DIVISION OF CELL MECHANISMS

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Higher plant cells contain several distinct organelles that play vital roles in cellular physiology. During proliferation and differentiation of the cells, the organelles often undergo dynamic changes. The biogenesis of new organelles may occur, existing organelles may undergo a transformation of function, while other organelles may degenerate. Because the dynamic transformation of organellar function (differentiation of organelles) is responsible for flexibility of differentiation events in higher plant cells, the elucidation of regulatory mechanisms underlying organelle transformation are currently studied in this division.

I. Regulation at the level of protein transport to microbodies during the microbody transition.

Dramatic metabolic changes which underlie the shift from heterotrophic to autotrophic growth occur in greening of seed germination. Accompanying these metabolic changes, many constitutive organelles are functionally transformed. For example, etioplasts differentiate into chloroplasts and mitochondria acquire the ability to oxidize glycine. Glyoxysomes which are microbodies engaged in the degradation of reserve oil via b-oxidation and the glyoxylate cycle, are transformed into leaf peroxisomes that function in several crucial steps of photorespiration. After the functional transition of glyoxysomes to leaf peroxisomes during the greening of pumpkin cotyledons, the reverse microbody transition of leaf peroxisomes to glyoxysomes occurs during senescence. The functional transformation between glyoxysomes and leaf peroxisomes is controlled by gene expression, protein translocation and protein degradation. In 1999, we showed that light dependent alternative splicing is involved in the microbody transition.

Hydroxypyruvate reductase (HPR) is a leaf peroxisomal enzyme that function in the glycolate pathway of photorespiration. We have obtained two highly similar cDNAs (HPR1 and HPR2) for pumpkin HPR. It has been revealed that two HPR mRNAs are produced by alternative splicing from a single type of pre-mRNA. The HPR1 protein, but not the HPR2 protein, was found to have a targeting sequence into leaf peroxisomes at the C-terminus, suggesting that alternative splicing control the subcellular localization of the two HPR proteins. Analysis of transgenic Arabidopsis expressing fusion proteins with green fluorescent protein (GFP) revealed the different subcellular localizations of the two HPR proteins (Fig. 1). RT-PCR analysis showed that the alternative splicing is regulated by light, indicating that the microbody transition is partly controlled in the level of splicing of the mRNA by the light dependent alternative splicing.

II. Microbody defective mutant of Arabidopsis.

It has been suggested that the functional conversion between glyoxysomes and leaf peroxisomes is controlled by gene expression, protein translocation, and protein degradation. A genetic approach is an effective strategy toward understanding the regulatory mechanism(s) of peroxisomal function at the level of gene expression, protein translocation, and protein degradation. We isolated and characterized 2,4-dichlorophenoxybutyric acid (2,4-DB)-resistant mutants. It has been demonstrated previously that 2,4-dichlorophenoxybutyric acid (2,4-DB) is metabolized to produce a herbicide, 2,4-D, by the action of peroxisomal fatty acid b-oxidation in higher plants. To isolate mutants that have defects in peroxisomal fatty acid β-oxidation, we screened mutant lines of Arabidopsis seedlings for growth in the presence of toxic levels of 2,4-DB. Genetic analysis revealed that these mutants can be classified as carrying alleles at three independent loci, which we designated ped1, ped2, and ped3, (where ped stands for peroxisome defective). The ped1 mutant lacks the thiolase protein, an enzyme involved in fatty acid b-oxidation during germination and subsequent seedling growth, whereas the ped2 mutant has a defect in the intracellular transport of thiolase from the cytosol to glyoxysomes. Further analysis on identification the mutated genes of these ped mutants are now in progress.

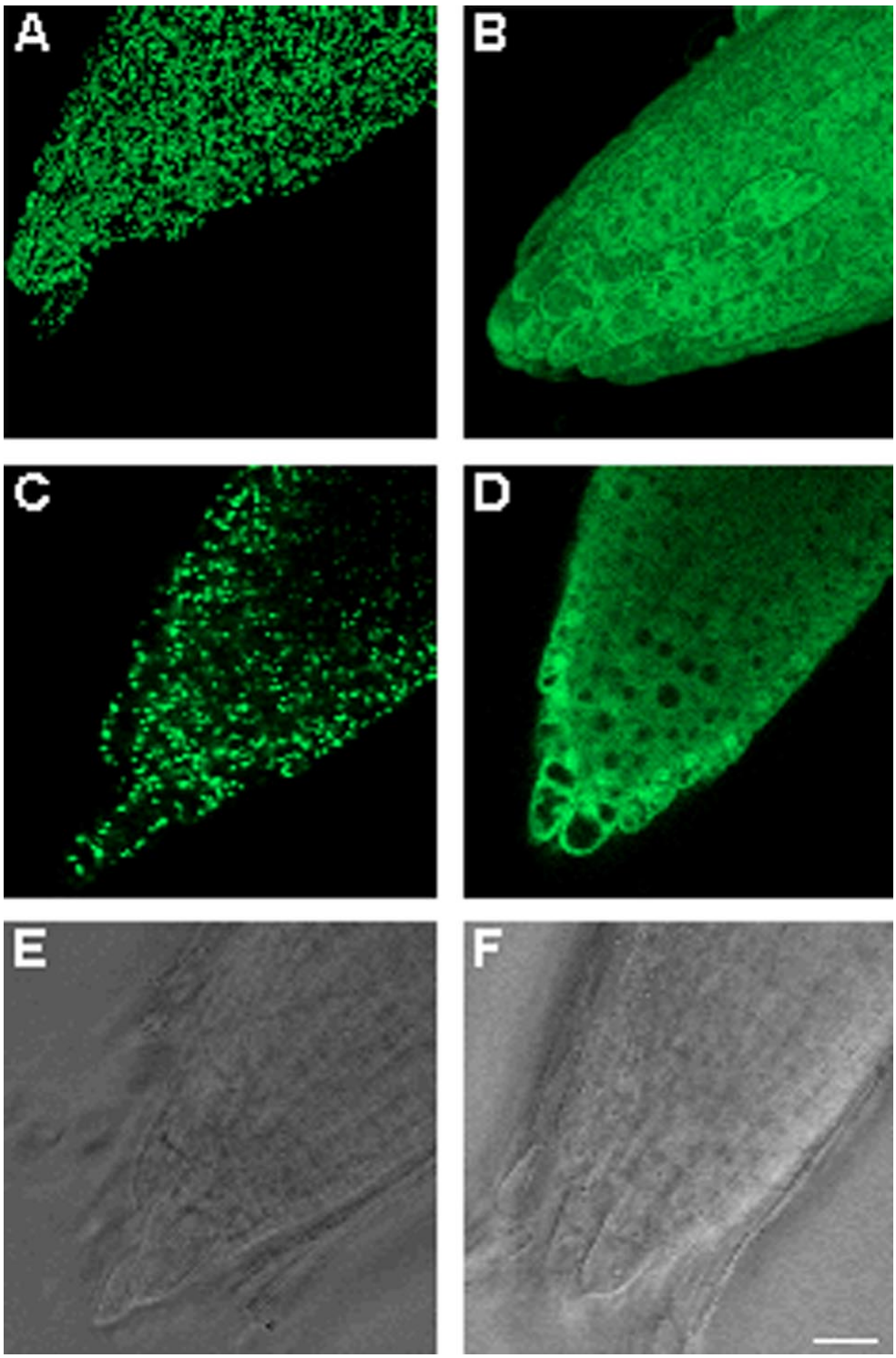

Figure 1. Subcellular localizations of green fluorescent protein (GFP) fusions in root cells of transgenic *Arabidopsis thaliana*.
Root cells were observed in light field (Nomarski optics; E and F) and with blue light excitation (470-490 nm; A, B, C and D). (A, B) Three-dimensional images of SGFP-H1 which has the peroxisomal targeting signal (PTS1) at the C-terminal end of GFP and SGFP-H2 which does not have PTS1, respectively. (C, D) The same samples viewed with a confocal microscope. (E, F) The same samples viewed with a light microscope. Peroxisomes were observed to be distributed diffusely throughout the cytosol of SGFP-H1 (A, C), whereas the fluorescence of GFP accumulated in the cytosol and the nucleus of SGFP-H2 (B, D). Bar indicates 10 μm.

In addition of these genetic approaches, we started to characterize the enzymes of b-oxidation cycle. In glyoxysomes, fatty acids are first activated to fatty acyl-CoA by fatty acyl-CoA synthetase. Fatty acyl-CoA is the substrate for fatty acid b-oxidation, which consists of four enzymic reactions. The first reaction is catalyzed by acyl-CoA oxidase. The second and the third enzymatic reactions are catalyzed by a single enzyme that possesses enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase activities. The fourth reaction is catalyzed by 3-ketoacyl-CoA thiolase. We have characterized acyl-CoA oxidase that is active on long-chain acyl-CoA and 3-ketoacyl-CoA thiolase in glyoxysomes. In 1999, we reported an evidence that glyoxysomes contain another acyl-CoA oxidase that can metabolize short-chain acyl-CoA. These findings revealed that the short-chain acyl-CoA oxidases function in fatty acid b-oxidation in glyoxysomes, and that by the cooperative action of long- and short-chain acyl-CoA oxidases, plant peroxisomes are capable to performing the complete b-oxidation of acyl-CoA, whereas mammal peroxisomes are not.

III. Transport of storage proteins to protein storage vacuoles is mediated by large PAC (precursor-accumulating) vesicles.

Novel vesicles that accumulate large amounts of proprotein precursors of storage proteins were purified from maturing pumpkin seeds. These vesicles were designated precursor-accumulating (PAC) vesicles and have diameters of 200 to 400 nm. We characterized them to answer the question of how seed protein precursors are accumulated in the vesicles to be delivered to protein storage vacuoles. They contain an electron-dense core of storage proteins surrounded by an electron-translucent layer, and some vesicles also contained small vesicle-like structures. An immunocytochemical analysis revealed numerous electron-dense aggregates of storage proteins within the endoplasmic reticulum. It is likely that these aggregates develop into the electron-dense cores of the PAC vesicles and then leave the endoplasmic reticulum. Immunocytochemical analysis also showed that complex glycans are associated with the peripheral region of PAC vesicles but not the electron-dense cores, indicating that Golgi-derived glycoproteins are incorporated into the PAC vesicles. These results suggest that the unique PAC vesicles might mediate a transport pathway for insoluble aggregates of storage proteins directly to protein storage vacuoles.

In order to investigate the mechanism of the PAC vesicle formation, we constructed chimeric genes that encode fusion proteins consisting to both various lengths of polypeptides derived from pumpkin 2S albumin and a selectable marker enzyme, phosphinothricin acetyltransferase and expressed in Arabidopsis. A fusion protein expressed by one of the chimeric genes is accumulated as a proprotein-precursor form, and localized in novel vesicles of vegetative cells, that show distinct features that well much to the PAC vesicles. Despite of the accumulation of the fusion protein, the transgenic Arabidopsis is still sensitive to phosphinothricin. Phosphinothricin acetyltransferase contained in the fusion protein is obviously compartmentalized in the PAC vesicles that do not permit the detoxification of this herbicide. These results indicate that PAC vesicle can be induced in vegetative cells by ectopic expression of the protein that is destined to be compartmentalized into the PAC vesicles. Arabidopsis mutants that defect vesicular transport of the fusion protein are screened and characterized by using the transgenic plants.

IV. Vacuolar processing enzymes in protein-storage vacuoles and lytic vacuoles.

Vacuolar processing enzyme (VPE) has been shown to be responsible for maturation of various seed proteins in plant vacuoles. *Arabidopsis* has three VPE homologues; βVPE is specific to seeds and αVPE and γVPE are specific to vegetative organs. We expressed the γVPE in a *pep4* strain of the yeast *Saccharomyces cerevisiae* and found that γVPE has the ability to cleave the peptide bond at the carbonyl side of asparagine residues. An immunocytochemical analysis revealed the specific localization of the γVPE in the lytic vacuoles of *Arabidopsis* leaves. These findings indicate that γVPE functions in the lytic vacuoles as the βVPE does in the protein-storage vacuoles. The βVPE promoter was found to direct the expression of the β-glucuronidase reporter gene in seeds of transgenic *Arabidopsis* plants. On the other hand, both the *αVPE* and *γVPE* promoters directed the expression in senescent tissues, but not in young intact tissues. The mRNA levels of both *αVPE* and *γVPE* were increased in the primary leaves during senescence in parallel with the increase of the mRNA level of a senescence-associated gene (*SAG2*). Treatment with wounding, ethylene and salicylic acid up-regulated the expression of *αVPE* and *γVPE*. Our results suggest that vegetative VPE might regulate the activation of some functional vacuolar proteins that are known to respond to these treatments.

To investigate a VPE system in protein-storage vacuoles, we isolated the PAC vesicles and characterized a 100-kDa component (PV100) of the vesicles. Isolated cDNA for PV100 encoded a 97,310-Da protein that was composed of a hydrophobic signal peptide and the following three domains: an 11-kDa Cys-rich domain with four CxxxC motifs (C, Cys), a 34-kDa Arg/Glu-rich domain composed of six homologous repeats, and a 50-kDa vicilin-like domain. Mass spectrometry and peptide sequencing of soluble proteins of the vacuoles showed that two Cys-rich peptides, three Arg/Glu-rich peptides and the vicilin-like protein were produced by cleaving Asn-Gln bonds of PV100 and that all these proteins had a pyroglutamate at their NH_2 terminus. VPE was responsible for cleaving Asn-Gln bonds of a single precursor, PV100, to produce multiple seed proteins. It is likely that the Asn-Gln stretches not only provide cleavage sites for VPE but also produce aminopeptidase-resistant proteins. Cys-rich peptide

function as a trypsin inhibitor and Arg/Glu-rich peptides function as cytotoxic peptides. Our findings suggested that PV100 is converted into different functional proteins in the vacuoles of seed cells.

V. Role of molecular chaperones in organelle differentiation.

Molecular chaperones are cellular proteins that function in the folding and assembly of certain other polypeptides into oligomeric structures but that are not, themselves, components of the final oligomeric structure. To clarify the roles of molecular chaperones on organelle differentiation, we have purified and characterized chaperonin and Hsp70s and analyzed their roles in the translocation of proteins into chloroplasts. In addition to mitochondrial chaperonin10 homologues, we isolated cDNAs for chloroplastic chaperonin 10 homologues from Arabidopsis thaliana. One of the cDNA insert was 958 bp long and encoded a polypeptide of 253 amino acids. The other cDNA insert was 603 bp and encoded a polypeptide of 139 amino acids. The former was comprised of two distinct Gro-ES domains whereas the latter had one Gro-ES domain. Further analyses on the roles of these chaperonin homologues in differentiation of plastids are under experiments by using transgenic Arabidopsis which was overexpressed or was reduced these chaperonin homologues

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