascular cylinder were never stained (**Fig. 2C-F**). In addition, it was notable that the specific GUS expression was well observed in the lateral roots but not in the taproot (**Fig. 2G**).

Timing and pattern of the Ids2 expression in tobacco

A time-course experiment was conducted to determine the exact timing and pattern of the iron-deficiency responsive expression of the *Ids2* promoter in tobacco roots. The chlorophyll content in the leaves of the iron-starved plant was also measured to estimate the level of iron deficiency. The specific GUS activity in roots began to increase within three days after the transplanting of seedlings to the iron-starved condition (**Fig. 3A**). The GUS activity in 3-days iron-starved tobacco roots was about 30 times higher than that in iron-sufficient tobacco roots. Then the GUS activity increased exponentially for at least 28 days after the transplanting, and reached about 260 times higher than that in the iron-sufficient roots. When iron was re-

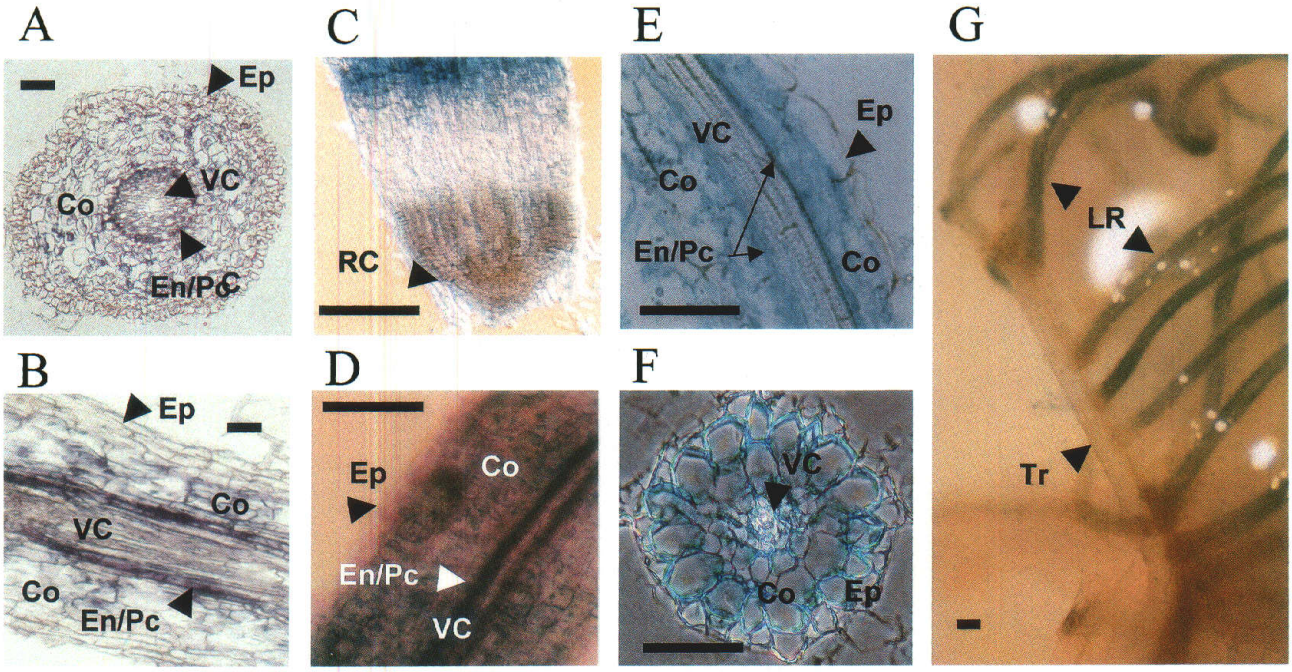

Fig. 2 In situ hybridization analysis for the endogenous *lds2* gene in barley and histochemical observation of the exogenous *lds2/GUS expression in* tobacco transformant

A horizontal (A) and a vertical (B) cross sections of iron-starved barley root were hybridized with a RNA probe of the barley *lds2* gene. Typical GUS staining pattern in iron-starved tobacco roots harboring *d2/GUS* (C, D, E, F, and G) are demonstrated. Bars are indicating a scale of 0.1mm. TR, tap root ; LR, lateral root ; VC, central vascular cylinder ; Co, cortex ; Ep, epidermis ; RC, root cap.

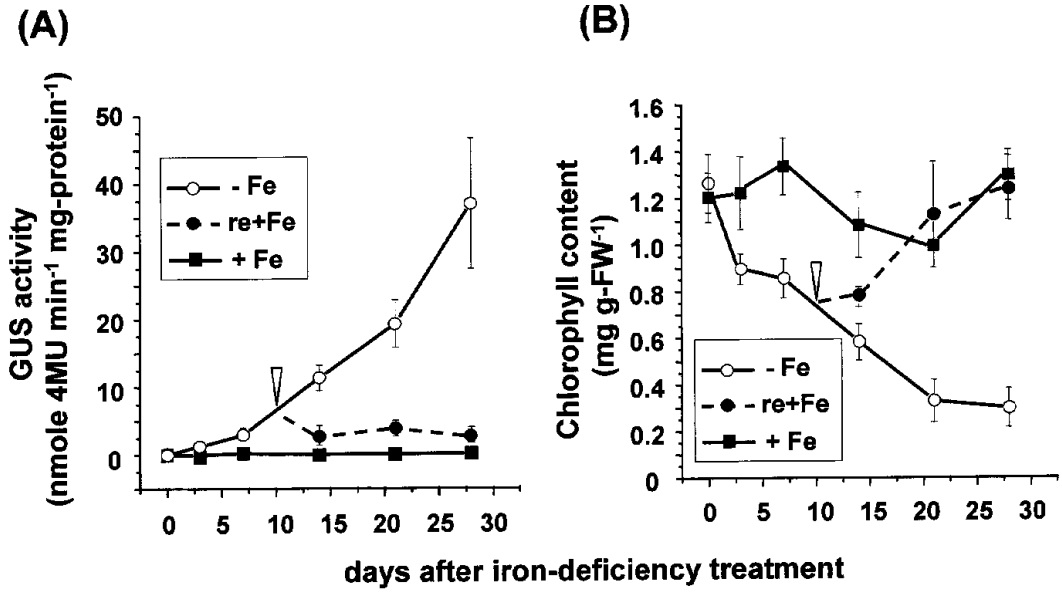

Fig. 3 Timing and pattern of *Ids2* expression in tobacco

Data displays an average ± S.E. of three independent experiments using the d2 construct. (A) Changes in the GUS activity of the transgenic tobacco roots after iron-deficiency treatment. Open circles and closed rectangles show the GUS activity regulated by the *Ids2* promoter (d2) in tobacco roots grown in iron-starved and iron-sufficient conditions, respectively. Closed circles show the GUS activity in tobacco roots after re-supplying Fe-EDTA. Open triangle indicates a timing of re-supplying Fe-EDTA (10 days after the iron-deficiency treatment). (B) Changes in the chlorophyll content in the leaves of the d2 transgenic tobacco grown under the iron-starved condition (open circles), iron-sufficient condition (closed rectangles), and after re-supplied condition (closed circles).

supplied to the iron-deficient tobacco at 10 days after the transplanting, the GUS activity quickly stopped increasing within four days at most after the re-supply. On the other hand, the chlorophyll content in the leaves decreased to about 75% of the normal level when the GUS activity began to increase (i.e. at 3 days after the transplanting, **Fig. 3B**). The content reached almost the lowest level within 21 days after the transplanting and remained at that level until the end of the experiment. Thus, the decrease of chlorophyll content in leaves and the GUS activity in roots of the iron-starved plant appeared to be inversely correlated within this duration. When iron was re-supplied, the chlorophyll content returned to the initial level within 11 days at most after the re-supply.

Expression of the Ids2 gene deletion clones/GUS in tobacco

The GUS activities regulated by every 5'-deletion clone of barley *Ids2* promoter were specifically observed in roots only when the tobacco was in an iron-deficient condition (**Fig. 4B**). This observation was carried out from averaged data of at least 11 independent lines per construct. The data of each line was based on the averaged data from four independent transformants, even though there was no significant variation among the data from these four transformants (data not shown). The induction of GUS activity by the longest promoter (d1, -1696/-47) in iron-starved roots was more than 160 times as much as that in the iron-sufficient roots. The specific GUS activity in iron-starved roots was significantly increased by the first deletion between -1696 and -1540 bp from the translational start site. The activity induced by d2 (-1540/-47) was the highest among the entire constructs. The averaged specific GUS activities tended to decline with further deletions although the differences among the constructs from d3 to d6 were not significant. Even the shortest clone among the constructs (d8, -272/-47) induced a distinct activity; the root specific and iron-deficiency responsive expression still remained in the d8 construct. However, the averaged specific GUS activity was significantly lower than that induced by the other constructs; it was about one-third of that of d1. Although the level of GUS expression varied depending on the constructs, tissue specificity was maintained among the deletion constructs used for the present assay.

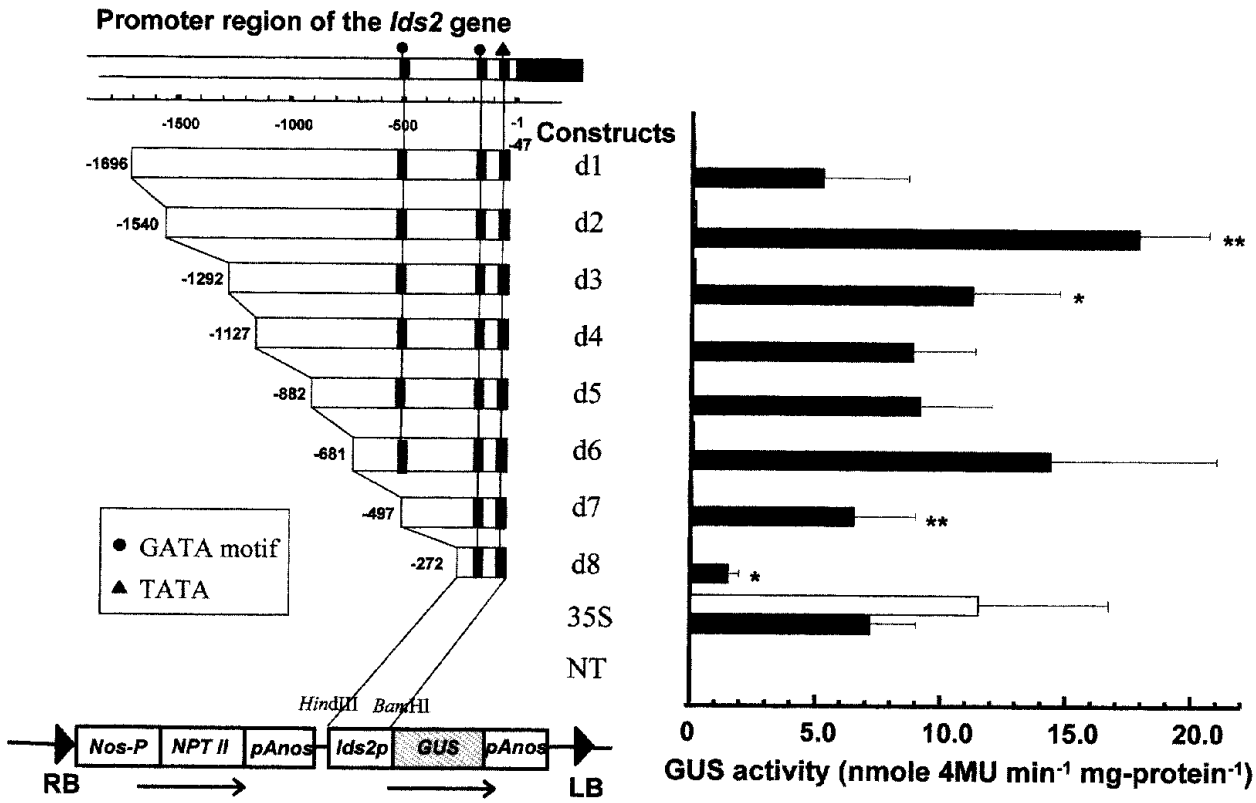

Fig. 4 Constructs and the GUS activity

Constructs of the 5'-deletion clones of the *Ids2* promoter/GUS and positions of a candidate motif (GATA motif) conferring the specific expression are indicated in the left side of the figure. 5'-deletion clones named d1 to d8 of the *Ids2* promoter were fused to the upstream region of the GUS gene in the binary vector pBI101.

The GUS activity regulated by the *Ids2* promoter in tobacco root are demonstrated in the right side of the figure. Open rectangles are indicating the results from seedlings grown under normal condition (+Fe) and closed rectangles are indicating the results from seedlings grown under iron-starved condition (-Fe). Data represent average ± S.E. of at least 11 independent experimental lines (4 lines for NT and 10 lines for 35S), which are described with the unit of nmole 4-MU min^{-1} mg-$protein^{-1}$. 35S: *CaMV* 35S promoter driven construct as a positive control, NT: Non-transformant. Significant differences between each construct and the neighboring longer construct were analyzed by non-parametrical Mann-Whitney test (*, $p<0.05$; **, $p<0.01$).

Discussion

The present study clarified that the *Ids2* gene expression responded exactly to iron starvation and was specific to the root tissues in the native barley, although the degree of expression varied according to the root position and the tissues: that is, secondary roots (lateral roots from an adventitious root) exhibited the most vigorous expression in the entire root system, while the intermediate position of adventitious roots and the root cap showed no or quite low expression. The endodermis and/or pericycle and cortex of the roots exhibited strong expression, but the central vascular cylinder and epidermis did not. These observations were reasonable, because the *Ids2* gene products, epiHMA and epiHDMA, play important roles in the acquisition of iron from soil in barley (Kobayashi *et al.*, 2000; Nakanishi *et al.*, 2000). Vigorous expression in the endodermis and cortex may contribute to the release of the product mainly in the apoplasmic space and that in the secondary roots may arise from the higher vital levels. Although the exact expression site at the tissue level has not yet been clarified in other MAs production related genes, it may be closely related to the roles of the products as in the case of the *Ids2* gene.

On the other hand, it is well known that tobacco and barley have different iron-acquisition strategies; tobacco is a Strategy I plant, whereas barley is a Strategy II plant (Moog and Brüggemann, 1995; Welch, 1995). Nevertheless, the present results clearly demonstrated that the *Ids2* gene of barley

could promote root specific and iron-deficiency responsive expression in heterogeneous tobacco. Furthermore, many aspects of the spatial specificity described above were also maintained in tobacco. The similarity is quite interesting in the view of the correlation between the role of *Ids2* gene products and the regulation of Strategy I gene expression. However, one major difference between barley and tobacco should be pointed out. In native barley, expression of the *Ids2* gene was found in all types of root although to varying degrees, whereas expression of the *Ids2* gene was observed in lateral roots but not in the taproot of tobacco. Such discrepancy may be due to the structure and/or the origin of the root tissue; all barley roots directly develop from the stem and are called adventitious roots, whereas the taproot of tobacco originates from the radicle and then lateral roots develop from the taproot (Rudall, 1992).

Aspects of the iron-deficiency responsiveness (e.g. sensitivity, stability and returnability) of the *Ids2* gene expression in tobacco were also similar to those in barley, although a few variations were recognized. For example, the *Ids2* expression in barley was scarcely detectable for at least the first 7 days after the start of iron-starved treatment (Okumura *et al.*, 1994), while that in tobacco was clearly detectable within 3 days. Even in view of the difference in the detection methods (Northern blot hybridization analysis for barley and GUS analysis for tobacco), the *Ids2* expression seemed to be more sensitive to iron deficiency in tobacco than in barley. On the other hand, the GUS activity in tobacco exponentially increased for at least 28 days from the start of iron-starved treatment, and the chlorophyll content decreased with elapse of time of iron-starved treatment. Together with the fact that the increase in GUS activity quickly stopped when iron was re-supplied, it is possible that the *Ids2* gene also stably expressed in tobacco as in barley whenever the tobacco was in a state of iron deficiency.

These observations suggest that barley and tobacco may have common mechanisms of regulating the *Ids2* expression. Also, it is probable that even the shortest *Ids2* promoter region (d8, -272/-47) in this experiment contains the iron-deficiency-inducible and the root-specific cis-element(s), which is recognized by the two different types of iron-acquisition strategy plants, barley and tobacco. Higuchi *et al.* (2001a, b) recently reported the similarities and differences in expression of the nicotianamine synthase (NAS) gene between barley and rice or barley and tobacco from this perspective. Although nicotianamine (NA) is known to play a critical role in Fe metabolism both in Strategy I and Strategy II plants, the expression control is different between the two strategies: the NAS gene expression in Strategy I plants is independent of iron-deficiency stress, whereas that in Strategy II plants sensitively responds to the stress (Higuchi *et al.*, 1995, 1999, 2001a). Interestingly, when Higuchi *et al.* (2001b) transformed the barley NAS promoter gene with the GUS gene into tobacco, the GUS expression clearly responded to iron-deficiency stress. It is possible that the endogenous NAS gene of tobacco does not contain the *cis*-element that responds to iron-deficiency stress; however, tobacco itself might have a *trans*-acting factor, which responds to its own iron-deficiency responsive gene and the factor could act with the exogenous NAS gene. In addition, even between the same Strategy II plants, barley and rice, the endogenous NAS gene expression varies; the NAS gene of barley only expresses in roots, while that of rice expresses in roots and leaves (Higuchi *et al.*, 2001a). Such a difference could also be explained by a simple relationship between *cis*-element(s) and *trans*-factor(s). Furthermore, a common regulation system among higher plant species, which may be based on a family of *cis*- and *trans*-factors, could also be a cause of the iron-deficiency responsive mechanism. This perspective would explain why the *Ids2* promoter works adequately in tobacco, in which there is no homologue. Marschner and Römheld (1995) suggested this possibility from a comparison of the internal level of Fe^{2+}-nicotianamine conjugates between Strategy I and II plants; however, no other supporting evidence has been found.

Except for a few examples such as the FUR (ferric iron uptake repressor) box system of prokaryotes (Straus, 1994), Aft1 and Aft2 transcription factors of yeast (Yamaguchi-Iwai *et al.*, 1995; Rutherford *et al.*, 2001) and 67 bp sequence of *idiA* gene of *Synechococcus elongates* (Michel *et al.*, 2001), little is known about the *cis*-elements and *trans*-factors involved in iron-deficiency responsive expression. Also, the proximal region of the *Ids2* gene promoter does not contain these known elements. Okumura *et al.* (1994) suggested some candidates for the *cis*-element responsible for the specific expression of the *Ids2* gene (root specific *cis*-element of the CaMV 35S promoter, Lam *et al.*, 1989, Benfey *et al.*, 1990; nodule-specific element of leghemoglobin, Stougaard *et al.*, 1990, Anderson *et al.*, 1997; a metal regulatory element and Cu regulatory elements of yeast metallothionein, Imbert *et al.*, 1990, Thiele, 1992). However, removal of these elements did not eliminate the ability of basic iron-deficiency responsive expression, suggesting

that most of these elements do not significantly contribute directly to the specific expression of *Ids2*. Another candidate existed in the sequence between -272 and -47 of *Ids2*, which is the shortest clone in the present analysis still showing the specific expression, and which is a complement of the GATA element situated at the positions of -157/-154 (**Fig. 4A**). The GATA element can bind a ferric uptake regulatory factor of Escherichia coli (Lucía *et al.*, 1998) and a fungal siderophores biosynthesis regulator (Zhou *et al.*, 1998; Haas *et al.*, 1999). Otherwise, a unique element might confer the specific expression of the *Ids2* gene. Further analyses may lead to a complete understanding of the molecular basis and evolution of the Fe-deficiency responsive mechanism.

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