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Cotton bracts are adapted to a microenvironment of concentrated CO_2 produced by rapid fruit respiration

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• *Background and Aims* Elucidation of the mechanisms by which plants adapt to elevated CO_2 is needed; however, most studies of the mechanisms investigated the response of plants adapted to current atmospheric CO_2. The rapid respiration rate of cotton (*Gossypium hirsutum*) fruits (bolls) produces a concentrated CO_2 microenvironment around the bolls and bracts. It has been observed that the intercellular CO_2 concentration of a whole fruit (bract and boll) ranges from 500 to 1300 μmol mol^{-1} depending on the irradiance, even in ambient air. Arguably, this CO_2 microenvironment has existed for at least 1·1 million years since the appearance of tetraploid cotton. Therefore, it was hypothesized that the mechanisms by which cotton bracts have adapted to elevated CO_2 will indicate how plants will adapt to future increased atmospheric CO_2 concentration. Specifically, it is hypothesized that with elevated CO_2 the capacity to regenerate ribulose-1,5-bisphosphate (RuBP) will increase relative to RuBP carboxylation.
• *Methods* To test this hypothesis, the morphological and physiological traits of bracts and leaves of cotton were measured, including stomatal density, gas exchange and protein contents.
• *Key results* Compared with leaves, bracts showed significantly lower stomatal conductance which resulted in a significantly higher water use efficiency. Both gas exchange and protein content showed a significantly greater RuBP regeneration/RuBP carboxylation capacity ratio (J_{max}/V_{cmax}) in bracts than in leaves.
• *Conclusions* These results agree with the theoretical prediction that adaptation of photosynthesis to elevated CO_2 requires increased RuBP regeneration. Cotton bracts are readily available material for studying adaption to elevated CO_2.

Key words: Bract, cotton, CO_2 acclimation, CO_2 adaptation, Cyt b_6f, *Gossypium hirsutum*, J_{max}/V_{cmax}, photosynthesis, respiration, Rubisco, stomatal conductance, water use efficiency.

INTRODUCTION

The atmospheric carbon dioxide concentration [CO_2] has been rising since the Industrial Revolution, from 280 to 400 μmol mol^{-1}, because of human activity, and is predicted to double by 2100 according to the Intergovernmental Panel on Climate Change (IPCC) (Meehl *et al.*, 2007). CO_2 is a substrate for photosynthesis; therefore, the increase in atmospheric [CO_2] generally enhances leaf photosynthesis, and increases plant growth and crop yield (Drake *et al.*, 1997; Nakano *et al.*, 1997; Kimball *et al.*, 2002). However, the enhancement of growth depends strongly on species (Long *et al.*, 2004). When plants are grown under prolonged elevated [CO_2], the enhancement is often offset by downregulation of photosynthetic capacity (Stitt, 1991; Gunderson and Wullschleger, 1994; Sage, 1994; Long *et al.*, 2004). This limits not only plant growth and production but also the capacity of vegetation to remove CO_2 from the atmosphere. Therefore, enormous research efforts have been devoted to studying the traits and mechanisms which enable a sustained enhancement of plant photosynthesis and growth.

Research has mostly focused on the response to elevated [CO_2] of plants which are adapted to the 'current' ambient [CO_2]. The effects of elevated [CO_2] have been studied in open top chambers (OTCs), by free air CO_2 enrichment (FACE) and in growth chambers, but the longest continuous study is at most two decades long (Mulchi *et al.*, 1992; McKee and Woodward, 1994; Ainsworth and Long, 2005). However, the plants analysed have not been adapted for very long periods (millennia or more) by growth in elevated [CO_2], so that understanding how long-term adaptation of mechanisms may occur is not possible. This 'short-term' response is not necessarily the same as the adaptive response to high [CO_2]. For example, plants can increase their photosynthesis rate just after the transfer to high [CO_2], but subsequently would decrease their photosynthetic capacity by the downregulation of photosynthesis triggered by the accumulation of carbohydrate (Webber *et al.*, 1994; Nie *et al.*, 1995).

Plants growing under high [CO_2] around natural CO_2 springs have been studied because they may have adapted to a high [CO_2]. In such conditions, the atomospheric [CO_2] is considered to have been enriched for many plant generations (Miglietta and Raschi, 1993; Bettarini *et al.*, 1998; Onoda *et al.*, 2009). Therefore, it is suggested that the plants from CO_2 springs have adapted to the elevated [CO_2] and are more likely to possess mechanisms that enhance photosynthesis continuously in high [CO_2]. Plants from CO_2 springs showed lower stomatal conductance and higher water use efficiency (WUE) in common garden experiments (Onoda *et al.*, 2009). These results agreed with the theoretical prediction of mechanisms for adaptation to high [CO_2] (Sage, 1994; Drake *et al.*, 1997), as plants can maintain a sufficient photosynthetic rate even with decreased stomatal conductance and so reduce the transpiration rate, which would result in the higher WUE of plants from CO_2 springs. From the photosynthesis model of Farquhar *et al.* (1980), the photosynthetic rate is limited by either the ribulose-1,5-bisphosphate (RuBP) regeneration rate or the RuBP carboxylation rate. Because the RuBP carboxylation rate is dependent on the intercellular [CO_2] (C_i), it is more enhanced in elevated [CO_2], and RuBP regeneration becomes the limiting factor of the rate of photosynthesis. Therefore, previous theoretical studies suggested that plants should increase their protein contents for RuBP regeneration and decrease their protein contents for RuBP carboxylation at high [CO_2], resulting in both increased photosynthetic nitrogen use efficiency and an increased RuBP regeneration/RuBP carboxylation capacity ratio (J_{max}/V_{cmax}) (Sage, 1994; Medlyn, 1996; Drake *et al.*, 1997; Hikosaka and Hirose, 1998), which is supported by elevated [CO_2] experiments (Long *et al.*, 2004; Ainsworth and Long, 2005). Accordingly, it is predicted that plants adapted to an elevated [CO_2] would show a higher J_{max}/V_{cmax}. However, Onoda *et al.* (2009) reported that plants from CO_2 springs did not show a significant difference in J_{max}/V_{cmax} compared with those from control sites.

The environment of CO_2 springs also has other characteristics which may affect plants, e.g. SO_2 emissions and water availability, and are difficult to distinguish from the CO_2 effects. To avoid such effects, we have looked at more familiar situations which offer a high CO_2 microenvironment in plants. In the present study, we focused on a high CO_2 microenvironment produced by rapid respiration rates in reproductive organs. Reproductive organs, especially fruits and associated organs, generally have high respiration rates (Wullschleger and Oosterhuis, 1990). These high respiration rates would cause a concentrated CO_2 microenvironment around the organs, a phenomenon which should have existed for millennia (evolutionary time scale). Cotton, *Gossypium hirsutum* L., providing the world's major natural fibre, bears fruits which have very rapid respiration. The fruits are covered by specialized leaves – bracts – which have a high photosynthetic capacity and contribute significantly to plant carbon gain especially in the later growth stages (Constable and Rawson, 1980; Wullschleger and Oosterhuis 1990; Hu *et al.*, 2012). Accordingly, we proposed the following hypotheses: (1) the very rapid respiration rate of cotton fruits would produce a high CO_2 microenvironment; and (2) the photosynthetic traits of bract have adapted to the elevated CO_2 microenvironment within the tissues, cells and chloroplasts. Bracts have many similarities to leaves in their morphology and anatomy, so that comparison of the two organs should provide insight into changes related to a long-term increase in [CO_2]. For morphological traits, stomatal density was measured because it relates to regulation of CO_2 influx and water vapour efflux from tissues. For physiological traits, RuBP carboxylation capacity and RuBP regeneration capacity, stomatal conductance and WUE were analysed with gas exchange measurements, and these capacities were complemented by measurements of photosynthetic protein contents.

MATERIALS AND METHODS

Study sites and species

Cotton (*Gossypium hirsutum* L. 'Deltapine 90') plants were grown in a glasshouse at approx. 28/18 °C (day/night) under natural light in Canberra (35°17′S, 149°08′E) from February to May 2011. The plants were provided twice per week with a nutrient solution of 'Aquasol' (23 % N:4 % P:18 % K; Hortico Ltd, New South Wales, Australia), supplemented by a slow-release fertilizer ('Osmocote', Scott Australia Pty. Ltd, Bella Vista, Australia). At anthesis, the main-stem leaves closest to the opening flowers were labelled (in April 2011). The gas exchange measurements of the leaves, bracts and fruits were conducted 10, 15, 20 and 30 d after anthesis (only measurements of fruit respiration were carried out 15 d after anthesis). Cotton (*G. hirsutum* 'Xinluzao 13') plants were also grown at an experimental field of Shihezi Agricultural College, Shihezi University, Xinjiang, China (45°19′N, 86°03′E) in 2011. Mean day/night temperature was 29·5/21·0 °C. Only the stomatal density of the field data was used in the main text (Table 1). The gas analyses data of field plants showed the same trend as did the data of glasshouse plants; therefore, they are given only as Supplementary information.

Stomatal density measurements

Stomatal density and the stomatal aperture size were measured on the leaves and bracts 20 d after anthesis in China. The organs were coated with a thick layer of cellulose acetate (nail polish), and the dried replicas were carefully peeled off and placed on microscope slides. Dried replicas were taken from a leaf and a bract of three separate plants, and three sub-samples per replica were used to determine stomatal sizes and densities.

Anatomical structures of leaves and bracts

Tissue samples for light microscopic photography were taken from a leaf and a bract of three separate plants 20 d after anthesis in China. The samples were fixed in formalin–acetic acid–alcohol (FAA) and then dehydrated in an ethanol series and embedded in paraffin. Transverse sections, 10 μm thick, were cut with a rotary microtome and double-stained with Safranin–Fast Green. To determine the thickness of the tissue, photomicrographs were taken with a light microscope (Vanox, Olympus, Tokyo, Japan) at a magnification of ×200.

Gas exchange measurements

Gas exchange was measured on the main leaves, bracts, bolls and whole fruits, using a portable photosynthesis measuring

instrument (LI6400, LI-COR, Lincoln, NE, USA). For whole fruits (including bracts and bolls, Fig. 1A) and 'only bolls' (without bracts, Fig. 1B), a conifer chamber (6400-05, LI-COR) with a white light-emitting diode (LED) light source (Luxeon LEDs; Electus Distribution, NSW, Australia) was used. For leaves and 'only' bracts, a normal 2 × 3 cm chamber with a 6400-02B (LI-COR) LED light source was used. 'Only bracts' means the bracts detached from bolls for the measurements of the CO_2 response of assimilation described below; they were still attached to the stem.

Response curves of the net CO_2 assimilation rate (A) and C_i in relation to photosynthetically active radiation (PAR) were measured by first determining the parameters at 2000 μmol m^{-2} s^{-1} PAR for leaves and at 1117 μmol m^{-2} s^{-1} for whole fruits and 'only bolls'. Then the light intensity was decreased in a stepwise manner. The net CO_2 assimilation rate in the dark was used as the respiration rate (Fig. 2). Leaf temperature and chamber CO_2 concentration were kept at about 25 °C and 400 μmol mol^{-1}, respectively. For whole fruits and 'only bolls', measurements were made of four separate plants, and two separate plants were used for leaves.

For measurements of the net CO_2 assimilation rate, stomatal conductance, C_i/C_a and instantaneous WUE (iWUE) as a function of C_i ($A-C_i$ curves), only leaves and bracts without bolls were used. The measurements were made with a leaf and a bract of four, five and three separate plants 10, 20 and 30 d after anthesis, respectively. Samples were kept at 400 μmol mol^{-1} of chamber [CO_2] for at least 30 min in the first step. In the next step, the parameters were measured at 0 μmol mol^{-1}, and then the [CO_2] was increased stepwise (9–12 steps) up to 2000 μmol mol^{-1}. Light intensity and leaf temperature were kept at 2000 μmol m^{-2} s^{-1} and 25 °C, respectively. Although the transpiration rate was higher in leaves than in bracts, the vapour pressure deficit (VPD) was similar during measurements on leaves and bracts. The iWUE was calculated as the net CO_2 assimilation rate divided by the transpiration rate.

Photosynthetic gas exchange measurements were made between 1000 and 1600 h. Data of samples of the same age (the same days after anthesis) were averaged at the same measurement light intensities or C_a. The surface area of the whole fruit was used when calculating the respiration rate of the whole fruit on an area basis.

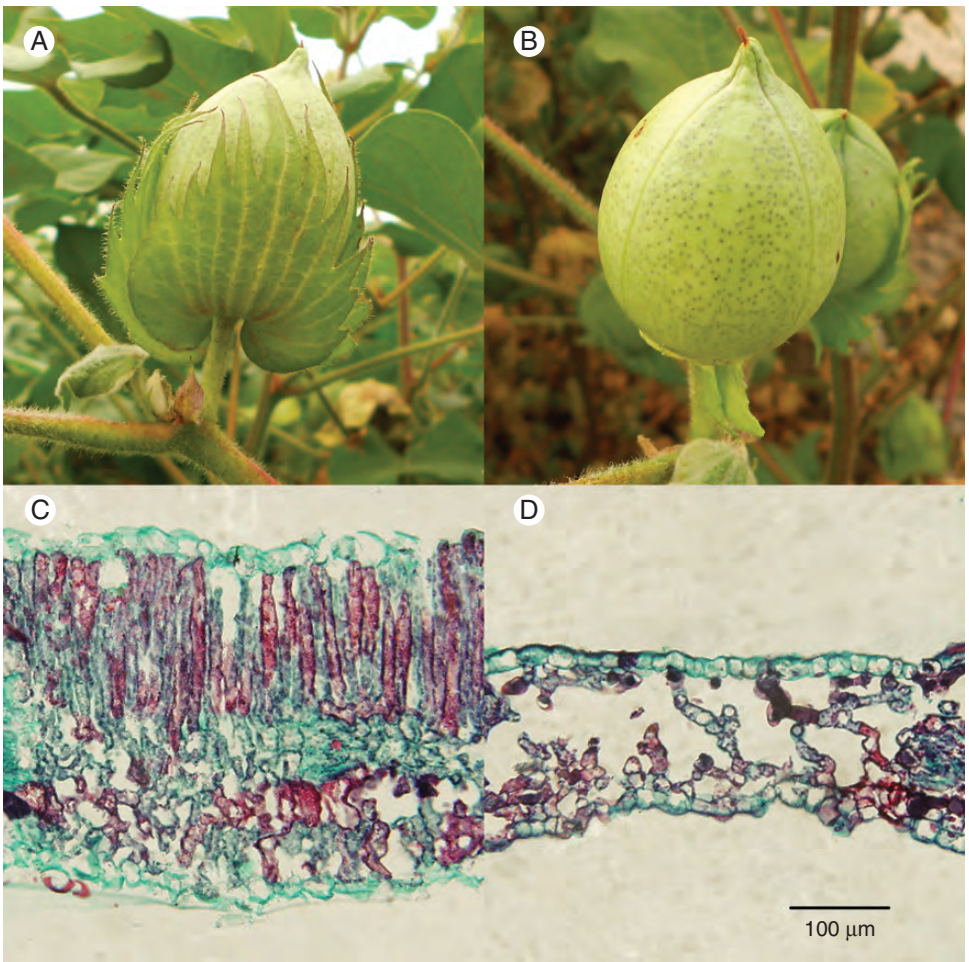

FIG. 1. (A, B) Photographs of fruit of cotton, *Gossypium hirsutum*. In (B), bracts were artificially detached from the boll. Photomicrographic cross-sections of (C) a leaf (×200) and (D) a bract (×200). In the photographs, the upper side of the tissue is the adaxial side and the lower side of the tissue is the abaxial side. In the case of the bract (D), the upper side (adaxial side) is facing the fruit (boll).

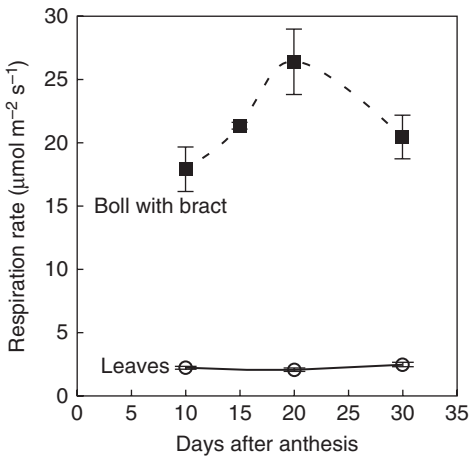

FIG. 2. Respiratory activity during ontogeny of whole fruits of cotton (boll with bract) and leaves expressed per unit surface area. Error bars represent the standard deviation. The sample number was four for 20 d after anthesis, and from two to three for other days. Respiratory activity of whole fruits measured in the field in China are given in Supplementary Data Fig. S1.

A–C_i *curve fitting*

The CO_2 response curve was fitted using the model of Farquhar *et al.* (1980). When RuBP is sufficient, the CO_2 assimilation rate limited by Rubisco activity (A_c) can be expressed as:

$$A_c = \frac{V_{c\,max}(C_i - \Gamma^*)}{C_i + K_c(1 + O/K_o)} - R_d \quad (1)$$

where V_{cmax} is the maximal velocity of RuBP carboxylation; K_c and K_o are the Michaelis constants of Rubisco activity for CO_2 and O_2, respectively; C_i and O are the partial pressure of CO_2 and O_2 in the intercellular space, respectively; R_d is the respiration rate in the light; and Γ^* is the CO_2 compensation point in the absence of R_d. The equation was fitted to the data with Rubisco kinetic parameters as reported by von Caemmerer *et al.* (1994).

The CO_2 assimilation rate limited by RuBP regeneration (A_j) can be expressed as:

$$A_j = \frac{J_{max}(C_i - \Gamma^*)}{4C_i + 8\Gamma^*} - R_d \quad (2)$$

where J_{max} is the maximum rate of electron transport driving RuBP regeneration.

Best-fit values of the parameters J_{max} and V_{cmax} were obtained by non-linear least squares regression for each leaf and bract. For leaves, values of V_{cmax} and R_d were estimated from the A–C_i curve where C_i <250 μmol mol^{-1}. J_{max} was calculated by fitting eqn (2) to the A–C_i curves above a C_i of 600 μmol mol^{-1}. For bracts, the values of V_{cmax} and R_d were estimated from the A–C_i curve where C_i <700 μmol m^{-2} s^{-1}, and J_{max} was estimated above a C_i of 1000 μmol mol^{-1}.

Measurements of photosynthetic pigments and proteins

Discs (6 mm diameter) were taken from the leaves and bracts of plants 20 d after anthesis. Chlorophyll contents (Chl*a*, Chl*b* and Chl*a* + *b*) were determined spectrophotometrically after extraction in 80 % (v/v) acetone for 24 h at room temperature in darkness (Porra *et al.*, 1989).

For protein analysis, frozen samples stored at −80 °C were ground in liquid nitrogen and homogenized in an extraction buffer, containing 50 mM EPPS-NaOH (pH 7·8), 10 mM $MgCl_2$, 2 mM EDTA, 10 mM dithiothreitol (DTT), 5 % polyvinyl polypyrrolidone (PVPP), 0·5 % Triton-X (v/v), 10 % glycerol, 1·5 % 'Complete Protease Inhibitor Cocktail' (Sigma, St Louis, MO, USA) and 2 % SDS. The protein concentration in the extract varied from 1·3 to 1·9 g L^{-1}. For the assay of all proteins, 90 μL of the extract was mixed with 30 μL of SDS loading buffer, which contains 225 mM Tris–HCl (pH 6·8), 36 % (v/v) glycerol, 7·2 % (w/v) SDS, 0·009 % (w/v) bromophenol blue and 10 % β-mercaptoethanol. The Rubisco content was determined with a calibration curve in which SDS-treated bovine serum albumin (BSA) was used as the standard. The content of Rieske FeS of the Cyt b_6f complex was quantified by immunoblotting with anti-Rieske FeS antibody (Agrisera, Vännäs, Sweden), following the method of Yamori *et al.* (2011). To calculate the absolute amount, Rieske FeS protein standard (Agrisera) was used to obtain the calibration curve.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) using SPSS statistical software (version 11.5, IBM, New York, USA). The data are present as the mean ± s.d. Differences at $P < 0{\cdot}05$ were considered significant.

RESULTS

Respiration rate and intercellular CO_2 concentration of leaves and bracts

The respiration rate per surface area of cotton fruit was more than eight times greater than the respiration rate of leaves throughout development (Fig. 2). The respiration rate of cotton fruit was so rapid that the net CO_2 assimilation rate of whole fruit (boll and bract) was negative even under the saturated light conditions (Fig. 3). This high respiration rate produced an enriched CO_2 microenvironment around cotton fruit (boll) and consequently increased the *C*i concentration (Fig. 4). Although the measurements were taken in ambient [CO_2] (400 μmol mol^{-1}), C_i of the boll (without bract) and the whole fruit (boll and bract) of cotton was 1279 and 500 μmol mol^{-1}, respectively, under saturated light conditions, which was significantly higher than that of leaves (187 μmol mol^{-1}).

Stomatal characteristics and water use efficiency

Total stomatal density (stomata per leaf area) in leaves was about twice that in bracts (Table 1), both values being greater than the stomatal density of bolls (47·12 ± 15·41 mm^{-2}). There was no consistent difference in the size of the stomatal aperture between leaves and bracts. Accordingly, the stomatal conductance of bracts was less than half of that of leaves

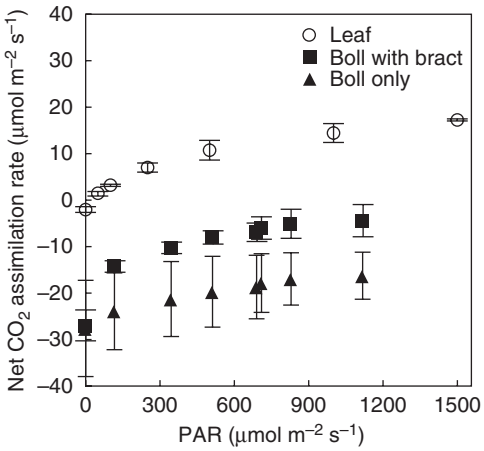

FIG. 3. Relationships between net CO_2 assimilation rate and photosynthetically active radiation (PAR) of leaf, boll with bract and 'only boll' of cotton, *Gossypium hirsutum*. These measurements were made in ambient CO_2 condition 20 d after anthesis in a glasshouse. Error bars represent the s.d. (the sample number was four for boll with bract and 'only boll', and two for leaf). Because the respiration rate of bolls was very rapid, the net CO_2 assimilation rate of whole fruit, and of bolls, was negative even in saturating light. Relationships between net CO_2 assimilation rate and PAR of leaf, boll with bract and 'only boll' measured in the field in China also showed the same trend (Supplementary Data Fig. S2).

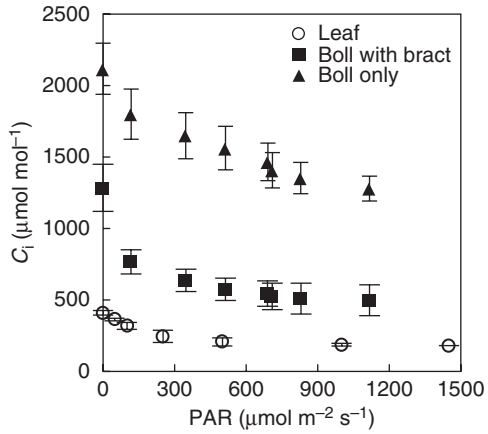

FIG. 4. Relationships between the intercellular CO_2 concentration (C_i) and photosynthetically active radiation (PAR) of leaf, boll with bract and 'only boll' of cotton, *Gossypium hirsutum*. These measurements were made in ambient CO_2 conditions at 20 d after anthesis. Error bars represent the s.d. (the sample number was four for boll with bract and 'only boll', and two for leaf). Relationships between C_i and PAR of leaf, boll with bract and 'only boll' measured in the field in China also showed the same trend (Supplementary Data Fig. S3).

(Fig. 5A). However, there was not a large difference between the C_i/C_a ratio of bracts and leaves, although it was significantly lower in bracts in the two highest C_a conditions (Fig. 5B). At a large [CO_2] in the chamber (>1200 μmol mol^{-1}), the iWUE in bracts was significantly higher than that in leaves (Fig. 5C).

Photosynthetic capacity and protein contents

Figure 6 shows an example of an $A-C_i$ curve in a leaf (open circles) and that of a bract (filled circles) 20 d after anthesis.

TABLE 1. *Stomatal density (number of stomata mm^{-2}) and stomatal aperture (μm) in leaf and bract of cotton,* Gossypium hirsutum, *grown in the field, 20 d after anthesis*

	Leaf	Bract
Stomatal density (mm^{-2})		
Adaxial side	176·4 ± 14·8	68·83 ± 6·41***
Abaxial side	224·1 ± 16·9	136·2 ± 20·9***
Total	400·5 ± 25·7	205·1 ± 21·3***
Stomatal aperture (μm)		
Breadth on adaxial side	4·00 ± 1·11	5·05 ± 0·49*
Length on adaxial side	18·86 ± 0·89	16·37 ± 1·44***
Breadth on abaxial side	4·09 ± 0·94	4·74 ± 1·94
Length on abaxial side	14·20 ± 1·19	12·57 ± 3·60

Data are presented as the mean ± s.d.
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, $n = 3$.

The photosynthesis rate was higher in the leaves than in the bracts. Accordingly leaves showed significantly higher values of V_{cmax}, J_{max}, Chl content, soluble protein content and all photosynthetic protein contents on an area basis (Table 2).

The ratio J_{max}/V_{cmax}, an indicator of protein allocation between RuBP regeneration and RuBP carboxylation processes (Hikosaka, 2005; Onoda *et al.*, 2005), was significantly higher in bracts than in leaves (Table 3), a difference which was maintained during fruit development (Fig. 7). This difference in J_{max}/V_{cmax} was accompanied by a difference in the C_i at which the photosynthetic rate was co-limited by RuBP carboxylation and RuBP regeneration. This co-limiting C_i (the crossing point between the solid line and the dashed line in Fig. 6) was 450 μmol mol^{-1} in leaves and 700 μmol mol^{-1} in bracts.

In accordance with the J_{max}/V_{cmax}, the ratio of a protein related to RuBP regeneration (Rieske FeS) and a protein related to RuBP carboxylation (Rubisco) was significantly higher in bracts than in leaves (Table 3). This higher J_{max}/V_{cmax} in bracts was mainly caused by a significant decrease in Rubisco content relative to the Chl content or soluble protein content, while the Rieske FeS protein contents showed no significant difference on the bases of Chl or soluble protein. The ratio of V_{cmax} to Rubisco content, or of J_{max} to Rieske FeS, also showed no significant difference in leaves and bracts. This indicated that there was no change in the protein activities in bracts compared with leaves, the effect coming from differences in amount of components.

DISCUSSION

High respiration rate of cotton fruit produces a high CO_2 microenvironment around the fruit

The cotton fruit (boll) showed a remarkably higher respiration rate per surface area than the leaf, attributable to the importance of respiration in developing seeds and fibres inside the boll (Fig. 2). Accordingly, the C_i of the fruit was >2100 μmol mol^{-1} in dark conditions and >1200 μmol mol^{-1} in photosynthetically saturated light conditions (Fig. 4). This high respiration rate produced an enriched CO_2 microenvironment around the boll, and the C_i of the whole fruit (with the bract) was >500 μmol mol^{-1} in saturating light conditions (it was even higher, 800 μmol mol^{-1}, in the Chinese field conditions; Supplementary Data Fig. S3).

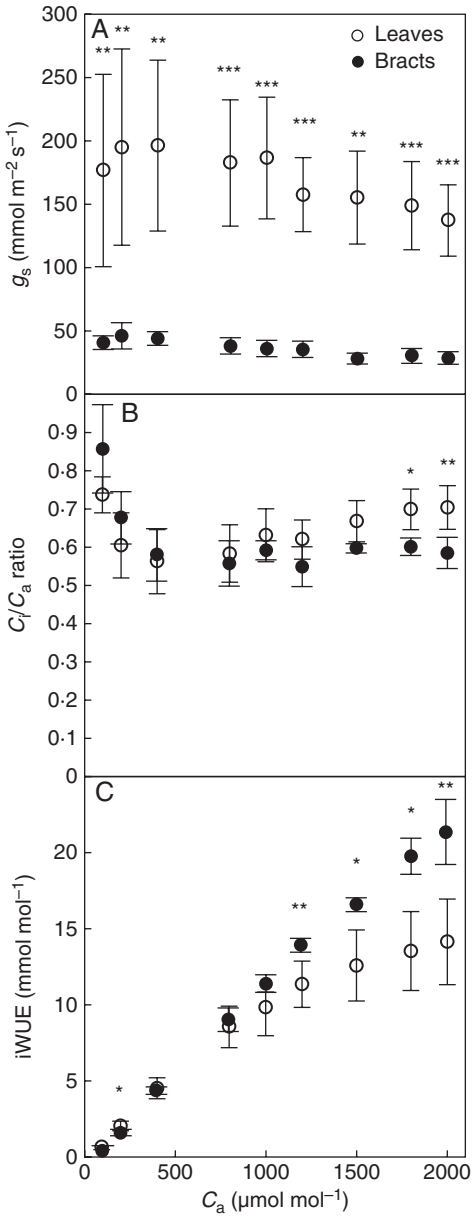

FIG. 5. (A) Stomatal conductance (g_s), (B) C_i/C_a ratio and (C) instantaneous water use efficiency (iWUE; photosynthetic rate divided by transpiration rate) at different C_a of leaves and bracts of cotton, *Gossypium hirsutum*, measured 20 d after anthesis. To see the CO_2 response, bracts were separated from bolls, but were still attached to the stem. Error bars represent the s.d. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$. CO_2 response curves measured in the field also showed the same trend (see Supplementary Data Fig. S4).

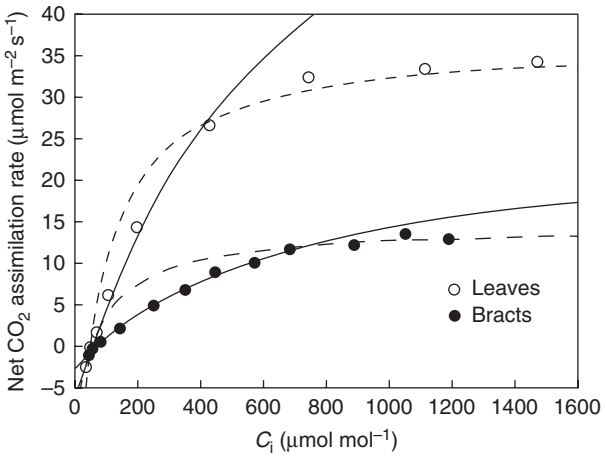

FIG. 6. Relationships between net CO_2 assimilation rate and intercellular CO_2 concentration (C_i) in leaves and bracts of cotton, *Gossypium hirsutum*., measured 20 d after anthesis. To see the CO_2 response, bracts were separated from bolls, but were still attached to the stem. The curves were fitted as described in the text: solid lines are when RuBP is sufficient, dashed lines are when RuBP is limiting.

TABLE 2. *Photosynthetic and physiological parameters of leaf and bract of cotton,* Gossypium hirsutum*, measured 20 d after anthesis*

Parameter	Leaf	Bract
V_{cmax} (μmol m^{-2} s^{-1})	99·03 ± 8·67	30·34 ± 10·91***
J_{max}(μmol m^{-2} s^{-1})	168·8 ± 13·5	59·5 ± 16·7***
Chl*a* + *b* content (μmol m^{-2})	409·5 ± 17·4	203·3 ± 46·4***
Soluble protein content (g m^{-2})	12·78 ± 1·19	4·47 ± 0·81***
Rubisco content (μmol m^{-2})	7·33 ± 1·30	2·14 ± 0·67***
Rieske FeS content (nmol m^{-2})	862·5 ± 144·9	339·8 ± 94·2***

Data are presented as the mean ± s.d.
$*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$, $n = 6$.

The total stomatal density of bracts was about half that of leaves (Table 1). Because the adaxial side of the bract faces the cotton fruit (boll), the adaxial stomata would allow CO_2 diffusion from the air, with concentrated CO_2 surrounding the tissue with the rapid respiration rate (boll), to the bract. On the other hand, the abaxial side of the bract faces the atmosphere; therefore, the low stomatal density would help to maintain a large $[CO_2]$ inside the bract while minimizing the rate of transpiration from the bract. In fact, the bract showed a significantly lower stomatal conductance (Fig. 5A), smaller C_i/C_a ratio at large C_a (Fig. 5B) and greater iWUE at large C_a (Fig. 6) than the leaf. A small stomatal conductance and large WUE are often observed in plants grown in elevated $[CO_2]$ (Eamus, 1991; Drake *et al.*, 1997; Royer, 2001). Plants growing at natural CO_2 springs also show low stomatal conductance (Tognetti *et al.*, 1996; Bettarini *et al.*, 1998; Paoletti *et al.*, 1998; Onoda *et al.*, 2007, 2009). At an Italian CO_2 spring, plants lost less water under elevated $[CO_2]$ and remained photosynthetically active in a dry season compared with control plants (Jones *et al.*, 1995; Hattenschwiler *et al.*, 1997). Therefore, the decrease in stomatal density and the accompanying increase in iWUE are considered to be a manifestation of adaptation of the bract to the elevated CO_2 produced by the rapid respiration rate of fruits, although the lower photosynthetic capacity might also be the cause of the lower stomatal density (Poole *et al.*, 1996; Drake *et al.*, 1997).

The balance between RuBP regeneration and RuBP carboxylation

The bract showed a significantly greater J_{max}/V_{cmax} and C_i at which photosynthesis was co-limited by RuBP carboxylation and RuBP regeneration than the leaf (Table 3, Fig. 7). These

TABLE 3. *Relative values of physiological parameters of leaf and bract of cotton,* Gossypium hirsutum*, measured 20 d after anthesis*

Organ	Leaf	Bract
J_{max}/V_{cmax}	1·71 ± 0·07	2·02 ± 0·25*
Rieske FeS/Rubisco (mmol mol^{-1})	118·6 ± 14·3	160·0 ± 16·5***
Rieske FeS/Chl (mmol mol^{-1}	2·11 ± 0·35	1·67 ± 0·46
Rieske FeS/soluble protein (nmol g^{-1})	67·66 ± 10·57	75·27 ± 14·09
J_{max}/Rieske FeS (mol s^{-1} mol^{-1})	195·7 ± 15·7	175·1 ± 49·2
Rubisco/Chl (mmol mol^{-1})	17·91 ± 3·19	10·54 ± 3·28**
Rubisco/soluble protein (μmol g^{-1})	0·57 ± 0·07	0·47 ± 0·08*
V_{cmax}/Rubisco (mol s^{-1} mol^{-1})	13·51 ± 1·18	14·15 ± 5·09

Data are presented as the mean ± s.d.
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, $n = 6$.

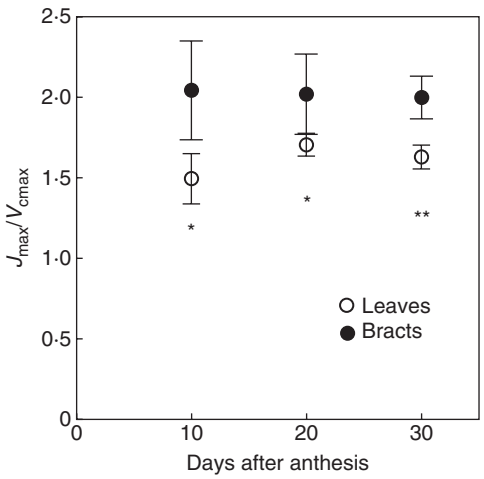

FIG. 7. J_{max} to V_{cmax} ratios in leaves and bracts of cotton, *Gossypium hirsutum*, grown in a glasshouse. They were measured at three different developmental stages. Error bars represent the s.d. The sample number was five for 20 d after anthesis, and from three to four for other days. J_{max} to V_{cmax} ratios measured in the field also showed the same trend (see Supplementary Data Fig. S5).

results indicate that chloroplasts in the bract were acclimated to the high CO_2 microenvironment produced by the rapid respiration rate of the boll (Fig. 4). As suggested in previous theoretical studies (Hogan *et al.*, 1991; Sage, 1994; Medlyn, 1996; Drake *et al.*, 1997; Hikosaka and Hirose, 1998), the RuBP carboxylation capacity of the leaf (solid line fitted to the filled circles) was redundant in elevated [CO_2] (>400 μmol mol^{-1} in Fig. 6). This means that the excess investment of nitrogen in RuBP carboxylation capacity leads to a decrease in nitrogen use efficiency in a high CO_2 environment. On the other hand, the RuBP carboxylation capacity of the bract (solid line fitted to the open circles) was redundant only above 800 μmol mol^{-1} CO_2. This increase in co-limiting C_i was adjusted by the balance between RuBP regeneration capacity and RuBP carboxylation capacity, namely the ratio J_{max}/V_{cmax} (Table 3).

The RuBP carboxylation capacity, V_{cmax}, is strongly correlated with the Rubisco content (Björkman, 1968, 1981; von Caemmerer and Farquhar, 1981). The RuBP regeneration capacity, J_{max}, is correlated with the content of Rieske FeS of the Cyt b_6f complex (Price *et al.*, 1998; Yamori *et al.*, 2011), since the content of the Cyt b_6f complex is considered to be a key rate-limiting step of electron transport and RuBP regeneration (Yamori *et al.*, 2011; Dwyer *et al.*, 2012). The ratio of Rieske FeS content to Rubisco content was significantly higher in the bract than in the leaf (Table 3), which indicates that the relative nitrogen investment in photosynthetic proteins in bract was related to adjustment of the RuBP regeneration/carboxylation balance.

At an organ level, the bract had smaller capacities for both RuBP regeneration and RuBP carboxylation (Table 2). This was probably caused by the thinner bracts (leaf, 374·9 ± 14·9 μm; bract, 178·5 ± 20·5 μm) with fewer chloroplasts per area than the leaf (Fig. 1C, D). This was accompanied by a lower content of Chl and soluble protein, although the Chl *a*/*b* ratio was almost the same (leaf, 3·60 ± 0·19; bract, 3·45 ± 0·35). On the other hand, chloroplasts of the bract had a similar RuBP regeneration capacity but a decreased RuBP carboxylation capacity compared with the leaf, which resulted in a greater RuBP regeneration/carboxylation capacity ratio. This was consistent with the result that only the Rubisco content relative to the Chl content or soluble protein content was significantly lower in the bract than in the leaf (Table 3).

Bracts as convenient material for studying long-term adaptation of photosynthesis to elevated [CO_2]

Bracts have been considered to provide protection for reproductive organs (Endress, 1987, 2011; von Balthazar and Endress, 1999). However, from the results of the present study, we suggest that the bract has evolved not only to protect the cotton fruit (boll) but also to supply carbohydrates, utilizing the CO_2 produced by the rapid respiration rate (Fig. 8). Photosynthesis of bracts contributes significantly to the yield of cotton (Constable and Rawson, 1980; Wullschleger and Oosterhuis 1990; Hu *et al.*, 2012). Therefore, covering the fruit with a bract and utilizing the released CO_2 should be a good strategy for plants which have large reproductive organs.

As bracts showed a smaller C_i/C_a ratio and greater iWUE than leaves at large C_a, we propose that the lower stomatal density of bracts is an adaptive mechanism. In the case of the adjustment of RuBP carboxylation capacity relative to RuBP regeneration capacity, we might need to consider the possibility of acclimation to high [CO_2]. According to the estimation of Wendel and Albert (1992) using sequence divergence, the first dichotomy inside the *Gossypium* genus is suggested to have occurred approx. 24–33 million years ago. They also estimated that the tetraploid *Gossypium*, namely *G. hirsutum*, originated from 1·1 to 1·9 million years ago (Wendel, 1989). Therefore, there should be ample time for stomatal density of bracts to have adapted to elevated [CO_2]. However, in the case of chloroplasts, adaptation may not be so simple, because chloroplasts (plastids) have their own genome which would be identical in all plant organs. If so, the chloroplast genome would have difficulty in adapting to the microenvironment existing only in one part of a plant. However, in many situations, the nucleus and nuclear-encoded proteins control chloroplast function (Woodson and Chory, 2008). For example, the nuclear genome also possesses genes for photosynthetic components, such as the Rieske FeS (apoprotein) gene (*petC*) and Rubisco small subunit gene (*rbcS*). In the synthesis of the Rubisco holoenzyme, both nuclear-encoded

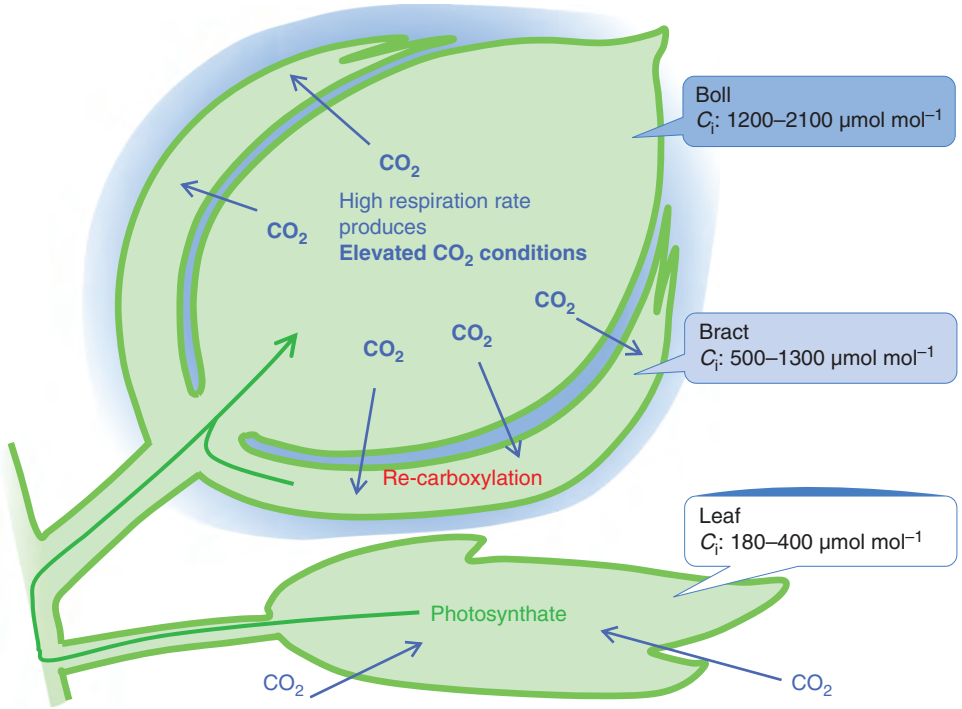

FIG. 8. Scheme of formation of elevated CO_2 conditions around a fruit of cotton, *Gossypium hirsutum* L. The cotton fruit (boll) has a rapid respiration rate (Fig. 2), attributable to its function in seed and fibre development: respiration utilizes photosynthate derived from leaves and bracts. Rapid respiration produces an elevated CO_2 microenvironment around the boll (Fig. 4). Because bracts usually cover almost all of a boll, here we assumed that the C_i measured for the boll with bracts is the same as the C_i of bracts. Compared with leaves, bracts had a lower stomatal density and higher J_{max}/V_{cmax} ratio (Table 3), which agree with the theoretical prediction of how photosynthetic mechanisms will adapt to elevated CO_2.

genes and chloroplast-encoded genes need to be expressed co-ordinately (Rodermel *et al.*, 1996; Suzuki and Makino, 2012). Accordingly, there might be a special signal which regulates plastids in bracts in a different way compared with leaves, and which may have helped to bring about adaptation of chloroplasts to the elevated CO_2 microenvironment. Thus the bract of the cotton boll may provide valuable material for studying the mechanisms by which chloroplasts and stomata adapt in the long term to elevated [CO_2].

Conclusions

The rapid respiration rate of whole cotton fruits produced a high [CO_2] surrounding cotton bolls and bracts. We show that bracts have several mechanisms to adapt to elevated [CO_2]. Compared with leaves, bracts had a lower stomatal density, resulting in lower stomatal conductance, a smaller C_i/C_a ratio at large C_a and greater iWUE at high C_a. The J_{max}/V_{cmax} ratio was greater in bracts than in leaves, thereby optimizing the balance between RuBP carboxylation and RuBP regeneration capacities in elevated [CO_2]. This is also supported by the protein content analysis. The greater Rieske FeS to Rubisco ratio in the bract indicated that more nitrogen was allocated to RuBP regeneration proteins than to RuBP carboxylation proteins, which is consistent with the adaptive response to elevated [CO_2] suggested by theoretical analyses. Because cotton bolls have evolved in this high intraplant CO_2 environment for between 1·1 and 1·9 million years (Wendel, 1989; Wendel and Albert, 1992), the bract may have adapted to the high CO_2 environment. Therefore, the cotton bract may be a convenient system for studying the mechanism of adaptation to elevated [CO_2].

SUPPLEMENTARY DATA

Supplementary data are available online at ww.aob.oxfordjournals.org and consist of the following. Figure S1: respiration rate of cotton whole fruits and leaves grown in the field in China, expressed on a unit surface area basis, during ontogeny. Figure S2: relationships between net CO_2 assimilation rate and PAR of leaf, boll with bract, and boll only of cotton in ambient CO_2 conditions 20 d after anthesis in the field. Figure S3: relationships between C_i and PAR of leaf, boll with bract, and boll only of cotton grown in the field in ambient CO_2 20 d after anthesis. Figure S4: stomatal conductance, C_i/C_a ratio and instantaneous iWUE at different air CO_2 concentrations of leaf and bract of cotton grown in the field at 20 d after anthesis. Figure S5: J_{max} to V_{cmax} ratios in leaf and bract of cotton grown in the field, at three different developmental stages.

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