RESEARCH PAPER

The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity

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Received 31 July 2006; Revised 7 October 2006; Accepted 16 October 2006

Abstract

In this study, the role of abscisic acid (ABA) in altered stomatal responses of *Tradescantia virginiana* leaves grown at high relative air humidity (RH) was investigated. A lower ABA concentration was found in leaves grown at high RH compared with leaves grown at moderate RH. As a result of a daily application of 20 \(\mu\)M ABA to leaves for 3 weeks during growth at high RH, the stomata of ABA-treated leaves grown at high RH showed the same behaviour as did the stomata of leaves grown at moderate RH. For example, they closed rapidly when exposed to desiccation. Providing a high RH around a single leaf of a plant during growth at moderate RH changed the stomatal responses of this leaf. The stomata in this leaf grown at high RH did not close completely in response to desiccation in contrast to the stomata of the other leaves from the same plant. The ABA concentration on a fresh weight basis, though not on a dry weight basis, of this leaf was significantly lower than that of the others. Moreover, less closure of stomata was found in the older leaves of plants grown at high RH in response to desiccation compared with younger leaves. This was correlated with a lower ABA concentration in these leaves on a fresh weight basis, though not on a dry weight basis. Stomata of leaves grown at moderate RH closed in response to short-term application of ABA or sodium nitroprusside (SNP), while for leaves grown at high RH there was a clear difference in stomatal responses between the leaf margins and main-vein areas. The stomatal aperture in response to short-term application of ABA or SNP at the leaf margins of leaves grown at high RH remained significantly wider than in the main-vein areas. It was concluded that: (i) a long-term low ABA concentration in well-watered plants during growth at high RH could be a reason for less or no stomatal closure under conditions of drought stress; and (ii) the long-term ABA concentration on a fresh weight basis rather than on a dry weight basis is likely to be responsible for structural or physiological changes in stomata during leaf growth.

Key words: Chlorophyll fluorescence, nitric oxide, PSII efficiency, stomata, vapour pressure deficit.

Introduction

Stomatal movement (producing changes in stomatal aperture) is a complex result of interactions of physiological factors and environmental conditions (Kearns and Assmann, 1993; Assmann and Wang, 2001; Hetherington and Woodward, 2003). Besides short-term conditions, stomatal movement also depends on the growth conditions in which the stomata developed, of which one of the most important is relative air humidity (RH). For example, a lack of stomatal closure under conditions of water stress has been reported in roses grown at RHs >85\% (Torre and Fjeld, 2001; Torre et al., 2003). Similarly, a failure of stomata to close in response to desiccation or abscisic acid (ABA) has been shown in leafy cuttings rooted at high RH (Fordham et al., 2001) and in plants propagated in vitro (Ziv et al., 1987; Santamaria et al., 1993). Recently,
it has been shown that *Tradescantia virginiana* plants grown at high (90%) RH had a higher leaf transpiration rate, and stomatal conductance and aperture than in plants grown at moderate RH (55%) under all treatments expected to cause stomatal closure (Rezaei Nejad and van Meeteren, 2005). The stomata of leaves grown at high RH were less sensitive to decreases in leaf relative water content and water potential (Rezaei Nejad et al., 2006). The reason why stomata of leaves grown at high RH are less hydrosensitive is not clear. There are several reports about the stomatal responses to short-term local changes in RH around the leaf (Haefner et al., 1997; Mott and Franks, 2001). However, to our knowledge, there has been no report where the effect of long-term local changes in RH around the leaf on stomatal response characteristics has been investigated.

ABA is a key component of the signal transduction pathway for stomatal closure (reviewed by Leung and Giraudat, 1998). The leaf ABA level is due not only to the synthesis and redistribution of ABA within leaves, but also to synthesis and transport from the roots (Zhang et al., 1997; Popova et al., 2000; Zhang and Outlaw, 2001). The rate at which ABA enters a leaf from the roots is determined by the ABA concentration in the xylem fluid and the transpiration flux of the leaf (Zhang et al., 1997). As the transpiration rate of plants growing at high RH [low vapour pressure deficit (VPD)] is low, it is likely that there is a low concentration of ABA in the leaves of these plants. However, the failure of stomata of excised leaves developed under high RH to close fully in response to ABA application suggests that short-term ABA deficiency was not responsible for the lack of stomatal closure in response to desiccation (Rezaei Nejad and van Meeteren, 2005). Moreover, many studies have suggested that though the short-term effects of elevated ABA concentrations on stomatal functioning are reversible (Ackerson, 1980; Trejo et al., 1995; Tardieu et al., 1996), its long-term effects on developmental changes and functioning of stomata are permanent (Brown et al., 1976; Cutler et al., 1977; Franks and Farquhar, 2001). For example, it has been shown that stomata of plants grown under water stress (which have higher ABA levels) are smaller than those of well-watered plants (Cutler et al., 1977; Spence et al., 1986; Xia, 1994). A daily application of ABA to leaves of well-watered *T. virginiana* plants during growth resulted in the production of smaller stomata with altered physiological properties (Franks and Farquhar, 2001). If high ABA concentrations during growth can change the stomatal anatomy and increase their responsivity to drought stress signals, it might be expected that when plants are subjected to low ABA concentrations during growth, the effect would be a lessening of stomatal responsivity to lowered hydration state. It is also unknown whether the stomata which do not respond to short-term ABA application are able to close in response to other signalling components of ABA-induced stomatal closure, such as nitric oxide (Neill et al., 2002; Garcia-Mata et al., 2003). Notably stomatal response characteristics of plants grown at high RH are not uniform within a leaf: in *Tradescantia*, stomata around the main vein remain open in response to desiccation while stomata at the leaf margins close (Rezaei Nejad et al., 2006). There is, however, not much information about the variability of ABA concentrations or ABA responses within a leaf or within a plant.

**Materials and methods**

**Plant material and growth conditions**

Young *T. virginiana* L. plants were grown in plastic pots (15 cm diameter) filled with a commercial potting compost (Potgrond 4, Hortimea, Lent, The Netherlands) in two growth chambers each with different RH (moderate: 55±5% and high: 90±5%) at Wageningen University. The temperature was 21±0.5 °C, resulting in VPDs of 1.12 kPa and 0.25 kPa for moderate and high RH conditions, respectively. The light intensity was 120±10 μmol m⁻² s⁻¹ (measured with an LI-250 Light Meter, Li-Cor, Lincoln, NE, USA) produced by fluorescent tubes (TLD 33 Philips) with a light period of 16 h per day. Though this light intensity is low, *T. virginiana* is a shade plant, and measurements of its CO₂ fixation/irradiance response showed that 120 μmol m⁻² s⁻¹ is ~40% saturating for CO₂ fixation. The plants were kept well watered and given a nutrient solution weekly (concentration: 2 g l⁻¹; Kristalon™, Yara, Rotterdam, The Netherlands). The CO₂ concentration in the growth chambers was 360±30 μmol mol⁻¹ (measured with a CO₂ analyser ADC225, MK3, Analytical Development Co. Ltd, Hoddesdon, UK).

**Measurements of ABA and water content**

The concentrations of ABA in fresh leaves (not desiccated) grown at moderate or high RH were measured. For ABA analysis, leaves were removed from the plants early in the morning, weighed (fresh weight), freeze-dried, reweighed (dry weight), and finely ground. Distilled water was added at ~3 ml per 50 mg dry weight, vortexed to mix the water and sample, and shaken overnight at 4 °C. The extracts were then centrifuged and the supernatant assayed in an enzyme-linked immunosorment assay (ELISA) for ABA using the MAC252 monoclonal antibody for ABA (Asch, 2000; Bahrun et al., 2002). No cross-reaction of antibody with other compounds was detected when tested (Quarrie et al., 1988; Asch, 2000). Water content was calculated using the following equation:

\[
\text{Water content} = \frac{\text{(fresh weight} - \text{dryweight}) \times 100}{\text{fresh weight}}
\]

**Changes in RH around a leaf of a plant during growth**

To investigate the long-term local effects of high RH on plants growing at moderate RH (55±5%), one emerging leaf from each plant growing at moderate RH was placed inside a glass tube (one leaf per tube). High RH (90±5%) was maintained inside half of the tubes by passing air through temperature-controlled columns of iron (II)-sulphate heptahydrate (Fluka). Air from the climate room (55±5%) was pumped directly into the remaining tubes with leaves. The CO₂ concentration inside the tubes was 360±40 μmol mol⁻¹. After 3 weeks, the leaf grown inside the tube and an adjacent leaf grown outside the tube at moderate RH in each plant...
were used for either chlorophyll fluorescence measurements or ABA analysis.

Stomatal responses to short-term application of ABA and SNP

The spatial heterogeneity of stomatal responses to short-term exogenous ABA in leaves grown at moderate or high RH was measured using a chlorophyll fluorescence imaging system (described further). Once in steady-state, an image of photosystem II (PSII) efficiency was taken from leaves in water (0 μM ABA as a control). Then 1 mM stock solution of (±)-ABA (Sigma) was added to the water to obtain the final concentration of 100 μM, and images were taken every 30 min for 150 min.

Stomatal aperture in response to exogenous ABA and sodium nitroprusside (SNP as a nitric oxide donor) (Neill et al., 2002) was determined with epidermal strips. Epidermal strips were removed from the margins and main-vein areas of the abaxial surfaces of eight leaves from eight randomly selected plants grown at moderate or high RH and cut into 5 mm × 10 mm pieces (Weyers and Meidner, 1990). One leaf per plant and one strip per location per leaf for each treatment were used. The strips were preincubated for 2 h in a stomata-opening medium (10 mM MES-KOH, pH 6.15, 50 mM KCl) in a test room (20 °C and 100 μmol m⁻² s⁻¹ irradiance). The strips were then incubated in a bath medium containing 10 mM MES-KOH, pH 6.15, 50 mM KCl and 0 μM ABA (control), 100 μM ABA, 200 μM SNP (Fluka), or 100 μM ABA + 200 μM SNP (only for leaves grown at high RH) for 1 h. Stomatal aperture of 10 randomly selected stomata in each strip was measured from digitized video images (×800 magnification) of stomata using a microscope (Leica, Aristoplan) connected to a Nikon digital imaging camera DXM-1200. Image processing was done using the free UTHSCSA ImageTool program (University of Texas Health Science Centre at San Antonio, TX, USA).

Stomatal response characteristics to long-term ABA application during growth

To investigate the effects of long-term ABA application on stomatal response characteristics, both sides of one emerging leaf from half of the plants in each climate room (moderate and high RH) were treated daily with a solution of 20 μM ABA in distilled water and two drops of Triton X-100 per litre. The remaining untreated plants (control) were painted with distilled water/Triton X-100 solution in the same manner as the treated plants. The treatment lasted 3 weeks in total. The treated leaves from seven plants (one leaf per plant) were used for chlorophyll fluorescence measurements. The ABA treatment was stopped 24 h prior to chlorophyll fluorescence measurements. Using an AP3 porometer (Delta-T Devices Ltd, Cambridge, UK), it was confirmed that the application of ABA induced short-term closure of stomata which lasted only for a few hours. Stomatal conductance completely recovered to the value of the control leaves within 24 h after the ABA treatment.

Mapping of PSII photochemical yield using chlorophyll fluorescence imaging

To study the effects of RH conditions or ABA treatments on stomatal response characteristics, leaves were removed from the plants, re-cut under water, and transferred to the laboratory. From these leaves, chlorophyll fluorescence images were made under an atmosphere of 20 mmol mol⁻¹ O₂, 350 μmol mol⁻¹ CO₂, and the remainder N₂ (a non-photorespiratory condition) as described elsewhere (Rezaei Nejad et al., 2006). Under non-photorespiratory conditions, ΦPSII is closely related to stomatal closure (Rezaei Nejad et al., 2006). To examine whether the steady-state was reached, the leaves were kept side by side inside a gas-tight cuvette under a continuous actinic irradiance of 100 μmol m⁻² s⁻¹ for ~20 min. Images of the leaves were then made at 5 min intervals and, when there was no significant difference between consecutive images, the leaves were considered to be at steady-state. After a steady-state was reached, the first image that was taken from the leaves (which were still in water) served as a control. The desiccation process was begun by removing the leaves from water, and images were then taken every 30 min for 150 min. The relative humidity in the air flowing through the cuvette was 40±2% and was produced by passing the air through a temperature-controlled column of iron (II)-sulphate heptahydrate (Fluka). The cuvette temperature was 22±1 °C. The experiments were repeated at least six times.

Statistical analysis

Each experiment was carried out with at least six leaves from six plants (one leaf per plant). Data were subjected to analysis of variance (ANOVA). Data in Figs 2, 4, and 6 were analysed using repeated measures ANOVA. The Student’s t-test was used for mean separation (P=0.05). GraphPad Prism 4 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analyses.

Results

The endogenous ABA level of the leaves was affected by the RH during growth (Table 1). The ABA concentration was significantly higher in fresh (not desiccated) leaves grown at moderate RH compared with leaves grown at high RH when expressed both on a dry weight basis (P=0.027) and on a fresh weight basis (P=0.0007). The water content was significantly lower in leaves grown at moderate RH compared with leaves grown at high RH (P<0.0001).

Figure 1 shows the images of ΦPSII of the second, fourth, and sixth leaf in basipetal sequence from the shoot tip in plants grown at high RH. Before desiccation and under a non-photorespiratory condition, ΦPSII was high and homogeneously distributed over the leaves irrespective of leaf age, implying the opening of stomata in all leaves (Fig. 1A). After 150 min of desiccation, ΦPSII decreased in all leaves but to different extents (Fig. 1B). The lower ΦPSII in the second leaf indicated a greater closure of stomata in younger leaves in response to desiccation. The different stomatal behaviour observed among the leaves from the three different positions (also corresponding to different ages) is shown by the changes in PSII efficiency.

<table>
<thead>
<tr>
<th>RH</th>
<th>ABA concentration pmol g⁻¹ DW</th>
<th>ABA concentration pmol g⁻¹ FW</th>
<th>Water content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55%</td>
<td>3904±392</td>
<td>408±38</td>
<td>89.5±0.1</td>
</tr>
<tr>
<td>90%</td>
<td>2772±192</td>
<td>190±24</td>
<td>93.2±0.6</td>
</tr>
</tbody>
</table>

Table 1. ABA level and water content in fresh (not desiccated) Tradescantia virginiana leaves grown at moderate (55%) or high (90%) RH

The ABA concentrations are expressed on dry weight (DW) and fresh weight (FW) bases. Values are the mean of six leaves ± SEM.
in average $\Phi_{\text{PSII}}$ measured under low O$_2$ concentration over time of desiccation (Fig. 2). Before desiccation (time 0), $\Phi_{\text{PSII}}$ in all leaves was high and there was no significant difference among them. With desiccation, $\Phi_{\text{PSII}}$ of younger leaves decreased sooner than it did for older leaves. The interaction between the effects of leaf age and duration of desiccation on $\Phi_{\text{PSII}}$ was significant ($P=0.001$). There were no significant differences in ABA concentration on a dry weight basis of leaves of the three different ages (Table 2). However, significantly higher ABA concentrations on a fresh weight basis were found in younger leaves compared with older leaves ($P=0.0001$).

Fig. 1. Images of $\Phi_{\text{PSII}}$ of leaves in water (A) and after 150 min desiccation (B) measured under 20 mmol mol$^{-1}$ O$_2$, 350 μmol mol$^{-1}$ CO$_2$ in the second (right leaf in each image), fourth (middle leaf in each image), and sixth (left leaf in each image) leaf in basipetal sequence from the shoot tip in Tradescantia virginiana plants grown at 90% RH.

Fig. 2. PSII efficiency ($\Phi_{\text{PSII}}$) of the second (squares), fourth (circles), and sixth (triangles) leaf in basipetal sequence from the shoot tip in Tradescantia virginiana plants grown at 90% RH over time of desiccation measured under 20 mmol mol$^{-1}$ O$_2$, 350 μmol mol$^{-1}$ CO$_2$. Values are the mean of six leaves ±SEM.

Table 2. ABA level and water content of the second, fourth, and sixth leaf in basipetal sequence from the shoot tip in Tradescantia virginiana plants grown at 90% RH.

<table>
<thead>
<tr>
<th>Leaf</th>
<th>ABA concentration (pmol g$^{-1}$)</th>
<th>Water content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DW</td>
<td>FW</td>
</tr>
<tr>
<td>Second</td>
<td>2451±148</td>
<td>281±15</td>
</tr>
<tr>
<td>Fourth</td>
<td>2593±164</td>
<td>231±13</td>
</tr>
<tr>
<td>Sixth</td>
<td>2842±137</td>
<td>182±12</td>
</tr>
</tbody>
</table>

The ABA concentrations are expressed on dry weight (DW) and fresh weight (FW) bases. Measurements were done on fresh (not desiccated) leaves. Values are the mean of eight leaves ±SEM.

The water content was significantly lower in younger leaves compared with older leaves ($P<0.0001$).

Figures 3 and 4 show how a high RH maintained around a single leaf of a plant grown at moderate RH can change the trend of $\Phi_{\text{PSII}}$ and thus stomatal behaviour, in response to desiccation. Following desiccation, leaves grown at high RH had both a greater heterogeneity and a higher average value of $\Phi_{\text{PSII}}$ compared with leaves grown at moderate RH or another plant. This implies a slower and smaller closure of stomata in these leaves compared with leaves grown at moderate RH. The interaction between the effects of RH conditions and duration of desiccation on $\Phi_{\text{PSII}}$ was significant ($P<0.0001$). There was no significant difference in ABA concentration on a dry weight basis of leaves grown at either high or moderate RH (Table 3). However, a significantly lower ABA concentration on a fresh weight basis was found in leaves grown in the tubes with high RH compared with leaves grown at moderate RH ($P<0.0001$). The water content was significantly higher in leaves grown in the tubes with high RH compared with leaves grown at moderate RH ($P<0.0001$).
Figure 5 shows the images of $\Phi_{\text{PSII}}$ of leaves treated daily with 0 l Mo or 20 l M ABA during growth at moderate or high RH. Before desiccation and under a non-photorespiratory condition, $\Phi_{\text{PSII}}$ was high and homogeneously distributed over the leaves irrespective of RH and ABA treatments, implying that stomata were open in all leaves (Fig. 5A). After 150 min of desiccation, although $\Phi_{\text{PSII}}$ decreased in all leaves, it was higher in control leaves grown at high RH (Fig. 5B). The ABA-treated leaves grown at high RH showed the same behaviour as did leaves grown at moderate RH with or without ABA application. The changes in average $\Phi_{\text{PSII}}$ over time of desiccation under low O$_2$ concentration in leaves treated with 0 l Mo or 20 l M ABA during growth at moderate or high RH is illustrated in Fig. 6. Before desiccation (time 0), the $\Phi_{\text{PSII}}$ in all leaves was high and there was no significant difference among them. With desiccation, the $\Phi_{\text{PSII}}$ of ABA-treated leaves grown at high RH decreased sooner than it did for control leaves grown at high RH, similar to leaves grown at moderate RH. These results show the similarity of stomatal responses to desiccation in leaves grown at high RH treated with ABA to those of leaves grown at moderate RH. In addition, no significant difference was found between $\Phi_{\text{PSII}}$ of control and ABA-treated leaves grown at moderate RH in response to desiccation.

In a previous study, it was shown that stomatal aperture around the main veins of leaves grown at high RH remained wider after 150 min of desiccation compared with leaf margins (Rezaei Nejad et al., 2006). Therefore, the endogenous ABA concentrations of these regions and also the response of stomata to short-term ABA application were investigated. There was no significant difference in ABA concentration on a dry weight basis between the margins and main-vein areas of the leaves grown at high RH (Table 4). However, significantly higher ABA concentration on a fresh weight basis was found in the margins compared with the main-vein areas ($P=0.04$). The water content was significantly lower in the margins compared with the main-vein areas ($P=0.0006$).

Figure 7 shows the images of $\Phi_{\text{PSII}}$ of leaves grown at moderate or high RH in response to short-term ABA application. Before applying ABA, when the bases of the

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**Fig. 3.** Images of $\Phi_{\text{PSII}}$ of leaves in water (A) and after 150 min desiccation (B) measured under 20 mmol mol$^{-1}$ O$_2$, 350 mmol mol$^{-1}$ CO$_2$ in *Tradescantia virginiana* plants grown at 55% RH. From each plant [plant (1) and plant (2)], there was one leaf inside a glass tube. The RH in this tube was kept at 90±5% [plant (1)] or at 55±5% [plant (2)].

**Fig. 4.** $\Phi_{\text{PSII}}$ of *Tradescantia virginiana* leaves over time of desiccation measured under 20 mmol mol$^{-1}$ O$_2$, 350 mmol mol$^{-1}$ CO$_2$. Triangles represent the $\Phi_{\text{PSII}}$ of the mature leaves from plants of which one of their leaves was grown in a glass tube with high RH (90±5%). Squares represent the $\Phi_{\text{PSII}}$ of the mature leaves from plants of which one of their leaves was grown in a tube with moderate RH (55±5%). From each plant, the leaf grown inside the tube (open symbols) and an adjacent leaf grown outside the tube at moderate RH (closed symbols) were used for the measurements. Values are the mean of eight leaves ±SEM.
leaves were in water and under a non-photorespiratory condition, \( \text{U} \)PSII was high and homogeneously distributed over the leaves irrespective of growth conditions (Fig. 7A). At 150 min after ABA application, \( \text{U} \)PSII decreased in almost all parts of the leaf grown at moderate RH. In the leaf grown at high RH, however, \( \text{U} \)PSII decreased only in the main-vein area and it remained high in the margins and in the tip of the leaf (Fig. 7B). Measurements on several leaves showed the same results, and the difference

<table>
<thead>
<tr>
<th></th>
<th>ABA concentration</th>
<th>Water content (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pmol g(^{-1}) DW</td>
<td>pmol g(^{-1}) FW</td>
</tr>
<tr>
<td>Leaf from plant (1) outside the tube under 55% RH</td>
<td>2265±155</td>
<td>243±12</td>
</tr>
<tr>
<td>Leaf from plant (1) inside the tube under 90% RH</td>
<td>2084±93</td>
<td>161±8</td>
</tr>
<tr>
<td>Leaf from plant (2) outside the tube under 55% RH</td>
<td>2077±95</td>
<td>223±13</td>
</tr>
<tr>
<td>Leaf from plant (2) inside the tube under 55% RH</td>
<td>1974±114</td>
<td>204±17</td>
</tr>
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Fig. 5. Images of \( \Phi_{\text{PSII}} \) of leaves in water (A) and after 150 min desiccation (B) measured under 20 mmol mol\(^{-1}\) \( \text{O}_2 \), 350 \( \mu \text{mol mol}^{-1}\) \( \text{CO}_2 \) in \( \text{Tradescantia virginiana} \) plants grown at 55% or 90% RH. One emerging leaf in each plant was treated daily with 0 (control) or 20 \( \mu \text{M} \) ABA for 3 weeks. The measurements of \( \Phi_{\text{PSII}} \) were done 24 h after the last application of ABA.

Table 3. \( \text{ABA level and water content of fresh (not desiccated) leaves in Tradescantia virginiana plants grown at 55\% RH} \)

The ABA concentrations are expressed on dry weight (DW) and fresh weight (FW) bases. From each plant [plant (1) and plant (2)], there was one leaf inside a glass tube. The RH in this tube was kept at 90±5% [plant (1)] or at 55±5% [plant (2)]. The leaf grown inside the tube and the adjacent leaf grown outside the tube at moderate RH in each plant were used for the measurements. Values are the mean of eight leaves ±SEM.

<table>
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<th>Water content (%)</th>
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<tr>
<td></td>
<td>pmol g(^{-1}) DW</td>
<td>pmol g(^{-1}) FW</td>
</tr>
<tr>
<td>Margin area</td>
<td>2766±393</td>
<td>219±30</td>
</tr>
<tr>
<td>Main-vein area</td>
<td>2298±274</td>
<td>138±17</td>
</tr>
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Fig. 6. \( \Phi_{\text{PSII}} \) efficiency of \( \text{Tradescantia virginiana} \) leaves grown at 55\% (squares) or 90\% (triangles) RH over time of desiccation measured under 20 mmol mol\(^{-1}\) \( \text{O}_2 \), 350 \( \mu \text{mol mol}^{-1}\) \( \text{CO}_2 \). Open and closed symbols represent the \( \Phi_{\text{PSII}} \) of the leaves treated daily with 0 \( \mu \text{M} \) (control) or 20 \( \mu \text{M} \) ABA for 3 weeks, respectively. Values are the mean of seven leaves ±SEM.

Table 4. \( \text{ABA level and water content in the margin and main-vein areas of fresh (not desiccated) Tradescantia virginiana leaves grown at 90\% RH} \)

The ABA concentrations are expressed on dry weight (DW) and fresh weight (FW) bases. Values are the mean of six leaves ±SEM.

<table>
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<th>ABA concentration</th>
<th>Water content (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pmol g(^{-1}) DW</td>
<td>pmol g(^{-1}) FW</td>
</tr>
<tr>
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</tr>
<tr>
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leaves were in water and under a non-photorespiratory condition, \( \Phi_{\text{PSII}} \) was high and homogeneously distributed over the leaves irrespective of growth conditions (Fig. 7A). At 150 min after ABA application, \( \Phi_{\text{PSII}} \) decreased in almost all parts of the leaf grown at moderate RH. In the leaf grown at high RH, however, \( \Phi_{\text{PSII}} \) decreased only in the main-vein area and it remained high in the margins and in the tip of the leaf (Fig. 7B). Measurements on several leaves showed the same results, and the difference
of stomatal closure in response to ABA between leaf margins and main-vein areas were still conspicuous when measurements ended 4 h after the start of the experiment. This implies that stomata in the margins of leaves grown at high RH do not close in response to the short-term ABA application. To ensure that there were no problems with ABA uptake, epidermal strips were bathed in ABA and SNP solutions. The application of ABA or SNP (Fig. 8) decreased stomatal aperture of the margins and main-vein areas of leaves grown at moderate or high RH ($P<0.0001$). There was no significant difference between average stomatal aperture at the margins and main-vein areas of leaves grown at moderate RH in control, ABA, or SNP treatments (Fig. 8A). There was a significant interaction ($P=0.01$) between the location of stomata and the effect of treatments in leaves grown at high RH (Fig. 8B). The average stomatal aperture at the margins of the leaves grown at high RH before application of ABA or SNP (control) was higher than around the main veins ($P=0.03$). Stomatal aperture decreased with the application of ABA or ABA+SNP in both parts of the leaf.

**Fig. 7.** Images of $\Phi_{\text{PSII}}$ of leaves in water (A) and after 150 min in 100 $\mu$M ABA (B) measured under 20 mmol mol$^{-1}$ O$_2$, 350 mmol mol$^{-1}$ CO$_2$ in *Tradescantia virginiana* plants grown at 55% (left leaf in each image) or 90% (right leaf in each image) RH.

**Fig. 8.** Stomatal aperture at the margins (closed bars) and main-vein areas (open bars) of *Tradescantia virginiana* leaves grown at 55% (A) or 90% (B) RH in response to ABA and sodium nitroprusside (SNP as a nitric oxide donor). The concentrations of ABA and SNP solutions were 100 $\mu$M and 200 $\mu$M, respectively. Values are the mean of eight leaves ±SEM. The measurements of stomatal aperture were made on 10 stomata at one location for each area type on each of eight leaves. The average stomatal aperture was then calculated per leaf and the averages from eight leaves were further averaged and the SEM of the mean was calculated.
(P <0.0001), but the average stomatal aperture was significantly wider in the margins (P <0.0001). Moreover, although application of SNP alone caused closure of stomata at the main-vein areas, it did not affect stomatal aperture at the leaf margins.

Discussion

The smaller amount of ABA in fresh leaves grown at high RH (Table 1) was due to (i) the higher water content found in leaves grown at high RH compared with leaves grown at moderate RH, and (ii) the lower amount of ABA expressed on a dry weight basis. It has been shown in well-watered Acer rubrum, an increase in VPD within a few hours resulted in higher accumulation of ABA in leaves (Bauerle et al., 2004). ABA can be synthesized by water-stressed roots and transferred to leaves via the transpiration stream (Davies and Zhang, 1991; Jackson, 1997; Zhang and Outlaw, 2001). However, ABA can be produced by the roots of even unstressed well-watered plants (Wilkinson and Davies, 2002, and references therein). The amount of ABA entering the leaves of plants grown at high RH via transpiration flux would be lower due to the lower transpiration rate of these plants than of plants grown at moderate RH. Another possible explanation would be the effect of VPD around the leaf on the ABA production by the leaf itself.

Drought stress during growth at high RH improves the vase life of cut roses due to a better control of water loss during periods of higher evaporative demands (Mortensen and Gislerød, 2005). Conditions that increase endogenous ABA levels of plants cultured in vitro, such as ventilation of the culture vessels or ABA addition to the medium during growth, improve the control of water loss (higher stomatal responsivity) after transferring them to low RH (Pospisilova, 1996; Aguilar et al., 2000; Talavera et al., 2001). Moreover, it has also been reported that the application of ABA during growth in T. virginiana results in the production of smaller stomata with altered physiological properties (Franks and Farquhar, 2001). The results show that the daily application of ABA to leaves during growth at high RH resulted in stomata whose behaviour in response to desiccation was similar to that found in stomata from plants grown at moderate RH (Figs 5, 6). After 3 weeks of ABA application, the endogenous ABA concentration in the ABA-treated leaves grown at high RH was the same as that of leaves grown at moderate RH (data not shown). It can be concluded from our results that a low ABA concentration during growth at high RH is likely to be the cause of less stomatal closure in response to drought stress.

The ABA concentration expressed on a fresh weight basis, although not on a dry weight basis, of older leaves (Table 2) was significantly lower than in younger leaves, which also showed faster closure of stomata (Figs 1, 2). The decrease in the ABA concentration on a fresh weight basis in the older leaves was correlated with a higher water content of these leaves (Table 2). The ABA concentration on a fresh weight basis, although not on a dry weight basis, of the leaf grown at high RH (Table 3) was significantly lower than that of the other leaves from the same plant grown at moderate RH, which also showed faster closure of stomata in response to desiccation (Figs 3, 4). The lower amount of ABA on a fresh weight basis in the leaves grown at high RH was also correlated with a higher water content (Table 3). According to these results, it seems that there is a correlation between ABA concentration on a fresh weight basis of leaves during growth and stomatal response characteristics. It also indicates that besides differences in ABA production or import, changes in water content of a leaf can be responsible for the differences in stomatal responses.

It has been previously shown that stomata at the leaf margins close faster in response to desiccation compared with main-vein areas in plants grown at a high RH (Rezaei Nejad et al., 2006). This phenomenon has been related to a substantially lower relative water content in these areas of leaves, resulting in a lower turgor of the guard cells rather than a proper functioning of stomata (Rezaei Nejad et al., 2006). A higher stomatal density and initial stomatal aperture would result in a higher transpiration rate and, consequently, lower water content in leaf margins compared with main-vein areas, as discussed by Rezaei Nejad et al. (2006). The failure of stomata at the margins of leaves grown at high RH to close in response to ABA and SNP treatments (Figs 7, 8) is consistent with this conclusion. However, the lack of stomatal closure in main-vein areas of excised leaves in response to desiccation (Rezaei Nejad et al., 2006) could be due to low ABA concentrations in leaves grown at high RH, as the stomata of these areas of leaves could close in response to ABA and SNP treatments (Figs 7, 8). The ABA measurements on the leaf margins and main-vein areas are in contrast to the other findings of this research which showed a correlation between ABA concentration on a fresh weight basis of leaves during growth and stomatal response characteristics. However, the ABA concentration of both parts of leaves grown at high RH are far lower than in leaves grown at moderate RH (Tables 1, 4).

The present results indicate faster responses of stomata to desiccation in younger leaves compared with older leaves (Figs 1, 2). In accordance with these results, it has been reported that the percentage of stomata that closed following exposure of epidermal strips to mannitol, coumarin, and darkness became progressively lower with increasing leaf age in in vitro plum shoots grown at high RH (Zacchini and Morini, 1998). It is also noteworthy that there is a gradient of decreased $\Phi_{PSII}$ from the tip to the base of the youngest leaf, implying differences in the stomatal responses to desiccation between the younger
and older parts of the same leaf (Fig. 1). This indicates that newly developed stomata are functional and need to be exposed to a high RH (or a low ABA level) for some time to become non-functional.

Growing one leaf from a plant grown at moderate RH under a high RH condition resulted in its stomata having a diminished response to drought stress (Figs 3, 4). The stomata of these leaves reacted more slowly and some remained open in response to desiccation, similar to the stomatal responses of the plants where whole plants were grown in a high RH climate room (Rezaei Nejad et al., 2006). The difference between stomatal behaviour of an individual leaf grown at high RH and the other leaves of the same plant grown at moderate RH shows the importance of the micro-climate around individual leaves and indicates the quantitative effect of RH during growth on stomatal functioning. The effect of a moderate RH on stomatal functioning of the whole plant is not transferred to a single leaf grown under a high RH.

In conclusion, according to the results presented here, (i) it is likely that a long-term low ABA concentration in well-watered plants during growth at high RH is a reason for less or no stomatal closure under conditions of drought stress; (ii) ABA concentration on a fresh weight basis rather than on a dry weight basis correlates with the changes in stomatal response characteristics; and (iii) stomata at the leaf margins of leaves grown at high RH are not able to close in the presence of short-term ABA or nitric oxide, which is involved in the signal transduction pathway linking the perception of ABA to reduced guard cell turgor, while stomata at the main-vein areas can.

Acknowledgement

This research was financed by the Ministry of Science, Research, and Technology of Iran.

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