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 \( \text{CO}_2 \) concentration, humidity, light, and pathogens.

A higher ambient \( \text{CO}_2 \) concentration increases leaf intercellular \( \text{CO}_2 \) concentration and mediates stomatal closure in plants, whereas a lower \( \text{CO}_2 \) concentration triggers stomatal opening. \( \text{CO}_2 \) 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 \( \text{CO}_2 \) concentration increases [1].

Despite the importance of stomatal responses to \( \text{CO}_2 \), little is known about the genetic and molecular mechanisms mediating stomatal development and movement in response to elevation in \( \text{CO}_2 \). \( \text{CO}_2 \) levels have been increasing steadily, and it is estimated that atmospheric \( \text{CO}_2 \) will reach 550 ppm in 2050 compared with 400 ppm presently [2], so it is increasingly urgent to discover
the underlying mechanisms of guard cell regulation in response to CO2 levels.

CO2 sensing in animals is mainly linked to α-carboxylic anhydrases (α-CAs) [3], which are also important for CO2 perception in fungi [3, 4]. Carbonic anhydrases (CAs) can accelerate the conversion of CO2 into HCO3− and H+, which in turn induce related responses. In plants, CO2 can also be converted into HCO3− and H+ by anhydrases [5]. The key question in understanding stomatal movement in response to CO2 is the mechanism for perception of changes in CO2 and/or HCO3− concentration. Despite the importance of anhydrase enzymes in CO2 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 CO2 in guard cells, whereas a ca1 ca4 double mutant exhibited insensitive stomatal movement response to CO2 [6]. Expression of a mammalian α-CA in the ca1 ca4 double mutant restored the stomatal response to CO2, implying that CA-mediated CO2 catalysis to HCO3− and H+ in guard cells is the key step for transmission of the CO2 signal [6].

Through isolation and analysis of genetic mutants, a number of proteins have been identified that function in CO2-controlled stomatal movement, including the SLAC1 anion channel [7, 8], the PATROL1 Munc 13-like protein [9], the AtALMT12/QUAC1 R-type anion channel [7, 8], and the PATROL1 Munc 13-like protein [9]. This characterization of these proteins has contributed to our understanding of the mechanisms of CO2-regulated guard cell behavior. For example, transporter protein RHC1 acts as a bicarbonate sensor, and the high-CO2-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 CO2 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-CO2-induced stomatal closure [13, 14]. But still, many points remain controversial, such as the mechanism underlying CO2 sensing; the identities of the CAs involved in this pathway; the function of CAs under low-CO2 conditions; and the interaction of CO2 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 hti, rhc1, and patrol1, were obtained using this method. Although this method has been effective in unraveling the regulation network in CO2-mediated stomatal movement, it is still not clear of this regulation network; thus, it is urgent to develop new screening methods.

Calcium ion (Ca2+) has been shown to act as a cellular second messenger in numerous plant processes. In Arabidopsis thaliana, abscisic acid (ABA), hydrogen peroxide, cold, and CO2 all can stimulate cytosolic Ca2+ ([Ca2+]c) oscillation, which causes stomatal closure [15]. CO2-induced stomatal closure is strongly Ca2+-dependent in Arabidopsis, consistent with previous findings in Commelina guard cells [16–18]. Cytosolic Ca2+ regulates stomatal closure by two mechanisms: short-term Ca2+-reactive closure and long-term Ca2+-programmed closure [15].

Extracellular CO2 induces changes of the [Ca2+]c in Arabidopsis guard cells. To further dissect this signaling pathway, new components in the CO2 response pathway that are related to the [Ca2+]c changes need to be identified. Here, we used a novel approach for screening genetic mutants to identify proteins involved in CO2 response. In this study, we used the Ca2+ reporter aequorin (AEQ) to record [Ca2+]c changes in Arabidopsis leaves in real time in order to visualize locally induced [Ca2+]c elevations in response to CO2 or HCO3− 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 CO2 or/and HCO3−. By using this system, we obtained several mci (mutant of HCO3−/CO2 insensitive) and mcs (mutant of HCO3−/CO2 sensitive) mutants. Further study with these mutants will be helpful for uncovering the mechanism for calcium-dependent CO2-regulated guard cell movement.

Results
[Ca2+]c changes induced by HCO3− 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 [Ca2+]c changes induced by HCO3−. As it is already known that the pH of incubation buffer (50 mM KCl, 0.1 mM CaCl2, 10 mM 2-[(N-morpholino) ethanesulfonic acid (MES) and 10 μM coelenterazine) cannot be stabilized at 7.0 when the concentration of KHCO3 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 KHCO3 and after 5 min, dramatic increases in [Ca2+]c were detected in the leaves by analyzing the AEQ luminescence image (Fig. 1a, left). The average
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 KHCO₃ 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 KHCO₃ is effective for checking the cytosolic calcium changes after treatment with incubation buffer. However, the stomata closed 5 min after 1 mM KHCO₃ treatment, and 60 min later, the 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 HCO₃⁻-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 [Ca²⁺]ₜقات transient change at 1 mM KHCO₃.

### High-throughput screening for CO₂/HCO₃⁻ 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. M₂ seeds were collected individually and screened as described in Fig. 3. The leaves of 3-week-old M₂ 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 leaf treatment with 1 mM KHCO₃ were then identified by using a luminescence reader (LB960, Berthold) (Fig. 3). So far, approximately 35,000 M₁ 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 KHCO₃ to narrow down the target mutants. HCO₃⁻/CO₂-induced stomatal closure of the putative mutants was assayed in M₂ and again in M₃, 6 out of 80 putative mutants with lower luminescence showed an insensitive stomatal response to HCO₃⁻/CO₂, 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 HCO₃⁻/CO₂ response mutants that appeared normal in both [Ca²⁺]ₜقات and stomatal movement. To exam the stability of the mutants obtained by this

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**Fig. 1** HCO₃⁻-induced [Ca²⁺]ₜقات increase in Arabidopsis leaves and guard cells. a HCO₃⁻-induced [Ca²⁺]ₜقات increase in Arabidopsis leaves. (Left) AEQ-transgenic Arabidopsis leaves were treated with 1 mM KHCO₃ and analyzed by AEQ imaging at 0 and 5 min. (Right) Time-course analysis of [Ca²⁺]ₜقات increases in guard cells. The YC3.6 transgenic plants were treated with 0.3% (w/v) ethyl methane sulfonate (EMS) and sown on soil. M₂ seeds were collected individually and screened as described in Fig. 3. The leaves of 3-week-old M₂ 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 leaf treatment with 1 mM KHCO₃ were then identified by using a luminescence reader (LB960, Berthold) (Fig. 3). So far, approximately 35,000 M₁ plants have been screened, and about 120 sensitive and 80 insensitive putative mutants have been identified.
a) 1 mM KHCO₃

0 min

5 min

Bar = 5 mm

Average aequorin luminescence intensity, RLU

Average aequorin luminescence intensity, cps

[Ca²⁺]₁ₑᵧₜ

Control
1 mM KHCO₃
1 mM KCl

Time (sec)

b) 0 min 1 min

1 mM KHCO₃

Bar = 20 μm

Average aequorin luminescence intensity, cps

[Ca²⁺]₁ₑᵧₜ

1 mM KHCO₃

Time (min)

0 1 2 3 4 5 6 7 8 9

0.6 1.2 1.8 2.4 3.0 3.6 4.2 4.8 5.4 6.0 6.6 7.2 7.8 8.4 9.0 9.6 10.2

1400 1200 1000 800 600 400 200 0

Control
1 mM KHCO₃
1 mM KCl

c) 0 min 1 min

1 mM KHCO₃

Bar = 10 μm

Average aequorin luminescence intensity, cps

[Ca²⁺]₁ₑᵧₜ
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 HCO3\(^-\)/CO2. The results clearly showed that 1 mM HCO3\(^-\) could induce stomatal closure within 30 min in mcs1 but not in wild type (Fig. 4a). For mci1, even 3 mM HCO3\(^-\) 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 HCO3\(^-\) treatment (Fig. 4c). These results further suggest that the products of MCS1 and MCI1 participate in HCO3\(^-\) signal transduction pathways regulating both [Ca\(^{2+}\)]\(_{cyt}\) 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

Conduct luminescence assay using a luminescence reader

Automatic injection of 100 µL of 2 mM KHCO₃ solution into each well to obtain final concentration of 1 mM.

Place the culture plate into automated microplate luminescence reader

Fig. 3 High-throughput strategy for isolation of CO₂/HCO₃⁻ 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 KHCO₃ (to give a final concentration of 1 mM), and AEQ luminescence was recorded for each well.

Fig. 4 mcs1 and mci1 exhibited abnormal responses to HCO₃⁻/CO₂ treatment. (a) The mcs1 mutant is hypersensitive to HCO₃⁻/CO₂ 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 KHCO₃ are shown. (Right) Changes in the apertures (width:length) of stomatal pores in wild type and mcs1 in response to 1 mM KHCO₃. 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 HCO₃⁻/CO₂ 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 KHCO₃ are shown. (Right) Changes to the apertures (width/length) of stomatal pores in wild type and mci1 in response to 3 mM KHCO₃. 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 KHCO₃. 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 KHCO₃ 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 \textit{mci1} or \textit{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 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ reflects most physiology processes including CO$_2$-regulated guard cell behavior. GROWTH CONTROLLED BY ABOYDIC ACID 2 (GCA2) has been proved to function downstream of both CO$_2$ signaling and ABA signaling by regulating $[\text{Ca}^{2+}]_{\text{cyt}}$. gca2 mutant plants display decreased sensitivity of stomata to elevated CO$_2$ and show an abnormal $[\text{Ca}^{2+}]_{\text{cyt}}$ pattern in guard cells [22]. This altered pattern of $[\text{Ca}^{2+}]_{\text{cyt}}$ in CO$_2$/HCO$_3^-$-treated guard cells prompted us to design a screening method to identify genes implicated in $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ 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

$[\text{Ca}^{2+}]_{\text{cyt}}$ 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 (Scilis; 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 M₁ seeds were rinsed thoroughly with tap water, sterilized with 10% bleach for 30 min, and washed with sterilized water 5–8 times. M₂ seeds were harvested separately from individual M₁ plants. For screening, M₂ seeds were individually planted in soil and grown for 3 weeks. Leaves from M₂ 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 KHCO₃ 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 KHCO₃ in light or dark, whole leaves 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 KHCO₃ and ratiometric Ca²⁺ imaging was performed using a confocal microscope (LSM710; Zeiss) as described previously [27]. The YC3.6 Ca²⁺ 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 KHCO₃.

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

We backcrossed mci1 or mcs1 to AEQ-expressing Col-0 and produced F₂ individuals. Plants with the mci1 or mcs1 phenotype were then subjected to MutMap analysis to find the mutated gene [28]. DNA of 30 F₂ 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 CO₂; OST1: OPEN STOMATA 1; HT1: HIGH LEAF TEMPERATURE 1; ABA: Abscisic acid; [Ca²⁺]cyt: Free calcium ion concentration in cytosol; AEQ: Aequorin photoprotein; Mci: Mutant of HCO₃⁻/CO₂ insensitive; MsC: Mutant of HCO₃⁻/CO₂ sensitive; EMS: Ethyl methane sulfonate; CAS: Carbonic anhydrases; GCA2: GROWTH CONTROLLED BY ABSICIC ACID 2.

**Acknowledgements**

We thank Marc R. Knight of Durham University (UK) for the kind gift of pMAQ2 and Simon Gilroy of the University of Wisconsin, Madison for the generous gift of the YC3.6 vector.

**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.
Funding
This work was supported by the National Natural Science Foundation of China (31900239 and 31570287) and the Henan Education Department Key Foundation (19A180012).

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
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

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

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Received: 15 September 2019 Accepted: 16 April 2020
Published online: 29 April 2020

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