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ABA Contents in the Guard-Cell Symplast and Guard-Cell Apoplast Are Not Correlated with Stomatal Aperture Size under Three Conditions of Water Sufficiency

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ABA CONTENTS IN THE GUARD-CELL SYMPLAST AND GUARD-CELL APOPLAST ARE NOT CORRELATED WITH STOMATAL APERTURE SIZE UNDER THREE CONDITIONS OF WATER SUFFICIENCY

By

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LIST OF ABBREVIATIONS

AAO3	Arabidopsis Aldehyde Oxidase 3
ABA	Abscisic Acid
AtSTP1	Arabidopsis Sugar Transporter 1
CytP450	Cytochrome P450
ELISA	Enzyme-linked ImmunoSorbent Assay
OST1	Open Stomatal 1
PAR	Photosynthetically Active Radiation
RH	Relative Humidity
SRK2E	SNF1-related Kinase 2E
VPD	Vapor Pressure Difference

ABSTRACT

Guard cells respond to environmental stimuli by opening and closing stomata, which balance CO_2 uptake and water conservation. Stomatal closure under water deficiency and the involvement of abscisic acid (ABA) in this response are well-known. However, whether ABA plays a role in stomatal regulation under some water-sufficient conditions, such as diurnal changes, humidity shift (i.e. transpiration-rate change) and brief flooding, is not clear. Using an enzyme-linked immunosorbent assay (ELISA) with sub-femtomole sensitivity for ABA assays, we studied the relationships of stomatal aperture size with the ABA contents in the symplast and apoplast of guard cells, as well as those in the leaf and the leaf apoplast in *Vicia faba* under the following three conditions. (1) Diurnal changes. Stomata opened in the morning, reached a maximum opening at 1400 h, and closed at 1800 h. Neither the leaf nor the leaf-apoplastic ABA content strongly correlated with stomatal aperture sizes. The ABA contents of the guard-cell compartments did not change over the course of the day, providing evidence that ABA is not involved in diurnal stomatal regulation. (2) Humidity-induced transpiration-rate changes. The transpiration rate of intact plants and that of detached leaves infused with 1μM ABA was decreased by shifting RH from 60% to 90%. However, the ABA contents of the four compartments were not changed by this humidity shift, in spite of an increase of 2–3 μm in stomatal aperture sizes. Thus, the guard-cell-apoplastic ABA content is not affected by transpiration rate, and ABA may not participate in the stomatal response to transpiration rate. (3) Brief flooding. Stomata closed after brief (4-h) flooding, when the leaf and the leaf apoplastic ABA increased 2–3 fold and the xylem pH increased 0.2 pH units. The leaf ABA increase did not correlate strongly with stomatal aperture size and the xylem ABA delivery rate remained unchanged. The ABA contents in the guard-cell compartments of flooded plants were not different from those of non-flooded plants. Therefore, ABA may not be an initiator of stomatal closure under brief flooding, and xylem alkalinization probably does not induce leaf ABA redistribution to guard cells.

INTRODUCTION

Stomata are surrounded by pairs of guard cells in the leaf surface and they form a major gas exchange pathway. The opening of stomata allows CO_2 uptake for photosynthesis and water is simultaneously lost through transpiration. Water is the limiting resource for terrestrial plants, thus plants must regulate stomatal aperture size in response to different signals to compromise between photosynthesis and water conservation.

Stomatal aperture size is regulated by a balance between guard cell turgor and epidermal turgor (Buckley & Mott 2002). Changing guard cell turgor is the most direct way for plants to control stomatal aperture, which is achieved by regulating ion channels and transporters on the guard-cell plasma membrane (reviewed in Outlaw 2003; Roelfsema & Hedrich 2005). In brief, when guard cells accumulate K^+ salts and sucrose, the subsequent osmotic water influx increases guard-cell turgor, causing guard-cell swelling and stomatal opening. When osmotica are dissipated from guard cells, the subsequent water efflux decreases guard-cell turgor, resulting in decreased stomatal aperture size.

Stomata respond to a variety of environmental and endogenous signals (Schroeder *et al.* 2001; Dodd 2003; Roelfsema & Hedrich 2005), and water deficiency is one of the most widely-studied signals. ABA participation in this stomatal response is well known (Davies & Zhang 1991; Sauter, Davies & Hartung 2001; Davies, Kudoyarova & Hartung 2005) and the guard-cell ABA signaling transduction is extensively documented (Luan 2002; Fan, Zhao & Assmann 2004; Pei & Kuchitsu 2005). However, how stomata respond to some water-sufficient conditions, such as humidity, diurnal changes, or brief flooding, is not well-known and whether ABA plays a role is not clear. Therefore, this dissertation focused on revealing the mechanisms underlying the stomatal responses to

these three water-sufficient conditions by examining the roles of ABA, using a model species in stomatal studies, *Vicia faba*.

The current understanding of whether ABA is involved in the stomatal response to a signal mainly derives from studies on ABA measurements at tissue level or in apoplastic sap (Jackson 1993; Wilkinson & Davies 2002), or from studies with ABA mutants. Since such ABA measurements are not close enough to guard cells and mutant behaviors are often complicated to interpret (Zeiger *et al.* 2002), these studies do not always provide consistent results (see below for more details). Therefore, it is critical to determine the ABA contents in ABA action sites, i.e. the symplast and apoplast of guard cells (reviewed in Pei & Kuchitsu 2005; Finkelstein 2006), to provide direct evidence on whether ABA plays a role in the stomatal regulation under certain conditions. Due to technical difficulties, the guard-cell-symplastic and -apoplastic ABA contents are not well-studied and not determined under any of the above three water-sufficient conditions, which may account for the conflicting conclusions from literature as stated below.

Diurnal changes. Diurnal patterns of stomatal movement were first observed in the 1920s (Loftfield 1921, cited in Dodd, Stikic & Davies 1996). However, the underlying mechanisms are not well-studied and the role of ABA is not certain. Earlier studies on diurnal changes of the leaf and the leaf-apoplastic ABA generally did not support ABA participation in stomatal diurnal regulation (reviewed in Dodd *et al.* 1996). However, Tallman recently proposed a hypothetical model in support of ABA participation in this stomatal response (Tallman 2004). In this model, the ABA contents in both the symplast and apoplast of guard cells both change during the day, which contributes to stomatal diurnal regulation. The proposed underlying mechanisms are: light favors ABA catabolism and darkness favors ABA biosynthesis in guard cells, and the high transpiration rate during midday controls ABA accumulation in the guard-cell apoplast. In the present study, the guard-cell-symplastic and -apoplastic ABA contents of *Vicia faba* were determined at three time points during the day (0600 h, 1400 h, 1800 h). The results showed that no changes in the guard-cell symplastic or -apoplastic ABA content were detected during the day.

Humidity. Stomata close at low humidity and open at high humidity (Sheriff 1979; Monteith 1995; Maier-Maercker 1983). However, it is not humidity *per se*, but

humidity-induced transpiration-rate changes that stomata sense (Mott & Parkhurst 1990; Monteith 1995; Jarvis *et al.* 1999). Among the two hypothetical models proposed for explaining the stomatal response to transpiration rate, the "osmotic regulation model" is preferred over the "drawdown model" by Buckley (2005). However, the underlying mechanisms for guard-cell osmotic regulation are not clear and whether ABA participates in this process is in debate as discussed in the following. As mentioned above, studies with *Arabidopsis* ABA mutants did not provide consistent results regarding whether ABA is involved in stomatal humidity response. Assmann, Snyder & Lee (2000) reported a wild-type humidity response in ABA-deficient (*aba1*) and -insensitive (*abi1-1*, *abi2-1*) mutants, whereas Xie *et al.* (2006) showed that the stomatal response was impaired at low humidity in ABA-deficient (*aba2*) and -insensitive (*ost1*) mutants. In addition, ABA accumulated around guard cells when a high level of ABA was introduced into the xylem of *Vicia faba* (Zhang & Outlaw 2001a), presumably resulted from transpiration. These results, together with results from Outlaw & De Vlieghere-He (2001), led us to hypothesize that ABA accumulates around guard cells at high transpiration rates, which inhibits further stomatal opening and provides a means for plants to measure transpiration rate. In the present study, the guard-cell-apoplastic and -symplastic ABA contents of intact *Vicia faba* after shifting relative humidity (RH) from 60% to 90% were determined and compared to those of intact plants kept at constant 60% RH. In addition, the ABA contents in the two guard-cell compartments of detached leaves infused with 1 μM ABA were determined respectively in the presence and absence of the same RH shift. The results indicate that ABA does not play a role in the stomatal response to transpiration rate, and the guard-cell-apoplastic ABA content is not affected by transpiration rate, even at a high level of xylem ABA.

Brief flooding. Stomatal closure starts within a few hours of flooding (brief flooding) and persists for days (sustained flooding) (reviewed in Sojka 1992; Jackson 2002). Although ABA functions in sustaining stomatal closure (Jackson 1993), a role of ABA in inducing stomatal closure under brief flooding has not been established (Jackson 2002). According to a postulate (Schurr, Gollan & Schulze 1992; Wilkinson & Davies 1997; Sauter *et al.* 2001; Davies *et al.* 2005), flooding- and other stress-induced xylem alkalinization (Wilkinson 1999; Jackson *et al.* 2003; Else, Taylor & Atkinson 2006) can

induce leaf ABA redistribution to guard cells and result in stomatal closure. Although stomata close when artificial xylem sap with a high pH is introduced to the transpiration stream of detached leaves (Wilkinson & Davies; 1997; Dodd *et al.* 2003; Jackson *et al.* 2003, but see the *Discussion* of *Chapter 2*), whether a high apoplastic pH indeed results in ABA accumulation around guard cells has not been tested. Therefore, the guard-cell-apoplastic and -symplastic ABA contents were determined under brief flooding. The results indicate that ABA is not an initiator of stomatal closure under brief flooding, and that xylem alkalinization does not induce leaf ABA redistribution to guard cells.

In summary, using an ELISA with sub-fmol sensitivity, we determined the ABA contents in the symplast and apoplast of guard cells upon diurnal changes, humidity-induced transpiration-rate changes, and brief flooding. Guard cells dissected from freeze-dried leaflets contain both symplastic and apoplastic ABA contents, whereas cells dissected from rinsed epidermal peels contain only symplastic contents. The guard-cell-apoplastic ABA content was calculated by subtraction of the guard-cell-symplastic ABA content from total-guard-cell ABA content (Zhang & Outlaw 2001a, b, c). Therefore, the roles of ABA in the stomatal responses to these water-sufficient conditions were clarified in the present study. The leaf and leaf-apoplastic ABA content, and leaf-apoplastic pH, which are both potential factors in regulating the guard-cell ABA pool, were also measured. The regulations of the guard-cell ABA pool by ABA transport, catabolism, and redistribution in whole plants under these three conditions are discussed.

CHAPTER 1

ABA CONTENTS OF THE GUARD-CELL SYMPLAST AND OF THE GUARD-CELL APOPLAST DO NOT CORRELATE WITH STOMATAL APERTURE SIZE UNDER TWO WELL-WATERED CONDITIONS

Introduction

The plant hormone ABA plays important roles in several physiological processes, such as seed germination, plant growth and development, and stomatal movement. Known as a "stress hormone," ABA is involved in water conservation by inducing stomatal closure under several water-stressed conditions. The underlying mechanisms for the ABA involvement have been widely studied (Davies & Zhang 1991; Sauter *et al.* 2001; Davies *et al.* 2005), and the guard-cell ABA signal transduction has been extensively documented (Luan 2001; Fan *et al.* 2004; Pei & Kuchitsu 2005). However, whether ABA participates in the stomatal response to well-watered conditions is not clear. This chapter focused on two well-watered conditions, diurnal changes and humidity-induced transpiration-rate changes, under which the role of ABA is under debate.

Diurnal changes. Diurnal stomatal movement was first observed in the 1920s (Loftfield 1921, cited in Dodd *et al.* 1996) and found repeatedly in many species (Tenhunen, Pearcy & Lange 1987): stomata typically open in the morning and close in the afternoon, with a peak in the middle of the day. Stomatal diurnal behavior may facilitate maximum use of daily environmental resources and increase plant fitness, just as stomatal circadian behavior does (Dodd *et al*. 2005). Therefore, it is important to

understand how stomatal diurnal movement is regulated. The major guard-cell osmotica (K^+ salts and sucrose), whose accumulations trigger stomatal opening, have been shown to exhibit diurnal changes (Lu *et al.* 1995; Talbott & Zeiger 1996; Talbott *et al.* 2006) and work coordinately in stomatal regulation during the day (Talbott & Zeiger 1998; Schroeder *et al.* 2001). However, how these two osmoregulation pathways are regulated and coordinated is not known and whether ABA is involved in these processes is not clear.

Earlier studies on the leaf and leaf-apoplastic ABA generally did not support ABA participation in stomatal diurnal regulation (reviewed in Dodd *et al.* 1996). However, a hypothetical model was recently proposed in support of ABA involvement in stomatal diurnal regulation (Tallman 2004). In this model, the ABA contents in ABA action sites, the symplast and apoplast of guard cells (reviewed in Pei & Kuchitsu 2005; Finkelstein 2006), both change diurnally. The proposed underlying mechanisms are: darkness favors guard-cell ABA biosynthesis whereas light favors the depletion of guard-cell ABA, and ABA accumulates around guard cells during midday because of high transpiration rate (vapor pressure difference (VPD)- and temperature-related). When the guard-cell-symplastic or -apoplastic ABA content reaches certain thresholds, ABA triggers K^+ efflux and/or overrides sucrose uptake, resulting in diurnal changes of K^+ and sucrose in guard cells and subsequent stomatal movement. This model is attractive because the ABA signal ties several daily environmental variables over a daily cycle. However, because of technical difficulties, no experimental evidence is available for evaluating this model. In the present study, the guard-cell-symplastic and -apoplastic ABA contents were determined at three time points during the day. The results indicate that ABA may not be involved in stomatal diurnal regulation, and that the Tallman's model (Tallman 2004) does not hold, at least under our experimental conditions.

Humidity-induced transpiration-rate changes. Stomata close under low atmospheric humidity and open at high atmospheric humidity (Sheriff 1979; Monteith 1995; Maier-Maercker 1983). The stomatal regulation at different RHs optimizes gas exchange and improves plant water-use efficiency (Farquhar, Schulze & Küppers 1980; Grantz, Moore & Zeiger 1987). Moreover, RH affects plant tolerance to stresses, i.e. high RH mitigates and low RH enhances the effect of water, salt and temperature stresses

(Asch, Dörffling & Dingkuhn 1995 and reference therein; Romero-Aranda, Soria & Cuartero 2002). Therefore, it is beneficial to understand how stomatal movement is regulated at different RHs. Current progress toward this understanding includes: first, stomata respond to humidity-induced transpiration-rate changes, rather than humidity *per se* (Mott & Parkhurst 1990; Monteith 1995; Jarvis *et al.* 1999). Second, two hypothetical models have been proposed in explaining this stomatal response. In the "osmotic regulation model," the stomatal response to transpiration rate involves active guard-cell osmoregulation, whereas in the "drawdown model," the stomatal response results from the water potential gradient between epidermal and guard cells (Buckley & Mott 2002). According to Buckley (2005), current evidence favors the "osmotic regulation model." However, the mechanisms for guard-cell osmotic regulation are not certain and a role of ABA in this process has not been established.

Assmann *et al.* (2000) suggested that ABA is not involved in this response because ABA-deficient (*aba1*) and ABA-insensitive mutants (*abi1-1*, *abi2-1*) of *Arabidopsis* have wild-type stomatal response to humidity. In contrast, Xie *et al.* (2006) recently showed that ABA probably plays a role because the two genes they identified in the stomatal regulation at low RH are both ABA-related. One encodes an ABA biosynthesis enzyme (ABA2) and the other encodes a guard-cell ABA signaling component (OST1). These researchers also found that the stomatal response was impaired at low humidity in *aba2* and *ost1* mutants. It is difficult to reconcile the above conflicting results with ABA mutants and further studies with other approaches are needed to clarify the role of ABA in the stomatal response to transpiration rate. We hypothesized that ABA accumulates around guard cells at high transpiration rates, which inhibits further stomatal opening and provides a means for plants to measure transpiration rate. This hypothesis was based on two observations: (1) Apoplastic ABA accumulated around *Vicia faba* guard cells when ABA was introduced to the petiole (Zhang & Outlaw 2001a), presumably by transpiration. (2) Endogenous apoplastic sucrose accumulates in the guard-cell apoplast under high transpiration rate (Lu *et al.* 1995; 1997), but not under low transpiration rate (Outlaw & De Vlieghere-He 2001). In the present study, the guard-cell-apoplastic ABA content was determined at two different transpiration rates (60% and 90% RH). Surprisingly, the results indicate that ABA probably does not play a

role in the stomatal response to transpiration rate. Even after a high level of ABA (1 μM) was introduced to the transpiration stream, the ABA accumulation around guard cells was not affected by transpiration rate.

Overall, the major objectives of this study were to clarify the roles of ABA in the stomatal response to two well-watered conditions, diurnal changes and humidity-induced transpiration rate changes. Using an ELISA with sub-fmol sensitivity, we determined the guard-cell-symplastic and -apoplastic ABA content under these conditions from pooled samples of individually dissected guard cells. The ABA contents of the leaf and the leaf apoplast, which are potential sources for the guard-cell ABA pool, were also determined. The results indicate that ABA does not play a role in stomatal regulation under these two well-watered conditions.

Materials and Methods

Plant material

Growth conditions

Broad bean (*Vicia faba* L. cv Longpod) plants were grown in a growth chamber (EGC, Chagrin Falls, OH). Day/night temperature was 25/20 ºC, and RH was kept at 60% unless otherwise indicated. Light was provided by fluorescent and incandescent lamps with a wattage ratio (fluorescent to incandescent) of ~ 6, the maximum photosynthetically active radiation (PAR) at plant level was ~ 550 μmol photons m^{-2} s^{-1}. Light and temperature were ramped, as in Lu *et al.* (1997).

Soil culture

Vicia faba plants were cultured in Fafard medium No. 2 in 1-L pots, at a density of two plants per pot. The watering was applied daily in the evening with half-strength of Peter's All Purpose fertilizer (J. R. Peters, Inc., Allentown, PA). Plants were used at 3-weeks old.

Hydroponic culture

Vicia faba seeds were surface sterilized and germinated for a week in the dark. Seedlings were then transferred to quarter-strength Hoagland's solutions and grown in the growth chamber. The solution was aerated all the time, and replaced every other day. Plants were used at 4-weeks old.

Humidity-shift treatments

Intact soil-cultured plants

Ambient RH was shifted at 1200 h from 60% to 90% for 2 h before physiological measurements and sample collections as mentioned below. Control plants were kept at 60% all the time. For the precision of humidity-shift treatments and associated effects, see Outlaw & De Vlieghere-He (2001).

Detached leaves (from hydroponic plants) infused with 1μM ABA

The youngest fully expanded leaves with petioles were detached under water at 0930 h. Ambient RH was then shifted from 60% to 90% then, and detached leaves were infused with 1μM (+) ABA buffer (5 mM K_2HPO_4/KH_2PO_4, 1 mM $CaCl_2$, pH 6.1) for 30 min before measurements and sample collections. Other leaves detached at 0930 h were infused with the same 1μM (+) ABA buffer but kept at 60% RH for 30 min. Samples for non-ABA-infused control were taken from intact plants at 0930 h.

The 30-min period of ABA infusion was chosen according to our preliminary experiments. We found that the decrease of stomatal aperture size was associated with the duration of the 1μM ABA infusion. After 30-min ABA infusion, stomata maintained ~ 50% of the original opening (~ 5 μm). Thus, the effects of transpiration rate could be tested. The reasons for choosing the concentrations of ABA (1μM) and other buffer components (5 mM K_2HPO_4/KH_2PO_4, 1 mM $CaCl_2$, pH 6.1) are explained in Zhang & Outlaw (2001a).

Leaf water potential and stomatal aperture measurements

Leaf water potential

The youngest fully expanded leaves with petioles were detached and water potential was measured using a pressure chamber (PMS Instrument Co., Corvallis, OR).

Stomatal aperture size

Aperture sizes of 30 stomata were measured from abaxial epidermis under a light microscope.

Sample collection and preparation for ABA assay

Leaf apoplastic ABA

After leaf-water-potential measurements, a 0.2–0.4 MPa in excess of the water-potential-balancing pressure was applied to the detached leaves to collect apoplastic sap (~15 µl) using a pressure chamber. The initial 2–3 µl (sufficient to empty petiolar tracheary elements, Kang *et al.* unpublished) was discarded.

Leaf, total-guard-cell, and guard-cell-symplastic ABA

A 4 cm^2 sample devoid of mid-vein and leaf edges was excised, frozen in liquid-N_2 slurry and broken into ~ 4 mm^2 fragments for leaf and total-guard-cell ABA assay. Abaxial epidermis (~ 1 cm^2) for the guard-cell symplastic ABA assay was peeled from the fresh leaflet and floated on ice water for 2 min to remove the apoplastic contents (Zhang & Outlaw 2001b). It was then frozen in liquid-N_2 slurry.

Frozen leaf fragments (~ 6 mg) for leaf ABA assay were homogenized in 80 µl extraction solution (80% (v/v) aqueous methanol containing 0.001% (w/v) 2, 6-di-*t*-butyl-*p*-cresol). The supernatant was collected and the homogenizer was rinsed two times. The 240 µl homogenate was placed in darkness at 4 ºC for 12 h, and then centrifuged at low speed for 2 min. The supernatant was dried under N_2 and redissolved in ~ 400 µl TBS (50 mM Tris, 1 mM $MgCl_2$, 150 mM NaCl, pH 8.1).

All frozen tissues for total-guard-cell- and guard-cell-symplastic-ABA assays were freeze-dried at -35 ºC and < 10 μm Hg. For each sample, guard cells from 4–6 plants (~ 200 pairs) were individually dissected from leaf and rinsed epidermal tissues in a controlled environment (18 ºC, 30–40% RH). The cells were placed in a cluster inside a horizontal 6 × 50-mm silanized borosilicate tube, covered with a small drop (~ 0.4 μl) of extraction solution (same as used for the leaf ABA extraction) and sealed with parafilm. The extraction was kept in the dark at 4 ºC overnight. After opening the tube, the small droplet of the extraction solution evaporated quickly and the dried guard-cell preparation was redissolved in 1.7 μl TBS.

Due to the labor-intensive processes of guard-cell dissection and transfer, only two complete replicates were conducted for each treatment. The guard-cell-symplastic ABA content in unstressed *Vicia faba* exhibited a ~ 20% difference at different growth conditions (Zhang & Outlaw 2001c), and differences in guard-cell ABA contents were also found among growth lots for unstressed *Vicia faba* (Zhang & Outlaw 2001b) and *Zea mays* (Zhang *et al.* unpublished). Therefore, samples from up to three plants each from two growth lots were pooled to minimize variation in each experiment. In addition, plants were carefully maintained, and cultured (e.g. widely spaced and away from the growth chamber walls) after selection of fully expanded young leaves 2–3 days before the experiment. Physiological measurements and the leaf apoplastic ABA assay described above were used as references to avoid inclusion of aberrant plants for guard-cell dissection.

ABA assay

Leaf and guard-cell preparations for ABA assay, and leaf apoplastic sap that was used directly without processing were analyzed in 0.5-μl triplicate using ELISA. The microscale enzyme-amplified immunoassay was a competitive, solid-phase assay (Harris *et al.* 1988; Harris & Outlaw 1990) with modifications (Zhang, Hite & Outlaw 1991).

Briefly, the assay can be divided into four parts. (1) Coating of plates with ABA-specific antibody using a "sandwich" method due to the poor binding of ABA antibody to plastic surfaces. First, Terasaki-type plates (10-μl capacity per well, NUNC

Serving Life Science, Roskilde, Denmark) were rigorously pre-washed (Zhang *et al.* 1991). Then, each well of the plates was filled with 1 µl solution of 250 µg/ml rabbit antimouse immunoglobulin in 50 mM carbonate buffer (pH 9.6) and incubated at 4 ºC for 24 h. The coating solution was decanted and the plates were rinsed four times with TBS containing 0.05% (v/v) Tween-20. Finally, 1 µl solution of ABA-specific, mouse monoclonal antibody was added to each well. The ABA antibody was reactive only with (+)-ABA, but not with other forms of ABA, ABA catabolites, or ABA conjugates (Mertens, Deus-Neumann & Weiler 1983). The plates were incubated at 4 ºC for 24 h, and rinsed as above after decanting the coating solution. (2) Competition for binding ABA-antibody between sample/standard ABA and alkaline-phosphatase-labelled ABA (AP-ABA). Solution of unknown samples or ABA standard (0.6 µl) was added to each well and incubated for 1 h at 18 ºC. Then, 0.6 µl of diluted AP-ABA (1:1000 dilution in TBS containing 0.1% gelatin) was added. After the plate was incubated for another 1.5 h, the competition solution was decanted and rinsed four times with TBS containing Tween-20. (3) A reaction step: the hydrolyzation of NADP to NAD by antibody-bound alkaline phosphatase. Each well was filled with 2 µl of 200 µM NADP in 50 mM diethanolamine (pH 9.5) containing 1 mM $MgCl_2$ and incubated at 37 ºC for 1.5 h. The reaction was terminated by moving the plate to -80 ºC for 20 min, and the solution of each well was then transferred to a 96-well assay plate (Corning Incorporated, Corning, NY). (4) An amplification step: NAD was enzymically cycled, which resulted in the accumulation of reduced iodonitrotetrazolium (INT) – formazan, a colored product. Cycling reagent (200 µl, 50 mM Tris (pH 7.5) containing diaphorase at 250 µg ml^{-1}, 1 mM INT, 300 mM ethanol, 0.25% bovine serum albumin and alcohol dehydrogenase at 50 µg ml^{-1}) was added to each well of the 96-well assay plate. Diaphorase was dissolved in 200 mM Tris containing 300 mM KCl, 0.5 mM FMN and 0.025% bovine serum albumin. The plate was incubated at 23 ºC for 20 min, and this colored reaction was terminated by adding 50 µl of 0.3 M HCl to each well. The absorbance was measured at 490 nm.

Calculations of the guard-cell-apoplastic ABA content, xylem ABA delivery rate, and VPD changes upon the RH shift

The guard-cell-apoplastic ABA content

Guard cells dissected from freeze-dried leaflets contain both symplastic and apoplastic ABA contents, whereas cells dissected from rinsed epidermal peels contain only symplastic contents. The guard-cell-apoplastic ABA content was calculated by subtraction of the guard-cell-symplastic ABA content from total-guard-cell ABA content (Zhang & Outlaw 2001a, b, c). Calculated errors for the apoplastic ABA contents are $S.E._{apoplast} = \sqrt{(S.E._{symplast})^2 + (S.E._{total})^2}$.

Changes of VPD and transpiration rate upon the RH shift

VPD is affected by leaf temperature. Thus, leaf temperature was measured with an infrared thermometer at the time of our sampling (ambient temperature was 25 ºC). Leaf temperature was 24.8 ºC at 60% RH, a temperature that was close to that measured with a CIRAS-1 Portable Photosynthesis System (24.7 ± 0.2 ºC) (Kang *et al.* unpublished). At 90% RH, leaf temperature was 25.4 ºC. The difference in VPD between the leaf and the atmosphere under these two conditions (60% and 90% RH) was calculated to be 3.2 fold.

Transpiration rate is the product of VPD and stomatal conductance. VPD was calculated as above, and stomatal conductance was converted from stomatal aperture size according to Zhang & Outlaw (2001a). Transpiration rate decreased ~ 2 fold by shifting RH from 60% to 90% RH (see Table 1.1 and Table 1.2 for stomatal aperture size).

The xylem ABA delivery rate

The xylem ABA delivery rate is the product of leaf apoplastic ABA concentration and transpiration rate. Transpiration rates were calculated as described above. Other parameters needed in this calculation were: guard cell density (5600 stomata cm^{-2} for *Vicia faba*, Poffenroth, Green & Tallman 1992), and the volume of the guard-cell apoplast (4.2 pL, Ewert *et al.* 2000).

Results

The potential role of ABA in stomatal diurnal regulation in well-watered *Vicia faba*

Diurnal stomatal movement not related to leaf water potential

Diurnal changes of stomatal aperture size were examined to identify representative time points for further mechanism studies. The determination of diurnal changes of leaf water potential would indicate whether hydraulic or chemical signals predominate in stomatal diurnal regulation, and having such information was a prerequisite for testing the role of a chemical signal (ABA) in this stomatal response.

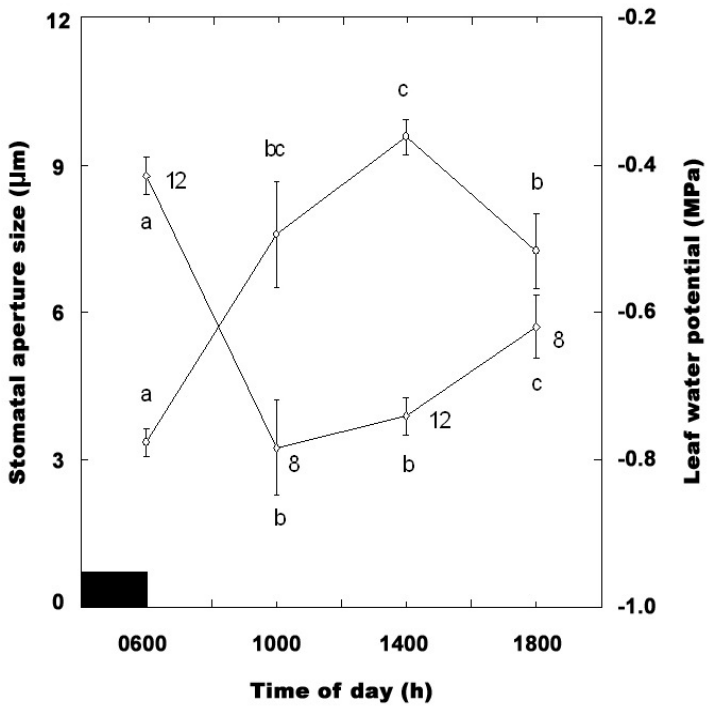

Figure 1.1 Diurnal changes of stomatal aperture size (○) and leaf water potential (◊) in well-watered *Vicia faba*. Photoperiod started at 0600 h. Number of plants is indicated by symbols (paired study). Error bars are SE. Data marked with different alphabetic characters are significantly different ($P < 0.05$) to each parameter.

In well-watered *Vicia faba*, stomatal aperture size exhibited typical diurnal changes (Fig. 1.1). Stomatal aperture size was 3.4 ± 0.3 μm before the light was on, and increased gradually to a maximum of 9.6 ± 0.4 μm in the early afternoon (1400 h). Stomata then closed in the late afternoon at 1800 h.

The diurnal change pattern of leaf water potential here (Fig. 1.1) was similar to that found in other species and other growth environments (e.g. Loveys & Düring 1984; Tardieu & Simonneau 1998). Leaf water potential was the highest (-0.4 ± 0.02 MPa) before the light was on, decreased with time to -0.7 – -0.8 MPa and recovered in the late afternoon. The highest leaf water potential was associated with the smallest stomatal aperture and low water potentials corresponded to large stomatal openings (Fig. 1.1), indicating that leaf water status is the effect and not the cause of stomatal movement. Therefore, hydraulic signals do not play important roles in stomatal diurnal regulation, and chemical signals, possibly ABA, are involved in stomatal diurnal response.

Diurnal changes of leaf ABA and leaf apoplastic ABA, and their relationships with stomatal aperture size

The essence of this study is diurnal changes of the guard-cell ABA pool and their correlations with stomatal aperture size. Therefore, the ABA contents in the leaf and the leaf apoplast that are potential sources for the guard-cell ABA pool were determined.

The leaf ABA content was relatively constant at a level of 300 nmol kg^{-1} fresh mass before early afternoon (Fig. 1.2). This content increased significantly to 478 ± 73 nmol kg^{-1} fresh mass at 1800 h. The correlation between the leaf ABA content and stomatal aperture size during the day was weak ($R^2 < 0.01$, Fig. 1.3a).

Compared to the other three time points tested during the day, the leaf-apoplastic ABA concentration was only lower (29 ± 5 nM) at 1400 h ($P \leq 0.02$, Fig. 1.2). The correlation between the leaf apoplastic ABA concentration and stomatal aperture size was not strong ($R^2 = 0.31$, Fig. 1.3b). The xylem ABA delivery rate, calculated by multiplying the leaf apoplastic ABA concentration with the transpiration rate (see the *Materials and Methods*), also did not correlate with stomatal diurnal changes ($R^2 = 0.14$, Fig. 1.3c).

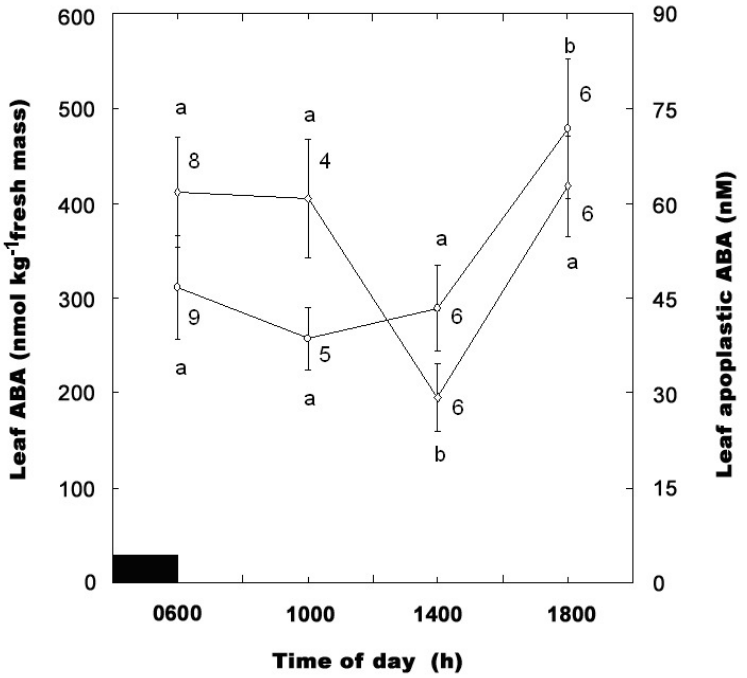

Figure 1.2 Diurnal changes of leaf ABA content (○) and leaf apoplastic ABA concentration (◊) in well-watered *Vicia faba.* Number of plants is indicated by symbols (paired study). Error bars are SE. Data marked with identical alphabetic characters are not significantly different (P > 0.05) to each parameter.

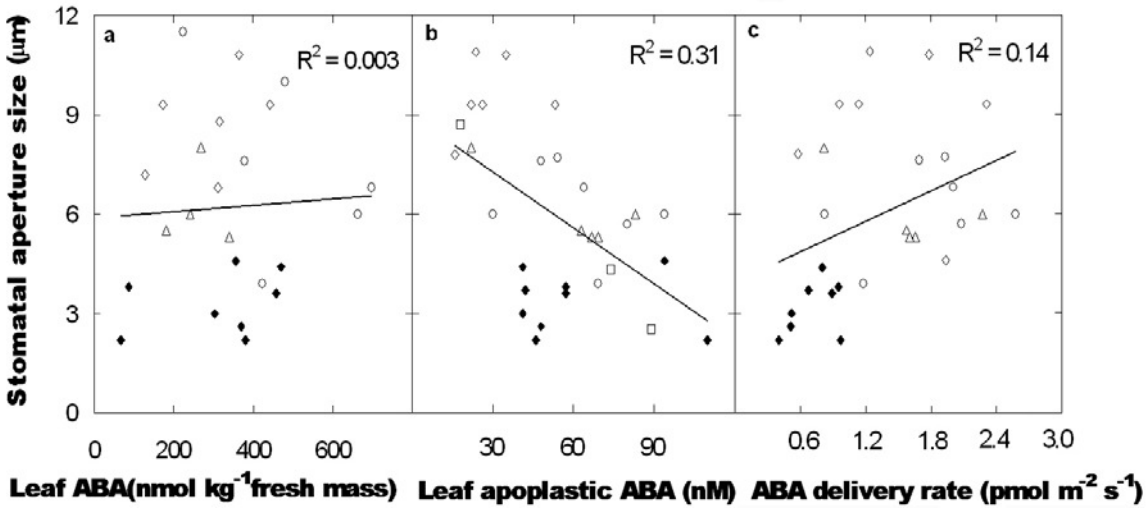

Figure 1.3 The relationship between leaf ABA content (a), leaf apoplastic ABA concentration (b) and ABA delivery rate (c) and stomatal aperture size during the day (♦, Δ, ◊, ○: 0600,1000,1400 and 1800 h) in well-watered *Vicia faba*.

No diurnal changes in the ABA contents of the guard-cell-symplast, guard-cell-apoplast, and total guard cells

The lack of correlation between stomatal aperture size and leaf ABA, leaf apoplastic ABA and xylem ABA delivery during the day (Fig. 1.3) indicates that these potential ABA sources may not contribute to diurnal changes of the guard-cell ABA pool. However, another potential source, the guard-cell ABA biosynthesis, is hypothesized to be regulated by light/darkness (Tallman 2004). Therefore, the guard-cell ABA contents were determined at three time points (0600 h, 1400 h, and 1800 h) during the day.

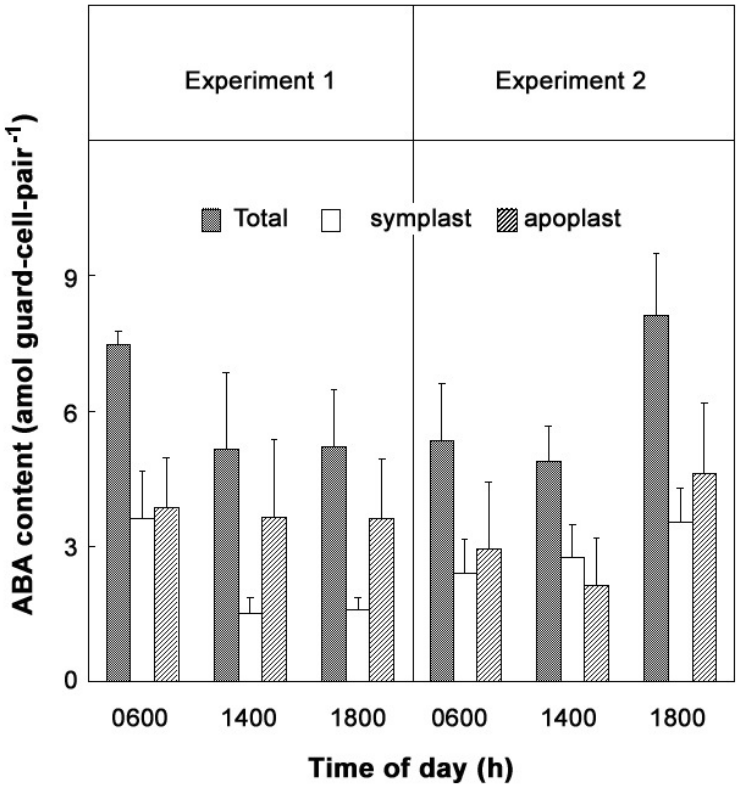

Figure 1.4 ABA contents in the guard-cell apoplast, the guard-cell symplast and total guard cells did not change diurnally in well-watered *Vicia faba*. Each column represents one assay conducted in triplicate (mean ± SE) for total-guard-cell and the guard-cell-symplastic ABA values. Individually dissected guard cells (120–178 pairs) were pooled from two growth lots in experiment 1 (2 plants) and experiment 2 (6 plants, 3 plants per lot).

In the two experiments conducted (Fig. 1.4), the guard-cell-symplastic ABA contents were 3.6 ± 1.1 and 2.4 ± 0.7 amol guard-cell-pair^{-1} and the guard-cell-apoplastic ABA contents were 3.8 ± 1.1 and 2.9 ± 1.4 amol guard-cell-pair^{-1} at 0600 h. These values were not different from those at 1400 or 1800 h in both experiments ($P \geq 0.13$ for the symplast, $P \geq 0.25$ for the apoplast), in contrast to large differences in stomatal aperture size ($P < 0.01$). The guard-cell-symplastic ABA contents were not different between any two points during the day in each experiment ($P \geq 0.13$). In addition, ABA contents in total guard cells were also not different between any two time points during the day ($P \geq 0.11$). These results indicate that ABA probably does not play a role in stomatal diurnal regulation.

The total-guard-cell and guard-cell-symplastic ABA contents in well-watered *Vicia faba* here are comparable to those from other studies (Harris *et al*. 1988; Popova *et al*. 2000; Zhang & Outlaw 2001b, c). The guard-cell apoplastic ABA content was only determined in Zhang & Outlaw (2001a, b, c), and lower than that detected in the present study. The difference is probably due to subtle changes in growth conditions, e.g. the guard-cell apoplastic ABA content was found to vary between different growth lots (Zhang & Outlaw 2001b). Therefore, up to six plants from two growth lots were pooled together for guard-cell dissection to minimize the variation in this study (see the *Materials and Methods*).

The potential role of ABA in the stomatal response to transpiration rate in well-watered *Vicia faba*

Stomatal aperture size increased without changes in leaf ABA and leaf apoplastic ABA at a lowered transpiration rate

We hypothesized that a decreased transpiration rate would diminish the apoplastic ABA accumulation in the guard-cell apoplast (see the basis for this hypothesis in the *Introduction*). Here, shifting RH from 60% to 90% was used to decrease transpiration rate. Such a RH shift increased stomatal aperture size (from 9 to 12.9 μm, Table 1.1), as expected. Leaf ABA and leaf apoplastic ABA were also measured at 90% RH to help

interpret whether the ABA accumulation around guard cells (see studies below) is controlled by lowered transpiration rate alone or also by ABA sources from leaf and leaf apoplast. The results showed that neither leaf ABA nor leaf apoplastic ABA was changed by lowering the transpiration rate (Table 1.1).

Table 1.1 Stomatal aperture size increased without changes of leaf ABA and leaf apoplastic ABA at a lowered transpiration rate in well-watered *Vicia faba*. RH was shifted from 60% to 90% at 1200 h and lasted for 2 h for lowered transpiration rates, and control plants were kept at constant 60% RH. The results are mean ± SE (n is indicated in parentheses). * indicates a significant difference ($P < 0.05$).

	Stomatal aperture size (μm)	Leaf ABA (nmol kg^{-1} fresh mass)	Leaf apoplastic ABA (nM)
60% RH	9.0 ± 0.8 (12)	289 ± 48 (6)	29 ± 5 (6)
90% RH	*12.9 ± 0.2 (12)	299 ± 36 (9)	25 ± 1 (6)

No changes of the guard-cell-apoplastic ABA content at a lowered transpiration rate

Since neither leaf ABA nor leaf apoplastic ABA was changed at 90% RH (Table 1.1), the effect of a lowered transpiration rate on ABA accumulation in the guard-cell apoplast could be tested by determining the guard-cell-apoplastic ABA contents at 90% RH as compared to those at 60% RH.

The guard-cell-apoplastic ABA contents were 2.4 ± 1.1 and 1.9 ± 0.8 amol guard-cell-$pair^{-1}$ at 90% RH in the two experiments. These values were not different from 3.6 ± 1.7 and 2.1 ± 1.0 amol guard-cell-$pair^{-1}$ at 60% RH (Fig. 1.5, $P \geq 0.29$). Total-guard-cell ABA contents were also unaffected by decreasing the transpiration rate (Fig. 1.5, $P \geq 0.23$). In contrast, the guard-cell-symplastic ABA content was found to differ between the two transpiration rates in experiment 1 (Fig. 1.5, $P = 0.02$). However, we attributed this change to plant variations rather than to the effect of transpiration rate, because the guard-cell-symplastic ABA also appeared to differ between the two experiments at 90% RH ($P = 0.01$). Incidentally, these results revealed

our ELISA's sensitivity. For a general assay with well-watered plants, we were able to detect a hypothetical change of 2.2-fold for both total-guard-cell and the guard-cell-symplastic ABA content. Changes of the guard-cell-apoplastic ABA content are more difficult to detect because of the way of calculating this ABA value and the standard error (see the *Materials and Methods*). In spite of the detection-limit issue, we consider the lack of changes in the guard-cell-apoplastic ABA content at a lowered transpiration rate to be real (see reasons in the *Discussion*).

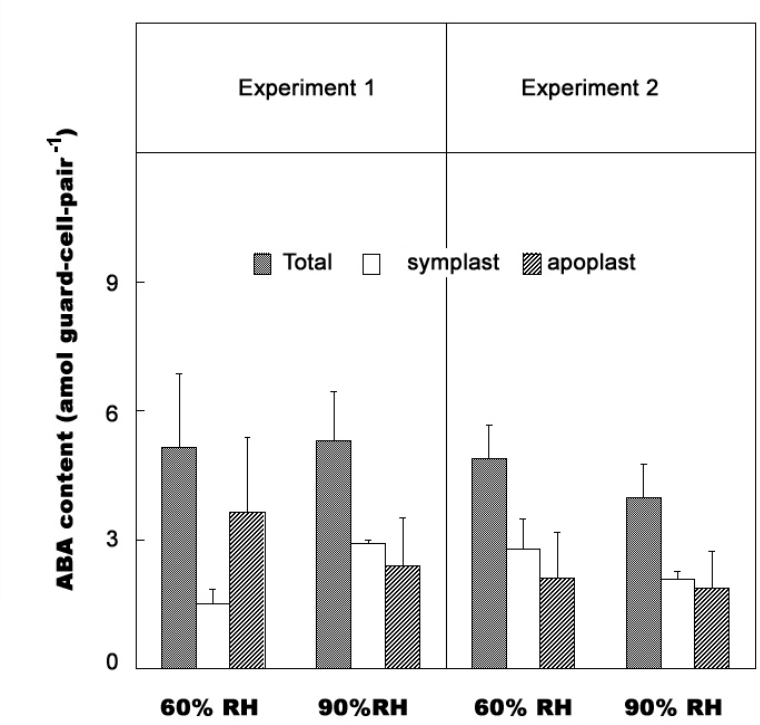

Figure 1.5 ABA contents in the guard-cell apoplast, the guard-cell symplast and total guard cells did not change at a lowered transpiration rate in well-watered *Vicia faba*. RH was shifted from 60% to 90% RH at 1200 h and lasted for 2 h for a lowered transpiration rate, and control plants were kept at constant 60% RH. Each column represents one assay conducted in triplicate (mean ± SE) for total-guard-cell and the guard-cell-symplastic ABA values. Individually dissected guard cells (149–249 pairs) were pooled from six plants (2 growth lots) for each assay in each experiment.

The regulation of ABA accumulation in the guard-cell apoplast: the roles of transpiration rate and xylem ABA concentration

As shown in Fig. 1.5, the ABA accumulation around guard cells was not affected by transpiration rate in well-watered *Vicia faba* (Fig. 1.5). This is probably because the ABA delivered via transpiration stream at a low xylem ABA concentration was modulated by leaf compartments in well-watered plants (see the *Discussion*). It would be interesting to know whether the ABA accumulation around guard cells is affected by transpiration rate at a high xylem ABA concentration. Therefore, we infused 1 μM ABA to detached leaves from hydroponic *Vicia faba* and decreased the transpiration rate by shifting RH from 60% to 90% concomitantly. The effect of transpiration rate on ABA accumulation around guard cells was then tested.

No changes of the guard-cell-apoplastic ABA content at a lowered transpiration rate with 1 μM ABA infusion to the transpiration stream

After 30 min ABA (1 μM) infusion to detached hydroponic leaves at 60% RH, the guard-cell-apoplastic ABA contents increased 5–8 fold to 17.9 ± 0.9 and 13.0 ± 1.7 amol guard-cell-pair^{-1} in the two experiments ($P \leq 0.02$; Fig. 1.6). This was similar to what was shown in Zhang & Outlaw (2001a). Surprisingly, a lowered transpiration rate by shifting RH to 90%, which occurred at the same time as 1 μM ABA infusion, did not affect the ABA accumulation around guard cells (14.0 ± 5.1 and 14.5 ± 2.6 amol guard-cell-pair^{-1}) ($P \geq 0.52$, Fig. 1.6). In addition, neither the guard-cell-symplastic nor total-guard-cell ABA contents were changed by decreasing transpiration rate at a high level of introduced xylem ABA (Fig. 1.6, $P \geq 0.36$ and $P \geq 0.58$).

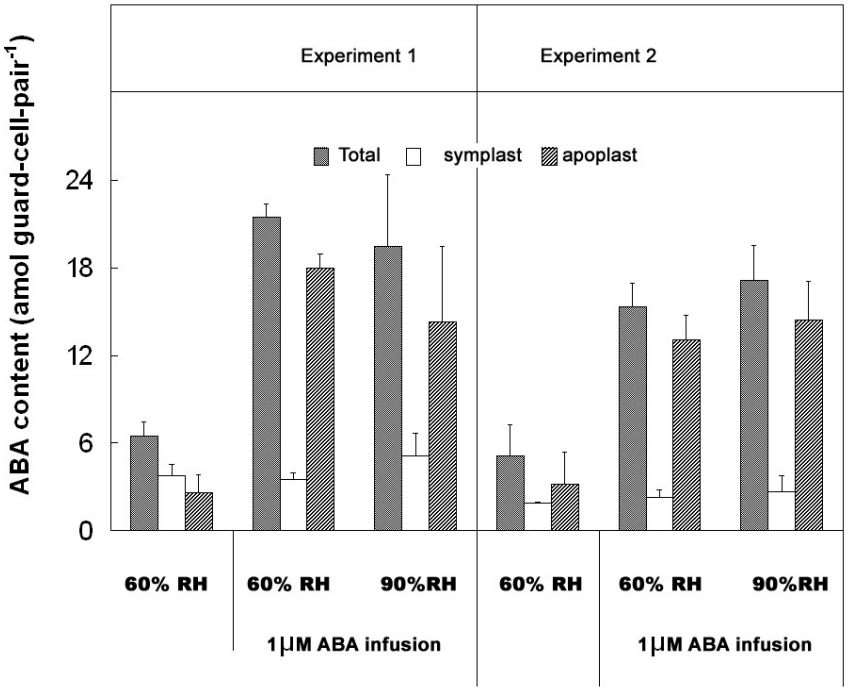

Figure 1.6 ABA contents in the guard-cell apoplast, the guard-cell symplast and total guard cells did not change at a lowered transpiration rate with 1 μM (+) ABA infusion to detached leaves from hydroponic *Vicia faba*. RH was shifted from 60% to 90% for a lowered transpiration rate and control plants (with ABA infusion) were kept at constant 60% RH. The 1 μM (+) ABA infusion and the RH shift both started at 0930 h and lasted for 30 min. Samples for non-ABA-infused control were taken at 0930 h from intact hydroponic plants. Individually dissected guard cells (165–269 pairs) were pooled from six plants for each column in each experiment.

Stomatal aperture size increased without changes in leaf water potential and leaf ABA content at a lowered transpiration rate, when 1μM ABA was infused to detached leaves of hydroponic Vicia faba

It is surprising that the ABA accumulation around guard cells was not affected by transpiration rate, even at a high xylem ABA concentration (Fig. 1.6). Therefore, we determined whether this result was due to the lack of the stomatal response to transpiration rate, or due to effects of other factors (leaf water status or leaf ABA content) in controlling the ABA accumulation around guard cells.

Although 1μM ABA infusion to detached leaves inhibited stomatal opening ~ 50% at 60% RH (Table 1.2), a lowered transpiration rate at 90% RH did result in a 2-μm increase in stomatal aperture ($P < 0.05$). This stomatal response was similar to that in well-watered *Vicia faba* (Table 1.1). A decrease of transpiration rate at a high xylem ABA concentration did not change leaf water potential or change the elevated leaf ABA content (Table 1.2). These results indicate that the unchanged guard-cell-apoplastic ABA content at 90% RH really reflected the lack of the effect of transpiration rate on the ABA accumulation around guard cells. Thus, ABA accumulation around guard cells might not provide a means for plants to sense transpiration rate.

Table 1.2 Stomatal aperture size increased without changes in leaf water potential and leaf ABA content at a lowered transpiration rate, when detached leaves from hydroponic *Vicia faba* were infused with 1 μM (+) ABA. RH was shifted from 60% to 90% RH for a lowered transpiration rate and control plants (with ABA infusion) were kept at constant 60% RH. Both the ABA infusion and the RH shift lasted for 30 min. Samples for non-ABA-infused control were taken at 0930 h from intact hydroponic plants. The results are mean ± SE (n = 14–16).

	Stomatal aperture size (μm)	Leaf water potential (MPa)	Leaf ABA (nmol kg^{-1} fresh mass)
60% RH (non-infused)	11.6 ± 0.5	-0.55 ± 0.01	229 ± 36
60% RH (ABA-infused)	5.1 ± 0.5	-0.45 ± 0.01	1058 ± 209
90% RH (ABA-infused)	6.9 ± 0.5	-0.50 ± 0.01	695 ± 67

Discussion

As a critical root-to-shoot chemical messenger for stresses, ABA has been an exciting topic in stomatal regulation studies. Biophysical, genetic, and molecular approaches are advancing the understanding of the guard-cell ABA signal transduction network (Leung & Giraudat 1998; Schroeder *et al*. 2001; Fan *et al.* 2004), but the inability to place this understanding in the context of the whole plant is a major

limitation. The understanding of ABA regulation of stomatal movement in whole plants mainly derives from measurements of ABA changes at plant tissue level or in apoplastic sap (Jackson 1993; Wilkinson & Davies 2002), or from studies with ABA mutants. Since such ABA measurements are not close enough to guard cells and mutant behaviors are often complicated to interpret (Zeiger *et al.* 2002), these studies do not always provide consistent results. In the present study, the analyses of the ABA contents in the guard-cell symplast and the guard-cell apoplast provided clear evidence that ABA is not involved in the stomatal response to two well-watered conditions, diurnal changes and humidity-induced transpiration-rate changes. The leaf and leaf apoplastic ABA assays allow us to discuss how ABA transport, metabolism and redistribution may participate in regulating the guard-cell ABA pool under these conditions.

ABA probably is not required in stomatal diurnal regulation in well-watered plants

In well-watered *Vicia faba*, the ABA contents in the symplast and apoplast of guard cells remained constant at three time points during the day (Fig. 1.4), in contrast to large differences in stomatal aperture size (Fig. 1.1). We conclude that ABA may not be required in stomatal diurnal regulation under well-watered conditions, which disagrees with the hypothetical model proposed by Tallman (2004).

In Tallman's model, the guard-cell-symplastic ABA content exhibits diurnal changes because light favors the depletion of guard-cell ABA, whereas darkness favors guard-cell ABA synthesis (Tallman 2004). The disagreement between this model and our results (Fig. 1.4) at least can be partially explained when his proposed mechanisms for the depletion of guard-cell ABA by light is examined as below (the regulation of the guard-cell ABA by darkness is essentially the reverse of that by light in the proposed model). First, the elevated O_2 concentration that resulting from mesophyll photosynthesis in the light activates a key enzyme in ABA catabolism (Cytochrome P450 (CytP450)), which degrades ABA in guard cells (Tallman 2004). However, according to Krochko *et al*. (1998), CytP450 activity is relatively stable when O_2 concentration exceeds 20%, although it increases dramatically from 0 to 10%. Therefore, the increased O_2 concentration in the light may not be able to change CytP450 activity significantly to

increase the guard-cell ABA catabolism. Second, the light-driven xanthophyll cycling, which reverses ABA biosynthesis, is also proposed to deplete guard-cell ABA (Tallman 2004). However, xanthophyll cycling occurs only when light is excessive and photosynthesis is saturated (Eskling, Arvidsson & Åkerlund 1997). The maximum light in growth chambers generally is below the light saturation point (e.g. Bassman & Zwier 1991, 600–800 μmol photons m^{-2} s^{-1}). Therefore, xanthopyll cycling may not occur in our study with plants grown in a growth chamber (maximum PAR ~ 550 μmol photons m^{-2} s^{-1}) and thus not cause guard-cell ABA depletion.

The guard-cell-apoplastic ABA content is proposed to change diurnally because the ABA accumulation around guard cells is hypothetically controlled by transpiration by midday (Tallman 2004). However, our data are inherently consistent in support of the absence of diurnal changes in the guard-cell-apoplastic ABA content. First, the correlation between the xylem ABA delivery rate and stomatal aperture size during the day was weak (Fig. 1.3c). Second, when ~ 50 nM ABA (a level close to the high leaf apoplastic ABA concentration during the day, Fig. 1.2) was infused to detached *Vicia faba* leaves at 1000 h, transpiration rate did not change during a 3-h infusion period (data not shown). Third, no changes in the guard-cell-apoplastic ABA contents were detected at three time points during the day (Fig. 1.4). Finally, the ABA accumulation around guard cells is not controlled by transpiration rate (Fig. 1.5, Fig. 1.6). The absence of diurnal changes in the guard-cell-apoplastic ABA content probably results from ABA modulation by leaf compartments in the transpiration pathway (see discussion below for more details).

Except for guard-cell ABA biosynthesis and xylem ABA import discussed above, leaf ABA redistribution (Popova *et al*. 2000; Wilkinson & Davies 1997; Sauter *et al.* 2001) is also a potential source for the guard-cell ABA pool. Although leaf ABA changed diurnally (Fig. 1.2) due to diurnally regulated hydrolysis of ABA conjugates (Lee *et al.* 2006), the leaf-ABA diurnal changes did not cause changes of the guard-cell ABA pool during the day (Fig. 1.4). Two reasons probably explain this. (a) The magnitude of leaf-ABA increase is too small (~ 2 fold, Fig. 1.2; 2–3 fold, Henson *et al.* 1982; Burschka, Tenhunen & Hartung 1983; Loveys & Düring 1984; Loveys, Robinson & Downton 1987; Lee *et al.* 2006, but see Nováková *et al.* 2005 (up to 8-fold increase)).

This small increase is probably unable to induce the ABA redistribution to guard cells as a large leaf-ABA increase does under stressed conditions (> 20-fold, Harris *et al*. 1988; Popova *et al*. 2000). (b) The leaf ABA redistribution upon stresses is often attributed to xylem alkalinization (Schurr *et al.* 1992; Sauter *et al.* 2001; Davies *et al.* 2005), but we did not detect a significant diurnal effect on apoplastic pH in *Vicia faba* (data not shown). Moreover, consistent with Else *et al.* (2006), we found that xylem alkalinization did not induce the ABA redistribution to guard cells (see *Chapter 2*).

In summary, the ABA contents in the symplast and apoplast of guard cells did not change during the day, indicating that ABA does not participate in regulating or coordinating the K^+ and sucrose accumulations in guard cells in stomatal diurnal regulation. Efforts in elucidating the mechanisms for this stomatal response should focus on other player(s). A recent study (Talbott *et al.* 2006) indicates that the morning K^+-based osmoregulation in guard cells is mediated by blue light. This is because a probe for characterizing the presence of blue-light-specific stomatal opening, green light reversibility (Talbott *et al.* 2002), only occurs in the K^+-dependent, morning phase of stomatal movement (Talbott *et al.* 2006). The afternoon osmoregulation phase, the sucrose accumulation in guard cells (Lu *et al.* 1995; Talbott & Zeiger 1996), is probably related to the expression of a sugar transporter gene (AtSTP1) (Stadler *et al.* 2003). A more complete understanding of stomatal diurnal regulation requires studies on the coordination of these osmoregulatory phases, i.e. whether and how the two phases communicate to each other and how the overlapping signals are translated into one physiological response, stomatal movement.

ABA probably is not required in the stomatal response to humidity-induced transpiration-rate changes in well-watered plants

In well-watered *Vicia faba*, no changes in the guard-cell-apoplastic ABA content were detected by shifting RH from 60% to 90% (Fig. 1.5), although stomatal aperture size increased ~ 3 μm (Table 1.1). We suggest that the ABA accumulation around guard cells is not affected by transpiration rate. Thus, ABA probably does not play a role in the stomatal response to transpiration rate under well-watered conditions.

Here, we attribute the lack of differences in the guard-cell-apoplastic ABA contents at different transpiration rates mainly to the leaf modulation of ABA delivered in the transpiration stream. For perspective, if all the ABA delivered via xylem accumulates around guard cells, the guard-cell-apoplastic ABA would reach 13 amol guard-cell-pair^{-1} at 90% RH and 42 amol guard-cell-pair^{-1} at 60% RH within 2 h (see the *Materials and Methods* for calculation). At these high levels of ABA, stomata would close permanently (Zhang & Outlaw 2001a, stomata close when the guard-cell apoplastic ABA is < 8 amol guard-cell-pair^{-1}). Therefore, the delivered ABA in the transpiration stream must be modulated by leaf compartments before it reaches guard cells in well-watered plants. The major mechanism for this ABA modulation is probably through leaf ABA metabolism because leaf ABA export is slow and insignificant (Zhang, Jia & Zhang 1997). Although it has long been known that leaves can metabolize ABA (Trejo, Davies & Ruiz 1993; Daeter & Hartung 1995; Zhang, Jia & Zhang 1997), the gene (*CYP707A*) that encodes the key enzyme in ABA catabolism (CytP450) was identified only recently (Kushiro *et al.* 2004; Saito *et al.* 2004). One member of this gene family, *CYP707A3*, is expressed abundantly in leaves (Kushiro *et al.* 2004; Saito *et al.* 2004) and manipulation of this gene indeed affects the endogenous ABA level (Umezawa *et al.* 2006). These results are consistent with the ABA modulation in the transpiration pathway via leaf metabolism. Overall, under well-watered conditions, the leaf ABA metabolism probably overrides the xylem ABA delivery, thus a slower (at 90% RH) or a faster (at 60% RH) xylem ABA delivery rate would not make a difference in the ABA accumulation around guard cells (Fig. 1.5).

The detection limit for the guard-cell ABA assays has been mentioned earlier (see the *Results*). Because of the method of calculating the standard error for the guard-cell-apoplastic ABA (see the *Materials and Methods*), it is difficult to detect changes in this ABA content with statistical confidence. For a general assay with well-watered plants, only an increase of ≥ 3.1-fold in the guard-cell-apoplastic ABA can be detected, and detecting a decrease in this ABA content requires an initial value of 10 amol guard-cell-pair^{-1}. However, the detection-limit issue would not affect our interpretations on the absence of changes in the guard-cell-apoplastic ABA contents at different transpiration rates (Fig. 1.5) for reasons listed below. First, if the ABA

accumulation around guard cells changed as significantly by the RH shift as the sucrose accumulation does (7-fold, Outlaw & De Vlieghere-He 2001), that magnitude of change would result in a detectable change in total-guard-cell ABA content. Second, the ABA delivery rate did not correlate with stomatal aperture size by the RH shift (figure not shown, $R^2 < 0.10$). Third, even when the guard-cell-apoplastic ABA reached a level > 10 amol guard-cell-pair^{-1}, no decreases were detected by decreasing transpiration rate (Fig. 1.6), thus confirming our above conclusion with well-watered plants.

Our conclusion that ABA is not involved in the stomatal response to transpiration rate in well-watered plants is consistent with that from Assmann *et al.* (2000), who showed that ABA-deficient and ABA-insensitive mutants of *Arabidopsis* have a wild-type stomatal response to RH. However, these conclusions seem to conflict with results from a recent study (Xie *et al.* 2006), in which both an ABA biosynthesis gene (*aba2*) and a guard-cell ABA signaling gene (*OST1*) are involved in the stomatal regulation at lowered RH. The conflict probably can be reconciled as follows. Although low RH up-regulates *OST1*/*SRK2E* expression (e.g. Yoshida *et al.* 2002) and OST1 protein kinase mediates ABA-regulated stomatal movement (Mustilli *et al.* 2002), the impaired stomatal response in *ost1* mutant at lowered RH (Xie *et al.* 2006) does not indicate an obligate role of ABA in the stomatal response to transpiration rate. The reason is that the activation of OST1 can be either ABA-dependent or ABA-independent (Yoshida *et al.* 2006). Thus, Yoshida *et al.* (2006) suggested that stomata respond to RH via both ABA-dependent and ABA-independent pathways. Having multiple responding pathways to the same signal would permit plants fine regulation of gas exchange under different conditions. For instance, depending on plant growth temperature, guard cells respond to external ABA via Ca^{2+}-dependent and Ca^{2+}-independent pathways (Allan *et al*. 1994). In the study of Xie *et al.* (2006), the VPD change is more extreme (RH was shifted to 5%) and probably less reversible compared to that in the present study and Assmann *et al.* (2000). At such a low RH (Xie *et al.* 2006), leaves possibly become desiccated (the plant water relations were not measured), thus triggering leaf ABA biosynthesis. This severe RH drop could also cause ABA production in leaves from conjugated ABA (Schroeder & Nambara 2006).

For a better and more complete understanding of the stomatal response to transpiration rate, one must seek explanations not requiring ABA involvement. One identified mechanism is the sucrose accumulation around guard cells. The guard-cell-apoplastic sucrose content decreases at a lower transpiration rate and the decrease is sufficient to increase stomatal aperture size (Outlaw & De Vlieghere-He, 2001). Apparently, the concentration of sucrose in the guard-cell apoplast is a more sensitive signal than that of ABA for plants to respond to transpiration rate (see Fig. 1.5). This is probably because sucrose accumulation not only provides a mechanism for measuring transpiration rate, but integrates transpiration rate, leaf photosynthesis rate and phloem translocation rate (Lu *et al*. 1997; Kang *et al.* unpublished), and thus functioning in the fine regulation of gas exchange. Other possible chemical signals involved in the stomatal response to transpiration rate include trienoic fatty acid (Poulson, Edwards & Browse 2002) and cytokinin (Aloni *et al*. 2005).

The regulation of ABA accumulation in the guard-cell apoplast: the roles of transpiration rate and xylem ABA concentration

As discussed above, the guard-cell-apoplastic ABA content was not affected by transpiration rate in well-watered plants (Fig. 1.5), probably due to leaf ABA metabolism of xylem-delivered ABA at the low xylem ABA concentration. However, when a high level of ABA was introduced to the petiole, ABA accumulated in the guard-cell apoplast (Zhang & Outlaw 2001a; Fig. 1.6), presumably by transpiration. Therefore, changing transpiration rate is likely to change the guard-cell-apoplastic ABA content at high xylem ABA concentration. To our surprise, the guard-cell-apoplastic ABA content was elevated similarly by 1 μM ABA infusion to xylem under two different transpiration rates (Fig. 1.6, $P \geq 0.52$), in spite of stomatal aperture changes (Table 1.2, $P < 0.05$). We suggest that the ABA accumulation in the guard-cell apoplast is not affected by transpiration rate and does not play a role in feedback regulation of stomatal movement.

In the present study, the guard-cell-apoplastic ABA content is presumably regulated by the balance between the rate of ABA arrival (xylem ABA delivery) and the rate of ABA removal (leaf ABA metabolism). After 30 min of 1 μM ABA infusion to

detached leaves, the ABA accumulation around guard cells theoretically would reach 65 and 180 amol guard-cell-pair^{-1} at 90% and 60% RH (see the *Materials and Methods* for calculation), respectively, if no ABA is removed. However, we found that the guard-cell-apoplastic ABA contents were both ~ 14 amol guard-cell-pair^{-1} at the two RHs (Fig. 1.6, $P \geq 0.52$) after 1 μM ABA infusion, compared to 2–3 amol guard-cell-pair^{-1} in non-ABA-infused plants. One way to explain these results is that when a stressed level of xylem ABA (1 μM, compared to non-stressed level ≤ 50 nM, Zhang, Outlaw & Aghoram 2001; our preliminary experiments) is infused to the transpiration stream, the dramatic increase (tens of fold) of xylem ABA delivery probably outbalances leaf metabolism. Thus, ABA is accumulated around guard cells to transduce the stress signal for plant gas exchange regulation. The balancing mechanism described above, however, is probably not sensitive enough to distinguish the small changes in xylem ABA delivery caused by a 2-fold transpiration-rate change (60% vs. 90% RH, see the *Materials and Methods*). Therefore, with the same 1 μM ABA infusion, the guard-cell-apoplastic ABA contents were not different under the two transpiration rates (Fig. 1.6). Another way to explain our results is that after the guard-cell-apoplastic ABA content reaches a certain high level, the above balancing mechanism may prevent excessive ABA accumulation around guard cells. The supporting evidence for this proposed mechanism was provided by Jia, Zhang & Zhang (1996), who showed that the leaf ABA metabolism is proportional to the ABA fed to the transpiration stream. Further support derived from recent studies (Kushiro *et al.* 2004; Saito *et al.* 2004; Umezawa *et al.* 2006) that high exogenous ABA activates the expression of the ABA metabolism genes *CYP707As*, and *CYP707A3* plays an important role in determining the threshold level of endogenous ABA under some conditions.

Overall, no role of ABA is identified in the stomatal response to diurnal or transpiration-rate changes under well-watered conditions. These results are thus consistent with the traditional understanding of ABA as a "stress hormone", even though ABA functions in plant developmental processes in the absence of stress.

CHAPTER 2

IS ABA INVOLVED IN INITIATING STOMATAL CLOSURE UNDER FLOODING IN *VICIA FABA*?

Introduction

Flooding is one of the major unfavorable growth conditions (Boyer 1982) that severely limit plant productivity (Dennis *et al.* 2000) and species distribution. To survive flooding, plants possess several adaptive responses (reviewed in Jackson 2002; Subbaiah & Sachs 2003; Visser & Voesenek 2004), including stomatal closure. Stomatal closure prevents large leaf water deficit and persistent wilting, which result from inhibited root hydraulic conductivity by flooding-induced anoxia (Bradford & Hsiao 1982; Else *et al.* 1995; Dell'Amico *et al.* 2001). Although stomatal closure in flooded plants was first observed in the 1970s (Moldau 1973; Pereira & Kozlowski 1977), the underlying mechanisms are not clear.

When plants experience flooding, stomatal aperture size decreases within hours (brief flooding) (Zhang & Davies 1986; Else *et al.* 1995, 2001; Yordanova, Uzunova & Popova 2005) and persists for days (sustained flooding) in many species (reviewed in Sojka 1992). Earlier studies of stomatal regulation in flooded plants have focused on the role of ABA in sustaining stomatal closure. Under sustained flooding, stomatal closure is generally associated with increased leaf-ABA content (reviewed in Sojka 1992; Jackson 1993; also see Olivella *et al.* 2000; Ahmed, Nawata & Sakuratani 2006). Further studies have confirmed this by demonstrating that the stomatal response to sustained flooding is impaired in ABA-deficient mutants (Jackson & Hall 1987; Jackson 1993). However, how stomatal closure is initiated under brief flooding is poorly studied and whether ABA is involved in this process is not clear.

In determining ABA involvement in the stomatal response to brief flooding, measurements of the ABA contents in the symplast and apoplast of guard cells are critical because ABA receptors are localized both in the cytosol and on the plasma membrane of guard cells (reviewed in Pei & Kuchitsu 2005; Finkelstein 2006). The guard-cell ABA pool has three potential sources: (1) xylem ABA import (Davies & Zhang 1991), which is the product of xylem ABA concentration and transpiration rate; (2) leaf ABA redistribution, which could result from increased leaf ABA content (Popova *et al.* 2000) or xylem alkalinization according to one postulate (Schurr *et al.* 1992; Wilkinson & Davies 1997; Sauter *et al.* 2001; Davies *et al.* 2005); (3) guard-cell ABA biosynthesis (Cornish & Zeevaart 1986; Koiwai *et al.* 2004). In this chapter, all the above factors that potentially contribute to the guard-cell ABA pool as well as stomatal aperture size were examined in a detailed time course after brief flooding. The guard-cell ABA contents were then determined at time identified by the time-course studies, when ABA in the leaf and the leaf apoplast increased, xylem became alkalinized, and stomatal aperture size decreased. The results not only provide direct evidence whether ABA is involved in initiating stomatal closure under brief flooding, but also provide evidence for evaluating the postulate that xylem alkalinization increases ABA fluxes to guard cells by leaf redistribution (Schurr *et al.* 1992; Wilkinson & Davies 1997; Sauter *et al.* 2001; Davies *et al.* 2005). *Vicia faba* was used in this study because it is a model species for stomatal studies and it has large guard cells that are easy to dissect for the ABA assay.

Materials and Methods

Plant material

Broad bean (*Vicia faba* cv Longpod) plants were grown in Fafard medium No. 2 in 1-L pots in a growth chamber (EGC, Chagrin Falls, OH), at a density of two plants per pot. Day/night temperature was 25/20 ºC, and RH was 60%. Light was provided by fluorescent and incandescent lamps with a wattage ratio (fluorescent to incandescent) of

~ 6, the maximum PAR at plant level was ~ 550 µmol photons m^{-2} s^{-1}. Temperature and light were both ramped as in Lu *et al.* (1997). Plants were watered daily with half-strength of Peter's All Purpose Fertilizer (J. R. Peters, Inc., Allentown, PA). Three-week-old plants were used in all experiments.

Flooding treatments

The pots were submerged in 25 ºC tap water in larger plastic containers, and the water level was maintained at the soil surface. Flooding started either at 0800 h or at 1000 h and lasted for one to six hours, as indicated.

Physiological measurements and tissue collections

After plants were flooded for 1–6 h (flooding started at 0800 h), the following parameters were measured and leaf apoplastic sap was collected for ABA assay.

Leaf water potential

The youngest fully expanded leaves with petioles were detached from plants, and water potential was measured using a pressure chamber (PMS Instrument Co., Corvallis, OR).

Leaf apoplastic pH and leaf apoplastic ABA

After leaf-water-potential measurements, a 0.2–0.4 MPa in excess of the water-potential-balancing pressure was applied to the detached leaves and leaf apoplastic sap (~15 µl) was collected using the pressure chamber. The initial 2–3 µl was discarded to remove the contaminants from damaged cells. The pH of the leaf apoplastic sap was measured with an Orion combination glass micro-pH electrode (VWR International, West Chester, PA) connected to a pH meter. Thereafter, leaf apoplastic sap was stored at -80 ºC until ABA assay (see below).

Stomatal aperture size

The aperture sizes of 30 stomata were measured from abaxial epidermis under a light microscope.

In accordance with the reasons mentioned in the *Introduction*, tissues for guard-cell ABA assays and leaf and root-tip samples were collected after 4-h flooding (flooding started at 1000 h). All the samples for ABA assays were stored at -80 ºC until use.

Sample collection and preparation for ABA assay

Leaf, root-tip, total-guard-cell, and the guard-cell-symplastic ABA

A 4 cm^2 sample devoid of mid-vein and leaf edges was excised, frozen in liquid-N_2 slurry, and broken into ~ 4 mm^2 fragments for leaf and total-guard-cell ABA assay, respectively. Abaxial epidermis (~1 cm^2) for the guard-cell-symplastic ABA assay was peeled from the fresh leaflet, brushed, and floated on ice water for 2 min to remove the apoplastic contents (Zhang & Outlaw 2001b). Then the abaxial epidermis was frozen in liquid-N_2 slurry. Root tips (~ 0.3 cm) were excised and frozen in liquid-N_2 slurry.

Frozen leaf fragments (~ 6 mg) and root tips (~ 10 mg) for leaf and root ABA assays were weighed quickly and homogenized three times in 80 µl extraction solution (80% (v/v) aqueous methanol containing 0.001% (w/v) 2, 6-di-*t*-butyl-*p*-cresol). The homogenate was placed in darkness at 4 ºC for 12 h, and then centrifuged at low speed for 2 min. The supernatant was dried under N_2 and redissolved in ~ 400 µl Tris-buffered saline (50 mM Tris, 1 mM $MgCl_2$, 150 mM NaCl, pH 8.1).

All frozen tissues for total-guard-cell- and guard-cell-symplastic-ABA assays were freeze-dried at -35 ºC and stored at -20 ºC in vacuumed tubes. For each sample, guard cells from 4–6 plants (~ 200 pairs) were randomly dissected from leaf and rinsed epidermal tissues, respectively, in a controlled environment (18 ºC, 30–40% RH). The cells were placed in a cluster inside a horizontal 6 × 50-mm silanized borosilicate tube, covered with a small drop (~ 0.4 µl) of extraction solution (same as used for the leaf ABA extraction) and sealed with parafilm. The extraction was kept in the dark at 4 ºC overnight. After opening the tube, the small droplet of the extraction solution evaporated

quickly and the dried guard-cell preparation was redissolved in 1.7 µl methanolic Tris-buffered saline.

ABA assay

Leaf, root and guard-cell preparations for ABA assay, and leaf apoplastic sap that was used directly without processing, were analyzed in 0.5 µl triplicate using ELISA (Harris *et al.* 1988, modified by Zhang, Hite & Outlaw 1991).

Guard cells dissected from freeze-dried leaflets contain both symplastic and apoplastic ABA contents, whereas cells dissected from rinsed epidermal peels contain only symplastic contents. The guard-cell-apoplastic ABA content was calculated by subtraction of the guard-cell-symplastic ABA content from total-guard-cell ABA content (Zhang & Outlaw 2001a, b, c). Calculated errors for the apoplastic ABA contents are $S.E._{apoplast} = \sqrt{(S.E._{symplast})^2 + (S.E._{total})^2}$.

Results

Stomatal responses to brief flooding in *Vicia faba*

After preliminary experiments to determine when stomatal closure occurs in flooded *Vicia faba*, studies focused on 1–6 h flooding period to identify the earliest stomatal response for further mechanism studies. Leaf water potential was also measured to determine whether hydraulic signals contribute to the stomatal regulation after brief flooding.

Stomatal aperture size did not change after the first 1 or 2-h of flooding (Fig. 2.1, $P \geq 0.12$), but it decreased from 8.5 ± 0.9 to 5.5 ± 0.8 µm after 4-h flooding ($P = 0.03$) and the closure persisted for another 2 h tested ($P = 0.03$).

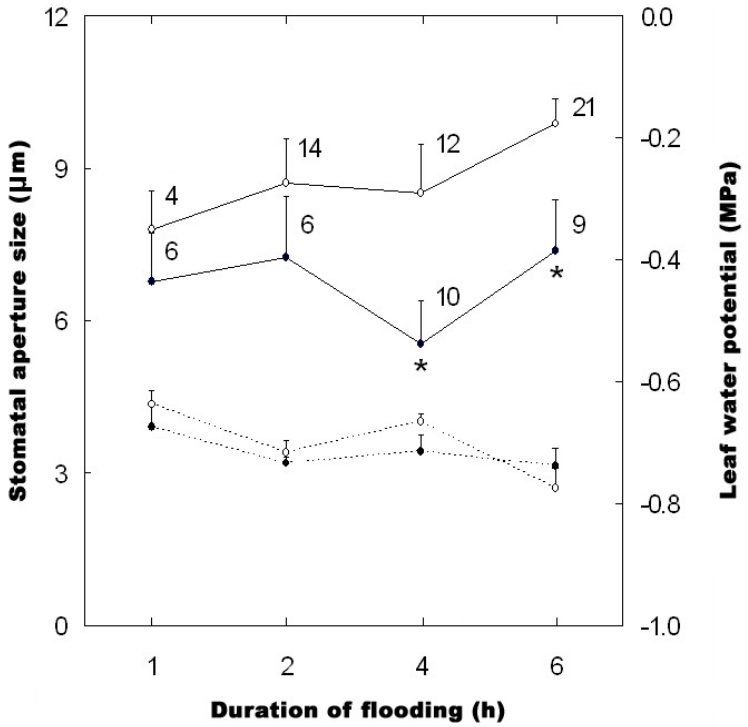

Figure 2.1 Effects of brief flooding on stomatal aperture size (solid lines) and leaf water potential (dotted lines) in *Vicia faba.* Flooding started at 0800 h. Open circles, non-flooded; filled circles, flooded. Number of plants is indicated in graph next to symbols (stomatal aperture size and leaf water potential were studied in pairs). Error bars are SE. * indicates a significant difference ($P < 0.05$) from its corresponding non-flooded control.

Leaf water potential was -0.7 ~ -0.8 MPa in non-flooded *Vicia faba* and remained unchanged after 1–6 h flooding (Fig. 2.1). These results indicate that chemical signals, not hydraulic signals, dominate in the stomatal regulation under flooding. The same conclusion applies to many of the species studied under both brief and sustained flooding (Jackson 1993 and references therein; Else *et al.* 1995). However, exceptions can be found (e.g. Else *et al.* 2001; Nicolás *et al.* 2005).

Changes of ABA contents in the leaf, leaf apoplast, and root after brief flooding in *Vicia faba*

The guard-cell ABA pool is critical in understanding whether ABA is involved in initiating stomatal closure in flooded plants. Therefore, the leaf and leaf apoplastic ABA

contents, which potentially contribute to the guard-cell ABA pool, were studied after brief flooding. The xylem ABA delivery rate was calculated by multiplying the leaf apoplastic ABA concentration with transpiration rate (see the *Materials and Methods* of Chapter 1). In addition, the root ABA was determined to help identify the sources for the leaf-ABA changes under brief flooding.

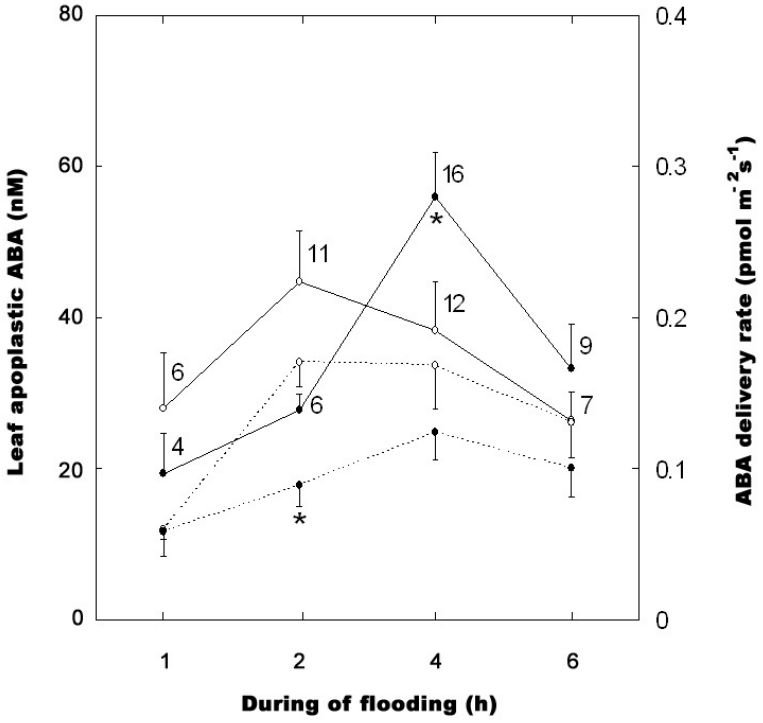

Figure 2.2 Changes of leaf apoplastic ABA concentration (solid lines) and the xylem ABA delivery rate (dotted lines) under brief flooding in *Vicia faba.* Flooding started at 0800 h. Open circles, non-flooded; filled circles, flooded. Number of plants is indicated next to symbols. Error bars are SE. * indicates a significant difference ($P < 0.05$) from its corresponding non-flooded control.

Within 1–6 h flooding, the leaf apoplastic ABA only transiently increased from 38 ± 6 nM to 55 ± 6 nM at 4-h flooding (Fig. 2.2, $P < 0.05$). The xylem ABA delivery rate was generally constant after 1–6 flooding (Fig. 2.2), except for a transient decrease at 2-h flooding ($P < 0.01$). The decrease resulted from the combination of the two insignificant decreases in stomatal aperture size and leaf apoplastic ABA concentration.

For non-flooded plants, both the leaf apoplastic ABA concentration and the xylem ABA delivery rate varied over the course of the day (Fig. 2.2). Their variation patterns are generally consistent with those from other studies (see the *Results* of Chapter 1; Correia *et al*. 1995).

When stomata closed after 4-h flooding (Fig. 2.1), the leaf ABA increased approximately 3-fold to 754 ± 110 nmol kg^{-1} fresh mass (Fig. 2.3, $P < 0.01$), but the root-ABA increase was not significant ($P = 0.13$). Taken together with the unchanged xylem ABA delivery rate (Fig. 2.2), these data indicate that the leaf-ABA increase under 4-h flooding did not originate from roots.

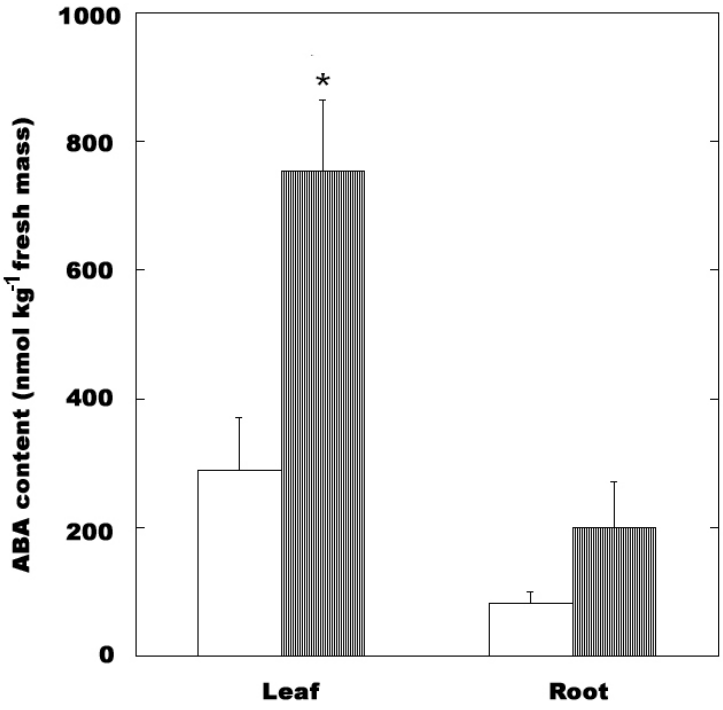

Figure 2.3 Brief flooding increased ABA contents in the leaf (8–16 plants), but not in the root (4–7 plants) of *Vicia faba*. Flooding started at 1000 h. Open bars, non-flooded; filled bars, flooded. Errors are SE. * indicates a significant difference ($P < 0.05$) from its corresponding non-flooded control.

Brief flooding increased leaf apoplastic pH in *Vicia faba*

The constant xylem ABA delivery rate (Fig. 2.2) indicates that xylem ABA import may not contribute to changes of the guard-cell ABA pool under brief flooding. The 3-fold leaf-ABA increase itself probably is not sufficient to increase the guard-cell ABA contents through ABA redistribution (see the *Discussion* of Chapter 1). However, leaf ABA redistribution could also result from xylem alkalinization as postulated (Schurr *et al.* 1992; Wilkinson & Davies 1997; Sauter *et al.* 2001; Davies *et al.* 2005), and xylem alkalinization has been shown to occur in flooded *Lycopersicon esculentum* (Jackson *et al.* 2003; Else *et al.* 2006). Unfortunately, guard cells in *Lycopersicon esculentum* are too small for micro-dissection for ABA assay. Therefore, the contribution of xylem alkalinization in changing the guard-cell ABA pool after brief flooding was examined here with *Vicia faba*. Although the apoplastic pH changes under several stress conditions, the certainty, magnitude, and even direction of such changes are unpredictable for some species and conditions (Schurr & Schulze 1996; Wilkinson 1999). Thus, the first step was to detect apoplastic pH changes after brief flooding in *Vicia faba*.

The leaf apoplastic pH in non-flooded *Vicia faba* was ~ 6.0, and it remained unchanged after 1-h flooding (Fig. 2.4). Within 2-h flooding and before stomata closed (Fig. 2.1), the leaf apoplastic pH increased ~ 0.2 pH units ($P = 0.03$). A similar magnitude of pH increase lasted for another 4 h ($P \leq 0.01$) (Fig. 2.4). According to Slovik & Hartung (1992) and Wilkinson & Davies (1997), an increase of 0.2 pH units in apoplastic pH is sufficient to increase the guard-cell ABA contents to induce stomatal closure. Therefore, xylem alkalinization is a possible initiator of stomatal closure in flooded *Vicia faba*.

The leaf apoplastic pH in non-treated *Vicia faba* from the present study is comparable to that detected in Mühling & Sattelmacher (1995), but it is higher than those from other studies (e.g. Mühling, Wimmer & Goldbach 1998; Mühling *et al.* 1995; Felle & Hanstein 2002). The differences are mainly attributed to different methods of measuring pH (reviewed in Yu, Tang & Kuo 2000). The apoplastic pH is heterogenous and the values measured with different methods probably refer to the pH in different apoplastic spaces**.**

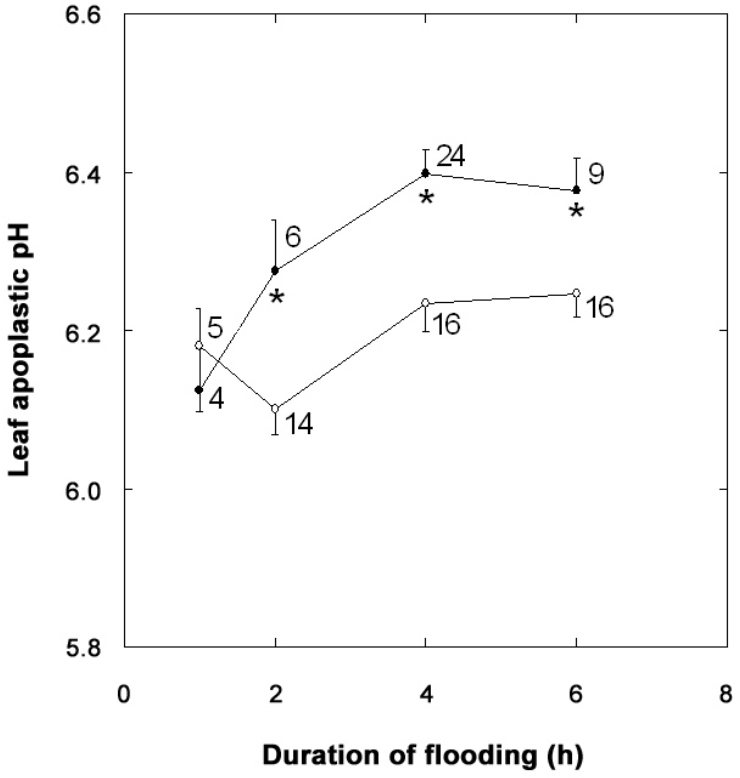

Figure 2.4 Brief flooding increased leaf apoplastic pH in *Vicia faba.* Flooding started at 0800 h. Open circles, non-flooded; filled circles, flooded. Numbers of plants are indicated by symbols. Errors are SE. * indicates a significant difference ($P < 0.05$) from its corresponding non-flooded control.

Brief flooding did not change the ABA contents in total guard cells, the guard-cell symplast, or the guard-cell apoplast in *Vicia faba*

According to the above kinetic studies (Fig. 2.1, Fig. 2.2, Fig. 2.4), flooding for 4 h is the time point at which the guard-cell ABA contents are most likely to increase if ABA is involved in the stomatal response to brief flooding. Since external and internal ABA receptors have been proposed (reviewed in Pei & Kuchitsu 2005; Finkelstein 2006), the ABA contents in the symplast and apoplast of guard cells were both determined after 4-h flooding.

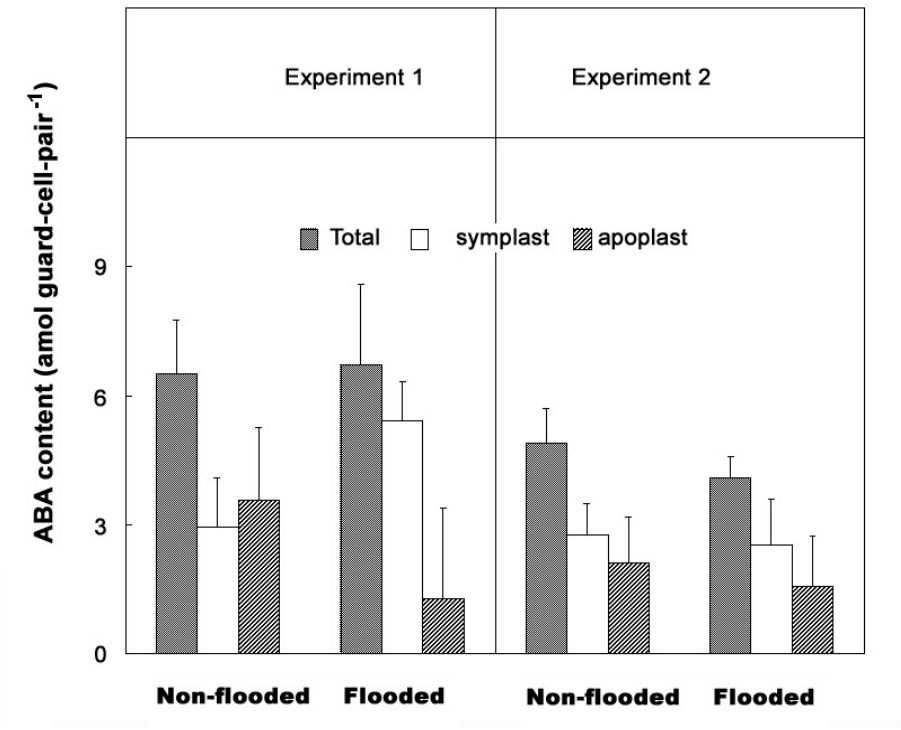

Figure 2.5 Flooding for 4 h did not change the ABA contents in the guard-cell apoplast, the guard-cell symplast, or total guard cells. Flooding started at 1000 h. Each column represents one assay conducted in triplicate (mean ± SE) for total-guard-cell and the guard-cell-symplastic ABA values. Individually dissected guard cells (208–344 pairs) were pooled from 4–6 plants for each assay.

In the two experiments conducted (Fig. 2.5), the guard-cell-apoplastic ABA contents were 1.3 ± 2.1 and 1.6 ± 1.1 amol guard-cell-pair^{-1} after 4-h flooding. The values were not different from those in non-flooded *Vicia faba*, 3.5 ± 1.6 and 2.1 ± 1.1 amol guard-cell-pair^{-1} ($P \geq 0.45$). In addition, 4-h flooding did not cause changes in the guard-cell-symplastic or total-guard-cell ABA content ($P \geq 0.16$ or $P \geq 0.44$). These results indicate that ABA is not an initiator of stomatal closure in flooded *Vicia faba*, and that xylem alkalinization may not induce leaf ABA redistribution to guard cells after brief flooding.

Discussion

Although plants vary greatly in their flooding sensitivities, most species can tolerate flooding for days by adaptive mechanisms in biochemistry, physiology and anatomy. Among the understandings towards these adaptive responses, the underlying mechanisms for stomatal closure are less well-known, especially for stomatal closure under brief flooding. In this study, we tested whether ABA is involved in the stomatal response to brief flooding using *Vicia faba*.

When stomata started to close after 4-h flooding (Fig. 2.1), the ABA contents in the symplast and apoplast of guard cells did not change (Fig. 2.5), although the leaf and leaf apoplastic ABA increased 2–3 fold (Fig. 2.2, Fig. 2.3) and the leaf apoplastic pH increased 0.2 pH units (Fig. 2.4). Thus, a modest increase of the leaf or leaf-apoplastic ABA in *Vicia faba* did not increase in the guard-cell ABA pool under brief flooding, and xylem alkalinization did not induce the leaf ABA redistribution to guard cells as postulated (Schurr *et al.* 1992; Wilkinson & Davies 1997; Sauter *et al.* 2001; Davies *et al.* 2005). The determination of the ABA contents in ABA action sites, the symplast and apoplast of guard cells (reviewed in Pei & Kuchitsu 2005; Finkelstein 2006), provided clear evidence that ABA may not be involved in the stomatal response to brief flooding in *Vicia faba*.

The leaf ABA increased ~ 3-fold after brief flooding in *Vicia faba* (Fig. 2.3). This magnitude of increase is similar to that in some brief-flooding/anoxia studies (Neuman & Smit 1991; Nan, Carman & Salisbury 2002), but larger than that in Else *et al.* (1996). A more dramatic (~ 10-fold) leaf-ABA increase was also reported in a brief-anoxia study. Unfortunately, whether this ABA increase is associated with stomatal closure was not examined (Emel'yanov *et al.* 2003). The leaf-ABA increase from the present study cannot be attributed to ABA import from the roots because neither the root ABA (Fig. 2.3) nor the xylem ABA delivery rate (Fig. 2.2) increased under brief flooding. Although reduced ABA export in the phloem is generally believed to account for the leaf-ABA increase under sustained flooding (Wadman-van Schravendijk & van Andel 1985; Jackson & Hall 1987; Jackson *et al.* 1988; Smit, Neuman & Stachowiak 1990; Castonguay, Nadeau & Simard 1993), it does not account for the leaf-ABA

increase under brief flooding here (preliminary experiments, data not shown). Therefore, it is likely that the leaf-ABA increase detected in the present study is due to the hydrolysis of ABA conjugates in leaves, as in Emel'yanov *et al.* (2003). Overall, the ~ 3-fold leaf-ABA increase was not able to induce the leaf ABA redistribution to guard cells (Fig. 2.5), even at an increased apoplastic pH (Fig. 2.4, see the discussion below for more details).

A transient 2-fold increase of the leaf apoplastic ABA after brief flooding (Fig. 2.2) is similar to that in Neuman & Smit (1991), but different from the findings in Else *et al.* (2001, 2006), in which the leaf apoplastic ABA decreases. These differences probably reflect variations in responses by roots to anoxia. Both ABA production and breakdown require O_2 (Cutler & Krochko 1999), and the balance between ABA production and breakdown determines the root ABA content and the amount of ABA carried in the xylem. This balance may differ in different species and/or under different experimental conditions. When combined with a decrease in stomatal aperture size after brief flooding (Fig. 2.1), even a modest increase of leaf apoplastic ABA did not result in an increase of xylem ABA delivery (Fig. 2.2; Smit *et al.* 1990). Hence, xylem ABA import does not contribute to the stomatal regulation under brief flooding, as under it does under sustained flooding (Smit *et al.* 1990; Else *et al.* 1996; 2001).

The leaf apoplastic pH increased before stomatal closure in flooded *Vicia faba* (Fig. 2.4), as in flooded *Lycopersicon esculentum* (Jackson *et al.* 2003; Else *et al.* 2006). Here the xylem alkalinization did not result in changes in the guard-cell ABA contents (Fig. 2.5). Thus, our results did not support the postulate (Schurr, Gollan & Schulze 1992; Wilkinson & Davies 1997; Sauter *et al.* 2001; Davies *et al.* 2005) that xylem alkalinization induces leaf ABA redistribution to guard cells and results in stomatal closure. Possible reasons for the disagreement between the postulate and our data are explained below.

There are three possible mechanisms for xylem alkalinization to induce leaf ABA redistribution to guard cells: ABA release from the leaf symplast, inhibited ABA uptake to the leaf symplast by ABA diffusion, and inhibited ABA uptake to the leaf symplast by ABA carriers. Firstly, xylem alkalinization does not induce ABA release from the leaf symplast to the guard-cell apoplast in the present study because ABA is released from the

leaf symplast only under severe stress conditions when leaves are dehydrated (Wilkinson 1999). However, leaf water potential did not change and leaves remained turgid under brief flooding in *Vicia faba* (Fig. 2.1). Secondly, inhibition of the ABA diffusive uptake to the leaf symplast by xylem alkalinization may not substantially affect the size of the guard-cell ABA pool. The ABA diffusive uptake to the leaf symplast is based on an anion-trapping model (Hartung & Slovik 1991; Sauter *et al.* 2001; Davies *et al.* 2005). According to this anion-trapping model, ABAH, a lipophilic form of ABA (pKa = 4.7), is present only at a relative low pH. After ABAH is taken up into the more alkaline symplast, it disassociates to ABA^- and is trapped there. At a higher xylem pH, a smaller portion of total ABA is in the ABAH form and ABA uptake into the symplast is decreased. However, 95% of ABA is already dissociated to ABA^- in the xylem at pH 6.0 (xylem pH of non-stressed plants, Fig. 2.4), according to the Henderson-Hasselbalch equation. Therefore, dissociation of the remaining ABA may not appreciably affect the size of the guard-cell ABA pool, regardless of how large the xylem pH increase is. Thirdly, whether the ABA uptake to the leaf symplast by ABA carriers is inhibited by xylem alkalinization is not certain. Although xylem alkalinization (1 pH unit higher, to 7.1) decreases the ABA uptake to isolated *Commelina communis* epidermis by ABA carriers (Wilkinson & Davies 1997), conflicting evidence exists. For example, a higher pH is shown to promote ABA uptake in barley epidermal protoplasts (Daeter & Hartung 1993). Due to the lack of studies conducted on ABA carriers in leaves, it is difficult to reconcile the above conflicting results. A further understanding of the effect of xylem alkalinization on the regulation of leaf ABA redistribution requires more work on the identification and pH regulation of ABA carriers in different species and different leaf compartments. In the present study, xylem alkalinization of 0.2 pH units in *Vicia faba* did not change the guard-cell ABA contents (Fig. 2.5). It is because either xylem alkalinization did not inhibit the ABA-carrier activity, or the magnitude of the xylem pH increase was not sufficient to inhibit the ABA-carrier activity.

Xylem alkalinization of 0.2 pH units is predicted to be sufficient to close stomata (Slovik & Hartung 1992; Wilkinson & Davies 1997). Our results, however, did not support this prediction and we suggest that the effect of xylem alkalinization of 0.2 pH units on stomatal regulation through ABA redistribution cannot be overstated. Although

xylem pH is reported to increase in a larger magnitude for many species and conditions (Wilkinson 1999), an increase of 0.2 pH units or less is not rare. Some examples are: maize (Bahrun *et al.* 2002; Goodger *et al.* 2005) and tobacco (Borel & Simonneau 2002) under drought, pepper under nitrate stress (Dodd, Tan & He 2003), and barley under cold stress (Felle *et al.* 2005). In addition, the xylem pH increase, which we presented as *an average,* seemed to be an underestimate compared to *the pH range* presented by other researchers. For example, xylem pH is reported to be 5.8 ~ 6.6 in non-stressed sunflowers and to reach 7.1 under drought conditions (Gollan, Schurr & Schulze 1992). Our calculation, on the other hand, showed an average increase of 0.2 ~0.3 pH units according to the researchers' data (Gollan, Schurr & Schulze 1992, Fig. 5).

Since xylem pH increases in response to several stress conditions that can induce stomatal closure (Wilkinson 1999, also see Wan *et al.* 2004 for cold), pH signals may not be simply categorized based on the magnitude of pH increases. It is also possible that xylem alkalinization may induce stomatal closure through other mechanisms other than through the regulation of leaf ABA redistribution. Xylem alkalinization, for instance, may change the stomatal sensitivity to ABA *in planta*, as in isolated epidermis (Paterson, Weyers & A'Brook 1988; Prokic *et al.* 2006), and lead to stomatal closure in flooded plants. However, a more thorough understanding of pH-affected stomatal sensitivity to ABA largely relies on the understanding of guard-cell ABA receptors, because the effect of pH on stomatal sensitivity to ABA is presumably determined by its influence on guard-cell ABA receptors. Recent progress on ABA receptors (Razem *et al.* 2006; Shen *et al.* 2006) may help characterize ABA receptors in guard cells, which would help resolve the mystery of the stomatal sensitivity to ABA affected not only by pH, but also by other factors, such as N/P-deficiency, CO_2, temperature, and drought (Peng & Weyers 1994 and references therein). Incidentally, N/P-deficiency also occurs after brief flooding (Jackson *et al.* 2003).

The effect of xylem alkalinization on the stomatal regulation is often tested by transpiration bioassays, i.e. detached leaves are infused with a high-pH buffer and a low-pH buffer, and transpiration rates are compared between those two infusions (e.g. Wilkinson & Davies 1997; Wilkinson *et al.* 1998; Dodd *et al.* 2003; Jackson *et al.* 2003). Using the same method, we did not measure an effect of high pH (1 pH unit

higher, to 7.1) on the transpiration rate of detached *Vicia faba* leaves (data not shown). However, the absence of an effect of high pH on transpiration rate could be due to the small effect of high-pH buffer infusion on the apoplastic pH. The apoplastic pH in the substomatal cavity changed less than 0.1 pH unit per pH unit imposed when the same transpiration bioassay systems were used by Felle's group (Felle & Hanstein 2002; Felle *et al.* 2005). Therefore, the effect of xylem alkalinization on the stomatal regulation may not be revealed by transpiration bioassays and the data from these bioassays have to be either confirmed or re-examined by other approaches.

To date, no clear evidence emerges for how stomatal closure is initiated under flooding. Except for the potential changes of the stomatal sensitivity to ABA by xylem alkalinization and/or N/P-deficiency as mentioned above, an unidentified xylem-sap component was recently seen implicated to account for stomatal closure under brief flooding (Else *et al.* 2006). In addition, recent (Liu *et al.* 2005) and future microarray studies on the early anoxia responses may be able to provide clues on which signaling components should be focused in understanding the stomatal response to brief flooding.

CONCLUSIONS

This dissertation focused on clarifying the roles of ABA in the stomatal response to three water-sufficient conditions: diurnal changes, humidity (i.e. transpiration rate), and brief flooding, by using a model species for stomatal studies (*Vicia faba*). The rationales for conducting these studies were: first, ABA is a well-known player in the stomatal regulation (Sauter *et al.* 2001; Davies *et al.* 2005). Second, the roles of ABA in the stomatal response to these three water-sufficient conditions have either been proposed (Tallman 2004; Jackson 2002) or tested (Assmann *et al.* 2000; Xie *et al.* 2006), but not yet established.

To provide unambiguous evidence, we determined the ABA contents in the ABA perception sites, i.e. the symplast and apoplast of guard cells (reviewed in Pei & Kuchitsu 2005; Finkelstein 2006). The determinations of leaf ABA and leaf apoplastic ABA, and leaf apoplastic pH furthered understanding of the regulation of the guard-cell ABA pool under the above three conditions.

Chapter one reported the absence of a role of ABA in the stomatal response to two well-watered conditions, i.e. diurnal changes and humidity-induced transpiration rate changes. (1) During the stomatal response to diurnal changes, no changes in the guard-cell-symplastic or -apoplastic ABA contents were detected at the three time points during the day (0600 h, 1400 h, and 1800 h), although the leaf and leaf apoplastic ABA exhibited 2–3-fold changes. We conclude that ABA is probably not involved in stomatal diurnal regulation. The guard-cell ABA data did not support Tallman's hypothetical model (Tallman 2004), in which the ABA contents in the symplast and apoplast of guard cells both change diurnally. Therefore, the regulation of ABA catabolism in guard cells by light/darkness, as proposed in this model, needs to be re-examined more carefully. The hypothetical changes of the guard-cell-apoplastic ABA content by midday (Tallman 2004), which are proposed to be transpiration-dependent, are unlikely to occur. This conclusion was confirmed by transpiration-rate studies as below. (2) In the stomatal

response to a transpiration-rate decrease induced by RH shift (60% to 90% RH), the ABA contents in the leaf, leaf xylem, and the guard-cell symplast and guard-cell apoplast of non-stressed intact plants did not change, in contrast to a 3-μm increase in stomatal aperture size. Similarly, the ABA contents of the four compartments were not changed by the same RH shift in detached leaves infused with 1 μM ABA. We conclude that ABA probably is not required in the stomatal response to transpiration rate. This conclusion is consistent with Assmann *et al.* (2000), but disagrees with Xie *et al.* (2006). The results indicate that the guard-cell-apoplastic ABA content is not affected by transpiration rate probably because of ABA modulation by leaf compartments in the transpiration pathway.

Chapter two reported the absence of a role of ABA in the stomatal response to brief flooding, a water-excessive stress. After brief (4-h) flooding, stomatal aperture size decreased 3 μm, when the leaf and the leaf xylem ABA increased 2–3 fold and the xylem pH increased 0.2 pH units. The ABA contents in the symplast and apoplast of guard cells in flooded *Vicia faba* were not different from those of non-flooded plants. We conclude that ABA is not an initiator of stomatal closure under flooding, and xylem alkalinization does not induce the leaf ABA redistribution to guard cells. These results, consistent with a recent report by Else *et al.* (2006), disagree with the postulate proposed earlier (Schurr *et al.* 1992; Wilkinson & Davies 1997; Sauter *et al.* 2001; Davies *et al.* 2005). Therefore, the theoretical basis for this postulation, the anion-trapping model (Hartung & Slovik 1991; Sauter *et al.* 2001; Davies *et al.* 2005), may deserve reconsideration.

Overall, ABA does not play a role in the stomatal regulation under two well-watered conditions (diurnal and transpiration-rate changes), which is consistent with the classical understanding of ABA as a "stress hormone". However, this classical understanding is challenged by our flooding study, i.e., ABA is not involved in the stomatal response to some stressed conditions. The same challenge is also imposed by Wilkinson, Clephan & Davies (2001), who demonstrated an absence of a role of ABA in the stomatal response to cold stress. In addition, we conclude that a modest increase (2–3 fold) of leaf or leaf-apoplastic ABA, or leaf apoplastic pH (0.2 pH units) is not able to change the size of the guard-cell ABA pool, and thus cannot contribute to stomatal regulation.

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Meng FX, Outlaw WH Jr. ABA contents in the guard-cell symplast and guard-cell apoplast do not correlate with stomatal aperture size under two well-watered conditions. (In preparation)

Meng FX, Outlaw WH Jr. Is ABA involved in initiating stomatal closure under flooding in *Vicia faba*? (In preparation)

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Meng FX, Liu X, Zhang SQ, Lou CH (2001) Localization of muscarinic acetylcholine receptor in plant guard cells. Chinese Science Bulletin 46, 586-588.

CONFERENCE PRESENTATIONS

Meng FX, Outlaw WH Jr., Du Z, Aghoram K, Riddle KA. The role of cytosolic alkalinization in the regulation of phosphorylation of guard-cell phosphoenolpyruvate carboxylase in *Vicia faba* L.
ASPB (Southern Section) annual meeting, Spring 2002

Meng FX, Outlaw WH Jr. No role for ABA identified in diurnal stomatal movement or transpiration-rate-dependent stomatal responses in non-stressed *Vicia faba*.
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