Biological Function of Calcium-Sensing Receptor (CAS) and Its Coupling Calcium Signaling in Plants

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Abstract: The calcium-sensing receptor (CAS), as a chloroplast thylakoid membrane protein, involved in the process of $[Ca^{2+}]_{ext}$-induced $[Ca^{2+}]_{cyt}$ increase (CICI) in the plant. However, the underlying mechanism regulating this process is lacking. Furthermore, recent evidence suggests that CAS may perform additional roles in the plant. Here, we provide an update covering the multiple roles of CAS in stomatal movement regulation and calcium signaling in the plant. We also analysis the possible phosphorylation mechanism of CAS by light and discuss the role of CAS in abiotic stress (drought, salt stress) and biotic stresses (plant immune signaling). Finally, we provide a perspective for future experiments which are required to fill gaps in our understanding of the biological function of CAS in the plant.

Keywords: stomatal movement; calcium sensing receptor; phosphorylation; abiotic stress; calcium signaling

1. Introduction

When we go into the field appreciating the beauty of different flowers and plants, do we realize that plants can sense their environment? Not only can they detect and respond to the environmental changes, they also use the messengers similar to those used animals in relaying the external signals to the cell, leading to responses at the cellular and whole-plant level. Calcium is a ubiquitous signaling molecule in the eukaryotic cell and calcium signal transduction is a key mechanism by which plants sense and responds to endogenous and environmental stimulus. So far, a variety of stimulus working in plants has been found, such as plant hormone, light, drought, cold, hypoxia. When these stimuli happen, the concentration of cytosolic calcium ion (Ca^{2+}_i)

will change almost at first [1, 2], which will cause the production of calcium signaling. Then, the calcium signaling will be received by some downstream target proteins of Ca^{2+} signal transduction, and a series of physiological and biochemical reactions will be generated to adapt or resist various adversity stresses. Up to now, there are three calcium signaling systems understood clearly in plant cells: calcium-dependent protein kinase (CDPK) [3]; Calcineurin B-like protein (CBL) [4], Calmodulin (CaM) [5]. Recently, a new calcium signaling system called Ca^{2+} sensing receptor (CAS), was found existing in plant [6]. However, many questions remain unanswered; for instance, as a chloroplast thylakoid membrane protein, how does the CAS sense extracellular high calcium signal? How does circadian rhythm effect on phosphorylation and dephosphorylation of CAS? Which did the protein kinase and phosphatase involve in the reversible phosphorylation process of CAS? Here we address these questions and review the new finding of CAS in the plant.

2. Modular Structure of CAS

In 2003, Han and his colleagues using a functional screening assay in human embryonic kidney cells (HEK293) isolated an *Arabidopsis* complementary DNA clone encoding a Ca^{2+}-sensing receptor (CAS). *CAS* is a single copy gene in the *Arabidopsis* genome. The *CAS* cDNA encodes a protein of 387 amino acids with a calculated molecular mass of 41,268 Da. The N-terminus of CAS, which contains the Ca^{2+}-binding domain, appears to be exposed to the stromal side of the thylakoid membrane [6, 7]. The C-terminus contains two motifs: a noncatalytic rhodanese homology domain (amino acids 231–352), with the putative active residue Cys309 substituted by Asp, and a motif that is involved in interaction with 14-3-3 proteins and proteins with the 'forkhead-associated' (FHA) domain. According to hydropathy analysis, CAS in higher plants has one transmembrane helix (amino acids 188–210 in *Arabidopsis*). The phosphorylation site of CAS is mapped to the stroma-exposed Thr380, located in a motif for interaction with 14-3-3 proteins and proteins with FHA domains [8], which suggests the CAS involves in stress responses and signaling pathways.

Initially, studies of CAS localization performed in onion epidermis and human

embryonic kidney cells indicated the plasma membrane as the site of CAS localization [6]. However, the succedent researches demonstrate that CAS is a plastid-specific protein but not a plasma membrane protein. The accurate localization of CAS is in the chloroplast thylakoid membrane, and CAS immunoblot analysis of thylakoid fractions revealed the presence of CAS both in grana and in stroma thylakoids, and it's clear enrichment in the stroma-exposed membranes [7-10]. Moreover, CAS has homologs in *Oryza sativa*, *Medicago truncatula*, the green algae *Chlamydomonas reinhardtii*, *Ostreococcus tauri* and *physcomitrellapatens,* as well as in *Brassica pekinensis,* but no proteins with significant sequence similarity to CAS were found in *cyanobacteria* [8, 11, 12].

3. Biological of Function of CAS in Plants

3.1. CAS Coupling Calcium Signaling in Plant

Calcium signal transduction is a primary mechanism of plants to sense and response to various stimuli. Calcium signaling is involved in almost all plants' developmental and physiological processes. According to recent researches, CAS has been shown to play a crucial role in the generation of Ca^{2+} oscillations in the cytosol. Han (2003) found that CAS could sense extracellular high calcium (Ca_o^{2+}) and converted the Ca_o^{2+} signal into cytosolic free Ca^{2+}. CAS may initiate the inositol 1,4,5-trisphosphate (IP_3) pathway in *Arabidopsis* [6], and demonstrated that $[Ca^{2+}]_i$ oscillations are coupled to the $[Ca^{2+}]_o$ oscillations–CAS–IP_3 pathway in *Arabidopsis thaliana*, $[Ca^{2+}]_i$ oscillations were synchronized to extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) oscillations largely through CAS. CAS regulates concentrations of IP_3, which in turn directs release of Ca^{2+} from internal stores. The oscillating amplitudes of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ are controlled by soil Ca^{2+} concentrations and transpiration rates, and the phase and period of oscillations are likely determined by stomata conductance [13]. However, these consequences are based on the wrong localization of CAS in the plasma membrane, which is not conclusive. Therefore, as a chloroplast protein localized in the thylakoid membrane of *Arabidopsis*, what does CAS play role in the cellular Ca^{2+} signal

transduction? The evidences suggested that CAS involved in the generation of $[Ca^{2+}]_{ext}$-induced $[Ca^{2+}]_{cyt}$ increase (CICI) in *Arabidopsis* [7, 10]. In addition, the *Oryza sativa* calcium-sensing receptor gene (*OsCAS*) could also sense an increase of extracellular Ca^{2+} concentration and mediate an increase in cytosolic Ca^{2+} concentration [14]. There is a further question. How does CAS in the chloroplast sense external Ca^{2+}? A recent study found CAU1 encoding the H4R3sme2 (for histone H4 Arg 3 with symmetric dimethylation)-type histone methylase protein arginine methytransferase5/Shk1 binding protein1. A mutation in CAU1 can significantly increase the expression level of the calcium signaling gene CAS, and CAU1 binds to the CAS promoter and modulates the H4R3sme2-type histone methylation of the CAS chromatin. In response to increases in $[Ca^{2+}]_o$, fewer CAU1 protein molecules bind to the CAS promoter, leading to decreased H4R3sme2 methylation and consequent derepression of the expression of CAS to mediate stomatal closure and drought tolerance [15]. Accumulation evidences suggested CAS as a kind of calcium sensor modulated calcium signaling in plant.

3.2. CAS Involved in Stomatal Movement Regulation

Ca^{2+} has been known to play a vital role in the stomatal movement as a secondary messenger. Many extracellular and cytosolic signals can evoke Ca^{2+} oscillations or increases in guard cells, such as ABA [16], H_2O_2 [17], Nitric oxide (NO) [18]. Previous researchers revealed that extracellular calcium (Ca^{2+}_o) can promote free cytosolic Ca^{2+} (Ca^{2+}_i) increase and finally lead to stomatal closure [19-21]. CAS is a crucial regulator of extracellular calcium-induced stomatal closure in *Arabidopsis* [6, 13]. The loss function of CAS impairs stomata closure that is induced by elevation of the extracellular Ca^{2+} concentration [10]. The overexpression of *CAS* promotes stomatal closure in the absence of externally applied Ca^{2+} [7]. In addition, the *Oryza sativa* calcium-sensing receptor gene (*OsCAS*) can also promote stomatal closure [14].

In order to further understand the mechanism of stomatal movement regulating by CAS, researchers have done a lot of work (Fig.1). Using multidisciplinary approaches to probe and different mutant, it exist crosstalk about hydrogen peroxide

(H_2O_2), ABA and nitric oxide (NO) in the CAS signaling pathway in guard cells in response to $Ca^{2+}{}_o$ [22, 23]. They proposed a hypothetical model where by $Ca^{2+}{}_o$ induces H_2O_2 and NO accumulation in guard cells through the CAS signaling pathway, which further triggers $Ca^{2+}{}_i$ transients and finally stomata closure. There is also study showing that decreased drought tolerance and WUE (water use efficiency) of *CASas* (the antisense of *CAS*) is associated with higher stomatal conductance due to improper regulation of stomatal aperture, rather than any change of stomatal density [24]. Moreover, the evidence shows that over-reduction of the plastoquinone (PQ) pool by dibromothymoquinone (DBMIB) was closely associated with stomatal closure in plants, where chloroplast H_2O_2 generation in the mesophyll is required. And in the PQ pool-H_2O_2 signaling pathway, CAS and LHCII phosphorylation are both in dispensable [25].

3.3. CAS Involved in Plant Light Signal Transduction

In plants, the resting circadian $[Ca^{2+}]_i$ oscillations are regulated by photoperiod and light intensity [26]. According to preamble description, CAS plays a crucial part in the CICI and the $[Ca^{2+}]_i$ oscillations synchronized to ($[Ca^{2+}]o$) oscillations procedures [13], particularly in the Ca^{2+}-CAS-IP_3 pathway. Clearly, transpiration-mediated soil Ca^{2+} uptake and transport synchronize the resting $[Ca^{2+}]_i$ throughout the plant. Transpiration rate is governed by stomatal conductance, as the same time, the stomatal conductance oscillations are regulated by photoperiod and the clock [9]. Thus, we can infer that photoperiod regulates flowering by Ca^{2+}-CAS-IP_3 pathway.

Such hypothesis is also in keeping with the phenomena that repression of CAS transcription impairs bolting (swift upward growth at the transition to seed production), and evenly hard to bolting, in response to Ca^{2+} deficiency [6, 8]. Moreover, it reported that the protein expression of CAS was higher in a 3-week rosette leaf than that in a 2-week rosette leaf of *A. thaliana* by western blot assay [7]. These results showed the loss function of CAS affects flowering time dramatically.

As a semi-autonomous organelle, chloroplast is not only the site of photosynthesis, but also functions as central hubs in plant metabolism [27]. Chloroplasts have their own genome although most chloroplast proteins are encoded

by nuclear genes [28]. The functional properties of chloroplasts are tightly regulated by the nuclear genome. Intracellular chloroplast retrograde signaling regulates the nuclear gene expression and is essential for the biogenesis of chloroplasts and for maintaining optimal chloroplast function in response to fluxes of metabolites and changes in environmental conditions [29]. As a chloroplast thylakoid membrane protein, CAS functions in de-etiolation and chloroplast development in *Arabidopsis thaliana* [30]. Thylakoid-localized CAS protein could cause to the generation of cytosolic calcium transients, and furtherly activate the MPK3/MPK6 signal system. Subsequently, activated MPK3/MPK6 into the nucleus induced phosphorylation of ABI4. Activation of ABI4 at both the transcriptional and posttranslational levels leads to the repression of *LHCB* [31]. These result showed that thylakoid-localized CAS involved in the plastid-to-nucleus retrograde signaling pathway.

As mentioned location of CAS and calcium signaling sensor responder, it is hypothesized that CAS play a critical role during photosynthesis regulation process. Previous works confirmed the highlight induced CAS diversification [8]. Response to increasing light intensities, the transcript and protein level of CAS is as well as its phosphorylation level significantly upregulated in *Arabidopsis thaliana* [32], while the phosphorylation is shown to be dependent on the state transition kinases STN8 protein kinase, with site mapped to Thr380 [8]. STN8 with other two protein kinases, chloroplast casein kinase2 (cpCK2) and the state transition kinases STN7, jointly dominate photosynthetic and environmental response related protein phosphorylation in the chloroplast [33]. The STN8 Antisense mutant performs an instantaneous influence in fine-tuning of cyclic electron flow (CEF) during a dark-to-light shift, whereas PGRL1-A, another substrate to STN8, confirms a link between protein phosphorylation and modulation of electron flow in plants [34]. It implies that as one of the others target of STN8 protein kinase, CAS possibly also connects with modulation of electron flow with the method like PGRL1.

Interestingly, by transfer *Chlamydomonas reinhardtii* to high-light growth conditions, researchers found that without CAS, alga was unable to properly induce the expression of LHCSR3 protein which is crucial for nonphotochemical quenching.

Prolonged exposure to high-light revealed a severe light sensitivity of alga and caused diminished activity and recovery of photosystem II (PSII) [35]. Soon after, more deeply study on CEF machinery in *Chlamydomonas reinhardtii* provides evidence for a Ca^{2+}-dependent regulation of CEF via the combined function of ANR1, CAS, PGRL1, associated with each other in a multiprotein complex [36]. This result further indicates the prominent role of CAS in calcium signaling regulation pathway, especially the control of photosynthesis at the chloroplast, in accordance with previously PGTL1 related research.

It is tempting to speculate that the whole CAS signaling network probably works like the following thedescription. For a response to high light, Under increased light intensities, the C-terminal of CAS protein be phosphorylated by STN8, and Ca^{2+} ion combined on CAS are released to chloroplast stroma [37], subsequently induced chloroplast Ca^{2+}-influx change, to created Ca^{2+} increased or oscillations signaling.

For consist of photosynthesis electron flow alteration, CAS work as a protein complex important in hypoxia condition. The CEF could turn out to have a Janus-faced function. On one hand, CEF is classically associated with ATP production without the production of NADPH; on the other hand, CEF leads to the reduction of the plastoquinone pool, thereby increasing the frequency of charge recombination events in PSII and as a result increasing O_2 production [38, 39]. Taking into account the conclusion that CAS associated with O_2-mediated retrograde signaling in *A. thaliana* [40], CAS and Ca^{2+} via CEF could activate O_2-mediated retrograde signaling. Likewise, activation of CEF via CAS and Ca^{2+} as observed in *C. reinhardtii* is in line with the inhibitory role of CAS in photosynthesis-driven CO_2 fixation [36, 41].

3.4. CAS and Abiotic Stresses

Ca^{2+} is known as a regulator in many cellular and physiological processes in plants and participates in plant responses to environmental stresses such as drought [42], high temperature [43], salinity [44] and cold injury [45]. As a Ca^{2+}-sensing receptor localized in the thylakoid membrane, CAS plays a crucial role in many physiological and biochemical processes in plants as well as stress resistance. CAS is beneficial to resist soil drought in *Arabidopsis*. Compared with the wild type, *CASas* plants were fewer

droughts tolerant due to excessive transpiration when grown under low soil moisture conditions [24]. In rice, the calcium-sensing receptor gene (*OsCAS*) is also closely associated with drought tolerance. The transgenic *Arabidopsis* overexpressed *OsCAS* gene shows better resistance to drought stress by decreasing damage to the cell membrane, increasing the number of osmoprotectants, and maintaining a relatively high photosynthetic capacity [14]. In addition, CAS is a crucial target of P. indica (Piriformospora indica, a root-colonizing endophytic fungus of Sebacinales) in Chinese cabbage leaves during the establishment of drought tolerance [46].

In *Chlamydomonas reinhardtii*, there is the orthologue of the plant CAS (CrCAS) which also localized in the thylakoids of chloroplast. CrCAS plays an important role in acclimation of photosynthesis to high light, is also involved in adaptation responses to low light at an intermediate time scale and also affects processes outside the chloroplast [35]. As aquatic photosynthetic organisms, *Chlamydomonas reinhardtii* possesses a CO_2-concentrating mechanism (CCM) to maintain photosynthetic activity in CO_2-limiting conditions by sensing environmental CO_2 and light availability [47]. Recently envidence indicated CrCAS could function in maintaining the expression levels of nuclear-encoded CO_2-limiting-inducible genes, including the HCO_3^- transporters high-light activated 3 (HLA3) and low-CO_2-inducible gene A (LCIA). CAS changed its localization from dispersed across the thylakoid membrane in high-CO_2 conditions or in the dark to being associated with tubule-like structures in the pyrenoid in CO_2-limiting conditions, along with a significant increase of the fluorescent signals of the Ca^{2+} indicator in the pyrenoid [25]. These results suggest that *Chlamydomonas* CAS is a Ca^{2+}-mediated regulator of CCM-related genes via a retrograde signal from the pyrenoid in the chloroplast to the nucleus.

3.5. CAS Mediated Plant Immune Signaling (Biotic Stresses)

In *Arabidopsis*, chloroplasts have a critical role in plant immunity as a site for the generation of chloroplast-derived reactive oxygen species (ROS) and the production of defence-related hormones, such as salicylic acid (SA) and jasmonic acid (JA). It's known that the pathogen-associated molecular pattern (PAMP) signals are quickly relayed to chloroplasts and evoke the increase of Ca^{2+} in the stroma. The calcium-

sensing receptor (CAS), a thylakoid membrane-associated Ca^{2+}-binding protein involved in the regulation of cytoplasmic Ca^{2+} oscillations and extracellular Ca^{2+} -induced stomatal closure, is still involved in stromal Ca^{2+} transients and responsible for both PAMP-induced basal resistance and *R* gene-mediated hypersensitive cell death. In addition, the synthesis of salicylic acid (SA) in the plant is dependent on CAS. Transcriptome analysis demonstrates that CAS is involved in the PAMP-induced expression of defense genes and suppression of chloroplast gene expression possibly through $^1O_2$-mediated retrograde signaling, allowing chloroplast-mediated transcriptional reprogramming during plant immune responses [40].

3.6. Other Physiological or Biochemical Processes with CAS in Plants

Light is very essential in plant growth and development, including germination, seedling growth and the control of flowering, particularly in the transition control of de-etiolation. The evidence shows that Ca^{2+} and CAS can promote *Arabidopsis thaliana* de-etiolation through effectively increasing chlorophyll accumulation, chloroplast development and the overall greening of leaves in *A. thaliana.* High Ca^{2+} significantly increases chlorophyll content and improves chloroplast development in both *A. thaliana* WT and *CASas* etiolated seedlings during de-etiolation. Etiolated *CASas* plants showes much lower chlorophyll content and delay of chloroplast development as compared with WT plants [30]. According to previous researches, it's known that calcium deficiency in the environment and low calcium utilization rate can cause tipburn in *Brassica pekinensis*. A recent research has found that the expression of CAS genes is affected by calcium deficiency, and associated with tipburn occurrence in seedling of Chinese cabbage [11].

4. Concluding Remarks

CAS is highly conserved in vascular plants. Although we have known its modular structure and many correlations with other cellular and physiological processes in plants, including $[Ca^{2+}]_{ext}$-induced $[Ca^{2+}]_{cyt}$ increase (CICI), stomatal closure, abiotic and biotic stresses etc (Fig.2), what specific function of CAS in those progresses remains not particularly clear. Therefore, the exploration of potential cross-talk between CAS and light signaling at the chloroplast thylakoid membranes is the key point to further

understand the function of CAS. In the future, it is important to identify the kinase and phosphatase which play a key role in reversible protein phosphorylation of CAS. On the other hand, as a chloroplast Ca^{2+}- binding protein, CAS might also bind calcium leading to a conformational change and play the role of transmitting signals. In the future, the functional impact of calcium-dependent phosphorylation on plant physiology can be estimated through quantitative phosphoproteomics and targeted mutation of the phosphorylation sites of CAS which will provide more comprehensive overview of biological function of CAS.

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Figure1. A model for CAS coupling calcium signaling in stomatal movement. PLC: phospholipase C; H_2O_2: hydrogen peroxide; IP_3: inositol 1, 4, 5-triphosphate; CAS: calcium sensing receptor.

Figure2. Biological function of CAS involved in biotic stresses, abiotic stress, photomorphogenesis and retrograde signaling in plants. PAMP: pathogen-associated molecular patterns; CAS: calcium sensing receptor; SA: salicylic acid; WUS: water use efficiency; ROS: reactive axygen species; MAPK: mitogen-activated protein kinase; *ABI4*: Abscisic acid-insensitive 4; *LHCB*: Chloroplast light-harvesting complex II.

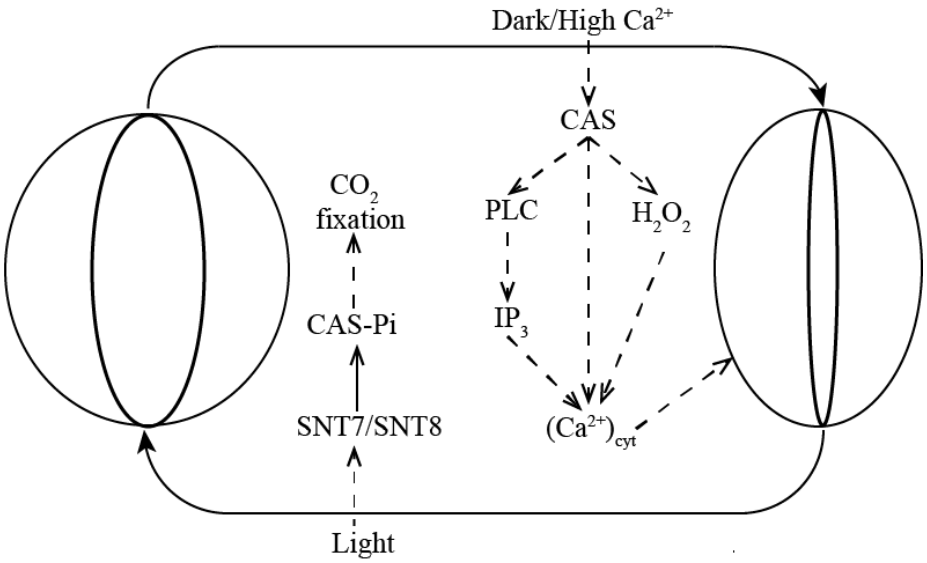

Figure1

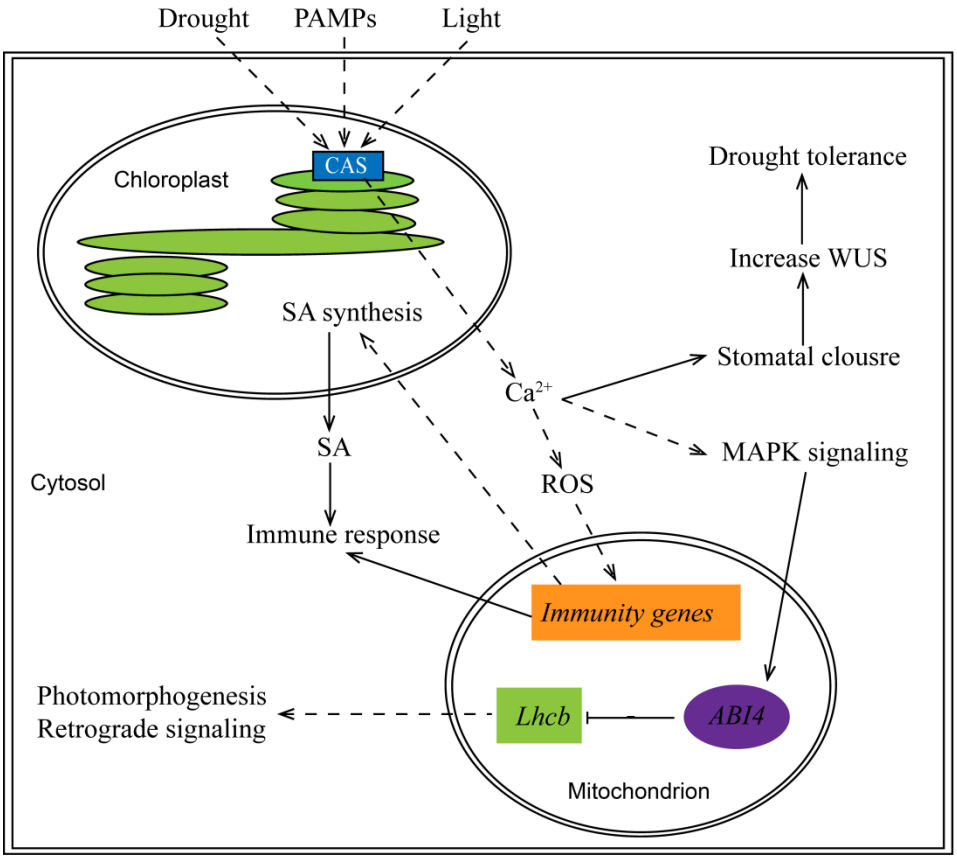

Figure2