THE FLORIDA STATE UNIVERSITY

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IS MALATE AN INTERMEDIATE IN THE SIGNAL-TRANSDUCTION NETWORK OF ELEVATED CO_2-INDUCED STOMATAL CLOSURE?

By

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ABSTRACT

Stomata close when plants are exposed to elevated CO_2 concentration and they open when plants are exposed to low CO_2 concentration. However, molecular elements of the signal-transduction network involved in CO_2 sensing have not been identified. In addition to the intrinsic CO_2 response that guard cells have, it has been proposed that high CO_2-evoked malate release from mesophyll cells activates the guard-cell anion channel, which initiates ion efflux. In our experimental system (*Vicia faba*, 3 h from the onset of illumination when stomatal aperture size is increasing), guard-cell K^+ content tracked the increase in aperture and therefore activation of the anion channel was the cardinal event in stomatal closure. The time course and concentration of malate required to close stomata were studied using epidermal peels (to determine guard-cell sensitivity to malate) and petiolar feeding (to infer apoplastic transport of malate to the guard cells). Isolation of the bulk-leaf apoplastic malate pool was additionally studied using a novel sequential sampling method. The malate contents of the guard-cell symplast and the guard-cell apoplast were determined using quantitative histochemical techniques. During the photoperiod, at stomatal aperture size $\leq$ 5 μm, guard-cell apoplastic malate content (maximum = 0.13 pmol, ~ 31 mM) was negatively correlated with stomatal aperture size (R^2 = 0.74), which indicates a regulatory role of malate at low apertures. As the essence of this study, the CO_2 concentration around whole plants was elevated two-fold from nominal pre-industrial CO_2 concentration (300 $\mu L \cdot L^{-1}$). Consistent with previous studies, the bulk-leaf apoplastic malate concentration increased (by 2.6-fold) within 30 min of exposure to elevated CO_2 concentration. However, stomata closed and the guard-cell K^+ contents declined before the modest increase in the guard-cell-apoplast malate content. These results do not support the hypothesis that elevated CO_2-induced malate release from mesophyll cells initiates stomatal closure, however, an elevated guard-cell-apoplast malate content could be a contributing factor in the maintenance of stomatal closure under high CO_2 concentration.

CHAPTER ONE

INTRODUCTION

Stomata are the major pathway for gas exchange of terrestrial plants. Closed stomata prevent water loss, but CO_2 cannot be taken up for photosynthesis. Open stomata permit CO_2 uptake but water is lost through them. Stomatal aperture size, which is sensitive to CO_2 concentration, is adjusted from moment-to-moment to balance the requirement for CO_2 for photosynthesis and the requirement to avoid excessive water loss. In addition, atmospheric CO_2 concentration is increasing—from 280 $\mu L \cdot L^{-1}$, at the beginning of the Industrial Revolution in the late 18th century, to its present value, 370 $\mu L \cdot L^{-1}$, and to a predicted value of 700 $\mu L \cdot L^{-1}$ by the end of the 21st century (e.g. Makino and Mae, 1999). In order to understand how plants respond to elevated CO_2 concentration, we have studied plants in which the ambient CO_2 concentration was increased two-fold, from 300 $\mu L \cdot L^{-1}$.

As alluded to, stomata close when plants are exposed to elevated CO_2 concentration and they open when plants are exposed to low CO_2 concentration (e.g. Mott, 1988; Assmann, 1999). The initial step of stomatal closure is the activation of the anion channel, which leads to K^+ efflux. As a result, water loss diminishes guard-cell volume and thus stomatal aperture size. Although molecular elements of the signal-transduction network involved in CO_2 sensing have not been identified, extracellular malate (an activator of the anion channel) has been proposed as an intermediate (Hedrich and Marten, 1993; Hedrich *et al*., 1994). Thus, Hedrich *et al*. (1994) reported that leaf-apoplast malate concentration increases from ~ 0.5 to ~ 2 mM within the first 15 min after *Vicia faba* is transferred from ambient (362 $\mu L \cdot L^{-1}$) to super-elevated CO_2 (10 000 $\mu L \cdot L^{-1}$) concentration. However, in addition to the difficulty of extrapolating these results to plausible Earth-based CO_2 concentrations, various values have been reported for the sensitivity of guard cells to extracellular malate. On one hand, transient activation of the

guard-cell anion channel was observed in response to 5 mM exogenous malate, which binds the extracellular face of the anion channel (Hedrich and Marten, 1993). Consistently, Raschke (2003) reported that 5 mM exogenous malate activates the R-type anion conductance in guard cells. On the other hand, Schroeder's lab (Schroeder *et al.*, 1993; Esser *et al.*, 1997) reported that 40 mM malate was required to activate the anion channel; if stomata were widely open (> 12 μm, *Vicia faba*) by light stimulation, applying malate did not result in stomatal closure. In *Commelina communis* (Cousson, 2000), 20 mM extracellular malate concentration is required to inhibit light-induced stomatal opening. Therefore, the attractive hypothesis that malate is an intermediate in CO_2 sensing remains in question, in part because of inconsistent values for the malate sensitivity of the anion channel.

Solutes accumulate in guard-cell apoplast when water evaporates from the guard-cell wall because it is the end-point of evaporative pathway (Ewert *et al.*, 2000; Outlaw and De Vlieghere-He, 2001). Regardless of the sensitivity of guard cells to extracellular malate, transpiration-linked malate accumulation in guard-cell wall could potentially reach an activating threshold. Therefore, quantitative localization of malate in the guard-cell wall following the initiation of CO_2-induced stomatal closure is also required to test the hypothesis.

In this study, the concentration of malate required to close "isolated" stomata was studied to determine guard-cell sensitivity to malate. Then, the transport and localization of xylem-source malate in excised leaflets were studied to infer malate transport to guard-cell wall. As the essence of this study, the guard-cell apoplast malate content was measured when the CO_2 concentration around whole plants was elevated two-fold from nominal pre-industrial CO_2 concentration (300 μL L^{-1}). Consistent with previous studies, the bulk-leaf apoplastic malate concentration increased (by 2.6-fold) within 30 min of exposure to elevated-CO_2 concentration. However, stomata closed and the guard-cell K^+ contents declined before the modest increase in the guard-cell-apoplast malate content. These results do not support the hypothesis that CO_2-induced malate release from mesophyll cells initiates stomatal closure, but an elevated guard-cell-apoplast malate content could be a contributing factor in the maintenance of stomatal closure under high CO_2.

CHAPTER TWO

RESULTS

2.1 Correlation of Guard-Cell K^+ Content and Stomatal Aperture Size in Intact Plants under Ambient CO_2 Concentration

The accumulation of osmotica in guard cells results in stomatal opening. Under some conditions, guard-cell sucrose accumulation is sufficient to support stomatal opening (e.g. Tallman and Zeiger, 1988; Talbott and Zeiger, 1996; Talbott and Zeiger, 1998; Outlaw and De Vlieghere-He, 2001). The molecular mechanisms for sucrose dissipation remain unknown, but theoretically, a role for anion efflux is improbable. Under most studied conditions (reviewed by Outlaw, 1983), however, fluctuations in K^+ salts are quantitatively sufficient to cause stomatal movements. The dissipation of K^+ salts, initiated by the activation of anion efflux, is then the cause of stomatal closure (for reviews, see Maathuis *et al.*, 1997; Schroeder *et al.*, 2001; Schroeder, 2003; Roelfsema and Hedrich, 2002; Outlaw, 2003). Therefore, as a means to establish relevancy to the hypothesized activation of the anion channel by malate in the guard-cell apoplast, we determined that K^+ accumulation indeed tracked the increase of stomatal aperture size under the conditions of our experiments. Guard-cell K^+ content increased during stomatal opening, reaching a maximum 4-to-6 h after the onset of illumination (Figure 1a). A summary plot of guard-cell K^+ content as a function of stomatal aperture size (Figure 2a) indicates a role of K^+ throughout the photoperiod ($R^2 = 0.49$).

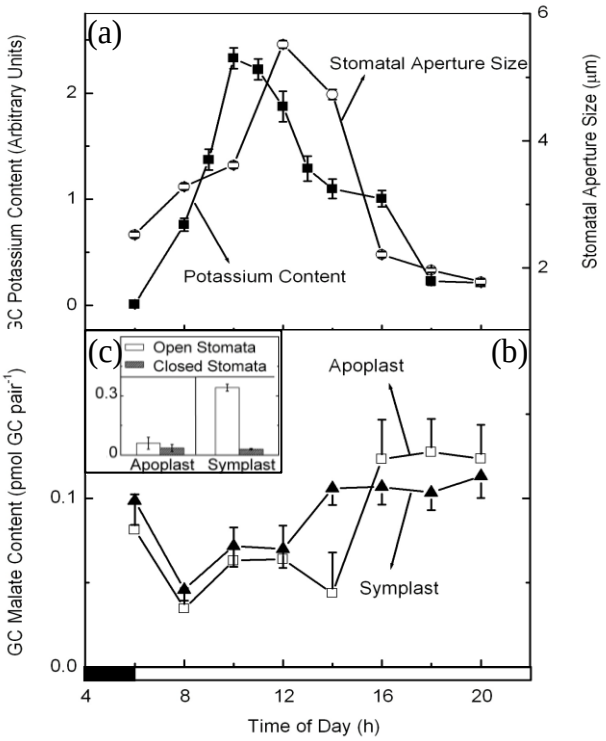

Figure 1: Daily time-course of guard-cell K^+ and malate contents in intact plants.
(a) Stomatal aperture size (○) and guard-cell K^+ content (■). Stomatal aperture size was measured using the same leaflets as for the malate assays; guard-cell K^+ content was measured using leaves from other intact plants. Data are means of 180 stomata (three independent experiments and 60 stomata per experiment) ± SE.
(b) Guard-cell symplast (▲) and guard-cell apoplast (□) malate contents. Samples were taken from intact plants at 2-h intervals during the photoperiod. Data are means of 22 guard-cell pairs (three independent experiments, 6 guard-cell pairs in one experiment and 8 guard-cell pairs in each of the other two experiments) ± SE.
(c) Comparison of guard-cell malate content in open stomata and closed stomata. Leaflets were cut under water and petioles were submerged in distilled water after 4-h from the onset of illumination. One leaflet was placed in darkness to induce stomata closure; the other was placed in a closed 600 mL container with 150 mL 0.1 N NaOH to induce stomata opening. Samples were taken for malate assay after 1.5-h treatments. Stomatal aperture size averaged 10.0 ± 0.4 μm for low CO_2-induced open stomata (n = 60), and 2.2 ± 0.3 μm for darkness-induced closed stomata (n = 60). Data are means of 8 guard-cell pairs ± SE.

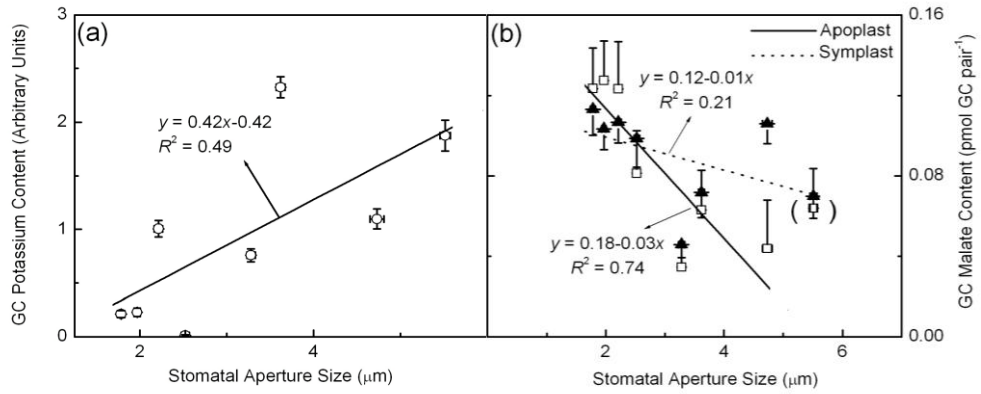

Figure 2: The relationship between guard-cell osmotica and stomatal aperture size in intact plants under ambient CO_2 concentration. Details are as in Figure 1.
(a) Stomatal aperture size vs. guard-cell K^+ content (○).
(b) Guard-cell malate contents in the apoplast (□) (parenthetical data are not used in the fitting line) vs. stomatal aperture size at ≤5 μm and in the symplast (▲) vs. stomatal aperture size over photoperiod.

Counter anions to K^+ accumulate in the guard-cell symplast during stomatal opening. If external Cl^- is sufficient, this anion accumulates in guard cells (Raschke and Schnabl, 1978; Schnabl and Raschke, 1980; Pemadasa, 1983), presumably by a Cl^--H^+ symporter (for reviews, see Schroeder *et al.*, 2001; Outlaw, 2003). In the absence of sufficient external permeant anions, malate accumulates to provide charge balance and cytosolic pH stabilization (Outlaw, 1990; refs. in Outlaw *et al.*, 2002). Differences in the anionic species that accumulate have also been reported for stomatal function in intact *Vicia faba* (Talbott and Zeiger, 1996), who reported that malate is the major counter anion to K^+ for growth-chamber-grown *Vicia faba* whereas Cl^- is the major counter anion for greenhouse-grown *Vicia faba*. However, they did not discriminate the guard-cell malate symplast pool and apoplast pool.

If malate accumulates in guard cells as the counter anion to K^+, its efflux resulting from activation of the anion channel would render ambiguous an interpretation that the guard-cell apoplast pool of malate resulted from mesophyll release. Therefore, we measured the guard-cell pools of malate over the photoperiod. Additionally, because the guard-cell apoplastic malate pool is calculated by subtracting malate content in guard-cell symplast from that in whole

guard-cell (see Experimental Procedures), precision improves if the symplastic pool is small and constant. Based on our previous studies (Outlaw and Lowry, 1977; Outlaw, 1978) on whole guard-cell malate content in excised leaflets (~ 0.5 pmol·GC pair^{-1} in open stomata; ~ 0.1 pmol·GC pair^{-1} in closed stomata), we confirmed that malate accumulated in the guard-cell symplast in open stomata (Δ_{sym} = ~ 0.3 pmol·GC pair^{-1}, Figure 1c) in a experiment that conducted in the same way as Outlaw and Lowry (1977). However, and serendipitously, in leaves of intact plants, the guard-cell symplastic malate pool was small before the onset of light (~ 0.1 pmol·GC pair^{-1}) and decreased ($p < 0.01$, n = 22) during stomatal opening. There was no overall correlation ($R^2 = 0.21$) between the size of the guard-cell symplastic malate pool and stomatal aperture size (Figure 1b, 2b). In particular, there was no decline in the guard-cell symplastic malate pool that correlated with stomatal closure. Therefore, charge balance to K^+ was provided by other anions (presumably Cl^- (for reviews, see Schroeder *et al.*, 2001; Outlaw, 2003), but possibly NO_3^- (Guo *et al*., 2003)) in our systems. Together, these data indicate that efflux of malate during stomatal closure from the guard-cell symplast will not confound an interpretation of the kinetics of the guard-cell apoplastic malate pool.

The guard-cell apoplast malate content decreased ($p < 0.01$, n = 22) following the onset of the photoperiod from ~ 0.08 pmol·GC pair^{-1} to ~ 0.03 pmol·GC pair^{-1}, or < 7 mM (Figure 1b). Interestingly, the guard-cell malate apoplastic pool increased by 1.8-fold ($p < 0.01$, n = 22) between 1400 h and 1600 h and this period corresponded to the largest decrease in stomatal aperture size (Figure 1a, 1b). Overall, there was a negative correlation between the stomatal aperture size over photoperiod and the guard-cell apoplast content ($R^2 = 0.56$) and a higher correlation exists at stomatal aperture size ≤ 5 μm (Figure 2b, $R^2 = 0.74$). Therefore, external guard-cell malate appears to have a regulatory role at low apertures.

2.2 Sensitivity of Guard Cells on Isolated Epidermal Peels to Extracellular Malate

Several laboratories (Hedrich and Marten, 1993; Raschke, 2003; Schroeder *et al.*, 1993; Esser *et al*., 1997; Cousson, 2000, see Introduction) have reported the sensitivity of stomata to extracellular malate. The reasons for differences among the results have not been elucidated fully (Assmann, 1999; Schroeder *et al*. 2001) so we have established the sensitivity under our conditions. Epidermal peels were floated in 10 mM MES/KOH (pH6.1) before transferred to artificial apoplastic solution, which was formulated based on recently reported values for the

guard-cell apoplast (see Experimental Procedures). According to the requirement for Ca^{2+} in CO_2-induced stomatal closure (Schwartz *et al.* 1998; Webb *et al.* 1996) and the activation of the anion channel by an enhancement of cytosolic free Ca^{2+} (Felle *et al.*, 2000), two Ca^{2+} concentrations, 0.1 mM and 1 mM, were used to determine the effect of Ca^{2+} on malate-induced stomatal closure.

In initial experiments, the time course for stomatal aperture size changes in response to 40 mM extracellular malate (with 0.1 mM Ca^{2+} in the incubation medium) was conducted. Stomatal closure appeared after a 20-min treatment and persisted for 60 min. Therefore, a 20-min incubation period was adopted for remaining experiments.

To rule out malate-induced stomatal closure by a simple osmotic effect, we conducted an osmotic control containing 120 mM mannitol (the osmolality of 40 mM Na_2malate equals that 120 mM mannitol). Inclusion of mannitol had a marginal or no effect on stomatal aperture size ($p = 0.15$ at 0.1 mM Ca^{2+}, n = 180; $p = 0.10$ at 1 mM Ca^{2+}, n = 180), indicating that malate-induced stomatal closure is a specific response.

In the incubation medium without malate, increasing Ca^{2+} concentration from 0.1 to 1 mM decreased stomatal aperture size ($p < 0.01$, n=180) as determined after 20-min incubation (Figure 3). Said differently, compared with initial values (T_0, not shown), stomatal aperture size did not change ($p = 0.52$, n = 180) with 0.1 mM Ca^{2+} in the incubation medium, whereas stomatal aperture size decreased ($p < 0.01$, n = 180) with 1 mM Ca^{2+}. Low malate concentration (2−4 mM) diminished stomatal aperture size with 0.1 mM Ca^{2+} in the incubation medium ($p < 0.01$ at both 2 and 4 mM malate, n = 180), whereas, low malate concentration only caused modest changes in stomatal aperture size with 1 mM Ca^{2+} in the incubation medium. However, application of $\geq$ 8 mM malate caused stomatal closure at both 0.1 and 1 mM Ca^{2+}. Therefore, these results indicate an interaction between malate and Ca^{2+}.

In general, above 8 mM malate, the concentration dependence of malate-induced stomatal closure on epidermal peels was the same with 1 mM or 0.1 mM Ca^{2+} in the incubation medium (Figure 3). At both Ca^{2+} concentrations, increasing malate from 4 mM to 8 mM had a large closing effect ($p < 0.01$ at both 0.1 and 1 mM Ca^{2+}, n = 180). Application of 40 mM malate did not cause further stomatal closure ($p = 0.31$ at 0.1 mM Ca^{2+}, n =180; $p = 0.73$ at 1 mM Ca^{2+}, n = 180). In summary, extracellular malate-induced stomatal closure was saturated at approximately 8 mM at both Ca^{2+} concentrations.

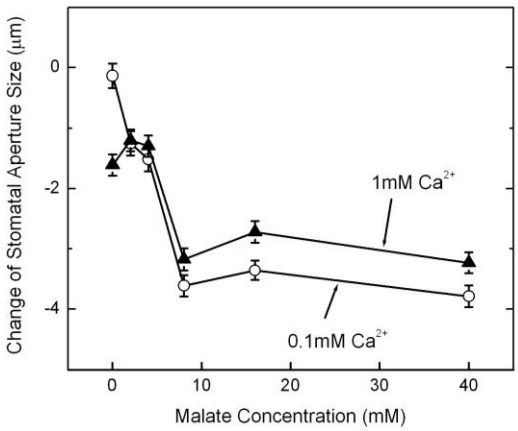

Figure 3: Concentration dependence of extracellular malate-induced stomatal closure on isolated epidermal peels.
Epidermal peels taken after 3-h illumination were incubated in a bathing medium (10 mM MES/KOH (pH 6.1, 6−8 mM K^+ depending on different malate concentrations) containing 0−40 mM malate or 120 mM mannitol (osmotic control), 0.1 mM (○) or 1 mM (▲) $CaSO_4$) at 25ºC, 60 % RH for 20 min. The initial stomatal aperture size averaged 6.3 ± 0.4 μm. Stomatal aperture size was measured at the end of the incubation period. Data represent means of stomatal aperture size change of 180 stomata (three independent experiments and 60 stomata per experiment) ± SE.

2.3 Transport and Localization of Xylem-Source Malate in Excised Leaflets

For CO_2-evoked malate release from mesophyll cells to activate the guard-cell anion channel, transport of malate to guard-cell wall is obviously required. Therefore, petiolar feeding was conducted to infer apoplastic transport of malate to guard cells. Artificial xylem sap was formulated based on the recently reported values for xylem sap (see Experimental Procedures). Initial experiments were conducted to establish the sensitivity of transpiration rate to infused malate (Figure 4). (In these experiments, sequential transpiration rates were measured on a single leaflet, whereas stomatal aperture size measurements are destructive and use of multiple leaflets introduces variability.) Control experiments were conducted by feeding artificial xylem sap without malate to excised leaflets.

Figure 4 shows the effect of infused malate on stomatal aperture size of excised leaflets following a 120-min infusion period. (The same general trend held after other incubation

periods, assayed at 15-min intervals up to 120 min as shown in part later.) At concentrations ≤ 8 mM, malate did not affect the transpiration rate of excised leaflets (for 8 mM, $p = 0.64$, n = 3; Figure 4). However, transpiration rate was inhibited by 41.1 % and 46.2 % when 16 mM ($p = 0.02$, n = 3) and 40 mM malate ($p < 0.01$, n = 3) were supplied to excised leaflets, respectively. Sixteen mM malate was saturating as 40 mM did not cause further inhibition of transpiration rate than it does ($p = 0.64$, n = 3). Therefore, in subsequent experiments, 16 mM malate was adopted. In addition, infusion of malate at low concentration (≤ 8 mM) did not affect transpiration rate, however, a relatively large stomatal aperture size change appeared when extracellular malate at low concentration applied (2−8 mM) (Figure 3), which indicates a different response of guard cells on excised leaflets and on isolated epidermal peels.

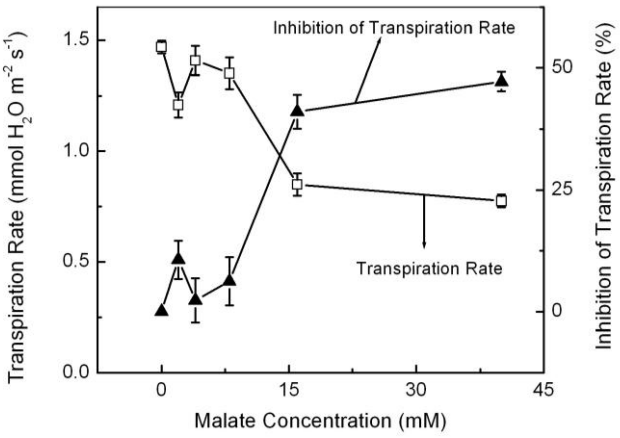

Figure 4: Concentration dependence of petiolar-fed-malate on transpiration rate (□) and inhibition of transpiration rate (▲) in excised leaflets. Petioles were excised under water 3 h after the onset of illumination and the petioles were immersed in feeding solutions (5 mM K_2HPO_4/KH_2PO_4 (pH 6.1) containing 1 mM $CaCl_2$, 0−40 mM malate). Approximately 600 μL of solution was infused into the transpiration stream during the 120-min infusion period. Transpiration rate was recorded at 15-min intervals and the average was used to represent the transpiration rate over the feeding period. For 2 mM malate-feeding treatment, data are the averages of six independent experiments ± SE; other data are the averages of three independent experiments ± SE.

The bulk-leaf apoplastic malate pool was studied by use of a novel sequential xylem-sap sampling method (Kang and Outlaw, unpublished). (With sucrose, this method appears to reproducibly sample different regions of the bulk-leaf apoplastic pool.) Specifically, 5-μL droplets were expressed sequentially by increased pressure application to the same leaflet (~ 5 droplets per leaflet). Although malate concentration varied somewhat among the different droplets, the differences were only a few percentage (Figure 5), indicating that the leaf apoplast is surprisingly homogeneous in malate concentration. Therefore, the average malate concentrations in all droplets from each leaflet are used to represent the average leaf-apoplast malate concentration in each leaflet (Figure 6).

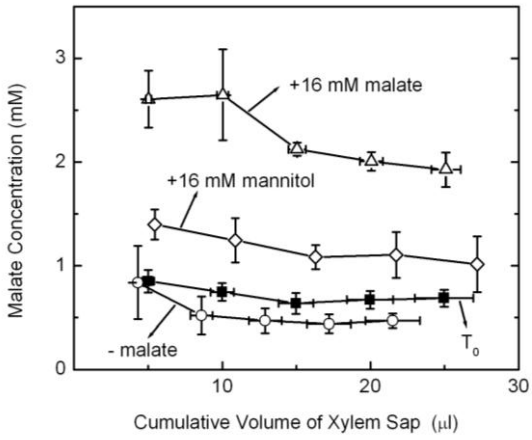

Figure 5: Variation of the bulk-leaflet-malate concentration with the cumulative volume of xylem sap.
Several 5-μL droplets were expressed sequentially from the same leaflet. Malate concentrations were measured separately in different droplets. T_0 (■) represents the initial xylem sap malate concentration. Excised leaflets were fed with artificial xylem sap (5 mM K_2HPO_4/KH_2PO_4 (pH 6.1) containing 1 mM $CaCl_2$, 0 mM malate (○), 16 mM malate (△) or 16 mM mannitol (◇)). Samples were taken after 120 min of infusion. Data are the means of three independent experiments ± SE. Other details are given in Figure 4.

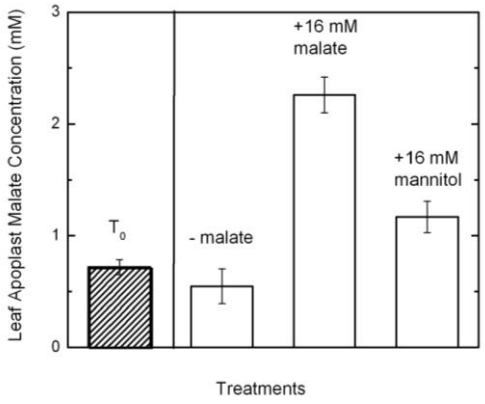

Figure 6: The effect of petiolar-fed-malate on average leaf-apoplast malate concentration in excised leaflets.
Data are the means of three independent experiments ± SE. The data are derived from Figure 5, where other details are given.

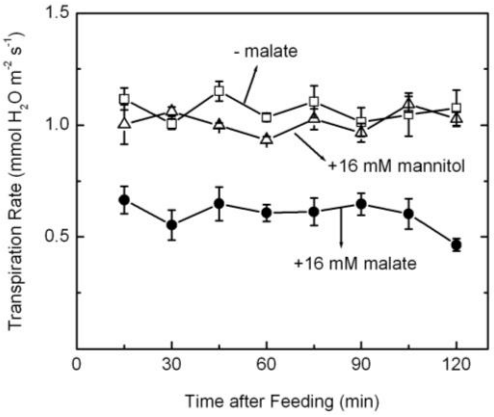

Figure 7: Time course of transpiration rate of excised leaflets in response to petiolar-fed-malate. Excised leaflets were fed with artificial xylem sap (5 mM K_2HPO_4/KH_2PO_4 (pH 6.1) containing 1 mM $CaCl_2$, 0 mM malate (□), 16 mM malate (●) or 16 mM mannitol (△)). Transpiration rate was recorded at 15-min intervals using the same leaflets in Figure 5. Data are the average of three independent experiments ± SE. Other details are given in Figure 4 and Figure 5.

Infusion of 16 mM mannitol to excised leaflets caused a moderate change of the leaf-apoplast malate concentration (p = 0.05, n = 3, compared with T_0; p = 0.04, n = 3, compared with non-osmotic control), whereas infusion of 16 mM malate to leaflets caused a 3.2-fold

increase in leaf-apoplast malate concentration ($p < 0.01$, n = 3, compared with T_0; $p < 0.01$, n = 3, compared with non-osmotic control). A significant difference between osmotic control and malate-feeding treatment ($p < 0.01$, n = 3) indicates that the increased leaf-apoplast malate concentration is a specific effect of malate. Transpiration rate was recorded at 15-min intervals; it decreased from ~ 1 mmol $H_2O \cdot m^{-2}s^{-1}$ to ~ 0.65 mmol $H_2O \cdot m^{-2}s^{-1}$ ($p < 0.01$, n = 8) when 16 mM malate was fed to excised leaflets (Figure 7), which is consistent with the initial experiments (Figure 4). Mannitol, the osmotic control, moves along transpiration stream and accumulates in guard-cell wall, thus diminishes the transpiration rate (Ewert, *et al*., 2000) as a slight change of transpiration rate over the 120-min infusion period shown in Figure 7 ($p = 0.05$, n = 8).

If infused malate were restricted to the leaf apoplast, its concentration would be 49 mM at the end of the infusion period (based on the average aqueous apoplastic-leaflet volume of *Vicia faba* (193 μL; Ewert *et al*. 2000), and the average infused volume and concentration). The much lower empirical concentration indicates that malate was removed from the leaf apoplast.

Guard-cell malate content was measured after infusion to examine the localization of xylem-source malate in guard cells (Figure 8). Exogenous malate increased the malate content of both the guard-cell apoplast and symplast. The guard-cell-apoplast malate content (0.26 pmol·GC $pair^{-1}$) resulting from malate infusion was 2.3-fold of that in the control ($p < 0.01$, n = 24). The guard-cell symplast malate content (0.14 pmol·GC $pair^{-1}$) resulting from malate infusion was 3.5-fold of that in the control ($p < 0.01$, n = 24).

The guard-cell-apoplast malate concentrations are shown in Table 1 (calculated based on the aqueous guard-cell apoplast volume (4.15×10^{-15} m^3; Ewert *et al.*, 2000), and the malate contents (Figure 8)).

Table 1: The effect of malate infusion into excised leaflets on the guard-cell apoplast malate concentration in excised leaflets. Values are means ± SE (n = 24). Details are as in Figure 8.

	− malate	+ 16 mM malate
Guard-cell apoplast malate concentration (mM)	27.6 ± 4.0	62.6 ± 4.7

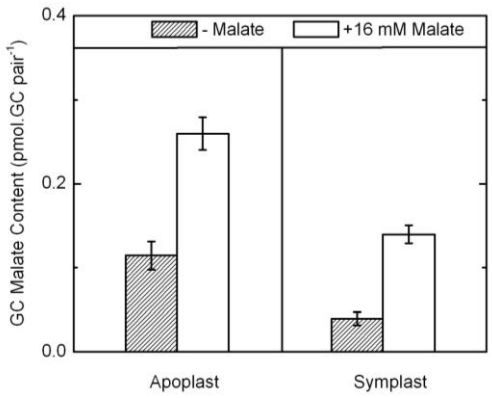

Figure 8: The effect of malate infusion into excised leaflets on the malate content of the guard-cell symplast and apoplast.
Samples were taken after 120-min feeding treatment. Data represent the means of 24 guard-cell pairs (8 guard-cell pairs per experiment, three independent experiments) ± SE. Other details are given in Figure 4.

Overall, the guard-cell apoplast malate concentration exceeded considerably the bulk-leaf apoplastic malate concentration (~2.3 mM after 120-min infusion of 16 mM malate, Figure 6), thus petiolar-fed-malate accumulated in the guard-call wall. It exceeded 8 mM, the saturating concentration for the sensitivity of guard cells on isolated epidermal peels to extracellular malate (Figure 3), and it also exceeded the value shown in Figure 1c, in which distilled water were used to fed excised leaflets. Explanations for these various malate values see Discussion.

In summary, supplying malate at concentration higher than or equal to 16 mM inhibited transpiration rate significantly and malate content increased in both the leaf and guard-cell apoplastic pools.

2.4 Effect of Elevated CO_2 Concentration on the Malate Content of Leaf Apoplast, Stomatal Aperture Size, K^+ Content of the Guard-Cell Symplast, and the Malate Content of the Guard-Cell Apoplast and Symplast of Intact Plants

Our main purpose was to examine the effect of elevated CO_2 concentration around whole plants on the guard-cell-apoplast malate pool. Plants were grown under ambient (growth room)

CO_2 concentration (~ 380−400 $\mu L \cdot L^{-1}$), and were then transferred to300 $\mu L \cdot L^{-1} CO_2$ concentration for 30 min (Figure 9a). The purpose of this transfer was to maximize stomatal aperture changes upon transition to elevated CO_2 and to set up initial conditions of low malate in the guard-cell apoplast (cf. Figure 1). Then, the CO_2 concentration was elevated to 600 $\mu L \cdot L^{-1}$ over 7 min (Figure 9a). Stomatal aperture size decreased from ~ 9.9 μm to ~ 6.3 μm within 5 min following the transition to elevated CO_2 concentration ($p < 0.01$, n = 180) (Figure 9b), which corresponded to a decrease in the guard-cell K^+ content (from ~ 2.9 arbitrary units (AU) to ~ 2.3 AU; $p < 0.01$, n = 180). Almost no K^+ stained (~ 0.06 AU) in guard cells after 30 min of high CO_2 concentration, when stomatal aperture size was diminished 75.5 %. In summary, significant stomatal closure correlated with a significant decrease of guard-cell K^+ content ($R^2 = 0.92$). This result implies the importance of the anion channel in stomatal closure in these experiments.

Exposure of intact plants to 600 $\mu L \cdot L^{-1}$ CO_2 for 5 min did not affect the bulk-leaf apoplastic malate concentration ($p = 0.25$, n = 3). The bulk-leaf apoplastic malate concentration appeared to increase moderately within 15 min (from ~ 0.4 mM to ~ 1.1 mM; $p = 0.07$, n = 3), which is qualitatively consistent with the previous studies (from ~ 0.5 to ~ 2 mM within the first 15 min in 10 000 $\mu L \cdot L^{-1}$ CO_2, Hedrich *et al*., 1994). Significant increase in the bulk-leaf apoplastic malate concentration ($p = 0.04$, n = 3; increased by 2.6-fold) took place within 30min of elevated CO_2 concentration (Figure 9c).

There is no significant change of guard-cell apoplastic malate ($p = 0.19$, n = 24) and symplastic malate ($p = 0.59$, n = 24) after 5 min of elevated CO_2 concentration (Figure 9d). Both the guard-cell-apoplast and symplast malate contents increased after 15 min of elevated CO_2 concentration ($p < 0.01$, n = 24, increase to 1.9-fold for apoplast; $p < 0.01$, n = 24, increase to 1.3-fold for symplast). The guard-cell-apoplast malate content declined between T_{30} and T_{15} ($p = 0.02$, n = 24). There was little correlation ($R^2 = 0.14$) between the change of guard-cell apoplastic malate pool and the change of bulk-leaf apoplastic malate pool. However, both bulk-leaf apoplastic malate concentration and transpiration rate affect transpiration-linked accumulation of malate in the guard-cell wall; since transpiration rate decreased, a product-precursor relationship is not expected.

The guard-cell-apoplast malate concentrations for the CO_2 transitions (Figure 9) are shown in Table 2 (calculated based on the aqueous guard-cell apoplast volume (4.15×10^{-15} m^3;

Ewert *et al.*, 2000), and the malate contents (Figure 9)). Overall, the guard-cell apoplast malate concentration in intact plants exceeded considerably the saturating malate concentration (8 mM) for the sensitivity of guard cells on isolated epidermal peels (Figure 3).

Table 2: The guard-cell-apoplast malate concentration of intact plants for the CO_2 transitions. Values are means ± SE (n = 24). Details are given in Figure 9.

	T_{-12}	T_5	T_{15}	T_{30}
Guard-cell-apoplast malate concentration (mM)	27.9 ± 5.6	35.5 ± 3.3	51.5 ± 6.3	33.9 ± 5.2

In summary, the decrease in stomatal aperture size preceded a moderate increase of guard-cell apoplast malate. If malate serves as a signal in the CO_2-sensing pathway, a significant increase of guard-cell-apoplast malate content should appear in advance of the decline of stomatal aperture size. Therefore, these results do not support the hypothesis that CO_2-induced malate release from mesophyll cells initiates stomatal closure, however, an elevated guard-cell apoplast malate content could be a contributing factor in the maintenance of stomatal closure induced by high CO_2 concentration.

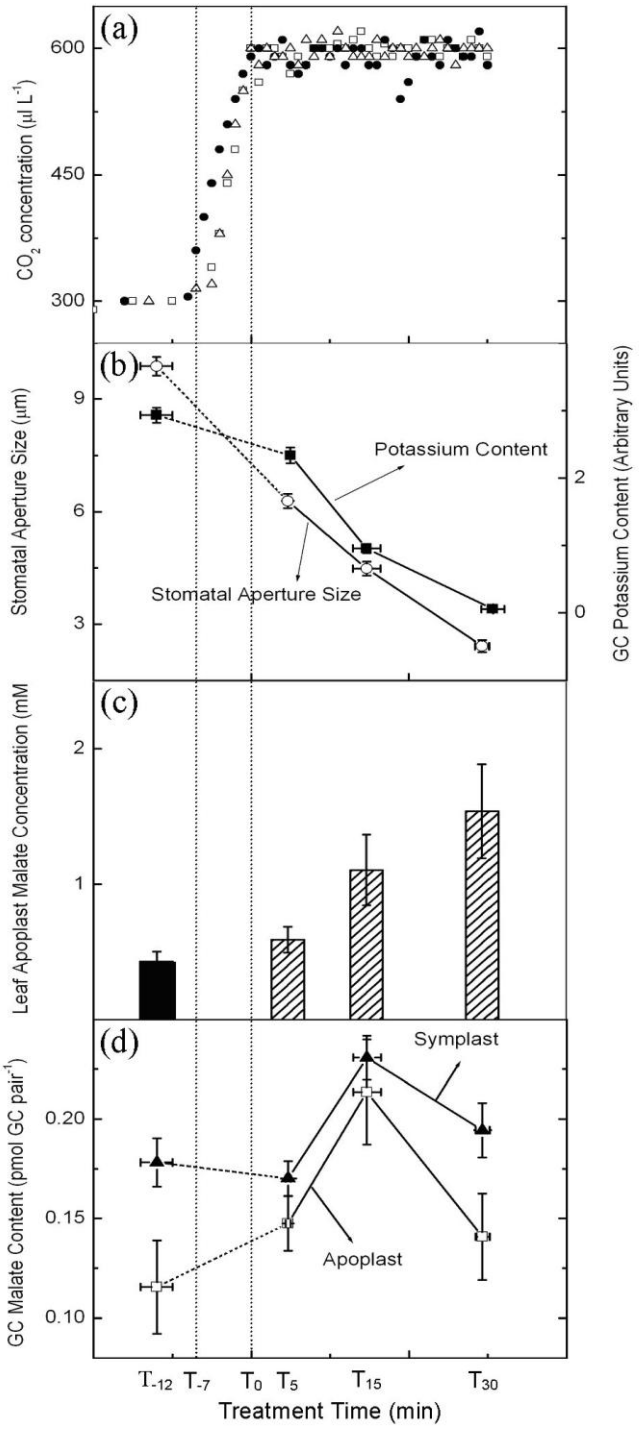
(a)
600
450
300
CO_2 concentration ($\mu l\ L^{-1}$)
(b)
9
6
3
Stomatal Aperture Size (μm)
Potassium Content
Stomatal Aperture Size
2
0
GC Potassium Content (Arbitrary Units)
(c)
2
1
Leaf Apoplast Malate Concentration (mM
(d)
0.20
0.15
0.10
GC Malate Content (pmol GC pair^{-1})
Symplast
Apoplast
T_{-12} T_{-7} T_0 T_5 T_{15} T_{30}
Treatment Time (min)

Figure 9: The effect of two-fold elevation of CO_2 concentration on the malate content of leaf apoplast, stomatal aperture size, K^+ content of the guard-cell symplast and the malate content of the guard-cell apoplast and symplast of intact plants. T_{-12} represents samples that were taken at 300 $\mu L \cdot L^{-1}$ CO_2. CO_2 concentration in growth chamber was increased from 300 (T_{-7}) to 600 $\mu L \cdot L^{-1}$ (T_0) over ~ 7 min. T_5, T_{15} and T_{30} represent samples that were taken after 5, 15 and 30 min in 600 $\mu L \cdot L^{-1}$ CO_2.
(a) CO_2 concentration change during overall experiment period. Three different icons (□, ●, △) represent data from three independent experiments.
(b) Stomatal aperture size (○) and guard-cell K^+ content (■). Data are the means of 180 stomata (three independent experiments, 60 stomata per experiment) ± SE.
(c) Leaf apoplast malate concentration. Data are the means of three independent experiments ± SE.
(d) Malate contents in guard-cell symplast (▲) and apoplast (□). Data are the means of 24 guard-cell pairs (three independent experiments and 8 guard-cell pairs per experiment) ± SE.

CHAPTER THREE

DISCUSSION

3.1 The Regulatory Role of Malate at Low Stomatal Aperture Size

Malate has a regulatory role in stomatal movement, especially in maintenance of stomatal closure. (1) Intact plants under normal conditions (ambient CO_2, RH 60% and 25ºC, see Experimental Procedures). In our experimental system, stomatal aperture size at ≤ 5 μm correlated negatively ($p = 0.74$) with the guard-cell-apoplast malate content (Figure 1b, 2b). Possible sources for the increased guard-cell-apoplast malate pool are malate release leaf cells or import via the xylem, but it is unlikely to be caused by release from the guard-cell-symplast pool, because malate symplast pool remained stable. Regardless of the source, malate appears to contribute to the maintenance of stomatal closure in intact plants under the circadian control. (2) Stomatal closure induced by elevated CO_2 concentration. An elevated guard-cell-apoplast malate content (increased to 1.9-fold after 15 min of elevated CO_2 concentration) could be a contributing factor in the maintenance of stomatal closure under high CO_2 concentration although our results showed that high CO_2-induced malate release from mesophyll cells does not initiate stomatal closure. (3) Stomatal closure induced by drought stress. Drought causes the increase of whole-leaf malate concentration during the dry seanon (*Fraxinus excelsior* L.; Guicherd *et al.,* 1997). In addition, an increase in xylem-malate concentration correlates with a decrease in the stomatal aperture size during drought stress and the stomatal opening is prevented by infusing 0.5 to 3 mM exogenous malate to excised leaflets (*Fraxinus excelsior* L.; Patonnier *et al*., 1999). These reports support the hypothesis that malate has a regulatory role in maintenance of stomatal closure induced by drought. Additionally, malate may serve as an intermediate in the signal-transduction pathway of drought induced stomatal closure. To verify

these hypotheses, guard-cell apoplast malate content needs to be investigated when plants are under drought stress, which has not been done before.

3.2 Sensitivity of Guard Cells on Isolated Epidermal Peels to Extracellular Malate

Various values have been reported for the sensitivity of guard cells to extracellular malate (Cousson, 2000; Esser et al., 1997; Hedrich and Marten, 1993; Hedrich *et al*., 1994; Schroeder et al., 1993; see Introduction). Extracellular malate-induced stomatal closure under our conditions was saturated at approximately 8 mM malate, which is less than the results reported from Schroeder's lab (40 mM in *Vicia faba*) and Cousson (20 mM in *Commelina communis*), but higher than that (5 mM in *Vicia faba*) from Hedrich's lab. These inconsistent results may result from the different ion concentrations used in experimental systems. Guard-cell apoplast ions play important roles in regulating stomatal movement (e.g. Roelfsema and Hedrich, 2002). (1) Ca^{2+} is required for elevated CO_2-induced stomatal closure (Schwartz *et al.* 1998; Webb *et al.* 1996). Additionally, an enhancement of cytosolic free Ca^{2+} induced by sudden darkness or ABA infusion activates the anion channel (Felle *et al.*, 2000). (2) K^+ at concentration higher than physiological level inhibits stomatal closure. In *Commelina communis* (Willmer and Mansfield, 1970), the presence of 300 mM K^+ in incubation medium stimulate stomatal opening; in *Vicia faba* (Wardle and Short, 1981), 100 mM K^+ stimulate stomatal opening even at the presence of 0.1 mM ABA. The ion concentration used in our experimental system was based on recently reported values for the guard-cell apoplast (6−8 mM K^+ depending on the malate concentrations; 0.1 or 1 mM Ca^{2+}; see Experimental Procedures), and was closer to physiological level than those previous studies (50 mM K^+; no Ca^{2+}).

3.3 Transportation and Localization of Petiolar-Fed Malate

Petiolar-fed malate caused a decrease of transpiration rate, and an increase of malate content in both the leaf and guard-cell apoplastic pool. The same phenomenon that petiolar-fed malate inhibits stomatal opening were reported in potato (Hedrich *et al.*,2001) and in *Fraxinus excelsior* L. (Patonnier *et al*., 1999). Hedrich *et al.* (2001) reported that feeding 2.5−20 mM malate into the potato transpiration stream lowers the transpiration rate in a malate-concentration-dependent manner. They also showed the effects of elevated CO_2-induced

stomatal closure and of malate-induced stomatal closure are additive. Supplying 0.5 to 3 mM malate to excised *Fraxinus excelsior* L. leaves effectively inhibit stomatal opening (Patonnier *et al*., 1999). The saturating concentration of petiolar-fed malate-induced inhibition of stomatal opening under our conditions is approximately 16 mM, 0.5 mM in Patonnier *et al*. (1999), and 10 mM in Hedrich *et al*. (2001). A possible reason is different species and feeding solutions were being used (feeding solutions were composed of malate and KOH at pH 6.25 in Patonnier *et al*. (1999), K_2malate at pH 6 in Hedrich *et al*. (2001)). Our malate infusion experiment showed the infused malate was removed from the leaf apoplast because the leaf apoplast concentration after infusion (~ 2.3 mM) was much lower than the concentration calculated (49 mM) if infused malate were restricted to the leaf apoplast. One possible pathway for malate enters into metabolism is being used in starch synthesis.

3.4 Multiple CO_2-Sensing Pathways Exist in the Signal-Transduction Pathway of Elevated CO_2-Induced Stomatal Closure and Environmental Stimuli Affect Stomatal Response to CO_2

Several pathways exist in guard-cell sensing CO_2. It is a controversial issue that CO_2 sensitivity is an intrinsic property of guard cells or mesophyll cells are required in this pathway. Stomatal closure occurs when epidermal peels are exposed to increased-CO_2 environment (e.g. Schwartz *et al*., 1988). Corroboratively, isolated guard-cell protoplasts swell in response to increased CO_2 concentration (Fitzsimons and Weyers, 1986). Thus, guard-cell can sense changes in CO_2 concentration by themselves. However, this cannot rule out the possibility that other sensing pathways, which require the presence of mesophyll cells, may exist. For example, mesophyll cells could influence stomatal response to CO_2 by photosynthetic rate change, which changed the intercellular CO_2 concentration (e.g. Mott, 1990). Therefore, elevated CO_2 concentration can be sensed by guard cells themselves in isolated epidermal peels or by mesophyll cells, which release signal(s) to guard cells in intact plants. However, which materials are intermediates in CO_2 sensing pathway have not been identified before.

In our experimental conditions, although an elevated guard-cell apoplast malate content (increased to 1.9-fold after 15 min of elevated CO_2 concentration) could be a contributing factor in the maintenance of stomatal closure, stomata closed and guard-cell K^+ contents declined before the modest increase in the guard-cell-apoplast malate content, which indicates that high

CO_2-induced malate release from mesophyll cells does not initiate stomatal closure. Other candidates are Cl^- and zeaxanthin. (1) Elevated CO_2 concentration (from 350 $\mu L \cdot L^{-1}$ to 600 $\mu L \cdot L^{-1}$) causes a transient rise of Cl^- concentration (from 3 to 14 mM within 10 min) in the guard-cell apoplast. Stomata are still open when guard-cell apoplast Cl^- concentration reach the peak (Hanstein and Felle, 2002), thus Cl^- may act as a signal for stomatal closure. However, more evidence needs to verify this hypothesis because a short time (2 min) CO_2 pulses does not cause Cl^- efflux. (2) Zeaxanthin concentration decreases from 180 $mmol \cdot mol^{-1}$Chl to 80 $mmol \cdot mol^{-1}$Chl and stomatal aperture size declines from 14 μm to 7 μm when CO_2 concentration increases from 400 $\mu L \cdot L^{-1}$ to 1200 $\mu L \cdot L^{-1}$ (Zhu *et al.*, 1998; Zeiger and Zhu, 1998). They proposed that CO_2 sensor is located in guard-cell chloroplasts and zeaxanthin is an intermediate in CO_2-sensing. However, the increase of zeaxanthin is the cause of stomatal closure or the result has not been clarified because zeaxanthin concentration and stomatal aperture size measured after in 1.5, 2, 2.5 and 3-fold of elevated CO_2 concentration for 1 h.

Some environment factors, e.g. temperature, light and humidity, affect stomatal response to CO_2. (1) Temperature. Both epidermal peels and leaves show stomatal sensitivity to CO_2 when atmospheric temperature is between 25ºC to 35ºC, whereas there is no stomatal response to the change of CO_2 concentration when atmospheric temperature above 35ºC during light period (Spence *et al*. 1984). (2) Light quality. Stomatal response to CO_2 of growth-chamber-grown *Vicia faba* plants is more sensitive than that of greenhouse-grown *Vicia faba* plants and the difference of light quality in these growth conditions might be the reason (Talbott *et al*. 1996). The stomatal response to CO_2 acclimates to the growth environment, because growth-chamber-grown *Vicia faba* plants lose the sensitivity to CO_2 after being transferred to greenhouse for 2−3 days, and stomatal response to CO_2 is observed in greenhouse-grown *Vicia faba* plants after being transferred to growth chamber for 5−7 days (Frechilla *et al*, 2002). (3) Relative humidity (RH). Change of RH caused the change of transpiration rate, and thus the changed solutes concentration in guard-cell apoplast (Outlaw and De Vlieghere-He, 2001), which may affect the stomatal response to CO_2. Increasing CO_2 concentration from 400 $\mu L \cdot L^{-1}$ to 900 $\mu L \cdot L^{-1}$ causes stomatal aperture size declines from ~ 16 μm to ~ 8 μm under normal RH (85 %), whereas no change of stomatal aperture size takes place under low (55 %) RH (Talbott *et al.* 2003). In summary, stomatal response to CO_2 is a multifactorial process being affected by many environment factors.

3.5 Guard-Cell Malate Content Various in Isolated Epidermal Peels, Excised Leaflets and Intact Plants

Various values for guard-cell malate content have been reported in this study (Table 3). (1) Excised leaflets in low CO_2 concentration, high RH and infused with distilled water vs. intact plants in ambient CO_2 concentration and 60 % RH. In the former, malate accumulated in the guard-cell symplast (Δ_{sym} = ~ 0.3 pmol·GC pair^{-1}, compare to darkness induced-closed stomata) and stomata opened widely (~ 10 μm) and rapidly (Figure 1c), whereas in the latter, the guard-cell symplast malate pool was small (0.05−0.11 pmol·GC pair^{-1}) (Figure 1b) and the maximum stomatal aperture size was ~ 6 μm (Figure 1a). For the former, pH and solutes (including Cl^-) concentrations changed in the guard-cell wall because distilled water was supplied and high RH caused a lower transpiration rate. (a) Guard-cell-apoplast Cl^- content affects guard-cell malate synthesis and accumulation (Raschke and Schnabl, 1978; Schnabl and Raschke, 1980; Pemadasa, 1983). A decrease of Cl^- concentration (insufficient Cl^- supply in xylem sap and low transpiration rate) in the guard-cell apoplast in excised leaflets resulted the accumulation of malate in guard-cell symplast. (b) The import of Cl^- to guard-cell symplast is presumed by a Cl^--H^+ symporter (for reviews, see Schroeder *et al.*, 2001; Outlaw, 2003). Thus, xylem sap pH change can act as an interacting regulatory element on guard-cell malate synthesis. In addition, pH itself is a signal in stomatal movement regulation (Wilkinson, 1999). Therefore, guard-cell apoplast Cl^- pool and the change of pH need to be investigated under our experimental conditions in the further studies.

(2) Excised leaflets in low CO_2 concentration, high RH and infused with distilled water vs. excised leaflets in ambient CO_2 concentration, 60 % RH and infused with non-malate artificial xylem sap. For both of them, guard-cell-apoplast malate contents around the same range (p = 0.05, n = 8 for the former, n = 24 for the latter). However, the guard-cell symplast malate contents in the latter (0.04 pmol·GC pair^{-1}) are much smaller than that in the former (0.34 pmol·GC pair^{-1}). The presence of 1 mM Cl^- in the artificial xylem sap might be the reason for the smaller malate content in guard-cell symplast as discussed above.

(3) Intact plants under ambient CO_2 vs. elevated CO_2 concentration. The guard-cell-apoplast malate contents in intact plants of closed stomata (0−3 μm) under ambient CO_2 (Figure 1b, 0.08−0.13 pmol·GC pair^{-1}, 19−31 mM) were lower than that in the intact plants

in 15 min of elevated CO_2 concentration (Table 2, ~ 52 mM). Therefore, malate contributes to the maintenance of elevated CO_2- induced stomatal closure.

(4) Isolated epidermal peels vs. intact plants in ambient CO_2 concentration, excised leaflets infused with malate and intact plants in elevated CO_2 concentration. (a) The saturating concentration (8 mM) for the sensitivity of guard cells on isolated epidermal peels to extracellular malate is in the range of guard-cell-apoplast malate in the open stomata (3−6 μm) of intact plants in ambient CO_2 concentration (7 mM−14 mM); it is lower than that in the closed stomata (0−3 μm) of intact plants (19 mM−31 mM). (b) The guard-cell-apoplast malate concentrations of excised leaflets (~ 63 mM) in which transpiration rate were inhibited by infused malate and of intact plants (~ 52 mM) in 15 min of elevated CO_2 concentration in which stomata opening was inhibited exceeded considerably the saturating concentration (8 mM) for the sensitivity of guard cells on isolated epidermal peels to extracellular malate (Figure 3). Therefore, the malate concentration required maintaining the stomatal closure in intact plants under circadian control, stomatal closure caused by infused malate to excised leaflets or high CO_2 concentration around intact plants is higher than the concentration that can induce stomatal closure on isolated epidermal peels.

In summary, malate has a regulatory role in stomatal movement and it was regulated by intrinsic factors of plants and environmental factors. The specific experimental protocols should be take into consider when comparing the guard-cell malate content.

Table 3: Guard-cell malate contents in excised leaflets and intact plants under different conditions.

		Excised leaflets (open stomata)		Excised leaflets (closed stomata)	
Feeding solution and other treatments		Distilled water Low CO_2 concentration High RH	Artificial xylem sap without malate	Distilled water Darkness	Artificial xylem sap with malate
		Figure 1c	Figure 8	Figure 1c	Figure 8
Guard-cell malate (pmol·GC pair^{-1})	Symplast	~ 0.34	~ 0.04	~ 0.03	~ 0.14
	Apoplast	~ 0.06	~ 0.11	~ 0.04	~ 0.26

		Intact plants		
CO_2 concentration (μL·L^{-1})		380−400		~ 600 for 15 min
Stomatal aperture size (μm)		3−6	0−3	~ 4.5
		Figure 1a, 1b		Figure 9b, 9d
Guard-cell malate (pmol·GC pair^{-1})	Symplast	0.05−0.11	0.10−0.11	~ 0.23
	Apoplast	0.03−0.06	0.08−0.13	~ 0.21

CHAPTER FOUR

EXPERIMENTAL PROCEDURES

4.1 Plant Material and Growth Conditions

Vicia faba L. was grown in a growth chamber (Environmental Growth Chambers Co., Chagrin Falls, OH, US) under the combination of fluorescent and incandescent light (PAR ~ 600 $\mu mol \cdot m^{-2}s^{-1}$) at 60 % RH and a 16-h photoperiod (25ºC in light and 20ºC in darkness). Details of plant growth and of light and temperature transitions of the growth chamber are in Ewert *et al*. (2000). The second fully expanded leaflets of 18–21 day-old plants were used in all experiments.

4.2 Sensitivity of Guard Cells on Isolated Epidermal Peels to Extracellular Malate

Epidermal strips were peeled from the abaxial surface of the second fully expanded leaflets 3 h after the onset of illumination. Strips were washed, brushed, cut into 5 mm × 5 mm-squares, and floated in 10 mM MES/KOH (pH 6.1) at 25ºC. An initial aperture measurement was taken; then the epidermal squares were transferred to Petri dishes that contained 5 mL incubation solution (10 mM MES/KOH (pH 6.1, 6−8 mM K^+ depending on malate concentration applied), containing 0−40 mM malate (or an osmotic control, 120 mM mannitol, the osmolality of 40 mM Na_2malate equals 120 mM mannitol), and $CaSO_4$ (0.1 mM or 1 mM)). This artificial apoplastic solution was formulated based on recent values for the guard-cell apoplast (K^+, 2.59 mM and Ca^{2+}, 0.064 mM; Felle *et al.*, 2000), the requirement for Ca^{2+} in CO_2-induced stomatal closure (Schwartz *et al.* 1998; Webb *et al.* 1996) and the activation of anion channels by an enhancement of cytosolic free Ca^{2+} (Felle *et al.*, 2000). Petri

dishes were then placed in the growth chamber as above. Measurement of stomatal aperture size, which required less than 2 min, was done by use of a light microscope after 20-min incubation. In initial experiments, the time course for stomatal aperture size changes in response to 40 mM extracellular malate (with 0.1 mM Ca^{2+}) was conducted. Stomatal closure appeared after a 20-min treatment and persisted for 60 min. Therefore, a 20-min incubation period was adopted here.

4.3 Guard-Cell Localization of Xylem-Source Malate in Excised Leaflets

Petioles were excised under water 3 h after the onset of illumination. Then, an individual petiole was immersed in a feeding solution (5 mM K_2HPO_4/KH_2PO_4 (pH 6.1) containing 1 mM $CaCl_2$, 0−40 mM malate) in a 6 mm (diameter) × 15 mm borosilicate tube. Solutions were quantitatively replenished at 15-min intervals. Artificial xylem sap was formulated on the recent reported values of ion concentration of xylem sap (K^+, ~ 22.5 mM and ~ 6.5 mM in abaxial leaf side and adaxial side, respectively, Mühling and Sattelmacher, 1997; the free Ca^{2+} in shoot-xylem sap, ~ 2.2 mM, Atkinson *et al*., 1992). A detailed explanation for the formulation of feeding solution was given by Zhang and Outlaw (2001b).

Transpiration rate was recorded at 15-min intervals over the feeding period. Samples of leaf apoplastic sap collected from one leaflet, of leaf and of epidermis were collected from another leaflet after 120 min as described below.

Samples of the leaf apoplast were collected using a pressure chamber (Model 1000, PMS Instrument Co., Corvallis, OR, USA). Specifically, 5-μL droplets were expressed sequentially from the same leaflet (~ 5 droplets per leaflet). These samples were stored at -80ºC before analysis.

A 0.5 cm × 1 cm sample was cut from one side of a leaflet and quick-frozen in liquid-N_2 slurry. An epidermal strip was peeled from the opposite side of the same leaflet; then, it was washed, brushed and floated on distilled water for 2 min at 25ºC to remove apoplastic content and quick-frozen in liquid-N_2 slurry. These tissue samples were freeze-dried at -35ºC, and stored at -20ºC in vacuum before malate analysis. Details are given in Zhang and Outlaw (2001a).

4.4 Histochemical Detection of Guard-Cell Malate and K^+ Contents

The malate content of xylem sap was measured enzymatically by coupling the oxidation of malate to the reduction of NAD essentially as described by Outlaw and Lowry (1977). The malate content of guard cells was measured by use of quantitative histochemical methods as described by Outlaw and Zhang (2001). In brief, guard cells were dissected from whole leaf or from epidermis and malate was extracted in sub-μL volumes under oil. Malate was oxidized enzymatically, with the resultant NADH being amplified by enzymatic cycling. The malate content of guard cells from rinsed epidermal peels is only the symplastic pool whereas the malate content of guard cells from whole leaf is both the apoplastic and symplastic pools. Therefore, the difference of these two values is the guard-cell apoplast malate content. Errors reported for the guard-cell apoplast malate contents were calculated by a means

[$SE_{Apo} = \sqrt{(SE_{Sym})^2 + (SE_{Whole})^2}$] that represents a maximum.

Guard-cell K^+ content was assessed semiquantitatively by staining with the hexanitrocobaltate reagent ($Na_3Co(NO_2)_6$) (Green *et al*. 1990). In brief, epidermal peels from leaves were brushed to remove mesophyll cells. Then, they were floated in ice-cold water for one minute to remove external K^+. After staining with hexanitrocobaltate reagent for 10 min, the peels were washed in ice-cold water for 1 min. The yellow precipitate were converted to black CoS for observation by a drop of 5 % $(NH_4)_2S$ solution. An arbitrary scale (0−5) imposed on a photographic reference was used to score guard-cell K^+ content.

4.5 Daily Time-Course of Stomatal Aperture Size and the Malate Content of the Guard-Cell Apoplast and Symplast

Stomatal aperture size was measured microscopically every two hours over the photoperiod. Guard cells from the same leaflets were analyzed for malate as described in the previous section.

4.6 Effect of Elevated CO_2 Concentration on the Malate Content of the Guard-Cell Apoplast of Intact Plants

Regulation of growth-chamber CO_2 concentration was achieved by manual control of inputs of CO_2-free air and 5 %-CO_2 air while monitoring the CO_2 concentration with an infra-red gas analyzer (Model 225 MK3, The Analytical Development Co., Hoddesdon, England).

Temperature and RH were kept at ~ 25ºC and ~ 60 %, respectively to eliminate the effect of temperature and RH change on stomatal response to CO_2. Samples of xylem sap, of leaf and of epidermis were collected from the same leaflet and assayed as described earlier.

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