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Growth in continuous high air humidity increases the expression of CYP707A-genes and inhibits stomatal closure

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ABSTRACT

To conserve water plants close the stomata in response to increased abscisic acid (ABA) content. Previous studies have shown that plants developed in high relative air humidity (RH > 85%) develop malfunctioning stomata and have lower ABA content. It has therefore been hypothesized that low ABA content during development results in malfunctioning stomata. In this study the stomatal functioning of *Arabidopsis thaliana* was evaluated and the content, biosynthesis and catabolism of ABA were quantified. It was found that even though they have lower ABA content during growth, plants developed under high RH were able to produce large amounts of ABA in detached leaves, but still had high water loss. Plants developed in high RH had increased ABA 8′-hydroxylase activity of cytochrome P450CYP707A. Also, plants developed in high RH that were sprayed with ABA or Abscinazole-E1, which inhibits the ABA 8′-hydroxylase activity, had reduced stomatal apertures. ABA deficient mutants had higher water loss in detached leaves than wild type plants in both high and moderate RH. From these results we therefore conclude that continuous low ABA content in high RH is due to increased ABA catabolism by the ABA 8′-hydroxylase activity of cytochrome P450CYP707A. The continuous low ABA content result in reduced ability to close the stomata.

1. Introduction

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

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

ABA is inactivated by two main pathways; oxidation or conjugation. The oxidation pathway is regulated by the ABA 8′-hydroxylase activity of cytochrome P450CYP707A, converting ABA to phaseic acid (PA) and further to 4′-dihydrophaseic acid (DPA) (Seki et al., 2007). This conversion is mostly catalyzed by CYP707A1 in guard cells and CYP707A3 in vascular tissues (Okamoto et al., 2009). The ABA 8′-hydroxylase activity can be inhibited by applying abscinazole-E1 (Okazaki et al., 2011). In the conjugation pathway, ABA is most commonly conjugated to ABA-β-D-glucosyl ester (ABA-GE) in a reaction catalyzed by ABA glucosyltransferase (Lee et al., 2006). ABA-GE is hypothesized to be a storage form of ABA, which can be converted back to ABA by β-glucosidase (Arve et al., 2013; Dietz et al., 2000; Lee et al., 2006).

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Extensive studies have previously been performed on plant responses and ABA production during drought. To retain as much water as possible, plants increase the ABA content which acts as a signal for stomatal closure (Monda et al., 2011; Seki et al., 2007). Especially NCED has been found to play an important role in regulating the ABA content by increasing the ABA biosynthesis during drought (Thompson et al., 2000). Another less-studied factor influencing stomata is the relative air humidity (RH). During *in-vitro* propagation, greenhouse production and some conditions in natural ecosystems plants exposed to under constant high RH. However, the effects of such conditions on ABA and ABA metabolism are not well understood. Previous studies of *Rosa x hybrida*,*Dianthus caryophyllus*, *Delphinium* spp. and *Wrightia tomentosa* have shown that plants developed under continuous high RH (>85%) develop larger stomata, that are unable to close in response to environmental conditions that usually lead to closure (Joshi et al., 2006; Santamaria et al., 1993; Torre et al., 2003; Ziv et al., 1987). This results in high water loss (Mortensen and Fjeld, 1998; Torre and Fjeld, 2001). The stomatal density in these plants is also higher or similar to plants grown under lower RH (Fanourakis et al., 2011; Sciutti and Morini, 1995; Torre et al., 2003).

Several studies have shown that compared to low RH, plants growing in high RH have lower ABA content (Nejad and van Meeteren, 2007). Similarly, after transfer from low to high RH, the ABA content is reduced due to catabolism of ABA into PA (Okamoto et al., 2009). It has also been shown in *Rosa x hybrida* that the plants need a minimum amount of ABA during development to develop fully functioning stomata (Giday et al., 2014). However, the ABA concentration needed to produce fully functional stomata probably varies between species and possibly also between cultivars. Since ABA is an important signal for stomatal closure, it could be hypothesized that development in high RH when endogenous ABA content is low, either reduces the plant's ability to produce ABA or the guard cells' ability to respond to ABA.

Most of the work done on high RH responses and malfunctioning stomata has been done on roses with limited possibilities to use genetic methods and mutants as a tool (Fanourakis et al., 2011; Mortensen, 2000; Torre and Fjeld, 2001). A recent paper deals with the genetic variation in stomatal closure following growth at high RH in 41 accessions of *Arabidopsis thaliana* (Aliniaeifard and van Meeteren, 2014). In this study, we therefore used *A. thaliana* as a model plant. The main aim of this study was to improve the understanding of the ABA regulation in plants developed in constant high and moderate RH during light and dark. The main hypothesis was that the CYP707A genes were expressed differently in constant high and moderate RH.

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana (L.) Heyhn. wild type (WT) (Columbia ecotype, Col-0) and the abscisic acid (ABA) deficient mutant *aba3-1* (N157, Nottingham Arabidopsis Stock Center (NASC), Nottingham, UK) seeds were placed in 0.1% agar in darkness at 4 °C for 4 days. The *aba3-1* mutant is mutated in the AAO3 gene (At1g16540) of the ABA biosynthesis and contains very low levels of ABA (Schwartz et al., 1997). The seeds were then germinated in 13 cm pots with peat (L.O.G. Gartnerjord, Rakkestad, Norway) or in water culture (Araphonics, Araphonics SA, Liège, Belgium). Three plants were allowed to grow in each pot and placed in a growth chamber with 8 h photoperiod, a light intensity of $100 \pm 5\ \mu mol\ m^{-2}\ s^{-1}$ from high pressure mercury lamps (Osram NAV T-400 W, Munich, Germany), 80% relative air humidity (RH), corresponding to 0.53 KPa water vapour deficit (vpd) and 22 °C. The irradiance was measured with a LI-COR Light Meter (LI-250, USA) and RH and temperature was set and regulated using a PRIVA system (Priva, Ontario, Canada). After two weeks, when the plants started to develop true leaves, the plants were transferred to the different RH treatments. There were two different treatments; (1) (Moderate RH): Constant RH at 60% (1.05 KPa vpd). (2) (High RH): Constant RH at 92% (0.26 KPa vpd). In both treatments the temperature was 22 °C, with a 20 h photoperiod and an irradiance of $100 \pm 5\ \mu mol\ m^{-2}\ s^{-1}$ and 4 h darkness. 12–20 plants in high RH were sprayed daily with water, 50 μM ABA or 10 μM abscinazole-E1 throughout the experiment. All plants were watered when needed to avoid any drought. Rosettes were sampled for analysis of ABA and its metabolites and expression of ABA biosynthesis and catabolism genes after three weeks of treatments. Additionally, water loss and stomatal opening were measured at the same time point. Plants grown in water culture were only used to test the water loss of detached leaves. Sampling was performed after 6.5 h of light and in the middle of the dark period for all analyses and treatments. In addition stomatal imprints were made and aperture was also measured after 8 and 12 h of light.

2.2. Water loss in detached leaves

To study the ability of detached leaves to retain water, detached rosettes without the inflorescence and roots were removed from the growth chambers and placed in a test environment with 50% RH, 22 °C and an irradiance of $15\ \mu mol\ m^{-2}\ s^{-1}$. The rosettes were weighed after 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 150 and 180 min and the relative weight of the rosettes at the different time points were calculated. Similar tests were done with single leaves that had been placed in 100 μM ABA or water for 1 h prior to weighing.

2.3. Stomatal measurements

Imprints were made in the mid-section of the 7th rosette leaf using SUMP liquid and SUMP plate B (SUMP Laboratory, Tokyo) as described previously (Tanaka et al., 2005). Imprints were made of three leaves from each treatment at each time point, in three independent experiments, during light and darkness. 15–20 images were taken from random sections of each imprint under a light microscope (Leitz, Labolux K, Type 0.2, Germany) using a Leica camera (Leica DC200, Switzerland) and the pore length, pore aperture (measured as the width of the stomatal pore, where it was widest) and stomatal density was measured on the images using UTHSCSA ImageTool 3.0 (University of Texas Health Science Center in San Antonio, USA).

2.4. Leaf transpiration

Leaf temperature is correlated to transpiration and can be measured using infrared imaging (Jones, 2004; Kümmerlen et al., 1999; Prytz et al., 2003). Since there were 3 plants per pot in this study, evaporation from the soil could not be avoided and therefore no direct transpiration measurements were possible. Infrared imaging was therefore used as an indirect measurement of leaf transpiration in the different treatments during light and darkness. Infrared images of leaves were captured using a ThermoVision™ A40 M infrared camera (FLIR Systems AB, Danderyd, Sweden) with a 3203240 pixels uncooled microbolometer focal plane array, a spectral range of 7.5–13 μm, and a thermal sensitivity of 0.08 °C at 30 °C ambient temperature. The images were analyzed using ThermaCam™ Researcher Pro 2.8 (FLIR Systems AB). Leaf emissivity was set to 0.95 (Jones, 2004). For each plant, the average temperature of the five largest leaves was used as an indirect measure of leaf transpiration. The temperature difference