Dynamics of adaptation of stomatal behaviour to moderate or high relative air humidity in *Tradescantia virginiana*

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Abstract

Chlorophyll fluorescence imaging was used to measure stomatal closure in response to desiccation of *Tradescantia virginiana* leaves grown under high (90%) and moderate (55%) relative humidities (RHs), or transferred between these humidities. Stomata in leaves grown at high RH were less responsive to desiccation than those of leaves grown at moderate RH. Stomata of plants transferred from moderate RH conditions to high RH showed the same diminished closure in response to desiccation as did stomata that developed at high RH. This response was found both when the leaves were fully expanded and when still actively expanding during the moderate RH pre-treatment. Four days of exposure to high RH was the minimal exposure time to induce the diminished closure response. When leaves were grown in high RH prior to a 10 d moderate RH treatment, the reduced stomatal closure response to desiccation was only reversed in leaves (regions) which were actively expanding during moderate RH treatment. This indicates that with respect to stomatal responses to desiccation, high RH leaf regions have a limited capacity to adapt to moderate RH conditions. The decrease in responsiveness to desiccation of the stomata, induced by long-term exposure to high RH, was not due to osmotic adjustment in the leaves. Within 1 d after transferring moderate RH-grown plants to a high RH, the abscisic acid (ABA) concentration of their leaves decreased to the low level of ABA found in high RH-grown leaves. The closure response in leaves exposed to high RH for 5 d, however, could not be fully restored by the application of ABA. Transferring plants from high to moderate RH resulted in increased ABA levels within 2 d without a recovery of the stomatal closing response. It is discussed that the diminished stomatal closure in plants exposed to high RH could be due to changes in the signalling pathway for ABA-related closure of stomata or to an increased sequestration of ABA by mesophyll tissue or the symplast in the epidermis, induced by a longer period (several days) of a low ABA level.

Key words: Abscisic acid, desiccation, PSII efficiency, relative water content, stomatal closure, vapour pressure deficit, water potential.

Introduction

Stomata regulate leaf diffusive conductance, and thereby influence two of the most important processes in terrestrial plants: photosynthesis and transpiration. They have to balance the uptake of CO₂ with the loss of water from the plant under various environmental conditions. To obtain the optimal response to multifactorial environmental changes, guard cells of stomata sense many environmental factors such as light (quantity and quality), temperature, humidity, intercellular CO₂ concentration, and drought-induced abscisic acid (ABA) (reviewed by Raschke, 1975; Zeiger, 1983; Schroeder *et al.*, 2001) and have the ability to integrate environmental and endogenous signals. Besides the short-term effects of many environmental factors, the history of growth conditions can affect the fine-tuning of the stomatal response, though these

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Abbreviations: ABA, abscisic acid; Φ<sub>PSII</sub>, relative quantum yield or efficiency for electron transport by photosystem II; PSII, photosystem II; RH, relative air humidity; RWC, relative water content; VPD, vapour pressure deficit.

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acclimation processes are still not completely described and understood. It has been demonstrated that stomata can adapt to water deficits by exposure of plants to water stress cycles. This results in more negative threshold leaf water potentials to initiate stomatal closure (Brown et al., 1976; Ackerson, 1980; Turner and Begg, 1981; Eamus, 1986; Torrecillas et al., 1988; Thomas et al., 2000). Exposing plants for longer periods to high air humidity [low vapour pressure deficit (VPD)] can disturb the control of stomatal opening and closing. For example, a lack of stomatal closure in response to water deficit has been reported in cut roses grown at relative air humidities (RHs) >85% (Torre and Fjeld, 2001; Torre et al., 2003). Similarly, a failure of stomata to close in response to desiccation or to ABA application has been shown in leafy cuttings rooted at a high RH (Fordham et al., 2001a, b) and in in vitro propagated plants (Wardle and Short, 1983; Ziv et al., 1987; Santamaria et al., 1993). It has been shown that Tradescantia virginiana plants grown at high (90%) RH and 20 °C had a higher leaf transpiration rate, and a higher stomatal conductance and aperture than plants grown at moderate RH (55%) when exposed to desiccation, darkness, or ABA application, all treatments that are expected to cause stomatal closure (Rezaei Nejad and van Meeteren, 2005). The stomata of high RH-grown T. virginiana leaves were less responsive to decreases in leaf relative water content (RWC) and water potential (Rezaei Nejad et al., 2006). Time curves during desiccation of the photochemical yield of photosystem II (ΦPSII) measured in non-photorespiratory conditions showed that ΦPSII of T. virginiana leaves grown at both moderate and high RH decreased with desiccation but to different extents; the ΦPSII of moderate RH-grown leaves decreased faster and the reduction was much higher during desiccation (Rezaei Nejad et al., 2006). ΦPSII measured in non-photorespiratory conditions is closely related to stomatal closure (Meyer and Genty, 1998, 1999; Harbinson et al., 2005; Rezaei Nejad et al., 2006).

Water loss and desiccation are the principal causes of plant death in rooted leafy cuttings and micropropagated plants transferred suddenly from a high to moderate or low RH, and of the shortened vase life in cut flowers which have been grown in greenhouses at high RH. In leafy cuttings and micropropagated plants it is common, therefore, to wean the plants gradually from high to low RH. There are many papers describing the adaptation process for both in vitro cultured plants (Wardle et al., 1983; Capellades et al., 1990; Koroch et al., 1997) and rooted leafy cuttings (Fordham et al., 2001a). However, beyond the fact that it occurs, little else is known about the phenomenon of stomatal acclimation to RH. Some authors have reported that the loss of stomatal responsiveness induced by acclimation to high RH can be reversed by placing the plants in a moderate RH (Brainerd and Fuchigami, 1981; Marin et al., 1988), whereas in other cases the loss has been irreversible (Sallanon et al., 1993; Fordham et al., 2001a). The reason why stomata developed at a high RH are less sensitive to water stress is also not clear. A significantly lower endogenous ABA concentration has been reported in T. virginiana leaves grown at a high RH compared with leaves grown at a moderate RH (Rezaei Nejad and van Meeteren, 2007). A daily application of ABA to T. virginiana leaves at a high RH for 3 weeks increased their stomatal closing response to desiccation. It was proposed that a long-term low ABA concentration in well-watered plants during growth at high RH could be a reason for a decreased response of stomata to desiccation. It is unknown to what extent the adaptation of stomatal closing behaviour by a changed RH is correlated with changes in leaf ABA concentration and other leaf hydraulic properties.

The main objectives of this study were as follows. (i) To investigate whether the closure response of stomata to water deficit present in leaves grown at a moderate RH diminishes in response to an increased RH (and vice versa), and if so, how quickly. (ii) To examine whether any decrease in the stomatal closure response induced by high RH is reversible. (iii) To measure the changes in ABA levels of leaves after transfer of plants from moderate to high RH, or vice versa. (iv) To investigate the correlation between the stomatal response characteristics of adapted plants and leaf water status.

Chlorophyll fluorescence imaging under low O2 concentration is a non-destructive and non-invasive technique which allows the temporal dynamics of stomatal closure to be studied. Using an imaging technique is also convenient to study stomatal behaviour of regions within one leaf formed under different environmental conditions when plants are transferred during their cultivation; therefore, the technique was used in this study.

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 RHs (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 the moderate and high RH conditions, respectively. The light intensity in both growth chambers 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 photoperiod of 16 h per day. Measurements of the CO2 fixation/irradiance response of T. virginiana plants 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).
Adaptation of different parts of a growing leaf to moderate or high RH

A young leaf (~1 week old) from each plant growing in a moderate RH climate room was marked and the plants were transferred to a high RH climate room. Ten days after the transfer, the marked leaves were used for the measurement of chlorophyll fluorescence in response to desiccation under a non-photosynthetic condition (described further). These leaves consisted of two parts: (i) the distal parts which were grown firstly at a moderate RH and then exposed to a high RH for 10 d; and (ii) the basal parts which were produced during the 10 d exposure to a high RH. This experiment was repeated with six leaves from six plants. A similar experiment was carried out with leaves which were grown first at a high RH and then transferred to a moderate RH.

Mapping of PSII photochemical yield using chlorophyll fluorescence imaging

The non-destructive and non-invasive chlorophyll fluorescence imaging technique allows the study of temporal dynamics of stomatal closure. In Tradescantia leaves subjected to water stress, measurement of \( \Phi_{\text{PSII}} \) under low \( O_2 \) concentration is very closely related to stomatal closure, as demonstrated by direct measurements of stomatal aperture (Rezaei Nejad et al., 2006). Moreover, the similarity of the mean \( \Phi_{\text{PSII}} \) of leaves before desiccation under normal \( CO_2 \) and after desiccation under high \( CO_2 \) demonstrated that the closure of stomata is the main factor causing lowering \( \Phi_{\text{PSII}} \) after desiccation (Rezaei Nejad et al., 2006). To study the effects of RH conditions during growth on stomatal responses to desiccation or ABA application, 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 m\(^{-1}\) \( O_2 \), 350 \( \mu \)mol m\(^{-1}\) \( CO_2 \), and the remainder \( N_2 \) (a non-photosynthetic condition) as described elsewhere (Rezaei Nejad et al., 2006). The first image of \( \Phi_{\text{PSII}} \) that was taken from the leaves (which were still in water) served as a control. The desiccation process was started by removing the leaves from water, and images were then taken every 30 min for 150 min. The RH 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). Cuvette temperature was 22±1 °C.

Adaptation of mature leaves to high or moderate RH

To examine the time course of adaptation of stomata, 11 plants grown at a moderate RH were randomly selected and labelled as M0, MH1, MH2, .... MH10. Eleven plants grown at a high RH were also randomly selected and labelled as H0, H1, .... H10. The labelled, high RH-grown plants were kept continuously at a high RH and served as control plants. Images of PSII efficiency of a mature leaf from M0 and a mature leaf from H0 were measured under non-photosynthetic conditions during desiccation (day 0). Then the MH1–MH10 plants were transferred to a high RH climate room, and images of PSII efficiency were made during the desiccation of leaves sampled from the appropriate H and MH plants on each of the 10 d after the transfer. To determine whether the adaptation of stomata to high RH can be reversed, after 4–10 d of exposure of MH4–MH10 to high RH, the plants were transferred back to a moderate RH. Six days after the return of the plants to a moderate RH (MH4M6–MH10M6), PSII efficiency images were made of leaf samples during desiccation. This experiment was repeated three times.

Stomatal responses to short-term application of ABA

The spatial heterogeneity of stomatal closure responses to short-term exposure to exogenous ABA in leaves which were grown at a moderate RH followed by 5 d exposure to a high RH, or of control leaves (moderate RH-grown leaves) was measured using the chlorophyll fluorescence imaging system. The first image of PSII efficiency (\( \Phi_{\text{PSII}} \)) was taken from leaves in water, and this served as the control. Then 1 mM stock solution of (+)-ABA (Sigma) was added to the water to obtain a final concentration of 100 \( \mu \)M, and images were taken every 30 min for 150 min. This experiment was repeated with six leaves from six plants in each RH treatment.

Measurements of leaf water potential and relative water content

The relationships between \( \Phi_{\text{PSII}} \), water potential, and RWC were measured on excised leaves which were first imaged while in water or during desiccation using the chlorophyll fluorescence imaging system. Leaf discs were then cut from the leaves and used for the measurements of water potential and RWC as described elsewhere (Rezaei Nejad et al., 2006).

ABA measurements

The ABA levels of leaves of plants transferred from moderate to high humidities and then back, and of leaves of high RH-grown plants (control) were measured daily from day 0 (before the transfer) until day 4 after the transfer. All transferred plants were kept at a high RH for 4 d and then they were transferred back to a moderate RH, and measurements of the ABA level were taken after 1, 2, 3, and 6 d re-exposure to a moderate RH. In total, two leaves per plant were used for the measurements: one leaf during exposure to a high RH and one leaf during re-exposure to a moderate RH (on day 0 only one leaf per plant was used). This experiment was repeated with eight leaves from eight plants for each RH treatment. For the ABA analysis, leaves were removed from the plants early in the morning (~1 h after the start of the daily light period), weighed, freeze-dried, reweighed, 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 immunosorbent 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).

Statistical analysis

Each experiment was carried out with at least three leaves from three plants (one leaf per plant). Data of ABA concentration and \( \Phi_{\text{PSII}} \) were subjected to analysis of variance (ANOVA). Data in Fig. 8 were analysed using repeated measures ANOVA. The Student’s \( t \)-test was used for mean separation (\( P<0.05 \)). The relationships between \( \Phi_{\text{PSII}} \) and time of desiccation (Fig. 2B), \( \Phi_{\text{PSII}} \) and RWC (Fig. 5A), and \( \Phi_{\text{PSII}} \) and water potential (Fig. 5B) were fitted using linear regressions; for the relationship between water potential and RWC (Fig. 5C), linear and non-linear regression were used. The parameters of fitted curves were compared statistically with the \( F \)-test. GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and curve fitting.

Results

Figure 1 shows the images of \( \Phi_{\text{PSII}} \) of leaves in water (A1 and B1) and after 150 min of desiccation (A2 and B2) under non-photosynthetic conditions. In Fig 1A1 and A2, the distal part of the leaf was first grown at a moderate
RH followed by 10 d at a high RH (M→H). The base of the leaf was produced at the high RH (H) during the period of exposure of the distal part to the high RH. In Fig. 1B1 and B2, the distal part of the leaf was first grown at a high RH followed by 10 d at a moderate RH (H→M). The base of the leaf was produced at the moderate RH (M) during the period of exposure of the distal part to the moderate RH. Before desiccation, the Φ_{PSII} was high and homogeneously distributed over the basal and distal regions of the leaves (i.e. irrespective

Fig. 1. Images of PSII efficiency (Φ_{PSII}) of Tradescantia virginiana leaves in water (A1 and B1) and after 150 min of desiccation (A2 and B2) under 20 mmol mol⁻¹ O₂ and 350 μmol mol⁻¹ CO₂. In A, the distal part of the leaf (the part above the black line) was grown at a moderate RH (55%) followed by exposure to a high RH (90%) for 10 d (M→H). The base of the leaf was grown at the high RH during this 10 d period (H). In B, the distal part of the leaf was grown at a high RH followed by exposure to a moderate RH for 10 d (H→M). The base of the leaf was grown at the moderate RH during this period (M).
of the RH treatment), implying that the stomata had opened in both parts of each leaf (Fig. 1A1, B1). After 150 min of desiccation, \( \Phi_{\text{PSII}} \) had decreased in both parts of each leaf (Fig. 1A2, B2). The decrease of \( \Phi_{\text{PSII}} \) in both parts of the leaf in Fig. 1A2 was of the same extent, while the response was different between the two parts of the leaf in Fig. 1B2. The higher \( \Phi_{\text{PSII}} \) in the distal part of the leaf in Fig. 1B2 (H \( \rightarrow \) M) indicated less closure of stomata in response to desiccation.

Irrespective of the duration of desiccation, the mean \( \Phi_{\text{PSII}} \) in the distal parts of the leaves which were first grown at a moderate RH followed by 10 d at a high RH (M \( \rightarrow \) H) was the same as that in the basal regions of the leaves even though the bases of the leaves had grown exclusively at a high RH (H) (Fig. 2A). In leaves which were first grown at a high RH followed by 10 d exposure to a moderate RH (H \( \rightarrow \) M), the decrease in \( \Phi_{\text{PSII}} \) during desiccation in the distal region was less than that in the basal regions which were grown exclusively at moderate RH.

Figure 3 shows how the duration of exposure of fully developed leaves to a high RH affected the trend of \( \Phi_{\text{PSII}} \) and thus stomatal behaviour, in response to desiccation. The high \( \Phi_{\text{PSII}} \) of all leaves in water showed that the stomata opened irrespective of RH treatments (Fig. 3A1, B1, C1). After 150 min desiccation, the leaf developed at a moderate RH and, when transferred to a high RH for 3 d (Fig. 3A2; leaf MH3) had a much lower \( \Phi_{\text{PSII}} \) than the high RH-grown leaf (Fig. 3A2; leaf H3), implying that the stomata of this M \( \rightarrow \) H leaf still closed more quickly in response to desiccation than those of the high RH-grown leaf. A similar rapid decrease of \( \Phi_{\text{PSII}} \) was observed in the moderate RH control plant (day 0) and in the leaves 1 d and 2 d after transfer to a high RH (data not shown).

However, the moderate RH-grown leaf transferred to a high RH for 4 d (Fig. 3B2; MH4) showed a response to desiccation similar to the high RH-grown leaf (H4): the \( \Phi_{\text{PSII}} \) of both leaves remained high in response to desiccation, implying the presence of non-closing stomata and the adaptation of stomatal behaviour of moderate RH-grown leaves to high RH. Similar results were observed 5–10 d after transferring the plants from a moderate to a high RH (data not shown). When a moderate RH-grown plant was kept at a high RH for 4 d and then transferred back to a moderate RH for a further 6 d, the response of \( \Phi_{\text{PSII}} \) to desiccation still remained similar to that of a control plant continuously grown at a high RH (H4). This indicated the irreversibility of the adaptation of the stomatal closure response to high RH (MH4M6; Fig. 3C2). Similar results were observed when moderate RH-grown plants were kept at a high RH for 5–10 d and then transferred back to a moderate RH for a further 6 d (data not shown).
The relative reductions of $\Phi_{PSII}$ [(\(\Phi_{PSII}\) in water - \(\Phi_{PSII}\) after 150 min desiccation)/\(\Phi_{PSII}\) in water] in moderate RH-grown leaves in response to 150 min desiccation from day 0 (before transferring) were significantly higher than in high RH-grown leaves until 3 d after their transfer to a high RH (Fig. 4A). However, the relative reductions of $\Phi_{PSII}$ of these plants 4–10 d after they had been transferred to a high RH were the same as those of high RH-grown plants, implying adaptation of stomatal behaviour to high RH. After 4–10 d exposure of moderate RH-grown
plants to a high RH, the plants were transferred back to a moderate RH and measurements were taken after a further 6 d of exposure to a moderate RH. The relative reductions of $\Phi_{\text{PSII}}$ of these ‘back-transferred’ plants were not significantly different from those of high RH-grown plants, indicating the irreversibility of the adaptation of stomatal closure response to high RH (Fig. 4B).

The adaptation of the stomatal closure response is paralleled by changes in the relationship between $\Phi_{\text{PSII}}$ and water status of the leaf whether measured as RWC or water potential (Fig. 5A, B). At the same values of RWC or water potential, the $\Phi_{\text{PSII}}$ in the leaves of adapted plants (MH5) was higher than in controls (M), as revealed in the significant differences between the slopes of regression lines ($P=0.0048$ and $P=0.0079$ for the relationships between $\Phi_{\text{PSII}}$ and RWC, and $\Phi_{\text{PSII}}$ and water potential, respectively). There was no difference in the relationship between water potential and RWC in the adapted and control plants, whether these data were fitted with linear (not shown) or non-linear (Fig. 5C) functions.

Before transfer of the plants to a high RH, the ABA concentration of moderate RH-grown leaves was significantly higher than that of the high RH-grown leaves ($P=0.0002$) (Fig. 6A). After transfer, the ABA level of the moderate RH-grown leaves (MH) decreased to the same level as high RH-grown leaves (H). After their 4 d exposure to high RH, transferring moderate RH-grown plants (MH) back to a moderate RH resulted in an increase of ABA concentration ($P <0.0001$) (Fig. 6B). Additionally, 5 d exposure of a mature moderate RH-grown leaf to a high RH changed the response of $\Phi_{\text{PSII}}$, and thus stomatal behaviour, to a short-term ABA application (Fig. 7). The high $\Phi_{\text{PSII}}$ of leaves in water implies that the stomata of the leaves opened irrespective of RH treatments (Fig. 7A). After feeding the leaves with ABA, the control leaf grown at a moderate RH (M) showed a rapid decrease of $\Phi_{\text{PSII}}$, while there was a clear lag in the response of the adapted leaf (MH5; i.e. grown at a moderate RH and exposed to a high RH for 5 d) (Fig. 7B–F). Figure 8 shows the changes of the mean $\Phi_{\text{PSII}}$ of images of control (M) and adapted leaves (MH5) during 150 min of ABA application. The $\Phi_{\text{PSII}}$ in both groups of leaves in water was high and there was no significant difference between them. Following ABA application, $\Phi_{\text{PSII}}$ of control leaves decreased earlier than it did for adapted leaves. The interaction between RH treatment and duration of ABA application was significant ($P <0.0001$).

**Discussion**

It has already been reported that the closure of stomata in leaves of T. virginiana grown at a high RH is less responsive to desiccation (RWC, water potential) than those grown at a moderate RH (Rezaei Nejad and van Meeteren, 2005; Rezaei Nejad et al., 2006). It was not known if this adaptation was permanent due to irreversible alterations in the properties of the guard cells following their development under high RH conditions, or if the adaptation could also have been induced in stomata that had developed at a moderate RH. In the case of leafy cuttings, Fordham et al. (2001a) demonstrated that the failure of stomata developed under a high RH to close could not be reversed by transferring the plants to...
Sallanon et al. (1993) found the same irreversibility in in vitro cultured plants. On the other hand, Brainerd and Fuchigami (1981) and Marin et al. (1988) showed that though initially incapable of closure, the stomata of in vitro propagated plants could develop a normal closure response when exposed to a low humidity. Thus it appears that changes in stomatal functioning due to development at a high RH are reversible in some cases, but not in others. The present results show that when a Tradescantia leaf is transferred during its development from a moderate to a high RH, the stomata of the two leaf regions that arise from this treatment (a distal part grown at a moderate RH and transferred to a high RH, and a basal part grown only at a high RH) displayed the same poor closure in response to desiccation (Figs 1A, 2A). This implies either that stomata grown at moderate RH can adapt to a high RH, or that the similar stomatal response of the two leaf regions is the
result of cross-talk between the two regions (e.g. due to the transport of ABA or another signalling compound). In the inverse experiment (leaves first grown at high RH and then transferred to moderate RH), the stomata of the two leaf regions still differed in their closure in response to desiccation 10 d after the transfer—which was the limit of the measurements (Figs 1B, 2B). This indicates that the stomata of the distal region could not acclimate to the moderate RH to an extent that their closure response matched that of stomata from the basal region or that the two leaf regions affected each other’s stomatal closing response. In a previous study (Rezaei Nejad and van
Within 1 d after the transfer of moderate RH-grown plants to a high RH the ABA concentration of their leaves decreased to that of the high RH-grown leaves and remained at this low level during the 4 d exposure to high RH conditions (Fig. 6A). Possible explanations for these changes could be: (i) less accumulation of leaf ABA due to a low transpiration rate under high RH conditions; or (ii) a decrease in the accumulated leaf ABA because of increased catabolism of ABA or increased phloem transport of ABA out of the leaf (Sauter et al., 2001). Transferring these high RH-exposed plants back to a moderate RH resulted in an increased ABA level in the leaves (Fig. 6B), possibly due to increased transpiration in the moderate RH environment. However, this increase in ABA level did not restore the stomatal closure response to

During a short-term desiccation treatment, the higher stomatal conductivity of leaves excised from high RH-grown plants or plants transferred from a moderate to high RH will result in their water content decreasing faster than that of leaves from moderate RH-grown plants. As argued in a previous study (Rezaei Nejad et al., 2006), desiccation will ultimately cause the stomata of high RH-grown leaves to close due to the decrease of guard cell turgor. This will result in a change of the $\Phi_{PSII}$ values. The stomatal closure that occurs in leaves of the high RH-grown leaves, and in leaves of plants transferred from a low to high RH, after $\sim$120–150 min of desiccation (Fig. 2A) is due to their low RWC at that time ($\sim$75–80%) (Fig. 5A). In contrast, the RWC of moderate RH-grown leaves had decreased to only $\sim$95% when stomatal closure occurred and $\Phi_{PSII}$ decreased (compare Figs 2A and B, and 5). This suggests that though the $\Phi_{PSII}$ values shown after 120 min of desiccation for moderate RH-grown leaves in Fig. 2B are rather similar to the values shown for high RH-grown leaves or leaves transferred from a moderate to high RH (Fig. 2A), this does not mean that the regulation of stomatal closure is identical.

ABA is a key component of the signal transduction pathway for stomatal closure (reviewed by Leung and Giraudat, 1998). Before the moderate RH plants were transferred from moderate to high RH, their bulk leaf ABA concentrations were higher than those in high RH-grown plants (Fig. 6A). This was possibly the result of them having a higher transpiration rate and thus more active ABA transport to, and accumulation in, the leaves (Trejo et al., 1995) or the result of less uptake of ABA by the tissue surrounding the xylem vessels during xylem transport to the leaves (Sauter et al., 2001). This agrees with the significantly lower endogenous ABA concentration that has been reported in *T. virginiana* leaves grown at a high RH compared with leaves grown at a moderate RH (Rezaei Nejad and van Meeteren, 2007). Higher leaf ABA concentration in response to an increase of VPD has been shown in *Acer rubrum* (Bauerle et al., 2004), and *Tagetes erecta* cultivated in *vitro* (Aguilar et al., 2000).
the level observed before the transfer to high RH. The change of stomatal behaviour that occurred during exposure to high RH seems to be irreversible, and it cannot be completely attributed to changes in whole leaf ABA levels. To test further whether the lack of stomatal closure of excised high RH-adapted leaves in response to desiccation is due to a deficiency of ABA, leaves were fed with ABA solution and the stomatal responses monitored. After feeding the leaves with ABA, the high RH-adapted leaves showed a stomatal closure response that was slower and less complete than that of the stomata of control leaves (Figs 7, 8). This reinforces the conclusion that the short-term low ABA level alone was not the reason for diminished closure response, but that the ABA-related closure of stomata has been impaired after a few days exposure of plants to a high RH. The lack of stomatal closure in response to exogenous ABA has also been shown in leafy cuttings rooted at a high RH (Fordham et al., 2001b), in in vitro propagated plants (Wardle and Short, 1983; Ziv et al., 1987; Santamaria et al., 1993), and in T. virginiana plants grown continuously at high RH (Rezaei Nejad and van Meeteren, 2005, 2007).

Although the leaf ABA level of moderate RH-grown plants decreased under high RH conditions within 1 d, the loss of stomatal closure response to desiccation developed over 4 d (Fig. 4). In water-stressed plants or leaves, there is often no straightforward, linear relationship between bulk leaf ABA concentrations and stomatal conductance (Beardsell and Cohen, 1975). Tardieu and Davies (1993) have suggested that it is more likely that stomatal conductance is controlled by the combination of chemical and hydraulic signals rather than chemical or hydraulic signals acting alone. In such a system, stomatal sensitivity to ABA would decrease at higher leaf water potentials. This is consistent with the poor stomatal closure in the high RH-grown leaves, which have low leaf ABA concentrations, but it cannot explain the lack of restoration of the stomatal closure response after the transfer of plants from high to moderate RH. Uneven distribution within the leaves could be another reason for discrepancy between bulk leaf ABA concentrations and stomatal conductance. Some of the ABA arriving in the transpiration stream will be removed by sequestration in the mesophyll or the epidermal symplast (Wilkinson and Davies, 2002). Drought-induced pH increases can result in redistribution of ABA from the symplastic to the apoplastic compartment of the leaf, inducing stomatal closure in the absence of any change in the bulk leaf ABA concentration (Davies et al., 2002; Wilkinson and Davies, 2002). ‘Trapping’ of ABA in the leaf mesophyll or in the cytoplasm of epidermal cells could be a possible explanation for the failure of the stomata to respond to the increase in bulk leaf ABA levels that develop when plants are transferred back to a moderate RH after the 4 d exposure to high RH, as well as for the slower response to exogenous applied ABA observed in plants exposed to a high RH for 5 d. The (absence of a) relationship between bulk leaf ABA and stomatal conductance could also be affected by the subcellular compartmentation of ABA in guard cells. According to Zhang and Outlaw (2001), ABA arriving from the roots accumulates in the guard cell apoplast, whereas a leaf subjected to water stress accumulates ABA in the guard cell symplast. Long-term exposure to a high RH, however, decreased the stomatal closure response both to leaf desiccation and to ABA applied via the cut surface of a leaf.

An immediate response of stomatal aperture to short-term changes in air humidity has been observed in many plant species (Sheriff, 1979; Monteith, 1995). In some woody ornamental plants, the aerial environment around the leaf can affect the pH inside the apoplast, resulting in changes in the ABA localization within the leaf (Davies et al., 2002). It has therefore been proposed that an increased VPD will result in lower stomatal conductance because of an increased pH in the leaf apoplast (Wilkinson and Davies, 2002; Wilkinson, 2004). However, based upon results obtained from ABA-deficient and ABA-insensitive mutants of Arabidopsis thaliana, Assman et al. (2000) suggested that an obligate role for ABA as the mediator of the short-term guard cell response to air humidity was unlikely in Arabidopsis. The lag between the reduction of ABA (day 1) and the loss of stomatal responsiveness (day 4) in the plants transferred to high RH, as well as the lack of restoration of this responsiveness following the increase in ABA level subsequent to transfer back to moderate RH, suggest that factors besides ABA are involved in the long-term RH effects on stomatal behaviour. Previous results, however, have shown that the daily application of ABA to leaves growing at a high RH largely prevents the loss of the stomatal closure response (Rezaei Nejad and van Meeteren, 2007). It appears that the ABA level must be below a certain level for a specific period if the stomatal dysfunction is to develop.

The diminished stomatal closure response to desiccation in high RH-grown plants and high RH-adapted plants could be due to changes in the signalling pathway for ABA-related closure of stomata. If this signalling pathway is impaired, the increased ABA level that develops in leaves in high RH-adapted plants after they are transferred back to a moderate RH would not result in stomatal closure in response to desiccation. Another explanation could be that after a prolonged period with a low ABA concentration, neither increased ABA transport from roots to leaves, increased foliar ABA biosynthesis, nor application of exogenous ABA results in an increase of ABA in the apoplast adjacent to the guard cells due to sequestration of ABA by the mesophyll tissue or the symplast in the epidermis, as discussed above.

Is this response to high RH in any way useful to the plant? In natural vegetation there are situations where long
periods of high RH can occur, for example in the forest understory. Often in these situations irradiance will be low and thus the stomatal conductance of a leaf with normal stomatal regulation will be low. Under conditions of high RH, especially when stomatal conductance is low, the potential for leaf temperature regulation by evaporative cooling will be poor, yet the risk of sudden leaf temperature increases due to increases in irradiance (e.g. sunflecks) will remain. Under these conditions, persistent stomatal opening may permit the leaf to maximize its potential for rapid evaporative cooling, with only a small extra cost in terms of water loss under conditions in which the VPD is typically low. In commercial greenhouse production, however, high humidities during the production phase are becoming more and more common, while in the post-harvest phase the harvested products are exposed to conditions with high VPD. The result is the production of cut flowers whose stomata are ill adapted to a low RH environment, which results in quality problems for the final consumer of the products.

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