Relative humidity is a key factor in the acclimation of the stomatal response to CO₂

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Abstract
Previous work has shown that stomata of growth chamber-grown Vicia faba leaves have an enhanced CO₂ response when compared with stomata of greenhouse-grown plants. This guard cell response to CO₂ acclimatizes to the environmental conditions on the transfer of plants between the two environments. In the present study, air relative humidity is identified as a key environmental factor mediating the changes in stomatal sensitivity to CO₂. In the greenhouse environment, elevation of relative humidity to growth chamber levels resulted in an enhanced CO₂ response, whereas a reduction in the light level to that comparable to growth chamber conditions had no effect on stomatal CO₂ sensitivity. The transfer of plants between humidified and normal greenhouse conditions resulted in an acclimation response with a time-course matching that previously obtained in transfers of plants between greenhouse and growth chamber environments. The high stomatal sensitivity to CO₂ of growth chamber-grown plants could be reduced by lowering growth chamber relative humidity and then restored with its characteristic acclimation time-course by an elevation of relative humidity. Leaf temperature was unchanged during this restoration, eliminating it as a primary factor in the acclimation response. Humidity regulation of stomatal CO₂ sensitivity could function as a signal for leaves inside dense foliage canopies, promoting stomatal opening under low light, low CO₂ conditions.

Key words: Acclimatization, carbon dioxide, relative humidity, stomata, Vicia faba.

Introduction
Light fluence rate and intercellular CO₂ concentration are two key environmental parameters sensed by guard cells and used to co-ordinate stomatal opening with the gas exchange requirements of photosynthesis (Assmann, 1993). Guard cells also sense other environmental signals such as humidity (Sheriff, 1979; Mott and Parkhurst, 1991; Assmann et al., 2000), temperature (Srivastava et al., 1995), and drought-induced ABA (Raschke, 1975; Tardieu and Davies, 1993). Substantial progress has been made in understanding the signal transduction pathways of the light response (Assmann, 1993; Assmann and Shimazaki, 1999; Zeiger, 2000; Schroeder et al., 2001). By contrast, the mechanism of CO₂ sensing is not well understood (Zhu et al., 1998; Assmann, 1999; Cousson, 2000), even though it has long been established that CO₂ sensing by guard cells is an intrinsic response independent of light sensing (Heath and Russell, 1954; Mouraviev, 1956). Interest in the stomatal response to CO₂ has been rekindled because of the uncertainty about the effect of recent increases in atmospheric CO₂ concentration on global climate change and plant function.

A compilation of independent studies covering some 60 species shows an extreme variability in reported stomatal response to changes in CO₂ concentration (Morison, 1987, 2001). Some of this variability undoubtedly results from interspecific variation and the well-documented ABA-mediated increase in stomatal CO₂ sensitivity in water-stressed plants (Raschke, 1975; Leymarie et al., 1998). However, independent studies using well-watered maize and Xanthium have reported both high and low CO₂ sensitivity within each species (Raschke et al., 1978; Mott, 1988; Farquhar et al., 1978; Sharkey and Raschke, 1981), indicating that factors other than interspecific variation and differences in water availability need to be taken into consideration.

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Recent studies have shown that a comparison of the CO₂ responses of stomata from leaves grown in greenhouse and growth chamber environments provides a valuable system for the characterization of differential CO₂ sensitivity (Talbott et al., 1996; Frechilla et al., 2002). The manipulation of ambient CO₂ concentration caused large aperture changes in stomata on growth chamber-grown *Vicia faba* leaves, while stomata on greenhouse-grown leaves were largely insensitive to changes in CO₂. The same responses can be observed in experiments manipulating ambient CO₂ around isolated stomata in epidermal peels, indicating that the differential CO₂ sensitivities are an intrinsic property of the guard cell and not due to a mesophyll signal (Frechilla et al., 2002).

Reciprocal transfer of plants between the greenhouse and growth chamber environments resulted in a shift of CO₂ sensitivity to that characteristic of the new environment, indicating that the stomatal CO₂ undergoes a plastic acclimation in response to environmental conditions (Frechilla et al., 2002). This acclimation had a characteristic time course; loss of CO₂ sensitivity occurred 2–3 d after transfer while acquisition of high CO₂ sensitivity required 5–7 d.

Previous studies have reported that stomata are sensitized to CO₂ by drought-induced ABA synthesis (Raschke, 1975; Leymarie et al., 1998), and that stomata of plants grown in constant elevated CO₂ (750 cm⁻³ m⁻³) show smaller changes in stomatal conductance in response to changes in CO₂ (Šantraček and Sage, 1996). However, neither drought or elevated CO₂ conditions were present in the greenhouse–growth chamber transfer experiments, indicating that the reversible acclimation response observed in this experiment is unlikely to result from either of these environmental factors.

The greenhouse and growth chamber environments differ in a number of environmental parameters that could cause the acclimation response. Maximum incident radiation in the greenhouse was twice that of the growth chamber and underwent both a daily cycle and weather-related variation. Differences in spectral quality also existed between the natural and artificial light sources of the two environments. Ambient temperature regimes of the two environments were comparable, although the growth chamber-grown plants experienced less variation than those in the greenhouse. Relative humidity was always higher in the growth chamber than in the greenhouse environment; dense canopy coverage combined with ample watering in the semi-closed growth chamber resulted in relative humidity of 85–90%, while greenhouse relative humidity typically ranged from 40–70%.

The present study investigated possible causal effects of key environmental variables in the acclimation of the CO₂ response of guard cells. Results showed that relative humidity is the main environmental factor mediating the reversible acclimation of the stomatal CO₂ response.

### Materials and methods

#### Plant material and growth conditions

Seeds of *Vicia faba* L., cv. Windsor Long Pod (Bountiful Gardens Seeds, Willits, CA, USA) were planted in pots with commercial potting mix (Sunshine mix No. 1, American Horticultural Supply, Camarillo, CA, USA). Planting sets of 24 pots were grown in a greenhouse under natural light or a 50% black shade cloth, 40–70% RH, 25–30/15–20 °C day/night, or in Conviron E8 growth chambers (Conviron Inc, Asheville, NC, USA), 85–90% RH, 23–15 °C 12/12 h day/night. Illumination (incandescent 40 W Philips; fluorescent: GTE Sylvania F48T12/CW/VHO) in the growth chamber was 375 μmol m⁻² s⁻¹. Light levels were measured with a quantum sensor (Li-Cor Inc., Lincoln, NE, USA). Plants were fertilized once a week (20–10-20 mix, Grow-More Research and Manufacturing Co, Gardena, CA, USA). Plants were watered three times a day with an automatic watering system, except in the case of low humidity in the growth chamber, in which the plants were watered daily by hand.

#### Relative humidity treatments

In both the greenhouse and growth chamber environments, high relative humidity was obtained with atomizer nozzles (Conviron, Inc) which supplied a fine water mist to the air moving through the plant canopy. Water supply to the atomizers was controlled by a solenoid valve operated by an interval timer (WW Grainger Inc, Commerce, CA, USA) set for 15 s of misting at 30 s intervals. This cycle was chosen because it yielded high relative humidity without the accumulation of liquid water on the leaf surfaces. Misting resulted in a relative humidity of around 95%.

A low humidity environment in a growth chamber was obtained by growing a small number of plants to reduce transpirational water input in the chamber. Plants were watered by hand to eliminate water spillage by the automatic irrigation system. This method reduced relative humidity values to around 55%.

All humidity measurements were made with a model 2200 hygrometer (Lab-Line Instruments Inc., Melrose Park, IL, USA). Temperature measurements were made using a TH85 digital thermocouple thermometer (Wescor Inc, Logan UT, USA).

#### Measurement of the stomatal response to CO₂

Stomatal sensitivity to CO₂ was assessed by measuring aperture changes in response to manipulated ambient CO₂ concentration around intact, attached leaves. For greenhouse experiments, plants were placed in an open top chamber located in the greenhouse that maintained temperature within 1 °C, relative humidity within 2% and the light level at 85% of ambient greenhouse conditions (Frechilla et al., 2002). Plants were transferred to the enclosure in the morning and allowed to equilibrate for 2 h before an initial aperture measurement was taken. Chamber CO₂ levels were then raised by injecting 100% CO₂ gas into the air stream supplying the chamber.

For growth chamber experiments, 100% CO₂ was injected directly into the fan compartment to ensure good mixing before the air stream reached the plants. In all experiments, carbon dioxide concentration was continuously monitored with an infrared gas analyser (EGM-1, PP systems, Hitchen-Herts, UK). Plants were maintained at each elevated CO₂ concentration for 1 h before measurement of stomatal apertures.

#### Acclimation time-course experiments

For greenhouse experiments, plants were grown for 4 weeks under either misted or unmisted conditions, then transferred to the alternate treatment. Stomatal response to ambient CO₂ was tested in the morning, starting on the morning of the day of transfer and continuing during the subsequent 7 d. For growth chamber transfer experiments, 4-week-old plants were subjected to misting at the start...
of the light cycle. Stomatal response to CO$_2$ was tested on the morning of the transition and followed for the subsequent 8 d.

**Stomatal aperture measurements**

Stomatal response was measured in fully expanded, recently matured leaves from the third and fourth nodes below the first expanded internode of 5-week-old plants. These leaves do not undergo significant further expansion, minimizing developmental effects during acclimation time-course experiments. Stomatal aperture was determined by selecting at least three leaves from three separate plants and quickly preparing epidermal peel sections for examination. Average aperture was determined from measurements of 30–40 digitized video images of abaxial stomata in at least three epidermal peels using an Olympus BH-2 microscope connected to a Javelin JE2362A digital imaging camera. Image processing was handled with an IBM PC-based MV-1 image analysis board (Metrabyte Corp., Taunton, MA, USA) and JAVA image analysis software (Jandel Scientific, Corte Madera, CA, USA). All experiments testing CO$_2$ sensitivity to an environmental condition were repeated a minimum of three separate days within a planting set, and with a minimum of two separate plantings. Each acclimation time-course experiment was repeated in three separate plantings.

**Results**

**Light and humidity effects on the CO$_2$ response of stomata from greenhouse-grown leaves**

Maximum light fluence rates in the greenhouse were typically 800 μmol m$^{-2}$ s$^{-1}$ while growth chamber fluence rates were 375 μmol m$^{-2}$ s$^{-1}$. In order to determine if the higher available light in the greenhouse was responsible for the differences in stomatal CO$_2$ response between growth chamber and greenhouse-grown leaves, *Vicia* sets were planted and grown in the greenhouse under 50% shade cloth so that the maximum light fluence rate approximated that prevailing in the growth chamber. Measurement of stomatal response to CO$_2$ showed that shading did not increase CO$_2$ sensitivity in greenhouse-grown plants (Fig. 1). Aperture values were approximately 4 μm lower in these leaves, consistent with the lower prevailing light fluences.

A second major environmental factor differing between the greenhouse and growth chamber environments is relative humidity. Typical relative humidity in the greenhouse ranged from 40% to 70%, while humidity in the growth chamber was typically 85–90%. To test the effect of relative humidity on CO$_2$ sensitivity, *Vicia* sets were planted and grown under a bank of misting spray nozzles that increased the relative humidity around these greenhouse-grown plants to levels comparable with the growth chamber. Tests in the open-topped chamber showed that stomata from misted plants responded with a 9 μm decrease in stomatal aperture when ambient CO$_2$ was increased from ambient levels (400 cm$^3$ m$^{-3}$) to 900 cm$^3$ m$^{-3}$ (Fig. 1). The magnitude of this response is similar to that seen in growth chamber-grown plants tested under greenhouse conditions (Frechilla *et al*., 2002). This increase in stomatal CO$_2$ sensitivity occurred under the high fluence rate, spectral distribution and variable illumination conditions typical of the greenhouse, indicating that these environmental variables were not causal factors in the acclimation response.

To confirm that changes in relative humidity could stimulate an acclimation of stomatal response to CO$_2$, plants were grown in misted and non-misted areas of the same greenhouse. After 3 weeks, plants in each area were transferred to the other growth condition. The stomatal response to CO$_2$ of plants from each group was tested daily in the open-top greenhouse chamber. Stomata from plants transferred from the misted to the non-misted area lost their high CO$_2$ sensitivity 2 d after the transfer (Fig. 2). Thereafter, their stomatal CO$_2$ response was indistinguish-
able from that of plants grown under non-misted greenhouse conditions. Stomata from plants transferred from non-misted to misted conditions acquired a high CO₂ sensitivity typical of misted plants 6 d after being transferred (Fig. 2). The time-course of these acclimation responses is identical to the time-course measured in transfer experiments between greenhouse and growth chamber environments (Frechilla et al., 2002).

Effect of humidity on the CO₂ response of stomata from growth chamber-grown plants

It was also investigated whether changes in relative humidity could alter stomatal response to CO₂ in a growth chamber environment. Stomata of growth chamber-grown plants, maintained at a constant 21/15 °C day/night temperature and >85% relative humidity typical of previous growth chamber experiments, showed a large aperture decrease in response to an increase in ambient CO₂: an increase from 400 to 900 cm³ m⁻³ resulted in a 6 μm decrease in aperture (Fig. 3). Stomata of plants grown under the same conditions, but with relative humidity lowered to 55%, showed little aperture change in response to the same increase in ambient CO₂ (Fig. 3). Stomata of plants grown under the same conditions, but with relative humidity lowered to 55%, showed little aperture change in response to the same increase in ambient CO₂ (Fig. 3). Stomata of growth chamber-grown plants with 500 μmol m⁻² s⁻¹ light showed a low response to CO₂, similar to the response seen in greenhouse and low-humidity growth chamber-grown plants (Fig. 4). This experiment yielded opposite results to the greenhouse experiments in which changes in light fluence had no effect on CO₂ sensitivity (Fig. 1). It was found, however, that increases in growth chamber light levels also resulted in elevation of both leaf temperature and temperature of the air surrounding the leaf (Fig. 5). These temperature increases would have the effect of raising leaf-air vapour pressure difference (VPD), both through increased vapour pressure of water in the leaf and through a reduction in relative humidity of the surrounding air. When relative humidity of the air surrounding the leaves was raised using a misting system similar to that employed in the previous greenhouse experiments, high CO₂ sensitivity was again observed (Fig. 4).

The misting treatment did not appreciably change the leaf temperature of plants grown under either 375 or 500 μmol m⁻² s⁻¹ light (Fig. 5). In particular, the misting treatment of plants grown under 500 μmol m⁻² s⁻¹ light did not lower leaf temperature values to those typical of plants grown under 375 μmol m⁻² s⁻¹ light. Thus the observed differences in CO₂ sensitivity of stomata caused by the misting treatment are unlikely to be mediated by changes in leaf temperature. This lack of leaf temperature change...
may be due to the fact that these leaves were grown in the relatively humid growth chamber environment in which leaf transpiration is probably a minor factor in the leaf energy balance.

In these growth chamber plants grown under 500 μmol m⁻² s⁻¹ light, the time-course of acclimation in CO₂ sensitivity following misting resembled the time-courses seen in previous experiments. Stomatal CO₂ sensitivity, typical of plants grown under 375 μmol m⁻² s⁻¹ light, was acquired 6 d after the start of the elevated humidity treatment (Fig. 6). The elevation of relative humidity by the misting system had no effect on the CO₂ response of plants grown under the normal 375 μmol m⁻² s⁻¹ light regime of the growth chamber (Fig. 6). Stomata from these plants maintained their high CO₂ sensitivity throughout the 8 d following the start of the misting treatment.

Discussion

The dramatic variability in stomatal CO₂ sensitivity found among previous studies (Morison, 1987, 2001) may result, at least in part, from a reversible acclimation process. This acclimation represents an ability of mature guard cells to vary CO₂ sensitivity in response to short-term changes in environmental conditions (Talbott et al., 1996; Frechilla et al., 2002), and not a developmental response such as changes in stomatal density resulting from long-term growth in elevated CO₂ (Woodward et al., 2002). Previous work has reported a reduced stomatal conductance response to changes in Cᵢ (Šantrůček and Sage, 1996) and reduced assimilation rates (Fletcher et al., 1988) in leaves of plants grown continuously in elevated CO₂. It is not known if these responses result from a reversible guard cell acclimation response or are developmental in nature.

The reversible acclimation response reported in the present paper appears to be independent of natural variation in ambient CO₂ concentration. Carbon dioxide concentration was not controlled in either the greenhouse or growth chamber environment and was thus determined by variation in ambient CO₂ concentration, which was the same for both environments. Ambient CO₂ concentration in the Los Angeles area showed a substantial daily variation in maximum concentration (360–600 cm⁻³ m⁻³) as well as a substantial variation of 100–150 cm⁻³ m⁻³ within a daily cycle (data not shown). Although photosynthetic activity during the light cycle resulted in a 30 cm⁻³ m⁻³ differential between growth chamber and ambient CO₂ concentration, this difference was minor compared to the natural variations in ambient CO₂ concentration. Most importantly, relative humidity manipulations led to high and low CO₂ sensitivities in both the greenhouse and growth chamber environments. There is, therefore, no evidence that ambient CO₂ is a primary environmental factor mediating the reversible acclimation of stomatal CO₂ sensitivity reported in this study.

An ABA-mediated enhancement of stomatal sensitivity to CO₂ is well established (Raschke, 1975; Leymarie et al., 1998). In the present study, however, the plants were well-watered throughout their growth cycle and the soil in the pots was always damp. It should also be noted that the greenhouse-grown plants had stomata with a lower CO₂ sensitivity compared with growth chamber-grown plants, even though they were in an environment with a higher evaporative demand, arguing against a role for ABA in the reversible acclimation process. Recent work with ABA-deficient and ABA-insensitive Arabidopsis mutants has failed to support a role for ABA in the normal stomatal humidity response (Assmann et al., 2000).

The shading experiments shown in Fig. 1 also rule out a major role for light intensity in the reversible acclimation response. In addition to maximum intensity, the greenhouse and growth chamber differed in other illumination parameters such as day length, spectral quality and shape of the illumination curve. However, both high and low stomatal CO₂ sensitivity were seen in plants grown under a greenhouse and two different growth chamber light regimes. Therefore it is not possible to assign to these light parameters a primary role in the mediation of the acclimation response.

The environmental parameter that was consistently effective in eliciting an acclimation of the CO₂ response was relative humidity. Increasing relative humidity in the greenhouse resulted in an acclimation response that increased stomatal sensitivity to CO₂ whereas reducing relative humidity in the growth chamber environment resulted in an acclimation response lowering stomatal CO₂ sensitivity. Loss of high sensitivity took 2–3 d while acquisition of the high sensitivity response required 5–6 d. These time-courses are identical to those measured in the
transfer experiments between the growth chamber and greenhouse environments (Frechilla et al., 2002), pointing to the operation of the same acclimation mechanism. Although air relative humidity was the controlled environmental factor mediating the acclimation response, the specific parameter sensed by the stomata remains to be determined. A change in air relative humidity brings concomitant changes in leaf-air vapour pressure difference (VPD) and transpiration rate. The complex interaction between these factors has hindered progress in the determination of the mechanism driving the well-documented stomatal response to relative humidity/VPD (Sheriff, 1979; Assmann et al., 2000). Experimental evidence has been advanced supporting roles for stomatal sensing of transpiration rate (Mott and Parkhurst, 1991; Bunce, 1996), cuticular transpiration (Meinzer, 1982; Meinzer et al., 1997), and leaf water potential (Comstock and Mencuccini, 1998).

A change in relative humidity and the concomitant change in transpiration rate often results in leaf temperature changes. However, leaf temperature does not seem to be involved in mediating the CO2 acclimation response since raising air relative humidity around leaves with elevated temperatures was effective in stimulating an acclimation response without a change in measured leaf temperature (Fig. 5).

The effect of long-term growth at different humidities was previously studied in *Oryza sativa* (Kawamitsu et al., 1993). Plants grown at 85% RH were found to have higher conductance rates and higher maximal assimilation rates than plants grown at 35% relative humidity. Stomatal responses to VPD changes were similar under both humidity conditions, but there were indications of a larger change in conductance in response to changes in ambient CO2 in the plants grown under the high humidity conditions. Interestingly, no differences in any of these parameters were noted in a C4 species, *Panicum maximum*, grown under the same differing relative humidity conditions.

**A role for the CO2 acclimation response under natural conditions**

A high air relative humidity appears to be a signal for an acclimation response generating a higher stomatal sensitivity to CO2. Under natural conditions, such a mechanism could function to acclimate leaves to growth under dense leaf canopies. Under dense, sheltered canopies, leaves would experience low light conditions, lowered ambient CO2 concentration (Francis and Parks, 1988), and elevated relative humidity. Under these conditions, light-stimulated stomatal opening would be reduced, possibly limiting photosynthesis through inadequate CO2 uptake or increased photorespiration. Using relative humidity as a signal for this condition, stomata would become sensitized to CO2 in order to track CO2 changes within the canopy, thus optimizing CO2 uptake and photosynthetic efficiency. In a closed canopy environment, water use efficiency would remain high since transpiration would be limited by the low VPD. A study of the CO2 response in plants growing under different densities or shade conditions might prove valuable in determining the actual role of this stomatal acclimation response under field conditions.

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**References**


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