Plant Cell Physiol. 43(3): 331–341 (2002)

Distribution and Characterization of Peroxisomes in Arabidopsis by Visualization with GFP: Dynamic Morphology and Actin-Dependent Movement

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Peroxisomes were visualized in living cells of various tissues in transgenic Arabidopsis by green fluorescent protein (GFP) through the addition of the peroxisomal targeting signal 1 (PTS1) or PTS2. The observation using confocal laser scanning microscopy revealed that the GFP fluorescence signals were detected as spherical spots in all cells of two kinds of transgenic plants. Immunoelectron microscopic analysis using antibodies against the peroxisomal marker protein, catalase, showed the presence of GFP in peroxisomes, confirming that GFP was correctly transported into peroxisomes by PTS1 or PTS2 pathways. It has been also revealed that peroxisomes are motile organelles whose movement might be caused by cytoplasmic flow. The movement of peroxisomes was more prominent in root cells than that in leaves, and divided into two categories: a relatively slow, random, vibrational movement and a rapid movement. Treatment with anti-actin and anti-tubulin drugs revealed that actin filaments involve in the rapid movement of peroxisomes. Moreover, abnormal large peroxisomes are present as clusters at the onset of germination, and these clusters disappear in a few days. Interestingly, tubular peroxisomes were also observed in the hypocotyl. These findings indicate that the shape, size, number and movement of peroxisomes in living cells are dynamic and changeable rather than uniform.

Key words: Arabidopsis — Confocal laser scanning microscope — GFP — Movement of peroxisome — Peroxisomal targeting signal (PTS) — Peroxisome.

Abbreviations: GFP, green fluorescent protein; PTS, peroxisomal targeting signal; DMSO, dimethyl sulfoxide.

Introduction

Peroxisomes are single membrane-bound organelles that exist ubiquitously in eukaryotic cells (Beevers 1979). In higher plants, peroxisomes are classified into three groups, namely glyoxysomes, leaf peroxisomes and unspecialized peroxisomes. Glyoxysomes in oil-rich tissues of etiolated cotyledons contain some enzymes for the β-oxidation of fatty acids and the glyoxylate cycle, and play a major role in utilizing the storage oils for gluconeogenesis. Leaf peroxisomes in the photosynthetic tissues such as green leaves and cotyledons contain enzymes for the glycolate cycle and participate in photorespiration. Unspecialized peroxisomes with undefined physiological function are present in other organs such as roots and stems. Interestingly, the peroxisome transition from glyoxysomes to leaf peroxisomes occurs directly when seedlings are exposed to light (Titus and Becker 1985, Nishimura et al. 1986). Moreover, the reverse transition from leaf peroxisomes to glyoxysomes is observed in senescing cotyledons (De Bellis and Nishimura 1991) and the reverse transition occurs also directly (Nishimura et al. 1993).

Most peroxisomal enzymes have a peroxisomal targeting signal at the C-terminus (PTS1) or at the N-terminus (PTS2). Peroxisomal enzymes containing PTS1 are synthesized as mature forms with the tripeptide at the C-terminus. Gould et al. (1987), Gould et al. (1988), Gould et al. (1989) reported that the unique tripeptide, Ser-Lys-Leu or derivations of this sequence, at the C-terminus could direct firefly luciferase to peroxisomes. It is assumed that these Ser-Lys-Leu-like sequences are involved in the transport of peroxisomal enzyme in eukaryotes since they function as PTS1 in insects, higher plants, yeast and protozoa (Gould et al. 1990, Blattner et al. 1992). In a higher plant (transgenic Arabidopsis), we have shown that fusion proteins consisting of β-glucuronidase and a Ser-Lys-Leu or similar sequence at the C-terminus are transported into glyoxysomes in etiolated tissues, leaf peroxisomes in greening tissues and unspecialized peroxisomes in root tissues (Hayashi et al. 1997). It has been reported that some peroxisomal enzymes have PTS1 at their C-terminus (Comai et al. 1989, Graham et al. 1989, Mori et al. 1991, Tsugeki et al. 1993, Mano et al. 1996, Mano et al. 1997, Hayashi et al. 1999). In contrast, PTS2 is a conserved N-terminal nonapeptide (R/K)(L/V/I)(X)5(H/Q)(L/A) that occurs in the presequences of several enzymes (3-ketoacyl-CoA thiolase, citrate synthase, malate dehydrogenase and long chain acyl-CoA oxidase) in higher plants (Kato et al. 1995, Kato et al. 1996b, Kato et al. 1998, Hayashi et al. 1998a). These proteins are synthesized as larger precursors and the presequence is cleaved after the import to peroxisomes. The PTS1 receptor (Pex5p) has been identified in yeasts (Leij et al. 1993), human (Fransen et al. 1995, Wiemer et al. 1995) and higher plants (Wimmer et al. 1998), and the PTS2 receptor (Pex7p) has been identified in yeast (Marzioch et al.

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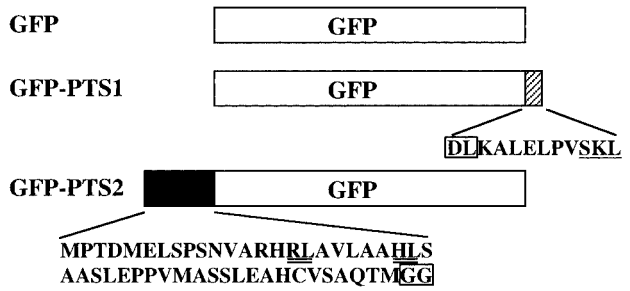


Fig. 1 Constructs for GFP, GFP-PTS1 and GFP-PTS2. GFP-PTS1 was produced by insertion of a nucleotide sequence encoding ten amino acids derived from pumpkin hydroxypyruvate reductase in the nucleotide sequence encoding the C-terminus of GFP. Underline represents PTS1. In the case of GFP-PTS2, the N-terminal nucleotide sequence encoding the presequence from pumpkin citrate synthase were inserted in front of the nucleotide sequence encoding GFP. Double underlines show the consensus sequences of PTS2. Boxes represent amino acids as spacers between GFP and each extra peptide.

1994). Peroxisomal membrane proteins such as peroxisomal ascorbate peroxidase, PMP38 and PMP47 do not contain typical PTS1 or PTS2 sequences (Purdue and Lazarow 1994, Fukao et al. 2001, Nito et al. 2001). This suggests the presence of one or more other targeting systems.

Peroxisomes seem to change in morphology, size, number and movement dramatically, but little is known about how these changes occur. To investigate the dynamics of peroxisomes, it is necessary to observe peroxisomes in living cells of various tissues and at different stages under the microscope. Unlike mitochondria, peroxisomes cannot be identified by dyes, and unlike chloroplasts, they cannot be identified by autofluorescence. Although it is possible to observe peroxisomes by indirect immunofluorescence staining (Hayashi et al. 1995, Mano et al. 1999), this technique is a little complicated and cannot be done with live cells.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used as a visible marker for gene expression, in vivo protein localization and time-lapse analysis in various organisms. GFP emits green fluorescence when it absorbs blue light without any substrates, co-factors and gene-products of the jellyfish. This protein can monitor organelles without disruption of cells since the small size of this protein makes it suitable for use in translational gene fusions. Various mutants of GFP optimized for each organism have been developed. sGFP-S65T, which was used in this study, is designed for high expression of eukaryotic proteins (Chiu et al. 1996, Niwa et al. 1999). In higher plants, GFP has been targeted to various organelles such as the nucleus (Kleiner et al. 1999, Stacey et al. 1999), chloroplasts (Niwa et al. 1999), mitochondria (Gálvez et al. 1998, Niwa et al. 1999), the cytoskeletons (Ueda et al. 1999), vacuoles (Mitsuhashi et al. 2000) and the tonoplast (Mitsuhashi et al. 2000).

We have generated transgenic Arabidopsis and suspension-cultured tobacco cells expressing the GFP-fusion protein

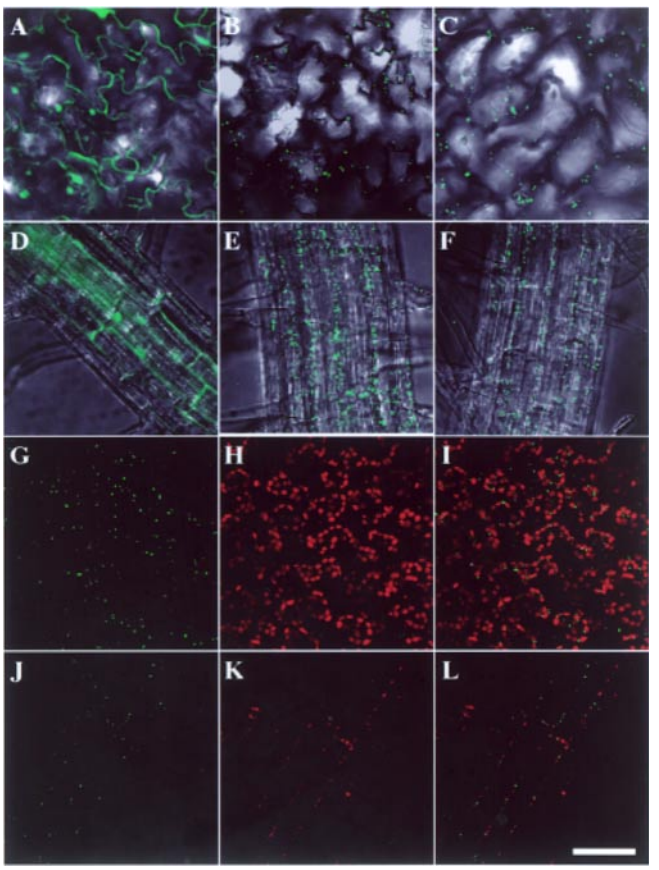


Fig. 2 Subcellular localization of GFP-fusion proteins in transgenic Arabidopsis. GFP (A and D), GFP-PTS1 (B and E) and GFP-PTS2 (C and F) were observed with blue light excitation. (A) to (C); rosette leaves, (D) to (F); the elongation zone of roots. To visualize chloroplasts, the fluorescence of 505–530 nm (G) and more than 530 nm (H) was detected simultaneously in 7-day-old cotyledons in GFP-PTS1. (I) represents the merged image of (G) and (H). For staining mitochondria, seedlings of GFP-PTS1 were treated with MitoTracker Orange and the roots were observed with blue light (J) and green light (K) excitation. (L) represents the merged image of (J) and (K). Each sample except (G) to (L) shows the merged images with blue light excitation and the difference interference contrast. Bar indicates 50 μm and refers to all samples.

that has PTS1 at the C-terminus (GFP-PTS1; Mano et al. 1999). In this paper, we created a new transgenic Arabidopsis that expresses a GFP-fusion protein consisting of the N-terminal PTS2 (GFP-PTS2). Using GFP-PTS1 and GFP-PTS2 fusion proteins, we examined the dynamics of peroxisomes, such as the shapes, size, the distribution, the spatial organization and movement, in various tissues and at the different stages.

Results

Visualization of peroxisomes in living cells of Arabidopsis by GFP

In order to characterize peroxisomes in vivo, we tried to visualize them with GFP because this non-disruptive method

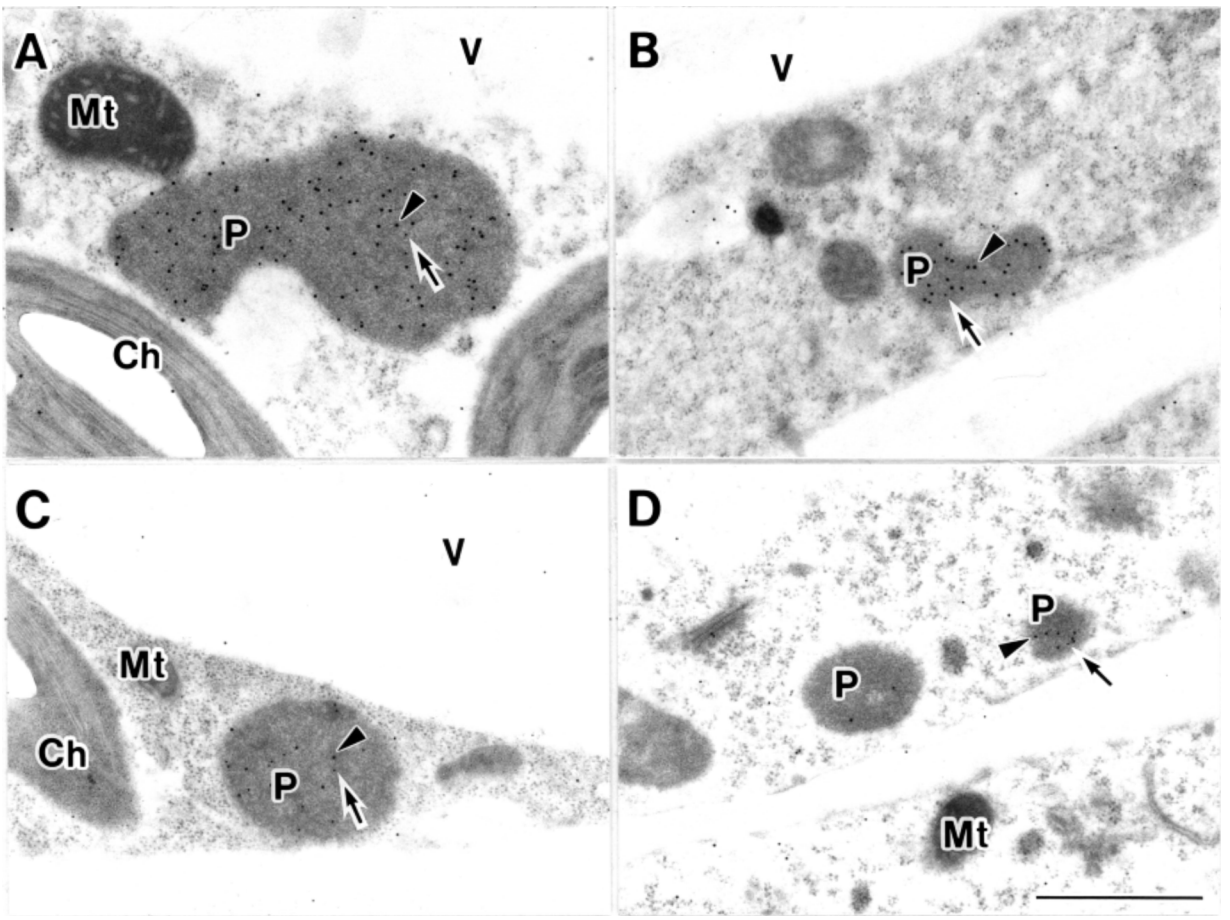


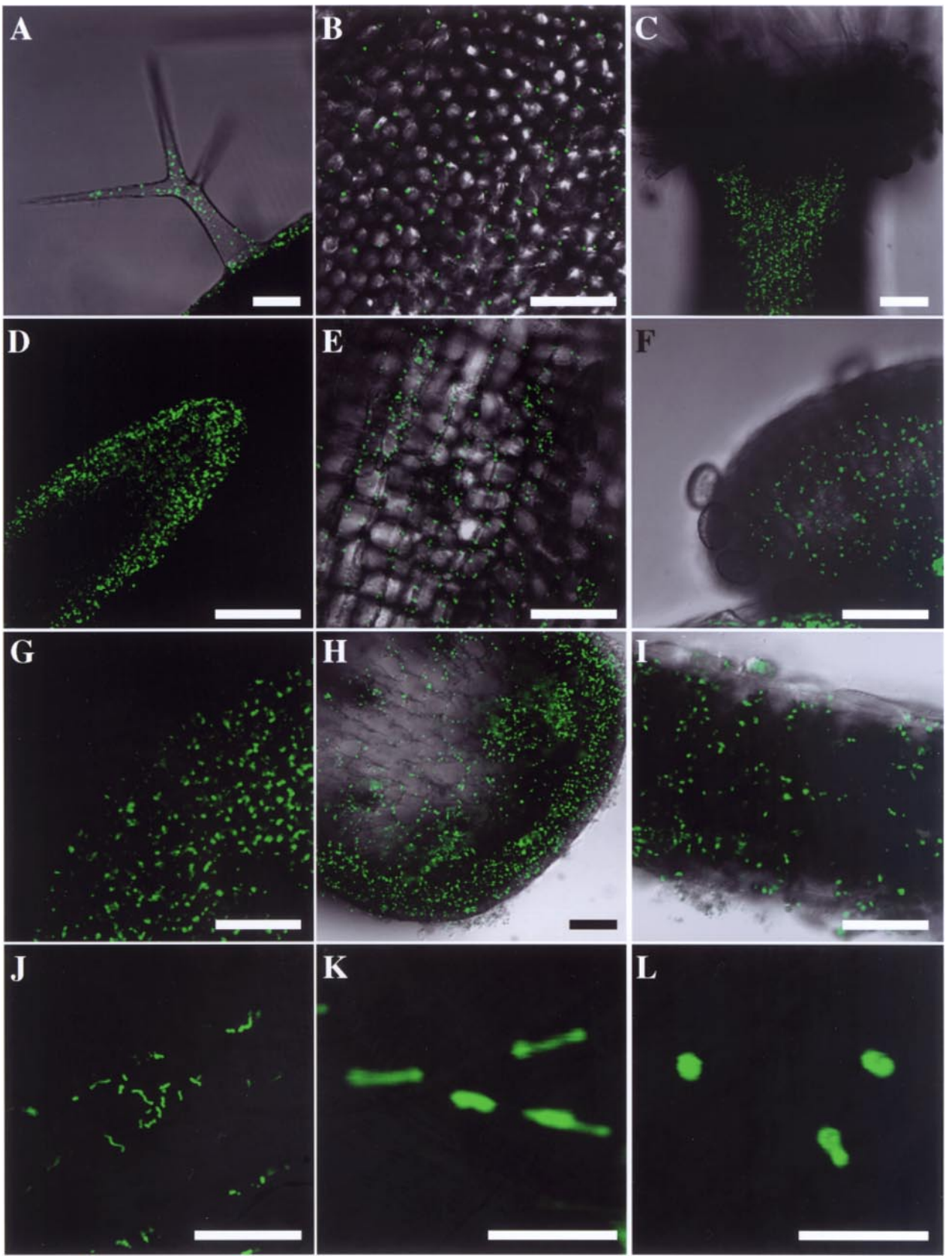
Fig. 3 Immunocytochemical localization of GFP-fusion protein in GFP-PTS1 and GFP-PTS2. Immunogold labeling of ultra-thin sections of 20-day grown rosette leaves (A and C) and roots (B and D) was carried out using antibodies against GFP (size 15 nm, arrowhead) and antibodies against catalase (size 10 nm, arrow). (A) and (B); GFP-PTS1, (C) and (D); GFP-PTS2. P, peroxisome; Mt, mitochondrion; Ch, chloroplast; V, vacuole. Bar indicates 1 μm and refers to all samples.

provides a vital stain without fixation. We constructed two chimeric proteins, GFP-PTS1 and GFP-PTS2. GFP-PTS1 contains PTS1, whose sequence is derived from the leaf-peroxisomal protein, hydroxypyruvate reductase (Mano et al. 1999), at the C-terminus of GFP (Fig. 1) and GFP-PTS2 has PTS2 from the glyoxysomal protein, citrate synthase (Kato et al. 1995), at the N-terminus of GFP (Fig. 1). It has been shown that these PTSs target the reporter protein, β-glucuronidase, to peroxisomes in higher plants (Kato et al. 1996a, Hayashi et al. 1997). Plasmid constructs were introduced into Arabidopsis via *Agrobacterium tumefaciens*. Several transgenic lines were generated, and the growth and morphology of all lines were nearly normal and completely fertile (data not shown).

The observation of GFP fluorescence excited with 488 nm was performed using a confocal laser scanning microscope. In control cells expressing GFP without a targeting sequence, diffuse fluorescence in the cytosol and on the surface of the nucleus was observed (Fig. 2A, D). In the case of GFP-PTS1 and GFP-PTS2, well-defined spherical spots were distributed diffusely throughout the cytosol (Fig. 2B, C, E, F).

Fig. 2G, H and I show the images of simultaneous detection of GFP fluorescence in peroxisomes and the autofluorescence from chloroplasts. As shown in these panels, the green fluorescence is clearly distinguishable from that in chloroplasts. Interestingly, most peroxisomes are localized near chloroplasts. Moreover, Arabidopsis seedlings were treated with the mitochondria-specific dye MitoTracker Orange. In Fig. 2, panel J, K and L clearly show that green fluorescence was not co-localized in mitochondria. These results show that GFP fluorescence is not localized in chloroplasts or mitochondria.

To confirm this result by another method, an immunocytochemical analysis was carried out using leaves and roots of GFP-PTS1 and GFP-PTS2. Fig. 3 shows peroxisomes in leaves and root cells of two transgenic Arabidopsis that are labeled with large gold particles for GFP (arrowhead) as well as with small gold particles for catalase, which is the peroxisomal marker enzyme (arrow). A few gold particles were observed in another organelles, especially in the root cells because of high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background labeling in the case of anti-catalase antibodies. However, most gold particles were localized in peroxisomes even in the root cells. This result confirmed the correct targeting of GFP into peroxisomes in Arabidopsis cells by way of the PTS1 and PTS2 pathways.

Visualization of peroxisomes in various tissues in transgenic Arabidopsis

All plant cells are believed to have peroxisomes. Since GFP-PTS1 and GFP-PTS2 were cloned under control of the constitutive 35S promoter from cauliflower mosaic virus, chimeric proteins were produced in all transgenic plant cells. We investigated GFP fluorescence of different organs in GFP-PTS1 (Fig. 4). Spherical spots were observed in all investigated tissues except stigmatic papillae (Fig. 4C) and pollen (data not shown), resulting in the visualization of three kinds of peroxisomes, namely, glyoxysomes, leaf peroxisomes and unspecialized peroxisomes. GFP fluorescence was even detected in dry seeds (Fig. 4G), showing that peroxisomes are already present in dry seed cells. Cells in other organs such as the stem, anther and sepal also showed the same fluorescence pattern and similar observations were made in GFP-PTS2-transformed Arabidopsis (data not shown).

In addition, we made transverse sections of the stem and rosette leaves in GFP-PTS1-transformed Arabidopsis. Stem (Fig. 4H) and rosette leaves (Fig. 4I) were cut with a razor blade and the sections were observed. Peroxisomes visualized with GFP were detected in all kinds of cells including the epidermis, parenchyma cells, xylem and phloem although the number and distribution in cells were different among cell types, showing that peroxisomes are present in all kinds of cells in Arabidopsis.

Interestingly, long peroxisomes, which looked like a dumbbell, as if two peroxisomes were connected with the tubule, were observed in the lower part of hypocotyls that were grown in darkness for 9 d (Fig. 4J, K). Their length was about 3–4.1 μm, whereas that of normal-shape peroxisomes was about 1.7 μm (Fig. 4L). These dumbbell-shape peroxisomes also exhibit a rapid movement as described below.

Movement of peroxisomes in living cells

The above observation of GFP-labeled peroxisomes showed that peroxisomes are motile organelles in vegetative tissues. Therefore, the behavior of peroxisomes was examined by time-lapse analysis in both root cells (Fig. 5A) and leaves (Fig. 5B) of GFP-PTS1-transformed Arabidopsis. Fig. 5A shows ten single-confocal images of 60 frames taken at 1-s intervals. These images revealed that peroxisomes exhibit two types of movement: a relatively slow, vibrational movement and a rapid movement (arrow in Fig. 5A). With regard to the latter type of movement, some peroxisomes moved longitudinally and others moved transversely. Furthermore, the moving peroxisomes categorized into two types seemed to be interchangeable in that slow moving peroxisomes could spontaneously exhibit rapid movement, and vice versa. Some peroxisomes were tracked over consecutive frames. The velocity was different for each peroxisome, although the range was 2.0–3.25 μm s^{-1}. Besides root cells, peroxisomes in trichomes also show the same movement fashion (data not shown).

In contrast, rapid movement of peroxisomes was hardly observed in leaves (Fig. 5B). Since most peroxisomes vibrate at the same position, images of cells taken over 1 min showed very little displacement of most peroxisomes. The same observation was made in the petal, the pistil and the stamen. In addition, peroxisomes in dry seeds did not move at all.

Microfilaments, but not microtubules, are involved in the movement of peroxisomes

The intracellular movement of organelles are modulated by a microfilament- and/or microtubule-based cytoskeleton. To investigate whether the movement of peroxisomes are controlled by actin and/or tubulin based cytoskeleton, we treated GFP-PTS1 with Latrunculin B, an inhibitor of actin polymerization (Fig. 5C, middle panels) and Nocodazole, a tubulin-disrupting drug (Fig. 5C, lower panels). In case of treatment with Nocodazole, the pattern of movement of peroxisomes did not change, showing two types of movement as described above. Peroxisomes marked by arrowheads moved even for 20 s under treatment with Nocodazole (Fig. 5C, lower panels) as well as under treatment without these drugs (Fig. 5C, upper panels). In contrast, treatment with Latrunculin B inhibited the rapid movement, but not vibrational movement. As a result, all peroxisomes exhibited the vibrational movement, and thus the distribution of peroxisomes in the cells did not change over 40 s. These data showed that the interaction with microfilaments, but not microtubules, is important for movement of peroxisomes in plant cells.

Large peroxisomes are present as clusters during post germination

Peroxisomes were needed for the early stage of post-germinative growth because plants need the activities of β-oxidation and the glyoxylate cycle to utilize the storage lipids. Therefore, the developmental changes of peroxisomes during the post-germinative growth were examined (Fig. 6). As stated above, GFP-labeled peroxisomes in almost all cells were observed as spherical forms and some of them were moving independently without clumping each other. At 1-day or 2-day

Fig. 4 Confocal microscopic images of various tissues in GFP-PTS1. Each sample except (D), (G), (J), (K) and (L) shows the merged images with blue light excitation and the difference interference contrast. (A) trichome, (B) petal, (C) pistil, (D) root tip, (E) silique, (F) stamen and (G) dry seed. (H) and (I) represent transverse sections of stem and rosette leaves, respectively. (J) and (K) show 9-day dark-grown hypocotyl. (L) shows the normal-shape peroxisomes as the reference. Each bar except (K) and (L) corresponds to 50 μm. Bars in (K) and (L) indicate 5 μm.

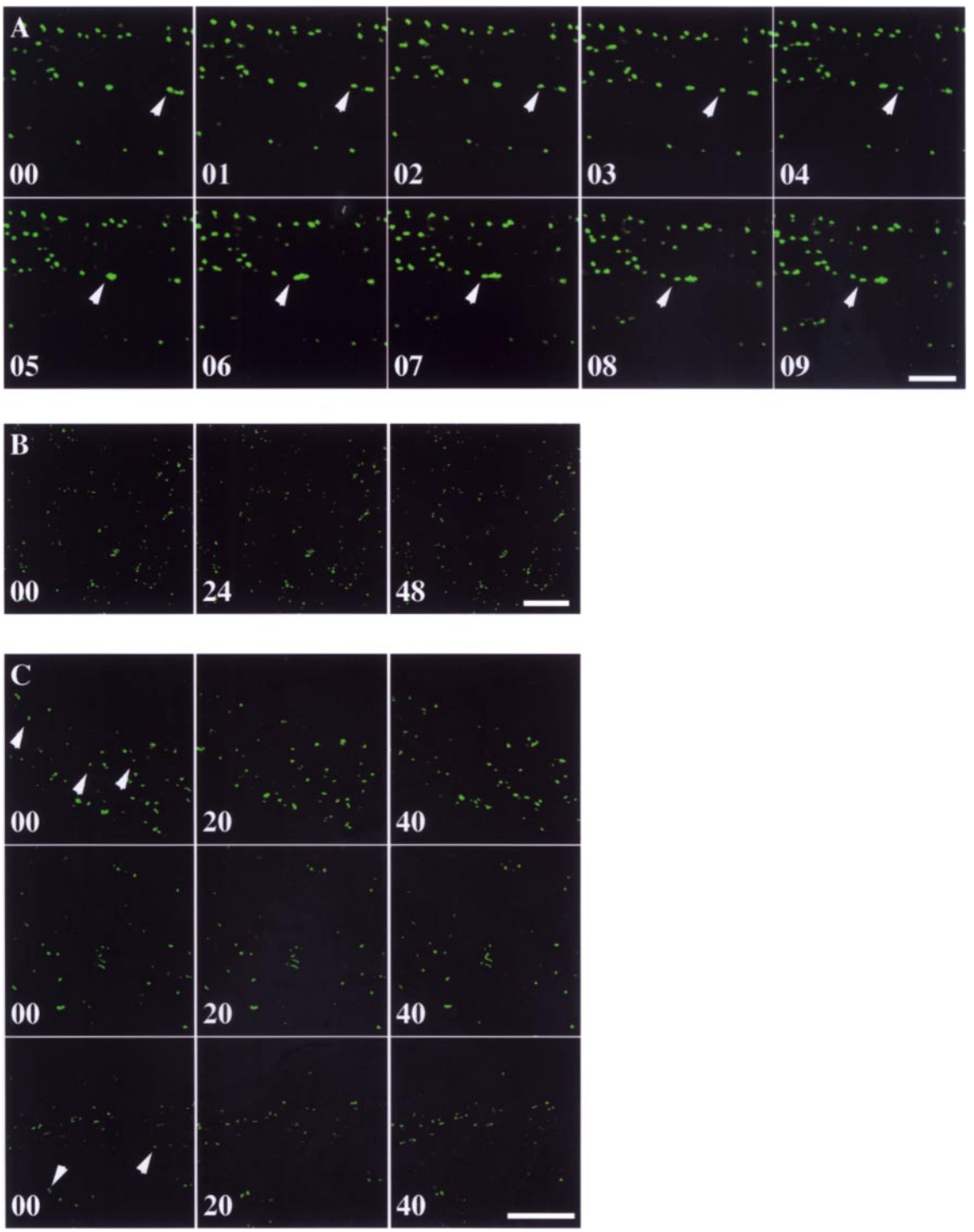

Fig. 5 Time-lapse analysis of the movement of individual peroxisomes in GFP-PTS1. Single-frame confocal images in the root (A and C) and the rosette leaves (B) were recorded at 1-s intervals for a total of 60 frames. (A) Ten confocal images from the series are shown at time =0 (frame 00) to time =9 s (frame 09). The arrow in each panel shows the same peroxisome. Bar indicates 10 μm and refers to all samples. (B) Three confocal images from the series are shown at time =0 s (frame 00), time =24 s (frame 24) and time =48 s (frame 48). Bar indicates 25 μm and refers to all samples. (C) The effects of Latrunculin B (middle panels) and Nocodazole (lower panels). Upper panels show the images of Arabidopsis root cells treated with only DMSO. Three confocal images from the series are shown at time =0 s (frame 00), time =20 s (frame 20) and time =40 s (frame 40). The arrow in each panel shows the representative of moving peroxisomes. Bar indicates 5 μm and refers to all samples.

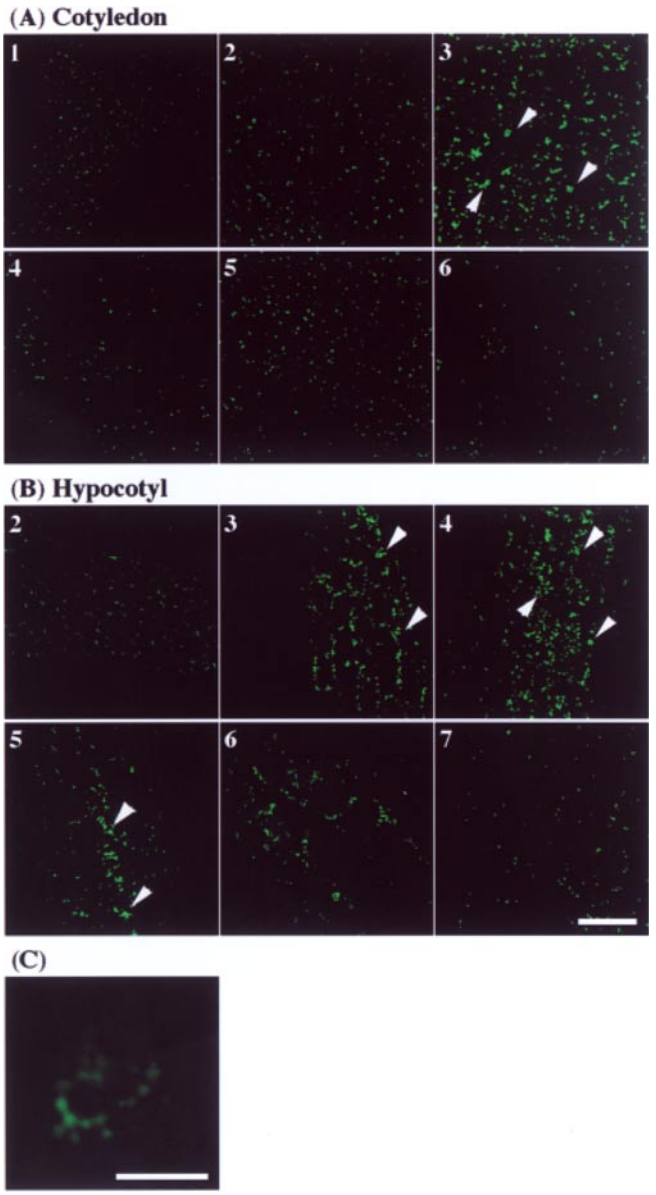

Fig. 6 Developmental changes of the fluorescent images during post germinative growth. GFP-PTS1 was grown in continuous light and observed at various stages. (A) cotyledons, (B) and (C) hypocotyls. The number in each panel represents the day after germination. Arrowheads show the representatives of peroxisomal clusters. Bar indicates 50 μm and refers to all samples in (A) and (B). Bar in (C) corresponds to 5 μm.

grown stages, however, peroxisomes in almost all cells did not move. On day 3, a few peroxisomes started moving. Interestingly, large peroxisomes were observed only at the 3-day and 4-day stages in the cotyledons (Fig. 6A) and from the 3-day to 5-day stages in the hypocotyls (Fig. 6B). Compared with normal peroxisomes with about 1.4–2.1 μm in diameter, these large peroxisomes were about 2.8–3.5 μm in diameter. In addition, these large peroxisomes existed as clusters (Fig. 6C). The clusters disappeared in a few days, so that only normal-sized peroxisomes remained. The peroxisomal clusters were especially abundant in the apical hook and the lower region of hypocotyl, and sometimes in the cotyledons and the roots. This phenomenon was observed in the root cells as well as in the other transgenic line, GFP-PTS2 (data not shown).

Discussion

Peroxisomes play an essential role during the plant cell cycle. To date, investigation of peroxisomes in higher plants has been mainly focused on the characterization of metabolic pathways. Although some cDNAs encoding peroxisomal proteins were cloned and their expression and subcellular localization were characterized (Tsugeki et al. 1993, Kato et al. 1995, Kato et al. 1996b, Kato et al. 1998, Mano et al. 1997, Hayashi et al. 1998a, Hayashi et al. 1999), little is known about the dynamics of peroxisomes in living cells. In this study, we were able to visualize peroxisomes in living cells of various tissues. This is the first report on the shapes, size, number and movement of peroxisomes in higher plants.

To stain peroxisomes, we used the mutant GFP, sGFP-S65T, as the reporter since this makes it possible to observe living cells. Although high level of GFP expression may be harmful to plant growth (Haseloff et al. 1997, Rouwendal et al. 1997), all transgenic plants in this study, including plants transformed with GFP without a targeting signal, showed morphologically normal growth and development, and were completely fertile. This was in good agreement with the results of Niwa et al. (1999) who found that GFP was targeted to chloroplasts and mitochondria.

Most peroxisomal proteins use PTS1 and PTS2 to enter the peroxisomes. GFP fused with PTS1 or PTS2 were detected as spherical spots in all transgenic Arabidopsis (Fig. 2). Besides peroxisomes, plant cells have plastids and mitochondria as multi organelles. Based on double staining with the autofluorescence from chloroplasts, most peroxisomes are localized near chloroplasts (Fig. 2I). Because peroxisomes, which are specially called leaf-peroxisomes in the photosynthetic tissues, play a role in the photorespiration concertedly with chloroplasts and mitochondria, the location of peroxisomes near chloroplasts might be important. In the comparison of peroxisomes with mitochondria (Fig. 2J, K, L), it is noteworthy that GFP-labeled mitochondria seem to resemble peroxisomes in shape, size and distribution in the cells (Gálvez et al. 1998, Niwa et al. 1999). Therefore, we tried to distinguish between peroxisomes and mitochondria using the mitochondria-specific dye MitoTracker Orange. As shown Fig. 2L, the GFP fluorescence was observed at positions different from the mitochondria-specific fluorescence. These results as well as the co-immunoelectron microscopic analysis (Fig. 3) demonstrate that GFP-PTS1 and GFP-PTS2 are correctly transported into peroxisomes in Arabidopsis cells.

As stated above, there are three types of peroxisome,

namely, glyoxysomes, leaf peroxisomes and unspecialized peroxisomes. Therefore, an intriguing question is whether all peroxisomes are visualized with GFP. The GFP fluorescence was detected in all cells except stigmatic papillae (Fig. 4C) and pollen (data not shown). However, this result does not mean that stigmatic papillae and pollen cells do not have peroxisomes, since the 35S promoter from cauliflower mosaic virus might not function in these cells. Plegt and Bino (1989) showed the disparity in the expression level of the 35S promoter depending on the tissues. If the 35S promoter is functioning and there are not any peroxisomes in the stigmatic papillae and pollen cells, the GFP fluorescence could be observed in another location such as the cytosol. A similar result was obtained in the case of GFP-PTS2, which shows that PTS1 and PTS2 signals are able to direct GFP to all types of peroxisomes. Although it is clear that various cells have peroxisomes, the function of peroxisomes except in cotyledons and green leaves remains to be determined. Peroxisomes are known to have roles in the biosynthesis of jasmonic acids (Vick and Zimmerman 1984) and the metabolism of uric acid (Lee et al. 1993).

It is difficult to compare the size, shape, number and distribution of peroxisomes in each tissue, because the confocal laser scanning microscopic analysis shows images of the thin sections but not the whole plant cell. Because peroxisomes are motile organelles (see next paragraph), it is difficult to obtain the many images that are needed to construct a three-dimensional object. However, Fig. 2 and 4 give a rough estimation of the number of peroxisomes in each tissue. With regard to epidermal cells, cells containing large volume such as leaves, especially trichomes, and roots contain more peroxisomes than small volume-sized cells such as petal cells. Cotyledonous and root cells in dry seeds already contain peroxisomes (Fig. 4G), which shows that the biogenesis of peroxisomes must have occurred during embryogenesis. This result is in good agreement with the case of GFP-labeled mitochondria (Niwa et al. 1999). Interestingly, tubular peroxisomes, as if two peroxisomes were connected, were observed in the hypocotyls when seedlings were grown in darkness (Fig. 4J, K). It has been already reported that plastids are interconnected with the tubular structure, and that these tubules allow the exchange of molecules (Köhler et al. 1997, Köhler and Hanson 2000). Mitochondria are dynamic structures that divide and fuse continually throughout the life of a cell (Bliek 2000) and some factors that regulate division and fusion have been characterized (Mozdy et al. 2000, Tieu and Nunnari 2000). Tubular peroxisomes are abundant in hypocotyl tissues in contrast with leaf tissues although the function is not determined. They might exchange molecules between peroxisomes, as do plastids. Cutler et al. (2000) constructed the library of plants expressing Arabidopsis cDNAs::GFP fusions, and as a result peroxisomes were visualized as torus structures in some lines, and thus they are referred to as Torus. Based on the sequence in Torus lines, PTS1 were present at the C-termini in some Torus lines, but not in other Torus lines. Probably, internal targeting signal(s), which are not determined, might direct GFP to peroxisomes in the latter lines. Torus structure has a central region lacking GFP fluorescence, indicating that peroxisomal membrane proteins might be visualized. In addition, tubular structures, which protruded from one peroxisome and fused to another peroxisome, were also observed in Torus lines. This structure might be related to tubular peroxisomes that we presented in this paper. These tubular peroxisomes might reflect the dynamic morphological changes of this organelle.

The advantage of vital staining with GFP is to follow the movement of each organelle. GFP-labeled peroxisomes are also motile organelles. The present results show that peroxisomes are divided into two types: those showing a slow, random, vibrational movement and those showing a rapid movement. The former resulted in little net movement of peroxisomes; images taken over 1 min showed very little displacement of peroxisomes (Fig. 5B). Epidermal cells in leaves, petals and stamen cells contain this type of peroxisome. In contrast, peroxisomes in root, root hair and trichome cells showed rapid movement. In these cells, the ratio of mobile peroxisomes to vibrating peroxisomes depended on the plantlet and developmental stages. That is, in some cases, most peroxisomes moved, and in the other cases only a few peroxisomes moved and most vibrated at the same position. Wiemer et al. (1997) found two types of peroxisomes in African green monkey kidney (CV1) cells. About 95% of peroxisomes exhibit a relatively random, vibrational movement and the rest displayed the fast, unidirectional movement. However, the peak velocity in CV1 cells (0.75 $\mu m\ s^{-1}$) was smaller than that in Arabidopsis (3.25 $\mu m\ s^{-1}$). It should be noted that the CV1 cells were cultured cells so that the movement of peroxisomes in individual cells from the whole body might be different. In Arabidopsis, the energy source for the rapid movement has not been determined. Wiemer et al. (1997) showed that treatment with sodium azide abolishes the rapid movement but not the slow vibrational movement in CV1 cells, indicating that ATP is involved in the rapid movement. In mammalian cells, the rapid movement is dependent on microtubules since microtubule-destabilizing agents such as Nocodazole, Vinblastine and Demecolcine blocked the movement (Wiemer et al. 1997). There was, however, no direct evidence about the involvement of the cytoskeleton in the movement of peroxisomes in plant cells. In case of treatment of GFP-PTS1 with anti-actin (Latrunculin B) and anti-tubulin (Nocodazole) drugs (Fig. 5C), Latrunculin B, but not Nocodazole, caused disruption of the rapid movement of peroxisomes, showing the involvement of microfilaments in the movement of plant peroxisomes. The association with actin and organelles in plant cells were reported in chloroplasts (Kandasamy and Meagher 1999), Golgi (Boevink et al. 1998) and ER (Grolig 1990). In yeasts, Smith et al. (1995) reported the actin-dependent rearrangement of mitochondria during cell division. These findings suggest an important role for actin in intracellular organelle movement.

We have been characterizing glyoxysomal enzymes

including proteins for β-oxidation because the β-oxidation and glyoxylate cycle are indispensable for plants during post-germinative growth. The mutants, which are devoid of glyoxysomal enzymes, are not able to germinate on medium without sucrose (Hayashi et al. 1998b). Therefore, the behavior of peroxisomes during post-germination period is intriguing. In this study, we were able to determine the timing of the beginning of the movement. In addition, of most interest is the appearance of large peroxisomes at the onset of germination (Fig. 6). The conditions under which each image was obtained (pinhole size, gain and offset for detection of the fluorescence) were identical. The peroxisomes in the cotyledon on day 3 and in the hypocotyls on days 3–5 were larger than those at another stages. The large peroxisomes formed clusters (Fig. 6C) that vibrated but did not move. Occasionally one or two large peroxisomes started to leave the cluster. These peroxisomal clusters were especially present in the apical hook, the lower region of the hypocotyls and the upper region of roots of plantlets, indicating that peroxisomal clusters might exist in the region of the activity for cell elongation. Kunce et al. (1984) reported that glyoxysomes from the analysis of cotton increase dramatically in volume (sevenfold) over a 37 h post-germinative growth period, and that this coincided with cotyledon expansion and cell enlargement. As shown in Arabidopsis, large peroxisomes were observed in the part of the hypocotyl where the activity of cell elongation is high. Therefore, large peroxisomes might appear in concert with cell division and cell elongation. Further analysis using Arabidopsis mutants that are defective in peroxisome biogenesis will provide the information on the mechanism(s) responsible for the dynamics of peroxisomes.

Materials and Methods

Plant materials

Arabidopsis thaliana (L.) Heynh. ecotype Columbia was used as the host for transformation. Germination was induced by a 48 h incubation at 4°C followed by white light at 22°C.

Plasmid construction and transformation of Arabidopsis

The construct of GFP-PTS1 has already been described in a previous study (Mano et al. 1999). GFP-PTS2 was prepared using pSGFP-HN, which has a *Hin*dIII and *Nco*I sites in the 5′ flanking region of the initiation codon and *Eco*RI site on either side of the stop codon in *sGFP* gene. pSGFP-HN was generated by PCR using the following primers SGFP-HiNc (5′-CCAAGCTTCCATGGGCGGCATGGTGAG-CAAGGGCGAGGAG-3′) and primer GFPsR (5′-GGGAATTCTCA-GAGATCTCCCTTGTACAGCTCGTCCAT-3′). The fragment was subcloned into a T-vector prepared using pBluescript KS^+ as described previously (Marchuk et al. 1990). The *Hin*dIII–*Nco*I fragment of the DNA fragment in pSGFP-HN was replaced with a 153-bp fragment that was produced by digesting ptCSC5 (Kato et al. 1995) with *Hin*dIII and *Nco*I, yielding pGFP-PTS2. The *Hin*dIII–*Eco*RI fragment, which contains the chimeric gene, was inserted into the *Hin*dIII–*Eco*RI site of a Ti-plasmid, pMAT137, which was kindly provided by Dr. Kenzo Nakamura (Nagoya University, Nagoya, Japan). The Ti-plasmid produced was then transformed into *Agrobacterium tumefaciens* (strain EHA101) by electroporation.

Transformation of *A. thaliana* (ecotype Columbia) was carried out by the infiltration method (Bechtold et al. 1993). Primary transformants were designated T0 plants. T1 seeds collected from T0 plants were surface sterilized in 2% NaClO plus 0.02% Triton X-100 and grown on germination media (2.3 μg ml^{-1} MS salts (Wako, Osaka, Japan), 1% sucrose, 100 μg ml^{-1} myo-inositol, 1 μg ml^{-1} thiamin-HCl, 0.5 μg ml^{-1} pyridoxine, 0.5 μg ml^{-1} nicotinic acid, 0.5 μg ml^{-1} MES-KOH (pH 5.7), 0.8% agar (INA, Nagano Japan)) containing 100 μg ml^{-1} of kanamycin. T2 seeds were collected from approximately 10 independent T1 plants. T2 plants that accumulated the highest amount of transgene product were selected based on the intensity of their fluorescence observed with fluorescence microscopy (see below).

Application of microfilament- and microtubule-disrupting drugs

Stock solutions of 2 mM Latrunculin B (Funakoshi, Tokyo, Japan) or Nocodazole (ICN, Ohio, U.S.A.) were prepared in DMSO. The concentrations (10 μM) of these drugs were made by diluting the solutions in Arabidopsis germination medium containing 0.002% Triton X-100 without antibiotics and agar (see above). Fourteen-day seedlings of GFP-PTS1 were treated by immersing them in the drug solution for 1 h. Control seedlings were treated identically but without the drugs.

Immunoelectron microscopy

Arabidopsis rosette leaves and roots were fixed, dehydrated and embedded in LR white resin (London Resin, U.K.) as described previously (Nishimura et al. 1993). Ultra-thin sections were cut on a Reichert ultramicrotome (Leica, Heidelberg, Germany) with a diamond knife and mounted on uncoated nickel grids. The protein A-gold labeling procedure was essentially the same as that described by Nishimura et al. (1993). Ultra-thin sections were incubated at room temperature for 1 h with antibodies against GFP diluted 1 : 200 and catalase diluted 1 : 2,000 and then with a 50-fold diluted suspension of protein A-gold (15 nm for GFP, 10 nm for catalase; Amersham, Japan) at room temperature for 30 min. The sections were examined with a transmission electron microscope (1200EX; JOEL, Tokyo, Japan) at 80 kV.

Confocal microscopy

Various tissues of transgenic Arabidopsis were sliced with razor blades and mounted between a slide and cover slip in tap water. For whole mounting, Arabidopsis seedlings were placed on glass slides and pressed gently. The specimens were examined using an LSM510 laser-scanning confocal microscope equipped with an argon laser and a filter set for a fluorescein filter (excitation filter 465–505 nm, emission filter BP505–550, Carl Zeiss, Germany).

To visualize chloroplasts, emission filters BP505–530 and BP560–615 were used for the detection of GFP and chloroplasts, respectively.

For staining mitochondria, Arabidopsis seedlings were soaked in 0.5 μM MitoTracker Orange CM-H2TMRos (Molecular Probes Inc., Netherlands) solution in MS medium for 15 min, washed three times in MS medium and observed using a rhodamine filter set (excitation filter 534–558 nm, emission filter BP560–615 Carl Zeiss, Germany).

Acknowledgments

We are most grateful to Dr. Yasuo Niwa (University of Shizuoka) for providing cDNA of sGFP-S65T and Dr. Kenzo Nakamura (Nagoya University) for the gift of pMAT137. This work was supported by a grant for “Research for the Future” Program (JSPS-RFTF 96L00407) from the Japan Society for the Promotion of Science and

Grants-in-Aid for Scientific Research (Nos. 09440271, 09274101 and 09274103) from the Ministry of Education, Science and Culture, Japan.

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(Received November 6, 2001; Accepted January 8, 2002)