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GH3 Auxin-Amido Synthetases Alter the Ratio of Indole-3-Acetic Acid and Phenylacetic Acid in Arabidopsis

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Auxin is the first discovered plant hormone and is essential for many aspects of plant growth and development. Indole-3-acetic acid (IAA) is the main auxin and plays pivotal roles in intercellular communication through polar auxin transport. Phenylacetic acid (PAA) is another natural auxin that does not show polar movement. Although a wide range of species have been shown to produce PAA, its biosynthesis, inactivation and physiological significance in plants are largely unknown. In this study, we demonstrate that overexpression of the *CYP79A2* gene, which is involved in benzylglucosinolate synthesis, remarkably increased the levels of PAA and enhanced lateral root formation in Arabidopsis. This coincided with a significant reduction in the levels of IAA. The results from auxin metabolite quantification suggest that the PAA-dependent induction of *GRETCHEN HAGEN 3* (*GH3*) genes, which encode auxin-amido synthetases, promote the inactivation of IAA. Similarly, an increase in IAA synthesis, via the indole-3-acetaldoxime pathway, significantly reduced the levels of PAA. The same adjustment of IAA and PAA levels was also observed by applying each auxin to wild-type plants. These results show that GH3 auxin-amido synthetases can alter the ratio of IAA and PAA in plant growth and development.

Keywords: Arabidopsis • Auxin • Biosynthesis • Inactivation • Indole-3-acetic acid • Phenylacetic acid.

Introduction

Auxin is a chemical signal that regulates embryogenesis, organogenesis, gravitropism and response to light by modulating the expression of various genes in plants (Davies 2010). Indole-3-acetic acid (IAA) is the most well-studied auxin, and a dynamic change in its cellular concentration triggers cell elongation and differentiation. Previous studies have demonstrated that IAA concentrations in plants are strictly controlled by its biosynthesis, inactivation and transport (Korasick et al. 2013, Kasahara 2016, Casanova-Sáez and Voß 2019). IAA is mainly synthesized by a two-step pathway in land plants. The TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family enzymes convert Trp to indole-3-pyruvic acid (IPA), and the YUCCA (YUC) family enzymes catalyze the subsequent reaction of IPA to IAA (**Fig. 1A**) (Stepanova et al. 2008, Tao et al. 2008, Mashiguchi et al. 2011, Won et al. 2011). Multiple knockout mutants of *TAA* or *YUC* genes display IAA-deficient phenotypes in embryogenesis, flower formation and root development (Cheng et al. 2006, Stepanova et al. 2008).

Phenylacetic acid (PAA) has been known as a plant growth substance for >80 years (Haagen-Smit and Went 1935). PAA has also been detected in a wide variety of plant species (Wightman and Lighty 1982, Korasick et al. 2013, Sugawara et al. 2015). PAA displays less auxin activity than IAA in most plant systems (Haagen-Smit and Went 1935, Muir et al. 1967, Cook 2019), although endogenous concentrations can be 10- to >100-fold greater than IAA in some plants (Wightman and Lighty 1982, Sugawara et al. 2015). Similar to IAA, PAA regulates the expression of various auxin responsive genes through a signaling pathway involving the auxin co-receptors, TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX and AUXIN/IAA (Shimizu-Mitao and Kakimoto 2014, Sugawara et al. 2015). In addition, both IAA and PAA are metabolized to corresponding amino acid conjugates by auxin-amido synthetases, which are encoded by the *GRETCHEN HAGEN 3* (*GH3*) genes (**Fig. 1A, B**) (Staswick et al. 2005, Sugawara et al. 2015). Intriguingly, several feeding studies have demonstrated that, in plants, ^{14}C-labeled PAA is not transported in a polar manner, unlike IAA (Prochàzka and Borkovec 1984, Suttle and Mansager 1986, Morris and Johnson 1987). Furthermore, endogenous IAA is distributed in a gravity-dependent manner in maize coleoptiles, whereas PAA is not, suggesting distinct physiological roles for these auxins in plant growth and development (Sugawara et al. 2015). Based on this evidence, we

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recently proposed that PAA may play a role in the maintenance of steady-state auxin levels required for the preservation of cellular activity in plants (Mashiguchi et al. 2019).

Although the main biosynthetic pathway of IAA has been solved, it is still unclear how plants produce PAA. Previous studies suggest that PAA is possibly produced from Phe via phenylpyruvate (PPA) by transamination and decarboxylation, similar to the IPA-dependent IAA biosynthesis pathway (Wightman and Rauthan 1974, Sugawara et al. 2015, Cook et al. 2016). Biochemical studies have shown that the TAA1 enzyme can catalyze the conversion of Phe to PPA and that YUC enzymes can mediate the subsequent conversion of PPA to PAA in vitro (**Fig. 1B**) (Tao et al. 2008, Dai et al. 2013). In addition, overexpression of *YUC* genes leads to the accumulation of PAA and its amino acid conjugates in Arabidopsis (Sugawara et al. 2015). However, endogenous levels of PAA were only slightly reduced in the *TAA1*-deficient *wei8-1* mutant and were not reduced in multiple *yuc1 yuc2 yuc6*-knockout mutants, suggesting that different genes or pathways may be involved in PAA biosynthesis (Sugawara et al. 2015). Cook et al. (2016) suggested that *TAA1*-related genes are unlikely to contribute to PAA synthesis in vivo, since the pea *tar2* mutant (*Pstar2*) shows a reduction in IAA, but not PAA, and proposed that other aminotransferase enzymes may be responsible for the conversion of Phe to PPA.

In Arabidopsis, IAA is partially produced from Trp via the indole-3-acetaldoxime (IAOx) pathway (**Fig. 1A**). In this pathway, cytochrome P450 monooxygenases, CYP79B2 and CYP79B3, convert Trp to IAOx (Hull et al. 2000, Mikkelsen et al. 2000). Sugawara et al. (2009) showed that the putative metabolites, indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM), are reduced in the *cyp79b2 cyp79b3* mutants. They also showed substantial ^{13}C labeling of these two compounds following a feeding of $^{13}C_6$-IAOx. The conversion of IAOx to IAN was also shown by Nafisi et al. (2007) who demonstrate that CYP71A13 catalyzes this reaction in vitro. The subsequent reactions of IAN and IAM to IAA are reportedly catalyzed by the nitrilase and amidase enzymes, respectively (Pollmann et al. 2002, Lehmann et al. 2010). While not the main biosynthetic pathway, the IAOx pathway has been suggested as a *Brassicaceae*-specific IAA biosynthesis pathway, since the *CYP79B* subfamily does not contain direct homologs, and IAOx has not been detected, in other higher plants (Hull et al. 2000, Mikkelsen et al. 2000, Zhao et al. 2002, Sugawara et al. 2009).

IAOx is also a key intermediate of indole glucosinolates (IG), plant defense compounds that deter pathogens and herbivores (Grubb and Abel 2006, Halkier and Gershenzon 2006). In the IG biosynthetic pathway, IAOx is further metabolized to indole *S*-alkylthiohydroximate and indole *S*-thiohydroximate by CYP83B1/SUPERROOT 2 (SUR2) and *C-S* lyase/SUPERROOT 1 (SUR1), respectively. (**Fig. 1A**) (Bak and Feyereisen 2001, Hemm et al. 2003, Mikkelsen et al. 2004, Malka and Cheng 2017). *sur1*- and *sur2*-knockout mutants accumulate IAA and show high-auxin phenotypes such as hypocotyl elongation and epinastic cotyledons (Bak and Feyereisen 2001, Sugawara et al. 2009). This evidence led to the suggestion that blocking metabolic flux to IG increases IAA synthesis from IAOx (Boerjan et al. 1995, Barlier et al. 2000, Maharjan et al. 2014). Recently, Kong et al. (2015) demonstrated that elevated temperature enhances IAA synthesis via IAOx. They showed that under high-temperature conditions, expression of *SUR1* was suppressed by endogenous miR10515, resulting in increased IAA levels. These findings indicate the physiological importance of the IAOx pathway in heat-dependent auxin biosynthesis in Arabidopsis (Kong et al. 2015).

Similar to IG, benzylglucosinolate (BG) is synthesized from phenylacetaldoxime (PAOx) as a plant defense compound in Arabidopsis (Halkier and Gershenzon 2006). It has been demonstrated that CYP79A2 selectively converts Phe to PAOx (Wittstock and Halkier 2000) and that SUR2 and SUR1 further metabolize PAOx to benzyl *S*-alkylthiohydroximate and benzyl *S*-thiohydroximate, respectively (**Fig. 1B**) (Halkier and Gershenzon 2006). The *CYP79A2* overexpression lines show enhanced herbivore resistance due to the accumulation of BG (Wittstock and Halkier 2000, Bejai et al. 2012). Based on the similarities of IG and BG biosynthesis (via IAOx and PAOx), and given the accumulation of IAA in *CYP79B* overexpression lines, PAA may also be produced from an analogous pathway in Arabidopsis as previously suggested (Irmisch et al. 2015).

In this article, we demonstrate the integrated feedback of endogenous levels of two auxins. We show that overexpression of *CYP79A2* increases the levels of PAA via a PAOx-dependent pathway and promotes lateral root formation, despite reducing the levels of IAA. In the same fashion, an increase in IAA levels, via upregulation of the IAOx pathway, reduces the levels of PAA. Finally, we conclude that the GH3 auxin-amido synthetases can alter the ratio of IAA and PAA levels in Arabidopsis.

Results

Overexpression of *CYP79A2* selectively accumulates PAA in Arabidopsis

To investigate whether PAA is synthesized from Phe via PAOx in Arabidopsis, we generated transgenic plants harboring the *CYP79A2* gene in an estradiol-inducible expression system using a pMDC7 vector (*CYP79A2ox*). In this system, the transgene is strongly and ubiquitously induced in plants (Curtis and Grossniklaus 2003). We analyzed *CYP79A2ox* plants in which the levels of *CYP79A2* transcripts were increased over 1,000-fold compared with vector control plants (*pER8*) in Murashige and Skoog (MS) medium containing β-estradiol (**Supplementary Fig. S1**). *CYP79A2ox* plants showed a significant increase in lateral root formation compared with *pER8* plants, which may suggest an accumulation of auxin (**Fig. 2A, B**). However, there was no difference in hypocotyl length or an obvious shoot phenotype (**Supplementary Fig. S2A**, B). We quantified the endogenous levels of the two auxins and their respective amino acid conjugates (Asp and Glu) in *CYP79A2ox* seedlings using liquid chromatography–tandem mass spectroscopy (LC–MS/

MS) (Fig. 2C). Analysis of auxin metabolites indicated that the levels of PAA were dramatically increased (18.6-fold) in *CYP79A2ox* (Fig. 2C). Moreover, PAA-aspartate (PAA-Asp) and PAA-glutamate (PAA-Glu) levels increased by nearly 100-fold. In contrast, IAA levels were decreased in *CYP79A2ox* (<70%), although IAA-aspartate (IAA-Asp) and IAA-glutamate (IAA-Glu) levels were slightly increased (1.6- and 1.3-fold, respectively).

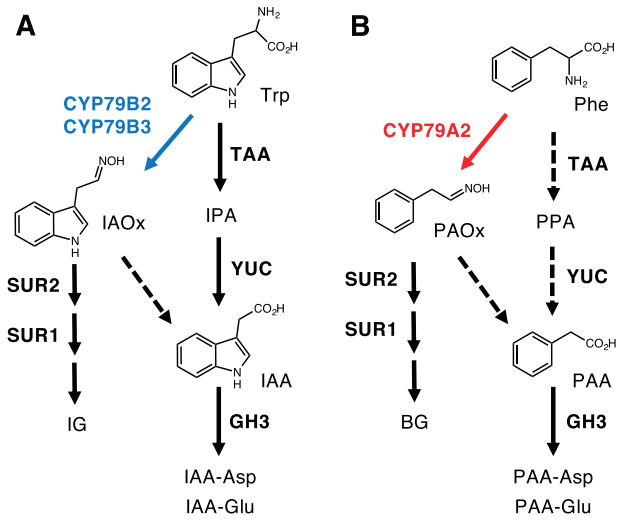

Fig. 1 Auxin biosynthesis and inactivation pathways and glucosinolate biosynthesis pathways in Arabidopsis. (A) Current IAA and IG pathways. (B) The PAA and BG pathways proposed in this study (dotted square). CYP79B2 and CYP79B3 catalyze the conversion of Trp to IAOx (blue arrow), and CYP79A2 mediates the conversion of Phe to PAOx (red arrow). The dashed arrows represent metabolic pathways in which enzymatic steps are still unknown.

PAA derived from the PAOx pathway induces the expression of *GH3* genes

A reduction in IAA levels and an increase in IAA- and PAA-amino acid conjugate levels in *CYP79A2ox* plants suggest that auxin metabolic genes are playing crucial roles in IAA and PAA homeostasis. To test this hypothesis, we analyzed the expression of *GH3* genes by quantitative RT-PCR (qPCR). In Arabidopsis, the group II *GH3*s are known to be early auxin responsive genes encoding the enzymes which catalyze the conversion of IAA to IAA-Asp in vitro (Staswick et al. 2005). We have previously shown that the expression of *GH3.2*, *GH3.3*, *GH3.4* and *GH3.5* was remarkably induced by both IAA and PAA treatments (Sugawara et al. 2015). Based on these findings, we analyzed the expression levels of *GH3.1* to *GH3.6* in *CYP79A2ox*. As expected, the expression levels of *GH3.3* and *GH3.5* were substantially elevated in *CYP79A2ox* plants by 4- and 2-fold, respectively, compared with *pER8* (Fig. 3). Specific analysis of the *GH3.2* and *GH3.4* transcripts was not possible due to high sequence similarity; however, their combined expression (*GH3.2*/*GH3.4*) was elevated 10-fold. The expression of both *GH3.1* and *GH3.6* was also slightly elevated, although not substantially (Fig. 3). Given that both PAA-Glu and IAA-Glu

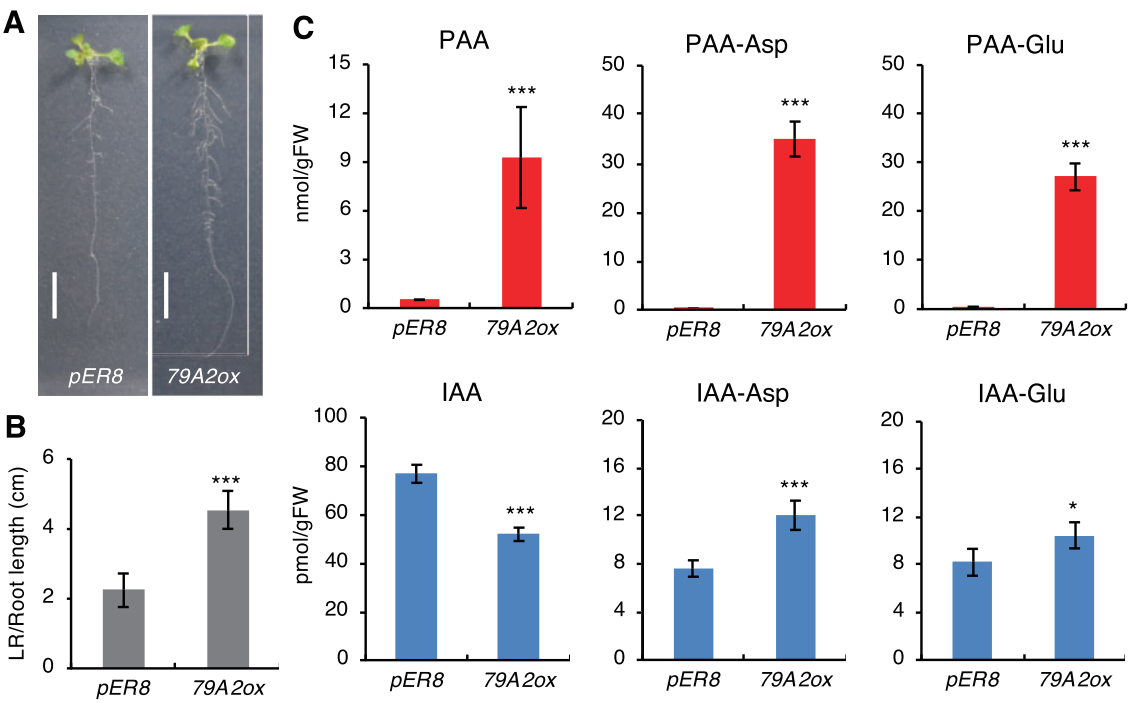

Fig. 2 Phenotypes and auxin metabolite levels of *CYP79A2ox* plants. (A) Phenotypes of *pER8* and *CYP79A2ox* (*79A2ox*) plants (10-day-old). Scale bars indicate 1 cm. (B) The number of lateral roots (LR) per the primary root length (cm). Values are mean ± SD ($n = 50$). (C) Auxin metabolite levels in *pER8* and *79A2ox* plants. Values are mean ± SD ($n = 4$). Differences between *pER8* and *79A2ox* plants are statistically significant at $P < 0.05$ (*$P < 0.05$ and ***$P < 0.001$, Student's *t*-test). Note that PAA metabolites are shown in nmol/gFW and IAA metabolites are in pmol/gFW. gFW, gram fresh weight.

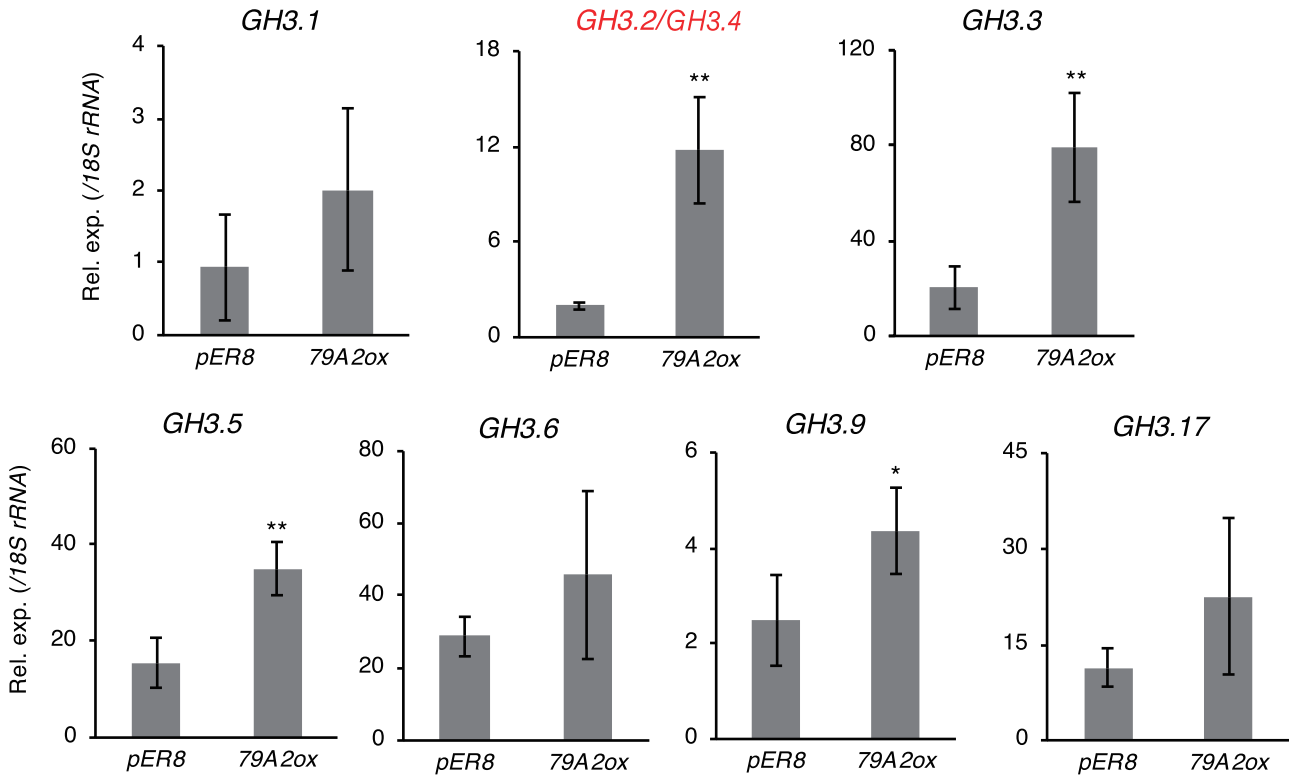

Fig. 3 *GH3* gene expression levels in *CYP79A2ox* plants. Expression levels of *GH3.1*, *GH3.2*, *GH3.3*, *GH3.5*, *GH3.6*, *GH3.9* and *GH3.17* genes in *pER8* and *CYP79A2ox* (*79A2ox*) plants (10-day-old). *18S rRNA* gene is used for normalization. Values are mean ± SD ($n = 4$). Differences between *pER8* and *79A2ox* plants are statistically significant at $P < 0.05$ (*$P < 0.05$ and **$P < 0.01$, Student's *t*-test).

accumulated in *CYP79A2ox* plants (**Fig. 2C**), we also analyzed the expression levels of *GH3.9* and *GH3.17* genes, which encode auxin-amido synthetases that preferentially conjugate IAA to Glu (Staswick et al. 2005, Sugawara et al. 2015, Zheng et al. 2016). We observed an upregulation of *GH3.9* transcripts in *CYP79A2ox* plants, indicating that GH3.9 likely inactivated PAA and IAA (**Fig. 3**). This result is consistent with our previous study demonstrating a significant accumulation of both PAA- and IAA-Glu in *GH3.9* overexpression lines (Sugawara et al. 2015). The expression of *GH3.17* was also slightly increased, although similar to *GH3.1* and *GH3.6*, this was not significant. As such, it is possible that *GH3.17* expression is not strongly controlled by the levels of endogenous auxins or that other hormones may be involved (**Fig. 3**).

Previous studies have reported that several IAA biosynthetic genes are downregulated following treatment with synthetic auxins (Suzuki et al. 2015, Takato et al. 2017). This could explain the reduction in IAA levels in the *CYP79A2ox* plants. To explore this, we analyzed the expression levels of *TAA1*, *TAR2*, *CYP79B2*, *YUC1*, *YUC2*, *YUC4* and *YUC6*. However, there was no obvious difference in their expression in *CYP79A2ox* plants compared with *pER8* plants (**Supplementary Fig. S3**). These results suggest that induction of the *GH3*s, rather than negative feedback on *TAA*s or *YUC*s, is the primary driver of auxin homeostasis in *CYP79A2ox* plants.

Overexpression of *CYP79B2* accumulates IAA, but not PAA

To assess if *CYP79B2* also contributes to PAA synthesis, we further generated transgenic Arabidopsis expressing the *CYP79B2* gene with the estradiol-inducible expression system (*CYP79B2ox*). The expression of *CYP79B2* was elevated 10-fold in *CYP79B2ox* lines compared with *pER8* plants (**Supplementary Fig. S1**). The *CYP79B2ox* plants also showed a significant increase in the number of lateral roots (**Fig. 4A, B**), similar to *CYP79A2ox* (**Supplementary Fig. S2A**, B). LC–MS/MS analysis showed that the endogenous levels of IAA and IAA-Asp were slightly increased in *CYP79B2ox* plants compared with *pER8* plants, confirming previous findings with *35S* overexpression lines (**Fig. 4C**) (Zhao et al. 2002). In contrast, PAA levels were not different in these experiments. This is consistent with previous findings on glucosinolate biosynthesis (Wittstock and Halkier 2000), where CYP79A2 and CYP79B2 possess strict substrate specificity toward PAA and IAA, respectively.

IAA levels are selectively increased in the glucosinolate-deficient *sur2* mutants

The *sur2* knockout mutants show high-auxin phenotypes including epinastic cotyledons, elongated hypocotyls and increased lateral and adventitious roots, due to a redirection of metabolic flux away from the IG biosynthetic pathway,

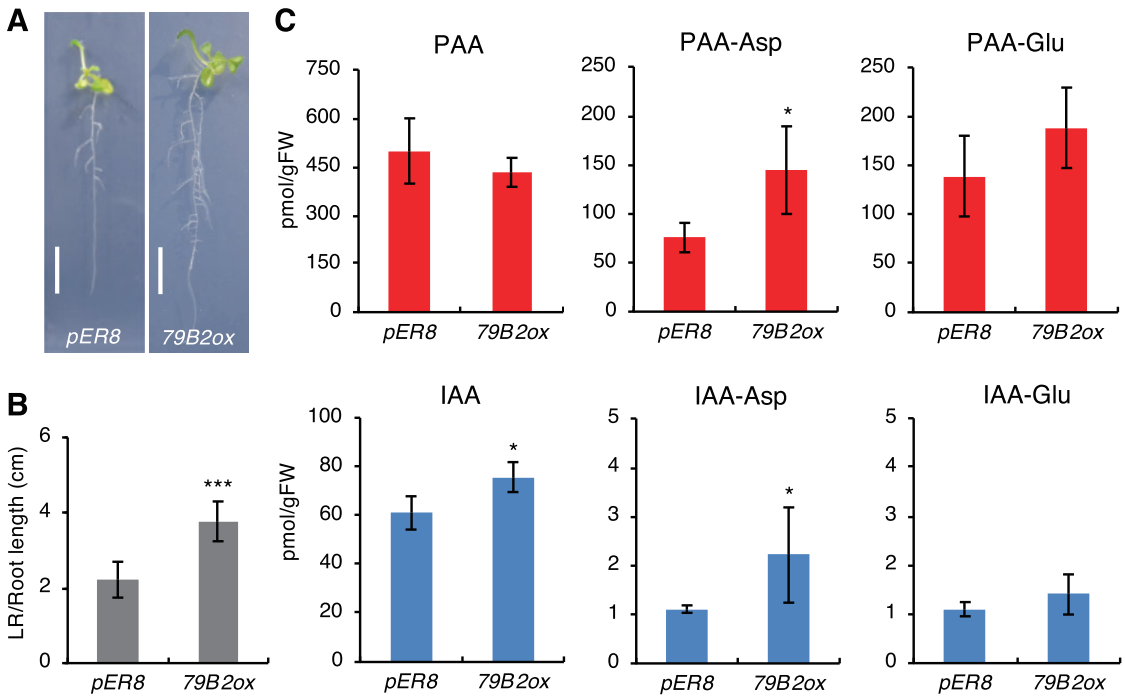

Fig. 4 Phenotypes and auxin metabolite levels of *CYP79B2ox* plants. (A) Phenotypes of *pER8* and *CYP79B2ox* (*79B2ox*) plants (10-day-old). Scale bars indicate 1 cm. (B) The number of lateral roots (LR) per the primary root length (cm). Values are mean ± SD ($n = 50$). (C) Auxin metabolite levels in *pER8* and *79B2ox* plants. Values are mean ± SD ($n = 4$). Differences between *pER8* and *79B2ox* plants are statistically significant at $P < 0.05$ (*$P < 0.05$ and ***$P < 0.001$, Student's *t*-test).

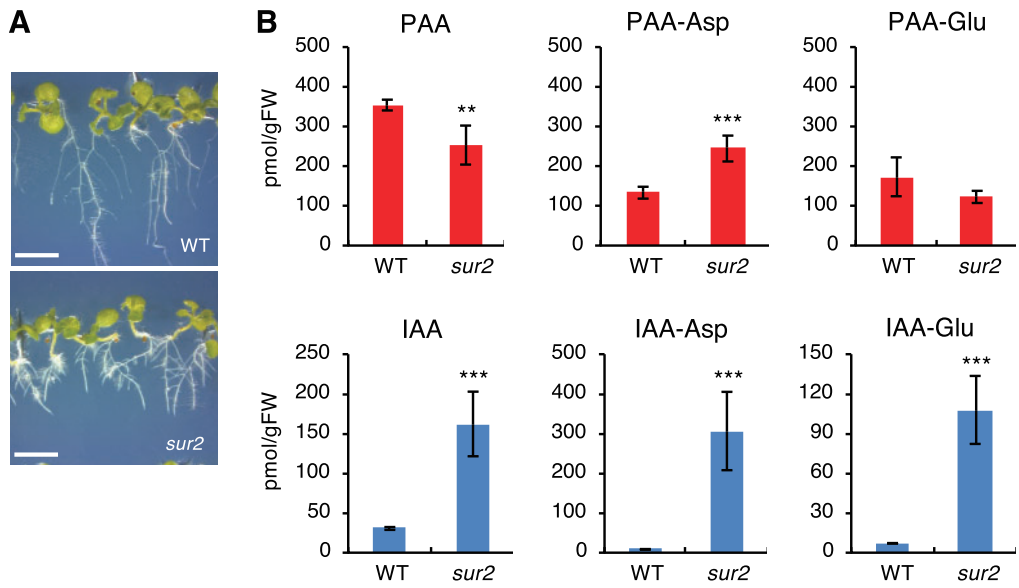

Fig. 5 Auxin metabolite levels in *sur2* mutants. (A) Phenotypes of WT and *sur2* mutants (8-day-old). Scale bars indicate 5 mm. (B) The levels of IAA, PAA and their conjugates (IAA-Asp, IAA-Glu, PAA-Asp, PAA-Glu) in WT and *sur2* mutants (8-day-old). Values are mean ± SD ($n = 4$). Differences between WT and *sur2* mutants are statistically significant at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, Student's *t*-test).

toward IAA from IAOx (Malka and Cheng 2017) (**Fig. 5A**). As such, we investigated whether accumulation of IAA from the IAOx pathway can reduce endogenous PAA levels. As predicted, the PAA levels were significantly reduced in *sur2* (**Fig. 5B**). We also detected a substantial increase in endogenous PAA-Asp in the same plants (**Fig. 5B**). The levels of IAA, IAA-Asp and IAA-Glu were dramatically increased in *sur2* mutants compared with wild-type (WT) plants, as previously reported (**Fig. 5B**)

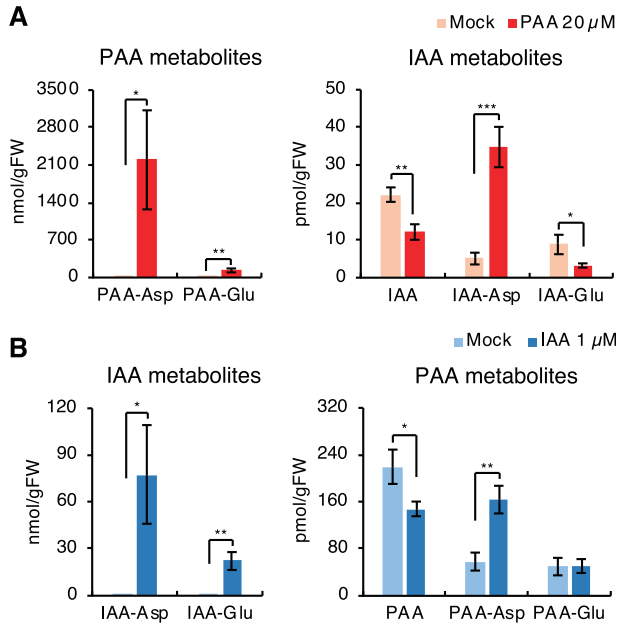

Fig. 6 Effect of PAA and IAA treatments on auxin metabolites in Arabidopsis. Twelve-day-old WT seedlings were treated with 20 μM of PAA or 1 μM of IAA for 24 h. (A) Auxin metabolites levels in PAA-treated plants. (B) Auxin metabolite levels in IAA-treated plants. Values are mean ± SD ($n = 3$). Differences between mock and auxin-treated plants are statistically significant at P-value <0.05 (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, Student's t-test).

(Barlier et al. 2000, Novák et al. 2012). These data are consistent with the hypothesis that induction of the *GH3*s by IAA promotes the metabolism of both IAA and PAA to their amino acid conjugates.

Application of PAA or IAA can reciprocally reduce auxin levels

Our current results from the analysis of *CYP79A2ox* plants and *sur2* mutants suggest that the accumulation of PAA (or IAA) can reduce the levels of IAA (or PAA) in a GH3-dependent manner. To confirm this metabolic feedback, we investigated whether application of one auxin would reduce the endogenous levels of the other. We treated WT plants with PAA (20 μM) or IAA (1 μM) for 24 h in liquid media and analyzed their metabolites using LC–MS/MS. Similar to the *CYP79A2ox* plants, PAA-Asp and PAA-Glu accumulated remarkably (38,000- and 2,700-fold, respectively) in PAA-treated plants (**Fig. 6A**). As we predicted, the levels of IAA-Asp were also substantially increased (6.3-fold), whereas IAA and IAA-Glu levels were reduced (55% and 40% of WT, respectively) following PAA treatment (**Fig. 6A**). This shows that the induction of particular GH3 enzymes by PAA can promote the metabolism of IAA. In the IAA-treated plants, both IAA-Asp and IAA-Glu were increased by 15,000- and 2,500-fold, respectively (**Fig. 6B**). Similar to the above results, when seedlings were treated with IAA, the levels of PAA were reduced by 33% while PAA-Asp was elevated 2.8-fold. These results provide further evidence that the PAA and IAA levels are coordinately regulated in Arabidopsis.

The PAOx pathway does not appear to be the main PAA biosynthesis pathway

Our evidence thus far suggested that CYP79A2 may play a role in PAA biosynthesis. Yet, as the PAOx pathway is analogous to the auxiliary IAOx pathway for IAA biosynthesis, it was important to confirm if the PAOx pathway is a major contributor to PAA levels. We carried out phenotypic analysis and auxin metabolite measurements of *cyp79a2*-knockout mutants. Although overexpression of *CYP79A2* resulted in high-auxin phenotypes, *cyp79a2* mutants did not show any phenotype (**Supplementary Fig. S4A**, B). In addition, the endogenous levels of auxin metabolites including PAA and IAA were not reduced in *cyp79a2* mutants compared with WT (**Supplementary Fig. S4C**). These results infer that PAA is not primarily produced through the CYP79A2-mediated PAOx pathway in Arabidopsis.

Discussion

The *GH3* family alters the ratio of IAA and PAA in Arabidopsis

The group II *GH3* gene family encodes auxin-amido synthetases that play a key role in auxin homeostasis. Some IAA-amino acid conjugates, including IAA-Ala and IAA-Leu, are recognized as storage forms of IAA (Staswick et al. 2005). Auxin-amido hydrolases such as IAA-Leu RESISTANT 1 and IAA-Ala RESISTANT 3 can hydrolyze IAA-Leu and IAA-Ala to IAA in Arabidopsis (Staswick et al. 2005, Korasick et al. 2013). Whereas IAA-Asp and IAA-Glu have been recognized as irreversible IAA metabolites (Korasick et al. 2013). Zheng et al. (2016) demonstrated that *vas2* mutants, which have a non-functional GH3.17, show hypocotyl elongation, due to an increase in IAA levels with a significant decrease in IAA-Glu levels in hypocotyl epidermal regions. The study of *vas2* mutants clearly showed that local auxin metabolism, mediated by GH3.17, in coordination with IAA biosynthesis, is crucial for modulation of the spatial distribution of IAA in response to environmental stimuli. Similarly, GH3.17 (and GH3.6) is crucial for the maintenance of root meristem size (Di Mambro et al. 2017). The same group also demonstrated that the expression of *GH3.17* and *GH3.6* was elevated when Arabidopsis roots were treated with the cytokinin, *trans*-zeatin (Pierdonati et al. 2019). The absence of a strong auxin response from *GH3.17* and *GH3.6* under elevated auxin conditions indicates a more complex regulation of these particular *GH3*s, whose expression may also be controlled by other plant hormones. Alternatively, our use of whole seedlings, which may obscure tissue specific response, could account for variation in gene expression. Especially considering other auxin related genes, the *YUC* genes vary considerably in their expression patterns (Cheng et al. 2006).

We have demonstrated here, for the first time, that the *GH3* family alters the ratio of IAA and PAA in plants. It is known that GH3 enzymes show low substrate specificity and can form both IAA- and PAA-amino acid conjugates (Staswick et al. 2005, Westfall et al. 2016). In addition, we recently demonstrated that

inducible expression of *YUC* genes or *iaaM*, an auxin biosynthesis gene from *Agrobacterium tumefaciens*, enhances the accumulation of both IAA- and PAA-amino acid conjugates in Arabidopsis and in barley (Sugawara et al. 2015, Mashiguchi et al. 2019). These findings indicate the importance of *GH3* genes in the metabolic regulation of IAA and PAA in higher plants. Our current study demonstrates that elevated production of PAA, through the PAOx pathway, results in the reduction in IAA and a significant increase in IAA-Asp, as well as an upregulation of the *GH3.2/4*, *GH3.3*, *GH3.5* and *GH3.9* genes (Figs 2C, 3). In contrast, downregulation of IAA biosynthetic genes was not observed. This shows that auxin metabolism, rather than biosynthesis, is responsible for the fine-tuning of active hormone levels by auxins themselves. We also found an accumulation of IAA (via the IAOx pathway) in *sur2* mutants, which coincided with a decrease in PAA and a substantial increase in PAA-Asp (**Fig. 5B**). Analysis of auxin metabolite profiles in PAA- and IAA-treated WT plants also supports the finding that GH3 enzymes can balance the ratio of two auxins (**Fig. 6A, B**). From these findings, we propose that the Arabidopsis group II *GH3* gene family is important in the integrated feedback of PAA and IAA.

Since the levels and the ratio of IAA and PAA vary depending on plant tissue type (Schneider and Wightman 1986, Sugawara et al. 2015), the GH3 enzymes likely have extensive control in the coordinated modulation of these two auxins. Comprehensive analysis of *GH3* expression and of auxin metabolic profiles in each plant organ/tissue would be important for understanding the distinct role of individual *GH3* genes in auxin metabolism in plants.

PAA biosynthesis via the PAOx pathway

In this study, we show that PAA can be produced through the CYP79A2-dependent PAOx pathway in Arabidopsis. Overexpression of *CYP79A2* elevated the levels of PAA almost 20-fold compared with *pER8* containing plants. Accumulation of PAA in *CYP79A2ox* plants was much higher than those previously observed in *YUC* overexpression plants (Sugawara et al. 2015). These results suggest that CYP79A2 catalyzes a rate-limiting step in a PAOx-dependent PAA biosynthesis pathway. If this pathway is analogous to the IAOx pathway (Sugawara et al. 2009), then PAOx could be converted to phenylacetonitrile (PAN) or phenylacetamide (PAM) and then to PAA. The characterization of CYP71A13 suggests that in addition to converting IAOx to IAN in camalexin biosynthesis, this enzyme can bind PAOx as a substrate in vitro (Nafisi et al. 2007), although the reaction has not yet been demonstrated.

The nitrilase-type enzymes are implicated in the conversion of IAN to IAA (Vorwerk et al. 2001, Pollmann et al. 2002). In addition, it has been recently shown that PAN is a substrate for the nitrilase enzymes in *Populus trichocarpa* and is converted to PAA (Günther et al. 2018). The PtNIT1 enzyme contains a higher affinity for PAN than IAN, although it is phylogenetically distinct from the Arabidopsis NIT1, 2 and 3. Interestingly, the PtNIT1 enzyme does not produce PAM as an end product, while the core NIT family from Arabidopsis can produce IAM (Pollmann et al. 2002).

Following the production of PAM by the NIT enzymes, it has been demonstrated that a bi-functional plant amidase is capable of converting PAM to PAA in vitro (Sánchez-Parra et al. 2014). These enzymes are widespread and also utilize IAM as a substrate, albeit at a substantially lower efficiency. This is supported by in vivo data in barley and Arabidopsis plants containing the *iaaM* gene, which encodes a tryptophan monooxygenase from *A. tumefaciens* (Mashiguchi et al. 2019). These plants produce PAM directly from Phe, resulting in a substantial increase in both PAA and its conjugates. These findings suggest potential elements of the PAOx-dependent pathway in Arabidopsis.

Despite support for all steps of the PAOx pathway, this method of PAA biosynthesis is not likely to be the main biosynthetic route in plants, as *cyp79a2* mutants display neither significant reduction in PAA levels nor obvious phenotypes under normal growth conditions (**Supplementary Fig. S4A**–C). However, under specific conditions, this pathway is a strong candidate for elevated production of PAA.

The PAOx-dependent PAA biosynthesis pathway is a defense response pathway

The IAOx pathway plays an auxiliary role for IAA biosynthesis in Arabidopsis under normal and high-temperature conditions (Zhao et al. 2002, Sugawara et al. 2009, Kong et al. 2015). *cyp79b2 cyp79b3* double mutants show reduced shoot phenotypes under normal growth conditions, and these phenotypes become more pronounced under high temperature (Zhao et al. 2002). In this article, we show that *cyp79a2* mutants do not show an obvious phenotype or chemotype (**Supplementary Fig. S4A**–C). As such, the physiological significance of PAOx-dependent PAA biosynthesis in plant growth and development remains undefined.

The *CYP79A* subfamily has a wider distribution than the *CYP79B* subfamily in the plant kingdom and has been characterized in Arabidopsis, sorghum and maize (Brader et al. 2006, Irmisch et al. 2015). Brader et al. (2006) demonstrated that Arabidopsis plants constitutively expressing *CYP79A2* or sorghum *SbCYP79A1* genes selectively accumulate BG or *p*-hydroxybenzyl glucosinolate, respectively. This results in increased resistance to the plant pathogen, *Pseudomonas syringae*. In addition, herbivore attack induces the expression of maize *CYP79A61*, resulting in the accumulation of PAOx, IAA and PAA (Irmisch et al. 2015). Although in this case, the levels of the amino acid precursors (Trp and Phe) were also substantially elevated. None the less, these findings indicate that the *CYP79A* subfamily mainly functions in the production of plant defense compounds. As *CYP79A* genes can be induced by herbivore attack, PAA generated from the CYP79A2-dependent pathway might also be elevated due to an increase in the PAOx precursor. Interestingly, the NIT enzymes are also upregulated following herbivory events (Günther et al. 2018). Günther et al. (2018) show that PAN accumulates in herbivore-damaged tissue, which, as previously discussed, is catabolized to PAA by the NIT enzymes. PAN itself also functions as a plant defense compound, but becomes phytotoxic at high concentrations (Urbancsok et al. 2018), explaining the need for PAN turnover.

This increased flux through PAOx (which branches toward benzyl-glucosinolates) and PAN to PAA implicates the PAOx pathway as a herbivory/pathogen-conditional auxin synthesis pathway. However, since Phe levels are also significantly elevated following herbivory, an upregulation of the main, as yet uncharacterized, PAA biosynthesis pathway may still contribute to the accumulation of PAA.

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Col-0 was used as WT plants. Arabidopsis seeds were stratified at 4°C for 2 d in the dark. Seedlings were grown on MS agar medium containing 1% sucrose at 23°C under long-day light conditions with 16-h photoperiod. The *cyp79a2* (GK-913H04) mutants were obtained from the Arabidopsis Biological Resource Center. T-DNA insertions were confirmed by genotyping using the primers described in **Supplementary Table S1**. Homozygous *cyp79a2* mutants were used in the phenotypic analysis and auxin metabolite quantification (**Supplementary Fig. S5**). *sur2*-knockout mutants used in this study were previously reported (Stepanova et al. 2005).

A DNA fragment of *CYP79A2* with its intron was amplified by PCR using genomic DNA from Arabidopsis seedlings as a template, and cDNA of *CYP79B2* was obtained from Arabidopsis seedlings using gene-specific primer pairs listed in **Supplementary Table S1**, respectively. Due to the low expression of *CYP79A2*, we were unable to isolate the coding sequence from synthesized cDNA. As such, it was necessary to use genomic DNA as a template for the *CYP79A2* overexpression construct. Each DNA was cloned into a pENTR/D-TOPO vector (Invitrogen, Waltham, MA, USA) and then transferred into pMDC7 using an LR clonase II (Invitrogen). The *pMDC7:CYP79A2* and *pMDC7:CYP79B2* plasmids were transformed into Arabidopsis plants by floral dipping (Clough and Bent 1998). The homozygous *CYP79A2ox* and *CYP79B2ox* lines with single-copy transgene were selected based on the segregation ratio of hygromycin resistant to susceptible seedlings. For the selection of overexpression lines that produce high levels of auxins, *CYP79A2ox*, *CYP79B2ox* and *pER8* plants were grown vertically on MS agar medium containing 2 μM β-estradiol with or without 5 μM L-kynurenine (Kyn), IAA synthesis inhibitor, for 6 d (He et al. 2011). The induction of auxin biosynthetic gene may restore the root growth defects by Kyn treatment. High and moderate *CYP79A2ox* and *CYP79B2ox* lines were first selected by measuring primary root length (**Supplementary Fig. S6A**). *CYP79A2ox* and *CYP79B2ox* lines were further isolated by analyzing gene expression levels (**Supplementary Fig. S6B**). *CYP79A2ox_23-1* and *CYP79B2ox_28-1* were used for phenotypic analysis, auxin metabolite measurements and gene expression analysis.

Whole seedlings were used for auxin metabolite measurements and gene expression analysis. For estradiol treatment, *CYP79A2ox*, *CYP79B2ox* and *pER8* plants (Mashiguchi et al. 2011) were grown for 6 d on MS agar medium, transferred to MS agar medium containing 2 μM β-estradiol and grown for 4 d.

Auxin treatments were conducted on 10-day-old WT seedlings. Plants were transferred to MS liquid medium (30 ml) and cultured for 2 d with rotation (150 rpm). Seedlings were then treated with PAA (20 μM), IAA (1 μM) or mock solution (100% ethanol) with three replications. After 24 h, seedlings were harvested and frozen at −80°C until auxin metabolite measurements.

qRT-PCR analysis

Total RNA was isolated from β-estradiol-treated *pER8*, *CYP79A2ox* and *CYP79B2ox* plants using RNeasy (Qiagen, Venlo, Netherlands). The PrimeScript™ RT reagent kit with gDNA eraser (Takara Bio Inc., Kusatsu, Japan) was used to generate first-strand cDNA. qPCR was performed on a G8830A AriaMx Real-time PCR system (Agilent Technologies, Inc., Santa Clara, USA) using a THUNDERBIRD SYBR qPCR mix (Toyobo) and specific primers listed in **Supplementary Table S1**.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, Mi, USA) unless otherwise stated. [phenyl-$^{13}C_6$]IAA was purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). [phenyl-$^{13}C_6$]PAA, [$^{13}C_4$, ^{15}N]IAA-Asp, [$^{13}C_5$, ^{15}N]IAA-Glu, [$^{13}C_4$, ^{15}N]PAA-Asp and [$^{13}C_5$, ^{15}N]PAA-Glu were previously synthesized (Mashiguchi et al. 2011, Sugawara et al. 2015).

LC–MS/MS analysis of auxin metabolites

Frozen plant materials (ca. 30 mg, 5–10 seedlings) were prepared in four replicates and homogenized with zirconia beads (3 mm) in 0.3 ml of 80% acetonitrile/1% acetic acid/H_2O containing [phenyl-$^{13}C_6$]IAA, [phenyl-$^{13}C_6$]PAA, [$^{13}C_4$, ^{15}N]IAA-Asp, [$^{13}C_5$, ^{15}N]IAA-Glu, [$^{13}C_4$, ^{15}N]PAA-Asp and [$^{13}C_5$, ^{15}N]PAA-Glu using a Tissue Lyser (Qiagen) for 3 min. Extracts were centrifuged at 13,000 × *g* for 5 min at 4°C, and the supernatant was collected. Extraction was repeated twice without internal standards. Extracts were combined, 1% acetic acid/H_2O (1 ml) was added and the total volume was reduced to <1 ml by evaporation using a Speed Vac (Thermo Fisher Scientific, Waltham, MA, USA). Concentrated extracts were loaded onto an Oasis HLB column (1 ml; Waters, Milford, MA, USA) and then washed with 1% acetic acid/H_2O (1 ml). IAA, PAA and their metabolites were eluted with 80% acetonitrile/1% acetic acid/H_2O (1 ml). After the addition of 1% acetic acid/H_2O (1 ml) to the eluted fraction, the volume was reduced to <1 ml by the evaporation of acetonitrile using a Speed Vac. The eluted fractions were loaded onto an Oasis WAX column (1 ml). After washing with 1% acetic acid/H_2O (1 ml) and subsequent washing with 80% acetonitrile/H_2O (2 ml), IAA, PAA and oxIAA were eluted with 80% acetonitrile/1% acetic acid/H_2O (1 ml). IAA-Asp, IAA-Glu, PAA-Asp and PAA-Glu were eluted from the column by 80% acetonitrile/0.8% formic acid/H_2O (2 ml). Each fraction was evaporated to dryness using a Speed Vac. Then, fractions were redissolved in 1% acetic acid/H_2O (30 μl) and injected into an Agilent 6420 Triple Quad system (Agilent Technologies, Inc., Santa Clara, CA, USA) with a ZORBAX Eclipse XDB-C18 column (1.8 mm, 2.1 mm × 50 mm). The HPLC separation and MS/MS analysis conditions are shown in **Supplementary Table S2**.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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