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Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO_2 signal transduction in guard cell

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Plants respond to elevated CO_2 via carbonic anhydrases that mediate stomatal closing, but little is known about the early signalling mechanisms following the initial CO_2 response. It remains unclear whether CO_2, HCO_3^- or a combination activates downstream signalling. Here, we demonstrate that bicarbonate functions as a small-molecule activator of SLAC1 anion channels in guard cells. Elevated intracellular $[HCO_3^-]_i$ with low $[CO_2]$ and $[H^+]$ activated S-type anion currents, whereas low $[HCO_3^-]_i$ at high $[CO_2]$ and $[H^+]$ did not. Bicarbonate enhanced the intracellular Ca^{2+} sensitivity of S-type anion channel activation in wild-type and *ht1-2* kinase mutant guard cells. *ht1-2* mutant guard cells exhibited enhanced bicarbonate sensitivity of S-type anion channel activation. The OST1 protein kinase has been reported not to affect CO_2 signalling. Unexpectedly, *OST1* loss-of-function alleles showed strongly impaired CO_2-induced stomatal closing and HCO_3^- activation of anion channels. Moreover, PYR/RCAR abscisic acid (ABA) receptor mutants slowed but did not abolish CO_2/HCO_3^- signalling, redefining the convergence point of CO_2 and ABA signalling. A new working model of the sequence of CO_2 signalling events in gas exchange regulation is presented.

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Introduction

Plants control CO_2 influx and water loss through stomatal pores, formed by guard cells in aerial tissues. Guard cells respond to abscisic acid (ABA), auxin, blue light, plant pathogens and CO_2 and have been developed as a powerful system for plant cell signal transduction research (Blatt, 2000; Evans and Hetherington, 2001; Schroeder *et al*, 2001; Sirichandra *et al*, 2009). Elevated intercellular CO_2 concentrations (C_i) that occur in leaves due to respiration in darkness and the continuing rise in atmospheric CO_2 concentrations due to anthropogenic fossil fuel burning cause a reduction in stomatal apertures (Medlyn *et al*, 2001; Frommer, 2010).

Stomatal closing is regulated by ion channel-mediated anion and K^+ efflux from guard cells and parallel organic solute metabolism (Schroeder *et al*, 1987; Schroeder and Hagiwara, 1989; Blatt and Armstrong, 1993; MacRobbie, 1998). Elevated CO_2 activates anion channels and K^+_{out} efflux channels in *Vicia faba* guard cells (Brearley *et al*, 1997; Raschke *et al*, 2003; Roelfsema *et al*, 2004) and triggers chloride release from guard cells causing guard cell depolarization in leaves (Hanstein and Felle, 2002; Roelfsema *et al*, 2002). Furthermore, cytosolic pH does not change in response to physiological $[CO_2]$ shifts in *V. faba* guard cells (Brearley *et al*, 1997).

Recently, we have shown that the β-carbonic anhydrases, βCA1 and βCA4, function in CO_2 regulation of stomatal movements. *ca1;ca4* double-mutant plants show impaired CO_2 induction of stomatal closing, whereas ABA-induced stomatal closing is functional (Hu *et al*, 2010). CO_2 is reversibly catalysed by carbonic anhydrases (CAs) into bicarbonate ions and protons. Cytoplasmic high CO_2 together with high HCO_3^- concentrations activates S-type anion channel currents in wild-type *Arabidopsis* guard cells (Hu *et al*, 2010). However, the mechanisms by which high CO_2 and/or HCO_3^- mediate this response were not further investigated. Whether high $[CO_2]$ and $[HCO_3^-]$ are able to activate anion channels in *ca1;ca4* double-mutant plants remains unknown. The concentrations of HCO_3^- and CO_2 required for channel regulation in patch-clamped guard cells are relatively high, necessitating genetic analyses to determine whether the high $[CO_2]$ plus $[HCO_3^-]$ activation are physiologically relevant. Moreover, genetic mechanisms downstream of this high $[HCO_3^-]$ plus $[CO_2]$ response and their position in the signalling cascade remain unknown and are dissected, with unexpected results, in the present study.

Activation of S-type anion channels at the plasma membrane of guard cells has been regarded as a critical step in stomatal closure (Schroeder and Hagiwara, 1989; Schmidt *et al*, 1995; Grabov *et al*, 1997; Pei *et al*, 1997). Mutations in the SLAC1 anion channel cause greatly reduced S-type anion current activities, whereas R-type anion channels and ABA-activated Ca^{2+} permeable channels remain intact in *slac1* mutants (Negi *et al*, 2008; Vahisalu *et al*, 2008). SLAC1 functions as an anion channel with permeabilities to Cl^- and NO_3^- when heterologously expressed in *Xenopus* oocytes (Geiger *et al*, 2009; Lee *et al*, 2009), consistent with *in vivo* Cl^- and NO_3^- permeabilities of S-type anion channels (Schmidt and Schroeder, 1994).

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The concentration of intracellular free calcium ions ($[Ca^{2+}]_i$) has been shown to function as a key signalling molecule in plants and mediates CO_2 signal transduction in guard cells of several plant species (Schwartz, 1985; Webb *et al*, 1996; Hetherington and Woodward, 2003; Young *et al*, 2006; Kim *et al*, 2010). Elevation of $[Ca^{2+}]_i$ in guard cells causes activation of S-type anion channels, downregulation of inward (rectifying) K^+_{in} channels and downregulation of proton ATPases, providing central mechanisms that mediate stomatal closing and inhibition of stomatal opening (Schroeder and Hagiwara, 1989; Kelly *et al*, 1995; Kinoshita *et al*, 1995; Grabov and Blatt, 1999; Siegel *et al*, 2009; Chen *et al*, 2010). Recent studies have suggested that the stomatal closing signals, CO_2 and ABA, enhance the $[Ca^{2+}]_i$ sensitivity of stomatal closing mechanisms (Young *et al*, 2006; Siegel *et al*, 2009) (for review see Hubbard *et al*, 2010). However, whether CO_2 activation of S-type anion channels requires $[Ca^{2+}]_i$ is not known. Furthermore, whether CO_2 primes Ca^{2+} regulation of ion channels remains unknown and no genetic mutants and mechanisms are known that mediate CO_2/HCO_3^- regulation of ion channels.

The HT1 protein kinase was identified as a major negative regulator of high CO_2-induced stomatal closure (Hashimoto *et al*, 2006) and is genetically epistatic to βCA1 and βCA4 in CO_2 response pathway (Hu *et al*, 2010). However, the cellular signalling mechanisms of HT1 have not yet been investigated and whether the HT1 kinase affects S-type anion channel regulation and/or Ca^{2+} signalling remains unknown.

The OST1 protein kinase, also named SnRK2.6 and SnRK2E, was identified as a key mechanism mediating ABA signal transduction (Mustilli *et al*, 2002; Yoshida *et al*, 2002; Vlad *et al*, 2009), but has no effect on low CO_2-induced stomatal opening (Mustilli *et al*, 2002). Recent findings have shown that OST1 activates SLAC1 anion currents when OST1 and SLAC1 are coexpressed in *Xenopus* oocytes (Geiger *et al*, 2009; Lee *et al*, 2009).

In the present study, we show that elevated bicarbonate, more so than elevated CO_2, acts as intracellular signalling molecule to activate SLAC1-mediated anion channels. Elevated bicarbonate enhances (primes) the $[Ca^{2+}]_i$ sensitivity of SLAC1 channel activation. The *ht1-2* kinase mutant is found to enhance the HCO_3^- sensitivity of anion channel activation but also requires cytosolic Ca^{2+} for S-type anion channel activation, further defining the placement of HT1 effects on the CO_2 signalling cascade. Surprisingly, our analyses of OST1 on CO_2 regulation of stomatal movements and anion channels demonstrate that the OST1 protein kinase is a major regulator of CO_2-induced stomatal closing and CO_2 activation of anion channels in guard cells, leading to a new model for CO_2 control of gas exchange in plants.

Results

Bicarbonate activates S-type anion currents in ca1;ca4 double-mutant guard cell protoplasts

The βCA1 and βCA4 CAs act as upstream regulators in CO_2-induced stomatal movements in guard cells (Hu *et al*, 2010). Elevated CO_2 together with bicarbonate concentrations activate S-type anion channel currents in wild-type *Arabidopsis* guard cells. Previous studies of CO_2 regulation of anion channels have only been pursued in wild-type guard cells (Brearley *et al*, 1997; Raschke *et al*, 2003; Hu *et al*, 2010). Therefore, we investigated whether elevated bicarbonate and intracellular CO_2 can by-pass the *ca1;ca4* mutant and activate S-type anion currents in *ca1;ca4* mutant guard cells. Addition of 13.5 mM total bicarbonate to the pipette solution (equivalent to 11.5 mM free bicarbonate ($[HCO_3^-]_i$)/2 mM free $[CO_2]$ at pH 7.1) activated anion currents in patch-clamped *ca1;ca4* guard cells (Figure 1B and C), compared with control currents in the absence of added intracellular bicarbonate (Figure 1A). Free $[HCO_3^-]_i$ and $[CO_2]$ were calculated using the Henderson–Hasselbalch equation as described in Materials and methods. These findings are consistent with CAs acting as upstream regulators of CO_2 signalling and that elevated bicarbonate and CO_2 together can activate S-type anion channel in *ca1;ca4* double-mutant guard cells.

Bicarbonate-activated S-type anion currents are greatly impaired in slac1 mutant guard cell protoplasts

The reversal potential of $CO_2 + HCO_3^-$ activated whole-cell currents was $+24.0 \pm 3.6$ mV ($n = 8$), which was close to the imposed chloride equilibrium potential of $+31.1$ mV, supporting the hypothesis that $CO_2 + HCO_3^-$ activate guard cell anion channels. The bicarbonate and CO_2 concentrations used for anion current activation were very high (Figure 1B and C) (Hu *et al*, 2010), giving rise to the question whether these anion currents correspond to physiological guard cell anion channel currents. SLAC1 is required for *Arabidopsis* ABA and Ca^{2+} activation of guard cell S-type anion channel function (Vahisalu *et al*, 2008). Therefore, we analysed whether high bicarbonate- and CO_2-activated anion currents are mediated by SLAC1. Guard cell protoplasts from the recessive *slac1-1* and *slac1-3* mutants displayed small anion currents in the presence of 11.5 mM free $[HCO_3^-]_i$ and 2 mM $[CO_2]$ in the pipette solution, similar to control currents in the absence of added bicarbonate (Figure 1D and E, $P > 0.05$). These data suggest that the high intracellular $[HCO_3^-] + [CO_2]$-mediated anion currents are largely mediated by the physiologically relevant SLAC1 anion channel (Figure 1).

Next, we analysed whether these anion currents show a clear HCO_3^- permeability in wild-type guard cells. The total bicarbonate was elevated to 50 mM in the pipette solution at pH 7.1 (corresponding to 43.4 mM free $[HCO_3^-]_i$ and 6.6 mM free $[CO_2]$). Under such high $[HCO_3^-]$, the reversal potential of whole-cell currents was $+26.0 \pm 0.9$ mV (Supplementary Figure S2, $n = 4$). A relative permeability ratio of $P_{HCO_3^-}/P_{Cl^-} = 0.06 \pm 0.01$ was estimated using the Goldman equation. This Cl^- over HCO_3^- selectivity of whole-cell anion currents is consistent with the anion selectivity of SLAC1 channels found in heterologous expression experiments in *Xenopus laevis* oocytes (Geiger *et al*, 2009).

High $[CO_2]$ and protons do not activate S-type anion currents in the absence of high bicarbonate levels in guard cells

CAs reversibly catalyse the conversion of CO_2 into bicarbonate ions and free protons (Supuran, 2008; Chandrashekar *et al*, 2009). Whether high $[CO_2]$, $[HCO_3^-]$, $[H^+]$ or a combination of these mediates activation of S-type anion channels in *Arabidopsis* guard cells remains to be investigated (Hu *et al*, 2010). We investigated whether intracellular acidification is capable of activating S-type anion currents in wild-type guard cell protoplasts. Intracellular acidification at pH 6.1

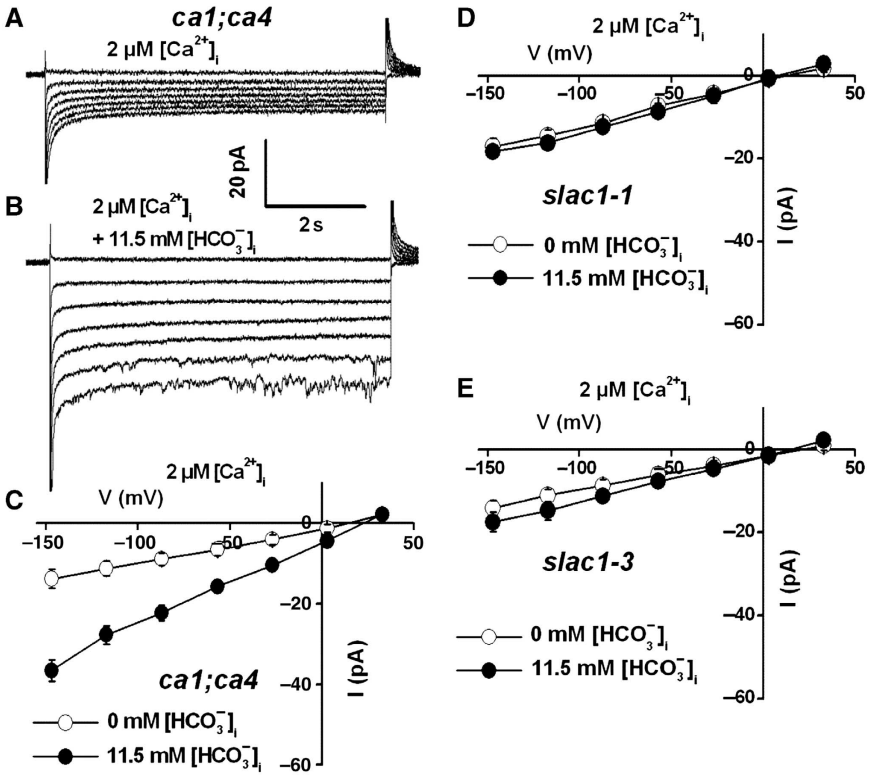

Figure 1 High intracellular [CO_2] and [HCO_3^-] activate S-type anion channel currents in *Arabidopsis ca1;ca4* double-mutant guard cells but do not activate S-type anion currents in *slac1* mutant guard cells with 2 μM [Ca^{2+}]$_i$. (**A**) Whole-cell currents without HCO_3^-/CO_2 and (**B**) with 11.5 mM free [HCO_3^-]$_i$/2 mM free CO_2 in the pipette solution (pH 7.1) in *ca1;ca4* double-mutant guard cells. (**C**) Steady-state current–voltage relationships of the whole-cell currents recorded in *ca1;ca4* mutant guard cells as in (**A**) (open circles, $n=4$ guard cells) and (**B**) (filled circles, $n=9$ guard cells). (**D**) Steady-state current–voltage relationships of whole-cell currents recorded in *slac1-1* mutant guard cells (open circles: 0 mM added [HCO_3^-]$_i$, $n=6$; filled circles: 11.5 mM free [HCO_3^-]$_i$ and 2 mM free [CO_2], $n=6$) and (**E**) in *slac1-3* mutant guard cells (open circles: 0 mM added [HCO_3^-]$_i$, $n=4$; filled circles: 11.5 mM free [HCO_3^-]$_i$ and 2 mM free [CO_2], $n=8$). Liquid junction potential was +1 mV. Data are mean ± s.e.

alone did not significantly activate S-type anion channel currents compared with control recordings at pH 7.1 (Figure 2A, $P>0.05$, Student's *t*-test). Interestingly, when the intracellular free [CO_2] was at a high concentration of 2 mM in the pipette solution (with 1.1 mM free [HCO_3^-]$_i$) at pH 6.1, S-type anion channel currents were not activated in wild-type guard cell protoplasts, despite the applied high [CO_2] and high proton concentrations (Figure 2B, $P>0.05$, Student's *t*-test).

Previous research has shown no intracellular pH shifts in *V. faba* guard cells in response to [CO_2] changes (Brearley *et al*, 1997). To further investigate whether cytosolic pH is affected in *Arabidopsis* guard cells in response to [CO_2] shifts, a ratiometric pH indicator *Pt-GFP* (Schulte *et al*, 2006) under the control of a strong guard cell preferential promoter *pGC1* (Yang *et al*, 2008) was transformed into *Arabidopsis* guard cells (Figure 2C). In control experiments, *in vivo* recordings of pH in fluorescent *pGC1*::*Pt-GFP* transgenic guard cells showed clear reversible shifts in ratiometric intracellular pH fluorescence when the extracellular pH was repeatedly shifted from pH 5.0 to pH 7.5 and back (Figure 2D; Supplementary Figure S3). Weak acids can control intracellular pH while maintaining a constant extracellular pH (Blatt and Armstrong, 1993; Grabov and Blatt, 1997). Therefore, the weak acid sodium butyrate was used to analyse whether Pt-GFP can report intracellular pH. Ratiometric fluorescence recordings of *Pt-GFP*-expressing guard cells showed clear shifts, when intact plant epidermes were perfused with defined concentrations of sodium butyrate-containing MES buffers (Figure 2E), indicating intracellular pH changes were easily detected in guard cells (Figure 2D and E). However, no clear shifts in guard cell intracellular pH fluorescence were observed when the concentration of CO_2 bubbled in the extracellular perfusion buffers was repeatedly shifted from 0 to 800 p.p.m. (Figure 2F), consistent with the findings in *V. faba* guard cells using a pH-sensitive dye (Brearley *et al*, 1997). Average changes in intracellular pH in response to extracellular pH changes appeared to be relatively rapid (Figure 2D), and slightly slower in response to weak acid treatments and without clearly discernable overshoots upon weak acid removal under the imposed conditions (Figure 2E). In conclusion, protons alone or in combination with elevated CO_2 could not activate S-type anion channels (Figure 2A and B) and [CO_2] changes did not cause measurable changes in intracellular pH of *Arabidopsis* guard cells (Figure 2F) (Brearley *et al*, 1997).

Bicarbonate activates S-type anion currents at low free CO_2 in guard cells

To analyse whether elevated intracellular [HCO_3^-] is sufficient to activate anion currents at low [H^+] and low [CO_2], 13.5 mM total $CsHCO_3$ was applied to the pipette solution and the free [HCO_3^-] was calculated as 13.04 mM with 0.46 mM free [CO_2] at pH 7.8. These analyses clearly

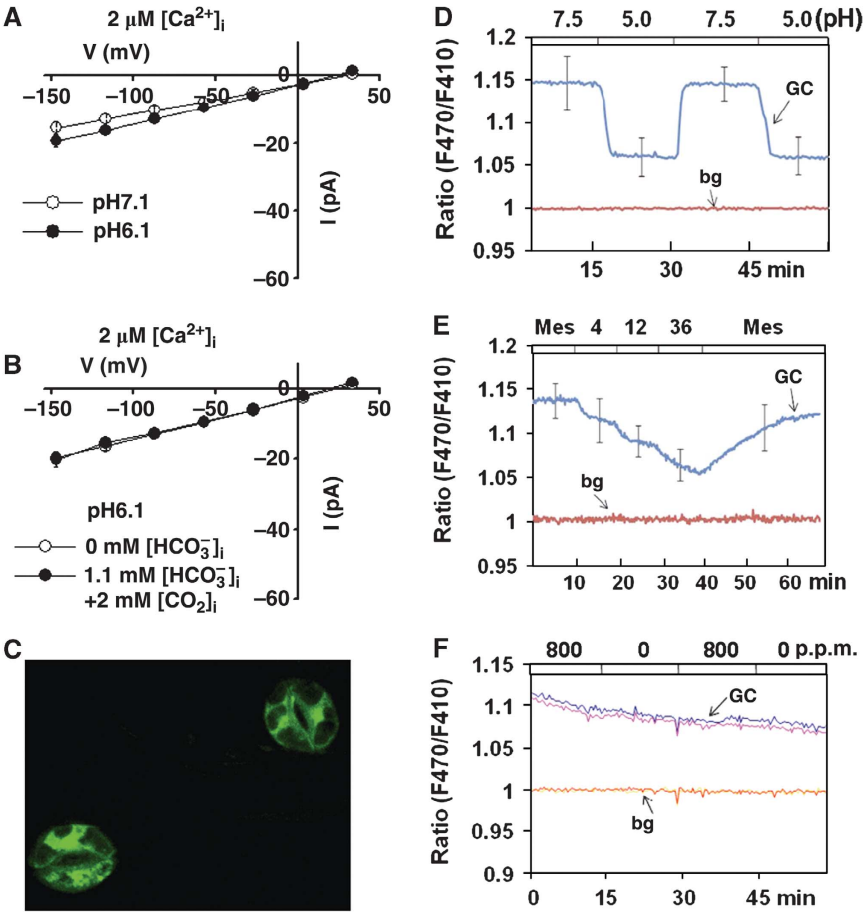

Figure 2 Elevated $[H^+]$ (pH 6.1) together with 2 mM intracellular free $[CO_2]$ did not activate S-type anion channel currents in wild-type Col-0 guard cells when bicarbonate levels were lower. (**A**) Steady-state current–voltage relationships of whole-cell currents recorded in guard cells at 2 μM $[Ca^{2+}]_i$ without bicarbonate in the pipette solution at pH 7.1 (open circles, $n=6$) and pH 6.1 (filled circles, $n=5$). (**B**) Steady-state current–voltage relationships of whole-cell currents at pH 6.1 without bicarbonate (open circles, $n=5$) and with 2 mM intracellular free $[CO_2]$ and 1.1 mM free $[HCO_3^-]_i$ (filled circles, $n=7$) in the pipette solution. Data are mean ± s.e. Liquid junction potential was +1 mV. (**C**) Example image of ratiometric pH sensitive *Pt-GFP* expressed guard cells. (**D**) Average fluorescence ratio time series of six guard cells expressing pH-sensitive reporter *Pt-GFP* during extracellular perfusion with buffers of different pH as indicated by the top bar. (**E**) Average fluorescence ratio time series of *Pt-GFP* expressed in guard cells perfused with MES buffer (10 mM MES, 10 mM KCl, 50 μM $CaCl_2$, pH 5.6) and supplemented with sodium butyrate at mM concentrations as indicated by the top bar of the graph. Data are mean ± s.e. The error bars presented in (**D**, **E**) were computed for the middle data points during each treatment, with the illustrated traces showing the averaged responses. (**F**) Fluorescence ratio time series of *Pt-GFP*-expressing guard cells perfused with extracellular buffers bubbled with 0 p.p.m. CO_2 and 800 p.p.m. CO_2. Raw data of two individual cells are depicted. GC denotes ratiometric fluorescence of guard cells and the ratio of non-guard cell background fluorescence (bg) is shown for the same experiments in (**D**–**F**).

showed that compared with the control recordings (Figure 3A), S-type anion currents were activated by the presence of high free HCO_3^- in the pipette solution (Figure 3B and C, $P<0.05$ at voltages from −146 to −26 mV, Student's *t*-test). Together, the above analyses show that elevated intracellular HCO_3^- is the main molecule that mediates activation of S-type anion currents in guard cells.

Extracellular bicarbonate was next tested on activation of S-type anion currents in wild-type guard cells. After obtaining whole-cell recordings in wild-type guard cells, the bath solution (200 μl) was perfused for 2 min at 1 ml/min with a solution that contained 11.5 mM free $[HCO_3^-]_i$ and 2 mM $[CO_2]$ at pH 7.1 (Supplementary Figure S1A). No large S-type anion currents were activated (Supplementary Figure S1B and C). A small increase in average anion current magnitude was not statistically significant and was not comparable to the clear activation of S-type anion currents by the same concentration of applied intracellular HCO_3^- (Supplementary Figure S1B and C).

Elevated intracellular $[Ca^{2+}]$ is required for bicarbonate activation of S-type anion channel currents in guard cells

The above analyses of activation of S-type anion currents were all conducted at 2 μM cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) (Figures 1–3). We investigated whether the elevated $[Ca^{2+}]_i$ (2 μM) was necessary for bicarbonate activation of S-type anion channel currents in *Arabidopsis* guard cells. At 2 μM $[Ca^{2+}]_i$, anion currents were not strongly activated in the absence of added $[HCO_3^-]_i$ (Figure 4A and G), consistent with previous studies (Allen *et al*, 2002; Siegel *et al*, 2009). In contrast, 11.5 mM free $[HCO_3^-]_i$ activated strong S-type anion channels (Figure 4C and G, $P<0.001$), while an intermediate free $[HCO_3^-]_i$ of 5.75 mM did not activate significant S-type

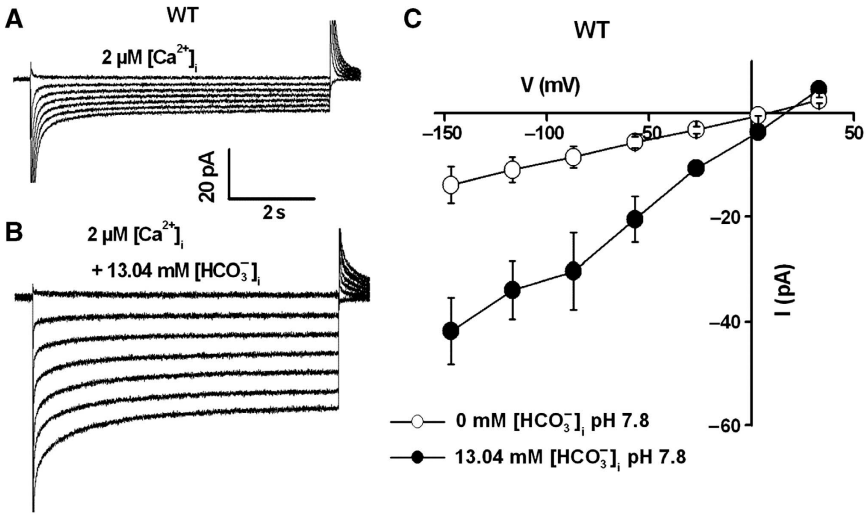

Figure 3 High intracellular $[HCO_3^-]$ at low $[H^+]$ and low free $[CO_2]$ activates S-type anion channel currents in wild-type Col-0 guard cells with 2 μM $[Ca^{2+}]_i$. (**A**) Typical recording of whole-cell currents in guard cell protoplasts without bicarbonate and (**B**) with 13.5 mM total bicarbonate (equivalent to 13.04 mM free $[HCO_3^-]_i$/0.46 mM free $[CO_2]$) added to the pipette solution at pH 7.8. (**C**) Average steady-state current–voltage relationships of whole-cell currents recorded as in (**A**) (open circles, $n=3$) and (**B**) (filled circles, $n=5$). Liquid junction potential was +1 mV. Data are mean ± s.e.

anion currents (Figure 4B and G, $P>0.05$, Student's *t*-test). When $[Ca^{2+}]_i$ was buffered to a baseline level of 0.15 μM even with high 11.5 mM free $[HCO_3^-]_i$ and 2 mM free $[CO_2]$ in the pipette solution (pH 7.1), S-type anion currents were not activated (Figure 4E and G). There was no significant difference between the average amplitudes of current recordings at 0.15 μM free $[Ca^{2+}]_i$ with or without added 11.5 mM free $[HCO_3^-]_i$ (Figure 4G, $P>0.05$, at voltages from −146 to +34 mV). In addition, an elevated cytosolic free $[Ca^{2+}]_i$ of 0.6 μM together with high 11.5 mM free $[HCO_3^-]_i$ and 2 mM free $[CO_2]$ in the pipette solution (pH 7.1) activated anion currents of intermediate average amplitudes (Figure 4F and G). A summary of cytosolic free Ca^{2+} and HCO_3^- activation of S-type anion channels are shown in Supplementary Table I. These data demonstrate a requirement for an elevated $[Ca^{2+}]_i$ in HCO_3^--mediated activation of guard cell anion channels and provide direct and mechanistic evidence for the model that CO_2-induced stomatal closing enhances the ability of $[Ca^{2+}]_i$ to activate stomatal closing mechanisms (Young *et al*, 2006).

Lower [bicarbonate] is sufficient for activation of S-type anion channel currents in ht1-2 guard cells

The *Arabidopsis* HT1 protein kinase functions as a negative regulator of CO_2-induced stomatal closing (Hashimoto *et al*, 2006). To test whether HT1 functions in the CO_2/HCO_3^- SLAC1 signalling pathway (Figures 1–3), the effects of bicarbonate on S-type anion currents in recessive *ht1-2* mutant guard cells were analysed. Whole-cell currents were recorded in guard cell protoplasts at lower intracellular $[HCO_3^-]_i$, 5.75 mM free $[HCO_3^-]_i$ and 1 mM free $[CO_2]$ at pH 7.1, compared with the above experiments (Figure 5A and B). In wild-type control guard cells, these intermediate $[HCO_3^-]_i + [CO_2]$ together with 2 μM free $[Ca^{2+}]_i$ showed small whole-cell current amplitudes that were slightly larger than wild-type guard cells in the absence of added HCO_3^- (Figure 5A, B and E, $P>0.05$, Student's *t*-test) (Hu *et al*, 2010). However, significant activation of S-type anion currents by intracellular addition of 5.75 mM free $[HCO_3^-]_i$ and 1 mM free $[CO_2]$ (pH 7.1) was observed in *ht1-2* guard cells (Figure 5D and E) compared with the control currents (Figure 5A–C and E, $P<0.01$ at voltages from −146 to −26 mV, Student's *t*-test). Note that 2 μM $[Ca^{2+}]_i$ alone in *ht1-2* guard cells was not sufficient to activate S-type anion currents (Figure 5C and E). While cytosolic $[Ca^{2+}]_i$ was buffered to a typical resting level of 0.15 μM in *ht1-2* guard cells, no significant S-type anion current activation was observed in the presence of 5.75 mM free $[HCO_3^-]_i$ (Figure 5F–H, $P>0.05$ at voltages from −146 to −26 mV, Student's *t*-test). Thus, *ht1-2* guard cells show an enhanced sensitivity to intracellular HCO_3^-, but this enhanced activation cannot by-pass the requirement for $[Ca^{2+}]_i$ in HCO_3^- activation of S-type anion currents.

The OST1 kinase functions in bicarbonate activation of S-type anion currents in guard cell protoplasts and CO_2-induced stomatal closure

The OST1 protein kinase was previously demonstrated to mediate ABA-induced stomatal closing. Recessive *ost1* mutants disrupt ABA-induced stomatal closure as well as ABA inhibition of light-induced stomatal opening, but low CO_2 induction of stomatal opening remained unaffected in the *ost1-2* mutant, indicating that OST1 does not participate in CO_2 signalling (Mustilli *et al*, 2002; Yoshida *et al*, 2002). Here, the effect of OST1 on bicarbonate activation of S-type anion channels was investigated. Using the same recording solutions as in Figure 1B, high $[HCO_3^-]_i$ (11.5 mM) and $[CO_2]$ (2 mM) activated only small S-type anion currents in Landsberg *erecta* (L*er*) *ost1-2* mutant guard cells (Figure 6A, B and F). Similar to Col-0 wild-type guard cells (Figures 1, 3 and 4), high HCO_3^- activated S-type anion channel currents in L*er* wild-type guard cells (Figure 6D, E and F). While HCO_3^--activated S-type anion currents in L*er* wild-type guard cells were larger ($I=-51\pm4.3$ pA at a voltage of −146 mV, $n=7$) than that in *ost1-2* mutant guard cells ($I=-25.2\pm1.9$ pA at a voltage of −146 mV, $n=6$) (Figure 6F, $P<0.001$, Student's *t*-test). Moreover, bicarbonate activation of S-type anion

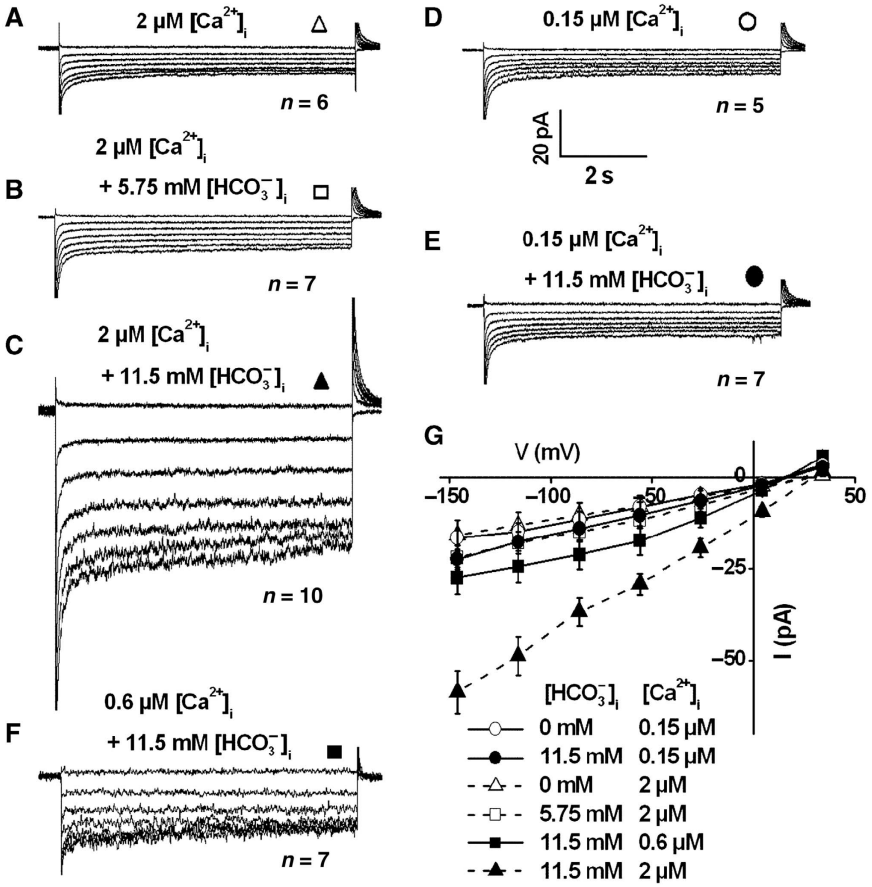

Figure 4 Both $[Ca^{2+}]_i$ and elevated bicarbonate are required for activation of S-type anion channel currents in wild-type (Col-0) guard cells. (**A**) Whole-cell currents in guard cell protoplasts at 2 μM $[Ca^{2+}]_i$ without bicarbonate, (**B**) with 5.75 mM intracellular free $[HCO_3^-]_i$/1 mM free $[CO_2]$ (6.75 mM total bicarbonate added) and (**C**) with 11.5 mM intracellular free $[HCO_3^-]_i$/2 mM free $[CO_2]$ (13.5 mM total bicarbonate added) in the pipette solution at pH 7.1. (**D**) Whole-cell currents in guard cell protoplasts at 0.15 μM $[Ca^{2+}]_i$ without bicarbonate, (**E**) with 11.5 mM free $[HCO_3^-]_i$/2 mM free $[CO_2]$ (13.5 mM total bicarbonate) in the pipette solution at pH 7.1. (**F**) Whole-cell currents in guard cell protoplasts with 0.6 μM $[Ca^{2+}]_i$ and 11.5 mM intracellular free $[HCO_3^-]_i$/2 mM free $[CO_2]$ in the pipette solution at pH 7.1. (**G**) Steady-state current–voltage relationships of whole-cell currents as recorded in (**A**) (open triangles, $n=6$), (**B**) (open square, $n=7$), (**C**) (filled triangles, $n=10$), (**D**) (open circles, $n=5$), (**E**) (filled circles, $n=7$), and (**F**) (filled squares, $n=7$). Average data shown by dashed lines in (**G**) with or without of 5.75 mM and 11.5 mM free $[HCO_3^-]_i$ at 2 μM $[Ca^{2+}]_i$ correspond to data reported in Hu *et al* (2010) and are included for comparison to 0.15 μM and 0.6 μM $[Ca^{2+}]_i$ data. Liquid junction potential was +1 mV. Data are mean ± s.e.

channels was also strongly impaired in Columbia based *ost1-3* T-DNA insertion allele guard cells (Figure 6C and F) compared with Col-0 wild type (Figure 4C and G). At a voltage of −146 mV, the current amplitude activated by bicarbonate in *ost1-3* mutant guard cells was −24 ± 1.9 pA (Figure 6F, $n=6$), and in Col-0 wild type, it was −59 ± 5.9 pA (Figure 4E, $n=10$, $P<0.001$, Student's *t*-test).

Elevated CO_2-induced stomatal closure was also impaired in *ost1-3* mutant leaf epidermes compared with wild-type controls in genotype-blind stomatal movement assays (Figure 7A, $P<0.05$ at 800 p.p.m. CO_2, Student's *t*-test). We next analysed the stomatal conductance changes in intact *ost1-3* mutant leaves in response to $[CO_2]$ shifts. Interestingly, stomatal conductance in *ost1-3* mutant leaves showed a very strong CO_2 insensitivity when the $[CO_2]$ was shifted to high concentrations (Figure 7B; Supplementary Figure S4A). To further investigate the unexpected strong CO_2 insensitivity of *ost1*, whole intact plant gas exchange experiments were pursued and the strong CO_2 insensitivity was observed in *ost1-1*, *ost1-2* and *ost1-3* plants (Figure 7C and D; Supplementary Figure S4B and C).

ABA receptor pyr1;pyl1;pyl2;pyl4 quadruple mutant maintains functional and slightly slower CO_2 response

The PYR/RCAR ABA receptor family was recently identified in *Arabidopsis* as major ABA receptors (Ma *et al*, 2009; Park *et al*, 2009). Since these ABA receptors tightly regulate and form complexes with SnRK2 kinases including OST1 (Fujii *et al*, 2009; Ma *et al*, 2009; Park *et al*, 2009; Nishimura *et al*, 2010), CO_2 regulation of gas exchange in intact *pyr1;pyl1;pyl2;pyl4* quadruple mutant leaves was analysed to determine the requirement of ABA receptors for this CO_2 response. Intact leaves of the *pyr1;pyl1;pyl2;pyl4* quadruple mutant showed clear CO_2 responses upon $[CO_2]$ changes (Figure 8A; Supplementary Figure S4D) and showed an average slight slowing of the CO_2 response, observed in independent experimental sets that was not highly significant ($P=0.1$, Student's *t*-test) at 18 min after 365 to 800 p.p.m. CO_2 transition. Upon shifting $[CO_2]$ from 365 to 800 p.p.m. for 30 min, the initial rates of stomatal conductance changes were −0.038 ± 0.014 mmol H_2O per m^2 per s per min for Col-0 wild-type plants and −0.035 ± 0.008 mmol H_2O per m^2 per s per min for *pyr1;pyl1;pyl2;pyl4* mutant plants ($P=0.24$,

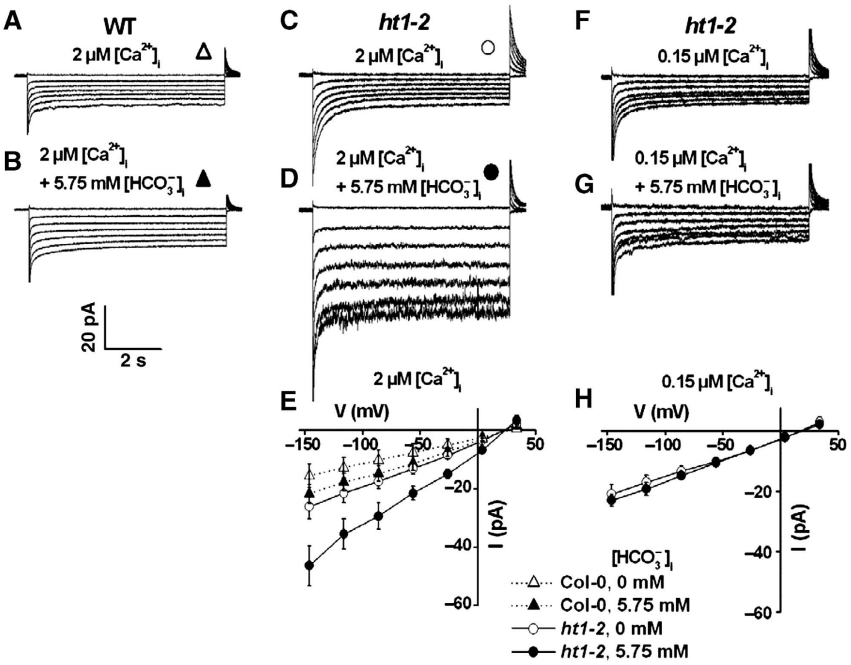

Figure 5 Enhanced bicarbonate sensitivity of S-type anion channel activation in *ht1-2* mutant guard cells only at elevated $[Ca^{2+}]_i$. (**A**) Whole-cell currents in wild-type Col-0 guard cells at 2 μM $[Ca^{2+}]_i$ without bicarbonate and (**B**) with 6.75 mM total bicarbonate (equivalent to 5.75 mM free $[HCO_3^-]_i$/1 mM free $[CO_2]$) added to the pipette solution. (**C**) Whole-cell currents in *ht1-2* mutant guard cells at 2 μM $[Ca^{2+}]_i$ without bicarbonate and (**D**) with 6.75 mM bicarbonate (equivalent to 5.75 mM free $[HCO_3^-]_i$/1 mM free $[CO_2]$) in the pipette solution. (**E**) Average steady-state current–voltage relationships of whole-cell currents as recorded in (**A**) (open triangles, $n=6$), (**B**) (filled triangles, $n=7$), (**C**) (open circles, $n=5$) and (**D**) (filled circles, $n=9$). Average data for wild-type Col-0 controls (WT) shown by dashed lines in (**E**) with 0 and 6.75 mM total bicarbonate (5.75 mM free $[HCO_3^-]$) with 2 μM $[Ca^{2+}]_i$ correspond to data reported in Hu *et al* (2010) and are included for comparison to *ht1-2* mutant data. (**F**) Whole-cell currents in *ht1-2* mutant guard cell protoplasts at low 0.15 μM $[Ca^{2+}]_i$ without bicarbonate and (**G**) with 6.75 mM bicarbonate (equivalent to 5.75 mM free $[HCO_3^-]_i$/1 mM free $[CO_2]$) added to the pipette solution. (**H**) Average steady-state current–voltage relationships of whole-cell currents as recorded in (**F**) (open circles, $n=5$) and (**G**) (filled circles, $n=5$). Liquid junction potential was +1 mV. Data are mean ± s.e.

Student's *t*-test). And during the first 30 min upon shifting $[CO_2]$ from 800 to 100 p.p.m., the initial rates were 0.042 ± 0.013 mmol H_2O per m^2 per s per min for Col-0 wild-type plants and 0.022 ± 0.002 mmol H_2O per m^2 per s per min for *pyr1;pyl1;pyl2;pyl4* mutant plants ($P=0.06$, Student's *t*-test). Further, genotype-blind stomatal movement imaging analyses of individually mapped stomata showed that high CO_2-induced stomatal closure was also retained in *pyr1;pyl1;pyl2;pyl4* mutant leaf epidermes 30 min after CO_2 elevation (Figure 8B).

Discussion

Elevated $[CO_2]$ in leaf intercellular spaces (C_i) and elevated atmospheric $[CO_2]$ cause closing of stomatal pores in diverse plant species (Medlyn *et al*, 2001). CAs have been identified that function early in CO_2 signal transduction (Hu *et al*, 2010). However, major questions in CO_2 signal transduction have arisen. Whether CO_2 or bicarbonate ions or a combination of these function in CO_2 signal transduction in guard cells remained unclear. The presented findings demonstrate that bicarbonate acts as an intracellular signalling molecule in CO_2 signal transduction, by activating SLAC1-mediated S-type anion channels in guard cells. We further found a synergistic action of intracellular HCO_3^- with cytosolic Ca^{2+} that requires both of these small molecules for CO_2 signalling to proceed. We also report the characterization of the cellular functions and relative positions within the CO_2 signal transduction cascade of mutants that strongly affect CO_2 control of stomatal movements, including *ca1;ca4*, *slac1* and *ht1-2*. *ht1-2* mutant guard cells show hypersensitivity to intracellularly applied HCO_3^-, but continue to require cytosolic Ca^{2+} for activation of SLAC1-dependent anion currents. In addition, we have unexpectedly found that loss-of-function mutations in the OST1 protein kinase cause a strong CO_2 insensitivity of stomatal regulation by analyses of S-type anion channel regulation, stomatal movements and gas exchange in intact leaves and in whole plants, which leads to a new model for early CO_2 signal transduction in guard cells.

Central function of the OST1 protein kinase in CO_2 signal transduction

Previous stomatal movement assays indicated that the OST1 protein kinase may not function in CO_2 inhibition of stomatal opening (Mustilli *et al*, 2002). We have found here that *ost1* mutant guard cells in both Col-0 and L*er* accessions show a dramatic impairment in CO_2 regulation of stomatal conductance in intact leaves. Recent studies have shown that the OST1 kinase activates SLAC1 channels via phosphorylation (Geiger *et al*, 2009; Lee *et al*, 2009; Vahisalu *et al*, 2010). Together, our findings of impairment in bicarbonate activation of S-type anion currents in *ost1-2* and *ost1-3* mutant guard cells (Figure 6A, B and D) and the strong impairment in CO_2-induced stomatal closing and stomatal conductance changes in intact leaves and in intact plants

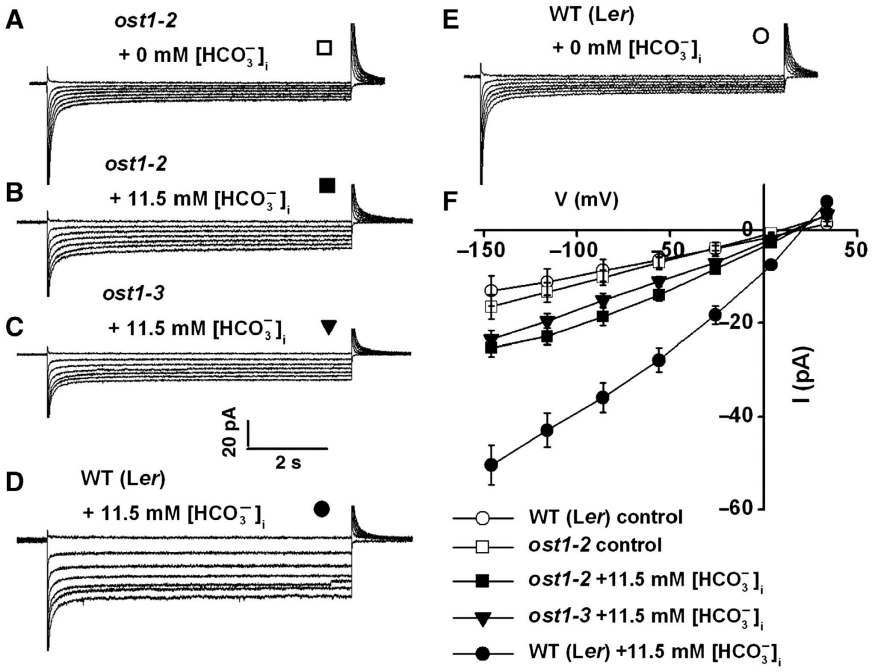

Figure 6 HCO_3^-/CO_2 activation S-type anion channel currents is disrupted in *ost1-2* and *ost1-3* mutant guard cells with 2 μM $[Ca^{2+}]_i$. (**A**) Whole-cell recording without and (**B**) with 13.5 mM total bicarbonate (11.5 mM free $[HCO_3^-]_i$ + 2 mM free $[CO_2]$) added to the pipette solution in *ost1-2* mutant guard cells. (**C**) Whole-cell recording with 13.5 mM total bicarbonate in the pipette solution in *ost1-3* mutant guard cells. (**D**) Whole-cell currents with 13.5 mM total bicarbonate and (**E**) without bicarbonate added to the pipette solution in wild-type L*er* guard cell protoplasts. (**F**) Steady-state current–voltage relationships of recordings as in (**A**) (open squares: *ost1-2*, $-[HCO_3^-]_i$, $n=5$), (**B**) (filled squares: *ost1-2*, $+[HCO_3^-]_i$, $n=6$), (**C**) (filled triangles: *ost1-3*, $+[HCO_3^-]_i$, $n=6$), (**D**) (filled circles: wild-type L*er*, $+[HCO_3^-]_i$, $n=7$) and (**E**) (open circles: wild-type L*er*, $-[HCO_3^-]_i$, $n=5$). The pipette solution was adjusted to pH 7.1 in all the recordings. Liquid junction potential was +1 mV. Data are mean ± s.e.

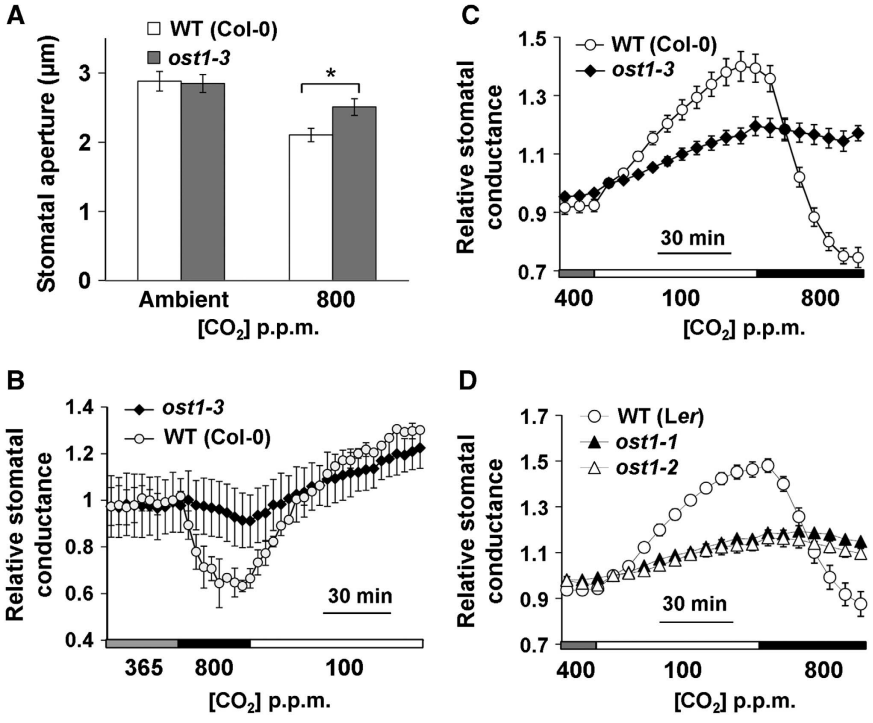

Figure 7 CO_2-induced stomatal closure is strongly impaired in *ost1* mutants. (**A**) Stomatal closure is impaired in *ost1-3* mutant leaves in response to elevated $[CO_2]$. *$P<0.05$, Student's *t*-test. (**B**) Time-resolved relative stomatal conductance in responses to $[CO_2]$ in *ost1-3* mutant and wild-type Col-0 intact leaves ($n=4$ for each genotype). (**C**) Patterns of whole-plant relative stomatal conductance in responses to changes in $[CO_2]$ in intact *ost1-3* and Col-0 wild-type plants ($n=8$ for *ost1-3*, $n=6$ for WT) and (**D**) in intact *ost1-1*, *ost1-2* and L*er* wild-type plants ($n=4$ for each genotype). Imposed CO_2 concentrations are shown at the bottom. Data are mean ± s.e.

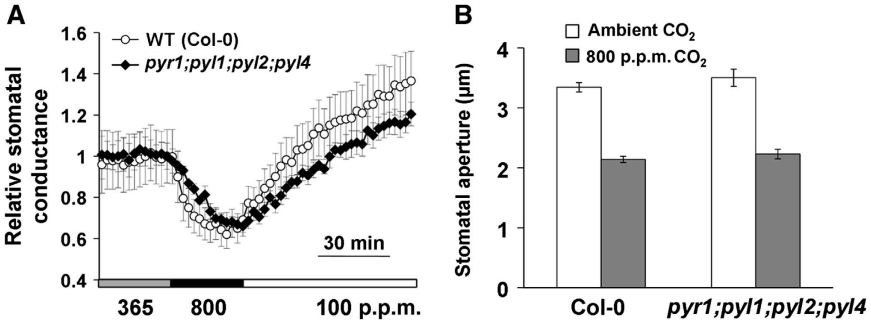

Figure 8 CO_2-induced stomatal closure is slightly slowed in ABA receptor *pyr1;pyl1;pyl2;pyl4* (Col based) quadruple mutant. (**A**) ABA receptor *pyr1;pyl1;pyl2;pyl4* quadruple mutant does not abrogate but slows CO_2 regulation of stomatal conductance in intact leaves ($n = 4$ for each genotype). (**B**) *pyr1;pyl1;pyl2;pyl4* quadruple leaf epidermes retained robust responses to elevated [CO_2] ($n = 30$ stomata for each genotype, genotype blind). For stomatal movement analyses in (**B**) individual stomata were imaged and tracked as previously reported (Siegel *et al*, 2009). Data are mean ± s.e.

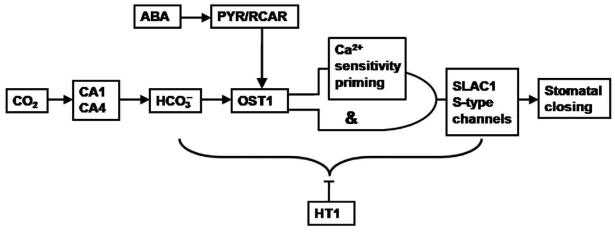

Figure 9 Model for the mechanisms characterized in the present study showing the sequence of events that mediate CO_2 regulation of S-type anion channels and stomatal closing. In this model, CA1/CA4, OST1 and SLAC1 function as positive mediators, and the HT1 protein kinase functions as a negative regulator of high CO_2-incuded stomatal closing. $[Ca^{2+}]_i$ sensitivity priming and $[Ca^{2+}]_i$-independent mechanisms are proposed to regulate SLAC1-dependent S-type anion currents in parallel via an 'AND'-like gate. In this 'AND'-like gate, one 'input' occurs via the OST1 pathway, and the other 'input' is mediated by the Ca^{2+} sensitivity priming pathway. Convergence with abscisic acid (ABA) signalling is also indicated here. CA, carbonic anhydrase; OST1, open stomata 1; HT1, high leaf temperature 1; SLAC1, slow anion channel; PYR/RCAR, ABA receptors.

(Figure 7B–D) show that the OST1 protein kinase is a central transducer of CO_2 signal transduction in guard cells.

The PYR/RCAR ABA receptors form a linear signal transduction module together with type 2C protein phosphatases and the OST1 protein kinase (Fujii *et al*, 2009; Ma *et al*, 2009; Park *et al*, 2009; Santiago *et al*, 2009; Umezawa *et al*, 2009; Nishimura *et al*, 2010). A quadruple mutant in four highly expressed guard cell ABA receptors *pyr1;pyl1;pyl2;pyl4* shows a strong impairment in ABA-induced stomatal closing (Nishimura *et al*, 2010). In contrast CO_2 regulation remained functional, albeit slowed, in intact leaves (Figure 8). These data lead to an updated model for early CO_2 signal transduction in which the convergence point of CO_2 and ABA signal transduction occurs earlier than previously thought at the level of the OST1 protein kinase or earlier (Figure 9). The CO_2 response of *pyr1;pyl1;pyl2;pyl4* quadruple mutant plants exhibited an average slowing compared with wild-type plants (Figure 8). This may be attributable to the early convergence of CO_2 and ABA signalling at the level of the OST1 protein kinase as revealed here. Thus, a degree of cross-talk between ABA and CO_2 signalling can be expected. Classical studies have shown that very low subthreshold concentrations of ABA do not cause an ABA response, but amplify CO_2-induced stomatal closing (Raschke, 1975). Our findings provide a mechanistic basis for this classical observation, with both CO_2 and ABA signal transduction occurring via the OST1 protein kinase (Figure 9), as *ost1* mutant alleles show both strong CO_2 (Figure 7) and ABA insensitivities (Mustilli *et al*, 2002; Yoshida *et al*, 2002).

The dominant protein phosphatase 2C (PP2C) mutants, *abi1-1* and *abi2-1*, have been reported to conditionally affect CO_2 signalling in guard cells (Webb and Hetherington, 1997; Leymarie *et al*, 1998a, b). ABI1 interacts with the OST1 protein kinase (Belin *et al*, 2006; Yoshida *et al*, 2006; Umezawa *et al*, 2009; Vlad *et al*, 2009; Nishimura *et al*, 2010). Further research is needed to determine whether the wild-type PP2C-protein phosphatases control the CO_2 response. The present study on CO_2 signalling and research indicating ABA-independent activation of the OST1 protein kinase (Yoshida *et al*, 2006; Zheng *et al*, 2010) indicates that the early ABA signalling module consisting of ABA receptors (Ma *et al*, 2009; Park *et al*, 2009), PP2Cs and OST1/SnRK2 kinases may be more complex than the present models (Fujii *et al*, 2009).

Bicarbonate activates S-type anion channels

In mammalian cells, CO_2 detection in olfactory receptor neurons require the expression of the receptor-type guanylate cyclase GC-D (Hu *et al*, 2007), which is activated by the CO_2 metabolite bicarbonate (Guo *et al*, 2009; Sun *et al*, 2009), that is catalytically produced by CAs (Hu *et al*, 2007). Here, elevated bicarbonate activation of S-type anion currents in *ca1;ca4* double-mutant guard cells (Figure 1) is consistent with the model that βCA1 and βCA4 act very early in the guard cell CO_2 signal transduction pathway (Figure 9). S-type anion channel activation by bicarbonate reported here (Figure 3) shows similar properties to SLC26A9 channels in mammalian epithelial cells. SLC26A9 encodes a Cl^- channel and is modulated by HCO_3^- (Loriol *et al*, 2008). Expression of SLC26A9 in *X. laevis* oocytes, produced Cl^- currents that increased in magnitude in the presence of 24 mM HCO_3^- compared with 2.4 mM HCO_3^-. Furthermore, the SLC26A9 channel has no HCO_3^- permeability and is not regulated by intracellular pH (Loriol *et al*, 2008). In *Arabidopsis* hypocotyl cells, bicarbonate is permeable through voltage-dependent anion channels (R-type anion channels) with a relative permeability ratio $P_{HCO_3^-}/P_{Cl-}$ of 0.8 (Frachisse *et al*, 1999).

Different from that, the SLAC1 channel is impermeable to HCO_3^- (Geiger *et al*, 2009), and our analyses of S-type anion currents also support this (Supplementary Figure S2). SLAC1 channels were not activated by bicarbonate when SLAC1 was heterologously expressed alone in *X. laevis* oocytes (Geiger *et al*, 2009). This can be explained by our findings that bicarbonate activation of S-type anion channel in planta requires other essential components, in particular the OST1 protein kinase and elevated $[Ca^{2+}]_i$, with the HT1 protein kinase functioning as a negative regulator within this module of the CO_2 signal transduction cascade (Figures 4–6 and 9). Further research will be needed to identify the bicarbonate-binding proteins that mediate this response.

The intracellular concentrations of bicarbonate and CO_2 used in patch-clamp experiments in the present study for S-type anion channel activation were higher than physiological concentrations in planta. Note that patch clamping of guard cells includes dialysis of the cytoplasm (Hamill *et al*, 1981) and it is possible that additional diluted small molecules or proteins are required for full sensitivity of this HCO_3^- response. Furthermore, typically high CO_2 and HCO_3^- concentrations are used in electrophysiological studies, up to 72 mM HCO_3^- (Loriol *et al*, 2008; Chandrashekar *et al*, 2009; Yarmolinsky *et al*, 2009), although these experiments were conducted in different systems. The close correlation of high HCO_3^- regulation of S-type anion channels in the present study and the impaired CO_2 response phenotypes in intact leaves of the *Arabidopsis ca1;ca4*, *slac1*, *ht1-2* and *ost1* mutants (Figures 6 and 7) and the $[Ca^{2+}]_i$ sensitivity of this response (Figure 4) suggest that the analysed intracellular HCO_3^- regulation responses are physiologically relevant (Schwartz, 1985; Webb *et al*, 1996; Hashimoto *et al*, 2006; Young *et al*, 2006; Negi *et al*, 2008; Vahisalu *et al*, 2008; Hu *et al*, 2010).

Intracellular acidification activates slow anion channel currents in the plasma membrane of *Arabidopsis* hypocotyl cells (Colcombet *et al*, 2005). However, intracellular acidification did not activate S-type anion currents in *Arabidopsis* guard cells, even in the presence of elevated 2 μM free $[Ca^{2+}]_i$ (Figure 2A). In animal chemosensitive neurons, intracellular pH is lowered in response to increasing CO_2 levels from 10% up to 50% $[CO_2]$ (Putnam *et al*, 2004). Using the pH-sensitive dye BCECF (2′,7′-bis-(2-carboxyethyl)-5,6-carboxyflourescein) and fluorescence microphotometry to measure cytosolic pH in *V. faba* guard cells, no significant pH change was observed during transition from 0 to 1000 p.p.m. CO_2 (Brearley *et al*, 1997). Our findings correlate with the previous study as no detectable pH changes were observed in guard cells expressing the ratiometric pH sensor *Pt-GFP* when intact leaf epidermes were perfused with buffers bubbled with 0 and 800 p.p.m. CO_2 (Figure 2F). These data are also compatible with models proposing (Raschke *et al*, 1988) and findings from pH measurements showing (Grabov and Blatt, 1997) a high pH buffering capacity of *V. faba* guard cells.

CO_2 enhances the $[Ca^{2+}]_i$ sensitivity of S-type anion channel activation

Calcium is a second messenger that transduces diverse stimuli in plants (Sanders *et al*, 1999; Blatt, 2000; Hetherington and Brownlee, 2004; Kim *et al*, 2010; Kudla *et al*, 2010). Elevated CO_2 caused an increase in $[Ca^{2+}]_i$ in *Commelina Communis* guard cells (Webb *et al*, 1996). Furthermore, elevated CO_2 caused a dampening of spontaneous repetitive $[Ca^{2+}]_i$ transients, whereas low CO_2 caused rapid $[Ca^{2+}]_i$ transients in *Arabidopsis* guard cells (Young *et al*, 2006), which can be attributed to CO_2-induced depolarization of guard cells (Grabov and Blatt, 1998; Staxen *et al*, 1999; Klusener *et al*, 2002). In both plant species, abolishment of $[Ca^{2+}]_i$ elevations abolished CO_2-induced stomatal closing (Schwartz, 1985; Webb *et al*, 1996; Young *et al*, 2006). Time-resolved $[Ca^{2+}]_i$ imaging experiments led to the Ca^{2+} sensitivity priming hypothesis, in which CO_2 was hypothesized to enhance (prime) the Ca^{2+} sensitivity of signalling mechanisms that relay CO_2-induced stomatal closure (Young *et al*, 2006). However, additional and direct evidence for this CO_2 signalling hypothesis has been lacking. Recent studies showed that ABA enhances (primes) the $[Ca^{2+}]_i$ sensitivity of S-type anion channel and K_{in}^+ channel regulation, strongly supporting the hypothesis that ABA primes $[Ca^{2+}]_i$ signal transduction (Siegel *et al*, 2009).

ABA increases cytosolic Ca^{2+} concentration by activating plasma membrane Ca^{2+} channels in *V. faba* and *Arabidopsis* guard cells (Schroeder and Hagiwara, 1990; Grabov and Blatt, 1998; Hamilton *et al*, 2000; Pei *et al*, 2000; Murata *et al*, 2001). Cytosolic $[Ca^{2+}]_i$ interacts with other signalling molecules including nitric oxide (Garcia-Mata *et al*, 2003) and cytosolic pH_i (Grabov and Blatt, 1997) in ion channel regulation in guard cells. Recently, Chen *et al* (2010) showed that cytosolic free $[Ca^{2+}]_i$ interacts with protein phosphorylation events during slow anion channel activation.

The present study shows that elevated bicarbonate enhances the $[Ca^{2+}]_i$ sensitivity in S-type anion channel activation (Figure 4). ABA- and Ca^{2+}-induced activation of S-type anion channels and stomatal closing are mediated by Ca^{2+}-dependent protein kinases (CDPKs) (Mori *et al*, 2006; Zhu *et al*, 2007; Geiger *et al*, 2010). Heterologous reconstitution analysis has proposed that ABA activates anion channels by the OST1 protein kinase, in parallel through a Ca^{2+}-dependent CDPK pathway (Geiger *et al*, 2010). Together with previous studies (Allen *et al*, 2002; Israelsson *et al*, 2006; Young *et al*, 2006; Siegel *et al*, 2009; Chen *et al*, 2010; Hu *et al*, 2010), the present findings provide strong evidence that Ca^{2+} sensitivity priming is a mechanism that controls both CO_2 and ABA regulation on S-type anion channels (Figure 9). Interestingly, here patch-clamped guard cell protoplasts were exposed to elevated HCO_3^-/CO_2 in the pipette solution for only ~3 to 5 min prior to analysing $[Ca^{2+}]$ activation of S-type anion currents (Figure 4C and G), whereas ABA signalling studies tested 30 min ABA pre-incubation (Siegel *et al*, 2009). This rapid 3 to 5 min $HCO_3^-/CO_2-[Ca^{2+}]_i$ response provides first evidence that Ca^{2+} sensitivity priming is a rapid modification and that transcriptional and translational mechanisms may not mediate Ca^{2+} sensitivity priming.

ht1 kinase mutant enhances bicarbonate sensitivity but requires $[Ca^{2+}]_i$

The HT1 protein kinase functions as a negative regulator of CO_2 signalling (Hashimoto *et al*, 2006) and our recent study showed that HT1 is epistatic to βCA1 and βCA4 in the CO_2 response pathway (Hu *et al*, 2010). However, the role of HT1 within the guard cell signalling network had not been further analysed. The *ht1-2* mutant exhibits a hypersensitive

response in bicarbonate activation of S-type anion currents, confirming that the HT1 kinase functions as a negative regulator and demonstrating that HT1 affects CO_2 signalling downstream of HCO_3^- production and upstream of anion channel activation (Figure 9). Cytosolic Ca^{2+} elevation is still required for S-type anion channel activation in *ht1-2* mutant guard cells, showing that HT1 kinase-mediated CO_2 signalling does not by-pass Ca^{2+} sensitivity priming (Figures 5 and 9).

In conclusion, the present study identifies the OST1 protein kinase and the synergistic roles of the intracellular small molecules HCO_3^- and Ca^{2+} in guard cell CO_2 signal transduction and anion channel regulation. Furthermore, characterization of the positions and roles of OST1, the HT1 protein kinase, the βCA1 and βCA4 CAs, PYR/RCAR ABA receptors, and SLAC1 in CO_2 regulation of S-type anion channels, leads to a revised model for CO_2 signal transduction (Figure 9). During CO_2-induced stomatal closing, CO_2 is first catalysed by CAs into bicarbonate. Elevated bicarbonate activates S-type anion channels via an 'AND'-like gate (Figure 9). In the 'AND'-like gate, one 'input' occurs via the OST1 pathway, and the other 'input' is mediated by the Ca^{2+} sensitivity priming pathway. The HT1 kinase acts as a negative regulator in the CO_2 signalling pathway downstream of HCO_3^- production and upstream of S-type anion channel activation, which continues to require $[Ca^{2+}]_i$. PYR/RCAR ABA receptors do not directly mediate guard cell CO_2 signalling, but mutation slows CO_2 responses, indicating that they function upstream of the convergence point of CO_2 and ABA signalling, while affecting common downstream signalling mechanisms (Figure 8). The OST1 protein kinase is an essential mediator of guard cell CO_2 signal transduction, providing evidence that mechanisms in addition to ABA can activate OST1-dependent signalling (Figures 6 and 7).

Materials and methods

Plant growth

The *Arabidopsis* mutant lines analysed in this study were *ca1;ca4* (Hu *et al*, 2010), *slac1-1*, *slac1-3* (Vahisalu *et al*, 2008), *ht1-2* (Hashimoto *et al*, 2006), *ost1-1*, *ost1-2* (Mustilli *et al*, 2002), *ost1-3* (Yoshida *et al*, 2002) and *pyr1;pyl1;pyl2;pyl4* in the backcrossed Col-0 background (Nishimura *et al*, 2010). Plants were grown in a Conviron growth chamber at 21°C, 65–85% humidity, except *abi1-1* and *abi2-1* were grown constantly at 75–85% humidity, and a 16-h light/8-h dark photoperiod regime at ~75 μmol/m^2 s.

Electrophysiology

Arabidopsis guard cell protoplasts were isolated as described previously (Leonhardt *et al*, 2004; Siegel *et al*, 2009). Whole-cell patch-clamp experiments were performed as described previously (Pei *et al*, 1997). During recordings of S-type anion currents, the membrane voltage was stepped to potentials starting at +35 to −145 mV for 7 s with −30 mV decrements and the holding potential was +30 mV. The interpulse period was 5 s. Liquid junction potentials were determined using Clampex 10.0. No leak subtraction was applied for all current–voltage curves. Steady-state currents were the average currents during the last 500 ms of pulses. Detail contents of solutions can be found in Supplementary data. Bicarbonate ($CsHCO_3$) was freshly dissolved in the pipette solution before patch-clamp experiments and pH was adjusted to the indicated values. The pipette solution was stored using air-tight precision glass syringes during patch-clamp experiments to slow CO_2 equilibration with the surrounding air and was not stored overnight. The concentrations of free CO_2 and bicarbonate in solutions were calculated using the Henderson–Hasselbalch equation ($pH = pK_1 + \log[HCO_3^-]/[CO_2]$) (Hauser *et al*, 1995). $[HCO_3^-]$ represents the free bicarbonate concentration; $[CO_2]$ represents the free CO_2 concentration. A value, $pK_1 = 6.352$, was used for calculations (Speight, 2005). To independently measure CO_2 concentrations in the solutions at different pH values, an InPro 5000 CO_2 sensor (Mettler Toledo 400, Mettler Toledo Inc.) was used for dissolved CO_2. The significance of differences between data sets was assessed by noncoupled double-tailed Student's *t*-test analysis. Values of $P < 0.05$ were considered statistically significant.

Expression of pH sensor Pt-GFP in Arabidopsis guard cells

The *Pt-GFP* cDNA was amplified with the primers PGF (5′-AACCATGGCGCAGACCCTTCCTCTAT-3′, with *Nco*I site) and PGR (5′-AACTGCAGAGGCGTCTCGCATATCTC-3′, with *Pst*I site) from the construct pART7-*Pt-GFP* (Schulte *et al*, 2006), kindly provided by Dr Christoph Plieth. The sequenced PCR product was digested with *Nco*I and *Pst*I and then subcloned into the binary expression vector *pGreenII 0179-pGCP(D1)-terminator* under the control of guard cell specific promoter *pGC1* (Yang *et al*, 2008). The construct *pGC1*::*Pt-GFP* was transformed to the *Agrobacterium* strain GV3101-containing helper plasmid *pSOUP* and then was introduced into *Arabidopsis* (Col-0) by the floral dip method (Clough and Bent, 1998).

Fluorescence imaging of guard cells expressing Pt-GFP

Fluorescence imaging was performed with a TE300 inverted microscope using a TE-FM Epi-Fluorescence attachment (Nikon) as previously described (Allen *et al*, 2000). Fluorescence images at excitation wavelengths of 470 and 440 nm were taken every 2 s using light from a 75-W xenon short arc lamp (Osram, Germany). In all, 32′ neutral density filters were used to reduce bleaching of fluorescent reporter. Metafluor software (MDS, Inc.) was used to control filter wheels, shutter and CoolSNAP CCD camera from Photomerics when recording and also processing raw data. The fluorescence ratio F470/F440 of Pt-GFP was analysed as a detection of pH shifts. Intact epidermes from *pGC1*::*Pt-GFP*-expressing leaves were prepared and affixed to glass Coverslips using Medical Adhesive (Hollister Incorporated Libertyville, IL) and then adhered to a glass slide with a hole in the middle generating a well, as described (Young *et al*, 2006; Siegel *et al*, 2009; Hu *et al*, 2010).

For recording intracellular *Pt-GFP* fluorescence in response to changes in extracellular pH incubation buffers, the pH of incubation buffers containing 10 mM MES, 10 mM KCl and 50 μM $CaCl_2$ at 5.0 and 7.5 was adjusted by adding Tris–HCl. The well was perfused with incubation buffer at pH 5.0 (or pH 4.5) for 15 min to obtain a background value and subsequently perfused with buffer at pH 7.5 for 15 min and returned to pH 5.0 (or pH 4.5) again. For recording intracellular *Pt-GFP* fluorescence in response to constant extracellular pH and added weak acid, the perfusion buffers contained 10 mM MES, 10 mM KCl and 50 μM $CaCl_2$, pH 5.6 supplemented with the indicated concentrations of sodium butyrate. For recording the *Pt-GFP* fluorescence of guard cells in response to CO_2 changes, the incubation buffer (10 mM MES, 10 mM KCl and 50 μM $CaCl_2$, pH 6.15) was continually bubbled with 800 p.p.m. CO_2 or bubbled with air through soda lime, which was considered as nominal 0 p.p.m. CO_2 inside the buffer. Note that the final CO_2 concentrations to which leaf epidermes were exposed were as reported previously using the same experimental set up and conditions (Young *et al*, 2006). The well was perfused with buffers shifting from 800 to 0 p.p.m. CO_2 via a peristaltic pump and teflon tubing. Background fluorescence intensities at 470 nm were measured in regions lacking guard cells and are also shown for the corresponding experiments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

UCSD has submitted a patent application based on some of the findings in this study.

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