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Very-long-chain fatty acids have an essential role in plastid division by controlling Z-ring formation in Arabidopsis thaliana

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Short title: VLCFAs control Z-ring formation

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Abstract

Recent studies have revealed the essential mechanisms for plastid division that have bacterial orthologues, such as FtsZ and Min system proteins; however, causal factors regulating plastid division in plant cells are poorly understood. Here, we show that plastid division is inhibited in Arabidopsis by reduced amounts of very-long-chain fatty acids (VLCFAs), which have an acyl chain length of more than 20 carbons and are used for cuticular wax formation. The number of amyloplasts and chloroplasts decreased in the mutant defective in VLCFA synthesis and in wild-type plants treated with an inhibitor of VLCFA synthesis. Although similar inhibition of plastid division was observed in transgenic plants that overexpressed PDV2, one of the outer-membrane proteins at the plastid division site, dot-like aggregates of FtsZ protein and disordered placement of multiple Z-rings were found in wild-type chloroplasts treated the inhibitor of VLCFA synthesis. Expression analysis revealed that ARC3, one of the Min system genes, was downregulated under low VLCFA conditions. Our results indicate that VLCFAs or VLCFA-containing lipids have an essential role in plastid division by controlling Z-ring formation, demonstrating a novel function of plant VLCFAs.
Introduction

Very-long-chain fatty acids (VLCFAs) are generally defined as fatty acids with an acyl chain length of more than 20 carbons. In plants, VLCFAs are used as components of cuticular wax, seed storage triacylglycerols, root suberins, phospholipids, and sphingolipids (Samuels et al. 2008; Worall et al. 2003). As a material for VLCFA synthesis, stearic acid (18:0), a long-chain fatty acid (LCFA), is synthesized \textit{de novo} in plastids. LCFA synthesis proceeds through the following continuous enzymatic reactions: (1) condensation of fatty acid precursor (C2 to C16-acyl carrier protein [ACP]) with a carbon donor (malonyl-ACP) catalyzed by ketoacyl-ACP synthase (KAS), (2) reduction of 3-ketoacyl-ACP by 3-ketoacyl-ACP reductase (KAR), (3) dehydration of 3-hydroxy acyl-ACP by 3-hydroxy acyl-ACP dehydratase (HAD), and (4) reduction of enoyl-ACP by enoyl-ACP reductase (ENR) (Ohlrogge & Jaworski 1997). In \textit{Arabidopsis}, \textit{had1 had2} double mutants show a lethal phenotype, suggesting that LCFA synthesis is indispensable for plant development (Wu & Xue 2010). The \textit{mod1} mutant, which has a mutation in \textit{ENR}, exhibits pleiotropic developmental defects, such as chlorotic and curly leaves, stunted growth, reduced fertility, and reduced number of chloroplasts (Mou et al. 2000). \textit{Arabidopsis kasI} mutants also display stunted growth, reduced fertility, and impaired chloroplast division in young leaves (Wu & Xue 2010).

Using LCFAs as an initial precursor, VLCFAs are synthesized in the endoplasmic reticulum (ER) by sequential addition of 2-carbon moieties to acyl-CoA precursors. The carbon donor malonyl-CoA is synthesized from acetyl-CoA by acetyl-CoA carboxylase. The elongation reactions for VLCFA synthesis consist of 4 enzymatic reactions: (1) condensation of acyl-CoA with malonyl-CoA catalyzed by ketoacyl-CoA synthase (KCS), (2) reduction of 3-ketoacyl-CoA by 3-ketoacyl-CoA reductase (KCR), (3) dehydration of 3-hydroxy acyl-CoA by 3-hydroxy acyl-CoA dehydratase (HCD), and (4) reduction of enoyl-CoA by enoyl-CoA reductase (ECR) (Bach & Faure 2010). The complete loss of HCD or KCR in \textit{Arabidopsis} is
lethal to embryos, demonstrating that VLCFAs are essential for plant growth (Bach et al. 2008; Beaudoin et al. 2009). Studies on Arabidopsis plants expressing reduced levels of KCS, KCR, and ECR have indicated that VLCFA synthesis plays an essential role in organ formation by producing cuticular wax on the plant’s surface (Millar & Kunst 1997; Millar et al. 1999; Todd et al. 1999; Yehrmov et al. 1999; Fiebig et al. 2000; Zheng et al. 2005; Lee et al. 2009; Beaudoin et al. 2009). On the other hand, VLCFA function other than that in cuticular wax formation has also been noted. For example, overexpression of FAE1 encoding KCS altered the thylakoid membrane shape in chloroplasts, suggesting a role in membrane dynamics (Millar et al. 1998). The leaky pas2-1 mutant, which contains reduced amounts of VLCFAs because of a defect in the HCD-encoding PAS2 gene, showed a higher sensitivity to cytokinin and exhibited ectopic cell proliferation (Faure et al. 1998; Bellec et al. 2002; Haberer et al. 2002; Harrar et al. 2003; Bach et al. 2008). Arabidopsis mutants for PAS3/GURKE and PAS1, which encode acetyl-CoA carboxylase and a scaffold protein for VLCFA elongation enzymes, respectively, also displayed similar phenotypes (Faure et al. 1998; Baud et al. 2004; Roudier et al. 2010), an indication that VLCFAs are involved in the control of cell division; however, the precise role of VLCFAs in multiple aspects of plant development remains elusive.

Plastids, such as amyloplasts and chloroplasts, are plant-specific symbiotic organelles and have essential roles in photosynthesis, and in amino acid and lipid biosynthesis (Galili 1995; Ohlrogge & Browse 1995). Amyloplasts are required for biosynthesis and storage of starch in sink organs, while chloroplasts are where photosynthesis in source organs is carried out. No plastid is generated de novo; they are produced from preexisting plastids through division processes.

The plastid division mechanism has an evolutionary origin in a bacterial cytokinetic apparatus; the filamentous temperature-sensitive (FtsZ) protein has a pivotal role in division
of both plant plastids and bacteria (Osteryoung & Vierling 1995; Strepp et al. 1998; reviewed by Yang et al. 2008). FtsZ is a tubulin-like GTPase that self-assembles into an FtsZ-ring (Z-ring) at the division site, and the Z-ring provides constriction force for division (Osawa et al. 2008). Two types of FtsZ proteins, FtsZ1 and FtsZ2, are encoded in the plant genome (Vitha et al. 2001). In prokaryotic cells, Min system proteins (MinC, MinD, and MinE), which restrict localization and polymerization of FtsZ proteins, allow the Z-ring to be formed at only the midsection of a cell (Margolin 2005). Plant ARC3, MinD (ARC11), and MinE (ARC12) are the counterparts of bacterial MinC, MinD, and MinE, respectively, and control the Z-ring formation on the inner membrane of plastids (reviewed by Yang et al. 2008). Nakanishi et al. (2009) reported that MCD1, a plant-specific membrane protein, interacts directly with MinE and controls proper placement of the Z-ring. After determination of the Z-ring assembly site, the Z-ring is stabilized on the inner membrane by the membrane proteins ARC6 and PARC6 (Vitha et al. 2003; Glynn et al. 2008; Glynn et al. 2009). PDV2 and PDV1, which are plant-specific coiled-coil transmembrane proteins, are localized to the outer membrane at the plastid division site and interact with ARC6 and PARC6, respectively (Glynn et al. 2008; Glynn et al. 2009). They then recruit a plant-specific dynamin-like GTPase, ARC5 (DRP5), to the outer membrane to achieve plastid division (Gao et al. 2003; Miyagishima et al. 2003; Yoshida et al. 2006). All of the Arabidopsis mutants with defects in the abovementioned components usually fail in proper Z-ring assembly, leading to incomplete chloroplast division (Vitha et al. 2003; Miyagishima et al. 2006; Glynn et al. 2007; Fujiwara et al. 2008; Glynn et al. 2009; Nakanishi et al. 2009; Wilson et al. 2011).

A recent report demonstrated that 2 mechanosensitive channel proteins, MSL2 and MSL3, are involved in the initial step of plastid division, suggesting a functional link between chloroplast division and membrane tension (Wilson et al. 2011); however, causal factors with host-cell origin that control plastid division remain relatively unknown. In this study, we
found that reduction of VLCFA content inhibited amyloplast and chloroplast division in *Arabidopsis*. The impaired plastid division was accompanied by misassembly of FtsZ proteins and improper placement of the Z-ring in chloroplasts. Our results demonstrate that plant VLCFAs have a novel function in Z-ring formation in plastids.

**Results**

*Reduced VLCFA synthesis inhibits amyloplast division and promotes starch accumulation*

*pas2-1* mutant seedlings, which contain reduced amounts of VLCFAs, exhibit pleiotropic phenotypes such as ectopic cell proliferation in shoots (Faure *et al*. 1998), fusion of aerial organs (Bellec *et al*. 2002; Harrar *et al*. 2003), shortened primary roots (Bach *et al*. 2011), and generation of callus-like structures from differentiated tissues (Bellec *et al*. 2002). We also found that hypocotyls and cotyledons reacted strongly to Lugol’s solution, the strong stain indicating the presence of starch (Fig. 1A). Our measurement of starch content revealed that *pas2-1* mutant seedlings contained approximately 10 times more starch than wild-type seedlings (Fig. 1B). Hypocotyl sections of *pas2-1* mutant seedlings showed that starch accumulation was prominent in the amyloplasts in cortical cells (Fig. 1C). We then quantified the number and size of amyloplasts and found that each was apparently bigger in *pas2-1* mutant seedlings than in wild-type seedlings, (Figs. 2A, B, +Suc), whereas the number in each cortical cell decreased to 1/3 in the *pas2-1* mutants (Figs. 2A, C, +Suc). These results indicate that reduction of VLCFA synthesis is accompanied by an increase in starch accumulation in less number of amyloplasts.

Starch is synthesized by photosynthesis and by using a carbon source absorbed as a nutrient. To test whether the carbon supply affects the number and size of amyloplasts, we depleted sucrose from the MS medium. As shown in Fig. 2A, amyloplasts were rarely stained by Lugol’s solution in both the wild-type and *pas2-1* seedlings, indicating the occurrence of a
carbon source deficiency; however, in the absence of sucrose, the size and number of amyloplasts in the pas2-1 seedlings were again increased and decreased, respectively, as compared to those in the wild-type seedlings (Figs. 2B, C, -Suc). These results suggest that reduced VLCFA synthesis inhibits amyloplast division independent of starch accumulation.

To further examine the above observation, we treated wild-type plants with cafestrole, which blocks the first step in VLCFA elongation reactions by targeting KCS (Trenkamp et al. 2004). Cafestrole-induced reduction of VLCFA content has been reported in barley and cucumber (Yang et al. 2010). In Arabidopsis, we found that the C22:0 and C24:0 VLCFA content was reduced by cafestrole in a dose-dependent manner, whereas no significant change was observed for C18 LCFAs or C20 VLCFAs (Fig. 3A). Although our experimental conditions did not allow us to detect C26 or longer fatty acids, this result indicates that cafestrole inhibits the biosynthesis of C22 and C24 fatty acids. As in the pas2-1 mutant, wild-type seedlings grown in the presence of cafestrole accumulated higher amounts of starch in the amyloplasts in shoot apices (Fig. 3B), suggesting that low VLCFA content is the cause of the amyloplast phenotype.

Previously, Faure et al. (1998) reported that pas2-1 mutants exhibit hypersensitivity to cytokinin, indicating a possibility that increased cytokinin signaling might lead to the amyloplast phenotype. To test this possibility, we used a transgenic line overexpressing cytokinin oxidase 2 (Pro35S:CKX2), in which active cytokinins are degraded and decreased by 10 times higher expression of CKX2 than wild-type (Werner et al. 2003). We grew Pro35S:CKX2 seedlings in the presence of cafestrole and observed sections of shoot apices stained with Lugol’s solution. As a result, starch accumulation in amyloplasts was detected at a level comparable to that in cafestrole-treated wild-type plants (Fig. 3B), indicating that cytokinin is not related to the amyloplast phenotype observed under low VLCFA conditions.
VLCFAs are required for proper chloroplast division

We then investigated the chloroplast phenotype in mesophyll cells, which were isolated from first leaves of 5-day-old and 2-week-old seedlings. Mesophyll cells from wild-type leaves contained chloroplasts with a globular shape, while those from pas2-1 or from cafenstrole-treated wild-type seedlings harbored oval-shaped chloroplasts regardless of leaf stage (Figs. 4A, B). Quantitative analyses showed that, although chloroplasts were larger in 5-day-old seedlings of pas2-1 and the cafenstrole-treated wild type, the size in the non-treated wild-type control reached a level comparable to that in the pas2-1 seedlings after 2 weeks (Figs. 4C, D). On the other hand, the number of chloroplasts per cell was significantly reduced in the pas2-1 and the cafenstrole-treated wild-type leaves for both 5-day-old and 2-week-old seedlings (Figs. 4E, F). These data indicate that lower VLCFA synthesis impairs chloroplast division and results in larger chloroplasts in young leaves, whereas chloroplasts mature regardless of VLCFA content. This and the abovementioned results demonstrate that VLCFAs are essential for proper plastid division.

A previous report has described that PDV proteins are recruited to the outer membrane at the chloroplast division site and determine the chloroplast division rate (Okazaki et al. 2009). This suggests that VLCFAs might control expression and/or function of PDV proteins. We applied cafenstrole to the pdv2-1 mutant and transgenic plants overexpressing PDV2 under the cauliflower mosaic virus 35S promoter. As described previously, pdv2-1 and Pro35S:PDV2 seedlings had reduced and increased numbers of chloroplasts, respectively (Figs. 5A, B; Miyagishima et al. 2006; Okazaki et al. 2009). Cafenstrole treatment of pdv2-1 did not further decrease the number of chloroplasts, indicating the essential role of PDV2 in chloroplast division (Fig. 5B); however, when Pro35S:PDV2 seedlings were grown in the presence of cafenstrole, the promoting effect of PDV2 overexpression on chloroplast division was nearly cancelled (Figs. 5A, B). Note that PDV2 expression level was not reduced by
cafenstrole treatment in either the wild-type and Pro35S:PDV2 seedlings (Fig. 5C), indicating a negligible effect of VLCFA content on PDV2 and the transgene expressions. Although we cannot deny the possibility that cafenstrole effectively inhibits the function of PDV2 or downstream regulators by unknown mechanisms, our results suggest that VLCFAs may control chloroplast division through another signaling pathway, which does not directly link to PDV functions.

**VLCFAs are involved in Z-ring formation in chloroplasts**

We then investigated whether the chloroplast Z-ring is properly formed under low VLCFA conditions. To visualize the Z-ring, we used an Arabidopsis line expressing GFP-fused FtsZ2 under the FtsZ2 promoter (Nakanishi et al. 2009). In the absence of cafenstrole, FtsZ2–GFP was localized at the middle of the chloroplast as a single ring (Fig. 6A; Nakanishi et al. 2009). In contrast, multiple rings were observed in seedlings treated with cafenstrole, and each ring was oriented toward a different direction (Fig. 6A). Measurement of the number of Z-rings revealed that cafenstrole treatment reduced the percentage of chloroplasts with a single ring, whereas those with more than 2 rings were significantly increased (Fig. 6B). Moreover, we detected dot-like fluorescence on chloroplasts in cafenstrole-treated leaves (Fig. 6A). These results imply that inhibition of VLCFA synthesis disturbs Z-ring formation, leading to impaired chloroplast division.

The appearance of multiple Z-rings is a commonly observed phenotype in mutants with defects in any step of FtsZ-associated chloroplast division such as in minD, minE (Vitha et al. 2003; Fujiwara et al. 2008), arc3 (Glynn et al. 2007), med1 (Nakanishi et al. 2009), msl2 msl3 (Wilson et al. 2011), arc6 (Vitha et al. 2003), parc6 (Glynn et al. 2009), pdv1, pdv2, and arc5/drp5b (Miyagishima et al. 2006). On the other hand, dot-like aggregates of FtsZ protein are especially found in mutants for the Min system, which is involved in the
initial step of Z-ring formation by restricting FtsZ protein localization (Vitha et al. 2003; Fujiwara et al. 2008; Wilson et al. 2011). To get insight into the role of VLCFAs, we measured expression levels of genes for chloroplast division, such as FtsZ (FtsZ1, FtsZ2), Min-related genes (MinD, MinE, ARC3, MCD1), and channel genes (MSL2, MSL3), in seedlings treated with cafenstrole. Quantitative RT-PCR showed that expression of FtsZ genes was slightly reduced by cafenstrole treatment, and that most of the genes for the Min system and channel proteins displayed only a small increase (Fig. 7). We noted that the transcripts of ARC3, one of the Min genes, were significantly decreased by cafenstrole treatment (Fig. 7), suggesting a possibility that VLCFAs might be involved in maintaining the Min system.

**Discussion**

In the present study, we showed that the pas2-1 mutant had a lower number of amyloplasts, while total starch content was dramatically elevated compared to that in the wild-type seedlings. In tobacco BY-2 cells, cytokinin application promotes starch accumulation in amyloplasts (Miyazawa et al. 1999). In addition, ectopic expression of Agrobacterium isopentenyltransferase (ipt) gene, which encodes a crucial enzyme for cytokinin synthesis, induced local overproduction of cytokinin and ectopic accumulation of starch grains in tobacco plants (Guivarc'h et al. 2002). Since pas2-1 mutants display hypersensitivity to cytokinin (Faure et al. 1998; Harrar et al. 2003), we speculated that higher starch accumulation under low VLCFA conditions might be associated with cytokinin signaling; however, starch accumulation was also enhanced by cafenstrole treatment in CKX2-overexpressing seedlings. It is therefore unlikely that a reduced VLCFA level promotes starch accumulation through the cytokinin-mediated pathway. One possibility is that structural changes of amyloplast might activate starch metabolic pathways under low VLCFA conditions. Indeed, it is known that starch metabolic processes are altered in the arc5 mutant,
which carries bigger amyloplasts (Yun & Kawagoe 2009). Moreover, higher accumulation of starch granules was observed in chloroplasts of some *arc* mutants (*arc3*, *arc5*, and *arc6*), which have bigger chloroplasts because of impaired plastid division (Austin II & Webber 2005). In this scenario, higher starch accumulation observed in this study may be an indirect outcome of inhibition of amyloplast division. Further studies will reveal how structural changes of plastids generally affect starch biosynthesis.

We revealed that reduction of VLCFA content inhibits division of amyloplasts and chloroplasts; whereas *PDV2* overexpression was not fully epistatic to the inhibitory effect of cafenstrole on plastid division, abnormal Z-ring formation, such as multiple Z-rings and dot-like FtsZ aggregates, was observed in cafenstrole-treated chloroplasts. These results imply that VLCFAs are prerequisite to proper Z-ring formation and play a crucial role in plastid division. Previous studies have also suggested the involvement of fatty acids in chloroplast division; *Arabidopsis kasI* and *mod1* mutants, which have defects in LCFA synthesis, displayed impaired chloroplast division and possessed less chloroplasts compared to wild-type plants (Mou et al. 2000; Wu & Xue 2010). Misplacement of the Z-ring was also observed in *kasI* mutants (Wu & Xue 2010). Because LCFAs are materials for VLCFA synthesis in ER, it is conceivable that, in *kasI* and *mod1* mutants, lower VLCFA content caused by the lack of LCFAs might result in impaired chloroplast division; therefore, we propose that VLCFAs are those that are essential for proper plastid division.

Our results demonstrated that VLCFAs are required for Z-ring formation and its localization at the middle of the chloroplast. We assume 3 possibilities to explain the role of VLCFAs: 1) VLCFAs are associated with signaling pathways that control gene expression for plastid division mechanisms, 2) VLCFAs are required for proper localization of division-related proteins, and 3) VLCFAs are involved in trafficking of division-related proteins through the endomembrane system.
The first possibility is suggested because the imbalance of gene expression related to Z-ring formation causes defects in chloroplast division. It was demonstrated that a strict balance between gene expressions for \textit{FtsZ1} and \textit{FtsZ2} is important to achieve proper chloroplast division (Osteryoung \textit{et al.} 1998; Stokes \textit{et al.} 2000). Fujiwara \textit{et al.} (2008) reported that multiple Z-rings and dot-like aggregates were differentially formed depending on relative expression levels of \textit{MinD} and \textit{MinE}; however, our expression analysis showed that stoichiometric balance between expression levels for \textit{FtsZ1} and \textit{FtsZ2} and for \textit{MinD} and \textit{MinE} did not change under low VLCFA conditions. Instead, we found that cafenstrole treatment significantly reduced the transcript level of \textit{ARC3}, one of the Min-related genes whose products control localization and polymerization of FtsZ proteins (Maple \textit{et al.} 2007); however, the \textit{arc3} mutant forms multiple Z-rings but not dot-like FtsZ aggregates (Glynn \textit{et al.} 2007), both of which were observed in this study under low VLCFA conditions. This suggests that VLCFAs may regulate multiple genes controlling Z-ring formation. In mammals, yeast, and bacteria, it is known that VLCFAs or VLCFA-containing lipids function in controlling gene transcription (Black \textit{et al.} 2000). In \textit{Arabidopsis}, arachidonic acid positively regulates stress-related genes (Savchenko \textit{et al.} 2010); therefore, although we cannot determine whether reduced expression of \textit{ARC3} is a cause or a consequence of impaired chloroplast division, it is possible that VLCFAs act as mediators or ligands controlling gene expression for plastid division mechanisms.

The second possibility is that VLCFAs might control proper localization of division-related proteins in plastid membranes. In maize mesophyll cells, VLCFA-containing lipids were detected in chloroplast envelope membranes (Poincelot 1976), probably residing as phospholipids. The role of VLCFAs and VLCFA-containing lipids in protein association has been described for the lipid raft, which is the micro-domain made of phospholipids, sphingolipids, cholesterol, and proteins, and functions in membrane signaling and trafficking.
Arabidopsis TOC75 protein, a component of the plastid outer membrane, was found in a fraction of detergent-resistant membranes (Borner et al. 2005), suggesting that specific proteins might be included in lipid raft-like domains in plastid membranes. Although the association of plastid division-related proteins with the lipid raft has not been described, it is likely that VLCFA-containing phospholipids mediate the interaction between plastid membrane components and division-related proteins. At the later stage of plastid division, the membrane curves at the constriction site. Considering that VLCFA-containing lipids have an important role in stabilizing highly curved membrane domains (Schneiter et al. 2004), they might function not only as a scaffold for assembling FtsZ-related proteins, but also as one of the division mechanisms that facilitates membrane dynamics during cell constriction.

The third possibility is that VLCFAs might be required for membrane trafficking of division-related proteins. Arabidopsis cer10, pas1, and pas3 mutants that contain reduced amounts of VLCFAs and VLCFA-containing lipids, exhibit impaired endomembrane trafficking (Zheng et al. 2005; Roudier et al. 2010). It has been demonstrated that VLCFA-containing phospholipids play an important role in stabilizing transport vesicles from the ER (Sturbois-Balcerzak et al. 1999). Moreover, Bach et al. (2011) demonstrated that VLCFAs are prerequisite to endomembrane dynamics during cytokinesis, which requires vesicle transport for cell-plate formation; therefore, it is probable that VLCFAs and/or VLCFA-containing lipids play an important role in endomembrane trafficking of plastid division-related proteins, such as ARC3, MinD, and MinE. Further studies will reveal how VLCFAs and their derivatives are associated with gene expression, protein localization, or endomembrane trafficking in plastid division mechanisms, and how these regulations are involved in FtsZ protein assembly and Z-ring formation and arrangement.
Because some of the enzymes for VLCFA elongation reactions are expressed specifically in the epidermis (Yephremov et al. 1999; Joubès et al. 2008), VLCFAs are likely to be synthesized predominantly in the epidermis; therefore, the present study suggests that chloroplast division in mesophyll cells is controlled by VLCFAs or VLCFA-containing lipids transported from the epidermis to the inner tissues. It is also probable that VLCFAs may be associated with some metabolic pathway in epidermal cells, and that inhibition of VLCFA synthesis might change the level of some metabolites that affect plastid division in internal tissues. pas mutants sometimes show ectopic cell proliferation in leaves (Faure et al. 1998), demonstrating that VLCFAs are also involved in the control of cell division. This raises an interesting hypothesis that VLCFAs are engaged in maintaining the plastid number by suppressing cell proliferation and promoting plastid division during the continuous growth and development of plants.

**Experimental procedures**

*Plant materials and growth conditions*

pas2 (Faure et al. 1998), pdv2-1 (Miyagishima et al. 2006), Pro35S:PDV2 (Okazaki et al. 2009), and ProFtsZ2:FtsZ2-GFP (Nakanishi et al. 2009) were described previously. All Arabidopsis plants used in this study are in the Columbia (Col-0) background. Arabidopsis plants were grown in Murashige and Skoog (MS) medium (1 × MS salts, 1 × MS vitamins, and 2% [w/v] sucrose [pH 6.3]) under continuous light conditions at 23°C. For the sucrose-depletion experiment, plants were grown in MS medium containing 1.064% (w/v) mannitol instead of sucrose. Cafenstrole (HPLC standard grade, Wako Pure Chemical Industries) was dissolved in dimethylsulfoxide at appropriate concentrations, and diluted 1,000 fold into the media.
Quantification of starch content

Approximately 100 mg of whole seedlings were frozen with liquid nitrogen and starch content was measured using the Starch Assay Kit (Sigma-Aldrich Corp.) according to the manufacturer’s instruction.

Histological analysis

Tissues were sectioned as described previously (Adachi et al. 2009). Briefly, tissue samples were fixed with glutaraldehyde and dehydrated with an ethanol series, after which ethanol was substituted with Technovit 7100 solution (Heraeus Kulzer). After solidification, the samples were sectioned to a thickness of a 4–5 μm and stained with Lugol’s solution (Sigma-Aldrich Corp.) to visualize starch granules.

Lipid analysis

Total fatty acid was extracted from whole seedlings using the Fatty Acid Methylation kit (Nacalai Tesque, Inc.), and purified using the Fatty Acid Methyl Ester Purification kit (Nacalai Tesque, Inc.) according to the manufacturer’s instruction. Fatty acid methyl ester samples were analyzed by gas chromatography/mass spectrometry (Hewlett Packard 5890 Series II/JEOL JMS-700 MStation) with an ULBON HR-SS-10 capillary column (φ0.25 mm × 30 m; Shinwa Chemical Industries). Peaks of fatty acid methyl esters were identified by comparing them with fatty acid methyl ester standards (GLC Reference Standard GLC62; NU-CHEK-PREP), and quantified using a selected ion monitor (SIM) mode with m/z 74.

Microscopy observation

Chloroplasts were observed as described by Pyke and Leech (1991). Leaves were fixed with 3.5% (v/v) glutaraldehyde for 1 hour, and incubated in a solution of 0.1 M Na₂EDTA (pH 9)
for 1–2 hours at 60°C. Mesophyll cells were then isolated by gentle tapping with tweezers on the cover glass, and observed with Nomarski differential interference contrast microscope. For quantification of plastid number and area, microscopic images were analyzed using NIH ImageJ 1.43u (http://rsb.info.nih.gov/nih-image/). GFP fluorescence in leaves was observed using a confocal laser-scanning microscope (FV1000, Olympus Corp.) without fixation.

**RT-PCR**

Total RNA was extracted from 5-day-old whole seedlings using an RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer’s instructions. cDNA was synthesized from 2 µg of total RNA with SuperscriptII reverse transcriptase (Invitrogen Corp.). Real-time RT-PCR was performed using the LightCycler system (Roche Applied Science) and SYBR Green I (TaKaRa Bio Inc.) with the following primers: 5′-AGCTCTTGGAGTCTCAGGCTG-3′ and 5′-GAGCCAGTTGCTCTCATATTGT-3′ for *PDV2*, 5′-TCCTCGGTATGATGCATTG-3′ and 5′-GCAAAAAGAGACAAAAATCTTCAAAA-3′ for *MinD*, 5′-CACCCTCTCGCTCTCA-3′ and 5′-TGCTTATGAAATCCGTGAAA-3′ for *MinE*, 5′-CTCTTCTGACTTGAATTTTGG-3′ and 5′-CTTCAAGGCATTATCTTGGTA-3′ for *ARC3*, 5′-CCCTCTGTGCTCTTCCTGA-3′ and 5′-AAAACAGTAGCCGTGATGCAG-3′ for *MCD1*, 5′-CACAAGGTTTTTGTAATCCAG-3′ and 5′-ATGTTGTCCCAACCGGAACAC-3′ for *MSL2*, 5′-AGTTTCTGGCAGAGGCAA-3′ and 5′-CCGGTCATCACCTCTGATA-3′ for *MSL3*, 5′-CATTCTCTGCCTAGTGTGGA-3′ and 5′-CCCTGACTCCTCTCTCT-3′ for *FtsZ1*, 5′-CTTAGATGTCAGTCCTGGG-3′ and 5′-ATTACAGGGGCACCA-3′ for *FtsZ2*, and 5′-CCGGTGGGTCTGACGAGATAGA-3′ and 5′-CCTCTTCTCCTGCCTAG-3′ for *TUBULIN4*. The PCR conditions were 1 cycle at 95°C for 45 sec; and 44 cycles at 95°C for 15 sec, at 60°C for 30 sec, and at 72°C for 45 sec.
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Referencess


Lee, SB., Jung, SJ., Go, YS., Kim, HU., Kim, JK., Cho, HJ., Park, OK. & Suh, MC. (2009) Two Arabidopsis 3-ketoacyl CoA synthase genes, KCS20 and KCS2/DAISY, are


Figure legends

Figure 1 Starch accumulation in pas2-1 seedlings.
(A) Lugol’s staining of 5-day-old wild-type seedlings (left) and pas2-1 seedlings (right). (B) Quantification of starch content in wild-type and pas2-1 seedlings. Values are mean ± s.d. of 3 biological replicates. FW, fresh weight. (C) Cross sections of hypocotyls of 5-day-old wild-type and pas2-1 seedlings. Starch granules were stained with Lugol’s solution. Bars, 1 mm (A) and 50 µm (C).

Figure 2 Amyloplasts in pas2-1 hypocotyls.
(A) Amyloplasts in cortical cells stained with Lugol’s solution. Hypocotyl sections were prepared at a thickness of 4 µm from 5-day-old seedlings grown in the presence (+Suc) or absence (-Suc) of sucrose. Bar, 10 µm. (B, C) Area of each amyloplast (B) and number of amyloplasts in a cortical cell (C). Images of cross sections were used for measurement. Values are mean ± s.d. (n ≥ 191 for (B) and n ≥ 50 for (C)). Significant differences between the wild-type and pas2-1 seedlings were determined using the Student’s t-test: ***, P < 0.001.

Figure 3 Higher starch accumulation after cafenstrole treatment.
(A) Reduction of VLCFA contents after cafenstrole treatment. Five-day-old seedlings grown in the absence (w/o) or presence of cafenstrole (30 nM or 3 µM) were subjected to lipid analysis. Fatty acid composition is represented as mol (%). Data are presented as mean ± s.d. of 3 biological replicates. Significant differences between untreated and cafenstrole-treated seedlings were determined using the Student’s t-test: ***, P < 0.001 and **, P < 0.01; the other differences were not significant (P > 0.05). (B) Longitudinal sections of shoot apices of 5-day-old seedlings grown in the presence or absence of 30 nM cafenstrole. Sections of wild-type and Pro35S:CKX2 shoot apices were stained with Lugol’s solution. Bar, 50 µm.
**Figure 4** Reduction of the number of chloroplasts under low VLCFA conditions.

(A, B) Chloroplasts in the wild-type seedlings, *pas2-1* mutants, and wild-type seedlings treated with 3 µM cafenstrole. Mesophyll cells were isolated from the first leaves of 5-day-old (A) and 2-week-old (B) seedlings. Bars, 10 µm. (C, D) Area of each chloroplast in 5-day-old (C) and 2-week-old (D) seedlings. The results of the measurement with each microscopic image were averaged. Values are mean ± s.d. (n ≥ 178). (E, F) Number of chloroplasts in a mesophyll cell of 5-day-old (E) and 2-week-old (F) seedlings. The results of the measurements with each microscopic image were averaged. Values are mean ± s.d. (n = 50). Significant differences between the wild-type and *pas2-1* or cafenstrole-treated wild-type seedlings were determined using the Student’s *t*-test: ***, P < 0.001; the other differences were not significant (P > 0.05). caf., cafenstrole.

**Figure 5** Cafenstrole treatment of *PDV2*-overexpressing plants and *pdv2-1* mutants.

(A) Chloroplasts of wild-type, *Pro35S:PDV2*, and *pdv2-1* seedlings. Mesophyll cells were isolated from the first leaves of 5-day-old seedlings grown in the presence (+) or absence (−) of 3 µM cafenstrole. Bar, 10 µm. (B) Number of chloroplasts in a mesophyll cell of 5-day-old seedlings grown in the presence (+) or absence (−) of 3 µM cafenstrole. The results of the measurements with each microscopic image were averaged. Values are mean ± s.d. (n = 50). Significant differences were determined using the Student’s *t*-test: ***, P < 0.001; the other differences were not significant (P > 0.05). (C) Expression level of *PDV2* in 5-day-old seedlings grown in the presence (+) or absence (−) of 3 µM cafenstrole. Total RNA was subjected to real-time RT-PCR. Expression levels were normalized to *TUBULIN4* and are indicated as relative values, with that for the nontreated wild-type seedlings set to 1. Data are presented as mean ± s.d. of 3 biological replicates.
Figure 6 Misplacement of Z-ring in cafenstrole-treated chloroplasts.

(A) Localization of FtsZ2-GFP in chloroplasts. Mesophyll cells were isolated from the first leaves of 7-day-old seedlings carrying ProFtsZ2:FtsZ2-GFP grown in the presence or absence of 3 μM cafenstrole. Lower photos are enlarged images of upper ones. Arrowheads indicate Z-rings. Autofluorescence of chloroplasts is displayed by pseudo-colored red. Bars, 5 μm. (B) Percentage of chloroplasts with 1, 2, or >2 Z-rings. Values are mean ± s.d. of 3 biological replicates. More than 90 chloroplasts were observed for each replicate.

Figure 7 Expression levels of chloroplast division-related genes.

RNA samples were prepared from 5-day-old wild-type seedlings grown in the absence (w/o) or presence of 3 μM cafenstrole. Total RNA was subjected to real-time RT-PCR. Expression levels were normalized to TUBULIN4 and are indicated as relative values, with those for the nontreated control set to 1. Data are presented as mean ± s.d. of 3 biological replicates.
Figure 2

(A) Wild-type vs. pas2-1 with +Suc and -Suc conditions.

(B) Graph showing Amyloplast area (µm²) with +Suc and -Suc conditions for WT and pas2-1.

(C) Graph showing Amyloplast number per cell with +Suc and -Suc conditions for WT and pas2-1.
Figure 3

(A)

(B)
Figure 4

(A) 5-day-old
Wild-type  pas2-1  cafenstrole

(B) 2-week-old
Wild-type  pas2-1  cafenstrole

(C) 5-day-old
Chloroplast area (μm²)

(D) 2-week-old
Chloroplast area (μm²)

(E) 5-day-old
Chloroplast number per cell

(F) 2-week-old
Chloroplast number per cell

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Figure 6

(A) - Cafenstrole + Cafenstrole

(B) Graph showing % Chloroplasts vs Cafenstrole with categories 1 ring, 2 rings, and ≥3 rings.
Figure 7