Plant adaptation to fluctuating environment and biomass production are strongly dependent on guard cell potassium channels


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At least four genes encoding plasma membrane inward K⁺ channels (Kᵢᵣ channels) are expressed in Arabidopsis guard cells. A double mutant plant was engineered by disruption of a major Kᵢᵣ channel gene and expression of a dominant negative channel construct. Using the patch-clamp technique revealed that this mutant was totally deprived of guard cell Kᵢᵣ channel (GCKᵢᵣ) activity, providing a model to investigate the roles of this activity in the plant. GCKᵢᵣ activity was found to be an essential effector of stomatal opening triggered by membrane hyperpolarization and thereby of blue light-induced stomatal opening at dawn. It improved stomatal reactivity to external or internal signals (light, CO₂ availability, and evaporative demand). It protected stomatal function against detrimental effects of Na⁺ when plants were grown in the presence of physiological concentrations of this cation, probably by enabling guard cells to selectively and rapidly take up K⁺ instead of Na⁺ during stomatal opening, thereby preventing deleterious effects of Na⁺ on stomatal closure. It was also shown to be a key component of the mechanisms that underlie the circadian rhythm of stomatal opening, which is known to gate stomatal responses to extracellular and intracellular signals. Finally, in a meteorological scenario with higher light intensity during the first hours of the photophase, GCKᵢᵣ activity was found to allow a strong increase (35%) in plant biomass production. Thus, a large diversity of approaches indicates that GCKᵢᵣ activity plays pleiotropic roles that crucially contribute to plant adaptation to fluctuating and stressing natural environments.

Arabidopsis | circadian rhythm | inward Shaker | stomata | transpirational water loss

The leaf epidermis is covered with a waxy cuticle that prevents water loss but also impedes diffusion of atmospheric CO₂ toward the inner photosynthetic tissues. Gas exchanges mainly occur through microscopic pores in the epidermis, named stomata. By controlling stomatal aperture, the plant copes with the conflict between the need for photosynthesis and transpiration, thereby preventing detrimental effects of Na⁺ on stomatal closure. It was also shown to be a key component of the mechanisms that underlie the circadian rhythm of stomatal opening, which is known to gate stomatal responses to extracellular and intracellular signals. Finally, in a meteorological scenario with higher light intensity during the first hours of the photophase, GCKᵢᵣ activity was found to allow a strong increase (35%) in plant biomass production. Thus, a large diversity of approaches indicates that GCKᵢᵣ activity plays pleiotropic roles that crucially contribute to plant adaptation to fluctuating and stressing natural environments.


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the effect of the mutations on GCK_{in} activity. The individual mutations kat2-1 and domneg-1 were found to result in partial reduction of GCK_{in} current, by ~60% and 55%, respectively, at −200 mV (Fig. 1). The fact that reduction in GCK_{in} activity was not total in the domneg-1 mutant suggested that the expression level of the domneg construct (under control of the KAT2 promoter) was not high enough to result in integration of at least one domneg polypeptide in every K_{in} channel (see Discussion). Interestingly, the combination of the two mutations kat2-1 and domneg-1 totally abolished the current (Fig. 1). The corresponding mutant was therefore named kincless (K^{+} inward current-less). It was used to investigate the role of GCK_{in} activity in stomatal and whole-plant physiology.

GCK_{in} Activity Plays an Essential Role in Stomatal Opening Triggered by Membrane Hyperpolarization. Stomatal opening is triggered by light at the beginning of the daily cycle. The blue component of white light acts as a specific signal (4, 17, 18). It activates guard cell plasma membrane H^{+}-ATPases, leading to membrane hyperpolarization and thereby to increased K^{+} uptake through K_{in} transport systems. To assess the actual contribution of GCK_{in} activity to this process, we evaluated the effects of fusicoccin (FC), a toxin known to up-regulate plant plasma membrane H^{+}-ATPases (17), of Cs^{+}, a classical inhibitor of K_{in} channel activity (19) and of blue, red, or white light on stomatal opening in epidermal peels from WT and kincless plants. The results (Fig. 2) can be summarized as follows. (i) Cs^{+} did not further affect stomatal opening in the kincless mutant, providing additional evidence for total absence of GCK_{in} activity. (ii) Stomatal opening was strongly dependent on GCK_{in} activity when it was triggered by fusicoccin or blue light, two treatments that have been extensively characterized as leading to membrane hyperpolarization. (iii) On the contrary, stomatal opening was poorly dependent on GCK_{in} activity when it was triggered by red light. The mechanisms of red light-induced stomatal opening are still unclear, and conflicting data have been reported on the actual contribution of H^{+}-ATPase activation and membrane hyperpolarization to the process (18).

GCK_{in} Activity Underlies Stomatal Responsiveness to Changes in Light, Air Humidity, and CO_{2} Availability in Intact Plants. Consequences of the absence of GCK_{in} activity on leaf transpiration were then investigated by using a setup that continuously monitors transpirational water loss in an intact plant. Consistent with the above results, absence of GCK_{in} activity affected the increase in transpiration induced by light (Fig. 3A). Fitting the increase in transpiration with monoexponential functions led to time constants approximately six times higher in the mutant (~20 h) than in the WT (~20 min) plants (Fig. 3A). These quantitative analyses demonstrate that stomatal opening induced by blue light in intact plants depends very strongly on GCK_{in} activity.

Besides light conditions, regulation of stomatal aperture integrates plant water status and CO_{2} availability. Transpiration recordings revealed that GCK_{in} activity improved stomatal responsiveness to a sudden reduction in leaf to air water vapor pressure difference (VPD) (Fig. 3B). Such sharp changes in micrometeorological conditions are likely to be frequent in natural environments, e.g., upon a decrease in wind velocity. GCK_{in} activity was also found to improve stomatal responsiveness to a reduction in CO_{2} availability (Fig. 3C).

Absence of Inward K^{+} Channel Activity Results in Impaired Control of Stomatal Aperture in the Presence of Na^{+} in the Medium. In far from physiological conditions, when epidermal peels were bathed in solutions containing high Na^{+} concentrations (30 mM NaCl) but no K^{+} at all, stomata were able to open upon light (Fig. S3), presumably by taking up Na^{+} as an osmoticum in place of K^{+} (4, 20). However, opening in such conditions subsequently precluded normal stomatal closure (upon ABA addition; Fig. S3), in agreement with previous reports in other plant species, probably because Na^{+} cannot be rapidly excreted from the cell (4, 20). Interestingly, introducing K^{+} in the bath solution (in the presence of Na^{+}, 20 mM NaCl, and 10 mM KCl) before inducing stomatal opening (by switching light on) rescued WT but not kincless stomata from the deleterious effects of Na^{+} on the subsequent stomatal closure (Fig. 4A and B). Thus, in the...
presence of Na\(^+\), stomatal closure itself (and not only stomatal opening) depends on K\(^+\)-selective GCK\(_{\text{in}}\) activity.

A second set of experiments was then performed on intact plants. WT and \textit{kincless} mutant plants were grown for 5 weeks in standard nutrient solution (containing 4.5 mM K\(^+\)). Then, the solution was supplemented with Na\(^+\) (70 mM). This treatment rapidly (12 h) affected stomatal physiology in the mutant plant: absence of GCK\(_{\text{in}}\) activity strongly impaired stomatal closure when the light was turned off (Fig. 4 C and D).

**Inward K\(^+\) Channel Activity Renders Stomatal Opening Independent of Previous Illumination Periods.** Stomatal opening in \textit{kincless} plants was strongly affected when illumination was reduced to 3 h during the two preceding photoperiods (compare Fig. 5 C and D) and totally inhibited after 48 h in darkness (Fig. 5 A), whereas the same treatments had no significant effect in the WT plants (see arrows in Fig. 5 A). It is worth noting that stomatal closure itself was affected in the mutant plants when the duration of the preceding photoperiods was reduced (Fig. 5 D).

**Absence of Inward K\(^+\) Channel Activity Affects Stomatal Circadian Rhythm.** In WT plants, significant stomatal preopening occurred during the last hours of the night period, as shown by recording transpirational water loss (Fig. 5 A and B), attesting circadian rhythm in stomatal movements. Furthermore, such circadian movements persisted under extended dark conditions (Fig. 5 A and B). These two features were strongly affected in \textit{kincless} mutant plants (Fig. 5 A and B).

**Dependence of Plant Growth on Inward K\(^+\) Channel Activity.** Based on the above results, we assessed the importance of GCK\(_{\text{in}}\) activity in plant growth under challenging light conditions, with higher light intensity during the first hours of the photophase. In
This suggested that GCKin activity may be much higher than CKing in darkness in the WT plant. (Enlargement from preceding photoperiods. (Periods, respectively. Two photoperiods were suppressed, on days 4 and 5. During 7 days. Black and white boxes under the curves indicate dark and light Continuous recording of the transpiration rate in a single WT or plants exposed to an 8-h photoperiod since sowing. (Rates recorded in the same plants after exposure to a 3-h photoperiod during 7.5% (n = 12) for the 650- Roles of GCKin Activity in Stomatal Opening upon Membrane Hyperpolarization and Adaptation to Environmental Conditions. Stomata can open in the absence of GCKin activity but with a time constant (~120 min) approximately six times greater than that measured in control WT plants. Fig. 2 clearly shows that signals inducing membrane hyperpolarization (fusicoccin, blue light) are poorly efficient in triggering stomatal opening in the mutant plant devoid of GCKin activity. This means that membrane hyperpolarization of the guard cell membrane due to H+-ATPase activation is unable, in the absence of GCKin activity, to trigger rapid accumulation of osmotica from the apoplast, for instance via H+-K+ or H+-sugar symporters. In other words, GCKin activity is an essential effector of rapid stomatal opening triggered by membrane hyperpolarization, a role that was generally assumed but which lacked the support of direct genetic and (electro)physiological tests described here. It is thus crucial for blue light-induced stomatal opening at dawn and control of opening during the first hours of the day period, when K+ salts are the dominant osmotica responsible for increased guard cell turgor. Accumulation of organic solutes resulting from photosynthesis has been shown to contribute to guard cell turgor and stomatal aperture during the second half of the daily cycle (6, 24).

GCKin activity is also involved in plant responses to a decrease in internal CO2 availability (Fig. 3C) or in evaporative demand (Fig. 3B). Such roles probably reflect the fact that GCKin activity is the sole transport activity that can enable membrane hyperpolarization to rapidly trigger stomatal opening. Conversely, the fact that GCKin activity plays a role in these responses indicates that they rely on membrane hyperpolarization.

Fig. 5. Disruption of inward K+ channel activity affects stomatal circadian rhythm and renders stomatal opening sensitive to the duration of the preceding photoperiods. (A and B) Effect on stomatal circadian rhythm. (A) Continuous recording of the transpiration rate in a single WT or kincless plant during 7 days. Black and white boxes under the curves indicate dark and light periods, respectively. Two photoperiods were suppressed, on days 4 and 5. (B) Enlargement from A (dotted boxed regions) highlighting stomatal preopening in darkness in the WT plant. (C and D) Sensitivity to the duration of the preceding photoperiods. (C) Transpiration rates recorded in 5-week-old WT or kincless plants exposed to an 8-h photoperiod since sowing. (D) Transpiration rates recorded in the same plants after exposure to a 3-h photoperiod during 2 days. Data are means ± SE; n = 15 plants per genotype.

standard growth chambers, with 250 μE m−2 s−1 light intensity, increasing light intensity to 650 or 1,000 μE m−2 s−1 (approximately two-thirds of light intensity on a bright sunny day) for the first 2 h of light period (8 h in total) resulted in large differences in growth between WT and kincless mutant plants: biomass production (shoot fresh weight of 6-week-old plants) was higher in WT plants than in mutant plants, by ∼9.6 ± 3.1% or 36 ± 7.5% (n > 12) for the 650-μE or 1,000-μE treatments, respectively. No significant difference in biomass production between the two genotypes was observed when light intensity was kept constant, at 250 μE m−2 s−1, during the whole light period.

Discussion

Large Reductions in GCKin Activity Do Not Affect Plant Transpiration in Standard Conditions. Disruption of the KAT2 gene and expression of a kat2 domneg construct (under control of the KAT2 promoter region) both resulted in a large decrease in GCKin activity, by ∼60%, but did not affect plant transpiration, consistent with previous analyses showing that disruption of the KAT1 gene, which leads to a >50% reduction in GCKin activity, did not affect stomatal opening (11). Also, when a kat1 domneg construct was overexpressed (by means of a tandem repeat of the CaMV 35S promoter) in Arabidopsis, impairment in stomatal opening was detectable only in the transgenic plants displaying the highest reduction rates in GCKin activity, by ∼70–80% (21). This suggested that GCKin activity may be much higher than expectedly required for physiological needs, because it could be strongly reduced without any apparent consequence on plant phenotype (8, 21). In this context, it was implicitly assumed that engineering plants with down-regulated GCKin activity could be a valuable strategy to decrease leaf transpirational water loss and improve plant tolerance to drought. However, based on the present report, it is very likely that such plants would display reduced adaptation to fluctuating and stressing environmental conditions, and ultimately decreased biomass production.

A Mutant Devoid of GCKin Activity. Two-hybrid experiments in yeast and functional analyses after coexpression in Xenopus oocytes have provided evidence that KAT2 can interact and form heteromeric channels with other Shaker subunits, at least KAT1 and AKT2 (15, 22). Expression of a domneg kat2 gene in a kat2 knockout background is shown to result in total suppression of GCKin activity. Shaker genes expressed in guard cells, besides KAT2, are KAT1 and, probably at a lower level, AKT1 and AKT2 (11). We propose that, in a kat2 knockout background, the domneg kat2 polypeptides interact with the other WT Shaker subunits expressed in guard cells, preventing formation of functional channels.

The domneg kat2 construct was expressed under control of KAT2 promoter, which is mainly active in guard cells and in leaf phloem (22). Based on quantitative real-time PCR, leaf phloem cells express AKT2 and KAT1 Shaker genes at much higher levels than KAT2 (23). Thus, expression of the kat2 domneg construct under control of KAT2 promoter may mainly affect KAT1 channel activity in guard cells, leaving phloem KAT1 channels largely unaffected. Consistent with this hypothesis, K+ assays revealed similar leaf K+ contents in kincless and control WT plants. It is also worth noting that lack of GCKin activity affected stomatal movements in epidermal peels, i.e., in the absence of control by the leaf and whole plant. Furthermore, all of the data obtained in intact plants are coherent with those obtained in epidermal peels, when comparisons can be made, regarding the kinetics of light-induced stomatal opening or of dark-induced stomatal closure, and the sensitivity to CO2 and to Na+. Thus, the whole set of data indicates that the kincless phenotype analyzed in this report, in terms of transpirational water loss by intact plants, reflects defects in stomatal movements rather than in plant K+ status.
GCK\textsubscript{in} Activity Protects Stomatal Closure Against Detrimental Effects of Na\textsuperscript{+}. Experiments using epidermal peels in K\textsuperscript+-free solution indicate that stomata can open by accumulating Na\textsuperscript{+} instead of K\textsuperscript{+}, but that stomatal closure is thereafter impaired, probably because cells are unable to rapidly release Na\textsuperscript{+} ions. This might be because of lack of a large outwardly directed Na\textsuperscript{+} concentration gradient able to drive rapid efflux upon membrane depolarization. Indeed, Na\textsuperscript{+} is compartmentalized into the vacuole because high concentrations of Na\textsuperscript{+} would be toxic in the cytosol, and thus the concentration gradient of Na\textsuperscript{+} across the plasma membrane cannot build up to become as steep as the K\textsuperscript{+} gradient. The present data show that, in physiological conditions, when K\textsuperscript{+} and Na\textsuperscript{+} are both present in the medium, GCK\textsubscript{in} activity is required for efficient stomatal functioning (Fig. 4). It allows stomata to open by preferentially using K\textsuperscript{+}, setting up the conditions for rapid stomatal closure, the large outward conductance of the membrane to K\textsuperscript{+} (10) and the steep electrochemical gradient of this cation between the cytoplasm and the cell wall, ensuring rapid efflux upon membrane depolarization. In other words, a prerequisite to efficient stomatal closure is selective K\textsuperscript{+} uptake in guard cells during stomatal opening, and this is guaranteed, in the presence of both K\textsuperscript{+} and Na\textsuperscript{+}, by the highly K\textsuperscript{+-}selective GCK\textsubscript{in} activity. Noteworthy is the observation that treating plants lacking GCK\textsubscript{in} activity with Na\textsuperscript{+}, resulted in higher transpirational water loss for several hours at night. Greater transpiration in the absence of photosynthesis would decrease plant water use efficiency. Increased transpiration may result in more Na\textsuperscript{+} being moved to the shoot through the xylem to an extent that is potentially toxic.

A Crucial Role of GCK\textsubscript{in} Activity in Circadian Rhythm. In plants, the circadian clock ensures an optimal phase relation between physiology and day/night cycle, providing the basis for anticipation of recurrent changes in environmental conditions and improving photosynthesis and water use efficiency (25, 26). In natural conditions, stomatal opening during the first part of the day promotes CO\textsubscript{2} fixation during the early part of the day, when solar radiation is already high but air temperature and VPD are not yet challenging the plant water status (26). Our results indicate that GCK\textsubscript{in} activity is central to the mechanisms that underlie the circadian rhythm of stomatal opening, thereby rendering stomatal functioning predictable of, and not merely reactive to, the fluctuating environment.

Conclusion
Rapid and selective K\textsuperscript{+} uptake by inward K\textsuperscript{+} channels from the Shaker family is shown to be the major transport process that converts membrane hyperpolarization into rapid guard cell swelling and stomatal opening. Disruption of this activity in guard cells appears to have more detrimental effects than that of outward K\textsuperscript{+} channel (GCK\textsubscript{out}) activity. For instance, absence of GCK\textsubscript{in} activity results in a 6-fold increase in the time constant of stomatal opening, from ∼20 min to 2 h, whereas the time constant of stomatal closure induced by disruption of GCK\textsubscript{out} activity is increased only 2-fold, from 10 to 20 min (10). Also, at least four Shaker genes code for inward K\textsuperscript{+} channels in guard cells (11), whereas a single one [GORK (10)] encodes the outward K\textsuperscript{+} conductance. This might mean that ensuring rapid and selective K\textsuperscript{+} uptake upon membrane hyperpolarization is more essential for stomatal physiology and/or more complex to achieve than wholesale K\textsuperscript{+} secretion upon membrane depolarization.

By enabling membrane hyperpolarization to rapidly trigger stomatal opening, GCK\textsubscript{in} activity improves stomatal reactivity to changes in internal, environmental, and micrometeorological conditions (CO\textsubscript{2} availability, light, and evaporative demand). GCK\textsubscript{in} activity also allows stomatal opening to be independent of past illumination period and photosynthesis, maybe because accumulation of K\textsuperscript{+} is likely to be less costly, from an energetic point of view, and/or more straightforward to achieve than accumulation of organic osmotica. Furthermore, GCK\textsubscript{in} activity underlies the circadian rhythm of stomatal opening, allowing stomatal opening to anticipate changes in light conditions and not merely to react to illumination. This might explain why GCK\textsubscript{in} activity strongly impacted biomass production when the plants were exposed to high light intensities only for a short period at the beginning of the day, a meteorological scenario used to mimic conditions that limit stomatal opening later in the day (such as decrease in light intensity from shadowing, high air temperature and VPD, or water shortage). Finally, under salt stress, large and selective GCK\textsubscript{in} activity prevents detrimental Na\textsuperscript{+} uptake by guard cells upon membrane hyperpolarization, thereby ensuring efficient stomatal function and decreasing transpirational water loss. In conclusion, a robust and K\textsuperscript{+-}selective inward channel activity at the guard cell plasma membrane is a major actor in plant adaptation to both atmospheric and soil fluctuating and challenging conditions.

Materials and Methods
Isolation of the T-DNA-Tagged Mutant kat2-1 Disrupted in the KAT2 Gene. The kat2-1 knockout line was obtained by PCR screening of 40,000 Arabidopsis thaliana T-DNA insertion mutants [Wassilevskija ecotype; library constructed by Institut National de la Recherche Agronomique, Versailles (27)]. A Southern blot with a probe targeting the T-DNA right border revealed a single insertion locus. The exact position of the T-DNA insertion was determined by sequencing the T-DNA flanking sequences. Plants homozygous for the disruption were selected by PCR in the F\textsubscript{2} progeny.

Generation of the domneg-1 and Kincless Mutants. Site-directed mutagenesis was performed on the KAT2 cDNA to replace the selectivity filter GlyTyrGlyAsp motif ( hallmark of K\textsuperscript{+-}selective channels) by ArgArgGlyAsp. The mutated cDNA, named domneg, was cloned downstream of the KAT2 promoter region (2.258 kb upstream from the initiation codon) into a binary vector (pBIB-HYGRO), and the resulting plasmid was introduced into Agrobacterium tumefaciens GV3010 (pMP90) strain as described (10). WT plants (Wassilevskija ecotype) and kat2-1 mutant plants were transformed by using the floral dip method (27). Selection on hygromycin allowed us to identify transformed lines and to obtain a fixed transgenic domneg-1 or kincless line, respectively.

Reverse Transcription Experiments. Total RNA was extracted from Arabidopsis leaves by using TRIzol reagent (Invitrogen). After conversion to first-strand cDNA, KAT2 and EF\textsubscript{1}α were amplified by PCR from the same amounts of cDNA by using the following couples of primers: KAT2-3000 (5'-ggttttagagtagtagtgc-3') and KAT2-3930 (5'-gggtgaagggcttgaagggg-3'). EF\textsubscript{1}α-350 (5'-ccacagtgtgggtgttagc-3') and EF\textsubscript{1}α-900 (5'-cattgacaccaagctgttcg-3').

Intact Plant Transpiration Measurements. Plants were individually grown on compost in plastic containers in a growth chamber (21°C, 70% relative humidity, 8-h/16-h light/dark, 300 μE m\textsuperscript{-2} s\textsuperscript{-1}). Each container was closed by a screw cap to avoid evaporation from the compost surface. After sowing, each plant grew through a hole pierced in the middle of the cap. This experimental device ensured that water loss (decrease in weight) could be ascribed to leaf transpiration. Periodic addition of water into the container maintained the compost water content close to 75% (wt/wt). The transpirational water loss was expressed on leaf area basis, estimated from daily plant photographs using OPTIMAS 6.1 software.

A controlled automated growth chamber [Phenopix robot (28)] was used to impose variable micrometeorological conditions (light and VPD; the containers were weighed every 15 min). To study the effect of Na\textsuperscript{+} on transpiration, the plants were hydropenonically grown in half-strength Hoagland solution using the same device (plastic containers) as described above for plants grown on compost. Na\textsuperscript{+} was added to the nutrient solution as a chloride salt.

Transpiration and CO\textsubscript{2} Assimilation in Individual Whole Plants. Plants were hydropenonically grown in a growth chamber (22°C, 65% relative humidity, 8-h/16-h light/dark, 300 μE m\textsuperscript{-2} s\textsuperscript{-1}) for 4 weeks before being transferred (a single plant per experiment) to an experimental chamber allowing continuous gas exchange measurements (as described in ref. 10).

Stomatal Conductance. Measurements of stomatal conductance were performed in intact leaves on intact plants using a Li-COR 6400 infrared gas analyzer-based gas exchange system (Li-COR). Leaves were kept at 75% ± 2% relative humidity and 22°C.
Stomatal Aperture Measurements. Epidermal peels were prepared from abaxial epidermis as described (10) and incubated in 30 or 10 mM KC1 and 10 mM Mes-iminodiacetic acid (pH 6.5) at 20°C, unless otherwise noted. To standardize the initial state, the epidermal strips were kept in the incubation solution for 30 min in darkness. Then, they were exposed to treatments inducing stomatal aperture: white light (300 μEm⁻²s⁻¹, 30 mM KC1), blue light (30 μEm⁻²s⁻¹, 30 mM KC1), red light (80 μEm⁻²s⁻¹, 30 mM KC1), fusicoccin (10 μM, 10 mM KC1), or CO₂-free air (30 mM KC1). Effects of Cs⁺ or Na⁺ on stomatal behavior were investigated either in 30 mM GNO₃ and 10 mM KC1, using fusicoccin (10 μM) to trigger stomatal opening, or in 20 mM NaCl and 10 mM KC1, using white light (300 μM) to induce stomatal opening. Stomatal apertures were measured (pore width; at least 40 measurements per epidermis for each experimental point) with an optical microscope (Optiphoto; Nikon) fitted with a camera lucida and a digitizing table (Houston Instruments) linked to a personal computer.

Guard Cell Protoplast Isolation and Electrophysiological Recordings. Plants were grown on compost for 5 weeks in a greenhouse. Guard cell protoplasts were enzymatically isolated, and patch-clamp experiments were carried out as described (10). The pipette solution contained 100 mM K-glutamate, 2 mM MgATP, 5 mM EGTA, 1 mM CaCl₂ (50 mM free Ca²⁺), 0.5 mM MgCl₂, 300 mM sorbitol, and 20 mM Hepes-KOH (pH 7.25). The bath solution contained 20 mM CaCl₂, 2 mM MgCl₂, 100 mM K-glutamate, 225 mM sorbitol, and 10 mM Mes-HCl (pH 5.5). Liquid junction potentials at the pipette–bath interface were measured and corrected.

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