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Abstract: Abscisic acid (ABA) catabolism is important for regulating endogenous ABA levels. Neophaseic acid (neoPA) is one of the ABA catabolites produced via ABA 9'-hydroxylation, but the gene encoding ABA 9'-hydroxylase has not been identified. We found that endogenous neoPA levels reduced in loss-of-function mutants defective in ABA 8'-hydroxylase (CYP707A) genes. In addition, levels of both neoPA and PA reduced similarly when wild-type plants were treated with uniconazole-P, a P450 inhibitor. Furthermore, in vitro enzyme assay using microsomal fraction from yeast expressing CYP707A showed that all four Arabidopsis CYP707As contained both ABA 8'- and 9'-hydroxylase activities, although ABA 9'-hydroxylase activity is minor. These results demonstrate that ABA 9'-hydroxylation is catalyzed by CYP707A as a side reaction in Arabidopsis.

Cover letter

Dear Editor,

Please find enclosed our manuscript entitled “ABA 9’-hydroxylation is catalyzed by CYP707A in Arabidopsis”. We would like to submit this manuscript for publication in the Phytochemistry. Abscisic acid (ABA) catabolism is important for regulating endogenous ABA levels in plant’s response to environmental or developmental cues. ABA is catabolized through hydroxylation at either C-8’, C-9’, or C-7’ and also through glycosylation. ABA 8’-hydroxyation is a key catabolic reaction, which is catalyzed by cytochrome P450 CYP707As. However, the gene coding ABA 9’-hydroxylases has not been identified. Here, we report that four Arabidopsis CYP707As catalyze both ABA 8’- and 9’-hydroxylation. This finding reveals the new enzymatic function of CYP707As, which ultimately helps to evaluate the role of each ABA catabolic pathway in other plant species in future.

We would like to suggest expert reviewers for reviewing this manuscript.

Possible reviewers are listed below.

1. Professor Peter Hedden (peter.hedden@bbsrc.ac.uk) Rothamsted Research

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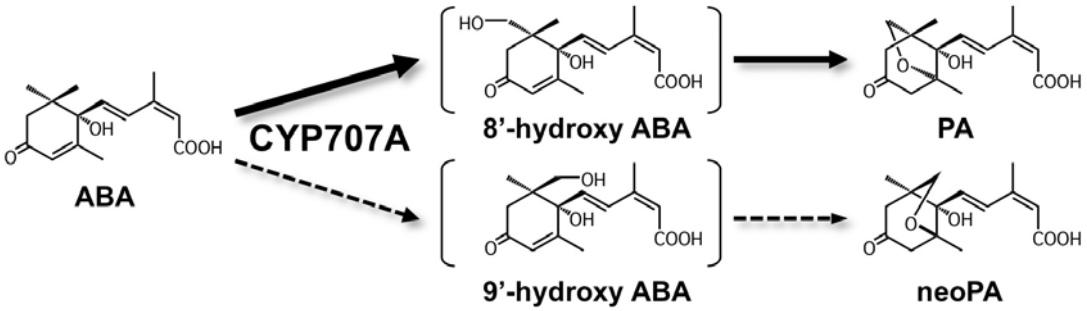
We hope that our manuscript is acceptable for publication in the Phytochemistry.

We look forward to hearing from you.

Sincerely,

Masanori Okamoto & Eiji Nambara

*Graphical Abstract



***Research Highlights**

Arabidopsis CYP707As catalyze both ABA 8'- and 9'-hydroxylation, but ABA 9'-hydroxylase activity is minor. Therefore, PA and neoPA are produced as major and minor catabolites of CYP707A, respectively.

Title:

ABA 9'-hydroxylation is catalyzed by CYP707A in Arabidopsis

Authors:

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Figures, 4 and supplemental figure, 1

Abstract

Abscisic acid (ABA) catabolism is important for regulating endogenous ABA levels. Neophaseic acid (neoPA) is one of the ABA catabolites produced via ABA 9'-hydroxylation, but the gene encoding ABA 9'-hydroxylase has not been identified. We found that endogenous neoPA levels reduced in loss-of-function mutants defective in ABA 8'-hydroxylase (CYP707A) genes. In addition, levels of both neoPA and PA reduced similarly when wild-type plants were treated with uniconazole-P, a P450 inhibitor. Furthermore, *in vitro* enzyme assay using microsomal fraction from yeast expressing CYP707A showed that all four Arabidopsis CYP707As contained both ABA 8'- and 9'-hydroxylase activities, although ABA 9'-hydroxylase activity is minor. These results demonstrate that ABA 9'-hydroxylation is catalyzed by CYP707A as a side reaction in Arabidopsis.

Keywords: Arabidopsis; abscisic acid; catabolism; P450; CYP707A; neophaseic acid; phaseic acid

1. Introduction

Abscisic acid (ABA) is a sesquiterpene plant hormone that regulates numerous processes during plant life cycle including seed maturation, induction and maintenance of seed dormancy and stress response including stomatal closure (Zeevaart and Creelman, 1988; Nambara and Marion-Poll, 2005). Endogenous ABA levels increase during seed maturation and during drought stress, whereas the levels reduce prior to germination and during rehydration of drought plants. In the stationary state, ABA levels are maintained as balance between biosynthesis and catabolism (Zeevaart, 1980). On the other hand, ABA biosynthesis and catabolism are specifically activated when plants respond to developmental and environmental stimulus (Yamaguchi-Shinozaki and Shinozaki, 2006; Nambara et al., 2010).

ABA is inactivated by the oxidation or conjugation, and several ABA catabolic pathways exist in plants (Fig. 1). Among them, ABA 8'-hydroxylation is a committed step in the major ABA catabolism (Nambara and Marion-Poll, 2005). Hydroxylation at C-8' of ABA is catalyzed by ABA 8'-hydroxylase to form 8'-hydroxy ABA. In the subsequent step, 8'-hydroxy ABA is spontaneous isomerized to (-)-phaseic acid (PA), and is further reduced by unidentified reductase to dihydrophaseic acid (DPA). ABA 8'-hydoxylase is encoded by the CYP707A family of the cytochrome P450 monooxygenase (Kushiro et al., 2004; Saito et al., 2004). ABA is also catabolized by the hydroxylation at C-7' and -9' of ABA to form 7'- and 9'-hydroxy ABAs, respectively (Hampson et al., 1992; Zhou et al., 2004). (-)-*R*-ABA, an ABA analog, is metabolized into (+)-phaseic acid in corn cell culture possibly by ABA 7'-hydroxylase (Balsevich et al., 1994). Neophaseic acid (neoPA), an isomer of 9'-hydroxy ABA is also observed in a variety of plant species (Zhou et al., 2004). However, the genes encoding 7'- and 9'-hydroxylases have not been identified. In contrast to oxidative catabolic pathways, ABA glucose ester (ABA-GE) is one of the major inactive forms of ABA and is thought to be a stored or long-distance-transport form of releasable ABA (Sauter et al., 2002). Glucosylation at the carboxyl group of ABA is triggered by glucosyltransferase (Xu et al., 2002; Lim et al., 2005).

ABA is mainly catabolized via ABA 8'-hydroxylation pathway in response to developmental and environmental signals such as drought stress, rehydration after drought stress, submergence or high humidity responses, seed maturation and germination process (Okamoto et al., 2006; Umezawa et al., 2006; Saika et al., 2007; Okamoto et al., 2009; Matakiadis et al., 2009). On the other hand, it is unclear how other catabolic pathways are involved in the regulation of ABA levels. There are numerous reports showing the differential accumulation of other catabolites. For example, ABA-GE levels are increased in *Xanthium strumarium* plant subjected to long-term drought stress (Zeevaart, 1983), moist-chilled Arabidopsis seeds (Chiwocha et al., 2005), whereas 7'-OH ABA levels are high in western pine seeds (Feurtado et al., 2004). Nonetheless, physiological roles of each catabolic pathway need to be evaluated by genetic, molecular and pharmacological analyses. In the present study, we report that neoPA was produced by CYP707A as a minor byproduct during ABA catabolic reaction process in Arabidopsis.

2. Results

2.1. ABA catabolite profiles in the *cyp707a* double mutant

ABA catabolites accumulate eminently when ABA increases reach a plateau under drought stress conditions. To examine the accumulation pattern of ABA catabolites, LC-MS/MS was used to measure the levels of ABA, ABA-GE, PA, DPA, neoPA and 7'-OH ABA in 6-h-dehydrated wild-type and *cyp707a1a3* double mutant plants (Fig. 2A). Six-h dehydrated wild-type plants accumulated catabolites of ABA 8'-hydroxylation pathway (PA and DPA) more abundantly than other catabolites (Fig. 2A). ABA-GE was less abundant than PA and DPA, but it was accumulated substantially in the dehydrated wild type. NeoPA was a minor catabolite detected in wild-type plants, whereas 7'-OH ABA levels were not determined due to its low abundance (Fig. 2A). The *cyp707a1a3* double mutant accumulated ABA 2-fold

more abundantly than wild type in 6 h-dehydrated plants (Fig. 2A). As expected, levels of PA and DPA were severely reduced in the *cyp707a1a3* double mutant compared with those in the wild type (Fig. 2A). The *cyp707a1a3* double mutant accumulated ABA-GE more abundantly than those in wild type. Interestingly, neoPA levels were 10-fold lower in *cyp707a1a3* double mutant than those in wild type. We could not detect 7'-OH ABA in the mutant similarly to the wild type.

We next analyzed levels of ABA catabolites in the siliques during mid-maturation. In siliques, ABA 8'-hydroxylation pathway was also predominant among ABA catabolic pathways (Fig. 2B, Okamoto et al., 2006). However, composition of ABA catabolites in the siliques was different from that in 6-h-drought-stressed plants (Fig. 2B). A large amount of DPA was detected in the siliques, but PA levels in siliques were much lower compared with 6-h-drought-stressed plants. In addition to detection of ABA-GE and neoPA, 7'-OH ABA was also detected in the siliques. In *cyp707a1a3* double mutant, ABA levels were 10-fold higher, whereas PA and DPA levels were 8-fold lower than wild type (Fig. 2B). On the other hand, levels of ABA-GE and of 7'-OH ABA in *cyp707a1a3* double mutant were higher than those in the wild type. Interestingly, neoPA levels in the *cyp707a1a3* double mutant siliques decreased compared with those in the wild type as the case in dehydrated plants.

2.2. Effects of P450 inhibitors on PA and neoPA accumulation

The *cyp707a* mutations inhibited neoPA accumulation as well as PA and DPA (Fig. 2). To test if an inhibitor for CYP707A also blocks neoPA accumulation, we examined levels of ABA catabolites in Arabidopsis plants treated with uniconazole-P. Uniconazole-P (Fig. 3A) was originally known as an inhibitor of CYP701, an enzyme catalyzing the oxidation of *ent*-kaurene to form *ent*-kaurenoic acid in gibberellin biosynthesis (Rademacher, 2000). However, it was reported that this compound also strongly inhibits other P450s including CYP707A (Kitahara et al., 2005; Saito et al., 2006). Uniconazole-P dose-dependently inhibited accumulation of both PA and neoPA in the 2-week-wild-type-plants supplied with exogenous 30 μM (+)-*S*-ABA

(Fig. 3B and C). In addition, dose-dependency of inhibiting neoPA accumulation by uniconalzole-P treatment was comparable to that of PA accumulation. Tebuconazole (Fig. 3A) is a P450 inhibitor for sterol 14α-demethylases (CYP51s), but not for ABA 8'-hydroxylase (Kwok et al., 1993; Kitahara et al., 2005; Saito et al., 2006). PA and neoPA accumulation was not affected by the tebuconazole treatment (Fig. 3B and C). These results suggest that uniconazole-P inhibits activities of both ABA 8'-hydroxylase and 9'-hydoxylase.

2.3. CYP707A contains both ABA 8'- and 9'-hydroxylase activity in vitro

To determine whether CYP707As have ABA 9'-hydroxylation activity, we examined enzymatic activity using yeast expressing CYP707A heterologously. Microsomal proteins were prepared from recombinant yeast and were incubated with (+)-*S*-ABA. As reported previously, microsomal proteins expressing CYP707A1 produced PA as a major product (Fig. 4A and B; Kushiro et al., 2004; Saito et al., 2004). In addition, we found that retention time and mass spectra of a minor product coincided with those of authentic neoPA (Fig. 4A, B, D and E). We also examined whether other Arabidopsis CYP707As have ABA 9'-hydoroxylase activity. In the result, CYP707A2, CYP707A3 and CYP707A4 also produced neoPA, although neoPA production was much less than that of PA as CYP707A1 (data not shown).

We also tested enzyme activity of splice variants of CYP707A1 and CYP707A3, designated as CYP707A1S and CYP707A3S, respectively, which were found in the TAIR9 gene model (http://www.arabidopsis.org/). These splice variants were distinct from corresponding primary forms only in the C-terminal region of CYP707A, which includes a substrate recognition site (SRS) (Fig. S1). According to previous reports, replacement of SRS alters the enzymatic nature or substrate recognition of P450s (Lindberg and Negishi, 1989; Cosme and Johnson, 2000). To examine the involvement of splice variants in hydroxylation at C-8' and -9' of ABA, CYP707A1S and CYP707A3S were expressed in yeast, and microsomal proteins were incubated with (+)-*S*-ABA. However, PA and neoPA were not detectable in the

reaction mixture by LC-MS/MS (Fig. 4C) and no visible peaks was seen by the HPLC analysis except for incubated 100 ng (+)-ABA.

3. Discussion

NeoPA, the cyclic isomer of 9'-hydroxy ABA, is an ABA catabolite and was first found in *Brassica napus* siliques (Zhou et al., 2004). Although it is minor, it exists in a wide range of plant species including orange, tomato, barley, and Arabidopsis (Zhou et al., 2004). In the present study, we report several lines of evidence that indicates neoPA is produced by CYP707A as a side reaction product of ABA 8'-hydroxylation in Arabidopsis. We found that neoPA levels were extremely reduced in the *cyp707a1a3* double mutant compared with those in the wild type. We also found that accumulation of neoPA was inhibited by uniconazole-P, a P450 inhibitor, with similar dose dependency to the PA accumulation. Moreover, CYP707As expressed in yeast contained a minor but remarkable activity for ABA 9'-hydroxylation *in vitro*. Both *in vivo* and *in vitro* data supports the notion that ABA 9'-hydroxylation is catalyzed by ABA 8'-hydroxylase (CYP707A) in Arabidopsis. Production of both PA and neoPA by CYP707A might reflect to the flexibility of substrate recognition of this enzyme. This means that ABA is able to turn in the pocket slightly along the side chain as an axis, which is consistent with prominent roles of the side chain in the ABA recognition by CYP707A (Ueno et al., 2005). It is noteworthy that all four Arabidopsis CYP707As contain ABA 9'-hydroxylation activity, suggesting that this is a conserved nature of CYP707As. These are consistent with the previous reports indicating that 9'-derivatives of ABA analogs are potent inhibitors of CYP707A equally to the 8'-derivatives (Cutler et al., 2000; Ueno et al., 2005).

NeoPA is known as a minor catabolite of ABA presents widely in many plant species. In general, abundance of such catabolite does not necessarily correlate

with either regulatory functions or with metabolic flow of the catabolic pathway, because these catabolites are not the end products. Indeed, the ratios of PA/DPA were different among the tissues examined (Fig. 2). It is also true for the ratios of PA/neoPA. Levels of neoPA were higher than those of PA in siliques. Similar observation is also reported in *Brassica napus* siliques (Zhou et al., 2004). This might be due to the difference in half-life of PA and neoPA, which are determined by downstream enzymes, such as unidentified reductases or glucosyltransferases. Therefore, these enzymes probably recognize PA and neoPA differentially.

ABA glucose ester and 7'-OH ABA levels were higher in the *cyp707a1a3* double mutants than in the wild type (Fig. 2). It is unclear whether or not this increase involves active regulatory mechanisms to activate or to induce these catabolic pathways as a homeostatic regulation. It is also possible that this is simply due to the increase of endogenous ABA without any regulatory functions of these pathways. ABA glucose ester is reportedly known as a storage form of ABA, which releases ABA quickly after hydrolysis (Lee et al., 2006). However, ABA glucosylation itself is not a regulatory step for ABA catabolism. Overexpression of CYP707A3 effectively reduced endogenous ABA levels (Millar et al., 2006; Umezawa et al., 2006), but overexpressing UGT71B6, an ABA glucosyl transferase gene, had only minor effect on reducing ABA levels, in which ABA 8'-hydroxylation pathway seems to involve in homeostatic regulation (Priest et al., 2006).

Recent development of inhibitors for ABA biosynthesis and catabolism might be useful to investigate regulatory functions of ABA metabolism among various plant species. More importantly, inhibitors of CYP707A lead to enhancing plant's stress tolerance, thus, this is a potentially useful target for biotechnology (Kitahata et al., 2005; Todoroki et al., 2009). The present work would help to understand the reaction mechanism of this enzyme, which ultimately leads to the development of specific inhibitors.

4. Conclusion

ABA catabolism is important to reduce the cellular concentrations of ABA, which is essential for its function as a signal molecule. CYP707A catalyzes ABA 8'-hydroxylation, which is the committed step in a major catabolic pathway. To date, however, enzyme(s) that catalyzes other ABA hydroxylation reactions at C-7' and C-9' positions were unknown. The present work reports that ABA 9'-hydroxylation is catalyzed by CYP707A as a side reaction.

5. Experimental

5.1. Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) wild type and mutant used in this study were of Columbia accession. The *cyp707a1-1 cyp707a3-1* double mutant used as previously described (Okamoto et al., 2006). For drought stress treatment, four-week-old plants were grown on soil in pots at 22°C, RH 60% under a 16-h light/8-h dark cycle, and detached leaves were put on the filter paper for 2 h and then were incubated into the plastic plate for 4 h. Siliques were harvested approximately 10-12 days after flowering and were used for LC-MS/MS analysis. For the inhibitor treatment, plants were grown for 2 weeks on 1% agar plates containing 1/2 MS salt and 0.5% sucrose under the continuous light condition. Two-week-old plants were incubated on 30 μM (+)-*S*-ABA solution containing various concentrations of uniconazole-P and of tebuconazole for 2 h.

5.2. Chemicals

Authentic neoPA, deuterium-labeled d_3-DPA, d_3-neoPA, d_3-PA, d_4-7'OH-ABA and d_5-ABA-GE were prepared as described in Zhou et al., 2005. Deuterium-labeled d_6-ABA was purchased from Icon Isotopes (Summit, NJ, USA). (+)-*S*-ABA was obtained from Dr. Tadao Asami (The University of Tokyo), uniconazole-P and tebuconazole were kindly provided from Dr. Masaharu Mizutani (Kobe University).

5.3. Determination of ABA and its catabolites

Extraction, purification and quantification of ABA and its catabolites were carried out as described in Okamoto et al. (2009).

5.4. Functional expression of CYP707A in yeast

Functional analyses of CYP707A and splice variants of CYP707A1 and CYP707A3 were performed as described previously (Kushiro et al., 2004) with some minor modifications. To obtain the splice variant forms, PCR-based mutagenesis technique was carried out as described in Picard et al. (1994). Amino acid sequences of CYP707A1, CYP7073 and of those variant forms are shown in supplemental figure 1. Microsomal fractions were suspended in 0.1 M potassium phosphate buffer (pH 7.6). One hundred ng of (+)-*S*-ABA was incubated with 2 µg of microsomal protein (in a volume of 100 *µ*L) containing 0.5 mM NADPH, 0.5% (w/v) Triton X-100 at 22°C for 12 h. The reaction was stopped by adding 1M HCl, and then products were extracted with ethyl acetate, and dried under vacuum. Extracts were dissolved in 100 µl of methanol and 900 µl of 1% acetic acid solution (v/v) was added. Oasis HLB (30 mg, 1 ml) solid-phase extraction cartridges (Waters, Milford, MA, USA) were conditioned with 1 ml of acetonitrile followed by 1 ml of methanol and equilibrated with 1 ml of 1% acetic acid solution (v/v). Samples were loaded, followed by a wash with 1 ml of 1% acetic acid solution (v/v). Sample was eluted using 1 ml 50% acetonitrile, 49% water: 1% acetic acid (v/v) and were dried under vacuum. The resulting sample was dissolved in water, and PA, neoPA and 7'OH-ABA were analyzed by LC-MS/MS. Retention time and MS/MS transitions of ABA catabolites and LC-MS/MS condition are described in Saika et al. (2007) and Okamoto et al. (2009).

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Figure Legends

Fig 1. ABA catabolic pathways in higher plants.

Fig 2. Profiles of ABA and its catabolites in wild type and *cyp707a* double mutant. Endogenous ABA and its catabolite levels in the wilted plants **(A)** and siliques at 10-12 DAF **(B)**. Experiment was performed four times using independent biological samples, and averages are shown with standard errors. N.D.; not detected due to low levels.

Fig 3. Effect of P450 inhibitors on accumulation of PA and neoPA. (A) Chemical structure of P450 inhibitors in this study. Inhibition of PA **(B)** and neoPA **(C)** accumulation by P450 inhibitors at various concentrations in wild type. Two-week-old plants were incubated with the indicated concentrations of inhibitors in the presence of 30 μM (+)-*S*-ABA for 2 h. *cyp707a1a3* double mutant were subjected to 30 μM of (+)-*S*-ABA without P450 inhibitors. Uni, Teb and M indicate uniconazole-P, tebuconazole, *cyp707a1a3* double mutant, respectively. Experiment was performed three times using independent biological samples, and averages are shown with standard errors.

Fig 4. LC-MS/MS analysis on reaction products of CYP707A recombinant protein with ABA. (A) Retention time of authentic ABA, PA, 7'-OH ABA and neoPA. Authentic compounds were injected 100 pg, and following MS/MS transition (*m/z*) was monitored: 263/153 (ABA), 279/139 (PA), 279/217 (7'-OH ABA), and 279/205 (neoPA). Reaction products of CYP707A1 **(B)** and of CYP707A1S **(C)**. One hundred ng of (+)-*S*-ABA was incubated with 2 μg of microsomal protein expressing CYP707A1 or CYP707A1S. Mass spectra of authentic neoPA **(D)** and minor reaction product from ABA by CYP707A1 **(E)**. **(F)** CYP707A mainly catabolize ABA to form PA and also produced neoPA as a result of minor enzymatic reaction.

Fig S1. Comparison of amino acid sequence of CYP707A and its splice variant form. CYP707A1S and CYP707A3S indicate splice variant forms of CYP707A1 and CYP707A3, respectively. SRS indicates putative substrate recognition site.

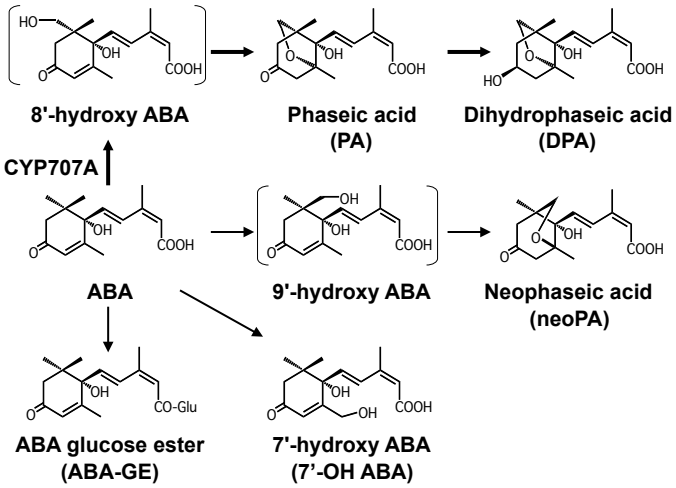
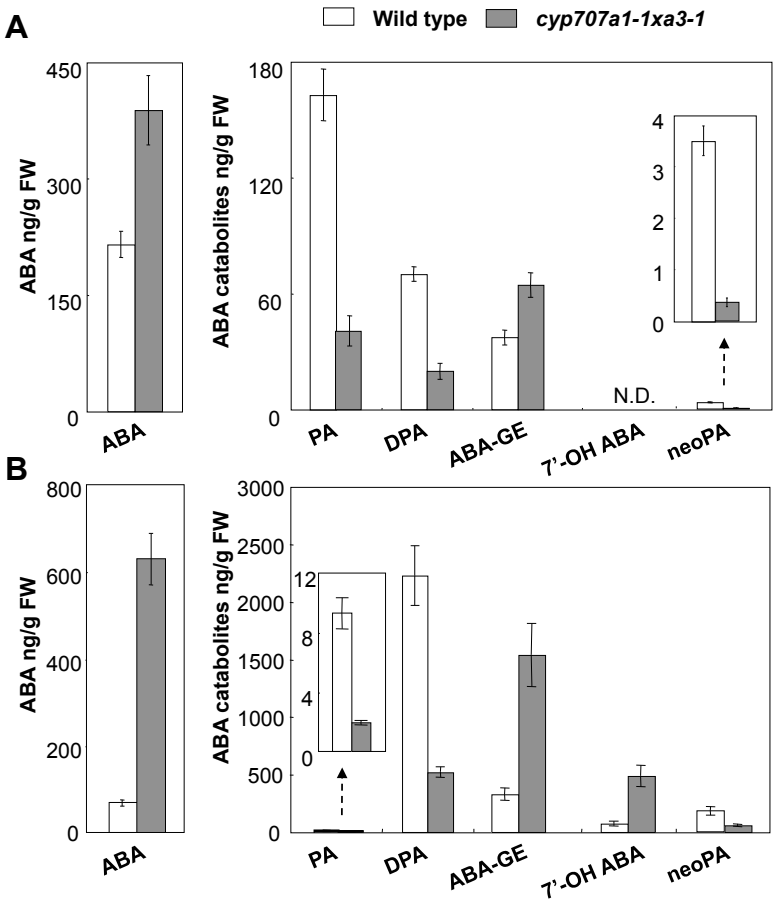


Figure 1. Okamoto et al.,



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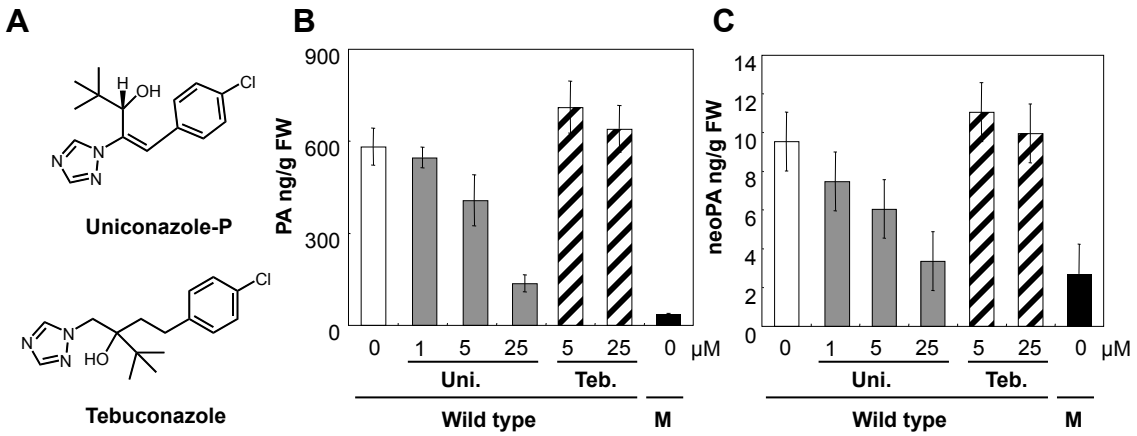

Figure 3. Okamoto et al.,

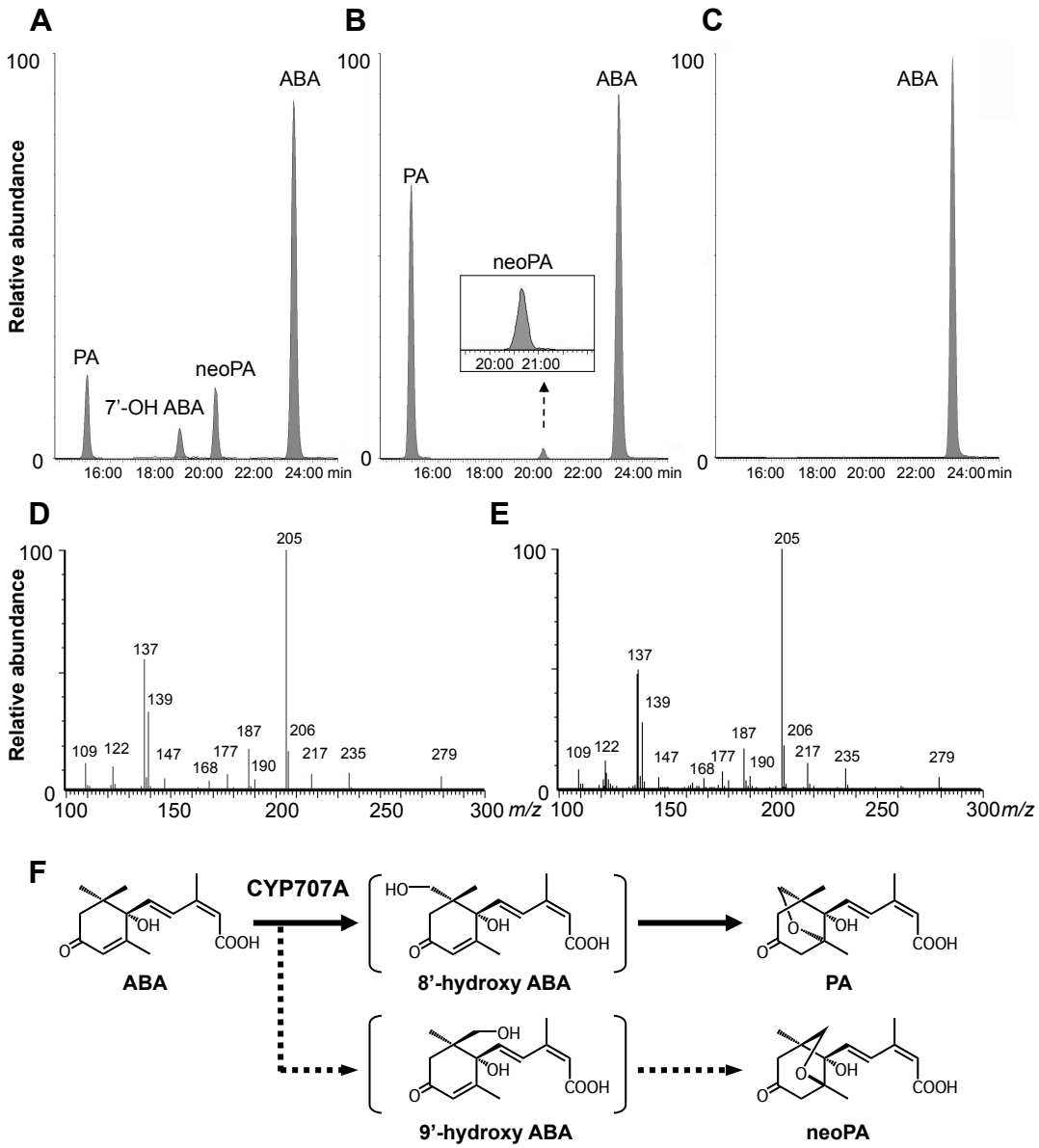

Figure 4. Okamoto et al.,

Figure S1. Okamoto et al.,