The BIG protein distinguishes the process of CO2-induced stomatal closure from the inhibition of stomatal opening by CO2.
Title: The BIG protein distinguishes the process of CO$_2$-induced stomatal closure from the inhibition of stomatal opening by CO$_2$.

Jingjing He$^{1,5}$, Ruo-Xi Zhang$^{1,5}$, Kai Peng$^2$, Cecilia Tagliavia$^3$, Siwen Li$^1$, Shaowu Xue$^5$, Amy Liu$^3$, Honghong Hu$^{4,5}$, Jingbo Zhang$^3$, Katherine E Hubbard$^{5,6}$, Katrin Held$^7$, Martin R McAinsh$^3$, Julie E Gray$^8$, Jörg Kudla$^7$, Julian I Schroeder$^5$, Yun-Kuan Liang$^1$* and Alistair M Hetherington$^2$*

$^1$State Key Laboratory of Hybrid Rice, Department of Plant Sciences, College of Life Sciences, Wuhan University, Wuhan 430072, China

$^2$School of Biological Sciences, Life Sciences Building, 24 Tyndall Avenue, Bristol BS8 1TQ, UK

$^3$Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK

$^4$College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

$^5$Cell and Developmental Biology Section, Division of Biological Sciences, University of California at San Diego, La Jolla, California 92093, USA

$^6$School of Environmental Sciences, University of Hull, HU6 7RX, UK.

$^7$Institut für Biologie und Biotechnologie der Pflanzen, Universität Münster, Schlossplatz 7, Münster 48149, Germany

$^8$Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, UK

$^*$These authors contributed equally to this work.

* Authors for correspondence:

Yun-Kuan Liang (ORCID ID 0000-0001-5869-5931), Tel. +86 27 68752363

Email: ykliang@whu.edu.cn

Alistair M Hetherington (ORCID ID 0000-0001-6060-9203), Tel. +44 117 3941188

Email: Alistair.Hetherington@bristol.ac.uk
Total word count:

Title: 18

Summary: 194

Main text (Introduction, Materials and Methods, Results, Discussion, and Acknowledgements): 4672

Introduction: 915

Results: 1436

Discussion: 1051

Materials and Methods: 1144

Acknowledgements: 124

Number of Figures: 5

Supplemental Figures: 3

Supplemental Text: 1
Summary

- We conducted an infrared thermal imaging-based genetic screen to identify Arabidopsis mutants displaying aberrant stomatal behavior in response to elevated concentrations of CO$_2$.

- This approach resulted in the isolation of a novel allele of the Arabidopsis BIG locus (At3g02260) that we have called cis1 (for CO$_2$ insensitive 1).

- BIG mutants are compromised in elevated CO$_2$-induced stomatal closure and bicarbonate activation of S-type anion channel currents. In contrast to wild type they fail to exhibit reductions in stomatal density and index when grown in elevated CO$_2$. However, like wild type, BIG mutants display inhibition of stomatal opening when exposed to elevated CO$_2$. BIG mutants also display wild type stomatal aperture responses to the closure-inducing stimulus ABA.

- Our results indicate that BIG is a signaling component involved in the elevated CO$_2$-mediated control of stomatal development. In the control of stomatal aperture by CO$_2$, BIG is only required in elevated CO$_2$-induced closure and not in the inhibition of stomatal by this environmental signal. These data show that, at the molecular level, the CO$_2$ mediated inhibition of opening and promotion of stomatal closure signalling pathways are separable and BIG represents a distinguishing element in these two CO$_2$-mediated responses.

**Key words:** Abscisic acid, BIG gene, CO$_2$ signalling, Stomatal function, S-type anion channel
**Introduction**

Stomata consist of a pair of guard cells that surround a central pore and serve to regulate water loss and the uptake of CO$_2$. Both the aperture of the stomatal pore and the number of stomata that develop on the leaf surface are controlled by environmental signals. By integrating external signals and local cues stomata “set” gas exchange to suit the prevailing environmental conditions (Hetherington & Woodward, 2003). One of the signals that controls stomatal aperture and influences stomatal development, in both the short and long term, is the atmospheric concentration of carbon dioxide ([CO$_2$]) (Kim *et al.*, 2010; Franks *et al.*, 2012). In response to an increase in [CO$_2$] stomatal aperture reduces, as in general, do the number of stomata that develop on the surface of leaves (Vavasseur & Raghavendra, 2005; Kim *et al.*, 2010; Franks *et al.*, 2012). Understanding how the plant perceives changes in [CO$_2$] and integrates this information with other internal and external signals, resulting in the adjustments of stomatal aperture and density is of key importance in the context of understanding the impact of global environment change on plants (Assmann & Jergla, 2016).

Recently we have begun to understand more about the underlying cellular mechanisms responsible for coupling increased [CO$_2$] to reduced stomatal conductance (Kim *et al.*, 2010; Assmann & Jergla, 2016; Engineer *et al.*, 2016). In this context it is important to recognize that elevated CO$_2$-induced reductions in stomatal conductance are the net result of two processes: these are the promotion of stomatal closure and the inhibition of stomatal opening (Assmann, 1993). These processes are separable; ABA induced-stomatal closure is distinct from ABA inhibited-stomatal opening (Allen *et al.*, 1999; Wang *et al.*, 2001; Mishra *et al.*, 2006). However, prior to the current work it was not known whether this also applied to [CO$_2$]-induced changes in stomatal aperture.
There is evidence that the guard cell ABA and CO$_2$ signaling responsible for the inhibition of light-induced stomatal opening pathways converge (Webb & Hetherington, 1997). It has been suggested that elevated [CO$_2$] brings about its effects on stomatal aperture and development by accessing the ABA signaling pathway because there is a requirement for both ABA and the ABA receptors of the PYR/RCAR family in these responses (Chater et al., 2015). There are other data suggesting that the early steps in CO$_2$-mediated closure converge with ABA signaling downstream of ABA receptors and the two pathways influence each other upon convergence (Xue et al., 2011; Merilo et al., 2013; Horak et al., 2016; Jakobson et al., 2016; Yamamoto et al., 2016). Obviously, these processes are not mutually exclusive. Although the mechanism(s) through which the guard cell ABA signaling pathway is accessed is not fully understood, it has been possible to distinguish, on a genetic basis, components that function in CO$_2$ mediated closure but not in guard cell ABA signaling. In *Arabidopsis* these include β-carboxy anhydrases which are encoded by the CA1 and CA4 genes (Hu et al., 2010), the protein kinase HT1 (HIGH LEAF TEMPERATURE 1) (Hashimoto et al., 2006), RHC1, a MATE transporter (Tian et al., 2015) and the MAP kinase MPK4 (Horak et al., 2016; Jakobson et al., 2016). Loss of the CAs, RHC1, MPK4 impairs CO$_2$-induced closure (Hashimoto et al., 2006; Hu et al., 2010; Tian et al., 2015; Horak et al., 2016; Jacobsen et al., 2016) whereas recessive *ht1* alleles show a constitutive high CO$_2$ response (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016).

In 1987, Woodward discovered an inverse relationship between atmospheric [CO$_2$] and stomatal density (Woodward, 1987). We know less about the operation of this developmental signaling pathway, however, the putative β-keto acyl CoA synthase encoded by the *HIC* gene is involved as are the CO$_2$ Response Secreted Protease (CRSP), the β-carboxy anhydrases CA1 and CA4 and the peptide, Epidermal Patterning Factor 2 (EPF2) (Gray et al., 2000; Doheny-Adams et al., 2012; Engineer
et al., 2014). Most recently, it has been shown that the activity of the ROS producing NADPH oxidases encoded by the *RBOHD* and *RBOHF* genes are involved in the CO$_2$-mediated reduction in stomatal density as is ABA and the ABA receptors encoded by the PYR/RCAR family (Chater et al., 2015).

During an infrared thermal imaging genetic screen in Arabidopsis (Wang et al., 2004) we isolated a novel allele of the *BIG* locus (*At3g02260*) that we name *cis1* (for CO$_2$ insensitive 1) that is compromised in both elevated [CO$_2$]-induced closure and reduction in stomatal density. However, when challenged with ABA *cis1* displays reductions in stomatal aperture that are indistinguishable from WT suggesting that *BIG* (CIS1) functions upstream of ABA or in an ABA-independent signaling pathway responsible for the control of stomatal aperture by CO$_2$. We also found that activation of the guard cell S-type anion channel by bicarbonate is compromised by the loss of *BIG* function. Furthermore, in contrast to elevated [CO$_2$]-mediated closure, the ability of elevated [CO$_2$] to inhibit stomatal opening was not affected in this mutant. In summary, we have identified BIG as a new component in the signaling pathway responsible for the control of stomatal development by elevated [CO$_2$]. We also show that *BIG* also features in the signaling pathway through which elevated [CO$_2$] controls stomatal aperture. Importantly, we show that BIG is only involved in elevated [CO$_2$]-induced stomatal closure and is not involved in the inhibition of stomatal opening by this environmental signal or in stomatal responses to ABA. These results show that, at the molecular level, these pathways are separable, with BIG representing a component that distinguishes these two CO$_2$-mediated responses.

**Results**

The *cis1* mutant is involved in the response of stomatal conductance to elevated CO$_2$. 

---

Manuscript submitted to New Phytologist for review
To understand the underlying cellular basis of the effect of elevated CO$_2$ on stomatal development and function we carried out a forward genetic screen using infrared thermography. We reasoned that mutants failing to exhibit reductions in aperture, in this case induced by exposure to elevated [CO$_2$], would be visible because they would exhibit reduced leaf temperature due to increased leaf evapotranspiration relative to WT (Darwin, 1904). Infrared thermography has been used previously to isolate mutants carrying lesions in stomatal responses to ABA (Raskin & Ladyman 1988; Merlot et al., 2002), reduced atmospheric relative humidity (Xie et al., 2006; Liang et al., 2010) and CO$_2$ (Hashimoto et al., 2006; Negi et al., 2008). Using this approach we screened M2 plants from an EMS-mutagenized population of Arabidopsis and identified cis1 (for CO$_2$ insensitive I) that displayed significantly lower leaf surface temperature (0.68 °C) relative to WT when challenged for 40 mins with 1,500 ppm [CO$_2$] (Fig. 1a,b). Genetic analysis revealed that this phenotype was caused by a single recessive Mendelian mutation (data not shown). To investigate the lesion in the cis1 mutant further, we measured stomatal conductance ($g_{s}$). Fig. 1c and d show that in WT, challenge with 800 ppm CO$_2$ results in a reduction in $g_{s}$, whereas the response was attenuated in cis1. In contrast both cis1 and WT displayed an increase in $g_{s}$ when exposed to low (100 ppm) CO$_2$. We confirmed this response in big-1, a second independent allele of cis1 (Supplemental Fig. S1). These data suggest that the cis1 mutant is compromised in the stomatal response to elevated [CO$_2$].

**Identification of the CIS1 gene locus**

We performed map-based gene cloning to identify the CIS1 locus, and mapped the mutation to a 107kb region of chromosome III close to the doc1 mutations (data not shown; Gil et al., 2001). Seeds for T-DNA insertion lines of all annotated genes within this region were obtained from NASC and screened using infrared thermal imaging. A T-DNA insertion line (SALK_105495) of At3g02260 was identified that displayed similar thermal behavior to the cis1 mutant. Sequencing of cis1 revealed a
single point mutation (G to A substitution) in locus $At3g02260$ localized at a splicing acceptor site at position +8,542 (GT...AG to GT...AA) (Fig. 2a) which resulted in alternative spliced mRNAs as shown in Fig. S2. Real-time quantitative (Q) PCR, revealed that compared with WT, $cis1$ ($At3g02260$) gene transcript abundance was reduced to a third (Fig. 2b).

$At3g02260$ has previously been named $BIG$ and is annotated as encoding a large protein of 5098 amino acids, containing multiple conserved functional domains including three putative Zn-finger domains (Kanyuka et al., 2003; Kasajima et al., 2007). Our sequencing revealed that the original annotation is incorrect, as the open reading frame of $BIG$ is 63bp shorter than predicted, because 30bp of the sequence of intron 1, 21 bp of intron 5 and 12bp of intron 7 had been annotated as part of the respective neighboring exons. Hence, the $BIG$ ORF is 15,234bp long encoding a putative 5,077-amino-acid peptide as predicted by Gil and coworkers (Gil et al., 2001).

Many alleles of $big$ mutants e.g. ga6, tir3, doc1, asr1, lpr1, elk1, asa1, umb1, crm1 and rao3 have been independently isolated. All mutants are characterized by deficient organ elongation (dwarfism) and have altered root architecture, reduced apical dominance, defects in light responses, aberrant auxin transport. They also show altered sensitivities to GA, cytokinin, ethylene, low phosphate and water withholding treatments (Li et al., 1994; Ruegger et al., 1997; Sponsel et al., 1997; Gil et al., 2001; Lease et al., 2001; Kanyuka et al., 2003; López-Bucio et al., 2005; Kasajima et al., 2007; Yamaguchi et al., 2007; Ivanova et al., 2014). Interestingly, insects and mammals possess homologs of the BIG protein and these are involved in signaling. Calossin/Pushover in $Drosophila melanogaster$ and mammalian p600/UBR4 are homologs of BIG, both of which have CaM-binding domain and are likely involved in $Ca^{2+}$ signaling (Xu et al., 1998; Parsons et al., 2015).
To confirm the identity of cis1 we obtained two additional mutant alleles of BIG. doc1-I was originally isolated in a genetic screen for components of light signaling and harbours a single base change from G to A at position +5,514 (Fig. 2a) resulting in change from a conserved Cys residue change to Tyr. This missense BIG mutation perturbs auxin transport and plant growth (Gil et al., 2001) but in our Q-PCR, analysis no change to the transcript abundance of BIG was detected (Fig. 2b). big-1 harbours a T-DNA insertion in exon 9 before position +13,617 of the BIG gene (Kasajima et al., 2007) (Fig. 2a). We detected no BIG transcript in this mutant by Q-PCR (Fig. 2b).

**BIG is also involved in the control of stomatal development by elevated CO₂.**

The data in Fig. 3a show that stomatal and epidermal pavement cell densities are greater in the BIG mutant alleles than WT (P≤0.001). This reflects the fact that both guard cells and epidermal cells were significantly smaller than WT (data not shown). Stomatal development is controlled by CO₂, with stomatal density and index typically reduced in plants grown under elevated [CO₂] (Woodward, 1987; Woodward & Kelly, 1995). We next investigated whether BIG has a role to play in the control of stomatal development by elevated [CO₂]. In WT growth at elevated [CO₂] resulted in a decrease in stomatal density and index (Fig. 3b,c). In marked contrast, under the same conditions, growth at elevated [CO₂] resulted in significant increases in both stomatal density and index in the BIG mutants (Fig. 3b,c). These data suggest that, in addition to controlling stomatal aperture, BIG is also required for the reduction in stomatal density and index caused by higher than ambient [CO₂].

**The BIG protein is involved in the signaling pathway by which elevated [CO₂] induces stomatal closure but not in the pathway through which elevated [CO₂] inhibits stomatal opening.**

The results from the gas exchange experiments (Fig. 1c,d) prompted us to make direct measurements of stomatal responsiveness by quantifying changes in stomatal aperture.
(Chater et al., 2015). Fig. 4a shows that in contrast to WT the stomata of *cis1*, *big-1* and *doc1-1* mutants failed to close when subjected to 700 ppm CO$_2$. These data indicate that BIG is required for elevated CO$_2$-induced stomatal closure. Elevated [CO$_2$] is also known to inhibit light-induced stomatal opening (Mansfield et al., 1990).

In contrast to CO$_2$-induced stomatal closure, the inhibition of light-induced stomatal opening of the BIG mutants was similar to WT (Fig. 4b). The specific role of the BIG gene in the pathway by which elevated [CO$_2$] brings about stomatal closure is highlighted by our observation that the series of allelic mutants all display WT behavior in response to ABA. This holds for both ABA-induced stomatal closure and the inhibition by ABA of light-induced stomatal opening (Fig. 4c,d). The intact stomatal ABA response as well as the impaired CO$_2$ response were both observed in more than one of our laboratories underlining the robustness of the CO$_2$ specificity of the stomatal phenotype in *big* mutant alleles.

**BIG is required for activation of S-type anion channels by elevated bicarbonates.**

S-type anion channels are recognized as one of the main players in guard cell signaling. They mediate the release of anions from guard cells and promote stomatal closure in response to diverse stimuli, including increased [CO$_2$] (Kollist et al., 2011; Wang et al., 2016). An increase in the cytoplasmic bicarbonate concentration activates S-type anion channels in guard cells and correlates with elevated [CO$_2$]-induced stomatal closure in diverse mutant backgrounds (Vahisalu et al., 2008; Xue et al., 2011; Merilo et al., 2013). To understand the role of BIG in guard cell signaling further we investigated whether the activation of S-type anion channels by applied bicarbonate was impaired by mutations in BIG. In WT guard cell protoplasts, large anion currents were recorded when the pipette solution contained 11.5 mM free bicarbonate (Fig. 5b). However, in guard cell protoplasts of the *doc1-1* and *big-1* mutant alleles, currents were activated by the same concentration of bicarbonate in the pipette solution (Fig. 5e,h). At a voltage of -145 mV, the average activated currents...
were $-39.7 \pm 4.6$ pA for WT (Fig. 5c), $-20.0 \pm 2.0$ pA for doc1-l mutant (Fig. 5f), $-16.8 \pm 1.8$ for big-l mutant (Fig. 5i). The differences between WT and each mutant allele of BIG were statistically significant ($P \leq 0.01$). These results demonstrate that the BIG protein is required for elevated intracellular bicarbonate-induced activation of guard-cell plasma-membrane S-type anion channel currents that function in CO$_2$-induced stomatal closure and further reinforce the importance of BIG in stomatal closure.

Discussion

BIG is involved in stomatal closure induced by elevated CO$_2$ but not in elevated CO$_2$-induced inhibition of stomatal opening.

We conducted a genetic screen that resulted in the identification of a novel allele of the BIG gene that we call CIS1 that plays a regulatory role in stomatal function and development. Our phenotypic analyses revealed that CIS1 is involved in the reduction in stomatal conductance induced by elevated CO$_2$ (Fig. 1b,c; Fig. S1). On the surface of a leaf, during the day, stomata are exposed to frequently conflicting signals from the environment. Guard cells integrate these signals and the overall result is the optimization of gas exchange under the prevailing environmental conditions. Looking at this more closely, in the case of stomatal closure it is necessary to stimulate the processes associated with the loss of guard cell turgor while simultaneously inhibiting the cellular reactions involved in solute accumulation and stomatal opening. The opening and closure responses are physiologically distinct and are not the reverse of each other (Assmann, 1993; Li et al., 2000). When we investigated the role of BIG in these processes we found, intriguingly, that it was only involved in elevated CO$_2$-induced stomatal closure. In marked contrast all of the BIG mutants exhibited WT behavior in our CO$_2$-inhibition of light-stimulated stomatal opening bioassay (Fig. 4a,b). To extend our investigation of the role of BIG in the regulation of stomatal aperture we also investigated whether it played a role in stomatal closure induced by
ABA. The data in Fig. 4 (c,d) clearly indicate that BIG is neither involved in ABA-promoted closure nor in ABA-inhibited light-induced opening. Because BIG encodes a protein that, in guard cells, is only involved in CO$_2$-induced closure and not CO$_2$-inhibited opening, this makes it possible at the molecular level to distinguish, and to start to define these different processes. In this sense these data fit well with the observation that in molecular terms ABA-induced stomatal closure is distinct from the inhibition of opening by ABA. Examples include GPA1, which is involved in ABA-inhibition of opening but not in closure (Wang et al., 2001), a sphingosine-1-phosphate phosphatase, long-chain base phosphate lyase double mutant (sppasedpl1) that displays WT behavior during ABA-induced closure but is slightly impaired in the ABA inhibition of stomatal opening response (Worrall et al., 2008), PI-phospholipase C which is involved in the ABA-inhibition of opening but not closure (Mills et al., 2004) and the observation that some members of PYR/PYL ABA receptor family involved in stomatal opening inhibition are different from those involved in stomatal closure induction (Yin et al., 2013). The second striking result to emerge from these experiments is that BIG is not involved in ABA-induced reductions in stomatal aperture (Fig. 4c,d). This suggests that the BIG protein lies upstream of the point of convergence of the guard cell CO$_2$ and ABA signaling pathways (Webb & Hetherington, 1997; Xue et al., 2011; Merilo et al., 2013; Chater et al., 2015; Jakobson et al., 2016; Yamamoto et al., 2016). Looking downstream of the point of convergence it is well known that both ABA- and CO$_2$-induced stomatal closure involve activation of slow anion channels (Kim et al., 2010; Assmann & Jergla, 2016; Engineer et al., 2016). Our data reveal that mutations in BIG depressed the activation of S-type anion channels by bicarbonate (Fig. 5) in line with the impaired elevated [CO$_2$]-induced stomatal closure. A recent study by Yamamoto and coworkers provided evidence that different parts of SLAC1 are separately responsible for sensing ABA and CO$_2$ signals (Yamamoto et al., 2016). It is the transmembrane domain of SLAC1 channels that perceives CO$_2$ signals in contrast to the N- and C terminal ends.
of SLAC1 which are responsible for ABA signaling in *Arabidopsis* (Brandt et al., 2015; Yamamoto et al., 2016). Further investigation is needed to determine whether the activation of S-type anion channels by ABA is affected by the loss of BIG gene function.

**BIG is also involved in the control of stomatal development by elevated CO$_2$.**

Fig. 3a shows that mutations in BIG result in significant increases in guard and epidermal pavement cell densities consistent with the findings of Guo et al. (2013). Growth at elevated [CO$_2$] typically results in a reduction in stomatal index and density (Hetherington & Woodward, 2003; Assmann & Jergla, 2016; Engineer et al., 2016). The results in Fig. 3b and c clearly show that, in marked contrast to WT, stomatal indices and density of BIG mutants increased when the plants were grown at 1,000 ppm CO$_2$. It is likely, as with *βca1ca4, epf2* and *hic* mutants (Gray et al., 2000; Engineer et al., 2014), that loss of BIG function relieves the elevated [CO$_2$]-mediated repression of stomatal development. How might BIG bring about an effect on CO$_2$-mediated stomatal development? One possibility that would merit future investigation is that this is an auxin-related response. The BIG gene has been reported to encode a protein associated with auxin transport (Gil et al., 2001; Kanyuka et al., 2003) and is specifically required in the process by which auxin inhibits endocytosis and promotes its own efflux from cells (Paciorek et al., 2005). In this context it is worth noting that evidence is emerging that auxin inhibits stomatal development. Mutants disrupted in the TAA1/TAR auxin biosynthesis pathway or polar auxin transport and strong perturbations in auxin signaling, as observed in multiple *tir1/afb* auxin receptor mutants, cause stomatal clustering (Balcerowicz et al., 2014; Le et al., 2014; Zhang et al., 2014). However, we observed no stomatal clustering in the *cis1* and related mutants. Further work will be required to reveal whether disruptions to auxin signaling underlie the BIG stomatal mutant phenotype.
In conclusion, we demonstrate that, in *Arabidopsis*, the BIG protein is involved in both the elevated [CO$_2$]-mediated control of stomatal closure and density. Our results reveal we have identified a component that is involved in the signaling pathway by which elevated CO$_2$ promotes stomatal closure. However, BIG is neither involved in the elevated [CO$_2$]-mediated inhibition of light induced opening nor stomatal closure initiated by ABA. These data indicate that elevated [CO$_2$]-mediated closure and inhibition of opening are, in molecular terms, distinguishable. Our data suggest that BIG lies upstream of the point of convergence of ABA and CO$_2$ or resides in an, as yet undefined parallel signaling pathway that converges at or above the SLAC1 ion channel.

**Materials and Methods**

**Plant Growth**

All *Arabidopsis thaliana* lines used were in the Columbia background (Col-0). Seeds of *doc1-1* and *big-1* were obtained from NASC (the European Arabidopsis Stock Centre, http://arabidopsis.org.uk). Seed germination and plant growth were as described previously (Liang *et al.*, 2010).

**Mutant screen**

To identify genes required for stomatal CO$_2$ responses we screened 20,000 seeds from an Arabidopsis EMS M2 population representing 40 independent pools (each pool corresponding to approximately 1,000 M1 plants) by infrared thermal imaging (Wang *et al.*, 2004; Xie *et al.*, 2006). Screening was carried out on 3-4 weeks old plants in a purpose-built chamber (84 x 68 x 20 cm), located inside a controlled environment room. CO$_2$ concentration inside the chamber was controlled externally from CO$_2$ cylinders. Air flow in the chamber was maintained at 0.03 m sec$^{-1}$ using fans. Relative humidity inside the chamber was about 60%, temperature was 22°C and light intensity 120 µmol m$^{-2}$ s$^{-1}$. Plants were placed in the chamber and exposed to 360
ppm [CO$_2$] cylinder (balanced air mixture). After 40 mins thermal images were captured and the plants then exposed to 1,500 ppm [CO$_2$] (1,500 ppm [CO$_2$] cylinder (balanced air mixture) for a further 40 minutes and thermal images captured. Pairs of images were compared to identify putative CO$_2$ response mutants. Infrared thermal imaging was performed using an Inframetrics middle infrared (3.4-5 μm) camera model SC1000E (FLIR Systems). Images were stored in a ThermaCam Image file format (IMG) and analysed with the ThermaCam$^\text{TM}$ Researcher 2001 software (FLIR Systems, 2001 Inc., USA). Mutants exhibiting altered leaf thermal profiles compared to WT were selected, self-pollinated and seeds (M3) were collected for further investigation. Backcross seeds (F1s) were obtained by using mutant lines as female and Col-0 as male. The F2 was used for segregation analysis. Mutants segregating in the F2 were backcrossed to WT Col-0 for another two generations before being used for fine mapping and phenotyping.

**Map based mutant gene cloning**

cis1 mutants were outcrossed to WT plants in the Landberg *erecta* background (Ler) and the segregating F2 seedlings were screened using infrared thermography. A total of 868 cis1 mutants were used for mapping. 22 SSLP markers were used for bulked segregant analysis as described (Lukowitz et al., 2000). The *Arabidopsis* SNP collections (http://www.arabidopsis.org/) were used for designing SSLP, CAPS and dCAPS markers for the fine mapping. The mutation was narrowed down into an approximate 100kb region at the top arm of Chromosome III between SSLP marker nga172 and CAPS marker CA1 and is adjacent to SSLP marker nga32. T-DNA insertion lines representing all the annotated genes within this region were obtained from NASC and screened using infrared thermal imaging. A T-DNA insertion line (SALK_105495) of *At3g02260* which also showed morphologically similarity to the mutant ‘cis1’ was identified. We performed an allelism tests using the F1 progeny of the cis1 and big-1 (SALK_105495) cross using thermal imaging. This confirmed that
cis1 and big-1 are allelic to each other. We used PCR based genotyping and gene sequencing to confirm the presence of a T-DNA insertion in gene At3g02260 of the SALK_105495 line and a single point mutation in gene At3g02260 of the cis1 mutant.

Measurements of stomatal density, index, aperture and cell viability

Stomatal density and index were measured on leaf abaxial surfaces as described (Chater et al., 2015). The effect of CO$_2$ on stomatal aperture was measured using the isolated epidermal strip bioassay technique as previously described (Chater et al., 2015). Forty stomatal pores were measured per treatment in three separate replicated tests. To avoid experimenter bias, all the aperture measurements were performed blind. Cell viability was assessed as described in Chater et al. (2015). Experiments on independently grown plant material were carried out three times and data analysed by SigmaPlot 10.

Gas exchange measurements

Time-resolved stomatal conductance analyses of intact leaves of five week-old plants were conducted using a Li-6400 gas exchange analyzer with a fluorometer chamber (Li-Cor Inc.) as described by Hu et al. (2010). The photon flux density was set at 150 µmol m$^{-2}$ s$^{-1}$, temperature and relative humidity were held at 21°C and approximately 60-70%, respectively. Stomatal conductance was stabilized at 400 ppm CO$_2$ (as ambient concentration) for 30 min and then shifted to 800 ppm for another 30 min before shifted to 100 ppm for 1.5h. Data shown are means ± SE, n=4 leaves for each genotype.

Patch clamp experiments

Arabidopsis guard cell protoplasts were isolated according to the procedure described previously (Siegel et al., 2009). The whole-cell currents were recorded using a patch clamp amplifier (Axopatch 200B) and a digitizer (Digidata 1550). CO$_2$/bicarbonate -
activated S-type anion currents were recorded as described before (Xue et al., 2011).
The bath solution contained 30 mM CsCl, 2 mM MgCl₂, 1mM CaCl₂ and 10 mM Mes/Tris pH 5.6. The pipette solution contained 150 mM CsCl, 2 mM MgCl₂, 6.7 mM EGTA, 6.03 mM CaCl₂ (2 µM free Ca²⁺), 5 mM Mg-ATP, 10 mM HEPES/Tris pH7.1. Bicarbonate was freshly added to the pipette solution before patching the protoplasts each day. At pH 7.1, 11.5 mM free bicarbonate was balanced with 2 mM free CO₂ in the pipette solution. For more details please consult with Xue et al. (2011).

RT-PCR and quantitative RT-PCR analysis

Total RNA from aerial parts of the plants was prepared using RNeasy total RNA mini kit (Qiagen) and digested with RNase-free DNase I (Thermo scientific), and the absence of genomic DNA contamination was confirmed by PCR using RNA as template without reverse transcription. First strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) and oligo d(T)₁₅₋₁₈ (Promega) mRNA primer with 1 µg of total RNA as the template. cDNA corresponding to 20 ng of total RNA and 300 nM of each primer were used in PCR reactions. The primers for RT-PCR amplification BIG fragments were: primer pair1, F1 (5'-CAGCAAGCTCTATACCTTCAG-3') and R1, (5'- TCCATCCATCCACTCAACTC-3'); primer pair 2, F2 (5'- GTCTTCTACTTCACTGACCAACTCC-3') and R2, (5'-TCCATCTTCTTCTTCTTCTTCTACTACCC-3'); Actin7 was amplified with forward primer (5'-TGTTCCCAAGTATTGTTGGTCGTC-3') and reverse primer (5'-TGCTGAGGGATGCAAGGATTGATG-3') as a loading control. The PCR conditions were as follows: 1 cycle (94°C, 5 min), 35 cycles (94°C, 30 s; 62°C, 30 s; 72°C, 1min), 1 cycle (72°C, 7 min). Q-PCR was carried out on a Mx3005P (Stratagene) or an ECO (Illumina) real-time PCR thermal cycler in a total reaction volume of 20µL using the SYBR green dye PCR Master Mix (Thermo scientific), using these conditions, 95°C for 10 min, 40 two-step cycles at 95°C for 15 sec and 60°C for 1 min, followed by dissociation melting curve analysis to determine the PCR
specificity. The gene-specific primers used for BIG are F: 5’-GAATGGGAAGGAGCTATGTTG-3’ and R: 5’-GATACTGTGCTAAGGGAACTG-3’; for Actin3 (At3g53750) are F: 5’-GGCAGAATATGATGAGTCAGG-3’ and R: 5’-AAAGAAGAGCAGAGAACGAAG3’. The relative RNA levels were calculated from cycle threshold (C_T) values according to the ΔC_T method, and relative target mRNA levels were normalized to Actin3 mRNA levels. Reactions were repeated independently three times with similar results.

Acknowledgements
The authors are grateful to Prof. HMO Leyser (University of Cambridge, UK) for the gift of the EMS-mutagenised Arabidopsis population. Y.-K.L. acknowledges National Key Research and Development Program (2016YFD0100600) and National Natural Science Foundation of China (31171356, 31470360) for providing research funding. A.M.H. and J.E.G. acknowledge the support of the UK Biotechnological and Biological Sciences Research Council. Research in J.I.S. laboratory was supported by National Science Foundation (MCB-16162360 and NIH (GM060396) grants. Research in J.K. laboratory was supported by grants from the Deutsche Forschungsgemeinschaft (DFG). S.X. was supported by NSFC (31670267) and the Fundamental Research Funds for the Central Universities (2662015PY213, 2014PY065). H.H. received support by the 1000-talents Plan for young researchers from China and the Fundamental Research Funds for the Central Universities (2662017PY034).

Author contributions
A.M.H. analyzed data. A.M.H., Y.-K.L. and J.E.G. wrote the manuscript. All authors read and approved the manuscript.
Reference


For Peer Review


Figure Legends:

Fig. 1. The cis1 mutant displays a lower leaf surface temperature under elevated CO$_2$ than WT.
(a) Infrared thermograms showing that the leaf surface temperature of the cis1 mutant is lower than that of WT when the plants are exposed to 1,500 ppm CO$_2$.
(b) The average leaf surface temperature of the cis1 mutant is approximate 0.68ºC lower than that of WT plant when both are exposed to 1,500 ppm CO$_2$. Bars=mean ±SE (Student’s $t$ test, **P ≤0.001, n=20).
(c) In contrast to WT, the cis1 mutant fails to display elevated (800 ppm) CO$_2$-induced reduction in stomatal conductance, but exhibits a WT response when exposed to low (100 ppm) CO$_2$ (representative data, n=4).
(d) Relative stomatal conductance in (c) (presented is representative data, n=4).

Fig. 2. cis1 is a new allele of the BIG gene.
(a) Schematic structure of the BIG gene. The intron and exon organization of the BIG gene shown was determined by comparison of the cDNAs obtained by RT-PCR and genomic sequences from the Arabidopsis WT Col-0. Closed boxes indicate exons, and lines between boxes indicate introns. The locations of the single base mutations and T-DNA insertion of cis1, doc1-1 and big-1 are indicated. Diagram not to scale.
(b) The relative mRNA levels of BIG mutant alleles quantified by Q-PCR with a pair primers (F and R) with binding sites shown on (a). Values are mean ± SE, n=3.

Fig. 3. BIG gene mutants have higher stomatal density than WT.
(a) Compared with WT, BIG mutants exhibit increased stomata and epidermal pavement cells (labelled as “Epidermis”) density when grown at ambient [CO$_2$]. Error bars represent ± SE (Mann-Whitney rank sum test, **P ≤ 0.001, n=72).
(b) Stomatal density of WT and BIG mutant seedlings grown at ambient 450 ppm and elevated 1,000 ppm [CO$_2$]. When grown at 1,000 ppm [CO$_2$] mean stomatal density of
WT was significantly reduced compared with growth at ambient [CO$_2$] (Mann-Whitney rank sum test, **p≤0.001, n>20), whereas in the BIG gene allele stomatal density increased in these conditions (Student’s t test, **p≤0.001, n>20).

(c) Stomatal index of WT and BIG mutant seedlings grown at 450 ppm and 1,000 ppm [CO$_2$]. When grown at 1,000 ppm mean stomatal index of WT was significantly reduced compared with growth at ambient [CO$_2$] (Student’s t test, **p≤0.001, n>20), whereas in the BIG gene mutants’ stomatal index increased in these conditions (Student’s t test, or Mann-Whitney rank sum test, **p≤0.001, n>20).

**Fig. 4. Stomatal responses of BIG gene mutants to elevated CO$_2$ or exogenous ABA.**

(a) Elevated CO$_2$-induced stomatal closure is impaired in BIG gene mutants. Values are mean ±SE (Mann-Whitney rank sum test, **P ≤ 0.001, n=40).

(b) Elevated CO$_2$ induced inhibition of stomatal opening is not compromised in BIG gene mutants. Error bars represent SE (n=40).

(c) ABA-induced stomatal closure is not compromised in BIG gene mutants.

Bars=mean ±SE (n=40).

(d) The inhibition of light-induced stomatal opening by ABA is not compromised in BIG gene mutants. Values are mean ± SE (n=40).

**Fig. 5. Bicarbonate-activated S-type anion currents were suppressed in BIG mutant guard cell protoplasts.**

(a) Typical recording in wild type guard cell protoplasts without bicarbonate.

(b) Typical recording of 11.5 mM [HCO$_3$]$^-$ activated S-type anion currents in wild type guard cell protoplasts.

(c) Average current-voltage relationships of whole-cell currents as recording in (a) (open circles, n=5) and (b) (filled circles, n=7).
(d) Representative recording in *doc1-1* mutant guard cell protoplasts without bicarbonate added in the pipette solution.

(e) Representative whole-cell current recording in *doc1-1* mutant guard cell protoplasts with 11.5 mM [HCO$_3^-$], added in the pipette solution.

(f) Average current-voltage relationships of whole-cell currents as recording in (d) (open circles, n=5) and (e) (filled circles, n=8).

(g) Representative recording in *big-1* mutant guard cell protoplasts without bicarbonate added in the pipette solution.

(h) Representative whole-cell current recording in *big-1* mutant guard cell protoplasts with 11.5 mM [HCO$_3^-$], bicarbonate added in the pipette solution.

(i) Average current-voltage relationships of whole-cell currents as recording in (g) (open circle, n=6) and (h) (filled circles, n=8).

**Fig. S1.** (a) In contrast to WT, the *big1* mutant fails to display elevated (800 ppm) CO$_2$-induced reduction in stomatal conductance, but exhibits a WT response when exposed to low (100 ppm) CO$_2$ (representative data, n=4). (b) Relative stomatal conductance in (a) (representative data, n=4).

**Fig. S2.** PCR amplification of the *BIG* fragment from cDNAs of WT and mutant plants showed a complex PCR band pattern of *cis1* and only truncated *BIG* transcript present in *big-1* whereas no change in mRNA abundance is detected in the *doc1-1* mutant. Primer binding sites as indicated in Fig. 2a. *ACTIN* was used as a reference gene.

**Fig. S3.** Epidermal cell density of WT and *BIG* gene mutant seedlings grown at elevated 1,000 ppm [CO$_2$].

**Supplementary Information.** Determination of the intron-exon structure of *BIG* by DNA sequencing.
Fig. 1. The cis1 mutant displays a lower leaf surface temperature under elevated CO2 than WT. (a) Infrared thermograms showing that the leaf surface temperature of the cis1 mutant is lower than that of WT when the plants are exposed to 1,500 ppm CO2. (b) The average leaf surface temperature of the cis1 mutant is approximately 0.68°C lower than that of WT plant when both are exposed to 1,500 ppm CO2. Bars=mean ±SE (Student’s t test, **P ≤0.001, n=20). (c) In contrast to WT, the cis1 mutant fails to display elevated (800 ppm) CO2-induced reduction in stomatal conductance, but exhibits a WT response when exposed to low (100 ppm) CO2 (representative data, n=4). (d) Relative stomatal conductance in (c)(presented is representative data, n=4).
Fig. 2.  cis1 is a new allele of the BIG gene. (a) Schematic structure of the BIG gene. The intron and exon organization of the BIG gene shown was determined by comparison of the cDNAs obtained by RT-PCR and genomic sequences from the Arabidopsis WT Col-0. Closed boxes indicate exons, and lines between boxes indicate introns. The locations of the single base mutations and T-DNA insertion of cis1, doc1-1 and big-1 are indicated. Diagram not to scale. (b) The relative mRNA levels of BIG mutant alleles quantified by Q-PCR with a pair primers (F and R) with binding sites shown on (a). Values are mean ± SE, n=3.
Fig. 3. BIG gene mutants have higher stomatal density than WT. (a) Compared with WT, BIG mutants exhibit increased stomata and epidermal pavement cells (labelled as “Epidermis”) density when grown at ambient [CO2]. Error bars represent ± SE (Mann-Whitney rank sum test, **P ≤ 0.001, n=72). (b) Stomatal density of WT and BIG mutant seedlings grown at ambient 450 ppm and elevated 1,000 ppm [CO2]. When grown at 1,000 ppm [CO2] mean stomatal density of WT was significantly reduced compared with growth at ambient [CO2] (Mann-Whitney rank sum test, **p ≤ 0.001, n>20), whereas in the BIG gene alleles stomatal density increased in these conditions (Student’s t test, **p≤0.001, n>20). (c) Stomatal index of WT and BIG mutant seedlings grown at 450 ppm and 1,000 ppm [CO2]. When grown at 1,000 ppm mean stomatal index of WT was significantly reduced compared with growth at ambient [CO2] (Student’s t test, **p≤0.001, n>20), whereas in the BIG gene mutants’ stomatal index increased in these conditions (Student’s t test, or Mann-Whitney rank sum test, **p≤0.001, n>20).
Fig. 4. Stomatal responses of BIG gene mutants to elevated CO2 or exogenous ABA. (a) Elevated CO2-induced stomatal closure is impaired in BIG gene mutants. Values are mean ±SE (Mann-Whitney rank sum test, **P ≤ 0.001, n=40). (b) Elevated CO2 induced inhibition of stomatal opening is not compromised in BIG gene mutants. Error bars represent SE (n=40). (c) ABA-induced stomatal closure is not compromised in BIG gene mutants. Bars=mean ±SE (n=40). (d) The inhibition of light-induced stomatal opening by ABA is not compromised in BIG gene mutants. Values are mean ± SE (n=40).
Fig. 5. Bicarbonate-activated S-type anion currents were suppressed in BIG mutant guard cell protoplasts. (a) Typical recording in wild type guard cell protoplasts without bicarbonate. (b) Typical recording of 11.5 mM [HCO₃⁻]- activated S-type anion currents in wild type guard cell protoplasts. (c) Average current-voltage relationships of whole-cell currents as recording in (a) (open circles, n=5) and (b) (filled circles, n=7). (d) Representative recording in doc1-1 mutant guard cell protoplasts without bicarbonate added in the pipette solution. (e) Representative whole-cell current recording in doc1-1 mutant guard cell protoplasts with 11.5 mM [HCO₃⁻] added in the pipette solution. (f) Average current-voltage relationships of whole-cell currents as recording in (d) (open circles, n=5) and (e) (filled circles, n=8). (g) Representative recording in big-1 mutant guard cell protoplasts without bicarbonate added in the pipette solution. (h) Representative whole-cell current recording in big-1 mutant guard cell protoplasts with 11.5 mM [HCO₃⁻] bicarbonate added in the pipette solution. (i) Average current-voltage relationships of whole-cell currents as recording in (g) (open circle, n=6) and (h) (filled circles, n=8).