博士論文（要約）

Identification of molecules regulating IDEF1-mediated iron deficiency responses in rice

（イネにおける IDEF1 を介した
鉄欠乏応答制御分子の同定）

Zhang Lixia

張 麗霞

Table of contents

Acknowledgments

Firstly, I would like to thank the China Scholarship Council for giving me the opportunity to study abroad and supporting in finance during my Ph.D study. It is very appreciated that my supervisor Prof. Dr. Yamakawa Takashi gave me constant encouragement and guidance during my Ph.D study. I owe my sincere gratitude to Prof. Dr. Naoko Nishizawa for her guidance and discussion for my research.

I thank Prof. Hiroshi Takatsuji, Dr. Haruhiko Inoue and Dr. Chang-Jie Jiang of the National Institute of Agrobiological Sciences for providing the facilities for the pull-down and protoplast transfection experiments, as well as for valuable discussion and help. I thank Dr. Yoshiaki Nagamura and colleagues at National Institute of Agrobiological Sciences for assistance with the microarray experiments. I thank Dr. Hiroaki Ichikawa at National Institute of Agrobiological Sciences for providing the FOX seed lines (K33309), Prof. Tsuyoshi Nakagawa of Shimane University for providing the gateway vectors pGWB541, pGWB542 and Dr. Haruhiko Inoue for providing the gateway vectors, modified pSAT4-Pubi-ADH-HA (or -Myc) -NRluc.

I thank Dr. Takanori Kobayashi for his guidance in the study and paper writing. I thank Dr. Reiko Nakanishi Itai for guiding and discussion with me for experiments and papers. I thank Dr. Satoshi Mori, Dr. Hiromi Nakanishi, Dr. Tomoko Nozoye, Dr. Khurram Bashir, Dr. Takeshi Senoura, Dr. Ryuichi Takahashi, Dr. Yusuke Kakei, Dr Yuko Ogo, Dr. May Sann Aung and Dr. Hongchun Xiong for valuable discussion and Dr. Hugo Massayoshi Shimo and Mr. Houdo Tanaka for help me in transgenic rice work. I thank Ms Aya Hayashi for help me with some daily life problems. I also owe my gratitude to my friends who gave me their help and time when I need. I really had a good time in Japan and I am thankful to all these friends.

Chapter 1

Introduction

1-1 Fe for plants and humans

Fe is an essential element for most organisms, including all animals and plants. In plants, Fe is essential in many cellular processes, such as respiration, chlorophyll biosynthesis, photosynthetic electron transport and synthesis of some proteins (Ishimaru et al. 2009). Fe is the second abundant metal element in the soil. However, plants cannot easily utilize Fe in soils as it mainly forms insoluble Fe (III) compounds under aerobic conditions. Calcareous soils make up approximately one third of the earth's arable area and Fe availability is especially in these soils limited because of high pH. Plants grown on these soils easily suffer from Fe deficiency (Fig. 1-1), resulting in reduced quality and quantity of crops (Guerinot and Yi, 1994; Mori, 1999).

Rice (*Oryza sativa* L.) is one of the major food crops in the world. As the world population increase, rice consumers will grow and 40% more rice will be needed to produce in 2030 (Khush, 2005). Substantial advances have occurred in the production of major crops including rice, wheat and maize due to the green revolution technology, which solved hunger problem for humans significantly. However, more progressions for increasing not only quantity but also quality of major crops are required.

According to Dutch State Mines report (http://www.dsm.com), two billion people are affected by the "hidden hungers" in the world and they may eat enough food to live, but the food fails to provide enough crucial vitamins and minerals allowing them to be mentally and physically healthy. Fe deficiency has become one of the top three "hidden hungers" globally and supplementation and fortification of micronutrient has been reported as the top development investments, by the Copenhagen Consensus in 2008. According to the report of World Health Orgnization (http://www.who.int/en/), Fe deficiency anemia affects a quarter of the world's population distribution of

malnourished people in the world and is spread widely in most developing countries population, especially children (Fig. 1-2) and pregnant women. It has become serious health problems for humans. According to Dutch State Mines report, dietary Fe deficiency is one of the main causes of the anemia and improvement of iron nutrition from main food is an inexpensive and effective solution, but poor family especially in developing countries cannot afford it (Bashir et al. 2010).

In recent years, molecular mechanism of Fe-deficiency responses in rice has begun to be revealed, which provide an approach for generating tolerant crops of Fe deficiency to enhance food and biomass production in calcareous soil, as well as crops accumulating higher Fe in edible parts. Understanding of the regulatory mechanisms of Fe-deficiency responses in rice will provide further improvement in the Fe-efficient and -fortified crops in molecular way.

1-2 Fe acquisition strategies in higher plants

To acquire Fe from the rhizosphere, two distinct mechanisms have evolved in higher plants (Römheld and Marschner, 1986; Marschner and Römheld, 1994) and the researches until now have uncovered the genes and proteins involved in each process. Responses in dicots and non-graminaceous monocots depend on reduction of rhizospheric ferric Fe to ferrous Fe via ferric-chelate reductase and transport of the ferrous ions through Fe-regulated transporter (IRT) across the root plasma membrane (Strategy I; Fig. 1-3). To support this reduction mechanism, these plants also drive soil acidification by increasing root H^+-ATPase activity, and secrete phenolic compounds to the rhizosphere to aid solubilization and reduction of ferric Fe. In *Arabidopsis thaliana*, ferric-chelate reductase FRO2, ferrous transporter IRT1, H^+-ATPase AHA2 and

coumarin exporter PDR9/ABCG37 are induced by Fe deficiency and play main roles in the Fe-deficiency responses (Robinson et al. 1999; Eide et al. 1996; Santi and Schmidt, 2009; Rodríguez-Celma et al. 2013; Fourcroy et al. 2014).

Graminaceous plants secrete natural Fe(III) chelators, designated mugineic acid family phytosiderophores (MAs) (Takagi, 1976; Römheld and Marschner, 1986; Marschner and Römheld, 1994), which are biosynthesized from S-adenosyl-L-methionine through a series of enzymatic reactions by nicotianamine synthase (NAS), nicotianamine aminotransferase (NAAT) and deoxymugineic acid synthase (DMAS) (Mori and Nishizawa, 1987; Shojima et al. 1990; Higuchi et al. 1999; Takahashi et al. 1999; Bashir et al. 2006). Genes involved in this biosynthetic pathway are transcriptionally induced under Fe-deficiency conditions (Higuchi et al. 1999; Takahashi et al. 1999; Bashir et al. 2006). The MAs are secreted to the rhizosphere by the efflux transporter of MAs, TOM1 in rice and HvTOM1 in barley (Nozoye et al. 2011), and chelate rhizospheric Fe(III) as Fe(III)-MAs complexes, which are transported into the root by YELLOW STRIPE 1 (YS1) transporter in maize (Curie et al. 2001) and the YS1-like (YSL) transporters in rice and barley (Inoue et al. 2009; Murata et al. 2006) (Strategy II; Fig. 1-3). Rice can also take up ferrous ion through OsIRT1 transporter in addition to distinct Strategy II Fe uptake system (Bughio et al. 2002; Ishimaru et al. 2006).

Rice possesses 18 members of *YSL* family genes (*OsYSL1-18*) and some of them have been characterized (Aoyama et al. 2009; Inoue et al. 2009; Kakei et al. 2012). Nicotianamine, the precursor of MAs, is also a natural chelator of heavy metal ions (Takahashi et al. 2003). Oryza Sativa Yellow Stripe Like transporter OsYSL2 is involved in Fe(II) and Mn(II)-nicotianamine transport in phloem and Fe and Mn

translocation to rice grains (Koike et al. 2004; Ishimaru et al. 2010).

1-3 Regulatory mechanism of Fe-deficiency responses

1-3-1 Regulatory mechanism of Fe-deficiency responses in Strategy I plants

Under conditions of low Fe availability, genes involved in Fe acquisition are transcriptionally upregulated in both non-graminaceous and graminaceous plants (Kobayashi and Nishizawa, 2012). Many basic helix-loop-helix (bHLH) transcription factors are involved in these Fe-deficiency responses. In *Arabidopsis* (non-graminaceous plant), FER-like transcription factor FIT (formerly FIT1/FRU /AtbHLH29), plays a central role in this response (Colangelo and Guerinot, 2004; Bauer et al. 2007). *FIT* is induced at the transcript level and FIT protein accumulates under Fe-deficient conditions (Sivitz et al. 2011). In addition, degradation of FIT mediated via the 26S proteasome pathway is also induced under Fe-deficient conditions and is required for a proper Fe-deficiency response (Sivitz et al. 2011). AtbHLH38, AtbHLH39, AtbHLH100 and AtbHLH101 can form heterodimers with FIT, activating downstream gene expression in *Arabidopsis* (Yuan et al. 2008; Wang et al. 2013). ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE1 (EIL1) interact with FIT and prevent its degradation (Lingam et al. 2011). Another bHLH transcription factor, POPEYE, induced by Fe deficiency in the pericycle, indirectly interacts with the Fe- and Zn-binding E3-ubiquitin ligase BRUTUS through POPEYE homologs, regulating Fe homeostasis in *Arabidopsis* (Long et al. 2010; Kobayashi et al. 2013). Knockout mutants of *POPEYE* are hypersensitive to Fe deficiency, whereas knockdown mutants of *BRUTUS* exhibit tolerance to Fe deficiency (Long et al. 2010).

In *Arabidopsis*, the ferrous transporter IRT1 is also regulated at protein levels.

Monoubiquitination of this protein controls subcellular localization, vacuolar sorting, and degradation (Barberon et al. 2011). This ubiquitination and degradation is mediated by an E3-ubiquitin ligase IRT1 degradation factor 1 (IDF1) (Shin et al. 2013).

1-3-2 Regulatory mechanism of Fe-deficiency responses in Strategy II plants

The first *cis*-acting elements related to microelement deficiency identified in plants were Fe deficiency-responsive element (IDE) 1 and IDE2 in barley (Kobayashi et al. 2003). Subsequently, transcription factors binding to IDE1 and IDE2 were identified, designated IDEF1 and IDEF2, which belong to the ABI3/VP1 and NAC transcription factor families, respectively (Kobayashi et al. 2007; Ogo et al. 2008). Transcription factor IDEF2 whose gene is constitutively expressed and activates downstream genes through binding CA(A/C)G(T/C)(T/C/A)(T/C/A) site (Ogo et al. 2008). *IDEF1* gene is also constitutively expressed in roots, leaves, flowers and seeds throughout the rice life cycle (Kobayashi et al. 2010a, 2010b). As shown in Fig. 1-4, during the early stages of Fe deficiency, IDEF1 upregulates various Fe uptake- and/or utilization-related genes, including *OsNAS1*, *OsNAS2*, *OsNAS3*, *OsDMAS1*, *TOM1*, *OsIRT1*, *OsYSL15*, *OsYSL2*, and a bHLH transcription factor gene *OsIRO2*. During subsequent Fe-deficiency stages, IDEF1-mediated regulation of Fe acquisition and/or utilization is less obvious. At this stage, IDEF1 regulates several Fe deficiency-inducible late embryogenesis abundant genes positively through RY elements (CATGCA) (Kobayashi et al. 2009, 2010a, 2010b). Transgenic rice lines expressing *IDEF1* under the control of Fe deficiency-inducible barley *IDS2* gene promoter exhibit tolerance to Fe deficiency at early stages, but not during prolonged Fe-deficient conditions (Kobayashi et al. 2007, 2009, 2012, 2013). IDEF1 is thought to sense cellular metal balance linked to Fe

availability through the histidine-asparagine repeats (HN region) and proline-rich regions (P-regions), which bind divalent metals such as ferrous Fe and zinc (Zn) (Kobayashi et al. 2012). Deletion of these metal-binding HN and P regions abolishes the induction activity of Fe deficiency-inducible genes during the early stages of Fe deficiency.

In graminaceous plants, the bHLH transcription factors OsIRO2 and OsIRO3 are transcriptionally induced in response to Fe deficiency and regulate Fe-deficiency responses (Ogo et al. 2006, 2007, 2011; Zheng et al. 2010). OsIRO2 plays a crucial role in inducing various Fe deficiency-responsive genes for sustainable Fe-deficiency responses involved in Fe acquisition and utilization. OsIRO2 protein is also strongly induced by Fe deficiency, binds to CACGTGG site, and upregulates the genes involved in biosynthesis and transport of deoxymugineic acid, such as *OsYSL15*, *OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1* and *TOM1* (Ogo et al. 2007, 2011).

Among the Fe deficiency-inducible regulators in rice, hemerythrin domain-containing RING and Zn-finger proteins OsHRZ1 and OsHRZ2 have recently been shown to bind Fe and Zn, and possess ubiquitination activity (Kobayashi et al. 2013). OsHRZ1 and OsHRZ2 are homologous to *Arabidopsis* BRUTUS. Expression of Fe deficiency-inducible genes involved in Fe uptake and/or utilization is enhanced in *OsHRZ*-knockdown plants, mostly under Fe-sufficient conditions. Therefore, OsHRZ1 and OsHRZ2 are thought to be possible Fe-binding sensors that negatively regulate Fe acquisition under conditions of Fe sufficiency. OsHRZ1 and OsHRZ2 are susceptible to 26S proteasome-dependent degradation in roots irrespective of Fe conditions. These results suggest possible importance of post-translational regulation of Fe regulators. However, this kind of regulation related to Fe homeostasis in plants remains unknown

except for *Arabidopsis* FIT, IRT1 and rice OsHRZs.

1-4 Summary of this study

The transcription factor IDEF1 plays an important role in regulation of Fe-deficiency response in rice. Even though *IDEF1* transcript expression is constitutive without alteration during progression of Fe deficiency (Kobayashi et al. 2007, 2009, 2010b; Itai et al. 2013), Fe-deficiency responses mediated by IDEF1 are shifted during this process (Kobayashi et al. 2009, 2010a, 2012). Therefore, IDEF1 expression and/or activity are thought to be regulated in response to Fe nutritional conditions.

In order to clarify the molecular mechanisms of IDEF1-mediated Fe-deficiency responses in more detail, I utilized yeast two-hybrid screening and identified proteins interacting with IDEF1, designating them IDEF1-Binding Proteins (IBP). Interaction between IDEF1 and IBP1, IBP2 or IBP3 was also confirmed by pull-down assays. The three factors interacting with IDEF1 may play different roles in response to Fe deficiency. Clarification of the factors interacting with IDEF1 would give us more information on the mechanisms of Fe-deficiency responses in graminaceous plants, which is important for generating tolerant crops of Fe deficiency to enhance quality and quantity of crops in calcareous soil.

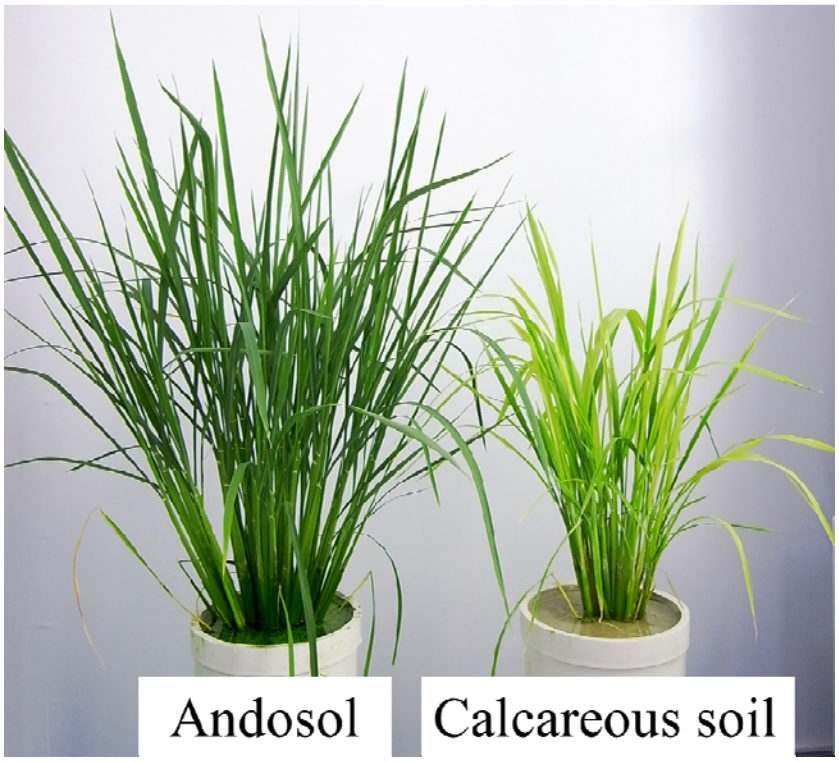

Figure 1-1. Fe deficiency symptom of rice in calcareous soil.

Rice grown on calcareous soil showed severe Fe-deficiency chlorosis and stunted growth compared with that on andosols.

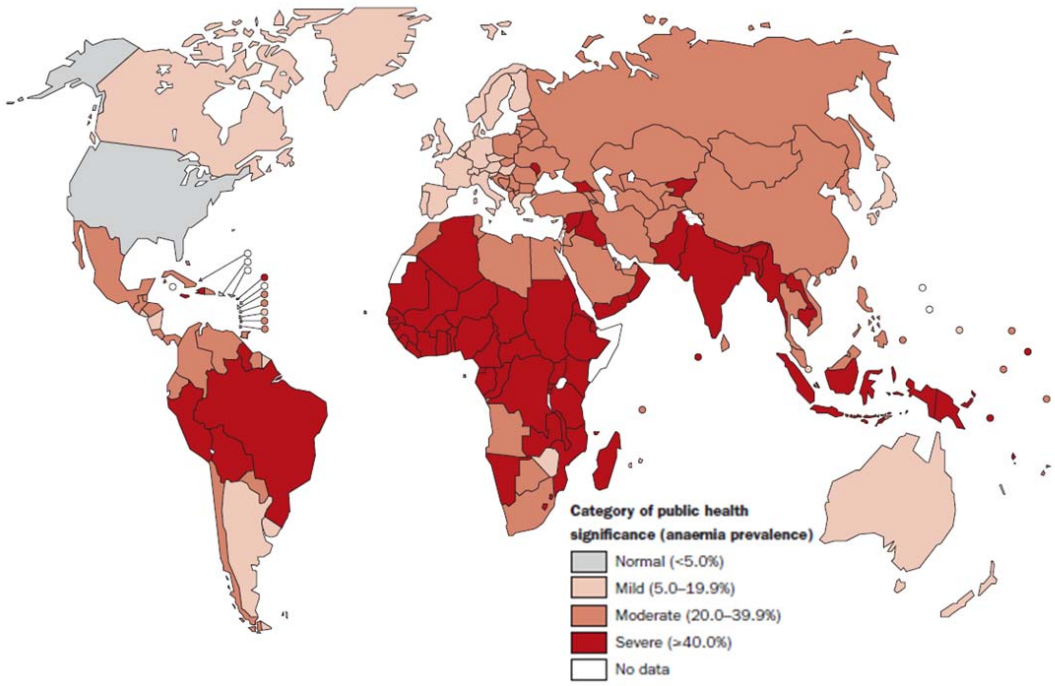

Figure 1-2. Anemia in preschool-age children.

Source: FAO hunger map.

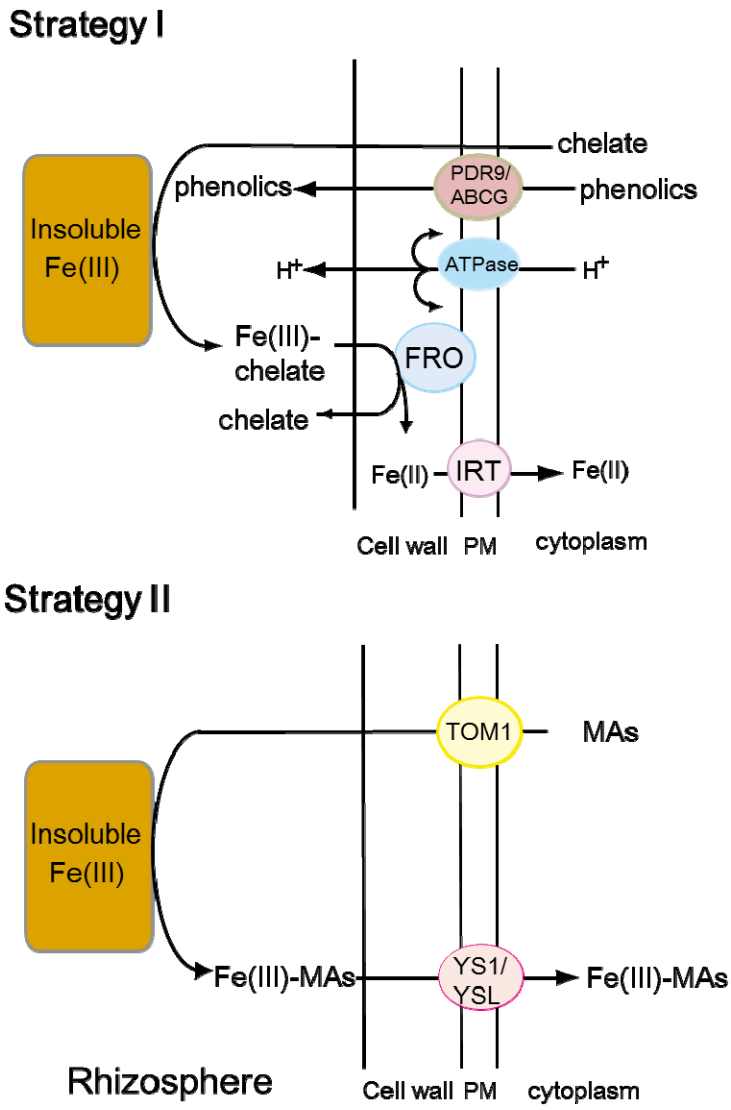

Figure 1-3. Fe acquisition strategies in higher plants.

Strategy I in nongraminaceous plants (upside) and Strategy II in graminaceous plants (downside). Ovals represent the transporters and enzymes that play central roles in these strategies, all of which are induced in response to Fe deficiency. Abbreviations: PDR/ABCG, pleiotropic drug resistance/ATP-binding cassette transporter G, a putative phenolics efflux transporter; FRO, ferric-chelate reductase oxidase; IRT, iron-regulated transporter; MAs, mugineic acid family phytosiderophores; TOM1, transporter of mugineic acid family phytosiderophores 1; YS1/YSL, YELLOW STRIPE 1/YELLOW STRIPE 1-Like; PM, plasma membrane.

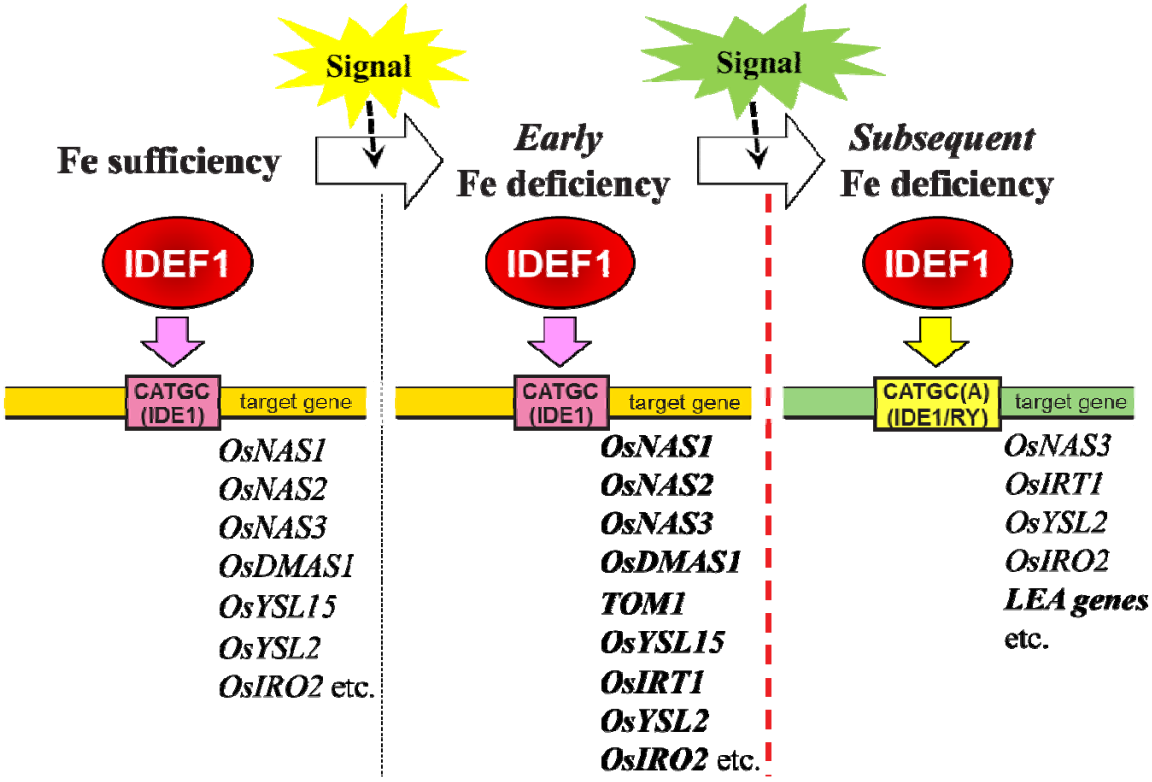

Figure 1-4. Regulatory pattern of IDEF1 under Fe-sufficient and -deficient conditions in rice.

During Fe sufficiency and the early stages of Fe deficiency, IDEF1 upregulates various Fe uptake- and/or utilization-related genes. Expression of these downstream genes is rapidly induced in response to Fe deficiency. During subsequent Fe-deficiency stages, IDEF1 regulates several Fe deficiency-inducible late embryogenesis abundant (LEA) genes in addition to some Fe uptake- and/or utilization-related genes (Kobayashi et al. 2009, 2010a, 2010b). Because of these shifts of regulation with the progression of Fe deficiency, IDEF1 is supposed to sense signals of Fe availability.

Chapter 2

The candidates interacting with IDEF1 screened by Yeast Two-Hybrid

本章の内容の一部は、学術雑誌論文として出版する計画があるため公表できない。5 年以内に出版予定である。

2.1 Introduction

In this Chapter, I utilized yeast two-hybrid screening to identify proteins interacting with IDEF1. The method of yeast two-hybrid was first established by Fields and Song (1989). As shown in Fig. 2-1a, the yeast transcription factor GAL4 containing DNA binding domain (BD) and activation domain (AD) was used for this system. These two domains are disconnected from each other and separately fused to bait and prey proteins of interest, respectively. When the two domains are brought to close by the interaction of fused bait and prey proteins, the GAL4 recovers its function and downstream reporter genes can be activated subsequently (Fields and Song, 1989). In the Matchmaker Gold Yeast Two-Hybrid System, there are four reporter genes *Aur1-C*, *HIS3*, *ADE2* and *MEL1*, whose expression can be screened on synthetic defined (SD) medium containing Aureobasidin A (+AbA), lacking of histidine (-His), lacking of adenine (-Ade) and containing X-α-galactoside (+X-α-Gal), respectively. I used this system to screen IDEF1-interacting candidates (Fig. 2-1b). The technology of yeast two-hybrid has already been employed to investigate the protein-protein interactions in various organisms and become an effective method for identifying the known interaction and screening the proteins to interact with protein of interest (Causier et al. 2002; Ferro et al. 2013). Other methods like pull-down and co-immunoprecipitation *in vitro* and bimolecular fluorescence complementation (BiFC) *in vivo* are also used widely for protein-protein interaction study.

Understanding the factors interacting with IDEF1 is important to clarify post-translational regulation of IDEF1 and understand molecular regulating Fe-deficiency responses in rice.

2.2 Materials and Methods

2.2.1 The screening by yeast two-hybrid

Yeast two-hybrid screening was carried out with the Matchmaker Gold Yeast Two-Hybrid System Kit (630489, Takara Bio, Shiga, Japan) following the manufacturer's protocol (PT4084-1). Complementary DNA (cDNA) synthesis was performed by PCR with CDSIII Primer, 5′-ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}$VN-3′, using the total RNA mixture extracted from Fe-deficient roots at day 1, 3 and 7, and Fe-deficient shoots at day 1 of hydroponically grown rice as described in Kobayashi et al. (2012). Rice (cv. Tsukinohikari) seeds were germinated on MS medium. After 2–3 weeks of culture followed by an acclimation period of 3 days, the plantlets were transferred to nutrient solution (Kobayashi et al. 2005) in a greenhouse with a 30°C light and 25°C dark cycle under natural light conditions. Fe-deficiency treatment was imposed on plantlets of similar size (shoot height 20–30 cm; 3–4 weeks old). Rice RNA was extracted and treated with DNase I using the NucleoSpin RNA Plant Mini Kit (Macherey-Nagel, Düren, Germany).

The full length Open Reading Frame (ORF) of IDEF1 in pGBKT7 (pGBKT7-IDEF1, Kobayashi et al. 2012), pGADT7-Rec linearized at the *Sma*I site and the cDNA pool were co-transformed into the yeast strain Y2HGold. In this system, co-transformed cDNA was inserted into pGADT7 through homologous recombination in yeast cells and the two-hybrid screening was carried out at once. A total of approximately 1.9×10^5 yeast transformants were screened. First screening was performed on the SD medium lacking tryptophan, leucine, histidine and adenine (SD/-Trp-Leu-His-Ade) at 30°C for 5 days. Approximately 1000 large colonies were

transferred to the second screening medium, SD/-Trp-Leu-His-Ade, containing both 125 ng/ml AbA and 5 μg/ml X-α-Gal. The yeast growth was observed from first to fourth day during culture at 30°C. Most of the colonies grew but some grew slowly and turned blue slowly and weakly. As a result, approximately 300 large blue colonies grew and 200 of larger blue colonies were chosen as primary candidate colonies of the second screening. The yeast colonies were cultured at 30°C for 5 days.

2.2.2 Sequencing of the candidates screened by yeast two-hybrid

Inserted cDNA in pGADT7 of the positive clones in the second screening was sequenced by two methods: direct sequencing by yeast colony and isolation of plasmid from yeast. Approximately 200 colonies which turned strongly blue and grew well were chosen for checking by colony PCR. The colonies chosen were first checked by colony PCR with the MATCHMAKER 5′ AD LD-Insert Screening Amplimer 5′-CTATTCGATGATGAAGATACCCCACCAAACCC-3', and MATCHMAKER 3′ AD LD-Insert Screening Amplimer 5′-GTGAACTTGCGGGGTTTTTCAGTATCTACGAT-3′. The 300-bp fragment was amplified from pGADT7 vector using these primers and one-hundred colonies were subjected to the direct PCR to check insertion. There are approximately 90 samples, showing single band which was bigger than 300-bp in length with these primers, and the corresponding colonies were chosen for primary candidates.

Approximately 60 colonies were chosen for direct sequencing by yeast colony PCR. Direct sequencing by yeast colony was performed with MATCHMAKER 5′ AD LD-Insert Screening Amplimer, annealing temperature for the sequencing reaction was 55°C.

Approximately 30 colonies the corresponding yeasts were transferred to new medium to get fresh colonies. After culturing the colonies in SD liquid medium lacking tryptophan for 3 days, plasmid isolation was performed. The yeast plasmid isolation was carried out according to ChargeSwitch Plasmid Mini Kit (Invitrogen, Carlsbad, CA). Then, plasmid extracted from yeast was transformed to *Escherichia coli* (*E.coil*) stain XL1 blue cells and extracted again. The extracted plasmids were cut by *Hind*III, and 12 candidate plasmids which showed possible inserted bands were chosen for sequencing.

Nucleotide sequencing using plasmids or direct sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using Applied Biosystems 3110 Genetic Analyzer with the T7 Sequencing primer 5′-TAATACGACTCACTATAGGGCGA-3′ and MATCHMAKER 5′ AD LD-Insert Screening Amplimer.

2.2.3 Confirmation of interaction using full-length ORF

The full-length ORF of the candidate genes were amplified using the combination of forward and reverse primers of each gene as described below by PCR from cDNA of Fe-deficient rice roots and cloned into pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA). The small letters indicate restriction sites and capital letters indicate target gene sequences.

IBP1.1 Forward primer (F), 5′-gaattcATGAGCAACACCACCAT-3′,

IBP1.1 Reverse primer (R), 5′-ggatccCTAGTTCTCCGCTCGG-3′;

After digestion by restriction enzymes and agarose gel electrophoresis, these fragments were purified with QIAEXII Gel Extraction Kit (Qiagen, Tokyo, Japan) and subcloned into pGADT7-rec at *Eco*RI and *Bam*HI or *Xho*I sites using DNA ligation kit

ver. 2.1 (Takara Bio). The insertion was checked by restriction enzyme cut and sequencing.

pGBKT7-IDEF1 which expresses IDEF1 fused to GAL4 BD (IDEF1-BD) and pGADT7-IBP which expresses IBP fused to GAL4 AD (IBP-AD) were co-transformed to Y2HGold and cultured on plates of SD/-Trp-Leu-His-Ade. A combination of p53-BD and Large T antigen-AD supplied by the manufacturer was used as a positive control. The yeast colonies were cultured at 30°C for 5 days.

2.3 Results

2.3.1 Testing IDEF1 for auto-activation in yeast

To check the auto-activation of pGBKT7-IDEF1 in yeast, pGBKT7-IDEF1 alone was transformed to Y2HGold and spread to the mediums SD/-Trp, SD/-Trp+AbA (12.5 ng / ml), SD/-Trp-His and SD/-Trp-His-Ade. After culture in 30°C for 5 days, some colonies appeared on SD/-Trp, a few on SD/-Trp+AbA, and no colonies on SD/-Trp-His or SD/-Trp-His-Ade medium which would be used during two-hybrid screening(Table 2-1), showing that auto-transactivation activity of the full length IDEF1 in yeast Y2HGold is very low or negligible. Thus, IDEF1-BD expressed from Y2HGold was judged to be applicable as a bait protein for further screening in this system.

2.3.2 Yeast two-hybrid screening

To isolate factors regulating IDEF1, a rice cDNA library was subjected to yeast two-hybrid screening by co-transformation of pGBKT7-IDEF1. The first screening was carried out on SD/-Trp-Leu-His-Ade medium (Fig. 2-2). Next big colonies grown in the first screening were chosen on SD/-Trp-Leu-His-Ade medium with AbA and X-α-Gal at the second screening. Approximately 200 colonies which were bigger, bluer, and more stable were checked in insertions and 90 colonies were tried to be sequenced.

Plasmids were isolated from approximately 20 candidates successfully. To confirm the interaction with the plasmids, co-transformation of each plasmid with IDEF1 to Y2HGold were performed on the SD/-Trp-Leu-His-Ade medium and most of them grew, while the combination of IDEF1 and large T antigen (negative control) did not grow (Fig. 2-3). Cells expressing between IDEF1 and candidates 2, 16 grew very weak or did not grow (Fig. 2-3). Approximately 60 colonies were chosen for direct

sequencing by yeast colony PCR. Sequencing of the plasmids and yeast colony was performed. Seventeen clones which were positive on the re-co-transformation had partial sequences in the correct open reading frame (ORF), as deduced from the rice genome database (Table 2-2) (KOME http://cdna01.dna.affrc.go.jp/cDNA/).

2.3.3 Confirmation of interaction with full-length ORF by yeast two-hybrid assay

To confirm the interaction further and remove some false positives, re-co-transformation between full-length ORF of each IBP and p53, large T antigen and IDEF1 was checked (Fig. 2-5). Similar to the positive control expressing p53-BD and Large T antigen-AD, cells expressing IDEF1-BD and IBP1.1, IBP2, IBP3, IBP4 or IBP5 grew on the SD/-Trp-Leu-His-Ade medium and turned blue on medium containing SD/-Trp-Leu/+X-α-Gal, while cells of negative controls expressing p53-BD and IBP-AD or IDEF1-BD and Large T antigen-AD failed to grow on the SD/-Trp-Leu-His-Ade medium and did not turn blue on medium containing SD/-Trp-Leu/+X-α-Gal. These results indicated these candidates did not have auto-activation in yeast Y2HGOLD and strong interactions between IDEF1-BD and IBP-AD.

Table 2-1. IDEF1 auto-activation in yeast.

SD/-Trp	SD/-Trp+AbA	SD/-Trp-His	SD/-Trp-His-Ade
++	+	-	-

pGBKT7-IDEF1 was transformed to Y2HGold and cultured on the mediums SD/-Trp, SD/-Trp+AbA (12.5 ng/ml), SD/-Trp-His and SD/-Trp-His-Ade at 30°C for 5 days. ++, + and - indicates that there are many, some and no colonies grown on the medium respectively.

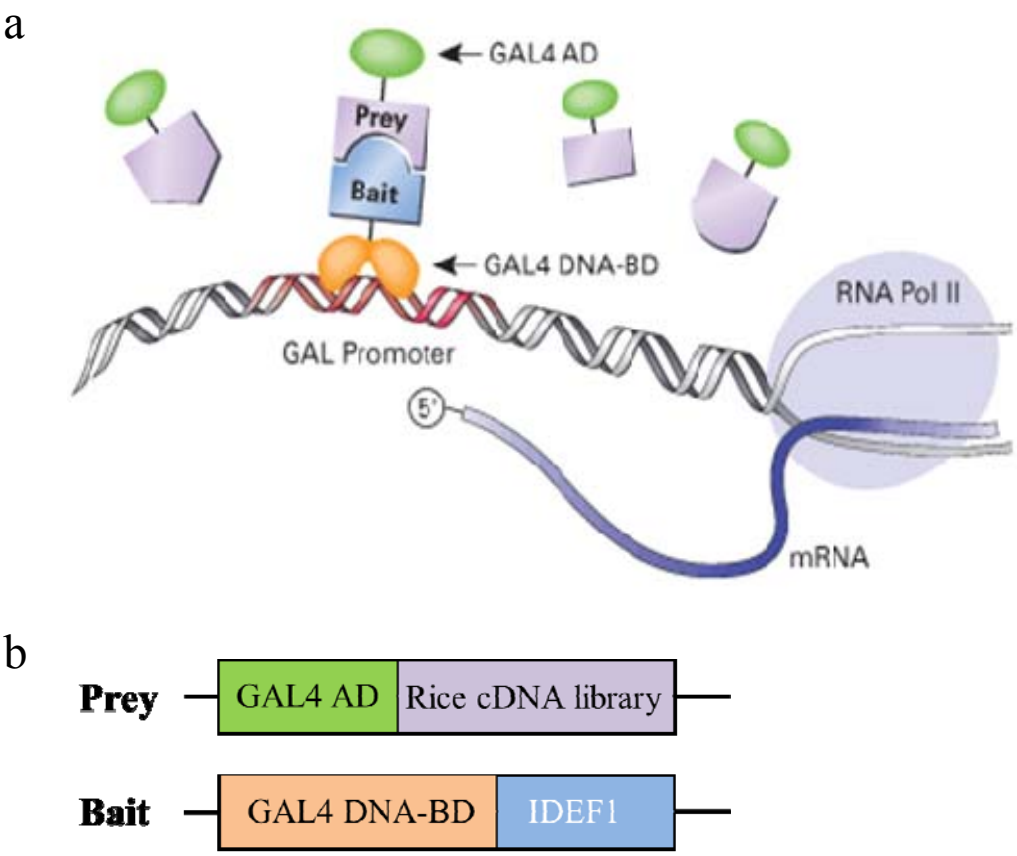

Figure 2-1. Molecular mechanism of yeast two-hybrid system and experimental design for screening IDEF1-interacting candidates.

(a) Molecular mechanism of yeast two-hybrid system. DNA binding domain (BD) and activation domain (AD) of the yeast transcription factor GAL4 are separately fused to bait and prey proteins of interest, respectively. When the two domains are brought to close by the interaction of fused bait and prey proteins, the GAL4 recovers its function and downstream reporter genes can be transcriptionally activated subsequently (Fields and Song, 1989).

(b) Experimental design for IDEF1 screening. IDEF1 was fused to GAL4 DNA BD as a bait protein (expressed by plasmid pGBKT7-IDEF1). Rice cDNA sequences constructed as a library were fused to GAL4 AD as prey proteins (expressed by plasmid pGADT7-cDNA). The size of both GAL4 DNA-BD and GAL4 AD is approximately 20 kDa. The size of IDEF1 is 40 kDa.

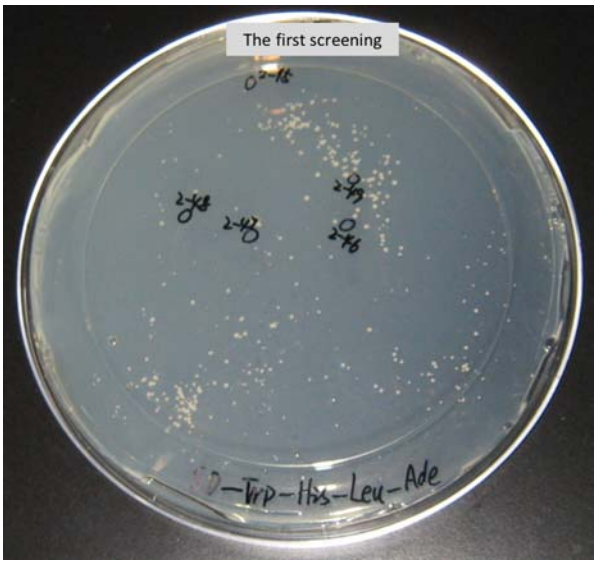

Figure 2-2. Colonies screened on SD/-Trp-Leu-His-Ade medium at the first screening by yeast two-hybrid.

Bigger colonies were chosen for further screening.

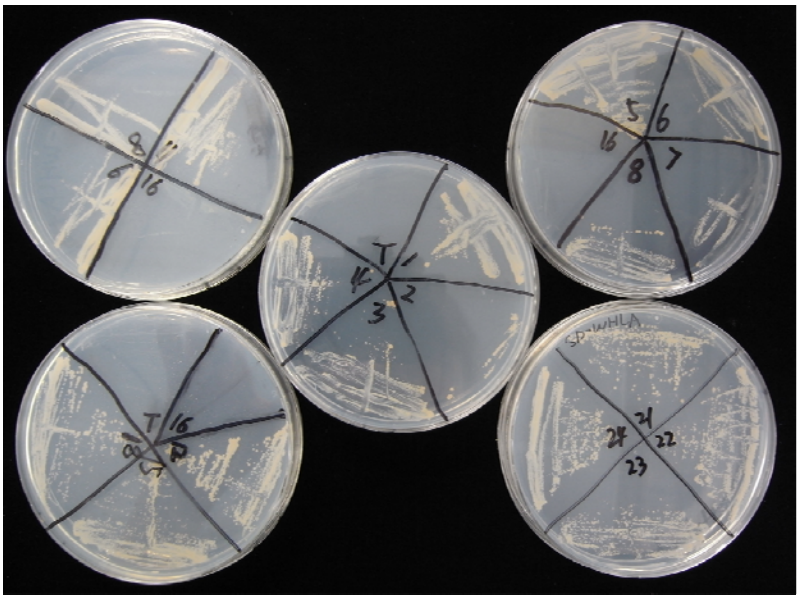

Figure 2-3. Two-hybrid analysis of IDEF1 and 12 candidates (partial cDNA sequences) on SD/-Trp-Leu-His-Ade medium.

T, large T antigen; 15, Os01g0124200 (IBP1.1); 21, Os01g0124401(IBP1.2).

Figure 2-4. Co-transformation of IDEF1 and some candidates (full-length ORF) on SD/-Trp-Leu-His-Ade medium by yeast two-hybrid.

T, large T antigen; 15, Os01g0124200 (IBP1.1).

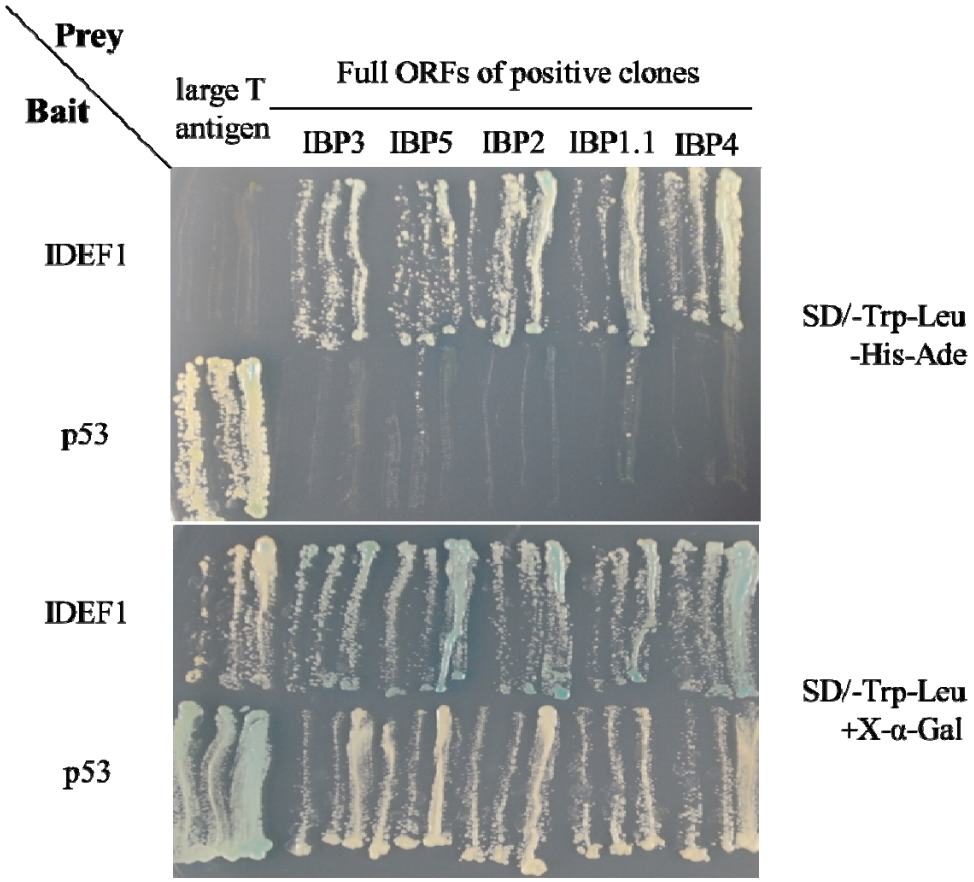

Figure 2-5. Interaction between IBP with full-length ORF and IDEF1 by yeast two-hybrid.

The bait and prey plasmids were co-transformed into yeast strain Y2HGold and cultured on SD/-Trp-Leu-His-Ade medium and SD/-Trp-Leu+X-α-Gal. A combination of p53-BD and Large T antigen-AD was used as a positive control, while IDEF1-BD and Large T antigen-AD and p53-BD and IBP-AD were used as negative controls.

2.4 Discussion

The transcription factor IDEF1 plays an important role in regulation of Fe-deficiency responses in graminaceous plants.

Gene expression profile of these candidates was investigated using microarray data of Ogo et al. (2008) which analyzed Fe-sufficient and -deficient rice roots and shoots, and publicly open databases. According to their relationship with Fe-deficiency responses and putative functions, I chose some of the positive clones for further confirmation. Full-length ORFs of IBP1.1 (Os01g0124200), IBP2, IBP3, IBP4 and IBP5 successfully conferred growth on the SD/-Trp-Leu-His-Ade medium with IDEF1-BD (Figs. 2-4, 2-5).

Based on their putative functions and/or the relationship of them with Fe deficiency according to microarray data, I focused on three candidates IBP1, IBP2 and IBP3, for further experiments. The three factors interacting with IDEF1 may play different roles in response to Fe deficiency.

Chapter 3

The Bowman-Birk trypsin inhibitor IBP1 interacts with and prevents degradation of IDEF1 in rice

Zhang L, Itai RN, Yamakawa T, Nakanishi H, Nishizawa NK, Kobayashi T.

The Bowman-Birk trypsin inhibitor IBP1 interacts with

and prevents degradation of IDEF1 in rice.

Plant Mol Biol Rep (2014) DOI: 10.1007/s11105-013-0695-8.

本章の内容の一部は、学術雑誌論文として出版する計画があるため公表できない。5 年以内に出版予定である。

3.1 Introduction

By yeast two-hybrid screening, I identified two homologous Bowman-Birk trypsin inhibitors, IBP1.1 and IBP1.2, which interact with IDEF1. In this chapter, I further characterized IBP1.1 in relation to Fe-deficiency responses. Bowman-Birk trypsin inhibitors are serine protease inhibitors which have been investigated extensively in legumes (Bowman, 1946) and graminaceous plants (Park et al. 2004). Bowman-Birk protease inhibitors are induced in response to injury, fungus, pathogens and in defense mediated by jasmonic acid and ethylene (Rakwal et al. 2001; Zavala et al. 2004). IBP1.1 was previously reported as RBBI3-1 which inhibits the activity of trypsin, but not that of chymotrypsin, in rice (Qu et al. 2003). A soybean Bowman-Birk inhibitor was reported to be involved in proteasome inhibition and effectively suppress carcinogenesis (Chen et al. 2005).

I also identified that IDEF1 protein is degraded in a 26S proteasome-dependent manner, and this degradation was prevented by IBP1.1. It is necessary for plants to remove some abnormal proteins and most short-lived regulatory proteins. Ubiquitin is specifically attached to these proteins and the resulting ubiquitin-protein conjugates are then recognized and degraded by the 26S proteasome (Smalle and Vierstra, 2004). This regulatory mechanism of ubiquitin/26S proteasome proteolytic pathway which affects various processes has been researched widely and revealed deeply in plants (Smalle and Vierstra, 2004). The discovery of ubiquitin-mediated protein degradation of Aaron Ciechanover, Avram Hershko, and Irwin Rose was awarded 2004 Nobel Prize in Chemistry.

In this chapter, it showed possible degradation way of IDEF1 protein and found the factors preventing the degradation, which is important for understand

post-translational regulatory mechanism mediated by IDEF1 in Fe-deficiency responses in rice.

3.2 Materials and methods

3.2.1 MBP pull-down assay for IBP1.1 and IDEF1

Production of recombinant tagged-proteins of IBP1.1 and IDEF1

The pEU-E01-MCS vector (CellFree Sciences, Yokohama, Japan) for wheat germ cell-free systems was modified to express three repeats of hemagglutinin (3×HA) tag fused protein. The 3×HA tag sequence was inserted at *Eco*RV and *Bam*HI sites of the pEU-E01-MCS vector (the resultant plasmid was designated pEU-3×HA) and the full-length ORF of *IBP1.1* was inserted at the end of the 3×HA tag at *Bam*HI and *Sal*I sites. HA-IBP1.1 proteins were produced in a wheat germ cell-free system (ENDEXT Technology WEPRO1240 Expression Kit, CellFree Sciences).

Maltose binding protein (MBP)-LacZ and MBP-IDEF1 (Kobayashi et al. 2012) were induced in *E.coil* BL21 (DE3) (Novagen, Tokyo, Japan) with 0.3 mM isopropyl β-D-thiogalactopyranoside at 18°C overnight and the cells were lysed by Bug Buster HT kit (Takara Bio). One hundred μl amylose resin (New England BioLabs, Tokyo, Japan) was washed with column buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl] and was added to the lysed crude proteins containing MBP-LacZ or MBP-IDEF1. The mixture was rotated at 4°C for 1 h for the MBP-tagged protein to bind the amylose resin. Then the resin was washed with washing buffer [20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1% Nonidet P-40] more than 3 times and with washing buffer containing 20 mM maltose once or twice to remove non-target proteins. Then SDS-PAGE was performed with the MBP-LacZ- and MBP-IDEF1-bound resin to check the protein quality. If the target protein still contained obvious unspecific or degraded proteins by staining of the SDS-PAGE gel, the resin was washed more with washing buffer containing 20 mM maltose. After final wash with column buffer, 50% slurry MBP-LacZ or MBP-IDEF1 protein-bound resin was made with column buffer.

The interaction of IBP1.1 and IDEF1 *in vitro*

Twenty μl of 50% slurry resin with bound MBP-LacZ or MBP-IDEF1 protein was incubated with HA-IBP1.1 at 4°C for 1 h in 700 μl of binding buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Nonidet P-40]. Then, the resin was washed with 400–700 μl of washing buffer six times. Finally, 30 μl of washing buffer containing 20 mM maltose was used to elute MBP-LacZ or MBP-IDEF1 proteins.

Western blotting

The eluates were diluted with SDS-PAGE sample buffer, heated at 70°C for 20 min and loaded onto a 10% SDS-PAGE gel. The electrophoresed proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Kyoto, Japan), blocked with Blocking One buffer (Nacalai Tesque, Kyoto, Japan), immunoblotted with anti-HA-tag (Medical & Biological Laboratories, Nagoya, Japan) or anti-MBP (New England Biolabs, Tokyo, Japan) monoclonal antibodies and washed with Tris-buffered saline containing 0.05% Tween 20 (TBST). Chemiluminescence detection was performed using the Chemi-Lumi one Ultra reagent (Nacalai Tesque) and imaged by ImageQuant LAS 4000 (GE Healthcare, Tokyo, Japan).

3.2.2 Rice transformation

3.2.3 Plant materials and hydroponic culture of the plants

T_2 seeds which constitutively overexpress *IBP1.1*, from two lines of Full-length cDNA Over-eXpressor (FOX) rice (K33309, cv. Nipponbare) were obtained from

Functional Plant Research Unit, National Institute of Agrobiological Sciences, and cultured for seed harvesting. T_3 lines of *IBP1.1*-FOX (157-2 and 157-3) and NT (cv. Nipponbare) were used for further experiments. NT (cv. Tsukinohikari and Nipponbare) and the transgenic seeds (*IBP1.1*-FOX) were germinated on Murashige and Skoog (MS) medium and MS medium with 50 mg/l hygromycin B, respectively, at 28°C in a chamber for 3 days in the dark. Then the light conditions were changed to a 14/10 h light/dark cycle. Thirteen days after imbibition, plants were transferred to hydroponic culture as described previously (Kobayashi et al. 2005). Half of the plants at 3 weeks old were subjected to Fe-deficiency treatment by transferring to medium without Fe(III)-EDTA. For analysis of the expressional dependence of *IBP1.1* and *IBP1.2* on IDEF1, NT (cv. Tsukinohikari), the full-length *IDEF1* induction line (*I2*pro-*IDEF1* FL-13) and overexpression lines of *IDEF1* lacking the metal-binding domains (*35S*pro-*IDEF1*ΔHNP-3, -7) were hydroponically cultured as described previously (Kobayashi et al. 2012). Both Fe-sufficient and Fe-deficient plants were harvested at days 1 and 7 of treatment.

3.2.4

3.2.5 Quantitative RT-PCR analysis

Total RNA was extracted from roots and shoots of rice, treated with DNase I and reverse-transcribed using a NucleoSpin RNA Plant Mini Kit, ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and priming with oligo-$d(T)_{17}$ or using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and ReverTra Ace qRT-PCR RT Master Mix with gDNA Remover (Toyobo). Real-time PCR was performed in a StepOnePlus Real-Time

PCR System (Applied Biosystems) with SYBR Green I and ExTaq Real-Time-PCR version (Takara Bio), TaqMan Gene Expression Assays (Applied Biosystems) or SYBR Premix DimerEraser Perfect Real Time version (Takara Bio). The TaqMan system was used for *IBP1.1* and *α*-2 tubulin quantification in Fig. 3-4 with probes TaqMan Gene Expression Assays Os03470892_g1 for *IBP1.1* and TaqMan Gene Expression Assays Os03562997_mH for *α*-2 tubulin. The SYBR system was used for the other quantification using the primers as follows:

IBP1.1 F, 5′-ATTGCCAGCGTGTGGAGTCGT-3′,

IBP1.1 R, 5′-AATCTGGATGGAGCTAAGCTCTAGCC-3′;

IBP1.2 F, GTGCACGGCGGCGCTGGAT,

IBP1.2 R, TCGGGC TTCGTCGGCAGCC;

OsYSL2 F, 5′-TCTGCTGGCTTCTTTGCATTTTCTG-3′,

OsYSL2 R, 5′-ACCATGTCGAACTCAGCATCCAGGA-3′;

α-tubulin F, 5′-TCTTCCACCCT GAGCAGCTC-3′,

α-tubulin R, 5′-AACCTTGGA GACCAGTGCAG-3′,

OsActin1 F, 5′-ACACCGGTGTCATGGTCGG-3′,

OsActin1 R, 5′-ACACGGAGCTCGTTGTAGAA-3′.

The transcript abundance was normalized against the *OsActin1* transcript level for the analysis of *IDEF1* dependence of *IBP1.1* and *IBP1.2* and against the *α-tubulin* transcript level for the other experiments. Results are shown as either numbers of transcript copies per 1 μg of total RNA or ratios relative to the levels in the NT plants.

3.2.6 Microarray analysis of gene expression in *IBP1.1*-FOX lines

3.2.7 Subcellular localization of IBP1.1

The gateway vectors pGWB541 and pGWB542 that were used to express enhanced yellow fluorescent proetin (EYFP) fusion protein under regulation of the *CaMV35S* promoter were kindly provided by Prof. Tsuyoshi Nakagawa of Shimane University. Full-length ORF of *IBP1.1* amplified by PCR with the F, 5′-caccATGAGCAACACCACCATG-3′ and the R 5′-GTTCTCCGCTCGGGGTTTGC-3′, was cloned into Gateway pENTR/D-TOPO vector and the sequence was verified. The ORF was subcloned into pGWB541 (IBP1.1-EYFP, EYFP fused to the C-terminus of IBP1.1) and pGWB542 (EYFP-IBP1.1, EYFP fused to the N-terminus of IBP1.1) by LR Clonase reaction. EYFP alone, IBP1.1-EYFP, and EYFP-IBP1.1 were introduced into onion (*Allium cepa*) epidermal cells using the Biolistic PDS-1000/He particle delivery system (Bio-Rad) as described by Mizuno et al. (2003) for transient expression. Fluorescence of EYFP was observed using a LSM 5 Pascal microscope (Carl Zeiss, Tokyo, Japan) following the manufacturer's instructions.

3.2.8 Transient expression of IDEF1 in rice protoplasts

The gateway expression vectors, pSAT4-Pubi-ADH-HA-NRluc and pSAT4-Pubi-ADH-Myc-NRluc, under the regulation of the ubiquitin promoter were modified for LR Clonase reaction and kindly donated by Dr. Haruhiko Inoue, National Institute of Agrobiological Sciences. The full-length ORFs of *IDEF1* and *IBP1.1* were cloned into Gateway pENTR/D-TOPO vector and subcloned into the HA- and Myc-containing vectors by LR Clonase reaction (HA-IDEF1 and Myc-IBP1.1). Then, as described by Inoue et al. (2013), rice protoplasts were produced from Oc cells and

transfected with HA-IDEF1 by electroporation. After incubating overnight at 28°C in a chamber, half of the protoplasts were treated with 70 μM MG132 for 3 h. For the co-expression experiment, 1 μg HA-IDEF1 was co-transfected with or without 1 μg Myc-IBP1.1. Then, the protoplasts were harvested and IDEF1 was detected by Western blotting with an anti-HA antibody, as described in 3.2.1.

3.2.9 Cell-free degradation assay

The cell-free degradation experiment was performed as described by Wang et al. (2009) using the recombinant MBP-IDEF1 and HA-IBP1.1 proteins. MBP-IDEF1 was produced as described in 3.2.1 and eluted by column buffer containing 10 mM maltose. Proteins were extracted from roots of Fe-deficient rice plants (cv. Nipponbare) grown hydroponically as described in 3.2.3. Purified MBP-IDEF1 (0.3 μg) and rice protein extracts (2 μg) were incubated at 28°C for 20 min with or without MG132 (100 μM), HA-IBP1.1 (approximately 1 μg as crude protein produced by wheat germ system as described in 3.2.1), or bovine serum albumin (BSA, 1 μg, Toyobo) in degradation buffer, containing 25 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 4 mM complete proteinase inhibitor (Roche, Tokyo, Japan), 5 mM DTT, and 10 mM ATP. Then SDS-PAGE sample buffer was added to the mixture and heated at 70°C for 20 min. MBP-IDEF1 was detected with an anti-MBP monoclonal antibody (Sigma-Aldrich, St. Louis, Missouri, USA). ECL prime (GE Healthcare) was used for chemiluminescence detection and imaged by ImageQuant LAS 3000 (Fujifilm, Tokyo, Japan).

3.3 Results

3.3.1 Sequence comparison of Bowman-Birk trypsin inhibitors IBP1.1 and IBP1.2

There are 11 homologs of Bowman-Birk trypsin inhibitor family in rice (Fig. 3-1a). IBP1.1 and IBP1.2 are highly homologous proteins with 87% identity in their amino acid sequences (Fig. 3-1b), and were previously designated RBBI3-1 and RBBI3-3, respectively (Qu et al. 2003). IBP1.1 and IBP1.2 are composed of 251 and 254 amino acid residues, respectively. Both IBP1.1 and IBP1.2 have three repeats of the Bowman-Birk domain (Fig. 3-1b). In the promoter region of *IBP1.1*, there are many IDEF1 binding sites and an IDEF2 binding site (Fig. 3-1c). For *IBP1.2*, I found IDEF1 binding sites but not IDEF2 binding sites (Fig. 3-1c). Since IBP1.1 and IBP1.2 were highly homologous, I analyzed IBP1.1 mainly in further experiments.

3.3.2 *In vitro* interaction between IDEF1 and IBP1.1

To confirm the interaction between IDEF1 and IBP1.1 *in vitro*, a pull-down assay was performed. To remove degraded proteins, the MBP-IDEF1 and MBP-LacZ proteins were washed with washing buffer containing maltose before incubating with HA-IBP1.1. HA-IBP1.1 proteins were pulled down with MBP-IDEF1 proteins bound to amylose resin (Fig. 3-2). Wheat germ cell-free system was chosen for the production of IBP1.1. HA-IBP1.1 was detected in the eluates of MBP-IDEF1-bound resin by Western blotting with an anti-HA antibody, while no band was detected in the eluates of MBP-LacZ-bound resin (Fig. 3-2), revealing that IBP1.1 interacts with IDEF1 *in vitro*. Taken together, results of yeast two-hybrid (Fig. 2-5) and pull-down assays (Fig. 3-2) confirm that IDEF1 and IBP1.1 interact *in vivo* and *in vitro*.

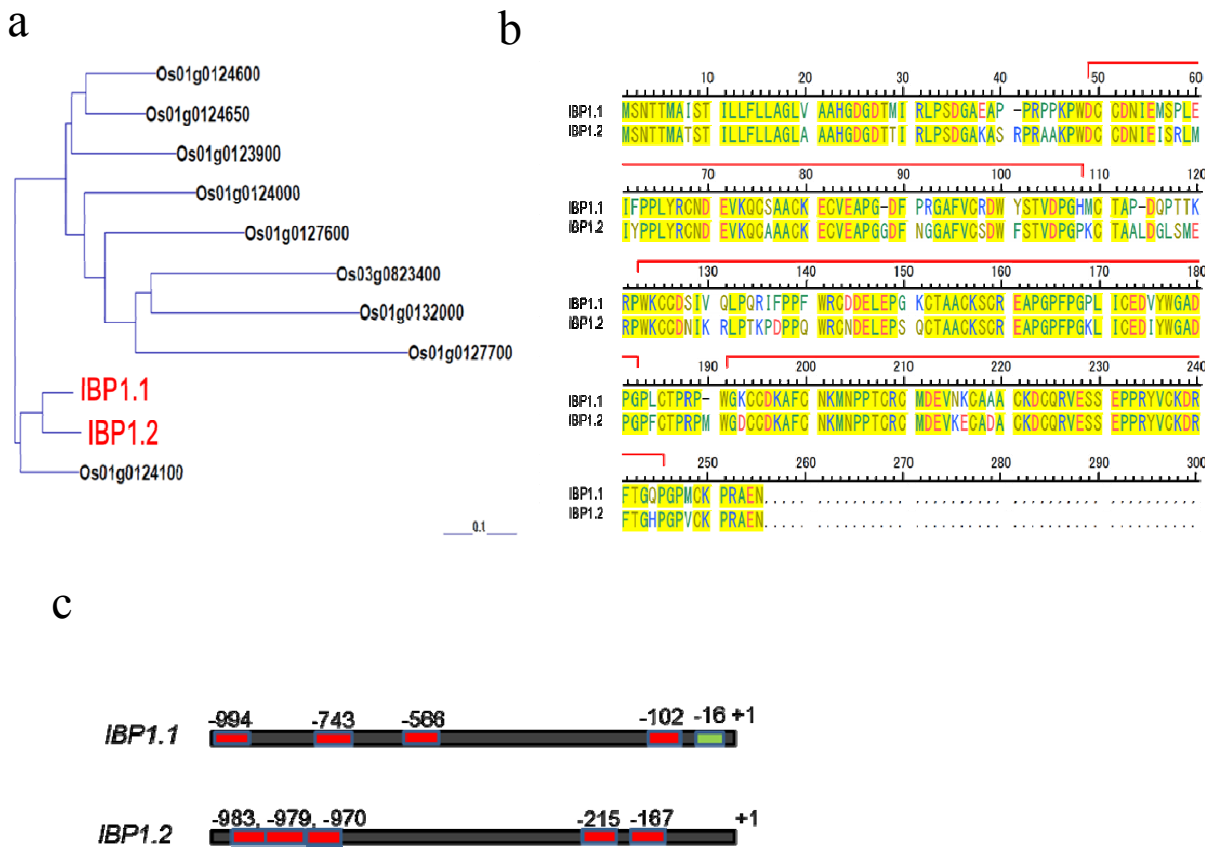

Figure 3-1. Sequence properties of IBP1.

(a) Phylogenic tree of the Bowman-Birk trypsin inhibitor family in rice.

(b) Sequence similarities between IBP1.1 and IBP1.2 proteins. Domain structure of IBP1.1 and IBP1.2 proteins were analyzed by SMART analysis (http:// smart.embl-heidelberg.de/). Three Bowman-Birk type proteinase inhibitor domains are shown in red lines.

(c) Localization of IDEF1 and IDEF2 binding sites in the promoter regions of *IBP1.1* and *IBP1.2*. The 1000-bp upstream regions of the predicted transcriptional initiation sites of IBP1.1 (AK067257) and IBP1.2 (AK243607) were searched. Red boxes indicate the IDEF1 binding site, CATGC (Kobayashi et al. 2007). The green box indicates the IDEF2 binding site, CA(A/C)G(T/C)(T/C/A)(T/C/A) (Ogo et al. 2008).

3.3.3 Subcellular localization of IBP1.1

To verify the subcellular localization of IBP1.1, IBP1.1-EYFP and EYFP-IBP1.1 fusion proteins were transiently expressed in onion epidermal cells. Both the IBP1.1-EYFP and EYFP-IBP1.1 localized to the nucleus and cytoplasm in onion epidermal cells, similar to EYFP alone (Fig. 3-3).

3.3.4 *IBP1.1* and *IBP1.2* expression is induced under Fe deficiency which is regulated by IDEF1

As there are many IDEF1 binding sites in the promoter regions of *IBP1.1* and *IBP1.2* (Fig. 3-1b), expression of *IBP1.1* and *IBP1.2* in response to Fe deficiency was analyzed by quantitative RT-PCR (Fig. 3-4). Expression of both *IBP1.1* and *IBP1.2* was significantly induced by Fe deficiency in both roots and leaves (Fig. 3-4). The induction of *IBP1.1* in roots and leaves was much higher at day 1 than at day 7 in Fe-deficiency treatment, while that of *IBP1.2* was higher at day 7 than at day 1 under Fe-deficiency treatment (Fig. 3-4).

I examined the effect of IDEF1 on the expression of *IBP1.1* and *IBP1.2* using overexpression lines of either the full-length or metal binding region-deleted *IDEF1* (Kobayashi et al. 2007, 2012). The gene expression of *IBP1.1* and *IBP1.2* was significantly higher in the full-length *IDEF1* overexpression line (*I2*pro-*IDEF1* FL) than in NT at both days 1 and 9 of Fe-deficiency treatment (Fig. 3-5). The induction of *IBP1.1* and *IBP1.2* in the metal binding region-deleted IDEF1 overexpression lines (*35S*pro-*IDEF1*ΔHNP-3, -7) was lower compared with that in the full-length *IDEF1* overexpression line at day 1 of Fe-deficiency treatment. At day 9, *IBP1.1* expression was higher in *35S*pro-*IDEF1*ΔHNP-3 and at a similar level in *35S*pro-*IDEF1*ΔHNP-7

compared with *I2*pro-*IDEF1* FL, whereas *IBP1.2* expression was significantly lower in *35S*pro-*IDEF1*△HNP-3 and *35S*pro-*IDEF1*△HNP-7 than in *I2*pro-*IDEF1* FL (Fig. 3-5). These results indicate that expression of *IBP1.1* and *IBP1.2* is induced under Fe deficiency under the control of IDEF1 and the metal-binding region of IDEF1 is required for this regulation, especially under the early stages of Fe deficiency.

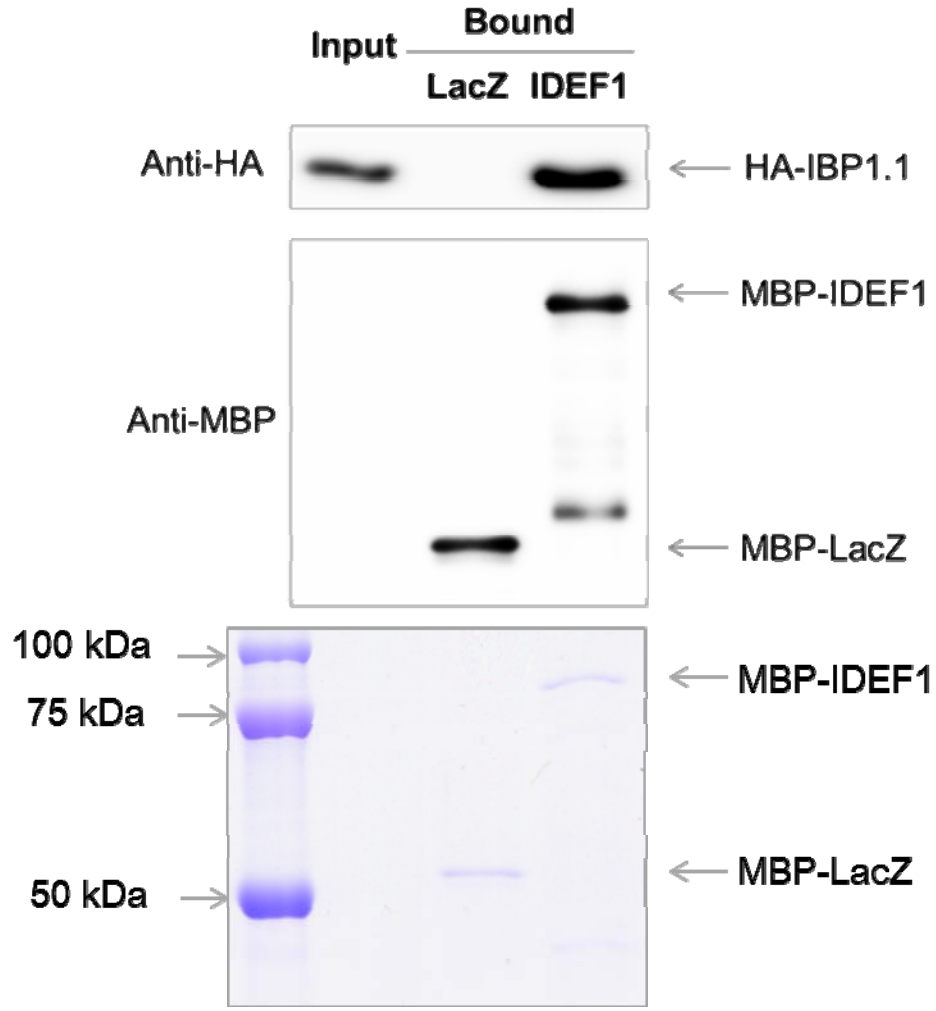

Figure 3-2. Interaction between IDEF1 and IBP1.1 *in vitro* by pull-down assay.
MBP-LacZ- or MBP-IDEF1-bound amylose resin was incubated with HA-IBP1.1. The HA-IBP1.1 and MBP-tagged proteins were detected with anti-HA and anti-MBP monoclonal antibodies, respectively. Coomassie brilliant blue staining was also performed. The sizes of HA-IBP1.1, MBP-IDEF1 and MBP-LacZ are 38, 81 and 52 kDa, respectively.

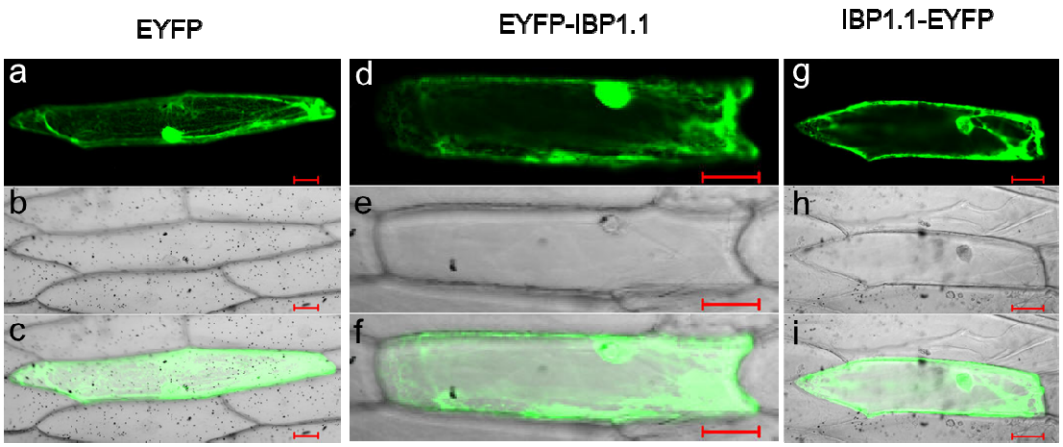

Figure 3-3. Subcellular localization of IBP1.1.

EYFP alone (a, b, c), EYFP-IBP1.1 (d, e, f) and IBP1.1-EYFP (g, h, i) were transiently expressed in onion (*Allium cepa*) epidermal cells. (a, d, g) YFP fluorescence. (b, e, h) Bright-field images. (c, f, i) Merged images. Scale bar = 50 μm.

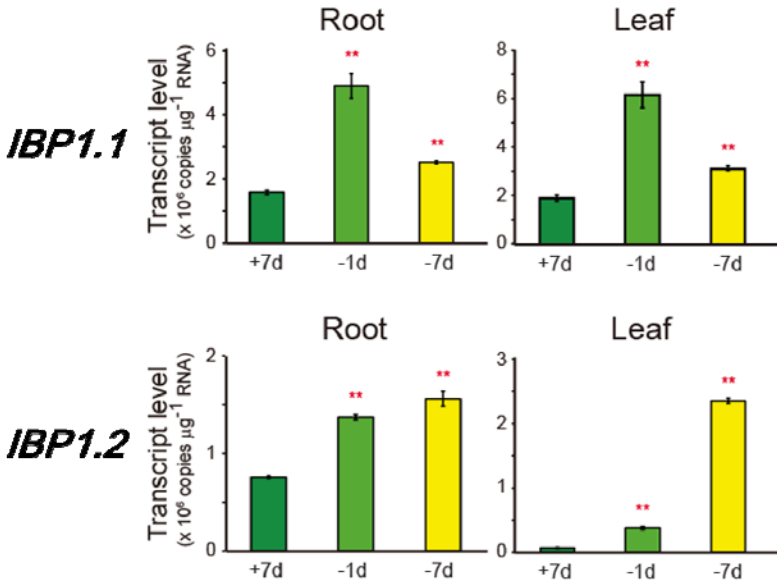

Figure 3-4. Expression of *IBP1* in response to Fe deficiency.

Expression of *IBP1.1* and *IBP1.2* in roots and leaves of hydroponically grown wild-type rice was analyzed by quantitative RT-PCR. +7d, Fe sufficiency for 7 days; –1d, Fe deficiency for 1 day; –7d, Fe deficiency for 7 days. Means ± s.d.; n = 3. Asterisks indicate significant differences from the +7d value (two-sample Student's *t*-test; **, $P < 0.01$).

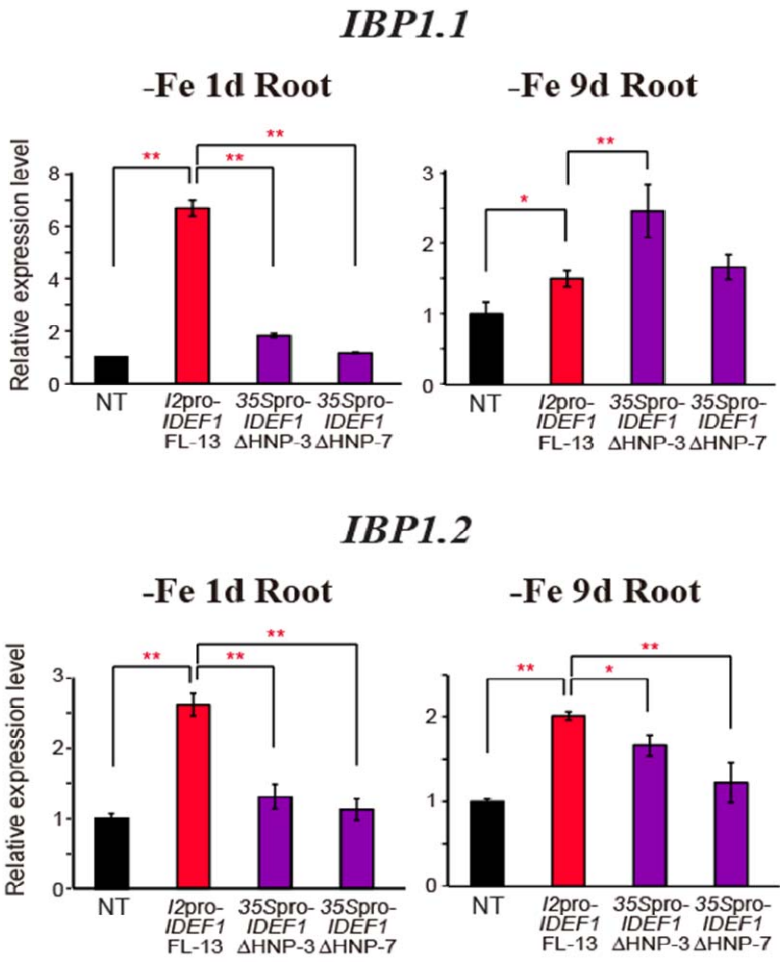

Figure 3-5. Expression of *IBP1* in response to IDEF1 overexpression.

IBP1.1 and *IBP1.2* for their expressional dependence on *IDEF1* and its metal-binding regions was analyzed by quantitative RT-PCR. NT, non-transformants; *I2*pro-*IDEF1* FL-13, the full-length *IDEF1* induction line (Kobayashi et al. 2007, 2012); *35S*pro-*IDEF1*ΔHNP-3 and 7, overexpression lines of *IDEF1* lacking the metal-binding domains (Kobayashi et al. 2012). The transcript levels in roots at day 1 (1d) or day 9 (9d) of Fe-deficiency treatment are expressed as ratios relative to the levels in the NT under each condition (means ± s.d.; n = 3). Asterisks indicate significant differences from the value in the *I2*pro-*IDEF1* FL line (two-sample Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$).

3.3.6 Overexpression lines of *IBP1.1* hyper-express the Fe(II)-nicotianamine transporter gene *OsYSL2*

I obtained and analyzed Full-length cDNA Over-eXpressor (FOX) rice lines that constitutively overexpress *IBP1.1* (lines 157-2 and 157-3). Hydroponic culture of these plants was performed under Fe-sufficient and -deficient conditions (Fig. 3-7). Expression of *IBP1.1* gene was highly enhanced in roots and shoots of FOX lines than in NT plants under both Fe-sufficient and Fe-deficient conditions (Fig. 3-8a).

I found that expression of *OsYSL2* gene, an Fe(II)- and manganese(II)-nicotianamine transporter gene facilitating Fe and Mn translocation in phloem and to grains of rice (Koike et al. 2004; Ishimaru et al. 2010), was much higher both in shoots and roots of *IBP1.1*-FOX lines under Fe-deficient conditions compared with NT plants according to the microarray data (Tables 3-1, 3-2). *OsYSL2* expression in *IBP1.1*-FOX lines was further confirmed by quantitative RT-PCR. The *OsYSL2* gene expression level was much higher in both shoots and roots of *IBP1.1*-FOX lines under Fe-deficient conditions compared with NT plants, and was also high in roots of Fe-sufficient *IBP1.1*-FOX lines, reaching a similar level to that induced under Fe-deficiency in NT (Fig. 3-8b).

3.3.7 IDEF1 is degraded via a 26S proteasome-dependent manner and IBP1.1 prevents its degradation

In the nucleus and cytosol of eukaryotic cells, degradation of most proteins occurs via the ubiquitin-proteasome pathway (Voges et al. 1999). I analyzed the possibility of IDEF1 degradation via the ubiquitin-proteasome pathway using both *in vivo* and *in vitro* systems. First, IDEF1 was transiently expressed in rice protoplasts and

treated with or without MG132, a 26S proteasome inhibitor. IDEF1 accumulated more under MG132 treatment (Fig. 3-9a), suggesting that IDEF1 is degraded via the 26S proteasome-dependent pathway. Interestingly, co-expression of IBP1.1 with IDEF1 in rice protoplasts enhanced IDEF1 accumulation (Fig. 3-9b), suggesting a protective effect of IBP1.1 on IDEF1 degradation.

Then, I confirmed IDEF1 stability and protective effect of IBP1.1 *in vitro* using a cell-free degradation assay, which can recapitulate the 26S proteasome-dependent pathway (Wang et al. 2009). In the reaction mixture containing protein extracts from Fe-deficient rice plants, MBP-IDEF1 were rapidly degraded (Fig. 3-9c). This degradation was effectively prevented by the addition of MG132 (Fig. 3-9c). Also, addition of HA-IBP1.1 in the reaction mixture resulted in a higher level of remaining MBP-IDEF1 protein than the addition of BSA, which is a non-specific protector of proteins (Fig. 3-9d), revealing that IBP1.1 prevents the degradation of IDEF1, which is probably mediated via the 26S proteasome pathway.

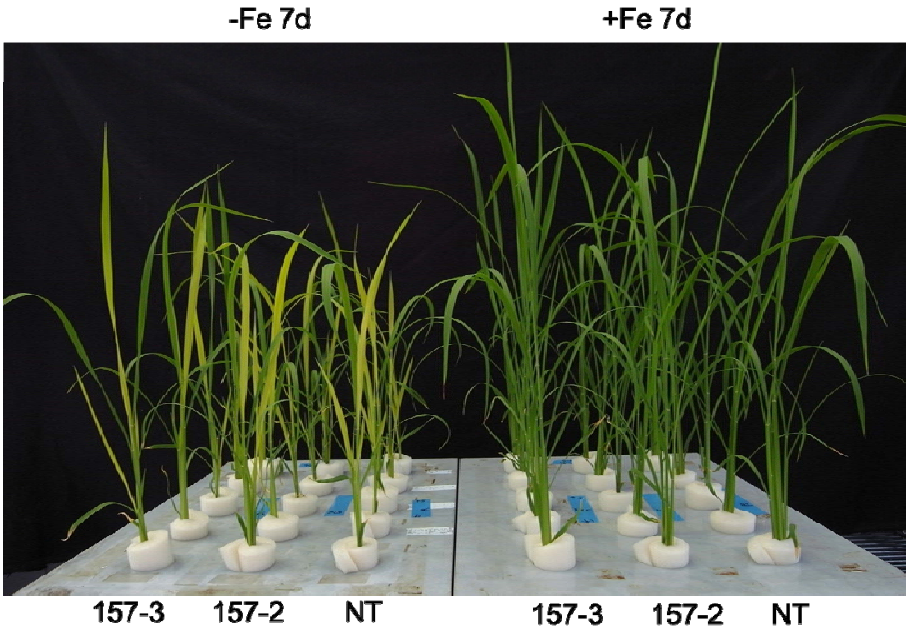

Figure 3-7. Hydroponic culture of *IBP1.1*-FOX lines.

The photograph was taken at day 7 under Fe-sufficient (+Fe) and -deficient (-Fe) conditions. 157-2 and 157-3, *IBP1.1*-FOX lines; NT, non-transformants.

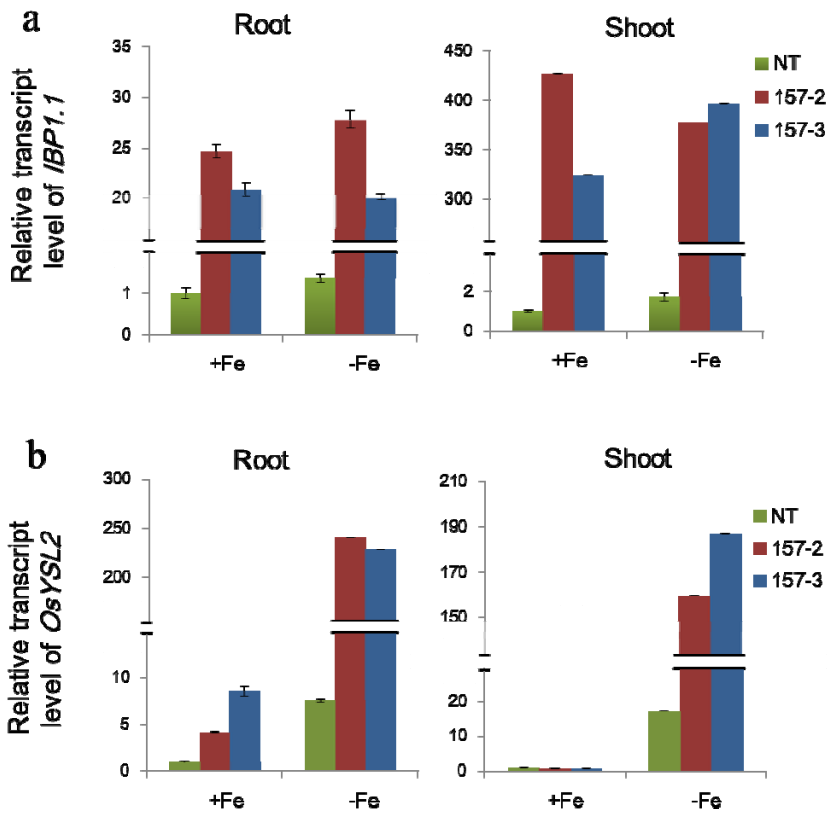

Figure 3-8. Gene expression analysis of *IBP1.1* and *OsYSL2* in roots and shoots of *IBP1.1*-FOX lines by quantitative RT-PCR.

Plants were grown hydroponically and harvested at day 7 under Fe sufficient (+Fe) and Fe-deficient (-Fe) conditions. 157-2 and 157-3, *IBP1.1*-FOX lines; NT, non-transformants. Means ± s.d.; n = 3. Transcript levels are expressed as ratios relative to NT under Fe-sufficient conditions.

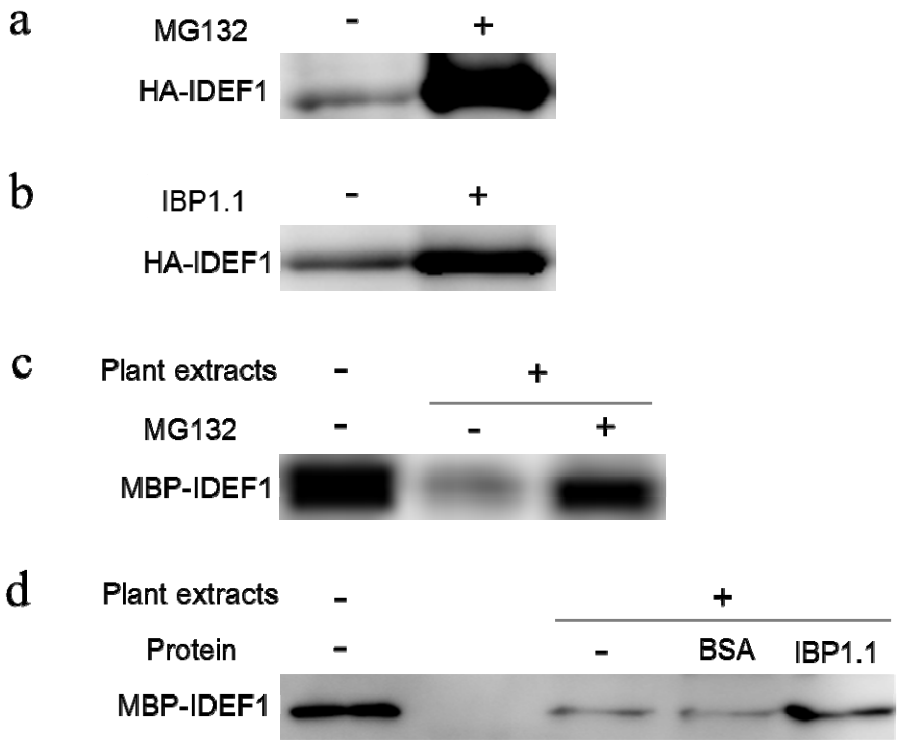

Fig. 3-9. Effect of proteasome-mediated degradation and IBP1.1 on the stability of IDEF1 protein.

(a, b) Accumulation of IDEF1 in rice protoplasts after expression. HA-IDEF1 was transiently expressed in rice protoplasts prepared from Oc cells. (a) The transfected protoplasts were incubated with (+) or without (–) MG132. (b) HA-IDEF1 was co-transfected with or without Myc-IBP1.1. Accumulation of IDEF1 protein was detected by Western blotting using an anti-HA antibody.

(c, d) Cell-free degradation assay of IDEF1. Purified MBP-IDEF1 and rice protein extracts were incubated with (+) or without (–) protein extracts from Fe-deficient rice roots. (c) Effect of MG132 treatment. The reaction was carried out with (+) or without (–) MG132. (d) Effect of IBP1.1. The reaction was carried out with or without recombinant HA-IBP1.1 or BSA as a negative control. IDEF1 protein was detected by Western blotting using an anti-MBP antibody.

3.4 Discussion

The Bowman-Birk protease inhibitor IBP1 interacts with IDEF1

In this study, we identified a Bowman-Birk protease inhibitor, designated IBP1.1, that interacts with IDEF1, as demonstrated by both the yeast two-hybrid and MBP pull-down assays (Figs. 2-5, 3-2). It seemed in the presence of glutathione, Bowman-Birk trypsin inhibitor is not stable (Jiao et al. 1992) and IDEF1 most likely binds to metals using histidine residues (Kobayashi et al. 2012), glutathione S-transferase (GST) or His tag was not suitable for the interaction. Therefore, wheat germ system was chosen for IBP1 protein expression.

In addition to IBP1.1, partial sequences of IBP1.2 were also identified as positive clones that interact with IDEF1 in yeast two-hybrid screening (Fig. 2-3). As IBP1.1 and IBP1.2 are highly homologous, full-length IBP1.2 is also thought to interact with IDEF1. IBP1.1 and IBP1.2 should exist in the same cells in which IDEF1 is expressed and functioning. The *IDEF1* gene is constitutively expressed in rice roots, leaves, flowers and seeds, regardless of Fe nutritional condition (Kobayashi et al. 2009, 2010a, 2010b). On the other hand, *IBP1.1/RBBI3-1* is reported to be expressed in leaves, flowers and more evidently in roots (Qu et al. 2003). When expressed in onion cells, IBP1.1 localized to the nucleus and cytoplasm (Fig. 3-3). As IDEF1 localizes mainly to the nucleus and to a lesser degree the cytoplasm (Kobayashi et al. 2007), the interaction between IDEF1 and IBP1.1 (and IBP1.2) could occur in both the nucleus and cytoplasm. This potential interaction in the cytoplasm is most likely to occur immediately after IDEF1 synthesis in the cytoplasm, before it is shuttled to the nucleus.

IBP1 was induced by Fe-deficiency through mediation of IDEF1 regulation

In the promoter regions of *IBP1.1* and *IBP1.2*, there are many IDEF1 binding

sites, whereas only one IDEF2 binding site was identified in the promoter region of *IBP1.1* (Fig. 3-1c). The expression of *IBP1.1* and *IBP1.2* was significantly induced by Fe deficiency at days 1 and 7 (Fig. 3-4) and the expression of *IBP1.1* and *IBP1.2* was significantly higher in *I2*pro-*IDEF1* FL than in NT at days 1 and 9 of Fe-deficiency treatment (Fig. 3-5), indicating that *IBP1.1* and *IBP1.2* were positively regulated by IDEF1 at both the early and subsequent stages of Fe deficiency, with greater effects at the early stage (Fig. 3-5). However, *IBP1.1* induction was higher at day 1 of Fe-deficiency treatment than at day 7 in both roots and leaves, whereas *IBP1.2* induction remained elevated in roots and much higher in leaves at day 7 (Fig. 3-4). These different patterns in *IBP1.1 vs. IBP1.2* gene expression at day 7 may relate to different IDEF1 binding sites in their promoter regions (Fig. 3-1c) or factors other than IDEF1 that may participate in the regulation of *IBP1.1* and *IBP1.2* under Fe-deficient conditions. In addition, the metal-binding regions of IDEF1 for possible Fe sensing had a marked effect on the regulation of *IBP1.1* and *IBP1.2* at the early stages of Fe deficiency (Fig. 3-5). These patterns of regulation are similar to some other Fe deficiency-inducible genes involved in Fe homeostasis, such as *OsIRO2*, *OsIRT1*, *OsYSL2*, and *OsNAS3* (Kobayashi et al. 2009, 2012).

The gene expression in *IBP1.1*-FOX lines

Transgenic rice plants overexpressing *IBP1.1* showed enhanced expression of the Fe(II)-nicotianamine transporter gene *OsYSL2* (Fig. 3-8b). *OsYSL2* gene expression is induced under Fe-deficient conditions and is positively regulated by both IDEF1 and IDEF2 (Koike et al. 2004; Ogo et al. 2008; Kobayashi et al. 2009).

IBP1 may play key roles in both jasmonic acid and Fe deficiency

Bowman-Birk protease inhibitors are induced in response to injury, fungus, pathogens, and in defense mediated by jasmonic acid and ethylene (Rakwal et al. 2001; Zavala et al. 2004). The *IBP1.1/RBBI3-1* transcript was induced by wounding and jasmonic acid treatment (Qu et al. 2003; Yoshii et al. 2010), and IBP1.1/RBBI3-1 protein levels were increased 12 h after wounding treatment in rice (Chen et al. 2006). Induction of IBP1.1 by both Fe deficiency and other stresses such as wounding may indicate an unidentified link between various stressors. Jasmonic acid is a key molecule in plant response to wounding (Howe, 2004). In *Arabidopsis*, jasmonate is reported to suppress basal expression of *IRT1* and *FRO2*, two major genes involved in the Fe-deficiency response (Maurer et al. 2011). In contrast, positive correlation between jasmonic acid and Fe-deficiency response is suggested in graminaceous plants. In maize intercropped with peanut, abundances of key enzymes in jasmonate biosynthesis were increased (Xiong et al. 2013a), and Fe-deficiency response was induced (Xiong et al. 2013b). The rice receptor-like protein OsRMC regulates both jasmonic acid-mediated root development and Fe-deficiency response, and its transcripts are induced by both jasmonic acid and Fe deficiency (Jiang et al. 2007; Yang et al. 2013). These results suggest substantial crosstalk between the Fe-deficiency response and the jasmonic acid-mediated stress response, in which IBP1 and OsRMC may play key roles.

IBP1 protects IDEF1 from degradation via the 26S proteasome-dependent pathway

IDEF1 accumulated to a greater degree under MG132 treatment both *in vivo* (Fig. 3-9a) and *in vitro* (Fig. 3-9c), suggesting that IDEF1 is degraded via the 26S

proteasome-dependent pathway.

Co-expression of IBP1.1 with IDEF1 in protoplasts enhanced IDEF1 accumulation (Fig. 3-9b) and the addition of HA-IBP1.1 to the reaction mixture reduced IDEF1 degradation (Fig. 3-9d), suggesting a protective effect of IBP1.1 on IDEF1 degradation. A soybean Bowman-Birk inhibitor was reported to be involved in proteasome inhibition and effectively suppress carcinogenesis (Chen et al. 2005). Because IBP1.1 and IBP1.2 are highly homologous, full-length IBP1.2 is also thought to protect IDEF1 against proteasome-mediated degradation.

A working model for the role of IBP1 in regulating IDEF1

Based on these results, I propose a working model for the role of IBP1 in regulating IDEF1 (Fig. 3-10). Under Fe-sufficient conditions, IDEF1 is vigorously degraded through the 26S proteasome-dependent pathway. Under Fe-deficient conditions, *IBP1* expression is induced under the regulation of IDEF1. Subsequently, the IBP1 protein is thought to accumulate and prevent the degradation of IDEF1. Accumulated IDEF1 is thought to induce the expression of downstream genes, such as *OsYSL2*.

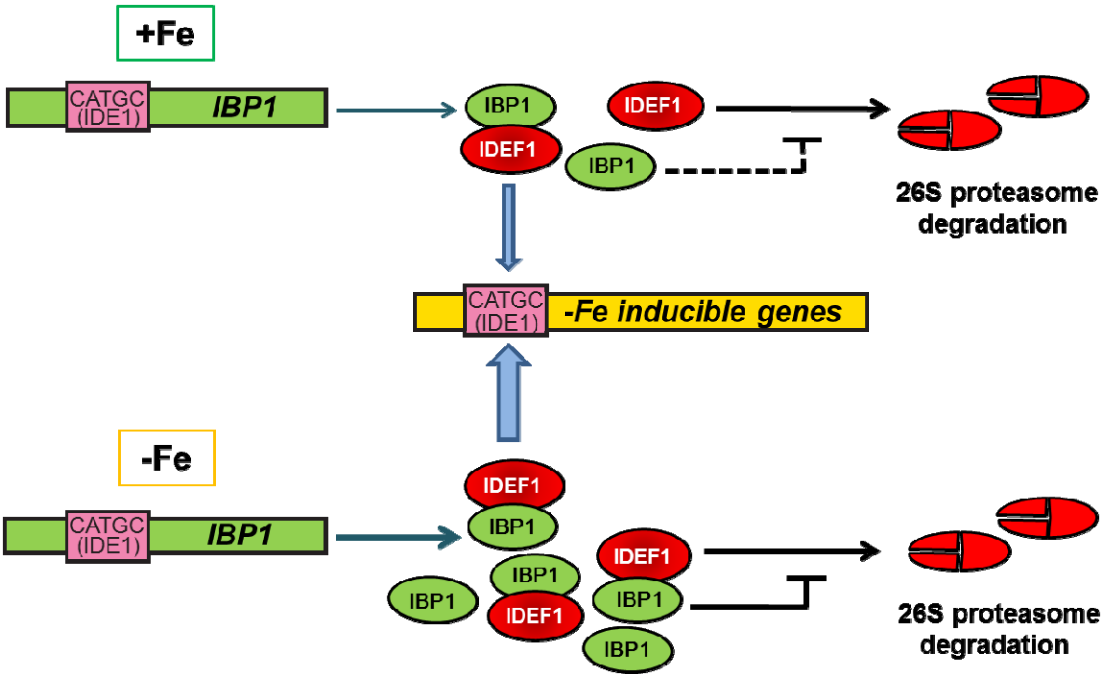

Figure 3-10. Hypothetical model of IBP1 regulation of IDEF1.

Under Fe-sufficient conditions, IDEF1 is degraded vigorously via the 26S proteasome-dependent pathway. Under Fe-deficient conditions, *IBP1* expression is induced under the control of IDEF1. IBP1 protein subsequently accumulates and prevents the degradation of IDEF1. Accumulated IDEF1 induces the expression of downstream genes, such as *OsYSL2*.

Chapter 4

IBP2 interacting with IDEF1 plays a role in Fe homeostasis

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。5 年以内に出版予定である。

Chapter 5

IBP3 interacting with IDEF1 plays a role in metal binding in rice

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。5 年以内に出版予定である。

Chapter 6

Final Discussion

本章の内容の一部は、学術雑誌論文として出版する計画があるため公表できない。5 年以内に出版予定である。

IDEF1 plays a key role as a positive regulator

Under Fe-deficient conditions, genes involved in Fe acquisition and utilization are transcriptionally upregulated in plants. In this responses, rice transcription factor IDEF1 plays a key role as a positive regulator and is a candidate Fe sensor through its metal-binding domain (Kobayashi et al. 2009, 2012). *IDEF1* gene is constitutively expressed regardless of Fe nutritional conditions (Kobayashi et al. 2007, 2009, 2010b; Itai et al. 2013). In the present study, I found that IDEF1 was vigorously degraded *via* a 26S proteasome-dependent manner (Fig. 3-9).

Analysis of *IDEF1* knockdown and overexpression rice plants revealed that *IDEF1* gene expression levels positively affect the tolerance to Fe deficiency during the early stage of Fe deficiency (Kobayashi et al. 2009). Fe-deficiency responses mediated by IDEF1 are shifted during progression of Fe deficiency (Kobayashi et al. 2009, 2010a, 2012). IDEF1 expression and/or activity are thought to be regulated in response to Fe nutritional conditions.

Post-translational regulatory mechanism of IDEF1 in Fe nutritional conditions

To clarify the underlying molecular mechanism mediated by IDEF1, I screened rice cDNA library by yeast two-hybrid assay and found candidates interacting with IDEF1 (Table 2-2, Fig. 2-5). Among these candidates, five IDEF1-binding proteins, IBP1-5, were identified to be capable of interacting with IDEF1 (Fig. 2-5). The interaction between IDEF1 and IBP1, IBP2 or IBP3 was confirmed by MBP pull-down assay (Figs. 3-2, 4-2, 5-2). Actually, co-expression of IDEF1 and IBP1.1 in Oc cells had effect on accumulation of IDEF1 protein (Fig. 3-9). Similar to IBP1, it is likely for IBP2 or IBP3 to interact with IDEF1 in rice also. Through the interaction with IDEF1, IBP1,

IBP2 and IBP3 are thought to work cooperatively in different ways such as prevention of IDEF1 degradation, regulating IDEF1-mediated Fe responses and Fe binding. Identification of IBP1 as a protecting factor of IDEF1 from its degradation is of great importance, because it is the first discovery of a post-translational regulatory mechanism in Fe-deficiency responses in graminaceous plants.

Final remarks and perspective

This research not only found the presence of post-translational regulation of IDEF1, but also indicated novel molecular mechanisms regulating Fe-deficiency responses in rice. More works will be needed to further elucidate the complicated regulation of IDEF1 activity required for Fe homeostasis in rice.

In graminaceous plants, post-translational regulation of other transcription factors IDEF2, OsIRO2, OsIRO3, OsHRZ1, OsHRZ2 in response to Fe-deficiency remains unknown (Ogo et al. 2006, 2007, 2008, 2011; Zheng et al. 2010; Kobayashi et al. 2013). Transgenic rice plants constitutively overexpressing OsIRO2 exhibit tolerance to Fe deficiency in both hydroponic culture and long-term culture in calcareous soils (Ogo et al. 2007, 2011). *OsHRZ*-knockdown rice lines with repressed expression of *OsHRZ1* and/or *OsHRZ2* exhibit tolerance to Fe deficiency in both hydroponic culture and long-term culture in calcareous soils, and accumulate high amounts of Fe in shoots and seeds (Kobayashi et al. 2013). In future, the work of screening factors interacting with these transcription factors will be needed to understand the regulation network involved in Fe-deficiency responses.

Underlying molecular mechanisms by which IDEF1 regulates Fe-deficiency responses remain largely unknown. Clarification of the factors interacting with IDEF1 would give us more information on the mechanisms of Fe-deficiency responses in rice.

References

Aoyama T, Kobayashi T, Takahashi M, Nagasaka S, Usuda K, Kakei Y, Ishimaru Y, Nakanishi H, Mori S, Nishizawa NK (2009) OsYSL18 is a rice iron(III)-deoxymugineic acid transporter specifically expressed in reproductive organs and phloem of lamina joints. Plant Mol Biol 70:681–692

Bashir K, Inoue H, Nagasaka S, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2006) Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants. J Biol Chem 43:32395–32402

Bashir K, Ishimaru Y, Nishizawa NK (2010) Iron Uptake and Loading into Rice Grains Rice 3-92–130

Bauer P, Ling HQ, Guerinot ML (2007) FIT, the FER-like iron deficiency induced transcription factor in *Arabidopsis*. Plant Physiol Biochem 45:260–261

Baxter I, Tchieu J, Sussman MR, Boutry M, Palmgren MG, Gribskov M, Harper JF, Axelsen KB (2003) Genomic comparison of P-type ATPase ion pumps in *Arabidopsis* and rice. Plant Physiol 132:618–628

Bowman DE (1946) Differentiation of soybean anti-tryptic factors. Proc Soc Exp Biol Med 63:547–550

Brunings AM, Datnoff LE, Ma JF, Mitani N, Nagamura Y, Rathinasabapathi B, Kirst M (2009) Differential gene expression of rice in response to silicon and rice blast fungus Magnaporthe oryzae. Ann Appl Biol 155:161–170

Causier B, Davies B (2002) Analysing protein-protein interactions with the yeast two-hybrid system. Plant Mol Biol 50:855–870

Chen J, Mao S, Xie Y, Cao Z, Zhang Y, Liu J, Chen Z, Qu L, Gu H (2006) Expression and inhibitory activity analysis of a 25-kD Bowman-Birk protease inhibitor in rice. Chin Sci

Bull 51:54–62

Chen YW, Huang SC, Lin-Shiau SY, Lin JK (2005) Bowman-Birk inhibitor abates proteasome function and suppresses the proliferation of MCF7 breast cancer cells through accumulation of MAP kinase phosphatase-1. Carcinogenesis 26:1296–1306

Colangelo EP, Guerinot ML (2004) The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. Plant Cell 16:3400–3412

Curie C, Panaviene Z, Loulergue C, Dellaporta SL, Briat JF, Walker EL (2001) Maize *yellow stripe 1* encodes a membrane protein directly involved in Fe(III) uptake. Nature 409:346 –349

Guerinot ML, Yi Y (1994) Iron: nutritious, noxious, and not readily available. Plant Physiol 104: 815–820

Hindt MN, Guerinot ML (2012) Getting a sense for signals:regulation of the plant iron deficiency response. Biochim Biophys Acta 1823:1521–1530

Higuchi K, Suzuki K, Nakanishi H, Yamaguchi H, Nishizawa NK, Mori S (1999) Cloning of nicotianamine synthase genes, novel genes involved in the biosynthesis of phytosiderophores. Plant Physiol 119:471–479

Howe GA (2004) Jasmonates as signals in the wound response. J Plant Growth Regul 23:223–237

Inoue H, Kobayashi T, Nozoye T, Takahashi M, Kakei Y, Suzuki K, Nakazono M, Nakanishi H, Mori S, Nishizawa NK (2009) Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. J Biol Chem 284:3470–3479

Inoue H, Hayashi N, Matsushita A, Liu XQ, Nakayama A, Sugano S, Jiang CJ, Takatsuji H (2013) Blast resistance of CC-NB-LRR protein Pb1 is mediated by WRKY45 through

protein–protein interaction. Proc Natl Acad Sci 110:9577–9582

Ishimaru Y, Masuda H, Bashir K, Inoue H, Tsukamoto T, Takahashi M, Nakanishi H, Aoki N, Hirose T, Ohsugi R, Nishizawa NK (2010) Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese. Plant J 62:379–390

Jiang J, Li J, Xu Y, Han Y, Bai Y, Zhou G, Lou Y, Xu Z, Chong K (2007) RNAi knockdown of *Oryza sativa root meander curling* gene led to altered root development and coiling which were mediated by jasmonic acid signaling in rice. Plant Cell Environ 30:690–699

Kobayashi T, Itai RN, Aung MS, Senoura T, Nakanishi H, Nishizawa NK (2012) The rice transcription factor IDEF1 directly binds to iron and other divalent metals for sensing cellular iron status. Plant J 69:81–91

Kobayashi T, Itai RN, Ogo Y, Kakei Y, Nakanishi H, Nishizawa NK (2009) The rice transcription factor IDEF1 is essential for the early response to iron deficiency, and induces vegetative expression of late embryogenesis abundant genes. Plant J 60:948–961

Kobayashi T, Nakanishi H, Nishizawa NK (2010) Dual regulation of iron deficiency response mediated by the transcription factor IDEF1. Plant Signal Behav 5:157–159

Kobayashi T, Nakayama Y, Itai RN, Nakanishi H, Yoshihara T, Mori S, Nishizawa NK (2003) Identification of novel *cis*-acting elements, IDE1 and IDE2, of the barley *IDS2* gene promoter conferring iron-deficiency-inducible, root-specific expression in heterogeneous tobacco plants. Plant J 36:780–793

Kobayashi T, Nishizawa NK (2012) Iron uptake, translocation, and regulation in higher plants. Ann Rev Plant Biol 63:131–152

Kobayashi T, Ogo Y, Itai RN, Nakanishi H, Takahashi M, Mori S, Nishizawa NK (2007) The novel transcription factor IDEF1 regulates the response to and tolerance of iron deficiency in plants. Proc Natl Acad Sci 104:19150–19155

Kobayashi T, Nagasaka S, Senoura T, Itai RN, Nakanishi H, Nishizawa NK (2013) Iron-binding haemerythrin RING ubiquitin ligases regulate plant iron responses and accumulation. Nat Commun 4:2792

Kobayashi T, Suzuki M, Inoue H, Itai RN, Takahashi, M, Nakanishi H, Mori S, Nishizawa NK (2005) Expression of iron-acquisition-related genes in iron-deficient rice is co-ordinately induced by partially conserved iron-deficiency-responsive elements. J Exp Bot 56:1305–1316

Koike S, Inoue H, Mizuno D, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. Plant J 39:415–424

Lingam P, Mohrbacher J, Brumbarova T, Potuschak T, Fink-Straube C, Blondet E, Genschik P, Bauer P (2011) Interaction between the bHLH transcription factor FIT and ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between the regulation of iron acquisition and ethylene signaling in *Arabidopsis*. Plant Cell 23:1815–1829

Long TA, Tsukagoshi H, Busch W, Lahner B, Salt DE, Benfey PN (2010) The bHLH transcription factor POPEYE regulates response to iron deficiency in *Arabidopsis* roots. Plant Cell 22:2219–2236

Marschner H, Römheld V (1994) Strategies of plants for acquisition of iron. Plant and Soil 165: 261–274

Maurer F, Müller S, Bauer P (2011) Suppression of Fe deficiency gene expression by jasmonate. Plant Physiol Biochem 49:530–536

Mizuno D, Higuchi K, Sakamoto T, Nakanishi H, Mori S, Nishizawa NK (2003) Three nicotianamine synthase genes isolated from maize are differentially regulated by iron

nutritional status. Plant Physiol 132:1989–1997

Mori S, Nishizawa NK (1987) Methionine as a dominant precursor of phytosiderophores in *Graminaceae* plants. Plant Cell Physiol 28:1081–1092

Murata Y, Ma JF, Yamaji N, Ueno D, Nomoto K, Iwashita T (2006) A specific transporter for iron(III)-phytosiderophore in barley roots. Plant J 46:563–572

Nozoye T, Nagasaka S, Kobayashi T, Takahashi M, Sato Y, Uozumi N, Nakanishi H, Nishizawa NK (2011) Phytosiderophore efflux transporters are crucial for iron acquisition in graminaceous plants. J Biol Chem 286:5446–5454

Ogo Y, Itai RN, Kobayashi T, Aung MS, Nakanishi H, Nishizawa NK (2011) OsIRO2 is responsible for iron utilization in rice and improves growth and yield in calcareous soil. Plant Mol Biol 75:593–605

Ogo Y, Itai RN, Nakanishi H, Inoue H, Kobayashi T, Suzuki M, Takahashi M, Mori S, Nishizawa NK (2006) Isolation and characterization of IRO2, a novel iron-regulated bHLH transcription factor in graminaceous plants. J Exp Bot 57:2867–2878

Ogo Y, Itai RN, Nakanishi H, Kobayashi T, Takahashi M, Mori S, Nishizawa NK (2007) The rice bHLH protein OsIRO2 is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions. Plant J 51:366–377

Ogo Y, Kobayashi T, Itai RN, Nakanishi H, Kakei Y, Takahashi M, Toki S, Mori S, Nishizawa NK (2008) A novel NAC transcription factor IDEF2 that recognizes the iron deficiency-responsive element 2 regulates the genes involved in iron homeostasis in plants. J Biol Chem 283:13407–13417

Park EY, Kim JA, Kim HW, Kim YS, Song HK (2004) Crystal structure of the Bowman-Birk inhibitor from barley seeds in ternary complex with porcine trypsin. J Mol Biol 343:173–186

Qu LJ, Chen J, Liu M, Pan N, Okamoto H, Lin Z, Li C, Li D, Wang J, Zhu G, Zhao X, Chen X, Gu H, Chen Z (2003) Molecular cloning and functional analysis of a novel type of Bowman-Birk inhibitor gene family in rice. Plant Physiol 33:560–570

Rakwal R, Kumar Agrawal G, Jwa NS (2001) Characterization of a rice (*Oryza sativa* L.) Bowman-Birk proteinase inhibitor: tightly light regulated induction in response to cut, jasmonic acid, ethylene and protein phosphatase 2A inhibitors. Gene 263:189–198

Römheld V, Marschner H (1986) Evidence for a specific uptake system for iron phytosiderophores
in roots of grasses. Plant Physiol 80:175–180

Shojima S, Nishizawa NK, Fushiya S, Nozoe S, Irifune T, Mori S (1990) Biosyntheisis of phytosiderophores. Plant Physiol 93:1497–1503

Sivitz A, Grinvalds C, Barberon M, Curie C, Vert G (2011) Proteasome-mediated turnover of the transcriptional activator FIT is required for plant Fe-deficiency responses. Plant J 66:1044–1052

Takagi S (1976) Naturally occurring iron-chelating compounds in oat- and rice-root washings:I. Activity measurement and preliminary characterization. Soil Sci Plant Nutr 22:423–433

Takahashi M, Yamaguchi H, Nakanishi H, Shioiri T, Nishizawa NK, Mori S (1999) Cloning two genes for nicotianamine aminotransferase, a critical enzyme in iron acquisition (Strategy II) in graminaceous plants. Plant Physiol 121:947–956

Voges D, Zwickl P, Baumeister W (1999) The 26S proteasome: a molecular machine designed for controlledproteolysis. Annu Rev Biochem 68:1015–1068

Wang F, Zhu D, Huang X, Li S, Gong Y, Yao Q, Fu X, Fan LM, Deng XW (2009) Biochemical insights on degradation of *Arabidopsis* della proteins gained from a cell-free assay system. Plant Cell 21:2378–2390

Wang N, Cui Y, Liu Y, Fan H, Du J, Huang Z, Yuan Y, Wu H, Ling HQ (2013) Requirement and functional redundancy of ib subgroup bHLH proteins for iron deficiency responses and uptake in *Arabidopsis thalian*a. Mol Plant 6:503–513

Xiong H, Shen H, Zhang L, Zhang Y, Guo X, Wang P, Duan P, Ji C, Zhong L, Zhang F, Zuo Y (2013a) Comparative proteomic analysis for assessment of the ecological significance of maize and peanut intercropping. J Proteomics 78:447–460

Xiong H, Kakei Y, Kobayashi T, Guo X, Nakazono M, Takahashi H, Nakanishi H, Shen H, Zhang F, Nishizawa NK, Zuo Y (2013b) Molecular evidence for phytosiderophore-induced improvement of iron nutrition of peanut intercropped with maize in calcareous soil. Plant Cell Environ 36:1888–1902

Yang A, Li Y, Xu Y, Zhang WH (2013) A receptor-like protein RMC is involved in regulation of iron acquisition in rice. J Exp Bot 64:5009–5020

Yuan Y, Wu H, Wang N, Li J, Zhao W, Du J, Wang D, Ling HQ (2008) FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in *Arabidopsis*. Cell Res18:385–397

Yoshii M, Yamazaki M, Rakwal R, Kishi-Kaboshi M, Miyao A, Hirochika H (2010) The NAC transcription factor RIM1 of rice is a new regulator of jasmonate signaling. Plant J 61:804-815

Zavala JA, Patankar AG, Gase K, Hui D, Baldwin IT (2004) Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. Plant Physiol 134:1181–1190

Zheng L, Ying Y, Wang L, Wang F, Whelan J, Shou H (2010) Identification of a novel iron regulated basic helix-loop-helix protein involved in Fe homeostasis in *Oryza sativa*. BMC Plant Biol 10:166