



RESEARCH PAPER

Species-dependent changes in stomatal sensitivity to abscisic acid mediated by external pH

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Abstract

The direct effects of pH changes and/or abscisic acid (ABA) on stomatal aperture were examined in epidermal strips of *Commelina communis* L. and *Arabidopsis thaliana*. Stomata were initially opened at pH 7 or pH 5. The stomatal closure induced by changes in external pH and/or ABA (10 μ M or 10 nM) was monitored using video microscopy and quantified in terms of changes in stomatal area using image analysis software. Measurements of aperture area enabled stomatal responses and, in particular, small changes in stomatal area to be quantified reliably. Both plant species exhibited a biphasic closure response to ABA: an initial phase of rapid stomatal closure, followed by a second, more prolonged, phase during which stomata closure proceeded at a slower rate. Changes in stomatal sensitivity to ABA were also observed. Comparison of these effects between *C. communis* and *A. thaliana* demonstrate that this differential sensitivity of stomata to ABA is species-dependent, as well as being dependent on the pH of the extracellular environment.

Key words: ABA, aperture area, *Arabidopsis thaliana*, *Commelina communis*, pH, stomatal closure.

Introduction

Stomata are pores on the leaf surface that regulate the uptake of CO₂ for photosynthesis and the loss of water via transpiration. Changes in the size of the stomatal pore occur in response to a wide range of environmental stimuli through the activation of signalling pathways that result

in alterations in guard cell turgor (for reviews, see Blatt, 2000; Hetherington, 2001; Schroeder *et al.*, 2001; Fan *et al.*, 2004). The plant hormone abscisic acid (ABA) plays a key role in the regulation of transpiration during drought conditions. As the soil dries, ABA builds up in the leaves, leading to stomatal closure through the inhibition of stomatal opening and the promotion of stomatal closure, enabling the plant to conserve water (Raschke, 1987). Many components of the signalling pathway by which ABA stimulates a reduction in the stomatal pore size have been identified. There is evidence for both an extracellular and an intracellular site of ABA perception (Allan *et al.*, 1994; Anderson *et al.*, 1994; Schwartz *et al.*, 1994; MacRobbie, 1995; Wilkinson and Davies, 1997). However, the identity of the ABA receptor(s) remains to be confirmed (Hornberg and Weiler, 1984), although the spatial distribution of extracellular ABA-perception sites (Yamazaki *et al.*, 2003), which in *Vicia faba* appear to correspond to ABA-binding proteins in the epidermis of leaves (Zhang *et al.*, 2002), has been clearly demonstrated. In addition, a number of components acting downstream of ABA have also been identified, including changes in ion channel activity, the organization of the cytoskeleton, membrane trafficking, protein kinases and phosphatases, organic acid/sugar metabolism, and cytosolic free Ca²⁺ (Blatt, 2000; Hetherington, 2001; Schroeder *et al.*, 2001; Fan *et al.*, 2004).

Apoplasmic pH is known to exert a marked effect on stomata (Wilkinson, 1999). The pH of the xylem sap has been shown to increase markedly in response to drought stress in a number of species: from 6.1 to 6.7 in *Commelina communis* (Wilkinson and Davies, 1997), from 6.3 to 7.2 in *Phaseolus vulgaris* (Hartung and Radin, 1989), and

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from between 5.8 and 6.6 to 7.1 in sunflower plants (Gollan *et al.*, 1992). A similar increase in guard cell apoplastic pH, from 6.3 to 7.1, has been reported in isolated epidermis of *C. communis* during dark-induced stomatal closure (Edwards *et al.*, 1988), whilst direct measurements of the apoplastic pH of the substomatal cavity have revealed values ranging from 6.3 in leaf sections of *Tradescantia virginiana* (Bowling and Edwards, 1984) to 4.7–5.2 in intact leaves of *Vicia faba* (Felle *et al.*, 2000; Felle and Hanstein, 2002), with only a transient small increase in pH of 0.4–0.5 pH units during dark- or ABA-induced stomatal closure (Bowling and Edwards, 1984; Felle *et al.*, 2000; Felle and Hanstein, 2002). The existence of a pH difference between the xylem sap and apoplast of the substomatal cavity highlights the dynamic pH barrier that exists as a result of active pH regulation, and clearly indicates that stomatal closure is associated with H⁺-ATPase activity. Results of Hartung and Radin (1989) demonstrated that an increase in pH of dehydrated leaves, determined by the change in H⁺-ATPase activity, may have an effect on the redistribution and compartmentation of ABA. ABA is a weak acid and will preferentially accumulate in the more alkaline compartments of the leaf; at an apoplastic pH of 5.2–6.5 more ABA will be present in its undissociated form (ABAH), which readily diffuses across the plasma membrane into the more alkaline cytoplasm where it dissociates into ABA⁻, which becomes trapped inside cells (Heilmann *et al.*, 1980; Kaiser and Hartung, 1981). Therefore, the tight regulation in this manner has the potential to affect the distribution of ABA between different leaf compartments, influencing the local concentration of ABA available to the guard cell receptors and, hence, ABA-induced stomatal closure. Furthermore, electrophysiological studies indicate that pH might also modulate the activity of K⁺ and anion channels that form part of the cohort of plasma membrane ion channels by which ABA regulates the ion content of guard cells and hence stomatal aperture (Blatt and Grabov, 1997). For example, Roelfsema and Prins (1998) have shown that apoplastic acidification increases the efflux of K⁺ in depolarized cells and decreases the influx in hyperpolarized cells. This provides an additional route by which pH may influence stomatal responses to ABA.

Isolated epidermis is routinely used to investigate the molecular machinery by which guard cells perceive and respond to external stimuli (McAinsh *et al.*, 1990; Peiter *et al.*, 2005), and studies using this experimental system continue to provide the basis for much of the current understanding of the individual components involved in guard cell signalling and how they are integrated into the signalling networks, by which stomata in intact plants respond to environmental stimuli (Hetherington and Woodward, 2003). Therefore, in this study, isolated epidermis of the two model species, *C. communis* and *Arabidopsis thaliana*, in which the external environment can be tightly and reproducibly

controlled, have been used as a tool to investigate whether differences in the mechanism by which stomata respond to ABA at the cellular level, including differences in the site(s) of ABA perception, might account, at least in part, for any species differences in stomatal responses to ABA observed *in planta*. In order to address this question, the direct effect of external pH on stomatal aperture and the interaction between external pH and ABA during stomatal closure were examined. Reports of the steady-state xylem sap and substomatal cavity apoplastic pH range from 5.8 to 7.2 and 4.7 to 6.3, respectively, depending on the species studied and techniques used (Bowling and Edwards, 1984; Edwards *et al.*, 1988; Hartung and Radin, 1989; Gollan *et al.*, 1992; Wilkinson and Davies, 1997; Felle *et al.*, 2000; Felle and Hanstein, 2002). Consequently, pH 5 and pH 7, representing the extremes of both ranges, were selected to test whether stomata exhibit a differential response between species to ABA under conditions of high and low pH, reflecting differences in the properties of the systems by which ABA is perceived at the cellular level, and to question whether this might help to explain any differences in stomatal responses observed under the more controlled pH conditions reported to exist in the apoplast of intact plants (Felle *et al.*, 2000; Felle and Hanstein, 2002). It is shown that both species exhibited a biphasic closure response to ABA: an initial phase of rapid stomatal closure followed by a second more prolonged phase during which stomatal closure proceeds at a slower rate. In addition, the sensitivity of stomata to ABA was both species-dependent and dependent on the pH of the extracellular environment. The data are discussed in the context of differences in the properties of ABA-binding sites between the two species.

Materials and methods

Plant material

Seeds of *C. communis* and *A. thaliana* (ecotype Landsberg *erecta*) were grown from seed (McAinsh *et al.*, 1991; Webb and Hetherington, 1997) and watered daily. Plants were grown in a controlled environment chamber under a 10 h light and 14 h dark cycle, with photon flux density of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and at a temperature of 22 °C.

Bioassay

Epidermal strips were isolated from the third, fully expanded leaf of 4–6-week-old *C. communis* plants and from 4–6-week-old *A. thaliana* plants. Abaxial epidermis was peeled from the leaves and floated on distilled water for the period between peeling and incubation. Strips were then transferred to 10 ml of 5 mM MES/TRIS buffer solutions at either pH 7 or pH 5, containing 50 mM KCl, and incubated under conditions promoting stomatal opening for 3 h (photon flux density of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$, CO₂-free air, 25 °C (for *C. communis*) or 22 °C (for *A. thaliana*) (McAinsh *et al.*, 1991; Webb and Hetherington, 1997). After the stomata reached their steady-state aperture value, the epidermal strips were transferred to a microscope slide and a series of digital images captured using a Nikon Eclipse TE300 microscope (Nikon, UK) fitted with a JVC colour CCD camera (JVC, Japan) imaging accessory and using

Ozaria 2.5 signal analysis software. Images were stored on the hard disk of the host PC for later analysis of stomatal aperture. Epidermal strips were subsequently transferred to fresh buffer at either the same pH as the opening buffer used or at an altered pH (i.e. strips initially incubated at pH 7 transferred to pH 5 and vice versa) in either the presence or absence of ABA (10 μM or 10 nM ABA). Time-course experiments were performed by incubating epidermal strips for 2 h under the appropriate conditions and capturing images of stomata every 20 min for later analysis of stomatal aperture.

Data analysis

The effects of external pH and/or ABA on stomata were determined in terms of changes in stomatal aperture area. This is directly proportional to stomatal aperture width and compensates for any non-synchronous changes in stomatal aperture width and length that may occur during time-course experiments. Stomatal aperture area measurements in Fig. 1 show a high linear dependence between pore area and width. Therefore, the aperture area provides a more reliable measurement of stomatal responses than aperture width. This was especially important when determining the responses of smaller stomata such as those of *A. thaliana*. For each treatment, five aperture areas were measured from each of four different epidermal strips. Unless otherwise stated, data are presented as the mean \pm standard error of the mean of 20 stomatal areas.

Results

Effect of external pH on stomatal aperture

External pH had a marked effect on stomatal opening, which was species-dependent (Fig. 2). The aperture area of *C. communis* was consistently greater than that of *A. thaliana* and was highly pH-dependent. After 3 h incubation under conditions promoting stomatal opening the aperture area of *C. communis* was 126.5 μm^2 at pH 7 and

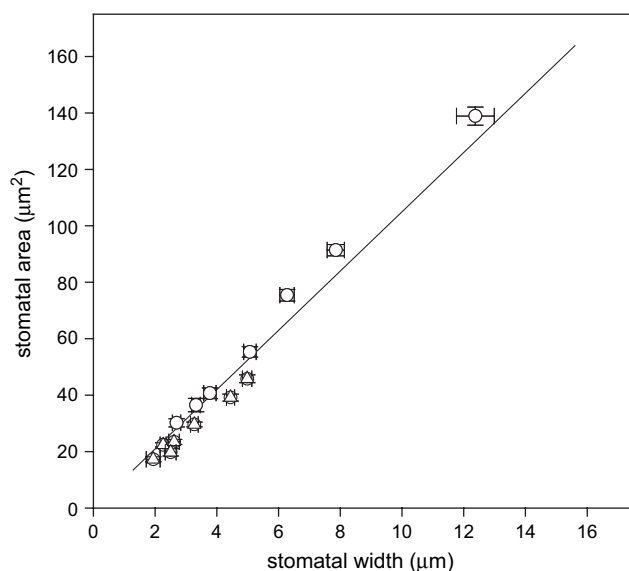


Fig. 1. The relationship between stomatal aperture area and width in *Commelina communis* (open circles) and *Arabidopsis thaliana* (open triangles). Values are the means of 20 separate measurements of aperture area \pm standard error of the mean.

92.8 μm^2 at pH 5, representing a 27% difference. This is consistent with the observations of Schwartz *et al.* (1994). By contrast, there was no significant difference ($P > 0.05$) in the aperture area of *A. thaliana* at pH 7 and pH 5 with values of 38.1 μm^2 and 38.6 μm^2 , respectively, representing a difference of only 1%, suggesting that in this species stomatal opening exhibits little sensitivity to external pH under these conditions. Schwartz *et al.* (1994) also found stomatal opening in *Vicia faba* to have a low sensitivity to external pH.

Stomatal closure was also markedly affected by changes in external pH in a species-dependent manner (Fig. 3). In *C. communis* (Fig. 3A), as expected from the data presented in Fig. 2 for acidification, i.e. transfer of epidermal strips from pH 7 to 5, resulted in a further decrease in aperture area from 136 μm^2 to 56.7 μm^2 . Interestingly, although the steady-state aperture area was greater at pH 7 than pH 5 (Fig. 2), alkalization, i.e. transfer of epidermal strips from pH 5 to pH 7, also resulted in a further decrease in aperture area from 88.1 μm^2 to 60.9 μm^2 rather than reopening of stomata as might be predicted. These data suggest that opening stomata of *C. communis* at pH 5 overrides the subsequent effect of transfer to a more alkaline pH, i.e. stomata are able to 'remember' opening at an external pH of 5. Stomatal closure was significantly ($P > 0.05$) greater in response to acidification compared with alkalization and, when expressed as a percentage of the initial aperture area of the open stomata, these changes represent a 58%

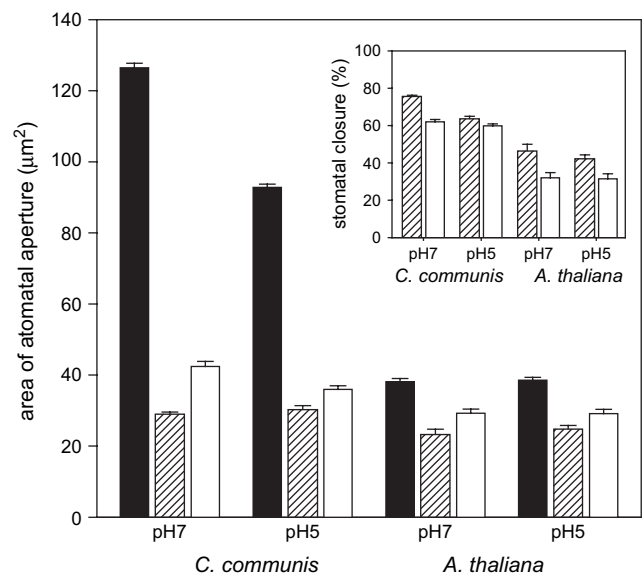


Fig. 2. Steady-state aperture areas of stomata in isolated epidermis of *Commelina communis* and *Arabidopsis thaliana* incubated at pH 7 or pH 5 under conditions promoting stomatal opening for 3 h (solid bars) and then incubated for a further 2 h in the presence of either 10 μM ABA (hatched bars) or 10 nM ABA (open bars). Inset: stomatal closure in response to either 10 μM ABA (hatched bars) or 10 nM ABA (open bars) expressed as the percentage change in aperture area relative to the initial aperture area. Values are the means of 150 separate measurements of aperture area \pm standard error of the mean.

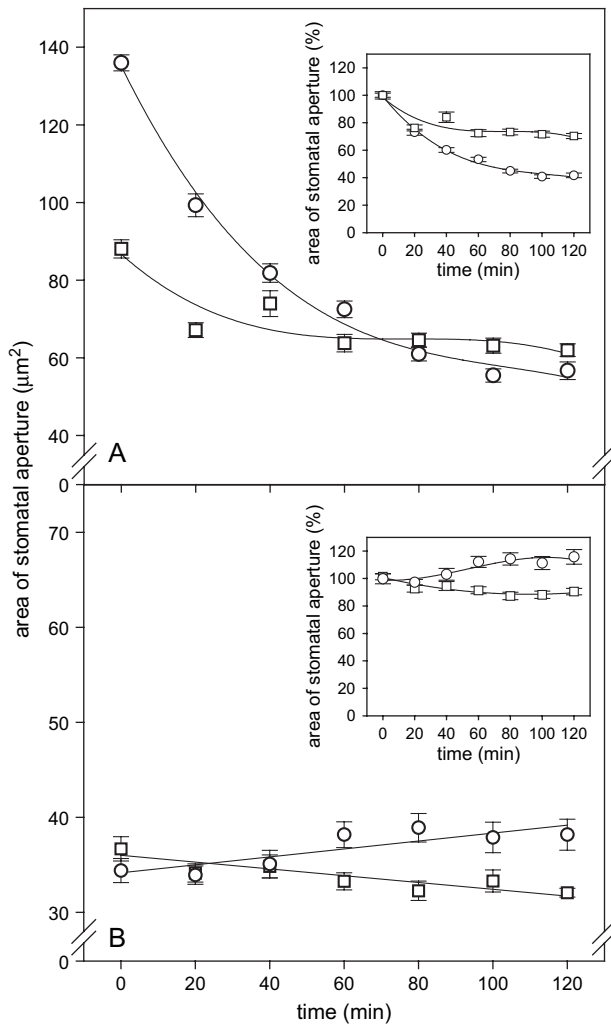


Fig. 3. The effect of external pH changes on stomatal aperture area in isolated epidermis of (A) *Commelina communis* or (B) *Arabidopsis thaliana*. Epidermal strips were incubated at pH 7 (circles) or pH 5 (squares) under conditions promoting stomatal opening for 3 h and then transferred to fresh buffer at pH 5 or pH 7, respectively, for a further 2 h. Insets: aperture area expressed as the percentage change relative to the initial aperture area: circles, opened at pH 7 and transferred to pH 5; squares, opened at pH 5 and transferred to pH 7. Values are the means of 20 separate measurements of aperture area \pm standard error of the mean.

and 31% decrease in aperture area, respectively (Fig. 3A, inset). In addition, there were marked differences in the temporal dynamics of acidification- and alkalization-induced stomatal closure. The aperture area decreased by $\sim 27\%$ during the first 20 min of acidification, followed by a gradual decrease up to 120 min, whilst alkalization resulted in steady-state aperture values, representing a decrease of $\sim 24\%$ in aperture area, within 20–40 min. By contrast, *A. thaliana* appeared to be less sensitive to external pH compared with *C. communis* under these conditions and exhibited a markedly different pattern of responses (Fig. 3B). Surprisingly, a decrease in external pH from 7 to 5 (acidification) resulted in a 15% increase ($5.0 \mu\text{m}^2$) in aperture area, whereas an increase in external pH from 5

to 7 (alkalization) resulted in a 13% decrease ($4.8 \mu\text{m}^2$) in aperture area. There were no marked differences in the temporal dynamics of acidification-induced stomatal opening and alkalization-induced stomatal closure, both occurring at a steady rate during the 60–120 min period (Fig. 3B, inset).

The interaction of ABA and external pH

External pH had a marked effect on ABA-induced stomatal closure, which was species-dependent. The stomata of *C. communis* were more sensitive to both 10 μM and 10 nM ABA at pH 7 compared with pH 5 (Fig. 2). Although the steady-state aperture areas were similar at pH 7 and pH 5, when the percentage closure was calculated, 10 μM ABA resulted in 76% and 63% closure, respectively, whilst there was no significant difference ($P > 0.01$) between the closures caused by 10 nM ABA (62% and 60%, respectively). This agrees with the results of Thompson *et al.* (1997). By contrast, the stomata of *A. thaliana* were less sensitive to ABA than stomata of *C. communis* at both pH 7 and pH 5 and there was no significant difference ($P > 0.05$) between the percentage closure caused by either 10 μM ABA (46% and 40%, respectively) or 10 nM ABA (32% and 30%, respectively) at the two pH values (Fig. 2, inset).

There were marked differences in the temporal dynamics of ABA-induced stomatal closure observed in response to ABA in the absence of an alkalization or acidification step and that observed in response to ABA accompanied by either alkalization (from pH 5 to pH 7) or acidification (from pH 7 to pH 5) of the external pH. In *C. communis*, the addition of ABA to stomata opened at pH 7 without a change in pH (i.e. epidermal strips remaining at the same external pH throughout) resulted in a steady rate of stomatal closure (Fig. 4A, open symbols). There was little difference in the degree of closure observed in response to 10 μM and 10 nM ABA during the first 20 min, both of which resulted in a decrease in aperture area of 29%, although after 120 min the aperture area observed in response to 10 μM was 14% smaller than with 10 nM ABA. However, when the addition of ABA was accompanied by alkalization (i.e. an increase in the external pH from 5 to 7) there was a marked difference in both the kinetics and degree of stomatal closure observed. ABA-induced stomatal closure was biphasic and highly dose-dependent (Fig. 4A, closed symbols). As previously, there was no significant difference ($P > 0.01$) in the closure induced by 10 μM and 10 nM ABA during the first 20 min, whilst the rate and magnitude of closure subsequent to this was 21% greater for 10 μM ABA after 120 min. Interestingly, although the percentage stomatal closure observed in response to ABA was greater at pH 7 than pH 5 (Fig. 2, inset) the stomata of *C. communis* did not close to the same extent in response to either 10 μM or 10 nM ABA following alkalization of the external pH from 5 to 7 compared with those maintained at pH 7 throughout (Fig. 4A). These data support the earlier

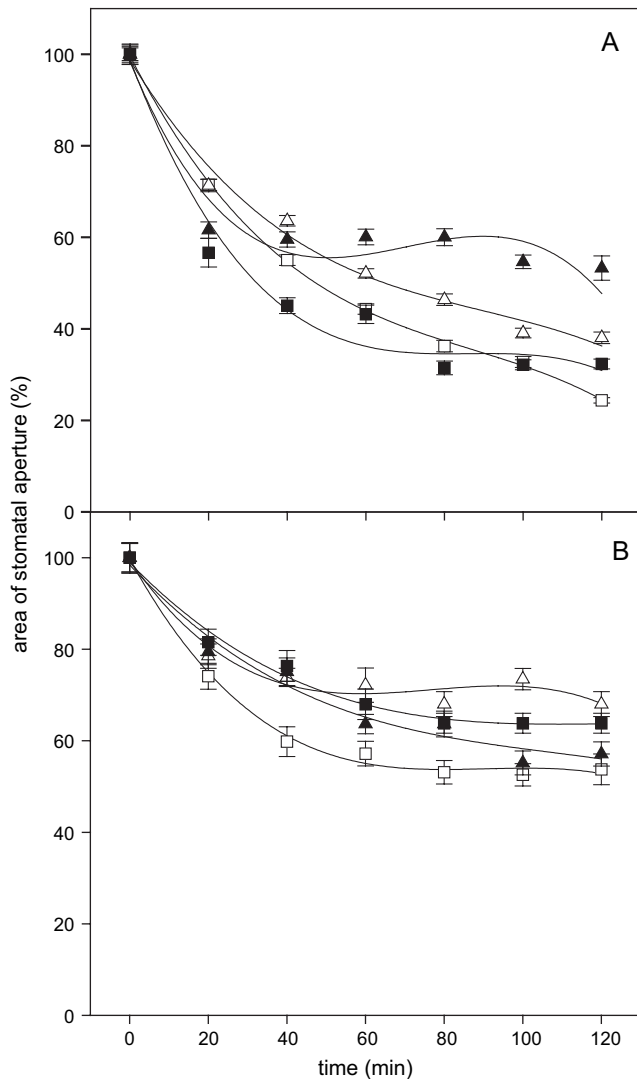


Fig. 4. The effect of external pH changes on the time-course of ABA-induced stomatal closure in isolated epidermis of (A) *Commelina communis* or (B) *Arabidopsis thaliana*. Epidermal strips were incubated at pH 7 (open symbols) or pH 5 (closed symbols) under conditions promoting stomatal opening for 3 h and then transferred to fresh buffer at pH 7 containing either 10 μ M ABA (squares) or 10 nM ABA (triangles) for a further 2 h. Values are the means of 20 separate measurements of aperture area and are expressed as the percentage change relative to the initial value \pm standard error of the mean.

suggestion that, in *C. communis*, stomata are able to ‘remember’ the external pH conditions under which they were opened in as much as opening at pH of 5 appears to ‘prime’ the guard cells to be less sensitive to ABA, and that the subsequent transfer to pH 7 cannot totally override this effect. By contrast, in *A. thaliana*, although stomata closed in response to ABA at pH 7, both in the absence of a shift in external pH and following alkalization from pH 5 to 7, an ABA dose-response was only observed in epidermal strips that had remained at the same external pH (pH 7) throughout (Fig. 4B, open symbols). Under these conditions, stomatal closure was biphasic; there was no

significant difference ($P > 0.05$) in ABA-induced stomatal closure during the first 20 min, whereas by 120 min 10 μ M ABA had induced a 17% greater reduction in aperture area than 10 nM ABA. The absence of an ABA dose-response in *A. thaliana* following alkalization of the external pH from 5 to 7 may provide further evidence for the desensitization of guard cells to ABA as a result of opening at pH 5.

ABA resulted in the closure of stomata in epidermal strips of *C. communis* that had been opened at pH 5 without a change of pH (i.e. epidermal strips remaining at the same external pH throughout) and in those that were subjected to acidification from pH 7 to 5, although in both cases stomata failed to exhibit an ABA dose-response so that there was no significant difference ($P > 0.05$) between the stomatal closure observed in response to 10 μ M and 10 nM ABA under either set of conditions (Fig. 5A). This is consistent with the steady-state ABA-induced stomatal closure observed at pH 5 (Fig. 2, inset). However, at an external pH of 5 throughout, ABA induced a steady rate of stomatal closure, resulting in an approximately 63% (10 μ M) and 60% (10 nM) reduction in aperture area after 120 min, whereas, following acidification from pH 7 to 5, stomatal closure was biphasic, resulting in a 63% reduction during the first 60 min relative to the initial area, after which little further stomatal closure was observed. These data suggest that, in this species, stomata respond more rapidly and are therefore more sensitive to ABA at pH 5 when opened at pH 7, although it is also evident that this shift in sensitivity has little effect on the final degree of closure observed under either set of conditions which show no significant difference ($P > 0.05$). This confirms the suggestion that the pH at which stomata of *C. communis* are opened exerts a marked influence over the sensitivity of the guard cells of this species to ABA priming. They are either less (when opened at pH 5; Fig. 4A) or more (when opened at pH 7; Fig. 5A) sensitive to ABA following alkalization or acidification, respectively, of the external pH. By contrast, *A. thaliana* stomata that had been opened at pH 5 exhibited a dose-response to ABA in the absence of a shift in external pH and following acidification from pH 7 to pH 5. Stomatal closure in epidermal peels maintained at pH 5 throughout was biphasic. The degree of stomatal closure observed in response to both 10 μ M ABA and 10 nM ABA was similar up to 40 min into the treatment (Fig. 5B, open symbols), although by 120 min the stomatal closure induced by 10 μ M ABA was significantly greater ($P > 0.01$) than that observed in response to 10 nM ABA, resulting in a 42% and 32%, respectively, reduction in aperture area. This is consistent with the steady-state ABA-induced stomatal closure observed in *A. thaliana* at pH 5 (Fig. 2, inset). The differential response of stomata to 10 μ M ABA and 10 nM ABA was detectable within 40 min, following acidification of the incubation buffer from pH 7 to pH 5, resulting in a reduction in aperture area which did not differ significantly ($P > 0.01$) from that observed in the absence

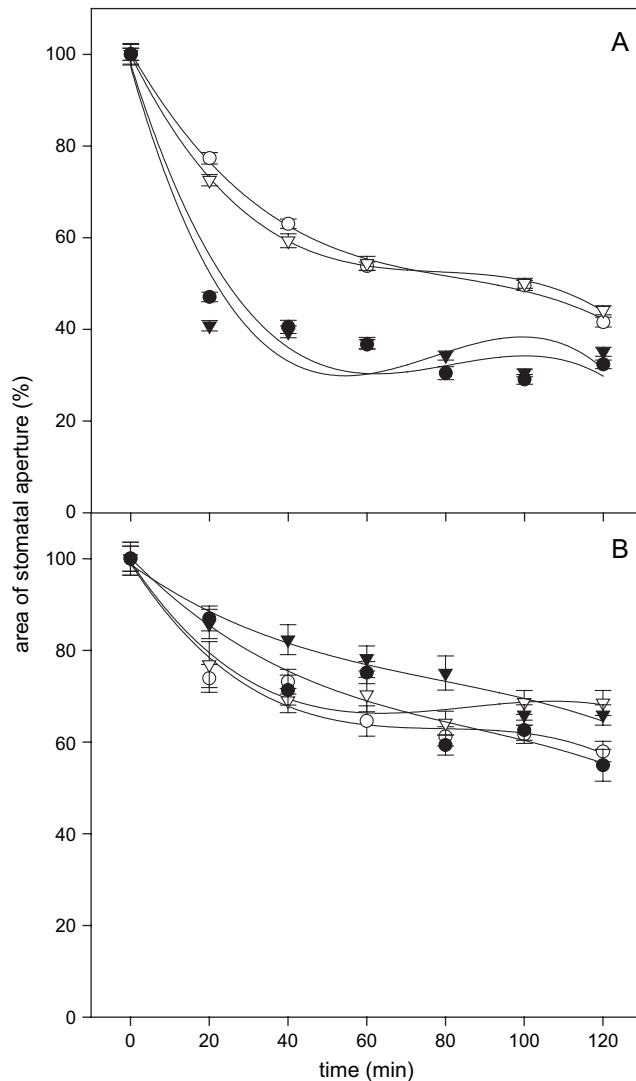


Fig. 5. The effect of external pH changes on the time-course of ABA-induced stomatal closure in isolated epidermis of (A) *Commelina communis* or (B) *Arabidopsis thaliana*. Epidermal strips were incubated at pH 5 (open symbols) or pH 7 (closed symbols) under conditions promoting stomatal opening for 3 h and then transferred to fresh buffer at pH 5 containing either 10 μ M ABA (circles) or 10 nM ABA (triangles) for a further 2 h. Values are the means of 20 separate measurements of aperture area and are expressed as the percentage change relative to the initial value \pm standard error of the mean.

of acidification after 120 min, being 55% and 42%, respectively (Fig. 5B, closed symbols). Taken together, these results clearly illustrate the differential sensitivity of stomata of *C. communis* and *A. thaliana* to ABA, and a marked influence of external pH on the response of guard cells to ABA.

Discussion

The present data show that the extracellular pH exerts a marked influence on stomatal responses in isolated

epidermis of *C. communis* and *A. thaliana* and they highlight the differential response of stomata of these two species to the pH of the extracellular environment. Stomatal opening in *C. communis* was, on average, 27% greater at pH 7 compared with at pH 5 (Fig. 2). These data agree with those of Wilkinson and Davies (1997) who pointed out that an alkaline external environment may act as a signal for stomatal opening in isolated epidermis of *C. communis*. By contrast, in *A. thaliana*, stomatal opening exhibited little sensitivity to external pH and there was little difference (1%) in stomatal opening at pH 7 and pH 5 (Fig. 2). The mechanistic basis for the difference in the response of stomata of *C. communis* and *A. thaliana* to external pH remains to be determined, although it has been suggested that the concentration of external cations may influence the sensitivity of stomata to external stimuli (Wilkinson and Davies, 1997). This raises the possibility that a differential sensitivity to the concentration of external potassium ions, the major cationic species in the perfusion buffer used in the current study, may be responsible, at least in part, for the differential response of *C. communis* and *A. thaliana* to external pH observed in this study. However, published data indicate that this is unlikely to be the case. Thompson *et al.* (1997) have reported that the stomatal aperture of *C. communis* varies with external pH in the presence of 50 mM KCl, the concentration used in the present study. Similarly, Roelfsema and Prins (1995) have shown that the stomata of *A. thaliana* respond maximally in the presence of 50 mM KCl, and studies of the guard cell inwardly rectifying potassium channel from *A. thaliana*, KST1, which is responsible for potassium uptake into guard cells during stomatal movements, have shown that this is activated at both pH 7 and pH 5 in the presence of 30–100 mM external potassium (Hoth and Hedrich, 1999; Hoth *et al.*, 2001). Consequently, an external potassium concentration of 50 mM is routinely used to determine stomatal responses in *A. thaliana* (Webb and Hetherington, 1997; Peiter *et al.*, 2005). Interestingly, comparison of the stomatal opening between the two species in the present study shows that the total aperture area of *A. thaliana* stomata represents only 30% of the total area measured in *C. communis* at pH 7 and 41% at pH 5. The higher percentage at pH 5 compared with pH 7 is due to a reduction in aperture area in *C. communis* at this pH relative to *A. thaliana*, in which the aperture area remains virtually unchanged. Factors that may help to compensate for this reduced dynamic range may include an increase in the number of stomata per unit area in *A. thaliana* (data not shown) and changes in the number of stomata reaching a given steady-state opening under different conditions.

It is possible that changes in external pH, both increases in alkalinity and acidity act as signals that induce alterations in guard cell turgor and, hence, stomatal aperture area, as a result of differences in the membrane potential of guard cells as the plasma membrane becomes more or less

hyperpolarized through changes in the extracellular pH. The effect of pH on the plasma membrane potential of plant cells has been clearly demonstrated (Miedema *et al.*, 1992). This in turn would affect the activity of plasma membrane ion channels and membrane-bound proteins. It is known that the activation of H⁺-ATPase, which in *Zea mays* has been shown to exhibit maximum activity at pH 6.5, with a pronounced increase of activity between pH 7.5 and 6.5 (Hager and Biber, 1984), would change the hyperpolarization of the guard cell plasma membrane that would lead to a reduction in K⁺ influx (Schwarz *et al.*, 1991), limiting increases in guard cell turgor and, therefore, preventing stomatal opening whilst enabling stomatal closure to occur. Other studies clearly demonstrate the potential differential impact of external pH on the intracellular machinery responsible for the regulation of guard cell turgor and stomatal aperture in different species. As long ago as 1976, Raghavendra and colleagues identified two H⁺-ATPase isoforms in epidermal tissue that were active during diurnal rhythms of stomata of *Commelina benghalensis*, one having an optimum pH of 7.5 associated with stomatal opening and the other with an optimal pH of 5.5 associated with stomatal closure (Raghavendra *et al.*, 1976). More recently there have been a number of reports that pH changes lead to alterations in the activity of ion channels that participate in the processes of stomatal closing and opening. These changes in activity result from both a change in the number of channels that are active, together with changes in their activation and deactivation times. For example, in *A. thaliana* acidification of the extracellular environment has been shown to result in a shortening of the activation time of slow outwardly rectifying channels followed by a decrease in their K⁺ selectivity (Ilan *et al.*, 1994; Roelfsema, 1997). Conversely, a decrease in the activity of the same channels following intracellular acidification has been reported for *Vicia faba* (Blatt and Armstrong, 1993) whilst, in the same plant species, alkalization is accompanied by a decrease in the activation and deactivation times for inwardly rectifying channels (Blatt, 1992).

ABA caused stomatal closure in high (pH 7) and low (pH 5) pH environments in both *Commelina* and *A. thaliana*, although stomata were more sensitive to ABA at pH 7 (Fig. 2). These data are consistent with the results of Paterson *et al.* (1988) who showed that stomata of *C. communis* were less sensitive to ABA *per se* at pH 5.5 compared with pH 6.8. They also agree with data obtained using the tomato *flacca* mutant which exhibits a reduced capacity to synthesize ABA but which retains the ability to respond to exogenous ABA. Measurements of the transpiration of these mutants showed the greatest decrease in response to ABA at pH 7.75 (Wilkinson *et al.*, 1998). In addition, Cousson and Vavasseur (1998) have shown that acidification with different concentrations of ABA in the bathing solutions induced a loss of sensitivity to calmodulin

antagonist during ABA-induced stomatal closure, implying a down-regulation of Ca²⁺-dependent signalling during the ABA response. These findings may help to explain the present results, whereby there is a greater sensitivity of the ABA-binding sites in *C. communis* under high pH (pH 7) conditions.

The kinetics of stomatal closure in response to ABA differed markedly between *C. communis* and *A. thaliana*. In the absence of a change in external pH, ABA-induced stomatal closure was monophasic in *C. communis* (Figs 4A, 5A) and biphasic in *A. thaliana* (Figs 4B, 5B), whereas the opposite trend was observed when the ABA treatment was accompanied by a change in the acidity of the extracellular environment. These data indicate that a number of factors are important when formulating the degree of stomatal closure achieved at any given time in response to ABA, including plant species, direction of pH change (i.e. acidification or alkalization), ABA concentration, and the time for the ABA response.

Several studies provide clues to the mechanisms underlying the effect of changes in external pH on the sensitivity of stomata to ABA. Hornberg and Weiler (1984) have identified high affinity ABA binding sites on the plasma membrane of *Vicia faba* guard cells, although the identity of this ABA receptor remains to be confirmed. They report that there is probably a single binding site for ABA⁻ and two for ABAH. The affinity of binding of the dissociated form decreases with acidification, while undissociated sites behaved in the opposite fashion. Studies using the *era1-2* mutant of *A. thaliana* have also shown a change in sensitivity to ABA under drought conditions (Pei *et al.*, 1998), which would be expected to result in alkalization of the apoplast (Wilkinson and Davies, 1997), although in these mutants hypersensitivity to ABA was expressed only within a certain range of concentrations (Allen *et al.*, 2002). Similarly, Peng and Weyers (1994) have demonstrated that medium- and long-term water-deficit stress can affect the sensitivity of *C. communis* stomata to ABA. Interestingly, Roelfsema *et al.* (2004) have shown that the rapid (R)-type anion channels which are activated during stomatal closure caused by ABA are most likely to be responsible for the fast responses to ABA, suggesting that variation in the sensitivity of stomata to ABA may be more pronounced during short ABA treatments. However, a reduction in the activity of slow (S)-type channels has also been observed in the AAPK mutant of *A. thaliana*, leading to inhibition of ABA-induced stomatal closure (Li *et al.*, 2000). Increases in guard cell cytosolic Ca²⁺ (McAinsh *et al.*, 1990; Gilroy *et al.*, 1991), including oscillations (Staxén *et al.*, 1999; Allen *et al.*, 2001), are an important component of the signalling pathway by which guard cells respond to ABA, and these may encode signalling information about the nature of a stimulus in the form of a stimulus-specific Ca²⁺-signature (Hetherington and McAinsh, 1998; Allen *et al.*, 2001). Therefore, it is possible that the differential response

of *C. communis* and *A. thaliana* to ABA, and the effects of external pH observed here, may result from differences in the ABA Ca^{2+} -signature. In addition, Allan *et al.* (1994) have suggested that ABA could affect stomata through a Ca^{2+} -independent signalling pathway. Shimazaki *et al.* (1992) and Li and Assmann (1996) have identified an ABA-activated and Ca^{2+} -independent protein kinase (AAPK), and have suggested that ABA could regulate the activity of the plasma membrane H^+ -ATPase through phosphorylation mediated by AAPK. Consequently, it is possible that the differential involvement of these Ca^{2+} -independent signalling pathways may also contribute to the ABA response of stomata at different external pH values and in different species.

To conclude, the present data demonstrate that stomata in isolated epidermis of *C. communis* and *A. thaliana* exhibit a differential response to external pH, which has a profound influence on ABA-induced stomatal closure, and provide an insight into the mechanistic basis for this difference. The pH at which stomata are opened exerts a marked influence over the sensitivity of the guard cells to ABA, priming them to be either more or less sensitive to ABA following alkalization or acidification of the external pH, suggesting that stomata are able to 'remember' the pH environment in which they were opened. It must be noted that the changes in apoplastic pH reported during ABA-induced stomatal closure (Felle *et al.*, 2000; Felle and Hanstein, 2002) may not be as large as the changes in external pH performed in the present study, and stomatal responses may therefore differ in the intact leaf. Nevertheless, these results clearly show that, at the cellular level, there are both species-dependent and external pH-dependent changes in stomatal sensitivity to ABA, which may reflect differences in the properties of the ABA-binding sites between species and/or differences in the uptake of ABA, and which may provide a possible mechanistic basis for differences between species in stomatal responses to ABA in intact plants.

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