CYP707A1 and CYP707A2, Which Encode Abscisic Acid 8′-Hydroxylases, Are Indispensable for Proper Control of Seed Dormancy and Germination in Arabidopsis[1]

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Endogenous abscisic acid (ABA) levels are regulated by both biosynthesis and catabolism of the hormone. ABA 8′-hydroxylase is considered to be the key catabolic enzyme in many physiological processes. We have previously identified that four members of the Arabidopsis (*Arabidopsis thaliana*) CYP707A gene family (*CYP707A1* to *CYP707A4*) encode ABA 8′-hydroxylases, and that the *cyp707a2* mutants showed an increase in ABA levels in dry and imbibed seeds. In this study, we showed that the *cyp707a1* mutant accumulated ABA to higher levels in dry seeds than the *cyp707a2* mutant. Expression analysis showed that the *CYP707A1* was expressed predominantly during mid-maturation and was down-regulated during late-maturation. Concomitantly, the *CYP707A2* transcript levels increased from late-maturation to mature dry seed. Phenotypic analysis of single and double *cyp707a* mutants indicates that the CYP707A1 is important for reducing ABA levels during mid-maturation. On the other hand, CYP707A2 is responsible for the regulation of ABA levels from late-maturation to germination. Moreover, *CYP707A1* and *CYP707A3* were also shown to be involved in postgermination growth. Spatial expression analysis suggests that *CYP707A1* was expressed predominantly in embryo during mid-maturation, whereas *CYP707A2* expression was detected in both embryo and endosperm from late-maturation to germination. Our results demonstrate that each CYP707A gene plays a distinct role during seed development and postgermination growth.

The phytohormone abscisic acid (ABA) plays an important role in a number of physiological processes such as seed maturation, seed dormancy, and adaptive responses to abiotic stress (Nambara and Marion-Poll, 2005). ABA is also involved in the regulation of growth and development, including shoot elongation, morphogenesis of submerged plants (Hoffmann-Benning and Kende, 1992; Kuwabara et al., 2003), and root growth maintenance (Sharp and LeNoble, 2002). Among numerous physiological functions of ABA, regulation of seed dormancy and plant responses to osmotic stress have been well documented in various plant species because ABA-deficient mutants commonly show defects in these processes (McCarty, 1995). In addition, progression of these events is reportedly associated with a change in endogenous ABA levels, which are con-

trolled by the precise balance between biosynthesis and catabolism rates of the hormone (Zeevaart, 1980).

Endogenous ABA levels change drastically during seed development, germination, and postgermination growth in response to developmental and environmental cues. Biphasic ABA accumulation has been reported to occur during Arabidopsis (*Arabidopsis thaliana*) seed development (Karssen et al., 1983). The ABA accumulated during mid-maturation is synthesized in both zygotic and maternal tissues; thus, the latter one is most likely transported from the mother plant. The maternal ABA is involved in the inhibition of precocious germination and in processes of seed maturation (Karssen et al., 1983; Koornneef et al., 1989; Raz et al., 2001). The ABA accumulated during late-maturation is derived from the zygotic tissues and is essential for the induction and maintenance of seed dormancy. On the other hand, the ABA accumulated in dry seed decreases rapidly after seed imbibition. After this reduction, endogenous ABA in imbibed seed is maintained at a given level that is correlated with the germination potential of the seed. ABA is also known to inhibit seedling growth after seed germination (Lopez-Molina et al., 2001). Recent molecular genetic analyses indicate that five members of the Arabidopsis 9-cis-epoxycarotenoid dioxygenase (*AtNCED*) family play distinct roles in the regulation of ABA biosynthesis during seed development and germination (Tan et al., 2003; Seo et al., 2004). AtNCED6 and AtNCED9 play a

major role in ABA biosynthesis during seed development and germination (Lefebvre et al., 2006). On the other hand, AtNCED3 is involved in germination under hyperosmotic conditions (Ruggiero et al., 2004). In spite of recent advances in the study of ABA biosynthesis, the regulation of ABA catabolism genes during seed development is still poorly understood.

ABA is catabolized via several pathways in plants. The committed steps in ABA catabolism are categorized into two types of reactions: hydroxylation and conjugation (Nambara and Marion-Poll, 2005). ABA is hydroxylated at positions C-7' and C-8' in many plant species, and recently an ABA 9'-hydroxylation pathway has been identified in several plants (Zhou et al., 2004). Among the catabolic pathways, the ABA 8'-hydroxylation pathway is thought to be predominant in many physiological processes. 8'-Hydroxy ABA is spontaneously isomerized to phaseic acid (PA) and is then further reduced to dihydrophaseic acid (DPA). ABA 8'-hydroxylation is catalyzed by a cytochrome P450 monooxygenase (Gillard and Walton, 1976; Krochko et al., 1998). Recently, it has been revealed that four members of the *CYP707A* family in Arabidopsis encode ABA 8'-hydroxylases (Kushiro et al., 2004; Saito et al., 2004). The transcript levels of all four *CYP707As* are induced by abiotic stress, dehydration, and exogenous ABA treatment (Kushiro et al., 2004; Saito et al., 2004). In contrast, the *CYP707A2* transcripts accumulate predominantly in the dry seed and this gene is up-regulated immediately after seed imbibition (Kushiro et al., 2004). Consistent with this expression pattern, the *cyp707a2* mutant overaccumulates ABA in dry and imbibed seeds and the mutant seed exhibits enhanced dormancy compared with its parent Columbia wild-type seed, which displays only weak dormancy (Kushiro et al., 2004). These results demonstrate that CYP707A2 plays a key role in the rapid decrease in ABA levels prior to seed germination. In contrast, physiological functions of other CYP707As remained unknown.

The aim of this study is to elucidate physiological functions of CYP707As during seed development and germination by characterizing loss-of-function mutants in these genes. Differential overaccumulation of ABA in each *cyp707a* mutant highlights a distinct role for each CYP707A associated with seed development and germination. Moreover, ABA overaccumulation in the *cyp707a* single and double mutants indicates that the ABA 8'-hydroxylation pathway plays an essential role in the regulation of endogenous ABA levels during seed development and germination.

RESULTS

CYP707A1 Is Involved in the Regulation of Endogenous ABA Levels and Dormancy in Seed

In Arabidopsis, there are four members of the *CYP707A* gene family that encode ABA 8'-hydroxylases. Our previous study has shown that *CYP707A2* mRNAs predominantly accumulate in dry seeds and that this

gene is up-regulated immediately after imbibition. The *cyp707a2* mutant seeds exhibit enhanced dormancy than the wild type (Kushiro et al., 2004). We have also shown that transcript levels of *CYP707A4* remain very low during seed germination, whereas *CYP707A1* and *CYP707A3* mRNA levels increase when the seed starts to germinate. However, the physiological function of CYP707A1 and CYP707A3 in seed has not been examined extensively in our previous study.

To determine the physiological roles of CYP707A1 and CYP707A3 in seed, we first isolated two *cyp707a1* mutants from SALK and Wisconsin T-DNA-tagged lines, designated *cyp707a1-1* and *cyp707a1-2*, respectively (Fig. 1A). Isolation of *cyp707a2* and *cyp707a3* mutants was reported previously (Kushiro et al., 2004). Endogenous ABA levels in dry seeds of the *cyp707a1* mutants
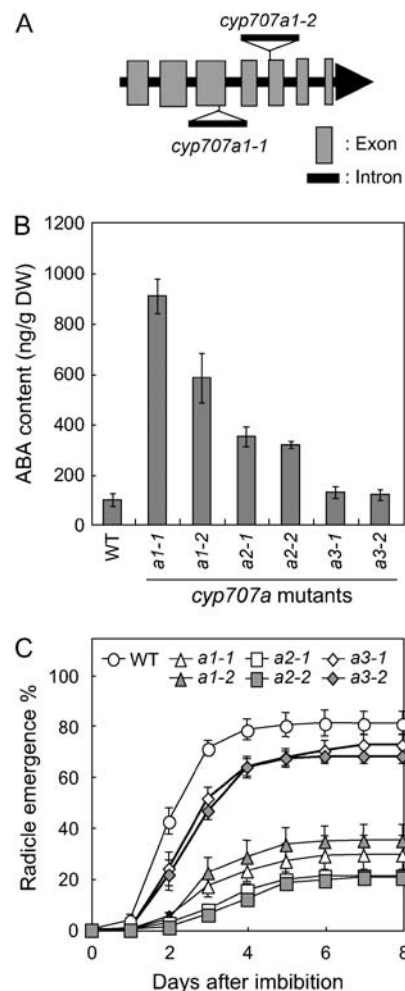

Figure 1. Phenotypic analysis of the *cyp707a* mutant seeds. A, Genomic structures of the *cyp707a1-1* (SALK_069127) and *cyp707a1-2* (WiscDsLox393-396J9) mutants. The *cyp707a1-1* and *cyp707a1-2* mutants harbor the T-DNA at the third and fifth exons, respectively. B, Dry seed ABA levels in the *cyp707a* mutants. Experiments were performed three times using independent seed batches and the averages are shown with SEs. DW, Dry weight. C, Dormancy of the freshly harvested seeds in *cyp707a* mutants. Experiments were performed five times using independent seed batches and the averages are shown with SEs.

were quantified to compare with those of wild-type and other *cyp707a* seeds harvested at the same time. We had expected that ABA levels in the *cyp707a1* dry seeds were comparable to those in the wild type because *CYP707A1* mRNA levels were low in dry seeds (Kushiro et al., 2004). Surprisingly, however, dry seeds for *cyp707a1-1* and *cyp707a1-2* mutants accumulated ABA to 10- and 7-fold higher levels than wild type, respectively (Fig. 1B). Endogenous ABA levels in *cyp707a1* dry seeds were much higher than that in the *cyp707a2* mutant, which also overaccumulates ABA in dry seeds as previously reported (Kushiro et al., 2004). In contrast, there was no difference in ABA levels between dry seeds of *cyp707a3* mutants and wild type. To examine whether *cyp707a1* mutant seeds exhibit dormancy, we carried out a germination test using freshly harvested seeds. The *cyp707a1-1* and *cyp707a1-2* mutant seeds exhibited a significant reduction in the percentage of germination unless seeds were stratified (Fig. 1C), suggesting that an increase in the ABA levels in the *cyp707a1* mutant affects degree of seed dormancy. Interestingly, dormancy in the *cyp707a1* mutant was weaker than in *cyp707a2* mutant that has lower amounts of ABA than the *cyp707a1* dry seeds. The *cyp707a3* mutant seeds exhibited slight

delayed germination compared to wild type. This result is different from our previous result, in which dormancy in the *cyp707a3* mutant is similar to wild type (Kushiro et al., 2004). This divergence in the results may be attributed to the subtle phenotype in *cyp707a3* seeds and the different experimental condition (i.e. paper filters moistened with water [Kushiro et al., 2004] versus agar plates supplemented with 0.5% Suc and 0.5× Murashige and Skoog salts [this work]).

Expression and Phenotypic Analyses of *CYP707As* during Seed Development

A recent study has indicated that the endogenous ABA levels in dry seed were affected by the ABA biosynthesis capacity during seed development (Lefebvre et al., 2006). We asked whether ABA overaccumulation in *cyp707a1* dry seeds is attributed to a defect in catabolism during seed development. To examine ABA biosynthesis and catabolism during seed development, we first measured endogenous levels of ABA, PA, and DPA in developing siliques (Fig. 2A). The levels of ABA in immature siliques reached a maximal level at 10 d after flowering (DAF). After this time, the
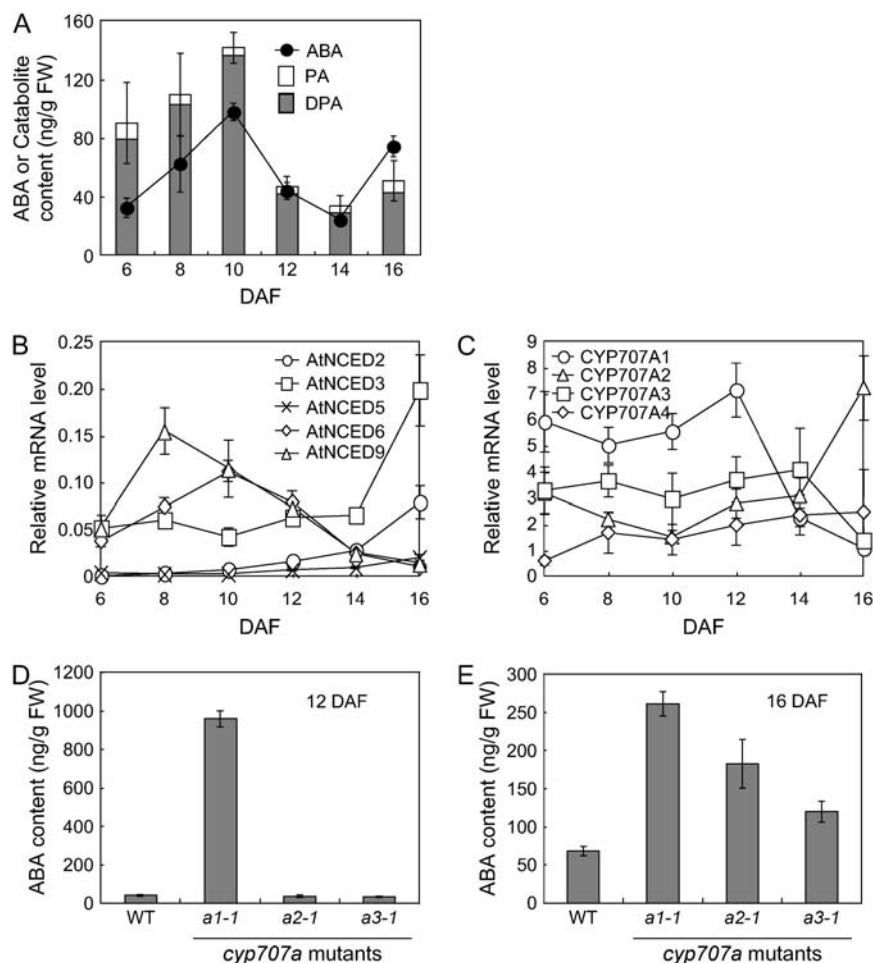

Figure 2. ABA biosynthesis and catabolism during seed development. A, Quantification of endogenous ABA, PA, and DPA levels in siliques during seed development. Experiments were performed three times using independent seed batches and the averages are shown with SES. FW, Fresh weight. B and C, Changes in the transcript levels of *AtNCEDs* and *CYP707As* in siliques determined by QRT-PCR. Experiments were performed four times and the averages are shown with SES. D and E, Quantification of endogenous ABA in siliques in *cyp707a* mutants 12 DAF (D) and 16 DAF (E). Experiments were performed three times and the averages are shown with SES.

ABA level decreased steadily until 14 DAF, and increased again at 16 DAF. This biphasic pattern is consistent with the previous observation (Gazzarrini et al., 2004). The levels of PA were maintained low throughout seed development, but the levels of DPA, the reduced form of PA, were high during seed development. This indicates that the ABA 8′-hydroxylation pathway is active during Arabidopsis seed development. Next, we examined the expression of the *AtNCED* and *CYP707A* genes in immature siliques during seed development by quantitative reverse transcription (QRT)-PCR. *AtNCED6* and *AtNCED9* transcripts were abundant at 8 and 10 DAF (Fig. 2B), as previously described (Tan et al., 2003; Lefebvre et al., 2006). An increase in the *AtNCED3* and *AtNCED2* transcripts after 14 DAF was predominantly detected in the silique envelopes rather than in the seed (Fig. 2B; data not shown). The *CYP707A1* transcripts were the most abundant among four *CYP707A*s until 12 DAF and decreased thereafter (Fig. 2C). Concomitant with the decrease in the *CYP707A1* transcript level, *CYP707A2* expression was induced at 16 DAF (Fig. 2C). These results indicate that ABA 8′-hydroxylation actively occurring during seed development is catalyzed essentially by two CYP707As, CYP707A1 at the mid-maturation stage and CYP707A2 at the late-maturation stage. To assess whether these expression patterns reflect physiological functions of each CYP707A, we measured ABA levels in immature siliques of the *cyp707a* mutants. The ABA levels in the *cyp707a1-1* mutant siliques at 12 DAF were 20-fold higher compared with those in wild type (Fig. 2D). The ABA levels in the *cyp707a1-1* siliques at 16 DAF were decreased compared with levels at 12 DAF, but were still higher than in the wild-type siliques at 16 DAF (Fig. 2, D and E). On the other hand, the ABA levels in *cyp707a2-1* and *cyp707a3-1* mutant siliques at 12 DAF were similar to those in wild type, but, at 16 DAF, the ABA levels in the *cyp707a2-1* mutant were higher than in wild type (Fig. 2, D and E). The *cyp707a3-1* mutant at 16 DAF also displayed slightly higher ABA levels than wild type, but this increase of ABA levels was much lower compared to what we measured in the *cyp707a1-1* and *cyp707a2-1* mutants.

Localization of *CYP707A* Expression during Seed Development

In developing seed, ABA is synthesized by both maternal and embryonic tissues (Groot and Karssen, 1992). A recent study has indicated that several *AtNCED* genes showed distinct tissue-specific expression patterns during seed development (Tan et al., 2003; Lefebvre et al., 2006). On the other hand, the expression site of *CYP707A* during seed development has not been determined.

To determine the site of *CYP707A* expression in siliques, QRT-PCR was performed on dissected embryo and endosperm/testa fractions at 10 and 15 DAF. We first assessed the purity of our fractions by analyzing

transcript levels of the *PROTODERMAL FACTOR1* gene (*PDF1*) and the extensin-like gene (*AtEPR1*), which are embryo- and endosperm-specific markers, respectively (Abe et al., 1999; Dubreucq et al., 2000; Penfield et al., 2004). The *PDF1* transcript was detected most abundantly in the embryo fraction, whereas *AtEPR1* mRNA was only detected in the testa/endosperm fraction, suggesting that these were not cross contaminated (Fig. 3A). Transcripts of *CYP707A1* were most abundant in the embryo at 10 DAF and were also detected in the testa/endosperm fraction and in the silique envelopes (Fig. 3B). The expression pattern of the *CYP707A3* was similar to that of *CYP707A1*, but the relative level of *CYP707A3* mRNA was lower than that of *CYP707A1* at 10 DAF. On the other hand, *CYP707A4* transcripts were detected mainly in the silique envelopes, but not in the embryo and the testa/endosperm fractions (Fig. 3B). At 15 DAF, *CYP707A2* was predominantly expressed in embryos, in contrast to other CYP707As, and was also highly expressed in the endosperm/testa fraction (Fig. 3C). *CYP707A4* was specifically expressed in the silique envelopes, but not in the seed at 15 DAF.

To analyze tissue-specific expression of *CYP707A1* and *CYP707A2*, we performed in situ hybridization. *CYP707A1* mRNA was detected in the vascular tissue of the 10-DAF embryo (Fig. 3D). In contrast, *CYP707A2* mRNA was detected mainly in the endosperm and also in the vascular tissue of the 15-DAF embryo (Fig. 3E). These observations are consistent with the data obtained by QRT-PCR (Fig. 3, B and C).

Analysis of Seed Germination in *cyp707a* Double Mutants

To investigate the role of ABA 8′-hydroxylase during seed development and germination in more detail, we constructed and analyzed *cyp707a* double mutants. ABA levels in dry seeds of *cyp707a1-1 cyp707a2-1* and *cyp707a1-1 cyp707a3-1* double mutants were 50- and 20-fold higher compared with those in wild type, respectively (Fig. 4A). In contrast, ABA levels in dry seeds of the *cyp707a2-1 cyp707a3-1* double mutant were 5-fold higher than in wild type and were comparable to that in the *cyp707a2-1* single mutant.

To examine whether the endogenous ABA levels in dry seeds correlate with the degree of seed dormancy, we performed a germination test using wild type and *cyp707a* single and double mutants. Because freshly harvested double-mutant seeds did not germinate without stratification or GA application, we could not distinguish differences in the degree of dormancy among *cyp707a* double mutants (data not shown). Therefore, seeds after ripened for 1 week were used for the germination test. Among these double mutants, the *cyp707a1-1 cyp707a2-1* double-mutant seed exhibited dormancy most prominently (Fig. 4B). The *cyp707a2-1 cyp707a3-1* double mutant exhibited a more pronounced dormancy compared to *cyp707a1-1 cyp707a3-1*, even though its dry seeds had lower
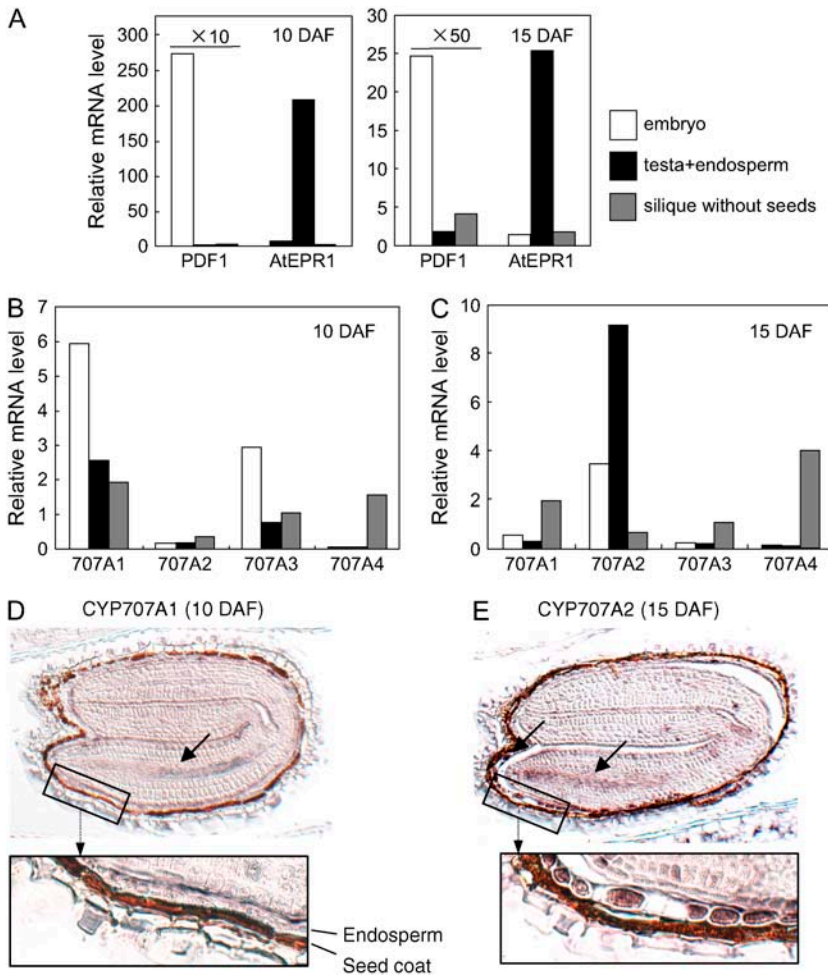
Figure 3. Localization of *CYP707A* transcripts during seed development. A, QRT-PCR quantification of mRNA levels of *PDF1* and *AtEPR1* in three silique fractions harvested at 10 DAF (left) and 15 DAF (right). QRT-PCR quantification of mRNA levels of *CYP707A* genes in three silique fractions harvested at 10 DAF (B) and 15 DAF (C). Results of QRT-PCR were confirmed in two independent assays. In situ hybridization of *CYP707A1* mRNA in 10-DAF (D) and *CYP707A2* mRNA in 15-DAF siliques (E). The arrows indicate site of strong signal in seeds (D and E).

levels of ABA. Next, we quantified ABA levels in the *cyp707a* single and double mutants after seed imbibition. The imbibed seed ABA content of wild type, *cyp707a1-1*, *cyp707a3-1*, and *cyp707a1-1 cyp707a3-1* mutants decreased approximately 70% at 12 h after imbibition, whereas in *cyp707a2-1* this decrease was much slower (Fig. 4C). Furthermore, the high ABA levels of *cyp707a1-2 cyp707a2-1* and *cyp707a2-1 cyp707a3-1* mutants were maintained even at 24 h after imbibition. In summary, the mutants that contained the *cyp707a1* mutation accumulated ABA in dry seeds to higher levels, whereas the mutants that contained the *cyp707a2* mutation exhibited slower reduction in ABA levels after imbibition. This result supports the idea that CYP707A1 plays an important role in determining ABA levels in dry seeds, whereas CYP707A2 plays a major role in the rapid decrease in ABA levels during early seed imbibition.

Localization of *CYP707A* Expression after Seed Imbibition

To determinate the site of *CYP707A* expression during seed germination, QRT-PCR was performed on dissected embryo and testa/endosperm fraction from 6-h-imbibed seeds. We first analyzed the transcript levels of *GA 3-oxidase1* (*AtGA3ox1*) and *AtEPR1*, an embryo- and endosperm-specific marker gene, respectively (Dubreucq et al., 2000; Yamaguchi et al., 2001; Penfield et al., 2004). The *AtGA3ox1* transcript was detected in the embryo, but only at low levels in the testa/endosperm fraction (Fig. 5A). In contrast, the *AtEPR1* transcript was detected only in the testa/endosperm fraction. This indicates that there was no significant cross contamination between the two fractions. We then analyzed transcript levels of *CYP707A* genes in these fractions. Our results showed that *CYP707A2* was predominantly expressed in both the embryo and the testa/endosperm fractions, whereas transcripts for other *CYP707As* were low in both tissues at 6 h after imbibition (Fig. 5B). To determine tissue-specific expression patterns of *CYP707A2*, in situ hybridization was carried out using 6-h-imbibed seeds. *CYP707A2* mRNA was detected in both embryo and endosperm (Fig. 5C). It is worth mentioning that the localization pattern of *CYP707A2* mRNA in 6-h-imbibed embryos differed from that in 15-DAF developing embryos. *CYP707A2* mRNA was localized in the

Figure 4. Phenotypic analysis of *cyp707a* double-mutant seeds. A, Dry seed ABA level in the *cyp707a* double mutants. Experiments were performed three times using independent seed batches and the averages are shown with SES. B, Dormancy of seeds after ripened for 1 week in *cyp707a* single and double mutants. Experiments were performed five times and the averages are shown with SES. C, Quantification of endogenous seed ABA levels in the *cyp707a* single and double mutants after seed imbibition. Two hundred milligrams of seeds were imbibed in 8.5-cm petri dishes containing two filter papers (approximately 7-cm diameter) and 4 mL of water. Experiments were performed twice using independent seed (after ripening for 1 week) batches.
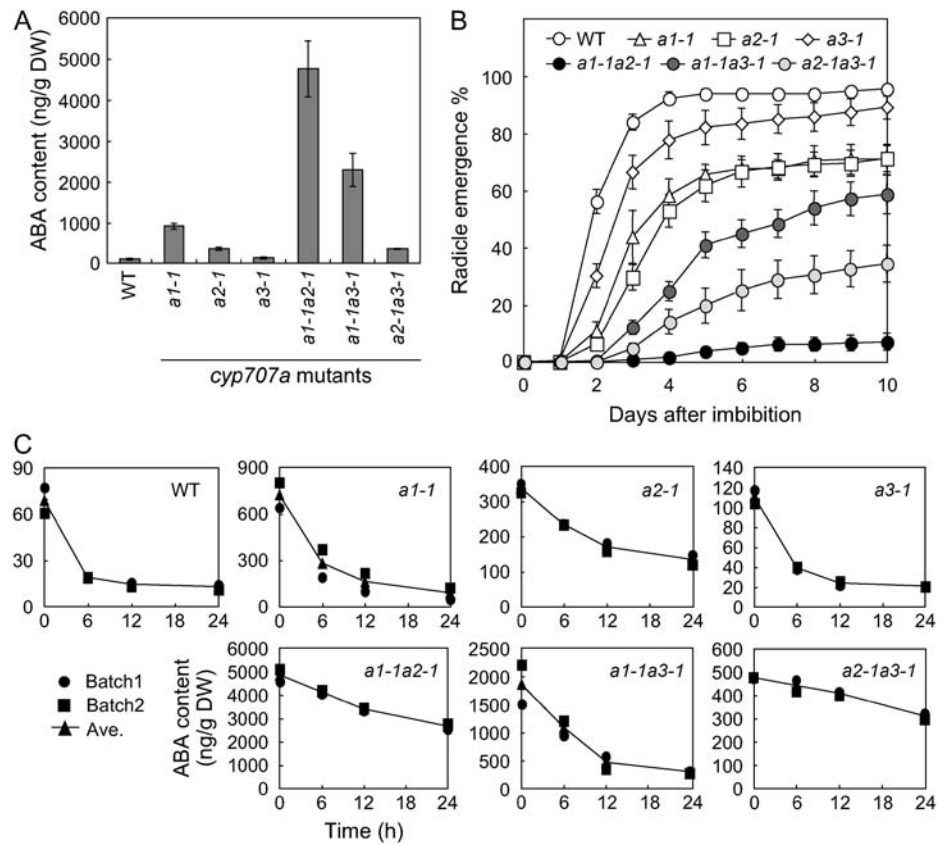
cortex and endodermis in the 6-h-imbibed embryo rather than vascular tissues observed at the late-maturation stage (Fig. 5C).

Physiological Function of *CYP707A* in Germination and Postgermination Growth

Seed is highly sensitive to ABA, but its sensitivity decreases after germination to accelerate seedling growth (Lopez-Molina et al., 2001). Such change in ABA sensitivity is possibly accompanied by alteration of both signaling and metabolism. We reported previously that *CYP707A2* is induced 6 h after imbibition, whereas *CYP707A1* and *CYP707A3* transcripts are increased 24 h after seed imbibition (Kushiro et al., 2004). We asked whether the difference in induction kinetics reflects mutant phenotypes.

To examine the role of CYP707A in germination and postgermination growth, we analyzed the responsiveness to exogenous (+)-ABA of *cyp707a* single and double mutants. Germination and postgermination growth in the presence of exogenous (+)-ABA were assessed by monitoring radicle emergence and cotyledon greening, respectively. Among the single mutants examined, germination of the *cyp707a2-1* mutant was inhibited most effectively by exogenous (+)-ABA, whereas seedling growth was inhibited more effectively in the *cyp707a1* and *cyp707a3* mutants (Fig. 6, A and B). These phenotypes observed in the single

mutants were more pronounced in the double mutants. Inhibition of germination by (+)-ABA was more effective in the double mutants that contained the *cyp707a2* mutation, namely, *cyp707a1-1 cyp707a2-1* and *cyp707a2-1 cyp707a3-1*, than in each single mutant (Fig. 6A). On the other hand, postgermination growth of the double mutants that contained the *cyp707a1* mutation, namely, *cyp707a1-1 cyp707a2-1* and *cyp707a1-1 cyp707a3-1*, was more ABA hypersensitive than each single mutant and the *cyp707a2-1 cyp707a3-1* double mutant (Fig. 6B). These results indicate that CYP707A1 and CYP707A3 are important for postgermination growth. In particular, CYP707A1 is involved in growth inhibition by ABA more prominently than CYP707A3 during early seedling development.

DISCUSSION

In this study, we conducted functional and expression analyses of Arabidopsis CYP707A during seed development and germination to elucidate their roles in growth and development. Our results indicate that CYP707A1 and CYP707A2 are the major isoforms for ABA 8′-hydroxylation during mid-maturation and late-maturation, respectively. During early seed imbibition, CYP707A2 plays a major role in the rapid decrease in ABA levels, whereas CYP707A1 and CYP707A3 are involved in early seedling growth after
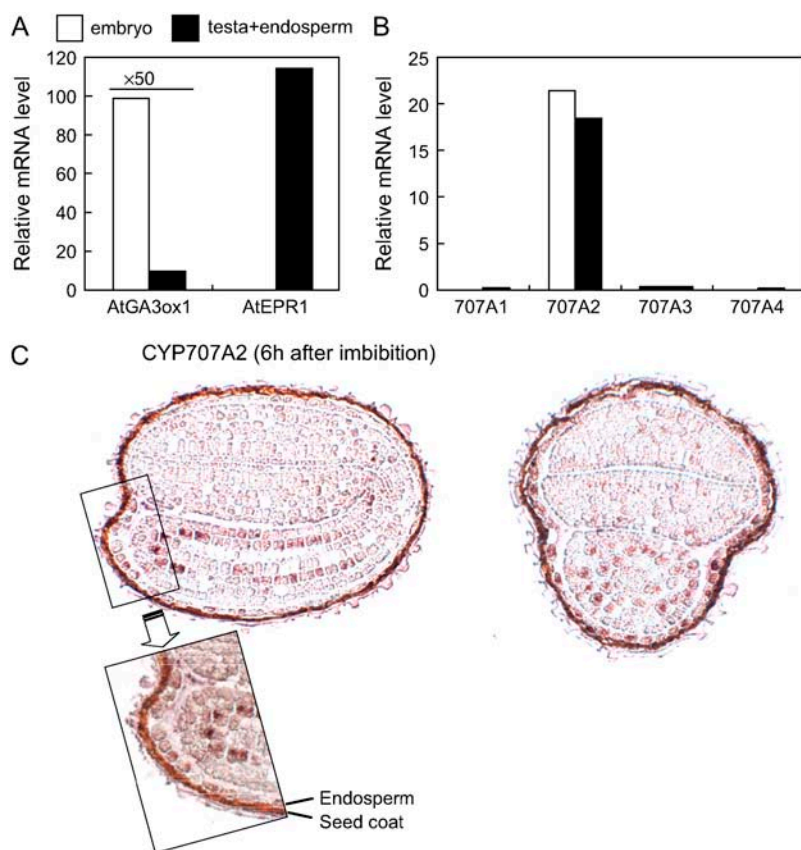
germination. Thus, each CYP707A plays a different role in seeds to control dormancy and germination.

Roles of CYP707As during Seed Development

The endogenous ABA levels in seed vary remarkably at each developmental stage (Karssen et al., 1983; Groot et al., 1991; Frey et al., 2004; Gazzarrini et al., 2004; Setha et al., 2004). In Arabidopsis, ABA is accumulated in immature siliques most abundantly at 10 DAF, and decreases gradually thereafter. Expression and phenotypic analyses of the *CYP707A* genes showed that CYP707A1 contributed predominantly to this reduction (Fig. 2, C and D). Although *CYP707A* genes other than *CYP707A1* were also expressed weakly at the mid-maturation stage, the ABA levels in these mutants were comparable to those in wild type (Fig. 3, C and D). Groot and Karssen (1992) reported that differences in the degree of dormancy in tomato (*Lycopersicon esculentum*) seeds are dependent on the ABA levels during seed development, but not on the levels in mature seeds or fruits. We also found that the *cyp707a1* seeds exhibited enhanced dormancy compared with the corresponding wild type. This suggests that ABA catabolism occurring at the mid-maturation stage affects seed dormancy.

Concomitant with the phase transition to the late-maturation stage, the role of CYP707A1 in regulation of ABA levels is taken over by the CYP707A2.

CYP707A2 was predominantly expressed during the late stage, and the *cyp707a2* mutants overaccumulated ABA at this stage (Fig. 2, C and E). Notably, ABA overaccumulation in the *cyp707a2-1* mutant was observed at 16 DAF, but not at 12 DAF, consistently in time with the expression patterns of the *CYP707A2*. In addition to CYP707A2, phenotypic analysis of the *cyp707a* single and double mutants showed that CYP707A family members play a distinct, but partially overlapping, role in regulating endogenous ABA during late-maturation to mature dry seeds. The *cyp707a1 cyp707a3* double mutant accumulated ABA to a higher level in dry seed (approximately 20-fold relative to the wild type) than the *cyp707a1* single mutant (10-fold relative to the wild type; Figs. 1B and 4A). Moreover, the *cyp707a1 cyp707a2* double mutant accumulated a huge amount of ABA, up to 50 times that of the wild type (Fig. 4A). These results indicate that these three CYP707As are involved more or less in the regulation of ABA levels at this stage. In contrast, because the expression of *CYP707A4* was found only in the silique envelopes, this gene appears not to be involved in the regulation of ABA levels in seeds. However, it would be interesting to examine whether or not *CYP707A4* expressed in tissues other than the seed influences the seed dormancy by using antisense or RNAi techniques.

In situ hybridization revealed that *CYP707A1* was expressed in the vascular tissue in the embryo during
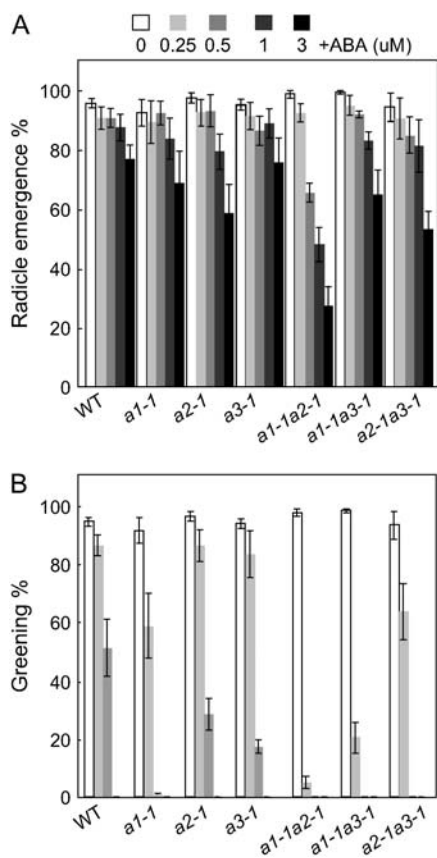
Figure 6. Germination and postgermination growth of the *cyp707a* single and double mutants in the presence of exogenous ABA. A, Germination was scored based on radicle emergence. B, Postgermination growth was scored based on greening of cotyledons. After-ripened seeds for 3 months were sown on agar plates containing several concentration of (+)-*S*-ABA and placed at 22°C under continuous light for 7 d after 4 d of stratification at 4°C. Experiments were performed four times and the averages are shown with SEs.

the mid-maturation stage (Fig. 3D). During late-maturation, the *CYP707A2* transcript was detected abundantly at both the embryo and the endosperm/testa (Fig. 3C). From in situ analysis, its signal was also observed in the vascular tissues in the embryo (Fig. 3, D and E). It is interesting to point out that vascular tissue appears to be the major site of *CYP707A* expression during the mid- and late-maturation stages. In a recent report, Arabidopsis aldehyde oxidase 3 (AAO3), an enzyme that catalyzes the last step of ABA biosynthesis, was shown to be localized abundantly in vascular tissues of roots, hypocotyls, and leaves, indicating that the vascular tissue is an important site of ABA biosynthesis in vegetative tissues (Koiwai et al., 2004). In addition, genes for the short-chain alcohol dehydrogenase/reductase (*ABA2*) and *AAO3* are also expressed in the vascular tissues of the embryo during the mid-maturation stage (M. Seo and E. Nambara, unpublished data), suggesting that ABA catabolism by CYP707A1 occurs closely at the site of ABA biosynthesis.

Physiological Roles of CYP707As during Germination and Postgermination Growth

Seed germination is a complex physiological event regulated by internal and external stimuli (Bewley, 1997). Germination starts with water uptake by seed imbibition and is completed when a radicle protrusion occurs. In typical angiosperm seeds, such as tomato and Arabidopsis, germination requires both activation of embryo growth potential and weakening of a mechanical constraint such as testa or endosperm, both of which appear to be regulated by antagonistic actions of ABA and GA. It has been shown that both dissected embryo and endosperm are able to respond to these hormones (Groot and Karssen, 1987, 1992; Debeaujon and Koornneef, 2000; da Silva et al., 2004); however, little is known concerning the site of biosynthesis and catabolism of the hormones. Our spatial expression analysis using 6-h-imbibed seeds indicates that both embryo and endosperm are possible sites for the regulation of endogenous ABA levels by CYP707A2 prior to germination. It has been shown that PA is detected in the embryo as well as in other tissues, such as barley (*Hordeum vulgare*) endosperm and western white pine (*Pinus monticola*) megagametophyte, in imbibed seeds in other plants (Jacobsen et al., 2002; Feurtado et al., 2004). Further studies on the site of hormone biosynthesis and catabolism will be necessary to clarify hormone actions during seed germination.

Expression of the *CYP707A2* was induced exclusively after seed imbibition by exogenous ABA or stratification treatments (Kushiro et al., 2004; Yamauchi et al., 2004), indicating that CYP707A2 plays a specific role in regulating germination in response to internal and external cues. Interestingly, *CYP707A2* expression in the embryo in 6-h-imbibed seeds was localized in the cortex and endodermis, where bioactive GAs are synthesized during germination (Yamaguchi et al., 2001). Furthermore, the moist chilling-treated western white pine seeds or GA-treated lettuce (*Lactuca sativa*) seeds had enhanced accumulation of PA and DPA (Feurtado et al., 2004; Gonai et al., 2004). This suggests that expression of *CYP707A*s is controlled by environmental cues or GAs. It will be necessary to explore factors that control the expression of *CYP707A* genes to fully understand the regulatory mechanism of dormancy and germination.

The degree of seed dormancy correlates with endogenous ABA levels in imbibed seeds rather than in dry seeds in various species, such as in Arabidopsis (Ali-Rachedi et al., 2004), lettuce (Gonai et al., 2004), barley (Jacobsen et al., 2002), and tobacco (*Nicotiana plumbaginifolia*; Grappin et al., 2000). Our mutant analyses also support this notion (Fig. 4). Moreover, the result of double-mutant analysis is consistent with our previous report that CYP707A2 is a key enzyme for breaking dormancy in Arabidopsis (Kushiro et al., 2004), even if the ABA levels in dry seed are determined predominantly by the CYP707A1.

Expression analysis of *CYP707A* genes after seed imbibition suggested that the physiological role of CYP707A1 and CYP707A3 is different from that of CYP707A2 (Kushiro et al., 2004). Induction of *CYP707A1* and *CYP707A3* expression was detected at 24 h after imbibition (Kushiro et al., 2004), and this induction was not observed when germination was inhibited by a high concentration of exogenous ABA (data not shown). This indicates that the induction of these genes might be triggered by germination events rather than a prerequisite for germination. Consistently, after stratification, *cyp707a1* and *cyp7070a3* single mutants germinated at the same rate as the wild type under low concentrations of exogenous (+)-ABA, in contrast to the postgermination growth of these mutants, which was arrested by ABA more effectively than the wild type (Fig. 6). This suggests that CYP707A1 and CYP707A3 also contribute to ABA catabolism during postgermination growth.

Physiological Roles of ABA 8′-Hydroxylase in ABA Catabolism in Arabidopsis Seeds

Physiological roles of the regulation of ABA biosynthesis and catabolism have been evaluated thus far by loss-of-function and gain-of-function experiments. In biosynthesis, a defect of any ABA biosynthesis genes causes a reduction in the ABA level unless the genome contains redundant genes. In contrast, only genes for a regulatory enzyme can increase the ABA level in gain-of-function experiments. For example, overexpression of the *NCED* gene is known to increase ABA levels in plants. On the other hand, in catabolism, the loss of function of only regulatory genes leads to increased ABA levels in plants. Therefore, to evaluate the physiological roles of ABA catabolism, phenotypes of the loss-of-function mutants affected in catabolic genes are extremely informative.

Our present work demonstrated the importance of the ABA 8′-hydroxylation pathway during seed development and germination in Arabidopsis. At this time, it is known that ABA is inactivated through several catabolic pathways (Nambara and Marion-Poll, 2005). Although previous reports suggest that ABA 8′-hydroxylation is predominant in the ABA catabolic pathways in many plants, a decisive conclusion could not be made. In this study, seeds of the *cyp707a* single or double mutants accumulated very large amounts of ABA. If catabolic pathways other than the ABA 8′-hydroxylation contribute to physiological roles in the regulation of endogenous ABA levels, accumulation of excessive ABA in the *cyp707a* single or double mutants would not be observed. Taken together, our results demonstrate that ABA 8′-hydroxylation is the predominant pathway during Arabidopsis seed development and germination.

However, it is also noteworthy that seed dormancy in *cyp707a* mutants was broken by after ripening,

application of GA, or stratification (Fig. 6; data not shown). *cyp707a* double mutants required much longer dormancy-breaking treatments to germinate, suggesting CYP707As are involved in the regulation of dormancy and germination responding to environmental stimuli. This also suggests that a CYP707A-independent mechanism might be involved in the regulation of germination responding to external cues, such as storage conditions, temperature, and other hormones. Recent reports described that ABA Glc esters were detected at significant levels in high-temperature-imbibed lettuce seeds or in Arabidopsis seeds treated by moist chilling (Gonai et al., 2004; Chiwocha et al., 2005). This suggests that CYP707A-independent germination responding to environmental factors is, in part, due to the ABA conjugation pathway. Furthermore, to fully understand the regulation of seed germination, it will be necessary to integrate the role of ABA catabolism into knowledge of the regulatory network for seed germination, such as regulation of ABA sensitivity or GA actions. For example, the hyper seed dormancy of the *enhanced ABA response 1* (*era1*) mutant defective in ABA signaling can be rescued effectively by the stratification treatment (Cutler et al., 1996). This suggests that a decrease in ABA sensitivity by stratification is sufficient to elicit germination. As an effect of after ripening, the deterioration of ABA biosynthetic capacity or increase of GA sensitivity has been reported in Arabidopsis (Ali-Rachedi et al., 2004), barley (Jacobsen et al., 2002), sunflower (*Helianthus annuus*; Bianco et al., 1994), and tobacco (Grappin et al., 2000). Moreover, the increase of GA sensitivity and biosynthetic ability or decrease of ABA sensitivity is reported as an effect of stratification (Groot and Karssen, 1992; Corbineau et al., 2002; Yamauchi et al., 2004).

In conclusion, our present study demonstrates that the CYP707A family plays a prominent role in regulating endogenous ABA levels during seed development and germination. The analysis of *cyp707a* triple or quadruple mutants will help in understanding the complex regulation of seed dormancy and germination controlled by the ABA and GA balance, including the role of ABA catabolic pathways other than the ABA 8′-hydroxylation pathway.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used in this study. The *cyp707a1-1* and *cyp707a1-2* mutants were isolated from SALK T-DNA lines, SALK_069127 (Alonso et al., 2003), and from Wisconsin T-DNA lines, WiscDsLox393-396J9, respectively. The *cyp707a2* and *cyp707a3* mutants used in this study were described previously (Kushiro et al., 2004). All plants were grown as described in Kushiro et al. (2004). To obtain silique samples for RNA extraction and ABA quantification, flowers were tagged on the days of flowering and sampled daily. Dissection of embryo, testa/endosperm, and other tissue from siliques and imbibed seeds was carried out under a stereoscopic microscope.

Germination Experiments

For dormancy analysis, freshly harvested seeds were sown on 1% agar plates containing $0.5 \times$ Murashige and Skoog salts and 0.5% Suc. Germination was scored daily for radicle emergence. For germination and postgermination growth analysis in the presence of exogenous ABA, seeds after ripened for 3 months were sown on agar plates containing several concentrations of (+)-*S*-ABA. After 4 d of stratification at 4°C, the plates were placed at 22°C under continuous light for 7 d. Germination was scored based on radicle emergence, whereas postgermination growth was scored for greening of cotyledons. In all experiments, approximately 50 seeds were used and replicated.

QRT-PCR

Total RNA was isolated using an RNAqueous column with plant RNA isolation aid (Ambion). cDNA was synthesized from 1 μg of total RNA using a QuantiTec reverse transcription kit (Qiagen) according to the manufacturer's instructions. QRT-PCR using Taq-Man probe or SYBR Green I was performed as described previously (Kushiro et al., 2004). The sequence of primers and Taq-Man probes for *AtNCED*s, *CYP707A*s, *AtGA3ox1*, and *AtEPR1* genes were used as described by Seo et al. (2004), Kushiro et al. (2004), Ogawa et al. (2003), and Penfield et al. (2004), respectively. QRT-PCR for the *PDF1* gene was as follows: forward primer, 5′-CACACACCCCTCCTTGTAACTG-3′; reverse primer, 5′-GTCACAACAACCGGAGTTCTTG-3′. For normalization of data, 18S rRNA was used as an internal standard.

Isolation of Double Mutants

The double-mutant lines were generated by crossing each *cyp707a1-1*, *cyp707a2-1*, and *cyp707a3-1* mutant. After-ripened F$_2$ seeds were placed in a cold room (4°C) for 7 d before transfer to the growth chamber, and genotypes of those mutants were confirmed by PCR. The following primer sets were used: for *cyp707a1-1*, A1F (5′-AAAGGTCAGTGCTTTAAGAACCG-3′) and A1R (5′-CACTTCGATCTCCGGCTTCC-3′); for *cyp707a2-1*, A2F (5′-CGCAACGG-CTTAAGTGATTC-3′) and A2R (5′-AATCGGGGTTACTCTTATTGGTAAACC-3′); and for *cyp707a3-1*, A3F (5′-GATACTCGGACCCAAATCATAC-3′) and A3R (5′-TGGTTTTCGTTCCAAGGCAATAGGC-3′). For identification of T-DNA insertion, left-border primer (5′-CTGGAACAACACTCAACCCTATCTC-3′) was used with A1F, A2R, and A3F primers, respectively.

In Situ Hybridization

In situ hybridization experiments were performed using digoxygenin-labeled RNA probes as described previously (Yamaguchi et al., 2001). To synthesize RNA probes in vitro, PCR-amplified cDNAs of *CYP707A1* and *CYP707A2* were cloned into pSTBlue-1 vector (Novagen). The following primer sets were used: for CYP707A1, 5′-GCCAGTGAATCTCCCTGGAA-CACTT-3′ and 5′-CAAGGGAAGTCTCAATCCAGGAGG-3′; for CYP707A2, 5′-ATGCAAATCTCATCTTCATCGTCTTCAAA-3′ and 5′-AATCGGGGTTA-CTCTTATTGGTAAACC-3′. The prepared CYP707A1 templates contained a 3′-untranslated region.

Determination of ABA, PA, and DPA Levels

Samples were homogenized in 80% (v/v) acetone containing 0.1 mg/mL 2,4-di-tert-butyl-4-methylphenol. [1,2-^{13}C$_2$]-(±)-ABA, deuterium-labeled PA, and DPA were used as internal standards (Asami et al., 1999; Hirai et al., 2003). After adding internal standards, the homogenate was shaken for 1 h on ice in darkness and then centrifuged at 1,970g for 10 min at 4°C. The precipitate was then reextracted, and the combined supernatant was evaporated to remove residual acetone. ABA, PA, and DPA were partially purified from the residual aqueous solution by partitioning, using hexane and ethyl acetate, followed by HPLC. ABA was methylated with diazomethane, whereas PA and DPA were trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide at 60°C for 10 min. Gas chromatography-electron impact ionization mass spectrometry (JMS-Automass SUN; JEOL) was carried out with a DB-1 column (250 m, i.d. 30 m, film thickness, 0.25 m; J&W Scientific). The following mass-to-charge ratio peaks were used for quantification: for ABA, 192 (labeled) and 190 (endogenous); for PA, 265 (deuterated) and 262 (endogenous); and for DPA, 429 (deuterated) and 426 (endogenous). Backup ions (labeled ABA 262 and 163, endogenous ABA 260 and 162; deuterated PA 355, 328, and 306; endog-enous PA 352, 325, and 303; deuterated DPA 339 and 250; endogenous DPA 336 and 247) were also monitored for peak confirmation. For only ABA determination, extraction and purification were performed as described previously (Cheng et al., 2002).

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