Genetic controls of short- and long-term stomatal CO$_2$ responses in
Arabidopsis thaliana

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INTRODUCTION

Stomata are microscopic pores in the epidermis, surrounded by two guard cells that regulate their aperture by changes in turgor pressure. Almost all gas exchange between plants and the atmosphere occurs through the stomata, hence the stomatal aperture is regulated to balance the trade-off between CO$_2$ uptake for photosynthesis and transpirational water loss. Elevated CO$_2$ concentration induces partial closure of stomata in most plant species (Morison, 1998; Ruszala et al., 2011; Franks and Britton-Harper, 2016). This reduces transpirational water loss and improves leaf-level water economy. With a projected doubling of the atmospheric CO$_2$ concentration within the next 100 years (IPCC, 2013), the stomatal CO$_2$ response could have a significant impact on global plant water use under future climatic conditions. However, the magnitude of the stomatal CO$_2$ response and hence the potential for water conservation under elevated CO$_2$ exhibit a large variation among and within species (Morison, 1998; Takahashi et al., 2015; Hörak et al., 2017). Significant variation in the stomatal CO$_2$ response among different accessions of the model plant Arabidopsis thaliana (Takahashi et al., 2015) provides an excellent opportunity to explore its genetic basis, as indicated by the recent discovery of a novel CO$_2$ signalling component using natural A. thaliana accessions (Jakobson et al., 2016). Knowledge about the genetic regulation of stomatal conductance (g) in response to elevated CO$_2$ could facilitate the improvement of crop water-use efficiency in a future climate.

The pathway for stomatal closure in response to elevated CO$_2$ consists of one CO$_2$-specific branch that converges downstream with the pathway for abscisic acid (ABA)-induced stomatal closure (Webb and Hetherington, 1997; Engineer et al., 2016).
The CO₂ response is initiated by the conversion of CO₂ to bicarbonate by the carbonic anhydrases CA1 and CA4 in guard cells (Hu et al., 2010), resulting in the activation of the mitogen-activated protein kinases MPK4 and MPK12 by a yet un-described mechanism (Marten et al., 2008; Hõrak et al., 2016). These two MPKs inhibit the protein kinase HT1 (Hashimoto et al., 2006; Hõrak et al., 2016; Jakobson et al., 2016). Downstream of the CO₂-specific branch are the kinases OST1 and GHR1. The inhibition of HT1 by MPK4/MPK12 releases the inhibition of OST1 and GHR1 (Hõrak et al., 2016), which results in the activation of the anion channel SLAC1 (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009; Hua et al., 2012) and other ion channels in the plasma and vacuolar membranes, leading to loss of turgor and stomatal closure (Kollist et al., 2014; Hedrich and Geiger, 2017; Jezek and Blatt, 2017). Recent research has identified the BIG protein as an additional component of the CO₂-specific branch of the signalling pathway. Although the exact molecular function of BIG is unknown, it was shown to induce anion currents in response to elevated HCO₃⁻ concentration (He et al., 2018). The mechanism by which changes in CO₂ and/or HCO₃⁻ concentration are sensed is currently unknown and it is likely that more components and interactions of the guard cell CO₂ response pathway remain to be discovered.

The current understanding of genetic and molecular controls of the stomatal CO₂ response is largely based on studies of the response to short-term fluctuations in CO₂ concentration, i.e. the change in gᵣ occurs within minutes to hours after a change in the atmospheric CO₂ concentration (Vahisalu et al., 2008; Engineer et al., 2016). It is, however, unclear whether the short-term responsiveness is a good predictor of long-term changes in gᵣ of plants grown under elevated CO₂ concentration, i.e. changes in gᵣ that occur over weeks to months (Morison, 1998; Haworth et al., 2013). Long-term responsiveness might represent both changes in aperture and density as it entails development of new leaves. Moreover, the potential links between short- and long-term gᵣ responses on a molecular level have not been explored. In a synthesis of data from free air CO₂ enrichment (FACE) experiments on trees, Hasper et al. (2017) observed a correlation between short-term stomatal responsiveness to changes in the CO₂ concentration and long-term reductions in gᵣ of plants grown under elevated CO₂. However, other studies have indicated that gᵣ may acclimate to growth under elevated CO₂ (Šantrůček and Sage, 1996; Morison, 1998; Lodge et al., 2001; Medlyn et al., 2001), possibly as a result of altered stomatal sensitivity to CO₂ (Onandia et al., 2011; Haworth et al., 2013, 2016). In addition, short- and long-term stomatal responses may be decoupled in cases where plants respond to prolonged CO₂ exposure by adjusting stomatal size or density rather than aperture (Haworth et al., 2013, 2015).

In this study, we investigated the genetic controls of both short-term (within an hour) and long-term (within a month) responses of gᵣ to elevated atmospheric CO₂ concentration in Arabidopsis thaliana. We identified genetic loci associated with short- and long-term gᵣ responses to elevated CO₂, and with several other traits related to stomatal regulation. We found that a major quantitative trait locus (QTL) associated with the short-term response to CO₂ was also involved in the long-term regulation of gᵣ in response to growth in elevated CO₂ concentration. This QTL was related neither to the ABA-induced stomatal closure pathway nor to any known genetic components of stomatal regulation.

MATERIALS AND METHODS

Plant material and growth conditions

Recombinant inbred lines (RILs) originating from a reciprocal cross between the Arabidopsis thaliana accessions C24 and Col-0 (Törjék et al., 2006) were used in this study. These two accessions were selected based on a pilot study of the short-term stomatal response to elevated CO₂ concentration among various A. thaliana accessions, where C24 was identified as a weak responder and Col-0 as a strong responder (Fig. 1). To confirm the location of a major QTL, we additionally used reciprocal near-isogenic lines (NILs) between C24 and Col-0 (Törjék et al., 2008).

The following growth conditions were used for all plants except the RILs used in ABA response measurements and the NILs used for confirmation of a major QTL: seeds were sown on soil–perlite mix, stratified at 4 °C for 2 d and cultivated under short-day conditions (8 h light/16 h darkness; 22/18 °C) at ~60 % relative humidity and a photosynthetic photon flux density of 150–170 μmol photons m⁻² s⁻¹ in growth chambers (model AR-82L2/DE, Percival Scientific, Perry, IA, USA). Seedlings were transplanted to individual pots 2 weeks after germination. In the CO₂ experiment, plants were grown in two separate, identical growth chambers (same as above) with contrasting CO₂ concentrations. The ambient treatment had an average daytime CO₂ concentration of 420 ppm and CO₂ in the elevated treatment was maintained at an average daytime concentration of 820 ppm using a TKG-CO2-3011C CO₂ control device (Tongdy Control Technology, Beijing, China). To avoid confounding effects of between- and within-chamber variation in environmental conditions, plants and CO₂ treatment levels were shifted between the two growth chambers twice a week and trays with pots were rotated 180 °C.

Seeds used to generate plants for ABA response measurements and for confirmation of a major QTL were stratified in water for 2 d at 4 °C, sown on peat–vermiculite mix and grown through a hole in a glass plate covering the pot as described previously (Kollist et al., 2007) under short-day conditions (12 h light/12 h darkness, 23/20 °C) at 70 % relative humidity and a light intensity of 100–150 μmol m⁻² s⁻¹ in growth chambers (Microclima Arabidopsis MCA1600-3LP6-E, Snijders Scientific, Tilburg, the Netherlands).

Study design

The study comprised three experiments to investigate various aspects of stomatal regulation (as illustrated in Fig. 2). QTL mapping of plants grown in ambient CO₂. The initial QTL mapping experiment was designed to identify genetic loci associated with the short-term (within minutes to hours) response of gᵣ to elevated CO₂, with absolute gᵣ at ambient and elevated CO₂, and with the ratio of mole fractions of CO₂ in the substomatal cavity and ambient air, c/ ca. The latter is a proxy for intrinsic water-use efficiency (iWUE), where low values represent high iWUE. We selected a subset of 100 RILs that displayed the largest number of chromosomal crossovers in the population, in order to maximize genetic variation (Supplementary Data Table S1).
Of these RILs, 51 originated from a cross using C24 as pollen donor and Col-0 as pollen acceptor, and 49 where Col-0 was used as pollen donor to C24, to account for potential cytoplasmic effects. The RILs and their parental accessions were grown at ambient CO2 concentration. The short-term CO2 response, absolute $g_s$, and $c_i/c_a$ were quantified and these data were used for QTL mapping. Fine mapping of a major QTL controlling the short-term $g_s$ response to elevated CO2 was performed using additional RILs with crossovers in the region of interest and the location and the effect of the QTL was confirmed using NILs.

Long-term CO2 experiment. The aim of this experiment was to study the effects of growth in elevated CO2 on stomatal regulation in A. thaliana. Specifically, we wanted to (1) investigate whether growth in elevated CO2 concentration affected the short-term (within minutes to hours) CO2 responsiveness and absolute $g_s$, as well as the detection of QTLs associated with CO2 responsiveness, and (2) map loci associated with the
long-term (within weeks to months) response to elevated CO2. We used 50 RILs from the cross where C24 was the pollen donor and Col-0 the acceptor, which had been used in the previous experiment. The RILs were grown together with their parental accessions in ambient or elevated CO2 in two separate treatments for 4 weeks, which constitutes a large proportion of the A. thaliana life cycle (Boyes et al., 2001). Data on short-term CO2 response and absolute gs of plants from both CO2 treatments were used for QTL mapping, as well as data on the long-term gs response to elevated CO2.

ABA experiment. The stomatal response to exogenously applied ABA was measured in ten RILs that showed the five strongest and the five weakest CO2 responses in the first experiment. The aim of the ABA experiment was to investigate the relationship between CO2- and ABA-induced stomatal closure in these lines.

Gas exchange measurements

In the first two experiments, gas exchange measurements on entire leaf rosettes of 4-week-old plants were conducted using two LI6400 systems (LI-COR Biosciences, Lincoln, NE, USA) fitted with 6400-17 Whole Plant Arabidopsis Chambers. Self-shading within rosettes was minimal at this growth stage. Leaf temperature was estimated using energy balance calculations (LI-COR Biosciences, 2011). The boundary layer conductance was estimated using a model of a leaf rosette made from filter paper, which was soaked in water that was allowed to evaporate inside the whole plant chamber. The boundary layer conductance was estimated to be 4 mol H2O m−2 s−1. The stomatal ratio (the ratio of gs on the leaf sides with lowest versus highest values) was assumed to be 0.5, which is recommended when the exact ratio is not known (LI-COR Biosciences, 2011). Fluxes of water vapour from the soil were prevented by covering the soil with household cling film. To test for the influence of water vapour exiting through the tiny gap in the plastic film surrounding the plant, we conducted measurements after cutting the plant stem, we conducted measurements after cutting the plant. The CO2 concentration was kept at 400 ppm until gs reached steady state (<2.5 % change in conductance over 5 min). When steady state had been reached, three measurements with 10 s between them were logged, after which the CO2 concentration was elevated to 800 ppm and the same procedure was repeated. Plant leaves were imaged using a flatbed scanner, leaf areas were calculated using the ROI manager function in ImageJ (version 1.48v, Schneider et al., 2012) and the conductance values were re-calculated to be expressed per unit leaf area. The percentage reduction in gs following elevation of the CO2 concentration was used as a measure of the short-term stomatal response to elevated CO2. In the CO2 experiment, we additionally calculated the long-term response to growth in elevated CO2 concentration. For the same genotype, we used gs values of plants from the two treatments measured at their respective growth CO2 concentration to calculate the percentage decrease in stomatal conductance resulting from growth in elevated CO2.

For the ABA response experiment and for the confirmation of a major QTL using NILs, gas exchange measurements were conducted on 25- to 30-d-old plants using a custom-made gas exchange system (Kollist et al., 2007). We quantified the percentage gs decrease in response to elevated CO2 (~800 ppm) in both experiments and in the ABA experiment also to spray application of 5 µm ABA solution (containing 0.012 % Silwet and 0.05 % ethanol). Measurements were conducted at a light intensity of 100–150 µmol photons m−2 s−1 and a temperature of 23–25 ºC. Stomatal conductance was allowed to stabilize at ambient CO2 concentration and 65–70 % relative humidity for ~40 min before the stimulus was applied. The stomatal response was calculated as the percentage gs decrease 28 min after application of the stimulus. Leaf areas were measured using the polygon tool in ImageJ (version 1.48v, Schneider et al., 2012) on photographs of intact leaf rosettes.

Stable isotope analyses

The ratio of mole fractions of CO2 in the substomatal cavity (c) and ambient air (c)a was used as a proxy for iWUE, where low c/ca corresponds to a high iWUE (Condon et al., 2004; Pérez-Harguindeguy et al., 2013) as shown by the relationship:

\[
\frac{c_i}{c_a} = 1 - \frac{iWUE \times 1.6}{c_a}
\]

A time-integrated measure of c/ca was determined by analysing leaf stable carbon isotope composition. Leaves were dried for at least 24 h at 70 ºC and homogenized with a pestle. The material (~1 mg per sample) was weighed into tin capsules and analysed for stable carbon isotope ratios using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (IRMS; Sercon, Crewe, UK) at the UC Davis Stable Isotope Facility, Davis, CA, USA. The photosynthetic 13C discrimination (Δ) was calculated from δ13C values according to the following equation:

\[
\Delta = \frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}}
\]

For this calculation we assumed a value of −8.44 ‰ for δ13Cair, based on the average δ13C ratio of CO2 in air measured at the Mauna Loa Observatory, HI, USA during 2014 (data downloaded from https://www.esrl.noaa.gov, Keeling et al., 2001). This value likely differed slightly from that in our experiment, but as the isotope data were only used to compare plants within the same experiment this error was considered negligible. The 13C discrimination was then used for the calculation of c/ca as follows:

\[
\frac{c_i}{c_a} = \frac{\Delta - a}{b - a}
\]
where $a$ is the isotopic fractionation caused by diffusion (4.4 ‰) and $b$ is the fractionation caused by carboxylation by Rubisco (27 ‰) (Farquhar et al., 1989). It should be noted that the above equations follow the simplified format presented by Farquhar et al. (1989), where ‰ is considered equivalent to 10⁻³; hence, all ‰ values were multiplied by 0.001 in our calculations.

Genotyping

The RIL population had previously been genotyped using SNP markers, as described by Törjék et al. (2003). The lines used in this study were partially re-genotyped in generations $F_1/F_2$ to confirm or correct double crossovers and to remove heterozygous regions. For this purpose, the SNaPshot® Multiplex System (Applied Biosystems, Waltham, MA, USA) was used according to the manufacturer’s protocol on an ABI 3730 Sequencer (Applied Biosystems). Peaks were identified using GeneMapper® (version 4.0, Applied Biosystems). In addition, simple sequence length polymorphism (SSLP) markers from the MSAT database (http://www7.inra.fr/vast/msat.php) were added to allow comparison with other A. thaliana RIL populations and single-feature polymorphism (SFP) markers were extracted from ATH1 GeneChip® (Affymetrix, Waltham, MA, USA) data as described by Schmidt et al. (2017). The SSLP fragments were PCR-amplified from genomic DNA and visualized on agarose gels. For large fragments and/or size differences above 10 bp, 1–2 % agarose gels (Carl Roth, Karlsruhe, Germany) were used. For smaller size differences, a 1:3 mixture of 4 % agarose/MetaPhor™ agarose (Lonza Group, Basel, Switzerland) was used. Fragment size was identified by comparison with the Generuler™ 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA) and genotypes were scored manually. Genetic maps for the two subsets of lines analysed in this study were constructed using the package R/qtl (version 1.41-6) in R (version 3.4.3) with the Kosambi mapping function (Broman et al., 2003). The NILs had been genotyped using the same set of SNP markers as the RILs, as described by Törjék et al. (2008).

For fine mapping of the major QTL on chromosome 2, 42 RILs with crossovers in the region of interest were used. Genotyping was performed using nine SSLP markers (Supplementary Data Table S2) in the QTL region following the methodology of Nilsson et al. (2016). Annealing temperatures were optimized for each primer pair using gradient PCR. PCR products were visualized on 3 % agarose gels (Seakem® LE, Lonza Group, Basel, Switzerland) and genotypes scored manually.

In order to confirm the lack of sequence variation in the MPK12 gene between C24 and Col-0 in publicly available sequence data (Berardini et al., 2015; Alonso-Blanco et al., 2016), a genomic fragment consisting of the coding region and 0.5-kb flanking region on both sides was PCR amplified from C24 using three sets of primers (Supplementary Data Table S2) and AccuPrime™ PFx polymerase (Thermo Fisher Scientific, Waltham, MA, USA) to produce three overlapping fragments. The products were sequenced (Eurofins Genomics, Ebersberg, Germany) using the same set of primers, with an additional sequencing primer for one of the products (Supplementary Data Table S2).

Data analysis

Differences in the short-term CO₂ response and absolute $g_s$ of accessions C24 and Col-0 were tested using Welch’s $t$-test. Welch’s $t$-test was also used to test for cytoplasmic effects by comparing trait averages from the reciprocal crosses. Differences in short-term response and absolute $g_s$ between RILs grown in ambient and elevated CO₂ treatments were tested using the paired $t$-test. The relationship between short- and long-term stomatal CO₂ responses was tested using linear regression and the paired $t$-test was used to test for a difference in magnitude of these responses. One-way ANOVA with Tukey’s post hoc test was used to test for differences between NILs and parental accessions. All statistical tests were performed with $\alpha = 0.05$ using JMP (version 12.0.1, SAS Institute, Cary, NC, USA).

For QTL mapping, data from both crosses were analysed together as no significant cytoplasmic effect on any of the traits had been detected. Data from the first experiment, where 100 RILs were grown in ambient CO₂, and the second experiment, where 50 RILs were grown in two CO₂ treatments (ambient and elevated), were analysed separately. To increase mapping power and enable the identification of QTLs with pleiotropic effects, we used multi-trait analysis combining all phenotype data from each experiment. A step size of 10 cm, minimum cofactor proximity of 50 cm, a minimum separation of selected QTLs of 30 cm and a threshold of −log10$P = 3.2$ (based on Li and Ji, 2005) were used for QTL analysis. First, the whole genome was scanned for significant polymorphisms using simple interval mapping. Then, based on the selected cofactors, two rounds of composite interval mapping were run. Thereafter, a final QTL model was selected using backward selection on the selected cofactors, where the allelic effect and explained phenotypic variance of each QTL were estimated for each trait. All QTL analyses were performed in GenStat for Windows (16th edition, VSN International, Hemel Hempstead, UK).

RESULTS

Stomatal regulation of parental accessions

We observed significantly weaker short-term stomatal CO₂ response, i.e. the percentage decrease in stomatal conductance ($g_s$) following a doubling of the CO₂ concentration (Welch’s $t$-test, $P = 0.01, n = 6$), as well as lower absolute $g_s$ at both 400 and 800 ppm CO₂ of C24 compared with Col-0 (Welch’s $t$-test, $P < 0.001$ and $P = 0.002$, respectively, $n = 6$; Fig. 1B), confirming the results of the pilot study. Furthermore, C24 demonstrated a significantly lower $c/l_c$ than Col-0, showing that C24 had a higher intrinsic water-use efficiency (iWUE) than Col-0 (Welch’s $t$-test, $P = 0.043, n = 5$; Fig. 1C). In summary, C24 generally has lower stomatal conductance and thus a more conservative regulation of transpirational water loss but at the same time its stomata are less responsive to increased CO₂ concentration.

QTL mapping of stomatal regulation in 100 RILs grown in ambient CO₂

To investigate the genetic basis for the variation in stomatal regulation between Col-0 and C24, we quantified several
stomata-related traits among 100 RILs originating from a reciprocal cross between these accessions and used these data for QTL mapping. The short-term g response to elevated CO₂ ranged from 13 to 64 % among the RILs (Supplementary Data Fig. S1, Table S3). The RILs also displayed a wide range in absolute CO₂ concentrations. After 4 weeks of the respective treatment, gas exchange measurements were conducted. The short-term response (Table 1) was mapped to the same marker as the short-term CO₂ response. The Col-0 allele at this QTL conferred a stronger CO₂ response on chromosome 2, explaining 51 % of the variation in this trait. The Col-0 allele was also more frequent in all cases, consistent with the observation of lower iWUE in Col-0. To test whether the short-term response could be used as a predictor of the long-term response, the short-term response of each genotype grown in ambient CO₂ was compared with the long-term response of the same genotype (Fig. 5). This showed a significant linear relationship between the responses (linear regression: r² = 0.53, F₁,₄₈ = 48.4). The long-term response was, however, significantly weaker than the short-term response (26 % versus 39 % decrease; paired t-test, P < 0.0001, n = 50).

Table 1. QTLs detected using data from measurements of gas exchange and stable isotope composition of recombinant inbred lines grown in ambient CO₂

| Trait                          | QTL location and confidence interval (cM) | Chromosome | Closest marker | Variance explained (%) | High-value allele |
|-------------------------------|-------------------------------------------|------------|----------------|------------------------|-------------------|
| Short-term CO₂ response       |                                           |            |                |                        |                   |
| g₄₀₀                          | 110 (105–110)                             | 2          | MASC02812/MSAT2.22 | 51                    | Col-0             |
| c/c                           | 110 (105–110)                             | 2          | MASC02812/MSAT2.22 | 13                    | Col-0             |
| g₈₀₀                          | 23 (0–108)                                | 3          | MASC04608      | 4                     | Col-0             |
| c/c                           | 23 (0–108)                                | 3          | MASC04608      | 9                     | Col-0             |
| g₁₀₀                          | 94 (0–108)                                | 3          | MASC03218      | 6                     | Col-0             |
| c/c                           | 94 (0–108)                                | 3          | MASC03218      | 4                     | Col-0             |
| Short-term CO₂ response       | 5 (0–35)                                  | 4          | FR1            | 3                     | C24               |
| g₄₀₀                          | 5 (0–35)                                  | 4          | FR1            | 3                     | C24               |
| g₈₀₀                          | 5 (0–35)                                  | 4          | FR1            | 14                    | Col-0             |
| c/c                           | 5 (0–35)                                  | 4          | FR1            | 4                     | Col-0             |
| g₁₀₀                          | 52 (6–92)                                 | 4          | MASC09213     | 7                     | C24               |
| g₈₀₀                          | 52 (6–92)                                 | 4          | MASC09213     | 12                    | C24               |

Among the tested RILs, growth in elevated CO₂ concentration resulted in an average g₄₀₀ reduction of 26 % (paired t-test, P < 0.0001, n = 50; Fig. 4A, Supplementary Data Table S4). When the g₄₀₀ of plants grown in ambient and elevated CO₂ was measured at the same CO₂ concentration, plants from the elevated treatment generally displayed higher g₄₀₀ than plants from the ambient treatment. On average, plants from the elevated treatment had 11 % higher g₄₀₀ than plants from the ambient treatment when measured at 400 ppm and 20 % higher g₄₀₀ when measured at 800 ppm (paired t-test of g₄₀₀, P = 0.004, n = 50; paired t-test of g₈₀₀, P < 0.0001, n = 50; Fig. 4A, Supplementary Data Table S4), indicating g₄₀₀ acclimation of plants grown in elevated CO₂.

Growth under elevated CO₂ concentration had a small but statistically significant effect on the short-term CO₂ response (paired t-test, P = 0.0004, n = 50). RILs grown in ambient CO₂ concentration showed an average g₄₀₀ decrease of 39 % in response to short-term elevation of the CO₂ concentration, whereas the average short-term g₄₀₀ response of RILs grown in elevated CO₂ was 34 % (Fig. 4B, Supplementary Data Table S4), indicative of a slight decrease in CO₂ sensitivity of plants grown in elevated CO₂.

Effects of long-term growth in elevated CO₂

We next sought to investigate the relationship between the control of short- and long-term g responses. To this end, 50 RILs from the cross where C24 was the pollen donor and Col-0 the pollen acceptor, which had been used in the previous experiment, were cultivated in ambient (~400 ppm) and elevated (~800 ppm) CO₂ concentrations. After 4 weeks of the respective treatment, gas exchange measurements were conducted. The short-term response was calculated as the percentage decrease in g₄₀₀ between 400 and 800 ppm measured in sequence for each individual. The long-term response was calculated as the percentage decrease in g₄₀₀ resulting from growth in elevated CO₂, i.e. for the same genotype we used g₄₀₀ values of plants from the two treatments measured at their respective growth CO₂ concentration.

When the g₄₀₀ of plants grown in ambient and elevated CO₂ was measured at the same CO₂ concentration, plants from the elevated treatment generally displayed higher g₄₀₀ than plants from the ambient treatment. On average, plants from the elevated treatment had 11 % higher g₄₀₀ than plants from the ambient treatment when measured at 400 ppm and 20 % higher g₄₀₀ when measured at 800 ppm (paired t-test of g₄₀₀, P = 0.004, n = 50; paired t-test of g₈₀₀, P < 0.0001, n = 50; Fig. 4A, Supplementary Data Table S4), indicating g₄₀₀ acclimation of plants grown in elevated CO₂.

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for QTL mapping. The major QTL on chromosome 2 associated with the short-term CO$_2$ response, identified in the previous experiment on plants grown in ambient CO$_2$, was detected in plants grown in both ambient and elevated CO$_2$. Using the subset of 50 RILs, this QTL mapped to the adjacent marker compared with the results from the previous experiment (Fig. 3, Table 2). Additionally, three other, minor QTLs for the short-term response were identified, explaining 7–10% of the variation in this trait. These QTLs were detected only in one of the CO$_2$ treatments (Table 2). For the long-term CO$_2$ response to elevated CO$_2$, one QTL explaining 14% of the variation was identified (Fig. 3, Table 2). This QTL mapped to the same marker as the major QTL for the short-term response, suggesting that these traits are regulated by the same genetic component. Furthermore, five QTLs for absolute g$_s$ were detected, of which two were associated with g$_s$ measured at both 400 and 800 ppm, two with g$_s$ at 400 ppm and one with g$_s$ at 800 ppm. Most QTLs for absolute g$_s$ were detected in plants from both CO$_2$ treatments (Fig. 3, Table 2).

ABA response

As the signalling pathway for the CO$_2$-induced closure response is known to converge downstream with the pathway for ABA-induced stomatal closure, we tested whether the main loci involved in CO$_2$-induced stomatal closure also affected the ABA-induced stomatal response. To this end, ten RILs representing contrasting genetic backgrounds and CO$_2$-induced closure phenotypes were cultivated at ambient CO$_2$ concentration and stomatal conductance was monitored after spray application of ABA. Measurements were performed on two or three replicates per line. This showed that the ability to close stomata in response to exogenous ABA was not correlated with the ability to respond to CO$_2$ (Fig. 6), suggesting that the QTL for stomatal CO$_2$ response identified in this study is not involved in ABA-induced stomatal closure.

Fine mapping of a locus on chromosome 2 controlling CO$_2$-induced closure

The major QTL associated with CO$_2$-induced stomatal closure among the RILs mapped to the end of chromosome 2. To narrow down the region of interest, nine new SSLP markers (Supplementary Data Table S2) spanning the area between markers MASC06025 and MASC02812 were developed and used to genotype a subset of RILs with crossovers at the end of chromosome 2. This approach narrowed the region to a physical distance of 410 kb between
markers MASC02812 and MASC00371 (Supplementary Data Table S5), consistent with previous mapping results that located this QTL to either of these two markers depending on the subset of RILs. This region contains the MPK12 gene, which encodes a kinase recently shown to be involved in CO₂-induced stomatal closure (Hõrak et al., 2016; Jakobson et al., 2016). However, neither publicly available sequence data (Berardini et al., 2015; Alonso-Blanco et al., 2016) nor results from our own sequencing show any sequence differences between C24 and Col-0 for this gene or 0.5-kb flanking regions, except for a single SNP 0.47 kb downstream of the coding sequence.

Confirmation of the major QTL on chromosome 2 using NILs

The short-term CO₂ response of nine NILs (Supplementary Data Table S1; Törjék et al., 2008) measured in triplicate was used to confirm the location and effect of the major QTL identified in previous experiments with RILs. In these lines, the genome was predominantly from one of the parents, with a small introgression of the opposite genotype at the end of chromosome 2. Three lines were on the C24 background and six on the Col-0 background (Fig. 7A). Lines on the Col-0 background with introgression from C24 changed their CO₂ response to the C24 phenotype and vice versa; lines on the C24 background with Col-0 introgression gained the Col-0 phenotype (Fig. 7B). One line (NIL number N1) could not be statistically distinguished from either of the parental accessions (Fig. 7B). The results of measurements of these independent lines confirm the location of the QTL between the two last markers on chromosome 2. Furthermore, these results show that the effect of the QTL was large enough to shift the phenotype from that of the background accession to one similar to the phenotype of the introgressed accession.

**DISCUSSION**

In this study, we used natural variation in stomatal regulation between the two A. thaliana accessions C24 and Col-0 to identify genetic loci associated with short- and long-term responses of CO₂ to elevated CO₂ concentration, as well as several other stomata-related traits. The short-term response represents the adjustment of gₛ that occurs within minutes to hours after change in atmospheric CO₂ concentration, whereas the long-term response represents a change in gₛ seen after weeks to months of cultivation under elevated CO₂ concentration. The use of RILs originating from a cross between C24 and Col-0 enabled the identification of a number of QTLs associated with stomatal regulation. Most notable was a QTL at the end of chromosome 2 explaining ~50% of the variation in the short-term gₛ response to elevated CO₂ concentration among the tested RILs.
Interestingly, this QTL was also associated with the long-term \( g_s \) response to growth under elevated CO\(_2\) concentration, suggesting that these traits are regulated by the same underlying gene. The same QTL was additionally associated with absolute \( g_s \) at ambient CO\(_2\) concentration and water-use efficiency. The Col-0 genotype at this locus conferred stronger CO\(_2\) responsiveness in both the short and the long term, as well as higher \( g_s \) at 400 ppm CO\(_2\). The C24 genotype was associated with higher water-use efficiency.

Exogenous application of ABA to a subset of RILs with the most extreme CO\(_2\) response phenotypes showed that there was no correlation between the stomatal closure responses to ABA and CO\(_2\). This implies that the identified major QTL is involved in the CO\(_2\)-specific branch of the signalling pathway for stomatal closure, upstream of the convergence point for CO\(_2\)- and ABA-induced responses. Analysis of short-term responsiveness to elevated CO\(_2\) in reciprocal NILs between C24 and Col-0 confirmed the location of the QTL at the end of chromosome 2. Introgressions in this region caused a significant change in responsiveness and shifted the phenotype to one similar to that of the introgressed parent. Fine mapping using RILs with crossovers in the region of interest allowed us to locate the QTL to a 410-kb region. This region contains the \textit{MPK12} gene, which was recently shown to have a pivotal role in CO\(_2\)-induced stomatal closure (Jakobson et al., 2016). However, no sequence polymorphisms were found between C24 and Col-0 in \textit{MPK12} that could explain the phenotypic difference. The phenotype of C24, i.e. weak CO\(_2\) response in combination with low \( g_s \), also differs from the phenotype resulting from known loss-of-function mutations in \textit{MPK12}, i.e. weak CO\(_2\) response in combination with very high \( g_s \) (Jakobson et al., 2016; Tõldsepp et al., 2018). Finally, C24 and Col-0 show only a moderate difference in expression of \textit{MPK12} (60% higher in C24; Xu et al., 2015). Taking these results together, it is thus unlikely that any polymorphism affecting the expression of \textit{MPK12} could explain the difference in CO\(_2\)-dependent stomatal closure between C24 and Col-0. Besides \textit{MPK12}, the mapped region does not contain any genes previously linked to stomatal behaviour. Further studies are required to accurately pinpoint the exact molecular difference underlying this QTL. Nevertheless, our data point to the presence of at least one gene in this region that encodes an important as yet unidentified component regulating \( g_s \) and its response to elevated CO\(_2\) in both the short and the long term.

### Table 2. QTLs detected using data from gas exchange measurements on recombinant inbred lines grown in ambient (A) or elevated (E) CO\(_2\) concentration

| Trait                        | QTL location and confidence interval (cM) | Chromosome | Closest marker   | Treatment | Variance explained (%) | High-value allele |
|------------------------------|-------------------------------------------|------------|------------------|-----------|------------------------|-------------------|
| Short-term CO\(_2\) response | 18 (0–138)                                | 1          | MASC09203        | E         | 8                      | C24               |
| \( g_s \) \(_{400}\)         | 18 (0–138)                                | 1          | MASC09203        | E         | 13                     | C24               |
| Short-term CO\(_2\) response | 123 (104–125)                              | 2          | MASC00371        | A and E   | 14                     | Col-0             |
| \( g_s \) \(_{400}\)         | 123 (104–125)                              | 2          | MASC00371        | A and E   | 14                     | Col-0             |
| Long-term CO\(_2\) response  | 38 (0–101)                                | 3          | MSAT3.19/MASC04516 | A         | 7                      | C24               |
| \( g_s \) \(_{400}\)         | 38 (0–101)                                | 3          | MSAT3.19/MASC04516 | A and E   | 18                     | Col-0             |
| \( g_s \) \(_{400}\)         | 6 (0–42)                                  | 4          | FRI/MASC04123    | A and E   | 11                     | Col-0             |
| \( g_s \) \(_{400}\)         | 6 (0–42)                                  | 4          | FRI/MASC04123    | A and E   | 11                     | Col-0             |
| Short-term CO\(_2\) response | 79 (0–103)                                | 4          | MASC02548/F24J7ID/G3883-1.4 | A         | 10                     | C24               |
| \( g_s \) \(_{400}\)         | 79 (0–103)                                | 4          | MASC02548/F24J7ID/G3883-1.4 | A and E   | 15                     | E 8               |

Fig. 6. There was no significant relationship between stomatal responses to exogenous abscisic acid (ABA) and elevated CO\(_2\). Five recombinant inbred lines displaying the weakest and five displaying the strongest CO\(_2\) responses were sprayed with 5 \( \mu \)m ABA. The percentage decrease in \( g_s \) following application was quantified using gas exchange measurements. Measurements were performed on two or three replicates per line.

\[
y = 0.17x + 29 \\
r^2 = 0.11, P = 0.34
\]
short-term responsiveness among plant species and/or varieties could thus be valuable for projections of plant water use under rising atmospheric CO₂ concentration. Cases where short- and long-term responses are decoupled due to a pronounced stomatal density response in plants with weak short-term responsiveness (Haworth et al., 2013) should, however, be a focus of further studies.

Growth under elevated CO₂ concentration resulted in an average gₛ decrease of 26 % among the tested RILs, which is similar to the average long-term response observed in field experiments (21 %, Medlyn et al., 2001; 22 %, Ainsworth and Rogers, 2007). Plants grown under elevated CO₂ exhibited slightly attenuated short-term responsiveness to CO₂ and higher gₛ compared with plants grown under ambient CO₂ when measured at the same CO₂ concentration. These results show that both guard cell CO₂ responsiveness and absolute gₛ acclimated to the CO₂ concentration during growth. Previous research has shown that guard cells of plants grown under elevated CO₂ may lose some of their sensitivity to short-term changes in CO₂ concentration (Morison, 1998; Lodge et al., 2001; Medlyn et al., 2001; Onandia et al., 2011). The direction of gₛ acclimation in our study differed from previous observations. A meta-analysis on trees subjected to long-term CO₂ exposure showed that photosynthetic capacity and gₛ were downregulated in parallel, resulting in lower gₛ of plants grown in elevated CO₂ when plants from both treatments were measured at the same CO₂ concentration (Medlyn et al., 1999, 2001). Similar results were observed in an experiment with A. thaliana in the reproductive stage (Teng et al., 2006). Experiments on earlier growth stages of A. thaliana, on the other hand, showed no downregulation of photosynthetic capacity or Rubisco content as long as plants were grown with an ample nitrogen supply (Tocquin et al., 2006; Jauregui et al., 2015). Tocquin et al. (2006) suggested that A. thaliana under controlled growth conditions simply responds to elevated CO₂ by growth rate adjustment. One potential explanation for the upregulation of gₛ in our experiment is that increased photosynthetic efficiency under elevated CO₂ stimulates leaf production and expansion in young A. thaliana plants, which not only maintains sink capacity but also increases the need for photosynthesis. Consequently, photosynthesis may be stimulated further and result in the observed upregulation of gₛ since changes in leaf CO₂ demand and supply are typically well coupled (Wong et al., 1979). Indeed, plants grown under elevated CO₂ in our experiment showed a 60 % increase in total leaf area (data not shown).

Stomatal regulation is a complex, tightly regulated trait of crucial importance for plant fitness and survival. As such, it can be expected to be controlled by the coordinated action of many genes with a certain degree of redundancy. Indeed, we identified numerous loci associated with stomatal regulation in addition to the major QTL on chromosome 2. Most of these QTLs explained minor proportions of the trait variation, but could potentially provide useful information about candidate genes if mapped more precisely. As the main focus of the present study was on the stomatal CO₂ response, for which phenotyping is very time-consuming, it was necessary to work with a reduced set of lines. However, traits such as water-use efficiency and absolute gₛ could be quantified in a larger population, which may increase mapping power and resolution (Keurentjes et al., 2007). For several traits we identified QTLs with allelic effects opposite to those predicted by the parental phenotypes, corroborating the observation of transgressive segregation in the RIL population.

The fact that short- and long-term CO₂ responses were significantly correlated and associated with the same locus suggests that knowledge about the signalling pathway for short-term gₛ regulation in response to elevated CO₂ concentration could be used for manipulation of long-term gₛ responses under rising atmospheric CO₂. Results from experimental field research corroborate the link between short- and long-term gₛ responses (Hasper et al., 2017), indicating that the short-term CO₂ response may be a useful predictor of the long-term CO₂ effect on gₛ also under ecologically realistic conditions. Data on
This study clearly demonstrates the large potential of using natural variation in *A. thaliana* to uncover the genetic basis of stomatal regulation and water economy in plants. The detection of two different QTLs in the same region using mapping populations with separate genetic backgrounds (the present study; Juenger et al., 2005; Brosché et al., 2010; Des Marais et al., 2014; Jakobson et al., 2016) highlights the importance of exploiting the variation among numerous accessions to fully resolve the genetic regulation of complex traits. The large differences in $g_s$ or water-use efficiency observed among cultivars of wheat (Lu et al., 1998; Condon et al., 2004), rice (Horie et al., 2006), maize (Ryan et al., 2016), legumes (Ehleringer et al., 1991; Ashok et al., 1999) cotton (Lu et al., 1998) and sugarcane (Basnayake et al., 2015) show that there is a large untapped potential in the genetic variation for stomatal traits in crop species as well. Breeding for low $g_s$ and high water-use efficiency may result in crop varieties suitable for cultivation in already dry areas (Araus et al., 2002), as shown by the successful development of transpiration-efficient wheat cultivars (Rebetzke et al., 2002; Condon et al., 2004). In the more moist and fertile areas currently suitable for highly productive crops with relatively high $g_s$, it would be advantageous to grow cultivars exhibiting a gradual but substantial shift towards lower $g_s$ as the atmospheric CO$_2$ concentration increases and the air, and perhaps also the soil, becomes progressively drier. While there is typically a trade-off between high gas exchange and high water-use efficiency, our results show that plants with high $g_s$ at the current atmospheric CO$_2$ concentration may also exhibit large improvements in water economy under rising atmospheric CO$_2$. In fact, high $g_s$ at present-day atmospheric CO$_2$ concentration and strong stomatal responsiveness to CO$_2$ were associated with the same QTL in the present study, and may even be regulated by the same gene.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: histogram plots showing the distribution of trait data among RILs. Table S1: genotype data and genetic maps of RILs and NILs. Table S2: sequences showing the distribution of trait data among RILs. Table S1: geno-com/aob and consist of the following. Figure S1: histogram plots regulated by the same gene. associated with the same QTL in the present study, and may even be rising atmospheric CO2. In fact, high $g_s$ as the atmos-

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LITERATURE CITED

Ainsworth EA, Rogers A. 2007. The response of photosynthesis and stomatal conductance to rising CO$_2$: mechanisms and environmental interactions. *Plant, Cell & Environment* 30: 258–270.

Alonso-Blanco C, Andrade J, Becker C, et al. 2016. 1,135 genomes reveal the global pattern of polymorphism in *Arabidopsis thaliana*. *Cell* 166: 481–491.

Araus JL, Slaper GA, Reynolds MP, Royo C. 2002. Plant breeding and drought in C3 cereals: what should we breed for? *Annals of Botany* 89: 925–940.

Ashok ISAH, Prasad TG, Wright GC, Kumar MU, Rao RCN. 1999. Variation in transpiration efficiency and carbon isotope discrimination in cowpea. *Functional Plant Biology* 26: 503–510.

Basnayake J, Jackson PA, Inman-Bamber NG, Lakshmanan P. 2015. Sugarcane for water-limited environments. Variation in stomatal conductance and its genetic correlation with crop productivity. *Journal of Experimental Botany* 66: 3945–3958.

Berardini TZ, Reiser L, Li D, et al. 2015. The Arabidopsis Information Resource: making and mining the “gold standard” annotated reference plant genome. *Genetics* 53: 474–485.

Boyes DC, Zayed AM, Ascenzi R, et al. 2001. Growth stage-based phenotypic analysis of *Arabidopsis*: a model for high throughput functional genomics in plants. *Plant Cell* 13: 1499–1510.

Broman KW, Wu H, Sen S, Churchill GA. 2003. R/qtl: QTL mapping in experimental crosses. *Bioinformatics* 19: 889–890.

Brosché M, Merilo E, Mayer F, et al. 2010. Natural variation in ozone sensitivity among *Arabidopsis thaliana* accessions and its relation to stomatal conductance. *Plant, Cell & Environment* 33: 914–925.

Condon AG, Richards RA, Rebetzke GJ, Farquhar GD. 2004. Breeding for high water-use efficiency. *Journal of Experimental Botany* 55: 2447–2460.

Ehleringer JR, Klasen S, Clayton C, et al. 1991. Carbon isotope discrimination and transpiration efficiency in common bean. *Crop Science* 31: 1611–1615.

Engineer CB, Hashimoto-Sugimoto M, Negi J, et al. 2016. CO$_2$ Sensing and CO$_2$ regulation of stomatal conductance: advances and open questions. *Trends in Plant Science* 21: 16–30.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 503–537.

Franks PJ, Britton-Harper JZ. 2016. No evidence of general CO$_2$ insensitivity in ferms: one stomatal control mechanism for all land plants? *New Phytologist* 211: 819–827.

Geiger D, Scherer S, Mumm P, et al. 2009. Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proceedings of the National Academy of Sciences of the USA* 106: 21425–21430.

Hashimoto M, Negi J, Young J, Israelsson M, Schroeder JI, Iba K. 2006. *Arabidopsis* HKT1 kinase controls stomatal movements in response to CO$_2$. *Nature Cell Biology* 8: 391–397.

Hasper TB, Dusenge ME, Breuer F, Uwizeye FK, Wallin G, Uddling J. 2017. Stomatal CO$_2$ responsiveness and photosynthetic capacity of tropical woody species in relation to taxonomy and functional traits. *Oecologia* 184: 43–57.

Haworth M, Elliott-Kingston C, McElwain JC. 2013. Co-ordination of physiological and morphological responses of stomata to elevated [CO$_2$] in vascular plants. *Oecologia* 171: 71–82.

Haworth M, Killi D, Materassi A, Raschi A. 2015. Coordination of stomatal physiological behavior and morphology with carbon dioxide determines stomatal control. *American Journal of Botany* 102: 677–688.
Johansson et al. — Genetic controls of short- and long-term CO₂ responses

Haworth M, Killi D, Materassi A, Raschi A, Centritto M. 2016. Impaired stomatal control is associated with reduced photosynthetic physiology in crop species grown at elevated [CO₂]. Frontiers in Plant Science 7: 1568.

He J, Zhang RX, Peng K, et al. 2018. The BIG protein distinguishes the process of CO₂-induced stomatal closure from the inhibition of stomatal opening by CO₂. BioMed Research International 210: 232–241.

Hedrich R, Geiger D. 2017. Biology of SLAC1-type anion channels – from nutrient uptake to stomatal closure. New Phytologist 216: 46–61.

Hörak H, Kollist H, Merilo E. 2017. Fern stomatal responses to ABA and CO₂ depend on species and growth conditions. Plant Physiology 174: 672–679.

Hörak H, Siera M, Töldsepp K, et al. 2016. A dominant mutation in the HT1 kinase uncovers roles of MAP kinases and GHR1 in CO₂-induced stomatal closure of forest species after long-term exposure to elevated CO₂ concentration: a synthesis. New Phytologist 149: 247–264.

Negi J, Matsuda O, Nagasawa T, et al. 2008. CO₂ regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature 452: 483–486.

Nilsson AK, Fahlgren P, Johansson ON, Hamberg M, Andersson MX, Ellerström M. 2016. The activity of HYDROPEROXIDE LYASE 1 regulates accumulation of galactolipids containing 12-oxo-phytodienoic acid in Arabidopsis. Journal of Experimental Botany 67: 5133–5144.

Onandia G, Olsson AK, Barth S, King JS, Uddling J. 2011. Exposure to moderate concentrations of tropospheric ozone impairs tree stomatal response to carbon dioxide. Environmental Pollution 159: 2350–2354.

Pérez-Harguindeguy N, Díaz S, Garnier E, et al. 2013. New handbook for standardised measurement of plant functional traits worldwide. Australian Journal of Botany 61: 167–234.

Rebetzke GJ, Condon AG, Richards RA, Farquhar GD. 2002. Selection for reduced carbon isotope discrimination increases aerial biomass and grain yield of rainfed bread wheat. Crop Science 42: 739–745.

Ruszala EM, Beering DJ, Franks PJ, et al. 2011. Land plants acquired active stomatal control early in their evolutionary history. Current Biology 21: 1030–1035.

Ryan AC, Dodd IC, Rothwell SA, et al. 2016. Gravimetric phenotyping of whole plant transpiration responses to atmospheric vapour pressure deficit identifies genotypic variation in water use efficiency. Plant Science 251: 101–109.

Šantrůček J, Sage R. 1996. Acclimation of stomatal conductance to a CO₂-enriched atmosphere and elevated temperature in Chenopodium album. Functional Plant Biology 23: 467–478.

Schmidt R, Boudichevskaia A, Cao HX, He S, Meyer RC, Reif JC. 2017. Extracting genotope information of Arabidopsis thaliana recombinant inbred lines from transcript profiles established with high-density oligonucleotide arrays. Plant Cell Reports 36: 1871–1881.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9: 671–675.

Takahashi S, Monda K, Negi J, et al. 2015. Natural variation in stomatal responses to environmental changes among Arabidopsis thaliana ecotypes. PLoS ONE 10: 1–13.

Teng N, Wang J, Chen T, Wu X, Wang Y, Lin J. 2006. Elevated CO₂ induces physiological, biochemical and structural changes in leaves of Arabidopsis thaliana. New Phytologist 172: 92–103.

Toquin P, Ormenes S, Piellun A,Detach N, Bernier G, Périlleux C. 2006. Acclimation of Arabidopsis thaliana to long-term CO₂ enrichment and nitrogen supply is basically a matter of growth rate adjustment. Physiologia Plantarum 128: 677–688.

Töldsepp K, Zhang J, Takahashi Y, et al. 2018. Mitogen-activated protein kinases MPK4 and MPK12 are key components mediating CO₂-induced stomatal movements. Plant Journal 96: 1018–1035.

Törjék O, Berger D, Meyer RC, et al. 2003. Establishment of a high-efficiency SNP-based framework marker set for Arabidopsis. Plant Journal 36: 122–140.

Törjék O, Witucka-Wall H, Meyer RC, et al. 2006. Segregation distortion in Arabidopsis C24/Col-0 and Col-0/C24 recombinant inbred line populations is due to reduced fertility caused by epistatic interaction of two loci. Theoretical and Applied Genetics 113: 1551–1561.

Törjék O, Meyer RC, Zehndorf M, et al. 2008. Construction and analysis of 2 reciprocal Arabidopsis introgression line populations. Journal of Heredity 99: 396–406.

Vahisalu T, Kollist H, Wang YF, et al. 2008. SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature 452: 487–491.

Webb AA, Hetherington AM. 1997. Convergence of the abscisic acid, CO₂ and extracellular calcium signal transduction pathways in stomatal guard cells. Plant Physiology 114: 1557–1560.

Wong SC, Cowan IR, Farquhar GD. 1979. Stomatal conductance correlates with photosynthetic capacity. Nature 282: 424–426.