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Gene Note

Characteristics of soybean 1-Cys peroxiredoxin and its behavior in seedlings under flooding stress

Keito Nishizawa*, Setsuko Komatsu

National Institute of Crop Science, Tsukuba, Ibaraki 305-8518, Japan
* E-mail: keito@affrc.go.jp Tel: +81-29-838-8693 Fax: +81-29-838-8694

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Abstract By previous proteomic analysis, the amount of proteins exhibiting similarity to 1-Cys peroxiredoxin (Prx), a thiol-dependent peroxidase, was shown to be higher in seedlings of soybean [*Glycine max* (L.) Merr.] suffering from flooding stress than in normally grown seedlings. In this study, we characterized soybean 1-Cys Prx to elucidate the relationship of the protein to flooding stress. In the soybean genome, two genes corresponding to 1-Cys Prx (designated as *GmPer1a*, *GmPer1b*) exist. *GmPer1a* encodes a polypeptide containing the putative catalytic site, and a recombinant GmPer1a protein exhibited peroxidase activity. On the other hand, the *GmPer1b* contains a stop codon inside the deduced polypeptide-coding region, indicating that *GmPer1b* might be a pseudogene. The *GmPer1a* was expressed in developing seeds and transiently in germinating seeds of soybean. It was no longer expressed in 2-day-old seedlings, and was not induced by flooding treatment. The GmPer1 protein was synthesized in developing seeds, and was degraded during germination and growth. In addition, two forms of GmPer1 protein existed in both submerged and normally grown seedlings, and the amount of both forms was higher in the submerged seedlings. These suggest that both normal and post-translationally modified forms of GmPer1 might remain in seedlings suffering from flooding stress as a result of growth retardation.

Key words: Antioxidant, 1-Cys peroxiredoxin, flooding stress, seed, soybean.

Flooding stress is one of the serious problems of soybean production in Japan, since much soybean is cultivated in ill-drained fields converted from paddy fields. Soybean most seriously suffers flooding stress during its early growth stage in the Japanese rainy season. We have been studying the responses of soybean seedlings to flooding stress using a proteome technique. We previously reported that 35 proteins were up-regulated and 16 proteins were down-regulated by a 24-h-flooding treatment of 2-day-old soybean seedlings (Hashiguchi et al. 2009). Of these proteins, two spots in 2-DE gels contain proteins showing similarity to 1-Cys peroxiredoxin (Prx) of barley (Per1, Swiss-Prot accession P52572) and *Medicago truncatula* (UniProt accession Q6E2Z6), and both were included in up-regulated proteins. To elucidate the involvement of 1-Cys Prx in the flooding response, we characterized soybean 1-Cys Prx (designated as GmPer1).

GmPer1 cDNA was cloned from 1-day-old soybean seedlings (cv. Enrei) by RT-PCR with primer 1 (5′-ATGCCAGGTCTTACCATC-3′) and primer 2 (5′-TCAAACTTTAGTTAAGCG-3′) designed according to the sequence of Glyma08g09310 (Figure 1A) found in a soybean genome database (http://www.phytozome.net/soybean.php, Schmutz et al. 2010) as a result of a BLAST search with the protein sequence of barley Per1 (P52572) and *M. truncatula* 1-Cys Prx (Q6E2Z6). The cloned cDNA sequence (designated as *GmPer1a*) is 100% identical to the deduced cDNA sequence of Glyma08g09310, and it encodes a polypeptide composed of 218 residues with a predicted molecular mass of 24.4 kDa and a pI of 6.44. The deduced primary structure contains peptide sequences which were observed by nano LC-MS/MS analysis (Figure 1B; Hashiguchi et al. 2009), and reveals about 70–85% of identity with 1-Cys Prxs from higher plants and about 50% of identity with 1-Cys Prxs from mammals and insects. The GmPer1a contains the putative catalytic residue (Cys 46) in a consensus surrounding sequence (DFTPVCTTEL) that is characteristic of 1-Cys Prxs (Figure 1B). Another gene for GmPer1 (Glyma05g26400, Figure 1A) was found in the soybean genome by a BLAST search of the soybean genome database. We designated this gene as *GmPer1b*. The deduced cDNA sequence of *GmPer1b* had 94% sequence identity to *GmPer1a* cDNA. *GmPer1b*, however, contained a stop

Abbreviations: Adh, alcohol dehydrogenase; Cys, cysteine; 2-DE, two-dimensional polyacrylamide gel electrophoresis; DTT, dithiothreitol; His, histidine; PAGE; polyacrylamide gel electrophoresis; pI, isoelectric point; Prx, peroxiredoxin.
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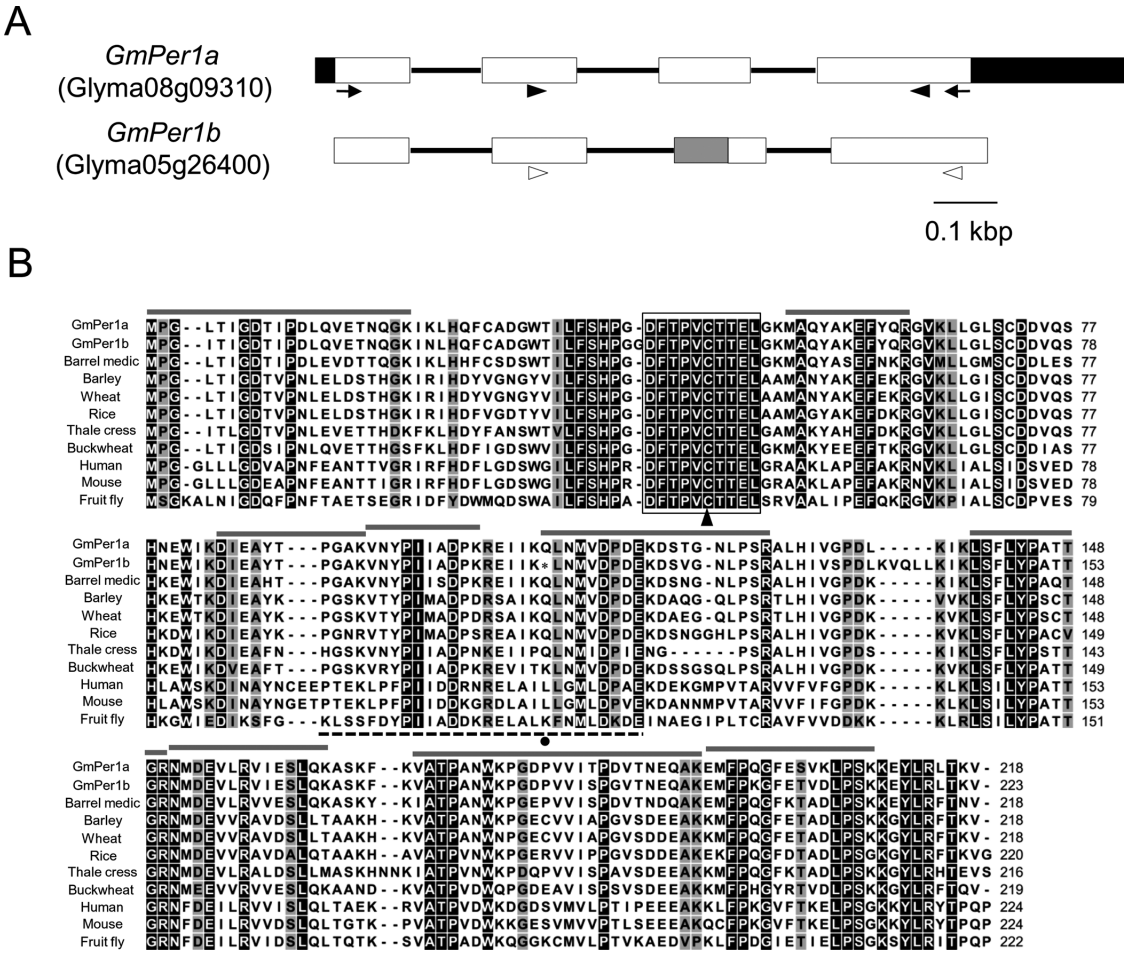

Figure 1. Characteristics of the soybean *GmPer1* genes and GmPer1 polypeptide. (A) Structures of the two *GmPer1* genes in the soybean genome. The black and white boxes represent untranslated regions and coding sequences, respectively. The gray box represents the portion eliminated by the gene prediction program associated with the soybean genome database, probably because this portion contains a stop codon. The lines between boxes are introns. The arrows indicate the position of primers used for cloning of *GmPer1a* cDNA. The arrowheads indicate the position of the primers for RT-PCR in Figure 3. (B) Alignment of amino acid sequences deduced from the two *GmPer1* genes in the soybean genome and 1-Cys Prxs from other organisms. Sequences were aligned using ClustalW (1.83) program. The arrowhead indicates the putative catalytic cysteine residue, and a consensus surrounding sequence that is characteristic of 1-Cys Prxs is enclosed. The dot indicates the position of the stop codon in the GmPer1b sequence. Dashed lines indicate regions encoded by the eliminated portion of the deduced transcript of Glyma05g26400 (gray box in Figure 1A). Gray lines above the GmPer1a sequence show peptide fragments detected by nano LC-MS/MS analysis (Hashiguchi et al. 2009). The accession numbers were: barrel medic (*Medicago truncatula*), Q6E2Z6; barley (*Hordeum vulgare*), P52572; wheat (*Triticum aestivum*), AAQ74769; rice (*Oryza sativa*), AAQ01200; thale cress (*Arabidopsis thaliana*), NP_175247; buckwheat (*Fagopyrum esculentum*), AAF12782; human (*Homo sapiens*), AAH53550; mouse (*Mus musculus*), NP_031479; fruit fly (*Drosophila melanogaster*), AAG47822.

codon inside the coding region (Figure 1B). By sequencing the genomic DNA of *GmPer1a* and *GmPer1b* from cv. Enrei, we confirmed that *GmPer1b* contains a stop codon at this position (data not shown). In the soybean genome database, based on a gene prediction program, a deduced transcript without the stop codon was inferred by considering this region as an intron (Figure 1A, gray box; Figure 1B, dashed line). In this case, some residues conserved among 1-Cys Prxs of other organisms were also eliminated; therefore, this deduction might not be true. In addition, by BLAST search of *G. max* ESTs (http://soybase.org/), only two clones (BM525652, BM519565), which are from a same library constructed with somatic embryos of cv. Jack, were identified as *GmPer1b* sequences; almost all clones (34 clones) corresponded to *GmPer1a*. These two clones, further, seemed to be due to contamination by genomic DNA, since introns remained and many stop codons existed. Therefore, *GmPer1b* might be a pseudogene.

It has been reported that 1-Cys Prx is an antioxidant enzyme having thiol-dependent peroxidase activity (Tripathi et al. 2009), although the physiological electron donor is still unclear (Dietz et al. 2006; Pulido et al. 2009). According to the method described by Pulido et al. (2009) with minor modification, we examined a peroxidase activity of recombinant GmPer1a protein with DTT as the electron donor. Recombinant His-tagged GmPer1a was synthesized with an ENDEXT Wheat Germ Expression H Kit (CellFree Sciences, Ehime, Japan), purified by Ni-Sepharose (GE Healthcare,

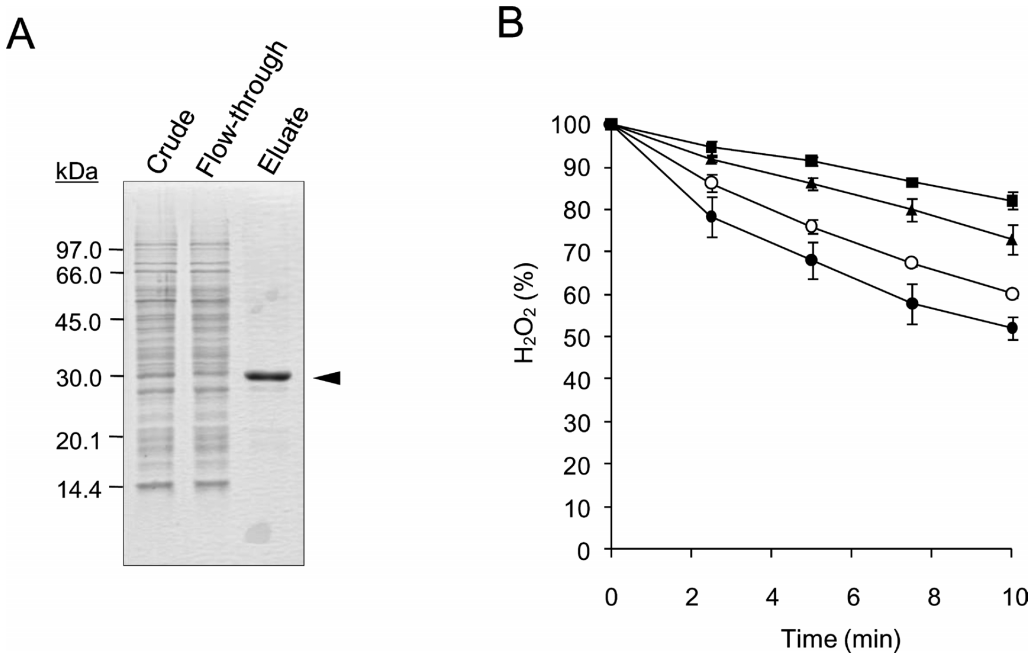

Figure 2. Peroxidase activity of GmPer1a. (A) SDS-PAGE of synthesized and purified recombinant GmPer1a. His-tagged GmPer1a was synthesized by a wheat germ expression system and then applied to a Ni-Sepharose column. His-tagged GmPer1a was eluted with 0.5 M imidazole. Crude, total extract after synthesis by wheat germ expression system; Flow-through, flow-through fraction; Eluate, fraction eluted with 0.5 M imidazole. Arrowhead, His-tagged GmPer1a. (B) Peroxidase activity of recombinant GmPer1a. The activity was assayed as reduction of H_2O_2 in a reaction mixture. The relative amount of H_2O_2 remaining in the mixture after the indicated incubation time is shown. Purified GmPer1a (Eluate shown in Figure 2A) was desalted and then used for the assay (filled circles). As a negative control, eluate after expression in the wheat germ expression system and purification without GmPer1a synthesis was desalted and used for the assay (squares). The assays without DTT (open circles) and with recombinant GmPer1a pretreated in 4 mM H_2O_2 (triangles) were also performed. Data are the means of triplicate experiments (±SD).

Buckinghamshire, UK) (Figure 2A). Apparent molecular size of His-tagged GmPer1a (about 31 kDa) was larger than the size expected from amino acid sequence (25.9 kDa). Such difference is not unusual since gel mobility is influenced by various properties of proteins such as amino acid composition (Shirai et al. 2008). The recombinant protein was then desalted through a Sephadex G-25 column (GE Healthcare). The activity was assayed at 25°C in a reaction mixture (100 mM phosphate buffer, pH 7.0, 3.2 μM His-tagged GmPer1a, 60 μM H_2O_2 and 2.2 mM DTT) by measuring the decrease of H_2O_2 in a colorimetric reaction with the PeroXOquant reagent (Thermo Fisher Scientific, Rockford, IL, USA). As shown in a study of wheat 1-Cys Prx (Pulido et al. 2009), GmPer1a exhibited unambiguous peroxidase activity compared with the negative control assayed in the absence of His-tagged GmPer1a (Figure 2B). Without DTT, the peroxidase activity of GmPer1a was decreased. In addition, incubation of GmPer1a in 4 mM H_2O_2 for 15 min at 25°C before the assay much lowered the activity. These results seem to be consistent with the assumed catalytic mechanism of 1-Cys Prx; catalytic cysteine is transformed into a sulphenic acid derivative after the reduction of the hydroperoxides and the cysteinyl residue relies on an external electron donor (in the present study, DTT) for regeneration to enter into next catalytic cycle (Tripathi et al. 2009).

To analyze expression of the *GmPer1a* and *GmPer1b* genes, total RNA prepared from each organ of soybean plants, various maturation stages of seeds and germinating seeds and seedlings was subjected to RT-PCR. Soybean plants were grown at 25°C with 14/10 h light/dark regime at 300 μmols s^{-1} m^{-2} light intensity. Primer sets specific to either *GmPer1a* (primer 3, 5′-AACGAGGAGTGAAGCTCTTA-3′ and primer 4, 5′-CATTGGTGACATCAGGGGTA-3′) or *GmPer1b* (primer 5, 5′-AACGAGGAGTGAAGTTATTG-3′ and primer 6, 5′-CTTTGATGGAAGATCCACAG-3′) (Figure 1A) or *18S rRNA* (primer 7, 5′-TGATTAACAGGGACAG-TCGG-3′ and primer 8, 5′-ACGGTATCTGATCGTCTT-CG-3′) as a control were used. Expression of the *GmPer1a* gene was detected mainly in developing and mature seeds, and its expression was detected both in hypocotyls and cotyledons (Figure 3A). In addition, expression of the *GmPer1a* was detected immediately after sowing, but was not detected 48 h after sowing. In contrast to *GmPer1a*, expression of the *GmPer1b* gene was barely detectable in soybean plants or seeds, suggesting again that *GmPer1b* is a pseudogene. It has been reported that *1-Cys Prx* genes of various plants, including *Per1* of barley (Aalen et al. 1994), *pBS128* of brome grass (Goldmark et al. 1992), *FePer1* of buckwheat (Lewis et al. 2000), *R1C-Prx* of rice (Lee et al. 2000) and *AtPer1* of *Arabidopsis* (Haslekås et al.

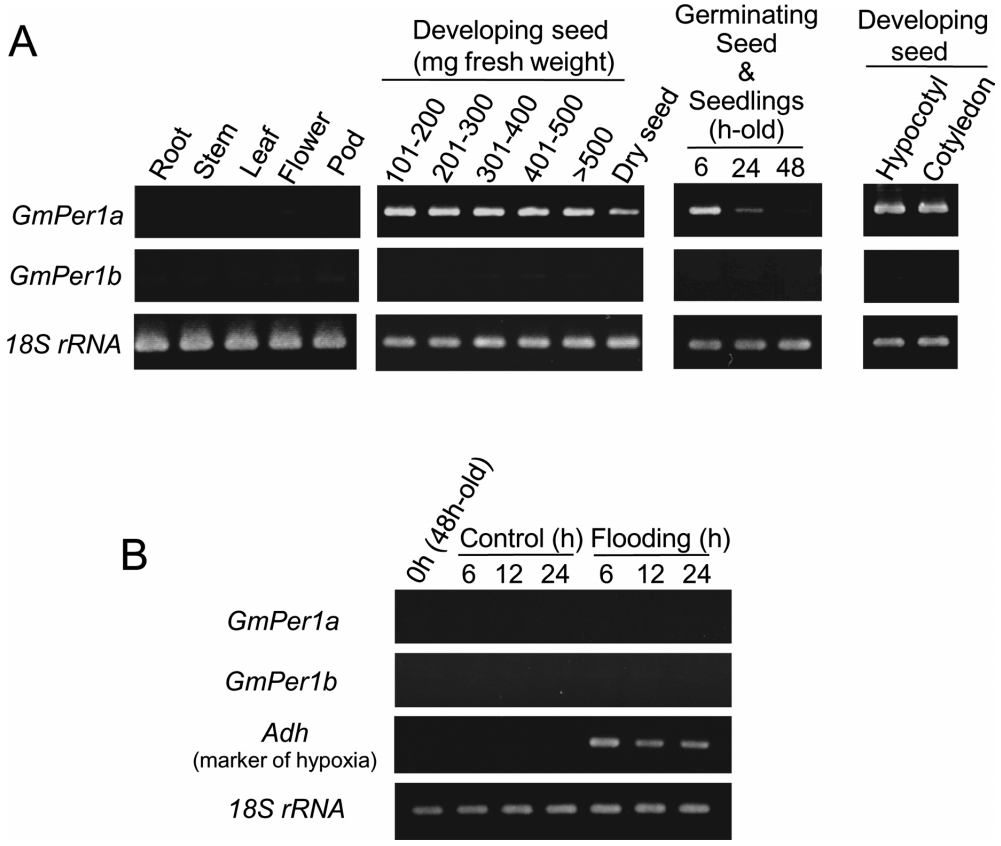

Figure 3. Expression analysis of the *GmPer1* genes by RT-PCR. (A) Seed-specific expression of the *GmPer1* gene. Total RNA prepared from various organs of soybean plants and various stages of soybean seeds and seedlings was subjected to RT-PCR. The maturation stage of developing seeds was represented by seed fresh weight. Age of germinating seeds and seedlings was represented by time after sowing (h-old). Expression of the *GmPer1* genes in either hypocotyl or cotyledon of developing seeds (301–400 mg fresh weight) is also shown. (B) Expression of the *GmPer1* and *Adh* genes under flooding stress. 48-h-old soybean seedlings (0 h treatment) were submerged for 6–24 h. Total RNA prepared from whole seedlings was subjected to RT-PCR. As a control, total RNA prepared from seedlings grown without flooding for the same period was also subjected to RT-PCR.

1998), are expressed in developing seeds. Expression of *AtPer1* gene was detected in both hypocotyls and cotyledons in *in situ* hybridization studies (Haslekås et al. 1998). In the present study, we also showed that the *GmPer1a* is expressed only in seeds, and expressed both in hypocotyls and cotyledons.

To analyze the synthesis and accumulation of GmPer1 protein, total protein extracted from either cotyledons or hypocotyls and roots of developing, dry and germinating seeds and seedlings of soybean with SDS-sample buffer [62 mM Tris-HCl (pH 6.8), 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.5 mM PMSF] was applied to SDS-PAGE and western blotting with a polyclonal antibody raised against recombinant GmPer1a. GmPer1 protein was detected in both hypocotyls and cotyledons of developing seeds (Figure 4A). The apparent size of GmPer1 (about 28 kDa, white arrowhead in Figure 4A) was larger than the size expected from amino acid sequence (24.4 kDa). The gel mobility might be influenced by some protein properties. GmPer1 protein was also detected in dry and germinating seeds, and gradually decreased concurrently with germination and seedling growth (24–72 h post-sowing). This indicates that the GmPer1 is synthesized and accumulates in developing seeds and is degraded during germination and seedling growth, as well as 1-Cys Prx of other plants, such as barley Per1 (Stacy et al. 1999) and rice R1C-Prx (Lee et al. 2000).

In our previous proteome analysis (Hashiguchi et al. 2009), GmPer1 was detected as an up-regulated protein after 24-h-flooding treatment of 48-h-old seedlings. To examine whether *GmPer1* genes are induced by flooding stress, 48-h-old soybean seedlings germinated on sand were submerged in water for 6–24 h at 25°C in a growth chamber (flooding treatment), and then total RNA was extracted from the seedlings. To confirm that the soybean seedlings really suffered flooding stress, expression of the *Adh* gene of soybean (Glyma04g41990), which is one of the marker genes for hypoxia (Newman and Vantoai 1992; Russell et al. 1990), was examined using *Adh*-specific primers (primer 9, 5′-ATGTCGAGCACAGC TGGCCAAG-3′ and primer 10, 5′-CCTGTGCAAATTC CACAACTGAGAAC-3′). Expression of the *Adh* gene was detected by flooding treatment, whereas expression of neither the *GmPer1a* nor *GmPer1b* was detected (Figure 3B) indicating that neither gene is induced by

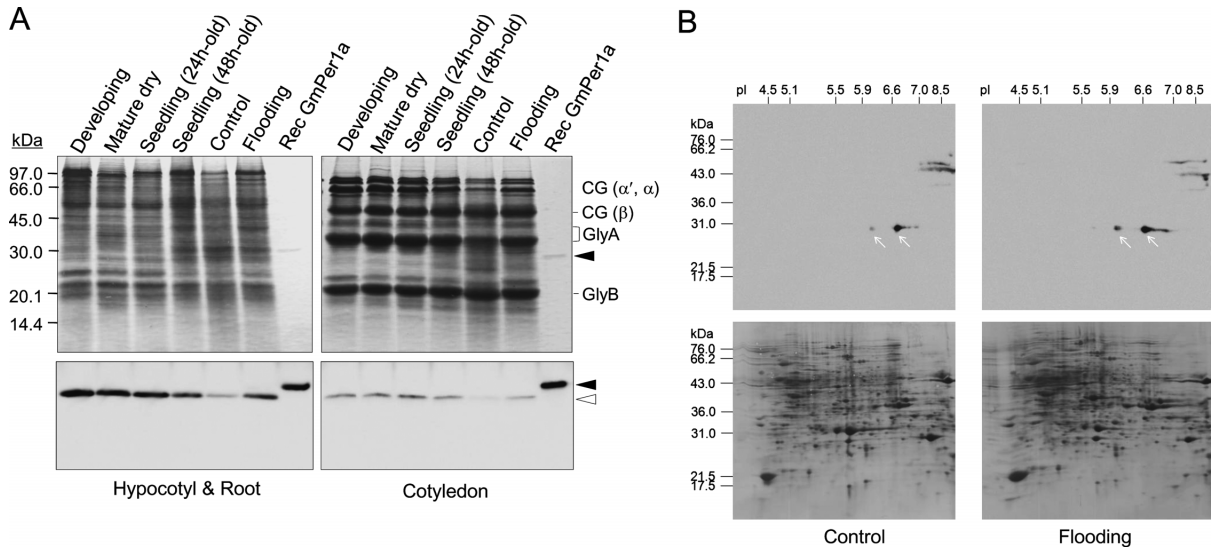

Figure 4. Behavior of GmPer1 in soybean seeds and seedlings, and effect of flooding stress on its behavior. (A) Accumulation and degradation of GmPer1 in soybean developing (301–400 mg) and mature dry seeds and seedlings (24 or 48 h after sowing), and suppression of degradation in seedlings submerged for 24 h. 48-h-old seedlings were submerged for 24 h (Flooding), whereas control seedlings without flooding treatment continued growing until 72 h after sowing (Control). Total protein (15 μg) extracted from either hypocotyl and root or cotyledon was applied to SDS-PAGE followed by CBB staining (upper panels) or western blotting with GmPer1 antibody (lower panels). To confirm the specificity of the antibody, 75 ng of purified His-tagged GmPer1a (Rec GmPer1a) was also applied. Black arrowhead, His-tagged GmPer1a; White arrowhead, GmPer1; CG (α', α), α' and α subunits of β-conglycinin; CG(β), β subunit of β-conglycinin; GlyA, A chains of glycinin; GlyB, B chains of glycinin. (B) Immunodetection of GmPer1 after 2-DE. Flooding treatment was performed for 24 h as described above. Total protein (400 μg) extracted from either roots and hypocotyls of submerged soybean seedlings or those of control seedlings was applied to 2-DE followed by western blotting with the antibody to GmPer1 (upper panels). After chemiluminescence detection, the PVDF membranes were stained with CBB (lower panels). Arrows indicate spots of GmPer1.

flooding stress.

The sensitivity and specificity of immunological detection are higher than gel-based proteome analysis. We therefore examined the behavior of GmPer1 in soybean seedlings under flooding stress by western blot analysis with the antibody to GmPer1. As shown in Figure 4A, the amount of GmPer1 in hypocotyls and roots and in cotyledons of seedlings suffered 24-h-flooding treatment (Flooding) was higher than in those of normally grown seedlings (Control), as was reported based on proteome analysis. In addition, the amount of GmPer1 was decreased in normally grown 72h-old seedlings (Control) compared with that in 48h-old seedlings, while the amount was not decreased in the seedlings suffered 24-h-flooding treatment started 48h after sowing (Flooding). Considering that expression of the *GmPer1* genes was not induced by flooding treatment (Figure 3B), GmPer1 protein was not newly synthesized but remained in seedlings because of a delay in degradation under flooding stress. Both β-conglycinin and glycinin, major seed storage proteins of soybean, are degraded during seedling growth (Wilson et al. 1986). In cotyledons of negative control seedlings for flooding treatment, which are 72-h-old, degradation of β-conglycinin and glycinin was prominent (Figure 4A, Control). On the other hand, in seedlings that had undergone flooding for 24 h, they remained much as in 48-h-old seedlings [compare "Seedling (48-h-old)" and "Flooding" in right panel of Figure 4A]. This suggests that soybean seedlings stopped growing under flooding conditions, and the degradation of GmPer1 therefore stopped. We next performed western blot analysis following 2-DE to examine the behavior of GmPer1 in more detail. Protein was extracted from hypocotyls and roots of soybean seedlings prepared as detailed in Figure 3 with extraction solution [7 M urea, 2 M thiourea, 5% (w/v) CHAPS, 2 mM tributylphosphine, 0.4% (v/v) Bio-Lyte 3/10 (Bio-Rad, Hercules, CA, USA)] and then separated first in immobilized pH gradient (IPG) strips (pH 3–10 NL, 11 cm long) and then in a 15% polyacrylamide gel according to the manufacturer's instructions. Proteins were transferred to PVDF membrane and probed with the GmPer1 antibody. At least two spots with the same molecular weight and different pI were observed not only in seedlings submerged for 24 h but also in control (normally grown) seedlings (Figure 4B); in our previous study, the existence of these spots was not clear in normally grown seedlings (Hashiguchi et al. 2009). Considering that only one *1-Cys Prx* gene (*GmPer1a*) in soybean genome likely encodes a full-length GmPer1 protein (Figures 1, 3), one of these protein spots must be a post-translationally modified form of GmPer1a. Pulido et al. (2009) reported that the peroxidatic Cys residue (Cys 46) of wheat 1-Cys Prx suffers overoxidation in the presence of hydrogen peroxide, resulting in a change in its pI, and

that two forms of 1-Cys Prx (one normal and the other probably overoxidized) exist in wheat seeds. In addition, it was also shown that the overoxidization of active-site cysteine of 1-Cys Prx into cysteine sulphinic or sulphonic acid results in a change in its pI in HeLa S3 cells (Wagner et al. 2002). Therefore, two spots of GmPer1 in the present study might also be normal and overoxidized forms of GmPer1a. However, the modification would not be specific for flooding stress, since the two forms of GmPer1a were detected not only in submerged seedlings but also in normally grown seedlings.

A transient increase and immediate reduction in hydrogen peroxide under low oxygen conditions has been reported (Banti et al. 2010). As 1-Cys Prx has antioxidant activity (Figure 2; Lee et al. 2000; Manevich and Fisher 2005; Tripathi et al. 2009), GmPer1 might function in the removal of hydrogen peroxide at the beginning of flooding treatment (hypoxia). In addition, GmPer1 remaining in submerged seedlings might somewhat alleviate postanoxic injury involving oxygen toxicity when the seedlings return to aerobic condition (VanToai and Bolles 1991). However, it is also assumed that GmPer1 is synthesized in developing seeds of soybean in preparation for removal of peroxides in germinating seeds, where reactive oxygen species are generated (Puntarulo et al. 1991; Wojtyla et al. 2006), and that the degradation of two forms of GmPer1 as well as several other proteins is suppressed just as a result of growth retardation of seedlings under flooding stress. A transgenic approach might elucidate physiological functions of GmPer1.

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