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Oxidative protein folding

Selective pressure for *prolamin* evolution in rice

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Abbreviations: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; PB, protein body; QSOX, quiescin sulfhydryl oxidase; TRX, thioredoxin; VKOR, vitamin K epoxide reductase

During seed development, endosperm cells of highly productive cereals, including rice, synthesize disulfide-rich proteins in large amounts and deposit them into storage organelles. Disulfide bond formation involves electron transfer and generates H_2O_2 as a by-product. To ensure proper development and maturation of seeds, the endosperm cells must supply large amounts of oxidizing equivalents to dithiols in nascent proteins in a controlled manner. This review compares multiple oxidative protein folding systems in yeast, cultured human cells and rice endosperm. We discuss possible roles of ERO1, other sulfhydryl oxidases, and the protein disulfide isomerase family in the formation of disulfide bonds in storage proteins and the development of protein bodies. Rice prolamins, encoded by a multigene family, are divided into Cys-rich and Cys-depleted subgroups. We discuss the potential importance of disulfide bond formation in the evolution of the *prolamin* family in *japonica* rice.

Introduction

Prokaryotes and eukaryotes have evolved distinct electron transfer systems for disulfide bond formation, such as DsbA-DsbB in the periplasm of *Escherichia coli*,[1] ERO1-PDI in the endoplasmic reticulum (ER),[2] and Erv1-Mia40 in the intermembrane space of mitochondria.[3] Protein disulfide isomerase (PDI), which is a ubiquitous housekeeping enzyme found in all eukaryotic cells, is the principal catalyst for the formation of native disulfide bonds, functioning as the direct donor of disulfides to polypeptides in dithiol-disulfide exchange reactions.[4] The genomes of rice (*Oryza sativa*), Arabidopsis (*Arabidopsis thaliana*), and maize (*Zea mays*) each encode about 20 members of the PDI family.[5] However, it remains unknown how the plant cells establish an ER redox environment favorable for oxidation of PDI and various substrates that are physicochemically and structurally diverse.

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Because rice endosperm synthesizes large amounts of disulfide-rich storage proteins during seed development, it provides an excellent system for studying how the cell can maintain an oxidizing environment in the ER. This review briefly summarizes the function of multiple systems for oxidative protein folding and focuses in particular on the functions of the oxidoreductase ERO1 and specific members of the PDI family. We then discuss the possible relationship between the formation of disulfides and the evolution of the *prolamin* family in *japonica* rice.

Rice Seed Proteins and Development of Protein Storage Organelles

During seed development, rice endosperm cells synthesize large amounts of disulfide-bonded storage proteins which differ considerably in structure and physicochemical properties: glutelins (acid- or alkaline-soluble 11S-type globulins), prolamins (highly hydrophobic and alcohol-soluble), and α-globulin (saline-soluble).[6]

Disulfide bond formation plays a critical role in accumulating storage proteins in protein bodies (PBs). Prolamins are polymerized through intermolecular disulfide bonds and packed into the ER-derived type-I PB (PB-I; spherical structure with a diameter of 1–2 μm).[7-9] The 10-kDa Cys-rich prolamins (Os03g0766100) are concentrated at the center core region of PB-I and the 13-kDa Cys-poor prolamins (Os05g0329100) are distributed mainly to the outer layers of PB-I.[10,11] In contrast, glutelin precursors (proglutelins) and α-globulin acquire intramolecular disulfide bonds and then exit the ER for delivery to the protein storage vacuole type-II PB (PB-II; crystalloid structure with a diameter of 2–4 μm). Proglutelins that are targeted to PB-II are processed into acidic and basic subunits by a vacuolar processing enzyme (VPE) and accumulate in the form of a higher-order complex through intermolecular disulfide bonds and hydrophobic interactions.[7,12-14] Inside PB-II, mature glutelins and α-globulin are predominantly segregated to the crystalloids and matrix, respectively.[9] Recent studies indicated that the processing of proglutelins is necessary for the formation of crystalloids[14] and that lowering the α-globulin level leads to malformed PB-II.[15]

In addition to oxidative protein folding, RNA targeting and intracellular trafficking systems play important roles in the

regulation of PB development in rice endosperm. mRNAs encoding glutelins and prolamins are targeted to discrete subdomains of the ER.[16,17] Proglutelins are targeted from the ER to PB-II via the Golgi apparatus.[18] Recent studies showed that a small GTPase protein, Rab5a, plays a critical role in the vesicular trafficking of proglutelins to PB-II.[19,20]

The ERO1-Dependent Electron Transfer Pathway: Roles in Disulfide Bond Formation and Development of Protein Bodies in Rice Endosperm

Oxidative protein folding of secretory proteins in the ER has been extensively studied in the yeast *Saccharomyces cerevisiae* and in cultured human cells.[2] As shown in **Figure 1A**, the oxidizing power for the oxidation of a pair of sulfhydryls in a nascent secretory protein is directly supplied by oxidized PDI.[4] The reduced form of PDI is then reoxidized by the FAD-containing sulfhydryl oxidase ERO1.[21,22] The net products of the electron transfer are one disulfide bond and one molecule of H_2O_2. Structural studies on yeast ERO1 have identified the active and shuttle Cys pairs.[23] Besides these catalytic Cys residues, ERO1 proteins contain regulatory Cys residues, which play an important role in redox-dependent feedback regulation of ERO1 activity in response to fluctuations in the ER redox environment.[24,25]

Whole-genome sequencing has identified members of sulfhydryl oxidase families, including ERO1 and PDI, in the genomes of rice, Arabidopsis and maize. When RNAi knockdown of rice *ERO1* (Os*ERO1*) is induced under the control of an endosperm-specific promoter, the formation of native disulfide bonds in proglutelins is inhibited, and instead the formation of nonnative intermolecular disulfide bonds is promoted, leading to the formation of proglutelin-containing aggregates.[26] Endosperm cells with severely reduced levels of OsERO1 exhibit defects in the development of PB-I and PB-II, and instead form abnormal aggregates of small particles.[26]

ERO1 proteins are tightly associated with the luminal face of the ER membrane in yeast[27] through the C-terminal tail of 127-aa residues,[28] whereas human ERO1α and ERO1β are retained in the ER lumen by covalent interactions with PDI and ERp44, another member of the PDI family.[29] In rice endosperm, OsERO1 is an ER-localized integral membrane protein, and the ER localization of OsERO1 depends on a hydrophobic sequence present in the N-terminal region.[26] The mechanism by which OsERO1 is retained in the ER membrane remains unclear.

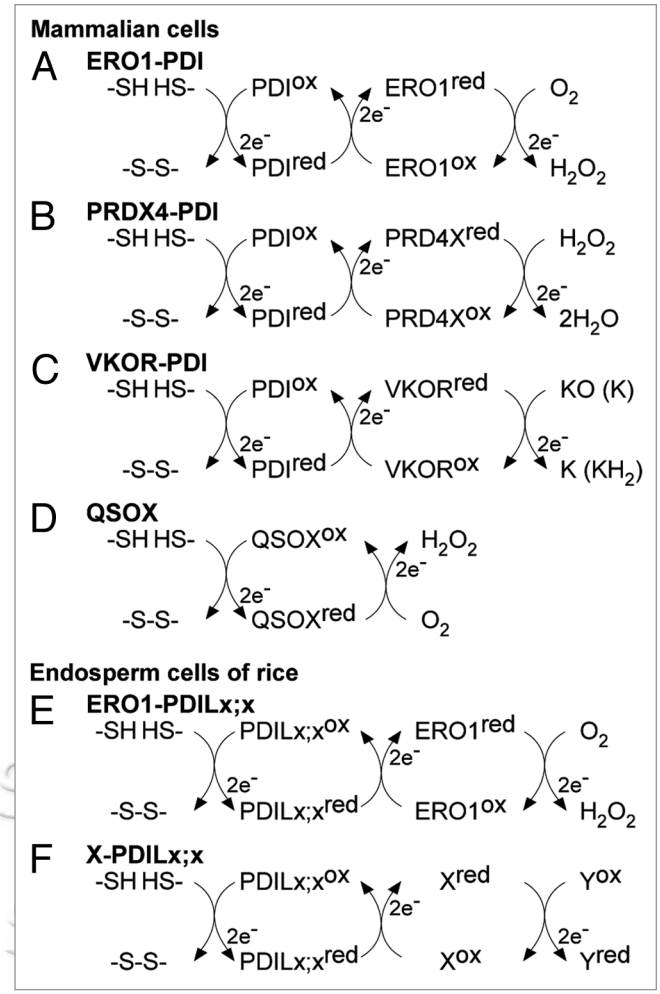

Figure 1. Multiple electron transfer pathways for oxidative protein folding in the ER of mammalian and endosperm cells. Listed pathways are (A) ERO1-PDI, (B) peroxiredoxin IV (PRDX4)-PDI, (C) vitamin K epoxide reductase (VKOR)-PDI and (D) quiescin sulfhydryl oxidase (QSOX), which all facilitate oxidative protein folding in the ER of mammalian cells. In the ER of rice endosperm, it is unlikely that OsERO1 directly oxidizes the active sites of OsPDIL1;1. Instead, it is plausible that OsERO1 oxidizes as-yet-unidentified members of the PDI family, indicated by PDILx;x, to promote the oxidative folding of storage proteins (E). Although there is no direct evidence, other oxidoreductases (X) and some members of the PDI family, indicated by PDILx;x, may also operate in oxidative folding of storage proteins (F). Y^{ox} represents unidentified electron acceptors, such as O_2, H_2O_2 and quinone (F). KO, vitamin K epoxide; K, quinone; KH_2, hydroquinone.

Distinct but Coordinated Functions of PDI Family Members in Rice Endosperm

The PDI family members vary widely in the organization of the catalytic thioredoxin (TRX)-fold domains (a and a′), which have redox-active Cys-x-x-Cys motifs, and the noncatalytic TRX-like domains (b and b′). The genomes of the higher plants, such as rice, Arabidopsis, and maize, each contain at least seven members of the PDI family with two redox-active sites, PDIL1;1–1;4 and PDIL2;1–2;3.[5] PDIL1;1–1;4 members have an a-b-b′-a′ domain organization, similar to that of human PDI and ERp57 and yeast Pdi1p, a U-shaped molecule in which the two active-site Cys pairs face each other.[30] PDIL2;1–2;3 members, however, have an a-a′-b domain organization, similar to that of human ER protein 5 (called P5), in which two redox-active TRX-like domains repeat in tandem followed by a single redox-inactive TRX domain.[31] Members of the PDI and P5 subfamilies are likely to have evolved to acquire distinct redox activities. Human P5 shows lower reductase activity (using insulin as substrate) and chaperone activity than human PDI.[32,33] Rice OsPDIL2;3, which belongs to the P5 subfamily, exhibits lower activity for oxidative folding of α-globulin in vitro than does OsPDIL1;1, a PDI

subfamily member.[10] However, recombinant OsPDIL2;3 exhibits higher sulfhydryl oxidase activity for the formation of nonnative disulfide bonds between α-globulin Cys79Phe mutant proteins.[10]

Multiple lines of evidence support specific functions of the PDI family members. In human cells, PDI prevents neurotoxicity associated with ER stress and with protein misfolding in Parkinson disease.[34] Human P5 promotes tumor immune evasion by reducing disulfide bonds in the major histocompatibility complex class I-related ligand MICA on the surface of tumor cells[35] and promotes breast cancer progression by activating ErbB2 and phosphoinositide-3-kinase signaling.[36] In Arabidopsis, AtPDIL1;1 regulates the timing of programmed cell death in the endothelial cells,[37] whereas AtPDIL2;1 plays a role in embryo sac development.[38] Although the distinct expression patterns and catalytic activities of the PDI members suggest a potential for specific functions, the actual specificity or redundancy of the individual PDI members in vivo remains an open question.

In a recent study, we demonstrated that OsPDIL1;1 and OsPDIL2;3, when expressed in the same rice cell, play distinct roles in the formation of the two types of PBs. OsPDIL1;1 is evenly distributed within the dilated ER and plays an important role in the formation of disulfide bonds in the PB-II-targeted storage proteins, including proglutelins and α-globulin.[26] In contrast, OsPDIL2;3 is mostly localized on the surface of PB-I and has a specific function in the intermolecular disulfide bond-assisted assembly of prolamin polypeptides in PB-I.[10,26] In addition to these distinct roles of OsPDIL1;1 and OsPDIL2;3, their coordinated functions are required for the development of PB-I and PB-II in the endosperm. The activity of OsPDIL1;1 indirectly supports the assembly of prolamin species in the ER.[10] Additionally, OsPDIL1;1 may be involved in the formation of a structural disulfide bond in the b domain of OsPDIL2;3.[10]

In yeast, among the active sites of PDI family members present in the ER, the CxxC active site in the N-terminal TRX domain of Pdi1p is preferentially oxidized by ERO1.[39] Surprisingly, complementation analysis suggests that the a and a′ domains of OsPDIL1;1 may independently assist the oxidative folding of proglutelins through reduction and/or isomerization reactions.[10] Because ERO1 oxidizes only the defined active sites of the PDI family members in yeast and mammalian cells, it is unlikely that OsERO1 directly oxidizes the active sites of OsPDIL1;1 (**Fig. 1E**). Alternatively, if OsERO1 does oxidize the active sites of OsPDIL1;1, it is likely that other oxidases also provide oxidizing power for proglutelins via electron transfer pathways that do not involve OsPDIL1;1 (**Fig. 1F**).

ERO1-Independent Disulfide Formation and Other Candidate Sulfhydryl Oxidases

In simple eukaryotes, such as yeast, the lack of ERO1 impairs cell viability.[27,40] Mammalian and plant ERO1 proteins also function in the process of disulfide bond formation in the ER. Nevertheless, when the functions of two mouse paralogues of human ERO1, ERO1α and ERO1β, are simultaneously impaired, the mice are still viable although having a mild diabetic phenotype.[41] *ERO1*-knockdown endosperm cells of rice also produce a reduced, but substantial, amount of disulfide bonds in storage proteins.

In addition to ERO1, other sulfhydryl oxidases have emerged as candidates for providing oxidizing power for disulfide bond formation in mammalian cells. Peroxiredoxin IV (PRDX4) oxidizes PDI to promote the formation of disulfide bonds in substrates, using H_2O_2 as the source of oxidizing power (**Fig. 1B**).[42] Plants also have the *peroxiredoxin* family, but no ER-localized peroxiredoxin has been identified yet.[43] Another system is the vitamin K epoxide reductase (VKOR)-dependent electron transfer system (**Fig. 1C**). Using oxidized vitamin K, VKOR interacts specifically with membrane-anchored PDI family members to transfer an oxidizing equivalent to PDI for disulfide bond formation in substrates.[44-46] Homologs of VKOR are present in a variety of organisms, spanning from bacteria, archaea, vertebrates and plants.[47] Additionally, quiescin sulfhydryl oxidase (QSOX) functions as a sulfhydryl oxidase in mammalian cells (**Fig. 1D**).[48] It is important to note that QSOX can directly oxidize dithiols of substrate proteins[49] and is localized in structures involved in the secretory pathway, including the Golgi and secretory granules.[50,51]

In rice endosperm, the redox-active form of OsPDIL2;3 is localized mainly on the PB-I surface, whereas the redox-inactive form dissociates from PB-I and is localized in the ER lumen.[10] These results indicate that not only the activity of OsPDIL2;3 but also the specific localization in the ER is regulated by the redox status at the active sites. As mentioned above, because ERO1 oxidizes only the defined active sites of PDI family members in yeast and mammalian cells, and because *S. cerevisiae* does not have an apparent P5 ortholog in its genome, it seems likely that an oxidase other than OsERO1 serves as a source of oxidizing power for OsPDIL2;3. Further studies are needed to identify the direct donor of oxidizing power to OsPDIL2;3 and to elucidate the distinct and overlapping roles of OsERO1 and other sulfhydryl oxidases (**Fig. 1F**).

Roles of O_2 and H_2O_2 in Oxidative Protein Folding

The source of the oxidizing power in the yeast ERO1-PDI system is O_2. ERO1 accepts electrons from the active site of PDI and transfers them to O_2 via the FAD cofactor, producing H_2O_2 as a by-product (**Fig. 1A**).[52] By using an H_2O_2-specific fluorescence probe (BES-H_2O_2) in the living cells of developing endosperm of rice, we showed that the oxidation of sulfhydryl groups is accompanied by the production of H_2O_2 in the ER. The homozygous mutant seeds of the rice EM49 line have fewer sulfhydryl groups for disulfide bonds than the wild-type because they accumulate Cys-rich prolamins at markedly reduced levels.[26,53] Homozygous EM49 mutant seeds produce H_2O_2 in the ER at a substantially reduced level compared with the wild-type.[26]

High concentrations of H_2O_2, a reactive oxygen species (ROS), may cause peroxidation of the membrane lipid and gradually deteriorate the membrane integrity, leading to the leakage of small molecules, including water.[54] Conversely, EM49 homozygous mutant seeds, which produce less H_2O_2, exhibit markedly slower desiccation and maturation than the wild type.[26]

Although ROS, including H_2O_2, are cytotoxic, they also play key roles as signal transduction messengers in eukaryote cells.[55] The 3n endosperm cells, but not the 2n embryo, are destined for programmed cell death during the late stages of seed maturation. The generated H_2O_2 in the ER may have a role as a signal for inducing programmed endosperm cell death and subsequent seed desiccation/maturation. If so, uncontrolled formation of disulfide bonds could lead to premature cell death in the endosperm; therefore, the quantity and timing of disulfide bond formation in storage proteins may affect the seed maturation, and hence the seed quality.

Disulfide Bond Formation and Evolution of the Cys-Depleted Prolamin Subfamily in japonica Rice

Rice prolamins are encoded by a multigene family and have been conventionally divided into Cys-rich and Cys-poor prolamins.[56] We searched the genome of japonica rice (Nipponbare) and conducted a phylogenetic analysis of prolamins together with protein allergens of RA16/17 [57] as an outgroup. RA16/17 are water-soluble monomers containing intramolecular disulfide bonds.[10] Surprisingly, although prolamins and RA16/17 proteins are substantially different in physicochemical properties, RA16/17 proteins are more closely related to prolamins than expected. The phylogenetic tree indicates that prolamins are clustered into three subgroups: (1) 13-kDa prolamins containing 0–8 Cys residues, (2) 10-kDa prolamins containing 9–11 Cys residues and (3) a 16-kDa prolamin containing 13 Cys residues (**Fig. 2A**). These findings suggest that the 10-kDa and the 13-Da/16-kDa subgroups diverged before the 13-kDa and the 16-kDa subgroups branched away from each other, and that the evolution and diversification of prolamins are relatively recent events.

Members of the prolamin superfamily, which includes the 2S albumin, contain conserved ABC peptide regions, and each region contains conserved sequence motifs: LxxC in the A region, CCxQL in the B region, and PxxC in the C region.[9] Interestingly, the 13-kDa Cys-poor prolamins of rice contain replacements or deletion of the Cys residues in these conserved sequence motifs: LxxY in the A region (Cys is substituted by Tyr), --xQL in the B region (Cys pair is deleted), and PxxY in the C region (Cys is substituted by Tyr) (**Fig. 2B**). We therefore hereafter call these Cys-poor prolamins Cys-depleted prolamins to reflect that the Cys residues were selectively mutated within otherwise conserved motifs. Ushijima et al.[59] identified a group of Cys-depleted *prolamin* genes on chromosomes 5 and 7 of *japonica* rice and elegantly showed that the cluster of Cys-depleted *prolamin* genes on chromosome 5 of *japonica* rice (Kinmaze) is not present in the corresponding chromosomal segment of *indica* rice (Kasalath). The phylogenetic tree indicates that the amino acid sequences of Cys-depleted prolamins show higher homology to the Cys-rich prolamins Os07g0206400 and Os07g0206500 than to the other Cys-rich prolamins (**Fig. 2A**). Although speculative, it is plausible that the evolution of Cys-depleted prolamins in *japonica* rice involved a series of mutations at the Cys residues on an ancestral Cys-rich prolamin closely related to Os07g0206400 and Os07g0206500. It is also likely that the resulting Cys-depleted, mutated *prolamin* gene was subsequently multiplied on chromosomes 7 and 5.

Because the critical mutations at the Cys residues that led to the generation of the Cys-depleted prolamin subfamily occurred at otherwise conserved regions in the Cys-rich prolamin (**Fig. 2B**), it is conceivable that these mutations were under strong selective pressure. The Cys-depleted prolamins constitute a high proportion of the total prolamin fraction, and their knockdown causes a reduction in the PB-I size.[60] The mutations at the Cys residues in prolamins and a rapid increase in copy numbers of Cys-depleted *prolamin* genes could reduce the concentration of H_2O_2 in the ER and increase storage substances in PB-I. The evolution of the Cys-depleted prolamin subfamily in *japonica* rice could have reduced the total amount of disulfide bonds in storage proteins, which may have contributed to the establishment of an appropriate redox environment in the secretory system for the development and maturation of seeds. The domestication of highly productive crops, including rice, has involved genetic modifications to meet the needs of humans.[61] We speculate that the dynamic genetic modifications that led to the generation of the Cys-depleted prolamin subfamily played a role in the natural or artificial selection of *japonica* rice. Further studies on the molecular evolution of *prolamin* in *Oryza sativa* subspecies, as well as the ancestors of rice, should provide new insight into how disulfide bond formation and H_2O_2 generation impact the development and quality of seeds and the natural or artificial selection of rice.

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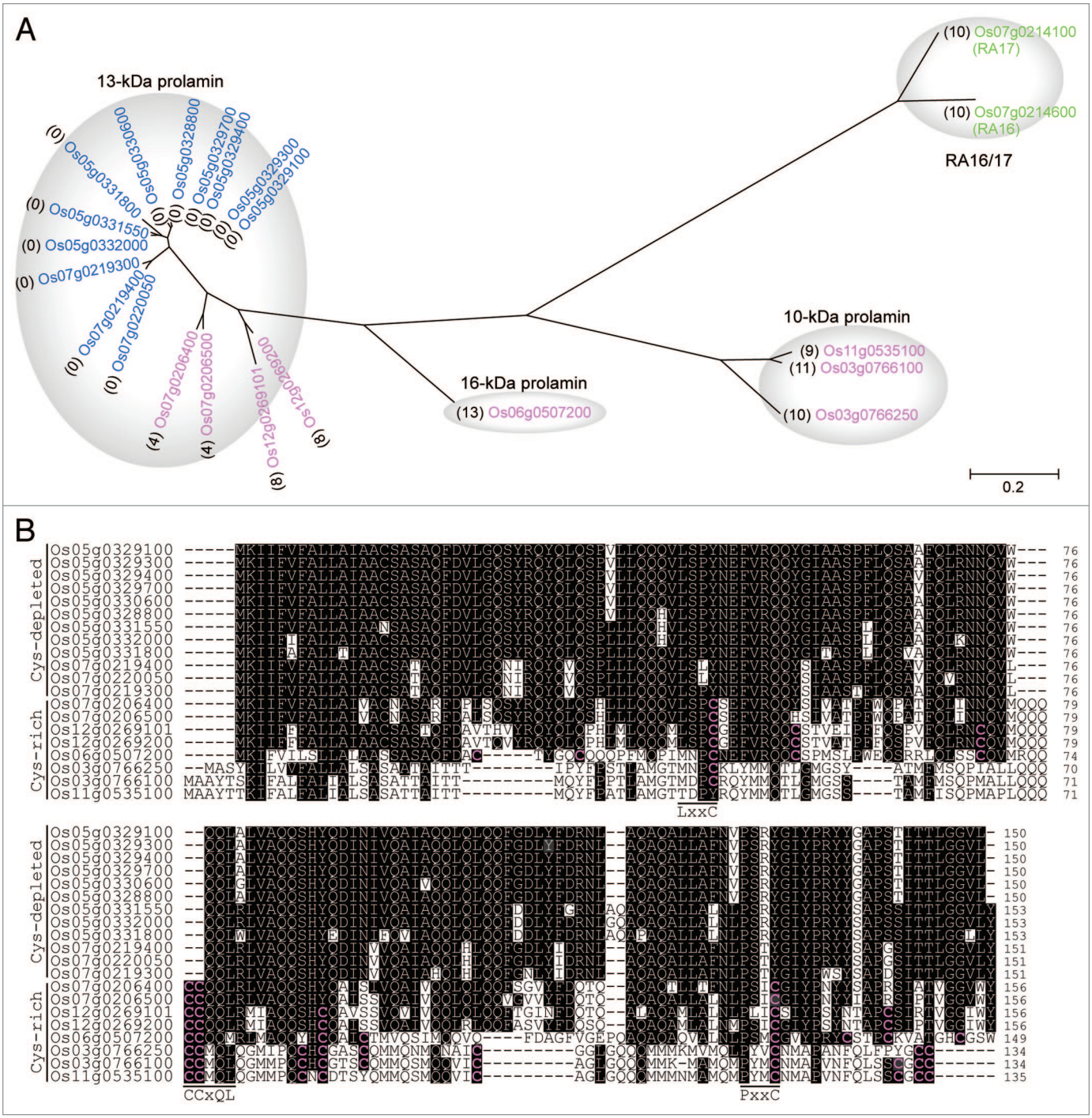

Figure 2. Comparison of the amino acid sequences of prolamins and protein allergens from rice seeds. (A) Phylogenetic analysis of Cys-rich prolamins, Cys-depleted prolamins and protein allergens of RA16/17. The phylogenetic tree was constructed by the neighbor-joining method using MEGA version 4 [58] (www.megasoftware.net). The reliability of different phylogenetic groupings was evaluated by the bootstrap test (1,000 replicates). Magenta, blue and green letters indicate Cys-rich prolamins, Cys-depleted prolamins and RA16/17 proteins, respectively. Numbers in parentheses indicate the numbers of Cys residues in the predicted mature-sized proteins. The NCBI accession numbers are Os03g0766100, NP_001051380; Os03g0766250, NP_001173654; Os05g0328800, NP_001055211; Os05g0329100, NP_001055213; Os05g0329300, NP_001055214; Os05g0329400, NP_001055215; Os05g0329700, NP_001055216; Os05g0330600, NP_001055218; Os05g0331550, NP_001174359; Os05g0331800, NP_001055221; Os05g0332000, NP_001055222; Os06g0507200, NP_001057724; Os07g0206400, NP_001059151; Os07g0206500, NP_001059152; Os07g0219300, NP_001059197; Os07g0219400, NP_001059198; Os07g0220050, NP_001175103; Os11g0535100, NP_001068024; Os12g0269101, NP_001176891; Os12g0269200, NP_001066544. (B) The amino acid sequences of Cys-rich and Cys-depleted prolamins were aligned with the CLUSTALW program. The genes of Os03g0766100 and Os05g0329100 encode the Cys-rich 10-kDa prolamin (crP10) and Cys-depleted 13-kDa prolamin (cpP13), respectively, analyzed in our recent paper.[10] Magenta-on-black letters indicate Cys residues in the predicted mature-sized proteins. White-on-black letters indicate amino acid residues conserved in more than 10 of the sequences analyzed.

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