<table>
<thead>
<tr>
<th>Title</th>
<th>Actin-Dependent Chloroplast Anchoring Mechanism in Spinach Mesophyll Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Takamatsu, Hideyasu</td>
</tr>
<tr>
<td>Citation</td>
<td></td>
</tr>
<tr>
<td>Issue Date</td>
<td></td>
</tr>
<tr>
<td>Text Version</td>
<td>ETD</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/11094/26855">http://hdl.handle.net/11094/26855</a></td>
</tr>
<tr>
<td>DOI</td>
<td></td>
</tr>
<tr>
<td>rights</td>
<td></td>
</tr>
</tbody>
</table>
Actin-Dependent Chloroplast Anchoring Mechanism in Spinach Mesophyll Cells

Hideyasu Takamatsu
Laboratory of Plant Cell Biology
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contents</td>
<td>2</td>
</tr>
<tr>
<td>General introduction</td>
<td>4</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>6</td>
</tr>
<tr>
<td>Chapter 1</td>
<td></td>
</tr>
<tr>
<td>Actin-Dependent Chloroplast Anchoring is Regulated by Ca^{2+}-Calmodulin in Spinach Mesophyll Cells</td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>7</td>
</tr>
<tr>
<td>Introduction</td>
<td>8</td>
</tr>
<tr>
<td>Results</td>
<td>11</td>
</tr>
<tr>
<td>Discussion</td>
<td>15</td>
</tr>
<tr>
<td>Material and methods</td>
<td>19</td>
</tr>
<tr>
<td>Figures</td>
<td>24</td>
</tr>
<tr>
<td>Chapter 2</td>
<td></td>
</tr>
<tr>
<td>A possible involvement of two kind of villin like proteins participates in chloroplast anchoring.</td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>35</td>
</tr>
<tr>
<td>Introduction</td>
<td>36</td>
</tr>
<tr>
<td>Results</td>
<td>38</td>
</tr>
<tr>
<td>Discussion</td>
<td>41</td>
</tr>
<tr>
<td>Material and methods</td>
<td>44</td>
</tr>
<tr>
<td>Figures</td>
<td>47</td>
</tr>
</tbody>
</table>
General Introduction

In plant, intracellular organelle positioning is very important for maintenance of cell activities (Takagi et al. 2011). For light requirement, photo-relocation movement of chloroplasts was induced under low-intensity light, while helping to avoid photo-damage under high-intensity light, respectively (Wada et al. 2003). On the other hand, to achieve maximal photosynthetic activity under continuous non-fluctuating illumination, intracellular positions of chloroplasts should be maintained at appropriate locations. For example, many microscopic studies have pointed out that chloroplasts are distributed along the cell wall adjacent to intercellular air spaces, but not to neighboring cells (Evans et al. 1994; Psaras et al. 1996). This distribution pattern may be advantageous not only in minimizing the diffusion pathway of CO₂ from the intercellular air space to chloroplast stroma, the site of CO₂ fixation, but also in maximizing the area available for CO₂ diffusion (Evans and von Caemmerer 1996, Terashima et al. 2006). Furthermore, Tholen et al. (2008) demonstrated that two parameters, namely, the area of chloroplast surface that faces the intercellular air space and the conductance of CO₂ in leaf tissues, change in parallel with photo-relocation movement of chloroplasts in Arabidopsis thaliana, suggesting that the intracellular positioning of chloroplasts plays a crucial role in rapid regulation of photosynthetic activity responding to sudden changes in light conditions.

Redistribution of chloroplasts is accompanied with anchoring and de-anchoring processes of chloroplasts (Takagi et al. 2009), while only a few reports suggest the involvement of microtubules (Chuong et al. 2006). Although structural basis for chloroplast anchoring on the cortical cytoplasm has not been elucidated, several reports
support the involvement of the actin cytoskeleton (Dong et al. 1998, Kandasamy and Meagher 1999, Kadota et al. 2009). However, it is not clear yet how actin cytoskeletons participate in chloroplast anchoring. In plants, as in animals, the actin organization was regulated many a variety of actin-binding proteins (MuCurdy et al. 2001). So, I assume the participation of actin-binding proteins to regulate actin-dependent chloroplast anchoring.

In plants, as in animals, Ca^{2+} signaling play very important role in response to several environmental stimuli (Batistič and Kudla 2011). Some reports suggest that Ca^{2+}-calmodulin participate in regulation of photo-relocation movement (Tlalka and Fricker 1999, Anielska-Mazur et al. 2009). However, it is not clear yet how Ca^{2+}-calmodulin participates in photo-relocation movement.

In this thesis, I first analyzed chloroplast anchoring in spinach mesophyll cells, focusing mainly the actin cytoskeleton and its Ca^{2+}-calmodulin regulation (Chapter 1). Since I have succeeded in verifying that chloroplast anchoring onto the cortical cytoplasm depends on the actin cytoskeleton and Ca^{2+}-calmodulin participates in the chloroplast de-anchoring, I further aimed to examine a possible involvement Ca^{2+}-calmodulin regulated actin-binding proteins in regulation of chloroplast anchoring (Chapter 2).
Abbreviations

ABP: actin binding protein, VLN: villin

BSA: bovine serum albumin, CHUP1: CHLOROPLAST UNUSUAL POSITIONING 1

EGTA: ethylene glycol tetraacetic acid, EGF: epidermal growth factor, LatB:
latrunculin B, LIM: LIM-domain containing proteins, PBS: phosphate buffered saline,
PIVES: piperazine-N,N'-bis(2-ethanesulfonic acid), PM: plasma membrane, PME:
PIVES, magnesium, and EGTA, SDS-PAGE: sodium dodecyl sulfate polyacrylamide
gel electrophoresis, TFP: trifluoperazine, TCA: trichloroacetic acid,
Chapter: 1

Actin-Dependent Chloroplast Anchoring is Regulated by \( \text{Ca}^{2+} \)-Calmodulin in Spinach Mesophyll Cells.

Abstract

Chloroplasts are actively anchored at the appropriate intracellular regions to maintain advantageous distribution patterns under specific environmental conditions. Redistribution of chloroplasts is accompanied with their de-anchoring and re-anchoring, respectively, from and to the cortical cytoplasm. In spinach mesophyll cells, high-intensity blue light and \( \text{Ca}^{2+} \) treatment induced the disappearance of meshwork-like array of actin filaments surrounding chloroplasts, which was suppressed by the calmodulin antagonist. Regulatory mechanisms of chloroplast anchoring were investigated using plasma-membrane ghosts (PM ghosts), on which the cortical cytoplasm underlying the PM was exposed. Addition of an actin-depolymerizing reagent or \( \text{Ca}^{2+} \) over 1 \( \mu \text{M} \) induced detachment of a substantial number of chloroplasts from the PM ghosts concomitant with disordered actin organization. The calmodulin antagonists and anti-calmodulin antibodies negated the effects of \( \text{Ca}^{2+} \). In addition, \( \text{Ca}^{2+} \)-induced detachment of chloroplasts was no longer evident on the calmodulin-depleted PM ghosts. I propose that chloroplasts are anchored onto the cortical cytoplasm through interaction with the actin cytoskeleton, and that \( \text{Ca}^{2+} \)-calmodulin-sensitized de-anchoring of chloroplasts is a critical early step in chloroplast redistribution induced by environmental stimuli.
Introduction

Intracellular redistribution of chloroplasts is accompanied with anchoring and de-anchoring processes of chloroplasts (Takagi et al. 2009). In leaf epidermal cells of the aquatic angiosperms Vallisneria spiralis (Seitz 1979) and V. gigantea (Takagi et al. 1991; Dong et al. 1998), the resistance to centrifugal force of chloroplasts located in the outer periclinal cytoplasm, namely, how firmly those chloroplasts are anchored, markedly increased and decreased, respectively, under low- and high-intensity light. In addition, in the anticlinal cytoplasm of V. gigantea epidermal cells exposed to high-intensity blue light, the resistance to centrifugal force of chloroplasts, which migrated from the periclinal cytoplasm due to the photo-relocation movement, transiently re-increased (Sakai and Takagi 2005). This transient anchoring of chloroplasts contributes to an efficient redistribution of chloroplasts induced by high-intensity blue light.

Several study support that chloroplast anchoring is regulated by an actin cytoskeleton (Takagi et al. 2003). Treatment of V. gigantea epidermal cells with the actin-depolymerizing reagent induced a decrease in resistance for the centrifugal force of chloroplasts (Dong et al. 1998). In A. thaliana mesophyll cells, the intracellular arrangement of chloroplasts was disordered when actin filaments, which form a basket-like array around the chloroplasts, were disrupted by the actin-depolymerizing reagent (Kandasamy and Meagher 1999). Furthermore, intact chloroplasts isolated from spinach leaves can interact with skeletal muscle filamentous actin (F-actin) in vitro (Kumatani et al. 2006). Moreover, a unique mutant of A. thaliana, chloroplast unusual positioning 1 (chup1), which is deficient in chloroplast photo-relocation
movement, was identified by Oikawa et al. (2003). Recombinant CHUP1 protein interacts with actin and profilin in vitro (Schmidt von Braun and Schleiff 2008). By overexpressing truncated CHUP1 proteins in the chup1 mutant plants, Oikawa et al. (2008) suggested that the coiled-coil region in CHUP1 protein plays an essential role in chloroplast anchoring on the cortical cytoplasm. However, the mode of regulation of CHUP1 activity still remains obscure.

The blue-light receptors responsible for photo-relocation movement of chloroplasts were identified as phototropins (Suetsugu and Wada 2007), which are also known to mediate a rapid blue-light induced increase in cytoplasmic Ca^{2+} levels (Harada and Shimazaki 2007). In several kinds of plant cells, a regulatory role of Ca^{2+} in chloroplast movement was suggested (Weidinger and Ruppel 1985; Russ et al. 1991; Kadota and Wada 1992; Tlalka and Gabryś 1993). On the other hand, chloroplast movement is also induced by mechanical stimuli, such as touch (Makita and Shihira-Ishikawa 1997; Sato et al. 1999; Sato et al. 2003), which have been frequently reported to cause a transient increase in the cytoplasmic level of Ca^{2+} (Knight 2000). Calmodulin antagonists inhibited chloroplast photo-relocation movement in *Lemna trisulca* L. (Tlalka and Fricker 1999) and *Nicotiana tabacum* (Anielska-Mazur et al. 2009). Ca^{2+}-calmodulin thus may participate in regulation of chloroplast movement, but its target processes have not been completely disclosed.

Plasma membrane (PM) ghosts have been a useful experimental tool to investigate the organization and function of cytoskeletal elements in the cortical cytoplasm of plant cells (Sonobe 1997; Collings and Allen 2000). PM ghosts can be prepared by rupturing or lysing protoplasts to expose the cortical cytoplasm that underlies the PM. Using such semi-intact preparations, microtubule organization (Cyr 1991; Sonobe and
Takahashi 1994; Murata et al. 2005), actin organization (Kobayashi 1996), and the interaction between the actin and microtubule cytoskeletons (Collings et al. 1998) have been examined extensively. We have been investigating regulatory mechanisms for chloroplast positioning and movement using spinach (Takagi et al. 2009). In spinach mesophyll cells, high-intensity blue light induces the actin-dependent photo-relocation movement of chloroplasts simultaneously with actin reorganization (Inoue and Shibata 1973; Kumatani et al. 2006). In chapter 1, to detect rapid actin reorganization in the cortical cytoplasm of the palisade cells, I took advantage of cryofixation known as a technique providing excellent preservation of actin filaments in plant cells (Lancelle et al. 1987; Ding et al. 1991). In addition, I established a method to prepare PM ghosts from spinach mesophyll cells to dissect the regulatory mechanism for chloroplast anchoring.
Results

Cortical actin organization in spinach mesophyll cells is regulated by Ca$^{2+}$-calmodulin. After spinach leaf sections were processed for cryofixation and indirect immunofluorescence microscopy using an anti-actin antibody as the primary antibody, samples were observed from the anticlinal side of palisade cells by a confocal laser scanning microscope. Along the Z axis of the samples, I sequentially took 30 optical images at intervals of 0.5 μm from the PM toward the cytoplasmic side. These images were super-imposed and shown as maximum projections. In palisade cells kept under dim white light (15 μmol m$^{-2}$ s$^{-1}$), actin signals were mainly observed like a meshwork surrounding each chloroplast (Fig. 1-1a). Under this condition, 85% of the palisade cells exhibited such a meshwork-like array of actin signals (Table 1-1). After treatment with latrunculin B (LatB), an actin-depolymerizing reagent, most of the actin signals disappeared (Fig. 1-1b). When the step of incubation with the primary antibody was omitted in the immunostaining procedures, little fluorescence signals were detected (data not shown). Continuous irradiation of palisade cells with high-intensity blue light (470 nm, 100 μmol m$^{-2}$ s$^{-1}$) from the adaxial side for 10 min induced the disappearance of meshwork-like array of actin filaments, and linearly-running, thicker actin bundles became prominent (Fig. 1-2a, Table 1-1). After continuous irradiation with blue light for 20 min, the actin bundles appeared to be arranged in more random directions (Fig. 1-3a).

Similar effects of blue-light irradiation for 10 min on the actin organization were induced by treatment of leaf pieces kept under dim white light with 2 mM CaCl$_2$ and 10-μM divalent cation ionophore A23187 (Fig. 1-2d, Table 1-1). CaCl$_2$ lower than 2
mM exhibited little effects (data not shown). The effects of Ca\(^{2+}\) treatment were not evident in the absence of A23187 (Fig. 1-3b). While the disappearance of meshwork-like array of actin filaments induced both by blue light and Ca\(^{2+}\) was suppressed by a calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (Figs. 1-2b, 1-2e, Table 1-1), a control reagent N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) never antagonized the effects of blue light and Ca\(^{2+}\) (Figs. 1-2c, 1-2f, Table 1-1). As predicted, W-7, but not W-5, substantially suppressed chloroplast photo-relocation movement in the palisade cells induced by high-intensity blue light (Fig. 1-4).

Chloroplast anchoring is actin dependent

On PM ghosts prepared from spinach mesophyll cells kept under dim white light by the present procedures, I noticed that many chloroplasts remained even after repeated washing with buffered solution (Fig. 1-5a). When observed by indirect immunofluorescence microscopy, actin signals seemed to be concentrated around each chloroplast on the PM ghost (Fig. 1-5b). Assuming a role of the actin filaments on the PM ghosts in anchoring the chloroplasts onto the cortical cytoplasm, I first examined the effects of LatB. To demonstrate the effects in a semi-quantitative manner, I determined the ratio of the total area occupied by chloroplasts to the whole area of PM on each PM ghost after various treatments. In Figure 1-6, the relative cumulative frequency of PM ghosts was plotted against the calculated area ratio of the PM occupied by chloroplasts under each test condition. The relative cumulative frequency for a certain value of the area ratio represents the total frequency of the PM ghosts exhibiting that value and smaller values than it. Therefore, I can see the distribution pattern of
values for the area ratio in PM ghosts prepared under each test condition without any bias.

In PM ghosts prepared in the absence of any additional reagents (untreated PM ghosts), the minimum area ratio of the PM occupied by chloroplasts was approximately 40%, with occupancy increasing linearly to a maximum area ratio of approximately 90% (Fig. 1-6a). In contrast, when PM ghosts were prepared in the presence of 0.1 or 1 μM LatB, the minimum area ratio began to increase at much lower values. A statistically significant difference was detected in the distribution patterns of values between the PM ghosts prepared in the presence of 1 μM LatB and the untreated PM ghosts (* in Fig. 1-6a). The effects of LatB appeared to be concentration dependent (Fig. 1-6a), suggesting that LatB induced detachment of chloroplasts from PM ghosts. On the PM ghosts prepared in the presence of 1 μM LatB (Fig. 1-5c), most of the actin signals disappeared, but occasionally, very faint signals were detectable on the surface of the remaining chloroplasts (Fig. 1-5d). When PM ghosts were prepared from protoplasts treated with LatB in advance, essentially identical results were obtained (data not shown).

On the other hand, the effects of propyzamide, a microtubule-depolymerizing reagent, on the microtubule organization in spinach palisade cells were examined by immunofluorescence microscopy. Most of the cortical microtubules were fragmented in the presence of propyzamide over 10 μM (Fig. 1-7). However, when chloroplast anchoring was assessed using PM ghosts, no statistically significant difference was detected between any combinations out of the untreated PM ghosts and the PM ghosts prepared in the presence of propyzamide at 10 or 100 μM (Fig. 1-8).
Chloroplast anchoring is regulated by Ca\(^{2+}\)-calmodulin

I next examined the effects of Ca\(^{2+}\) to help understand its role in chloroplast anchoring. In PM ghosts prepared in the presence of 1 μM Ca\(^{2+}\), detachment of a substantial number of chloroplasts from the PM ghosts was observed (Fig. 1-5e). A statistically significant difference was detected between the PM ghosts prepared in the presence of 1 μM Ca\(^{2+}\) (Ca\(^{2+}\)-treated PM ghosts) and the untreated PM ghosts (* in Fig. 1-6b). Actin signals in the vicinity of the remaining chloroplasts markedly reduced on the Ca\(^{2+}\)-treated PM ghosts (Fig. 1-5f), similar to the case of LatB treatment (Fig. 5d). Ca\(^{2+}\) at 10 μM showed similar effects to Ca\(^{2+}\) at 1 μM, while Ca\(^{2+}\) at 0.1 μM neither induced detachment of chloroplasts from PM ghosts nor disappearance of actin signals on PM ghosts (data not shown).

Since Ca\(^{2+}\) over 1 μM exhibited marked effects, I hypothesized an involvement of calmodulin as a regulatory factor. As predicted, W-7 exhibited a statistically significant effect to suppress a Ca\(^{2+}\)-induced detachment of chloroplasts from PM ghosts (Fig. 1-5g, # in Fig. 1-6b), while a control reagent W-5 never antagonized the effect of Ca\(^{2+}\) (Fig. 1-5i, *, $ in Fig. 1-6b). The actin organization on the Ca\(^{2+}\)+W-7-treated PM ghosts (Fig. 1-5h) did not seem to change much from that of untreated PM ghosts (Fig. 1-5b). On the contrary, disorder of actin organization was obvious on the Ca\(^{2+}\)+W-5-treated PM ghosts (Fig. 1-3j). Another calmodulin antagonist trifluoperazine (TFP) also significantly suppressed a Ca\(^{2+}\)-induced detachment of chloroplasts from PM ghosts (Fig. 5k, # in Fig. 6b). The actin organization on the Ca\(^{2+}\)+TFP-treated PM ghosts (Fig. 1-5l) was very similar to that of untreated PM ghosts (Fig. 1-5b).

Finally, using anti-calmodulin antibodies, which were proven to specifically recognize spinach calmodulin (Muto and Miyachi 1984), I aimed to verify the involvement of
calmodulin in regulation of chloroplast anchoring. I confirmed that calmodulin is localized uniformly on the untreated PM ghosts by immunofluorescence microscopy (Figs. 1-9a, 1-9b). When PM ghosts pretreated with the anti-calmodulin antibodies were further treated with 1 μM Ca$^{2+}$, Ca$^{2+}$-induced detachment of chloroplasts (Fig. 1-10a) and actin disorganization (Fig. 1-10b) were significantly suppressed. On the other hand, to ask whether the presence of calmodulin is essential to the effects of Ca$^{2+}$, I prepared calmodulin-depleted PM ghosts. Ca$^{2+}$-induced detachment of chloroplasts was no longer evident on the calmodulin-depleted PM ghosts (Figs. 1-9c, 1-9d). These results strongly support my hypothesis that calmodulin plays a regulatory role in the Ca$^{2+}$-induced detachment of chloroplasts from PM ghosts.

Discussion

Chloroplast anchoring is actin dependent

In spinach palisade cells, both high-intensity blue light (Fig. 1-2a) and Ca$^{2+}$ treatment (Fig. 1-2d) induce the disappearance of meshwork-like array of actin filaments surrounding each chloroplast. I also unequivocally demonstrated that depolymerization and/or severing of actin filaments, regardless of whether it was produced by treatment with LatB (Fig. 1-5d) or Ca$^{2+}$ (Fig. 1-5f), induces detachment of the chloroplasts from PM ghosts (Figs. 1-5c, 1-5e, 1-6). On the other hand, depolymerization of microtubules by propyzamide (Fig. 1-7) dose not induce any sign of chloroplast detachment (Fig. 1-8). These results strongly suggest that, in spinach palisade cells, the meshwork-like array of actin filaments surrounding chloroplasts, not microtubule cytoskeleton, contributes to chloroplast anchoring, and that chloroplast de-anchoring is induced by
depolymerization and/or severing of actin filaments.

My hypothesis may be supported by the following reports. Treatment of *V. gigantea* epidermal cells with the actin-depolymerizing reagent induced fragmentation of the actin filaments surrounding chloroplasts concomitant with a decrease in resistance for the centrifugal force of chloroplasts (Dong et al. 1998). In *A. thaliana* mesophyll cells, the intracellular arrangement of chloroplasts was disordered when actin filaments, which form a basket-like array around the chloroplasts, were disrupted by the actin-depolymerizing reagent (Kandasamy and Meagher 1999). Furthermore, in *A. thaliana* leaf petiole cells, the organization of cp-actin filaments, which are short actin filaments associated with the chloroplast surface on the PM side, is under the control of light (Kadota et al. 2009). The amount of cp-actin filaments increased concomitant with a decrease in chloroplast motility under low-intensity blue light, whereas it markedly decreased upon irradiation with high-intensity blue light preceding the initiation of photo-relocation movement. In addition, the organization of cp-actin filaments appears to depend on the presence of CHUP1.

Chloroplast anchoring is regulated by Ca$^{2+}$-calmodulin

The calmodulin antagonist W-7 suppressed chloroplast photo-relocation movement (Fig. 1-4), blue-light- and Ca$^{2+}$-induced actin reorganization (Figs. 1-2b, 1-2e), and Ca$^{2+}$-induced chloroplast detachment from PM ghosts (Figs 1-5g, # in Fig. 1-6b). Muto and Miyachi (1984) demonstrated that a partially-purified nicotinamide adenine dinucleotide kinase activity from spinach leaves was inhibited only after removal of the complex of calmodulin and anti-calmodulin antibodies. In this study, when calmodulin was depleted from PM ghosts using the anti-calmodulin antibodies at a slightly high
concentration, Ca$^{2+}$-induced chloroplast detachment was hampered (Figs. 1-9c, 1-9d). Furthermore, the anti-calmodulin antibodies appeared to directly suppress the Ca$^{2+}$-induced chloroplast detachment from PM ghosts (Fig. 1-10a) and actin disorganization (Fig. 1-10b). However, I could not exclude the possibility that inhibitory effects of the antibodies are attributable to a partial depletion of the complex of calmodulin and anti-calmodulin antibodies during preparation of the samples. Taken together, these evidences strongly suggest that early process of chloroplast movement induced by environmental stimuli is the actin-mediated chloroplast de-anchoring regulated by Ca$^{2+}$-calmodulin.

Anielska-Mazur et al. (2009) reported that the calmodulin antagonist TFP induced fragmentation of actin filaments in dark-adapted *N. tabacum* mesophyll cells. However, the cytoplasmic Ca$^{2+}$ level in dark-adapted leaf cells of *A. thaliana* was lower than 50 nM, and the level increased upon irradiation with blue light (Harada et al. 2003). Therefore, it is unlikely that calmodulin is activated in leaf cells kept under dark condition. It may be necessary to examine whether TFP exerted its effects as the calmodulin antagonist in the dark-adapted *N. tabacum* mesophyll cells.

Actin reorganization is regulated by Ca$^{2+}$

In spinach mesophyll cells, Ca$^{2+}$ treatment exhibited quite similar effects to high-intensity blue light on the actin organization (Figs. 1-2a, 1-2d). In dayflower guard cells, high-intensity white-light irradiation and ABA treatment induced fragmentation of the actin filaments (Eun and Lee 1997). Ca$^{2+}$ treatment without a divalent cation ionophore, which had been reported to increase the cytoplasmic Ca$^{2+}$ level (McAinsh et al. 1995), also induced a similar phenomenon (Hwang and Lee 2001). Though the
cytoplasmic Ca\(^{2+}\) level in the spinach palisade cells was not examined in this study, these evidences may indicate that cytoplasmic Ca\(^{2+}\) plays important roles in the actin reorganization induced by environmental stimuli.

On PM ghosts prepared from *Zinnia* mesophyll cells (Kobayashi 1996) and tobacco suspension cultured cells (Collings et al. 1998), high concentrations of Ca\(^{2+}\) did not produce a detectable change in the actin organization. In the spinach PM ghosts treated with Ca\(^{2+}\), although the actin filaments in the vicinity of chloroplasts were substantially disorganized, I often observed actin filaments remaining in the region of cortical cytoplasm between the chloroplasts (Fig. 1-5f). This might suggest that actin filaments associated with chloroplasts and those with cortical cytoplasm and/or PM have different sensitivity to Ca\(^{2+}\). Furthermore, the retention of Ca\(^{2+}\)-insensitive actin signals may also indicate that the Ca\(^{2+}\)-induced disorganization of actin cytoskeleton was not a nonspecific result, for example, caused by Ca\(^{2+}\)-dependent proteolytic activities.

There is a possibility that actin-binding proteins regulated by Ca\(^{2+}\)-calmodulin participate in the Ca\(^{2+}\)-calmodulin-sensitized actin reorganization. For example, P-135-ABP was identified as plant villin in lily pollen, which exhibits the actin-bundling activity at lower Ca\(^{2+}\) concentrations while Ca\(^{2+}\)-calmodulin-dependent actin-depolymerizing or -severing activities at higher Ca\(^{2+}\) concentrations (Yokota et al. 2005). Furthermore, Hwang and Lee (2001) reported that inhibitors for protein kinase and phosphatase suppressed the Ca\(^{2+}\)-induced actin reorganization in dayflower guard cells. In mammals, the Ca\(^{2+}\)-dependent actin-depolymerizing or -severing activities of proteins belonging to the gelsolin superfamily, which includes villin, are regulated by phosphorylation (Silacci et al. 2004). Phosphorylation and
dephosphorylation of actin-binding proteins may also be involved in the Ca^{2+}-dependent actin reorganization in plant cells.

In spinach palisade cells, thick, linearly-running actin bundles became prominent after blue light irradiation (Fig 1-2a, Kumatani et al. 2006) or Ca^{2+} treatment (Fig. 1-2d). Anielska-Mazur et al. (2009) reported that actin bundles in *N. tabacum* mesophyll cells became thicker after Ca^{2+} treatment without a divalent cation ionophore. In *A. thaliana*, THRUMIN1 was recently identified as an actin-bundling protein, which is involved in the regulation of photo-relocation movement of chloroplasts (Whippo et al. 2011). The actin bundles observed during blue-light irradiation or Ca^{2+} treatment might be protected from Ca^{2+}-induced actin-depolymerizing or -severing activities by such actin-bundling proteins.

Materials and Methods

Plant material

*Spinacia oleracea* L. cv. Torai plants were grown under an 8-h light (280 µmol m^{-2}s^{-1}) period at 20 °C and a 16-h dark period at 15 °C. Leaves from plants at least 90 days after sowing were used for all experiments.

Light irradiation and Ca^{2+} treatment

Plants were kept in darkness for 16 h. Leaf pieces of 1-cm square were prepared from the dark-adapted leaves removing the midvein, floated on pure water, and then kept under dim white light (15 µmol m^{-2}s^{-1}) from fluorescent lamps (FLR20SD/M; NEC, Tokyo, Japan) for 10 min. In the case of blue-light treatment, leaf pieces were irradiated
with blue light (470 nm, 100 μmol m⁻² s⁻¹) from the adaxial side using a light-emitting diode light source system (MIL-C1000T for the light source controller, MIL-U200 for the light source frame, and MIL-B18 for the light-emitting diode; SMS, Osaka, Japan). Light intensity was measured with a quantum sensor and data logger (LI-1400; LI-COR, Lincoln, Nebraska, USA). In the case of Ca²⁺ treatment, leaf pieces were floated for 1 h under white dim light on pure water containing 2 mM CaCl₂ and 10 μM divalent cation ionophore A23187, with or without the addition of 30 μM W-7 or 30 μM W-5. A23187 was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was adjusted to 0.1%. After these treatments, transverse leaf sections less than 1-mm thickness were prepared from the leaf pieces using a razor blade.

Cryofixation of leaf sections and visualization of cytoskeleton

Leaf sections were rapidly frozen by plunging them into liquid propane held at -180°C, and then immersed in methanol at -80°C for more than 72 h. After substitution, the leaf sections were brought to room temperature during over an 8-h period, rehydrated through graded methanol series, and fixed with 1.5% p-formaldehyde in a PME [piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), magnesium, ethylene glycol tetraacetic acid (EGTA)] solution (2 mM EGTA, 2 mM MgCl₂, and 50 mM PIPES-KOH at pH 7.0) for 20 min. After washing with the PME solution three times every 10 min, the fixed sections were digested with 0.1% Macerozyme R-10 (Yakult, Tokyo, Japan) in the PME solution for 20 min at 37°C, washed as described above, and permeabilized with 1% Triton X-100 in PME solution for 1 h. After washed as described, leaf sections were incubated with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄) containing 1% bovine
serum albumin (BSA) and anti-actin (C-4; ICN Biomedicals, Aurora, Ohio, USA) or anti-tubulin (Monoclonal anti-a-tubulin clone DM1A mouse ascites fluid; Sigma-Aldrich, St Louis, Missouri, USA) antibodies at 1/1000 dilution at 4 °C for 16 h, washed with PBS three times every 10 min, and further incubated with PBS containing 1% BSA and the secondary antibodies (Alexa488-labeled goat anti-mouse IgG; Molecular Probes, Oregon, USA) at 1/1000 dilution at 37 °C for 1 h. After washed as described above, samples were examined under a laser scanning confocal microscope system (LSM710; Carl Zeiss, Oberkochen, German).

Isolation of mesophyll protoplasts

Mesophyll protoplasts were isolated following the method of Walker (1987) with minor modifications. Spinach leaves were cut off and kept under dim white light for 10 min to allow recovery from damage. Then leaves were cut into small pieces using a razor blade. Leaf pieces were incubated in 4% Cellulase Onozuka RS (Yakult, Tokyo, Japan), 0.6% Macerozyme R-10, 330 mM sorbitol, 1 mM CaCl₂, and 10 mM Mes-KOH at pH 5.5 for 2 h on a rotary shaker at 60 rpm at 25 °C. The enzyme solution including protoplasts was centrifuged at 100 g for 3 min at 20 °C. The pellet was suspended in a solution of 500 mM sucrose, 1 mM CaCl₂, and 10 mM Mes-KOH at pH 6.8. The suspended protoplasts were laid on two layers of the suspending media; one contained only 500 mM sucrose and the other contained 400 mM sucrose and 100 mM sorbitol, and centrifuged at 250 g for 5 min at 20 °C. After centrifugation, intact mesophyll protoplasts were recovered from the interface between the two suspending media. All the procedures were carried out under dim white light.
Preparation of PM ghosts

PM ghosts were prepared essentially following the method of Kobayashi (1996). Isolated protoplasts were attached onto poly-L-Lys-coated cover slips and incubated in an actin-stabilizing solution (2 mM EGTA, 5.6 mM KCl, 10 mM MgCl₂, 3 mM dithiothreitol, 200 mM mannitol, 3.2 μg/ml APMSF, and 7 mM PIPES-KOH at pH 6.8) for 20 min. The protoplasts were gently ruptured by pressing down with a nylon mesh. The resultant PM ghosts were washed three times every 10 min with the actin-stabilizing solution. When effects of various kinds of reagents or various concentrations of Ca²⁺ were examined, PM ghosts were prepared using actin-stabilizing solutions containing proper reagents. LatB and propyzamide were dissolved in DMSO and diluted to appropriate concentrations with the actin-stabilizing solution. The final concentration of DMSO was adjusted to 0.1%. The actin-stabilizing solution of 0.1, 1, and 10 μM Ca²⁺ was prepared by adding 0.3, 1.25, and 1.9 mM of CaCl₂, respectively. Each Ca²⁺ concentration was calculated on the basis of the association constant between EGTA and Ca²⁺ in the presence of Mg²⁺ (Fabiato and Fabiato 1978). Calmodulin antagonists were dissolved in pure water and diluted to appropriate concentrations with the actin-stabilizing solution.

When I examined the effects of pretreatment with anti-calmodulin antibodies, PM ghosts were incubated with PBS containing 1% BSA or anti-spinach calmodulin antibodies (Muto and Miyachi 1984) at 1/1000 dilution for 30 min at room temperature. The pretreated PM ghosts were then washed three times every 10 min with the actin-stabilizing solution containing 1 μM Ca²⁺. To prepare immunodepleted PM ghosts, PM ghosts were incubated with PBS containing the anti-spinach calmodulin antibodies at 1/330 dilution for 30 min at room temperature, and then washed three times every 10 min.
min with the actin-stabilizing solution. The calmodulin-depleted PM ghosts were further washed three times every 10 min with the actin-stabilizing solution containing 1 μM Ca^{2+}.

Light microscopy of PM ghosts

PM ghosts prepared under various conditions were examined under an epifluorescence light microscope (BX-50; Olympus, Tokyo, Japan). To demonstrate the extent of chloroplast anchoring semi-quantitatively, the ratio of the total area occupied by chloroplasts to the whole area of PM was determined on the bright-field image of each PM ghost using the NIH image software (National Institute of Health, Maryland, USA). Statistical differences in the distribution patterns of the ratios obtained under different experimental conditions were analyzed using Chi-square test. When I compared two distribution patterns, I controlled comparison wise error rate of Chi-square test according to Sidak method (p < 0.00512).

For indirect immunofluorescence microscopy, PM ghosts were fixed with the actin-stabilizing solution supplemented with 4% formaldehyde and 1% glutaraldehyde for 30 min at room temperature. Actin cytoskeleton on PM ghosts was visualized by immunostaining as described above. Calmodulin on PM ghosts was visualized similarly using the anti-spinach calmodulin antibodies as the primary antibody at 1/10000 dilution. The optical images were captured digitally by charge-coupled device camera (RETIGA 2000RV; Roper Industries, Florida, USA).
Fig. 1-1. Actin organization in the cortical cytoplasm of spinach palisade cells. Leaf pieces kept under white dim light (15 \text{\mu mol m}^{-2} \text{s}^{-1}) were treated with 1 \text{\mu M LatB} for 1 h. Actin cytoskeleton in the control (a) or LatB-treated (b) palisade cells was visualized by indirect immunofluorescence microscopy after cryofixation. Bar = 10 \text{\mu m}. 
Fig. 1-2. Effects of blue light, Ca$^{2+}$, and calmodulin antagonists on actin organization in spinach palisade cells. Palisade cells were irradiated with high-intensity blue light (470 nm, 100 μmol m$^{-2}$ s$^{-1}$) from the adaxial side for 10 min in the absence of any additional reagents (a), and in the presence of 30 μM W-7 (b) or 30 μM W-5 (c). Palisade cells kept under white dim light were treated for 1 h with 2 mM CaCl$_2$ and 10 μM divalent cation ionophore A23187 in the absence of any additional reagents (d), and in the presence of 30 μM W-7 (e) or 30 μM W-5 (f). Actin cytoskeleton was visualized by indirect immunofluorescence microscopy after cryofixation. Bar = 10 μm.
Table 2-1 Frequency of spinach palisade cells harboring meshwork-like array of actin filaments surrounding chloroplasts under different experimental conditions.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Frequency of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17/20 (85%)</td>
</tr>
<tr>
<td>Blue light 10 min</td>
<td>5/20 (25%)</td>
</tr>
<tr>
<td>Blue light 10 min with W-7</td>
<td>9/20 (45%)</td>
</tr>
<tr>
<td>Blue light 10 min with W-5</td>
<td>4/20 (20%)</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2/20 (10%)</td>
</tr>
<tr>
<td>Ca²⁺ plus W-7</td>
<td>11/20 (55%)</td>
</tr>
<tr>
<td>Ca²⁺ plus W-5</td>
<td>4/20 (20%)</td>
</tr>
</tbody>
</table>

Actin cytoskeleton in spinach palisade cells was visualized by indirect immunofluorescence microscopy after cryofixation. The number of cells harboring meshwork-like array of actin filaments was counted in 20 cells in several leaf pieces derived from one leaf under each experimental condition.
Fig. 1-3. Effects of high-intensity blue-light irradiation for 20 min (a) or 2 mM CaCl₂ in the absence of A23187 (b) on actin organization in spinach palisade cells. Actin cytoskeleton in the treated palisade cells was visualized by indirect immunofluorescence microscopy after cryofixation. Bar = 10 μm
Fig. 1-4. Effects of calmodulin antagonists on chloroplast photo-relocation movement in spinach palisade cells. After dark-adapted leaf sections were mounted on a glass slide, palisade cells were irradiated with high-intensity blue light from the anticlinal side for 0 min (a, d, g), 10 min (b, e, h), or 20 min (c, f, i), in the absence of any additional reagents (a, b, c), and in the presence of 30 μM W-7 (d, e, f) or 30 μM W-5 (g, h, i). After leaf sections were fixed with 1.5% p-formaldehyde, chloroplast distribution was examined by light microscopy. Bar = 10 μm.
Fig. 1-5. Effects of LatB, Ca\(^{2+}\), and calmodulin antagonists on actin organization in spinach PM ghosts. PM ghosts were prepared in the absence of any additional reagents (a, b), in the presence of 1 \(\mu\) M LatB (c, d), 1 \(\mu\) M Ca\(^{2+}\) (e, f), 1 \(\mu\) M Ca\(^{2+}\) plus 30 \(\mu\) M W-7 (g, h), 1 \(\mu\) M Ca\(^{2+}\) plus 30 \(\mu\) M W-5 (i, j), or 1 \(\mu\) M Ca\(^{2+}\) plus 15 \(\mu\) M TFP (k, l). Actin on the PM ghosts was visualized by indirect immunofluorescence microscopy. (a, c, e, g, i, k) Bright-field images of the PM ghosts. (b, d, f, h, j, l) Merged images of actin staining (green) and chlorophyll autofluorescence (red) of the PM ghosts. Bars = 10 \(\mu\)m.
Fig. 1-6. Semi-quantitative analyses of chloroplast anchoring on spinach PM ghosts. In (a), PM ghosts were prepared in the presence or absence of LatB at 0.1 mM and 1 mM. In (b), PM ghosts were prepared in the presence or absence of 1 mM Ca$^{2+}$, and in the presence of 1 mM Ca$^{2+}$ plus 30 mM W-7, 30 mM W-5, or 15 mM TFP. The relative cumulative frequency of PM ghosts was plotted against the values for the relative area occupied by chloroplasts on PM ghost determined on each digitized bright-field image of PM ghost. A statistically significant difference in the distribution patterns of the values was detected from the untreated PM ghosts (*), the Ca$^{2+}$-treated PM ghosts (#), and the Ca$^{2+}$+W-7-treated PM ghosts ($$), respectively, according to Chi-square test.
Fig. 1-7. Effects of propyzamide on microtubule organization in spinach palisade cells.

Leaf pieces kept under white dim light were treated for 30 min with 0 μM (a), 1 μM (b), 10 μM (c), or 100 μM (d) propyzamide. Microtubules were visualized by indirect immunofluorescence microscopy after cryofixation. Bar = 10 μm.
Fig. 1-8. Effects of propyzamide on chloroplast anchoring on spinach PM ghosts. PM ghosts were prepared in the presence or absence of propyzamide (Propyz) at 10 μM and 100 μM. Chloroplast anchoring was semi-quantitatively analyzed as in Fig. 1-6. No statistically significant difference in the distribution patterns of the values was detected between any combinations out of the three experimental conditions according to Chi-square test. About 25 cells were examined under each experimental condition.
Fig. 1-9. Localization of calmodulin on spinach PM ghosts. PM ghosts were prepared in the absence of any additional reagents (a, b). Calmodulin-depleted PM ghosts were treated with 1 μM Ca²⁺ (c, d). In (e, f), the step of incubation with primary antibodies was omitted in the immunostaining procedures for untreated PM ghosts. (a, c, e)

Bright-field images of the PM ghosts. (b, d, f) Calmodulin signals visualized by indirect immunofluorescence microscopy. Bar = 10 μm.
Fig. 1-10. Effects of anti-calmodulin-antibodies pretreatment on Ca$^{2+}$-induced chloroplast detachment from spinach PM ghosts and the actin disorganization. (a) PM ghosts pretreated with BSA or anti-calmodulin antibodies were further treated with 1 μM Ca$^{2+}$. Chloroplast anchoring was semi-quantitatively analyzed as in Fig. 4. A statistically significant difference in the distribution patterns of the values was detected between PM ghosts pretreated with BSA and those pretreated with anti-calmodulin antibodies according to Chi-square test (*). About 25 cells were examined under each experimental condition. (b) Actin on the PM ghosts pretreated with anti-calmodulin antibodies was visualized by indirect immunofluorescence microscopy after 1 μM Ca$^{2+}$ treatment. A merged image of actin staining (green) and chlorophyll autofluorescence (red). Bar = 10 μm.
Chapter 2

A possible involvement of two kind of villin like proteins participates in chloroplast anchoring.

Abstract

In Chapter 1, my results suggested that the actin-mediated chloroplast de-anchoring was regulated by Ca\(^{2+}\)-calmodulin. Therefore, I assumed an involvement of Ca\(^{2+}\)-regulated actin-binding proteins in the regulation of chloroplast anchoring. Villin is known as an actin binding protein, which has actin bundling and Ca\(^{2+}\)-dependent actin-depolymerizing activities. Addition of anti-P-115-ABP and anti P-135-ABP antibodies, both are anti-lily-villin antibodies, induced detachment of a substantial number of chloroplasts from the PM ghosts prepared from spinach mesophyll cells. Using DNase I affinity column chromatography and liquid chromatography-tandem mass spectrometry, two villin-like proteins were identified in protein extracts from spinach leaves. The localization of each villin-like protein was different in palisade cells. Signals detected by anti-P-115-ABP antibodies were mainly observed like dots randomly in the cell. Almost isolated chloroplasts were not stained positive by using anti-P-115-ABP antibodies. In contrast, Signals detected by anti-P-135-ABP antibodies were observed on many chloroplasts. I propose that at least two different kinds of villin play important roles in actin-mediated chloroplast anchoring and de-anchoring in spinach palisade cells.
Introduction

Results in Chapter 1 strongly suggested that actin-dependent chloroplast anchoring is regulated by Ca^{2+}-calmodulin. So I assumed that actin-binding proteins, which are regulated by Ca^{2+}-calmodulin, participate in the regulation of actin-dependent chloroplast anchoring. In plants, Ca^{2+}-regulated actin-binding proteins such as LIM protein (Thomas et al. 2006, Wang et al. 2008), PrABP80 (Huang et al. 2004), and villin (Nakayasu et al. 1998, Yokota and Shimmen 1998, Huang et al. 2005, Khurana et al. 2010, Zhang et al. 2010, Zhang et al. 2011) were reported. Among them, villin has actin-bundling and Ca^{2+}-dependent actin-depolymerizing activities. Villin was first identified as an actin-bundling protein from microvilli (Bretscher and Weber 1979).

Villin belongs to the gelsolin/severin superfamily. Gelsolin is composed of six conserved gelsolin/severin domains (G1 to G6) and possesses Ca^{2+}-sensitive G-actin-binding and F-actin-severing activities. Villin also has six gelsolin/severin domains and, in addition, a C-terminal headpiece domain, which is an actin-binding domain (Silacci et al. 2004). Recently, it was reported that tyrosine-phosphorylation and interaction with phospholipids regulate activities of villin in human cells, and such regulation participated in actin reorganization induced by EGF stimuli (Khurana and George 2009).

In plants, villin was first identified from lily pollen tubes as P-115-ABP and P-135-ABP (Yokota et al. 1998). Both of them have actin-bundling and Ca^{2+}-calmodulin-dependent actin-depolymerizing activities (Yokota et al. 2003, 2005). In *Arabidopsis thaliana*, there are five villin genes. Biochemical analysis using recombinant proteins showed that AtVLN3, AtVLN4, and AtVLN5 exhibit
actin-bundling and Ca^{2+}-dependent actin-depolymerizing activities (Khurana et al. 2010, Zhang et al. 2010, Zhang et al. 2011). While on the other hand, AtVLN1 exhibit actin bundling activity, but it does not exhibit Ca^{2+}-dependent actin-depolymerizing activities (Huang et al. 2005). Zhang et al. (2010) reported that loss of function of AtVLN5, which is a pollen specific villin, resulted in retardation of pollen tube growth. Zhang et al. (2011) reported that loss of function of AtVLN4 resulted in a short-root-hair phenotype, alteration in cytoplasmic streaming routes and reduction of both axial and apical actin bundles. As described above, functions of plant villin were analyzed about participation in the maintenance of actin organization in cells exhibiting tip growth. I focus the participation of villin in actin-mediated chloroplast anchoring in mesophyll cells which did not exhibit tip growth.
Results

Lily-villin antibodies induce chloroplast detachment from spinach PM ghost

Using anti-lily-villin antibodies, anti-P-115-ABP and anti-P-135-ABP antibodies (Yokota et al. 1998), I examined about the involvement of villin in regulation of chloroplast anchoring. Treatment with anti-P115-ABP and/or anti-P135-ABP antibodies induced detachment of chloroplasts (Figs. 2-1b, 2-1c, 2-1d, Fig. 2-2). Actin signals in the vicinity of the remaining chloroplasts reduced on the antibodies-treated PM ghosts (2-1f, 2-1g, 2-1h). Treatment with a preimmune serum exhibited little effect on PM ghosts (Figs. 2-1a, 2-1e, Fig. 2-2). In addition, additive action was not seen in the effects of two kinds of antibodies (Fig. 2-2). These results raise the possibility that villin-like proteins play a regulatory role in chloroplast anchoring in spinach mesophyll cells.

At least two kinds of villin-like proteins exist in spinach leaves

To verify the existence of villin-like proteins in spinach, I performed immunoblotting of a TCA precipitation prepared from the leaves of spinach. Anti-P-115-ABP antibodies recognized 120-kDa polypeptides (Fig. 2-3b), while anti P-135-ABP antibodies recognized 135-kDa polypeptides (Fig. 2-3c). To examine whether 120-kDa and 135-kDa polypeptides are Ca^{2+}-sensitive actin-binding proteins, the crude protein extract prepared from spinach leaves in the presence of 1 mM CaCl_{2} was applied to a DNase I affinity column. DNase I interact with G-actin. And villin was known that interaction with G-actin was Ca^{2+}-dependent (Bretscher and Weber 1980). So, using a DNase I affinity column, polypeptides that interact with G-actin can isolate. When the fraction eluted with a solution containing 5 mM EGTA was separated
by SDS-PAGE and stained with silver, polypeptides of 120 and 135 kDa were clearly detected (Fig. 2-4d). A large amount of polypeptides of 48 kDa was eluted from the column by a further wash with 50% formamide (Fig. 2-4e), which was most probably spinach G-actin. On immunoblotting of the EGTA eluate, the anti-P-115-ABP antibodies recognized 120-kDa polypeptides (Fig. 2-4f), while anti-P-135-ABP antibodies recognized 135-kDa polypeptides (Fig. 2-4g).

To reveal the identity of 120-kDa and 135-kDa polypeptides, protein elution containing with 120-kDa and 135-kDa polypeptides were subjected to LC/MS/MS and compared with registered proteins in Swiss-Prot database. The partial amino acid sequences of the 120-kDa polypeptides were identical to that deduced from *A. thaliana* AtVLN4 (Table 2-1). A relatively high score was obtained after comparison of partial amino acid sequences obtained from 120-kDa polypeptides with those from AtVLN4 (Table 2-2). Similarly, a high score was obtained between 135-kDa polypeptides and AtVLN2, and AtVLN3 (Table 2-1, 2-2).

Intracellular localization of two villin-like proteins is different.

After spinach leaf sections were processed for cryofixation and indirect immunofluorescence microscopy using anti-P-115-ABP or anti-P-135-ABP antibodies as the primary antibodies, samples were observed from the anticlinal side of palisade cells by a confocal laser scanning microscope. Along the Z axis of the samples, I sequentially took 30 optical images at intervals of 0.5 mm from the PM toward the cytoplasmic side. These images were super-imposed and shown as maximum projections. Signals detected by anti-P-115-ABP antibodies were mainly observed like-dots and their distribution pattern was random (Fig. 2-5a). Signals detected by
anti-P-135-ABP antibodies were mainly observed like a meshwork surrounding each chloroplast (Fig. 2-5b). This pattern was similar to that of signals detected by an anti-actin antibody (Fig. 2-5c).

To examine the localization of villin-like proteins on chloroplasts in more detail, isolated chloroplasts were stained by indirect immunofluorescence microscopy and the number of with fluorescence signals was counted. Almost isolated chloroplasts were not stained positive by preimmune serum and anti-P-115-ABP antibodies (Fig. 2-6a, Fig. 2-7). In contrast, Signals detected by anti-P-135-ABP antibodies were observed on many chloroplasts (Fig. 2-6b, Fig. 2-7). In addition, pretreatment of isolated chloroplasts with 500 mM KCl significantly decreased the amount of fluorescence signals, while LatB pretreatment exhibited a smaller but significant effect to deplete signals associated with chloroplasts (Fig. 2-7).
Discussion

Plant villin participates in the actin reorganization induced by environmental stimuli. Plant villin has actin-bundling and Ca$^{2+}$-dependent actin-depolymerizing activities (Yokota et al. 2003, 2005, Khurana et al. 2010, Zhang et al. 2010, Zhang et al. 2011). Treatment of spinach PM ghosts with antibodies against lily villin induced chloroplast detachment (Figs. 2-1b, 2-1c, 2-1d, 2-2) and a concomitant reduction in actin signals (Figs. 2-1f, 2-1g, 2-1h). Tominaga et al. (2000) showed that microinjection of anti-P-135-ABP antibodies into living root hair cells of Hydrocharis dubia induced the disappearance of the transvacuolar strand, which is maintained by thick actin bundles. Though it was not determined whether anti-villin antibodies induced inhibition of actin bundling or activation of actin depolymerization or severing by mimicking the interaction of target proteins with Ca$^{2+}$-calmodulin, my present results suggest that villin-like proteins play an important role in the maintenance of actin bundles associated with chloroplasts in the cortical cytoplasm.

To date, roles of plant villin have been studied only in cells exhibiting the tip growth, for example pollen tubes (Khurana et al. 2010) and root hairs (Tominaga et al. 2000, Zhang et al. 2010). Their results indicated that villin generally functions to maintain the intracellular architecture of actin bundles. On the other hand, my results suggested that plant villin plays important roles in the regulation of chloroplast anchoring and de-anchoring through the actin reorganization in palisade cells. Using human villin-knockout cells, it was revealed that villin participates in the cell migration induced by EGF (Tomar et al. 2004). Consequently, animal villin functions not only in the maintenance of actin bundles in microvilli but also in the actin reorganization induced...
by external stimuli (Khurana and George 2008). In A. thaliana, AtVNL2 and AtVNL3 are expressed ubiquitously in whole plant bodies (Klahre et al. 2000). Plant villin may play general important roles in the actin reorganization induced by environmental stimuli.

At least two different villin-like proteins exist in spinach leaves

On immunoblotting of the crude protein extract prepared from spinach leaves, anti-P-115-ABP antibodies recognized 120-kDa polypeptides (Fig. 2-3B), while the anti-P-135-ABP antibodies recognized 135-kDa polypeptides (Fig. 2-3C). In addition, using DNase I affinity column chromatography, which was used to isolate and identify chicken villin (Bretscher and Weber 1980) and lily P-135-ABP (Yokota et al. 2005), both proteins eluted out with an EGTA solution (Fig 2-4f, 2-4g). These evidences indicated that 120-kDa and 135-kDa polypeptides are equipped with Ca^{2+}-dependent G-actin-binding activity. This characteristics is shared with P-135-ABP (Yokota et al. 2005). Furthermore, LC/MS/MS analyses revealed that the partial amino acid sequences of both 120-kDa and 135-kDa polypeptides were identical to those deduced from A. thaliana villin genes (Table 2-1). Taken together, we tentatively concluded that 120-kDa and 135-kDa proteins are spinach villin.

Signals detected by anti-P-115-ABP antibodies were mainly observed like dots randomly distributed in the cortical cytoplasm (Fig. 2-5a). Signals detected by anti-P-135-ABP antibodies were mainly observed like a meshwork surrounding each chloroplast (Fig. 2-5b). In addition, isolated chloroplasts were stained positively using anti-135-kDa antibodies (Fig. 2-6b, Table 2-2), but not using anti-115-kDa antibodies (Fig. 2-6a, Table 2-2). These results strongly suggest that the intracellular localization
of 120-kDa polypeptides and 135-kDa polypeptides is different. Though KCl treatment and LatB treatment of isolated chloroplasts decreased fluorescence signals detected by anti-P-135-ABP antibodies, the effect of LatB was much smaller (Table 2-2). This may suggest that at least some population of 135-kDa protein interacts with chloroplast outer envelope independent of actin filaments. In animal, it was known that villin interacts with some phospholipids, especially phosphatidylinositol 4,5-bisphosphate (Khurana and George 2008). It is necessary to verify the mechanism and significance for the association of 135-kDa proteins with chloroplasts.

A relatively high score was obtained after comparison of partial amino acid sequences obtained from 120-kDa polypeptides with those from AtVLN4 (Table 2-2). Similarly, a high score was obtained between 135-kDa polypeptides and AtVLN2, and AtVLN3 (Table 2-1, 2-2). As described above, the localization of 120-kDa and 135-kDa polypeptides is apparently different (Fig. 2-5, Fig. 2-6). In phylogenetic analysis of A. thaliana villin genes, AtVLN1 is designated as the group I, AtVLN2 and 3 are as the group II, and those with AtVLN4 and 5 are as the group III (Khurana et al. 2010). In amino acid sequences, group I harbors three putative Ca$^{2+}$-binding sites. And the group II harbors four putative Ca$^{2+}$-binding sites, while the group III dose three putative Ca$^{2+}$-binding sites (Khurana et al. 2010). These evidences suggest that there are multiple groups of villin in plants and their intracellular functions are different among different groups.
Materials and methods

Immunoblotting of TCA precipitate from spinach leaves

A protein extract of spinach leaves for immunoblotting was prepared according to a TCA precipitation method of Yokota et al. (2005). Spinach leaves were homogenized in cold 15% TCA using a grass homogenizer on ice. After the homogenate was centrifuged at 4500 g for 5 min at 2 °C, the pellet was suspended in cold 80% acetone and 25 mM Tris-HCl at pH 8.0). The suspended pellet was centrifuged at 4500 g for 5 min at 2 °C. These washing processes by centrifugation were repeated three times. Finally, the pellet suspended in 100 mM Tris-HCl at pH 6.8 was centrifuged at 4500 g for 5 min at 2 °C. The pellet suspended in an SDS-lysis buffer (2% SDS, 4% mercaptoethanol, 40 mM Tris-HCl at pH6.8) was boiled for 10 min, and then centrifuged at 18000 g for 5 min at 2 °C. The resultant supernatant was applied to SDS-PAGE. The protein content was determined by the method of Bradford (1976), using bovine serum albumin as a standard. Proteins separated by SDS-PAGE were electrophoretically transferred onto a polyvinylidenedifluoride membrane. After being blocked with 5% (w/v) skim milk over night, the polyvinylidenedifluoride membranes were reacted with the anti-ABP-135 and/or anti-ABP-115 antibodies at 1/1000 dilution. After rinsing, the membranes were reacted with the goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase. The reaction was developed by using 5-bromo-4-chloro-3-indoyl-phosphoate and nitroblue tetrazolium.

DNase I affinity column chromatography
The method of DNase I affinity column chromatography followed that described in Yokota et al. (2005). Spinach leaves were homogenized in a buffer solution (60 mM KCl, 1 mM MgCl₂, 0.2 mM ATP, 2 mM DTT, 1 mM PMSF, 0.2 mg/ml leupeptin, and 30 mM HEPES-KOH at pH 7.5) supplemented with 1% casein, 0.2 M sucrose, and 0.2 mM CaCl₂. After centrifugation at 15,000 g for 10 min, the supernatant was further centrifuged at 100,000 g for 30 min. A resulting supernatant was applied to a DNase I affinity column pre-equilibrated with the buffer solution supplemented with 1 mM CaCl₂. After the column was thoroughly washed with the pre-equilibrated solution, the adsorbed materials were eluted successively with a buffer solution supplemented with 5 mM EGTA, and then with 50% formamide.

Mass spectrometry

The 120-kDa and 135-kDa polypeptides were separated by SDS-PAGE and digested with 12.5 μg ml⁻¹ of trypsin (Sequencing Grade, Roche, Indianapolis, USA) at 37°C for 16 h. Digested peptides were extracted with formic acid and acetonitrile. Peptide mixtures were separated and analyzed using an EASY-nLC (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and their mass spectra were analysed with a micrOTOF-QII (Bruker Daltonics, Billerica, Massachusetts, USA). Mass spectrum data generated by the LC/MS/MS were used to search the swiss prot protein database with Mascot MS/MS Ion Search software (Matrix Science, Boston, Massachusetts, USA).

Chloroplast isolation
Spinach leaves were homogenized four times every 1 sec using a blender in a chloroplast isolation buffer (0.3 M sucrose, 5 mM EGTA, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM DTT, 3.2 μg/ml APMSF, 0.2 mg/ml leupeptin, and 50 mM HEPES-KOH at pH 7.0) supplemented with 0.2% casein. After centrifugation at 1,000 g for 1 min, the pellet was suspended in a chloroplast isolation buffer. This fraction was loaded on layers of 40 and 80% (v/v) Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) that had been made in a suspension medium containing 330 mM sorbitol, 10 mM NaCl, 1 mM MnCl₂, 1 mM MgCl₂, and 50 mM HEPES-KOH at pH 7.0. After centrifugation at 1000 g for 3 min, intact chloroplasts banded at the interface between the 40 and 80% Percoll were carefully recovered. The isolated intact chloroplasts were washed with the suspension medium by centrifugation at 1000 g for 1 min. The pellet resuspended in the medium was designated as the intact-chloroplast fraction. All procedures were carried out at 0 to 4 °C. Isolated chloroplasts were treated with 500 mM KCl or 1 mM LatB. LatB was dissolved in DMSO and diluted to appropriate concentrations with the suspension medium. The final concentration of DMSO was adjusted to 0.1%.

For indirect immunofluorescence microscopy, isolated chloroplasts were fixed with the suspension medium supplemented with 4% formaldehyde and 1% glutaraldehyde for 30 min at room temperature. Fixed chloroplasts were subjected to immunofluorescence microscopy as described in Chapter 1.
Fig. 2-1 Effects of anti-villin-antibodies treatment on the actin organization in spinach PM ghosts. PM ghosts were treated with preimmune serum (a, e), anti-P-115-ABP antibodies (b, f), anti-P-135-ABP antibodies (c, g), or anti-P-115-ABP plus anti-P-135-ABP antibodies (d, h). Actin on the PM ghosts was visualized by indirect immunofluorescence microscopy. Bright-field images of the PM ghosts (a, b, c, d). A merged image of actin staining (green) and chlorophyll autofluorescence (red) of PM ghosts (e, f, g, h). Bar = 10 μm.
Fig. 2-2 Effects of anti-villin-antibodies treatment on chloroplast anchoring on spinach PM ghosts. PM ghosts were treated with preimmune serum (◇), anti-P-115-ABP antibodies (□), anti-P-135-ABP antibodies (▲), or anti-P-115-ABP plus anti-P-135-ABP antibodies (×). Chloroplast anchoring was semi-quantitatively analyzed as in the legend for Fig. 1-6. About 30 cells were examined under each experimental condition.
Fig. 2-3 Immunoblots of crude protein extract prepared from spinach leaves. The crude protein fraction prepared with a TCA-precipitation method was applied to SDS-PAGE using a 7% polyacrylamide gel. Samples were immunoblotted with either anti-P-115-ABP antibodies (b) or anti-P-115-ABP antibodies (c). (a) CBB stained gel. The molecular masses of standard proteins are indicated on the left in kDa. Arrowheads with 120 and 135 indicate the position of main bands detected in b and c, respectively.
Fig. 2-4 Elution pattern from DNase I affinity chromatography of spinach proteins and immunoblotting with anti-lily-villin antibodies. The crude protein extract prepared in the presence of 1 mM CaCl₂ and casein (a) was applied to DNase I affinity column. (b) Flow-through fraction. Adsorbed materials were eluted successively by solutions containing 1 mM CaCl₂ (c), 5 mM EGTA (d), and 50% formamide (e), respectively. Immunoblotting was conducted using the fraction (d) with anti-P-115-ABP antibodies (f) or anti-P-115-ABP antibodies (g). The molecular masses of standard proteins are indicated on the left in kDa. Arrowheads with 48, 120 and 135 indicate the position of main bands detected in e, f and g, respectively.
Table 2-1 LC/MS/MS analyses of spinach 120- and 135-kDa polypeptides.

a

<table>
<thead>
<tr>
<th>120-kDa polypeptides</th>
<th>Detected amino acid sequence</th>
<th>Matching in database</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFQFNGSNSSIQER</td>
<td>AtVLN4: headpiece domain</td>
</tr>
<tr>
<td></td>
<td>DLDPAFQGAGQK</td>
<td>AtVLN4</td>
</tr>
<tr>
<td></td>
<td>TVELDAALGGR</td>
<td>AtVLN4</td>
</tr>
<tr>
<td>135-kDa polypeptides</td>
<td>IYQFNGANSNQIER</td>
<td>AtVLN2, AtVLN3</td>
</tr>
<tr>
<td></td>
<td>SENPVTGIDFK</td>
<td>AtVLN2</td>
</tr>
<tr>
<td></td>
<td>ALEVQYILK</td>
<td>AtVLN2</td>
</tr>
<tr>
<td></td>
<td>SSLNHDDVIFLDTK</td>
<td>AtVLN3</td>
</tr>
</tbody>
</table>

b

<table>
<thead>
<tr>
<th>120-kDa polypeptides</th>
<th>Score</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>At VLN4</td>
<td>110</td>
<td>villin-4 in <em>A. thaliana</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>135-kDa polypeptides</th>
<th>Score</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>At VLN2</td>
<td>102</td>
<td>villin-2 in <em>A. thaliana</em></td>
</tr>
<tr>
<td>At VLN3</td>
<td>82</td>
<td>villin-3 in <em>A. thaliana</em></td>
</tr>
<tr>
<td>AT RDR3</td>
<td>28</td>
<td>probable RNA dependent RNA polymerase 3 in <em>A. thaliana</em></td>
</tr>
</tbody>
</table>

120- and 135-kDa polypeptides were isolated by DNaseI affinity column chromatography. Results of LC/MS/MS analyses were searched on Swiss-Prot database. (a) Detected amino acid sequences from 120- and 135-kDa polypeptides and their matching sequences in database. (b) Score and matching proteins in database for 120- and 135-kDa polypeptides.
Fig. 2-5 Localization of villin-like proteins and actin in the cortical cytoplasm of spinach palisade cells. Palisade cells were irradiated with white dim light (15 μmol m$^{-2}$ s$^{-1}$). 120-kDa protein (a), 135-kDa protein (b) and actin (c) were visualized by indirect immunofluorescence microscopy after cryofixation. Bar = 10 μm.
Fig. 2-6 Localization of 120-kDa and 135-kDa proteins on isolated spinach chloroplasts. 120-kDa (a) and 135-kDa proteins (b) were visualized by indirect immunofluorescence microscopy after fixation. Preimmune serum was used in substitution for primary antibodies (c). Merged images of staining by anti-villin-antibodies (green) and chlorophyll autofluorescence (red) of isolated chloroplasts. Bar = 5 μm.
Isolated spinach chloroplasts were subjected to immunofluorescence microscopy using anti-P-115-ABP antibodies, anti-P-135-ABP antibodies, and preimmune serum. When necessary, isolated chloroplasts were pretreated with 500 mM KCl or 1 mM LatB before fixation. The number of chloroplast stained by anti-villin antibodies was counted in 50 each experimental condition. And I made same experiment at three times. I controlled comparison wise error rate of t-test.
Acknowledgement

I wish to express my gratitude to Dr. S. Takagi (Osaka Univ.) for invaluable discussion and continuous encouragement throughout this work. As Dr. S. Takagi supported me, I was able to do it to here. And I am grateful to the late Professor S. Muto for the kind gift of anti-spinach calmodulin antibodies, Professor T. Shimmen and Dr. E. Yokota (Hyogo Univ.) for help with biochemical experiments, Professor S. Kuramitsu and Dr. K. Kim (Osaka Univ.) for help LC-MS-MS analysis, Professor Dr. K. Noguchi (Tokyo Univ.) for help with statistical analyses, and Professor I. Tsutsui (Hitotsubashi Univ.) for his help in calculation of Ca^{2+} concentrations. I thank for all the member of the Laboratory of Plant Cell Biology of Osaka University.
References


56


Khurana, S. and George, S.P. (2008) Regulation of cell structure and function by


