Studies on the structural and functional diversities of soybean ferritin subunits

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Abbreviations

ATP	adenosine 5'-triphosphate
bp	base pair
cDNA	complementary deoxyribonucleic acid
CP	creatine phosphate
DTT	dithiothiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β-aminoethylether)- *N,N,N',N'*-tetraacetic acid
EP	extension peptide
GTP	guanosine 5'-triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
sfer, SFER	soybean ferritin
TBS	tris-buffered saline
TP	transit peptide
Tris	tris(hydroxymethyl)aminomethane

General Introduction

Iron is essential for all living kingdoms, however, when exist in excess level, they are sometimes harmful because iron is highly reactive as a pro-oxidant in Harber-Weiss reactions to generate free radicals (Halliwel and Gutteridge 1988). Therefore, iron homeostasis involving both of metal uptake and storage must be under highly controlled regulation. Especially, plants require tightly regulated iron homeostasis to prevent both iron toxicity and deficiency, because of their immobility. Although iron is abundant in many types of soil, it is present as insoluble form in neutral pH (ferric oxide or ferric hydroxide) which is not accessible and available to plants (Guerinot and Yi, 1994). In dicotyledonous plants, chelated ferric ions are reduced by a root plasma membrane-bound ferric reductase, and then absorbed via a metal ion transporter (Romheld and Marschner 1986). Recently, genes involved in iron uptake have been cloned from *Arabidopsis thaliana*: root ferric reductase (*fro2*) (Robinson *et al.* 1999), and ferrous transporter (*irt1*) (Eide *et al.* 1996). On the other hand, inside the plant cell, the iron atoms are usually stored in iron storage protein 'ferritin' as ferrihydrite form in mammal (Bauminger *et al.* 1993, St. Pierre *et al.* 1990, Perrira et al. 1997, Zhao *et al.* 2003) or ferric phosphate form in plant (Wade *et al.* 1993).

Ferritin is a class of iron storage protein distributed to plant as well as bacteria and vertebrate, which can store a few thousands of iron atoms as non-toxic and biologically available form (Harrison and Arosio 1996,

Carrondo 2003). Each of known ferritin is a multimeric protein composed of 24 subunits, which is assembled to a symmetrical cage with inner cavity. The three dimensional structures of ferritin are more conserved than their primary structure. Three-dimensional structural analyses of ferritins from vertebrate and bacteria reveal that each subunit is composed of a four helix bundle (helices A, B, C and D) and fifth short helix (E-helix), in which the helices B and C are linked by a loop sequence (BC-loop) composed of about fifteen residues (Lawson *et al.* 1991, Trikha *et al.* 1994, 1995, Hempstead *et al.* 1997, Cobessi *et al.* 2002, Stillman *et al.* 2001). Three-dimensional structure of plant ferritins have not been solved yet, however, Lobreaux and co-worker have predicted that they can adopt the conformation similar to the mammals' on the basis of computational analysis for pea ferritin (Lobreaux *et al.* 1992). Ferritin subunits in the assembled oligomer are related by operation of 4-, 3- and 2-fold symmetry axes. In mammal, two types of ferritin subunits, H and L, are characterized. The H chain catalyzes the Fe(II) oxidation, which is the first step in iron uptake (Lawson *et al.* 1991). On the other hand, the L chain does not have ferroxidase activity, however, the L chain, which has the function of iron core formation, is necessary for stable iron storage (Levi *et al.* 1992, Liu and Theil 2004). These subunits have about 50 % identity of the amino acid sequence. The two types of subunit co-assemble into a mixed subunit 24-mers (hetero-polymer) because H and L subunits have the close conformations and the many identical or similar residues in H-H, H-L and L-L inter-subunit interaction region enable the formation of hetero-polymers with the

complete range of subunit compositions. The co-assembled isoferritin have distinctive composition ratio of the two subunits in different tissue and organ in mammal (Arosio *et al.* 1978). For example, L rich ferritins are predominant in organs storing iron such as liver and spleen, whereas H rich ferritins are predominant in heart and brain.

Among ferritins identified from many species, plant ferritin has unique characteristics in its primary sequence, that is, extension regions in their N-terminus. The first part of which is called a transit peptide and the other is an extension peptide (Ragland *et al.* 1990). The transit peptide is a plastid targeting sequence, which is processed on entry into the plastid. Similar to the plant ferritin, a novel ferritin gene with N-terminal leader sequence has been identified in human, and the leader sequence was suggested to be responsible for mitochondrial targeting of this ferritin (Levi *et al.* 2001). In contrast to cytoplasmic location of mammal ferritin H and L chains, plant ferritin is localized in plastid because of the presence of the plastid targeting signal (Ragland *et al.* 1990) although plant and vertebrate ferritins are derived from a common ancestor (Proudhon *et al.* 1996). On the other hand, a role of the extension peptide, which is highly conserved among plant ferritin sequences, remains unclear (Van Wuytswinkel *et al.* 1995). Since the 'extension peptide' is present at the N-terminus of plant ferritin, the apparent molecular mass of its mature form is 28 kDa in the case of legume plant, whereas the apparent molecular mass of mammal H and L chains are 21 and 19 kDa, respectively.

During the last decade, some ferritin genes have been identified in

maize, *Arabidopsis*, cowpea, beans and peas, and suggested to form a gene family (Sanvino *et al.* 1997, Wardrop *et al.* 1999, Petit *et al.* 2001a). Cowpea had at least four ferritin genes and their primary structures were suggested to be highly conserved (80 to 90 % similarity) (Wardrop *et al.* 1999). Similarly, Petit and co-workers reported that gene family composed of four genes were present in *Arabidopsis thaliana*, and that each of them showed the specific patterns of expression in response to environmental stimulation (Petit *et al.* 2001a). The expression of ferritin gene family has been suggested to be regulated via at least two pathways (Lobreaux *et al.* 1992, Fobis-Loisy *et al.* 1995, Gaymard *et al.* 1996): Iron dependent regulatory pathway and abscisic acid (ABA) dependent pathway (Lobreaux *et al.* 1993). Recently, it has been suggested that nitric oxide plays an important role as a signaling molecule in iron dependent regulatory pathway of ferritin synthesis (Murgia *et al.* 2002). In plant, synthesis of ferritin genes in response to iron is regulated at the transcriptional level, in contrast to translational control in mammal (Dickey *et al.* 1988). As iron-dependent regulatory sequences of plant ferritin, "iron regulatory element" from soybean and "iron-dependent regulatory sequence" from maize and *Arabidopsis* were identified in the promoter region of the ferritin genes and well characterized (Wei and Theil 2000, Petit *et al.* 2001b). In contrast to the recent progress in genetic studies of plant ferritin, knowledge about plant ferritin subunits from the gene families are still restricted. In order to clarify whether functional diversity is developed among plant ferritin subunits, the author has investigated the ferritin gene family in soybean.

In this thesis, the author demonstrated that soybean ferritin subunits were functionally distinguishable. In addition, a mechanism for iron release from assembled ferritin shell was proposed. Chapter I demonstrated that soybean seed ferritin was present as hetero-polymer composed of two types of subunit, one of which was easy to be degraded and promoted the iron release from the ferritin shell. The author has cloned four ferritin genes from soybean plantlet and shown their expression patterns specific to tissues or developmental stages of seeds (Chapter II). In Chapter III, the ability of assembly to homo- or hetero-polymer of the ferritin subunit was investigated using an *in vitro* protein expression system. Crystallization and preliminary X-ray crystallographic analysis for solving the three-dimensional structure of recombinant soybean ferritin was described in Chapter IV.

Chapter I

A novel plant ferritin subunit from soybean that is related to a mechanism in iron release

1-1 Abstract

Ferritin is a multimeric iron storage protein composed of 24 subunits. Ferritin purified from dried soybean seed resolves into two peptides of 26.5 and 28 kDa. To date, the 26.5 kDa subunit has been supposed to be generated from the 28 kDa subunit by cleavage of the N-terminal region. The author performed amino acid sequence analysis of the 28 kDa subunit, and found that it had a different sequence to the 26.5 kDa subunit, thus rendering it novel among known soybean ferritins. The author cloned a cDNA encoding this novel subunit from ten-days-old seedlings, each of which contained developed bifoliates, an epicotyl and a terminal bud. The 26.5 kDa subunit was found to be identical to that identified previously lacking the C-terminal 16 residues that correspond to the "E-helix" of mammalian ferritin. However, the corresponding region in the 28 kDa soybean ferritin subunit identified in this study was not susceptible to cleavage. The author present evidence that the two different ferritin subunits in soybean dry seeds show differential sensitivity to protease digestions, and that the novel, uncleaved 28 kDa ferritin subunit appears to stabilize the ferritin shell by co-existing with the cleaved 26.5 kDa subunit. These data demonstrate that soybean ferritin is composed of at least two different subunits, which have co-operative functional roles in soybean seeds.

1-2 Introduction

The iron storage protein ferritin has a structure highly conserved among plants, animals and bacteria. Ferritin has 24 subunits that are assembled into a spherical shell characterized by 4-fold, 3-fold and 2-fold symmetry (432 symmetry). Up to 4500 Fe(III) atoms can be stored as an inorganic complex in the inner cavity of assembled ferritin, rendering these atoms non-toxic and biologically available (Harrison and Arosio 1996). Structural analyses of vertebrate and bacterial ferritins indicate that each subunit consists of a four-helix bundle (helices A, B, C and D) and a fifth short helix (E helix). The E-helix exists around the 4-fold inter subunit symmetry axes of the protein shell, and forms a hydrophobic pore (Harrison and Arosio 1996, Trikha *et al.* 1994, Hempstead *et al.* 1997, Frowlow *et al.* 1994). In mammals, two distinct ferritin subunits (H and L) are found (Arosio *et al.* 1978). These subunits have 50% identity and similar 3-dimensional structures. The H subunit has ferroxidase activity (Lawson *et al.* 1989, 1991) and catalyzes oxidation of Fe(II), which is the first step in iron storage. The L subunit promotes nucleation of the iron core (Levi *et al.* 1992). The synthesis of ferritin in vertebrates is regulated during translation. It was suggested that both the 5'- and 3'- untranslated regions of ferritin mRNA contributed to translational control (Dickey *et al.* 1988). The iron responsive element (IRE), which was first identified in the 5'- untranslated region of human ferritin mRNA (Hentze *et al.* 1987), is highly conserved among mammals and other vertebrates. Despite the probable common

ancestry of plant and vertebrate ferritins (Proudhon *et al.* 1996), expression of plant ferritin is regulated primarily at the transcriptional level, in response to iron administration (Lescure *et al.* 1991). A sequence with similarity to the IRE is absent in the 5'-untranslated region of plant ferritin mRNA, however, Wei and Theil (2000) recently suggested the existence of an "iron regulatory element (FRE)" in the promoter region of the soybean ferritin gene, which controls the transcription together with a *trans*-acting factor (Wei and Theil 2000).

Only one polypeptide chain type has been identified as a functional subunit of plant ferritins (Lescure *et al.* 1991). Like the mammalian H subunit, with which it shares about 40 % sequence homology, this subunit has ferroxidase activity (Briat et al. 1995, Laulhere and Briat 1993). During the last decade, evidence for ferritin multigene families in maize (Lobreaux et al. 1992), cowpea (Wicks and Entsch 1993, Wardrop *et al.* 1999) and soybean (Wardrop *et al.* 1999) was provided. Cowpea has at least four different ferritin genes, one that encodes a protein with 97% sequence identity to soybean ferritin (Lescure *et al.* 1991, Ragland *et al.* 1990). The peptides encoded by the other cowpea genes in the ferritin family are more divergent. Despite the evidence that multiple ferritin genes exist in plants, it is not clear whether the products of these different genes are functionally divergent. This is in contrast to the situation reported in pigs, frogs and salmon, where two functional H-type subunits showing different tissue specificity are found (Collawn and Fish 1984, Dickey *et al.* 1987, Andersen *et al.* 1995).

The ferritin subunits of soybean and many other legumes are synthesized as 32 kDa precursor proteins (van der Mark *et al.* 1983) which contain an unique two-domain N-terminal sequence (Ragland *et al.* 1990). These N-terminal domains are not present in mammalian or other ferritins. The first domain, which consists of 40-50 residues and is known as the 'transit peptide' (TP), is presumed to facilitate transport of the ferritin precursor to plastids (Briat *et al.* 1995). The function of the second domain, which is a part of the mature protein and is termed extension peptide (EP), is currently unclear. The ferritin subunit purified from soybean seed is 28 kDa, but it is apparently converted in significant amounts to a 26.5 kDa subunit (Ragland *et al.* 1990, Laulhere *et al.* 1989). In 1991, Lobreaux and colleagues (Lobreaux *et al.* 1991) suggested that such truncation occurs by cleavage of the EP during germination or free radical damage. They considered the 28 kDa subunit to be an iron containing form, and the 26.5 kDa subunit to be an iron releasing form (van der Mark *et al.* 1983, van Wuytswinkel *et al.* 1995). This proposal has yet to be unsubstantiated by experimental evidence.

Here the author report evidence that the iron conversion mechanism of soybean seed ferritin involves two distinct subunits. One of these is novel and presented here for the first time, while the other is identical to that previously identified. These data about soybean ferritin maturation allow us to hypothesize a novel mechanism by which iron is released from the assembled ferritin molecule.

1-3 Materials and Methods

1-3-1 Purification of native soybean ferritin

500g of soybean dry seeds (*Glycine max* Merr cv. Kita-no-shiki) were crushed into flour by a mill. The soybean seed flour was suspended in 50mM Tris-HCl buffer (pH7.5) containing 1mM EDTA and 10mM 2-mercaptoethanol (2-ME), homogenized, and centrifuged at 10,000 *g* for 10min. The supernatant was fractionated using 20% saturation of ammonium sulfate. An amber colored precipitant was collected by centrifugation and dialyzed against 50mM Tris-HCl (pH7.5) buffer. The dialyzed sample was applied to a DEAE-Toyopearl (TOSOH, Tokyo, Japan) column previously equilibrated by 50mM Tris-HCl (pH7.5) containing 1mM EDTA. The column was washed with a buffer containing 0.15M sodium chloride, and amber colored ferritin was eluted. The eluate was again fractionated with 20% saturated ammonium sulfate. The supernatant was collected by centrifugation, applied to a butyl-Toyopearl (TOSOH) column and eluted with a linear gradient of 20-0% saturated ammonium sulfate. Fractions containing soybean ferritin were pooled and dialyzed against 50 mM Tris-HCl (pH7.5) containing 1mM EDTA and applied to Q-sepharose columns (Amersham- Pharmacia). Proteins were eluted with a linear gradient of sodium chloride from 0 to 0.7 M. Fractions containing soybean ferritin were pooled and concentrated, and finally loaded to Superdex 75 pg gel filtration columns (Amersham- Pharmacia) equilibrated before use in 10 mM Tris-HCl buffer (pH7.5) containing 0.15 M NaCl.

Apo-ferritin was obtained using methods described by Chasteen and Theil (1982). Purified ferritin was dialyzed against 50 mM HEPES/NaOH

buffer (pH7.0) containing 1 % thioglycolic acid, followed by successive changes of HEPES buffer with (0.1 %) or without thioglycolic acid. Protein was then dialyzed against HEPES buffer containing 13g/litre of Chelex-100 (Bio-Rad) and 0.2M NaCl, and finally dialyzed against deionized water. The concentration of purified proteins was determined using a 'protein assay kit' (Bio-Rad) and densitometer (Amersham-Pharmacia).

1-3-2 Amino acid sequence analysis

Soybean ferritin subunits were separated electrophoretically using a 12.5% SDS-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was stained with 0.1% Ponceau-S (SIGMA) in 2%(v/v) acetic acid and two distinctive bands of 28.0 and 26.5 kDa were independently cut from the membrane. N-terminal amino acid sequence analysis was performed by automatic Edman degradation using an Applied Biosystems' model 477A pulse-liquid sequencer system.

C-terminal amino acid sequence analysis of the 26.5 kDa subunit was performed using *p*-phenylene diisothiocyanate controlled-pore glass (DITC-CPG Sigma) (Nokihara *et al.* 1995). Briefly, the 26.5 kDa subunit was digested with lysyl endopeptidase, and the resulting peptide fragments were covalently bound to DITC-CPG. Only the C-terminal fragment, which contained no lysyl residue, was detached by cleavage of the first residue with trifluoroacetic acid (TFA) (Nokihara *et al.* 1995). The sequence of the C-terminal fragment was determined by automatic Edman degradation, as

described above.

1-3-3 Cloning of a novel ferritin gene cDNA from soybean

Primers for amplification of the subunit containing a novel N-terminal amino acid sequence were designed using the expressed sequence tag (EST) sequences (AW185525, AI966037, AW397605, AI443722, AI900240) of soybean registered in GenBank. 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE was carried out using a 'SMART RACE cDNA Amplification kit' (Clontech) according to the manufacturer's instructions; total RNA extracted from 10 days-old seedlings contained developed bifoliates, an epicotyl and a terminal bud was used as a template. About 10 candidate sequences of a target sequence coding the novel subunit were determined.

1-3-4 Preparation of recombinant soybean ferritin

DNA sequences encoding the mature region of soybean ferritin, previously reported by Lescure *et al.* (Lescure *et al.* 1991), were amplified by PCR using the primers -TP (5'-GCGCATATGTCAACGGTGCCTCTCAC -3') and C (5'-GCGGGATCCTAATCAAGAAGTCTTTG-3'). -TP and C contained *Nde* I and *Bam*H I restriction sites, respectively. The resulting fragments, which were missing the TP sequence, were ligated to the *Nde* I and *Bam*H I sites on the expression vector pET3a (Novagen) to generate pESF. The expression plasmid pESF was transformed into the *E. coli* strain BL21(DE3)pLys. An *E. coli* strain harboring the expression plasmid was cultured in LB medium supplemented with ampicillin (50 μg/ml) at 37 °C.

Bacterial growth was monitored with a spectrophotometer at 600 nm. When an absorbance of 0.6 was reached, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1mM. The cells were harvested by centrifugation, and proteins were extracted using 'BugBuster' protein extraction reagent (Novagen). Recombinant ferritin was purified using the same methods described above for native ferritin.

1-3-5 Soybean ferritin degradation analysis

Soluble protein was extracted from 200 mg of soybean leaves by homogenization with 1 ml of extraction buffer [50 mM K_2PO_4, 10 mM 2-ME, 0.1% TritonX-100, 0.1% Sarcosine] and sea sand followed by centrifugation at 12000 rpm. The supernatant was used for degradation experiments of soybean ferritin. Recombinant ferritin or native soybean ferritin (150 ng each) were added to 20 μl of the leaf extract and incubated at room temperature for 0, 1, 10, 30, 60 and 120 minutes, respectively. After SDS-PAGE and electroblotting to a PVDF membrane, protein-immunodetection was performed. Antiserum was raised against the soybean ferritin as previously described (Goto *et al.* 1998). Probing of blots with soybean ferritin antibody was carried out using anti-rabbit IgG sheep immunoglobulin coupled with biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Labs. USA). Immunostain HRP-1000 (Konica, Japan) was used for visualizing the signal (Goto *et al.* 1998, 1999).

1-3-6 Iron incorporation and iron release measurement

Reactions examining iron uptake by native soybean and recombinant

ferritin were performed in 100mM HEPES/NaOH buffer (pH7.0) with a Fe^{2+}/ferritin molar ratio of 1000:1 (0.1 mM ferrous sulfate and 0.1 μM ferritin) at room temperature. Iron incorporation by each type of ferritin was monitored by measuring the absorbance at 310 nm (Levi *et al.* 1988, Paques *et al.* 1980) using an UV spectrophotometer (U3000, Hitachi, Tokyo, Japan).

For iron-release experiments, native soybean and recombinant apoferritins (2μM each) were mineralized using freshly prepared ferrous sulfate (1mM) in 0.1 M MOPS containing 0.2 M NaCl (van der Mark and van den Briel 1985, Mertz and Theil 1983) for 2 hours at room temperature and then over night at 4 °C. The excess iron which was not incorporated into the ferritin shell was precipitated by the centrifugation and separated from the mineralization mixture. External iron was then removed using 'Econo-Pac 10 DG' (Bio-Rad). Iron release was initiated by addition of 1 mM sodium ascorbate and 4 mM ferrozine (van der Mark and van den Briel 1985); the former. was previously reported to enhance the reductive release of iron from the ferritin shell (van der Mark and van den Briel 1985, Laulhere *et al.* 1995). Exogenous Fe^{2+} was measured by the absorbance of the Fe^{2+}/ferrozine complex at 560 nm using the same spectrophotometer described above. The data of iron uptake and release were obtained from 3 different preparations. The levels of reductive iron release by the two subunits were compared statistically using t-tests ($p = 0.05$).

1-4 Results

1-4-1 Isolation of soybean ferritin from seeds

Soybean ferritin was extracted from dry seeds and isolated using the following sequential chromatographic purification steps: anion exchange, hydrophobicity and gel filtration. Soybean ferritin was eluted in a single peak after each chromatographic step. Purified ferritin subunits were separated as two peptides (28 and 26.5 kDa) by SDS-polyacrylamide gel electrophoresis (Fig.1A). Densito-metric analysis indicated that the 28 and 26.5 kDa subunits are present in purified native ferritin in nearly equivalent amounts (28:26.5 kDa = 1:1.09). Non-denaturing gel electrophoresis (Native PAGE) resolved purified soybean ferritin as a single complex, estimated to be about 550-560 kDa (Fig1B).

1-4-3 Amino acid sequence analyses of ferritin subunits

Comparisons between the N-terminal sequences of the 28 kDa subunit (Ala-Ser-Asn-Ala-Pro-Ala) and the 26.5 kDa subunit (Ala-Ser-Thr-Val-Pro-Leu) revealed that the 28 kDa subunit was different to any previously reported. Further determination for the N-terminal sequence verified that the 28 kDa subunit was a novel subunit (Fig.2). The N-terminal sequence of the 26.5 kDa subunit was identical to that of the EP region of the sequence reported by Lescure *et al.* (Lescure *et al.* 1991). The analyses revealed that the transit peptide is cleaved at the carboxyl side of the 48th cysteine in the 26.5 kDa subunit, which contrasts to the previously reported cleavage site at position 49 (alanine) (Fig.2) (Lescure *et al.* 1991, van der Mark *et al.* 1983).

To detect the site of cleavage for hypothesized conversion of the 28 kDa

subunit to the 26.5 kDa subunit (Lescure *et al.* 1991, Wei and Theil 2000), the C-terminal amino acid sequence of the 26.5 kDa subunit was determined. The sequence of peptide fragment containing the C-terminal residue was Ser-Glu-Tyr-Val-Ala-Gln-Leu- Arg-Arg (Fig.2), which was the same as that previously reported (Lescure *et al.* 1991). The C-terminal residue was determined to be the 234th arginine, which is situated 17 residues upstream from the C-terminus. These data demonstrated that the 26.5 kDa subunit in the soybean seed ferritin is generated as a result of proteolytic cleavage of the C-terminal 16 residues of the previously described subunit.

1-4-4 cDNA isolation and the deduced amino acid sequence of a novel ferritin subunits

A cDNA encoding a newly identified subunit (28 kDa) of soybean ferritin was cloned using a PCR-based strategy. The predicted amino acid sequence was compared with the previously reported sequence of ferritin subunit (Lescure *et al.* 1991) and several differences were found (Fig.2). Based on the conservation of several residues, thought to comprise the ferroxidase site (Lawson *et al.* 1989, 1991), the author defined the 26.5 and the 28 kDa subunits as SFER1 and SFER2, respectively. The mature region of the SFER2 subunit, in which the TP is detached from the precursor, is composed of 209 residues. It is seven residues longer than the SFER1 subunit (202 residues). The amino acid sequence of the mature region of the SFER2 subunit has 82 % identity with that of the SFER1 subunit. In contrast to a high identity in the mature regions, low sequence identity between the TP sequences of SFER1

and SFER2 is found (41 %). In the sequence of the mature region, the deduced helical regions (A to E helices) (Frolow *et al.* 1994) are highly conserved between the SFER1 and SFER2 subunits, while the loop region between the B and C helices, as well as the EP region, are relatively variable. Specifically, the amino acid sequence identities of the helical, loop and EP regions are 90 %, 81 %, and 63 %, respectively (Table1). The SFER2 subunit has C-terminal extension consisting of five residues (four of them charged). A comparison between the C-terminal cleavage site of SFER1 (α-amino side of position 234) with the putatively homologous position in SFER2 (position 235) revealed the presence of an arginine in SFER1 and a leucine in SFER2 (Fig.2). The unsusceptibility of the SFER2 subunit to C-terminal cleavage is likely due to the presence of leucine instead of arginine at this position.

1-4-5 Degradation of the soybean ferritin SFER1 subunit

The full mature region of the soybean ferritin SFER1 subunit was expressed in *E. coli*. Native-PAGE (Fig.1B) and gel filtration (data not shown) analysis showed that the recombinant soybean ferritin subunit assembles into a 24-mer, presumably in a similar fashion to native ferritin. The author abbreviated the recombinant SFER1 subunit as 'rSFER1' and native soybean ferritin purified from seeds as 'seed SFER1/SFER2'. The rSFER1 was composed of only the SFER1 subunit in the original, uncleaved form (28 kDa), while the seed SFER1/SFER2 included both the SFER2 and cleaved SFER1 (26.5 kDa) subunits in nearly equal amounts (Fig.1 (A)). The rSFER1 was incubated with soybean leaf extract (Fig.3). Prior to the incubation with the

soybean leaf extract, the rSFER1 subunit remained in its 28 kDa form, however it was degraded quickly following addition of leaf extract. About half the amount of the rSFER1 subunit was degraded to the cleaved form (26.5 kDa) in ten minutes, and after one hour of incubation, the original 28 kDa form had completely disappeared; only a small amount of the cleaved 26.5 kDa form was detected. Subsequently, the cleaved form degraded completely after two hours of incubation. In contrast, the seed SFER1/ SFER2 was still detectable after two hours incubation, although the 28 kDa form was not present. Since the author used an anti-serum raised against the SFER1 subunit, specific detection of the SFER2 subunit in seed SFER1/ SFER2 was not expected to occur; this may explain the absence of a 28 kDa band in Fig.3.

1-4-6 Effect of cleavage on iron uptake and release

To investigate the effects of C-terminal cleavage in SFER1 on the multimeric complex, the author compared iron uptake and release activities between seed SFER1/ SFER2 and rSFER1. A recombinant form of the cleaved SFER1 subunit (whose C-terminus was deleted) did not assemble into a 24-mer (data not shown). Both rSFER1 and seed SFER1/ SFER2 showed iron uptake activity (Fig.4A). Progression plots indicated that the uptake rate of the seed SFER1/ SFER2 was slower than that of the rSFER1, despite the presence in all the subunits of ferroxidase sites. Both the uptake rates were faster than that of Fe(II) autoxidation (control).

The rate of reductive release of iron from seed SFER1/ SFER2 and rSFER1 was assessed (Fig4B). The level of ferrous atoms released from the

ferritin shell was calculated via absorbance measurement of the Fe(II)-ferrozine complex. At every assessed time, the amounts of the Fe(II)-ferrozine complex were significantly larger with seed SFER1/ SFER2 than with rSFER1. This result suggested that cleavage of C-terminal 16 residues accelerated iron release from the protein shell.

1-5 Discussion

In this study, the author demonstrated that two polypeptide chains with different sequences are found in soybean ferritin purified from seeds. Previously, it was hypothesized that only one type of polypeptide chain, which was in either a cleaved or uncleaved form, was present in the multimer (Lescure *et al.* 1991). The author found that the two different subunits exist in nearly equal amounts in the ferritin of soybean seeds. Two kinds of subunits with different amino acid sequences were also detected previously in clover seed (Barcelo *et al.* 1997), though one of them existed only at very low levels, and its amino acid sequence was not unambiguously identified. From amino acid sequence and cDNA analysis, the author designated the two soybean ferritin subunits as SFER1 and SFER2. SFER1 had a sequence identical to that previously described (Lescure *et al.* 1991), and experiments involving rSFER1 and leaf extracts revealed that this 28 kDa subunit could be readily converted to 26.5 kDa by cleavage of the 16 residues at the C-terminus. In the amino acid sequence profiles, no contaminating peaks were detectable, indicating that the SFER1 subunit was perfectly converted to the cleaved 26.5 kDa form. The SFER2 subunit had a novel amino acid sequence, and unlike

SFER1, appeared unsusceptible to cleavage, remaining in its 28 kDa mature form. These data are in conflict with the previous hypothesis that the 26.5 kDa subunit is generated by cleavage of the N-terminal extension peptide (EP) of the 28 kDa subunit, and that the 28 kDa and 26.5 kDa subunit are identical apart from this cleavage (Lobreaux and Briat 1991).

In degradation experiments using the extract of soybean leaves, the homo 24-mer of rSFER1 was unstable compared with the seed SFER1/ SFER2 (Fig.3). The instability of the SFER1 multimer is likely due to cleavage of the C-terminal 16 residues in each subunit. In support of this hypothesis, a mutant recombinant form of SFER1, which lacked the C-terminal 16 residues, could not assemble into a native-ferritin like complex. Luzzago and Cesareni (1989), and Levi *et al.* (1989) also remarked on the importance of the C-terminal region for shell stability in human H-chain ferritin (Luzzago and Cesareni 1989, Levi *et al.* 1989). Notably, the SFER2 subunit was not susceptible to cleavage of the C-terminal region, and was stable during incubation with the leaf extraction. These data indicate that the SFER2 subunit stabilizes the native ferritin 24-multimer in soybean seeds.

In vertebrate ferritins, the 3-dimensional structures of the single subunit and assembled 24-mer have been analyzed in detail (Trikha *et al.* 1994, Hempstead *et al.* 1997, Lawson *et al.* 1991). Ferritin subunits in the spherical 24-mer are related by 432 symmetry. There are narrow channels around the 3-fold and the 4-fold symmetry axes (Harrison and Arosio 1996). Residues around the 3-fold channels are mainly hydrophilic and the channels are proposed as the main entrance for iron atoms (Stefanini *et al.* 1989,

Yablonski and Theil 1992, Treffry *et al.* 1993). In contrast, four subunits are tightly packed around the 4-fold channels, with hydrophobic interactions occurring among the non-polar residues in the E-helices (Harrison and Arosio 1996, Trikha *et al.* 1994, Hempstead *et al.* 1997). Our results indicate that conversion of the SFER1 subunit from 28 kDa to 26.5 kDa is due to the cleavage of C-terminal 16 amino acid residues. From amino acid sequence alignment data and the deduced three dimensional structure of plant ferritin subunits (Lobreaux *et al.* 1992), the cleaved 16 residues correspond to the "E-helix" of vertebrate ferritin, which is a short helix forming the narrow channels around the 4-fold intersubunit interaction axes. In the case of amphibian red-cell L-chain ferritin, it was reported that the diameter of this channel is about 1.5 Å, while that of the 3-fold channel is about 3.7 Å at its narrowest point (Trikha *et al.* 1994). Thus, the channel around the 4-fold axes does not appear to have enough pore size to allow ions to move freely (Trikha *et al.* 1994). What might the functional significance of cleavage of the E-helices in soybean ferritin be? It is likely that the pore size of the channel around the 4-fold axes would be expanded drastically. Even though half of the subunits (SFER2) in soybean ferritin are not susceptible to proteolysis, the pore generated by cleavage of the SFER1 E-helices is expected to be large enough to allow iron atoms to pass freely. Indeed, the rate of iron release from seed SFER1/ SFER2 was faster than that from the rSFER1 (uncleaved 28 kDa form) (Fig.4B). These data suggest that the rate of iron release is accelerated by cleavage of the E-helix. In contrast to the case of iron entrance in mammalian ferritin, few studies have been performed on iron release

pathways in either plant and animal ferritin (Takagi *et al.* 1998). Therefore, the large pore generated by the cleavage of the E-helix provides a novel hypothesis for iron release from ferritin. Conversely, the iron uptake activity of seed SFER1/ SFER2 was relatively low compared with the rSFER1, despite the fact that both the SFER1 and SFER2 subunits possess the predicted ferroxidase center. The apparent difference in the rate of iron uptake is probably due to the C-terminal cleavage in SFER1, which facilitates reductive release of incorporated iron from native ferritin. C-terminal mutation in the human H-chain ferritin has also been reported to affect iron incorporation and ferroxidase activity (Levi *et al.* 1989).

In plant ferritin, some functional genes have already been identified (Lobreaux *et al.* 1992, Wicks and Entsch 1993, Wardrop *et al.* 1999). Here, we have identified a novel soybean ferritin subunit (SFER2), whose maturation process is different to that of the originally described subunit (SFER1). The novel subunit proved to be one of the major subunits of soybean seed ferritin. The primary structure of the novel ferritin subunit was closely related to that of the known (Lescure *et al.* 1991) subunit (82% identity). However, the difference in the maturation process of SFER2 appears to promote the novel function of "iron release". The 28 kDa (presumably SFER2-like) and partially cleaved 26.5 kDa (presumably SFER1-like) subunits are also found in pea and other legume ferritins (Andersen *et al.* 1995, van der Mark *et al.* 1983, van Barcelo *et al.* 1997). Notably, a leucine is found at a similar position in one of cowpea ferritin subunits to that of the leucine at position 235 in SFER2 (Fig.2) (Wardrop *et al.* 1999). Thus, like the case for soybean, it is possible that the

different sized subunits observed in many legume ferritins derive from different genes, and play co-operative roles in the storage and release of iron atoms.

In agreement with the results of Lobréaux and Briat (Lobréaux and Briat 1991), concentration of ferritin subunits in soybean cotyledon decreased gradually during germination and finally disappeared after about 10 days (data not shown). To date, many studies have examined expression of plant ferritin in legume plants and maize. While ferritin accumulates in developing nodules, cotyledons and embryo axes (Lobréaux and Briat 1991, Ragland and Theil 1993, Kimata and Theil, 1994) of soybean and pea, it has not been detected in green leaves in bean (Lobréaux and Briat 1991). In conflict with the latter result, van der Mark and colleagues reported that ferritin was detectable in normal green leaves of bean (van der Mark *et al.* 1981, van der Mark *et al.* 1982). Interestingly, these authors provided preliminary evidence for the existence of multiple subunits of ferritin in bean (van der Mark *et al.* 1982). A consensus on patterns of ferritin expression in plants has not yet been reached, and the author suggest that a contributing factor to this has been the exclusive study of the SFER1 gene and its product during analysis. The author have analyzed the expression and tissue distribution of the newly identified SFER2 subunit, as well as the SFER1 subunit, in order to elucidate their respective functions and to obtain information on the significance of the multi gene family of soybean ferritin (Chapter II).

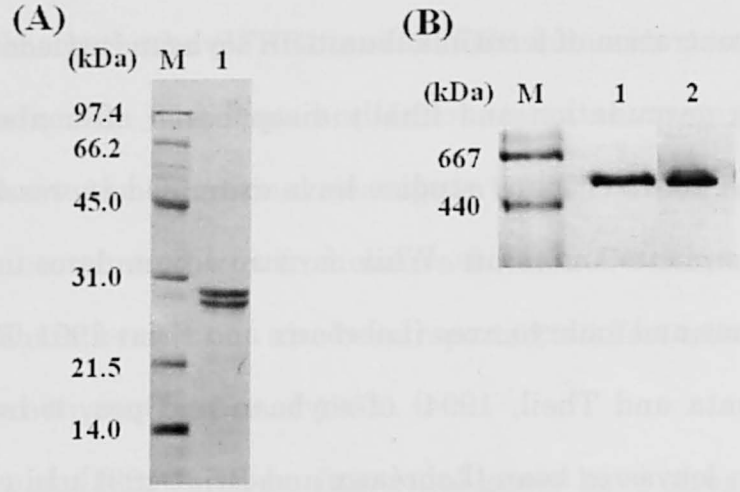

Fig.1 SDS-PAGE and native PAGE analysis of purified soybean ferritin.

Samples were added after the final gel filtration step (see methods).

(A) Purified soybean ferritin was analyzed by SDS-PAGE and stained with CBB (lane 1).

(B) Native PAGE analysis of soybean ferritin. Lane 1: native soybean ferritin purified from seed; lane 2: recombinant soybean ferritin expressed in *E. coli*. M: protein markers and their corresponding molecular masses.

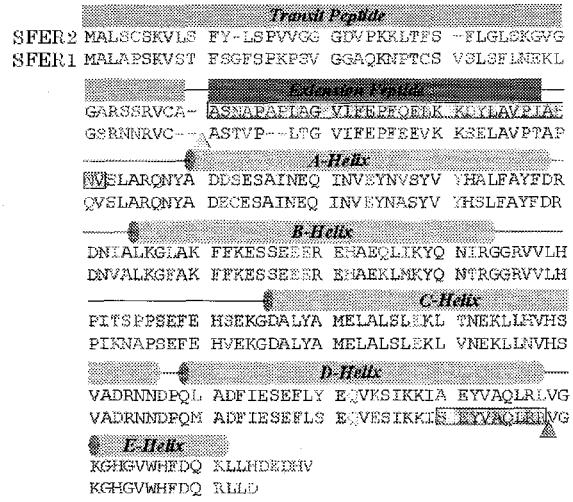

Fig.2 Deduced amino acid sequences of soybean ferritin subunits.

Upper: amino acid sequence of the novel, SFER2 subunit identified during this study. Lower: amino acid sequence of the SFER1 subunit, which is identical to the sequence previously reported by Ragland *et al.* (1991). Conserved residues between the two subunits are shown in black. Residues in green indicate those which have been suggested to form the deduced ferroxidase center. The N-terminal 32 residues of the SFER2 subunit determined during amino acid sequence analysis are shown boxed in green. The peptide fragment containing the C-terminal residue of the cleaved 26.5 kDa form of the SFER1 subunit is shown boxed in red. The cleavage sites of the transit peptides (TP) in both the SFER2 and SFER1 subunits are indicated by a yellow arrowhead. The mature regions of both subunits are downstream from here. The cleavage site for conversion of the SFER1 subunit from 28 kDa to 26.5 kDa is indicated by a green arrowhead.

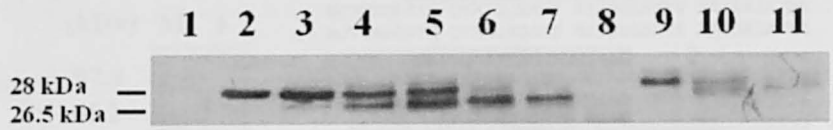

Fig.3 Degradation of recombinant and native soybean ferritin.

The rSFER1 and seed SFER1/SFER2 were incubated separately in soybean leaf extract. Ferritin subunits were detected by anti SFER1 subunit anti-serum. Lane 1: soybean leaf extract, lane 2: 150 ng of the rSFER1 subunit, lane 3 to 8: 150 ng of rSFER1 subunit after incubation with leaf extract for 0, 1, 10, 30, 60 and 120 minutes, respectively, lane 9: 150 ng of seed SFER1/SFER2, lanes 10 and 11: 150 ng of seed SFER1/SFER2 with leaf extract for 60 and 120 minutes, respectively.

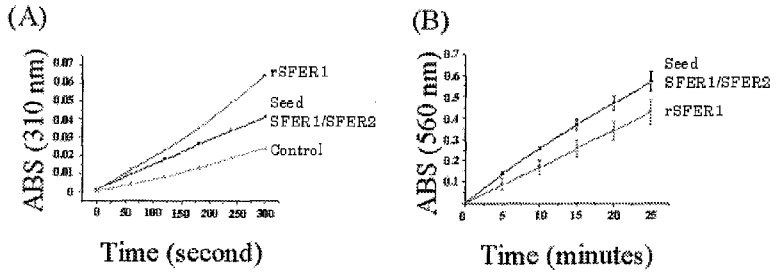

Fig.4 (A) Progression plots of iron uptake in soybean ferritin.

Experiments were performed in 0.1 M HEPES-Na, pH 7.0, containing 0.1 μM of each ferritin type (rSFER1 and seed SFER1/SFER2) and 0.1 mM ferrous sulfate. The control indicates the rate of Fe(II) auto-oxidation.

(B) Rates of iron release from assembled ferritin shells.

Seed SFER1/SFER2 and rSFER1 (2 μM) were mineralized *in vitro* by mixing with 1mM ferrous surfate in 0.1 M MOPS (pH 7.0) and 0.2 M NaCl. Iron release from mineralized ferritins was initiated by the addition of 1 mM ferrozine and 4 mM sodium ascorbate. Released iron was detected in the Fe^{2+}/ferrozine complex form by monitoring the absorbance at 560 nm. The results were obtained from three experiments.

Region	Identitiy (%)
TP	41
EP	63
Helix A	90
Helix B	86
Helix C	94
Helix D	88
Helix E	91
BC loop	81
Total	82

Table 1 The partial amino acid sequence identity between the SFER1 and SFER2 subunit.

The amino acid sequences of both subunits were compared with each other in eight parts.

TP: transit peptide. EP: extension peptide

Chapter II

Differential expression in tissues and response to metal treatments of four soybean ferritin genes

2-1 Abstract

It has been recently found that iron storage protein ferritin in plants is encoded to a gene family. Only a few attempts have been made so far to understand the expression patterns in the ferritin gene family. To investigate the role of each ferritin gene and its expression in plants, the author first analyzed copy numbers of 4 ferritin genes containing 3 novel genes (*sfer2*, *sfer3* and *sfer4*) and characterized the sequences of the genes. All of them were classified to H/L hybrid type of ferritin from predicted amino acid sequences. Next, the author examined expression of ferritin genes during the developmental stage of seed, its tissue specificity and its response to metal treatments through Northern blot analysis. Three expression patterns of ferritin genes were observed during seed development: continuous (*sfer1*, *sfer4*), declining (*sfer2*), and no expression (*sfer3*). The tissue-specific expression of each gene was shown in radicles (*sfer1*, *sfer4*), terminal buds (*sfer1*, *sfer2*), and bifoliate leaves (*sfer3*). Furthermore, each gene expressed mRNA in leaves differently in response to metal treatments; genes responded to most kinds of metals (*sfer1*, *sfer2*), to metals other than iron (*sfer3*), or to no metals (*sfer4*). These results suggest that the expression of each ferritin gene was variedly regulated during growth, in specific tissue, and against exogenous stimulus in order to control iron metabolism although each ferritin

subunit encoded in the 4 genes had a very similar structure.

2-2 Introduction

Ferritin is widely distributed among plants, animals and bacteria. Ferritin is a huge protein composed of 24 subunits, and functions as an iron reservoir. Plant ferritin locates in chloroplast and other plastids. Plant ferritin is not or only slightly detected in the leaves grown normally (van der Mark *et al.* 1982, Lobreaux and Briat 1991) though ferritin is accumulated in the leaves when iron is supplied to iron deficient plants (van der Mark *et al.* 1982, Seckbach 1969). The induction of ferritin is observed in soybeans (Lescure *et al.* 1991), maize (Lobreaux *et al.* 1992) and *Arabidopsis* (Gaymard *et al.* 1996). Therefore, ferritin synthesis in response to iron is thought to be controlled at a transcriptional level in plants, while mainly at a translational level in animals. There is no observation of ferritin gene expression in plants against abiotic stress such as non-ferrous metals. However, ferritin can bind to some metals other than iron. Regarding animal ferritin, the ability of ferritin to bind to Be, Cd, Zn and Cu in rats is reported (Price and Joshi 1982, 1983). Zaman and Verwilghen (Zaman and Verwilghen 1981) showed that ferritin in liver can bind to at least 50 mol of Zn^{2+} per mol. In plants, it is reported that ferritin is able to bind to Al, Be, Ca, Zn, Cd and Cu *in vitro* (Sczekan and Joshi 1989, Rama Kumar and Prasad 1999).

It is still controversial when and where the ferritin gene is expressed. Van der Mark and his colleagues indicated that there is ferritin in normal

green leaves of *Phaseolus vulgaris* (van der Mark *et al.* 1982, van der Mark and van der Briel 1985). On the contrary, Lobreaux and Briat (1991) reported that ferritin is not detected in total protein extracts from roots or leaves of peas, but detected from flowers, pods and seeds. Furthermore, ferritin disappears during germination, within the first 4 days of radicle and epicotyl growth. With respect to the expression of ferritin mRNA, Ragland *et al.* (Ragland *et al.* 1990) showed that the concentration of mRNA extracted from soybean plants 16 days after planting has increased during leaf maturation. Lobreaux *et al.* (Lobreaux and Briat 1991, Lobreaux *et al.* 1993) showed 2 opposite patterns of ferritin gene expression in roots and leaves. They attempted to explain their findings by circadian regulation that affects ferritin gene expression. Buchanan-Wollaston and Ainsworth (1997) showed that ferritin mRNA accumulates in young green and old leaves during a senescence in *Brassica napus*. Wardrop *et al.* (1999) demonstrated that 4 ferritin genes of cowpeas express mRNA in leaves and roots under normal growth condition using RT-PCR methods. These reports showed that an integrated understanding of ferritin accumulation in each organ and during development had not been achieved yet.

Animal ferritin consists of 2 types of subunits called H and L. H subunit has a ferroxidase site and contributes to iron uptake into protein shell. L subunit functions to nucleate iron (Harrison and Arosio 1996). On the other hand, plant ferritin has been thought to be composed of only one type of subunits, i.e. L/H hybrid. More than 10 genes of plant ferritin have been cloned and registered in a database for 10 years (Goto *et al.* 2001). Only

one ferritin gene and its product in almost all the plants have been investigated except for cowpeas, maize and soybeans. The author has shown that soybean ferritin in seeds consisted of 2 kinds of subunits which were coded by two different genes (Chapter I). One gene was the same as that reported previously but the other was a novel one. The differential expression of 2 ferritin genes in maize shoots was observed in response to iron, exogenous abscisic acid and water stress (Lobreaux *et al.* 1993, Lobreaux *et al.* 1995, Fobis-Loisy *et al.* 1996, Savino *et al.* 1997). Wardrop *et al.* (1999) claimed the existence of a family of 4 ferritin genes in cowpeas. These ferritin genes differentially express mRNA in leaves and roots under normal growth. However, it is not clear whether each gene expression varies depending on the type of exogenous stimulus and developmental stage. Furthermore, the functions of individual genes and interactions within the ferritin gene family are mostly unclear.

In this chapter, the author compared the profile of ferritin genes reported previously with that of 2 novel ferritin genes cloned from soybeans and tested the hypothesis that expression pattern of each ferritin gene varied depending on development stage of seeds, type of tissues, and metal treatments.

2-3 Materials and Methods

2-3-1 cDNA cloning

Total RNA was extracted from 10-day-old seedlings of soybeans (*Glycine max Merr* c.v. Kita-no-shiki) containing an epicotyl with a part of the

bifoliate leaves and terminal buds. The seedling was grown with water. The sequences of the expressed sequence tag (EST) of soybean, AI966037 and AW397605, registered in the GenBank were used to design primers which amplified a part of *sfer3* and *sfer4*, respectively. The primer sets were (5'-CTGTCGTTGGAGAAGCTA-3') and (5'-CAACGCCCTCCTCATGCA-3') for *sfer3*, and (5'-GCAGATAAGGGAGATGCG-3'), (5'-CTTCCACCTGTTCACCCA-3') for *sfer4*. 5'RACE and 3'RACE were carried out using a ' SMART RACE cDNA Amplification kit' (Clonetech). Regenerated DNA fragments were cloned into plasmid Top10 F' using a Topo TA cloning kit (invitrogen). Parts of sequences obtained were used to design a second primer to obtain a perfect sequence. Amino acid sequences deduced from cDNA sequences were analyzed using the GENETYX-WIN software (Software Development; Japan). Evolutionary tree was constructed from the matrix of amino acid sequence deduced from DNA sequence similarities calculated with the UPGMA (the unweighted pair group method with an arithmetic mean) program.

2-3-2 Southern blot analysis

Total DNA was extracted from leaves using the methods of Shure *et al.* (1983). 10 μg of DNA was digested by *Eco*R I, *Hin*d III and *Bam*H I, subjected to agarose gel electrophoresis, blotted to a nylon membrane and hybridized with ferritin cDNAs labelled with digoxigenin (DIG system, Rosch diagnostics, Germany) using PCR. The sequence coding transit peptide (TP) region was used as probes to identify 4 ferritin genes. The

primer sets to synthesis probes were

5'-ATGGCTCTTGCTCCATCCAAAG-3' and

5'-AGAGGCACCGTTGAGGCACAAA-3' for *sfer1*, and

5'-CAAATGGCCCTTTCTTGCTCCAA-3' and

5'-AATATGACCCCAGCGAGTGGTG-3' for *sfer2*, and

5'-CATGCTTCTCAGAACAGCTTCA-3' and

5'-TTCAAAAACGACGCCGGTTAAGG-3' for *sfer3*, and

5'-ATGCTTCTCCGAACCGCTGCT-3' and

5'-TGGTTCGTGGATCCTTTCGCT-3' for *sfer4*.

Before using probes, the specificity of each probe was examined through dot blot analysis.

2-3-3 Metal treatment

Soybeans were grown in perlite with water at 25 °C under the 12 hour light of approximately 406 $\mu molm^{-2}s^{-1}$ for 3 weeks, after the seeds were sown in water for 24 hours. The plants were transferred to glass vials containing 100 ml water. Twenty-four hours later, the plants whose roots were cut off were dipped into 100 ml of metal solutions. Metal compounds were NaCl, $MgSO_4$, $AlCl_3$, $MnSO_4$, Fe-EDTA, $CoCl_2$, $NiCl_2$, $CuSO_4$, $ZnSO_4$ and $CdCl_2$. The concentration of each metal other than Fe-EDTA (0.5 mM) and NaCl (50 mM) was 1 mM. The pH levels of metal solution except for $AlCl_3$ (pH=4.0) was adjusted between 5.8-6.0 with HCl. The first trifoliate leaf was harvested 24 hours after the metal treatments, and total RNA was extracted from it.

2-3-4 Developing seeds

Pods were harvested from soybean plants grown in a greenhouse 2 weeks after the first flower was opened. The diameter of young seeds were measured and classified according to the size: more than 2.5 mm to less than or equal to 3 mm (mean weight of 41 seeds was 2.8 mg), more than 3mm to less than or equal to 4 mm (41 seeds; 5.8 mg), more than 4mm to less than or equal to 5 mm (16 seeds; 13.9 mg), more than 5mm to less than or equal to 6 mm (19 seeds; 29.3 mg), more than 6mm to less than or equal to 7 mm (5 seeds; 40.8 mg), more than 7mm to less than or equal to 8 mm (5 seeds; 58.7 mg) and more than 8mm to less than or equal to 9 mm (1 seed; 81.6 mg). Each of them was reserved in a freezer until RNA and protein extraction.

2-3-5 Tissue specific expression

Radicles and cotyledons of seeds soaked in water in dark for 2 days, and epicotyls, terminal buds, roots and bifoliate leaves of 10-day-old seedlings grown in perlite under a 12-h light-dark cycle were used for total RNA extraction.

2-3-6 RNA gel blot analysis

For RNA gel blot analysis, total RNA was isolated from samples using Trizol (Life Technologies). 15 μg of total RNA was fractionated by electrophoresis through a 1.2% agarose gel containing formaldehyde, blotted on a positive charged nylon membrane and hybridized with the same probes

as those used in Southern hybridization. Signals were quantified using an imageanalyzer, and normalized to the background (Bio-Rad; max2).

2-3-7 Protein extraction and Western blot analysis

Total protein was extracted from 0.1 g of immature seeds mentioned above, dry seeds, seeds soaked in water for 24 hours, and for 72 hours by homogenizing with extraction buffer (80 mM Tris-HCl (pH 7), 2% SDS, 2% 2-ME and 20% glycerol) and sea sand. The protein concentration of the extract was determined by the Bradford's method (Protein assay kit; Bio-Rad) using bovine-globulin as a standard. The protein was separated by SDS-PAGE, electroblotted to a polyvinylidene difluoride (PVDF) membrane and detected with antiserum raised against SFER1 subunit expressed in *E.coli.* (Goto *et al.* 1998).

2-4 Results

2-4-1 Soybean ferritin gene family

Two novel ferritin genes were cloned from soybean seedlings. The genes *sfer3* and *sfer4* had 768 and 747 bp of open reading frame (ORF), respectively. *sfer4* corresponded to about 200 bp fragment that was cloned as homologue to the cowpea ferritin gene using the PCR cloning method (Wardrop *et al.* 1999). *sfer3* had no identical sequences to those registered in databases. The amino acid sequences deduced from novel ferritin genes were compared with the sequences (*sfer1*) reported previously (Lescure *et al.* 1991) (Fig. 1). All of the sequences were divided into 2 obvious regions: a low

homology part located at the up-stream region of the sequences and a high homology part at the down-stream. The low homology part corresponded to TP reported by Lescure *et al.* (1991). The high homology part was a mature region of ferritin. All of them had residues to form the deduced ferroxidase center and the acidic residues, which is located in B, D and E helix, facing the inside cavity of the protein shell were also conserved. Figure 2 showed that these genes were conserved among legume plants. The genetically distance between SFER1 and SFER2 and between SFER3 and SFER4 were relatively short, respectively.

Southern blot analysis was carried out to determine the copy number of each gene in the soybean genome (Fig. 3). The cDNA sequences of the TP region having a low homology among the investigated genes were used for templates of probes. The existence of one copy of *sfer1* and *sfer3* was suggested because a single band was detected in *Bam*H I digestion. In *sfer2* and *sfer4*, one or two copies might exist because two bands were detected in all the lanes.

2-4-2 Ferritin gene expression depending on developmental stage of seed

The relation between seed development and ferritin gene expression was investigated (Fig. 4). Since total RNA was extracted from developing seeds 2 weeks after the first flower bloomed, the diameter of seed would reflect the stage of seed development. The expression pattern of *sfer1* seemed similar to that of *sfer4*. Both of them were almost constantly expressed in various size of seeds except the weak expression of *sfer1* in seeds of 3 mm or less. *sfer2* strongly expressed in seeds of 5 mm or less though clear reduction of

expression was observed in seeds of 6 mm or more. Very little expression of *sfer3* was observed. The pattern of mRNA synthesis of *sfer1* was significantly different from that of *sfer2*. SFER1 accumulation during seed development was determined using Western blot analysis with antiserum raised against SFER1. While 26.5 kDa peptide of SFER1 was often detected in total protein harvested from early stage development seeds to matured dry seeds, the 28-kDa peptide of SFER1 was seldom detected (Fig. 5).

2-4-3 Tissue specific expression of ferritin

Figure 6 showed tissue specific expression of 4 ferritin genes. The gene expression pattern of *sfer1* was almost same as that of *sfer4*, as seen in the case of seed development. However, while *sfer1* was more strongly expressed in tissues which had high cell activity, such as a radicle and terminal bud, *sfer4* was expressed in root tissues including radicle. *sfer3* was expressed in bifoliate only and trifoliate leaves (control in Fig. 7). Furthermore, *sfer3* was expressed rather constantly in a bifoliate leaf and the 2, 4, 6 and 7th (half-expanded leaf) trifoliate leaves of 1-month-old plant (data not shown). *sfer2* was expressed in a terminal bud and an epicotyl. The accumulation of *sfer2* mRNA in the terminal bud was much more than that in the epicotyl. Any ferritin genes were not expressed in a cotyledon.

2-4-4 Response to metal treatments

mRNA of *sfer1* accumulated in response to many different kinds of metals, especially in Fe, Ni, Cu and Cd, 24 hours after treatment (Fig. 7).

sfer2 was expressed in response to metals similar to those of *sfer1* though there was no response to Ni or Cd. *sfer3* was expressed in the metals other than iron. Interestingly, the gene expression pattern of *sfer3* was almost opposite to that of *sfer1*; e.g., *sfer3* responded to the control (water), Mg and Mn, and *sfer1* to Fe, Ni, Cu and Cd. *sfer4* was not expressed through metal treatment.

2-5 Discussion

The author found that 3 different ferritin genes were expressed during seed maturation. *sfer1* was expressed (Fig. 4), and its product was accumulated in both immature and mature seeds (Fig. 5). If the expression of *sfer1* in seeds was controlled at a transcriptional level as in leaves stimulated by excess iron, the results shown in Fig. 4 would not contradict those shown in Fig. 5. *sfer1* was strongly expressed during seed maturation while the expression of *sfer2* was clearly reduced when seeds grew more than 6 mm in diameter. One of the reasons why *sfer1* was expressed stronger and longer might be that parts of the product of *sfer1* accumulated as phytosiderin. Ferritin is composed of SFER1 and SFER2 evenly in soybean seeds (Chapter I). However, the pattern and amount of mRNA accumulation in *sfer1* and *sfer2* investigated in the present study were notably different from that of SFER1 and SFER2 subunits shown in lane6 of Fig5. Phytosiderin are considered to be derived from ferritins by pertial degradation of its shell. Laulhere *et al.* show that ferritin built up from 28 kDa subunits converts to phytosiderin which consisted of 26.5 kDa

subunits in pea seeds (Laulhère *et al.* 1989). However, fig. 5 showed that most of *sfer1* products were 26.5 kDa in developing seeds. This result indicated that SFER1 subunit was cleaved at an early seed developmental stage, suggesting phytosiderin was probably formed from SFER1 subunits without a formation of ferritin. Since each subunit has ferroxidase activity and phytosiderin can bind to as much iron as the ferritin (Laulhère *et al.* 1989, Sczekan and Joshi 1987), phytosiderin may be useful to protect cell damage derived from rapid increases of iron in seeds. *sfer4* might function in the same manner as *sfer1* in seeds because the expression pattern of *sfer4* was similar to that of *sfer1*. Thus, in mature soybean seeds, at least 3 ferritin genes might be related to the formation of ferritin and phytosiderin.

Each ferritin gene was expressed not only in developing seeds but also in other tissues specifically. *sfer1* was strongly expressed in radicles, terminal buds and young roots (Fig. 6). These tissues have high metabolic activities and iron seems to flow into the tissues (Dickey *et al.* 1987). Therefore, the expression of *sfer1* in these tissues was presumably controlled by the mechanism similar to that in developing seeds. *sfer2* was strongly expressed in the terminal bud as *sfer1* though *sfer2* mRNA slightly accumulated in the roots and the radicles. On the contrary, expression of *sfer4* was relative strong in the roots and the radicles. Interestingly copea2 ferritin gene which had the most similar sequence to *sfer4* (Fig. 2) is strongly expressed in roots (Wardrop *et al.* 1999). *sfer3* was expressed in normal leaves (Fig. 6, control in Fig. 7). Present showed the constitutive expression of a plant ferritin gene in normal leaves for the first time. In animals, Dickey

et al. (1987) show that existence of a specialized–cell ferritin (stored iron is used for the other cell types) and a housekeeping ferritin (stored iron is used for intracellular purpose) in red cells. The expression of these ferritins was differentially regulated. Hence, the function of SFER3 might be a micro-level regulation of iron in cells, as a housekeeping ferritin be in animal cells. If so, it would not be need to build up 24mer of ferritin because each subunit having ferroxydase activity might work alone without formation of ferritin. The regulation of this *sfer3* gene should be analyzed in a further work. Taken together, ferritin genes were expressed tissue-specifically and at least one ferritin gene was expressed in each tissue except cotyledons which disappeared overtime with the development of plants. Ferritin was expressed adequately as a primary iron reservoir (*sfer1*, *2* and *4*) to protect cells from iron damage during normal growth. Furthermore, ferritin such as *sfer3* presumably related to micro iron regulation in cells.

Each ferritin gene charactically expressed mRNA against metal stimulation (Fig. 7) though the author could not clearly distinguish between the effects of metals and secondary stimulus such as oxidative stress on ferritin gene expression. The expression of *sfer1* varied according to the type of metal. On the contrary, Lobreaux *et al.* (1995) show that iron specific induction of ferritin occurs in maize. This discrepancy could be due to a difference in plant species or to a difference in period of metal treatments (24 hours and 6 hours of treatment in soybeans and maize, respectively). A tendency of accumulation of *sfer2* mRNA was similar to that of *sfer1* in the metal treatment except in Ni and Cd. In general, ferritin mRNA synthesis is

regulated at a transcriptional level. However, the regulation system against metals other than iron different from that in *sfer1* most likely existed in *sfer2*. *sfer3* had no response to iron overload though mRNA synthesis was detected in the other metals such as Na, Mg and Al. The expression level of *sfer3* was low against metals which were thought to be hazardous including Fe, Ni, Cu and Cd, resulting in showing the expression pattern well contrastive to that of *sfer1* expression pattern. *sfer3* would not be induced by injury generated by metals; namely, *sfer3* would be expressed in normal condition constitutively in leaves. However, it must be reduced when leaves were injured through metal treatment. It is reported that mRNA accumulation of maize ferritin (*FM2*) is detected neither in leaves treated with excess iron nor in normal leaves (Savino *et al.* 1997), in contrast with the expression of *sfer3* described here. *sfer4* mRNA was not detected in leaves stimulated by metals. The expression of *sfer4* was probably reduced in leaves (Fig. 6). Therefore, it can be concluded that the expression of each ferritin gene was independently regulated against metal treatments.

In spite of the variation of ferritin genes expression, a mature sequence of amino acid deduced from cDNA was highly conserved among a gene family. The results of Southern blot analysis indicated that from 5 to 6 ferritin genes existed in soybeans. At least 5 genes existed because a PCR amplified fragment of ferritin gene reported by Wardrop *et al.* (1999) had a different sequence from the 4 ferritin genes. The sequences of the ferritin gene family had the highly conserved and non-conserved regions (Fig. 1). The non-conserved region was corresponded to TP (transit peptide) of *sfer1* and

most likely functioned as TP because plant ferritin genes cloned from other species previously had TP sequence, containing putative one (Lescure *et al.* 1991, Lobreaux *et al.* 1992, Buchanan-Wollaston and Ainsworth 1997, Wardrop *et al.* 1999). The author has not yet clarified whether the non-conserved regions of the novel ferritin genes had a function as TP and the presumed TP transferred ferritin precursor to plastids only. The ferritin subunits encoded in all the 4 ferritin genes reported here would have the function of catalyzing oxidation of Fe (II), because they had a ferroxidase center. And like L type ferritin in animal, acidic residues that contribute to mineral core formation are conserved (Harrison and Arosio 1996). Therefore, all the genes were classified into H/L type. However, when ferritin was composed of more than 2 kinds of subunits, these subunits might have different functions, as *sfer1* and *sfer2* did. We had previously shown that ferritins derived from the two genes played co-operative roles in the storage and release of iron atoms (Chapter I).

In conclusion, the expression of the soybean ferritin gene family showed diversification. Varied expressions of highly conserved ferritin genes responded to iron and other stimulus in cells and tissues at individual levels. In other words, since one type of ferritin genes and its regulational elements could not react to iron stimulation of either internal (e.g., physiological demand in matured leaves and in tissues having high activity) or external (e.g., iron overload) cells at the same time, many ferritin genes with independent regulation were needed. To understand the function of ferritin encoded in novel genes, the gene regulation elements and the relationship

among each gene might contribute to understanding iron metabolism in plants.

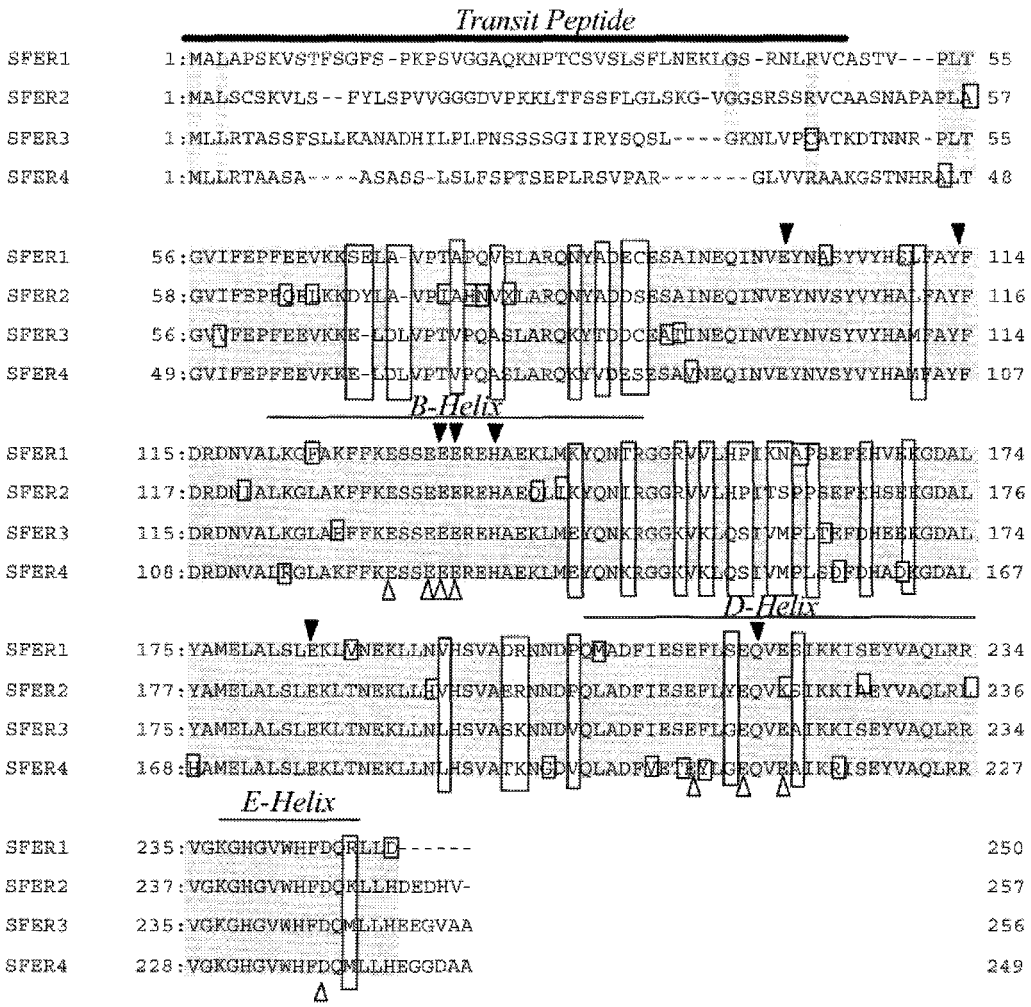

Fig. 1. **Comparison of amino acid sequences of ferritins in soybeans.**

Identical residues are shadowed. Black arrowheads and white arrowheads indicate residues which were thought to comprise the ferroxidase site and conserved residues to process mineral core formation, respectively. The bold line indicates a transit peptide of SFER1 ferritin precursor (Lescure *et al.* 1991).

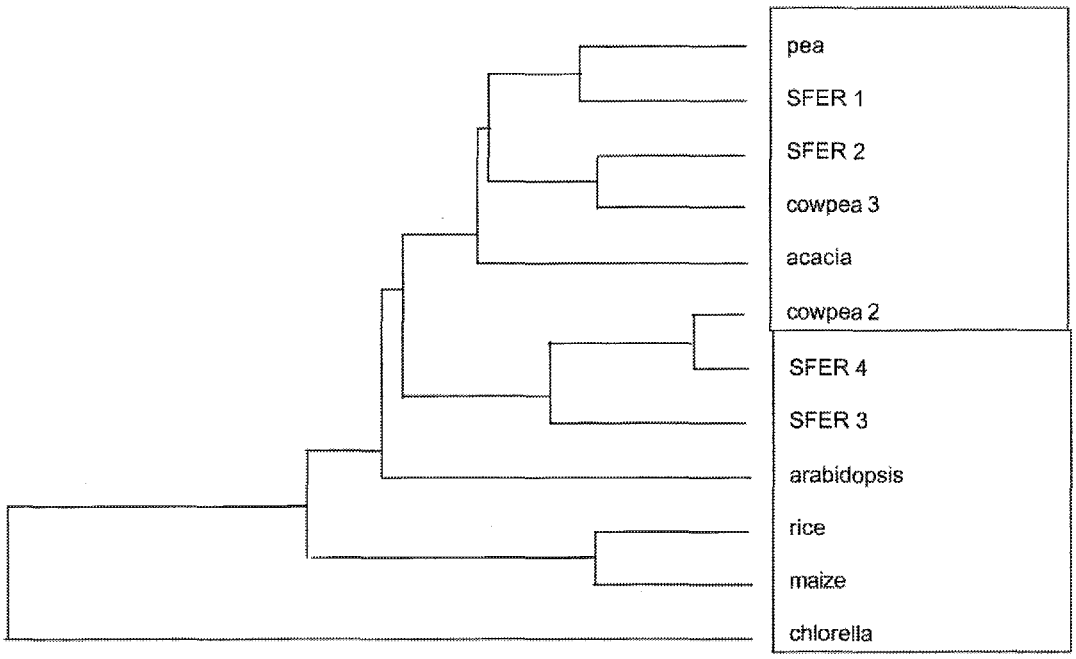

Fig. 2. **Evolutionary tree of plant ferritins.**

The phylogenetic tree was based on the aligned deduced amino acid sequences of plant ferritin. The calculation was performed using the UPGMA method. The accession numbers for the proteins are given below in parenthesis: pea (X73369), cowpea 3 (AF052057), Acacia (E13168), cowpea 2 (AF052058), arabitopsis (AF339691), rice (AF370029), maize (X61391) and chlorella (AJ238628)

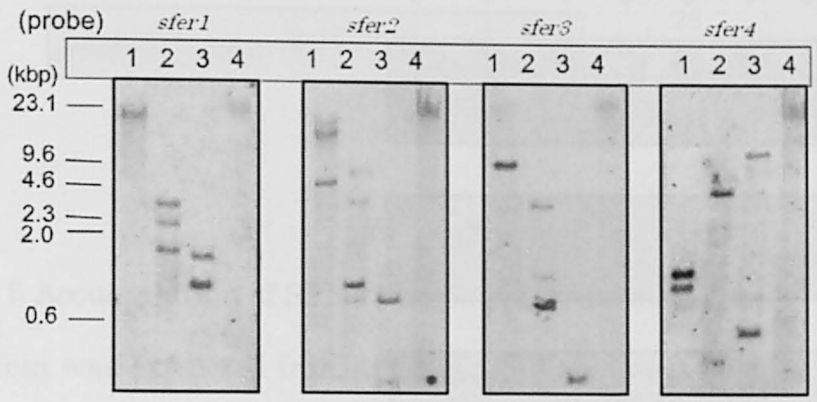

Fig. 3. **Southern blot analysis of ferritin genes in soybean plants.**

Total DNA (10 μg) was digested with *Bam*H I (Lane 1), *Eco*R I (Lane 2) and *Hin*d III (Lane 3). Uncut DNA was present in Lane 4. Low conserved regions of 5' up-stream of each gene were used to synthesize probes.

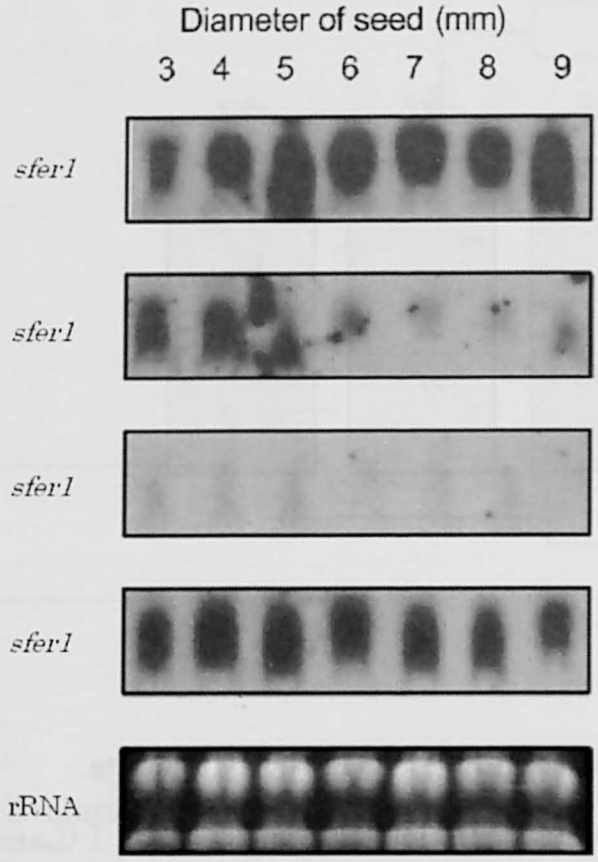

Fig. 4. **Ferritin gene expression during bean development.**

RNA was extracted from various-sized immature soybean seeds and 15 μg RNA of each sample was analyzed through Northern blot hybridization. The ethidium bromide-stained rRNA bands were shown as a loading control. Diameter of seeds are described in Materials and Method.

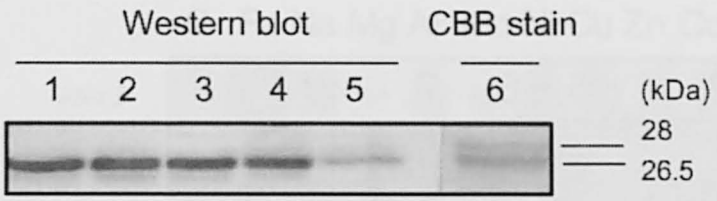

Fig. 5 **Accumulation of SFER1 during seed development.**

Protein was extracted from 100 mg of immature seed (lane 1, 2) (more than 4 mm to less than or equal to 5 mm, and more than 7 mm to less than or equal to 8 mm, respectively), dry seed (lane 3), seed soaked in water for 24 hours, and for 72 hours. 10 μl of extract was loaded on 12.5 % polyacrylamide gel and detected by anti SFER1 anti-serum. Lane 6; 1 μg of purified ferritin from dry seed was loaded of the 12.5 % gel and stained by CBB.

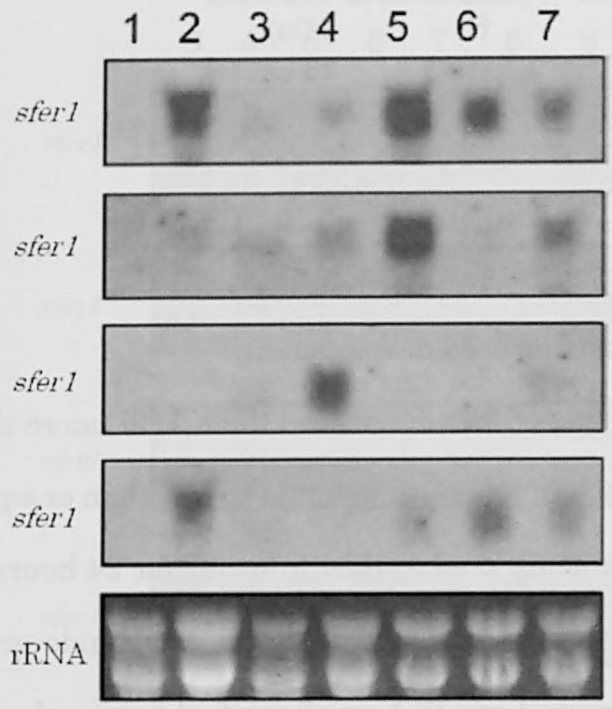

Fig. 6 **Tissue specific expression of ferritin gene.**

RNA (15 μg) was prepared from cotyledons (Lane 1) and radicle (Lane 2) of 2-day-old seedling, and cotyledons (Lane 3), bifoliate leaves (Lane 4), terminal buds (Lane 5), roots (Llane 6) and epicotyls (Lane 7) of 10-day-old seedling. Total RNA of 15 μg was lorded The ethidium bromide-stained rRNA band was shown as a loading control. RNA blot was scanned using a digital densitometer, and relative signals were plotted for each lane.

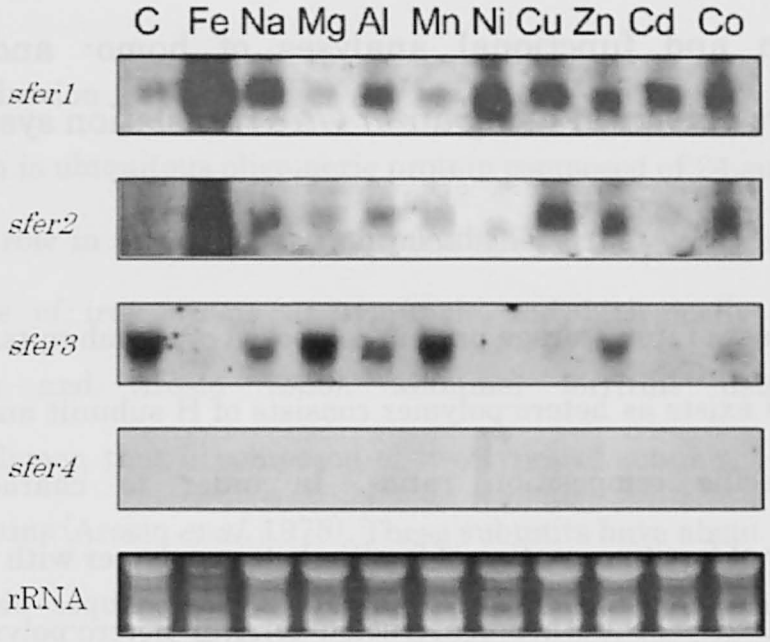

Fig. 7 **Induction of ferritin mRNA synthesis using metals.**

Plantlets were cut the potion under cotyledon and dipped into the various metal treatments and water (control) for 24 hours. After the metal treatments, total RNA (15 μg) was extracted from trifoliate leaves and analyzed through Northern blot hybridization.

Chapter III

Construction and functional analyses of homo- and hetero-polymers of ferritin using an *in vitro* translation system

3-1 Abstract

Ferritin is a class of iron storage protein composed of 24 subunits. In general, human ferritin exists as hetero-polymer consists of H subunit and L subunit in tissue specific composition ratios. In order to characterize the hetero-polymer of ferritin, creation of ferritin hetero-polymer with various H/L ratios is required. The author constructed ferritin hetero-polymers which mimic the natural heterogeneity of the protein using a novel *in vitro* translation technology. This system enabled to express two or more cDNAs simultaneously in one batch. The SDS-PAGE analysis revealed that the expression of cDNAs of the H and L chains could be quantitatively regulated by the amounts of added mRNAs of both proteins. The H/L composition ratios of resulting hetero-polymers were in proportion to the amounts of added mRNA, therefore, all the theoretically possible H/L hetero-polymers could be created by the method. The author demonstrated that four cDNAs of ferritin from soybean, *sfer1*, *sfer2*, *sfer3* and *sfer4* could be expressed and assembled to homo- or hetero-polymer in this system. Prussian blue stain of resulting soybean ferrtin oligomer revealed that homo-polymer of SFER1 and SFER2, and hetero-polymer of SFER1/SFER2 and SFER1/SFER3 could be produced as active oligomeric ferritin using the system. These results suggest that it was possible to characterize the role of individual component of multi subunit

protein by the *in vitro* translation strategy described here.

3-2 Introduction

Ferritin is ubiquitous oligomeric protein composed of 24 subunits. It plays a crucial role in intracellular iron metabolism, that is, it can storage some thousands of iron atoms as non-toxic and biologically available form (Harrison and Arosio 1996). Mammal ferritins usually exist as hetero-polymer, that is composed of two types of subunit, H (heavy) and L (light) chains (Arosio *et al.* 1978). These subunits have about 50 % identity in amino acid sequences and similar three-dimensional structures. The H subunit has ferroxidase activity (Lawson *et al.* 1989, 1991) and responsible for the first step of the iron incorporation, whereas the L subunit promotes nucleation of the iron core (Levi *et al.* 1992). In general, the composition of ferritin hetero polymer is variable in a consistent and tissue specific manner, e.g. brain or heart contains ferritins composed of predominantly the H subunit, whereas L subunit rich ferritins are predominant in liver and spleen (Harrison and Arosio 1996). In order to characterize the biochemical property of these hetero-polymers, many studies have been attempted to produce a series of recombinant ferritin hetero-polymer with broad H/L composition ratio in homogeneously. In 1993, Santambrogio and co-workers reported that ferritin hetero polymers with broad range of subunit composition were produced from the recombinant H and L homo-polymer by mixing the unfolded H and L subunits. When the mixed unfolded subunits were put to the similar condition to form homo-polymers, subunits renatured

to expected hetero-polymers. The resulting hetero-polymers exhibited low heterogeneity, moreover, these hetero polymers were properly folded and biochemically active. However, denaturation and renaturation process were required to prepare the ferritin heteropolymer. Recently, co-expression methods were explored using *E. coli* expression system (Rucker *et al.* 1997, Grace *et al.* 2000). Using these methods, it was possible to prepare the hetero-polymer composed of the H and L chain ferritins, however, the resulting heteropolymer of ferritin exhibited a restricted range of the H/L ratio in subunit composition.

In contrast to vertebrate ferritin, functional diversity among subunits has not been observed in plant ferritin species, although more than two genes encoding ferritin were cloned from maize, *Arabidopsis* and cowpea (Lobreaux *et al.* 1992, Wicks and Entsch 1993, Petit *et al.* 2001a, Wardrop *et al.* 1999). In previous chapter, the author cloned four ferritin genes, *sfer1*, *sfer2*, *sfer3* and *sfer4*, from soybean. Researches about plant ferririn have been performed mainly using the *sfer1* type gene, and knowledge about other type of plant ferritin genes was very rare except the mRNA transcriptional analysis of them in previous chapter. Moreover, the soybean ferritin purified from dry seeds was composed of SEFR1 and SFER2 only (Chapter I). Thus, the function and property in subunit assembly of SFER3 and SFER4 remains unclear.

Nowadays, novel *in vitro* translation system has been developed from cultured insect cell lysate (Ezure *et al.* unpublished data). Using this system, catalytically active proteins can be produced up to the concentration of 100

μg/ml within five hours by only adding the mRNA of desired protein. Since proteins are produced from added mRNA in this system, it can be expected to produce two or more kinds of protein simultaneously in one batch. Here, the author demonstrated that human ferritin heteropolymer, which mimic the tissue specific heterogeneity, could be created using the *in vitro* translation system. The author also carried out the preliminary characterization in subunit assembly of four soybean ferritin subunits, SFER1, SFER2, SFER3 and SFER4 using this system.

3-3 Materials and Methods

3-3-1 Plasmid construction

The plasmid used in this study was constructed by modifying the pTNT vector (Promega). In brief, the EoNPV 5'-polyhedrin reader sequence (5'-TACGGTGTATT TTAGATTTCACAAACAATTA-3'), U95014 registered in the GenBank, was inserted into the site between the 3' side of T7 promoter and the 5' side of the *Kpn*I restriction site in the multi cloning site of the pTNT vector, in stead of the 5'-β-globin leader sequence (Fig.1). The resulting modified pTNT vector was named as EoNPV/pTNT. The cDNAs of human ferritin H chain and L chain, BC016009 and BC016346, registered in the GenBank were purchased from Open Biosystems (Cat. No.MHS1011). Primer sets used here for the amplificaton of ferritin cDNAs are shown in Table 1. In order to construct the expression plasmid of the human H chain ferritin, the EoNPV/pTNT and the human H chain ferritin cDNA were amplified by the polymerase chain reaction using the primer set of

(5'-AGTATTGTAGTCCTTTCG-3') and (5'-GGATCCCTCGAGAATTCAC-3') for the amplification of EoNPV/pTNT, and the set of 'Human H' (Table 1) for the amplification of the cDNA of human H chain ferritin. The resultant PCR fragments were digested by *Kpn*I (Takara) at 37 °C for 2 hours. The digested vector and the human ferritin H chain cDNA fragment were ligated using Ligation Kit version 2 (Takara), followed by the transformation of the *E. coli* DH-5α strain. The resultant vector for expression of the human ferritin H chain was named as pHuFH. The plasmid for human ferritin L chain expression was constructed in above mentioned method, using the primer set of the 'Human L' (Table 1) for the amplification of the ORF of the human L chain ferritin. The constructed plasmid for the expression of the human L chain ferritin was named as pHuFL.

DNA sequences encoding the mature region of *sfer1*, previously reported by Lescure *et al.* (1991), were amplified by PCR using the primer set of *sfer1* (Table 1). The resulting fragments were ligated with the PCR fragments of EoNPV/pTNT to generate the pTSF1 for the expression of the mature region of the *sfer1*. The expression vectors of SFER2, SFER3 and SFER4, named as pTSF2, pTSF3 and pTSF4, respectively, were constructed using the primer set of 'Sfer-2', 'Sfer-3' and 'Sfer-4' (Table 1) for the amplification of the mature regions of *sfer2*, *sfer3* and *sfer4*, respectively. All the plasmids constructed here were sequenced to confirm the sequences of each ferritin ORF.

3-3-2 Preparation of cell extract for *in vitro* translation system

The insect High Five (Invitrogen) cells, about 3×10^6cells/ml, were cultured in Express Five SFM (Invitrogen) serum free medium, to which L-glutamine was added prior to use according to the manufactures protocol, at 27 °C for four days. The cells (about 3×10^6cells/ml) were precipitated by centrifugation at 700 *g* for ten minutes at 4 °C. Then, the cell pellet was re-suspended in extraction buffer (40 mM HEPES-KOH pH 7.9, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM calcium chloride, 20 % (v/v) glycerol, 1mM dithiothreitol, 1mM phenylmethansulfonylfluoride). This centrifugation and re-suspension step was repeated for additional two times. The cell pellet was weighed, following to re-suspend in extraction buffer (0.8 ml buffer/ 1g cells). Then, the cell pellet was frozen by pouring liquid nitrogen. Frozen cell pellet was put on ice for 30 minutes to melt and re-suspend the cell. The cell suspension was centrifuged at 45,000 *g* for 60 minutes. The resulting supernatant (cell lysate) was used for the *in vitro* translation experiments.

3-3-3 Preparation of mRNAs encoding the ferritin molecules

The DNA sequences, which were composed of T7 promoter, EoNPV 5'-polyhedrin reader sequence, ORF of each ferritin gene, poly A and T7 terminator, were amplified using the common primer set of (5'-TACGGTTCCTGGCCTTTTGCTGGCCTTTTG-3') and (5'-GCCATTCAGGCTGCGCAACTGTTGGGAAGG-3'). Each PCR product was ethanol precipitated, and then, was used as a template of mRNA production. Transcription reactions were performed at 37 °C for 3 hours using the RiboMax T7 *in vitro* transcription system (Promega) according to the

manufactures manual. The generated mRNAs were desalted using the Nick column (Amersham), followed by phenol/ chloroform extraction and ethanol precipitation. The concentrations of mRNAs were adjusted to 2.0 mg/mL.

3-3-4 Production of ferritin subunits of human and soybean *in vitro*

Translation reactions were carried out in the 20 μl reaction mixture at room temperature for 5 hours. The composition of the mixture was as follows; 3.2 μl above mentioned mRNA, 0.5 μl ribonuclease inhibitor (Takara), 10 μl HFL and 5.3 μl of premix, which consisted of 40mM HEPES-KOH (pH=7.9), 100mM potassium acetate, 2mM magnesium acetate, 0.5 mM adenosine 5'-triphosphate (ATP), 0.25 mM guanosine 5'-triphosphate (GTP), dipotassium creatine phosphate (CP), 200 μg/ml creatine kinase, 2 mM dithiothreitol (DTT), 80 μM amino acids mixture, 0.25 mM ethylene glycol bis(b-aminoethylether)-*N,N,N,N*-tetraacetic acid (EGTA). The mixture of amino acids contained 80 μM of twenty amino acids, alanine, glycine, cysteine, methionine, histidine, serine, threonine, tyrosine, tryptophan, phenylalanine, valine, leucine, isoleucine, proline, lysine, arginine, glutamine, asparagines, glutamic acid and asparaginic acid. For purpose of the fluorescent labeling of *in vitro* translation products, 0.5 μl of Fluoro Tect $Green_{Lys}$ *in vitro* Translation Labeling reagent (Promega) were added to the reaction mixture. In the co-expression experiments, mRNAs were added to the translation reaction mixtures in the desired ratio, e.g. equal volume (1.6 μl each) of the mRNA of human ferritin H chain and L chain were added to the reaction mixture, if equal volume of the both subunit were desired. When three or four soybean

ferritin cDNAs were expressed simultaneously, equal amounts (1.1 μl or 0.8 μl, respectively) of mRNA solutions of desired cDNA were added to the reaction mixture. The *in vitro* translated ferritin proteins were separated by the SDS-PAGE and non-denatured polyacrylamide gel electrophoresis, followed by the detection using FLA-5000 image analyzer (Fuji Film) with a 532 nm excitation.

3-3-5 Iron incorporation to the *in vitro* translated ferritin proteins

Large scale *in vitro* translation reactions were carried out in the 50 μl of reaction mixtures, which included 13.3 μl of above mentioned premix, 1.25 μl of ribonuclease inhibitor, 25 μl of insect cell lysate and 9 μl of prepared mRNA solution. Each reaction mixture was dialyzed against the 1200 μl of dialysis buffer including 40 mM of HEPES-KOH (pH=7.9), 100 mM of potassium acetate, 2mM of magnesium acetate, 0.5 mM ATP, 0.25 mM of GTP, 20 mM of CP, 2mM of DTT, 80 μM of amino acids mixture and 0.25 mM EGTA until whole reaction time. Four hours after initiation of translation reactions, 9 μl of mRNA solutions were added to the reaction mixtures. Then, fifteen hours after initiation of reactions, iron incorporation were started by adding $Fe(NH_4)_2(SO_4)_26H_2O$ to the dialysis buffer to the final concentration of 2mM. Three hours after addition of Fe(II), 10 μl of reaction mixture were mixed with sample buffer for Native PAGE. Non-denaturing polyacrylamide gel electrophoresis was carried out using 6 % acrylamide gel. In order to detect the iron incorporated ferritin, the gel was dipped on Prussian Blue staining reagent, which contained 1 % of potassium ferrocyanide and 1% hydrochloride,

for 30 minutes.

3-4 Results

3-4-1 Sybthesis of human ferritin H chain and L chain in various composition ratio

The human ferritin H chain, L chain and mixture of them were expressed in the *in vitro* translation system of the cultured insect cell lysate in the presence of FluoroTect^{TM} Green$_{Lys}$ tRNA (Promega) (Fig.2A, B). The produced hetero or homo ferritin proteins were separated by SDS-PAGE (Fig.2A) and non-denatred polyacrilamide gel electrophoresis (Native PAGE) (Fig.2B). Proteins on acrylamide gels were visualized using laser imager. The apparent molecular masses of the expressed H chain and L chain were 21 kDa and 20.5 kDa, respectively (Fig.2A, lane 1 and 13). The individually expressed H chain and L chain were assembled to 24 mer, which is ordinal form of ferritin oligomer (Fig.2B, lane 1 and 13). The mobility of the H chain homo-polymer (Fig.2B, lane 1) is faster than that of the L chain (Fig.2B, lane 13) although the molecular mass of the H chain is slightly larger than that of the L chain. That is due to the differences in charge of outer surfaces of these proteins (Rucker *et al.* 1996). Fig.2A shows that when the H/L ratios of mRNAs were altered from 24/0, 22/2, 20/4, to 4/20, 2/22, 0/24, the ratios of produced subunits were estimated to about 24/0, 22/2, 20/4, to 4/20, 2/22, 0/24. When the ferritin 24-mer composed of the H chain and L chain in various ratios were separated by the non-denaturing polyacrylamide gel electrophoresis, the

mobility of them were between that of the H chain homo-polymer and the L chain homo-polymer; *e.g.* the H chain homopolymer was most acidic (easiest to move), the second acidic was the ferritin heteromer composed in H/L = 22/2 ratio, and the third acidic was H/L = 20/4 (Fig.2B). When the ratio of the L chain was getting higher, the isoelectric points of ferritin hetero 24 mer were seemed to getting higher and, finally, converged on the individually expressed L chain (Fig.4B).

3-4-2 Expression of soybean ferritin, *sfer1*, *sfer2*, *sfer3* and *sfer4*

Soybean ferritin SFER1, SFER2, SFER3 and SFER4 were expressed in *in vitro* translation system of high five cultured cell lysate, followed by separation by SDS-PAGE (Fig.4A) or Native-PAGE (Fig.4B). Proteins on the acrylamide gels were visualized using the laser imager. Fig.4A shows that the SFER1, SFER2, SFER3 and SFER4 were produced when only one type of mRNA was added to the translation system (lane 1-4). Although all of the soybean ferritin subunits, SFER1, SFER2, SFER3 and SFER4 were equally produced and visualized on the SDS-polyacryl amide gel, only the SFER1, SFER2 and SFER3 were detected as assembled oligomeric protein on the non denaturing acrylamide gel (Fig.4B, lane 1-4). The SFER4 was detected as slight bands compared with the bands of SFER1, SFER2 and SFER3 (Fig4B, lane 4). When two types mRNAs of ferritin subunits were added to the *in vitro* translation system in 1/1 ratio (1.6 μl of 2 mg/ml mRNA solution of each ferritin gene), each soybean ferritin subunits were produced in nearly equal amounts (Fig.5A lane 5-10). Since the molecular masses of the SFER2, SFER3

and SFER4 were very close (estimated to 23.2, 24.0, 24.4 and 24.2 kDa ,respectively), the bands of produced SFER2, SFER3 and SFER4 could not to be fully separated when they were co-expressed in the *in vitro* translation system. When the *sfer1* was co-expressed with the *sfer2* or *sfer3*, SFER1/SFER2 hetero polymer and SFER1/SFER3 hetero polymer were detected as a prominent single band on the non-denatured acryl amide gel (Fig.4B, lane 5 and 6). However, when the *sfer1* was co-expressed with the *sfer4*, the SFER1/SFER4 hetero polymer was detected as smear band (Fig.4B, lane 7). The SFER2/SFER3 hetero polymer was detected as a single band (Fig.4B, lane 8), although slightly smear compared with the cases of the SFER1/SFER2 or the SFER1/SFER3. On the other hand, the hetero polymers of the SFER1/SFER4, SFER2/SFER4 and SFER3/SFER4 were detected as smear bands, not as single bands (Fig.4B, lane 7, 9 and 10). When three or four cDNAs of soybean ferritin were co-expressed, only SFER1/SFER2/SFER3 hetero polymer was detected as a single band (Fig.4B, lane 11). Other hetero polymers, all of which contained SFER4, were not detected as a convergent single band. They have shown smear bands with high heterogeneity (Fig.4B, lane 12-15).

3-4-3 Iron incorporation in soybean ferritin homo- and hetero-polymer

The iron incorporated soybean ferritin, which were *in vitro* translated using cultured insect cell lysate, were visualized by Prussian Blue stain. In order to produce more protein, the reaction mixtures were dialyzed against the dialysis buffer containing amino acids, energy source (ATP, GTP, CP etc. see

experimental procedures). After 15 hours incubation, Fe^{2+} was added to the dialysis buffers to final concentration of 1 mM, then 10 μl of aliquot of each reaction mixture was separated by non-denaturing acrylamide gel electrophoresis using 6 % acrylamide gel, followed by Prussian blue stain (Fig.5). When translated *in vitro* individually, both of the SFER1 and SFER2, which were able to assemble to 24 mer, were also detected as single bands, suggesting that they incorporated iron efficiently (Fig.5, lane 1 and lane 2). The individually expressed SFER3 could be stained by Prussian blue, however, the signal was faint compared with that of the SFER1 and SFER2 (Fig.5 lane 3). The SFER4 alone was not detected as any visible band (Fig.5 lane 4).

The co-expression experiments were also performed using the same experimental system. When the *sfer1* and *sfer2* were co-expressed *in vitro*, the SFER1/SFER2 hetero polymer was formed and the resultant heteropolymer could incorporate the iron atom (Fig.5, lane 5). The mobility of the SFER1/SFER2 hetero polymer was in the middle of that of the SFER1 homopolymer and the SFER2 homopolymer (Fig.5, lane 1, 2 and 5), consistent with the result of fluorescent detection (Fig.4B, lane 1, 2 and 5). When the *sfer1* and *sfer3* were co-expressed *in vitro*, the SFER1/ SFER3 heteropolymer was detected as a single prominent band (Fig.5, lane 6), although the SFER3 was detected as only a faint band (Fig.5, lane 3). The mobility of the SFER1/ SFER3 (Fig.5, lane 6) was in the middle of the SFER1 homopolymer (Fig.5, lane 1) and SFER3 homopolymer (Fig.5, lane 3). When the *sfer1* and *sfer4* were co-expressed *in vitro*, the SFER1/ SFER4 hetero polymer detected as a

faint band (Fig.5, lane 7). The SFER2/ SFER3 hetero polymer was detected as a faint band (Fig.5, lane 9), while the SFER2/SFER4 and the SFER3/SFER4 hetero polymer were not observed as any detectable bands (Fig.5, lane 9 and 10).

3-5 Discussion

3-5-1 Assembly of human ferritin H chain and L chain *in vitro*

Present data confirm that human ferritin hetero-polymers composed of variable ratio of the H chain and L chain can be produced using the *in vitro* translation system of the cultured insect cell lysate. Previously, it has been attempted to produce the isoferritin, that contain the H chain and L chain in various ratio using several kinds of expression systems; e.g. *E. coli* (Grace et al. 2000), cultured cell (Guo *et al.* 1998, Corsi *et al.* 1998) and yeast expression system (Shin *et al.* 2001). In general, when the H chain and L chain are co-expressed using the *in vivo* expression system, the produced ferritin is usually L chain rich ferritin, because of the toxicity of the H chain to cell growth (Guo *et al.* 1998). In co-expression experiments of ferritin H chain and L chain, Rucker and co-leagues (1997) succeeded in producing isoferritin, which had relatively high variety of H/ L composition ratios, using a bicistronic vector system and dual vector system in *E. coli* . Another experiment was performed by Grace *et al.* (2000), who manipulated the *E. coli* cultivation condition to increase the ratio of the H chain ferritin, for example, added the antibiotics, rifampicin. However, the heterogeneity of the resultant

ferritin hetero-polymers was still restricted in subunit composition. In the present study, we constructed the *in vitro* translation system of cultured insect cell lysate. Using this protein expression system, variable ferritin hetero-polymer, which mimic the natural heterogeneity, could be created by controlling the H/L ratio of their mRNAs added to this system. In addition to the ability of various hetero-polymer creation, the homo- and hetero ferritin oligomer incorporated iron atoms (data not shown), accordingly, this protein synthesis system becomes a powerful tool for characterization of iso-ferritin in mammal organs.

3-5-2 Assembly of soybean ferritin, SFER1, SFER2, SFER3 and SFER4

The SFER1 and SFER2 assembled into hetero-polymer in the co-expression experiment of SFER1/ SFER2 (Fig.4B, lane 5), moreover, the resulting SFER1/SFER2 hetero-polymer has incorporated iron atoms (Fig.5, lane 5). The author has indicated that soybean ferritn was present as hetero-polymer in iron containing form, that consisted of SFER1 and SFER2 in equal amounts (Chapter I). Further investigation suggested that the *sfer1* and the *sfer2* were expressed in nearly equal amounts in transcriptional level in the formation state of seeds (Chapter II). These data demonstrated that the *in vitro* translation system could imitate the formation of mature protein. The single gene expression experiment presented here suggested that the SFER1 and SFER2 assembled to active homo polymer (Fig.4B lane 1 and 2, Fig.5 lane 1 and 2). Until now, many studies have been performed using the *sfer1* type genes for the purpose of characterization of plant ferritin (Laulhere *et al.*

1988, Lobreaux and Briat 1989,1991, Ragland *et al.* 1990). As results, the expressed SFER1 type of plant ferritin was correctly folded and catalytically active *in vivo*. However, recombinant proteins of other types of plant ferritin have not ever been expressed *in vivo* or *in vitro*. Present data suggested that the SFER2 as well as the SFER1 was produced as active oligomeric form (Fig.5, lane 2). The SFER3 was assembled into homo-polymer when expressed *in vitro* individually (Fig.4B, lane 3), however, the iron incorporating activity of resulting homo-polymer was very low, compared with the activity of SFER1 and SFER2 (Fig.5, lane 3), although all of four ferritin genes possessed ferro-oxidase site. It has been suggested that the mammal H subunit ferritin required for assistance of celuroplasmin in iron incorporating process (Guo *et al.* 1996, Juan *et al.* 1997). The celuroplasmin or other unknown factor might be required for iron incorporation by the SFER3 homo-polymer. When the *sfer3* was co-expressed with the *sfer1*, the catalytically active SFER1/SFER3 hetero-polymer was formed with low heterogeneity (Fig.4B lane 6 and Fig.5 lane 6). The SFER2/SFER3 was also assembled to hetero-polymer, however, the iron incorporate activity of it was very lower than the hetero polymers of SFER1/SFER2 or SFER1/SFER3. Thus, these data suggested that the SFER3 could not incorporate iron unless co-expression with the SFER1 in this condition, however, *sfer3* was not always co-expressed with *sfer1 in vivo* (Chapter II). The role of the *sfer3* remains unclear.

In contrast to other soybean ferritin subunits, SFER4 could not be assembled to active homo- or hetero-polymer (Fig.5 lane 4, 7 and 9). Thus,

SFER4 would not function as oligomeric protein although the primary structures were highly conserved among the four subunits of soybean ferritin. It is possible that the SFER4 subunit functions as phytosiderin, which is insoluble form of plant ferritin in soybean seed (Laulhere *et al.* 1989). In the previous chapter, the author indicated that the ferritin cDNAs, *sfer1*, *sfer2* and *sfer4*, were transcribed actively in seed maturing process. In chapter I, the author suggested that ferritin was composed of the SFER1 and SFER2 in nearly equal amount in soybean dry seed. However, SFER1/SFER2 hetero-polymer or any specific band of oligomeric ferritin was not detected in non-denaturing polyacrylamide gel when the three cDNAs (*sfer1*, *sfer2* and *sfer4*) were co-expressed in this system (Fig4B, lane 12). It might be due to differential localization between SFER1/SFER2 and SFER4, because the N-terminal of transit peptide of the *sfer4* was more hydrophobic than that of *sfer1*, *sfer2* and *sfer3*. In fact, the N-terminal amino acid sequence of eighteen residues in transitpeptide of the *sfer4* was deduced to be a "signal peptide (for endoplasmic reticulum)", while the others were deduced to transit peptide for mitochondrial or chloroplast using the iPSORT program (http://biocaml.org/ipsort/iPSORT).

In present study, the author demonstrated that the *in vitro* translation system made from the cultured insect cell lysate would become a powerful tool for analyzing the subunit stoichiometry and functions of homo- or hetero- polymer of ferritin. More generally, the *in vitro* translation strategy described here may be applicable to studies of the role of component subunit in the assembly or function of other miltimeric proteins or protein complex.

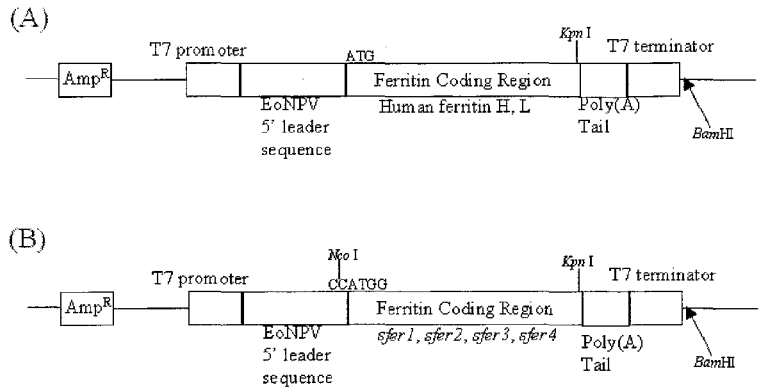

Fig. 1 Design of constructs used for *in vitro* expression of recombinant human and soybean ferritin cDNAs.

The ferritin coding regions were inserted into 3'- side of EoNPV 5'- leader sequence in EoNPV/pTNT vector. In case of expression plasmid of soybean ferritin, the mature region (absent of transit peptide) of each ferritin cDNA was inserted between *Nco* I restriction site and *Kpn* I restriction site, while the human ferritin ORFs were inserted without *Nco* I restriction.

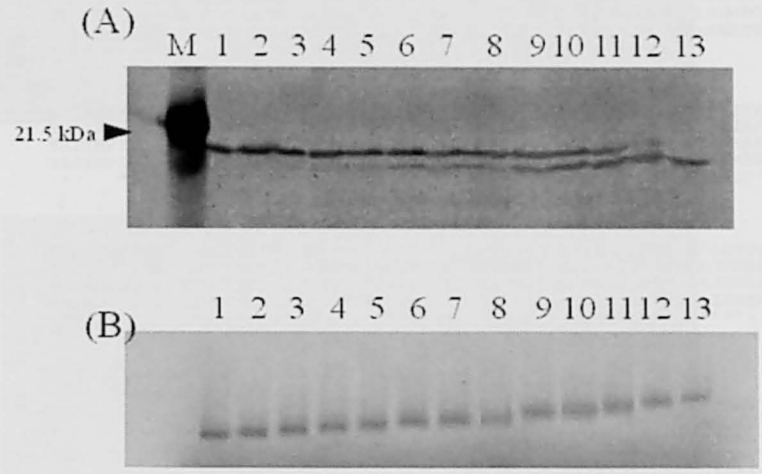

Fig. 2 SDS-PAGE and native PAGE analysis of human ferritin homo- and hetero-polymers.

Human ferritin H chain and L chain were translated *in vitro* and 10 μl of *in vitro* translation mixture was loaded on each lane and separated by the SDS-PAGE (12.5 % gel) (A) and non-denaturing acrylamide gel electrophoresis (native PAGE) (6 % gel) (B). Signals of translated proteins were visualized using laser imager. The H/L ratios of added mRNAs were 24/0 (lane 1), 22/2 (lane 2), 20/4 (lane 3), 18/6 (lane 4), 16/8 (lane 5), 14/10 (lane 6), 12/12 (lane 7), 10/14 (lane 8), 8/16 (lane 9), 6/18 (lane 10), 4/20 (lane 11), 2/22 (lane 12) and 0/24 (lane 13). Lane M; protein marker and its molecular mass.

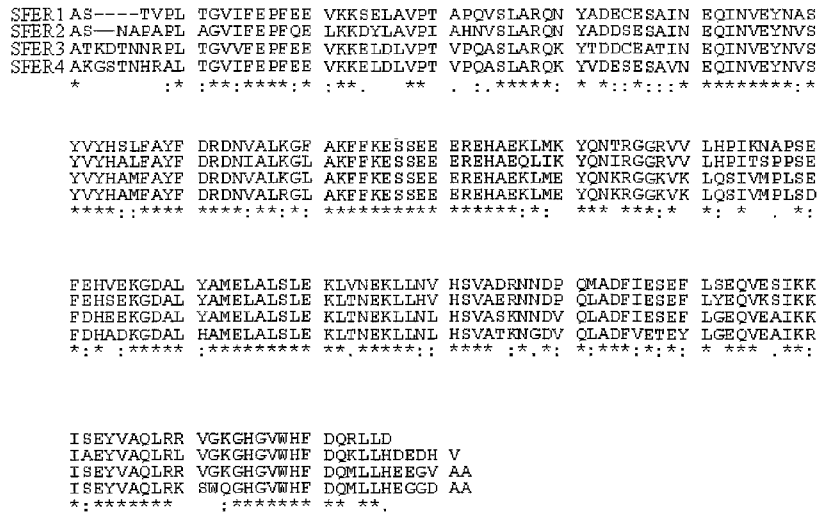

Fig.3 Deduced amino acid sequences of mature regions of four soybean ferritin subunits, SFER1, SFER2, SFER3 and SFER4. The conserved amino acid residues among four subunits were asterisked below.

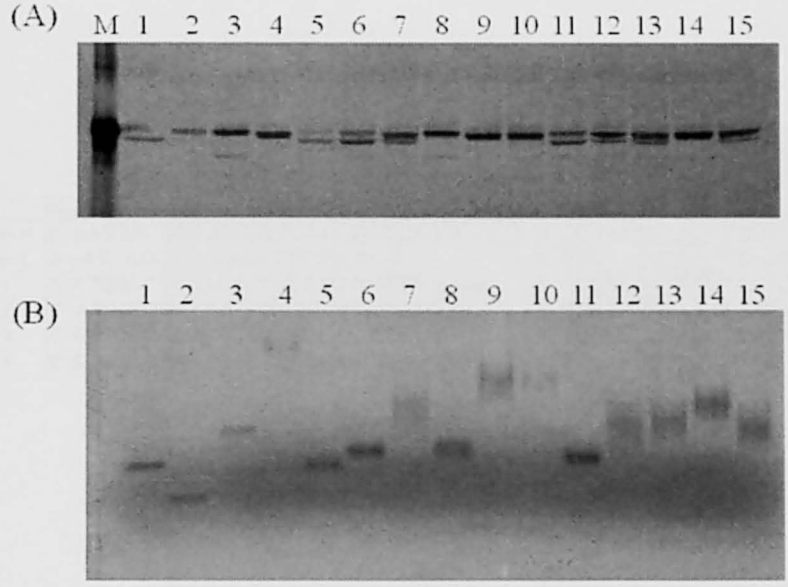

Fig.4 SDS-PAGE and native PAGE analysis of soybean ferritin homo- and hetero-polymers.

Soybean ferritin *sfer1*, *sfer2*, *sfer3* and *sfer4* were expressed *in vitro* and 10 μl of *in vitro* translation mixture was loaded on each lane and separated by the SDS-PAGE (12.5 % gel) (A) and non-denaturing acrylamide gel electrophoresis (native PAGE) (6 % gel) (B). Signals of translated proteins were visualized using laser imager. Added mRNAs were as follow. Lane 1, *sfer1*; Lane 2, *sfer2*; Lane 3, *sfer3*; Lane 4, *sfer4*; Lane 5, *sfer1*/*sfer2*; Lane 6, *sfer1*/*sfer3*; Lane 7, *sfer1*/*sfer4*; Lane 8, *sfer2*/*sfer3*; Lane 9, *sfer2*/*sfer4*; Lane 10, *sfer3*/*sfer4*; Lane 11, *sfer1*/*sfer2*/*sfer3*; Lane 12, *sfer1*/*sfer2*/*sfer4*; Lane 13, *sfer1*/*sfer3*/*sfer4*, Lane 14, *sfer2*/*sfer3*/*sfer4*; Lane 15, *sfer1*/*sfer2*/*sfer3*/*sfer4*.

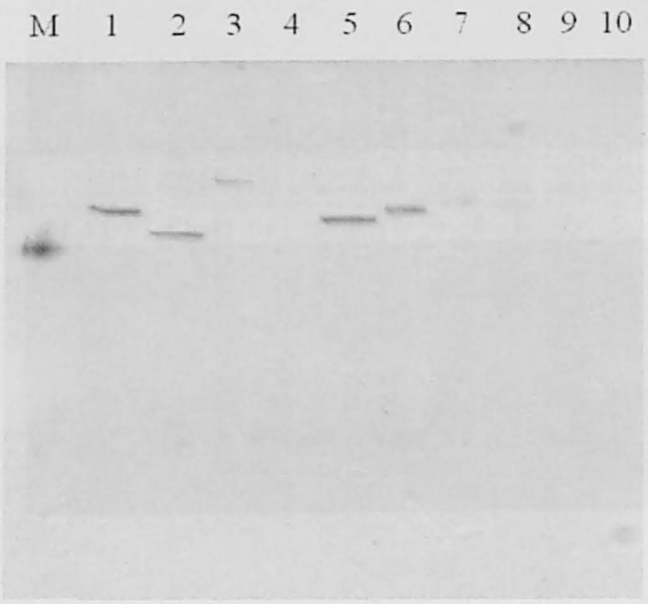

Fig.5 Prussian blue staining of *in vitro* expressed soybean ferritins.

Soybean ferritin *sfer1*, *sfer2*, *sfer3* and *sfer4* were expressed *in vitro* and 10 μl of *in vitro* translation mixture was loaded on each lane and separated by native PAGE (6 % gel). Iron incorporated ferritin oligomers were visualized by Prussian blue stain. Added mRNAs were as follow. Lane 1, *sfer1*; Lane 2, *sfer2*; Lane 3, *sfer3*; Lane 4, *sfer4*; Lane 5, *sfer1/sfer2*; Lane 6, *sfer1/sfer3*; Lane 7, *sfer1/sfer4*; Lane 8, *sfer2/sfer3*; Lane 9, *sfer2/sfer4*; Lane 10, *sfer3/sfer4*. Lane M; horse spleen ferritin (H/L = 2/22) (Sigma).

	Foward Primers	Reverse Primers
Human H	5'- pATGACGACCGCGTCCACCTC GCAG-3'	5'- GCGAAGGTACCTTAGCTTTCATTATCACTG-3'
Human L	5'-pATGAGCTCCCAGATTCGTCAG-3'	5'- GCGAAGGTACCTTAGTCGTGCTTGAGAGTG-3'
Sfer-1	5'-ATGGCCTCAACGGTGCCTCTCAC-3'	5'- GCGAAGGTACCTAATCAAGAAGTCTTTGAT-3'
Sfer-2	5'- ATGGCTTCAAATGCACCCGCACCAC-3'	5'- GCGAAGGTACCTCATACATGATCTTCATCG-3'
Sfer-3	5'-ATGGCTACCAAGGACACCAAC-3'	5'- GCGAAGGTACCTCAAGCAGCAACTCCTTCC-3'
Sfer-4	5'-ATGGCGAAAGGATCCACGAACC-3'	5'- GCGAAGGTACCTCAAGCTGCATCTCCTCCC-3'

Table 1 Primer sets used for amplification of ferritin ORF

	SFER1	SFER2	SFER3	SFER4
SFER1	100	83.2	78.7	73.3
SFER2	83.2	100	73.7	68.9
SFER3	78.7	73.7	100	85.8
SFER4	73.3	68.9	85.8	100

Table 2 The amino acid sequence identity among the SFER1, SFER2, SFER3 and SFER4. The mature region of four soybean ferritin were compared.

Chapter IV

Crystallization and preliminary X-ray crystallographic analysis of plant ferritin from soybean

4-1 Abstract

The iron storage protein ferritin from soybean (*Glycine max*) was expressed in *E. coli* and crystallized using the hanging drop vapor diffusion method with sodium tartrate as the precipitant. The crystals belong to the tetragonal $I4_122$ space group, with unit cell parameters a=b=324.0, c=182.7 Å. The diffraction data were collected up to a resolution of 3.0 Å with a multi-wire area detector.

4-2 Introduction

Ferritin is a class of iron storage protein widely distributed in the plant and animal kingdoms. It is comprised of twenty-four subunits assembled into a spherical cage within which up to 4500 atoms of iron can be stored in a bio-available and nontoxic form (Harrison and Arosio 1996). Exceptionally, dodecameric ferritins have been identified in some bacteria species (Ilari *et al.* 2000, 2002, Reindel *et al.* 2002). They have been suggested to be induced via oxidative stress and capable of incorporating iron (Papinutto *et al.* 2002, Yamamoto *et al.* 2002, Zhao *et al.* 2002, Ceci *et al.* 2004). Two subunit types, H and L, have been characterized in vertebrates. Their primary structures show about 54% identity, and each has a particular

physiological function: the H subunit, which possesses the ferroxidase site, can incorporate iron at a much faster rate than the L subunit (Lawson *et al.* 1989), whereas the L subunit, which shows iron-nucleation capacity, can retain iron stably in the inner cavity of the ferritin molecule as ferrihydrite or complexed with phosphate (Levi *et al.* 1992). The three dimensional structure of ferritin, which has been analyzed in vertebrates (Lawson *et al.* 1991, Hempstead *et al.* 1997) and bacteria (Frowlo *et al.* 1994, Stillman *et al.* 2001), is highly conserved among these species. Each subunit is composed of four α-helix bundles (A, B, C, D) and one short α-helix (E helix), and within the assembled 24-mer, subunits are characterized by the operation of 2-fold, 3-fold and 4-fold symmetry (432 symmetry).

Plant ferritin has been well characterized in legumes (Briat *et al.* 1995, Ragland *et al.* 1990, Lescure *et al.* 1991, Laulhere *et al.* 1988, Wardrop *et al.* 1999) and other plants (Lobreaux *et al.* 1992, Petit *et al.* 2001). In general, plant ferritin is synthesized as a precursor protein with an N-terminal transit peptide (TP) responsible for its transportation into plastids. The apparent molecular mass of the mature form of soybean ferritin is 28 kDa (Ragland *et al.* 1990), whereas that of the mammalian H and L chains is 21 kDa and 19 kDa, respectively (Arosio *et al.* 1978). One major reason for the difference in the sizes of plant and mammalian ferritin is the presence of an N-terminal extension peptide (EP) in the plant form (Harrison and Arosio 1996, Briat *et al.* 1995, Ragland *et al.* 1990, Lescure *et al.* 1991). The EP, which in soybean consists of 28 amino acid residues, is a plant-specific domain whose function remains unknown. An EP deletion

mutant remained able to assemble into a ferritin like protein; moreover, the mutant protein showed slightly greater ferroxidase activity than the wild-type protein containing the EP sequence (van Wuytswinkel *et al.* 1995). Based on computational analysis of pea ferritin, Lobréaux and co-workers (Lobréaux *et al.* 1992) predicted that this domain formed a three-turn helix, termed a "P-helix;" however, the crystal structure of plant ferritin has yet to be characterized.

Another notable feature of plant ferritin is the primary structure of the C-terminal domain. This domain is thought to perhaps correspond to vertebrate helix E (Lobréaux *et al.* 1992), which is positioned around the 4-fold inter-subunit interaction axes in the assembled ferritin. In plant ferritin, this domain mainly consists of hydrophilic residues, whereas in mammalian and other ferritins, it forms a hydrophobic channel in the assembled ferritin shell (Harrison and Arosio 1996). Despite the sequence differences, the C-terminal domain of plant ferritin was also predicted to form a short helix similar to that in vertebrate ferritin (Lobreaux *et al.*). The author has shown that two types of ferritin subunit, SFER1 and SFER2, co-exist in soybean seeds; the C-terminal domain of the SFER1 subunit is cleaved, likely to facilitate further degradation of soybean ferritin (Chapter I).

To investigate the structure and function of the domains unique to plant ferritin, including the N-terminal EP and C-terminal E-helix, the author crystallized the SFER1 subunit from soybean (*Glycine max*) expressed in *E. coli*. Here we present the preliminary X-ray crystallographic data.

4-3 Materials and Methods

4-3-1 Preparation of recombinant SFER1

The ferritin cDNA (Lescure *et al.*) was isolated from soybean (*Glycine max* Merr cv. Kita-no-shiki) leaves using RT-PCR (Goto *et al.* 1998, 1999). The PCR product was then cloned into plasmid pCRII (Invitrogen), and the cDNA sequence of the mature region of soybean ferritin in pCRII was subjected to PCR with primers

ΔTP (5'-GCGCATATGTCAACGGTGCCTCTCAC-3') and

C-term (5'-GCGGGATCCTAATCAAGAAGTCTTTG-3'). The amplified product was cleaved using *Nde*I and *Bam*HI, and the resultant fragment was ligated into the *Nde*I and *Bam*HI restriction sites of pET3a vector (Novagen) to generate pESF (Chapter I). *E. coli* strain BL21 (DE3)pLys were transformed with the pESF, grown at 37 °C in LB medium supplemented with ampicillin and induced with 100 μM IPTG. Eventually, the cells were harvested by centrifugation, and the proteins extracted using BugBuster protein extraction reagent (Novagen). The crude extract was dialyzed against buffer A (10 mM Tris-HCl pH 7.5, 1 mM EDTA) containing 10 mM 2-ME, after which the dialyzate was centrifuged, and the supernatant was collected and applied to a DEAE-Toyopearl column previously equilibrated in buffer A. Proteins were eluted with buffer A containing 0.2 M sodium chloride. The resultant protein sample was fractionated using a 20% saturation of ammonium sulfate, after which it was centrifuged, and the supernatant was loaded onto a butyl-Toyopearl column. In this case, proteins

were eluted with a descending 20 to 0 % ammonium sulfate gradient. Finally, the ferritin was loaded onto a Superdex 200 pg (Amersham Pharmacia) previously equilibrated with 10 mM Tris-HCl (pH 7.5) and 0.15 M NaCl and then concentrated to 15 mg/ml using a Centriprep (Millipore).

4-3-2 Crystallization of recombinant SFER1

Crystallizations were performed using the hanging drop vapor diffusion method. The crystallization conditions were initially tested using a Crystal Screen (Hampton Research), but no crystals were obtained. The author then tested several conditions, and polyhedral shaped crystals were obtained using 0.75 M sodium tartrate as a precipitant. The best crystal was grown when 5 μl of protein solution (15 mg protein per ml in 10 mM Tris-HCl and 0.15 M sodium chloride, pH 7.5) were mixed with 5 μl of reservoir solution (0.75 M sodium tartrate in 0.1 M Tris-HCl, pH 8.2) at 20 °C. Crystals used for data collection were obtained after about one month (Fig.1).

4-4 Results and Discussion

X-ray diffraction data were collected at room temperature on a multi-wire area detector (Bruker) with Cu *K*α X-rays produced by a rotating anode generator (M18XHF MacScience) operating at 40 kV and 90 mA. The crystals mounted in thin-walled glass capillaries diffracted to better than 3.0 Å resolution, and the collected data set was processed using the SADIE and SAINT software packages (Bruker). The crystals contained a tetragonal lattice ($I4_122$ or $I4_322$) and the unit cell dimensions were a=324.0, b=324.0,

c=182.7 Å, $\alpha=\beta=\gamma=90°$, yielding a unit cell volume of 1.92×10^7 Å^3. A native data set was collected to 3.0 Å with an R-sym of 10.4% and 85.4% completeness for 61,825 unique reflections. The density of the crystal and of the total solvent compartment were measured at 25 °C in a density gradient of bromobenzene and *m*-xylene (Low and Richards 1952a, 1952b, 1954) and found to be 1.16 g/cm^3 and 1.10 g/cm^3, respectively. Together with the estimated partial specific volume of soybean ferritin (0.734 cm^3/g), the number of molecules per asymmetric unit was calculated to be 10.5. When 12 subunits of soybean ferritin exist in an asymmetric unit, the solvent volume fraction (V_{sol}) and the crystal volume per unit molecular mass (V_M) were calculated to be 73.5 % and 4.47 Å^3/Da, respectively. The V_M value of the soybean ferritin crystal is similar to the value calculated for other tetragonal crystals of ferritin (Cobessi *et al.* 2002, Stillman *et al.* 2001).

The author has now diffracted the soybean ferritin crystal to a resolution of 2.5 Å using SPring-8 synchrotron radiation. To determine its three dimensional structure, we are presently attempting to carry out molecular replacement using human H-chain ferritin as a search model.

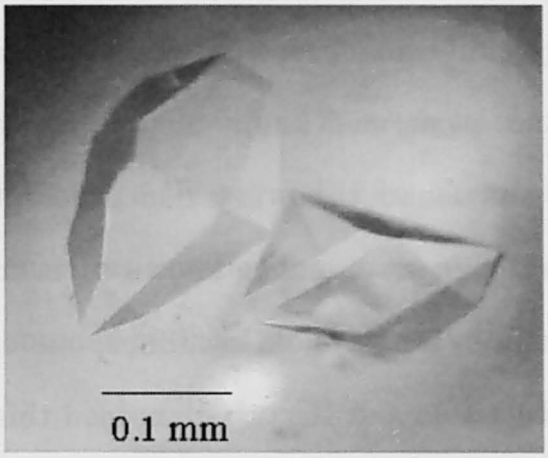

Fig. 1 Crystals of soybean ferritin

Summary

Ferritin purified from dried soybean seeds were resolved into two subunits of 26.5 and 28 kDa. The author performed amino acid sequence analysis of the two ferritin subunits, and found that they had different sequences, thus rendering the 28 kDa subunit novel among known soybean ferritins. The author cloned two cDNAs from ten-days-old seedlings, one of which encoded the previously identified ferritin and the other encoded this novel subunit. The 26.5 kDa subunit (SFER1) was found to be identical to that previously identified to be lacking the C-terminal 16 residues that correspond to the "E-helix" of mammalian ferritin. However, the corresponding region in the 28 kDa (SFER2) ferritin subunit identified in this study was not susceptible to cleavage. The author presents evidence that the two different ferritin subunits in soybean dry seeds show differential sensitivity to protease digestions, and that the novel, uncleaved SFER2 subunit appears to stabilize the ferritin shell by co-existing with the cleaved SFER1 subunit. These data demonstrate that soybean ferritin is composed of at least two different subunits, SFER1 and SFER2, which have co-operative functional roles in soybean seeds (Chapter I).

In Chapter II, the author cloned two novel genes of soybean ferritin (*sfer3* and *sfer4*). To investigate the role of each ferritin gene and its expression in plant, the author first analyzed copy number of four ferritin genes containing two novel genes (*sfer3* and *sfer4*) and characterized the sequences of the genes. All of them classified to H/L hybrid type of ferritin

from predicted amino acid sequences. Next, the author examined the expression of ferritin genes during the developmental stage of seed, its tissue specificity and its response to metal treatments by Northern blot analysis. These data obtained here suggested that the expression of each ferritin gene was variedly regulated in tissue specific manner and against exogenous stimulus in order to control iron metabolism although each ferritin subunit encoded by the 4 genes had a very similar structure.

In Chapter III, the author demonstrated that four cDNAs of ferritin from soybean, *sfer1*, *sfer2*, *sfer3* and *sfer4* could be expressed and assembled to homo- or hetero-polymer using the *in vitro* translation system. Native-PAGE and Prussian blue stain of resulting ferrtin revealed that homo-polymer of SFER1 and SFER2, and hetero-polymer of SFER1/SFER2 and SFER1/SFER3 could be produced as active oligomeric ferritin using this system, whereas SFER4 never assembled to oligomer although every possible combination is tested. These results indicated that SFER1 and SFER2 could function as homo- and hetero-polymer, on the other hand, SFER4 would not assemble to oligomeric form although the *sfer4* is a functional gene.

The iron storage protein ferritin from soybean (*Glycine max*) was expressed in *E. coli* and crystallized using the hanging drop vapor diffusion method with sodium tartrate as the precipitant. The crystals belong to the tetragonal $I4_122$ space group, with unit cell parameters a=b=324.0, c=182.7 Å. The diffraction data were collected up to a resolution of 3.0 Å with a multi-wire area detector (Chapter IV).

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List of Publications

1. Masuda T., Goto F. & Yoshihara T. (2001) A novel plant ferritin subunit from soybean that is related to a mechanism in iron release. *J. Biol. Chem.* **276**: 19575-19579
2. Goto F. Yoshihara T. & Masuda T. Differential expression in tissue and response to metal treatments of four soybean ferritin genes. In preparation.
3. Masuda T., Ezure T., Ito M., Goto F., Yoshihara T. & Shikata M. Construction of homo- and hetero-polymers of ferritin using novel *in vitro* protein expression system. In preparation.
4. Masuda T., Mikami B., Goto F., Yoshihara T. & Utsmi S. (2003) Crystallization and preliminary X-ray crystallographic analysis of plant ferritin from *Glycine max*. *Biochim. Biophys. Acta* **1645**: 113-115

Related

1. Goto F., Yoshihara T. Masuda T. & Takaiwa F. (2001) Genetic improvement of iron content and stress adaptation in plants using ferritin gene. *Biotechnol. Genet. Eng. Rev.* **18**: 351-371
2. Yoshihara T., Masuda T. & Goto F. (2001) A genetic approach for phytoremediation of heavy metals. An approach for increasing the accumulation capacity within plants. *Recent Res. Plant Biol.* **1**: 163-181
3. Yoshihara T., Kobayashi T., Goto F., Masuda T., Higuchi K., Nakanishi H., Nishizawa N.K. & Mori S. (2003) Regulation of the iron deficiency

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