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Arabidopsis guard cell CO_2/HCO_3^- response mutant screening by an aequorin-based calcium imaging system

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Abstract

Background: The increase in atmospheric CO_2 is causing a number of changes in plant growth such as increases in leaf area and number, branching, plant size and biomass, and growth rate. Despite the importance of stomatal responses to CO_2, little is known about the genetic and molecular mechanisms that mediate stomatal development and movement in response to CO_2 levels. Deciphering the mechanisms that sense changes in CO_2 and/or HCO_3^- concentration is critical for unraveling the role of CO_2 in stomatal development movement. In *Arabidopsis*, CO_2-induced stomatal closure is strongly Ca^{2+}-dependent. To further dissect this signaling pathway and identify new components in the CO_2 response pathway, we recorded $[Ca^{2+}]_{cyt}$ changes in mutagenized *Arabidopsis* leaves and screened for mutants with abnormal guard cell behavior in response to CO_2/HCO_3^-.

Results: We observed that 1 mM HCO_3^- induces $[Ca^{2+}]_{cys}$ transient changes in guard cells and stomatal closure both in light and darkness. The changes in $[Ca^{2+}]_{cys}$ induced by HCO_3^- could be detected by an aequorin-based calcium imaging system. Using this system, we identified a number of *Arabidopsis* mutants defective in both $[Ca^{2+}]_{cyt}$ changes and the stomatal response to CO_2/HCO_3^-.

Conclusions: We provide a sensitive method for isolating stomatal CO_2/HCO_3^- response genes that function early in stomatal closure and that have a role in regulating $[Ca^{2+}]_{cyt}$. This method will be helpful in elucidating the Ca^{2+}-dependent regulation of guard cell behavior in response to CO_2/HCO_3^-.

Keywords: CO_2/HCO_3^-, Stomatal movement, Aequorin (AEQ), Ca^{2+}, High-throughput screening, *Arabidopsis thaliana*

Background

The stomata, which are formed by pairs of guard cells, can be considered the gas-exchange valves of plants. Stomatal aperture is regulated by several factors including phytohormone levels, carbon dioxide (CO_2) concentration, humidity, light, and pathogens.

A higher ambient CO_2 concentration increases leaf intercellular CO_2 concentration and mediates stomatal closure in plants, whereas a lower CO_2 concentration triggers stomatal opening. CO_2 influences not only the stomatal response, but also the number of stomata per unit leaf. This number is decreasing due to the long-term effect of continuing CO_2 concentration increases [1].

Despite the importance of stomatal responses to CO_2, little is known about the genetic and molecular mechanisms mediating stomatal development and movement in response to elevation in CO_2. CO_2 levels have been increasing steadily, and it is estimated that atmospheric CO_2 will reach 550 ppm in 2050 compared with 400 ppm presently [2], so it is increasingly urgent to discover

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the underlying mechanisms of guard cell regulation in response to CO_2 levels.

CO_2 sensing in animals is mainly linked to α-carbonic anhydrases (α-CAs) [3], which are also important for CO_2 perception in fungi [3, 4]. Carbonic anhydrases (CAs) can accelerate the conversion of CO_2 into HCO_3^- and H^+, which in turn induce related responses. In plants, CO_2 also can be converted into HCO_3^- and H^+ by anhydrases [5]. The key question in understanding stomatal movement in response to CO_2 is the mechanism for perception of changes in CO_2 and/or HCO_3^- concentration. Despite the importance of anhydrase enzymes in CO_2 perception in mammalian and fungal systems [3, 4], no orthologous α-CAs has been identified in plants. There are six β-CAs in *Arabidopsis thaliana*. CA1- and CA4-related stomatal movements were controlled by CO_2 in guard cells, whereas a *ca1 ca4* double mutant exhibited insensitive stomatal movement response to CO_2 [6]. Expression of a mammalian α-CA in the *ca1 ca4* double mutant restored the stomatal response to CO_2, implying that CA-mediated CO_2 catalysis to HCO_3^- and H^+ in guard cells is the key step for transmission of the CO_2 signal [6].

Through isolation and analysis of genetic mutants, a number of proteins have been identified that function in CO_2-controlled stomatal movement, including the SLAC1 anion channel [7, 8], the PATROL1 Munc 13-like protein [9], the AtALMT12/QUAC1 R-type anion channel [10], and the RHC1 MATE transporter [11]. The characterization of these proteins has contributed to our understanding of the mechanisms of CO_2-regulated guard cell behavior. For example, transporter protein RHC1 acts as a bicarbonate sensor, and the high-CO_2-induced stomatal closure mediated by RHC1 is controlled by inhibition of HT1 (HIGH LEAF TEMPERATURE1) activity [11, 12].

HT1 is regarded as a negative regulator in the CO_2 signaling pathway: it functions by promoting phosphorylation of OST1 and thus inhibiting its kinase activity [11]. Furthermore, OST1 protein kinase has been proved essential for high-CO_2-induced stomatal closure [13, 14]. But still, many points remain controversial, such as the mechanism underlying CO_2 sensing; the identities of the CAs involved in this pathway; the function of CAs under low-CO_2 conditions; and the interaction of CO_2 with light, temperature, humidity, and phytohormones in influencing stomatal movement.

The primary requirement for solving these questions is the isolation of mutants. Screening dependent on thermal imaging is quite common for isolating *Arabidopsis* mutants with abnormal guard cell behavior. Almost all of the mutants obtained until now, including *ht1*, *rhc1*, and *patrol1*, were obtained using this method. Although this method has been effective in unraveling the regulation network in CO_2-mediated stomatal movement, it is still not clear of this regulation network; thus, it is urgent to develop new screening methods.

Calcium ion (Ca^{2+}) has been shown to act as a key cellular second messenger in numerous plant processes. In *Arabidopsis thaliana*, abscisic acid (ABA), hydrogen peroxide, cold, and CO_2 all can stimulate cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) oscillation, which causes stomatal closure [15]. CO_2-induced stomatal closure is strongly Ca^{2+}-dependent in *Arabidopsis*, consistent with previous findings in *Commelina* guard cells [16–18]. Cytosolic Ca^{2+} regulates stomatal closure by two mechanisms: short-term Ca^{2+}-reactive closure and long-term Ca^{2+}-programmed closure [15].

Extracellular CO_2 induces changes of the $[Ca^{2+}]_{cyt}$ in *Arabidopsis* guard cells. To further dissect this signaling pathway, new components in the CO_2 response pathway that are related to the $[Ca^{2+}]_{cyt}$ changes need to be identified. Here, we used a novel approach for screening genetic mutants to identify proteins involved in CO_2 response. In this study, we used the Ca^{2+} reporter aequorin (AEQ) to record $[Ca^{2+}]_{cyt}$ changes in *Arabidopsis* leaves in real time in order to visualize locally induced $[Ca^{2+}]_{cyt}$ elevations in response to CO_2 or HCO_3^- stimulus. Although this screening method had already been used for analyzing the responses of *Arabidopsis* to different stimuli such as salt stress, ABA, sorbitol, and cold [15], it had not been tried for screening mutants with altered stomatal responses to CO_2 or/and HCO_3^-. By using this system, we obtained several *mci* (mutant of HCO_3^-/CO_2 insensitive) and *mcs* (mutant of HCO_3^-/CO_2 sensitive) mutants. Further study with these mutants will be helpful for uncovering the mechanism for calcium-dependent CO_2-regulated guard cell movement.

Results

$[Ca^{2+}]_{cys}$ changes induced by HCO_3^- can be detected by an aequorin-based calcium imaging system

In our first experiment, we tested whether AEQ-transgenic Arabidopsis plants could be used to detect $[Ca^{2+}]_{cys}$ changes induced by HCO_3^-. As it is already known that the pH of incubation buffer (50 mM KCl, 0.1 mM $CaCl_2$, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) and 10 μM coelenterazine) cannot be stabilized at 7.0 when the concentration of $KHCO_3$ is above 5 mM, a lower concentration (1 mM) that has previously been used for analyzing guard cell behavior [2] was selected to avoid the putative influence of pH.

The AEQ-transgenic *Arabidopsis* leaves were treated with 1 mM $KHCO_3$, and after 5 min, dramatic increases in $[Ca^{2+}]_{cys}$ were detected in the leaves by analyzing the AEQ luminescence image (Fig. 1a, left). The average

(See figure on next page.)
Fig. 1 HCO_3^--induced $[Ca^{2+}]_{cys}$ increase in *Arabidopsis* leaves and guard cells. **a** HCO_3^--induced $[Ca^{2+}]_{cys}$ increase in *Arabidopsis* leaves. (Left) AEQ-transgenic *Arabidopsis* leaves were treated with 1 mM $KHCO_3$, and analyzed by AEQ imaging at 0 and 5 min. (Right) Time-course analysis of $[Ca^{2+}]_{cys}$ changes after treatment with incubation buffer, 1 mM KCl and 1 mM $KHCO_3$. Leaves were put individually into the wells of a 96-well plate and treated with incubation buffer, 1 mM KCl or 1 mM $KHCO_3$. Luminescence recording began 4 s before treatment and was conducted at intervals of 0.2 s for a total of 12.4 s. Data for 59 leaves are shown (mean ± SE; n = 59). Bar = 5 mm. RLU, relative luminescence units. **b** HCO_3^--induced $[Ca^{2+}]_{cys}$ increase in *Arabidopsis* guard cells. (Left) AEQ images of AEQ-transgenic *Arabidopsis* epidermal strips after 1 mM $KHCO_3$ treatment. Red circles indicated guard cells. (Right) Time-course analysis of $[Ca^{2+}]_{cys}$ changes after 1 mM $KHCO_3$ treatment. The luminescence data were quantified from guard cell pairs (red circles) in the left side of the figure (n = 8). Bar = 20 μm. **c** Emission images (FRET-dependent Venus, 526–536 nm; CFP, 473–505 nm) of epidermal strips expressing YC3.6 were taken before and 1 min after addition of 1 mM $KHCO_3$ solution. Bar = 10 μm

luminescence values increased from about 200 to 2300 RLU (Relative luminescence units, which represents the electrical signal values generated by stimulated photons) within 2 s of $KHCO_3$ addition (Fig. 1a, right). We also added incubation buffer and same concentration of KCl as controls, and found that these only caused small changes of calcium (Fig. 1a, right). These suggested that 1 mM $KHCO_3$ is effective for checking the cytosolic calcium changes with the AEQ system. Furthermore, when we analyzed individual guard cells within leaf epidermal strips under 1 mM $KHCO_3$ treatment, a visible increase in luminescence of the guard cell was found after 1 min; when luminescence values were collected continuously for about 10 min, the quantified data of luminescence images of the guard cells confirmed the visually increase in $[Ca^{2+}]_{cys}$ to $KHCO_3$ (Fig. 1b).

To further validate the AEQ-based screening method, we adopted another Ca^{2+} indicator, yellow Cameleon 3.6 (YC3.6), for measuring CO_2/HCO_3^--induced $[Ca^{2+}]_{cyt}$ increases in guard cells. The YC3.6 transgenic plants showed a marked increase in $[Ca^{2+}]_{cyt}$ when treated with 1 mM $KHCO_3$. This was consistent with the results of AEQ, suggesting that the aequorin-based system is a reliable method of $[Ca^{2+}]_{cyt}$ measurement (Fig. 1c).

1 mM HCO_3^- induces closure of *Arabidopsis thaliana* stomata whether in light or darkness

Since the aequorin-based system requires that samples first be incubated in incubation buffer in the dark for several hours, we measured the stomatal responses in both light and darkness. For this experiment, detached leaves of 3-week-old plants were incubated in a glass chamber with quicklime to remove CO_2 from the chamber.

After 2 h of incubation in the chamber under light conditions, almost all stomata had opened very well. The opened stomata were closing after 20 min of 1 mM $KHCO_3$ treatment, and 60 min later, the stomatal apertures (width/length) were 0.71 ± 0.01 and 0.56 ± 0.01 for control and treated leaves, respectively (Fig. 2a).

Compared with the responses in light, the stomatal apertures were less after incubation in darkness without CO_2. However, the stomata closed 5 min after 1 mM $KHCO_3$ was added, and stomatal aperture decreased to 0.20 ± 0.01 for treated leaves at 60 min; while stomatal aperture was 0.42 ± 0.01 for the untreated at this time (Fig. 2b). The results showed that HCO_3^--induced stomatal closure whether in light or darkness; thus, by using the aequorin-based system, it will be possible to indentify abnormal-response mutants for both stomatal movement and $[Ca^{2+}]_{cys}$ transient change at 1 mM $KHCO_3$.

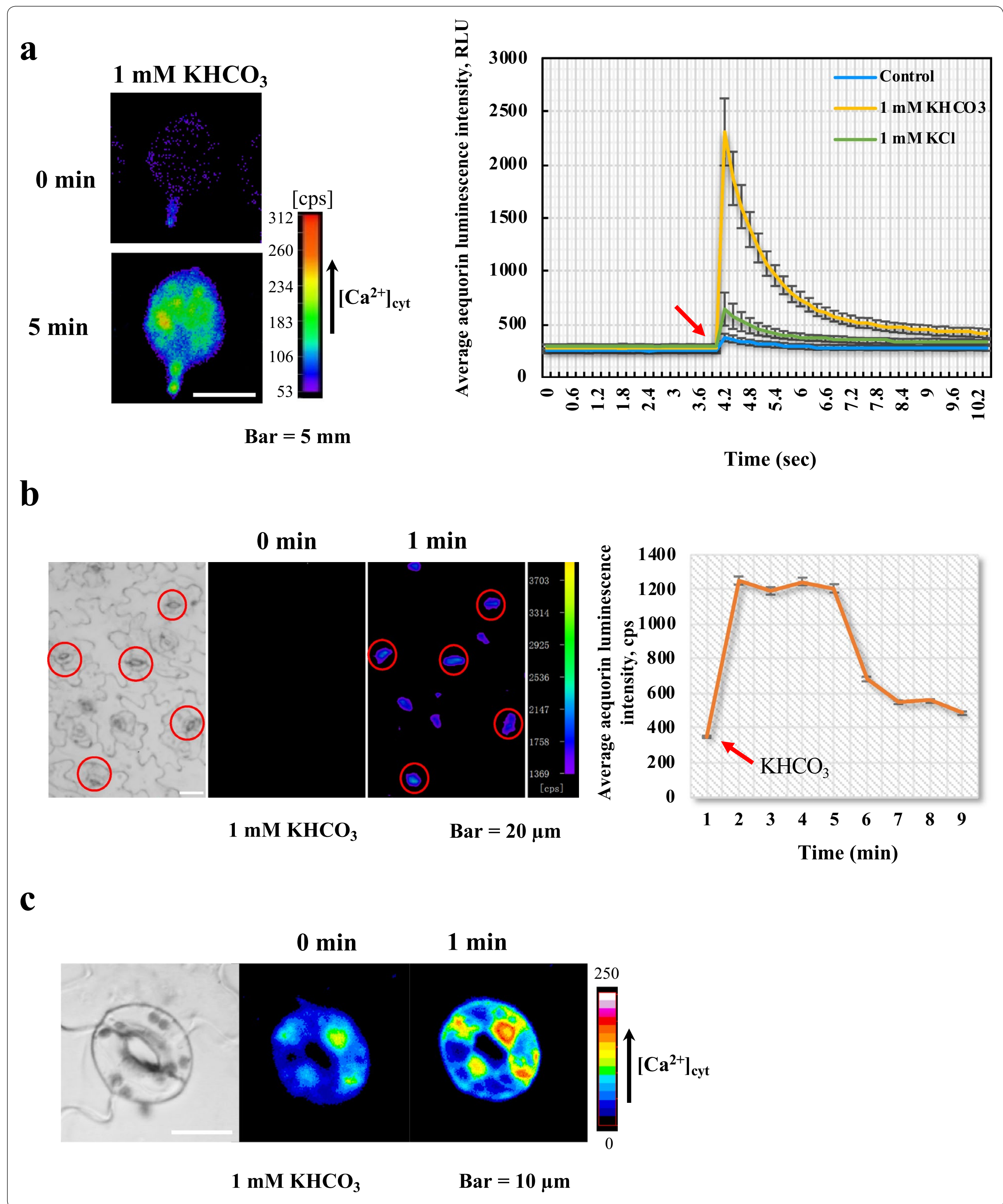
High-throughput screening for CO_2/HCO_3^- response mutants

For high-throughput genetic screening with the aequorin-based system, we used the protocol shown schematically in Fig. 3. About 5000 AEQ-expressing *Arabidopsis* seeds were treated with 0.3% (w/v) ethyl methane sulfonate (EMS) and sown on soil. M_2 seeds were collected individually and screened as described in Fig. 3. The leaves of 3-week-old M_2 plants were placed in a 96-well plate and 100 μL of freshly prepared incubation buffer was added to each well for 4-6 h. AEQ luminescences of leave treatment with 1 mM $KHCO_3$ were then identified by using a luminescence reader (LB960, Berthold) (Fig. 3). So far, approximately 35,000 M_2 plants have been screened, and about 120 sensitive and 80 insensitive putative mutants have been identified.

The selected plants were examined further for their stomatal response to $KHCO_3$ to narrow down the target mutants. HCO_3^-/CO_2-induced stomatal closure of the putative mutants was assayed in M_2 and again in M_3, 6 out of 80 putative mutants with lower luminescence showed an insensitive stomatal response to HCO_3^-/CO_2, and 4 out of 120 putative mutants with higher luminescence displayed a hypersensitive response.

Characterization of mutants obtained by the aequorin-based screening method

By using the aequorin-based screening procedure, we identified HCO_3^-/CO_2 response mutants that appeared abnormal in both $[Ca^{2+}]_{cys}$ and stomatal movement. To examin the stability of the mutants obtained by this


a
1 mM KHCO3
0 min
5 min
[cps]
312
260
234
183
106
53
[Ca2+]cyt
Bar = 5 mm
Average aequorin luminescence intensity, RLU
3000
2500
2000
1500
1000
500
0
Control
1 mM KHCO3
1 mM KCl
0 0.6 1.2 1.8 2.4 3 3.6 4.2 4.8 5.4 6 6.6 7.2 7.8 8.4 9 9.6 10.2
Time (sec)
b
0 min
1 min
3703
3314
2925
2536
2147
1758
1369
[cps]
1 mM KHCO3
Bar = 20 µm
Average aequorin luminescence intensity, cps
1400
1200
1000
800
600
400
200
0
KHCO3
1 2 3 4 5 6 7 8 9
Time (min)
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0 min
1 min
250
0
[Ca2+]cyt
1 mM KHCO3
Bar = 10 µm

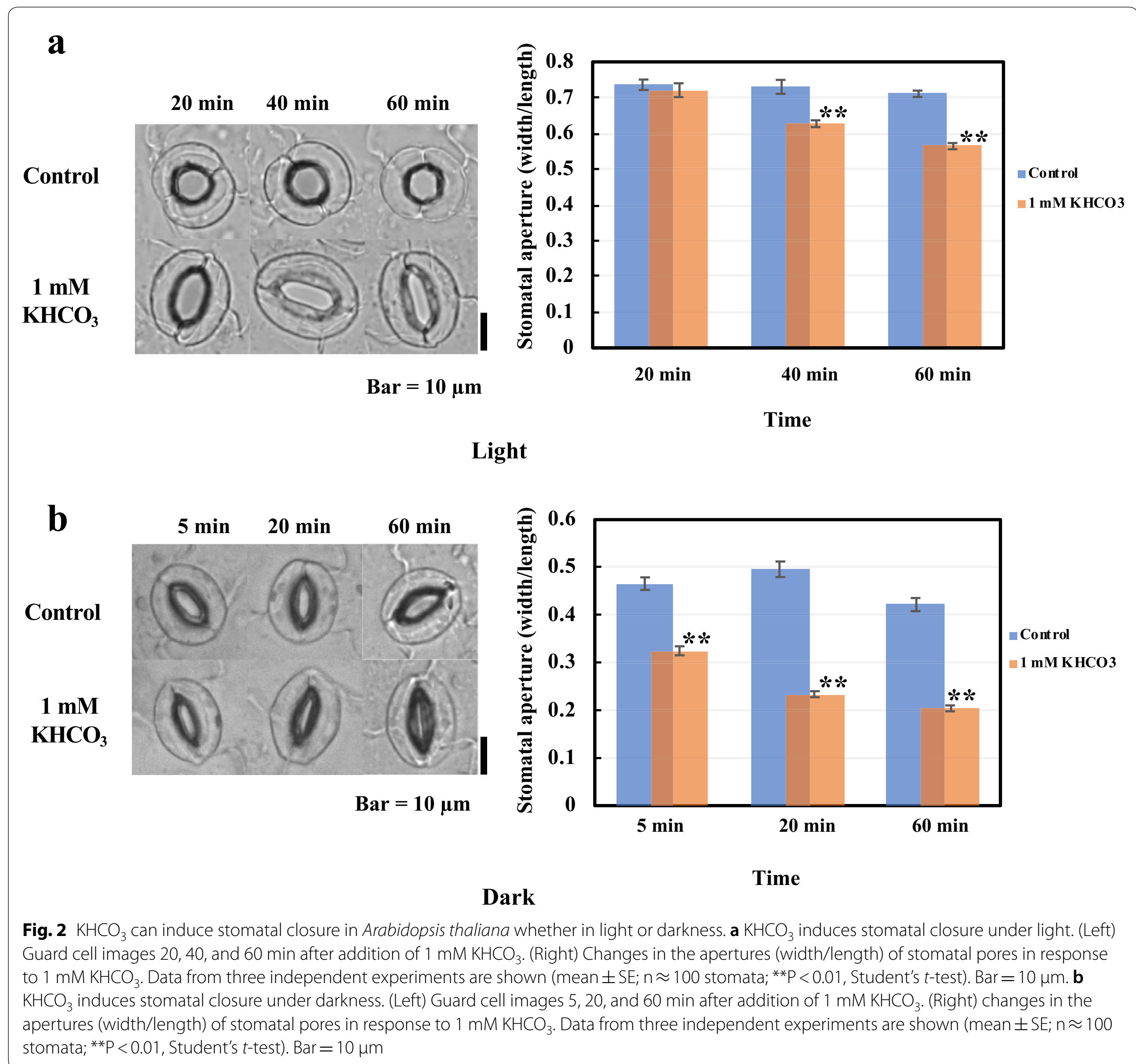


Fig. 2 $KHCO_3$ can induce stomatal closure in *Arabidopsis thaliana* whether in light or darkness. **a** $KHCO_3$ induces stomatal closure under light. (Left) Guard cell images 20, 40, and 60 min after addition of 1 mM $KHCO_3$. (Right) Changes in the apertures (width/length) of stomatal pores in response to 1 mM $KHCO_3$. Data from three independent experiments are shown (mean ± SE; n ≈ 100 stomata; **P < 0.01, Student's *t*-test). Bar = 10 µm. **b** $KHCO_3$ induces stomatal closure under darkness. (Left) Guard cell images 5, 20, and 60 min after addition of 1 mM $KHCO_3$. (Right) changes in the apertures (width/length) of stomatal pores in response to 1 mM $KHCO_3$. Data from three independent experiments are shown (mean ± SE; n ≈ 100 stomata; **P < 0.01, Student's *t*-test). Bar = 10 µm

method, two were selected for further analysis and named *mci1* (insensitive response) and *mcs1* (hypersensitive response).

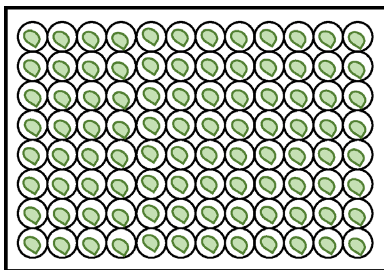
We first monitored the stomatal movement of *mci1* and *mcs1* in response to HCO_3^-/CO_2.The results clearly showed that 1 mM HCO_3^- could induce stomatal closure within 30 min in *mcs1* but not in wild type (Fig. 4a). For *mci1*, even 3 mM HCO_3^- could not induce stomatal closure after 1 h (Fig. 4b).

Consistent with the results of the screen, by comparing with wild type, AEQ luminescence intensities increased dramatically in *mcs1*, while no significant change was observed in *mci1* in response to HCO_3^- treatment (Fig. 4c). These results further suggest that the products of *MCS1* and *MCI1* participate in HCO_3^- signal transduction pathways regulating both $[Ca^{2+}]_{cys}$ and stomatal movement.

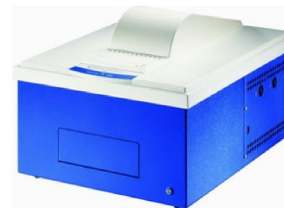
It is necessary to make sure that only a single gene locus functions in controlling a phenotype of interest before conducting gene mapping. After crossing each of the mutants with wild type, we analyzed the segregation of the F_2 progeny. Phenotypes of F_2 plants showed

Obtain leaves from 3-week-old plants

Place samples in 96-well white culture plate
(leaf + 100 μL of incubation buffer in each well)



Incubation in dark at RT for 4–6 h



Place the culture plate into automated microplate luminescence reader

Automatic injection of 100 μL of 2 mM $KHCO_3$ solution into each well to obtain final concentration of 1 mM.

Conduct luminescence assay using a luminescence reader

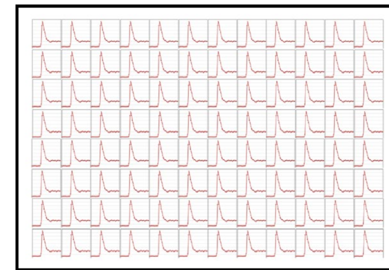
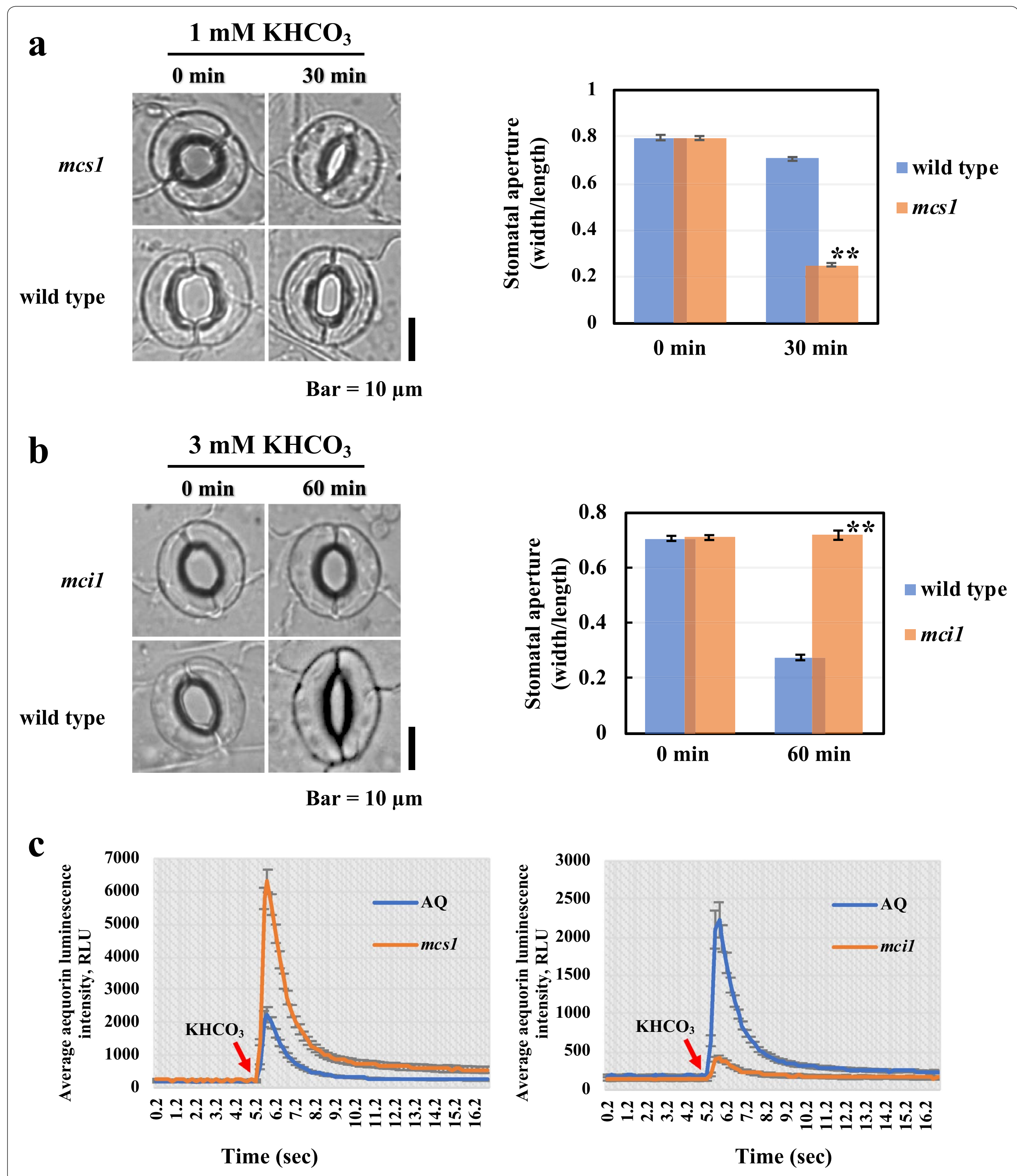


Fig. 3 High-throughput strategy for isolation of CO_2/HCO_3^- response mutants. Schematic of the screening strategy with 96-well culture plates. The leaves (red arrows) of 3-week-old AEQ-transgenic *Arabidopsis* were placed in a 96-well culture plate and 100 μL of incubation buffer was added to each well. Plates were incubated in the dark at 25 °C for 4 to 6 h. The wells were automatically injected with 100 μL of 2 mM $KHCO_3$ (to give a final concentration of 1 mM), and AEQ luminescence was recorded for each well

(See figure on next page.)
Fig. 4 *mcs1* and *mci1* exhibited abnormal responses to HCO_3^-/CO_2 with respect to $[Ca^{2+}]_{cys}$ changes and stomatal movement. **a** The *mcs1* mutant is hypersensitive to HCO_3^-/CO_2 treatment. (Left) Images of wild-type and *mcs1* epidermal strips were taken, and guard cell images before and 30 min after addition of 1 mM $KHCO_3$ are shown. (Right) Changes in the apertures (width:length) of stomatal pores in wild type and *mcs1* in response to 1 mM $KHCO_3$. Data from three independent experiments are shown (mean ± SE; n ≈ 100 stomata; $^{**}P < 0.01$, Student's *t*-test). Bar = 10 μm. **b** The *mci1* mutant is insensitive to HCO_3^-/CO_2 treatment. (Left) Images of wild-type and *mci1* epidermal strips were taken, and guard cell images before and 60 min after addition of 3 mM $KHCO_3$ are shown. (Right) Changes to the apertures (width/length) of stomatal pores in wild type and *mci1* in response to 3 mM $KHCO_3$. Data from three independent experiments are shown (mean ± SE; n ≈ 100 stomata; $^{**}P < 0.01$, Student's *t*-test). Bar = 10 μm. **c** *mcs1* (left) and *mci1* (right) exhibited abnormal AEQ luminescence intensities changes in response to 1 mM $KHCO_3$. Leaves were put individually into the wells of a 96-well plate, and luminescence values were recorded at intervals of 0.2 s after 1 mM $KHCO_3$ was added. Data for 59 leaves are shown (mean ± SE). Orange lines indicate mutants; blue lines indicate wild type (AQ). RLU, relative luminescence units



3:1 (wild-type:*mci1* or *mcs1*) segregation, suggesting that *mci1* or *mcs1* was resulted from a recessive mutation. These two mutants are appropriate for subsequent gene mapping work.

Together, these data demonstrated that the high-throughput methods developed in this study are valuable for identifying new calcium-related components in the

HCO_3^-/CO_2-mediated stomatal closure signaling network pathway.

Discussion

CO_2 influences both stomatal movement and stomatal development; however, the mechanisms of guard cell perception and transduction are still not fully clear, and the sensors that mediate CO_2-controlled stomatal movement remain enigmatic. Previous studies have suggested that intracellular bicarbonate acts as a second messenger in guard cells involved in mediating CO_2 signal transduction [19–21]. To date, a number of proteins with critical roles in this signaling pathway have been identified, such as CA1 and CA4, HT1, SLAC1, RHC1, and others.

Because Ca^{2+} is a key cellular second messenger, transient change in $[Ca^{2+}]_{cyt}$ reflects most physiology processes including CO_2-regulated guard cell behavior. GROWTH CONTROLLED BY ABSCISIC ACID 2 (GCA2) has been proved to function downstream of both CO_2 signaling and ABA signaling by regulating $[Ca^{2+}]_{cyt}$. *gca2* mutant plants display decreased sensitivity of stomata to elevated CO_2 and show an abnormal $[Ca^{2+}]_{cyt}$ pattern in guard cells [22]. This altered pattern of $[Ca^{2+}]_{cyt}$ in CO_2/HCO_3^--treated guard cells prompted us to design a screening method to identify genes implicated in $[Ca^{2+}]_{cyt}$ regulation during stomatal response to CO_2.

AEQ photoprotein has been extensively used in the Ca^{2+} signaling field for almost 40 years. Because it is convenient, fast, sensitive, easy to use, and applicable to real-time measurement of $[Ca^{2+}]_{cyt}$ changes, we chose an AEQ-based system for our genetic screen. According to our data showing that the CO_2/HCO_3^--induced increase of $[Ca^{2+}]_{cyt}$ happened no more than 1 s after CO_2/HCO_3^- application, almost climbed to the highest value, then dropped almost back to the baseline; the whole process only lasted for about 3 s (Fig. 1a, right), suggesting that the variation of $[Ca^{2+}]_{cyt}$ happened both early and rapidly in this physiological process. To identify components underlying this response, *Arabidopsis* mutants were usually isolated by analyzing their leaf temperature through thermal imaging. This traditional method was convenient and common, however thermal imaging takes hours to reach a steady state before detection, which may miss some important components that function earlier in the response to CO_2.

According to a previous report about detecting stomatal responses to bicarbonate, 1 mM $KHCO_3$ has been used for screening [2]. As shown in Figs. 1, 2, both significantly increased bioluminescence and remarkable stomatal closure can be detected at this concentration. Before the screen, a period of dark treatment for AEQ incubation is necessary, so we conducted another preliminary test because dark can influence guard cell status. We found that even in dark treatment, 1 mM $KHCO_3$ still can cause closure of the stomata (Fig. 2b), further suggesting the suitability of this screening method.

By using this AEQ-based method and treatment with 1 mM $KHCO_3$, we obtained *mci* and *mcs* mutants from about 35,000 M_2 seeds. We will continue to analyze these mutants and characterize the function of these genes. This series of experiments will shed light on the mechanism of calcium-mediated CO_2/HCO_3^- response in the guard cell, which appears to occur early during CO_2/HCO_3^--induced stomatal closure.

Conclusions

We have developed a sensitive method for isolating stomatal CO_2/HCO_3^- response genes that function early in the response and play a role in regulating $[Ca^{2+}]_{cyt}$ transient changes. This method will be helpful in elucidating the Ca^{2+}-dependent regulation of stomatal response.

Methods

Plant material and growth conditions

Lines of *Arabidopsis thaliana* ecotype *Col-0* constitutively expressing the intracellular Ca^{2+} indicator AEQ (pMAQ2; a gift from Marc R. Knight) or Cameleon (YC3.6; a gift from Simon Gilroy) were used. Plants homozygous for the AEQ-transgenic *Arabidopsis* plant were selected from the second generation after transformation (T1 plants). One such plant, expressing a high level of AEQ, was selected for subsequent experiments.

Plants were grown in soil or in medium containing Murashige and Skoog salts (MS; PhytoTechnology Laboratories), 3% (w/v) sucrose (Sigma), and 0.6% agar (Solarbio) in controlled environmental rooms at 20 ± 2 °C. The fluency rate of white light was ~ 80–100 μmol m^{-2} s^{-1}. The photoperiod was 16 h light/8 h dark. Seeds were sown on MS medium, placed at 4 °C for 3 days in the dark, and then transferred to growth rooms.

AEQ bioluminescence-based Ca^{2+} imaging

$[Ca^{2+}]_{cys}$ was measured using *Arabidopsis* plants expressing AEQ. Leaves (1 per well) were treated evenly with 150 μL of 10 μM coelenterazine (Sigma, C2230) in 96-well white culture plates 4 to 6 h before imaging and placed in the dark and in the glass chamber to remove CO_2. AEQ bioluminescence imaging was performed using a Berthold LB985 system equipped with a light-tight box and a cryogenically cooled, back-illuminated CCD camera. The recording of luminescence was started 30 s prior to treatment and lasted for 5 min. All the treatments were carried out in the dark, and the experiments were carried out at room temperature (22–24 °C).

Similarly, guard cells were used for AEQ bioluminescence imaging. Rosette leaf epidermal peels from 3- to 4-week-old plants were placed in a microwell chamber in incubation buffer for 4–6 h in the dark. AEQ bioluminescence imaging of guard cells was performed using a bioluminescence microscope (Sclis; Biocover) equipped with a light-tight box and a cryogenically cooled, back-illuminated CCD camera. The recording of luminescence was started 60 s prior to treatment and lasted for 5 min. Bright-field images were taken after AEQ imaging. All treatments were carried out in the dark, and the experiments were carried out at room temperature (22–24 °C).

Mutant screening

Arabidopsis seeds expressing AEQ were mutagenized with EMS as described previously [23]. Briefly, about 5000–10,000 seeds were imbibed overnight and then shaken in 0.3% EMS (v/v) for 15 h. The M_1 seeds were rinsed thoroughly with tap water, sterilized with 10% bleach for 30 min, and washed with sterilized water 5–8 times. M_2 seeds were harvested separately from individual M_1 plants. For screening, M_2 seeds were individually planted in soil and grown for 3 weeks. Leaves from M_2 plants were placed in a 96-well plate and 100 µL of freshly prepared incubation buffer was added to each well. Kinetic luminescence measurements were performed with an automated microplate luminescence reader (LB960; Berthold) every 0.2 s. After 3 s of luminescence counts, 100 µL of 2 mM $KHCO_3$ solution was automatically injected into each well to obtain a final concentration of 1 mM. Bioluminescence was recorded for 30 s per well.

Stomatal aperture bioassay

Leaves of 3- to 4-week-old seedlings were used in the stomatal aperture assays [24]. Leaves were detached before the light period started. For monitoring stomatal response to $KHCO_3$ in light or dark, whole leaves were incubated in stomatal buffer and then exposed to light (100 µmol m^{-2} s^{-1}) or dark for 2 h at 25 °C in the glass chamber.

The stomatal buffer contained 50 mM KCl, 0.1 mM $CaCl_2$, and 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), adjusted to pH 7.0 with Tris (hydroxymethyl) aminomethane (Tris) [25, 26]. Two hours later, the effects of 1 mM $KHCO_3$ on stomatal closure were tested. For characterization of stomatal response mutants, 3 mM $KHCO_3$ was also used. Prior to measuring the stomatal aperture, the adaxial epidermis and mesophyll layers were gently separated, and the epidermal strips were placed on microslides containing a drop of stomatal buffer with the desired concentration of $KHCO_3$ and covered with coverslips. Pictures of stomata were acquired using an inverted microscope (IX73; Olympus) at 40× magnification. Approximately 100 stomatal apertures from different leaves of each plant type were measured using Image J software (Broken Symmetry Software), and three independent experiments were performed.

Cameleon-based $[Ca^{2+}]_{cys}$ imaging in guard cells

The wild-type plants constitutively expressing GFP fluorescence resonance energy transfer (FRET)-based Ca^{2+} sensor YC3.6, and 10 homozygous lines were generated. Rosette leaf epidermal peels from 3-week-old plants were placed in a microwell chamber in the stomatal buffer for 2 h under light (100 µmol m^{-2} s^{-1}). Epidermal peels were treated with 1 mM $KHCO_3$ and ratiometric Ca^{2+} imaging was performed using a confocal microscope (LSM710; Zeiss) as described previously [27]. The YC3.6 Ca^{2+} sensor was excited with the 458 nm line of the argon laser. The cyan fluorescent protein (CFP; 473–505 nm) and FRET-dependent Venus (562–536 nm) emission were collected using a 458 nm primary dichroic mirror and the Meta detector of the microscope. Emission images (562–536 nm and 473–505 nm) of epidermal peels were taken, and ratiometric images before and 10 s after addition of 1 mM $KHCO_3$.

Identification of *MCI1* and *MCS1* by MutMap analysis

We backcrossed *mci1* or *mcs1* to AEQ-expressing *Col-0* and produced F_2 individuals. Plants with the *mci1* or *mcs1* phenotype were then subjected to MutMap analysis to find the mutated gene [28]. DNA of 30 F_2 progeny showing the mutant phenotype was isolated and then bulked using an equal amount of DNA from each plant. This bulked DNA was then subjected to MutMap analysis.

Abbreviations

CAs: Carbonic anhydrases; SLAC1: SLOW ANION CHANNEL-ASSOCIATED 1; PATROL1: A MUNC 13 ortholog in *Arabidopsis* controls the tethering of an H^+-ATPase; ALMT12: ALUMINUM-ACTIVATED MALATE TRANSPORTER 12; RHC1: RESISTANT TO HIGH CO_2; OST1: OPEN STOMATA 1; HT1: HIGH LEAF TEMPERATURE 1; ABA: Abscisic acid; $[Ca^{2+}]_{cyt}$: Free calcium ion concentration in cytosol; AEQ: Aequorin photoprotein; *Mci*: Mutant of HCO_3^-/CO_2 insensitive; *Mcs*: Mutant of HCO_3^-/CO_2 sensitive; EMS: Ethyl methane sulfonate; CAs: Carbonic anhydrases; GCA2: GROWTH CONTROLLED BY ABSCISIC ACID 2.

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Authors' contributions

XM, LB and C-PS designed the experiments. MT, MZ, KW, YH and ND performed the experiments. XZ analyzed the data. XM and LB wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The raw data from all experiments as well as the material used in this manuscript can be obtained from the corresponding authors upon reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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