Control of stomatal opening after growth at high relative air humidity

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Contents

To my dear wife, Mahnaz, and my lovely daughter, Sara

Chapter 1

General introduction

As a result of contemporary horticultural practice the normal functioning of stomata is sometimes disturbed. This happens especially when plants are grown at high relative air humidities (RH) and transferred to low RH conditions. Disturbance of stomatal movements results in a negative water balance in plants and quality problems due to excessive water loss after harvest. As a result of energy saving practices (less ventilation, totally closed greenhouses) it may be expected that very high RH will become more and more common in commercial greenhouses. At the end of the production chain - at the consumer - the RH will be low. Thus, more quality problems can be foreseen in the near future. Understanding the physiology of stomatal behavior in response to long-term growth at high RH is therefore important.

Stomatal physiology and structure

Stomata (Fig. 1) are small pores on the surfaces of leaves and stems, bordered by a pair of highly specialized guard cells. Stomatal movement (opening and closing of stomata) is regulated in response to the complex integration of numerous signals that ultimately act upon a network of ion channels in the plasma and vacuolar membranes of guard cells. Stomata open due to an increase in guard cell volume driven by an influx of water following the increase in the concentration of K^+, Cl^-, and organic solutes in the guard cell (Blatt, 2000; Schroeder *et al.*, 2001). Conversely, stomatal closure results from efflux of solutes to the apoplast, which drives osmotic water loss and the decrease of guard cell turgor pressure (Blatt, 2000; Schroeder *et al.*, 2001). The transport of K^+ across the plasma membrane is dominated by two classes of K^+ channels: inward-rectifying K^+ channels ($I_{K,in}$) which facilitate K^+ uptake during stomatal opening, and outward-rectifying K^+ channels ($I_{K,out}$) which allow the release of K^+ during stomatal closure (Blatt, 2000). Factors influencing the activity of channels affect stomatal movement. For example, an increase of cytosolic Ca^{2+} concentrations inhibits $I_{K,in}$ and inhibits stomatal opening (Hedrich *et al.*, 1990; Schroeder and Hagiwara, 1989).

There are two morphologically different types of guard cells: kidney shaped and dumb-bell shaped. The most common form is kidney shaped which is found in dicotyledons and some monocotyledons, whereas dumb-bell shaped cells are found in grasses (Atwell *et al.*, 1999; Willmer and Fricker, 1996). Both types of guard cells are surrounded by epidermal cells which may or may not have a distinctive

shape. If they are morphologically the same as other epidermal cells they are called neighboring cells, but if they are clearly distinct they are known as subsidiary cells (Willmer and Fricker, 1996). The shapes, sizes, arrangements and cell wall characteristics of neighboring or subsidiary cells are also important in stomatal aperture changes (Raschke, 1975; Zeiger, 1983). Directly beneath each pair of guard cells inside the leaf is the substomatal cavity. Air in this cavity in living leaves is virtually saturated with water vapour due to evaporation from adjacent cell walls (Atwell *et al.*, 1999). Besides their shapes, guard cells are different from other epidermal cells in having chloroplasts (Zeiger, 1983). *Paphiopedilum* (an orchid) is the only reported genus with guard cells containing undeveloped chloroplasts (Rutter and Willmer, 1979; Talbott *et al.*, 2002b). Cell walls around each of the guard cells are more thickened on the side facing the pore, so an increase in guard cell turgor leads to an outward bowing of the guard cells, which opens the stomata, while a decrease in guard cell turgor results in stomatal closure (Blatt, 2000; Zeiger, 1983). Mature guard cells also lack plasmodesmata (Wille and Lucas, 1984). Therefore, all the solute fluxes that drive cell volume changes must take place across the plasma membrane (Blatt, 2000).

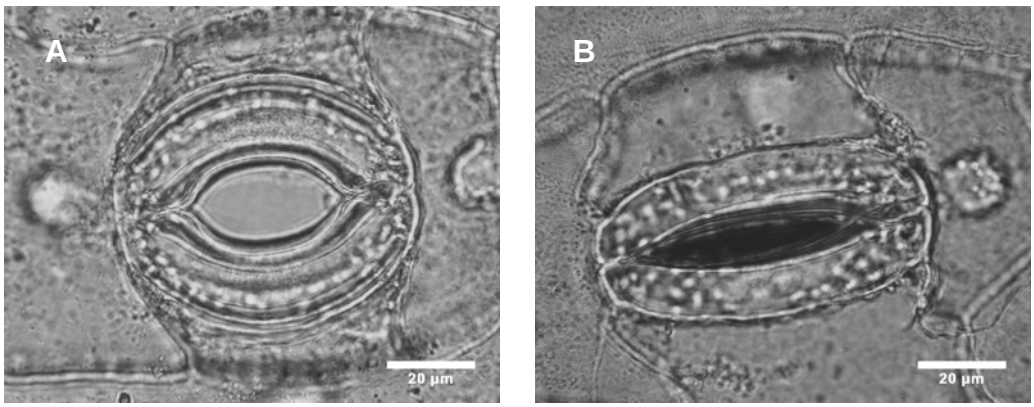

Fig. 1 An open (A) and a closed (B) stoma in *Tradescantia virginiana.*

Stomatal responses to environmental and hormonal stimuli

The primary role of stomata is to regulate leaf gas exchange, in particular maximizing the uptake of CO_2 for photosynthesis in relation to the loss of water vapor by transpiration. To do this, stomata open or close in response to a wide range

of environmental factors such as light, temperature, atmospheric CO_2 concentration, atmospheric humidity, and hormonal stimuli such as abscisic acid (ABA).

In most plants, in the absence of stresses, stomata open in response to light and close in response to darkness. Stomata remain open during the day and close rapidly as light levels fall towards evening. In contrast, stomata in crassulacean acid metabolism (CAM) plants open at night for CO_2 fixation (Black and Osmond, 2003). Light is a direct factor for stomatal opening. In general stomatal closure upon darkening is faster than opening in response to increasing light levels (Willmer and Fricker, 1996). Light quality also directly affects stomatal movements. At equal energy levels, light of different wavelengths promotes stomatal opening in epidermal strips in the following order of effectiveness: blue > red > green (Willmer and Fricker, 1996). Guard cells have two well-characterized light responses, one mediated by chlorophyll-dependent, guard cell photosynthesis, often called a "red light response" (Sharkey and Ogawa, 1987; Tallman, 1992) and a second response specific for blue light (Assmann, 1993; Assmann and Shimazaki, 1999; Tallman, 1992; Zeiger, 1990). Recently it has been shown that though green light alone does not cause stomatal closure, it reverses blue light-specific opening of stomata in a dose-response fashion, with full reversal obtained with a 2:1 ratio of green to blue light (Eisinger *et al.*, 2003; Frechilla *et al.*, 2000; Talbott *et al.*, 2002a). Stomata also have their own endogenous controls which lead to a circadian rhythm of opening and closing (Mencuccini *et al.*, 2000; Webb, 2003).

Stomata appear to respond to intercellular CO_2 concentrations (Mott, 1988), and this is determined by both the CO_2 concentration at the leaf surface and by the mesophyll CO_2 assimilation rate (Morison, 1998). In general, within the physiological range of CO_2 levels, stomata open as CO_2 levels decrease and reduce their aperture as the levels rise (Mansfield *et al.*, 1990; Morison, 1998; Morison, 2001).

Temperature has some direct and indirect effects on stomatal behaviour. As the leaf temperature increases, the metabolic activity within the leaf and guard cells increases, reaches an optimum, and then decreases as more and more cell damage occurs (Raschke, 1975; Willmer and Fricker, 1996; Zeiger, 1983). At moderate temperature (13-20 ^{o}C), the activity of both $I_{K,in}$ and $I_{K,out}$ in *Vicia* increases with increasing temperature. At temperature from 20 to 28 ^{o}C, the activity of $I_{K,out}$ decreases with increasing temperature, whereas the activity of $I_{K,in}$ continues to

increase (Ilan *et al.*, 1995). This difference in temperature response between $I_{K,in}$ and $I_{K,out}$ at high temperatures would favor K^+ influx and stomatal opening, and thus could allow cooling of leaves via a higher transpiration rate (Ilan *et al.*, 1995).

The effects of certain environmental factors on stomatal behaviour may be mediated by hormones. For example, drought stress, salt stress and chilling of plants can result in elevated ABA levels within leaves with subsequent stomatal closure.

Stomatal responses to short-term changes in RH

Stomatal closure in response to a short-term decrease in air relative humidity (RH) or an increase in vapor pressure difference (VPD) at the leaf surface has been observed in a wide variety of species (Eamus and Shanahan, 2002; Monteith, 1995; Sheriff, 1979). As VPD increases, stomatal conductance decreases (Eamus and Shanahan, 2002; Yong *et al.*, 1997). Also, there are three phases in the stomatal response to transpiration rate (Eamus and Shanahan, 2002; Monteith, 1995). Initially, at low values of VPD with an increasing VPD, stomatal conductance is high and the transpiration rate increases. At intermediate values of VPD with an increasing VPD, transpiration rate remains relatively constant because stomatal conductance now declines with increasing VPD. Finally, at larger values of VPD with an increasing VPD, stomatal closure is more extreme and transpiration rate declines with increasing VPD (Eamus and Shanahan, 2002; Monteith, 1995). Though the mechanism by which guard cells actually sense air humidity is unknown, two mechanisms have been proposed: feedforward and feedback. In a feedforward mechanism, stomata respond to VPD independently of the bulk leaf water status and respond only to a change in the ambient evaporative demand (Cowan, 1977; Farquhar, 1978; Lange *et al.*, 1971; Schulze *et al.*, 1972). In contrast, a feedback system for stomatal response to VPD is based on the effect of whole-leaf transpiration rate on leaf water status or the gradient of water potential between guard cells and other epidermal cells (Monteith, 1995; Mott and Parkhurst, 1991). There is also still no accepted mechanism to explain how guard cells sense VPD directly (Grantz, 1990).The results of Mott and Parkhurst (1991) in experiments using air and helox (a helium: oxygen mixture) were consistent with a mechanism for stomatal responses to humidity based on the rate of water loss from the leaf. Monteith (1995), in reviewing gas exchange data in 52 sets of

measurement on 16 species, concluded that evidence for feedforward control was rare and most data were consistent with a feedback system. In gas exchange experiments using additional 13 species, in rainforest canopy ranging from tree seedlings to herbaceous crop plants, Franks et al (1997) observed an apparent feedforward response in only two. They concluded that the VPD response may be readily explained by a feedback system. But, over the course of a day, additional factors, possibly metabolic, could be acting to enhance the VPD response, giving rise to what they referred to as an apparent feedforward response. Eamus and Shanahan (2002) described a model for stomatal responses to increasing VPD based on cellular water relations and concluded that feed-forward behaviour of stomata does not explain stomatal responses to VPD, but that feedback control, involving water loss from guard cells, can explain these responses. They mentioned cuticular transpiration as an important feature of stomatal responses to VPD and the cause of the three phase response to VPD.

Stomatal behavior after long periods of high RH

In contrast to studies exploring stomatal regulation under conditions where the plant is exposed to a water deficit, stomatal regulation in plants with an ample supply of water and a long-term exposure to a low VPD (high RH) has scarcely ever been examined in detail, in spite of some reports of unusual phenomena in such plants. In recent years, a shorter vase life of cut roses has been reported in plants grown at high RH above 85% (Mortensen and Fjeld, 1998; Mortensen and Gislerød, 1999; Torre and Fjeld, 2001; Torre *et al.*, 2001). At a high VPD typical of domestic conditions, the symptoms that develop during vase life are characteristics for roses suffering from water stress: bent neck, wilting of the leaves and improper opening and wilting of flowers (Torre and Fjeld, 2001). In severe cases cut stems developed dried and dead patches in the leaves (brittle leaves). Recently, a symptom very similar to this caused large problems in cut lily flowers after harvest. Torre and Fjeld (2001) reported only negligible differences in growth and morphology between moderate and high RH grown roses, though an uncontrolled water loss is characteristic for roses grown at high RH. Stomata of leaves grown in high RH do not respond to decreasing water potentials or to darkness (Torre *et al.*, 2003).

Vegetative propagation from leafy cuttings is an important and widely used method for producing large numbers of genetically identical woody plants. After

transfer of the rooted cuttings from the low VPD in the rooting environment to one of increased evaporative demand, the young plants may shrivel and die. It is common, therefore, to slowly adapt the rooted cuttings to an environment with a high VPD so as to avoid desiccation. The reason why this hardening is necessary in some species is unclear but may be related to an inability of leaves to control water loss following adaptation to the high RH rooting environment. Stomatal conductance in expanding leaves of *Corylus maxima* cuttings increased almost 10-fold over the first 14 days in the rooting environment (Fordham *et al.*, 2001b).

There is some evidence that stomata developed during *in vitro* propagation have a reduced capacity to control water loss in response to desiccation (Santamaria *et al.*, 1993). They showed 2-3-fold greater stomatal apertures in plants grown *in vitro* than in those that had developed *ex vitro*. During *in vitro* propagation VPD is very low. Changes in stomatal morphology in *in vitro* propagated plants have been reported in several species such as apples (Blanke and Belcher, 1989), *Delphinium* (Santamaria *et al.*, 1993) and roses (Ghashghaie *et al.*, 1992). The development of stomata *in vitro* leads to progressively more rounded stomatal pores instead of the elliptical pores observed in *ex vitro* stomata (Zacchini *et al.*, 1997; Ziv *et al.*, 1987), and these rounded pores are associated with irregular stomatal function (Marin *et al.*, 1988). Similarly, measurements of stomatal pore dimensions indicated more rounded stomatal pores in cuttings of *Corylus maxima* than in intact plants (Fordham *et al.*, 2001b). Torre (2003) reported a higher stomatal density, and bigger stomata in high RH grown roses. In contrast, a lower density of stomata in leafy *C. maxima* cuttings in higher RH was reported by Fordham *et al.* (2001b).

Several authors have shown that stomatal aperture in distinct areas of a leaf can be well below the mean stomatal aperture of the rest of the leaf. This phenomenon has been called ‘patchy’ stomatal closure, and it can be induced by changes in environmental factors, like water and salt stress, changes in light intensity, changes in ambient CO_2 partial pressure and low air humidity (Beyschlag and Eckstien, 2001; Beyschlag and Pfanz, 1990; Downton *et al.*, 1988; During, 1992; Eckstien *et al.*, 1996; Mott *et al.*, 1993). However the effect of long-term exposure to high RH on the heterogeneity of stomatal closure over a leaf surface is unknown. It is also unknown to what extent the effect of long-term high RH on stomatal responses correlates with changes in other leaf hydraulic properties. Such

correlations could point to either compensatory mechanisms or co-adaptation of hydraulic properties.

The reason why stomata of high RH grown leaves are less hydrosensitive is not clear. The role of ABA in the control of stomatal functioning is well known (reviewed by Leung and Giraudat, 1998). When plants are exposed to soil drying, ABA concentration is increased in the guard cells of the stomata and influences the influx and efflux of K^+, along with either Cl^- or malate, across the plasma and tonoplast membranes, which decrease guard cell turgor, causing stomatal closure (Assmann, 1993; Hartung *et al.*, 1998). This allows the plant to retain water. In addition to the reduction in guard cell turgor, changes in cytoskeletal organization (actin filaments) and alterations in gene expression take place when stomata are exposed to ABA (Hetherington and Woodward, 2003; Leung and Giraudat, 1998).
Many studies suggest that the short-term effects of elevated ABA concentrations are reversible (Ackerson, 1980; Tardieu *et al.*, 1996; Trejo *et al.*, 1995). In addition, there is some literature about the effect of long-term elevated ABA concentration on developmental changes and functioning of stomata. Brown *et al.* (1976) showed that when plants were subjected to frequent or long-term drought, their stomata reopened more readily upon rewatering than did stomata in plants experiencing only a single, brief period of drought. Cutler *et al*. (1977) showed stomata from plants grown under water stress were smaller than those of well-watered plants. Franks and Farquhar (2001) showed that ABA-treated plants had significantly smaller stomata but with a higher density. They also found that plants grown under ABA treatment had significantly lower stomatal conductance and operated with lower stomatal conductance for any given guard cell turgor. They concluded that ABA not only regulates short-term, reversible adjustments to rates of carbon uptake and water loss, but through its effect on stomatal structure has the potential to permanently alter the photosynthetic operating point in the direction of improved water use efficiency.

In contrast to drought stress, there is little information about the role of ABA on stomatal development and functioning when well-watered plants are exposed for prolonged periods to high RH and thus a low VPD. The amount of leaf ABA depends on:

1. ABA produced by the roots and transported through the xylem by the transpiration stream (Liang and Zhang, 1999; Zhang *et al.*, 1997; Zhang and Outlaw, 2001). The rate at which ABA enters a leaf is determined by xylem ABA

concentration and transpiration flux (Zhang *et al.*, 1997). The transpiration flux is positively related to VPD.

2. ABA produced by the leaf (Zeevaart and Creelman, 1988). Turgor loss has been proposed as the event that initiates ABA formation in detached leaves (Pierce and Raschke, 1980).
3. Intraleaf ABA redistribution. During water stress, ABA stores within the leaf symplast are released to the apoplast (Hartung *et al.*, 1998). As mature guard cells lack plasmodesmatal connections with other cells, the elevated ABA level in the apoplast is a source for the accumulation of ABA by guard cells (Popova *et al.*, 2000).

As the transpiration rate in plants growing at high RH (low VPD) is low, it might be that under these conditions low concentrations of ABA develop in leaves. If high ABA concentrations during growth can change the stomatal anatomy and increase their responsivity to drought stress signals, low ABA concentrations during growth might lessen stomatal responsivity to a lowered hydration state.

There are many papers reporting the adaptation process from a high to a low RH especially in *in vitro* cultured plants (e.g. Capellades *et al.*, 1990; Koroch *et al.*, 1997; Wardle *et al.*, 1983). However, there is not much information available regarding the rate and the reversibility of adaptation of stomatal behaviour to high RH. There are contradictory findings regarding the possibility of improving the behaviour of stomata developed at high RH after transferring to low RH, as both adaptation (Brainerd and Fuchigami, 1981; Marin *et al.*, 1988) and a failure to adapt (Fordham *et al.*, 2001a; Sallanon *et al.*, 1993; Ziv *et al.*, 1987) have been reported. It is also unknown how this adaptation process is related to changes in leaf ABA concentration and leaf hydraulic properties in the adapting plants.

Aim and outline of the thesis

The general aim of this study is to investigate the effects of high RH during growth on the stomatal response characteristics of *Tradescantia virginiana* L. *T. virginiana* is a plant of the Commelinaceae. Ease of propagation and cultivation, and the large size of its stomata have made *T. virginiana* a model plant for stomatal research. The experiments and results described in this thesis provide insights into the physiological effects of high RH during growth on stomatal functioning.

Chapter 2 shows the quantitative effects of moderate and high RH during growth on the stomatal anatomy and responses of well-watered plants in response to desiccation, ABA application and light/dark transition. The results in this chapter show higher variability of stomatal closure and the presence of some non-closing stomata in high RH grown plants.

Chapter 3 describes the dynamics of spatial heterogeneity of stomatal closure altered by growth at high RH. In this chapter the estimates of stomatal closure obtained by means of Φ_{PSII} measurements under low O_2 concentration is correlated with direct measurements of stomatal closure and water relation parameters in plants subjected to water stress.

Chapter 4 describes the role of ABA in the disturbed stomatal responses of plants during growth at high RH. In this chapter the concentration of ABA in individual leaves grown at moderate and high RH is compared and stomatal responses to short-term ABA application, and stomatal responses to long-term ABA application is analysed. The results of this chapter reinforce the proposal that a very low ABA concentration in well-watered plants during growth at high RH could be a reason for the structural or physiological changes of stomata. In addition, the consequences for stomatal responses of maintaining a high or low RH around only one leaf are described.

Chapter 5 describes the dynamics of adaptation of stomatal behaviour to moderate or high RH. In this chapter the rate and the reversibility of adaptation of stomatal behaviour to moderate or high RH is investigated and correlated with leaf ABA concentration and leaf hydraulic properties of the adapted plants.

In the **general discussion (Chapter 6)** the quantitative effects of high RH on stomatal behaviour and leaf hydraulic properties, and the role of ABA is discussed on the basis of the present results. Suggestions for further research are highlighted.

References

Ackerson RC. 1980. Stomatal response of cotton to water stress and abscisic acid as affected by water stress history. *Plant Physiology* **65**, 455-9.

Assmann SM. 1993. Signal transduction in guard cells. *Annual Review of Cell Biology* **9**, 345-75.

Assmann SM, Shimazaki K-i. 1999. The multisensory guard cell. Stomatal responses to blue light and abscisic acid. *Plant Physiology* **119**, 809-16.

Atwell B, kriedemann P, Turnbull C. 1999. Plants in Action. South yarra: Macmillan Publishers Australia pty Ltd.

Beyschlag W, Eckstien J. 2001. Towards a causal analysis of stomatal patchiness: the role of stomatal size variability and hydrological heterogeneity. *Acta Oecologica* **22**, 161-73.

Beyschlag W, Pfanz H. 1990. A fast method to detect the occurrence of nonhomogenous distribution of stomatal aperture in heterobaric plant leaves. *Oecologia* **82**, 52-5.

Black C, Osmond C. 2003. Crassulacean acid metabolism photosynthesis: `working the night shift'. *Photosynthesis Research* **76**, 329-41.

Blanke MB, Belcher AR. 1989. Stomata of apple leaves cultured *in vitro*. *Plant Cell, Tissue and Organ Culture* **19**, 85-9.

Blatt MR. 2000. Cellular signaling and volume control in stomatal movements in plants. *Annual Review of Cell and Developmental Biology* **16**, 221-41.

Brainerd KE, Fuchigami LH. 1981. Acclimatization of aseptically cultured plants to low relative humidity. *Jornal of the American Society for Horticultural Science* **106**, 515-8.

Brown KW, Jordan WR, Thomas JC. 1976. Water stress induced alternations of the stomatal response to decreases in leaf water potential. *Physiologia Plantarum* **37**, 1-5.

Capellades R, Fontanau C, Carulla C, Debergh P. 1990. Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa moltiflora*. *Journal of the American Society for Horticultural Science* **115**, 141-5.

Cowan IR. 1977. Stomatal behaviour and environment. *Advances in Botanical Research* **4**, 117-228.

Cutler JM, Rains DW, Loomis RS. 1977. The importance of cell size in the water relations of plants. *Physiologia Plantarum* **40**, 255-60.

Downton WJS, Loveys BR, Grant WJR. 1988. Stomatal closure fully accounts for the inhibition of photosynthesis by abscisic acid. *New Phytologist* **108**, 263-6.

During H. 1992. Low air humidity causes non-uniform stomatal closure in heterobaric leaves in *Vitis* species. *Vitis* **31**, 1-7.

Eamus D, Shanahan ST. 2002. A rate equation model of stomatal responses to vapour pressure deficit and drought. *BMC Ecology* **2**, 8.

Eckstien J, Beyschlag W, Mott KA. 1996. Changes in photon flux can induce stomatal patchiness. *Plant, Cell and Environment* **19**, 1066-75.

Eisinger WR, Bogomolni RA, Taiz L. 2003. Interactions between a blue-green reversible photoreceptor and a separate UV-B receptor in stomatal guard cells *American Journal of Botany* **90**.

Farquhar GD. 1978. Feedforward responses to stomata to humidity. *Australian Journal of Plant Physiology* **5**, 787-800.

Fordham MC, Harrison-Murray RS, Knight L, Clay CM. 2001a. Decline in stomatal response to leaf water deficit in *Crylus maxima* cuttings. *Tree Physiology* **21**, 489-96.

Fordham MC, Harrison-Murray RS, Knight L, Evered CE. 2001b. Effects of leaf wetting and high humidity on stomatal function in leafy cuttings and intact plants of *Corylus maxima*. *Physiologia Plantarum* **113**, 233-40.

Franks PJ, Cowan IR, Farquhar GD. 1997. The apparent feedforward response of stomata to air vapour pressure deficit: information revealed by different experimental procedures with two rainforest trees *Plant, Cell and Environment* **20**, 142-5.

Franks PJ, Farquhar GD. 2001. The effect of exogenous abscisic acid on stomatal development, stomatal mechanics and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology* **125**, 935-42.

Frechilla S, Talbott LD, Bogomolni RA, Zeiger E. 2000. Reversal of blue light-stimulated stomatal opening by green light. *Plant Cell Physiology* **41**, 171-6.

Ghashghaie J, Brenkmann F, Saugier B. 1992. Water relations and growth of rose plants cultured *in vitro* under various relative humidities. *Plant Cell, Tissue and Organ Culture* **30**, 51-7.

Grantz DA. 1990. Plant response to atmospheric humidity. *Plant, Cell and Environment* **13**, 667-79.

Hartung W, Wilkinson S, Davies WJ. 1998. Factors that regulate abscisic acid concentrations at the primary site of action at the guard cell. *Journal of Experimental Botany* **49**, 361-7.

Hedrich R, Busch H, Raschke K. 1990. Ca^{2+} and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. *EMBO Journal* **9**, 3889-92.

Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. *Nature* **424**, 901-8.

Ilan N, Moran N, Schwartz A. 1995. The role of potassium channels in the temperature control of stomatal aperture. *Plant Physiology* **108**, 1161-70.

Koroch AR, Juliani HRJr, Juliani HR, Trippi VS. 1997. Micropropagation and acclimatization of *Hedeoma multiflorum*. *Plant Cell, Tissue and Organ Culture* **48**, 213-7.

Lange OL, Lösch R, Schulze ED, Kappen L. 1971. Responses of stomata to changes in humidity. *Planta* **100**, 76-86.

Leung J, Giraudat J. 1998. Abscisic acid signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 199-222.

Liang J, Zhang J. 1999. The relations of stomatal closure and reopening to xylem ABA concentration and leaf water potential during soil drying and rewatering. *Plant Growth Regulation* **29**, 77-86.

Mansfield TA, Hetherington AM, Atkinson CJ. 1990. Some current aspects of stomatal physiology. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 55-75.

Marin JA, Gella R, Herrero M. 1988. Stomatal structure and functioning as a response to environmental changes in acclimtized micropropagated *Prunus cerasifera* L *Annals of Botany* **62**, 663-70.

Mencuccini M, Mambelli S, Comstock J. 2000. Stomatal responsiveness to leaf water status in common bean (*Phaseolus vulgaris* L.) is a function of time of day. *Plant, Cell and Environment* **23**, 1109-18.

Monteith JL. 1995. A reinterpretation of stomatal responses to humidity. *Plant, Cell and Environment* **18**, 357-64.

Morison JIL. 1998. Stomatal response to increased CO_2 concentration. *Journal of Experimental Botany* **49**, 443-52.

Morison JIL. 2001. Increasing atmospheric CO_2 and stomata *New Phytologist* **49**, 154-6.

Mortensen LM, Fjeld T. 1998. Effects of air humidity, lighting period and lamp type on growth and vase life of roses. *Scientia Horticulturae* **73**, 229-37.

Mortensen LM, Gislerød HR. 1999. Influence of air humidity and lighting period on growth, vase life and water relations of 14 rose cultivars. *Scientia Horticulturae* **82**, 289-98.

Mott KA. 1988. Do stomata respond to CO_2 concentrations other than intercellular? *Plant Physiology* **86**, 200-3.

Mott KA, Cardon ZG, Berry JA. 1993. Asymmetric patchy stomatal closure for the two surfaces of *Xanthium strumarium* L. leaves at low humidity. *Plant, Cell and Environment* **16**, 25-34.

Mott KA, Parkhurst DF. 1991. Stomatal responses to humidity in air and helox. *Plant, Cell and Environment* **14**, 509-15.

Pierce M, Raschke K. 1980. Correlation between loss of turgor and accumulation of abscisic acid in detached leaves. *Planta* **148**, 174-82.

Popova LP, Outlaw WH, Aghoram K, Hite DRC. 2000. Abscisic acid - an intraleaf water-stress signal. *Physiologia Plantarum* **108**, 376-81.

Raschke K. 1975. Stomatal action. *Annual Review of Plant Physiology* **26**, 309-40.

Rutter JC, Willmer CM. 1979. A light and electron microscopy study of the epidermis of *Paphiopedilum* spp. with emphasis on stomatal ultrastructure. *Plant, Cell and Environment* **2**, 211-9.

Sallanon H, Tort M, Courdret A. 1993. The ultrastructure of micropropagated and greenhouse rose plant stomata. *Plant Cell, Tissue and Organ Culture* **32**, 227-33.

Santamaria JM, Davies WJ, Atkinson CJ. 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. *Journal of Experimental Botany* **44**, 99-107.

Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D. 2001. Guard cell signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 627-58.

Schroeder JI, Hagiwara S. 1989. Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* **338**, 427-30.

Schulze ED, Lange OL, Buschbom U, Kappen L, Evenari M. 1972. Stomatal responses to changes in humidity in plants growing in the desert. *Planta* **108**, 259-70.

Sharkey TD, Ogawa T. 1987. Stomatal responses to light. In: Zeiger G, Farquhar D, Cowan IR eds. *Stomatal function*. Stanford, California, USA: Stanford University Press, 195-208.

Sheriff DW. 1979. Stomatal aperture and the sensing of the environment by guard cells. *Plant, Cell and Environment* **2**, 15-22.

Talbott LD, Nikolova G, Ortiz A, Shmayevich I, Zeiger E. 2002a. Green light reversal of blue-light-stimulated stomatal opening is found in a diversity of plant species. *American Journal of Botany* **89**, 366-8.

Talbott LD, Zhu J, Han SW, Zeiger E. 2002b. Phytochrome and blue light-mediated stomatal opening in the orchid, *Paphiopedilum*. *Plant Cell Physiology* **43**, 639-46.

Tallman G. 1992. The chemiosmotic model of stomatal opening revisited. *Critical Reviews in Plant Science* **11**, 35-57.

Tardieu F, Lafarge T, Simonneau TH. 1996. Stomatal control by fed or endogenous xylem ABA in sunflower: interpretation of correlations between leaf water potential and stomatal conductance in anisohydric species. *Plant, Cell and Environment* **19**, 75-84.

Torre S, Fjeld T. 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. *Scientia Horticulturae* **89**, 217-26.

Torre S, Fjeld T, Gislerød HR. 2001. Effects of air humidity and K/Ca ratio in the nutrient supply on growth and postharvest characteristics of cut roses. *Scientia Horticulturae* **90**, 291-304.

Torre S, Fjeld T, Gislerød HR, Moe R. 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. *Journal of the American Society for Horticultural Science* **128**, 598-602.

Trejo CL, Clephan AL, Davies WJ. 1995. How do stomata read abscisic acid signals? *Plant Physiology* **109**, 803-11.

Wardle K, Dobbs EB, Short KC. 1983. *In vitro* acclimatization of aseptically cultured plantlets to humidity. *Journal of the American Society for Horticultural Science* **108**, 386-9.**Webb AAR.** 2003. The physiology of circadian rhythms in plants *New Phytologist* **160**, **Wille AC, Lucas WJ.** 1984. Ultrasructural and histochemical studies on guard cells. *Planta* **160**, 129-42.

Willmer C, Fricker M. 1996. Stomata. London, UK: Chapman and Hall.

Yong JWH, Wong SC, Farquhar GD. 1997. Stomatal responses to changes in vapour pressure difference between leaf and air. *Plant, Cell and Environment* **20**, 1213-6.

Zacchini M, Morini S, Vitagliano C. 1997. Effect of photoperiod on some stomatal characteristics of *in vitro* cultured fruit tree shoots *Plant Cell, Tissue and Organ Culture* **49**, 195-200.

Zeevaart JAD, Creelman RA. 1988. Metabolism and physiology of ascisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology* **39**, 439-73.

Zeiger E. 1983. The biology of stomatal guard cells. *Annual Review of Plant Physiology* **34**, 441-74.

Zeiger E. 1990. Light perception in guard cells. *Plant, Cell and Environment* **13**, 739-47.

Zhang J, Jia W, Zhang D. 1997. Re-export and metabolism of xylem-delivered ABA in attached maize leaves under different transpirational fluxes and xylem ABA concentrations. *Journal of Experimental Botany,* **48**, 1557-64.

Zhang SQ, Outlaw WH. 2001. Abscisic acid introduced into the transpiration stream accumulates in the guard cell apoplast and causes stomatal closure. *Plant, Cell and Environment* **24**, 1045-54.

Ziv M, Schwartz A, Fleminger D. 1987. Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; implication for hardening. *Plant Science* **52**, 127-34.

Chapter 2

Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity

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Abstract

Plants produced at high relative air humidity (RH) show poor control of water loss after transferring to low RH, a phenomenon which is thought to be due to their stomatal behavior. The stomatal anatomy and responses of moderate (55%) and high (90%) RH grown *Tradescantia virginiana* plants to treatments that normally induce stomatal closure i.e. desiccation, abscisic acid (ABA) application and exposure to darkness were studied using attached or detached young, fully expanded leaves. Compared to plants grown at moderate RH the transpiration rate, stomatal conductance and aperture of high RH grown plants measured at the same condition (40% RH) were, respectively, 112%, 139% and 132% in light and 141%, 188% and 370% in darkness. Besides the differences in stomatal size (guard cell length was 56.7 and 73.3 µm for moderate and high RH grown plants, respectively), there was a clear difference in stomatal behavior. The stomata responded to desiccation, ABA and darkness in both moderate and high RH grown plants, but the high variability of stomatal closure in high RH grown plants was striking. Some stomata developed at high RH closed in response to darkness or to a decrease in relative water content (RWC) to the same extent as did stomata from moderate RH grown plants, whereas others closed only partly or did not close at all. Evidently some as yet unidentified physiological or anatomical changes during development disrupt the normal functioning of some stomata in leaves grown at high RH. The failure of some stomata to close fully in response to ABA suggests that ABA deficiency was not responsible for the lack of stomatal closure in response to desiccation.

Key words: Abscisic acid, aperture, desiccation, stomata, stomatal conductance, stomatal index, stomatal size

Abbreviations: ABA, abscisic acid; RH, relative air humidity; RWC, relative water content, VPD, vapour pressure deficit

Introduction

Stomata control leaf gas exchange, especially CO_2-uptake for photosynthesis and water vapor loss via transpiration in aerial plant organs. Stomatal movement (producing changes in stomatal aperture) depends on the

swelling and shrinking of the guard cells caused by changes in cell turgor (Assmann, 1993; Wang *et al.*, 1998). This behavior is the result of interactions between physiological factors and environmental conditions (Assmann and Wang, 2001; Hetherington and Woodward, 2003; Kearns and Assmann, 1993). Moreover, stomatal responses to leaf water potential are altered by preceding environmental conditions. In general, the threshold leaf water potential required initiating stomatal closure shifts to a lower value by subjecting plants to repeated cycles of water stress by soil drying (Ackerson, 1980; Brown *et al.*, 1976; McCree, 1974). It has also been shown that stomata of plants grown under water stress are smaller than in well-watered plants (Cutler *et al.*, 1977; Spence *et al.*, 1986; Xia, 1994). When low water potentials during drought stress can change the stomatal anatomy and response characteristics, some opposite changes might be expected when plants are subjected to high water potentials i.e. growing well-watered plants at high RH. In contrast to studies exploring stomatal regulation under conditions where the plant is exposed to a water deficit, stomatal regulation in plants with an ample supply of water and a long-term exposure to a low VPD (high RH) has scarcely ever been examined in detail, in spite of some interesting reports of unusual phenomena in such plants. Of the few studies that have been made, most of them have been carried out on *in vitro* plantlets. It has been shown that high relative air humidity (RH > 85%) during greenhouse cultivation is a critical environmental factor in reducing the post-harvest life of cut roses, mainly as a result of uncontrolled water loss from the cut stems (Mortensen and Fjeld, 1998; Torre and Fjeld, 2001; Torre *et al.*, 2001). It has been proposed that the stomata of roses grown at high RH show differences in size, density, function and that they are insensitive to water stress and darkness (Torre and Fjeld, 2001; Torre *et al.*, 2001). However there are not much data available to support these claims. There is also the experience that stomata developed during *in vitro* propagation have a reduced capacity to control water loss. The stomata of plantlets produced in this way fail to reduce their aperture in response to turgor-reducing treatments such as abscisic acid (ABA) and darkness (Wardle and Short, 1983). The problem is thought to be due to the high RH in the culture containers, but it is also clearly affected by the cytokinins in the culture medium (Santamaria *et al.*, 1993). Furthermore, a failure of stomata to close properly in response to desiccation or ABA has been shown in rooted leafy cuttings after transferring from

the humid conditions of the propagator to one of increased evaporative demand (Fordham *et al.*, 2001a; Fordham *et al.*, 2001b).

Despite many papers on the short-term response of stomata to high RH, a comprehensive study of the changes in stomatal response characteristics of well-watered plants grown under prolonged conditions of low vapor pressure deficits is lacking. The aim of this work was to elucidate the differences in stomatal anatomy and response characteristics between well-watered *Tradescantia virginiana* plants grown at moderate and high RH in response to desiccation, ABA application and light/dark transition.

Materials and methods

Plant material and growth conditions

Young *T. virginiana* L. plants were grown in plastic pots filled with a commercial potting compost (Potgrond 4, Hortimea, Lent, The Netherlands) in two growth chambers each with different relative air humidity (moderate: 55 ± 5% and high: 90 ± 5%) at Wageningen University. The temperature was 21 ± 0.5 ^{o}C resulting in VPDs of 1.12 and 0.25 kPa for moderate and high RH conditions, respectively. The light intensity was 120 ± 10 µmol m^{-2} s^{-1} (measured with LI-250 Light Meter, LI-COR, Lincoln, USA) produced by fluorescent tubes (TLD 33 Philips) with a light period of 16 h per day. The plants were kept well-watered and given a nutrient solution weekly (concentration: 2 g l^{-1}; Kristalon^{TM}, Yara, Rotterdam, The Netherlands). The CO_2 concentration in the growth chambers was 360 ± 30 µmol mol^{-1} (measured with a CO_2 analyzer ADC-225-MK3, The Analytical Development Co. Ltd, Hoddesdon, England). Young fully expanded leaves were used in the experiments. All experiments were started 3 h after the beginning of the light period.

Measurements on stomata were only performed on the abaxial surface of the leaves, because some preliminary observations indicated that the ratio of stomatal density of the adaxial and abaxial side of the leaves used is about 1:6.

Experiment 1: Stomatal responses to desiccation

To study the effect of desiccation on transpiration rate, leaves were removed and transferred to a test room (40% RH, 20 ^{o}C, 1.40 kPa VPD and 50 µmol m^{-2} s^{-1}

irradiance). The leaves were re-cut under water, placed in small vials filled with water, covered with a plastic bag and incubated in darkness for 1 h to establish their maximum fresh weight. The leaves were then placed in empty vials in the light and their transpiration rate was measured gravimetrically by weighing the vials and leaves every 10 seconds and calculating the rate of water loss over time. This measurement procedure lasted for 5 h after which the leaf area was determined using a leaf area meter (LI-COR, model 3100 Area Meter; Lincoln, USA) and the leaves were dried at 80 ^{o}C. The transpiration rate was then calculated per unit leaf area and relative water content (RWC) was calculated according to Slavik (1974).

Stomatal conductance was measured every 75 minutes during 5 h desiccation using an AP3 porometer (Delta-T Devices Ltd, Cambridge, U.K.) in the test room.

Stomatal aperture was determined using a silicon rubber impression technique (Smith *et al.,* 1989). Impressions were made immediately after cutting and 2 h after the start of the desiccation treatment. Stomatal aperture 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, Texas).

Experiment 2: Stomatal responses to ABA application

To study the effect of ABA on transpiration rate, leaves were removed and transferred to the test room. The leaves were re-cut under water containing 0 or 100 µM (±)-ABA (Sigma, St. Louis, MO) and placed in small vials filled with 0 or 100 µM ABA solution. The vials were covered with aluminum foil to reduce evaporation. The transpiration rate was measured gravimetrically for 5 h and then the leaf area above the aluminum foil was determined to calculate transpiration rate per unit leaf area.

Stomatal conductance was measured once every hour for 5 h on leaves placed in vials containing 0 or 100 µM ABA in the test room.

Stomatal aperture in response to ABA application was determined with epidermal strips. The epidermal strips were removed from the abaxial surface of four leaves from randomly selected plants (one leaf per plant) and cut into 5mm ×10mm pieces using the technique of Weyers and Meidner (1990). The strips were

pre-incubated for 2 h in a stomata-opening medium (10 mM MES-KOH, pH 6.15, 50 mM KCl) in the test room. Some strips were picked at random from this pool of epidermal strips and incubated in a bath medium containing 10 mM MES-KOH, pH 6.15, 50 mM KCl and 0, 10, 100, 1000 µM ABA for 1 h (Iwai *et al.*, 2003). Stomatal aperture measurement was done on four randomly selected epidermal strips in each ABA concentration. Stomatal aperture of 10 randomly selected stomata in each epidermal strip was measured from digitized video images (×800 magnification) of stomata.

Experiment 3: Stomatal responses to light/dark transition

The transpiration rate was measured as described earlier during 2 h light followed by 2 h darkness treatment. The leaves were kept in water through the measurements.

Stomatal conductance and aperture were measured as described in experiment 1 after 2 h in light and then after 2 h in darkness in intact leaves.

Stomatal size, density and index

Using images taken from epidermal strips incubated in the stomatal opening medium used in experiment 2, guard cell length and width of stomata were measured. The measurement of stomatal size was done in 12 randomly selected strips in each RH treatment from three experiments (four strips per experiment and 10 randomly selected stomata in each strip). The stomatal density was also calculated from counts of the number of stomata in 30 randomly selected strips in each RH treatment from three experiments (10 strips per experiment). Stomatal index was calculated using the following equation (Weyers and Meidner, 1990):

$$Stomatal\ index = \frac{stomatal\ density \times 100}{stomatal\ density + density\ of\ subsidiary\ and\ epidermal\ cells}$$

Data collection and statistical analysis

Each experiment was carried out with at least four leaves from randomly selected plants (one leaf per plant) in each RH treatment. All experiments were repeated at least three times. Stomatal aperture was determined from 10 randomly selected stomata per leaf. To obtain the normal distribution of data for stomatal

aperture, it was necessary to use the square root of data. For stomatal size, density, index, aperture, transpiration rate and stomatal conductance (only in the experiment 3) data were subjected to analysis of variance (ANOVA) and $P>0.05$ was considered as not significant. The decline with time of transpiration rate and stomatal conductance during desiccation or ABA application (Figs. 1 and 3) was fitted using a nonlinear regression with one-phase exponential decay, [Y= a*exp (-k*X) + b]. The mean value of at least three experiments was used for the curve fitting with $1/SD^2$ to weigh the data points. For transpiration rate in relation to RWC a sigmoid was used. The parameters of fitted curves were compared statistically with F-test. GraphPad Prism 4 for Windows (GraphPad Software, San Diego, California) was used for all statistical analyses and curve fittings.

Results

Stomatal size, density and index

Significantly bigger stomata were found in plants grown at high RH (Table 1) compared to plants grown at moderate RH ($P=0.0002$ for stomatal length and $P=0.03$ for stomatal width). The stomata in plants grown at moderate RH were more rounded and their stomatal density was significantly higher than in plants grown at high RH ($P=0.02$). However, there was no significant difference between stomatal indices in the moderate and high RH grown plants ($P=0.43$).

Table 1 Stomatal length, width, density and index in *Tradescantia virginiana* leaves grown at 55% or 90% RH. Figures are mean values of three experiments ± SEM. For stomatal density and index 10 strips per experiment and for stomatal size four strips per experiment and 10 stomata in each strip were used.

	RH=55%	RH=90%
Guard cell length (µm)	56.7 ± 0.78	73.3 ± 1.05
Guard cell width (µm)	19.7 ± 0.20	21.9 ± 0.64
Stomatal density (mm^{-2})	22.8 ± 0.7	19.69 ± 0.6
Stomatal index	10.4 ± 0.2	10.6 ± 0.1

Experiment 1: Stomatal responses to desiccation

With desiccation, leaf transpiration rate (Fig. 1A) decreased in both groups of plants, grown at high or moderate RH. However, transpiration rate in high RH grown plants was higher than in moderate RH grown plants, as expressed in a significant difference (P<0.0001) between the asymptotic values of the regression curves (0.07 and 0.17 mmol $m^{-2}s^{-1}$ for moderate and high RH grown plants, respectively) . Moreover, the transpiration rate was less sensitive to decreases of RWC in high RH grown plants (Insert of Fig. 1A): at a RWC of 83%, the transpiration rate was 0.07 and 0.22 mmol $m^{-2}s^{-1}$ for moderate and high RH grown plants, respectively. The transpiration rate decreased to 0.17 mmol $m^{-2}s^{-1}$ at 65% RWC in high RH grown plants after 5 h desiccation. There is a sinusoid fluctuation in transpiration rate superimposed upon the exponential decay of transpiration rate. This correlated with fluctuations of temperature and humidity in the test room.

The drying curves of stomatal conductance (Fig. 1B) showed almost the same trend as transpiration rate. There was also a significant difference (P<0.003) between the asymptotic values of the fitted curves (2.11 and 6.65 mmol $m^{-2}s^{-1}$ for moderate and high RH grown plants, respectively). Therefore, stomatal conductance in high RH grown plants was significantly higher than in moderate RH grown plants.

The mean stomatal aperture was decreased with desiccation (P<0.0001) in both groups of plants (Fig. 2A), but the aperture was significantly wider in stomata from high RH grown plants (P<0.0001). The interaction between RH and desiccation on stomatal aperture was not significant.

Besides the difference in mean stomatal aperture, high RH grown plants differed from moderate RH grown plants in the frequency distribution of the number of stomata in different stomatal aperture ranges (Figs. 2B and 2C). In desiccated leaves the distribution of stomatal aperture in moderate RH grown plants became highly skewed to the right, while in high RH grown plants it became skewed to the left. In addition, the stomatal aperture distribution in high RH grown plants was more in the wider aperture ranges as compared to moderate RH grown plants before as well as after desiccation.

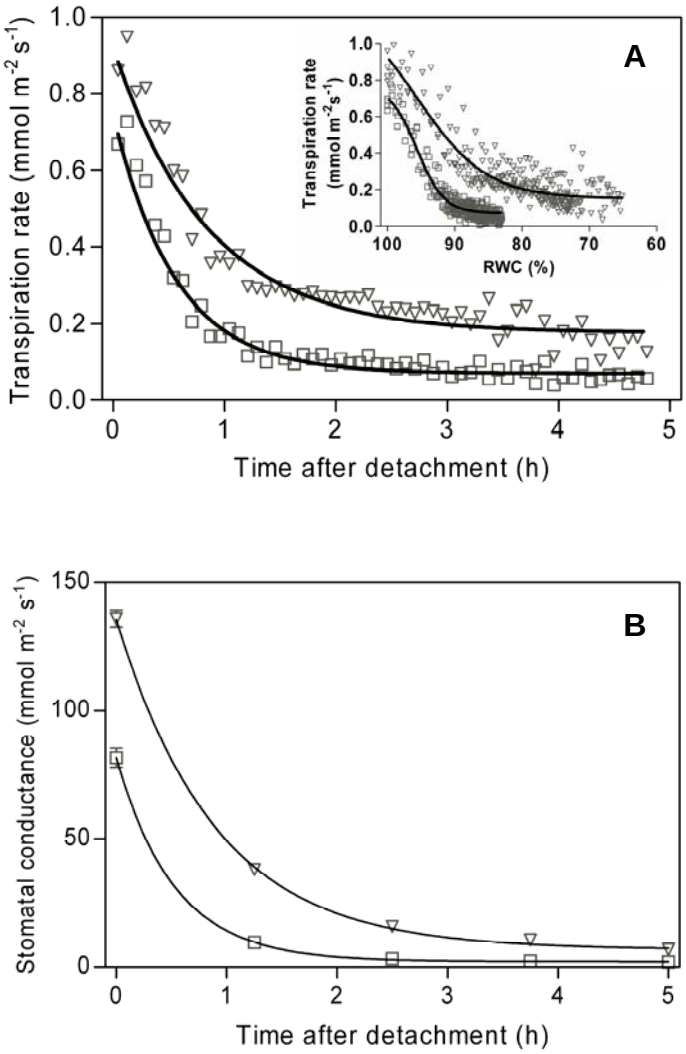

Fig.1 Changes of transpiration rate (A) and stomatal conductance (B) in *Tradescantia virginiana* grown at moderate (square symbols) or high RH (triangle symbols) during 5 h desiccation at VPD of 1.40 kPa. The relationship between transpiration rate and RWC is shown as an insert of Fig. 1A. Each point is the mean value of four experiments (eight leaves per experiment) for transpiration rate and three experiments (eight leaves per experiment) for stomatal conductance. In stomatal conductance bars indicate standard errors larger than symbols.

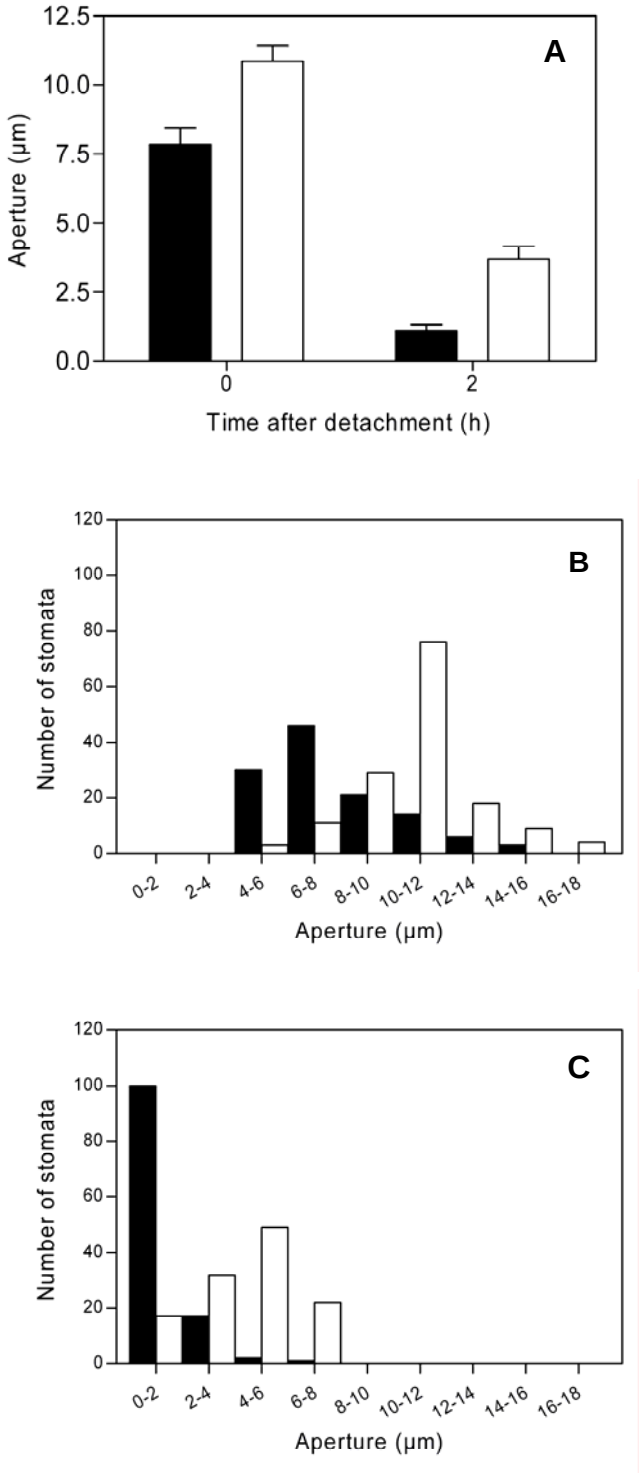

Fig.2 Stomatal aperture in *Tradescantia virginiana* leaves grown at moderate (closed bars) or high (open bars) RH immediately after detachment and after 2 h desiccation (A). Values are the average of measurements from 12 leaves (10 stomata per leaf) ± SEM. Frequency distribution of number of stomata in different stomatal aperture ranges immediately after detachment (B) and 2 h after desiccation (C) are indicated (n=120).

Experiment 2: Stomatal responses to ABA application

Transpiration rate (Fig. 3A) and stomatal conductance (Fig. 3B) from detached leaves placed in ABA solutions decreased with time for all treatments. After 2-3 hours transpiration rate and stomatal conductance tended to a constant rate. There were significant differences in all asymptotic values of the fitted curves (P<0.0001). Therefore, transpiration rate and stomatal conductance in high RH grown plants in controls (0 µM ABA) were significantly higher than in moderate RH grown plants. Application of 100µM ABA decreased transpiration rate and stomatal conductance in both high and moderate RH grown plants compared to controls. However, transpiration rate and stomatal conductance in high RH grown plants treated with ABA were significantly higher than in moderate RH grown plants treated with ABA. There is a sinusoid fluctuation in transpiration rate superimposed upon the exponential decay of transpiration rate. This correlated with fluctuations of temperature and humidity in the test room.

The effect of ABA on stomatal aperture was determined using epidermal strips. The stomatal aperture decreased with increasing ABA concentration (P<0.0001) in both groups of plants (Fig. 4A), but it was significantly wider in stomata from high RH grown plants (P<0.0001). The interaction between RH and ABA concentration was not significant.

Figs. 4B and C show the frequency distribution of the number of stomata in different stomatal aperture ranges in 0 and 1000 µM ABA, respectively. In 0 µM ABA the distributions of stomatal aperture in high RH grown plants were more in the wider aperture ranges as compared to moderate RH grown plants. In 1000 µM ABA most of stomata in moderate RH grown plants were in the 0-2 µm class and the others distributed in other size-classes up to 6-8 µm, whereas in high RH grown plants, although the greatest population of stomata was in the low size-classes, there was a small population of stomata in all other size-classes up to 18-20 µm.

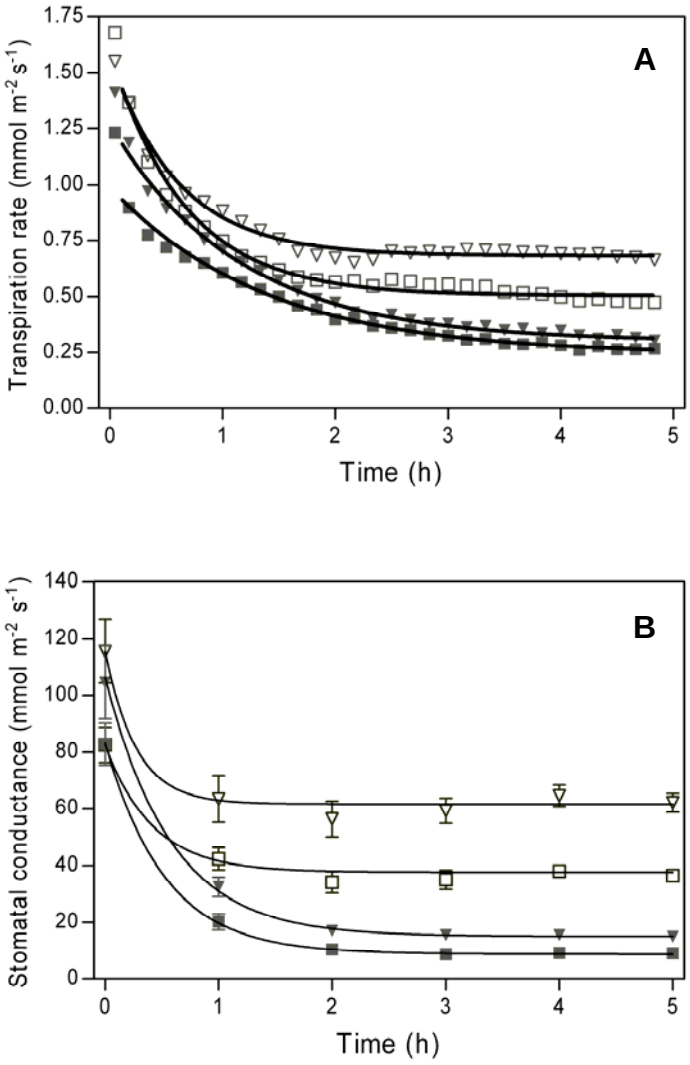

Fig.3 Changes of transpiration rate (A) and stomatal conductance (B) in *Tradescantia virginiana* grown at moderate (square symbols) or high RH (triangle symbols) during 5 h application of 0 (open symbols) or 100 µM (closed symbols) ABA. Measurements were conducted at VPD of 1.40 kPa. Each point is the mean value of three experiments (eight leaves per experiment). In stomatal conductance bars indicate standard errors larger than symbols.

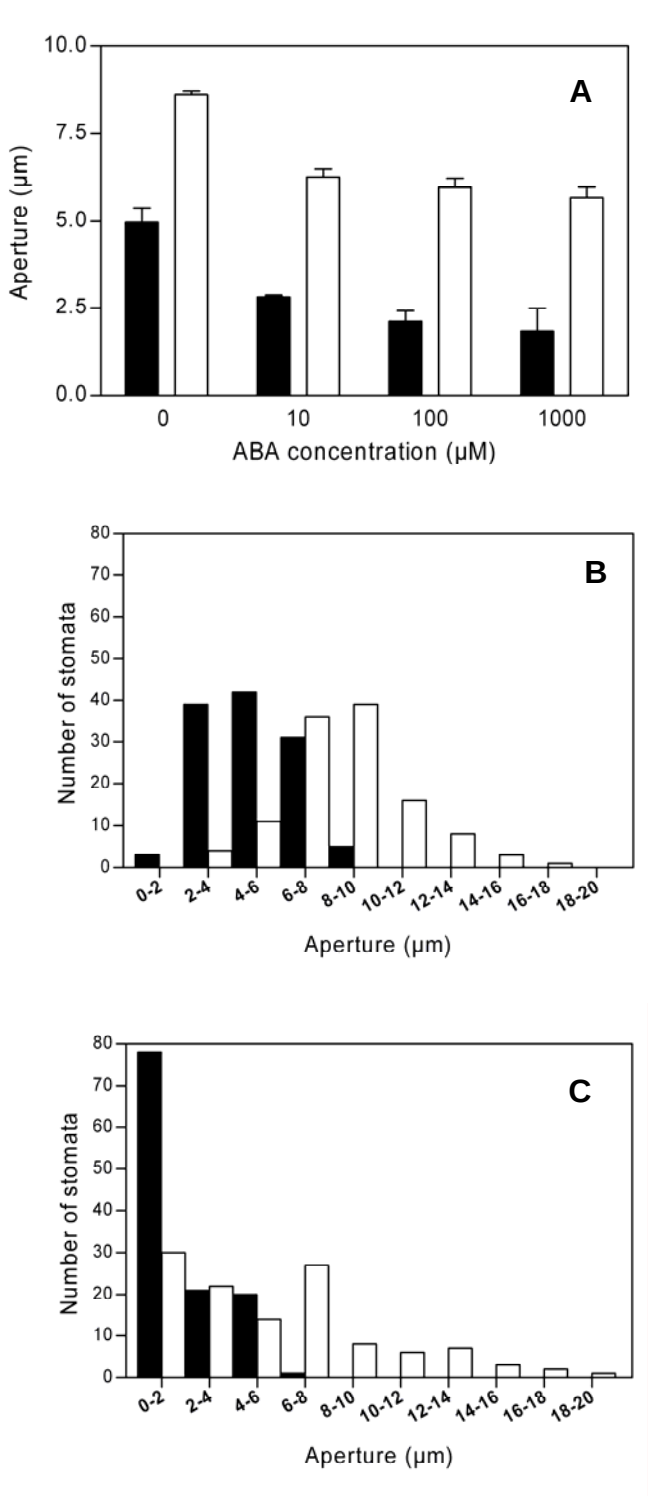

Fig.4 Stomatal aperture of epidermal strips in *Tradescantia virginiana* plants grown at moderate (closed bars) or high (open bars) RH in response to ABA application (A). Values are the average of measurements from three experiments (four strips per experiment and 10 stomata per strip) ± SEM. Distribution of number of stomata in different stomatal aperture ranges in 0 (B) and 1000 µM (C) ABA are indicated (n=120).

Experiment 3: Stomatal responses to light/dark transition

Though the transpiration rate and stomatal conductance decreased with light/dark transition (P<0.0001) in both groups of plants (Table 2), they were significantly higher in leaves from high RH grown plants (P=0.001 for transpiration rate and P=0.0002 for stomatal conductance). The interactions between RH and light/dark transition were not significant.

Stomatal aperture decreased with light/dark transition (P<0.0001) in both groups of plants (Fig. 5A), but it was significantly wider in stomata of high RH grown plants (P<0.0001). The interaction between RH and light/dark regime was not significant.

Figs. 5B and C show the distribution of the number of stomata in different stomatal aperture ranges in light and darkness. Similar to the results of stomatal aperture distribution of controls in experiments 1 and 2, stomatal aperture distribution of high RH grown plants in light was more in the wider aperture ranges as compared to moderate RH grown plants. In darkness almost all stomata of moderate RH grown plants were in the 0-2 µm class, whereas in high RH grown plants, although the greatest population of stomata was in the 0-2 µm class, there was a small population of stomata in all other size-classes up to 18-20 µm.

Table 2 Average transpiration rate and stomatal conductance in *Tradescantia virginiana* leaves grown at 55% or 90% RH in light and darkness. Figures are the mean values of three experiments (eight leaves per experiment) ± SEM.

	Transpiration rate (mmol $m^{-2}s^{-1}$)		Stomatal conductance (mmol $m^{-2}s^{-1}$)	
	RH=55%	RH=90%	RH=55%	RH=90%
Light	0.68± 0.01	0.76±0.03	104.7±9.3	145.6±8.5
Darkness	0.22±0.01	0.31±0.01	27.8±3.7	52.2±5.6

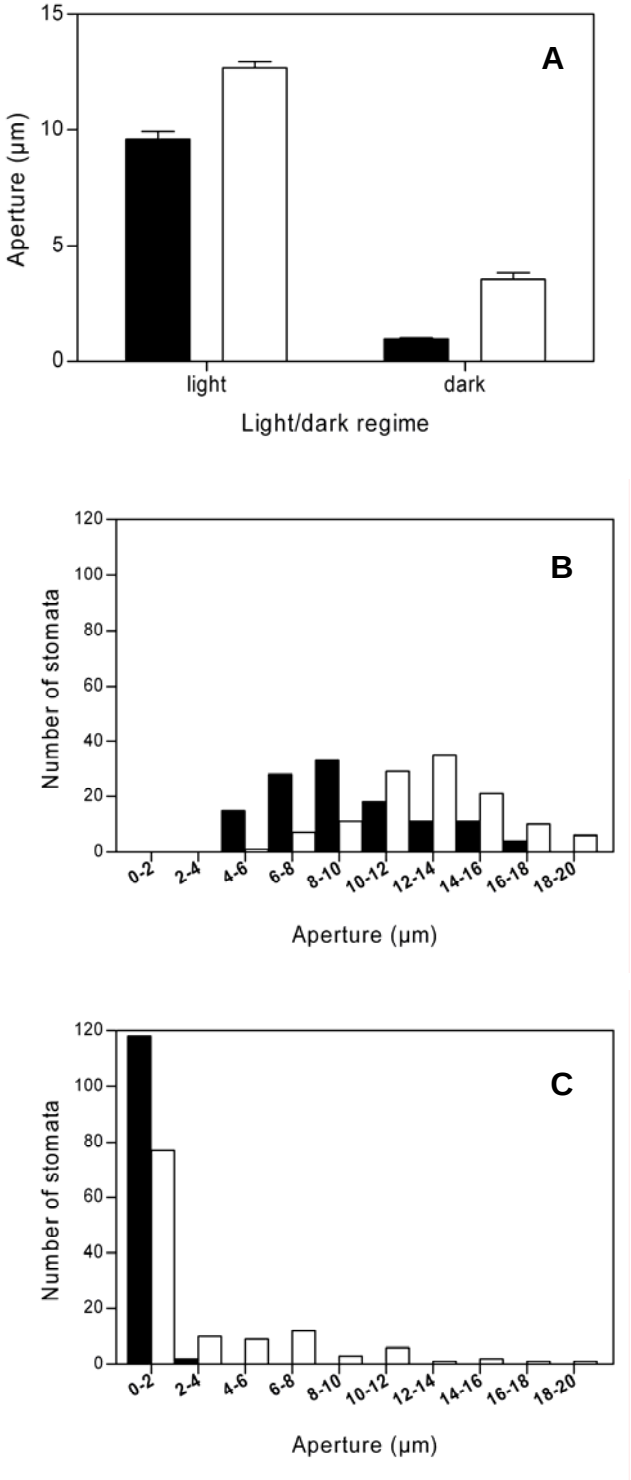

Fig.5 Stomatal aperture in *Tradescantia virginiana* leaves grown at moderate (closed bars) or high (open bars) RH in response to light and darkness (A). Values are the average of measurements from 12 leaves (10 stomata per leaf) ± SEM. Frequency distribution of number of stomata in different stomatal aperture range in light (B) and darkness (C) are indicated (n=120).

Discussion

The length of guard cell in *T. virginiana* plants grown at high RH was about 29% more than in moderate RH grown plants. Bigger stomata were also reported by Torre *et al.* (2003) for rose plants. In addition, larger stomata have been reported in plants cultivated *in vitro* grown under high RH [i.e. *Delphinium* (Santamaria *et al.*, 1993) and *Rosa multiflora* (Capellades *et al.*, 1990)]. However, the decrease of stomatal density observed in our high RH grown plants (Table 1), is in contrast with the report of Torre *et al.* (2003) on rose grown at high RH which showed an increased stomatal density. As our results showed no significant difference between stomatal indices in high and moderate RH grown plants (Table 1), it can be concluded that the lower stomatal density in high RH grown plants was because of bigger stomata as well as bigger epidermal cells due to cell enlargement, which is in agreement with the larger leaf area of plants grown at high RH (data not shown).

Under the same VPD (1.40 kPa) the transpiration rate per leaf unit area in high RH grown plants was higher than in moderate RH grown plants, even after exposure to stomatal closing treatments. Leaf drying curves (Fig. 1A) showed that transpiration rate in high RH grown plants was less hydrosensitive than in moderate RH grown plants. This could (partly) be the result of higher stomatal aperture found in the high RH grown plants. A greater average stomatal aperture has also been observed in micro-propagated plants (Santamaria *et al.*, 1993; Wardle *et al.*, 1983; Ziv *et al.*, 1987) and roses grown at high RH (Torre and Fjeld, 2001; Torre *et al.*, 2003). Therefore, an increased stomatal aperture seems to be a common feature of plants grown at high RH.

The decline in leaf transpiration rate and overall stomatal conductance of both moderate and high RH grown plants in our experiments was the result of stomatal responses to stomatal closing treatments as well as lower RH and irradiance in the test room. However, after steady-state was reached, there were significantly higher leaf transpiration rate and overall stomatal conductance in leaves grown at high RH. This could be due to two possibilities: 1) difference in stomatal apertures 2) difference in cuticular transpiration rates. Our results showed higher average stomatal aperture in high RH grown plants. Moreover, the distributions of stomatal aperture in response to desiccation (Fig. 2), ABA application (Fig. 4) and exposure to darkness (Fig. 5) showed higher variability of stomatal aperture in high RH grown plants than in moderate RH plants. The

distribution histograms revealed that some partially or completely non-closing stomata were present amongst normal stomata in high RH grown plants. Looking at the observations of individual leaves, we found that these non-closing stomata were present amongst normal stomata in each individual leaf. Cuticular transpiration rate can easily be measured after application of stomatal closing treatments. However, none of the applied treatments (desiccation, ABA application, exposure to darkness) resulted in total closure of all stomata in high RH grown plants. Although differences in cuticular transpiration rate cannot be excluded, the 3 to 4-fold stomatal aperture in high RH grown leaves seems likely to be the explanation for the higher transpiration rate in these leaves. Moreover, Torre *et al* (2003) reported no difference between cuticular structure, shape and size in leaves of roses grown at moderate and high RH.

Numerous authors have shown that stomatal patchiness or heterogeneity can be influenced by short-term changes in environmental factors (Beyschlag and Eckstien, 2001; Beyschlag and Pfanz, 1990; Downton *et al.*, 1988; During, 1992; Eckstien *et al.*, 1996; Mott *et al.*, 1993). It can be concluded from our results that long-term exposure to high RH during growth increased stomatal heterogeneity in response characteristics to short-term exposure to stomatal closing treatments. However, more information is needed to conclude if the non-closing stomata are randomly distributed, show a patchy distribution or are located in specific areas of the leaf.

Compared to smaller stomata, bigger stomata have a larger aperture area due to their longer guard cells even when both have an identical inner width of stomatal aperture. Therefore, stomatal size could affect leaf transpiration rate and stomatal conductance. However, as discussed by some authors (Santamaria *et al.*, 1993; Torre *et al.*, 2003), this does not explain why high RH grown leaves are less hydrosensitive and dehydrate rapidly after detachment. As the rate at which ABA enters a leaf from the roots is determined by xylem ABA concentration and the transpiration flux (Zhang *et al.*, 1997), it might be that there was a low concentration of ABA in the plants grown at high RH. The lack of root-sourced ABA might be limiting the strength of stomatal closing signals generated during desiccation or darkness. However, the failure of some stomata to close fully in response to ABA suggests that ABA deficiency was not responsible for the lack of stomatal closure in response to desiccation. This is supported by the frequency

distribution histograms, which show that many stomata in high RH grown leaves react in the same way as do the stomata in moderate RH grown leaves. Ziv et al. (1987) in experiments with epidermal strips and guard cell protoplasts from *in vitro* cultured carnation, concluded that the cause for the failure of stomata to close in response to ABA, darkness and Ca^{2+} lies mainly in the guard cell wall and not in the protoplast. However, these stomata were largely affected by the cytokinins used in the growing medium. Many studies have suggested that the short-term effects of elevated ABA concentrations on stomatal functioning are reversible (Ackerson, 1980; Tardieu *et al.*, 1996; Trejo *et al.*, 1995) but 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). All these studies concern increased ABA levels resulting in lower conductance and opening with lower stomatal conductance at any given guard cell turgor. We propose that a very low ABA concentration in well-watered plants during growth at high RH might also cause the structural or physiological malfunctioning of stomata. However, further research is needed to explore why only some stomata developed at high RH fail to function normally.

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References

Ackerson RC. 1980. Stomatal response of cotton to water stress and abscisic acid as affected by water stress history. *Plant Physiology* **65**, 455-9.

Assmann SM. 1993. Signal transduction in guard cells. *Annual Review of Cell Biology* **9**, 345-75.

Assmann SM, Wang X-Q. 2001. From milliseconds to millions of years: guard cells and environmental responses. *Current Opinion in Plant Biology* **4**, 421-8.

Beyschlag W, Eckstien J. 2001. Towards a causal analysis of stomatal patchiness: the role of stomatal size variability and hydrological heterogeneity. *Acta Oecologica* **22**, 161-73.

Beyschlag W, Pfanz H. 1990. A fast method to detect the occurrence of nonhomogenous distribution of stomatal aperture in heterobaric plant leaves. *Oecologia* **82**, 52-5.

Brown KW, Jordan WR, Thomas JC. 1976. Water stress induced alternations of the stomatal response to decreases in leaf water potential. *Physiologia Plantarum* **37**, 1-5.

Capellades R, Fontanau C, Carulla C, Debergh P. 1990. Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa moltiflora*. *Journal of the American Society for Horticultural Science* **115**, 141-5.

Cutler JM, Rains DW, Loomis RS. 1977. The importance of cell size in the water relations of plants. *Physiologia Plantarum* **40**, 255-60.

Downton WJS, Loveys BR, Grant WJR. 1988. Stomatal closure fully accounts for the inhibition of photosynthesis by abscisic acid. *New Phytologist* **108**, 263-6.

During H. 1992. Low air humidity causes non-uniform stomatal closure in heterobaric leaves in *Vitis* species. *Vitis* **31**, 1-7.

Eckstien J, Beyschlag W, Mott KA. 1996. Changes in photon flux can induce stomatal patchiness. *Plant, Cell and Environment* **19**, 1066-75.

Fordham MC, Harrison-Murray RS, Knight L, Clay CM. 2001a. Decline in stomatal response to leaf water deficit in *Crylus maxima* cuttings. *Tree Physiology* **21**, 489-96.

Fordham MC, Harrison-Murray RS, Knight L, Evered CE. 2001b. Effects of leaf wetting and high humidity on stomatal function in leafy cuttings and intact plants of *Corylus maxima*. *Physiologia Plantarum* **113**, 233-40.

Franks PJ, Farquhar GD. 2001. The effect of exogenous abscisic acid on stomatal development, stomatal mechanics and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology* **125**, 935-42.

Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. *Nature* **424**, 901 - 8.

Iwai S, Shimomura N, Nakashima A, Etoh T. 2003. New fava been guard cell signaling mutant impaired in ABA-induced stomatal closure. *Plant Cell Physiology* **44**, 909-13.

Kearns EV, Assmann SM. 1993. The guard cell-environment connection. *Plant Physiology* **102**, 711-5.

McCree KJ. 1974. Changes in the stomatal response characteristics of grain sorghum produced by water stress during growth. *Crop Science* **14**, 273-8.

Mortensen LM, Fjeld T. 1998. Effects of air humidity, lighting period and lamp type on growth and vase life of roses. *Scientia Horticulturae* **73**, 229-37.

Mott KA, Cardon ZG, Berry JA. 1993. Asymmetric patchy stomatal closure for the two surfaces of *Xanthium strumarium* L. leaves at low humidity. *Plant, Cell and Environment* **16**, 25-34.

Santamaria JM, Davies WJ, Atkinson CJ. 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. *Journal of Experimental Botany* **44**, 99-107.

Slavik B. 1974. Methods of Studying Plant Water Relations. London: Chapman & Hall Ltd.

Smith S, Weyers JDB, Berry WG. 1989. Variation in stomatal characteristics over the lower surface of *Commlina communis* leaves. *Plant, Cell and Environment* **12**, 653-9.

Spence RD, Wu H, Sharpe PJH, Clark KG. 1986. Water stress effects on guard cell anatomy and the mechanical advantage of the epidermal cells. *Plant, Cell and Environment* **9**, 197-202.

Tardieu F, Lafarge T, Simonneau TH. 1996. Stomatal control by fed or endogenous xylem ABA in sunflower: interpretation of correlations between leaf water potential and stomatal conductance in anisohydric species. *Plant, Cell and Environment* **19**, 75-84.

Torre S, Fjeld T. 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. *Scientia Horticulturae* **89**, 217-26.

Torre S, Fjeld T, Gislerød HR. 2001. Effects of air humidity and K/Ca ratio in the nutrient supply on growth and postharvest characteristics of cut roses. *Scientia Horticulturae* **90**, 291-304.

Torre S, Fjeld T, Gislerød HR, Moe R. 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. *Journal of the American Society for Horticultural Science* **128**, 598-602.

Trejo CL, Clephan AL, Davies WJ. 1995. How do stomata read abscisic acid signals? *Plant Physiology* **109**, 803-11.

Wang X-Q, Wu W-H, Assmann SM. 1998. Differential responses of abaxial and adaxial guard cells of broad bean to abscisic acid and calcium. *Plant Physiology* **118**, 1421-9.

Wardle K, Dobbs EB, Short KC. 1983. *In vitro* acclimatization of aseptically cultured plantlets to humidity. *Journal of the American Society for Horticultural Science* **108**, 386-9.

Wardle K, Short KC. 1983. Stomatal response of *in vitro* cultured plantlets. I. Responses in epidermal strips of Chrysanthemum to environmental factors and growth regulators. *Biochemie und Physiologie der Pflanzen* **178**, 619-24.

Weyers JDB, Meidner H. 1990. Methods in Stomatal Research. Harlow, England: Longman Scientific & Technical.

Xia MZ. 1994. Effects of soil drought during the generative development phase of faba been (*Vicia faba*) on photosynthetic characters and biomass production. *Journal of Agricultural Science* **122**, 67-72.

Zhang J, Jia W, Zhang D. 1997. Re-export and metabolism of xylem-delivered ABA in attached maize leaves under different transpirational fluxes and xylem ABA concentrations. *Journal of Experimental Botany,* **48**, 1557-64.

Ziv M, Schwartz A, Fleminger D. 1987. Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; implication for hardening. *Plant Science* **52**, 127-34.

Chapter 3

Dynamics of spatial heterogeneity of stomatal closure in *Tradescantia virginiana* altered by growth at high relative air humidity

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Abstract

The spatial heterogeneity of stomatal closure in response to rapid desiccation of excised well-watered *Tradescantia virginiana* leaves grown at moderate (55%) or high (90%) relative air humidity (RH) was studied using a chlorophyll fluorescence imaging system under non-photorespiratory conditions. Following rapid desiccation, excised leaves grown at high RH had both a greater heterogeneity and a higher average value of PSII efficiency (Φ_{PSII}) compared to leaves grown at moderate RH. Larger decreases in relative water content (RWC) resulted in smaller decreases in water potential and Φ_{PSII} of high RH grown leaves compared to moderate RH grown leaves. Moreover, the Φ_{PSII} of excised high RH grown leaves decreased less with decreasing water potential implying that the stomata of high RH grown leaves are less sensitive to decreases in leaf water potential compared to moderate RH grown leaves. After desiccation, some non-closing stomata were distributed around the main vein in high RH grown leaves. Direct measurements of stomatal aperture showed 77% stomatal closure in the margins after 2 h desiccation compared to 40% closure of stomata in the main-vein areas in high RH grown leaves. Faster closure of stomata in leaf margins compared to main-vein areas of leaves grown at high RH was related to a substantial lower RWC in these areas of the leaves.

Key words: desiccation, patchiness, PSII efficiency, relative water content, stomata, water potential

Abbreviations- ABA, abscisic acid; F_t, the steady state value of fluorescence at a certain light level; F'_m, maximum fluorescence at a certain light level; $\Delta F'/ F'_m$, the ratio of the difference between (F'_m- F_t) and F'_m equals to Φ_{PSII} if during the saturating light pulse the Q_A pool is completely reduced; Φ_{PSII}, relative quantum yield or efficiency for electron transport by photosystem II; PSII, photosystem II; Q_A, primary quinone acceptor of photosystem II; RWC, relative water content; VPD, vapour pressure deficit.

Introduction

Stomata play a dominant role in the control of plant water relations and photosynthesis. Stomatal behaviour is the result of interactions between

physiological factors and environmental conditions (Assmann, 1993; Hetherington and Woodward, 2003; Kearns and Assmann, 1993). Moreover, stomatal response characteristics depend on the growing conditions in which the stomata developed. One of the most important growing conditions affecting stomatal response is relative air humidity (RH). For example, stomatal malfunctioning has been reported in roses grown at a relative air humidity above 85% (Torre and Fjeld, 2001; Torre *et al.*, 2003). Furthermore, 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 vitro* propagated plants (Santamaria *et al.*, 1993; Ziv *et al.*, 1987). Recent research has shown that whether grown at moderate (55%) or high (90%) RH, the stomata in the leaves of *Tradescantia virginiana* decreased their aperture in response to desiccation, ABA application and darkness-exposure (Rezaei Nejad and van Meeteren, 2005). However, transpiration rate and stomatal conductance and aperture in the high RH grown plants remained higher than in the moderate RH grown plants (Rezaei Nejad and van Meeteren, 2005), indicating a quantitative effect of RH during growth on stomatal functioning. This difference was because some of the stomata that developed in a high RH closed only partially, or not at all. The distribution over a leaf surface of these less responsive stomata is unknown. Several authors have shown that stomatal aperture in distinct areas of a leaf can be well below the mean stomatal aperture of the leaf. This phenomenon has been called 'patchy' stomatal closure, and it can be induced by changes in a range of environmental factors, such as water and salt stress, changes in light intensity, changes in ambient CO_2 partial pressure and low air humidity (Beyschlag and Eckstien, 2001; Beyschlag and Pfanz, 1990; Downton *et al.*, 1988; During, 1992; Eckstien *et al.*, 1996; Mott *et al.*, 1993). However, the effect of long-term development in high RH on the heterogeneity of stomatal closure over a leaf surface is unknown. It is also unknown to what extent the developmentally determined differences in stomatal responses correlate with changes in other leaf hydraulic properties. Such correlations could point to either compensatory mechanisms or co-adaptation of hydraulic properties.

In recent years, chlorophyll fluorescence has been widely used to study the photosynthetic performance of many plant species in response to stress (Lichtenthaler and Babani, 2000; Lu and Zhang, 1998; Maxwell and Johnson, 2000; Meyer and Genty, 1999). Stomatal closure causes lower availability of CO_2 inside

the leaf and thus a decrease in the rate of carboxylation (Cornic, 2000; Cornic and Massacci, 1996), but its effect on PSII efficiency (Φ_{PSII}) is not proportional to the decrease in carboxylation because of photorespiration in normal air. Photorespiration can be almost completely eliminated by placing the leaf in an atmosphere with an O_2 concentration of 2% or less (Genty *et al.*, 1990). Moreover, if the loss of Φ_{PSII} is due solely to stomatal closure, exposing desiccated leaves to a CO_2 concentration sufficiently high to overcome the stomatal closure should restore Φ_{PSII} to the control value, as shown by Meyer and Genty (1999). Thus, a measurement of Φ_{PSII} under low O_2 concentration may be used to detect the closure of stomata, and non-destructive chlorophyll fluorescence imaging techniques allow the spatial and temporal dynamics of this phenomenon to be studied (e.g. Daley *et al.*, 1989; Harbinson *et al.*, 2005; Meyer and Genty, 1998; Meyer and Genty, 1999). In previous studies attention was directed to the measurements of stomata that closed rapidly following water stress. In this study we focus more on how the closure in response to water stress of some stomata is delayed or entirely prevented. Moreover, to our knowledge, there has been no report where the estimates of stomatal closure obtained by means of Φ_{PSII} measurements has been correlated with direct measurements of stomatal closure and water relation parameters in plants subjected to water stress.

Materials and methods

Plant material and growth conditions

Young *Tradescantia 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 relative air humidity (moderate: 55 ± 5% and high: 90 ± 5%) at Wageningen University. The temperature was 21 ± 0.5 ^{o}C resulting in VPDs of 1.12 and 0.25 kPa for moderate and high RH conditions, respectively. The light intensity was 120 ± 10 µmol m^{-2} s^{-1} (measured with LI-250 Light Meter, LI-COR, Lincoln, USA) produced by fluorescent tubes (TLD 33, Philips) with a photoperiod of 16 h per day. Though this light intensity is low, *T. virginiana* is a shade plant and measurements of its CO_2 fixation irradiance response showed that 120 µmol m^{-2} s^{-1} is about 40% saturating for CO_2 fixation. The plants were kept well-watered and given a nutrient solution weekly

(concentration: 2 g l^{-1}; Kristalon™, Yara, Rotterdam, The Netherlands). The CO_2 concentration in the growth chambers was 360 ± 30 μmol mol^{-1} (measured with a CO_2 analyzer ADC225, MK3, Analytical Development Co. Ltd, Hoddesdon, England). Young fully expanded leaves were used in the experiments.

Mapping of PSII photochemical yield using chlorophyll fluorescence imaging

One intact leaf (attached to the plant) from a high RH grown plant and another leaf from a moderate RH grown plant were put side by side inside a gas-tight cuvette that was placed in the imaging area of a commercial chlorophyll fluorescence imaging system (FluorCam 700MF, Photon Systems Instruments, Brno, Czech Republic). In the imaging system, an 8-bit, 512 × 512 pixel, black and white CCD camera equipped with an F1.2/2.8-6 mm objective was used to record fluorescence images. Images were recorded during short measuring flashes superimposed upon a continuous actinic irradiance of 100 μmol m^{-2} s^{-1}. These flashes and the continuous actinic irradiance were provided by two panels each containing 345 orange light emitting diodes (LED's). Saturating white light pulses with 2500 μmol m^{-2} s^{-1} intensity were generated with a 250 W halogen lamp. While this is a relatively low irradiance for a saturating irradiance, it was sufficient to saturate Q_A reduction in these leaves as the $\Delta F'/F'_m$ obtained with 2500 μmol m^{-2} s^{-1} irradiance was 97% of that obtained with a saturating irradiance of 6000 μmol m^{-2} s^{-1}. Once the leaves had reached steady-state, two successive series of fluorescence images were digitized and averaged, one just before (I_t), and the other (I'_m) during the saturating light flash that causes a transitory saturation of photochemistry. The averaged fluorescence intensities obtained for each related pixel in images I_t and I'_m were used directly as relative measurements of fluorescence yield and used to calculate values for Φ_{PSII} (Genty *et al.*, 1989). The frequency distribution histogram, average value and standard deviation of Φ_{PSII} per image were calculated using the FluorCam software (version 5.0). The fluorescence measurements were conducted under an atmosphere of 20 mmol mol^{-1} O_2, 350 μmol mol^{-1} CO_2 and remainder N_2 (normal CO_2; a non-photorespiratory condition) in the cuvette. 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)-sulfate heptahydrate (Fluka). Cuvette temperature was 22 ± 1 ^oC. After a steady state was

reached, the first image that was taken from the leaves (which were still attached) served as a control. The desiccation process was begun by excising both leaves from their plants, and images were then taken every 30 min for 150 min. At the end of the desiccation period an image was made after 5 min exposure to 20 mmol mol^{-1} O_2, 5000 μmol mol^{-1} CO_2 and remainder N_2 (high CO_2) to test for the recovery of Φ_{PSII} under high CO_2 conditions. The experiment was repeated with seven leaves from seven randomly selected plants in each RH treatment.

Stomatal aperture measurements

Based on many preliminary observations we established that margins always had lower Φ_{PSII} than main-vein areas. Measurements of stomatal aperture were made from these two areas on leaves that had not themselves been imaged. Using a silicon rubber impression technique (Smith *et al.*, 1989), stomatal aperture was determined from young fully expanded leaves at approximately two-thirds of the distance from the base to the tip. One leaf per plant and one impression (5 mm × 10 mm) per location on the leaf were used. Impressions were made on attached leaves, and on detached leaves 2 h after their separation from the plants. All plants and leaves were kept in a test room (40% RH, 20 ^{o}C, 1.40 kPa VPD and 100 μmol m^{-2} s^{-1} irradiance) for the duration of this treatment. Stomatal aperture was determined with digitized video images (×800 magnification) of abaxial stomata (10 stomata per impression) 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, Texas).

Measurements of water potential and relative water content (RWC)

Guided by the images obtained from the imaging system, leaf discs or segments were cut from the different regions of a leaf and were used for the measurements of either water potential or RWC. Leaf discs were cut with a 5 mm diameter cork-borer and the edges of cut discs swabbed with tissue to remove the liquid released by the localized cell crushing. Leaf water potential was measured using a Wescor Vapro (Wescor, Model 5520, Logan, Utah) vapour pressure osmometer. Leaf discs were sealed inside the chamber for at least 2 h to ensure water vapour equilibrium. To obtain RWC, leaf discs or segments were first

weighed, and then floated in distilled water for 4 h, then reweighed after which their dry weight was determined.

Measurements of stomatal size and density

Stomatal size and density were measured on epidermal strips from young fully expanded leaves at approximately two-third of the distance from the base to the tip. The epidermal strips were removed from the margins and main-vein areas of abaxial surface of leaves and cut into 5 mm × 10 mm pieces using the technique of Weyers and Meidner (1990). One leaf per plant and one strip per location of the leaf were used. The strips were pre-incubated for 2 h in a stomata-opening medium (10 mM MES-KOH, pH 6.15, 50 mM KCl) in the test room. The measurement of guard cell length was done on 10 strips in each location and 10 randomly selected stomata in each strip from digitized video images of stomata. The stomatal density was calculated from the counts of the number of stomata in 20 strips in each location of the leaf.

Statistical analysis

For Φ_{PSII}, stomatal aperture, stomatal size and density, data were subjected to analysis of variance (ANOVA). Data in figure 4 were analysed using repeated measures ANOVA. The student's t-test was used for mean separation (P=0.05). The relationships between Φ_{PSII} and RWC, water potential and RWC, Φ_{PSII} and water potential were fitted using linear regressions. The parameters of fitted lines were compared statistically with F-test. GraphPad Prism 4 for Windows (GraphPad Software, San Diego, California) was used for all statistical analyses and curve fitting.

Results

Figure 1 shows the mean Φ_{PSII} (ie the mean of the average Φ_{PSII} values that were obtained from individual images) of leaves from moderate and high RH grown plants: i) before excision (control) under normal CO_2, ii) 150 min after excision under normal CO_2, and iii) 150 min after excision under high CO_2. There was a significant interaction between RH during growth and rapid desiccation after excision of the leaves (P=0.003). In moderate RH grown plants, there was a significant difference in the Φ_{PSII} of leaves in different treatments (P<0.001) and

leaves before excision had a high Φ_{PSII}. With rapid desiccation after excision Φ_{PSII} decreased significantly and with the transition to high CO_2, Φ_{PSII} almost completely recovered to the value of the control images. Qualitatively the same results were found in leaves grown at high RH. The average recovery of Φ_{PSII} in both groups of the plants in high CO_2 treatments was around 95%. Moreover, there was a significant difference between the Φ_{PSII} of leaves grown at moderate and high RH after desiccation under normal CO_2 (P=0.01).

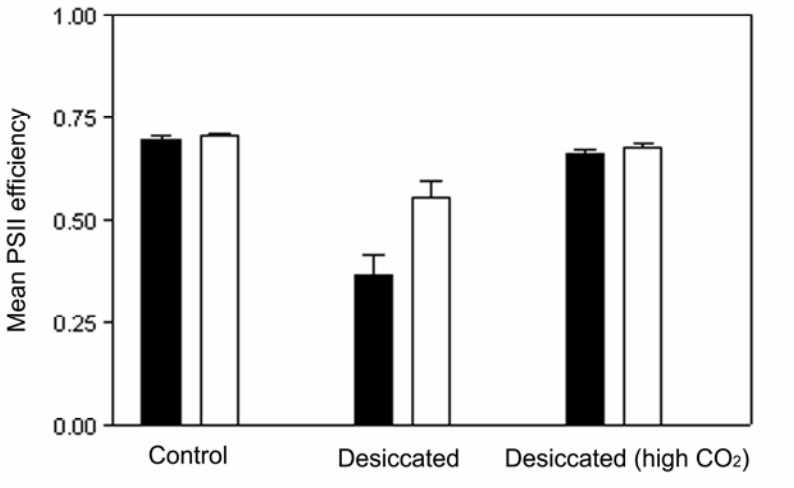

Fig.1 PSII efficiency (Φ_{PSII}) of *Tradescantia virginiana* leaves grown at moderate (closed bars) or high (open bars) RH: i) before excision (control) under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 (normal CO_2), ii) 150 min after excision under normal CO_2 and iii) 150 min after excision under 20 mmol mol^{-1} O_2, 5000 µmol mol^{-1} CO_2. Values are the mean of seven leaves ± SEM.

Images of Φ_{PSII} (Fig. 2) and corresponding frequency distributions (Fig. 3) show different trends in the distribution of photosynthetic activity of *T. virginiana* leaves grown at moderate or high RH. Before excision and in an atmosphere of normal CO_2, Φ_{PSII} was both high and homogeneously distributed over the leaves irrespective of whether they have been grown at a moderate or a high RH (Fig. 2A). The frequency distributions of both leaves had an almost identical shape: a narrow distribution around 0.7, negatively skewed (Fig. 3A). During desiccation after excision, leaves grown at moderate RH showed a rapid decrease of Φ_{PSII}, especially during the second hour of desiccation (Fig. 2B-2E). Though leaves grown at moderate RH showed some heterogeneity after 90 min of desiccation (Fig. 2C), the frequency distribution of their Φ_{PSII} was unimodal and symmetrical (Fig. 3C). With increasing duration of desiccation the Φ_{PSII} of the moderate RH leaves remained homogeneously distributed over the leaf surface (Fig. 2D-2E) and the frequency distributions shifted progressively towards the lower Φ_{PSII} values (Fig. 3D-3E). In leaves grown at high RH Φ_{PSII} decreased less during desiccation (Fig. 2B-2E). The Φ_{PSII} distributions initially became more negatively skewed and after 90 min of desiccation the distribution became rather broad (Fig. 3B-3E). After 2 h of desiccation the distribution of Φ_{PSII} in leaves grown at high RH was much more heterogeneous than in leaves grown at moderate RH with clear differences between different areas of a leaf. In marginal parts of the high RH grown leaves, Φ_{PSII} decreased during desiccation, while around the main vein, Φ_{PSII} always remained high. The average Φ_{PSII} in the desiccated leaves of both leaf types recovered almost completely after 5 min exposure to high CO_2 (Fig. 2F), and the frequency distributions were negatively skewed (Fig. 3F). The tips of the leaves were located outside the cuvette and figure 2 showed that Φ_{PSII} in normal air with photorespiration was high.

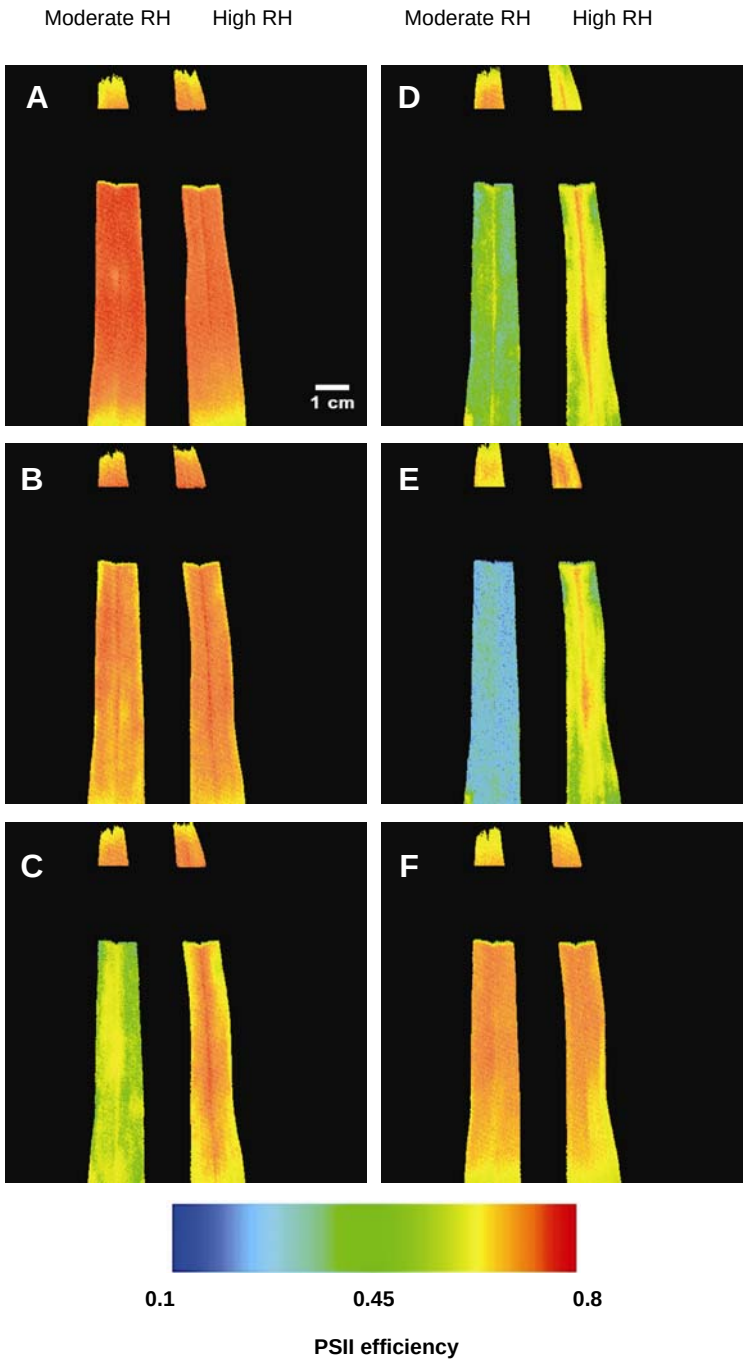

Fig.2 Images of PSII efficiency (Φ_{PSII}) for control (A) and 60 min (B), 90 min (C), 120 min (D) and 150 min (E) desiccation after excision under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 and 150 min desiccation under 20 mmol mol^{-1} O_2, 5000 µmol mol^{-1} CO_2 (F) in *Tradescantia virginiana* leaves grown at moderate (left leaf in each image) or high (right leaf in each image) RH. The tips of the leaves were located outside the cuvette and provided an indication of Φ_{PSII} under normal air where photorespiration could take place.

Figure 4 shows how the mean Φ_{PSII} of images of leaves grown at moderate and high RH changes with time of desiccation. Before excision (time 0), Φ_{PSII} in both groups of leaves was high and there was no significant difference between them. With desiccation after excision, Φ_{PSII} of leaves from moderate RH grown plants decreased earlier than it did for leaves from high RH grown plants. The interaction between RH during growth and time of desiccation after excision was significant (P<0.0001).

Figure 5 shows in more details the effect of rapid desiccation after excision on Φ_{PSII} and its frequency distribution in two different regions (leaf margin and main-vein area) of a leaf grown at high RH. The skewed frequency distribution of Φ_{PSII} from the entire leaf image is the result of a clear difference in the frequency distributions of the two regions. The RWC in the margin area which has a low Φ_{PSII}, was much lower than in the main-vein area where the Φ_{PSII} was higher.

In leaves grown at high RH, the interaction between the location of stomata and the effect of rapid desiccation after excision on stomatal aperture was significant (P<0.0001) (Fig. 6A). The average stomatal aperture at the margins of non-desiccated attached leaves grown at high RH was higher than around the main veins (P=0.02). After excision stomatal aperture decreased with desiccation (P<0.0001) in both parts of the leaf, but after 2 h of desiccation the average stomatal aperture was significantly wider around the main veins (P=0.0003). Stomatal aperture at the leaf margins decreased by 77% after 2 h of desiccation, while around the main veins the decrease was only 40%. Before excision, the stomatal aperture distribution at the leaf margins was more in the wider aperture classes compared to the areas around the main vein (Fig. 6B). Two hours after excision, more than 50% of stomata in the margins were in the 0-2 μm size class and the distribution was positively skewed. In the main-vein areas although the stomata on average decreased their aperture, they still remained slightly or completely open (Fig. 6C).

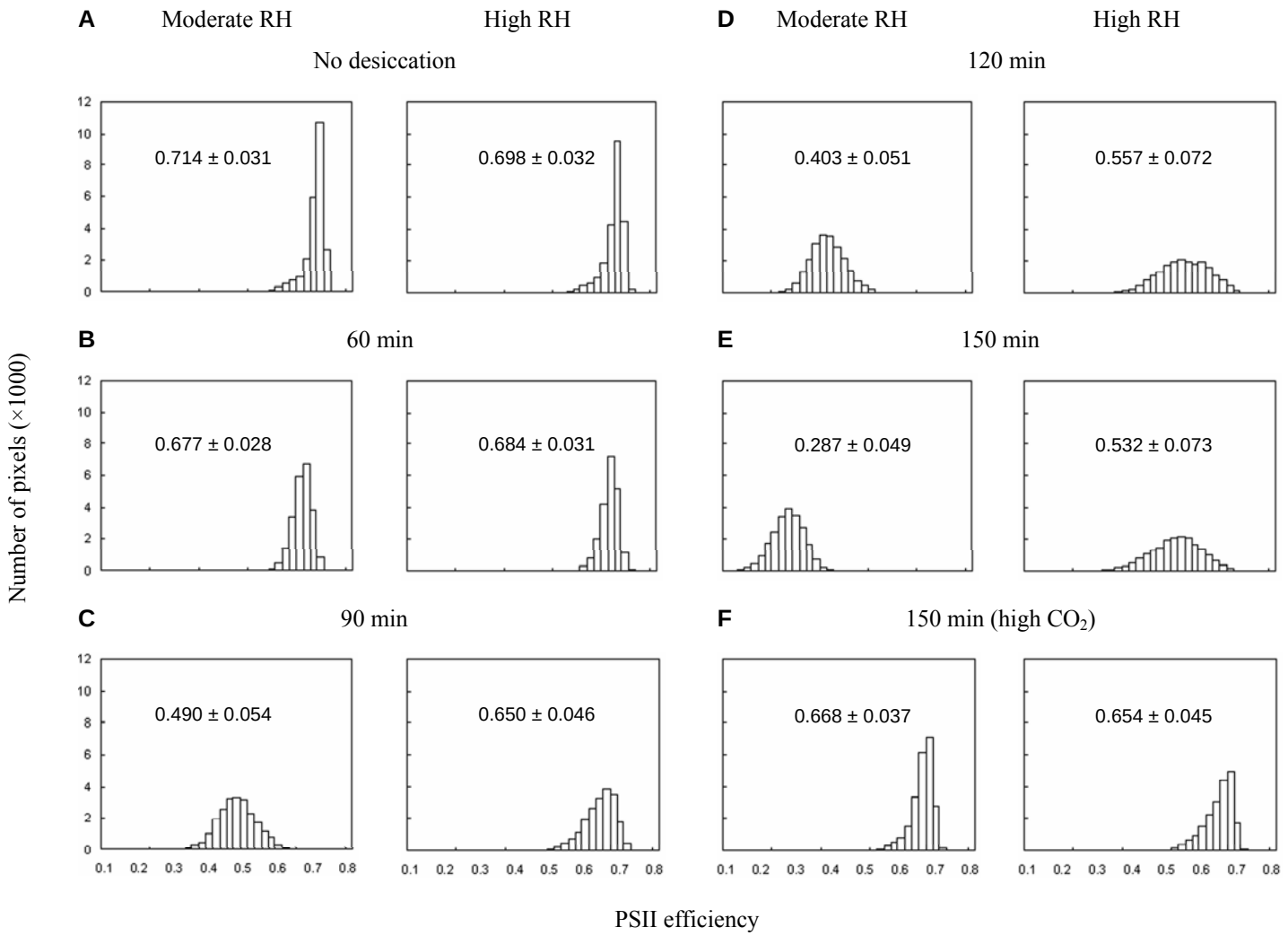

Fig.3 Frequency distributions of PSII efficiency (Φ_{PSII}) corresponding to the images shown in Fig. 2. The measurements in panels A-E were made under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 and the measurements in panel F were made under 20 mmol mol^{-1} O_2, 5000 µmol mol^{-1} CO2 (high CO_2). The duration of desiccation is indicated for each panel. The size class of the frequency distributions was 0.02. Average values of Φ_{PSII} ± SD are indicated above the histograms.

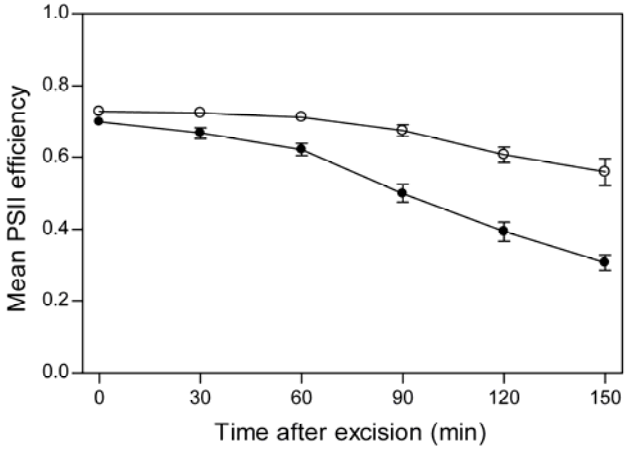

Fig.4 PSII efficiency (Φ_{PSII}) of *Tradescantia virginiana* leaves grown at moderate (closed symbols) or high (open symbols) RH over time of desiccation after excision under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2. Values are the mean of seven leaves ± SEM.

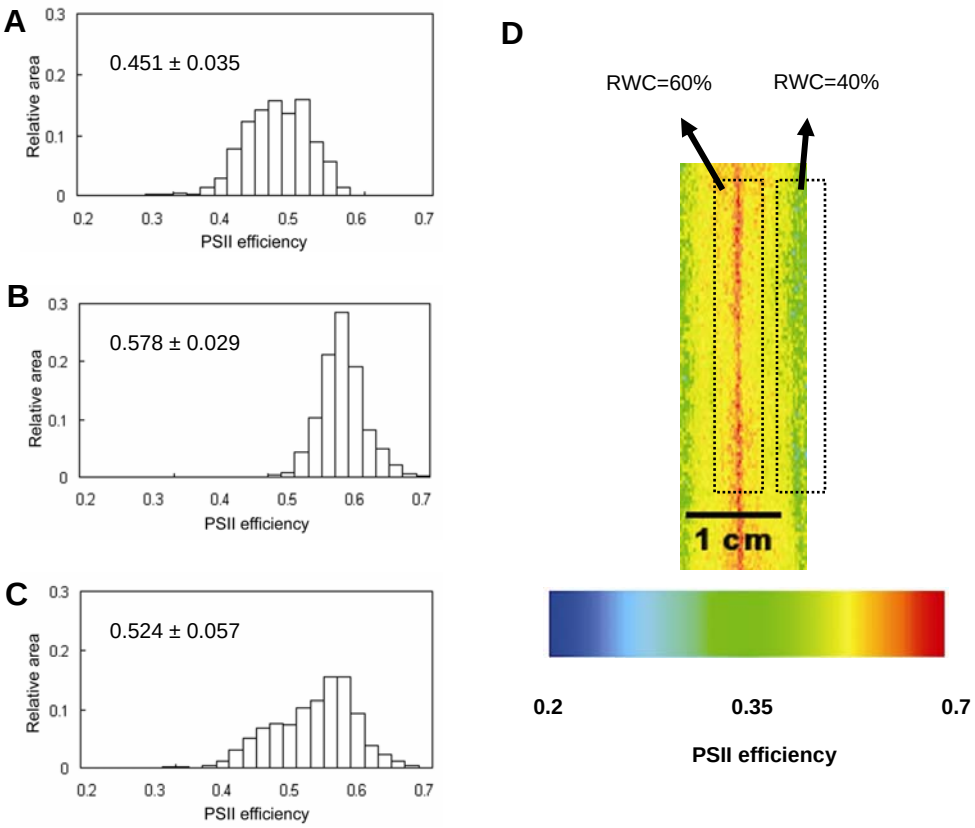

Fig.5 Image of PSII efficiency (Φ_{PSII}) in an excised leaf of *Tradescantia virginiana* grown at high RH (90%) after 130 min desiccation under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 (D) and its frequency distribution at the margin (A), main-vein area (B), and whole leaf (C). The size class of the frequency distributions was 0.02. Average values of Φ_{PSII} ± SD are indicated above the histograms.

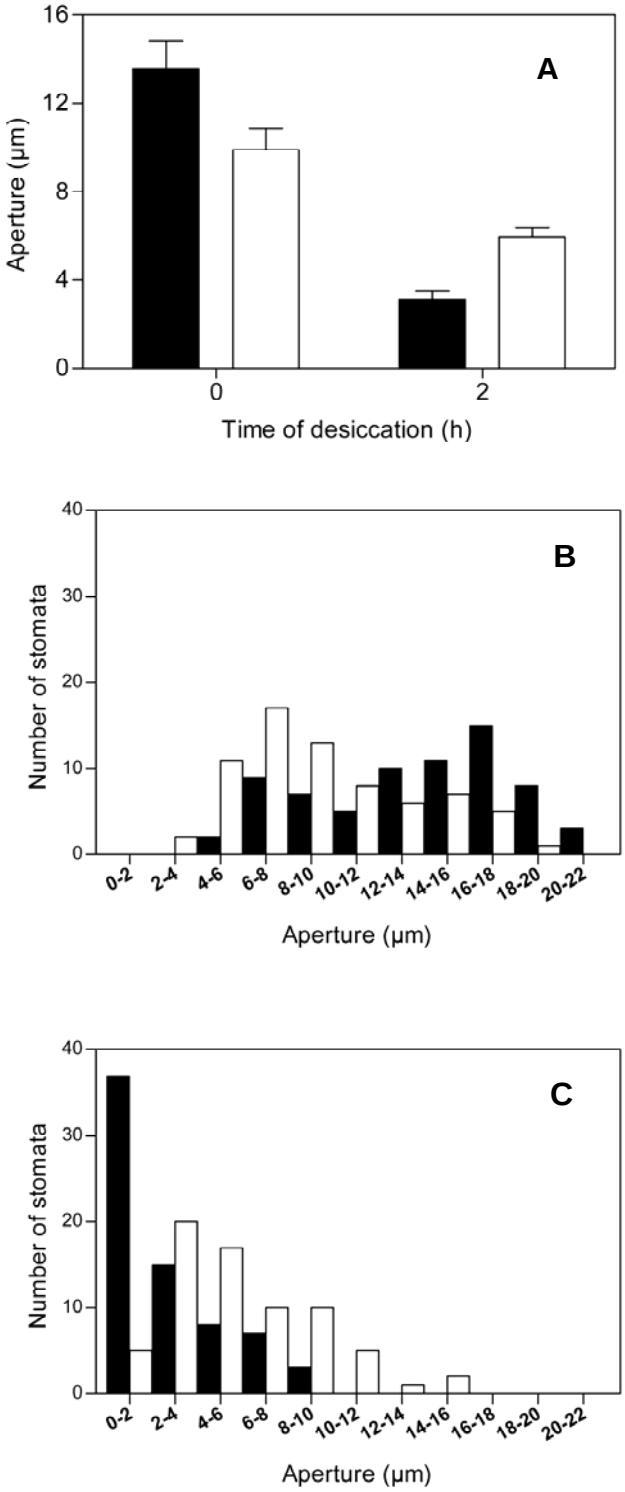

Fig.6 Stomatal aperture at the margins (closed bars) and around the main veins (open bars) in *Tradescantia virginiana* leaves grown at high RH before excision and 2 h after excision (A). Values are the mean of seven leaves ± SEM. The measurements of stomatal aperture were made on 10 stomata at one location for each area type on each of seven leaves. The average stomatal aperture was then calculated per leaf and the averages from seven leaves were further averaged and the SEM of the mean was calculated. Frequency distribution of number of stomata in different stomatal aperture ranges in non-desiccated intact (B) and desiccated(C) leaves are indicated (n=70).

There were decreases in Φ_{PSII} (Fig. 7A) and leaf water potential (Fig. 7B) as RWC decreased in moderate or high RH grown leaves. However, at the same values of RWC, the Φ_{PSII} and water potential in high RH grown leaves were higher than in the moderate RH grown leaves. Considering either the moderate or the high RH grown plants, there were no significant differences between the slopes of the regression lines calculated from data obtained from the leaf margins and main-vein areas. However, when the moderate and high RH grown plants were compared, there were significant differences in both the relationships of Φ_{PSII} and RWC, and leaf water potential and RWC (P<0.0001). Furthermore, Φ_{PSII} decreased as leaf water potential decreased in both groups of plants (Fig. 8). However, compared to moderate RH grown leaves, the Φ_{PSII} of high RH grown leaves decreased less with decreasing water potential, as revealed in the significant difference (P=0.0003) between the slopes of regression lines (0.35 ± 0.05 and 0.12 ± 0.02 for moderate and high RH grown plants, respectively).

Regardless of the growth conditions, there was no significant difference between the length of guard cells (Table 1) in the margins and main-vein areas of leaves. However, stomatal density in the leaf margins was significantly higher than in main-vein areas in both groups of leaves grown at moderate and high RH (P<0.0001).

Table 1 The length of guard cell and stomatal density in the leaf margins and main-vein areas of *Tradescantia virginiana* leaves grown at moderate or high RH. Values are the mean of 10 leaves for guard cell length and 20 leaves for stomatal density ± SEM. The measurements of stomatal length were made on 10 stomata at one location for each area type on each of 10 leaves. The average stomatal length was then calculated per leaf and the averages from 10 leaves were further averaged and the SEM of the mean was calculated.

	RH=55%		RH=90%	
	Margin area	Main-vein area	Margin area	Main-vein area
Guard cell length (μm)	68.6 ± 1.2	70.2 ± 1.5	76.0 ± 0.8	78.8 ± 1.2
Stomatal density (mm^{-2})	20.7 ± 0.8	17.3 ± 0.7	16.5 ± 0.7	12.0 ± 0.5

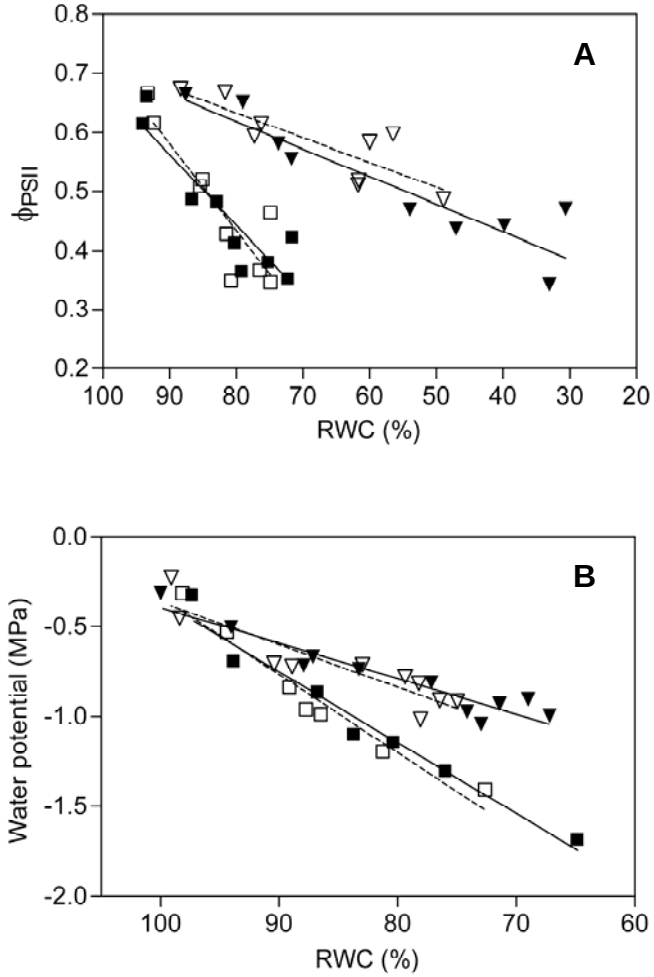

Fig.7 Relationships between Φ_{PSII} and RWC (A), and leaf water potential and RWC (B) at the margins (closed symbols) and around the main veins (open symbols) in *Tradescantia virginiana* leaves grown at moderate (squares) or high (triangles) RH.

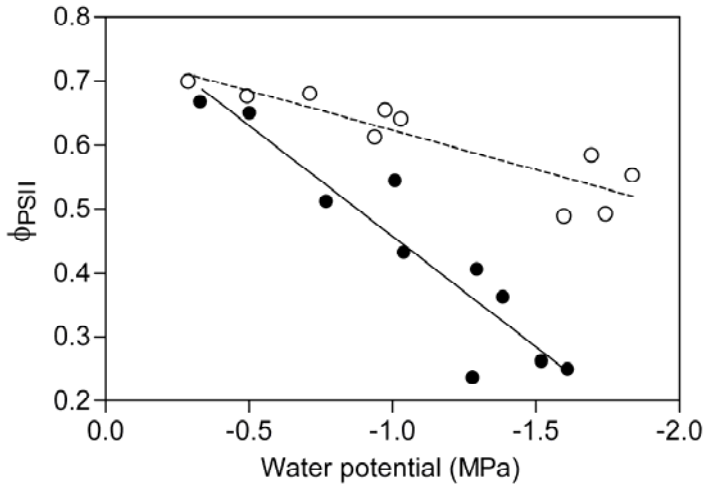

Fig.8 Relationship between Φ_{PSII} and leaf water potential in *Tradescantia virginiana* leaves grown at moderate (closed symbols) or high (open symbols) RH.

Discussion

The similarity of the mean Φ_{PSII} of leaves before desiccation under normal CO_2 and after desiccation under high CO_2 suggests that the closure of stomata is the main factor causing lower Φ_{PSII}, via a decrease in the CO_2 concentration inside the leaf, as has been shown in *Rosa rubiginosa* (Meyer and Genty, 1998; Meyer and Genty, 1999). However, as high CO_2 did not completely restore the Φ_{PSII} distributions to the pre-desiccation pattern also suggests that desiccation has other direct effects on photosynthesis. An explanation would be a higher mesophyll resistance to CO_2 uptake in desiccated leaves due to water loss (Harbinson *et al.*, 2005).

In both leaf types stomata responded to rapid desiccation after excision. However, the homogeneity, speed and degree of stomatal closure were less in high RH grown plants (Figs 2 and 3).

The result of Φ_{PSII} measurements in this study is in accordance with the results of a previous study which showed higher leaf conductance, leaf transpiration rate and average stomatal aperture in leaves grown at high RH in response to 2 h desiccation compared to leaves grown at moderate RH (Rezaei Nejad and van Meeteren, 2005). The higher Φ_{PSII} and stomatal aperture around the main vein in leaves grown at high humidity revealed that the stomata in this area did not close in response to desiccation, while in the area near leaf margins the stomata closed and thus Φ_{PSII} was reduced.

Some studies have shown that the heterogeneity of photosynthesis induced by dehydration (Meyer and Genty, 1999) or ABA treatment (Meyer and Genty, 1998) was due to heterogeneous distribution of stomatal closure over the leaflet of *Rosa rubiginosa* which is heterobaric. Though we observed heterogeneity of stomatal closure in high RH grown *Tradescantia* plants, which are monocotyledons, the pattern is different to that observed in the leaves of dicotyledons i.e. *Rosa* and *Xanthium*. There are differences between the venation (especially minor veins) and stomatal arrangement in homobaric (*Tradescantia*) and heterobaric (*Rosa*) leaves. In *Rosa* stomata are distributed and oriented randomly over the leaf surface, and the minor veins sub-divide the leaf into heterobaric zones. Stomata of *Tradescantia* are distributed in intercostal areas and in linear arrays with the orientation of stomatal pores generally parallel to the long axis of the leaf. Nonetheless, it is clear that a

heterogeneous stomatal response can be induced in monocotyledonous homobaric leaves, resulting in an uneven distribution of Φ_{PSII}. It is also noteworthy that the large differences in Φ_{PSII} can be established over relatively small lateral distances. The width of the leaves used in these experiments was about 1 cm, which implies that gradients of Φ_{PSII} can be maintained over lateral distances of millimeters, indicating that lateral diffusion of CO_2 through the mesophyll air spaces is not efficient over these distances (Morison *et al.*, 2005).

Our results suggest that the heterogeneity of stomatal resistance, and consequently of photosynthesis, induced by dehydration is affected both by the history of growth conditions in which stomata developed and the duration of desiccation. In main-vein areas of leaves grown at high RH, stomatal closure is much delayed or even absent. Moreover, it is not clear to what extent the mechanism proposed for stomatal patchiness induced by, for example, drought stress (Mott and Buckley, 2000) will be useful in explaining the behaviour of less-responsive stomata found in leaves grown at high RH.

Our results show that the relationships of Φ_{PSII} and RWC, and water potential and RWC have been affected by the high RH during growth (Fig. 7). Though Φ_{PSII} and water potential decreased as RWC decreased in both groups of plants, they both decreased less with decreasing RWC in the high RH grown plants than in the moderate RH grown plants. Regardless of the growth conditions, the leaf margins had the same responses of Φ_{PSII} and water potential to decrease in RWC as the main-vein areas, in spite of the fact that growth at high RH resulted in different responses between the leaf margins and main-vein areas. Moreover, Φ_{PSII} of high RH grown leaves decreased less with decreasing water potential compared to moderate RH grown leaves (Fig. 8). From these observations the following can be concluded. First, growth at high RH greatly and uniformly modifies the leaf tissue properties such that leaf water potential becomes less sensitive to RWC. Second, growth at high RH greatly and uniformly modifies the response of stomatal aperture such that it also becomes less sensitive to decrease in RWC and leaf water potential. Third, the differences in stomatal aperture and Φ_{PSII} that developed between the leaf margins and main-vein areas in the high RH grown leaves during desiccation implies that gradients of RWC, and thus water potential, can be established and maintained between main vein and leaf margin zones, a distance of around 5 mm.

The changes in the relationship between water potential and RWC during growth at high RH could be due to changes in the osmotic potential and/or pressure potential. It has been shown that cell size has a major effect on changes in cellular osmotic potential due to water loss. The bigger the cell size, the higher (less negative) osmotic potential can be with the same water loss (Cutler *et al.*, 1977). Bigger stomata and epidermal cells have been reported in *Tradescantia virginiana* grown at high RH (Rezaei Nejad and van Meeteren, 2005). Therefore, leaves grown at high RH would need to have a lower water content to reach the same value of osmotic potential as leaves grown at moderate RH.

Though the bigger guard cells found in leaves grown at high RH may have an effect on the osmotic potential in stomata, it does not completely explain why stomata of leaves grown at high RH do not close in response to prolonged desiccation. Stomatal movement is the result of changes in guard cell turgor. These are regulated in response to the complex integration of numerous signals that ultimately act upon a network of ion channels in the plasma and vacuolar membranes of guard cells. The movement of water in response to these fluxes results in changes of the turgor pressure of the guard cells. ABA is a key component of the signal-transduction pathway for stomatal closure (Leung and Giraudat, 1998). Many studies have suggested that the short-term effects of elevated ABA concentrations on stomatal functioning are reversible (Ackerson, 1980; Tardieu *et al.*, 1996; Trejo *et al.*, 1995), but 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). All of these studies were concerned with increased ABA levels resulting in lower conductance and opening with lower stomatal conductance at any given guard cell turgor. We propose that a very low ABA concentration in well-watered plants during growth at high RH might also cause structural or physiological changes in stomata, reducing stomatal responsivity to lowered hydration state. However, further research is needed to support this suggestion.

The Φ_{PSII} of the leaf margins in high RH grown leaves was lower than found in main-vein areas after the same duration of desiccation (Fig. 5). A lower Φ_{PSII} was also correlated with a lower RWC (Figs 5 and 7A). There were no differences in the relationships between RWC and Φ_{PSII} or water potential in the leaf margins and the main-vein areas in leaves grown at either moderate or high RH. So, it can be

concluded that the lower Φ_{PSII} that develops in the leaf margins after desiccation is not due to faster response of stomata. Rather it is a consequence of the lower water content of this tissue resulting in lower turgor of the guard cells. The difference between the RWCs of the two areas of leaf might be the result of differences in transpiration rates of these two areas. The stomatal density (Table 1) and the initial stomatal aperture (Fig. 6A) are higher in leaf margins which would result in a higher transpiration rate. When the leaf is detached from the plant, the water flux from the leaf would therefore be faster in the leaf margin compared to the main-vein area. This greater rate of water loss would only result in a lower water potential if the diffusive resistance for lateral water movement from the main vein to margins were sufficiently high, thus preventing equilibration of the two tissue zones (Fig. 7). It was surprising to us that such a large lateral diffusive resistance was present in these leaves.

In conclusion, the results of this research have provided further support for the effectiveness of chlorophyll fluorescence imaging systems in the study of stomatal behaviour, and the heterogeneity of this behaviour. Direct measurements of stomatal aperture and its distribution in this research confirmed the indication of stomatal closure obtained from the chlorophyll fluorescence images under low O_2 concentration. Different trends of stomatal closure and heterogeneity in response to rapid desiccation after excision in leaves grown at moderate and high RH suggest that high RH during the development of stomata affected stomatal behaviour in response to rapid water stress. The higher average values of Φ_{PSII} and stomatal aperture in response to desiccation in the main-vein areas compared to leaf margins of leaves grown at high RH confirmed the non-uniform closure of stomata and the location of non-closing stomata mostly around the main vein of the leaves.

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References

Ackerson RC. 1980. Stomatal response of cotton to water stress and abscisic acid as affected by water stress history. *Plant Physiology* **65**, 455-9.

Assmann SM. 1993. Signal transduction in guard cells. *Annual Review of Cell Biology* **9**, 345-75.

Beyschlag W, Eckstien J. 2001. Towards a causal analysis of stomatal patchiness: the role of stomatal size variability and hydrological heterogeneity. *Acta Oecologica* **22**, 161-73.

Beyschlag W, Pfanz H. 1990. A fast method to detect the occurrence of nonhomogenous distribution of stomatal aperture in heterobaric plant leaves. *Oecologia* **82**, 52-5.

Brown KW, Jordan WR, Thomas JC. 1976. Water stress induced alternations of the stomatal response to decreases in leaf water potential. *Physiologia Plantarum* **37**, 1-5.

Cornic G. 2000. Drought stress inhibits photosynthesis by decreasing stomatal aperture-not by affecting ATP synthesis. *Trends in Plant Science* **5**, 187-8.

Cornic G, Massacci A. 1996. Leaf photosynthesis under drought stress. In: Baker NR ed *Advances in Photosynthesis: Photosynthesis and the Environment*. Dordrecht: Kluwer Academic Publishers, 347-66.

Cutler JM, Rains DW, Loomis RS. 1977. The importance of cell size in the water relations of plants. *Physiologia Plantarum* **40**, 255-60.

Daley PF, Raschke k, Ball JT, Berry JA. 1989. Topography of photosynthetic activity of leaves obtained from images of chlorophyll fluorescence. *Plant Physiology* **90**, 1233-8.

Downton WJS, Loveys BR, Grant WJR. 1988. Stomatal closure fully accounts for the inhibition of photosynthesis by abscisic acid. *New Phytologist* **108**, 263-6.

During H. 1992. Low air humidity causes non-uniform stomatal closure in heterobaric leaves in *Vitis* species. *Vitis* **31**, 1-7.

Eckstien J, Beyschlag W, Mott KA. 1996. Changes in photon flux can induce stomatal patchiness. *Plant, Cell and Environment* **19**, 1066-75.

Fordham MC, Harrison-Murray RS, Knight L, Evered CE. 2001. Effects of leaf wetting and high humidity on stomatal function in leafy cuttings and intact plants of *Corylus maxima*. *Physiologia Plantarum* **113**, 233-40.

Franks PJ, Farquhar GD. 2001. The effect of exogenous abscisic acid on stomatal development, stomatal mechanics and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology* **125**, 935-42.

Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochemica et Biophysica Acta* **990**, 87-92.

Genty B, Harbinson J, Baker NR. 1990. Relative quantum efficiencies of photosystem I and II of leaves in photorespiratory and non-photorespiratory conditions. *Plant Physiology and Biochemistry* **28**, 1-10.

Harbinson J, van Meeteren U, van Rensen R. 2005. The use of imaging of the efficiency of photosystem II electron transport to visualise the effect of dry storage on the photosynthesis and stomatal closure of cut rose stems. *Acta Horticulturae* **669**, 57-69.

Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. *Nature* **424**, 901 - 8.

Kearns EV, Assmann SM. 1993. The guard cell-environment connection. *Plant Physiology* **102**, 711-5.

Leung J, Giraudat J. 1998. Abscisic acid signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 199-222.

Lichtenthaler HK, Babani F. 2000. Detection of photosynthetic activity and water stress by imaging the red chlorophyll fluorescence. *Plant Physiology and Biochemistry* **38**, 889-95.

Lu C, Zhang J. 1998. Effects of water stress on photosynthesis, chlorophyll fluorescence and photoinhibition in wheat plants. *Australian Journal of Plant Physiology* **25**, 883-92.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany* **51**, 659-68.

Meyer S, Genty B. 1998. Mapping intercellular CO_2 mole fraction (C_i) on *Rosa rubiginosa* leaves fed with abscisic acid by using chlorophyll fluorescence imaging. *Plant Physiology* **116**, 947-57.

Meyer S, Genty B. 1999. Heterogeneous inhibition of photosynthesis over the leaf surface of *Rosa rubiginosa* L. during water stress and abscisic acid treatment: induction of a metabolic component by limitation of CO_2 diffusion. *Planta* **210**, 126-31.

Morison JIL, Gallouet E, Lawson T, Cornic G, Herbin R, Baker NR. 2005. Lateral diffusion of CO_2 in leaves is not sufficient to support photosynthesis. *Plant Physiology* **139**, 254-66.

Mott AM, Buckley TN. 2000. Patchy stomatal conductance: emergent collective behaviour of stomata. *Trends in Plant Science* **5**, 258-62.

Mott KA, Cardon ZG, Berry JA. 1993. Asymmetric patchy stomatal closure for the two surfaces of *Xanthium strumarium* L. leaves at low humidity. *Plant, Cell and Environment* **16**, 25-34.

Rezaei Nejad A, van Meeteren U. 2005. Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. *Physiologia Plantarum* **125**, 324-32.

Santamaria JM, Davies WJ, Atkinson CJ. 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. *Journal of Experimental Botany* **44**, 99-107.

Smith S, Weyers JDB, Berry WG. 1989. Variation in stomatal characteristics over the lower surface of *Commlina communis* leaves. *Plant, Cell and Environment* **12**, 653-9.

Tardieu F, Lafarge T, Simonneau TH. 1996. Stomatal control by fed or endogenous xylem ABA in sunflower: interpretation of correlations between leaf water potential and stomatal conductance in anisohydric species. *Plant, Cell and Environment* **19**, 75-84.

Torre S, Fjeld T. 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. *Scientia Horticulturae* **89**, 217-26.

Torre S, Fjeld T, Gislerød HR, Moe R. 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. *Journal of the American Society for Horticultural Science* **128**, 598-602.

Trejo CL, Clephan AL, Davies WJ. 1995. How do stomata read abscisic acid signals? *Plant Physiology* **109**, 803-11.

Weyers JDB, Meidner H. 1990. Methods in Stomatal Research. Harlow, England: Longman Scientific & Technical, 129-55.

Ziv M, Schwartz A, Fleminger D. 1987. Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; implication for hardening. *Plant Science* **52**, 127-34.

Chapter 4

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

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Abstract

In this study we have investigated the role of abscisic acid (ABA) in altered stomatal responses of *Tradescantia virginiana* leaves grown at high RH. A lower ABA concentration was found in leaves grown at high RH compared to leaves grown at moderate RH. As a result of a daily application of 20 μM ABA to leaves for three 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 high RH grown leaf 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 the others. Moreover, less closure of stomata was found in the older leaves of high RH grown plants in response to desiccation compared to 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 high RH grown leaves remained significantly wider than in the main-vein areas. It was concluded that: (1) 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; (2) 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: stomata, chlorophyll fluorescence, nitric oxide, PSII efficiency, vapour pressure deficit

Abbreviations- ABA, abscisic acid; Φ_{PSII}, relative quantum yield or efficiency for electron transport by photosystem II; PSII, photosystem II; RH, relative air humidity; SNP, sodium nitroprusside; VPD, vapour pressure deficit.

Introduction

Stomatal movement (producing changes in stomatal aperture) is a complex result of interactions of physiological factors and environmental conditions (Assmann and Wang, 2001; Hetherington and Woodward, 2003; Kearns and Assmann, 1993). 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 relative air humidities above 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 *in vitro* propagated plants (Santamaria *et al.*, 1993; Ziv *et al.*, 1987). Recently, it has been shown that *Tradescantia virginiana* plants grown at high (90%) RH had higher leaf transpiration rate, and stomatal conductance and aperture than in moderate RH (55%) grown plants under all treatments expected to cause stomatal closure (Rezaei Nejad and van Meeteren, 2005). The stomata of high RH grown leaves were less sensitive to decreases in leaf relative water content and water potential (Rezaei Nejad *et al.*, 2006). The reason why stomata of high RH grown leaves 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 (Popova *et al.*, 2000; Zhang *et al.*, 1997; 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 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; Tardieu *et al.*, 1996; Trejo *et al.*, 1995), 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 in well-watered plants (Cutler *et al.*, 1977; Spence *et al.*, 1986; Xia, 1994). A daily application of ABA to leaves of well-watered *Tradescantia 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 (Garcia-Mata *et al.*, 2003; Neill *et al.*, 2002). 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 *Tradescantia 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 relative air humidity (moderate: 55 ± 5% and high: 90 ± 5%) at Wageningen University. The temperature was 21 ± 0.5 ^{o}C resulting in VPDs of 1.12 and 0.25 kPa for moderate and high RH conditions, respectively. The light intensity was 120 ± 10 µmol m^{-2} s^{-1} (measured with LI-250 Light Meter, LI-COR, Lincoln, 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 CO_2 fixation/irradiance response showed that 120 µmol m^{-2} s^{-1} is about 40% saturating for CO_2 fixation. The plants were kept well-watered and given a nutrient solution weekly (concentration: 2 g l^{-1}; Kristalon™, Yara, Rotterdam, The Netherlands). The CO_2 concentration in the growth chambers was 360 ± 30 µmol mol^{-1} (measured with a CO_2 analyzer ADC225, MK3, Analytical Development Co. Ltd, Hoddesdon, England).

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 about 3 ml per 50 mg dry weight, vortexed to mix the water and sample and shaken overnight at 4 ^{o}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 (Asch, 2000; Quarrie *et al.*, 1988). Water content was calculated using the following equation:

$$Water\ content = \frac{(fresh\ weight - dry\ weight) \times 100}{fresh\ weight}$$

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)-sulfate heptahydrate (Fluka). Air from the climate room (55 ± 5%) was pumped directly into the remaining tubes with leaves. The CO_2 concentration inside the tubes was 360 ± 40 µmol mol^{-1}. After three 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 were measured using a chlorophyll fluorescence imaging system (described further). Once in steady state, an image of 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 pre-incubated for 2 h in a stomata-opening medium (10 mM MES-KOH, pH 6.15, 50 mM KCl) in a test room (20 ^{o}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 (control), 100 μM ABA, 200 μM SNP (Fluka) or 100 μM ABA+200 μM SNP (only for high RH grown leaves) 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, Texas).

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 three 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 lab. From these leaves chlorophyll fluorescence images were made under an atmosphere of 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 and remainder N_2 (a non-photorespiratory condition) as described elsewhere (Rezaei Nejad *et al.*, 2006). Under non-photorespiratory conditions, Φ_{PSII} 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 about 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)-sulfate heptahydrate (Fluka). Cuvette temperature was 22 ± 1 ^oC. The experiments were repeated at least six times.

Statistical analysis

Each experiment was carried out at least with six leaves from six plants (one leaf per plant). Data were subjected to analysis of variance (ANOVA). Data in figures 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, California) was used for statistical analyses.

Results

The endogenous ABA level of the leaves was affected by the relative air humidity during growth (Table 1). The ABA concentration was significantly higher in fresh (not desiccated) leaves grown at moderate RH compared to high RH grown leaves 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 to high RH grown leaves (P<0.0001).

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 and fresh weight bases. Values are the mean of six leaves ± SEM.

	ABA concentration		Water content (%)
	pmol g^{-1} DW	pmol g^{-1} FW	
RH=55%	3904 ± 392	408 ± 38	89.5 ± 0.1
RH=90%	2772 ± 192	190 ± 24	93.2 ± 0.6

Figure 1 shows the images of Φ_{PSII} 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, Φ_{PSII} 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, Φ_{PSII} decreased in all leaves but to different extents (Fig. 1B). The lower Φ_{PSII} 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 average Φ_{PSII} measured under low O_2 concentration over time of desiccation (Fig. 2). Before desiccation (time 0), Φ_{PSII} in all leaves was high and there was no significant difference among them. With desiccation, Φ_{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 Φ_{PSII} was significant (P=0.001).

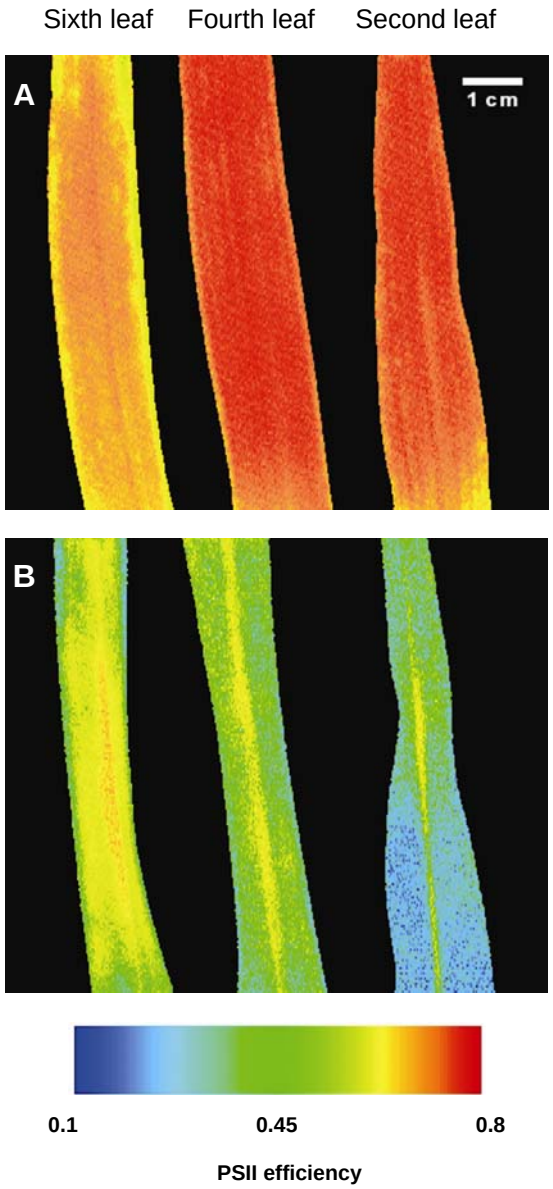

Fig.1 Images of Φ_{PSII} of leaves in water (A) and after 150 min desiccation (B) measured under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 in second (right leaf in each image), fourth (middle leaf in each image) and sixth (left leaf in each image) leaf in basipetal sequence from the shoot tip in *Tradescantia virginiana* plants grown at 90% RH.

There were no significant differences in ABA concentration on a dry weight basis of leaves in the three different ages (Table 2). However, significantly higher ABA concentrations on a fresh weight basis were found in younger leaves compared to older leaves (P=0.0001). The water content was significantly lower in younger leaves compared to older leaves (P<0.0001).

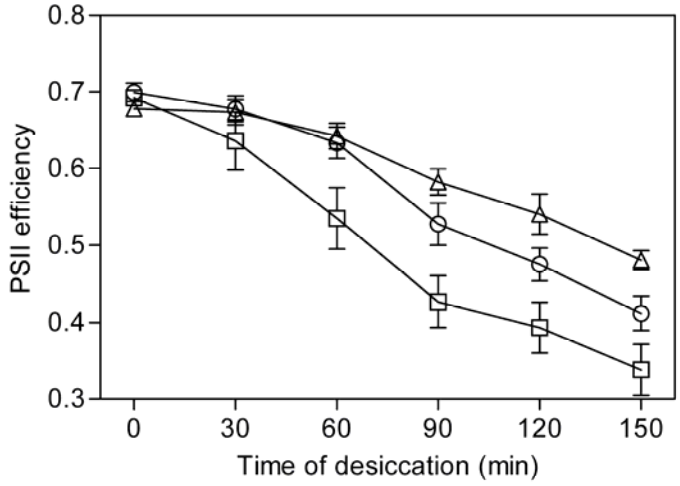

Fig.2 PSII efficiency (Φ_{PSII}) of second (squares), fourth (circles) and sixth (triangles) leaf in basipetal sequence from the shoot tip in *Tradescantia virginiana* plants grown at 90% RH over time of desiccation measured under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2. Values are the mean of six 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. The ABA concentrations are expressed on dry and fresh weight bases. Measurements were done on fresh (not desiccated) leaves. Values are the mean of eight leaves ± SEM.

	ABA concentration		Water content (%)
	pmol g^{-1} DW	pmol g^{-1} FW	
Second leaf	2451 ± 148	281 ± 15	88.5 ± 0.3
Fourth leaf	2593 ± 164	231 ± 13	91 ± 0.2
Sixth leaf	2842 ± 137	182 ± 12	93.6 ± 0.3

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 Φ_{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 Φ_{PSII} compared to leaves grown at moderate RH on either the same or another plant. This implies a slower and smaller closure of stomata in these leaves compared to moderate RH grown leaves. The interaction between the effects of RH conditions and duration of desiccation on Φ_{PSII} was significant (P<0.0001).

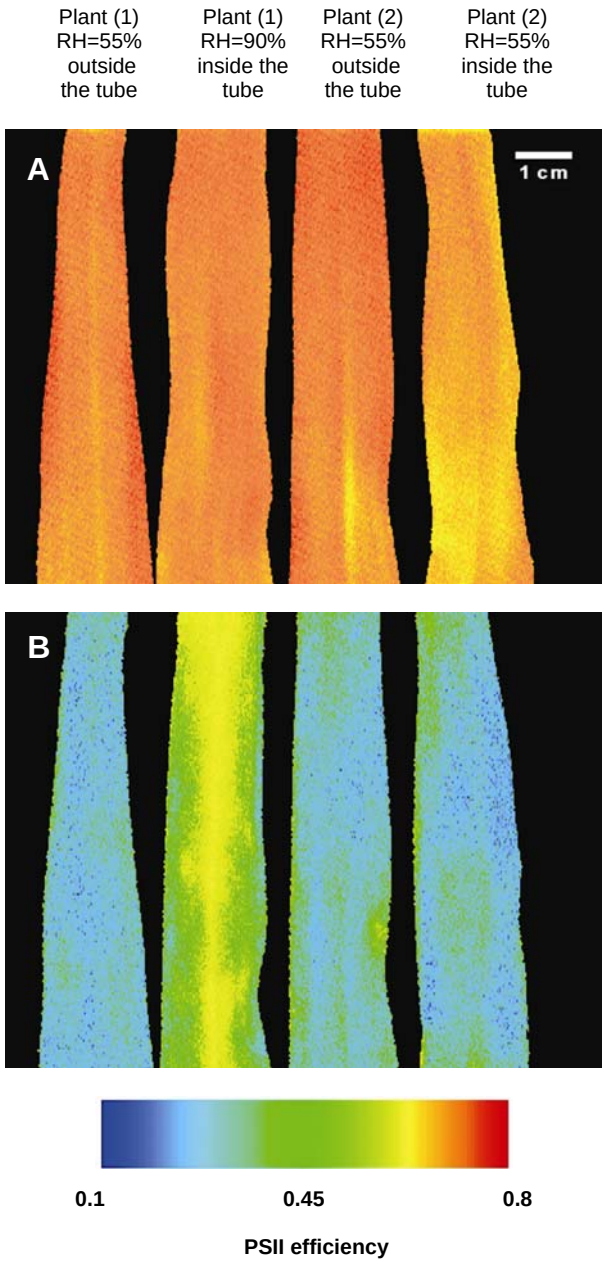

Fig.3 Images of Φ_{PSII} of leaves in water (A) and after 150 min desiccation (B) measured under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 in *Tradescantia virginiana* plants grown at 55% RH. From each plant [plant (1) and plant (2)], there was one leaf inside a glass tube. The RH in this tube was kept at 90 ± 5% [plant (1)] or at 55 ± 5% [plant (2)].

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 to moderate RH grown leaves (P<0.0001). The water content was significantly higher in leaves grown in the tubes with high RH compared to moderate RH grown leaves (P<0.0001).

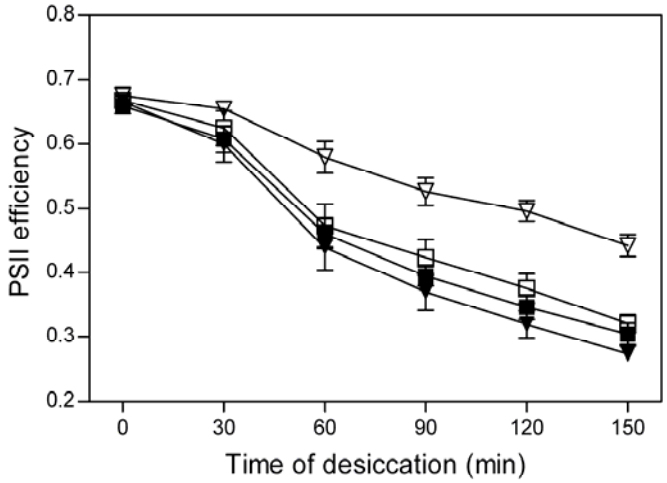

Fig.4 PSII efficiency (Φ_{PSII}) of *Tradescantia virginiana* leaves over time of desiccation measured under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2. Triangle symbols represent the Φ_{PSII} of the mature leaves from plants of which one of their leaves was grown in a glass tube with high RH (90 ± 5%). Square symbols represent the Φ_{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.

Table 3 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 and fresh weight 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.

	ABA concentration		Water content (%)
	pmol g^{-1} DW	pmol g^{-1} FW	
Leaf from plant (1) outside the tube under 55% RH	2265 ± 155	243 ± 12	89.2 ± 0.3
Leaf from plant (1) inside the tube under 90% RH	2084 ± 93	161 ± 8	92.3 ± 0.2
Leaf from plant (2) outside the tube under 55% RH	2077 ± 95	223 ± 13	89.3 ± 0.4
Leaf from plant (2) inside the tube under 55% RH	1974 ± 114	204 ± 17	89.8 ± 0.5

Figure 5 shows the images of Φ_{PSII} of leaves treated daily with 0 or 20 µM ABA during growth at moderate or high RH. Before desiccation and under a non-photorespiratory condition, Φ_{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, though Φ_{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.

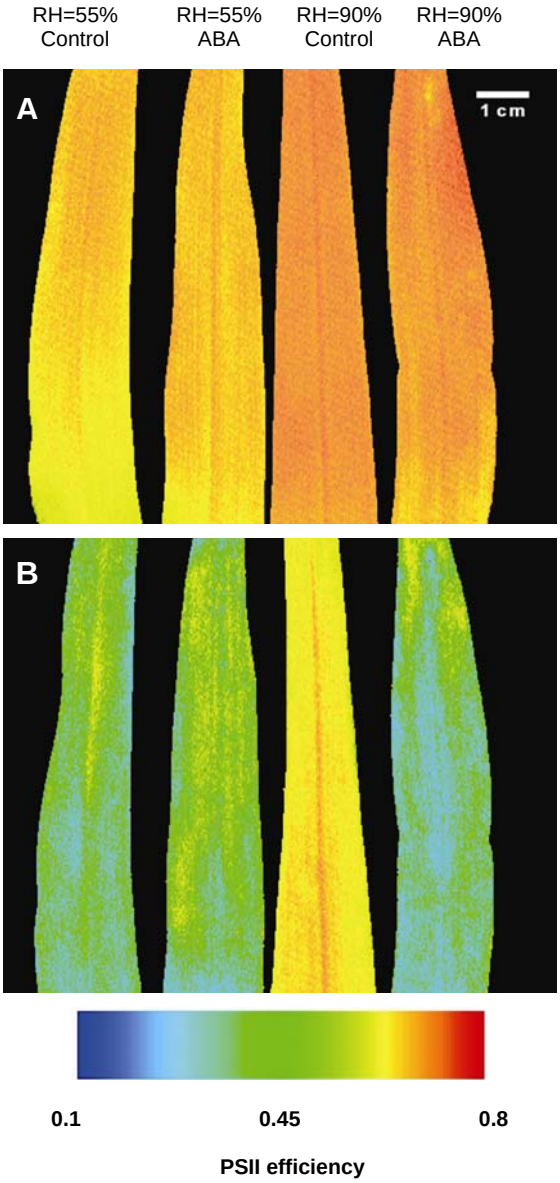

Fig.5 Images of Φ_{PSII} of leaves in water (A) and after 150 min desiccation (B) measured under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 in *Tradescantia virginiana* plants grown at 55% or 90% RH. One emerging leaf in each plant was treated daily with 0 (control) or 20 µM ABA for three weeks. The measurements of Φ_{PSII} were done 24 h after the last application of ABA.

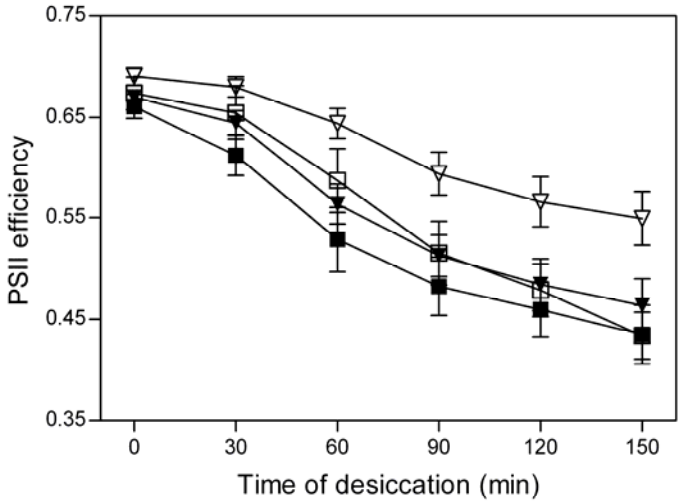

Fig.6 PSII efficiency (Φ_{PSII}) of *Tradescantia virginiana* leaves grown at 55% (squares) or 90% (triangles) RH over time of desiccation measured under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2. Open and closed symbols represent the Φ_{PSII} of the leaves treated daily with 0 (control) or 20 µM ABA for three weeks, respectively. Values are the mean of seven leaves ± SEM.

The changes in average Φ_{PSII} over time of desiccation under low O_2 concentration in leaves treated with 0 or 20 µM ABA during growth at moderate or high RH are illustrated in figure 6. Before desiccation (time 0), the Φ_{PSII} in all leaves was high and there was no significant difference among them. With desiccation, the Φ_{PSII} of ABA-treated high RH grown leaves decreased sooner than it did for control high RH grown leaves similar to leaves grown at moderate RH. These results show the similarity of stomatal responses to desiccation in high RH grown leaves treated with ABA to moderate RH grown leaves. In addition, no significant difference was found between Φ_{PSII} of control and ABA-treated leaves grown at moderate RH in response to desiccation.

In a previous paper we have showed that stomatal aperture around the main veins of high RH grown leaves remained wider after 150 min of desiccation compared to leaf margins (Rezaei Nejad *et al.*, 2006). Therefore, we have investigated the endogenous ABA concentrations of these regions and also the response of stomata to short-term ABA application. 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 a significantly higher ABA concentration on a fresh weight basis was found in the margins

compared to the main-vein areas (P=0.04). The water content was significantly lower in the margins compared to the main-vein areas (P=0.0006).

Table 4 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 and fresh weight bases. Values are the mean of six leaves ± SEM.

	ABA concentration		Water content (%)
	pmol g^{-1} DW	pmol g^{-1} FW	
Margin area	2766 ± 393	219 ± 30	92 ± 0.3
Main-vein area	2298 ± 274	138 ± 17	94 ± 0.2

Figure 7 shows the images of Φ_{PSII} of leaves grown at moderate or high RH in response to short-term ABA application. Before applying ABA when the bases of the leaves were in water and under a non-photorespiratory condition, Φ_{PSII} was high and homogeneously distributed over the leaves irrespective of growth conditions (Fig. 7A). 150 min after ABA application, Φ_{PSII} decreased in almost all parts of the moderate RH grown leaf. In the high RH grown leaf, however, Φ_{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 four hours 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 moderate RH grown leaves 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 high RH grown leaves (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 (P<0.0001), but the average stomatal aperture was significantly wider in the margins (P<0.0001). Moreover, though application of SNP alone caused closure of stomata at the main-vein areas it did not affect stomatal aperture at the leaf margins.

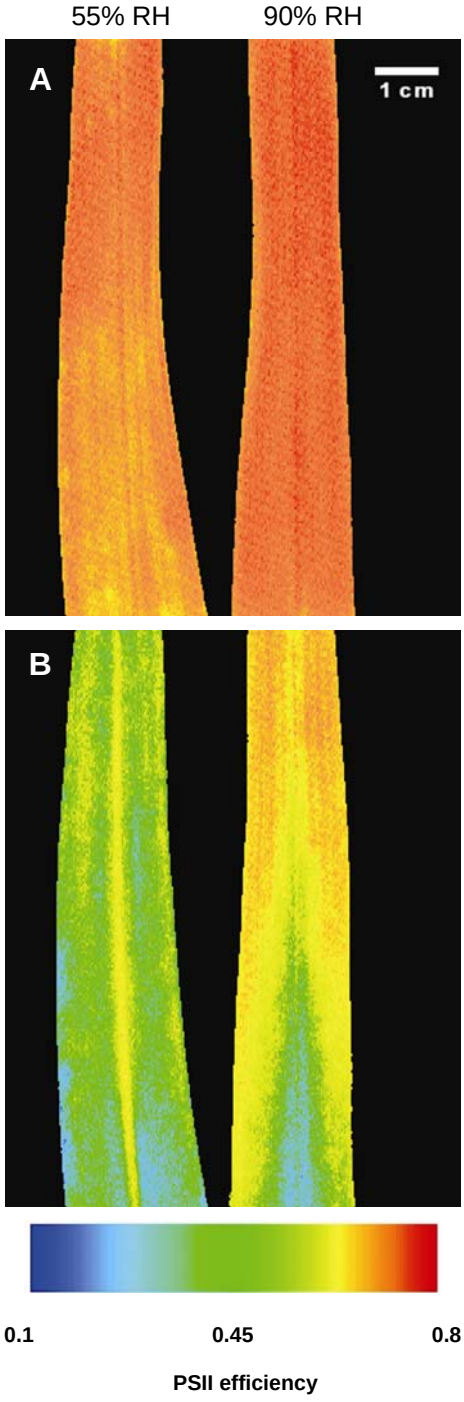

Fig.7 Images of Φ_{PSII} of leaves in water (A) and after 150 min in 100 µM ABA (B) measured under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 in *Tradescantia virginiana* plants grown at 55% (left leaf in each image) or 90% (right leaf in each image) RH.

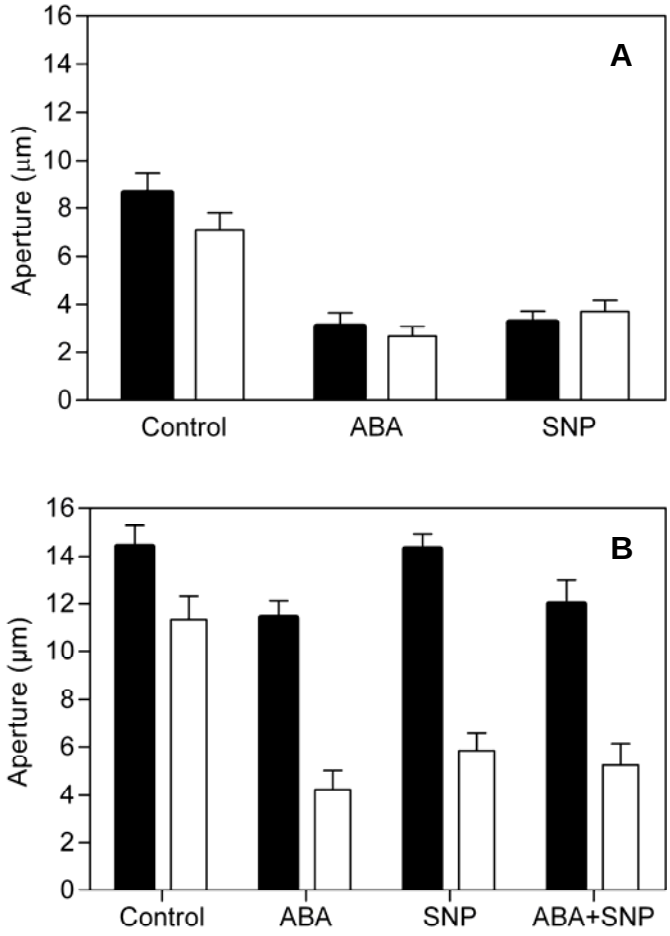

Fig.8 Stomatal aperture at the margins (closed bars) and main-vein areas (open bars) of *Tradescantia virginiana* leaves grown at 55% (A) or 90% (B) RH in response to ABA and sodium nitroprusside (SNP as a nitric oxide donor). The concentration of ABA and SNP solutions were 100 μM and 200 μM, respectively. 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.

Discussion

The smaller amount of ABA in fresh leaves grown at high RH (Table 1) was due to: 1) the higher water content found in high RH grown leaves compared to moderate RH grown leaves, and 2) the lower amount of ABA expressed on a dry weight basis. It has been shown that 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 in 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 *in vitro* cultured plants, 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 (Aguilar *et al.*, 2000; Pospisilova, 1996; Talavera *et al.*, 2001). Moreover, it has also been reported that the application of ABA during growth in *Tradescantia virginiana* results in the production of smaller stomata with altered physiological properties (Franks and Farquhar, 2001). Our 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 moderate RH grown plants (Figs 5 and 6). After three 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, though 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 and 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, though not on a dry weight basis, of the high RH grown leaf (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 and 4). The lower amount of ABA on a fresh weight basis in the high RH grown leaves 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 to 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 to main-vein areas as discussed by Rezaei Nejad *et al.* (2006). The failure of stomata at the margins of high RH grown leaves to close in response to ABA and SNP treatments (Figs 7 and 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 high RH grown leaves, as the stomata of these areas of leaves could close in response to ABA and SNP treatments (Figs 7 and 8). The ABA measurements on the leaf margins and main-vein areas are in contrast with 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 and 4).

Our results indicate faster responses of stomata to desiccation in younger leaves compared to older leaves (Figs 1 and 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 Φ_{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 moderate RH grown plant under a high RH condition resulted in its stomata having a diminished response to drought stress (Figs 3 and 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 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 (1) 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, (2) ABA concentration on a fresh weight basis rather than on a dry weight basis correlates with the changes in stomatal response characteristics, and (3) Stomata at the leaf margins of high RH grown leaves 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.

Acknowledgements

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References

Ackerson RC. 1980. Stomatal response of cotton to water stress and abscisic acid as affected by water stress history. *Plant Physiology* **65**, 455-9.

Aguilar ML, Espadas FL, Coello J, Maust BE, Trejo C, Robert ML, Santamaria JM. 2000. The role of abscisic acid in controlling leaf water loss, survival and growth of micropropagated *Tagetes erecta* plants when transferred directly to the field. *Journal of Experimental Botany* **51**, 1861-6.

Asch F. 2000. Determination of abscisic acid by indirect Enzyme Linked Immuno Sorbent Assay (ELISA). Technical report. Taastrup, Denmark: Laboratory for

Agrohydrology and Bioclimatology, Department of Agricultural Sciences, The Royal Veterinary and Agricultural University.

Assmann SM, Wang X-Q. 2001. From milliseconds to millions of years: guard cells and environmental responses. *Current Opinion in Plant Biology* **4**, 421-8.

Bahrun A, Jensen CR, Asch F, Mogensen VO. 2002. Drought-induced changes in xylem pH, ionic composition, and ABA concentration act as early signals in field-grown maize (*Zea mays* L.). *Journal of Experimental Botany* **53**, 251-63.

Bauerle WL, Whitlow TH, Setter TL, Vermeylen FM. 2004. Abscisic acid synthesis in *Acer rubrum* L. leaves- A vapor pressure deficit mediated response. *Journal of the American Society for Horticultural Science* **129**, 182-7.

Brown KW, Jordan WR, Thomas JC. 1976. Water stress induced alternations of the stomatal response to decreases in leaf water potential. *Physiologia Plantarum* **37**, 1-5.

Cutler JM, Rains DW, Loomis RS. 1977. The importance of cell size in the water relations of plants. *Physiologia Plantarum* **40**, 255-60.

Davies WJ, Zhang JH. 1991. Root signals and the regulation of growth and development of plants in drying soil. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 55-76.

Fordham MC, Harrison-Murray RS, Knight L, Evered CE. 2001. Effects of leaf wetting and high humidity on stomatal function in leafy cuttings and intact plants of *Corylus maxima*. *Physiologia Plantarum* **113**, 233-40.

Franks PJ, Farquhar GD. 2001. The effect of exogenous abscisic acid on stomatal development, stomatal mechanics and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology* **125**, 935-42.

Garcia-Mata C, Gay R, Sokolovski S, Hills A, Lamattina L, Blatt MR. 2003. Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signalling pathways. *Proceedings of the National Academy of Sciences of the USA* **100**, 11116-21.

Haefner JW, Buckley TN, Mott KA. 1997. A spatially explicit model of patchy stomatal responses to humidity. *Plant, Cell and Environment* **20**, 1087-97.

Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. *Nature* **424**, 901-8.

Jackson M. 1997. Hormones from roots as signals for the shoots of stressed plants. *Trends in Plant Science* **2**, 22-8.

Kearns EV, Assmann SM. 1993. The guard cell-environment connection. *Plant Physiology* **102**, 711-5.

Leung J, Giraudat J. 1998. Abscisic acid signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 199-222.

Mortensen LM, Gislerød HR. 2005. Effect of air humidity variation on powdery mildew and keeping quality of cut roses. *Scientia Horticulturae* **104**, 49-55.

Mott KA, Franks PJ. 2001. The role of epidermal turgor in stomatal interactions following a local perturbation in humidity. *Plant, Cell and Environment* **24**, 657-62.

Neill SJ, Desikan R, Clarke A, Hancock JT. 2002. Nitric oxide is a novel component of abscisic acid signalling in stomatal guard cells. . *Plant Physiology* **128**, 13-6.

Popova LP, Outlaw WH, Aghoram K, Hite DRC. 2000. Abscisic acid - an intraleaf water-stress signal. *Physiologia Plantarum* **108**, 376-81.

Pospisilova J. 1996. Hardening by abscisic acid of tobacco plantlets grown *in vitro*. *Biologia Plantarum* **38**, 605-9.

Quarrie SA, Whitford PN, Appleford NEJ, Wang TL, Cook SK, Henson IE, Loveys BR. 1988. A monoclonal antibody to (S)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. *Planta* **173**, 330-9.

Rezaei Nejad A, Harbinson J, van Meeteren U. 2006. Dynamics of spatial heterogeneity of stomatal closure in *Tradescantia virginiana* altered by growth at high relative air humidity *Journal of Experimental Botany* **57**, 3669-3678.

Rezaei Nejad A, van Meeteren U. 2005. Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. *Physiologia Plantarum* **125**, 324-32.

Santamaria JM, Davies WJ, Atkinson CJ. 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. *Journal of Experimental Botany* **44**, 99-107.

Spence RD, Wu H, Sharpe PJH, Clark KG. 1986. Water stress effects on guard cell anatomy and the mechanical advantage of the epidermal cells. *Plant, Cell and Environment* **9**, 197-202.

Talavera CR, Espadas FL, Aguilar ML, Maust BE, Oropeza CM, Santamaria JM. 2001. The control of leaf water loss by coconut plants cultured *in vitro* depends

on the type of membrane used for ventilation *Journal of Horticultural Science and Biotechnology* **76**, 569-74.

Tardieu F, Lafarge T, Simonneau TH. 1996. Stomatal control by fed or endogenous xylem ABA in sunflower: interpretation of correlations between leaf water potential and stomatal conductance in anisohydric species. *Plant, Cell and Environment* **19**, 75-84.

Torre S, Fjeld T. 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. *Scientia Horticulturae* **89**, 217-26.

Torre S, Fjeld T, Gislerød HR, Moe R. 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. *Journal of the American Society for Horticultural Science* **128**, 598-602.

Trejo CL, Clephan AL, Davies WJ. 1995. How do stomata read abscisic acid signals? *Plant Physiology* **109**, 803-11.

Weyers JDB, Meidner H. 1990. Methods in Stomatal Research. Harlow, England: Longman Scientific & Technical, 129-55.

Wilkinson S, Davies WJ. 2002. ABA-based chemical signalling: the co-ordination of response to stress in plants. *Plant, Cell and Environment* **25**, 195-210.

Xia MZ. 1994. Effects of soil drought during the generative development phase of faba been (*Vicia faba*) on photosynthetic characters and biomass production. *Journal of Agricultural Science* **122**, 67-72.

Zacchini M, Morini S. 1998. Stomatal functioning in relation to leaf age in in-vitro-grown plum shoots. *Plant Cell Reports* **18**, 292-6.

Zhang J, Jia W, Zhang D. 1997. Re-export and metabolism of xylem-delivered ABA in attached maize leaves under different transpirational fluxes and xylem ABA concentrations. *Journal of Experimental Botany,* **48**, 1557-64.

Zhang SQ, Outlaw WH. 2001. Abscisic acid introduced into the transpiration stream accumulates in the guard cell apoplast and causes stomatal closure. *Plant, Cell and Environment* **24**, 1045-54.

Ziv M, Schwartz A, Fleminger D. 1987. Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; implication for hardening. *Plant Science* **52**, 127-34.

Chapter 5

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

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Abstract

The rate and the reversibility of adaptation of stomatal closing behaviour in *Tradescantia Virginiana* leaves to a moderate (55%) or a high (90%) relative air humidity (RH) were investigated. Stomatal closure in response to desiccation of the distal parts of leaves which were grown firstly at a moderate RH followed by 10 days at a high RH was the same as that of the bases of the leaves grown fully at the 10-day period of high RH. However, stomatal closure of the distal parts of leaves which were grown firstly at a high RH followed by 10 days at a moderate RH was less than in the bases of the leaves grown at the moderate RH. Four days after transferring fully expanded leaves to a high RH, stomata of leaves grown at a moderate RH showed the same behaviour as did stomata of high RH grown leaves in response to desiccation: they did not close fully. Transferring the plants back to a moderate RH did not result in the recovery of stomatal closure. Exposure of moderate RH grown plants to a high RH for five days modified the response of stomatal aperture such that it became less sensitive to decrease in leaf relative water content (RWC) and leaf water potential. However, the relationship between leaf water potential and RWC was not affected by the exposure to a high RH. Leaves grown and fully expanded at a moderate RH and exposed to a high RH for five days showed slower and less closure of stomata in response to short-term application of ABA than control leaves (not exposed to high RH). Within one day after transferring moderate RH grown plants to a high RH, the ABA concentration of their leaves decreased to the level of ABA in high RH grown leaves. The decrease in leaf ABA concentration in the transferred plants occurred at least three days prior to the occurrence of altered stomatal closure. It seems that ABA level should stay above a certain level to prevent the stomatal dysfunction. The altered stomatal closure in high RH grown plants or adapted plants could be due to changes in the signalling pathway for ABA-related closure of stomata. If this signalling pathway is impaired, an increased ABA level of the leaves in adapted plants (after transferring them back to moderate RH) would not result in stomatal closure in response to desiccation.

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

Abbreviations- ABA, abscisic acid; Φ_{PSII}, 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.

Introduction

Stomata are largely responsible for gas exchange, especially CO_2 uptake and water vapour loss, between plant and atmosphere. Stomatal aperture must be finely tuned to allow sufficient CO_2 uptake for photosynthesis while preventing excessive water loss via transpiration. Beside the short-term effects of many environmental and physiological factors (reviewed by Raschke, 1975; Schroeder *et al.*, 2001; Zeiger, 1983), the history of growth conditions can also affect this fine-tuning process. There are some reports suggesting that this process can be negatively affected by very high relative air humidities (RH) and thus very low vapour pressure deficits (VPD) during growth. For example, a lack of stomatal closure in response to water stress has been reported in cut roses grown at relative air humidities above 85% (Torre and Fjeld, 2001; Torre *et al.*, 2003). Similarly, a failure of stomata to close in response to desiccation or to abscisic acid (ABA) application has been shown in leafy cuttings rooted at a high RH (Fordham *et al.*, 2001a; Fordham *et al.*, 2001b) and in *in vitro* propagated plants (Santamaria *et al.*, 1993; Wardle and Short, 1983; Ziv *et al.*, 1987). Recently, it has been shown that *Tradescantia virginiana* plants grown at high (90%) RH had higher leaf transpiration rate, and stomatal conductance and aperture than in moderate RH (55%) grown plants under all treatments expected to cause stomatal closure (Rezaei Nejad and van Meeteren, 2005). The stomata of high RH grown leaves were less sensitive to decreases in leaf relative water content and water potential (Rezaei Nejad *et al.*, 2006).

Water loss and desiccation are the principal causes of plant death in rooted leafy cuttings and micro-propagated plants transferred suddenly from high RH and of the shortened vase life in cut flowers which have been grown at high RH. In leafy cuttings and micro-propagated plants it is common, therefore, to wean the plants gradually from high to low RH. There are many papers describing the adaptation process both in *in vitro* cultured plants (e.g. Capellades *et al.*, 1990; Koroch *et al.*, 1997; Wardle *et al.*, 1983) and in rooted leafy cuttings (Fordham *et al.*, 2001a). However, there is not much information available to understand how

stomatal behaviour adapts to alterations in RH. Stomatal behaviour may remain unaltered following a transfer from a high RH to a low RH (Fordham *et al.*, 2001a; Sallanon *et al.*, 1993), or it may be able to adapt (Brainerd and Fuchigami, 1981; Marin *et al.*, 1988). The reason why stomata developed at a high RH are less hydrosensitive is not clear. Rezaei Nejad and van Meeteren (2006) have reported a significantly lower endogenous ABA concentration in *Tradescantia virginiana* leaves grown at a high RH compared to leaves grown at a moderate RH. A daily application of ABA to *T. virginiana* leaves at a high RH for three weeks improved their stomatal behaviour in response to desiccation. The authors proposed that a long-term low ABA concentration in well-watered plants during growth at high RH could be a reason for altered functioning of stomata in response to desiccation. It is unknown to what extent the adaptation of stomatal behaviour to a changed RH is correlated with changes in leaf ABA concentration and other leaf hydraulic properties.

The main objectives of this study were:

1) To investigate whether the closing behaviour of stomata can change following alterations in RH, and if so, how quickly.

2) To examine whether the changes in stomatal behaviour induced by alterations in RH are reversible.

3) To elucidate the changes in ABA levels of leaves with changes in RH.

4) To investigate the correlation between stomatal response characteristics of adapted plants with leaf water status.

Materials and methods

Plant material and growth conditions

Young *Tradescantia 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 relative air humidity (moderate: 55 ± 5% and high: 90 ± 5%) at Wageningen University. The temperature was 21 ± 0.5 ^{o}C resulting in VPDs of 1.12 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, USA) produced by fluorescent tubes (TLD 33, Philips) with a photoperiod of 16 h per day. Measurements of the

CO_2 fixation/irradiance response of *T. virginiana* plants showed that 120 μmol m^{-2} s^{-1} is about 40% saturating for CO_2 fixation. The plants were kept well-watered and given a nutrient solution weekly (concentration: 2 g l^{-1}; Kristalon™, Yara, Rotterdam, The Netherlands). The CO_2 concentration in the growth chambers was 360 ± 30 μmol mol^{-1} (measured with a CO_2 analyzer ADC225, MK3, Analytical Development Co. Ltd, Hoddesdon, England).

Adaptation in different parts of a growing leaf to moderate or high RH

A young leaf (approximately one week old) from each plant growing at 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-photorespiratory condition (described further). These leaves consisted of two parts: (1) the distal parts of the leaves which were grown firstly at a moderate RH and then exposed to a high RH for 10 days, and (2) the base of the leaves which were produced during the 10-day exposure of the distal parts 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 firstly at a high RH and thereafter exposed to a moderate RH.

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 labeled as M0, MH1, MH2, …, MH10. Eleven plants grown at a high RH were also randomly selected and labeled as H0, H1, …, H10. The labeled high RH grown plants were kept continuously at a high RH and served as control plants. First, PSII efficiency of a mature leaf from M0 together with a mature leaf from H0 was measured in response to desiccation under a non-photorespiratory condition (day 0). Then MH1-MH10 plants were transferred to a high RH climate room and daily measurements of PSII efficiency in response to desiccation were made 1-10 days after transferring. So, one day after transferring, measurements were made on a leaf from MH1 together with a leaf from H1 until 10 days after transferring, when measurements were made on MH10 and H10. To determine whether the adaptation of stomata to high RH can be reversed, after 4-10 days of exposure of MH4-MH10 to high RH, they were transferred back to a

moderate RH and labeled as MH4M6-MH10M6 and then after six days exposure to a moderate RH the plants were re-measured. This experiment was repeated three times.

Stomatal responses to short-term application of ABA

The spatial heterogeneity of stomatal responses to short-term exogenous ABA in leaves which were grown at a moderate RH followed by five days exposure to a high RH together with control leaves (moderate RH grown leaves) were measured using a chlorophyll fluorescence imaging system (described further). The first image of PSII efficiency (Φ_{PSII}) 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. This experiment was repeated with six leaves from six plants in each RH condition.

Mapping of PSII photochemical yield using chlorophyll fluorescence imaging

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 lab. From these leaves chlorophyll fluorescence images were made under an atmosphere of 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2 and remainder N_2 (a non-photorespiratory condition) as described elsewhere (Rezaei Nejad *et al.*, 2006) . Under non-photorespiratory conditions, Φ_{PSII} is closely related to stomatal closure (Rezaei Nejad *et al.*, 2006). The first image of Φ_{PSII} 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)-sulfate heptahydrate (Fluka). Cuvette temperature was 22 ± 1 ^{o}C.

Measurements of leaf water potential and relative water content (RWC)

To obtain the relationships of Φ_{PSII}, water potential and RWC, excised leaves were imaged while in water or in response to desiccation using the chlorophyll fluorescence imaging system and thereafter leaf discs were cut from the

leaves and were used for the measurements of water potential and RWC as described elsewhere (Rezaei Nejad and van Meeteren, 2006).

ABA measurements

The effect on leaf ABA levels of transferring plants from a moderate to a high RH was measured. The ABA levels of leaves in transferred plants and in high RH grown plants (control) were measured daily from day 0 (before transferring) until four days after transferring. All transferred plants were kept at a high RH for four days and then they were transferred back to a moderate RH and measurements of ABA level were done after 1, 2, 3 and 6 days 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 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 in each RH condition. For ABA analysis, leaves were removed from the plants early in the morning, weighed, freeze-dried, reweighed and finely ground. Distilled water was added at about 3 ml per 50 mg dry weight, vortexed to mix the water and sample, and shaken overnight at 4 ^{o}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 (Asch, 2000; Quarrie *et al.*, 1988)

Statistical analysis

Each experiment was carried out at least with three leaves from three plants (one leaf per plant). Data of ABA concentration and Φ_{PSII} were subjected to analysis of variance (ANOVA). Data in figures 2 and 8 were analysed using repeated measures ANOVA. The student's t-test was used for mean separation (P=0.05). The relationships between Φ_{PSII} and RWC, water potential and RWC, Φ_{PSII} and water potential were fitted using linear regressions. The parameters of fitted lines were compared statistically with F-test. GraphPad Prism 4 for Windows (GraphPad Software, San Diego, California) was used for all statistical analyses and curve fitting.

Results

Figure 1 shows the images of Φ_{PSII} of leaves in water (A1 and B1) and after 150 min of desiccation (A2 and B2) under a non-photorespiratory condition. In figure 1A the distal part of the leaf (M→H) was grown firstly at a moderate RH followed by 10 days at a high RH. The base of the leaf (H) was produced at the high RH during the period of exposure of the distal part to the high RH. In figure 1B the distal part of the leaf (H→M) was grown firstly at a high RH followed by 10 days at a moderate RH. The base of the leaf (M) was produced at the moderate RH 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 leaves irrespective of RH conditions implying the opening of stomata in both parts of each leaf (Figs 1A1 and 1B1). After 150 min of desiccation, the Φ_{PSII} decreased in both parts of each leaf (Figs 1A2 and 1B2). The decrease of Φ_{PSII} in both parts of the leaf in figure 1A2 was to the same extent, while the response was different between the two parts of the leaf in figure 1B2. The higher Φ_{PSII} in the distal part of the leaf in figure 1B2 (H→M) indicated less closure of stomata in response to desiccation. The decline in the mean Φ_{PSII} with duration of desiccation in the distal parts of the leaves which were grown firstly at a moderate RH followed by 10 days at a high RH (M→H) was the same as that of the bases of the leaves grown completely at the high RH (H) indicating the adaptation of stomatal behaviour of the moderate RH grown part to the high RH (Fig. 2A). The decrease in Φ_{PSII} with duration of desiccation in the distal parts of the leaves which were grown firstly at a high RH followed by 10 days exposure to a moderate RH (H→M) was less than that for the bases of the leaves grown at the moderate RH (M) indicating that stomata developed at the high RH did not adapt fully to the moderate RH (Fig. 2B).

Figure 3 shows how the duration of the exposure to a high RH condition in fully developed leaves can change the trend of Φ_{PSII}, and thus stomatal behaviour, in response to desiccation. The high Φ_{PSII} of leaves while in water showed the opening of stomata in all leaves irrespective of RH treatments (Figs 3A1, 3B1 and 3C1). After 150 min desiccation the leaf developed at a moderate RH and transferred to a high RH for three days (MH3; Fig. 3A2) showed much lower Φ_{PSII} than in high RH grown leaf (H3) implying the faster closure of stomata in response to desiccation. Similar results were observed from day 0 (before transferring) until two days after transferring the plants from a moderate to a high RH (not shown). The moderate RH

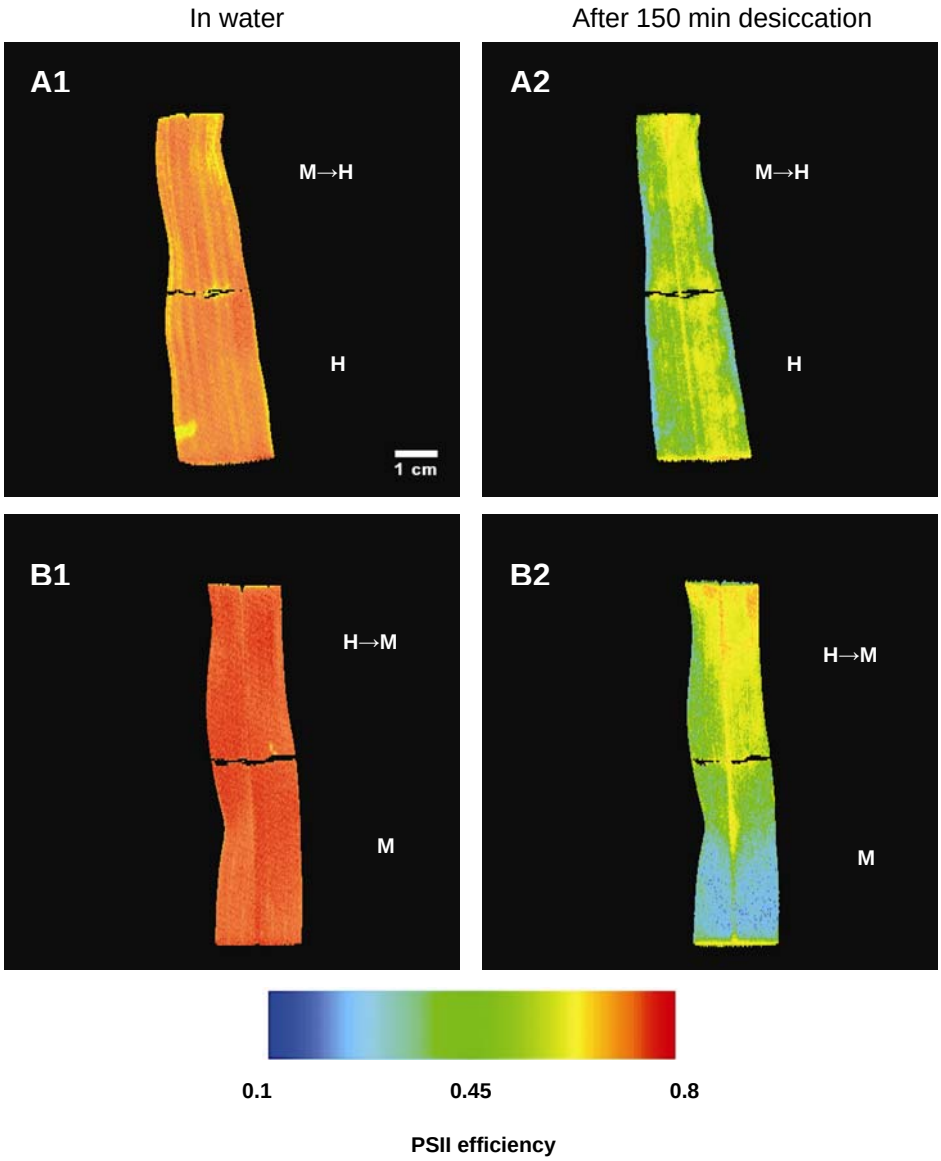

Fig.1 Images of PSII efficiency (Φ_{PSII}) of *Tradescantia virginiana* leaves in water (A1 and B1) and after 150 min of desiccation (A2 and B2) under 20 mmol mol^{-1} O_2 and 350 µmol mol^{-1} CO_2. In figure (A) the distal part of the leaf (the part above the black line) was grown at a moderate RH (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 period (H). In figure (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).

grown leaf transferred to a high RH for four days (MH4; Fig. 3B2) showed the same response as the high RH grown leaf (H4): the Φ_{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 days after transferring the plants from a moderate to a high RH (not shown). When a moderate RH grown plant was kept at

a high RH for four days and then transferred back to a moderate RH for further six days, the response of Φ_{PSII} to desiccation still remained the same as control plant continuously grown at a high RH (H4) indicating the irreversibility of adaptation of stomata to high RH (MH4M6; Fig. 3C2). Similar results were observed when moderate RH grown plants were kept at a high RH for 5-10 days and then transferred back to a moderate RH for a further six days (not shown).

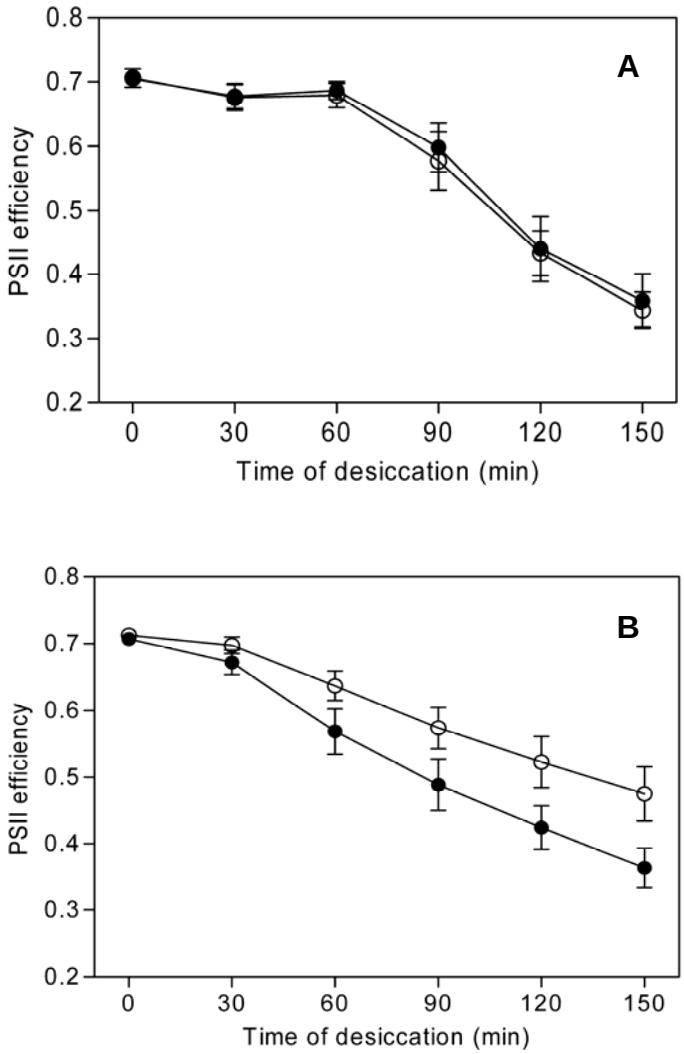

Fig.2 PSII efficiency (Φ_{PSII}) of two parts of *Tradescantia virginiana* leaves over time of desiccation under 20 mmol mol^{-1} O_2 and 350 µmol mol^{-1} CO_2. In figure (A) closed symbols represent the distal parts which were grown at a moderate RH (55%) followed by exposure to a high RH (90%) for 10 d (M→H). Open symbols represent the bases of the leaves which were grown at the high RH during this period (H). In figure (B) open symbols represent the distal parts which were grown at a high RH followed by exposure to a moderate RH for 10 d (H→M). Closed symbols represent the bases of the leaves which were grown at the moderate RH during this period (M). Values are the mean of six leaves ± SEM.

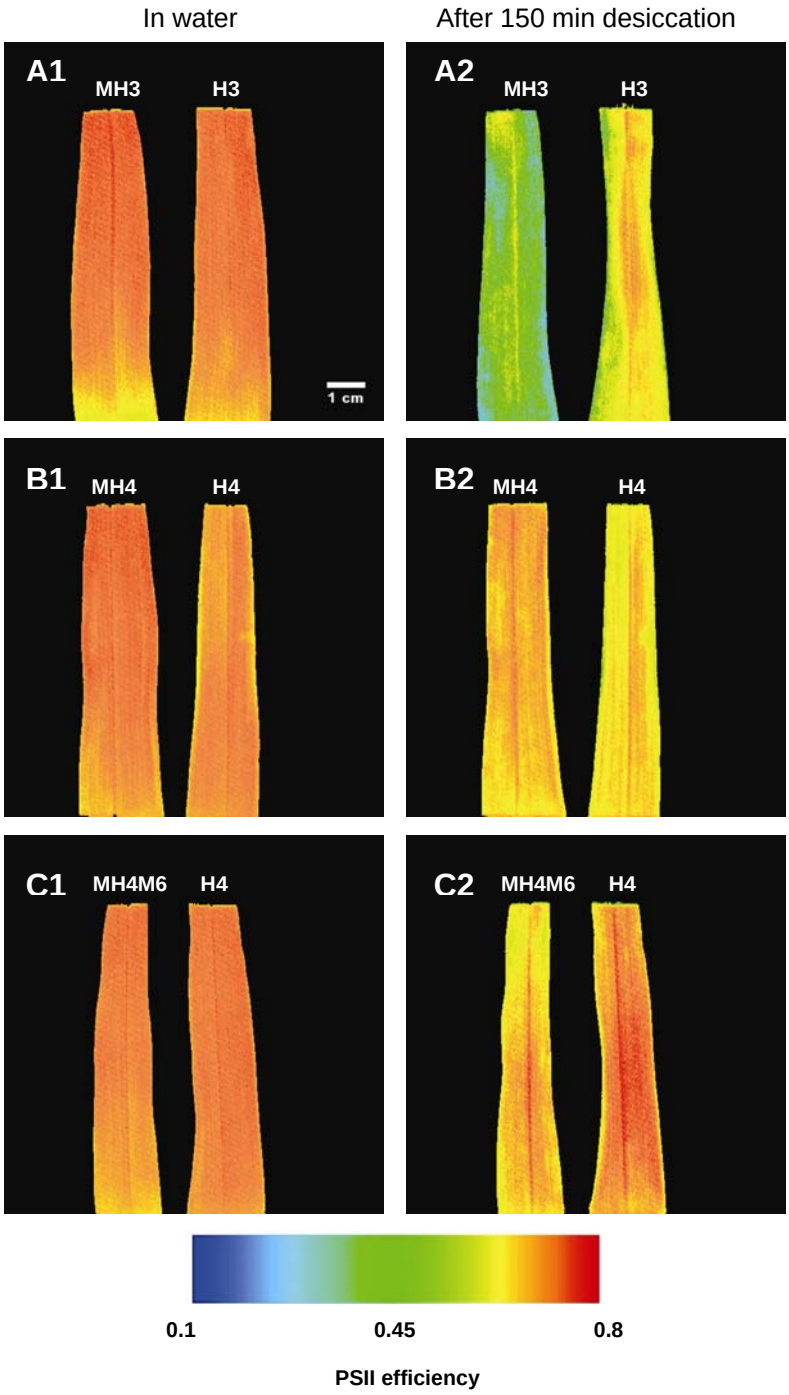

Fig.3 Images of PSII efficiency (Φ_{PSII}) of *Tradescantia virginiana* leaves in water (A1, B1 and C1) and after 150 min desiccation (A2, B2 and C2) under 20 mmol mol^{-1} O_2 and 350 µmol mol^{-1} CO_2. The right leaves in the images (H3 and H4) were grown at a high RH (90%) and used in this experiment as controls. The left leaves in the images were grown and fully developed at a moderate RH (55%) followed by exposure to a high RH: (A) for 3 d (MH3), (B) for 4 d (MH4), and (C) for 4 d and to a moderate RH for a further 6 d (MH4M6).

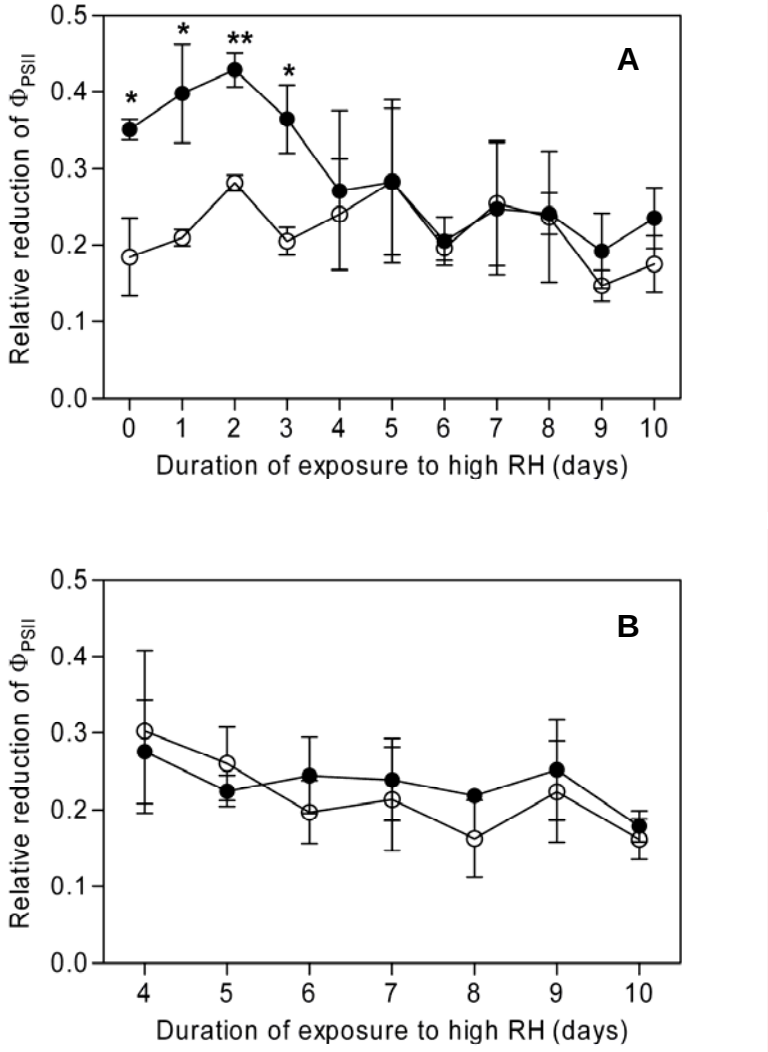

Fig.4 Relative reduction of PSII efficiency (Φ_{PSII}) in *Tradescantia virginiana* leaves grown at a moderate (55%) or a high (90%) RH after 150 min desiccation under 20 mmol mol^{-1} O_2 and 350 µmol mol^{-1} CO_2. Open symbols represent the relative reduction of Φ_{PSII} of mature leaves grown continuously at a high RH and used in this experiment as controls. Closed symbols represent the relative reduction of Φ_{PSII} of leaves grown and fully expanded at a moderate RH followed by exposure to a high RH for 1-10 days (A). Measurements were started before moving the plants from a moderate to a high RH (day 0). After 4-10 days of exposure of moderate RH grown plants to a high RH, the plants were transferred back to a moderate RH and after six days of exposure to a moderate RH the plants were re-measured (B). One leaf of each plant type was measured each day (see materials and methods). Values are the mean of three leaves ± SEM. Significant differences between the treatments are indicated according to student's t-test (* $P< 0.05$ and ** $P<0.01$).

The relative reductions of Φ_{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) until three days after transferring to a high RH were significantly higher than in high RH grown leaves (Fig. 4A). However, the relative reductions of Φ_{PSII} of these plants 4-10 days after transferring 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 days exposure of moderate RH grown plants to a high RH, the plants were transferred back to a moderate RH and measurements were done after a further six days of exposure to a moderate RH. The relative reductions of Φ_{PSII} of the transferred plants were also not significantly different from those of high RH grown plants indicating the irreversibility of the adaptation of stomata to high RH (Fig. 4B).

The adaptation of stomatal closure to high RH is also shown by the relationship between Φ_{PSII} and water status of the leaf measured as either RWC or water potential (Figs 5A and 5B). At the same values of RWC or water potential, the Φ_{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 Φ_{PSII} and RWC, and Φ_{PSII} and water potential, respectively). There was no difference in the relationship between water potential and RWC in the adapted and control plants (Fig. 5C).

The ABA concentration of moderate RH grown leaves was significantly higher than in high RH grown leaves (P=0.0002) before transferring of the plants to a high RH (Fig. 6A). After transferring, the ABA level of the moderate RH grown leaves (MH) decreased to the same level as high RH grown leaves (H). Transferring the moderate RH grown plants (MH) back to a moderate RH resulted in an increase of ABA concentration (P<0.0001) (Fig. 6B).

Five days exposure of a mature moderate RH grown leaf to a high RH could change the response of Φ_{PSII}, and thus stomatal behaviour, to short-term ABA application (Fig. 7). The high Φ_{PSII} of leaves while in water showed the opening of stomata in both leaves 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 Φ_{PSII}, while there was a clear lag-phase in the response of the adapted leaf (MH5) grown at a moderate RH and exposed to a high RH for five days (Fig. 7B-7F). Figure 8 shows the changes of the mean Φ_{PSII} of images of control (M) and adapted leaves (MH5) during 150 min of ABA application. The Φ_{PSII} in both groups of leaves in water was high and there was no significant difference between them. With ABA application, Φ_{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).

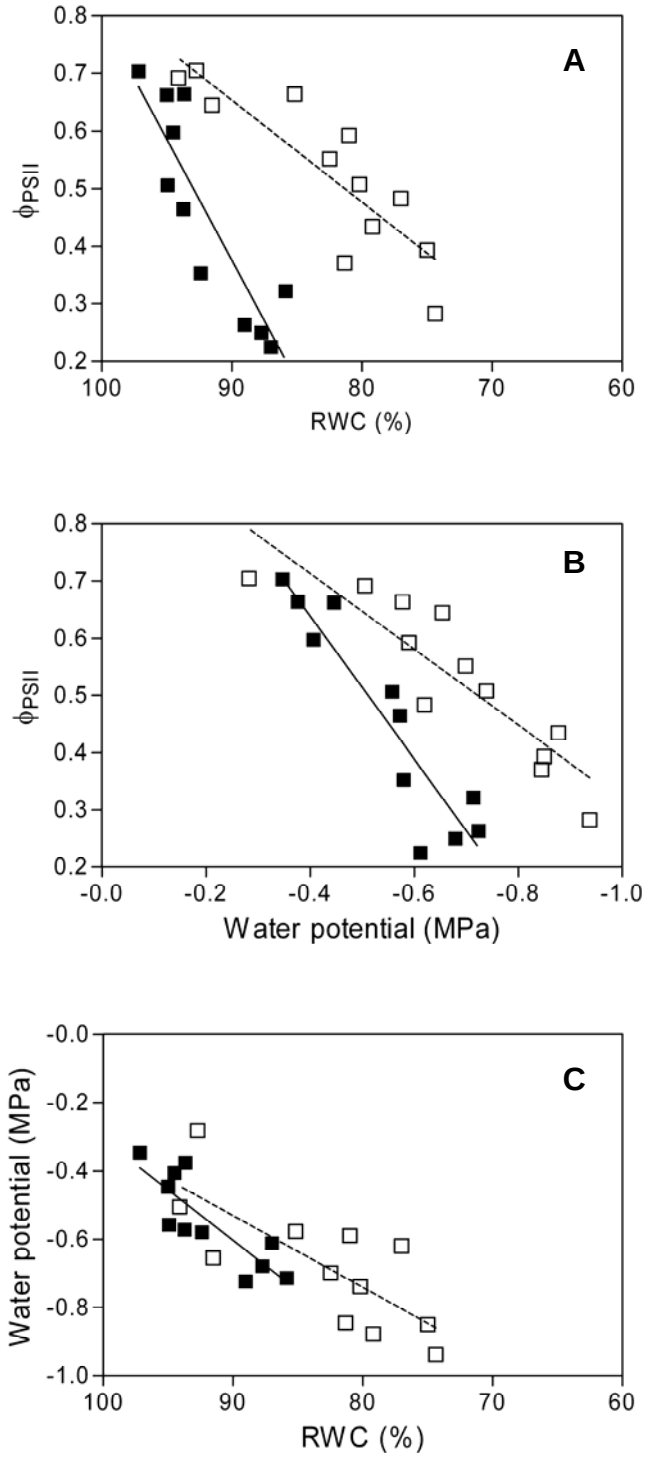

Fig.5 Relationships between Φ_{PSII} and RWC (A), Φ_{PSII} and leaf water potential (B), and leaf water potential and RWC (C) in *Tradescantia virginiana* leaves. Closed symbols represent the data obtained from leaves grown at 55% RH as controls (M). Open symbols represent the data obtained from leaves grown and fully expanded at 55% RH followed by 5 d exposure to 90% RH (MH5).

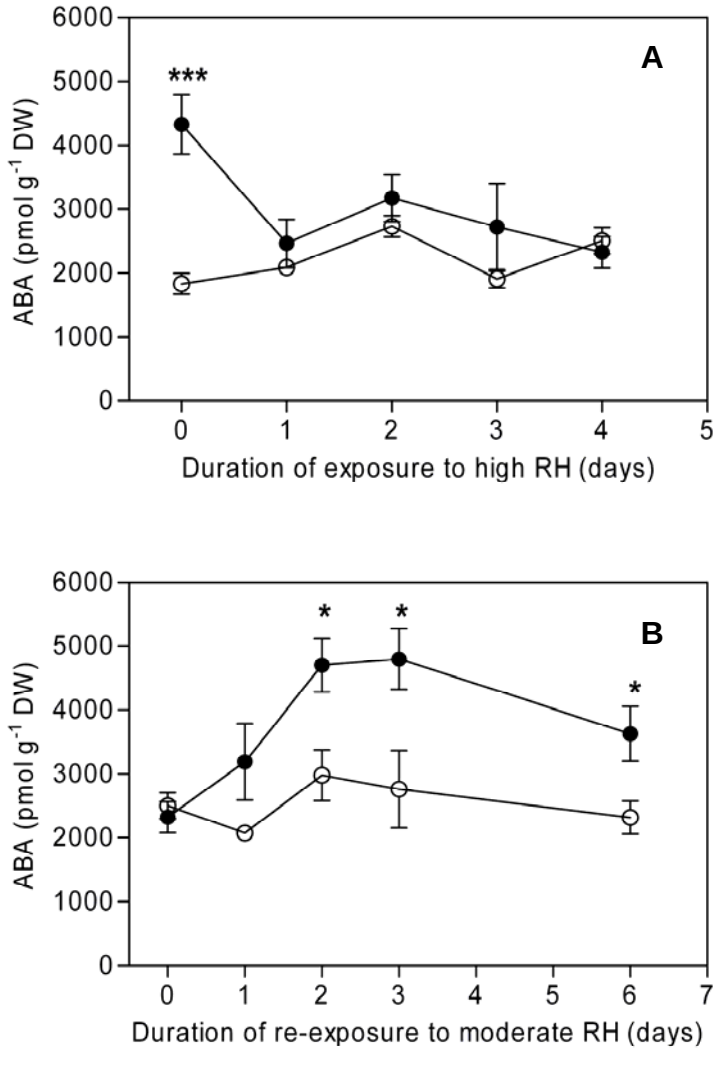

Fig.6 The ABA levels of *Tradescantia virginiana* leaves grown at a moderate (55%) or a high (90%) RH. Open symbols represent the ABA levels of leaves grown at a high RH as controls (H). Closed symbols represent the ABA levels of leaves grown and fully expanded at a moderate RH followed by exposure to a high RH for 1-4 days (MH) (A). Measurements were started before transferring of the plants from a moderate to a high RH (day 0). After four days exposure of moderate RH grown plants to a high RH, they were transferred back to a moderate RH and measurements were done after 1, 2, 3 and 6 days exposure to a moderate RH (B). Data of day 0 in Fig. B are the same as data of day 4 in Fig. A. In total, two leaves per plant were used for the measurements: one leaf during exposure to high RH (A) and one leaf during exposure to moderate RH (B) except in day 0 that one leaf per plant were used. Values are the mean of eight leaves ± SEM. Significant differences between the treatments are indicated according to student's t-test (* $P< 0.05$ and *** $P<0.001$).

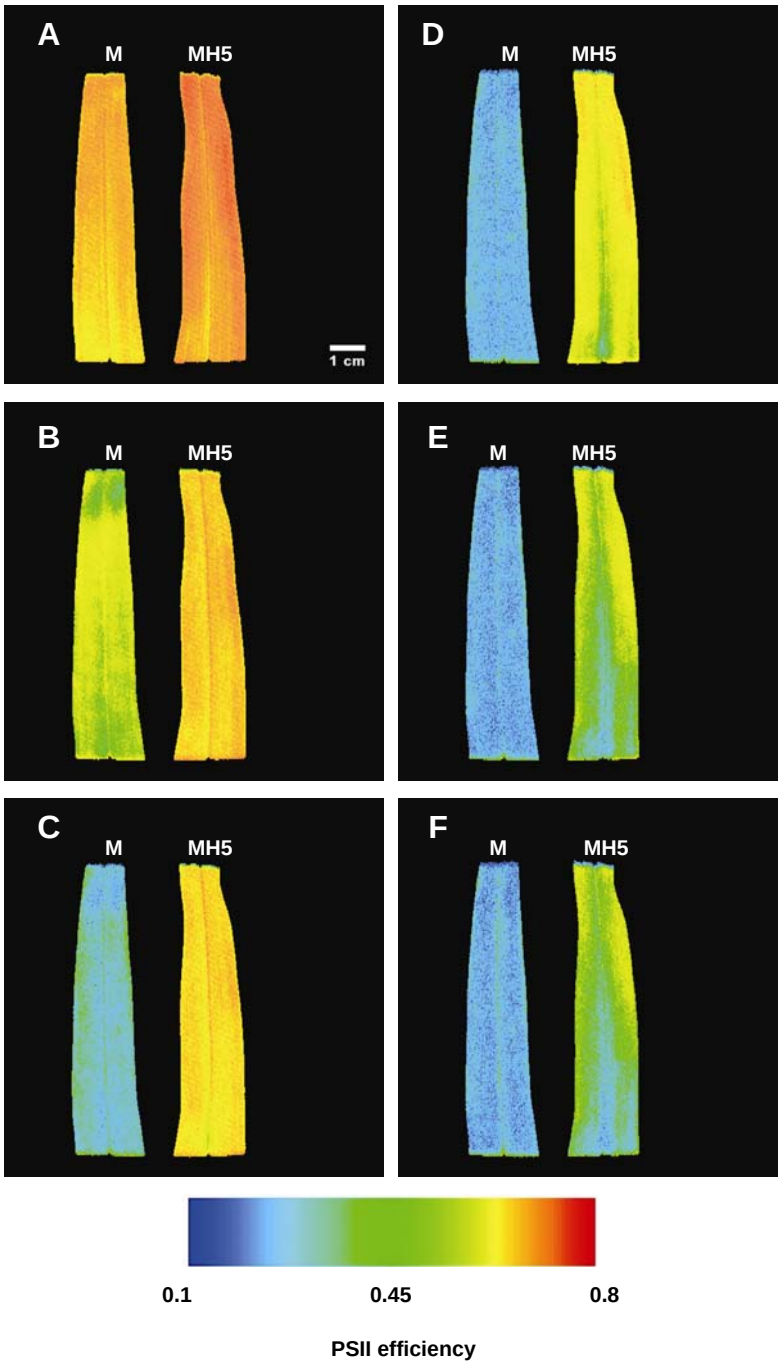

Fig.7 Images of PSII efficiency (Φ_{PSII}) of *Tradescantia virginiana* leaves in water (A) and after 30 min (B), 60 min (C), 90 min (D), 120 min (E) and 150 min (F) in 100 µM ABA solution measured under 20 mmol mol^{-1} O_2 and 350 µmol mol^{-1} CO_2. The left leaf in the images was grown at 55% RH (M). The right leaf in the images was grown and fully developed at 55% RH followed by 5 d exposure to 90% RH (MH5).

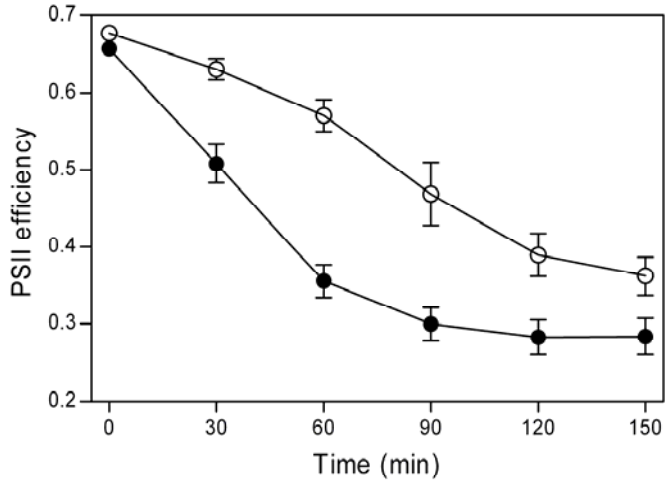

Fig.8 Changes of PSII efficiency (Φ_{PSII}) in *Tradescantia virginiana* leaves during 150 min application of 100µM ABA measured under 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2. Closed symbols represent the Φ_{PSII} of the leaves grown at 55% RH (M). Open symbols represent the Φ_{PSII} of the leaves grown and fully expanded at 55% RH followed by 5 d exposure to 90% RH (MH5). Values are the mean of six leaves ± SEM.

Discussion

The results demonstrate not only that stomata developed at a high RH cannot improve their closure when transferred to a moderate RH, but also that stomata developed at a moderate RH can adapt their behaviour to a high RH within four days and this adaptation is not reversible within six days (Figs 1-4). The irreversibility of stomatal behaviour in leaves developed at a high RH to a low humidity has been shown in rooted leafy cuttings (Fordham *et al.*, 2001a) and *in vitro* cultured plants (Sallanon *et al.*, 1993). Our results are in conflict with those of Brainerd and Fuchigami (1981) and Marin et al (1988), which showed the adaptation of high RH grown micropropagated plants to low humidity.

Exposure of moderate RH grown plants to a high RH for five days modifies the response of stomatal aperture such that it becomes less sensitive to decreases in RWC and leaf water potential (Figs 5A and 5B). Similar responses have been reported previously in high RH grown *Tradescantia* leaves when compared to moderate RH grown leaves (Rezaei Nejad *et al.*, 2006). This indicates that even stomata of leaves which have been fully developed at a moderate RH can show a fast adaptation to high humidity. The relationship between water potential and RWC was not affected by five days exposure of moderate RH grown leaves to high RH (Fig. 5C). When *Tradescantia* plants are grown continuously at a high RH, the

leaf tissue properties modify such that leaf water potential becomes less sensitive to RWC (Rezaei Nejad *et al.*, 2006). This has been attributed to the bigger cell size of high RH grown plants compared to moderate RH grown plants (Rezaei Nejad *et al.*, 2006), as cell size has been shown to have a major effect on changes in cellular osmotic potential (Cutler *et al.*, 1977). For leaves already developed at a moderate RH this change in cell size would not be possible. Another conclusion from these results is that even stomata with smaller guard cells can adapt to a high RH such that they become less hydrosensitive. Therefore, a large guard cell is not required for the occurrence of altered stomatal behaviour under high RH.

Before transferring the plants, leaf ABA concentration of moderate RH grown plants was higher than in high RH grown plants (Fig. 6A), possibly due to increased transpiration that could have promoted increased accumulation of leaf ABA (Rezaei Nejad and van Meeteren, 2006). Higher leaf ABA concentration in response to increase of VPD has been shown in *Acer rubrum* (Bauerle *et al.*, 2004), and *Tagetes erecta* cultivated *in vitro* (Aguilar *et al.*, 2000). Within one day after transferring of moderate RH grown plants to a high RH, ABA concentration of their leaves decreased to the level of ABA in high RH grown leaves and remained at this low level during the four day exposure to high RH conditions (Fig. 6A). Possible explanations for these changes would be: (1) less accumulation of leaf ABA due to low transpiration rate in high RH, (2) a decrease in the leaf ABA already accumulated. It has been shown that conditions that increase endogenous ABA levels of *in vitro* cultured plants, 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 a low RH (Aguilar *et al.*, 2000; Pospisilova, 1996; Pospisilova *et al.*, 1998; Talavera *et al.*, 2001). Moreover, it has also been reported that frequent application of ABA during growth in *Tradescantia virginiana* results in the production of smaller stomata with altered physiological properties (Franks and Farquhar, 2001). A significantly lower endogenous ABA concentration has been reported in *Tradescantia virginiana* leaves grown at a high RH compared to leaves grown at a moderate RH (Rezaei Nejad and van Meeteren, 2006). A daily application of ABA to leaves during growth at a high RH improved their stomatal behaviour in response to desiccation (Rezaei Nejad and van Meeteren, 2006). Therefore, it has been proposed that a long-term low ABA concentration in well-watered plants during growth at high RH could be a reason

for less functioning of stomata in response to desiccation (Rezaei Nejad and van Meeteren, 2006). The adaptation of stomata to a high RH occurred within four days after transferring of moderate RH grown plants to a high RH (Fig. 4). Transferring the plants back to a moderate RH after four days at a high RH resulted in increased ABA level of the leaves (Fig. 6B), possibly due to increased transpiration in moderate RH. However, this increase in ABA level could not recover the stomatal behaviour, as the adaptation occurred during exposure to high RH seems to be irreversible.

To determine whether the lack of stomatal closure of excised adapted leaves in response to desiccation is due to the deficiency of ABA, leaves were fed with ABA solution and the stomatal responses monitored using chlorophyll fluorescence system under a non-photorespiratory condition. After feeding the leaves with ABA, the adapted leaves showed slower and less closure of stomata than control leaves (Figs 7 and 8). This indicates that the ABA-related closure of stomata has been impaired after a few days exposure of moderate RH grown 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) and *in vitro* propagated plants (Santamaria *et al.*, 1993; Wardle and Short, 1983; Ziv *et al.*, 1987) and *Tradescantia virginiana* plants grown at a high RH (Rezaei Nejad and van Meeteren, 2005; Rezaei Nejad and van Meeteren, 2006).

In conclusion, the results of this research demonstrate that the stomata developed at a moderate RH adapt their behaviour to a high RH within four days and this adaptation is not reversible. The stomatal aperture in adapted plants is less sensitive to decreases in RWC and leaf water potential. The adapted plants show slower and less closure of stomata in response to short-term application of ABA than in control plants. Within one day after transferring moderate RH grown plants to a high RH, the ABA concentration of their leaves decreases to the level of ABA in high RH grown leaves. The decrease in leaf ABA concentration in transferred plants occurs at least three days prior to the occurrence of altered stomatal behaviour. It seems that ABA level should stay above a certain level to prevent the stomatal dysfunction. The altered stomatal closure in high RH grown plants or adapted plants could be due to changes in the signalling pathway for ABA-related closure of stomata. If this signalling pathway is impaired, an increased ABA level

of the leaves in adapted plants (after transferring them back to moderate RH) would not result in stomatal closure in response to desiccation.

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References

Aguilar ML, Espadas FL, Coello J, Maust BE, Trejo C, Robert ML, Santamaria JM. 2000. The role of abscisic acid in controlling leaf water loss, survival and growth of micropropagated *Tagetes erecta* plants when transferred directly to the field. *Journal of Experimental Botany* **51**, 1861-6.

Asch F. 2000. Determination of abscisic acid by indirect Enzyme Linked Immuno Sorbent Assay (ELISA). Technical report. Taastrup, Denmark: Laboratory for Agrohydrology and Bioclimatology, Department of Agricultural Sciences, The Royal Veterinary and Agricultural University.

Bahrun A, Jensen CR, Asch F, Mogensen VO. 2002. Drought-induced changes in xylem pH, ionic composition, and ABA concentration act as early signals in field-grown maize (*Zea mays* L.). *Journal of Experimental Botany* **53**, 251-63.

Bauerle WL, Whitlow TH, Setter TL, Vermeylen FM. 2004. Abscisic acid synthesis in *Acer rubrum* L. leaves- A vapor pressure deficit mediated response. *Journal of the American Society for Horticultural Science* **129**, 182-7.

Brainerd KE, Fuchigami LH. 1981. Acclimatization of aseptically cultured plants to low relative humidity. *Jornal of the American Society for Horticultural Science* **106**, 515-8.

Capellades R, Fontanau C, Carulla C, Debergh P. 1990. Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa moltiflora*. *Journal of the American Society for Horticultural Science* **115**, 141-5.

Cutler JM, Rains DW, Loomis RS. 1977. The importance of cell size in the water relations of plants. *Physiologia Plantarum* **40**, 255-60.

Fordham MC, Harrison-Murray RS, Knight L, Clay CM. 2001a. Decline in stomatal response to leaf water deficit in *Crylus maxima* cuttings. *Tree Physiology* **21**, 489-96.

Fordham MC, Harrison-Murray RS, Knight L, Evered CE. 2001b. Effects of leaf wetting and high humidity on stomatal function in leafy cuttings and intact plants of *Corylus maxima*. *Physiologia Plantarum* **113**, 233-40.

Franks PJ, Farquhar GD. 2001. The effect of exogenous abscisic acid on stomatal development, stomatal mechanics and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology* **125**, 935-42.

Koroch AR, Juliani HRJr, Juliani HR, Trippi VS. 1997. Micropropagation and acclimatization of *Hedeoma multiflorum*. *Plant Cell, Tissue and Organ Culture* **48**, 213-7.

Marin JA, Gella R, Herrero M. 1988. Stomatal structure and functioning as a response to environmental changes in acclimtized micropropagated *Prunus cerasifera* L *Annals of Botany* **62**, 663-70.

Pospisilova J. 1996. Hardening by abscisic acid of tobacco plantlets grown *in vitro*. *Biologia Plantarum* **38**, 605-9.

Pospisilova J, Wilhelmova N, Synkova H, Catsky J, Krebs D, Ticha I, Hanackova B, Snopek J. 1998. Acclimation of tobacco plantlets to *ex vitro* conditions as affected by application of abscisic acid. *Journal of Experimental Botany* **49**, 863-9.

Quarrie SA, Whitford PN, Appleford NEJ, Wang TL, Cook SK, Henson IE, Loveys BR. 1988. A monoclonal antibody to (S)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. *Planta* **173**, 330-9.

Raschke K. 1975. Stomatal action. *Annual Review of Plant Physiology* **26**, 309-40.

Rezaei Nejad A, Harbinson J, van Meeteren U. 2006. Dynamics of spatial heterogeneity of stomatal closure in *Tradescantia virginiana* altered by growth at high relative air humidity *Journal of Experimental Botany* **57**, 3669-3678.

Rezaei Nejad A, van Meeteren U. 2005. Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. *Physiologia Plantarum* **125**, 324-32.

Rezaei Nejad A, van Meeteren U. 2006. The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity. *Journal of Experimental Botany,* **In press**.

Sallanon H, Tort M, Courdret A. 1993. The ultrastructure of micropropagated and greenhouse rose plant stomata. *Plant Cell, Tissue and Organ Culture* **32**, 227-33.

Santamaria JM, Davies WJ, Atkinson CJ. 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. *Journal of Experimental Botany* **44**, 99-107.

Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D. 2001. Guard cell signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 627-58.

Talavera CR, Espadas FL, Aguilar ML, Maust BE, Oropeza CM, Santamaria JM. 2001. The control of leaf water loss by coconut plants cultured *in vitro* depends on the type of membrane used for ventilation *Journal of Horticultural Science and Biotechnology* **76**, 569-74.

Torre S, Fjeld T. 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. *Scientia Horticulturae* **89**, 217-26.

Torre S, Fjeld T, Gislerød HR, Moe R. 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. *Journal of the American Society for Horticultural Science* **128**, 598-602.

Wardle K, Dobbs EB, Short KC. 1983. *In vitro* acclimatization of aseptically cultured plantlets to humidity. *Journal of the American Society for Horticultural Science* **108**, 386-9.

Wardle K, Short KC. 1983. Stomatal response of *in vitro* cultured plantlets. I. Responses in epidermal strips of Chrysanthemum to environmental factors and growth regulators. *Biochemie und Physiologie der Pflanzen* **178**, 619-24.

Zeiger E. 1983. The biology of stomatal guard cells. *Annual Review of Plant Physiology* **34**, 441-74.

Ziv M, Schwartz A, Fleminger D. 1987. Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; implication for hardening. *Plant Science* **52**, 127-34.

Chapter 6

General discussion

Plants grown at high relative air humidity (RH) conditions lose much more water than moderate RH grown plants after transferring them to a low RH. The transpiration rate in high RH grown plants remains higher than in moderate RH grown plants, even in response to desiccation (Torre and Fjeld, 2001). This phenomenon has been attributed to the effects of RH during plant growth on stomatal functioning (Fordham *et al.*, 2001; Torre *et al.*, 2003; Ziv *et al.*, 1987), although this connection has not been fully quantified or tested. Thus, one of the aims of this thesis was to elucidate the differences in stomatal anatomy and response characteristics between well-watered plants grown at a moderate (55%) or a high (90%) RH in response to treatments that normally induce stomatal closure [i.e. desiccation, abscisic acid (ABA) application and exposure to darkness]. The results of this research showed that besides the differences in stomatal size and density, overall stomatal conductance and the transpiration rate per leaf unit area in non-desiccated high RH grown leaves was higher than in moderate RH grown leaves (chapter 2). This could be the result of the higher stomatal aperture found in the high RH grown plants (chapter 2 and 4). A greater average stomatal aperture has also been observed in micro-propagated plants (Santamaria *et al.*, 1993; Wardle *et al.*, 1983; Ziv *et al.*, 1987) and in roses grown at a high RH (Torre and Fjeld, 2001; Torre *et al.*, 2003). Therefore, an increased stomatal aperture seems to be a common feature of plants grown at a high RH. More interesting is that whether grown at a moderate or a high RH, the stomata in the leaves of *Tradescantia virginiana* decreased their aperture in response to desiccation, ABA application and darkness-exposure (chapter 2). However, transpiration rate, and stomatal conductance and aperture in the high RH grown plants remained higher than in the moderate RH grown plants (chapter 2), indicating a quantitative effect of RH during growth on stomatal functioning. The use of a chlorophyll fluorescence imaging system allowed visualization of the heterogeneity of stomatal closure over the leaf surface (Chapter 3), and showed that the homogeneity, speed and degree of stomatal closure in response to desiccation or ABA application were less in high RH grown plants than in moderate RH grown plants (Chapter 3 and 4). Some of the stomata that developed at a high RH closed only partially, or not at all (Chapter 2). While these non-closing stomata were mainly distributed around the main vein in response to desiccation (Chapter 3), they were located in the leaf margin in response to short-term ABA application (Chapter 4). Faster closure of stomata in leaf margins

compared to main-vein areas in response to desiccation had a mechanical explanation related to a substantially lower RWC in margin areas (Chapter 3), most probably resulting in a low turgor pressure in these areas of the leaves, and was not because of a fast response to water loss signalling. In fact, stomata at the leaf margins neither responded to water stress signals nor ABA and nitric oxide, the latter being a component of the ABA signalling pathway that leads to closure of stomata (Garcia-Mata *et al.*, 2003; Neill *et al.*, 2002). However, the lack of stomatal closure in main-vein areas in response to desiccation (Chapter 3) was likely due to low ABA concentrations in high RH grown leaves (Chapter 4).

The results of this study provide new insights into the field of leaf heterogeneity. Though we observed heterogeneity of stomatal closure in high RH grown *Tradescantia* plants, which are monocotyledons, the pattern was different to that observed in the leaves of dicotyledons i.e. *Rosa* (Meyer and Genty, 1998; Meyer and Genty, 1999) and *Xanthium* (Beyschlag and Eckstien, 2001; Mott *et al.*, 1993). Nonetheless, it is clear that a heterogeneous stomatal response could be induced in monocotyledonous homobaric leaves, resulting in an uneven distribution of Φ_{PSII} (Chapter 3). In addition, the differences in stomatal aperture and Φ_{PSII} that developed between the leaf margins and main-vein areas in the high RH grown leaves during desiccation implies that gradients of RWC, and thus water potential, could be established and maintained over relatively small lateral distances of the leaf (Chapter 3). This also indicates that lateral diffusion of CO_2 through the mesophyll air spaces was not efficient over these distances (Morison *et al.*, 2005).

It is noteworthy that the poor control of water loss in plants grown at high RH conditions has also been related to poor cuticular development (in particular in micro-propagated plantlets) resulting in a higher cuticular transpiration rate (Pospisilova *et al.*, 1998; Sutter and Langhans, 1979; Ziv *et al.*, 1983). Though the effects of high RH on leaf cuticlar properties has not been investigated in the present thesis, substantial changes in cuticlar transpiration rate during only a few days exposure of moderate RH grown plants to high RH (chapter 5) would not be expected. The stomatal density would also not have been changed during adaptation of the leaves. Thus, it can be concluded that the main reason for higher water loss in moderate RH grown leaves exposed to a high RH is stomatal dysfunction. Moreover, higher stomatal aperture found in high RH grown plants during exposure to desiccation, ABA application and light/dark transition (chapter 2, 3 and 4), shows the importance

of stomatal dysfunction resulting in a higher water loss in high RH grown leaves, although the differences in cuticular transpiration rate cannot be excluded. Cuticular transpiration rate can easily be measured after application of stomatal closing treatments. However, none of the applied treatments (desiccation, ABA application, exposure to darkness) resulted in total closure of all stomata in high RH grown plants (Chapter 2). Moreover, Torre *et al* (2003) reported no difference between cuticular structure, shape and size in leaves of roses grown at a moderate and a high RH.

Compared to the stomata of moderate RH grown leaves, the larger stomata found in high RH grown leaves (chapter 2 and 3) have a larger aperture area due to their longer guard cells even when both have an identical inner width of stomatal aperture. Therefore, stomatal size could affect leaf transpiration rate and stomatal conductance. However, this does not explain why stomata of high RH grown leaves are less hydrosensitive. Moreover, the fast adaptation of stomatal behaviour in mature leaves developed at a moderate RH (with smaller stomata) to a high RH (chapter 5) showed that the larger guard cell is not required for the occurrence of stomatal dysfunction under high RH.

Growing leaves from moderate RH grown plants under high RH conditions resulted in their stomata having a diminished response to drought stress (Chapter 4). The stomatal responses to desiccation in such leaves were similar to the stomatal responses of the plants where whole plants were grown in a high RH climate room (chapter 3). The difference between stomatal behaviour of an individual leaf grown at a high RH and the other leaves of the same plant grown at a moderate RH shows the importance of the micro-climate around individual leaves. The fast adaptation of moderate RH grown leaves to high RH and the irreversibility of this adaptation (Chapter 5) emphasize the importance of greenhouse management to prevent negative effects of high RH microclimates around the leaves when the average RH in the greenhouse may not be very high.

Stomatal responses to desiccation became progressively slower and diminished with increasing leaf age in high RH grown plants (Chapter 4). In addition, there was a gradient of stomatal responses to desiccation from the tip to the base of the youngest leaf. The younger parts of the youngest leaf showed a faster closure of stomata in response to desiccation compared to the older parts (Chapter 4). This indicates that newly developed stomata are functional and need to be exposed to a high RH for some time to become non-functional.

The study of the relationships between Φ_{PSII} under a non-photorespiratory condition [which is closely related to stomatal closure (Chapter 3)] and RWC and water potential showed that growth at a high RH modifies the response of stomatal aperture such that it becomes less sensitive to a decrease in leaf RWC and water potential (Chapter 3). Interestingly, this phenomenon occurred even in mature and fully expanded moderate RH grown leaves when transferred to a high RH for a few days (chapter 5). It is not clear to what extent a few days exposure of moderate RH grown leaves to a high RH can change stomatal structure. However, stomatal responses to short-term ABA application showed that although stomata of adapted plants reacted very slowly and to a lesser extent, most of them were eventually able to close (chapter 5). Stomata of main-vein areas of high RH grown leaves [which did not close in response to desiccation (chapter 3)] closed in response to short-term ABA application (chapter 4). Stomata in the margins of high RH grown leaves [which did not respond to ABA application (chapter 4)] were also able to close in response to a prolonged desiccation, although the reason for the closure of stomata was plasmolysis due to a very low RWC (chapter 3). Taken all together, it seems that in high RH grown or adapted plants the reason for the altered stomatal closure in response to treatments that normally induce closure of stomata could not be due to the rigidity of cell wall. This conclusion is in contrast with the report of Ziv *et al.* (1987) which showed that stomata of *in vitro* cultured carnations did not close even when the turgor was reduced to zero by plasmolysis. They concluded that the cause for the failure of stomata to close in response to ABA, darkness and Ca^{2+} lies mainly in the guard cell wall structure. One explanation for this discrepancy could be growth conditions in *in vitro* cultured plants which might have an effect on guard cell wall elasticity.

ABA is a key component of the signal-transduction pathway for stomatal closure (reviewed by Leung and Giraudat, 1998). As the transpiration rate of plants growing at a high RH (a low VPD) is low, it was hypothesized that there is a low concentration of ABA in the leaves of these plants. Based on the observation that high ABA concentrations during growth can change the stomatal anatomy and increase their responsivity to drought stress signals (as discussed in chapter 4), it was hypothesized that when plants are subjected to low ABA concentrations during growth, the effect would be a lessening of stomatal responsivity to lowered hydration state. Therefore one of the aims of this study was to investigate the role of

ABA in producing the altered stomatal closure in response to desiccation in high RH grown plants. A lower ABA concentration was found in leaves grown at a high RH compared to leaves grown at a moderate RH (chapter 4). As a result of a daily application of 20 μM ABA to leaves for three weeks during growth, the stomata of ABA-treated leaves grown at a high RH showed the same closing behaviour as did the stomata of leaves grown at a moderate RH i.e. they closed rapidly when exposed to desiccation (chapter 4). Within one day after transferring of moderate RH grown plants to a high RH, ABA concentrations of their leaves decreased to the level of ABA in high RH grown leaves (chapter 5). This decrease in leaf ABA concentration in transferred plants occurred at least three days prior to the occurrence of altered stomatal behavior (Chapter 5). Thus, a conclusion from the results of this study is that a long-term low ABA concentration during growth or exposure to a high RH would be a reason for the altered stomatal functioning in response to desiccation. This conclusion is in line with the reports of some authors showing that conditions that increase endogenous ABA levels of *in vitro* cultured plants, 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 a low RH (Aguilar *et al.*, 2000; Pospisilova, 1996; Pospisilova *et al.*, 1998; Talavera *et al.*, 2001). In young parts of leaves, stomata reacted fast in response to desiccation even in high RH grown plants. When the leaves became older at the same RH condition, the closing behaviour in response to desiccation was disturbed (Chapter 4). It seems that the ABA level should stay above a certain level to prevent the occurrence of stomatal dysfunction. The altered stomatal closure in high RH grown or adapted plants could be due to changes in the signalling pathway for ABA-related closure of stomata. If this signalling pathway is impaired, an increased ABA level of the leaves in adapted plants after transferring them back to moderate RH (Chapter 5) would not result in stomatal closure in response to desiccation. In other words, besides the fact that ABA is a key component of the signal transduction pathway for stomatal closure, it seems that the ABA level should remain above a certain level to prevent impairment of this signalling pathway.

The finding that the ABA level was low at least three days prior to the occurrence of altered stomatal behaviour (Chapter 5) suggests that practices could be developed to prevent the irreversible adaptation of stomatal behaviour to high RH in commercial greenhouses for plant production. For example, the RH could be

periodically reduced for a few hours (e.g. a few hours every three days). Mortensen and Gislerørd (2005) have shown that a daily period of 6 h with moderate RH (75%) during growth was enough to prevent the negative effects of high RH during plant growth on the vase life of cut roses.

To investigate the stomatal responses to water stress, leaves were detached from the plants. This method might have had some effects on stomatal functioning in response to water stress. For example, the lack of root-sourced signalling (e.g. ABA) might have limited the closure of stomata. In order to overcome this problem, plants were grown in a nutrient solution so that they could easily be water stressed without cutting the leaves. Surprisingly, stomata of plants grown in nutrient solution did not behave the same as those of soil culture in response to desiccation (unpublished results). Stomata of excised leaves from plants grown at a high RH in nutrient solution showed a fast response to water stress, and their transpiration rate per unit leaf area was lower than in high RH grown plants in soil. In a split-root system with nutrient solution and soil it was shown that some signals from the nutrient solution roots during growth influenced stomatal responses.

It is well documented that the guard cell cytoskeleton (actin filaments) is involved in stomatal movement and it can modify the ability of guard cells to respond to environmental and hormonal stimuli (reviewed by Dzierzynska, 2006; Galatis and Apostolakos, 2004). Actin filaments in guard cells are assembled in a radial pattern when stomata are induced to open under light, but the actin filaments are disassembled when stomata are closed under darkness or by ABA (Eun and Lee, 1997; Hwang and Lee, 2001; Kim *et al.*, 1995). In particular, the rapid disruption of the radial actin filaments arrays in stomata after ABA treatment implies their involvement in the signal transduction pathway of ABA-related closure of stomata (Eun and Lee, 1997; Hwang and Lee, 2001; Lemichez *et al.*, 2001). We have proposed that disturbed stomatal closure in high RH grown plants in response to treatments that normally induce stomatal closure (this thesis) could be due to changes in the signalling pathway for ABA-related closure of stomata. The stomata of high RH grown plants fail to close in response to ABA and nitric oxide which is also involved in the signal-transduction pathway linking the perception of ABA to reduced guard cell turgor (Chapter 4). Further research is needed to examine whether growth at high RH affects guard cell cytoskeleton as a part of this transduction pathway. Moreover, altered stomatal closure in high RH grown leaves could also be due to

changes in the relation between guard cell hydrostatic pressure and stomatal aperture. It has been shown that long-term application of ABA altered the physical properties of stomata in *Tradescantia virginiana*, and for any given guard cell pressure, stomatal pore width in the ABA-treated plants was less than half of that in control plants (Franks and Farquhar, 2001). The effects of long-term low ABA concentrations, to the kind found in high RH grown leaves (Chapter 4), on the physical properties of stomata and on the relation between guard cells hydrostatic pressure and stomatal aperture is unknown. In addition, the stomatal aperture is also determined by the balance between forces generated within the guard cells and those in neighbouring epidermal cells (Franks *et al.*, 1998). Thus, the higher stomatal aperture in high RH grown plants in response to desiccation (Chapter 2) could also be due to changes in the relationship between guard cell turgor and epidermal turgor. Unfortunately, due to technical problems, we were not able to measure the physical properties of the stomata and to measure turgor pressure in the guard cells and epidermal cells. Therefore, further research could be directed to the effect of high RH on: 1) physical properties of stomata and the relation between guard cell hydrostatic pressure and stomatal aperture 2) the relationship between guard cell turgor and epidermal turgor.

In conclusion, our findings provide insights into the physiological effects of high RH during plant growth on stomatal functioning and they are another step towards preventing problems caused by high RH conditions during plant growth on the water balance and ultimately the quality of plants.

References

Aguilar ML, Espadas FL, Coello J, Maust BE, Trejo C, Robert ML, Santamaria JM. 2000. The role of abscisic acid in controlling leaf water loss, survival and growth of micropropagated *Tagetes erecta* plants when transferred directly to the field. *Journal of Experimental Botany* **51**, 1861-6.

Beyschlag W, Eckstien J. 2001. Towards a causal analysis of stomatal patchiness: the role of stomatal size variability and hydrological heterogeneity. *Acta Oecologica* **22**, 161-73.

Dzierzynska A. 2006. The role of cytoskeleton in stomata functioning. *Acta Physiologiae plantarum* **28**, 59-79.

Eun SO, Lee Y. 1997. Actin filaments of guard cells are reorganized in response to light and abscisic acid. *Plant Physiology* **115**, 1491-8.

Fordham MC, Harrison-Murray RS, Knight L, Evered CE. 2001. Effects of leaf wetting and high humidity on stomatal function in leafy cuttings and intact plants of *Corylus maxima*. *Physiologia Plantarum* **113**, 233-40.

Franks PJ, Cowan IR, Farquhar GD. 1998. A study of stomatal mechanics using the cell pressure probe. *Plant, Cell and Environment* **21**, 94-100.

Franks PJ, Farquhar GD. 2001. The effect of exogenous abscisic acid on stomatal development, stomatal mechanics and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology* **125**, 935-42.

Galatis B, Apostolakos P. 2004. The role of the cytoskeleton in the morphogenesis and function of stomatal complexes. *New Phytologist* **161**, 613-39.

Garcia-Mata C, Gay R, Sokolovski S, Hills A, Lamattina L, Blatt MR. 2003. Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signalling pathways. *Proceedings of the National Academy of Sciences of the USA* **100**, 11116-21.

Hwang J-U, Lee Y. 2001. Abscisic acid-induced actin reorganization in guard cells of dayflower is mediated by cytosolic calcium levels and by protein kinase and protein phosphatase activities. *Plant Physiology* **125**, 2120-8.

Kim M, Hepler PK, Eun SO, Ha KS, Lee Y. 1995. Actin filaments in mature guard cells are radially distributed and involved in stomatal movement. *Plant Physiology* **109**, 1077-84.

Lemichez E, Wu Y, Sanchez J-P, Mettouchi A, Mathur J, Chua N-H. 2001. Inactivation of AtRac1 by abscisic acid is essential for stomatal closure. *Genes and Development* **15**, 1808-16.

Leung J, Giraudat J. 1998. Abscisic acid signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 199-222.

Meyer S, Genty B. 1998. Mapping intercellular CO_2 mole fraction (C_i) on *Rosa rubiginosa* leaves fed with abscisic acid by using chlorophyll fluorescence imaging. *Plant Physiology* **116**, 947-57.

Meyer S, Genty B. 1999. Heterogeneous inhibition of photosynthesis over the leaf surface of *Rosa rubiginosa* L. during water stress and abscisic acid treatment: induction of a metabolic component by limitation of CO_2 diffusion. *Planta* **210**, 126-31.

Morison JIL, Gallouet E, Lawson T, Cornic G, Herbin R, Baker NR. 2005. Lateral diffusion of CO_2 in leaves is not sufficient to support photosynthesis. *Plant Physiology* **139**, 254-66.

Mortensen LM, Gislerød HR. 2005. Effect of air humidity variation on powdery mildew and keeping quality of cut roses. *Scientia Horticulturae* **104**, 49-55.

Mott KA, Cardon ZG, Berry JA. 1993. Asymmetric patchy stomatal closure for the two surfaces of *Xanthium strumarium* L. leaves at low humidity. *Plant, Cell and Environment* **16**, 25-34.

Neill SJ, Desikan R, Clarke A, Hancock JT. 2002. Nitric oxide is a novel component of abscisic acid signalling in stomatal guard cells. . *Plant Physiology* **128**, 13-6.

Pospisilova J. 1996. Hardening by abscisic acid of tobacco plantlets grown *in vitro*. *Biologia Plantarum* **38**, 605-9.

Pospisilova J, Wilhelmova N, Synkova H, Catsky J, Krebs D, Ticha I, Hanackova B, Snopek J. 1998. Acclimation of tobacco plantlets to *ex vitro* conditions as affected by application of abscisic acid. *Journal of Experimental Botany* **49**, 863-9.

Santamaria JM, Davies WJ, Atkinson CJ. 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. *Journal of Experimental Botany* **44**, 99-107.

Sutter E, Langhans RW. 1979. Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. *Journal of American Society for Horticultural Science* **104**, 493-6.

Talavera CR, Espadas FL, Aguilar ML, Maust BE, Oropeza CM, Santamaria JM. 2001. The control of leaf water loss by coconut plants cultured *in vitro* depends on the type of membrane used for ventilation *Journal of Horticultural Science and Biotechnology* **76**, 569-74.

Torre S, Fjeld T. 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. *Scientia Horticulturae* **89**, 217-26.

Torre S, Fjeld T, Gislerød HR, Moe R. 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. *Journal of the American Society for Horticultural Science* **128**, 598-602.

Wardle K, Dobbs EB, Short KC. 1983. *In vitro* acclimatization of aseptically cultured plantlets to humidity. *Journal of the American Society for Horticultural Science* **108**, 386-9.

Ziv M, Meir G, Halevy AH. 1983. Factors influencing the production of hardened glaucous carnation plantlets *in vitro*. *Plant Cell, Tissue and Organ Culture* **2**, 55-65.

Ziv M, Schwartz A, Fleminger D. 1987. Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; implication for hardening. *Plant Science* **52**, 127-34.

Appendixes

Summary

The primary role of stomata is to allow sufficient uptake of CO_2 by the leaves for photosynthesis while preventing excessive water loss via transpiration. To do this, stomata open or close in response to a wide range of environmental factors and hormonal stimuli. As a result of contemporary horticultural practice the normal functioning of stomata is sometimes disturbed. This happens especially when plants are grown at high relative air humidities (RH) and transferred to low RH conditions. Disturbance of stomatal movements results in a negative water balance in plants and quality problems due to excessive water loss after harvest. As a result of energy saving practices (less ventilation, totally closed greenhouses) it may be expected that very high RH will become more and more common in commercial greenhouses. At the end of the production chain (at the consumer) the RH will be low. Thus, more quality problems can be foreseen in the near future. Understanding the physiology of stomatal behavior in response to long-term growth at high RH is therefore important. The general aim of this study is to investigate the effects of high RH during growth on the stomatal response characteristics of *Tradescantia virginiana* L.

In **Chapter 2**, the stomatal anatomy and responses of moderate (55%) and high (90%) RH grown *Tradescantia virginiana* plants to treatments that normally induce stomatal closure i.e. desiccation, abscisic acid (ABA) application and exposure to darkness was studied. Bigger stomata and lower stomatal density were found in high RH grown leaves compared to moderate RH grown leaves. However, the stomatal indices were the same in both plant types. Whether grown at moderate (55%) or high (90%) RH, the stomata in the leaves of *Tradescantia virginiana* decreased their aperture in response to desiccation, ABA application and darkness-exposure. However, transpiration rate and stomatal conductance and aperture in the high RH grown plants remained higher than in the moderate RH grown plants, indicating a quantitative effect of RH during growth on stomatal functioning. This difference was because some of the stomata that developed in a high RH closed only partially, or not at all.

In **Chapter 3**, the spatial heterogeneity of stomatal closure in response to rapid desiccation of excised well-watered *T. virginiana* leaves grown at moderate (55%) or high (90%) relative air humidity (RH) was studied using a chlorophyll

fluorescence imaging system under non-photorespiratory conditions. Following rapid desiccation, excised leaves grown at high RH had both a greater heterogeneity and a higher average value of PSII efficiency (Φ_{PSII}) compared to leaves grown at moderate RH. Larger decreases in relative water content (RWC) resulted in smaller decreases in water potential and Φ_{PSII} of high RH grown leaves compared to moderate RH grown leaves. Moreover, the Φ_{PSII} of excised high RH grown leaves decreased less with decreasing water potential implying that the stomata of high RH grown leaves are less sensitive to decreases in leaf water potential compared to moderate RH grown leaves. After desiccation, some non-closing stomata were distributed around the main vein in high RH grown leaves. Direct measurements of stomatal aperture showed 77% reduction in average stomatal aperture in the margins after 2 h desiccation compared to 40% closure of stomata in the main-vein areas in high RH grown leaves. Faster closure of stomata in leaf margins compared to main-vein areas of leaves grown at high RH was related to a substantially lower RWC in these areas of the leaves.

In **Chapter 4**, the role of abscisic acid (ABA) in the altered stomatal responses of *T.virginiana* leaves grown at high RH was investigated. A lower ABA concentration was found in leaves grown at high RH compared to leaves grown at moderate RH. As a result of a daily application of 20 μM ABA to leaves for three 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 e.g. they closed rapidly when exposed to desiccation. Providing 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 high RH grown leaf did not close completely in response to desiccation in contrast to the stomata of the other leaves from the same plant. While there was no significant difference in ABA concentration on a dry weight basis between this leaf and other leaves, the ABA concentration on a fresh weight basis of this leaf was significantly lower than the others. Moreover, less closure of stomata was found in the older leaves of high RH grown plants in response to desiccation compared to younger leaves. This was correlated with a lower ABA concentration in these leaves on a fresh weight basis. However, there was no significant difference in ABA concentration on a dry weight basis between the younger and older leaves. Furthermore, all 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 high RH grown leaves remained significantly wider than in the main-vein areas. It was concluded that: (1) a long-term low ABA concentration in well-watered plants during growth at high RH could be a reason for altered functioning of stomata in response to desiccation; (2) the long-term ABA concentration on a fresh weight basis rather than on a dry weight basis could be responsible for structural or physiological changes in stomata during leaf growth; and (3) stomata at the leaf margins of high RH grown leaves are physiologically 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.

In **Chapter 5**, the rate and the reversibility of adaptation of stomatal closing behaviour in *T. virginiana* leaves to a moderate (55%) or a high (90%) relative air humidity (RH) were investigated. Stomatal closure in response to desiccation of the distal parts of leaves which were grown firstly at a moderate RH followed by 10 days at a high RH was the same as that of the bases of the leaves grown at the 10-day period of high RH. However, stomatal closure of the distal parts of leaves which were grown firstly at a high RH followed by 10 days at a moderate RH was less than in the bases of the leaves grown at the moderate RH. Four days after transferring fully expanded leaves to a high RH, stomata of leaves grown at a moderate RH showed the same behaviour as did stomata of high RH grown leaves in response to desiccation: they did not close fully. Transferring the plants back to a moderate RH did not result in the recovery of stomatal closure. Exposure of moderate RH grown plants to a high RH for five days modified the response of stomatal aperture such that it became less sensitive to decrease in relative water content (RWC) and leaf water potential. However, the relationship between leaf water potential and RWC was not affected by the exposure to a high RH. The leaves grown and fully expanded at a moderate RH and exposed to a high RH for five days showed slower and less closure of stomata in response to short-term application of ABA than in control plants (not exposed to high RH). Within one day after transferring moderate RH grown plants to a high RH, the ABA concentration of their leaves decreased to the level of ABA in high RH grown leaves. The decrease in leaf ABA concentration in the transferred plants occurred at least three days prior

to the occurrence of altered stomatal closure. It seems that the ABA level should stay above a certain level to prevent the occurrence of stomatal dysfunction. The altered stomatal closure in high RH grown or adapted plants could be due to changes in the signalling pathway for ABA-related closure of stomata. If this signalling pathway was impaired, an increased ABA level of the leaves in adapted plants (after transferring them back to moderate RH) would not result in stomatal closure in response to desiccation.

The experiments and results described in this thesis provide insights into the physiological effects of high RH during growth on stomatal functioning. The main achievements and practical implications of this study are discussed in **Chapter 6**, and suggestions for further research are presented.

Samenvatting

De belangrijkste rol van stomata is om voldoende opname van CO_2 door de bladeren mogelijk te maken voor fotosynthese en tegelijkertijd overmatig verlies van water door verdamping te voorkomen. Om dit te realiseren reageren stomata op een groot aantal omgevingsfactoren en hormonale prikkels met openen of sluiten. Als gevolg van ontwikkelingen in de hedendaagse tuinbouwpraktijk is het functioneren van stomata soms verstoord. Dit gebeurd vooral als planten zijn geteeld bij hoge relatieve luchtvochtigheden (RV) en daarna overgebracht worden naar omstandigheden met lage RV condities. Als gevolg van bovenmatig waterverlies leidt de verstoring van de sluiting van de stomata tot een negatieve waterbalans in de plant en kwaliteitsproblemen na de oogst. In commerciële kassen zullen door het toepassen van energiebesparende maatregelen (minder ventilatie, geheel gesloten kassen) zeer hoge luchtvochtigheden steeds vaker voorkomen. Aan het einde van de productieketen (bij de consument) zal de RV laag zijn. Er kan dan ook verwacht worden dat er zich in de toekomst meer kwaliteitsproblemen zullen voordoen. Het is daarom van belang om te begrijpen hoe de fysiologie van de sluitcellen van stomata wordt beïnvloed door groei bij voortdurend hoge RV. De algemene doelstelling van deze studie is om de gevolgen te onderzoeken van hoge RV tijdens de teelt op de sluitings karakteristieken van stomata van *Tradescantia virginiana* L.

In **Hoofdstuk 2** wordt de anatomie beschreven van stomata afkomstig van *Tradescantia virginiana* planten die geteeld waren bij een gematigde (55%) en een hoge (90%) RV, alsmede hun reacties op behandelingen die normaliter sluiting van de stomata induceren, zoals uitdroging, toediening van abscissinezuur (ABA) en blootstelling aan donker. In bladeren gegroeid bij hoge RV werden grotere stomata en lagere stomata-dichtheden vastgesteld dan in bladeren gegroeid bij gematigde RV. De stomata index was echter gelijk voor beide planttypen. Zowel in bladeren gegroeid bij gematigde (55%) als bij hoge (90%) RV verkleinden de stomata hun opening in reactie op uitdroging, toediening van ABA of blootstelling aan donker. Echter, de verdampingssnelheid, en de geleidbaarheid en opening van stomata van planten gegroeid bij hoge RV bleef hoger dan van planten gegroeid bij gematigde RV. Hieruit blijkt dat de RV tijdens de groei van planten een kwantitatief effect heeft op het sluiten van stomata. Het verschil werd veroorzaakt doordat sommige

stomata die ontwikkeld waren bij hoge RV slechts gedeeltelijk of helemaal niet sloten.

Hoofdstuk 3 beschrijft de ruimtelijke heterogeniteit van de sluiting van stomata als reactie op snelle uitdroging van afgesneden *T. virginiana* bladeren die afkomstig waren van planten die zijn opgegroeid bij een gematigde (55%) of hoge (90%) relatieve luchtvochtigheid (RV). Hiervoor werd gebruik gemaakt van een chlorofyl fluorescentie imaging systeem waarbij gemeten werd onder condities waaronder er geen fotorespiratie plaats vindt in de bladeren. Na snelle uitdroging vertoonden afgesneden bladeren gegroeid bij hoge RV zowel een grotere heterogeniteit in PSII efficiëntie (Φ_{PSII}) als ook een hoger gemiddelde waarde van Φ_{PSII} in vergelijking met bladeren gegroeid bij gematigde RV. Grotere dalingen van relatief water gehalte (RWC) resulteerden in kleinere dalingen van de water potentiaal en van Φ_{PSII} in bladeren gegroeid bij hoge RV in vergelijking tot bladeren gegroeid bij gematigde RV. Bovendien daalde de Φ_{PSII} minder met dalende waterpotentiaal van afgesneden bladeren gegroeid bij hoge RV, wat impliceert dat de stomata van bladeren gegroeid bij hoge RV minder gevoelig zijn voor dalingen van waterpotentiaal dan die van bladeren gegroeid bij gematigde RV. Na uitdroging waren er een aantal niet-sluitende stomata aanwezig rondom de hoofdnerf van bladeren gegroeid bij hoge RV. Directe metingen van stomata-opening toonden aan dat na 2 uur uitdroging de gemiddelde opening met 77% was afgenomen in de buitenste marges van de bladeren terwijl dit rondom de hoofdnerf 40% was. De snellere sluiting van stomata in de buitenste marges in vergelijking met gebieden rondom de hoofdnerf van bladeren gegroeid bij hoge RV was gerelateerd aan substantieel lagere RWC in deze delen van de bladeren.

Vervolgens werd de rol van abscissinezuur (ABA) onderzocht in de veranderde reacties van stomata van *T. virginiana* bladeren gegroeid bij hoge RV (**Hoofdstuk 4**). Er werd een lagere ABA concentratie vastgesteld in bladeren tijdens de groei bij hoge RV in vergelijking met bladeren gegroeid bij gematigde RV. Een dagelijkse toediening gedurende drie weken van 20 μM ABA aan bladeren gegroeid bij hoge RV resulteerde erin dat de stomata van deze bladeren zich net zo gedroegen als die van bladeren gegroeid bij gematigde RV, d.w.z. dat zij snel sloten indien ze werden blootgesteld aan uitdroging. Het blootstellen van een enkel blad aan hoge RV bij een plant die groeide in gematigde RV veranderde het sluitingsgedrag van de stomata van dit enkele blad. De stomata van dit enkele bij

hoge RV gegroeide blad sloten slechts gedeeltelijk in reactie op uitdroging in tegenstelling tot de stomata van de overige bladeren van deze plant. Terwijl er geen verschil was in het ABA gehalte op basis van drooggewicht tussen dit specifieke blad en de andere bladeren van dezelfde plant, was het ABA gehalte op basis van versgewicht van dit blad significant lager dan het gehalte in de overige bladeren. Stomata in oudere bladeren gegroeid bij hoge RV sloten minder als reactie op uitdroging dan jongere bladeren. Dit gedrag correleerde met lagere ABA gehalten op basis van versgewicht in deze oudere bladeren. Op basis van drooggewicht was er echter geen verschil in ABA gehalte tussen jongere en oudere bladeren. Bovendien sloten alle stomata van bladeren gegroeid bij gematigde RV in reactie op een kort durende toediening van ABA of natrium nitroprusside (SNP), terwijl in bladeren gegroeid bij hoge RV er een duidelijk verschil in stomata reactie was tussen de marges van de bladeren en de gebieden rondom de hoofdnerf. De opening van stomata in reactie op een kortdurende toediening van ABA of SNP aan de marges van bladeren gegroeid bij hoge RV bleef significant groter dan die in de gebieden bij de hoofdnerf. Er werd geconcludeerd dat: (1) een langdurige lage ABA concentratie in planten gedurende hun groei bij hoge RV waarschijnlijk de oorzaak is van het veranderde functioneren van stomata in reactie op uitdroging; (2) eerder de langdurige ABA concentratie gebaseerd op het versgewicht verantwoordelijk is voor veranderingen in structurele of fysiologische eigenschappen van stomata gedurende de bladgroei dan de ABA concentratie gebaseerd op het drooggewicht; en (3) stomata in de marges van bladeren gegroeid bij hoge RV niet in staat zijn te sluiten t.g.v. de kortdurende aanwezigheid van ABA of stikstof oxide.

Tenslotte werden de snelheid en de reversibiliteit van de aanpassing van het sluitingsgedrag van stomata aan een gematigde (55%) of een hoge (90%) relatieve luchtvochtigheid (RV) onderzocht (**Hoofdstuk 5**). De sluiting van stomata als reactie op uitdroging van de distale gedeelten van bladeren die eerst gegroeid zijn bij gematigde RV en daarna gedurende 10 dagen zijn blootgesteld aan een hoge RV was dezelfde als de sluiting van stomata in de bases van dezelfde bladeren die geheel gevormd waren tijdens de 10-daagse periode bij hoge RV. De sluiting van stomata in de distale gedeelten van bladeren die eerst gegroeid zijn bij hoge RV en daarna gedurende 10 dagen zijn blootgesteld aan een gematigde RV was echter minder dan de sluiting van stomata in de bases van dezelfde bladeren die geheel gevormd waren tijdens de 10-daagse periode bij gematigde RV. Vier dagen nadat

volledig uitgegroeide bladeren waren verplaatst van gematigde naar hoge RV vertoonden de stomata het zelfde gedrag als stomata van bladeren die geheel bij hoge RV waren gegroeid: zij sloten gedeeltelijk. Terug plaatsen van de planten naar een gematigde RV leidde niet tot een herstel van de sluiting van de stomata. Een blootstelling aan hoge RV gedurende 5 dagen van planten gegroeid bij gematigde RV verminderde de reactie gevoeligheid van stomata-sluiting voor een daling van relatief water gehalte (RWC) en water potentiaal van de bladeren. De correlatie tussen blad water potentiaal en RWC werd echter niet beïnvloed door blootstelling aan een hoge RV. De bladeren volledig volgroeid bij een gematigde RV en daarna vijf dagen blootgesteld aan een hoge RV vertoonden een langzamere en minder sluiting van stomata als reactie op een kort durende toediening van ABA dan controle planten (niet blootgesteld aan hoge RV). Binnen een dag na het overbrengen naar een hoge RV van planten gegroeid bij gematigde RV daalde het ABA gehalte in de bladeren tot het niveau in bladeren gegroeid bij hoge RV. De daling in het ABA gehalte in bladeren in planten die overgebracht werden gebeurde tenminste drie dagen voordat de sluiting van stomata werd beïnvloed. Het lijkt er op dat het ABA gehalte boven een minimum niveau moet blijven om het disfunctioneren van stomata te voorkomen. Het veranderde sluitingsgedrag van stomata in planten gegroeid bij hoge RV of aangepast aan hoge RV zou het gevolg kunnen zijn van veranderingen in de signaaltransductie weg van ABA-gerelateerde sluiting van stomata. Als deze signaaltransductie weg niet goed functioneert zal een verhoogd ABA gehalte in bladeren van aan hoge RV aangepaste planten (na terugplaatsen bij gematigde RV) niet leiden tot sluiting van stomata als reactie op uitdroging.

De experimenten en resultaten beschreven in dit proefschrift geven inzicht in de fysiologische effecten op het functioneren van stomata als gevolg van hoge RV tijdens de groei. De belangrijkste uitkomsten van deze studie en gevolgen voor de tuinbouwpraktijk zijn bediscussieerd in **Hoofdstuk 6**, waarin tevens enkele suggesties voor verder onderzoek worden gegeven.

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1- Omidbaigi R and **Rezaei Nejad A.** 2000. The influence of nitrogen-fertilizer and harvest time on the productivity of *Thymus vulgaris* L. *International Journal of Horticultural Science* 6, 43-46.

2- **Rezaei Nejad A** and van Meeteren U. 2005. Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. *Physiologia Plantarum* 125, 324-32.

3- **Rezaei Nejad A,** Harbinson J, van Meeteren U**.** 2006. Dynamics of spatial heterogeneity of stomatal closure in *Tradescantia virginiana* altered by growth at high relative air humidity *Journal of Experimental Botany* 57, 3669-3678.

4- **Rezaei Nejad A,** van Meeteren U. 2006. The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity. *Journal of Experimental Botany*, In press.

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Abstracts:

1- **Rezaei Nejad A** and van Meeteren U. 2004. Stomatal behaviour in *Tradescantia virginiana* grown at high relative air humidity. Proceedings of the 14th Congress of the Federation of European Societies of Plant Biology, Cracow, Poland. *Acta Physiologia Plantarum* 26(3), Page 84.

2- **Rezaei Nejad A,** Harbinson J, van Meeteren U**.** 2005. Detection of stomatal behaviour using chlorophyll fluorescence imaging system. Proceedings of the 12th Congress of the Scandinavian Plant Physiology Society, Page 16, Umeå, Sweden.

3- **Rezaei Nejad A,** Harbinson J, van Meeteren U**.** 2005. Effects of long-term exposure of stomata to high relative air humidity. Proceedings of the Annual Meeting of the American Society of Plant Biologists, Page 66, Seattle, USA.

4- **Rezaei Nejad A,** Harbinson J, van Meeteren U**.** 2005. Stomata and long-term exposure to high relative air humidity. Proceedings of the Annual

Meeting of the American Society of Plant Biologists, Pages 131-132, Seattle, USA.

5- **Rezaei Nejad A** and van Meeteren U. 2006. Irreversible adaptation of stomata in *Tradescantia virginiana* grown at moderate relative air humidity (RH) to high RH. Proceedings of the 27th International Horticultural Congress, Page 201, Seoul, South Korea.

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With the educational activities listed below the PhD candidate
has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 22 credits (= 32 ECTS = 22 weeks of activities)

Review of Literature (4 credits)

- Literature and theoretical study on stomatal behaviour in ornamental plants in relation to growth condition and post-harvest quality (2003)
- Presentation as a seminar of the above mentioned theoretical study to HPC (2003)

Writing of Project Proposal (5 credits)

- Control of stomata opening after prolonged exposure to high air humidity (2003)

Post-Graduate Courses (2.6 credits)

- Excursion and discussion: Aalsmeer research centre (2003)
- Excursion: flower auction and several companies (2003)
- Scientific publishing (2004)
- Excursion and discussion: bulb flower research centre, Lisse (2005)
- Excursion: flower production (2005)
- The 27th IHC symposium, workshop and excursion, South Korea (2006)
- Excursion: flower production (2006)

Deficiency, Refresh, Brush-up and General Courses (9.7 credits)

- English language course (Intermediate level) (2003)
- English language course (Upper-Intermediate level) (2003)
- Techniques for writing and presenting scientific papers (2003)
- Basic statistics (2004)
- Greenhouse technology (2004)
- Principles of horticulture (2005)
- Post-harvest physiology (2005)

PhD Discussion Groups (3 credits)

- Frontier literature in plant physiology (FLOP) (2003- 2006)

PE&RC Annual Meetings, Seminars and Introduction Days (0.6 credit)

- PE & RC weekend (2004)
- Excursion (closed greenhouse and orchid production) (2006)
- PE & RC annual meeting “The Scientific Agenda: Who pulls the strings?” (2006)

International Symposia, Workshops and Conferences (4.4 credits)

- The 8th international symposium on post-harvest physiology of ornamentals, The Netherlands (2003)
- The 14th FEBS congress (poster), Poland (2004)
- BSHS mini-symposium (The Netherlands, 2004 // Belgium, 2005)
- The 12th SPPS congress (poster), Sweden (2005)
- Plant Biology (ASPB) (oral and poster), US (2005)
- One day seminar horticultural production chains, The Netherlands (2005)

Laboratory Training and Working Visits (3 credits)

- ELISA Technique & Chlorophyll fluorescence imaging system, HPC, The Netherlands (2004)

گرفتند روزنه ها در برگهای بالغشان در مقایسه با روزنه برگهای گیاهان شاهد که در معرض رطوبت بالا قرار نگرفته بودند بسیار کندتر و کمتر به هورمون آبسیزیک اسید واکنش نشان دادند. طی یک روز پس از انتقال گیاهان تولید شده در رطوبت متوسط به رطوبت بالا غلظت هورمون آبسیزیک اسید آنها کاهش یافت و مشابه غلظت هورمون آبسیزیک اسید در گیاهان تولید شده در رطوبت بالا شد. کاهش غلظت هورمون آبسیزیک اسید در این گیاهان سه روز قبل از اختلال در رفتار روزنه ها رخ داد. بنظر می رسد که غلظت هورمون آبسیزیک اسید باید از یک حد معینی بالاتر بماند تا از اختلال در رفتار روزنه ها جلوگیری شود. اختلال در رفتار روزنه ها در گیاهان تولید شده در رطوبت بالا می تواند به علت اختلال در مسیر سیگنالی بسته شدن روزنه ها توسط هورمون آبسیزیک اسید باشد. اگر این مسیر دچار اختلال شود حتی افزایش غلظت هورمون آبسیزیک اسید در این گیاهان منجر به بسته شدن روزنه ها در واکنش به استرس خشکی نمی شود.

آزمایشات انجام شده و نتایج به دست آمده از این تحقیق درک بهتری از اثرات فیزیولوژیکی رطوبت بالای هوا بر رفتار روزنه ها به دست می دهد. مهمترین نتایج به دست آمده از این تحقیق و استفاده عملی از این نتایج در **فصل ششم** این رساله بحث شده و پیشنهادهای لازم برای انجام تحقیقات بعدی ارائه گردیده است.

مسن همراه بود درحالیکه میزان این هورمون در وزن خشک برگهای پیر و جوان تفاوت معنی داری نداشت. همچنین در پاسخ به استعمال هورمون آبسیزیک اسید و سدیم نیتروپروسید[1] همه روزنه های گیاهان تولید شده در رطوبت متوسط بسته شدند در حالیکه در برگ گیاهان تولید شده در رطوبت بالا تفاوت واضحی بین رفتار روزنه ها در حاشیه برگ در مقایسه با اطراف رگبرگ اصلی دیده شد. روزنه ها در حاشیه برگ این گیاهان کمتر از روزنه های اطراف رگبرگ اصلی در واکنش به استعمال هورمون آبسیزیک اسید و سدیم نیتروپروسید بسته شدند. لازم به ذکر است که سدیم نیتروپروسید آزاد کننده نیتریک اکسید[2] در گیاه است و نیتریک اکسید یکی از عوامل موثر در مسیر سیگنالی[3] بسته شدن روزنه ها توسط هورمون آبسیزیک اسید می باشد. از این نتایج بصورت زیر نتیجه گیری شد: (۱) غلظت پایین هورمون آبسیزیک اسید در طی رشد گیاه در رطوبت بالا می تواند عامل مهمی در اختلال رفتار روزنه ها در واکنش به خشکی باشد. (۲) غلظت هورمون آبسیزیک اسید در وزن تر گیاه بهتر از وزن خشک می تواند تغییر رفتار روزنه ها را توجیه کند. (۳) روزنه ها در حاشیه برگ گیاهان تولید شده در رطوبت بالا قادر به واکنش به هورمون آبسیزیک اسید و سدیم نیتروپروسید نیستند.

در **فصل پنجم** سرعت سازگاری[4] رفتار روزنه ها به رطوبت نسبی متوسط و بالا و برگشت پذیری[5] این سازگاری مورد بررسی قرار گرفته است. میزان بسته شدن روزنه ها در واکنش به استرس خشکی در نوک برگی که ابتدا بمدت یک هفته در رطوبت متوسط رشد داده شده بود و بعد به مدت ده روز در معرض رطوبت بالا قرار گرفته بود مشابه رفتار روزنه ها در قاعده همان برگ بود که در این مدت ده روز در رطوبت بالا رشد کرده بود. اما وقتی که نوک برگ ابتدا در رطوبت بالا رشد داده شد و بعد در معرض رطوبت متوسط قرار گرفت روزنه های نوک برگ در مقایسه با قاعده برگ که در رطوبت پایین رشد کرده بود کمتر در واکنش به استرس خشکی بسته شدند. در آزمایشی دیگر چهار روز بعد از انتقال گیاهان از رطوبت متوسط به رطوبت بالا روزنه های برگهای بالغ این گیاهان به رطوبت بالا سازگاری نشان داده و رفتاری مشابه روزنه های گیاهان شاهد که فقط در رطوبت بالا رشد کرده بودند در پاسخ به استرس خشکی از خود نشان دادند، یعنی بطور کامل بسته نشدند. برگرداندن این گیاهان به رطوبت متوسط باعث بهبود در رفتار روزنه های این گیاهان نشد. در آزمایشی دیگر نشان داده شد که قراردادن گیاهان بالغ تولید شده در رطوبت متوسط بمدت پنج روز در رطوبت بالا باعث کاهش حساسیت روزنه ها به کاهش محتوای نسبی آب و پتانسیل آب برگها می شود اما رابطه بین محتوای نسبی آب و پتانسیل آب تغییر نمی کند. همچنین وقتی گیاهان بالغ تولید شده در رطوبت متوسط بمدت پنج روز در رطوبت بالا قرار

[1] Sodium nitroprusside (SNP)
[2] Nitric oxide (NO)
[3] Signaling pathway
[4] Adaptation
[5] Reversibility

نشاندهنده اثر کمی رطوبت بالا بر رشد و نمو روزنه هاست. این تفاوت به علت این بود که بعضی روزنه های تولید شده در رطوبت بالا بسته شده در حالیکه بقیه قادر به بسته شدن نبودند.

در **فصل سوم** هتروژنیتی[1] رفتار روزنه ها در پاسخ به استرس خشکی در برگ گیاهان تولید شده در رطوبت متوسط و بالا با استفاده از سیستم عکسبرداری فلورسانس کلروفیلی[2] تحت شرایط بدون تنفس نوری[3] بررسی شده است. با استرس خشکی برگ گیاهان تولید شده در رطوبت بالا هتروژنیتی بالاتر و متوسط عملکرد فتوسیستم دو[4] بالاتری از خود نشان دادند. کاهش بیشتر محتوای نسبی آب[5] در برگ گیاهان تولید شده در رطوبت بالا منجر به کاهش کمتری در پتانسیل آب[6] و عملکرد فتوسیستم دو شد. علاوه بر این، عملکرد فتوسیستم دو در برگ گیاهان تولید شده در رطوبت بالا با کاهش پتانسیل آب کاهش کمتری پیدا کرد. این نشان می دهد که روزنه ها در گیاهان تولید شده در رطوبت بالا حساسیت کمتری به کاهش پتانسیل آب از خود نشان می دهند. بعد از تیمار استرس خشکی روزنه های اطراف رگبرگ اصلی برگ بسته نشدند. اندازه گیری مستقیم قطر دهانه روزنه ها هم نشان داد که کاهش درمتوسط قطر روزنه ها در ناحیه حاشیه ای برگ ۷۷% بود در حالیکه این میزان در اطراف رگبرگ اصلی فقط ۴۰% بود. سریع تر بسته شدن روزنه ها در حاشیه برگ ناشی از محتوای نسبی کمتر در این ناحیه از برگ بود.

در **فصل چهارم** نقش هورمون آبسیزیک اسید در اختلال رفتار روزنه های گیاهان تولید شده در رطوبت بالا بررسی شده است. درگیاهان تولید شده در رطوبت بالا میزان هورمون آبسیزیک اسید در مقایسه با گیاهان تولید شده در رطوبت متوسط کمتر بود. با استعمال محلول ۲۰ میکرومولار هورمون آبسیزیک اسید بطور روزانه بمدت سه هفته بر روی برگ گیاهان طی رشد در رطوبت بالا، روزنه ها در این برگها رفتاری مشابه رفتار روزنه ها درگیاهان تولید شده در رطوبت متوسط از خود نشان دادند. مثلا" این روزنه ها بسرعت در واکنش به استرس خشکی قادر به بسته شدن بودند. افزایش رطوبت هوا در اطراف یک برگ از یک گیاه در حال رشد در رطوبت متوسط باعث تغییر در رفتار روزنه ها در این برگ در مقایسه با برگهای دیگر این گیاه شد. روزنه ها در این برگ قادر به بسته شدن کامل در واکنش به استرس خشکی نبودند. در حالیکه میزان هورمون آبسیزیک اسید بیان شده در وزن خشک این برگ با برگهای دیگر این گیاه تفاوتی نداشت میزان این هورمون در وزن تر این برگ بطور معنی داری پایین تر بود. علاوه بر این، در گیاهان تولید شده در رطوبت بالا میزان بسته شدن روزنه ها در برگهای مسن تر کمتر از برگهای جوان بود. این مسئله با میزان پایین تر هورمون آبسیزیک اسید بیان شده در وزن تر در برگهای

[1] Heterogeneity
[2] Chlorophyll fluorescence imaging system
[3] Photorespiration
[4] PSII efficiency (Φ_{PSII})
[5] Relative water content (RWC)
[6] Water potential

خلاصه

روزنه های هوایی[1] در کنترل تبادلات گازی بین گیاه و اتمسفر اطراف گیاه نقشی اساسی ایفا می کنند بطوریکه به دی اکسید کربن مورد نیاز برای فتوسنتز اجازه ورود به گیاه می دهند در حالیکه از هدر رفتن آب زیادی از گیاه از طریق تعرق جلوگیری به عمل می آورند. به این منظور روزنه های هوایی به طیف وسیعی از فاکتورهای محیطی و هورمونی واکنش نشان می دهند. امروزه در نتیجه عملیات باغبانی عمل عادی روزنه ها گاهی از حالت تعادل خارج شده و در مواقع لازم بسته نمی شوند. این اتفاق بخصوص زمانی اتفاق می افتد که گیاهان در رطوبتهای بالای هوا رشد داده شده و به رطوبتهای پایین انتقال داده می شوند. اختلال در رفتار روزنه های هوایی باعث عدم تعادل آبی[2] گیاه و ازدست رفتن آب زیاد شده و در نتیجه باعث کاهش کیفیت می شود. با توجه به فعالیتهایی که امروزه در راستای کاهش مصرف انرژی و یا ایزوله کردن گلخانه ها انجام می شود مانند کاهش تهویه یا گلخانه های با سیستم کاملا" بسته پیش بینی می شود که رطوبت بالا در گلخانه های تجاری بیش از پیش معمول گردد. با توجه به اینکه در انتهای چرخه تولید محصولات باغبانی مصرف کننده قرار دارد و در نزد مصرف کننده همیشه رطوبت هوا پایین است افزایش مشکل کیفیت قابل پیش بینی است. بنابراین درک فیزیولوژیکی رفتار روزنه های هوایی در گیاهان تولید شده در رطوبت بالای هوا اهمیت ویژه ای دارد. هدف از انجام این تحقیق مطالعه اثرات رطوبت بالای هوا بر رفتار روزنه های هوایی در گیاه برگ بیدی[3] است.

در **فصل دوم** این رساله خصوصیات آناتومیکی و فیزیولوژیکی روزنه های هوایی در گیاهان تولید شده در رطوبتهای متوسط (۵۵%) و بالا (۹۰%) در پاسخ به تیمارهایی که در حالت عادی باعث بسته شدن روزنه ها می شوند مانند استرس خشکی[4]، هورمون آبسیزیک اسید[5] و تاریکی مطالعه شده است. در گیاهان تولید شده در رطوبت بالا اندازه روزنه ها بزرگتر و تراکم روزنه ای[6] در مقایسه با گیاهان تولید شده در رطوبت متوسط پایین تر بود ولی شاخص روزنه ای[7] در هر دو نوع گیاه یکسان بود. در هر دو نوع گیاه روزنه ها به استرس خشکی، هورمون آبسیزیک اسید و تاریکی واکنش نشان دادند اما متوسط سرعت تعرق[8] و هدایت روزنه ای[9] برگ و همچنین متوسط قطر دهانه روزنه ها[10] در برگ گیاهان تولید شده در رطوبت بالا بیشتر بود که

[1] Stomata
[2] Water balance
[3] *Tradescantia virginiana*
[4] Desiccation
[5] Abscisic acid (ABA)
[6] Stomatal density
[7] Stomatal index
[8] Transpiration rate
[9] Stomatal conductance
[10] Stomatal aperture

کنترل باز و بسته شدن روزنه های هوایی پس از رشد در رطوبت نسبی بالای هوا

عبدالحسین رضایی نژاد

بهمن 1385

رساله دکترای تخصصی

دانشگاه واخنینگن

هلند

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Layout and design: by the author.

Background: Stomata of the abaxial surface of a *Tradescania virginiana* L. leaf

Front page: An image of photosystem II efficiency (Φ_{PSII}) of *Tradescania virginiana* L. leaves grown at 55% (left leaf) or 90% (right leaf) relative air humidity 150 min after excision. The image was taken while the leaves were in an atmosphere of 20 mmol mol^{-1} O_2, 350 µmol mol^{-1} CO_2. The tips of the leaves were located outside the cuvette and provided an indication of Φ_{PSII} under normal air where photorespiration could take place.

The training and supervision plan was completed at the graduate school “Production Ecology and Resource Conservation”