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PHYSIOLOGICAL AND MOLECULAR MECHANISMS OF STOMATAL FUNCTIONING IN PLANTS GROWN AT HIGH HUMIDITY

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Dália Rosa Alves Carvalho

Supervisor: Dr. Susana M.P. Carvalho
Co-supervisors: Dr. Marta W. Vasconcelos
Dr. Ir. Ep Heuvelink

October 2015

In memory of my father
Abílio Pinto de Carvalho

Abstract

Stomata from several plant species developed at high relative air humidity (RH ≥ 85%) may become malfunctional, leading to excessive water loss in conditions of high evaporative demand. In ornamental plants, including rose, this results in a low postharvest longevity. The factors underlying a poor stomatal functioning under high RH are not yet fully understood, but the magnitude of this effect is genotype dependent which broadens the possibilities for plant breeding. The aim of this thesis is to analyze the physiological and molecular mechanisms of stomatal malfunctioning in plants cultivated at high RH, focusing on abscisic acid (ABA) regulation and its effects on stomatal closure. The identification of genomic regions associated with the control of water loss is also a subject of this study. *Rosa* × *hybrida* was used as the model system since in this species there are cultivars with a contrasting sensitivity to high RH.

Stomatal movements are to a large extent regulated by ABA concentration ([ABA]), which is determined by its metabolism within the leaf, as well as by its import from the roots. The role of root-to-shoot ABA signaling in inducing stomatal closure and its role in inducing genotypic differences in stomatal functioning was investigated in ten genotypes grown at contrasting RH conditions. It was found that xylem sap [ABA] did not explain genotypic differences in stomatal functioning, since sensitive and tolerant genotypes to high RH had a similar estimated [ABA] in the root xylem sap and a similar ABA delivery rate and [ABA] in the leaf petiole sap.

Elevated air movement (MOV) and moderate salinity are two environmental stress factors known to induce stomatal closure. In two different studies, we combined a high RH level with high MOV or with a moderate salinity throughout plant growth in order to test if these physiological stresses would counteract the negative effect of high RH on stomatal functioning due to an increased [ABA]. Indeed, stomata developed at high RH with additional MOV or moderate salinity closed faster in response to leaf desiccation when compared to plants grown at high RH without either of the imposed stresses. While salinity enhanced stomatal functioning due to an increase in leaf [ABA], high MOV improved stomatal responsiveness due to a higher stomatal sensitivity to ABA.

The two parents and the 108 offspring of the K5 tetraploid cut rose population grown at high RH were phenotyped for stomatal responsiveness to desiccation, showing large genotypic differences [i.e., relative water content after 4h of leaflet desiccation (RWC_4h) varied between 7 and 62%]. Three QTLs (two major and one putative minor)

explained 32% of the genotypic variability and low RWC_4h proved to be a good proxy for eliminating the offspring with shorter vase life. These particular findings contribute to speeding up genotype selection using marker-assisted selection programs for breeding cultivars with more responsive stomata after cultivation at high RH, minimizing the negative impacts associated with excessive water loss during postharvest.

The transcriptional analysis of nine ABA-related genes (involved in ABA biosynthesis, oxidation and conjugation) and two non-ABA related genes (involved in water stress response) in four contrasting genotypes selected from the K5 tetraploid cut rose population revealed that stomatal responsiveness to desiccation is a polygenic trait forming a highly complex regulatory network acting towards tolerance to high RH. The large majority of the studied genes had a relevant role on stomatal functioning (*NCED1*, *AAO3*, *UGT75B2*, *BG2*, *OST1*, *ABF3* and *Rh-APX*) while three others showed a minor contribution (*CYP707A1*, *CYP707A3* and *BG1*) and *DREB1B* did not contribute to the tolerance trait.

Overall, this study has helped identifying major factors responsible for the reduction of potential vase life of cut roses due to uncontrolled water loss, and represents a major step forward in developing future tools to mitigate this phenomenon. In summary, it highlights that: (1) roses have a large genotypic variation in terms of stomatal functioning; (2) xylem sap [ABA] does not explain genotypic differences in stomatal functioning; (3) additional MOV or moderate salinity enhances stomatal functioning due to higher sensitivity to ABA or higher leaf [ABA], respectively; (4) three QTLs can explain 32% of the genotypic variability in stomatal functioning of the K5 tetraploid cut rose population in response to desiccation; (5) multiple genes form a highly complex regulatory network acting together towards the genotypic tolerance to high RH.

Resumo

Plantas cultivadas em humidade relativa elevada (HR ≥ 85%) apresentam frequentemente estomas pouco funcionais, conduzindo a uma perda de água excessiva quando transferidas para condições de elevada transpiração. Em plantas envasadas e flores de corte, incluindo rosas, estas condições de crescimento resultam numa baixa longevidade pós-colheita. As causas responsáveis por esta disfunção estomática são ainda desconhecidas, mas sabe-se que este problema está fortemente relacionado com o genótipo, o que viabiliza o melhoramento genético para esta característica. Esta tese tem como objetivo analisar os mecanismos fisiológicos e moleculares relacionados com o mau funcionamento estomático em plantas cultivadas em HR elevada, abordando a regulação do ácido abscísico (ABA) e os seus efeitos no fecho dos estomas. Paralelamente, pretendeu-se ainda identificar as regiões genéticas associadas ao controlo da perda de água. A rosa foi utilizada como espécie modelo devido à existência de cultivares com sensibilidade contrastante à HR elevada.

O funcionamento dos estomas é regulado pela concentração de ABA ([ABA]), a qual é determinada pelo seu metabolismo na folha, bem como pela sua importação das raízes. Investigámos o papel do ABA transportado das raízes até às folhas no fecho dos estomas e na determinação das diferenças genotípicas em dez genótipos cultivados em condições contrastantes de HR. A [ABA] estimada no exsudado do xilema das raízes, a taxa de transporte de ABA das raízes para as folhas, e a [ABA] no exsudado do xilema do pecíolo foliar foram semelhantes entre genótipos sensíveis e tolerantes, indicando que a [ABA] na seiva xilémica não explica as diferenças genotípicas na sensibilidade dos estomas à HR elevada.

Um acentuado movimento do ar (MOV) e a salinidade moderada são dois fatores de stresse ambiental que induzem o fecho dos estomas. Em dois estudos distintos, combinámos a HR elevada com MOV elevado ou a HR elevada com salinidade moderada, durante todo o crescimento das plantas, de modo a testar se esses stresses fisiológicos poderiam contrariar os efeitos negativos da HR elevada no funcionamento dos estomas devido a um aumento na [ABA]. De facto, estomas desenvolvidos em HR elevada com MOV elevado ou salinidade moderada fecharam mais rapidamente em resposta à desidratação foliar quando comparados com estomas desenvolvidos em HR elevada na ausência desses stresses. Porém, enquanto a salinidade melhorou o funcionamento

estomático devido a um aumento na [ABA] foliar, o MOV elevado aumentou a sensibilidade dos estomas ao ABA.

A resposta fenotípica dos estomas à desidratação foliar dos dois progenitores e de 108 descendentes da população tetraploide de rosas K5 cultivada em HR elevada apresentou grandes diferenças entre genótipos, uma vez que o conteúdo relativo em água após 4 h de desidratação foliar (CRA_4h) variou entre 7 e 62%. Três QTLs (dois ‘major’ e um ‘putative minor’) explicaram 32% da variabilidade genotípica e o baixo CRA_4h mostrou ser um indicador fidedigno do funcionamento estomático, que poderá ser utilizado para excluir genótipos com baixa longevidade pós-colheita. Estes resultados contribuem para acelerar a seleção de genótipos, em programas de melhoramento usando seleção assistida por marcadores, para a obtenção de cultivares com estomas mais funcionais após o cultivo em HR elevada, minimizando os impactos negativos associados à perda de água excessiva durante a pós-colheita.

A avaliação dos níveis de transcrição de nove genes relacionados com o metabolismo do ABA (biossíntese, oxidação e conjugação) e dois genes independentes do ABA (relacionados com a resposta ao stresse hídrico) em quatro genótipos contrastantes selecionados da população tetraploide K5 revelou que a resposta estomática à desidratação é uma característica poligénica formando um complexo sistema regulatório contribuindo para a tolerância à HR elevada. A grande maioria dos genes estudados teve um papel relevante no funcionamento estomático (*NCED1*, *AAO3*, *UGT75B2*, *BG2*, *OST1*, *ABF3* and *Rh-APX*), enquanto três tiveram uma contribuição reduzida (*CYP707A1*, *CYP707A3* and *BG1*) e *DREB1B* não contribuiu para a tolerância à HR elevada.

Este estudo contribuiu para a identificação de fatores responsáveis pela redução da longevidade pós-colheita em rosas de corte devido à perda de água excessiva, e representa um passo importante no desenvolvimento de futuras ferramentas para mitigar este fenómeno. Em suma, esta tese destaca que: (1) as rosas apresentam uma larga variabilidade genotípica em termos de funcionamento estomático; (2) a [ABA] no exsudado do xilema não explica as diferenças no funcionamento estomático entre genótipos; (3) MOV elevado ou uma salinidade moderada melhoram o funcionamento estomático devido, respetivamente, a um aumento da sensibilidade ao ABA ou devido ao aumento da [ABA] foliar; (4) três QTLs explicam 32% da variabilidade genotípica do funcionamento dos estomas de uma população tetraploide de rosas K5 em resposta à desidratação; (5) vários genes formam um complexo sistema regulatório atuando em conjunto para a tolerância à HR elevada.

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Dália Rosa

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Abbreviations

10_D	10 min after the beginning of the dark period
10_L	10 min after the beginning of the light period
180_D	180 min after the beginning of the dark period
180_L	180 min after the beginning of the light period
AAO	Abscisic-aldehyde oxidase
ABA	Abscisic acid
ABA-GE	Abscisic acid-β-D-glucosyl ester
ABF	Abscisic acid-responsive element-binding factor
APX	Ascorbate peroxidase
BG1	β-glucosidase homolog1
BG2	β-glucosidase homolog2
DPA	4'-dihydrophaseic acid
DREB	Dehydration responsive element binding
EC	Electrical conductivity
FLE	Full leaflet expansion
g_s	Stomatal conductance
ICM	Integrated consensus map
MAS	Marker-assisted selection
MOV	Air movement
NCED	9-cis epoxycarotenoid dioxygenase
OST1	Open stomata 1
PA	Phaseic acid
QTL	Quantitative trait locus
RGE	Relative gene expression
RH	Relative air humidity
RWC	Relative water content
RWC_4h	Relative water content after 4 h of leaflet desiccation
SNP	Single-nucleotide polymorphism
UGT	Uridine diphosphate glucosyltransferase
VPD	Vapor pressure deficit

Keywords

Abscisic acid
Breeding
Drought
Electrical conductivity
Flower fresh weight
Gene expression
Marker-assisted selection
Mechanical stimuli
Plant growth
Postharvest longevity
QTL mapping
Relative air humidity
Relative water content
Rosa x *hybrida*
Salinity
Stomatal anatomy
Stomatal conductance
Stomatal functioning
Stomatal physiology
Storability
Tetraploid roses
Transpiration rate
Vapor pressure deficit
Vase life
Water loss
Wind speed

CHAPTER 1

General Introduction

Long postharvest longevity of ornamentals is a very important factor to ensure consumers' satisfaction and reinforce their choice (Reid and Evans, 1986). High relative air humidity (RH $\geq$ 85%) during growth is the environmental factor that mostly reduces postharvest longevity (Fanourakis et al., 2013a). RH is defined as the ratio of the actual amount of water vapor relative to the amount of water vapor that would be present at saturation and it is given as a percentage. By increasing the temperature, the maximum water holding capacity of the air increases, therefore the RH depends on water vapor pressure and air temperature. Vapor pressure deficit (VPD) combines the effects of RH and temperature, and the VPD between stomatal cavity (where the air is saturated with water vapor) and the surrounding air is considered as the driving force for plant transpiration. In conditions of constant temperature, VPD only depends on the RH.

In northern latitude countries, greenhouse production represents a significant fraction of the total agricultural sector. In these regions, greenhouses are continuously heated throughout the winter, which represents a very high cost in the greenhouse management. To save energy costs and to avoid heat loss, growers drastically reduce the opening of the greenhouse windows, leading to build up of the RH level, frequently reaching values above (Max et al. 2009; Arve et al. 2013). In the Mediterranean area, winters are milder but the greenhouses are less technologically developed and often not heated, thus leading to high RH levels in the rainy and colder days (Baptista et al., 2012). Other examples of growing systems where high RH takes place is in *in vitro* culture vessels and in the early phase of plantlets propagation, where high RH is intentionally maintained to avoid tissue dehydration (Ziv et al. 1987; Santamaria et al. 1993; Aguilar et al. 2000). Additionally, in natural environments such as tropical and subtropical areas or northern latitudes high RH has been reported during certain periods of the year (Fanourakis et al., 2009; Sáez et al., 2012; Tullus et al., 2012). Also in the microclimate of a dense canopy the RH is usually higher compared to the surrounding air in the rest of the greenhouse (Mortensen & Gislerød 2005). Since high RH does not generally affect plant growth, growers tend to neglect this environmental factor as long as this is kept below 94% in order to avoid the incidence of *Botrytis cinerea* (Williamson et al., 1995). However, it has been shown that RH ≥ 85% negatively affects postharvest quality of several ornamental plants (Mortensen & Gislerød, 1999; Fanourakis et al., 2012a). In order to reduce RH inside greenhouses, rather than passive ventilation, other methods such as forced ventilation in combination with a heat exchanger, condensation on a cold surface and hygroscopic dehumidification could be implemented, however, their application is not easy due to high investment costs and elevated energy consumption (Campen et al., 2003). Moreover, as a result of energy saving practices (e.g. use of energy screens, temperature integration climate control and totally closed greenhouses) it may be expected that high RH levels frequently occur in commercial greenhouses. Therefore, high RH causing negative effects on plants is still an ongoing problem.

Stomatal malfunctioning in high RH grown plants

Stomata are small pores bounded by a pair of guard cells located in the epidermis of leaves, stems, flowers and fruits. Most of the stomata are located in the leaves, and it has been shown that leaf transpiration rate was responsible for 75% of the total water loss of well-watered cut roses, while 20% originated from the flowers and only 5% from the stems (Carpenter and Rasmussen, 1974). These cells are kidney-shaped in dicots and some monocots, or dumbbell-shaped in grasses (Willmer and Fricker, 1996). Stomata from several plant species have been reported to become malfunctional [i.e., fail to close in response to closing stimuli like high evaporative demand, light-dark transition and abscisic acid (ABA)] after growth at high RH, leading to excessive plant water loss (Torre & Fjeld 2001; Rezaei Nejad & van Meeteren 2005; Fanourakis et al. 2011; Aliniaeifard et al. 2014; Arve et al. 2015). Stomatal physiology has been pointed out as the major cause of this negative water balance (water loss > water uptake) (Fanourakis et al. 2013b; Aliniaeifard et al. 2014). In contrast, the role of stomatal and leaf anatomy on the increased water loss in leaves developed at high RH seems to be more controversial. Some authors refer that stomatal anatomy (size of the stomata) and density (number of stomata per area) contribute *per se* to the increased water loss in leaves developed at high RH (Drake et al. 2013; Giday et al., 2013a), while other reject this conclusion (Fanourakis et al. 2013a; Aliniaeifard et al. 2014). Several hypotheses such as low [ABA] (Rezaei Nejad & van Meeteren 2007; Arve et al. 2013; Giday et al. 2013b; Aliniaeifard et al. 2014), reduced stomatal sensitivity to

ABA (Pantin et al., 2013), reduced Ca^{2+} levels due to reduced transpirational water uptake (Felle et al., 2000) and changes in guard cell anatomy (Giday et al. 2013a; Drake et al. 2013) have been proposed to explain the less responsive stomata. However, the reasons why stomata fail to close fully during water stress periods in plants grown under high RH remain unclear.

Stomatal functioning – the role of ABA metabolism, signaling and perception

In vascular plants, the primary function of stomata is to control plant hydration, i.e., stomata close as plant water potential decreases, reducing plant transpiration rate and avoiding excessive water loss (Brodribb and McAdam, 2011). Additionally, stomata also regulate the entry of CO_2 for photosynthetic carbon fixation and the transpiration stream ensuring the nutrient uptake (Hetherington and Woodward, 2003). During the night, since there is no need for CO_2 uptake for photosynthesis, stomata close to maximize the rehydration before the following day. It has been found in a study on deciduous trees that hydraulic factors (leaf water potential and air humidity) prevail over photosynthetic factors ($[CO_2]$ and light intensity) in determining stomatal conductance, guaranteeing plant survival in conditions of drought stress (Aasamaa and Sõber, 2011). Moreover, the cuticle layer protects plants from drying due to its airtight properties. In some plant species including rose and *A. thaliana* the cuticle has been reported to have only a minor contribution to the total leaf evapotranspiration (Fanourakis et al. 2013b; Pantin et al. 2013) indicating that stomata represent the most important pathway for water loss.

Stomata respond to environmental signals through hydro passive or active pathways (Buckley and Mott, 2002; Buckley, 2005; Lawson, 2009). Both mechanisms operate together but the hydro passive mechanism is more common in non-seed plants (e.g. ferns and lycophytes), while the active pathway prevails in seed plants (e.g. angiosperms) (Franks, 2013; Mcadam and Brodribb, 2013). The active mechanism usually involves the hormone ABA which regulates ion channels in the plasma membrane and vacuoles of guard cells and consequently ion fluxes (Kim et al., 2010). ABA is perceived by external and possibly internal ABA receptors located in the plasma membranes of stomatal guard cells (Wilkinson and Davies, 2002). These include PYR/PYL/RCAR (pyrabactin resistance/pyrabactin resistance-like/regulatory component of ABA response) and GCR2 (G protein coupled receptor) (Liu et al. 2007; Klingler et al. 2010). Then, $[Ca^{2+}]$ increases in the guard cell due to influx through the plasma membrane and release from vacuoles, leading to the inactivation of inward K^+ channels and activation of outward anion channels

(Felle et al., 2000). This causes depolarization of the plasma membrane, resulting in K^+ efflux from the guard cell which reduces the water content and the turgor pressure leading to stomatal closure (Felle et al., 2000). Sucrose removal and metabolism of malate to osmotically inactive starch also contribute to the osmotic water loss leading to stomatal closure (Schroeder et al., 2001). Stomata open due to an increase in the guard cell volume determined by water influx, following an increase in the osmotic potential via the accumulation of K^+, Cl^-, and/or organic solutes (Blatt, 2000; Schroeder et al., 2001).

Stomatal movements are to a large extent regulated by ABA concentration ([ABA]). Leaf [ABA] is determined by its metabolism within the leaf (biosynthesis vs. catabolism; Fig. 1), as well as by its import from the roots, an important ABA production site (Wilkinson and Davies, 2002; Nambara and Marion-Poll, 2005). ABA is synthesized from isopentenyl diphosphate followed by a cascade of reactions, with zeaxanthine, violaxanthin, xanthoxin and abscisic aldehyde as intermediate precursors (Seo and Koshiba, 2002). The major cause of ABA inactivation is oxidation by ABA 8'-hydroxylase to form 8'-hydroxy ABA which spontaneously isomerizes to phaseic acid (PA) and is further reduced to dihydrophaseic acid (DPA) by phaseic acid reductase (Cutler and Krochko, 1999). Moreover, ABA can also be inactivated by covalent conjugation with monosaccharides (e.g. glucose) resulting in ABA-β-D-glucosyl ester (ABA-GE) which does not easily pass through bio-membranes and has therefore been hypothesized to be a storage (in vacuoles) and transport form of ABA (Cutler and Krochko, 1999; Dietz et al., 2000; Arve et al., 2015). ABA-GE can be cleaved by β-D-glucosidase releasing ABA when needed (Dietz et al., 2000; Lee et al., 2006). Several studies relate stomatal malfunctioning to lower leaf [ABA] in *Trasdescantia virginiana* (Rezaei Nejad and van Meeteren, 2007), *Vicia faba* (Aliniaeifard et al. 2014) and *Rosa × hybrida* (Arve et al., 2013; Giday et al., 2013b) developed at high (90%) RH, compared to plants grown at moderate (60%) RH. High RH has been found to reduce the ABA availability by inactivating ABA to PA in *Arabidopsis thaliana* (Okamoto et al., 2009). Moreover, at high RH the ABA-GE levels remain high during the night indicating that conversion to ABA does not occur (Arve et al., 2013). Furthermore, also tobacco plants grown *in vitro* have shown lower [ABA] compared to plants grown *ex vitro* (Hronková et al., 2003). Even though most studies have shown a correlation between [ABA] and RH, a study in *A. thaliana* showed that ABA-deficient and ABA-insensitive mutants have the same response to short-term changes in RH as the wild-type, excluding an obligate role of ABA in stomatal responses to RH (Assmann et al., 2000).

Despite recent studies showing ABA synthesis in the mesophyll cells of leaves (Christmann et al., 2005; Endo et al., 2008; Seo and Koshiba, 2011; McAdam et al., 2015) it is undeniable that roots are a very important production site of ABA which can be translocated via the xylem vessels to the leaves, driven by the transpiration stream (Dodd, 2005). By studying ABA-deficient mutants, Dodd et al. (2009) showed that root ABA synthesis affects leaf [ABA], when leaf ABA synthesis is compromised. Giday et al. (2014) found that leaf [ABA] increased in leaves of a sensitive rose cultivar (i.e., a cultivar with reduced stomatal closure in response to closing stimuli after growth at high RH) grafted onto a tolerant one (i.e., a cultivar maintaining the stomatal closure capacity in response to closing stimuli after growth at high RH) grown at high RH, and speculated that this leaf [ABA] increase was due to a higher root ABA biosynthesis. Nonetheless, experimental evidence to support this claim is currently lacking and the role of the root-to-shoot ABA signaling in the functioning of stomata developed under contrasting RH conditions still has to be evaluated, as the majority of studies is associated with drought stress (Christmann et al., 2007; Schachtman and Goodger, 2008; Martin-Vertedor and Dodd, 2011). A very low evaporative demand (i.e. low VPD) in conditions of high RH results in a very low plant transpiration rate (Fanourakis et al., 2011). This can lead to lower ABA import from the roots via the xylem (i.e., attenuated root-to-shoot ABA signaling) (Zhang and Outlaw, 2001). Moreover, it has been proposed that leaf transpiration rate positively influences leaf ABA biosynthesis (Heilmeier et al., 2007).

It seems that stomatal sensitivity to ABA also plays a role in stomatal functioning rather than only [ABA] *per se* (Pantin et al., 2013). Changes in guard cell sensitivity to ABA occur over the light-dark cycles and during plant development, frequently in response to changes in electrical and osmotic membrane potentials generated by the guard cells themselves (Assmann and Shimazaki, 1999; Blatt, 2000; Pantin et al., 2013). In detached *A. thaliana* leaves subjected to desiccation an increase in [ABA] has been reported, independently of the RH during growth, but only stomata from moderate RH-grown leaves were able to close (Arve et al., 2015) suggesting that high RH-grown plants could not perceive the ABA despite the increase of its concentration. Furthermore, several studies have suggested that stomatal malfunctioning in high RH-grown plants is strongly related to a long-term low [ABA] during leaf development because a daily exogenous ABA application during leaf development originated functional stomata, while a short-term exogenous ABA application in fully developed leaves did not increase stomatal functionality (Rezaei Nejad and Van Meeteren, 2008; Fanourakis et al., 2011). In rose plants grown at high RH, it was shown that after full leaf expansion stomatal function is no longer affected either by RH (when plants were moved from high to moderate RH) or [ABA] (Fanourakis et al., 2011). Thus, it remains unclear whether the lack of stomatal responsiveness to short-term ABA feeding is due to leaf developmental stage or due to the duration of this stimulus. Still in roses, it was concluded that in expanding leaves the degree of stomatal adaptation depends on the duration and timing of exposure to high RH, i.e., in general, the longer the exposure to high RH the higher the loss of stomatal functionality in response to desiccation (Fanourakis et al., 2011). Furthermore, it was found that stomatal malfunctioning, as a result of plant growth under high RH, is strongly determined during the last part of leaf expansion (Fanourakis et al., 2011), but the stomatal response to a short-term exogenous ABA application has not been tested yet.

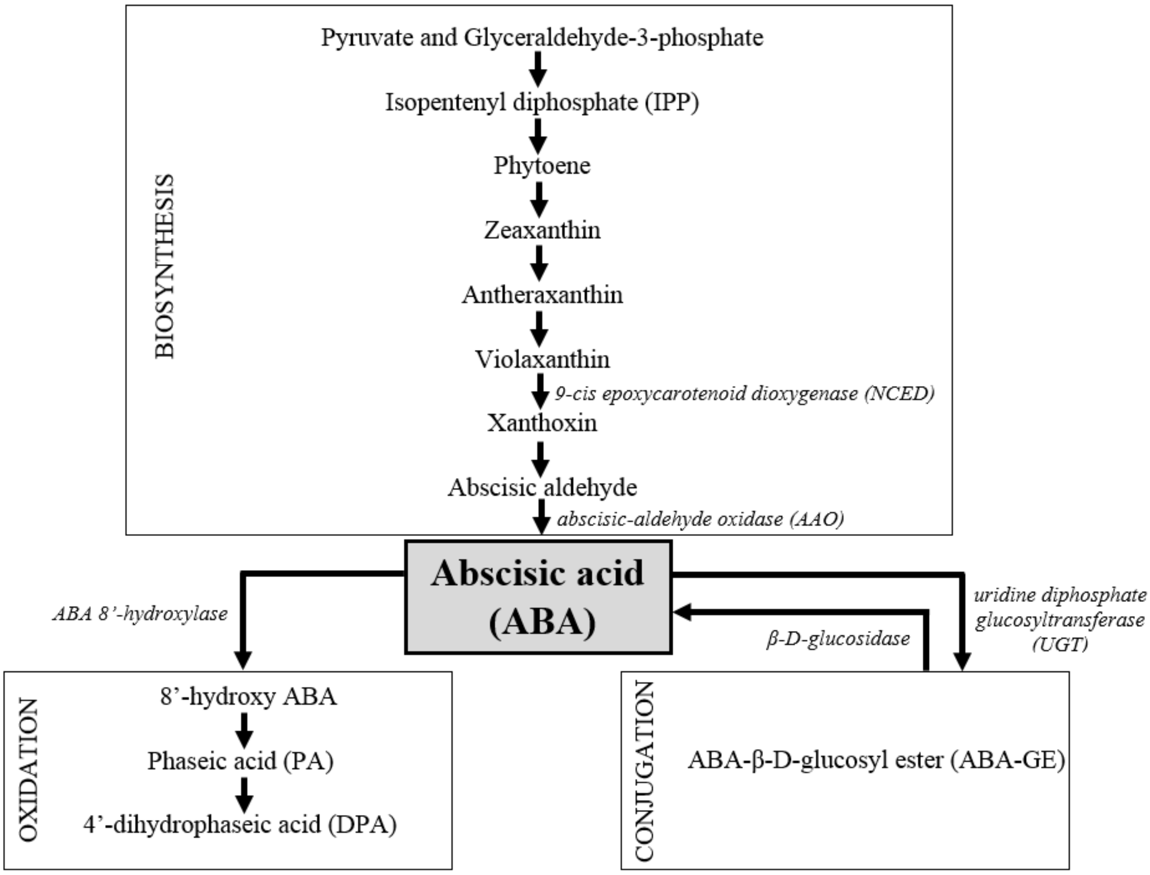

Fig. 1. Abscisic acid (ABA) metabolism (biosynthesis, oxidation and conjugation) with reference to the most important enzymes (in italic) involved in the metabolic pathway and which expression of the codifying genes has been studied in Chapter 6. Adapted from: Arve et al. (2011).

Enhancing stomata functioning in high RH-grown plants

To counteract the negative effects of high RH on the stomatal functioning, several strategies exploring the interaction of RH with other environmental factors such as temperature, drought and ventilation have been explored. It has been shown in roses that a daily 6 h reduction in RH or an increase in temperature (both consecutive or divided into three periods of 2 h daily) resulted in improved stomatal functioning (Mortensen and Gislerod, 2011; Mortensen et al., 2007). A severe drought stress during cultivation applied to high RH-grown plants improved vase life (16–118%) in five out of six rose cultivars (Mortensen and Gislerød, 2005). The effect of increased ventilation (0.08, 0.21, and 0.35 m s^{-1}) combined with RH (70 and 90%) revealed that these ventilation levels had little effect on water loss of detached leaves, indicating that stomatal functioning was not improved (Mortensen and Gislerød, 1997). Nonetheless, some of these strategies are not favored by horticulturists because of the negative impact on productivity and the increased energy consumption with resultant high production costs. It is known that [ABA] increases in vegetative tissues subjected to stress, triggering adaptive responses essential to plant survival and productivity (Zeevaart and Creelman, 1988; Leung and Giraudat, 1998), but the [ABA] has not been quantified in the approaches mentioned above.

Stomatal responses to wind as a mechanical stimulus

In higher plants, a thigmomorphogenic response (i.e., touch- induced morphological change; e.g., wind and rain) is a slow, intensity-dependent, and saturating systemic response, that translocates from the stimulated plant regions to the non- disturbed distal areas (Jaffe, 1976; Beryl and Mitchell, 1977; Erner et al., 1980). Wind is an environmental factor having several effects on plants (Grace 1977; Ennos 1997), depending on leaf characteristics and on its speed (Schuepp, 1993; Lambers et al., 2008). These effects include a reduction of the boundary layer thickness, enhancing gas diffusion (CO_2 and H_2O; Schuepp, 1993; Lambers et al., 2008). Moreover, wind flow exerts drag forces causing mechanical stress (Anten et al., 2010), and high wind speed has been suggested to have a positive effect on [ABA], reducing stomatal aperture (Whitehead, 1962; Weyers and Hillman, 1979), although this effect has not yet been quantified. One of the few studies that investigated the effect of air movement (MOV; 0.08, 0.21, and 0.35 m s^{-1}) combined with RH (70 and 90%) found that increasing wind speed at high RH had little effect on water loss of detached leaves of cut rose (Mortensen and Gislerød, 1997). However, the air speed levels used in that study were relatively low. To the best of our knowledge, the combined effects of high MOV and high RH on plant growth and development as well as on stomatal functioning and ABA metabolism have not yet been properly explored.

Stomatal responses to salinity

Plant responses to salinity during cultivation depend on plant species, salt concentration, duration of exposure, plant developmental stage and other environmental conditions (Munns, 2002). During the initial phase of root exposure to excessive [NaCl], water uptake may be inhibited causing a physiological drought stress (Shalhevet and Bernstein, 1968). Osmotic stress is caused by the presence of salt outside the roots and immediately reduces plant growth and causes stomatal closure (Munns and Tester, 2008). An increased [ABA] associated with a reduced stomatal conductance has been reported in tomato plants as a response to osmotic stress (Lovelli et al., 2012; Maggio et al., 2007). Moreover, a decreased transpiration rate in chrysanthemum (Lee and van Iersel, 2008), wheat (Sharma et al., 2005), salvia (Kang and Van Iersel, 2004), *Sophora secundiflora* and *Cercis canadensis* (Niu et al., 2010) was also observed. Additionally, salinity can affect stomatal morphology. In cotton a decrease in stomatal density was compensated by an increase in stomatal size and mesophyll surface area (Jafri and Ahmad, 1995) and strawberry showed reduced transpiration flux due to low stomatal density (Orsini et al., 2012). Beyond the described osmotic stress, plants subjected to long-term salinity may suffer from ionic stress due to ion accumulation in the shoot (Munns and Tester, 2008). This can cause premature senescence of adult leaves and thus a reduction in the available photosynthetic area to ensure plant growth (Cramer and Nowak, 1992). To the best of our knowledge, the combined effects of moderate salinity and high RH on plant growth and development, as well as on stomatal functioning and ABA metabolism, still need to be revealed.

Postharvest longevity in ornamentals

Postharvest longevity of ornamentals is the period comprising product's harvest and the loss of its ornamental value. In cut flowers, long vase life longevity is very important to ensure consumers' satisfaction and reinforce their choice (Reid and Evans, 1986). A vase life of at least 14 days is desirable for cut roses, considering 7 days from production to selling and another 7 days vase life at consumer level to ensure the so-called vase life guarantee label (van Kooten and Kuiper, 2009). Recently, pre-harvest and postharvest conditions affecting vase life longevity of ornamentals have been reviewed by Fanourakis et al. (2013a). The potential (maximum) vase life is the result of the complex interaction between the genotype and the environmental conditions during cultivation (Fig. 2; van Meeteren et al., 2005; Fanourakis et al. 2013a). Thus, from the moment the plant is cut, the potential vase life has already been established, as the plants physiological and anatomical traits are determined during cultivation (Fig. 2; Fanourakis et al., 2013a). Among these traits, having functional stomata to prevent the occurrence of a negative water balance is seen as a key trait for a proper postharvest longevity (Fanourakis et al., 2012a). The vase life of a new cultivar is always tested before it is released to the market, and this is often done only in the final phase of the breeding program, despite the fact that a short vase life will lead to the rejection of a promising cultivar that has been selected for other reasons, e.g., a high productivity. Therefore, an early identification and elimination of offspring with a very poor vase life will increase the efficiency of breeding programs (Fanourakis et al., 2012b). High RH during growth is the environmental factor that mostly reduces postharvest longevity (Fanourakis et al., 2013a). In cut roses, high RH during growth caused lethal wilting symptoms such as desiccated leaves and the pedicel bending (so-called 'bent-neck'), drastically shortening the postharvest longevity (Mortensen and Gislerød, 1999; Fanourakis et al., 2012a). However, the magnitude of these effects are highly cultivar dependent (Fanourakis et al., 2012a). The negative effects of high RH on postharvest have also been observed in pot plants such as *Begonia × cheimantha*, *Chrysanthemum morifolium*, *Euphorbia pulcherrima* and *Kalanchoe blossfeldiana* that showed excessive water loss in conditions of high evaporative demand (Mortensen, 2000).

After harvest, efforts should be done to minimize any decrease in the vase life. For example, it is known that preservative solutions containing, e.g., 8-hydroxyquinoline can reduce bacterial growth responsible for blocking the xylem vessels and limit water uptake, while solutions containing, e.g., silver thiosulfate are described to block the action of ethylene and sometimes reduce its production, given that ethylene has a negative effect on vase life longevity (Veen 1979; Elhindi 2012). In addition, low postharvest storage temperature reduces plant metabolism (respiration) and transpiration, delaying plants senescence (Reid and Jiang, 2012).

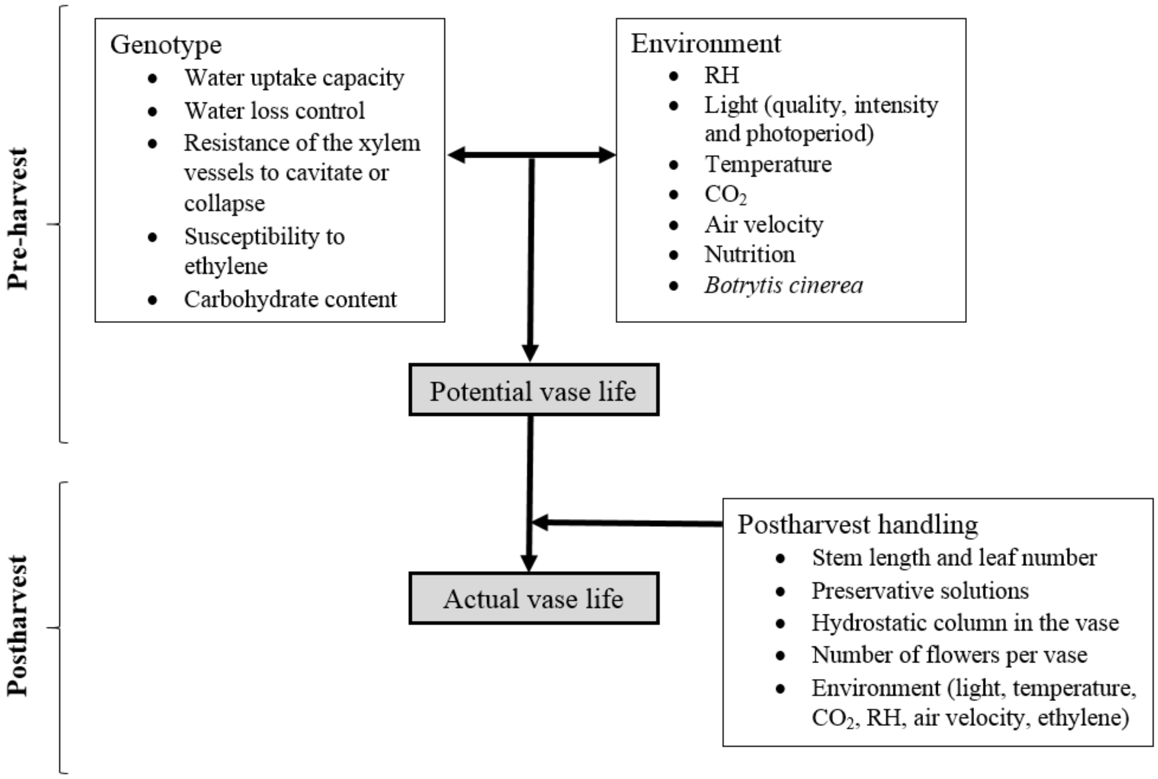

Fig. 2. Schematic representation of the factors involved in the potential and actual vase life of cut flowers.

Genotypic variability

Genotypic variability is of extreme importance for plant breeding. In cut roses, the poor stomatal functioning in response to closing stimuli observed in high RH grown plants has been shown to be highly cultivar dependent (Mortensen and Gislerød, 1999; Fanourakis et al., 2013b; Giday et al., 2013b). This variability can be useful when breeding for enhanced stomatal functioning, but the genetic basis of this trait has not been studied in most crops, including ornamental plants.

Quantitative trait locus (QTL) analysis is used to detect an association between the phenotype and the genotype of markers (Collard et al., 2005), allowing the identification of genomic regions involved in the quantitative variability in the phenotypic trait. Moreover, it also identifies markers linked to those QTL regions that can be used for marker-assisted selection (MAS) in plant breeding (Ribaut and Hoisington, 1998). Research reporting on QTL mapping for the stomatal response to environmental conditions is very scarce. In the model species *A. thaliana*, the natural variability in mechanisms responsible for stomatal functioning has been studied by several authors, who highlighted the importance of identifying QTLs associated to water loss (Bouchabke et al. 2008; Brosché et al. 2010). In this species, screening a recombinant inbred line population (Col-0 × Cvi-0) identified a single QTL for high water loss rates (Brosché et al., 2010). In rice, two QTLs affecting stomatal conductance have been identified (Zhao et al., 2008). In field-grown barley, nine chromosomal regions were found to control the leaf relative water content (RWC) (Teulat et al., 2003). In roses, QTL studies have focused on morphological traits (e.g., flower and leaf size, thorn number), days to flowering or disease resistance (e.g., powdery mildew) (Crespel et al., 2002; Dugo et al., 2005; Linde et al., 2006). Aliniaeifard and van Meeteren (2014) were the first authors to focus on the natural variability in stomatal malfunctioning of *A. thaliana* following growth at high RH, however, no QTL analysis was conducted. Recently, Fanourakis et al. (2015) identified seven QTLs associated with stomatal responsiveness to water loss in tomato, though their analysis was limited to a moderate RH environment. To the best of our knowledge, there are no studies on identifying QTLs related to the stomatal functioning mediating the control of water loss after growth at high RH, associated with postharvest longevity of cut roses.

Molecular mechanisms involved in stomatal functioning

Besides the plant physiological and genetic factors, also the molecular mechanisms must be carefully evaluated aiming to understand the reasons of poor stomatal functioning in conditions of high evaporative demand. Many stress-related genes are induced by ABA leading to stomatal closure (Kang et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). The function of ABA is accomplished via a complex and refined cascade of biochemical and molecular events (Fig. 1; Hauser et al. 2011). Regarding the ABA biosynthesis, the enzyme 9-cis epoxycarotenoid dioxygenase (NCED) has been related to the conversion of violaxanthin to xanthoxin (Fig. 1; Schwartz et al. 1997) while the enzyme abscisic-aldehyde oxidase (AAO) acts on the conversion of abscisic aldehyde to ABA (Fig. 1; Seo et al. 2000). In the *A. thaliana* genome there are five members of the NCED family (*AtNCED* 2, 3, 5, 6, and 9) (Iuchi et al., 2001; Toh et al., 2008). In tomato and strawberry, *NCED1* was described to be involved in the first step of ABA biosynthesis (Jia et al., 2011; Sun et al., 2012). *AAO3*, one of the four *A. thaliana* aldehyde oxidase genes (*AAO1-4*), is the most likely candidate to encode the enzyme that catalyzes the oxidation of abscisic aldehyde to ABA (Seo et al., 2000). Concerning ABA catabolism, the genes encoding ABA 8'-hydroxylase (Fig. 1) were identified to be members of the subfamily CYP707A (Kushiro et al., 2004; Saito et al., 2004). *CYP707A1* and *CYP707A3* have been described to be involved in the oxidation of ABA to phaseic acid when *A.*

thaliana plants were transferred from moderate to high RH, however the first is more important in guard cells while the latter plays a main role in vascular tissues (Okamoto et al., 2009). The conjugation of ABA with monosaccharides generating ABA-GE is mediated by the enzyme uridine diphosphate glucosyltransferase (UGT) (Fig. 1). More than 90 glucosyltransferase families have been classified based on similarities in amino acid sequence and stereochemistry (Coutinho et al., 2003). *UGT75B2* has been annotated with activity related to ABA-glucosyltransferase in *A. thaliana* (Hong et al., 2001). ABA-GE can be hydrolyzed back to ABA by β-glucosidase (Fig. 1; Dietz et al. 2000; Lee et al. 2006; Arve et al. 2013). Arve et al. (2013) showed that in darkness ABA levels were decreased and ABA-GE levels were increased in high RH compared to moderate RH-grown plants indicating a low activity of β-glucosidase. In *A. thaliana*, β-glucosidase homolog1 (*BG1*) and β-glucosidase homolog2 (*BG2*) are described to hydrolyze ABA-GE to ABA during dehydration (Lee et al., 2006; Xu et al., 2012).

Besides the genes directly involved in ABA biosynthesis and catabolism, there are others related to the ABA signal transduction. Open stomata 1 (*OST1*), a serine/threonine protein kinase belonging to the family SnRK2, is a positive regulator of downstream ABA signaling (Merlot et al., 2002; Mustilli et al., 2002). When present, ABA bounds its receptor (PYR/PYL/RCAR) blocking the activity of PP2Cs (protein phosphatases type 2C that operate as negative modulators of the ABA signaling pathway) and, consequently, allowing SnRK2/OST1 activation. In the absence of ABA, PP2C inactivates SnRK2/OST1 via dephosphorylation resulting in a repression of downstream ABA signaling (Fujii et al., 2009; Geiger et al., 2009; Park et al., 2009; Umezawa et al., 2009). Furthermore, ABA-responsive elements (ABREs) that control ABA- and/or stress-responsive gene expression have been determined in numerous studies (Giraudat et al., 1994). *ABF3* is one of the four ABRE-Binding Factors 1 to 4, belonging to the family basic leucine zipper (bZIP) (Choi et al., 2000; Uno et al., 2000). In *A. thaliana OST1* is involved in the phosphorylation of the abscisic acid-responsive element-binding factor 3 (*ABF3*) meaning that the long-term responses to ABA that require sustained gene expression are, in part, mediated by the stabilization of ABFs driven by ABA-activated SnRK2s (Sirichandra et al., 2010). In *A. thaliana*, the overexpression of *ABF3* caused ABA hypersensitivity resulting in partial stomatal closure, reduced transpiration and enhanced drought tolerance (Kang et al., 2002).

Dehydration responsive element binding (DREB) is an important plant transcription factor, belonging to the ERF/AP2 family that regulates the expression of many water stress-inducible genes, mostly in an non-ABA related manner (Lata and Prasad, 2011;

Yamaguchi-Shinozaki and Shinozaki, 2005). The rice *OsDREB1B* was reported to have a stress-dependent activity and its overexpression in tobacco (*Nicotiana plumbaginifolia* Viv.) enhanced tolerance to dehydration, osmotic and oxidative stresses, and tobacco streak virus infection, demonstrating that *OsDREB1B* has a unique role in stress response protecting the plants from a range of stresses (Gutha and Reddy, 2008). In roses, it was found that *Rh-DREB1B* was induced by water deficit in petals of *Rosa* × *hybrida* 'Samantha' (Ting et al., 2009).

Plants utilize antioxidant enzymes, such as ascorbate peroxidase (APX), to scavenge active oxygen species (Ito et al., 1999), which may also have a role in the regulation of water stress response. It has been shown that, in cut roses, changing patterns of *Rh-APX1* expression were consistent with the changes of APX activity indicating that the improved tolerance to water stress induced by ascorbic acid pre-treatment may be related to the regulation of *Rh-APX1* at the transcriptional level (Jin et al., 2006). In transgenic plants from other species, is has also been shown that over-expression of APX can enhance tolerance to oxidative stress (Pitcher et al., 1994; Webb and Allen, 1996; Wang et al., 2005) and water deficit (Badawi et al., 2004).

To the best of our knowledge, the regulation of the aforementioned genes and their effect on stomatal functioning has not yet been analyzed in genotypes with contrasting tolerance to high RH.

Aim and outline of the thesis

The aim of this thesis is to understand the physiological and molecular mechanisms behind stomatal malfunctioning in plants cultivated at high RH focusing on the ABA regulation and its effects on stomatal closure. Moreover, the identification of genomic regions associated with the control of water loss is also a subject of this study. *Rosa* × *hybrida* was used as the model crop because: (i) it is a species showing genotypic differences regarding tolerance to high RH during growth either in commercial cultivars as well as within the available inbred K5 tetraploid cut rose population; (ii) it is one of the economically most important ornamental crops worldwide.

Studies have described a correlation between foliar [ABA] and genotypic variability regarding tolerance to high RH during growth, however, the causes for these differences in [ABA] are not yet understood. In **Chapter 2** we investigated the role of the root and leaf [ABA] in the ABA signaling inducing stomatal closure and in determining the genotypic variability of stomatal functioning in ten genotypes grown at contrasting RH conditions.

Elevated air movement (MOV) and moderate salinity are two environmental factors known to induce stomatal closure. Here, we combined each of them with high RH throughout leaf development in order to test if these physiological stresses would counteract the negative effect of high RH on stomatal functioning. To this end, the interactive effects of MOV and RH (**Chapter 3.1**) and of salinity and RH (**Chapter 4.1**) on the stomatal physiology, leaf [ABA] and its metabolites, plant growth and visual quality parameters are analyzed. Furthermore, **Chapter 3.2** describes the effects of MOV and RH on the postharvest quality and stress tolerance to dark and drought of pot roses (simulating shipping and/or retailing) while **Chapter 4.2** analyzes the effects of salinity and RH on vase life longevity of cut roses.

Chapter 5 assesses the genotypic variability of a subset of the K5 tetraploid cut rose population grown at high RH and phenotypically evaluated for stomatal responsiveness to desiccation. Genomic regions associated with the control of water loss in plants grown at high RH are identified. This work contributes to speeding up the selection of offspring using MAS programs in breeding for cultivars with more responsive stomata after cultivation at high RH, minimizing the negative impacts associated with excessive water loss during the postharvest phase.

Chapter 6 investigates the transcript levels of ABA-related genes (i.e., involved in the biosynthesis, catabolism, conjugation and signaling) and non-ABA related genes (involved in the water stress response) in four contrasting genotypes selected from the K5 tetraploid cut rose population (Chapter 5) aiming to get more insight into the molecular mechanisms underlying contrasting stomatal functioning in plants grown at high RH.

The results of the previous chapters are discussed in **Chapter 7**. The main causes for stomatal malfunctioning in plants grown at high RH are highlighted and topics for future research are proposed. A graphic representation of the outline of this thesis and its association with the problem statement is represented in Figure 3.

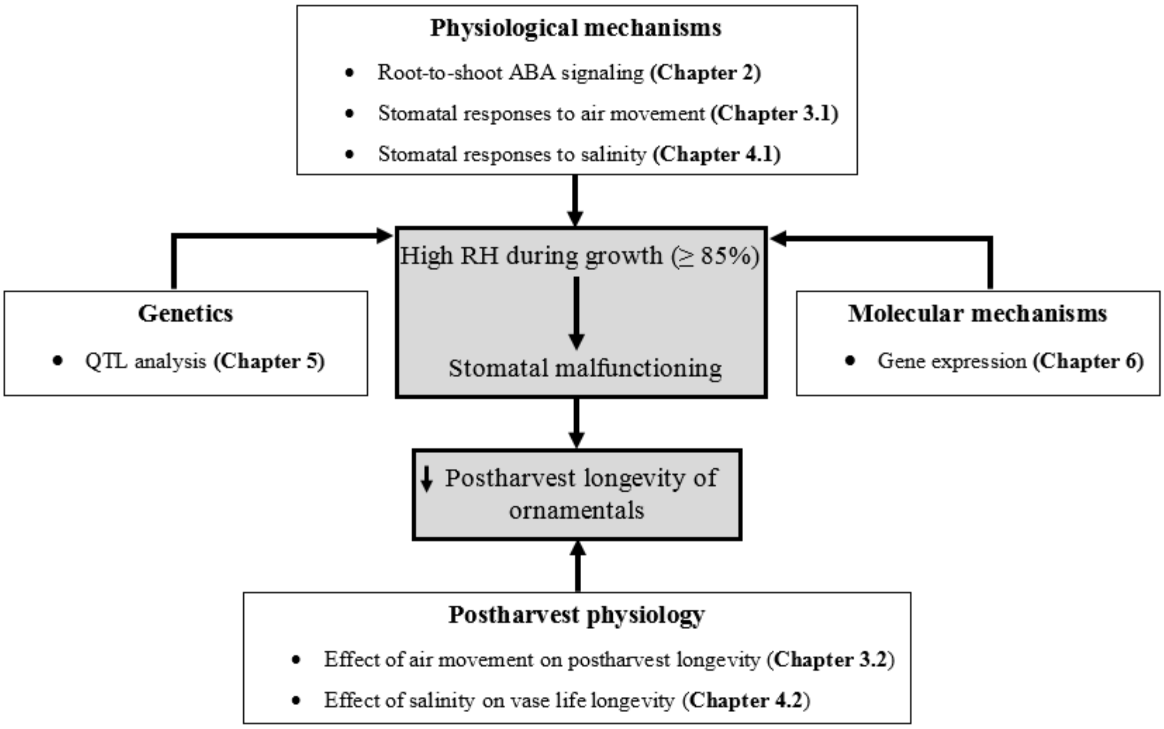

Fig. 3. Schematic representation of the thesis outline and its association with the problem statement.

References

Aasamaa, K., Sõber, A., 2011. Stomatal sensitivities to changes in leaf water potential, air humidity, CO_2 concentration and light intensity, and the effect of abscisic acid on the sensitivities in six temperate deciduous tree species. Environ. Exp. Bot. 71, 72–78.

Aguilar, M.L., Espadas, F.L., Coello, J., Maust, B.E., Trejo, C., Robert, M.L., Santamaría, J.M., 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. J. Exp. Bot. 51, 1861–1866.

Aliniaeifard, S., Malcolm Matamoros, P., van Meeteren, U., 2014. Stomatal malfunctioning under low VPD conditions: Induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? Physiol. Plant. 152, 688–699.

Aliniaeifard, S., van Meeteren, U., 2014. Natural variation in stomatal response to closing stimuli among *Arabidopsis thaliana* accessions after exposure to low VPD as a tool to recognize the mechanism of disturbed stomatal functioning. J. Exp. Bot. 65, 6529–6542.

Anten, N.P.R., Alcalá-Herrera, R., Schieving, F., Onoda, Y., 2010. Wind and mechanical stimuli differentially affect leaf traits in *Plantago major*. New Phytol. 188, 554–564.

Arve, L., Torre, S., Olsen, J., Tanino, K., 2011. Stomatal responses to drought stress and air humidity. In: Shanker, P.A. (Ed.), Abiotic Stress in Plants - Mechanisms and Adaptations.

Arve, L.E., Terfa, M.T., Gislerød, H.R., Olsen, J.E., Torre, S., 2013. High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves. Plant, Cell Environ. 36, 382–392.

Arve, L.E., Kruse, O.M.O., Tanino, K.K., Olsen, J.E., Futsæther, C., Torre, S., 2015. Growth in continuous high air humidity increases the expression of CYP707A-genes and inhibits stomatal closure. Environ. Exp. Bot. 115, 11–19.

Assmann, S.M., Shimazaki, K., 1999. The multisensory guard cell. Stomatal responses to blue light and abscisic acid. Plant Physiol. 119, 809–816.

Assmann, S.M., Snyder, J.A., Lee, Y.J., 2000. ABA-deficient (aba1) and ABA-insensitive (abi1-1, abi2-1) mutants of *Arabidopsis* have a wild-type stomatal response to humidity. Plant, Cell Environ. 23, 387–395.

Badawi, G.H., Kawano, N., Yamauchi, Y., Shimada, E., Sasaki, R., Kubo, A., Tanaka, K., 2004. Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiol. Plant. 121, 231–238.

Baptista, F.J., Bailey, B.J., Meneses, J.F., 2012. Effect of nocturnal ventilation on the occurrence of *Botrytis cinerea* in Mediterranean unheated tomato greenhouses. Crop Prot. 32, 144–149.

Beryl, C.A., Mitchell, C.A., 1977. Characterization of mechanical stress dwarfing in chrysanthemum. J. Am. Soc. Hortic. Sci. 102, 591 – 594.

Blatt, M., 2000. Cellular signaling and volume control in stomatal movements in plants. Annu. Rev. Cell Dev. Biol. 16, 221–241.

Bouchabke, O., Chang, F., Simon, M., Voisin, R., Pelletier, G., Durand-Tardif, M., 2008. Natural variation in *Arabidopsis thaliana* as a tool for highlighting differential drought responses. PLoS One 3, e1705.

Brodribb, T.J., McAdam, S.A.M., 2011. Passive origins of stomatal control in vascular plants. Science 331, 582–585.

Brosché, M., Merilo, E., Mayer, F., Pechter, P., Puzõrjova, I., Brader, G., Kangasjärvi, J., Kollist, H., 2010. Natural variation in ozone sensitivity among *Arabidopsis thaliana* accessions and its relation to stomatal conductance. Plant, Cell Environ. 33, 914–925.

Buckley, T.N., 2005. The control of stomata by water balance. New Phytol. 168, 275–292.

Buckley, T.N., Mott, K.A., 2002. Dynamics of stomatal water relations during the humidity response: Implications of two hypothetical mechanisms. Plant, Cell Environ. 25, 407–419.

Campen, J.B., Bot, G.P.A., Zwart, H.F. de, 2003. Dehumidification of greenhouses at northern latitudes. Biosyst. Eng. 86, 487–493.

Carpenter, W.J., Rasmussen, H.P., 1974. The role of flower and leaves in cut flower water uptake. Sci. Hortic. (Amsterdam). 2, 293–298.

Choi, H., Hong, J., Ha, J., Kang, J., Kim, S.Y., 2000. ABFs, a family of ABA responsive element binding factors. J. Biol. Chem. 275, 1723–1730.

Christmann, A., Hoffmann, T., Teplova, I., Grill, E., Müller, A., 2005. Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed *Arabidopsis*. Plant Physiol. 137, 209–219.

Christmann, A., Weiler, E.W., Steudle, E., Grill, E., 2007. A hydraulic signal in root-to-shoot signalling of water shortage. Plant J. 52, 167–174.

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B., Pang, E.C.K., 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142, 169–196.

Coutinho, P.M., Deleury, E., Davies, G.J., Henrissat, B., 2003. An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. 328, 307–317.

Cramer, G.R., Nowak, R.S., 1992. Supplemental manganese improves the relative growth, net assimilation and photosynthetic rates of salt-stressed barley. Physiol. Plant. 84, 600–605.

Crespel, L., Chirollet, M., Durel, C.E., Zhang, D., Meynet, J., Gudin, S., 2002. Mapping of qualitative and quantitative phenotypic traits in *Rosa* using AFLP markers. Theor. Appl. Genet. 105, 1207–1214.

Cutler, A.J., Krochko, J.E., 1999. Formation and breakdown of ABA. Trends Plant Sci. 4, 472–477.

Dietz, K.J., Sauter, A., Wichert, K., Messdaghi, D., Hartung, W., 2000. Extracellular beta-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. J. Exp. Bot. 51, 937–944.

Dodd, I.C., 2005. Root-to-shoot signalling: Assessing the roles of “up” in the up and down world of long-distance signalling in planta. Plant Soil 274, 251–270.

Dodd, I.C., Theobald, J.C., Richer, S.K., Davies, W.J., 2009. Partial phenotypic reversion of ABA-deficient flacca tomato (*Solanum lycopersicum*) scions by a wild-type rootstock: Normalizing shoot ethylene relations promotes leaf area but does not diminish whole plant transpiration rate. J. Exp. Bot. 60, 4029–4039.

Drake, P.L., Froend, R.H., Franks, P.J., 2013. Smaller, faster stomata: Scaling of stomatal size, rate of response, and stomatal conductance. J. Exp. Bot. 64, 495–505.

Dugo, M.L., Satovic, Z., Millán, T., Cubero, J.I., Rubiales, D., Cabrera, A., Torres, a. M., 2005. Genetic mapping of QTLs controlling horticultural traits in diploid roses. Theor. Appl. Genet. 111, 511–520.

Elhindi, K.M., 2012. Evaluation of several holding solutions for prolonging vase-life and keeping quality of cut sweet pea flowers (*Lathyrus odoratus* L.). Saudi J. Biol. Sci. 19, 195–202.

Endo, A., Sawada, Y., Takahashi, H., Okamoto, M., Ikegami, K., Koiwai, H., Seo, M., Toyomasu, T., Mitsuhashi, W., Shinozaki, K., Nakazono, M., Kamiya, Y., Koshiba, T., Nambara, E., 2008. Drought induction of *Arabidopsis* 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. Plant Physiol. 147, 1984–1993.

Ennos, A.R., 1997. Wind as an ecological factor. Trends Ecol. Evol. 12, 108–111.

Erner, Y., Biro, R., Jaffe, M.J., 1980. Thigmomorphogenesis: Evidence for a translocatable thigmomorphogenetic factor induced by mechanical perturbation of beans (*Phaseolus vulgaris*). Physiol. Plant. 50, 21–25.

Fanourakis, D., Tapia, a., Heuvelink, E., Carvalho, S.M.P., 2009. Cultivar differences in the stomatal characteristics of cut roses grown at high relative humidity. Acta Hortic. 847, 251–258.

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F., Heuvelink, E., 2011. Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. Physiol. Plant. 142, 274–286.

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F., van Kooten, O., van Doorn, W.G., Heuvelink, E., 2012a. Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biol. Technol. 64, 64–73.

Fanourakis, D., Carvalho, D.R.A., Gitonga, V.W., Van Heusden, A.W., Almeida, D.P.F., Heuvelink, E., Carvalho, S.M.P., 2012b. Breeding cut roses for better keeping quality : First steps. In: Acta Horticulturae 937, 875–882.

Fanourakis, D., Pieruschka, R., Savvides, A., Macnish, A.J., Sarlikioti, V., Woltering, E.J., 2013a. Sources of vase life variation in cut roses: A review. Postharvest Biol. Technol. 78, 1–15.

Fanourakis, D., Heuvelink, E., Carvalho, S.M.P., 2013b. A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. J. Plant Physiol. 170, 890–898.

Fanourakis, D., Giday, H., Milla, R., Pieruschka, R., Kjaer, K.H., Bolger, M., Vasilevski, A., Nunes-Nesi, A., Fiorani, F., Ottosen C-O, 2015. Pore size regulates operating stomatal conductance , while stomatal densities drive the partitioning of conductance between leaf sides. Ann. Bot. 115, 555–565.

Felle, H.H., Hanstein, S., Steinmeyer, R., Hedrich, R., 2000. Dynamics of ionic activities in the apoplast of the sub-stomatal cavity of intact *Vicia faba* leaves during stomatal closure evoked by ABA and darkness. Plant J. 24, 297–304.

Franks, P.J., 2013. Passive and active stomatal control: Either or both? New Phytol. 198, 325–327.

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.-Y., Cutler, S.R., Sheen, J., Rodriguez, P.L., Zhu, J.-K., 2009. *In vitro* reconstitution of an abscisic acid signalling pathway. Nature 462, 660–4.

Geiger, D., Scherzer, S., Mumm, P., Stange, A., Marten, I., Bauer, H., Ache, P., Matschi, S., Liese, A., Al-Rasheid, K. a S., Romeis, T., Hedrich, R., 2009. Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. Proc. Natl. Acad. Sci. U. S. A. 106, 21425–21430.

Giday, H., Kjaer, K.H., Fanourakis, D., Ottosen, C.O., 2013a. Smaller stomata require less severe leaf drying to close: A case study in *Rosa hydrida*. J. Plant Physiol. 170, 1309–1316.

Giday, H., Fanourakis, D., Kjaer, K.H., Fomsgaard, I.S., Ottosen, C.O., 2013b. Foliar abscisic acid content underlies genotypic variation in stomatal responsiveness after growth at high relative air humidity. Ann. Bot. 112, 1857–1867.

Giday, H., Fanourakis, D., Kjaer, K.H., Fomsgaard, I.S., Ottosen, C.O., 2014. Threshold response of stomatal closing ability to leaf abscisic acid concentration during growth. J. Exp. Bot. 65, 4361–4370.

Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P.C., Bouvier-Durand, M., Vartanian, N., 1994. Current advances in abscisic acid action and signalling. Plant Mol. Biol. 26, 1557–1577.

Grace J, 1977. Plant responses to wind. Academic Press, London, UK.

Gutha, L.R., Reddy, A.R., 2008. Rice *DREB1B* promoter shows distinct stress-specific responses, and the overexpression of cDNA in tobacco confers improved abiotic and biotic stress tolerance. Plant Mol. Biol. 68, 533–555.

Hauser, F., Waadt, R., Schroeder, J.I., 2011. Evolution of abscisic acid synthesis and signaling mechanisms. Curr. Biol. 21.

Heilmeier, H., Schulze, E.D., Fan, J., Hartung, W., 2007. General relations of stomatal responses to xylem sap abscisic acid under stress in the rooting zone - A global perspective. Flora 202, 624–636.

Hetherington, A.M., Woodward, F.I., 2003. The role of stomata in sensing and driving environmental change. Nature 424, 901–8.

Hong, Z., Zhang, Z., Olson, J.M., Verma, D.P., 2001. A novel UDP-glucose transferase is part of the callose synthase complex and interacts with phragmoplastin at the forming cell plate. Plant Cell 13, 769–779.

Hronková, M., Zahradníčková, H., Šimková, M., Šimek, P., Heydová, A., 2003. The role of abscisic acid in acclimation of plants cultivated in vitro to ex vitro conditions. Biol. Plant. 46, 535–541.

Ito, O., Ella, E., Kawano, N., 1999. Physiological basis of submergence tolerance in rainfed lowland rice ecosystem. F. Crop. Res. 64, 75–90.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., 2001. Regulation of drought tolerance by gene manipulation of 9- cis -epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. Plant J. 27, 325–333.

Jaffe, M.J., 1976. Thigmomorphogenesis: Electrical resistance and mechanical correlates of the early events of growth retardation due to mechanical stimulation in beans. Zeitschrift für Pflanzenphysiologie 78, 24–32.

Jafri, A.Z., Ahmad, R., 1995. Effect of soil salinity on leaf development, stomatal size and its distribution in cotton (*Gossypium hirsutum* L.). Pak J Bot 27, 297–303.

Jia, H.-F., Chai, Y.-M., Li, C.-L., Lu, D., Luo, J.-J., Qin, L., Shen, Y.-Y., 2011. Abscisic acid plays an important role in the regulation of strawberry fruit ripening. Plant Physiol. 157, 188–199.

Jin, J., Shan, N., Ma, N., Bai, J., Gao, J., 2006. Regulation of ascorbate peroxidase at the transcript level is involved in tolerance to postharvest water deficit stress in the cut rose (*Rosa hybrida* L.) cv. Samantha. Postharvest Biol. Technol. 40, 236–243.

Kang, J., Choi, H., Im, M., Kim, S.Y., 2002. Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell 14, 343–357.

Kang, J.G., Van Iersel, M.W., 2004. Nutrient solution concentration affects shoot:root ratio, leaf area ratio, and growth of subirrigated salvia (*Salvia splendens*). HortScience 39, 49–54.

Kim, T.-H., Böhmer, M., Hu, H., Nishimura, N., Schroeder, J.I., 2010. Guard cell signal transduction network: Advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu. Rev. Plant Biol. 61, 561–591.

Klingler, J.P., Batelli, G., Zhu, J.K., 2010. ABA receptors: The START of a new paradigm in phytohormone signalling. J. Exp. Bot. 61, 3199–3210.

Kushiro, T., Okamoto, M., Nakabayashi, K., Yamagishi, K., Kitamura, S., Asami, T., Hirai, N., Koshiba, T., Kamiya, Y., Nambara, E., 2004. The Arabidopsis cytochrome P450 CYP707A encodes ABA 8’-hydroxylases: key enzymes in ABA catabolism. EMBO J. 23, 1647–1656.

Lambers, H., Chapin, F.S., Pons, T.L., 2008. Plant physiological ecology, Zhurnal Eksperimental’noi i Teoreticheskoi Fiziki. Springer, New York, USA.

Lata, C., Prasad, M., 2011. Role of DREBs in regulation of abiotic stress responses in plants. J. Exp. Bot. 62, 4731–4748.

Lawson, T., 2009. Guard cell photosynthesis and stomatal function. New Phytol. 181, 13–34.

Lee, K.H., Piao, H.L., Kim, H.Y., Choi, S.M., Jiang, F., Hartung, W., Hwang, I., Kwak, J.M., Lee, I.J., Hwang, I., 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. Cell 126, 1109–1120.

Lee, M., van Iersel, W., 2008. Sodium chloride effects on growth, morphology, and physiology of chrysanthemum (*Chrysanthemum* × *morifolium*). Hortscience 43, 1888–1891.

Leung, J., Giraudat, J., 1998. Abscisic Acid Signal Transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 199–222.

Linde, M., Hattendorf, a., Kaufmann, H., Debener, T., 2006. Powdery mildew resistance in roses: QTL mapping in different environments using selective genotyping. Theor. Appl. Genet. 113, 1081–1092.

Liu, X., Yue, Y., Li, B., Nie, Y., Li, W., Wu, W.-H., Ma, L., 2007. A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science (80). 315, 1712–1716.

Lovelli, S., Scopa, A., Perniola, M., Di Tommaso, T., Sofo, A., 2012. Abscisic acid root and leaf concentration in relation to biomass partitioning in salinized tomato plants. J. Plant Physiol. 169, 226–233.

Maggio, A., Raimondi, G., Martino, A., De Pascale, S., 2007. Salt stress response in tomato beyond the salinity tolerance threshold. Environ. Exp. Bot. 59, 276–282.

Martin-Vertedor, A.I., Dodd, I.C., 2011. Root-to-shoot signalling when soil moisture is heterogeneous: Increasing the proportion of root biomass in drying soil inhibits leaf growth and increases leaf abscisic acid concentration. Plant, Cell Environ. 34, 1164–1175.

Max, J.F.J., Horst, W.J., Mutwiwa, U.N., Tantau, H.J., 2009. Effects of greenhouse cooling method on growth, fruit yield and quality of tomato (*Solanum lycopersicum* L.) in a tropical climate. Sci. Hortic. (Amsterdam). 122, 179–186.

Mcadam, S.A.M., Brodribb, T.J., 2013. Ancestral stomatal control results in a canalization of fern and lycophyte adaptation to drought. New Phytol. 198, 429–441.

McAdam, S.A.M., Sussmilch, F.C., Brodribb, T.J., 2015. Stomatal responses to vapour pressure deficit are regulated by high speed gene expression in angiosperms. Plant. Cell Environ.

Merlot, S., Mustilli, A.-C., Genty, B., North, H., Lefebvre, V., Sotta, B., Vavasseur, A., Giraudat, J., 2002. Use of infrared thermal imaging to isolate Arabidopsis mutants defective in stomatal regulation. Plant J. 30, 601–609.

Mortensen, L., Gislerød, H., 1997. Effects of air humidity and air movement on the growth and keeping quality of roses. Gartenbauwissenschaf 62, 273–277.

Mortensen, L.M., Gislerød, H.R., 1999. Influence of air humidity and lighting period on growth, vase life and water relations of 14 rose cultivars. Sci. Hortic. (Amsterdam). 82, 289–298.

Mortensen, L.M., 2000. Effects of air humidity on growth, flowering, keeping quality and water relations of four short-day greenhouse species. Sci. Hortic. (Amsterdam). 86, 299–310.

Mortensen, L.M., Gislerød, H.R., 2005. Effect of air humidity variation on powdery mildew and keeping quality of cut roses. Sci. Hortic. (Amsterdam). 104, 49–55.

Mortensen, L., Pettersen, R., Gislerod, H., 2007. Air humidity variation and control of vase life and powdery mildew in cut roses under continuous lighting. Eur. J. Hortic. Sci. 72, 255–259.

Mortensen, L., Gislerod, H., 2011. Vase life: The influence of variation in air humidity, temperature and super-elevated CO2 concentration in roses grown under continuous light. Eur. J. Hortic. Sci. 76, 63–68.

Munns, R., 2002. Comparative physiology of salt and water stress. Plant. Cell Environ. 25, 239–250.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681.

Mustilli, A.-C., Merlot, S., Vavasseur, A., Fenzi, F., Giraudat, J., 2002. *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell 14, 3089–3099.

Nambara, E., Marion-Poll, A., 2005. Abscisic acid biosynthesis and catabolism. Annu. Rev. Plant Biol. 56, 165–185.

Niu, G., Rodriguez, D.S., Gu, M., 2010. Salinity tolerance of *Sophora secundiflora* and *Cercis canadensis* var . mexicana. Hortscience 45, 424–427.

Okamoto, M., Tanaka, Y., Abrams, S.R., Kamiya, Y., Seki, M., Nambara, E., 2009. High humidity induces abscisic acid 8’-hydroxylase in stomata and vasculature to regulate local and systemic abscisic acid responses in *Arabidopsis*. Plant Physiol. 149, 825–834.

Orsini, F., Alnayef, M., Bona, S., Maggio, A., Gianquinto, G., 2012. Low stomatal density and reduced transpiration facilitate strawberry adaptation to salinity. Environ. Exp. Bot. 81, 1–10.

Pantin, F., Renaud, J., Barbier, F., Vavasseur, A., Le Thiec, D., Rose, C., Bariac, T., Casson, S., McLachlan, D.H., Hetherington, A.M., Muller, B., Simonneau, T., 2013. Developmental priming of stomatal sensitivity to abscisic acid by leaf microclimate. Curr. Biol. 23, 1805–1811.

Park, S., Fung, P., Nishimura, N., Jensen, D.R., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Alfred, S.E., Bonetta, D., Finkelstein, R., Provart, N.J., Rodriguez, P.L., Mccourt, P., Zhu, J., Schroeder, J.I., Volkman, B.F., Cutler, S.R., 2009. Abscisic acid inhibits PP2Cs via the PYR/PYL family of ABA- binding START proteins. Science (80). 324, 1068–1071.

Pitcher, L.H., Repetti, P., Zilinskas, B.A., 1994. Overproduction of ascorbate peroxidase protects transgenic tobacco plants against oxidative stress. Plant Physiol. 105 (Suppl, 116).

Reid, M.S., Evans, R.Y., 1986. Control of cut flower opening. Acta Hortic. 181, 45–54.

Reid, M.S., Jiang, C.Z., 2012. Postharvest biology and technology of cut flowers and potted plants. Hortic. Rev. (Am. Soc. Hortic. Sci). 40, 1–54.

Rezaei Nejad, A., van Meeteren, U., 2005. Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. Physiol. Plant. 125, 324–332.

Rezaei Nejad, A., van Meeteren, U., 2007. The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity. J. Exp. Bot. 58, 627–636.

Rezaei Nejad, A., Van Meeteren, U., 2008. Dynamics of adaptation of stomatal behaviour to moderate or high relative air humidity in *Tradescantia virginiana*. J. Exp. Bot. 59, 289–301.

Ribaut, J.-M., Hoisington, D., 1998. Marker-assisted selection: new tools and strategies. Trends Plant Sci. 3, 236–239.

Sáez, P.L., Bravo, L.A., Latsague, M.I., Sánchez, M.E., Ríos, D.G., 2012. Increased light intensity during *in vitro* culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels. Sci. Hortic. (Amsterdam). 138, 7–16.

Saito, S., Hirai, N., Matsumoto, C., Ohigashi, H., Ohta, D., 2004. Arabidopsis CYP707As encode (+)-abscisic acid 8’-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. Plant Physiol. 134, 1439–1449.

Santamaria, J.M., Davies, W.J., Atkinson, C.J., 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. J. Exp. Bot. 44, 99–107.

Schachtman, D.P., Goodger, J.Q.D., 2008. Chemical root to shoot signaling under drought. Trends Plant Sci. 13, 281–287.

Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M., Waner, D., 2001. Guard cell signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 627–658.

Schuepp, P.H., 1993. Tansley Review No. 59 Leaf boundary layers. New Phytol. 125, 477–507.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart, J.A., McCarty, D.R., 1997. Specific oxidative cleavage of carotenoids by VP14 of maize. Science (80). 276, 1872–1874.

Seo, M., Koshiba, T., 2002. Complex regulation of ABA biosynthesis in plants. Trends Plant Sci. 7, 41–48.

Seo, M., Koshiba, T., 2011. Transport of ABA from the site of biosynthesis to the site of action. J. Plant Res. 124, 501–507.

Seo, M., Peeters, a J., Koiwai, H., Oritani, T., Marion-Poll, a, Zeevaart, J. a, Koornneef, M., Kamiya, Y., Koshiba, T., 2000. The Arabidopsis aldehyde oxidase 3 (*AAO3*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc. Natl. Acad. Sci. U. S. A. 97, 12908–12913.

Shalhevet, J., Bernstein, L., 1968. Effects of vertically heterogeneous soil salinity on plant growth and water uptake. Soil Sci. 106, 85–93.

Sharma, N., Gupta, N.K., Gupta, S., Hasegawa, H., 2005. Effect of NaCl salinity on photosynthetic rate, transpiration rate, and oxidative stress tolerance in contrasting wheat genotypes. Photosynthetica 43, 609–613.

Sirichandra, C., Davanture, M., Turk, B.E., Zivy, M., Valot, B., Leung, J., Merlot, S., 2010. The arabidopsis ABA-activated kinase OST1 phosphorylates the bZIP transcription factor ABF3 and creates a 14-3-3 binding site involved in its turnover. PLoS One 5, 1–13.

Sun, L., Sun, Y., Zhang, M., Wang, L., Ren, J., Cui, M., Wang, Y., Ji, K., Li, P., Li, Q., Chen, P., Dai, S., Duan, C., Wu, Y., Leng, P., 2012. Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. Plant Physiol. 158, 283–298.

Teulat, B., Zoumarou-Wallis, N., Rotter, B., Ben Salem, M., Bahri, H., This, D., 2003. QTL for relative water content in field-grown barley and their stability across Mediterranean environments. Theor. Appl. Genet. 108, 181–188.

Ting, W., Zheng, T., Nan, M., JunPing, G., 2009. Isolation and expression analysis of *Rh-DREB1s* gene in cut roses (*Rosa hybrida*) under ethylene treatment and water deficit stress. Acta Hortic. Sin. 36, 65–72.

Toh, S., Imamura, A., Watanabe, A., Nakabayashi, K., Okamoto, M., Jikumaru, Y., Hanada, A., Aso, Y., Ishiyama, K., Tamura, N., Iuchi, S., Kobayashi, M., Yamaguchi, S., Kamiya, Y., Nambara, E., Kawakami, N., 2008. High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in *Arabidopsis* seeds. Plant Physiol. 146, 1368–1385.

Torre, S., Fjeld, T., 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. Sci. Hortic. (Amsterdam). 89, 217–226.

Tullus, A., Kupper, P., Sellin, A., Parts, L., Sõber, J., Tullus, T., Lõhmus, K., Sõber, A., Tullus, H., 2012. Climate change at Northern latitudes: Rising atmospheric humidity decreases transpiration, N-uptake and growth rate of hybrid aspen. PLoS One 7, 1–11.

Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., Shinozaki, K., 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. Proc. Natl. Acad. Sci. U. S. A. 106, 17588–17593.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., Yamaguchi-Shinozaki, K., 2000. *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc. Natl. Acad. Sci. U. S. A. 97, 11632–11637.

Van Kooten, O., Kuiper, E., 2009. Consumer acceptability in flower chains: How can we determine what the final customers really want? Acta Hortic. 847, 17–26.

Van Meeteren, U., van Gelder, A., van Ieperen, W., 2005. Effect of growth conditions on post harvest rehydration ability of cut chrysanthemum flowers. Acta Hortic. 669, 287–296.

Veen, H., 1979. Effects of silver on ethylene synthesis and action in cut carnations. Planta 145, 467–470.

Wang, Y., Wisniewski, M., Meilan, R., Cui, M., Webb, R., Fuchigami, L., 2005. Overexpression of cytosolic ascorbate peroxidase in tomato confers tolerance to chilling and salt stress. J. Am. Soc. Hortic. Sci. 130, 167–173.

Webb, R.P., Allen, R.D., 1996. Overexpression of pea cytosolic ascorbate peroxidase confers protection against oxidative stress in transgenic *Nicotiana tabacum*. Plant Physiol. 111 (Suppl, 48).

Weyers, J.D., Hillman, J.R., 1979. Sensitivity of *Commelina* stomata to abscisic acid. Planta 146, 623–8.

Whitehead, F., 1962. Experimental studies of the effect of wind on plant growth and anatomy. New Phytol. 61, 59–62.

Wilkinson, S., Davies, W.J., 2002. ABA-based chemical signalling : the co-ordination of responses to stress in plants. Plant, Cell Environ. 25, 195–210.

Williamson, B., Duncan, G.H., Harrison, J.G., Harding, L. a., Elad, Y., Zimand, G., 1995. Effect of humidity on infection of rose petals by dry-inoculated conidia of *Botrytis cinerea*. Mycol. Res. 99, 1303–1310.

Willmer, C., Fricker, M., 1996. Stomata, 2nd ed. Chapman & Hall, London, UK.

Xu, Z.-Y., Lee, K.H., Dong, T., Jeong, J.C., Jin, J.B., Kanno, Y., Kim, D.H., Kim, S.Y., Seo, M., Bressan, R. a., Yun, D.-J., Hwang, I., 2012. A vacuolar β-glucosidase homolog that possesses glucose-conjugated abscisic acid hydrolyzing activity plays an important role in osmotic stress responses in *Arabidopsis*. Plant Cell 24, 2184–2199.

Yamaguchi-Shinozaki, K., Shinozaki, K., 2005. Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci. 10, 88–94.

Zeevaart, J.A.D., Creelman, R.A., 1988. Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 439–473.

Zhang, S.Q., Outlaw, W.H., 2001. Abscisic acid introduced into the transpiration stream accumulates in the guard-cell apoplast and causes stomatal closure. Plant, Cell Environ. 24, 1045–1054.

Zhao, X.Q., Xu, J.L., Zhao, M., Lafitte, R., Zhu, L.H., Fu, B.Y., Gao, Y.M., Li, Z.K., 2008. QTLs affecting morph-physiological traits related to drought tolerance detected in overlapping introgression lines of rice (*Oryza sativa* L.). Plant Sci. 174, 618–625.

Ziv, M., Schwartz, A., Fleminger, D., 1987. Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; Implications for hardening. Plant Sci. 52, 127–134.

CHAPTER 2

Root-to-shoot ABA signaling does not contribute to genotypic variation in stomatal functioning induced by high relative air humidity

Abstract

High relative air humidity (RH ≥ 85%) during leaf expansion hampers stomatal responsiveness to closing stimuli, a genotype-dependent effect. Genotypes with reduced stomatal closure in response to closing stimuli (i.e., sensitive genotypes) show low bulk leaf abscisic acid concentration ([ABA]). We hypothesized that the amount of ABA produced in the roots and delivered with the transpiration stream to the leaves is a critical step for a proper stomatal functioning in high RH-grown plants. Ten genotypes from a cut rose tetraploid population were grown at moderate (62%) or high (89%) RH. Stomatal anatomy and responsiveness to desiccation or ABA feeding were evaluated. Root and leaf petiole xylem sap [ABA] were quantified, and ABA delivery rate from root-to-shoot was estimated. High RH reduced stomatal responsiveness to both closing stimuli in the sensitive genotypes, whereas it had a non-significant effect in the tolerant ones. Estimates of [ABA] in root xylem sap at transpirational flow rate were not related to the genotypic differences in the sensitivity to high RH. However, high RH increased this concentration irrespective of the genotype, probably due to a reduced dilution of the [ABA] in the xylem sap as a result of a lower transpiration rate compared to moderate RH-grown plants. Leaf petiole xylem sap [ABA] was neither related to the genotypic differences in the sensitivity to high RH nor it was affected by RH. We concluded that genotypic differences in the stomatal sensitivity to high RH cannot be explained by changes in the [ABA] delivered with the transpiration stream from the roots to the leaves.

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Introduction

Plants lose water through stomata, which are actively regulated pores situated on the leaf surface. Genotypic factors interact with environmental conditions during growth determining the ability of stomata to regulate their aperture (Fanourakis et al., 2012b). An illustrative case of this interaction is the plant exposure to long-term high relative air humidity (RH ≥ 85%). Such RH levels can often occur in horticultural production systems (e.g., protected cultivation during the winter period and *in vitro* culture vessels), but also in natural environments such as tropical and subtropical areas or northern latitudes (Fanourakis et al., 2009; Sáez et al., 2012; Tullus et al., 2012). At high RH, plants develop stomata that fail to close in response to closing stimuli (e.g., light-dark transition, desiccation or abscisic acid feeding), leading to excessive water loss following transfer to conditions of high evaporative demand (Torre et al., 2003; Rezaei Nejad and van Meeteren, 2005; Fanourakis et al., 2012a). Nonetheless, poor stomatal functioning has been shown to be highly cultivar dependent (Mortensen and Gislerød, 1999; Fanourakis et al., 2013; Giday et al., 2013), but the reasons of such genotypic differences remain elusive.

Recent studies relate stomatal malfunctioning to lower leaf ABA concentration ([ABA]) in Tr*asdescantia virginiana* (Rezaei Nejad and van Meeteren, 2007), *Vicia faba* (Aliniaeifard et al., 2014a) and *Rosa* × *hybrida* (Arve et al., 2013; Giday et al., 2013) developed at high (90%) RH, compared to plants cultivated at moderate (60%) RH. Moreover, lower stomatal sensitivity to ABA has also been suggested to be involved in stomatal malfunctioning (Carvalho et al., 2015; Pantin et al., 2013). Leaf [ABA], is determined by its metabolism within the leaf (biosynthesis *vs.* catabolism), as well as by its import from the roots, an important ABA production site (Wilkinson and Davies 2002). ABA is translocated from the roots via the xylem vessels to the leaves driven by the transpiration stream (Dodd, 2005). Recently it has been shown that the differential decrease of bulk leaf [ABA] was closely related to the genotypic variation in stomatal functioning of rose plants grown at high RH (Giday et al., 2013). Yet, it is still to be evaluated the role of the root-to-shoot ABA signaling in the functioning of stomata developed under contrasting RH conditions, as the majority of studies is associated with drought stress (Christmann et al., 2007; Schachtman and Goodger, 2008; Martin-Vertedor and Dodd, 2011). By employing ABA-deficient lines, Dodd et al. (2009) showed that root ABA synthesis affects leaf xylem [ABA], when leaf ABA synthesis is compromised. Since high RH decreases leaf [ABA] (Arve et al., 2013), it may be expected that root ABA synthesis also plays a role in determining leaf [ABA] under these conditions. Giday et al.

(2014) found that leaf [ABA] increased in leaves of a sensitive rose cultivar grafted onto a tolerant one grown at high RH, and speculated that this leaf [ABA] increase was due to a higher root ABA biosynthesis. Nonetheless, experimental evidence to support this claim is currently lacking. Therefore, assessing the root-to-shoot ABA signaling in plants grown at contrasting evaporating demands is of significant interest to understand the reasons underlying the high RH-induced stomatal malfunctioning, as well as the noted intraspecific variation in this effect.

The main objective of the present work was to understand the role of the root and leaf [ABA] in determining the genotypic variation of stomatal functioning in plants developed under contrasting RH environments. We hypothesize that tolerant genotypes have a higher ABA transport from the roots towards the leaves as compared to the sensitive ones, leading to a higher [ABA] in the petiole xylem sap and to a proper stomatal functioning when grown at high RH.

Materials and methods

Plant material and growth conditions

Ten genotypes (K148, K123, P867, K085, K039, K015, K107, K121, K059 and K099) were selected from a segregating tetraploid cut rose population previously phenotyped for stomatal responsiveness to desiccation after growth at high (85%) RH (Fanourakis et al., 2012b). Fourteen rooted cuttings per genotype were randomly distributed over two growth chambers (length × width × height = 2.0 × 1.6 × 2.0 m) (5000 EH, Aralab, Albarraque, Portugal) at a density of 29 plants m^{-2} (one plant per pot, single stem) and planted in 3.5 L pots containing a mixture (2/1, v/v) of sphagnum peat (Floragard, Oldenburg, Germany) and perlite (Otavi, Neuss, Germany). During growth, the RH in one growth chamber was 62 ± 2% (moderate RH), while in the other one it was 89 ± 4% (high RH). Temperature was kept constant at 19.0 ± 1.0 °C in both chambers, resulting in vapor pressure deficits (VPDs) of 0.83 ± 0.02 and 0.24 ± 0.07 kPa for moderate and high RH, respectively. The CO_2 concentration was 350 ± 20 µmol mol^{-1} (IAQ 910, TSI Incorporated, Shoreview, Minnesota, USA). Fluorescent lamps (Osram L58W/840, Lumilux, Cool White, Munich, Germany) provided 18 h photoperiod of 200 ± 10 µmol m^{-2} s^{-1} photosynthetic active radiation (Li-1000 datalogger, Li-Cor, Lincoln, Nebraska, USA), measured 50 cm above the root-shoot intersection. Plants were watered daily, automatically using a drip system, with a nutrient solution. The irrigation volume was increased in the course of the growth period, targeting at 30 to 35% drainage throughout cultivation. The nutrient solution contained both (i) macronutrients (mM) [NH_4 1.0, K 4.0, Ca 3.5, Mg 1.38, NO_3 10.5, SO_4 1.5, H_2PO_4 1.25] and (ii) micronutrients (μM) [Fe 25, Mn 5, Zn 3.5, B 20, Cu 0.75, Mo 0.5]. Electrical conductivity and pH of the nutrient solution were 2 dS m^{-1} and 5.4, respectively.

Stomatal responsiveness to desiccation

Stomatal responsiveness to desiccation was assessed gravimetrically in fully developed terminal leaflets (first penta-foliate counting from the apex) detached from fully grown plants (i.e., flower bud with cylindrical shape and pointed tip). Leaflets were sampled at the onset of the light period. Immediately after excision, leaflet petioles were recut under water (to prevent cavitation induced-embolism) and placed in flasks filled with degassed water. Leaflets were further incubated in a saturated RH (≈ 100%) environment at 21.0 ± 0.5 ºC (i.e., VPD close to 0 kPa) for 1 h to establish their saturated fresh weight (Fanourakis et al., 2011). The rehydration process took place under light (40 ± 3 μmol m^{-2} s^{-1}) to induce stomatal opening of leaflets previously exposed to prolonged darkness (Drake et al., 2013). After rehydration, the leaflets were allowed to desiccate for 4 h on a bench (abaxial surface down) in a test room with 1.14 ± 0.07 kPa VPD (54 ± 1% RH, 21 ± 0.5 ˚C) and 45 ± 2 μmol m^{-2} s^{-1} light intensity. Leaflet weight was recorded every 5–30 min (ABJ, Kern & Sohn GmbH, Balingen-Frommern, Germany). After evaluation, leaflet area was measured (AM 300, ADC BioScientific, Hoddesdon, England) and dry weight was determined (24 h at 70 ºC). Transpiration rate was calculated according to Equation 1. For the calculation of the relative water content (RWC), Equation 2 was employed (Slavík, 1974). Seven (one leaflet per plant) replicates per treatment were evaluated.

$$\text{Transpiration rate } (mmol\ m^{-2}\ s^{-1}) = \left(\left(\frac{\Delta\ \text{leaflet fresh weight } (g)}{\text{molar mass water } (g\ mol{-1})} \times 1000\ (mmol\ mol^{-1})\right) / \text{measurement frequency } (s)\right) / \text{leaflet area } (m^2) \quad \text{(Eqn. 1)}$$

$$\text{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{saturated fresh weight} - \text{dry weight}} \times 100 \quad \text{(Eqn. 2)}$$

Assessing the stomatal closing ability by gravimetric determination of the transpiration rate in detached leaflets has been shown to be an adequate quantitative description of the stomatal functionality since it shows the same trend as the stomatal conductance evaluated with a porometer (Rezaei Nejad and van Meeteren, 2005). This simple procedure has been widely adopted in stomatal studies (Rezaei Nejad and van Meeteren, 2005; Giday et al., 2013; Aliniaeifard and van Meeteren, 2014b; Fanourakis et al., 2015a) also due to its effectiveness under conditions of low stomatal conductance (i.e., excessively desiccated leaflets; e.g. RWC < 20%), which fall below the detection limit of the porometer.

Stomatal responsiveness to ABA feeding through the transpiration stream

The effect of RH during growth on stomatal responsiveness to ABA feeding through the transpiration stream was assessed in three genotypes. These were selected based on stomatal responsiveness to desiccation, and divided into sensitive (K148, K123) or tolerant (K121) genotypes (i.e., at high RH, sensitive genotypes show hampered stomatal response to closing stimuli, whereas the tolerant ones hold their closing ability). The terminal leaflets of the third or fourth penta-foliated leaf were excised and rehydrated, as described above (section 2.2). Following 1 h rehydration, leaflets were left in the vials to transpire for 30 min under the same controlled conditions. Vials were sealed with water-proof parafilm, ensuring that water loss could only occur through transpiration. Subsequently, the leaflets were transferred to solutions containing 0 (control) or 200 µM (±) ABA (Sigma, St. Louis, Missouri, USA). This [ABA] has been selected based on previous studies which tested the effect of an [ABA] dose response on stomatal closure in *Arabidopsis* (0, 50, 100 and 200; Aliniaeifard and van Meeteren, 2014b) and in rose cv. ‘Toril’ (0, 100, 200 and 300; Carvalho et al., data not published). Leaflet weight was recorded every 5–10 min for 2.5 h and at the end of the evaluation the leaflet area was measured. ABA intake was calculated as the product of the leaflet transpiration rate and the concentration of the feeding solution (Fanourakis et al., 2013). The changes in transpiration rate in response to ABA showed the features of a dose-response curve (ABA intake was considered as the dose), and was fitted with a four parameter logistic model as described by Giday et al. (2013). The model fitting (Equation 3) was performed using GraphPad Prism (version 6.00 for Windows, GraphPad Software, San Diego, California, USA). In Equation 3, the coefficients maximum and minimum value correspond to the transpiration rate before (t = 0) and after (t = 2.5 h) ABA feeding, respectively. EC_{50} refers to the amount of ABA required to reduce the transpiration rate half-way between the maximum and the minimum values. Hill slope represents the steepness of the curve. Five leaflets (one leaflet per plant) were evaluated per treatment.

$$\text{Transpiration rate} = \text{minimum value} + \frac{(\text{maximum value} - \text{minimum value})}{(1+10^{\wedge}((\text{LogEC50} - \text{ABA intake}) * \text{hill slope})} \text{(Eqn. 3)}$$

Stomatal anatomical characteristics

The effect of RH during growth on the stomatal anatomical characteristics was evaluated in the same three genotypes employed in section 2.3. Stomatal features included: stomatal density (i.e., number per unit area), index (i.e., stomatal density per total epidermal cell density; Salisbury, 1927), stomatal and pore length (i.e., longest diameter), stomatal and pore width (i.e., shortest diameter), pore area (i.e., π × (pore length/2) × (pore width/2)) and pore area per leaf area (i.e., stomatal density × pore area). Stomatal traits were analyzed on one lateral leaflet from the first fully expanded penta-foliated leaf, sampled 4 h after the onset of the light period. Epidermal impressions were made by the silicon rubber impression technique (Xantopren® VL Plus and Activator Universal, Heraeus Kulzer, Hanau, Germany; Smith et al., 1989) on the abaxial leaflet side, since rose is hypostomatous. The sampling area was located midway the leaflet base and tip, as well as between the midrib and lateral margin (Fanourakis et al., 2015b). A light microscope (Eclipse 55i, Nikon, Tokyo, Japan) connected to a digital camera (DS-Fi1, Nikon, Tokyo, Japan) was used to record the images. Stomatal and epidermal cell densities were assessed with 100× magnification, while for stomatal and pore dimensions a magnification of 400× was used. Image analysis was performed using the UTHSCSA ImageTool for windows version 3.00 (The University of Texas Health Science Center at San Antonio, Texas, USA). For stomatal and epidermal cell counting, 35 images (five images per leaflet, one leaflet per plant) were analyzed per treatment. Regarding stomatal and pore dimensions, 70 stomata (ten stomata per leaflet, one leaflet per plant) were evaluated per treatment.

Root and leaf petiole xylem sap ABA quantification

The effect of RH during growth on the [ABA] in the xylem sap of roots and leaf petioles of fully grown plants was quantified in the ten cultivated genotypes using six plants (root xylem sap [ABA]) and four leaves (petiole xylem sap [ABA]) per treatment. Both quantifications were done on the same plant. The employed protocol for collecting the root xylem sap was based on natural root exudation (Schurr, 1998). At first, well-watered plants (watered till container capacity in the beginning of the dark period) were decapitated approximately 2 cm above the soil level, immediately before the beginning of the light period. After discarding the first aliquot of the fluid (i.e., after 25 min), to avoid contamination from the symplast of the cells in the cut surface, the exudate produced during the next 40 min was collected, immediately frozen in liquid nitrogen and stored at –80 °C for further analysis. The [ABA] loaded into the root xylem has been found to be dependent on the flow of water through the roots (Jokhan et al., 1996; Schurr, 1998). In accordance, we found that [ABA] in root xylem sap decreased with increased root exudation rate (Fig 4B). Since the xylem sap flow is lower (95%) in detopped plants compared to intact ones, we estimated the concentration of ABA in root xylem sap of intact plants by multiplying the [ABA] in root exudate by the ratio between the exudation rate of detopped plants and the 'in vivo' transpiration per plant. Moreover, we have found a negative correlation between ABA delivery rate and 'in vivo' transpiration rate (P = 0.021; data not shown). Thus, although this methodology has not yet been validated, the possible errors associated to this calculation are consistent across genotypes. The ABA delivery rate per unit leaf area was calculated by multiplying the [ABA] in the root xylem sap at transpirational flow rate by the transpiration rate per unit of leaf area. 'In vivo' plant transpiration rate was measured gravimetrically during three consecutive days using fully developed plants. Well-watered plant pots were wrapped into impermeable plastic bags to avoid evaporation from the substrate, remaining with this set-up during measurements. During this period plants weight was recorded every 24 h. At the end of the 3-days period total leaf area per plant was measured to calculate transpiration rate per unit leaf area. Leaf petiole xylem sap was collected according to Correia et al. (1997). In short, leaf water potential was determined using a Scholander pressure chamber (Model 600, PMS Instrument Company, Albany, Oregon, USA), where an overpressure of 0.4 MPa was gradually applied and held constant during 3–4 min. After discarding the first drop of the fluid to prevent contamination from the symplast, the leaf xylem sap exudate was collected, immediately frozen in liquid nitrogen and stored at –80 °C for further analysis. In all treatments, leaf petiole xylem sap collection started 1 h after the onset of the light period and was completed 2 h thereafter. The tri-foliated leaf, above the first penta-foliated leaf, was used in this procedure because of its long petiole, facilitating the sap collection using the Scholander pressure chamber. [ABA] in the xylem sap of roots and leaf petioles was quantified by the ELISA immunoassay using monoclonal antibodies (Idetek, San Bruno, California, USA) following the instructions of the Idetek manual. This methodology has also been used in recent studies for ABA quantification in *Tradescantia virginiana* grown at moderate or high RH (Rezaei Nejad and Van Meeteren, 2008) and in *Arabidopsis* subjected to osmotic stress (Xu et al., 2012). Two replicate standard calibrations were made for each ELISA plate, using synthetic (±)-cis,trans-ABA (Sigma, St Louis, MO, USA), 50% of which was assumed to be the (+) form. Samples were analyzed without any previous purification. The validation of the ELISA method for accurate ABA measurements of non-purified samples was confirmed by assaying a replicate pooled sample of xylem exudate in the presence of increasing amounts of synthetic (±)-ABA. This showed that the ELISA estimate increased in proportion to the amount of ABA assayed, which indicates that non-specific interference was absent (Pengelly, 1985). Therefore, it was assumed that what has been measured in crude sap is ABA, although the identity of the immunoreactive compound present in xylem sap as true ABA was not checked by any other method.

Statistical design and analysis

The experimental set-up was a complete randomized design. Analysis of variance was conducted, considering individual plants as experimental units. Main effects and interactions were tested at $P = 0.05$. When relevant, Fisher's least significant difference (LSD) at $P = 0.05$ was calculated to separate means. Correlations were evaluated using Spearman's rank correlation coefficient. The Genstat software (15th edition; VSN International Ltd., Herts, UK) was used for the analysis. F-probabilities for the main effects of RH and genotype as well as for their interaction are provided in Table A1 (Appendix) for each variable evaluated in this study.

Results

High RH reduces stomatal responsiveness to desiccation and to ABA feeding depending on the genotype

The transpiration rate dynamics during 4 h of leaflet desiccation is shown for two sensitive (K148, K123) and one tolerant (K121) genotypes (Fig. 1). In general, plants grown at high RH showed higher initial transpiration rate and lower rate of stomatal closure (shallower slope of the curve) in comparison with plants grown at moderate RH. However, the difference in transpiration rate between plants grown at moderate and high RH was greater in the sensitive genotypes (Figs. 1A and 1B) and not significant in the tolerant one (Fig. 1C). When analyzing the relationship between leaflet transpiration rate and RWC, the difference between genotypes became more apparent (Fig. 1, inserts). High RH strongly reduced the RWC_4h in K148 (from 54 to 8%, insert of Fig. 1A) and K123 (from 58 to 11%, insert of Fig. 1B), whereas no significant decrease was noted in K121 (from 72 to

70%, insert of Fig. 1C). These results confirm the interest of further using RWC_4h as an indicator for discriminating between responsive and non-responsive stomata to water deprivation.

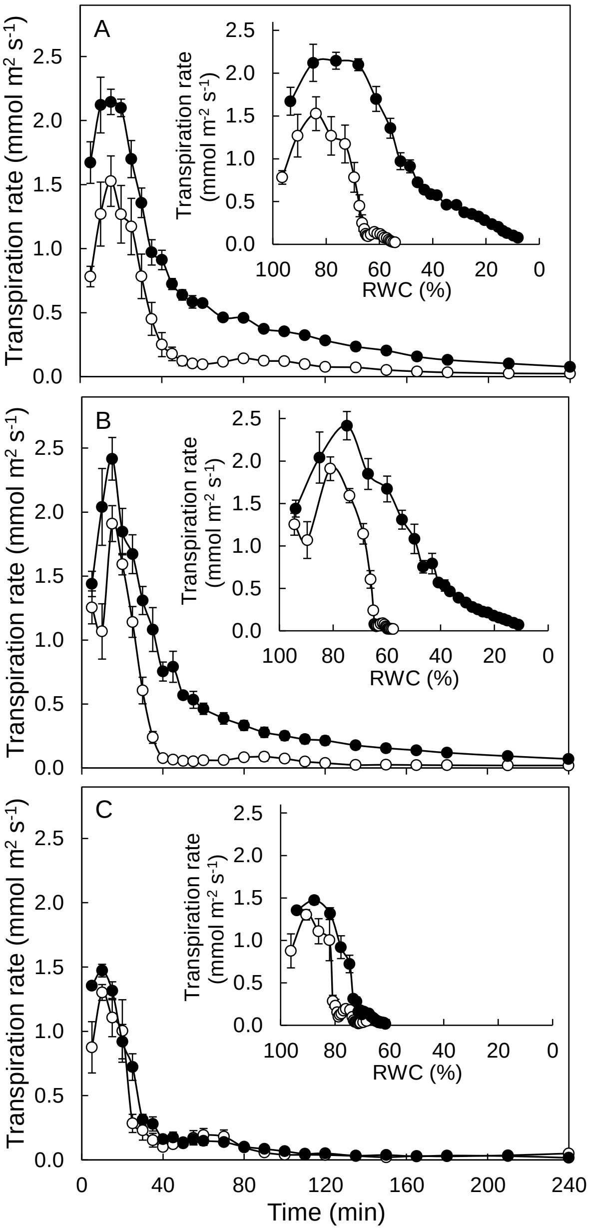

Fig. 1. Transpiration rate as a function of time and relative water content (RWC; inserts) during leaflet desiccation in two sensitive (A, K148; B, K123) and one tolerant (C, K121) genotypes to high relative air humidity (RH). Plants were grown at moderate (62%; open symbols) or high (89%; solid symbols) RH. Values are the mean of seven leaflets ± SEM

The effect of high RH on the RWC_4h was strongly genotype-dependent (Fig. 2). High RH during growth significantly decreased the RWC_4h in the six sensitive genotypes

(K148, K123, P867, K085, K039 and K015), while in the four tolerant ones (K107, K121, K059, K099) the RWC_4h was not significantly different from the values found in desiccated leaflets detached from plants grown under moderate RH (Fig. 2). In moderate RH-grown plants, the RWC_4h ranged between 50 and 72% while in high RH-grown plants it varied between 8 and 70%, representing a much wider variation (Fig. 2).

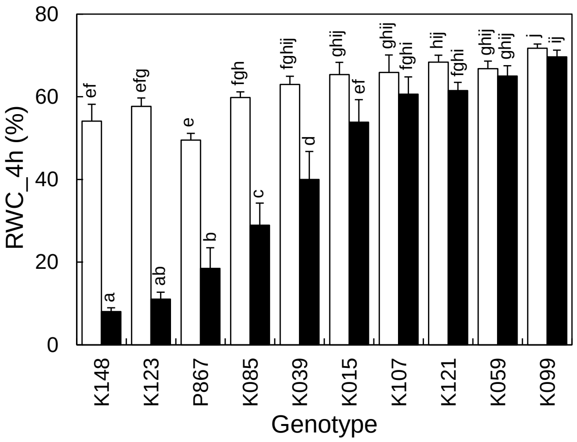

Fig. 2. Relative water content after 4 h of leaflet desiccation (RWC_4 h) in ten genotypes grown at moderate (62%; open columns) or high (89%; solid columns) relative air humidity. Values are the mean of seven leaflets and bars represent the SEM. Different letters indicate significant differences according to Fisher's LSD test (*P* = 0.05)

The stomatal response to ABA feeding was assessed in the same three genotypes used for evaluating the transpiration rate dynamics. A significant interaction between RH and genotype (*P* = 0.012) was found for the amount of ABA required to reduce the transpiration rate half-way between the maximum and the minimum values, the so-called EC_{50} (Fig. 3). High RH during growth significantly increased the EC_{50} in the sensitive genotypes K148 and K123 (77 and 386%, respectively), whereas this effect was not significant in the tolerant one (Fig. 3), indicating its higher responsiveness to the ABA closing stimulus.

High RH resulted in larger stomata with wider pore areas

The influence of high RH during growth on stomatal anatomical features was evaluated in two sensitive (K148 and K123) and one tolerant (K121) genotypes. Although high RH significantly increased stomatal size (i.e., length and width), pore width, pore area and pore area per leaf area in the three studied genotypes, the magnitude of this effect was genotype dependent (Table 1). The increase in those anatomical characteristics was 2 to 7-fold higher in the sensitive genotypes as compared to the tolerant one ($P < 0.001$; Table 1). High RH also resulted in 14% higher stomatal index and 32% longer pore length. Moreover, there was a significant effect of the genotype ($P < 0.001$) on those parameters as well as on stomatal density (Table 1). K148 and K123, showed lower (33 and 15%, respectively) stomatal density ($P < 0.001$) and lower (19 and 11%, respectively) stomatal index ($P < 0.001$), as compared to K121 (Table 1). Pore length was longer (≈ 8%) in the two sensitive (K148 and K123) genotypes than in the tolerant one (K121) ($P < 0.001$; Table 1).

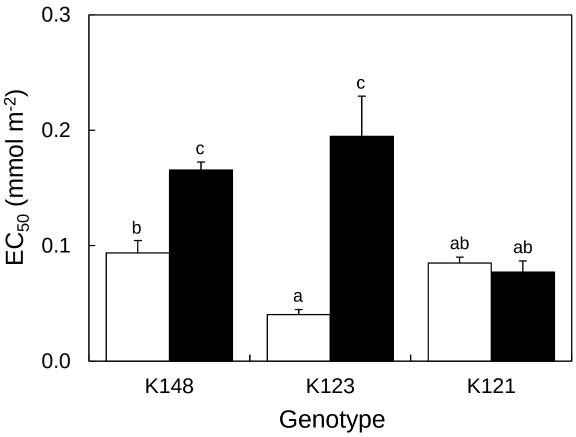

Fig. 3. Abscisic acid (ABA) intake required to reduce the transpiration rate to 50% between the maximum and the minimum values (EC_{50}) in two sensitive (K148, K123) and one tolerant (K121) genotypes grown at moderate (62%; open columns) or high (89%; solid columns) relative air humidity. ABA was fed (200 µM) through the leaflet petiole for 160 min. Values are the mean of five leaflets and bars represent the SEM. Different letters indicate significant differences according to Fisher's LSD test ($P = 0.05$)

Table 1. Stomatal anatomical characteristics from two sensitive (K148 and K123) and one tolerant (K121) genotypes grown at moderate (62%) or high (89%) relative air humidity (RH). Sampling took place 4 h after the onset of the light period. Values are the mean of 35 fields of view (stomatal density and index) and 70 stomata (stomatal and pore dimensions). Different letters represent significant differences according to Fisher's LSD test ($P = 0.05$) (comparison in rows).

	Genotype	K148			K123			K121			*F pr.*	*F pr.*	*F pr.*
	RH	62%	89%	Mean	62%	89%	Mean	62%	89%	Mean	RH	G	RH × G
Stomatal	Density (no mm^{-2})	51.3	54.7	53.0^{a}	68.2	65.6	66.9^{b}	79.7	77.6	78.6^{c}	0.771	< 0.001	0.192
	Index (%)	10.5	12.7	11.6^{a}	11.9	13.6	12.8^{b}	13.8	15.0	14.4^{c}	< 0.001	< 0.001	0.175
	Length (µm)	24.9^{a}	30.1^{c}	27.5	24.9^{a}	31.1^{d}	28.0	25.5^{a}	28.9^{b}	27.2	0.070	< 0.001	< 0.001
	Width (µm)	16.8^{a}	24.6^{d}	20.7	17.7^{ab}	25.3^{d}	21.5	18.8^{b}	22.6^{c}	20.7	< 0.001	0.054	< 0.001
Pore	Length (µm)	15.4	19.7	17.5^{b}	15.2	20.2	17.7^{b}	13.8	18.6	16.2^{a}	< 0.001	< 0.001	0.561
	Width (µm)	4.83^{a}	9.93^{d}	7.38	5.29^{a}	9.76^{d}	7.52	6.51^{b}	7.52^{c}	7.01	< 0.001	0.092	< 0.001
	Area (µm^{2})	60.8^{a}	154.8^{c}	107.8	63.1^{a}	157.7^{c}	110.4	72.4^{a}	110.2^{b}	91.3	< 0.001	< 0.001	< 0.001
	Area per leaf area (‰)	6.5^{a}	17.1^{d}	11.8	8.5^{b}	20.9^{e}	14.7	11.5^{c}	16.9^{d}	14.2	< 0.001	< 0.001	< 0.001

High RH increased [ABA] in root xylem sap at transpirational flow rate but did not affect [ABA] in leaf petiole xylem sap

In nine out of the ten studied genotypes, high RH had no significant effect on root xylem sap [ABA] and it was significantly increased in the tolerant genotype K121 (P = 0.002; Fig. 4A). The [ABA] measured in the root xylem sap collected from natural root exudation was, in general, lower in the five sensitive genotypes (K148, K123, P867, K085 and K039) as compared to the tolerant ones (K015, K107, K121, K059 and K099) (Fig. 4A). The root exudation rate was, in general, higher in the five sensitive genotypes as compared to the tolerant ones (Fig. 4B). Genotypes with higher root exudation rate showed lower [ABA] in the root xylem sap, i.e., [ABA] depended on the exudation rate (R^2 = 0.78) (Fig. 4C). In general, high RH during growth reduced the transpiration rate per plant, but no contrasting transpiration rate was found between sensitive and tolerant genotypes (P = 0.013; Fig. 4D).

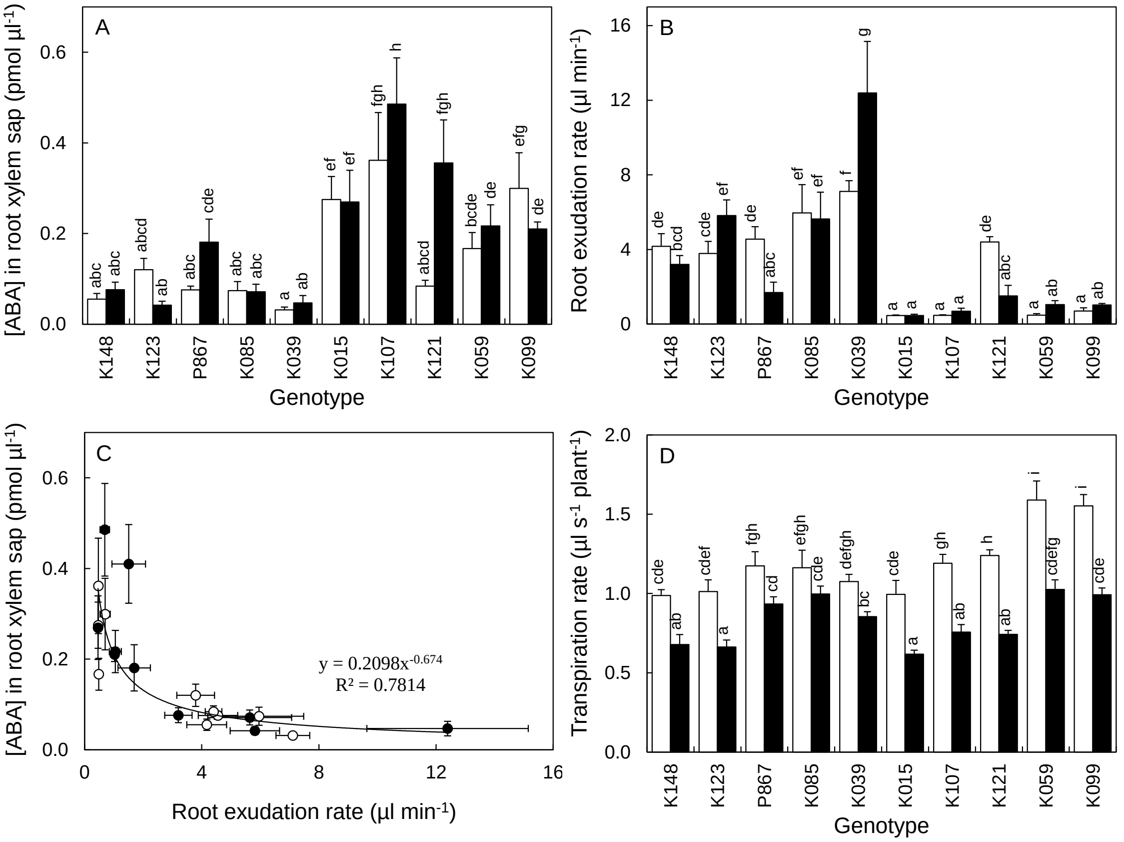

Fig. 4. Abscisic acid concentration ([ABA]) in root xylem sap quantified by Elisa (A), root exudation rate (B), [ABA] in the root xylem sap as a function of root exudation rate (C) and transpiration rate per plant (D) in ten genotypes grown at moderate (62%; open columns and symbols) or high (89%; solid columns and symbols) relative air humidity. Values are the mean of six plants and bars represent the SEM. Different letters indicate significant differences according to Fisher's LSD test (P = 0.05)

There was a significant effect of the genotype on [ABA] in root xylem sap at transpirational flow rate ($P = 0.001$) but these differences did not relate to the contrasting genotypic stomatal responsiveness (Fig. 5A). Moreover, high RH increased the [ABA] in root xylem sap at transpirational flow rate by 68% compared to moderate RH-grown plants ($P < 0.001$; Fig. 5B). The [ABA] in the leaf petiole xylem sap varied about three-fold between genotypes ($P = 0.009$; Fig. 5C), but there was no significant effect of the RH ($P = 0.135$; Fig. 5D).

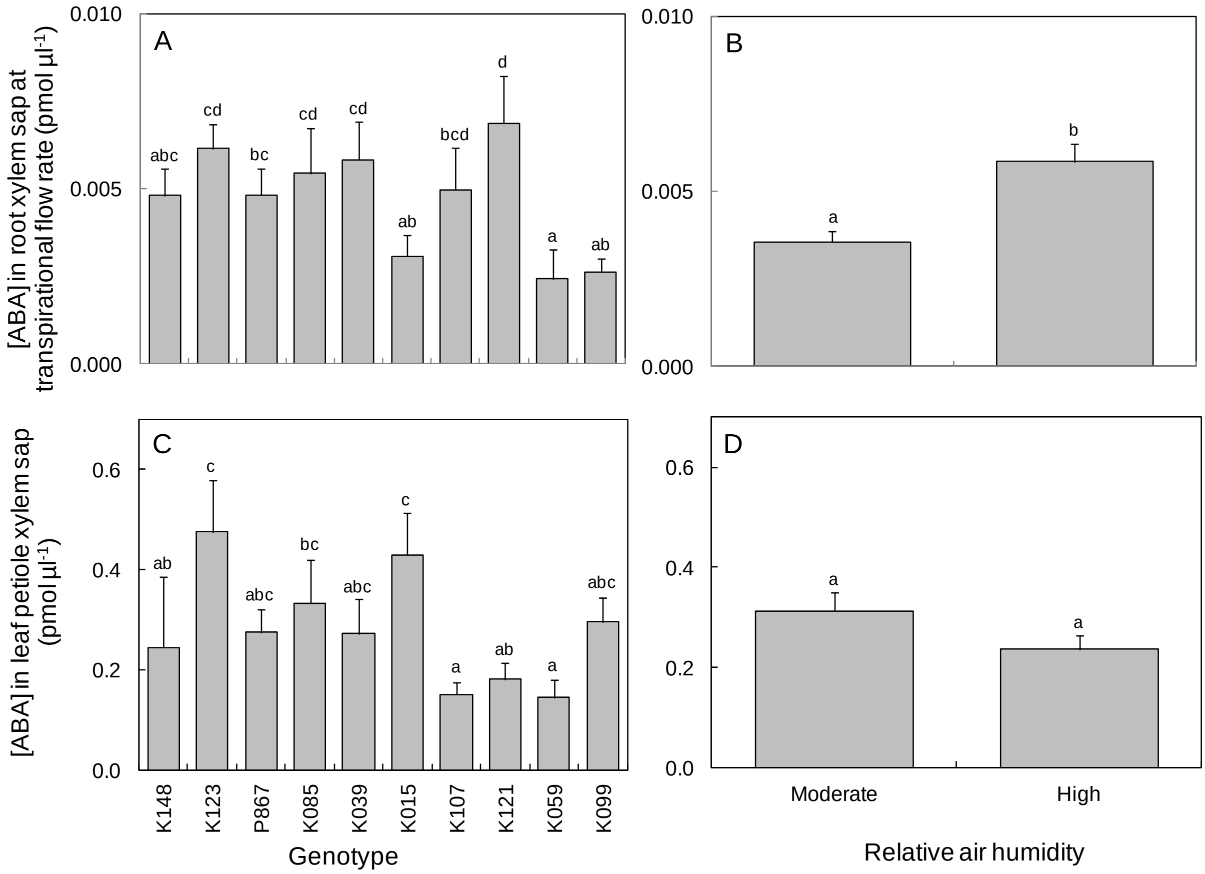

Fig. 5. Abscisic acid concentration ([ABA]) in root xylem sap at transpirational flow rate (A, B) and [ABA] in the leaf petiole xylem sap (C, D) as a function of the genotype (A, C) and the relative air humidity (RH; B, D) in ten genotypes grown at moderate (62%) or high (89%) RH. Values are the average of 62% and 89% RHs per genotype (A, C) and the average of all ten genotypes per RH level (B, D), as no significant interactions between genotype and RH were found in either [ABA] in root xylem sap at transpirational flow rate ($P = 0.279$) or [ABA] in the leaf petiole xylem sap ($P = 0.286$). Values are the mean of 12 (A) or 60 (B) plants and eight (C) or 40 (D) leaves and bars represent the SEM. Different letters indicate significant differences according to Fisher's LSD test ($P = 0.05$)

In spite of a significant genotypic effect on the ABA delivery rate per unit leaf area, this rate was rather similar among most of the analyzed genotypes (*P* = 0.008; Fig. 6), and it was not significantly affected by the RH (*P* = 0.180; data not shown). No-significant correlation was noted between ABA delivery rate and the [ABA] in the leaf petiole xylem sap (*P* = 0.993; data not shown). Moreover, none of those variables were correlated with the RWC_4h (Fig. 7). For instance, at an ABA delivery rate close to 0.05 ± 0.01 pmol m^{-2} s^{-1} some genotypes showed poor stomatal responsiveness to desiccation while others showed a proper stomatal functioning (i.e., RWC_4h at that ABA delivery rate level ranged between 8 and 70%; Fig. 7A).

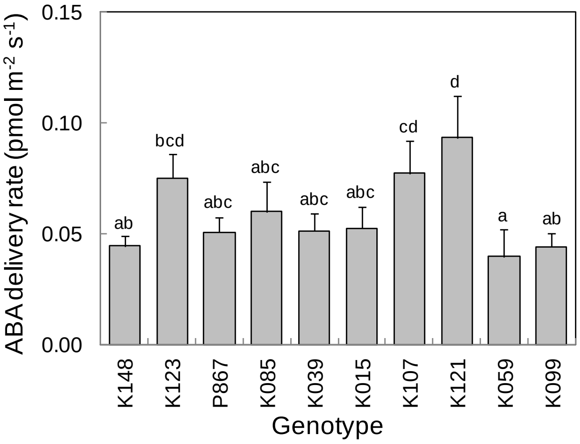

Fig. 6. Abscisic acid (ABA) delivery rate per unit leaf area in ten genotypes grown at moderate (62%) and high (89%) relative air humidity (RH). Values are the average of 62% and 89% RHs per genotype, as no significant interaction between genotype and RH was found (*P* = 0.087). Values are the mean of 12 plants and bars represent the SEM. Different letters indicate significant differences according to Fisher's LSD test (*P* = 0.05)

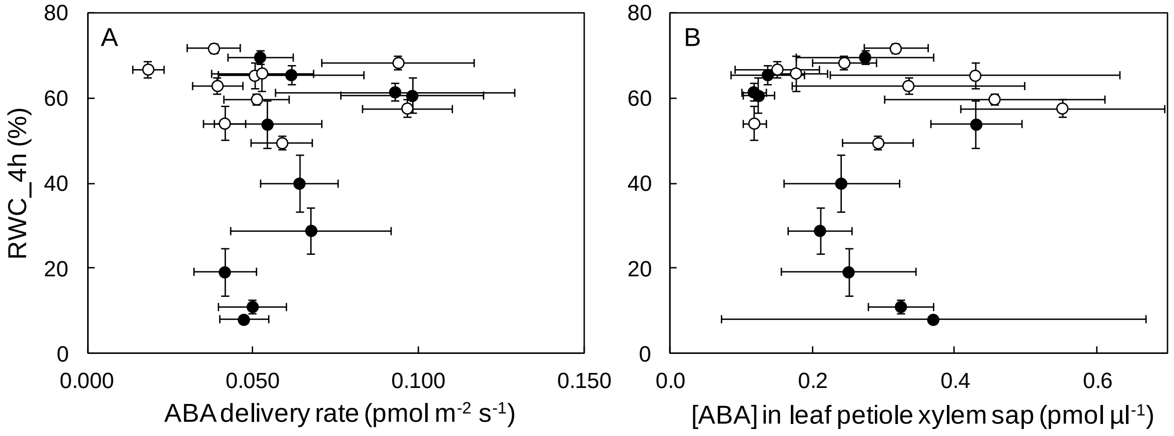

Fig. 7. Relative water content after 4 h of leaflet desiccation (RWC_4h) as a function of abscisic acid (ABA) delivery rate (A) or abscisic acid concentration ([ABA]) in leaf petiole xylem sap (B) of ten genotypes (K148, K123, P867, K085, K039, K015, K107, K121, K059 and K099) grown at moderate (62%; open symbols) or high (89%; solid symbols) relative air humidity. Values are the mean of six (ABA delivery rate), four ([ABA] in leaf petiole xylem sap) or seven (RWC_4h) samples ± SEM

Discussion

In this study we found a very wide genotypic variation in the stomatal responsiveness to both desiccation (i.e., 8–72% RWC_4h; Fig. 2) and petiole-fed ABA (Fig. 3) in plants cultivated at high RH, which has been previously described (Fanourakis et al., 2013; Giday et al., 2013). Our findings confirm that the selected genotypes based in a previous study (Fanourakis et al., 2012b) represent a good basis for analyzing the role of the root-sourced and leaf petiole xylem sap [ABA] on the genotypic variation of stomatal functioning following growth at high RH.

In rose, an increase in the leaf transpiration rate largely reflects a higher stomatal pore area per unit of leaf area, since the cuticle has a minor contribution to the total leaf water loss (Fanourakis et al., 2013). Here, it was found that pore area per leaf area, determining the transpiring surface, was greatly enhanced following growth at high RH, being the sole result of increased pore dimensions, since stomatal density was not affected by RH during growth (Table 1). Previous work also reports larger stomata with wider pores on leaflets developed at high RH as compared to moderate RH, whereas a minor effect on stomatal density was noted (Fanourakis et al., 2013). Although stomatal anatomy is clearly affected by high RH during leaf expansion, and that this contributes *per se* to an enhanced water loss when plants are subject to conditions of high evaporative demand

(Fanourakis et al., 2013), it has been shown that stomatal and pore sizes are not crucial in this process as long as stomata are able to function properly (Fanourakis et al., 2013; Aliniaeifard et al., 2014a; Giday et al., 2014).

Several studies suggest that slow responsive stomata to closing stimuli on leaflets expanded at high RH are a consequence of long-term low leaf [ABA] (Rezaei Nejad and van Meeteren, 2007; Fanourakis et al., 2011; Arve et al., 2013). Giday and co-workers (2013) recently showed that bulk leaf [ABA] was lower in *Rosa hybrida* grown at high RH, an effect that was more pronounced in the sensitive cultivars resulting in impaired stomatal functionality. However, the reasons behind this differential decrease of bulk leaf [ABA] remain unknown. In spite the fact that roots are the most important ABA production site (Wilkinson and Davies, 2002), there are no studies on the role of root [ABA] in determining genotypic differences in plants grown under contrasting RHs. In this study, we found for sensitive and tolerant genotypes a similar estimated [ABA] in the root xylem sap of transpiring plants and a similar ABA delivery rate per unit leaf area (Figs. 5A and 6). Hence, our results suggest that the ABA delivered from the roots does not contribute to the genotypic variability in stomatal functioning following growth at high RH. The 68% increase in the [ABA] in root xylem sap at transpirational flow rate of high RH-grown plants compared to the ones grown at moderate RH (Fig. 5B) is most likely due to a reduced dilution of the [ABA] in the xylem sap as a result of the lower plant transpiration rate of high RH-grown plants (Fig. 4D). To estimate the [ABA] in root xylem sap of transpiring plants (Fig. 5A) from the ratio between the exudation rate and the transpiration rate per plant, we assumed that the [ABA] was dependent of the exudation rate, as shown in Fig. 4C. A negative correlation between sap flow rate and [ABA] has also been previously noted in other studies where a root pressure was applied to mimic the transpiration rate of intact plants (Jokhan et al., 1996; Schurr, 1998). The lower sap exudation rate from the roots of the tolerant genotypes compared to the sensitive ones (Fig. 4B) can be due to a smaller root system and/or an increased thickness of the Casparian bands in the sclerenchyma layer of the former compared to the latter (Darwent et al., 2003; Shi et al., 2015). In the current study, we also found that the amount of exogenous ABA fed through the leaflet petiole that was required for inducing stomatal closure in plants cultivated at high RH was lower in the tolerant genotype compared to the sensitive ones (Fig. 3). These results reinforce the hypothesis, also supported by other authors (Carvalho et al., 2015; Pantin et al., 2013), that the studied tolerant genotype (K121) seem to be more sensitive to ABA as compared to the two studied sensitive ones (K148 and K123).

The lack of correlation between ABA delivery rate and the [ABA] in the leaf petiole xylem sap can be explained by the fact that [ABA] in the xylem sap can be modified on its path between the roots and the leaves (Berger et al., 1994). Recent molecular studies have shown that ABA synthesized autonomously by the guard cells is the predominant mechanism for stomatal responses to increased VPD (Bauer et al., 2013; Okamoto et al., 2009). However, others have found that ABA synthesized and transported by the vascular tissue, in particular the phloem (Koiwai et al., 2004; Kuromori et al., 2014; Okamoto et al., 2009; Seo and Koshiba, 2011) and ABA synthesized by the leaf only, yet outside of the guard cells (McAdam and Brodribb, 2015) may be the dominant signal for stomatal closure. In our study, similar root-to-shoot ABA delivery rates between contrasting genotypes led to comparable [ABA] measured in the leaf petiole xylem sap among genotypes (Fig. 5C). Furthermore, high RH during growth did not affect [ABA] in the leaf petiole sap [ABA] (Fig. 5D). Additionally, no significant correlation was noted between [ABA] in the leaf petiole xylem sap and RWC_4h (Fig. 7B). All together these results indicate that xylem sap [ABA] does not explain genotypic differences in the stomatal sensitivity to high RH; ABA homeostasis within the leaf is the main determinant in this process (Giday et al., 2013; McAdam and Brodribb, 2015). Future work should explore the expression of relevant genes related to the ABA metabolism at leaf level (e.g. *NCED1*, *AAO3*, *CYP707A1* and *CYP707A3*).

Conclusions

This is the first study that evaluates the role of root-to-shoot ABA signaling on genotypic variation in stomatal functioning following growth at high RH. Here we clarified that the [ABA] in root xylem sap at transpirational flow rate and in leaf petiole xylem sap was not related to the genotypic differences in the sensitivity to high RH. It is concluded that genotypic differences in the stomatal sensitivity to high RH cannot be explained by changes in [ABA] in the xylem sap.

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References

Aliniaeifard, S., Malcolm Matamoros, P., van Meeteren, U., 2014a. Stomatal malfunctioning under low VPD conditions: Induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? Physiol. Plant. 152, 688–699. doi:10.1111/ppl.12216

Aliniaeifard, S., van Meeteren, U., 2014b. Natural variation in stomatal response to closing stimuli among *Arabidopsis thaliana* accessions after exposure to low VPD as a tool to recognize the mechanism of disturbed stomatal functioning. J. Exp. Bot. 65, 6529–6542. doi:10.1093/jxb/eru370

Arve, L.E., Terfa, M.T., Gislerød, H.R., Olsen, J.E., Torre, S., 2013. High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves. Plant, Cell Environ. 36, 382–392. doi:10.1111/j.1365-3040.2012.02580.x

Bauer, H., Ache, P., Lautner, S., Fromm, J., Hartung, W., Al-Rasheid, K. a S., Sonnewald, S., Sonnewald, U., Kneitz, S., Lachmann, N., Mendel, R.R., Bittner, F., Hetherington, A.M., Hedrich, R., 2013. The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. Curr. Biol. 23, 53–57. doi:10.1016/j.cub.2012.11.022

Berger, A., Oren, R., Schulze, E.D., 1994. Element concentrations in the xylem sap of *Picea abies* (L.) Karst. seedlings extracted by various methods under different environmental conditions. Tree Physiol. 14, 111–128

Carvalho, D.R.A., Torre, S., Kraniotis, D., Almeida, D.P.F., Heuvelink, E., Carvalho, S.M.P., 2015. Elevated air movement enhances stomatal sensitivity to abscisic acid in leaves developed at high relative air humidity. Front. Plant Sci. 6, 1–11. doi:10.3389/fpls.2015.00383

Christmann, A., Weiler, E.W., Steudle, E., Grill, E., 2007. A hydraulic signal in root-to-shoot signalling of water shortage. Plant J. 52, 167–174. doi:10.1111/j.1365-313X.2007.03234.x

Correia, M.J., Rodrigues, M.L., Ferreira, M.I., Pereira, J.S., 1997. Diurnal change in the relationship between stomatal conductance and abscisic acid in the xylem sap of field-grown peach trees. J. Exp. Bot. 48, 1727–1736. doi:10.1093/jxb/48.9.1727

Darwent, M.J., Paterson, E., McDonald, A.J.S., Tomos, A.D., 2003. Biosensor reporting of root exudation from *Hordeum vulgare* in relation to shoot nitrate concentration. J. Exp. Bot. 54, 325–334. doi:10.1093/jxb/54.381.325

Dodd, I.C., 2005. Root-to-shoot signalling: Assessing the roles of "up" in the up and down world of long-distance signalling in planta. Plant Soil 274, 251–270. doi:10.1007/s11104-004-0966-0

Dodd, I.C., Theobald, J.C., Richer, S.K., Davies, W.J., 2009. Partial phenotypic reversion of ABA-deficient flacca tomato (*Solanum lycopersicum*) scions by a wild-type rootstock: Normalizing shoot ethylene relations promotes leaf area but does not diminish whole plant transpiration rate. J. Exp. Bot. 60, 4029–4039. doi:10.1093/jxb/erp236

Drake, P.L., Froend, R.H., Franks, P.J., 2013. Smaller, faster stomata: Scaling of stomatal size, rate of response, and stomatal conductance. J. Exp. Bot. 64, 495–505. doi:10.1093/jxb/ers347

Fanourakis, D., Tapia, a., Heuvelink, E., Carvalho, S.M.P., 2009. Cultivar differences in the stomatal characteristics of cut roses grown at high relative humidity. Acta Hortic. 847, 251–258.

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F., Heuvelink, E., 2011. Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. Physiol. Plant. 142, 274–286. doi:10.1111/j.1399-3054.2011.01475.x

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F., van Kooten, O., van Doorn, W.G., Heuvelink, E., 2012a. Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biol. Technol. 64, 64–73. doi:10.1016/j.postharvbio.2011.09.016

Fanourakis, D., Carvalho, D.R.A., Gitonga, V.W., Van Heusden, A.W., Almeida, D.P.F., Heuvelink, E., Carvalho, S.M.P., 2012b. Breeding cut roses for better keeping quality : First steps, in: Acta Hortic. 937, 875–882.

Fanourakis, D., Heuvelink, E., Carvalho, S.M.P., 2013. A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. J. Plant Physiol. 170, 890–898. doi:10.1016/j.jplph.2013.01.013

Fanourakis, D., Giday, H., Milla, R., Pieruschka, R., Kjaer, K.H., Bolger, M., Vasilevski, A., Nunes-Nesi, A., Fiorani, F., Ottosen C-O, 2015a. Pore size regulates operating stomatal conductance , while stomatal densities drive the partitioning of conductance between leaf sides. Ann. Bot. 115, 555–565. doi:10.1093/aob/mcu247

Fanourakis, D., Heuvelink, E., Carvalho, S.M.P., 2015b. Spatial heterogeneity in stomatal features during leaf elongation : an analysis using *Rosa hybrida*. Funct. Plant Biol. doi:10.1071/FP15008

Giday, H., Fanourakis, D., Kjaer, K.H., Fomsgaard, I.S., Ottosen, C.O., 2013. Foliar abscisic acid content underlies genotypic variation in stomatal responsiveness after growth at high relative air humidity. Ann. Bot. 112, 1857–1867. doi:10.1093/aob/mct220

Giday, H., Fanourakis, D., Kjaer, K.H., Fomsgaard, I.S., Ottosen, C.O., 2014. Threshold response of stomatal closing ability to leaf abscisic acid concentration during growth. J. Exp. Bot. 65, 4361–4370. doi:10.1093/jxb/eru216

Jokhan, A.D., Else, M.A., Jackson, M.B., 1996. Delivery rates of abscisic acid in xylem sap of *Ricinus communis* L. plants subjected to part-drying of the soil. J. Exp. Bot. 47, 1595–1599. doi:10.1093/jxb/47.10.1595

Koiwai, H., Nakaminami, K., Seo, M., Mitsuhashi, W., Toyomasu, T., Koshiba, T., 2004. Tissue-specific localization of an abscisic acid biosynthetic enzyme, AAO3, in *Arabidopsis*. Plant Physiol. 134, 1697–1707. doi:10.1104/pp.103.036970.1

Kuromori, T., Sugimoto, E., Shinozaki, K., 2014. Intertissue signal transfer of abscisic acid from vascular cells to guard cells. Plant Physiol. 164, 1587–92. doi:10.1104/pp.114.235556

Martin-Vertedor, A.I., Dodd, I.C., 2011. Root-to-shoot signalling when soil moisture is heterogeneous: Increasing the proportion of root biomass in drying soil inhibits leaf growth and increases leaf abscisic acid concentration. Plant, Cell Environ. 34, 1164–1175. doi:10.1111/j.1365-3040.2011.02315.x

McAdam, S.A.M., Brodribb, T.J., 2015. The evolution of mechanisms driving the stomatal response to vapor pressure deficit. Plant Physiol. 167, 833–843. doi:10.1104/pp.114.252940

Mortensen, L.M., Gislerød, H.R., 1999. Influence of air humidity and lighting period on growth, vase life and water relations of 14 rose cultivars. Sci. Hortic. (Amsterdam). 82, 289–298. doi:10.1016/S0304-4238(99)00062-X

Okamoto, M., Tanaka, Y., Abrams, S.R., Kamiya, Y., Seki, M., Nambara, E., 2009. High humidity induces abscisic acid 8’-hydroxylase in stomata and vasculature to regulate local and systemic abscisic acid responses in Arabidopsis. Plant Physiol. 149, 825–834. doi:10.1104/pp.108.130823

Pantin, F., Renaud, J., Barbier, F., Vavasseur, A., Le Thiec, D., Rose, C., Bariac, T., Casson, S., McLachlan, D.H., Hetherington, A.M., Muller, B., Simonneau, T., 2013. Developmental priming of stomatal sensitivity to abscisic acid by leaf microclimate. Curr. Biol. 23, 1805–1811. doi:10.1016/j.cub.2013.07.050

Pengelly, W., 1985. Validation of immunoassays, in: Bopp, M. (Ed.), Plant growth substances, Proceedings in Life Sciences. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 35–43. doi:10.1007/978-3-642-71018-6

Rezaei Nejad, A., van Meeteren, U., 2005. Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. Physiol. Plant. 125, 324–332. doi:10.1111/j.1399-3054.2005.00567.x

Rezaei Nejad, A., van Meeteren, U., 2007. The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity. J. Exp. Bot. 58, 627–636. doi:10.1093/jxb/erl234

Rezaei Nejad, A., Van Meeteren, U., 2008. Dynamics of adaptation of stomatal behaviour to moderate or high relative air humidity in *Tradescantia virginiana*. J. Exp. Bot. 59, 289–301. doi:10.1093/jxb/erm308

Sáez, P.L., Bravo, L. a., Latsague, M.I., Sánchez, M.E., Ríos, D.G., 2012. Increased light intensity during in vitro culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels. Sci. Hortic. (Amsterdam). 138, 7–16. doi:10.1016/j.scienta.2012.02.005

Salisbury, E., 1927. On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. Philos. Trans. R. Soc. London 216, 1–65.

Schachtman, D.P., Goodger, J.Q.D., 2008. Chemical root to shoot signaling under drought. Trends Plant Sci. 13, 281–287. doi:10.1016/j.tplants.2008.04.003

Schurr, U., 1998. Xylem sap sampling - new approaches to an old topic. Trends Plant Sci. 3, 293–298. doi:10.1016/S1360-1385(98)01275-8

Seo, M., Koshiba, T., 2011. Transport of ABA from the site of biosynthesis to the site of action. J. Plant Res. 124, 501–507. doi:10.1007/s10265-011-0411-4

Shi, L., Guo, M., Ye, N., Liu, Y., Liu, R., Xia, Y., Cui, S., Zhang, J., 2015. Reduced ABA accumulation in the root system is caused by ABA exudation in upland rice (*Oryza sativa* L. var. Gaoshan1) and this enhanced drought adaptation. Plant Cell Physiol. 56, 951–64. doi:10.1093/pcp/pcv022

Slavík, B., 1974. Methods of studying plant water relations. Chapman and Hall, London, UK.

Smith, S., Weyers, J.D.B., Berry, W.G., 1989. Variation in stomatal characteristics over the lower surface of *Commelina communis* leaves. Plant, Cell Environ. 12, 653–659. doi:10.1111/j.1365-3040.1989.tb01234.x

Torre, S., Fjeld, T., Gislerod, H.R., Moe, R., 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. J. Am. Soc. Hortic. Sci. 128, 598–602.

Tullus, A., Kupper, P., Sellin, A., Parts, L., Sõber, J., Tullus, T., Lõhmus, K., Sõber, A., Tullus, H., 2012. Climate change at Northern latitudes: Rising atmospheric humidity decreases transpiration, N-uptake and growth rate of hybrid aspen. PLoS One 7, 1–11. doi:10.1371/journal.pone.0042648

Wilkinson, S., Davies, W.J., 2002. ABA-based chemical signalling : the co-ordination of responses to stress in plants. Plant, Cell Environ. 25, 195–210.

Xu, Z.-Y., Lee, K.H., Dong, T., Jeong, J.C., Jin, J.B., Kanno, Y., Kim, D.H., Kim, S.Y., Seo, M., Bressan, R. a., Yun, D.-J., Hwang, I., 2012. A vacuolar β-glucosidase homolog that possesses glucose-conjugated abscisic acid hydrolyzing activity plays an important role in osmotic stress responses in *Arabidopsis*. Plant Cell 24, 2184–2199. doi:10.1105/tpc.112.095935

Appendix

Table A1 – F-probabilities (*F pr.*) for the main effects of RH, genotype and their interaction resulting from the ANOVA for each variable evaluated in this study. RWC_4h, relative water content after 4 h of leaflet desiccation; EC50, amount of ABA required to reduce the transpiration rate half-way between the maximum and the minimum values

	F pr. RH	*F pr.* G	*F pr.* RH × G
RWC_4h (Fig. 2)	< 0.001	< 0.001	< 0.001
EC_{50} (Fig. 3)	< 0.001	0.025	< 0.001
[ABA] in root xylem sap (Fig. 4A)	0.031	< 0.001	0.002
Root exudation rate (Fig. 4B)	0.710	< 0.001	< 0.001
Tranpiration rate per plant (Fig. 4C)	< 0.001	< 0.001	0.013
[ABA] in root xylem sap at transpirational flow rate (Figs. 5A and 5B)	< 0.001	0.001	0.279
[ABA] in leaf petiole xylem sap (Figs. 5C and 5D)	0.135	0.009	0.286
ABA delivery rate (Fig. 6)	0.180	0.008	0.087

CHAPTER 3

Effect of elevated air movement on stomatal functioning and postharvest stress tolerance

3.1 Elevated air movement enhances stomatal sensitivity to abscisic acid in leaves developed at high relative air humidity

3.2 Effects of air humidity and air movement on growth, visual quality and post-production stress tolerance of pot rose ‘Toril’

CHAPTER 3.1

Elevated air movement enhances stomatal sensitivity to abscisic acid in leaves developed at high relative air humidity

Abstract

High relative air humidity (RH ≥ 85%) during growth leads to stomata malfunctioning, resulting in water stress when plants are transferred to conditions of high evaporative demand. In this study, we hypothesized that an elevated air movement (MOV) 24 h per day, during the whole period of leaf development would increase abscisic acid concentration ([ABA]) enhancing stomatal functioning. Pot rose ‘Toril’ was grown at moderate (61%) or high (92%) RH combined with a continuous low (0.08 m s^{-1}) or high (0.92 m s^{-1}) MOV. High MOV reduced stomatal pore length and aperture in plants developed at high RH. Moreover, stomatal function improved when high MOV-treated plants were subjected to leaflet desiccation and ABA feeding. Endogenous concentration of ABA and its metabolites in the leaves was reduced by 35% in high RH, but contrary to our hypothesis this concentration was not significantly affected by high MOV. Interestingly, in detached leaflets grown at high RH, high MOV increased stomatal sensitivity to ABA since the amount of exogenous ABA required to decrease the transpiration rate was significantly reduced. This is the first study to show that high MOV increases stomatal functionality in leaves developed at high RH by reducing the stomatal pore length and aperture and enhancing stomatal sensitivity to ABA rather than increasing leaf [ABA].

Carvalho DRA, Torre S, Kraniotis D, Almeida DPF, Heuvelink E, Carvalho SMP (2015) Elevated air movement enhances stomatal sensitivity to abscisic acid in leaves developed at high relative air humidity. Frontiers in Plant Science 6:383, DOI: 10.3389/fpls.2015.00383

Introduction

Stomatal opening and closing are influenced by environmental factors such as light, temperature, CO_2 concentration, drought, relative air humidity (RH) and their interactions (Tallman, 2004; Reynolds-Henne et al., 2010; Feller and Vaseva, 2014). Long-term high RH (i.e. RH $\geq$ 85% throughout leaf ontogeny) is regarded as the environmental factor that mostly disturbs the capacity of stomata to close in response to water stress and darkness, leading to uncontrolled water loss (Torre et al., 2003; Rezaei Nejad and van Meeteren, 2005; In et al., 2006; Fanourakis et al., 2012). Recent studies have shown that although stomatal anatomy and density do contribute *per se* to the increased water loss in leaves developed at high RH, stomatal physiology is the major cause for this negative water balance (Fanourakis et al., 2013; Aliniaeifard et al., 2014a). However, the reasons why stomata fail to close fully during water stress periods in plants grown under long-term high RH remain unclear.

Stomatal movement is to a large extent regulated by abscisic acid concentration ([ABA]). Lower [ABA], associated with higher stomatal aperture during growth, has been measured in leaves of *Trasdescantia virginiana* (Rezaei Nejad and van Meeteren, 2007), *Vicia faba* (Aliniaeifard et al., 2014a) and *Rosa×hybrida* (Arve et al., 2013; Giday et al., 2013) developed at high RH (90%) compared to those developed at moderate RH (60%). The active hormone concentration in the tissue depends on its biosynthetic and catabolic rates (Nambara and Marion-Poll, 2005). The major cause of inactivation of free ABA is oxidation. Free ABA is firstly hydroxylated to phaseic acid (PA), which is reduced to dihydrophaseic acid (DPA) (Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005). Free ABA is also inactivated by covalent conjugation with monosaccharides, e.g. ABA-β-D-glucosyl ester (ABA-GE), which is hypothesized to be a storage form of ABA (Cutler and Krochko, 1999). It has been shown in roses that ABA-GE is converted to free ABA during the night inducing stomatal closure (Arve et al., 2013). High RH has been found to reduce the ABA availability by inactivating the ABA to PA in *Arabidopsis thaliana* (Okamoto et al., 2009). Moreover, at high RH the ABA-GE levels remain high during the night indicating that the conversion to free ABA does not occur (Arve et al., 2013).

In higher plants, a thigmomorphogenic response (i.e. touch-induced morphological change; e.g. wind and rain) is a slow, intensity-dependent, and saturating systemic response, that translocates from the stimulated plant regions to the non-disturbed distal regions (Jaffe, 1976; Beryl and Mitchell, 1977; Erner et al., 1980). Wind is an environmental factor having several effects on plants (Grace, 1977; Ennos, 1997), depending on leaf characteristics and on its speed (Schuepp, 1993; Lambers et al., 2008). These effects include a reduction of the boundary layer thickness enhancing gas diffusion (CO_2 and H_2O) (Schuepp, 1993; Lambers et al., 2008). Moreover, wind flow exerts drag forces causing mechanical stress on plants (Anten et al., 2010) and high wind speed has been suggested to have a positive effect on the endogenous [ABA] reducing stomatal aperture (Whitehead, 1962; Weyers and Hillman, 1979), although this effect has not yet been quantified. To the best of our knowledge, the combined effects of high MOV and high RH on plant growth and development as well as on stomatal functioning have not yet been properly explored. One of the few studies that investigated the effect of MOV (0.08, 0.21 and 0.35 m s^{-1}) combined with RH (70% and 90%) found that increasing wind speed at high RH had little effect on water loss of detached leaves of cut rose (Mortensen and Gislerød, 1997). However, the air speed levels used in that study were relatively low.

Several studies have suggested that stomatal malfunctioning in high RH-grown plants is strongly related to a long-term low [ABA] during leaf development as a short-term exogenous ABA application did not increase stomatal functionality (Rezaei Nejad and Van Meeteren, 2008; Fanourakis et al., 2011). However, in those studies exogenous ABA was applied only on fully developed leaves. In rose plants it was shown that after full leaf expansion stomatal function is no longer affected either by RH (when plants were moved from high to moderate RH) or ABA levels (Fanourakis et al., 2011). Thus, it remains unclear whether the lack of stomatal responsiveness to short-term ABA feeding is due to leaf developmental stage or due to the duration of this stimulus.

In this study we aimed at understanding the physiological effect of MOV on stomatal functioning in plants developed under high RH and whether the stage of leaflet development has an influence on stomatal sensitivity to ABA. It is our hypothesis that: (1) high MOV applied 24 h per day during the whole period of leaf development increases [ABA] improving stomatal closure in high RH-grown plants; and (2) non-fully developed leaflets close better their stomata than fully developed ones in response to exogenous ABA application. Additionally, we aimed at studying the combined effect of high MOV and high RH on plant growth and visual quality parameters.

Materials and methods

Plant material and growth conditions

Rooted cuttings of pot rose cultivar 'Toril' (*Rosa* × *hybrida*) were planted in 12 cm (0.66 l) pots containing a standard fertilized *Sphagnum* peat (Floralux, Nittedal, Norway). When the broken buds were 1-1.5 cm long, fifty six plants were randomly distributed over four climate controlled growth cabinets (length × width × height = 1.5 × 1.0 × 2.2 m). Plants were grown as single shoot, one plant per pot. During the cultivation period, the RH was 61 ± 3% (moderate RH) in two growth cabinets and 92 ± 2% (high RH) in the other two. In two growth cabinets (one per RH level), two fans (HT – 112 E, Honeywell, Lausanne, Switzerland) were located equidistant (70 cm) from the 14 plants that were placed in a semi-circle side by side, and were on during 24 h per day. Plants were rotated 90˚ daily in the horizontal plane to ensure that exposure to high MOV was similar in all directions (Anten et al., 2010). In the cabinets without fans (i.e. with no additional air movement) the plants were distributed similarly and were also rotated. An ultrasonic anemometer (Ultrasonic anemometer, Model 81000, Young, Traverse City, MI, USA) registered automatically the three dimensional air velocity and turbulence intensity (i.e. the percentage value calculated as the standard deviation of the fluctuations of the air velocity divided by the mean wind velocity) at plant level. Although traditionally the air speed is measured as the air flow in a specified direction (Downs and Krizek, 1997), in a closed environment, such as the one used in this study, the deflections of a high MOV on the cabinet's wall amplify the air movement making the flow regime more turbulent than in the open field. Thus, under such environment, turbulence intensity should also be quantified. In our study, we guaranteed that plants were subjected to a strong mechanical stimuli because in addition to their visible continuous strong waving, the measured turbulence intensity ranged between 92% and 240% with an average of 142%, which is 5.7-fold higher compared to that registered in standard growth cabinets (Downs and Krizek, 1997). The air velocity in the high MOV-treated plants was 0.92 ± 0.03 m s^{-1}, being 2.6-fold higher as compared to the maximum level applied by Mortensen and Gislerød (1997). In the absence of additional MOV the measured air velocity at plant level was 0.08 m s^{-1}. Temperature was 21 ± 0.5 ˚C (day and night), resulting in VPD of 0.97 ± 0.03 kPa (moderate RH) and 0.20 ± 0.01 kPa (high RH). The CO_2 concentration was 400 ± 50 μmol mol^{-1} and high pressure sodium lamps (Plantastar 400W, Osram, Münich, Germany) provided 20 h photoperiod of 160 ± 10 μmol m^{-2} s^{-1} photosynthetic active radiation (Li-250 Light Meter, LI-COR, Lincoln, NE, USA). Climate data were recorded automatically every 5 minutes (Priva, De Lier, The Netherlands). Plants were watered daily until draining with a nutrient solution (Arve et al., 2013). The pH and EC levels of the nutrient solution were 5.7 and 1.75 dS m^{-1}, respectively.

Plant growth and plant transpiration rate

The effects of MOV and RH on plant growth and visual quality parameters were evaluated in fully developed plants (i.e. flower bud with cylindrical shape and pointed tip). Total plant dry weight (stem, leaves and flower), leaf area, plant height, number of internodes, average internode length, peduncle length and diameter, flower dry weight and time to flowering (number of days from planting till full developed plant) were assessed in fourteen plants per treatment. Moreover, plant transpiration rate during the light and the dark period was measured gravimetrically during three consecutive days using fully developed plants. Plants were watered until container capacity and pots were wrapped into impermeable plastic bags to avoid evaporation from the substrate. During this period the weight of seven plants per treatment was recorded at the beginning of the light and dark periods (Model PG503DR Delta Range, Mettler-Toledo, Greifensee, Switzerland). At the end of the three-day period total leaf area per plant was measured using a leaf area meter (Model 3100 Area Meter, LI-COR, Lincoln, NE, USA) to calculate transpiration rate per unit leaf area.

Stomatal characteristics and leaf surface morphology

Stomatal density, index, length, width, pore length and pore aperture were analyzed in one of the two uppermost lateral leaflets from the first fully expanded penta-foliated leaf. Epidermal impressions were made by Suzuki's Universal Micro-Printing (SUMP) method using SUMP liquid 1 and SUMP plate B (SUMP Laboratory, Tokyo, Japan) as described by Tanaka et al. (2005). Samples were taken from the abaxial side of intact leaflets, midway between the tip and the base, away from the edge and avoiding veins, 4 h after the light period started. The imprints were observed under a light microscope (Eclipse 55i, Nikon, Tokyo, Japan) and stomatal images were obtained with a 5.24 megapixel camera (DS-Fi1, Nikon, Tokyo, Japan). To quantify stomatal density and index a magnification of 100× was used and 70 images per treatment were analyzed. The stomatal index was calculated according to Equation 1 (Salisbury, 1927).

$$\text{Stomatal index} = \frac{\text{stomatal density}}{\text{stomatal density} + \text{epidermal cell density}} \times 100 \qquad (1)$$

To measure stomatal and pore size, a magnification of 400× was used and 140 stomata per treatment were evaluated. Image analysis was performed using the UTHSCSA ImageTool for windows version 3.00 (The University of Texas Health Science Center at San Antonio, Texas, USA).

To study leaf surface morphology (namely stomatal deepness, i.e. the deepness of stomatal insertion in the leaf epidermis, and leaf epidermal cells shape/undulation) 0.5 × 0.5 cm leaf sections excised close to the midrib, midway between the tip and the base, away from the edge and avoiding veins were observed under a scanning electron microscope (Zeiss EVO – 50 – EP, Carl Zeiss SMT Ltd, Cambridge, United Kingdom). Samples were fixed in 1.25% glutaraldehyde and 2% paraformaldehyde in 0.05 M PIPES buffer, pH 7.2, and kept in PIPES buffer (0.1 M, pH 7). After fixation, samples were dried by the use of a critical point dryer (Bal-Tec CPD 030, Bal-Tec AG, Balzers, Germany) with dehydration series of 70, 90, 96 and 100% ethanol. Samples were mounted on aluminium stubs and coated in a sputter coater (Polaron SC 7640, Quorum Technologies Ltd, Ringmer, East Sussex, United Kingdom). Four biological replicates per treatment were analyzed.

Stomatal responsiveness to leaflet desiccation

Stomatal responsiveness to leaflet desiccation (i.e., stomatal closing stimulus) was evaluated by determining the transpiration rate and relative water content (RWC) in detached terminal leaflets. Determination of the transpiration rate by gravimetry is an adequate quantitative description of the stomatal functionality as demonstrated by Rezaei Nejad and van Meeteren (2005). This simple procedure has been thoroughly used in this type of studies (Rezaei Nejad and van Meeteren, 2005; Giday et al., 2013; Fanourakis et al., 2015) due to its effectiveness under conditions of low stomatal conductance, e.g. excessively desiccated leaflets (i.e., RWC < 20%), which fall below the detection limit of the porometer. Fully developed leaflets (first penta-foliated, counting from the apex) were detached from the plants and their petioles were recut under MilliQ-water to avoid cavitation-induced embolism. To establish leaflet saturated fresh weight, leaflets were placed with their petioles in a vial with MilliQ-water and were incubated in light (11.2 ± 0.2 µmol m^{-2} s^{-1}; Philips TL 58W, colour 84) for 1 h at about 100% RH (23.7 ± 1.3 ˚C; VPD close to 0) (Fanourakis et al., 2011). Because leaflets were detached from the plants at the beginning of the light period, the rehydration was also conducted in light, since following darkness the light-induced stomatal opening might require up to 1 h (Blom-Zandstra et al., 1995; Drake et al., 2013). After rehydration, petioles were removed from the water and leaflets were allowed to desiccate under constant conditions (abaxial surface down; 1.68 kPa VPD [42.7 ± 7.3% RH, 23.7 ± 1.3 ˚C] and 11.2 ± 0.2 μmol m^{-2} s^{-1} light intensity). Leaflets were weighted every 5-30 min for 4 h. Leaflet area, dry weight (24 h at 70 ˚C) and transpiration rate were determined, and RWC was calculated using Equation 2 (Slavík, 1974). One leaflet per plant was evaluated (14 plants per treatment).

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{saturated fresh weight} - \text{dry weight}} \times 100 \qquad (2)$$

Endogenous ABA quantification

Fully developed tri-foliated leaves (just above the first penta-foliated) were sampled five hours after the beginning of the light period, immediately frozen in liquid nitrogen and stored at -80 ˚C till analysis. Two composite samples (each with seven biological replicates) per treatment were evaluated.

Chemicals & calibration curves

Standard ABA-catabolites (PA, DPA, ABA-GE, 7'-OH-ABA, neoPA, and trans-ABA), deuterated forms of ABA-catabolites used as internal standards (i.e. d3-DPA, d5-ABA-GE, d3-PA, d4-7'-OH-ABA, d3-neoPA, d4-ABA, and d4-trans-ABA) according to Abrams et al. (2003) and Zaharia et al. (2005) and the deuterated forms of the selected compounds used as recovery standards (i.e. d6-ABA and d2-ABA-GE), were synthesized and prepared at the National Research Council of Canada (NRCC, Saskatoon, SK, Canada). *Cis*-ABA was purchased from Sigma–Aldrich (Sigma Chemicals, St Louis, MO, USA).

Extraction & purification

Samples were freeze dried and homogenized before analysis. A 100 μL aliquot containing the deuterated internal standards, each at a concentration of 0.2 pg μL^{-1}, was added to approximately 50 mg of homogenized plant tissue; 3 mL of isopropanol:water:glacial acetic acid (80:19:1, v/v/v) was added and the samples were agitated in the dark for 24 h at 4 ºC. Samples were then centrifuged and the supernatant was isolated and dried on a Büchi Syncore Polyvap (Büchi, Flawil, Switzerland). Samples were reconstituted in 100 μL acidified methanol, adjusted to 1 mL with acidified water, and then partitioned against 2 mL hexane. After 30 min, the aqueous layer was isolated and dried as above. Dry samples were reconstituted in 100 μL acidified methanol and adjusted to 1 mL with acidified water. The reconstituted samples were loaded onto equilibrated Oasis HLB cartridges (Waters, Mississauga, ON, Canada), washed with acidified water and eluted with acetonitrile:water:glacial acetic acid (30:69:1, v/v/v). The eluate was then dried on a LABCONCO centrivap concentrator (Labconco Corporation, Kansas City, MO, USA). An internal standard blank was prepared with 100 µL of the deuterated internal standards mixture. Quality control standards (QC) were prepared by adding 100 µL and 30 µL (separately) of a mixture containing the analytes of interest, each at a concentration of 0.2 pg μL^{-1}, to 100 µL of the internal standard mix. Finally, samples, blanks, and QCs were reconstituted in an aqueous solution of 40% methanol (v/v), containing 0.5% acetic acid and 0.1 pg μL^{-1} of each of the recovery standards.

Hormone quantification by HPLC-ESI-MS/MS

The samples were subjected to UPLC-ES-MS/MS analysis and quantification (Ross et al., 2004). Samples were injected onto an ACQUITY UPLC® HSS C18 column (2.1x100 mm, 1.8 µm) with an ACQUITY HSS C18 VanGuard Pre-column (2.1x5 mm, 1.8 µm) and separated by a gradient elution of water containing 0.025% acetic acid against an increasing percentage of acetonitrile containing 0.025% acetic acid. Briefly, the analysis utilizes the Multiple Reaction Monitoring (MRM) function of the MassLynx v4.1 (Waters Inc) control software. The resulting chromatographic traces are quantified off-line by the QuanLynx v4.1 software (Waters Inc) wherein each trace is integrated and the resulting ratio of signals (non-deuterated/internal standard) is compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample). Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross et al. (2004). The QC samples, internal standard blanks and solvent blanks were also prepared and analyzed along each batch of tissue samples.

Stomatal responsiveness to ABA feeding through the transpiration stream

Stomatal responsiveness to exogenous ABA feeding (i.e., stomatal closing stimulus) through the transpiration stream was evaluated in detached terminal leaflets in two stages of expansion: 100% full leaflet expansion (FLE) and 70-80% FLE. The percentage of FLE was defined as the proportion of leaflet length at harvest relative to its final length (i.e. when the midrib stopped elongating for three consecutive days) (Fanourakis et al., 2011). Terminal leaflets from the second penta-foliated leaves were used as 100% FLE samples.

The developmental stage of the terminal leaflet from the third penta-foliated leaf (intended to be 70 to 80% of the FLE) was estimated based on its length and the elongation curve of the terminal leaflet from the fourth penta-foliated leaf, of which its length was recorded daily from unfolding till 100% FLE. Leaflet detachment and rehydration were conducted as described above for evaluation of the stomatal responsiveness to desiccation. After 30 minutes of rehydration in vials with MilliQ-water, leaflets were transferred to a vial with 0 or 100 µM (±) ABA solution (Sigma, St. Louis, MO, USA) and were weighted every 5-10 min during 150 min. At the end, leaflet area was measured and transpiration rate was calculated. ABA intake was calculated as the product of leaflet transpiration rate and the concentration of the feeding solution (Fanourakis et al., 2013). The changes in transpiration rate in response to ABA showed the features of a dose-response curve (ABA intake was considered as the dose), and was fitted with a four parameter logistic model as described by Giday et al. (2013). The model fitting (Equation 3) was performed using GraphPad Prism (version 6.00 for Windows, GraphPad Software, San Diego, California, USA).

$$\text{Transpiration rate} = \text{minimum value} + \frac{(\text{maximum value} - \text{minimum value})}{(1+10\hat{}((\text{LogEC50} - \text{ABA intake}) * \text{hill slope})} \quad (3)$$

In Equation 3, the coefficients maximum and minimum values correspond to the transpiration rate before (t = 0) and after (t = 2.5 h) ABA feeding, respectively. EC_{50} describes the amount of ABA required to reduce the transpiration half-way between its maximum and minimum values. Hill slope represents the steepness of the curve. One leaflet per plant was evaluated from five or six plants per treatment.

Statistical design and analysis

The experimental set-up was a 2 by 2 factorial design and the experiment was repeated once. Analysis of variance was conducted, considering individual plants as experimental units. Main effects and interactions were tested at $P = 0.05$. When relevant, Fisher's least significant difference (LSD) at $P = 0.05$ was calculated to separate interaction means. The Genstat software (15th edition; VSN International Ltd., Herts, UK) was used for the analysis.

Results

Plant growth, visual quality and plant transpiration rate

The only significant effect of MOV on plant growth and visual quality parameters was a 6% increase on peduncle diameter ($P = 0.022$) (Table 1). High RH during growth did not significantly affect total dry weight ($P = 0.174$), number of internodes ($P = 0.250$), or time to flowering ($P = 0.480$). However, it significantly increased plant height ($P < 0.001$), resulting in 9% taller plants, longer peduncle length (12%) ($P < 0.001$) and higher average internode length (6%) ($P = 0.010$) (Table 1). Additionally, flower dry weight and peduncle diameter were significantly reduced in high RH-grown plants: 13% ($P = 0.002$) and 12% ($P < 0.001$), respectively (Table 1).

The effect of MOV on plant transpiration rate at growth conditions depended on the RH level (Fig. 1). At growth conditions during the light period, high MOV increased the transpiration rate by 12% in intact plants grown at moderate RH, while it was decreased by 17% in high RH-grown plants ($P < 0.001$; Fig. 1A). The same trend was observed in the dark period where high MOV increased the transpiration rate by 57% in moderate RH-grown plants, whereas the transpiration rate was 19% lower in high RH-grown plants ($P < 0.001$; Fig. 1B). In all treatments, darkness led to a lower transpiration rate, but this reduction was stronger in plants grown at moderate (84%) than at high RH (56%). Moreover, there was no MOV effect on the transpiration rate reduction in response to darkness. These results indicate that stomatal response to darkness is lower in high RH-grown plants compared to moderate RH-grown plants and that high MOV did not improve the dark-induced stomatal closure.

Stomatal physiology and morphology

Desiccated leaves from moderate RH-grown plants showed lower transpiration rates, irrespective of MOV, when compared to leaflets from plants developed at high RH (Fig. 2A). Leaflets grown under high RH with or without additional MOV had a similar initial transpiration rate, but stomata from high MOV leaflets closed faster in response to leaf desiccation (Fig. 2B). This resulted in a RWC after 4 h of desiccation 2-fold higher in high RH-grown plants with high MOV as compared to high RH-grown plants without additional MOV (Fig. 2C).

Table 1. Plant growth and visual quality parameters in fully developed plants (i.e. flower bud with cylindrical shape and pointed tip) of pot rose cv. Toril grown at moderate (61%) or high (92%) relative air humidity without (– MOV) or with (+ MOV) air movement. Different letters indicate significant differences according to Fisher's LSD test (*P* = 0.05).

	RH	- MOV	+ MOV	Mean
Total dry weight (g)	61%	6.4	6.5	6.5
	92%	6.9	7.0	6.9
	Mean	6.6	6.8	
Total leaf area (cm^2)	61%	545.9	572.5	559.2
	92%	538.6	535.8	537.2
	Mean	542.2	554.1	
Plant height (cm)	61%	34.3	35.2	34.8^a
	92%	37.3	38.2	37.8^b
	Mean	35.8	36.7	
Number of internodes	61%	11.7	11.9	11.8
	92%	11.9	12.6	12.3
	Mean	11.8	12.3	
Average internode length (cm)	61%	2.2	2.2	2.2^a
	92%	2.4	2.3	2.3^b
	Mean	2.3	2.3	
Peduncle length (cm)	61%	5.4	5.4	5.4^a
	92%	6.0	6.1	6.1^b
	Mean	5.7	5.8	
Peduncle diameter (mm)	61%	4.1	4.5	4.3^b
	92%	3.7	3.8	3.8^a
	Mean	3.9^a	4.2^b	
Flower dry weight (g)	61%	1.8	2.0	1.9^b
	92%	1.7	1.7	1.7^a
	Mean	1.8	1.9	
Time to flowering (days)	61%	31.4	31.3	31.4
	92%	30.5	31.6	31.1
	Mean	31.0	31.5	

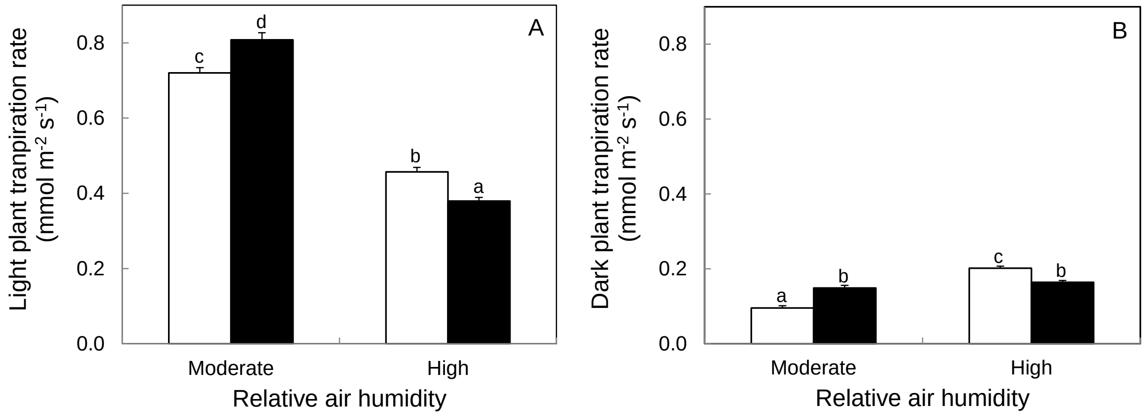

Fig. 1. Transpiration rate in intact plants during light (A) and dark (B) period in pot rose cv. ‘Toril’ grown at moderate (61%) or high (92%) RH, without (open columns) and with (solid columns) additional air movement. Measurements were conducted throughout three consecutive days in fully grown plants, starting when the flower bud had cylindrical shape and pointed tip. Values are the mean of 14 intact plants and bars represent the SEM. Different letters indicate significant differences according to Fisher’s LSD test ($P = 0.05$).

High MOV reduced the pore aperture by 16% ($P = 0.002$) and the pore length by 6% ($P = 0.022$) in stomata developed at high RH, while there was no significant effect on stomata developed at moderate RH (Table 2). Moreover, MOV had no significant effect on stomatal density ($P = 0.060$), index ($P = 0.719$), length ($P = 0.189$) and width ($P = 0.970$), but increasing the RH significantly increased these features by 4%, 13%, 20% and 26% ($P < 0.001$), respectively (Table 2).

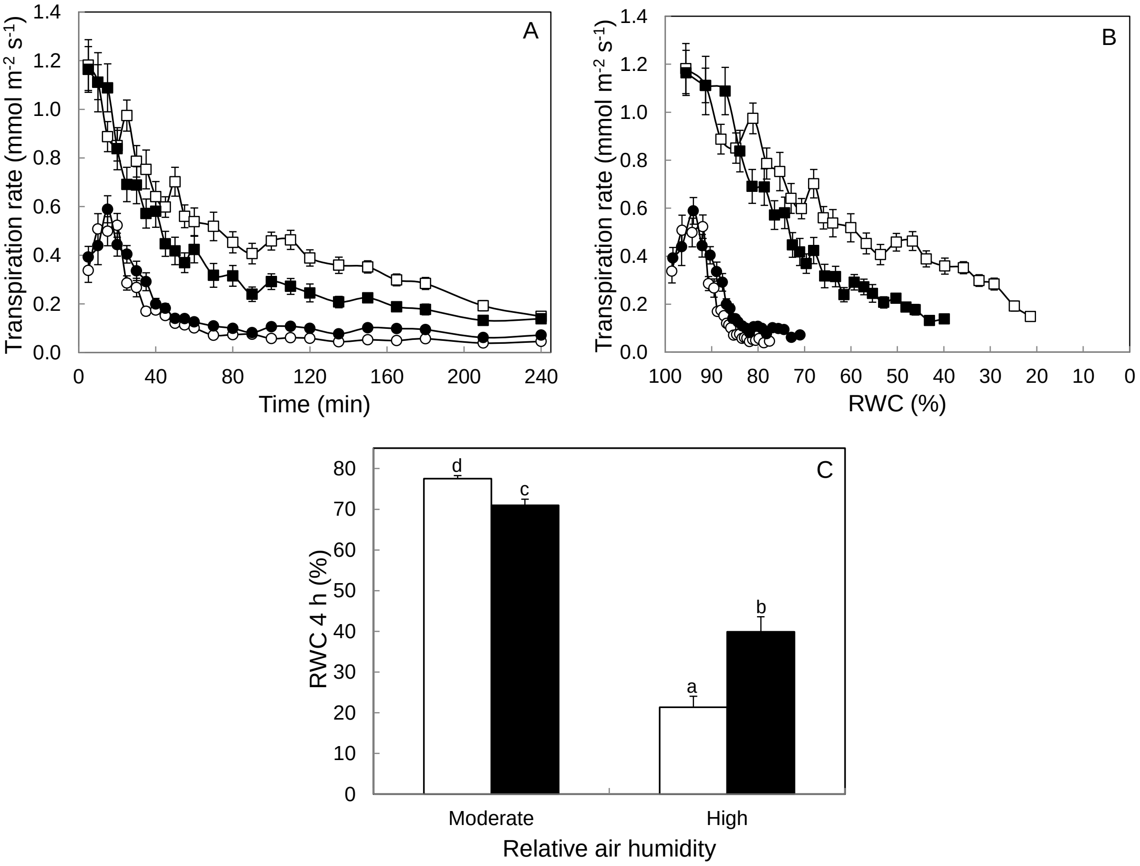

Fig. 2. Transpiration rate as a function of time of desiccation (A) and as a function of relative water content (RWC) (B) during 4 h of leaflet desiccation. Relative water content (RWC) after 4 h of leaflet desiccation (C). All measurements were conducted in pot rose cv. 'Toril' grown at moderate (circles; 61%) or high (squares; 92%) relative air humidity, without (open symbols) or with (solid symbols) additional air movement. Values are the mean of 28 detached leaflets and bars represent SEM. Different letters indicate significant differences according to Fisher's LSD test (*P* = 0.05).

Exposure to high MOV did not induce visual morphological changes on the leaf epidermal cells (e.g. shape or undulations) neither on the adaxial (data not shown) nor on the abaxial surfaces (Fig. 3). Additionally, high MOV did not visually affect stomatal deepness i.e. how deep the stomata are inserted in the leaf epidermis. However, a clear increase in stomatal size at high RH, already described above, was also apparent when analyzing leaf surface using the scanning electron microscope.

Table 2. Stomatal characteristics of pot rose cv. 'Toril' grown at moderate (61%) or high (92%) relative air humidity (RH), without (– MOV) or with (+ MOV) additional air movement in intact fully expanded leaves, 4 h after the beginning of the light period. Values are the mean of 70 field views (stomatal density and index) and 140 stomata (stomatal length and width, pore length and aperture). Different letters represent significant differences according to Fisher's LSD test (P = 0.05) (comparison in rows).

	Moderate RH			High RH		
	- MOV	+ MOV	Mean	- MOV	+ MOV	Mean
Stomatal density (no mm^{-2})	57.2	55.8	56.5^{a}	59.8	57.8	58.8^{b}
Stomatal index (%)	12.4	12.4	12.4^{a}	13.9	14.0	14.0^{b}
Stomatal length (µm)	27.4	26.8	27.1^{a}	32.6	32.4	32.5^{b}
Stomatal width (µm)	15.4	15.5	15.4^{a}	19.6	19.5	19.5^{b}
Pore length (µm)	17.3^{a}	17.2^{a}	17.2	24.5^{c}	23.1^{b}	23.8
Pore aperture (µm)	3.4^{a}	3.2^{a}	3.3	6.8^{c}	5.7^{b}	6.2

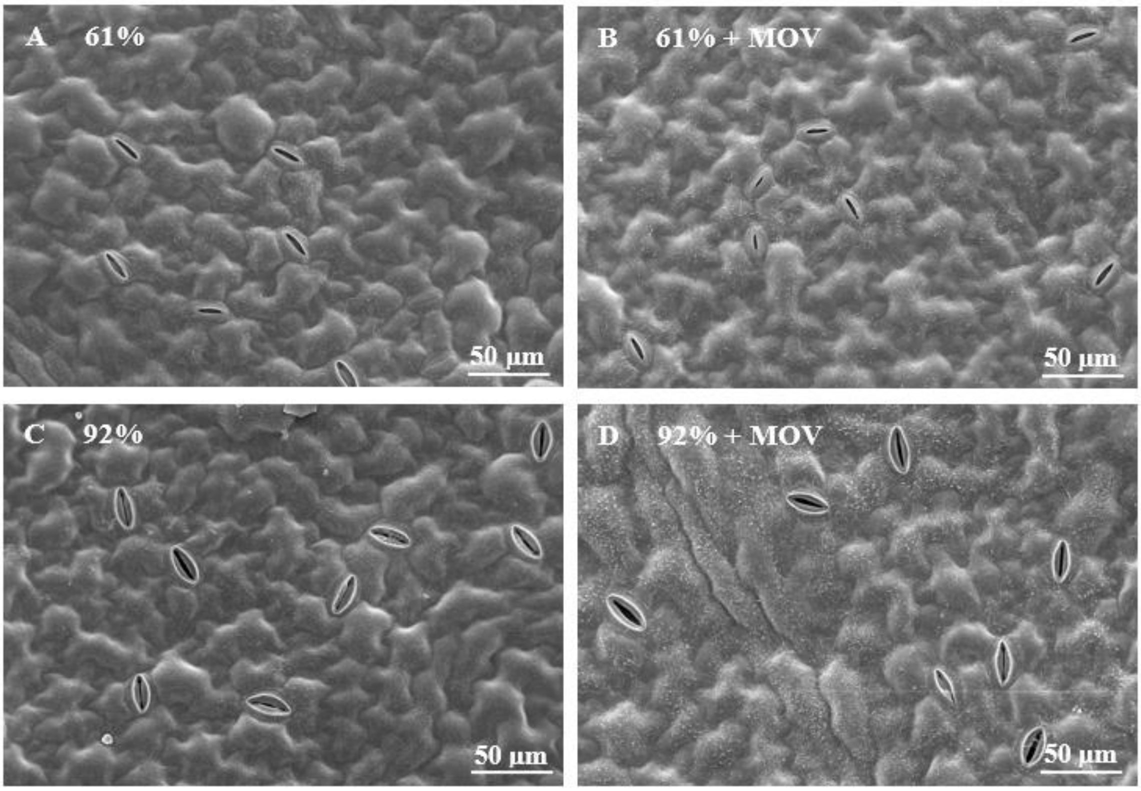

Fig. 3. Morphology of the abaxial leaf surface in pot rose cv. 'Toril' grown at moderate (61%; A, B) or high (92%; C, D) relative air humidity combined with no additional air movement (A, C) or with additional air movement (+MOV; B, D). Images were obtained by scanning electron microscope.

Stomatal responsiveness to ABA

ABA and its metabolites (PA, DPA, ABA-GE, 7'OH-ABA, *neo*PA, *trans*-ABA and *cis*-ABA) were quantified in fully developed leaves. In all treatments, the levels of 7'OH-ABA, *neo*PA, *trans*-ABA and *cis*-ABA were very low (data not shown), hence, they have only been included in the combined amount of ABA and its metabolites when quantification was possible. High RH reduced the concentrations of ABA by 52% (P = 0.005; Fig. 4A), PA by 46% (P = 0.008; Fig. 4B), DPA by 48% (P = 0.004; Fig. 4C), ABA-GE by 23% (P = 0.184; Fig. 4D) and the combination of ABA and its metabolites by 35% (P = 0.013; Fig. 4E). High MOV did not significantly affect the concentration of ABA (P = 0.764, Fig. 4F), PA (P = 0.224, Fig. 4G), DPA (P = 0.234, Fig. 4H), ABA-GE (P = 0.488, Fig. 4I) or combined ABA and its metabolites (P = 0.671, Fig. 4J).

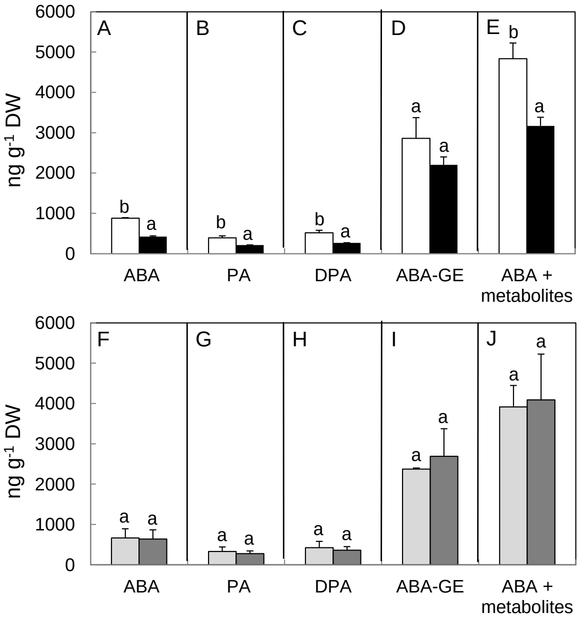

Fig. 4. Effect of moderate (61%; open columns) and high (92%; solid columns) relative air humidity (A-E), combined with no additional air movement (light grey columns) or with additional air movement (grey columns) (F-J) on the levels (ng g^{-1} DW) of abscisic acid (ABA) (A, F), phaseic acid (PA) (B, G), 4'-dihydrophaseic acid (DPA) (C, H), ABA-β-D-glucosyl ester (ABA-GE) (D, I) and total concentration of ABA and its metabolites (E, J) in leaves of rose plants cv. 'Toril'. Sampling occurred five hours after the beginning of the light period. Each sample consisted of a composite of seven leaflets from seven biological replicates. Values are the mean of two biological repeats and bars represent the SEM. Different letters indicate significant differences according to Fisher's LSD test (P = 0.05).

In fully developed leaflets (100% FLE) there was a significant interaction between RH and MOV (*P* = 0.028; Table 3). When these leaves were grown at high RH, high MOV reduced by 48% the EC_{50}, while it was not significantly affected in moderate RH-grown plants. In non-fully developed leaflets (70-80% FLE) grown at high RH without MOV stomatal response to ABA feeding was practically absent (i.e. the stomatal remained open with no reduction in the transpiration rate), making it impossible to determine the EC_{50} (Table 3). However, high RH-grown plants with high MOV showed an EC_{50} of 0.431, which was about 2.3 times higher than the one observed in moderate RH-grown plants (Table 3). In moderate RH-grown plants, MOV did not affect EC_{50} (Table 3). EC_{50} was not significantly different when comparing non-fully developed leaflets with fully developed leaflets (moderate RH without MOV, *P* = 0.060; moderate RH with high MOV, *P* = 0.064; high RH with MOV, *P* = 0.592) (Table 3).

Table 3. Abscisic acid (ABA) intake required to reduce the transpiration rate to half-way (50%) between the maximum and minimum values (EC_{50}) in fully developed leaflets (100% FLE) and non-fully developed leaflets (70–80% FLE) of pot rose cv. Toril grown at moderate (61%) or high (92%) relative air humidity (RH), without (– MOV) or with (+ MOV) air movement. ABA feeding (100 µM) through the leaflet petiole lasted for 150 min. Values are the mean of five detached leaflets. Different letters indicate significant differences according to Fisher's LSD test (*P* = 0.05) (comparison within leaf developmental stage).

Leaf developmental stage (% FLE)		Moderate RH	High RH
100%	- MOV	0.121^{a}	0.758^{c}
	+ MOV	0.106^{a}	0.395^{b}
70-80%	- MOV	0.212^{a}	*
	+ MOV	0.169^{a}	0.431^{b}

(*) In high RH (92%) without additional MOV the stomatal response to ABA was practically absent, making it impossible to determine the EC_{50}.

Discussion

Effects of MOV and RH on plant transpiration rate

It is well known that high wind-speed reduces leaf boundary layer, which results in enhanced transpiration rate (Schuepp, 1993; Lambers et al., 2008). Thus, the increased transpiration rate found in intact plants grown under moderate RH and subjected to high MOV can possibly be explained by the reduction of the leaf boundary layer (Mortensen and Gislerød, 1997; Anten et al., 2010). Nevertheless, at high RH the saturated air present on the leaf boundary layer (nearly 100% RH) was replaced also by very moist air existing in the growth cabinets (92 ± 2%), which explains why high MOV did not increase the transpiration rate also under high RH levels (Mortensen and Gislerød, 1997). Actually, in these plants, the lower stomatal pore dimensions (Table 2) might have contributed to their reduced plants transpiration rate (Fig. 1), via a reduction in the total transpiration area.

Effects of MOV and RH on the stomatal responsiveness to closing stimuli

In *Arabidopsis* the cuticle wax deposition contributes to enhance the water loss tolerance (Seo et al., 2011; Yang et al., 2011; Zhu et al., 2014). However, in R. hybrida, the cuticle has a minor contribution to the total leaf water loss while an increase in the leaf transpiration rate seems to largely reflect a higher stomatal pore area per leaf area (Fanourakis et al., 2013). Long-term high RH is known to decrease stomatal responsiveness to closing stimuli leading to high transpiration rate and lower RWC upon desiccation (Torre et al., 2003; Rezaei Nejad and van Meeteren, 2005) and darkness (Arve et al., 2013; Fanourakis et al., 2013). Our results confirm those findings (Figs. 1 and 2) and demonstrated for the first time that MOV is effective in increasing stomatal responsiveness to desiccation in high RH-grown leaflets, resulting in a 2-fold higher RWC as compared to leaflets without additional MOV (Fig. 2C). In spite of the improved stomatal functioning in high RH-grown leaflets subjected to MOV compared to still air, the RWC of the former was still far below the one of moderate RH-grown plants (Fig. 2A-B). This can be partly explained by their initial higher transpiration rate contributing to a large water loss in the first phase of leaflet desiccation, before the stomata trigger the closure response.

ABA is a very important hormone inducing stomatal closure under different abiotic stress conditions (Xiong et al., 2002; Davies et al., 2005; Schachtman and Goodger, 2008). Unlike our hypothesis, it was found that despite the increased stomatal responsiveness to desiccation in high RH-grown plants subjected to high MOV (Fig. 2), this did not significantly increase the endogenous [ABA] and its metabolites in the bulk leaves (Figure

4F-J). Other reasons such as (1) perception and/or sensitivity of ABA receptors (Anderson et al., 1994; Schwartz et al., 1994) which may differ in fully expanded and in expanding leaves and (2) [ABA] in the guard cells (Harris and Outlaw, 1991) might also be involved in stomatal closure. High MOV decreased the required amount of exogenous ABA to reduce in 50% the half-maximal effective concentration (EC_{50}) in detached fully expanded leaflets grown at high RH (Table 3). Although in *Arabidopsis*, Aliniaeifard and van Meeteren, (2014b) did not find a correlation between stomatal responsiveness to desiccation and stomatal sensitivity to ABA, our results suggest that in high RH-grown plants, high MOV increased stomatal tolerance to desiccation due to increased stomatal sensitivity to ABA, rather than an increase in the leaf [ABA]. Pantin et al. (2013) suggested that stomatal sensitivity to ABA is related to the leaf developmental stage in *A. thaliana*. Here we found no difference in the stomatal responsiveness to ABA feeding between leaf developmental stages (Table 3) indicating that even non-fully mature stomata grown at high RH do not respond to a short-term ABA application.

Concerning the effect of high RH on the free [ABA], ‘Toril’ reduced by 35% the concentration of ABA and its metabolites (Fig. 4E). These findings are in agreement with Giday et al. (2013) who found a 25-35% decrease in the [ABA] in the sensitive cultivars. Similarly, the concentrations of the metabolites PA (Fig. 4B) and DPA (Fig. 4C) followed the pattern of ABA (Fig. 4A). Arve et al. (2013) described the same tendency and suggested that low PA and DPA levels seem to be a result of low ABA levels at high RH and a constant inactivation rate of ABA to PA and consequently to DPA.

Effects of MOV and RH on stomatal anatomy and plant morphology

The absence of high MOV effect in most of the stomatal anatomical features and in the leaf ultrastructure (Table 2 and Fig. 3) is in contrast with a previous study in *Picea sitchensis* and *Pinus sylvestris* (van Gardingen et al., 1991) which described modifications on the leaf surface in plants grown under high MOV (11 m s^{-1}). This might indicate that different species, with contrasting leaf morphology, respond differently to MOV but it can also be due to the extremely high MOV intensity applied to those trees as compared to the MOV used in this study. Stomatal density and index were significantly increased at high RH, but according to Fanourakis et al. (2013) this effect seems to be cultivar dependent. Moreover, our study confirmed that a sensitive cultivar (‘Toril’) responds to high RH enhancing their stomatal and pore dimensions (Table 2), as previously reported for other sensitive rose cultivars (Torre et al., 2003; Arve et al., 2013; Fanourakis et al., 2013; Giday et al., 2013).

In contrast, high RH did not affect the leaf ultrastructure (Fig. 3) and it had only a minor effect on plant growth and visual quality parameters (Table 1), which is in agreement with previous studies (Mortensen and Gislerød, 1997; Torre and Fjeld, 2001; Torre et al., 2003). The thinner peduncles observed in high-RH grown plants can partly contribute to the higher incidence of bent-neck symptoms during post-harvest, which is typically found in plants developed at high RH (Fanourakis et al., 2012). Here, we show that this positive effect of MOV on peduncle diameter (Table 1) might partly reduce the bent-neck incidence in high RH-grown plants. Mortensen and Gislerød (1997) also reported little effect of MOV on plant height, no effect on total dry weight but an increased time to flowering. It is concluded that unlike MOV, high RH has a strong effect on stomatal anatomy in *R. hybrida*, whereas leaf surface morphology as well as plant growth and visual quality parameters are not affected by either RH of MOV, evidencing that rose plants seem to be well adapted to high MOV.

Conclusions

The current work shows for the first time that high MOV during leaf development improves stomatal functioning of leaves developed at high RH. Unlike our hypothesis, we have shown that this is due to an increased sensitivity to ABA (evidenced by a lower transpiration rate in leaflets fed with exogenous ABA) and reduced stomatal pore length and aperture rather than an increase in the leaf [ABA] or in the concentration of its metabolites. Finally, in this study we showed that leaf developmental stage had no effect in the stomatal responsiveness to ABA feeding demonstrating that even non-fully mature stomata grown at high RH do not respond to a short-term ABA application.

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References

Abrams, S. R., Nelson, K., and Ambrose, S. J. (2003). Deuterated abscisic acid analogs for mass spectrometry and metabolism studies. *J. Label. Compd. Radiopharm.* 46, 273–283. doi:10.1002/jlcr.670.

Aliniaeifard, S., Malcolm Matamoros, P., and van Meeteren, U. (2014a). Stomatal malfunctioning under low VPD conditions: Induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? *Physiol. Plant.* 152, 688–699. doi:10.1111/ppl.12216.

Aliniaeifard, S., and van Meeteren, U. (2014b). Natural variation in stomatal response to closing stimuli among *Arabidopsis thaliana* accessions after exposure to low VPD as a tool to recognize the mechanism of disturbed stomatal functioning. *J. Exp. Bot.* 65, 6529–6542. doi:10.1093/jxb/eru370.

Anderson, B. E., Ward, J. M., and Schroeder, J. I. (1994). Evidence for an extracellular reception site for abscisic acid in *Commelina* guard cells. *Plant Physiol.* 104, 1177–1183.

Anten, N. P. R., Alcalá-Herrera, R., Schieving, F., and Onoda, Y. (2010). Wind and mechanical stimuli differentially affect leaf traits in *Plantago major*. *New Phytol.* 188, 554–564. doi:10.1111/j.1469-8137.2010.03379.x.

Arve, L. E., Terfa, M. T., Gislerød, H. R., Olsen, J. E., and Torre, S. (2013). High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves. *Plant, Cell Environ.* 36, 382–392. doi:10.1111/j.1365-3040.2012.02580.x.

Beryl, C. A., and Mitchell, C. A. (1977). Characterization of mechanical stress dwarfing in chrysanthemum. *J. Am. Soc. Hortic. Sci.* 102, 591 – 594.

Blom-Zandstra, M., Pot, C. S., Maas, F. M., and Schapendonk, A. H. C. M. (1995). Effects of different light treatments on the nocturnal transpiration and dynamics of stomatal closure of two Rose cultivars. *Sci. Hortic. (Amsterdam).* 61, 251–262. doi:10.1016/0304-4238(94)00751-Z.

Cutler, A. J., and Krochko, J. E. (1999). Formation and breakdown of ABA. *Trends Plant Sci.* 4, 472–477. doi:10.1016/S1360-1385(99)01497-1.

Davies, W. J., Kudoyarova, G., and Hartung, W. (2005). Long-distance ABA signaling and its relation to other signaling pathways in the detection of soil drying and the mediation of the plant’s response to drought. *J. Plant Growth Regul.* 24, 285–295. doi:10.1007/s00344-005-0103-1.

Downs, R. J., and Krizek, D. T. (1997). “Air movement,” in *Plant growth chamber handbook*, eds. R. W. Langhans and T. W. Tibbitts (Iowa Agricultural and Home Economics Experiment Station), 87–104.

Drake, P. L., Froend, R. H., and Franks, P. J. (2013). Smaller, faster stomata: Scaling of stomatal size, rate of response, and stomatal conductance. *J. Exp. Bot.* 64, 495–505. doi:10.1093/jxb/ers347.

Ennos, A. R. (1997). Wind as an ecological factor. *Trends Ecol. Evol.* 12, 108–111. doi:10.1016/S0169-5347(96)10066-5.

Erner, Y., Biro, R., and Jaffe, M. J. (1980). Thigmomorphogenesis: Evidence for a translocatable thigmomorphogenetic factor induced by mechanical perturbation of beans (*Phaseolus vulgaris*). *Physiol. Plant.* 50, 21–25. doi:10.1111/j.1399-3054.1980.tb02677.x.

Fanourakis, D., Carvalho, S. M. P., Almeida, D. P. F., and Heuvelink, E. (2011). Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. *Physiol. Plant.* 142, 274–286. doi:10.1111/j.1399-3054.2011.01475.x.

Fanourakis, D., Carvalho, S. M. P., Almeida, D. P. F., van Kooten, O., van Doorn, W. G., and Heuvelink, E. (2012). Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. *Postharvest Biol. Technol.* 64, 64–73. doi:10.1016/j.postharvbio.2011.09.016.

Fanourakis, D., Heuvelink, E., and Carvalho, S. M. P. (2013). A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. *J. Plant Physiol.* 170, 890–898. doi:10.1016/j.jplph.2013.01.013.

Fanourakis, D., Giday, H., Milla, R., Pieruschka, R., Kjaer, K. H., Bolger, M., Vasilevski, A., Nunes-Nesi, A., Fiorani, F., and Ottosen C-O (2015). Pore size regulates operating stomatal conductance , while stomatal densities drive the partitioning of conductance between leaf sides. *Ann. Bot.* 115, 555–565. doi:10.1093/aob/mcu247.

Feller, U., and Vaseva, I. I. (2014). Extreme climatic events: impacts of drought and high temperature on physiological processes in agronomically important plants. *Front. Environ. Sci.* 2, 1–17. doi:10.3389/fenvs.2014.00039.

Giday, H., Fanourakis, D., Kjaer, K. H., Fomsgaard, I. S., and Ottosen, C. O. (2013). Foliar abscisic acid content underlies genotypic variation in stomatal responsiveness after growth at high relative air humidity. *Ann. Bot.* 112, 1857–1867. doi:10.1093/aob/mct220.

Grace J (1977). *Plant responses to wind*. London, UK: Academic Press.

Harris, M. J., and Outlaw, W. H. (1991). Rapid adjustment of guard-cell abscisic acid levels to current leaf-water status. *Plant Physiol.* 95, 171–173. doi:10.1104/pp.95.1.171.

In, B.-C., Sato, K., Ito, K., Inamoto, K., Doi, M., and Mori, G. (2006). Influences of preharvest relative humidity on yield, vase life and transpiration of cut roses. *Environ. Control Biol.* 44, 257–263. doi:10.2525/ecb.44.257.

Jaffe, M. J. (1976). Thigmomorphogenesis: Electrical resistance and mechanical correlates of the early events of growth retardation due to mechanical stimulation in beans. *Zeitschrift für Pflanzenphysiologie* 78, 24–32. doi:10.1016/S0044-328X(76)80136-5.

Lambers, H., Chapin, F. S., and Pons, T. L. (2008). *Plant physiological ecology*. New York, USA: Springer doi:10.1007/978-0-387-78341-3.

Mortensen, L., and Gislerød, H. (1997). Effects of air humidity and air movement on the growth and keeping quality of roses. *Gartenbauwissenschaf* 62, 273–277.

Nambara, E., and Marion-Poll, A. (2005). Abscisic acid biosynthesis and catabolism. *Annu. Rev. Plant Biol.* 56, 165–185. doi:10.1146/annurev.arplant.56.032604.144046.

Okamoto, M., Tanaka, Y., Abrams, S. R., Kamiya, Y., Seki, M., and Nambara, E. (2009). High humidity induces abscisic acid 8’-hydroxylase in stomata and vasculature to regulate local and systemic abscisic acid responses in arabidopsis. *Plant Physiol.* 149, 825–834. doi:10.1104/pp.108.130823.

Pantin, F., Renaud, J., Barbier, F., Vavasseur, A., Le Thiec, D., Rose, C., Bariac, T., Casson, S., McLachlan, D. H., Hetherington, A. M., et al. (2013). Developmental priming of stomatal sensitivity to abscisic acid by leaf microclimate. *Curr. Biol.* 23, 1805–1811. doi:10.1016/j.cub.2013.07.050.

Reynolds-Henne, C. E., Langenegger, A., Mani, J., Schenk, N., Zumsteg, A., and Feller, U. (2010). Interactions between temperature, drought and stomatal opening in legumes. *Environ. Exp. Bot.* 68, 37–43. doi:10.1016/j.envexpbot.2009.11.002.

Rezaei Nejad, A., and van Meeteren, U. (2005). Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. *Physiol. Plant.* 125, 324–332. doi:10.1111/j.1399-3054.2005.00567.x.

Rezaei Nejad, A., and van Meeteren, U. (2007). The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity. *J. Exp. Bot.* 58, 627–636. doi:10.1093/jxb/erl234.

Rezaei Nejad, A., and Van Meeteren, U. (2008). Dynamics of adaptation of stomatal behaviour to moderate or high relative air humidity in *Tradescantia virginiana*. *J. Exp. Bot.* 59, 289–301. doi:10.1093/jxb/erm308.

Ross, A. R. S., Ambrose, S. J., Cutler, A. J., Feurtado, J. A., Kermode, A. R., Nelson, K., Zhou, R., and Abrams, S. R. (2004). Determination of endogenous and supplied deuterated abscisic acid in plant tissues by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry with multiple reaction monitoring. *Anal. Biochem.* 329, 324–333. doi:10.1016/j.ab.2004.02.026.

Salisbury, E. (1927). On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. *Philos. Trans. R. Soc. London* 216, 1–65.

Schachtman, D. P., and Goodger, J. Q. D. (2008). Chemical root to shoot signaling under drought. *Trends Plant Sci.* 13, 281–287. doi:10.1016/j.tplants.2008.04.003.

Schwartz, a, Wu, W. H., Tucker, E. B., and Assmann, S. M. (1994). Inhibition of inward K^+ channels and stomatal response by abscisic acid: an intracellular locus of phytohormone action. *Proc. Natl. Acad. Sci. U. S. A.* 91, 4019–4023. doi:10.1073/pnas.91.9.4019.

Schuepp, P. H. (1993). Tansley Review No. 59 Leaf boundary layers. *New Phytol.* 125, 477–507.

Seo, P. J., Lee, S. B., Suh, M. C., Park, M.-J., Go, Y. S., and Park, C.-M. (2011). The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in *Arabidopsis*. *Plant Cell* 23, 1138–1152. doi:10.1105/tpc.111.083485.

Slavík, B. (1974). *Methods of studying plant water relations*. London, UK: Chapman and Hall.

Tallman, G. (2004). Are diurnal patterns of stomatal movement the result of alternating metabolism of endogenous guard cell ABA and accumulation of ABA delivered to the apoplast around guard cells by transpiration? *J. Exp. Bot.* 55, 1963–1976. doi:10.1093/jxb/erh212.

Tanaka, Y., Sano, T., Tamaoki, M., Nakajima, N., and Kondo, N. (2005). Ethylene inhibits abscisic acid-induced stomatal closure in arabidopsis. *Plant Physiol.* 138, 2337–2343. doi:10.1104/pp.105.063503.plants.

Torre, S., and Fjeld, T. (2001). Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. *Sci. Hortic. (Amsterdam).* 89, 217–226. doi:10.1016/S0304-4238(00)00229-6.

Torre, S., Fjeld, T., Gislerod, H. R., and Moe, R. (2003). Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. *J. Am. Soc. Hortic. Sci.* 128, 598–602.

van Gardingen, P. R., Grace, J., and Jeffree, C. E. (1991). Abrasive damage by wind to the needle surfaces of *Picea sitchensis* (Bong.) Carr. and *Pinus sylvestris* L. *Plant, Cell Environ.* 14, 185–193. doi:10.1111/j.1365-3040.1991.tb01335.x.

Weyers, J. D., and Hillman, J. R. (1979). Sensitivity of *Commelina* stomata to abscisic acid. *Planta* 146, 623–8. doi:10.1007/BF00388842.

Whitehead, F. (1962). Experimental studies of the effect of wind on plant growth and anatomy. *New Phytol.* 61, 59–62. doi:10.1111/j.1469-8137.1963.tb06316.x.

Xiong, L., Schumaker, K. S., and Zhu, J. K. (2002). Cell signaling during cold, drought, and salt stress. *Plant Cell* 14, S165. doi:10.1105/tpc.000596.S166.

Yang, J., Isabel Ordiz, M., Jaworski, J. G., and Beachy, R. N. (2011). Induced accumulation of cuticular waxes enhances drought tolerance in *Arabidopsis* by changes in development of stomata. *Plant Physiol. Biochem.* 49, 1448–1455. doi:10.1016/j.plaphy.2011.09.006.

Zaharia, L. I., Galka, M. M., Ambrose, S. J., and Abrams, S. R. (2005). Preparation of deuterated abscisic acid metabolites for use in mass spectrometry and feeding studies. *J. Label. Compd. Radiopharm.* 48, 435–445. doi:10.1002/jlcr.939.

Zhu, L., Guo, J., Zhu, J., and Zhou, C. (2014). Enhanced expression of EsWAX1 improves drought tolerance with increased accumulation of cuticular wax and ascorbic acid in transgenic *Arabidopsis*. *Plant Physiol. Biochem.* 75, 24–35. doi:10.1016/j.plaphy.2013.11.028.

CHAPTER 3.2

Effects of air humidity and air movement on growth, visual quality and post-production stress tolerance of pot rose 'Toril'

Abstract

This work aimed at assessing whether a high level of air movement would improve stomatal closure, counteracting the negative effect of high relative air humidity (RH > 85%) on plant growth, visual quality and postharvest stress tolerance to dark and drought (simulating shipping and/or retailing). Pot rose 'Toril' was grown at moderate (60%) and high (90%) RH, combined with no additional air movement or with a continuous additional air flow of 0.92 ± 0.03 m s^{-1} (MOV). At flowering stage, plants were transferred to continuous darkness and without irrigation. Total dry weight and time to flowering were not significantly affected by RH or MOV. MOV did not affect plant height nor flower dry weight, but it significantly increased peduncle diameter by 6%. Plants grown at elevated RH showed a six-fold higher initial transpiration rate at the start of the postharvest period and a reduced longevity (71%) as compared to moderate RH-grown plants. MOV decreased the initial transpiration rate in high RH-grown plants by 23%. In conclusion, high RH had a minimal effect on plant growth and visual quality parameters but it drastically reduced postharvest storability. MOV during growth slightly improved post-production storability in high RH-grown plants, having a minor effect on visual quality.

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Introduction

Long-term high relative air humidity (RH > 85%) during growth reduces postharvest life of roses due to stomata malfunctioning and negative water balance (Fanourakis *et al.*, 2011; Fanourakis *et al.*, 2012). However, little is known about its effects on plant growth, visual quality and postharvest stress tolerance of pot roses during shipping and/or retailing. Wind is an environmental factor having several effects on plants. It reduces the thickness of the leaf boundary layer, enhancing gas diffusion (CO_2 and H_2O) and heat exchange rate depending on leaf characteristics and wind speed (Anten *et al.*, 2010). Moreover, wind flow exerts drag forces on plants that cause mechanical stress (Anten *et al.*, 2010). Several plant species respond to mechanical stress by closing stomata (Biddington 1986). Mechanical stress also leads to changes in plant morphology mainly by inhibiting stem elongation and increasing stem diameter (Telewski and Pruyn, 1998). Dark and drought conditions are very common during shipping and/or retailing of ornamentals. These conditions lead to a reduction in the storability of pot plants that is usually worsened by the combination with high temperatures during transport (Reid and Jiang, 2012). The objective of this work was to assess whether a high level of additional air movement (MOV) throughout leaf development – mechanical stress – would reduce water loss, counteracting the negative effect of high RH during growth on postharvest quality. Additionally, its impact on plant growth, visual quality and post-production stress tolerance to dark and drought (simulating shipping and/or retailing) was evaluated.

Materials and methods

Single node rooted cuttings from pot rose 'Toril' (*Rosa* × *hybrida*) with one mature leaf were planted in 0.66 l pots containing a standard fertilized *Sphagnum* peat (Floralux, Nittedal, Norway). Fifty six plants were randomly distributed over four climate controlled growth chambers (length × width × height = 1.5 × 1.0 × 2.2 m). Plants were grown as single shoot, one plant per pot. During the cultivation period, the RH was 61 ± 3% (moderate RH) in two growth chambers and 92 ± 2% (high RH) in the other two. In two growth chambers (one per RH level), two fans were on during 24 h (HT – 112 E, Honeywell, Lausanne, Switzerland), being equidistant (70 cm) from the 14 plants that were placed in a semi-circle side by side. In the chambers without fans the plants were distributed similarly. Plants were rotated 90˚ daily in the horizontal plane to ensure that exposure to MOV was similar in all directions (Anten *et al.* 2010). In the growth chambers with fans (MOV), the turbulence intensity was 1.42 ± 0.13 and the air velocity was 0.921 ±

0.033 m s^{-1} (Ultrasonic anemometer, Model 81000, Young, Traverse City, MI, USA), being absent in the growth chambers without fans. Temperature was 21 ± 0.5 ˚C (day and night), resulting in vapour pressure deficits of 0.97 ± 0.03 kPa (moderate RH) and 0.20 ± 0.01 kPa (high RH). The CO_2 concentration was 400 ± 50 µmol mol^{-1} and high pressure sodium lamps (Plantastar 400W, Osram, Münich, Germany) provided 20 h photoperiod of 160 ± 10 µmol m^{-2} s^{-1} photosynthetic active radiation (Li-250 Light Meter, LI-COR, Lincoln, NE, USA). Climate data was recorded automatically every 5 minutes (Priva, De Lier, The Netherlands). Plants were watered daily until draining with a standard nutrient solution. The pH and EC levels of the nutrient solution were 5.7 and 1.75 dS m^{-1}, respectively.

Two types of measurements were conducted in this study. Firstly, the effects of the RH and MOV during growth on total dry weight, plant height, peduncle diameter, flower dry weight and time to flowering (number of days till flower stage 2 i.e. flower bud with a cylindrical shape and pointed tip) were assessed in seven plants per treatment. This experiment was repeated twice. Secondly, plants at flower stage 2 were moved to cabinets (Termaks KB 2324V, Termaks, Bergen, Norway) in continuous darkness at 40 ± 2% RH and 20 ± 0.5 ˚C, without watering throughout the post-production period (to simulate shipping and/or retailing). Darkness was briefly interrupted twice per day for plants evaluation. Pots were placed in plastic bags tight at soil level to prevent evaporation from the substrate. Plant transpiration rate was gravimetrically recorded on a daily basis. The termination of storage-life was determined based on the occurrence of at least one of the following criteria: (i) edges of five or more petals desiccated, i.e. turned brown or black and may dry out; (ii) flower wilted, i.e. dropping or the petals became wrinkled; (iii) more than 50% of the total leaf area and/or 50% of the total number of leaves desiccated or wilted (VBN, 2005). At the end of storage life the total leaf area per plant was recorded (Model 3100 Area Meter, LI-COR, Lincoln, NE, USA) and plant transpiration rate was normalized per unit leaf area. Seven plants per treatment were evaluated. This experiment was performed once.

The experimental set-up was a complete randomized block design where individual plants were considered as independent experimental units. Analysis of variance was conducted and mean separation was done using the least significant difference (LSD) at P=0.05. The Genstat software (15th edition; VSN International Ltd., Herts, UK) was used for the analysis.

Results and discussion

In general, high RH during the growing period had only a minor effect on plant growth and visual quality parameters (Table 1), which is consistent with previous studies (Mortensen and Gislerød, 1997; Torre and Fjeld, 2001). For instance, high RH during growth did not significantly affect total dry weight (*P*=0.174), number of internodes (*P*=0.250), or time to flowering (*P*=0.480). However, it significantly increased plant height (*P*<0.001), resulting in 9% taller plants due to a significantly longer peduncle length (12%) (*P*<0.001) and a higher average internode length (6%) (*P*=0.010) (Table 1). Additionally, flower dry weight and peduncle diameter were significantly reduced in high RH-grown plants: 13% (*P*=0.002) and 12% (*P*<0.001), respectively (Table 1). Thinner peduncles can partly contribute to higher incidence of bent-neck symptoms during the post-harvest of high-RH grown plants (Fanourakis *et al.*, 2012). In this study, bent-neck was not observed and the termination of storability was mostly due to wilted or desiccated petals and/or leaves. A frequently overlooked environmental factor that affects plant growth is air movement. Mortensen and Gislerød (1997) reported little or no effect of continuous air movement on plant growth and vase life during cultivation of cut roses Although in the current study a much higher level of MOV was used (0.92 m s^{-1}, compared to up to 0.35 m s^{-1}), the only significant effect of MOV on plant growth was a 6% increase on peduncle diameter (*P*=0.022) (Table 1). MOV had no significant effect on plant height (*P*=0.161), total dry weight (*P*=0.727) or on time to flowering (*P*=0.219) (Table 1). Mortensen and Gislerød (1997) also reported little effect of MOV on plant height, no effect on total dry weight but an increased time to flowering.

During the postproduction storage period in continuous darkness, plants grown at elevated RH had a six-fold higher initial transpiration rate than plants grown at moderate RH (day 1; Fig. 1A). Although the transpiration rate of the former plants decreased throughout the storage period, it still remained very high compared to the moderate RH-grown plants. Confirming our results (Fig 1A), several studies described a higher water loss in detached high RH-grown leaves allowed to desiccate during 4 h (Mortensen and Gislerød, 1997; Fanourakis *et al.*, 2011). High transpiration rates of leaves developed under elevated RH and further transferred to conditions of high evaporative demand have already been described in cut roses (Mortensen and Gislerød, 1997; Fanourakis *et al.*, 2011). This excessive water loss, that could not be compensated by an increased water uptake (as plants were not irrigated in order to mimic shipping and/or retailing conditions), can explain the strong reduction in the storability of high RH-grown plants (from 25 to 7 days; *P*<0.001) (Fig. 1B). Although not significantly different (*P*=0.684), we found that MOV enhanced the storability from 6 to 8 days in plants grown at high RH (Fig. 1B). The positive but limited effect of MOV on storability of high RH-grown plants might be partly explained by the 23% lower initial transpiration rate (day 1; Fig. 1A) whereas throughout the remaining storage period there was no significant effect of MOV on this parameter (Fig. 1A).

Table 1. Plant growth and visual quality parameters at harvest stage of pot rose 'Toril' grown at moderate (60%) or high (90%) relative air humidity without (-MOV) or with (+MOV) additional air movement. Different letters represent significant differences according to LSD test (*n*=14).

		– MOV	+ MOV	Mean
Total dry weight (g)	60%	6.4	6.5	6.5
	90%	6.9	7.0	6.9
	Mean	6.6	6.8	
Plant height (cm)	60%	34.3	35.2	34.8^{a}
	90%	37.3	38.2	37.8^{b}
	Mean	35.8	36.7	
Number of internodes	60%	11.7	11.9	11.8
	90%	11.9	12.6	12.3
	Mean	11.8	12.3	
Average internode length (cm)	60%	2.2	2.2	2.2^{a}
	90%	2.4	2.3	2.3^{b}
	Mean	2.3	2.3	
Peduncle length (cm)	60%	5.4	5.4	5.4^{a}
	90%	6.0	6.1	6.1^{b}
	Mean	5.7	5.8	
Peduncle diameter (mm)	60%	4.1	4.5	4.3^{b}
	90%	3.7	3.8	3.8^{a}
	Mean	3.9^{a}	4.2^{b}	
Flower dry weight (g)	60%	1.8	2.0	1.9^{b}
	90%	1.7	1.7	1.7^{a}
	Mean	1.8	1.9	
Time to flowering (days)	60%	31.4	31.3	31.4
	90%	30.5	31.6	31.1
	Mean	31.0	31.5	

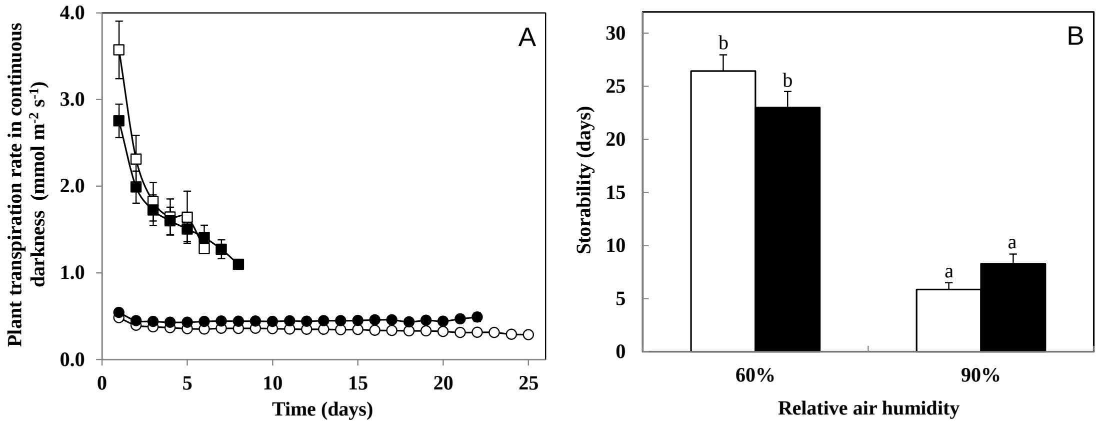

Fig 1. Plant transpiration rate during storage (A) and postharvest storability (B) of pot rose ‘Toril’ which was grown at moderate (circles, 60%) or high (squares, 90%) relative air humidity without (white symbols) or with (black symbols) additional air movement and then stored in continuous darkness at 40 ± 2% RH and 20 ± 0.5 ˚C. Values are the mean of 7 plants ± SEM. Different letters represent significant differences according to LSD test (*n*=7).

In conclusion, long-term high RH during growth with or without MOV had a minimal effect on plant growth and visual quality parameters but drastically reduced postharvest storability. In this study we showed that a continuous high level of air movement throughout leaf development in high RH-grown plants only slightly reduced water loss during postharvest, resulting in a limited capacity to counteract the negative effect of high RH during growth on the post-production storability. Thus, cultivation methods other than additional air movement are necessary to improve postharvest stress tolerance of pot roses grown under high RH.

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References

Anten N.P.R., Alcalá-Herrera R., Schieving F., Onoda Y. 2010. Wind and mechanical stimuli differentially affect leaf traits in *Plantago major*. New Phytol. 188: 554–564.

Biddington N.L. 1986. The effects of mechanically-induced stress in plants – a review. Plant Growth Regul. 4: 103–123.

Fanourakis D., Carvalho S.M.P., Almeida D.P.F., Heuvelink E. 2011. Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. Physiol. Plant. 142: 274–286.

Fanourakis D., Carvalho S.M.P., Almeida D.P.F., van Kooten O., van Doorn W.G., Heuvelink E. 2012. Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biol. Tech. 64: 64–73.

Mortensen L.M. and Gislerød H.R. 1997. Effects of air humidity and air movement on the growth and keeping quality of roses. Gartenbauwissenschaf. 62: 273–277.

Reid M.S. and Jiang C.Z. 2012. Postharvest biology and technology of cut flowers and potted plants. p. 1-54. In: J. Janick (Ed.), Horticultural Reviews, Vol. 40, First Edition, Wiley-Blackwell, John Wiley & Sons, Inc.

Telewski F.W. and Pruyn M.L. 1998. Thigmomorphogenesis: a dose response to flexing in *Ulmus americana* L. seedlings. Tree Physiol. 18: 65–68.

Torre S. and Fjeld T. 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. Sci. Hort. 89: 217–226.

VBN, 2005. Evaluation cards for cut flowers. VBN, Leiden, The Netherlands.

CHAPTER 4

Effect of salinity on stomatal functioning and postharvest longevity

4.1 Moderate salinity triggers stomatal functioning in high relative air humidity grown plants

4.2 Effect of salinity on plant growth and vase life of cut roses grown at high relative air humidity

CHAPTER 4.1

Moderate salinity triggers stomatal functioning in high relative air humidity grown plants

Abstract

Plants grown at high relative air humidity (RH ≥ 85%) show hampered stomatal closure in response to closing stimuli. We hypothesized that a moderate salinity during growth could trigger a stress response and stimulate stomatal functioning due to an increase in the leaf abscisic acid concentration ([ABA]). Cut rose 'Prophyta' was grown at moderate (63%) or high (89%) RH combined with three electrical conductivities (EC) in the nutrient solution (2, 4 and 6 dS m^{-1}; using NaCl). High RH resulted in higher pore area per leaf area in intact leaves, and higher stomatal conductance (g_s) both in leaves subjected to desiccation and to light/dark transition, as compared to moderate RH. Increasing EC in high RH-grown plants enhanced stomatal closure in response to leaflet desiccation, but not in response to light-dark transition. This enhanced stomatal functioning was associated with increased [ABA] and increased [ABA-GE], although the latter did not increase significantly. Moreover, plants grown at high EC showed a significantly lower chlorophyll content, total plant dry weight and total leaf area. Sodium concentration ([Na^+]) increased 111-fold in plants grown at EC6 compared to EC2, but only at moderate RH. Chloride concentration ([Cl^-]) increased eight- and 14-fold in EC4 and EC6, respectively, compared to EC2, irrespective of the RH level. This is the first study on the interactive effects of RH and salinity and it provides new insight on the stomatal functioning when environmental factors that influence stomatal responsiveness in an opposite way are combined during leaf development.

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Introduction

High relative air humidity (RH ≥ 85%) during leaf development is well described to disturb the stomata's capacity to close in response to water stress, darkness and abscisic acid (ABA), leading to uncontrolled water loss when plants are further subjected to conditions of high evaporative demand (Torre et al., 2003; Fanourakis et al., 2012; Carvalho et al., 2015). Stomatal physiology has been pointed out as the major cause for this negative water balance (Fanourakis et al., 2013; Aliniaeifard et al., 2014) and the phytohormone ABA is closely involved in this process. Lower ABA concentrations ([ABA]), associated with poor stomatal functioning, were measured in leaves of *Trasdescantia virginiana* (Rezaei Nejad and van Meeteren, 2007), *Vicia faba* (Aliniaeifard et al., 2014) and *Rosa×hybrida* (Arve et al., 2013; Giday et al., 2013; Carvalho et al., 2015) developed at high RH compared to moderate RH. The tissue [ABA] is determined by its biosynthesis and catabolism (Nambara and Marion-Poll, 2005). ABA can be permanently inactivated via oxidation to form 8'-hydroxy ABA which rearranges to phaseic acid (PA) and is further reduced to dihydrophaseic acid (DPA) (Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005). In addition, ABA can also be inactivated by covalent conjugation with monosaccharides (e.g. ABA-β-D-glucosyl ester; ABA-GE), which accumulates in vacuoles and it is, hence, hypothesized to be a storage form of ABA (Dietz et al., 2000; Lee et al., 2006; Arve et al., 2013). High RH has been shown to increase the inactivation of ABA to PA in *Arabidopsis thaliana* leaves (Okamoto et al., 2009) and in roses ABA-GE remained high during the night, indicating that the conversion to ABA following a light-dark transition did not occur (Arve et al., 2013). Besides RH, there are other environmental factors, such as salinity, that play a role on ABA metabolism (Maggio et al. 2007; Lovelli et al. 2012). An increased [ABA] associated with a higher stomatal resistance has been reported in tomato plants in response to salinity (Maggio et al., 2007; Lovelli et al., 2012).

A soil is considered saline when the EC is above 4 dS m^{-1} (George et al., 2012). There are three major constrains for plant growth under salinity: (i) osmotic stress, i.e., water deficit arising from the low water potential in the rhizosphere; (ii) ionic stress, i.e., ion toxicity resulting from the excessive uptake mainly of Na^+ and Cl^-; and (iii) nutrient imbalance by reduction in uptake and/or shoot transport and impaired distribution of nutrients (Greenway and Munns, 1980). During the initial phase of root exposure to excessive [NaCl], water uptake may be inhibited causing a physiological drought stress (Shalhevet and Bernstein, 1968) leading to stomatal closure and a reduction in plant growth (Munns and Tester, 2008). Similarly, a decreased transpiration rate was also observed in chrysanthemum (Lee and van Iersel, 2008), wheat (Sharma et al., 2005), salvia (Kang and Van Iersel, 2004), *Sophora secundiflora* and *Cercis canadensis* (Niu et al., 2010). Moreover, salinity can affect stomatal and leaf anatomy, as in cotton a decrease in stomatal density was compensated by an increase in stomatal size and mesophyll surface area (Jafri and Ahmad, 1995) and in strawberry salinity reduced transpiration flux due to low stomatal density (Orsini et al., 2012). Beyond the described osmotic stress, plants subjected to long-term salinity may suffer from ionic stress due the accumulation of ions in the shoots and an inability to tolerate this ionic imbalance (Munns and Tester, 2008). This can cause premature senescence of adult leaves with a direct negative effect on leaf photosynthetic rate or in extreme cases leading to leaf abscission (Cramer & Nowak 1992; Rivelli et al. 2002; Niu et al. 2010). Moreover, ionic stress resulted in toxicity symptoms (i.e., chlorosis, necrosis) in mature leaves due to high Na^+ which disrupted protein synthesis and interfered with enzymatic activity (Munns & Termaat 1986; Hasegawa & Bressan 2000; Munns 2002). Furthermore, salinity may also affect the homeostasis of certain elements, e.g. K, Ca, Mn and P (George et al., 2012). Efflux of K from roots may occur due to decreased membrane integrity under salinity and K efflux rate has been negatively correlated with salinity tolerance (Chen et al., 2007). Calcium is important to membrane and cell wall stabilization maintaining tissue integrity and contributing to Na exclusion and decrease in K efflux, however a low uptake of Ca is likely to occur in response to salinity (Francois et al., 1991). Manganese uptake is usually not impaired in response to salinity, but in barley supplied with low [Mn], salinity reduced growth mainly by inhibiting Mn uptake and inducing Mn deficiency (Cramer and Nowak, 1992). Salinity may induce P deficiency in cotton (Martinez and Lachli, 1991) and reduce P utilization efficiency in tomato (Awad et al., 1990). Plant responses to salinity during cultivation depend on plant species, salt concentration, duration of exposure, plant developmental stage and other environmental conditions (Munns, 2002). The modern rose cultivars are regarded as moderately tolerant to salinity in soilless culture (Cabrera and Perdomo, 2003). The exclusion mechanism for Na^+ and Cl^- seem to be rather efficient in roses due to a reduced ion uptake by the roots and further loading into the xylem avoiding toxic concentrations within the leaves (Sonneveld et al. 1999; Davenport et al. 2005).

To the best of our knowledge, the effect of a moderate salinity combined with high RH on ABA metabolism has not yet been studied. The objective of this work was to investigate the interactive effects of moderate salinity and high RH during plant development on stomatal physiology and anatomy of cut roses. We hypothesized that when grown at high RH, the increased salinity during growth could trigger a mild stress response and stimulate stomatal functioning in response to closing stimuli due to an increase in the leaf [ABA].

Materials and methods

Plant material and growth conditions

Rooted cuttings of the cut rose cultivar ‘Pink Prophyta’ (*Rosa* × *hybrida*) (hereafter named as ‘Prophyta’) were planted in 3.5 L pots containing a mixture (2/1, v/v) of cocopeat (Pelemix, Murcia, Spain) and perlite (Otavi, Neuss, Germany). Sixty plants were randomly distributed over two walk-in climate controlled growth chambers (length x width x height = 2.0 x 1.6 x 2.0 m; 5000 EH, Aralab, Albarraque, Portugal) at a density of 19 plants m^{-2} (one plant per pot; single stem). During cultivation, the RH in one of the growth chambers was 63 ± 2% (moderate RH) while in the other one it was 89 ± 3% (high RH). Constant day and night temperature was 22.2 ± 1.5 °C in both growth chambers, resulting in vapor pressure deficits (VPDs) of 0.99 ± 0.02 kPa (moderate RH) and 0.29 ± 0.06 kPa (high RH). The CO_2 concentration was 350 ± 20 µmol mol^{-1} (IAQ 910, TSI Incorporated, Shoreview, MN, USA). Fluorescent lamps (Osram L58W/840, Lumilux, Cool White, Munich, Germany) provided a 20 h photoperiod of 130 ± 5 µmol m^{-2} s^{-1} photosynthetic active radiation (Li-1000 datalogger, Li-Cor, Lincoln, Nebraska, USA) measured 20 cm above the root-shoot interface. Plants were watered daily with a nutrient solution containing both (i) macronutrients (mM) [NH_4 1.0, K 4.0, Ca 3.5, Mg 1.38, NO_3 10.5, SO_4 1.5, H_2PO_4 1.25] and (ii) micronutrients (µM) [Fe 25, Mn 5, Zn 3.5, B 20, Cu 0.75, Mo 0.5]. The nutrient solution had an electrical conductivity (EC) of 2 dS m^{-1} (EC2; control) (Cond 6+, Eutech Instruments, Eutech Instruments Europe bv, Nijkerk, The Netherlands). In each growth chamber 10 plants were directly watered with the standard nutrient solution (EC2, control), while the other 20 plants were watered with the standard nutrient solution corrected to an EC of 4 dS m^{-1} (EC4) or 6 dS m^{-1} (EC6) using NaCl. To prevent EC build-up in the substrate throughout plant development, the drained solution was monitored twice per week. When the registered values were above the target EC level, plants were watered with their corresponding nutrient solution to lower the EC values until reaching EC2, EC4 or EC6. A pH of 5.3 (pH 5+, Eutech Instruments, Eutech Instruments Europe bv, Nijkerk, The Netherlands) was kept in all the treatments.

Stomatal conductance and responsiveness to desiccation

Stomatal conductance (g_s) was measured in intact plants on the terminal leaflets of fully grown tri-foliated leaves (i.e., tri-foliate immediately above the first penta-foliated counting from the apex), with a porometer (AP4, Delta-T Devices, Cambridge, United Kingdom), 2 h after the beginning of the light and of the dark periods**.**

Stomatal responsiveness to desiccation was assessed in fully grown terminal leaflets (i.e., first penta-foliated counting from the apex) detached from fully grown plants (i.e., flower bud with cylindrical shape and pointed tip). Leaflet petioles were recut under water (to prevent cavitation induced-embolism) and placed in flasks filled with degassed water. Leaflets were further incubated in a saturated RH (≈ 100%) environment at 20.4 ± 1.0 ºC (i.e., VPD close to 0) for 1 h to establish their saturated fresh weight (Fanourakis et al., 2011). The rehydration took place under fluorescent light (40 **± 2** µmol m^{-2} s^{-1}) to induce stomatal opening of leaflets previously exposed to prolonged darkness (Drake et al., 2013). After rehydration, the leaflets were allowed to desiccate for 4 h on a bench (abaxial surface down) in controlled conditions of evapotranspiration: 1.10 ± 0.05 kPa VPD (20.4 ± 1.0 ºC, 54 ± 4% RH) and 40 ± 2 µmol m^{-2} s^{-1}. Leaflet weight was gravimetrically recorded every 5-30 min during the 4 h (ABJ, Kern & Sohn GmbH, Balingen-Frommern, Germany). Simultaneously, g_s was measured on the same leaves with the porometer every 10-30 min during the initial 2 h of leaflet desiccation (measurements during the last 2 h of desiccation were not possible due to high stomatal closure leading to g_s values below the detection limit of the porometer). Following the evaluation of stomatal responsiveness, leaflet area (AM 300, ADC BioScientific, Hoddesdon, England) and dry weight (24 h at 70 ˚C) were measured. Transpiration rate was calculated according to Equation 1 and for the calculation of the relative water content (RWC), Equation 2 (Slavík, 1974) was employed. In both measurements (g_s and RWC) one leaflet per plant was evaluated in seven plants per treatment.

$$\text{Transpiration rate } (mmol\ m^{-2}\ s^{-1}) = \left(\left(\frac{\Delta\ \text{leaflet fresh weight } (g)}{\text{molar mass water } (g\ mol{-}1)} \times 1000\ (mmol\ mol^{-1})\right) / \text{measurement frequency } (s)\right) / \text{leaflet area } (m^2) \qquad \text{(Eqn. 1)}$$

$$\text{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{saturated fresh weight} - \text{dry weight}} \times 100 \qquad \text{(Eqn. 2)}$$

Stomatal anatomical characteristics

The effects of RH and EC on stomatal density (i.e., number of stomata per unit leaf area), stomatal index (i.e., ((stomatal density / (stomatal density + epidermal cell density)) × 100); Salisbury, 1927), stomatal and pore length (i.e., longest diameter), stomatal and pore width (i.e., shortest diameter) and pore area per leaf area [i.e., stomatal density × π × (pore length/2) × (pore width/2)] were determined. Stomatal traits were analyzed in one of the intact lateral leaflets from the fully grown first penta-foliated leaf, 4 h after the onset of the light period. Epidermal imprints were made by silicon rubber impression technique (Xantopren® VL Plus and Activator Universal, Heraeus Kulzer, Hanau, Germany) as described by Smith et al. (1989). Samples were taken from the abaxial side of the leaflets, midway between the tip and the base, away from the edge and avoiding veins (Fanourakis et al., 2015). A light microscope (Eclipse 55i, Nikon, Tokyo, Japan) connected to a digital camera (DS-Fi1, Nikon, Tokyo, Japan) was used to record the images. Stomatal and epidermal cell densities were assessed with 100× magnification, while for stomatal and pore dimensions a magnification of 400× was used. Image analysis was performed using UTHSCSA ImageTool for windows version 3.0 (The University of Texas Health Science Center at San Antonio, Texas, USA). For stomatal and epidermal cell counting, 35 images (five images per leaflet, one leaflet per plant) were analyzed per treatment. Regarding stomatal and pore dimensions, 70 stomata (ten stomata per leaflet, one leaflet per plant) were evaluated per treatment.

Endogenous ABA quantification

ABA was quantified in penta-foliated leaves, i.e., second penta-foliated counting from the apex, sampled 5 h after the beginning of the light period. Leaves were immediately frozen in liquid nitrogen and stored at -80 ˚C and freeze dried afterwards. Three composite samples (each one consisting of three biological replicates) per treatment were analyzed.

Extraction and purification of ABA metabolites

Samples were prepared according to Saika et al. (2007), with modifications. Approximately 5 mg of lyophilized tissue (powder) was extracted twice with 2 ml of acetone:water (80:20, v/v) in the presence of antioxidant 2,6-di-tert-butyl-4-methylphenol (0.1 mg ml^{-1}) and 100 pmol of deuterium-labelled internal standards as each counterpart of ABA, ABA-GE, PA and DPA, followed by centrifugation (15 min, 3000 rpm, 4℃). Supernatants were pooled and evaporated to dryness under vacuum. The extracts were suspended in 1 ml of isopropanol:formic acid (99:1, v/v, pH 3.3) and dried under vacuum. Reconstitutes in 1 ml of 10% (v/v) methanol containing 0.1% (v/v) formic acid (pH 2.6) were partitioned twice with 1 ml of n-hexane. Dried samples were dissolved in 1 ml of 10% methanol containing 0.1% formic acid (pH 2.6) and purified with Oasis-HLB cartridge (150 mg/6 cc, Waters). The sorbent was pre-conditioned with 3 ml of methanol and equilibrated with 3 ml of methanol containing 0.1% formic acid. After loading the sample, the column was washed with 1 ml of water and ABA was eluted with two series of eluents: 1 ml of acetonitrile:water:formic acid (50:49.9:0.1, v/v/v) and 2.5 ml of acetonitrile:water:formic acid (90:9.9:0.1, v/v/v), respectively. Both eluates were combined and dried under vacuum.

Quantitative analysis of ABA metabolites

Quantification of ABA metabolites was performed by ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS-MS; Xevo™ TQ-S, Waters, Milford, Massachusetts, USA). Purified samples were re-dissolved in 200 µl of mobile phase, filtered with 0.45 µm PTFE membrane filter (Phenomenex) and separated on Acquity UPLC CSH C_{18} column (100 x 2.1 mm, 1.7 µm; Waters, Milford, Massachusetts, USA). Analytes were eluted using a binary gradient consisting of 15 mM formic acid in water (A) and acetonitrile (B), for 7 min at a flow rate of 0.7 ml min^{-1} and constant column temperature of 45°C. The linear gradient elution was performed as follows: 30 s, 15% eluent B; 3 min, 60% eluent B; 1 min, 80% eluent B; 75 s, 100% eluent B. At the end, the column was equilibrated to initial conditions for 75 s. The effluent was introduced into the electrospray ion source of the mass spectrometer with optimized operating parameters: capillary voltage 3 kV, cone voltage 25 V, source / desolvation temperature 150 °C / 600 °C, cone / desolvation gas flow 150 / 600 L h^{-1} and collision energy 10 V. Compounds were quantified in negative mode as $[M-H]^-$ and multiple reaction monitoring transitions (MRM: MS1 > MS2) were: ABA (263.15 > 153.1), $[^2H_6]$-ABA (269.15 > 159.1); ABA-GE (425 > 262.72), $[^2H_5]$-ABA-GE (430 > 267.72); PA (279 > 138.9), $[^2H_3]$-PA (282 > 142.17); DPA (281 > 171.17), $[^2H_3]$-DPA (284 > 174). Data were processed by TargetLynx^{TM} Software, version 4.1 (Waters, Milford, Massachusetts, USA).

Mineral concentration, chlorophyll and plant growth parameters

Mineral concentration was assessed in young fully expanded leaves located in the central part of the flowering stem. Chloride was extracted with deionized water (1:50) (Walinga et al., 1989) from dried leaves (fifth penta-foliated) and quantified by molecular absorption (Zall et al., 1956) after dialysis in a segmented analyzer (SanPlus, Skalar, Breda , The Netherlands). Four composite samples (each one composed by three biological replicates) per treatment were analyzed. For the remaining minerals, dried leaf material was mixed with 5 ml of 65% HNO_3 in a Teflon reaction vessel and digested in a microwave system (Speedwave MWS-3+, Berghof, Eningen, Germany). Digestion was conducted in five steps: 130 °C for 10 min; 160 °C for 15 min; 170 °C for 12 min; 100 °C for 7 min and 100°C for 3 min. The resulting solutions of the digestion were then cleared by filtration and brought up to 20 ml with ultrapure water for further analysis. Nutrient concentration was analyzed by inductively coupled plasma argon spectrometry (ICP; ICP-OES Optima 7000 DV, PerkinElmer, Waltham, Massachusetts, USA). Four biological replicates, each with two leaves per plant: third and fourth penta-foliated (pooled) were analyzed in duplicate.

Leaf chlorophyll was measured non-destructively with a SPAD meter (502-Plus Chlorophyll Meter, Minolta corporation, Ltd., Osaka, Japan) on the third to fifth penta-foliated leaves (three readings per terminal leaflet), using seven plants per treatment.

The effects of RH and EC on plant growth and visual quality parameters were evaluated in fully grown plants (i.e., flower bud with cylindrical shape and pointed tip). Total plant dry weight (48 h at 70 ˚C; stem, leaves and flower), total leaf area, specific leaf area, plant height, number of internodes, average internode length, peduncle length and diameter were assessed in seven plants per treatment.

Statistical design and analysis

The experimental set-up was a complete randomized design. Analysis of variance was conducted considering individual plants as experimental units. Main effects and interactions were tested at $P = 0.05$. When relevant, Fisher's least significant difference (LSD) at $P = 0.05$ was calculated to separate interaction means. The Genstat software (15th edition; VSN International Ltd., Herts, UK) was used for the analysis.

Results

Stomatal functioning and anatomy

Intact plants grown at high RH showed higher g_s, compared to plants grown at moderate RH, but g_s was significantly lower at increased EC both in light (Fig. 1A) and dark (Fig. 1B) conditions. For instance, plants grown at EC6 showed 40 and 60% lower g_s in the light

(*P* = 0.012; Fig. 1A) and in the dark (*P* = 0.008; Fig. 1B) periods, as compared to plants grown at EC2. Moreover, high RH reduced the percentage of dark-induced stomatal closure from 87% (moderate RH) to 55% (high RH), irrespective of the EC level (*P* < 0.001; Fig. 1C). In spite of the non-significant effect of the EC (*P* = 0.075) on the percentage of dark-induced stomatal closure, plants grown at high RH and EC6 showed a tendency of higher stomatal responsiveness to darkness as compared to EC2 or EC4 (Fig. 1C).

When evaluating the stomatal responsiveness to desiccation, either with a porometer or by gravimetry, a similar pattern of leaflet water loss was observed using both methods (Fig. 2A and 2B). Plants grown at high RH combined with higher EC levels showed a lower g_s and lower transpiration rate in detached leaflets reflecting more responsive stomata (Fig. 2). In contrast, increasing the EC level in plants grown at moderate RH resulted in a negative effect on stomatal functioning. For instance, at the end of desiccation period (4 h), increasing EC increased the RWC_4h in high RH grown-leaflets (18, 30 and 42% in EC2, EC4 and EC6, respectively), whereas it was decreased in leaflets grown at moderate RH (70, 60 and 48% in EC2, EC4 and EC6, respectively) (*P* < 0.001; Fig. 2C).

Concerning the effects of higher EC levels on stomatal anatomy it was found that, in high RH- grown plants, EC6 significantly reduced stomatal and pore length (6 and 10%, respectively) but it significantly increased stomatal density, pore aperture and pore area per leaf area (15, 10 and 15%, respectively) as compared to EC2 (Table 1). In plants grown at moderate RH, EC6 significantly decreased stomatal density and index (9 and 12%, respectively) as well as pore aperture and pore area per leaf area (19 and 24%), while stomatal width and length and pore length were not significantly affected when compared to EC2-grown plants (Table 1).

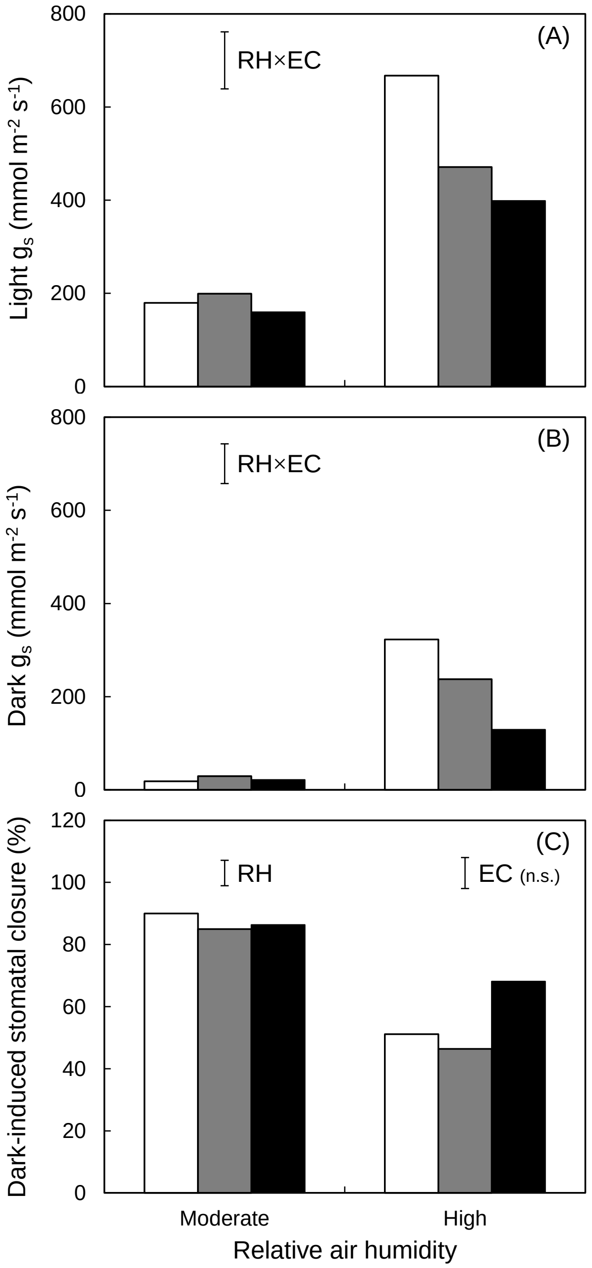

Fig. 1. Stomatal conductance (g_s) in intact plants 2 h after the beginning of the light (A) and of the dark (B) periods and percentage of dark-induced stomatal closure (C) in cut rose cv. 'Prophyta' grown at moderate (63%) or high (89%) relative air humidity with an electrical conductivity (EC) of the nutrient solution of 2, 4 or 6 dS m^{-1} (white, grey or black columns, respectively). Values are the mean of seven intact plants. Bars represent Fisher's LSD (P = 0.05) for the significant interaction 'RH × EC' (A and B) and for the independent effects when the interaction was not statistically significant (C; P = 0.069). n.s. = non-significant difference of a given independent effect

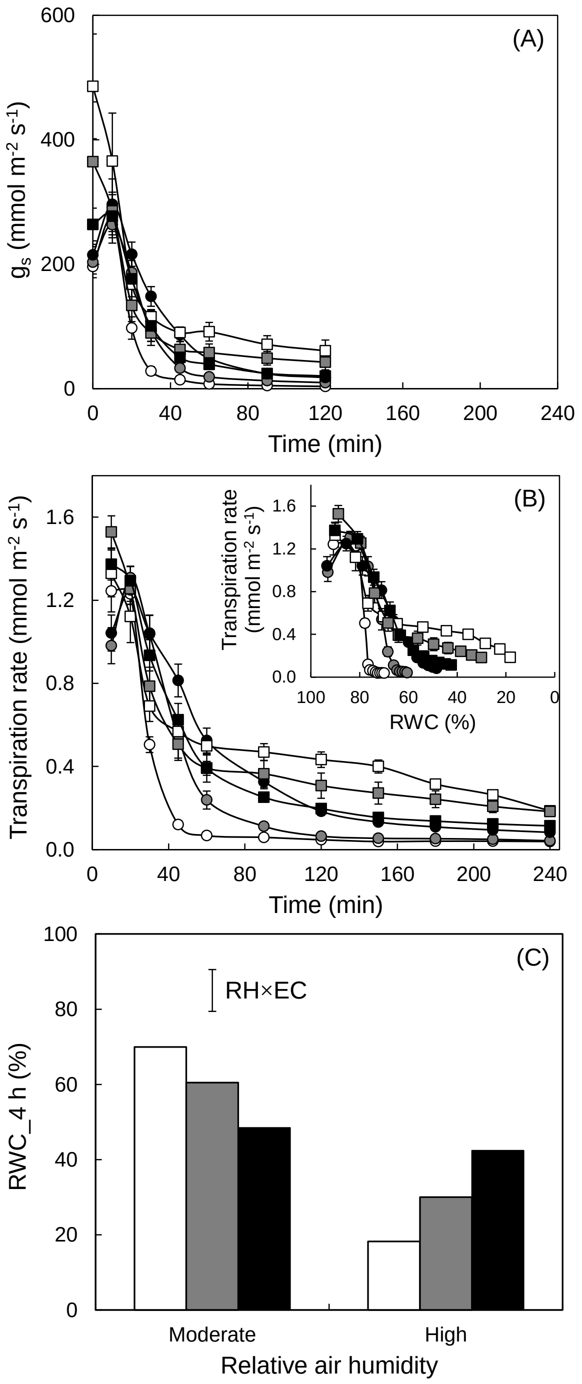

Fig. 2. Stomatal conductance (g_s; A), transpiration rate as a function of time (B) and of relative water content (RWC; insert) and RWC after 4 h of leaflet desiccation (RWC_4h; C) in detached leaflets from cut rose cv. 'Prophyta' grown at moderate (circles; 63%) or high (squares; 89%) relative air humidity with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (white, grey or black symbols, respectively). Values are the mean of seven detached leaflets. Bars represent the SEM (A and B) and Fisher's LSD (P = 0.05) for the significant interaction 'RH × EC' (C)

Table 1. Stomatal anatomical characteristics in intact plants from cut rose cv. 'Prophyta' grown at moderate (63%) or high (89%) relative air humidity (RH) with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (EC2, EC4 or EC6, respectively). Sampling took place 4 h after the onset of the light period. Values are the mean of 35 images (stomatal density and index) and 70 stomata (stomatal and pore size). Different letters represent significant differences according to LSD test (P = 0.05; significant F probabilities are presented in bold; comparison in rows)

		Moderate RH (63%)			High RH (89%)			*F pr.*
		EC2	EC4	EC6	EC2	EC4	EC6	RH × EC
Stomatal	Density (number mm^{-2})	51.5^{c}	46.2^{ab}	47.0^{b}	43.7^{a}	47.0^{b}	50.4^{c}	**<0.001**
	Index (%)	11.9^{b}	10.8^{a}	10.5^{a}	12.2^{bc}	12.0^{bc}	12.5^{c}	**0.002**
	Length (µm)	28.8^{a}	28.7^{a}	28.2^{a}	32.1^{c}	30.7^{b}	30.2^{b}	**0.009**
	Width (µm)	19.0^{a}	19.7^{b}	18.6^{a}	21.8^{c}	21.8^{c}	22.3^{c}	**0.004**
Pore	Length (µm)	17.8^{a}	17.9^{ab}	18.1^{ab}	20.5^{d}	19.3^{c}	18.5^{b}	**<0.001**
	Aperture (µm)	3.81^{b}	4.12^{b}	3.08^{a}	5.01^{c}	5.39^{cd}	5.50^{d}	**<0.001**
	Area per leaf area (‰)	2.68^{b}	2.72^{b}	2.05^{a}	3.54^{c}	3.94^{cd}	4.08^{d}	**<0.001**

Endogenous ABA quantification

There was no significant interaction between RH and EC in [ABA] (P = 0.917; Fig. 3A) and [PA] (P = 0.427, Fig. 3B). [ABA] was not significantly affected by RH (P = 0.681) but it increased by 45 and 100% in plants grown at EC4 and EC6 as compared to EC2 (P = 0.014; Fig. 3A). [PA] was not significantly affected by EC (P = 0.456), but in moderate RH-grown plants there was a tendency for the [PA] to increase with increasing EC (Fig. 3B). High RH reduced [PA] in 61% (P = 0.003; Fig. 3B) irrespective of EC. [DPA] was significantly decreased by increasing EC in plants grown at moderate RH while it was not affected in high RH-grown plants (P = 0.042; Fig. 3C). [ABA-GE] was neither significantly affected by EC (P = 0.137) nor by RH (P = 0.109), however, in high RH-grown plants there was a tendency for the [ABA-GE] to be higher in plants grown at EC6 compared to the ones grown at EC2 or EC4 (Fig. 3D). When analyzing the total amount of ABA and its metabolites it was found that increasing EC significantly increased [ABA + metabolites] in high RH-grown plants, while this concentration was not affected in moderate RH-grown ones (P = 0.038; Fig. 3E).

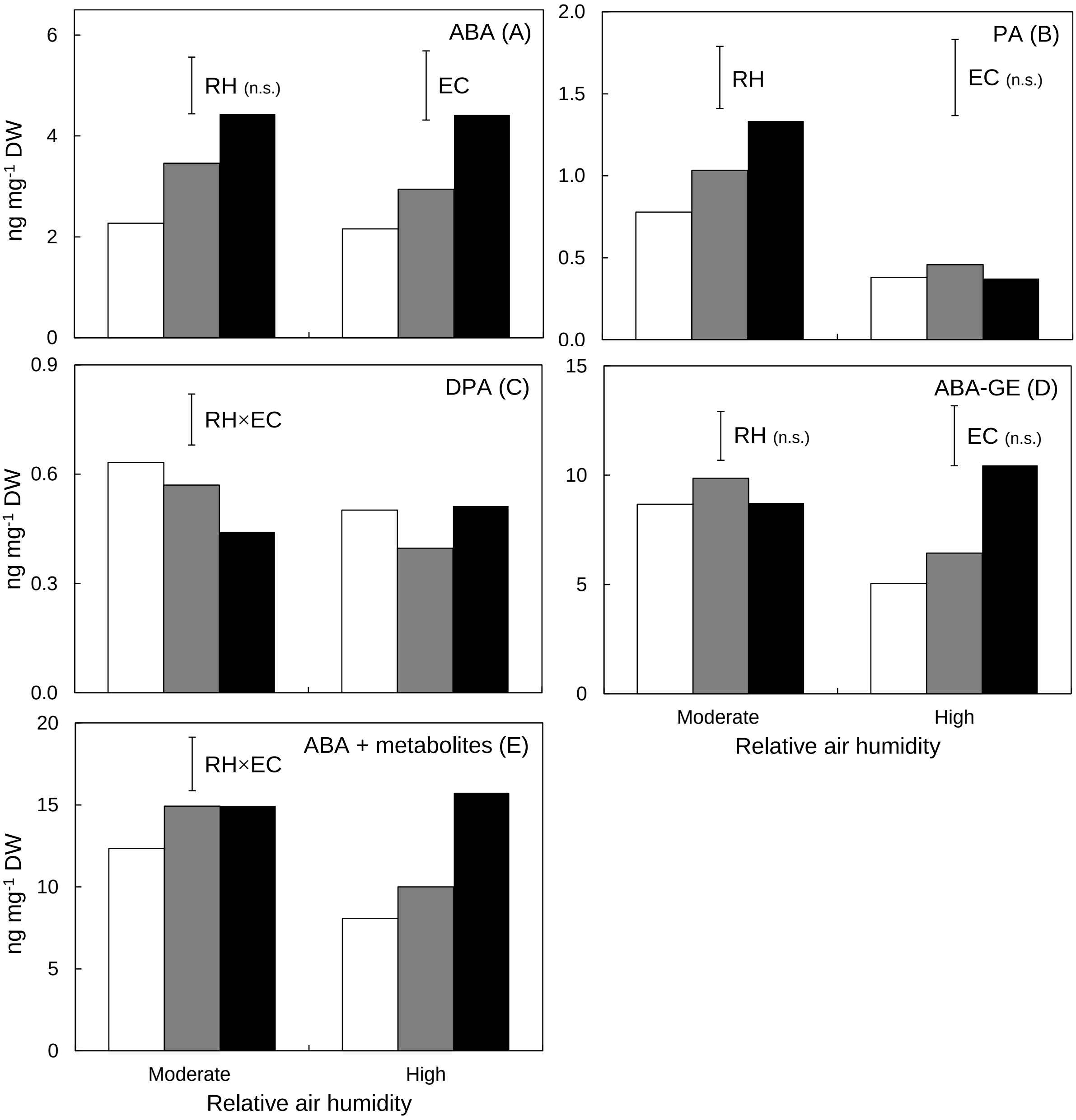

Fig. 3. Concentration (ng mg^{-1} DW) of abscisic acid (ABA; A), phaseic acid (PA; B), 4'-dihydrophaseic acid (DPA; C), ABA-β-D-glucosyl ester (ABA-GE; D) and total amount of ABA and its metabolites (E) in intact leaves harvested from cut rose cv. 'Prophyta' grown at moderate (63%) or high (89%) relative air humidity with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (white, grey or black columns, respectively). Values are the mean of three leaves. Bars represent Fisher's LSD (P = 0.05) for the significant interaction 'RH × EC' (C and E) and for the independent effects when the interaction was not statistically significant (A; P = 0.917), PA (B; P = 0.427) or ABA-GE (D; P = 0.094). n.s. = non-significant difference of a given independent effect

Mineral concentration, chlorophyll and plant growth parameters

The analysis of the mineral concentration in young fully expanded leaves revealed that [Na$^+$] significantly increased in plants grown at EC6 and moderate RH, while this ion concentration was not significantly different in the other treatments ($P = 0.013$; Fig. 4A). Compared to EC2, [Cl$^-$] increased by eight- and 14-fold in EC4 and EC6, respectively ($P < 0.001$), but it was not significantly affected by the RH during plant growth ($P = 0.728$) (Fig. 4B). There was no significant interaction between RH and EC in the concentration of any of the minerals presented in Table 2, except for Zn. Compared to EC2, plants grown at EC6 significantly decreased [Mg] (11%), whereas high EC level increased [K] (23%), [Fe] (15%) and [Cu] (61%) (Table 2). High RH significantly decreased [B] (12%) and increased [K] (18%) (Table 2). Concentrations of P, Ca, Mn, Ni and Mo were not significantly affected by RH or EC (Table 2).

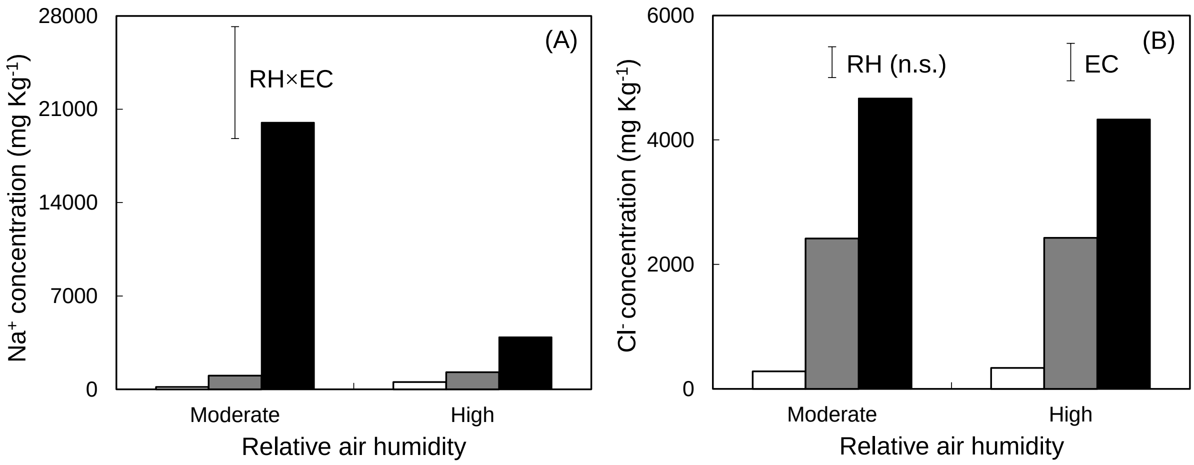

Fig. 4. Concentration (mg kg^{-1}) of the ions sodium (Na$^+$; A) and chloride (Cl$^-$; B) of young fully-expanded leaves located in the central part of the flowering stem of cut rose cv. 'Prophyta', grown at moderate (63%) or high (89%) relative air humidity (RH) with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (white, grey or black columns, respectively). Values are the mean of four samples. Bars represent Fisher's LSD ($P = 0.05$) for the significant interaction 'RH × EC' (A) and for the independent effects when the interaction was not statistically significant (B; $P = 0.748$). n.s. = non-significant difference of a given independent effect

Table 2. Mineral concentration (mg kg^{-1}) of young fully-expanded leaves located in the central part of the flowering stem (third and fourth penta-foliated leaves counting from the apex, pooled) of cut rose cv. 'Prophyta', grown at moderate (63%) or high (89%) relative air humidity (RH) with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (EC2, EC4 or EC6, respectively). Different letters represent significant differences according to LSD test ($P = 0.05$; significant F probabilities are presented in bold; comparison in rows)

		Moderate RH (63%)				High RH (89%)				*F pr.*	*F pr.*	*F pr.*
		EC2	EC4	EC6	Mean	EC2	EC4	EC6	Mean	RH	EC	RH × EC
Macronutrients	K	28482	33835	33428	31915^{a}	32594	39067	41482	37715^{b}	**0.004**	**0.010**	0.648
	P	10429	10837	11117	10795	9077	9920	9978	9658	0.116	0.619	0.967
	Ca	10614	10223	10750	10529	9911	9490	9338	9580	0.071	0.801	0.805
	Mg	2582	1985	2287	2285	2296	2184	2051	2177	0.273	**0.018**	0.100
Micronutrients	Fe	75.5	80.9	84.8	80.4	73.9	87.6	86.5	82.7	0.417	**0.008**	0.483
	Mn	239	193	230	221	216	209	189	205	0.244	0.267	0.234
	Cu	1.88	2.56	2.72	2.38	1.62	2.55	2.93	2.37	0.892	**<0.001**	0.286
	Zn	12.6^{a}	12.1^{a}	13.9^{ab}	12.9	12.3^{a}	15.0^{b}	13.5^{ab}	13.6	0.175	0.110	**0.030**
	Ni	0.142	0.148	7.784	2.692	0.176	0.175	0.335	0.229	0.350	0.378	0.407
	Mo	0.211	0.318	0.325	0.285	0.397	0.187	0.388	0.324	0.434	0.250	0.050
	B	155	146	137	146^{b}	127	128	130	128^{a}	**0.008**	0.606	0.412

Increasing EC significantly decreased the chlorophyll levels in rose leaves from 61.3 SPAD units in plants grown at EC2 to 58.4 and 56.6 in the ones grown at EC4 and EC6, respectively (*P* < 0.001; Fig. 5). Moreover, RH did not affect chlorophyll levels (*P* = 0.356).

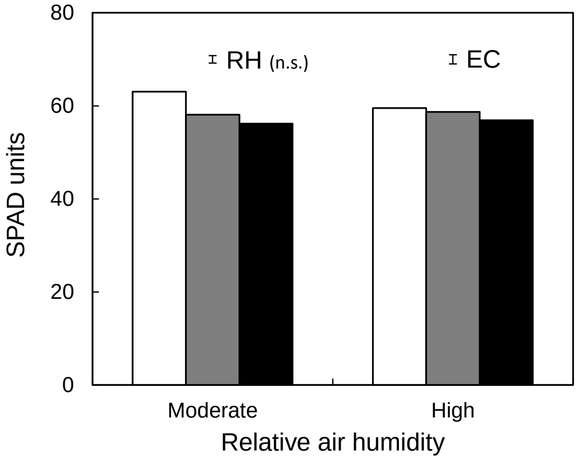

Fig. 5. SPAD units measured on young fully-expanded leaves located in the central part of the flowering stem of cut rose cv. 'Prophyta', grown at moderate (63%) or high (89%) relative air humidity (RH) with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (white, grey or black columns, respectively). Values are the mean of seven samples. Bars represent Fisher's LSD (*P* = 0.05) for the independent effects when the interaction was not statistically significant (*P* = 0.054). n.s. = non-significant difference of a given independent effect

None of the evaluated plant growth and visual quality parameters were significantly influenced by the interaction between RH and EC (Table 3). Comparatively to EC2, EC6-grown plants reduced their total dry weight (25%), via a reduction in stem (29%), leaf (26%) and flower (22%) dry weight (Table 3). Moreover, plants grown at high EC had a lower total leaf area (17%) and plant height (10%), although the number of internodes and the average internode length were not affected (Table 3). Specific leaf area increased by 10% in EC6-grown plants compared to EC2-grown plants. Plants grown at EC6 decreased both peduncle length and peduncle diameter by 15%, comparing to EC2-grown plants (Table 3). High RH significantly increased peduncle length by 13% (Table 3).

Table 3. Plant growth and visual quality parameters of cut rose cv. ‘Prophyta’ grown at moderate (63%) or high (89%) relative air humidity (RH) with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (EC2, EC4 or EC6, respectively). Since the interaction ‘RH × EC’ was not statistically significant for any parameter, the independent effect of EC is presented. Different letters represent significant differences according to LSD test (P = 0.05; significant F probabilities are presented in bold; comparison in rows)

	EC2	EC4	EC6	*F pr.* RH	*F pr.* EC	*F pr.* RH×EC
Total dry weight (g)	6.11^b	5.69^b	4.56^a	0.909	**<0.001**	0.934
Stem dry weight (g)	1.24^b	1.21^b	0.88^a	0.764	**0.003**	0.732
Leaf dry weight (g)	3.23^b	2.95^b	2.40^a	0.909	**0.002**	0.938
Flower dry weight (g)	1.64^b	1.53^b	1.28^a	0.815	**<0.001**	0.485
Total leaf area (cm^2)	674^b	641^b	558^a	0.261	**0.018**	0.352
Specific leaf area (cm^2 g^{-1})	211^a	221^{ab}	233^b	0.056	**0.014**	0.059
Plant height (cm)	40.6^b	41.2^b	36.7^a	0.093	**0.019**	0.340
Number of internodes (no.)	10.4	10.6	10.2	0.455	0.741	0.144
Average internode length (cm)	2.87	2.89	2.63	0.257	0.078	0.482
Peduncle length (cm)	6.33^c	5.80^b	5.37^a	**<0.001**	**<0.001**	0.357
Peduncle diameter (mm)	5.75^c	5.33^b	4.91^a	0.067	**<0.001**	0.713

Discussion

Increased EC enhances stomatal functioning in high RH-grown plants due to increased leaf [ABA]

An appropriate stomatal closure in response to closing stimuli, such as light-dark transition or desiccation, is important to prevent excessive water loss in conditions of high evaporative demand (Arve et al., 2015). In roses, leaf transpiration rate is mainly affected by the stomatal conductance, since the cuticle has a minor contribution to the total leaf water loss (Fanourakis et al., 2013). In this study, a larger pore area per leaf area was found (Table 1) associated with higher g_s (Figs. 1A and 1B) and lower dark-induced stomatal closure (Fig. 1C) in high RH-grown plants compared to plants grown at moderate RH. This poor stomatal functioning was also confirmed in response to leaf desiccation, where the transpiration rate of detached leaflets grown at high RH was significantly higher resulting in a low RWC after 4 h of leaflet desiccation than those grown at moderate RH (Fig. 2). Rather than stomatal anatomy or density, recent studies have shown that stomatal physiology is the major cause for the negative water balance in high RH-grown plants (Fanourakis et al., 2013; Aliniaeifard et al., 2014).

To the best of our knowledge, this is the first study investigating the interactive effects of salinity and RH during plant growth on the stomatal functioning of cut roses. It was our hypothesis that in cut roses grown at high RH, the increased EC could trigger a stress response and stimulate stomatal functioning in response to closing stimuli due to increased leaf [ABA]. This hypothesis was partly validated since in high RH-grown plants, increasing EC enhanced stomatal closure in response to leaflet desiccation (Fig. 2C) but such improvement did not occur in response to light-dark transition, despite the tendency of higher dark-induced stomatal closure in EC6-grown plants compared to EC2 and EC4 (Fig. 1C). The enhanced stomatal closure in plants grown at high RH and EC6 appears to originate from the increased [ABA] as a response to salinity (Fig. 3A). An increase in [ABA] associated with a reduction in stomatal conductance, has also been reported in tomato plants (Maggio et al., 2007; Lovelli et al., 2012). In detached *A. thaliana* leaves the [ABA] increased during desiccation independently of the RH during growth, but only stomata from moderate RH-grown leaves were able to close, evidencing that high RH-grown plants could not perceive the ABA signal (Arve et al., 2015), which suggests that stomatal malfunctioning is also related to stomatal sensitivity to ABA and not only to low [ABA] *per se* (Pantin et al. 2013; Carvalho et al. 2015). In addition, increasing EC, significantly increased [ABA + metabolites] in high RH-grown plants (Fig. 3E) mainly due to a tendency for increased [ABA-GE], although this increase in response to high EC was not significant (Fig. 3D). In roses, it has been suggested that conjugation of ABA to ABA-GE and subsequent release is more relevant for a proper stomatal functioning rather than a lower oxidation to PA and DPA (Arve et al., 2013; Arve et al., 2015). However, in moderate RH-grown plants, the light and dark g_s were not affected by increased EC (Figs. 1A and 1B) and the stomatal functioning was diminished in response to leaflet desiccation (reduced RWC_4h; Fig. 2C), in spite of the increase in [ABA] in response to increased EC (Fig. 3A). Qin et al. (2010) also found a significant reduction in the RWC of leaves from *Shepherdia argentea* (Pursh) Nutt. seedlings treated with ≈ 40 and 60 dS m^{-1} NaCl compared to the control (0 dS m^{-1} NaCl). These authors suggested that the high salinity caused dehydration at cellular level. We hypothesize that cell membranes were weakened by the increased Na^+ toxicity (Fig. 4A) and, therefore, they became more permeable to water loss.

Roses are moderately tolerant to increased EC

Plants adapt to salinity via three distinct mechanisms: (1) tolerance to osmotic stress; (2) Na^+ or Cl^- exclusion, and; (3) tolerance of tissue to accumulated Na^+ or Cl^- (Munns and Tester, 2008). Plants exclude Na^+ reducing its uptake by the roots and its consequent loading into the xylem ensuring that this ion does not accumulate to toxic concentrations within the leaves (Davenport et al., 2005). The modern rose cultivars have been described to have a very efficient exclusion mechanism for Na^+ and Cl^- (Sonneveld et al. 1999). It has been suggested that the exclusion of Na^+ is compensated by the uptake of K^+ to maintain the osmotic adjustment (Munns and Tester, 2008). In the present study it was found an increase in $[K^+]$ in plants grown at EC4 and EC6 compared to those grown at EC2, which supports the Na^+ exclusion mechanism described in roses (Sonneveld et al., 1999). However, during a long-term exposure to salinity, such as the one applied in the current study, the exclusion mechanism was not sufficient to keep all the Na^+ and Cl^- outside the plant and accumulation of these ions at leaf level have occurred (Fig. 4). At moderate RH, $[Na^+]$ increased 111-fold in EC6-grown plants compared to plants grown at EC2 (Fig. 4A). $[Cl^-]$ increased eight- and 14-fold in EC4 and EC6, respectively, compared to EC2, and it was not significantly affected by RH (Fig. 4B). The high evaporative demand present at moderate RH increased $[Na^+]$ in this treatment due to high plant transpiration rate and consequent ion uptake. In the majority of plant species grown under saline conditions, Na^+ appears to reach a toxic concentration before Cl^- does (Munns and Tester, 2008). The maximum ion toxicity accumulation occurs in old leaves, when plants are no longer expanding and so no longer diluting the accumulated salts.

The osmotic effect of salinity limits plant growth irrespective the capacity of the plants to exclude salt, resulting in reduced growth rates (James et al., 2008). In this study, increased EC strongly affected plant growth and visual quality parameters (Table 3). Compared to plants grown at EC2, plants grown at EC6 showed lower total plant dry weight via a reduction of all its organs (i.e., stem, leaf and flower) and lower total leaf area (Table 3). In the same plants, lower plant height was also measured and although the number of internodes and the average internode length were not affected, the reduced plant height was mostly due to a lower average internode length (Table 3). These findings are in agreement with previous studies in roses where high salinity up to 8.2 dS m^{-1} also resulted in lower total dry weight, reduced leaf area, reduced stem length and weight and decreased flower quality and yield (De Kreij and Van Den Berg, 1990; Brun and Settembrino, 1996; Cabrera, 2003; Oki and Lieth, 2004; Niu and Rodriguez, 2008). The occurrence of bent-neck during post-harvest is a typical symptom in plants developed at high RH (Fanourakis et al., 2012). It can be hypothesized that increased EC would increase the bent-neck incidence in high RH-grown plants because the peduncle diameter was also negatively affected by the EC level increasing the susceptibility of this region for bending (Table 3).

Conclusions

This is the first study that analysis the interactive effect of RH and salinity. It is expected that these two environmental factors act on stomatal functioning in opposite ways (high RH impairing stomatal responsiveness to closing stimuli and high EC enhancing stomatal closure). Here we have shown that the higher EC during leaf development has overruled the negative impact of high RH on stomatal functioning due to increased [ABA].

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References

Aliniaeifard, S., Malcolm Matamoros, P. and Meeteren, U. van (2014) Stomatal malfunctioning under low VPD conditions: Induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? *Physiol. Plant.*, 152, 688–699.

Arve, L.E., Terfa, M.T., Gislerød, H.R., Olsen, J.E. and Torre, S. (2013) High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves. *Plant, Cell Environ.*, 36, 382–392.

Arve, L.E., Kruse, O.M.O., Tanino, K.K., Olsen, J.E., Futsæther, C. and Torre, S. (2015) Growth in continuous high air humidity increases the expression of CYP707A-genes and inhibits stomatal closure. *Environ. Exp. Bot.*, 115, 11–19.

Awad, A.S., Edwards, D.G. and Campbell, L.C. (1990) Phosphorus enhancement of salt tolerance of tomato. *Crop Sci.*, 30, 132–128.

Brun, R. and Settembrino, A. (1996) Production and quality of *Rosa hybrida* cv Sonia in rockwool at three levels of electrical conductivity combined with two levels of relative humidity. *Acta Hortic.*, 424, 115–117.

Cabrera, R.I. (2003) Demarcating salinity tolerance in greenhouse rose production. *Acta Hortic.*, 609, 51–57.

Cabrera, R.I. and Perdomo, P. (2003) Reassessing the salinity tolerance of greenhouse roses under soilless production conditions. *HortScience*, 38, 533–536.

Carvalho, D.R.A., Torre, S., Kraniotis, D., Almeida, D.P.F., Heuvelink, E. and Carvalho, S.M.P. (2015) Elevated air movement enhances stomatal sensitivity to abscisic acid in leaves developed at high relative air humidity. *Front. Plant Sci.*, 6, 1–11.

Chen, Z., Zhou, M., Newman, I.A., Mendham, N.J., Zhang, G. and Shabala, S. (2007) Potassium and sodium relations in salinised barley tissues as a basis of differential salt tolerance. *Funct. Plant Biol.*, 34, 150–162.

Cramer, G.R. and Nowak, R.S. (1992) Supplemental manganese improves the relative growth, net assimilation and photosynthetic rates of salt-stressed barley. *Physiol. Plant.*, 84, 600–605.

Cutler, A.J. and Krochko, J.E. (1999) Formation and breakdown of ABA. *Trends Plant Sci.*, 4, 472–477.

Davenport, R., James, R.A., Zakrisson-Plogander, A., Tester, M. and Munns, R. (2005) Control of sodium transport in durum wheat. , 137, 807–818.

Dietz, K.J., Sauter, A., Wichert, K., Messdaghi, D. and Hartung, W. (2000) Extracellular beta-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. *J. Exp. Bot.*, 51, 937–944.

Drake, P.L., Froend, R.H. and Franks, P.J. (2013) Smaller, faster stomata: Scaling of stomatal size, rate of response, and stomatal conductance. *J. Exp. Bot.*, 64, 495–505.

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F. and Heuvelink, E. (2011) Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. *Physiol. Plant.*, 142, 274–286.

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F., Kooten, O. van, Doorn, W.G. van and Heuvelink, E. (2012) Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. *Postharvest Biol. Technol.*, 64, 64–73.

Fanourakis, D., Heuvelink, E. and Carvalho, S.M.P. (2013) A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. *J. Plant Physiol.*, 170, 890–898.

Fanourakis, D., Heuvelink, E. and Carvalho, S.M.P. (2015) Spatial heterogeneity in stomatal features during leaf elongation : an analysis using *Rosa hybrida*. *Funct. Plant Biol.*, 42, 737–745.

Francois, L.E., Donovan, T.J. and Maas, E.V. (1991) Calcium deficiency of artichoke buds in relation to salinity. *HortScience*, 26, 549–553.

George, E., Horst, W. and Neumann, E. (2012) Adaptation of plants to adverse chemical soil conditions. In P. Marschner, ed. *Marschner's Mineral Nutrition of Higher Plants*. Academic Press.

Giday, H., Fanourakis, D., Kjaer, K.H., Fomsgaard, I.S. and Ottosen, C.O. (2013) Foliar abscisic acid content underlies genotypic variation in stomatal responsiveness after growth at high relative air humidity. *Ann. Bot.*, 112, 1857–1867.

Greenway, H. and Munns, R. (1980) Mechanisms of salt tolerance in non-halophytes. *Annu. Rev. Plant Physiol.*, 31, 149–190.

Hasegawa, P. and Bressan, R. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 51, 463–499.

Jafri, A.Z. and Ahmad, R. (1995) Effect of soil salinity on leaf development, stomatal size and its distribution in cotton (*Gossypium hirsutum* L.). *Pak J Bot*, 27, 297–303.

James, R.A., Caemmerer, S. von, Condon, A.G. (Tony), Zwart, A.B. and Munns, R. (2008) Genetic variation in tolerance to the osmotic stress componentof salinity stress in durum wheat. *Funct. Plant Biol.*, 35, 111–123.

Kang, J.G. and Iersel, M.W. Van (2004) Nutrient solution concentration affects shoot:root ratio, leaf area ratio, and growth of subirrigated salvia (*Salvia splendens*). *HortScience*, 39, 49–54.

Kreij, C. De and Berg, T.H.J.M. Van Den (1990) Nutrient uptake, production and quality of *Rosa hybrida* in rockwool as affected by electrical conductivity of the nutrient solution. In M. L. van Beusichem, ed. *Plant Nutrition — Physiology and Applications*. Dordrecht: Springer Netherlands, pp. 519–523.

Lee, K.H., Piao, H.L., Kim, H.Y., Choi, S.M., Jiang, F., Hartung, W., Hwang, I., Kwak, J.M., Lee, I.J. and Hwang, I. (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell*, 126, 1109–1120.

Lee, M. and Iersel, W. van (2008) Sodium chloride effects on growth, morphology, and physiology of chrysanthemum (*Chrysanthemum × morifolium*). *Hortscience*, 43, 1888–1891.

Lovelli, S., Scopa, A., Perniola, M., Tommaso, T. Di and Sofo, A. (2012) Abscisic acid root and leaf concentration in relation to biomass partitioning in salinized tomato plants. *J. Plant Physiol.*, 169, 226–233.

Maggio, A., Raimondi, G., Martino, A. and Pascale, S. De (2007) Salt stress response in tomato beyond the salinity tolerance threshold. *Environ. Exp. Bot.*, 59, 276–282.

Martinez, V. and Lachli, A. (1991) Phosphorus translocation in salt-stressed cotton. *Physiol. Plant.*, 83, 627–632.

Munns, R. (2002) Comparative physiology of salt and water stress. *Plant. Cell Environ.*, 25, 239–250.

Munns, R. and Termaat, A. (1986) Whole-plant responses to salinity. *Funct. Plant Biol.*, 13, 143–160.

Munns, R. and Tester, M. (2008) Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.*, 59, 651–681.

Nambara, E. and Marion-Poll, A. (2005) Abscisic acid biosynthesis and catabolism. *Annu. Rev. Plant Biol.*, 56, 165–185.

Niu, G. and Rodriguez, D.S. (2008) Responses of growth and ion uptake of four rose rootstocks to chloride- or sulfate-dominated salinity. *J. Am. Soc. Hortic. Sci.*, 133, 663–669.

Niu, G., Rodriguez, D.S. and Gu, M. (2010) Salinity tolerance of Sophora secundiflora and *Cercis canadensis* var . mexicana. *Hortscience*, 45, 424–427.

Okamoto, M., Tanaka, Y., Abrams, S.R., Kamiya, Y., Seki, M. and Nambara, E. (2009) High humidity induces abscisic acid 8’-hydroxylase in stomata and vasculature to regulate local and systemic abscisic acid responses in Arabidopsis. *Plant Physiol.*, 149, 825–834.

Oki, L.R. and Lieth, J.H. (2004) Effect of changes in substrate salinity on the elongation of *Rosa hybrida* L. “Kardinal” stems. *Sci. Hortic. (Amsterdam).*, 101, 103–119.

Orsini, F., Alnayef, M., Bona, S., Maggio, A. and Gianquinto, G. (2012) Low stomatal density and reduced transpiration facilitate strawberry adaptation to salinity. *Environ. Exp. Bot.*, 81, 1–10.

Pantin, F., Renaud, J., Barbier, F., Vavasseur, A., Le Thiec, D., Rose, C., Bariac, T., Casson, S., McLachlan, D.H., Hetherington, A.M., Muller, B. and Simonneau, T. (2013) Developmental priming of stomatal sensitivity to abscisic acid by leaf microclimate. *Curr. Biol.*, 23, 1805–1811.

Qin, J., Dong, W.Y., He, K.N., Yu, Y., Tan, G.D., Han, L., Dong, M., Zhang, Y.Y., Zhang, D., Li, A. Z. and Wang, Z. L. (2010) NaCl salinity-induced changes in water status, ion contents and photosynthetic properties of *Shepherdia argentea* (Pursh) Nutt. seedlings. *Plant, Soil Environ.*, 56, 325–332.

Rezaei Nejad, A. and Meeteren, U. van (2007) The role of abscisic acid in disturbed stomatal response characteristics of Tradescantia virginiana during growth at high relative air humidity. *J. Exp. Bot.*, 58, 627–636.

Rivelli, A.R., Lovelli, S. and Perniola, M. (2002) Effects of salinity on gas exchange, water relations and growth of sunflower (*Helianthus annuus*). *Funct. Plant Biol.*, 29, 1405.

Saika, H., Okamoto, M., Miyoshi, K., Kushiro, T., Shinoda, S., Jikumaru, Y., Fujimoto, M., Arikawa, T., Takahashi, H., Ando, M., Arimura, S., Miyao, A., Hirochika, H., Kamiya, Y., Tsutsumi, N., Nambara, E. and Nakazono, M. (2007) Ethylene promotes submergence-induced expression of *OsABA8ox1*, a gene that encodes ABA 8'-hydroxylase in rice. *Plant Cell Physiol.*, 48, 287–298.

Salisbury, E. (1927) On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. *Philos. Trans. R. Soc. London*, 216, 1–65.

Shalhevet, J. and Bernstein, L. (1968) Effects of vertically heterogeneous soil salinity on plant growth and water uptake. *Soil Sci.*, 106, 85–93.

Sharma, N., Gupta, N.K., Gupta, S. and Hasegawa, H. (2005) Effect of NaCl salinity on photosynthetic rate, transpiration rate, and oxidative stress tolerance in contrasting wheat genotypes. *Photosynthetica*, 43, 609–613.

Slavík, B. (1974) *Methods of studying plant water relations*, London, UK: Chapman and Hall.

Smith, S., Weyers, J.D.B. and Berry, W.G. (1989) Variation in stomatal characteristics over the lower surface of Commelina communis leaves. *Plant, Cell Environ.*, 12, 653–659.

Sonneveld, C., Baas, R., Nijssen, H.M.C. and Hoog, J. de (1999) Salt tolerance of flower crops grown in soilless culture. *J. Plant Nutr.*, 22, 1033–1048.

Torre, S., Fjeld, T., Gislerod, H.R. and Moe, R. (2003) Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. *J. Am. Soc. Hortic. Sci.*, 128, 598–602.

Walinga, I., Vark, W. van, Houba, V.J.G. and Lee, J.J. van der (1989) Soil and Plant Analysis, Sillabus part 7, Plant Analysis Procedures. In Wageningen Agricultural University, Wageningen, The Netherlands.

Zall, D.M., Fisher, D. and Garner, M.Q. (1956) Photometric determination of chlorides in water. *Anal. Chem.*, 28, 1665–1668.

CHAPTER 4.2

Effect of salinity on plant growth and vase life of cut roses grown at high relative air humidity

Abstract

Cultivation at high relative air humidity (RH ≥ 85%) frequently occurs in greenhouses during winter period leading to stomatal malfunctioning. In cut roses, this results in a severe vase life longevity reduction due to excessive water loss. High salinity of the irrigation water is a serious problem in arid and semiarid regions. Plants respond to salinity reducing stomatal conductance and transpiration rate often having a negative impact on growth. In this study, we aimed to investigate the interactive effects of moderate salinity and RH on plant growth and vase life of cut roses. Cut rose 'Prophyta' grown at moderate (63%) or high (89%) RH was subjected to three electrical conductivities (EC) in the nutrient solution (2, 4 and 6 dS m^{-1}). Total dry weight, leaf area and plant height were evaluated at harvest. Light and dark transpiration rate, flower stalk relative fresh weight and vase life longevity were assessed during vase life. Increased EC reduced total plant dry weight (23%), total leaf area (15%) and plant height (10%) whereas high RH had no significant effect in any of these parameters. In high RH-grown flower stalks, transpiration rate during vase life increased by 1.7- and 2.5-fold in light and darkness, respectively, compared to moderate RH-grown flower stalks, reflecting the poor dark-induced stomatal closure in flowers developed at high RH. Increased EC enhanced relative fresh weight especially in high RH-grown flower stalks, which is probably due to a lower transpiration rate during the dark period at higher EC. Nonetheless, higher salinity during growth did not promote vase life longevity in high RH-grown flowers. In contrast, increased EC significantly reduced vase life longevity in moderate RH-grown flower stalks.

Carvalho DRA, Vasconcelos MW, Heuvelink E, Carvalho SMP (2015) Effect of salinity on plant growth and vase life of cut roses grown at high relative air humidity. Acta Horticulturae (in press)

Introduction

High relative air humidity (i.e. RH ≥ 85%) regularly occurs in greenhouses during the winter period. It is well known that vase life longevity is reduced in plants cultivated at high RH due to a negative water balance (Fanourakis et al., 2012). Poor stomatal closure in conditions of high evaporative demand, has been pointed out as the major cause for this water stress (Fanourakis et al., 2013; Aliniaeifard et al., 2014). The need for using low quality irrigation water due to increased salinity is a serious problem especially in arid and semiarid regions. Plants subjected to high salinity during cultivation may inhibit water uptake causing physiological drought stress during the initial phase of exposure (Shalhevet and Bernstein, 1968). Such osmotic stress reduced stomatal conductance in roses and tomato plants (Jimenez et al., 1997; Maggio et al., 2007; Lovelli et al., 2012) and decreased transpiration rate in chrysanthemum (Lee and van Iersel, 2008). Moreover, plant growth is frequently reduced under increased salinity conditions (Oki and Lieth, 2004; Munns, 2002). In soilless culture, the modern rose cultivars are considered moderately sensitive to salinity (Cabrera and Perdomo, 2003) but these effects on vase life longevity of roses have been poorly studied (De Kreij and Van Den Berg, 1990). We hypothesized that increased salinity during growth in cut rose developed at high RH could stimulate stomatal functioning leading to a higher vase life longevity. The objective of this work was to analyze the interactive effects of moderate salinity and RH on plant growth and vase life longevity of cut roses.

Materials and methods

Rooted cuttings of the cut rose cultivar ‘Pink Prophyta’ (*Rosa* × *hybrida*) (hereafter named as ‘Prophyta’) were planted in 3.5 L pots containing a mixture (2/1, v/v) of cocopeat (Pelemix, Murcia, Spain) and perlite (Otavi, Neuss, Germany). Sixty plants were randomly distributed over two walk-in climate controlled growth chambers (length x width x height = 2.0 x 1.6 x 2.0 m; 5000 EH, Aralab, Albarraque, Portugal) at a density of 19 plants m^{-2} (one plant per pot; single stem). During cultivation, the RH in one of the growth chambers was 63 ± 2% (moderate RH) while in the other one it was 89 ± 3% (high RH). Constant day and night temperature was 22.2 ± 1.5 °C in both growth chambers, resulting in vapor pressure deficits (VPDs) of 0.99 ± 0.02 kPa (moderate RH) and 0.29 ± 0.06 kPa (high RH). The CO_2 concentration was 350 ± 20 µmol mol^{-1} (IAQ 910, TSI Incorporated, Shoreview, MN, USA). Fluorescent lamps (Osram L58W/840, Lumilux, Cool White, Munich, Germany) provided 20 h photoperiod of 130 ± 5 µmol m^{-2} s^{-1} photosynthetic active radiation (Li-1000 datalogger, Li-Cor, Lincoln, Nebraska, USA) measured 20 cm above the root-shoot interface. Plants were watered daily with a nutrient solution containing both (i) macronutrients (mM) [NH_4 1.0, K 4.0, Ca 3.5, Mg 1.38, NO_3 10.5, SO_4 1.5, H_2PO_4 1.25] and (ii) micronutrients (µM) [Fe 25, Mn 5, Zn 3.5, B 20, Cu 0.75, Mo 0.5]. This nutrient solution had an electrical conductivity (EC) of 2 dS m^{-1} (EC2, control) (Cond 6+, Eutech Instruments, Eutech Instruments Europe bv, Nijkerk, The Netherlands). In each growth chamber 10 plants were watered with that nutrient solution (control), while the other 20 plants were watered with the control nutrient solution corrected to an EC of 4 dS m^{-1} (EC4) or 6 dS m^{-1} (EC6) using NaCl. To prevent EC build-up in the substrate, twice per week, abundant nutrient solution was given to the plants to guarantee that the target EC values were present in the drained solution. A pH of 5.3 (pH 5+, Eutech Instruments, Eutech Instruments Europe bv, Nijkerk, The Netherlands) was kept in all the treatments.

The experiment consisted of two growing cycles. In the first growing cycle, the stomatal anatomy and physiology (Carvalho et al., 2015a) and the growth parameters of plants in response to elevated EC were determined at harvest stage (i.e., cylindrical flower bud with pointed tip). Total plant dry weight (48 h at 70 ˚C), leaf area, plant height, number of internodes and average internode length were assessed in seven plants per treatment. Thereafter, plants were pruned and a second growing cycle also with a single stem per plant was used to evaluate the vase life longevity in response to EC. Flower stalks were harvested at the length of: 34 ± 0.5 cm (60%; EC2 and EC4), 26 ± 2.5 cm (60%; EC6), 31.5 ± 1.3 cm (90%; EC2 and EC4) and 25 ± 3.3 cm (90%; EC6). Flower stalks were allowed to aspire air for 2–3 minutes, the cut end was cleaned with a solution of sodium hypochlorite (5%, pH 13; commercial bleach) and were then placed in 200 ml flasks covered with water-proof parafilm to ensure that water loss occurred only via the flower stalks (Fanourakis et al., 2012). Flasks contained a vase solution with the following composition: 0.7 mM $CaCl_2.2H_2O$, 1.5 mM $NaHCO_3$, 0.005 mM $CuSO_4.5H_2O$ (Van Meeteren et al., 1999). Flasks with flower stalks were kept in controlled conditions (20 ± 0.5 °C; 54 ± 1% RH; 10 ± 2 µmol m^{-2} s^{-1} photosynthetic active radiation, provided by fluorescent lamps during 12 h photoperiod) until loss of commercial value. During vase life, flasks and flowers were weighed (balance Kern PB, Kern & Sohn GmbH, Balingen-Frommern, Germany) twice per day, at the beginning and at the end of the photoperiod. At the end of vase life evaluation, leaf area per flower stalk was measured and the transpiration rate in the light and in the dark periods during vase life were calculated according to Equation 1. Relative fresh weight of the flower stalks during vase life was also calculated.

Transpiration rate (mmol m^{-2} s^{-1}) =

$$((\frac{\Delta\ \text{leaflet fresh weight (g)}}{\text{molar mass water (g mol}-1)}\times 1000\ \text{(mmol mol}^{-1}))/\text{measurement frequency (s))/leaflet area (m}^{2})$$

(Eqn. 1)

Vase life termination was considered when one of the following symptoms were present: (1) ‘bent-neck’: stem desiccated and bended beneath the bud forming an angle higher than 90°; (2) desiccated edges: the edges of five or more petals turned brown or black (> 1 cm), and some dried out; (3) end of flowering: the flower reached its maximum open stage and the petals started to fall (> two fallen petals); (4) wilted flower: the flower was visibly wilted or the petals became wrinkled; (5) leaf wilted foliage: more than 50% of the total number of leaves wilted; (6) leaf abscission: more than 50% of the total number of leaves fell from the stem (VBN, 2005).

The experimental set-up was a complete randomized design having seven plants as replicates in the first growing cycle. However, due to NaCl accumulation causing toxicity, two plants from EC4 and eight from EC6 grown at moderate RH as well as five plants from EC6 grown at high RH died during the time course of the experiment leading to an unbalanced number of replicates. Therefore, an unbalanced analysis of variance was conducted considering individual plants as experimental units where two to 10 flower stalks were evaluated per treatment. Main effects and interactions were tested at $P = 0.05$. When relevant, least significant difference (LSD) at $P = 0.05$ was calculated to separate interaction means. The Genstat software (17th edition; VSN International Ltd., Herts, UK) was used for the analysis.

Results and discussion

For all the evaluated plant growth parameters there was no significant effect of the interaction between EC and RH and no significant effect of the RH (Table 1). The lack of effect of RH on plant growth parameters (including total dry weight and total leaf area) was also previously described in roses and only a minor increase in plant height (9%) due to an increase in the average internode length (6%) was noted (Carvalho et al., 2015b). In contrast, increased EC strongly influenced plant growth. Plants grown at EC6 showed lower total plant dry weight (23%; *P* < 0.001), lower total leaf area (15%; *P* = 0.018) and lower plant height (10%; *P* = 0.019) as compared to plants grown at EC2 (Table 1). Although the number of internodes and the average internode length were not significantly affected by the EC level (*P* = 0.741 and *P* = 0.078, respectively) the reduced plant height was mostly due to a lower average internode length (Table 1). In previous studies, high salinity levels also resulted in lower rose stem length and stem weight (De Kreij and Van Den Berg, 1990; Brun and Settembrino, 1996; Cabrera, 2003; Oki and Lieth, 2004).

Table 1. Plant growth parameters at harvest stage of cut rose 'Prophyta' grown at moderate (63%) or high (89%) relative air humidity with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (EC2, EC4 or EC6, respectively). Values are the mean of EC2, EC4 and EC6 per RH (n = 14) as no significant interaction between EC and RH was found for any of the studied parameters. Different letters indicate significant differences according to Fisher's LSD test (*P* = 0.05)

	EC2	EC4	EC6	*F pr.* RH	*F pr.* EC	*F pr.* RH×EC
Total dry weight (g)	6.11^b	5.69^b	4.56^a	0.909	<0.001	0.934
Total leaf area (cm^2)	674^b	641^b	558^a	0.261	0.018	0.352
Plant height (cm)	40.6^b	41.2^b	36.7^a	0.093	0.019	0.340
Number of internodes (no.)	10.4	10.6	10.2	0.455	0.741	0.144
Average internode length (cm)	2.87	2.89	2.63	0.257	0.078	0.482

There was no significant interaction between EC and RH in the transpiration rate in the light (*P* = 0.870) or in the dark (*P* = 0.444) periods during vase life (Fig. 1). Flower stalks cultivated at high RH showed 1.7- and 2.5-fold higher transpiration rate in light (Fig. 1A) and darkness (Fig. 1B), respectively, when compared to moderate RH-grown flower stalks. These findings reflect the poor stomatal closure of plants developed at high RH and further transferred to conditions of high evaporative demand. Similar findings were reported by Fanourakis et al. (2012) who evaluated the transpiration rate during postharvest in three rose cultivars grown at contrasting RH levels. Concerning the effect of EC on transpiration rate in light (*P* = 0.471) or darkness (*P* = 0.409), there was a tendency for a reduced transpiration rate with increased EC in light (Fig. 1A) as well as in darkness (Fig. 1B). Lee and van Iersel (2008) found in chrysanthemum a reduction in transpiration rate induced by increased salinity. Moreover, the light-dark transition reduced the transpiration rate of flower stalks cultivated at moderate RH by 63% while it was only decreased by 45% in high RH-grown flower stalks (Fig. 1). This also confirms a poor dark-induced stomatal closure in flower stalks developed at high RH, as it has been previously shown in cut roses as well as in intact plants (Fanourakis et al., 2012; Carvalho et al., 2015b).

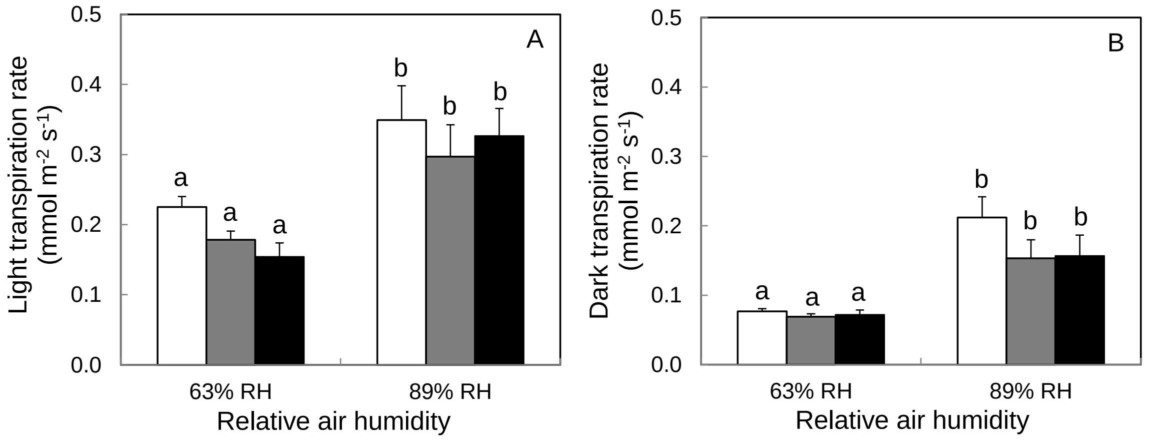

Fig. 1. Transpiration rate in the light (A) and in the dark (B) periods during vase life of cut rose 'Prophyta' grown at moderate (63%) or high (89%) relative air humidity (RH) with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (white, grey or black columns, respectively). Evaluation started when flower stalks had cylindrical flower bud with pointed tip. Values are the mean of two to 10 replicates per treatment (unbalanced analysis of variance due to plant mortality at high EC) and bars represent the SEM. Different letters indicate significant differences according to Fisher's LSD test (*P* = 0.05).

During vase life, relative fresh weight of flower stalks grown at high RH was lower compared to moderate RH-grown ones (Fig. 2). Increasing the EC level led to an increase of relative fresh weight irrespective the RH (Fig. 2). In addition, during the initial four days of evaluation, the relative flower fresh weight increased with increasing EC mainly in high RH grown plants (Fig. 2). In particular, for EC6, high RH-grown flower stalks reached the relative fresh weight of the ones grown at moderate RH (Fig. 2C). Vase life longevity was significantly reduced in moderate RH-grown plants in response to increased EC, whereas it was not affected in high RH-grown plants (*P* = 0.036; Fig. 3). In general, vase life longevity was lower in flower stalks developed at high RH compared to the ones grown at moderate RH (Fig. 3). Also Fanourakis et al. (2012) found lower vase life longevity in high RH-grown plants compared to plants grown at moderate RH. Studies evaluating the effects of salinity on vase life longevity of ornamentals are lacking. De Kreij and Van Den Berg (1990) found a negative effect of high EC on vase life longevity, which is in agreement with our findings for flower stalks developed at moderate RH (Fig. 2). The present study is thus the first evaluating the effect of increased EC in flower stalks developed at high RH.

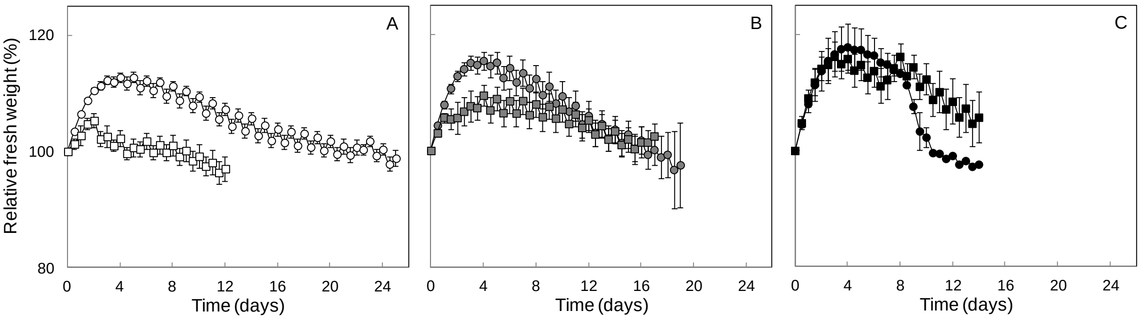

Fig. 2. Relative fresh weight of flower stalks during vase life of cut rose 'Prophyta' grown at moderate (63%; circles) or high (89%; squares) relative air humidity with an electrical conductivity of the nutrient solution of 2 dS m^{-1} (A, white symbols), 4 dS m^{-1} (B, grey symbols) or 6 dS m^{-1} (black symbols). Evaluation started when flower stalks had cylindrical flower bud with pointed tip. Values are the mean of two to 10 replicates per treatment (unbalanced analysis of variance due to plant mortality at high EC) and bars represent the SEM.

In conclusion, our results indicated that increasing EC levels in the nutrient solution of high RH-grown plants reduced the transpiration rate of the flower stalks (Fig. 1) leading to an increase in their relative fresh weight (Fig. 2), but contrary to our hypothesis this was not sufficient to promote an increase on the vase life longevity (Fig. 3). Contrariwise, in moderate RH-grown flower stalks the stomata appeared to be functional (i.e., good dark-induced stomatal closure; Fig. 1) and thus the reduction of the relative fresh weight of the flower stalks (Fig. 2) is hypothesized to have another origin rather than stomatal malfunctioning. We suggest that the accumulation of Na^+ and Cl^- ions during plant growth result in saline toxicity damaging cell membranes and reducing vase life. The effect of high EC was more harmful in moderate RH-grown plants evidenced by the severe decrease on vase life longevity. In high RH-grown plants, contrary to our expectations, the vase life longevity did not increase.

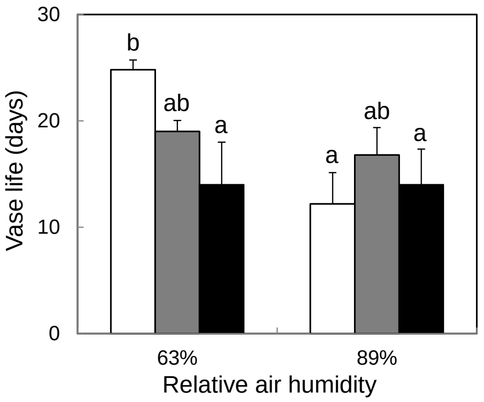

Fig. 3. Vase life longevity of cut rose 'Prophyta' grown at moderate (63%) or high (89%) relative air humidity with an electrical conductivity of the nutrient solution of 2, 4 or 6 dS m^{-1} (white, grey or black columns, respectively). Evaluation started when flower stalks had cylindrical flower bud with pointed tip. Values are the mean of two to 10 replicates per treatment (unbalanced analysis of variance due to plant mortality at high EC) and bars represent the SEM. Different letters indicate significant differences according to Fisher's LSD test (*P* = 0.05).

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References

Aliniaeifard, S., Malcolm Matamoros, P., van Meeteren, U., 2014. Stomatal malfunctioning under low VPD conditions: Induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? Physiol. Plant. 152, 688–699. doi:10.1111/ppl.12216

Brun, R., Settembrino, A., 1996. Production and quality of *Rosa hybrida* cv Sonia in rockwool at three levels of electrical conductivity combined with two levels of relative humidity. Acta Hortic. 424, 115–117.

Cabrera, R.I., 2003. Demarcating salinity tolerance in greenhouse rose production. Acta Hortic. 609, 51–57.

Cabrera, R.I., Perdomo, P., 2003. Reassessing the salinity tolerance of greenhouse roses under soilless production conditions. HortScience 38, 533–536.

Carvalho, D.R.A., Vasconcelos, M.W., Lee, S., Vreugdenhil, D., Heuvelink, E., Carvalho, S.M.P., 2015a. Increased electrical conductivity of the nutrient solution improves stomatal functioning in plants grown at high relative air humidity (in preparation)

Carvalho, D.R.A., Torre, S., Kraniotis, D., Almeida, D.P.F., Heuvelink, E., Carvalho, S.M.P., 2015b. Elevated air movement enhances stomatal sensitivity to abscisic acid in leaves developed at high relative air humidity. Front. Plant Sci. 6, 1–11. doi:10.3389/fpls.2015.00383

De Kreij, C., Van Den Berg, T.H.J.M., 1990. Nutrient uptake, production and quality of *Rosa hybrida* in rockwool as affected by electrical conductivity of the nutrient solution, in: van Beusichem, M.L. (Ed.), Plant Nutrition — Physiology and Applications. Springer Netherlands, Dordrecht, pp. 519–523. doi:10.1007/978-94-009-0585-6

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F., van Kooten, O., van Doorn, W.G., Heuvelink, E., 2012. Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biol. Technol. 64, 64–73. doi:10.1016/j.postharvbio.2011.09.016

Fanourakis, D., Heuvelink, E., Carvalho, S.M.P., 2013. A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. J. Plant Physiol. 170, 890–898. doi:10.1016/j.jplph.2013.01.013

Jimenez, M., Gonzalez-Rodriguez, A., Morales, D., Cid, M. C., Socorro, a. R., & Caballero, M., 1997. Evaluation of chlorophyll fluorescence as a tool for salt stress detection in roses. *Photosynthetica*. http://doi.org/10.1023/A:1022176700857

Lee, M., van Iersel, W., 2008. Sodium chloride effects on growth, morphology, and physiology of chrysanthemum (*Chrysanthemum* × *morifolium*). Hortscience 43, 1888–1891.

Lovelli, S., Scopa, A., Perniola, M., Di Tommaso, T., Sofo, A., 2012. Abscisic acid root and leaf concentration in relation to biomass partitioning in salinized tomato plants. J. Plant Physiol. 169, 226–233. doi:10.1016/j.jplph.2011.09.009

Maggio, A., Raimondi, G., Martino, A., De Pascale, S., 2007. Salt stress response in tomato beyond the salinity tolerance threshold. Environ. Exp. Bot. 59, 276–282. doi:10.1016/j.envexpbot.2006.02.002

Munns, R., 2002. Comparative physiology of salt and water stress. Plant. Cell Environ. 25, 239–250. doi:10.1046/j.0016-8025.2001.00808.x

Oki, L.R., Lieth, J.H., 2004. Effect of changes in substrate salinity on the elongation of *Rosa hybrida* L. “Kardinal” stems. Sci. Hortic. (Amsterdam). 101, 103–119. doi:10.1016/j.scienta.2003.09.013

Shalhevet, J., Bernstein, L., 1968. Effects of vertically heterogeneous soil salinity on plant growth and water uptake. Soil Sci. 106, 85–93.

Van Meeteren, U., Van Gelder, H., Van Ieperen, W., 1999. Reconsideration of the use of deionized water as vase water in postharvest experiments on cut flowers. Postharvest Biol. Technol. 17, 175–187. doi:10.1016/S0925-5214(99)00050-2

VBN, 2005. Evaluation cards for cut flowers. VBN, Leiden, The Netherlands.

CHAPTER 5

QTL analysis for stomatal functioning in tetraploid *Rosa* × *hybrida* grown at high relative air humidity and its implications on postharvest longevity

Abstract

High relative air humidity (RH ≥ 85%) during leaf development disturbs stomatal functioning leading to excessive water loss in conditions of high evaporative demand, resulting in severe reduction of postharvest longevity. In roses, this effect depends on the genotype, opening the possibility for breeding cultivars with more responsive stomata. In this study, we aim at identifying genomic regions associated with the control of water loss following growth at high RH. The F1 generation (108 offspring) and the two parents (P540 and P867) of a tetraploid cut rose population grown at high (85%) RH were phenotyped for stomatal control to water loss by assessing the relative water content after four hours of leaflet desiccation (RWC_4h). The RWC_4h varied between 7 and 62% across the 110 studied individuals, with parents P540 and P867 showing 51 and 20% RWC_4h, respectively. Based on these data, a quantitative trait locus (QTL) analysis was performed. The impact of the identified QTLs on postharvest longevity of ten selected offspring was further evaluated. Three QTLs were identified: two major [positioned on linkage group 5 of the integrated consensus map (ICM 5) of both parents and on ICM 2 of the parent P867] and one putative minor (mapped to ICM 6 of both parents), explaining 32% of the variability in the RWC_4h. Low RWC_4h was found to be a good proxy for eliminating the offspring with short vase life. This study constitutes a first step towards identifying the most likely regions for genes of interest controlling stomatal functioning in high RH-grown plants.

Carvalho DRA, Koning-Boucoiran CFS, Fanourakis D, Vasconcelos MW, Carvalho SMP, Heuvelink E, Krens FA, Maliepaard C (2015) QTL analysis for stomatal functioning in tetraploid *Rosa* x *hybrida* grown at high relative air humidity and its implications on postharvest longevity. Molecular Breeding, 35:172, DOI: 10.1007/s11032-015-0365-7

Introduction

Vase life of a cut flower refers to the duration between placement in the vase and loss of ornamental value. The potential (maximum) vase life is the result of the complex interaction between genotype and environment during cultivation (van Meeteren et al. 2005). The physiological and anatomical traits involved in the potential vase life are established during plant cultivation (Fanourakis et al. 2012b) and among them stomatal functioning is seen as a key trait for a proper control of water loss during postharvest (Fanourakis et al. 2012a). For several plant species, high relative air humidity (RH ≥ 85%) throughout leaf development is the most important environmental factor during growth attenuating the stomatal response to closing stimuli such as leaflet desiccation (Torre et al. 2003; Rezaei Nejad and van Meeteren 2005; Fanourakis et al. 2012a). This stomatal malfunction leads to excessive water loss under conditions of high evaporative demand, causing lethal wilting symptoms such as desiccated leaves and the pedicel bending in roses (so-called ‘bent-neck’), thus drastically shortening the postharvest longevity (Mortensen and Gislerød 1999; Fanourakis et al. 2012a). A similar stomatal malfunctioning has been noted in several plant species (e.g., rose, carnation and delphinium) propagated *in vitro* and suddenly transferred to conditions of high evaporative demand (Ziv et al. 1987; Ghashghaie et al. 1992; Santamaria et al. 1993; Sáez et al. 2012). Nevertheless, the effect of high RH on stomatal responsiveness to closing stimuli is highly cultivar dependent (Mortensen and Gislerød 1999; Fanourakis et al. 2013a; Giday et al. 2013). This genotypic variation can be easily tracked by evaluating the relative water content after four hours of leaflet desiccation (RWC_4h) (Fanourakis et al. 2013a; Giday et al., 2013). Based on the noted genotypic differences, it appears to be feasible to breed for cultivars with more responsive stomata following cultivation at high RH (Fanourakis et al. 2012b), a procedure that will be highly promoted by identifying the genomic regions containing genes that control this physiological trait.

Quantitative trait locus (QTL) analysis is used to detect an association between the phenotype and the genotype of markers (Collard et al. 2005), allowing the identification of genomic regions involved in the quantitative variability in the phenotypic trait. Moreover, it also identifies markers linked to those QTL regions, that can be used for marker-assisted selection (MAS) in plant breeding (Ribaut and Hoisington 1998). Research reporting on QTL mapping for the stomatal response to environmental conditions is very scarce. In *Arabidopsis thaliana*, screening a recombinant inbred line population (Col-0 × Cvi-0) identified a single QTL for high water loss rates (Brosché et al. 2010). In rice, two QTLs affecting stomatal conductance have been identified (Zhao et al. 2008). In field-grown barley, nine chromosomal regions were found to control the relative water content (RWC) (Teulat et al. 2003). In roses, QTL studies focus on morphological traits (e.g., flower and leaf size, thorn number), days to flowering or disease resistance (e.g., powdery mildew) (Crespel et al. 2002; Dugo et al. 2005; Linde et al. 2006). To the best of our knowledge, there are no studies on identifying QTLs related to the stomatal functioning mediating the control of water loss after growth at high RH, associated with plant survival following transfer to high evaporative demand conditions.

Rosa × *hybrida* is a challenging crop for genetic studies because its genome has not yet been fully sequenced, it has a high heterozygosity (Berninger 1992; Gudin and Mouchotte 1996), it is a polyploid species (Berninger 1992; Jacob et al. 1996) and it has small progeny sizes (Buck 1960; Gudin 1995; Gudin and Mouchotte 1996). However, roses are one of the economically most important ornamental crops worldwide (Yu et al. 2015) often suffering from a short vase-life which is highly cultivar dependent. To better characterize the rose genome and to find markers for important economical characters, Koning-Boucoiran et al. (2012) initially constructed a genetic linkage map of two tetraploid parents, i.e., P540 (mother) and P867 (father), resulting in the K5 population of 184 offspring. More recently, a new genetic linkage map became available for the same parental lines with an improved marker distribution and with 25 linkage groups (a number closer to the 28 expected total) (Koning-Boucoiran, pers. comm.). Since the majority of cut roses are tetraploid, each marker can be present in different dosages, corresponding with the number of copies of an allele. In turn, different allele dosages can result in differences in RNA levels of a particular allele and possibly also phenotypic differences (Voorrips et al. 2011). By employing the above-mentioned improved genetic linkage map, we here attempt to identify genetic regions that are related to poor control of water loss, owing to malfunctional stomata following growth at high RH, frequently underlying short vase life.

In this study, a subset of the K5 tetraploid cut rose population grown at high RH was phenotypically evaluated for stomatal responsiveness to desiccation. We aimed to identify the genomic regions associated with the control of water loss in plants grown at high RH. This work contributes to speeding up the selection of offspring using MAS programs in breeding for cultivars with more responsive stomata after cultivation at high RH, minimizing the negative impacts associated with excessive water loss during the postharvest phase.

Materials and methods

Plant material and growth conditions

A subset of the K5 tetraploid cut rose population (Yan et al. 2006), consisting of 108 progeny individuals plus the two parents (P540 and P867), was cultivated in a multi-span Venlo-type glasshouse (144 m^2) located in Wageningen, The Netherlands (N 51°58’, E 5°39’). Two cuttings of the same individual from the K5 population were planted in 10 L pots filled with cocopeat (Jongkind Grond BV, Aalsmeer, The Netherlands) and placed at a density of six plants m^{-2}. Each individual had four replicates (two plants per pot). The plants on the two outer beds and on the edges of the inner beds acted as borders. Canopy was conducted according to the bending technique, allowing multiple stems per plant to grow. The RH was 85.0 ± 0.4% (high), while day and night temperatures were 19.6 ± 0.1 and 18.2 ± 0.1 ºC, respectively, resulting in vapor pressure deficits (VPD) of 0.34 ± 0.01 and 0.31 ± 0.01 kPa, respectively. A 12 h photoperiod (from 08h00 till 20h00) was achieved using supplementary light provided by high-pressure sodium lamps (SON-T Agro, Philips, Eindhoven, The Netherlands) when the outside global radiation was lower than 200 W m^{-2}. Lamps were switched off when natural radiation was higher than 300 W m^{-2}. Throughout the experimental period climate data were automatically recorded every 5 min by a commercial computer system (Hoogendoorn, Vlaardingen, The Netherlands) and average values were calculated daily. Plants were irrigated using a drip system with a nutrient solution described by Fanourakis et al. (2009) and a drainage rate of 30%. Plant protection was applied according to an integrated pest management scheme (IPM), using both biological and chemical agents.

Phenotyping and data collection

Fully-expanded terminal leaflets from the first to third penta-foliated leaves (counting from the apex) were detached from the upper sunlit canopy layer of the flower stalk with a cylindrical shape and pointed bud. Sampling took place during the first hour of the light period. Immediately after excision, leaflet petioles were recut under water (to prevent cavitation induced-embolism) and placed in flasks filled with degassed water. Leaflets were further rehydrated under light (15 µmol m^{-2} s^{-1}) to keep the stomata open (Drake et al. 2013), in a saturated (≈ 100%) RH environment at 21.0 ± 0.5 ºC (i.e., VPD close to 0 kPa) for 1 h to establish their saturated fresh weight (Fanourakis et al. 2011). After rehydration, the leaflets were allowed to desiccate for four hours on a bench (abaxial surface down) in a test room with 1.24 ± 0.07 kPa VPD (50 ± 3% RH, 21 ± 0.5 ºC) and 50 ± 2 µmol m^{-2} s^{-1}

light intensity. Leaflet weight was gravimetrically recorded before (0 h) and after (4 h) desiccation (Model LP 3200D, Sartorius AG, Goettingen, Germany). At the end of the desiccation period, leaflet dry weight was determined (24 h at 105 ºC). RWC was calculated according to Slavík (1974) (Equation 1) in 12 replicates (one leaflet per stem) per studied individual from the K5 population.

$$\text{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{saturated fresh weight} - \text{dry weight}} \times 100 \ \text{(Eqn. 1)}$$

Data analysis and QTL identification

An improved linkage map of the K5 rose population was used in the current study. It was constructed by Koning-Boucoiran et al. using JoinMap 4.1 (Van Ooijen 2006) for each parent separately and per homolog. Each chromosome was numbered (1–7) according to the numbering in the integrated consensus map (ICM) of Spiller et al. (2011). The numbering of the homologs per chromosome (1–4) within the same ICM number is arbitrary. QTL analysis for the control of water loss was conducted using 15154 single-nucleotide polymorphisms (SNP) markers generated by genotyping the K5 population with the newly developed WagRhSNP 68k Axiom SNP array (Koning-Boucoiran et al. 2015) and allele dosages were scored using fitTetra (Voorrips et al. 2011). Since the K5 cut rose population is tetraploid, marker alleles are present in different dosages, i.e., 0 (nulliplex), 1 (simplex), 2 (duplex), 3 (triplex) or 4 (quadruplex). According to the parental configurations, these markers segregate differently in the progeny, i.e., there are SNPs with: (1) single dosage, whose minor allele (i.e., least common allele) is present in only one parent (simplex × nulliplex) or in both parents (simplex × simplex), (2) double dosage, whose minor allele is present in only one parent (duplex × nulliplex) or in both parents (duplex × duplex), or (3) double dosage in one parent and singe dosage in the other parent (duplex × simplex).

Firstly, an analysis of variance (ANOVA) was conducted for each independent marker on dosage classes using an in-house R script (R–3.1.2) at a 99.9% confidence level for each single test ($\alpha = 0.001$). Thereafter, an ANOVA was performed for the combination of the most significant marker from each of the three identified QTL regions. Main effects and interactions among these three markers were tested at $\alpha = 0.05$ using Genstat (17th edition). The most significant markers were taken as indicators of the QTL positions. The regions of the identified QTLs were visually represented on the ICM of each parental line separately using MapChart 2.2 (Voorrips 2002).

Postharvest evaluation

In order to evaluate whether the most explanatory markers could predict postharvest longevity, a subset of ten offspring (K148, K039, K121, K161, K225, K119, K107, K059, K015 and K099) from the K5 population were evaluated for vase life. These offspring were selected based on their contrasting stomatal responsiveness to desiccation. Flower stalks were harvested, when the flower bud had cylindrical shape and pointed tip. Following harvest, cut flowers were left to aspire air for 2-3 minutes. The cut end of the stems was then surface-sterilized with NaClO [5% (w/v); commercial bleach] and placed in 200 ml flasks containing an artificial vase solution: 0.7 mM $CaCl_2.2H_2O$, 1.5 mM $NaHCO_3$, 0.005 mM $CuSO_4.5H_2O$ (Van Meeteren et al. 1999). Cut flowers were kept under controlled conditions: 20 ± 0.5 ºC, 54 ± 1% RH and 12 h photoperiod of 10 ± 2 µmol m^{-2} s^{-1} photosynthetic active radiation (Li-1000 datalogger, Li-Cor, Lincoln, Nebraska, USA) provided by fluorescent lamps. The end of postharvest longevity was determined according to the following VBN criteria (VBN 2005): (i) 'bent-neck': bending of the peduncle (i.e., > 90°); (ii) desiccated petal edges: ≥ 5 petals turned brown or black on the edges (> 1 cm) or dried out; (iii) end of flowering: flower reached the maximum opening and more than two petals fell; (iv) flower wilting: the flower lost turgor or the petals became wrinkled; (v) foliage wilting: > 50% of the foliage lost turgor; (vi) leaf abscission: > 50% of the leaves dropped. Seven flower stalks were evaluated per studied individual. Data were analyzed by one-way ANOVA tested at $\alpha = 0.05$ using Genstat (17th edition).

Results

Genotypic variability in stomatal response to water loss

Detached leaflets from the two parents and the 108 offspring grown at high RH were desiccated to investigate stomatal responsiveness to water loss. The RWC_4h, used as the indicator of stomatal responsiveness, varied between 7 and 62%, with the parent P540 showing considerably higher RWC_4h (51%) compared to the parent P867 (20%) (Fig. 1a). The F1 generation revealed transgressive segregation for this trait, however most of the offspring (72%) showed RWC_4h in between those of the parents (Fig. 1a). Moreover, the RWC_4h of the studied offspring approximately followed a normal distribution, with a median of 37.4% (Fig. 1b).

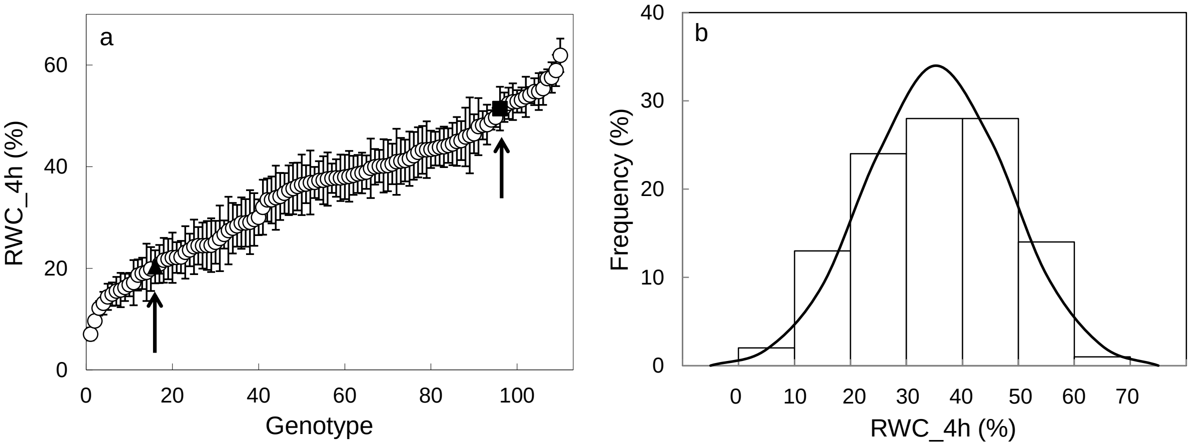

Fig. 1 Relative water content after four hours of leaflet desiccation (RWC_4h) (a) and its frequency distribution (b) in 110 genotypes of the K5 tetraploid cut rose population grown at high (85%) relative air humidity. Circles represent the F1 generation, while square and triangle illustrate the parents P540 and P867, respectively (marked with an arrow). Values are the mean of 12 detached leaflets ± SEM

QTLs associated with stomatal responsiveness to water loss

QTL analysis revealed 66 significant markers (α = 0.001) for the water loss trait (RWC_4h). Twenty-seven of these markers were not mapped on the K5 population, while the other 39 were mapped to three different ICM linkage groups, i.e., ICM 5 and ICM 6 (both parental maps) and ICM 2 (P867) (Table A1, Appendix). In the ANOVA analysis combining the most significant marker in each ICM [i.e., K19678_920R11[1] (ICM 5), K10792_5574R01[1] (ICM 2) and K403_2848R11[1] (ICM 6)] and their interactions, none of the interactions were significant (data not shown). The marker K19678_920R11 individually explained the largest percentage of phenotypic variability (27.5%) in the RWC_4h, followed by K10792_5574R01 and K403_2848R11 that individually explained 10.3 and 11.2%, respectively (Table 1). However, when conducting a stepwise regression analysis, the marker K403_2848R11 was not significant (P = 0.209), when the other two significant markers K19678_920R11 (P < 0.001) and K10792_5574R01 (P = 0.010) had been already included in the model (data not shown). This resulted in the classification of two major QTLs (in ICM 5 and ICM 2) and one putative minor QTL (in ICM 6) associated with RWC_4h. These three markers together explained 32.2% of the phenotypic variability

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[1] The last two numbers in the marker name refer to the segregation type where 01 = nulliplex × simplex and 11 = simplex × simplex.

(data not shown). The markers K19678_920R11 and K403_2848R11 were linked to higher RWC_4h in the presence of a double dosage (dosage 2) ($P < 0.001$; Table 1; Figs. 2a and 2c), while lower dosages (dosage 0 or 1) of these markers were associated with low RWC_4h. In contrast, in the absence (i.e., dosage 0) of the marker K10792_5574R01, higher RWC_4h values were noted, as compared to its presence (i.e., dosage 1) ($P < 0.001$; Table 1; Fig. 2b).

Table 1. The most significant marker associated with stomatal responsiveness to water loss mapped to each integrated consensus map (ICM 5, ICM 2, ICM 6) and respective homolog of the parents P540 and P867. -^{10}log (*P*-value), percentage of phenotypic variability explained by each marker (% Expl) and average relative water content after four hours of leaflet desiccation (RWC_4h) per dosage class per marker are shown. Different letters represent significant differences (LSD test, $\alpha = 0.05$) (comparison in rows). The two last numbers in the marker name refer to the segregation type where 01 = nulliplex × simplex, 11 = simplex × simplex.

Marker	ICM_homolog		-10log (*P*-value)	% Expl	RWC_4h per dosage class		
	P540	P867			0	1	2
K19678_920R11	5_1	5_4	7.61	27.5	32.2^a	31.4^a	46.8^b
K10792_5574R01	-	2_3	3.38	10.3	39.2^b	30.8^a	-
K403_2848R11	6_1	6_3	3.15	11.2	32.6^a	32.7^a	42.6^b

The most explanatory QTL was mapped to both parental lines on ICM 5 to homologs 1 and 4 in P540 and P867, respectively (Figs 3a and 3b), with the numbering of the homologs within a linkage group being arbitrarily chosen. The second most important QTL, according to the statistical analysis, was only mapped to ICM 2, homolog 3, from the parent P867 (Fig. 3e), since this marker segregated as nulliplex × simplex only from that parent. The minor QTL was mapped to ICM 6, homologs 1 and 3, respectively in P540 and P867 (Figs. 3c and 3d).

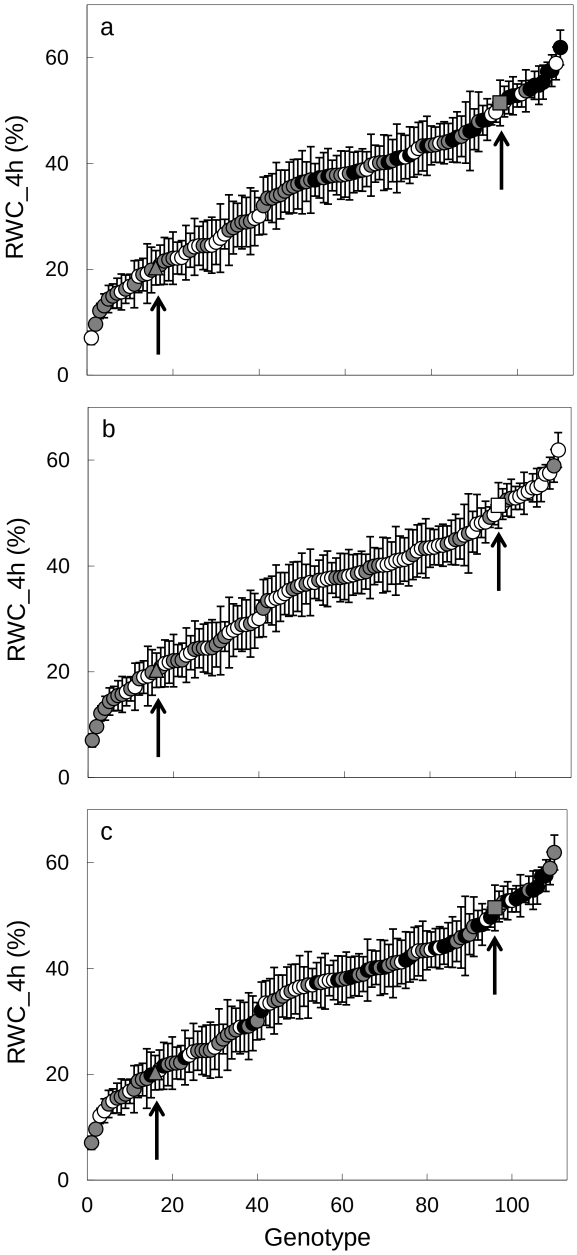

Fig. 2 Dosage of the markers K19678_920R11 (a), K10792_5574R01 (b) and K403_2848R11 (c) as a function of relative water content after four hours of leaflet desiccation (RWC_4h) in 110 genotypes of the K5 tetraploid cut rose population grown at high (85%) relative air humidity. White, grey and black symbols represent dosages 0, 1 and 2, respectively. Circles represent the F1 generation, while square and triangle illustrate the parents P540 and P867, respectively (marked with an arrow). Values are the mean of 12 detached leaflets ± SEM. The two last numbers in the marker name refer to the segregation type where 01 = nulliplex × simplex, 11 = simplex × simplex

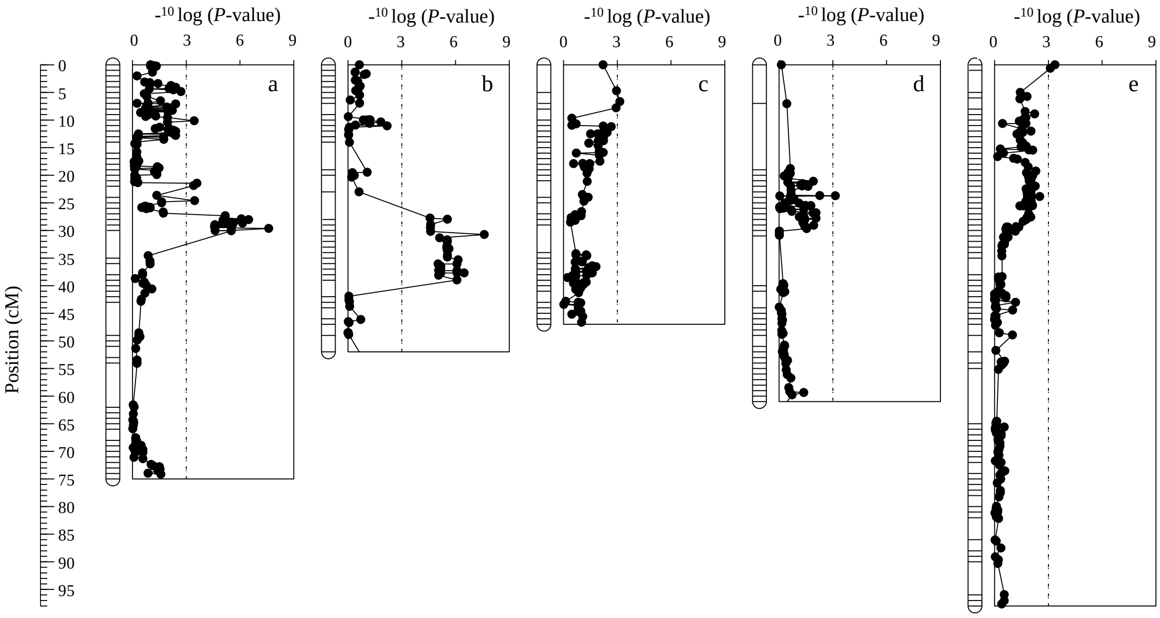

Fig. 3 Integrated consensus map (ICM) of the parental lines representing the QTL regions for stomatal responsiveness to water loss corresponding to the significant peaks above the threshold -^{10}log (*P*-value) of three (indicated by the dashed line). The represented significant markers segregated from parents P540 (a, c) and P867 (b, d, e) and were mapped to: ICM 5, homolog 1 (a); ICM 5, homolog 4 (b); ICM 6, homolog 1 (c); ICM 6, homolog 3 (d); and ICM 2, homolog 3 (e)

Assessment of postharvest longevity

In offspring with high RWC_4h (i.e., > 54%), the vase life varied largely between 10 and 26 days (for K225 and K099, respectively), whereas for the ones with lower RWC_4h, it varied from 4 to 9 days (Fig. 4).

A preliminary evaluation of the QTLs showed that when the markers K19678_920R11 and K403_2848R11 had dosage 2 and marker K10792_5574R01 had dosage 0, the vase life was very long for K099, but moderate for K225 and K119 (Table 2). K148 showed the shortest vase life, and had the two former markers at a dosage of 1 in combination with dosage 1 of the latter marker (Table 2).

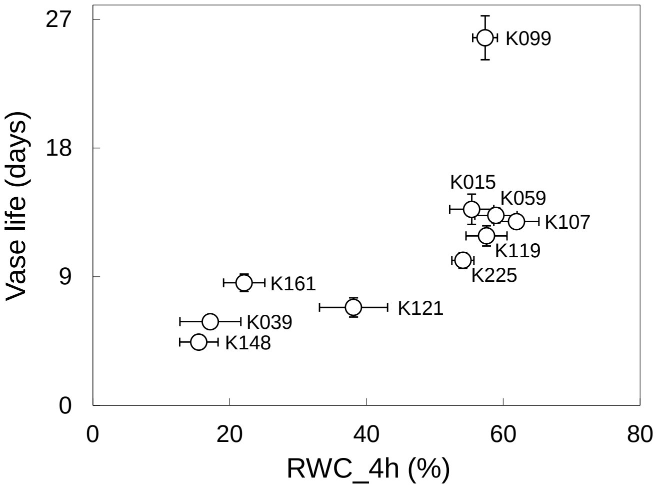

Fig. 4 Vase life as a function of relative water content after four hours of leaflet desiccation (RWC_4h) in ten genotypes from the K5 rose population. Values are the mean of 12 detached leaflets ± SEM (RWC_4h) or seven flower stalks ± SEM (vase life)

Table 2. Dosage of the most significant marker (i.e., K19678_920R11, K10792_5574R01 and K403_2848R11) from each integrated consensus map in ten genotypes from the K5 rose population, ordered by increasing vase life. The two last numbers in the marker name refer to the segregation type where 01 = nulliplex × simplex, 11 = simplex × simplex.

	Marker (dosage)		
Genotype	K19678_920R11	K10792_5574R01	K403_2848R11
K148	1	1	1
K039	1	0	1
K121	1	0	1
K161	0	1	1
K225	2	0	2
K119	2	0	2
K107	2	0	1
K059	0	1	1
K015	2	0	2
K099	2	0	2

Discussion

Two major and one putative minor QTLs were associated with stomatal responsiveness

This is the first study identifying QTLs related to the stomatal functioning and the control of water loss following cultivation at high RH, which is of utmost importance for plants grown in greenhouses as well as *in vitro* propagated species.

RWC_4h has been used as a tool to quantify stomatal responsiveness to desiccation (Fanourakis et al. 2012b; Giday et al. 2014). In the current study, we found a large genotypic variability for stomatal functioning in response to water loss during desiccation (i.e., RWC_4h; Fig. 1), providing evidence that the K5 tetraploid cut rose population offers a good genetic basis for QTL analysis linked to this trait. The observed transgressive segregation in both directions (Fig. 1a) suggested that the stomatal responsiveness to water loss is a polygenic trait, i.e., multiple genes may contribute to the phenotypic variability, in addition to environmental effects.

The three most explanatory markers from the three significant ICMs together explained 32% of the phenotypic variance (data not shown). Two QTLs linked with stomatal conductance in rice collectively explained only 14.9% (Zhao et al. 2008), while nine QTLs associated with RWC in field-grown barley individually explained a maximum of 15.0% (Teulat et al. 2003). In the present study, the identified genomic regions were located on different chromosomes, supporting the expectations that stomatal responsiveness to desiccation is a polygenic trait derived from a combination of different genomic regions. Similarly, the QTLs identified in rice and barley, were mapped on two and six different chromosomes, respectively (Teulat et al. 2003; Zhao et al. 2008).

The majority of the QTLs noted in our study showed a single peak above the significance threshold $-^{10}\log$ (*P*-value) of three comprising multiple neighboring markers (Fig. 3). An exception to this was the QTL mapped to ICM 5 from the parent P540 (Fig. 3a) which seems to be constituted by three peaks. Since these three peaks are within 19.5 cM, we assume that this is still only evidence of a single QTL associated with RWC_4h. Gaps between peaks in the same region can be caused by differences in numbers of missing values of markers, different homologous chromosomes being tagged by individual markers, and different segregation types of markers.

In our study, we performed single marker analyses considering possible dosage effects of markers, which is a valuable tool for QTL studies in polyploid populations. Interestingly, the most significant marker from the major QTL, mapped to ICM 5 (K19678_920R11), and the marker from the putative minor QTL, mapped to ICM 6 (K403_2848R11), showed indeed a dosage effect: if the favorable allele was present in double dosage, i.e. on two of the four homologs, the progeny showed a higher RWC_4h (Table 1; Figs. 2a and 2c). The individuals showing zero or only one copy of the marker allele (dosage 0 and 1, respectively) were not significantly different from each other (Table 1) and had lower average RWC_4h compared to dosage 2 (Figs. 2a and 2c). These results indicate that it is not enough to study the presence or the absence of this marker to infer about stomatal responsiveness to water loss. It is important to know about the dosage, since at least for these two QTLs only a double dosage is associated with a higher RWC_4h and consequently an increased stomatal functioning. For marker K10792_5574R01, which is a simplex × nulliplex marker, it is enough to study the presence (dosage 1) or the absence (dosage 0) of the scored allele where the RWC_4h was lower in the former compared to the latter (Table 1; Fig. 2b). In case of using MAS to breed for rose genotypes with higher stomatal responsiveness to water loss, a combination of the QTLs would be more favorable. Although the dosage of the markers K19678_920R11 and K403_2848R11 is the same in both parental lines (i.e., dosage 1; Figs. 2a and 2c, respectively), P540 showed higher RWC_4h compared to the P867 (Fig. 1a). This can partly be explained by the absence of the marker K10792_5574R01 in the parent P540 leading to a higher RWC_4h for this line (Fig. 2b). This finding highlights, once more, the importance of considering these QTLs together in future breeding programs. However, direct use of the identified markers in MAS is not straightforward yet, since the applicability of the markers should first be validated in other germplasm. Based on the QTL analyses, it is not possible yet to propose candidate genes, first of all because of the large number of genes located in the QTL regions and second because of the lack of annotation of genes involved in stomatal functioning in rose and related species within the family Rosaceae (e.g. the diploid *Fragaria vesca* genome). The present work represents an initial step towards identifying the most promising regions in which genes of interest for stomatal malfunctioning might be located.

QTL implications on postharvest longevity

Reduced cut flower water loss during the postharvest phase is associated with an adequate stomatal response to closing stimuli (e.g., leaflet desiccation or darkness), and is a prerequisite for a long vase life (Fanourakis et al. 2012a). Low RWC_4h was indeed found to be a good proxy for limited vase life (Fig. 4). In this case, the vase life appears to be limited by the excessive water loss during the postharvest phase, owing to less responsive stomata. Contrary to this, high RWC_4h was not always related to long vase life (Fig. 4). This may be explained by the fact that although water loss is crucial for vase life, it is not the only determining factor (Fanourakis et al. 2013b). A vase life of at least 14 days is desirable for cut roses, considering seven days from production to selling and another seven days vase life at consumer level to ensure the so-called vase life guarantee label (van

Kooten and Kuiper 2009). For most of the ornamental plants and cut flowers, breeding programs are rather limited to morphological traits or disease resistance (Crespel et al. 2002; Dugo et al. 2005; Linde et al. 2006). Although the vase life of a new cultivar is always tested before it is released to the market, this is often done only in the final phase of the breeding program, despite the fact that a short vase life will lead to the rejection of a promising cultivar that has been selected for other reasons, e.g. a high productivity. Therefore, an early identification and elimination of offspring with a very poor vase life will increase the efficiency of the breeding programs (Fanourakis et al. 2012b).

In the present study, a dosage 0 or 1 from both markers K19678_920R11 and K403_2848R11 in combination with dosage 1 from marker K10792_5574R01 would point towards the lowest vase life, as a result of a poor stomatal functioning (i.e. low RWC_4h). This was confirmed for K148 and K161 (and partly for K039 and K121) (Table 2; Fig. 4). In a similar way, it was expected that offspring having dosage 2 from each of the former two markers in combination with a dosage 0 for the latter marker would show the longest vase life as a result of adequate stomatal functioning (i.e., high RWC_4h). This was confirmed on K015 and K099 (and partly on K107), but not for K225 and K119 (expected to have long vase life based on markers' scores) and K059 (expected to have low vase life based on markers' scores, despite the high RWC_4h) (Table 2; Fig. 4). A likely reason for the aforementioned exceptions is that the three QTLs identified in this study explain 32% of the variability, with a 68% of variability attributed to other (genetic or non-genetic) factors.

Conclusions

This is the first work identifying QTLs related to stomatal functioning following cultivation at high RH. Here, we found a very large phenotypic variability with the RWC_4h of the K5 tetraploid cut rose population varying between 7 and 62% across the 110 studied individuals from the K5 population. Three QTLs, two major and one putative minor, explaining 32% of the variability in the RWC_4h, were identified. The two major QTLs were mapped to ICM 5 (contribution of both parents) and to ICM 2 of parent P867; the putative minor QTL was mapped to ICM 6 (both parents). Although the QTLs' regions identified in the present study still need to be validated in other environments and mapping populations, the information achieved so far will contribute to further genetic analyses and future breeding programs. An early identification and elimination of offspring with a very poor potential vase life can increase the efficiency of breeding programs. At present, the rose genome is not yet fully sequenced, but this work represents an initial approach towards identifying the most promising regions in which genes of interest for stomatal malfunctioning might be located.

Acknowledgements

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References

Aliniaeifard S, van Meeteren U (2014) Natural variation in stomatal response to closing stimuli among *Arabidopsis thaliana* accessions after exposure to low VPD as a tool to recognize the mechanism of disturbed stomatal functioning. J Exp Bot 65:6529–6542. doi: 10.1093/jxb/eru370

Berninger E (1992) Le rosier de serre pour fleurs à couper. In: Gallais A BH (ed) Amélioration des espèces végétales cultivées, objectifs et critères de sélection. INRA Editions, Paris, pp 490 – 504

Bouchabke O, Chang F, Simon M, et al (2008) Natural variation in *Arabidopsis thaliana* as a tool for highlighting differential drought responses. PLoS One 3:e1705. doi: 10.1371/journal.pone.0001705

Brosché M, Merilo E, Mayer F, et al (2010) Natural variation in ozone sensitivity among *Arabidopsis thaliana* accessions and its relation to stomatal conductance. Plant, Cell Environ 33:914–925. doi: 10.1111/j.1365-3040.2010.02116.x

Buck GJ (1960) Progress report on breeding hardy ever-blooming roses. Am Rose Annu 45:95–99.

Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142:169–196. doi: 10.1007/s10681-005-1681-5

Crespel L, Chirollet M, Durel CE, et al (2002) Mapping of qualitative and quantitative phenotypic traits in *Rosa* using AFLP markers. Theor Appl Genet 105:1207–1214. doi: 10.1007/s00122-002-1102-2

Drake PL, Froend RH, Franks PJ (2013) Smaller, faster stomata: Scaling of stomatal size, rate of response, and stomatal conductance. J Exp Bot 64:495–505. doi: 10.1093/jxb/ers347

Dugo ML, Satovic Z, Millán T, et al (2005) Genetic mapping of QTLs controlling horticultural traits in diploid roses. Theor Appl Genet 111:511–520. doi: 10.1007/s00122-005-2042-4

Fanourakis D, Tapia a., Heuvelink E, Carvalho SMP (2009) Cultivar differences in the stomatal characteristics of cut roses grown at high relative humidity. Acta Hortic 847:251–258.

Fanourakis D, Carvalho SMP, Almeida DPF, Heuvelink E (2011) Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. Physiol Plant 142:274–286. doi: 10.1111/j.1399-3054.2011.01475.x

Fanourakis D, Carvalho SMP, Almeida DPF, et al (2012a) Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biol Technol 64:64–73. doi: 10.1016/j.postharvbio.2011.09.016

Fanourakis D, Carvalho DRA, Gitonga VW, et al (2012b) Breeding cut roses for better keeping quality : First steps. Acta Hortic. 937:875–882

Fanourakis D, Heuvelink E, Carvalho SMP (2013a) A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. J Plant Physiol 170:890–898. doi: 10.1016/j.jplph.2013.01.013

Fanourakis D, Pieruschka R, Savvides A, et al (2013b) Sources of vase life variation in cut roses: A review. Postharvest Biol Technol 78:1–15. doi: 10.1016/j.postharvbio.2012.12.001

Fanourakis D, Giday H, Milla R, et al (2015) Pore size regulates operating stomatal conductance, while stomatal densities drive the partitioning of conductance between leaf sides. Ann Bot 115:555–565. doi: 10.1093/aob/mcu247

Ghashghaie J, Brenckmann F, Saugier B (1992) Water relations and growth of rose plants cultured *in vitro* under various relative humidities. Plant Cell Tissue Organ Cult 30:51–57. doi: 10.1007/BF00040000

Giday H, Fanourakis D, Kjaer KH, et al (2013) Foliar abscisic acid content underlies genotypic variation in stomatal responsiveness after growth at high relative air humidity. Ann Bot 112:1857–1867. doi: 10.1093/aob/mct220

Giday H, Fanourakis D, Kjaer KH, et al (2014) Threshold response of stomatal closing ability to leaf abscisic acid concentration during growth. J Exp Bot 65:4361–4370. doi: 10.1093/jxb/eru216

Gudin S (1995) Rose improvement, a breeder’s experience. Acta Hortic 420:125–128.

Gudin S, Mouchotte J (1996) Integrated research in rose improvement - A breeder’s experience. Acta Hortic 424:285–292.

Jacob Y, Teyssier C, Reynders-Aloisi S, Brown S (1996) Use of flow cytometry for the rapid determination of ploidy level in the genus *Rosa*. Acta Hortic 424:273–278.

Koning-Boucoiran CFS, Gitonga VW, Yan Z, et al (2012) The mode of inheritance in tetraploid cut roses. Theor Appl Genet 125:591–607. doi: 10.1007/s00122-012-1855-1

Koning-Boucoiran CFS, Esselink GD, Vukosavljev M, et al (2015) Using RNA-Seq to assemble a rose transcriptome with more than 13,000 full-length expressed genes and to develop the WagRhSNP 68k Axiom SNP array for rose (*Rosa* L.). Front Plant Sci. doi: 10.3389/fpls.2015.00249

Linde M, Hattendorf a., Kaufmann H, Debener T (2006) Powdery mildew resistance in roses: QTL mapping in different environments using selective genotyping. Theor Appl Genet 113:1081–1092. doi: 10.1007/s00122-006-0367-2

Mortensen LM, Gislerød HR (1999) Influence of air humidity and lighting period on growth, vase life and water relations of 14 rose cultivars. Sci Hortic (Amsterdam) 82:289–298. doi: 10.1016/S0304-4238(99)00062-X

Rezaei Nejad A, van Meeteren U (2005) Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. Physiol Plant 125:324–332. doi: 10.1111/j.1399-3054.2005.00567.x

Ribaut J-M, Hoisington D (1998) Marker-assisted selection: new tools and strategies. Trends Plant Sci 3:236–239. doi: 10.1016/S1360-1385(98)01240-0

Sáez PL, Bravo LA, Latsague MI, et al (2012) Increased light intensity during *in vitro* culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels. Sci Hortic (Amsterdam) 138:7–16. doi: 10.1016/j.scienta.2012.02.005

Santamaria JM, Davies WJ, Atkinson CJ (1993) Stomata of micropropagated *Delphinium* plants respond to ABA, CO2, light and water potential, but fail to close fully. J Exp Bot 44:99–107.

Slavík B (1974) Methods of studying plant water relations. Chapman and Hall, London, UK

Spiller M, Linde M, Hibrand-Saint Oyant L, et al (2011) Towards a unified genetic map for diploid roses. Theor Appl Genet 122:489–500. doi: 10.1007/s00122-010-1463-x

Teulat B, Zoumarou-Wallis N, Rotter B, et al (2003) QTL for relative water content in field-grown barley and their stability across Mediterranean environments. Theor Appl Genet 108:181–188. doi: 10.1007/s00122-003-1417-7

Torre S, Fjeld T, Gislerod HR, Moe R (2003) Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. J Am Soc Hortic Sci 128:598–602.

Van Kooten O, Kuiper E (2009) Consumer acceptability in flower chains: How can we determine what the final customers really want? Acta Hortic 847:17–26.

Van Meeteren U, van Gelder A, van Ieperen W (2005) Effect of growth conditions on post harvest rehydration ability of cut chrysanthemum flowers. Acta Hortic 669:287–296.

Van Meeteren U, Van Gelder H, Van Ieperen W (1999) Reconsideration of the use of deionized water as vase water in postharvest experiments on cut flowers. Postharvest Biol Technol 17:175–187. doi: 10.1016/S0925-5214(99)00050-2

Van Ooijen J (2006) JoinMap, software for the calculation of genetic linkage maps, 4 edn. BVK, Wageningen, The Netherlands

VBN (2005) Evaluation cards for cut flowers. VBN, Leiden, The Netherlands

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78. doi: 10.1093/jhered/93.1.77

Voorrips RE, Gort G, Vosman B (2011) Genotype calling in tetraploid species from bi-allelic marker data using mixture models. BMC Bioinformatics 12:172. doi: 10.1186/1471-2105-12-172

Yan Z, Dolstra O, Prins TW, et al (2006) Assessment of partial resistance to powdery mildew (*Podosphaera pannosa*) in a tetraploid rose population using a spore-suspension inoculation method. Eur J Plant Pathol 114:301–308. doi: 10.1007/s10658-005-5995-x

Yu C, Luo L, Pan H, et al (2015) Filling gaps with construction of a genetic linkage map in tetraploid roses. Front Plant Sci 5:1–9. doi: 10.3389/fpls.2014.00796

Zhao XQ, Xu JL, Zhao M, et al (2008) QTLs affecting morph-physiological traits related to drought tolerance detected in overlapping introgression lines of rice (*Oryza sativa* L.). Plant Sci 174:618–625. doi: 10.1016/j.plantsci.2008.03.009

Ziv M, Schwartz A, Fleminger D (1987) Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophyllus*) plants propagated *in vitro*; Implications for hardening. Plant Sci 52:127–134. doi: 10.1016/0168-9452(87)90114-2

Appendix

Table A1 – Significant markers associated with stomatal responsiveness to water loss, mapped to the integrated consensus map (ICM) and respective homolog of the parents P540 and P867. Significance (*P*-value), $-^{10}\log$ (*P*-value) and percentage of phenotypic variability explained by each marker (% Expl) are shown. The two last numbers in the marker name refer to the segregation type where 01 = nulliplex × simplex and 11 = simplex × simplex (continues)

Marker name	ICM_homolog		*P*-value	$-^{10}\log$ (*P*-value)	% Expl
	P540	P867			
K19678_920R11	5_1	5_4	2.44E-08	7.61	27.5
G12826_1991R11	5_1	5_4	3.30E-07	6.48	24.9
K17337_1631R11	5_1	5_4	7.18E-07	6.14	23.8
G13746_429R11	5_1	5_4	8.16E-07	6.09	23.6
K14259_337R11	5_1	5_4	8.16E-07	6.09	23.6
G60359_1361R11	5_1	5_4	8.16E-07	6.09	23.6
G76971_171R11	5_1	5_4	8.16E-07	6.09	23.6
M5926_1121R11	5_1	5_4	8.16E-07	6.09	23.6
K379_1141R11	5_1	5_4	2.30E-06	5.64	22.4
K5269_1324R11	5_1	5_4	2.81E-06	5.55	21.7
K12380_179R11	5_1	5_4	2.82E-06	5.55	21.7
K3600_497R11	5_1	5_4	2.82E-06	5.55	21.7
K379_2105R11	5_1	5_4	2.82E-06	5.55	21.7
K379_418R11	5_1	5_4	2.82E-06	5.55	21.7
M9540_846R11	5_1	5_4	2.82E-06	5.55	21.7
K2112_1024R11	5_1	5_4	2.92E-06	5.54	21.6
K2112_793R11	5_1	5_4	2.92E-06	5.54	21.6
K3749_911R11	5_1	5_4	2.95E-06	5.53	21.6
K1948_135R11	5_1	5_4	3.00E-06	5.52	21.6
K1948_354R11	5_1	5_4	3.00E-06	5.52	21.6
G19068_681R11	5_1	5_4	6.56E-06	5.18	20.4
K15803_214R11	5_1	5_4	6.56E-06	5.18	20.4
K3946_743R11	5_1	5_4	6.56E-06	5.18	20.4
K7382_193R11	5_1	5_4	6.56E-06	5.18	20.4
M1663_1173R11	5_1	5_4	7.70E-06	5.11	20.3
K3567_887R11	5_1	5_4	8.71E-06	5.06	19.9
K3567_1496R11	5_1	5_4	8.75E-06	5.06	20.3
K14975_456R11	5_1	5_4	9.29E-06	5.03	20.2
K11531_279R11	5_1	5_4	2.42E-05	4.62	18.3
K4289_862R11	5_1	5_4	2.42E-05	4.62	18.3
K4289_420R11	5_1	5_4	2.52E-05	4.60	18.2
K6493_408R11	5_1	5_4	2.61E-05	4.58	18.3
K3240_1070R11	5_1	-	2.52E-04	3.60	14.4
K12386_669R11	5_1	-	3.36E-04	3.47	13.9

(Continuation) **Table A1**

Marker name	ICM_homolog		*P*-value	$-^{10}\log$ (*P*-value)	% Expl
	P540	P867			
M13641_208R11	5_1	-	3.51E-04	3.45	13.8
K292_539R11	5_1	-	3.84E-04	3.42	13.6
K10792_5574R01	-	2_3	4.21E-04	3.38	10.3
K403_2848R11	6_1	6_3	7.10E-04	3.15	11.2
D6495_2389R01	-	2_3	7.69E-04	3.11	9.3

CHAPTER 6

Gene expression and physiological responses associated to stomatal functioning in *Rosa* × *hybrida* grown at high relative air humidity

Abstract

High relative air humidity (RH ≥ 85%) during growth disturbs stomatal functioning, resulting in excessive water loss in conditions of high evaporative demand. Here, it was investigated the expression of nine abscisic acid (ABA)-related and two non-ABA related genes involved in the water stress response aiming to unravel the mechanisms underlying contrasting stomatal functioning in plants grown at high RH. Four rose genotypes with contrasting sensitivity to high RH [one sensitive (K023), one tolerant (K099) and two intermediate (P540 and P867)] were grown at moderate (62 ± 3%) or high (89 ± 4%) RH. The sensitive genotype grown at high RH showed a significantly higher stomatal conductance (g_s) and water loss in response to closing stimuli and an increased stomatal and pore size, as compared to the other genotypes. Moreover, high RH reduced the leaf ABA concentration and its metabolites to a greater extent in the sensitive genotype as compared to the tolerant one. Multiple genes were found to be involved in determining tolerance to high RH as in the tolerant genotype it was observed an increased expression in the ABA biosynthesis (*NCED1*, *AAO3*), conjugation (*UGT75B2*), mobilization (*BG2*) and signaling (*OST1*, *ABF3*) as well as in the antioxidant system (*Rh-APX*). In contrast, the ABA oxidation (*CYP707A1*, *CYP707A3*) and mobilization (*BG1*) had a minor contribution and *DREB1B* did not contribute to the tolerance of K099. These results show that several genes forming a highly complex gene network are involved in the tolerance mechanisms related to stomatal functioning in leaves grown at high RH.

Carvalho DRA, Vasconcelos MW, Lee S, Koning-Boucoiran CFS, Vreugdenhil D, Krens FA, Heuvelink E, Carvalho SMP (2015) Gene expression and physiological responses associated to stomatal functioning in *Rosa* × *hybrida* grown at high relative air humidity (Submitted)

Introduction

Environmental factors such as light, temperature, CO_2 concentration and relative air humidity (RH) affect stomatal opening and closure (Tallman, 2004). High relative air humidity (RH ≥ 85%) can often occur in horticultural production systems (e.g., protected cultivation during the winter period, or *in vitro* culture vessels), but also in natural environments such as tropical and subtropical areas or northern latitudes (Fanourakis *et al.*, 2009; Sáez *et al.*, 2012; Tullus *et al.*, 2012). At high RH, plants develop stomata that fail to close in response to closing stimuli (e.g., light-dark transition, desiccation or abscisic acid feeding), leading to excessive water loss following transfer to conditions of high evaporative demand (Fanourakis *et al.*, 2012; Giday *et al.*, 2013a; Carvalho *et al.*, 2015a). Nonetheless, poor stomatal functioning has been shown to be highly cultivar dependent (Fanourakis *et al.*, 2013; Giday *et al.*, 2013a), but the reasons of such genotypic differences remain elusive. Furthermore, the molecular and physiological mechanisms underlying contrasting stomatal functioning in plants grown at high RH are not yet understood (Fanourakis *et al.*, 2013; Aliniaeifard *et al.*, 2014).

Stomatal movements are mainly regulated by abscisic acid concentration ([ABA]), which is determined by its metabolism within the leaf (biosynthesis *vs.* catabolism) (Wilkinson and Davies, 2002). ABA increases in vegetative tissues subjected to stress, triggering adaptive responses essential to plant survival and productivity (Zeevaart and Creelman, 1988; Leung and Giraudat, 1998). Low [ABA] was measured in leaves of *Tradescantia virginiana* (Rezaei Nejad and van Meeteren, 2007), *Vicia faba* (Aliniaeifard *et al.*, 2014), *Arabidopsis thaliana* (Arve *et al.*, 2015) and *Rosa×hybrida* (Arve *et al.*, 2013; Giday *et al.*, 2013*a*, Carvalho *et al.*, 2015a) grown at high RH, compared to the ones grown at moderate RH. Many stress-related genes are induced by ABA leading to stomatal closure, but many other stress-related genes are not associated with ABA regulation (Kang *et al.*, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). ABA is synthesized from isopentenyl diphosphate followed by a cascade of reactions, with important reference being made to the conversion of violaxanthin to xanthoxin by the enzyme 9-cis epoxycarotenoid dioxygenase (NCED) (Schwartz *et al.*, 1997) and to the conversion of abscisic aldehyde to ABA by the enzyme abscisic-aldehyde oxidase (AAO) (Seo *et al.*, 2000). Recently, it was found that stomata respond to changes in VPD due to an extremely rapid *de novo* ABA biosynthesis mediated by the *NCED* gene (McAdam *et al.*, 2015). In tomato and strawberry, *NCED1* was described to be involved in the first step of ABA biosynthesis (Jia *et al.*, 2011; Sun *et al.*, 2012). *AAO3*, one of the four *Arabidopsis* aldehyde oxidase genes

(*AAO1-4*), is the most likely candidate to encode the enzyme that catalyzes the oxidation of abscisic aldehyde to ABA (Seo *et al.*, 2000). Concerning ABA catabolism, the hormone can be permanently inactivated via oxidation by ABA 8'-hydroxylase to form 8'-hydroxy ABA which spontaneously isomerizes to phaseic acid (PA) and is further reduced to dihydrophaseic acid (DPA) by phaseic acid reductase (Cutler and Krochko, 1999). In *Arabidopsis*, the genes *CYP707A1* and *CYP707A3* were identified as responsible for the oxidation of ABA to phaseic acid when plants were transferred from moderate to high RH (Okamoto *et al.*, 2009). The conjugation of ABA with monosaccharides (e.g. glucose) generates ABA-β-D-glucosyl ester (ABA-GE) which accumulates in vacuoles and is, therefore, hypothesized to be a storage form of ABA (Arve *et al.*, 2013). This process is mediated by the enzyme uridine diphosphate glucosyltransferase (UGT) and *UGT75B2* has been annotated with activity related to ABA-glucosyltransferase in *Arabidopsis* (Hong *et al.*, 2001). In roses, ABA conjugates with glucose during the day, forming ABA-GE, which is hydrolyzed by β-glucosidase to free ABA during the night, increasing the [ABA] and inducing stomatal closure (Arve *et al.*, 2013). In *Arabidopsis*, β-glucosidase homolog1 (*BG1*) and β-glucosidase homolog2 (*BG2*) are described to hydrolyze ABA-GE to ABA during dehydration (Lee *et al.*, 2006; Xu *et al.*, 2012). However, a recent study with three angiosperm species (*Solanum lycopersicon*, *Pisum sativum* and *Arabidopsis thaliana*) has shown that the stomatal closure and the rapid increase in foliar [ABA] could not be explained by the release of ABA from internal stores in the leaf or the hydrolysis of the conjugate ABA-GE as a significant expression of BG1 or BG2 was not found (McAdam *et al.*, 2015). Open stomata 1 (*OST1*) is a positive regulator of downstream ABA signaling (Merlot *et al.*, 2002; Mustilli *et al.*, 2002) and in *Arabidopsis* it is involved in the phosphorylation of the abscisic acid-responsive element-binding factor 3 (*ABF3*) (Sirichandra *et al.*, 2010). In *Arabidopsis*, the overexpression of *ABF3*, caused ABA hypersensitivity resulting in partial stomatal closure, reduced transpiration, and enhanced drought tolerance (Kang *et al.*, 2002).

DREBs (dehydration responsive element binding) are important plant transcription factors regulating the expression of many water stress-inducible genes, mostly in an non-ABA related manner, i.e., via an ABA-independent stomatal closing response (Yamaguchi-Shinozaki and Shinozaki, 2005; Lata and Prasad, 2011). For instance, in rose, it was found that *Rh-DREB1B* was induced by water deficit in petals of *Rosa* × *hybrida* 'Samantha' (Ting *et al.*, 2009). Ascorbate peroxidase (APX) is an antioxidant enzyme that scavenges active oxygen species (Ito *et al.*, 1999). It has been shown that, in cut roses, changing patterns of *Rh-APX1* expression were consistent with the changes of APX activity indicating that the improved tolerance to water stress induced by ascorbic acid pre-treatment may be related to the regulation of *Rh-APX1* at transcript level (Jin *et al.*, 2006). In transgenic plants from other species, is has also been shown that over-expression of *APX* can enhance tolerance to oxidative stress (Pitcher *et al.*, 1994; Webb and Allen, 1996; Wang *et al.*, 2005) and water deficit (Badawi *et al.*, 2004).

The present work aimed to better understand the physiological and molecular mechanisms behind the contrasting stomatal malfunctioning between genotypes grown at high RH. It is hypothesized that: (1) high RH reduces ABA biosynthesis to a greater extent in the sensitive genotypes as compared to the tolerant ones; (2) [ABA] correlates positively with the expression of genes associated with ABA biosynthesis and negatively with the expression of those associated with ABA catabolism and conjugation; (3) high RH reduces the expression of non-ABA related genes involved in the water stress response more in the sensitive genotypes compared to the tolerant ones. In this study, the concentrations of ABA and of its metabolites were determined and linked to the expression of ABA-related genes (i.e. genes involved in the ABA metabolism). The expression of non-ABA related genes involved in the water stress response was also evaluated. Roses were used in this study due to their great economic importance worldwide, because of the contrasting genotypic sensitivity to high RH during growth present within the available tetraploid population and based on genetic factors (Carvalho *et al.*, 2015b).

Materials and methods

Plant material and growth conditions

Four genotypes, two parents (P540 and P867) and two F1 offspring (K023 and K099), were selected from the segregating K5 tetraploid cut rose population (*Rosa* × *hybrida*; Carvalho *et al.*, 2015b). The two offspring were selected because of their contrasting stomatal responsiveness to leaflet desiccation following growth at high RH, with K023 being sensitive and K099 being tolerant to high RH (i.e., when plants are grown at high RH, sensitive genotypes show hampered stomatal closure in response to light-dark transition and leaflet desiccation while the tolerant ones maintain their closing ability) (Carvalho *et al.*, 2015b). Compared to these offspring, the two parents showed intermediate stomatal responsiveness, with the stomata from P540 responding better to leaflet desiccation than the ones from P867 (Carvalho *et al.*, 2015b). Rooted cuttings of the four genotypes were planted in 3 L pots containing a mixture (2/1, v/v) of black peat

(Jongkind B.V., Aalsmeer, The Netherlands) and perlite (Agra-perlite, Rhenen, The Netherlands). Plants were cultivated as single stem having one plant per pot, at a density of 40 plants m^{-2}. Fifty-six plants were randomly distributed over two growth cabinets (length × width × height = 146 × 77 × 126 cm; Biochim 1600 SP, Weiss Technik, Leicestershire, United Kingdom), both with a constant temperature of 20 ± 0.7 **°C.** One cabinet was maintained at 62 ± 3% RH and the other at 89 ± 4% RH, resulting in vapor pressure deficits (VPDs) of 0.89 ± 0.03 kPa (moderate RH) and 0.26 ± 0.06 kPa (high RH), respectively. Fluorescent tubes (TLD 58W/84, Philips, Eindhoven, The Netherlands) provided 20 h photoperiod of 310 ± 15 µmol m^{-2} s^{-1} photosynthetic active radiation **(**LI-1400, LI-COR, Lincoln, Nebraska, USA) measured 60 cm above the root-shoot intersection. Plants were watered daily with a standard nutrient solution (Fanourakis *et al.*, 2009).

Stomatal conductance and responsiveness to desiccation

Stomatal conductance (g_s) was measured with a porometer (AP4, Delta-T Devices, Cambridge, United Kingdom) in intact plants, 2 h after the beginning of the light and of the dark periods in the terminal leaflets of fully grown trifoliate leaves (i.e., tri-foliate immediately above the first penta-foliated leaf counting from the apex).

Stomatal responsiveness to desiccation was assessed in fully grown terminal leaflets (i.e., tri-foliate immediately above the first penta-foliated leaf counting from the apex; previous non-published results demonstrated that both tri- and penta-foliated leaves show a very similar stomatal responsiveness to desiccation) detached from fully grown plants (i.e., with a flower bud with cylindrical shape and pointed tip). Leaflet petioles were recut under water (to prevent cavitation induced-embolism) and placed in flasks filled with degassed water. Leaflets were further incubated in a saturated RH (≈ 100%) environment at 21.0 ± 0.5 ºC (i.e., VPD close to 0) for 1 h to establish their saturated fresh weight (Fanourakis *et al.*, 2011). The rehydration took place under fluorescent light (10 **±** **2** µmol m^{-2} s^{-1}) to induce stomatal opening of leaflets previously exposed to prolonged darkness (Drake *et al.*, 2013). After rehydration, the leaflets were allowed to desiccate for 4 h on a bench (abaxial surface down) in controlled conditions of evapotranspiration (21.5 ± 0.5 ºC, 47 ± 5% RH, 10 ± 2 µmol m^{-2} s^{-1}). Leaflet weight was gravimetrically recorded before (0 h) and after (4 h) desiccation (LP 3200D, Sartorius AG, Goettingen, Germany). After evaluation, leaflet dry weight was determined (24 h at 70 ºC) and relative water content after 4 h of leaflet desiccation (RWC_4h) was calculated according to Equation 1 (Slavík

1974). In both measurements (g_s and RWC_4h) one leaflet per plant was evaluated in seven plants per treatment.

$$\text{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{saturated fresh weight} - \text{dry weight}} \times 100 \qquad \text{(Eqn. 1)}$$

Stomatal anatomical characteristics

The effect of RH during growth on stomatal density (i.e., number per unit area), index (i.e., stomatal density per total epidermal cell density; Salisbury, 1927), stomatal and pore length (i.e., longest diameter), stomatal and pore width (i.e., shortest diameter) and pore area per leaf area [i.e., stomatal density × π × (pore length/2) × (pore width/2)] were determined. Stomatal traits were analyzed in one of the intact lateral leaflets from the fully grown tri-foliated leaf immediately above the first penta-foliated counting from the apex, 150 min after the onset of the light period. Pore aperture and pore area per leaf area were also evaluated 150 min after the beginning of the dark period. Epidermal imprints were made by Suzuki's Universal Micro-Printing (SUMP) method using SUMP liquid 1 and SUMP plate B (SUMP Laboratory, Tokyo, Japan) as described by Tanaka *et al.* (2005). Samples were taken from the abaxial side of the leaflets, midway between the tip and the base, away from the edge and avoiding veins (Fanourakis *et al.*, 2015). A light microscope (Eclipse 55i, Nikon, Tokyo, Japan) connected to a digital camera (DS-Fi1, Nikon, Tokyo, Japan) was used to record the images. Stomatal and epidermal cell densities were assessed with 100× magnification, while for stomatal and pore dimensions a magnification of 400× was used. Image analysis was performed using Digimizer version 4.3.4.0 (MedCalc software, Ostend, Belgium). For stomatal and epidermal cell counting, 35 images (five images per leaflet, one leaflet per plant) were analyzed per treatment. Regarding stomatal and pore dimensions, 70 stomata (ten stomata per leaflet, one leaflet per plant) were evaluated per treatment.

Endogenous ABA quantification

ABA analysis was conducted in penta-foliated leaves, i.e., fifth and sixth counting from the apex, sampled 2 h after the beginning of the light and of the dark periods, respectively. Leaves were immediately frozen in liquid nitrogen and stored at -80 ˚C. Frozen samples were grinded in liquid nitrogen using mortar and pestle and freeze dried before analysis. Three biological replicates per treatment were analyzed.

Extraction and purification of ABA metabolites

Samples were prepared according to Saika *et al.* (2007), with modifications. Approximately 5 mg of lyophilized tissue (powder) was extracted with 2 ml of acetone:water (80:20, v/v) in the presence of antioxidant 2,6-di-tert-butyl-4-methylphenol (0.1 mg ml^{-1}) and 100 pmol of deuterium-labelled internal standards as each counterpart of ABA, ABA-GE, PA and DPA. After centrifugation (15 min, 3000 rpm, 4℃) the supernatant was collected and the pellet was re-extracted with 2 ml of the extraction solvent as previously described and subsequently centrifuged. Both supernatants were pooled and evaporated to dryness under vacuum. The extracts were suspended in 1 ml of isopropanol:formic acid (99:1, v/v, pH 3.3) and dried under vacuum. Reconstitutes in 1 ml of 10% (v/v) methanol containing 0.1% (v/v) formic acid (pH 2.6) were partitioned twice with 1 ml of n-hexane each time. Dried samples were dissolved in 1 ml of 10% methanol containing 0.1% formic acid (pH 2.6) and purified using Oasis-HLB cartridge (150 mg/6 cc, Waters). The sorbent was pre-conditioned with 3 ml of methanol and equilibrated with 3 ml of methanol containing 0.1% formic acid. After loading the sample, the column was washed and ABA was eluted with two series of eluents: 1 ml of acetonitrile:water:formic acid (50.0 : 49.9 : 0.1, v/v/v) and 2.5 ml of acetonitrile:water:formic acid (90.0 : 9.9 : 0.1, v/v/v), respectively. Both eluates were combined and dried under vacuum.

Quantitative analysis of ABA metabolites

Quantification of ABA metabolites was performed by ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS-MS; Xevo™ TQ-S, Waters). Purified samples were re-dissolved in 200 µl of mobile phase, filtered with 0.45 µm PTFE membrane filter (Phenomenex) and separated on Acquity UPLC CSH C18 column (100 x 2.1 mm, 1.7 µm; Waters). Analytes were eluted using a binary gradient consisting of 15 mM formic acid in water (A) and acetonitrile (B), for 7 min at a flow rate of 0.7 ml min^{-1} and constant column temperature of 45°C. The linear gradient elution was performed as follows: 30 s, 15% eluent B; 3 min, 60% eluent B; 1 min, 80% eluent B; 75 s, 100% eluent B. At the end, the column was equilibrated to initial conditions for 75 s. The effluent was introduced into the electrospray ion source of the mass spectrometer with optimized operating parameters: capillary voltage 3 kV, cone voltage 25 V, source / desolvation temperature 150 °C / 600 °C, cone / desolvation gas flow 150 / 600 L h^{-1} and collision energy 10 V. Compounds were quantified in negative mode as $[M-H]^-$ and multiple reaction monitoring transitions (MRM: MS1 > MS2) were: ABA (263.2 > 153.1), [$^2H_6$]-ABA (269.2 > 159.1); ABA-GE (425.0 > 262.7), [$^2H_5$]-ABA-GE (430.0 > 267.7); PA (279.0 > 138.9), [$^2H_3$]-PA (282.0 > 142.2); DPA (281.0 >171.2), [$^2H_3$]-DPA (284.0 > 174.0). Data were processed by TargetLynx^{TM} Software, version 4.1 (Waters).

RNA extraction and cDNA synthesis

Fully grown penta-foliated leaves (first to fourth counting from the apex) were sampled 10 and 180 min after the beginning of the light (10_L and 180_L, respectively) and of the dark (10_D and 180_D, respectively) periods to evaluate the effects of the light-dark transition on stomatal closure and consequently on the expression of genes of interest. Leaflets without petioles were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Two biological replicates were grinded and homogenized in liquid nitrogen using mortar and pestle. Each replicate resulted from a pool of two leaves, i.e., first and third leaves (counting from the apex) each one from two different plants were pooled together as well as the second and fourth leaves, each one from two additional plants. The RNA was isolated using a modified CTAB method (Chang *et al.*, 1993). In short, 1.0 to 1.5 g of grinded leaves was added to 7.5 ml extraction buffer, with further volumes proportionally adjusted. The RNA quantity and quality were checked using a nanodrop (NanoDrop 1000 Spectrophotometer, Thermo Scientific, Wilmington, Delaware, USA) and running a 1.5% (w/v) agarose electrophoresis gel, respectively. The RNA was then purified with DNase I kit (Invitrogen, Life Technologies, Carlsbad, USA) to eliminate eventual contamination by genomic DNA and its integrity was confirmed again running a 1.5% (w/v) agarose electrophoresis gel. One microgram of RNA was converted to cDNA using iScript cDNA Synthesis kit containing a blend of oligo(dT) and random hexamer primers (Bio-Rad Laboratories, Inc., Hercules, USA).

Quantitative real-time PCR (qRT-PCR) and data analysis

Eleven genes of interest, nine ABA-related (*NCED1*, *AAO3*, *CYP707A1*, *CYP707A3*, *UGT75B2*, *BG1*, *BG2*, *OST1*, *ABF3*) and two non-ABA related (*DREB1B*, *Rh-APX*) involved in the water stress response were selected from the literature, taking into account their possible involvement in the trait as described above in the introduction. The sequences of the genes of interest were blasted against an existing EST database derived from both parents of the K5 tetraploid cut rose population obtained with Illumina platform (Koning-Boucoiran *et al.*, 2015). Amplified fragments from selected primers pairs for each gene were isolated, sequenced and blasted against the NCBI database for confirmation. Primers were designed using DNASTAR (DNASTAR, Madison, USA) and Primer3Plus (Untergasser *et al.*, 2007) and were produced by Biolegio BV (Biolegio BV, Nijmegen, The Netherlands; Table A1, Appendix). *Actin* and *Ubiquitin* were used as reference genes (Meng *et al.*, 2013; Table A1, Appendix). The quantitative real-time PCR (qRT-PCR) analysis was performed in a thermocycler (CFX96^{TM} Real-Time PCR, Bio-Rad Laboratories, Inc., Hercules, USA). The qRT-PCR reactions occurred in a total volume of 10 µl containing: 5 µl of SYBR Green PCR Mix, 2 µl cDNA (diluted 10 times), 0.3 µl of each primer and 2.4 µl of ultra-pure water. The amplification conditions were: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, finalizing with 95 °C for 10 s and 65 °C for 5 s for melting curve construction. Two biological and two technical replicates were analyzed per gene. Firstly, ΔCt was calculated by: $\Delta Ct = (Ct_{\text{interest gene}} - Ct_{\textbf{average of the two reference genes}})$. Thereafter, the relative gene expression (RGE) was calculated by: $RGE = 2^{-\Delta Ct}$ with the subsequent transformation: Final value = Log_2 (RGE x 100000) (Wahyuni *et al.*, 2014). To construct the heat map, MeV (Multiexperiment Viewer, version 4.9) from TM4 was utilized (Saeed *et al.*, 2003).

Statistical design and analysis

The experimental set-up was a complete randomized design. Analysis of variance was conducted, considering individual plants as experimental units. Main effects and interactions were tested at $P = 0.05$. When relevant, Fisher’s least significant difference (LSD) at $P = 0.05$ was calculated to separate interaction means. The Genstat software (16th edition; VSN International Ltd., Herts, United Kingdom) was used for the analysis.

Results

Stomatal physiological and anatomical parameters

In general, intact plants grown at high RH showed a higher g_s as compared to plants grown at moderate RH, both during the light and the dark periods (Figs. 1A and 1B). Moreover, this effect is more pronounced in the sensitive (K023) and intermediate (P867 and P540) genotypes. For instance, in K023 grown at high RH the g_s increased 5.4-fold in light and 5.6-fold in darkness as compared to K023 grown at moderate RH, whereas no significant differences were found in the tolerant (K099) genotype (Figs. 1A and 1B). When analyzing the dark-induced stomatal closure (Fig. 1C) and the stomatal responsiveness to leaflet desiccation (Fig. 2) in plants grown at high RH it was also found that the tolerant genotype had the highest stomatal responsiveness to these closing stimuli as compared to the remaining genotypes.

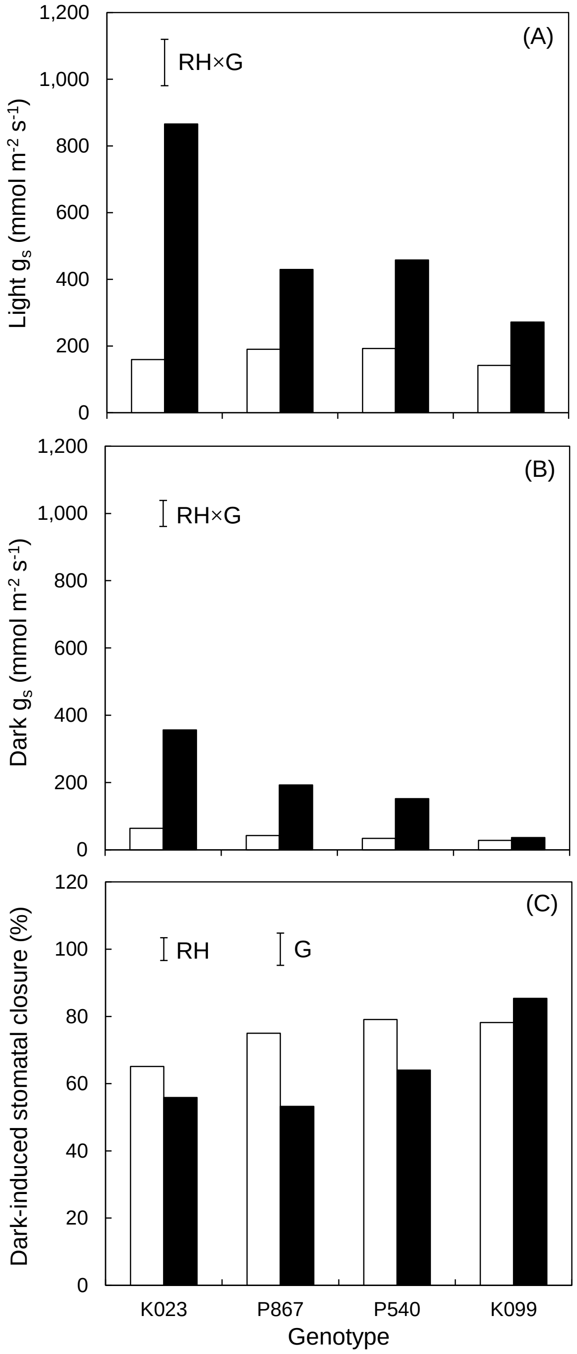

Fig. 1. Stomatal conductance (g_s) in intact plants, 2 h after the beginning of the light (A) and of the dark (B) periods and percentage of dark-induced stomatal closure (C) in four rose genotypes (G; K023, P867, P540 and K099) grown at moderate (62%; open columns) or high (89%; solid columns) relative air humidity (RH). Values are the mean of seven intact plants and bars represent Fisher's LSD ($P = 0.05$) for the interaction 'RH × G' (A and B) and the independent effects when the interaction was not statistically significant (C; $P = 0.058$)

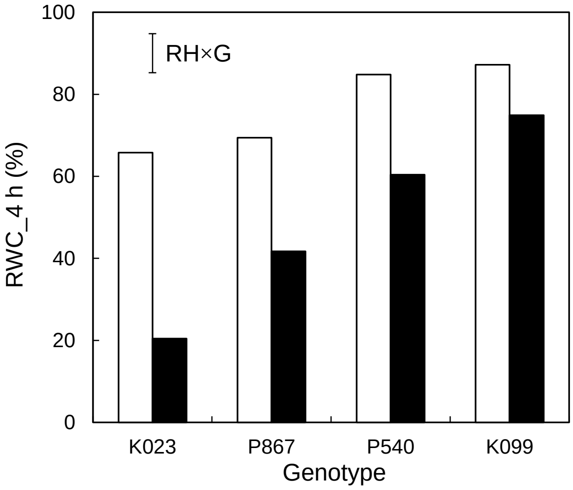

Fig. 2. Relative water content after 4h of leaflet desiccation (RWC_4h) in four genotypes (K023, P867, P540 and K099) grown at moderate (62%; open columns) or high (89%; solid columns) relative air humidity. Values are the mean of seven leaflets and bars represent Fisher's LSD (*P* = 0.05) for the interaction 'RH × G'

An interaction between RH and genotype was observed in the majority of the evaluated stomatal parameters, apart from stomatal length and width (Table 1). Concerning those parameters, K099 presented the highest stomatal length and P867 the largest width, irrespective of the RH (Table 1) and high RH increased stomatal length (11%) and width (2%), irrespective of the genotype (data not shown). The generalized interaction in the stomatal parameters reflects a non-systematic effect of the RH which was dependent on the genotype (Table 1). Though, an exception was found in the dark period where high RH consistently increased the pore aperture and the pore area per leaf area in all genotypes, although to a lower extent in the tolerant one (K099) compared to the other genotypes (Table 1).

Table 1. Stomatal characteristics from genotypes K023, P867, P540 and K099 grown at moderate (62%) or high (89%) relative air humidity (RH). Sampling took place 150 min after the onset of the light and of the dark periods. Values are the mean of 35 fields of view (stomatal density and index) and 70 stomata (stomatal and pore dimensions). Different letters represent significant differences according to Fisher's LSD test (P = 0.05; significant F probabilities are presented in bold; comparison in columns)

Genotype	RH	Stomatal					Light period		Dark period	
		Density (no mm^{-2})	Index (%)	Length (µm)	Width (µm)	Pore length (µm)	Pore aperture (µm)	Pore area per leaf area (‰)	Pore aperture (µm)	Pore area per leaf area (‰)
K023	62%	70.7^{c}	17.0^{bc}	25.0	17.9	15.8^{a}	4.34^{cde}	3.80^{b}	2.37^{c}	2.25^{b}
	89%	69.3^{c}	18.2^{d}	28.0	18.6	17.3^{c}	4.60^{ef}	4.36^{c}	3.72^{e}	3.78^{d}
	Mean	70.0	17.6	26.5^{a}	18.2^{b}	16.5	4.47	4.08	3.05	3.01
P867	62%	76.6^{d}	17.0^{bc}	25.3	19.0	15.7^{a}	4.76^{f}	4.54^{c}	2.29^{bc}	2.14^{b}
	89%	59.9^{a}	15.9^{ab}	28.0	19.2	17.8^{c}	4.52^{def}	3.81^{b}	3.71^{e}	3.24^{c}
	Mean	68.3	16.4	26.6^{a}	19.1^{c}	16.7	4.64	4.18	3.00	2.69
P540	62%	59.9^{a}	16.4^{b}	25.7	17.0	15.6^{a}	3.40^{a}	2.52^{a}	1.90^{ab}	1.48^{a}
	89%	75.1^{d}	18.1^{cd}	28.6	17.7	18.7^{d}	4.17^{cd}	4.66^{c}	3.24^{d}	3.72^{d}
	Mean	67.5	17.3	27.2^{b}	17.4^{a}	17.1	3.79	3.59	2.57	2.60
K099	62%	64.8^{b}	15.0^{a}	26.7	17.9	16.4^{b}	4.09^{bc}	3.47^{b}	1.60^{a}	1.48^{a}
	89%	62.2^{ab}	16.1^{ab}	28.8	18.0	18.8^{d}	3.73^{ab}	3.46^{b}	2.11^{bc}	1.98^{b}
	Mean	63.5	15.6	27.8^{c}	17.9^{b}	17.6	3.91	3.47	1.86	1.73
	F pr.									
	RH	0.185	0.012	**< 0.001**	**0.008**	< 0.001	0.277	< 0.001	< 0.001	< 0.001
	G	< 0.001	< 0.001	**< 0.001**	**< 0.001**	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	RH × G	**< 0.001**	**0.004**	0.192	0.381	**0.005**	**< 0.001**	**< 0.001**	**0.005**	**< 0.001**

Endogenous ABA quantification

ABA was quantified in leaves harvested 2 h after the beginning of the light and 2 h after the beginning of the dark periods. However, no significant effect of the sampling time was observed for any of the metabolites (ABA, $P = 0.115$; PA, $P = 0.416$; DPA, $P = 0.352$; ABA-GE, $P = 0.508$; ABA + metabolites, $P = 0.465$; see caption of Fig. 3). High RH decreased the levels of all the determined ABA metabolites (Fig. 3). Except for PA, the strongest effect was observed in the sensitive genotype K023, while the reduction in the other genotypes was lower and often not significant (Figs. 3A, 3C, 3D and 3E). In contrast, [PA] was higher in the tolerant genotypes (P540 and K099) compared to the sensitive ones (K023 and P867) (Fig. 3B).

Gene expression analysis

The RGE of the nine ABA-related and the two non-ABA related genes are represented on a heat-map (Fig. 4), with the RGE values being displayed in Table A2 (Appendix). There was no clear expression pattern that discriminated between genotypes with contrasting tolerance to high RH (Fig. 4), but significant differences in individual genes were found that might contribute to the understanding of the stomatal functioning and thus these differences must be carefully analyzed (Table 2).

During the light period most of the effects on gene expression occurred at 180_L (180 min after the beginning of the light period) (Table 2). Looking in more detail at this time point and at the genes involved in ABA biosynthesis *NCED1* was significantly affected by both the RH and the genotype (Table 2), being down-regulated in P867 compared to the other genotypes and down-regulated by high RH (Fig. 4); *AAO3* was up-regulated by high RH in K099 while it was not affected in the other genotypes (Table 2; Fig. 4). Regarding the genes involved in ABA oxidation, *CYP707A1* was up-regulated in P867, irrespective the RH (Table 2; Fig. 4) and *CYP707A3* was down-regulated by high RH in P867, whereas it was slightly up-regulated in K099 (yet not significant) (Table 2; Fig. 4). Considering the conjugation of ABA with glucose, *UGT75B2* was up-regulated by high RH in K099 while it was not significantly affected in the other genotypes (Table 2; Fig. 4). In addition, *BG1* was more down-regulated in K099, irrespective the RH, compared with the other genotypes and *BG2* was more down-regulated by high RH in K023, P867 and P540 than in K099, yet this interaction was not significant (Table 2; Fig. 4). *OST1* was down-regulated by high RH in P867 and *ABF3* was up-regulated by high RH in K099, while they were not significantly affected in the other genotypes (Table 2; Fig. 4).

Furthermore, at 10_L (10 min after the beginning of the light period) only the expressions of *BG1* and *BG2* were affected (Table 2). *BG1* and *BG2* were more down-regulated in K099 and in P540, respectively, compared to the other genotypes (Table 2; Fig. 4).

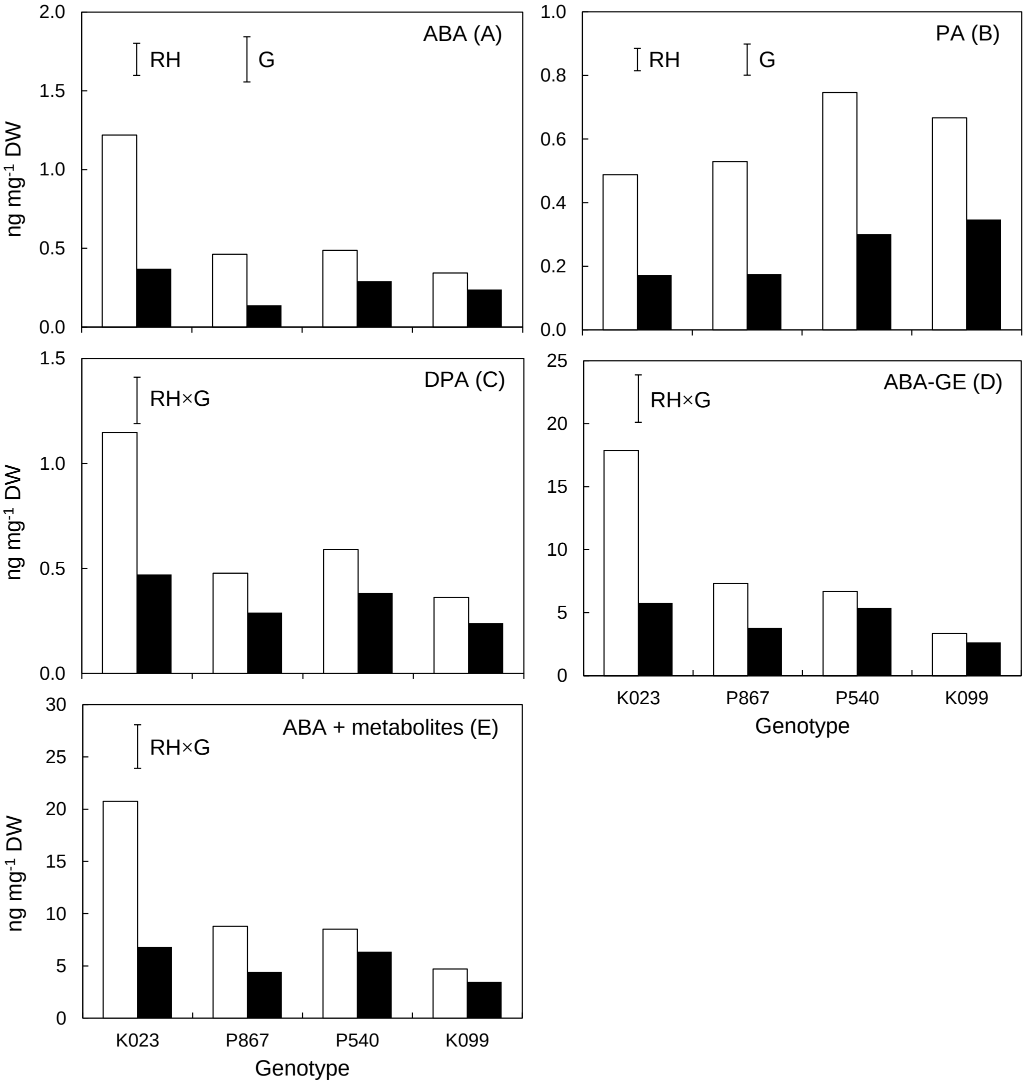

Fig. 3. Concentration (ng mg^{-1} DW) of abscisic acid (ABA; A), phaseic acid (PA; B), 4'-dihydrophaseic acid (DPA; C), ABA-β-D-glucosyl ester (ABA-GE; D) and total amount of ABA and its metabolites (E) measured in leaves of four rose genotypes (G; K023, P867, P540 and K099) grown at moderate (62%; open columns) or high (89%; solid columns) relative air humidity (RH). Values are the mean of six leaves and bars represent Fisher's LSD (P = 0.05) for the interaction 'RH × G' (C, D and E) and the independent effects when the interaction was not statistically significant (A; P = 0.058 and B; P = 0.514). The results are the average of sampling 2 h after the beginning of the light and 2 h after the beginning of the dark periods, as no significant effect of the sampling time was observed for any of the metabolites (ABA, P = 0.115; PA, P = 0.416; DPA, P = 0.352; ABA-GE, P = 0.508; ABA + metabolites, P = 0.465)

Table 2. Statistical analysis (P-values) from the relative expression of ABA-related (*NCED1*, *AAO3*, *CYP707A1*, *CYP707A3*, *UGT75B2*, *BG1*, *BG2*, *OST1*, *ABF3*) and non-ABA related (*DREB1B*, *Rh-APX*) genes in fully expanded leaflets from four genotypes (K023, P867, P540 and K099) cultivated at moderate (62%) or high (89%) relative air humidity (RH). Samples were collected 10 and 180 min after the beginning of the light (10_L and 180_L) and of the dark (10_D and 180_D) periods

Gene	Sampling time			
	10_L	180_L	10_D	180_D
NCED1	n.s.	$P = 0.034$[1] $P = 0.002$[2]	$P = 0.028$[3]	$P = 0.024$[2]
AAO3	n.s.	$P = 0.034$[3]	n.s.	n.s.
CYP707A1	n.s.	$P = 0.010$[2]	n.s.	n.s
CYP707A3	n.s.	$P = 0.021$[3]	$P = 0.014$[2]	$P = 0.002$[2]
UGT75B2	n.s.	$P = 0.039$[3]	n.s.	$P = 0.022$[3]
BG1	$P < 0.001$[2]	$P = 0.042$[2]	n.s.	n.s.
BG2	$P = 0.049$[2]	$P = 0.002$[1]	$P = 0.012$[2]	$P = 0.043$[2]
OST1	n.s.	$P = 0.045$[3]	$P = 0.040$[1]	$P = 0.037$[3]
ABF3	n.s.	$P = 0.009$[3]	$P = 0.041$[3]	$P = 0.010$[1]
DREB1B	n.s.	n.s.	n.s.	n.s.
Rh-APX1	$P = 0.011$[3]	$P = 0.015$[3]	n.s.	n.s.

n.s., not significant (i.e., $P \geq 0.05$);

[1] Independent effect of the RH;

[2] Independent effect of the genotype;

[3] Effect of the interaction 'RH × genotype'.

Considering the ABA-related genes during the dark period, *NCED1* was up-regulated by high RH in K099 whereas it was down-regulated or not affected in the other genotypes (at 10_D, i.e. 10 min after the beginning of the dark period; Table 2; Fig. 4). In addition, the same gene was up-regulated in K023 and down-regulated in K099, irrespective the RH (180_D, i.e., 180 min after the beginning of the dark period; Table 2; Fig. 4). At 10_D and 180_D, *CYP707A3* was up-regulated in K023 and down-regulated in K099, irrespective the RH (Table 2; Fig. 4). *UGT75B2*, showed the same expression pattern in 180_D as described above for 180_L, i.e., it was up-regulated by high RH in K099 while it was not significantly affected in the other genotypes (Table 2; Fig. 4). In both dark time points, *BG2* was down-regulated in P540 compared to the other genotypes, irrespective the RH (Table 2; Fig. 4). In general, *OST1* and *ABF3* were down-regulated by high RH in both dark time points in all the genotypes but not in K099 (Table 2; Fig. 4).

Regarding the non-ABA related genes, *DREB1B* was only dependent on the time, i.e., it was up-regulated at 180_D being gradually down-regulated in the course of the light period reaching the minimal expression level at 10_D (Fig. 4). *Rh-APX* was the highest expressed gene (Fig. 4). In general, under light *Rh-APX* was up-regulated by high RH in the tolerant genotype K099 while it was down-regulated or not affected in the other genotypes (Table 2; Fig. 4).

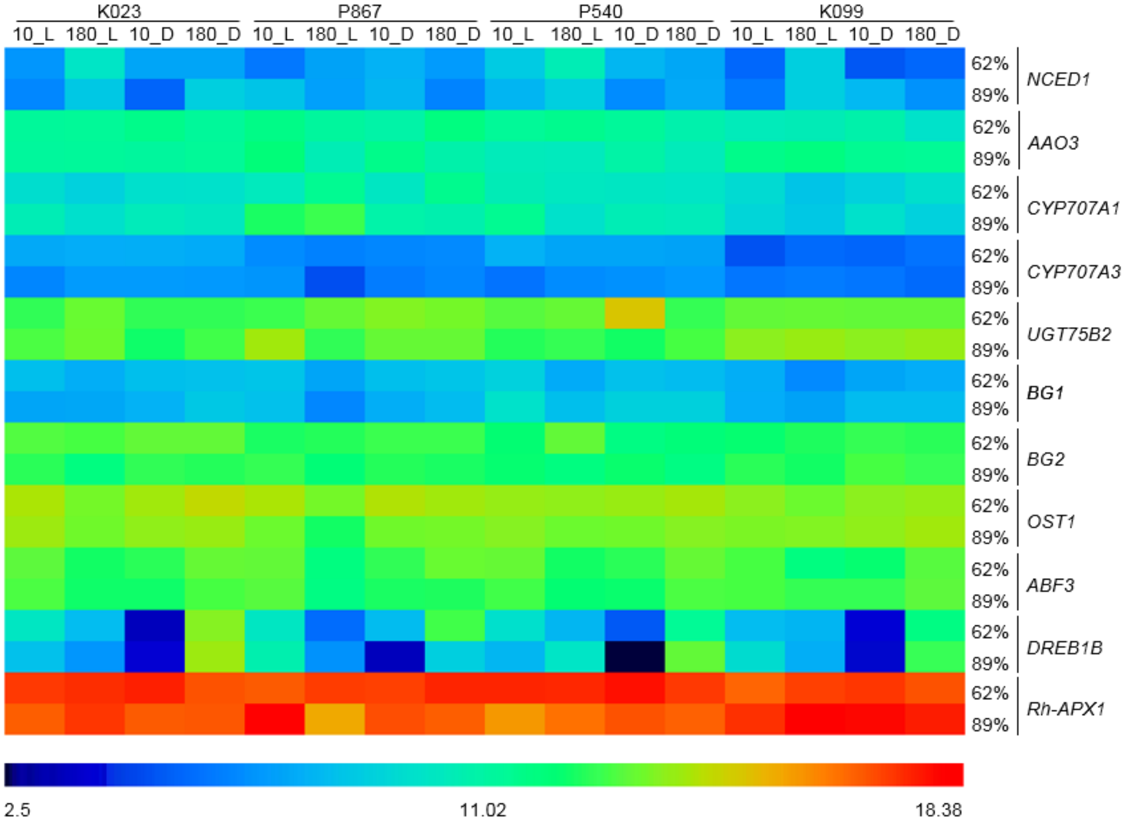

Fig. 4. Relative gene expression (RGE) [i.e., Log_2 (RGE × 100000)] of the ABA-related genes (*NCED1*, *AAO3*, *CYP707A1*, *CYP707A3*, *UGT75B2*, *BG1*, *BG2*, *OST1*, *ABF3*) and of the non-ABA related genes involved in the water stress response (*DREB1B*, *Rh-APX1*) evaluated in fully expanded leaves from four genotypes (K023, P867, P540 and K099) cultivated at moderate (62%) or high (89%) relative air humidity. Samples were collected 10 and 180 min after the beginning of the light (10_L and 180_L) and of the dark (10_D and 180_D) periods

Discussion

The genotypes evaluated in this study were selected from the segregating K5 tetraploid cut rose population because of their contrasting stomatal responsiveness to leaflet desiccation following growth at high RH (Carvalho *et al.*, 2015b). Our results confirmed K023 as a sensitive genotype to high RH, K099 as tolerant, and P867 and P540 showed intermediate tolerance levels (Figs. 1 and 2). Genotype K023 showed poor stomatal functioning in plants grown at high RH evidenced by the low responsiveness to light-dark transition (Fig. 1C) alongside with a strong decrease of the RWC_4h following leaflet desiccation (Fig. 2). This is in agreement with Arve *et al.* (2013) who found 85% dark-induced stomatal closure in plants grown at moderate RH compared to only 29% in high RH-grown plants. Additionally, the large pore area per leaf area measured in high RH-grown plants contributed to their higher g_s compared to moderate RH-grown plants in darkness as well as in light (Table 1; Figs 1a and 1b). Stomatal anatomical parameters (i.e., density, index and size) did not correlate to RH and genotype in a consistent manner (Table 1) suggesting that although stomatal anatomy contributes *per se* to an increased water loss these parameters are not decisive in this process as long as stomata are able to function properly, i.e., to close avoiding plant's dehydration (Fanourakis *et al.*, 2013; Giday *et al.*, 2013*b*; Aliniaeifard *et al.*, 2014). Furthermore, in rose, an increase in the leaf transpiration rate points to a higher stomatal pore area per leaf area, since the cuticle has only a minor contribution to the total leaf water loss (Fanourakis *et al.*, 2013).

Several studies suggest that long-term low leaf [ABA] is responsible for the low response of stomata grown at high RH (Rezaei Nejad and van Meeteren, 2007; Fanourakis *et al.*, 2011; Arve *et al.*, 2013). This work evaluates the expression of a broad range of ABA-related and non-ABA related genes involved in the water stress response, in response to a closing stimulus (i.e., light-dark transition) in rose genotypes with contrasting tolerance to high RH. It was expected that [ABA] would correlate positively with the expression of genes associated with ABA biosynthesis and negatively with the expression of those associated with ABA catabolism and conjugation, being this effect dependent on the genotype and the time of the day. Indeed, the studied genes were differently expressed between light and dark periods (Table 2), but the concentrations of ABA and of its metabolites were not significantly affected by the time of the day (see *P*-values in the caption of Fig. 3). Similarly, in *Arabidopsis* the concentrations of ABA and of its metabolites were not significantly different between light and dark periods (Arve *et al.*, 2015), while in rose cultivar Rebecca grown at moderate RH a higher [ABA] and a lower

[ABA-GE] were quantified in darkness compared to light period (Arve *et al.*, 2013). It is suggested that the [ABA] required to produce functional stomata depends not only on the species but also on the cultivar within a species (Arve *et al.*, 2015). Aiming to close the stomata in response to light-dark transition, the relative expression levels of *NCED1* indicated that the tolerant genotype K099 grown at high RH increased the ABA biosynthesis within the initial 10 min of the dark period, while the sensitive genotype K023 required a longer time (180 min) (Fig. 4). In light, the up-regulation of *AAO3* in K099 grown at high RH (Fig. 4) also suggested an increased ABA biosynthesis in tolerant genotypes leading to enhanced stomatal closure. Nevertheless, the expression of these two genes did not correlate with the [ABA] since the sensitive genotype (K023) showed the highest [ABA] compared to K099 (Fig. 3A). It seems that [ABA] in the leaves is not a limiting factor regarding stomatal tolerance to high RH since in spite of the high [ABA] in K023, this genotype is still not responsive to closing stimuli such as darkness (Fig. 1) or desiccation (Fig. 2), which can be due to a reduced stomatal sensitivity to ABA rather than to the [ABA] itself. Regarding the ABA metabolism, Okamoto *et al.* (2009) found that *CYP707A1* and *CYP707A3* were up-regulated in *Arabidopsis* transferred to high RH. This was confirmed by Arve *et al.* (2015) in darkness, whereas in light the high RH down-regulated *CYP707A1* and did not affect *CYP707A3*. Our results were also not affected by high RH in darkness (Table 2). Moreover, still in darkness, *CYP707A3* was up-regulated in K023 and down-regulated in K099 (Table 2; Fig. 4) which might contribute to the higher ABA catabolism via oxidation in the sensitive genotype compared to the tolerant one. Nevertheless, it was found higher [PA] in the tolerant genotypes compared to the sensitive ones (Fig. 3B) which does not hold for [DPA] (Fig. 3C). It can be hypothesized that the degradation to PA and later to DPA can also be affected by the RH and be genotype-dependent explaining the measured concentrations. Furthermore, in roses, it has been suggested that conjugation of ABA with glucose forming ABA-GE, and its subsequent release to ABA, is a more relevant process for a proper stomatal functioning, compared to the oxidation of ABA to PA and DPA, which was found to play a more important role in *Arabidopsis* (Arve *et al.*, 2013, 2015). This indicates that these two species might have a different ABA regulation mechanism in response to light-dark transition and RH. In K099, high RH similarly up-regulated *UGT75B2* in light and darkness (Table 2; Fig. 4), which may reflect the increased capacity of the tolerant genotype to cope with high RH by increasing the conversion of ABA into ABA-GE, as this has been hypothesized to be a storage form of ABA (Cutler and Krochko, 1999). However, [ABA-GE] was higher in the sensitive genotype compared to the tolerant one (Fig. 3 D). The advantage of storing ABA as ABA-GE is that this can be converted back to ABA when needed, but only if the enzyme β-glucosidase is available to act in this process. Arve *et al.* (2013) found a reduced β-glucosidase activity in high RH-grown plants. In our study, the evidence that in darkness high RH did not reduce the *BG2* activity in K099, while it did reduce in the other genotypes, may further contribute to the tolerance of this genotype to high RH. The higher down-regulation of *BG1* in K099 compared to the other genotypes may suggest that this gene does not play a role in the tolerance of this genotype to high RH, with *BG2* being of higher importance in this process. *OST1* is a positive regulator of downstream ABA signalling belonging to the family SnRK2 (Merlot *et al.*, 2002; Mustilli *et al.*, 2002). In the presence of ABA, the plant hormone binds to its receptor (PYR/PYL/RCAR) blocking the activity of PP2Cs and consequently allowing SnRK2/OST1 activation. In the absence of ABA, the PP2C inactivates SnRK2/OST1 resulting in a repression of downstream ABA signalling (Geiger *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009; Fujii *et al.*, 2009). Moreover, it has been suggested that *OST1* phosphorylates *ABF3* in *A. thaliana* meaning that the long term responses to ABA that require sustained gene expression is, in part, mediated by the stabilization of ABFs driven by ABA-activated SnRK2s (Sirichandra *et al.*, 2010). In this study, it is suggested that the maintenance of the ABA-downstream cascade via *OST1* and *ABF3* contributes to the tolerance to high RH in K099, since in darkness the expression of these two genes was not affected by high RH in K099 while it was reduced in the other genotypes (Fig. 4).

Hydraulic signalling rather than only chemical signalling may also play a role in stomatal functioning (Schachtman and Goodger, 2008) and non-ABA related genes can be activated. *OsDREB1B* was reported to have a stress-dependent activity in rice and its overexpression in tobacco enhanced tolerance to dehydration, osmotic and oxidative stresses and to tobacco streak virus infection, demonstrating that *OsDREB1B* has a unique role in stress response and protects the plants from a range of stresses (Gutha and Reddy, 2008). In the present study, *DREB1B* was not significantly affected by RH nor genotype (Table 2) indicating that its expression does not contribute to explain the contrasting genotypic tolerance to high RH. In maize leaves, the water stress-induced ABA accumulation activates the production of reactive oxygen species, which in turn, leads to the up-regulation of antioxidant enzymes such as APX (Jiang and Zhang, 2002). *Rh-APX* was the highest expressed gene in all the treatments and appeared to be over-expressed in K099 grown at high RH (Fig. 4), suggesting the contribution of that gene to the increased water stress tolerance of the tolerant genotype. Jin *et al.* (2006) suggested improving the tolerance to water deficit stress in cut roses through transferring *APX* genes.

The genes evaluated in this study have been carefully selected from the literature and their sequences blasted against an existing EST database derived from both parents of the K5 tetraploid cut rose population. However, it is not excluded the possibility that other genes belonging to the same gene families considered in this study can also contribute to unravel the causes of the stomatal malfunctioning in high RH grown plants. Also, it might be the case that the function of a gene in a given species (e.g. *Arabidopsis*) does not correspond to the exact same function in roses as they are phylogenetically apart. The K5 cut rose population showed transgressive segregation in both directions for RWC_4h (Carvalho *et al.*, 2015b) indicating that stomatal responsiveness to desiccation is a polygenic trait, i.e., multiple genes are likely to contribute to the phenotypic variation of this trait in addition to environmental effects forming a highly complex gene network. Moreover, the occurrence of extreme genotypes is a result of several genes acting in the same direction, via increasing the ABA biosynthesis (*NCED1*, *AAO3*), conjugation (*UGT75B2*), mobilization (*BG2*), signaling (*OST1*, *ABF3*) and antioxidant system (*Rh-APX*), as it seems to be evident here for genotype K099. However, the individual contribution of each gene depended on the time of the day (light *vs.* dark). Additionally, the genes involved in ABA oxidation (*CYP707A1* and *CYP707A3*) and mobilization (*BG1*) had only a minor contribution and *DREB1B* did not contribute to the tolerance of the tolerant genotype to high RH during growth. Finally, our results showed that high RH reduced the concentrations of ABA and of its metabolites to a greater extent in the sensitive genotype compared to the tolerant one. Still, unlike our hypothesis, it is shown that simply increasing ABA biosynthesis or reducing break-down is not enough to achieve tolerance to high RH during growth as stomatal sensitivity to ABA rather than [ABA] plays an important role. This study unravels the complexity of the stomatal functioning trait under high RH and represents a first step towards understanding the molecular mechanisms underlying the stomatal functioning in high RH-grown plants.

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References

Aliniaeifard S, Malcolm Matamoros P, van Meeteren U. 2014. Stomatal malfunctioning under low VPD conditions: Induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? Physiologia Plantarum 152, 688–699.

Arve LE, Terfa MT, Gislerød HR, Olsen JE, Torre S. 2013. High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves. Plant, Cell and Environment 36, 382–392.

Arve LE, Kruse OMO, Tanino KK, Olsen JE, Futsæther C, Torre S. 2015. Growth in continuous high air humidity increases the expression of CYP707A-genes and inhibits stomatal closure. Environmental and Experimental Botany 115, 11–19.

Badawi GH, Kawano N, Yamauchi Y, Shimada E, Sasaki R, Kubo A, Tanaka K. 2004. Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiologia Plantarum 121, 231–238.

Carvalho DRA, Torre S, Kraniotis D, Almeida DPF, Heuvelink E, Carvalho SMP. 2015*a*. Elevated air movement enhances stomatal sensitivity to abscisic acid in leaves developed at high relative air humidity. Frontiers in Plant Science 6, 1–11.

Carvalho DRA, Koning-Boucoiran CFS, Fanourakis D, Vasconcelos MW, Carvalho SMP, Heuvelink E, Krens FA, Maliepaard C. 2015*b*. QTL analysis for stomatal functioning in tetraploid Rosa × hybrida grown at high relative air humidity and its implications on postharvest longevity. Molecular Breeding 35, 172.

Chang S, Puryear J, Cairney J. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11, 113–116.

Cutler AJ, Krochko JE. 1999. Formation and breakdown of ABA. Trends in Plant Science 4, 472–477.

Drake PL, Froend RH, Franks PJ. 2013. Smaller, faster stomata: Scaling of stomatal size, rate of response, and stomatal conductance. Journal of Experimental Botany 64, 495–505.

Fanourakis D, Tapia a., Heuvelink E, Carvalho SMP. 2009. Cultivar differences in the stomatal characteristics of cut roses grown at high relative humidity. Acta Horticulturae 847, 251–258.

Fanourakis D, Carvalho SMP, Almeida DPF, Heuvelink E. 2011. Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. Physiologia Plantarum 142, 274–286.

Fanourakis D, Carvalho SMP, Almeida DPF, van Kooten O, van Doorn WG, Heuvelink E. 2012. Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biology and Technology 64, 64–73.

Fanourakis D, Heuvelink E, Carvalho SMP. 2013. A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. Journal of Plant Physiology 170, 890–898.

Fanourakis D, Heuvelink E, Carvalho SMP. 2015. Spatial heterogeneity in stomatal features during leaf elongation : an analysis using *Rosa hybrida*. Functional Plant Biology 42, 737–745.

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park S-Y, Cutler SR, Sheen J, Rodriguez PL, Zhu J-K. 2009. In vitro reconstitution of an abscisic acid signalling pathway. Nature 462, 660–4.

Geiger D, Scherzer S, Mumm P, Stange A, Marten I, Bauer H, Ache P, Matschi S, Liese A, Al-Rasheid KAS, Romeis T, Hedrich R. 2009. Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. Proceedings of the National Academy of Sciences of the United States of America 106, 21425–21430.

Giday H, Fanourakis D, Kjaer KH, Fomsgaard IS, Ottosen CO. 2013*a*. Foliar abscisic acid content underlies genotypic variation in stomatal responsiveness after growth at high relative air humidity. Annals of Botany 112, 1857–1867.

Giday H, Kjaer KH, Fanourakis D, Ottosen CO. 2013*b*. Smaller stomata require less severe leaf drying to close: A case study in *Rosa hydrida*. Journal of Plant Physiology 170, 1309–1316.

Gutha LR, Reddy AR. 2008. Rice DREB1B promoter shows distinct stress-specific responses, and the overexpression of cDNA in tobacco confers improved abiotic and biotic stress tolerance. Plant Molecular Biology 68, 533–555.

Hong Z, Zhang Z, Olson JM, Verma DP. 2001. A novel UDP-glucose transferase is part of the callose synthase complex and interacts with phragmoplastin at the forming cell plate. The Plant cell 13, 769–779.

Ito O, Ella E, Kawano N. 1999. Physiological basis of submergence tolerance in rainfed lowland rice ecosystem. Field Crops Research 64, 75–90.

Jia H-F, Chai Y-M, Li C-L, Lu D, Luo J-J, Qin L, Shen Y-Y. 2011. Abscisic acid plays an important role in the regulation of strawberry fruit ripening. Plant physiology 157, 188–199.

Jiang M, Zhang J. 2002. Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. Journal of Experimental Botany 53, 2401–2410.

Jin J, Shan N, Ma N, Bai J, Gao J. 2006. Regulation of ascorbate peroxidase at the transcript level is involved in tolerance to postharvest water deficit stress in the cut rose (*Rosa hybrida* L.) cv. Samantha. Postharvest Biology and Technology 40, 236–243.

Kang J, Choi H, Im M, Kim SY. 2002. Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. The Plant cell 14, 343–357.

Koning-Boucoiran CFS, Esselink GD, Vukosavljev M, van 't Westende WPC, Gitonga VW, Krens FA, Voorrips RE, van de Weg WE, Schulz D, Debener T, Maliepaard C, Arens P, Smulders MJM. 2015. Using RNA-Seq to assemble a rose transcriptome with more than 13,000 full-length expressed genes and to develop the WagRhSNP 68k Axiom SNP array for rose (Rosa L.). Frontiers in Plant Science 6.

Lata C, Prasad M. 2011. Role of DREBs in regulation of abiotic stress responses in plants. Journal of Experimental Botany 62, 4731–4748.

Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee IJ, Hwang I. 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. Cell 126, 1109–1120.

Leung J, Giraudat J. 1998. Abscisic Acid Signal Transduction. Annual Review Plant Physiology Plant Molecular Biology 49, 199–222.

McAdam SAM, Sussmilch FC, Brodribb TJ. 2015. Stomatal responses to vapour pressure deficit are regulated by high speed gene expression in angiosperms. Plant, Cell & Environment. doi.wiley.com/10.1111/pce.12633.

Meng Y, Li N, Tian J, Gao J, Zhang C. 2013. Identification and validation of reference genes for gene expression studies in postharvest rose flower (*Rosa hybrida*). Scientia Horticulturae 158, 16–21.

Merlot S, Mustilli A-C, Genty B, North H, Lefebvre V, Sotta B, Vavasseur A, Giraudat J. 2002. Use of infrared thermal imaging to isolate Arabidopsis mutants defective in stomatal regulation. The Plant Journal 30, 601–609.

Mustilli A-C, Merlot S, Vavasseur A, Fenzi F, Giraudat J. 2002. Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. The Plant cell 14, 3089–3099.

Okamoto M, Tanaka Y, Abrams SR, Kamiya Y, Seki M, Nambara E. 2009. High humidity induces abscisic acid 8'-hydroxylase in stomata and vasculature to regulate local and systemic abscisic acid responses in Arabidopsis. Plant physiology 149, 825–834.

Park S, Fung P, Nishimura N, Jensen DR, Zhao Y, Lumba S, Santiago J, Rodrigues A, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Rodriguez PL, Mccourt P, Zhu J-K, Schroeder JI, Volkman BF, Cutler SR. 2009. Abscisic acid inhibits PP2Cs via the PYR/PYL family of ABA- binding START proteins. Science 324, 1068–1071.

Pitcher LH, Repetti P, Zilinskas BA. 1994. Overproduction of ascorbate peroxidase protects transgenic tobacco plants against oxidative stress. Plant physiology 105 (Suppl.) 116.

Rezaei Nejad A, van Meeteren U. 2007. The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity. Journal of Experimental Botany 58, 627–636.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J. 2003. TM4: a free, open-source system for microarray data management and analysis. BioTechniques 34, 374–378.

Sáez PL, Bravo LA, Latsague MI, Sánchez ME, Ríos DG. 2012. Increased light intensity during in vitro culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels. Scientia Horticulturae 138, 7–16.

Saika H, Okamoto M, Miyoshi K, Kushiro T, Shinoda S, Jikumaru Y, Fujimoto M, Arikawa T, Takahashi H, Ando M, Arimura S, Miyao A, Hirochika H, Kamiya Y, Tsutsumi N, Nambara E, Nakazono M. 2007. Ethylene promotes submergence-induced expression of *OsABA8ox1*, a gene that encodes ABA 8'-hydroxylase in rice. Plant and Cell Physiology 48, 287–298.

Salisbury E. 1927. On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. Philosophical Transactions of the Royal Society of London 216, 1–65.

Schachtman DP, Goodger JQD. 2008. Chemical root to shoot signaling under drought. Trends in Plant Science 13, 281–287.

Schwartz SH, Tan BC, Gage DA, Zeevaart JA, McCarty DR. 1997. Specific oxidative cleavage of carotenoids by VP14 of maize. Science 276, 1872–1874.

Seo M, Peeters AJM, Koiwai H, Oritani T, Marion-Poll a, Zeevaart J a, Koornneef M, Kamiya Y, Koshiba T. 2000. The Arabidopsis aldehyde oxidase 3 (*AAO3*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proceedings of the National Academy of Sciences of the United States of America 97, 12908–12913.

Sirichandra C, Davanture M, Turk BE, Zivy M, Valot B, Leung J, Merlot S. 2010. The arabidopsis ABA-activated kinase OST1 phosphorylates the bZIP transcription factor ABF3 and creates a 14-3-3 binding site involved in its turnover. PLoS ONE 5, 1–13.

Slavík B. 1974. *Methods of studying plant water relations*. London, UK: Chapman and Hall.

Sun L, Sun Y, Zhang M, Wang L, Ren J, Cui M, Wang Y, Ji K, Li P, Li Q, Chen P, Dai S, Duan C, Wu Y, Leng P. 2012. Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. Plant Physiology 158, 283–298.

Tallman G. 2004. Are diurnal patterns of stomatal movement the result of alternating metabolism of endogenous guard cell ABA and accumulation of ABA delivered to the apoplast around guard cells by transpiration? Journal of Experimental Botany 55, 1963–1976.

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N. 2005. Ethylene inhibits abscisic acid-induced stomatal closure in Arabidopsis. Plant Physiology 138, 2337–2343.

Ting W, Zheng T, Nan M, JunPing G. 2009. Isolation and expression analysis of Rh-DREB1s gene in cut roses (*Rosa hybrida*) under ethylene treatment and water deficit stress. Acta Horticulturae Sinica 36, 65–72.

Tullus A, Kupper P, Sellin A, Parts L, Sõber J, Tullus T, Lõhmus K, Sõber A, Tullus H. 2012. Climate change at Northern latitudes: Rising atmospheric humidity decreases transpiration, N-uptake and growth rate of hybrid aspen. PLoS ONE 7, 1–11.

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K. 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 106, 17588–17593.

Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM. 2007. Primer3Plus , an enhanced web interface to Primer3. Nucleic Acids Research 35, 71–74.

Wahyuni Y, Stahl-Hermes V, Ballester A-R, de Vos RCH, Voorrips RE, Maharijaya A, Molthoff J, Zamora MV, Sudarmonowati E, Arisi ACM, Bino RJ, Bovy AG. 2014. Genetic mapping of semi-polar metabolites in pepper fruits (*Capsicum* sp.): Towards unravelling the molecular regulation of flavonoid quantitative trait loci. Molecular Breeding 33, 503–518.

Wang Y, Wisniewski M, Meilan R, Cui M, Webb R, Fuchigami L. 2005. Overexpression of cytosolic ascorbate peroxidase in tomato confers tolerance to chilling and salt stress. Journal of the American Society for Horticultural Science 130, 167–173.

Webb RP, Allen RD. 1996. Overexpression of pea cytosolic ascorbate peroxidase confers protection against oxidative stress in transgenic *Nicotiana tabacum*. Plant physiology 111 (Suppl.) 48.

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

Xu Z-Y, Lee KH, Dong T, Jeong JC, Jin JB, Kanno Y, Kim DH, Kim SY, Seo M, Bressan RA, Yun D-J, Hwang I. 2012. A vacuolar β-glucosidase homolog that possesses glucose-conjugated abscisic acid hydrolyzing activity plays an important role in osmotic stress responses in Arabidopsis. The Plant Cell 24, 2184–2199.

Yamaguchi-Shinozaki K, Shinozaki K. 2005. Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends in Plant Science 10, 88–94.

Zeevaart JAD, Creelman RA. 1988. Metabolism and physiology of abscisic acid. Annual Review of Plant Physiology and Plant Molecular Biology 39, 439–473.

Appendix

Table A1. List of the primers used for qRT-PCR analysis

Gene	Forward primer sequence (5'- 3')	Reverse primer sequence (5'- 3')
NCED1	GAATTCGGAGAACGAAGACG	CTTCGAGCTTCAAAGTCATGG
AAO3	ATACCCGAAGCAAGTTGGTG	AGCAAGGGTAGTTTGTGTCTCC
CYP707A1	TTGGGATGTCCTTGCGTGATGATT	CTTGGCGTGGTAGTCTCCTTGGTG
CYP707A3	GCAGGCAGCGAAGATGATAAGGA	AGCCATGTAAGAGCAGCAGTCGTG
UGT75B2	GGTATGGTGGTTTCATGGTG	TGGAACCCCTAAACTCAAAGC
BG1	GTGCCATTACCCTTCCTTTC	TTAAGCTTCTCATCCCCTCAG
BG2	TCGAATCTTCCCTGATGGTC	ATGCCTTTTTCCAGGAGAGC
OST1	TTCTGGAGGAGAGCTTTTCG	CCGGTGACATACTTGCATTG
ABF3	GCAGGAGGCGCCGCTTGATAA	GGCGTGCATTTTCCTCCTCCAG
DREB1B	GGCCGACCGGGGAGGAA	GTCATCGCCGCCGTAGCCATTAT
Rh-APX	TCTTGGAGCCAATCAAGGAG	TGGGTGGAATGGAACATCAG
Actin	TTCCTTGCTCATCCTGTCTG	AAGTCCTCTTCCAACCATCC
Ubiquitin	GGTGAAGGCGTGCGTAGC	AGGCTCCGTGGTGGTATTATTG

Table A2. Relative gene expression (RGE) values [i.e., Log_2 (RGE × 100000)] of the ABA-related genes (*NCED1*, *AAO3*, *CYP707A1*, *CYP707A3*, *UGT75B2*, *BG1*, *BG2*, *OST1*, *ABF3*) and of the non-ABA related genes involved in the water stress response (*DREB1*, *Rh-APX1*) evaluated in fully expanded leaves from four genotypes (K023, P867, P540 and K099) cultivated at moderate (62%) or high (89%) relative air humidity. Samples were collected 10 and 180 min after the beginning of the light (10_L and 180_L) and of the dark (10_D and 180_D) periods

		K023				P867				P540				K099			
		10_L	180_L	10_D	180_D	10_L	180_L	10_D	180_D	10_L	180_L	10_D	180_D	10_L	180_L	10_D	180_D
NCED1	62%	6.73	9.50	7.12	7.17	5.88	7.05	7.49	6.81	8.38	9.99	7.73	7.19	5.45	8.54	5.05	5.44
	89%	6.34	8.28	5.36	8.48	8.06	6.99	7.67	6.22	7.63	8.53	6.47	7.28	5.93	8.51	7.77	6.56
AAO3	62%	10.78	10.83	11.06	10.76	11.11	10.61	10.38	11.32	10.81	10.99	10.75	10.22	9.85	9.97	10.28	9.37
	89%	10.70	10.73	10.61	10.84	11.47	9.99	11.05	10.28	9.90	9.82	10.46	9.87	11.05	11.38	10.96	10.88
CYP707A1	62%	9.14	8.61	9.25	9.32	9.83	10.95	9.57	11.00	9.94	9.74	9.65	9.53	8.99	8.08	8.63	9.19
	89%	10.03	9.26	9.89	9.68	12.01	12.46	10.32	10.15	10.91	9.32	10.01	9.89	8.84	8.26	9.35	8.66
CYP707A3	62%	7.28	7.37	7.45	7.35	6.47	6.15	6.36	6.38	7.49	7.13	7.15	7.07	4.95	5.49	5.38	5.77
	89%	6.28	6.83	6.83	6.78	6.65	4.81	5.99	6.30	5.80	6.47	6.50	6.75	5.83	6.01	5.81	5.53
UGT75B2	62%	12.25	12.98	12.26	12.26	12.44	12.94	13.32	13.20	12.73	12.94	14.79	12.33	12.88	12.95	12.88	12.87
	89%	12.59	13.08	11.89	12.52	13.82	12.26	12.91	12.91	12.13	12.32	11.96	12.56	13.43	13.70	13.43	13.60
BG1	62%	7.98	7.43	7.96	8.02	8.16	7.12	7.95	8.12	8.61	7.30	8.00	7.81	7.42	6.38	7.15	7.38
	89%	7.16	7.20	7.51	8.23	8.01	6.34	7.47	7.85	9.40	7.96	8.56	8.60	7.40	7.10	7.85	7.84
BG2	62%	12.67	12.53	12.84	12.88	11.98	12.12	12.44	12.44	11.66	12.85	11.18	11.47	11.77	12.07	12.31	12.19
	89%	12.21	11.36	12.25	12.14	12.28	11.55	12.12	12.00	11.74	11.30	11.80	11.16	12.20	11.92	12.53	12.39
OST1	62%	13.93	13.20	13.79	14.24	13.92	13.19	13.97	13.81	13.63	13.47	13.69	13.88	13.46	13.04	13.46	13.61
	89%	13.72	13.12	13.51	13.67	13.04	11.92	13.09	13.21	13.34	13.07	13.10	13.38	13.25	13.32	13.47	13.78
ABF3	62%	12.78	11.91	12.17	12.93	12.86	11.38	12.26	13.00	12.92	11.91	12.21	12.91	12.58	11.32	11.73	12.75
	89%	12.63	11.85	11.89	12.55	12.74	11.20	12.02	12.05	12.52	11.66	11.82	12.59	12.54	12.32	12.32	12.78
DREB1B	62%	9.60	7.89	3.46	13.37	9.58	5.60	7.83	12.52	9.28	7.72	5.09	10.85	7.90	7.64	3.98	11.23
	89%	8.04	6.72	3.93	13.77	10.13	6.58	3.49	8.50	7.70	9.52	2.50	12.88	9.09	7.44	4.18	12.38
Rh-APX1	62%	17.21	17.41	17.63	16.86	16.72	17.16	17.08	17.53	17.52	17.46	17.85	17.21	16.57	17.10	17.29	16.88
	89%	16.64	17.32	16.75	16.77	18.38	15.36	16.94	16.67	15.65	16.37	16.87	16.61	17.36	18.33	17.96	17.69

CHAPTER 7

General Discussion

An appropriate stomatal closure in response to closing stimuli (such as desiccation, light-dark transition and [ABA] increase) is a key trait to prevent plant excessive water loss in conditions of high evaporative demand (Arve et al., 2015). It has been demonstrated in several species that stomata developed at high RH fail to close fully in response to those stimuli (Torre & Fjeld 2001; Fanourakis et al. 2011; Aliniaeifard et al. 2014). Although the magnitude of this problem is highly cultivar dependent (Mortensen and Gislerød, 1999; Fanourakis et al., 2013a; Giday et al., 2013a), the causes behind a contrasting stomatal behavior are not yet fully understood. For this reason, contrasting genotypes from the segregating K5 tetraploid cut rose population were used to analyze the genetic, physiological (stomatal features and ABA regulation) and molecular mechanisms underlying stomatal malfunctioning in plants cultivated at high RH (Chapters 2, 5 and 6). Moreover, the commercial cultivars 'Toril' (pot rose) and 'Prophyta' (cut rose), previously reported as sensitive to high RH, were used for testing the hypothesis that increasing MOV (Chapter 3) or salinity (Chapter 4) could trigger a mild stress response and stimulate stomatal functioning due to an increase in leaf [ABA]. To the best of our knowledge, the combination of high RH with each of these two physiological stresses was not yet properly studied. Below we discuss the main findings obtained in this study.

Stomatal physiology and anatomy

Stomatal morphological and physiological parameters have been described to contribute to the increased water loss in leaves developed at high RH, despite the controversy about the relative importance of the morphological parameters in that process (Fanourakis et al. 2013a; Giday et al., 2013b; Aliniaeifard et al. 2014). In this study, it was found that pore width, pore area and pore area per leaf area were highly increased at high RH in sensitive genotypes as compared to tolerant ones, while stomatal density, index and size were not influenced by RH or genotype in a consistent manner (Chapters 2 and 6). This suggests that although stomatal anatomy contributes *per se* to an increased water loss, these parameters are not decisive in this process as long as stomata are able to function properly. Thus, we confirmed that stomatal physiology has a major contribution to the control of water loss, but stomatal anatomy should not be disregarded as it also plays a relevant role (Fanourakis et al. 2013a; Aliniaeifard et al. 2014). When analyzing the effect of the two studied physiological stresses (MOV and salinity) it was found that increasing MOV in plants grown at high RH reduced pore aperture and length compared to low MOV (Chapter 3.1). Increasing EC in plants grown at high RH resulted in lower stomatal and pore length but in a higher pore aperture and stomatal density, which lead to an increased pore area per leaf area compared to plants developed at an EC of 2 dS m^{-1} (EC2) (Chapter 4.1). In spite of the higher pore area per leaf area in plants developed at an EC of 4 dS m^{-1} (EC4) and 6 dS m^{-1} (EC6), stomatal functionality was improved which is in line with the conclusions above.

Stomatal functioning in high RH grown plants – the role of ABA

Stomatal responsiveness to closing stimuli can be assayed gravimetrically or using a porometer. The first procedure has been thoroughly used in studies assessing stomatal responsiveness to water loss after growth at high RH (Rezaei Nejad and van Meeteren, 2005; Giday et al., 2013a; Fanourakis et al., 2015) due to its effectiveness under conditions of low stomatal conductance, e.g. excessively desiccated leaflets (i.e., RWC < 20%), which fall below the detection limit of the porometer. In Chapter 4.1 (Fig. 2) RWC was determined after 4 h of leaflet desiccation (RWC_4h) and this parameter was found to be a good indicator for discriminating between responsive and non-responsive stomata to water deprivation.

High RH during growth significantly decreased RWC after 4 h of leaflet desiccation in the sensitive genotypes as compared to the tolerant ones indicating a poor stomatal functioning in leaves developed at high RH (Chapters 2-4 and 6). Besides the leaflet desiccation we also tested the light-dark transition as a stomatal closing stimulus and obtained a similar response, with the sensitive genotypes showing reduced stomatal closure in response to darkness as compared to the tolerant ones. Several studies suggest that these slow responsive stomata to closing stimuli are a consequence of long-term low leaf [ABA] (Rezaei Nejad and van Meeteren, 2007; Fanourakis et al., 2011; Arve et al., 2013). Although several studies have shown the relevance of ABA synthesis in the mesophyll cells of leaves (Christmann et al., 2005; Endo et al., 2008; Seo and Koshiba, 2011; McAdam et al., 2015), it is undeniable that roots are a very important production site of ABA which can be translocated via the xylem vessels to the leaves driven by the transpiration stream (Dodd, 2005). Giday et al. (2014) found that leaf [ABA] increased in leaves of a sensitive rose cultivar grafted onto a tolerant one grown at high RH, and speculated that this leaf [ABA] increase was due to a higher root ABA biosynthesis. However, these authors did not quantify the [ABA] at root level. In Chapter 2, we clearly show that sensitive and tolerant genotypes had a similar estimated [ABA] in the root xylem sap of transpiring plants and a similar ABA delivery rate per unit leaf area, suggesting that the ABA delivered from the roots does not contribute to the genotypic differences in stomatal functioning following growth at high RH. At leaf level it was found that (i) a similar root-to-shoot ABA delivery rate between contrasting genotypes led to a comparable [ABA] measured in the leaf petiole xylem sap among genotypes; (ii) high RH during growth did not affect the leaf petiole sap [ABA]; and (iii) no significant correlation was noted between [ABA] in the leaf petiole xylem sap and RWC_4h. All together our results indicate that xylem sap [ABA] does not explain genotypic differences in the stomatal sensitivity to high RH; the ABA homeostasis within the leaf (Giday et al., 2013a; McAdam and Brodribb, 2015) or a distinct genotypic sensitivity to ABA are the main determinants in this process. Based in our findings, the hypothesis of a distinct genotypic sensitivity to ABA seems to be valid because: (1) the amount of exogenous ABA fed through the leaflet petiole required to induce stomatal closure in plants cultivated at high RH was lower in the tolerant genotype compared to the sensitive ones (Chapter 2; Pantin et al., 2013); (2) simply increasing ABA leaf biosynthesis or reducing its break-down was not enough to reach tolerance to high RH (Chapter 6). This conclusion is in agreement with Arve et al. (2015) who found that detached *Arabidopsis thaliana* leaves subjected to desiccation increased [ABA] independently of the RH during growth, but only stomata from moderate RH-grown leaves were able to close, evidencing that high RH-grown plants could not perceive the ABA signal.

In Chapters 3 and 4 we tested the hypothesis that increasing MOV or salinity could trigger a mild stress response and stimulate stomatal functioning due to an increase in the leaf [ABA]. Stomata developed at high RH with additional MOV or high EC (EC6) closed faster in response to leaf desiccation, resulting in a RWC_4h two-fold higher when compared to plants grown at high RH with low MOV or low EC (Chapters 3.1 and 4.1). This shows that MOV and EC were effective in enhancing stomatal responsiveness to desiccation, although due to different reasons: in high MOV there was no increase in the leaf [ABA] but stomata were more sensitive to ABA; while under salinity there was an actual increase in leaf [ABA]. Pantin et al. (2013) suggested that stomatal sensitivity to ABA is related to leaf developmental stage in *A. thaliana* but in Chapter 3.1 no difference was found in the stomatal responsiveness to ABA feeding between leaf developmental stages indicating that even non-fully mature stomata grown at high RH do not respond to a short-term ABA application. Reasons such as (1) perception and/or sensitivity of ABA receptors (Anderson et al., 1994; Schwartz et al., 1994) which may differ in fully expanded and in expanding leaves and (2) ABA compartmentation in the leaf that can affect [ABA] in the guard cells (Harris and Outlaw, 1991) might also be involved in stomatal closure.

Genotypic variability associated with the control of water loss

In *A. thaliana*, the natural variability in mechanisms responsible for stomatal functioning has been studied by several authors and the importance of identifying QTLs associated to water loss has been highlighted (Bouchabke et al. 2008; Brosché et al. 2010). In rose, we identified three QTLs, two major and one putative minor, explaining 32% of the variability in the RWC_4h. The two major QTLs were mapped to linkage group 5 of the integrated consensus map (ICM5) (contribution of both parents) and to ICM 2 of parent P867; the putative minor QTL was mapped to ICM 6 (both parents). Two QTLs linked with stomatal conductance in rice collectively explained only 14.9% (Zhao et al. 2008), while nine QTLs associated with RWC in field-grown barley individually explained a maximum of 15.0% (Teulat et al. 2003). In the present study, the identified genomic regions were located on different chromosomes, supporting the expectations that stomatal responsiveness to desiccation is a polygenic trait derived from a combination of different genomic regions. Similarly, the QTLs identified in rice and barley, were mapped on two and six different chromosomes, respectively (Teulat et al. 2003; Zhao et al. 2008). In our study, a single marker analysis considering possible dosage effects of markers was performed, which is a valuable tool for QTL studies in polyploid populations. Interestingly, the most significant marker from the major QTL, mapped to ICM 5, and the marker from the putative minor QTL, mapped to ICM 6, showed indeed a dosage effect: if the favorable allele was present in double dosage, i.e. on two of the four homologs, the progeny showed a higher RWC_4h. The individuals showing zero or only one copy of the marker allele were not significantly different from each other and had lower average RWC_4h compared to dosage 2. These results indicate that it is not enough to study the presence or the absence of this marker to infer about stomatal responsiveness to water loss. It is important to know about the dosage, since at least for these two QTLs only a double dosage is associated with a higher RWC_4h and, consequently, an increased stomatal functioning. For simplex × nulliplex markers (i.e., markers that segregate differently in the progeny whose least common allele is present in only one parent), such as the most significant marker mapped to ICM 2, it is enough to study the presence or the absence of the scored allele and the RWC_4h was lower in the former compared to the latter.

The direct use of the identified markers in marker-assisted selection (MAS) is not straightforward yet, since the applicability of the markers should first be validated in other germplasm. However, the information achieved so far will contribute to further genetic analyses and future breeding programs aiming an early identification and elimination of offspring with a very poor stomatal functioning which is associated with a reduced potential vase life (Chapter 1, Fig. 2). Furthermore, at present, the rose genome is not yet fully sequenced, but this work represents an initial approach towards identifying the most promising regions in which genes of interest for stomatal malfunctioning might be located. Based on the QTL analyses, it is not possible yet to propose candidate genes, first of all because of the large number of genes located in the QTL regions and second because of the lack of annotation of genes involved in stomatal functioning in rose and related species within the family Rosaceae (e.g. the diploid *Fragaria vesca* genome). For this reason, the genes studied in Chapter 6 were selected from the literature.

Gene expression associated to stomatal functioning

The expression of nine ABA-related and two non-ABA related genes involved in water stress response was investigated to unravel the mechanisms underlying contrasting stomatal functioning in plants grown at high RH. The expression of a broad range of ABA-related genes in response to light-dark transition revealed that the variations at metabolic level do not always correlate with the variations at transcriptional level (Chapter 6). Genes evaluated in this study were carefully selected based on literature and their sequences blasted against an existing EST database derived from both parents of the K5 tetraploid cut rose population. The K5 cut rose population showed transgressive segregation in both directions for RWC_4h (Chapter 5) indicating that stomatal responsiveness to desiccation is a polygenic trait, i.e., multiple genes are likely to contribute to the phenotypic variation of this trait in addition to environmental effects forming a highly complex gene network. Moreover, the occurrence of extreme genotypes, such as K099, is a result of the high expression of several genes acting together towards the tolerance to leaflet desiccation.

This means that an increase in ABA biosynthesis (*NCED1*, *AAO3*), an increase in ABA conjugation, i.e., storage form of ABA as ABA-GE (*UGT75B2*) and further mobilization of ABA-GE to ABA (*BG2*), as well as an enhanced ABA signaling (*OST1*, *ABF3*) and an improved antioxidant system (*Rh-APX*) seem to contribute to the tolerance of K099 to leaflet desiccation (Chapter 6). The individual contribution of each gene depended on the time of the day (light *vs.* dark). Additionally, the genes involved in ABA oxidation (*CYP707A1* and *CYP707A3*) and mobilization (*BG1*) had only a minor contribution while *DREB1B* did not contribute to the tolerance to high RH during growth. However, other genes belonging to the same or to other gene families could also contribute to clarify the causes of the stomatal malfunctioning in high RH grown plants.

Effects of MOV and salinity on postharvest longevity and plant growth

The ultimate aim of obtaining ornamental plants with functional stomata following growth at high RH is to have plants with a long postharvest longevity (Fanourakis et al. 2012a). Low RWC_4h was found to be a good proxy for identifying genotypes with a limited vase life when vase life is limited by an excessive water loss during the postharvest phase, owing to less responsive stomata. Nevertheless, high RWC_4h was not always related to long vase life which can be explained by the fact that although water loss is crucial for vase life, it is not the only determining factor (Fanourakis et al. 2013b). Although the vase life of a new cultivar is always tested before it is released to the market, this is often done only in the final phase of the breeding program. Since a short vase life will lead to the rejection of a promising cultivar, that has been selected for other relevant traits (e.g. a high productivity, flower color, flower size, etc.), an early elimination of the offspring with a very poor vase life will increase the efficiency of the breeding programs (Fanourakis et al. 2012b). The dosage score of the most significant marker from each QTL correlated with the vase life longevity of several genotypes but not with all of them (Chapter 5). A likely reason for this is that the three QTLs identified explain 32% of the variability, with a 68% of variability attributed to other (genetic or non-genetic) factors.

High MOV had a minor effect on plant growth and visual quality parameters (Chapter 3), whereas salinity had a strong negative impact on plant growth (Chapter 4.2). High MOV only increased the peduncle diameter in 6% irrespective the RH, which can contribute to higher tolerance to bent-neck occurrence during postharvest especially in high-RH grown plants (Fanourakis et al., 2012a). Plants grown at EC6 showed lower plant dry weight, total leaf area and plant height compared to plants grown at EC2, which is very critical since it depreciates plants commercial value. Other authors have also reported lower total dry weight, reduced leaf area, reduced stem length and weight and decreased flower quality and yield in roses grown under salinity (De Kreij and Van Den Berg, 1990; Brun and Settembrino, 1996; Cabrera, 2003; Oki and Lieth, 2004; Niu and Rodriguez, 2008). Therefore, it can be interesting to explore the advantages of growing the plants at a saline level of 4 dS m^{-1}, where stomatal functioning of high RH grown plants was improved while plant growth parameters were not significantly affected, comparing to EC2 (Chapter 4.1). Under salinity, the maximum ion toxicity accumulation occurs in old leaves, when plants are no longer expanding. In this study (Chapter 4), plants were grown as single stem, i.e., lower vegetation when compared to plants in a commercial greenhouse, where the bending technic is often used, leading to a high leaf area. Whether a moderate saline stress applied in plants grown at high RH but with large foliar mass is able to enhance stomatal responsiveness while attenuating the negative effects of ion accumulation is still to be evaluated. High RH mildly affected plant growth and visual quality parameters in the two independent experiments (Chapters 3 and 4.2) confirming similar findings from other authors (Santamaria et al., 1993; Mortensen & Gislerød, 1999; Aguilar et al., 2000; Fanourakis et al., 2012a).

Main conclusions and future research

In this thesis we explored the sources of stomatal malfunctioning in plants developed at high RH using rose as a model system. To that end we have evaluated stomatal anatomy and physiology, [ABA] and the concentration of its metabolites, as well as the molecular and genetic mechanisms involved in this process. Several major conclusions can be drawn based on the obtained findings.

The large genotypic variability for stomatal functioning in response to water loss present in the 110 studied genotypes of the K5 tetraploid cut rose population is valuable to breed for better stomatal functioning in high RH-grown plants (**Chapter 5**). The genotypic differences in the stomatal sensitivity to high RH cannot be explained by changes in [ABA] in the xylem sap (**Chapter 2**). This conclusion reinforces the suggestion made by other researchers that ABA homeostasis within the leaf is the main determinant in this process. For this reason, we have evaluated the [ABA] and the concentration of its metabolites at leaf level. We concluded that an increase in leaf [ABA] is not needed to induce stomatal functioning, since leaf [ABA] was not affected in plants subjected to high MOV but an increased sensitivity to the endogenous ABA did occur (**Chapter 3**).

However, when [ABA] was increased there was always a simultaneous increase in the stomatal response to closing stimuli (**Chapter 4**). In future studies it is relevant to monitor the expression profiles of genes involved in plant responses to salinity and ABA in order to better understand the mechanisms that confer enhanced stomatal functioning and increased [ABA] under salinity.

The transcriptional analysis of nine ABA-related and two non-ABA related genes (involved in the response to water stress) in four contrasting genotypes selected from the K5 tetraploid cut rose population revealed that multiple of these genes formed a highly complex gene network acting together towards tolerance to high RH (**Chapter 6**). The regulatory mechanism of these genes in the stomatal functioning of high RH-grown plants should be validated using mutant lines. However, such mutants do not exist in roses, thus other species such as *A. thaliana* should be used.

We suggest that stomatal sensitivity to ABA plays an important role in the stomatal functioning of high RH-grown plants. For this reason, the expression of genes related to ABA receptors should also be considered. The expression of such genes could be evaluated in desiccated leaves, as this stomatal closing stimulus may be more effective in inducing more contrasting gene expression related to stomatal functioning. However, when evaluating the gene expression in the time course of desiccation it is important to be aware that moderate- *vs*. high-RH grown plants acquire different hydration status after a given time since the first have a better control of water loss than the second one.

Three QTLs explaining 32% of the variability in the RWC_4h, were identified in the K5 tetraploid cut rose population (**Chapter 5**). This information will contribute to further genetic analyses and future breeding programs, but the QTLs' regions identified need to be validated in other mapping populations. Moreover, at present, the rose genome is not yet fully sequenced, but in the future this work can facilitate the identification of the most promising regions in which genes of interest for stomatal malfunctioning might be located.

References

Aguilar, M.L., Espadas, F.L., Coello, J., Maust, B.E., Trejo, C., Robert, M.L., Santamaría, J.M., 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. J. Exp. Bot. 51, 1861–1866.

Aliniaeifard, S., Malcolm Matamoros, P., van Meeteren, U., 2014. Stomatal malfunctioning under low VPD conditions: Induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? Physiol. Plant. 152, 688–699.

Anderson, B.E., Ward, J.M., Schroeder, J.I., 1994. Evidence for an extracellular reception site for abscisic acid in *Commelina* guard cells. Plant Physiol. 104, 1177–1183.

Arve, L.E., Terfa, M.T., Gislerød, H.R., Olsen, J.E., Torre, S., 2013. High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves. Plant, Cell Environ. 36, 382–392.

Arve, L.E., Kruse, O.M.O., Tanino, K.K., Olsen, J.E., Futsæther, C., Torre, S., 2015. Growth in continuous high air humidity increases the expression of CYP707A-genes and inhibits stomatal closure. Environ. Exp. Bot. 115, 11–19.

Bouchabke, O., Chang, F., Simon, M., Voisin, R., Pelletier, G., Durand-Tardif, M., 2008. Natural variation in *Arabidopsis thaliana* as a tool for highlighting differential drought responses. PLoS One 3, e1705.

Brosché, M., Merilo, E., Mayer, F., Pechter, P., Puzõrjova, I., Brader, G., Kangasjärvi, J., Kollist, H., 2010. Natural variation in ozone sensitivity among *Arabidopsis thaliana* accessions and its relation to stomatal conductance. Plant, Cell Environ. 33, 914–925.

Brun, R., Settembrino, A., 1996. Production and quality of *Rosa hybrida* cv Sonia in rockwool at three levels of electrical conductivity combined with two levels of relative humidity. Acta Hortic. 424, 115–117.

Cabrera, R.I., 2003. Demarcating salinity tolerance in greenhouse rose production. Acta Hortic. 609, 51–57.

Christmann, A., Hoffmann, T., Teplova, I., Grill, E., Müller, A., 2005. Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed Arabidopsis. Plant Physiol. 137, 209–219.

De Kreij, C., Van Den Berg, T.H.J.M., 1990. Nutrient uptake, production and quality of *Rosa hybrida* in rockwool as affected by electrical conductivity of the nutrient solution. In: van Beusichem, M.L. (Ed.), Plant Nutrition — Physiology and Applications. Springer Netherlands, Dordrecht, pp. 519–523.

Dodd, I.C., 2005. Root-to-shoot signalling: Assessing the roles of “up” in the up and down world of long-distance signalling in planta. Plant Soil 274, 251–270.

Endo, A., Sawada, Y., Takahashi, H., Okamoto, M., Ikegami, K., Koiwai, H., Seo, M., Toyomasu, T., Mitsuhashi, W., Shinozaki, K., Nakazono, M., Kamiya, Y., Koshiba, T., Nambara, E., 2008. Drought induction of Arabidopsis 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. Plant Physiol. 147, 1984–1993.

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F., Heuvelink, E., 2011. Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. Physiol. Plant. 142, 274–286.

Fanourakis, D., Carvalho, S.M.P., Almeida, D.P.F., van Kooten, O., van Doorn, W.G., Heuvelink, E., 2012a. Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biol. Technol. 64, 64–73.

Fanourakis, D., Carvalho, D.R.A., Gitonga, V.W., Van Heusden, A.W., Almeida, D.P.F., Heuvelink, E., Carvalho, S.M.P., 2012b. Breeding cut roses for better keeping quality : First steps. In: Acta Horticulturae 937. pp. 875–882.

Fanourakis, D., Heuvelink, E., Carvalho, S.M.P., 2013a. A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. J. Plant Physiol. 170, 890–898.

Fanourakis, D., Pieruschka, R., Savvides, A., Macnish, A.J., Sarlikioti, V., Woltering, E.J., 2013b. Sources of vase life variation in cut roses: A review. Postharvest Biol. Technol. 78, 1–15.

Fanourakis, D., Giday, H., Milla, R., Pieruschka, R., Kjaer, K.H., Bolger, M., Vasilevski, A., Nunes-Nesi, A., Fiorani, F., Ottosen C-O, 2015. Pore size regulates operating stomatal conductance , while stomatal densities drive the partitioning of conductance between leaf sides. Ann. Bot. 115, 555–565.

Giday, H., Fanourakis, D., Kjaer, K.H., Fomsgaard, I.S., Ottosen, C.O., 2013a. Foliar abscisic acid content underlies genotypic variation in stomatal responsiveness after growth at high relative air humidity. Ann. Bot. 112, 1857–1867.

Giday, H., Kjaer, K.H., Fanourakis, D., Ottosen, C.O., 2013b. Smaller stomata require less severe leaf drying to close: A case study in *Rosa hydrida*. J. Plant Physiol. 170, 1309–1316.

Giday, H., Fanourakis, D., Kjaer, K.H., Fomsgaard, I.S., Ottosen, C.O., 2014. Threshold response of stomatal closing ability to leaf abscisic acid concentration during growth. J. Exp. Bot. 65, 4361–4370.

Harris, M.J., Outlaw, W.H., 1991. Rapid adjustment of guard-cell abscisic acid levels to current leaf-water status. Plant Physiol. 95, 171–173.

McAdam, S.A.M., Brodribb, T.J., 2015. The evolution of mechanisms driving the stomatal response to vapor pressure deficit. Plant Physiol. 167, 833–843.

McAdam, S.A.M., Sussmilch, F.C., Brodribb, T.J., 2015. Stomatal responses to vapour pressure deficit are regulated by high speed gene expression in angiosperms. Plant. Cell Environ.

Mortensen, L.M., Gislerød, H.R., 1999. Influence of air humidity and lighting period on growth, vase life and water relations of 14 rose cultivars. Sci. Hortic. (Amsterdam). 82, 289–298.

Niu, G., Rodriguez, D.S., 2008. Responses of growth and ion uptake of four rose rootstocks to chloride- or sulfate-dominated salinity. J. Am. Soc. Hortic. Sci. 133, 663–669.

Oki, L.R., Lieth, J.H., 2004. Effect of changes in substrate salinity on the elongation of *Rosa hybrida* L. “Kardinal” stems. Sci. Hortic. (Amsterdam). 101, 103–119.

Pantin, F., Renaud, J., Barbier, F., Vavasseur, A., Le Thiec, D., Rose, C., Bariac, T., Casson, S., McLachlan, D.H., Hetherington, A.M., Muller, B., Simonneau, T., 2013. Developmental priming of stomatal sensitivity to abscisic acid by leaf microclimate. Curr. Biol. 23, 1805–1811.

Rezaei Nejad, A., van Meeteren, U., 2005. Stomatal response characteristics of *Tradescantia virginiana* grown at high relative air humidity. Physiol. Plant. 125, 324–332.

Rezaei Nejad, A., van Meeteren, U., 2007. The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity. J. Exp. Bot. 58, 627–636.

Santamaria, J.M., Davies, W.J., Atkinson, C.J., 1993. Stomata of micropropagated *Delphinium* plants respond to ABA, CO2, light and water potential, but fail to close fully. J. Exp. Bot. 44, 99–107.

Schwartz, A., Wu, W.H., Tucker, E.B., Assmann, S.M., 1994. Inhibition of inward K^+ channels and stomatal response by abscisic acid: an intracellular locus of phytohormone action. Proc. Natl. Acad. Sci. U. S. A. 91, 4019–4023.

Seo, M., Koshiba, T., 2011. Transport of ABA from the site of biosynthesis to the site of action. J. Plant Res. 124, 501–507.

Teulat, B., Zoumarou-Wallis, N., Rotter, B., Ben Salem, M., Bahri, H., This, D., 2003. QTL for relative water content in field-grown barley and their stability across Mediterranean environments. Theor. Appl. Genet. 108, 181–188.

Torre, S., Fjeld, T., 2001. Water loss and postharvest characteristics of cut roses grown at high or moderate relative air humidity. Sci. Hortic. (Amsterdam). 89, 217–226.

Zhao, X.Q., Xu, J.L., Zhao, M., Lafitte, R., Zhu, L.H., Fu, B.Y., Gao, Y.M., Li, Z.K., 2008. QTLs affecting morph-physiological traits related to drought tolerance detected in overlapping introgression lines of rice (*Oryza sativa* L.). Plant Sci. 174, 618–625.