The BIG protein distinguishes the process of CO2-induced stomatal closure from the inhibition of stomatal opening by CO2

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Summary

- We conducted an infrared thermal imaging-based genetic screen to identify Arabidopsis mutants displaying aberrant stomatal behavior in response to elevated concentrations of CO2.
- This approach resulted in the isolation of a novel allele of the Arabidopsis BIG locus (At3g02260) that we have called CO2 insensitive 1 (cis1).
- BIG mutants are compromised in elevated CO2-induced stomatal closure and bicarbonate activation of S-type anion channel currents. In contrast with the wild-type, they fail to exhibit reductions in stomatal density and index when grown in elevated CO2. However, like the wild-type, BIG mutants display inhibition of stomatal opening when exposed to elevated CO2.
- BIG mutants also display wild-type stomatal aperture responses to the closure-inducing stimulus abscisic acid (ABA).
- Our results indicate that BIG is a signaling component involved in the elevated CO2-mediated control of stomatal development. In the control of stomatal aperture by CO2, BIG is only required in elevated CO2-induced closure and not in the inhibition of stomatal opening by this environmental signal. These data show that, at the molecular level, the CO2-mediated inhibition of opening and promotion of stomatal closure signaling pathways are separable and BIG represents a distinguishing element in these two CO2-mediated responses.

Introduction

Stomata consist of a pair of guard cells that surround a central pore and serve to regulate water loss and the uptake of CO2. 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 ([CO2]) (Kim et al., 2010; Franks et al., 2012). In response to an increase in [CO2], stomatal aperture reduces, as, in general, do the number of stomata that develop on the surface of the leaves (Vavasseur & Raghavendra, 2005; Kim et al., 2010; Franks et al., 2012). Understanding how the plant perceives changes in [CO2] 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 environmental change on plants (Assmann & Jugla, 2016).

Recently, we have begun to understand more about the underlying cellular mechanisms responsible for coupling increased [CO2] to reduced stomatal conductance (Kim et al., 2010; Assmann & Jugla, 2016; Engineer et al., 2016). In this context, it is important to recognize that elevated CO2-induced reductions in stomatal conductance are the net result of two processes: the promotion of stomatal closure and the inhibition of stomatal opening (Assmann, 1993). These processes are separable; abscisic acid (ABA)-induced stomatal closure is distinct from ABA-inhibited stomatal opening (Allen et al., 1999; Wang et al., 2001; Mishra et al., 2006). However, before the current work, it was not known whether this also applied to [CO2]-induced changes in stomatal aperture.

There is evidence that the guard cell ABA and CO2 signaling responsible for the inhibition of light-induced stomatal opening...
pathways converge (Webb & Hetherington, 1997). It has been suggested that elevated [CO₂] 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 CO₂-mediated closure converge with ABA signaling downstream of ABA receptors and the two pathways influence each other on convergence (Xue et al., 2011; Merilo et al., 2013; Höрак 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 CO₂-mediated closure, but not in guard cell ABA signaling. In Arabidopsis, these include β-carbonic 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), RHCl, a MATE transporter (Tian et al., 2015), and the MAP kinase MPK4 (Hötrak et al., 2016; Jakobson et al., 2016). Loss of the CAs, RHCl and MPK4 impairs CO₂-induced closure (Hashimoto et al., 2006; Hu et al., 2010; Tian et al., 2015; Hötrak et al., 2016; Jakobson et al., 2016), whereas recessive bt1 alleles show a constitutive high CO₂ response (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016).

In 1987, Woodward discovered an inverse relationship between atmospheric [CO₂] and stomatal density (Woodward, 1987). We know less about the operation of this developmental signaling pathway, but the putative β-keto acyl CoA synthase encoded by the HIC gene is involved, as are the CO₂ Response Secreted Protease (CRSP), the β-carbonic anhydrases CA1 and CA4, and the peptide Epidermal Patterning Factor 2 (EPF2) (Gray et al., 2000; Dohney-Adams et al., 2012; Engineer et al., 2014). Most recently, it has been shown that the activity of the reactive oxygen species (ROS)-producing NADPH oxidases, encoded by the RBOHD and RBOHF genes, is involved in the CO₂-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 CO₂ insensitive 1 (cis1), which is compromised in both elevated [CO₂]-induced closure and reduction in stomatal density. However, when challenged with ABA, cis1 displays reductions in stomatal aperture that are indistinguishable from the wild-type (WT), suggesting that BIG (CIS1) functions upstream of ABA or in an ABA-independent signaling pathway responsible for the control of stomatal aperture by CO₂. We also found that the activation of the guard cell S-type anion channel by bicarbonate is compromised by the loss of BIG function. Furthermore, in contrast with elevated [CO₂]-mediated closure, the ability of elevated [CO₂] 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 [CO₂]. We also show that BIG features in the signaling pathway through which elevated [CO₂] controls stomatal aperture. Importantly, we show that BIG is only involved in elevated [CO₂]-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 CO₂-mediated responses.

Materials and Methods

Plant growth

All Arabidopsis (Arabidopsis thaliana L.) 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 performed as described previously (Liang et al., 2010).

Mutant screen

To identify genes required for stomatal CO₂ responses, we screened 20,000 seeds from an Arabidopsis EMS (ethyl methane-sulfonate) M2 population representing 40 independent pools (each pool corresponding to c. 1000 M1 plants) by infrared thermal imaging (Wang et al., 2004; Xie et al., 2006). Screening was carried out on 3–4-wk-old plants in a purpose-built chamber (84 × 68 × 20 cm³), located inside a controlled environment room. The CO₂ concentration inside the chamber was controlled externally from CO₂ cylinders. Air flow in the chamber was maintained at 0.03 m s⁻¹ using fans. Relative humidity inside the chamber was c. 60%, temperature was 22°C and light intensity was 120 μmol m⁻² s⁻¹. Plants were placed in the chamber and exposed to 360 ppm [CO₂] (360 ppm [CO₂] cylinder (balanced air mixture)). After 40 min, thermal images were captured and the plants were then exposed to 1500 ppm [CO₂] (1500 ppm [CO₂] cylinder (balanced air mixture)) for a further 40 min, and thermal images were captured. Pairs of images were compared to identify putative CO₂ response mutants. Infrared thermal imaging was performed using an Inframetrics middle infrared (3.4–5 μm) camera model SC1000E (FLIR Systems Inc., Wilsonville, OR, USA). Images were stored in a ThermaCam Image file format (IMG) and analysed with the ThermaCam™ RESEARCHER 2001 software (FLIR Systems). Mutants exhibiting altered leaf thermal profiles compared with WT were selected, self-pollinated, and seeds (M3) were collected for further investigation. Backcross seeds (F1s) were obtained using mutant lines as female and Col-0 as male. F2 was used for segregation analysis. Mutants segregating in 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. Twenty-two simple sequence length
polymorphism (SSLP) markers were used for bulked segregant analysis as described previously (Lukowitz et al., 2000). The Arabidopsis single nucleotide polymorphism (SNP) collections (http://www.arabidopsis.org/) were used to design SSLP, cleaved amplified polymorphic sequences (CAPS) and derived CAPS (dCAPS) markers for fine mapping. The mutation was narrowed down to a 100-kb 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 line 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 morphological similarity to the mutant ‘cis1’ was identified. We performed an allelism test 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 previously (Chater et al., 2015). The effect of CO2 on stomatal aperture was measured using the isolated epidermal strip bioassay technique as described previously (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 were analysed by SIGMAPLOT 10 (Systat Software Inc., San Jose, CA, USA).

Gas exchange measurements

Time-resolved stomatal conductance analyses of intact leaves of 5-wk-old plants were conducted using a Li-6400 gas exchange analyzer with a fluorometer chamber (Li-Cor, Lincoln, NE, USA), 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 c. 60–70%, respectively. Stomatal conductance was stabilized at 400 ppm CO2 (as ambient concentration) for 30 min and then shifted to 800 ppm for another 30 min before being shifted to 100 ppm for 1.5 h. Data shown are the 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) (Molecular Devices LLC, Sunnyvale, CA, USA). CO2/bicarbonate-activated S-type anion currents were recorded as described previously (Xue et al., 2011). The bath solution contained 30 mM CsCl, 2 mM MgCl2, 1 mM CaCl2 and 10 mM Mes/Tris, pH 5.6. The pipette solution contained 150 mM CsCl, 2 mM MgCl2, 6.7 mM ethylene glycol-bis(β-aminoethylether)-N,N′-tetraacetic acid (EGTA), 6.03 mM CaCl2 (2 μM free Ca2+), 5 mM Mg-ATP, 10 mM HEPES/Tris, pH 7.1. 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 Xue et al. (2011).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR analysis

Total RNA from aerial parts of the plants was prepared using an RNeasy total RNA mini kit (Qiagen, Hilden, Germany) and digested with RNase-free DNase I (Thermo Fisher Scientific Inc., Waltham, MA, USA); 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, Thermo Fisher Scientific) and oligo d(T)15–18 (Promega (Beijing) Biotech Co. Ltd, Beijing, China) 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 PCRs. The primers for RT-PCR amplification of BIG fragments were: primer pair1, F1 (5′-CAGCAAGCTTATACTTCCAG-3′) and R1 (5′-TCCATCC ATCCACTCAACTCTC-3′); primer pair 2, F2 (5′-GTCTTCT ACTTCACTGGAACACTCC-3′) and R2 (5′-TCCATCTTC TCTTCTCTCATGTC-3′); Actin7 was amplified with forward primer (5′-TGTCTCCAACTATTTGTTGCTGC-3′) and reverse primer (5′-TGCTGAGGATGCAAGGA TTA -TTGGA TGTC-3′) as a loading control. The PCR conditions were as follows: one cycle (94°C, 5 min), 35 cycles (94°C, 30 s; 62°C, 30 s; 72°C, 1 min), one cycle (72°C, 7 min). Quantitative PCR was carried out on an Mx3005P (Stratagene, La Jolla, CA, USA) or an ECO (Illumina Inc., San Diego, CA, USA) real-time PCR thermal cycler in a total reaction volume of 20 μl using the SYBR green dye PCR Master Mix (Thermo Fisher Scientific) and the conditions 95°C for 10 min, 40 two-step cycles at 95°C for 15 s and 60°C for 1 min, followed by dissociation melting curve analysis to determine the PCR specificity. The gene-specific primers used for BIG were: F, 5′-GAATGGGAAGGAGCTAGTGTG-3′; R, 5′-GATACTGTG CTAAGGGAACTG-3′; for Actin3 (At3g53750), the primers were: F, 5′-GCCAGAATAG ATGAGTCAGG-3′; R, 5′-AAAAGAAGGACAGAAGACG-3′.

The relative RNA levels were calculated from cycle threshold (Ct) values according to the ΔCt method, and relative target mRNA levels were normalized to Actin3 mRNA levels. Reactions were repeated independently three times with similar results.

Results

The cis1 mutant is involved in the response of stomatal conductance to elevated CO2

To understand the underlying cellular basis of the effect of elevated CO2 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 [CO₂], would be visible because they would exhibit reduced leaf temperature as a result of 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 CO₂ (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 that displayed significantly lower leaf surface temperature (0.68°C) relative to WT when challenged for 40 min with 1500 ppm [CO₂] (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 the stomatal conductance (g). Figure 1(c,d) shows that, in WT, challenge with 800 ppm CO₂ results in a reduction in g, whereas the response is attenuated in cis1. By contrast, both cis1 and WT display an increase in g when exposed to low (100 ppm) CO₂. We confirmed this response in big-1, a second independent allele of cis1 (Supporting Information Fig. S1). These data suggest that the cis1 mutant is compromised in the stomatal response to elevated [CO₂].

Identification of the CIS1 gene locus

We performed map-based gene cloning to identify the CIS1 locus, and mapped the mutation to a 107-kb 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 +8542 (GT...AG to GT...AA) (Fig. 2a), which resulted in alternative spliced mRNAs as shown in Fig. S2. Real-time quantitative 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 63 bp shorter than predicted, because 30 bp of the sequence of intron 1, 21 bp of intron 5 and 12 bp of intron 7 had been annotated as part of the respective neighboring exons (Notes S1). Hence, the BIG open reading frame (ORF) is 15 234 bp long, encoding a putative 5077-amino-acid peptide, as predicted by Gil et al. (2001).

Many alleles of big mutants, for example gao6, tir3, doc1, ar1, lpr1, elk1, asa1, umb1, cmd1 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 and 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; Lopez-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 a calmodulin (CaM) -binding domain and are probably involved in Ca²⁺ signaling (Xu et al., 1998; Parsons et al., 2015).

To confirm the identity of cis1, we obtained two additional mutant alleles of BIG, doc1-1 was originally isolated in a genetic screen for components of light signaling and harbors a single base change from G to A at position +5514 (Fig. 2a), resulting in a change from a conserved cysteine (Cys) residue to tyrosine (Tyr). This missense BIG mutation perturbs auxin transport and plant growth (Gil et al., 2001) but, in our quantitative PCR analysis, no change to the transcript abundance of BIG was detected (Fig. 2b). big-1 harbors 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 quantitative PCR (Fig. 2b).

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

The data in Fig. 3(a) show that stomatal and epidermal pavement cell densities are greater in the BIG mutant alleles than in WT (P≤0.001). This reflects the fact that both guard cells and epidermal cells were significantly smaller than in 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). Figure 4(a) shows that, in contrast with WT, the stomata of cis1, big-1 and doc1-1 mutants failed to close when subjected to 700 ppm CO₂. These data indicate that BIG is required for elevated CO₂-induced stomatal closure. Elevated
[CO2] is also known to inhibit light-induced stomatal opening (Mansfield et al., 1990). In contrast with CO2-induced stomatal closure, the inhibition of light-induced stomatal opening of the BIG mutants was similar to that of WT (Fig. 4b). The specific role of the BIG gene in the pathway by which elevated [CO2] 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 CO2 response were both observed in more than one of our laboratories, underlining the robustness of the CO2 specificity of the stomatal phenotype in big mutant alleles.

BIG is required for the 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 [CO2] (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 [CO2]-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, lower anion currents were recorded.
activated by the same concentration of bicarbonate in the pipette solution (Fig. 5c,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 the \(doc1-1\) mutant (Fig. 5f) and \(-16.8 \pm 1.8\) pA for the \(big-1\) 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 \(CIS1\), which 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 \(CO_2\) (Figs 1b,c, 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, whilst 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 bioassay of \(CO_2\) inhibition of light-stimulated stomatal opening (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 not involved in ABA-promoted closure or in ABA-inhibited light-induced opening. Because \(BIG\) encodes a protein which, 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

![Fig. 3 BIG gene mutants have higher stomatal density than wild-type (WT) Arabidopsis. (a) Compared with WT, BIG mutants exhibit increased stomata and epidermal pavement cells (Epidermis) density when grown at ambient [CO2]. Error bars represent ± SE (Mann–Whitney rank sum test, **, \(P \leq 0.001\), \(n=72\)). (b) Stomatal density of WT and BIG mutant seedlings grown at ambient (450 ppm) and elevated (1000 ppm) [CO2]. When grown at 1000 ppm [CO2], the mean stomatal density of WT was significantly reduced compared with growth at ambient [CO2] (error bars represent ± SE (Mann–Whitney rank sum test, **, \(P \leq 0.001\), \(n>20\)), whereas, in the BIG gene alleles, stomatal density increased in these conditions (error bars represent ± SE (Student’s t-test, **, \(P \leq 0.001\), \(n>20\)). (c) Stomatal index of WT and BIG mutant seedlings grown at 450 ppm and 1000 ppm [CO2]. When grown at 1000 ppm, the mean stomatal index of WT was significantly reduced compared with growth at ambient [CO2] (error bars represent ± SE (Student’s t-test, **, \(P \leq 0.001\), \(n>20\)), whereas, in the BIG gene mutants, the stomatal index increased in these conditions (error bars represent ± SE (Student’s t-test, or Mann–Whitney rank sum test, **, \(P \leq 0.001\), \(n>20\)).]
opening, but not in closure (Wang et al., 2001), a sphingosine-1-phosphate phosphatase, long-chain base phosphate lyase double mutant (sppasedpl1), which 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 the 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 CO2 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 CO2-induced stomatal closure involve the activation of slow anion channels (Kim et al., 2010; Assmann & Jegla, 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 [CO2]-induced stomatal closure. A recent study by Yamamoto et al. (2016) has provided evidence that different parts of SLAC1 are separately responsible for sensing ABA and CO2 signals. It is the transmembrane domain of SLAC1 channels that perceives CO2 signals, in contrast with 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 CO2

Figure 3(a) 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 [CO2] typically results in a reduction in stomatal index and density (Hetherington & Woodward, 2003; Assmann & Jegla, 2016; Engineer et al., 2016). The results in Fig. 3(b,c) clearly show that, in marked contrast with WT, the stomatal indices and density of BIG mutants increased when the plants were grown at 1000 ppm CO2. The epidermal cell densities in the mutants remained significantly higher than those of WT at this elevated [CO2] (Fig. S3). It is likely, as with ßca1ca4, epf2 and bic mutants (Gray et al., 2000; Engineer et al., 2014), that loss of BIG function relieves the elevated [CO2]-mediated repression of stomatal development. How might BIG bring about an effect on CO2-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 or polar auxin
transport and 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 the elevated [CO2]-mediated control of stomatal closure and density. Our results reveal that we have identified a component which is involved in the signaling pathway by which elevated CO2 promotes stomatal closure. However, BIG is not involved in the elevated [CO2]-mediated inhibition of light-induced opening or in stomatal closure initiated by ABA. These data indicate that elevated [CO2]-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 CO2, or resides in an as yet undefined parallel signaling pathway that converges at or above the SLAC1 ion channel.

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References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 The big-1 mutant fails to display elevated (800 ppm) CO2-induced reduction in stomatal conductance.

Fig. S2 PCR amplification of the BIG fragment from cDNAs of wild-type (WT) and mutant plants.

Fig. S3 Epidermal cell density of wild-type (WT) and BIG gene mutant seedlings grown at elevated (1000 ppm) [CO2].

Notes S1 Determination of the intron–exon structure of BIG by DNA sequencing.

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