# Stomatal functioning and abscisic acid (ABA) regulation in plants developed in different air humidity regimes

Stomatafunksjon og abscisinsyre- (ABA) regulering hos planter dyrket ved ulike luftfuktighetsregimer

Philosophiae Doctor (PhD) Thesis

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## Abstract

Greenhouse as a production system is important in the production of vegetables, herbs and ornamental plants. However, plant production in continuous high relative air humidity (RH) may results in poor plant quality, due to uncontrolled water loss after harvest. This water loss is caused by less functioning stomata that are larger in size and unable to close properly in environments that normally induce closure, like darkness and dry air. Since stomatal movement is regulated by abscisic acid (ABA) it has been hypothesized that low levels of ABA is the cause of the less functioning stomata.

In this study the ability of *Rosa x hybrida*, *Arabidopsis thaliana* and *Vicia faba* developed in continuous high (90%) and moderate RH (60%) to close the stomata was evaluated. Further, the ABA content and regulation was studied in *Rosa x hybrida* and *A. thaliana*. Since both ABA and darkness are signals for stomatal closure and induce the production of the secondary messenger hydrogen peroxide ($H_2O_2$) the ability of *V. faba* to initiate $H_2O_2$ production when treated with ABA or transferred to darkness was also assessed.

This study shows that stomata developed in continuous high RH have reduced response to closing stimuli compared to plants grown under moderate RH. The results also show that *Rosa x hybrida* and *A. thaliana* have slightly different ABA regulation. In *Rosa x hybrida* ABA-glucose ester (ABA-GE) is an important storage form of ABA, which can be released when needed. In *A. thaliana* the level of ABA is mostly regulated at the level of biosynthesis and inactivation to phaseic acid (PA). Compared to high RH, *Rosa x hybrida* developed in moderate RH (60%) and 20 h photoperiod contained higher levels of ABA and had higher *β*-glucosidase activity. Also, the amount of ABA increased during darkness simultaneously as the ABA-GE levels decreased. In contrast, plants developed under high RH and a 20 h photoperiod showed no increase in ABA levels during darkness, and had low *β*-glucosidase activity. Continuous lighting (24 h) resulted in low levels of *β*-glucosidase activity irrespective of RH, indicating that a dark period is essential to activate *β*-glucosidase in roses. It has been hypothesized that plants developed in high RH are unable to produce large amounts of ABA. However, this study clearly shows that *A. thaliana* developed under high RH were able to produce large amounts of ABA during desiccation. However, they still had high water loss in the desiccation test. The difference in water loss between wild type and ABA-deficient mutants were similar in both RH treatments indicating that it is not the lower ABA levels per se that result in less functioning stomata in high RH. The results from *V. faba*

developed in high RH show that the plants are able to increase the $H_2O_2$ production when the ABA levels are increased. However, they do not increase the $H_2O_2$ production during darkness.

These results suggest that the reduced stomatal response in plants developed in continuous high RH is caused either by one or more factors downstream of $H_2O_2$ in the signaling pathway towards stomatal closure or might possibly be a result of changed guard cell anatomy. The results also show that plants developed in high RH, that are given a daily 2 h temperature increase/RH decrease have improved stomatal function. However, the stomatal functioning is still not as good as in moderate RH.

Key words: abscisic acid (ABA), relative air humidity (RH), stomata, $\beta$-glucosidase, hydrogen peroxide ($H_2O_2$)

## Sammendrag

Veksthus som produksjonssystem er viktig i produksjon av grønnsaker, urter og prydvekster. Imidlertid gir dyrking i kontinuerlig høy relativ luftfuktighet (RH) dårlig kvalitet på grunn av ukontrollert vanntap etter høsting. Det høye vanntapet er en konsekvens av store, ufunksjonelle spalteåpninger, som ikke lukker seg fullstendig under forhold som normal fører til lukking. Siden spalteåpningsbevegelse er regulert av plantehormonet abscisinsyre (ABA), er det fremsatt en hypotese om at høy RH resulterer i lave ABA nivåer i plantene og at de lave ABA-nivåene gir ufunksjonelle spalteåpninger.

I denne studien ble lukkeevnen til spalteåpninger fra *Rosa x hybrida*, *A. thaliana* og *V. faba* utviklet i kontinuerlig høy (90%) og moderat RH (60%) undersøkt. Videre ble ABA-nivå og regulering hos *Rosa x hybrida* and *A. thaliana* studert. Siden både ABA og mørke er signaler som fører til lukking av spalteåpningene gjennom økning av hydrogenperoksid ($H_2O_2$) produksjon ble evnen til å produsere $H_2O_2$ undersøkt i *V. faba* etter ABA-tilførsel eller etter flytting til mørke.

Denne studien viser at planter utviklet i kontinuerlig høy RH har nedsatt evne til å lukke spalteåpningene. Resultatene viser også at *Rosa x hybrida* og *A. thaliana* har ulik ABA-regulering. I *Rosa x hybrida* er ABA-glukoseester (ABA-GE) viktig i lagring av ABA, som siden kan frigis når det trengs. I *A. thaliana* er ABA-nivået avhengig av biosyntesen og inaktivering til phaseinsyre (PA). *Rosa x hybrida* utviklet i moderat RH og 20 timer lys inneholdt mer ABA og hadde høyere *β*-glucosidase-aktivitet. I tillegg ble ABA-nivået økt i mørke samtidig som ABA-GE-nivået ble redusert. I motsetning hadde planter utviklet i høy RH med 20 timer lys ingen økning av ABA-nivå i mørke og lavere *β*-glucosidase-aktivitet. Kontinuerlig lys ga lavere *β*-glucosidase-aktivitet uavhengig av RH, noe som viser at mørke (natt) er viktig for å aktivere *β*-glucosidase. En hypotese har vært at planter utviklet i høy RH ikke kan produsere tilstrekkelige mengder ABA for stomatalukking. Imidlertid viser denne studien tydelig at *A. thaliana* utviklet i høy RH kan produsere store mengder ABA under dehydrering. Til tross for dette har plantene fortsatt stort vanntap. Forskjellen i vanntap mellom villtype og mutanter som ikke inneholder ABA var lik i begge RH-behandlingene, noe som indikerer at lavt ABA-nivå ikke alene er grunnen til de ufunksjonelle spalteåpningene i høy RH. Resultatene fra *V. faba* utviklet i høy RH viser at plantene øker $H_2O_2$-nivået når ABA-nivået øker, men de øker ikke $H_2O_2$-produksjonen i mørke.

Disse resultatene indikerer at de ufunksjonelle spalteåpningene i planter utviklet i kontinuerlig høy RH er en konsekvens av påvirkning på enten en eller flere faktorer nedstrøms for $H_2O_2$-produksjon i signalveien til lukking av spalteåpningene eller er et resultat av endret lukkecelleanatomi. Resultatene viser også at planter utviklet i høy RH, men som er gitt 2 timer daglig med høy temperatur/lav RH utvikler bedre evne til å lukke spalteåpningene. Men, de har fortsatt ikke like god lukkeevne som planter utviklet i moderat RH.

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## List of papers

I. Louise E. Arve, Meseret T. Terfa, Hans Ragnar Gislerød, Jorunn E. Olsen and Sissel Torre (2013)
High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves,
Plant, Cell and Environment 36 (2), 382-392

II. Louise E. Arve, Ole Mathis Opstad Kruse, Karen Tanino, Jorunn E. Olsen, Cecilia Futsæther, Sissel Torre (2013)
ABA regulation and stomatal malfunctioning in *Arabidopsis thaliana* developed in continuous high air humidity (Manuscript)

III. Louise E. Arve, Hans Ragnar Gislerød, Jorunn E. Olsen and Sissel Torre (2013)
ABA, but not darkness increases the $H_2O_2$ production in *Vicia faba* developed in continuous high relative air humidity (Manuscript)

IV. Arve LE, Torre S, Olsen JE and Tanino KK (2011).
Stomatal Responses to Drought Stress and Air Humidity,
Abiotic Stress in Plants - Mechanisms and Adaptations, Prof. Arun Shanker (Ed.),
ISBN: 978-953-307-394-1, InTech, DOI: 10.5772/24661.
Available from: http://www.intechopen.com/books/abiotic-stress-in-plants-mechanisms-and-adaptations/stomatal-responses-to-drought-stress-and-air-humidity

## Abbreviations

RH = Relative air humidity

ABA = Abscisic acid

ROS = Reactive oxygen species

$H_2O_2$ = Hydrogen peroxide

NO = Nitric oxide

ZEP = zeaxanthin epoxidase

NCED = 9-cis-epoxycarotenoid dioxygenase

AAO = abscisic aldehyde oxidase

PA = Phaseic acid

DPA = Dihydrophaseic acid

ABA-GE = ABA-glucose ester

WUE = Water use efficiency

# 1. General introduction

Plant growth and development is affected by climate factors such as temperature, $CO_2$, light, photoperiod, soil moisture and air humidity. Relative air humidity (RH) is a measure of how much water vapor the air contains. RH is defined as the actual vapor pressure of water in the air divided by the saturation vapor pressure. In nature the RH in dry areas can be less than 30% while moist areas can have a RH close to 100% (New et al., 2002). In Scandinavia the average outdoor summer RH is 70%, while the average RH during early winter can be higher than 95% (New et al., 2002). In greenhouses the RH can vary between 30% and 100% depending on the season, geographical location and the greenhouse system (Del Bosque-Villarreal et al., 2012). The microclimate around the plants can also be very different than the surrounding air. Inside a dense canopy it is usually a higher RH than in the rest of the greenhouse (Mortensen and Gislerød, 2005). RH is the most difficult climate factor to control in a greenhouse and the most common methods until now has been to regulate the RH by opening and closing the vents and to warm up humid air. However, these methods lead to high energy consumption. To save energy new strategies in greenhouse production are continuously developed. One such strategy is to ventilate less, thus reducing the amount of $CO_2$ enriched air lost and reducing the need for heating in winter. Unfortunately, reducing the ventilation will also increase the RH. Other methods to reduce the RH inside the greenhouses exist. Such methods include air exchange, condensation and dehumidifying, (Campen et al., 2003). However, high RH is still a problem and can cause negative effects on greenhouse grown plants.

## 1.1. Greenhouse production

Greenhouse as a production system is important in the production of vegetables, herbs and ornamental plants, but plant production in continuous high RH (>85%) may results in poor plant quality, nutrient deficiency due to reduced transpiration and problems with diseases like botrytis and powdery mildew (Hannusch and Boland, 1996; Torre et al., 2001; Tullus et al., 2012). Furthermore, the shelf life of ornamental plants has been found to be lower. For example, *Rosa x hybrida* (roses) developed under continuous high RH have been found to have greater transpirational water loss and 6-8 days shorter shelf life than plants grown under lower humidities (Mortensen and Fjeld, 1998; Torre and Fjeld, 2001). These negatives effects of high RH on water loss and shelf life have also been observed in *Begonia*

*x cheimantha*, *Chrysanthemum morifolium*, *Euphorbia pulcherrima* and *Kalanchoe blossfeldiana* developed in high RH (Mortensen, 2000).

The natural day length can be extended with artificial light. This is common in greenhouses and is important to increase productivity in periods with low natural irradiance. If plants in addition to high RH are grown under continuous lighting the negative effects of high RH are worsened, resulting in further reduced postharvest life (Mortensen and Gislerød, 1999).

One of the most important factors influencing the shelf-life of a plant is the water balance, which is dependent on the water uptake and transpirational water loss. Negative water balance occurs when the water uptake is lower than the water loss. This results in poorer quality plants and reduced shelf-life. Plants developed at high RH have been found to have higher water loss and reduced shelf life due to increased transpiration (Mortensen and Fjeld, 1998; Mortensen, 2000; Torre and Fjeld, 2001; Nejad and van Meeteren, 2005).

Transpirational water loss through the leaves occur through two main pathways. Most of the water is lost through stomatal transpiration. However, some water is also lost through cuticular transpiration. Leaves of plants developed in high RH are thinner, commonly with only one layer of palisade parenchyma (Torre et al., 2003). It could be hypothesized that these thinner leaves result in more cuticular transpiration. However, the cuticular transpiration is very small compared to the stomatal transpiration. Although the cuticular transpiration is higher in plants developed in high RH it is still too small to be the main cause for the large increase in water loss in high RH (Fanourakis, 2011). The increased water loss from plants developed in high RH must therefore be largely caused by increased stomatal transpiration.

## 1.2. Stomatal regulation of water loss

The stomatal complex consists of two guard cells surrounding the stomatal pore (Berry et al., 2010). The guard cells regulate the size of the stomatal aperture by increasing/decreasing the turgor pressure within the guard cells and thus increasing/decreasing the stomatal aperture.

The water lost through transpiration is closely regulated by the opening and closing of the stomata (Tallman, 2004). The main signal for stomatal closure is abscisic acid (ABA), which in turn is regulated by the $O_2$:$CO_2$ ratio, air humidity, drought, temperature, light and biotic stresses (Correia et al., 1997; Wilkinson and Davies, 2002; Tallman, 2004; Acharya

and Assmann, 2009; Reynolds-Henne et al., 2010). Throughout the day the stomatal aperture is closely regulated depending on the plant's needs (Tallman, 2004). During the night, when there is no need for $CO_2$ uptake for photosynthesis, the stomata are closed to maximize the rehydration before the next day. In the early daylight the stomata are opened to take up $CO_2$ for photosynthesis and to increase the transpiration to increase the nutrient uptake and transport. Throughout the rest of the day the stomatal opening is regulated depending on the plant`s need to conserve water and the need for $CO_2$ uptake for photosynthesis (Tallman, 2004).

In a study on deciduous trees it was found that the regulation of stomatal conductance is more dependent on hydraulic factors, such as leaf water potential or air humidity, than on photosynthetic factors (Aasamaa and Sober, 2011, 2011). This ensures survival of the plants during drought conditions.

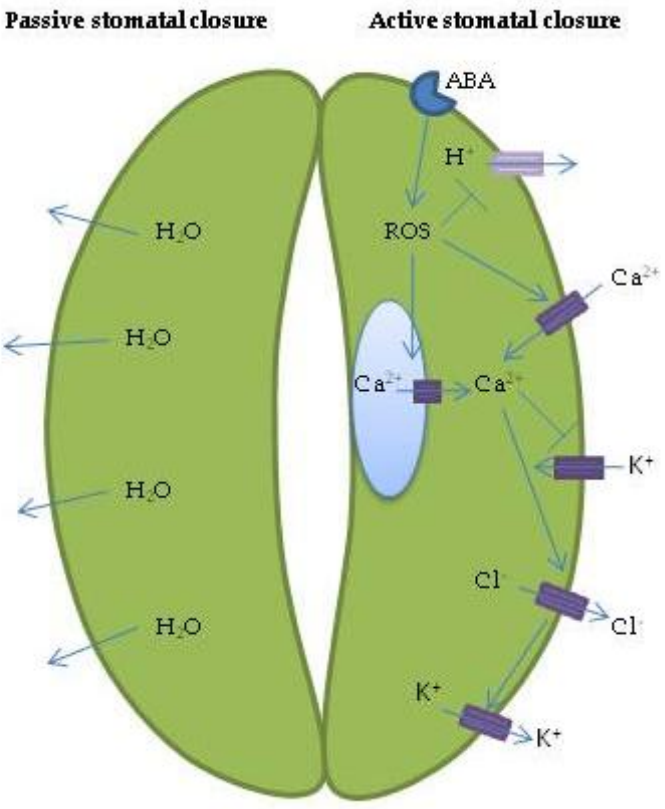


Figure 1: Hydro passive and active stomatal closure pathways (Figure from paper IV).

### 1.3. Mechanisms behind stomatal closure

The stomatal opening is regulated by ABA and increased levels of ABA induce stomatal closure (Figure 1) (Fan et al., 2004; Parent et al., 2009). ABA also acts on the roots and xylem, where increased levels of ABA increase the hydraulic conductivity and thus increase the water uptake and transportation (Parent et al., 2009).

The transport of ABA through the plant occurs in the vascular tissue. Transport through the cell wall is facilitated by an ATP-binding cassette (ABC) transporter, which is regulated by the AtABCG25 and AtABC40 in *A. thaliana* (Kuromori et al., 2010; Ye et al., 2012). AtABCG22 has also been found to be an ABA importer and is mostly located in guard cells (Kuromori et al., 2011).

Increased levels of ABA is recognised by PYR/PYL/RCAR (PYRABACTIN RESISTANCE/ PYRABACTIN RESISTANCE –LIKE/REGULATORY COMPONENT OF ABA RESPONSE) and GCR2 (G protein coupled receptor), which have been shown to function as ABA receptors (Liu et al., 2007; Klingler et al., 2010). The increased ABA levels increase the production of reactive oxygen species (ROS) (Desikan et al., 2004; Cho et al., 2009). First the level of hydrogen peroxide ($H_2O_2$) is increased, most likely by NADPH oxidase (Zhang et al., 2001; Desikan et al., 2004). $H_2O_2$ then increases the levels of nitric oxide (NO) and $Ca^{2+}$ (Kohler et al., 2003; Bright et al., 2006). The increased levels of $Ca^{2+}$ are due to increased influx of $Ca^{2+}$ through $Ca^{2+}$ channels in the plasma membrane and from $Ca^{2+}$ release from the vacuole (Felle et al., 2000). The increased $Ca^{2+}$ levels then inactivate inward $K^+$ channels and activate outward anion channels (Felle et al., 2000). The increased $Ca^{2+}$ levels and reduced anion levels in the guard cells depolarize the plasma membrane, which activates outward $K^+$ channels (Fan et al., 2004). The reduced amount of ions and solutes in the guard cells reduce the water content in the cells, decreasing the turgor pressure and thus closing the stomata.

### 1.4. ABA regulation

ABA is a plant hormone regulating seed development, seed dormancy, desiccation tolerance, xylem conductance and stomatal closure (Seo and Koshiba, 2002; Wilkinson and Davies, 2002; Parent et al., 2009). The level of ABA in plants is therefore closely regulated through biosynthesis and inactivation pathways (Figure 2).

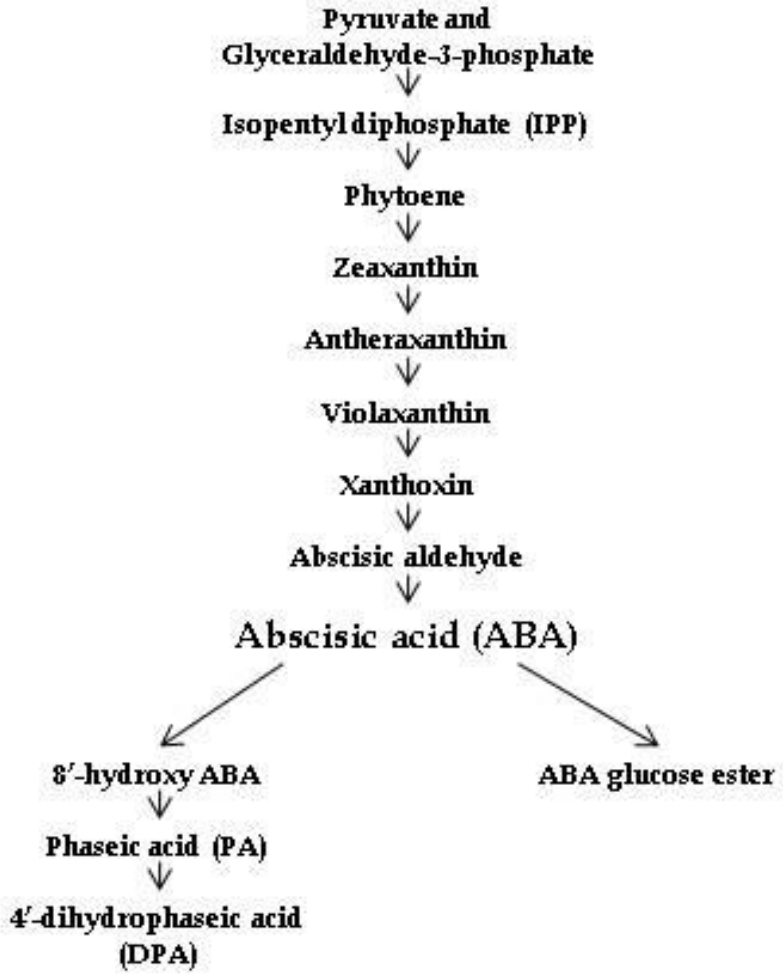


Figure 2: Biosyntehesis of abscisic acid (ABA) from pyruvate and glyceraldehyde-3-phosphate and ABA metabolism by oxidation to PA and DPA and conjugation to ABA glucose ester (Figure from paper IV).

### *1.4.1. ABA biosynthesis*

The biosynthesis of ABA have previously been thought to occur only in the roots (Simonneau et al., 1998). However, more recent studies show that ABA is also synthesized in mesophyll cells in leaves (Christmann et al., 2005; Endo et al., 2008; Seo and Koshiba, 2011).

ABA is synthesized in plastids from the carotenoid phytoene, which is produced from pyruvate and glyceraldehyde-3-phosphate (Cutler and Krochko, 1999; Liotenberg et al., 1999). Phytoene is then converted to ζ-carotene by phytoene desaturase and then to *β*-carotene, lycopene and zeaxanthin (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). Zeaxanthin is further converted to antheraxinthin and then to violaxanthin by zeaxanthin epoxidase (ZEP) (Cutler and Krochko, 1999; Seo and Koshiba, 2002; Nambara

and Marion-Poll, 2005). Violaxanthin is then converted to xanthoxin by 9-cis-epoxycarotenoid dioxygenase (NCED) (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). The ABA biosynthesis pathway from xanthoxin is continued in the cytosol (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). The main pathway from xanthoxin to ABA is through abscisic aldehyde. In this pathway xanthoxin is converted to abscisic aldehyde by an enzyme related to a short-chain dehydrogenase/reductase (SDR) (Seo and Koshiba, 2002). Abscisic aldehyde is further oxidized to ABA by abscisic aldehyde oxidase (AAO) (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005).

During drought the most important genes in up-regulating the ABA biosynthesis are NCED and AAO (Qin and Zeevaart, 1999; Seo et al., 2004; Zhang et al., 2009). The significant up-regulation of these genes during water stress shows the important role ABA plays in rapid stress responses (Qin and Zeevaart, 1999; Seo et al., 2000).

### *1.4.2. ABA inactivation*

ABA is inactivated by two main pathways, oxidation and conjugation (Nambara and Marion-Poll, 2005). The first pathway starts with oxidation of ABA to 8'-hydroxy ABA. This oxidation is catalyzed by the enzyme (+)-ABA 8'-hydroxylase (Cutler and Krochko, 1999; Kushiro et al., 2004). This enzyme is encoded by the CYP707A genes and is closely regulated by environmental factors, such as air humidity (Okamoto et al., 2009; Okamoto et al., 2011). Of the CYP707A genes the CYP707A1 gene is most important in ABA inactivation in the guard cells, while CYP707A3 is more important in ABA inactivation in vascular tissues (Okamoto et al., 2009). 8'-hydroxy ABA is converted spontaneously to phaseic acid (PA) (Nambara and Marion-Poll, 2005). PA is then reduced to 4'dihydrophaseic acid (DPA) by the enzyme phaseic reductase (Cutler and Krochko, 1999).

The second main inactivation pathway is conjugation with glucose to form ABA glucose ester (ABA-GE) (Cutler and Krochko, 1999). The conjugation to ABA-GE is catalyzed by ABA glucosyltransferase (Lee et al., 2006). ABA-GE does not easily pass through biomembranes and has therefore been hypothesized to be a storage and transport form of ABA (Kleczkowski and Schell, 1995; Cutler and Krochko, 1999; Dietz et al., 2000; Ye et al., 2012). Several studies have shown that ABA-GE can later be cleaved by $\beta$-D-glucosidase and release ABA when needed (Dietz et al., 2000; Lee et al., 2006).

## 1.5. Less responsive stomata in high RH

Several studies show that the increased water loss of plants developed in high RH is due to a reduced ability to close the stomata in response to darkness, desiccation or ABA (Torre et al., 2003; Nejad and van Meeteren, 2005; Fanourakis et al., 2012). The stomata at the margins of the leaves developed in high RH have the poorest ability to close the stomata, while stomata further toward the main-vein have a better closing ability (Nejad and van Meeteren, 2007).

When rose leaves developed under high RH were studied under a microscope they were found to have more stomata and larger stomata than roses developed at lower RH (Torre et al., 2003). Both the stomatal aperture and stomatal length were found to be larger in high RH, resulting in a larger pore area (Torre et al., 2003; Nejad and van Meeteren, 2005).

It has also been shown that when plants developed in high RH have been transferred to low RH, darkness or given an ABA treatment the stomata do not close as much as the stomata of plants developed in lower RH (Fordham et al., 2001; Fanourakis et al., 2011; Fanourakis et al., 2012). Several hypotheses have been proposed to explain the less responsive stomata. Reduced $Ca^{2+}$ levels due to reduced transpirational water uptake, changes in the guard cell anatomy, low ABA levels and reduced sensitivity to ABA are some of the proposed explanations.

Micropropagated plants are also often grown in high RH. These plants therefore often develop similar problems with the malfunctioning stomata as plants in high RH in the greenhouse. Studies on *in vitro* grown plants have shown that the stomata do not close fully in response to closing stimuli such as ABA, darkness, mannitol, low leaf water potential and high $CO_2$ concentrations (Brainerd and Fuchigami, 1982; Ziv et al., 1987; Santamaria et al., 1993; Sciutti and Morini, 1995; Fordham et al., 2001). It has also been found that *Wrightia tomentosa* plants grown *in vitro* had 29.4 % malformed stomata, which were large, spherical, wide open and unable to close (Joshi et al., 2006). In these studies it has been speculated that the stomatal size has an effect on the closing ability. Such a lack of stomatal closure response and thus more water loss has also been reported in leaf cuttings placed in a foggy environment (Fordham et al., 2001).

### *1.5.1. $Ca^{2+}$ levels in high RH*

One hypothesis to the less functioning stomata in high RH has been that the reduced closing ability is due to lower $Ca^{2+}$ levels. $Ca^{2+}$ is an important secondary messenger in ABA-

induced stomatal closure and reduced levels of $Ca^{2+}$ will reduce the ability of the stomata to close (Palta, 1996; Felle et al., 2000; Hubbard et al., 2012).

Plants growing in high RH have lower transpiration, which causes reduced uptake and transport of $Ca^{2+}$ (Marschner, 1995). The $Ca^{2+}$ content in roses grown in continuous high RH has been found to be lower than in roses grown in moderate RH. Also, the conductance of these plants regardless of RH was higher in plants with lower $Ca^{2+}$ levels (Torre et al., 2001). However, the difference in stomatal conductance between plants treated with high and low levels of $Ca^{2+}$ was not enough to explain the much larger difference in conductance between plants developed in high and moderate RH (Torre et al., 2001).

### *1.5.2. Stomatal anatomy*

Another hypothesis to why the stomata in high RH do not close properly is that the anatomy of the guard cells makes them unable to move. The cytoskeleton and flexibility of the guard cells is important in establishing the shape of the guard cells and making them able to move (Jones et al., 2003; Dzierzynska, 2006).

The cytoskeleton consists of microtubules and actin filaments (Dzierzynska, 2006). In tip and cell growth in general microtubuli are important in growth polarity, determining the direction of growth and actin filaments deliver materials required for growth (Mathur and Hulskamp, 2002). In guard cells the microtubuli have a radial patterning, where they radiate from the ventral side to the dorsal side (Galatis and Apostolakos, 2004; Dzierzynska, 2006). This patterning gives the shape of the cells and results in a pore between the guard cells when the turgor pressure in the cells increase (Galatis and Apostolakos, 2004). When the stomata close the microtubuli are reorganized or broken down (Fukuda et al., 1998; Galatis and Apostolakos, 2004). However, some studies have found a similar radial patterning of the microtubuli in both open and closed stomata (Eun and Lee, 1997).

Similarly, in open stomata the actin filaments also have a radial patterning. However, when the stomata close the actin filaments are reorganized in different directions (Eun and Lee, 1997; Galatis and Apostolakos, 2004; Higaki et al., 2010; Zhao et al., 2011).

For the stomata to close in response to the decrease in turgor pressure the cell wall needs to be flexible, allowing the cells to move. A previous study showed that for the cell wall to be flexible it must contain arabinan (Jones et al., 2003). Arabinan prevents the homogalacturonan polymers in the cell wall from forming rigid structures, thus keeping the cell wall flexible and making the stomata able to move (Jones et al., 2003).

### *1.5.3. Plants contain less ABA in high RH*

It has been believed that the less functioning stomata found in plants developed in high RH are caused by lower ABA levels. Several studies show that plants growing in continuous high RH have lower levels of ABA than plants growing in lower RH (Zeevaart, 1974; Nejad and van Meeteren, 2007; Okamoto et al., 2009). This was first discovered in *Spinacia oleracea* where plants growing in 35% RH had higher ABA levels than plants growing in 75% RH (Zeevaart, 1974). In later studies of *Tradescantia virginiana* it was found that plants growing in 90% RH had lower levels of ABA than plants growing in 55% RH (Nejad and van Meeteren, 2007). A subsequent study on *T. virginiana* showed that when plants were transferred from moderate to high RH the ABA level decreased (Nejad and van Meeteren, 2008). When the plants were later transferred back to moderate RH the ABA levels were increased again, but the stomata would not return to a fully functioning state after the high RH treatment (Nejad and van Meeteren, 2008). Thus, the less responsive state of the stomata developed in high RH is irreversible. In another study, when *A. thaliana* plants were moved from moderate to high RH the ABA levels were decreased, due to increased expression of CYP707A genes which increased the ABA catabolism (Okamoto et al., 2009). It has also been found that plants grown *in vitro* under high RH have low ABA levels (Hronkova et al., 2003). When these plants are later transferred to an *ex vitro* environment with lower RH the ABA levels increased (Hronkova et al., 2003).

In contrast to these studies, another study have shown similar ABA levels in different RH conditions and yet another study on *A. thaliana* also found that ABA-deficient and ABA-insensitive mutants showed the same response to changes in RH as wild type (WT) plants (Assmann et al., 2000; Hronkova et al., 2003). Even though most studies have found an interaction between ABA and RH, the study by Assmann et al. (2000) rules out an obligate role of ABA in stomatal responses to RH. A recent study has also shown that ABA both has a direct effect on the stomata, but also an indirect hydraulic effect through decreasing the water permeability within leaf vascular tissues (Pantin et al., 2013).

Thus, the relationship between low ABA levels and malfunctioning stomata is still unclear. However, there is still considered to be a connection between the low ABA levels and the development of malfunctioning stomata.

## 1.6. Avoiding malfunctioning stomata in high RH

By growing plants in semi-closed greenhouses that are rarely ventilated, less energy is needed for heating. However, reducing the ventilation will also increase the RH. Several studies have therefore been performed to find ways to avoid the development of malfunctioning stomata, while still growing the plants in high RH.

In a study on roses, plants growing in high RH were given a daily 6 hour RH reduction. Desiccation tests of detached leaves showed significantly reduced water loss in the plants given the RH reduction compared with continuous high RH (Mortensen et al., 2007). A 6 h temperature increase can be used to decrease the RH and result in more functioning stomata (Mortensen and Gislerød, 2011). It has also been found that dividing the 6 hour RH reduction into three daily 2 hour RH reductions also improved the stomatal functioning (Mortensen and Gislerød, 2011).

Continuous lighting in high RH reduces the stomatal functionality even further and reduces the plants ability to retain water during desiccation tests (Mortensen et al., 2007). To avoid this a light period of 18 h gives stomata that are significantly more responsive to desiccation, increases the water retention and result in plants with longer shelf life, compared with continuous lighting (Mortensen et al., 2007).

Elevated $CO_2$ concentrations have been found to partly close the stomatal opening (Morison, 1998). However, elevated $CO_2$ concentrations had little effect on the stomatal functioning on roses grown in high RH, although a small improvement was seen on cv. 'Amadeus' during low irradiance (Mortensen and Gislerød, 2011).

One of the main signals for stomatal closure is ABA (Fan et al., 2004). It can therefore by hypothesized that ABA application should result in more functional stomata. Studies of *T. virginiana* and roses have shown that leaves developing in continuous high RH which are given a daily ABA treatment during development will develop smaller and fully functional stomata (Nejad and van Meeteren, 2007; Fanourakis et al., 2011). However, the ABA application only gave functional stomata in young expanding leaves. If the leaves were already fully developed when the treatment started the ABA applications had no effect on the stomata (Nejad and van Meeteren, 2008). Similar results have also been found in *Salvia splendens* and a number of other ornamentals, where continuous ABA application reduced the transpiration rate and increased the shelf life (Pompodakis et al., 2004; Waterland et al., 2010a; Waterland et al., 2010b; Kim and van Iersel, 2011). However, application of too high

ABA concentrations can cause early leaf abscission in *S. splendens* (Kim and van Iersel, 2011).

### 1.7. Critical stages for stomatal functioning

In a previous study on *T. virginiana* it was found that when plants from high RH were transferred to moderate RH after the leaves were fully developed the malfunctioning stomata could not be reversed. However, in actively expanding leaves the stomatal functionality could be improved by moving the plants to lower RH (Nejad and van Meeteren, 2008).

In a study of roses, plants were transferred from high to moderate RH when the leaves were at different expansion stages. It was found that the stomatal functionality was improved more the earlier the plants were transferred to lower RH and that after the leaves were fully expanded the stomatal functionality could not be improved (Fanourakis et al., 2011). However, the most important stage when most of the stomatal functionality was determined was the last stage of leaf expansion, when the leaves had reached 70% of their full size (Fanourakis et al., 2011).

### 1.8. Stomatal density

The water loss from stomata is not only determined by the size and functionality of the stomatal openings, but also by the number of stomata (Metwally et al., 1971). A larger number of stomata can take up more $CO_2$ and transpire more water.

In studies determining the number of stomata and stomatal density in different humidities it has been found that the number of stomata per leaf increased in higher soil humidities, but when calculated as number of stomata per area the number decreased in higher humidities (Metwally et al., 1970; Metwally et al., 1971). In another study, the stomatal index increased with soil moisture (Schürmann, 1959). In *V. faba* drought and salinity stress have been found to increase the stomatal density and stomatal index, facilitating water uptake under water-stressed conditions (Gan et al., 2010).

Similar experiments have been performed with RH, where increased RH results in increased stomatal density (Sciutti and Morini, 1995). When the stomatal density is compared with the endogenous ABA concentrations, the stomatal density was found to increase with decreased ABA levels (Lake and Woodward, 2008). In roses, one study show that plants developed in high RH have higher stomatal density compared with moderate RH (Torre et al.,

2003). However, another study on roses found that RH had little effect on the stomatal density, which was slightly, but not statistically higher in high RH (Fanourakis, 2011).

## 2. Aim of the present study

The main aim of this study was to improve the understanding of why plants developed in continuous high RH have stomata with reduced response to closing stimuli. As this is a very large topic it was narrowed down to focus on the ABA regulation and effects of ABA on stomatal closure.

Specifically, the ABA-regulation in leaves was studied. Also, the effect of ABA and other closing stimuli on the stomatal aperture in plants developed in high and moderate RH was investigated (Paper I).

Further, the genetic regulation of ABA and the importance of ABA in stomatal closure were investigated. The effects of a daily period with temperature increase/RH decrease while keeping the average daily RH high was simultaneously studied (Paper II).

Another aim was to investigate the ability of plants developed under high RH to sense ABA and initiate the ABA-dependent pathway to stomatal closure. Also, the ability to close the stomata in response to increased levels of $H_2O_2$ was investigated (Paper III).

# 3. Materials and methods

Several different methods were used in this study. In all three papers desiccation tests and stomatal measurements were used to make sure the different species showed the same response to development in continuous high RH. Different methods were used to measure the diurnal conductance and quantify the ABA and *β*-glucosidase levels in *Rosa x hybrida*, cv. Rebecca (Paper I). In *Arabidopsis thaliana* (Columbia ecotype, Col-0) methods to determine the leaf transpiration, water use efficiency (WUE), ABA quantification and expression of genes in the ABA biosynthesis and inactivation pathways were used (Paper II). Finally in *Vicia faba* methods to determine the stomatal closing ability in response to ABA and $H_2O_2$ and $H_2O_2$ quantification were used (Paper III).

In this study, three different species was used. In paper I, the effect on high RH was studied in *Rosa x hybrida*, cv. Rebecca. The reason for choosing roses was the fact that roses have been an important model for shelf life studies. Roses are among the most important ornamentals in the floricultural industry worldwide. Further, roses have been used in high RH experiments since the early 1990-ies and a lot of knowledge is available on their response to high RH. There is a lot of genetic variation between rose cultivars (Mortensen and Gislerød, 1999), cv. Rebecca was used in this study because it is sensitive to RH. In the second paper *A. thaliana* was used since we wanted to investigate whether this could be used as a model species for high RH responses. Since the entire genome of *A. thaliana* has been sequenced and many mutants are available, several methods can be used on *A. thaliana* that are difficult to apply on other species, such as roses. In the third paper epidermal peels of *V. faba* plants were used for the fluorescence microscope. To be able to compare the results more readily with previous studies it would have been preferable to use roses. However, it is very difficult to take epidermal peels from rose leaves and even if the guard cells in the peels can be observed, there are still too many cell layers present and too much interference from auto-fluorescence from chlorophyll. *A. thaliana* could also have been used, but since we found that to be a poor model species in paper II we chose not to continue working with this species. *V. faba*, was used since it is very easy to obtain very good quality peels with almost no auto-fluorescence. Also, a lot of research on ROS signaling and stomata closure has been done on this species (Zhang et al., 2001; She et al., 2004; Yan et al., 2007; Song et al., 2008; Wang and Song, 2008; Song et al., 2011).

# 4. Main results and discussion

Stomata developed in continuous high RH have been found to be larger and less responsive to closing stimuli (Santamaria et al., 1993; Mortensen, 2000; Torre and Fjeld, 2001; Nejad and van Meeteren, 2005; Fanourakis et al., 2011). The less functional stomata result in rapid postharvest water loss and highly reduced stress tolerance (Mortensen, 2000; Torre and Fjeld, 2001).

## 4.1. Malfunctioning stomata in high RH

The easiest way to assess the plant`s ability to retain water is to do a desiccation test. The studies of *Rosa x hybrida* (Paper I), *A. thaliana* (Paper II) and *V. faba* (Paper III) all clearly showed that plants developed in high RH had much larger water loss during three hours of desiccation compared to plants grown in moderate RH. Previous studies have shown the cuticular transpiration to be very low compared with the stomatal transpiration (Fanourakis, 2011). It can therefore be assumed that most of the water lost in the desiccation test is lost through the stomata and much of the difference in water loss is a result of reduced stomatal closure in plants developed in high RH. When *Rosa x hybrida* were developed in continuous lighting they showed the same trend during the desiccation test. However, they initially lost more water, indicating that it takes longer for these plants to initiate stomatal closure. Similar results have previously been found in *Rosa x hybrida*, *T. virginiana*, *Begonia x cheimantha*, *E. pulcherrima*, *K. blossfeldiana* and *C. morifolium* plants (Mortensen, 2000; Torre and Fjeld, 2001; Nejad and van Meeteren, 2005). Since it has been shown in several species that development in high RH results in higher water loss during desiccation it can be concluded that this is a general plant response to high RH at least in C3 plants.

In all three species, it was found that plants developed in continuous high RH developed larger stomata. This is similar to previous studies performed on *Corylus mamima*, *Rosa x hybrida* and *Zamioculcas zamiifolia* (Fordham et al., 2001; Torre et al., 2003; Karbulkova et al., 2008). Several studies have shown that these larger stomata are unable to close properly (Ziv et al., 1987; Santamaria et al., 1993; Mortensen, 2000; Fordham et al., 2001; Torre et al., 2003; Nejad and van Meeteren, 2005). It has been hypothesized that the larger, less functioning stomata found in plants developed under high RH is a result of low endogenous ABA levels. Supporting this is also a study on *Populus x canescens* showing that ABA insensitive plants had larger stomata (Arend et al., 2009).

Water loss is not only determined by the size of the stomatal pore, but also by the number of stomata. In this study we found the stomatal density to be significantly lower in *A. thaliana* plants developed in high RH compared to moderate. However, the difference was small. The higher transpirational water loss must therefore be a consequence of the larger stomatal opening. These results contrast with what has previously been found in some other studies. A study on *Prunus cerasifera* found that increased RH resulted in increased stomatal density (Sciutti and Morini, 1995). Two other studies on *Rosa x hybrida* found that the stomatal density was higher in plants developed in high RH compared with moderate RH (Torre et al., 2003; Fanourakis, 2011).

Darkness is known to induce stomatal closure, although the degree of closure varies between species (Tallman, 2004; Caird et al., 2007). When comparing the aperture in light and darkness, both *Rosa x hybrida* and *A. thaliana* developed in high RH showed no or very little closure during darkness. One possible explanation for this could be that the moist conditions in high RH is a stronger signal than darkness and overrides the need for stomatal closure during darkness. In contrast, the plants developed in moderate RH had smaller stomata and clearly closed the stomata during darkness. Further evidence for reduced stomatal closure during the dark in plants developed in high RH are the infrared (IR) images of *A. thaliana*, showing higher transpiration in plants from high RH during the dark. In *Rosa x hybrida* the diurnal conductance was decreased during late day and night in plants from both high and moderate RH, but remained higher in plants from high RH throughout both the day and night. A previous study on the diurnal conductance of *Rosa x hybrida* showed a similar pattern where plants from both high and moderate RH reduce the conductance during the dark period (Fanourakis et al., 2012). However, both the present and the previous (Fanourakis et al. 2012) studies showed a larger relative change in conductance in plants developed in moderate RH compared with high RH.

Other stimuli that usually cause stomatal closure are ABA and $H_2O_2$ (Zhang et al., 2001; Tallman, 2004; Seo and Koshiba, 2011). When examining the plant`s ability to close the stomata in response to these stimuli, we found that *V. faba* developed in high RH had much smaller response and closed the stomata very little compared to plants developed under moderate RH. Other studies have indicated that stomata of *in vitro* grown plants, which also experience high RH, fail to close fully in response to ABA (Santamaria et al., 1993), low leaf water potential (Fordham et al., 2001) and darkness (Ziv et al., 1987). In a study on *T. virginiana* the stomata of plants developed in high RH had reduced response to ABA (Nejad

and van Meeteren, 2005). Similarly, another study of *Rosa x hybrida* showed that ABA-application on fully developed plants in high RH have some effect on the transpiration, but less effect than on plants developed in moderate RH (Fanourakis et al., 2012). All these studies show the same trend, where plants developed in high RH have reduced response to ABA. If plants developed in high RH are subjected to daily ABA application during leaf expansion the stomata will become fully functional (Nejad and van Meeteren, 2008; Fanourakis et al., 2011).

### 4.2. Effect of RH on ABA levels

ABA is an important signal for stomatal closure and low levels of ABA has been hypothesized to be the reason for the malfunctioning stomata. The ABA levels were measured in both *Rosa x hybrida* and *A. thaliana*. The total level of ABA and its metabolites PA, DPA, ABA-GE, t-ABA, neo-PA and7'OH-ABA was significantly lower in plants developed under high RH, compared with moderate RH in both species. Similarly, when only the ABA levels were considered it was also significantly lower in plants developed under high RH. In *Rosa x hybrida* grown in moderate RH the ABA levels were increased during the dark, while the levels remained constant in high RH. In *A. thaliana* there was no change in the ABA levels between light and darkness in either of the RH treatments. This may indicate that *A. thaliana* and *Rosa x hybrida* have slightly different ABA responses to darkness and RH. Previous studies on the diurnal variation of ABA in *N. tabaccum* and *S. oleracea* demonstrated a peak in ABA concentration in the beginning of the dark period, before decreasing and remaining low throughout the rest of the dark period (Zeevaart, 1974; Novakova et al., 2005). The sampling time will therefore influence whether an increase in ABA levels will be found or not.

The increased ABA concentration during dark in moderate RH is believed to act as a signal for stomatal closure during darkness (Tallman, 2004; Novakova et al., 2005). Previous studies of *T. virginiana* and *A. thaliana* also found lower ABA concentrations in plants developed under high RH or when moved from low to high RH (Zeevaart, 1974; Nejad and van Meeteren, 2007, 2008; Okamoto et al., 2009). It is believed that the decrease in ABA levels when plants are moved from low to high RH is a result of increased inactivation and not altered biosynthesis (Okamoto et al., 2009). However, the involvement of ABA in RH responses is contradicted by experiments showing that ABA-insensitive mutants responded similarly as WT plants to changes in RH (Assmann et al., 2000). Thus, despite the general

belief that there is a connection between low ABA concentrations and malfunctioning stomata, the relationship is still unclear.

ABA is inactivated by oxidation to PA and further to DPA or by conjugation to ABA-GE (Nambara and Marion-Poll, 2005). In *Rosa x hybrida* the PA levels were significantly lower in plants developed in high RH. However, there was no difference in the ABA:PA ratio between plants from high and moderate RH. This indicates that the lower PA levels in high RH are probably a result of the lower ABA levels and a constant inactivation rate of ABA to PA. The amount of ABA-GE in *Rosa x hybrida* was similar in both RH treatments in the light. However, as the level of ABA was increased during dark in moderate RH, the level of ABA-GE was simultaneously decreased, but both the ABA and ABA-GE levels remained unchanged in high RH. In *A. thaliana* there was no difference in the PA levels in high and moderate RH. However, the DPA levels were lower in plants developed in high RH.

When *Rosa x hybrida* were grown under continuous lighting the levels of ABA and its metabolites and the PA levels were lower in high RH than in moderate RH, while there was no difference in ABA, ABA-GE or $\beta$-glucosidase levels between the two treatments. The levels from both high and moderate RH in continuous light were in general more similar to the levels from high RH in 20 h photoperiod and lower than the levels from moderate RH in 20 h photoperiod. There were also no interaction effects between photoperiod and RH, except for PA, indicating that continuous lighting has the same effect irrespective of the RH. The low ABA levels in continuous light also show that the higher ABA levels in moderate RH in 20 h light is caused by increased ABA levels during the dark period.

It has been hypothesized that plants developed in high RH are unable to produce large amounts of ABA. In this study the ABA levels in *A. thaliana* leaves after three hours of desiccation had increased 10-fold in plants from both moderate and high RH. Even though the ABA levels were increased also in leaves from high RH during desiccation, they still had uncontrolled water loss during the desiccation test and did not close the stomata properly. One hypothesis that must therefore be considered is that the reduced stomata closure in plants developed in high RH might be due to reduced sensitivity to ABA.

## 4.3. ABA regulation

### *4.3.1. Rosa x hybrida*

In roses the total amount of ABA and its metabolites PA, DPA, ABA-GE, t-ABA, neo-PA and7'OH-ABA did not change between light and dark in either moderate RH or high RH. However, in moderate RH the amount of ABA was increased, simultaneously as the levels of ABA-GE was decreased. ABA-GE is therefore likely the source of the increased ABA levels. ABA-GE has been hypothesized to be a storage form of ABA, which can be converted to ABA when needed (Dietz et al., 2000; Sauter et al., 2002). *β*-glucosidase is an enzyme that converts ABA-GE into ABA (Dietz et al., 2000; Lee et al., 2006). The activity of *β*-glucosidase in the *Rosa x hybrida* study was about five fold higher in plants grown in moderate RH, than in plants grown in high RH. However, there was no significant difference between light and dark within either of the treatments, only a tendency of higher activity during the dark. These data are supported by a previous study, where the amount of *β*-glucosidase and ABA was highest during light and after an initial peak during darkness remained low the rest of the dark period (Novakova et al., 2005). These results indicate that more ABA-GE is converted to ABA in leaves developed under moderate compared to high RH and that the ABA-GE could be the source of the increased ABA levels.

In high RH there was no change in total ABA and metabolites, ABA or ABA-GE levels between light and dark. This might be due to the favorable conditions in high RH, which might make it unnecessary for the plants to close the stomata during darkness. The lack of change in ABA levels in high RH also indicates that high RH overrides the influence of darkness, keeping the stomata open. Supporting this is a study showing that RH responses dominated over photosynthetic responses in stomatal movement (Aasamaa and Sober, 2011).

### *4.3.2. A. thaliana*

The genetic regulation of ABA in *A. thaliana* showed that the levels of the ABA biosynthesis genes ZEP, NCED3 and AAO3 were similar in plants from high and moderate RH. The RNA levels of these genes were significantly decreased during darkness in plants from both treatments, keeping the ABA levels low.

During light, the expression of the ABA inactivation gene CYP707A1 was significantly lower in plants from high compared to moderate RH. In moderate RH, the expression of CYP707A1 was similar in light and darkness. However, in plants grown in high

RH, the CYP707A1 transcript levels were significantly higher during darkness. The relative expression of another ABA inactivation gene CYP707A3 was also similar in plants from both moderate and high RH during light. During darkness, the expression remained unchanged in moderate RH, but was significantly higher in plants from high RH. A study of the CYP707A3 gene has indicated that this gene is especially important in ABA regulation (Umezawa et al., 2006). These results clearly show that that the expression of ABA biosynthesis genes in the leaves is down-regulated and the ABA inactivation genes up-regulated in darkness in high RH, while only the biosynthesis genes are changed in moderate RH.

Although there was no change in the ABA levels in the leaves during the dark, the transcript levels of ABA biosynthesis genes (ZEP, NCED3 and AAO3) were reduced and those of the ABA inactivation genes (CYP707A1, CYP707A3) increased. It is probable that during the dark ABA production in the roots and subsequent transport to the leaves is more important than ABA produced in leaves. It has previously been observed that ABA in the xylem increases the hydraulic conductivity, which is important for rehydration during darkness (Parent et al., 2009). ABA production in the leaves could then be reduced and inactivation increased to keep the amount of ABA levels constant. In a previous study the highest expression of CYP707A3 gene was found in vascular tissues, and this might result in inactivation of ABA from the roots during darkness before it reaches the stomata (Okamoto et al., 2009).

The expression of CYP707A3 is regulated by several environmental factors. CYP707A3 expression has previously been found to increase when plants are moved from low to high RH, before returning to lower levels again (Okamoto et al., 2009). In addition, in this study we found that the expression of CYP707A3 was dependent on the RH regime and light/dark conditions. A similar dependence was found in the expression of CYP707A1, which was only increased during darkness in plants grown in high RH. CYP707A1 is mostly expressed in the guard cells (Okamoto et al., 2009) and can therefore more specifically regulate the ABA levels that affect the stomatal movement. The increased levels of CYP707A1 during dark in high RH might therefore reduce the ABA levels in the guard cells and prevent stomatal closure.

The lack of an increase in ABA levels during dark in plants developed under moderate RH may reduce the degree of stomatal closure, keeping the stomata slightly open. Since *A. thaliana* is a rosette plant, it is also possible that the microclimate around the rosette

leaves had reduced air movement and higher RH. The stomata in the moderate RH treatment might therefore have been exposed to a somewhat higher RH than was actually measured in the air of the growth chamber. This may have led to incomplete closure during darkness. However, it is also known that stomata of many species remain partially open during the night (Caird et al., 2007).

### 4.4. Importance of ABA in stomatal development

It has been hypothesized that a minimum level of ABA is required for the development of fully functional stomata. Plants developed in high RH have never experienced high ABA content during leaf development and this might cause the less responsive stomata. Supporting this theory is studies showing that ABA applications during growth or transfer from high to moderate RH before the leaves are fully expanded result in fully functional stomata (Fanourakis et al., 2011).

To further investigate the importance of ABA in stomatal development the stomata of the *A. thaliana* mutant *aba3-1*, which is mutated in the AAO3 gene (At1g16540) in the ABA biosynthesis and contains very little ABA (Schwartz et al., 1997), was studied.

During a desiccation test the water loss of the ABA deficient mutants were significantly higher than that of the WT counterparts within the same RH treatment. However, the difference in water loss between the WT and mutants within each RH treatment was almost similar. If the low ABA levels were the cause of the reduced stomatal functioning in plants developed in high RH similar water loss in the mutants from both RH treatments and the WT plants developed in high RH should have been observed. The similar difference in water loss between WT and *aba3-1* mutants indicates that the lower ABA level found in WT plants developed in high RH is not the reason for lower desiccation tolerance.

Looking at the stomatal size, stomata from both WT and *aba3-1* mutants developed under high RH and *aba3-1* mutants developed in moderate RH were larger than the stomata of WT plants developed under moderate RH. An earlier study of *Populus x canescens* also found that ABA insensitive plants had larger stomata (Arend et al., 2009). This indicates that lack of ABA, reduced sensitivity to ABA or low ABA contents result in the development of large stomata.

These results showed that both lacking ABA and development in high RH had negative effects on water loss and that these effects were additive.

### 4.5. Stomatal apertures during development

Plants developed in a controlled environment with high RH never experienced unfavorable conditions. This might result in reduced ABA production and larger stomatal apertures during development. Continuously large apertures during development may result in stomata that are not able to close fully at a later stage. The ABA deficient *aba3-1* mutants contained no ABA to reduce the stomatal aperture during development. Lack of stomatal closure during development may be the cause of the less responsive stomata.

As stated above, daily application of ABA in high RH has been shown to produce functional stomata (Fanourakis et al., 2011). However, it is not known whether it is the ABA in itself that changes the stomatal development or whether it is an indirect effect of the closing stimulus reducing the stomatal apertures. To test the importance of stomatal movement during development a treatment where *A. thaliana* plants developed in high RH were given a 2 hour temperature increase/RH decrease during the day was tested. Plants given this treatment were found to have smaller stomatal apertures than plants in continuous high RH. However, the stomatal apertures were still larger in the stress treatment than in continuous moderate RH. If the stomatal aperture during development is important in producing functioning stomata, plants from the stress treatment should have improved desiccation tolerance compared with plants developed in continuous high RH.

Plants given the stress treatment had better desiccation tolerance than those from continuous high RH. However, the desiccation tolerance was still not as good as in plants developed under continuous moderate RH. The length of the stomatal pore during light was significantly smaller in the moderate RH treatment, while there was no difference between the high RH and stress treatments. Using IR imaging the leaf temperature after 65 minutes in a dark environment was lower in plants developed under high RH than in plants from moderate RH and the stress treatments, indicating similar higher leaf temperatures due to more functioning stomata. The plants from the stress treatment were also found to have similar water use efficiency (WUE) as plants developed under moderate RH and better WUE than plants developed under constant high RH. These results indicates a higher transpiration rate due to more open stomata in plants developed under constant high RH, while plants from the other treatments were able to close the stomata in response to dry air and darkness. The plants given the stress treatment developed larger stomata, but also showed reduced transpiration during darkness. This indicates that it is not the larger stomata per se that cause

the reduced closing ability. These results support the hypothesis that the stomatal aperture during development influences the stomatal functionality.

### 4.6. Causes for the less functioning stomata

There could be several causes for the malfunctioning stomata under high RH. One possible explanation may be that the stomata are less sensitive or insensitive to the ABA signals, either due to fewer ABA receptors or inhibition of one of the steps of the signaling pathway. It is also possible that plants developed under high RH conditions develop stomata with a structural anatomy making them unable to close completely, in spite of receiving signals for stomatal closure. One such change might be a lack of arabinan in the cell wall, which has been shown to be essential for stomatal movement (Jones et al., 2003).

It has previously been found that *Rosa x hybrida* exposed to a six hour stress period in the middle of the day have decreased water loss and increased vase life compared with constant high RH (Mortensen and Gislerød, 2005). Previous studies on roses and *T. virginiana* showed that when plants developed under constant high RH are given daily ABA applications during development, they develop functioning stomata (Nejad and van Meeteren, 2007; Fanourakis et al., 2011). In these studies it was hypothesized that the malfunctioning stomata were due to the long term low ABA levels and that ABA application resulted in functional stomata. However, when combining these results with the results from this study, it may be hypothesized that it is not the low ABA level per se that result in malfunctioning stomata, but the large stomatal aperture during development. Accordingly, the ABA application would reduce the stomatal aperture resulting in better functioning stomata.

The results discussed above support the hypothesis that to produce functional stomata the stomatal aperture must be small or closing and opening regularly during development. However, *aba3-1* mutants from high and moderate RH behaved differently during desiccation, which suggests that these mutants developed under moderate RH have some stomatal movement or that the turgor pressure in the guard cells has been reduced, possibly during the dark. This might be caused by other ABA independent pathways, possibly involving $H^+$/ATPase or an indirect hydraulic effect through decreasing the water permeability within leaf vascular tissues (Netting, 2000; Pantin et al., 2013). *A. thaliana* WT plants in moderate RH did not show any increase in ABA levels in the leaves during darkness, but the stomata still closed to a certain degree compared with the *aba3-1* mutants. This indicates that increased ABA in the dark is not necessary for stomatal movement.

However, it cannot be excluded that there was an ABA increase in the guard cells themselves, since only the bulk ABA level in the leaves was measured. Whatever the signal for dark induced stomatal closure, other factors may override this signal in plants growing in high RH. As stated earlier, stomatal responses to RH have been found to dominate over responses to photosynthetic signals (Aasamaa and Sober, 2011). High RH might therefore be hypothesized to override the dark-induced stomatal closure signals, due to conditions that make stomatal closure unnecessary.

### 4.7. Stomatal signaling

There are several environmental stimuli that usually result in stomatal closure. Many of these stimuli have been found to increase the amount of ABA in the plants, which acts as a chemical signal for stomatal closure (Fan et al., 2008). ABA signals stomatal closure through a series of steps, including the production of $H_2O_2$ in the guard cells (Bright et al., 2006). In this study stomata of *V. faba* from both high and moderate RH responded to ABA or $H_2O_2$ treatments by closing the stomata. However, the stomata developed in high RH had a very weak response. The reduced amount of closure found in stomata developed in high RH could therefore be a result of reduced sensitivity to ABA.

We also quantified the amount of $H_2O_2$ produced after treatment with ABA or darkness. In the light there was no significant difference in the amount of $H_2O_2$ between high and moderate RH. When the guard cells had been given a dark treatment, the amount of $H_2O_2$ was significantly increased in guard cells from moderate RH. However, there was no change in $H_2O_2$ production in response to darkness in guard cells from high RH. After the ABA treatment the $H_2O_2$ levels increased in guard cells from both moderate and high RH. The lack of $H_2O_2$ production during darkness in plants developed in high RH might be a result of the high RH conditions, which inhibits stomatal closure during darkness.

These results show that plants developed in high RH are able to initiate the ABA dependent pathway toward stomatal closure. The reduced stomatal movement must therefore be caused either by a later step than $H_2O_2$ in the ABA dependent pathway or possibly by altered guard cell morphology, which could make them physically unable to close completely.

# 5. Conclusions and further perspectives

*Rosa x hybrida, A. thaliana* and *V. faba* plants developed under high RH had larger stomata and higher transpiration during desiccation stress, compared with plants developed in moderate RH. Plants developed in high RH had reduced or no stomatal response to closing stimuli, such as darkness, ABA and $H_2O_2$. The results from all three species also show that plants developed in high RH do not increase the ABA levels during darkness and therefore have reduced stomatal response during darkness.

The results also show that plants developed in high RH kept the stomata open during development, which resulted in malfunctioning stomata. However, daily periods with temperature increase/RH decrease reduced the stomatal apertures and can improve the stomatal functionality.

*Rosa x hybrida* developed in moderate RH showed increased levels of ABA during darkness and simultaneously a similar decrease in ABA-GE, indicating that the increased levels of ABA is due to conversion of ABA-GE. In contrast, plants developed under high RH showed no increase in ABA levels during dark, and had very little *β*-glucosidase activity converting ABA-GE to ABA. Further, in *Rosa x hybrida* developed under continuous lighting the *β*-glucosidase activity was low irrespective of the RH, indicating that a dark period is essential to activate this enzyme. The results clearly show that *β*-glucosidase has a central role and is a key enzyme in regulating the ABA pool in *Rosa x hybrida*.

*A. thaliana* developed under high RH had lower ABA levels in the leaves, but were able to produce large amounts of ABA in response to water stress. Plants developed in high RH showed increased ABA inactivation during darkness.

This show that *Rosa x hybrida* and *A. thaliana* regulate the ABA levels differently. In *Rosa x hybrida* inactivation to ABA-GE and subsequent release is important, while inactivation to PA is more important in *A. thaliana*.

Furthermore this study clearly shows that *V. faba* plants developed in high RH are able to increase the $H_2O_2$ production when the ABA levels are increased. However, they do not increase the ABA levels during darkness and therefore do not initiate the ABA dependent pathway. These results suggest that the reduced stomatal response found in plants developed in continuous high RH is caused either by a step downstream of $H_2O_2$ in the pathway toward stomatal closure or might be a result of changed anatomy, making the stomata unable to close properly.

To fully understand why stomata developed in continuous high RH have reduced ability to close further research is necessary. Only the initiation of the ABA dependent pathway to stomatal closure has been investigated in this study. It would be interesting to investigate the remaining steps and measure the turgor pressure inside the guard cells. The stomatal anatomy in plants developed in high RH has also not been well studied. The cell wall composition and arrangement of microfibrils and microtubule could give further information about the guard cells ability to close.

Cultivation strategies have to be developed for greenhouse systems in order to produce high quality plants with functional stomata in periods when the RH is high. A daily stress treatment (high temperature/ low RH) improves the closure significantly. However, the responsiveness, the degree and duration of a stress treatment needs further investigation in greenhouse cultivated plant species before any recommendations can be made.

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# Paper I

Plant, Cell & Environment

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# High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves

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## ABSTRACT

**Plants developed under high (90%) relative air humidity (RH) have previously been shown to have large, malfunctioning stomata, which results in high water loss during desiccation and reduced dark induced closure. Stomatal movement is to a large extent regulated by abscisic acid (ABA). It has therefore been proposed that low ABA levels contribute to the development of malfunctioning stomata. In this study, we investigated the regulation of ABA content in rose leaves, through hormone analysis and β-glucosidase quantification. Compared with high RH, rose plants developed in moderate RH (60%) and 20 h photoperiod contained higher levels of ABA and β-glucosidase activity. Also, the amount of ABA increased during darkness simultaneously as the ABA-glucose ester (GE) levels decreased. In contrast, plants developed under high RH with 20 h photoperiod showed no increase in ABA levels during darkness, and had low β-glucosidase activity converting ABA-GE to ABA. Continuous lighting (24 h) resulted in low levels of β-glucosidase activity irrespective of RH, indicating that a dark period is essential to activate β-glucosidase. Our results provide new insight into the regulation of ABA under different humidities and photoperiods, and clearly show that β-glucosidase is a key enzyme regulating the ABA pool in rose plants.**

*Key-words*: abscisic acid; ABA-glucose ester; β-glucosidase.

## INTRODUCTION

Stomatal opening plays a critical role in regulating gas exchange required for photosynthesis and transpirational water loss needed for nutrient uptake and cooling. Throughout the day, there is a constant regulation of the stomata as the water loss through stomata is balanced against the need for $CO_2$ uptake (Tallman 2004). During the night, most C3 plants close the stomata to maximize the hydration when there is no need for $CO_2$ uptake for photosynthesis. In the early morning, when the plant water potential is the least negative, stomata open so that transpirational nutrient uptake can occur. Later in the day, the stomatal opening is closely regulated to make sure the plants retain enough water to maintain turgor. The control of stomatal opening and closing is regulated by the plant hormone abscisic acid (ABA), which in turn is regulated by the $O_2$:$CO_2$ ratio, air humidity, drought, temperature and light (Tallman 2004; Reynolds-Henne *et al.* 2010). Increased levels of ABA activate $Ca^{2+}$ inwards channels and $K^+$ and $Cl^-$ outwards channels, which decrease the turgor pressure, resulting in stomatal closure.

The regulation of endogenous ABA levels in plant tissues is mediated by the balance between biosynthesis and inactivation (Zeevaart 1980). ABA is synthesized through a series of steps from isopentenyl diphosphate (IPP), with zeaxanthin and then violaxanthin as two of the intermediate precursors (Seo & Koshiba 2002). Zeaxanthin and violaxanthin are also parts of the light-driven xanthophyll cycle, which regulates the dispatching of excess energy through non-photochemical quenching (Jahns, Latowski & Strzalka 2009). During light, violaxanthin is converted to zeaxanthin, reducing the amount of violaxanthin available for ABA biosynthesis (Tallman 2004). In *Nicotiana tabaccum*, the concentration of ABA in unstressed plants was at its maximum 3 h into the dark period, before decreasing and remaining low the rest of the dark period (Novakova *et al.* 2005). During the light period, the ABA concentration in unstressed plants was regulated by the need to conserve water, but never reached the level of the maximum peak during darkness. The increase in ABA levels in the beginning of the dark period is believed to ensure stomatal closure in order to preserve water and rehydrate when no $CO_2$ uptake is needed (Tallman 2004; Novakova *et al.* 2005).

ABA is inactivated by two main pathways: either oxidation or conjugation of free ABA (Nambara & Marion-Poll 2005). The oxidation pathway involves the hydroxylation of ABA to phaseic acid (PA), which is further reduced to dihydrophaseic acid (DPA) (Cutler & Krochko 1999; Nambara & Marion-Poll 2005). The second pathway is conjugation with monosaccharides, most commonly with glucose creating ABA-glucose ester (ABA-GE) (Lim *et al.* 2005; Priest *et al.* 2006). ABA-GE is hypothesized to be a storage form of ABA, which can be stored in the vacuoles and released when ABA is needed (Dietz *et al.* 2000). In a number of plant species like *Arabidopsis thaliana*, *Hordeum vulgare* (barley) and *Triticum spp.* (wheat),

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ABA-GE has been found to be hydrolyzed in response to water stress by β-glucosidase, leading to an increase in the active ABA pool (Dietz *et al*. 2000; Sauter, Dietz & Hartung 2002; Lee *et al*. 2006). β-glucosidases are also known to hydrolyze other conjugated plant hormones such as cytokinins, auxin, gibberellins and salicylic acid, as well as to degrade cellulose and anthocyanin (Minic 2008; Morant *et al*. 2008; Oren-Shamir 2009; Verma *et al*. 2010).

ABA plays an important role in environmental stress responses and the levels increase when plants experience adverse environmental conditions like drought, salt and suboptimal temperatures (Luan 2002; Zhu 2002; Reynolds-Henne *et al*. 2010). However, environmental factors such as air humidity are also known to affect the endogenous ABA levels of plants. For instance, in *Spinacia oleracea* (spinach), *Tradescantia virginiana* (Virginia spiderwort) and *A. thaliana*, ABA levels were lower in leaves developed under high compared with moderate humidity (Zeevaart 1974; Nejad & Van Meeteren 2007; Okamoto *et al*. 2009). *Arabidopsis* also showed lower ABA content due to increased inactivation of ABA to PA and DPA when moved from low to high relative air humidity (RH) (Okamoto *et al*. 2009).

Previous studies have shown that continuous high RH (>85%) during growth also results in the development of malfunctioning stomata, which are unable to close (Torre & Fjeld 2001; Torre *et al*. 2003). Similar observations have been obtained in experiments with leafy cuttings rooted at high RH (Fordham *et al*. 2001) and in micro propagated plants (Santamaria, Davies & Atkinson 1993). It has also been shown that when transferred to low RH conditions, *T. virginiana* plants developed under high RH (90%) had a higher leaf transpiration rate and stomatal conductance as well as larger stomatal apertures than plants developed under moderate RH (55%) (Nejad & Van Meeteren 2005). The development of malfunctioning stomata leads to significant water loss, low post-harvest stress tolerance, and limited survival of cuttings and *in vitro* grown plantlets upon transplanting (Torre *et al*. 2003). However, in roses, daily ABA applications could overcome the negative effect of high RH and produce functional stomata (Fanourakis *et al*. 2011).

Much of the work done on RH responses on stomata function and stress resistance has been performed on roses (*Rosa x hybrida*). Roses are one of the world's most economically important commercial ornamentals produced in greenhouses either as cut flowers or as pot plants. To save energy, ventilation of humid air is avoided and as a consequence the RH can increase to above 90% in some greenhouse systems and in certain periods of the year (Max *et al*. 2009). The critical threshold is found to be 85%, and an RH above this level affects the behaviour and anatomical features of stomata, stomatal function and post-harvest behaviour (Mortensen & Gislerød 1997; Pettersen, Moe & Gislerød 2007).

Further, to increase the productivity in greenhouse systems, extension of the natural photoperiod with the use of artificial lighting is common in periods when the natural irradiance is low. In some plant species, continuous lighting (24 h) induces severe injuries like leaf chlorosis and necrosis, and results in lower photosynthesis and accelerated leaf senescence (Velez-Ramirez *et al*. 2011). However, roses are tolerant to continuous lighting, and high growth rate and productivity are commonly observed (Mortensen & Gislerød 1999). The effect of high RH is clearly aggravated under continuous lighting and results in extremely high water loss and limited drought tolerance (Mortensen & Gislerød 1999; Mortensen, Pettersen & Gislerød 2007).

As daily application of ABA to roses results in functional stomata under high RH (Fanourakis *et al.* 2011) and the fact that ABA is a key regulator of the signal transduction pathway in stomatal closure in response to water deficit, it can be hypothesized that ABA is involved in RH responses. However, despite the progress in research on ABA metabolism, the mechanisms by which the ABA content is regulated by air humidity are less understood. The aim of this study was to study the effect of high air humidity on the ABA regulation and responses in rose leaves. To improve the understanding of plant responses to interactive effects of air humidity and photoperiod, the effects of high humidity under continuous lighting on ABA regulation were also investigated.

## MATERIALS AND METHODS

### Pre-cultivation and experimental growth conditions

*Rosa x hybrida*, cv. Rebecca plants were grown from a single node stem segment with one mature leaf. The cuttings were taken from the middle and lower positions of fully developed stems with open flowers. After 2–3 weeks, the cuttings were rooted and transferred to 12 cm pots containing a standard fertilized *Sphagnum* peat media (Floralux, Nittedal, Norway). The pH and EC level were 5.7 and 1.75, respectively, in all experiments (Superba: NPK 9-5-25 + Mg + S + Mikro and calcinit, Yara, Norway). During pre-cultivation, the plants were kept in a greenhouse compartment (glass roof and polycarbonate walls) at a temperature of 21 °C, and average daily RH of 70% [corresponding to a water vapour deficit (vpd) of 0.74 kPa], at the Center for Plant Research in Controlled Climate at the Norwegian University of Life Sciences, Ås, Norway (N 59°40.120′, E 10°46.232′). Supplementary light by high-pressure-sodium-lamps (HPS, Osram NAVT- 400W, Munich, Germany) was given daily for 20 h at a photon flux density of 100 (±10) $\mu$mol $m^{-2}$ $s^{-1}$ at 400–700 nm (measured with a Li-Cor, Model L1-185, quantum sensor, Lincoln, NE, USA). The pre-cultivation ended when the plants had 1–1.5 cm long shoots. The plants were then transferred to the different humidity treatments in growth chambers.

During the experimental period, the plants were exposed to 100 $\mu$mol $m^{-2}$ $s^{-1}$ for 20 h $day^{-1}$ (4 h darkness) or continuous lighting (24 h) by Mercury lamps (Osram NAV T-400W). The temperature was 20 ± 0.5 °C; the RH in the

growth chambers was either 60 ± 3% (moderate humidity, vpd: 0.7 kPa) or 90 ± 2% (high humidity, vpd: 0.23 kPa); and the $CO_2$ concentration was 0.4 mg $g^{-1}$. A PRIVA (Priva, Ontario, Canada) greenhouse computer was connected for recording, controlling and storing the climate data in growth chambers and the greenhouse. Three different repeats of the experiments were carried out.

Leaf samples for analysis of ABA, its metabolites and $\beta$-glucosidase activity as well as stomata imprints, were taken from the first fully expanded leaves with five leaflets. The sampling was done after 6 weeks of treatment when the plants had flowered, in the middle of the light and dark periods in both humidity treatments with 20 h photoperiod. In continuous lighting, the samples were taken between 1700 and 1800 h. Samples were immediately frozen in liquid nitrogen and stored at −80 °C prior to extraction for ABA and $\beta$-glucosidase quantification.

## Measurement of stomatal conductance

Stomatal conductance of the intact leaves was measured for 24 h during a diurnal cycle using a CIRAS-2 Portable Photosynthesis System with PLC6 (U) Automatic Universal Leaf Cuvette (PP Systems, 2001, Amesbury, MA, USA). During all measurements, the humidity and light in the leaf cuvette was the same as in the growth chamber, the $CO_2$ concentration was 400 $\mu$mol $mol^{-1}$, the airflow was 250 $\mu$mol $s^{-1}$ and the temperature was 22 °C. Measurements were taken every 15 min for 24 h.

## Stomata response to desiccation

To study the stomata response to dehydration, desiccation tests were done with detached upper leaves from 10 plants grown under high (90%) and moderate (60%) RH. The tests were performed in a test room with 50% RH, 15 $\mu$mol $m^{-2}$ $s^{-1}$ light intensity and 22 °C. The leaves were weighed 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120 and 180 min after detachment. After the desiccation test, the leaf area was determined with a leaf area meter (Li-Cor, LI-3100). The rate of water loss (transpiration rate) per leaf area was calculated using the following equation:

$$Transpiration\ rate = \frac{Change\ in\ leaf\ weight\ during\ the\ desiccation\ time}{leaf\ area}$$

## Microscopy analysis of stomata

Epidermal impressions were made of fresh intact upper leaves by Suzuki's Universal Micro-Printing (SUMP) method using SUMP liquid and SUMP plate B (SUMP Laboratory, Tokyo, Japan) as described previously (Tanaka *et al*. 2005). All samples were taken interveinally close to the mid-rib on the abaxial side of the leaf from 12 leaves from each air humidity treatment during both light and dark. The SUMP imprints were observed under a light microscope (Leitz, Labolux K, Type 0.2, Wetzlar, Germany) and stomata images were obtained with a Leica camera (Leica DC200, Heerbrugg, Switzerland). Stomatal morphology (length, width and area) were measured with the use of UTHSCSA ImageTool for windows version 3.00 (The University of Texas Health Science center, San Antonio, TX, USA).

## ABA quantification

### *Chemicals and calibration curves*

ABA-catabolites 4′-dihydrophaseic acid (DPA), ABA-$\beta$-D-glucosyl ester (ABA-GE), PA, 7′-hydroxy-ABA (7′OH-ABA), neophaseic acid (*neo*PA) and *trans*-ABA were synthesized and prepared at the Plant Biotechnology Institute of the National Research Council of Canada (PBI-NRC Saskatoon, SK, Canada), while *cis*-ABA was purchased from Sigma-Aldrich (Sigma Chemicals, St Louis, MO, USA). Deuterated forms of the hormones, which were used as internal standards, that is, *d*3-DPA, *d*5-ABA-GE, *d*3-PA, *d*4-7′-OH-ABA, *d*3-*neo*PA, *d*4-ABA, *d*4-*trans*-ABA, were synthesized and prepared at PBI-NRC (Abrams, Nelson & Ambrose 2003; Zaharia *et al*. 2005). The deuterated forms of selected compounds used as recovery standards, d6-ABA and d2-ABA-GE, were also prepared and synthesized at PBI-NRC. Calibration curves were created for all compounds of interest. Quality control samples (QCs) were run along with the tissue samples.

### *Extraction and purification*

The samples were freeze dried and homogenized before analysis. A 100 $\mu$L aliquot containing the deuterated internal standards, each at a concentration of 0.2 pg $\mu L^{-1}$, was added to approximately 50 mg of homogenized plant tissue; 3 mL of isopropanol:water:glacial acetic acid (80:19:1, v/v) was then added, and the samples were agitated in the dark for 24 h at 4 °C. Samples were then centrifuged and the supernatant was isolated and dried on a Büchi Syncore Polyvap (Büchi, Flawil, Switzerland). Samples were reconstituted in 100 $\mu$L 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 $\mu$L 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). The eluate was then dried on a LABCONCO centrivap concentrator (Labconco Corporation, Kansas City, MT, USA). An internal standard blank was prepared with 100 $\mu$L of the deuterated internal standard mixture. QC standards were prepared by adding 100 and 30 $\mu$L (separately) of a mixture containing the analytes of interest, each at a concentration of 0.2 pg $\mu L^{-1}$, to 100 $\mu$L of the internal standard mix. Finally, samples, blanks and

QCs were reconstituted in a solution of 40% methanol (v/v), containing 0.5% acetic acid and 0.1 pg $\mu L^{-1}$ of each of the recovery standards.

### *Hormone quantification by UPLC-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 SB column (2.1 × 100 mm, 1.8 $\mu$m, Waters, Milford, MA, USA) with an in-line filter and separated by a gradient elution of water containing 0.02% formic acid against an increasing percentage of a mixture of acetonitrile and methanol (volume ratio: 50:50). 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 analysed along each batch of tissue samples. MassLynx™ and QuanLynx™ (Micromass, Manchester, UK) were used for data acquisition and data analysis.

### *β*-glucosidase quantification

*β*-glucosidase was measured as described by Dietz *et al*. (2000), with some modifications. The leaf samples were taken from the freezer (–80 °C) and immediately homogenized in liquid nitrogen using a mortar and pestle. Samples (1.00 g) were extracted for 1.5 h at 4 °C in 10 mL 100 mM citrate buffer, containing 5% (w/v) poly (vinylpolypyrrolidone) (PVPP), 1 mM ethylenediaminetetraacetic acid (EDTA), 14 mM mercaptoethanol and 10% (w/v) glycerol. The samples were then centrifuged at 201 *g* for 2 min (Eppendorf 5810 centrifuge, Hamburg, Germany). The supernatant (100 µL) was mixed with 1 mL 100 mM citrate buffer containing 4 mM pNPG (p-nitrophenol-*β*-D-glucopyranoside) and incubated at 37 °C for 60 min (Termaks B 8054 Incubator, Bergen, Norway). The reaction was then terminated with 2 mL 1 M $Na_2CO_3$ and the amount of liberated p-nitrophenol was measured spectrophotometrically at 405 nm (Helios Alpha Spectrophotometer, Thermo Scientific, Surrey, UK). The concentration was calculated using the Beer-Lambert law, Absorbance = ε × length × concentration, and the molar extinction coefficient for p-nitrophenol ε = 18300 (Dietz *et al*. 2000). One unit of enzyme is then defined as the amount of enzyme needed to yield 1 nmol of p-nitrophenol per hour at 37 °C.

### Statistical analyses

Significant differences between means were tested for normally distributed general linear models (GLM) and Tukey's test. Differences with $P < 0.05$ were considered significantly different. All statistical tests were performed in Minitab 16.1.1 (Minitab 16.1.1, windows version, State College, PA, USA).

## RESULTS

### Increased diurnal stomatal conductance and decreased response to desiccation in leaves developed at high RH under a 20 h photoperiod

We analysed the diurnal stomatal conductance of plants growing at high (90%) and moderate (60%) RH with a gas exchange analyser (CIRAS-2). A considerably higher conductance ($g_{sw}$) was measured throughout both the day and night in plants growing in high RH, compared with plants in moderate RH ($P = 0.041$, Fig. 1). However, the $g_{sw}$ of plants grown under both humidity treatments showed a similar pattern with the conductance decreasing throughout the day and the lowest values during the dark, before increasing again when the light was turned on. The $g_{sw}$ of plants growing in high RH was still high during darkness (30 mmol $m^{-2} s^{-1}$), while the plants grown under moderate RH showed very low $g_{sw}$ (3.5 mmol $m^{-2} s^{-1}$), indicating stomatal closure. The difference in stomata conductance between light and dark was similar in both treatments (≈30 mmol $m^{-2} s^{-1}$). To estimate the relative change in the stomatal aperture between day and night, the day:night ratio was calculated. The relative change in conductance was much larger in moderate RH (85% change) compared with high RH (28.5% change, $P = 0.032$). This indicates that stomata developed under moderate RH close better during darkness

To further test the ability of the plants to close the stomata and retain water, a desiccation test was performed. After a 3 h desiccation, treatment plants grown at high RH had lost significantly more of their original weight (≈50%), while plants grown under moderate humidity had only lost 10–15% due to stomatal closure ($P < 0.001$, Fig. 2a). The transpiration rate was highest in the beginning, before the stomata had closed, and decreased throughout the

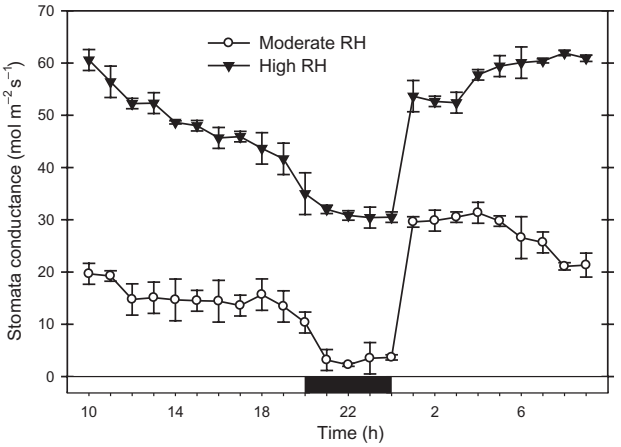


**Figure 1.** Diurnal stomata conductance (mol $m^{-2} s^{-1}$) measured on the adaxial side of the first fully expanded leaves with five leaflets of rose plants grown at moderate (60%) or high (90%) relative air humidity (RH). The measurements were done on fully grown plants with open flowers during a light/dark cycle of 24 h. Mean ± SE. $n = 9$.

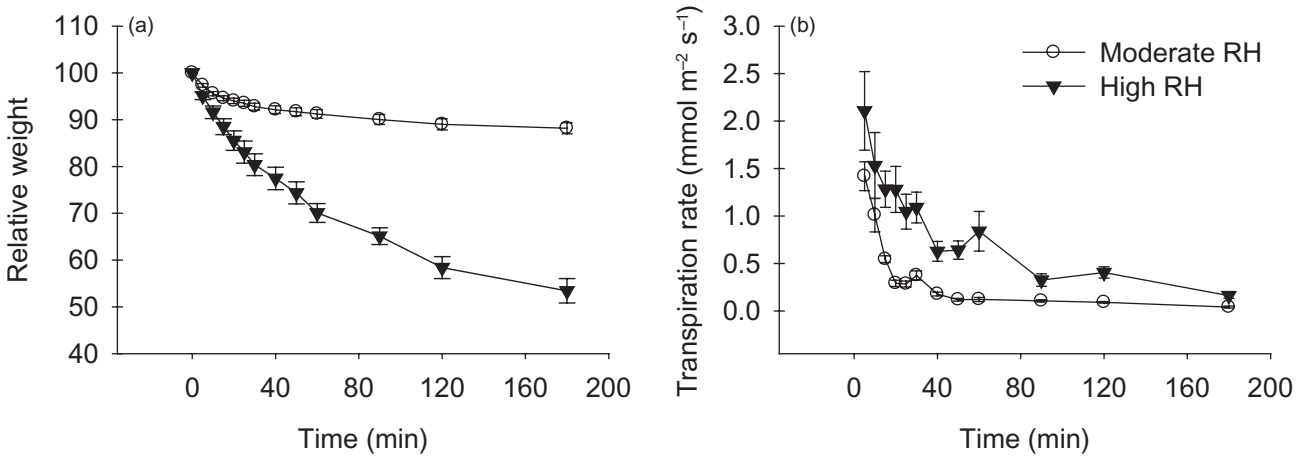


**Figure 2.** Relative weight (a) and transpiration rate (b) of leaves of rose plants grown under moderate (60%, circles) or high (90%, triangles) relative air humidity (RH) during 3 h of desiccation. Mean ± SE. $n = 10$.

desiccation test in plants developed in both moderate and high relative humidity (Fig. 2b). During the first 10 min, there was no statistically significant difference in the transpiration rate between the plants developed under the different RH conditions. However, after the first 10 min and throughout the rest of the test, a significantly higher transpiration rate was measured in plants developed under high RH ($P \leq 0.004$). This shows that the stomata of plants developed under moderate RH have better stomatal functioning and are able to retain more water.

To better understand the effect of high RH on the stomatal development and the ability to close the stomata, imprints were made of the leaves during both light and dark, and stomatal characteristics were measured. In general, the stomatal pore aperture and length were larger in plants grown at high RH than in plants grown at moderate RH (Table 1, Fig. 3). The length of the stomatal pore of plants grown under high RH, measured during light and dark, was 1.3 and 1.6 times larger than those grown under moderate RH ($P < 0.001$). Likewise, the stomatal pore aperture was also larger in plants grown under high RH ($P < 0.001$). The pore aperture during light and dark in plants grown under high RH was 1.9 and 3 times larger than in plants grown under moderate RH, respectively. As a consequence of the larger stomatal pore length and aperture, the pore area was also larger in plants grown under high RH ($P < 0.001$). During light, the pore area was 2.2 times larger and during dark 2.6 times larger in high RH than in moderate RH. Plants grown under moderate RH had larger pore aperture during light than during darkness, showing that the stomata developed in moderate RH close during the dark ($P < 0.001$, Table 1). In contrast, the pore aperture of plants developed under high RH did not change between light and dark, but remained open.

## Reduced ABA content and no difference between light and dark under high RH in 20 h photoperiod

As ABA is an important signal for stomatal closure, the amount of ABA and its metabolites (DPA, ABA-GE, PA, 7′OH-ABA, *neo*PA, *trans*-ABA and *cis*-ABA) in the leaves was quantified. However, the levels of DPA, 7′OH-ABA, *neo*PA and *trans*-ABA were in all treatments mostly too low to be quantified (data not shown), and have therefore only been included in the value for total ABA metabolite content (i.e. in those cases where they could be quantified). The combined amount of ABA and its metabolites was significantly higher (in average 69%) in plants from moderate compared with high RH ($P < 0.001$). However, there was no significant difference in the combined amount of ABA and its metabolites between light and dark within

**Table 1.** Stomatal pore characteristics of pot rose cv. Rebecca grown at moderate (60%) or high (90%) RH

| | Moderate RH (60%) | | High RH (90%) | |
|---|---|---|---|---|
| | Light | Dark | Light | Dark |
| Pore length ($\mu$m) | 31.2 ± 0.1$^{b}$ | 25.7 ± 0.2$^{c}$ | 41.7 ± 0.2$^{a}$ | 41.0 ± 0.5$^{a}$ |
| Pore aperture ($\mu$m) | 11.5 ± 0.1$^{b}$ | 7.0 ± 0.2$^{c}$ | 21.8 ± 0.2$^{a}$ | 20.7 ± 0.7$^{a}$ |
| Pore area ($\mu$m$^2$) | 174.3 ± 0.9$^{b}$ | 144.4 ± 0.9$^{c}$ | 378.4 ± 4.6$^{a}$ | 376.0 ± 3.5$^{a}$ |

$n = 28$–42.
Different superscript letters indicate significant differences.
Mean ± SE.
RH, relative air humidity.

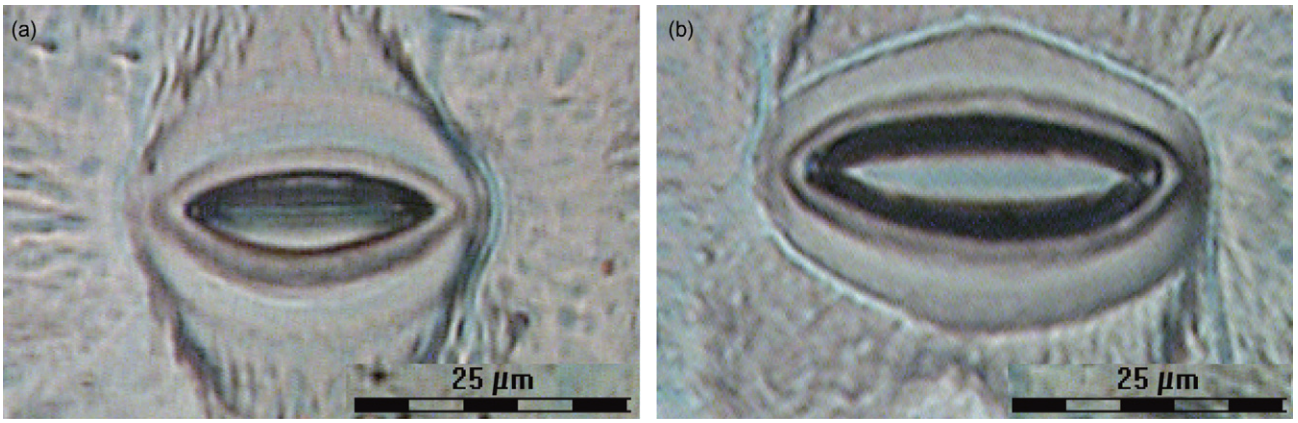


**Figure 3.** Light microscope images of stomata in 60% relative air humidity (RH) (a) and 90% RH (b). The images are of imprints of the abaxial side of the leaves during the light period.

either treatment (Fig. 4d). If only the amount of ABA is considered, the levels were still significantly higher in moderate RH both during light and dark ($P < 0.05$, Fig. 4a). During dark, the amount of ABA was significantly increased in moderate RH (approximately doubled, $P = 0.006$). However, there was no significant change between light and dark in high RH, indicating that the diurnal ABA level was constant and that unlike in moderate RH there was no signal to induce closure in the dark.

ABA-GE has been hypothesized to be a storage form of ABA, which can be converted to ABA (Dietz *et al*. 2000). The amount of ABA-GE was similar in both humidity

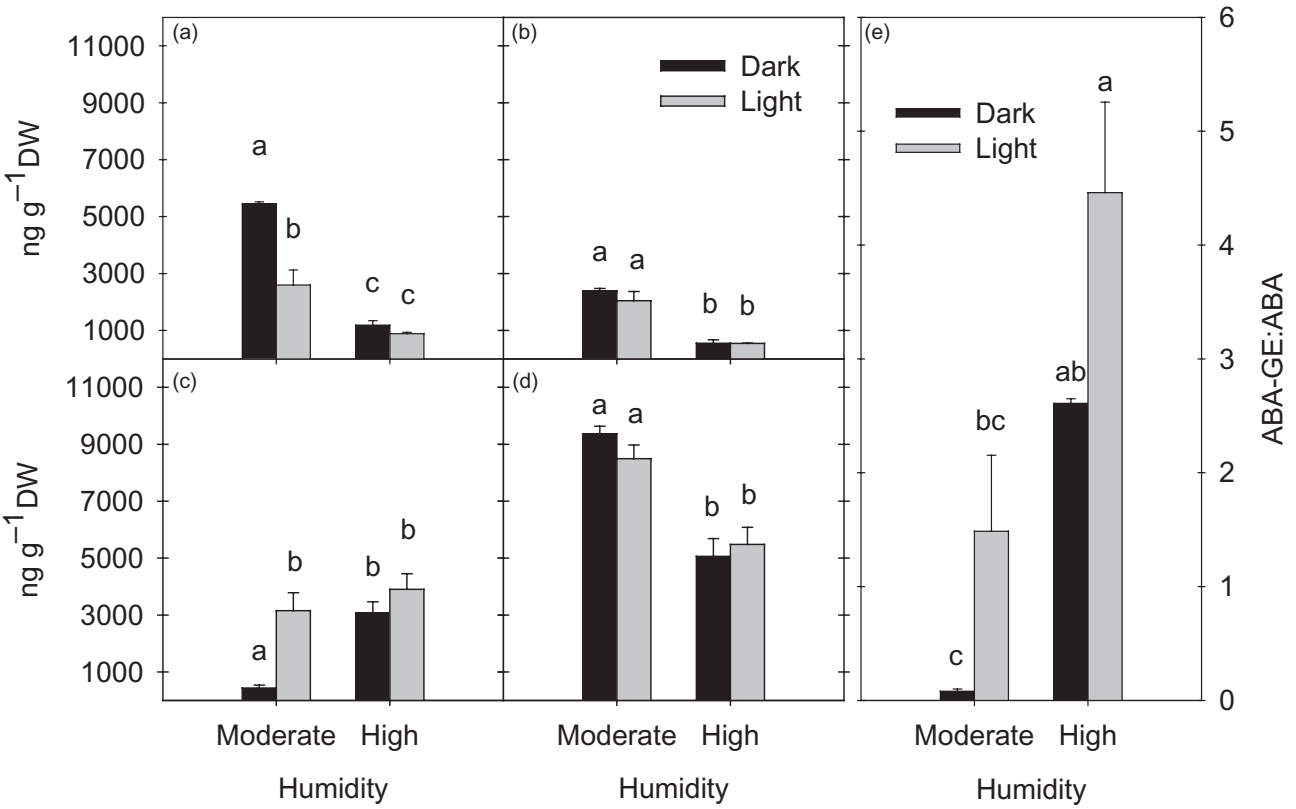


**Figure 4.** The effect of moderate (60%) and high (90%) relative air humidity on amounts of abscisic acid (ABA) (a) and its metabolites phaseic acid (PA) (b), and ABA-$\beta$-D-glucosyl ester (ABA-GE) (c), the total combined amount of ABA and its metabolites (d), and the ABA-GE:ABA ratio in leaves of rose plants (e). Measurements were done in the middle of the photoperiod and in the middle of the dark period. Different letters within each figure indicate significantly different values ($P < 0.05$). Mean ± SE. $n = 3$. Each sample consisted of 5–6 leaves from a single plant.

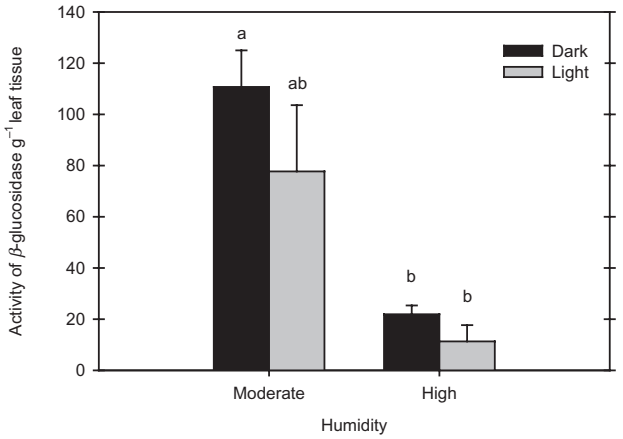


**Figure 5.** The activity of $\beta$-glucosidase in the middle of the light and dark periods in leaves of rose plants growing in high (60%) and moderate (90%) relative air humidity. Different letters indicate significantly different values ($P < 0.05$). Mean ± SE. $n = 3$. Each sample consisted of 5–6 leaves from a single plant.

treatments during light (Fig. 4c). However, as the level of ABA was increased [2800 ng $g^{-1}$ dry weight (DW)] during dark in moderate RH, the level of ABA-GE was decreased similarly (2700 ng $g^{-1}$ DW, $P = 0.013$), but remained unchanged in high RH. ABA-GE might therefore be the source of the increased ABA levels.

In moderate RH, the amount of ABA was similar or higher than that of ABA-GE, while in high RH the amount of ABA was lower than that of ABA-GE (Fig. 4e). This is consistent with the measured activity of $\beta$-glucosidase, converting ABA-GE into ABA. The activity of $\beta$-glucosidase was about fivefold higher in plants grown in moderate RH, than in plants grown in high RH ($P = 0.001$, Fig. 5). This indicates that more ABA-GE was converted to ABA in leaves developed under moderate RH. However, there was no significant difference between light and dark within either of the treatments, only a slight tendency of higher activity during the dark in both treatments.

When ABA is inactivated by oxidation, it is converted into 8′-hydroxy ABA and PA by the enzyme 8′-hydroxylase (Cutler & Krochko 1999). The amount of PA was significantly higher in plants from moderate RH ($P < 0.001$), where the amount of ABA was higher as well, but there was no difference in PA between light and dark in either treatment (Fig. 4b). The ratio between ABA and PA was not significantly different in either treatment or in light or darkness. Thus, inactivation of ABA to PA was similar in light and dark, and the relative amount of ABA being inactivated to PA was the same in both moderate and high RH.

## Continuous light reduces the desiccation tolerance and affects the ABA regulation

Extension of the natural daylength with artificial light is important to increase productivity in greenhouses in periods with low natural irradiance. However, a combination of very long days and high RH significantly decreases post-harvest life of roses (Mortensen & Gislerød 1999). To further understand the effect of daylength and the importance of a dark period in stomatal development, we studied the effects of RH under continuous lighting.

During the desiccation test, plants developed under continuous light and high RH exhibited significantly higher water loss at the end compared with plants developed under continuous light and moderate RH ($P = 0.004$, Fig. 6a). Thus, the leaves developed under continuous lighting showed a similar behaviour as the leaves developed under 20 h photoperiod. However, during the first hour of desiccation, the plants developed under continuous lighting showed significantly higher ($P < 0.040$) water loss than their counterparts developed under 20 h photoperiod. On the other hand, during the last 2 h of desiccation, this difference was reduced and not statistically significant.

To get a better understanding of the increased water loss in continuous light, we examined the ABA levels. In general, most of the levels of ABA and its metabolites in continuous light were more similar to the levels from high RH in 20 h photoperiod and lower than the levels from moderate RH in 20 h photoperiod. There were also no interaction effects between photoperiod and RH, except in PA, indicating that continuous lighting has the same effect irrespective of the RH.

The levels of the combined amount of ABA and its metabolites in continuous light was significantly higher in moderate RH, compared to high RH ($P = 0.036$, Fig. 6f). However, when only the ABA levels were considered, there was no significant difference between the two treatments (Fig. 6c). Compared with the 20 h light treatment, these ABA values were not significantly different from the high RH values. On the other hand, the concentrations in high RH and continuous light were significantly lower than the moderate RH values from 20 h light ($P = 0.0336$). Similarly, the ABA concentrations from moderate RH in continuous light appeared slightly, although statistically insignificantly ($P = 0.085$) lower than the values from moderate RH with 20 h photoperiod.

The ABA-GE concentrations in continuous light were similar in both humidity regimes (Fig. 6e). There was no significant difference between any of these values and those in the 20 h light experiment. The amount of ABA-GE converted back to ABA by $\beta$-glucosidase, was also similar in both humidity treatments in continuous light (Fig. 6b).

The PA concentrations in continuous light were higher in moderate RH than in high RH ($P = 0.003$, Fig. 6d). However, there was no significant difference between these values and those in high RH in 20 h light. However, the PA levels in both treatments in continuous light were significantly lower than those from moderate RH and 20 h light ($P < 0.0008$).

Overall, the plants developed under continuous light behaved similarly as plants developed under a 20 h photoperiod. However, they had lower desiccation tolerance, with increased water loss the first hour of desiccation than their counterparts growing in 20 h photoperiod. Also, their ABA

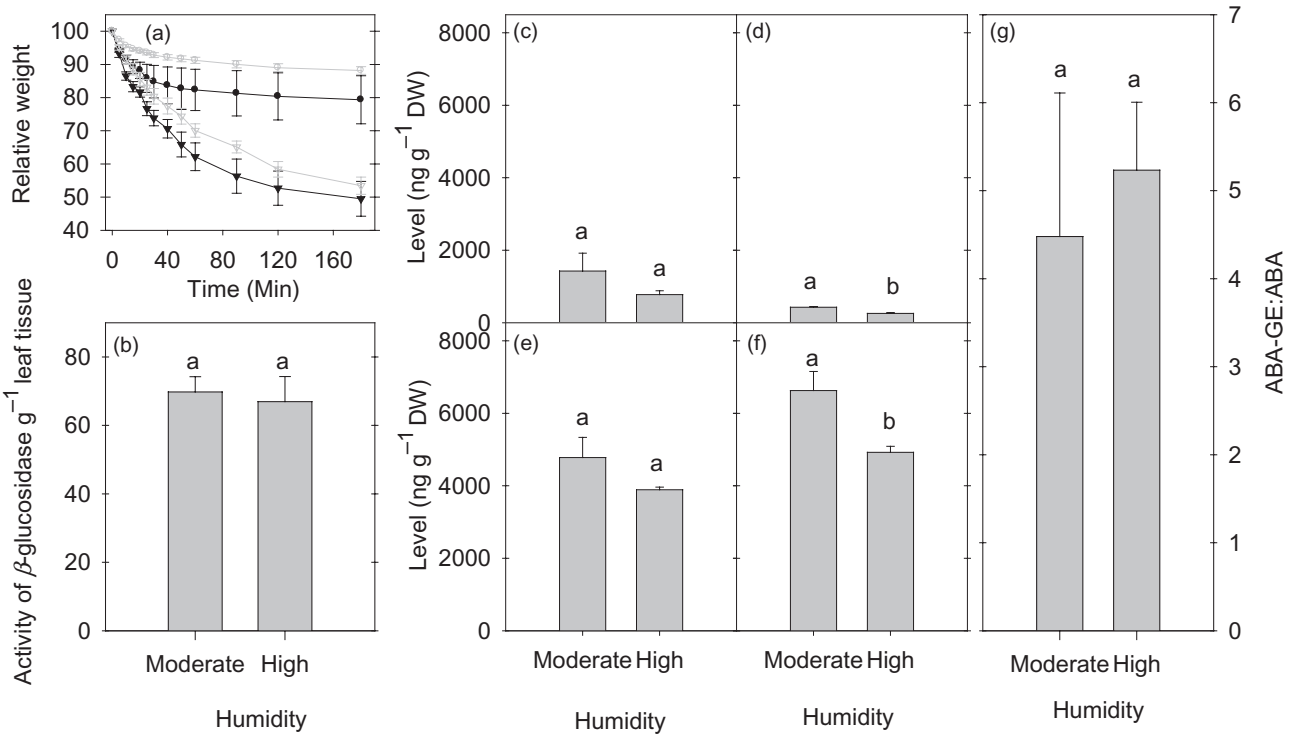


**Figure 6.** Relative weight during a 3 h desiccation (a) and levels of abscisic acid (ABA) and its metabolites in leaves of plants developed under moderate (60%, circles) and high (90%, triangles) relative air humidity (RH) in continuous light (dark lines). For comparison, in (a) the results from 20 h light (above) moderate (circles) and high (triangles) RH is added and shown in grey. (b–g) The amount of $\beta$-glucosidase (b), ABA (c), phaseic acid (PA) (d), ABA-$\beta$-D-glucosyl ester (ABA-GE) (e), the total combined amount of ABA and its metabolites (f), and ABA-GE:ABA ratio (g). Different letters within each figure indicate significantly different values. Mean ± SE. $n = 10$ (a), 5 (b), 3 (c–g). Each sample consisted of 5–6 leaves from a single plant (b–g) and were taken between 1700 and 1800 h.

levels were more similar to those of plants developed under high RH and 20 h photoperiod.

## DISCUSSION

High RH induces malfunctional stomata in different growing systems and several different plant species (Santamaria *et al*. 1993; Mortensen 2000; Torre & Fjeld 2001; Nejad & Van Meeteren 2005). Most studies in this respect has been performed on roses (*Rosa x hybrida*), which are among the economically most important greenhouse-grown ornamental plants. Malfunctional stomata result in rapid post-harvest water loss and highly reduced stress tolerance, thus strongly reduced plant quality. ABA application and ABA quantification in plants from different RH regimes have suggested an involvement of ABA (Nejad & Van Meeteren 2007; Okamoto *et al*. 2009; Fanourakis *et al*. 2011), but the effect of RH on ABA regulation has not been studied. In this study, we demonstrate for the first time that plants growing under constant high RH regulate the ABA levels differently than plants grown at constant moderate RH.

### High RH affects stomata morphology and physiology in roses

The diurnal response and desiccation test confirmed that roses developed under high RH have higher transpiration rate than roses developed under moderate RH (Fig. 2). Previous studies have shown similar results in several other species, such as *T. virginiana*, *Begonia x cheimantha*, *Euphorbia pulcherrima*, *Kalanchoe blossfeldiana* and *Chrysanthemum morifolium*, indicating that uncontrolled water loss induced by high RH during development is a universal response in plants (Mortensen 2000; Torre & Fjeld 2001; Nejad & Van Meeteren 2005). The uncontrolled loss of water has been suggested to either be due to alteration in stomata morphology or physiology, which causes the stomata to remain open when subjected to stimuli normally inducing closing.

The plants developed under high RH had larger stomatal length and aperture, which in turn resulted in a larger stomatal area (Table 1). This is similar to previous studies in a number of species (Fordham *et al*. 2001; Torre *et al*. 2003; Karbulkova *et al*. 2008). Furthermore, we found that the stomata of plants developed under high RH did not respond to darkness or drought, but remained open. In contrast, the stomata of plants developed under moderate RH responded by closing their stomata when subjected to darkness or drought. Several studies have indicated that stomata of *in vitro* grown plants, which also experience high RH, fail to close fully in response to ABA (Santamaria *et al*. 1993), low leaf water potential (Fordham *et al*. 2001) and darkness (Ziv, Schwartz & Fleminger 1987). In addition,

plants developed under high RH have higher stomatal density (Torre *et al*. 2003; Nejad & Van Meeteren 2005). The increased area of the stomatal pore in combination with higher number of stomata might explain at least parts of the increased transpiration found in plants developed under high RH. It has previously been discussed if the large stomata found in plants developed under high RH are a result of low endogenous ABA levels. Supporting this is a study on *Populus x canescens* where it was found that ABA insensitive plants had larger stomata (Arend *et al*. 2009).

## High RH during growth in 20 h photoperiod affects the ABA content in light and darkness

ABA is an important stress hormone that induces stomatal closure. The regulation of ABA is well known and its two main pathways of inactivation result in formation of PA and ABA-GE (Nambara & Marion-Poll 2005). It has been suggested that the lack of stomatal response in plants developed under high RH is partly due to low ABA concentrations. In this study, we measured the ABA concentrations in leaves of plants developed under high and moderate RH under a 20 h photoperiod, in light and darkness, and compared the conversion rates of ABA with PA and ABA-GE with ABA.

The amount of ABA and the catabolite PA was significantly lower in plants developed under high RH, compared with moderate RH (Fig. 4). The lower PA levels in high RH are apparently a result of the lower ABA levels and a constant inactivation rate of ABA to PA. Previous studies on *T. virginiana* and *A. thaliana* also found lower ABA concentrations in plants developed under high RH or when moved from low to high RH (Zeevaart 1974; Nejad & Van Meeteren 2007, 2008; Okamoto *et al*. 2009). It is believed that the decrease in ABA levels when plants are moved from low to high RH is a result of increased inactivation and not altered biosynthesis (Okamoto *et al*. 2009).

In our study, the amount of ABA increased in the dark in moderate RH, while it remained unchanged in high RH (Fig. 4). The increased ABA concentration during dark in moderate RH is believed to act as a signal for stomatal closure during darkness (Tallman 2004; Novakova *et al*. 2005). Plants from high RH did not increase their ABA levels, and the stomata thus lack a signal for closure during darkness. A previous study on the diurnal variation of ABA in *N. tabaccum* demonstrated a peak in ABA concentration after 3 h of darkness, before decreasing, and remaining low throughout the rest of the dark period (Novakova *et al*. 2005). Similarly, a study of *S. oleracea* showed decreased levels of ABA at the end of an 8 h dark period compared with at the onset of darkness (Zeevaart 1974).

The amount of ABA-GE during light was similar in the two humidity treatments under 20 h photoperiod. However, it was reduced during dark in moderate RH (Fig. 4). Several studies, including this one, have hypothesized that ABA-GE is a storage form of ABA and can be converted back to ABA when needed (Dietz *et al*. 2000; Sauter *et al*. 2002). The increased levels of ABA and reduced levels of ABA-GE in moderate humidity during dark support this, indicating that the increased ABA levels have been converted from ABA-GE.

## $\beta$-glucosidase has a central role in ABA regulation in moderate RH

The constant amount of ABA metabolized to PA during light and dark in both RH treatments indicates that the changes in ABA levels are due to either conversion to ABA-GE or increased biosynthesis. A study on *Nicotiana plumbaginifolia* showed that light regulates the ABA concentration through affecting degradation rate and not biosynthesis (Kraepiel *et al*. 1994). Although the regulation of ABA through biosynthesis has not been quantified, the combined amount of ABA and its metabolites did not differ between light and dark (Fig. 4). This indicates that there is no difference in the biosynthesis or degradation by oxidation between light and dark (Fig. 5). The $\beta$-glucosidase assay also showed that there are higher concentrations of enzyme converting ABA-GE into ABA in moderate humidity, while there was very little of this enzyme in high humidity. In a previous study, the amount of $\beta$-glucosidase was highest during light, and after an initial peak during darkness, remained low the rest of the dark period (Novakova *et al*. 2005). Thus, it appears very probable that the increased levels of ABA during darkness arise from ABA-GE. To increase the amount of enzymes in the ABA biosynthesis during drought stress, turgor has to decrease significantly (Liu *et al*. 2005). In this study, there was no loss of turgor in the leaves during darkness in either of the treatments, indicating that $\beta$-glucosidase does not require a large reduction of turgor before converting ABA-GE to ABA. The release of ABA from ABA-GE is therefore a quicker response to stress than an increase in biosynthesis. However, although the enzyme has been found to be most active at hydrolyzing ABA-GE, it is presumed that it has a broader substrate specificity, hydrolyzing also other hormones (Dietz *et al*. 2000; Minic 2008).

The lack of change in the amount of ABA and ABA-GE between light and dark in plants developed under high RH might be due to the extremely favourable conditions for rapid growth in high RH and long photoperiod (20 h). These conditions might make it unnecessary for the plants to close the stomata during darkness and reduce the amount of $\beta$-glucosidase, reducing the plant's ability to convert ABA-GE to ABA. It is also possible that plants developed under high RH are insensitive to or not receiving the signals inducing the production of $\beta$-glucosidase.

In another study of *Arabidopsis*, we have shown that the ABA-GE concentration is very low (Arve *et al*. unpublished results) in both moderate and high RH. The regulation of ABA through biosynthesis and degradation to PA therefore appears more important in *Arabidopsis* (Okamoto *et al*. 2009). In light of this, it can be hypothesized that the pathways of ABA regulation might be different in different species.

### A dark period and reduced RH (<85%) are important for the development of functional stomata

As shown here, stomata developed under continuous light are poorer at retaining water than those developed under a 20 h photoperiod, regardless of RH treatment (Fig. 6). This is consistent with previous studies on roses (Mortensen & Gislerød 1999). The ABA content in plants developed under continuous light was also lower than in plants developed in moderate RH under 20 h photoperiod and more similar to that of the plants developed in high RH under 20 h photoperiod. This shows that the dark period is important for the development of fully functional stomata in rose plants. The lack of stomatal closure during dark in high RH is an indication that high RH overrides the signals from the dark period. In a study on six temperate deciduous tree species, the leaf water potential was found to be the most important signal for stomatal responses, and responses to air humidity dominated over responses to photosynthetic signals (Aasamaa & Sober 2011).

One possibility is that the higher ABA concentrations during dark in moderate RH are important in stomatal development. Plants developed under high RH have never experienced high ABA content, which might be necessary to produce functional stomata. Applications of ABA during growth or transfer from high to moderate RH before the leaves are fully expanded, have been shown to result in production of functional stomata (Fanourakis *et al*. 2011). It is also possible that it is not the high ABA concentrations *per se* that are important, but the fact that the stomata are closing. If so, the stomata must open and close during development to be able to close properly when they are fully developed. ABA application in high RH has been shown to produce functional stomata (Fanourakis *et al*. 2011), but it is not known whether it is the ABA in itself that changes the stomatal development or whether it is an indirect effect of the closing stimulus.

## CONCLUSION

The present study confirms that rose plants developed under high RH have higher transpiration both during growth and during desiccation stress, and that there is no stomatal response to darkness. We have further shown that in moderate RH, the amount of ABA was increased during darkness and that there was simultaneously a similar decrease in ABA-GE, indicating that the increased levels of ABA is due to conversion of ABA-GE. In contrast, plants developed under high RH show no increase in ABA levels during dark, and have very little $\beta$-glucosidase activity converting ABA-GE to ABA. As ABA is a signal for stomatal closure, the low ABA levels in high RH might explain some of the lack of stomatal closure during darkness and development of malfunctioning stomata. However, further study is still needed to fully understand the lack of stomatal responses in plants developed under high RH.

Further, in plants developed under continuous lighting, the $\beta$-glucosidase activity was low irrespective of the RH, indicating that a dark period is essential to activate this enzyme. On the other hand, as stomata developed under high RH lack dark-induced closure mechanisms, it can be hypothesized that high RH overrides the signals and importance of the dark period. Thus, these data provide new insight into the regulation of ABA under high RH and continuous lighting. The results clearly show that $\beta$-glucosidase has a central role and is a key enzyme in regulating the ABA pool in rose plants and that $\beta$-glucosidase does not require a large reduction in turgor before releasing ABA.

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# Paper II

# ABA regulation and stomatal malfunctioning in *Arabidopsis thaliana* developed in continuous high air humidity

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**Abstract**

To conserve water plants close the stomata in response to increased levels of abscisic acid (ABA). Previous studies have shown that plants developed under high relative air humidity (RH>85%) develop malfunctioning stomata and contain lower ABA levels. It has therefore been hypothesized that low ABA levels during development results in malfunctioning stomata. In this study the stomatal functioning of *Arabidopsis thaliana* was evaluated and the concentration, biosynthesis and metabolism of ABA were quantified. It was found that even though they contained lower ABA levels during growth, plants developed under high RH were able to produce large amounts of ABA during desiccation, but still had high water loss. ABA deficient mutants had lower desiccation tolerance than wild type plants in both high and moderate RH. The difference in water loss between wild type and ABA-deficient mutants were similar in both RH treatments. Plants developed at high RH that were exposed to a daily stress treatment of 2 h increased temperature and decreased RH produced more functional stomata. From these results we conclude that it was not the lower ABA levels per se that resulted in malfunctioning stomata in high RH.

**Key words**

abi3-1, abscisic acid, ZEP, NCED3, AAO3, CYP707A1, CYP707A3

## Introduction

The stomata complex can be considered among the most important adaptive developments in land plants. It consists of two guard cells surrounding the stomatal pore, which regulates the amount of $CO_2$ entering and $H_2O$ transpired by the leaf. To balance $CO_2$ uptake with the need to reduce transpirational water loss, the guard cells are able to regulate the stomatal aperture in response to several environmental factors such as air humidity, light, temperature, leaf water status and intracellular $CO_2$ concentration (Tallman, 2004).

The plant hormone abscisic acid (ABA) is a highly important chemical signal for stomatal closure. ABA is produced in roots and leaves of plants (Hartung et al., 2002). The biosynthesis occurs in chloroplasts and other plastids, where isopentyl diphosphate (IPP) is transformed to ABA through a series of steps. These steps include the conversion of zeaxanthin to violaxanthin, which is regulated by zeaxantin epoxidase (ZEP). Violaxanthin is further converted to xanthoxin by 9-cis-epoxycarotenoid dioxygenase (NCED), and then to ABA-aldehyde by a short chain alcohol dehydrogenase and finally to ABA by abscisic aldehyde oxidase (AAO) (Liotenberg et al., 1999; Xie et al., 2006; Seki et al., 2007).

ABA is inactivated by two main pathways; oxidation or conjugation. The oxidation pathway is regulated by the ABA 8`hydroxylase activity of cytochrome P450 CYP707A, converting ABA to phaseic acid (PA) and further to 4'-dihydrophaseic acid (DPA) (Seki et al., 2007). This conversion is mostly catalyzed by CYP707A1 in guard cells and CYP707A3 in vascular tissues (Okamoto et al., 2009). In the conjugation pathway, ABA is most commonly conjugated to ABA-*β*-D-glucosyl ester (ABA-GE) in a reaction catalyzed by ABA glucosyltransferase (Lee et al., 2006). ABA-GE is hypothesized to be a storage form of ABA, which can be converted back to ABA by *β*-glucosidase (Dietz et al., 2000; Lee et al., 2006; Arve et al., 2013).

Extensive studies have previously been performed on plant responses and ABA production during drought. To retain as much water as possible, plants produce increased levels of ABA which acts as a signal for stomatal closure (Seki et al., 2007; Monda et al., 2011). Especially NCED has been found to play an important role in regulating the ABA content by increasing the ABA biosynthesis during drought (Thompson et al., 2000). Another less-studied factor influencing stomata is the relative air humidity (RH). During *in-vitro* propagation, greenhouse production systems and some conditions in natural ecosystems plants develop under constant high RH. However, the effects of such conditions on ABA and ABA metabolism are not well understood. Previous studies of *Rosa x hybrida*, *Dianthus*

*caryophyllus*, *Delphinium* spp. and *Wrightia tomentosa* have shown that plants developed under continuous high RH (>85%) develop larger stomata, that are unable to close in response to environmental conditions that usually lead to closure (Ziv et al., 1987; Santamaria et al., 1993; Torre et al., 2003; Joshi et al., 2006). This results in high water loss and lower desiccation tolerance (Mortensen and Fjeld, 1998; Torre and Fjeld, 2001). The stomatal density in these plants is also higher, compared to plants grown under lower RH (Sciutti and Morini, 1995; Torre et al., 2003).

The negative effects of high RH can be counteracted by daily application of ABA, a daily temperature increase, a decrease in RH for a few hours or by transferring plants to lower RH before the leaves are fully developed (Pompodakis et al., 2004; Joshi et al., 2006; Pettersen et al., 2007; Waterland et al., 2010a; Waterland et al., 2010b; Fanourakis et al., 2011; Kim and van Iersel, 2011). Also, it appears that the critical stage for determination of the functionality of the stomata is the developmental stage when the leaves are at 70% of full leaf expansion (Fanourakis et al., 2011). Thus, leaves that develop fully under constant high RH conditions cannot be rescued from malfunctioning stomata.

Several studies have shown that compared to lower RH, plants growing in higher RH have lower ABA contents (Nejad and van Meeteren, 2007). Also, after transfer from lower to higher RH, the ABA level is reduced due to inactivation of ABA into PA (Okamoto et al., 2009). However, other studies have also shown similar ABA levels in different RH conditions, and a wild type-like response to decreased RH in ABA-deficient and ABA-insensitive mutants of *Arabidopsis thaliana* (Assmann et al., 2000; Hronkova et al., 2003). Thus, the relationship between ABA levels and stomatal function under high RH is still not clear. Nevertheless, since ABA is an important signal for stomatal closure, it has been hypothesized that development in high RH when endogenous ABA content is low, either reduces the plant`s ability to produce ABA or the guard cells` ability to respond to ABA.

Most of the work done on high RH responses and malfunctioning stomata has been done on commercial crops with limited possibilities to use genetic methods and mutants as a tool. In this study, we therefore used *Arabidopsis thaliana* as a model plant. The main aim of this study was to improve the understanding of the ABA regulation in plants developed in constant high and moderate RH during light, dark and desiccation. Further, the effects of a daily period with temperature increase/RH decrease while keeping the RH high was studied.

## Materials and methods

### Plant material and growth conditions

*Arabidopsis thaliana* (L.) Heyhn. wild type (WT) (Columbia ecotype, Col-0) and the abscisic acid (ABA) deficient mutant *aba3-1* (N157, Nottingham Arabidopsis Stock Centre (NASC), Nottingham, UK) seeds were placed in 0.1% agar in darkness at 4˚C for 4 days. The *aba3-1* mutant is mutated in the AAO3 gene (At1g16540) of the ABA biosynthesis (Schwartz et al., 1997). The seeds were then germinated in 13 cm pots with peat (L.O.G. Gartnerjord, Rakkestad, Norway) or in water culture (Araphonics, Araphonics SA, Liège, Belgium). Three plants were allowed to grow in each pot and placed in a growth chamber with 8 h photoperiod, a light irradiance of 100 ± 5 µmol $m^{-2}$ $s^{-1}$ from high pressure mercury lamps (Osram NAV T-400 W, Munich, Germany), 80% relative air humidity (RH), corresponding to 0.53 KPa water vapour deficit (vpd) and 22˚C. The irradiance was measured with a LI-COR Light Meter (LI-250, USA) and RH and temperature was set and regulated using a PRIVA system (Priva, Ontario, Canada). After two weeks, when the plants started to develop true leaves, the plants were transferred to the different RH and temperature treatments (Figure S1). There were three different treatments; 1 (Moderate RH): Constant RH at 60% (1.05 KPa vpd) and 22˚C. 2 (High RH): Constant RH at 92% (0.26 KPa vpd) and 22˚C. 3 (Stress): Variable RH from 95% (0.12 KPa vpd) at 20.7˚C for 20.5 h and 50% RH (2.37 KPa vpd) at 32˚C for 2 h in the middle of the light period, with an additional 0.5 h to increase the temperature /lower the RH and 1 h to reduce the temperature/increase the RH again. All treatments had 20 h photoperiod with an irradiance of 100 ± 5 µmol $m^{-2}$ $s^{-1}$ and 4 h darkness. Rosettes were sampled for analysis of ABA and its metabolites, expression of ABA biosynthesis and metabolism genes and water use efficiency after three weeks of treatments. Additionally, water loss and stomatal opening were measured at the same time point. Plants grown in water culture were only used in the desiccation test. Sampling was performed at the same time point for all analyses and treatments. In the stress treatment sampling was done in the middle of the dark period, 1 h before the temperature increase, after 1 h of 32˚C and 2 h after the temperature is back at 20.7˚C.

**Response to desiccation**

To study the ability of the plants to retain water during desiccation, detached rosettes without the inflorescence and roots were removed from the growth chambers and placed in a test environment with 50% RH, 22˚C and an irradiance of 15 µmol $m^{-2}$ $s^{-1}$. The rosettes were detached and weighed after 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 150 and 180 minutes and the relative weight of the rosettes at the different time points was calculated.

**Stomatal measurements**

Imprints were made in the mid-section of the 7$^{th}$ rosette leaf using SUMP liquid and SUMP plate B (SUMP Laboratory, Tokyo) as described previously (Tanaka et al., 2005). Imprints were made of three leaves at each time point, in three independent experiments. In the treatments with no temperature increase, imprints were made at the same time points as in the stress treatment. 15 - 20 images were taken from random sections of each imprint under a light microscope (Leitz, Labolux K, Type 0.2, Germany) using a Leica camera (Leica DC200, Switzerland) and the pore length, pore aperture and stomatal density was measured on the images using UTHSCSA ImageTool 3.0 (University of Texas Health Science Center in San Antonio, USA).

**Leaf transpiration**

As leaf temperature is correlated to transpiration (Kümmerlen et al., 1999; Prytz et al., 2003; Jones, 2004), infrared imaging was used as an indirect measurement of leaf transpiration in the different treatments after transfer to a test chamber with 40% RH, 22 ˚C and no light. Infrared images of leaves were captured using a ThermoVision$^{TM}$ A40 M infrared camera (FLIR Systems AB, Danderyd, Sweden) with a 3203240 pixels uncooled microbolometer focal plane array, a spectral range of 7.5–13 µm, and a thermal sensitivity of 0.08 °C at 30 °C ambient temperature. IR images were taken after 5, 35 and 65 minutes in the test environment. To investigate the response to darkness, IR images were also taken in the different treatments during light and dark. The images were analyzed using ThermaCam$^{TM}$ Researcher Pro 2.8 (FLIR Systems AB). Leaf emissivity was set to 0.95 (Jones, 2004). For each plant, the average temperature of the five largest leaves was used as an indirect measure of leaf transpiration. The temperature change between light and darkness was calculated and used as a measure of stomatal movement.

**Water use efficiency (WUE)**

Carbon isotope discrimination (Δ) was used as a measure of WUE, as the two are strongly correlated (Farquhar and Richards, 1984). Samples were dried and 2 mg samples were analyzed for their 13C:12C ratio ($\delta^{13}C$), using Gas Isotope Ratio Mass Spectrometry (Isotech, Middlewich, UK). PDB PeeDee Belemnite was used as a standard and the 13C:12C ratio of PDB PeeDee Belemnite corresponds to $\delta^{13}C = 0$ in the samples. The $\delta^{13}C$ values were converted into Δ as described by (Farquhar et al., 1989), assuming the $\delta^{13}C$ for atmospheric $CO_2$ is -8.0‰ on the PDB scale.

**ABA quantification**

*Chemicals & Calibration Curves*

ABA catabolites dihydrophaseic acid (DPA), ABA-*β*-D-glucosyl ester (ABA-GE), phaseic acid (PA), 7'OH-ABA, *neo*PA and *trans*-ABA were synthesized and prepared at the Plant Biotechnology Institute of the National Research Council of Canada (PBI-NRC Saskatoon, SK, Canada), while *cis*-ABA was purchased from Sigma–Aldrich (Sigma Chemicals, St Louis, MO, USA). Deuterated forms of ABA and ABA metabolites which were used as internal standards were synthesized and prepared at PBI-NRC (Abrams et al., 2003; Zaharia et al., 2005). These included *d6*-ABA and *d2*-ABA-GE, *d*3-DPA, *d*5-ABA-GE, *d*3-PA, *d*4-7'-OH-ABA, *d*3-*neo*PA, *d*4-ABA, *d*4-*trans*-ABA. Calibration curves were generated for all compounds of interest. Quality control samples (QCs) were run along with the tissue samples.

*Extraction & purification*

The samples, each consisting of rosettes from three plants, were sampled after 2 h of darkness and after 6.5 h of light, frozen in liquid nitrogen and kept frozen at -80˚C, before being freeze-dried and homogenized. 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 (accurately weighed and recorded) of homogenized plant tissue; 3 ml of isopropanol:water:glacial acetic acid (80:19:1, v/v) was then added, and the samples were agitated in the dark for 24 h at 4 ºC. Samples were then centrifuged and the supernatant isolated and dried on a Büchi Syncore Polyvap (Büchi, 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). 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. QC (quality control) standards 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 a 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 UPLC-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 SB column (2.1x100 mm, 1.8 µm) with an in-line filter and separated by a gradient elution of water containing 0.02% formic acid against an increasing percentage of a mixture of acetonitrile and methanol (volume ratio: 50:50). 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. MassLynx™ and QuanLynx™ (Micromass, Manchester, UK) were used for data acquisition and data analysis.

**RNA extraction and purification**

Rosettes were harvested during light, dark and 3 h after detachment in both high and moderate RH. Each sample contained three rosettes and was immediately frozen in liquid nitrogen and stored at -80 °C until used. Total RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Austin, Texas, USA) according to the manufacturer's instructions. Residual DNA was removed through a RNase free DNase treatment using TURBO DNA-Free™ Kit (Ambion, Austin, Texas, USA) according to the manufacturer's instructions. The RNA samples were then purified using PureLink™ RNA Mini Kit (Ambion) according to the manufacturer's instructions. Quality and quantity of RNA were analyzed by

spectrophotometric measurements (Nanodrop®, ND-1000 Spectrophotometer, Wilmington, Delaware, USA).

**Quantitative real-time PCR analyses**

cDNA synthesis was performed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions, with 1000 ng RNA in a 20 µl reaction volume. The Quantitative real-time PCR (qRT-PCR) analyses were performed using the Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen) using 2.5 µl of the diluted cDNA in 25 µl reactions in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA). The cycling program for qPCR was as follows: 50°C for 2 min, 95°C for 2 min and 45 cycles of 95°C for 15 s and 60°C for 35 s. Reaction products were then confirmed by melting curve analysis and agarose gel electrophoresis. PCR reactions were conducted in triplicate for each sample and a minus RT reaction was included to detect any remains of genomic DNA. The primers used for qRT-PCR were tested using gel electrophoresis and standard curve analysis. The primers used are listed in Table 1. The transcript levels of the ABA-related genes were normalized to that of two endogenous standard genes (Actin 2 and EF-1α) which showed stable transcript levels under all conditions. For each gene all samples were related to the light-harvested sample of the moderate RH treatment.

Table 1: List of the primers used in the qPCR analysis of abscisic acid biosynthesis and metabolism genes *Arabidopsis thaliana*, Columbia ecotype.

| Gene | Forward primer (5`to 3`) | Reverse primer (5`to 3`) | AGI number |
|---|---|---|---|
| **CYP707 A1** | cgatcttcggaaaagacgag | ttccttccgagctttcattg | At4g19230 |
| **CYP707A3** | cggccagtaaagagaggatg | tgattcaattgtgagggacca | At5g45340 |
| **NCED3** | gagctgcagccggtatagtc | gaaccgaccaacggttttta | At3g14440 |
| **AAO3** | gaaggtcttggaaacacgaagaa | gaaatacacatccctggtgtacaaac | At2g27150 |
| **ZEP** | ttgtttggccgtagtgaagct | agactcgatatccgctggtataaaa | At5g67030 |
| **Actin 2** | tcagatgcccagaagtcttgttcc | ccgtacagatccttcctgatatcc | At3g18780 |
| **EF-1α** | cccaggctgattgtgctgt | gggtagtggcatccatcttgtt | At5g60390 |

### Statistical analyses

Significant differences between means were tested for, by using normally distributed general linear models (GLM) and Tukey's test. Differences with $p \leq 0.050$ were considered significantly different. All statistical tests were performed in Minitab 16.1.1 (Minitab 16.1.1, Windows version, State College, Pennsylvania, USA).

## Results

### Development under high RH resulted in larger stomatal aperture and poor desiccation tolerance

It has previously been shown that several plant species develop malfunctioning stomata when grown under constant high RH (Santamaria et al., 1993; Mortensen, 2000; Torre and Fjeld, 2001; Nejad and van Meeteren, 2005). *Arabidopsis* responded similarly and plants developed in constant moderate RH had a better ability than plants developed in high RH to retain water during desiccation ($p<0.001$, Figure 1A). Plants developed in moderate RH lost on average 30% of the original water content. In contrast, plants developed in constant high RH lost on average 45% of the original water content. Similar water loss results were obtained with plants grown both in soil and in water culture, showing that the differences between the two treatments were not due to any differences in soil moisture (data from water culture not shown).

Epidermal imprints were used to directly evaluate stomatal aperture in light and darkness (Figure 1C). The stomatal aperture was significantly larger in plants developed in high RH at all time points ($p<0.050$). In high RH the aperture was significantly larger at the earliest time point ($p<0.050$), while there was no significant differences between the other time points. In moderate RH there was no significant difference in aperture between the time points in the light, while the aperture was significantly smaller during darkness ($p<0.050$). This shows that the stomatal aperture is reduced during darkness in moderate RH, but not in high RH.

IR imaging was used to evaluate the change in temperature and hence transpiration during light and darkness (Figure 1B). During light there were no differences in leaf temperature between the high and moderate RH treatments. However, during darkness the leaf temperature was significantly higher in moderate than high RH ($p=0.001$). The change in leaf temperature between darkness and light was significantly different in the two RH

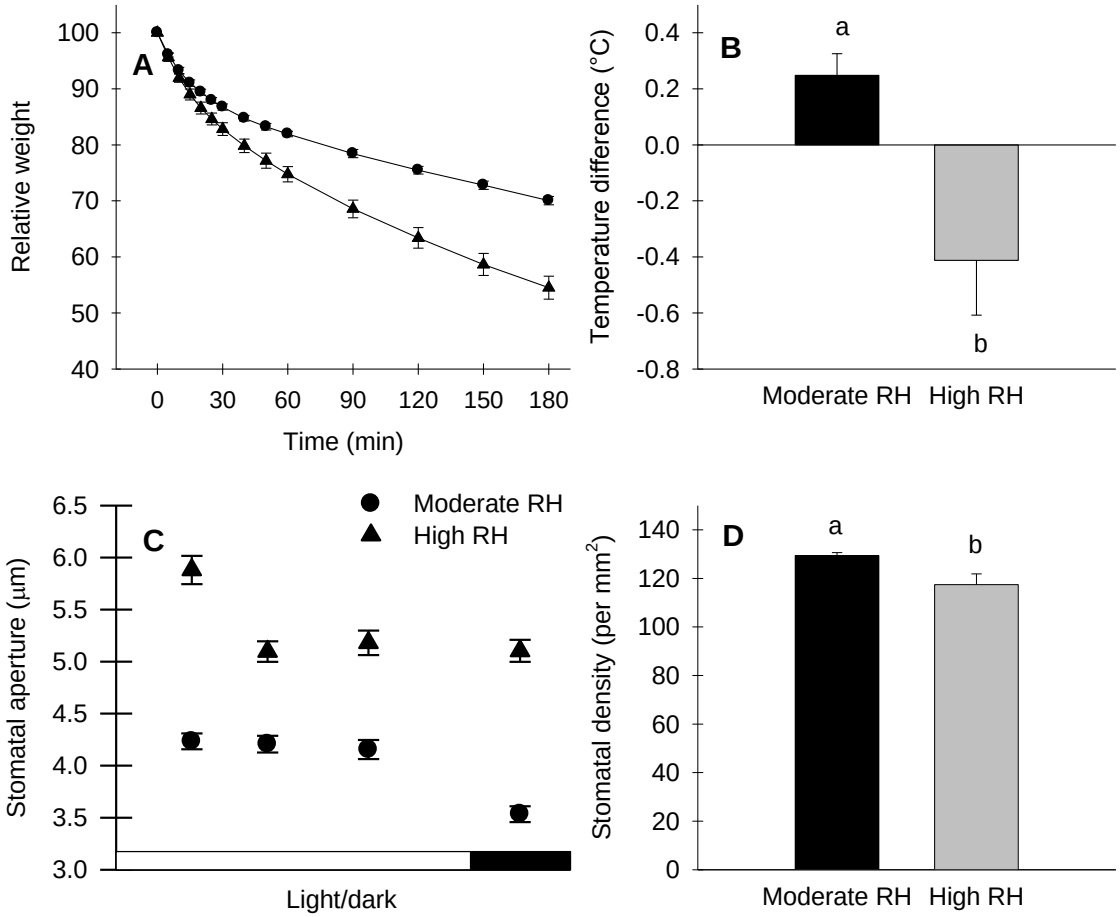


Figure 1: Effects of moderate and high relative air humidity (RH) on *Arabidopsis*. Water loss curves during a 3 h desiccation test of detached rosettes, n=20 (A). The difference in leaf temperature ($\Delta T = T_{dark} - Tl_{ight}$ where T is the leaf temperature) between darkness and light in the RH treatments, n=5 (B). Stomatal aperture at three time points during the light period and one in the dark, n=103-115 (C). Stomatal density, n=5 (D). Mean values ± SE are shown. Different letters indicate significantly different values from the GLM and Tukey's test.

treatments (p=0.014), with higher and lower temperature during dark in moderate and high RH, respectively. The higher temperature in moderate RH during darkness suggests reduced transpirational cooling due to better stomatal closing ability.

As the transpirational water loss is not only determined by the stomatal aperture, but also by the number of stomata the stomatal density was measured. The stomatal density was significantly higher in plants developed in moderate RH, compared with plants developed in high RH ($p<0.050$, Figure 1D). However, the difference was not very large.

### *Arabidopsis* developed under high RH had low levels of ABA and its metabolite DPA

As low ABA levels have been hypothesized to be the reason for the malfunctioning stomata, the levels of ABA and its metabolites in *Arabidopsis* rosette leaves were measured during light and darkness.

The total level of ABA and its metabolites PA, DPA, ABA-GE, t-ABA, neo-PA and7'OH-ABA was significantly lower in plants developed under high RH, compared with moderate RH ($p<0.001$, Figure 2A, Table 2). Similarly, the content of only ABA was also significantly lower in plants developed under high RH (Figure 2B, Table 2).

When ABA is oxidized it is converted into PA and then further converted to DPA. There were no differences in the PA levels between plants from moderate and high RH (Figure 2C, Table 2). However, the level of DPA was significantly lower in plants developed under high RH ($p<0.001$, Figure 2D, Table 2). There was no change in the ABA levels or any of its metabolites between light and dark within either of the RH treatments (Figure 2, Table 2).

The quantitative analysis of ABA catabolites showed no difference between the levels of PA in the two treatments, but a clear difference in DPA. However since the reductase converting PA to DPA is still unknown, this step was not studied further, but is probably important in high RH responses. The levels of ABA-GE, 7'OH-ABA, *neo*PA and *trans*-ABA were mostly too low to be quantified in all treatments (data not shown), and have therefore only been included in the value for total ABA metabolite content (i.e. in those cases where they could be quantified).

### ABA biosynthesis genes were not affected by RH, but inactivation genes were

To estimate the regulation of ABA biosynthesis by RH, we investigated relative transcript levels of several genes in the pathway. The transcript levels of ZEP, NCED3 and AAO3 were similar in plants from both RH treatments (Figure 3A, B, C). However, the RNA levels of these genes were significantly decreased during darkness in plants from both treatments ($p<0.050$). Also, the expression of AAO3 were significantly higher in the dark in plants from high compared to moderate RH ($p<0.050$, Figure 3C).

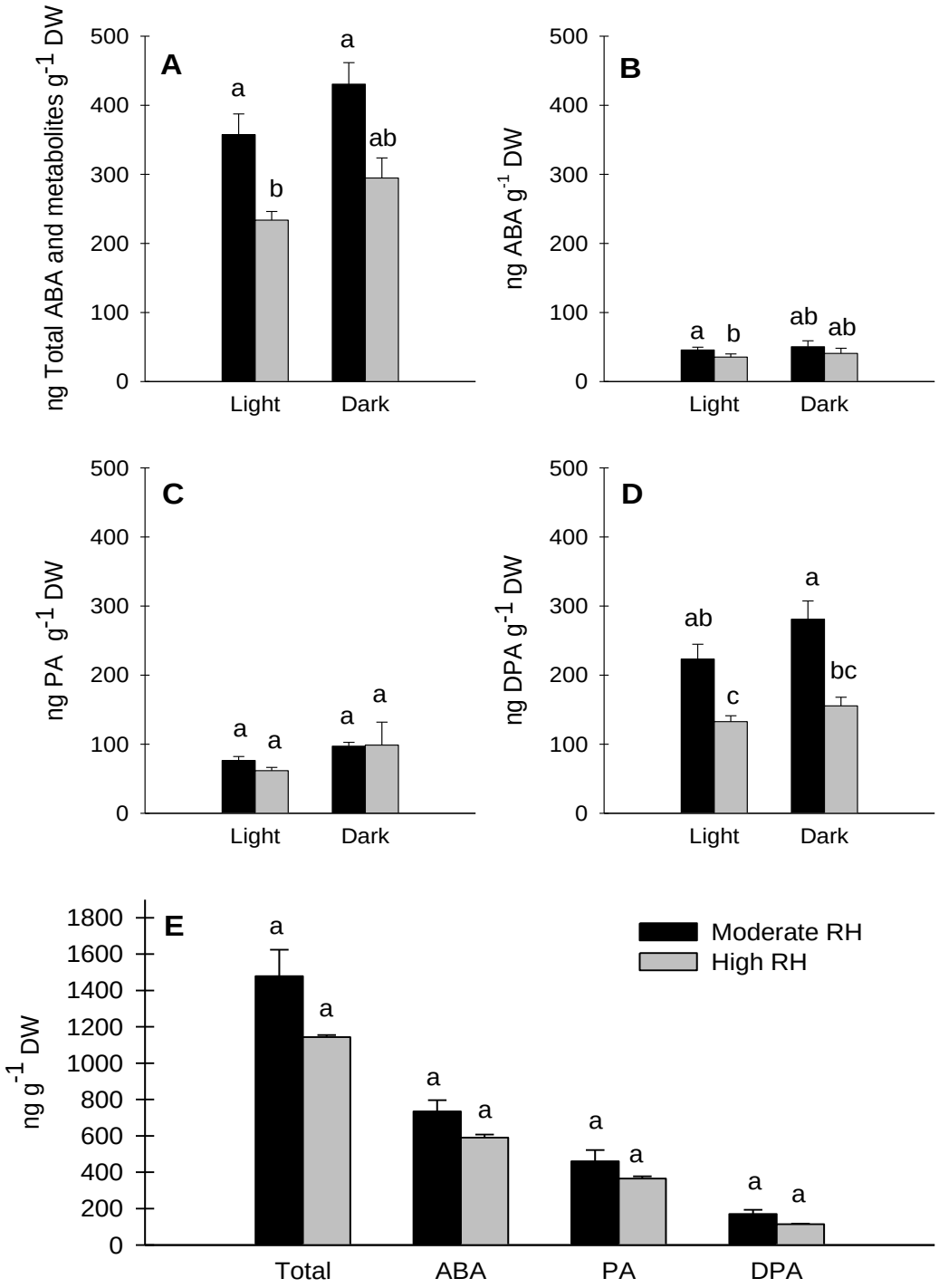


Figure 2: The levels of abscisic acid (ABA) and its metabolites in *Arabidopsis* leaf rosettes during light and darkness in moderate (black) and high (gray) relative air humidity (RH). Total ABA and metabolites (A), ABA (B), phaseic acid (PA; C), dihydrophaseic acid (DPA; D) and levels after 3 h desiccation (E). n=3-10, each sample contains 3 rosettes. Mean ± SE. Different letters indicate significantly different values in the Tukey's test (A-D) and GLM analysis (E).

Table 2: General linear model analysis comparing the level of total abscisic acid and its metabolites (Total), abscisic acid (ABA), dihydrophaseic acid (DPA) and phaseic acid (PA) during light and dark in leaves developed under constant moderate or high RH. P-values higher than 0.05 were considered not significant.

| | **Total** | **ABA** | **DPA** | **PA** |
|---|---|---|---|---|
| **RH** | <0.001 | <0.001 | <0.001 | ns |
| **Time** | ns | ns | ns | ns |
| **RH*Time** | ns | ns | ns | ns |

The relative transcript levels of genes involved in the inactivation of ABA were also quantified. During light, the expression of CYP707A1 was significantly higher in plants from moderate compared to high RH ($p<0.050$, Figure 3D). In moderate RH, the expression of CYP707A1 was similar in light and darkness. However, in plants in high RH, CYP707A1 transcript levels were significantly higher ($p<0.050$) during darkness.

The relative expression of CYP707A3 was similar in plants from both moderate and high RH during light (Figure 3E). During darkness, the expression remained unchanged in moderate RH, but was significantly higher in plants from high RH. Accordingly, the expression of CYP707A3 in darkness was significantly higher in high RH compared to moderate RH ($p<0.050$), clearly showing that the expression of CYP genes were up-regulated in the darkness in high but not moderate RH.

**ABA increased during desiccation independently of previous RH but stomata closure only occurred in moderate RH**

One of the main hypotheses concerning ABA and high RH is that plants developed under constant high RH are unable to produce ABA. We therefore studied the plants ability to produce ABA during a desiccation test (Figure 2E). As stated above, the ABA levels were significantly higher in plants developed under moderate RH compared to high RH ($p<0.001$, Figure 2B). After three hours of desiccation of the leaf rosettes, significantly higher ABA levels were observed in plants from both moderate and high RH ($p<0.001$). However, the ABA levels were not significantly different between plants developed in moderate and high RH (Figure 2E). The ABA levels were more than 10 times higher after 3 h of detachment in plants from both treatments, clearly showing that plants from both treatments were able to produce large amounts of ABA. However, even though they produced large amounts of

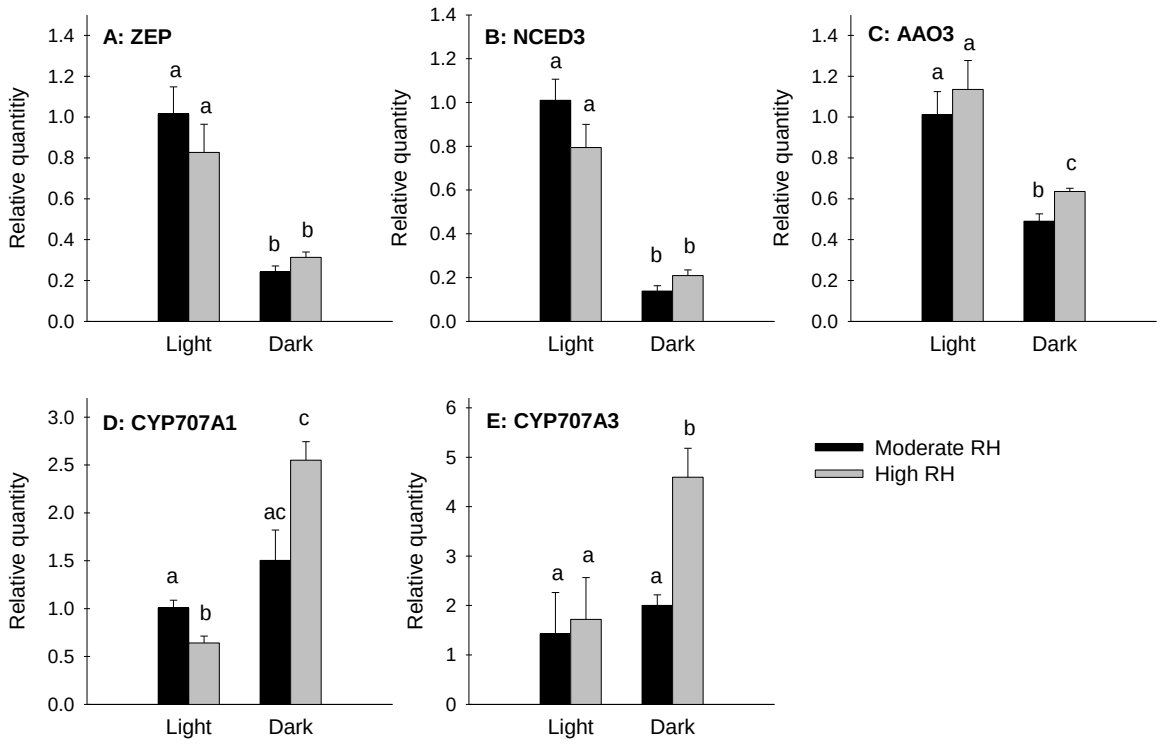


Figure 3: Relative transcript levels of abscisic acid (ABA) biosynthesis and metabolism genes in *Arabidopsis* leaf rosettes during light and darkness in moderate and high relative air humidity (RH). ABA biosynthesis genes: zeaxantin epoxidase (ZEP; A), 9-cis-epoxycarotenoid dioxygenase (NCED3; B) and abscisic aldehyde oxidase (AAO3; C). ABA metabolism genes: the 8`hydroxylases cytochrome 450 CYP707A1 (D) and CYP707A3 (E). Results were normalized against Actin 2 and EF-1α, and for each gene to the moderate RH treatment in light., Each sample contains 3 rosettes (n=3). Mean ± SE. Different letters indicate significantly different values in the Tukey's test.

ABA, plants from high RH still had uncontrolled water loss (Figure 1A) during the desiccation test and did not close the stomata properly.

The levels of PA were similar in plants from both moderate and high RH before detachment (Figure 2C). After 3 h of desiccation, levels were significantly higher in plants from both treatments ($p<0.001$). However, there were still no significant differences between plants from the two RH treatments (Figure 2E). Before detachment, DPA levels were significantly higher in plants developed under moderate RH ($p<0.001$, Figure 2D). However, there was no significant difference between the levels in the two RH treatments after 3 h of desiccation (Figure 2E). Interestingly, there was also no significant change in the DPA levels in any of the RH treatments after 3 h of desiccation.

**ABA deficient mutants had large stomata, but similar difference in desiccation response between moderate and high RH as the WT**

To further study the effect of low ABA content on stomatal function an ABA deficient mutant was grown under high and moderate RH. The *aba3-1* mutant is mutated in the AAO3 gene (At1g16540) in the ABA biosynthesis and contains very little ABA (Schwartz et al., 1997).

In detached leaf rosettes, the water loss of the ABA deficient mutant was significantly higher than that of the WT counterparts within the same RH treatment ($p<0.001$, Figure 4A). However, the difference in water loss between the WT and ABA deficient mutants within each RH treatment was almost similar (20% in high RH and 16% in moderate RH). Like the WT, the ABA deficient mutants from moderate RH lost significantly less water after three hours than the mutants from high RH ($p<0.001$, Figure 4A). The similar difference in water loss between WT and *aba3-1* mutants show that the effects of RH and lacking ABA are additive, which indicates that lower ABA level was not the reason for lower desiccation tolerance of WT plants developed under high RH. These results also indicate that WT plants developed under high RH had some stomatal response to desiccation, although they still lost more water than plants from moderate RH.

To study the effect of low ABA on stomatal size, imprints were made of fully developed leaves and the length of the stomatal pores was measured. We found that the stomatal pore from both WT and *aba3-1* mutants developed under high RH were the same size (10 µm, Figure 4B). However, the stomatal pore of WT plants developed under moderate RH were significantly smaller (7.5 µm, $p<0.001$, Figure 4B). The stomatal pores in *aba3-1* mutants in moderate RH were larger than those of the WT in the same treatment, but not significantly different from the WT plants developed in high RH (9 µm, Figure 4B). Taken together, these results showed that plants lacking ABA to induce stomatal closure (ABA deficient mutants) developed large stomata and had higher water loss during desiccation. It also showed that WT plants developed in high RH had reduced water loss compared with ABA deficient mutants during desiccation, indicating some stomatal movement.

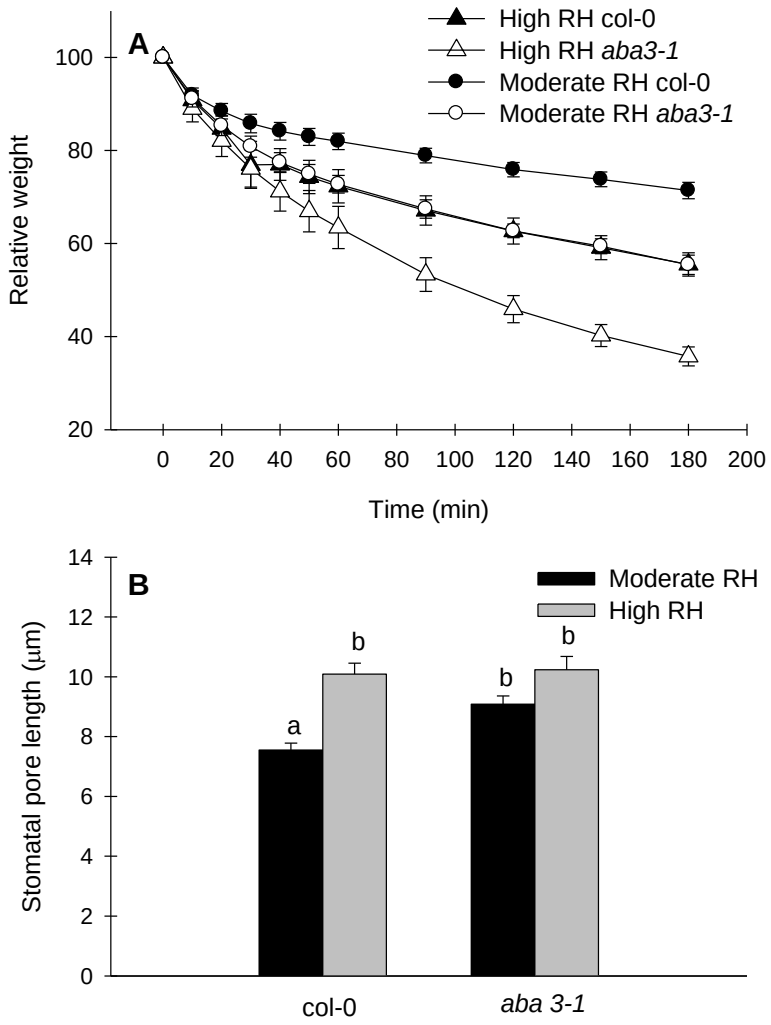


Figure 4: Effects of moderate and high relative air humidity (RH) on the wild type (Col-0) and the *aba3-1* ABA deficient mutant in *Arabidopsis*. Water loss curve from a 3 hour desiccation test of detached leaves, n=8 (A). Length of the stomatal pore during light, n=20 (B). Mean ± SE. Different letters indicate significantly different values in the Tukey's test.

**A short temperature increase/RH decrease induced more functional stomata**

Considering the above results, it can be hypothesized that plants growing in a constant environment with high RH develop large, malfunctioning stomata. We therefore tested if inducing a short term (2 h) daily stress treatment with temperature increase/RH decrease while keeping the diurnal average RH high (>90%) could improve the stomatal functioning.

In a desiccation test plants from the stress treatment had significantly lower water loss than the plants from high RH ($p<0.001$, Figure 5A). However, plants from the stress treatment still had significantly higher water loss than the plants from moderate RH ($p<0.001$, Figure 5A).

To test the ability of plants from the stress treatment to close the stomata compared with plants from constant moderate or high RH, plants were placed in a dry and dark environment and IR imaging was used to measure the leaf temperature as an estimate for the transpiration and stomatal opening. The leaf temperature after 65 minutes was lower in plants developed under high RH ($p<0.050$) than in plants from moderate RH and the stress treatments, which showed similar leaf temperatures (Figure 5B). This indicates a higher transpiration rate due to more open stomata in plants developed under constant high RH, while plants from the other treatments were able to close the stomata in response to dry air and darkness.

The stomatal pore length was measured to determine if the better functioning stomata from the stress treatment were due to smaller stomata. The length of the stomatal pore during light was significantly smaller in the moderate RH treatment ($p<0.001$), while there was no difference between the high RH and stress treatments (Figure 5C). The stomatal aperture was also measured in the middle of the dark period, before the temperature increase, in the middle of the temperature increase and after the temperature increase (Figure 5E). Within the stress treatment the aperture was significantly larger before the temperature increase ($p<0.050$), while there is no difference between the other time points. Compared with plants from high RH the stomatal aperture in plants from the stress treatment was significantly smaller at all time points ($p<0.050$). In contrast, compared with plants from moderate RH plants from the stress treatment have larger apertures ($p<0.050$) at all time points, except 2 hours after the temperature increase where there is no significant difference.

To determine the efficiency of the stress-exposed plants in $CO_2$ uptake per $H_2O$ molecule lost, carbon isotope discrimination ($\Delta$) was used as a measure of WUE. Plants from the stress treatment had similar $\Delta$ values as the moderate RH treatment (Figure 5D). However, the $\Delta$ values from high RH were significantly higher than the stress treatment ($p<0.050$). This indicates that plants from the stress treatment had similar WUE as plants developed under moderate RH and better WUE than plants developed under constant high RH.

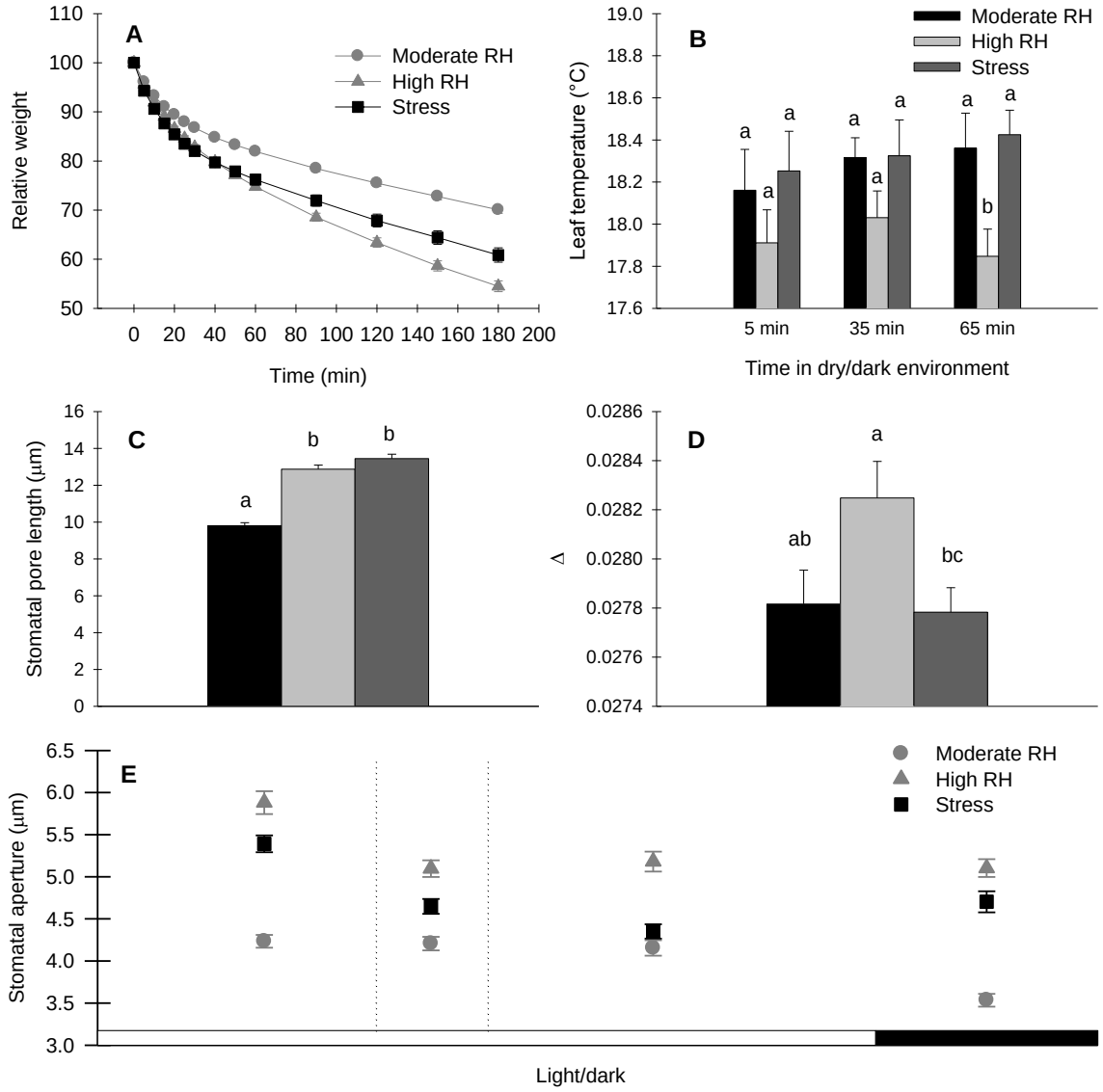


Figure 5: Effect of moderate and high relative air humidity (RH) as well as a stress treatment with 2 h increased temperature and reduced RH (see figure S1). Water loss curve from a 3 hour desiccation test of detached leaves, where the curves from high and moderate RH (gray) are copied from figure 1, n=20 (A). Leaf temperature was measured 5 min, 35 min and 65 min after transfer of the plants to the same dry/dark environment, n=12 (B). Stomatal length, n=93 (C). Carbon isotope discrimination (Δ), n=15 (D). Stomatal aperture measurements from 3 time points in the light and one in the dark. The period with the stress treatment is between the dotted lines. Datapoints from high and moderate RH are copied from figure 1. N=82-115. (E) Mean ± SE. Different letters indicate significantly different values in the Tukey's test.

## Discussion

It was shown that the malfunctioning stomata in *Arabidopsis* developed under high RH was not due to the low ABA levels in the leaves. However, the ABA levels were significantly lower in high RH, compared with moderate RH. Also, increased ABA inactivation during darkness was found in high RH. We also show that *Arabidopsis* developed under both moderate and high RH were able to produce large amounts of ABA during water stress. The results also show that giving plants growing in high RH a daily temperature increase/RH decrease will result in more desiccation tolerant plants.

### *Arabidopsis* developed under high RH have low ABA levels and develop malfunctioning stomata

Water loss is determined by the size of the stomatal pore and by the number of stomata. In this study we found the stomatal density to be lower in *Arabidopsis* plants developed in high RH (Figure 1D). The higher transpirational water loss must therefore be a consequence of a larger stomatal opening. These results contrast what has previously been found in RH studies. A study on *Prunus cerasifera* found that increased RH resulted in increased stomatal density (Sciutti and Morini, 1995). Two other studies on *Rosa x hybrida* found that the stomatal density was higher in plants developed in high RH compared with moderate RH (Torre et al., 2003; Fanourakis, 2011).This study also showed that when *Arabidopsis* was grown under constant high RH, they developed larger stomata, that did not close during darkness (Figure 1C). This resulted in low stress tolerance and uncontrolled water loss when the plants were moved to lower RH. Similar responses to RH have been observed in *Rosa x hybrida* and *Tradescantia virginiana* (Mortensen and Fjeld, 1998; Torre and Fjeld, 2001; Nejad and van Meeteren, 2005; Arve et al., 2013). In these studies, detached leaves from plants grown under high RH lost more water than leaves from plants grown under lower RH. Roses grown under high RH were also found to have low stress tolerance in general and shorter post-harvest life.

There may be several reasons for the reduced stress tolerance and development of malfunctioning stomata. During stress, plants produce ABA, which is a signal for stomatal closure. It has been hypothesized that plants developed under high RH contain lower levels of ABA and that these low ABA levels make the stomata unable to close. In this study we found that *Arabidopsis* plants developed under high RH had significantly lower ABA content

during growth than plants developed under moderate RH (Figure 2). Similarly, the combined content of ABA and its metabolites was lower in high RH, showing that there was both less ABA and less ABA degradation in the leaves from high RH. This is similar to studies of *T. virginiana* showing that plants developed under constant high RH contain lower levels of ABA than plants grown at moderate RH (Nejad and van Meeteren, 2007). Similarly, a rapid decrease in ABA content in plants transferred from low to high RH has also been reported, showing a distinctive and quick ABA response to different RH treatments (Okamoto et al., 2009). However, the involvement of ABA in RH responses is contradicted by experiments showing that ABA insensitive mutants responded similarly to the WT to changes in RH (Assmann et al., 2000). Thus, despite the general belief that there is a connection between low ABA concentrations and malfunctioning stomata, the relationship is still unclear.

**No increase in ABA in the dark under moderate and high RH, but large production of ABA during subsequent desiccation**

Few studies have investigated the diurnal levels of ABA in plants. However, a study has shown that the ABA levels in *Nicotiana tabacum* peak about two hours into the dark period (Novakova et al., 2005).

In our experiment with *Arabidopsis,* there was no increase in ABA levels in either RH treatment after 2 h of darkness (Figure 2), indicating that darkness in itself was not enough to increase the ABA production in *Arabidopsis*. However, in a recent study *Rosa x hybrida* developed under constant moderate RH, but not high RH, showed increased ABA levels during the dark (Arve et al., 2013). This may indicate that *Arabidopsis* and *Rosa x hybrida* have slightly different ABA responses to darkness and RH. In *Rosa x hybrida* developed in moderate RH the levels of ABA-GE were considerably higher and were reduced during darkness as the ABA levels increased (Arve et al., 2013). It was then hypothesized that ABA-GE was the source of the increased ABA levels during darkness.

Although there was no change in the ABA levels in the leaves during the dark, the transcript levels of ABA biosynthesis genes (ZEP, NCED3 and AAO3) were reduced and those of the ABA inactivation genes (CYP707A1, CYP707A3) increased (Figure 3). It is probable that during the dark, ABA production in the roots and subsequent transport to the leaves is more important than ABA produced in leaves. Supporting this is the observation that ABA in the xylem increases the hydraulic conductivity, which is important for

rehydration during darkness (Parent et al., 2009). ABA production in leaves could then be reduced and inactivation increased to keep the amount of ABA levels constant.

The lack of an increase in ABA levels during dark may reduce the degree of stomatal closure, keeping the stomata slightly open (Figure 1C). Since *Arabidopsis* is a rosette plant, one possibility could be that the microclimate around the rosette leaves had reduced air movement and higher RH. The stomata in the moderate RH treatment might therefore have been exposed to a somewhat higher RH than was actually measured in the air of the growth chamber. This may have led to incomplete closure during darkness. However, it is known that stomata of many species remain partially open during the night (Caird et al., 2007).

The lower ABA levels found in plants developed under high RH have previously been proposed to be a result of the plants inability to produce ABA. However, during the desiccation test in this study, plants developed under both moderate and high RH produced large amounts of ABA (Figure 2E). This clearly shows that plants developed under high RH were perfectly able to produce ABA in response to water stress. However, the lack of stomata closure might be due to reduced sensitivity to ABA.

**CYP707As are upregulated in darkness in high but not moderate RH**

Several other studies have shown that increased ABA levels during drought is a result of increased biosynthesis and increased expression of NCED3 (Thompson et al., 2000; Endo et al., 2008). In this study, reduction in expression of ABA biosynthesis genes (ZEP, NCED3 and AAO3) and increased expression of ABA inactivation genes (CYP707A1 and CYP707A3) were found in high RH during the dark (Figure 3), obviously keeping the ABA levels low (Figure 2). The reductase converting PA to DPA is still unknown and has therefore not been investigated in this study. However, the levels of DPA were found to be lower in plants developed under high RH, while there were no differences in the PA levels in the two RH treatments, indicating that the reductase converting PA to DPA was affected by RH. A study on the CYP707A3 gene has indicated that this gene is especially important in ABA regulation (Umezawa et al., 2006). In this study, the expression of CYP707A genes during dark was significantly higher in high RH while it remained low in moderate RH (Figure 3D-E). This increased inactivation may have caused the lower ABA levels found in high RH. In a previous study the highest expression of CYP707A3 gene was found in vascular tissues, and this might result in inactivation of ABA from the roots during darkness before it reaches the stomata (Okamoto et al., 2009). This may explain the low ABA biosynthesis activity but

constant ABA levels in the leaves during dark compared to light. The increased levels of CYP707A genes during darkness in high RH should have increased the PA levels. However, in this study there was no difference in the PA levels in high and moderate RH. The degradation of PA and DPA could also be affected by RH. If the degradation of PA and DPA are increased in high RH, this could explain the similar PA values and low DPA levels in high RH. In this study only the levels of PA and DPA in the entire leaves were measured. However, the levels might differ in different localizations within the leaves.

The expression of CYP707A3 is regulated by several environmental factors. CYP707A3 expression has previously been found to increase when plants are moved from low to high RH, before returning to lower levels again (Okamoto et al., 2009). In addition, in this study we found that the expression of CYP707A3 was dependent on the RH regime and light/dark conditions (Figure 3). A similar dependence was found in the expression of CYP707A1, which was increased during darkness in plants grown in high RH. CYP707A1 is mostly expressed in the guard cells (Okamoto et al., 2009) and can therefore more specifically regulate the ABA levels that affect the stomatal movement. The increased levels of CYP707A1 during dark in high RH might therefore reduce the ABA levels in the guard cells and prevent stomatal closure.

**Low ABA level per se is not the main reason for developing malfunctioning stomata**

In this study, the ABA deficient mutants (*aba3-1*), lost more water during desiccation than the WT plants grown in the same RH regimes (Figure 4). The difference between the mutants and the WT was also similar in both RH treatments, indicating that there was some stomatal movement induced by ABA production in WT plants developed in high RH. The similar difference between mutants and WT plants also show that lack of ABA had similar effects in both high and moderate RH and was therefore not the cause of the increased water loss in high RH. If low/no ABA alone had been the main reason for the malfunctioning stomata, the water loss during desiccation would have been similar in both genotypes in high RH and in the mutants from the two RH treatments. Since the relative difference between WT and ABA deficient mutant was the same in both treatments, it is probable that the lower ABA levels in plants developed in high RH was not the main cause of the malfunctioning stomata. We also found that the size of the stomata was larger in WT and *aba3-1* mutants developed in high RH and in *aba3-1* mutants from moderate RH showing a correlation between stomatal size and ABA levels (Figure 4). An earlier study on *Populus x canescens* also found that

ABA insensitive plants had larger stomata (Arend et al., 2009). This indicates that lack of ABA, reduced sensitivity to ABA or low ABA contents results in the development of large stomata.

Another possible hypothesis is therefore that plants developed under constant high RH never experience unfavorable conditions during development and therefore have larger stomatal apertures throughout leaf development. This may then result in stomata that are not able to close at a later stage. Similarly, the ABA deficient *aba3-1* mutants contained no ABA to induce stomatal closure. This lack of stomatal closure may result in the development of malfunctioning stomata. There could be several causes for the malfunctioning. One possible explanation may be that the stomata are less sensitive or insensitive to the ABA signals, either due to fewer ABA receptors or inhibition of one of the steps of the signaling pathway. It is also possible that plants developed under high RH conditions develop stomata with a structural anatomy making them unable to close completely, in spite of receiving signals for stomatal closure. One such change might be a lack of arabinan in the cell wall. Arabinan has previously been shown to be essential for stomatal movement (Jones et al., 2003).

**Does the stomatal aperture during development affect the stomatal functionality?**

Treating plants grown at high RH with a daily two hour period with high temperatures and low air humidity during development still resulted in large stomata, but the stomatal apertures were smaller (Figure 5). If the reduced stomatal functionality is a result of larger stomatal apertures during development, plants from the stress treatment should have improved desiccation tolerance. Indeed, these plants developed more functional stomata and higher desiccation tolerance than those from constant high RH (Figure 5). However, the desiccation tolerance was still not as good as in plants developed under constant lower RH. The size of the stomata from the stress treatment was still as large as those from constant high RH, indicating that it was not the stomatal size alone that made them unable to close in high RH.

It has previously been found that roses exposed to a six hour stress period in the middle of the day have decreased water loss and increased vase life compared with constant high RH (Mortensen and Gislerød, 2005). Previous studies on roses and *T. virginiana* showed that when plants developed under constant high RH are given daily ABA applications during development, they develop functioning stomata (Nejad and van Meeteren, 2007; Fanourakis et al., 2011). In these studies it was hypothesized that the malfunctioning stomata were due to

the long term low ABA levels and that ABA application resulted in functional stomata. However, when combining these results with the results from this study, it may be hypothesized that it is not the low ABA level per se that result in malfunctioning stomata, but the larger stomatal apertures during development. Accordingly, the ABA application would reduce the stomatal apertures resulting in fully functioning stomata.

The results discussed above support the hypothesis that continuously larger stomatal apertures during development result in malfunctional stomata. However, *aba3-1* mutants from high and moderate RH behaved differently during desiccation, which suggests that these mutants developed under moderate RH have some stomatal movement or that the turgor pressure in the guard cells has been reduced, possibly during the dark. This might be caused by other ABA independent pathways, possibly involving $H^+$/ATPase or an indirect hydraulic effect through decreasing the water permeability within leaf vascular tissues (Netting, 2000; Pantin et al., 2013). WT plants in moderate RH did not show any increase in ABA levels in the leaves during darkness, but the stomata still closed to a certain degree. This indicates that increased ABA in the dark is not necessary for stomatal movement. However, it cannot be excluded that there was an ABA increase in the guard cells themselves, since only the bulk ABA level in the leaves was measured. Whatever the signal for dark induced stomatal closure, other factors may override this signal in plants growing in high RH causing the stomata to remain open. A study on six temperate deciduous tree species showed that responses to RH dominated over responses to photosynthetic signals (Aasamaa and Sober, 2011). High RH might therefore be hypothesized to override the dark-induced stomatal closure signals, due to conditions that make stomatal closure unnecessary.

## Conclusion

This study shows that *Arabidopsis thaliana* developed under continuous high RH had a lower desiccation tolerance and larger stomata that did not close during darkness, compared with plants developed under moderate RH. The results also clearly showed that plants developed under high RH had lower ABA levels in the leaves, but were able to produce large amounts of ABA in response to water stress. Plants developed under high RH also showed increased ABA inactivation during darkness. The results also indicated that constant high RH kept the stomata open during development also in darkness, which resulted in malfunctioning stomata. However, daily periods with temperature increase/RH decrease reduced the stomatal apertures and improved the stomatal functionality.

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## SUPPLEMENTARY INFORMATION

**S1**:

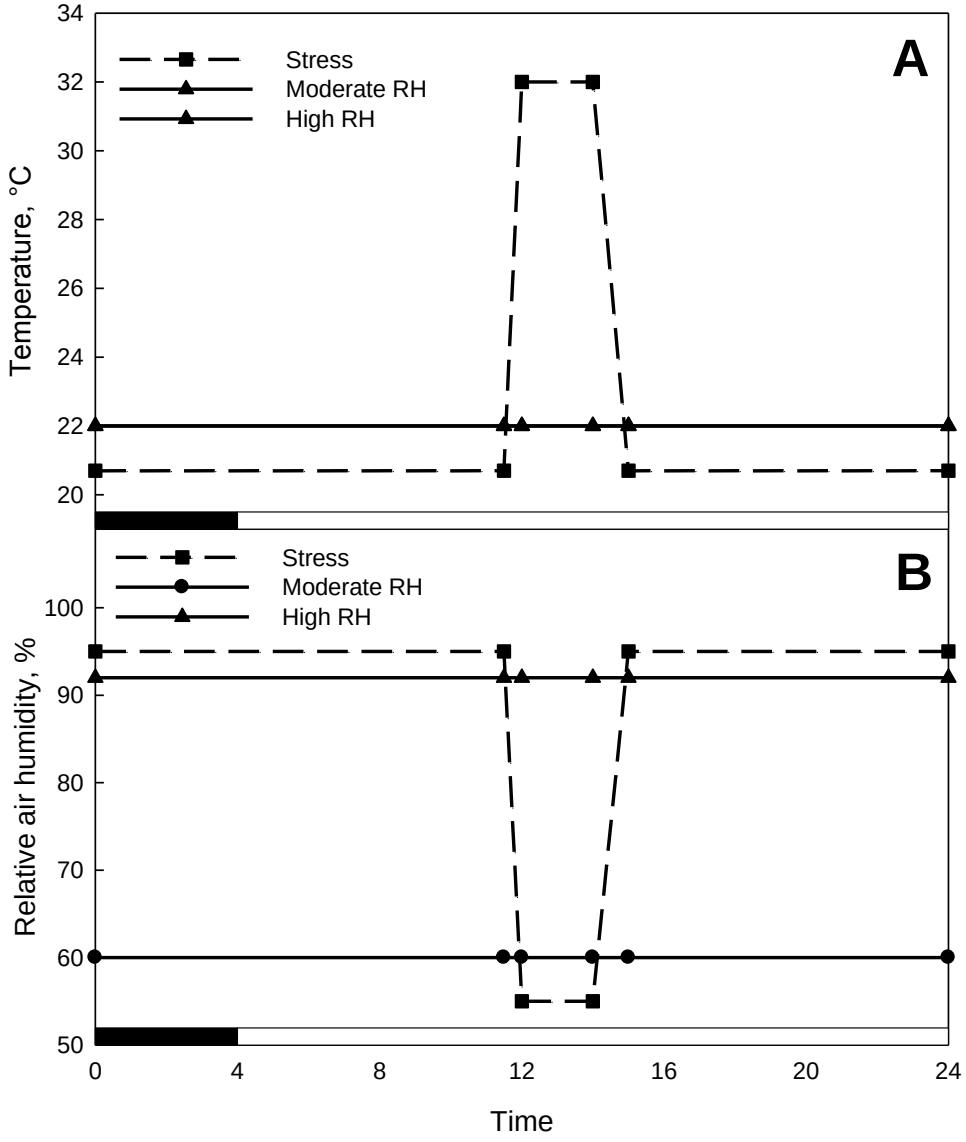


Figure S1: The temperature (A) and RH (B) in each treatment during 24 h. The diurnal average temperature was 22°C inn all three treatments and the diurnal average air humidity was 92% for both the high and the stress treatment and 60% for the moderate RH treatment. During stress, the temperature was increased to 32°C and the RH reduced to 55%.

# Paper III

# ABA, but not darkness increases the $H_2O_2$ production in *Vicia faba* developed in continuous high relative air humidity

**Authors**

Louise E. Arve, Dália R. Carvalho, Jorunn E. Olsen, Sissel Torre

## Abstract

Plants developed under constant high (>85%) relative air humidity (RH) have larger stomata that are unable to close completely in response to closing stimuli. One of the hypotheses for the less responsive stomata is that the plants have reduced sensitivity to abscisic acid (ABA). Both ABA and darkness are signals for stomatal closure and induce the production of the secondary messenger hydrogen peroxide ($H_2O_2$). In this study, the ability of *Vicia faba* plants developed in continuous high and moderate RH to close the stomata in response to ABA and $H_2O_2$ was investigated. Moreover, the ability of the plants to produce $H_2O_2$ when treated with ABA or transferred to darkness was also assessed. Our results show that *V. faba* plants developed in continuous high RH are able to increase the $H_2O_2$ production when the ABA levels are increased. However, they do not increase the $H_2O_2$ production during darkness. Plants from high RH also show reduced stomata closure in response to ABA or $H_2O_2$. Our results suggest that the reduced stomatal response in plants developed in continuous high RH is caused either by one or more factors downstream of $H_2O_2$ in the signaling pathway toward stomatal closure or might be a result of changed guard cell anatomy.

**Key-words**

Abscisic acid, hydrogen peroxide, darkness, relative air humidity

## Introduction

The stomatal complex consists of two guard cells surrounding the stomatal pore and functions as a gate for $CO_2$ uptake for photosynthesis and transpirational water loss. Through regulation of the stomatal opening the need for $CO_2$ uptake and water preservation can be closely balanced (Tallman, 2004).

Previous studies have shown that plants developed under constant high (>85%) relative air humidity (RH) have larger stomata that are unable to close completely in response to closing stimuli like desiccation, darkness and the hormone abscisic acid (ABA) (Torre and Fjeld, 2001; Fanourakis et al., 2011; Arve et al., unpublished manuscript). These plants therefore have reduced ability to preserve water, leading to higher water loss (Torre et al., 2003). Similar results have been found in micro propagated plants and leaf cuttings rooted at high RH (Santamaria et al., 1993; Fordham et al., 2001).

This kind of stomatal malfunctioning has previously been hypothesized to be a result of low ABA levels (Nejad and van Meeteren, 2007). It has further been hypothesized that these plants are unable to produce ABA. However, it has recently been shown that plants developed under high RH are able to produce large amounts of ABA during desiccation (Arve et al., unpublished manuscript). It is therefore likely that the reduced ability to close the stomata is either a result of reduced sensitivity to ABA or an impaired ABA signalling pathway.

The ABA levels in the leaves are dependent on the ABA transported from the roots and the ABA that is produced in the leaves themselves (Thompson et al., 2000; Endo et al., 2008). ABA in the guard cells is percepted by PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) (Joshi-Saha et al., 2011; Gonzalez-Guzman et al., 2012). This in turn induces the formation of reactive oxygen species (ROS). Hydrogen peroxide ($H_2O_2$) is formed first, most likely by NADPH oxidase, which generates superoxide and $H_2O_2$ (Desikan et al., 2004). $H_2O_2$ induces nitrogen oxide (NO) production (Bright et al., 2006). Both $H_2O_2$ and NO are essential for stomatal closure and removal of either result in reduced stomatal closure (Zhang et al., 2001; Bright et al., 2006; Neill et al., 2008). Downstream of NO in the signaling pathway is the influx of $Ca^{2+}$ into the cytosol, which activates $anion_{out}$ channels and $K^{+}_{out}$ channels and inhibits the proton pumps (Fan et al., 2004). This depolarizes the plasma membrane and decreases the turgor pressure in the guard cells leading to stomatal closure.

Normally most plants close the stomata in response to darkness, drought or ABA treatments (Luan, 2002; Tallman, 2004; Wilkinson and Davies, 2010). During the dark most plants close the stomata in order to retain water and rehydrate (Tallman, 2004). However, the degree of stomatal closure is species dependent (Caird et al., 2007). Previous studies have also shown that the extent of stomatal closure during darkness is dependent on the environmental conditions, such as temperature, drought and air humidity (Reynolds-Henne et al., 2010; Arve et al., 2013). Both *Arabidopsis thaliana* and *Rosa x hybrida* growing in high RH (>85%) have less stomatal movement during darkness than plants growing in moderate RH (Arve et al., 2013; Arve et al., unpublished manuscript). The signalling pathway for stomatal closure in darkness does not necessarily involve ABA, but $H_2O_2$ is still required (Desikan et al., 2004).

The aim of this study was to investigate if plants developed under constant high RH have reduced sensitivity to ABA. To shed light on this, the ability of the plants to initiate $H_2O_2$ production in response to ABA or darkness was tested. Previous work on responses of the stomatal complex to RH and possible signalling molecules involved has only been performed on intact plants or detached leaves In this study we used epidermal peels from *Vicia faba*, which emit very little auto-fluorescence that could interfere with the fluorescence analysis, to study the stomatal responses of plants grown at high and moderate RH to darkness, ABA and $H_2O_2$.

## Materials and methods

### Plant material and growth conditions

*Vicia faba* L. seeds were germinated in 12 cm pots containing peat (L.O.G. Gartnerjord, Rakkested, Norway) and grown in a greenhouse during fall and winter at 20˚C, 80 % RH and 20 h supplementary light of 100 µmol $m^{-2}$ $s^{-1}$ supplementary light from high pressure sodium lamps (HPS, Osram NAVT- 400W, Munich, Germany) at the Center for plant research in controlled climate at the Norwegian University of Life Sciences, Ås, Norway (N 59° 40.120', E 10° 46.232'). Plants were grown in the greenhouse for about 1 week, until they were about10 cm tall.

The plants were then transferred to environmentally controlled growth chambers with high RH at 92% (0.26 KPa vpd) or moderate RH at 60% (1.05 KPa vpd). Both treatments had

a temperature of 22˚C, regulated by a PRIVA system (Priva, Ontario, Canada). A photoperiod of 20 h light with a photosynthetic photon flux (PPF) at 400-700 nm of 100 ± 10 µmol $m^{-2}$ $s^{-1}$ (LI-COR Light Meter, LI-250, USA) was provided by HPS lamps (Osram NAVT- 400W, Munich, Germany).

Leaflets of the first trifoliate leaf at the onset of flowering were used for stomatal conductance measurement and sampled for desiccation test, stomatal size measurements and $H_2O_2$ staining.

**Response to desiccation**

To evaluate the ability of plants to retain water during desiccation one of the leaflets on the first trifoliate leaf was detached and weighed after 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 150 and 180 minutes. The relative water content at time 0 was set to 100% and the relative water loss at the different time points was calculated. The test was performed in a room with 40% RH, 15 µmol $m^{-2}$ $s^{-1}$ irradiance at the surface of the leaves and 22°C.

**Stomatal size and closing ability**

Peels were taken from the lower side of leaflets of the first trifoliate leaves of plants grown at high and moderate RH. The individual plants within each RH treatment were cultivated at separate times. The peels were placed in MES buffer (10 mM MES, 50 mM KCl, 100 µM CaCl2, pH 6.15) for 2.5 h while receiving light from Metal Halide lamps (HPI, MBID250/T/H, Kolorarc, Hungary) at an irradiance of 100 µmol $m^{-2}$ $s^{-1}$. First half of the peels from a leaf from 4 replicate plants were then moved to another buffer containing 10 µM ABA (10 mM MES, 50 mM KCl, 100 µM $CaCl_2$, 10µM ABA, pH 6.15). In a subsequent experiment half of the peels from each leaf from 4 replicate plants were after 2.5 h of MES buffer moved to a buffer containing 1mM $H_2O_2$ (10 mM MES, 50 mM KCl, 100 µM $CaCl_2$, 1mM $H_2O_2$, pH 6.15). All peels were then incubated for another 2.5 h receiving light from HPI lamps (MBID250/T/H) of an irradiance of 100 µmol $m^{-2}$ $s^{-1}$. Control peels were similarly incubated for another 2.5 h in MES buffer under the same conditions. Images of the stomata from ABA, $H_2O_2$ as well as the control treatment were then taken with a Leica camera (Leica DFC 425, Leica Microsystems GmbH, Wetzlar, Germany) connected to a light microscope (Leitz, Labolux K, Wetzlar, Germany). The stomatal aperture was recorded in all images of peels from all treatments, while the pore length was only measured in the light. All

measurements were done using the UTHSCSA ImageTool 3.0 (University of Texas Health Science center in San Antonio, USA).

### $H_2O_2$ quantification

Separate MES-buffer-incubated peels (2.5 h) from plants grown at high and moderate RH, all as described above, were cut into squares of 0.5 x 0.5 cm. These were then moved to either a buffer containing 10 µM ABA (as described above), fresh MES buffer which were placed in the dark for 2.5 h or kept in the same MES buffer in light. After 2.5 h the peels from each of the buffer treatments were placed in a Tris buffer (10 mM Tris, 50mM KCl, pH 7.2) containing 50 µM $H_2$DCF-DA (dichlorodihydrofluorescein diacetate) dissolved in dimethyl sulfoxide, for 10 minutes in the dark. Excess dye was then removed by washing twice with Tris buffer for 1 minute. The peels were then photographed through a confocal laser scanning microscope (Leica TCS SP 5 mounted on a Leica DMI 6000 microscope, Wetzlar, Germany), with excitation 488 nm and emission 495-595 nm. Eight images were taken of each peel and all stomata on the images were used in the analysis. The fluorescent images were analyzed using Fiji (Fiji Is Just ImageJ, http://fiji.sc/). The mean fluorescence within the outer edges of the guard cells was used in the statistical analyses.

### Statistical analysis

The results were analyzed using normally distributed general linear model (GLM) and Tukey's test. Differences with $p \leq 0.050$ were considered significantly different. All statistical analyses were performed in Minitab 16.1.1 (Minitab 16.1.1, windows version, State College, Pennsylvania, USA).

## Results

### Development of *Vicia faba* leaves in high RH resulted in larger stomata with reduced responses to closing stimuli

Previous studies have shown larger, less functional stomata in species such as *Rosa x hybrida*, *Tradescantia virginiana* and *A. thaliana* when grown in high RH compared to moderate RH (Torre et al., 2003; Nejad and van Meeteren, 2005; Arve et al., unpublished manuscript). The effect of RH on stomata was therefore also investigated in *V. faba* in order to study signaling involved. When developed under high RH *V. faba* stomata in epidermal

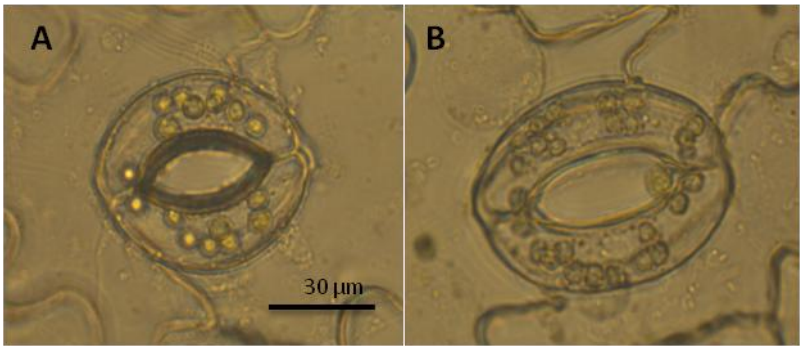


Figure 1: Light microscope images of stomata in 60 % relative air humidity (RH) (A) and 90 % RH (B). The images are of epidermal peels from the abaxial side of *V. faba* leaves in MES buffer.

peels were larger in size compared with stomata developed under moderate RH ($p<0.001$, Fig. 1). The average length of the stomatal pore in high and moderate RH was 57 µm and 48 µm, respectively (Fig. 2). Thus, the stomata of *V. faba* developed in high RH showed a similar response as previously studied species.

A desiccation test was performed to test the ability of *V. faba* plants from high and moderate RH to close the stomata. A significant difference in water loss was found after only 5 min of desiccation ($p<0.001$). The difference increased throughout the test and after 180 min the plants from high RH had lost approximately 70% of their initial weight (Fig. 3). In contrast, plants from moderate RH had only lost about 20% of their initial weight (Fig. 3).

To evaluate the involvement of ABA and $H_2O_2$ as signaling molecules in stomatal response to RH we measured the stomatal aperture in epidermal peels from plants grown at high and moderate RH after treatment with ABA and $H_2O_2$. The average stomatal aperture in control peels from plants grown under moderate RH in the two different experiments were 9.8 µm and 7.9 µm in the light and closed to approximately 6.3 and 3.2 µm after ABA and $H_2O_2$ treatment, respectively (Fig. 4). The stomatal aperture in control peels from plants grown under high RH was approximately 11.2 µm and 9.9 µm in the light and approximately 10.7 µm and 8.4 µm after the ABA and $H_2O_2$ treatment, respectively (Fig. 4). Thus, both treatments showed stomatal closure in response to ABA and $H_2O_2$. However, plants from high RH only exhibited a 5 % reduction in stomatal aperture in response to ABA ($p<0.050$), while plants from moderate RH showed a 36 % reduction ($p<0.001$). Similarly, plants from high RH only had a 15 % reduction in stomatal aperture in response to $H_2O_2$ ($p<0.001$), while plants from moderate RH exhibited a 59 % reduction ($p<0.001$). This shows that plants

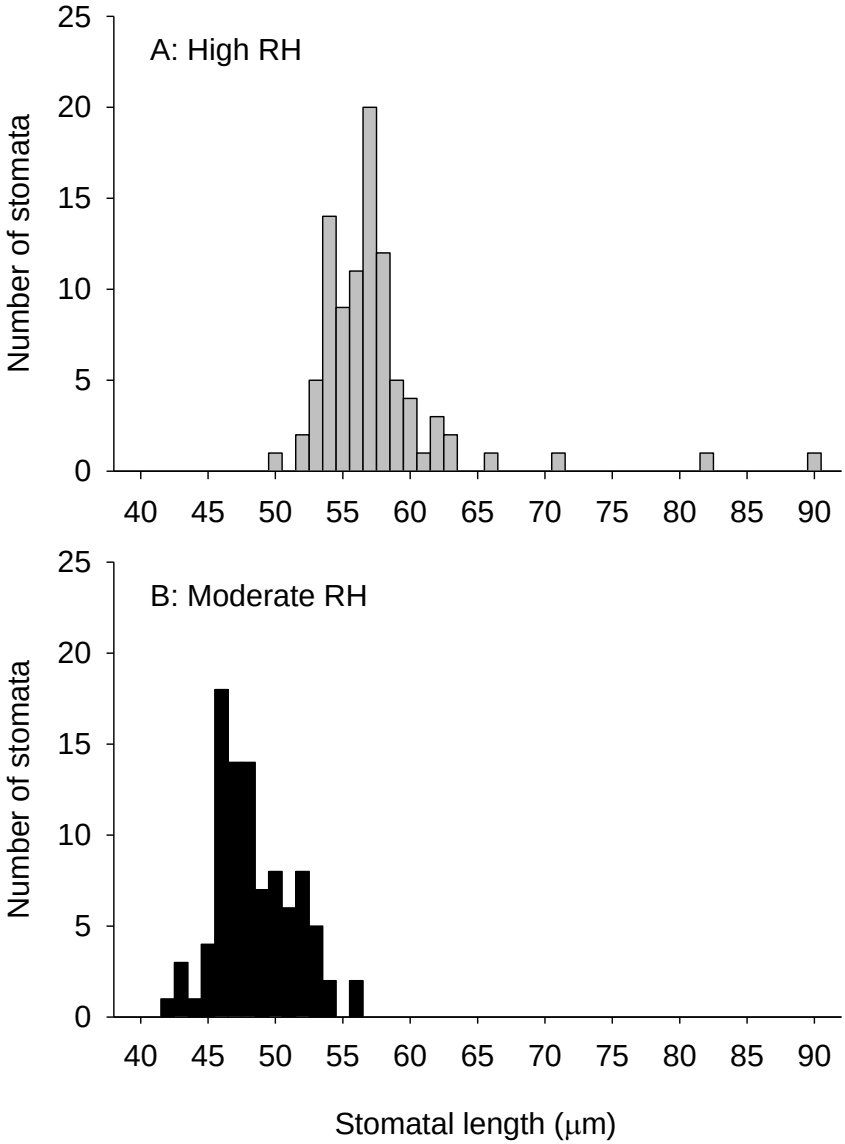

Figure 2: Frequency distribution of stomatal pore length in light. Stomatal length was measured on epidermal peels of *V. faba* developed in high relative air humidity (RH) (A) and moderate RH (B). n=93.

developed in high RH responded to a smaller extent to ABA and $H_2O_2$ than plants developed in moderate RH. Thus, although plants developed in high RH showed a certain response to closing stimuli, they showed a much weaker response than plants developed in moderate RH.

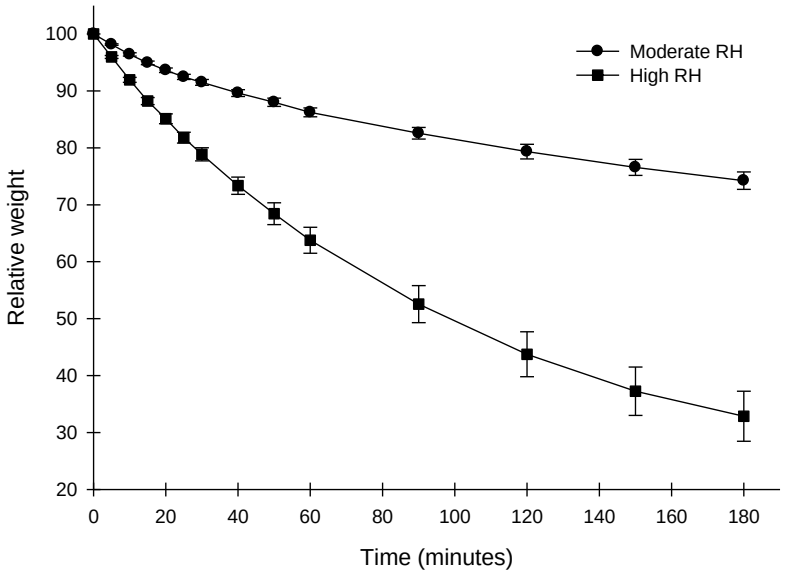

Figure 3: Weight loss of detached *Vicia faba* leaves, developed under high and moderate relative air humidity (RH), during 180 minutes of desiccation in a dry environment (40% RH), n=11. Mean values ± SE are shown.

**Plants developed in high RH increased $H_2O_2$ production in response to ABA but not during darkness**

Since induction of stomatal closure by ABA involves production of $H_2O_2$ (Bright et al., 2006) $H_2O_2$ was quantified in guard cells from plants grown under moderate and high RH kept in light, darkness and after ABA treatment.

The light treatment was used as a control to represent the amount of $H_2O_2$ in open stomata. In the light there was no significant difference in the amount of $H_2O_2$ between high and moderate RH (Fig. 5). After exposure to darkness, the amount of $H_2O_2$ was significantly higher in guard cells of plants from moderate RH compared to those from high RH ($p<0.001$). Also, compared to the light control the amount of $H_2O_2$ increased significantly in guard cells from moderate RH ($p<0.001$), but did not change significantly in guard cells from high RH (Fig. 5). Furthermore, ABA treatment of guard cells from moderate as well as high RH-grown plants resulted in significantly higher $H_2O_2$ levels compared to the control ($p<0.001$, Fig. 5). After the ABA treatment the amount of $H_2O_2$ was significantly higher in guard cells from high RH, than in guard cells from moderate RH ($p<0.001$). In summary, the $H_2O_2$ levels in guard cells from moderate RH increased after treatment with ABA and in darkness whereas in high RH the $H_2O_2$ levels increased in response to ABA treatment, but not in darkness.

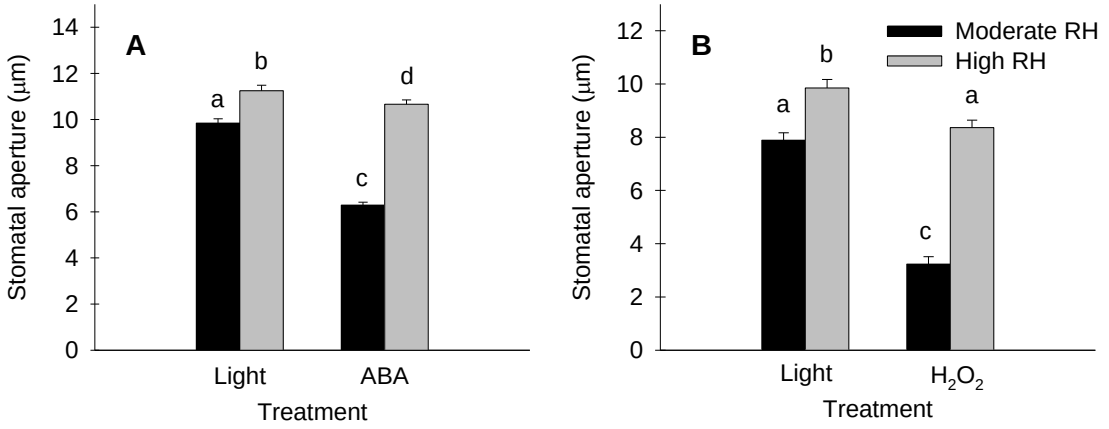


Figure 4: Stomatal aperture measured on epidermal peels taken from leaves developed in moderate and high relative air humidity (RH). The peels were either treated with abscisic acid (ABA) (A) or $H_2O_2$ (B) or kept as a control in the light. The ABA and $H_2O_2$ applications were done in two different experiments. Different letters indicate significant differences, n=216 (A), 68-75(B). Mean values ± SE are shown.

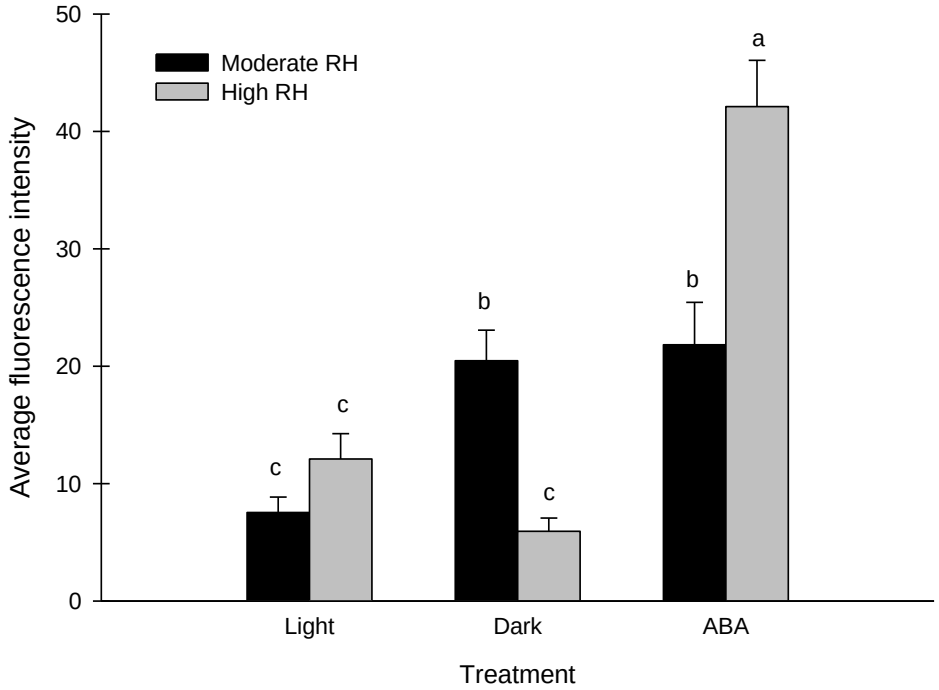


Figure 5: The amount of $H_2O_2$ in the guard cells in epidermal peels from plants grown in high and moderate relative air humidity (RH) after incubation in light (control), darkness and after treatment with abscisic acid (ABA) as measured by fluorescence microscopy after addition of dichlorodihydrofluorescein diacetate. The different letters indicate significant differences, n=72-98. Mean values ± SE are shown.

## Discussion

### Stomata from plants developed in high RH show weaker response to closing stimuli than those from moderate RH

In this study we found that *V. faba* developed in continuous high RH have significantly larger stomata compared with plants developed in moderate RH (Fig. 2). This is consistent with previous studies of *Rosa x hybrida*, *A. thaliana* and *T. virginiana* showing that plants developed in continuous high RH have lower ABA content and larger stomata (Torre et al., 2003; Nejad and van Meeteren, 2005; Arve et al., 2013; Arve et al., unpublished manuscript). An earlier study on *Populus x canescens* also found that ABA- insensitive mutants had larger stomata (Arend et al., 2009). This indicates a connection between development in high RH, ABA and stomatal size.

We also found that leaves from high RH lost more water during a desiccation test than those from moderate RH (Fig. 3). This is also consistent with previous studies of *A. thaliana*, *Rosa x hybrida*, *Begonia x cheimantha*, *Chrysanthemum morifolium*, *Euphorbia pulcherrima*, *Kalanchoe blossfeldiana* and *T. virginiana* (Mortensen, 2000; Torre and Fjeld, 2001; Nejad and van Meeteren, 2005; Arve et al., unpublished manuscript). Thus, plants developed in continuous high RH have a reduced ability to close the stomata and the reduced stomatal functionality is similar in several different species. It can therefore be concluded that this is a general plant response to high RH, at least in C3 plants.

Darkness is known to induce stomatal closure, although the amount of closure varies between species (Tallman, 2004; Caird et al., 2007). In a previous study of *V. faba* the stomatal aperture was reduced by about 60% after 2 h of darkness (Desikan et al., 2004). Another study also been found that there was a small reduction in pore area after only 5 minutes of darkness (Kaiser and Kappen, 1997). Previous studies of the diurnal conductance of roses have shown a pattern where plants from both high and moderate RH reduce the conductance during the dark period (Fanourakis et al., 2012; Arve et al., 2013). However, both studies showed a much larger relative change in conductance in plants developed in moderate RH compared with high RH. Also, studies on *A. thaliana* and *Rosa x hybrida* showed that plants developed in moderate RH reduced the stomatal aperture in darkness, while plants developed in high RH did not (Arve et al., 2013; Arve et al., unpublished manuscript).

Other stimuli that usually cause stomatal closure are ABA and $H_2O_2$ (Zhang et al., 2001; Tallman, 2004; Seo and Koshiba, 2011). In this study the stomata from both high and moderate RH responded to both ABA and $H_2O_2$ by reducing the stomatal aperture (Fig. 4). However, the stomata developed in moderate RH showed a much stronger response to both ABA and $H_2O_2$ than those from high RH which showed a very weak response. In a study on *T. virginiana* it was found that the stomata of plants developed in high RH had reduced response to ABA (Nejad and van Meeteren, 2005). Similarly, a study of roses showed that ABA-application on fully developed plants grown in high RH had some effect on the transpiration, but the effect was smaller than for plants developed in moderate RH (Fanourakis et al., 2012). Furthermore, daily application of ABA during leaf expansion in plants developed in high RH resulted in fully functional stomata (Nejad and van Meeteren, 2008; Fanourakis et al., 2011). All these studies show the same trend, where plants developed in high RH have reduced response to ABA. However, they show some stomatal response to closing stimuli.

**Signaling pathway involved in stomatal closure**

When plants are placed in the dark they generally produce $H_2O_2$ and close the stomata (Desikan et al., 2004). In this study, plants from moderate RH showed an increase in $H_2O_2$ levels after transfer to darkness (Fig. 5). However, plants developed in high RH did not increase the $H_2O_2$ levels when transferred to the dark (Fig. 5). This shows that darkness only induces active stomatal closure through $H_2O_2$ production in plants developed in moderate RH, not in plants developed in high RH. In a previous study of six temperate deciduous tree species it was shown that responses to RH dominated over responses to photosynthetic signals (Aasamaa and Sober, 2011). The lack of an increased $H_2O_2$ production during darkness in plants developed in high RH might therefore be a result of the high RH conditions, which would not induce stomatal closure during darkness. Other studies of *A. thaliana*, *Rosa x hybrida* and *T. virginiana* showed that plants developed in high RH did not close the stomata during darkness (Nejad and van Meeteren, 2005; Arve et al., 2013; Arve et al., unpublished manuscript). The same studies also showed that the ABA levels in leaves developed in high RH did not increase during darkness (Arve et al., 2013; Arve et al., unpublished manuscript). In this study we showed that increased levels of ABA increased the amount of $H_2O_2$ (Fig. 5). Since there was no increase in $H_2O_2$ levels in plants from high RH during darkness, it can be assumed that the ABA levels then remained low. The previous and

present studies indicate that plants developed in high RH do not increase the ABA levels during darkness to induce stomatal closure. This is in accordance with previous studies that clearly show that *A. thaliana* and *Rosa x hybrida* plants developed in high RH do not close the stomata during darkness (Arve et al., 2013; Arve et al., unpublished manuscript).

One of the most important signals for stomatal closure is ABA (Tallman, 2004). The reduced degree of closure found in stomata developed in high RH could therefore be a result of reduced sensitivity to ABA. In this study stomata from both high and moderate RH responded to ABA or $H_2O_2$ treatments by closing the stomata (Fig. 4). However, the stomata developed in high RH had a very weak response. To investigate if this reduced response is due to a reduced ability to sense the ABA the relative amount of the secondary messenger $H_2O_2$ was quantified. Plants from both moderate and high RH showed an increase in $H_2O_2$ levels after treatment with ABA (Fig. 5). This shows that ABA can initiate the signaling pathway toward stomatal closure in plants developed in both moderate and high RH. However, even though the stomata of plants developed in high RH produced $H_2O_2$ in response to ABA, they showed reduced stomatal closure compared to plants from moderate RH (Fig. 4). The reduced stomatal movement in high RH may therefore be caused either by a later step in the ABA dependent-pathway or alternatively by alteration in cell wall structure affecting the guard cell morphology, which could make them physically unable to close properly (Jones et al., 2003).

## Conclusion

This study clearly shows that *V. faba* plants developed in continuous high RH are able to increase the $H_2O_2$ production when treated with ABA. However, they show reduced stomatal closing ability. These results suggest that the reduced stomatal response is caused either by a step downstream of $H_2O_2$ in the pathway toward stomatal closure or is a result of changed anatomy, making the stomata unable to close properly. Darkness as a signal for closure did not initiate the ROS signaling pathway in plants developed at high RH because no increase in $H_2O_2$ production was detected.

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# Paper IV

12

# Stomatal Responses to Drought Stress and Air Humidity

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## 1. Introduction

Water is one of the most important substances for both plant and animal survival. Plants require water for photosynthesis, nutrient uptake and transportation as well as cooling (Farooq et al., 2009). Plants are sessile organisms and in contrast to most animals they are unable to move when the environment becomes unfavorable. Accordingly, plants have to be able to respond and adapt to the local environmental changes. Since water is essential for plant survival, the ability to tolerate water stress is crucial.

To be able to grow plants need to take up water from the soil and $CO_2$ from the atmosphere and use it in photosynthesis. This is done by $CO_2$ uptake through the stomatal pore, where water is simultaneously transpired. Water transpiration drives the water uptake by the roots and transport through the xylem. When the stomata are open $CO_2$ is taken up while water is transpired. When the stomata are closed little $CO_2$ is taken up and the transpiration is lowered. By opening and closing the stomata plants can regulate the amount of water lost, by sacrificing $CO_2$ uptake, when the environmental conditions are unfavorable.

Water stress can be defined as reduced water availability; either by water scarcity (drought) or osmotic stress (high salt concentrations) or water logging; too much water. Water stress may reduce photosynthesis, respiration and ion uptake, change the metabolic and growth patterns in the plant and in severe cases result in plant death (Jaleel et al., 2009a). In nature water stress is common either for long or short periods of time, depending on the local climate. Most plants therefore have some adaptation or response to enhance the growth and survival rate during water stress and subsequent recovery.

In agriculture and horticulture drought stress is one of the major problems, causing major crop losses every year as well as loss of aesthetic value in ornamentals. In agriculture crop loss is due to reduced numbers of tillers, spikes and grains per plant and reduced grain weight (Farooq et al., 2009). With the global human population rapidly increasing, simultaneously as water scarcity increases, the loss of crop will be even more serious than before. The discovery and development of stress tolerant crops to avoid yield loss during water stress is therefore very important. In the greenhouse industry, energy saving for economic profit is important to be able, but it also affects the plants. To reduce the amount of energy needed for $CO_2$ and heating in the greenhouses, energy-efficient semi-closed

greenhouses can be used. In these greenhouses the ventilation is reduced to a minimum, which consequently results in increased relative air humidity inside. This increase in air humidity affects the plants in different ways and might result in plants that are less tolerant to water stress (Torre and Fjeld, 2001).
In this review different plant responses to water stress will be discussed, with most attention to drought and the role for abscisic acid (ABA) as a plant stress hormone. In addition, consequences of plant development under high relative air humidity, which reduces the plants ability to respond to water stress, will be discussed.

## 2. Plant responses to water stress

Plants growing in deserts or high salinity habitats are all exposed to more or less constant water stress. To survive such conditions plants have developed growth strategies such as increased water use efficiency with $C_4$- or CAM metabolism (Keeley and Rundel, 2003), succulent growth and extensive root systems (Henry et al., 2011). These strategies are good in a dry environment, but in more "favourable" conditions at least some of these plants may, due to lower growth rates, more easily be outcompeted by other less drought tolerant plants. Other adaptations to plant life in dry environments are thick cuticula and wax layers, depressed stomata and high density of trichomes. Thick cuticula and wax layers reduce extra-stomatal transpiration, and depressed stomata and trichomes create a thicker boundary layer outside the stomata, where the humidity gradient is more gradual, thereby reducing the stomatal transpiration.
Plants living in saline environments (e.g. beaches, salt marches) commonly keep a low osmotic potential in their cells, which facilitates water uptake. They usually also have the ability to exclude or excrete salt from their cells to avoid to too high salt concentrations. A variety of perennials commonly avoid water stress during the winter by entering dormancy and often shedding leaves (deciduous woody species) before the onset of the harsh conditions when water is unavailable due to frost. However, plants keeping the leaves on through the winter commonly face water stress in the spring when air temperatures are high while the soil is still frozen.
Even if they do not live in particularly dry places, most plants will occasionally encounter water stress for shorter or longer periods of time. Most of these plants do not have many of the adaptations of desert plants and must respond to the water stress in other ways. When these plants are exposed to water stress, such as drought or saline conditions, to survive they must be able to retain as much water as possible. If the plants are not able to cope with the water stress, they will not be able to survive. The sensitivity and response time to drought differs between different species and slow growing species have been found to be more sensitive (Aasamaa and Sober, 2011). Repeated drought encounters increases the sensitivity to environmental changes that induce stomatal closure, while the sensitivity to changes that induce stomatal opening is decreased (Aasamaa and Sober, 2011). In response to water stress plants have developed several different mechanisms that increase the desiccation tolerance and water retention. These responses can be divided into short term and long term responses (Figure 1).

### 2.1 Long term responses

During prolonged water stress plants must be able to survive with low water content and maintain a minimum amount of water, through water uptake and retention. To cope with

prolonged drought stress plants respond with energy demanding processes that alter the growth pattern, chemical content of the plants and the up or down regulation of genes.

### 2.1.1 Biochemical changes

When the water availability is reduced, plants change the biochemistry to be able to retain as much water as possible and take up whatever water they can. During water stress plants produce and accumulate compatible solutes such as sugars, polyols and amino acid to lower the osmotic potential in the cells to facilitate water absorption and retention (Xiong and Zhu, 2002). Some of the compatible solutes also contribute to maintaining the conformation of macromolecules by preventing misfolding or denaturation (Xiong and Zhu, 2002). Plants also produce higher levels of the plant stress hormone ABA during water stress and this affects their growth pattern and stress tolerance (details under growth changes and stomatal functioning).

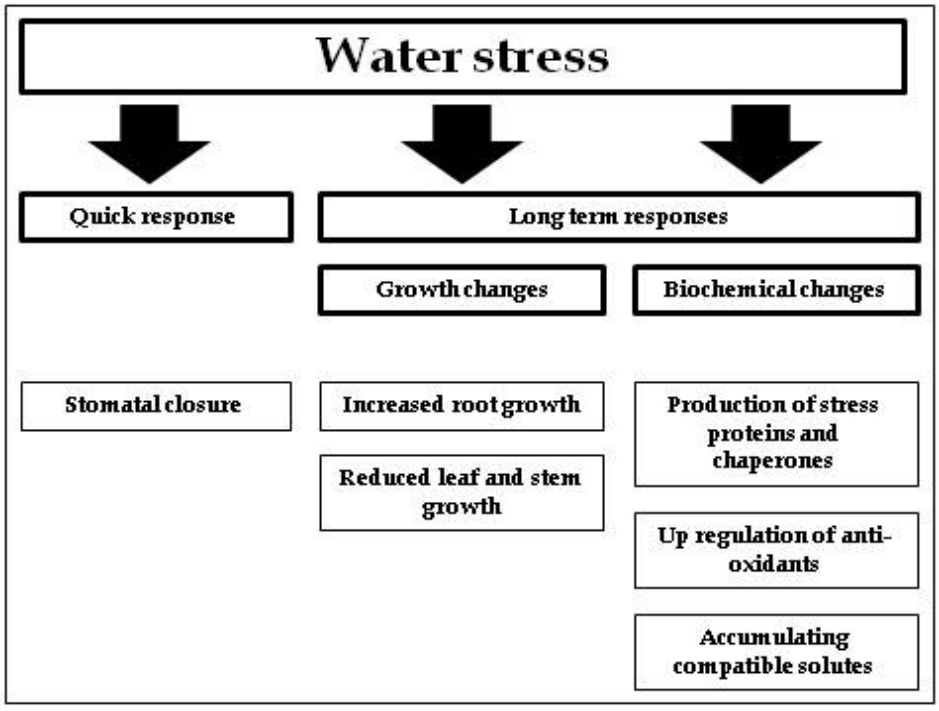


Fig. 1. Plant responses to water stress.

A group of proteins called late embryogenesis abundant like (LEA) proteins are also produced during water stress. These LEA-like proteins are highly hydrophilic, glycine-rich and highly soluble and have been found to be regulated by ABA (Xiong and Zhu, 2002). The LEA-like proteins are thought to act as chaperones, protecting enzymatic activities (Reyes et al., 2005) and preventing misfolding and denaturation of important proteins (Xiong and Zhu, 2002). Some of the LEA-like proteins have similar features as ribosomal proteins and are thought to interact with RNA (Garay-Arroyo et al., 2000).

Decreased transpiration and decreased $CO_2$ and nutrient uptake during water stress result in changes in metabolic pathways such as photosynthesis and respiration, as well as changes in ion uptake, transport and extrusion (Xiong and Zhu, 2002). Some of these changes can lead to oxidative damage. Reactive oxygen species, such as $H_2O_2$, $O_2^-$, OH and $OH_2$, are by-products in electron transport chains and have unpaired electrons that can attract electrons from other components. Reactive oxygen species can therefore cause damage to a variety of compounds such as DNA, RNA, proteins, lipids and chlorophyll and thus damage membranes and change cell metabolism and eventually lead to senescence. Many antioxidant systems, both enzymatic and non-enzymatic, are up-regulated in response to the increased reactive oxygen species levels during water stress. These antioxidants scavenge the reactive oxygen species and reduce the oxidative damage. The enzymatic antioxidants, such as superoxide dismutase, peroxidase, ascorbate peroxidase, catalase, polyphenol oxidase and gluthathione reductase can detoxify reactive oxygen species (Prochazkova et al., 2001; Jaleel et al., 2009b). The non-enzymatic anti oxidants, including vitamins (A, C and E), glutathione, carotenoids and phenolic compounds, can scavenge reactive oxygen species by donating an electron or a hydrogen atom (Prochazkova et al., 2001; Jaleel et al., 2009b).

### 2.1.2 Growth changes

During water stress the water content of the plant decreases, which causes the cells to lose turgor pressure and shrink. The loss of turgor pressure in the cells inhibits turgor dependent activities such as cell expansion, which affects the growth of the whole plant. Some studies show that ABA can function as a signal to reduce leaf growth rate, both when ABA is applied exogenously or generated by water stress (Wilkinson and Davies, 2010). Reduced cell growth during water stress has e.g. been found to decrease the stem length in *Arabidopsis thaliana* soybean (*Glycine max*), potato (*Solanum tuberosum*), ocra (*Abelmoschus esculentus)* and parsley (*Petroselinum crispum*) (Heuer and Nadler, 1995; Specht et al., 2001; Park et al., 2007; Petropoulos et al., 2008; Sankar et al., 2008). Similarly reduced cell enlargement reduces the leaf expansion in *Populus* (Ren et al., 2007). By reducing the leaf expansion the leaves become smaller and therefore transpire less. In some cases water stress can even lead to leaf abscission. This has e.g. been seen in *Populus* and paper birch (*Betula papyrifera)* (Giovannelli et al., 2007; Gu et al., 2007). The reduction of cell volume also concentrates the solutes in the cells and compresses the plasma membranes causing them to increases in thickness.

To increase water uptake and maintain a minimum osmotic pressure during drought many plants increase their root growth, either deeper or laterally. By increasing the root growth the area for water uptake becomes larger and water further away and deeper in the soil may be reached. This growth response has been found in e.g. maize, madagaskar periwinkle (*Catharanthus roseus)* and date palm (*Phoenix dactylifera)* (Djibril et al., 2005; Jaleel et al., 2008; Trachsel et al., 2010).

## 2.2 Short term response

When plants suddenly encounter drought it is important to respond as quickly as possible. A faster drought response means that less water is lost and the survival rate of the plants is increased. The most important quick response is stomatal closure. Stomata consist of two guard cells surrounding the stomatal pore. When the stomata are open water is transpired and $CO_2$ enter the leaf through the stomatal pore. During water stress the stomatal pore can be closed to reduce water loss. By closing the stomatal pore the

water use efficiency is increased (Farooq et al., 2009), reducing the amount of water lost per $CO_2$ molecule assimilated. Several mechanisms work together to close the stomata, such as hydro passive closure and chemical signals from the plant stress hormone ABA.
Increased levels of ABA also causes increased hydraulic conductivity in the roots and xylem, enabling the plants to transport more water and thereby recover more rapidly after water stress (Kudoyarova et al., 2011).

## 3. Stomatal functioning

Development of stomata if often considered one of the most important developments in plant evolution (Brodribb and McAdam, 2011). By being environmentally controlled gateways into the plants controlling $CO_2$ uptake and transpiration they are central determinants of photosynthesis, cooling and nutrient uptake (Farooq et al., 2009). To be able to balance $CO_2$ uptake and water transpiration through stomatal movement is therefore an important response to changes in the environmental conditions. Low transpiration due to stomata closure means less cooling of the leaves and less uptake and transportation of nutrients.

### 3.1 Stomatal signaling and movement

Stomatal closure occur when the two guard cells surrounding the stomatal opening lose turgor pressure and close the opening (Outlaw, 2003). There are many signals that induce stomatal closure, among these the best known signal is probably ABA. In the signaling pathway towards stomatal closure there are several secondary messengers, such as $Ca^{2+}$, $H_2O_2$ and NO (Atkinson et al., 1990; Zhang et al., 2001; Neill et al., 2002; Garcia-Mata and Lamattina, 2009) that contribute to the stomatal closure. Passive loss of turgor pressure also results in stomatal closure.
Since stomatal closure has negative effects on $CO_2$ uptake, photosynthesis, transpirational cooling as well as water and nutrient uptake it is important to close the stomata only when the benefit of water retention outweighs the negative effects. To be able to close the stomata during unfavourable conditions there are several mechanisms and signalling pathways leading to stomatal closure. These pathways can be divided into hydro passive and active stomatal closure (Figure 2).

#### 3.1.1 Hydro passive stomatal closure

Hydro passive stomatal closure occurs when the water evaporation from the guard cells is too low to be balanced by water movement into these cells. The water content in the cells is then rapidly reduced to the extent where the osmotic pressure is reduced and the cells lose turgor pressure and shrink (Luan, 2002). When this happens the guard cells are unable to maintain the shape and the stomatal pore is covered.
Some studies have shown that passive stomatal closure is important in ferns and Lycopods, but not in Angiosperms and Gymnosperms (Franks and Farquhar, 2007; Brodribb and McAdam, 2011). This is because in Angiosperms and Gymnosperms the guard cells closely interact with their subsidiary cells. When the guard cells lose turgor pressure the subsidiary cells also lose turgor pressure and the force from the subsidiary cells pulls the guard cells apart, opening the stomata. This hydro passive opening is called the "wrong-way" response (Franks and Farquhar, 2007). In contrast the guard cells of ferns and Lycopods do not interact closely with their subsidiary cells.

The loss of turgor pressure in the subsidiary cells in these plants does therefore not result in the guard cells being pulled apart. The simultaneous loss of turgor in the guard cells will in these plants be enough to close the stomata.

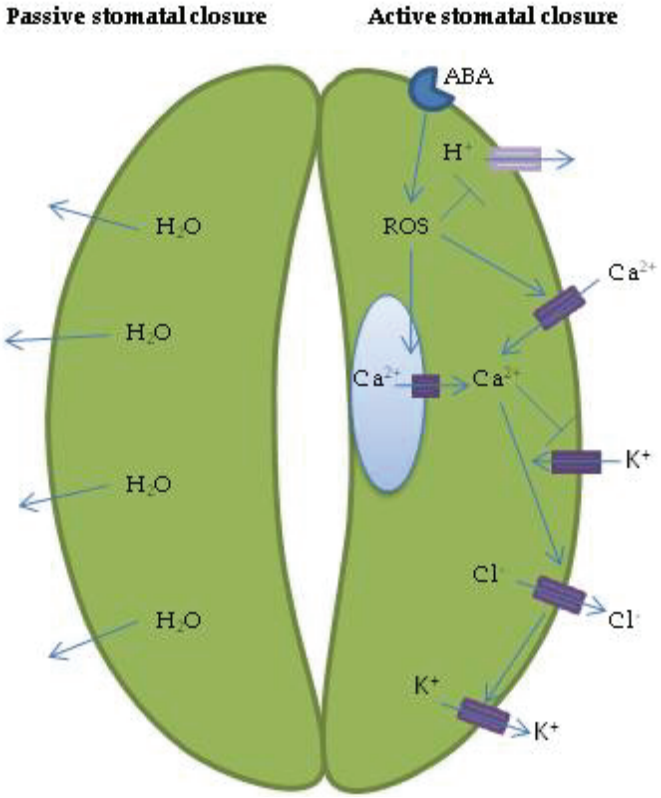


Fig. 2. Hydro passive and active stomatal closure pathways.

### 3.1.2 Active stomatal closure

ABA as well as elevated levels of $CO_2$ activates signalling pathways leading to stomatal closure (Kim et al., 2010). ABA is produced in the roots and leaves during water stress and is transported to the guard cells. ABA is transported into the guard cells by ATP-binding cassette (ABC) transporters that are located in the plasma membrane (Kang et al., 2010). When the ABC transporters are knocked out the ABA uptake is lower, stomata remain more open during drought and the stress tolerance is decreased (Kang et al., 2010). The ABA signals are first recognised by several receptors. PYR/PYL/RCAR (PYRABACTIN RESISTANCE/ PYRABACTIN RESISTANCE –LIKE/REGULATORY COMPONENT OF ABA RESPONCE) proteins have been shown to function as ABA receptors (Klingler et al., 2010). Another protein GCR2 (G protein coupled receptor) has also been shown to be a ABA receptor (Liu et al., 2007).

The size of the stomatal opening is regulated by the turgor pressure and cell volume of the guard cells (Schroeder et al., 2001; Kim et al., 2010). Regulation of stomatal opening is linked

to transport of ions and water through channel proteins across the plasma and vacuole membrane (Kim et al., 2010). ABA induces the production of reactive oxygen species (e.g. $H_2O_2$), which in turn acts as a trigger for NO production, inhibition of membrane proton pumps and $Ca^{2+}$ influx across both the plasma and vacuole membranes. $H^+$-ATPases that are hyperpolarizing the plasma membranes must be inhibited to induce ABA mediated stomatal closure (Merlot et al., 2007). The increased $Ca^{2+}$ levels activate slow and rapid type anion channels, generating an anion efflux from the cells. The anion efflux depolarizes the membrane, which in turn causes $K^+$ efflux through $K^+_{out}$ channels across both the vacuole and the plasma membrane. Simultaneously $Ca^{2+}$ also inhibits $K^+_{in}$ channels (Wasilewska et al., 2008). Malate is also converted to starch reducing the osmotic potential and turgor pressure further (Kim et al., 2010). The plasma membrane is thus depolarised, the turgor pressure and cell volume reduced and the stomata close (Kim et al., 2010).

## 4. ABA biosynthesis and metabolism

Increased content of ABA during water stress has been found in all photosynthetic organisms. The biosynthesis of ABA have previously been thought to occur only in the roots, but more recent studies show that ABA is also synthesized in mesophyll cells, vascular tissue and stomata. As stated above increased levels of ABA in leaves induces and regulates stomatal closure, while the increased levels of ABA in roots increase the hydraulic conductivity increasing the water uptake and transportation (Parent et al., 2009). The amount of ABA in the tissue is regulated in several metabolic steps, both in the biosynthesis and inactivation steps.

ABA is synthesized from phytoene (Figure 3), a carotenoid produced from pyruvate and glyceraldehydes-3-phosphate (Cutler and Krochko, 1999; Liotenberg et al., 1999). In the plastids phytoene is converted to ζ-carotene by phytoene desaturase and then to β-carotene, lycopene and zeaxanthin. Zeaxanthin is converted first to antheraxinthin and then to violaxanthin by zeaxanthin epoxidase (ZEP). Violaxanthin is then converted to xanthoxin by 9-cis-epoxycarotenoid dioxygenase (NCED). Xanthoxin is then converted further in the cytosol. The main pathway from xanthoxin to ABA is through abscisic aldehyde. Xanthoxin is then converted to abscisic aldehyde by an enzyme related to a short-chain dehydrogenase/reductase SDR). Abscisic aldehyde is further oxidized to ABA by abscisic aldehyde oxidase (AAO) (Seo and Koshiba, 2002). It has been found that genes regulating at least the last steps in the ABA biosynthesis (NCED and AAO) are the most important and are strongly up regulated during water stress, showing the important role of ABA as a rapid stress response (Qin and Zeevaart, 1999; Seo et al., 2000).

ABA is further regulated by several inactivation pathways (figure 3) (Cutler and Krochko, 1999). There are two main such pathways. The first is inactivation by oxidation. ABA is then oxidized to 8′-hydroxy ABA and subsequently to phaseic acid (PA) and 4′dihydrophaseic acid (DPA). The conversion of ABA to 8′-hydroxy ABA is catalysed by the enzyme (+)-ABA 8′-hydroxylase (Kushiro et al., 2004) and the enzyme phaseic reductase catalyzes the conversion of PA to DPA (Cutler and Krochko, 1999). (+)-ABA 8′-hydroxylase is highly regulated by environmental factors, such as air humidity (Okamoto et al., 2009). The other inactivation pathway is by conjugation to ABA glucose ester, which is hypothesised to be a storage form of ABA (Cutler and Krochko, 1999). This conjugation is catalyzed by ABA glucosyltransferase (Lee et al., 2006). Several experiments provide evidence that ABA

glucose ester can be cleaved enzymatically by β-D-glucosidase (Dietz et al., 2000; Lee et al., 2006). The liberated ABA can then induce metabolic and changes and stomatal closure.

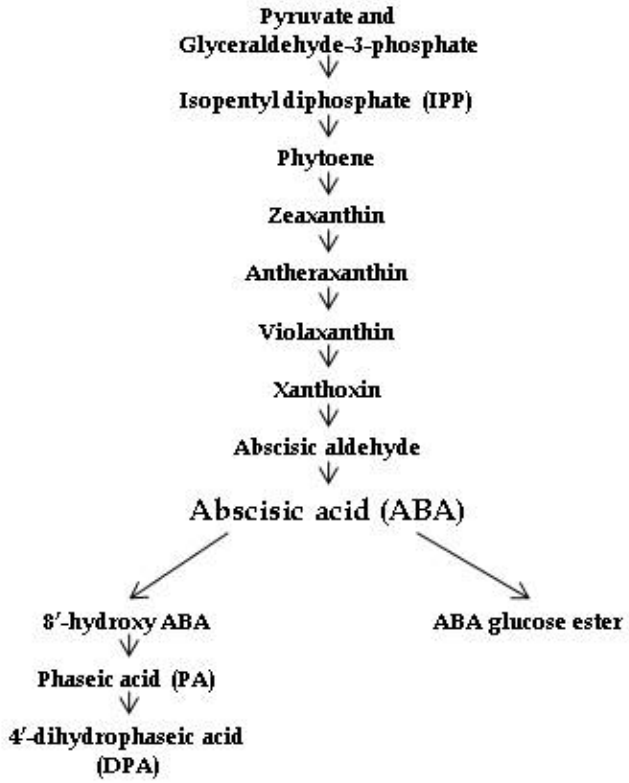


Fig. 3. Biosyntehesis of ABA from pyruvate and glyceraldehydes-3-phosphate and ABA metabolism by oxidation to PA and DPA and conjugation to ABA glucose ester.

## 5. Stomatal development under high relative air humidity

Plants grown under high relative air humidity have malfunctioning stomata that are unable to close in response to darkness, ABA and desiccation (Fordham et al., 2001). This results in high stomatal conductance and frequent leaf drying. Also, plants grown *in vitro* under high relative air humidity have low ABA levels, but when moved to an *ex vitro* environment with lower relative air humidity the ABA levels increase (Hronkova et al., 2003). Furthermore, *Wrightia tomentosa* plants grown under high relative air humidity *in vitro*, had 29.4 % malformed stomata (Joshi et al., 2006). These stomata were described as large, spherical and wide open, lacking the ability to close. In comparison stomata of *in vivo* developed plants were smaller, elliptical and depressed. Other studies have shown similar results, where *in vitro* propagation has resulted in stomata that are unable to close in response to environmental and biochemical stimuli (Brainerd and Fuchigami, 1982; Santamaria et al., 1993; Sciutti and Morini, 1995).

The efficiency of stomatal openings for $CO_2$ uptake and water transpiration is not only determined by the size of the opening, but also by the number of stomata (Metwally et al., 1971). More stomata can take up more $CO_2$ and transpire more. In research done in different humidities it has also been found that the number of stomata per leaf increased with development in higher soil humidities, but when calculated as number of stomata per area the number decreased in higher humidities (Metwally et al., 1970; Metwally et al., 1971). The stomatal index, the number of stomata relative to the number of epidermal cells, was also found to increase with soil moisture (Schürmann, 1959). Similar experiments have been preformed with air humidity, increased air humidity results in increased stomatal density (Sciutti and Morini, 1995). The stomatal density has been found to increase in plants with decreased ABA concentrations, which also have increased transpiration (Lake and Woodward, 2008). In *Vicia* faba drought and salinity stress has been found to increase the stomatal density and stomatal index, facilitating water uptake under water stressed conditions (Gan et al., 2010).

In the greenhouse industry the stomatal functioning and transpiration influences the post harvest quality of the plants. The value of ornamental plants is dependent on the aesthetic condition. Loss of aesthetic value can be due to water stress, where high transpiration rates shorten the shelf life. When ornamental plants are grown in large scale industries it is important to produce stress tolerant plants that have long shelf lives. In greenhouses there is an artificial environment, where the day length, temperature, relative air humidity (RH) and watering regimes are controlled to be able to produce as many plants as possible with as little cost as possible, without reducing the quality of the plants. This has resulted in energy-efficient greenhouses, which conserve energy ($CO_2$ and temperature) by rarely opening the ventilation. This consequently increases the relative humidity inside the greenhouses. Furthermore, much of the plant breeding is done in greenhouses, particularly when it comes to ornamentals.

Roses developed under high relative humidity (>85%) have 6-8 days shorter shelf life and greater water loss than plants grown under lower humidities (Mortensen and Fjeld, 1998; Torre and Fjeld, 2001). When roses are cultivated in high relative humidity environments in greenhouses they develop large, malfunctioning stomata, similar as the malfunctioning stomata produced under *in vitro* conditions (Torre and Fjeld, 2001; Torre et al., 2003). When these plants are moved to a dryer environment the stomata are unable to close, which results in high water loss and less stress tolerant plants that quickly lose their ornamental value (Torre and Fjeld, 2001).

The shorter shelf life of plants developed under high humidity is a major problem in the greenhouse industry. One of the important challenges is therefore to find new environmental regimes that save energy, but still produce high quality and stress tolerant plants. When plants grown in high relative humidity are treated with a 6 hour low humidity period in the middle of every day, the stomata remain functional (Mortensen et al., 2007; Pettersen et al., 2007). Similarly using 18 hour light period instead of 24 hours also result in more water retention and longer shelf life in roses (Mortensen et al., 2007).

Plants grown under constant high relative humidity contain less ABA than plants grown under lower relative humidities and some of the stomata of these plants are larger and malfunctioning (Nejad and van Meeteren, 2005, 2007). One of the main hypotheses explaining the malfunctioning stomata in high humidity is development with low ABA concentrations (Nejad and van Meeteren, 2007; Okamoto et al., 2009). If the plants developed under high relative humidities are treated with ABA during development, the

stomata respond as if they were developed under lower relative humidities (Nejad and van Meeteren, 2007). In plants moved from high humidity to lower humidities regained stomatal functioning in leaves that were still actively expanding, but not in fully developed leaves (Nejad and van Meeteren, 2008). Similarly if leaves developed under high relative humidity were given ABA application, the stomatal functioning was restored in young expanding leaves, but not in fully developed leaves (Nejad and van Meeteren, 2008).These experiments implicate that ABA is involved in the development of functioning and malfunctioning stomata, although there is also contradicting results. In *Arabidopsis thaliana* it has been shown that ABA-deficient and ABA-insensitive mutants responded similarly as wild type plants to changes in humidity (Assmann et al., 2000). Plants developed under low ABA conditions also have higher stomatal density (Lake and Woodward, 2008), indicating that ABA is important in both the development of stomata size and density.
ABA application in lower concentrations, applied to plants can reduce transpiration rate and increase the shelf life of *Salvia splendens* and a number of other ornamentals, by inducing stomatal closure (Pompodakis et al., 2004; Waterland et al., 2010a; Waterland et al., 2010b; Kim and van Iersel, 2011). On the other hand, application of high ABA concentration caused early leaf abscission in *Salvia* (Kim and van Iersel, 2011). Also, ABA application decreased the shelf life of miniature potted roses (Muller et al., 1999), possibly due to high concentrations.

## 6. Conclusion

The ability of plants to be able to regulate the size of the stomatal opening is a very important mechanism to control water loss and survive. This ability is especially important during water stress, when loss of water can have serious consequences for the plants. Water stress can cause reduced growth and in severe cases plant death. To minimize the negative effects of water stress the plants respond by changing their growth pattern, producing stress proteins and chaperones, up-regulation of anti-oxidants, accumulation of compatible solutes, increasing the amount of transporters involved in water and ion uptake and transport and by closing the stomata. If the plants are unable to quickly respond to water stress, by closing the stomata and thereby conserve as much water as possible, the consequences are more severe and plants wilt and die more quickly. This is a major problem in plant propagation of ornamentals. Plants developed under high relative air humidity develop malfunctioning stomata, which are unable to close in response to water stress. When these plants are later placed in dryer conditions they quickly lose their ornamental value and wilt. Treatments with ABA or periods of high temperature or low relative air humidity during development can offset this malfunctioning and produce functioning stomata, even in high humidity.

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