Novel Soybean Enzymes Involved in the Oxidative Protein Folding in the Endoplasmic Reticulum

Aya OKUDA
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ABBREVIATIONS

DTT  dithiothreitol

E. coli  *Escherichia coli*

ER  endoplasmic reticulum

ERO1  endoplasmic reticulum oxidoreductin 1

PDI  protein disulfide isomerase

GSH  glutathione

GSSG  oxidized glutathione

GST  glutathione-S-transferase

HEPES  2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid

IEF  isoelectric focusing

mPEG  methoxypolyethylene glycol maleimide

PAGE  polyacrylamide gel electrophoresis

PDI  protein disulfide isomerase

QSOX  quiescin sulfhydryl oxidase

RNaseA  ribonuclease A

Tris  Tris (hydroxymethyl) aminomethane
GENERAL INTRODUCTION

Soybean is a protein rich crop with 35–50% of its dry weight being protein, suggesting that, in the future, soybean seeds could be used in a variety of applications, including for the production of oral vaccines, monoclonal antibodies, and therapeutic proteins, in addition to their normal function as seed storage proteins. The total proteins in soybeans are mainly storage proteins consisting of the two major seed storage proteins, glycinin and β-conglycinin. The proteins are synthesized and folded in the endoplasmic reticulum (ER). In most cases, storage proteins are folded with accompanying intramolecular disulfide bonds (oxidative folding) prior to transport from the ER via the Golgi apparatus, where they accumulate in the protein storage vacuoles. More than 90% of the proteins synthesized in the ER have intramolecular disulfide bonds, which are essential for the conformational stability and function of proteins (Braakman and Bulleid, 2011). If proteins fail to fold, the unfolded and misfolded proteins are degraded by protein quality control systems in the cell (Smith et al., 2011) (Fig. 1). Therefore, it is important that the mechanisms are clarified for the oxidative folding of nascent seed storage proteins before their subsequent accumulation in protein storage vacuoles.

**Fig. 1. Protein synthesis in the ER.**
Disulfide bond formation in nascent polypeptide chains in the ER is accompanied by dithiol/disulfide transfer reactions that are catalyzed by members of the protein disulfide isomerase (PDI) family (Hatahet and Ruddock, 2009). In humans, there are approximately twenty proteins that belong to the PDI family, a subset previously studied in detail (Kozlov et al., 2010). In higher plants, including Arabidopsis, soybean (Glycine max) (Fig. 2), wheat (Triticum aestivum), and rice (Oryza sativa), there are ten groups of proteins that belong to the PDI family (Houston et al., 2005; d’Aloisio et al., 2010; Selles et al., 2011). In Arabidopsis, a set of 22 orthologs of the known PDI family proteins were discovered by a genome-wide search and separated into phylogenetic groups designated I–X (Houston et al., 2005). Among them, PDI family proteins of group I, including a plant PDI ortholog, have been studied in a wide variety of plants.

Fig. 2. Soybean PDI family proteins
The group I–V soybean PDI family proteins of, GmPDIL-1, GmPDIL-2, GmPDIL-3a, GmPDIS, and GmPDIM have been identified and have enzymatic activities (Wadahama et al., 2007, 2008; Kamauchi et al., 2008; Iwasaki et al., 2009). The group VI–VIII soybean PDI family proteins, GmPDIL6, GmPDIL7, and GmPDIL8, have not been studied. The PDI family proteins contain one or two thioredoxin domains, a, and a’, which have an active center motif (Cys-X-X-Cys). The group I–III, V and VII PDI family proteins contain one or two thioredoxin domains, b, and b’, which lack catalytically active cysteines. The group VI PDI family proteins have an a domain structure similar to mammalian ERp18 (Alanen et al., 2003; Jeong et al., 2008). The enzymatic characteristics of the plant group VI PDI family proteins have not yet been determined. The group VII PDI family proteins have an a-b-b’ domain structure with a C-terminal transmembrane region similar to mammalian TMX3 (Haugstetter et al., 2005; Haugstetter et al., 2007). However, only the oxidase activity of recombinant human TMX3 (HsTMX3) has been confirmed, and the enzymatic characteristics of the plant group VII PDI family proteins have not been determined.

Disulfide bond formation in a nascent polypeptide by the PDI family proteins is accompanied by the reduction of an active center to dithiol. Because the active centers of the PDI family proteins cannot become oxidized, alone, PDI family proteins require other oxidizing molecules. In yeast and mammalian cells, ER oxidoreductin 1 (Ero1) directly transfers disulfide bonds to PDI (Frand and Kaiser, 1998; Pollard et al., 1998; Cabibbo et al., 2000; Dias-Gunasekara et al., 2005; Araki and Inaba, 2012). Ero1 orthologs are present universally in eukaryotes. Plant orthologs of Ero1p, rice Ero1 (OsERO1), and soybean Ero1 (GmERO1) have been identified (Onda et al., 2009; Matsusaki et al., 2016). OsERO1 is necessary for disulfide bond formation in rice endosperm, whereas recombinant GmERO1 reportedly has broad substrate specificity and oxidizes GmPDIL-1, GmPDIM, GmPDIS-1, and GmPDIS-2, but not GmPDIL-2.

Although knockout experiments in yeast showed that Ero1p was an essential protein (Frand and Kaiser, 1998; Pollard et al., 1998), Ero1 knockouts in mice did not produce severe
phenotypes (Zito et al., 2010a), prompting further studies of additional pathways for disulfide bond generation in mammalian cells. At present, four additional pathways have been shown to generate disulfide bonds de novo in the secretary apparatus: peroxiredoxin 4 (Tavender et al., 2010; Zito et al., 2010b), glutathione (GSH) peroxidase 7 and 8 (Nguyen et al., 2011; Kakihana et al., 2012; Wang et al., 2014), vitamin K epoxide reductase (Wajih et al., 2007; Rutkevich et al., 2012), and quiescin sulfhydryl oxidase (QSOX) (EC 1.8.3.2) (Chakravarthi et al., 2007; Kodali and Thorpe, 2010a). In plants, genes encoding Ero1p and QSOX orthologs have been identified (Kodali and Thorpe, 2010a).

Animal QSOXs were first isolated as unknown flavoproteins that catalyzed the oxidation of low molecular mass thiol compounds (Ostrowski et al., 1979) by coupling disulfide oxidation to the reduction of oxygen, forming hydrogen peroxide (Ostrowski and Kistler, 1980). QSOXs directly transfer disulfide bonds to unfolded proteins, but they cannot isomerize non-native disulfides (Hoober et al., 1999; Jaje et al., 2007; Heckler et al., 2008; Kodali and Thorpe, 2010b). Hence, one may assume the existence of an oxidative folding pathway, in which QSOX acts as an oxidase and reduced PDI corrects mispaired disulfide bonds introduced by QSOX. The reduced and denatured pancreatic RNase A and the reduced avian riboflavin-binding protein are effectively refolded in vitro, with the correct four and nine disulfide bonds being formed via cooperation between human or avian QSOX and PDI (Hoober et al., 1999; Raney and Thorpe, 2008).

There are two previous reports regarding QSOX orthologs in plants (Alejandro et al., 2007; Limor-Waisberg et al., 2012). Limor-Waisberg et al. (2012) reported that recombinant Arabidopsis thaliana QSOX1 (AtQSOX1) expressed in E. coli oxidized dithiothreitol but did not oxidize reduced ribonuclease (RNase) A. They concluded that AtQSOX1 does not show the same interdomain electron transfer activity as its animal counterpart (Limor-Waisberg et al., 2012).

The current study identified GmPDIL6 and GmPDIL7, novel members of the soybean PDI family, and soybean QSOX [Glycine max QSOX (GmQSOX)]. Chapter 1 describes the
identification of GmPDIL6 and GmPDIL7, novel soybean PDI family members, the cloning of cDNAs encoding these proteins, and their expression and cellular localization in soybean tissues. Chapter 2 describes the enzymatic properties of recombinant GmPDIL6 and GmPDIL7. Chapter 3 describes the cooperative oxidative folding by GmPDIL7 in the soybean ERO1 pathway. In Chapter 4, recombinant GmQSOX1 (rGmQSOX1) is described as having sulfhydryl oxidase activity, and rGmQSOX1 and the soybean PDI family proteins could refold the reduced and denatured RNase A without GSH redox buffer.
CHAPTER 1
Identification of GmPDIL6 and GmPDIL7, Novel Soybean Protein Disulfide Isomerase Family Proteins

Introduction
In higher plants including, Arabidopsis, soybean (Glycine max), wheat (Triticum aestivum), and rice (Oryza sativa), there are ten groups of proteins that belong to the PDI family (Houston et al., 2005; d’Aloisio et al., 2010; Selles et al., 2011). In Arabidopsis, a set of 22 orthologs of known the PDI family proteins were discovered by a genome-wide search and separated into phylogenetic groups I–X (Houston et al., 2005).

The group I PDI family proteins contain two thioredoxin domains, $a$ and $a'$, which have an active center motif (Cys-Gly-His-Cys) and two thioredoxin domains, $b$ and $b'$, which lack catalytically active cysteines. An Arabidopsis ortholog of the group I PDI family proteins is essential for seed development and regulates the timing of programmed cell death by chaperoning and inhibiting Cys proteases (Andème Ondzighi et al., 2008). A PDI in the coffee plant family (Rubiaceae), has been shown to be involved in the folding of knotted circular proteins (Gruber et al., 2007). PDIL1-1, the rice ortholog of group I PDI family proteins, is reportedly related to the maturation of the major seed storage protein glutelin and to the regulatory activities of various proteins involved in the synthesis of grain components (Takemoto et al., 2002; Satoh-Cruz et al., 2010). The oxidative refolding abilities of group I and II PDI family proteins were confirmed using recombinant soybean GmPDIL-1 and GmPDIL-2 and wheat proteins expressed in Escherichia coli (E. coli) (Kamauchi et al., 2008; Kimura et al., 2015). The group II PDI family proteins have an $a\text{--}b\text{--}b'\text{--}a'$ domain structure and an acidic amino acid-rich sequence in the N-terminal region adjacent to the $a$ domain. Group III also have an $a\text{--}b\text{--}b'\text{--}a'$ domain structure; however, the recombinant soybean PDI family protein of group III, GmPDIL-3 and wheat proteins lack oxidative refolding activities in vitro (Iwasaki et al., 2009; Kimura et al., 2015), most likely because they contain nonclassical
active-center CXXS/C motifs in the a and a’ domains. The group IV PDI family proteins, which have an a–a’–ERp29 domain structure, are unique to plants (Wadahama et al., 2007). Recombinant soybean PDI family proteins of group IV, GmPDIS-1 and GmPDIS-2, and wheat PDI family proteins of group IV have an oxidative refolding activity. However, the activity is weaker than the activity apparent of group I and II (Wadahama et al., 2007; Kimura et al., 2015). The group V PDI family proteins have an a–a’–b domain structure that is similar to mammalian P5. As well, a rice PDI family protein of group V has been shown to play an important role in the accumulation of Cys-rich 10-kD prolamin (crP10) (Onda et al., 2011). Further, recombinant soybean PDI family proteins of group V, GmPDIM, and wheat PDI family proteins of group V have an oxidative refolding activity that is similar to that of group IV (Wadahama et al., 2008; Kimura et al., 2015). The group VI, VII, and VIII PDI family proteins have a single catalytically active domain. Among them, group VII and VIII have a putative transmembrane region that could act as an ER anchor. The group VIII PDI family proteins are unique to plants and have an a-COPII domain structure with two predicted transmembrane regions at the N-terminal and C-terminal regions. The Arabidopsis PDI family proteins of group VIII PDI7, PDI12, and PDI13 have been identified as hybrid PDI-like and cargo receptor-like proteins (Yuen et al., 2015). The group VII PDI family proteins have an a-b-b’ domain structure with a C-terminal transmembrane region similar to mammalian TMX3 (Haugstetter et al., 2005; Haugstetter et al., 2007). However, only the oxidase activity of recombinant human TMX3 (HsTMX3) has been confirmed as the enzymatic characteristics of the plant group VII PDI family proteins have not yet been determined. The group VI PDI family proteins have an a domain structure similar to mammalian ERp18 (Alanen et al., 2003; Jeong et al., 2008). The enzymatic characteristics of the group VI plant PDI family proteins have not yet been determined.

In this chapter, GmPDIL6 and GmPDIL7, novel members of the soybean PDI family were identified, their encoding cDNAs were synthesized, and their expression and cellular localizations in soybean tissues were determined.
Materials and Methods

Plants and cell culture

Soybean (*G. max* L. Merrill cv. Jack) seeds were planted in 5-L pots and grown in a controlled environmental chamber at 25 °C under 16-h day/8-h night cycles. All samples collected, with the exception of the leaves used for the ER stress experiment, were immediately frozen and stored in liquid nitrogen until use. Soybean DG330 (rpc00051) cells (Asano and Otobe, 2011) were provided by RIKEN BioResource Center (Tsukuba, Japan), and cultured, propagated, and passaged in a 200-mL Erlenmeyer flask containing 80 mL of 0.25% gellan gum-solidified Murashige and Skoog medium at 25 °C under 5000 lx continuous light.

Cloning of GmPDIL6 and GmPDIL7 cDNA

The cloning of the cDNA for *GmPDIL6* and *GmPDIL7* was performed by RT-PCR. Soybean leaves were frozen in liquid nitrogen and then ground into a fine powder using an SK-100 micropestle (Tokken, Chiba, Japan). Total RNA was isolated using an SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) according to the manufacturer’s protocol and the cDNA was prepared from total RNA using a Prime Script™ RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan). The amplification of *GmPDIL6* cDNA was performed using PrimeSTAR® HS DNA Polymerase (TaKaRa Bio Inc.) and oligonucleotide primers, 5’-GGGTAGGGTAGATCAGGCAGGCTTCAGTTCAGCC-3’ and 5’-GAGCTAGTTGTGTAATCCAGATAATGACTGCACC-3’. The amplification of *GmPDIL7* cDNA was performed using PrimeSTAR® HS DNA Polymerase (TaKaRa Bio Inc.) and oligonucleotide primers, 5’-CCGATCTGATGATTATTGAGTAAAAAGG-3’ and 5’-CAGCTACTTTCTAGAATAATCCTGACCACGTTC-3’. The resulting cDNA fragment was subcloned into the pMD20-T vector using the Mighty TA-cloning Reagent Set for PrimeSTAR® (TaKaRa Bio Inc.). The sequencing of the inserts in the plasmid vectors was performed by Eurofins Genomics (Tokyo, Japan).
Quantitative real-time PCR analysis

Soybean roots, stems, and leaves were collected from plants 20 days after seeding. For the treatment of the leaves to induce ER stress, the leaves were divided into two halves and 5 mL of H$_2$O with 0.1% DMSO alone or with 20 µg·mL$^{-1}$ tunicamycin or 1 mM dithiothreitol (DTT) was administered to the inner surface of one-half of each of the leaves and incubated at 25 °C for 10 h. Total RNA was isolated from the plant tissues using the SV Total RNA Isolation System. The cDNA was prepared from the total RNA using the Prime Script™ RT reagent kit. The quantification of the mRNA was conducted using SYBR® Premix Ex Taq™ II (TaKaRa Bio Inc.) and a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.). The GmPDIL6 mRNA was measured using the forward primer, 5’-TGAGAACTCACACTCACACTTTACAC-3’ and the reverse primer 5’-CAAATCATCCCACAATGAACCAAAGA-3’. The GmPDIL7 mRNA was measured using the forward primer, 5’-GTTTTGGCTTTGGAGGCTG-3’ and the reverse primer 5’-GAGGCTATGCGGAATCGAA-3’. The GmBiP mRNA was measured using the forward primer 5’-TGGACTTTCTCTTCGACATG-3’ and the reverse primer 5’-CAATGACCGTCCCAACTTG-3’. The mRNA CT values were obtained from the second derivative maximum value and the expression levels were normalized using the CT values obtained for GmActin3. The GmActin3 mRNA was detected using the forward primer 5’-TGGAACAGGCAGGATTTGC-3’ and the reverse primer 5’-CCCATCCACAACCACC-3’.

Western blot analysis

Roots, leaves, and stems were collected from the plants 20 days after seeding. The seeds were also collected from the plants and the cotyledons were isolated. The tissues were frozen in liquid nitrogen and then ground into a fine powder using an SK-100 micropestle. The proteins were extracted by boiling for 5 min in SDS-PAGE buffer (Laemmli, 1970) containing a 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). To cleave the N-glycans, the proteins were extracted from the cotyledons in 0.1% SDS / 50 mM phosphate buffer (pH 5.5) and the
concentration of the proteins in the sample was measured using a reducing agent and detergent compatible (*RC DCTM*) protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The proteins (0.4 mg) were then treated with 10 mU PNGase F (Sigma-Aldrich) or endoglycosidase H (Sigma-Aldrich) at 37 °C for 16 h. To separate the supernatant and the membrane fractions, the frozen cotyledons from three 100 mg seeds were crushed in 200 mM Tris (hydroxymethyl) aminomethane (Tris)-HCl (pH 7.8) using a Dounce homogenizer (Wheaton, Millville, NJ, USA) and disrupted by sonication on ice. The homogenate was then placed into a cell strainer (BD Biosciences, San Jose, CA, USA) and centrifuged at 824 g for 40 min at 4 °C. The filtered suspension was divided two fractions, diluted with 200 mM Tris-HCl (pH 7.8) with or without 1% Triton X100, and centrifuged at 100,000 g for 1 h at 4 °C. The proteins were subjected to SDS-PAGE (Laemmli, 1970) and blotted onto a polyvinylidene difluoride membrane. The blots were then immunostained with antiserum specific to the proteins detected as the primary antibody followed by horseradish peroxidase-conjugated IgG antiserum (Promega, Fitchburg, WI, USA) as the secondary antibody. Anti-GmPDIL6 and anti-GmPDIL7 guinea pig serum were prepared by Japan Bio Serum (Hiroshima, Japan) using recombinant GmPDIL6 and GmPDIL7 (Chapter 2, Materials and Methods). Anti-GmPDIL-1 and anti-soybean calnexin (GmCNX) serum were prepared as previously described (Kamauchi *et al.*, 2005). The blots were developed using the Western Lightning® Chemiluminescence Reagent (Perkin Elmer Life Sciences, Waltham, MA, USA).

**Confocal microscopy**

Cultured soybean DG330 (rpc00051) cells were fixed in 4% formaldehyde for 1 h at room temperature. The fixed tissues were then washed twice for 1 min each in PBS and clarified using ClearSee optical clearing reagent at room temperature for 3 days (Kurihara *et al.*, 2015). The ClearSee-treated cells were dehydrated in a series of ethanol dilutions, embedded in Technovit® 7100 resin (Heraeus Kulzer, Wehrheim, Germany), and sliced into sections using a rotary RP-50 microtome (Yamato, Saitama, Japan). The sections were then stained with anti-
GmPDIS-1 rabbit serum (Wadahama et al., 2007), followed by staining with Cy5-conjugated anti-(rabbit IgG) goat serum (Rockland Immunochemicals Inc., Limerick, PA, USA). For the detection of GmPDIL7 or GmBiP (Wadahama et al., 2007), the specimens were stained with guinea pig antiserum against recombinant GmPDIL7 or guinea pig antiserum against recombinant GmBiP, followed by staining with Cy3-conjugated anti-guinea pig IgG donkey serum (Chemicon International, Temecula, CA, USA). The specimens were examined on an FV1000D confocal laser scanning imaging system (Olympus, Tokyo, Japan).
Results and Discussion

cDNA cloning of GmPDIL6

To clone *GmPDIL6* cDNA, the novel member of the soybean PDI family, I performed a BLAST search with the *TaPDIL6-1a* cDNA nucleotide sequence from the National Center for Biotechnology Information and identified a predicted mRNA sequence (NM_001252899). I then designed primer sets based on NM_001252899 and generated a cDNA derived from young soybean leaves by RT-PCR. The cDNA (LC205738) encoded GmPDIL6, which is a protein composed of 147 amino acids (Fig. 1-1A, B). A BLAST genome search of *G. max* in the Phytozome database identified the nucleotide sequences for *GmPDIL6*, which is located on chromosome 13, and is composed of five exons and four introns (Fig. 1-1C). GmPDIL6 encoded by the cDNA possesses a putative N-terminal signal sequence, a thioredoxin domain with an active center motif (CKHC) (Fig. 1-1A). The domain structure of GmPDIL6 is similar to that of HsERp18 (Alanen *et al*., 2003; Jeong *et al*., 2008). However, the identity between the amino acid sequences of GmPDIL6 and HsERp18 is only 17%. 


Fig. 1-1. Primary structure of GmPDIL6. (A) The boxes indicate the a thioredoxin domain boundaries predicted by an NCBI conserved domain search. SP, signal peptide; CKHC, putative active site. The numbers indicate amino acid residues. (B) Amino acid sequence of GmPDIL6. The SP is underlined by a solid line. The gray highlighting represents the a domain region. (C) The intron-exon structure of GmPDIL6. White boxes and black bars indicate exons and introns, respectively.
Expression of GmPDIL6 in soybean tissue

The levels of GmPDIL6 mRNA in the roots, stems, leaves and cotyledon were measured (Fig. 1-2A). GmPDIL6 mRNA was ubiquitously expressed in all of the tissues but the mRNA levels were the highest in the cotyledon. The 14 kDa band corresponding to GmPDIL6 was detected only in the cotyledon by western blot analysis with anti-GmPDIL6 serum, which revealed that the expression of GmPDIL6 was higher in the cotyledon than in the other tissues (Fig. 1-2B). The GmPDIL6 mRNA levels in the soybean cotyledon were measured during seed development (Fig. 1-2C). The mRNA level was higher in beans weighing 70–90 and 160–220 mg, corresponding to the periods during which the seed storage proteins, such as β-conglycinin and glycinin, are synthesized the most (Dixon et al., 2008). Those results suggest that GmPDIL6 may play important roles in the folding of storage proteins. As well, GmPDIL6 protein was expressed in the latter half of the cotyledon during seed development (Fig. 1-2D).

Many ER-resident proteins that assist in the folding of nascent polypeptides are upregulated by the unfolded protein response in plants, which is triggered by the accumulation of unfolded proteins in the ER (i.e., ER stress) (Martinez and Chrispeels, 2003; Kamauchi et al., 2005; Urade, 2007; Howell et al., 2013). To examine the effect of ER stress on the expression of GmPDIL6, leaves were divided into two halves and each was treated or not treated with tunicamycin and DTT, which induce ER stress (Martinez and Chrispeels, 2003; Kamauchi et al., 2005). mRNA levels were then measured. The expression of GmPDIL6 mRNA increased strikingly following treatment with tunicamycin and DTT. Furthermore, the level of increase was higher than that of GmBiP, a known unfolded protein response gene (Fig. 1-2E). As well, one of the unfolded protein response elements (Oh et al., 2003; Iwata and Koizumi, 2005; Iwata et al., 2008; Hayashi et al., 2013; Sun et al., 2013), UPRE-III(CGATGA) sequences were found in the 1236-bp and 1425-bp downstream sequences of the GmPDIL6 open reading frame.
Fig. 1-2. Expression of *GmPDIL6* mRNA and *GmPDIL6*. (A) The amounts of *GmPDIL6* mRNA in the root, stem, leaf and cotyledon were quantified by qRT-PCR. Each value was standardized using *actin 3* mRNA and indicates a relative transcription level (gene/actin 3 ratio). (B) Expression of *GmPDIL6* in the root, stem, leaf and cotyledon. Proteins (20 µg) extracted from each tissue were separated by SDS-PAGE and evaluated by immunostaining with anti-*GmPDIL6* serum. The arrowhead indicates the *GmPDIL6* band. (C) The amounts of *GmPDIL6* mRNA in the cotyledons during maturation were quantified by qRT-PCR. Each value indicates a relative transcription level (gene/actin 3 ratio). (D) Expression of the *GmPDIL6* protein in the cotyledons during maturation. The cotyledon proteins (20 µg) were separated by SDS-PAGE and evaluated by immunostaining with anti-*GmPDIL6* serum. (E) Expression of *GmPDIL6* and *GmBiP* mRNA under ER stress induced by tunicamycin (TM) or dithiothreitol (DTT) in the leaf. Each value was standardized using *actin 3* mRNA. The fold-change in expression was calculated as the ratio of the amount of mRNA in the samples treated with TM (+) or DTT (+) to that in the sample treated without the ER stress trigger (−). The values represent the mean ± SD of three experiments.
cDNA cloning of *GmPDIL7*

To clone *GmPDIL7* cDNA, the novel member of the soybean PDI family, I performed a BLAST search with the *TaPDIL7-1a* cDNA nucleotide sequence from the National Center for Biotechnology Information and identified a predicted mRNA sequence (XM003536342). I then designed primer sets based on XM003536342 and generated a cDNA derived from young soybean leaves by RT-PCR. The cDNA (LC158001) encoded GmPDIL7, which is a protein composed of 433 amino acids (Fig. 1-3A,B). A BLAST genome search of *G. max* in the Phytozome database identified the nucleotide sequences for *GmPDIL7*, which is located on chromosome 10 and is composed of five exons and four introns (Fig. 1-3C). GmPDIL7 possesses a putative N-terminal signal sequence, a thioredoxin domain with an active center motif (CGHC), two thioredoxin domains lacking catalytically active cysteines, and a putative C-terminal transmembrane region (Fig. 1-3A). The domain structure of GmPDIL7 is similar to that of HsTMX3 (Haugstetter et al., 2007). However, the identity between the amino acid sequences of GmPDIL7 and HsTMX3 is only 21%.
Fig. 1-3. Primary structure of GmPDIL7. (A) The boxes indicate the a, b, and b’ thioredoxin domain boundaries predicted by an NCBI conserved domain search. SP, signal peptide; TM, transmembrane region; CGHC, putative active site. The numbers indicate amino acid residues.
(B) Amino acid sequence of GmPDIL7. The SP is underlined by a solid line and the TM is underlined by a dotted line. The square box indicates an N-glycan consensus sequence. The gray, yellow, and light green highlighting represent the a, b, and b’ domain regions, respectively.
(C) The intron-exon structure of GmPDIL7. White boxes and black bars indicate exons and introns, respectively.
Expression of GmPDIL7 in soybean tissue and cellular localization

The levels of *GmPDIL7* mRNA in the roots, stems, and leaves were measured (Fig. 1-4A). *GmPDIL7* mRNA was ubiquitously expressed in all of the tissues but the mRNA levels were the highest in the roots. I used plants 20 days after seeding. During this time of relatively rapid growth, GmPDIL7 might play an important role in root growth and protein folding in the roots. The 47 kDa band corresponding to GmPDIL7 was detected in the roots, stems, and leaves by western blotting with anti-GmPDIL7 serum, which revealed that the concentration of GmPDIL7 was higher in the roots than in the other tissues (Fig. 1-4B). The *GmPDIL7* mRNA levels in the soybean cotyledon were measured during seed development (Fig. 1-4C), which revealed that the mRNA level increased from the initial period of seed filling (bean weight of 20-50 mg). The *GmPDIL7* mRNA levels were also high in beans weighing 140–250 mg, which corresponded to the periods during which the synthesis of seed storage proteins, such as glycinin, is the highest (Wadahama *et al*., 2007). As well, the GmPDIL7 protein was continuously expressed in the cotyledon during seed development (Fig. 1-4D).

To examine the effect of ER stress on the expression of GmPDIL7, leaves were divided into two halves and each was treated or not treated with tunicamycin and DTT, which induce ER stress (Kamauchi *et al*., 2005; Martínez and Chrispeels, 2003). mRNA levels were then measured. Leaves were used instead of roots for this purpose because they can be easily divided and the physiologic conditions in each half should be the same. It is difficult, however, to divide roots in this manner. The expression of *GmPDIL7* mRNA increased slightly following treatment with tunicamycin and DTT. However, the level of increase was very small compared to that of *GmBiP*, a known unfolded protein response gene (Fig. 1-4E). As well, the unfolded protein response elements (Oh *et al*., 2003; Iwata and Koizumi, 2005; Iwata *et al*., 2008; Hayashi *et al*., 2013; Sun *et al*., 2013) were not found within the 2500-bp upstream and downstream sequences of the *GmPDIL7* open reading frame.
Fig. 1-4. Expression of GmPDIL7 mRNA and GmPDIL7. (A) The amounts of GmPDIL7 mRNA in the root, stem, and leaf were quantified by qRT-PCR. Each value was standardized using actin 3 mRNA and indicates a relative transcription level (gene / actin 3 ratio). (B) Expression of GmPDIL7 in the root, stem, and leaf. Proteins (20 μg) extracted from each tissue were separated by SDS-PAGE and evaluated by immunostaining with anti-GmPDIL7 serum. The arrowhead indicates the GmPDIL7 band. (C) The amounts of GmPDIL7 mRNA in the cotyledons during maturation were quantified by qRT-PCR. Each value indicates a relative transcription level (gene / actin 3 ratio). (D) Expression of the GmPDIL7 protein in the cotyledons during maturation. The cotyledon proteins (20 μg) were separated by SDS-PAGE and evaluated by immunostaining with anti-GmPDIL7 serum. (E) Expression of GmPDIL7 and GmBiP mRNA under ER stress induced by tunicamycin (TM) or dithiothreitol (DTT) in the leaf. Each value was standardized using actin 3 mRNA. The fold-change in expression was calculated as the ratio of the amount of mRNA in the samples treated with TM (+) or DTT (+) to that in the sample treated without the ER stress trigger (−). The values represent the mean ± SD of three experiments.
GmPDIL7 has a putative transmembrane region (Ile376–Phe395) near the C terminus and one putative N-glycosylated asparagine residue. Hence, it was confirmed that GmPDIL7 is a membrane-bound glycoprotein targeted to the ER. When the extract from the cotyledon was treated with endoglycosidase H or PNGase F, the size of the GmPDIL7 protein did not change (Fig. 1-5A), suggesting that no high-mannose-type N-glycan was attached to GmPDIL7. GmPDIL7 was recovered in the precipitate from the cell homogenate following sonication, but not in the supernatant after ultracentrifugation, and pretreatment with Triton X-100 caused the solubilization of GmPDIL7 in the supernatant along with soybean calnexin (Fig. 1-5B). In the confocal microscopy images, the colocalization of GmPDIL7 with GmPDIS-1, which colocalized with the ER protein GmBiP, was observed upon immunostaining (Fig. 1-5C,D). These results revealed that GmPDIL7 was a membrane-bound protein targeted to the ER. The presence of a putative N-terminal signal sequence suggests that GmPDIL7 is a type I membrane-bound protein.
**Fig. 1-5.** GmPDIL7 is a membrane-bound protein located in the ER. (A) GmPDIL7 is not N-glycosylated. GmPDIL7 was detected in the immature cotyledon (30 mg) by western blotting with anti-GmPDIL7 (L7) or anti-GmERO1 (ERO1, a glycosylated protein control) serum after treatment with (+) or without (−) endoglycosidase H (Endo H) or PNGase F. The GmPDIL7 band was not shifted after glycosidase treatment. (B) An immature cotyledon (100 mg) was homogenized using a Dounce homogenizer and by sonication. The homogenate was centrifuged at 100,000 g for 2 h at 4 °C in the absence (−) or presence (+) of 1% Triton X-100. GmPDIL7 in the supernatant (sup) and pellet (ppt) was detected by western blotting with anti-GmPDIL7 serum (L7), anti-soybean calnexin (CNX, a marker for ER membrane-bound protein) serum, or anti-GmPDIL-1 (L-1, a marker for ER lumen protein). (C, D) GmPDIL7 localizes in the ER. Cultured soybean DG330 (rpc00051) cells were fixed and embedded in resin. The sections were cut with a microtome and evaluated by immunostaining with antiGmPDIL7 (L7), or anti-GmBiP guinea pig serum (BiP) and anti-GmPDIS-1 rabbit serum (S-1), and observed under a confocal microscope. DIC, Nomarski image.
CHAPTER 2
Enzymatic Properties of GmPDIL6 and GmPDIL7

Introduction
In Chapter 1, I have described the identification of GmPDIL6 and GmPDIL7, novel members of the soybean PDI family, cloned cDNAs encoding these proteins, and shown expression and cellular localization of them in soybean tissues. The expression of *GmPDIL6* mRNA was increased by ER stress and GmPDIL7 was an ER membrane-bound protein, suggesting that GmPDIL6 and GmPDIL7 may have played important roles in the folding of storage proteins. The roles of GmPDIL6 and GmPDIL7 in the enzymatic oxidative protein folding processes were then determined.

Oxidative folding occurs *via* two steps, the first is the introduction of transient and non-native disulfide bonds, and the second includes isomerization into native disulfide bonds (Kojima *et al.*, 2014) (Fig. 2-1). In the first step, the oxidized PDI family proteins, in which active cysteines are oxidized, form non-native disulfide bonds within their substrates, and then the reduced PDI family proteins rearrange into native disulfide bonds. The first step of oxidative folding is catalyzed by the oxidation activity of the PDI family proteins, and the second step is catalyzed by reductase and oxidation activities.

In this chapter, I have described the dithiol oxidase activities, the reductase activities, and the oxidative refolding activities of recombinant GmPDIL6 and GmPDIL7.
**Materials and Methods**

**Preparation of recombinant GmPDIL6 and GmPDIL7**

Expression plasmids encoding glutathione-S-transferase (GST)-fused GmPDIL6 (His25–Leu147) lacking the signal peptide predicted by SOSUI (Hirokawa *et al.*, 1998). The DNA fragment was amplified from GmPDIL6 cDNA by PCR using the oligonucleotide primers 5’-ATTTCGGGAATTCCATTCCGGTTATAACGTAAACC-3’ and 5’-TGTAATCGTCCGACATGACTGCACCTCAAAAGTTG-3’. Expression plasmids encoding GST-fused GmPDIL7 (Glu25–Asp375) lacking the signal peptide predicted by SOSUI, the putative transmembrane region predicted by TMHMM (Krogh *et al.*, 2001) and the subsequent C-terminal flanking region were constructed.

The DNA fragment was amplified from *GmPDIL7* cDNA by PCR using the oligonucleotide primers 5’-GGCTTAGGAATTCCCGAGACATTCTCGGTGGATG-3’ and 5’-TGTAACGTCGACTTAGTCAAAGGATCGATGGATGAA-3’. The resulting DNA fragments were then digested with *Eco*R1 and *Sal*I (TaKaRa Bio Inc.) and subcloned into the pGEX6p-2 vector (GE Healthcare, Little Chalfont, Buckinghamshire, UK), which had been cleaved with *Eco*R1 and *Sal*I. The recombinant proteins had GST linked to the N terminus. The GST fusion vectors were transformed into *E. coli* BL21(DE3) cells (Takara Bio Inc.) as described above. The expression of recombinant GST-GmPDIL6 and GST-GmPDIL7 were induced in lysogeny broth (LB) containing 0.5 mM isopropyl-β-D-thiogalactoside and 100 μg·mL⁻¹ ampicillin at 15 °C for 120 h. The cells in 1 L of culture broth were collected by centrifugation at 6,500 g for 20 min at 4 °C, disrupted by sonication in 50 mL of 20 mM sodium phosphate-buffered saline (pH 7.4), and then centrifuged at 26,000 g for 20 min at 4 °C. The supernatant was applied to a column packed with a Glutathione Sepharose™ 4B and digested by PreScission Protease. The eluted recombinant GmPDIL6 and GmPDIL7 were then purified by gel filtration chromatography on a TSKgel® G3000SW column (Tosoh, Tokyo, Japan) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 10% glycerol.
The concentration of the purified recombinant GmPDIL6 and GmPDIL7 were determined from its absorbance at 280 nm using a molar extinction coefficient of 21,220 M$^{-1}$·cm$^{-1}$ and 35,995 M$^{-1}$·cm$^{-1}$ respectively, which were calculated by the modified method of Gill and von Hippel (Pace et al., 1995). Typically, 2 mg of recombinant GmPDIL6 and 10 mg of recombinant GmPDIL7 were obtained from 1 L of LB broth respectively. The purity of the recombinant GmPDIL6 and GmPDIL7 were confirmed by SDS-PAGE (Laemmli, 1970).

**Measurement of the PDI family protein redox equilibrium constant**

Recombinant soybean PDI family proteins (1 μM) was incubated with 0.1 mM oxidized glutathione (GSSG) and 0.015–28 mM GSH at 25 °C for 1 h in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.15 M NaCl. After incubation under N$_2$ at 25 °C for 1 h, further thiol-disulfide exchange was prevented by the addition of 10% trichloroacetic acid. The aggregated proteins were precipitated by centrifugation, washed with 100% acetone, and the protein pellet was solubilized and incubated in 0.1 M sodium phosphate buffer (pH 7.0) containing 2% SDS and 3 mM methoxypolyethylene glycol maleimide (Fluka/Sigma-Aldrich) at 25 °C for 30 min. The proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The values for the reduced fraction and oxidized fraction were quantified using IMAGE J software (National Institutes of Health, Bethesda, MD, USA) and the values for the completely oxidized or reduced states were regarded as 0 or 100, respectively. The $K_{eq}$ was calculated by fitting the recalibrated fraction of the apparently reduced form to the following equation: 

$$R = \frac{([\text{GSH}]^2 / [\text{GSSG}]) / \{K_{eq} + ([\text{GSH}]^2 / [\text{GSSG}])\}}$$

in which R is the relative ratio of the reduced forms. The equilibrium redox potential of the proteins was calculated using the Nernst equation 

$$E' = E'_0 (\text{GSH/GSSG}) - (RT/nF) \ln K_{eq}$$

using the GSH standard potential $E'_0$ (GSH/GSSG) of −0.240 V at pH 7.0 and 25 °C.
Dithiol oxidase activity assay

The dithiol oxidase activity was measured using a synthetic peptide, NH₂-NRCSQGSCWN-COOH (Operon Biotechnologies, Tokyo, Japan), as previously described (Ruddock et al., 1996; Alanen et al., 2006). Briefly, the recombinant proteins (0.56 μM) were incubated in McIlvaine buffer (0.2 M disodium phosphate/0.1 M citrate buffer, pH 6.0), 2 mM reduced GSH, 0.5 mM oxidized GSH, and 5 μM synthetic peptide at 25 °C, and fluorescence was monitored on an FP-750 fluorescence spectrophotometer (JASCO Corporation, Tokyo, Japan) at 350 nm with excitation at 280 nm. The half-time was determined by calculating the midpoint fluorescence as the mean of the initial and final fluorescence intensities. A measure of the rate of the reaction was determined from the inverse of the half-time of the reaction.

Disulfide reductase activity assay

Disulfide reductase activity was assessed by measuring the glutathione-dependent reduction of insulin according to the method as described previously (Morjana and Gilbert, 1991). Briefly, recombinant proteins were incubated at 25°C in 1 mL of 0.2 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 3.7 mM glutathione, 120 μM NADPH, 1 U of glutathione reductase (Sigma-Aldrich Inc.) and 30 μM bovine insulin (Sigma-Aldrich), and absorbance was monitored at 340 nm.

Oxidative refolding assay with the reduced and denatured RNase A

The recombinant soybean PDI family proteins (GmPDIL-1, GmPDIL-2, GmPDIM, GmPDIS-1, and GmPDIS-2) were prepared as previously described (Wadahama et al., 2007, 2008; Kamauchi et al., 2008). The reduced and denatured RNase A was likewise prepared as previously described (Creighton, 1977). The reduced and denatured RNase A (10 μM) was incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA with the recombinant soybean PDI family protein (3 μM) in the presence of GSH redox buffer (2 mM GSH and 0.5 mM GSSG) at 25 °C. An aliquot (20 μL) of the reaction mixture was removed and added to 5
μL of 10 mM N-ethylmaleimide solution, and then incubated at 4 °C for 10 min. The aliquots were diluted with 180 μL of Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.1 mg·mL⁻¹ cCMP, incubated at 25 °C, and the absorbance of the mixture was subsequently monitored at 284 nm. Using GmERO1a as a disulfide donor, each reaction mixture containing 100 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES; pH 7.5), 150 mM NaCl, 2 mM CaCl₂, 2 mM cCMP, reduced RNase A (8 μM), recombinant PDI family proteins (3 μM), and recombinant GmERO1a (1 μM) were incubated at 25 °C. The formation of active RNase A was measured by spectrophotometry over time at 284 nm. The recombinant GmERO1a was prepared as previously described (Matsusaki et al., 2016).
Results and Discussion

Preparation of recombinant GmPDIL6 and GmPDIL7

To determine the enzymatic properties of GmPDIL6 and GmPDIL7, I expressed recombinant GST-fused GmPDIL6 (His25–Leu147) lacking the putative N-terminal signal sequence (Fig. 2-2A) and GST-fused GmPDIL7 (Glu25–Asp375) lacking the putative N-terminal signal sequence and the putative C-terminal transmembrane region (Fig. 2-2C). The recombinant GST-fused GmPDIL6 and GST-fused GmPDIL7 were expressed as soluble proteins in E. coli (Fig. 2-2B, lane 1 and Fig. 2-2D, lane 1), isolated by GST-tag affinity chromatography, and digested using PreScission Protease (Fig. 2-2B, lane 2 and Fig. 2-2D, lane 2). The eluted GmPDIL6 and GmPDIL7 were purified by gel filtration column chromatography (Fig. 2-2B, lane 3 and Fig. 2-2D, lane 3). It was revealed that the recombinant GmPDIL7 was eluted from the gel filtration column in a monomeric position (data not shown). On the other hand, the recombinant GmPDIL6 was eluted from the gel filtration column in a monomeric position, and dimeric position (data not shown). Monomeric GmPDIL6 was collected and used for enzymatic assays.
Fig. 2-2. GmPDIL7 has oxidative refolding activity. (A) Schematic representation of the recombinant GmPDIL6. (B) Expression and purification of recombinant GmPDIL6 in *E. coli*. Recombinant GST-GmPDIL6 (GST-L6) in *E. coli* (lane 1) was adsorbed onto a glutathione resin column and the GmPDIL6 fragment (L6) was eluted from the resin by cleavage with PreScission™ Protease (lane 2), followed by gel filtration chromatography (lane 3). Proteins in each eluate were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Purified recombinant GmPDIL6 (100 ng) (lane 4) was analyzed by western blotting with anti-GmPDIL6 serum. (C) Schematic representation of the recombinant C-terminal-truncated GmPDIL7. (D) Expression and purification of recombinant GmPDIL7 in *E. coli*. Recombinant GST-GmPDIL7 (GST-L7) in *E. coli* (lane 1) was adsorbed onto a glutathione resin column and the GmPDIL7 fragment (L7) was eluted from the resin by cleavage with PreScission™ Protease (lane 2), followed by gel filtration chromatography (lane 3). Proteins in each eluate were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Purified recombinant GmPDIL7 (100 ng) (lane 4) was analyzed by western blotting with anti-GmPDIL7 serum.
Enzymatic properties of recombinant GmPDIL6 and GmPDIL7

The direction of a redox reaction between functional groups depends on their redox potentials, that is to say, a functional group with higher redox potential oxidizes a functional group with lower redox potential. To examine whether GmPDIL6 and GmPDIL7 could oxidize unfolded proteins, the redox potential of the GmPDIL6 and GmPDIL7 active center were determined. Recombinant GmPDIL6, GmPDIL7 and other soybean PDI family proteins were incubated in GSH buffer that contained various ratios of GSH/GSSG and the cysteine residue-free thiols were modified by methoxypolyethylene glycol maleimide (mPEG 5000mal). The reduced and oxidized forms of soybean PDI family proteins were then separated by SDS-PAGE (Fig. 2-3A, B, upper panels), which revealed that the redox state of GmPDIL6 and GmPDIL7 changed according to the GSH/GSSG ratio. The calculated GmPDIL6 and GmPDIL7 redox equilibrium constant were $K_{eq} = 7.4$ mM and $K_{eq} = 17$ mM, respectively (Fig. 2-3A, B, lower panel). The redox potential values of GmPDIL6 and GmPDIL7 calculated using the Nernst equation were $-175$ mV and $-187$ mV, respectively. It is known that the total GSH concentration in the ER is approximately 9-10 mM and that the ratio of [GSH]/[GSSG] in the ER is approximately 3-5 : 1 (Dixon et al., 2008; Hatahet and Ruddock, 2009). From these values, the reduction potential for GSH in the ER was estimated at $-191$ mV, indicating that GmPDIL6 and GmPDIL7 have a higher redox potential than unfolded proteins with a redox potential between $-220$ and $-200$ mV (Hatahet and Ruddock, 2009). These suggest that GmPDIL6 and GmPDIL7 will act as a protein dithiol oxidant toward an unfolded protein. In addition, if oxidized GmPDIL6 and GmPDIL7 are continually provided as a result of efficient oxidation by GmERO1 or other redox equivalents, GmPDIL6 and GmPDIL7 would oxidize substrates with higher oxidizing redox potentials to an even greater extent.
**Fig. 2-3. The redox potential of the GmPDIL6 and GmPDIL7 active center.** (A) Recombinant GmPDIL6 redox equilibrium constant assay. Coomassie Brilliant Blue staining of the SDS-PAGE is shown above the redox graphs. Data are represented as the mean of triplicate experiments. (B) Recombinant GmPDIL7 redox equilibrium constant assay. Coomassie Brilliant Blue staining of the SDS-PAGE is shown above the redox graphs. Data are represented as the mean of triplicate experiments.
As expected from the redox potential value, the recombinant GmPDIL6 and GmPDIL7 possessed the dithiol oxidation activities of a synthetic decapeptide containing two cysteine residues (Fig. 2-4A) and their thiol oxidation activities were similar to other proteins in the soybean PDI family. Next, disulfide reductase activities of recombinant GmPDIL6 and GmPDIL7 were assessed by measuring the glutathione-dependent reduction of insulin (Fig. 2-4B), which revealed that GmPDIL6 had no disulfide reductase activity and GmPDIL7 had low activity. And then, the abilities of recombinant GmPDIL6 and GmPDIL7 to refold reduced and denatured RNaseA were measured in the presence of GSH redox buffer. GmPDIL6 had hardly oxidative refolding activity, because GmPDIL6 had no disulfide reductase activity. Generally, oxidative refolding of unfolded protein is accompanied by formation of disulfide bonds and rearrangement to native ones through reduction and oxidation. Since GmPDIL6 has no disulfide reductase activity, GmPDIL6 may cannot rearrange disulfide bond. The oxidative refolding activity of GmPDIL7 was 31 mmol RNaseA·mmol⁻¹PDI·min⁻¹ (Fig. 2-4C), which was the lower activity observed in the PDI family proteins evaluated. The low folding activity observed was likely due to the fact that GmPDIL7 has only one active center because the active center of GmPDIL-2 was mutated to an inactive form and the refolding activity was decreased to approximately half (Matsusaki et al., 2016).
Fig. 2-4. Enzymatic properties of GmPDIL6 and GmPDIL7. (A) Dithiol oxidase activity of recombinant soybean PDI family proteins. Dithiol oxidase activity of recombinant GmPDIL-1 (L-1), GmPDIL-2 (L-2), GmPDIM (M), GmPDIS-1 (S-1), GmPDIS-2 (S-2), GmPDIL6 (L6) or GmPDIL7 (L7) was assayed using a synthetic decapeptide containing two cysteine residues. (B) Disulfide reductase activity of recombinant soybean PDI family proteins. Reductase activity of recombinant L-1, L-2, M, S-1, S-2, L6 or L7 was assayed using insulin. (C) Oxidative refolding of denatured and reduced RNase A in GSH redox buffer was assayed in the presence of recombinant L7, L-1, L-2, M, S-1, or S-2. Data are represented as mean ± SEM from at least three replicates.
CHAPTER 3
Cooperative Oxidative Folding by GmPDIL7 and Other Soybean PDI Family Proteins in the ERO1 pathway

Introduction
Disulfide bond formation in a nascent polypeptide by the PDI family proteins is accompanied by the reduction of an active center to dithiol. Because the active centers of the PDI family proteins cannot become oxidized, alone, the PDI family proteins require other oxidizing molecules. In yeast and mammalian cells, ER oxidoreductin 1 (Ero1) directly transfers disulfide bonds to PDI (Frand and Kaiser, 1998; Pollard et al., 1998; Cabibbo et al., 2000; Dias-Gunasekara et al., 2005; Araki and Inaba, 2012). Ero1 orthologs are present universally in eukaryotes, and plant orthologs of Ero1p, rice Ero1 (OsERO1), and soybean Ero1 (GmERO1a) have been identified (Onda et al., 2009; Matsusaki et al., 2016). OsERO1 is necessary for disulfide bond formation in rice endosperm, whereas recombinant GmERO1a reportedly has broad substrate specificity and oxidizes GmPDIL-1, GmPDIM, GmPDIS-1, and GmPDIS-2, but not GmPDIL-2.

In Chapter 3, the cooperative oxidative folding by GmPDIL7 in the soybean ERO1 pathway was described. I have determined whether recombinant GmPDIL6 and GmPDIL7 were oxidized by recombinant GmERO1a. The associations of GmPDIL6 and GmPDIL7 with other PDI family proteins in the soybean cotyledon ER were identified, and the effects of the coexistence of GmPDIL7 and other PDI family proteins on the oxidative refolding of denatured RNase A were also determined.
Materials and Methods

Oxidative refolding assay with the reduced and denatured RNase A

The recombinant soybean ERO1 (GmERO1a) were prepared as previously described (Matsusaki et al., 2016). The reduced and denatured RNase A was likewise prepared as previously described (Creighton, 1977). The reduced and denatured RNase A (10 µM) was incubated in 50 mM Tris-Cl buffer (pH 7.4) containing 1 mM EDTA with the recombinant soybean PDI family protein (3 µM) in the presence of GSH redox buffer (2 mM GSH and 0.5 mM GSSG) at 25 ºC. An aliquot (20 µL) of the reaction mixture was removed and added to 5 µL of 10 mM N-ethylmaleimide solution, and then incubated at 4 ºC for 10 min. The aliquots were diluted with 180 µL of Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.1 mg·mL⁻¹ cCMP, incubated at 25 ºC, and the absorbance of the mixture was subsequently monitored at 284 nm. Using GmERO1a as a disulfide donor, each reaction mixture containing 100 mM HEPES; pH 7.5, 150 mM NaCl, 2 mM CaCl₂, 2 mM cCMP, reduced RNase A (8 µM), recombinant PDI family proteins (3 µM), and recombinant GmERO1a (1 µM) were incubated at 25 ºC. The formation of active RNase A was measured by spectrophotometry over time at 284 nm.

Oxidation of recombinant PDI family proteins by GmERO1a assay

Oxygen consumption was measured using a Clark-type oxygen electrode system (OXYT-1; Hansatech Instruments, King’s Lynn, Norfolk, UK). All of the experiments were performed at 25 ºC in 100 mM HEPES (pH 7.5), 2 mM CaCl₂, and 0.15 M NaCl. Catalytic oxygen consumption was initiated by the addition of GmERO1a in a reaction mixture containing 3 µM recombinant soybean PDI family proteins and 10 mM GSH. The reaction was started by the addition of GmERO1a (1 µM) and the activity was measured using a coupled assay following the decrease in A340 nm due to the consumption of NADPH by GSH reductase (Sigma-Aldrich) (Nguyen et al., 2011; Sato et al., 2013; Matsusaki et al., 2016). A molar extinction
coefficient of 6200 M$^{-1}$-cm$^{-1}$ for NADPH was used for the calculations. All of the experiments were performed in 100 mM HEPES (pH 7.5) containing 0.15 M NaCl, 2 mM CaCl$_2$, 120 μM NADPH, 1 U·mL$^{-1}$ GSH-disulfide reductase, 3 μM recombinant PDI family protein, and 3 mM GSH.

**Immunoprecipitation experiments**

The cotyledons (100 mg) were homogenized using a Dounce homogenizer at 4 °C in 20 mM HEPES buffer (pH 7.2) containing 0.15 M NaCl, 1% digitonin, and 1% protease inhibitor cocktail. The homogenate was placed on ice for 1 h then into a cell strainer and centrifuged at 824 g for 40 min at 4 °C. The filtered suspension was then centrifuged for 30 min at 10,000 g at 4 °C. Immunoprecipitation was performed at 4 °C overnight with preimmune serum or anti-GmPDIL7 serum or other anti-soybean PDI family protein serum (anti-PDIM, anti-PDIS-1, and anti-PDIS-2). The immunoprecipitate was collected with protein A conjugated Sepharose beads (Sigma-Aldrich), washed with 20 mM HEPES buffer (pH 7.2) containing 0.15 M NaCl, and subjected to SDS-PAGE. Western blot analysis using a specific antiserum against soybean PDI family proteins (anti-GmPDIL-1, anti-GmPDIL-2, anti-PDIM, anti-PDIS-1, and anti-PDIS-2) as primary antibodies and a Pure-IP™ Western Blot Detection Kit (Cell Biolabs, Inc., San Diego, CA, USA) was performed. For detection of GmPDIL7, the immunoprecipitate was subjected to two-dimensional gel electrophoresis. The samples were treated using a 2D clean-up kit (GE Healthcare) and subjected to isoelectric focusing (IEF) using a Protean IEF Cell (Bio-Rad Laboratories), using the 7-cm ReadyStrip™-immobilized pH gradient (IPG) strips. The IPG strips were then subjected to SDS-PAGE. GmPDIL7 was detected by western blot analysis with anti-GmPDIL7 serum.
Results and Discussion

Oxidation of GmPDIL6 and GmPDIL7 by GmERO1a

The active centers of PDI family proteins are reduced when the disulfide bonds in an unfolded protein are catalyzed. Hence, a disulfide donor such as Ero1 is needed for the continuous oxidation reaction catalyzed by PDI family proteins in vivo (Frand and Kaiser, 1998; Pollard et al., 1998). Previously, I found that soybean ERO1 (GmERO1a) had broad specificity for members of the soybean PDI family (Matsusaki et al., 2016). Consequently, I examined whether recombinant GmPDIL6 and GmPDIL7 were oxidized by recombinant GmERO1a. Because the oxidation of PDI family proteins by GmERO1a is accompanied by the consumption of O$_2$, the oxidation of GmPDIL6 and GmPDIL7 by GmERO1a were monitored by oxygen consumption (Fig. 3-1A). The reaction was performed in the presence of GSH, which served as a substrate for the GmPDIL6 and GmPDIL7 oxidized by GmERO1a, and the results indicated that GmPDIL7 was rapidly oxidized by GmERO1a but GmPDIL6 was not oxidized by GmERO1a (Fig. 3-1B). The oxidation of GmPDIL6 or GmPDIL7 by GmERO1a was also measured by a method that involved GSH disulfide reductase and NADPH (Fig. 3-1C), which likewise revealed that GmPDIL7 was oxidized by GmERO1a but GmPDIL6 was not oxidized by GmERO1a (Fig. 3-1D). Furthermore, the oxidation rate of GmPDIL7 was similar to the rates observed for GmPDIS-1 and GmPDIS-2. In contrast to plants, human Ero1α and Ero1β preferentially oxidize PDI but do not oxidize other PDI family proteins. In humans, there are other oxidation pathways such as the peroxiredoxin-4 pathway, which complicates the function of Ero1α and Ero1β by oxidizing other PDI family proteins such as ERp46 and P5 using the H$_2$O$_2$ generated during the oxidation of PDI by human Ero1α (Sato et al., 2013). To date, a peroxiredoxin-4 ortholog has not been found in plants. Plant Ero1 might be the primary disulfide bond donor for oxidative folding in the ER and could supply disulfide bonds to several PDI family proteins. The oxidative refolding of RNaseA by recombinant GmPDIL7 was determined in the presence of GmERO1a without GSH redox buffer (Fig. 3-1E,F), which
revealed that GmPDIL7 was able to refold the reduced and denatured RNase A in the presence of GmERO1a. However, its oxidative refolding activity was lower than that of GmPDIL-1, although GmPDIL7 was oxidized faster than GmPDIL-1 by GmERO1a, indicating that its isomerization activity was inferior to the activity of GmPDIL-1.

**Association of GmPDIL6 and GmPDIL7 and other soybean PDI family proteins**

Previously, it was found that GmPDIS-1, GmPDIM, and GmPDIL-2 associate with nascent proglycinin in the cotyledon cell ER (Wadahama et al., 2007, 2008; Kamauchi et al., 2008). In addition, GmPDIM and GmPDIL-2, GmPDIM and GmPDIS-1, and GmPDIM and GmPDIS-2 have been shown to form complexes in the ER, suggesting that these PDI family proteins cooperatively fold proglycinin (Wadahama et al., 2007, 2008). Among the complexes, it has been shown *in vitro* that GmPDIM and GmPDIL-2 cooperatively refold RNaseA that has been reduced and denatured (Matsusaki et al., 2016). Accordingly, I examined the associations of GmPDIL6 and GmPDIL7 with other PDI family proteins in the soybean cotyledon ER by coimmunoprecipitation with anti-GmPDIL6 and anti-GmPDIL7 serum (Fig. 3-2A), which revealed that GmPDIM, GmPDIS-1, and GmPDIS-2 coprecipitated with GmPDIL7. GmPDIL6 did not coprecipitated with other PDI family proteins. When the experiments were performed with each of the anti-PDI family protein sera, the 47kDa GmPDIL7 band was difficult to detect by western blot analysis after SDS-PAGE, owing to the overlap with the IgG heavy chains of the antibodies used for the immunoprecipitation. In order to overcome the problem, the proteins immunoprecipitated with each of the anti-PDI family protein sera were separated by two-dimensional gel electrophoresis of involving IEF (first dimension) and SDS-PAGE (second dimension), which revealed the coimmunoprecipitation of GmPDIL7 with GmPDIM, GmPDIS-1, or GmPDIS-2 (Fig. 3-2B). Because the coimmunoprecipitation experiments were performed without treatment for protein crosslinking, the associations of GmPDIL7 and GmPDIM, GmPDIS-1, or GmPDIS-2 were stable.
Fig. 3-1. GmPDIL7 is a substrate of GmERO1a. (A) Schematic representation of the continuous oxidation of the PDI family protein by GmERO1a in the presence of GSH and the reduction of O$_2$. (B) Oxygen consumption by GmERO1a (1 µM) in the presence of 3 µM GmPDIL-1 (L-1), GmPDIL-2 (L-2), GmPDIM (M), GmPDIS-1 (S-1), GmPDIS-2 (S-2), GmPDIL6 (L6) or GmPDIL7 (L7) and 10 mM GSH at 25 °C (solid line). Little oxygen was consumed without GmERO1a (dotted line). (C) Schematic representation of the coupling reaction of oxidation by GmERO1a and the reduction of GSSG by glutathione disulfide reductase (GR) in the presence of GSH and NADPH. (D) NADPH oxidation rate in the presence of 1 µM GmERO1a and 3 µM L-1, L-2, M, S-1, S-2, L6 or L7, in the presence of 3 mM GSH, 120 µM NADPH, and 1 U·mL$^{-1}$ GR. Data are represented as mean ± SEM from at least three replicates. (E) Reconstitution of oxidative protein folding with recombinant L-1, L-2, M, S-1, S-2, L6 or L7 and GmERO1a in vitro. GmERO1a (1 µM) with 3 µM of each PDI family protein, was incubated with the reduced and denatured RNase A (8 µM), and the recovered RNase A activity was assayed. (F) The maximum refolding rate of the reduced and denatured RNase A (8 µM) by 3µM of each PDI family protein in the presence of GmERO1a. Data are represented as mean ± SEM from at least three.
Fig. 3-2. GmPDIL7 associates with GmPDIM, GmPDIS-1, or GmPDIS-2 in the ER. (A) Detection of PDI family protein complexes. Immunoprecipitation (IP) from the cotyledon extract was conducted using anti-GmPDIL6 (L6), anti-GmPDIL7 (L7) or preimmune serum. The immunoprecipitate was subjected to western blotting with anti-GmPDIL-1 (L-1) serum, anti-GmPDIL-2 (L-2) serum, anti-GmPDIM (M) serum, anti-GmPDIS-1 (S-1) serum, anti-GmPDIS-2 (S-2) or anti-GmPDIL6 (L6) serum. (B) Immunoprecipitation from the cotyledon extract was conducted using anti-L-1 serum, anti-L-2 serum, anti-M serum, antiS-1 serum, or anti-S-2 serum. The cotyledon extract (input) and the resulting immunoprecipitates were subjected to two-dimensional gel electrophoresis, involving IEF (first dimension) and SDS-PAGE (second dimension), was followed by western blotting (WB) with anti-L7 serum. Dotted circles represent L7 spots.
Cooperative oxidative folding by GmPDIL7 and other soybean PDI family proteins in the ERO1 pathway

Because GmPDIL-2 accelerates the oxidation of GmPDIM by GmERO1a in a manner to transfer oxidative equivalents from GmPDIM to GmPDIL-2 (Matsusaki et al., 2016), I examined the oxidation rates of the coexisting PDI family proteins by GmERO1a (Fig. 3-3A), which revealed that the oxidation rates of GmPDIL7 and GmPDIM or GmPDIL7 and GmPDIL-2 were nearly the same as the sum of the oxidation rates of each of the PDI family proteins. However, the oxidation rates of GmPDIL7 and GmPDIS-1, GmPDIL7 and GmPDIS-2, or GmPDIL7, GmPDIM, and GmPDIL-2 were lower than the sum of the oxidation rates of each of the PDI family proteins. These results suggested that there was no transfer of oxidative equivalents between GmPDIL7 and GmPDIM, GmPDIS-1, GmPDIS-2, or GmPDIL-2. Thus, these soybean PDI family proteins, which form complexes including GmPDIL7, might be oxidized by GmERO1a independently in the ER. It is possible that these complexes play another role in vivo.

I determined the effects of the coexistence of GmPDIL7 and other PDI family proteins on the oxidative folding of denatured RNase A. The coexistence of GmPDIL7 and GmPDIM, GmPDIS-1, or GmPDIS-2 shortened their lag times, which occurred prior to the initiation of refolding (Fig. 3-3F, purple bars). However, no additive effect on the refolding rate was observed (Fig. 3-3F, red bars). The oxidative refolding of denatured RNase A by GmPDIM in

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**Fig. 3-3. Effects of the coexistence of GmPDIL7 and PDI family proteins in vitro.** (A) Effect on oxidation by GmERO1a. The oxidation rate of NADPH in the presence of 1 μM GmERO1a and 3 μM GmPDIL7 (L7), GmPDIL-2 (L-2), GmPDIM (M), GmPDIS-1 (S-1), or GmPDIS-2 (S-2) in the presence of 3 mM GSH, 120 μM NADPH, and 1 U mL⁻¹ GR was determined. Values are shown as the ratio of the measured value (MV) for the coexistence of soybean PDI family proteins (black bars) to the calculated sum (CS) of the rate of oxidation of individual PDI family proteins (shown in Fig. 3-1D) by GmERO1a. The measured oxidation rates were 12.9 (L7 + M), 14.0 (L7 + S-1), 17.2 (L7 + S-2), 9.5 (L7 + L-2), 5.3 (M + L-2), and 12.3 μM NADPH min⁻¹ (L7 + L-2 + M). Data are represented as mean ± SEM from at least three replicates. (B–E) Oxidative refolding of the reduced and denatured RNase A (8 μM) by L7, M, S-1, S-2, and L-2 alone or in combination, as indicated, was assayed in the presence of GmERO1a. The concentration of each PDI family protein was 3 μM. (F) The maximum reduced and denatured RNase A refolding rate (red bars, left vertical scale) and lag time (purple bars, right vertical scale) for L7, M, S-1, S-2, and L-2 alone or in combination are shown. Data are represented as mean ± SEM from at least three replicates.
the presence of GmERO1a is reportedly synergistically accelerated by GmPDIL-2 (Fig. 3-3F) (Matsusaki et al., 2016). The coexistence of GmPDIL7 with GmPDIM and GmPDIL-2 shortened the lag time, but caused a significant decrease in the refolding rate (Fig. 3-3B,F), suggesting that GmPDIL7 and GmPDIL-2 compete against each other for the formation of a complex with GmPDIM. Thus, the GmPDIL7/GmPDIM and GmPDIL-2/GmPDIM complexes in the ER might act separately during oxidative folding. The coexistence of GmPDIL7 and GmPDIL-2 caused an increase in the refolding rate (Fig. 3-3C–F), suggesting that GmPDIL7 initially forms non-native disulfide bonds in the substrate, and then GmPDIL-2 rearranges them into native bonds. The coexistence of GmPDIL7 and GmPDIL-2 with GmPDIS-1 or GmPDIS-2 shortened the lag time but caused a decrease in the refolding rate (Fig. 3-3C,D,F). In addition, the coexistence of GmPDIL7 with GmPDIL-2, GmPDIM, GmPDIS-1, and GmPDIS-2 also shortened the lag time, but it did not affect the refolding rate (Fig. 3-3E,F). These results suggest that GmPDIL7 and other soybean PDI family proteins do not synergistically fold substrates, but rather, the coexistence of GmPDIL7 and other soybean PDI family proteins might accelerate the initiation of oxidative protein folding.

In this study, I found that GmPDIL7, an ER membrane-bound protein, was rapidly oxidized by GmERO1a, which is also an ER membrane protein (Matsusaki et al., 2016). Even though GmPDIL7 has only one active center, its oxidation activity was similar to the activity of other PDI family proteins that have two active centers. However, the oxidative refolding activity of GmPDIL7 was very low. Based on these results, I believe that a major role of GmPDIL7 could be to cotranslationally introduce the disulfide bonds transferred from GmERO1a to nascent polypeptides in the vicinity of the ER membrane. In addition, GmPDIL7 might oxidize luminal portions of membrane-bound proteins in the ER. Furthermore, the quick formation of disulfide bonds prior to folding might expedite the initiation of the folding by other PDI family proteins such as GmPDIL-2. In addition, GmPDIL7 associated with GmPDIM, GmPDIS-1, and GmPDIS-2. However, no synergistic oxidative folding was observed by the coexistence of GmPDIL7 and GmPDIM, GmPDIS-1, or GmPDIS-2, which was in contrast to
the synergistic action that occurred by the coexistence of GmPDIM and GmPDIL-2. Hence, the formation of complexes of GmPDIM, GmPDIS-1, or GmPDIS-2 with membrane-bound GmPDIL7 might operate in favor of disulfide bond formation on nascent polypeptides. Oxidative folding occurs via two steps, the first of which is the introduction of transient, non-native disulfide bonds and the second, which includes their isomerization into native disulfide bonds (Kojima et al., 2014). For example, PDI family proteins such as GmPDIL7 form non-native disulfide bonds in the substrate and then expert PDI family proteins at isomerization such as GmPDIL-2 fold and rearrange them into native bonds. Thus, the division of the two steps between PDI family proteins might be sufficient for effective oxidative folding \textit{in vivo}. 
CHAPTER 4
Identification of Soybean QSOX and Cooperative Oxidative Folding by Soybean QSOX and GmPDIL-2

Introduction
Although knockout experiments in yeast showed that Ero1p is an essential protein (Franda and Kaiser, 1998; Pollard et al., 1998), both Ero1α and Ero1β knockouts in mice did not produce severe phenotypes (Zito et al., 2010a), prompting explorations of additional pathways for disulfide bond generation in mammalian cells. At present, four additional pathways have been shown to generate disulfide bonds de novo in the secretary apparatus: peroxiredoxin 4 (Zito et al., 2010b; Tavender et al., 2010), GSH peroxidase 7 and 8 (Nguyen et al., 2011; Kakihana et al., 2012; Wang et al., 2014), vitamin K epoxide reductase (Wajih et al., 2007; Rutkevich et al., 2012), and QSOX (EC 1.8.3.2) (Kodali and Thorpe, 2010a; Chakravarthi et al., 2007). In plants, genes encoding Ero1 and quiescin sulfhydryl oxidase (QSOX) orthologues have been found (Kodali and Thorpe, 2010a) but no other pathway has been found.

Animal QSOXs were first isolated as unknown flavoproteins that catalyze the oxidation of low molecular mass thiol compounds such as DTT (Ostrowski et al., 1979) by coupling disulfide oxidation to the reduction of oxygen, forming hydrogen peroxide (Ostrowski and Kistler, 1980). QSOXs directly transfer disulfide bonds to unfolded proteins, but they cannot isomerize non-native disulfides (Hoober et al., 1999; Jaje et al., 2007; Heckler et al., 2008; Kodali and Thorpe, 2010b). Hence, one may presume an oxidative folding pathway, in which QSOX acts as an oxidase and reduced PDI corrects mispaired disulfide bonds introduced by QSOX. The reduced and denatured pancreatic RNase A and the reduced avian riboflavin-binding protein are effectively refolded in vitro, with the correct four and nine disulfide bonds being formed via cooperation between human or avian QSOX and PDI (Hoober et al., 1999; Rancy and Thorpe, 2008).
QSOXs have an N-terminal signal peptide, a thioredoxin domain related to the redox-active domain of PDI, a helix-rich region, an Erv/ALR (flavin-binding) domain, followed by a C-terminal transmembrane region (Kodali and Thorpe, 2010a). *Trypanosoma brucei* QSOX (TbQSOX) and plant QSOXs have only one redox-active thioredoxin domain (Kodali and Thorpe, 2010a), whereas human QSOX (HsQSOX) has an additional redox-inactive thioredoxin domain. QSOXs have one redox-active CxxC motif within the first thioredoxin domain and two redox-active CxxC motifs within the Erv/ALR domain. The CxxC motif in the thioredoxin domain oxidizes unfolded and reduced proteins, and then the pair of reducing equivalents is transferred to the CxxC motifs in the Erv/ALR domain (Raje and Thorpe, 2003; Heckler et al., 2008; Kodali and Thorpe, 2010b). Next, the electron pair is transferred to oxygen via FAD bound to the Erv/ALR domain. The crystal structures of an intact TbQSOX and a TbQSOX intermediate trapped in the disulfide hand-off showed that a 165° domain rotation relative to the original structure brings the two CxxC centres within disulfide-bonding distance (Alon et al., 2012).

There are two previous reports about QSOX orthologues in plants (Alejandro et al., 2007; Limor-Waisberg et al., 2012). Limor-Waisberg et al. (2012) reported that recombinant *Arabidopsis thaliana* QSOX1 (AtQSOX1) expressed in *E. coli* oxidized DTT but did not oxidize reduced RNase A. They concluded that AtQSOX1 does not show the same interdomain electron-transfer activity as its animal counterpart (Limor-Waisberg et al., 2012).

QSOXs localize in the ER, in the Golgi, at the cell surface, and in the extracellular space, and may play multiple physiological roles (Kodali and Thorpe, 2010a). Humans have two QSOX genes (*HsQSOX1* and *HsQSOX2*) (Coppock et al., 1993; Wittke et al., 2003). Two splice variants, *HsQSOX1a* and *HsQSOX1b*, are derived from *HsQSOX1* (Coppock et al., 1998). *HsQSOX1a* has a C-terminal transmembrane region and is localized in the Golgi when overexpressed in Chinese hamster ovary cells (Chakravarthi et al., 2007). *HsQSOX1a* restored disulfide bond formation, as assayed by the folding of the secretory protein carboxypeptidase Y in *Ero1*-null yeast (Chakravarthi et al., 2007), and was recently shown to be processed and
secreted from cells (Rudolf et al., 2013). HsQSOX1b, the shorter variant, lacks a C-terminal transmembrane region. A mouse orthologue of HsQSOX was shown to transit from the ER to the Golgi before being secreted from the cell (Tury et al., 2006; Portes et al., 2008). AtQSOX1 (QSOX2) is expressed predominantly in the root, and is thought to function as a positive regulator, directly or indirectly, of root K⁺ efflux systems involved in xylem loading, because K⁺ deficiency induces its expression (Alejandro et al., 2007).

In this chapter, I showed that recombinant GmQSOX1 (rGmQSOX1) had sulfhydryl oxidase activity. I explored the reactivity of rGmQSOX1 with the reduced and denatured RNase A, and showed that rGmQSOX1 could introduce intramolecular disulfide bonds into unfolded proteins. In addition, by monitoring oxidative refolding, I showed that rGmQSOX1 and reduced soybean PDI family proteins could promptly refold the reduced and denatured RNase A without GSH redox buffer.
Materials and Methods

Plants

Soybean (G. max L. Merrill cv. Jack) seeds were planted in 5-L pots and grown in a controlled environmental chamber at 25 °C under 16-h day/8-h night cycles. All samples taken, except for the leaves used for the ER stress experiment, were immediately frozen and stored in liquid nitrogen until use.

cDNA cloning of GmQSOX1a and GmQSOX1b mRNAs

Cloning of the cDNAs for GmQSOX1a and GmQSOX1b was performed with 3’-RACE and 5’-RACE. Soybean trifoliate leaf centres were frozen in liquid nitrogen and then ground into a fine powder with an SK-100 micropestle (Tokken, Chiba, Japan). Total RNA was isolated with the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s protocol. mRNA was isolated from the total RNA with the PolyATtract® mRNA Isolation System (Promega, Madison, WI, USA). 3’-RACE and 5’-RACE were performed with the SMART RACE cDNA Amplification kit (Clontech Laboratories, Mountain View, CA, USA), according to the manufacturer’s protocol. First, 3’-RACE was carried out with the primer 5’-GCCCATCATCCTTCTAGGAGATGCC-3’, which was designed from the nucleotide sequence of expressed sequence-tag contig TC199187 in the Institute for Genomic Research Soybean Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/tgi/gimain.pl?gudb=soybean). Next, 5’-RACE was carried out with the primer 5’-GTCCTCCTTTCC CCCAAATTCCTAGC-3’, which was designed on the basis of the sequence obtained by 3’-RACE. The amplified DNA fragment was subcloned into pT7 Blue T-vector (TaKaRa Bio, Shiga, Japan). The inserts in the plasmid vectors were sequenced with the fluorescence dideoxy chain-termination method and an ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA). A neighbor joining tree was created with CLUSTALX (Larkin et al., 2007), with the seed set to 111 and the number of bootstraps set to 1000.
Real-time PCR analysis
Leaves were collected from plants 20 days after seeding. For treatment of the leaves under conditions causing ER stress, the leaves were divided into two halves, and 5 mL of H₂O with or without 20 μg·mL⁻¹ tunicamycin was administered to the inner surface of the divided half of each leaf and incubated at 25 °C for 10 h. Total RNA was isolated from the plant tissues with the ST Total RNA Isolation System (Promega). cDNA was prepared from the total RNA with the Prime Script RT reagent kit (TaKaRa Bio). Quantification of the mRNA was carried out in SYBR Premix Ex Taq II (TaKaRa Bio) with a Thermal Cycler Dice Real Time System (TaKaRa Bio). The \textit{GmQSOX1a}, \textit{GmQSOX1b}, \textit{GmQSOX2a} and \textit{GmQSOX2b} mRNAs were detected with the following primers: \textit{GmQSOX1a} forward, 5'CTTGGCTGATTACTACAGTAAAACA-3'; \textit{GmQSOX1a} reverse, 5'-TAGGTGGTGAAAATACTTCCGACTC-3'; \textit{GmQSOX1b} forward, 5'-TGCTTTTGCTATTTGCTTGCTA-3'; \textit{GmQSOX1b} reverse, 5'-CACGTTTTCTAGGGCTTCCGACTC-3'; \textit{GmQSOX2a} forward, 5'-GTTTTTGACTGATTACTACGGTAAAATG-3'; \textit{GmQSOX2a} reverse, 5'-TAGGTGGTGAAAATACTTCCGACTC-3'; \textit{GmQSOX2b} forward, 5'-GTTTTTGACTGATTACTACGGTAAAATG-3'; and \textit{GmQSOX2b} reverse, 5'-CACGTTTTCTAGGGCTTCCGACTC-3'. \textit{GmActin3} mRNA was detected with forward primer 5'-TGTCCTGGTTTCACGGGTTGTC-3' and reverse primer 5'-GCCCTAGGAGCATCGTCACCAGCA-3'. The \textit{GmBiP} mRNA was detected with forward primer 5'-TCTGCTTCTCCTGCTCAGCCATC-3' and reverse primer 5'-CAATGACCACGCTCCCACTTGG-3'. The CT value of each mRNA was obtained from the second derivative maximum value. The expression levels were normalized by use of the CT values obtained for \textit{GmActin3}.

Construction of His-tagged GmQSOX1 expression plasmids
Expression plasmids encoding His-tagged GmQSOX1a (Ser23–Val473) without the signal peptide predicted by SOSUI (Hirokawa et al., 1998), the putative transmembrane region
predicted by TMHMM (Krogh et al., 2001) and the subsequent C-terminal flanking region were constructed as follows. The DNA fragment was amplified from GmQSOX1a cDNA by PCR with the oligonucleotide primers 5’-GACGACGACAAGATGTCTTCTTCCCTCGTGCGCCGTT-3’ and 5’-GAGGAGAAAGCCCGTTACACACATTGCATTAGTGCTAC-3’. The amplified DNA fragment was then subcloned into the ligation-independent cloning site of the pET46Ek/LIC vector (EMD Biosciences, San Diego, CA, USA).

Expression and purification of recombinant GmQSOX1

Rosetta-gami B(DE3) cells (Merck Millipore, Darmstadt, Germany) were transformed with expression plasmids. Expression of recombinant GmQSOX1 (rGmQSOX1) was induced by the addition of 0.5 mM IPTG at 15 °C for 7 days in LB broth containing 100 μg·mL⁻¹ ampicillin, 15 μg·mL⁻¹ kanamycin, 12.5 μg·mL⁻¹ tetracycline, and 5 μM riboflavin. The cells in 1 L of culture broth were collected by centrifugation at 6500 g for 20 min at 4 °C, disrupted by sonication in 50 mL of 20 mM sodium phosphate buffer (pH 7.4) / 0.5 M NaCl, and then centrifuged at 26,000 g for 20 min at 4 °C. The supernatant was filtered with a Millex Filter Unit (0.2 μm) (Merck Millipore), and applied to a column packed with Ni Sepharose 6 Fast Flow (GE Healthcare, Uppsala, Sweden). After the column had been washed with 20 mM sodium phosphate buffer (pH 7.4) / 0.5 M NaCl containing 20 mM imidazole, recombinant proteins were eluted with 20 mM sodium phosphate buffer (pH 7.4) / 0.5 M NaCl containing 100 mM imidazole, concentrated with a Vivaspin 6-10K (GE Healthcare), and then subjected to gel filtration chromatography on a TSK gel G3000SW column (Tosoh, Tokyo, Japan) equilibrated with 10 mM Tris / HCl buffer (pH 7.4) containing 0.15 M NaCl and 10% glycerol. Typically, 0.1 mg of rGmQSOX was obtained from 1 L of LB broth. The protein-bound FAD concentration was determined by absorbance at 456 nm, assuming a molar absorption coefficient of 12,400 M⁻¹·cm⁻¹ (Limor-Waisberg et al., 2012). The concentration of the purified rGmQSOX1 was determined from its absorbance at 280 nm with a molar extinction coefficient
of 100,555 M\(^{-1}\text{-cm}^{-1}\), which was calculated with the modified method of Gill and von Hippel (Pace et al., 1995) and includes the absorbance of FAD at that wavelength (21,300 M\(^{-1}\text{-cm}^{-1}\)) (Limor-Waisberg et al., 2012). The purity of the rGmQSOX1 was confirmed by SDS-PAGE (Laemmli, 1970).

**Identification of FAD bound to rGmQSOX1**

The purified rGmQSOX1 was boiled in 10 mM Tris / HCl buffer (pH 7.4) containing 0.15 M NaCl and 10% glycerol for 10 min, and centrifuged at 10,000 g and 4 °C for 30 min. The supernatant was subjected to HPLC with a μBondusphere C18 column (300 Å, 5 μm, 3.9 × 150 mm) (Waters Corporation, Milford, MA, USA) equilibrated with methanol (20%) / 5 mM acetate ammonium (pH 6.0) (80%). The chromatography was carried out at a flow rate of 0.5 mL min\(^{-1}\) at 20 °C, and the absorbance of the effluent was monitored at 264 nm. The fraction of peak 1 was lyophilized and dissolved in distilled water. The UV-visible absorption spectra of native rGmQSOX1, denatured rGmQSOX1, FAD and the material in peak 1 obtained from the column HPLC were measured with an Ultrospec 2100 pro (GE Healthcare).

**Expression and purification of GST-fused wild-type and mutant GmPDIL2 variants**

Expression plasmids for GST-fused GmPDIL-2 and mutant GmPDIL-2 were constructed (Matsusaki et al., 2016). Briefly, DNA fragments of GST-fused GmPDIL-2 and mutant GmPDIL-2 variants were amplified by PCR with the oligonucleotide primers. The DNA fragments were subcloned into pGEX-6P-2 (GE Healthcare). Recombinant proteins were expressed in *E. coli* BL21(DE3) in the presence of 0.5 mM IPTG at 15 °C for 72 h. The recombinant proteins applied to a GSH Sepharose 4B column (GE Healthcare), cleaved from GST with PreScission protease (GE Healthcare), and eluted from the column with 50 mM Tris / HCl (pH 7.5) containing 0.15 M NaCl, 1 mM EDTA, and 1 mM DTT. The eluted recombinant proteins were purified by gel filtration chromatography on a TSK gel G3000SW column equilibrated with 10 mM Tris / HCl buffer (pH 7.4) containing 0.15 M NaCl and 10% glycerol.
Typically, 9.5 mg of the C104A/C443A mutant, 6.8 mg of the C101A/C104A/C440A/C443A mutant and 13 mg of the wild-type and other GmPDIL-2 mutants were obtained from 1 L of LB broth. The concentrations of the purified wild-type GmPDIL-2 and mutant GmPDIL-2 variants were determined from their absorbance at 280 nm, with a molar extinction coefficient of 40,304 M\(^{-1}\)·cm\(^{-1}\).

**Construction of His-tagged precursor A1aB1b expression plasmids**

Expression plasmids encoding His-tagged precursor A1aB1b without the putative N-terminal signal peptide and the next three amino acids were constructed as described by Kim et al. (1990). The DNA fragment was amplified from precursor *A1aB1b* cDNA by PCR with the oligonucleotide primers

5’-GACGACGACAAGATGAGAGAGCAGCCTCAGCAAAACGAGTGCCAG-3’ and 5’-GAGGAGAAGCCCGGTAAAGCCACAGCTCTCTTTCTGAGACTCCTGAGG-3’ and then subcloned into the pET46Ek/LIC vector.

**Expression and purification of recombinant precursor A1aB1b**

*E. coli* BL21(DE3) cells previously transformed with pTf16 (TaKaRa Bio) plasmids were transformed with the expression plasmids. Expression of the recombinant proteins was induced by the addition of 2 mM IPTG at 25 °C for 3 days in LB broth containing 50 μg·mL\(^{-1}\) ampicillin, 20 μg·mL\(^{-1}\) chloramphenicol, and 0.5 mg·mL\(^{-1}\) L-arabinose. Recombinant precursor A1aB1b was purified by affinity chromatography with a column packed with Ni Sepharose 6 Fast Flow and gel filtration chromatography on a TSK gel G3000SW column equilibrated with 10 mM Tris / HCl buffer (pH 7.4) containing 0.15 M NaCl and 10% glycerol. Typically, 1.5 mg of recombinant precursor A1aB1b was obtained from 1 L of LB broth.
Assay of rGmQSOX1 sulfhydryl oxidase activity

The sulfhydryl oxidase activity of rGmQSOX1 was assayed with 90 nM rGmQSOX1 and 1-100 mM DTT or GSH in 500 μL 50 mM potassium phosphate (pH 7.4) containing 1 mM EDTA equilibrated with air at 25 °C, with an Oxytherm oxygen electrode control unit equipped with a Clark type electrode (Hansatech Instruments, Norfolk, UK). In the case of RNase A and precursor A1aB1b, the reduced and denatured RNase A or precursor A1aB1b was incubated in 50 mM potassium phosphate buffer (pH 7.4) with or without rGmQSOX1 (50 nM) at 25°C. The amount of free thiol groups was measured with 0.5 mM 5,5'-dithiobis (2-nitrobenzoic acid) at 412 nm (Ellman, 1959). The reduced and denatured RNase A and precursor A1aB1b were prepared as described previously (Creighton, 1977). The $k_{cat}$ and $K_M$ values were estimated by nonlinear curve fitting with Microsoft Excel 2007 solver. To confirm the association of precursor A1aB1b into trimers, the sample was subjected to two dimensional PAGE with blue native PAGE (Wittig et al., 2006) and SDS-PAGE (Laemmli, 1970). Proteins on the gel were detected by western blot analysis with antisera specific for the glycinin acidic subunit (Wadahama et al., 2007).

Oxidative refolding assay with the reduced and denatured RNase A

The recombinant soybean PDI family proteins (GmPDIL1, GmPDIL-2, GmPDIL-3a, GmPDIM, GmPDIS-1, and GmPDIS-2) were prepared as described previously (Wadahama et al., 2007, 2008; Kamauchi et al., 2008; Iwasaki et al., 2009). The active centers of these recombinant proteins were reduced in the presence of a 40-fold molar excess of DTT at 25°C for 1 h. Next, the DTT was removed with a 7K MWCO, 0.5-mL Zeba spin desalting column (Thermo Fisher Scientific, Waltham, MA, USA). The reduced and denatured RNase A (10 μM) was incubated in 50 mM Tris / HCl buffer (pH 7.4) containing 1 mM EDTA with or without rGmQSOX1 (50 nM) in the presence or absence of the reduced recombinant soybean PDI family protein and mutant GmPDIL-2 (3 μM) at 25°C. The refolding activity of each PDI family protein without rGmQSOX1 was determined in the absence or presence of GSH redox
buffer (2 mM GSH and 0.5 mM GSSG). An aliquot (20 μL) of the reaction mixture was removed and added to 5 μL of 10 mM or 5 mM N-ethylmaleimide solution, and then incubated at 25 °C for 10 min. The aliquots were diluted with 180 μL of Tris / HCl buffer (pH 7.4) containing 1 mM EDTA and 0.1 mg·mL⁻¹ cCMP, and then incubated at 25 °C; the absorbance was subsequently monitored at 284 nm (Lyles and Gilbert, 1991).
Results and Discussion

Identification of GmQSOX1a and GmQSOX1b

To clone the soybean orthologue of QSOX, I performed a BLAST search with the HsQSOXs cDNA nucleotide sequence from the Institute for Genomic Research Soybean Index. As a result, TC199187, a tentative consensus sequence, was found. I designed primer sets based on TC199187, and generated two cDNAs derived from young soybean leaves by 3’RACE and 5’-RACE. The two cDNAs encoding GmQSOX1a and GmQSOX1b, which are proteins composed of 516 and 511 amino acids, respectively (Fig. 4-1). The amino acid sequences are well conserved, and differ only in 11 amino acids and six amino acids being present at the C termini of GmQSOX1a and GmQSOX1b, respectively.

A BLAST genome search of G. max in the Phytozome database identified the nucleotide sequences for GmQSOX1a and GmQSOX1b in GmQSOX1, which is located on chromosome 20 and is composed of 12 exons and 11 introns (Fig. 4-2). The GmQSOX1a and GmQSOX1b mRNAs are assumed to be splicing variants generated by alternative splicing of exon 11. A BLAST genome search identified GmQSOX2, a gene with 97% sequence similarity to GmQSOX1, on chromosome 10. GmQSOX1 and GmQSOX2 might have resulted from a chromosomal duplication (Schmutz et al., 2010). GmQSOX2 is predicted to produce two mRNAs, GmQSOX2a and GmQSOX2b, by alternative splicing.

GmQSOX1a, GmQSOX1b, GmQSOX2a and GmQSOX2b each possess a putative N-terminal signal sequence, a thioredoxin domain with an active-centre motif (CPAC), a helix-rich region, an Erv/ALR domain with two sequences (CEEC and CSSC) that may act in an internal electron transfer leading to the reduction of FAD to FADH₂, and a putative C-terminal transmembrane region (Fig. 4-3A). Hence, the proteins may be type I membrane-bound proteins targeted to the ER. The domain structures of GmQSOX1a, GmQSOX1b, GmQSOX2a and GmQSOX2b are similar to that of TbQSOX, which has only one thioredoxin domain, and not to that of HsQSOX, which has two thioredoxin domains (Fig. 4-3A). Phylogenetic trees, in
which metazoan and plant QSOXs cluster separately, demonstrate the separation (Fig. 4-3B). Higher plant QSOXs separate into two branches: monocot QSOXs and dicot QSOXs. Soybean QSOXs and *Arabidopsis* QSOXs belong to the latter group. GmQSOX1a/GmQSOX1b and GmQSOX2a/GmQSOX1b share 62%/61% and 59%/59% amino acid sequence identity with AtQSOX1, respectively (Fig. S1), and they share 65%/66% and 64%/64% amino acid sequence identity with AtQSOX2, respectively. Alignment between the amino acid sequences of GmQSOX1 and AtQSOX1 revealed that AtQSOX1 is richer in cysteine than GmQSOX1 (Fig. 4-3C). The Cys257/Cys262 disulfide pair is seen only in AtQSOX.
Fig. 4-1. Putative amino acid sequences of GmQSOX1a, GmQSOX1b, GmQSOX2a, GmQSOX2b, AtQSOX1, and AtQSOX2. The numbers refer to the amino acid number. The putative signal sequence (underlined), active centre CXXC motifs (shaded in black), Trx domain (shaded in grey), FAD binding domain (box), and transmembrane region (dotted, underlined) are indicated.
Fig. 4-2. Comparison of the intron-exon structures of GmQSOX1a, GmQSOX1b, GmQSOX2a, and GmQSOX2b. Open boxes indicate exons, and solid black lines denote introns. The numbers represent exon and intron sizes (bp).
Fig. 4-3. Comparison of the domain structures of soybean QSOX, trypanosomal QSOX, and human QSOX. (A) The boxes indicate the domain boundaries predicted by an NCBI conserved domain search. Trx, HRR and Erv/ALR represent the thioredoxin domain, helix-rich region, and FAD-binding domain, respectively. SP, signal peptide; TM, transmembrane region. The CxxC motifs are indicated by the solid circles. (B) Plant QSOX neighbor-joining tree. (C) Cysteines (black circles) are aligned across GmQSOX1 and AtQSOX1.
Expression of *GmQSOX1a, GmQSOX1b, GmQSOX2a* and *GmQSOX2b* mRNAs

I measured the levels of *GmQSOX1a, GmQSOX1b, GmQSOX2a* and *GmQSOX2b* mRNAs in the soybean cotyledon during seed development (Fig. 4-4A-D). The level of each of the mRNAs increased from the initial period of seed filling (bean weight of 120–140 mg). The levels of *GmQSOX1a, GmQSOX1b, GmQSOX2a* and *GmQSOX2b* mRNAs were highest in beans weighing 200–220, 180–200, 200, and 180 mg, respectively, corresponding to the periods during which the seed storage proteins, such as glycinin, are synthesized the most (Wadahama *et al.*, 2007). Those results suggest that GmQSOX1a, GmQSOX1b, GmQSOX2a and GmQSOX2b may play important roles in the folding of glycinin precursor and other storage proteins. In addition, *GmQSOX1a, GmQSOX1b, GmQSOX2a* and *GmQSOX2b* mRNAs were also expressed in the roots, stems, and leaves (Fig. 4-4E). The levels of *GmQSOX1a, GmQSOX1b* and *GmQSOX2b* mRNAs were lower in the leaf than in other tissues. In contrast to the GmQSOXs, AtQSOX1 is expressed mostly in the roots (Alejandro *et al.*, 2007). AtQSOX1 is thought to act as a regulator of ion homeostasis, because mutant and transgenic plants overexpressing *AtQSOX1* cDNA show higher tolerance to toxic cations (Alejandro *et al.*, 2007). There is, however, no information about the physiological function of AtQSOX1 in other tissues.

To examine the effect of ER stress on the expression of GmQSOX1a, GmQSOX1b, GmQSOX2a and GmQSOX2b mRNAs, leaves were treated or not treated with tunicamycin, a trigger of ER stress (Kamauchi *et al.*, 2005; Martínez and Chrispeels, 2003), and mRNA levels were measured. The expression of *GmQSOX1a, GmQSOX1b* and *GmQSOX2a* mRNAs was substantially upregulated following tunicamycin treatment, similar to that of *GmBiP* (Fig. 4-4F). The expression of *GmQSOX2b* mRNA did not increase under ER stress conditions, indicating that *GmQSOX2* hnRNA was exclusively spliced to form the *GmQSOX2a* mRNA under conditions causing ER stress. These results suggest that *GmQSOX1a, GmQSOX1b* and *GmQSOX2a* play important roles in protein folding in the ER.
Fig. 4-4. Expression of GmQSOX1a, GmQSOX1b, GmQSOX2a and GmQSOX2b mRNAs. The amounts of GmQSOX1a (A), GmQSOX1b (B), GmQSOX2a (C) and GmQSOX2b mRNAs (D) in the cotyledon during maturation were quantified by real-time RT-PCR. Each value was standardized with actin 3 mRNA. (E) The amounts of GmQSOX1a, GmQSOX1b, GmQSOX2a and GmQSOX2b mRNAs in the root, stem, leaf, and cotyledon (bean weight, 200 mg). Each value shows a relative transcription level (gene/actin 3 ratio). (F) Expression of GmQSOX1a, GmQSOX1b, GmQSOX2a, GmQSOX2b and GmBiP mRNAs under ER stress induced by tunicamycin (TM) in the leaf. Each value was standardized with actin 3 mRNA. The fold expression change was calculated as the ratio of the amount of mRNA in the samples treated with TM (+) to that in the sample not treated with TM (–). Values represent the mean ± standard deviation for three experiments.
Sulfhydryl oxidase activity of recombinant GmQSOX1

To examine the enzymatic characteristics of GmQSOX1a and GmQSOX1b, I first tried to express recombinant GmQSOX1a and GmQSOX1b without the putative signal peptide by using an *E. coli* expression system. The recombinant proteins were expressed as insoluble proteins. Next, I used the same expression system to express a recombinant GmQSOX1a from which the putative N-terminal signal sequence and the putative C-terminal transmembrane region were removed. Because its amino acid sequence was the same as that of GmQSOX1b, I called the recombinant protein rGmQSOX1. rGmQSOX1 was expressed as a soluble protein in *E. coli* (Fig. 4-5A, lane 1), so I purified it by His-tag affinity chromatography (Fig. 4-5A, lane 2) and gel filtration column chromatography (Fig. 4-5A, lane 3), and eluted it in a monomeric position from a gel filtration column (data not shown). Recombinant HsQSOX, TbQSOX and AtQSOX have been shown to be monomeric forms (Heckler *et al.*, 2008; Kodali and Thorpe, 2010b; Limor-Waisberg *et al.*, 2012). The UV–visible absorption spectrum of the purified rGmQSOX1 had a maximum absorbance at 455 nm and a shoulder at 485 nm (Fig. 4-5B). The soluble materials released from rGmQSOX1 by boiling were separated by HPLC with a C18 column, and two peaks were detected (Fig. 4-5C). The major peak (peak 1) passed through the column with times corresponding to those of authentic FAD. The material in that peak showed a spectrum identical to that of free FAD (Fig. 4-5B). Those results indicated that, similarly to HsQSOX and TbQSOX, rGmQSOX1 possesses FAD as a cofactor. On the other hand, the material of peak2 was identified as 6-hydroxy-FAD by mass spectrometry analysis.
**Fig. 4-5. rGmQSOX1 was expressed as a flavin-containing protein.** (A) rGmQSOX1 expressed in *E. coli* (lane 1) was purified by His-tag column chromatography (lane 2) followed by gel filtration chromatography (lane 3). Proteins in each effluent were separated by 10% SDS-PAGE and stained with Coomassie Blue. (B) UV spectra of 25 µM rGmQSOX1 (solid line), free FAD (dotted line), and peak 1 from a C18 HPLC column of materials released from rGmQSOX1 by boiling (dashed line). The spectra were measured in 10 mM Tris / HCl buffer (pH 7.4) containing 0.15 M NaCl and 10% glycerol. The black and white arrows show the maximum peak (455 nm) and the shoulder peak (485 nm), respectively, of the rGmQSOX1 spectrum. (C) C18 HPLC column chromatogram of the material released from rGmQSOX1 by boiling (solid line). The arrows show the retention times of free FAD and free FMN.
HsQSOXs and TbQSOX reduce oxygen to hydrogen peroxide with small-molecule substrates such as DTT and GSH (Chakravarthi et al., 2007; Kodali and Thorpe, 2010b). rGmQSOX1 oxidized DTT (Fig. 4A), although its activity was lower than those of TbQSOX and HsQSOX1b (Table 4-1). The $k_{cat}$ of rGmQSOX1, monitored by oxygen consumption when DTT was the substrate, was less than one-third that of TbQSOX. The $K_M$ of rGmQSOX1 for DTT ($\sim$ 50 mM) was higher than those of TbQSOX and HsQSOX1b ($\sim$ 0.1 mM). High $K_M$ values for DTT were reported previously for other QSOX mutants lacking thioredoxin domain CXXC cysteines (Kodali and Thorpe, 2010b) and for AtQSOX1 (Limor-Waisberg et al., 2012). In the QSOX mutants lacking thioredoxin domain CXXC cysteines, DTT directly reduces the FAD-proximal Erv/ALR CXXC disulfide; in AtQSOX1, however, DTT readily reduces thioredoxin domain CXXC (Limor-Waisberg et al., 2012). Hence, Limor-Waisberg et al. concluded that electrons are not efficiently transferred from the thioredoxin domain to the Erv/ALR domain in AtQSOX1 (Limor-Waisberg et al., 2012). A lower affinity for DTT may be a common property of plant QSOXs. The sulfhydryl oxidase activity of rGmQSOX1 on GSH was very low (Fig. 4-6A).

I determined the sulfhydryl oxidase activity of rGmQSOX1 on the reduced and denatured RNase A (Fig. 4-6B). Eighty per cent of the free thiols of the reduced and denatured RNase A were oxidized during a 120-min incubation with rGmQSOX1. The $k_{cat}$ of rGmQSOX1 was comparable to that of TbQSOX and approximately one-quarter that of HsQSOX1b (Table 4-1). The $K_M$ of rGmQSOX1 for RNase A was almost the same as those of TbQSOX and HsQSOX1b.
Fig. 4-6. rGmQSOX1 catalyzes disulfide-bond formation but is unable to refold the reduced and denatured RNase A and precursor A1aB1b into a native form. (A) The oxidase activity of rGmQSOX1 was determined with dithiothreitol (DTT) or GSH as a substrate. GSH (100 mM) was incubated with (-----) or without (······) 90 nM rGmQSOX1. DTT (100 mM) was incubated with (—) or without (-----) 90 nM rGmQSOX1. rGmQSOX1 was added at the time point indicated by the arrow. Oxygen consumption during the reaction was monitored with an oxygen electrode. (B) The sulfhydryl oxidase activity of rGmQSOX1 was determined with the reduced and denatured RNase A (12.5 μM) as a substrate. The reaction was carried out in the presence (black circles) or absence (white circles) of rGmQSOX1. The decrease in free thiol groups during the reaction was measured with Ellman’s reagent. Values represent the mean ± standard error for three experiments.
Table 4-1. Comparison of steady-state kinetic parameters for the oxidation of thiol substrates between rGmQSOX1, AtQSOX1, TbQSOX, and HsQSOX1b. $k_{cat}$ values are expressed in terms of the number of thiols oxidized, and not in terms of the number of disulfides generated per second. $K_M$ values are expressed on a per-thiol basis. The $k_{cat}$ measurement with dithiothreitol (DTT) was performed with an oxygen electrode. The $k_{cat}$ values for the reduced and denatured RNase A were calculated from the thiol oxidation rate. Values represent the mean ± standard error for three experiments. The turnover number of AtQSOX1, which was calculated from the value at 20 mM DTT, was 5.3 s$^{-1}$ (Matsusaki et al., 2016).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>QSOX</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>GmQSOX1</td>
<td>13.9 ± 0.2</td>
<td>52.23 ± 2.41</td>
<td>2.7 × 10$^2$</td>
</tr>
<tr>
<td></td>
<td>AtQSOX1</td>
<td>-</td>
<td>&gt;100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TbQSOX</td>
<td>45</td>
<td>0.086</td>
<td>5.2 × 10$^5$</td>
</tr>
<tr>
<td></td>
<td>HsQSOX1b</td>
<td>21</td>
<td>0.10</td>
<td>2.0 × 10$^5$</td>
</tr>
<tr>
<td>RNase A</td>
<td>GmQSOX1</td>
<td>17.5 ± 2.5</td>
<td>0.22 ± 0.05</td>
<td>8.4 × 10$^4$</td>
</tr>
<tr>
<td></td>
<td>TbQSOX</td>
<td>22</td>
<td>0.36</td>
<td>6.0 × 10$^4$</td>
</tr>
<tr>
<td></td>
<td>HsQSOX1b</td>
<td>72</td>
<td>0.32</td>
<td>2.2 × 10$^5$</td>
</tr>
</tbody>
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a) Values are quoted from reference (Limor-Waisberg et al., 2012). b) Values are quoted from reference (Kodali and Thorpe, 2010b). c) Values are quoted from reference (Heckler et al., 2008).
GmQSOX1, which has only one thioredoxin domain, may transfer electrons in a similar way to TbQSOX. Recombinant AtQSOX1 has been reported to have no oxidizing activity on the cysteines of the reduced and denatured RNase A (Limor-Waisberg et al., 2012). Recombinant AtQSOX1 does not efficiently transfer electrons from the thioredoxin domain to the Erv/ALR domain (Limor-Waisberg et al., 2012). The difference in the intramolecular disulfide transfer abilities of rGmQSOX1 and recombinant AtQSOX1 may be the cause of the difference in the abilities of these proteins to oxidize RNase A.

After the reaction with rGmQSOX1, most of the RNase A molecules were monomers, and little crosslinked RNase A was detected by non-reducing SDS-PAGE (Fig. 4-7A), suggesting that most of the disulfide bonds formed in RNase A by rGmQSOX1 were intramolecular ones. Because rGmQSOX1 had no refolding activity on the reduced and denatured RNase A (Fig. 4-7B), similarly to TbQSOX and HsQSOXs, the disulfide bonds that formed were non-native ones.

RNase A is a well-known model protein that is relatively small and easy to refold in vitro. In the ER, larger and more complicated proteins are synthesized. To determine whether GmQSOX1 is capable of introducing disulfide bonds into a larger and more complicated protein, I examined its activity with a precursor of the soybean seed storage protein glycinin A1aB1b as a substrate. Precursor A1aB1b is synthesised as a 60kDa polypeptide with eight cysteines, and its folding involves the formation of three disulfide bonds in the ER (Ereken-Tumer et al., 1982). The folded protomers are held together as trimers by noncovalent interactions in the ER (Nam et al., 1997). Precursor A1aB1b trimers are transported to protein storage vacuoles via the Golgi bodies, and then processed by protease to form mature A1aB1b (Scott et al., 1992). I prepared recombinant precursor A1aB1b with an E. coli expression system according to the design reported by Kim et al. (1990). The recombinant precursor A1aB1b occurred as trimers and hexamers, which were detected as approximately 180 and 360-kDa complexes, respectively, by blue native PAGE (Fig. 4-7D, panel 1). Kim et al. also reported the existence of trimers and hexamers (Kim et al., 1990). I detected 2.6 ± 1.6 (n = 3) free thiol groups per molecule of
recombinant pro A1aB1b, suggesting that two to three disulfide bonds were formed for each molecule. The recombinant precursor A1aB1b was dissociated to monomers of ~ 60 kDa by reduction and denaturation (Fig. 4-7D, panel 2). I detected 8.3 ± 1.6 (n = 3) free thiol groups per molecule of the reduced and denatured precursor A1aB1b, suggesting that all of the disulfide bonds were reduced.

I subjected the reduced and denatured recombinant precursor A1aB1b to assays measuring disulfide formation by rGmQSOX1. When the reduced and denatured precursor A1aB1b was incubated with rGmQSOX1, the amount of free thiol groups in the reaction mixture decreased (Fig. 4-7C). After 60 min, the amount of free thiol groups decreased to 36% of the initial amount. This indicates that rGmQSOX1 formed disulfide bonds in the denatured precursor A1aB1b, because most of the free thiol groups in the reaction mixture were from the cysteines of the precursor A1aB1b. The precursor A1aB1b did not form trimers in the presence of rGmQSOX1 (Fig. 4-7D, panel 3), suggesting that it did not refold. When native precursor A1aB1b was incubated with rGmQSOX1, the number of free thiol groups in the mixture did not decrease, suggesting that rGmQSOX1 does not oxidize native proteins.
Fig. 4-7. rGmQSOX1 catalyzes disulfide-bond formation but is unable to refold the reduced and denatured RNase A and precursor A1aB1b into a native form. (A) rGmQSOX1 formed entirely intramolecular disulfide bonds. The reduced and denatured RNase A was incubated with rGmQSOX1. The reaction mixtures were subjected at the indicated times to nonreducing and reducing SDS-PAGE. Proteins were stained with Coomassie Blue. The arrow shows monomeric RNase A. (B) Refolding activity was assayed by measuring the activity of the refolded RNase A. The reduced and denatured RNase A was incubated with rGmQSOX1 (squares) or GmPDIL-1 (circles) in the presence (white) or absence (black) of 2 mM GSH and 0.5 mM GSSG. Values represent the mean ± standard error for three experiments. (C) GmQSOX1 formed disulfide bonds on the reduced and denatured precursor A1aB1b. The native precursor A1aB1b (triangles) and the denatured and reduced precursor A1aB1b (circles) were incubated with (black) or without (white) rGmQSOX1 at 25 °C. The decrease in free thiol groups in 1 mL of reaction mixture during the reaction was measured with Ellman’s reagent. Data represent the mean ± standard error for three experiments. (D) Precursor A1aB1b (panel 1), the reduced and denatured precursor A1aB1b (panel 2) and the reduced and denatured precursor A1aB1b (2.8 μM) incubated in the presence of 90 nM rGmQSOX1 at 25 °C for 120 min (panel 3) were first separated onto blue native PAGE (BN-PAGE) gel and then applied to an SDS-PAGE gel for resolution in the second dimension. Precursor A1aB1b was immunostained with antiserum against the glycinin acidic chain.
Cooperative refolding of unfolded RNase A by rGmQSOX1 and soybean PDI family proteins

rGmQSOX1 introduced non-native intramolecular disulfide bonds into the reduced and denatured RNase A and precursor A1aB1b expeditiously and without a disulfide donor. PDI family proteins can rearrange incorrect disulfide bonds to native ones and can fold substrate polypeptides, but they cannot introduce additional disulfide bonds into proteins without a disulfide donor such as Ero1 (Araki and Inaba, 2012). Therefore, I expect GmQSOX and PDI family proteins to cooperate in the refolding of reduced and unfolded proteins. Avian QSOX cooperates with human PDI to generate native disulfide bonds in unfolded RNase A and riboflavin binding protein in vitro (Hoober et al., 1999; Rancy and Thorpe et al., 2008). I investigated the cooperative refolding of the reduced and denatured RNase A by rGmQSOX1 and soybean PDI family proteins belonging to group I (GmPDIL-1), group II (GmPDIL-2), group III (GmPDIL3a and GmPDIL3b), group IV (GmPDIS-1 and GmPDIS-2), and group V (GmPDIM) (Wadahama et al., 2007, 2008; Kamauchi et al., 2008; Iwasaki et al., 2009). These PDI family proteins contain two classic CGHC motifs in the a and a’ domains, except for the group III PDI family proteins, which have nonclassic active centre CXXC motifs (Fig. 4-8A) (Iwasaki et al., 2009). The PDI family proteins in groups I, II, VI, and V, but not those in group III, showed RNase A-refolding activities in GSH redox buffer (Fig. 4-8B, grey bars). None of the PDI family proteins treated with DTT showed activity in the absence of GSH redox buffer (Fig. 4-8B, white bars). Each of the PDI family proteins other than those in group III showed refolding activity when incubated together with rGmQSOX1 and the reduced and denatured RNase A in the absence of GSH redox buffer (Fig. 4-8B, black bars). The refolding activities of all of the PDI family proteins in GSH redox buffer in the presence of rGmQSOX1 were higher than those in the absence of GmQSOX1. The combination of rGmQSOX1 and GmPDIL-2 with an a–b–b’–a’ domain structure showed the highest level of refolding activity. The combination of GmQSOX1 and GmPDIL-1, also with an a–b–b’–a’ domain structure, showed the second highest level of refolding activity.
Fig. 4-8. rGmQSOX1 cooperates with soybean PDI family proteins to refold the reduced and denatured RNase A. (A) Diagram of soybean PDI family proteins. CxxC motifs are indicated by the solid circles. (B) Refolding of the reduced and denatured RNase A by rGmQSOX1 and PDI family proteins. The reduced and denatured RNase A (10 μM) and 3 μM GmPDIL-1, GmPDIL-2, GmPDIL-3a, GmPDIM, GmPDIS-1 or GmPDIS-2 were incubated with (+, black bars) or without (−, white and gray bars) 50 nM rGmQSOX1 in the presence (+, gray bars) or absence (−, white and black bars) of 2 mM GSH and 0.5 mM GSSG at 25 °C for 1 h. The activity of the refolded RNase A was determined. Values represent the mean ± standard error for three experiments.
To elucidate the roles of the active centers of GmPDIL-2 in the cooperative refolding with rGmQSOX1, the activities of GmPDIL-2 active center mutants were determined (Fig. 4-9A,B). The active center mutant C101A/C104A/C440A/C443A, in which all of the cysteines in the two active centers were replaced with alanine, was devoid of the cooperative oxidative refolding activity with rGmQSOX1. The removal of the active center cysteine residues in the \( \alpha' \) domain (C440A/C443A) or the \( \alpha \) domain (C101A/C104A) reduced the cooperative activity with rGmQSOX1 to 59% or 60%, respectively, of that of the wild-type GmPDIL-2, indicating that both active centers are essential for the full cooperative refolding activity of rGmQSOX1 and GmPDIL-2, and that both active centers can function independently.

There are two possible ways in which rGmQSOX1 and GmPDIL-2 could cooperate for oxidative folding. One is that rGmQSOX1 introduces non-native disulfide bonds into the protein substrate, and GmPDIL-2 then rearranges those disulfide bonds to native ones. The other is that rGmQSOX1 oxidizes the thiol group pair in the active centers of GmPDIL-2, and the activated GmPDIL-2 then performs the oxidative refolding.

I determined the ability of rGmQSOX1 to oxidize GmPDIL-2 and other PDI family proteins (Fig. 4-9C). The amount of free thiol groups in the reaction mixture did not decrease during the incubation of GmPDIL-2 with rGmQSOX1. In addition, I detected no oxygen consumption during the incubation of GmPDIL-2 with rGmQSOX1 in the presence of GSH (Fig. 4-9D), suggesting that rGmQSOX1 does not oxidize GmPDIL-2 directly. Therefore, GmPDIL-2 must fold the unfolded RNase A as an isomerase in a manner similar to the shuffling of the non-native disulfide bonds introduced by rGmQSOX1. Because neither GmPDIL-1 nor GmPDIM was oxidized by rGmQSOX1 (Fig. 4-9C), those PDI family proteins may also act as isomerases in cooperation with rGmQSOX1. On the other hand, when GmPDIS-1 or GmPDIS-2, which have two or three cysteines in addition to the four active center cysteines, was incubated with GmQSOX1 for 60 min, there was a modest decrease in the amount of free thiol residues in the reaction mixture, corresponding to the formation of 0.34 or 0.62 disulfides per molecule of GmPDIS-1 or GmPDIS-2, respectively. Therefore, the observed cooperative
refolding activity cannot be explained by the activation of the active centers of those PDI family proteins by rGmQSOX1. Furthermore, it has been shown that reduced human PDI and avian PDI are also very poor substrates for avian QSOX (Rancy and Thorpe, 2008).

For the rearrangement of disulfide bonds in the substrate protein by PDI family proteins, there are two possible pathways: direct isomerization and reduction–oxidation cycles (Hatahet and Ruddock, 2009). In order to determine which pathway is important for the disulfide rearrangement reaction, I generated GmPDIL-2 mutants (C104A/C443A, C104A, and C443A) in which the C-terminal cysteine in both or one active center CGHCs in the a and a’ domains was replaced by alanine (Fig. 4-9B). The mutation of the C-terminal cysteine of CGHC to alanine causes defects in the abilities of GmPDIL-2 to oxidize thiols to disulfide and to reduce disulfide bonds, but does not alter the ability of the protein to isomerize disulfide bonds (Hatahet and Ruddock, 2009). The refolding activity of rGmQSOX1 combined with the C104A/C443A mutant was 18% of that of rGmQSOX1 combined with wild-type GmPDIL-2. In addition, the folding activities of rGmQSOX1 combined with the C413A mutant or C104A mutant were lower than those of rGmQSOX1 combined with the C440A/C443A mutant or the C101A/C104A mutant. These results suggest that the substrate that is bound to the cysteine of the mutated active center CGHA via a mixed disulfide is trapped, because the direct isomerization reaction is very slow. Hence, I can reasonably assume that the wild-type GmPDIL-2 rearranges non-native disulfide bonds to native ones mainly through a reduction–oxidation cycle.

In conclusion, I characterized the enzymatic properties of rGmQSOX1 by using the reduced and denatured RNase A and precursor A1aB1b as substrates. Although rGmQSOX1 can introduce non-native disulfides into unfolded polypeptides, it cannot refold the polypeptides alone, and instead refolds them by cooperating with ER-resident soybean PDI family proteins. In addition, ER stress increased the expression of GmQSOX1a, GmQSOX1b and GmQSOX2a mRNAs. On the basis of these findings, I presume that GmQSOX1a and GmQSOX1b, and possibly GmQSOX2a, act as disulfide donors in the oxidative folding of
reduced, unfolded proteins in the ER, especially when the reduced, unfolded proteins are overabundant in the ER. Unfortunately, the localization of GmQSOX proteins in soybean tissues is still unclear. I could not identify endogenous GmQSOX proteins by western blot experiments with anti-rGmQSOX1 serum (data not shown). Only a small amount of GmQSOX proteins may be present in soybean tissues. Further in vivo studies should help to clarify the physiological functions of GmQSOXs in soybeans.

Fig. 4-9. The cysteines of GmPDIL-2 are essential for the refolding activity of the rGmQSOX1–GmPDIL-2 system. (A) Diagram of the wild type and active center cysteine mutants of GmPDIL-2. (B) Effects of mutations in the active centers of GmPDIL-2 on the refolding activity of rGmQSOX1 and GmPDIL-2. Refolding was assayed with the reduced and denatured RNase A (10 μM) as a substrate in the presence of rGmQSOX1 (50 nM) and wild-type or active center mutants of GmPDIL-2 (3 μM) at 25°C for 1 h. Values represent the mean ± standard error for three experiments. (C) rGmQSOX1 does not oxidize the thiol groups of soybean PDI family proteins, except for GmPDIS-1 and GmPDIS-2. GmPDIL-1, GmPDIL-2, GmPDIL-3, GmPDIM, GmPDIS-1 and GmPDIS-2 (20 μM) were incubated with 100 nM rGmQSOX1 at 25°C. (D) Oxygen consumption in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 10 mM GSH without (--) or with rGmQSOX1 ( - - - - ). GmPDIL-2 (·····) or rGmQSOX1 and GmPDIL2 (- - -) was monitored during incubation. rGmQSOX1 was added at the time point indicated by the arrow.
REFERENCES


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SUMMARY

CHAPTER 1
The cDNAs encoding GmPDIL6 and GmPDIL7, novel members of the soybean PDI family, were cloned. The expression levels of GmPDIL6 protein and the mRNA levels were elevated when a seed storage protein, such as β–conglycinin or glycinin, was synthesized in the cotyledon. The expression of GmPDIL6 mRNA strikingly increased following treatment with ER stress inducers. The GmPDIL7 protein was ER membrane-bound. These results suggested that GmPDIL6 and GmPDIL7 played important roles in the oxidative folding of seed storage proteins in the ER.

CHAPTER 2
The enzymatic properties of recombinant GmPDIL6 and GmPDIL7 were described. The recombinant GmPDIL6 and GmPDIL7 possessed dithiol oxidation activities and the thiol oxidation activities were similar to that of other proteins in the soybean PDI family. However, GmPDIL6 had no disulfide reductase activity and GmPDIL7 had low disulfide reductase activity. GmPDIL6 had low oxidative refolding activity, because it had no disulfide reductase activity. The oxidative refolding activity of GmPDIL7 was lower than that of other soybean PDI family proteins. These results suggested that GmPDIL6 and GmPDIL7 played a role in oxidative protein folding.

CHAPTER 3
GmPDIL7 was well oxidized by GmERO1a. However, GmPDIL6 was hardly oxidized by GmERO1a. GmPDIL7 was found to be associated with GmPDIM, GmPDIS-1, and GmPDIS-2 in vivo. The effects of the coexistence of GmPDIL7 and other PDI family proteins on the oxidative folding of denatured RNase A were assessed, but no additive effects on the refolding rates were observed. These results suggested that GmPDIL7 and other soybean PDI family
proteins did not synergistically fold substrates, but rather, the coexistence of GmPDIL7 and other soybean PDI family proteins might have accelerated the initiation of oxidative protein folding. Taken together, these results indicated that GmPDIL7 primarily played a role as a supplier of disulfide bonds in nascent proteins for oxidative folding on the ER membrane.

CHAPTER 4
Two GmQSOX1 cDNAs, GmQSOX1a and GmQSOX1b, were cloned and generated by alternative splicing. The GmQSOX1a, GmQSOX1b, and GmQSOX2 mRNA levels increased during seed storage protein synthesis in the cotyledon, and were also upregulated during conditions causing ER stress. Recombinant GmQSOX1 expressed in E. coli formed disulfide bonds in reduced and denatured RNase A, but did not show any refolding activity. The reduced and denatured RNase A was effectively refolded by recombinant GmQSOX1 in the presence of the soybean protein disulfide isomerase family protein, including GmPDIL-2 in the absence of glutathione redox buffer, further suggesting that GmQSOX1 and the soybean PDI family proteins cooperatively played a role in oxidative protein folding in the ER.

I propose a new model. The PDI family proteins, GmPDIL6, GmPDIL7, and GmQSOX, form non-native disulfide bonds in their substrates. The GmPDIL-1 and GmPDIL-2 isomerize, fold, and rearrange them into native bonds. Therefore, I hypothesize that the two steps between the PDI family proteins and QSOX might be sufficient for effective oxidative folding in vivo.
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PAPERS

Original Papers


Okuda, A., Matsusaki, M., Masuda, T., Urade, R. Identification and characterization of GmPDIL7, a soybean ER membrane-bound protein disulfide isomerase family protein. FEBS J in press.

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