High Humidity Induces Abscisic Acid 8′-Hydroxylase in Stomata and Vasculature to Regulate Local and Systemic Abscisic Acid Responses in Arabidopsis[1][OA]

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Levels of endogenous abscisic acid (ABA) are changed dynamically in response to environmental conditions. The ABA 8′-hydroxylase is a key enzyme in ABA catabolism and is encoded by *CYP707A* genes. In this study, we examined physiological roles of Arabidopsis (*Arabidopsis thaliana*) CYP707As in the plant's response to changes in humidity. The *cyp707a1* and *cyp707a3* mutants displayed lower stomatal conductance under turgid conditions (relative humidity 60%) than the wild type. When wild-type plants were transferred to high-humidity conditions (relative humidity 90%), *CYP707A1* and *CYP707A3* transcript levels increased, followed by the reduction of ABA levels. The *cyp707a3* mutant exhibited high ABA levels even after transferring to high-humidity conditions, whereas, under similar conditions, the *cyp707a1* mutant exhibited low ABA levels comparable to the wild type. Analysis of spatial expression patterns by using transgenic plants harboring a promoter::*β*-glucuronidase gene indicated that high-humidity-induced expression of *CYP707A1* and *CYP707A3* occurred primarily in guard cells and vascular tissues, respectively. Furthermore, stomatal closure of the *cyp707a1* mutant, but not *cyp707a3* mutant, was ABA hypersensitive when epidermal peel was treated with exogenous ABA, suggesting that CYP707A1 is essential for ABA catabolism inside the guard cells. These results implicate that CYP707A3 reduces the amount of mobile ABA in vascular tissues in response to high humidity, whereas CYP707A1 inactivates local ABA pools inside the guard cells. Taken together, ABA catabolism in both vascular tissues and guard cells participates in the systemic ABA action that controls stomatal movement in response to high humidity.

The phytohormone abscisic acid (ABA) has multiple roles in numerous physiological processes during the plant life cycle (Zeevaart and Creelman, 1988). In seeds, ABA promotes the accumulation of storage proteins and the acquisition of seed dormancy (Hilhorst, 1995; McCarty, 1995). In vegetative tissues, ABA mediates adaptive responses to abiotic and biotic stresses, such as drought, cold, high salinity, heat exposure, and pathogen attack (Robertson et al., 1994; Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; de Torres-Zabala et al., 2007). ABA functions not only as a stress signal but also as a growth regulator that controls vegetative growth and determines cell fate (Sharp and LeNoble, 2002; Kuwabara et al., 2003; Benschop et al., 2005; Saika et al., 2007). The regulation of these processes is mediated by a change in endogenous ABA levels that are controlled by the balance between biosynthesis and catabolism (Zeevaart, 1980).

The biosynthetic pathway of ABA in higher plants has been established by forward-genetic approaches (Seo and Koshiba, 2002; Zhu, 2002; Nambara and Marion-Poll, 2005). Biochemical and molecular genetic studies of ABA-deficient mutants from various plant species contributed to the identification and characterization of a number of enzymes of ABA biosynthesis. The 9-cis-epoxycarotenoid dioxygenase (NCED) cleaves 11, 12 double bonds of C_{40} carotenoids and produces xanthoxin. This is a key regulatory step in de novo ABA biosynthesis (Qin and Zeevaart, 1999; Thompson et al., 2000; Iuchi et al., 2001). In the Arabidopsis (*Arabidopsis thaliana*) genome, there are five members (*AtNCED2*, *3*, *5*, *6*, and *9*) of the NCED family (Iuchi et al., 2001; Toh et al., 2008). Transcript levels of *AtNCED3* rapidly increase in response to drought stress, and its protein levels are correlated with drought-induced ABA accumulation. These results indicate that AtNCED3 plays a crucial role in ABA accumulation during dehydration (Iuchi et al., 2001; Endo et al., 2008).

In the case of ABA catabolism, ABA is converted to inactive forms by oxidation or conjugation (Nambara

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and Marion-Poll, 2005). The major ABA catabolic pathway in higher plants involves oxidation of ABA by ABA 8′-hydroxylase to form 8′-hydroxy ABA. In the subsequent step, 8′-hydroxy ABA is spontaneously isomerized to phaseic acid (PA), which is further reduced to dihydrophaseic acid (DPA) by an unknown reductase (Gillard and Walton, 1976; Krochko et al., 1998; Cutler and Krochko, 1999). The genes encoding ABA 8′-hydroxylase were identified to be members of the subfamily of CYP707A (Kushiro et al., 2004; Saito et al., 2004). There are four members of CYP707A family in the Arabidopsis genome. The transcript levels of all four *CYP707A*s are induced by dehydration and exogenous ABA treatment (Kushiro et al., 2004; Saito et al., 2004). *CYP707A3* is induced strongly by rehydration after dehydration conditions. The *cyp707a3* mutant contains higher ABA levels than the wild type during dehydration and rehydration, suggesting that CYP707A3 plays a predominant role in ABA catabolism in these conditions (Umezawa et al., 2006). On the other hand, ABA is also inactivated by conjugation with Glc (Xu et al., 2002; Chiwocha et al., 2005; Lim et al., 2005; Priest et al., 2006). ABA-Glc ester (ABA-GE) is one of the predominant ABA conjugates and may function as a storage form of releasable ABA (Dietz et al., 2000). In Arabidopsis, ABA-GE is hydrolyzed in response to dehydration by β-glucosidase (AtBG1), leading to an increase in the active ABA pool (Lee et al., 2006).

ABA and its catabolites are transported through the phloem and xylem flow (Zeevaart and Boyer, 1984; Sauter et al., 2002). There are many reports concerning ABA synthesis in root and its possible transport from root to shoot (Davies et al., 2005). Recent studies have highlighted the importance of drought- and osmotic stress-induced ABA synthesis in shoots (Holbrook et al., 2002; Christmann et al., 2005, 2007). Grafting experiments using a tomato (*Lycopersicum esculentum*) ABA-deficient mutant showed that shoot genotype is the primary determinant of stomatal closure during soil drying (Holbrook et al., 2002). Christmann et al. reported that the induction of a reporter gene driven by an Arabidopsis ABA-inducible *ATHB6* promoter was observed in vascular tissues of shoots when the roots were subjected to osmotic stress (Christmann et al., 2005). Consistent with these findings, dehydration-induced AtNCED3 protein accumulation was observed in vascular parenchyma cells in shoots (Endo et al., 2008). These results indicated that the major tissues that supply dehydration-induced ABA pools are the vascular tissues in the shoot. Indeed, three de novo ABA biosynthesis enzymes, AtNCED3, short-chain dehydrogenase/reductase (ABA2), and abscisic aldehyde oxidase (AAO3) are localized in vascular tissues (Koiwai et al., 2004; Endo et al., 2008). Moreover, the expression of an ABA-inducible reporter gene is observed in vascular tissues after a short period of osmotic stress, followed by the reporter gene expression in guard cells (Christmann et al., 2005). These findings suggest that the vascular tissues provide a major ABA pool to regulate stomatal closure during dehydration. Nonetheless, the competence of the guard cells to regulate ABA metabolism in response to environmental signals remains unknown.

Stomatal aperture changes in response to humidity. This response is complex and is controlled by both ABA-dependent and ABA-independent mechanisms. Xie et al. (2006) reported that stomata of Arabidopsis ABA-deficient *aba2* mutants are defective in the rapid response (within minutes) to low humidity (Xie et al., 2006). In contrast, Arabidopsis *aba1* mutant and tomato *flacca* mutant reportedly change stomatal conductance in response to humidity (Bradford et al., 1983; Assmann et al., 2000). Humidity is also known to affect endogenous ABA levels. In spinach (*Spinacia oleracea*) and *Tradescantia virginiana*, ABA levels were lower in leaves grown in high-humidity conditions, compared with those grown in moderate humidity conditions (Zeevaart, 1974; Rezaei Nejad and van Meeteren, 2007, 2008). In contrast to recent progress in understanding the regulation of ABA biosynthesis, our knowledge of ABA catabolism in response to environmental cues is still limited. The aim of this study is to elucidate physiological roles of CYP707As in the humidity response in vegetative tissues by characterizing loss-of-function mutants in these genes. Our results indicate that CYP707A family members play differential roles in the systemic plant responses to changes in humidity.

RESULTS

CYP707A1 and CYP707A3 Are Involved in the Regulation of Transpiration in Turgid Plants

In Arabidopsis, there are four members of *CYP707A* genes that encode ABA 8′-hydroxylases (Kushiro et al., 2004; Saito et al., 2004). A previous study showed that the *CYP707A3* was induced predominantly among the *CYP707A* genes during dehydration and subsequent rehydration in plants. In addition, endogenous ABA levels in the *cyp707a3* mutant were found to be higher than those in the wild type, and *cyp707a3* mutants showed reduced transpiration compared to wild-type plants (Umezawa et al., 2006). This indicates that CYP707A3 is the major isoform for ABA 8′-hydroxylation in these conditions. However, it is still unclear whether other CYP707As contribute to the regulation of transpiration.

To examine the physiological role of other CYP707As in transpirational control, we first examined the rate of transpiration in *cyp707a* mutant plants in turgid conditions. Currently, allelic series of the *cyp707a1*, *cyp707a2*, and *cyp707a3* mutants, except for the *cyp707a4* mutant, are available from the Arabidopsis Biological Resource Center. A Leaf Porometer was used to measure stomatal conductance of these mutants and wild type. The stomatal conductance of *cyp707a1* mutants was significantly reduced compared

to that of wild type, and its value was similar to the *cyp707a3* mutants (Fig. 1A). In contrast, there was no difference in stomatal conductance between wild-type and *cyp707a2* mutants. Next, we examined the *cyp707a* double mutants. Stomatal conductance of *cyp707a1 cyp707a3* double mutant was reduced more strongly than that of each single mutant. On the other hand, transpiration of *cyp707a1 cyp707a2* and *cyp707a2 cyp707a3* double mutants was comparable with that of *cyp707a1* and *cyp707a3* mutants, respectively. These results suggest that CYP707A1 and CYP707A3, but not CYP707A2, are involved in the control of transpiration.

Leaf surface temperature is a useful indicator to detect differences in water loss from rosettes leaves (Merlot et al., 2002). We examined whether thermography would show if differences of leaf surface temperatures of the *cyp707a* mutants reflected the rates of transpirational water loss. Leaf surface temperature of *cyp707a1* and *cyp707a3* mutants was significantly higher than that of wild type (Fig. 1B). Moreover, leaf temperature of the *cyp707a1 cyp707a3* double mutant drastically increased compared to the parental single mutants. As shown in a typical thermal image (Fig. 1C), rosette leaves of the wild type were predominantly blue (low temperature area). On the other hand, rosette leaves of the *cyp707a1 cyp707a3* double mutant had drastically reduced blue color. These results support the notion that CYP707A1 and CYP707A3 regulate transpiration, which in turn regulate leaf surface temperature.

Regulation of ABA Biosynthesis and Catabolism in Response to High Humidity

We then examined whether high humidity affects endogenous ABA levels in Arabidopsis wild-type plants. High-humidity treatment was performed by transferring plants from normal humidity (relative humidity [RH] 60%) to high-humidity conditions (RH 90%) at 22°C. The ABA levels decreased by 40% at 30 min, and then by 80% at 1 h after initiation of the high-humidity treatment (Fig. 2A). The levels of PA and

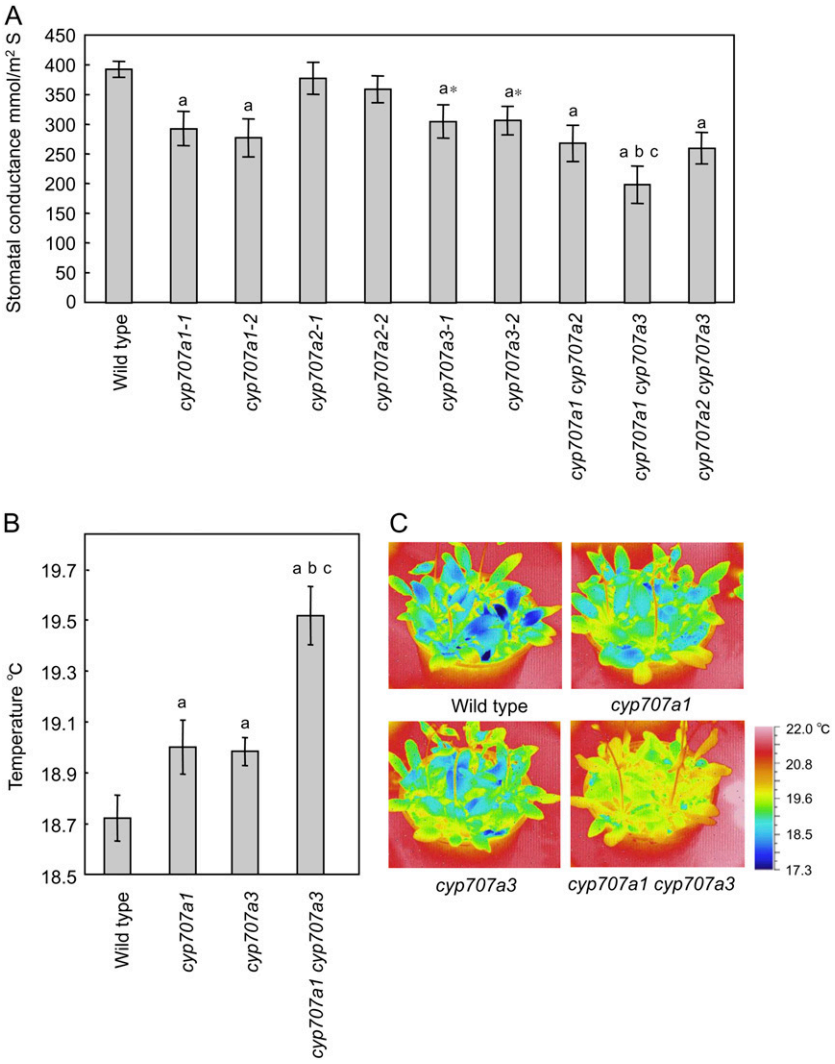

Figure 1. Stomatal conductance and leaf surface temperatures of the *cyp707a* mutants. A, Stomatal conductance of wild-type and *cyp707a* plants. Stomatal conductance was measured with the intact 4-week-old plants of wild type, *cyp707a* mutants under 22°C, RH 60% condition. At least six leaves were measured in each experiment and stomatal conductance is shown by an average with SE from four experiments. B and C, Leaf surface temperatures of nonstress plants under well-watered conditions. Plants were grown on soil under well-watered conditions (22°C, RH 75%) for 4 weeks. B, Leaf surface temperature in *cyp707a* mutant. At least four plants were used in each experiment. Leaf surface temperature is shown by an average with SE from seven experiments. C, Typical thermal images of rosette leaves in *cyp707a* mutants. A lowercase letter in A and B indicates a significant difference judged by Student's *t* test; a, b, and c indicate the significant difference ($P < 0.01$) relative to wild type, *cyp707a1-1* mutant, and *cyp707a3-1* mutant, respectively. An asterisk indicates the significant difference when less stringent cutoff value was used ($P < 0.05$).

DPA, catabolites of ABA 8′-hydroxylation pathway, increased, followed by the ABA reduction. The levels of these catabolites increased significantly within 10 min of humidity treatment and their levels remained at the high level until 30 min and decreased gradually thereafter (Fig. 2B). This suggests that high-humidity-induced ABA reduction occurs through ABA 8′-hydroxylation, not by alteration of ABA biosynthesis. Endogenous levels of other catabolites, such as ABA-GE and neoPA were much lower than PA and DPA levels. In addition, endogenous 7′-OH ABA could not be detected in this experiment (data not shown). These results suggest that, in response to high humidity, ABA is predominantly converted to PA and DPA. PA and DPA levels decline, indicating that DPA is further metabolized. Next, we examined the expression of *AtNCED3* and *CYP707A* genes during the high-humidity treatment by quantitative reverse transcription (QRT)-PCR. The levels of *AtNCED3* transcript were low and did not change significantly under the high-humidity treatment (Fig. 2C), suggesting that *AtNCED3* expression is not responsive to high humidity. As for the *CYP707A* genes, the *CYP707A3* transcript was the most abundant under normal humidity conditions. The transcript level of the *CYP707A3* increased immediately after onset of the high-humidity treatment, reached a maximum within 10 min, and decreased thereafter. Although *CYP707A1* transcript levels were relatively lower than those of *CYP707A3*, induction of *CYP707A1* was also observed during the high-humidity treatment. The *CYP707A1* transcript levels reached a maximum in 30 min, slower than the *CYP707A3* induction, and decreased thereafter. On the other hand, *CYP707A2* and *CYP707A4* transcript levels were much lower than *CYP707A1* and *CYP707A3* transcript levels and were not changed significantly during high-humidity treatment. These results suggest that high humidity reduces the ABA levels by the activation of *CYP707A1* and *CYP707A3* rather than the down-regulation of the *AtNCED3*.

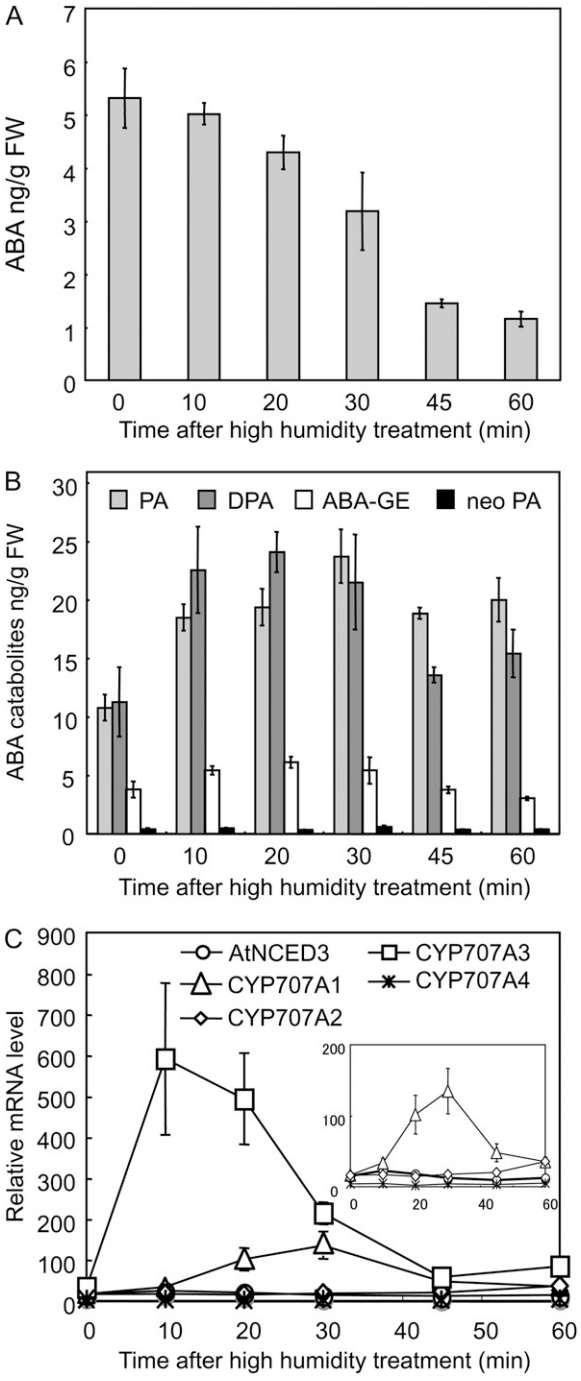

Figure 2. ABA biosynthesis and catabolism in response to high humidity. Changes of ABA (A) and its catabolite (B) levels after transferring to high-humidity treatment. C, Changes of *AtNCED3* and *CYP707A* transcript levels in response to high-humidity treatment. Four-old-week plants were transferred from RH 60% to 90%. Rosette leaves were collected every 10 min after the onset of the treatment. Experiments were performed three times and an average is shown with SE. FW, Fresh weight.

Physiological Roles of CYP707A1 and CYP707A3 in Response to High Humidity

To examine stomatal opening after transferring to high-humidity conditions, we measured stomatal apertures. Stomatal aperture in wild-type plants increased by approximately 25% under the high-humidity treatment (from RH 60% to 90% at 22°C) for 1 h (Fig. 3). We then compared stomatal apertures of *cyp707a* mutants to test if this stomatal opening could be attributed to the activation of *CYP707A1* and *CYP707A3*. Stomata of *cyp707a1* and *cyp707a3* mutants, but not *cyp707a2*, were more closed compared to that of wild type under normal conditions (Fig. 3). After 1 h of high-humidity treatment, the degree of stomatal opening in *cyp707a2* was similar to that in wild type, whereas stomatal opening of *cyp707a1* and *cyp707a3* was repressed compared to that of wild type. Next, we examined stomatal aperture in the *cyp707a* double mutants. The *cyp707a1 cyp707a3* double mutant exhibited more drastic stomatal closure than other mutants under normal conditions. In addition, stomatal

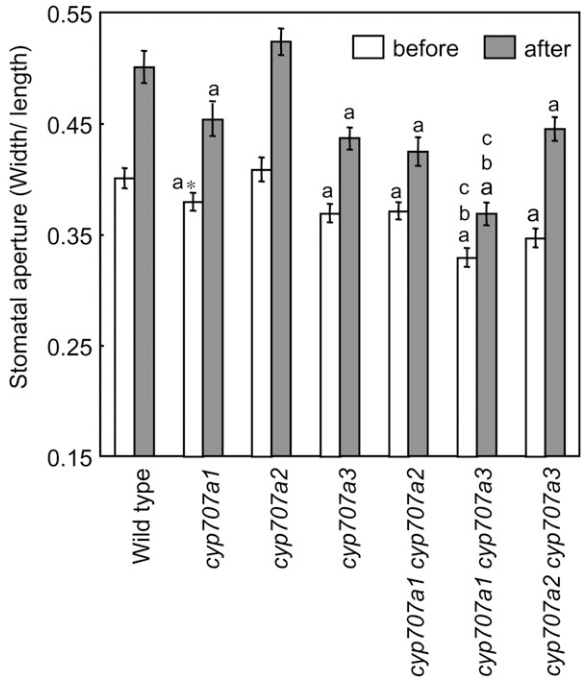

Figure 3. Stomatal aperture of the *cyp707a* mutants in response to high humidity. Stomatal aperture in *cyp707a* single and double mutants before and 1 h after transferring to high-humidity conditions. Four-week-old plants were transferred from RH 60% to 90% at 22°C. Images of stomatal aperture from intact leaves were obtained using SUMP method. Images were observed by light microscopy. Experiments were performed at least four times using rosette leaves from independent plants. An average ($n \geq 60$) is shown with SE. A lowercase letter indicates a significant difference judged by Student's *t* test. a, b, and c indicate the significant difference ($P < 0.01$) relative to wild type, *cyp707a1-1* mutant, and *cyp707a3-1* mutant, respectively. An asterisk indicates the significant difference when less stringent cutoff value was used ($P < 0.05$).

opening of *cyp707a1 cyp707a3* double mutant was strongly repressed under high-humidity conditions. On the other hand, *CYP707A2* mutation did not affect stomatal response to humidity in *cyp707a1 cyp707a2* and *cyp707a2 cyp707a3* double mutants relative to their parental single mutants, *cyp707a1* and *cyp707a3*, respectively. This result indicates that both *CYP707A1* and *CYP707A3* are important for stomatal opening under high-humidity treatment.

Next, we assessed whether the *cyp707a* mutations alter ABA levels under high-humidity conditions. Among single mutants, the levels of ABA in the *cyp707a3* mutant were highest after 1 h of the high-humidity treatment. The *cyp707a1* mutants had much lower ABA levels than the *cyp707a3* mutant, although ABA levels in the *cyp707a1* mutant were slightly higher than those of the wild type. The effect of the *cyp707a1* mutation was obvious when we analyzed the *cyp707a1 cyp707a3* double mutants. The *cyp707a1 cyp707a3* double mutant contained higher ABA levels than the parental single mutants under high-humidity conditions (Fig. 4A). *ATHB6* and *ABF3* are the known ABA-responsive genes (Christmann et al., 2005; Kang et al. 2002), and the transcript levels of these genes were analyzed in the high-humidity-treated plants. The relative levels of mRNA of *ATHB6* and *ABF3* were comparable to the ABA levels in shoots among the genotypes (Fig. 4B). These results indicate that shoot CYP707A3 plays a major role in regulating ABA levels, whereas CYP707A1 plays a minor role in regulating ABA levels in shoots.

Spatial Expression Patterns of the *CYP707A1* and *CYP707A3* in Response to High Humidity

To investigate the site of CYP707As expression in response to high humidity, the promoter of *CYP707A1* or *CYP707A3* was fused to a GUS gene and transgenic Arabidopsis plants were generated. Under normal humidity conditions, both *pCYP707A1*::*GUS* and *pCYP707A3*::*GUS* lines showed only faint GUS staining at the edge of the veins (Fig. 5, A and E). The *pCYP707A1*::*GUS* lines also showed weak GUS staining in guard cells at the leaf edges. Coincident with the transcript levels, high-humidity induction of the reporter gene was observed (Fig. 5, B and F). After 2 h of high-humidity treatment, GUS staining for *pCYP707A1*::*GUS* was observed in guard cells of rosette leaves (Fig. 5, C and D). In addition, weak but reproducible GUS staining of *pCYP707A1*::*GUS* was also in vascular tissues of rosettes leaves (Fig. 5C). On the other hand, strong GUS staining for *pCYP707A3*::*GUS* was observed in vascular tissues of high-humidity-treated plants (Fig. 5G). It is worth mentioning that GUS staining in guard cell was observed in *pCYP707A1*::*GUS*, but not in *pCYP707A3*::*GUS* (Fig. 5, D and H). These results indicate that the major sites of *CYP707A1* and *CYP707A3* expression in response to high humidity are the guard cells and vascular tissues, respectively.

CYP707A1 Is Essential for ABA Catabolism Inside Guard Cells

CYP707A1 and CYP707A3 are involved in stomatal opening in response to high humidity (Fig. 3), but only *CYP707A1* was expressed in guard cells (Fig. 5, D and H). To investigate the function of CYP707A in guard cells, we examined ABA-induced stomatal closure using epidermal peels. In this experiment, we used natural type of ABA [(+)-*S*-ABA] rather than racemic ABA, because (−)-*R*-ABA was found not to be a substrate of CYP707As (Kushiro et al., 2004; Saito et al., 2004), and its weak biological activity in guard cells but strong activity in other ABA-related processes (for review, see Zaharia et al., 2005). Among the *cyp707a* mutants, ABA-induced stomatal closure of the *cyp707a1* mutant was hypersensitive to (+)-*S*-ABA (Fig. 6, A and B). The stomata of the *cyp707a2* and *cyp707a3* mutants responded to exogenous (+)-*S*-ABA similarly to the wild type. These results indicate that CYP707A1 is involved in ABA catabolism inside

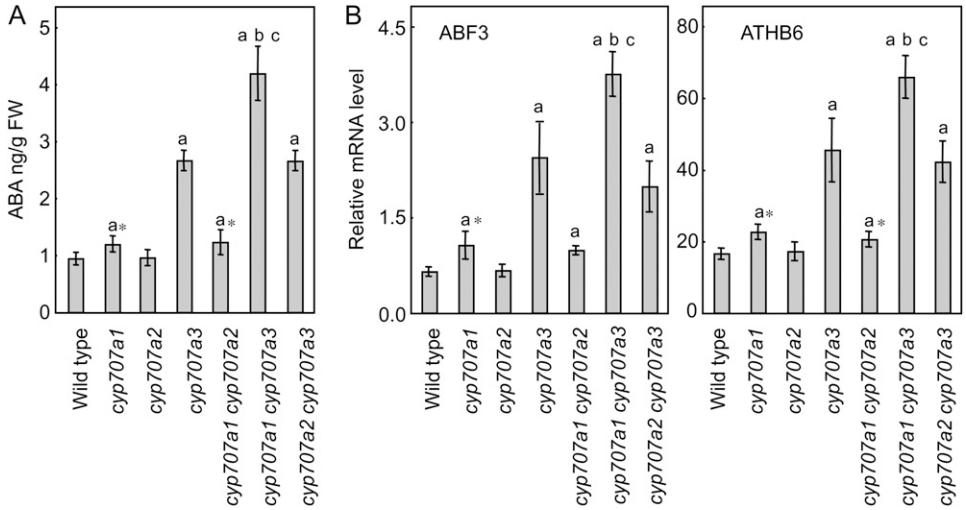

Figure 4. Phenotypic analysis of the *cyp707a* mutants in high-humidity conditions. A, ABA levels of the *cyp707a* mutants under high-humidity conditions for 1 h. B, Expression of ABA-responsive genes in *cyp707a* single and double mutants under high-humidity conditions for 1 h. *ABF3* and *ATHB6* expression in *cyp707a* mutants are shown in right and left graph, respectively. Experiments were performed at least four times and an average is shown with SE. A lowercase letter indicates a significant difference judged by Student's *t* test. a, b, and c indicate the significant difference ($P < 0.01$) relative to wild type, *cyp707a1-1* mutant, and *cyp707a3-1* mutant, respectively. An asterisk indicates the significant difference when less stringent cutoff value was used ($P < 0.05$).

guard cells, whereas CYP707A3 is likely to inactivate ABA outside the guard cells.

DISCUSSION

Regulation of ABA Metabolism in Response to High Humidity

Humidity can change rapidly (within minutes) in the natural environment. To date, there are a number of reports about the regulation of ABA metabolism in response to low humidity, specifically dehydration (Qin and Zeevaart, 1999; Iuchi et al., 2001; Tan et al., 2003; Lee et al., 2006; Endo et al., 2008). On the other hand, information of physiological roles of ABA metabolism and its regulatory mechanism in response to high humidity is limited. Previous reports described that endogenous ABA levels in leaves grown under high humidity was lower than under moderate humidity (Zeevaart, 1974; Rezaei Nejad and van Meeteren, 2007, 2008). However, these previous reports studied the effect of the long term under high humidity. In addition, long-term treatment under high humidity

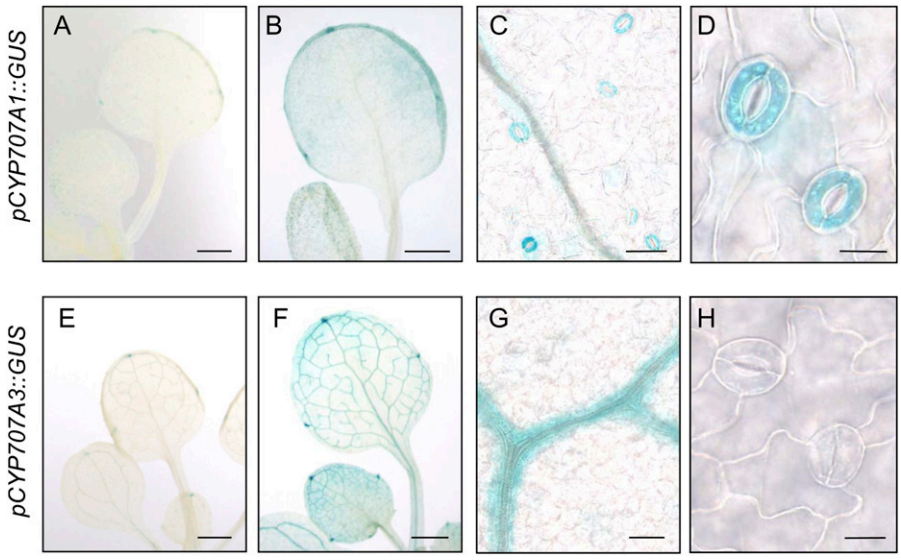

Figure 5. Spatial expression patterns of *CYP707A1* and *CYP707A3* in response to high humidity. Expression of promoter *CYP707A1*::*GUS* before (A) and after transferring to high humidity conditions (B–D). Expression of promoter *CYP707A3*::*GUS* before (E) and after transferring to high-humidity conditions (F–H). Approximately 3-week-old plants were grown on soil in pots under RH 60% at 22°C condition. High-humidity treatment (from RH 60% to 90% at 22°C) was performed for 2 h. Bars = 1 mm for A, B, E, and F; 50 μm for C and G; 10 μm for D and H.

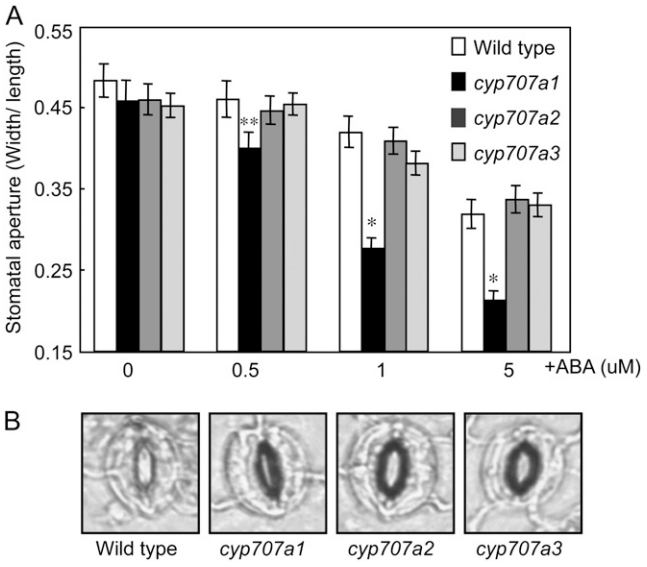

Figure 6. The effect of exogenous ABA treatment on the stomatal apertures of *cyp707a* mutants in epidermal peels. A, ABA sensitivity of epidermal peels in *cyp707a* mutants. B, Typical images of ABA-induced stomatal closure on 1 μM (+)-*S*-ABA. Epidermal peels were incubated with opening buffer under light condition for 2 h to open stomata. The opened epidermal peels were incubated with (+)-*S*-ABA solution for 2 h. Experiments were performed using at least four independent plants' rosettes leaves. An average ($n \geq 60$) is shown with SE. An asterisk indicates a significant difference relative to wild type by Student's *t* test (**$P < 0.05$ and *$P < 0.01$).

causes changes of stomatal properties such as stomatal development and response to ABA (Spence et al., 1986; Xia, 1994; Rezaei Nejad and van Meeteren, 2007, 2008). In contrast, our present study revealed that the endogenous ABA levels decreased after 20 min of high-humidity treatments (Fig. 2A). Concomitant with the reduction of ABA levels, stomata were opened in response to high-humidity treatment (Fig. 3). Expression and phenotypic analyses indicate that ABA levels were reduced primarily by activation of ABA catabolism (Fig. 2B). Rapid activation of ABA 8′-hydroxylation pathway in response to high humidity is important for the reduction of ABA levels. Our analysis revealed that CYP707A3 plays a major role, whereas CYP707A1 has a relatively minor role, in regulating ABA levels in shoots in response to high humidity (Figs. 2C and 4). It is interesting to note that plants' response to dehydration is triggered by the rapid induction of the *AtNCED3*. Dehydration induction of the *CYP707A* genes is slow, and is involved in the fine tuning of ABA levels (Kushiro et al., 2004; Umezawa et al., 2006). This study indicates that the expression of the *CYP707A3* and *CYP707A1*, but not *AtNCED3*, responded rapidly to high humidity. The defects in the *cyp707a3* and *cyp707a1* mutants support the notion that ABA 8′-hydroxylation is the principal pathway for ABA metabolism to respond to high humidity. The metabolic balance between ABA biosynthesis and catabolism is differentially regulated under different environmental cues to control the hormone levels properly.

ABA Catabolism Inside Guard Cells

Guard cells are one of the well-known target cells for the ABA action. Although a number of genes have been identified as ABA-signaling components in guard cells, ABA metabolism inside guard cells has not been characterized (Schroeder et al., 2001; Nilson and Assmann, 2007; Wasilewska et al., 2008). It has been reported that the expression of AAO3, last step enzyme for ABA biosynthesis, is detected in guard cells of dehydrated Arabidopsis plants (Koiwai et al., 2004). Moreover, a transient expression of *AtNCED3* or *AAO3* fused to a GFP in *Vicia faba* guard cells led to stomatal closure, suggesting that guard cell itself is competent to synthesize ABA (Melhorn et al., 2008). Endo et al. (2008) reported that drought-induced accumulation of mRNA and protein of *AtNCED3* is observed in the vascular tissues, but not in guard cells. It remains unclear whether the guard cell itself contributes to the change in ABA levels in response to environmental signals. In this study, our results showed that *CYP707A1* was induced in guard cells in response to high humidity (Fig. 5), and the *cyp707a1* mutant, but not *cyp707a3* mutant, showed the ABA-hypersensitive stomatal closure when applied exogenous ABA was applied to epidermal peels (Fig. 6). These results indicate that CYP707A1 has an essential role for ABA catabolism in the guard cell. It is also interesting to note that ABA responsiveness in stomata is reportedly shown to be regulated by environmental conditions as light intensity and quality, atmospheric concentration of CO_2, and by phytohormones other than ABA (for review, see Zeiger, 1983). Some of known ABA-signaling components possibly regulate ABA catabolism in guard cells rather than signaling pathways. Elucidation of cross talk between CYP707A1 and these factors will be the next challenge for fully understanding stomatal movement.

ABA Metabolism in Vegetative Tissues and Its Involvement in Systemic ABA Responses

ABA is a mobile signal and activates various adaptive responses in stressed conditions (Davies et al., 2005; Schachtman and Goodger, 2008). Recent work reported that osmotic- and dehydration-inducible gene expression occurs in a systemic manner (Christmann et al., 2005; Endo et al., 2008). Recent advances in the identification of key regulatory genes enable linkage of the metabolic and signaling pathways of ABA action. Nevertheless, it remains unclear at the molecular levels how ABA is transported and how it acts in particular tissues/cell types. Identification of the cell type that synthesizes and inactivates ABA provides an insight into the nature of ABA transport and its dynamic mode of action in a plant system.

Recent studies indicated that major ABA pools are supplied mainly by vascular tissues in dehydrated plants. De novo ABA biosynthesis enzymes (*AtNCED3*, *ABA2*, and *AAO3*) are abundantly localized in vascular tissues of shoots during dehydration (Endo et al., 2008). In addition, the early expression of *ATHB6*, an ABA-inducible gene, is observed in vascular tissues of shoots (Christmann et al., 2005). Interestingly, the expression of *ATHB6* is also observed in guard cell after prolonged osmotic stress (Christmann et al., 2005). It is likely that vascular tissues of shoots are the main site of ABA biosynthesis, and ABA is transported to other cells, including guard cells. This study showed that the *CYP707A3*, the most abundant member of the *CYP707A* genes, was predominantly expressed in vascular tissues in response to high humidity (Fig. 5, F and G). The prominent effect of the *CYP707A3* in transpiration is likely due to inactivation of the major ABA pool in the vascular tissues. We propose that the vascular tissue is the main site for regulating the levels of major ABA pool for mobile ABA. It is also worth mentioning that the *pCYP707A1*::*GUS* lines showed an intense staining in guard cells (Fig. 5D), but GUS staining was also observed reproducibly in vascular tissues (Fig. 5C). Therefore, it is likely that the *CYP707A1* also play a minor role in regulating bulk ABA levels in vascular tissues.

Our present study suggests that ABA is transferred from vascular tissues to guard cell for controlling stomatal movement. The ABA movement from xylem strands to the guard cells takes place likely through the apoplast, because of the absence of plasmodesmata between guard cells and neighboring cells (Plaeviz and Hepler, 1985). In the future, as microtracer techniques or chemical probe methods become available, it would be important to determine the actual movement of ABA, because ABA levels are determined by not only the balance between synthesis and catabolism, but also by mobilization (Seo and Koshiba, 2002; Davies et al., 2005; Schachtman and Goodger, 2008).

In conclusion, we have characterized the physiological role of ABA 8′-hydroxylase by expression analysis of *CYP707A*s and phenotypic analysis of *cyp707a* single and double mutants in response to high humidity. Our result indicates that CYP707A3 plays a role in regulating the levels of major ABA pool provided by the vascular tissues, whereas CYP707A1 is essential for ABA catabolism inside guard cells. ABA catabolism in both vascular tissues and guard cells is indispensable for controlling stomatal movement.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild-type and all mutant plants were Columbia accession of Arabidopsis (*Arabidopsis thaliana*). The *cyp707a* single and double mutants used in this study were described previously (Kushiro et al., 2004; Okamoto et al., 2006). In all experiments except for Figure 1A, *cyp707a* single mutants used were *cyp707a1-1*, *cyp707a2-1*, and *cyp707a3-1*, whereas *cyp707a1-1 cyp707a2-1*, *cyp707a1-1 cyp707a3-1*, and *cyp707a2-1 cyp707a3-1* double mutants were used in this study. Plants were grown for 2 weeks on 1% agar plates containing one-half Murashige and Skoog and 0.5% Suc, transferred to soil in 200-mL pots, and grown under 22°C, RH 60% condition. Soil-grown plants were watered every 3 d from the bottom. For high-humidity treatment, 4-week-old plants were transferred from RH 60% to 90% under 22°C by using two humidity-controlled growth chambers (model LPH-350S, NK system, Nihonika Co.).

Measurement of Stomatal Conductance

Stomatal conductance was measured for intact 4-week-old plants of wild type, *cyp707a* single, and double mutants using Leaf Porometer (Decagon Devices, Inc.) under 22°C, RH 60% condition into growth chamber (model MCP-201CP system, SANYO). Experiments were repeated four times and at least six leaves were measured for each experiment.

Thermal Imaging

Thermal images of nonstress plants were obtained using thermal video system TVS-8500 (Nippon Avionics Co., Ltd.). Temperature range of thermal image was restricted to between 22.0°C and 17.3°C. Plants used in this experiment were grown on soil pot under well-watered condition (22°C, RH 75%) for 4 weeks. Growth chamber used model LHP-350S (NK system, Nihon-ika Co.). Images and leaf surface temperature of plants were analyzed by PE Professional software (Nippon Avionics Co., Ltd.). Experiments were repeated seven times and at least four plants were used for each experiment.

QRT-PCR

Four-week-old plants were grown on soil in pots at 22°C, RH 60%. High-humidity treatment was performed at 22°C, RH 90% condition. Total RNA was isolated using TRIZOL Reagent (Invitrogen) according to manufacturer's protocol. cDNA synthesized from 2 μg of total RNA using QuantiTec reverse transcription kit (QIAGEN) as described previously (Okamoto et al., 2006). QRT-PCR using Taq-Man probe was performed as described previously (Kushiro et al., 2004). The sequences of primers and Taq-Man probe for *AtNCED3* and *CYP707A1-A4* were used as described in Kushiro et al. (2004). The following primer sets were used: for *ABF3*, forward primer (5′-TGGTGGGAGTTTGCAGAGAC-3′) and reverse primer (5′-TCCCTAACCACACCAGCCCT-3′); for *ATHB6*, forward primer (5′-GGGCTTGTCGGAGAAGAAGAGAA-3′) and reverse primer (5′-CTTCCACCGAGCACGACGGT-3′). For normalization of data, 18S rRNA was used as an internal standard.

Determination of ABA and Its Catabolites

For determination of ABA and its catabolites, approximately 300 mg of plants materials (fresh weight) was used. Deuterium-labeled d_6-ABA purchased from ICON SERVICES and deuterium-labeled d_3-PA, d_3-DPA, d_5-ABA-GE, d_3-neoPA, and d_4-7′OH-ABA (Priest et al., 2006) were used as internal standards. After adding 1 ng of internal standards, extraction and purification of ABA and its catabolites were performed as described previously (Saika et al., 2007). The hormone and metabolites were quantified by liquid chromatography (LC)- (ACQUITY UPLC system, Waters) tandem mass spectrometry (MS/MS; Q-Tof premier, Micromass) system. The LC conditions were same as described previously (Saika et al., 2007). The retention times of the compounds were 9.1 min (DPA and d_3-DPA), 15.5 min (PA and d_3-PA), 16.5 min (ABA-GE and d_5-ABA-GE), 19.2 min (7′OH-ABA and d_4-7′OH-ABA), 20.7 min (neoPA and d_3-neoPA), 23.3 min (d_6-ABA), and 23.6 min (ABA). MS/MS conditions were as follows: Capillary (kV) = 2.6, source temperature (°C) = 80, desolvation temperature (°C) = 400, cone gas flow (l/h) = 0, desolvation gas flow (l/h) = 500, collision energy (eV) = 8.0 (ABA and d_6-ABA), 12.0 (PA and d_3-PA, 7′OH-ABA and d_4-7′OH-ABA, neoPA and d_3-neoPA, ABA-GE and d_5-ABA-GE), 18.0 (DPA, d_3-DPA). MS/MS transitions (m/z) were: 263/153 (ABA), 269/159 (d_6-ABA), 279/139 (PA), 282/142 (d_3-PA), 281/171 (DPA), 284/174 (d_3-DPA), 425/263 (ABA-GE), 430/268 (d_5-ABA-GE), 217/279 (7′OH-ABA), 221/283 (d_4-7′OH-ABA), 279/205 (neoPA), and 282/208 (d_3-neoPA). Data analysis was performed using the spectrometer software (MassLynx v. 4.1, Micromass).

Measurement of Stomatal Aperture

For analysis of intact plants, images of stomatal apertures were obtained by Suzuki's Universal Micro-Printing (SUMP) method using SUMP liquid and

SUMP plate B (SUMP Laboratory) as described previously (Tanaka et al., 2005). The copied SUMP images were observed by light microscopy (model BX60, Olympus). For analysis of epidermal peels, abaxial epidermis was peeled from rosettes leaves of 4-week-old plants 6 h after the beginning of morning in 16/8-h light/dark cycles. Epidermal peels were floated on opening buffer (5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES, pH 6.15) and incubated under light condition for 2 h to open the stomata. Subsequently, the epidermal peels with preopened stomata were transferred to the same buffer containing (+)-*S*-ABA (Toray Co., Ltd.) and incubated for 2 h to close the stomata. Stomatal image was obtained by light microscopy and the height and width of stomatal aperture were measured with ImageJ 1.37v software (National Institutes of Health).

Generation of Transgenic Plants

For reporter gene analysis, a promoter fragment (for *CYP707A1*, 2,244 bp; for *CYP707A3*, 2,776 bp) of the translational start of each *CYP707A* was amplified by PCR and cloned into pENTR/D-TOPO vector (Invitrogen). The following primer sets were used: for *CYP707A1*, 5′-*CACC*TATTTTTC-TCCGCTCCGCCAAGTCAATTGA-3′ and 5′-TTTTGTTTTTGTTCTTTGATC-AGAAAAAAACCCACTTCG-3′ for *CYP707A3*, 5′-*CACC*CACATAAGCGG-GTTCCCTTACT-3′ and 5′-TATCTTCGTCTTTTAAACAATGGGAATTC-GAAG-3′. The adaptor sites of pENTR/D-TOPO are shown in italic. After nucleotide sequences were checked, the cloned cDNAs were cloned into the binary vector, pGWB3, with a recombination cassette for the expression of GUS-fused protein (Nakagawa et al., 2007). The resulting plasmids were electroporated into *Agrobacterium* strain GV3101, which was used to transform wild-type Arabidopsis accession Columbia plants by floral-dipping method (Desfeux et al., 2000). Transgenic plants were obtained by selection with kanamycin and hygromycin. GUS staining was performed as described previously (Tan et al., 2003). Approximately 20 T_2-independent transgenic lines were analyzed. Detail analysis was performed using at least between five and 10 T_3 independent transgenic lines.

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