Stomatal responses to carbon dioxide and light require ABA catabolism in Arabidopsis

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In plants, stomata control water loss and CO_2 uptake. The aperture and density of stomatal pores, and hence the exchange of gases between the plant and the

atmosphere, is controlled by internal factors such as the plant hormone abscisic acid (ABA) and external signals including light and CO_2. In this study we examine the importance of ABA catabolism in the stomatal responses to CO_2 and light. By using the ABA 8′-hydroxylase-deficient *Arabidopsis thaliana* double mutant *cyp707a1 cyp707a3* which is unable to break down and instead accumulates high levels of ABA, we reveal the importance of the control of ABA concentration in mediating stomatal responses to CO_2 and light. Intriguingly, our experiments suggest that endogenously produced ABA is unable to close stomata in the absence of CO_2. Furthermore, we show that when plants are grown in short day conditions ABA breakdown is required for both the modulation of elevated [CO_2]-induced stomatal closure, and elevated [CO_2]-induced reductions in leaf stomatal density. ABA catabolism is also required for the stomatal density response to light intensity, and for the full range of light-induced stomatal opening, suggesting that ABA catabolism is critical for the integration of stomatal responses to a range of environmental stimuli.

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Introduction

Stomata are pores found on the aerial surfaces of land plants. Each stoma is bound by a pair of guard cells which, through changes in turgor, regulate the aperture of the central pore. Controlling stomatal apertures allows the plant to regulate water loss and the uptake of CO_2 from the atmosphere[1, 2]. Thus, stomata play important roles in optimising plant growth, survival and reproductive capacity through regulating photosynthesis and water loss and influencing nutrient uptake and leaf cooling. The optimisation of stomatal gas exchange relies on two important processes; one physiological and the other developmental. Firstly, changes in guard cell turgor bring about stomatal opening in the light when soil water is plentiful and closure in the dark or when soil water availability is

limited[3, 4]. Secondly, plants are able to adjust their stomatal density so that when they are growing under conditions where high levels of gas exchange could be advantageous (*e.g.* at high light levels or low $[CO_2]$) they produce leaves with a high density of stomata, and when growing under conditions where lower levels of gas exchange could be advantageous (*e.g.* at low light levels or high $[CO_2]$) they produce leaves with a lower density of stomata[5]. Light intensity and quality regulate stomatal density through the red far-red absorbing phytochromes[6] and the cryptochrome blue light photoreceptors[5, 7, 8]. How plants integrate the multitude of environmentally variable signals to optimally adjust their stomatal apertures, and also over a longer timeframe their stomatal density, are current topics of research which have wide implications for responses to predicted anthropogenic changes in global $[CO_2]$ and water availability. Furthermore, such studies have the potential to identify useful targets for future crop breeding under climate change.

The plant hormone abscisic acid (ABA) is a major regulator of plant responses to environmental stresses, and has long been known to reduce stomatal apertures under drought conditions[8]. ABA-induced guard cell closure mechanisms and their cross-talk with CO_2 signalling are becoming well-characterised[1, 9, 10]. *Arabidopsis thaliana* (Arabidopsis) plants deficient in ABA biosynthesis or ABA perception and signalling can be compromised in their ability to shut their stomatal pores in response to elevated $[CO_2]$[10, 11]; although other studies while supporting a role for ABA, suggest that there is not a role for ABA receptors and some ABA signalling proteins[12-15]. ABA has also emerged as an important regulator of stomatal development[1, 13, 16-18]. Growth of plants at elevated $[CO_2]$ or in the presence of exogenous ABA generally results in plants with lower stomatal densities[17, 19-22]. Mutants deficient in ABA biosynthesis and signalling have high stomatal densities[13] and can fail to reduce stomatal densities in response to elevated $[CO_2]$[10, 23]. In Arabidopsis, $[CO_2]$ perception feeds into the core signalling pathway controlling stomatal development[17] via a pathway that remains to be fully characterised but involves the activity of putative β-keto acyl CoA synthase encoded by

the *HIC* gene[21], the Epidermal Patterning Factor 2 (EPF2) peptide, the CO_2 Response Secreted Protease (CRSP) and the carbonic anhydrases CA1 and CA2[24, 25]. ABA's role in the stomatal density response to [CO_2] has been shown[10, 17], but the mechanisms by which it acts are unknown.

Although ABA and ABA signalling are required for stomatal [CO_2] signalling[10, 11], the role of ABA catabolism in [CO_2] perception and stomatal responses remains contentious[14, 15, 26]. Plants are known to tightly control the metabolism of ABA to modulate responses to changes in humidity and water availability[17], and ABA catabolism is important for reducing endogenous ABA concentrations upon the cessation of water deficit[27]. Under water deficit stress, ABA is synthesised in and imported to guard cells where it induces stomatal closure and inhibits re-opening[28]. This reduces transpirational water loss, but also restricts the uptake of CO_2 through stomata, thus inhibiting photosynthesis and growth. Upon rehydration, the export of ABA, the breakdown of ABA in the guard cell by ABA 8′-hydroxylases, or the conjugation of ABA by ABA glucosyl transferase activity must occur to permit re-opening of stomata and increased take up of CO_2[14, 26]. ABA is catabolized to phaseic acid (PA) and dihydrophaseic acid (DPA) by the action of CYP707A family cytochrome P450 monooxygenases (P450s)[29, 30]. PA and DPA accumulate under prolonged water deficit and may underpin the plant's resilience to longer-term drought stress by maintaining some of the biological activity of non-hydroxylated ABA[23, 29, 31].

Humidity has been shown to be a strong regulator of the ABA catabolic enzyme, ABA 8′-hydroxylase[27, 32]. In Arabidopsis, two ABA 8′-hydroxylases, CYP707A1 and CYP707A3, play key roles in modulating ABA levels in stomata and vascular tissues, respectively, in response to water stress and humidity[32-34]. The double mutant *cyp707a1 cyp707a3* (*cyp707a1/a3*) has approximately 30%[34] to 400%[27] higher levels of ABA, and a lower stomatal index and density in comparison to wild-type Arabidopsis[13, 16]. Merilo *et al.*

reported wild-type-like aperture responses to [CO_2] in *cyp707a1* and *cyp707a3* single mutants[15], and in *cyp707a1/a3* double mutants[13], despite the fact that they contain substantially higher levels of ABA. The authors argue that because they observed no differences in aperture widths between *cyp707a1/a3* and wild-type, other traits beside aperture must be responsible for the reduced stomatal conductances they reported in these mutants[13]. Merilo *et al.* (2015) also show that other mutants in ABA transport and recycling are not impaired in their stomatal responses to [CO_2] or humidity, including ABA exporter mutants *abcg22*, *abcg25*, *abcg27*, and *abcg40*, ABA importer mutant *ait*, and ABA recycling mutants *bg1* and *bg2* [14].

Here we use the double mutant *cyp707a1/a3* to further investigate the role of ABA catabolism in stomatal responses to CO_2 and light. We show that the capacity for ABA catabolism is required for elevated [CO_2]-induced stomatal closure and elevated [CO_2]-induced reductions in leaf stomatal density. We also report that ABA 8′-hydroxylases CYP707A1 and CYP707A are required for the stomatal density response to light intensity, suggesting that the level of ABA in vegetative tissues is critical for the integration of several environmental responses.

Results and Discussion

Stomatal aperture response to [CO_2]

We measured the apertures of stomatal pores following exposure of isolated epidermal strips to differing levels of [CO_2] using a previously described bioassay[35]. The results in Fig. 1a and 1b show that stomata of the *cyp707a1/a3* mutant, which has impaired ability to break down ABA, were significantly more closed at both ambient and elevated levels of CO_2 than WT (ANOVA, *P* = 0.0211, *P* = 0.0240 respectively). As the stomatal apertures of both WT and *cyp707a1/a3* mutant were open to the same extent under low CO_2, these data suggest that the *cyp707a1/a3* mutant exhibits a hypersensitive response to [CO_2] because of higher endogenous levels of ABA[27, 32]. This stomatal

hypersensitivity appears to be in contrast to previous reports of relatively normal $[CO_2]$-mediated stomatal responses in this and other ABA catabolism mutants. The discrepancies between our results and the investigations of Merilo *et al.*[15] may arise from differences between whole rosette gas exchange measurements and epidermal peel measurements of apertures (although both labs used peels), but it more probably arises from the different plant growth conditions. In the single mutants, wild-type stomatal aperture responses to CO_2 could be attributed to their ability to break down ABA within guard cells in the case of *cyp707a3* and within the vasculature in the case of *cyp707a1* [27]; this spatially distinct activity potentially masking the effect of high ABA concentrations on guard cell $[CO_2]$ responses. However, this does not account for the *cyp707a1/a3* aperture differences. Given the guard cell $[CO_2]$ hypersensitivity observed in our experiments at ambient and elevated $[CO_2]$, it is surprising that *cyp707a1/a3* apertures were indistinguishable from wild-type in $[CO_2]$-free air conditions (Fig. 1). This observation is intriguing as exogenous ABA has previously been shown to close stomata in many similar experiments using epidermal strips provided with CO_2-free air[36]. This further suggests that the precise location of the ABA signal is important for the guard cell $[CO_2]$-response, and that under CO_2-limiting conditions exogenous application of ABA may be more potent in triggering stomatal closure than endogenous ABA accumulated within guard cells. These results may reflect the relative contributions of mesophyll versus guard cell ABA production and breakdown[37], and suggest that the location of ABA biosynthesis is likely to be as important as the location of degradation, for aperture control. This is supported not only by vascular-localised NCED3 for ABA production, but also by the relative contributions of vascular-localised CYP707A3 versus guard-cell localised CYP707A1 to whole leaf ABA break-down[27].

The importance of ABA levels in the modulation of stomatal $[CO_2]$ responses was first demonstrated almost forty years ago in a series of experiments performed by Raschke, Farquhar, Dubbe and others. Raschke observed that open stomata of *Xanthium*

strumarium are insensitive to [CO_2] but become [CO_2] -responsive when either supplied with ABA or subjected to mild water stress[38]. The work of Raschke and co-workers revealed that, under some conditions, stomatal [CO_2]-responsiveness is conditional on the presence, or level of, ABA[38-42]. Our results not only support the role of ABA levels in modulating stomatal aperture response to CO_2, but also show that the inability to reduce ABA levels interferes with these responses. We suggest that minor excursions of VPD or other environmental conditions could result in semi-irreversible increases in ABA in the *cyp707a1/a3*. This dependence on ABA levels is the most likely reason for the differences observed between this work and the results of Merilo *et al.*.

An increase in guard cell reactive oxygen species (ROS) has been shown to be required in both ABA- and [CO_2]-induced stomatal closure[10, 43-46]. To determine the role of ROS in the *cyp707a1/a3* mutant's response to elevated [CO_2], we used the fluorescent ROS indicator H_2DCF-DA[43]. Challenge of guard cells with elevated [CO_2] resulted in a significant (ANOVA, *P* =0.0093) increase in H_2DCF-DA fluorescence, consistent with a significantly greater ABA- and [CO_2]-induced increase in guard cell ROS production in the double mutant (Fig. 1 c and d). Thus, the inability to catabolise ABA causes both an increased production of ROS at elevated [CO_2] and an enhanced stomatal closure response to [CO_2]. Although in the experiment reported here, the increase in ROS was not significant in the Co-0 controls, these data support previous work suggesting that guard cell sensitivity to [CO_2] is associated with an ABA-induced signalling pathway involving ROS[47, 48].

Stomatal density response to [CO_2]

Stomatal densities of mature leaves are influenced by both ABA and [CO_2]. To determine the extent to which the ABA 8′-hydroxylase mutations affect stomatal density we grew the single *cyp707a1* and *cyp707a3* mutants and the double *cyp707a1/a3* mutant and counted the number of stomata that developed on the abaxial surface of mature leaves

grown under ambient CO_2 (Fig. 2a). Interestingly, whilst neither the *cyp707a1* nor the *cyp707a3* single mutants showed significantly (ANOVA, *P* = 0.2258 and *P* = 0.4763, respectively) altered stomatal density in comparison to wild-type, the *cyp707a3* single mutant had significantly lower stomatal density than the *cyp707a1* mutant (ANOVA, *P* = 0.0163). The *cyp707a1/a3* double mutant, however, displayed a more significant (ANOVA, *P* = 0.0001) reduction in stomatal density compared to the single mutants and the wild-type, presumably because of its greatly elevated endogenous ABA levels[27].

We then grew the double mutant and wild-type at ambient and elevated [CO_2] to look at any possible interaction between endogenous ABA and [CO_2]. We found that, unlike the wild-type plants, the *cyp707a1/a3* plants that were deficient in ABA breakdown were unable to further reduce their leaf stomatal density in response to elevated [CO_2] (Fig. 2b). Furthermore, we found that the *cyp707a1/a3* plants had 24 % lower stomatal density than wild-type when grown at ambient [CO_2]. Presumably, this reflects higher levels of ABA in the double mutant. Thus, we conclude that, the ability to break down ABA is essential for the maintenance of wild-type density responses to [CO_2], and that ABA levels regulate leaf stomatal density.

Comparison of the density responses of the single mutants suggests that vascular-specific *CYP707A3* expression contributes more strongly to wild-type modulation of stomatal density than the guard cell associated expression of *CYP707A1*. As vascular *CYP707A3* expression contributes more greatly to ABA turnover than guard cell *CYP707A1* expression[27] the single mutant phenotypes could be attributable to the respective plants' total ABA pool. Together, these findings strongly support and add to previous reports which suggest both a role for ABA in stomatal density[13, 16, 18, 49] and in the stomatal density response to [CO_2][10, 22].

Stomatal density response to light intensity

ABA turnover is also implicated in the interaction of stomatal CO_2 responses and light signalling[18]. Having shown that the stomatal density response of mutants with defective ABA catabolism is unresponsive to [CO_2] we carried out an experiment to test whether the inability to synthesise or breakdown ABA also impaired stomatal density responses to different light intensities (Fig. 3). Growth of plants under reduced light intensities (PPFD of 100 µmol m^{-2}s^{-1} versus 200 µmol m^{-2}s^{-1}) resulted in significantly (ANOVA, P = 0.0356) lower stomatal densities in wild-type. However, *cyp707a1/a3* plants maintained a consistently low stomatal density and were not able to significantly (ANOVA, P = 0.7162) reduce their stomatal density under lower irradiances. This result contrasts with the density response to low light in mutants that produce reduced ABA; the *nced3 nced5* double mutant, which produces very low levels of ABA, displayed high stomatal densities and maintained a strong density response to light intensity (Supplementary Fig. S1). We also observed an interaction between elevated ABA levels and the stomatal aperture response to light; although *cyp707a1/a3* was not significantly affected in the extent of light-induced stomatal opening (Fig. 3a) or ROS production at any individual timepoint compared with wild-type (Supplementary Fig. S2), the mutant's dynamic range of stomatal movement (or speed of stomatal movement) was significantly reduced in response to light across the experimental time-course (t test T_0 vs T_{135}, P = 0.0001). For example, after 90 mins in the light, wild-type stomatal apertures had increased by an average of 3.1 µm while *cyp707a1/a3* apertures had only increased by 1.3 µm. Thus, ABA catabolism, but not an attenuation of ABA-induced ROS production, appears to be required for maximal light-induced stomatal opening.

Next, we investigated the differential expression of *CYP707A* transcripts in response to light intensity by quantitative RT-PCR analysis. Interestingly, *CYP707A1* as well as *CYP707A2* are upregulated in wild-type Col-0 in response to transfer from growth at low light (50 µmol m^{-2}s^{-1}) to high light (250 µmol m^{-2}s^{-1}) conditions, as shown by quantitative RT-PCR (Supplementary Fig. S3). This suggests that guard cell- and whole plant- ABA

catabolism is involved in acclimation to high light levels, and as shown in Fig. 3a is required for appropriate light-induced stomatal opening dynamics. This is in broad agreement with the result of Huang *et al.* (2019) who found that all four *CYP707s* and nine ABA biosynthesis genes including three *NCEDs* are upregulated under high light stress (1,200 mmol m^{-2} s^{-1})[50]. Our results indicate that ABA biosynthesis is not required to reduce stomatal density at low light intensity, but that active ABA catabolism is required to increase stomatal density at high light. This impaired density response also suggests that the effect of ABA on stomatal density is stronger than the effect of light intensity, with ABA signals overriding light signals. In this way, water limitation could be a stronger driver of the stomatal density response than light. Although this is evidence for the primacy of water conservation (in contrast to carbon acquisition) in leaf development, there remains the possibility that endogenous ABA levels in the mutant could interfere with the integration of light and ABA signalling pathways. In addition, the restricted dynamic range of light-mediated stomatal opening (Fig. 3a), and the possible influence of daylength, underlines the complexity of stomatal controls and the need for further deconstruction of closely-linked pathways.

Conclusions and future directions

In summary, our results suggest that plants exhibiting a lesion in the ability to break down ABA exhibit hypersensitivity to CO_2 in their stomatal closure most likely because of high endogenous levels of ABA. We also show that ABA catabolism is required for optimal stomatal opening in the absence of CO_2. Similarly, these plants with impaired ABA catabolism exhibit markedly lower stomatal densities than normal (a result consistent with high endogenous levels of ABA) and fail to exhibit elevated CO_2-induced reductions in stomatal density. Furthermore, we demonstrate that these plants also fail to increase stomatal density when grown under high irradiance. This suggests that ABA signals and water limitation override other environmental signals in leaf development, or because of

a requirement for ABA in plant adaptation to high light (Fig. 4). The disruption of ABA catabolism therefore prevents modulation- and integration- of various environmental signals and thereby partially uncouples growth and abiotic stress response (Fig. 4). It remains to be seen whether ABA levels themselves or the failure to produce PA and DPA is responsible for some of these CO_2 and light phenotypes and/or whether their absence further disrupts responses to prolonged abiotic signals. In conclusion, by using mutants impaired in ABA breakdown and synthesis we show that elevated $[CO_2]$-mediated control of stomatal aperture and density and the control of stomatal density by light require precise control of both the level and cellular location of ABA. Furthermore, the reduced stomatal apertures and densities observed in this study, suggest that ABA turnover provides a potential target for improving water use efficiency in crops under future predicted CO_2 environments.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 was used in this investigation. Seeds of *cyp707a1 cyp707a3* in the Col-0 background were obtained from Dr Eiji Nambara (University of Toronto)*,* and have been previously described[27, 34]. Seeds for experiments shown in Figs. 1, 2, 3a and S2 were sown onto M3 and perlite in a 4:1 ratio in plug-trays and transferred into a growth cabinet (Conviron) with growth conditions 22°C (day)/ 16°C (night) temperature cycle; 9/15hr light/dark cycle; 70 % relative humidity, PPFD 150 µmol $m^{-2}s^{-1}$, at ambient $[CO_2]$ (approx. 400ppm) or elevated $[CO_2]$ (approx. 1,000 ppm). Seeds for experiments shown in Fig. 3b and Supplementary Fig. S1 were sown and grown under either PPFD 100 or 200 µmol $m^{-2}s^{-1}$ at ambient $[CO_2]$. Plants used in the qRT-PCR light transfer experiment shown in Supplementary Fig. S3 were grown in a 12-hour photoperiod at 22°C, 60 % relative humidity, with 50 µmol $m^{-2}s^{-1}$ light at ambient $[CO_2]$.

Measurement of stomatal aperture responses

Abaxial epidermis was removed from the youngest, fully expanded leaves of 5 to 6 week old plants and floated, cuticle-side up, on CO_2-free 10 mM MES/KOH (pH 6.15) in 5 cm Petri dishes (Sterilin, UK) at 22°C. For [CO_2]-concentration sensitivity experiments (Fig. 1) epidermal peels were transferred to fresh Petri dishes and incubated for 2h in the light under a PPFD of 150 µmol $m^{-2}s^{-1}$ in 50 mM KCl, 10 mM MES/KOH (pH 6.15) at 22°C whilst being aerated with CO_2-free air by bubbling through the buffer solution. This treatment brought about stomatal opening. The use of CO_2-free air treatment is a long-standing and standard technique in stomatal bioassays to induce maximal stomatal opening[51, 52]. Peels were then either aerated with lab air (ambient, approx. 400 ppm CO_2- as measured by IRGA), CO_2 scrubbed air (0 ppm), or CO_2 from a pressurised cylinder containing 1,000 ppm CO_2 in air (BOC, Special Gasses, UK) by bubbling directly into the buffer. To determine the effect of Calcium ion supplementation on [CO_2]-concentration sensitivity, experiments were performed as above with and without the addition of 10 or 50 µM $CaCl_2$ in the buffer solutions; no effect of $CaCl_2$ was found at these concentrations, suggesting that endogenous guard cell calcium levels or residual calcium in buffer solutions are sufficient to ensure stomatal closure (Supplementary Fig. S4). For the light-induced opening assay plants were kept in the dark overnight and in the morning before the lights came on epidermal strips were prepared in dim-light and stored in 10 mM MES/KOH (pH 6.15) for one hour in the dark. Strips were then transferred to 50 mM KCl, 10 mM MES/KOH (pH 6.15) supplied with CO_2 free air in the light. Peels were removed at 0, 45, 90, and 135 minute time-points, mounted on slides and measurements of stomatal aperture recorded using an inverted microscope (Leica DM-IRB, Leica UK) or a microscope (Olympus BX51), fitted camera (Olympus DP70), and ImageJ software v. 1.43u. The maximal widths of forty stomatal pores were measured per treatment in three separate replicated experiments (total stomatal number

= 120; n = 3). To avoid experimenter bias, measurements were performed without the researcher being aware of the sample identity. Data were tested for normality and analysed using one-way or two-way ANOVA (MINITAB and Sigmaplot 12).

Measurement of ROS

To estimate ROS generation abaxial epidermal peels were prepared as described above and incubated in 50mM KCl, 10 mM MES/KOH (pH 6.15) at 22°C, PPFD of 100 μmol m^{-2}s^{-1} whilst being aerated with CO_2-free air for 2h. For the experiments shown in Fig. 1, they were then transferred to 50mM KCl, 10 mM MES/KOH (pH 6.15) at 22°C, PPFD of 100 μmol m^{-2}s^{-1} and either aerated with ambient $[CO_2]$ (400ppm) or 1,000 ppm CO_2 for 2h. For the experiment shown in Fig. 3a and Supplementary Fig. S2, the peels were removed at 0, 45, and 90-minute time-points. For both experiments the epidermal peels were then loaded (by pipetting) with 25 μM (final concentration) H$_2$DCF-DA (2',7'-dichlorodihydrofluoresceindiacetate) (Invitrogen, UK) from a 25 mM stock in DMSO for 10 minutes in dark. They were then washed in 50mM KCl, 10 mM MES/KOH (pH 6.15) at 22°C for 10 min in the light (PPD 150 μmol m^{-2}s^{-1}) to remove excess H$_2$DCF-DA and the fluorescence intensity was measured as using an Olympus BX51 fluorescence microscope and ImageJ as described in[53]. For ROS analysis, pixel intensities of forty stomatal areas (fluorescence zone of two guard cells) relative to their background intensities (four equivalent sized areas surrounding each stoma) were measured per treatment in three separate replicated experiments (total stomatal number = 120; n = 3). Fluorescence intensities were normalised to those of controls. Data were tested for normality and analysed using two-way ANOVA (MINITAB and Sigmaplot 12).

Stomatal density measurements

Dental resin (Coltene Whaledent, Switzerland) was applied to the abaxial surfaces of fully expanded leaves and nail varnish peels were taken from set resin. Cell counts were taken from four fields of view from the widest area of four leaves each from five plants of each genotype from both growth conditions (400 and 1,000 ppm CO_2 for experiments shown in Fig. 2 conditions, and PPFD of 100 or 200 µmol m^{-2}s^{-1} for experiments shown in Fig. 3b and Supplementary Fig. S1). Data were tested for normality and analysed using two-way ANOVA (MINITAB and Sigmaplot 12).

Quantitative RT-PCR analyses

For quantitative RT-PCR analysis (qRT-PCR), RNA was extracted from plants grown for 12 days post germination in 50 µmol m^{-2}s^{-1} light before being transferred to 250 µmol m^{-2}s^{-1} light. Tissue was collected at 3 and 6 hours after transfer and RNA extracted using the Quick-RNA™ MiniPrep (Zymo Research) plant RNA extraction kit with on-column DNase treatment according to the manufacturer's instructions. RNA was reverse transcribed with High Capacity Reverse Transcriptase (Applied Biosystems). Transcript abundance of target genes was assayed using SYBR Green/JumpStart Taq Ready Mix qPCR Master Mix (Sigma Aldrich) on a Biorad CFX qPCR machine. The ACT2 and UBC21 genes were used as controls, as transcript levels remained constant under all treatments and relative expression levels were calculated using the ΔΔCt method. All primers used are in Table 1. Expression was calculated relative to that of Col-0 seedlings grown at 50 µmol m^{-2}s^{-1} light. Three biological repeats and three technical repeats were performed for each sample and used to calculate s.e.m. values. Reaction conditions were (1 x 95°C - 10 mins; 40 x 95°C - 15s/57°C – 20s/72°C – 30s).

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Data, code and materials

The datasets supporting this article have been uploaded as part of the supplementary material.

Author contributions

MM and CC contributed equally to this work by designing and performing experiments and analysing data. NZ and SC performed reciprocal light and qRT-PCR experiments. PS and Y-KL performed $CaCl_2$ bioassays. MM, CC, JEG, NZ, SC, and AMH wrote the manuscript and interpreted data. CC, JEG and AMH conceived the project. All authors reviewed the manuscript.

Fig. 1. Impaired ABA catabolism sensitises *cyp707a1 cyp707a3* stomatal closure in response to [CO_2], but does not induce aperture closure in the absence of CO_2.

a. [CO_2]-induction of stomatal closure is hypersensitive in the *cyp707a1 cyp707a3* mutant (labelled as *cyp707a1/a3*; ANOVA, P = 0.0211), but there is no significant (ANOVA, P =0.1001) difference in stomatal apertures in the absence of CO_2. b. Representative bright-field images used to determine stomatal apertures from (a), showing differences between *cyp707a1 cyp707a3* and wild-type guard cells under zero, ambient and elevated [CO_2]. c. [CO_2]-induction of reactive oxygen species (ROS) is hypersensitive in the *cyp707a1 cyp707a3* mutant (ANOVA, P = 0.0240), but there is no significant (ANOVA, P = 0.9990, P = 0.9978) difference in ROS production in the absence of CO_2 or in ambient [CO_2]. d. Representative images showing fluorescence of *cyp707a1 cyp707a3* and wild-type guard cells under zero, ambient and elevated [CO_2] as in (c).

Fig. 2. The stomatal density response to [CO_2] requires ABA catabolism.

a. Mean stomatal density was significantly (ANOVA, P = 0.0001) lower in the double mutant *cyp707a1 cyp707a3* leaves compared to wild-type grown under ambient conditions. Mean stomatal density was significantly (ANOVA, P = 0.0163) lower in the single *cyp707a3* compared to the single *cyp707a1* mutant. b. Mean stomatal density was significantly (ANOVA, P = 0.0010) lower in wild-type leaves grown under 1,000 ppm [CO_2] compared to 400 ppm, but the stomatal density of *cyp707a1 cyp707a3* mutant was unaffected by [CO_2]. Stomatal densities of the *cyp707a1 cyp707a3* mutant were significantly (ANOVA, P = 0.0094) lower than wild-type when grown under ambient [CO_2] and did not reduce further when grown at elevated [CO_2].

Fig. 3. The dynamic range of stomatal aperture response to light is reduced, and the stomatal density response to light intensity is inhibited, when ABA catabolism is impaired.

a. The range of light-induced stomatal opening is reduced in the *cyp707a1/a3* mutant. b. Mean stomatal density was significantly (ANOVA, P = 0.0356) higher in wild-type leaves grown under 200 µmol m^{-2}s^{-1} PPFD than under 100 µmol m^{-2}s^{-1}, but did not increase significantly (ANOVA, *P* = 0.7162) in the *cyp707a1 cyp707a3* mutant at the higher light intensity.

Fig. 4. Disruption of abscisic acid (ABA) catabolism and feedback on stomatal phenotypes.

The disruption of the cytochrome P450 CYP707A1 and CYP707A3 prevents the vascular and guard cell hydroxylation of ABA, respectively. ABA biosynthesis, regulated principally by the activity of the 9-cis-epoxycarotenoid dioxygenase (NCED) remains intact. ABA concentrations are therefore maintained at a higher level and the balance of the free ABA pool can be altered by reversible production of the less physiologically active ABA-glucose ester (ABA-GE). The resulting imbalance of ABA metabolism results in defective guard cell physiology and stomatal density responses to [CO_2] and light intensity. The effect of elevated ABA appears to cancel out the effect of high light on stomatal density. Thick solid lines show upregulation of specific pathways and thin solid lines show downregulation, whereas dashed lines show inhibition of processes.

Table 1. Details of primer sequences

Target	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
ACT2	TCAGATGCCCAGAAGTCTTGTTCC	CCGTACAGATCCTTCCTGATA
UBC21	GAATGCTTGGAGTCCTGCTTG	CTCAGGATGAGCCATCAATGC
HT1 kinase	GGGCTAAGCTTGAACAACAGT	GCGAGTAAGGCTCTTTCTTG
CYP707A1	CCATCAAGATTCGAGGTGGCT	GGACACGAGTGGGTTCCATT
CYP707A2	GCTCTCAGACCAACCGTCTC	CGAGGGTGTTGATGGACTTT
CYP707A3	CAATTCTTGGGATGGAACTCAA	GTCTTTGCCGAGTATTGAGATT
CYP707A4	CTATCCATGCGTGATGTTGG	GTGCAGAGGGTCCTATCAGC

a

Stomatal aperture (μm)

Col-0   *cyp707a1/a3*

- CO$_2$-free
- ambient CO$_2$
- elevated CO$_2$

b

	Col-0	*cyp707a1a3*
CO$_2$-free		
ambient CO$_2$		
elevated CO$_2$		

c

ROS Production Fluorescence (a.u)

Col-0   *cyp707a1/a3*

- CO$_2$-free
- ambient CO$_2$
- elevated CO$_2$

d

	Col-0	*cyp707a1a3*
CO$_2$-free		
ambient CO$_2$		
elevated CO$_2$		

Figure 1

Figure 2

a

b

Figure 3

ABA sequestration: conjugation

ABA:GE

ABA biosynthesis

NCED3

light

stomatal density

ABA catabolism: hydroxylation

CYP707A1,A3

8'HO ABA

ABA

CO_2

ROS

stomatal closure

Figure 4

SUPPLEMENTARY INFORMATION

Stomatal responses to carbon dioxide and light require ABA catabolism in Arabidopsis

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# Supplementary Information
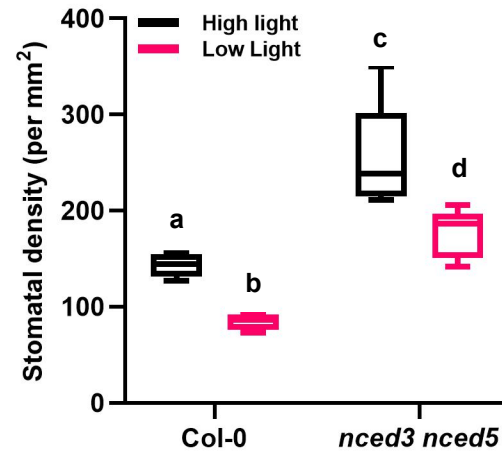
Supplementary Fig. S1. The stomatal density response to light intensity is not altered when ABA biosynthesis is impaired. The stomatal density response to light intensity was not impaired in the ABA biosynthesis double mutant *nced3 nced5* compared to wild-type leaves.

Supplementary Information
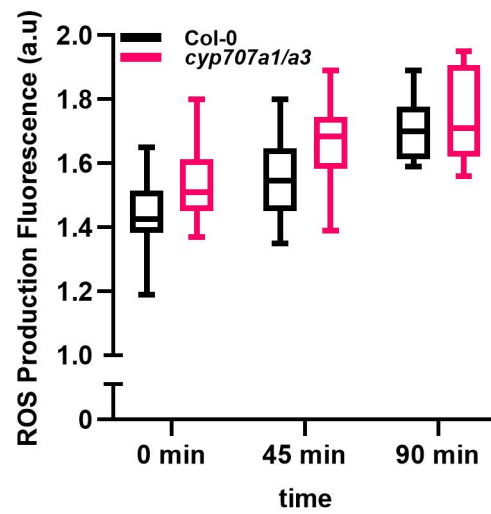
Supplementary Fig. S2. ROS production in response to light is not significantly altered when ABA catabolism is impaired. There is only marginally elevated reactive oxygen species (ROS) production in the *cyp707a1/a3* mutant in response to light.
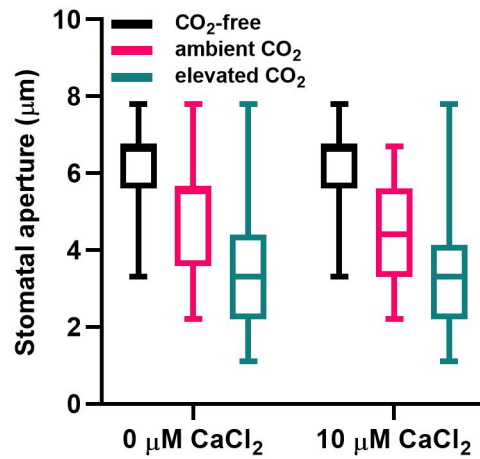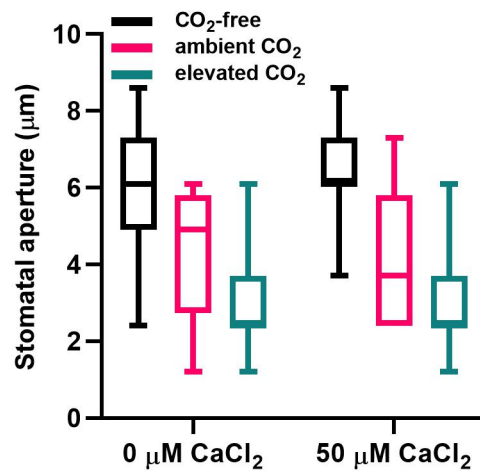
Supplementary Information
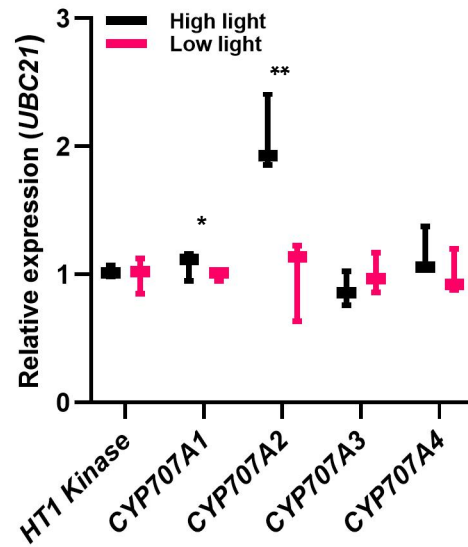
Supplementary Fig. S3. *CYP707A1* and *CYP707A2* **are upregulated in response to high light.** Differential expression analysis by quantitative RT-PCR shows upregulation of *CYP707A1* and *CYP707A2* in wild-type Col-0 in response to transfer to high light from growth at low light.

a

b

Supplementary Fig. S4. Supplemental Calcium ions do not affect the stomatal aperture response to changes in carbon dioxide concentration. The addition of (a) 10 μM or (b) 50 μM $CaCl_2$ to the epidermal strip buffer does not affect the stomatal aperture response to $[CO_2]$.