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Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning

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Plants of several species, if grown at high relative air humidity (RH ≥85%), develop stomata that fail to close fully in case of low leaf water potential. We studied the effect of a reciprocal change in RH, at different stages of leaf expansion of *Rosa hybrida* grown at moderate (60%) or high (95%) RH, on the stomatal closing ability. This was assessed by measuring the leaf transpiration rate in response to desiccation once the leaves had fully expanded. For leaves that started expanding at high RH but completed their expansion after transfer to moderate RH, the earlier this switch took place the better the stomatal functioning. Leaves initially expanding at moderate RH and transferred to high RH exhibited poor stomatal functioning, even when this transfer occurred very late during leaf expansion. Applying a daily abscisic acid (ABA) solution to the leaves of plants grown at continuous high RH was effective in inducing stomatal closure at low water potential, if done before full leaf expansion (FLE). After FLE, stomatal functioning was no longer affected either by the RH or ABA level. The results indicate that the degree of stomatal adaptation depends on both the timing and duration of exposure to high RH. It is concluded that stomatal functionality is strongly dependent on the humidity at which the leaf completed its expansion. The data also show that the effect of ambient RH and the alleviating role of ABA are restricted to the period of leaf expansion.

Introduction

From the time that plants grow on land, the proper regulation of water loss is essential for their survival. Stomatal opening is often a compromise between the required uptake of carbon dioxide and the prevention of excessive loss of water. Stomatal movement is regulated by external environmental conditions, such as light, carbon dioxide concentration and vapour pressure deficit (VPD) via a complex signalling network (reviewed by Hetherington and Woodward 2003, Schroeder et al. 2001). These environmental stimuli control not only the opening and closing of stomatal pores on a short-term basis (min to h), but can also permanently alter the stomatal functioning (long-term responses). For example, it is well documented for several plant species that the capacity of the stomata to close in response to water stress is strongly influenced by the relative air humidity (RH) level during growth (Rezaei Nejad and van Meeteren 2005, Torre et al. 2003). Stomata developed under long-term high RH (≥85%) show distinct anatomical features and are less sensitive to low leaf water potential compared with those that develop at moderate RH. This phenomenon has been reported in plants

Abbreviations – ABA, abscisic acid; FLE, full leaf expansion; RH, relative air humidity; RWC, relative water content; VPD, vapour pressure deficit.

grown under high RH and transferred suddenly to conditions of increased evaporative demand, namely leafy cuttings rooted at high RH (Fordham et al. 2001), in vitro propagated plants (Santamaria et al. 1993) and cut flowers grown in greenhouses at high RH (Mortensen and Gislerød 2000). Lack of proper stomatal closure has been identified as the major cause leading to disturbed water relations in high RH-grown plants (Aquilar et al. 2000, Mortensen and Gislerød 2005).

Although stomatal acclimation to RH has been largely described for plants grown continuously at high RH, this acclimation process is not yet fully understood (Rezaei Nejad and van Meeteren 2008). According to Schoch et al. (1980), there is a brief time during which stomatal development is most sensitive to environmental changes. The dynamics of stomatal adaptation to long-term alterations in the RH during cultivation, i.e. the adaption capacity of stomatal apparatus when transferring plants from one RH level to a contrasting one, has been poorly addressed. From the few studies available, most were only focussed on stomatal adaptation to a contrasting RH after full leaf expansion (FLE; Mortensen and Gislerød 2000, Pospisilova 1996). It was shown that transfer of the plants, after leaf expansion, to a new RH level (i.e. moderate to high RH or vice versa) resulted in a full stomatal adaptation to the new RH environment in *Phaseolus vulgaris* (Pospisilova 1996), whereas it had no effect on stomatal responsiveness in *Rosa hybrida* (Mortensen and Gislerød 2000). Recently, Rezaei Nejad and van Meeteren (2008) went one step further by comparing the degree of stomatal adaptation to contrasting RH levels in expanding and in fully expanded leaves. It was shown that stomata of *Tradescantia virginiana* were able to adapt to a change in RH, but the degree of adaption was higher in expanding leaves. However, these authors only examined one stage of leaf development. Thus, it is still unclear whether stomatal functioning is mostly determined during the first stages of leaf development or during later stages.

The role of abscisic acid (ABA) in the control of stomatal functioning has been well described for several species grown under drought stress conditions (Zhang and Davies 1990, Zhang and Outlaw 2001). However, the reasons why stomata fail to close fully in response to leaf dehydration in plants subjected to prolonged exposure to high RH are not completely clear. As the stomata respond to the rate of transpiration rather than to RH itself (Mott and Parkhurst 1991), stomatal malfunctioning could be a consequence of the low transpiration rate. This in turn creates a situation where the plants are subjected to a long-term abnormally high leaf water potential (weaker hydraulic signalling) and to a long-term low leaf ABA concentration (weaker chemical signalling). As the leaf ABA concentration is influenced by the ABA import via the xylem from the roots (Zhang and Outlaw 2001), it is expected that in plants growing at high RH this import will decrease because of the relatively low rate of transpiration. Increasing evidence supports the hypothesis that long-term low ABA concentration plays an important role in the loss of stomatal functionality in plants grown at high RH (Rezaei Nejad and van Meeteren 2007, 2008). For instance, it was found that a daily exogenous ABA application during leaf development increased stomatal response to desiccation of high RH-grown *T. virginiana* leaves (Rezaei Nejad and van Meeteren 2007). Furthermore, Mortensen and Gislerød (2005) showed that severe drought stress, which is expected to intensify the ABA signals (Schachtman and Goodger 2008), increased the vase life of five out of six high RH-grown cultivars. Therefore, it is unclear if the aforementioned lack of stomatal adaptation in fully expanded leaves of *R. hybrida*, upon transfer to a contrasting RH level, is because of insufficient changes in the leaf ABA concentration or because the stomatal functionality in fully expanded rose leaves is independent of the ABA level. Moreover, to the best of our knowledge there are no studies on the effect of exogenous ABA application on fully expanded leaves, which would allow investigating whether ABA has an active role in stomatal functioning after FLE.

The aims of this work were (1) to investigate the dynamics of stomatal adaptation at various stages of leaf ontogeny (accessed during and after FLE) in response to long-term high RH, (2) to test whether long-term exogenous ABA application could improve stomatal functionality during or after complete leaf expansion at high RH and (3) to analyse whether root signalling is a prerequisite for sustaining stomatal functionality in leaves fully expanded at moderate RH. We hypothesize that the RH level during critical stages, rather than throughout leaf expansion, is decisive for stomatal functionality and that drastic changes in the ABA concentration after leaf expansion can influence stomatal functionality.

Materials and methods

Plant material and growth conditions

Rooted cuttings of the rose cultivar 'Pink Prophyta' (*R. hybrida*) were obtained from a commercial propagator (Kordes, De Kwakel, The Netherlands), and planted in 3.6 l pots containing a mixture of cocopeat (Jongkind Grond B.V., Aalsmeer, The Netherlands) and perlite (Agraperlite nr. 3, Pull, Rhenen, The Netherlands) (3:1, v/v). The cultivar 'Pink Prophyta' was selected because of its sensitivity to high RH (Fanourakis et al. 2009).

A total of five experiments were conducted. In each experiment, plants were grown in four growth chambers (length × width × height = 1.3 × 0.8 × 1.3 m) as a single shoot (one plant per pot) at a density of 30 plants m^{-2}. In two growth chambers, the RH was 60 ± 3% (moderate RH) and in two others it was 95 ± 1% (high RH) during the cultivation period. The four chambers had constant day and night temperatures (19 ± 1°C), resulting in a VPD of 0.88 ± 0.12 kPa (moderate RH) or 0.11 ± 0.03 kPa (high RH). Climate parameters were recorded automatically every 5 min, using data loggers (Fourier MicroLog EC650, MicroDAQ.com Ltd., Contoocook, NH). The CO_2 concentration during the light period was 370 ± 50 μmol mol^{-1} (determined using Indoor Air Quality Meter, Model 8760, TSI Incorporated, Shoreview, MN). Fluorescent tubes (TLD 58W/84, Philips, Eindhoven, The Netherlands) provided an 18h on–off cycle and 300 ± 20 μmol m^{-2} s^{-1} photosynthetically active radiation (Model LI-250, LI-COR, Lincoln, NE). The light intensity was measured at 70 cm height, which corresponds to the top of the plants at harvest stage. Plants were watered automatically with a nutrient solution, as described by Fanourakis et al. (2009).

Water relations in intact plants during cultivation (experiment 1)

Eight weeks after planting, when the plants were fully developed and had a flower bud with a cylindrical shape and pointed tip, plant transpiration rate was gravimetrically recorded on a daily basis for 5 days, using nine intact plants at each humidity level. During this period, the pots were covered with aluminium foil to prevent evaporation from the substrate, and the amount of solution used for irrigation and that drained from the pots were recorded daily. At the end of the measurements, the total leaf area per plant was determined using a leaf area meter (Model 3100 Area Meter, LI-COR, Lincoln, NE). The plant transpiration rate was normalized per unit leaf area, whereas the stomatal conductance (g_s) was calculated as a daily average per plant according to the equation of von Caemmerer and Farquhar (1981) (Eqn 1):

$$g_s = \frac{\text{Transpiration rate}}{W_L - W_A} \quad (1)$$

The water vapour gradient between leaf interior and air ($W_L - W_A$) was calculated using the difference of the saturated mole fraction of water vapour inside the leaf [W_L; 21.7 mmol H_2O mol^{-1} of air at 19°C, according to Nobel (1991)] and the mole fraction of water vapour in the bulk air outside the leaf (W_A; 13.0 and 20.6 mmol H_2O mol^{-1} of air at 19°C, at 60 and 95% RH, respectively). The leaf temperature was assumed to be equal to the air temperature. However, if this assumption would be incorrect, and a leaf with a relatively high rate of transpiration (at moderate RH) would be 1°C cooler while a leaf with a relatively low rate of transpiration (at high RH) would be 1°C warmer than ambient air, the stomatal conductance would be about 6% higher at moderate RH and 6% lower at high RH than the one quoted here.

Additionally, leaf water potential was measured destructively at regular intervals during the light period (3, 6 and 9 h after the onset of the light period), using plants from both RH levels. The measurements were conducted in three different sets of eight fully expanded leaves each (using the first penta-foliate leaf counting from the apex of stalks with a flower bud at the stage described above), using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA).

Stomatal adaptation to a new RH during leaf expansion (experiment 2)

To determine stomatal adaptation capacity to a long-term alteration in the RH during leaf expansion, a reciprocal transfer experiment was conducted. Eighteen plants were transferred to the contrasting humidity level (i.e. 60→95% RH and 95→60% RH), whereas 14 plants were kept at their original humidity level (controls). Just before transferring the plants, leaves were tagged and the length of the terminal leaflets of the penta-foliate leaves (from first to fifth order counting from the apex) was followed in time. This allowed the calculation of the proportion of the leaf that expanded at the new humidity level in relation to its final length (whereby final length was defined as the length when the midrib did not elongate significantly for three consecutive days). The developmental stage of the leaves at the moment of transfer was expressed as a percentage of full leaf expansion by measuring leaf length (FLE; defined as the proportion of leaf length at transfer, relative to its final length, Woodall et al. 1998). The moment of transfer corresponded to 90 ± 5% FLE of the fifth penta-foliate leaf counting from the apex, whereas the upper leaves were at various percentages of their final length. Therefore, after final length had been achieved, leaves were sorted into eight groups: 21–30%, 31–40%, 41–50%, 51–60%, 61–70%, 71–80%, 81–90% and 91–95% FLE. For example, 20% FLE refers to a leaf which at the moment of transfer had 20% of its final length, whereas the remaining 80% developed under the new RH level, after transfer. The stomatal adaptation ability was assessed by determining the transpiration rate of the terminal leaflets in response to desiccation. These leaflets were detached, their petioles were immediately recut

under degassed water (to prevent cavitation-induced embolism), placed in flasks filled with water and further incubated for 1 h at about 100% RH (21°C; VPD close to 0) to establish their saturated fresh weight. As the leaflets were detached during the light period in the growth chamber, the rehydration process was therefore conducted in light (15 μmol m^{-2} s^{-1}), as following darkness the light-induced stomatal opening requires up to 1 h (Blom-Zandstra et al. 1995). Subsequently, the leaflets were removed from water and placed in the test room on a table (abaxial surface down; 50 ± 3% RH, 21°C, 1.24 kPa VPD and 50 μmol m^{-2} s^{-1}) where the leaf transpiration rate was measured gravimetrically every 5–30 min during 4 h. The leaf area was then determined and the leaflets were dried at 80°C for 24 h. The leaf relative water content (RWC) was calculated according to Slavik (1974) (Eqn 2). This experiment was repeated once.

$$\text{RWC} = \frac{\text{Fresh weight} - \text{dry weight}}{\text{Saturated fresh weight} - \text{dry weight}} \times 100 \quad (2)$$

Effect of ABA application during leaf expansion (experiment 3)

To estimate the effect of long-term ABA application on the stomatal functionality during leaf expansion, the terminal leaflets of the penta-foliate leaves (from first to third order counting from the apex) were gently brushed with an aqueous solution containing 30 μ*M* (±)ABA (Sigma, St. Louis, MO) on both the abaxial and adaxial surfaces twice a day. In control plants, leaves were brushed with water in a similar manner. ABA was applied from the start of leaf unfolding (i.e. when the midrib of the terminal leaflet was visible, corresponding to about 20% of its final length) to the time of FLE, in plants grown continuously at moderate and high RH. This ABA application lasted about 14 days and was terminated 48 h before the measurements took place.

After the final length had been achieved, the transpiration rate in response to desiccation was determined as described previously (experiment 2) using the terminal leaflets of the ABA-treated leaves. Additionally, some anatomical features of the stomata were measured on a leaflet of the first pair of lateral leaflets from the first penta-foliate leaf (counting from the apex). The measurements were performed on the abaxial surface, using a part of the leaflet that was midway between the tip and the base and away from the edge. Sites overlying veins were also avoided as they support no stomata. Five fields of view per leaflet were counted for determining the stomatal density using 12 leaflets (one leaflet per plant) and a magnification of 100×. In 20 randomly selected stomata per leaflet, the stomatal length, stomatal width, pore length and pore aperture were measured immediately after leaf detachment using a magnification of 1000×. Stoma width was chosen instead of guard cell width, as the latter undergoes changes up to 50% as stomata close (Shope and Mott 2006). These anatomical features were determined using the silicon rubber impression technique (Smith et al. 1989). The leaf was held horizontally and upside-down until the impression material hardened (<2 min) to ensure better replication of the pores (Weyers and Meidner 1990). The impression material was then detached and a positive replica was made on clear nail varnish and observed on a microscope slide. Digitized video images were taken using a microscope (Leitz Aristoplan, Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) which was connected to a digital imaging camera (Nikon DXM-1200, Nikon Corp., Tokyo, Japan). Image processing was done using the freeware UTHSCSA IMAGETOOL program (University of Texas Health Science Centre, San Antonio, TX).

Stomatal adaptation to a new RH in fully expanded leaves (experiment 4)

To study the effect of a changed RH level on stomatal functionality in young fully expanded leaves, 12 plants were kept at the humidity level in which they had been grown (controls), whereas 20 other plants were transferred to the new RH (i.e. 60→95% RH and 95→60% RH). Transfer took place when the flower bud had a cylindrical shape and pointed tip. To follow the time course of stomatal adaptation to the new RH environment, the transpiration rate in response to desiccation was recorded (as described for experiment 2) using terminal leaflets that were detached every 2 days for a period of 14 days.

Effect of ABA manipulation in fully expanded leaves (experiment 5)

To investigate the importance of ABA on the dynamics of stomatal functionality in fully expanded leaves, two treatments were performed: (1) long-term ABA application, twice a day, after complete leaf expansion under continuous high RH and (2) root excision in plants grown continuously at moderate RH.

Both treatments were initiated when stalks had a flower bud with a cylindrical shape and pointed tip. ABA was applied using the same method as described above (experiment 3), and it lasted for 14 days while keeping the plants at high RH. Root removal was carried out in plants grown at moderate RH, while care was taken that the water uptake was undisturbed. The night before the experiment, plants were well irrigated and placed in darkness for 12 h to ensure maximal turgidity and to minimize the presence of natural air emboli

(van Doorn and Suiro 1996). Pots with the plants were placed in buckets which contained degassed sterilized water, whereby the water level was about 5 cm above the soil surface. Flowering stems were cut under water to prevent air entrance at the cut surface. Each cut was made using secateurs that had been sterilized in 98% ethanol and through an internode that had been surface-sterilized by rubbing with a cloth drenched in the same solution (sterile treatment, van Doorn et al. 1991). The flowering stalks were normalized for stem length (47 ± 2 cm) and leaf number (four penta-foliate leaves, resulting in a leaf area of 340 ± 20 cm^2). Subsequently, the shoots were placed in sterilized flasks (one flower per flask) containing sterilized artificial vase solution (0.7 m*M* $CaCl_2 \cdot 2H_2O$, 1.5 m*M* $NaHCO_3$ and 5 µ*M* $CuSO_4 \cdot 5H_2O$, van Meeteren et al. 2000), with a pH adjusted to 3 by addition of citric acid. The cut flowering stems were placed in a climate-controlled room at 20°C, 60% RH and light for 12 subsequent h per day at a photon flux density of 10–12 µmol m^{-2} s^{-1}.

In both treatments, the transpiration rate in response to leaf desiccation was recorded every 2days for a period of 14 days.

Statistical design and analysis

Data were subjected to analysis of variance using GENSTAT software (10th edition; VSN International Ltd., Herts, UK). Experiment 1 was analysed by one-way ANOVA. Experiment 2 was analysed using the Linear Mixed Models method (Crawley 2002), because there were an unbalanced number of replicates. Experiments 3, 4 and 5 were analysed by two-way ANOVA, where the RH level was the main factor, whereas ABA application (experiment 3), duration of new RH level (experiment 4), ABA application or root excision (experiment 5) were the split factors, respectively. Treatment effects were tested at 5% probability level and the mean separation was done using least significant differences based on Student's *t*-test ($P = 0.05$).

Results

Plant water relations when grown at continuously high or moderate RH

Water relations were measured in intact plants grown continuously at 60 or 95% RH. As expected, because of the lower VPD, plants grown continuously at high RH had a significantly lower transpiration rate during cultivation than plants grown at moderate RH ($P < 0.001$; Fig. 1A). The estimated stomatal conductance (Eqn 1) was about threefold higher in the plants grown at high RH compared with those from moderate RH (Fig. 1B). The leaf water potential decreased during the light period, but remained significantly higher in the plants grown at high RH ($P < 0.001$; Fig. 1C).

Concerning the stomatal anatomical features it was found that stomata in plants grown at high RH were bigger than in plants grown at moderate RH, but their density was not affected by the RH level during leaf

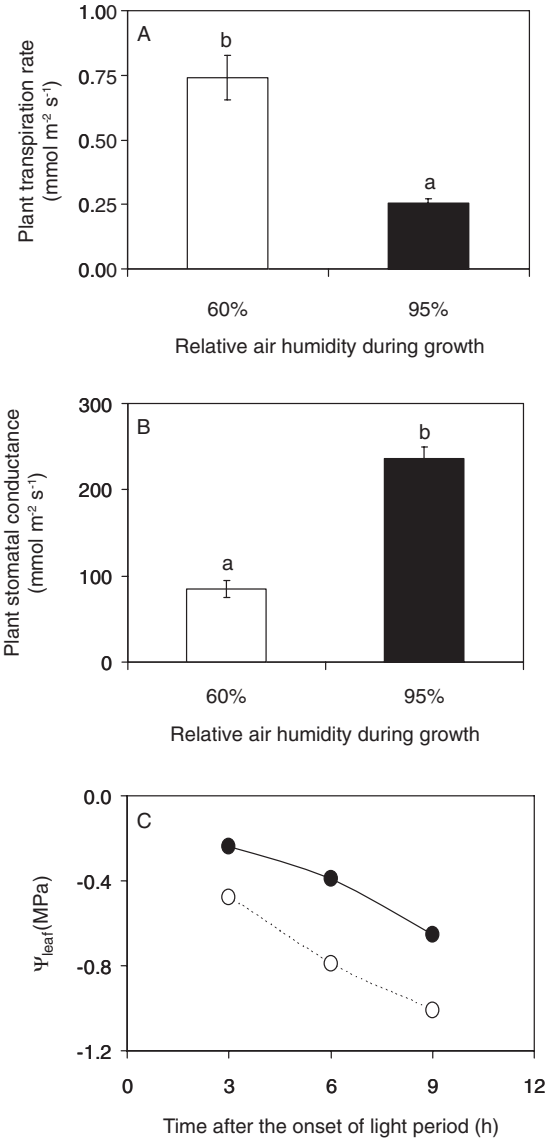

Fig. 1. Plant transpiration rate (A), plant stomatal conductance (B), and leaf water potential (Ψ) at different times after the onset of the light period (C) during cultivation of cut rose cv. 'Pink Prophyta' grown at moderate RH (open symbols, 60%) and high RH (closed symbols, 95%). The measurements were conducted using fully grown plants when the flower bud had a cylindrical shape and pointed tip. Values are the daily mean of nine intact plants (A and B) or the mean of eight leaves (C) ± SEM. Different letters indicate significant differences according to LSD test (experiment 1).

Table 1. Stomatal characteristics of cut rose cv. 'Pink Prophyta' grown continuously at moderate (60%) or high (95%) RH in response to long-term ABA application (0 or 30 μ*M* twice a day throughout leaf expansion). Values are the mean of 60 fields of view (stomatal density) and 240 stomata (stomatal anatomical features) measured on fully expanded leaves. Different letters indicate significant differences according to LSD test (comparison in columns; experiment 3). [a]Significant levels < 0.05.

RH	ABA application (μ*M*)	Stomatal density (number mm^{-2})	Stomatal length (μm)	Stomatal width (μm)	Pore length (μm)	Pore aperture (μm)
60%	0	43.1	42.6^{c}	31.8^{b}	23.7^{c}	4.83^{b}
	30	43.3	36.0^{a}	22.5^{a}	19.3^{a}	4.10^{a}
95%	0	45.3	50.6^{d}	36.0^{c}	30.7^{d}	7.08^{c}
	30	44.5	37.6^{b}	22.9^{a}	21.1^{b}	4.35^{a}
F pr.[a]						
RH		0.05	<0.001	<0.001	<0.001	<0.001
ABA		0.70	<0.001	<0.001	<0.001	<0.001
RH × ABA		0.57	<0.001	<0.001	<0.001	<0.001

development (Table 1). The pore length and aperture, when the lights were on for 2 h, were also greater in plants grown at high RH.

To determine the stomatal response to desiccation, terminal leaflets cut from fully developed leaves were allowed to dehydrate for 4 h. The transpiration rate was taken as a measure of stomatal opening (Fig. 2). After 2 h of desiccation, the transpiration rate in leaves expanded at high RH was about eight times higher than in those expanded at moderate RH (Fig. 2A). When the leaves were allowed to desiccate for 4 h, their transpiration rate became similar at both RH levels (Fig. 2A), but the leaf hydration levels were very different (58 and 11% RWC values for leaves of plants grown at moderate and high RH, respectively; Fig. 2B). These results show that stomata that developed at high RH were less sensitive to a decrease in leaf RWC, as they had higher transpiration rates throughout the RWC range (Fig. 2B). On the basis of these findings, the stomatal closure capacity in response to leaf desiccation was assessed in the subsequent experiments by measuring both the transpiration rate and the leaf RWC 2 h after the onset of desiccation, once leaves were fully expanded.

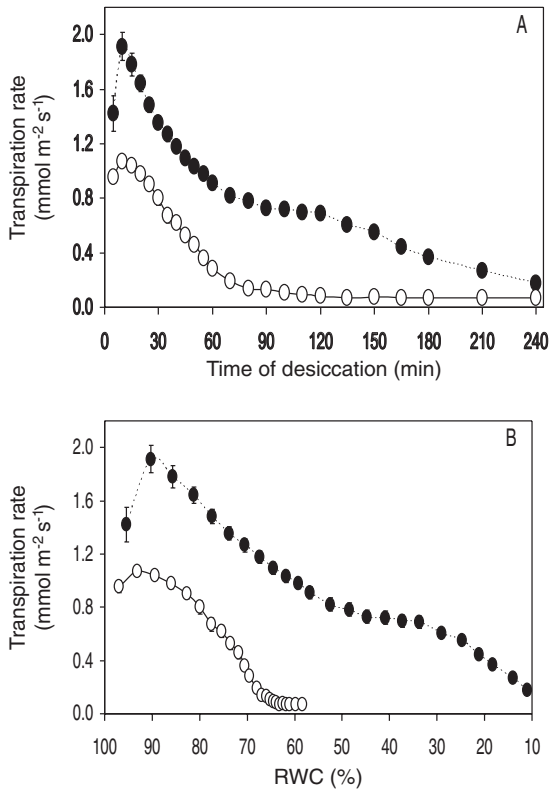

Fig. 2. Transpiration rate as a function of time of desiccation (A) and RWC (B) during 4 h of leaf desiccation in cut rose cv. 'Pink Prophyta' grown continuously at moderate RH (O, 60%) and high RH (●, 95%). Values are the mean of 14 leaves ± SEM (experiment 2).

Stomatal adaptation to a new RH during leaf expansion

Leaf elongation growth, expressed as a percentage of FLE measured in length (i.e. actual leaf length as a percentage of final length), followed an S-shape pattern in time (Fig. 3A). The percentage of leaf area increased exponentially with the percentage of FLE (Fig. 3B). Interestingly, leaf expansion, both in length and in area, was independent of the humidity level during growth (Fig. 3).

To investigate the dynamics of stomatal adaptation throughout leaf expansion in response to long-term high RH, plants grown at moderate and at high RH were transferred to the other RH level (i.e. 60→95% RH and vice versa). At the moment of transfer, the percentage of FLE of individual leaves varied between 25 and 93% (Fig. 4A, B). This resulted in different durations of exposure to high RH until complete leaf expansion, which reached a maximum of 9 days (Fig. 4C, D). To assess the stomatal response to dehydration, the rate of transpiration of cut leaflets and their RWC, measured

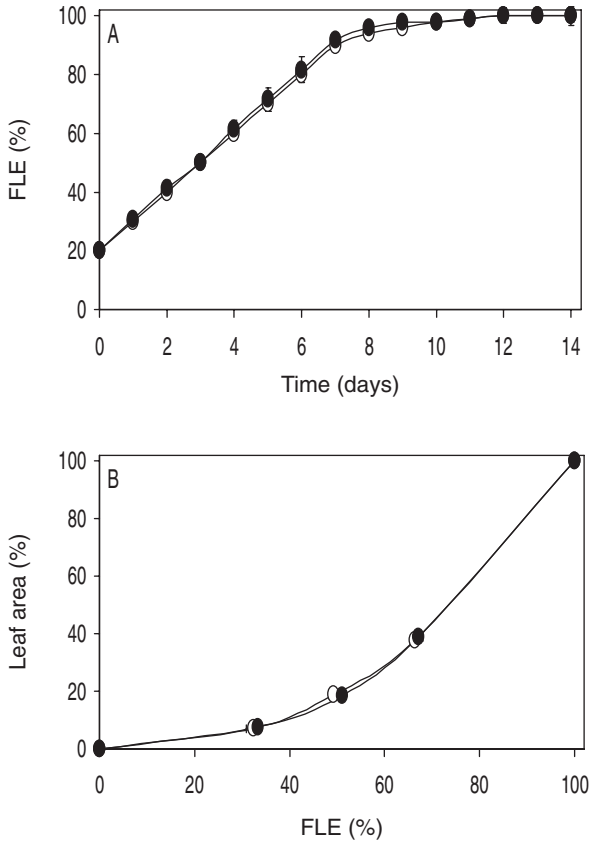

Fig. 3. Percentage of full leaf expansion (FLE; measured in length) as a function of time (A) and relationship between the percentage of leaf area and the percentage of FLE (B) in cut rose cv. 'Pink Prophyta' grown continuously at moderate RH (○, 60%) and high RH (●, 95%), under a constant day and night temperature (19°C). Time 0 corresponds to the beginning of leaf unfolding (i.e. when the midrib of the terminal leaflet was visible). Values are the mean of 12 leaves ± SEM (experiment 2).

2 h after the onset of dehydration, were evaluated. It was found that the leaves of transferred plants always showed intermediate values between the ones observed for leaves grown continuously at moderate or at high RH (controls; Fig. 4). The only exception was found in leaves that had expanded up to 25–35% FLE under high RH and whereby the plants had subsequently been transferred to moderate RH. These had a transpiration rate similar to the moderate RH control leaves. These results revealed that stomata from leaves initially expanded at a certain RH level and that attached to plants subsequently transferred to the contrasting RH (at 25–93% FLE) always had the capacity to partly adapt to that new RH level. Before 25–35% FLE, a high RH had no effect on stomatal functioning. However, the degree of stomatal adaptation was strongly dependent on the timing and duration of exposure to high RH. In expanding leaves, the stomatal adaptation capacity was mostly dependent on the RH level at which the last part of leaf expansion took place. For leaves that started expanding at high RH but had completed their expansion after transfer to moderate RH (i.e. 95→60% RH), the moment of transfer had a significant effect on the stomatal capacity to adapt to the new environment (Fig. 4A, B). The younger the leaves when the shift to moderate RH took place (Fig. 4A, B), i.e. the shorter the period the leaf was exposed to high RH (Fig. 4C, D), the higher the stomatal capacity to become functional. On the other hand, in leaves that started expanding at moderate RH and were attached to plants that had been transferred to high RH (i.e. 60→95% RH), different percentages of FLE at the moment of transfer showed similar stomatal response characteristics, once leaves were fully expanded (Fig. 4A, B). Surprisingly, the stomatal functionality of a leaf transferred to high RH at 93% FLE was strongly affected. The same trends as those described here were observed, though the differences were more pronounced when the RWC after 4 h of desiccation was considered (data not shown). These results indicate that the stomatal capacity to respond to desiccation by closing is not completely determined until leaf expansion has finished.

Effect of ABA application during leaf expansion

In plants that were grown continuously at moderate or high RH, an aqueous ABA solution (0 or 30 μ*M* ABA) was applied on the leaves twice a day, throughout the period of leaf expansion. This long-term ABA application did not influence stomatal density (Table 1). In contrast, it resulted in significantly smaller stomata with lower pore dimensions at either RH level (Table 1). There was no significant difference in stomatal width and pore aperture between ABA-treated leaves at the two RH levels (Table 1). Besides the effect of ABA application on stomatal anatomy, stomatal closure during desiccation was largely enhanced in ABA-treated leaves from plants grown continuously at high RH (Fig. 5). In these leaves, the transpiration rate after 2 h of desiccation was about 85% lower compared with leaves that developed at high RH, reaching the same values as those from leaves grown continuously at moderate RH (Fig. 5A). This resulted in 34% RWC after 2 h of dehydration in untreated leaves, whereas the RWC was 75% in high RH leaves treated with ABA (Fig. 5B). Leaves treated daily with water behaved in a similar way as untreated leaves, at both RH levels (data not shown).

Stomatal adaptation to a new RH in fully expanded leaves

To evaluate the dynamics of stomatal adaptation in fully expanded leaves, terminal leaflets were cut every 2 days

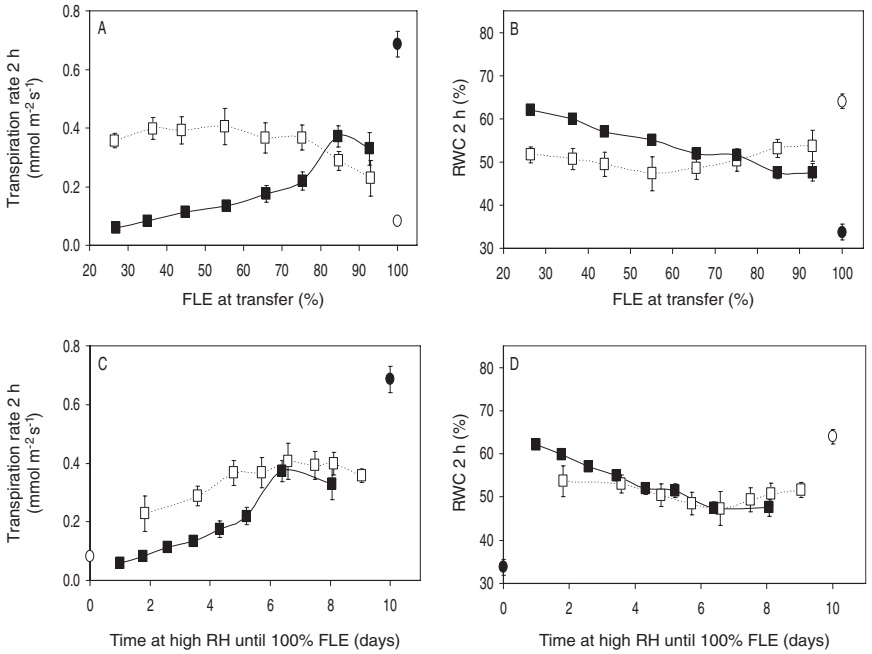

Fig. 4. Transpiration rate (A and C) and RWC (B and D) after 2 h of leaf desiccation in cut rose cv. 'Pink Prophyta' grown continuously at moderate (○, 60%) and high (●, 95%) RH (controls) or transferred to the new humidity environment (□, 60→95% RH; ■, 95→60% RH) at different percentages of full leaf expansion (FLE) (A and B), corresponding to different duration of exposure to high RH (C and D). The measurements were conducted after complete leaf expansion. Values are the mean of at least 18 leaves ± SEM (experiment 2).

from plants that had been transferred to a contrasting RH, for a period between 0 and 14 days. The transpiration rate 2 h after the onset of dehydration did not differ significantly from the controls (leaves kept at humidity level of expansion), irrespective of the period that the plants had been placed in the new RH environment (Fig. 6A). This was also reflected in the RWC after 2 h of desiccation. The leaf RWC had an inverse correlation with the rate of transpiration (Fig. 6B). Thus, leaves that had expanded under moderate RH showed a normal stomatal closing response to desiccation, even when the plants were subsequently exposed to high RH for as much as 14 days. Similarly, stomata on leaves fully expanded at high RH were unable to close rapidly during dehydration, even when the plants were further placed at moderate RH for as much as 2 weeks.

Role of ABA in fully expanded leaves

To test the hypothesis that drastic changes in ABA concentration after leaf expansion can influence stomatal functioning, ABA levels were exogenously manipulated both by ABA application in plants grown at high RH and by root excision in plants grown at moderate RH, as described above for experiment 3. During treatments' application, leaflets were sampled every 2 days for a period of 2 weeks.

Effect of ABA application on fully expanded leaves

An ABA solution (30 μ*M* ABA) was applied twice per day on fully expanded leaves from plants grown continuously at high RH. The stomata of leaves expanded at high RH did not improve their closing ability even after 2 weeks of exogenous ABA application (Fig. 7).

Effect of root excision

The effect of root excision was evaluated in plants developed at moderate RH. Care was taken that the water uptake in the tested plants was undisturbed. The stomata of leaves expanded at moderate RH maintained, for at least 2 weeks, their functionality despite the removal of root signals (Fig. 8).

Taken together the results of these two treatments suggest that after leaf expansion there were no changes in stomatal responses to desiccation, even when drastic changes in leaf ABA concentration occurred.

Discussion

Stomatal closing behaviour at continuously high or moderate RH

It is well established that the long-term high RH negatively affects stomatal regulation of water loss in several

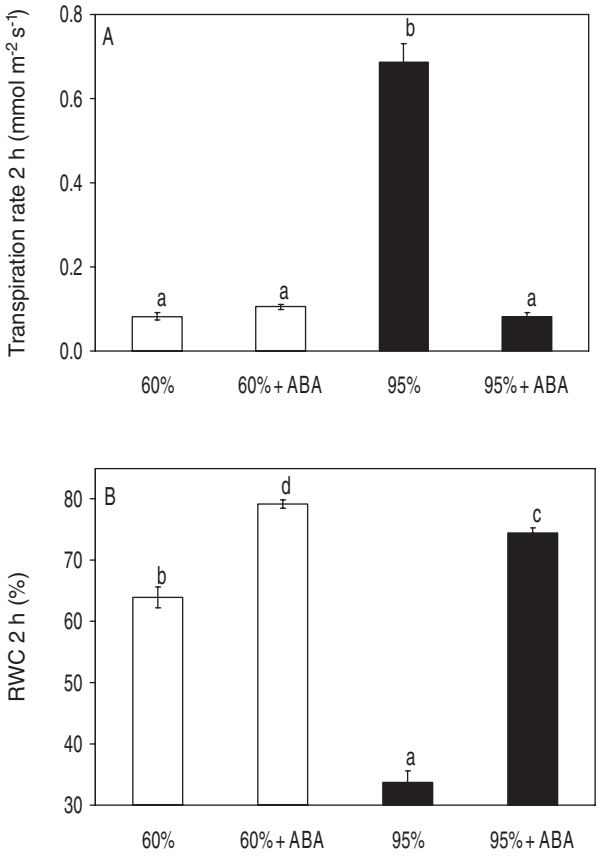

Fig. 5. Transpiration rate (A) and RWC (B) after 2 h of leaf desiccation in cut rose cv. 'Pink Prophyta' grown continuously at moderate (open columns, 60%) and high (closed columns, 95%) RH and treated with long-term ABA application (0 or 30 μ*M* twice a day throughout leaf expansion). The measurements were conducted after complete leaf expansion. ABA application ceased 48 h before the measurements. Values are the mean of 10 leaves ± SEM. Different letters indicate significant differences according to LSD test (experiment 3).

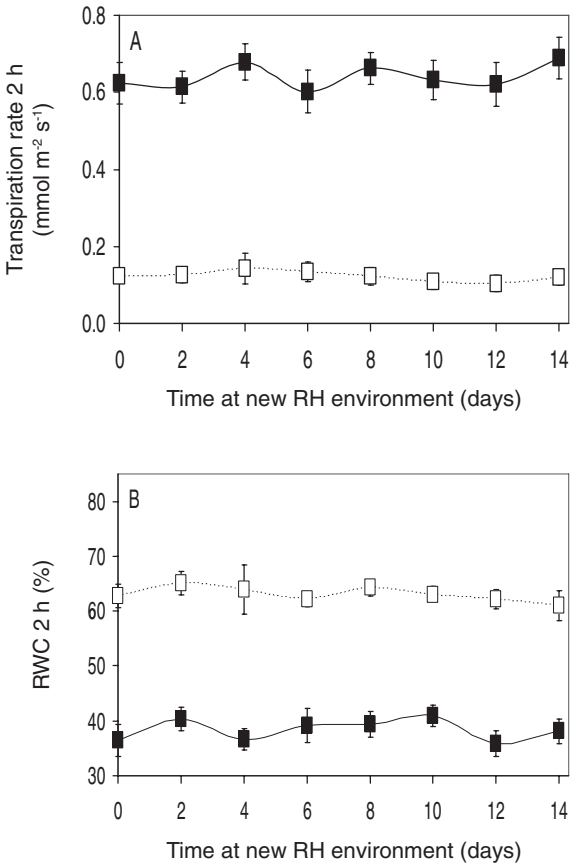

Fig. 6. Transpiration rate (A) and RWC (B) after 2 h of leaf desiccation in cut rose cv. 'Pink Prophyta' grown at moderate and high RH and further transferred to a new humidity environment (□, 60→95% RH; ■, 95→60% RH) after complete leaf expansion. Values are the mean of 14 leaves ± SEM (experiment 4).

species such as *Corylus maxima* (Fordham et al. 2001), *Oryza sativa* (Kawamitsu et al. 1993), *R. hybrida* (Torre and Fjeld 2001) and *T. virginiana* (Rezaei Nejad and van Meeteren 2005). The present results confirm those findings in rose, as stomata developed at high RH during cultivation under well-watered conditions remained open in response to desiccation until very low leaf hydration levels (Fig. 2). By then the leaf had visibly wilted and is not expected to recover when rehydrated (Lawlor and Cornic 2002).

When leaves are exposed to conditions of a rapid dehydration, because of a fast increase in the transpiration rate, a reduction of the epidermal cell turgor takes place which causes a lower backpressure exerted on the guard cells, leading to a transient increase of stomatal aperture (hydropassive stomatal opening). This temporary response of stomata to rapid changes in leaf hydration, the so-called 'wrong way' response, is well described in literature and usually precedes stomatal closure (Buckley 2005, Mott and Franks 2001). The 'wrong-way' response has also been shown in Fig. 2 at the onset of leaf desiccation. This effect was stronger in leaves grown under high RH because of their higher transpiration rates.

Leaves subjected to continuously high RH during leaf expansion also had bigger stomata with longer pores, compared with stomata on moderate RH-grown leaves (Table 1). Previous studies have shown that when comparing different species, the ones having a larger stomatal size were the ones with the longer response time with respect to water loss (Franks and Farquhar 2007, Hetherington and Woodward 2003). Therefore, it can be hypothesized that within a given species the longer stomata, induced by high RH, are strongly implicated in the inefficient stomatal closure. However, further research is needed to confirm this hypothesis.

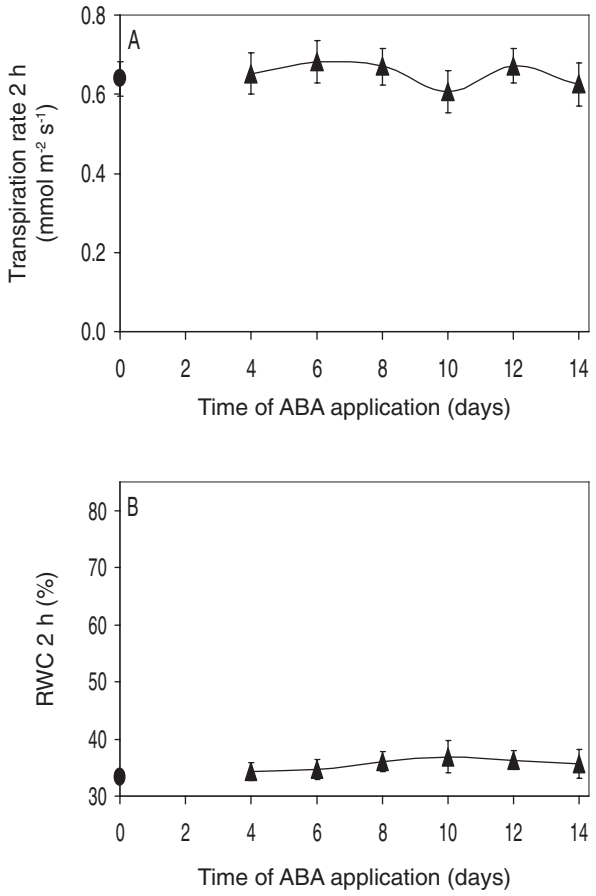

Fig. 7. Transpiration rate (A) and RWC (B) after 2 h of leaf desiccation in cut rose cv. 'Pink Prophyta' grown continuously at high RH (95%) and treated with 30 μ*M* ABA twice a day (▲) after complete leaf expansion during a period of 0 days [controls; i.e. non-treated leaves (⬮)] until 14 days. ABA application ceased 48 h before the measurements. Values are the mean of 14 leaves ± SEM (experiment 5).

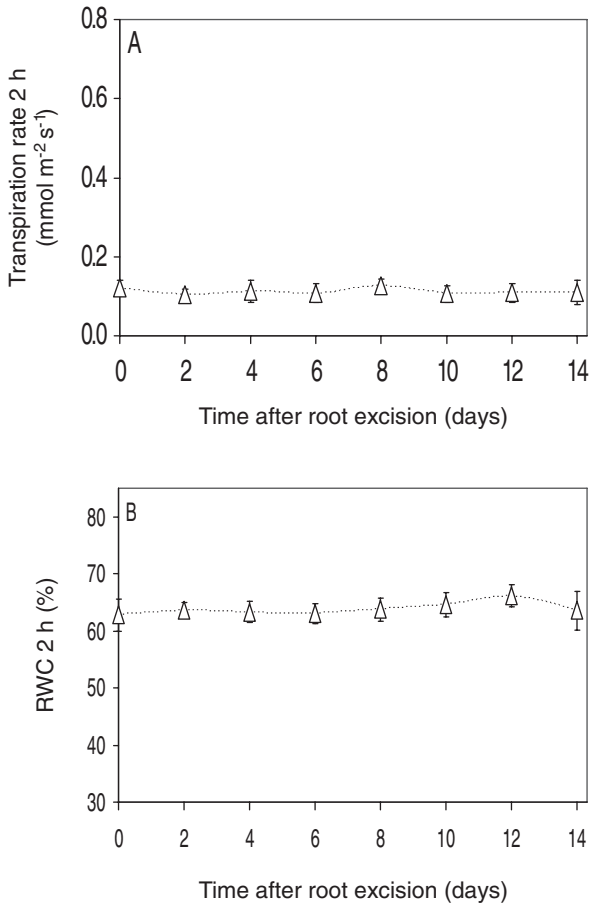

Fig. 8. Transpiration rate (A) and RWC (B) after 2 h of leaf desiccation in cut rose cv. 'Pink Prophyta' grown continuously at moderate RH (60%) and further subjected to root excision after complete leaf expansion starting at 0 days (controls) until 14 days. Values are the mean of 14 leaves ± SEM (experiment 5).

Stomatal adaptation to long-term alterations in RH

Although a constant high RH level during leaf growth clearly results in a poor stomatal closure in response to dehydration, its impact at various stages of leaf ontogeny was not analysed previously. In this study, it was found that stomatal functionality in rose leaves is completely determined during leaf expansion. Stomata from leaves that started expanding at a certain RH level and were subsequently transferred to a contrasting one (moderate to high RH and vice versa) between 35 and 93% FLE were always able to partly adapt to the new RH level (Fig. 4). In contrast, out of this range of leaf developmental stages subjecting the plants to high RH had no effect on stomatal functioning (Figs 4 and 6). Moreover, this study has shown that in expanding leaves, the degree of stomatal adaptation was closely related to the timing and duration of exposure to high RH (Fig. 4). It was found that the stomatal adaptation capacity was strongly dependent on the humidity level at which the leaf completed its expansion and, therefore, high RH should be avoided particularly during the last stages of leaf ontogeny. These findings are in agreement with our hypothesis that the RH level during critical stages, rather than throughout leaf expansion, is decisive for stomatal functionality.

In fully expanded leaves, the stomatal size and stomatal population (i.e. absolute number of stomata = density × leaf area) are fixed. In contrast, in expanding leaves, the stomatal size is either increasing or does not change anymore (Tichá 1982). Stomatal development often stops before 60–80% of leaf expansion has occurred (Rawson and Craven 1975). It might be speculated that developing stomata have a better ability to adapt to a new environment than fully developed ones (i.e. stomata in which the final length has been achieved). As rose is a dicotyledonous species, stomatal initiation is

not synchronous (Larkin et al. 1997). Therefore, during the early phases of leaf expansion, different stomatal developmental stages can be found. This might explain why the sensitivity to high RH is spread over such a large range of leaf developmental stages (35–100% FLE).

When our findings are translated to conditions of changing RH, as in plants grown in natural environments or in protected cultivation, they indicate that only a few days of high RH can induce lack of proper stomatal closure in response to desiccation, at least in the leaves that are in the last phase of elongation. Depending on the RH levels over time, plants grown under such conditions are expected to have some leaves that respond properly to dehydration and others that do not.

ABA manipulation during and after FLE

Long-term application of exogenous ABA during leaf expansion fully counteracted the problem of lack of proper stomatal response to dehydration in rose plants grown at high RH (Fig. 5). The same was shown previously in *T. virginiana* (Rezaei Nejad and van Meeteren 2007). The fact that ABA application in moderate RH leaves resulted in a significantly better stomatal closure when the leaves became dehydrated (Fig. 5B), as compared with ABA-treated leaves expanded in high RH, might be taken to support the hypothesis that the endogenous leaf ABA concentration in rose is lower at high RH leaves. These results provide additional evidence that the weaker hydraulic signal during cultivation at high RH (i.e. low transpiration rates and/or the high leaf water potential; Fig. 1) is not per se the dominant factor involved in poor stomatal functioning. It is concluded that a long-term low leaf ABA concentration during stomatal development is the main cause of stomatal malfunctioning in well-watered rose plants grown at high RH.

Unlike the results for expanding leaves (Fig. 5), we show here that daily exogenous ABA application after complete leaf expansion – keeping the same application conditions (i.e. ABA concentration and treatment duration) – did not have any effect on the stomatal functionality in plants grown continuously at high RH (Fig. 7). Thus, it could be speculated that in fully expanded leaves the cuticula has worked as a barrier for ABA penetration. However, this is not likely because exogenously applied ABA has been shown to penetrate to the leaf interior via the cuticle (Blumenfeld and Bukovac 1972), but also solute uptake takes place through the stomatal pores (Eichert and Burkhardt 2001). Moreover, in the latter study it was found that this uptake is facilitated at elevated humidity because of the increased stomatal opening. Additionally, in our study, a lack of stomatal responses to a change in RH in fully expanded rose leaves [Fig. 6, and also described by Mortensen and Gislerød (2000)] was shown. Taken together, these results bring us to the conclusion that after FLE, stomatal functioning is no longer affected either by the ABA or RH level. In *T. virginiana,* the loss of stomatal functionality after transfer to high RH was related to a decrease in the leaf ABA concentration, and the authors proposed that a certain ABA level is required not only to induce, but also to sustain stomatal functionality (Rezaei Nejad and van Meeteren 2008). As our study shows that stomatal functioning in rose is independent of post-development RH level (Fig. 6), two hypotheses arise: (1) although the root to shoot ABA signalling is weakened after transfer to high RH, this is still sufficient to sustain stomatal responsivity or (2) the level of ABA after the development of stomatal apparatus (i.e. in fully developed leaves) is not relevant for a proper stomatal functioning in rose. We tested this hypothesis by excising the root (hormonal and hydraulic) signals for a period of 2 weeks using plants grown continuously at moderate RH and assessing the response to desiccation in fully expanded leaves. Similarly to the non-excised control plants, stomata in leaves fully expanded at moderate RH sustained their functionality despite the complete absence of root signals (Fig. 8). Therefore, these results suggest that in rose, the functionality of the stomatal apparatus is determined during leaf expansion, and is independent from the ABA level after that period, even when ABA concentration is drastically changed.

Besides the role of ABA on stomatal physiology it was shown that exogenous ABA application resulted in smaller stomatal dimensions (Table 1). Similar results were observed in *T. virginiana* (leaves treated daily with ABA; Franks and Farquhar 2001) and wheat (ABA was injected regularly in the nutrient solution; Quarrie and Jones 1977). However, exogenous ABA application did not affect stomatal density, whereas in other studies a higher stomatal density has been reported in ABA-treated leaves (Bradford et al. 1983, Franks and Farquhar 2001).

Conclusions

It was shown that the capacity of stomata to close in response to leaf dehydration is completely established during the period of leaf expansion. Moreover, it was concluded that in expanding leaves the degree of stomatal adaptation depends on the duration and timing of exposure to high RH. In general, the longer the exposure to high RH the higher the loss of stomatal functionality in response to desiccation. Furthermore, it was found that stomatal malfunctioning, as a result of plant growth under long-term high RH, is strongly determined during the last part of leaf expansion. A long-term

exogenous ABA application (twice a day), throughout leaf expansion, is able to counteract the negative effect of high RH on stomatal functioning. Nevertheless, once the leaves have fully expanded, the stomatal closure capacity is no longer affected either by the RH or the ABA level, even when drastic changes in the leaf ABA concentration take place (i.e. long-term exogenous ABA application in high RH-grown plants and root removal in moderate RH-grown plants). Thus, these results suggest that the role of ABA on stomatal hydrosensitivity is restricted to the period of leaf expansion.

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