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Elevated carbon dioxide affects the patterning of subsidiary cells in *Tradescantia* stomatal complexes

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Abstract

The influence of elevated CO_2 concentration (670 ppm) on the structure, distribution, and patterning of stomata in *Tradescantia* leaves was studied by making comparisons with plants grown at ambient CO_2. Extra subsidiary cells, beyond the normal complement of four per stoma, were associated with nearly half the stomatal complexes on leaves grown in elevated CO_2. The extra cells shared characteristics, such as pigmentation and expansion, with the typical subsidiary cells. The position and shape of the extra subsidiary cells in face view differed in the green and purple varieties of *Tradescantia*. Substomatal cavities of complexes with extra subsidiary cells appeared larger than those found in control leaves. Stomatal frequency expressed on the basis of leaf area did not differ from the control. Stomatal frequency based on cell counts (stomatal index) was greater in leaves grown in CO_2-enriched air when all subsidiary cells were counted as part of the stomatal complex. This difference was eliminated when subsidiary cells were included in the count of epidermal cells, thereby evaluating the frequency of guard cell pairs. The extra subsidiary cells were, therefore, recruited from the epidermal cell population during development. Stomatal frequency in plants grown at elevated temperature (29 °C) was not significantly different from that of the control (24 °C). The linear aggregations of stomata were similar in plants grown in ambient and elevated CO_2. Since enriched CO_2 had no effect on the structure or patterning of guard cells, but resulted in the formation of additional subsidiary cells, it is likely that separate and independent events pattern the two cell types. Plants grown at enriched CO_2 levels had significantly smaller internode lengths, but leaf area and the time interval between the appearance of successive leaves were similar to that of control plants. Porometric measurements revealed that stomatal conductance of plants grown under elevated CO_2 was lower than that of control leaves and those grown at elevated temperature. *Tradescantia* was capable of regulating stomatal conductance in response to elevated CO_2 without changing the relative number of stomata present on the leaf.

Key words: Elevated CO_2, stomata, subsidiary cells, patterning.

Introduction

The distribution of stomata on leaves is not random, but ordered. Although the mechanism of their patterning is not known, environmental conditions reportedly influence their frequency (Bristow and Looi, 1968; Woodward, 1987; El-Hashani and Pearson, 1995). Since stomatal complexes regulate gas exchange between the plant and the atmosphere, changes in stomatal number that correlate with variable CO_2 conditions may be an essential, adaptive response (Woodward and Bazzaz, 1988; Beerling and Woodward, 1995). However, since each stomatal complex can adjust the size of its pore opening, the number of stomata might not need to change in response to increasing or decreasing levels of CO_2. The available range in pore size may be great enough for the leaf adequately to regulate gas exchange over a wide range of CO_2 concentrations and may account for the absence of changes in stomatal frequency in certain species (Knapp *et al.*, 1994; Radoglou and Jarvis, 1990, 1992).

The growth and developmental responses of plants to elevated CO_2 are known not to be uniform, but species specific (Taylor *et al.*, 1994). Some species respond with stimulated leaf initiation and expansion, others with increased leaf cell expansion without a change in cell

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number, and still others with greater cell numbers accompanied by decreased cell size (Taylor *et al.*, 1994). Given this variation, the method of evaluating stomatal frequency must be selected with the goal of the research in mind. Since variable CO_2 levels may induce changes in cell number or size, either of which could markedly affect stomatal measurements, the investigator must be cognizant of these possibilities when designing experiments and interpreting data. There is no single measure of stomatal frequency that would be appropriate in all circumstances.

The objective was to study the effects of elevated CO_2 on stomatal patterning in hypostomatous *Tradescantia* leaves by (1) determination of stomatal frequency on two bases (leaf area and cell count), (2) measurement of the linear distribution of stomata, (3) microscopic examination of stomata, and (4) porometric measurements.

Materials and methods

Tradescantia (species unknown but likely to be *T. fluminensis* Vell.) was grown in a matrix consisting of vermiculite, peat moss, bark ash, and washed sand (Metro-mix 360, Grace Sierra, Milpitas CA) and watered with half-strength Hoagland's solution (Hoagland and Arnon, 1938) as needed, approximately 3 times each week. Green and purple varieties of *Tradescantia* were propagated vegetatively for use in this study. Propagules of uniform size and number of expanded leaves were placed in pots and grown under the experimental conditions for a minimum of 3 weeks before sampling. This allowed the initiation and development of leaves to take place entirely under the new environmental conditions. Once the propagules were established, the older leaves that were present on the propagule at the time of planting were removed. The procumbent shoots of *Tradescantia* have never flowered in seven years of propagation nor shown changes in development; quantitative measures of their stomatal patterning have remained uniform during this period. Observations were made on both varieties, but measurements were taken only from the green variety of *Tradescantia*.

Propagules were maintained in growth chambers capable of providing an environment of regulated atmospheric gasses, temperature, humidity, and light levels (Conviron, Model E15 from Controlled Environments, Inc., Pembina, North Dakota, USA). The photoperiod (16 h), photon flux density (ramped from 50 μmol at the beginning of the light period to a maximum of 250 μmol m^{-2} s^{-1} during the middle 12 h followed by a decline in the last 2 h), and relative humidity (84%) were the same in all chambers. Growth chambers provided ambient (360 ppm) or elevated (670 ppm) CO_2 levels at 24 °C and a third chamber provided ambient CO_2 at 29 °C. Average internode length ($n = 20$) and leaf area ($n = 10$) were determined from eight shoots of each treatment.

Stomatal frequency was determined from cyanoacrylate glue replicas of the abaxial leaf surface, the only surface on which stomata occur (Croxdale *et al.*, 1992). Data were collected from three leaves of each treatment by means of an image analysis system. Measurements were taken from five fields of each leaf with each field containing ten or more stomatal complexes; the number of epidermal cells, and in some cases the number of subsidiary cells, was counted also. The number of stomata in each field of known area (1967 μm^2) was counted to determine stomatal frequency on an area basis. Stomatal frequency based on cell counts was evaluated in two ways: (1) each stoma with all its associated subsidiary cells was considered a discrete unit (a stomatal complex), and (2) stomata were redefined as guard cell pairs excluding all subsidiary cells. For the first calculation, the number of stomatal complexes was divided by the number of complexes plus the number of epidermal cells, and then multiplied by 100. For the second calculation, the number of guard cell pairs was divided by the number of pairs plus the number of subsidiary and epidermal cells, and then multiplied by 100. Each of these calculations is derived from the equation for stomatal index, defined by Salisbury (1927).

The percentage of stomatal complexes with extra subsidiary cells was calculated from replicas of three leaves for each treatment. In fields containing at least ten complexes, the number of subsidiary cells around each pair of guard cells was tallied. Fifteen fields, five each from the base, middle, and tip of the leaf, were counted on each of the three leaves.

The linear distribution of stomatal complexes was determined by counting the number of stomata occurring singly and in series. Stomata in series have only one epidermal cell between complexes while single stomata are separated by two or more epidermal cells (Chin *et al.*, 1995). Representative samples (greater than 100 series of stomata per leaf) on three leaves of each treatment was used to evaluate distribution.

Whole mounts and 50 μm transections of leaves were viewed by brightfield and fluorescence microscopy with a Zeiss Axiophot using filter pack 395–440/FT 460/LP 470.

Stomatal conductance was measured under the experimental conditions at which the plants were grown using a LI-1600 Steady State Porometer (Li-Cor, Lincoln, NE), with a 0.5 [illegible] cuvette. Measurements were taken from nine leaves for each plant at equivalent times during the light period, and the results from two collection periods were combined for analysis.

Results

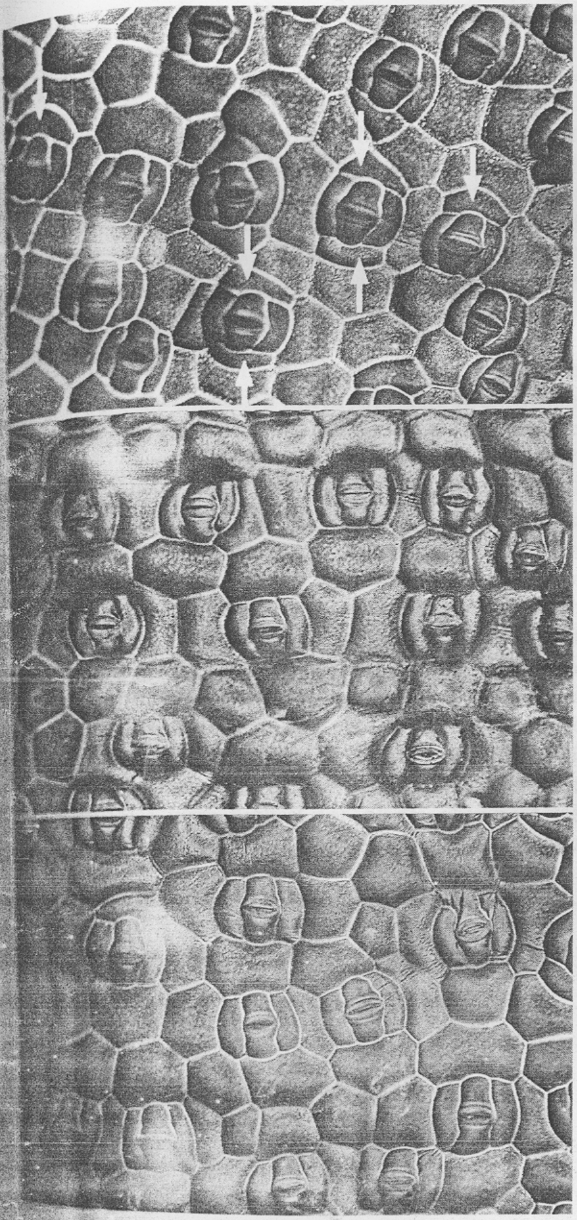
Forty-four per cent of stomatal complexes in leaves of the green variety of *Tradescantia* grown at elevated CO_2 had more than four subsidiary cells (Table 1; Fig. [illegible]). This percentage was significantly greater than the percentage in ambient CO_2 (99% confidence level, $P \leq 0.001$). Eleven per cent of the stomatal complexes in leaves grown at increased CO_2 had six or more subsidiary cells, which also was significantly different from the control at [illegible]

Table 1. *The occurrence of subsidiary cells associated with stomatal complexes on* Tradescantia *leaves grown under [illegible] conditions*

Treatment	Percentage of stomatal complexes with variable number of subsidiary cells [illegible]		
	4 Subsidiary cells	5 or 6 Subsidiary cells	6 Subsidiary cells
Elevated CO_2	56	44 (22)[a]	11 [illegible]
Elevated temperature	71	29 (21)	[illegible]
Ambient CO_2 (control)	73	27 (17)	[illegible]

[a] Values for elevated CO_2 were significantly different at the [illegible] confidence level from those for ambient CO_2 ($P = 0.001$). Values for elevated temperature were not significantly different from the control; $n = 45$ for each treatment.

[b] Values for elevated CO_2 were significantly different at the [illegible] confidence level from those for ambient CO_2 ($P = 0.004$); $n =$ [illegible]



Leaf replicas from plants grown in elevated CO_2 (top panel), CO_2 (middle panel), and elevated temperature (bottom panel). subsidiary cells (arrows in top panel) are associated with that form under enriched CO_2 while complexes in ambient elevated temperature typically have four subsidiary cells.

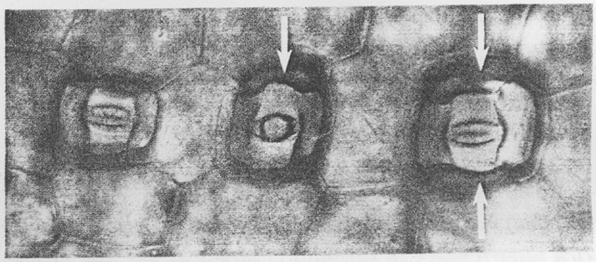


Fig. 2. Abaxial surface of a *Tradescantia* leaf (green variety) grown in elevated CO_2. The complexes with extra subsidiary cells (arrows), are curvilinear in shape and positioned at right angles to the second pair of subsidiary cells.

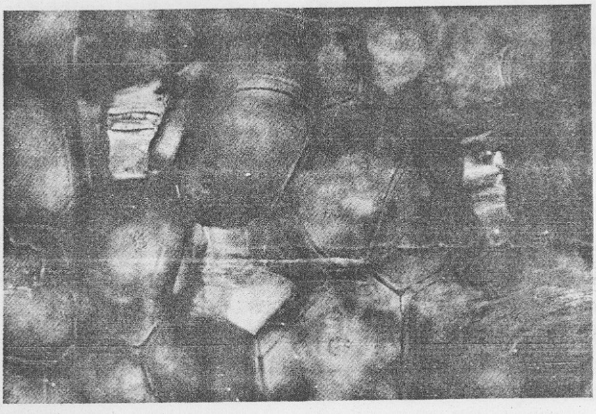


Fig. 3. Abaxial surface of a *Tradescantia* leaf (purple variety) grown in elevated CO_2. The position and shape of the extra subsidiary cells in the middle stomatal complex differ from those on the sides and those in the green variety of *Tradescantia* (Fig. 2). The extra subsidiary cells in the purple variety are angular and found in the same position as a typical subsidiary cell or attached to the short wall of one of the second pair of subsidiary cells. The size of the substomatal cavity in the middle complex appears larger than those of complexes with the typical number of subsidiary cells located on each side.

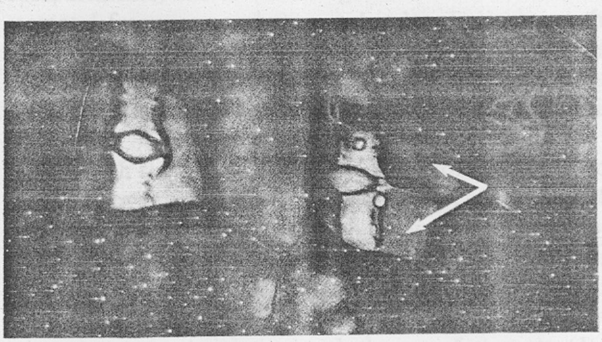


Fig. 4. Abaxial surface of an anthocyanin-producing leaf grown at elevated CO_2 showing a stoma with the typical complement of subsidiary cells (left) and one with five subsidiary cells (right). In the complex on the right two subsidiary cells (arrows) are found in the space normally occupied by one cell. Although the subsidiary cells appear to contain anthocyanin, changing the plane of focus during examination reveals that the pigment is contained in neighbouring epidermal cells bulging into the substomatal cavity (see Fig. 5).

fidence level (Table 1, $P \leq 0.004$). The percentage plexes with extra subsidiary cells was similar in grown under elevated temperature and control (Table 1; Fig. 1).

position and shape (in face view) of the extra cells associated with stomata differed in the purple varieties of *Tradescantia* (Figs 2, 3, 4). variety the extra cells were located immediately outside the typical group of four subsidiary cells, with one or more cells found at right angles to the outermost pair of subsidiary cells (Fig. 2). The shape of the extra cells in the green variety was curved in face view (Fig. 2). In the purple variety, and rarely in the green variety, two cells were found in the space normally occupied by one of the typical subsidiary cells (Fig. 4). The shape of these extra cells was angular in face view (Figs 3, 4).

When grown in ambient CO_2 conditions, epidermal cells of the purple variety accumulate anthocyanin whereas guard cells and subsidiary cells do not (Boetsch *et al.*, 1995). In elevated CO_2 conditions, epidermal cells bulged into the substomatal cavity, giving the appearance that the extra subsidiary cells contained anthocyanin when seen in face view (Fig. 4). However, changing the plane of focus during examination revealed that the pigment was actually contained in the nearby epidermal cells (Fig. 5) and not in the subsidiary cells.

In ambient CO_2, the substomatal cavity is created passively when the neighbouring epidermal cells expand radially and cells of the stomatal complex do not (Fig. 6; Boetsch *et al.*, 1995). In plants grown at elevated CO_2 the size of the substomatal chamber appeared to reflect the number of subsidiary cells in the complex. Complexes with more than the typical number of subsidiary cells seemed to have larger cavities (Figs 3, 4).

When stomatal complexes were defined as units that included all subsidiary cells, stomatal frequency based on cell counts was significantly greater for leaves grown at elevated CO_2 than for those grown in ambient CO_2 (Table 2). When frequency was re-evaluated by tallying subsidiary cells with epidermal cells, thereby considering the frequency of guard cell pairs alone, there was no significant difference between the elevated CO_2 and control treatments (Table 2). Stomatal frequency in plants grown at elevated temperature was not significantly different from the control (Table 2). On an area basis

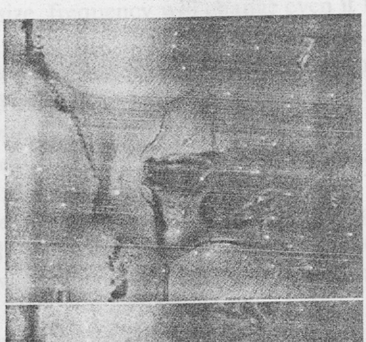


Fig. 5. Abaxial leaf surface focused at the level of the stomatal complex (upper panel) and on the epidermal cell wall (lower panel) beneath it. The anthocyanin that appears to be in subsidiary cells on the right-hand side of the complex (upper panel) actually is contained in adjacent epidermal cells (lower panel) that bulge into the substomatal cavity. All subsidiary cells are intact, but the epidermal cells on the left side of the stomatal complex are at the cut edge of the preparation and no longer contain anthocyanin.

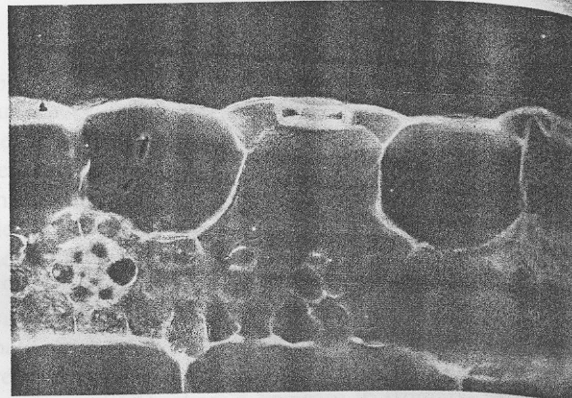


Fig. 6. Autofluorescence of a mature stomatal complex in a leaf transection (abaxial surface at top) from a plant grown at ambient CO_2. The cell walls fluoresce blue and the chlorophyll in chloroplasts of guard cells and mesophyll cells fluoresces red. The complex is bounded on the left and right by large epidermal cells that abut with the mesophyll layer. The expansion of the guard and subsidiary cells toward the mesophyll is less than 20% that of the neighbouring epidermal cells. The differential expansion between these cells of the epidermal layer passively creates the substomatal cavity, which lies beneath the interior walls of the complex's cells.

Table 2. *Stomatal frequency for leaves of the green variety of* Tradescantia, *grown under varied conditions*

Treatment	Stomatal frequency		
	Cell count (±SD)[a]	Cell count (±SD)[b]	Area (±SD)[c]
Elevated CO_2	27.2 (3.05)	23.1 (2.24)	10.3 ([illegible])
Elevated temperature	22.3 (3.01)	20.9 (3.12)	11.1 ([illegible])
Ambient CO_2	23.9 (2.42)	22.7 (2.32)	11.1 ([illegible])

[a] Each stomatal complex regardless of its number of subsidiary cells was counted as one unit. Frequency based on cell count was calculated using the equation for stomatal index (Salisbury, 1927): Stomatal index = no. of stomatal complexes/no. of stomatal complexes + no. of epidermal cells × 100. Stomatal frequency in elevated CO_2 was significantly different at the 99% confidence level from the control (p = 0.001); $n = 15$. Frequency for elevated temperature was not statistically different from the control.

[b] Stomatal frequency was re-evaluated by dividing the number of guard cell pairs by the sum of epidermal cells, subsidiary cells, and guard cell pairs, and then multiplying by 100. There was no significant difference between the treatments and the control when frequency was calculated in this manner.

[c] Area used in frequency determinations was 1967 μm^2. Stomatal frequency by area was similar in all treatments.

stomatal frequency in the green variety of *Tradescantia* was similar in all treatments (Table 2).

One measure of stomatal distribution is the arrangement of stomata in linear units (strings), where each stoma is separated from the next by a single epidermal cell (Chin *et al.*, 1995). These linear groups of stomata may contain from one to eight or more stomata in series. The incidence of increasing string lengths follows a characteristic curve that does not change during leaf development (Chin *et al.*, 1995). The incidence curves were similar for plants grown at elevated and ambient CO_2 levels (Fig. 7).

When the green variety of *Tradescantia* was grown at elevated CO_2, the expanded internodes were twice as long as those of plants grown in ambient CO_2 (57 ± 9.9 mm versus 33 ± 6.6 mm, significantly different at the 99.9% level of confidence, $P = 0.001$). There was no appreciable difference in internode length between plants grown at ambient CO_2 and elevated temperature. Leaf area and the time interval between the appearance of successive leaves varied little among treatments and no significant differences were found. For example, leaf area was 16 ± 1.6 cm^2 for plants grown at ambient CO_2 and 175 ± 1.8 cm^2 for those grown in enriched CO_2.

Porometric measurements of stomatal conductance, taken under the experimental conditions at which the plants were grown, differed for each treatment. Conductance values were highest for plants grown at elevated temperature (0.398 ± 0.135 mol m^{-2} s^{-1}), and lowest for plants grown at elevated CO_2 (0.269 ± 0.038 mol m^{-2} s^{-1}). Each of these values was significantly different from the control (0.313 ± 0.044 mol m^{-2} s^{-1}) at the 95% confidence level.

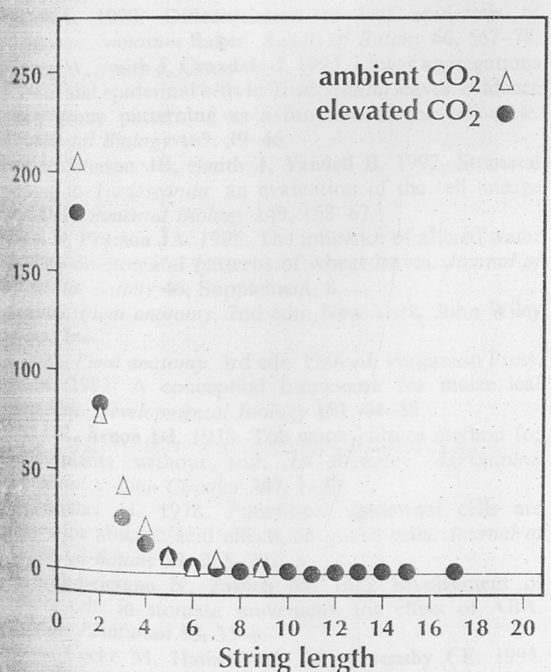


Fig. 7. Stomatal string incidence in order of increasing string length on plants grown in ambient (△) and elevated (●) CO_2.

Discussion

One or more extra subsidiary cells were associated with nearly half the stomatal complexes in *Tradescantia* leaves grown in elevated CO_2. In the green variety of *Tradescantia* the extra subsidiary cells were bean-shaped in face view and positioned at right angles to the second pair of subsidiary cells. In the purple (anthocyanin-producing) variety, the members of the second pair of subsidiary cells divided again to form two cells in the space usually occupied by one subsidiary cell. These cells were angular in face view. The extra subsidiary cells had expansion characteristics that parallel those of typical subsidiary cells. Epidermal cells expand radially about five times more than cells of the stomatal complex and are the only cells in the epidermis to accumulate anthocyanin (Boetsch *et al.*, 1995). Anthocyanin was not found in the additional subsidiary cells formed in elevated CO_2.

An apparent increase in the volume of substomatal cavities was observed when extra subsidiary cells are present and although measuring the cavities was considered, their irregularity and concealed nature make accurate determinations difficult. An increase in cavity volume might be a functional modification associated with gas exchange in elevated CO_2. There are indications that structural changes alter internal gas relations in leaves. The size of mesophyll compartments affects the homogeneity of photosynthesis, the distribution of open stomata, and the localization of starch (Terashima *et al.*, 1988). When the effect of elevated CO_2 was studied in variegated homobaric (large compartments) and heterobaric (small compartments) leaves (Beerling and Woodward, 1995), stomatal frequency and stomatal index decline over the green regions and only modest increases occur over white or light red areas of homobaric leaves. Collectively, these results indicate that various structural adjustments in leaves may occur in response to elevated CO_2.

Although the precise function of subsidiary cells is not known, they are integral for stomatal movement. Possible roles include serving as a location for starch or carbon storage, as repositories for inorganic ions, and as a means of structural support during stomatal function. It is known that subsidiary and epidermal cells must be alive to ensure normal opening and closing of stomata (Itai and Meidner, 1978; Itai *et al.*, 1982; Weyers and Meidner, 1990). It seems likely that the role of the extra subsidiary cells in *Tradescantia* is similar to that of the typical subsidiary cells.

The growing body of literature on plants exposed to elevated CO_2 indicates that stomatal frequency is adjusted in some (Bristow and Looi, 1968; Woodward, 1987, 1995; Woodward and Bazzaz, 1988), but not other species (Taylor *et al.*, 1994; Knapp *et al.*, 1994; Radoglou and Jarvis, 1990, 1992; Radoglou *et al.*, 1992). Even within an ecological community, species respond differently to increases in this atmospheric gas (Knapp *et al.*, 1994).

In the green variety of *Tradescantia*, elevated CO_2 had no significant effect on the number of stomata patterned per leaf, as evidenced by area-based stomatal frequency and the frequency of guard cells by cell count. However, when subsidiary cells were included with guard cell pairs in determining frequency by cell count, frequency was higher in plants grown in elevated CO_2. The extra subsidiary cells associated with stomata in elevated CO_2 account for this difference in frequency. Extra cells in the green variety, recruited from the epidermal cell population, reduce the denominator in the equation for stomatal index. This, in turn, artificially boosts the stomatal frequency when all subsidiary cell are counted as part of the stomatal complex.

Since variable CO_2 concentrations may affect the size or number of leaf cells (Taylor *et al.*, 1994), stomatal frequency measurements must be made on a basis that allows accurate comparison to plants grown in ambient CO_2. Commonly, stomatal frequency is based on the number of stomata in a given area of epidermis. The difficulty is that if an experimental treatment alters epidermal cell size, frequency will change even if the relative number of stomata remains the same. Conversely, stomatal frequency based on cell counts has the potential for this kind of misinterpretation as well. As encountered in *Tradescantia*, any treatment that affects the number of subsidiary cells at the expense of the epidermal cell population must be evaluated with caution. The results illustrate the importance of specifying whether subsidiary cells are included in the stomatal count or in the epidermal cell count when using the equation for stomatal index (Salisbury, 1927).

The porometric measurements of stomatal conductance demonstrate that conductance varied under the experimental conditions within a range commonly reported at ambient CO_2 (Mansfield *et al.*, 1990). Since the relative number of stomata did not change in *Tradescantia*, the lower conductance of leaves growing in enriched CO_2 indicates that stomata respond by not opening as widely as those in control conditions. On the other hand, the increased conductance at elevated temperature indicates stomata respond by opening wider than those in the control plants. No differences were observed in guard cells or the size of the stomatal pore to account for the changes in conductance. Therefore, in *Tradescantia* the available range in pore size is great enough for the leaf adequately to regulate gas exchange when growing in CO_2 concentrations ranging from 360 ppm (ambient) to 670 ppm.

Stomatal patterning involves the mechanism responsible for initiating the development of guard cells and subsidiary cells, and for regulating the proximity of neighbouring stomatal complexes. Patterning is also responsible for the ratio of guard cells to subsidiary cells in a complex. Given that subsidiary cells are sometimes derived from the guard mother cell and other times from adjacent epidermal cells (Esau, 1965; Payne, 1978; Fahn, 1982), and that the number of cells within a complex can vary, patterning changes can easily be overlooked unless patterning is the focus of the study. Although not directly reported by North *et al.* (1995), *Opuntia* cladodes exposed to elevated CO_2 show a decrease in stomatal frequency based on cell counts, that results from an increase in the number of epidermal cells. In this instance, the ratio of stomatal complexes to epidermal cells has been modified, indicating a change in patterning. Patterning events are under genetic control in *Arabidopsis* (Yang and Sack, 1993) and probably in other species as well.

Since extra subsidiary cells were recruited from the epidermal cell population in *Tradescantia*, while no significant changes occurred in the structure or frequency of guard cell pairs, separate and independent events likely determine these two cell types. Because elevated CO_2 affects the patterning of subsidiary cells, it will be a useful tool in understanding the patterning mechanisms that produce stomatal complexes. It seems likely that the production of extra subsidiary cells would prolong the process of forming mature stomatal complexes. These additional steps may provide an opportunity for studying subsidiary cell formation and development in detail. An important feature to note would be the timing of guard cell formation relative to that of the additional subsidiary cells. Normally in *Tradescantia* the guard mother cell divides after the two pairs of subsidiary cells form (Croxdale *et al.*, 1992). If this progression is maintained when extra subsidiary cells form, then it would be evidence for communication between the subsidiary cells and the guard mother cell during development. Alternatively, if division of the guard mother cell always occurs after the second pair of subsidiary cells is produced, then the critical event is independent of subsidiary cell formation or it is coupled in some way with the second set of subsidiary cells.

Since stomatal placement is not random but ordered (Bünning, 1956; Sachs, 1974; Korn, 1981; Charlton, 19[illegible]; Croxdale *et al.*, 1992), a particular treatment could affect the arrangement of stomata on the leaf surface without affecting stomatal number. The frequencies of local aggregations of stomata were analysed in order to examine distribution, and found to be roughly the same in all treatments.

Patterning in leaves is the centre of much current research (Nelson and Langdale, 1989; Sylvester *et al.*, 1990; Croxdale *et al.*, 1992; Freeling, 1992; Lincoln *et al.*, 1994; Smith and Hake, 1994; Boetsch *et al.*, 1995; Chin *et al.*, 1995). The accessibility and limited number of differentiated cell types in the epidermis make it ideal for study. In this study the focus has been on stomata for clues to epidermal patterning processes. The current study indicates that elevated CO_2 affects stomatal patterning in two important ways: augmentation of the number of subsidiary cells associated with stomatal complexes, and recruitment of these cells from the epidermal cell population.

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