Signal transduction pathway(s) in guard cells after prolonged exposure to low vapour pressure deficit

Sasan Ali niaei fard

Thesis committee

Promotor

Prof. Dr E.J. Woltering

Professor of Physiology and Quality of Fresh Produce

Wageningen University

Co-promotor

Dr U. van Meeteren

Associate professor, Horticulture and Product Physiology Group

Wageningen University

Other members

Prof. Dr T. Bisseling, Wageningen University

Prof. Dr P.C. Struik, Wageningen University

Prof. Dr M. De Proft, Catholic University Leuven, Belgium

Dr C.O. Ottosen, Aarhus University, Denmark

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Abstract

In short-term, guard cells close stomata in response to an increase in vapour pressure deficit (VPD) and they open the stomata after exposure to low VPDs. However, in long-term responses to low VPD, adaptation processes occur which make stomata less sensitive to stimuli which usually induce stomatal closure (stomatal malfunctioning). Cellular mechanism(s) leading to occurrence of stomatal malfunctioning is (are) still unknown. The aim of this project was to elucidate the processes that are involved in the malfunctioning of stomata after long-term exposure to low VPD. To elucidate whether the problem of stomatal malfunctioning is due to alterations in stomatal morphology and leaf anatomy or in the ABA signalling pathway, fava bean plants were grown at low or moderate VPDs and some plants that developed their leaves at moderate VPD were then transferred for four days to low VPD. Leaf anatomical and stomatal morphological alterations due to low VPD were not the main reason of stomatal malfunctioning in response to ABA and desiccation. Within one day exposure to low VPD, the level of foliar ABA decreased to the same level as in low VPD-grown plants, while the level of ABA-glucose ester was not affected. Spraying ABA during a 4-day exposure to low VPD maintained closing ability of the stomata after 4-day low VPD-exposure. Therefore, alteration in the signalling pathways due to low foliar ABA level was recognized as the main reason for stomatal malfunctioning after long-term low VPD-exposure. Coincidence in changes of Ca^{2+}, ABA receptors, and positive and negative regulators of ABA signalling are proposed as early steps for stomatal malfunctioning induced by low VPD-exposure. Transcriptional activators, transcriptional repressors as well as E3 ligases are proposed for long-term adaptation of cellular processes which consequently cause decreased stomatal response to closing stimuli afterwards. In order to find the molecular mechanism(s) of stomatal malfunctioning, possible variation in stomatal response to closing stimuli was studied among *Arabidopsis thaliana* accessions after a 4-day low VPD-exposure. Accessions could be grouped to very sensitive, moderately sensitive and less sensitive to closing stimuli using principle component analysis. A positive correlation was found between foliar ABA level (before desiccation) and stomatal closure response to ABA (but not to desiccation) after exposure to different VPDs. Stomatal response to desiccation was positively correlated with the foliar ABA level after desiccation. In order to elucidate the molecular network underlying stomatal malfunctioning in response to ABA due to long-term low VPD-exposure, two groups of *Arabidopsis* accessions were used as accessions that maintained responsiveness to ABA after low VPD-exposure and accessions with low VPD induced non-ABA-responsive stomata. The foliar ABA content in all accessions correlated with the stomatal response to ABA: only when the ABA level was above a threshold value, stomata responded to ABA. After low VPD-exposure, mainly due to catabolism of ABA, the foliar ABA content decreased. This decrease in ABA level resulted in down regulation of *RD29A*, which caused decreased stomatal responsiveness to ABA.

Keywords: Abscisic acid, *Arabidopsis thaliana*, calcium, *CYP707As*, desiccation, environmental factors, guard cells' signalling pathway, hydrogen peroxide, natural variation, nitric oxide, photosystem II efficiency, *RD29A*, relative water content, secondary messengers, stomata, vapour pressure deficit, *Vicia faba*

To my dear wife, Maryam, and my lovely son, Avash

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CHAPTER 1

General Introduction

Climatic conditions in both protected and open field cultivation can influence postharvest performance of horticultural products. However, when the optimum conditions are applied for the production of plants, it does not necessarily guaranty the best postharvest quality of the horticultural products. Sometimes, growing plants in certain conditions result in optimum growth of the plants, but after harvest several problems for the product occur which lead to decreased short shelf or vase life and in the worse situations lead to deterioration of horticultural products.

Regarding the quality of horticultural products, although, on the one hand studies focused on the production of high quality plant products by selecting suitable cultivars and using proper environmental and agronomical conditions in the preharvest stage, on the other hand, studies focused on the postharvest performance of products by inventing highly sophisticated storage techniques and finding the best conditions to maximize the performance of the product. Nonetheless, some studies also focused on the influence of preharvest conditions on postharvest quality (Sams, 1999; Linke and Kläring, 2004; Murray *et al.*, 2005; Hewett, 2006; Akbudak and Murat, 2012; Burchi and Prisa, 2013). The results of the previous studies highlighted the importance of pre-harvest factors such as cultivar (genetic factors), agronomic practices, climatic conditions, degree of maturity at harvest time, time of harvest, and prevalence of diseases and pests (Sams, 1999; Lee and Kader, 2000; Tijskens *et al.*, 2003; Hewett, 2006; Moretti *et al.*, 2010; Tibaldi *et al.*, 2011; Fanourakis *et al.*, 2013b; Luna *et al.*, 2013;Tudela *et al.*, 2013) and postharvest factors such as fast handling after harvest, storage conditions, packaging and processing, and transport and distribution for the postharvest quality of horticultural products (Watada *et al.*, 1996; Lee and Kader, 2000; Chiesa, 2003; Moretti *et al.*, 2010). Management of these elements is the critical point to obtain a product with maximum nutritional and visual quality. One of the important preharvest factors that influences postharvest performance of horticultural crops is relative humidity (RH) (Rezaei Nejad and van Meeteren, 2005; Islam *et al.*, 2010; Fanourakis *et al.*, 2011; Fanourakis *et al.*, 2013b). The plant structure that makes the connection between growing plant in pre-harvest to its quality performance at the postharvest stage is the stoma.

Role of stomata in plants

Stomata are pores in the epidermis consisting of a pair of kidney-shaped cells in dicots (and some monocots) or dumb-bell shaped cells in monocots (Sack, 1987). The presence of waxy cuticles at the interface between plant internal tissue and the surrounding environment, make the stomata the only openings connecting internal leaf space to outside environment. The cuticular layer protects plants from drying via its airtight properties. Guard cells regulate opening and closing of stomata to control gas and water vapour exchange between plant and the surrounding environment. The stomata provide an entry channel for CO_2 and an exit for water vapour to the environment. Main role of stomata is providing enough CO_2 for plant photosynthesis, while at the same time protecting the water status of the plant by preventing excess water loss via its opening. Excessive transpiration slows down plant growth and leads to deterioration of plants due to dehydration. However, stomata have the capacity of special adaptations to the environmental conditions to minimise water loss while promoting the acquisition of CO_2.

Leaf temperature increases directly through exposure to high temperatures or indirectly when stomata are closed. High leaf temperatures can decrease the photosynthesis or can cause damage to the components of photosynthetic apparatus (Schreiber and Berry, 1977; Wise *et al.*, 2004; Camejo *et al.*, 2005). Leaf temperature depends on air temperature and RH, absorbed net radiation, boundary layer conductance, stomatal conductance and leaf morphological traits such as presence of trichomes on the leaf (Jones, 1999; Nobel, 1999). Energy is released when water evaporates at the leaf cell to the surrounding atmosphere. The energy is required to break down the hydrogen bonds in the liquid phase of water molecules. The released energy is taken from the leaf and transferred to the water molecules and released as gas molecules. In the substomatal cavity, the water vapour pressure is in equilibrium with the apoplast fluid facing to the gas phase. When water vaporizes from the substomatal cavity a new equilibrium establishes between water vapour pressure of cavity and cells. Consequently, the water vapour and associated energy are released into the atmosphere via stomata. Therefore, transpiration of water vapour from the stomata is associated with cooling of the leaf (Hetherington and Woodward, 2003). For example, using infrared thermal imaging, it has been shown that mutants with open stomata have lower leaf surface temperature than wild-type plants (Merlot *et al.*, 2002). Therefore, proper functioning of stomata is vital for balancing leaf temperature and water loss. In *Arabidopsis* plants which had developed at high temperature (28 °C), increased water loss was associated with enhanced leaf cooling capacity. The leaves of high temperature-developed plants possess lower stomatal

density and reduced stomatal size. In this case, to cool down the leaf, plant architectural adaptions such as petiole elongation, leaf elevation and decrease in leaf thickness may enhance diffusion of water vapour from stomata (Crawford *et al.*, 2012; Murata and Mori, 2013).

As another role of stomata: it is generally accepted that transport of nutrients in plants by the transpirational flux is the main mechanism of transport of water and nutrients in plants (Mengel and Kirkby, 1982; Novák and Vidovič, 2003).

Human activity in the recent decades causes increase in the concentration of greenhouse gases such as carbon dioxide (CO_2), ozone (O_3) and sulphur dioxide (SO_2). The greenhouse gases can enter the plants mainly via the aperture of the stomata (Krupa and Manning, 1988; Mauzerall and Wang, 2001; Overmyer *et al.*, 2008; Hoshika *et al.*, 2012). Therefore, concerning the importance of stomata on global issues, more research is required to understand the influence of environmental variables on stomatal function (Roelfsema and Kollist, 2013).

Stomatal malfunctioning and problems with low vapour pressure deficit (VPD) in horticulture

RH is the ratio of the partial pressure of water vapour of the air to the saturated water vapor pressure, expressed in percentages. Air saturates when it holds the water with its maximum capacity which depends on temperature. More moisture beyond this capacity would lead to condensation of water vapour molecules to water. By increasing the temperature, the maximum water holding capacity of the air increases. Therefore, the RH depends on water vapour pressure of the air and air temperature. Vapour pressure deficit (VPD) considers the effect of temperature on the water holding capacity of the air as well. Therefore, VPD is the combination of RH and temperature and defined as the difference between the saturation water vapour pressure and the actual water vapour pressure at a certain temperature. VPD is the driving force for plant transpiration.

Plants that have been produced under low VPD conditions grow often normally, but after harvest the stomata cannot function in a normal way. In this situation, due to the prolonged exposure to low VPD, a habituation process occurred in guard cells of the stomata. As a result stomata stay open after harvest of the plants which makes the stomata insensitive to stimuli that would normally provoke stomatal closure (stomatal malfunctioning). This disturbance in the normal functioning of the stomata due to previous exposure to low VPD has horticultural consequences. For example, growing rose plants at low VPD conditions often results in a

decrease in vase life after harvest of the plants (Fanourakis *et al.*, 2013b). Low VPD-grown plants often have a higher rate of water loss than the moderate VPD-grown plants, also during desiccation or hampered water uptake (postharvest stage) or when they are exposed to high VPDs (Rezaei Nejad *et al.*, 2006; Rezaei Nejad and van Meeteren, 2005; Fanourakis *et al.*, 2013a). Such a transfer from low to high VPD is common after vegetative propagation by leafy cuttings, after *in vitro* propagation and at the end of the cultivation period of ornamentals when plants or cut flowers are transferred to domestic conditions. Although plants can be cultured *in vitro* in large scale under low VPD conditions, *in vitro*-produced plants are usually susceptible to wilting upon transfer to normal atmospheric VPDs (Brainerd and Fuchigami, 1982; Ghashghaie *et al.*, 1992; Santamaria *et al.*, 1993; Aguilar *et al.*, 2000; Hronková *et al.*, 2003; Hazarika, 2006; Aracama *et al.*, 2008; Khan *et al.*, 2010). This is also because of malfunctioning of the stomata in response to a wide range of closing stimuli such as darkness, abscisic acid (ABA) and elevated calcium (Ca^{2+}) levels (Brainerd and Fuchigami, 1982; Ziv *et al.*, 1987; Santamaria *et al.*, 1993). It has been shown that higher rates of water loss after desiccation in the plants grown at low VPD conditions is mainly caused by stomata compared to the role of the cuticle (Ziv *et al.*, 1987; Santamaria and Kerstiens, 1994; Fanourakis *et al.*, 2013a).

Cultivation at low VPD not only influences the vase life and visual appearance of cut flowers, it can also influence the postharvest life and nutritional quality of leafy vegetables via the stomata. Higher rate of water loss after harvest of plant leaves was observed in basil (*Ocimum basilicum* L.) and lemon balm (*Melissa officinalis* L.) as a result of exposure during cultivation to low VPD compared with plants grown at higher VPDs (Islam *et al.*, 2010). Postharvest life was negatively correlated to the rate of water loss via stomata after harvest of the plants (Islam *et al.*, 2010). Uncontrolled water loss by leaves due to malfunctioning of stomata after production of vegetables at low VPD conditions resulted in declined nutritional quality such as decreased vitamin C content of the leaf (Ezell and Wilcox, 1959; Lee and Kader, 2000). Leaves of plants that lose their moisture easer are more vulnerable to lose of vitamin C compared to plants that are resistant to wilting (Ezell and Wilcox, 1959; Lee and Kader, 2000).

It can be concluded that production of flowers and vegetables in low VPD renders the stomata incapable of suitable response to closing stimuli afterwards, which results in a negative water balance of the leaf and flower after harvest: in the flowers their rate of water uptake becomes lower than their transpiration rate and leafy vegetables have a reduced capacity to keep water after harvest, resulting in decreased relative water content and water potential and

consequently wilting which reduces postharvest life and the nutritional quality of the horticultural products.

Mechanism of stomatal closure and opening

Movements of the stomata depend on many factors including environmental factors such as light, temperature and RH (VPD), CO_2 concentrations, water availability, pathogens, etc., and endogenous factors such as phytohormones and their interactions and secondary messengers. Stomatal aperture changes over diurnal cycles. To facilitate CO_2 assimilation, stomata stay open during the day especially in response to blue light and tend to be closed at night (Talbott and Zeiger, 1998; Schroeder *et al.*, 2001; Tallman, 2004). However, to conserve water, crassulacean acid metabolism (CAM) plants close their stomata during the daytime and open at night to take up CO_2 (Bohnert *et al.*, 1995; Black and Osmond, 2005).

Stomatal opening and closing are controlled by guard cells swelling and shrinking, respectively. Stomatal opening is initiated by extrusion of H^+ from guard cell membrane through H^+-ATPases. H^+ extrusion induces plasma membrane hyperpolarization and apoplast acidification. The voltage gradient activates the inward-rectifying K^+ channels. Influx of K^+ together with Cl^- and inorganic solutes such as malate through guard cells membrane enhances guard cells osmotic potential, therefore water pumped into the guard cells causes swelling of the guard cells and as a result stomatal opening occurs. While, stomatal closing is initiated by inhibition of the H^+-ATPases which depolarize the plasma membrane. Following depolarization, outwardly rectifying K^+-channels enhance the driving force for K^+ efflux and decrease the K^+ level inside the guard cells. Efflux of K^+ and Cl^- ions or malate through guard cells membrane decreases osmotic potential, as a result water exit from the guard cells which causes shrinking of them, resulting in stomatal closure (Blatt, 2000; Schroeder *et al.*, 2001; Outlaw, 2003).

Plants dynamically respond to changes in environmental conditions by regulating the aperture of the stomata. Plant responses to environmental stresses (e.g. drought) are usually associated with induction of abscisic acid (ABA) production. ABA through its signal transduction pathway causes stomatal closure (Hu *et al.*, 2006; Endo *et al.*, 2008; Lee and Luan, 2012; Sreenivasulu *et al.*, 2012).

Change of ABA level by low VPD

Levels of endogenous ABA [ABA] are altered dynamically in response to environmental conditions. It has been known that exposure to different VPDs also influences [ABA] (Rezaei

Nejad and Van Meeteren, 2007, 2008; Okamoto *et al.*, 2009; Arve *et al.*, 2012). In many plant species such as spinach (*Spinacia oleracea*) (Zeevaart, 1974), rose (Arve *et al.*, 2012; Giday *et al.*, 2013a), *Arabidopsis* (Okamoto *et al.*, 2009) and spiderwort (*Tradescantia virginiana*) (Rezaei Nejad and Van Meeteren, 2007, 2008) foliar [ABA] decreases as a result of exposure to low VPD conditions. In *Tradescantia*, one day after transferring moderate VPD-grown plants to a low VPD condition, foliar [ABA] decreased to the ABA level found in plants grown at low VPD. Reciprocal transfer of moderate VPD-grown plants from low to moderate VPD, increased foliar [ABA] again to its level in moderate VPD-grown plants (Rezaei Nejad and Van Meeteren, 2008). In rose plants, contrary to moderate VPD-grown plants, exposure of low VPD-grown plants to darkness did not result in elevation of foliar [ABA] (Arve *et al.*, 2012). In *Arabidopsis* the [ABA] decreases sharply even one hour after exposure to a low VPD condition (Okamoto *et al.*, 2009). Moreover, *in vitro*-propagated plants which were produced under low VPD conditions were deficient in ABA (Hronková *et al.*, 2003). Therefore, in the absence of stresses, the foliar [ABA] depends on the VPD as well; decreasing the VPD will result in decrease in [ABA]. Although, it has been reported that foliar [ABA] underlies genotypic variation in stomatal responsiveness of rose cultivars after growth at low VPD (Giday *et al.*, 2013a), it is still unclear if foliar [ABA] is the only determinant for the response of stomata of low VPD-exposed plants to different closing stimuli such as desiccation or exogenous ABA application.

ABA production and degradation

Stomata react very fast to changes in the environmental conditions through internal signals (Martin and Meidner, 1971; Wigger *et al.*, 2002; Tallman, 2004; Neill *et al.*, 2008; Kim *et al.*, 2010; Hao *et al.*, 2011; Hossain *et al.*, 2011). In natural conditions, plants are continuously encountered to changes in the environment. Therefore, in order to survive, they should have the ability to react fast to the changing environment accordingly. ABA acts as an internal signal in response to changes in environmental conditions and triggers changes in various plant physiological and developmental processes, which results in adaptation to the stress conditions (Aguilar *et al.*, 2000; Chen *et al.*, 2010; Lee and Luan, 2012). In general, ABA level is regulated by the balance between its biosynthesis and its catabolism (Nambara and Marion-Poll, 2005; Lee and Luan, 2012). The first step in the specific ABA production pathway is the synthesis of violaxanthin through zeaxanthin epoxidase. Neoxanthin synthase and an isomerase may be required for formation of cis-isomers of violaxanthin and neoxanthin. 9-cis-epoxycarotenoid dioxygenases (NCED) cleave the cis-xanthophylls to yield

xanthoxin. The *NCED* genes encode key enzymes for ABA biosynthesis. It has been shown that NCED is the rate-limiting factor in the ABA biosynthetic pathway (Qin and Zeevaart, 1999; Iuchi *et al.*, 2001; Tan *et al.*, 2003). Through a short-chain alcohol dehydrogenase (ABA2), xanthoxin is then converted to abscisic aldehyde. Finally, abscisic aldehyde oxidase (AAO3) catalyses the last step of ABA biosynthesis by oxidation of abscisic aldehyde into ABA (Nambara and Marion-Poll, 2005).

However, the place of ABA production and the place of its action is still under debate (Christmann *et al.*, 2005; Davies *et al.*, 2005; Endo *et al.*, 2008; Jiang and Hartung, 2008; Melhorn *et al.*, 2008). Following drought stress, ABA acts as a signal between roots and shoots (Holbrook *et al.*, 2002; Davies *et al.*, 2005; Jiang and Hartung, 2008). It has been indicated that synthesis of ABA in the shoot is a response to a long-distance hydraulic signal in xylem vessels due to low water potential in the soil (Holbrook *et al.*, 2002; Christmann *et al.*, 2005; Christmann *et al.*, 2007; Christmann *et al.*, 2013). Nonetheless, it has been shown that *NCED3* is mainly expressed and localized in vascular parenchyma of leaves (Cheng *et al.*, 2002; Endo *et al.*, 2008). The expression of *AAO3* in the guard cells has also been reported (Koiwai *et al.*, 2004; Nambara and Marion-Poll, 2005; Melhorn *et al.*, 2008). Moreover, transient expression of *NCED3* or *AAO3* in guard cells promote stomatal closure, suggesting the possibility of ABA synthesis by guard cells (Melhorn *et al.*, 2008). Bauer *et al.* (2012) showed that guard cells possess the entire ABA biosynthetic pathway. Guard cells are able to autonomously synthesize ABA and there is a positive feedback loop for ABA production when they have been exposed to high VPDs around the leaves (Bauer *et al.*, 2012). It has been suggested that foliar ABA production is capable of inducing stomatal closure and influencing ABA signalling and there is no need for root–shoot transport of ABA (Osakabe *et al.*, 2013). Moreover, increasing VPD around the leaves of well-watered plants resulted in a higher ABA level in the leaf (Rezaei Nejad and Van Meeteren, 2007, 2008).

ABA is inactivated mainly through oxidation or conjugation processes. Hydroxylation of ABA is the main process for ABA inactivation (Nambara and Marion-Poll, 2005). Oxidation of ABA is catalysed by 8'-hydroxylases to form 8'-hydroxy ABA. In the next step, 8'-hydroxy ABA spontaneously isomerizes to phaseic acid (PA), and is further reduced to dihydrophaseic acid (DPA) through an unknown reductase (Krochko *et al.*, 1998; Cutler and Krochko, 1999). Similar to PA, Neophaseic acid (neoPA) can be formed from hydroxy ABA through isomerization (Zhou *et al.*, 2004). ABA 8'-hydroxylases are the members of CYP707A subfamily of cytochrome P450 monooxygenases (Kushiro *et al.*, 2004; Saito *et al.*,

2004). It has been reported that exposure of plants to low VPD induces catabolism of ABA via *CYP707A*s genes (Okamoto *et al.*, 2009).

Apart from the oxidative catabolic pathways, the ABA can be inactivated via conjugation with glucose to form its glucose ester (ABA-GE) (Xu *et al.*, 2002; Priest *et al.*, 2006). ABA-GE is readily reversible but not easily permeable through biomembranes and may function as a realizable (storage form) and transportable form of ABA (Dietz *et al.*, 2000; Sauter *et al.*, 2002). When ABA is needed, ABA-GE is hydroxylated through β-glucosidase to increase the active form of ABA (Lee *et al.*, 2006). The activity of β-glucosidase was decreased in rose plants which had been grown in low VPD compared with its activity in moderate VPD-grown plants. This resulted in higher ratio of ABA-GE to ABA (Arve *et al.*, 2012). However, it is still not clear whether the lower ABA level after long-term exposure to low VPD is due to lower production or due to higher catabolism of ABA.

Signal transduction pathways in guard cells for stomatal closure

Guard cells perceive multiple signals from the environment and integrate them to internal signals and, by following complex transduction pathways respond to them by regulating stomatal aperture in order to adapt to the environment (Bohnert *et al.*, 1995; Qin and Zeevaart, 2002; Lebaudy *et al.*, 2008; Oh *et al.*, 2009; Kim *et al.*, 2010; Trontin *et al.*, 2011; Lee and Luan, 2012; Zhu *et al.*, 2012; Christmann *et al.*, 2013; Kuromori *et al.*, 2014).

Over the past several years, many internal signals have been recognized in guard cells in response to different environmental signals. For example calcium, reactive oxygen species (ROS), phosphatidic acid, cyclic guanosine 3', 5'-monophosphate (cGMP), nitric oxide and pH has been recognized as essential signals mediated in stomatal closure (Suhita *et al.*, 2004; Li *et al.*, 2006; Wang and Song, 2008; Xue *et al.*, 2009; Kim *et al.*, 2010; Dubovskaya *et al.*, 2011; Stael *et al.*, 2011). Although an ABA-independent pathway for closure of the stomata has also been proposed (Yoshida *et al.*, 2006; Huang *et al.*, 2009; Montillet *et al.*, 2013; Roychoudhury *et al.*, 2013), ABA is considered to be the main phytohormone that promotes stomatal closure, which helps to minimize water loss by decreasing transpiration via stomata. This function of ABA is accomplished through modulating a complex and sophisticated cascade of biochemical and molecular events (Hauser *et al.*, 2011). Two ABA-inducible *RD29* (Responsive to Desiccation) genes, *RD29A* and *RD29B*, are induced by abiotic stresses such as drought and salinity. *RD29B* functions in an ABA-dependent pathway, while *RD29A* functions in both ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki et al., 1995; Narusaka et al., 2003; Kasuga et al., 2004; Hua et al., 2006; Ma et al., 2010). The

role of ABA-inducible *RD29* genes in stomatal closure is largely unknown. Almost all of the previous mentioned internal signals are involved in guard cells' ABA signalling pathway for closure of the stomata (Leung *et al.*, 1997; Kwak *et al.*, 2002; Suhita *et al.*, 2004; Li *et al.*, 2006; Zhu *et al.*, 2007; Neill *et al.*, 2008; Wang and Song, 2008; Hubbard *et al.*, 2010; Kim *et al.*, 2010; Dubovskaya *et al.*, 2011; Joshi-Saha *et al.*, 2011; Hossain *et al.*, 2011; Hubbard *et al.*, 2012). The ABA-induced stomatal closure is often associated with an increase in guard cells calcium concentration. However, calcium-dependent and calcium-independent ABA signalling pathways have been suggested for ABA-induced stomatal closure (Fig. 1) (MacRobbie, 1990; Li and Assmann, 1996; Levchenko *et al.*, 2005; Marten *et al.*, 2007; Sutter *et al.*, 2007; Geiger *et al.*, 2009; Siegel *et al.*, 2009; Geiger *et al.*, 2010; Joshi-Saha *et al.*, 2011).

To initiate ABA signal transduction, guard cells are equipped with ABA receptors to bind to ABA (Moes *et al.*, 2008; Fujita *et al.*, 2009; Ma *et al.*, 2009; Santiago *et al.*, 2009; Cutler *et al.*, 2010; Raghavendra *et al.*, 2010; Lee *et al.*, 2013). Binding of ABA with its receptors inhibits the activity of group A protein phosphatase 2C (PP2C) (Moes *et al.*, 2008; Fujita *et al.*, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; Santiago *et al.*, 2009; Raghavendra *et al.*, 2010). OST1 is an *Arabidopsis* SnRK2-type protein kinase that, together with several other SnRK2-type protein kinases, is also known to function in ABA responses (Yoshida *et al.*, 2002; Yoshida *et al.*, 2006; Belin *et al.*, 2006; Fujita *et al.*, 2009; Lee *et al.*, 2013). In contrast to A-type PP2Cs, SnRK2-type protein kinases are positive regulators of ABA signalling (Fujii *et al.*, 2009; Fujii and Zhu, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Lee and Luan, 2012).

Downstream of ABA receptors, PP2Cs, and SnRKs are ion channels that control stomatal movements (Fujii *et al.*, 2009; Geiger *et al.*, 2009; Lee *et al.*, 2009). The guard cell slow-type anion channel (SLAC1), may represent an essential component for stomatal closure induced by ABA or other signals (Negi *et al.*, 2008; Vahisalu *et al.*, 2008; Geiger *et al.*, 2009; Vahisalu *et al.*, 2010). SLAC1 acts as a substrate for and is activated by OST1 (Geiger *et al.*, 2009; Lee *et al.*, 2009; Lee *et al.*, 2013).

Calcium dependent protein kinases (CDPK's) function as essential elements of the calcium-dependent ABA signalling (Zhu *et al.*, 2007). It has been shown that SLAC1 can be activated by CDPK's, which leads to stomatal closure (Mori *et al.*, 2006; Geiger *et al.*, 2010) (Fig. 1).

However, what happens with guard cells' ABA signalling pathway after long-term exposure to low VPD is still fully unknown.

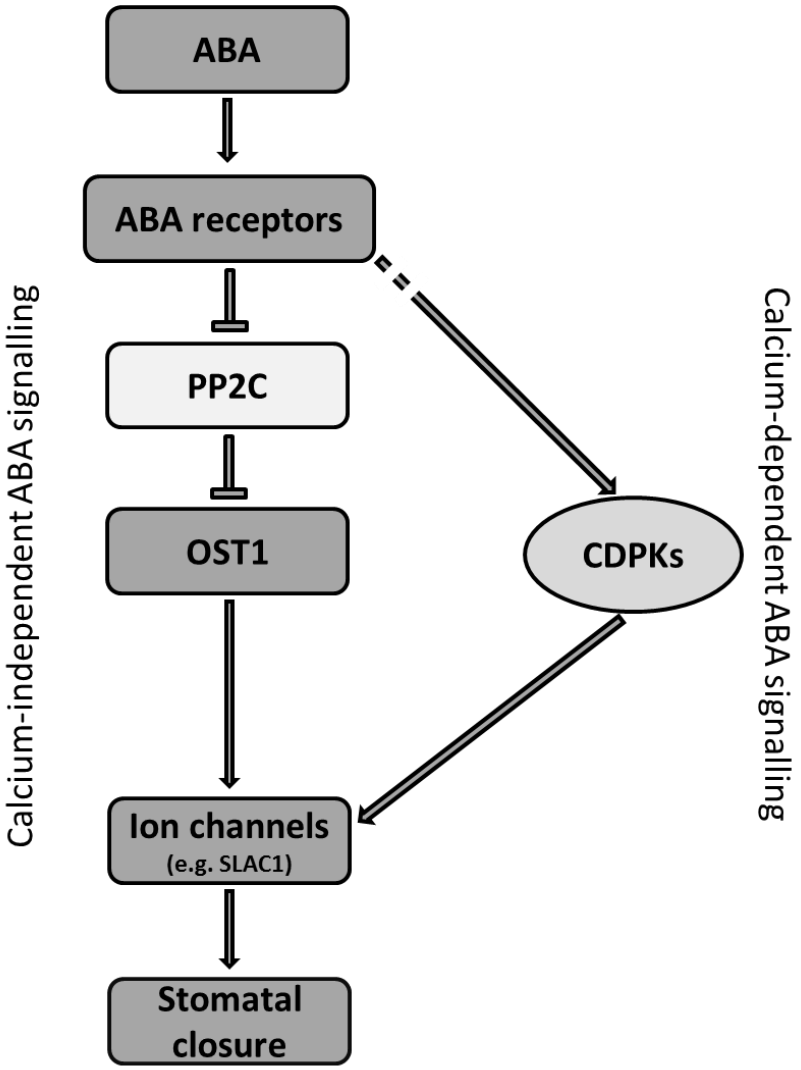

Fig 1. Simplified ABA signal transduction pathway in guard cells for closure of the stomata. In calcium-independent ABA signalling pathway, perception of ABA by receptors leads to inactivation of type-2C protein phosphatases (PP2C), as a result S-type anion channels (SLAC1) will be activated by SnRK2-type protein kinase (OST1). Consequently, stomatal closure occurs. In the calcium-dependent ABA signalling pathway, calcium dependent protein kinases (CDPK's) via activation of SLAC1 can induce stomatal closure (Hubbard *et al.*, 2010; Kim *et al.*, 2010; Antoni *et al.*, 2011; Sreenivasulu *et al.*, 2012; Lee *et al.*, 2013).

Induction of stomata morphological changes by low VPD

Stomatal size and density can be influenced by VPD (Fordham *et al.*, 2001; Torre *et al.*, 2003; Tricker *et al.*, 2012; Fanourakis *et al.*, 2013a). Plants which developed their leaves in low VPD conditions are characterized by large stomata and wide aperture area (Torre *et al.*, 2003; Rezaei Nejad and van Meeteren, 2005; Fanourakis *et al.*, 2011; Fanourakis *et al.*, 2013a). Since, in comparison with moderate VPD-grown plants, the stomatal closing ability of low VPD-grown plants decreased in response to water deficit, a question arises: are stomatal

morphological alterations involved in the decreased stomatal closing ability of low VPD-grown plants?

A connection between stomatal function and structural features for various species has been previously suggested (Franks and Farquhar, 2007; Doheny-Adams *et al.*, 2012; Drake *et al.*, 2013; Giday *et al.*, 2013b). Because of higher ratio of guard cell's membrane surface to volume, species with smaller stomata may respond faster compared with species with larger stomata. On the other hand, smaller stomata are usually associated with higher stomatal density per leaf area (Hetherington and Woodward, 2003). To optimize the trade-off between carbon gain and transpirational water loss, these characteristics (smaller stomata and higher stomatal density) allow the leaf to attain high stomatal conductance under favourable conditions, and to promptly reduce stomatal conductance when conditions are unfavourable, which help the plant to cope with stress conditions (Xu and Zhou, 2008; Doheny-Adams *et al.*, 2012). Using five closely related species of the genus *Banksia*, it has been demonstrated that the rate of stomatal response was negatively correlated with stomatal size (Drake *et al.*, 2013).

Similar to low VPD, decreased ABA levels as well as absence of changes in ABA level during leaf developments of *in vitro* plants may result in alterations in wall structures of guard cells (Mansfield, 1994). Stomata of *in vitro* plants are usually large and their guard cells have thinner cell walls (Marin *et al.*, 1988). It has been suggested that as a consequence of this structural alterations in guard cells, the stomata of *in vitro* plants cannot close in response to water deficit (Mansfield, 1994). It has been indicated that smaller stomata require less leaf drying to close and that plants stomatal size underlays much of the variation in the regulation of transpiration upon desiccation after growth of the plants in low and moderate VPDs (Franks and Farquhar, 2007; Doheny-Adams *et al.*, 2012; Drake *et al.*, 2013; Giday *et al.*, 2013b). On the other hand, it has been shown that within one species longer stomata is not the key factor for reduced hyposensitivity in low VPD-grown plants (Fanourakis *et al.*, 2013a).

It is not still clear whether the decreased stomatal closing ability of low-VPD grown plants is a physical process due to alterations in stomatal morphology or that it is related to alterations in the guard cell signalling pathways.

Scope and outline of the thesis

The main aim of this project was to understand the disturbed stomatal closing mechanism in plants that have been exposed for long-term to low VPD. Fava bean and *Arabidopsis* were used for the experiments. Fava bean was used because: (i) it emerges as a model plant for stomatal research, (ii) ease of cultivation and growing, (iii) the large size of its stomata. *Arabidopsis* was used since: (i) it is the model plant for investigating cellular and molecular processes, (ii) it has a wide number of accessions from different places which make it suitable to study the natural variation for different aspects of plant responses, (iii) the availability of a large number of plants with modified gene activity.

In order to identify the reasons for decreased closing ability of stomata in long-term low VPD-exposed plants, it was important to determine whether the decreased stomatal closing ability in long-term low VPD-exposed plants is because of changes in the signalling pathway or because of morphological alterations. **Chapter 2** describes the stomata morphological and leaf anatomical alterations due to growth at low VPD and during a 4-day exposure to low VPD. In addition, stomatal responses of low VPD-grown or low VPD-exposed plants were investigated in response to ABA and desiccation. The results of this chapter exclude involvement of the stomata morphological and leaf anatomical alterations in the occurrence of stomatal malfunctioning after exposure to low VPD and it highlights the involvements of signalling alterations in the occurrence of stomatal malfunctioning due to prior exposure to low VPD.

Chapter 3 describes a literature study of stomatal malfunctioning by long-term exposure of plants to several environmental factors such as low VPD. The magnitude of induced-stomatal malfunctioning by long-term exposure to environmental factors such as ozone and continuous light is more pronounced when the exposure is accompanied by low VPD. In this chapter alterations in the signalling pathway of ABA and secondary messengers such as calcium and reactive oxygen species are discussed.

Chapter 4 assesses the natural variation in the stomatal responses of 41 natural accessions of *Arabidopsis thaliana* to ABA and to desiccation after long-term exposure to low VPD. It was found that the *Arabidopsis* accessions can be categorized into 3 different groups according to their stomatal responses to ABA and desiccation.

Chapter 5 investigates the transcript levels of candidate genes which are important in production, catabolism, perception and signaling of ABA or in signaling of secondary massengers or ethylene in three accessions that belong to the three groups which were identified in chapter 4. The transcript levels of candidate genes were investigated not only in

moderate and low VPD-exposed plants, but also in daily ABA-sprayed low VPD-exposed plants to have more indication for involved genes in the malfunctioning of stomata after prior exposure to low VPD. It highlights the importance of an ABA responsive gene and of genes involved in catabolism of ABA in the occurence of stomatal malfunctioning after exposure to low VPD. Also a threshold level of ABA in the leaf was found to be important in order to have responsive stomata to ABA after prior exposure to low VPD.

Chapter 6 is the general discussion. The main findings of the previous chapters are combined and discussed in this chapter. The main reasons for occurrence of stomatal malfunctioning after exposure to low VPD and the areas that require further research are highlighted.

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CHAPTER 2

Stomatal malfunctioning under low VPD conditions: Induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling?

Abstract

Exposing plants to low VPD reduces leaf capacity to maintain adequate water status thereafter. To find the impact of VPD on functioning of stomata, stomatal morphology and leaf anatomy, fava bean plants were grown at low (L, 0.23 kPa) or moderate (M, 1.17 kPa) VPDs and some plants that developed their leaves at moderate VPD were then transferred for four days to low VPD (M→L). Part of the M→L-plants were sprayed with ABA during exposure to L. L-plants showed bigger stomata, larger pore area, thinner leaves and less spongy cells compared with M-plants. Stomatal morphology (except aperture) and leaf anatomy of the M→L-plants were almost similar to the M- plants, while their transpiration rate and stomatal conductance were identical to that of L-plants. The stomatal response to ABA was lost in L-plants, but also after 1-day exposure of M-plants to low VPD. The level of foliar ABA sharply decreased within 1-day exposure to L, while the level of ABA-GE was not affected. Spraying ABA during the exposure to L prevented loss of stomatal closing response thereafter. The effect of low VPD was largely depending on exposure time: the stomatal responsiveness to ABA was lost after 1-day exposure to low VPD, while the responsiveness to desiccation was gradually lost during 4-days exposure to low VPD. Leaf anatomical and stomatal morphological alterations due to low VPD were not the main cause of loss of stomatal closure response to closing stimuli.

Abbreviations – ABA, Abscisic acid; VPD, vapour pressure deficit; RH, relative humidity; SLA, specific leaf area; g_s, Stomatal conductance; E, transpiration rate; RWC, Relative water content; Φ_{PSII}, relative quantum yield or efficiency for electron transport by photosystem II; PSII, photosystem II.

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Introduction

Regulation of stomatal aperture in leaves is crucial for CO_2 import, a substrate of photosynthesis, and at the same time decisive to prevent excessive water loss through transpiration. Guard cells continuously sense signals from the environment and respond via their turgor pressure changes; these changes result in stomatal opening or closure (Schroeder et al. 2001a, 2001b, Kim et al. 2010, Monda et al. 2011). In general stomata close in response to closing-stimuli such as drought and ABA. It is known for a long time that stomata respond rapidly to air humidity, resulting in higher stomatal conductance at low vapour pressure deficit (VPD) (Hall et al. 1975, Morison and Gifford 1983). The fine regulation of stomatal movements can be influenced by preceding environmental conditions. As example, plants that were grown at low VPD had thereafter a reduced capacity to control water loss in response to high VPD (Rezaei Nejad and van Meeteren 2005). As a result, plants frequently shrivel and die when a condition of water stress follows upon the growth of plants at low VPD conditions. Although the mechanisms involved in the stomatal movements usually provide a robust and fault-tolerant system, this control system can be disturbed under certain environmental conditions, leading to a reduced closing capacity of stomata in response to stimuli that usually induce stomatal closure (Aliniaeifard and van Meeteren 2013). Reduced closing ability of stomata has been shown in plants produced *in vitro* (Brainerd and Fuchigami 1982, Ziv et al. 1987, Santamaria et al. 1993, Hazarika 2006), after prolonged exposure to some environmental pollutants such as ozone, sulphur dioxide and hydrogen sulphide (Maier-Maercker and Koch 1986, Wilkinson and Davies 2009, Paoletti 2005, Lisjak et al. 2010, Aliniaeifard and van Meeteren 2013), after growing plants under continuous light (Slootweg and van Meeteren 1991, Mortensen and Gislerød 1999, Pettersen et al. 2007, Arve et al. 2012), or after growing at low VPD (Torre and Fjeld 2001, Rezaei Nejad et al. 2006, Rezaei Nejad and van Meeteren 2005, 2007, 2008, Fanourakis et al. 2011, Arve et al. 2012, Aliniaeifard and van Meeteren 2013). From these factors, low VPD showed the strongest negative effect on the stomatal closing response and the magnitude of stomatal malfunctioning induced by the other above mentioned environmental factors is more pronounced when these are applied together with low VPD (Aliniaeifard and van Meeteren 2013). It is astonishing that a single environmental condition, like low VPD, can influence the robust network of stomata control.

Alteration in leaf morphology due to growth of plants at low VPD conditions was previously reported for roses. Torre et al. (2003) showed that growing plants at low VPD caused alterations in some of the leaf anatomical and stomata morphological traits. During

development of plants stomatal size and density can be influenced by VPD (Fordham et al. 2001, Torre et al. 2003, Tricker et al. 2012, Fanourakis et al. 2013). Dependency of stomatal function on structural features has been recently reported (Doheny-Adams et al. 2012, Giday et al. 2013b). Torre et al. (2003) concluded that the weak ability for controlling water loss in low VPD-grown rose plants is due to increased stomatal density and size. It has been shown in rose plants that it is the development of the leaves at low VPD that determines stomatal malfunctioning to occur (Fanourakis et al. 2011). However, in *Tradescantia virginiana*, already after four days exposure to low VPD of leaves that were full grown at moderate VPD, the stomata were not responsive anymore to closing stimuli (Rezaei Nejad and van Meeteren 2008).

Production, hydroxylation and inactivation of ABA can be influenced by VPD (Kushiro 2004, Okamoto et al. 2009, Arve et al. 2012). In response to changing environmental conditions, ABA-glucose ester (ABA-GE) functions as the main conjugate form of ABA. It provides a releasable pool of ABA during water stress (Lee et al. 2006). Arve et al. (2012) showed that in rose plants, the level of ABA-GE was increased in plants that were grown at low VPD conditions, indicating that conjugation was involved in the decreased ABA levels induced by low VPD. However, in *Arabidopsis thaliana* time course analysis of ABA-GE during exposure to low VPD for 1 hour did not show a significant change in the ABA-GE level. It is unknown what would happen to the ratio of ABA and ABA-GE levels during few days exposure to low VPD.

The current study was carried out in order to find whether leaf anatomical alterations or stomatal morphological changes due to exposure to low VPD are involved in the reduced ability of the stomata to respond to closing stimuli. The aims of the study were to evaluate (i) the stomatal response of fava bean (*Vicia faba* L.) to moderate and low VPD conditions, (ii) whether changes in the stomatal density are involved in the reduced ability of leaves to control water loss, (iii) the contribution of stomata morphological changes to the malfunctioning of stomata, and (iv) the involvement of leaf anatomical changes in the stomatal response of low and moderate VPD exposed leaves. For that reason plants were grown at low (L, 0.23 kPa) or moderate (M, 1.17 kPa) VPDs, but also some plants, that developed their leaves at moderate VPD, were then transferred for four days to low VPD (M→L). Besides anatomical and morphological features, stomatal response to ABA and desiccation was tested after 1, 2, 3, or 4 days or continues exposure to low VPD.

Material and methods

Plant material and growth conditions

Fava bean (*Vicia faba* L. cv Longpod) plants were grown in 15 cm diameter plastic pots containing commercial potting compost (Potgrond 4, Hortimea, Lent, the Netherlands) in two growth chambers with different VPD conditions. One of them with 20±1 °C temperature, 55±5% relative humidity (RH), resulting in a VPD of 1.05 kPa [moderate VPD (M)]. Another one with 20±1 °C temperature, 90±5% RH, resulting in a VPD of 0.23 kPa [low VPD (L)]. The light intensity in the chambers was 300 µmol m^{-2} s^{-1} (measured with an LI-250 light meter, Li-Cor, Lincoln, NE, USA) produced by fluorescent tubes (TLD 58W/84 Philips), the lighting period was 12h/12h day night cycle; 380 µmol mol^{-1} CO_2 (determined using Indoor Air Quality Meter, Model 8760, TSI Incorporated, Shoreview, USA) was kept in the chambers. Temperature and RH in the growth chambers were automatically recorded every 5 min using data loggers (Fourier MicroLog EC650, MicroDAQ.com, Ltd. Contoocook, New Hampshire, USA).

To investigate the involvement of leaf anatomical and stomatal morphological changes on the stomatal closing ability, 4 weeks after germination, some of the plants which were grown in moderate VPD growth chambers, were transferred to low VPD growth chambers with conditions as described before (M→L). After four days of exposure to low VPD conditions, leaves were used for analysing the response of stomata to closing stimuli. For measuring specific leaf area (SLA), leaf area from plants of different treatments were measured and then the leaves dried at 80 ºC. For all measurements fully developed leaves (the fourth and fifth leaves in acropetal order) were used.

Stomatal conductance and transpiration rate

Stomatal conductance (g_s) and transpiration rate (E) were recorded using a porometer (Delta-T Devices Ltd, Cambridge, UK) in an environment with a 20 °C temperature, 55% RH and 300 µmol m^{-2} s^{-1} illumination. In Fig. 1 g_s was measured at 1.40 kPa VPD and 35 µmol m^{-2} s^{-1} irradiance.

Mapping of PSII photochemical efficiency using chlorophyll fluorescence

For analysing the stomatal response of plants to ABA feeding, chlorophyll fluorescence imaging under non-photorespiratory condition was used as described by Rezaei Nejad et al. (2006). The petiole of the leaves were cut under water, placed in 2 ml eppendorf vials containing 25 mM KCl, 5 mM MES-KOH, pH 6.15, 25 µM $CaCl_2$ and placed in a flow-

through cuvette. The temperature in the cuvette was 22±1 °C. The cuvette was placed under a chlorophyll fluorescence imaging system (FuorCam 700MF, PSI, Brno, Czech republic). The imaging measurement was conducted with an atmosphere with 20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and the rest N_2 (non-photorespiratory condition) in the cuvette. The RH was set to 40±3% via passing the gas mixture through a temperature-controlled column of iron (II)-sulphate heptahydrate (Fluka St. Gallen, Switzerland). The leaf that was placed in the cuvette was exposed to a continuous irradiance of 100 µmol m^{-2} s^{-1}. Once reaching the steady state, an image of photosystem II efficiency (Φ_{PSII}) was taken from leaves in water. Then the vial was replaced by another vial with the same volume of an ABA solution (100 µM ABA). Every 30 min the protocol for the FluorCam run and images were taken for 150 min. The average value of Φ_{PSII} per leaf was calculated by using version 5 of FluorCam software. Values for F_t and F_m' in the generated image were averaged over all pixels per leaf and then the Φ_{PSII} was calculated using the ratio of the difference between (F_m'-F_t) and F_m'. To investigate if all stomata of one leaf responded similarly, frequency distributions were analysed by using the individual values for F_t and F_m' in the generated image. To ensure that the decreased Φ_{PSII} was due to stomatal closure, at the end of imaging Φ_{PSII} for each treatment an image was taken in an atmosphere with a high CO_2 concentration (20 mmol mol^{-1} O_2, 50000 µmol mol^{-1} CO_2) to test the recovery of Φ_{PSII}.

ABA extraction and quantification

For determination of ABA and ABA-GE, leaves were excised from the plants and were ground in a mortar using liquid nitrogen. One leaf of four plants per treatment were used as four repetitions. The samples (around 0.2 g of ground leaf material) were extracted with one ml of cold ethyl acetate containing [$^2H_6$]-ABA as internal standard to have 0.1 nmol internal standard in the extraction. The samples were vortexed (1 min), then sonicated (15 min) in a Branson 3510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA). Samples were centrifuged for 10 min at 450 g in an MSE Mistral 2000 centrifuge (Mistral Instruments, Leicester, UK). The supernatant was carefully transferred to a 4-ml glass vial. The pellets were re-extracted with 1 ml of methanol without sonication. The solvent fractions were pooled in a 4-ml glass vial. Then the samples were dried using a speedvac and the residue was dissolved with 50 µl methanol. 3 ml MQ water was added to the samples and the extracts were purified using 500 mg C18 columns. The samples were eluted with 1 ml acetone. Then the acetone was evaporated under N_2. The residue was dissolved with 200 µl of acetonitrile:water:formic acid (10:90:0.1, v:v:v). Samples were filtered into vials with

Minisart 0.2 µm filters (Sartorius, Goettingen, Germany) and were used for LC-MS ⁄MS analysis according to López-Ráez et al. (2010).

Stomatal response to desiccation

To study the effect of desiccation on leaf transpiration rate, leaves (3-6 repetitions) were detached after one, two, three and four days exposure to low VPD and an image was taken to measure the leaf area. Leaves of the same age were also taken from fully L and M grown plants. After cutting, the leaf was placed with its petiole in tap water for one h at 21 °C, 100% RH (VPD≈0); under this condition the leaves gained maximum fresh weight. For desiccation, the petioles were removed out off the water and the leaves were placed upside down on balances connected to a PC in a test room (40±3% RH, 20 ºC, resulting in 1.40 kPa VPD and 35 µmol $m^{-2}s^{-1}$ irradiance). Water loss was recorded gravimetrically every 10 s and lasted for three hours. Leaf area was calculated by using the public domain image processing program ImageJ (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). The data were used to calculate the rate of water loss over time per unit leaf area. After the desiccation period, the leaves were dried for 48 h at 80 ºC. Relative water content (RWC) during the desiccation period was calculated according to Slavik (1974).

Leaf sectioning

After four days exposure to low VPD condition in the M→L treatment, leaves of plants grown at L, M and M→L VPDs were fixed in sterile Phosphate buffer solution (PBS) with 0.25% glutaraldehyde included. Vacuum was applied for 1-2 h until tissues sat on the bottom and then tissues were incubated at 4°C overnight. After two times washing with PBS, dehydration steps were performed with 10%, 30%, 50%, 70%, 90% and 100% ETOH respectively for 10 min at room temperature for each step. Plastic infiltration was done in 4 steps, which included solution A (100ml Technovit7100, 1pack HrdnerI, 2.5ml PEG400):100% ETOH in 1:3, 1:1, 3:1 ratio respectively for 30-60 min in room temperature and finally treated with 100% solution A for overnight at the 4°C. All material was transferred into cupules, solution A was removed and polymerization solution (15ml Solution A, 1ml Hardener II) was added immediately. To remove air from the samples, cupules were covered with parafilm and left for overnight at room temperature. After polymerization, holders were put on the blocks and holding solution (technovit3040: 2part powder, 1 part liquid) was added from the hole located in the centre of holder and kept for 15 min at room temperature. Finally sectioning of leaves was performed using a microtome and the samples were analysed by

microscopy (Leica, Rijswijk, Netherlands) after staining with toluidine blue (0.5%) buffer and washing with tap water for 5 minutes.

Stomatal morphology

The stomatal morphology (i.e. stomatal length, stomatal width, stomatal density, stomatal index, pore length, pore area and pore aperture) were measured in leaves of the three mentioned treatments (M, L and M→L). Images were taken from epidermal strips incubated in stomatal opening medium (50 mM KCl, 10 mM MES-KOH, pH 6.15, 50 µM $CaCl_2$) using a Nicon digital camera (DXM-1200) attached to a microscope (Leica, Aristoplan). For stomatal density, images from 31-51 different epidermal strips were used. For stomatal features images from 20 randomly selected leaves from 10 plants (n=131-191) were used. Images were analysed by using ImageJ. Stomatal index was calculated using the following equation (Weyers and Meidner 1990):

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

Statistical analysis

For stomata morphological and leaf anatomical traits, data were subjected to analysis of variance (ANOVA) and $P \leq 0.05$ was considered as not significant. Homogeneity of variances was tested with Levene's test. When normalization of data was necessary, data were transformed using the square root of the data. The change of transpiration (E) as a function of RWC was fitted using a sigmoidal dose-response curve with a variable slope [E=Bottom+((Top-Bottom)/(1+$10^{(RWC50-RWC).Slope}$))]. Data in Fig. 3 were fitted with segmental linear regression and the F-test was used for comparing the slope of the curves. GraphPad Prism 5 for Windows (GraphPad software, Inc. San Diego, CA) and IBM SPSS Statistics version 19 were used for analyzing the data.

Results

Low VPD induced changes in transpiration rate and stomatal conductance afterwards

When exposed to the same VPD, *Vicia faba* plants that had been grown at low VPD (L plants), showed a higher transpiration rate and stomatal conductance compared to moderate VPD-grown plants (M plants) (Table 1). Plants of which full grown leaves had been exposed for only 4 d to low VPD (M→L plants) had the same transpiration rate and stomatal conductance as L plants that were continuously grown at low VPD.

Table. 1. Transpiration rate (E) and stomatal conductance (g_s) of *Vicia faba* leaves exposed to different VPDs. Plants grown at low VPD (0.23 kPa) (L), moderate VPD (1.05 kPa) (M) or at moderate VPD and then transferred for 4 days to low VPD (M→L). E and g_s measured at 20°C, RH 55% (VPD is 1.05kPa), and 300 µmol m^{-2} s^{-1} irradiance.

	VPD during growth of the plants		
	L	**M**	**M→L**
E (mmol m^{-2} s^{-1})	3.97 b	2.07 a	3.71 b
g_s (mmol m^{-2} s^{-1})	355.3 a	208.7 b	344.9 a

Low VPD induced fundamental changes in stomatal morphology

Stomatal morphological features were significantly influenced by growth at different VPDs (Table 2). Leaves grown in low VPD (L plants) had significantly ($P \leq 0.001$) longer and wider stomata compared with the stomata of the plants that were grown in moderate VPD (M plants). There was no effect of VPD on stomata size when plants were exposed for 4 d to low VPD (M→L plants) (Table 2). Moreover, length of the pore was larger in L plants in comparison with the stomata of M and M→L plants (Table 2) and no significant differences were found between pore length of M and M→L plants. Wider aperture and bigger area were observed in the stomata of the L plants compared with the stomata of M and M→L plants. Pore aperture of M→L plants was wider and its area was larger compared with stomata of M plants (Table. 2). Different VPDs also affected the density of stomata on the leaf (Table. 2). Growing plants in L condition caused a decrease in the number of stomata per leaf area compared to M plants. The density of the stomata in M→L plants was not significantly different of that of both M and L plants (Table. 2). On the other hand, the stomatal index of the leaves of L plants was similar to the index in the M plants. The highest stomatal index was observed in the M→L plants (Table. 2). These observations revealed that continuous L conditions induce essential changes in the morphology and density of stomata on the leaf.

Table. 2. Stomatal traits of leaves of *Vicia faba* plants exposed to different VPDs. *Vicia faba* plants developed their leaves at low VPD (0.23 kPa) (L), moderate VPD (1.05 kPa) (M) or developed their leaves at moderate VPD then transferred for four days to low VPD (1.05 kPa→0.23 kPa) (M→L). The plants used in the experiment were well watered. The irradiance in all treatments during exposure to VPDs was 300 µmol m^{-2} s^{-1}. The gas composition during the experiment was kept in ambient concentrations. Epidermal strips were incubated in stomatal opening medium (50 mM KCl, 10 mM MES-KOH, pH 6.15, 50 µM $CaCl_2$) under 35 µmol m^{-2} s^{-1} irradiance.

Stomatal traits	L	M	M→L
Stomatal length (µm)	44.08 a	38.14 b	39.64 b
Stomatal width (µm)	31.32 a	22.95 b	24.60 b
Pore length (µm)	33.32 a	26.33 b	28.13 b
Pore aperture (µm)	12.30 a	8.59 c	10.59 b
Pore area (µm^2)	256.23 a	170.17 c	206.84 b
Stomatal density (no. mm^{-2})	37.36 b	42.83 a	39.80 ab
Stomatal index	20.47 b	21.04 b	25.68 a

Different letters show significant difference at 0.01 probability level according to least significance difference (LSD) test.

Low VPD induced changes in leaf anatomy

The number of palisade cells was not influenced by different VPD conditions, while the number of spongy cells was significantly ($P \leq 0.05$) influenced by different VPDs (Table 3). The number of spongy cells in L-grown leaves was lower than their number in the M-grown leaves. However, leaves of the M→L plants did not show statistical differences for number of spongy cells with both L and M plants. There were no differences between VPD conditions for leaf intercellular air space. The thinnest leaves were observed in L-grown leaves; the thickest leaves were found in M-grown leaves (Table 3). In accordance with this result, the highest specific leaf area (SLA) was also detected in the L-grown leaves, while the lowest SLA was found in the M-grown leaves (Table 3).

Exposure of leaves for a few days to low VPD condition changed the stomatal responses to closing stimuli

With desiccation, leaf transpiration rate (E) decreased in leaves of all treatments (L, M and M→L plants). However, E decreased less strong in response to desiccation for leaves of L and M→L plants compared to that of M plants (Table 4). After 4 days exposure to low VPD, the slopes of the E*RWC correlation curves for L and M→L leaves were similar to each other, while they were different from the slope for M leaves (Table 4). E of M leaves sharply

decreased at a RWC between 80 and 70%. The RWC50 of the curve fits of E versus RWC was 76% for M leaves and 64% for L and M→L leaves. The stomatal conductance (g_s) decreased over 150 min desiccation in M, L and M→L leaves (Fig. 1). However, the slope of the g_s curve over desiccation time was sharper in M leaves compared with L and M→L leaves. There was no significant difference in the response to desiccation between L and M→L leaves. The g_s of L and M→L desiccated leaves stayed approximately 4 times higher after 60 min desiccation as compared with M leaves.

To test the effect of exposure time to low VPD on the responses of stomata to desiccation, RWC and E were measured in M-grown plants after 1-4 d exposure to L condition. E responded less to leaf water content when plants had previously been exposed for 1, 2, 3 or 4 d to L conditions, as can be seen in the slopes of the curves of E as function of RWC (Fig. 2). Already after 1 d of exposure to L, the slope of the curve was significantly shallower than the slope of the M plants ($P \leq 0.0001$). Longer exposure to L condition affected stomatal response to desiccation more and more. Although there were no statistical differences between the slopes of plants that had been exposed for 2 and 3 d to L condition, the steepness of their E*RWC curves were shallower compared to 1 d exposed-plants. The shallowest slope was found after 4 d exposure to L condition (Fig. 2).

Table. 3. Leaf anatomical features of *Vicia faba* plants grown at different VPDs. *Vicia faba* plants developed their leaves at low VPD (0.23 kPa) (L), moderate VPD (1.05 kPa) (M) or developed their leaves at moderate VPD then transferred for four days to low VPD (1.05 kPa→0.23 kPa) (M→L). The plants used in the experiment were well watered. The irradiance in all treatments during exposure to VPDs was 300 µmol m^{-2} s^{-1}. The gas composition during the experiment was kept in ambient concentrations.

Leaf anatomy	L	M	M→L	Sig
Palisade cell number (no. mm^{-2})	60.27	55.14	58.95	0.827 ^{ns}
Spongy cell number (no. mm^{-2})	211.5 b	246.4 a	233.1 ab	0.045 *
Leaf intercellular space (%)	41.02	43.91	42.98	0.704 ^{ns}
Leaf thickness (µm)	419.1 b	477.8 a	464.1 ab	0.033 *
Specific leaf area (cm^2 g^{-1})	391.2 a	370.5 b	380.1 ab	0.042 *

^{ns} Non significance, * Significance at 0.05 probability level according to least significance difference (LSD) test.

Table. 4. Slope and RWC50 for transpiration rate × RWC in plants exposed to different VPDs during desiccation. *Vicia faba* plants developed their leaves at low VPD (0.23 kPa) (L), moderate VPD (1.05 kPa) (M) or developed their leaves at moderate VPD then transferred for four days to low VPD (1.05 kPa→0.23 kPa) (M→L). The transpiration rate × RWC curves of different treatments are fitted as a sigmoidal dose-response curve with a variable slope [E=Bottom+((Top-Bottom)/(1+$10^{(RWC50-RWC).Slope}$))] during 3 h desiccation. The measurements were carried out at 1.40 kPa VPD. Values shown by different letters indicating significant differences at $P \leq 0.0001$, n=6

	VPD during growth of the plants		
	L	**M**	**M→L**
Slope	0.0766 a	0.2167 b	0.0635 a
RWC50	64.63 b	76.06 a	64.18 b

Application of ABA to the petioles of the L, M and M→L exposed-leaves led to a gradual decrease in ϕ_{PSII} at low oxygen (non-photorespiratory condition) (Fig. 3). A significant interaction was found between the effect of previous VPD conditions and ABA feeding on ϕ_{PSII} ($P \leq 0.001$). In L and M→L leaves the decline in ϕ_{PSII} started after 30 min from the start of the application of ABA, but in M leaves the decline in ϕ_{PSII} started already between 0 and 30 min of ABA-application. The slopes of the time curves for ϕ_{PSII} of L and M→L leaves were similar, while it was significantly steeper in M leaves compared to L and M→L leaves (Fig. 3). With 5 min exposing the L, M and M→L leaves to high CO_2 at the end of ABA feeding, ϕ_{PSII} recovered approximately to 70 to 86% of the original values, indication that the decrease in ϕ_{PSII} was mainly due to stomatal closure. The frequency distributions of leaf images of ϕ_{PSII} (Figs. 4, 5) had similar trends for the control (before ABA feeding) of all 3 plant types (M, L, and M→L), showing a normal distribution around an average ϕ_{PSII} of 0.65. After ABA feeding, the frequency distribution of ϕ_{PSII} in M leaves shifted to lower values and stayed almost unimodal (Fig. 4A). However feeding of ABA to the L and M→L leaves caused a distribution of ϕ_{PSII} with a double peak (Fig. 4B, C). The ϕ_{PSII} in part of the leaf in L and M→L leaves decreased to low values, while in another part of the leaf they remained almost unchanged. The frequency distributions of both leaves (L and M→L) were almost identical in shape. Also when M leaves had been exposed for only 1 d to low VPD, the decrease of ϕ_{PSII} due to ABA feeding was less than that of M leaves without any exposure to low VPD (Fig. 5). Spraying leaves every day with ABA during the 4 d exposure to low VPD, resulted thereafter in the same ϕ_{PSII} response to ABA feeding as that of M leaves (Fig. 6).

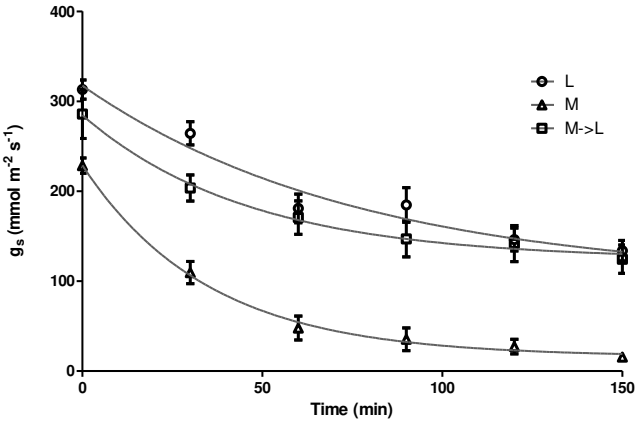

Fig. 1. Stomatal conductance (g_s) of *Vicia faba* plants exposed to different VPDs during desiccation. *Vicia faba* plants developed their leaves at low VPD (0.23 kPa) (L), moderate VPD (1.05 kPa) (M) or developed their leaves at moderate VPD then transferred for four days to low VPD (1.05 kPa→0.23 kPa) (M→L).The measurements were carried out at 1.40 kPa VPD and 35 µmol m^{-2} s^{-1} irradiance.

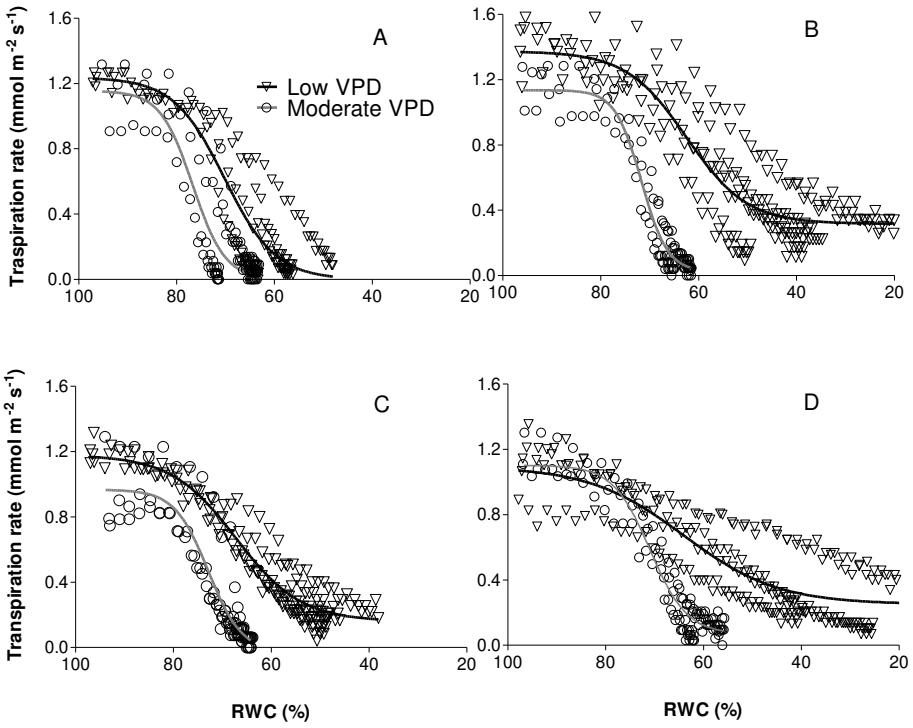

Fig. 2. Changes of transpiration rate as a function of RWC in *Vicia faba* leaves during desiccation. Leaves were taken from plants exposed to one (A), two (B), three (C), four (D) days to low VPD (triangle symbols) (0.23 kPa) or continuously grown at moderate VPD (circle symbols) (1.05 kPa). Measurements were made every 10 seconds. The symbols represent data from individual leaves (n=3-5 per treatment).The grey and black lines represent fitted curves for the moderate and low VPD data sets, respectively. The measurements were carried out at 1.40 kPa VPD and 35 µmol m^{-2} s^{-1} irradiance. Each point represents the mean value of transpiration rate over 5 minutes and RWC measured at that time point.

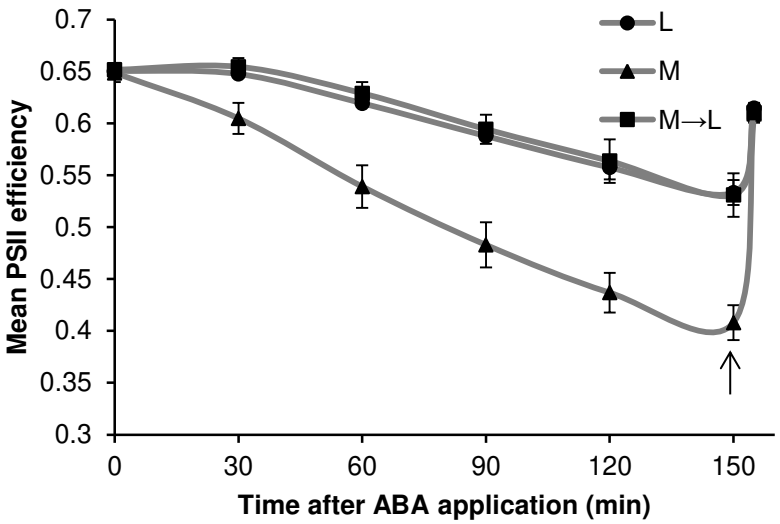

Fig. 3. PSII efficiency of *Vicia faba* leaves exposed to different VPDs in response to ABA. Vicia faba plants developed their leaves at low VPD (0.23 kPa) (L), moderate VPD (1.05 kPa) (M) or developed their leaves at moderate VPD then transferred for four days to low VPD (1.05 kPa→0.23 kPa) (M→L). The PSII efficiency was measured over 150 min of 100 µM ABA feeding in an atmosphere with 20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and the reminder N_2. The black arrow represent start of exposure to 20 mmol mol^{-1} O_2, 50000 µmol mol^{-1} CO_2 for 5 min (t=150). Values are the mean of seven leaves ± standard error of the mean.

Exposure to low VPD decreased the bulk foliar ABA level

The level of ABA in the leaves of L plants was significantly lower than that of the M plants ($P \leq 0.05$) (Fig. 7). After 1 d exposure of plants to L conditions, the level of the bulk foliar ABA was sharply decreased in M-grown plants and below the level in leaves of L-grown plants. At the second day of exposure to L, the level of ABA reached the same level as L-grown plants and stayed at this level after 3 and 4 d exposure to L conditions. Although the level of ABA-GE increased after 1 d exposure of M-grown plants to L conditions, no statistical differences were found between treatments (Fig. 7).

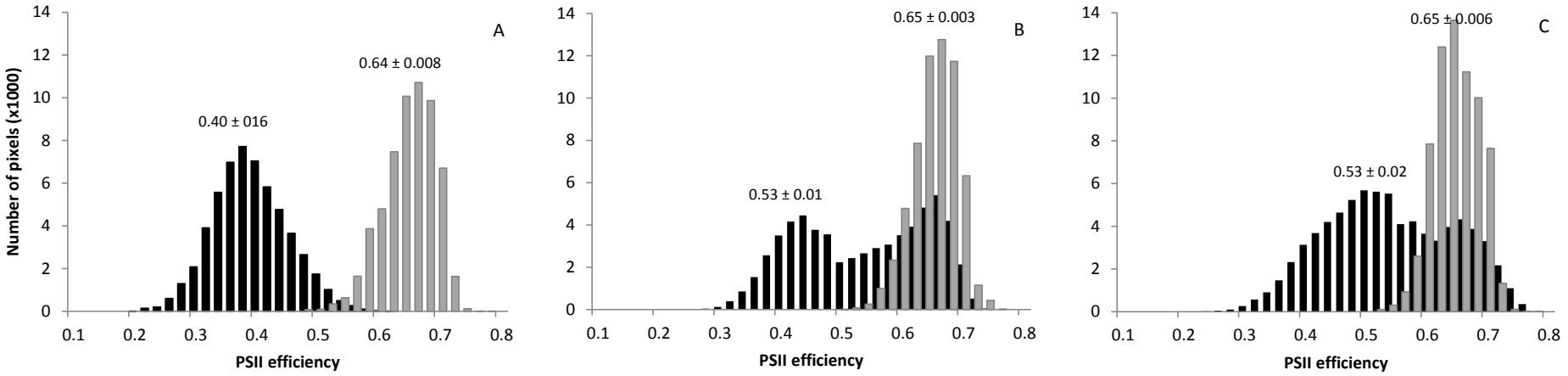

Fig. 4. PSII efficiency distributions in *Vicia faba* leaves exposed to different VPDs in response to ABA. The leaves were developed at moderate VPD (A) (1.05 kPa), low VPD (B) (0.23 kPa) or developed their leaves at moderate VPD then transferred for four days to low VPD (C) before (grey bars) and after 100 µM ABA feeding (black bars) in an atmosphere with 20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and the reminder N_2. Average values of PSII efficiency ± SE are indicated above the corresponding bar sets.

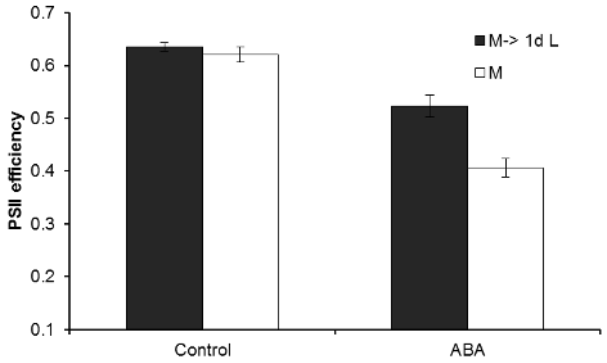

Fig. 5. PSII efficiency of *Vicia faba* leaves exposed to different VPDs in response to ABA. The plants were grown at moderate VPD (open bars) (1.05 kPa) or developed their leaves at moderate VPD then transferred for one day to low VPD (0.23 kPa) (black bars) before (control) and after 150 min of 100 µM ABA feeding (ABA) in an atmosphere with 20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and the reminder N_2. Values are the mean of four leaves ± standard error of the mean.

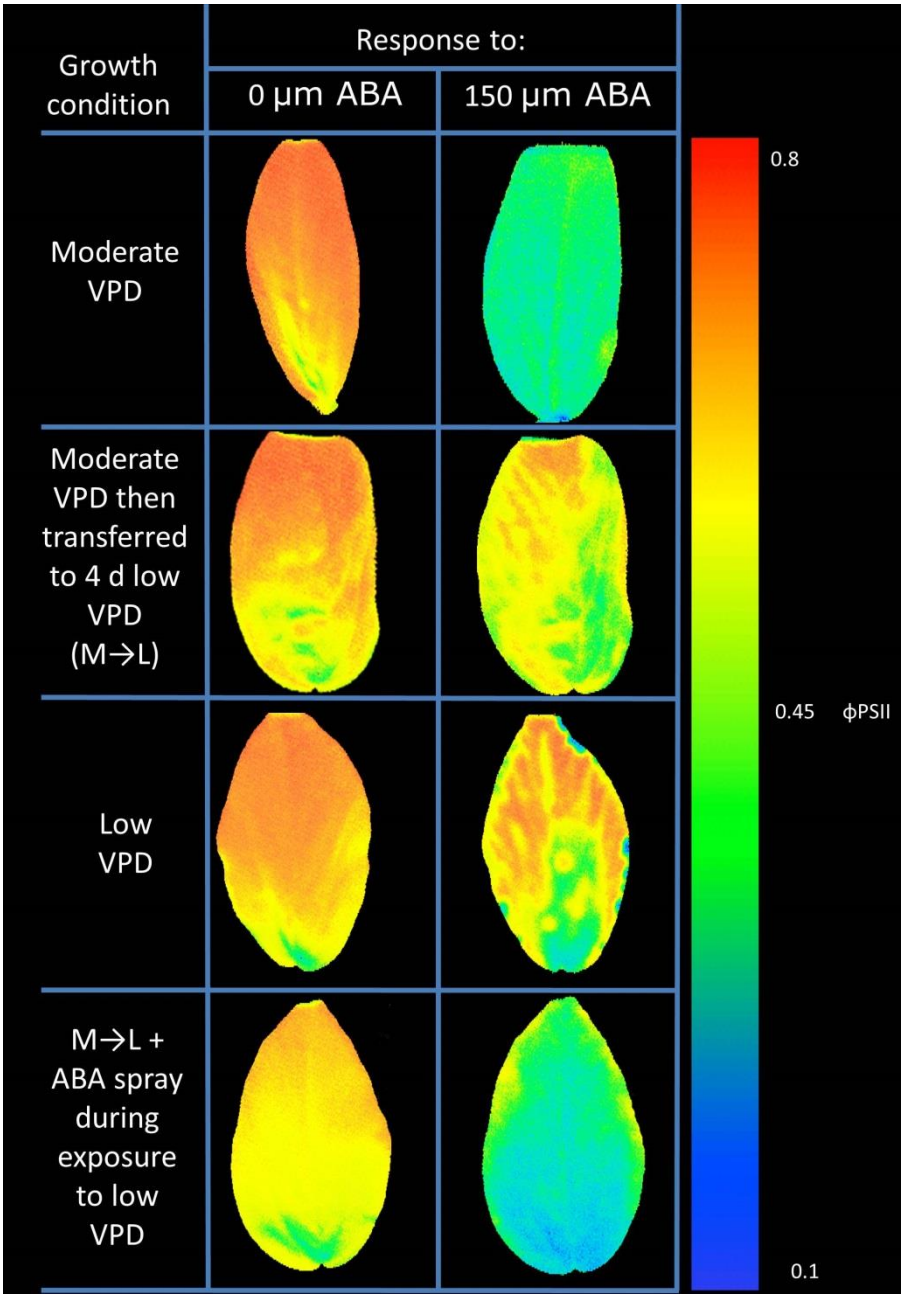

Fig. 6. Images of PSII efficiency in *Vicia faba* leaves exposed to different VPDs in response to ABA feeding. *Vicia faba* plants grown at moderate VPD (1.05 kPa), low VPD (0.23 kPa), developed their leaves at moderate VPD then transferred for four days to low VPD (M→L) or sprayed every day with 5 µM ABA during exposure to low VPD in M→L. PSII efficiency was recorded before and after 150 min 100 µM ABA feeding in an atmosphere with 20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and the reminder N_2. The mean value ± SE after ABA feeding were 0.4±0.01 for moderate VPD, 0.53 ± 0.01 for M→L, 0.53 ± 0.02 for low VPD and 0.38 ± 0.02 for M→L + ABA spray during exposure to low VPD plants.

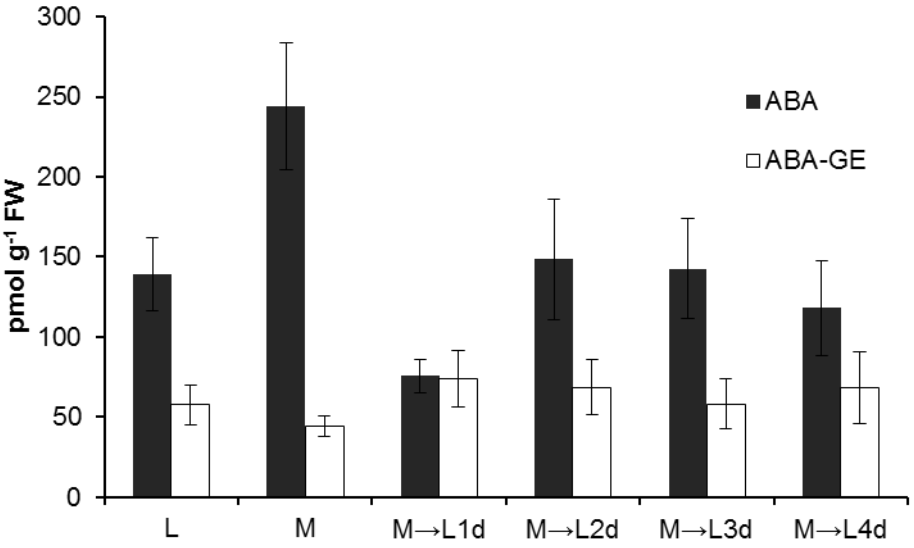

Fig. 7. Concentration of bulk ABA and ABA-GE in *Vicia faba* leaves exposed to different VPDs. ABA (black bars) and ABA-GE (open bars) was measured in the leaves that fully grown at low VPD (0.23 kPa) (L), moderate VPD (1.05 kPa) (M) or developed their leaves at moderate VPD then transferred for one (M→L1d), two (M→L2d), three (M→L3d) and four days (M→L1d) to low VPD. The plants used in the experiment were well watered. The irradiance in all treatments during exposure to VPDs was 300 µmol m^{-2} s^{-1}. The gas composition during the experiment was kept in ambient concentrations. The leaf samples were taken in the mid-time of the lighting period.

Discussion

Morphological aspects of disturbed stomatal response after prolonged exposure to low VPD

After exposure of *Vicia faba* plants to low VPD continuously or for 4 d (L and M→L plants), their leaves showed a higher transpiration rate and stomatal conductance compared to leaves of moderate VPD-grown plants (M plants) (measured at the same VPD). The higher stomatal conductance of the L plants will be the result of the larger pore area in the leaves of these plants. The larger pore area could (partly) be the result of the increase in size of the stomata of L plants (stomatal length and width) compared with the stomata in M plants. Generation of bigger stomata has been shown for leaves that were subjected to continuous low VPD during their development for roses (Torre et al. 2003, Fanourakis et al. 2011, Arve et al. 2012) and *Tradescantia virgiana* (Rezaei Nejad and van Meeteren 2005). Although previous studies showed an increase in stomatal density in low VPD-grown roses (Torre et al. 2003, Fanourakis et al. 2011), we showed that growing bean plants at low VPD reduced the number of stomata per leaf area which is in agreement with the study of Rezaei Nejad and van Meeteren (2005) in *Tradescantia*. However, the decrease in stomata density was too small to compensate for the effect of the increase in aperture area on transpiration rate (Table. 2).

Moreover, the stomatal index was not affected by the VPD during growth, indicating that stomatal density was decreased due to increase in epidermal cell size, as was previously reported in *Tradescantia* (Rezaei Nejad and van Meeteren 2005). As result stomata number/leaf was not affected by VPD.

High RH during the development of rose leaves influenced leaf anatomical traits like intercellular air-space and number of spongy and palisade mesophyll cells (Torre et al. 2003). However significant differences were not found in our study with bean plants for palisade cell numbers and intercellular air space. On the other hand, in agreement with Torre et al. (2003), leaf thickness was significantly decreased by growing leaves at low VPD (Table. 3), which resulted in increased specific leaf area in the L-grown plants, likely because of cell enlargement at low VPD (Rezaei Nejad and van Meeteren 2005).

When plants were grown at moderate VPD and thereafter transferred for 4 d to low VPD (M→L), the anatomical and morphological characteristics of leaves and stomata were not affected by this 4-days transfer and were identical to that of M plants (Tables. 2, 3); also stomatal density was the same as that of M plants. However, the stomata were more opened (pore aperture, pore area) as compared with M plants (Table. 2) and the transpiration rate and stomatal conductance of M→L plants was identical to that of L plants (Table. 1).

Stomatal size has been associated with responsiveness to closing stimuli across species, where smaller stomata have been related to shorter response times and vice versa (Hetherington and Woodward 2003, Franks and Farquhar 2007, Drake et al. 2013, Giday et al. 2013b). Moreover, within one species, rose, it has been discussed that differences in stomatal size determine variation in the stomatal responsiveness to closing stimuli (Giday et al. 2013b). However, analyzing different rose cultivars, Fanourakis et al. (Fanourakis et al. 2013) showed that stomatal length and closing ability (response to desiccation) were not correlated to each other, both in moderate and high RH grown leaves. Our results showed within one species (fava bean), that the M-grown plants had smaller sized-stomata compared to L-grown plants, and they react faster to closing stimuli (desiccation and ABA) and vice versa. However in M→L plants, the size of the stomata were more similar to M-grown plants, but they respond to closing stimuli in a way that L-grown plants did. It has been reported that higher water loss in rose plants grown in high RH is because of higher stomatal density of these plants and also wider stomatal aperture (Torre et al. 2003). In our study, although the stomatal density was the same between M→L and M-grown plants, their response to closing stimuli was different. It can be concluded that stomatal malfunctioning and higher water loss induced by growing or exposure of the plant to L condition is not solely due to differences in stomatal density.

It seems that occurring of stomatal malfunctioning is species-dependent. For instance, it has been shown that the ability of rose stomata to close in response to water stress is fully established during the time of leaf development. After development of the leaves, it is not possible to induce a change in the stomatal closing ability by VPD (Fanourakis et al. 2011). However the obtained results in this study showed that after development of the fava bean leaves in moderate VPD, one day exposure to L condition resulted in a decreased sensitivity of the stomata to closing stimuli and after four days the stomatal response would be the same as in L-grown plants. Also in *Tradescantia* was shown that 4 d exposure of full grown leaves to low VPD resulted in a decreased sensitivity of the stomata to desiccation and ABA (Rezaei Nejad and van Meeteren 2008).

Physiological aspects of disturbed stomatal response after prolonged exposure to low VPD

Our results imply that 4 d exposure to low VPD resulted in an adaptation of the stomata that was not related to morphological changes but to the physiology of the guard cells. Growing plants at low VPD resulted in a decreased bulk foliar ABA concentration compared with M-grown plants; after exposing M plants for only 1 day to low VPD the foliar ABA concentration was decreased to about 50% of that in M plants and even lower than the ABA concentration in leaves of L plants (Fig. 7). Since no significant differences were found in ABA-GE levels after different times of exposure to L condition, it is likely that the decreased ABA levels were mainly due to increased hydroxylation of ABA. It has been shown that in response to high RH, transcript levels of genes encoding ABA 8'-hydroxylase increases which reduces the amount of mobile and local ABA (Kushiro et al. 2004, Okamoto et al. 2009). The low ABA level in the leaves could possibly explain the larger stomata opening in plants grown at moderate VPD and transferred to low VPD. That stomatal aperture and area of M→L plants were between that of M and L plants could be the combined effect of low endogenous ABA levels and the effect of the moderate VPD during growth on stomatal length.

However, application of ABA to the petiole of the leaves of L grown plants did not result in a decreased ϕ_{PSII} in contrast with M plants (Fig. 3), although feeding leaves of L plants with exogenous ABA even resulted in four times higher ABA intake compared with leaves of M plants (data not shown). Because ϕ_{PSII} was measured while photorespiration was inhibited by low oxygen, induced ϕ_{PSII} is closely related to stomatal closure (Rezaei Nejad et al. 2006). Aliniaeifard and van Meeteren (2013) hypothesized that a long period of low ABA as a result

of a prolonged exposure to low VPD, will result in ABA desensitisation. Spraying leaves with ABA during the exposure to low VPD prevented the loss of stomatal response to ABA feeding afterwards (Fig.6), confirming that 1 d or longer low endogenous ABA levels (due to exposure to low VPD) resulted in a decreased capacity of stomatal response to exogenous ABA. As concluded by Bauer et al. (2012), their transcriptomic data are suggestive of a positive ABA-mediated feedback on ABA production. It seems that there is also a positive ABA-mediated feedback on ABA sensitivity. Giday et al. (2013a) showed that rose cultivars that differed in their stomatal response to growth at high RH had different foliar ABA levels. The cultivars with decreased stomatal response to closing stimuli had lower ABA levels compared with tolerant ones after growth at high RH.

Analysis of leaf responses to desiccation showed that prolonging the time of exposure to L condition, slows down more and more the response of stomata to water stress afterwards. Four days exposure to L condition reduced strongly the stomatal capacity to close in response to water deficit (Fig. 2). In *Tradescantia* has been shown that 3 d exposure of moderate VPD-grown plants to low VPD did not result in stomatal malfunctioning (their response to desiccation was the same as that of moderate VPD-grown plants). However, after 4 d exposure to low VPD, the response of stomata changed and they became less sensitive to desiccation (Rezaei Nejad and van Meeteren 2008). The stomatal closing response to exogenous ABA of bean leaves that had been exposed for 1 or 4 d to L condition were both strongly diminished and was similar to the response of leaves of plants that were continuously grown at L condition (Table. 4). This indicates that the responses to desiccation and to ABA feeding were not affected in the same way by the exposure to low VPD. In the analysis of Fanourakis et al. (2013) of four different rose cultivars, they showed that in one of the cultivars the response to ABA feeding was strongly affected by growth at low VPD, while the response to desiccation was only minimally affected. A possibility could be that after exposing leaves for 1 d to low VPD, ABA feeding did not result in an increase in ABA levels inside the guard cells (hampered transport, low mobility because of binding, sequestered or hydrolysed) while ABA produced by the guard cells themselves as a result of desiccation can still induce stomatal closure after 1 d exposure to L condition. Bauer et al. (2012) showed that guard cell-autonomous ABA synthesis is required for and is sufficient for stomatal closure in response to low RH. Another explanation for the different effect of low VPD-exposure on the stomatal response to ABA and to desiccation can be that desiccation controls stomata closure (also) via a non-ABA controlled pathway. Both ABA-dependent and independent pathways

have been proposed for stomatal response to dehydration (Seu et al. 2012, Aliniaeifard and van Meeteren 2013).

Heterogeneity of stomatal responses to desiccation was previously shown for L-grown *Tradescantia* plants (Rezaei Nejad et al. 2006). In our study, we showed that heterogeneity in the stomatal responses to exogenous ABA is present in both M→L and L-grown plants (Fig. 4). It seems likely that the reduced stomatal response to desiccation in leaves of L and M→L plants is the result of the strong diminished ABA sensitivity in a part of the stomata within a leaf. In *Tradescantia* it has been shown that different parts of the leaf can have different ABA and RWC levels (Rezaei Nejad et al. 2006, 2007), which can result in variation in the stomatal response to closing stimuli. We did not analyse the endogenous ABA distribution within bean leaves of L or M→L plants.

Stomatal responses to low VPD in dependency of exposure time

In conclusion, effects of low VPD around leaves on their transpiration rate can have different causes depending on the length of the exposure time of the leaves to low VPD. An immediate response of stomata to a decrease in VPD is further opening of the stomata. Outlaw and De Vlieghere-He (2001) suggested that differences in transpiration rate due to differences in VPD, may result in differences in the guard cell apoplast sucrose concentration, which affects stomatal aperture size. Another explanation can be that the endogenous ABA level in guard cells decreases fast under low VPD as shown in *Tradescantia* (Rezaei Nejad and van Meeteren 2007), *Arabidopsis* (Okamoto et al. 2009) and in this study in *Vicia faba* (1 day). When exposed for one day (*Vicia faba*, this study) or four days (*Tradescantia*, (Rezaei Nejad and van Meeteren 2008)) to low VPD, stomata also lost their responsiveness to ABA; when exposed for four days to low VPD as well *Vicia faba* as *Tradescantia* lost their response to desiccation. Changes in size of stomata, and in stomatal density or index occur when leaves develop during growth at low VPD. These morphological changes are not the main reason for occurrence of malfunctioning stomata after long-term exposure to low VPD.

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CHAPTER 3

Can prolonged exposure to low VPD disturb the ABA signalling in stomatal guard cells?

Abstract

The response of stomata to many environmental factors is well-documented. Multiple signalling pathways for abscisic acid (ABA)-induced stomatal closure have been proposed over the last decades. However, it seems that exposure of a leaf for a long time (several days) to some environmental conditions generate a kind of memory in the guard cells that results in the loss of suitable responses of the stomata to closing stimuli, like desiccation and ABA. In this review paper we discuss changes in the normal pattern of signal transduction that could account for disruption of guard cell signalling after long-term exposure to some environmental conditions with special emphasis on long-term low vapour pressure deficit (VPD).

Keywords: Abscisic acid, calcium, environmental factors, guard cells' signalling pathway, hydrogen peroxide, nitric oxide, secondary messengers, stomata, Vapour Pressure Deficit.

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Introduction

A range of environmental and endogenous signals trigger a complex network of signalling pathways that regulate ion channels and solute transporters to drive stomatal movements. Although the mechanisms behind stomatal movements usually provide a robust and fault-tolerant system, it is susceptible to disruption under certain conditions, leading to a reduced ability of the stomata to close in response to stimuli that normally provoke stomatal closure. This disruption of stomatal behaviour has been observed in plants grown *in vitro* (Brainerd and Fuchigami, 1982; Ziv *et al.*, 1987; Santamaria *et al.*, 1993; Hazarika, 2006) and also after long-term exposure to some environmental conditions like continuous light (Slootweg and van Meeteren, 1991; Mortensen and Gislerød, 1999; Pettersen *et al.*, 2007; Arve *et al.*, 2012), ozone (O_3) (Paoletti, 2005; Wilkinson and Davies, 2009), hydrogen sulphide (H_2S) (Lisjak *et al.*, 2010), sulphur dioxide (SO_2) (Maier-Maercker and Koch, 1986) and, especially, low vapour pressure deficit (VPD) (Rezaei Nejad and van Meeteren, 2005, 2007, 2008; Rezaei Nejad *et al.*, 2006; Torre and Fjeld, 2001; Fanourakis *et al.*, 2011; Arve *et al.*, 2012). It is rather surprisingly that a single factor, like low VPD, can disturb the robust system of stomata control. The main consequence of this stomatal dysfunction is a reduced capacity of leaves to maintain an adequate water status, which often results in a lethal degree of water stress (Fanourakis, Pieruschka *et al.*, 2013). Despite the recent advances in our understanding of the signalling in guard cells (Nambara and Marion-Poll, 2005; Li *et al.*, 2006; Kim *et al.*, 2010; Kline *et al.*, 2010; Raghavendra *et al.*, 2010; Umezawa *et al.*, 2010; Lee and Luan, 2012), the signal transduction elements which are disturbed or disrupted in guard cells of malfunctioning stomata are still not understood. As highlighted at 'Stomata 2012' (29th New Phytologist symposium, Manchester, UK), considering the impact of stomata on global issues, more information is required for environmental influences on guard cell responses (Roelfsema and Kollist, 2013). In this review paper we discuss changes in the normal pattern of signal transduction that could probably account for disruption in guard cell signalling after long term exposure to low VPD.

Role of exposure duration to environmental factors in stomatal malfunctioning

Guard cells continuously sense signals from the plant and the environment and respond via changes in turgor pressure; these changes result in stomatal opening or closing (Schroeder *et al.*, 2001a; Schroeder *et al.*, 2001b; Kim *et al.*, 2010; Monda *et al.*, 2011). Besides the short-term effects of many environmental factors, the history of growth conditions can influence the response of the stomata. For example growing plants at continuous low VPD, 24h light

period, SO_2 or O_3, will modify stomatal functioning (Table 1). It has been observed in a wide range of species, that stomatal apertures are narrowed as an immediate response to high VPD and widened due to a VPD decrease around the leaf (Outlaw and De Vlieghere-He, 2001; Okamoto *et al.*, 2009). If subjected to a prolonged exposure to a low VPD, however, a habituation process occurs which renders the stomata insensitive to stimuli that would otherwise provoke stomatal closure. Stimuli shown to become ineffective in this way includes desiccation (Rezaei Nejad and van Meeteren, 2005; Rezaei Nejad *et al.*, 2006; van Meeteren *et al.*, 2009), high VPD (Torre *et al.*, 2003; Rezaei Nejad and van Meeteren, 2008; Mortensen and Gislerød, 2011), darkness (Mortensen and Fjeld, 1998; Fanourakis, Heuvelink *et al.*, 2013), abscisic acid (ABA) (Ziv *et al.*, 1987; Rezaei Nejad and van Meeteren, 2005, 2007) and the nitric oxide (NO) donor sodium nitroprusside (SNP) (Rezaei Nejad and van Meeteren, 2007). Similar to stomata of *Tradescantia virginiana* plants grown at low VPD, the loss of stomatal functioning took place 4 days after transfer of fully-grown leaves (grown at moderate VPD) to low VPD conditions (Rezaei Nejad and van Meeteren, 2008). Interestingly, transfer of plants back to a moderate VPD after long exposure (4-10 days) to low VPD, did not result in recovery of the stomatal closure response to desiccation (Rezaei Nejad and van Meeteren, 2008). Moreover, patchy stomatal dysfunction can be induced by high and low VPD (Mott *et al.*, 1993; Rezaei Nejad *et al.*, 2006). In *T. virginiana* grown at low VPD non-closing stomata were distributed around the main vein after desiccation of the leaves (Rezaei Nejad *et al.*, 2006). Furthermore, *in vitro*-propagated plants, which are produced under low VPD conditions, are susceptible to wilting upon transfer to normal atmospheric VPDs (Brainerd and Fuchigami, 1982; Ghashghaie *et al.*, 1992; Santamaria *et al.*, 1993; Aguilar *et al.*, 2000; Hronková *et al.*, 2003; Hazarika, 2006; Aracama *et al.*, 2008; Khan *et al.*, 2010). This is due to malfunctioning of the stomata, which are no longer able to close in response to closing stimuli such as darkness, ABA and elevated calcium (Ca^{2+}) levels (Brainerd and Fuchigami, 1982; Ziv *et al.*, 1987; Santamaria *et al.*, 1993). Increasing the VPD *in vitro* during the entire cultivation period maintained normal stomatal functioning (Ziv *et al.*, 1987; Ghashghaie *et al.*, 1992; Majada *et al.*, 1998, 2002; Ivanova and van Staden, 2010). Although poor cuticular development under low VPD conditions partly contributes to the poor resistance to desiccation shown by *in vitro* plants (Ziv *et al.*, 1987; Zacchini *et al.*, 1997; Hazarika, 2006), the contribution of increased cuticular water loss is small in comparison to the role of stomata (Ziv *et al.*, 1987; Santamaria and Kerstiens, 1994). Similar to plants generated *in vitro*, higher water loss of low VPD-grown roses was mainly due to malfunctioning of the stomata and to a lesser extent to an increased cuticular transpiration rate (Fanourakis, Heuvelink *et al.*, 2013).

The same authors also recognized stomatal malfunctioning as the main source of genotypic variation in water loss of rose cultivars grown under low VPD conditions as compared to the involvement of cuticular water loss.

As well stomata size as stomatal index are affected by VPD during plant development (Fordham *et al.*, 2001; Lake and Woodward, 2008; Torre *et al.*, 2003; Tricker *et al.*, 2012); this could (partly) account for higher transpiration rates of leaves grown at low VPD. However, it is difficult to explain the lower sensitivity to drought, darkness, ABA and SNP by these morphological changes. Since stomatal malfunctioning also takes place after a few days exposure to low VPD of fully developed leaves, it is more likely that changes in signalling pathways play an important role in the malfunctioning of stomata.

The impact of long-term exposure to environmental factors on stomatal regulation can be illustrated by several practical examples. Greenhouse crop production frequently makes use of supplementary lighting to improve plant productivity in periods of the year when natural irradiance is low; in some occasions continuous lighting is used (Mortensen and Fjeld, 1998; Dodd *et al.*, 2005; Pettersen *et al.*, 2006; Velez-Ramirez *et al.*, 2011). Although growing plants under continuous light has several advantages, it also adversely influences post-harvest leaf water loss (Mortensen and Fjeld, 1998; Mortensen and Gislerød, 1999, 2011) due to poor stomatal closure under conditions of decreasing leaf water potential and turgor. Notably, however, in those experiments that revealed a possible link between growth under continuous light and malfunctioning of the stomata, the plants were also grown under low VPD conditions. It has been shown that it is possible to maintain normal stomatal responses when plants are grown under continuous light by increasing the VPD (Mortensen and Fjeld, 1998; Mortensen and Gislerød, 1999, 2011; Pettersen *et al.*, 2006; Arve *et al.*, 2012).

Plant responses to environmental pollutants also involve effects on stomatal regulation, which are depended on the exposure time. Although short-term exposure to H_2S did not change the transpiration rate in maize, pumpkin, spruce and spinach (De Kok *et al.*, 1989), long-term application of H_2S donor-compounds to *Arabidopsis* leaves induced stomatal opening and exposing these leaves to darkness did not result in stomatal closure (Lisjak *et al.*, 2010). Similarly, stomata of plants that have been exposed for several days to O_3 are unable to close in response to ABA and drought stress; therefore O_3 renders stomata incapable of controlling transpiration (Mills *et al.*, 2009; Wilkinson and Davies, 2009). In *Arbutus unedo* slow stomatal closure persisted for 10 days after a 90 days exposure to O_3 (Paoletti, 2005). In *Leontodon hispidus* exposure to O_3 for at least 48 h resulted in the loss of stomatal closure response in the presence of ABA (Wilkinson and Davies, 2009), whilst short term exposure of

the leaves to O_3 would trigger a rapid stomatal closure (Leipner *et al.*, 2001; Torsethaugen *et al.*, 1999; Overmyer *et al.*, 2008; Vahisalu *et al.*, 2010). Stomatal malfunctioning was more pronounced when the exposure to O_3 took place at low VPD conditions (Costonis and Sinclair, 1969; Maier-Maercker and Koch, 1986; Maier-Maercker, 1989).

These observations illustrate that certain environmental conditions can make stomata incapable of responding to stimuli that would normally produce stomatal closure. The duration of exposure is critical in determining if an environmental condition will cause stomatal dysfunction; prolonged exposure to pollutants or low VPD result in abnormal stomatal regulation while short term exposure does not. The magnitude of stomatal malfunctioning induced by factors such as continuous light and O_3, is much more pronounced when these are applied simultaneously with low VPD.

As CO_2 and light around mature leaves can affect the stomatal density in developing leaves of the same plant, these factors seem to have a systemic effect on stomatal density (Lake *et al.*, 2001). However, providing a low VPD around an individual leaf of a plant which was kept at moderate VPD made the stomata of this low-VPD leaf incapable of suitable response to closing stimuli, but the other leaves from the same plant responded still adequately to closing stimuli (Rezaei Nejad and van Meeteren, 2007). The same authors showed that the response of stomata in different parts of one leaf which were exposed for long term to low or moderate VPDs were different in their response to closing stimuli. Stomata of the part of the leaf that developed at moderate VPD closed and stomata of the part of the leaf that developed at low VPD stayed open in response to closing stimuli (Rezaei Nejad and van Meeteren, 2008). This indicates that the effect of long-term low VPD on stomata signalling is only local. This is another indication that the long-term low VPD effect on stomata closing is not related to effects of environmental factors on morphological aspects like stomatal density.

Table 1. Examples of stomatal response to various closing stimuli that are altered after short (a) or long-term (b) exposure to environmental variables.

Species	Environmental variable (duration)	Closing stimuli (duration)	Reaction of the stomata	Ref
a. Short-term exposure				
Tradescantia virginiana	Low VPD (1-3 d)	Desiccation (150 min)	Closure of the stomata	(Rezaei Nejad and van Meeteren, 2008)
Arabidopsis thaliana	Low VPD (1 h) O_3 (3min & 6 h)	ABA (2 h) exposure to ozone (3min & 6 h)	Closure of the Stomata Closure of the stomata and decrease in stomatal conductance	(Okamoto *et al.*, 2009) (Overmyer *et al.*, 2008; Vahisalu *et al.*, 2010)
Polypodium vulgar	Low VPD (1 h)	Dry air (15-30 min)	Closure of the stomata	(Lange *et al.*, 1971)
Phaseolus vulgaris	O_3 (3 h)	exposure to ozone (3 h)	Decrease in stomatal conductance	(Leipner *et al.*, 2001)
Vicia faba	Light (30-120 min)	ABA, Ca^{2+} and SNAP (30-120 min)	Inhibition of stomatal opening	(Garcia-Mata and Lamattina, 2007)
b. Long-term exposure				
Tradescantia virginiana	Low and moderate VPD (>4 d)	ABA, SNP and desiccation (150 min)	In moderate VPD exposed leaves stomata close, but in low VPD exposed leaves stomata remain to some extent open	(Rezaei Nejad *et al.*, 2006; Rezaei Nejad and van Meeteren, 2008; Rezaei Nejad and van Meeteren, 2007, 2005)
Rosa hybrida	Low VPD (during growth) Continuous light (24 & 20 h d^{-1})	Desiccation (>2 h) Leaf detachment (3 h) and darkness (4 h)	Slow reduction in transpiration rate High water loss and stomata remain open	(Fanourakis *et al.*, 2011; Torre and Fjeld, 2001) (Mortensen and Gislerød, 1999; Slootweg and van Meeteren, 1991)
Leontodon hispidus	O_3 (1-29 d) (20 weeks)	ABA (1 h) ABA and leaf desiccation	reduction of stomatal Sensitivity for closure response Impaired stomatal control	(Wilkinson and Davies, 2009) (Mills *et al.*, 2009)
Arbutus unedo	O_3 (90 d)	Abrupt reduction of light intensity and water stress (20 min)	Sluggish stomatal response	(Paoletti, 2005)

Role of ABA in malfunctioning of stomata

Role of ABA in the stomatal reaction to evaporative demand

ABA is a phytohormone that plays an important role in reducing transpiration by provoking stomatal closure (Lake and Woodward, 2008). The participation of ABA in drought-induced stomatal response is well known (Sauter *et al.*, 2001; Luan, 2002; Davies *et al.*, 2005) and guard cell ABA signal transduction has been extensively documented (Luan, 2002; Fan *et al.*, 2004; Pei and Kuchitsu, 2005; Li *et al.*, 2006; Hubbard *et al.*, 2010; Antoni *et al.*, 2011; Joshi-Saha *et al.*, 2011). Recently, a double role for ABA-induced stomatal closure was proposed: a direct biochemical mechanism in guard cells of stomata and an indirect effect via decreased leaf hydraulic conductance (Pantin *et al.*, 2013). Whether ABA participates in the direct response of stomata to VPD is still debated. Studies with *Arabidopsis* ABA mutants have not provided consistent results regarding the involvement of ABA in the immediate stomatal VPD response (Assmann *et al.*, 2000; Xie *et al.*, 2006). however, it has been shown that guard cells can autonomously produce ABA and elicit stomatal closure in response to an increase in VPD (Bauer *et al.*, 2012). There is also an immediate effect of VPD on ABA catabolism. In *Arabidopsis thaliana*, the leaf ABA level decreased by 80% one hour after the transfer of plants from moderate (60%) to high (90%) relative humidity (RH) (Okamoto *et al.*, 2009); this decrease was primarily due to an increased ABA catabolism by the cytochrome P450 mono-oxygenase (CytP450) ABA 8'-hydroxylase. The foliar ABA content of *Tradescantia virginiana* plants was decreased within one day after increasing the RH from 55% to 90% (Rezaei Nejad and van Meeteren, 2008). The ABA 8'-hydroxylase is encoded by genes of the CYP707A family (Kushiro *et al.*, 2004). In response to high RH, transcript levels of two CYP707A genes increased; CYP707A1 catabolises local ABA pools inside guard cells, whereas CYP707A3 reduces the amount of mobile ABA in vascular tissues (Okamoto *et al.*, 2009). It seems likely that part of the immediate stomatal response to an increasing VPD is ABA independent and another is ABA dependent (Yoshida *et al.*, 2006).

Opening of stomata is strongly controlled by light. Circadian rhythms for stomatal movement by light and dark periods as well as involvement of photoreceptors (such as phytochromes, cryptochromes, and phototropins) for stomatal movements has been largely documented (Gorton *et al.*, 1993; Shimazaki *et al.*, 2007; Wang *et al.*, 2010). For example it has been demonstrated that phytochrom B and cryptochroms are involved in stomatal opening through regulation of the transcription factor AtMYB60 (see transcription factor section of this paper) expression (Wang *et al.*, 2010). Tallman (2004) has proposed a model based on changes in guard cell apoplastic and symplastic ABA levels to explain diurnal stomatal movements that

many plants show in temperate or dry conditions. In this model, the diurnal movement is the result of a triphasic alternation of (i) depletion of endogenous guard cell ABA in the morning; (ii) transfer of root-source ABA through transpiration to the guard cell apoplast in the midday; and (iii), increase of ABA production in the guard cells in the dark period. The depletion of endogenous guard cell ABA early in day time (phase 1) is the result of activation of ABA 8'-hydroxylase. This NADPH-requiring CytP450 is activated by elevated O_2 and reduced CO_2 concentrations resulting from mesophyll photosynthesis. Simultaneously, the ABA precursor violaxanthin will be removed through light-driven xanthophyll cycling and the stomata start to open (Tallman, 2004).

As discussed before, besides the effect of changes in O_2 and CO_2 due to photosynthesis, there is an effect of VPD on ABA 8'-hydroxylase. Therefore it is likely that during phase 1 of the Tallman model in low VPD-exposed plants the effect of light (activation of ABA 8'-hydroxylase) is strengthened by low VPD. It would be interesting to investigate a possible interaction between light/dark and low VPD in causing stomatal malfunctioning.

In phase 2 of the Tallman model, root-source ABA should accumulate in the apoplast around guard cells. Infusion of ABA into the xylem stream of water-sufficient *Vicia faba* plants, indicated that root-source guard-cell ABA accumulation occurs solely in the apoplastic compartment of the guard cells (Zhang and Outlaw, 2001a). The apoplastic accumulation of ABA was strongly correlated with stomatal aperture in the leaf epidermis (Zhang and Outlaw, 2001b). However, the increase of osmotic potential due to ion loss from guard cells as result of the increased ABA level by midday will be compensated by osmotic potential decrease due to sucrose synthesis by photosynthesis and stomata remain open over the afternoon. At the end of the day when symplastic ABA exceeds the sucrose threshold level (concentration of sucrose in guard cell cytosol required for keeping stomata open), stomatal closure will take place (Tallman, 2004). We can expect that when transpiration is limited for a longer period due to low VPD the apoplastic accumulation of ABA in phase 2 is hampered, and the rise in apoplastic ABA will not occur.

In phase 3 of the Tallman model, the ABA 8'-hydroxylase activity will decrease in darkness due to the decrease of O_2-levels as result of lack of photosynthesis and ABA levels will further increase. As low VPD will increase the ABA 8'-hydroxylase activity, it seems likely that under low VPD conditions this decrease in ABA-hydroxylase activity in darkness will be absent. Rose plants that developed under high (90%) RH with 20h photoperiod showed no increase in ABA levels during darkness in contrast to plants that developed under moderate (60%) RH (Arve *et al.*, 2012). Arve *et al.* (2012) also showed that moderate RH-grown plants

had higher activities of β-glucosidase during darkness as compared to high RH-grown plants. ABA levels can rise by conversion of ABA-glucose ester to ABA by β-glucosidase.

After the reciprocal transfer of moderate RH-grown *Tradescantia* plants from 90% to 55% RH, the ABA increased to levels found before the high RH exposure (Rezaei Nejad and van Meeteren, 2008). When the exposure to 90% RH was for one or two days, the increase in endogenous leaf ABA after transferring back to 55% RH was accompanied by stomatal closure in response to desiccation. However, when the plants had been exposed to 90% RH for 4 days or longer, the increase in endogenous ABA after re-exposure to 55% RH was not accompanied by stomatal closure in response to desiccation nor did the stomata respond to exogenous ABA application (Rezaei Nejad and van Meeteren, 2008). These results indicate that, although leaf ABA concentration decreases rapidly under low VPD conditions, the actual ABA concentration is not the reason for the malfunctioning of stomata after transferring of low VPD-grown plants to moderate VPD, but it is the diminished response to ABA that causes the stomata malfunctioning. Although stomata of low VPD-grown plants are not able to close fully in response to short-term application of ABA (Rezaei Nejad and van Meeteren, 2005, 2007), long-term (daily) ABA application during leaf expansion at low VPD prevented the development of ABA-insensitive stomata (Rezaei Nejad and van Meeteren, 2007; Fanourakis *et al.*, 2011). Therefore, we can hypothesize that a long period of low ABA as a result of a prolonged exposure to low VPD, will result in ABA desensitisation. This agrees with the suggestion of Montillet and Hirt (2013) that long-term ABA accumulation is essential to regulates the efficiency of both its own and also other biotic signals for closure of the stomata.

Changes in stomatal sensitivity to ABA have been reported for different methods of ABA application and for modifications of the bathing solution of epidermal strips in stomatal aperture-assays (Snaith and Mansfield, 1982; Trejo *et al.*, 1993; Prokic *et al.*, 2006). However, how prolonged exposure to environmental factors provoke stomatal insensitivity to ABA have not been discussed. The question arises: why do long term low ABA levels make the stomata insensitive to ABA? Is it because of changes in the signalling pathway or because of sequestration of ABA in the leaf mesophyll or other parts?

Changes in ABA signal transduction

The action of ABA in guard cells begins with the transport of ABA from the vascular tissue or mesophyll to the guard cell apoplast and thereafter from the guard cell membrane to its receptors in the cytosol (Hirayama and Shinozaki, 2007; Pandey *et al.*, 2009; Kang *et al.*,

2010; Kuromori and Shinozaki, 2010; Kuromori *et al.*, 2010, 2011). ABC transporter genes such as *AtABCG40, AtABCG25* and *AtABCG22* are involved in both ABA transport and responses (Kang *et al.*, 2010; Kuromori *et al.*, 2010; Kuromori *et al.*, 2011). The *AtABCG40* and *AtABCG22* genes are mostly expressed in the guard cells, and probably function as ABA importers (Kang *et al.*, 2010; Kuromori *et al.*, 2011). On the other hand, the *AtABCG25* is a plasma membrane-localized protein which may function as an ABA exporter from vascular tissues (Kuromori *et al.*, 2010). Moreover, receptors have been identified that bind to extracellular (Anderson *et al.*, 1994) and intracellular ABA (Allan *et al.*, 1994; Assmann and Wu, 1994). The Receptor-like Kinase1 (RPK1) is localized in the plasma membrane and is involved in early ABA perception and possibly acts as an extracellular ABA receptor (Osakabe *et al.,* 2005). Also two G proteins (GTG1 and GTG2) have been identified as plasma membrane-localized extracellular ABA receptors which modulate ABA responses (Pandey *et al.*, 2009). Recently, however, Urano and Jones (2013) questioned the role of G proteins as plant hormone receptors. If long-term exposure to low VPD leads to sequestration of ABA in leaf mesophyll, it will be of interest to unravel the role of extracellular ABA receptors and transporters when plants have been exposed for long time to low VPD conditions. After transport of ABA from guard cell apoplast to cytosol, the earliest events of the ABA signalling pathway inside guard cells occur through a central signalling module made up of three protein classes: i) the PYR (PYRabactin Resistance)/PYL (PYR1-like)/RCARs (Regulatory Components of ABA Receptor)-family, ii) type 2C protein phosphatases (PP2Cs), and iii) the SNF1-related protein kinase (SnRK2) Open Stomata 1 (OST1). The current model for direct (short-term) ABA action through the PYR/PYL/RCAR receptors has been reviewed by Cutler *et al.* (2010) and is summarized in Figure 1. The proteins of class (i) (PYR/RCARs) operate as ABA receptors, the proteins of class (ii) (PP2Cs) operate as negative modulators of the ABA signalling pathway, while the proteins of class (iii) (SnRK2s/OST1) operate as positive modulators of downstream signalling (Mustilli *et al.*, 2002; Belin *et al.*, 2006; Park *et al.*, 2009; Ma *et al.*, 2009; Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Hubbard *et al.*, 2010). All of these components are present in both cytosol and nucleus and can induce long term as well as temporary changes in ABA responses (Moes *et al.*, 2008; Fujita *et al.*, 2009; Ma *et al.*, 2009; Santiago *et al.*, 2009; Raghavendra *et al.*, 2010). The combination of ABA receptors, PP2Cs and SnRK2/OST1 determine the activation or inactivation of downstream ABA signalling. Under long-term low VPD, the ABA level is continuously low in the guard cell cytosol. In this situation of low ABA concentration, the PP2C/ABI1 (ABA-Insensitive 1) inactivates SnRK2/OST1 via dephosphorylation; as a result

ABI1 repress ABA downstream signalling components. On the other hand in high VPD conditions, because of presence of ABA, the ABA is bound by intracellular PYR/RCAR dimers and they dissociate to form ABA receptor–PP2C complexes. The ABA-PYR/RCARs-PP2C complexes inhibit phosphatase activity, allowing SnRK2 activation and phosphorylation of target proteins (Fujii *et al.*, 2009; Geiger *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009). In this way, a double-negative regulatory pathway is established in which ABA-bound PYR/RCARs inhibit PP2C activity, while in a condition such as low VPD, PP2C inactivate SnRK2s (Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009; Vlad *et al.*, 2009; Lee and Luan, 2012). Moreover, in response to ABA the phospholipase Dα1 (PLDα1) produces phosphatidic acid (PA) that binds to ABI1, which in turn releases the inhibition of OST1 by ABI1 (Zhang *et al.*, 2004; Mishra *et al.*, 2006; Takemiya and Shimazaki, 2010) (Fig. 1) and strengthen the ABA induced OST1 activity. PA also acts as a lipid secondary messenger (see next section). Yoshida and colleagues (Yoshida *et al.*, 2002) found that SnRK2 can be activated by high VPD. By a T-DNA knockout mutation in a SnRK2-type protein kinase gene, stomata failed to close completely in response to ABA and high water loss took place after a rapid decrease in humidity (Yoshida *et al.*, 2002). Whether low VPD causes the opposite effect on SnRK2 is unknown. If so, low VPD will result in a decreased ABA-sensitivity.

We can hypothesize that a long period of low ABA as a result of a prolonged exposure to low VPD, as discussed above, will result in ABA desensitisation due to a strong negative regulation of ABA responses via activated PP2Cs (lack of inhibitory interaction of PYR/RCARs with PP2Cs) and weak positive regulation of ABA signalling via the inhibitory effect of PP2Cs on SnRK2s (Fig. 1).

ABA, transcription factors and stomatal malfunctioning

Transcription Factors (TFs) are proteins involved in the regulation of cellular processes via initiating and controlling the transcription of genes. AtMYB60 is the first TF characterized for a role in stomatal opening (Cominelli *et al.*, 2005). The involvement of TFs in ABA responses has been well documented. As example, in the presence of ABA phosphorylation of bZip TFs by SnRK2 leads to closure of the stomata (Yoshida *et al.*, 2002; Raghavendra *et al.*, 2010; Umezawa *et al.*, 2010). It has been reported that high VPD promotes the expression of bZip TFs such as ABI5 (Bauer *et al.*, 2012). An APETALA2/EREBP-type TF (AtERF7) downregulates the expression of ABA-induced genes. *AtERF7* overexpressed plants are dysfunctional to ABA and have less control over transpiration after desiccation (Song *et al.*,

2005). Also, Nuclear Protein X1 (NPX1) acts as a transcriptional repressor of ABA-regulated genes. Plants overexpressing *NPX1* are hypersensitive to drought because of a decreased ability for closing their stomata (Kim *et al.*, 2009). All above mentioned studies were related to abiotic stress conditions like drought and salinity. However, there is not any research about the role of relevant transcription factors in stomata that are malfunctioning due to long-term exposure to low VPD. Therefore, it will be relevant to unravel the role of transcription factors in guard cells of plants exposed for long time to low VPD conditions.

When *Tradescantia* plants were grown for a long time (3 weeks) in a low VPD condition, the problem of stomatal malfunctioning increased by leaf age; the stomata of older leaves were less responsive to desiccation compared with the younger leaves (Rezaei Nejad and van Meeteren, 2007). Interestingly, in *Arabidopsis* the rate of water loss by desiccation increases by leaf age; the expression of Senescence-Associated Gene113 (*SAG113*) and of *AtNAP* (gene encoding for a NAC family transcription factor, AtNAP) are co-induced during leaf senescence (Zhang *et al.*, 2012; Zhang and Gan, 2012). *SAG113*, a gene that encodes a protein phosphatase belonging to the PP2C family, functions as a negative regulator of the ABA signalling pathway and prevents stomatal closure in response to closing stimuli such as ABA and desiccation (Zhang *et al.*, 2012). The TF AtNAP physically interacts with the promoter region of *SAG113* and promotes its expression at transcriptional level (Zhang and Gan, 2012) and keeps the stomata less functional to closing stimuli. Therefore it could be of interest to unravel the role of the AtNAP-SAG113 PP2C regulatory node in the plants when they are exposed for a long time to low VPD conditions. Can stomata of long-term low VPD exposed plants of *atnap* or knockout SAG113 close in response to closing stimuli?

By ubiquitin-mediated regulation of protein stability, E3 ubiquitin ligases play a role in post-translational control of protein degradation (the ubiquitin proteasome system or UPS) (Lyzenga and Stone, 2012). Through modulating the abundance of TFs, E3 ubiquitin ligases facilitate plant adaptation to adverse environmental conditions. Several E3 ubiquitin ligases have been suggested as negative regulators of ABA signalling (Zhang *et al.*, 2008; Peng *et al.*, 2012; Seo *et al.*, 2012). E3 ligases may reduce the sensitivity to ABA via degradation of ABI3 and ABI5 TFs (Lyzenga and Stone, 2012). As other example, a negative feedback loop for the F-Box protein DOR in ABA responses has been demonstrated. DOR functions as negative regulator of ABA, while the *DOR* gene is suppressed by ABA (Zhang *et al.*, 2008). DOR inhibits ABA-induced stomatal closure under drought conditions independently from PLDα1. Another F-Box protein, FOA1, also plays a negative role in ABA signal transduction (Peng *et al.*, 2012). AtPUB18 and AtPUB19, which are U-box E3 ubiquitin ligases, negatively regulate

ABA signalling downstream of H_2O_2. On the other hand, other U-box E3 ubiquitin ligases, AtPUB22 and AtPUB23, are negative regulators of drought responses which act independently from ABA (Seo *et al.*, 2012). Expressions of *AtPUB18* and *AtPUB19* depends on SnRK2, while expressions of *AtPUB22* and *AtPUB23* are independent from SnRK2. Since several E3 ligases, that function as negative regulators of ABA, are suppressed by ABA (as discussed above), the extended period with low ABA levels under conditions of low VPD will result in abundant presence of these negative regulators. Therefore, it would be interesting to investigate the involvement of UPS in the stomatal response of low VPD grown plants to closing stimuli.

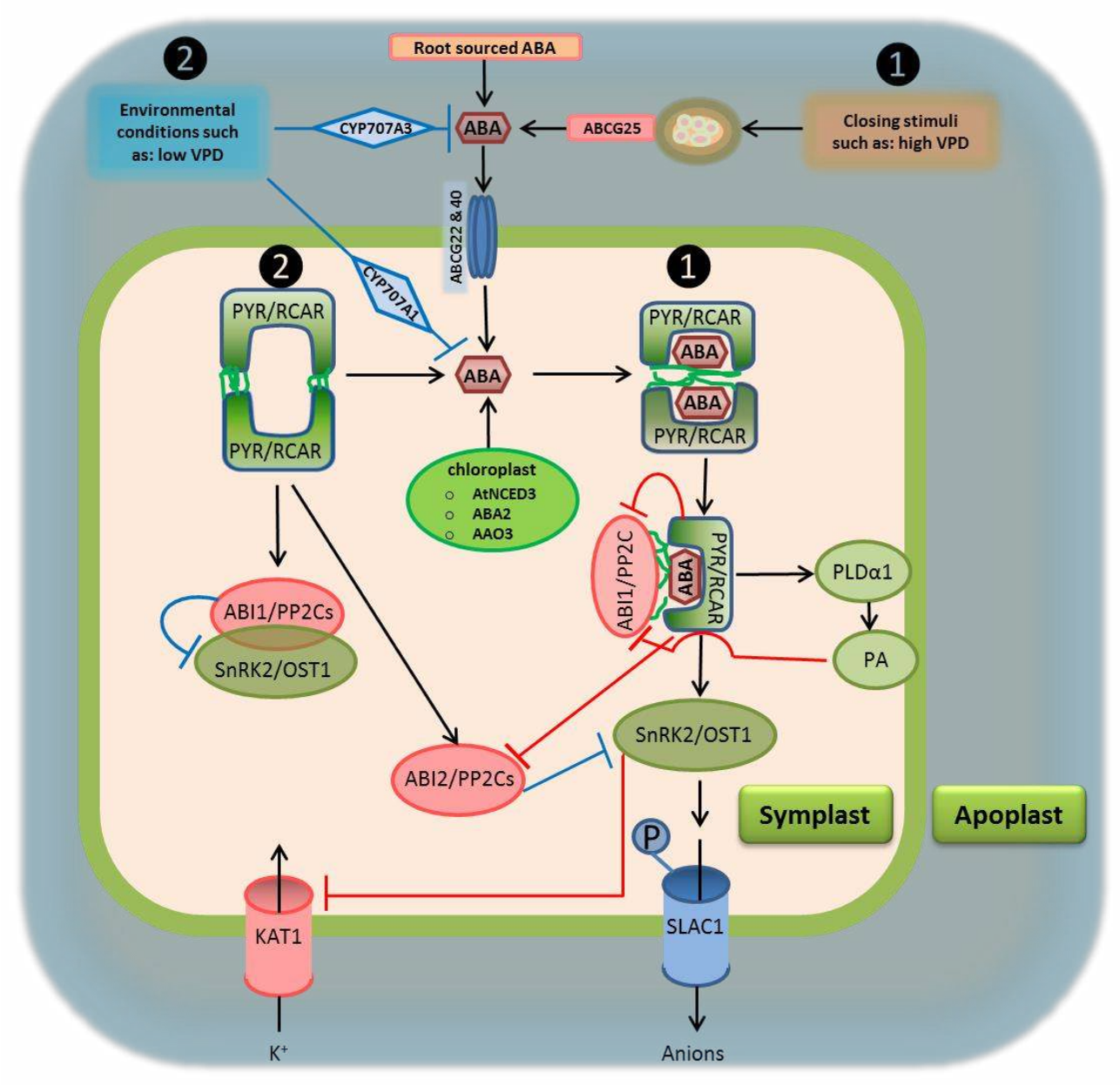

Figure 1. Schematic overview of the perception of ABA in stomata guard cells. In conditions which favour ABA production (*right*), such as high VPD ❶, the produced ABA accumulate in the apoplast. Through the function of importers (AtABCG22&40) its level increases in the guard cell symplast. By binding of ABA to its receptor PYR/PYL/RCAR, it is able to block ABI1&2/PP2C activity; as a result SnRK2/OST1 protein kinase will be activated. Also production of PA through PLDα1 will be increased, inhibiting ABI1/PP2C activity even more. Consequently, SnRK2/OST1 will stimulate SLAC1 as well inhibit KAT1; as a result stomatal closure will take place. On the other hand in the conditions which don't favour ABA production (*left*), such as low VPD ❷, the rest ABA will be catabolized by *CYP707A1* inside the guard cells and by *CYP707A3* outside of the guard cells; in this situation PYR/PYL/RCAR is unable to block ABI1&2/PP2C activity. As a result ABI1/PP2C will inactivate SnRK2/OST1 protein kinase; therefore the ion channels such as KAT1 continue to import K^+ which causes stomata to remain open. Red bars show blockage effects in the presence of ABA. Blue bars show blockage effects in the absence of ABA. Arrows indicate positive effects.

Involvement of crosstalk between secondary messengers

A variety of second messengers have been implicated in the perception of stimuli of stomatal closure (Fig. 2), like cytosolic calcium ($[Ca^{2+}]_{cyt}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Pei *et al.*, 2000; Zhang *et al.*, 2001; Siegel *et al.*, 2009; Kim *et al.*, 2010; Wang *et al.*, 2011; Distéfano *et al.*, 2012; Hubbard *et al.*, 2012). Therefore, disturbances in the regulation of these secondary messengers can be other candidates to explain stomatal malfunctioning due to long-term exposure to low VPD. $[Ca^{2+}]_{cyt}$ is one of the most important secondary messengers in stomatal guard cells (Leckie *et al.*, 1998; Siegel *et al.*, 2009; Hubbard *et al.*, 2012). For example, stomata close in response to ABA due to cytosolic calcium oscillation (Allen *et al.*, 2000), and increases in $[Ca^{2+}]_{cyt}$ have been observed in response to closing stimuli like elevated CO_2, oxidative stress and external calcium (Neill *et al.*, 2008; Kim *et al.*, 2010; Wang *et al.*, 2011). ABA-induced anion channel activation and potassium channel inactivation can be calcium-independent as well as calcium-dependent (Li and Assmann, 1996; Levchenko *et al.*, 2005; Marten *et al.*, 2007; Sutter *et al.*, 2007; Geiger *et al.*, 2009, 2010; Siegel *et al.*, 2009; Joshi-Saha *et al.*, 2011). ABI1 and OST1 are Ca^{2+} independent proteins. OST1 provides an ABA-sensitive, but Ca^{2+}-independent element for activation of anion channels (Li and Assmann, 1996). Calcium Dependent Protein Kinases (CDPK's) are elements of the Ca^{2+}-dependent ABA responses (Zhu *et al.*, 2007). SLow Anion Channel-associated 1 (SLAC1), which is the main anion channel in the ABA signalling pathway, can be activated by CDPK's (Geiger *et al.*, 2010; Mori *et al.*, 2006); CDPK's also inhibit the inward-rectifying K^+ channel (KAT1) by phosphorylation (Fig. 2) (Li *et al.*, 1998). ABA activates guard cell plasma membrane Ca^{2+}-permeable cation (I_{Ca}) channels, which mediate Ca^{2+} influx from extracellular space (Hamilton *et al.*, 2000), and also induces Ca^{2+} release from intracellular stores (Blatt, 2000). Both effects of ABA will increase $[Ca^{2+}]_{cyt}$ and thus activate SLAC1 via CDPK.

According to Weinl et al. (Weinl *et al.*, 2008), guard cells possess a Ca^{2+}-sensing receptor (CAS) localized in chloroplasts that is crucial for proper stomatal regulation. CAS is required for an increase in $[Ca^{2+}]_{cyt}$ as response to an increase in the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) (Han *et al.*, 2003). It has been demonstrated that removal of external Ca^{2+} inhibited increases in $[Ca^{2+}]_{cyt}$ (Klüsener *et al.*, 2002).

H_2O_2 is an essential intermediate in guard cell ABA signalling. ABA activation of I_{Ca} channels requires H_2O_2 production by membrane bound NADPH oxidases AtrbohD and F (Pei *et al.*, 2000). ABI1 and OST1 act upstream of H_2O_2 (Mustilli *et al.*, 2002). NO functions as a downstream intermediate of H_2O_2 signalling to mediate ABA-induced stomatal closure

(Murata *et al.*, 2001; Wang *et al.*, 2011) (Fig. 2). Recently, it has been shown that $[Ca^{2+}]_o$ increases H_2O_2 and NO levels inside the guard cells via CAS (Wang *et al.*, 2011). Therefore, increase in the $[Ca^{2+}]_o$ can result in increase in the activation of SLAC1. Moreover, a combination of an extracellular Ca^{2+}-sensor, extracellular calmodulin (ExtCaM), and $[Ca^{2+}]_o$ can activate a signalling pathway that results in activation of GPA1 (G Protein Alpha subunit 1), and thereafter H_2O_2 and NO generation, resulting in changes in $[Ca^{2+}]_{cyt}$ and then stomatal closure (Fig. 2) (Chen *et al.*, 2004; Li *et al.*, 2009; Zhang *et al.*, 2011).

As a result of low Ca^{2+} transport in the xylem due to low transpiration rate, the $[Ca^{2+}]_o$ will be low after long term exposure to low VPD. This will result in low activity of CAS and ExtCaM. Moreover, I_{Ca} channels will have low activity due to the low ABA concentration at low VPD (see above). Therefore, it can be expected that long exposure to low VPD results in a low $[Ca^{2+}]_{cyt}$ and, as result of that, in low H_2O_2 and NO concentrations. This will result in low ABA sensitivity and a diminished closure response. It has been shown that ABA enhances the $[Ca^{2+}]_{cyt}$ sensitivity of stomatal closure mechanisms (Siegel *et al.*, 2009).

A bifurcating signalling pathway for PA is demonstrated: besides interacting with ABI1 (Fig. 1), PA also stimulates GPA1 which can induce production of H_2O_2 and NO (Fig. 2) (Mishra *et al.*, 2006; Zhang *et al.*, 2011); in this way there is a Ca^{2+} independent signalling pathway for NO-induced stomatal closure. In guard cells of stomata that malfunction, due to long term exposure to H_2S, NO production in response to ABA application was reduced (Lisjak *et al.*, 2010). After prolonged exposure to 90% RH, guard cells of *Tradescantia virginiana* were not fully responsive to short term application of the NO-donor SNP (Rezaei Nejad and van Meeteren, 2007). These observations indicate that in malfunctioning stomata the signalling pathway was disrupted downstream of ABI1 and OST1.

ABI2 is considered to exert a negative regulation on ABA action downstream of H_2O_2 (Murata *et al.*, 2001; Mustilli *et al.*, 2002). The protein phosphatase activity of ABI2 is very sensitive to H_2O_2. Therefore, the ABA signalling pathway will be activated by H_2O_2-induced transient inactivation of ABI2 (Meinhard *et al.*, 2002). ABI2 represents a likely target for redox-regulation of a hormonal signalling pathway. It physically interacts with the glutathione peroxidase GPX3, which regulate the redox state of guard cells (Meinhard *et al.*, 2002; Miao *et al.*, 2006). Oxidized GPX3 converted the reduced form of ABI2 into the oxidized form; this reduces the phosphatase activity of ABI2 approximately five-fold. GPX3 is also a key enzyme in scavenging H_2O_2 (Fig. 2) by catalysing the reduction of H_2O_2 by glutathione (GSH). Thus, GPX3 functions in both H_2O_2 sensing and scavenging (Miao *et al.*, 2006). Ascorbate (Asc) is another major antioxidant that scavenges H_2O_2 resulting in dehydroascorbate (DHA)

(oxidixed ascorbate). Dehydroascorbate reductase (DHAR) catalyses the reduction of DHA to Asc and thus contributes to the regulation of the Asc redox state (ratio of Asc/DHA). DHA is reduced to Asc by the expense of GSH (glutathione-ascorbate cycle) (Noctor and Foyer, 1998; Gallie, 2013). Chen and Gallie (2004) demonstrated for tobacco that the levels of H_2O_2 and the Asc redox state are diurnally regulated such that H_2O_2 in guard cells increases during the afternoon, whereas the Asc redox state decreases. An increase in H_2O_2 and increased oxidation of Asc coincided with stomatal closure. Guard cells with an increase in Asc redox state as a result of DHAR overexpression were less responsive to H_2O_2 or ABA signalling. A more reduced state of Asc and GSH will result in a higher scavenging capacity on H_2O_2 as well as in a high negative regulatory effect by ABI2, both resulting in less ABA sensitivity. However, it is not clear why a prolonged exposure to low VPD should result in an increase in the redox state of Asc and/or GSH and by that in less ABA sensitivity.

DST (Drought and Salt Tolerance) is a C2H2 zinc finger transcription factor which is involved in stomatal movement regulation. It has been shown that DST employs an ABA-independent pathway for regulating stomatal aperture (Huang *et al.*, 2009). DST influences the transcription of genes involved in the H_2O_2 homeostasis. Therefore stomata stay open when DST is in an active state through inhibition of H_2O_2 accumulation. In the ABA-independent pathway, it can be expected that the redox state of guard cells will be higher in low VPD compared to high VPD-exposed plants, due to the lack of $[Ca^{2+}]_o$-induced H_2O_2 production. Moreover, it is feasible to assume that, because of the low level of ABA in long-term low VPD-exposed plants, the ABI2 will be in its active form resulting in low H_2O_2 and NO production.

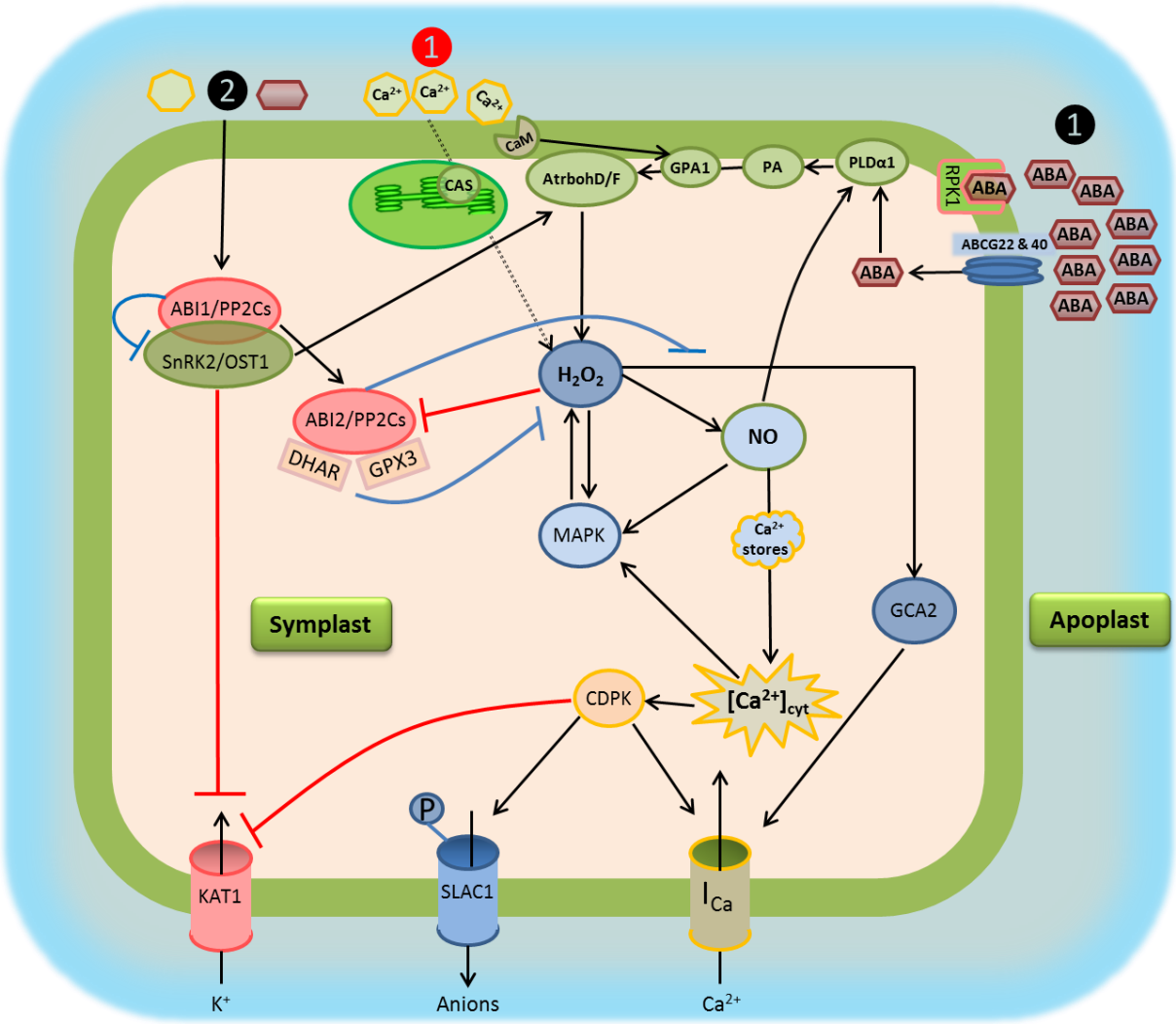

Figure 2. Schematic of secondary messengers cross-talk in stomata guard cells. Under the conditions which lead to high transpiration rate (*right)*, such as high VPD, Ca^{2+} and ABA will accumulate in the guard cells apoplast. In the case of ABA ❶ after increasing its concentration in the guard cells symplast, it leads to activation of NADPH oxidases, AtrbohD and AtrbohF, through PA-activated GPA1. As a result, the level of H_2O_2 increases which leads to: 1. NO production, 2. MAPK activation, 3. I_{Ca} channels activation via GCA2. Consequently, stomatal closure takes place through the regulation of ion channels. When Ca^{2+} accumulate in the guard cell apoplast ❶ its concentration will increase in the guard cells symplast via I_{Ca} channel, also CAS activation will results in $[Ca^{2+}]_{cyt}$ transients and H_2O_2 accumulation which cause activation of MAPK as well as CDPK. Besides, Extracellular CalModulin (CaM) can activate the signalling pathways leading to H_2O_2 and NO generations. As a result, export of anions via SLAC1 will be accelerated and import of K^+ via KAT1 will be inhibited; therefore membrane potential depolarizes and stomatal closure occurs. On the other hand under conditions which lead to lower transpiration rate (*left)*, such as low VPD ❷, the concentration of ABA and Ca^{2+} will be low in apoplast and symplast of the guard cells which leads to activation of PP2C/ABI2 via the inhibitory effect of PP2Cs/ABI1 on SnRK2/OST1. The ABI2 can prevent H_2O_2 signal transduction; consequently the downstream components will not be activated and stomata remain open. Red bars show blockage effect under high apoplastic and symplastic ABA and Ca^{2+} concentrations. Blue bars show blockage effect under low apoplastic and symplastic ABA and Ca^{2+} concentrations. Arrows indicate positive effects.

Is there a role for other phytohormones in the malfunctioning of stomata?

In addition to ABA other phytohormones, as well as interplay between them, regulate stomatal movements. An extensive overview of hormone interactions in stomatal function was given by Acharya and Assmann (2009). Brassinosteriods (BRs), salicylic acid (SA) and jasmonic acid (JA) trigger stomatal closure (Mori *et al.*, 2001; Haubrick *et al.*, 2006; Gonugunta *et al.*, 2009; Ashraf *et al.*, 2010; Sun *et al.*, 2010; Hossain *et al.*, 2011; Khokon *et al.*, 2011; Munemasa *et al.*, 2011), while promotion of stomatal opening has been reported for auxin and cytokinin (Song *et al.*, 2006; Tanaka *et al.*, 2006). Application of BRs induces water stress tolerance in many plant species by closing stomata (Acharya and Assmann 2009). However, stomatal closure was more sensitive to ABA in *Arabidopsis* loss-of-function mutant *bsk5* plants (BSK5 encoding a brassinosteroid-signaling kinase protein) (Li et al. 2012). According to Acharya and Assmann (2009) it is likely that interactions between BRs, ABA, and guard cell responses are species-specific. Whether water stress or air humidity causes changes in endogenous BR is not clear. SA induces stomatal closure, likely via stimulation of ROS production; it plays a key role in pathogen defense and accumulates in water-stressed plants (Acharya and Assmann 2009). It has been reported that both short-term and long-term O_3 application induced production of SA in different plant species (Overmyer *et al.*, 2008; Cui *et al.*, 2012). We are not aware of papers describing an effect of RH on SA. JA mediates plant defense against necrotic pathogen and insects and is often recognized as a biotic stress hormone (Liechti and Farmer 2002; Fujita *et al.,* 2006). JA accumulates during drough stress and has a postive role in stomatal closure. It is suggested that JA-mediated stomatal response requires ABA and that JA and ABA employe common signaling components (Acharya and Assmann 2009; Zhu *et al.,* 2012). However significant induction in JA production was not observed after long-term and short-term O_3 application (Overmyer *et al.*, 2008; Cui *et al.*, 2012). Therefore there are no indications of the involvement of JA in the malfunctioning of stomata. It has been reported that cytokinins and auxins influence stomatal movements via ethylene. These plant hormones can promote stomatal opening via the antagonistic effects of ethylene on ABA-induced stomata closure, likely through the modulation of ethylene biosynthesis (Tanaka *et al.*, 2006). However, there are contradictory reports regarding stomatal responses to ethylene. It seems that stomata close in response to ethylene in the absence of ABA and open in the presence of ABA (Desikan *et al.*, 2006; Tanaka *et al.*, 2006). It has been shown that ethylene or ACC (the precursor of ethylene) can prevent ABA-induced stomatal closure (Tanaka *et al.*, 2005). Under drought conditions, application of ethylene increases stomatal aperture of wild type *Arabidopsis* (Tanaka *et al.*, 2005). Ethylene seems to

act in the later steps in the ABA-induced stomatal closure but not in the early steps (Tanaka *et al.*, 2005). The interaction between AtERF7 (which is a member of ethylene-responsive binding factors) and PKS3, a Ser/Thr protein kinase which interact with ABI2 and to some extent ABI1 (Guo *et al.*, 2002), reduces the sensitivity of guard cells to ABA and induces water loss through transpiration (Song *et al.*, 2005). It seems that ethylene acts via an ABA-independent pathway that leads to stomatal closure. The ethylene receptor ETR1 mediates H_2O_2 signalling in guard cells and is maybe a sensor for H_2O_2 perception in guard cells (Desikan *et al.*, 2005). So ETR1 maybe the site of ethylene and H_2O_2 cross-talk leading to stomatal closure (Desikan *et al.*, 2005). Ethylene antagonizes ABA-induced stomatal closing response after long term O_3 application in wild-type *Arabidopsis* plants. Exposure of plants for a long time to elevated O_3 concentrations (70 ppb) leads to stomatal malfunctioning in response to ABA and water stress (Wilkinson and Davies, 2009). In this case the malfunctioning of the stomata coincided with an induction of ethylene production. On the other hand, when the plants were exposed for a short time to O_3, after 6 hr the level of ACC and ethylene decreased to the control level; at the same time stomata start to close (Overmyer *et al.*, 2008). Consistent with this hypothesis, it has been shown that pre-treatment of ozone-treated plants by 1-MCP (a blocker of ethylene receptors) restores the closing response of stomata to ABA or water stress (Wilkinson and Davies, 2009). However, there is not any information about effects of ethylene on behaviour of stomata after prolonged exposure to low VPD conditions. Most of the ethylene responses are Ca^{2+} dependent (Raz and Fluhr, 1992). Therefore, in low VPD conditions together with low ABA and Ca^{2+}, cross-talk between plant hormones could also be an effector in stomatal response to the environmental conditions which result in stomatal dysfunction. In addition to interaction of ABA and ethylene on stomatal regulation, it has been shown that auxin can stimulate stomatal opening and is able to inhibit stomatal closure in response to ABA and other closing stimuli such as darkness and CO_2 (Řicánek and Vicherková, 1992). Exogenous application of the naturally occurring auxin indolyl-3-butyric acid (IBA) to epidermal peels can open stomata under darkness, likely via a Ca^{2+}dependent signalling in the guard cell. However the interaction of phytohormones in the malfunctioning of stomata under low VPD condition has not been demonstrated.

Conclusion and future challenges

Through reviewing literature to link stomatal malfunctioning with signalling pathways in guard cells, we have attempted to connect signalling components inside the guard cells to the signalling elements outside of the guard cells. Although a number of experiments have shown

the occurrence of stomatal malfunctioning under some prolonged environmental conditions, the mechanisms that are involved in the guard cells of malfunctioning stomata are still poorly recognized. We propose alterations in signalling pathways due to long-term low transpiration rate under long-term exposure to environmental conditions especially low VPD, which lead to depletion of ABA, Ca^{2+} and H_2O_2 in the guard cells, as well depletion of extracellular ABA and Ca^{2+}. This will be accompanied by a low sensitivity for ABA due to a long negative regulation of the ABA signalling pathway by the PP2Cs ABI1 and ABI2 and a low positive regulation of the ABA signalling pathway by OST1. These effects will be strengthened by a low sensitivity of the anion channel SLAC1 for Ca^{2+}. This coincidence in changes of Ca^{2+}, ABA receptors, and positive and negative regulators of ABA signalling is proposed as an explanation for the stomatal malfunctioning induced by long-term exposure to low VPD. Among essential experiments that could help to understand the signalling pathway in malfunctioning stomata, are:

- The effect of short term and long term exposure to low VPD on the activity of ABA transporters and ABA perception in stomata guard cells.
- Characterization of transcription factors such as transcriptional activators (for example: AtMYB60 and AtNAP) and transcriptional repressors (for example: NPX1 and AtERF7) when plants have been exposed for long time to low VPD.
- The up or down-regulation of E3 ligases by long term exposure to low VPD and their role in responsiveness to stomata closing stimuli
- Interaction of phytohormones such as ABA and ethylene, ABA and auxin for controlling stomatal movements when plants have been exposed for a long time to low VPD.
- Using a reverse genetic approach for identifying the place of stomatal malfunctioning in guard cell signal transduction pathway.

These experiments, together with other approaches (such as transcriptome profiling and QTL mapping) can help us to understand the disturbed signal transduction in guard cells of stomata of plants that have been exposed to long-term environmental conditions, like low VPD.

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CHAPTER 4

Natural variation in stomatal response to closing stimuli among *Arabidopsis thaliana* accessions after exposure to low VPD as a tool to recognise the mechanism of disturbed stomatal functioning

Abstract

Stomatal responses to closing stimuli are disturbed after long-term exposure of plants to low VPD. The mechanism behind this disturbance is not fully understood. Genetic variation between naturally occurring ecotypes can be helpful to elucidate the mechanism controlling stomatal movements in different environments. We characterized the stomatal responses of 41 natural accessions of *Arabidopsis thaliana* to closing stimuli (ABA and desiccation) after they had been exposed for 4 days to moderate VPD (1.17 kPa) or low VPD (0.23 kPa). A fast screening system was used to test stomatal response to ABA, using chlorophyll fluorescence imaging under low O_2 concentrations of leaf discs floating on ABA solutions. In all accessions stomatal conductance (g_s) was increased after prior exposure to low VPD. After exposure to low VPD, stomata of 39 out of 41 of the accessions showed a diminished ABA closing response; only stomata of low VPD-exposed Map-42 and C24 were as responsive to ABA as moderate VPD-exposed plants. In response to desiccation, most of the accessions showed a normal stomata closing response following low VPD exposure. Only low VPD-exposed Cvi-0 and Rrs-7 showed significant less stomatal closure compared to moderate VPD-exposed plants. Using Principle Component Analysis (PCA) accessions could be categorized to very sensitive, moderately sensitive and less sensitive to closing stimuli. In conclusion we present evidence for different stomatal responses to closing stimuli after long-term exposure to low VPD across *Arabidopsis* accessions. The variation can be a useful tool for finding the mechanism of stomatal malfunctioning.

Keywords: *Arabidopsis thaliana*, stomata, vapour pressure deficit (VPD), abscisic acid, natural variation, desiccation

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Introduction

Stomata pores in the epidermis of leaves are largely responsible for gas exchange, especially CO_2 uptake and water loss, between plant and atmosphere. A fine regulation of the stomata aperture is required to allow sufficient CO_2 uptake for photosynthesis, while preventing excessive water loss through transpiration under various environmental conditions.

It is well known that as a short term response, stomata widen their aperture when the atmospheric vapour pressure deficit (VPD) is low and decrease the aperture after an increase of VPD (Outlaw and De Vlieghere-He, 2001; Shope *et al.*, 2008; Okamoto *et al.,* 2009; Aliniaeifard and van Meeteren, 2013; Aliniaeifard *et al.*, 2014). The mechanism of stomatal responses to VPD has been the subject of many researches during the last few decades (Farquhar, 1978; Appleby and Davies, 1983; Assmann and Gershenson, 1991; Mott and Parkhorst, 1991; Bunce, 1997; Mott and Peak, 2012; Fanourakis *et al.*, 2013). As well 'feedforward' as 'feedback' hypotheses have been proposed for the stomatal response to VPD (Farquhar, 1978; Grantz, 1990; Saliendra *et al.*, 1995). In the 'feedforward' hypothesis the stomatal response to VPD is a result of direct sensing of the VPD and independent from leaf water status (Farquhar, 1978; Franks *et al.*, 1997). It was shown that ABA can act as intermediary between stomatal responses and VPD (Grantz, 1990; Zhang and Davies, 1991; Buncec, 1998; Tardieu and Simonneau, 1998). A close relationship has been observed between VPD and the ABA level in the leaf. Increasing VPD results in ABA accumulation in the leaf (Bauerle *et al.*, 2004) and decreasing VPD causes catabolism of ABA (Okamoto *et al.*, 2009). Mott and Parkhurst (1991) proposed that stomata respond to VPD via transpiration rate rather than humidity *per se*. In the 'feedback' hypothesis, stomatal response to VPD is a result of a negative feedback of transpiration on leaf water status (Raschke, 1970; Saliendra *et al.*, 1995). In this hypothesis indirect induction of ABA production by increased transpiration has been proposed (Buckley, 2005). However, the involvement of ABA in the stomatal response to VPD is still debated. Assmann *et al.* (2000) showed both ABA insensitive (*abi1-1* and *abi2-1*) and ABA deficient mutants (*aba1*) of *Arabidopsis* have a similar stomatal response to increased VPD compared with the wild-type *Arabidopsis* plants, which make the role of ABA more complicated. Recently feedback and feedforward mechanisms together have been taken into account for stomatal response to VPD (Peak and Mott, 2011). Accordingly, a dual role for ABA-induced stomatal closure has been proposed: (1) a direct biochemical mechanism on guard cells of stomata and (2) an indirect effect of ABA via a decreased leaf hydraulic conductance (Pantin *et al.*, 2013).

In all mentioned studies, the short-term response of the stomata to VPD has been investigated and they focused on the stomatal response to high VPD. However, when plants were grown at low VPD, the behaviour of the stomata in response to desiccation or ABA changed and the stomata showed a diminished response to closing stimuli (Fordham *et al.*, 2001a, b; Rezaei Nejad and van Meeteren, 2005, 2007, 2008;Rezaei Nejad *et al.*, 2006; Fanourakis *et al.*, 2011; Arve *et al.*, 2012; Aliniaeifard and van Meeteren, 2013; Aliniaeifard *et al.*, 2014). Even when full grown leaves were transferred from high to low VPD this loss of stomatal response to closing stimuli can be induced (Rezaei Nejad and van Meeteren, 2008). The occurrence of stomatal malfunctioning depends on the duration of the exposure to low VPD and it is species dependent (Fanourakis *et al.*, 2011; Aliniaeifard and van Meeteren, 2013; Aliniaeifard *et al.*, 2014). We previously proposed that after prolonged exposure to low VPD a perturbation in the ABA signalling pathway inside the guard cells leads to the malfunctioning of the stomata. However, the altered signalling pathway in the guard cells of dysfunctional stomata is still unknown (Aliniaeifard and van Meeteren, 2013).

Variation in sensitivity of stomatal conductance to VPD has been observed at intraspecific levels. In red maple, for example, wet site ecotypes responded quicker to water stress than dry site ecotypes by biosynthesizing ABA and by closing their stomata (Bauerle *et al.*, 2004). *Arabidopsis* is widely distributed around the world and large variation has been found in this species for many aspects. Genetic variation between accessions of *Arabidopsis* under stress conditions has been found for responses to high light (Jung and Niyogi, 2009; Athanasiou *et al.*, 2010), ozone (Brosché *et al.*, 2010), freezing (Hannah *et al.*, 2006), drought (Bouchabke *et al.*, 2008), high temperature (Edwards *et al.*, 2006), and salinity (Katori *et al.*, 2010). Brosche *et al.* (2010) investigated the ozone sensitivity between *Arabidopsis* accessions and correlated it to stomatal conductance. Bouchabke *et al.* (2008) showed differences in cut rosette water loss between accessions under drought stress and assumed that these differences were related to differences in stomatal aperture. The ABA signalling pathway in guard cells comprises a network of many components. In order to find the effect of prior exposure to low VPD on guard cell signalling, it will be very helpful to identify variation in stomatal response to closing stimuli in a collection of *Arabidopsis* accessions after exposure of the plants to different VPDs. However, to the best of our knowledge there is not any publication available in relation to natural variation in the stomatal response of *Arabidopsis* to closing stimuli nor in the stomatal response after prolonged exposure to different VPDs.

In this paper, we analysed the stomatal response of 41 distinct accessions of *Arabidopsis* to ABA and to desiccation after growing them at moderate VPD as well as after transfer of the

plants for four days to low VPD. For efficient large scale screening of stomatal responses to ABA in plants that have been exposed to different environmental conditions, we developed a system in which we used chlorophyll fluorescence imaging under a non-photorespiratory condition for leaf discs floating on ABA solutions. For screening of stomatal responses to desiccation we used the rate of water loss as function of leaf relative water content (RWC) from excised leaves to characterize the water loss parameters of the *Arabidopsis* accessions. We asked the following questions: (1) how large is the variation in the stomatal response of the *Arabidopsis* accessions to closing stimuli (ABA and desiccation) after acclimation to moderate (M) and low (L) VPDs? (2) are there relationships between the stomatal conductance after long-term exposure to M and L conditions (without closing stimuli) and responses of the stomata to closing stimuli? (3) is there a relationship between foliar ABA content before or during desiccation after long-term exposure to M and L conditions and stomatal response to closing stimuli?

Materials and methods

An overview of treatments and measurements is given in Appendix A (page 100).

Plant material and growth conditions

41 natural accessions of *Arabidopsis thaliana* obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio State University, USA were used. The accessions were chosen in such a way that there was a large genetic and geographical diversity among them. The names and geographical characterisations of the accessions are given in Table 1.

After stratification of seeds at 4 °C for 4 days, the seeds were sown in a pot filled with a soil developed for *Arabidopsis* (Arabidopsis soil, Horticoop, the Netherlands). After germination, in the stage of 2 leaves, the plants were transplanted to pots filled with a mixture of fine and course sands. The bottom of the pots were covered with net-like plastic sheets and the top of the sand mixture was covered with 0.5 cm Arabidopsis soil. The surface of the soil was covered with a black plastic sheet to prevent contact of the leaves with wet soil and to prevent a micro-climate with low VPD around the rosette of the plants. The plants were placed in a tray and irrigated 4 times per week using a nutrient solution developed for *Arabidopsis*. All plants were grown in a climate chamber with a constant temperature of 20°C, 60% relative humidity (RH), resulting in a VPD of 0.94 kPa, 12h/12h day night lighting period, 150 µmol m^{-2} s^{-1} light (measured with an LI-250 light meter, Li-Cor, Lincoln, NE, USA) produced by fluorescent tubes (TLD 58W/84 Philips) and 380 µmol mol^{-1} CO_2 (determined using Indoor

Air Quality Meter, Model 8760, TSI Incorporated, Shoreview, USA). When the plants produced fully developed leaves in the stage between 3.9 and 5 (stages as indicated by Boyes *et al.* (2001)), they were transferred to other growth chambers with the same temperature and light conditions but with different VPDs. One of them with 50±5% RH, resulting in a VPD of 1.17 kPa (M); another one with 90±5% RH, resulting in a VPD of 0.23 kPa (L). Temperature and RH in the climate room and growth chambers were recorded every 5 min using data loggers (Fourier MicroLog EC650, MicroDAQ.com, Ltd. Contoocook, New Hampshire, USA). After four days exposure to the two VPD conditions, fully developed leaves were used for analysing the response of stomata to ABA and desiccation.

Stomatal conductance

Stomatal conductance (g_s) was recorded in fully developed leaves after exposure to different VPDs, using a porometer (Delta-T Devices Ltd, Cambridge, UK) in an environment with a 20°C temperature, 50% RH and 150 µmol m^{-2} s^{-1} illumination.

Mapping of stomatal response to ABA using chlorophyll fluorescence

To investigate the stomatal response of M and L-exposed plants to ABA, chlorophyll fluorescence imaging under a non-photorespiratory condition (low O_2 concentration) was used. Because PSII photochemical efficiency (Φ_{PSII}) was measured while photorespiration was inhibited, a decreased Φ_{PSII} is closely related to stomatal closure (Rezaei Nejad *et al.*, 2006). Leaf discs (0.5 cm diameter) were prepared from 8 leaves (one disc/leaf) of 8 individual plants (one leaf/plant). Middle of the leaf between main vein and leaf margin was chosen for making the leaf discs. The leaf discs were put with their adaxial surface down in petri dishes filled with stomata-opening medium (50 mM KCl, 10 mM MES-KOH, pH 6.15, 50 µM $CaCl_2$ in degassed distilled water) with different concentrations of ABA (0, 50, 100, 200 µM ABA). To obtain fast and uniform uptake of the solutions, 3 min vacuum infiltration (75 mbar) was used. After vacuum infiltration, the leaf discs were pre-incubated for 3 h in the above mentioned ABA-solutions at 20°C and 40 µmol m^{-2} s^{-1} irradiance. Thereafter the petri dishes were placed in a flow-through cuvette. Four petri dishes could be placed simultaneously in the cuvette. The cuvette was placed under a chlorophyll fluorescence imaging system (FluorCam 700MF, PSI, Brno, Czech republic). The temperature in the cuvette was 22±1°C. The imaging measurement was conducted while flowing an atmosphere with 20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and the rest N_2 (non-photorespiratory condition) into the cuvette. The RH was set to 40±3% via passing the air in a temperature-controlled

column of iron (II)-sulphate heptahyrate (Fluka). The leaf discs in the stomata-opening medium were exposed to a continuous irradiance of 100 µmol m^{-2} s^{-1}. Preliminary experiments showed that 10 min was sufficient to reach the steady state Φ_{PSII}. Therefore, after 10 min the protocol for the FluorCam was run and the average value of Φ_{PSII} per leaf disc was calculated by using version 5 of FluorCam software. Values for F_t and F_m' in the generated image were averaged over all pixels per leaf disc and the Φ_{PSII} was calculated using the ratio (F_m'-F_t) / F_m'. To ensure that the decreased Φ_{PSII} was due to stomatal closure, at the end of the imaging of Φ_{PSII} for the different treatments, an image was taken in an atmosphere with high CO_2 concentration (20 mmol mol^{-1} O_2, 50000 µmol mol^{-1} CO_2) to test the recovery of Φ_{PSII}.

Stomatal response to desiccation

To study the effect of desiccation on leaf transpiration rate of the *Arabidopsis* accessions, fully developed leaves from 8 plants (one leaf/plant) were detached and an image was taken to determine the leaf surface area. Then the leaves were placed in closed petri dishes with a layer of degassed deionized water. The leaves were incubated for one hour at 21°C. Under this condition the leaves gained maximum fresh weight. For desiccation the leaves were removed from the petri dishes and placed with the abaxial side up on balances in a test room (40±3% RH, 21ºC, resulting in 1.40 kPa VPD and 35 µmol $m^{-2}s^{-1}$ irradiance). The water loss of the leaves was recorded gravimetrically every 10 s for a period of 10000 s. The leaf area was calculated by using the public domain image processing program ImageJ (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). After the desiccation period, the leaves were dried for 48 h at 80ºC. The relative water content (RWC) during the desiccation period was calculated according to Slavik (1974).

ABA extraction and quantification

One fully developed leaf was detached from M and L-exposed plants and incubated in a petri dish for one hour at 21°C, 100% RH (VPD≈0). Three plants per treatment were used as repetitions. Samples for ABA analysis were taken before and after 45 min desiccation of the leaves. For desiccation the leaves were removed from petri dishes and then placed upside down in a test room (40±3% RH, 20 ºC, resulting in 1.40 kPa VPD, and 35 µmol $m^{-2}s^{-1}$ irradiance). 0.5 g of leaf tissue was ground in a mortar using liquid nitrogen. The samples were extracted with 1 ml of cold ethyl acetate containing [$^2H_6$]-ABA as internal standard to have 0.1 nmol internal standard in the extraction. The samples were vortexed (1 min), then sonicated (15 min) in a Branson 3510 ultrasonic bath (Branson Ultrasonics, Danbury, CT,

USA). Samples were centrifuged for 10 min at 2200 rpm in an MSE Mistral 2000 centrifuge (Mistral Instruments, Leicester, UK). The supernatant was transferred to a 4-ml glass vial. The pellets were re-extracted with 1 ml of methanol without sonication. The solvent fractions were pooled in a 4-ml glass vial. Then the samples were dried using a speedvac (SPD2010-230, Thermo Scientific, USA) and the residue was dissolved by 50 µl methanol. 3 ml MQ water was added to the samples and the extracts were purified using 500 mg C18 columns. The samples were eluted with 1 ml acetone. Then the acetone was evaporated under N_2. The residue was dissolved in 200 µl of acetonitrile:water:formic acid (10:90:0.1, v:v:v). Samples were filtered into vials with Minisart 0.2 µm filters (Sartorius, Goettingen, Germany) and were used for LC-MS/MS analysis according to López-Ráez *et al.* (2010).

Statistical analysis

Data for stomatal response to ABA, ABA content and g_s were subjected to analysis of variance (ANOVA). Treatment means were compared using least significant difference (LSD) test and $P>0.05$ was assumed as not significant. The change of transpiration rate (E) as a function of RWC was fitted using a sigmoidal dose-response curve with a variable slope [$E=Bottom+((Top-Bottom)/(1+10^{(RWC50-RWC).Slope}))$]. The parameters RWC50 and Slope of the fitted curves were used for the analyses of ecotype differences in the relationship between transpiration rate and RWC. GraphPad Prism 5 for Windows (GraphPad software, Inc. San Diego, CA) and IBM SPSS Statistics version 19 were used for statistical analyzing the data. RWC50, Slope, and stomatal response to 200 µmol ABA (as measured by changes in Φ_{PSII}) for moderate and low VPD-exposed plants were used for principle component analysis (PCA) to compare the differences between accessions. The free software environment for statistical computing R (version 3.0.0) was used for PCA and hierarchical cluster classification.

Table 1. Geographical characterisations of the accessions used in the current experiment

Accession	PCA number	ABRC Stock number	Latitude	Longitude	Country
1-Pn	1	CS76197	50	10	Switzerland
Aa-0	2	CS28007	50.9	9.5	Germany
Ag-0	3	CS76087	45	1.3	France
Bur-0	4	CS76105	54.1	-6.2	Ireland
C24	5	CS76106	41.2	-8.4	Portugal
Bs-2	6	CS28097	47.5	7.5	Switzerland
Cvi-0	7	CS76116	15.1	-23.6	Cape Verde island
Eri-1	8	CS22548	56.4	15.3	Sweden
Ler-1	9	CS76164	52.7	15.2	Poland
Lis-1	10	CS76169	56	14.7	Sweden
Lis-2	11	CS76170	56	14.7	Sweden
Lm-2	12	CS76173	48	0.5	France
Lp2-2	13	CS76176	49.3	16.8	Czech republic
Map-42	14	CS76180	42.1	-86.4	USA
Mib-15	15	CS76181	47.3	5.3	France
MNF-Pot-68	16	CS76188	43.5	-86.2	USA
Mt-0	17	CS76192	32.3	22.4	Libya
Mz-0	18	CS76193	50.3	8.3	Germany
NFA-10	19	CS76198	51.4	-0.6	UK
Ost-0	20	CS76202	60.2	18.3	Sweden
Pa-1	21	CS76204	38	13.2	Italy
Par-5	22	CS76207	46.6	-0.2	France
Pent-1	23	CS76209	43.7	-86.3	USA
Per-1	24	CS76210	58	56.3	Russia
Petergof	25	CS76211	59	29	Russia
Pla-0	26	CS28640	41.5	2.2	Spain
Pog-0	27	CS28650	49.2	-123.2	Canada
Pro-0	28	CS76214	43.2	-6	Spain
Pu2-23	29	CS76215	49.4	16.3	Czech republic
Ren-1	30	CS76218	48.5	-1.4	France
Sapporo-0	31	CS28724	43	141.3	Japan
Shahdara	32	CS76227	38.3	68.4	Tajikistan
T10-60	33	CS76234	55.6	13.2	Sweden
Ta-0	34	CS76242	49.5	14.5	Czech republic
Ws-0	35	CS76303	52.3	30	Russia
Zdrl 2-25	36	CS76308	49.3	16.2	Czech republic
Col-0	37	CS76113	-	-	Unknown
Kas-1	38	CS76150	35	77	India
Bay-0	39	CS76094	49	11	Germany
Ba-1	40	CS28053	56.5	-4.7	UK
RRS-7	41	CS28713	41.5	-86.4	USA

Results

Stomatal conductance increased in all Arabidopsis accessions after prolonged exposure to low VPD

Prior exposure to low VPD (L) for 4 days caused a significant increase in stomatal conductance (g_s) in all tested *Arabidopsis* accessions (Fig. 1). The relative effect of low VPD on g_s differed per accession. Highest g_s among the studied accessions was found in Cvi-0 after exposure to L. Similarly, Cvi-0 showed highest g_s among *Arabidopsis* accessions that were not exposed to low VPD (M). The lowest g_s was observed in C24 in both M and L plants (Fig. 1).

Stomata of 39 out of 41 Arabidopsis accessions strongly lost their responsiveness to ABA after prior exposure to low VPD

For most of the accessions, stomatal closure response to ABA was less after prior exposure to low VPD as shown by higher PSII efficiency (Φ_{PSII}) after ABA application compared to that of previously M-exposed plants, when measured under a non-photorespiratory condition. An example is given for Col-0 in Fig. 2A-B. Although the response to ABA was strongly affected by the previous VPD to which the plants had been exposed, the lowest Φ_{PSII} for both the L- and the M-exposed Col-0 was observed in 200 µM ABA. For all treatments, application of high CO_2 (50000 µmol mol^{-1} CO_2) to the leaf discs resulted in the recovery of Φ_{PSII}; this indicates that the reduction of Φ_{PSII} was mainly due to stomatal closure. The effect of different concentrations of ABA (50, 100, 200 µM) on Φ_{PSII} of the 41 *Arabidopsis* accessions which had been exposed for 4 days to M or L conditions can be seen in Supplementary Table S1. To be able to compare the accessions, the effect of ABA was expressed in relative values as $\Phi_{PSII\ x\ ABA}$ / $\Phi_{PSII\ C}$, which is the ratio of Φ_{PSII} measured of leaf discs at one of the ABA concentrations and Φ_{PSII} measured without ABA application. The 'x' indicates the ABA concentration in µM. Substantial variation was found in stomatal response to ABA among accessions after exposure to different VPDs (Fig. 3). By application of different ABA concentrations (Fig. 3A-C), heterogeneity was observed in $\Phi_{PSII\ x\ ABA}$ / $\Phi_{PSII\ C}$ in both M and L plants. In 50 µM ABA, $\Phi_{PSII\ 50\ ABA}$ / $\Phi_{PSII\ C}$ in L-plants was partly overlapped by M plants (Fig. 3A). The overlapping accessions for their $\Phi_{PSII\ ABA}$ / $\Phi_{PSII\ C}$ responses were decreased by increasing the ABA concentration to 100 (Fig. 3B) and 200 (Fig. 3C) µM ABA, and two distinct patterns of distribution between M and L plants were recognized. Especially at 200 µM ABA, the distribution for L plants was much broader than the distribution for M plants.

In all accessions the $\Phi_{PSII\ ABA}/\Phi_{PSII\ C}$ was decreased in an ABA concentration dependent manner for both VPDs (Supplementary Table S1). Significant differences were found between M and L plants for Φ_{PSII} in response to ABA for 39 of the tested accessions. In all 39 accessions, the Φ_{PSII} was reduced less by ABA for L plants in comparison with that it was for M plants (Supplementary Table S1).

In contrast to the other accessions, in Map-42, C24, Pent1, Lis1 and Ost-0, also the Φ_{PSII} of L plants strongly responded to ABA; that was also true for the lowest ABA concentration tested (50 µM). However, in Pent1, Lis1 and Ost-0, M and L plants significantly differed in their response to ABA. In the case of two accessions, Map-42 and C24, no significant differences were found between M and L plants for their response to ABA; for both plant types (M and L) the Φ_{PSII} showed a comparable strong decrease by application of ABA (Supplementary Table S1).

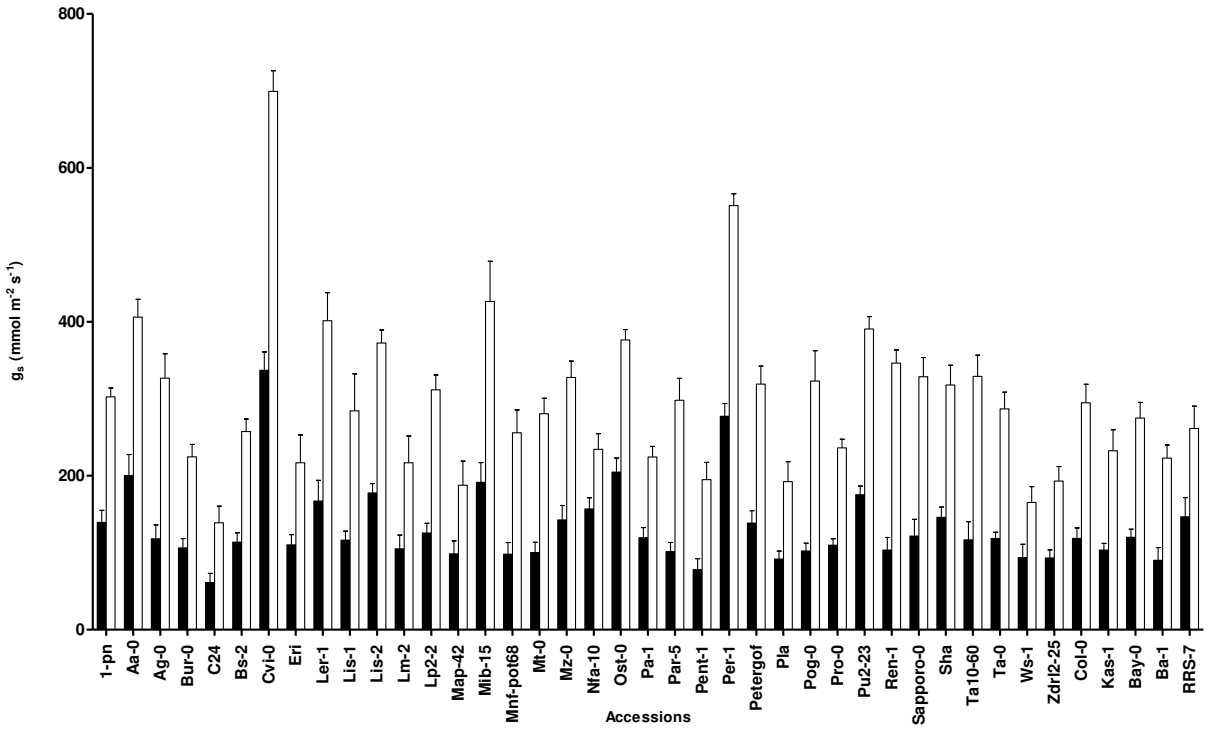

Fig. 1. Stomatal conductance (g_s) of 41 *Arabidopsis* accession after exposure to different vapour pressure deficits (VPDs). Plants had been exposed to moderate (1.17 kPa; filled bars) or to low (0.23 kPa; open bars) VPD. The measurements were carried out at 1.40 kPa VPD and 35 µmol m^{-2} s^{-1} irradiance.

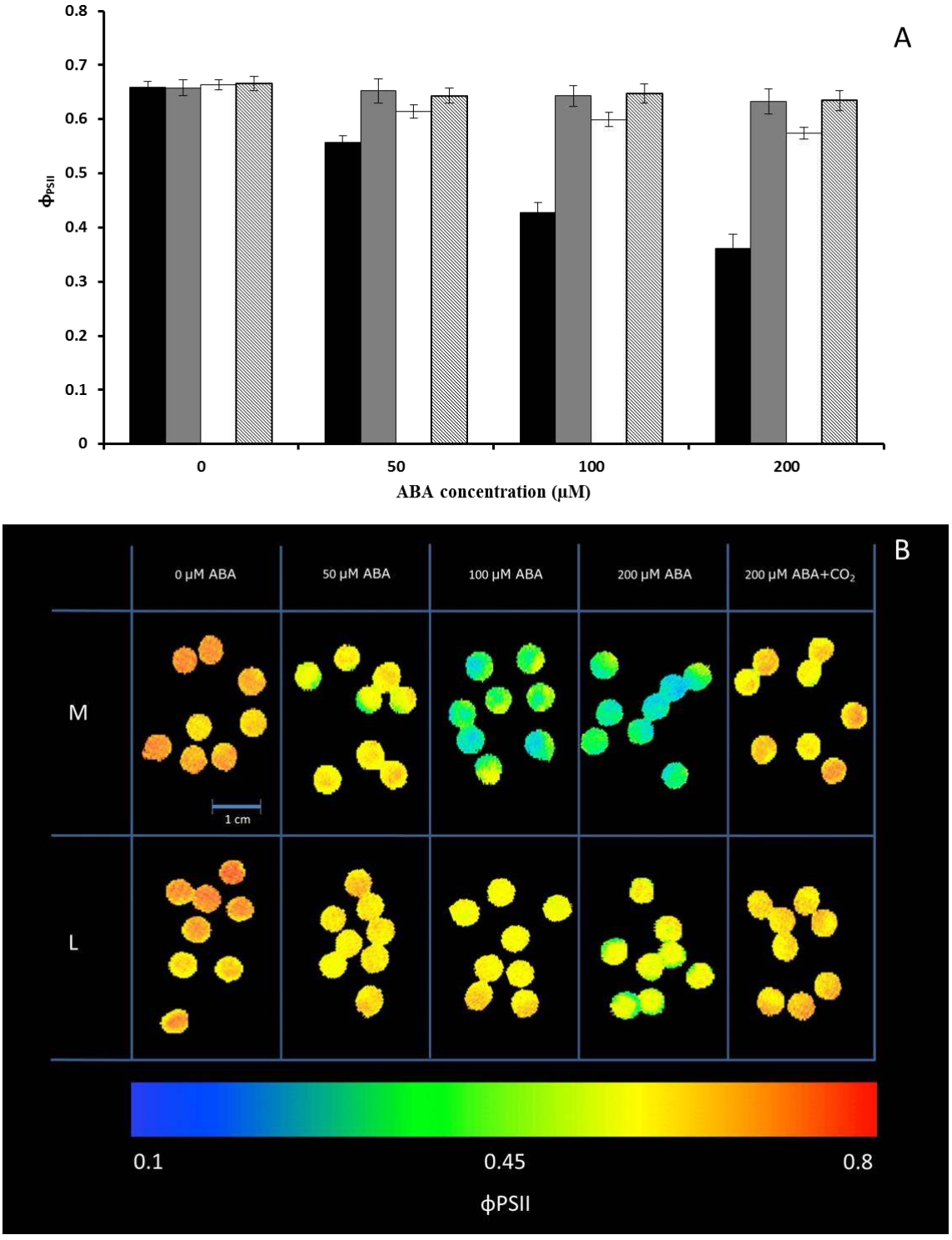

Fig. 2. Average PSII efficiency (Φ_{PSII}) (A) and representative images of Φ_{PSII} (B) for Col-0 leaf discs in response to ABA after prior exposure to different VPDs. Φ_{PSII} was measured under non-photorespiratory conditions (20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and reminder N_2) in plants that had been exposed for 4 days to moderate [1.17 kPa; black bars in (A)] or to low [0.23 kPa; L; open bars in (A)] VPD in response to ABA. At the end, an image was made after 5 min exposure to 20 mmol mol^{-1} O_2 and 50000 µmol mol^{-1} CO_2 (grey bars for M, hedged bars for L in Fig. 2A and $+CO_2$ in Fig. 2B). Leaf discs (0.5 cm diameter) were put with the adaxial surface down in petri dishes filled with stomata-opening medium with different concentrations of ABA (0, 50, 100, 200 µM ABA), and Φ_{PSII} was recorded 3 hr after application of the ABA.

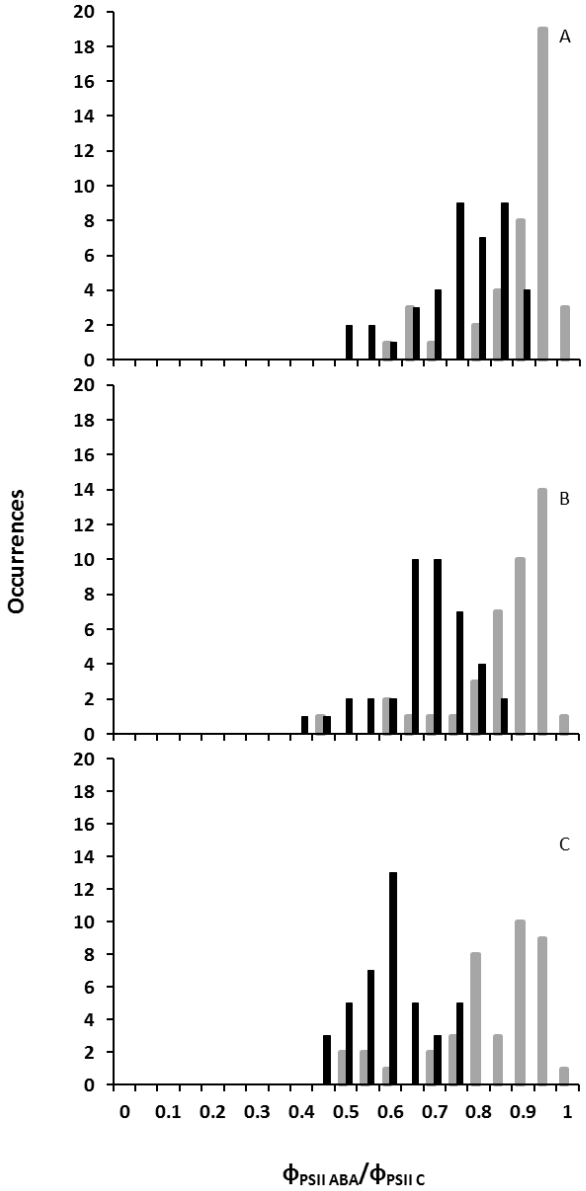

Fig. 3. Frequency distribution of different accessions according to the relationships between PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions in response to 50 (A), 100 (B) and 200 µM ABA (C) relative to no ABA ($\Phi_{PSII\ ABA}$/ $\Phi_{PSII\ C}$) after 4 days exposure of plants to moderate VPD (1.17 kPa; black bars) or to low VPD (0.23 kPa; grey bars).

Stomata of 39 out of 41 Arabidopsis accessions kept their responsiveness to desiccation after prior exposure to low VPD

Although during desiccation, the rate of water loss decreased in leaves of both M and L-exposed accessions, in some of them the amount of water loss was higher in L-exposed compared with M-exposed plants. The transpiration rate (E) for M and L Col-0 and Cvi-0 is presented as an example of the water loss in response to desiccation (Supplementary Fig. S1). E followed an exponential decay over desiccation time. In both Col-0 and Cvi-0 significant differences were found during desiccation between plants exposed to M and L conditions.

However, a larger difference was found between M and L exposed Cvi-0 during desiccation time in comparison with Col-0.

The influence of water status of the leaf during desiccation on the stomata opening was expressed using the relationship between E and RWC (E×RWC). In all accessions, E followed a sigmoidal decay as a function of RWC. RWC50 and Slope of the fitted curves of E×RWC were used for analysing the response of stomata to RWC during desiccation of the ecotypes after prior exposure to M and L conditions. Higher RWC50 or larger Slope means stomata close at higher RWC. Analysis showed that RWC50 and Slope were strongly correlated (r^2 = 0.94 for L and r^2 = 0.96 for M plants). For that reason only data of Slope are shown.

The E×RWC for M and L-exposed Col-0 and Cvi-0 are presented as examples (Fig. 4). Although L-exposed Col-0 exhibited higher E at certain RWC, no statistical difference were found for Slope of the curves between M and L-exposed Col-0 plants. While, in the case of Cvi-0, Slope of the E×RWC for L plants was significantly less compared with Slope in M plants. Fig. 5 shows the Slope of the E×RWC in all accessions when they had been previously exposed to M and L conditions. Most of the *Arabidopsis* accessions responded in the same way in both M and L-exposed plants. In contrast to ABA, accessions were similarly distributed for their Slope of E×RWC after exposure to M and L conditions (Supplementary Fig. S2). However the Slope for Cvi-0 and Rrs-7 was different between their M and L plants. Cvi-0 and Rrs-7 plants that been exposed to L condition showed slower rate for stomatal closure compared with M plants. This indicates that Cvi-0 and Rrs-7 plants lost more water in response to desiccation after prior exposure to L condition in comparison with M plants.

Stomatal response to closing stimuli after prior exposure to different VPDs reveals natural variation among Arabidopsis accessions

In order to group all tested accessions according to the effect of a prior exposure to different VPDs on their stomatal response to ABA and to desiccation, a global principle component analysis (PCA) was performed on the plants that had been exposed to M and L conditions. For the stomatal response to ABA, the relative effect of ABA on Φ_{PSII} was used and for the response of stomata to desiccation the Slopes as given in Fig. 5 were used. The result showed that PCA1 and PCA2 explained 86.8% of the point variation between *Arabidopsis* accessions (Fig. 6). PCA1 accounted for 63.5% and PCA2 accounted for 23.3% of the observed variation. Since the correlations between RWC50 and Slope were more than 0.9 for both M and L plants, only Slope of the fitted curves was used for the PCA. The PCA showed that also

adding g_s (stomatal conductance after exposure to M and L conditions) to the analysis did not increase the explained part of the point variation. The PCA showed 3 distinct groups for the stomatal responses to closing stimuli in all accessions when they had prior been exposed to M and L conditions (Fig. 6). Most of the accessions including Col-0 (accession number 37) belong to one group (number 3). Fig. 7 shows the classification of 41 accessions using cluster algorithms of the dataset. Group number 2 shows the accessions with extreme responses, Map-42, C24, Pent1, Lis1 and Ost-0, characterized as accessions with maximum response of stomata to closing stimuli, after prior exposure to as well moderate as low VPD. Moreover, two other big groups (number 1 and 3 in Fig. 6) can be categorized into two distinct clusters for their stomatal response to closing stimuli (Fig. 6 and 7).

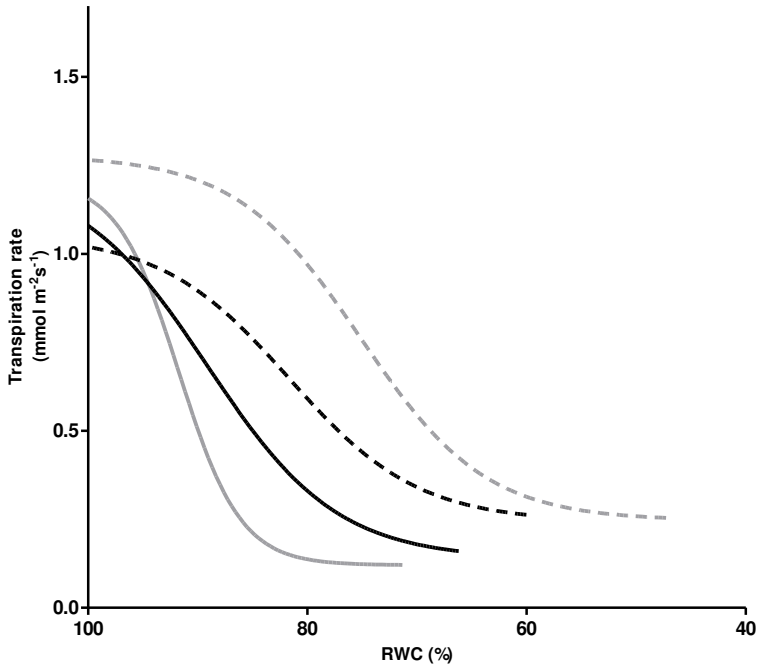

Fig. 4. Fitted curves of the relationship between transpiration rate (E) and leaf relative water content (RWC) for Col-0 (black lines) and Cvi-0 (grey lines) *Arabidopsis* accessions of leaves of plants that had been exposed for 4 days to moderate (solid lines) or to low (broken lines) VPD. The leaves were first saturated in degassed deionized water and after 1 hr measurements were conducted during desiccation at VPD of 1.40 kPa. The R square of goodness of fits was 0.9±0.1. The raw data are not shown for clarity of the figure.

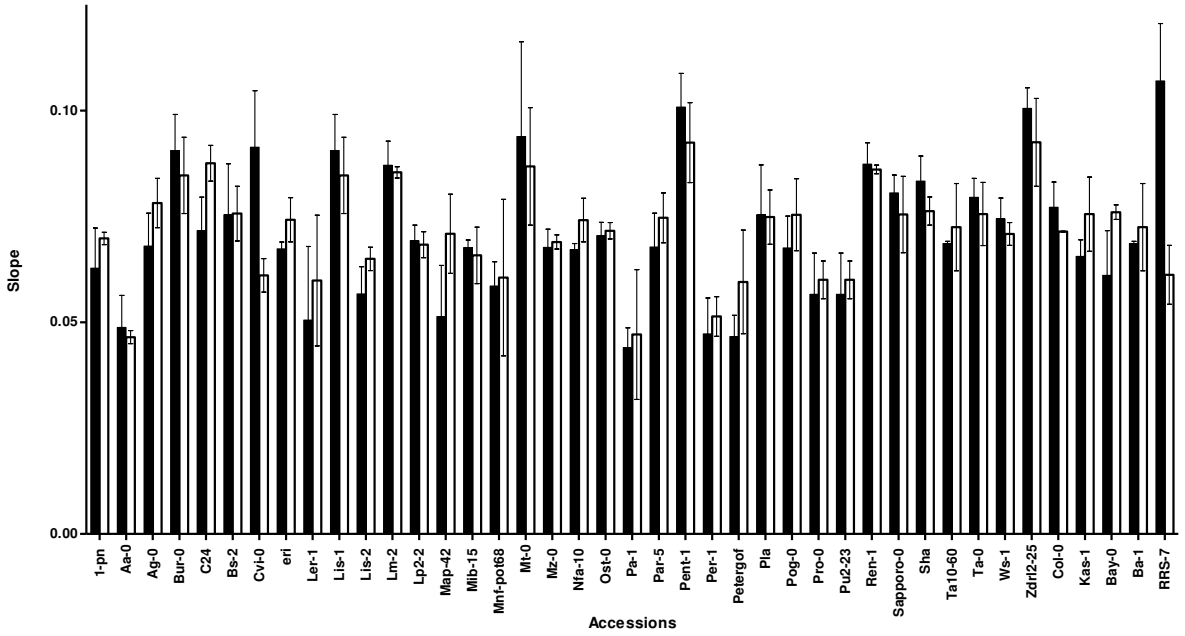

Fig. 5. Slopes of the curves for relationship between transpiration rate (E) and leaf relative water content during 10000 s desiccation of the leaves of plants that had been exposed for 4 days to moderate (1.17 kPa; filled bars) or to low (0.23 kPa; open bars) vapour pressure deficits (VPD). The leaves were first saturated in degassed deionized water and after 1 hr measurements were conducted during desiccation at VPD of 1.40 kPa.

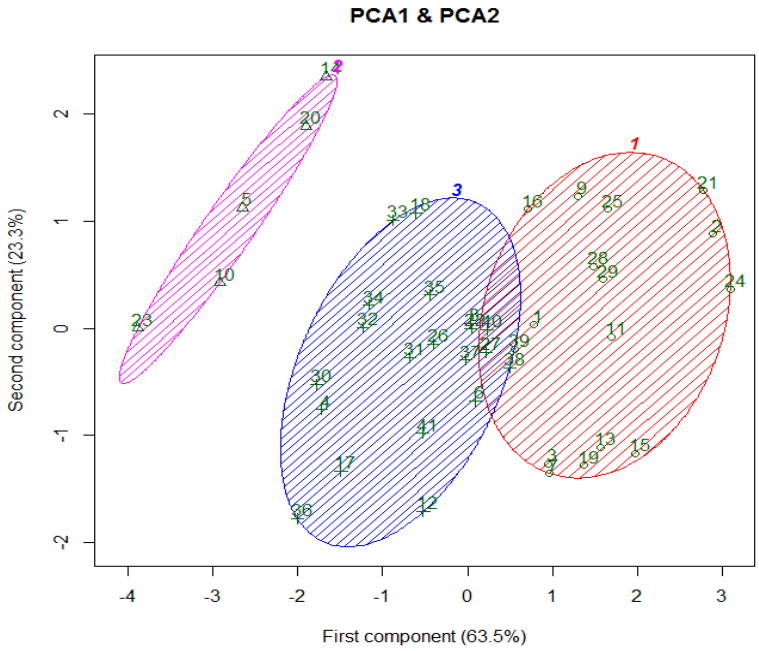

Fig. 6. Principle component analysis (PCA) for 41 *Arabidopsis* accessions that had been exposed for 4 days to moderate vapour pressure deficit (VPD) (1.17 kPa) or to low VPD (0.23 kPa). The numbers indicate the accessions according to the numbering in Table 1. The PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions at 200 µM ABA relative to Φ_{PSII} of the control (0 µM ABA), together with Slope of the fitted sigmoidal relationship between transpiration rate and RWC of the leaves, were used for the analysis. Component one and two explain 86.3% of the point variability.

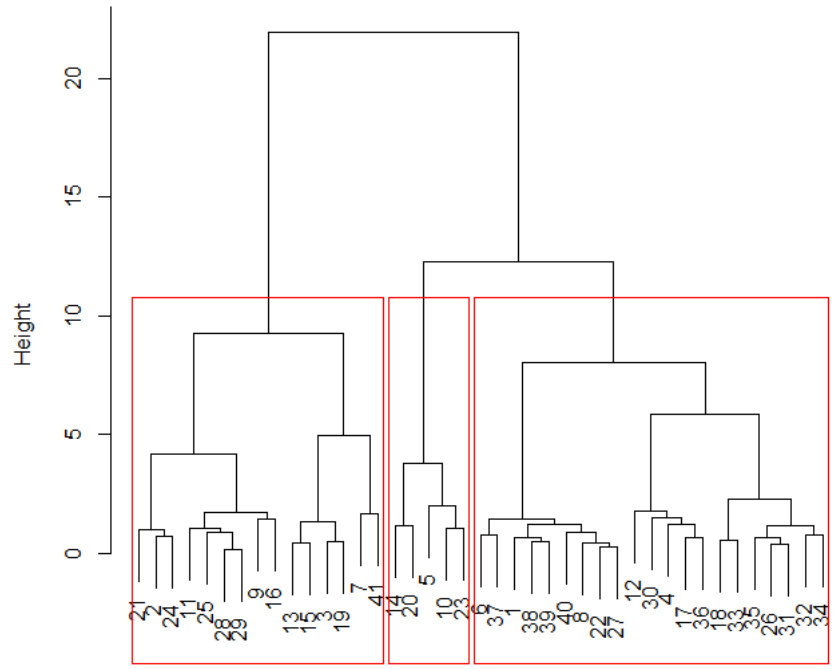

Fig. 7. Dendrogram classification for 41 *Arabidopsis* accessions that had been exposed for 4 days to moderate vapour pressure deficit (VPD) (1.17 kPa) or to low VPD (0.23 kPa). The PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions at 200 µM ABA relative to Φ_{PSII} of the control (0 µM ABA), together with Slope of the fitted sigmoidal relationship between transpiration rate and RWC of the leaves, were used for classification. The red box showing accessions with three different type of responses to closing stimuli.

VPD and desiccation considerably influenced foliar ABA level

From the results obtained from screening of the stomatal response of *Arabidopsis* accessions to closing stimuli (after prior exposure to two different VPDs), two extreme accessions [Map-42 (group 2) and Cvi-0 (group1)] together with a 'control' accession [Col-0 (group3)] were used for measuring the bulk foliar ABA levels before and after desiccation. Before desiccation, lower ABA levels were found in the leaves of all three accessions as a result of exposure to L condition (Fig. 8; A, B, and C). After exposure to L condition the ABA level in the Map-42 (Fig. 8C) was 44% and 32% higher than the level in Col-0 and Cvi-0, respectively. Desiccation led to a sharp increase ($P \leq 0.001$) in the bulk foliar ABA level in all three accessions. In all three accessions, the level of ABA after desiccation was more in the plants that had prior been exposed to M condition, but there was a large difference in the after effect of VPD on the increase in ABA due to desiccation. In Col-0 the [ABA] in L plants was 88% of that of M plants after desiccation, while in L plants of Cvi-0 it was 49% of that of M

plants. The highest bulk foliar ABA level following desiccation was found in the M-exposed Cvi-0 plants (Fig. 8B).

In these three accessions there were no significant correlation between the desiccation response (Slope of the E×RWC) and the foliar ABA level before desiccation (Fig. 9). However, Slope of the E×RWC positively correlated with the amount of ABA produced due to desiccation (Fig. 9).

Of the 3 accessions tested, the response to ABA ($\Phi_{PSII\ 200\ ABA}$ / $\Phi_{PSII\ C}$) was inversely correlated to the foliar ABA level (before desiccation) of M and L plants (Fig 10). A high $\Phi_{PSII\ 200\ ABA}$ / $\Phi_{PSII\ C}$ indicates no closing of stomata.

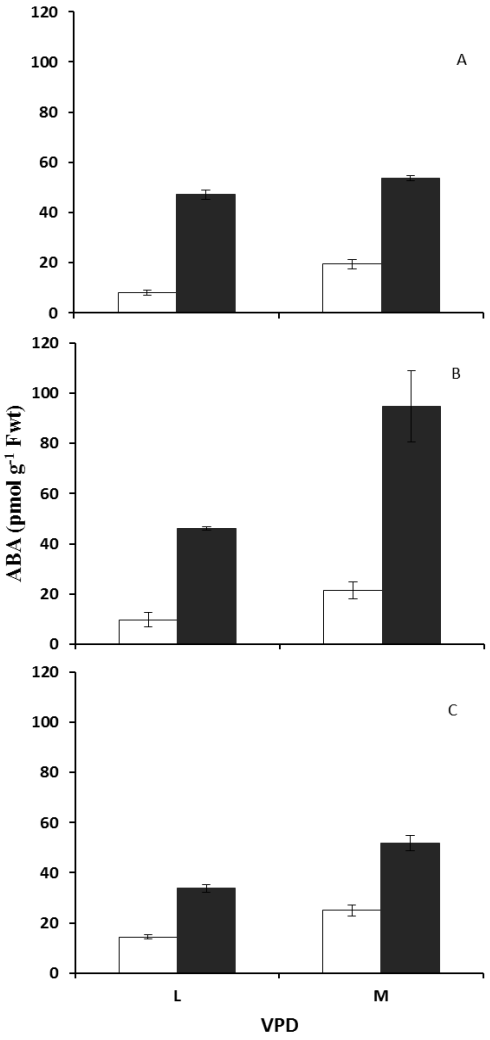

Fig. 8. Concentration of ABA in Col-0 (A), Cvi-0 (B) and Map-42 (C) *Arabidopsis* accessions before (white bars) and after 45 minutes desiccation (black bars). The plants had been exposed for 4 days to moderate VPD (M) (1.17 kPa) or to low VPD (L) (0.23 kPa) prior to ABA measurements and desiccation treatment. The desiccation was conducted at VPD of 1.40 kPa.

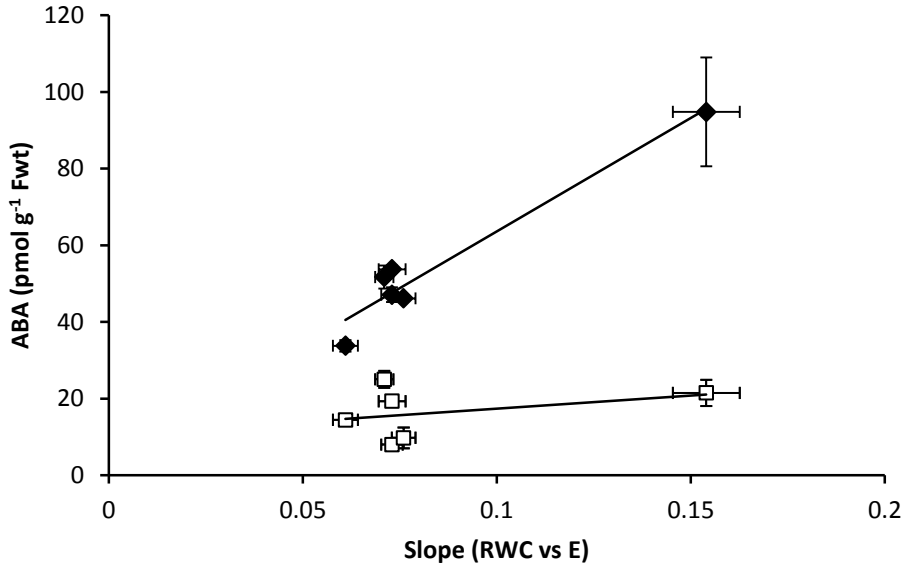

Fig. 9. Relationship between desiccation response (Slope of the E × RWC relationship) and the ABA-concentration before (open symbols) and after (closed symbols) 45 min desiccation of the leaves in Col-0, Cvi-0 and Map-42 accessions.

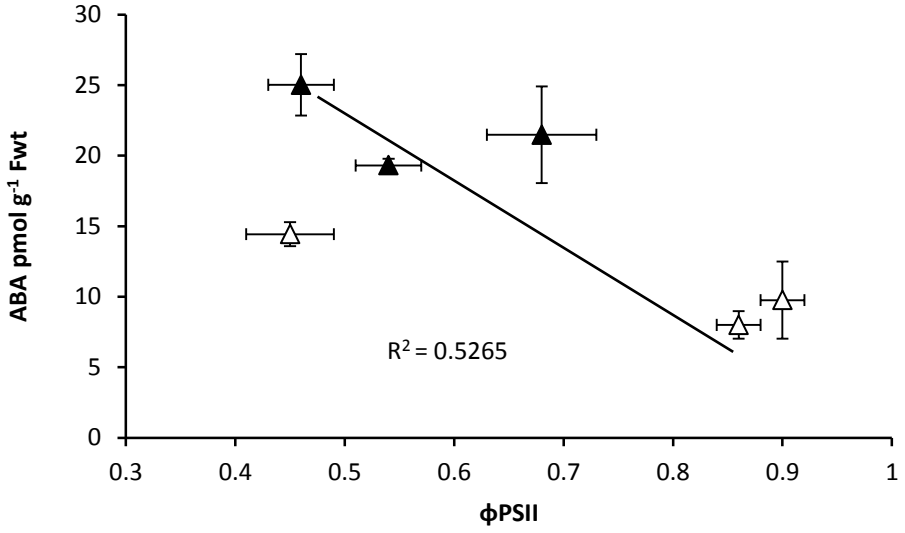

Fig. 10. Relationship between PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions in response to 200 µM ABA relative to no ABA ($\Phi_{PSII\ 200\ ABA}$ / $\Phi_{PSII\ C}$) and foliar ABA level for plants that had been exposed for 4 days to moderate (1.17 kPa) (closed symbols) or to low VPD (0.23 kPa) (open symbols).

Discussion

Fast screening procedure for ABA sensitivity of stomatal closing

To analyse the response of stomata to exogenous ABA, we developed an efficient and fast technique based on fluorescence of chlorophyll. In this technique, leaf discs were prepared from the leaves of plants that had been exposed to different VPDs and were floated in petri dishes (filled with stomata opening medium together with different concentrations of ABA). The PSII efficiency (Φ_{PSII}) of the leaf discs was measured under non-photorespiratory conditions (low O_2). In this situation, the only source for CO_2 assimilation is the ambient CO_2 which will be provided through stomata. Therefore, the closure of the stomata is the main reason for decreased Φ_{PSII} of the leaf discs. To test whether the decreased Φ_{PSII} is via stomatal closure, at the end an image was taken after 5 min exposure to 50000 ppm CO_2 for recovering Φ_{PSII}. The recovery of Φ_{PSII} by exposure to high CO_2 confirmed that the decreased Φ_{PSII} is because of stomatal closure. In the imaging area of the system it was feasible to investigate 32 samples simultaneously. Therefore, the developed method provides a fast and efficient way for investigating the response of the stomata to ABA.

Arabidopsis showed remarkable natural genotypic variation for stomatal response to closing stimuli after prior exposure to different VPDs

Natural genetic variation between accessions is advantageous to study, because it facilitates to understand which processes within a trait are subjected to natural selection (Alonso-Blanco *et al.*, 2009; Trontin *et al.*, 2011). Stomata response to environmental conditions is a complex trait involving a complex network of signalling pathways. Natural variations in plant sensitivity to ozone (Brosché *et al.*, 2010) and mild water stress (Bouchabke *et al.*, 2008) were reported among *Arabidopsis* accessions which indirectly can be related to the stomata. In this study we compared the stomatal response to closing stimuli after the plants had prior been exposed to moderate and low VPD conditions, in order to reveal natural variation among *Arabidopsis* accessions. We have demonstrated that there is remarkable natural variation among *Arabidopsis* accessions for adaptation (or disturbance) of the stomatal responses to closing stimuli after long-term exposure to low VPD. The studied accessions can be categorized in 3 different groups according to the adaptation of their stomatal response to ABA and desiccation by low VPD: sensitive to ABA and desiccation, sensitive to desiccation but not anymore to ABA, and non-sensitive to ABA and desiccation after low VPD-exposure.

Outliers from screening of Arabidopsis accessions can be used to identify new molecular constituents involved in the stomatal response to closing stimuli

The results of our study revealed that there is a genotypic variation in the after effect of long-term exposure to low VPD on the stomatal response among 41 distinct *Arabidopsis* accessions. The current screening revealed that Map-42 and C24 are accessions which maintained their response to ABA and desiccation, while Cvi-0 is an accession that lost its response to desiccation and ABA after prior exposure to low VPD. Most of the accessions, including Col-0, were recognized as responsive to desiccation but non-responsive to ABA after long-term exposure to L condition. To confirm that Map-42, C24 and Cvi-0 were outliers, the stomatal responses of these accessions to ABA and to desiccation were further analysed (2-4 times) as separate repetitions. The outliers with extreme responses can be used for building up promising RIL populations for identification of the involved QTLs in the malfunctioning stomata. QTL mapping for the stomatal response to environmental conditions are scarce. Screening 164 plants of a Col-0 × Cvi-0 RIL population for ozone and water loss phenotypes showed three QTLs for ozone and one QTL for water loss (Brosché *et al.*, 2010). The strongest QTL for ozone sensitivity was close to the same position as the QTL for water loss. Therefore, it is likely there is a correlation between stomatal functioning and plant injury response to the ozone stress (Brosché *et al.*, 2010). Moreover natural genetic variation was found between 24 accessions of *Arabidopsis* under a water deficit condition (Bouchabke *et al.*, 2008). The mentioned studies are the only ones that showed a variation in response of the plants to environmental conditions that were indirectly related to variation in stomatal functioning. Natural variation in stomatal density and stomatal index has been found among 62 wild *Arabidopsis* accessions (Delgado *et al.*, 2011). However, stomatal morphological alterations due to long-term exposure to L condition is not the main reason for stomatal malfunctioning after exposure to low VPD (Aliniaeifard *et al.*, 2014). To the best of our knowledge, the current study is the only one which focus on the natural variation in stomatal response of *Arabidopsis* accessions when they have been exposed for long-term to low VPD condition. RILs from different *Arabidopsis* parents (such as RIL populations for Col-0, Ler-1, Cvi-0, C24 and Te-0) have been used for QTL mapping for traits such as flowering time, seed dormancy and resistance to disease which participate in plant response and adaptation to different environmental conditions (Shindo *et al.*, 2007; Brosché *et al.*, 2010). The recognized natural variation in the current study can be used for QTL mapping and finding the genes involved in the malfunctioning of stomata due to low VPD.

Low VPD condition reduced the stomatal response to ABA, but did not highly affect stomatal response to desiccation

The disturbed ABA signalling pathway due to long-term exposure to low VPD was reviewed by Aliniaeifard and van Meeteren (2013) in more detail. The results of the current study showed that most of the *Arabidopsis* accessions were not capable of full stomatal closure in response to different ABA concentrations after exposure to L (Supplementary Table S1). As a result of long-term exposure to low VPD, habituation occurs which renders the stomata insensitive to ABA (Aliniaeifard and van Meeteren, 2013).

In response to desiccation most of the *Arabidopsis* accessions showed stomatal closure after exposure to both, moderate and low VPDs. However in this study Cvi-0 was recognized as an accession with malfunctioning stomata in response to desiccation after prior exposure to low VPD. Compared with other *Arabidopsis* accessions Cvi-0 had the highest stomatal conductance after exposure to different VPDs (Fig. 1). It has been shown that high stomatal conductance in Cvi-0 caused a high rate of ozone uptake by the leaf, resulting in more sensitivity of this accession to ozone (Brosché *et al.*, 2010). Moreover, long-term exposure to ozone reduced the sensitivity of the stomata in response to different closing stimuli (Paoletti, 2005; Mills *et al.*, 2009; Wilkinson and Davies, 2009; Aliniaeifard and van Meeteren, 2013), resulting in more damage by ozone in the long term. Bouchabke *et al.* (2008) showed that compared to other 23 *Arabidopsis* accessions, Cvi-0 had the highest leaf water loss in well-watered and water deficit conditions. In our study L-exposed Cvi-0 lost more water compared with M-exposed Cvi-0. Similar to the current study with prior exposure to different VPDs, a difference between well-watered and water-deficit grown Cvi-0 plants was found for water loss after two hours desiccation (Bouchabke *et al.*, 2008). QTL mapping in a core Col-0 × Cvi-0 RIL population identified one QTL for high water loss trait (Bouchabke *et al.*, 2008).

Why most of the *Arabidopsis* accessions were still responsive to desiccation after exposure to low VPD while they lost their responsiveness to ABA? Analysing the stomatal response of four different rose cultivars, Fanourakis *et al.* (2013) showed that in one of the cultivars stomatal response to exogenous ABA was considerably influenced by growth at low VPD, while its response to desiccation was only minimally affected. In a research using full grown leaves of bean plants, Aliniaeifard *et al.* (2014) found that as a result of exposure to low VPD, stomatal responsiveness to ABA was decreased before a diminished response to desiccation occurred. They concluded that the stomatal responses to desiccation and to ABA were not affected in the same way by exposure to low VPD. They suggested that signals induced by desiccation were capable of increasing ABA levels in the guard cells, but ABA feeding to the

petiole was not or that desiccation controls stomata closure (also) via a non-ABA controlled pathway (Aliniaeifard *et al.*, 2014). Exposure to different VPDs affected as well g_s as the desiccation response (Slope of RWC×E) of the ecotypes, but these changes (g_{sL}/g_{sM} and $Slope_L/Slope_M$) were not correlated to each other (Supplementary Fig. S3A). Nevertheless, although the correlation was not strong, the effect of VPD on the stomatal response to ABA significantly correlated positively with the effect of VPD on g_s (Supplementary Fig. S3B). This strengthens the concept that desiccation controls stomata closure (also) via a non-ABA controlled pathway.

Stomatal conductance is an important indicator of stomatal response to ABA

In general, prior exposure to low VPD led to higher stomatal conductance and less stomatal response to ABA. Similarly, increased stomatal conductance and decreased stomatal responsiveness to ABA due to long-term exposure to low VPD has been reported in *Vicia faba* (Aliniaeifard *et al.*, 2014), *Tradescantia virginiana* (Rezaei Nejad and van Meeteren, 2005, 2007) and *Rosa hybrida* (Fanourakis *et al.*, 2011; Fanourakis *et al.*, 2013).

The involvement of ABA in the stomatal response to water stress is extensively studied. It has been well documented that drought induced ABA production results in stomatal closure (Larque-Saavedra and Wain, 1974; Luan, 2002; Giday *et al.*, 2013). Accordingly, in the current study a positive correlation (R^2=0.94) was found between foliar ABA level after desiccation and transpiration rate (Slope of E×RWC). In *Vicia faba* and *Tradescantia* long-term exposure to low VPD decreased the ABA level and thereafter stomata are no longer responsive to closing stimuli (Rezaei Nejad and van Meeteren, 2008; Aliniaeifard *et al.*, 2014). It was concluded that low foliar ABA level for long time could be the main reason for malfunctioning of the stomata in response to closing stimuli (Rezaei Nejad and van Meeteren, 2007; Aliniaeifard and van Meeteren, 2013). After exposure to low VPD, foliar ABA level decreased via ABA 8'-hydroxylases (Kushiro *et al.*, 2004; Okamoto *et al.*, 2009). It was suggested that as a result of long-term low ABA level, the ABA receptors are unable to block ABA negative regulators inside the guard cells which consequently leads to stomatal insensitiveness to ABA (Rezaei Nejad and van Meeteren, 2007; Aliniaeifard and van Meeteren, 2013). Overcoming the low ABA level due to exposure to low VPD via daily application of ABA during leaf development in rose (*Rosa hybrida*) (Fanourakis *et al.*, 2011) and *Tradescantia* (Rezaei Nejad and van Meeteren, 2007) or during 4 days exposure to low VPD in *Vicia faba* (Aliniaeifard *et al.*, 2014) maintained functional stomata that are responsive to closing stimuli (e.g. ABA). In the three accessions tested, as representatives of

the three clusters of the PCA, a positive correlation was found between foliar ABA level and stomatal closure response to ABA after exposure to different VPDs (Fig. 10). Cvi-0 showed the largest decrease in foliar ABA level after exposure to low VPD (Fig. 8) and lost its response to ABA, while MAP-42 showed the smallest decrease of ABA and kept its response to ABA after low VPD-exposure.

In conclusion, we have shown that there is natural variation in the effect of long-term exposure to low VPD on the sensitivity to closing stimuli among 41 accessions of *Arabidopsis thaliana*. This variation can be exploited to identify genes involved in the signalling pathways in malfunctioning stomata. RILs can be generated from extreme accessions for using in QTL mapping to identify the main modules involved in the function of stomata after low VPD-exposure. Accessions can be categorized in three groups according to their stomatal response to closing stimuli after prior exposure to low VPD. Stomata of most of the *Arabidopsis* accessions were not fully responsive to ABA when the plants had been exposed to low VPD, but most of them were responsive to desiccation after exposure to low VPD. Stomatal response to ABA, but not to desiccation was related to the stomatal conductance after exposure to low VPD.

Acknowledgements

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Supplementary data

Table S1. The effect of different ABA concentrations (50, 100, 200 µM) on PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions for 41 *Arabidopsis* accessions which have been exposed for 4 days to moderate (1.17 kPa; M) or to low (0.23 kPa; M) VPD. Φ_{PSII} is expressed as relative effect of ABA to the control treatment (without ABA). Leaf discs (0.5 cm diameter) were put with the adaxial surface down in petri dishes with stomata-opening medium with different ABA concentrations and Φ_{PSII} was recorded 3 hr after application of ABA. Numbers are mean values of 8 leaf disks ±SEM.

Accession	PCA number	VPD	φ_{PSII} 50 µM ABA/φ_{PSII} C	φ_{PSII} 100 µM ABA/φ_{PSII} C	φ_{PSII} 200 µM ABA/φ_{PSII} C
1-pn	1	M	0.73±0.04	0.67±0.03	0.58±0.05
		L	0.91±0.01	0.93±0.02	0.89±0.01
Aa-0	2	M	0.80±0.02	0.74±0.01	0.67±0.03
		L	0.91±0.01	0.91±0.03	0.90±0.03
Ag-0	3	M	0.83±0.02	0.76±0.02	0.70±0.03
		L	0.92±0.02	0.90±0.02	0.89±0.03
Bur-0	4	M	0.66±0.03	0.66±0.04	0.56±0.05
		L	0.82±0.03	0.80±0.02	0.68±0.04
C24	5	M	0.63±0.08	0.54±0.04	0.46±0.03
		L	0.64±0.07	0.56±0.07	0.49±0.05
Bs-2	6	M	0.71±0.05	0.68±0.04	0.60±0.05
		L	0.88±0.03	0.86±0.03	0.85±0.03
Cvi-0	7	M	0.87±0.03	0.77±0.03	0.68±0.05
		L	0.92±0.02	0.92±0.02	0.90±0.02
Eri-1	8	M	0.75±0.03	0.67±0.04	0.55±0.05
		L	0.91±0.01	0.86±0.04	0.83±0.03
Ler-1	9	M	0.85±0.02	0.62±0.03	0.51±0.04
		L	0.93±0.01	0.92±0.02	0.91±0.01
Lis-1	10	M	0.51±0.03	0.49±0.04	0.43±0.04
		L	0.63±0.02	0.64±0.03	0.58±0.04
Lis-2	11	M	0.86±0.03	0.75±0.04	0.66±0.04
		L	0.92±0.03	0.89±0.03	0.89±0.03
Lm-2	12	M	0.81±0.05	0.67±0.03	0.62±0.04
		L	0.94±0.02	0.91±0.05	0.86±0.03
Lp2-2	13	M	0.86±0.02	0.85±0.03	0.72±0.07
		L	0.95±0.02	0.95±0.03	0.91±0.02
Map-42	14	M	0.64±0.03	0.54±0.04	0.45±0.03
		L	0.65±0.04	0.56±0.03	0.46±0.04
Mib-15	15	M	0.83±0.04	0.81±0.03	0.73±0.05
		L	0.98±0.02	0.94±0.03	0.96±0.02
Mnf-pot68	16	M	0.66±0.07	0.64±0.05	0.57±0.04
		L	0.84±0.05	0.84±0.03	0.75±0.05
Mt-0	17	M	0.71±0.03	0.58±0.05	0.55±0.06
		L	0.93±0.02	0.89±0.02	0.80±0.05
Mz-0	18	M	0.59±0.04	0.58±0.04	0.49±0.04
		L	0.76±0.06	0.79±0.03	0.71±0.06

Nfa-10	19	M	0.71±0.04	0.74±0.02	0.72±0.05
		L	0.92±0.02	0.92±0.02	0.91±0.02
Ost-0	20	M	0.49±0.05	0.44±0.01	0.42±0.03
		L	0.69±0.04	0.58±0.04	0.55±0.03
Pa-1	21	M	0.79±0.05	0.68±0.04	0.62±0.07
		L	0.93±0.02	0.95±0.01	0.93±0.01
Par-5	22	M	0.66±0.04	0.70±0.05	0.53±0.05
		L	0.88±0.03	0.89±0.02	0.87±0.02
Pent-1	23	M	0.48±0.02	0.47±0.03	0.40±0.03
		L	0.64±0.08	0.66±0.04	0.54±0.04
Per-1	24	M	0.80±0.05	0.76±0.04	0.70±0.06
		L	0.97±0.02	0.99±0.03	0.94±0.02
Petergof	25	M	0.77±0.04	0.66±0.05	0.59±0.04
		L	0.90±0.02	0.88±0.03	0.84±0.02
Pla	26	M	0.72±0.02	0.63±0.05	0.56±0.04
		L	0.90±0.03	0.80±0.04	0.77±0.03
Pog-0	27	M	0.73±0.03	0.62±0.04	0.55±0.04
		L	0.91±0.03	0.91±0.02	0.88±0.03
Pro-0	28	M	0.80±0.03	0.69±0.05	0.60±0.05
		L	0.90±0.03	0.91±0.03	0.86±0.02
Pu2-23	29	M	0.78±0.05	0.72±0.06	0.61±0.06
		L	0.86±0.03	0.88±0.01	0.87±0.01
Ren-1	30	M	0.64±0.07	0.64±0.03	0.46±0.03
		L	0.89±0.02	0.84±0.05	0.79±0.04
Sapporo-0	31	M	0.78±0.03	0.73±0.02	0.56±0.03
		L	0.88±0.02	0.81±0.03	0.75±0.03
Shahdara	32	M	0.73±0.02	0.64±0.04	0.50±0.03
		L	0.84±0.02	0.80±0.03	0.73±0.03
Ta10-60	33	M	0.61±0.07	0.54±0.04	0.45±0.03
		L	0.81±0.03	0.779±0.04	0.74±0.06
Ta-0	34	M	0.69±0.06	0.63±0.02	0.54±0.03
		L	0.77±0.04	0.71±0.05	0.65±0.06
Ws-0	35	M	0.74±0.04	0.62±0.03	0.53±0.04
		L	0.89±0.03	0.81±0.06	0.75±0.03
Zdrl2-25	36	M	0.77±0.05	0.63±0.03	0.55±0.04
		L	0.88±0.03	0.82±0.04	0.78±0.03
Col-0	37	M	0.84±0.02	0.65±0.03	0.54±0.03
		L	0.92±0.02	0.90±0.02	0.86±0.02
Kas-1	38	M	0.81±0.04	0.70±0.07	0.57±0.06
		L	0.96±0.03	0.93±0.04	0.90±0.05
Bay-0	39	M	0.84±0.03	0.70±0.06	0.59±0.03
		L	0.91±0.04	0.89±0.04	0.86±0.03
Ba-1	40	M	0.88±0.02	0.69±0.05	0.51±0.03
		L	0.95±0.02	0.93±0.02	0.92±0.03
RRS-7	41	M	0.75±0.03	0.73±0.05	0.59±0.03
		L	0.92±0.01	0.90±0.03	0.78±0.02

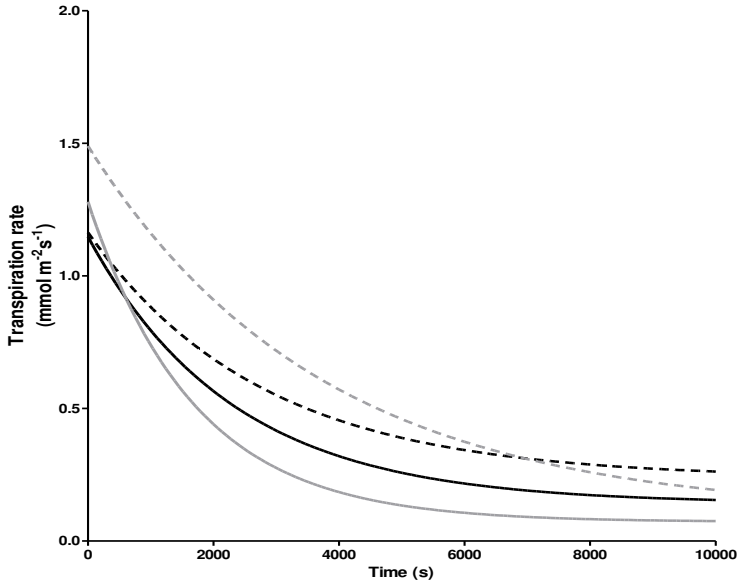


Figure S1. Fitted curves of transpiration rate (E) for Col-0 (black lines) and Cvi-0 (grey lines) *Arabidopsis* accessions during 10000 s desiccation of leaves of plants that have been exposed for 4 days to moderate (1.17 kPa; solid lines) or to low (0.23 kPa; broken lines) VPD. The leaves were first saturated in degassed deionized water and after 1 hr measurements were conducted at VPD of 1.40 kPa. The R^2 of goodness of fits was 0.9±0.1. The raw data are not shown for clarity of the figure.

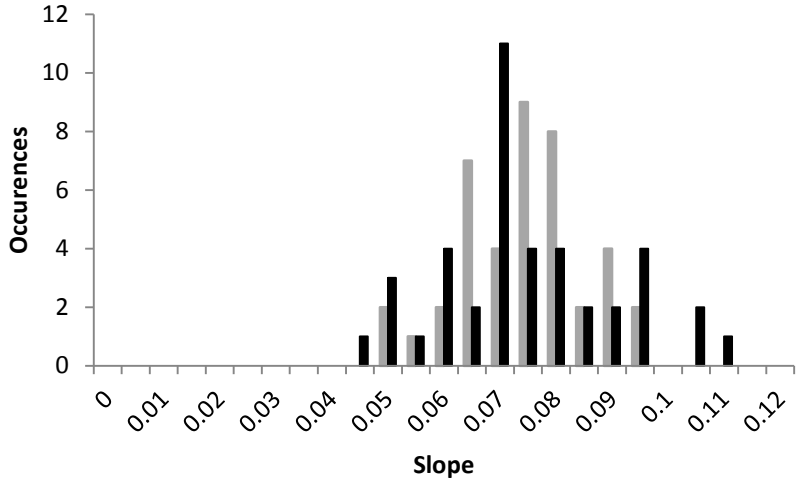


Figure S2. Distribution of 41 *Arabidopsis* accessions that have been exposed for 4 days to moderate (1.17 kPa; black bars) or to low (0.23 kPa; grey bars) VPD according to Slope of E×RWC during 10000 s desiccation of the leaves. The leaves were first saturated in degassed deionized water and after 1 hr measurements were conducted at VPD of 1.40 kPa.

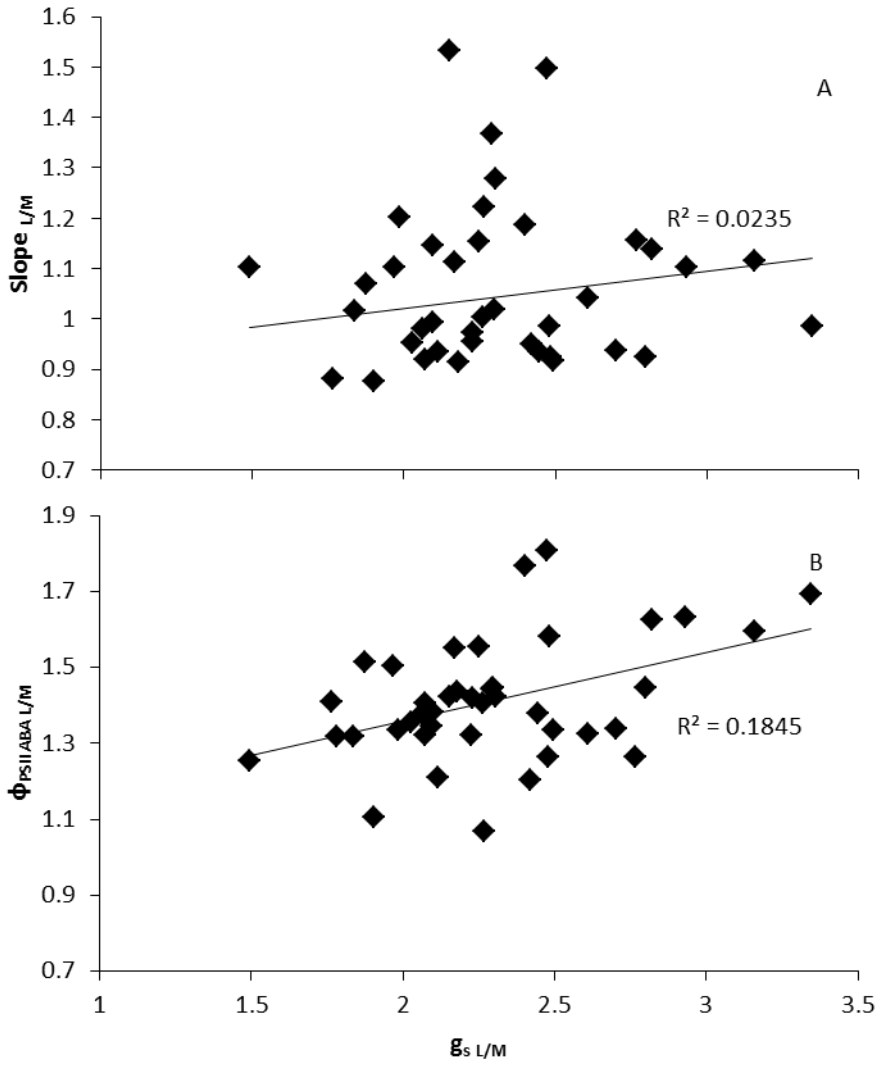
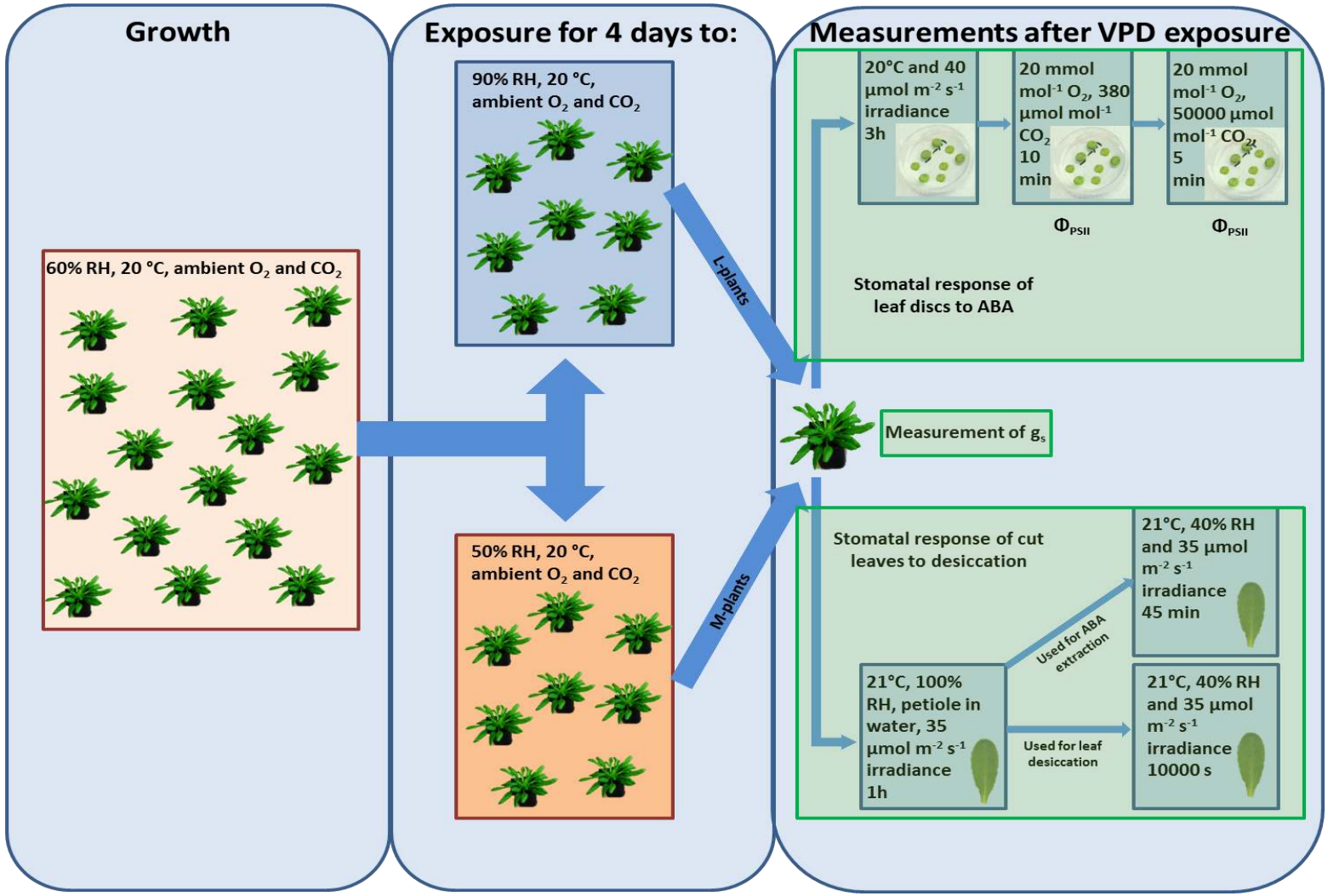


Figure S3. Relation between the effect of prior VPD-exposure on stomatal conductance (g_s) and desiccation response (A) or ABA response (B) of 41 *Arabidopsis* accessions. Plants had been exposed for 4 days to low VPD (0.23 kPa) or to moderate VPD (1.17 kPa). The effect of prior VPD on stomatal conductance was expressed as g_s at L/g_s at M ($g_{s\ L/M}$), on desiccation response as the ratio of the Slopes of RWC×E at L and M (Slope $_{L/M}$), and on ABA response as the ratio of the relative effects of 200 µM ABA to Φ_{PSII} ($\Phi_{PSII\ 200\ ABA}$ / $\Phi_{PSII\ C}$) at low and moderate VPD ($\Phi_{PSII\ ABA\ L/M}$). Measurements of g_s were conducted at a VPD of 1.40 kPa.

Appendix A. Schematic representation of the experimental setup and conditions which were used for growing plants and measurements. Boxes describe the conditions used for growing plants and measurements. The arrows shows transferring to new conditions.



CHAPTER 5

Abscisic acid-induced *RD29A* is crucial for keeping stomatal functionality after long-term exposure to low vapour pressure deficit

Abstract

Abscisic acid (ABA) is a key component controlling stomatal closure. However, long-term exposure to low vapour pressure deficit (VPD) decreases the stomatal closing response to ABA. Exogenous application of 5 µM ABA during the low VPD-exposure (ABA-treated plants) prevented the loss of stomatal response to ABA. To elucidate the molecular network underlying this stomatal malfunctioning due to long-term low VPD, two groups of *Arabidopsis* accessions were selected as responsive (Map-42 and C24) and non-responsive (Col-0, Cvi-0 and Rrs-7) to ABA after a 4-day exposure to low VPD. Neither genes involved in ABA transport and perception nor genes involved in secondary messengers and ethylene signalling were responsible for the lack of stomatal responsiveness to ABA. In contrast, transcript levels of *CYP707A* genes, which are involved in ABA catabolism, increased by low VPD in Col-0 and Rrs-7, but not in the accessions which remained responsive to ABA. Transcript levels of *RD29A* (Responsive to Desiccation) genes decreased by low VPD in the accessions with non-responsive stomata to ABA (Col-0, Cvi-0 and Rrs-7), while its expression increased in the ABA-treated plants and in the accessions which remained responsive to ABA after low VPD-exposure (Map-42 and C24). Stomata of an *RD29A* over-expressing line and of a *cyp707a1 cyp707a3* double mutant remained responsive to ABA after exposure to low VPD. Only when the ABA level was above a threshold value, stomata responded to exogenously applied ABA. In conclusion, down-regulation of ABA regulated *RD29A* gene expression causes diminished stomatal response to ABA after exposure to low VPD.

Keywords: Stomata functioning, vapour pressure deficit, abscisic acid, *RD29A*, *CYP707A*

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Introduction

The aperture of stomata is controlled by two guard cells which continuously receive endogenous signals due to exposure to various environments. The environment-induced signals change the turgor pressure in the guard cells, causing changes in stomatal aperture (Schroeder et al., 2001; Schroeder et al., 2001; Kim et al., 2010; Monda et al., 2011). Stomata regulate the crucial balance between beneficial CO_2 import, a substrate of photosynthesis, and at the same time detrimental excessive water loss via transpiration. To obtain an optimal balance between water loss and CO_2 uptake, stomata dynamically respond to environmental factors such as temperature, light, CO_2, relative humidity (RH) and water availability (Schroeder et al., 2001; Schroeder et al., 2001; Okamoto et al., 2009; Kim et al., 2010; Wilkinson and Davies, 2010; Monda et al., 2011; Chen et al., 2012) as well as to endogenous signals such as phytohormones, especially abscisic acid (ABA) which plays an important role under stress conditions (reviewed by (Acharya and Assmann, 2009)). Although guard cells are equipped with a complex signalling network for suitable responses to environmental factors, a reduced capacity of stomata to close in response to closing stimuli has been reported after plants had been exposed to some environmental factors (reviewed by (Aliniaeifard and van Meeteren, 2013)). Especially, it has been shown that stomata are not capable of suitable response to different closing stimuli when plants have grown at a low vapour pressure deficit (VPD) as occurs in humid conditions (Rezaei Nejad and van Meeteren, 2005, 2007; Fanourakis et al., 2011; Aliniaeifard and van Meeteren, 2013; Giday et al., 2013; Giday et al., 2013; Aliniaeifard et al., 2014). Even a few days exposure to a low VPD results in a decreased capacity of stomata to close in response to subsequent desiccation or ABA treatment (Rezaei Nejad and van Meeteren, 2008; Aliniaeifard and van Meeteren, 2013; Aliniaeifard et al., 2014).

Growing plants at low VPD results in alterations in leaf anatomical and stomatal morphological traits (Torre et al., 2003; Aliniaeifard and van Meeteren, 2013; Aliniaeifard et al., 2014). However, it is likely that alterations in leaf anatomy and stomata morphology are not involved in the decreased ability of stomatal closure in response to closing stimuli such as ABA and desiccation (Aliniaeifard et al., 2014). Aliniaeifard and colleagues suggested that alterations in the stomatal guard cell's signalling pathway are the main reason for stomatal malfunctioning after a few days exposure to low VPD (Aliniaeifard et al., 2014). It is astonishing that a single environmental condition, like low VPD, can disrupt the normally so robust network of stomata control. The aim of the current study was to elucidate the

molecular mechanism of decreased stomatal responsiveness to ABA after exposure to low VPD.

After transport of ABA from vascular tissue or the mesophyll to its receptors in the guard cell cytosol (Allan et al., 1994; Anderson et al., 1994; Assmann and Wu, 1994; Hirayama and Shinozaki, 2007; Pandey et al., 2009; Kang et al., 2010; Kuromori et al., 2010; Kuromori and Shinozaki, 2010; Kuromori et al., 2011), the ABA signalling pathway inside the guard cells starts through three protein classes: i) the PYR (PYRabactin Resistance)/PYL (PYR1-like)/RCARs (Regulatory Components of ABA Receptor)-family, ii) the type 2C protein phosphatases (PP2Cs), and iii) the SNF1-related protein kinase (SnRK2) Open Stomata 1 (OST1). The model for short-term ABA action through the PYR/PYL/RCAR receptors has been reviewed by Cutler *et al.* (2010). These three types of proteins operate as ABA receptors, negative modulators of the ABA signalling pathway, and positive modulators of downstream signalling, respectively (Mustilli et al., 2002; Belin et al., 2006; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). All of these components are present in both cytosol and nucleus and can induce long term as well as temporary changes in ABA responses (Moes et al., 2008; Fujita et al., 2009; Ma et al., 2009; Santiago et al., 2009; Raghavendra et al., 2010). Combined, these three types of proteins determine the activation or inactivation of ABA signalling. It has been shown that the level of ABA decreased as a result of exposure to low VPD (Rezaei Nejad and van Meeteren, 2007; Aliniaeifard and van Meeteren, 2013; Aliniaeifard et al., 2014). As reviewed by Aliniaeifard and van Meeteren (2013), under low ABA level, the PP2C/ABI1 (ABA-Insensitive 1) inactivates SnRK2/OST1 via dephosphorylation; as a result ABI1 represses signalling components downstream of ABA. Under high ABA levels, for example under high VPD conditions, the ABA is bound by intracellular PYR/RCAR dimers which then inhibits phosphatase activity of PP2Cs, allowing SnRK2 activation and phosphorylation of target proteins (Fujii et al., 2009; Geiger et al., 2009; Park et al., 2009; Umezawa et al., 2009). Therefore, a long period of low concentration of ABA (as a result of a prolonged exposure to low VPD) will result in ABA desensitisation. Stomatal closure during water deprivation maintains water status of the plants, two ABA-inducible *RD29* (Responsive to Desiccation) genes, *RD29A* and *RD29B*, are induced by abiotic stresses such as drought and salinity. *RD29B* functions in an ABA-dependent pathway, while *RD29A* functions in both ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki et al., 1995; Narusaka et al., 2003; Kasuga et al., 2004; Hua et al., 2006; Ma et al., 2010).

Secondary messengers such as H_2O_2 and calcium (Ca^{2+}) are also involved in the guard cell's signalling pathway for closure of the stomata. NADPH oxidases (e.g. AtrbohD and AtrbohF) involved in H_2O_2 production, mediate activation of Ca^{2+} channels by ABA (Pei et al., 2000). It has been proposed that a more reduced state of ascorbate and glutathione increases the H_2O_2 scavenging capacity resulting in less stomatal closure (Chen and Gallie, 2004; Aliniaeifard and van Meeteren, 2013). Closure of stomata by ABA requires nitric oxide (NO) which acts downstream of H_2O_2 (Murata et al., 2001; Wang et al., 2011). Aliniaeifard and van Meeteren (Aliniaeifard and van Meeteren, 2013) hypothesized that due to a low transpiration rate at low VPD, Ca^{2+} transport in the xylem stream slows down, resulting in low activity of calcium sensors and consequently low ABA sensitivity and a diminished stomatal closure response.

Also other phytohormones are involved in the regulation of stomatal aperture. Ethylene is one of the phytohormones that attracted a lot of attention in respect to its interaction with ABA for regulating stomatal aperture under abiotic stress conditions (Tanaka et al., 2005; Wilkinson and Davies, 2009; Wilkinson and Davies, 2010). It has been shown that stomatal response to ethylene can be different in the presence or absence of ABA (Desikan et al., 2006; Tanaka et al., 2006). Although, ethylene or its precursor (ACC) can inhibit the closure of stomata by ABA (Tanaka et al., 2005), in the absence of ABA ethylene can induce stomatal closure (Desikan et al., 2006; Tanaka et al., 2006). There is a lack of information regarding the role of ethylene in stomatal behaviour in respect to VPD conditions.

Our knowledge of disturbed ABA signalling components for a decreased stomatal response to closing stimuli is far from complete. It is not clear if the disturbance in the signalling pathway occurs at the level of ABA biosynthesis and catabolism, transport, perception, or signal transduction or if it is due to changes in the secondary messengers or ethylene signalling.

Based on our previous study, which analysed stomatal responses after a 4-day exposure to moderate and low VPD of 41 natural accessions of *Arabidopsis thaliana* (Aliniaeifard and van Meeteren, 2014), three accessions were chosen according to the type of their stomatal response: Map-42 as an accession with stomata that maintained responsiveness to desiccation and ABA after prior exposure to low VPD, Col-0 as an accession with stomata that lost their responsiveness to exogenous ABA after prior low VPD exposure, but maintained responsive to desiccation, and Cvi-0 as an accession with non-responsive stomata to ABA and less-responsive stomata to desiccation after prior exposure to low VPD. Stomatal response to desiccation is a complicated process, likely involving ABA and non-ABA controlled pathways (Aliniaeifard and van Meeteren, 2014). In the current study we focused on the stomatal response to ABA.

Material and methods

Plant Material and Growth Conditions

Seeds of Map-42, C24, Cvi-0, Rrs-7 and Col-0 were obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio State University, USA. *rd29a*, *cyp707a1*, *cyp707a3* mutant plants were in Columbia background and were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The *cyp707a1 cyp707a3* double mutant was provided by Eiji Nambara, department of Cell & Systems Biology (CSB), University of Toronto, Toronto, Ontario, Canada; it was also in Columbia background. The plants were grown as described before (Aliniaeifard and van Meeteren, 2014). In short: After stratification, the seeds were sown in a pot filled with a soil developed for *Arabidopsis* (Arabidopsis soil, Horticoop, the Netherlands). After germination, in the stage of 2 leaves, the plants were transplanted to pots filled with a mixture of fine and course sands. The top of the sand mixture was covered with 0.5 cm Arabidopsis soil. The surface of the soil was covered with a black plastic sheet to prevent contact of the leaves with wet soil and to prevent a micro-climate with low VPD around the rosette of the plants. The plants were irrigated 4 times per week using a nutrient solution. All plants were grown in a climate chamber with a constant temperature of 20°C, 60% relative humidity (RH), resulting in a VPD of 0.94 kPa, 12h/12h day/night lighting period, 150 µmol m^{-2} s^{-1} light produced by fluorescent tubes (TLD 58W/84 Philips) and 380 µmol mol^{-1} CO_2. When the plants had produced fully developed leaves in the stage between 3.9 and 5 (stages as indicated by (Boyes et al., 2001)), they were transferred to growth chambers with the same temperature and light conditions but with different VPDs. One of them with 50±5% RH, resulting in a VPD of 1.17 kPa (moderate VPD); another one with 90±5% RH, resulting in a VPD of 0.23 kPa (low VPD). After 4 days exposure to the two VPD conditions, fully developed leaves were used for analyses.

Generation of Transgenic Plants

The full length of *Arabidopsis thaliana RD29A* cDNA was generated using the Arabidopsis genome sequence in NCBI (8048 bp and 710 a.a residues) as a template (D13044.1). PCR products are directionally cloned by adding four bases (CACC) to the forward primer. The cDNA was isolated by polymerase chain reaction (PCR) using the forward primer 5' CACCACAAATATGCAAACTAGA and reverse primer 5' CTCCTTCTGCACCGGAACAACAG. The PCR products were sequenced and the open reading frame (ORF) from the cDNA of the *RD29A* gene (D13044.1) was cloned into pENTR/D-TOPO vector (Invitrogen) creating pENTR-RD29A. The UBQ promoter was also

cloned to a pENTR4-1 vector (Invitrogen). The pENTR/D-TOPO containing *RD29A* gene, pENTR vector including the ubiquitin-10 gene promoter (PUBQ10) and pENTR p2rp3 (Invitrogen) containing GFP Stop-Term, were recombined in a multisite Gateway reaction (Invitrogen) into a binary destination pBnRGW vector. This vector contained DsRED1 (as visual selection marker) and spectinomycin (as bacterial resistance). The resulting plasmids were transferred by electroporation to *Agrobacterium tumefaciens* strain C58, which was used for transformation of *Arabidopsis thaliana* (Columbia, CS76113) plants by floral-dipping. Transgenic plants were obtained by selection for red florescent seed coats with stereomicroscopy. Approximately 10% of the seeds were transgenic and 20 seeds selected to generate F2 line as independent homozygote line for final stomatal response.

Transcript Analysis

Transcript levels of genes involved in ABA biosynthesis, catabolism, perception and signalling (*ABCG25*, *ABCG40*, *PYL4*, *PYR1*, *RCAR*, *GTG1*, *CYP707A1*, *CYP707A3*, *GCA2*, *NCED3*, *ABI1*, *ABI2*, *RCN1*, *OST1*, *RD29A*, *RD29B*, *GTG2*) or in signalling of secondary messengers and ethylene (*CAS*, *rbohD*, *rbohF*, *CPK3*, *CPK4*, *CPK6*, *NOA1*, *NIA1*, *NOS1*, *GPX3*, *DHAR*, *PLD@1*, *SLAC1*, *ETR1*, *EIN2*) were analysed in Col-0, Cvi-0 and Map-42 accessions after a 4-day exposure to low or moderate VPD. Moreover, ABA was sprayed to the leaves of Col-0 and Cvi-0 during the 4-day exposure to low VPD. Also in these ABA sprayed plants, the transcript levels of the mentioned genes were analysed. The expression of *RD29A*, *CYP707A1* and *CYP707A3* was further analysed in C24 and Rrs-7 accessions and also in *rd29a*, *cyp707a1 cyp707a3* mutant plants.

From *Arabidopsis* plants exposed for 4 days to moderate and low VPD, 50 mg of fresh leaves is collected by dipping in liquid nitrogen. The tissue was grinded (by Tissue Lyser LT, Qiagen®) and total RNA was isolated according to the RNA extraction protocol by the E.Z.N.A. ® Plant RNA Kit (Omega® bio-tek). cDNAs were synthesized from 2 µg of total RNA using iScript TM cDNA Synthesis Kit (BIO RAD®). The quantification of RNA transcripts was analysed using Ubiquitin C as housekeeping gene. The primer sets that were used are listed in Table 1 of the supplementary data. Analysis of RNA transcript level was performed using single color real time PCR detection system icycler (BIO RAD®) and the Bio-rad iQ5 Software. All experiments were repeated at least three times with three biological independent repetitions.

Short-term ABA application after exposure to different VPDs

The stomatal response to ABA at the end of a 4-day exposure to low and moderate VPDs was measured as described before (Aliniaeifard and van Meeteren, 2014). Leaf discs (0.5 cm diameter) were prepared from 8 leaves from 8 individual plants. The discs were put with the adaxial surface down in petri dishes filled with stomata-opening medium (50 mM KCl, 10 mM MES-KOH, pH 6.15, 50 µM $CaCl_2$) with different concentrations of ABA (0, 50, 100, 200 µM ABA). After 3 min vacuum infiltration, the leaf discs were pre-incubated for 3 hr in the above mentioned ABA-solutions at 20°C and 40 µmol m^{-2} s^{-1} irradiance. To investigate stomatal closure, φ_{PSII} under a non-photorespiratory condition was measured.

Mapping of PSII photochemical efficiency using chlorophyll fluorescence

To analyse stomatal closing response to ABA, chlorophyll fluorescence imaging under a non-photorespiratory condition was used as described before (Aliniaeifard and van Meeteren, 2014). The petri dishes containing the leaf discs in solutions with different ABA concentrations, were placed in a gas-tight cuvette. The temperature in the cuvette was 22±1 °C. The cuvette was placed under a chlorophyll fluorescence imaging system (FuorCam 700MF, PSI, Brno, Czech republic). The imaging measurement was conducted while flowing an atmosphere with 20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and the rest N_2 (non-photorespiratory condition) into the cuvette. The RH of the gas mixture was set to 40±3% via passing the air in a temperature-controlled column of iron (II)-sulphate heptahyrate (Fluka). The leaf discs were exposed to a continuous irradiance of 100 µmol m^{-2} s^{-1}. After 10 min (when steady state Φ_{PSII} was reached) the protocol for FluorCam was run and the average value of Φ_{PSII} per leaf disc was calculated by using version 5 of FluorCam software. Values for F_t and F_m' in the generated image were averaged over all pixels per leaf disc and then the Φ_{PSII} was calculated using ratio F_m'-F_t / F_m'. At the end of the imaging of Φ_{PSII}, an image was taken in an atmosphere with a high CO_2 concentration (20 mmol mol^{-1} O_2, 50000 µmol mol^{-1} CO_2) to test the recovery of Φ_{PSII} when stomatal closure is not the limiting factor for CO_2 entrance into the mesophyll.

Long-term ABA application during exposure to low VPD condition

In order to analyse the effect of enhanced ABA level during exposure to low VPD on the transcript level of the genes and on stomatal response characteristics, the leaves of Col-0 and Cvi-0 accessions were daily sprayed with a solution of 5 µM ABA in distilled water and two drops of Triton X-100 per litre during a 4-day exposure to low VPD. Control plants were

sprayed with distilled water/Triton X-100 solution similar to the treated plants. The ABA spray was stopped 24 h prior to the measurement of the ABA response. For analysis of the stomatal response to ABA, the protocol of short term ABA application was used.

ABA extraction and quantification

Fully developed leaves of Col-0, Cvi-0, Map-42, C24 and Rrs-7 accessions and *RD29A-OE*, *cyp707a1 cyp707a3* mutant plants were used after a 4-day exposure to moderate and low VPD. 0.5 g of leaf tissue was ground in a mortar using liquid nitrogen. The samples were extracted with 1 ml of cold ethyl acetate containing 0.1 nmol [$^2H_6$]-ABA as internal standard. The samples were vortexed for 1 min and then sonicated for 15 min in a Branson 3510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA). Samples were centrifuged for 10 min at 2200 rpm in an MSE Mistral 2000 centrifuge (Mistral Instruments, Leicester, UK). The supernatant was transferred to a 4-ml glass vial. The pellets were re-extracted with 1 ml of methanol without sonication. The solvent fractions were pooled in a 4-ml glass vial. Then the samples were dried using a speedvac (SPD2010-230, Thermo Scientific, USA) and the residue was dissolved in 50 µl methanol. 3 ml MQ water was added to the samples and the extracts were purified using 500 mg C18 columns. The samples were eluted with 1 ml acetone. Then the acetone was evaporated under N_2. The residue was dissolved in 200 µl of acetonitrile:water:formic acid (10:90:0.1, v:v:v). Samples were filtered into vials with Minisart 0.2 µm filters (Sartorius, Goettingen, Germany) and were analysed using LC-MS /MS analysis as described by López-Ráez *et al.* (2010) with minor modifications and using a Waters Xevo TQ tandem mass spectrometer (Waters, USA) equipped with an electrospray ionization (ESI) source and coupled to an Acquity UPLC system (Waters). Chromatographic separation was done on an Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm) (Waters). Data acquisition and analysis were performed using Masslynx 4.1 software (Waters).

Results

Disturbance in stomatal response to ABA (short-term) due to exposure to low VPD can be prevented by ABA application during the exposure to low VPD

To study the stomatal response of *Arabidopsis* accessions to low VPD, we exposed leaf discs of Col-0, Cvi-0 and Map-42, to a regime of different ABA concentrations, after prior exposure to moderate and low VPD. Measuring of Φ_{PSII} under a non-photorespiratory condition, showed that stomatal aperture was decreased by application of different

concentrations of ABA (50, 100, 200 µM) to leaf discs in moderate VPD-exposed as well as low VPD-exposed plants of Col-0, Cvi-0 and Map-42 *Arabidopsis* accessions (Fig. 1). However, in Col-0 (Fig. 1a) and Cvi-0 (Fig. 1b) different responses were observed between plants exposed to moderate and low VPD. In Col-0 and Cvi-0 the Φ_{PSII} was less reduced in low VPD than in moderate VPD-exposed plants after application of ABA. At higher ABA concentrations, larger differences were observed between moderate and low VPD-exposed Col-0 (Fig 1a) and Cvi-0 (Fig 1b) plants. However, the lowest value of Φ_{PSII} was observed in 200 µM ABA for both VPDs in all accessions. These results indicate that the closing response to ABA of Col-0 and Cvi-0's stomata decreased as a result of a prior 4-day exposure to low VPD.

In order to investigate the role of leaf ABA level in stomatal functioning of low VPD-exposed plants, 5 µM ABA was daily sprayed during plant exposure to low VPD on accessions which lost stomatal ABA responsiveness after low VPD exposure (Col-0 and Cvi-0). Spraying leaves with ABA during the exposure of Col-0 and Cvi-0 plants to low VPD altered the behaviour of their stomata afterwards (Fig. 1a-b). Contrary to water-sprayed Col-0 and Cvi-0 plants, stomata of Col-0 and Cvi-0 closed in response to ABA (short term) when they had been sprayed with ABA during the prior 4-day exposure to low VPD, as shown by the decrease of Φ_{PSII}. In long-term ABA-sprayed plants, the Φ_{PSII} decreased in an ABA concentration dependent manner, similar as the Φ_{PSII} of Col-0 and Cvi-0 plants that had been exposed to moderate VPD (Fig 1a-b). These results indicate that ABA application during exposure to low VPD maintains the stomatal closing response to ABA afterwards.

In Map-42 the effect of a 4-day exposure to low VPD on the stomatal response to ABA differed from the response of Col-0 and Cvi-0. The Φ_{PSII} decreased as a result of short-term ABA application in moderate VPD as well as low VPD-exposed Map-42 plants and no difference was found between the low VPD and moderate VPD-exposed plants after application of different concentrations of ABA (0, 50, 100, 200 µM) (Fig. 1c). In all of the experiments Φ_{PSII} was recovered to its original value before ABA application by 5 min exposure to a high CO_2 concentration (50000 µmol mol^{-1}), confirming that the decrease in Φ_{PSII} was because of stomatal closure (data not shown). Therefore these results are indicative of different effects of a prior exposure to low VPD between *Arabidopsis* accessions: (1) Map-42 with stomata that maintained responsive to ABA and (2) Col-0 and Cvi-0 accessions with low VPD induced non-ABA-responsive stomata.

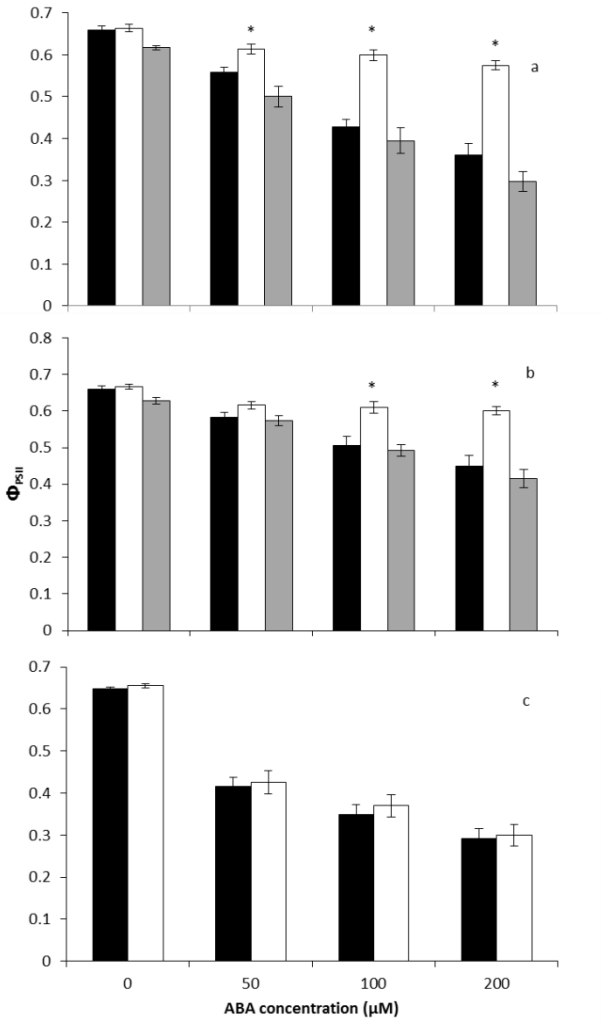

Figure 1. PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions (20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and reminder N_2) for Col-0 (a), Cvi-0 (b) and Map-42 (c) accessions after a 4-day exposure to moderate (black bars) or low (white bars) VPD or sprayed every day with 5 µM ABA during the exposure to low VPD (grey bars). Leaf discs (0.5 cm diameter) were put with the adaxial surface down in petri dishes filled with stomata-opening medium (50 mM KCl, 10 mM MES-KOH, pH 6.15, 50 µM $CaCl_2$) with different concentrations of ABA (0, 50, 100, 200 µM ABA). Φ_{PSII} was recorded 3 hr after application of ABA. Asterisks show the significant differences compared with Φ_{PSII} of moderate VPD-exposed plants ($P < 0.05$).

Transcript levels of genes involved in ABA production, catabolism, perception and signalling considerably differ after exposure to different VPDs

To find which steps in the guard cell-ABA pathway are involved in the loss of the stomatal closing response to ABA after long-term exposure to low VPD, the transcript levels of genes involved in ABA transport (*ABCG25, ABCG40*), ABA perception (*PYL4*, *PYR1*, *RCAR*, *GTG1, GTG2*), ABA biosynthesis and catabolism (*NCED3, CYP707A1*, *CYP707A3*) and ABA signal transduction (*ABI1*, *ABI2*, *OST1*, *RD29A*, *SLAC1*, *RCN1, GCA2*) were analysed

after a 4-day exposure to low VPD in Col-0 and Cvi-0 (as accessions with non-responsive stomata to ABA) and Map-42 (as an accession with responsive stomata to ABA). Since spraying ABA during low VPD-exposure of Col-0 and Cvi-0 accessions (ABA-treated plants) sustained the stomatal closing response to short-term ABA treatment afterwards, transcript levels were determined by qPCR on RNA isolated from ABA- treated and untreated leaves. The transcript levels of *ABI1* and *ABI2* were significantly higher in Col-0 due to exposure to low VPD in comparison with their levels in plants that had been exposed to moderate VPD (Fig. 2a). *ABI1* and *ABI2* transcript levels were doubled after exposure to low VPD, while the transcript level of *ABCG25* was significantly decreased (Fig. 2a). The transcript level of *CYP707A1* was approximately 3-fold, and of *CYP707A3* approximately 5-fold higher as result of low VPD-exposure. Application of ABA during the exposure to low VPD inhibited the increase in transcript levels of *CYP707A1* and *CYP707A3*. In ABA-sprayed Col-0 plants, the transcript level of *CYP707A3* remained at the same level as that of moderate VPD-exposed plants. These results showed that in Col-0, the genes involved in catabolism of ABA were highly induced by exposure to long-term low VPD and that this induction was prevented by increased ABA levels, indicating a negative regulation of ABA catabolism by (long-term) ABA.

Exposure to low VPD decreased the transcript level of *RD29A* 17-fold, while the transcript level of *RD29A* remained at the same level as that of moderate VPD-exposed plants by spraying ABA during the 4-day exposure of Col-0 plants to low VPD (Fig. 2a). In Cvi-0, exposure to low VPD significantly increased the *RCAR* transcript level in comparison with moderate VPD-exposed plants (Fig. 2b). Long-term application of ABA during the low VPD-exposure of Cvi-0 plants led to a decline in the transcript levels of *CYP707A1* and *CYP707A3* in comparison with moderate VPD. The transcript level of *ABCG40* significantly increased due to long-term ABA application during low VPD exposure in comparison with its level in non-ABA sprayed plants and with plants exposed to moderate VPD (Fig. 2b). The transcript level of *RD29A* decreased approximately 3-fold as a result of exposure to low VPD. Similar to Col-0, spraying ABA during low VPD-exposure of Cvi-0, maintained the transcript level of *RD29A* at the same level as the Cvi-0 plants exposed to moderate VPD (Fig. 2b).

In the case of Map-42 (an accession which kept its stomatal response to ABA after low VPD-exposure), exposure to low VPD significantly decreased the transcript levels of *CYP707A1*, *PYR1* and *ABCG25* in comparison with their levels in moderate VPD-exposed plants (Fig. 2c). The transcript level of *PYL4* increased as a result of a exposure to low VPD in Map-42

accession (Fig. 2c). There were no significant changes in the other measured transcript levels in Map-42 after a 4-day exposure to low VPD.

These results indicate that only transcript levels of *RD29A* correspond to the responsiveness to ABA in the three studied accessions.

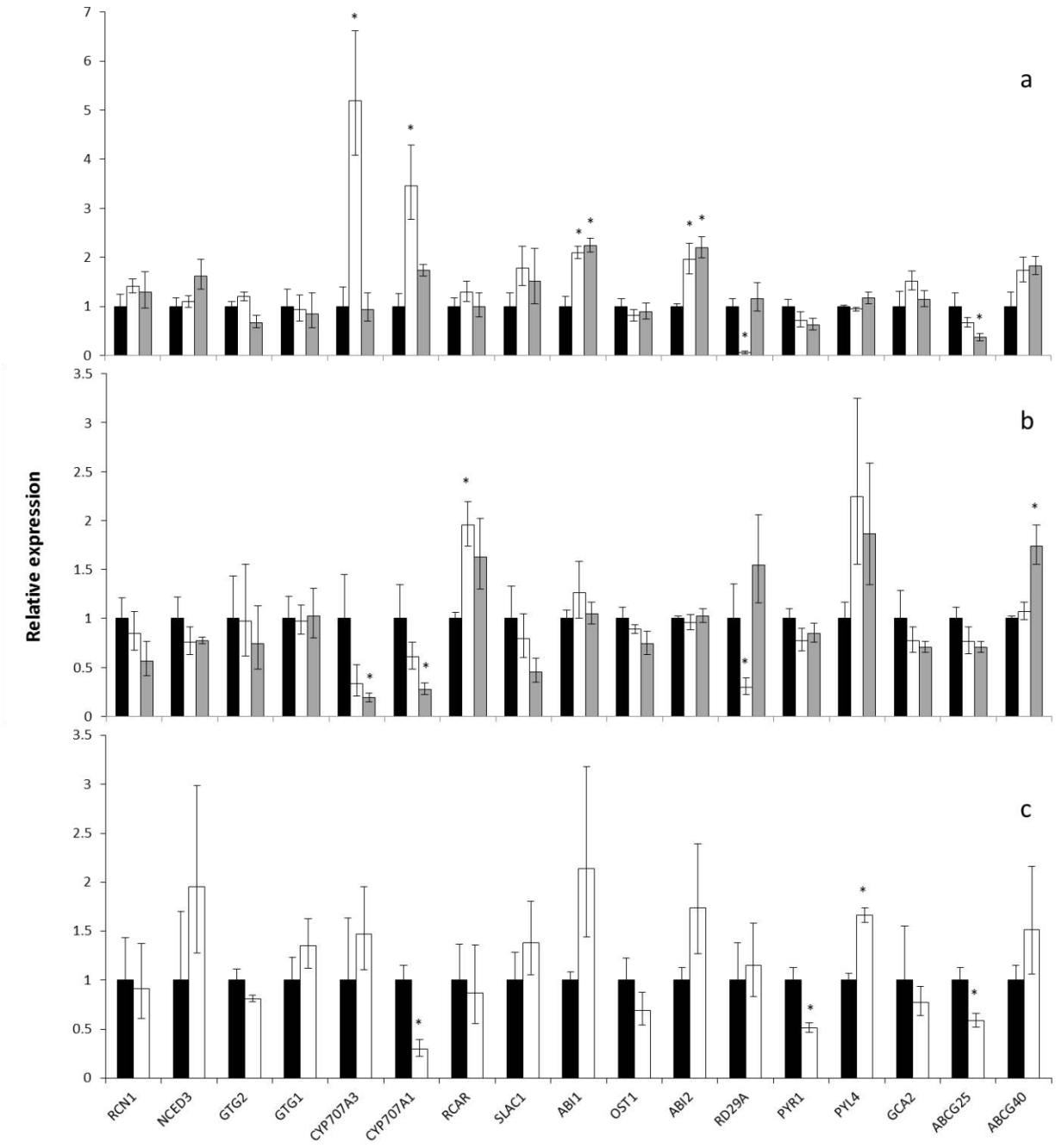

Figure 2. Transcript levels of genes involved in ABA production, catabolism, perception and signaling. Col-0 (a), Cvi-0 (b) and Map-42 (c) *Arabidopsis* accessions were exposed for 4 days to moderate (black bars), or low (white bars) VPD or sprayed every day with 5 µM ABA during low VPD-exposure (grey bars). Ubiquitin C was used as housekeeping gene in quantitative RT-PCR analysis. Each gene was normalized against plants in moderate VPD. Data are the mean values of three biological replicates ±SE. Asterisks show the significant differences compared with transcript level in the moderate VPD-exposed plants ($P < 0.05$).

Transcript levels of genes involved in secondary messengers and ethylene signalling after exposure to different VPDs

To investigate whether disruption in the responsiveness of stomata to ABA, due to long-term exposure to low VPD, occurs because of changes in the guard cell-secondary messengers pathways or ethylene signalling, the transcript levels of genes involved in calcium signalling (*CAS*, *CPK3*, *CPK4, CPK6*), nitric oxide production and signalling (*NOA1*, *NIA1*, *NOS1, PLDα1*), H_2O_2 production and catabolism (*rbohF*, *rbohD*, *DHAR, GPX3*) and ethylene signalling (*ETR1, EIN2*) were analysed in Col-0, Cvi-0, Map-42 after 4 days exposure to moderate and low VPD. The same transcript levels were also analysed in ABA-treated plants of Col-0 and Cvi-0 after a 4-day exposure to low VPD. In Col-0, the transcript level of *rbohF*, *NOS1*, *CPK4*, *CPK6* and *CAS* were significantly decreased after exposure to low VPD in comparison with their levels in moderate VPD-exposed plants (Fig 3a). Spraying leaves with ABA during the exposure of Col-0 to low VPD only restored the transcript level of *rbohF* to the level in low VPD-exposed plant (Fig. 3a). In Cvi-0, the transcript level of *rbohD* was 6 and 16 times higher in moderate VPD-exposed plants than its level after exposure to low VPD in non-treated and ABA-treated plants, respectively (Fig. 3b). Also the transcript level of *CPK6* decreased by 2.7 times as a result of a 4-day exposure to low VPD in both non-treated and ABA-treated Cvi-0 plants. In the case of Map-42, there were no significant differences for transcript levels of the genes involved in secondary messengers and ethylene signalling except for *EIN2* for which exposure to low VPD doubled the transcript level of *EIN2* in comparison with its transcript level in moderate VPD-exposed plants (Fig 3c).

Overall, these results suggest that from the secondary messenger signalling components, mostly calcium signalling was influenced by long-term exposure to low VPD. However, this alteration in calcium signalling was not restored by ABA treatment during the low VPD treatment.

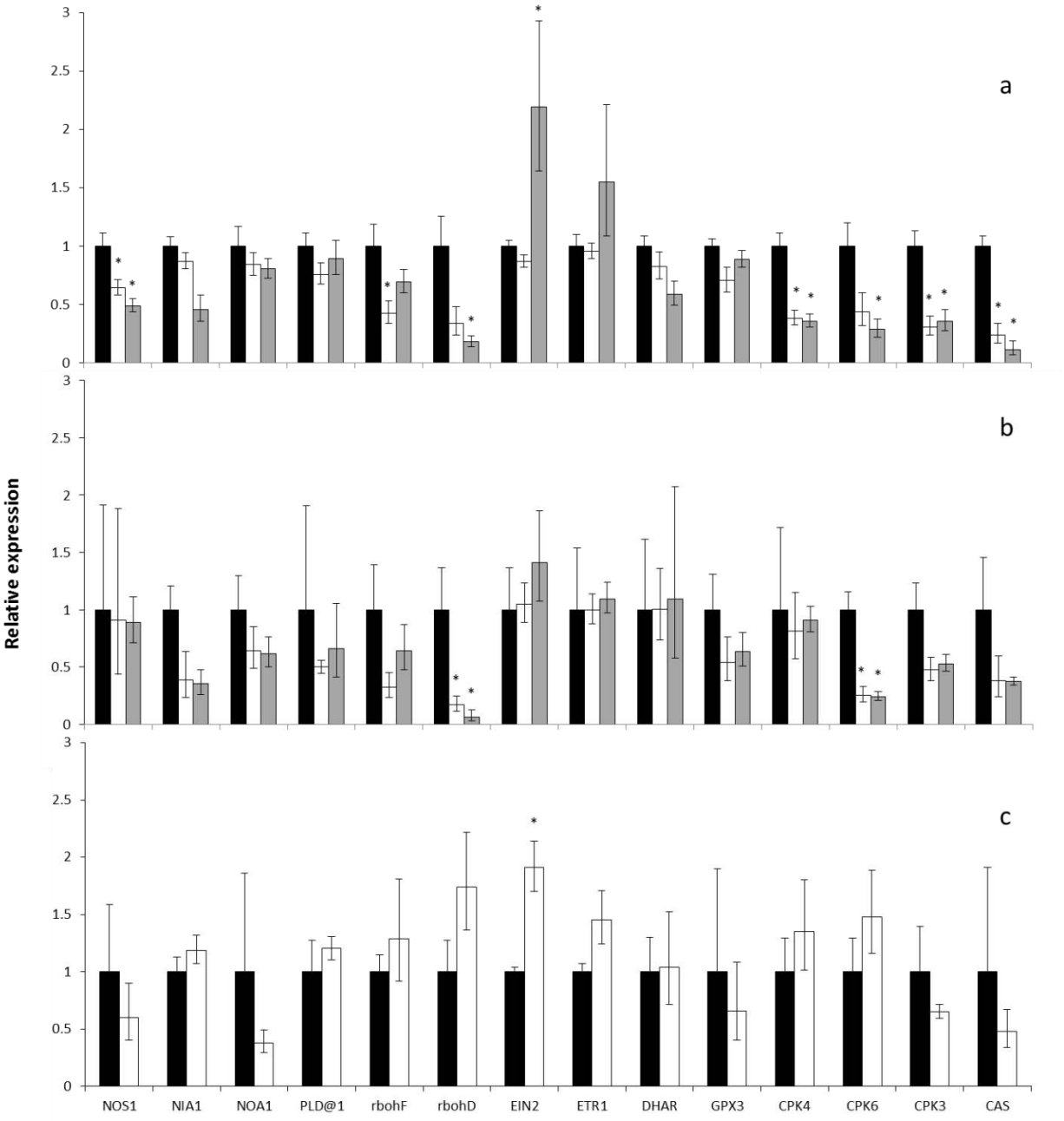

Figure 3. Transcript levels of genes involved in secondary messengers or ethylene signaling. Col-0 (a), Cvi-0 (b) and Map-42 (c) *Arabidopsis* accessions were 4 days exposed to moderate (black bars), or low (white bars) VPD or sprayed every day with 5 µM ABA during low VPD-exposure (grey bars). Ubiquitin C was used as housekeeping gene in quantitative RT-PCR analysis. Each gene was normalized against plants in moderate VPD. Data are the mean values of three biological replicates ±SE. Asterisks show the significant differences compared with transcript level in the moderate VPD-exposed plants ($P < 0.05$).

Stomatal response to ABA and transcript levels of RD29A, CYP707A1 and CYP707A3 in accessions with contrasting sensitivity to low VPD

Analysing the transcript level of genes involved in ABA biosynthesis, catabolism, perception and signalling or secondary messengers (and ethylene) signalling suggested that *RD29A* and *CYP707A*s (especially in Col-0) are involved in decreased stomatal response to ABA after low VPD-exposure. To find support for this, the transcript levels of these genes were also analysed in two accessions (C24 and Rrs-7) with a different response to ABA after exposure to different VPDs. In C24 the stomatal closing response to ABA was not influenced by a prior 4-day exposure to low VPD; the Φ_{PSII} of low VPD-exposed plants decreased in the same way after application of different ABA concentrations as the Φ_{PSII} in moderate VPD-exposed plants (Fig. 4a). In contrast, the Φ_{PSII} of low VPD-exposed Rrs-7 decreased less after application of different concentrations of ABA compared with the Φ_{PSII} of moderate VPD-exposed plants (Fig. 4b). If *RD29A* and *CYP707A*s are involved in the low VPD induced decreased stomatal response to ABA, we expect that the transcription of these genes is not altered in C24 plants, but is in Rrs-7 plants after low VPD exposure.

Indeed qPCR analyses showed that the transcript levels of *RD29A*, *CYP707A1* and *CYP707A3* were not significantly changed after exposure to low VPD in C24 (Fig. 5a). However, in low VPD-exposed Rrs-7 plants the transcript level of *RD29A* was considerably decreased (Fig. 5b) and the *CYP707A1* transcript level significantly increased (Fig. 5b). There was no effect of VPD exposure on *CYP707A3* in the Rrs-7 accession (Fig. 5b).

These results confirm that ABA responsive and non-responsive accessions after a prior low VPD-exposure, differed in the expression patterns of *RD29A* and *CYP707A* genes, particularly *RD29A* showed a consistent pattern.

RD29A and CYP707As are involved in the ABA closing response of stomata after long-term exposure to low VPD

We then wondered whether the expression of *RD29A* and *CYP707A* genes are essential for the ABA closing response of stomata after long-term exposure to low VPD. Since we had a *cyp707a1 cyp707a3* double mutant, the *cyp707a1* and *cyp707a3* single mutants and also a *rd29a* mutant available, we tested the response of these mutants to ABA after a 4-day exposure to low VPD. After exposure of the plants to moderate as well as low VPD, the Φ_{PSII} under non-photorespiratory conditions was significantly lower in a *cyp707a1 cyp707a3* double mutant in comparison with the wild-type, and the *cyp707a1* and *cyp707a3* single mutants (0 µM ABA in Fig. 6). This difference in Φ_{PSII} values only partially disappeared

when the CO_2 concentration (during measuring chlorophyll fluorescence) was increased to 50000 µmol mol^{-1} (data not shown), indicating that it was only partly due to lower stomatal conductance. The response to low VPD-exposure of the *cyp707a1* and *cyp707a3* single mutants did not differ from the moderate VPD-exposed wild-type, *cyp707a1* and *cyp707a3* single mutants. However, after applying ABA, increasing differences were observed between moderate and low VPD-exposed *cyp707a1* and *cyp707a3* single mutants particularly at the higher ABA concentrations (Fig. 6). After low VPD-exposure, no significant differences were found between Φ_{PSII} of wild-type and the *cyp707a1* and *cyp707a3* single mutants. Application of ABA (50, 100 or 200 µM) significantly decreased the Φ_{PSII} in moderate compared to low VPD-exposed plants of the wild-type, and the *cyp707a1* and *cyp707a3* single mutants. In the *cyp707a1 cyp707a3* double mutant, the value of Φ_{PSII} considerably decreased as a result of short-term ABA application in both low and moderate VPD-exposed plants compared with its value in wild-type, *cyp707a1* and *cyp707a3* single mutants. Although increasing ABA concentrations enlarged the differences in Φ_{PSII} between low and moderate VPD-exposed plants in wild-type, *cyp707a1* and *cyp707a3* single mutants, no significant differences were found between low and moderate VPD-exposure across different concentrations of ABA for the *cyp707a1 cyp707a3* double mutant (Fig. 6). The value of Φ_{PSII} was recovered to its value without ABA application by 5 min exposure to a high CO_2 concentration at the end of the Φ_{PSII} measurements of the different concentrations of ABA in all the mutants and wild-type plants, confirming that the decrease in Φ_{PSII} was because of stomatal closure (data not shown). Because *CYP707A1* and *CYP707A3* are involved in catabolism of ABA, these results indicate that catabolism of ABA during exposure to low VPD reduces afterwards the sensitivity of stomata to ABA.

No significant difference in Φ_{PSII} between moderate and low VPD-exposed *rd29a* mutant plants was detected after application of 50 µM ABA, but increasing differences were observed between moderate and low VPD-exposed *rd29a* mutants by increasing the ABA concentration. The difference in Φ_{PSII} between the moderate and low VPD-exposed *rd29a* mutant was smaller than in the wild-type. In the moderate VPD-exposed *rd29a* mutant, the response to ABA was less compared with the wild-type (Fig. 7).

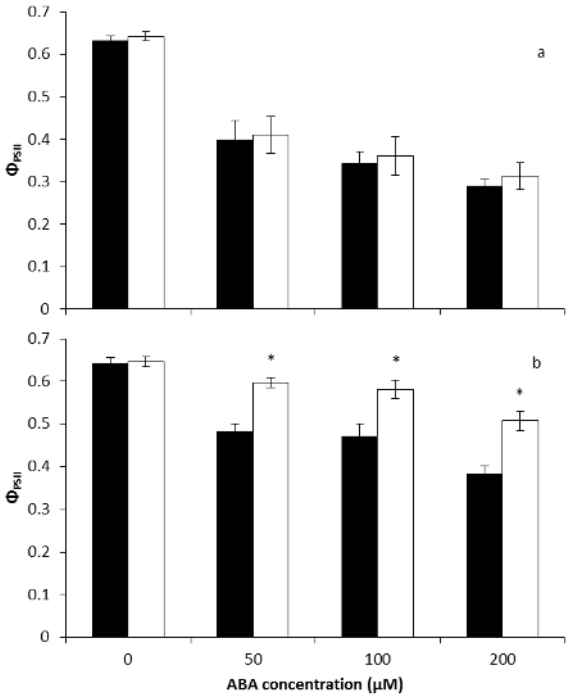

Figure 4. PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions (20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and reminder N_2) for C24 (a) and Rrs-7 (b) accessions exposed to moderate (black bars), or low (white bars) VPD. Leaf discs (0.5 cm diameter) were put with the adaxial surface down in petri dishes filled with stomata-opening medium (50 mM KCl, 10 mM MES-KOH, pH 6.15, 50 µM $CaCl_2$) with different concentrations of ABA (0, 50, 100, 200 µM ABA). Φ_{PSII} was recorded 3 hr after application of ABA. Asterisks show the significant differences compared with Φ_{PSII} of moderate VPD-exposed plants ($P < 0.05$).

Overexpression of RD29A maintained closing response of the stomata after long-term low VPD-exposure

We then wondered whether an increased level of *RD29A* transcript confers an increased sensitivity of stomata to ABA exposed to low VPD. To test this we generated transgenic *Arabidopsis* carrying the *RD29A* gene fused to GFP under the control of the UBQ10 promoter. In transgenic plants the location of the *RD29A*-GFP protein is confined to the trichomes and stomata's guard cells (Fig. 8).

Overexpression of *RD29A* significantly decreased the Φ_{PSII} in comparison with the wild-type (Fig. 7). In contrast to the wild-type and the *rd29a* mutant, no significant differences were found in Φ_{PSII} between plants exposed to moderate and low VPD in the *RD29A* overexpression line across different concentrations of ABA (Fig. 7a-b). After exposure to moderate as well as low VPD, the Φ_{PSII} decreased in response to ABA, indicating that stomata are responsive to ABA. The value of Φ_{PSII} recovered by 5 min exposure to a high CO_2 concentration at the end of the Φ_{PSII} measurements of the different concentrations of ABA in the *RD29A* overexpressed line and in *rd29a* (Fig. 7b), confirming that the decrease in Φ_{PSII} was because of stomatal closure.

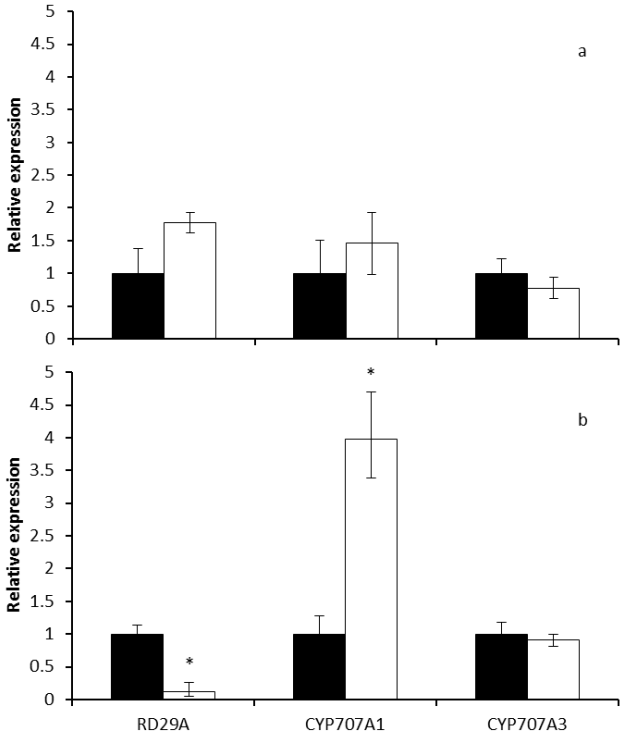

Figure 5. Transcript levels of *RD29A*, *CYP707A1* and *CYP707A3* for C24 (a) and Rrs-7 (b) accessions exposed to moderate (black bars), or low (white bars) VPD. Ubiquitin C was used as housekeeping gene in quantitative RT-PCR analysis. Each gene was normalized against plants in moderate VPD. Data are the mean values of three biological replicates ±SE. Asterisks show the significant differences compared with transcript level of moderate VPD-exposed plants (P< 0.05).

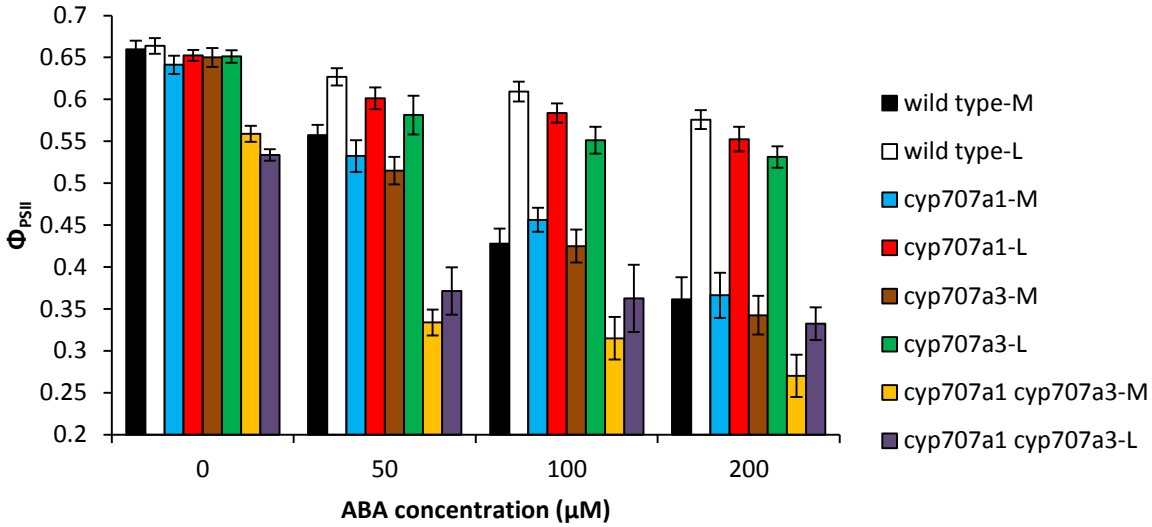

Figure 6. PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions (20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and reminder N_2) for wild-type, *cyp707a1*, *cyp707a3* and *cyp707a1 cyp707a3* mutants exposed to moderate (M) or to low (L) VPD. For measuring Φ_{PSII}, leaf discs (0.5 cm diameter) were put with the adaxial surface down in petri dishes filled with stomata-opening medium (50 mM KCl, 10 mM MES-KOH, pH 6.15, 50 µM $CaCl_2$) with different concentrations of ABA (0, 50, 100, 200 µM ABA), and Φ_{PSII} was recorded 3 h after application of ABA. Data are the mean value of Φ_{PSII}±SE.

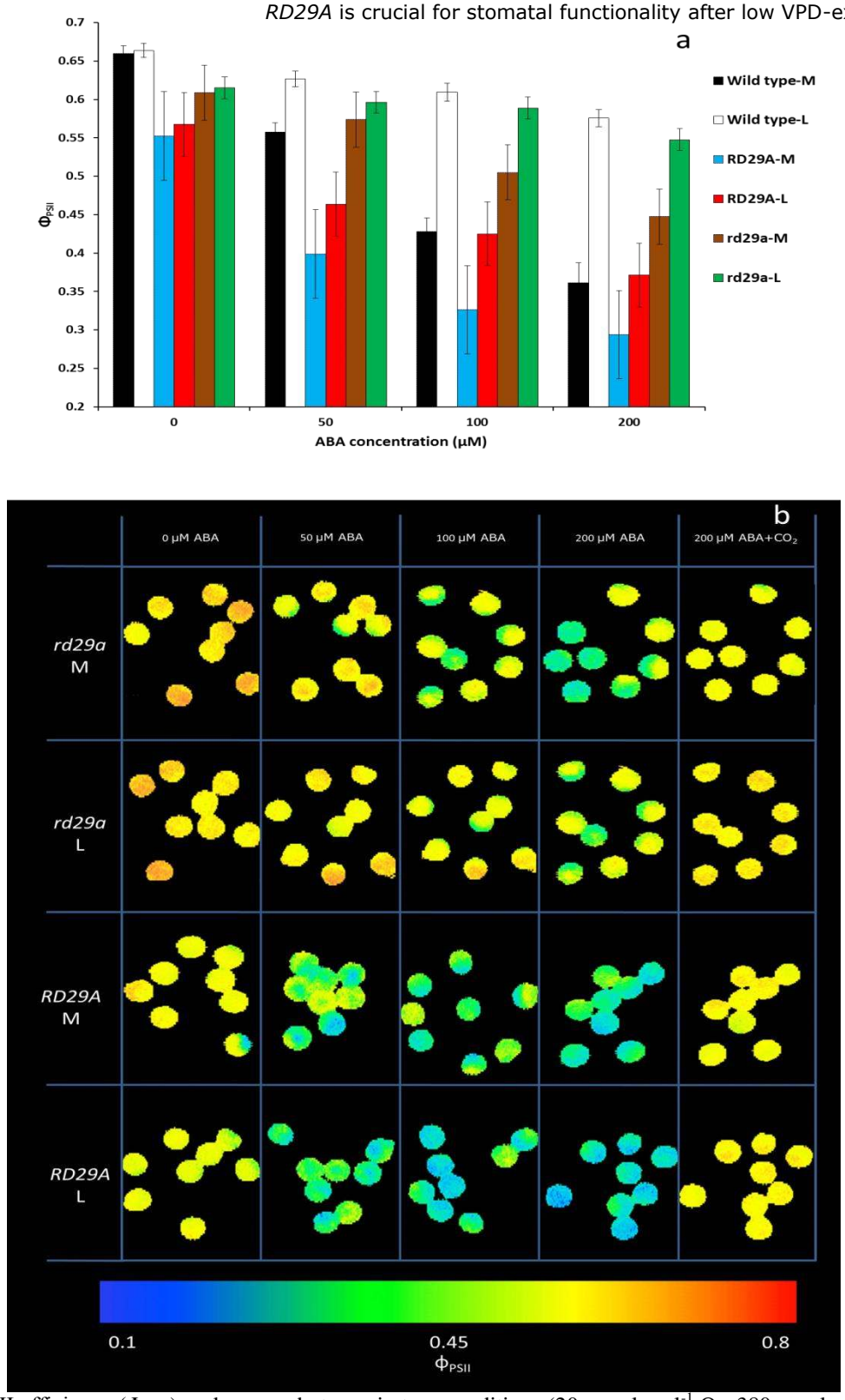

Figure 7. PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions (20 mmol mol^{-1} O_2, 380 µmol mol^{-1} CO_2 and reminder N_2) and representative images of Φ_{PSII} for *rd29a* mutant and *RD29A* overexpression line exposed to moderate (M) or to low (L) VPD. At the end of the imaging of leaf discs with 200 µM ABA, an image was made after 5 min exposure to an environment with high CO_2 concentration (20 mmol mol^{-1} O_2, 50000 µmol mol^{-1} CO_2) (200 µM ABA+ CO_2) (b). For measuring Φ_{PSII}, leaf discs (0.5 cm diameter) were put with the adaxial surface down in petri dishes filled with stomata-opening medium (50 mM KCl, 10 mM MES-KOH, pH 6.15, 50 µM $CaCl_2$) with different concentrations of ABA (0, 50, 100, 200 µM ABA), and Φ_{PSII} was recorded 3 hr after application of treatments. Data are the mean value of Φ_{PSII}±SE.

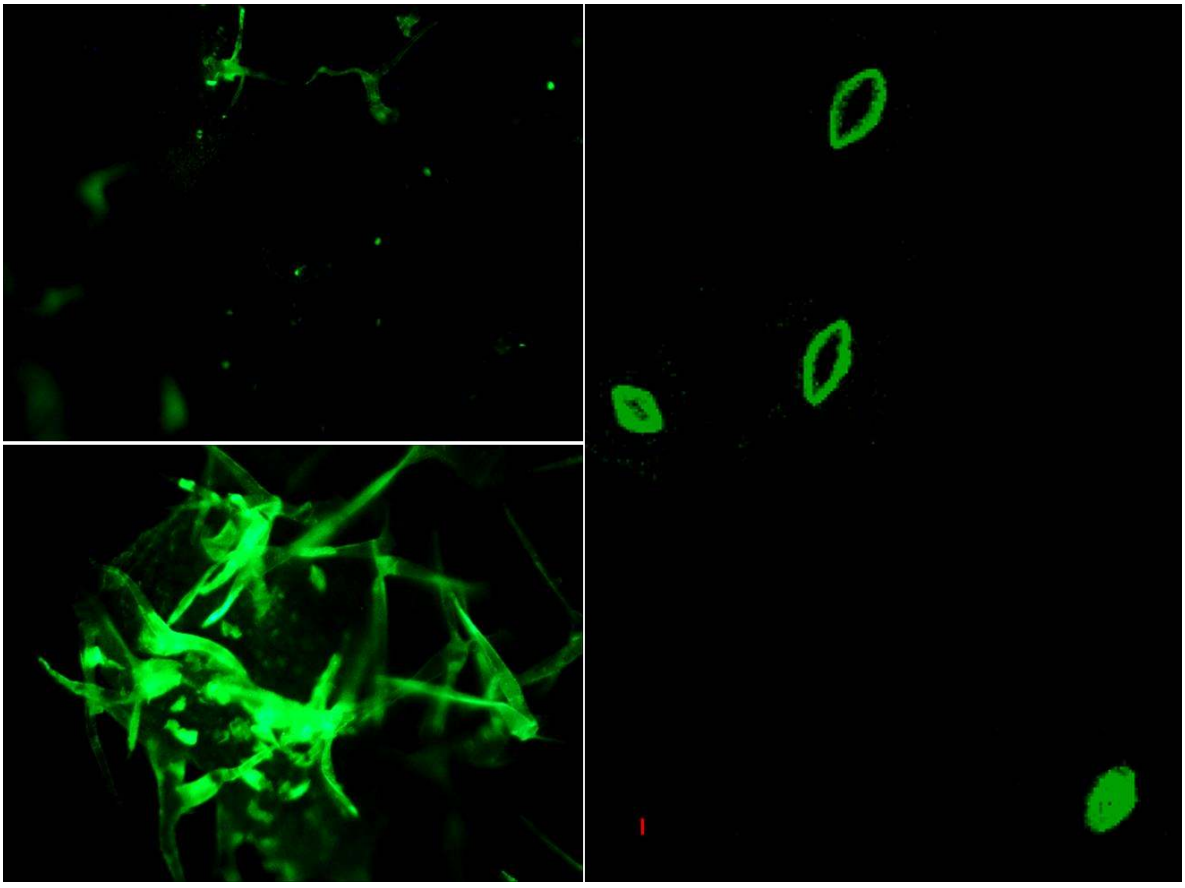

Figure 8. Site of *RD29A* expression. An over expression vector with GFP-*RD29A* fusion protein fused to the C-terminus of *RD29A* (GFP-*RD29A*) driven by UBQ10 promoter was used for constructing transgenic plants. The red bar is indicative of 1 µm.

To determine the effect of *RD29A* overexpression on genes involved in ABA-catabolism, we analysed the effect of VPD-exposure on *CYP707A*s gene expression in the overexpressing line. The transcript level of *CYP707A1* was not affected by VPD, but the transcript level of *CYP707A3* increased as a result of exposure to low VPD of plants of the *RD29A* overexpressing line (Fig. 9a). The transcript level of *RD29A* was not decreased in the *cyp707a1 cyp707a3* double mutants exposed to low VPD (Fig. 9b). Since CYP707A proteins are involved in ABA catabolism, these results indicate that *RD29A* transcription is down regulated because of deficiency in ABA under low VPD conditions. From studies in the *rd29a* mutant it can be concluded that a decreased transcript level of *RD29A* correlates with a reduced sensitivity of stomata to ABA after exposure to low VPD.

Taken together, these results suggest that the ABA concentration is critical for the *RD29A* mediated stomata response under low VPD condition.

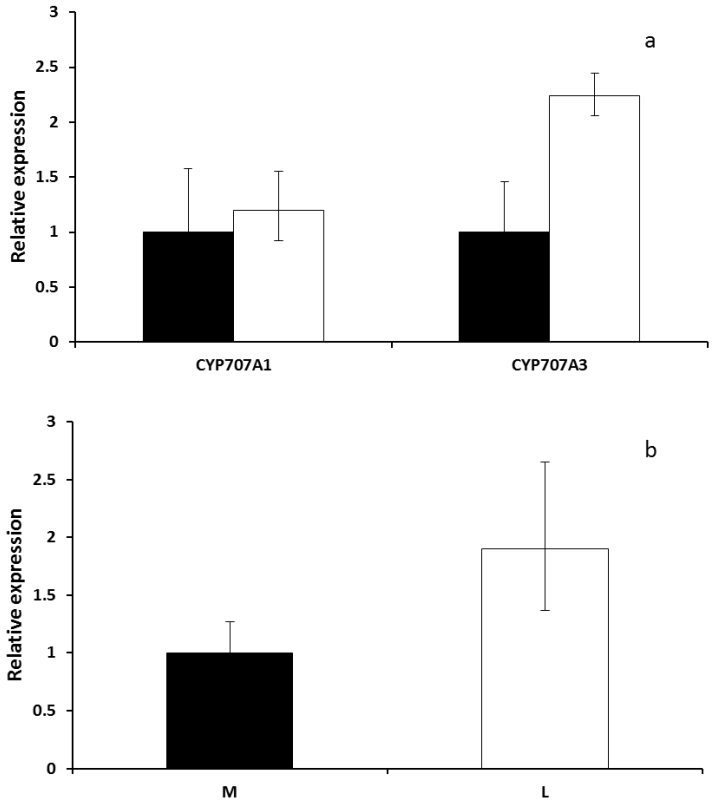

Figure 9. Transcript levels of the genes involved in catabolism of ABA in *RD29A* overexpression line (a) and transcript levels of *RD29A* in the *cyp707a1 cyp707a3* double mutant (b) which have been exposed for 4 days to moderate (black bars) or low (white bars) VPD.

Foliar ABA level determines responsiveness of stomata to ABA after long-term exposure to low VPD

In order to find the role of foliar ABA level in stomatal responsiveness, the level of ABA was determined in Col-0, Cvi-0, Rrs-7, Map-42 and C24 after exposure to moderate and low VPD (Fig. 10). A 4-day exposure to low VPD significantly decreased the ABA level in leaves of Col-0, Cvi-0, Rrs-7 and Map-42, but not in C24. After exposure to low VPD, the highest ABA levels were found in Map-42 and C24, while the lowest ABA levels were found in Col-0 and Cvi-0.

It was tested whether the foliar ABA level underlays variation in stomatal responsiveness to ABA [as expressed by the Φ_{PSII} response to 200 µM ABA relative to control (0 µM ABA); $\varphi_{PSII\ 200\ ABA}/\varphi_{PSII\ C}$)]. No correlation was found between foliar ABA level and the ABA response for moderate VPD-exposed plants (Fig. 11). However, after a 4-day exposure to low VPD, a highly significant correlation (R^2=0.91) was found between foliar ABA level and the $\varphi_{PSII\ 200\ ABA}/\varphi_{PSII\ C}$. Although the ABA level considerably increased in the *RD29A* overexpression line and *cyp707a1 cyp707a3* double mutants (Fig. 12), no significant differences were found between moderate and low-VPD exposed plants. These results suggest

that the foliar ABA level influences the stomatal response to ABA after a prior exposure to low VPD, likely because a threshold level of ABA is required for keeping the stomata functional. After exposure to low VPD, the ABA level can fall below this threshold level, depending on the accession, and induce subsequent ABA-insensitivity.

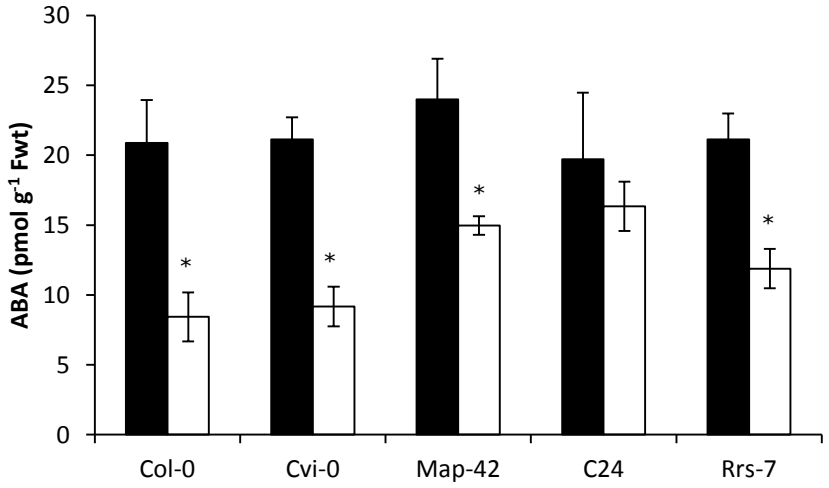

Figure 10. Foliar concentration of ABA in fully developed leaves of Col-0, Cvi-0, Map-42, C24 and Rrs-*7* *Arabidopsis* accessions which have been exposed for 4 days to moderate (black bars) or low (white bars) VPD. Asterisks show the significant differences compared with ABA level of moderate VPD-exposed plants ($P < 0.05$).

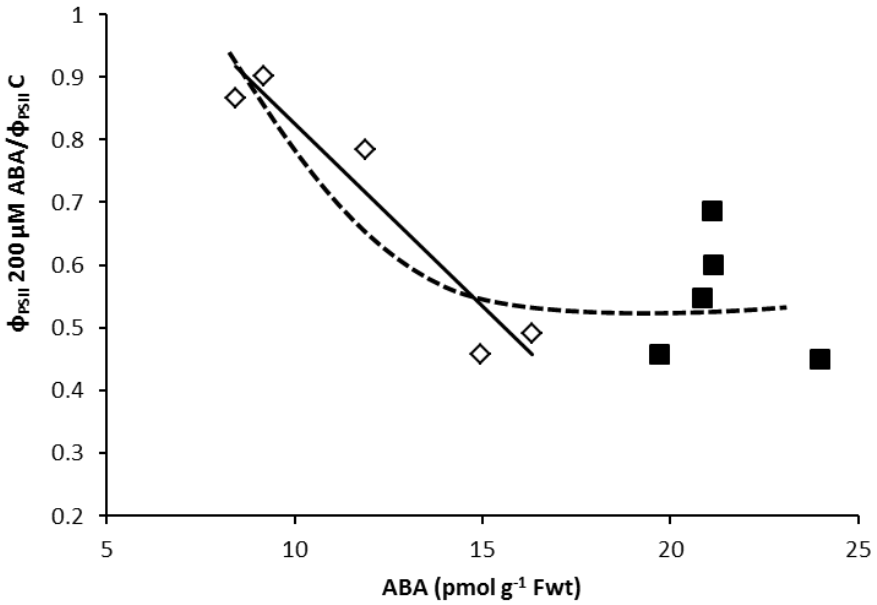

Figure 11. Relationship between PSII efficiency (Φ_{PSII}) under non-photorespiratory conditions in response to 200 µM ABA relative to no ABA ($\Phi_{PSII\ 200\ ABA}$ / $\Phi_{PSII\ C}$) and the foliar ABA level of plants that had been exposed for 4 days to moderate (black symbols) or to low VPD (white symbols). The solid line represents the linear correlation between $\Phi_{PSII\ 200\ ABA}$ / $\Phi_{PSII\ C}$ and ABA of the low VPD-exposed plants, the dashed curve is fitted using all data points (moderate and low VPD-exposed plants).

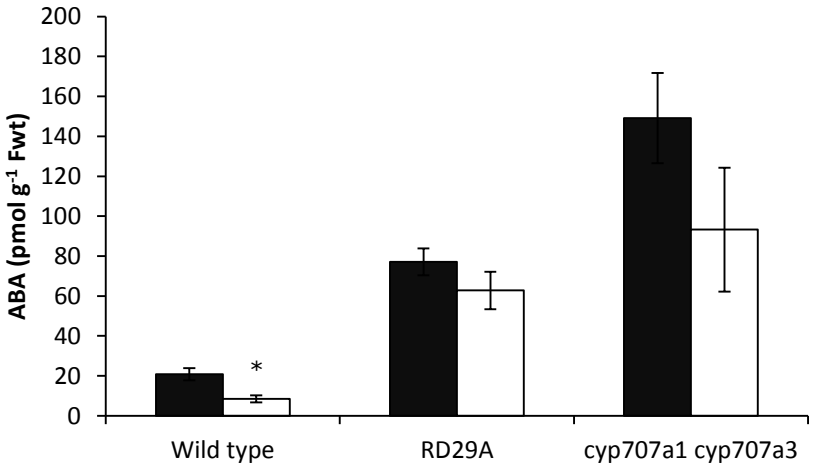

Figure 12. Foliar concentration of ABA in fully developed leaves of wild-type (Colombia), *RD29A* overexpression line and *cyp707a1 cyp707a3* double mutant which have been exposed for 4 days to moderate (black bars) or low (white bars) VPD. Asterisk shows the significant differences compared with ABA level of moderate VPD-exposed plants ($P < 0.05$).

Discussion

Although growing plants at low VPD induces fundamental changes in stomatal morphology, it has been shown that stomatal malfunctioning after long time exposure to low VPD is not dependent on the morphological changes, but occurs because of a change in ABA signalling (Aliniaeifard et al., 2014). Compared to the moderate VPD-exposed plants, after a 4-day exposure to low VPD, stomata of Col-0 (Fig 1a), Cvi-0 (Fig 1b) and Rrs-7 (Fig. 4b) are less responsive to ABA, while the ABA response of stomata of Map-42 and C24 was not affected by prior low VPD-exposure. Aliniaeifard and van Meeteren (2014) found substantial natural variation for low VPD-exposure sensitivity between *Arabidopsis* accessions. They showed that except for Map-42 and C24, stomata of other *Arabidopsis* accessions largely lost their responsiveness to ABA after a 4-day exposure to low VPD. To study the effect of prior exposure to VPD on the expression of genes known to be involved in ABA production, catabolism, perception and signalling, in the current study Col-0 and Cvi-0 were used as accessions with non-responsive stomata to ABA and Map-42 as accession with ABA-responsive stomata after prior exposure to low VPD. For further confirmation of the genes that showed a likelihood to be involved in the effect of VPD on stomatal ABA-responsiveness, Rrs-7 and C24 were used. These were considered as accessions with non-responsive and responsive stomata to ABA after prior exposure to low VPD, respectively. As a result of exposure to low VPD, foliar ABA levels decreased in all accessions except C24;

there were differences in the relative decrease due to low VPD between the accessions (Fig. 10 and 11). In low VPD-exposed plants, the foliar ABA level correlated (R^2=0.91) with the stomatal response to ABA (Fig. 11); in accessions with higher ABA levels, the stomata kept a stronger closing response to ABA. It seems there is a foliar ABA threshold level for stomatal response to ABA; ABA levels higher than this threshold keep stomata responsive to ABA. As a result of exposure to low VPD, in most of the tested accessions, the foliar ABA level decreased to a level lower than the threshold which resulted in a decreased stomatal response to ABA. Application of ABA during exposure to low VPD is capable of maintaining the normal functioning of stomata after exposure to low VPD, likely because it increases the foliar ABA level in low VPD-exposed plants. In low VPD-exposed C24 and Map-42, the ABA levels stayed above the threshold level and these accessions kept their stomata closing response to ABA. Low ABA levels due to exposure to low VPD has been reported in *Tradescantia* (Rezaei Nejad and van Meeteren, 2007), rose (Arve et al., 2012; Giday et al., 2013) and *Vicia faba* (Aliniaeifard et al., 2014). In agreement with the current results, daily application of ABA during leaf development in rose (*Rosa hybrida*) (Fanourakis et al., 2011) and *Tradescantia* (Rezaei Nejad and van Meeteren, 2007) or during a 4-day exposure to low VPD in *Vicia faba* (Aliniaeifard et al., 2014) maintained the normal response of the stomata to closing stimuli (e.g. ABA). Since transcript levels of many genes can be influenced by low VPD, in the current study the transcript levels of the candidate genes were investigated not only in moderate and low VPD-exposed plants, but also in daily ABA-sprayed low VPD-exposed plants of accessions that lost ABA responsiveness under low VPD (Col-0 and Cvi-0). Aliniaeifard and van Meeteren (2013) hypothesized that a low foliar ABA level for long time could be the main reason for malfunctioning of the stomata in response to closing stimuli. However it was still unknown in which part of the signalling pathway the malfunctioning occurs. They suggested that as a result of a long-term low ABA level after long-term exposure to low VPD, the ABA receptors (such as *PYR*, *PYL*, *RCAR* and etc.) cannot inhibit ABA negative regulators (such as *ABI1* and *ABI2*) inside the guard cells, therefore resulting in stomata desensitization to ABA (Rezaei Nejad and van Meeteren, 2007; Aliniaeifard and van Meeteren, 2013). Our results in Col-0, Cvi-0 and Map-42 did not show an important role for ABA receptors and transporters for occurrence of stomatal malfunctioning due to exposure to low VPD.

ABA signalling in the guard cells can be calcium dependent as well as calcium independent (Li and Assmann, 1996; Levchenko et al., 2005; Marten et al., 2007; Sutter et al., 2007; Geiger et al., 2009; Siegel et al., 2009; Geiger et al., 2010; Joshi-Saha et al., 2011). In a

situation of high extracellular calcium concentration ($[Ca^{2+}]_0$) (such as under moderate VPD), calcium sensing receptor (CAS) is required for cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation (Han et al., 2003). Calcium dependent protein kinases are involved in the calcium dependent pathway for regulation of S type anion channels and stomatal closure (Mori et al., 2006). The transcript levels of *CAS*, *CPK3* and *CPK6* (which are in the calcium dependent pathway) decreased after exposure to low VPD compared with their transcript levels in moderate VPD-exposed Col-0 and Cvi-0 accessions (Fig. 3). As a result of low transpiration rate the $[Ca^{2+}]_0$ will be low at low VPD; therefore after exposure to low VPD, the calcium dependent pathway will not take a big part in the ABA signalling pathway (Aliniaeifard and van Meeteren, 2013). Moreover, there was no effect of VPD on the transcript levels of *CAS*, *CPK3*, and *CPK6* in Map-42. This could indicate that changes in calcium signalling are involved in the effect of VPD on stomatal closing response to ABA. However, spraying Col-0 and Cvi-0 plants with ABA during exposure to low VPD did not counteract the decrease in transcript levels of *CAS*, *CPK3* and *CPK6.* Also spraying of calcium to the plants during exposure to low VPD did not result in the recovery of stomatal closing capacity in response to ABA (data not shown). Since ABA enhances calcium sensitivity of stomatal closure mechanisms (Siegel et al., 2009), we can conclude that long-term application of calcium alone would not result in ABA responsive stomata because of the low ABA concentration in the leaves of low VPD-exposed plants.

The results of the current study suggest that the genes involved in ABA catabolism (*CYP707A1* and *CYP707A3* especially in Col-0) (Fig. 6) and response (*RD29A*) (Fig. 7) are involved in the occurrence of stomatal malfunctioning after exposure to low VPD. A decreased ABA level after exposure to low VPD seems to be caused mainly by catabolism of ABA. Although no significant differences were found for *NCED3*, its transcript level was lower in low VPD-exposed Cvi-0 compared with its level in moderate VPD-exposed plants, suggesting that the low ABA level in low VPD-exposed Cvi-0 was due to lower ABA production. In Map-42, the combined effect of ABA production (high after low VPD) and catabolism (low after low VPD) determined the relatively high ABA level in low VPD-exposed plants.

The genes of the CYP707A family encode ABA 8′-hydroxylase (Kushiro et al., 2004). Due to exposure to low VPD, *CYP707A1* and *CYP707A3* reduce the amount of ABA inside guard cells and in vascular tissues, respectively (Okamoto et al., 2009). Similar to our finding which showed increased *CYP707A1* and *CYP707A3* transcript levels after a 4-day exposure to low VPD in Col-0, increased transcript levels of *CYP707A1* and *CYP707A3* after a few minutes exposure to low VPD has been reported (Kushiro et al., 2004; Okamoto et al., 2009).

High levels of ABA were found in the *cyp707a1 cyp707a3* double mutant (Fig. 12). Moreover, after exposure to low VPD stomata of the *cyp707a1 cyp707a3* double mutant had a similar response to ABA as the stomata of moderate VPD-exposed plants. Since the response of stomata correlated with the ABA level (Fig. 11), keeping a high ABA level maintained normal function of stomata in the *cyp707a1 cyp707a3* double mutant plants after exposure to low VPD.

RD29 genes including *RD29A* and *RD29B*, are genes which are induced by stress conditions (Hua et al., 2006; Msanne et al., 2011; Jia et al., 2012). The coding regions of both *RD29* genes are 55.42% identical and 32.87% similar (Yamaguchi-Shinozaki et al., 1995; Jia et al., 2012). These genes (especially *RD29A*) can be involved in both ABA-dependent and ABA-independent signal transduction pathways under abiotic stress conditions (Jia et al., 2012). In the current study, after exposure to low VPD, the transcript level of *RD29A* was considerably decreased in Col-0 and Cvi-0 accessions (with non-responsive stomata to ABA after low VPD-exposure); the level was maintained by ABA spraying during the low VPD-exposure (Fig. 2). But in Map-42 (with responsive stomata to ABA after low VPD-exposure), no differences were found between the transcript level of *RD29A* in moderate and low VPD-exposed plants (Fig. 2). However, the transcript level of *RD29B* did not differ between moderate and low VPD-exposed Col-0 (data not shown).

It has been shown that *RD29A* is involved in tolerance to abiotic stresses such as drought (Yamaguchi-Shinozaki et al., 1995; Narusaka et al., 2003; Kasuga et al., 2004; Hua et al., 2006; Ma et al., 2010), cold (Kasuga et al., 2004; Behnam et al., 2007; Ma et al., 2010) and high salt stresses (Narusaka et al., 2003; Hua et al., 2006; Qiu et al., 2012). Although one of the mechanisms for tolerance to abiotic stresses is regulation of stomatal functioning, information regarding the role of *RD29A* in this regulation is lacking.

In response to several closing stimuli, stomata close due to an increases in $[Ca^{2+}]_{cyt}$ (Neill et al., 2008; Kim et al., 2010; Wang et al., 2011). It has been shown in seedlings, that the circadian rhythm in low temperature-induced increases in whole plant $[Ca^{2+}]_{cyt}$ correlated with the circadian pattern of *RD29A* induction (Dodd et al., 2006). However, increases in $[Ca^{2+}]_{cyt}$ in guard cells was not correlated with diurnal variation in low temperature-induced stomatal closure (Dodd et al., 2006). In another study, using a non-protein amino acid β-aminobutyric acid (BABA), which is a resistance inducer against plant infection by a wide number of pathogens, plant tolerance to drought stress could be induced. The increase in drought tolerance by BABA was because of ABA accumulation in the leaf resulting in

accelerated ABA-dependent gene expression (e.g. *RD29A*) and stomatal closure (Jakab et al., 2005).

Therefore, we can conclude that after exposure to low VPD, due to enhanced catabolism (or maybe decreased biosynthesis of ABA as suggested for Cvi-0), the foliar ABA content will be low which results in down regulation of *RD29A*, and as a result stomata are not responsive anymore to ABA. On the other hand by application of ABA during exposure to low VPD, foliar ABA content increases which results in induction of *RD29A* expression. *RD29A* through its inhibitory effects on *CYP707A* genes (especially *CYP707A1*) will further increase the ABA level and consequently the stomata will be responsive to ABA afterwards (Fig. 13). In agreement with this conclusion, BABA treatment on ABA deficient (*aba1*) or ABA insensitive (*abi4*) mutants could not induce stomatal closure or protect the plants against drought stress. Further studies should show how *RD29A* affects closure of stomata.

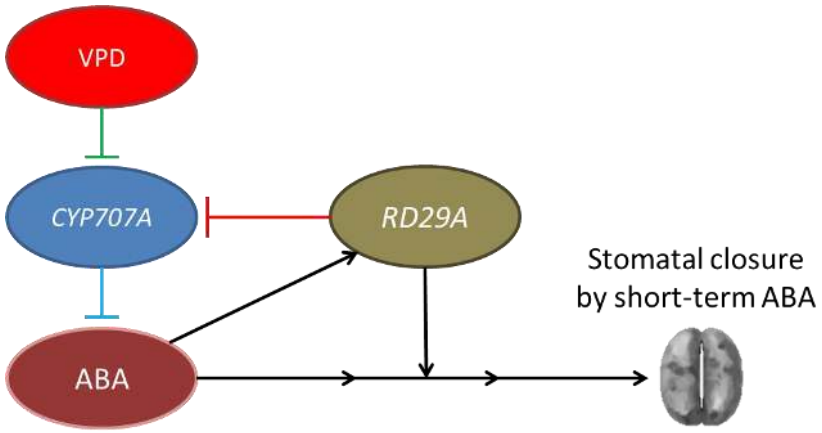

Figure 13. Schematic diagram for signalling pathway leading to different response of the stomata in different VPDs. In low VPD condition due to high catabolism of ABA (high expression of *CYP707A* genes), the ABA level will be low (blue blockage effect), as a result the *RD29A* is not induced by ABA, leading to a low response of the stomata to ABA afterwards. In moderate VPD condition, due to low activity of ABA 8′-hydroxylase (green blockage effect), the ABA level would be above a threshold level, leading to induction of *RD29A*; as a result stomata would be responsive to ABA afterwards (black arrows). When ABA was sprayed to the plant during low VPD-exposure, the exogenous ABA leads to induction of *RD29A*. The inhibitory effect of *RD29A* on *CYP707A* genes (red blockage effect) would result in higher ABA level, as a result stomata would be responsive to ABA afterwards.

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Supplementary Table S1. Primers in this Study.

primers Used for qPCR analysis for expression of the genes involved in secondary messengers and ethylene signalling

CPK6:

Forward primer: 5'-CGAGGAGAATTCCAAATCCA-3'

Reverse primer: 5'-CCCGAATTGTCCTTGTCCTA-3'

CPK3:

Forward primer: 5'-AAACTTCAAGACGGCGCTTA-3'

Reverse primer: 5'-CTGCCGTTGCTATCTCTTCC-3'

CPK4:

Forward primer: 5'-AACTTGGTGGTTGCGTTTTC-3'

Reverse primer: 5'-TCCCAACACCATCTCCTTTC-3'

RBOHD:

Forward primer: 5'-CCTATGAGCCGATGGAAAAA-3'

Reverse primer: 5'-TACCAAAAGGCGTTGAAACC-3'

RBOHF:

Forward primer: 5'-GGATTCGATCTCGGATTTCA-3'

Reverse primer: 5'-AGCAGAACGAGCATCACCTT-3'

ETR1:

Forward primer: 5'-TCCGCTTCTCCACCTTTCTA-3'

Reverse primer: 5'-TGATCAGCGACGACTTCAAC-3'

DHAR:

Forward primer:5'-TAATGACGGATCCGAGAAGG-3'

Reverse primer: 5'-AAGCTCTCAGGGACAGACCA-3'

GPX3:

Forward primer: 5'-ATCGACGGTGGAACAATCAT-3'

Reverse primer: 5'-CTGCCTGGCTCTTGACTACC-3'

NOS1:

Forward primer: 5'-ACAAACTTCCGACGTCGATT-3'

Reverse primer: 5'-CCTCCATTACCACCAACTGC-3'

NOA1:

Forward primer: 5'-AATGGCGCTACGAACACTCT-3'

Reverse primer: 5'-AGCTTCATGAGCTCGTTGGT-3'

NIA1:

Forward primer: 5'-GGGATCTATAGCCGGAGAGG-3'

Reverse primer: 5'-CCATTTAACCATCCGACCAC-3'

PLDa1:

Forward primer: 5'-TGCAATCAGACGTGCTAAGG-3'

Reverse primer: 5'-TGCACTGATCCACTCTCTGG-3'

CAS:

Forward primer: 5'-TGCTTCATCGACCATGGATA-3'

Reverse primer: 5'-CGGCGTAAGATCACCTTTGT-3'

EIN2:

Forward primer: 5'-CTTGGCTTCATCGTGCTACA-3'

Reverse primer: 5'-ACCCCAGAAATCCCAAAAAC-3'

primers Used for qPCR analysis for expression of the genes involved in ABA production, catabolism, perception and signalling

ABI1:

Forward primer: 5'-TGTCAAAGCTGGCGATACAG-3'

Reverse primer: 5'-ACCCTCTCTGCCTCAGTTCA-3'

ABI2:

Forward primer: 5'-AGGATGCATCTGGCTTTGAC-3'

Reverse primer: 5'-GAGCATGAGCCACAGTTTCA-3'

OST1:

Forward primer: 5'-GGAAAGAGGGGGAGAAAATG-3'

Reverse primer: 5'-GGAGCCAATATCCTTGACGA-3'

ABCG25:

Forward primer: 5'-CCAATCACCCTCAAGTTCGT-3'

Reverse primer: 5'-CTCATCGGACGGTTTTTGTT-3'

ABCG40:

Forward primer: 5'-TGCCCCAGGAAATGATAGAG-3'

Reverse primer: 5'-GTTTTGCCAGCTCCAGAGAC-3'

GTG1:

Forward primer: 5'-GATGCTGCACTCCTCTCACA-3'

Reverse primer: 5'-GACCCACTTCCCACTCTTGA-3'

GTG2:

Forward primer: 5'-AACTTGGAAGGGTCATGTGC-3'

Reverse primer: 5'-TCGTGACAGGATCTTTCGTG-3'

PYR1:

Forward primer: 5'-GACGTGATCGTCATCAGTGG-3'

Reverse primer: 5'-CGCCTCCGATGATACTGAAT-3'

PYL4:

Forward primer: 5'-CTTCTTCCGCCGTATCAGAC-3'

Reverse primer: 5'-ACCAACCTCGTGTGTGTGAA-3'

RCAR:

Forward primer: 5'-AATCGGTGATCCTGAAATCG-3'

Reverse primer: 5'-TGTGATCACCACCGATGATT-3'

CYP707A1:

Forward primer: 5'-GGGATGTCCATGTGTGATGA-3'

Reverse primer: 5'-TGTTTCCCCAACATCCTCTC-3'

CYP707A3:

Forward primer: 5'-ACGAACAAATCGCCGATAAC-3'

Reverse primer: 5'-TTGCCATTTGCTCTTCAGTG-3'

NCED3:

Forward primer: 5'-TCTGTTTCGTTCACGACGAG-3'

Reverse primer: 5'-TCCGATGAATGTACCGTGAA-3'

RD29A:

Forward primer: 5'-GACAAGGACGCGAAGAAGAC-3'

Reverse primer: 5'-TCCATCCCAGCTTTTGATTC-3'

SLAC1:

Forward primer: 5'-CGGGCTCTAGCACTCACTCT-3'

Reverse primer: 5'-AAGATCGTTTGGGAACAACG-3'

GCA2:

Forward primer: 5'-GAAGAAGGGATTGGGCTTTC-3'

Reverse primer: 5'-CCTTGAGGCTAGTCGGAGTG-3'

RCN1:

Forward primer: 5'-CCGACGCCTGGATCGTGATTTGATTCGA-3'

Reverse primer: 5'-CAATTCAGGATTGTGCTGCTGTGGAACCA-3'

RD29B:

Forward primer: 5'-ACATACCAGCAATCGCAACA-3'

Reverse primer: 5'-CCACAAGACCACCACCTCTT-3'

CHAPTER 6

General discussion

Proper functioning of stomata is vital for plants in order to survive under unfavourable conditions. However, growing or even long-term (a few days) exposure of plants to some environmental conditions such as low VPD, ozone, continuous light and some air pollutants attenuates closing ability of stomata in response to stimuli that normally provoke stomatal closure (stomatal malfunctioning). Reduced closing ability of stomata has negative consequences for plants especially when they encounter a period of water shortage which results in wilting of plants due to excessive water loss. Low VPD is recognized as the most detrimental environmental condition leading to stomatal malfunctioning. Moreover, low VPD exacerbates the detrimental effects of other environmental conditions such as ozone and continuous light on the decreased stomatal closing ability (**Chapter 3**).

Dynamic stomatal responses to changes in the environment have been reported by many researchers. To enable these dynamic responses, guard cells are equipped with a complex network of signalling pathways to respond promptly to environmental and endogenous signals. However, it is astonishing that long-term exposure to low VPD can disturb the fine tuning of stomatal control. Therefore, this study aimed to find: (i) whether changes in the signalling pathways and/or changes in morphological traits are responsible for disturbed stomatal response to closing stimuli (e.g. desiccation and ABA) after long-term low VPD-exposure, (ii) natural variation in stomatal response to closing stimuli in a collection of *Arabidopsis thaliana* accessions after long-term low VPD-exposure, in order to have tools to find molecular reasons of stomatal malfunctioning, (iii) and, if alterations in the signalling pathway are the main reason for occurrence of stomatal malfunctioning, what specific changes in the signalling pathways have been occurred which lead to decreased closing ability of the stomata.

Stomatal malfunctioning depends on duration of low VPD-exposure

As mentioned before, stomata often dynamically respond to changes in VPD. They respond in such a way that they decrease their pore area during exposure to high VPD, while they increase their aperture area due to exposure to low VPD (Outlaw and De Vlieghere-He, 2001; Okamoto *et al.*, 2009). However, depending on the species, the prompt responses of stomata can only occur when exposure-time to low VPD is not too long (Rezaei Nejad and van

Meeteren, 2008; Fanourakis *et al.*, 2011). For example, in *Arabidopsis* a 4-day exposure to low VPD decreases the stomatal closing ability in response to ABA (**Chapter 4 and 5**), while exposure to low VPD for one hour will not result in attenuated stomatal response to ABA (Okamoto *et al.*, 2009). In rose plants, it has been reported that development of a leaf at low VPD is critical for occurrence of stomatal malfunctioning (Fanourakis *et al.*, 2011). Our results showed that in fava bean plants stomatal malfunctioning can be observed in similar magnitude in the plants that have been exposed for only 4 days to low VPD as in the plants that were grown at low VPD. Similar results have been found in *Tradescantia* (Rezaei Nejad and van Meeteren, 2008). Therefore, not only growing the plants but also a few days exposure to low VPD is enough to disturb normal functioning of the stomata (**Chapter 2**). Stomatal malfunctioning due to exposure to air pollutants, such as ozone and hydrogen sulphide, also depends on the duration of exposure: short-term exposure to air pollutants will not change the stomatal closing response, while long-term exposure will reduce closing response of the stomata (**Chapter 3**). We can expect two reasons to explain why stomatal malfunctioning only occurs when the exposure-time to low VPD is extended: one reason can be changes in stomatal morphology or leaf anatomy due to low VPD exposure, another reason can be alteration at the molecular level and signalling pathways.

Morphological changes induced by low VPD are not the main reason for occurrence of stomatal malfunctioning

Growing plants at low VPD conditions induces fundamental changes in stomatal morphology and leaf anatomy. It has been reported that growing plants at low VPD leads to an increase in stomatal density of the leaves (Bakker, 1991; Torre *et al.*, 2003; Fanourakis *et al.*, 2013). It can be assumed that higher stomatal densities enlarge the stomatal pore area per leaf area leading to increase in the transpiration rate in low VPD-grown plants. However, in fava bean, stomatal density decreased as a result of growing them at low VPD. On the other hand, stomata of low VPD-grown plants are larger for all guard cells dimensions compared with its size in moderate VPD-grown plants (**Chapter 2**). Therefore, high stomatal conductance in low VPD-grown plants can be attributed to their larger stomata with wider pore area in comparison with the stomata in moderate VPD-grown plants. Moreover, stomata of low VPD-grown plants were less responsive to desiccation and exogenous ABA compared with the stomata of moderate VPD-grown plants (**Chapter 2**) (Rezaei Nejad *et al.*, 2006; Rezaei Nejad and van Meeteren, 2007; Fanourakis *et al.*, 2011; Fanourakis *et al.*, 2013). It has been previously reported that the function of stomata can be determined by the size of the stomata,

in a way that smaller stomata perform a faster response-time than larger stomata (Hetherington and Woodward, 2003; Franks and Farquhar, 2007; Doheny-Adams *et al.*, 2012; Drake *et al.*, 2013; Giday *et al.*, 2013b). The faster response-time in smaller stomata can be related to higher surface area to volume ratio, improving water balance and water use efficiency in plants (Drake *et al.*, 2013). In the previous studies, investigating the impact of stomatal morphological traits on stomatal functionality, the stomatal responsiveness of different species in general was investigated or plants from one or several species or from different genotypes of one species were exposed to contrasting environments which usually induced changes in both stomatal morphology and responsiveness (Hetherington and Woodward, 2003; Torre *et al.*, 2003; Franks and Farquhar, 2007; Drake *et al.*, 2013; Fanourakis *et al.*, 2013; Giday *et al.*, 2013b). Therefore, in order to find the importance of the stomatal morphological traits on the stomatal functionality, in this study the closing ability of the stomata was tested not only in moderate and low-VPD grown plants, but also in plants that had developed their leaves in moderate VPD and thereafter transferred for one to four days to low VPD. Our findings confirmed that stomata morphological traits are not always determinant of stomatal functionality (**Chapter 3**), because the morphology of stomata in plants that had developed their leaves at moderate VPD and were then transferred for 4 days to low VPD was more similar to moderate VPD-grown plants, while their response to ABA and desiccation was similar to the stomatal response of low VPD-grown plants (**Chapter 2**). Daily application of a low concentration of ABA (5 μM) to the leaves during exposure to low VPD, maintained the stomatal closing ability in 4-day low VPD-exposed plants.

Plants produced *in vitro* are usually characterized by large guard cells, decreased epicuticular wax and decreased stomatal responses to water deprivation. Although the cuticle is involved in reduced ability of *in vitro* plants to control water loss during drought stress, the cuticular water loss covers only a small proportion of the high water loss characteristics of *in vitro* plants (Santamaria and Kerstiens, 1994) (**Chapter 3**). Similar findings have been reported in rose plant developed under low VPD conditions (Fanourakis *et al.*, 2013).

In conclusion, we suggest that changes in the signalling related to ABA is the determining factor in the decreased closing ability of stomata after long-term exposure to low VPD (**Chapter 2, 3 and 5**).

Reasons for low foliar ABA content due to long-term exposure to low VPD

Plants that have been exposed to low VPD usually contain lower foliar ABA level ([ABA]) compared with the foliar [ABA] in moderate VPD-exposed plants (**Chapter 2, 4 and 5**)

(Rezaei Nejad and van Meeteren, 2007; Rezaei Nejad and van Meeteren, 2008; Okamoto *et al.*, 2009; Arve *et al.*, 2012; Giday *et al.*, 2013a). The question is why the foliar [ABA] is low after exposure to low VPD?

Several processes are involved in determination of the ABA level in plant cells: (i) ABA biosynthesis, (ii) ABA oxidation, (iii) ABA conjugation, (iv) ABA re-distribution in the leaf and (v) long-distance transport of ABA. From these processes ABA oxidation and ABA conjugation inactivate ABA. Next question is: what is the importance of these processes in determination of foliar ABA level after exposure to low VPD?

Opening and closing of stomata is closely related to water availability in the root zone. The stress signal which originates from roots affects the leaf so that even without or before considerable change in leaf water potential the signal induces closing of the stomata (Blackman and Davies, 1985; Gollan *et al.*, 1986; Jiang and Hartung, 2008; Christmann *et al.*, 2013). ABA is considered to be the major biochemical signal which acts as the long-distance signal from root to shoot (Davies *et al.*, 2005; Jiang and Hartung, 2008; Christmann *et al.*, 2013). Therefore, water shortage in the root leads to increase in foliar [ABA] through the xylem stream (Davies and Zhang, 1991; Hartung *et al.*, 2002; Wilkinson and Davies, 2002; Jiang and Hartung, 2008). It can be hypothesized that the ABA delivery from root to leaf decreases due to low transpiration rate during exposure to low VPD (**Chapter 3**). Supporting this hypothesis, it has been suggested that root to shoot ABA delivery via xylem sap is the main reason for low foliar [ABA] in the rose cultivars which showing high water loss after growing at low VPD (Giday *et al.*, 2013a).

ABA-Glucose Ester (ABA-GE) is the most widespread conjugate form of ABA that functions as storage and transportable form of ABA between root and shoot (Dietz *et al.*, 2000; Sauter *et al.*, 2002; Davies *et al.*, 2005). Since, the permeability of biomembranes for ABA-GE is very low, ABC transporters are involved in the release of ABA-GE into the xylem vessels and also in the delivery from vascular tissue to the guard cells apoplast (Dietz *et al.*, 2000; Sauter *et al.*, 2002; Ye *et al.*, 2012). In rose plants, the ABA-GE level increased as a result of growing plants at low VPD (Arve *et al.*, 2012). However, no significant differences were found in ABA-GE levels between moderate VPD-grown *Vicia faba* plants and plants that had been exposed for one to four days or continuously to low VPD (**Chapter 2**). Moreover, the expression patterns for ABC transporter genes (such as *ABCG25* and *ABCG40*) in different *Arabidopsis* accessions did not support their role in lowering foliar [ABA] due to low VPD-exposure (**Chapter 5**). Therefore, we can exclude the role of ABA long-distance transport from the root and also ABA re-distribution in low VPD-reduced foliar [ABA] in *Arabidopsis*

and bean plants. The transcript levels of genes which are involved in ABA oxidation, such as *CYP707A1* and *CYP707A3*, considerably increased after low VPD-exposure in the accessions which had reduced stomatal-closing response to ABA (non-responsive accession after low VPD-exposure) (except Cvi-0). Furthermore, the expression of *CYP707A1* and *CYP707A3* remained unchanged in the accessions with responsive stomata to ABA after low VPD-exposure (Map-42 and C24). In accordance with this finding, after exposure to low VPD, the foliar [ABA] in the ABA-responsive accessions stayed higher than foliar [ABA] in the low VPD-induced non-responsive accessions. Therefore, among the process determining the [ABA], our findings highly supported the involvement of the ABA oxidation process in the decreased ABA level due to exposure to low VPD. Confirming this finding, the ABA level was considerably increased in a *cyp707a1 cyp707a3* double mutant (**Chapter 5**). However, in Cvi-0 it seems that ABA oxidation is not the main process in reducing foliar [ABA] due to low VPD-exposure. It is likely that in this accession a decreased ABA biosynthesis process, decrease in the ABA transport and re-distribution (excluding ABA catabolism) determined the ABA level in low VPD-exposed plants.

In rose plants, it seems that ABA inactivation is also taking part in lowering the foliar [ABA] at low VPD. However, there is no information regarding ABA oxidation processes in low VPD-exposed rose plants.

Differences between stomatal response to ABA and desiccation after long-term exposure to low VPD

After exposure to low VPD, stomata responded differently to desiccation and exogenous ABA application. In fava bean plants, decline in stomatal-closing ability in response to desiccation usually occurred after longer low VPD exposure-times in comparison with the duration of low VPD-exposure that was required to see a decline in the stomatal-closing ability in response to ABA (**Chapter 2**). Moreover, in *Arabidopsis*, only two accessions were responsive to both desiccation and ABA after a 4-day exposure to low VPD. 39 out of 41 of the studied *Arabidopsis* accessions maintained responsive to desiccation but lost their responsiveness to ABA after a 4-day exposure to low VPD (**Chapter 4**). Some questions arise: 1) why are stomata less responsive to ABA than to desiccation after exposure to low VPD? 2) what is the role of ABA in desiccation-induced stomatal closure? 3) whether desiccation closes the stomata via an ABA-independent pathway?

In our methodology in *Arabidopsis* plants, we vacuum infiltrated the ABA solution into leaf discs floated on ABA solutions, while in fava bean plants we fed the petiole with an ABA

solution. Two hypotheses can be presented for the decreased stomatal response to ABA after exposure to low VPD:

(i) In accordance with the stomatal-closing response to ABA feeding in moderate VPD-exposed plants in our study, it has been shown that ABA feeding into the xylem of well-watered bean plants induces apoplastic ABA accumulation in the leaf which results in stomatal closure (Zhang and Outlaw, 2001a, b). Tallman (2004) hypothesized that during the daytime, although apoplastic [ABA] can be high, the guard cell [ABA] decreases through ABA oxidation by activated CytP450 due to increased O_2/CO_2 ratio in the guard cells. CytP450 catalyses the first step in endogenous guard cell ABA catabolism to 8'-hydroxy-ABA. Therefore, we hypothesize that, although the [ABA] can be increased in the apoplast by ABA feeding, it is not capable of inducing guard cells ABA accumulation in low VPD-exposed plants.

(ii) In our methodology for ABA feeding on the petiole of fava bean leaves, from the amount of solution that was taken up, we were able to calculate the amount of ABA that entered into the leaf. The result confirmed that after exposure to low VPD due to higher stomatal conductance, the amount of ABA taken up by the leaves was considerably higher than the amount of ABA taken up by leaves of moderate VPD-exposed plants (data not shown, **Chapter 2**). Therefore, short-term foliar [ABA] deficiency cannot be the reason for stomatal malfunctioning after exposure to low VPD. It can be hypothesized that as a result of long-term low ABA concentration due to long-term low VPD-exposure, the ABA signalling pathway was disturbed which results in attenuated stomatal response to short-term exogenously ABA application (**Chapter 3**).

In low VPD-exposed plants desiccation appeared to be a stronger signal for closure of the stomata than ABA (**Chapter 2 and 4**). Induction of ABA production after experiencing water deficit by plants and consequently stomatal closure has been extensively documented (Larque-Saavedra and Wain, 1974; Luan, 2002;Davies *et al.*, 2005; Hu *et al.*, 2006; Hirayama and Shinozaki, 2007; Endo *et al.*, 2008; Lee and Luan, 2012; Sreenivasulu *et al.*, 2012; Dodd, 2013; Giday *et al.*, 2013a; Osakabe *et al.*, 2013). Accordingly, in our study the foliar [ABA] was considerably increased as a result of desiccation, and stomatal response to desiccation was positively correlated to the foliar [ABA] after desiccation (**Chapter 4**). Upon rapid water deficit conditions, such as leaf desiccation, ABA accumulates in the guard cells through de-novo ABA biosynthesis and re-distribution of the existing pool of foliar ABA inside the leaf

(Harris and Outlaw, 1991; Popova *et al.*, 2000). Therefore, it is possible to assume that desiccating the leaves causes not only elevation in foliar [ABA], but also accumulation of ABA inside the guard cells, which leads to stronger closure response of stomata to desiccation. In contrast to desiccation, a positive correlation was found between foliar [ABA] and stomatal-closure response to ABA in general (**Chapter 4**) and especially after exposure to low VPD (**Chapter 5**). It seems that there is a foliar threshold level for ABA in order to keep the stomata responsive to ABA. Since the foliar [ABA] in moderate VPD-exposed plant is always higher than this threshold, their stomata are always responsive. In those *Arabidopsis* accessions which had higher ABA levels than this threshold after VPD-exposure, they maintained responsive to ABA after prior VPD exposure (**Chapter 5**). In accordance with this conclusion, a daily spray of ABA during growth (development) of spiderwort (Rezaei Nejad and van Meeteren, 2007) and rose (Fanourakis *et al.*, 2011) leaves, maintained normal functioning of stomata in response to desiccation. However, because the duration of ABA spray was long enough to induce stomata morphological changes, it is not clear whether maintaining the stomatal closing-response was because of stomatal morphological alterations or because of signalling alterations. In our study, a daily spray of ABA was carried out only during a 4-day exposure to low VPD, which resulted in maintaining the stomatal closing-response to ABA in fava bean and *Arabidopsis* accessions which otherwise become non-responsive to ABA (**Chapter 2 and 5**).

Plant responses to water deficit consist of both ABA-dependent and ABA-independent pathways for controlling stomatal aperture (Luan, 2002; Chaves *et al.*, 2003; Liang *et al.*, 2005; Umezawa *et al.*, 2006; Fujita *et al.*, 2009; Huang *et al.*, 2009; Planchet *et al.*, 2011; Seo *et al.*, 2012). At least two pathways for drought-induced stomatal closure have been proposed. One of them is through an ABA signalling pathway; and the other performs its action on stomatal closure via direct osmotic stress. ABA accumulation is considered as a slower response to water stress, whereas, induced hyperosmotic shock is considered as a rapid signal sensed by guard cells for closure of stomata (Raschke, 1975; Luan, 2002). Therefore, it would be rational to expect that by desiccation not only ABA-induced, but also osmotic stress-induced stomatal closure occurred, which caused a stronger closing-response of the stomata to desiccation and was still present in most of the tested low VPD-exposed *Arabidopsis* accessions (**Chapter 4**).

Natural variation in stomatal response to closing stimuli after exposure to low VPD

In our study substantial variation was found in stomatal closure response to ABA and desiccation between different accessions of *Arabidopsis thaliana* when they had been exposed to moderate or low VPD. In response to desiccation, similar distributions for stomatal response were found between moderate and low VPD-exposed *Arabidopsis* accessions. This indicates that in *Arabidopsis*, low VPD does not considerably influence stomatal response of the accessions to desiccation. It has been reported that in rose plants, genotypic variation is present in stomatal responsiveness to desiccation after growth at low VPD, determined by foliar [ABA] of the rose genotypes during growth at low VPD (Giday *et al.*, 2013a). In our study, no correlation was found between stomatal response to desiccation and foliar [ABA] in accessions which were representative of 3 different groups of stomatal responses to closing stimuli after exposure to low VPD. However, in response to desiccation the foliar [ABA] was considerably increased in the studied accessions. This increase in foliar [ABA] was positively correlated with their stomatal response to desiccation (**Chapter 4**). Since both ABA-dependent and ABA-independent pathways control stomatal closure under water deficit conditions, therefore, foliar [ABA] elevation strengthened stomatal closure response due to desiccation.

In contrast to stomatal response to desiccation, the distribution of *Arabidopsis* accessions in response to ABA was changed by exposure to low VPD. By increasing the applied ABA concentration, two discrete patterns of distribution were observed between moderate and low VPD-exposed *Arabidopsis* accessions. This indicated that low VPD considerably influences stomatal response of the *Arabidopsis* accessions to ABA. The foliar [ABA], especially at low VPD, was positively correlated with their stomatal response to ABA (**Chapter 4 and 5**). We suggested a threshold for foliar [ABA] to maintain the stomatal-closing response to ABA (**Chapter 5**); in moderate VPD-exposed plants the foliar [ABA] is always higher than this level across different accessions, which resulted in closure of the stomata in response to ABA. Supporting this hypothesis, there is not a considerable variation between *Arabidopsis* accessions in response to ABA for moderate VPD-exposed plants, especially at higher concentrations of ABA.

Induction of RD29A by abscisic acid is crucial for normal functioning of stomata after exposure to low VPD

Foliar [ABA] decreased as a result of exposure to low VPD (**Chapter 2, 4 and 5**). As discussed before, ABA oxidation is the main reason for low foliar [ABA] at low VPD-

exposed plants. Moreover, low foliar [ABA] (lower than a threshold level) is the primary reason for occurrence of stomatal malfunctioning in low VPD-exposed plants. Increasing the foliar [ABA] by means of daily spraying of ABA or by means of disturbing the genes involved in the oxidation of ABA (using a *cyp707a1 cyp707a3* double mutant) is capable of maintaining the normal functioning of the stomata after exposure to low VPD (**Chapter 5**). The question is: how increased foliar [ABA] during exposure to low VPD maintains normal functioning of stomata afterwards?

Our study showed that when the ABA level stayed above a threshold level in plants (e.g. in moderate VPD-exposed plants, low VPD ABA-treated plants, or a *cyp707a1 cyp707a3* double mutant) the transcript level of *RD29A* increased accordingly, which consequently facilitated the closing-response of stomata to ABA. In *Arabidopsis*, *RD29A* and *RD29B* are closely located on its genome, which are differentially induced by abiotic stresses such as drought, low temperature and high salt concentrations (Yamaguchi-Shinozaki *et al.*, 1995; Yoshida *et al.*, 2002; Narusaka *et al.*, 2003; Kasuga *et al.*, 2004; Hua *et al.*, 2006; Behnam *et al.*, 2007; Ma *et al.*, 2010; Msanne *et al.*, 2011; Qiu *et al.*, 2012). From perception of the signals to gene expression in the signalling pathways under abiotic stress conditions, *cis*-acting elements in the stress-responsive promoters act as final step in the signalling cascades. Two different *cis*-acting elements: DRE (Dehydration-Responsive Element) and ABRE (ABA-Responsive Element), are the main *cis*-acting elements for induction of gene expression by abiotic stresses (Yamaguchi-Shinozaki *et al.*, 1995; Uno *et al.*, 2000; Narusaka *et al.*, 2003; Kasuga *et al.*, 2004; Yamaguchi-Shinozaki and Shinozaki, 2005; Behnam *et al.*, 2007; Ma *et al.*, 2010; Msanne *et al.*, 2011; Jia *et al.*, 2012). DRE acts in early stress signalling, while ABRE acts after the accumulation of ABA during drought and salinity stress (Yamaguchi-shinozaki *et al.*, 1992; Yamaguchi-Shinozaki *et al.*, 1995; Narusaka *et al.*, 2003; Jakab *et al.*, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005; Hua *et al.*, 2006; Ma *et al.*, 2010). From these *cis*-acting elements, ABREs are involved in ABA-dependent gene expression responses and DREs are involved in both ABA-dependent and ABA-independent gene expression responses to drought and osmotic stresses (Yamaguchi-Shinozaki *et al.*, 1995; Jakab *et al.*, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005; Msanne *et al.*, 2011). It has been found that *RD29A* has both cis-acting elements in ABA-dependent (ABREs) and ABA-independent (DREs) pathways, while the regulation of *RD29B* expression is through only the ABA-dependent pathway (ABREs) (Yamaguchi-Shinozaki *et al.*, 1995). In our study, no considerable influence of low VPD was found for the transcript level of *RD29B* (data not shown), while the transcript level of *RD29A* was considerably decreased by low VPD and

maintained to the same level as in the moderate VPD-exposed plants by daily spraying of ABA to low VPD-exposed plants (**Chapter 5**). On the one hand, the regulation of *RD29B* expression is through only the stress–induced ABA and, on the other hand, regulation of *RD29B* expression by ABA is a slow and late response to the increase in the [ABA]. Probably the difference between [ABA] in moderate VPD and low VPD-exposed plants or the duration of high [ABA] in moderate VPD-exposed plants was not sufficient to induce *RD29B* expression. *RD29A* is frequently employed as a marker gene for stress tolerance (Ma *et al.*, 2010; Jia *et al.*, 2012). There are many drought-inducible transcription factors that act downstream of stress and ABA responses. These transcription factors participate mainly in adaptive processes during stress responses (Kasuga *et al.*, 2004; Jakab *et al.*, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005; Behnam *et al.*, 2007; Jung *et al.*, 2008; Ma *et al.*, 2010; Jia *et al.*, 2012). Gene expression cascades can be constituted by *cis*-acting elements during plant responses to abiotic stresses and regulate the molecular events in order to adapt to abiotic stresses. It has been found that *DREB1A* transcription factor specifically interacts with the DRE and promotes expression of stress tolerance genes for adaptation (Kasuga *et al.*, 2004; Behnam *et al.*, 2007; Ma *et al.*, 2010). Since the promoter of *RD29A*, include both types of cis-acting elements, DREs and ABREs, through DREs elements, it has the capability to integrate different signals (Tuteja and Gill, 2013). One of the important mechanisms of tolerance to dehydration stress is stomatal closure. Induction of *RD29A* via ABA accumulation and stomatal closure has been reported (Jakab *et al.*, 2005). We can hypothesize that down regulation of *RD29A*, due to low VPD-exposure, resulted in decreased stomatal closing ability and consequently the decreased stomatal response to ABA. Confirming this hypothesis, *rd29a* mutant exhibited decreased stomatal closing response to ABA, while the stomata of a low VPD-exposed *RD29A* overexpression line closed normally in response to ABA (**Chapter 5**). As mentioned before, desiccation is a stronger signal for stomatal closure compared to ABA alone. As previously proposed, ABA-dependent and ABA-independent pathways are involved in stomatal closure by desiccation (**Chapter 2 and 4**), it would be rational to hypothesize that induction of *RD29A* (which has both ABA-dependent and ABA-independent elements) by desiccation helps stomata to close after exposure to both moderate and low VPDs. In the case of short-term application of ABA after long-term low VPD-exposure probably just ABA dependent elements are involved which are not sufficient for closure of the stomata. However, by daily application of ABA, because the level of ABA was always high, it induces a high transcript level of *RD29A*; therefore the stomata can close in response to short-term ABA application (**Chapter 2 and 5**). In accordance, the foliar [ABA]

of a *cyp707a1 cyp707a3* double mutant was high, even in low VPD-exposed plants, which resulted in up-regulation of *RD29A*. As a result stomata were responsive to ABA afterwards (**Chapter 5**).

Conclusions and suggestions for future research

Guard cells are equipped with complex signalling cascades for proper responses to a changing environment. However, long-term exposure to some environmental factors, especially low VPD, reduces the ability of stomata to close in response to closing stimuli such as desiccation and ABA (stomatal malfunctioning). Especially, plants that have been grown or exposed for a few days to low VPD are not capable of suitable closing response to desiccation and ABA (**Chapter 3**). Stomatal morphological and leaf anatomical alterations are not the main reason for occurrence of stomatal malfunctioning by low VPD (**Chapter 2**). The foliar [ABA] is usually high in moderate VPD-exposed plants, while due to mainly ABA oxidation, its concentration is low in the low VPD-exposed plants (**Chapter 2, 4 and 5**). Foliar [ABA] lower than a threshold level in low VPD-exposed plants causes down regulation of *RD29A*, which attenuates the stomatal closing response to ABA afterwards (**Chapter 5**).

In our study, ABA application to the leaf petiole causes four times higher uptake of ABA solution by low VPD-exposed plants in comparison with the amount of ABA solution sucked by moderate VPD-exposed plant. This type of ABA feeding causes stomatal closure in moderate VPD- but not in low VPD-exposed plants (**Chapter 2**). Although ABA feeding increases the apoplastic ABA concentrations, it is still unclear whether decreased sensitivity of stomata of low VPD-exposed plants is due to low ABA level in the guard cells' cytosol or is due to insensitivity in the signalling pathway of the ABA.

In **Chapter 4** substantial natural variation was identified among *Arabidopsis* accessions after long-term exposure to low VPD for their stomatal responses to closing stimuli. The studied accessions can be categorized in 3 different groups based on their stomatal responses to ABA and desiccation. The outliers with extreme responses from different groups can be used for construction of promising recombinant inbred line populations for identification of the involved quantitative trait loci for breeding of the stomata malfunctioning problem due to low VPD-exposure in plants.

In the current project we found that ABA-induced *RD29A* is crucial for keeping normal functioning of stomata after exposure to low VPD. Since in the *RD29A*-overexpressing line the level of ABA was also increased (**Chapter 5**), in order to find whether *RD29A* is the only component that keeps closing response of the stomata after low VPD-exposure, one

interesting experiment could be preventing ABA accumulation in the *RD29A*-overexpressing line (e.g. overexpression of *RD29A* in ABA-deficient mutants) during low VPD-exposure and then investigating its stomatal response to ABA.

The promoter of *RD29A* includes both types of cis-acting elements: ABREs and DREs for both ABA-dependent and ABA-independent pathways. We hypothesize that the strong impact of desiccation on closure of stomata is because it uses both ABA-dependent and ABA-independent pathways for closure of the stomata. Therefore, another experiment can be investigating the role of *RD29A* (as well as DRE and ABRE elements) after leaf desiccation in both responsive and non-responsive accessions. Moreover, our knowledge regarding how *RD29A* is involved in stomatal closure is highly limited; future research may be directed towards the mechanism of stomatal closure by *RD29A*.

In this project, we focused mainly on the genes which are mostly in the early step in the signalling pathways of ABA, secondary messengers and ethylene (**Chapter 5**). Transcription factors are proteins which are involved in the regulation of cellular processes for long-term response of the plants and also for adaptation processes. Since, it has been shown that many of them are involved in the opening and closing of stomata (**Chapter 3**), it may be of interest to unravel the role of different transcription factors in the occurrence of stomatal malfunctioning after long-term plant exposure to low VPD.

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SUMMARY

Stomata are pores spreading over the leaf surface responsible for two crucial gas diffusion processes (CO_2 uptake and water vapour release) between plants and the surrounding environment. Apart from stomatal pores, the rest of the leaf surfaces are covered with a waxy cuticle. Stomata's position at the interface between plant internal tissue and the surrounding environment, make them the only openings connecting the internal leaf space to the outside environment. The aperture of the stoma is controlled by swelling and shrinking of two guard cells. The processes which are responsible for regulating swelling and shrinking of guard cells are extremely sensitive to changes in environmental conditions. As a short-term reaction, in response to an increase in vapour pressure deficit (VPD) (dry air), guard cells start to shrink and as a result stomatal closure occurs. On the other hand, guard cells swelling and stomatal opening takes place in response to low VPDs. However, when guard cells face to low VPD for a prolonged time, adaptation processes occurs which render stomata incapable of suitable closure response to stimuli which usually provoke stomatal closure (stomatal malfunctioning). The occurrence of stomatal malfunctioning can have negative consequences for the plants as it causes wilting when plants encounter drought stress. Despite of considerable efforts over the past 25 years regarding finding the consequence and reasons of stomatal malfunctioning in plants (especially in horticulture), the molecular mechanism(s) leading to occurrence of stomatal malfunctioning is still unknown. Therefore the general aim of this project was to elucidate the altered signalling pathway in guard cells of malfunctioning stomata after long-term exposure to low VPD.

In **Chapter 2,** in order to recognize whether the problem of stomatal malfunctioning is due to alterations in stomatal morphology and leaf anatomy or in the ABA signalling pathway, fava bean plants were grown at low or moderate VPDs and some plants, that had developed their leaves at moderate VPD, were transferred for four days to low VPD. Growing plants at low VPD induced fundamental changes in stomata morphology and leaf anatomy: stomata were bigger with larger pore area compared with moderate VPD-grown plants. Moreover, higher specific leaf area (SLA) and less spongy cells were found in the leaves of low VPD-grown plants compared with the leaves of moderate VPD-grown plants. Besides the morphological and anatomical changes in stomata and leaf, the response of the stomata to closing stimuli of low VPD-grown plants was changed. Stomata of low VPD-grown plants closed less and slower in response to ABA compared with the stomata of the moderate VPD-grown plants. Moreover, leaves of the plants that were grown under low VPD conditions, transpired much

more water compared to moderate VPD-grown leaves at the same RWC levels; low VPD-grown leaves also desiccate to lower RWC levels. Stomatal morphology (except stomatal aperture), stomatal density and leaf anatomy of the leaves that were expanded at moderate VPD and were then transferred for 4 days to low VPD were almost similar to the leaves that had fully developed at moderate VPD. However, the stomatal response to desiccation and ABA after a 4-day exposure to low VPD was similar to the stomata of the plants that were fully grown at low VPD. Therefore, leaf anatomical and stomatal morphological alterations due to low VPD were not the main reasons for decreased stomatal closure response to desiccation and ABA. The stomatal responsiveness to ABA was lost after a 1-day exposure to low VPD, while the responsiveness to desiccation was gradually lost during 4-days exposure to low VPD. The level of foliar ABA sharply decreased within 1-day exposure to low VPD, while the level of ABA-glucose ester was not affected by low VPD. Spraying ABA during the 4-day exposure to low VPD maintained the closure ability of the stomata. These results indicate that alteration in the signalling pathways, due to low foliar ABA level, is the reason for stomatal malfunctioning after long-term low VPD-exposure.

Since alteration in the signalling pathway(s) was found as the main reason for the occurrence of stomatal malfunctioning, in **Chapter 3** we discussed, by literature review, possible changes in the signalling pathway(s) after prolonged exposure to some environmental conditions. The duration of plant exposure to conditions such as ozone, hydrogen sulphide, sulphur dioxide and especially low VPD was found to be critical for occurrence of stomatal malfunctioning: during or after a short-term exposure, stomata respond normally to closing stimuli, while a long-term exposure to the mentioned environmental conditions results in decreased stomatal closing ability. The magnitude of stomatal malfunctioning induced by some environmental factors, such as continuous light and ozone, is more pronounced when these factors are applied simultaneously with low VPD. In conditions which favour ABA accumulation, such as high VPD, ABA and calcium accumulate in the guard cells apoplast and thereafter in the guard cell symplast. When ABA is available in guard cells symplast, through binding to its receptor (PYR/PYL/RCARs), it causes hydrogen peroxide and nitric oxide accumulation and also blocks protein phosphatases type 2C, which are negative regulators of ABA signalling. As a result, SnRK2/OST1 protein kinase activates slow type anion channels (e.g. SLAC1) as well as inhibits potassium channels (e.g. KAT1), consequently stomatal closure will occur. In contrast, in conditions which do not favour ABA accumulation, such as low VPD, the concentrations of ABA and calcium will be low due to low transpiration rate and increased

*CYP707A*s activity (ABA oxidation). In this situation, ABI1/PP2C will inactivate SnRK2/OST1 protein kinase, therefore, there will not be an inhibitory effect on ion channels; as a result stomata stay open. Concomitant changes in Ca^{2+}, ABA receptors, and positive and negative regulators of ABA signalling are proposed as early steps for stomatal malfunctioning induced by long-term exposure to low VPD. Transcriptional activators (e.g. AtMYB60 and AtNAP) and transcriptional repressors (e.g. NPX1 and AtERF7) as well as E3 ligases can lead to long-term adaptation of cellular processes which consequently cause decreased stomatal response to closing stimuli afterwards. Besides ABA, other phytohormones and interplay between them, regulate stomatal movements as well. Cytokinins and auxins influence stomatal movements via ethylene. It is proposed that stomata close in response to ethylene in the absence of ABA, but open in response to ethylene in the presence of ABA. Hence, possibly interactions between phytohormones also influence the stomatal responses after long-term exposure to low VPD.

In order to find the molecular mechanism(s) of stomatal malfunctioning after long-term exposure to low VPD, it was important to identify possible variation in stomatal response to closing stimuli between *Arabidopsis thaliana* accessions after long-term low VPD-exposure. Therefore, in **Chapter 4** stomatal responses of a collection of *Arabidopsis* accessions to different closing stimuli (ABA and desiccation) were analysed after a long-term exposure to moderate and low VPD. For efficient large scale screening of stomatal responses to ABA, we used chlorophyll fluorescence imaging under a non-photorespiratory condition for leaf discs floating on ABA solutions. For screening of stomatal responses to desiccation, the rate of water loss as function of leaf relative water content (RWC) from excised leaves was used to characterize the water loss parameters of the *Arabidopsis* accessions after a long-term exposure to low VPD. In all accessions stomatal conductance (g_s) was increased after prior exposure to low VPD. However, stomata of 39 out of 41 of the accessions showed a diminished ABA closing response after exposure to low VPD. Only stomata of low VPD-exposed Map-42 and C24 were responsive to ABA after exposure to low VPD. On the other hand, only low VPD-exposed Cvi-0 and Rrs-7 exhibited less stomatal closure response to desiccation compared to moderate VPD-exposed plants. Stomatal response to ABA (but not to desiccation) negatively correlated with their stomatal conductance after prior exposure to low VPD. Accessions could be grouped to very sensitive, moderately sensitive and less sensitive to closing stimuli using Principle Component Analysis (PCA). Bulk foliar ABA levels were measured before and after desiccation in the leaves of three accessions, as representatives of

the three clusters of the PCA. A positive correlation was found between foliar ABA level (before desiccation) and stomatal closure response to ABA (but not to desiccation) after exposure to different VPDs. Stomatal response to desiccation was positively correlated with the bulk foliar ABA level after desiccation. In conclusion substantial natural variation in stomatal response to closing stimuli was found between *Arabidopsis* accessions, especially after a long-term exposure to low VPD.

In **Chapter 5**, in order to elucidate the molecular network underlying stomatal malfunctioning in response to ABA due to long-term low VPD-exposure, two groups of *Arabidopsis* accessions were used as accessions that maintained responsiveness to ABA after low VPD-exposure (Map-42 and C24) and accessions with low VPD induced non-ABA-responsive stomata (Col-0, Cvi-0 and Rrs-7). Transcript levels of genes involved in ABA transport, perception, biosynthesis, catabolism, and signal transduction as well as in secondary messengers pathways and ethylene signal transduction were analysed in the leaf of Col-0, Cvi-0, and Map-42 after a 4-day exposure to low and high VPD. Since spraying ABA during low VPD-exposure of Col-0 and Cvi-0 (ABA-treated plants) sustained the stomatal closing response to short-term ABA afterwards, transcript levels of mentioned genes were analysed in ABA-treated plants as well. Activity of genes involved in calcium signalling was influenced by long-term exposure to low VPD. However, this alteration in calcium signalling was not influenced by long term ABA spraying. Neither genes involved in ABA transport and perception nor genes involved in pathways of secondary messengers and ethylene signalling were involved in the lack of stomatal responsiveness to ABA. In contrast, transcript levels of *CYP707A* genes, which are involved in ABA catabolism, were increased by low VPD in Col-0 and Rrs-7, but not in the accessions which maintained responsiveness to ABA (Map-42 and C24). Catabolism of ABA is the main process regulating foliar ABA level after exposure to long-term low VPD (except for Cvi-0). Transcript levels of *RD29A* were decreased by low VPD in the accessions with low VPD induced non-ABA-responsive stomata (Col-0, Cvi-0 and Rrs-7), while its expression was increased in the ABA-treated plants and in the accessions which maintained responsive to ABA after low VPD-exposure. To test the role of *RD29A* in stomatal functioning of low-VPD exposed plants, we generated transgenic *Arabidopsis* carrying the *RD29A* gene fused to Green Fluorescence Protein (GFP). In transgenic plants the location of the *RD29A*-GFP protein is confined to the trichomes and stomata's guard cells. The foliar ABA level was considerably increased in the *RD29A* over-expressing line and in a *cyp707a1 cyp707a3* double mutant. Stomata of the *RD29A* over-expressing line and the *cyp707a1 cyp707a3* double mutant maintained responsive to ABA after exposure to low

VPD, while decreased closing ability was observed for the stomata of *rd29a* mutant after 4-day exposure to low VPD. The foliar ABA content in all accessions correlated with the stomatal response to ABA: only when the ABA level was above a threshold value, stomata responded to ABA. In conclusion, regulation of *RD29A* is under control of ABA. After low VPD-exposure, the foliar ABA content decreased, mainly due to catabolism of ABA. This decrease in ABA level resulted in down regulation of *RD29A*, which caused decreased stomatal responsiveness to ABA.
Finally, the results obtained in this study provide new insights into the main reasons for the occurrence of stomatal malfunctioning after long-term low VPD-exposure.

In **Chapter 6**, the main achievements of this study are discussed and directions for future experiments are highlighted.

SAMENVATTING

Huidmondjes zijn openingen die zich verspreid over het bladoppervlak bevinden en die een belangrijke rol spelen bij de diffusie van twee belangrijke gassen (CO_2 opname en waterdamp afgifte) van en naar planten en de hun omringende omgeving. Naast de huidmondjes is het bladoppervlak bedekt met een wasachtige cuticula. Doordat de huidmondjes zich op het grensvlak tussen het inwendige plantenweefsel en de omringende omgeving bevinden, zijn zij de enige openingen die de inwendige ruimte in het blad verbinden met de omgeving buiten de plant. De opening van de huidmondjes wordt gecontroleerd door zwellen en krimpen van twee sluitcellen. De processen die verantwoordelijk zijn voor het zwellen en krimpen van de sluitcellen zijn uitermate gevoelig voor veranderingen in omgevingscondities. Als onmiddellijke (korte termijn) reactie krimpen sluitcellen als het waterdampdruk deficit (VPD) stijgt (droge lucht) met als gevolg dat de huidmondjes zich sluiten. Hier tegenover staat dat sluitcellen zwellen en huidmondjes zich openen als de VPD daalt. Als huidmondjes echter langere tijd worden blootgesteld aan een lage VPD treden er aanpassingsverschijnselen op waardoor de huidmondjes niet meer reageren op prikkels die normaal gesproken sluiting van huidmondjes veroorzaken (disfunctioneren van huidmondjes). Het disfunctioneren van huidmondjes kan negatieve gevolgen hebben voor planten doordat ze verwelken tijdens droogte. Ondanks de aanzienlijke inspanning gedurende de afgelopen 25 jaar (vooral in de tuinbouw) om de oorzaken van het disfunctioneren van huidmondjes te ontrafelen zijn de moleculaire mechanismen hiervan nog steeds onbekend. De doelstelling van dit project was om de veranderingen in de signaaloverdracht te ontrafelen in disfunctionerende huidmondjes ten gevolge van langdurige blootstelling aan lage VPD.

Om na te gaan of het disfunctioneren een gevolg is van veranderingen in de morfologie van huidmondjes en bladanatomie of in de signaaloverdracht van abscissinezuur (ABA) zijn in **Hoofdstuk 2** een aantal tuinboonplanten opgegroeid bij een lage en een gematigde VPD en zijn bovendien een aantal planten, die hun bladeren hadden gevormd bij een gematigde VPD, voor een periode van vier dagen overgeplaatst naar een lage VPD. Planten die zijn gegroeid bij lage VPD vertoonden een aantal verschillen in huidmondjes morfologie en bladanatomie t.o.v planten gegroeid bij gematigde VPD: de huidmondjes waren groter en hadden een grotere opening. Bovendien hadden de bladeren gegroeid bij lage VPD een groter specifiek bladoppervlak (SLA) en minder spons parenchym. Naast de verschillen in morfologie en anatomie van huidmondjes en blad was de reactie veranderd van sluitcellen op prikkels die normaal sluiting van de huidmondjes oproepen. Als reactie op toediening van ABA sloten de

huidmondjes van planten gegroeid bij lage VPD minder en langzamer in vergelijking met planten gegroeid bij een gematigde VPD. Bovendien, bij een zelfde relatief watergehalte, verdampten de bladeren van planten gegroeid bij lage VPD meer in vergelijking met bladeren gegroeid bij een gematigde VPD; bladeren van lage VPD-planten droogden ook uit tot lagere relatief watergehaltes. Planten die waren gegroeid bij een gematigde VPD en daarna vier dagen waren blootgesteld aan lage VPD hadden nagenoeg dezelfde huidmondjes morfologie (m.u.v. de huidmondjesopening), huidmondjesdichtheid en bladanatomie als de planten die volledig waren opgegroeid bij een gematigde VPD. Na een blootstelling van vier dagen aan lage VPD waren de reacties van de huidmondjes op uitdroging en op toediening van ABA echter gelijk aan die van planten die gegroeid waren bij lage VPD. Daarom werd geconcludeerd dat veranderingen in bladanatomie en huidmondjes morfologie als gevolg van lage VPD niet de belangrijkste oorzaken waren waardoor huidmondjes gegroeid bij lage VPD minder reageren op uitdroging en toediening van ABA. De reactie van huidmondjes op ABA was na 1 dag blootstelling aan lage VPD verloren, terwijl hun reactie op uitdroging geleidelijk minder werd tijdens 4 dagen bloostelling aan lage VPD. Binnen een dag blootstelling aan lage VPD daalde het gehalte aan ABA in het blad scherp, terwijl het gehalte aan ABA-glucose ester niet werd beïnvloed door blootstelling aan een lage VPD. Het vermogen van huidmondjes om te sluiten bleef behouden wanneer de bladeren werden bespoten met ABA gedurende de 4-daagse blootstelling aan een lage VPD. Deze resultaten vormen een aanwijzing dat het disfunctioneren van huidmondjes na langdurige blootstelling aan een lage VPD veroorzaakt wordt door een verandering in de signaaltransductie ten gevolge van het lage ABA gehalte in de bladeren.

Omdat was vastgesteld dat veranderingen in de signaaltransductie de belangrijkste oorzaken zijn voor disfunctioneren van huidmondjes bediscussiëren we in **Hoofdstuk 3**, aan de hand van een literatuur overzicht, de mogelijke veranderingen in de signaaltransductie na een langdurige blootstelling aan enkele omgevingscondities. De duur van de blootstelling van planten aan omgevingsfactoren als ozon, waterstofsulfide, zwavel dioxide en vooral lage VPD bleek kritiek te zijn voor het optreden van disfunctionaliteit van huidmondjes: gedurende of na een kortstondige blootstelling aan genoemde factoren reageerden huidmondjes normaal op prikkels die gewoonlijk hun sluiting induceren, terwijl na een langdurige blootstelling huidmondjes een verminderde sluitingsreactie vertoonden. De mate waarin disfunctionaliteit optrad na blootstelling aan enkele omgevingsfactoren, zoals continu licht en ozon, was sterker als deze factoren gelijktijdig aanwezig waren met een lage VPD. ABA en calcium stijgen in

de apoplast en daarna in de symplast van de sluitcellen onder omstandigheden die een accumulatie van ABA in het blad bevorderen, zoals een hoge VPD. Als ABA beschikbaar is in de symplast van de sluitcellen, veroorzaakt dit, na binding aan zijn receptor (PYR/PYL/RCARS), een stijging in waterstofperoxide en stikstofoxide en blokkeert het tevens eiwit fosfatasen type 2C; deze laatste zijn negatieve regulatoren van de ABA signaaloverdracht. Als resultaat activeert een SnRK2/OST1 eiwit kinase een anion kanaal (SLAC1) (voor efflux) en remt het een kalium kanaal (KAT1) (voor influx) met als gevolg sluiting van het huidmondje. In tegenstelling hiermee zullen onder omstandigheden die de accumulatie van ABA niet bevorderen, zoals lage VPD, de concentraties van ABA en calcium laag zijn t.g.v. een geringe verdampingssnelheid en een verhoogde activiteit van *CYP707As* (oxideren ABA). In deze situatie zal ABI1/PP2C het SnRK2/OST1 eiwit kinase inactiveren en zal er geen remmend effect zijn op ionen kanalen; het resultaat is dat huidmondjes open blijven. Voorgesteld wordt dat het gelijktijdig optreden van veranderingen in Ca^{2+}, ABA receptoren, en positieve en negatieve regulatoren van ABA signaaltransductie de eerste stappen zijn die leiden tot het disfunctioneren van huidmondjes t.g.v. langdurige blootstelling aan lage VPD. Transcriptie activators (bijv. AtMYB60 en AtNAP) en transcriptie onderdrukkers (bijv. NPX1 en AtERF7), alsook E3 ligasen, kunnen resulteren in een lange termijn aanpassing van cellulaire processen die daarna een verminderde reactie van huidmondjes op sluitings-prikkels veroorzaken. Naast ABA reguleren ook andere plantenhormonen en hun interacties de reacties van huidmondjes. Cytokininen en auxinen beïnvloeden huidmondjes gedrag via ethyleen. Er wordt gesuggereerd dat huidmondjes sluiten als reactie op ethyleen indien ABA afwezig is, maar openen als ABA aanwezig is. Vandaar dat mogelijke interacties tussen plantenhormonen ook het huidmondjes gedrag na langdurige blootstelling aan lage VPD kunnen beïnvloeden.

Om het moleculaire mechanisme te kunnen vinden van het disfunctioneren van huidmondjes dat optreedt na een langdurige blootstelling aan lage VPD, was het belangrijk om mogelijke variatie vast te stellen in de reactie van huidmondjes op sluiting-inducerende prikkels tussen accessies van *Arabidopsis thaliana* na langdurige blootstelling aan lage VPD. Daarom zijn in **Hoofdstuk 4** de reacties van huidmondjes vastgesteld van een verzameling accessies van *Arabidopsis* op prikkels die sluiting van huidmondjes oproepen (ABA en uitdroging) nadat de planten langere tijd waren blootgesteld aan een gematigde of een lage VPD. Om een efficiënte screening te kunnen uitvoeren van de huidmondjesreactie op ABA van grote aantallen bladmonsters hebben we een methode toegepast van chlorofyl fluorescentie imaging van

bladschijfjes die dreven op ABA oplossingen, onder een omstandigheid waarbij geen fotorespiratie optreedt. Voor het screenen van huidmondjesreactie op uitdroging werd de verdampingssnelheid van afgesneden bladeren gebruikt als functie van hun relatief watergehalte, nadat de planten van de geteste *Arabidopsis* accessies aan lage VPD waren blootgesteld. In alle geteste accessies was de huidmondjes geleidbaarheid (g_s) gestegen nadat de planten waren blootgesteld aan een lage VPD. Echter in 39 van de 41 geteste accessies vertoonden de huidmondjes een verminderde sluitingsreactie door ABA na blootstelling aan lage VPD. Alleen de huidmondjes van Map-42 en C24 reageerden in gelijke mate op ABA, zowel na blootstelling aan een gematigde als een lage VPD. Aan de andere kant vertoonden alleen Cvi-0 en Rrs-7 een verminderde sluitingsreactie van de huidmondjes als reactie op uitdroging nadat planten waren blootgesteld aan lage VPD in vergelijking met planten afkomstig van een gematigde VPD. De sluiting van huidmondjes als reactie op ABA (maar niet als reactie op uitdroging) was negatief gecorreleerd met de huidmondjes geleidbaarheid onmiddellijk na een voorafgaande blootstelling aan lage VPD. Met behulp van een hoofdcomponentenanalyse (PCA) konden de accessies worden ingedeeld in groepen van zeer gevoelig, gematigd gevoelig en minder gevoelig voor sluiting-inducerende prikkels. Zowel voor als na uitdroging werden de ABA gehalten in bladeren gemeten van drie accessies als representanten van de drie clusters van de PCA. Na blootstelling aan de beide VPD's werd er een positieve correlatie gevonden tussen ABA gehalte van het blad (voor uitdroging) en de huidmondjes reactie op ABA (maar niet met de reactie op uitdroging). De huidmondjes reactie op uitdroging was positief gecorreleerd met ABA gehalte van het blad na uitdroging. Geconcludeerd werd dat er een grote natuurlijke variatie aanwezig is tussen *Arabidopsis* accessies in het effect van langdurige blootstelling aan lage VPD op de reactie van huidmondjes op prikkels die sluiting van huidmondjes oproepen.

Om het moleculaire netwerk op te helderen dat een rol speelt bij het niet reageren van huidmondjes op ABA nadat planten langdurig aan een lage VPD zijn blootgesteld, werden in **Hoofdstuk 5** twee groepen van *Arabidopsis* accessies gebruikt; een groep bleef gevoelig voor ABA na langdurige blootstelling aan lage VPD (Map-42 en C24) en een groep waar de reactie op ABA verdween na langdurige bloostelling aan lage VPD (Col-0, Cvi-0 en Rrs-7). De transcriptie niveaus van genen betrokken bij ABA transport, perceptie, biosynthese, afbraak, en signaal overdracht als ook bij secundaire boodschappers en ethyleen signaal overdracht werden geanalyseerd in bladeren van Col-0, Cvi-0, en Map-42 na een 4-daagse blootstelling aan een lage en hoge VPD. Omdat het bespuiten van bladeren met ABA gedurende de

blootstelling aan lage VPD van Col-0 en Cvi-0 (ABA behandelde planten) de sluitingsrespons van huidmondjes intact hield, werden de transcriptie niveaus van eerder genoemde genen ook geanalyseerd van deze ABA behandelde planten. De transcriptie van genen betrokken bij calcium signalering werd beïnvloed door langdurige blootstelling aan lage VPD. Deze transcriptie werd echter niet beïnvloed door de ABA behandeling. Noch genen betrokken bij ABA transport en perceptie als genen betrokken in de signalering van secundaire boodschappers en ethyleen respons waren betrokken bij het ontbreken van de huidmondjes reactie op ABA. In tegenstelling hiermee, waren de transcriptie niveaus van *CYP707A* genen, die een rol spelen bij afbraak van ABA, verhoogd na blootstelling aan lage VPD in Col-0 en Rrs-7, maar niet in de accessies die hun reactie op ABA behielden (Map-42 en C24). Afbraak van ABA is het belangrijkste proces dat het ABA gehalte in bladeren verlaagd na langdurige blootstelling aan lage VPD (behalve voor Cvi-0). Transcriptie niveaus van *RD29A* werden verlaagd door lage VPD in de accessies waarin lage VPD veroorzaakte dat huidmondjes niet meer reageerden op ABA (Col-0, Cvi-0 en Rrs-7), terwijl de expressie werd verhoogd in ABA behandelde planten en in de accessies die hun ABA-reactie behielden na lage VPD-blootstelling. Om de rol van *RD29A* in het disfunctioneren van huidmondjes na blootstelling aan lage VPD te testen, zijn er transgene *Arabidopsis* gemaakt waarin het *RD29A* gen was gefuseerd met Green Fluorescence Protein (GFP). In de transgenen planten was de aanwezigheid van het *RD29A*-GFP eiwit beperkt tot de trichomen en de sluitcellen van de huidmondjes. In de bladeren van de planten met een overexpressie van *RD29A* als ook in de dubbel mutant *cyp707a1 cyp707a3* was het ABA gehalte aanzienlijk hoger. Huidmondjes van deze planten bleven reageren op ABA na blootstelling aan lage VPD, terwijl er verminderde sluiting werd waargenomen in huidmondjes van de *rd29a* mutant na een 4-daagse blootstelling aan lage VPD. Het ABA gehalte van de bladeren correspondeerde in alle accessies met de reactie van de huidmondjes op ABA: alleen als het ABA gehalte boven een drempelwaarde was reageerden de huidmondjes op ABA toediening. Geconcludeerd werd dat *RD29A* onder controle staat van ABA. Na blootstelling aan lage VPD daalt het ABA gehalte in het blad, vnl. door afbraak van ABA. Deze daling van het ABA niveau veroorzaakt een verminderde transcriptie van *RD29A*, dat op zijn beurt een verlaagde reactie van huidmondjes op ABA veroorzaakt.

In **Hoofdstuk 6** worden de belangrijkste resultaten van deze studie bediscussieerd en worden richtingen voor toekomstig onderzoek besproken.

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About the author

Sasan Ali niaei fard was born on 22nd March 1981 in Khorram abad, Iran. After completing high school in his home town he started his higher education in 1999 and finished his bachelor in horticulture in 2003 at University of Kurdistan. From 2003 to 2005 he did military service and thereafter he started his MSc-program in Horticultural sciences at Tabriz University. He received his MSc certificate with distinguished degree in 2007. He accomplished several research studies funded by different institutes between 2008 and 2010. One of his research with the title of "improving tolerance to boron toxicity by using of salicylic acid in peppermint" was selected as best national research plan in 2010. he was awarded a full scholarship from the Ministry of Science, Research and Technology of Iran to pursue a PhD in the Horticultural Production Chains Group (new name: Horticulture and Product Physiology group), Wageningen University, the Netherlands. During his PhD he worked on the signal transduction pathways in guard cells of stomata after prolonged exposure to low vapour pressure deficit. The result of this study is presented in this thesis. After the PhD graduation, he will carry on his career as an assistant professor in Tehran University.

Publication list

Refereed journals

Aliniaeifard S, van Meeteren U (2013) Can prolonged exposure to low VPD disturb the ABA signalling in stomatal guard cells? *Journal of Experimental Botany* 64: 3551-3566

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Aliniaeifard S, Seifi Kalhor M, Geurts R, Dépré S, Franssen H, Bouwmeester H, and van Meeteren U (2014) Abscisic acid-induced *RD29A* is crucial for keeping stomatal functionality after long-term exposure to low vapour pressure deficit. Submitted to *Molecular plant*

Conference proceeding and other scientific journals

Aliniaeifard S, and van Meeteren U (2014). Dynamics of stomatal response to abscisic acid in *Arabidopsis thaliana* under different VPDs. Plant signalling: dynamic properties conference. Breckenridge, Colorado, USA

Aliniaeifard S, van Meeteren U (2012) Stomatal functioning of fava bean in response to different closing stimuli influenced by preceding relative air humidity. 29th new Phytologist symposium. Page 36. Manchester, United Kingdom.

Aliniaeifard S, van Meeteren U, Bouwmeester H (2013) Root Signals can maintain stomatal functionality of low Vapour Pressure Deficit-exposed fava bean plants. American Society of Plant Biology (ASPB 2013). Providence, Rhode island, USA.

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Shahlaei A, Alemzadeh Ansari N, **Aliniaeifard S** (2009) Osmopriming Eggplant (*Solanum melongena* L.) seeds by Using Salt Solutions. *Middle Eastern and Russian Journal of Plant Science and Biotechnology* **3:** 41-43

PE&RC Training and Education Statement

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

Review of literature (6 ECTS)

- Review of literature regarding mechanism behind different responses of stomata after exposure to different VPDs

Writing of project proposal (4.5 ECTS)

- Signal transduction pathway in guard cells after prolonged exposure to low VPD

Post-graduate courses (4.3 ECTS)

- Molecular advances in ecology: WUR (2012)
- Introduction to R; WUR (2013)
- Plant signalling: dynamic properties; poster presentation; Colorado, USA (2014)
- LI-6400 Training Course; LI-COR Biosciences GmbH Bad Homburg (2013)

Laboratory training and working visits (1.2 ECTS)

- Scientific visiting flower auctions and several companies related to vegetable and flower production; Aalsmeer Flower Auction and several greenhouse companies (2010-2013)

Invited review of (unpublished) journal manuscript (3 ECTS)

- Acta Horticulturae: effect of moderately saline water and water deficit on the content of antioxidants in paprika (*Capsicum annuum*) at different ripening stages (2014)
- Acta Horticulturae: impact of salinity and water deficiency on the fluorescence signature of tomato leaves (2014)
- Acta Horticulturae: near-infrared spectroscopy: a promising sensor technique for quality assessment of ornamental cuttings (2014)

Deficiency, refresh, brush-up courses (3 ECTS)

- Greenhouse technology (2013)
- Gene technology (2012)
- Crop ecology (2011)

Competence strengthening / skills courses (3.2 ECTS)

- Techniques for writing and presenting a scientific paper; WUR (2012)
- Reviewing scientific paper; WUR (2012)
- Interactive workshop "how to write a convincing research proposal"; WUR (2013)
- Scientific writing; WUR (2014)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.8 ECTS)

- PE&RC Weekend (2010 and 2014)
- PE&RC Day (2010-2011)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)

- Frontier Literature in Plant Physiology (FLOP) (2010-2014)
- EPS Flying seminar (2011-2013)
- Evolution of chemical diversity in plants (2012)
- 1st Wageningen PhD Symposium "Healthy Food & Living Environment", 10 Des; Wageningen, the Netherlands (2013)
- Minisymposium: how to write a world-class paper (2013)

International symposia, workshops and conferences (4.1 ECTS)

- Stomata Symposium; poster presentation; Manchester, UK (2012)
- Plant Biology; poster presentation; Rhode Island, USA (2013)

Lecturing / supervision of practical's / tutorials (3 ECTS)

- Physiology and development of plants in horticulture (2012-2013)

Supervision of 5 MSc students

- Disturbance of the stomata control pathway in Tradescantia by long term exposure to high relative humidity
- Is there an effect of low VPD on salicylic acid induced stomata closure
- The role of NO in the stomatal behavior of *Vicia Faba* plants grown at high and moderate relative humidity
- Interaction between plant wound response and stomatal sensitivity to ABA in plants exposure to low VPD
- RD29 Is involved in the malfunctioning of stomata in low VPD-grown *Arabidopsis thaliana*

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Front page: Production of hydrogen peroxide in guard cells after application of 100 µM ABA to epidermal strips of *Vicia faba* plants. Green fluorescence (488–515 nm) corresponds to H_2DCFDA and red fluorescence corresponds to chlorophyll autofluorescence.

Background: Production of hydrogen peroxide in guard cells after application of 100 µM ABA to epidermal strips of *Vicia faba* using different filters. Green fluorescence (488–515 nm) corresponds to H_2DCFDA and red fluorescence corresponds to chlorophyll autofluorescence.

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