Coupling Diurnal Cytosolic Ca\textsuperscript{2+} Oscillations to the CAS–IP\textsubscript{3} Pathway in Arabidopsis

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Various signaling pathways rely on changes in cytosolic calcium ion concentration ([Ca\textsuperscript{2+}]\textsubscript{i}). In plants, resting [Ca\textsuperscript{2+}]\textsubscript{i} oscillates diurnally. We show that in Arabidopsis thaliana, [Ca\textsuperscript{2+}]\textsubscript{i} oscillations are synchronized to extracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{o}) oscillations largely through the Ca\textsuperscript{2+}-sensing receptor CAS. CAS regulates concentrations of inositol 1,4,5-trisphosphate (IP\textsubscript{3}), which in turn directs release of Ca\textsuperscript{2+} from internal stores. The oscillating amplitudes of [Ca\textsuperscript{2+}]\textsubscript{o} and [Ca\textsuperscript{2+}]\textsubscript{i} are controlled by soil Ca\textsuperscript{2+} concentrations and transpiration rates. The phase and period of oscillations are likely determined by stomatal conductance. Thus, the internal concentration of Ca\textsuperscript{2+} in plant cells is constantly being actively revised.

Organisms, from single-celled to multicellular, exploit the unique physical and chemical properties of the calcium ion to carry out essential biological functions. [Ca\textsuperscript{2+}]\textsubscript{i} increases transiently and/or repetitively in response to many abiotic and biotic stimuli (1–3) and also displays diurnal oscillations in animals and plants (4, 5). In plants, the resting circadian [Ca\textsuperscript{2+}]\textsubscript{i} oscillations occur at the whole-tissue level (6), in contrast to those seen in specific neurons in animals (4, 7), and are regulated by photoperiod and light intensity (8). This oscillating feature implies a robust regulatory machinery that synchronizes [Ca\textsuperscript{2+}]\textsubscript{i} throughout the plant. However, due to the lack of the knowledge of sensory receptors and Ca\textsuperscript{2+} channels (2, 5, 9), the underlying mechanisms for the resting [Ca\textsuperscript{2+}]\textsubscript{i} and its oscillations remain largely unknown.

We have cloned a receptor for external Ca\textsuperscript{2+} (Ca\textsuperscript{2+}-o), CAS, from Arabidopsis (10). CAS is expressed in the shoot, localizes to the plasma membrane, binds to Ca\textsuperscript{2+}, and mediates Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} increases (CICI) in stomatal guard cells. We have generated CAS antisense lines (CAS\textsubscript{s}s) and shown that its mRNA and protein levels are reduced and that CICI is abolished (10). Identification of CAS and genetic manipulation of its activity may provide a powerful tool to dissect the mechanisms controlling [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. We hypothesized not only that Ca\textsuperscript{2+}-o serves as a signal triggering [Ca\textsuperscript{2+}]\textsubscript{i} increases, but also that Ca\textsuperscript{2+}-o and CAS control the resting [Ca\textsuperscript{2+}]\textsubscript{i}. To test this hypothesis, we measured the resting [Ca\textsuperscript{2+}]\textsubscript{i}, using aequorin bioluminescence-based Ca\textsuperscript{2+} imaging (6, 8, 11) and found that the resting [Ca\textsuperscript{2+}]\textsubscript{i} was lower in CAS\textsubscript{s}s than in wild-type plants (Fig. 1A).

Next, we asked whether [Ca\textsuperscript{2+}]\textsubscript{i} oscillations were affected in CAS\textsubscript{s}s. Biological oscillations can be described by three parameters: amplitude, phase, and period (12). Quantitative analysis of leaf aequorin luminescence showed that the amplitudes of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations were reduced in CAS\textsubscript{s}s throughout a long day (Fig. 1B; P < 0.001). The average resting [Ca\textsuperscript{2+}]\textsubscript{i} and absolute amplitude (peak trough) were reduced by 46.0 ± 2.3% and 50.2 ± 3.5% (P < 0.001), respectively. However, the phase and period were not altered in CAS\textsubscript{s}s (P > 0.1), indicating that CAS is required for maintaining appropriate oscillating amplitudes. Similar results were seen in three CAS\textsubscript{s}s lines, CAS\textsubscript{s}s1 to CAS\textsubscript{s}s3 (10).

The reduced luminescence in CAS\textsubscript{s}s was not due to low abundance of cytosolic aequorin. Aequorin-expressing CAS\textsubscript{s}s lines were generated from a cross between CAS\textsubscript{s}s and a wild-type line carrying the 35S::aequorin construct. The stability of aequorin expression in wild-type plants was confirmed for eight generations. The CAS\textsubscript{s}s and wild-type lines had similar aequorin protein levels (fig. S1A), which remained stable throughout a long day (fig. S1B). Finally, the maximum luminescence was identical in both genotypes, as estimated by discharge in excess Ca\textsuperscript{2+}.

Our data prompted us to investigate how [Ca\textsuperscript{2+}]\textsubscript{o} is regulated. Ca\textsuperscript{2+} is dissolved in water in the apoplast (extracellular spaces) and transported primarily from the root to the shoot through the transpiration stream (13, 14). The transpiration rate is governed by stomatal conductance, which displays diurnal oscillations (15). We reasoned that [Ca\textsuperscript{2+}]\textsubscript{o} is synthesized to stomatal-conductance oscillations, and by Ca\textsuperscript{2+}, oscillations may be perceived by CAS and converted into [Ca\textsuperscript{2+}]\textsubscript{i}, oscillations. Thus, the soil Cas2+ signaling cascade would be as follows: soil Ca2+ → Cr2+ uptake and transport → Ca2+ oscillations → CAS → [Ca\textsuperscript{2+}]\textsubscript{i}, oscillations.

To test this hypothesis, we analyzed whether media Ca\textsuperscript{2+} affects [Ca\textsuperscript{2+}]\textsubscript{i}. In wild-type plants, [Ca\textsuperscript{2+}]\textsubscript{o} was elevated with increases in media Ca\textsuperscript{2+}, whereas in CAS\textsubscript{s}s this response was reduced (Fig. 1C; P < 0.001). We monitored [Ca\textsuperscript{2+}]\textsubscript{o}, oscillations in plants grown under physiological (1 mM) and elevated (30 mM) Ca\textsuperscript{2+} concentrations. The overall amplitudes of [Ca\textsuperscript{2+}]\textsubscript{o} were elevated in both wild-type and CAS\textsubscript{s}s plants with increases in media Ca\textsuperscript{2+}, but much more so in the wild type, although the phases were not altered (Fig. 1D).

We assessed how media Ca\textsuperscript{2+} regulates [Ca\textsuperscript{2+}]\textsubscript{o}, and whether [Ca\textsuperscript{2+}]\textsubscript{i}, oscillates. We monitored [Ca\textsuperscript{2+}]\textsubscript{o} directly by expressing aequorin in the apoplast as described previously (16). We found that [Ca\textsuperscript{2+}]\textsubscript{i} also displayed diurnal oscillations, which were similar in wild-type and CAS\textsubscript{s}s plants (Fig. 2A), [Ca\textsuperscript{2+}]\textsubscript{o} increased with increases in media Ca\textsuperscript{2+}, and both genotypes showed virtually identical responses (Fig. 2B). To ensure equivalent activity of apoplastic aequorin in both genotypes, we measured extracellular aequorin under conditions similar to those for cytosolic aequorin (17). The aequorin protein abundance was similar in wild-type and CAS\textsubscript{s}s plants (fig. S2).
Finally, we determined whether stomatal-conductance oscillations are correlated with Ca$^{2+}$ uptake and transport. Ca$^{2+}$, oscillations, and [Ca$^{2+}$]$_i$, oscillations. As expected, the stomatal conductance of wild-type plants was high in the day and low at night (Fig. 2C). We also found that wild-type and CASas plants displayed similar diurnal oscillations in $^{45}$Ca uptake and transport (Fig. 2D). These experiments confirmed previous reports that Ca$^{2+}$ uptake and transport to leaves are controlled mainly by transpiration that is regulated by stomatal conductance (13, 14).

The shoot Ca content of seedlings grown at various concentrations of media Ca$^{2+}$ was similar between wild-type and CASas plants (Fig. 2E). Thus, the reduced [Ca$^{2+}$] oscillations in CASas were due not to reduced Ca content, but rather to a defect in Ca$^{2+}$ sensing.

It appears that the phases and periods of oscillations in stomatal conductance, $^{45}$Ca uptake, [Ca$^{2+}$]$_o$, and [Ca$^{2+}$]$_i$, were well correlated. CAS seems to control the amplitude of [Ca$^{2+}$] oscillations, but not Ca uptake or [Ca$^{2+}$]$_i$, oscillations. Media Ca$^{2+}$ concentration regulates the oscillating amplitudes of both [Ca$^{2+}$]$_i$, and [Ca$^{2+}$]$_o$. In addition, the expression patterns of CAS, noticeably in mesophyll cells and vascular tissues in the shoot (Fig. 2F), fit well with the route of Ca$^{2+}$ transport and distribution in the shoot (17), supporting the function proposed for CAS.

Next, we asked how CAS couples [Ca$^{2+}$]$_o$ with [Ca$^{2+}$]$_i$, i.e., whether CAS mediates Ca$^{2+}$ influx or release from internal stores. In animals, although diurnal [Ca$^{2+}$]$_o$, oscillations at the whole-tissue level have not been observed (4, 7), fast [Ca$^{2+}$]$_i$, spiking at the cellular level has been well studied (1). External signals are perceived by cell-surface receptors, which activate phospholipase C (PLC), increasing inositol 1,4,5-trisphosphate (IP$_3$) concentration. IP$_3$ activates IP$_3$ receptors (IP$_3$Rs) in the endoplasmic reticulum (ER), resulting in Ca$^{2+}$ release. The Ca$^{2+}$ ions are reabsorbed by the ER, leading to [Ca$^{2+}$]$_i$, oscillations. In plants, although cell-surface receptors and IP$_3$Rs are unknown (18), it is possible that CAS might serve as a receptor triggering Ca$^{2+}$ release. To explore this possibility, we investigated the function of CAS in CICI in a heterologous system as well as in planta.

CAS confers CICI in human embryonic kidney 293 (HEK293) cells (10). We observed that the CAS–green fluorescent protein (GFP) was localized in the vicinity of the plasma membrane in HEK293 cells (Fig. 3A), consistent with the subcellular localization in plants (10). We found that Ca$^{2+}$$_o$, triggered IP$_3$ production in CAS expressing HEK293 cells (Fig. 3B). The PLC blockers, neomycin and U-73122, inhibited CICI (Fig. 3C). These results indicate that CAS can function as a receptor in the IP$_3$ pathway in HEK293 cells.

Next, we assessed whether CAS also serves as a receptor in the putative IP$_3$ pathway in Arabidopsis. In plants, some of the components seen in the animal IP$_3$ pathways have been identified (17, 18). For instance, increases in IP$_3$ have been detected in response to several stimuli, which enhance the activities of PLCs. High-affinity IP$_3$ binding sites in internal membranes have been detected, although it is not clear whether plants have IP$_3$Rs. IP$_3$ can also trigger Ca$^{2+}$ release from internal stores. Nonetheless, the molecular nature of corresponding cell-surface receptors remains obscure.

We found that neomycin abolished CICI in Arabidopsis guard cells (Fig. 3D). Similarly, we observed CICI in mesophyll cells and also detected CICI in intact leaves using aequorin imaging, which suggests that CICI occurs in cells other than guard cells and at the whole-leaf level. To analyze further whether IP$_3$ participates in CICI, we measured the IP$_3$ content of leaves. Ca$^{2+}$$_o$, induced IP$_3$ generation (Fig. 3E; $P < 0.01$), and neomycin eliminated the IP$_3$ production (Fig. 3F; $P > 0.5$). Together, these results suggest that Ca$^{2+}$$_o$, may evoke the IP$_3$ pathway in Arabidopsis.

To verify Ca$^{2+}$$_o$, induced IP$_3$ production, we carried out single-cell imaging of IP$_3$. The com-
Fig. 3. CAS is a cell-surface receptor in the IP₃ pathway. (A) Plasma membrane localization of CAS in HEK293 cells expressing a CAS-GFP construct (upper) with GFP as a control (lower). Scale bar, 5 μm. (B) The effect of Ca²⁺ on IP₃ generation in HEK293 cells expressing CAS (red). The control (blue) was cells transfected with empty vector. Cells were bathed in 0.1 mM Ca²⁺ before addition of 2.5 mM Ca²⁺ and harvested at the times indicated. The IP₃ content was determined by [³H]IP₃ radioreceptor assay (17) (mean ± SEM; n = 6). (C) Inhibition of CICr by PLC blockers in HEK293 cells expressing CAS. Cells were incubated with 0.1 mM Ca²⁺ without (white circles) or with 100 μM neomycin (black circles) before Ca²⁺ stimulation. Ca²⁺ at 2.5 mM was added at the time indicated (arrow), and [Ca²⁺]o was monitored by Fura-2 imaging (17). (D) Inhibition of CICr by neomycin in guard cells. The leaf epidermis was incubated with 50 μM Ca²⁺ for 2 hours, and then treated with 2.5 mM Ca²⁺ (10). Neomycin (100 μM) was added 15 min before addition of Ca²⁺. [Ca²⁺]o is shown as changes in emission fluorescence ratios (Δ[Ca²⁺]; n = 15 cells; P < 0.001). (E) Ca²⁺, induces IP₃ generation in Arabidopsis leaves. Leaves were incubated in the solution containing 50 μM Ca²⁺ for 2 hours, then transferred to the same solution (white circles) or the solution containing 10 mM Ca²⁺ (black circles), and harvested at the times indicated. IP₃ was determined by [³H]IP₃ assay (mean ± SEM; n = 6). (F) Neomycin inhibits Ca²⁺-induced IP₃ generation in leaves. The samples were collected before addition of Ca²⁺ (white bars) or 60 s after addition of 10 mM Ca²⁺ (black bars). Neomycin (100 μM) was added 15 min before Ca²⁺ stimulation (mean ± SEM; n = 6). pmol/gFW, picomole per gram fresh weight.

Fig. 4. CAS is required for the Ca²⁺o-evoked IP₃ pathway. (A and B) Ca²⁺o-induced IP₃ production in single WT guard cells. The leaf epidermis was incubated with 50 μM Ca²⁺ before and treated with 10 mM Ca²⁺. Ca²⁺o triggered PHPLC-GFP translocation from the plasma membrane (blue) to the cytosol (red). The images in (B) correspond to before (○) and after (●) Ca²⁺o stimulation in (A). (C) IP₃ content in WT and CASas leaves with (black bars) and without (white bars) addition of 10 mM Ca²⁺ (mean ± SEM; n = 6; P < 0.02 for WT; P > 0.5 for CASas1 and CASas2). (D and E) Defect in Ca²⁺o-induced IP₃ generation in CASas guard cells. The CASas line expressing PHPLC-GFP was generated from a cross between CASas and the wild-type PHPLC-GFP line, as in (A). (F) Increases in PHPLC-GFP fluorescence in the cytosol with addition of Ca²⁺o were analyzed from experiments as in (A) and (D). Thirty-eight out of 133 guard cells (28.6%) for the wild type (A) and 4 out of 76 (5.3%) for CASas (E) showed increases. Data are shown as the mean ± SEM (P < 0.001). Scale bars, 5 μm.

We have revealed that [Ca²⁺]o oscillations are coupled to the [Ca²⁺]i oscillations—CAS–IP₃ pathway. Our data, together with previous studies, may explain how [Ca²⁺]i oscillations are determined: the amplitude by the soil Ca²⁺ level and stomatal conductance, and the phase and period largely by stomatal-conductance oscillations. We can simulate [Ca²⁺]i oscillations using a schematic model (fig. S4). First, CAS and a Ca²⁺-influx channel, such as guard cell ICₐ (25), perceive [Ca²⁺]o and convert it into [Ca²⁺]i. Second, [Ca²⁺]i, oscillates. The [Ca²⁺]i, is determined by two opposite processes: supplying fresh Ca²⁺ to the apoplast and sequestrating it into stores. For the sequestration, fresh Ca²⁺ ions bind...
Odor Cues During Slow-Wave Sleep Prompt Declarative Memory Consolidation

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Sleep facilitates memory consolidation. A widely held model assumes that this is because newly encoded memories undergo covert reactivation during sleep. We cued new memories in humans during slow-wave sleep by presenting an odor that had been presented as context during prior learning, and so showed that reactivation indeed causes memory consolidation during sleep. Re-exposure to the odor during slow-wave sleep (SWS) improved the retention of hippocampus-dependent declarative memories but not of hippocampus-independent procedural memories. Odor re-exposure was ineffective during rapid eye movement sleep or wakefulness or when the odor had been omitted during prior learning. Concurring with these findings, functional magnetic resonance imaging revealed significant hippocampal activation in response to odor re-exposure during SWS.

Sleep facilitates the consolidation of newly acquired memories for long-term storage (1–3). The prevailing model assumes that this consolidation relies on a covert reactivation of the novel neuronal memory representations during sleep after learning (3–6). In rats, hippocampal neuronal assemblies implicated in the encoding of spatial information during maze learning are reactivated in the same temporal order during slow-wave sleep (SWS) as during previous learning (7, 8). The consolidation of hippocampus-dependent memories benefits particularly from SWS (9–11), and reactivation of the hippocampus in SWS after spatial learning has also been seen in humans observed with positron emission tomography (12). However, none of these studies experimentally manipulated memory reactivation during sleep. Therefore, its causal role in memory consolidation is still unproven.

We used an odor to reactivate memories in humans during sleep, because odors are well known for their high potency as contextual retrieval cues not only for autobiographic memories, as delicately described in Marcel Proust’s Remembrance of Things Past, but also for various other types of memory, including visuo-spatial memories (13, 14). Notably, in the brain, primary olfactory processing areas bypassing the thalamus project directly to higher-order regions, including the hippocampus (15), which enables them to modulate hippocampus-dependent declarative memories (16). The use of olfactory stimuli for cueing memories during sleep is particularly advantageous because odors, in contrast to other stimuli, can be presented without disturbing ongoing sleep (17).

To establish a robust association between learning stimuli and a smell, we applied a purely olfactory stimulus (the smell of a rose) (18) repetitively while volunteers (n = 18) learned object locations in a two-dimensional (2D) object-location memory task in the evening before sleep. During the first two periods of subsequent SWS, the odor was presented again (in an alternating 30 s on/30 s off mode). In a control condition, odorless vehicle was delivered. The object-location task required visually learning the locations of 15 card pairs on a computer screen to a criterion of 60% correct responses (Fig. 1A). The task is sensitive to the memory-improving effect of sleep (18) and involves hippocampal function (19).

At retrieval testing after sleep, memory of the card locations was distinctly enhanced when the odor had been presented during SWS as compared to presentation of the vehicle alone. After the odor night, participants remembered 97.2 ± 4.1% of the card pairs they had learned before sleep, but they remembered only 85.8 ± 3.8% after the vehicle night (P = 0.001; Fig. 2A, 18)}