Visualizing organelles in living cells is a powerful method to analyze their intrinsic mechanisms. Easy observation of chlorophyll facilitates the study of the underlying mechanisms in chloroplasts, but not in other plastid types. Here, we constructed a transgenic plant enabling visualization of plastids in pollen grains. Combination of a plastid-targeted fluorescent protein with a pollen-specific promoter allowed us to observe the precise number, size and morphology of plastids in pollen grains of the wild type and the *ftsZ1* mutant, whose responsible gene plays a central role in chloroplast division. The transgenic material presented in this work is useful for studying the division mechanism of pollen plastids.

**Keywords:** FtsZ1 • Fluorescent protein • Male gametophyte • Plastid division • Pollen.

**Abbreviations:** ARC, accumulation and replication of chloroplasts; GFP, green fluorescent protein; IF2, translation initiation factor 2; PD, plastid-dividing; RFP, red fluorescent protein; VC, vegetative cell.

Plastids develop into various types according to plant development and perform various cellular activities. Proplastids are undifferentiated plastids in stem cells and are capable of giving rise to different plastid types. Chloroplasts for photosynthesis are one plastid type in which thylakoid membranes are organized as granal stacks in mesophyll cells. Other plastid types include, for example, amyloplasts for storage synthesis in roots, and chromoplasts for carotenoid in fruits (Sakamoto et al. 2008). Plastids are not created de novo, but arise from pre-existing plastids by fission (Aldridge et al. 2005). Chloroplast division occurs by the action of the plastid-dividing (PD) ring. Several components have been identified as being associated with the site of the PD ring. These include proteins with a GTPase domain such as the dynamin-like ARC5 (accumulation and replication of chloroplasts 5), ARC6 and FtsZ1 (Robertson et al. 1995, Osteryoung et al. 1998, Gao et al. 2003). In contrast to extensive studies in chloroplasts, the division machinery in other plastid types is poorly understood. Very little is known about the division of plastids in reproductive organs including pollen grains.

In *Arabidopsis*, a tricellular mature pollen grain consists of one vegetative and two sperm cells. The vegetative cell, which makes up the bulk of a mature pollen grain, contains plastids that accumulate starch (Van Aelst et al. 1993). At the initial stage of pollen formation from microspores, plastids are poorly differentiated, with an indistinguishable inner membrane, in contrast to the double membrane structure of proplastids in the meristem (Robertson et al. 1995, Kuang and Musgrave 1996). Plastid differentiation and division occur alongside pollen maturation. In mature pollen, the final plastid structure contains a double membrane structure with several starch grains and simple thylakoid structures (Kuang and Musgrave 1996). Pollen grains exist in the homogenous developmental stage in anthers, whereas the shoot meristem contains cell layers where the cells contain plastids with various morphologies and nucleoid structures (Mascarenhas 1989, Fujie et al. 1994). Thus, pollen can provide a homogenous tissue appropriate for the study of plastid division. However, a technical problem is that the unpigmented plastids in pollen can only be visualized using fixed samples.

To achieve direct visualization of plastids in pollen, we attempted to express green fluorescent protein (GFP; Haseloff et al. 1997) in plastids. A chimeric GFP gene was constructed in which GFP was fused to the DNA fragment...
corresponding to a transit peptide sequence derived from plastid translation initiation factor 2 (IF2) (Miura et al. 2007). The LATS2 promoter from tomato was used to drive transgene expression in vegetative cells (VCs) (Twell 1990). The resulting chimeric construct was designated as VC-ptoGFP (Fig. 1A). Transgenic Arabidopsis plants were generated and GFP signals in mature pollen grains were examined in the T3 generation onwards by fluorescence microscopy. Under the normal light microscope, plastids in Arabidopsis pollen are not visible because of the absence of chlorophyll. Under fluorescence microscopy, plastids are present in a variety of shapes while the size did not show large variations (Fig. 1D–G). Occasionally, dumbbell-shaped plastids were observed (Fig. 1G). The existence of dumbbell-shaped plastids indicates that the division is active in pollen vegetative cells.

The area of a single plastid in wild-type pollen grains was 1.90 ± 0.54 µm² (mean ± SD; n = 233). To estimate the plastid number, three image planes were captured from a single pollen grain and the total number of non-overlapping GFP signals was scored. On average, each wild-type pollen grain contained approximately 43 ± 15 plastids (mean ± SD; n = 16). To confirm the correct targeting of the VC-ptoGFP transgene, we introduced the VC-mtRFP transgene into VC-ptoGFP plants by conventional crossing. The VC-mtRFP transgene allows visualization of mitochondria in pollen VCs with red fluorescent protein (RFP) (Matsushima et al. 2008). Double visualization of plastids and mitochondria was achieved in the same pollen (Fig. 1H–J). No overlapping signals of mtRFP and pGFP were detected in these pollen grains. This confirmed that the GFP signals were indeed localized in plastids. During pollen germination, VC plastids were observed throughout the pollen tube, except at the tip region (Fig. 1K, L). This is consistent with previous observations that the pollen tip region is free of large organelles (Rosen et al. 1964, Pierson et al. 1990).

Although the components of the plastid division machinery have been identified by previous molecular genetic studies, most of them are involved in chloroplasts rather than other types of plastids such as proplastids and amyloplasts (Aldridge et al. 2005). Studying plastid division in the plastid types other than chloroplasts is difficult, because they are not visible by chlorophyll autofluorescence. We thus considered that VC-ptoGFP allows us to study plastid division. The plant ftsZ1 gene is a critical factor in the chloroplast division machinery. The Arabidopsis ftsZ1 mutants showed enlarged chloroplasts in mesophyll cells (Yoder et al. 2007).

Fig. 1 Fluorescent images of the pollen of VC-ptoGFP plants. (A) Plasmid construct of plastid-targeted GFP that is expressed under the control of pollen-specific promoter, LATS2-P (LATS2 promoter). ‘TP’ represents plastid-targeted transit peptide. ‘Nos-T’ is a terminator of nopaline synthase. The plasmid name is VC-ptoGFP. (B) Light microscopic image of a mature pollen grain. Bar = 20 µm. (C) GFP-labeled plastids in a VC-ptoGFP plant. Bar = 20 µm. (D–G) Different morphologies of each plastid. Bars = 1 µm. (H–J) Visualization of mitochondria and plastids in the same pollen by crossing VC-mtRFP and VC-ptoGFP plants. (H) RFP-labeled mitochondria. (I) GFP-labeled plastids. (J) Merged image of (H) and (I). Bar = 20 µm. (K) Fluorescent GFP-labeled plastids in a vegetative cell during pollen germination. Bar = 20 µm. (L) Differential interference contrast image of the same field of (G). Bar = 20 µm.

The mutant line that has a T-DNA insertion at the FtsZ1 locus, SALK_124371, was found using the SIGnal ‘T-DNA Express’ database search. As the T-DNA was inserted at the first exon, this line is likely to be a null allele (producing no FtsZ1 protein). We checked the genotype of the FtsZ1 locus based on PCR analysis, and the homozygous line for T-DNA insertion was obtained (Fig. 2A). We named this line ftsZ1. The homozygous ftsZ1 plants showed enlarged chloroplasts in leaf mesophyll cells compared with wild-type plants (Fig. 2B), consistent with previous work. No FtsZ1 protein was detected in the ftsZ1 mutant when analyzed through immunoblot using AtFtsZ1-specific antibodies (Fig. 2C) (Stokes et al. 2000). We introduced the VC-ptoGFP gene into the ftsZ1 mutant by crossing, and captured pollen images (Fig. 3A, B).
The average area size of a *ftsZ1* pollen plastid was 3.11 ± 1.15 µm² (*n* = 233), showing a 64% significant increase compared with the wild type (Mann–Whitney U-test, *P* < 0.05). In addition, the plastid number was reduced to 15 ± 4 in *ftsZ1* pollen (*n* = 16). This result clearly shows that plastid division occurs in pollen grains, and *FtsZ1* is involved in the division process.

To compare the ultrastucture of plastids between wild-type and *ftsZ1* pollen, we performed electron microscopic observation ([Fig. 4A–D](#fig4)). Plastids in both lines had an oval shape surrounded by double membranes, and multiple starch grains were present in each plastid. Occasionally, simple thylakoid structures were also observed in both lines ([Fig. 4C, D](#fig4)). Thus, pollen plastids of the *ftsZ1* mutant showed an enlarged size but no other significant changes.

Before this report, *Arabidopsis* proplastids were observed through microscopy sections of meristematic tissues (Robertson et al. 1995, Robertson et al. 1996). *Arabidopsis* plastids were observed by electron microscopy of fixed materials (Van Aelst et al. 1993, Kuang and Musgrave 1996). The transgenic material presented here provides a convenient alternative for rapid determination of sizes, numbers and morphology of pollen plastids. Due to the evident structural differences between plastid types, it is likely that the division machinery is also different. This is supported by the observation that proplastid division is normal in the *arc5* mutant where chloroplast division is severely affected (Robertson et al. 1995, Robertson et al. 1996). Studying pollen plastids in other chloroplast division mutants using VC-ptGFP will be promising for a deeper understanding of plastid division.

### Materials and Methods

*Arabidopsis* plants were germinated and grown according to Miura et al. (2007). The *ftsZ1* T-DNA seeds were obtained through the Arabidopsis Biological Resource Center (SALK_124371). T-DNA mutants were identified by PCR. The presence of the wild-type allele was determined using primer sets *FtsZ1*-1u2 (5′-CTGAGATTCCTTGTACTCC TTG-3′) and *FtsZ1*-1d2 (5′-CTTCGTCGACACTAAAACC CTA-3′) that flanked the insertion site, while the combination of the primer sets *FtsZ1*-1u2 and LBb1 (LBb1, 5′-GCG TGGACCGCTTGTGCACTAAACC CTA-3′) was used to identify the mutant allele.

Western blot of *FtsZ1* protein was performed as described previously (Nishihama et al. 2001). We used the *FtsZ1*-specific antibody that was described in Stokes et al. (2000) at 1:3,000 dilution. The secondary antibody, that was described in Nishihama et al. (2001), was used at 1:2,000 dilution.

To generate transgenic plants with plastid-localized GFP signals in the VCs of pollen, chimeric gene constructs were introduced into *ftsZ1* backgrounds by crossing. The constructs were all derived from the LAT52-GFPN plasmid.
donated by Dr. Sheila McCormick (University of Berkeley, USA) and Dr. Shinichi Nishikawa (Nagoya University, Japan). LAT52-GFPN plasmid contains the GFP gene inserted between the LAT52 promoter and the nopaline synthase terminator. In this vector, SalI and NcoI restriction sites are located between the LAT52 promoter and the GFP gene. To construct VC-ptGFP, a DNA fragment containing the plastid-targeted transit peptide of IF2 (Miura et al. 2007) was produced by PCR amplification using the Columbia genome as template and the primers 5'-TTTGTCGACATGCCATCGACA TCGATGCCCGAGTCGAAGTCAG-3' and 5'-TGTCCATGGCGTCAGCAATGAAGTCAG-3'. The fragment was treated with SalI and NcoI and inserted into the SalI and NcoI site of LAT52-GFPN. To generate transgenic Arabidopsis plants, Columbia wild-type plants were transformed with the aforementioned VC-ptGFP by the in planta method (Clough and Bent 1998).

Pollen grains were released onto glass slides by gentle tapping and supplemented with 5% (w/v) mannitol (Nacalai Tesque, Kyoto, Japan). GFP signals were detected with a Disk Scanning Unit (DSU-BX61, Olympus, Tokyo, Japan) fluorescence microscope using a GFP filter set (U-MGFPHQ, Olympus), an excitation filter (460–480 nm), a dichroic mirror (DM485) and a barrier filter (495–540 nm). RFP signal detection and pollen germination were performed according to Matsushima et al. (2008).

The homozygous plants with the ftsZ1 mutation were selected based on an enlarged plastid in leaf tissues. To view plastids in leaf, tissue samples were placed on a glass slide, supplemented with 50% glycerol (Nacalai Tesque) and covered with a coverslip. True leaves were sampled from 4- to 6-week-old plants. Autofluorescence signals were detected by a filter set (U-MWIG3, Olympus), an excitation filter...
(530–550 nm), a dichroic mirror (DM570) and a barrier filter (570 nm long pass).

The anthers of flower buds were fixed for 20 h in 4% glutaraldehyde and 4% paraformaldehyde, and then post-fixed with 2% osmium tetroxide for 2 h. The fixed samples were dehydrated through an ethanol series and embedded in Spurr’s resin. Ultra-thin sections were double-stained with uranyl acetate and lead citrate, and then observed using a JEM-1200 EX transmission electron microscope (Jeol, Tokyo, Japan).

Images were analyzed using Metamorph (Molecular Devices, Downingtown, PA, USA) and Adobe Photoshop (Adobe Systems, Tokyo, Japan). Statistical analysis was performed using JMP7 (SAS Institute, wwwjmp.com).

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**References**


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