Differential Localization of Tonoplast Intrinsic Proteins on the Membrane of Protein Body Type II and Aleurone Grain in Rice Seeds

Hideyuki TakaHashi,1 Mika Rai,1 Tomoya Kitagawa,1 Shigeto Morita,1,2 Takehiro Masumura,1,2,1 and Kunisuke Tanaka1,2

1Laboratory of Genetic Engineering, Graduate School of Agriculture, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan
2Kyoto Prefectural Institute of Agricultural Biotechnology, Seika, Kyoto 619-0244, Japan

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Tonoplast intrinsic proteins (TIPs) belong to an aquaporin family of proteins that function as water-transport channels. In this study, we isolated and characterized three novel rice cDNAs for OsTIP1, OsTIP2, and OsTIP3 that are homologous to rice γ-TIP cDNA. Northern blot hybridization analyses revealed that rice γ-TIP was expressed in all plant organs. OsTIP1 was expressed in mature seed embryos and during early seed germination. OsTIP2 was expressed exclusively in roots. OsTIP3 was specifically expressed in seeds. These results suggest that the OsTIP1, OsTIP2, and OsTIP3 genes encode discrete, functionally specialized TIPs. Immunocytochemical analysis in rice endosperm cells revealed that rice γ-TIP was localized only on the protein body type II (PB-II) membranes, whereas OsTIP3 was localized on the PB-II and the aleurone grain membranes. Although both the PB-II and the aleurone grain are derived from vacuoles, these results suggest that they may be derived from different types of vacuoles.

Key words: tonoplast intrinsic protein; protein body; aleurone grain; vacuole; rice

The plant vacuole is a multifunctional organelle that plays important roles in space filling, osmotic adjustment, storage, and digestion. Although previous models have hypothesized that these diverse functions occur within a single vacule, it is now clear that protein storage vacuoles (PSVs), which contain seed-type storage proteins, and lytic vacuoles (LVs), which contain active proteases, are separate organelles. In addition, Jauh et al. defined a third functionally distinct type of vacuole that stores pigments and vegetative storage proteins synthesized in response to developmental and environmental cues in various plant tissues.

Studies of vacuole function and biogenesis were greatly assisted by the finding of tonoplast intrinsic proteins (TIPs). TIPs are integral membrane proteins that were originally found in plant seeds and belong to the major intrinsic protein (MIP) family, the members of which are widely distributed in bacteria, animals, and plants. It has been proposed that TIPs function as aquaporins to regulate water transport in various vacuolar functions. But, TIPs are very abundant proteins, and the level of their expression appear to be in excess of the requirement for physiological water transport, suggesting that these proteins have an additional structural function.

Paris et al. gave reason to conclude that the tonoplasts of PSVs contain α-TIP whereas those of LVs contain γ-TIP. Both types of vacuoles are found in the same cell types in barley roots and maturing pea cotyledons. Although it remains to be determined whether the physiological role of a specific isoform of TIP is associated with a specific functional type of vacuole, it has been proposed that the presence of a specific TIP isoform may define the function of the vacuole.

To date, genes encoding plant TIPs have been identified almost exclusively in Arabidopsis thaliana (10 genes) and maze (11 genes), and have been divided into five groups. The TIP1 group has been extensively analyzed with γ-TIP of Arabidopsis, which is expressed in the vegetative tissues. The TIP2 group corresponds to δ-TIP of Arabidopsis. Vacuoles containing δ-TIP protein may act as storage compartments for pigments and vegetative proteins. The TIP3 group corresponds to α-TIP of Arabidopsis, which is expressed exclusively in seeds. The TIP4 group contains NtTIPa of tobacco, a protein that transports water and glycerol in X. leavis oocytes. The TIP5 group includes not-yet-characterized proteins. Several TIP isoforms may be
expressed in tissue- and time-specific manners and the others may be expressed constitutively. Some isoforms are expected to respond to internal and external stimuli.

In rice, only a single TIP-encoding cDNA, which corresponds to the γ-TIP of *Arabidopsis thaliana*, has been isolated. The γ-TIP was expressed in shoots and roots of seedlings and expression was enhanced by salt and water stresses and exogenous abscisic acid, but only limited information on rice TIPS is available. For this reason, we attempted to isolate and characterize genes for TIPs of rice. We isolated and characterized three novel rice cDNAs that are homologous to rice γ-TIP cDNA. These proteins encoded by the three novel rice cDNAs were designated OsTIP1, OsTIP2, and OsTIP3. We also analyzed expression of these genes in different tissues and developmental stages. Furthermore, we performed an immunocytochemical analysis to examine the localization of rice γ-TIP and OsTIP3 in rice endosperms.

Materials and Methods

Plant materials. Rice (*Oryza sativa* L. cv Nipponbare) was grown with soil in a greenhouse at the experimental farm of the Kyoto Prefectural Institute of Agricultural Biotechnology. Rice dwarf mutant (*Oryza sativa* L. cv Hosetsu d.) was sown in plastic pots filled with soil from the experimental farm. The plants were grown in a growth chamber under fluorescent lamps of 170 μmol m⁻² s⁻¹ under a 16 h light/8 h dark regime at a temperature of 28 °C. Rice seedlings (*Oryza sativa* L. cv Nipponbare) were grown hydroponically in a growth chamber under fluorescent lamps and purified with ProbeQuant G-50 Micro Columns (Amersham Biosciences, Buckinghamshire, U.K.) according to the manufacturer’s instructions. Genomic DNA was extracted from rice leaves of 14-d-old seedlings using the cetyltrimethylammonium bromide method according to standard procedures. The coding region of rice γ-TIP obtained by PCR with a pair of primers (forward, 5'-ATTGCGATCCCGACAAATA-TGCC, reverse, 5'-GTAGTCCGTTGTTGGGAGC) was used as a probe to detect related sequences. The hybridization probes were labeled with [α-³²P] dCTP using a *BcaBEST™ Labeling Kit* (Takara, Otsu, Japan) and purified with ProbeQuant G-50 Micro Columns (Amersham Biosciences, Buckinghamshire, U.K.) according to the manufacturer’s instructions. Genomic DNA was extracted from rice leaves of 14-d-old seedlings using the cetyltrimethylammonium bromide method according to standard procedures. The coding region of rice γ-TIP probe was washed twice at 55 °C for 20 min with 6 × SSC, 0.1% SDS. The membrane hybridized with the probes as described above at 65 °C. The membrane hybridized with the gene-specific probe was washed twice at 55 °C for 20 min with 6 × SSC, 0.1% SDS. The membrane hybridized with the coding region of the rice γ-TIP probe was washed twice at 55 °C for 20 min with 0.5 × SSC, 0.1% SDS. Hybridization signals were visualized with a bio-image analyzer (Molecular Imager; Bio-Rad, Hercules, California, U.S.A.).

DNA sequencing and analysis. Complete nucleotide sequencing of the cDNA clones was performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, U.S.A.) with an ABI PRISM™ 310 genetic analyzer (Applied Biosystems).

Southern blot hybridization analysis. To prepare the gene-specific probe for each rice TIP gene, the 3'-untranslational region (UTR) of each gene was used. The 3'-UTR fragments were obtained by polymerase chain reaction (PCR) with the following primer pairs: for the cDNA of rice γ-TIP (forward, 5'-TAAGCTCATTGCCGCCGCC, reverse, 5'-ACTGTGGCGGACATCG); for OsTIP1 cDNA (forward, 5'-CGCCGACTCTGATCATATC, reverse, 5'-GAACACACAGACAGATTTTGG); for OsTIP2 cDNA (forward, 5'-CGCCTTTAAAGTGATGAACCG, reverse, 5'-GATTTCCTTCTCCAGACAC); and for OsTIP3 cDNA (forward, 5'-AGGACTACTAGACGGCCGCC, reverse, 5'-GGCGGTGATTTTCACTTTATAATGG). In addition, the coding region of rice γ-TIP obtained by PCR with a pair of primers (forward, 5'-ATTGCGATCCCGACAAATA-TGCC, reverse, 5'-GTAGTCCGTTGTTGGGAGC) was used as a probe to detect related sequences. The hybridization probes were labeled with [α-³²P] dCTP using a *BcaBEST™ Labeling Kit* (Takara, Otsu, Japan) and purified with ProbeQuant G-50 Micro Columns (Amersham Biosciences, Buckinghamshire, U.K.) according to the manufacturer’s instructions. Genomic DNA was extracted from rice leaves of 14-d-old seedlings using the cetyltrimethylammonium bromide method according to standard procedures. The coding region of rice γ-TIP probe was washed twice at 55 °C for 20 min with 6 × SSC, 0.1% SDS. The membrane hybridized with the probes as described above at 65 °C. The membrane hybridized with the gene-specific probe was washed twice at 55 °C for 20 min with 6 × SSC, 0.1% SDS. Hybridization signals were visualized with a bio-image analyzer (Molecular Imager; Bio-Rad, Hercules, California, U.S.A.).

Northern blot hybridization analysis. Total RNA was extracted from rice seeds and seedlings of various developmental stages by the phenol-SDS method. Extraction of total RNA from roots, shoots, and suspension-cultured cells was performed by the guanidine-thiocyanate method. The RNA (20 μg) was denatured with formamide, fractionated through 1.2% agarose gels, and transferred to nitrocellulose mem-
branes (Hybond-C extra; Amersham Biosciences). The membrane was hybridized with the same 32P-labeled gene-specific probes used for Southern blot hybridization analysis at 42 °C, and washing was performed twice at 42 °C for 20 min with 2 x SSC, 0.1% SDS. Hybridization signals were visualized with a bio-image analyzer (Molecular Imager; Bio-Rad).

Production of anti-TIP peptide antibodies. Synthetic peptides of rice γ-TIP and OsTIP3 and their related affinity-purified rabbit anti-peptide polyclonal antibodies were produced by Qiagen (Hilden, Germany). The synthetic peptides were based on the carboxyl-terminal, cytoplasmic-tail sequences of each: rice γ-TIP, ISHTHEQLPTTDY; OsTIP3, GGAHQPLAPEDY.

Immunoblot analysis. Total proteins from rice seeds were extracted by the method previously described.5) Rice seeds were homogenized directly in denaturing buffer [60 mM Tris–HCl (pH 6.8), 15% (v/v) glycerol, 4% (w/v) SDS, 4% (v/v) β-mercaptoethanol] (5 ml/g of seeds fresh weight) at 4 °C with mortar and pestle. The homogenate was centrifuged twice for 15 min at 15,000 × g. The supernatant (total extractable protein) was incubated for 15 min at 37 °C. The total protein samples (2 μl/lane) were separated by SDS-PAGE with a 16% gel. For immunoblot analysis, the proteins separated by SDS-PAGE were electro-transferred from a gel to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences). The anti-TIP peptide primary antibodies and the alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody (Promega, Madison, Wisconsin, U.S.A.) were used at a 1:10,000 dilution. The band was stained with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (BCIP/NBT Color Development Substrate; Promega) according to the manufacturer’s instructions.

Immunocytochemical analysis. Developing rice seeds were vacuum infiltrated for 10 min with a fixative that consisted of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), and treated for another 3 h at room temperature with the fixative. After washing with the same buffer, the seeds were dehydrated in a graded ethanol series and cut into slices. The samples were then embedded in LR White resin (London Resin Co., Ltd., Hampshire, U.K.). Blocks were polymerized at 55 °C for 48 h. Ultrathin sections were cut with a diamond knife using a Leica Ultracut UCT (Leica, Heidelberg, Germany) and mounted on nickel grids.

For immunocytochemical analysis, the sections were incubated with a solution of each anti-TIP peptide primary antibody diluted 1:1,000 in the blocking solution for 2 h at room temperature. After washing with 0.1 M phosphate buffer (pH 7.2), the sections were incubated with a solution of 10 nm gold-labelled goat anti-rabbit IgG secondary antibody (Amersham Biosciences) diluted 1:50 in the blocking solution for 2 h at room temperature. The sections were washed with distilled water and then stained with 2% uranyl acetate. After staining, all sections were examined with a transmission electron microscope (JEM-1220; Tokyo, Japan) at 100 kV.

Results

Molecular cloning and sequencing of three novel rice TIP isoform cDNAs

The rice proteins registered in the GenBank, PDB, SwissProt, PIR, and PRF databases were searched against the amino acid sequence of rice γ-TIP (accession no. D25534) using the program blastp. Three putative proteins (accession nos. BAB63833, CAC39073, and AAG13544) were selected from seven hits with significant similarity on the query sequences. Then Oryza sativa-ESTs in the GenBank, EMBL, and DDBJ databases were searched with the blastn program using nucleotide sequences encoding each protein. Three EST clones, E1680, R1507, and E11967, were selected as the respective EST clones encoding these proteins. These three clones were provided by the Rice Genome Research Program of the National Institute of Agrobiological Sciences (Tsukuba, Japan).

The proteins encoded by the cDNA clones for E1680 and R1507 were designated OsTIP1 and OsTIP2 respectively. A full-length cDNA encoding the EST clone E11967 was isolated using the 3’-RACE technique, and the protein encoded by this clone was designated OsTIP3. The nucleotide sequence for OsTIP1 was 970 bp in length without a polyadenylate tail, and was made up of a 10 bp 5’ leader sequence followed by 756 bp of an open reading frame encoding 252 amino acids and a 204 bp 3’ noncoding region. The nucleotide sequence for OsTIP2 was 1013 bp in length without a polyadenylate tail, and was made up of a 73 bp 5’ leader sequence followed by 744 bp of an open reading frame encoding 248 amino acids and a 196 bp 3’ noncoding region. The nucleotide sequence for OsTIP3 was 1178 bp in length without a polyadenylate tail, and was made up of a 140 bp 5’ leader sequence followed by 792 bp of an open reading frame encoding 264 amino acids and a 246 bp 3’ noncoding region.

Structural characteristics of the deduced OsTIP1, OsTIP2, and OsTIP3 polypeptides

A comparison of the amino acid sequences predicted from rice γ-TIP, OsTIP1, OsTIP2, and OsTIP3 showed the presence of several conserved regions (Fig. 1). An analysis with the computer program “Prediction of Transmembrane Regions and Orientation” (TMpred)24) indicated that every amino acid sequence contained six putative transmembrane-spanning regions. Moreover,
two Asn-Pro-Ala (NPA) motifs that participate in forming an aqueous channel were perfectly conserved. The phylogenetic tree of the rice TIPs and other plant TIP isoforms is shown in Fig. 2. The rice TIPs had the highest sequence identity with maize TIPs at the amino acid level. Rice OsTIP1 and OsTIP3 had 95.2% and 92.8% identity with ZmTIP1-1 and ZmTIP1-2 respectively. OsTIP1 also had homology to ZmTIP2-1 and homology to root-specific TobRB7 from tobacco (81.2% and 76.2% identity respectively). OsTIP3 had 63.3% identity.

It has been reported that rice OsTIPs are encoded by a single-copy gene. Southern blot hybridization analysis with gene-specific probes from the 3'UTR indicated that OsTIP1 (Fig. 3A), OsTIP2 (Fig. 3B), and OsTIP3 (Fig. 3C) were single-copy genes. Southern blot hybridization using the coding region of rice OsTIP as a probe indicated that rice has many TIP-related sequences (Fig. 3D).

Expression in different tissues of rice plants and in suspension-cultured cells was analyzed by Northern blot hybridization with a DNA probe specific for an individual TIP isoform (Fig. 4). Total RNA was extracted from roots and shoots of 10-d-old seedlings, embryos, and endosperms of 10 DAF seeds, mature seed embryos, and suspension-cultured cells. Rice OsTIP1 was expressed in all plant organs, and the roots of 10-d-old seedlings showed the highest amounts of the transcripts among these organs. In 10 DAF seeds, the transcripts were detected predominantly in embryos. The transcripts of OsTIP2 were detected exclusively in roots. OsTIP3 was expressed in 10 DAF seeds and in mature seed embryos. The transcripts of OsTIP3 were detected in both embryos and endosperms of the 10 DAF seeds. In addition, rice OsTIP1 and OsTIP3 were expressed in suspension-cultured cells.

**Tissue-specific expression of rice TIP isoforms**

Developmental changes of transcripts of rice TIP isoforms during maturation and germination of seeds

The developmental changes of the rice TIP isoforms during the maturation and germination of seeds were analyzed by Northern blot hybridization using the gene-specific probes described above (Fig. 5). The transcripts of rice γ-TIP were detected at 3 DAF in seed maturation (Fig. 5A). The level increased up to 10 DAF, then decreased. OsTIP1 was detected in mature seed embryos, but not in 10 DAF seeds. OsTIP2 was expressed exclusively in roots. OsTIP3 was expressed in 10 DAF seeds and in mature seed embryos. The transcripts of OsTIP3 were detected in both embryos and endosperms of the 10 DAF seeds. In addition, rice γ-TIP and OsTIP3 were also expressed in suspension-cultured cells.

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decreased to 20 DAF. The level increased remarkably again at 30 DAF. By the stage of full maturation, starchy endosperm cells had turned into lifeless tissues. Thus the total RNA from 30 DAF seeds was composed of the RNA from the tissues, mainly embryos, except for the starchy endosperms. And as shown in Fig. 4, the relative mRNA levels of rice $\gamma$-TIP in embryos were higher than those in endosperms (Fig. 4). Here, the remarkable increase in relative mRNA levels of rice $\gamma$-TIP may be observed at 30 DAF. The transcripts of OsTIP1 were detected only at 30 DAF. OsTIP2 was not expressed at any of the developmental stages during seed maturation. OsTIP3 was expressed beginning at some point between five and seven DAF and reached a peak at about 10 DAF. The level remained unchanged to 20 DAF, then decreased slightly at 30 DAF.

With respect to seed germination, rice $\gamma$-TIP was expressed beginning at about two d of age in seedlings (Fig. 5B). The relative level of rice $\gamma$-TIP mRNA had decreased at 10 d of age in seedlings. OsTIP1 was expressed beginning at about two d of age in seedlings. The expression continued until five d of age and subsequently disappeared. Moreover, the expression of OsTIP1 was detected in both roots and shoots (Fig. 5C). The level of OsTIP2 mRNA in seedlings was below the level of detection (Fig. 5B), but the transcripts of OsTIP2 were detected in roots of five-d-old seedlings and gradually increased thereafter (Fig. 5C). The transcripts of OsTIP3 decreased rapidly after imbibition and were not detectable in at three-d-old seedlings.

**Accumulation of rice $\gamma$-TIP and OsTIP3 proteins in rice seeds during seed maturation**

We prepared affinity-purified antipeptide antibodies specific to the deduced C-terminal amino acid sequences from the cDNA sequences of rice $\gamma$-TIP and OsTIP3 according to the method described previously. The deduced C-terminal cytoplasmic tail sequences were diverse enough to allow generation of antibodies specific to the different TIPs (Fig. 1, double underline). No cross-reaction was observed between rice $\gamma$-TIP antibody and the C-terminal peptide of OsTIP1 (data not shown). To analyze changes in the levels of rice $\gamma$-TIP and OsTIP3 proteins during seed maturation, we
prepared homogenates from rice seeds at various stages of seed development and subjected them to immunoblot analysis using anti-TIP peptide antibodies (Fig. 6). The anti-rice γ-TIP and OsTIP3 peptide antibodies identified bands for two peptides in the total protein extracted from rice seeds. They were approximately 26- and 28-kDa in sizes (Fig. 6). Each size of polypeptide detected by the anti-rice γ-TIP and OsTIP3 antibodies coincided with that deduced from the cDNA sequences of rice γ-TIP (25,699 Da) and OsTIP3 (27,514 Da) respectively. Rice γ-TIP was detected at 3 DAF in seed maturation (Fig. 6A). The level of rice γ-TIP was unchanged up to 7 DAF, and decreased thereafter to 30 DAF. In contrast, OsTIP3 was detected at 5 DAF, reached a peak at about 10 DAF, and then remained unchanged to 30 DAF (Fig. 6B).

Immunocytochemical localization of rice γ-TIP and OsTIP3 in rice endosperm cells
Rice endosperm is composed of starchy endosperm tissue and an aleurone layer. The starchy endosperm cells contain protein body type II (PB-II) that is the accumulation site for glutelins, and the aleurone cells contain aleurone grain that is the accumulation site for phytin. But both the PB-II and the aleurone cells are derived from vacuoles. To examine the localization of rice γ-TIP and OsTIP3 in the starchy endosperm and aleurone cells, we performed an immunocytochemical analysis using anti-TIP peptide antibodies. The anti-rice γ-TIP antibodies were localized on the membranes of the PSV (thought to form PB-II by accumulating storage proteins) in the starchy endosperms of 10 DAF seeds (Fig. 7A), but were not detected on the membranes of aleurone grain in aleurone cells (Fig. 7B). In contrast, anti-OsTIP3 antibodies were localized on the membranes of PSV and aleurone grain (Fig. 7C, D).
Discussion

We isolated and characterized three novel rice cDNAs that are homologous to rice γ-TIP cDNA. OsTIP1, OsTIP2, and OsTIP3 had structures typical of the aquaporin family, namely, six transmembrane-spanning regions and two NPA motifs. Plant aquaporins can be classified into different subfamilies based on their sequence similarity. Two of the subfamilies, plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs), are named after their main location in the cell. Two cDNAs for the plasma membrane-type aquaporin of rice have been isolated. Based on their amino acid sequences, OsTIP1, OsTIP2, and OsTIP3 were assigned to the TIP subfamily (data not shown).

To date, genes encoding plant TIPs have been identified almost exclusively in Arabidopsis thaliana (10 genes) and maize (11 genes), and have been divided...
into five groups.\(^{11,12}\) The TIP1, TIP2, and TIP3 groups correspond to \(\gamma\)-TIP, \(\delta\)-TIP, and \(\alpha\)-TIP of \(\textit{Arabidopsis}\) respectively. The phylogenetic tree of the rice TIPs and other plant TIP isoforms showed that TIPs with similar expression patterns in different plant species are more closely related than the TIPs of one organism. Rice \(\gamma\)-TIP and OsTIP1 had homology to \(\gamma\)-TIP of \(\textit{Arabidopsis}\) and were classified into the TIP1 group, but rice \(\gamma\)-TIP and OsTIP1 had different expression patterns. Rice \(\gamma\)-TIP was expressed in all organs, similarly to \(\textit{Arabidopsis}\) \(\gamma\)-TIP.\(^{13}\) On the other hand, OsTIP1 was specifically expressed in mature seed embryos and during early seed germination. As shown in the phylogenetic tree in Fig. 2, OsTIP1 was considered to form a novel subgroup in the maize TIP1 showing a higher homology with ZmTIP1-2. OsTIP2 was classified into the TIP2 group, corresponding to \(\delta\)-TIP of \(\textit{Arabidopsis},\)\(^{14}\) but OsTIP2 had a higher homology to root-specific TobRB7 of tobacco\(^{16}\) than to \(\delta\)-TIP of \(\textit{Arabidopsis}\). The expression of OsTIP2 was detected exclusively in roots, similarly to TobRB7.\(^{16}\) Here, we propose that the TIP2 group can be divided into two smaller groups, \(\delta\)-TIP and TobRB7, based on the expression patterns. OsTIP3 had homology to \(\alpha\)-TIP and \(\beta\)-TIP of \(\textit{Arabidopsis}\) and was classified into the TIP3 group. Northern blot hybridization analysis showed that OsTIP3 was expressed exclusively in seeds, similarly to \(\textit{Arabidopsis}\) \(\alpha\)-TIP.\(^{13}\) The different expression patterns and evolutionary relationships among rice \(\gamma\)-TIP, OsTIP1, OsTIP2, and OsTIP3 suggest that these rice TIP isoforms might have specific individual functions.

To analyze changes in levels of rice \(\gamma\)-TIP and OsTIP3 proteins during seed maturation, we performed an immunoblot analysis using the affinity-purified antipeptide antibodies specific to rice \(\gamma\)-TIP or OsTIP3. In maturing seeds, the protein level of rice \(\gamma\)-TIP decreased whereas OsTIP3 increased with seed maturation. We also examined the immunocytochemical localization of rice \(\gamma\)-TIP and OsTIP3 in rice endosperm cells. The anti-rice \(\gamma\)-TIP antibodies were localized on the membranes of PSV (thought to form PB-II by accumulating storage proteins) in starchy endosperm (Fig. 7A), but were not detected on the membranes of aleurone grain in aleurone cells (Fig. 7B). In contrast, anti-OsTIP3 antibodies were localized on the membranes of PSV and the aleurone grain (Fig. 7C, D). These results suggest that PB-II and aleurone grain may be derived from different types of vacuoles, although both PB-II and aleurone grain are derived from vacuoles and function as storage compartments.

Paris \textit{et al.} gave reason to conclude that the tonoplasts of PSVs contain \(\alpha\)-TIP whereas those of LVs contain \(\gamma\)-TIP.\(^{3}\) Both types of vacuoles are found in the same cell types of barley roots\(^{3,8,9}\) and maturing pea cotyledons.\(^{10}\) It has been reported that in some pea and barley root tip cells, however, vacuoles are present where both \(\alpha\)-TIP and \(\gamma\)-TIP colocalize in the tonoplast.\(^{3,9}\) Jauh \textit{et al.} have been proposed that plant cells have the ability to generate and maintain separate vacuole organelles, each being marked by a different TIP, and that the functional diversity of the vacuolar system may be generated from different combinations of TIPS.\(^{9}\) How cells change vacuoles marked by individual TIPS to make functionally mature vacuoles marked by combinations of TIPS is unknown, but this process probably involves some type of fusion of the different precursors.\(^{9}\) In accordance with this model, we propose that PB-II and aleurone grain might be generated by different systems, although the physiological role of different combinations of TIPS is unknown. The fusion process of separate membranes marked by \(\gamma\)-TIP and OsTIP1 might be involved in the biogenesis of PB-II.

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