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Elevated CO_2 Concentration and Leaf Growth: A Biophysical and Biochemical Analysis of Cell Expansion

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ABSTRACT. The impact of exposure to elevated CO_2 concentration on the processes of leaf cell production and leaf cell expansion was studied using primary leaves of Phaseolus vulgaris L.. Cell division and expansion were separated temporarily by exposing seedlings to dim red light (7 μmol $m^{-2}s^{-1}$) for 10 days (where leaf cell division was completed) followed by exposure to bright white light (300 μmol $m^{-2}s^{-1}$) for 14 days (when leaf growth was entirely dependent on cell expansion). When plants were exposed to elevated CO_2 during the phase of cell expansion, epidermal, palisade and spongy parenchyma cell sizes and rate of leaf growth were stimulated. Two pieces of evidence suggest that this occurred as a result of increased cell wall loosening and extensibility, (i) cell wall extensibility (WEx, measured as tensiometric extension using an Instron) was significantly increased, and (ii) xyloglucan endotransglycosylase (XET) enzyme activity was significantly increased. Assessments were made of the spatial patterns of WEx across the expanding leaf lamina and the data suggest that exposure to elevated CO_2 during the phase of leaf expansion may lead to enhanced extensibility particularly at basal leaf margins which may result in altered leaf shape. The data show that both cell production and expansion were stimulated by elevated CO_2 but that leaf growth was only enhanced in the cell expansion phase of leaf development. Increased leaf cell expansion is, therefore, an important mechanism for enhanced leaf growth in elevated CO_2, whilst the importance of increased leaf cell production in elevated CO_2 remains to be elucidated.

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INTRODUCTION

Many reports reveal that plant growth is stimulated by exposure to elevated CO_2 including studies on trees (Ceulemans and Mousseau, 1994) and a range of herbaceous species (Radoglou and Jarvis, 1992). However, our understanding of the underlying growth mechanisms and how they are altered when plants are exposed to elevated CO_2 remains poor. Leaf growth is an important component determining total plant productivity (Monteith, 1977) and is often enhanced following exposure to elevated CO_2 (Taylor *et al.*, 1994). As leaf growth involves both the production and expansion of leaf cells, it is possible that either or both processes could be influenced by additional carbon dioxide, although a number of recent reports have suggested that cell expansion may be of overriding importance.

Leaves of dicotyledons contain no spatially separated zones of meristematic and expansion activity, and cell division and expansion normally occur simultaneously during leaf expansion (Sunderland, 1960). This has made it difficult to discriminate, in these leaves, between the effects of environmental factors on cell division and cell expansion. Studies on the control of cell expansion are only conclusive if cell expansion can be separated from cell division. An exception to this was found in beans (*Phaseolus vulgaris* L.), where it has been possible to study the regulation of cell expansion in primary leaves by temporarily separating cell division from expansion (Van Volkenburgh and Cleland, 1979), achieved by growing seeds in dim red light for ten days, during which time cell division is completed.

Leaf cell expansion may be studied by analysing the biophysical components of growth in a model proposed by Lockhart (1965). The model shows that expansive growth is co-regulated by the rate at which cell walls loosen and extend and by the rate at which water and solutes are accumulated by the growing cell. However, considerable evidence suggests that biophysical properties of the cell wall may be of overriding importance in controlling leaf expansion (Taylor and Davies, 1985; Cosgrove, 1993; Taylor *et al.*, 1994). A putative wall loosening enzyme, xyloglucan endotransglycosylase (XET) (Smith and Fry, 1991; Fry *et al.*, 1992) has been shown recently to be correlated with increased growth in a number of plant cells and organs (Pritchard *et al.*, 1993; De Silva *et al.*, 1994), and may contribute to the cell wall loosening occurring in rapidly expanding leaves. However, there is little data on XET and leaf growth to substantiate this point. The objectives of this study were to assess the influence of elevated CO_2 on leaf growth and to identify the mechanism responsible for the altered rates of leaf growth.

MATERIALS AND METHODS

Plant material, growing conditions and exposure to CO_2 treatment

Plants of dwarf bean, *Phaseolus vulgaris* L. (cultivar: tender green) were surface sterilised with 1% NaOCl (Bleach solution) for 15 minutes, washed thoroughly, and then soaked in distilled water for 24 h to induce germination. Seeds were sown in plastic pots, 2 seeds per pot filled with vermiculite saturated with deionized water. Two Perspex chambers were used to expose plants to one of the two CO_2 concentrations, either ambient or elevated. The chambers which were covered with red filters to generate dim red light (7 μmol $m^{-2}s^{-1}$) were placed in an illuminated growth cabinet; temperature 25°C day and 22°C night with a 14 h photo period. Air was ducted into the chambers from outside of the building. One chamber was maintained at a CO_2 concentration of 330-350 μmol mol^{-1} whilst the second chamber received CO_2 at a target concentration of 620-640 μmol mol^{-1}. Pure CO_2 was added from a cylinder regulated by a gap flow meter. The air was rapidly mixed by a fan inside each chamber. The CO_2 concentration was continuously monitored with a bench-top IRGA, (ADC Ltd., Hoddesdon, UK) with a chart recorder attached. One hundred and twenty pots were randomly placed within the chambers and watered daily with full strength Hoagland's solution. After the seedlings were raised in dim red light for 10 days, cell division had ceased in the primary leaves (Van Volkenburgh and Cleland, 1979), and the leaves began to unfold. Rapid cell expansion was then stimulated by exposing the plants to relatively bright, white light (300 μmol $m^{-2}s^{-1}$) at 25°C day and 22°C night as described previously (Van Volkenburgh and Cleland, 1979). To record the individual effects of elevated CO_2 on cell division and cell expansion, on day 10, half of the plants from elevated CO_2 chamber were transferred to ambient CO_2 chamber and those from ambient chamber to elevated$_2$CO chamber, respectively. The remaining plants in ambient CO_2 chamber were used as control and those in elevated CO_2 chamber were used to determine the combined effect of elevated CO_2 on both cell division and cell expansion. The plants were maintained in bright white light for 14 days. During this time the primary leaves expanded and reached maturity.

Non-destructive measurement of leaf growth

Leaf area was measured using a Li Cor 3000 A (Li Cor Inc., Nebraska, U.S.A.) area meter throughout the experimental period at 2-4 d intervals. Leaf growth rates were calculated after completion of cell division (day 10) and cell expansion (day 20).

Leaf surface imprints

Epidermal impressions of the leaves were made according to Ferris and Taylor (1994a) with appropriate modifications. After 20 days of growth (mature leaf), a thin layer of nail varnish was applied to the abaxial and adaxial surfaces of the leaves, allowed to dry for 20-30 min and then gently peeled off, placed on a microscopic slide and covered with a cover slip. The number of stomata and number of other epidermal cells per half field was counted using a light microscope. From the data, stomatal density (SD) and stomatal index (SI) were calculated. Epidermal cell sizes were drawn using a camera lucida attached to a light microscope and mean cell areas measured using a calibrated Image Analyser (Delta -T Devices Ltd, Cambs., UK). Epidermal cell number was calculated using the average epidermal cell area and average leaf area of the particular day.

Leaf anatomy

Detail work to study the anatomy of the leaves was carried out on transverse sections of leaves from all four treatments. Sampling was done just before exposing to white light (day 10), during the period of rapid cell expansion (day 13) and maturing leaves (day 20). Leaf sections which were fixed in 2.5% glutaraldehyde and 1% osmium tetraoxide were subsequently dehydrated in an ethanol series and embedded in Spurr's resin (Ranasinghe, 1995). Using 4 μm sections, the area of palisade parenchyma and spongy parenchyma cells were measured from projected images, using a camera lucida and an image analyser.

Cell wall properties, plasticity and elasticity

Cell wall extensibility (WEx) was assessed as percent plasticity from a mechanical load extension analysis using a home-made Instron apparatus, similar in principle to the Instron technique used by Cleland (1967). Primary leaves of bean were sampled on seven occasions during the course of leaf development and stored in methanol for subsequent determination of cell wall extensibility. Leaf strips (4 x 2 mm) were cut parallel to the mid vein and rehydrated for 20 min in distilled water. Each strip was stretched twice allowing plastic and elastic components to be defined. The first stretch gave the combined values of percentage plasticity and percentage elasticity and the second stretch gave the percentage elasticity of the leaf cell wall. The percentage plasticity was calculated by the difference. Results were corrected

for difference in leaf thickness (Cleland, 1967; Cosgrove, 1993) and are expressed as percent extension per 10 g load. To study the effect of CO_2 on the spatial pattern of leaf growth, leaf strips were taken from 9 different areas of the each leaf (Figure 1) on days 6, 10, 12, 16, 20 and 24.

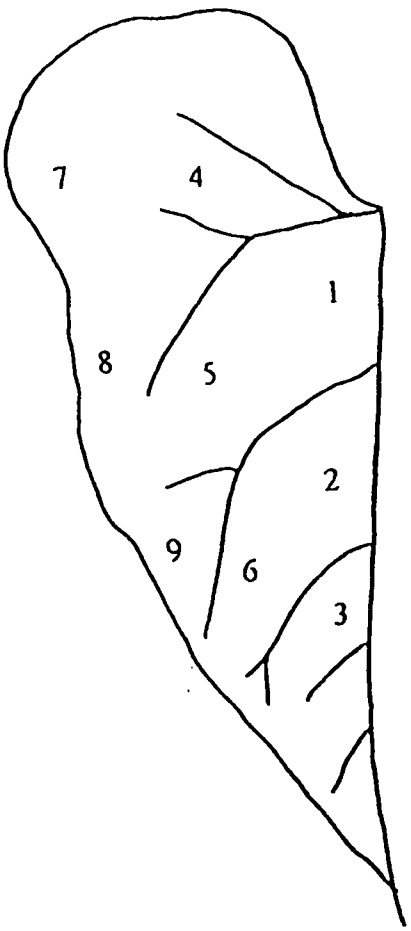

Figure 1. **Nine different positions of the leaf which were assayed for measurement of cell wall % plasticity and % elasticity across the leaf lamina. (In the respective groups, means with same letter are not significantly different by DNMRT at p= 0.05).**

Xyloglucan endotransglycosylase (XET) enzyme activity

The enzyme assay was carried out according to Fry *et al.* (1992). Quickly frozen leaf material was stored in -20°C and the buffer extract (ascorbate/$CaCl_2$/Mes (Na^+), pH 6.0) was used as the enzyme solution. Substrate solution which contains xyloglucan (XG7) and [^{3}H]XG7-ol in pH 5.5, 20 µl was mixed with 10 µl of enzyme extract and incubated at 25°C for 1h. After stopping the reaction using 100 µl of 15% formic acid, the whole 130 µl was dried on to a chromatography paper, the unreacted [^{3}H]XG7-ol was washed out and assayed for paper-bound [^{3}H]xyloglucan by scintillation counting. The XET activity was reported as Bq of [^{3}H]polymer formed per kBq of [^{3}H]oligosaccharide added, per h per mg fresh weight.

Statistical analysis of data

Using the SPSS statistical package, the results were analysed with one-way ANOVA. The difference between treatment means was compared using Duncan's multiple range test for each measurement day at $p = 0.05$ level.

RESULTS AND DISCUSSION

Figure 2 illustrates the leaf growth rates which were measured after completion of cell division (cell div) (day 10) and after completion of cell expansion (cell exp) (day 20). During the period of leaf expansion, growth rates of all the leaves were tremendously increased. There was an increase in growth rate for the 'cell exp' in leaves while there was a reduction in growth rates for the 'cell div' and 'cell exp + div' in leaves, respectively, compared to the control plants.

Neither the stomatal density, nor the stomatal index were affected by CO_2 concentration on either leaf surfaces (Table 1). In contrast, differences in abaxial epidermal cell sizes and numbers were observed for all four treatments On day 20, abaxial epidermal cell sizes were significantly larger for the leaves which were exposed to ambient CO_2 during the period of cell division and elevated CO_2 during cell expansion. This finding confirms a number of other reports where rapid leaf growth in elevated CO_2 was linked to the production of larger epidermal cells, including a study of native herbs (Ferris and Taylor. 1994b) and of hybrid poplar leaves (Gardner *et al.*, 1995). For the leaves exposed to elevated CO_2 during the phases of either cell division or cell

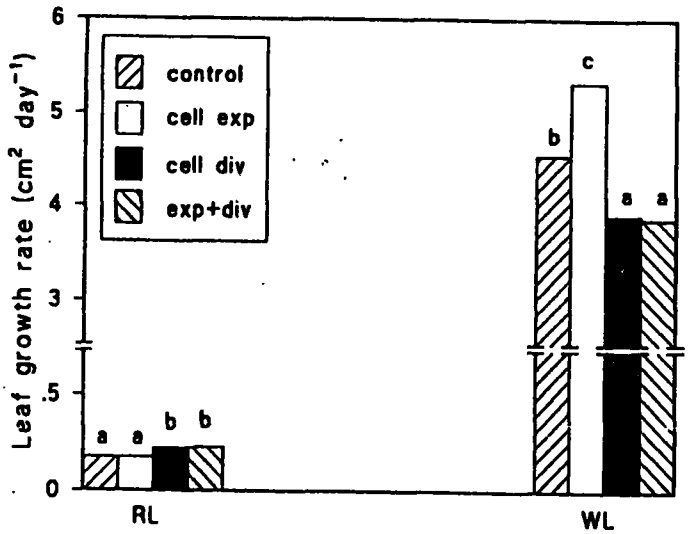

Figure 2. **Average rate of leaf expansion (cm^2 day^{-1}) of *P. vulgaris* measured after completion of cell division period (RL) and cell expansion period (WL). Plants were exposed to elevated CO_2 during cell expansion (cell exp), cell division (cell div) or expansion and division (exp+div), n = 12. (In the respective groups, means with same letter are not significantly different by DNMRT at p= 0.05).**

division and expansion the abaxial epidermal cell sizes were significantly reduced whilst the numbers of epidermal cells were increased.

Areas of the palişade parenchyma and spongy parenchyma cells are presented in Figure 3 a and b. When the leaves were exposed to bright white light to influence cell expansion (day 13), palisade cell areas (Figure 3a) of all four sets were increased and the values were atleast 2,3 times greater than those in dim red light. But for the 'cell div' in leaves, palisade cell areas were significantly reduced compared to the control plants whilst there was no effect on other two sets (cell exp and cell exp + div). On day 20, regardless of the CO_2 concentration during cell division, the leaves exposed to elevated CO_2 during cell expansion (cell exp and cell exp + div) showed significantly greater cell areas in comparison to the control plants. The highest value which was shown by 'cell exp' leaves was 70% higher than the control values and significantly different to the value of 'cell exp + div' treatment. During the

Table 1. **Leaf surface characteristics of *P. vulgaris* L. seedlings. Mean stomatal density, stomatal index on both the adaxial and abaxial leaf surfaces, abaxial epidermal cell size and abaxial epidermal cell number after exposed to ambient or elevated CO_2 for 20 days, n=50.**

	Control	Cell exp	Cell div	div + exp
Leaf Stomatal Density (No. mm^{-2})				
adaxial	58[a]	48[a]	58[a]	67[a]
abaxial	280[a]	348[a]	135[a]	194[a]
Leaf Stomatal Index (%)				
adaxial	10.79[a]	9.64[a]	9.35[a]	280.00[a]
abaxial	34.64[a]	43.21[a]	21.63[a]	27.80[a]
Leaf Epidermal Cell Size (μm^2) Abaxial	2456.55[b]	2969.01[a]	1741.06[c]	1813.73[c]
Leaf Epidermal Cell s $surface^{-1}$ ($X 10^5$) Abaxial	6.70[a]	7.25[a]	8.74[b]	8.53[b]

period of rapid cell expansion (day 13) areas of spongy parenchyma cells (Figure 3b) were significantly increased in 'cell exp' treatment while it was decreased in 'cell div' treatment. There was no significant effect on the 'cell exp + div' in leaves. On day 20 the cell areas of 'cell exp' treatment was almost doubled that of the control plants whilst the other two sets of leaves (cell div and cell exp + div) showed no difference to the control plants.

The influence of elevated CO_2 during the phases of cell division and/or cell expansion on the plastic properties of cell walls was assessed, on samples taken from 9 different positions across the expanding lamina as depicted in Figure 1. Corrected percentage plasticity values are presented in Figure 4. The most striking characteristic of this information is the effect of elevated CO_2 on cell wall percentage plasticity during cell expansion (Figure 4a). For the leaves exposed to elevated CO_2 during cell expansion, percentage plasticity values for all positions of the leaves were significantly increased ($p \leq 0.05$) when compared to the controls (Figure 4c). When exposed to elevated CO_2 during cell division, percentage plasticity was not affected by CO_2 enrichment (Figure 4b), except

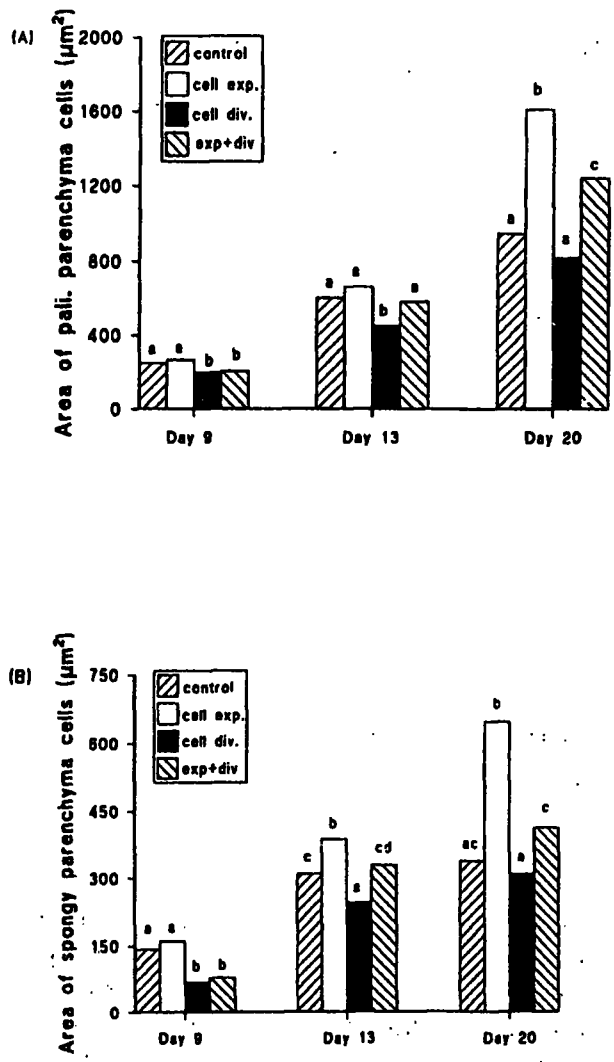

Figure 3. The influence of CO_2 concentration on the parenchyma cell expansion. Mean cell area (µm²) of (a) Palisade and (b) Spongy parenchyma for young (day 9), developing (day 13) and mature (day 20) leaves of *P. vulgaris* exposed to elevated CO_2 during cell expansion (cell exp), cell division (cell div) or expansion and division (exp + div), n = 30. (In the respective groups, means with same letter are not significantly different by DNMRT at p= 0.05).

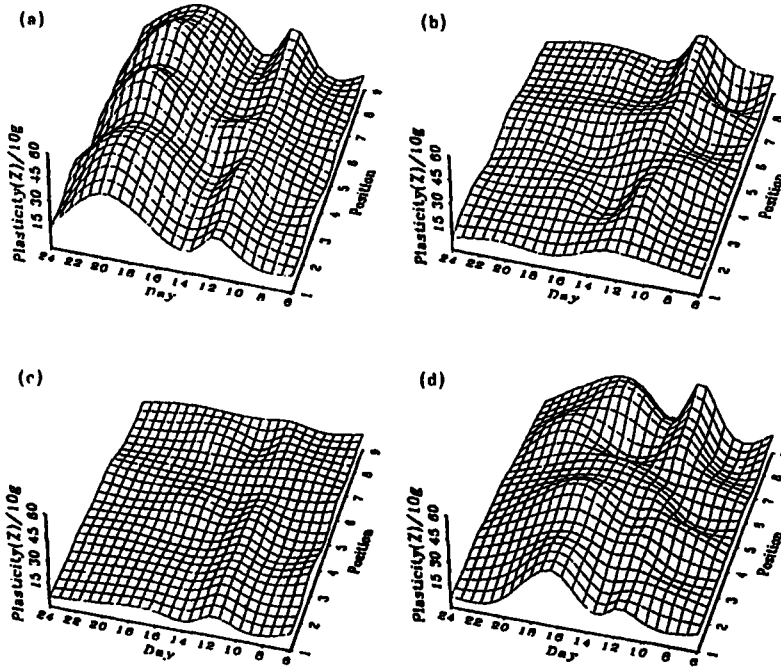

Figure 4. Elevated CO_2 and spatial patterns of cell wall extensibility (% plasticity). (a) leaves exposed to elevated CO_2 during cell expansion, (b) leaves exposed to elevated CO_2 during cell division, (c) control leaves exposed to ambient CO_2 during cell expansion and division, (d) leaves exposed to elevated CO_2 during cell expansion and division. Three axes of each graph indicate 9 different positions of the leaf, different harvesting days and the relevant % plasticity values, n = 12. (In the respective groups, means with same letter are not significantly different by DNMRT at p= 0.05).

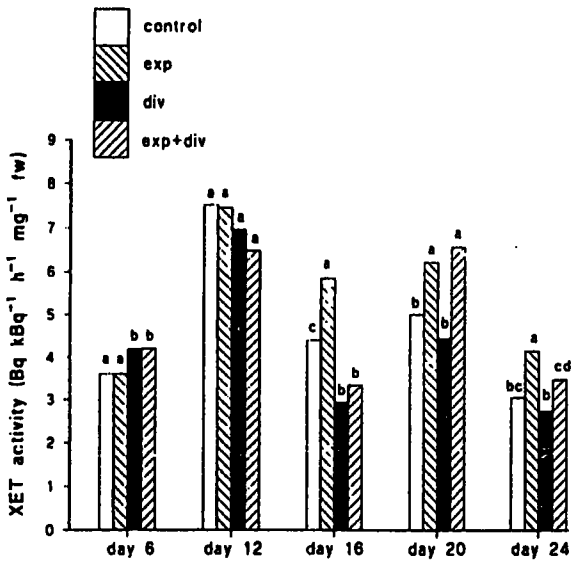

Figure 5. **Elevated CO_2 and XET activity. Activity of the enzyme xyloglucan endotransglycosylase of *P. vulgaris* leaves harvested on days 6, 12, 16, 20, and 24, following exposure to elevated CO_2 during the phase of either cell expansion (exp), cell division (div) or expansion and division (cell exp + div), n = 8. (In the respective groups, means with same letter are not significantly different by DNMRT at p=.05).**

on days 10 and 12, which showed a significant increase ($p \leq 0.05$) when compared to control plants. For leaves exposed to elevated CO_2 during the periods of both cell division and expansion, the effect was not consistent (Figure 4d). The effect on cell expansion is not confined to leaves since it has also been shown that root cell expansion is promoted following exposure of shoots to elevated CO_2 (Taylor *et al.*, 1995) and that this also occurs as a result of enhanced cell wall loosening and extensibility.

Figure 5 illustrates the influence of elevated CO_2 on the activity of the cell wall loosening enzyme xyloglucan endotransglycosylase (XET). Exposure to elevated CO_2 during the phase of cell expansion (Figure 5), resulted higher XET activity for expanding and maturing leaves when compared to the control plants. In contrast XET activity was lower for leaves exposed to elevated CO_2 during cell division (Figure 5) on days 16, 20 and 24. For the leaves exposed

to elevated CO_2 during both cell division and expansion (Figure 5), the CO_2 treatment influenced the activity of XET on days 16 and 20 although the magnitude and the direction of the effect appeared to vary. In the plant cell wall, this enzyme is thought to cut and rejoin xyloglucan molecules spanning adjacent microfibrils, some of which are load-bearing and form 'tethers' (Smith and Fry, 1991). An increase in the activity of this enzyme would loosen the wall allowing faster extension growth for a given turgor pressure. The highest XET activities were shown by the leaves which recorded the highest growth rates (cell exp). At present it is unclear whether XET is the sole wall loosening enzyme, but it has been found in several tissues assayed and is correlated closely with growth rates for roots (Pritchard *et al.*, 1993) and stems (Potter and Fry, 1993).

CONCLUSION

The present experiment has illustrated an important mechanism for increased plant growth following exposure to elevated CO_2. Rapid leaf growth in elevated CO_2 occurred as a result of increased leaf cell expansion and this was independent of leaf cell production. We observed that when cell division was completed in ambient CO_2 and expansion in elevated CO_2, leaf growth rate was greatly enhanced; the sizes of epidermal, palisade and spongy cells were bigger whilst epidermal cell numbers remained similar to controls. According to the results it seems likely that increased leaf growth was the result of enhanced cell wall loosening and extensibility. The data also suggest that this was linked to an increase in the activity of the putative wall loosening enzyme, XET. Rapid leaf growth in elevated CO_2 was associated exclusively with cell expansion, suggesting that this process may be more important in determining plant growth responses to elevated CO_2.

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