Short report

Galactose oxidase/kelch repeat-containing protein is involved in the iron deficiency stress response in the roots of *Hyoscyamus albus*

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Abstract: To confirm the involvement of galactose oxidase/kelch repeat-containing protein (Glx) in response to iron (Fe) deficiency in *Hyoscyamus albus*, we cloned a putative full-length *HaGlx* cDNA, which contained an open reading frame (975 bp, 324 amino acids). *HaGlx* was confirmed by homology searches, molecular phylogeny analysis, and domain search. *HaGlx* was expressed in the roots but not in the leaves, and the expression significantly increased under Fe deficiency. Sequencing of ~1.9 kb of the 5'-upstream region of the *HaGlx* gene, followed by the analysis of promoter elements, resulted in the identification of multiple root-specific elements together with stress-induced elements, including the Fe deficiency-induced element (*IDE1*) core motif. This suggests that *HaGlx* plays a key role in stress responses induced under Fe deficiency in the roots. To our knowledge, this is the first report confirming, in a plant other than *Arabidopsis thaliana*, that Glx is involved in the stress response to Fe deficiency.

Keywords: *cis*-acting element, galactose oxidase/kelch repeat-containing protein, *Hyoscyamus albus*, *IDE1* core motif, iron deficiency, root

Introduction

Iron (Fe) deficiency is a major nutrient constraint for plant growth and development in neutral and alkaline soils, and it has expanded owing to over-irrigation and recent climate change. Therefore, studies investigating tolerance to Fe deficiency stress and maintenance of Fe homeostasis have been conducted (Vigani et al. 2013). Our previous studies have shown that root morphological changes such as root-tip swelling are observed in *Hyoscyamus albus* roots under conditions of Fe deficiency (Kawahara and Kitamura 2015). Secretion of riboflavins from the roots and a shift in the mitochondrial respiratory system are characteristic metabolic changes that occur in this plant's roots (Higa et al. 2010). In addition, proteomic analysis revealed an increased accumulation of proteins involved in carbohydrate metabolism and proteolysis and a decreased accumulation of proteins involved in Fe/ATP-containing metabolism and *de novo* amino acid synthesis in *H. albus* roots under Fe deficiency (Khandakar et al. 2013).

Furthermore, some proteins of unknown function, including galactose oxidase/kelch repeat-containing protein (Glx), which exhibits an increased accumulation in response to Fe depletion, were identified in *H. albus* roots (Khandakar et al. 2013). Galactose oxidase, a copper enzyme, oxidizes primary alcohols, including D-galactose and its constituent polysaccharides, to aldehydes by the reduction of O_2 to H_2O_2 (Whittaker 2005). The kelch motif was first identified in *Drosophila* kelch protein as being related to galactose oxidase, for which a structure was resolved and then recognized to be distributed widely from prokaryotes to eukaryotes (Adams et al. 2000). Although the function of bacterial and fungus Glx has been studied relatively well (Paukner et al. 2014, Yin et al. 2015), the function of plant Glx remains elusive.

Recently, the involvement of Glx or the *Glx* gene in the response to Fe deficiency has been studied

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closely (Ivanov et al. 2012, Khandakar et al. 2013, Mai et al. 2016). Despite this, the *Glx* sequence information is available only for *Arabidopsis thaliana* and *Medicago truncatula* among higher plants so far, and no functional study is available. Therefore, the aims of this study were to confirm the presence of Glx in *H. albus*, to determine the involvement of Glx in the stress response to Fe deficiency, and to clarify the phylogenetic relationship between HaGlx and other plant Glxs using a gene cloning approach. In addition, the promoter region of *HaGlx* was analysed to determine *cis*-elements involved in the Fe deficiency stress response.

Materials and Methods

Culture methods

Hairy roots of *H. albus* L. (Solanaceae) used in these experiments were established previously (Higa et al. 2008). A primary root tip with a few lateral roots (ca. 2 cm in length) was pre-propagated in normal liquid MS medium (Murashige and Skoog 1962) containing 3% sucrose for 2 weeks. After pre-propagation, root cultures were separated into subsets by exchanging the medium for fresh MS medium containing 3% sucrose, with or without Fe; the culture was then continued for 5 days. All cultures were incubated at 25°C with agitation at 80 rpm on a rotary shaker.

RNA extraction and RT-PCR

Total RNA was isolated from the roots (ca. 100 mg) using an RNeasy® Plant Mini Kit (Qiagen, Tokyo, Japan). RT-PCR was performed using a Prime Script™ RT-PCR kit (Takara Bio, Shiga, Japan) with gene-specific primers. A sample aliquot containing 0.5 μg RNA was subjected to reverse transcription (30 min at 42°C, 5 min at 65°C). The PCR conditions were 94°C for 3 min for the initial cycle; 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and an extra cycle with a 7-min extension step at 72°C. Primers used to determine gene expression, organ specificity, and stress responses were as follows: forward (5′-GCAGCAACACAAGGCA AATGGATCAAG-3′) and reverse (5′-CCGCTT CCAAACTAGTGTCTCGGTGTCA-3′). A universal primer pair for *18S* ribosomal RNA (315 bp) was added in the same reaction mixture as a control for semi-quantification according to the manufacturer's recommendations (Applied Biosystems, Foster City, USA).

cDNA cloning

Initial primers for *H. albus Glx* (*HaGlx*) were designed by aligning the conserved cDNA sequences of these genes from *A. thaliana* (NM_111651) and *M. truncatula* (XM_003596116) previously deposited in the database (GenBank). RT-PCR was performed as described in section 2.2, using the primers as follows: forward (5′-GCTACCACTCGATGACTGC-3′) and reverse (5′-CACTAGCAAAGGCGCACCA-3′). After PCR amplification, products were cloned using a TOPO TA Cloning kit (Invitrogen, Tokyo, Japan). The full-length cDNA sequence was analysed by 3′ RACE and 5′ RACE methods using a SMARTer™ RACE cDNA Amplification Kit (Clontech, Shiga, Japan). Based on the partially revealed sequence, the new primers for 3′ RACE and 5′ RACE were designed as follows: forward for 3′ RACE (5′-GGTTGTGGAA ATGCTGGAAGG-3′) and reverse for 5′ RACE (5′-CCTTCCAGCATTTCCACAACCACCAAAG-3′). After cloning and sequencing, the data were submitted to GenBank (the accession number of *HaGlx* is LC155941).

DNA extraction and cloning of the HaGlx promoter region

Genomic DNA was isolated from the roots (ca. 100 mg) using the ISOPLANT II kit (Nippon Gene, Tokyo, Japan). Before the *HaGlx* promoter sequence was cloned, intron insertion in genomic *HaGlx* near the start codon and 5′-UTR was determined by PCR. Primers were designed based on the sequence of *HaGlx* cDNA (LC155941): forward (5′-GCA GCAACACAAGGCAAATGGATCAAG-3′) and reverse (5′-ACATGGTCCCCTTTTAACATTTGGA TCTCC-3′). PCR was performed using a Prime Script™ PCR kit (Takara Bio) and the thermal cycling conditions were 30 cycles of 95°C for 1 min and 60°C for 1 min.

On the basis of the sequence information of the partial genomic *HaGlx*, inverse PCR was performed to analyse the sequence of the *HaGlx* promoter using the digested and self-ligated genome. The restriction enzymes *Vsp* I (Promega) and *Rsa* I (Takara Bio) were used for the first and the second inverse PCR, respectively. The thermal cycling profile was as follows: 35 cycles of 94°C for 1 min, 60°C (54°C in the second inverse PCR) for 1 min, and 72°C for 1 min. Primers used for the first and second inverse PCRs were as follows: forward 1 (5′-CAGC AACACAAGGCAAATGGATCAAG-3′), reverse 1 (5′-TCGTAGTCCTATCATCCGTACCGAC-3′), forward 2 (5′-CTTGTCCACGCCCCTACATT-3′), and reverse 2 (5′-CGTATCACTAATCACTCAGT CC-3′). After cloning and sequencing, the DNA sequence information of the promoter region was submitted to GenBank (the accession number of genomic *HaGlx* is LC155942).

Bioinformatics analysis

Homologies of *HaGlx* cDNA and the deduced amino acids were determined by BLAST search using conserved cDNA sequences previously deposited in the database (GenBank). A phylogenetic tree was produced using BLAST pairwise alignments or MEGA7. Domains were searched using CDART and Batch Web CD-Search Tool from the NCBI database. Regulatory elements in promoter regions were analysed using PLACE and SOGO by NIAS.

Plasmid construction and transformation

HaGlxORF was fused into the overexpression vector pRI 201-AN DNA (Takara Bio) using the In-Fusion Cloning Kit (Clontech). cDNA was obtained from *H. albus* hairy roots cultured under Fe deficiency for 5 days. Then, using the cDNA, *HaGlxORF* was amplified by In-Fusion PCR. Primers used were as follows: forward (5′-CACTGTTGATACATATGGC TGCAGCAACACA-3′) and reverse (5′-GAGACCA CCTCTGAACTCAGCTGTTAAGACTTA-3′). The PCR conditions were 35 cycles of 94°C for 10 s, 55°C for 15 s, and 72°C for 1 min. The PCR products and the digested overexpression vector were fused using In-Fusion HD enzyme.

The vector plasmids were inserted into Stellar competent cells by heat shock. Then, the vector plasmids were further introduced into the *Agrobacterium rhizogenes* strain ATCC15834 by electroporation with a MicroPulser™ (Bio-Rad, Hercules, USA). Using the *A. rhizogenes* harbouring pRI 201-AN DNA fused with *HaGlxORF*, *H. albus* seedlings were transformed to induce overexpression in hairy roots, as previously described (Higa et al. 2008).

Results and Discussion

Cloning of HaGlx cDNA

In a previous proteomic study, Glx was identified by matching with Glx of *M. truncatula* (XP_ 003596164.1) as a major protein, the levels of which are increased in *H. albus* roots under conditions of Fe depletion (Khandakar et al. 2013). Thus far, *Glx* sequences of higher plants have only been registered for *M. truncatula* and *A. thaliana*. Using a suitable primer set designed from the homologous region of *MtGlx* and *AtGlx* cDNAs by sequence alignment, the RT-PCR product was cloned and sequenced. The sequence (495 bp) showed high homology with the cDNA of the aforementioned species. Therefore, based on this sequence, the 3′- and 5′-ends were cloned, and a full-length putative *HaGlx* cDNA, together with a few additional nucleotides at the end, was obtained. After obtaining the promoter sequence described in section 3.3, the 5′-end of the cDNA was determined (1,178 bp, LC155941). This sequence contained an ORF (975 bp, 324 amino acids), which was used for a homology search against the GenBank database. A putative *HaGlx* showed 90% or more identity with *predicted nitrile-specifier protein 5* (*NSP5*) of Solanaceae plants, such as *Solanum*

Table 1. Similarity comparison of registered genes and the deduced amino acids of nitrile-specifier proteins 5/5-like and galactose oxidase/kelch repeat (-containing) proteins (Glxs) against putative *Glx*/Glx obtained from *Hyoscyamus albus* roots

NCBI definition		gene (mRNA)				Protein (Amino acid)			
Name	Species	Accession No.	query cover (%)	E value	identity (%)	Accession No.	query cover (%)	E value	identity (%)
predicted: nitrile-specifier protein 5	*Solanum tuberosum*	XM_006364748.2	100	0.0	92	XP_006364810.1	99	0.0	92
predicted: nitrile-specifier protein 5-like	*Capsicum annuum*	XM_016713728.1	100	0.0	91	XP_016569214.1	99	0.0	92
predicted: nitrile-specifier protein 5-like	*Nicotiana tabacum*	XM_016637315.1	100	0.0	90	XP_016470848.1	99	0.0	92
galactose oxidase/ kelch repeat protein	*Medicago truncatula*	XM_003596116.2	91	6.38E-127	71	XP_003596164.1	97	3.05E-160	71
galactose oxidase/ kelch repeat-containing protein	*Arabidopsis thaliana*	NM_111651.3	96	2.72E-87	68	NP_566316.1	98	6.63E-160	69
nitrile-specifier protein 5	*Arabidopsis thaliana*	NM_124193.4	94	1.32E-40	65	NP_568692.1	96	3.33E-119	55

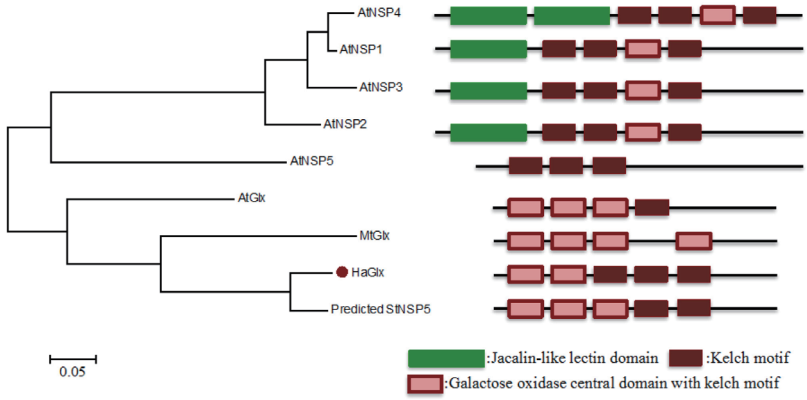

Fig. 1. Phylogenetic relationships and domain comparisons between galactose oxidase/kelch repeat containing proteins (Glxs) and nitrile-specifier proteins (NSPs) of higher plants. At, *A. thaliana*; Ha, *H. albus*; Mt, *M. truncatula*; Nt, *N. tabacum*; St, *S. tuberosum*.

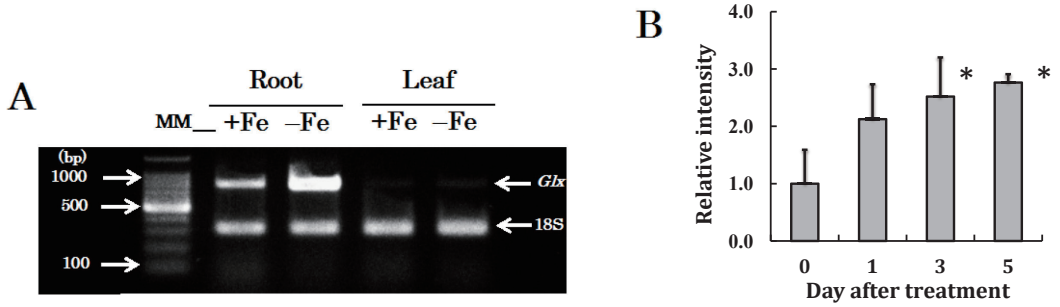

Fig. 2. RT-PCR analysis of *HaGlx* in *H. albus* seedlings. A: Seedlings subjected to Fe-replete (control, +Fe) and Fe-deficient (−Fe) conditions for 5 days under sterile conditions were used. B: Changes in expression were surveyed during 5-day treatment under Fe deficiency. Results are means of 3 replicates (n = 3), and bars indicate standard deviations. Data were analysed by Dunnett's test; * denotes a significant difference at $P < 0.05$ compared with the control.

tuberosum, *Capsicum annuum*, and *Nicotiana tabacum*: NSP5 catalyses the hydrolysis of glucosinolate (Kong et al. 2012) (Table 1). Conversely, there was around 70% identity with *MtGlx* and *AtGlx*. Since many *NSP5* sequences have been registered as 'predicted' or 'like' proteins, and both *NSPs* and *Glxs* showed relatively high identity, our gene was compared with *Glx* and *NSP5* from *A. thaliana*, which is the most widely studied dicot model plant (Table 1). Among five *NSPs* (At3g16400/NSP1, At2g33070/NSP2, At3g16390/NSP3, At3g16410/NSP4, and At5g48180/NSP5), *NSP5* had the highest identity (65%) with *HaGlx*.

To distinguish between Glx and NSP, molecular phylogeny was investigated and a domain search was performed using the amino acid sequences of HaGlx, predicted StNSP5, MtGlx, and AtGlx, in addition to AtNSP1–NSP5 (Fig. 1). The results indicated that NSPs and Glxs may have evolved differently, with the exception of the predicted StNSP5. The predicted StNSP5, which was closest to HaGlx, was grouped into Glxs in the phylogenetic tree. The putative HaGlx domain was searched, and two galactose oxidase central domains, together with five kelch motifs, were identified: these galactose oxidase domains were mostly overlapped with kelch motifs. AtGlx, MtGlx, and the predicted StNSP5 also contained both galactose oxidase central domains and kelch motifs. On the other hand, AtNSP1–AtNSP4 were found to contain Jacalin domains in addition to galactose oxidase domains and kelch motifs, while AtGlx did not possess a Jacalin domain. AtNSP5 lacked both galactose oxidase and Jacalin domains, and contained only kelch motifs.

Although the galactose oxidase central domain is absent in AtNSP5, AtNSP5 and AtGlx possess very similar sequences, making it difficult to distinguish between NSP5 and Glx. In fact, the name of *MtGlx* was once changed to *MtNSP5* (XM_003596116.1), but it was later changed back to the original name (XM_003596116.2) (Tang et al. 2014). Because the gene cloned here lacks a Jacalin domain but contains galactose oxidase domains, we concluded that the gene cloned in our laboratory was *HaGlx*.

Table 2. Important *cis*-acting elements in the *HaGlx* promoter region analysed by the PLACE database

Category	Name	Sequence†	No. of repeats (+) strand	No. of repeats (−) strand	Major function
Stress response	GT1GMSCAM4	GAAAAA	5	5	Responsive to salt
	WRKY71OS	TGAC	7	4	Responsive to defense signaling
	PREATPRODH	ACTCAT	1	2	Responsive to hypoosmolarity
	ANAERO1CONSENSUS	AAACAAA	4	1	Responsive to anoxia
	IDE1COREMOTIF	CATGC	2	1	Responsive to Fe deficiency
	MYBIAT	WAACCA	3	2	Responsive to dehydration
	MYCCONSENSUSAT	CANNTG	4	4	
	ACGTATERD1	ACGT	4	4	
Root specifity	RAV1AAT	CAACA	1	3	Roots
	ROOTMOTIFTAPOX1	ATATT	8	10	
	OSE1ROOTNODULE	AAAGAT	2	2	Root/root nodules
	RHERPATEXPA7	KCACGW	1	1	Root hairs
Transcription	GATABOX	GATA	12	9	Transcriptional enhancer
	CAATBOX1	CAAT	8	9	
	ARRIAT	NGATT	5	7	Transcriptional activator
	TATABOX2	TATAAAT	4	2	Essential for transcription start
	TATABOX5	TTATTT	6	11	

†K (G or T); N (A, C, G or T); W (A or T).

RT-PCR analysis of HaGlx

On the basis of *HaGlx* cDNA sequence information (LC155941), the expression in major organs and dependence on Fe deficiency were determined (Fig. 2). *H. albus* seedlings cultivated *in vitro* and treated with/without Fe were used for RT-PCR analysis. The primer set that amplified an 810-bp sequence within the ORF (975 bp) was used, resulting in the corresponding bands being conspicuously detected in the roots but not in the leaves of rosette-stage seedlings, regardless of Fe availability, and the expression in the roots clearly increased following Fe depletion (Fig. 2A). Changes in *HaGlx* expression following Fe deficiency were surveyed for 5 days, which showed that *HaGlx* expression significantly increased from day 3 onwards ($P < 0.05$) (Fig. 2B).

These results indicate that Glx mainly functions in the roots and is induced under conditions of Fe depletion in the seedlings; this is consistent with our previous proteomic data obtained using hairy roots (Khandakar et al. 2013).

Cloning and sequence analysis of the HaGlx promoter

To predict the function of HaGlx, we proceeded to analyse *cis*-elements of its promoter. Before we sequenced the 5′-upstream region of the *HaGlx* genome, PCR was performed to confirm the presence of an intron near the 5′-end of the genome based on the sequence of *Glx* cDNA (LC155941), from which an initial primer set was designed. The PCR product (1,555 bp) contained an intron (1,037 bp) (LC155942). On the basis of the sequence information, inverse PCRs were performed twice to obtain the 5′-upstream sequence of the *HaGlx* genome, resulting in the successful determination of the 1,877 bp upstream sequence (LC155942). Compared with the sequence of *HaGlx* cDNA (LC155941), the transcription start site (TSS) was determined, and the first nucleotide of TTS was designated as +1.

This promoter sequence was analysed for *cis*-elements using the PLACE database (Higo et al. 1999), revealing the presence of important elements such as transcriptional regulatory elements, root-specific elements, and stress response elements (Table 2). *TATA* boxes are important transcriptional regulatory elements where transcription factors bind to initiate transcription in eukaryotes and archaea; the closest one to TSS was *box 2* (TATAAAT), which was located between -29 and -35. As root- and root hair-specific elements, *RAV1AAT*, *ROOTMOTIFTAPOX1*, *OSE1ROOTNODULE*, and *RHERPATEXPA7* were identified. As stress response elements, *ACGTATERD1*, *MYBIAT*, and *MYCCONSENSUSAT*, which are involved in dehydration stress, and the *IDE1* core motif, which is involved in responses to Fe deficiency, were detected in the searches. The core motif, CATGC, within the iron deficiency responsive element *IDE1*, has been recognized as the binding site of a rice transcription factor, IDEF1 (Kobayashi and Nishizawa 2014). Since Fe deficiency occurs easily in arid and semiarid areas, the detection of the dehydration response

elements must be reasonable.

The presence of multiple *cis*-elements involved in dehydration stress, together with the *IDE1* core motif, indicates that HaGlx must have an essential role in stress responses induced by Fe deficiency in *H. albus* roots. Factually, recent transcriptomic analyses in *A. thaliana* have indicated that *Glx* is one of the most reliable marker genes to assess Fe deficiency (Mai et al. 2016). Including the *Glx* reported by us, the gene has only been reported in dicot plants thus far. Although the function of Glx remains still elusive, it might play an important role in the survival strategy of dicot plants under Fe deficiency.

HaGlx overexpression study

To dominate the function of HaGlx, we tried to establish an overexpression line of *H. albus* hairy roots. The ORF of *HaGlx* fused to the expression vector pRI 201-AN DNA was introduced into *A. rhizogenes* via electroporation, and three *HaGlxORF* positive clones were successfully obtained. Using these clones, the transformation of *H. albus* seedlings was repeatedly attempted. However, we failed to establish any hairy root clones expressing homologous *GlxORF*, although 16 negative clones were obtained after selection through antibiotic resistance.

This result suggests that HaGlx overexpression is fatal or inhibits root growth, and that during selection, all positive clones were eliminated, possibly due to their slow growth and therefore, bacterial contamination. In fact, selection was repeated to eliminate bacteria completely. As reported earlier, *H. albus* hairy roots grew less under conditions of Fe deficiency (Kawahara and Kitamura, 2015). In addition, the expression vector we used is known to be very powerful in dicot plants (Nagaya et al. 2010).

For further investigation on the function of Glx, more careful approaches using different technologies are necessary.

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