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

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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 generates a sort of memory in the guard cells that results in the loss of suitable responses of the stomata to closing stimuli, such as 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).

Key words: Abscisic acid, calcium, environmental factors, guard cell signalling pathway, hydrogen peroxide, nitric oxide, secondary messengers, stomata, vapour pressure deficit.

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 such as continuous light (Slootweg and van Meeteren, 1991; Mortensen and Gislerød, 1999; Pettersen et al., 2007; Arve et al., 2012), ozone (O$_3$) (Paolletti, 2005; Wilkinson and Davies, 2009), hydrogen sulphide (H$_2$S) (Lisjak et al., 2010), sulphur dioxide (SO$_2$) (Maier-Maercker and Koch, 1986), and, in particular, low vapour pressure deficit (VPD) (Torre and Fjeld, 2001; Rezaei Nejad and van Meeteren, 2005, 2007, 2008; Rezaei Nejad et al., 2006; Fanourakis et al., 2011; Arve et al., 2012). It is rather surprisingly that a single factor, such as low VPD, can disturb the robust system of stomatal 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 et al., 2013). Despite the recent advances in our understanding of 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 on 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 duration of exposure 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, b; 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, with a 24 h light period, or with 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 include 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 et al., 2013a), 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 d 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 d) 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; Majeda 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.

### 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                                                                 | Reference                          |
|--------------------------|----------------------------------|---------------------------------------------|----------------------------------------------------------------------------------------|------------------------------------|
| (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)                    | ABA (2 h)                                   | Closure of the stomata                                                                 | Okamoto et al. (2009)              |
|                          | O$_3$ (3 min and 6 h)            | Exposure to ozone (3 min and 6 h)           | Closure of the stomata and decrease in stomatal conductance                             | Overmyer et al. (2008); Vahisalu et al. (2010) |
| Polypodium vulgaris      | 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 remain open to some extent open                           | Rezaei Nejad et al. (2006); Rezaei Nejad and van Meeteren (2005, 2007, 2008) |
| Rosa hybrida             | Low VPD (during growth)          | Desiccation (>2 h)                          | Slow reduction in transpiration rate                                                    | Torre and Fjeld (2001); Fanourakis et al. (2011) |
|                          | Continuous light (24 and 20 h d$^{-1}$) | Leaf detachment (3 h) and darkness (4 h)     | High water loss and stomata remain open                                                  | Slootweg and van Meeteren (1991); Mortensen and Gislerød (1999) |
| Leontodon hispidus       | O$_3$ (1–29 d)                   | ABA (1 h)                                   | Reduction of stomatal sensitivity for closure response                                  | Wilkinson and Davies (2009)         |
| Arbutus unedo            | O$_3$ (20 weeks)                 | ABA and leaf desiccation                    | Impaired stomatal control                                                               | Mills et al. (2009)                |
|                          | O$_3$ (90 d)                     | Abrupt reduction of light intensity and water stress (20 min) | Sluggish stomatal response                                                             | Paoletti (2005)                    |
(Ziv et al., 1987; Zacchini et al., 1997; Hazarika, 2006), the contribution of increased cuticular water loss is small in comparison with 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 et al., 2013a). 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 with the involvement of cuticular water loss.

Stomatal size and stomatal index are also affected by VPD during plant development (Fordham et al., 2001; Torre et al., 2003; Lake and Woodward, 2008; 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; on 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 Gislerod, 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 Gislerod, 1999, 2011; Pettersen et al., 2006; Arve et al., 2012).

Plant responses to environmental pollutants also involve effects on stomatal regulation, which are dependent on the exposure time. Although short-term exposure to H\textsubscript{2}S did not change the transpiration rate in maize, pumpkin, spruce, and spinach (De Kok et al., 1989), long-term application of H\textsubscript{2}S 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\textsubscript{3} are unable to close in response to ABA and drought stress; therefore, O\textsubscript{3} renders stomata incapable of controlling transpiration (Mills et al., 2009; Wilkinson and Davies, 2009). In Arbutus unedo, slow stomatal closure persisted for 10 d after a 90 d exposure to O\textsubscript{3} (Paolletti, 2005). In Leontodon hispidus, exposure to O\textsubscript{3} for at least 48 h resulted in the loss of the stomatal closure response in the presence of ABA (Wilkinson and Davies, 2009), whilst short-term exposure of the leaves to O\textsubscript{3} would trigger a rapid stomatal closure (Torsethaugen et al., 1999; Leipner et al., 2001; Overmyer et al., 2008; Vahisalu et al., 2010). Stomatal malfunctioning was more pronounced when the exposure to O\textsubscript{3} took place under 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 results in abnormal stomatal regulation, while short-term exposure does not. The magnitude of stomatal malfunctioning induced by factors such as continuous light and O\textsubscript{3} is much more pronounced when these are applied simultaneously with low VPD.

As CO\textsubscript{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 still responded adequately to closing stimuli (Rezaei Nejad and van Meeteren, 2007). The same authors showed that the stomata in different parts of one leaf which were exposed for a long time 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 stomatal signalling is only local. This is another indication that the long-term low VPD effect on stomatal closing is not related to effects of environmental factors on morphological aspects such as stomatal density.

**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 the 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% h 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 T. virginiana plants was decreased within 1 d 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 catabolizes 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 part 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 have been widely documented (Gorton et al., 1993; Shimazaki et al., 2007; Wang et al., 2010). For example, it has been demonstrated that phytochrome B and cryptochromes are involved in stomatal opening through regulation of the transcription factor (TF) AtMYB60 (see ‘ABA, transcription factors, and stomatal malfunctioning’) 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 at midday; and (iii) increase in ABA production in the guard cells in the dark period. The depletion of endogenous guard cell ABA early in the daytime (phase 1) is the result of activation of ABA 8'-hydroxylase. This NADPH-requiring CytP450 is activated by elevated O2 and reduced CO2 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 O2 and CO2 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 a result of the increased ABA level by midday will be compensated by the osmotic potential decrease due to sucrose synthesis by photosynthesis, and stomata remain open throughout the afternoon. At the end of the day when symplastic ABA exceeds the sucrose threshold level (the concentration of sucrose in guard cell cytosol required for keeping the 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 O2 levels as a 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 a 20 h 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 with 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 1 d or 2 d, 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 d, 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 low VPD-grown plants to moderate VPD, but it is the diminished response to ABA that causes the stomatal 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 desensitization. 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 provokes stomatal insensitivity to ABA has not been discussed. The question arises of why 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, 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, 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). 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 a long time to low VPD conditions. After transport of ABA from the guard cell apoplast to the 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 Fig. 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; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Hubbard et al., 2010). All of these components are present in both the 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, determines 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, 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 the 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 D D1 (PLD1) 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; Takemini and Shimazaki, 2010) (Fig. 1) and strengthens the ABA-induced OST1 activity. PA also acts as a lipid secondary messenger (see the following section). Yoshida et al. (2002) found that SnRK2 can be activated by high VPD. By a T-DNA knockout mutation in an 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 desensitization 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

TFs are proteins involved in the regulation of cellular processes via initiating and controlling the transcription of genes. AtMYB60 is the first TF characterized as having a role in stomatal opening (Cominelli et al., 2005). The involvement of TFs in ABA responses has been well documented. As an 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) down-regulates the expression of ABA-induced genes. AtERF7-overexpressing 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 to close their stomata (Kim et al., 2009). All the above-mentioned studies were related to abiotic stress conditions such as drought and salinity. However, there is no research on the role of relevant TFs
in stomata that are malfunctioning due to long-term exposure to low VPD. Therefore, it will be relevant to unravel the role of TFs in guard cells of plants exposed for a 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 with 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 with leaf age; the expression of senescence-associated gene113 (*SAG113*) and of AtNAP (the gene encoding a NAC family TF, AtNAP) is co-induced during leaf senescence (Zhang and Gan, 2012; Zhang et al., 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 the transcriptional level (Zhang and Gan, 2012), and keeps the stomata less susceptible 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 another example, a negative feedback loop for the F-box protein DOR in ABA responses has been demonstrated. DOR functions as a 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 of 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$_2$O$_2$. On the other hand, other U-box E3 ubiquitin ligases, AtPUB22 and AtPUB23, are negative regulators of drought responses which act independently of ABA (Seo et al., 2012). Expression of *AtPUB18* and *AtPUB19* depends on SnRK2, while expression of *AtPUB22* and *AtPUB23* is independent of 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 the 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.

### Involvement of cross-talk between secondary messengers

A variety of second messengers have been implicated in the perception of stimuli of stomatal closure (Fig. 2), such as cytosolic calcium ([Ca$^{2+}$]$_{cyt}$), hydrogen peroxide (H$_2$O$_2$), and NO (Pei et al., 2000; Zhang et al., 2001; Siegel et al., 2009; Kim et al., 2010; Wang et al., 2011; Distefano 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 such as 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 (CDPKs) 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 CDPKs (Mori et al., 2006; Geiger et al., 2010). CDPKs 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 the 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 CDPKs.

According to 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 a 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$_2$O$_2$ is an essential intermediate in guard cell ABA signalling. ABA activation of I$_{Ca}$ channels requires H$_2$O$_2$ production by membrane-bound NADPH oxidases AtRbohD and F (Pei et al., 2000). ABI1 and OST1 act upstream of H$_2$O$_2$ (Mustilli et al., 2002). NO functions as a downstream intermediate of H$_2$O$_2$ 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$_2$O$_2$ and NO levels inside the guard cells via CAS (Wang et al., 2011). Therefore, an increase in the [Ca$^{2+}$]$_{o}$ can result in an increase in the activation of SLAC1. Moreover, a combination of an extracellular Ca$_{2+}$ sensor, extracellular calmodulin (ExCaM),
and $[\text{Ca}^{2+}]_o$ can activate a signalling pathway that results in activation of GPA1 (G protein alpha subunit 1), and thereafter $\text{H}_2\text{O}_2$ and NO generation, resulting in changes in $[\text{Ca}^{2+}]_{cyt}$ and then stomatal closure (Fig. 2) (Chen et al., 2004; Li et al., 2009; Zhang et al., 2011).

As a result of low $\text{Ca}^{2+}$ transport in the xylem due to a low transpiration rate, the $[\text{Ca}^{2+}]_o$ will be low after long-term exposure to low VPD. This will result in low activity of CAS and ExtCaM. Moreover, $I_{\text{Ca}}$ channels will have low activity due to the low ABA concentration at low VPD (see above). Therefore, it can be expected that long-term exposure to a low VPD results in a low $[\text{Ca}^{2+}]_{cyt}$, and, as result of that, in low $\text{H}_2\text{O}_2$ 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\(_2\)O\(_2\) and NO (Fig. 2) (Mishra et al., 2006; Zhang et al., 2011); in this way, there is a Ca\(^{2+}\)-dependent signalling pathway for NO-induced stomatal closure. In guard cells of stomata that malfunction due to long-term exposure to H\(_2\)S, NO production in response to ABA application was reduced (Lisjak et al., 2010). After prolonged exposure to 90% RH, guard cells of T. 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\(_2\)O\(_2\) (Murata et al., 2001; Mustilli et al., 2002). The protein phosphatase activity of ABI2 is very sensitive to H\(_2\)O\(_2\). Therefore, the ABA signalling pathway will be activated by H\(_2\)O\(_2\)-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 regulates 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 ABI2~5-fold. GPX3 is also a key enzyme in scavenging H\(_2\)O\(_2\) (Fig. 2) by catalysing the reduction of H\(_2\)O\(_2\) by glutathione (GSH). Thus, GPX3 functions in both H\(_2\)O\(_2\) sensing and scavenging (Miao et al., 2006). Ascorbate (Asc) is another major antioxidant that scavenges H\(_2\)O\(_2\), resulting in dehydroascorbate (DHA) (oxidized ascorbate). Dehydroascorbate reductase (DHAR) catalyses the reduction of DHA to Asc and thus contributes to the regulation of the Asc redox state (Asc/DHA ratio). DHA is reduced to Asc at 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\(_2\)O\(_2\) and the Asc redox state are diurnally regulated such that H\(_2\)O\(_2\) in guard cells increases during the afternoon, whereas the Asc redox state decreases. An increase in H\(_2\)O\(_2\) 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\(_2\)O\(_2\) or ABA signalling. A more reduced state of Asc and GSH will result in a higher scavenging capacity on H\(_2\)O\(_2\) 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 regulation of stomatal movement. 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 H\(_2\)O\(_2\) homeostasis. Therefore, stomata stay open when DST is in an active state through inhibition of H\(_2\)O\(_2\) accumulation. In the ABA-independent pathway, it can be expected that the redox state of guard cells will be higher in low VPD-compared with high VPD-exposed plants, due to the lack of [Ca\(^{2+}\)]\(_{cyt}\)-induced H\(_2\)O\(_2\) 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\(_2\)O\(_2\) and NO production.

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). Brassinosteroids (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; Hussain 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-signalling 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, probably via stimulation of reactive oxygen species (ROS) production; it plays a key role in pathogen defence 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 defence against necrotic pathogens and insects, and is often recognized as a biotic stress hormone (Lieciti and Farmer, 2002; Fujita et al., 2006). JA accumulates during drought stress and has a positive role in stomatal closure. It is suggested that JA-mediated stomatal response requires ABA and that JA and ABA employ common signalling components (Acharya and Assmann, 2009; Zhu et al., 2012). However, significant induction of 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 stomatal closure, probably 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
It has been shown that ethylene or ACC (1-aminocyclopropane-1-carboxylic acid; the precursor of ethylene) can prevent ABA-induced stomatal closure (Tanaka et al., 2006; Tanaka et al., 2006). 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 ABA-induced stomatal closure but not in the early steps (Tanaka et al., 2005). The interaction between AtERF7 (which is a member of the 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 H₂O₂ signalling in guard cells and may be a sensor for H₂O₂ perception in guard cells (Desikan et al., 2005). So ETR1 may be the site of ethylene and H₂O₂ cross-talk leading to stomatal closure (Desikan et al., 2005). Ethylene antagonizes the ABA-induced stomatal closing response after long-term O₂ application in wild-type Arabidopsis plants. Exposure of plants for a long time to elevated O₂ 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 O₂, after 6h 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 (1-methylcyclopropene; 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 Ca²⁺ dependent (Raz and Fluhr, 1992). Therefore, in low VPD conditions together with low ABA and Ca²⁺, cross-talk between plant hormones could also be an effecter of the 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 CO₂ (Ricáněk and Vícherková, 1992). Exogenous application of the naturally occurring auxin indolyl-3-butyric acid (IBA) to epidermal peels can open stomata under darkness, probably via a Ca²⁺-dependent signalling in the guard cell. However, the interaction of phytohormones in the malfunctioning of stomata under low VPD conditions has not been demonstrated.

**Conclusion and future challenges**

Through reviewing the 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 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 that alterations in signalling pathways due to a long-term low transpiration rate under long-term exposure to environmental conditions, especially low VPD, lead to depletion of ABA, Ca²⁺, and H₂O₂ in the guard cells, as well depletion of extracellular ABA and Ca²⁺. 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 Ca²⁺. This coincidence in changes of Ca²⁺, 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 following:

- 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 TFs such as transcriptional activators (e.g. AtMYB60 and AtNAP) and transcriptional repressors (e.g. NPX1 and AtERF7) when plants have been exposed for a 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 stomatal closing stimuli.
- Interaction of phytohormones such as ABA and ethylene, and 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 site of stomatal malfunctioning in the guard cell signal transduction pathway.

These experiments, together with other approaches (such as transcriptome profiling and quantitative trait locus 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, such as low VPD.

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