Evidence for chloroplast control of external Ca\textsuperscript{2+}-induced cytosolic Ca\textsuperscript{2+} transients and stomatal closure

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Received 30 September 2007; revised 10 November 2007; accepted 21 November 2007.
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Summary

The role of guard cell chloroplasts in stomatal function is controversial. It is usually assumed that stomatal closure is preceded by a transient increase in cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) in the guard cells. Here, we provide the evidence that chloroplasts play a critical role in the generation of extracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{ext})-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} transients and stomatal closure in Arabidopsis. CAS (Ca\textsuperscript{2+} sensing receptor) is a plant-specific putative Ca\textsuperscript{2+}-binding protein that was originally proposed to be a plasma membrane-localized external Ca\textsuperscript{2+} sensor. In the present study, we characterized the intracellular localization of CAS in Arabidopsis with a combination of techniques, including (i) in vivo localization of green fluorescent protein (GFP) fused gene expression, (ii) subcellular fractionation and fractional analysis of CAS with Western blots, and (iii) database analysis of thylakoid membrane proteomes. Each technique produced consistent results. CAS was localized mainly to chloroplasts. It is an integral thylakoid membrane protein, and the N-terminus acidic Ca\textsuperscript{2+}-binding region is likely exposed to the stromal side of the membrane. The phenotype of T-DNA insertion CAS knockout mutants and cDNA mutant-complemented plants revealed that CAS is essential for stomatal closure induced by external Ca\textsuperscript{2+}. In contrast, overexpression of CAS promoted stomatal closure in the absence of externally applied Ca\textsuperscript{2+}. Furthermore, using the transgenic aequorin system, we showed that [Ca\textsuperscript{2+}]\textsubscript{ext}-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} transients were significantly reduced in CAS knockout mutants. Our results suggest that thylakoid membrane-localized CAS is essential for [Ca\textsuperscript{2+}]\textsubscript{ext}-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} transients and stomatal closure.

Keywords: chloroplasts, stomatal movement, calcium signaling, CAS, extracellular Ca\textsuperscript{2+}.

Introduction

Stomata control the exchange of gases and H\textsubscript{2}O between a plant and the atmosphere. In most species, stomatal guard cells contain well-developed chloroplasts, while typical epidermal cells have poorly differentiated plastids. It is usually assumed that guard cell chloroplasts have an important role in stomatal opening and closing through photosynthetic production of ATP, reductants, osmotically active sugars, and accumulation and degradation of starch (Melis and Zeiger, 1982; Talbott and Zeiger, 1993; Tallman and Zeiger, 1988; Tominaga et al., 2001). However, the role of guard cell chloroplasts in signal transduction pathways is not clear.

In plant cells, Ca\textsuperscript{2+} has a key role as a second messenger in intracellular signal transduction in a range of events, including phytochrome action, hormone responses, various stress responses, plant-microbe interactions, tip growth, and stomatal movement. The complexity of cellular signals related to cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) is well illustrated in stomatal guard cells. ABA (McAinsh et al., 1990; Schroeder and Hagiwara, 1990), reactive oxygen species (ROS; McAinsh et al.,1996; Pei et al., 2000), low temperature (Allen et al., 2000), and external Ca\textsuperscript{2+} (Allen et al., 1999, 2000) produce high-frequency [Ca\textsuperscript{2+}]\textsubscript{cyt} oscillations in Arabidopsis guard cells that are mediated by two sources: Ca\textsuperscript{2+} influx from extracellular space and intracellular Ca\textsuperscript{2+} mobilization during signaling. Vacuoles and endoplasmic reticulum are the most prominent intracellular sinks for Ca\textsuperscript{2+} in most plant cells. Although chloroplasts contain high concentrations (i.e. 4–23 mm) of total Ca\textsuperscript{2+} (Brand and Becker, 1984; Evans et al., 1991), it is not clear that they have the capacity to sequester Ca\textsuperscript{2+} or are involved in the generation of Ca\textsuperscript{2+} signals in guard cells.
Recently, molecular genetic studies have suggested that chloroplasts may be involved in the generation of cytosolic Ca\textsuperscript{2+} signals in plant cells. A putative thylakoid membrane-localized Ca\textsuperscript{2+} carrier protein, PPF1, may regulate flowering time by modulating the Ca\textsuperscript{2+} storage capacity of chloroplasts (Li et al., 2004; Wang et al., 2003). It was also proposed that chloroplast-localized CASTOR and POLLUX, homologues of the Methanobacterium thermoautotrophicum Ca\textsuperscript{2+}-gated K\textsuperscript{+} channel are involved in a Ca\textsuperscript{2+} spiking response in Rhizobium-legume symbiosis (Imaizumi-Anraku et al., 2005).

Furthermore, Sai and Johnson (2002) demonstrated that the dark-induced large increase of stromal Ca\textsuperscript{2+} levels precedes the generation of [Ca\textsuperscript{2+}]\textsubscript{cyt} transients in tobacco leaf cells. These results suggest that chloroplasts act as intracellular Ca\textsuperscript{2+} stores that release Ca\textsuperscript{2+} in response to various stimuli and play a crucial role in the generation of [Ca\textsuperscript{2+}]\textsubscript{cyt} transients.

CAS (Ca\textsuperscript{2+} sensing receptor) encodes a novel, plant-specific 42 kDa Ca\textsuperscript{2+}-binding protein that contains a single predicted transmembrane domain (Han et al., 2003). The N-terminus has a low-affinity/high-capacity Ca\textsuperscript{2+} binding site, and the C-terminus contains a rhodanese-like domain, which is probably involved in protein-protein interactions. CAS was originally identified as a Ca\textsuperscript{2+}-sensor protein located in the plasma membrane. However, CAS is predicted to be localized in chloroplasts, but not in plasma membrane by a number of subcellular localization prediction programs.

Furthermore, we recently found that CAS had been identified in various proteomes of chloroplast thylakoid membranes, but not in plasma membrane or stroma proteomes (Friso et al., 2004; Peltier et al., 2004). In the present study, we re-examined the intracellular location of CAS, using subcellular fractionation, protein gel blots, and in vivo localization using green fluorescent protein (GFP) fusions. Our results demonstrate that CAS protein is localized primarily in chloroplast membranes. T-DNA insertion CAS knockout mutants were impaired in extracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{ext})-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} transients and subsequent stomatal closure. In contrast, CAS overexpression promoted stomatal closure in the absence of externally applied Ca\textsuperscript{2+}. The results provide the evidence that chloroplasts have a critical role in [Ca\textsuperscript{2+}]\textsubscript{ext}-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} increase and subsequent stomatal closure in Arabidopsis.

**Results**

**CAS is essential for [Ca\textsuperscript{2+}]\textsubscript{ext}-induced stomatal closure**

To understand the role of CAS in stomatal movement, we examined CAS knockout and CAS overexpressing genotypes. Firstly, we isolated two CAS T-DNA insertion mutant alleles, SALK_070416 (cas-1) and 665G12 (cas-2; Figure 1a). Homozygous individuals were identified by PCR analysis (Figure S1). Sequencing the T-DNA flanking region showed that the insertions were localized 224 bp (cas-1) and 857 bp (cas-2) downstream from the ATG start codon. Although both T-DNA insertions were located in introns, Northern (Figure 1b) and Western (Figure 1c) blots showed that CAS expression was undetected in those lines. The progeny of cas-1 and cas-2 homozygous individuals grow normally under standard conditions.

Elevating external Ca\textsuperscript{2+} induces stomatal closure in several plants, including Commelina communis (McAinsh et al., 1995) and Arabidopsis (Allen et al., 1999). As shown in Figure 1(d), 2 mM CaCl\textsubscript{2} induced stomatal closure within 2 h in wild-type Arabidopsis. On the other hand, [Ca\textsuperscript{2+}]\textsubscript{ext}-induced stomatal closure was completely impaired in cas-1 (Figure 1d) and cas-2 (Figure S3) mutant alleles. This is consistent with a previous report that [Ca\textsuperscript{2+}]\textsubscript{ext}-induced stomatal closure was inhibited in CAS antisense transgenic plants (Han et al., 2003). To confirm that the CAS-deficient phenotype is caused by a disruption of the CAS gene, we introduced CAS cDNA driven by a cauliflower mosaic virus (CaMV) 35S promoter in cas-1 mutants. Expression of CAS mRNA at wild type levels (CAS/cas-1 #5) restored [Ca\textsuperscript{2+}]\textsubscript{ext}-induced stomatal closure in cas-1 mutant plants to the levels of wild-type plants (Figure 1d; Figure S2a). Moreover, slight overexpression of CAS partially promoted stomatal closure in cas-1 mutants (Figure S3). Thus, we concluded that CAS is essential for [Ca\textsuperscript{2+}]\textsubscript{ext}-induced stomatal closure. On the other hand, ABA- and dark-induced stomatal closure and light-induced stomatal opening was not suppressed in cas-1 and cas-2 mutant lines (Figure S4), suggesting that the molecular machinery involved in stomatal movement was not impaired by CAS inactivation.

To further test the role of CAS in stomatal movement, we generated transgenic Arabidopsis plants that overexpressed CAS (designated CASox) under the control of a CaMV 35S promoter. A comparison of CAS expression in seedlings showed that CASox #4 and #11 expressed the highest levels of CAS (Figure S2b). Expression of CAS protein was ~10 times greater in CASox #4 plants compared with wild-type plants (Figure 1c). Interestingly, CAS overexpression promoted stomatal closure in the absence of external Ca\textsuperscript{2+} (Figure 1d). The stomatal opening was apparently dependent on CAS expression levels (Figure S3). On the other hand, stomata in CASox #4 and #11 leaves closed normally in response to external Ca\textsuperscript{2+} treatment (Figure 1d; Figure S3). Taken together, these results demonstrate that CAS is essential for [Ca\textsuperscript{2+}]\textsubscript{ext}-induced stomatal closure in Arabidopsis.

**CAS is localized primarily to chloroplast membranes**

In a previous paper, Han et al. (2003) reported that CAS targets plasma membranes. However, we recently found that CAS had been identified in various proteomes of chloroplast thylakoid membranes (Friso et al., 2004; Peltier et al., 2004).
This prompted us to examine the subcellular localization of CAS in detail. Our results revealed that the N-terminus of CAS exhibits features typical of chloroplast transit peptides, as indicated by several subcellular localization prediction programs, including TargetP, ChloroP, PSORT, Predotar and iPSORT. To determine whether the CAS protein is localized to chloroplasts, we examined the intracellular localization of CAS-GFP fusion proteins. First, 70 N-terminal amino acid residues containing the predicted transit peptide were fused to the GFP protein and expressed transiently in Arabidopsis protoplasts. Confocal microscopy revealed co-localization of GFP fluorescence with chlorophyll autofluorescence in chloroplasts (Figure 2a), suggesting that the N-terminal sequence of CAS acts as a chloroplast transit peptide. Furthermore, we observed that GFP-tagged full-length CAS was predominantly localized to chloroplasts (Figure 2b). GFP fluorescence was detected in the central region of the chloroplasts, but not in the stromules or periphery of the chloroplasts (data not shown), suggesting that CAS-GFP fusion is localized to inner thylakoid membranes. Since GFP fluorescence did not disappear after disruption of protoplasts by osmotic shock, CAS is unlikely a stroma-localized soluble protein (data not shown). On the other hand, GFP fused syntaxin SYP132 (plasma membrane maker) was localized exclusively to plasma membrane (Figure 2e). Additionally, the localization of GFP-Sec22 (endoplasmic reticulum maker) fusion proteins was apparently different from that of CAS-GFP fusions (Figure 2d). GFP fluorescence was detected mainly in the cytoplasm in control GFP-expressing cells (Figure 2c). To confirm the localization of CAS-GFP in chloroplasts, we generated transgenic Arabidopsis plants that constitutively expressed CAS-GFP under control of a CaMV 35S promoter. GFP fluorescence was detected exclusively in chloroplasts in all types of leaf cells, including mesophyll, epidermal, and guard cells (Figure 3), and in non-photosynthetic plastids in flower petals and roots (data not shown). On the other hand, no GFP fluorescence was detected in the plasma membranes of any cell. We next examined subcellular localization of CAS protein by cell fractionation and Western blotting. In crude protein extracts from wild-type plants, rabbit anti-Arabidopsis CAS polyclonal antiserum detected a 34 kDa protein, corresponding to the predicted size of the mature CAS protein, but slightly the 42 kDa CAS precursor protein, suggesting that the CAS precursor is imported into chloroplasts and processed to mature protein (Figure 4a). Furthermore, we isolated chloroplast thylakoid membrane (S6k)- and cytoplasmic microsomal (S100k)-containing fractions and analyzed them in Western blots. The plasma membrane marker H^+-ATPase was detected in microsomal fraction but not in the chloroplast fraction (Figure 4b). In contrast, CAS was detected exclusively in the chloroplast membrane fraction, as was an integral protein of the chloroplast thylakoid membrane, CP43. CAS signal was not detected in the plasma membrane fraction. To further determine the localization of CAS in chloroplasts, we isolated intact chloroplasts from spinach and fractionated them into soluble, envelope and thylakoid fractions. Each fraction was obtained with little cross-contamination, as determined by western analysis of specific marker proteins. We used GS2, Toc75 and CP43 as the stromal, outer envelope and the thylakoid membrane markers, respectively (Figure 4c). CAS was exclusively detected in the thylakoid fraction, but not in the envelope
and stroma fractions. Furthermore, CAS was also detected in the chloroplast membrane fraction from Arabidopsis (Figure S5). The results clearly demonstrated that CAS is localized mainly to chloroplast thylakoid membranes and is not associated with plasma membranes.

To further describe the topology of CAS protein in the thylakoid membrane, isolated thylakoids were treated with thermolysin or trypsin. This treatment is expected to digest proteins exposed to the stromal side of the thylakoid membrane. The protease treated thylakoids were analyzed by western blot using N-terminal specific anti-CAS antibody. CAS was degraded by proteases in a concentration dependent manner (Figure 5a), suggesting that the CAS N-terminus is exposed to the stromal side of the thylakoid membrane. In contrast, the thylakoid lumen peripheral protein, PsbP, was not affected by protease treatments, indicating that thylakoids remained sealed and retained their correct sidedness (Figure 5c). It has been shown that EGFP fluorescence could not be detected in the photosynthetically active thylakoid lumen due to its acidic pH (Marques et al., 2004), suggesting that GFP fused to the C-terminus of CAS may also be exposed to the stromal side. However, we could not determine the location of the CAS C terminus immunologically, since our C-terminal-specific antibody could not detect CAS proteins in plants. Furthermore, we treated thylakoid membranes with high salt (1 M NaCl), alkaline (0.1 M Na₂CO₃), denaturing (4 M urea) or non-ionic detergent (1% Triton X-100) solutions (Figure 5b). PsbP was released from the membranes after treatment with NaCl and Na₂CO₃ (Figure 5c), but membrane-associated CAS resisted high salt alkaline and denaturing treatment, suggesting that CAS is an integral membrane protein.

**Figure 2.** Subcellular localization of CAS in Arabidopsis protoplasts. Confocal images of Arabidopsis protoplasts transfected with 3SS-traCAS-GFP (a) and 3SS-CAS-GFP (b), 3SS-GFP (c), 3SS-GFP-Sec22 (d), and 3SS-GFP-SYP132 (e) plasmids are shown. Scale bar represents 20 μm.

**Figure 3.** Confocal images of 3-week-old transgenic Arabidopsis plants expressing CAS-GFP fusion proteins. Chlorophyll, GFP and merged images from mesophyll (a), epidermal (b), and guard (c) cells are shown. Scale bar represents 10 μm.

### Chloroplast-localized CAS mediates cytoplasmic Ca²⁺ signaling

We were interested in knowing how chloroplasts regulate [Ca²⁺]ₘₜₚ- induced stomatal closure. It has been shown that [Ca²⁺]ₘₜₚ- induced stomatal closure is usually preceded by a transient elevation in [Ca²⁺]ₘₜₚ. To examine the involvement of chloroplast-localized CAS in the generation of [Ca²⁺]ₘₜₚ- induced [Ca²⁺]ₘₜₚ transients, we generated several wild-type or cas-1 mutant plants that expressed the apoaequorin gene under the control of the CaMV 35S promoter. Wild-type and cas-1 mutants that expressed the same amount of apoaequorin protein were selected for further evaluation (Figure 6a). As shown in Figure 6(b), 10 mM CaCl₂ induced transient [Ca²⁺]ₘₜₚ elevation within 2 min, after which the signal gradually declined. The initial [Ca²⁺]ₘₜₚ elevation was
significantly reduced in cas-1 mutant plants. The integrated amount of \([\text{Ca}^{2+}]_{\text{ext}}\)-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation (\(\Delta[\text{Ca}^{2+}]_{\text{cyt}}\)) over 500 sec (3.74 ± 0.69 \(\mu\text{M}\); \(n = 10\)) in wild-type plants was greatly reduced in cas-1 mutant plants (1.15 ± 0.36 \(\mu\text{M}\); \(n = 5\); Figure 6c). These results, together with the previous finding that \([\text{Ca}^{2+}]_{\text{ext}}\)-induced high-frequency \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations were not detected in guard cells in CAS antisense plants (Han et al., 2003), led us to conclude that chloroplast-localized CAS is involved in the generation of \([\text{Ca}^{2+}]_{\text{ext}}\)-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation in Arabidopsis.

**CAS protein accumulation is tissue specific and regulated during senescence**

We examined CAS protein levels during plant development and senescence with Western blot. CAS protein was detected in rosette leaves, cauline leaves, stems, and flowers, but not in roots (Figure 7a), suggesting that CAS is upregulated with chloroplast development. To determine whether tissue-specific and developmental expression of CAS protein is controlled at the level of transcription, we histochemically examined the localization of CAS promoter-driven beta-glucuronidase (GUS) expression in transgenic Arabidopsis. In 2-week-old plants, GUS was detected in rosette leaves and sepals, but not in roots (Figure 7b–d). The results suggest that tissue-specific CAS protein accumulation is regulated at the level of transcription. On the other hand, high levels of GUS and low levels of CAS were detected in 1-week old cotyledons (Figure 7a,b), suggesting that post-transcriptional regulation has a role in CAS gene expression in cotyledons. Furthermore, we found that CAS declined to very low levels in rosette and cauline leaves in 6-week-old plants. The decrease in CAS coincided with the

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**Figure 4.** Subcellular and suborganellar fractionation and Western blot analysis of CAS in the fractions.

(a) Detection of CAS-GFP fusion proteins in transgenic Arabidopsis. Total proteins (10 \(\mu\text{g} \text{ lane}^{-1}\)) isolated from Arabidopsis wild type and CAS-GFP transgenic plants were electrophoresed on 10% SDS-PAGE and detected with anti-GFP (1:500 dilution) or anti-CAS (1:3000 dilution) antibodies. Asterisks indicate possible degradation products.

(b) Chloroplast and microsome fractions were prepared as described in Materials and Methods. Protein was resolved on 10% SDS-PAGE and immunologically detected with the indicated antibodies.

(c) Western blot analysis of chloroplast fractions. The spinach chloroplast total lysate fraction were fractionated into envelope (Env), thylakoid (Thy) and soluble (Sol) fractions. Fractions were resolved on a 10% SDS-PAGE and detected with the indicated antibodies. Ten micrograms of protein of T, Env and Thy fractions, and 2 \(\mu\text{g}\) protein of Sol fraction were loaded.

**Figure 5.** Association of CAS with chloroplast thylakoid membranes.

(a) Thylakoid membrane (0.1 mg chlorophyll \(\text{ml}^{-1}\)) was suspended in an isolation buffer containing thermolysin (TL), trypsin (Try), or no protease. Upper numbers indicate the protease concentration. Thylakoid membrane was separated by 12% SDS-PAGE and proteins were detected with anti-CAS (a) or anti-PsbP (c) antibodies.

(b) Thylakoid membrane (0.1 mg chlorophyll \(\text{ml}^{-1}\)) was suspended in an isolation buffer containing 1 M NaCl, 0.1 M Na\(_2\)CO\(_3\), 4 M urea, 1% Triton X-100 or no treatment (control). Protein was separated into membrane (P) and supernatant (S) fractions by centrifuging. Thylakoid membrane protein was detected with anti-CAS (b) or anti-PsbP (c) antibodies.
disappearance of GS2, which is known to be quickly degraded during senescence (Kamachi et al., 1991). Thus, CAS, like GS2, is downregulated quickly and specifically during leaf senescence. Finally, to examine cell-specific CAS promoter activity, we analyzed transgenic plants expressing CAS promoter-GFP gene fusion. GFP fluorescence was detected in the palisade, mesophyll, epidermal, and guard cells in leaves (Figure 7e,f). It is likely that CAS is expressed in all types of leaf cells and may also have a role in chloroplast function in mesophyll and epidermal cells.

Since CAS is localized to chloroplast membranes, we examined the effects of CAS gene disruption and overexpression on chlorophyll and photosystem II (PSII) activity. We found that suppression and overexpression of CAS protein did not result in significant decreases or increases in chlorophyll or the chlorophyll a/b ratio (Table 1). Thus, it is unlikely that CAS is directly involved in photosynthesis activity. However, it should be noted that non-photochemical quenching (NPQ) values were slightly, but significantly increased and decreased in the CAS knockout and overexpressing mutants, respectively.

Discussion

CAS is localized primarily to chloroplast membranes

CAS was originally identified as a novel Ca\(^{2+}\)-binding protein involved in extracellular Ca\(^{2+}\) sensing and was shown to be associated with plasma membranes (Han et al., 2003). The results of the present study, however, demonstrate that CAS is mainly localized to chloroplast thylakoid membranes. First, we showed that CAS-GFP fusions were localized to chloroplasts, most likely at thylakoid membranes in transiently transformed protoplasts and transgenic Arabidopsis. In transgenic plants, no GFP fluorescence was detected on the cell surface, suggesting that the fusion proteins were not localized to plasma membranes (Figure 3). On the other hand, it was previously shown that CAS-GFP fusion protein was localized to the plasma membranes of onion epidermal (Han et al., 2003) and HEK 293 cells (Tang et al., 2007). We repeated the experiment and found no evidence that CAS-GFP fusion is targeted to the plasma membrane of onion epidermal cells. GFP fluorescence was localized in plastid-like dotted organelles apparently different from the plasma membrane in onion cells (Figure S6). Second, CAS protein was detected in chloroplast membranes, but not in the microsomal fraction that contained plasma membranes (Figure 4b). Third, subfractionation of intact chloroplasts revealed that CAS protein was detected in the thylakoid membrane fraction of chloroplasts. Finally, CAS has been identified in various proteomes of chloroplast thylakoid membrane, but not in plasma membrane or stroma proteomes (Friso et al., 2004; Peltier et al., 2004; Tsunoyama et al., unpublished data). Based on those findings, we concluded that CAS is localized mainly to chloroplast thylakoid membranes. Interestingly, the distribution of CAS suggests that it is an integral thylakoid membrane protein, and the N-terminus of CAS is localized to plasma membranes (Han et al., 2003). The CAS-2+-binding domain, which contains the Ca\(^{2+}\)-binding domain, appears to be the stromal side of the thylakoid membrane (Figure 5a). Furthermore, CAS expression coincides with chloroplast development (Figure 7a), suggesting a role for CAS in chloroplasts.

CAS plays a critical role in the regulation of stomatal movement

Guard cells contain chloroplasts that are smaller and have less granal stacking than mesophyll cell chloroplasts. It has been proposed that guard cell chloroplasts are photosynthetically active, provide the ATP required for H\(^{+}\)

**Figure 8.** CAS and [Ca\(^{2+}\)]\(_{\text{ext}}\)-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) increase.  
(a) Western blot analysis of aequorin in transgenic plants. Twenty micrograms of total extracted protein/lane was separated by 12% SDS-PAGE. The transgenic aequorin protein (24 kDa) was detected with anti-AEQ antibody. Rubisco was stained with CBB to confirm equal loading of the proteins.  
(b) [Ca\(^{2+}\)]\(_{\text{ext}}\)-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) increase in transgenic Arabidopsis expressing the aequorin gene in wild-type and cas-1 mutant plants. Detached leaves were used for measurements. [Ca\(^{2+}\)]\(_{\text{ext}}\)-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) increase resulted from the addition of 10 mM CaCl\(_2\) (arrow) to the external medium. A significant increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) was observed in half of the plants examined (wild-type, 10/18; cas-1, 5/11).  
(c) Time-integrated increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) for the first 500 sec of the Ca\(^{2+}\) transient was plotted for wild-type (n = 10) and cas-1 (n = 5) plants. Data are mean ± SEM of relative values. The similar result was obtained by another two individual experiments.
pumping in the plasma membrane and stomatal opening, and produce sugars by photosynthetic carbon assimilation. Furthermore, guard cell chloroplasts accumulate starch in the dark and hydrolyze starch in the light to synthesize malate, the major osmoticum in guard cells (Melis and Zeiger, 1982; Talbott and Zeiger, 1993; Tallman and Zeiger, 1988; Tominaga et al., 2001). However, the role of guard cell chloroplasts in signal transduction pathways is not clear. In the present study, we demonstrated that disrupting the CAS gene impaired stomatal closure induced by \([Ca^{2+}]_{ext}\) elevation (Figure 1d). Complementation experiments with a CAS cDNA clone confirmed that chloroplast-localized CAS has a role in regulating stomatal movement. In contrast, CAS overexpression promoted stomatal closure in the absence of externally applied \([Ca^{2+}]_{ext}\) (Figure 1d; Figure S3). Taken together, the results suggest that chloroplast-localized CAS is essential for \([Ca^{2+}]_{ext}\)-induced stomatal closure in Arabidopsis. Our results provide the evidence that chloroplasts play a crucial role in the regulation of stomatal movement.

It is unlikely that \([Ca^{2+}]_{ext}\)-induced stomatal closure was impaired in CAS knockout mutants due to defects in photosynthesis activity and/or decreased production of ATP, since maximum PSII efficiency was not affected in CAS mutants (Table 1), and plant growth was not significantly impaired by knockout or overexpression of CAS. Rather, CAS may be involved in the regulation of stomatal responses, namely environmental sensing or intracellular signal transduction. We consider here three possible roles for chloroplast-localized CAS in signal transduction pathways leading to stomatal closure in guard cells. First, thylakoid membrane-associated CAS may be involved in the transduction of blue light signals received by zeaxanthin in chloroplasts (Talbott et al., 2003). However, light-depen-

### Table 1 Chlorophyll content and chlorophyll fluorescence of wild-type, cas-1 and CASox plants

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<th>Chl (a + b) (mg g(^{-1}) fresh weight)</th>
<th>Chl (a/b)</th>
<th>Fv/Fm</th>
<th>(\Phi_{PSII})</th>
<th>NPQ</th>
<th>1–qP</th>
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<tr>
<td>Wild-type</td>
<td>2.55 ± 0.25</td>
<td>2.68 ± 0.11</td>
<td>0.78 ± 0.01</td>
<td>0.62 ± 0.01</td>
<td>0.33 ± 0.02</td>
<td>0.12 ± 0.02</td>
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<tr>
<td>cas-1</td>
<td>2.59 ± 0.05</td>
<td>2.79 ± 0.03</td>
<td>0.80 ± 0.01</td>
<td>0.62 ± 0.01</td>
<td>0.45 ± 0.03</td>
<td>0.12 ± 0.01</td>
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<tr>
<td>CASox</td>
<td>2.90 ± 0.16</td>
<td>2.65 ± 0.01</td>
<td>0.78 ± 0.01</td>
<td>0.65 ± 0.01</td>
<td>0.27 ± 0.03</td>
<td>0.10 ± 0.01</td>
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Chl \(a + b\), chlorophyll \(a\) and \(b\) contents in plant leaves (mg g\(^{-1}\) fresh weight); Chl \(a/b\), chlorophyll \(a/b\) ratio; Fv/Fm, maximum quantum yield of PSII; \(\Phi_{PSII}\), effective quantum yield of PSII; NPQ, non-photochemical quenching; 1–qP, fraction of QA present in the reduced state. Data are mean values ± SEM of six separate experiments.
dent stomatal movement was normal in CAS knockout mutants, indicating that CAS is not involved in light-induced stomatal movement (Figure S4). Second, several studies have suggested that ROS are produced by various stimuli, such as ABA, salicylic acid, and fungal elicitor, and function as second messengers in regulating stomatal closure (Lee et al., 1999; Mori et al., 2001; Murata et al., 2001). Chloroplasts are major producers of ROS during photosynthesis. Thus, we investigated the effects of external CaCl$_2$ on the production of ROS in chloroplasts in cas-1 plants. ROS-sensitive fluorescent probes revealed that ROS was produced in chloroplasts by [Ca$^{2+}$]$_{ext}$ stimulation in wild-type and cas-1 plants, suggesting that CAS is not directly involved in the production of ROS (data not shown).

CAS is essential for [Ca$^{2+}$]$_{ext}$-induced [Ca$^{2+}$]$_{cyt}$ elevation

Thirdly, we further investigated a role for CAS in intracellular Ca$^{2+}$ mobilization. It has been shown that external Ca$^{2+}$ triggers a transient increase in [Ca$^{2+}$]$_{cyt}$ that leads to stomatal closure. The rise in [Ca$^{2+}$]$_{cyt}$ was proposed to be caused by Ca$^{2+}$ influx across the plasma membrane and Ca$^{2+}$ release from internal stores (Sathyanarayanan and Poovaiah, 2004). Previous studies on the role of plant organelles in cytosolic Ca$^{2+}$ signals have focused on the endoplasmic reticulum and the vacuole. However, the role of chloroplasts in the generation of [Ca$^{2+}$]$_{cyt}$ transients has been underappreciated. In the present study, we demonstrated that [Ca$^{2+}$]$_{ext}$-induced [Ca$^{2+}$]$_{cyt}$ increase was significantly impaired in CAS knockout mutants (Figure 6b,c). That result is consistent with a previous report that [Ca$^{2+}$]$_{ext}$-induced [Ca$^{2+}$]$_{cyt}$ was abolished in guard cells of CAS antisense plants (Han et al., 2003). Based on our finding that CAS is localized mainly in chloroplast thylakoid membranes, we propose that chloroplast-localized CAS plays a critical role in [Ca$^{2+}$]$_{ext}$-induced [Ca$^{2+}$]$_{cyt}$ elevation in Arabidopsis.

Although chloroplasts contain high concentrations (4–23 mM) of total Ca$^{2+}$ (Portis and Heldt, 1976), stroma free Ca$^{2+}$ concentration is maintained at sub-$\mu$M (Johnson et al., 2006). Thus, it has been assumed that most of the Ca$^{2+}$ is sequestered at unidentified binding sites within the chloroplast stroma or thylakoid lumen, as discussed by Sai and Johnson (2002). However, the identity of the relevant Ca$^{2+}$ store in the stroma is not known. Interestingly, the distribution of CAS suggests that it is an integral thylakoid membrane protein, and the N-terminus of CAS, which contains the Ca$^{2+}$-binding domain, appears to be exposed to the stromal side of the thylakoid membrane (Figure 5a). Thus, CAS may act as a Ca$^{2+}$ store that is involved in sequestering stromal Ca$^{2+}$. On the other hand, thylakoid membranes are capable of accumulating Ca$^{2+}$ and reducing stromal Ca$^{2+}$ levels (Ettinger et al., 1999). Alternatively, CAS may act as a Ca$^{2+}$ transporter in chloroplast thylakoid membranes. CAS conferred [Ca$^{2+}$]$_{ext}$-induced [Ca$^{2+}$]$_{cyt}$ increases to transfected human embryonic kidney (HEK293) cells (Tang et al., 2007), suggesting that CAS is involved in extracellular Ca$^{2+}$ sensing or Ca$^{2+}$ transport across the membrane. In conclusion, we speculate that extracellular Ca$^{2+}$ may activate cell signal transduction pathways that eventually lead to the release of Ca$^{2+}$ from chloroplasts and subsequent stomatal closure, although the molecular mechanism underlying this action remains largely unknown. Interestingly it has been proposed that CAS may be involved in inositol 1,4,5-trisphosphate signaling (Tang et al., 2007).

As shown in Figure 7a,e,f, CAS is expressed in all types of leaf cells and may also have a role in chloroplast function in non-stomatal leaf cells. It has been shown that extracellular Ca$^{2+}$ affects various cellular functions in plant cells. Especially it is reported that extracellular Ca$^{2+}$ prevents Na$^{+}$ toxicity and maintains intracellular K$^{+}$ homeostasis in both leaf and root cells (Shabala et al., 2006). Addressing the molecular nature of the CAS function will be critical to understanding the [Ca$^{2+}$]$_{ext}$-induced cell signaling in plant cells.

Several lines of evidence suggest that chloroplasts contain other proteins involved in cytosolic Ca$^{2+}$ signaling. PPF1 is a putative Ca$^{2+}$-carrier protein in thylakoid membranes and may influence Ca$^{2+}$ storage capacities in chloroplasts (Wang et al., 2003). It was demonstrated that PPF1 is involved in the regulation of flowering time and programmed cell death in apical meristem cells (Li et al., 2004). Recently, Imaizumi-Anraku et al. (2005) demonstrated that CASTOR and POLLUX act as Ca$^{2+}$-permeable channels in chloroplasts and regulate the cytosolic Ca$^{2+}$ spiking response in Rhizobium-legume symbiosis. These findings suggest that chloroplasts may be involved in the generation of cytosolic Ca$^{2+}$ signals in various plant cells. Further molecular analyses of chloroplast-localized Ca$^{2+}$ signal-related proteins, including CAS, will provide novel insights into the role of chloroplasts in cytosolic Ca$^{2+}$ signaling and regulation in a variety of basic cellular processes, including stress responses, flowering, cell proliferation, cell differentiation, plant-microbe interactions, and stomatal movement.

Ca$^{2+}$ signals in chloroplasts

Ca$^{2+}$ may have various roles in chloroplast function, including assembly of the PSII/oxygen-evolving complex, repair of PSII reaction centers, stromal enzyme activities, protein translocation (Chigri et al., 2006) and plastid division (Aldridge and Möller, 2005). Each of those processes may be regulated by fluctuations in stromal Ca$^{2+}$ concentration. When plants are transferred from light to darkness, chloroplasts have large transient increases in stromal Ca$^{2+}$ levels (Sai and Johnson, 2002). Furthermore, our preliminary results suggest that superoxide induces a transient increase in stromal Ca$^{2+}$ levels (Komori et al., unpublished data). The possible roles of CAS in the generation of stromal Ca$^{2+}$ signals are under investigation.

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Experimental procedures

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia were germinated and grown on MS medium containing 0.8 % (w/v) agar and 1 % (w/v) sucrose at 22°C with 80–100 µmol m⁻² sec⁻¹ illumination for a daily 16 h light period. The T-DNA insertion lines (SALK_070416, 665012) were ordered from the Arabidopsis Biological Resource Center and GABI-Kat, respectively. The presence, nature, and location of the T-DNA insertions were confirmed by PCR and sequencing. The consequence of T-DNA insertion on the expression of the affected gene was checked by northern and western blot analyses. Col was used as the control.

Plant transformation constructs and plant transformations

CAS and GFP fusion genes were constructed as follows. First-strand cDNA was synthesized from total RNA prepared from Arabidopsis seedlings using AMV reverse transcriptase (TaKaRa). cDNA was amplified by PCR using KOD-plus-DNA polymerase (TOYOBO) according to the manufacturer’s protocol. Transient expression vectors were constructed using the GFP reporter plasmid 35S-sGFP (S6ST). The PCR fragment containing 70 N-terminal amino-acids of CAS (35S-trCAS-GFP) or full length CAS (35S-CAS-GFP) was ligated in frame into the 35S-sGFP (S6ST) plasmid. Transient expression of the GFP fusion proteins in Arabidopsis protoplasts was performed as previously described (Kovtun et al., 2000).

To generate transgenic plants, the binary vector pBI121 was used for transformation. To obtain CAS or CAS-GFP overexpression constructs under the control of CaMV 35S promoter, the GUS gene in the binary vector was replaced with the CAS or CAS-GFP fusion genes described above. CAS promoter-GUS expression plasmid was constructed as follows. The 1500 bp CAS promoter fragment was fused to a promoterless GUS gene of pBI121 binary vector to give a CAS promoter-GUS construct. Subsequently, GUS gene was replaced to GFP(S6ST) gene to generate CAS promoter-GFP construct. The resulting constructs were introduced into Agrobacterium tumefaciens and used to transform the wild-type (Col-0) or cas-1 plants by flower dipping (Clough and Bent, 1998). Kanamycin-resistant T1 transformants were then selected and used for further analyses. Furthermore, transgenic Arabidopsis plants that express apoaequorin specifically in cytosol were obtained by transforming wild-type (Col-0) and cas-1 mutant plants with an aequorin expression vector pMAQ02 (Harada et al., 2003; Knight et al., 1991) via A. tumefaciens.

Subcellular fractionation

Cell membranes were prepared as previously described (Lu and Hirak, 2002) with some modifications. Three-week-old, plate-grown Arabidopsis plants were homogenized with a mortar and pestle in 2 ml of homogenization buffer 50 mM HEPES-KOH, pH 7.5; 5 mM MgCl₂; 5 mM EGTA; 330 mM sorbitol; 1% polyvinylpyrolidon K-30; 1% ascorbic acid; 1 mM dithiothreitol; protease inhibitor cocktail (Nakarai Tesque, Kyoto, Japan) per gram tissue. Homogenates were filtered through two layers of Miracloth and centrifuged at 6000 g for 10 min at 4°C. Pellets (chloroplast fraction) were resuspended in 50 mM HEPES-KOH, pH 7.5; 2 mM MgCl₂; 5 mM EGTA, and 13.7% sucrose. The supernatant was centrifuged at 100 000 g for 60 min at 4°C. The pellets (microsome fraction) were resuspended in 1 ml resuspension buffer 10 g⁻¹ wet weight of starting materials.

Intact chloroplasts were isolated from 3- to 4-week-old plate-grown Arabidopsis leaves and spinach leaves using a Percoll two-step gradient, as previously described (Napier and Barnes, 1995). Subsequently, Arabidopsis intact chloroplasts were lysed and separated into supernatant (soluble) and pellet (membrane) fractions. For fractionation of chloroplasts, intact chloroplasts from Spinach were lysed in a hypotonic buffer and were loaded onto a sucrose step gradient of 0.33 and 0.6 m sucrose, centrifuged at 70 000 g for 1 h at 4°C. Soluble, envelope and thylakoid fractions were retrieved from the supernatant, the 0.6/1 m sucrose interface, and the pellet, respectively. Proteins in the soluble fraction were concentrated by acetone precipitation. The envelope fraction was pelleted by centrifugation at 150 000 g for 1 h at 4°C. Thylakoid fraction was washed by centrifugation at 7000 g for 5 min at 4°C. All procedures were performed at 4°C.

Thylakoid membrane isolation and protease treatment

Three-week-old plate-grown Arabidopsis leaves were homogenized for 30 sec in a waring blender with isolation buffer (50 mM Tricine-KOH, pH 7.5; 350 mM sucrose, 2 mM MgCl₂; 10 mM NaCl). Homogenates were filtered through two layers of Miracloth and centrifuged at 5000 g for 3 min at 4°C. The thylakoid membrane pellets were resuspended in isolation buffer and washed twice with same buffer. Protease treatment was performed as previously described (Karnaucho et al., 1997) with a slight modification. Thylakoid membrane (0.1 mg chlorophyll ml⁻¹) was suspended in an isolation buffer containing thermolysin (TL), trypsin (Try), or no protease, and incubated on ice for 30 min. Proteolysis was stopped by the addition of the protease inhibitor cocktail (Nakarai Tesque). Membrane association experiments were performed as previously described (Karnaucho et al., 1997). Thylakoid membrane (0.1 mg chlorophyll ml⁻¹) was suspended in an isolation buffer containing 1 mM NaCl, 0.1 mM Na₂CO₃, 4 mM Urea, 1% Triton X-100 or no treatment (Control) and incubated on ice for 30 min. Protein was separated into membrane (P) and supernatant (S) fractions by centrifuging at 15 000 g for 30 min at 4°C. All procedures were performed at 4°C.

Antibodies and Western blotting

The DNA encoding 182 N-terminal amino-acids of CAS was amplified by PCR and inserted into the expression vector pET-16b, which generates histidine-tagged fusion protein in E. coli. The CAS N-terminal fragment was overexpressed in E. coli, purified and used to immunize a rabbit. Polyclonal antisera were used in Western blotting experiments. Total protein extracts were prepared from various Arabidopsis tissues as described previously (Martinez-Garcia et al., 1999). Electrophoresed proteins resolved by SDS-PAGE were electrophoretically transferred to polyvinylpyroliodine (immobilon; Millipore) membrane. The membranes were subsequently probed with antibodies. The Western blots were developed with the enhanced chemi-luminescence (ECL) kit.

Cytosolic Ca²⁺ measurement

Detached rosette leaves from 3-week-old, plate-grown Arabidopsis were floated on MS liquid medium, containing 50 µM CaCl₂ and 2.5 µM coelenterazine, overnight in the dark. Aequorin lumines-
Ca2+ concentration was increased to 2 mM, and the leaves were incubated for an additional 2 h. After the incubation period, epidermal strips of the leaves were observed by microscopy. Stomatal apertures were also measured in rosette leaves from soil-grown, Arabidopsis plants, and similar results were obtained (data not shown).

Stomatal aperture measurement

Stomatal aperture measurements were performed as previously described (Pei et al., 1997) with modifications. Rosette leaves from 3- to 4-week-old plate-grown plants were detached and floated on the incubation buffer (10 mM MES–KOH, pH 6.15; 10 mM KCl; 50 μM CaCl2) for 2 h in 80–100 μmol m−2 sec−1 light. After 2 h, extracellular Ca2+ concentration was increased to 2 mM, and the leaves were incubated for an additional 2 h. After the incubation period, epidermal strips of the leaves were observed by microscopy. Stomatal apertures were also measured in rosette leaves from soil-grown, Arabidopsis plants, and similar results were obtained (data not shown).

Acknowledgements

We thank the Salk Institute and GABI-Kat for providing Arabidopsis insertion mutants. We thank Dr T. Furuichi of Nagoya University for helpful discussion and donating AEQ antibody, and Dr M. Isobe and Dr M. Kuse of Nagoya University for coelenterazine, Dr K. Okada of NIBB for the pMAQ2 binary vector and Dr MH Sato of Kyoto Prefectural University for the 35S-GFP-Sec22 and 35S-GFP-SYP132 plasmids. We also thank Dr Y. Kashino of University of Hyogo, Dr M. Nakai of Osaka University, Drs F. Sato and K. Ifuku of Kyoto University, and Dr Y. Kasahara of National Agricultural Research Center for the Hokkaido Region for donating spinach CP43 antibody, guard cells.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Characterization of the CAS insertional mutants.

Figure S2. RT-PCR analysis of CAS mRNA abundance in CAS/cas-1 complement lines.

Figure S3. [Ca2+]ext-induced stomatal closure in other CAS/cas-1 and CASox plants.

References


