

Foliar abscisic acid content underlies genotypic variation in stomatal responsiveness after growth at high relative air humidity

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Received: 5 April 2013 Returned for revision: 1 July 2013 Accepted: 7 August 2013 Published electronically: 25 October 2013

- **Background and Aims** Stomata formed at high relative air humidity (RH) respond less to abscisic acid (ABA), an effect that varies widely between cultivars. This study tested the hypotheses that this genotypic variation in stomatal responsiveness originates from differential impairment in intermediates of the ABA signalling pathway during closure and differences in leaf ABA concentration during growth.
- **Methods** Stomatal anatomical features and stomatal responsiveness to desiccation, feeding with ABA, three transduction elements of its signalling pathway (H_2O_2 , NO, Ca^{2+}) and elicitors of these elements were determined in four rose cultivars grown at moderate (60 %) and high (90 %) RH. Leaf ABA concentration was assessed throughout the photoperiod and following mild desiccation (10 % leaf weight loss).
- **Key Results** Stomatal responsiveness to desiccation and ABA feeding was little affected by high RH in two cultivars, whereas it was considerably attenuated in two other cultivars (thus termed sensitive). Leaf ABA concentration was lower in plants grown at high RH, an effect that was more pronounced in the sensitive cultivars. Mild desiccation triggered an increase in leaf ABA concentration and equalized differences between leaves grown at moderate and high RH. High RH impaired stomatal responses to all transduction elements, but cultivar differences were not observed.
- **Conclusions** High RH resulted in decreased leaf ABA concentration during growth as a result of lack of water deficit, since desiccation induced ABA accumulation. Sensitive cultivars underwent a larger decrease in leaf ABA concentration rather than having a higher ABA concentration threshold for inducing stomatal functioning. However, cultivar differences in stomatal closure following ABA feeding were not apparent in response to H_2O_2 and downstream elements, indicating that signalling events prior to H_2O_2 generation are involved in the observed genotypic variation.

Key words: Abscisic acid signal transduction, calcium, hydrogen peroxide, methyl jasmonate, nitric oxide, *Rosa hybrida*, salicylic acid, stomatal closure, stomatal malfunction, transpiration.

INTRODUCTION

Abscisic acid (ABA) has been defined as a stress hormone because a rapid increase in its synthesis is elicited by various stresses, mediating plant responses to cope with the applied stress (Leung and Giraudat, 1998; Zhang *et al.*, 2006). It is now well accepted that ABA also exerts an important regulatory role in stomatal functioning in the absence of stress (Franks and Farquhar, 2001; Wigger *et al.*, 2002). For instance, plants grown at high relative air humidity (RH \geq 85 %) display very low transpirational water loss and are minimally exposed to water deficit, as manifested by the higher leaf water potential, compared with well-watered plants grown at moderate atmospheric humidity (Fanourakis *et al.*, 2011). However, long-term high RH also results in decreased leaf ABA concentration ([ABA]) (Rezaei Nejad and van Meeteren, 2007; Arve *et al.*, 2013), which in turn induces phenotypes with weak stomatal responsiveness (Fanourakis *et al.*, 2012; Rezaei Nejad and van Meeteren, 2008). As a consequence, in environments where evaporative demand exceeds transport capacity, high-RH-grown plants frequently wilt even when soil water is readily available due to their inability to limit transpiration (Arve *et al.*, 2013; Fanourakis *et al.*, 2013b). Recently, a wide variation in the

degree to which this effect appears was found among cultivars of the same species (Fanourakis *et al.*, 2013a; Giday *et al.*, 2013). One illustrative result is that high atmospheric humidity decreased the leaflet hydration level at which the maximum stomatal closure occurred by between 12 and 143 % (Giday *et al.*, 2013). Fanourakis *et al.* (2013a) discuss the idea that cultivar differences are related to variation in [ABA] or the [ABA] threshold required for inducing stomatal functioning, but do not offer experimental evidence. Assessment of the reasons underlying the described genotypic variation is of significant interest for our understanding of the relationship between [ABA] during growth and the control of water loss by plants.

Like plants with long-term decreased [ABA], a number of ABA-insensitive mutants displayed weak stomatal responsiveness and were thus highly prone to lethal wilting (Koornneef *et al.*, 1984). Later studies demonstrated that certain signalling events of the ABA transduction cascade were impaired or disrupted, and when these were experimentally activated the stomata of ABA-insensitive mutants reacted similarly to wild type (Allen *et al.*, 1999; Iwai *et al.*, 2003). These results indicate that lack or attenuation of intermediate elements in the ABA signalling network do not necessarily affect the signalling components downstream from these regions. ABA induction of

stomatal closure is mediated by two responses operating on different time scales (Geiger *et al.*, 2011; Brandt *et al.*, 2012). The slower response (minutes to hours) involves gene expression under transcriptional regulation (Fujii *et al.*, 2009; Kline *et al.*, 2010; for potential effects of high RH see Aliniaiefard and van Meeteren, 2013). The fast response (seconds to minutes) encompasses the ABA receptors [PYRabactin Resistance (PYR)/PYR1-like (PYL)/regulatory components of ABA receptor (RCARs)] together with positive [SNF1-related protein kinase (SnRK2)/open stomata 1 (OST1)] and negative [type 2C protein phosphatases (PP2Cs)] pathway modulators (reviewed by Cutler *et al.* 2010; for potential effects of high RH see Aliniaiefard and van Meeteren, 2013). Key intermediates, acting downstream of the fast response pathway components, on sequence include hydrogen peroxide (H₂O₂) (Pei *et al.*, 2000), nitric oxide (NO) (Garcia-Mata *et al.*, 2003; Bright *et al.*, 2006) and calcium (Ca²⁺) (McAinsh, 1990). Growing evidence suggests that these three elements serve similar functions in the stomatal response pathways of methyl jasmonate (MJ) (Suhita *et al.*, 2004; Munemasa *et al.*, 2011a, b) and salicylic acid (SA) (Mori *et al.*, 2001; Khokon *et al.*, 2011), regulating physiological processes such as plant defence against microorganisms (Suhita *et al.*, 2004; Zeng *et al.*, 2011). Despite a well-documented genotypic effect on the degree to which stomatal sensitivity to ABA is attenuated by elevated atmospheric humidity (Fanourakis *et al.*, 2013a), it is less clear whether it originates from variation in fast response pathway components or downstream intermediates. Given that the ABA, MJ and SA networks converge on the regulation of stomatal closure, it may be expected that the latter two pathways are also differentially impaired by high RH during growth.

Four cultivars with contrasting sensitivity to elevated RH were selected to determine the relationship between [ABA] and regulation of water loss in high-RH-grown plants. We also examined whether the ability of the leaf to accumulate ABA contributed to the intraspecific variation in [ABA]. Another objective of this study was to examine whether intermediate elements (H₂O₂, NO, Ca²⁺) in the ABA signalling network contribute to the genotypic variation in stomatal response after leaf development in more humid air. We tested the hypotheses that genotypic variation in the control of water loss is primarily driven by differential effects on [ABA] rather than variation in the [ABA] threshold required for inducing stomatal functioning, and that it originates from intermediate elements in the ABA signalling pathway.

MATERIALS AND METHODS

Plant material and growth conditions

Rooted cuttings of four pot rose cultivars (*Rosa hybrida* ‘Apache Kordana’, ‘Pearl Kordana’, ‘Escimo Kordana’ and ‘Mandarina Kordana’) were planted in 0.55 L pots (four cuttings of the same cultivar per pot) containing a mixture of peat and perlite (9:1, v/v; Meegaa Substrates BV, Rotterdam, the Netherlands) at a commercial nursery (Rosa Danica, Marslev, Denmark). In subsequent parts of this paper, cultivars will be referred to without the (common) second part of their name (e.g. ‘Apache’ in place of ‘Apache Kordana’). Cultivar selection was based on their contrasting water loss rates following growth at high RH: ‘Apache’ and ‘Pearl’ showed transpirational

water loss rates similar to plants grown at moderate RH (defined as tolerant to high RH), whereas ‘Escimo’ and ‘Mandarina’ had considerably higher water loss compared with moderate RH-grown plants (defined as sensitive to high RH) (Giday *et al.*, 2013). Four weeks after planting, the plants were pruned, leaving two or three leaves on the shoot. Subsequently, 20 pots per cultivar were obtained from the nursery and placed in two walk-in growth chambers at a density of 25 pots m⁻². Each chamber accommodated two tables, established as plots, every plot having five randomly distributed pots of each cultivar. In one chamber the RH was 60 ± 3 % (moderate RH) and in the other it was 90 ± 2 % (high RH). Air temperature was kept constant (20.5 ± 1.4 °C) in both chambers, resulting in vapour pressure deficits (VPDs) of 0.94 ± 0.07 and 0.23 ± 0.05 kPa for moderate and high RH chambers, respectively. Light was provided by fluorescence lamps (HQI-BT 400W/D Pro, Slovakia) at 400 ± 15 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD; determined with a LI-250A; LI-COR, Lincoln, NE, USA) for 18 h per day. Air temperature and RH were measured continuously with sensors (Humitter 50U/50Y(X), Vaisala, Helsinki, Finland) placed at the top of fully grown plants (i.e. 60 cm from the root–shoot interface), and data were automatically recorded by loggers (Dataaker; Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Australia). Plants were fertigated twice a day (>20 % drainage day⁻¹). Electrical conductivity and pH of the drain water were monitored daily and adjusted to 2 dS m⁻¹ and 5.5, respectively. Border plants (adjacent to chamber walls) were not sampled. All measurements were conducted on fully expanded sunlit leaves, sampled from fully grown plants (defined as bearing at least two flower buds with cylindrical shape and pointed tip).

Stomatal and pore anatomy

To study the effect of high RH on stomatal anatomy, the length, width and density (i.e. number per unit leaf area) of stomata, together with pore length and aperture, were assessed in the four cultivars. The stomatal and pore lengths correspond to the major axes (i.e. longest diameter) of the respective ellipse, while stomatal width and pore aperture were taken as the minor axes (i.e. shortest diameter) (Savvides *et al.*, 2012). Measurements were made by the silicon rubber impression technique (Giday *et al.*, 2013) using a lateral leaflet of the first pinnate leaf (counting from the apex). The abaxial (lower) leaflet surface was assessed, since the rose is a hypostomatous species (Fanourakis *et al.*, 2013a). Sampling took place 2 h following the onset of the light period, since this time is required for plants exposed to prolonged darkness (i.e. night-time period) to open stomata and reach a steady-state operating stomatal conductance (Drake *et al.*, 2013). Stomatal (length, width) and pore (length, aperture) anatomical features were determined on 30 randomly selected stomata per leaflet, and stomatal density was counted on five non-overlapping fields of view. Five leaflets (one leaflet per plant, one plant per pot) were assessed.

Stomatal responsiveness to seven closing stimuli

To determine the effect of high RH on stomatal responsiveness to closing stimuli in the four cultivars, terminal leaflets of the first and second five-leaflet leaves (counting from the apex) were sampled, re-cut by submerging their petiole under water

(to prevent cavitation of xylem vessels that were opened by cutting) and placed in flasks filled with degassed water. Leaflets were harvested at least 2 h after the beginning of the photoperiod. The environmental conditions in the test room, where all closing stimuli were applied, were as follows: air temperature 21.0 ± 1.2 °C, RH 50 ± 4 % (i.e. VPD 1.24 ± 0.03 kPa), and $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD provided by fluorescent lamps (T5 fluorescent lamp, GE Lighting, Cleveland, OH, USA).

For the desiccation stimulus, leaflets were further incubated at 21 °C, about 100 % RH (i.e. VPD close to 0) and under $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 1 h to establish their maximum fresh weight (Fanourakis *et al.*, 2011). The leaflets were then placed on a bench (abaxial surface facing down) in the test room and transpiration rate was recorded for 4 h by gravimetry. At the end of the measurement, leaflet area was determined (LI-250A, LI-COR, Lincoln, NE, USA) and leaflets were dried in a forced-air oven at 80 °C for at least 48 h prior to determining dry weight. Leaflet relative water content (RWC) was calculated using the following equation (Slavik, 1974):

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

The hormones ABA, MJ and SA, together with three common elements of their signalling pathways (H_2O_2 , NO and Ca^{2+}), were also applied through the transpiration stream, as stomatal closing treatments. For these stimuli, the leaflets were left for 1 h with their petioles in water (based on preliminary experiments) in the test room in order to reach a steady state prior to measurements. Subsequently, the transpiration rate of the leaflets in water was recorded by regular weighings (every 20 min) for 1 h. The leaflets were then transferred to flasks containing water (as a control), 100 μM ABA (Fanourakis *et al.*, 2013a), 200 μM MJ (Herde *et al.*, 1997), 1 mM SA (Manthe *et al.*, 1992), 200 μM H_2O_2 (Bright *et al.*, 2006), 200 μM sodium nitroprusside [SNP (NO donor)] (He *et al.*, 2004; Rezaei Nejad *et al.*, 2006) or 8 mM Ca^{2+} (Atkinson *et al.*, 1990). The leaflet transpiration rate was further determined by weighing the flasks every 20 min for 4 h 40 min. Throughout the measurements, leaflets in the vials were maintained inclined at 10 ° to the horizontal, and flasks were sealed with parafilm to prevent evaporation. Following the measurements, leaflet area was determined as mentioned above. Closing stimulus intake was calculated as the product of leaflet transpiration rate and the closing stimulus concentration of the feeding solution (Fanourakis *et al.*, 2013a).

Measurements were carried out on eight (petiole-fed closing stimuli) or nine (desiccation) leaflets (one leaflet per plant, one plant per pot).

Deriving the closing stimulus intake ($\text{EC}_{1/2}$) inducing 50 % of stomatal response

For the xylem-fed closing stimuli (i.e. ABA, MJ, SA, SNP, H_2O_2 , Ca^{2+}), the change in transpiration rate in response to closing stimulus intake showed the typical features of a dose-response curve (stimulus intake was treated as the dose), and was fitted with a four-parameter logistic model

$$y = c + \frac{d - c}{1 + \left(\frac{x}{\text{EC}_{1/2}}\right)^b} \quad (2)$$

(Secchi *et al.*, 2013). The coefficients d (maximum value) and c (minimum value) correspond to the transpiration rates prior to ($t = 0$) and following ($t = 4$ h 40 min) feeding, respectively. The half-maximal effective concentration ($\text{EC}_{1/2}$) refers to the stimulus intake required to induce 50 % of the change between the maximum and minimum values (known as the inclination point), while b is the relative slope at $\text{EC}_{1/2}$ (the so-called Hill slope). The $\text{EC}_{1/2}$ therefore represents the fed amount of a closing stimulus at which 50 % of its maximal effect is observed. By using eqn (2), values for b , c , d and $\text{EC}_{1/2}$ were determined in response to the six closing stimuli applied through the petiole for each leaflet ($n = 8$) in the four studied cultivars. In all cases, an F -test for lack of fit was performed, indicating that data were well described ($P > 0.05$) by eqn (2).

[ABA] during growth and following desiccation

The effect of ambient humidity on [ABA] was determined in the four cultivars at three different times during the photoperiod. Five-leaflet leaves were collected at 0, 4 and 8 h following the onset of the light period, immediately frozen in liquid nitrogen and stored at -80 °C for further analysis.

In addition, it was assessed whether the [ABA] response to desiccation was affected by the RH of growth in 'Escimo' (the most sensitive) and 'Apache' (the most tolerant). Terminal leaflets were collected 2 h following the beginning of the photoperiod, and the same procedure as that applied for leaflet desiccation was followed, as described above. Leaflets were allowed to dehydrate to 90 % of the initial fresh weight (corresponding to 85 ± 2 % RWC). Immediately afterwards, the leaflets were sealed in polyethylene bags (to prevent further water loss) and stored in darkness (21.0 ± 0.1 °C) for 6 h. The darkening was applied to prevent ABA degradation, since ABA is light-sensitive (Davies and Jones, 1991). Following the dehydration event, leaflets were sampled at three different times (0, 3 and 6 h), immediately frozen in liquid nitrogen and stored at -80 °C for further analysis. Following 6 h of incubation, further weight loss was less than 1 % of leaflet weight, indicating the effectiveness of the sealing method.

Liquid nitrogen-frozen samples were freeze-dried, ground and homogenized (Genogrinder 2000; Metuchen, NJ, USA). Deuterated internal standard (2.4 ng mL^{-1}) was then added to the homogenized samples, before extraction with an accelerated solvent extraction system (ASE 350, Dionex, Hvidovre, Denmark) as described by Pedersen *et al.* (2011). All extractions were duplicated and extracts were diluted with an equal volume of water before analysis.

Chromatographic separation was performed using an HPLC system (Agilent 1200; Agilent, Horsholm, Denmark) equipped with a 150×2.1 mm column (Kinetex 2.6 μm PFP, 100Å; Phenomenex, Macclesfield, UK). Gradient elution was performed with 7 % acetonitrile in 20 mM acetic acid (solvent A) and 78 % acetonitrile in 20 mM acetic acid (solvent B) at a constant flow rate of $200 \mu\text{L min}^{-1}$ and injection volume of $20 \mu\text{L}$. A gradient profile with the following proportions of solvent B was applied (t (min), %B): (0, 35), (6, 35), (7, 100), (8, 35), (18, 35). Prior to analysis, the system was equilibrated (8–18 min) with 35 % solvent B.

The chromatographic system was interfaced to a liquid chromatography triple quadrupole mass spectrometer (Sciex 3200;

Applied Biosystems, Foster City, CA, USA). The analysis was performed using electrospray ionization in negative mode. Multiple reaction monitoring of unlabelled and labelled ABA analogues was based on the 263 > 153 and 267 > 156 mass transitions for *cis*-ABA (Sigma-Aldrich, Brøndby, Denmark) and its deuterated analogue (d_4 *trans*-ABA; Plant Biotechnology Institute of the National Research Council of Canada, Saskatoon, SK, Canada), respectively. The calibration curve was prepared from seven ABA standard solutions (0.097–6.250 ng mL⁻¹) to which equal amounts (1.2 ng mL⁻¹) of internal standard were added. Subsequently, for each standard solution the analyte area (divided by the internal standard area) was plotted against the known analyte concentration (divided by the internal standard concentration). Data were analysed using the Analyst software v.1.5.1 (Applied Biosystems). The limits of detection (232.8 pmol ABA g⁻¹ dry weight plant tissue) and quantification (620.8 pmol ABA g⁻¹ dry weight plant tissue) were determined based on a recovery experiment consisting of four replicates (0.0195 ng ABA mL⁻¹).

In these measurements, first and second five-leaflet leaves (counting from the apex) were sampled, and eight replicate leaves (one leaf per plant, one plant per pot) were analysed per treatment. The whole leaf (i.e. the five leaflets were pooled) was analysed for the determination of [ABA] at different times in the photoperiod, whereas only the terminal leaflet was sampled when applying desiccation and subsequently measuring [ABA].

Statistical analysis

Data were subjected to analysis of variance using R software (version 2.14.2; www.r-project.org). Because no significant plot effects were revealed by ANOVA (i.e. minimal microsite influence), data for the two plots were allowed to pool (i.e. individual plants were treated as independent observations) for further

analysis. Treatment effects were tested at the 5 % probability level and the mean separation was done using least significant differences based on the Bonferroni-adjusted LSD ($P = 0.05$).

RESULTS

Stomatal and pore anatomy

The effect of high (90 %) RH on stomatal anatomical features was investigated in the four cultivars (Table 1). Leaves expanded at high RH had a higher (5 %) number of stomata per leaf area. Moreover, stomata formed at high RH not only showed enhanced length (3–8 %, though not significant for ‘Apache’) and width (2 %), but also possessed longer (9–16 %) and wider (7 %) pores.

Stomatal response to desiccation

Water loss through the stomatal pores has been identified as the principal determinant of the high-RH-induced increase in leaf transpiration in various rose cultivars, since cuticular water loss makes a minor contribution (Fanourakis *et al.*, 2013a). Against this background, the transpiration rate in response to leaflet desiccation was measured to evaluate the effect of growth RH on stomatal opening in the four cultivars (Fig. 1). Following a transient increase in water loss (known as a wrong-way response), transpiration rate decreased as leaflets dehydrated. In general, stomata on leaves expanded at high RH remained more open, leading to higher transpirational water loss and more dehydrated leaves (i.e. lower RWC) following desiccation. However, the extent to which this effect was observed depended strongly on the cultivar. For instance, the RWC at the end of desiccation was significantly affected by the interaction between RH and cultivar ($P < 0.01$). RWC at the end of desiccation was slightly decreased (≈ 7 %; Fig. 1A, B) by high

TABLE 1. Stomatal and pore anatomical features of four pot rose cultivars grown at moderate (60 %) or high (90 %) relative air humidity (RH)

		Stomatal			Pore	
		Density (mm ⁻²)	Length (μm)	Width (μm)	Length (μm)	Aperture (μm)
RH	60	49 ^b	40	31 ^b	22	8.1 ^b
	90	51 ^a	43	32 ^a	25	8.7 ^a
Cultivar ‘Apache’	60	45	39 ^d	29	21 ^c	6.4
	90	45	40 ^{cd}	29	23 ^b	6.8
‘Pearl’	60	55	40 ^c	31	20 ^c	6.7
	90	58	42 ^b	32	23 ^b	7.4
‘Escimo’	60	37	43 ^b	34	24 ^b	11.0
	90	40	45 ^a	34	26 ^a	11.3
‘Mandarina’	60	59	40 ^{cd}	32	23 ^b	8.4
	90	62	43 ^b	32	27 ^a	9.3
F probability						
Cultivar		***	***	***	***	***
RH		**	***	*	***	***
Cultivar × RH		NS	***	NS	**	NS

Measurements were made 2 h following the onset of the light period. Five fields of view (stomatal density) and 30 stomata (stomatal and pore dimensions) were analysed per leaf. Values are the means of five leaves. Means followed by different letters indicate significant differences according to the Bonferroni-adjusted LSD test (comparison in columns).

NS, not significant; *significant at the 0.05 probability level; **significant at the 0.01 probability level; ***significant at the 0.001 probability level.

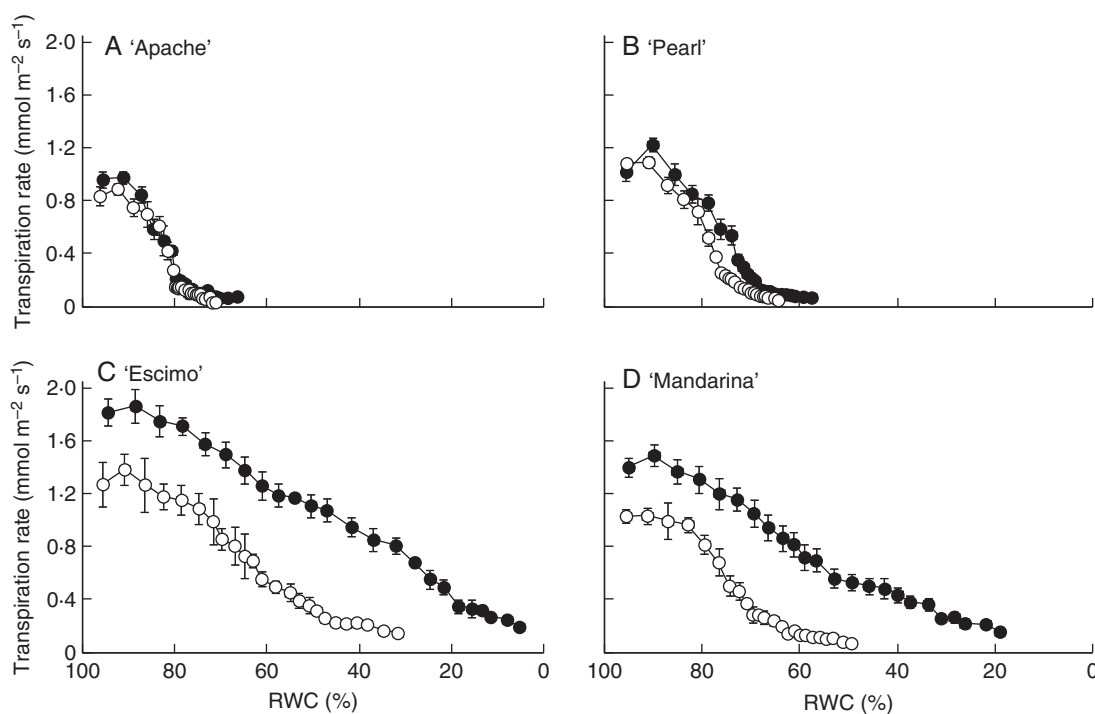


FIG. 1. Transpiration rate as a function of relative water content (RWC) during leaflet desiccation of four pot rose cultivars grown at moderate (60 %, open symbols) or high (90 %, closed symbols) relative air humidity. Leaflets were left to desiccate for 4 h. Values are means of nine leaflets \pm SEM.

RH in ‘Apache’ and ‘Pearl’ (defined as tolerant to high RH), whereas RWC was considerably lower (52–75 %; Fig. 1C, D) in high-RH-grown leaves of ‘Escimo’ and ‘Mandarina’ (defined as sensitive to high RH).

Relationship between stomatal responsiveness and [ABA] in growth conditions

[ABA] was determined at different times during the photo-period in the studied cultivars grown (and assessed) under both RH conditions. [ABA] was found to be significantly affected by the interaction between RH and cultivar ($P < 0.01$; Fig. 2). In the tolerant cultivars (‘Apache’, ‘Pearl’), [ABA] was slightly lower (7–11 %; Fig. 2A, B) in leaves developed at high RH, though high-RH-grown leaves of the sensitive cultivars (‘Escimo’, ‘Mandarina’) had a substantially decreased [ABA] (25–32 %; Fig. 2C, D). These differences were similar throughout the light period, since [ABA] did not change significantly during this time (Fig. 2).

It was also investigated whether [ABA] at growth conditions underlay variation in stomatal responsiveness, as expressed by dehydration curves. The correlation between [ABA] under growth conditions and RWC following desiccation was found to be highly significant (Fig. 3).

[ABA] in response to desiccation

Since [ABA] at growth conditions was lower in high-RH-grown leaves compared to plants developed at moderate RH (Fig. 2), it was tested whether [ABA] response to water deprivation was impaired by high RH. This analysis was performed in the most tolerant (‘Apache’) and most sensitive (‘Escimo’) cultivars. Leaflets

were slightly desiccated (to 85 % RWC), and subsequently stored under conditions where further water loss was prevented. In all cases, dehydration induced an increase in [ABA] (Fig. 4). Leaves expanded at high RH had lower [ABA] immediately after desiccation ($t = 0$), but later on they reached [ABA] similar to that of leaves developed at moderate RH. The sensitive cultivar showed lower [ABA] than the tolerant cultivar following desiccation, but this difference was not noticeable after 6 h.

Stomatal responsiveness to closing stimuli applied through the transpiration stream

Stomatal closing ability in response to three hormones (ABA, MJ and SA) and three downstream elements of their signalling pathways [H_2O_2 , NO (induced by SNP) and Ca^{2+}] was investigated in the four cultivars grown at both RH levels. As an example, the temporal dynamics of transpiration rate in response to all six closing stimuli is presented in ‘Apache’ grown at moderate RH (Fig. 5). ABA induced the highest decrease in transpiration rate ($P < 0.001$), followed by the three assessed signalling elements, which induced similar responses. MJ and SA elicited the least decrease in transpiration ($P < 0.001$) compared with the rest closing stimuli. Figure 6 shows the changes in transpiration rate in response to closing stimulus intake, the latter being the product of closing stimulus concentration in the feeding solution and the leaflet transpiration rate. It becomes apparent here that ABA induced stomatal closure by the least intake (i.e. the fed amount), while the Ca^{2+} intake required to elicit the stomatal response was enormously higher (≈ 80 -fold) ($P < 0.001$). By fitting eqn (2) to these data, the fed amount of a given closing stimulus (EC_2) required to generate 50 % of its maximal decrease in the transpiration rate was calculated.

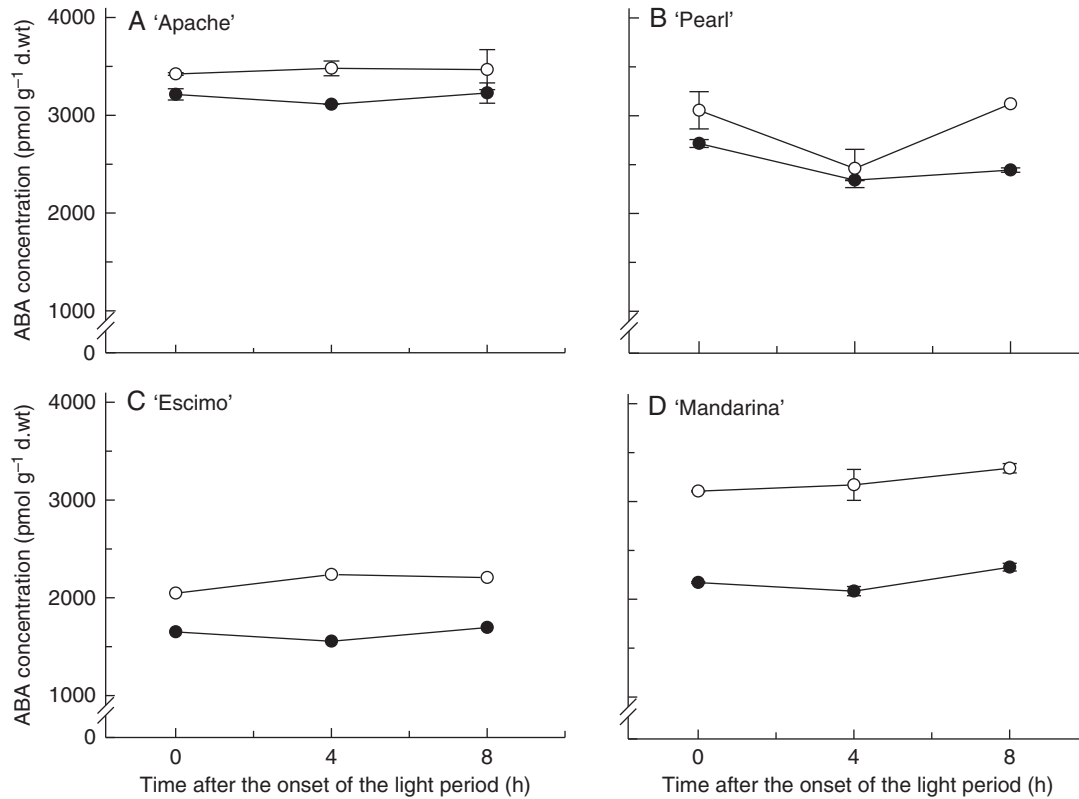


FIG. 2. Endogenous leaflet ABA concentration, expressed on a dry weight (d.wt) basis, at different times after the onset of the light period of four pot rose cultivars grown at moderate (60 %, open symbols) or high (90 %, closed symbols) relative air humidity. Values are the means of either one (C) or two (A, B, D) extractions. Eight leaflets were pooled for extraction.

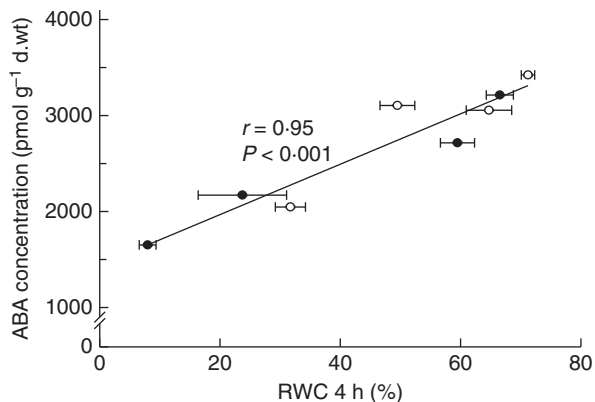


FIG. 3. Endogenous leaflet ABA concentration, expressed on a dry weight (d.wt) basis, before the onset of the light period as a function of relative water content (RWC) 4 h after leaflet desiccation of four pot rose cultivars grown at moderate (60 %, open symbols) or high (90 %, closed symbols) relative air humidity. ABA concentrations refer to either one ('Escimo') or two (eight leaflets per extraction) extractions, and RWC values are the means of nine leaflets.

Table 2 shows the effect of growth RH on stomatal closing behaviour in response to the assessed closing stimuli. When examining the decrease in stomatal closure in response to ABA or MJ feeding, a clear separation between tolerant ('Apache', 'Pearl') and sensitive ('Escimo', 'Mandarina') cultivars was observed. The decrease in stomatal closure in response to Ca^{2+} (Fig. 7) or the remaining closing treatments was lower in leaves

developed at high RH, but there was no RH by cultivar interaction (i.e. tolerant cultivars reacted like sensitive cultivars; Table 2). Comparable effects were observed when the effect of high RH on $\text{EC}_{1/2}$ was assessed (Table 2). The $\text{EC}_{1/2}$ values for ABA and MJ were significantly increased by high RH in the sensitive cultivars, but no significant difference was observed between moderate and high RH in the tolerant cultivars. For the remaining stimuli, there was no effect of high RH on $\text{EC}_{1/2}$, other than for Ca^{2+} (26 % higher $\text{EC}_{1/2}$ in high-RH-grown leaves compared to moderate RH).

DISCUSSION

Plants grown in elevated atmospheric humidity conditions show limited control of water loss and increased lethality upon transfer to environments of increased evaporative demand (reviewed by Aliniaefard and van Meeteren, 2013; Fanourakis *et al.*, 2013b). The incidence of adverse water relations, however, strongly depends on the cultivar (Giday *et al.*, 2013). This is the first report in which the immediate origin (i.e. during closure) and long-term cause (i.e. in the course of leaf expansion) of genotypic variation in stomatal responsiveness, caused by high growth RH, are revealed.

Genotypic variation is related to [ABA] during growth

Longer stomata with larger pores have been noted following growth at high RH (Table 1); this is an anatomical adaptation

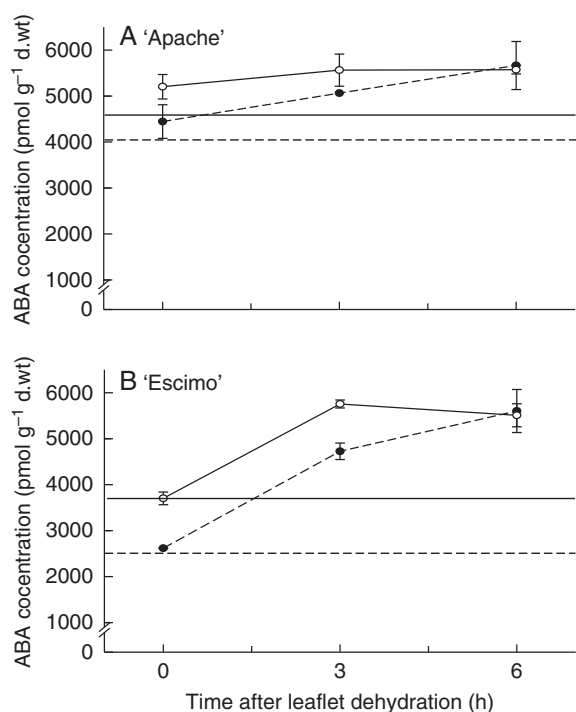


FIG. 4. Endogenous leaflet ABA concentration, expressed on a dry weight (d.wt) basis, at different times after leaflet dehydration to 85 % relative water content, of two pot rose cultivars grown at moderate (60 %, open symbols) or high (90 %, closed symbols) relative air humidity (RH). Following dehydration, leaflets were sealed in polyethylene bags and incubated in darkness. Horizontal lines (solid and dashed for 60 and 90 % RH, respectively) denote leaflet ABA concentration before dehydration. Leaflets were sampled 2 h after the onset of the light period, and ABA concentration was calculated as the average of the concentrations at 0 and 4 h (see Fig. 2). Values are the means of two extractions. Eight leaflets were pooled for extraction.

typical of several species, in which leaves expanded in more humid air (Fanourakis *et al.*, 2013a). Like stomatal anatomy, stomatal functionality is established during growth (Fanourakis *et al.*, 2011; Pantin *et al.*, 2013b). The effect of high RH on stomatal responsiveness, expressed both by desiccation curves (Fig. 1) and by feeding through the petioles with ABA (Table 2), was clustered into two distinct groups. Stomata of tolerant cultivars (i.e. 'Apache' and 'Pearl') were little affected by high RH, whereas stomata of sensitive cultivars (i.e. 'Escimo' and 'Mandarina') were much less responsive after growth at high RH. Observations of reduced stomatal closure in plants grown at high RH and consistent cultivar differences upon desiccation or ABA feeding have also been reported previously (Fanourakis *et al.*, 2013a).

Environmental conditions (e.g. *in vitro* culture) or genetic factors (e.g. ABA-biosynthetic mutants or transgenic plants expressing an anti-ABA antibody) that cause a long-term decline in [ABA] also result in impaired stomatal closing ability (Artsaenko *et al.*, 1995; Leon-Kloosterziel *et al.*, 1996; Aguilar *et al.*, 2000). By examining a single cultivar, recent reports indicated that plants grown at high RH also have decreased [ABA], which mediates the weak stomatal responsiveness of these plants (Arve *et al.*, 2013; Rezaei Nejad and van Meeteren, 2007). In the present study it was found that the decrease in [ABA], as a result of high RH during growth, primarily

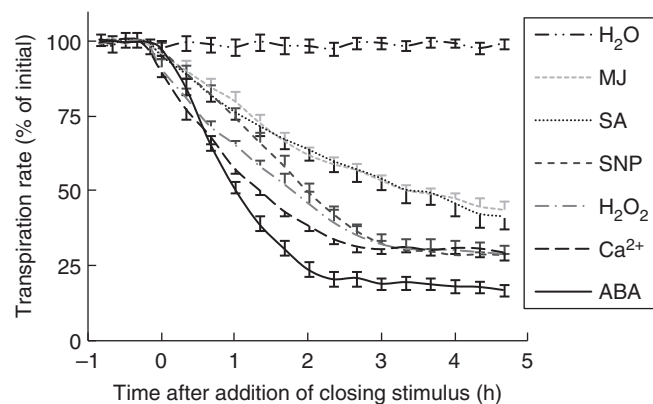


FIG. 5. Leaflet transpiration rate as a function of time following feeding through the petiole with a range of closing stimuli (concentrations are given in Table 2) of pot rose 'Apache' grown at moderate (60 %) relative air humidity. The transpiration rate of leaflets placed in water was recorded for 1 h prior to the closing stimulus feeding ($1.26 \pm 0.02 \text{ mmol m}^{-2} \text{ s}^{-1}$), and subsequent transpiration rates were expressed relative to that value, i.e. $[(\text{transpiration rate in water}) - (\text{transpiration rate})] / (\text{transpiration rate in water})$. Data are averages of eight replications \pm SEM.

occurred in the sensitive cultivars (Fig. 2). More importantly, stomatal responsiveness was positively related to [ABA] during growth among cultivars and RHs (Fig. 3). This indicates that the [ABA] threshold for inducing stomatal functioning was similar among cultivars that differ in their sensitivity to high RH.

Whether the reduced [ABA] of high-RH-grown leaves persisted following water stress was also examined. Following very mild desiccation (i.e. to 85 % RWC), [ABA] increased in high-RH grown leaves and reached the [ABA] of leaves developed at moderate RH (Fig. 4). Consequently, although high RH resulted in lower [ABA] during growth, it did not affect the [ABA] in response to water deficit. Moreover, it was shown that a mild stress during growth at high RH is sufficient to induce [ABA] that is similar to [ABA] of plants developed at moderate RH.

Stomata on desiccating angiosperm leaves respond to both hydraulic (i.e. decrease in water potential) and biochemical (i.e. increase in endogenous ABA) signals, triggering the hydropassive and hydroactive stomatal closure mechanisms, respectively (Brodrick and McAdam, 2011; Franks, 2013). Upon desiccation, ABA redistribution from existing pools and synthesis of new ABA increase endogenous [ABA] (Harris and Outlaw, 1991; Finkelstein and Rock, 2002). We show that although resting [ABA] may be smaller prior to desiccation in high-RH-grown leaves depending on the cultivar (Fig. 2), ABA levels rise substantially following water deprivation (Fig. 4). Consequently, the impaired stomatal closure in response to desiccation observed in leaves developed at high RH (Fig. 1) is the sole result of reduced stomatal sensitivity to hydraulic and biochemical closing signals, rather than a combination of this and reduced ABA accumulation (Fig. 8).

Genotypic variation originates from differential impairment of signalling components between ABA perception and H₂O₂ generation

ABA elicits stomatal closure via a complex signalling cascade (reviewed by Dodd *et al.*, 2010; Hubbard *et al.*, 2010; Kim *et al.*,

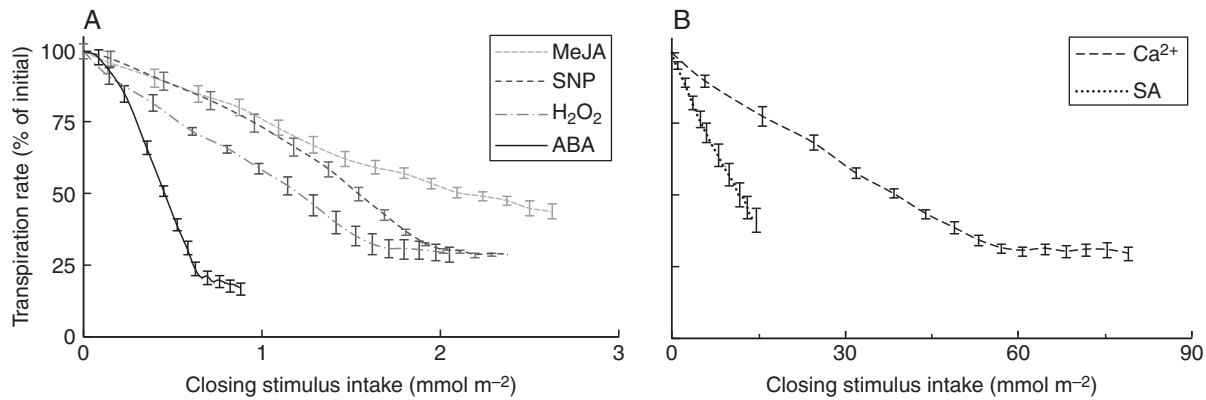


FIG. 6. Leaflet transpiration rate as a function of different closing stimuli intake through the petiole (concentrations are given in Table 2) of pot rose ‘Apache’ grown at moderate (60 %) relative air humidity. Closing stimulus intake was calculated as the product of leaflet transpiration rate and closing stimulus concentration. The transpiration rate of leaflets placed in water was recorded for 1 h prior to the closing stimulus feeding ($1.26 \pm 0.02 \text{ mmol m}^{-2} \text{ s}^{-1}$), and subsequent transpiration rates were expressed relative to that value, i.e. $[(\text{transpiration rate in water}) - (\text{transpiration rate})] / (\text{transpiration rate in water})$. Data are averages of eight replications \pm SEM.

TABLE 2. Transpiration rate of leaflets placed in water, change in transpiration rate following 4 h 40 min feeding through the petiole (i.e. final value relative to the value prior to feeding) with a range of closing stimuli [$100 \mu\text{M}$ ABA, $200 \mu\text{M}$ MJ, 1 mM SA, $200 \mu\text{M}$ H_2O_2 , $200 \mu\text{M}$ SNP (NO donor), 8 mM Ca^{2+}] and the closing stimulus intake (EC_{50}) required to induce 50 % of this change in four pot rose cultivars grown at moderate (60 %) or high (90 %) relative air humidity (RH)

	RH	Transpiration rate prior to feeding ($\text{mmol m}^{-2} \text{ s}^{-1}$)	EC_{50} (mmol m^{-2})						Decrease in transpiration rate (%)					
			ABA	MJ	SA	H_2O_2	SNP	Ca^{2+}	ABA	MJ	SA	H_2O_2	SNP	Ca^{2+}
	60	1.27	0.4	1.1	6.6	1.1	1.3	39 ^b	79	57	54 ^a	61 ^a	66 ^a	59 ^a
	90	1.32	0.6	1.6	6.7	1.0	1.5	49 ^a	66	40	37 ^b	42 ^b	45 ^b	45 ^b
Cultivar														
‘Apache’	60	1.26	0.4 ^b	1.3 ^{abc}	7.5	1.1	1.5	33	83 ^{ab}	55 ^a	58	70	71	71
	90	1.33	0.4 ^b	1.3 ^{abc}	6.5	1.1	1.7	49	77 ^{ab}	49 ^a	46	54	49	56
‘Pearl’	60	1.17	0.4 ^b	1.1 ^{bc}	5.6	0.7	1.1	31	81 ^{ab}	63 ^a	66	55	70	60
	90	1.19	0.5 ^b	1.0 ^{bc}	6.3	0.7	1.2	33	82 ^{ab}	56 ^a	42	45	56	50
‘Escimo’	60	1.40	0.4 ^b	0.9 ^c	6.1	1.5	1.1	65	67 ^{bc}	54 ^a	51	65	63	49
	90	1.44	0.9 ^a	1.9 ^a	5.7	1.4	1.8	71	50 ^d	25 ^b	34	34	38	33
‘Mandarina’	60	1.23	0.4 ^b	1.0 ^c	7.1	0.9	1.4	29	84 ^a	58 ^a	43	54	61	56
	90	1.33	0.7 ^a	1.9 ^{ab}	8.2	0.8	1.5	43	54 ^{cd}	31 ^b	25	36	36	39
F probability														
Cultivar		**	***	NS	NS	*	NS	***	***	***	***	*	***	***
RH		NS	***	**	NS	NS	NS	*	***	***	***	***	***	***
Cultivar \times RH		NS	***	**	NS	NS	NS	NS	***	**	NS	NS	NS	NS

EC_{50} was calculated by fitting a four-parameter logistic model [a dose–response relationship, where stimulus intake was treated as the dose; eqn (2)] to the transpiration rate versus closing stimulus intake data. Leaflets were placed in water for 1 h prior to closing stimulus feeding. Data refer to eight replications. Means followed by different letters indicate significant differences according to the Bonferroni-adjusted LSD test (comparison in columns).

NS, not significant; *significant at the 0.05 probability level; **significant at the 0.01 probability level; ***significant at the 0.001 probability level.

2010). Small signalling molecules (the so-called secondary messengers) are key elements of this pathway. For instance, ABA-induced stomatal closure has been associated with increases in cytosolic free Ca^{2+} (McAinsh, 1990), preceded by generation of both H_2O_2 (Pei *et al.*, 2000) and NO (Garcia-Mata *et al.*, 2003). Although Ca^{2+} -independent signalling pathways exist, Ca^{2+} -related mechanisms largely (around 70 %) account for stomatal closure (Siegel *et al.*, 2009). H_2O_2 , NO and Ca^{2+} have been proposed to also act as secondary messengers in the stomatal response pathways of both MJ (Suhita *et al.*, 2004; Munemasa *et al.*, 2007) and SA (Mori *et al.*, 2001; Khokon *et al.*, 2011).

This study shows that the genotypic variation in stomatal response, following growth at high RH, was expressed when ABA or MJ were employed as closing signals, but not in response

to SA (Table 2; see also Fig. 8). This suggests that the signalling components that were differentially impaired by high RH likely belong to the common branch of the ABA and MJ pathways and are not required in the SA signalling network. Despite the fact that the SA pathway is the least understood, transduction elements that are independent of the ABA and MJ pathways have already been revealed (Khokon *et al.*, 2011; Zeng *et al.*, 2011). For instance, although H_2O_2 belongs to the cross-talk among the ABA, MJ and SA pathways, its production is elicited by different oxidase families [NAD(P)H oxidases for ABA and MJ; SHAM-sensitive peroxidases for SA] (Khokon *et al.*, 2011).

When leaves were fed with downstream elements of the ABA, MJ and SA signalling networks [i.e. H_2O_2 , NO (elicited by SNP), and Ca^{2+}], stomata formed at high RH closed less compared to

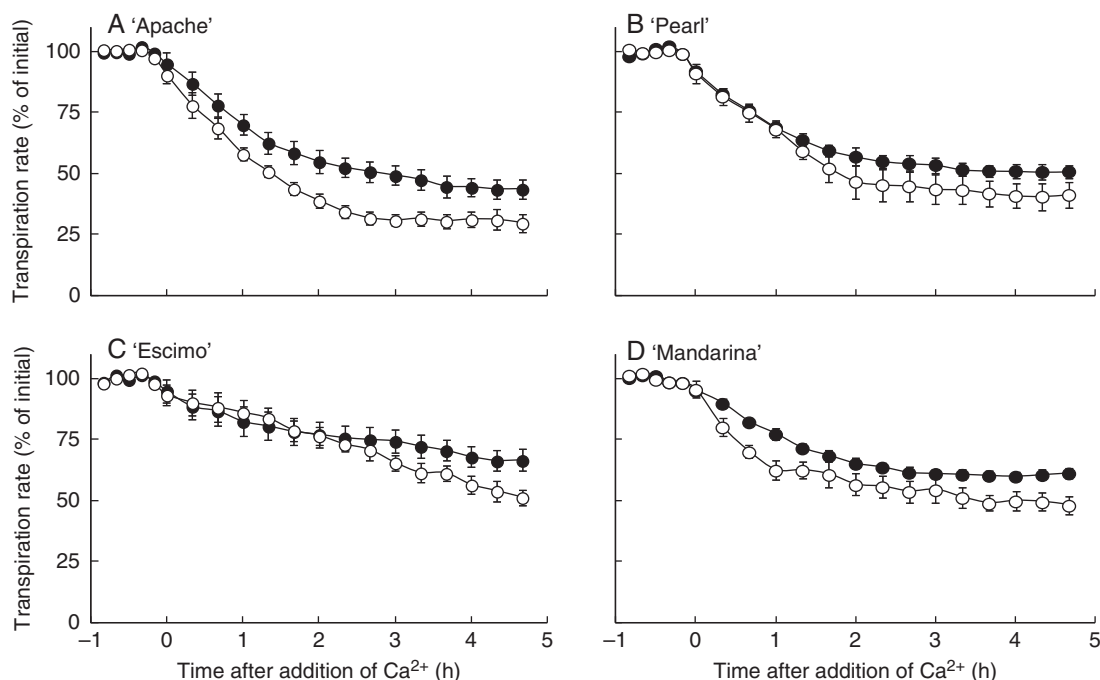


FIG. 7. Leaflet transpiration rate as a function of time following feeding through the petiole with 8 mM Ca^{2+} of four pot rose cultivars grown at moderate (60 %, open symbols) or high (90 %, closed symbols) relative air humidity. The transpiration rate of leaflets placed in water was recorded for 1 h prior to the closing stimulus feeding (values are given in Table 2), and subsequent transpiration rates were expressed relative to that value i.e. [(transpiration rate in water) – (transpiration rate)] / (transpiration rate in water). Data are averages of eight replications \pm SEM.

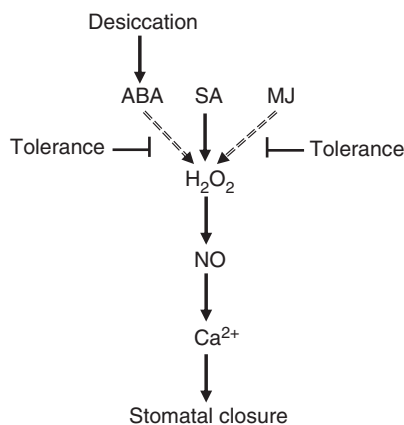


FIG. 8. Model summarizing the biochemical signalling elements generating genotypic variation in stomatal closure following growth at high (90 %) relative air humidity. Components connected with dashed arrows reveal cultivar differences. The model does not include the hydraulic signals induced by desiccation and petiole-fed ABA stimuli.

stomata on leaves developed at moderate RH, though this RH effect was consistent among cultivars differing in their sensitivity to high RH (Table 2; see also Fig. 8). Consequently, the high-RH-induced reductions in stomatal closure cannot be reversed by applying signalling elements downstream of the fast response pathway, as described in ABA-insensitive mutants (Allen *et al.*, 1999; Iwai *et al.*, 2003). These results also indicate that high growth RH impairs differentially the signalling components upstream of H_2O_2 generation, and that these elements contribute to the genotypic variation in stomatal

closure. Figure 8 summarizes the proposed model for the high RH-imposed genotypic variation in stomatal responsiveness through defects in ABA signalling. Identification of which specific element(s) is responsible will further advance our understanding of how long-term high RH impairs stomatal functioning in plants.

Our method of assessing ABA-induced stomatal closure could have missed the possibility that xylem-fed ABA also exerts an indirect hydraulic effect on stomata, mediated via decreases in leaf hydraulic conductivity (Pantin *et al.*, 2013a). Similarly to desiccation, it is not possible to distinguish whether hydraulic or biochemical signals elicit cultivar differences in ABA-induced stomatal response. However, the fact that xylem-fed MJ induces genotypic variation in the stomatal response (Table 2) clearly indicates that the biochemical signals were differentially affected by high growth RH.

Conclusions

High (90 %) RH during leaf expansion attenuates stomatal functionality to a cultivar-dependent extent. We have demonstrated here that this genetic variation in stomatal responsiveness is related to leaf ABA concentration during growth rather than the ABA concentration required to stimulate stomatal functioning. Very mild water stress applied to high-RH-grown leaves normalized differences in leaf ABA concentration between plants grown at moderate and high RH. The substantially large cultivar differences in stomatal closure when fed with ABA or MJ did not occur when H_2O_2 or downstream signalling elements were fed through the petiole. This suggests that genotypic variation in ABA-induced stomatal closure is associated with components

lying upstream of H₂O₂ generation. The differential stomatal responses triggered by both ABA and MJ were not induced by SA, providing further evidence in favour of diverging signalling pathways eliciting H₂O₂ production by these hormones.

ACKNOWLEDGEMENTS

We thank Ruth Nielsen, Kaj Ole Dideriksen and Helle Kjærsgaard Sørensen for their help in conducting the measurements, as well as Anne Garfield Mortensen and Bente Birgitte Laursen for assisting in ABA analysis. The valuable comments of two anonymous reviewers are greatly acknowledged. This work formed part of a PhD project supported by a grant from Aarhus University and linked to the project GreenGrowing, an Interreg NorthSea project.

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