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ORIGINAL ARTICLE

The phenylalanine ammonia-lyase inhibitor AIP induces rice defence against the root-knot nematode *Meloidogyne graminicola*

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Abstract

The phenylalanine ammonia-lyase (PAL) enzyme catalyses the conversion of L-phenylalanine to *trans*-cinnamic acid. This conversion is the first step in phenylpropanoid biosynthesis in plants. The phenylpropanoid pathway produces diverse plant metabolites that play essential roles in various processes, including structural support and defence. Previous studies have shown that mutation of the *PAL* genes enhances disease susceptibility. Here, we investigated the functions of the rice *PAL* genes using 2-aminoindan-2-phosphonic acid (AIP), a strong competitive inhibitor of PAL enzymes. We show that the application of AIP can significantly reduce the PAL activity of rice crude protein extracts in vitro. However, when AIP was applied to intact rice plants, it reduced infection of the root-knot nematode *Meloidogyne graminicola*. RNA-seq showed that AIP treatment resulted in a rapid but transient upregulation of defence-related genes in roots. Moreover, targeted metabolomics demonstrated higher levels of jasmonates and antimicrobial flavonoids and diterpenoids accumulating after AIP treatment. Furthermore, chemical inhibition of the jasmonate pathway abolished the effect of AIP on nematode infection. Our results show that disturbance of the phenylpropanoid pathway by the PAL inhibitor AIP induces defence in rice against *M. graminicola* by activating jasmonate-mediated defence.

KEYWORDS
diterpenoids, flavonoids, jasmonate, phenylpropanoids, phytoalexins, plant defence, salicylic acid

1 | INTRODUCTION

Phenylalanine ammonia-lyase (PAL) is a well-studied enzyme in the phenylpropanoid pathway. PAL catalyses the nonoxidative elimination of ammonia from L-phenylalanine to produce *trans* (*t*)-cinnamic acid and it is the first committed enzyme in the phenylpropanoid pathway (Jun et al., 2018). This pathway produces a wide array of specialized plant metabolites, including phenolic acids, hydroxycinnamic acid esters, monolignols, coumarins, stilbenes and flavonoids. This pathway is very important for most land plants because

monolignols serve as building blocks for lignin, which is an essential polymer needed for structural support, vascular integrity and pathogen resistance (Boerjan et al., 2003). Furthermore, many phytoalexins, metabolites that rapidly accumulate upon pathogen infection and play a role in plant defence, are also derived from the phenylpropanoid pathway (Deng & Lu, 2017).

After the deamination of phenylalanine by PAL, the product *t*-cinnamic acid is subsequently hydroxylated at the *para* (*p*) position to form *p*-coumaric acid by the enzyme cinnamate 4-hydroxylase (C4H). Then, *p*-coumaric acid is converted to *p*-coumaroyl-CoA by 4-coumarate-CoA ligase (4CL). These first three steps are conserved in plants, which provides the basis for all subsequent branches and downstream metabolites (Vogt, 2010; Yadav et al., 2020). The lignin biosynthesis pathway starts from the common precursor *p*-coumaroyl-CoA following multistep enzymatic reactions, synthesizing different monolignols. The monolignols coniferyl alcohol and sinapyl alcohol generate the guaiacyl (G) and syringyl (S) units, respectively, while *p*-coumaryl alcohol generates the *p*-hydroxyphenyl (H) units for the lignin polymer (Miedes et al., 2014). Several phenolic acids and hydroxycinnamic acid esters are synthesized in the lignin pathway as side products.

PAL also plays a role in the biosynthesis of the plant hormone salicylic acid (SA). In plants, SA can be synthesized through the isochorismate synthase (ICS) or the PAL pathway (Lefevere et al., 2020). The relative importance of these two pathways in SA biosynthesis differs between plant species. In the rice PAL pathway for SA biosynthesis, *t*-cinnamic acid is converted to benzoic acid by abnormal inflorescence meristem1 (AIM1) (Xu et al., 2023). Benzoic acid is then converted to SA by a presumed benzoic acid 2-hydroxylase (BA2H) (León et al., 1995). PAL is therefore a branch point, after which *t*-cinnamic acid is directed either to the biosynthesis of SA and other benzoic acid derivatives or to the general phenylpropanoid pathway that produces lignin, phenolic acids, coumarins, stilbenes and flavonoids. Some of these secondary metabolites are directly and indirectly involved in plant defence (Zaynab et al., 2018). In many plants, interactions between the phenylpropanoid pathway and the jasmonic acid (JA) have been reported. Jasmonates (JA and its derivatives) are well-studied plant hormones that can enhance the accumulation of several classes of secondary metabolites (Taheri & Tarighi, 2010; Wasternack & Strnad, 2019). Sharan et al. (1998) reported that JA increases the activity of PAL enzymes in tobacco and the accumulation of phenylpropanoids like coumarin and scopoletin. In rice, JA is known to enhance the accumulation of phenylpropanoid pathway-derived phytoalexins, including naringenin and sakuranetin, enhancing defence against blast fungus (Lahari et al., 2024; Ogawa et al., 2017). On the other hand, an earlier study shows that the activation of the phenylpropanoid pathway leads to a decrease in JA concentration and downstream signalling (Alon et al., 2013), indicating complex relationships between both pathways.

The *PAL* genes have been extensively studied for their role in resistance to plant pathogens. In *Arabidopsis*, the *pal1/pal2/pal3/pal4* quadruple knockout mutant contains only 10% of the wild-type PAL activity, reduced levels of SA, and it displays increased susceptibility to the bacterial pathogen *Pseudomonas syringae* (Huang et al., 2010). The rice *pal4* mutant (Zonghua11 background) exhibits a significant reduction of PAL activity and SA content, and also an increased susceptibility to the blast fungus *Pyricularia oryzae* (synonym *Magnaporthe oryzae*) (Duan et al., 2014). The rice IR64 *pal4* mutant displays increased susceptibility to three pathogens, including *P. oryzae*, *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *Rhizoctonia solani* (Tonnessen et al., 2015). Overexpression of *OsPAL1* in a susceptible cultivar shows enhanced resistance to *P. oryzae* and higher lignin accumulation (Zhou et al., 2018). Knockdown of *OsPALs* (2–8) in a resistant cultivar (Rathu Heenati) significantly reduces PAL activity, lignin and SA content, and brown planthopper resistance (He et al., 2020). These results indicate that PAL is involved in plant defence against multiple pathogens, at least partially via lignin accumulation and SA biosynthesis. Hence, PAL is an attractive target to enhance disease resistance in plants.

Aminoindan-phosphonic acid (AIP) was synthesized as a PAL inhibitor for the first time in 1992 (Zoń & Amrhein, 1992). AIP competitively inhibits PAL in a time-dependent manner and this inhibition is reversible (Appert et al., 2003). Consistent with genetic *PAL* inactivation, increased susceptibility to *Botrytis cinerea* was observed in *Arabidopsis thaliana* after AIP application (Ferrari et al., 2003). Furthermore, AIP treatment significantly decreased levels of SA and reactive oxygen species (ROS), and suppressed *PR* gene expression in *Gossypium hirsutum* (Chai et al., 2017). In rice, the inhibition of PAL using α-aminooxy-β-phenylpropionic acid (AOPP) does not affect the susceptibility of the root-knot nematode *Meloidogyne graminicola* (Huang et al., 2016; Ji et al., 2015; Singh et al., 2021). However, these studies do not rule out the involvement of PAL in rice defence against pathogens. Therefore, we investigated the effect of PAL inhibition in rice using AIP. In contrast to our expectations, AIP was found to act as an effective inducer of resistance in rice to enhance defence against *M. graminicola*. We examined how AIP affects rice susceptibility to this pathogen by studying transcriptome and metabolite changes.

2 | RESULTS

2.1 | AIP induces rice resistance against the root-parasitic nematode *M. graminicola*

Previous studies revealed that AOPP, a widely used PAL inhibitor, caused no significant difference in rice susceptibility to the root-knot nematode *M. graminicola* compared to the controls (Huang et al., 2016; Ji et al., 2015; Singh et al., 2021). To investigate whether AIP, another PAL inhibitor, could affect rice defence against pathogens, rice seedlings were either foliar sprayed or soil drenched with AIP 24 h before *M. graminicola* inoculation. From a preliminary experiment, we selected the concentration of 100 μM AIP for further experiments. The effect of AIP on the number of galls and total

nematodes was evaluated at 14 days post-inoculation (dpi). When AIP was applied by soil drenching on rice seedlings, the shoot length was significantly reduced (Figure S1a), and the root length was similar to the mock treatment (Figure S1b). There were significantly lower numbers of galls and nematodes in AIP root-drenched rice plants (Figures 1a and S2a). Furthermore, in AIP-treated plants, the development of nematodes from the second-stage juveniles (J2s) to young females and egg-laying females inside rice roots was reduced (Figures 1b and S2b).

Considering that *M. graminicola* is a soil pathogen, there is a possibility that AIP is directly toxic to the nematode. To look for a direct negative effect, infective J2 nematodes were soaked in 100 μM AIP for 48 h. There was no significant difference in nematode mortality between AIP treatment and control (Figures 1c and S2c). To test the AIP effect on the mobility and infectivity of nematodes, rice plants were inoculated with AIP-treated and mock-treated J2 nematodes. There was no significant difference between the number of galls and total nematodes counted at 3 dpi, indicating that AIP-treated J2s of *M. graminicola* had the same infectivity as untreated J2s (Figures 1d and S2d). These results indicate that the reduced susceptibility of the AIP-treated rice seedlings is probably due to an AIP-induced effect on plant defence.

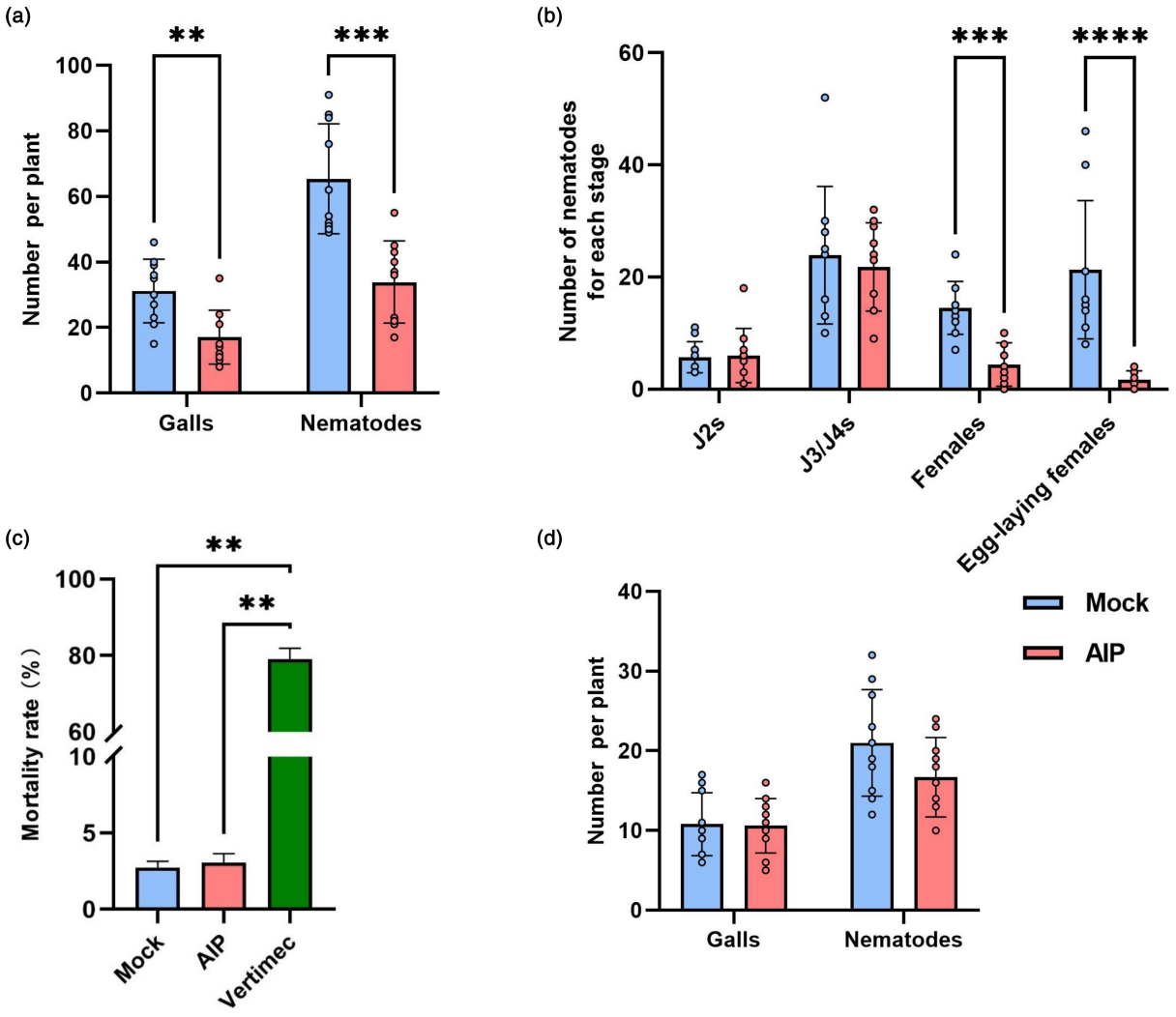

FIGURE 1 Root drenching with the phenylalanine-lyase (PAL) inhibitor aminoindan-phosphonic acid (AIP) (100 μM) reduced rice susceptibility to the root-knot nematode *Meloidogyne graminicola*. (a) Number of galls and nematodes in control and AIP-drenched rice plants at 14 days post-inoculation (dpi). (b) Number of nematodes in different stages in control and AIP-drenched plants at 14 dpi. Data shown in (a) and (b) were analysed using a Student's *t* test (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Bars represent means ± *SD* ($n = 10$) (c) The effect of AIP pretreatment on the viability of *M. graminicola* second-stage juveniles (J2s). J2s were soaked in 100 μM AIP for 48 h, and the nematicide Vertimec was used as positive control. Data were analysed using using a Student's *t* test (**$p < 0.01$). Bars represent means + *SD* ($n = 50$). (d) The effect of AIP pretreatment on infectivity of *M. graminicola*. Infective J2 stage nematodes were presoaked with and without 100 μM AIP for 48 h, followed by wild-type plant inoculation. Galls and total number of nematodes were analysed after 2 weeks. Bars represent means ± *SD* ($n = 10$). Data were analysed using a Student's *t* test (non-significant). The data show one representative of three independent biological replicates.

2.2 | AIP application to rice plants evokes higher PAL activity, while adding AIP in vitro to rice protein extracts inhibits PAL activity

As a PAL inhibitor, AIP was expected to increase rice susceptibility to pathogens by suppressing PAL activity and interfering with the production of downstream defence-related compounds. However, we found the opposite effect on rice susceptibility to *M. graminicola*. This surprising result led us to investigate PAL activity directly after AIP application on rice plants by foliar spraying or soil drenching at different concentrations (30, 100 and 300 μM) followed by protein extraction 1 day post-treatment (dpt), 3 and 5 dpt. The crude protein extracts were incubated with the substrate L-phenylalanine to monitor the formation of the product *t*-cinnamic acid. Upon foliar application, AIP treatments did not change the PAL activity at all three time points in shoots. In the systemic roots, 300 μM AIP treatment led to a significant increase in PAL activity at 5 dpt (Figure S3). For soil drenching, AIP treatments did not change the PAL activity in roots and systemic shoots at 1 dpt (Figure 2a). However, the PAL activity was significantly increased by 100 μM and 300 μM AIP treatment in both roots and systemic shoots at 3 dpt (Figure 2b). Drenching with 100 μM AIP resulted in higher PAL activity in shoots and roots at 5 dpt (Figure 2c). These data suggest that AIP soil drenching is more effective than foliar spraying in changing PAL activity in rice crude protein extracts. This is consistent with our nematode infection results.

Increased PAL activity after AIP treatment via root drenching raised the question of whether AIP is an effective inhibitor. Therefore, we analysed PAL activity after adding AIP to protein extracts in vitro. In this experiment, extracts were made from shoots of 2-week-old rice seedlings. Instead of applying AIP to the rice plant, a 100 μM AIP solution was added directly to a rice crude protein extract. AIP induced a significant decrease in PAL activity by about 70% (Figure 2d), suggesting AIP is an effective PAL inhibitor in vitro.

2.3 | AIP drenching induces local transcriptional changes in the roots at 1 day after treatment, but not in systemic shoots

To understand the molecular mechanisms of AIP-induced defence in rice, the transcriptomes of shoots and roots were investigated 1 day after AIP treatment. Both AIP foliar-sprayed and soil-drenched plants were analysed. The expression levels of all genes in the AIP-treated shoots and roots were compared to those in the corresponding mock treatments. All differentially expressed genes (DEGs) and their expression changes can be found in Tables S1 and S2. When comparing AIP soil-drenched plants to the corresponding control plants, there were more DEGs in roots (169 up, one down); however, no genes were differentially expressed in shoots (Figure 3a). In AIP foliar-sprayed plants, minor changes in gene expression were found, with only six genes that were differentially expressed in shoots (one up, five down), and no DEGs were observed in the systemic roots (Figure 3b).

When specifically focusing on AIP soil-drenched plants, gene ontology (GO) enrichment analysis showed the 10 top over-represented categories in AIP-drenched roots were mostly related to plant immunity, including regulation of defence response, regulation of the JA-mediated signalling pathway, diterpenoid biosynthesis, response to wounding and oxidoreductase activity (Figure 3c). RNA-seq data showed that the *PAL4* gene (Os02g0627100) was transcriptionally upregulated in roots after AIP treatment. Two early phenylpropanoid pathway genes, *C4H1* (Os02g0467600) and *4CL5* (Os08g0448000), were also induced in rice roots treated with AIP. Pathogenesis-related (PR) proteins are well-known to be involved in plant defence against pathogens (Mitsuhara et al., 2008; van Loon, 1985). Ten *PR* genes were upregulated in AIP-treated roots, including *PR1b*, *PR5* and *PR10*. With regard to plant defence hormone pathways, the JA biosynthetic genes *AOS2* and *OPR5*, and the JA response gene *JAMyb* had higher transcript levels in AIP-treated roots than in the control. JAZs (JASMONATE ZIM-DOMAIN proteins) are known to act as transcriptional repressors of JA responses (Pauwels & Goossens, 2011). In our analysis, six *JAZ* genes were upregulated in AIP-treated roots. In AIP-drenched roots, the SA response gene *PAD4* was upregulated, as well as the ethylene (ET) biosynthetic gene *ACO5*, and the ET-response transcription factor genes *ERF* and *AP2-EREB*. Some rice diterpenoids are known to have antimicrobial activities and play an important role in plant defence (Desmedt et al., 2022). Several genes (*CPS4*, *KSL4*, *KSL7*, *CYP71Z2*, *CYP99A2*, *CYP76M7* and *MAS*) involved in the biosynthesis of these diterpenoids were upregulated in AIP-treated roots. AIP treatment also induced seven *WRKY* transcription factor genes involved in defence.

To validate the transcriptome data, 11 genes were chosen for reverse transcription-quantitative PCR (RT-qPCR) analysis. We performed RT-qPCR analysis using samples collected at 1 and 3 dpt. Eight of them were DEGs in the transcriptome of roots after AIP treatment, and are *WRKY* transcription factors, *PR* genes or involved in the phenylpropanoid pathway, JA biosynthesis and response, or diterpenoid biosynthesis pathway. In addition, *PAL1*, *PAL7* and *WRKY45* (not DEGs) were selected to check their transcriptional response to AIP treatment. In the systemic shoots, none of the genes showed a significant change, except *PR5* (Figure S4a,c). *PR5* was significantly upregulated in the systemic shoots in the RT-qPCR, while it was not differentially expressed in the RNA-seq data. In the AIP-drenched roots, transcripts of *PAL1*, *PAL7* and *WRKY45* were similar to the control at 1 dpt, confirming the RNA-seq results. The phenylpropanoid pathway genes *PAL4* and *4CL5*, the JA-responsive gene *JAMyb*, *PR10*, and the diterpenoid biosynthetic gene *KSL7* were upregulated in AIP-drenched roots (Figure S4c). At 3 dpt, only the phenylpropanoid pathway genes *PAL1*, *PAL4* and *4CL5* were upregulated in roots (Figure S4d). Overall, our RNA-seq data are supported by the RT-qPCR results for most selected genes.

2.4 | AIP treatment causes significant changes in the metabolic profile of rice

To assess the effects of AIP treatment on the metabolic profile of rice plants at 1 dpt and 3 dpt, we used a targeted ultra-high-performance liquid chromatography (UHPLC) coupled to a tandem mass spectrometry (MS/MS) approach. Because the PAL enzyme catalyses the

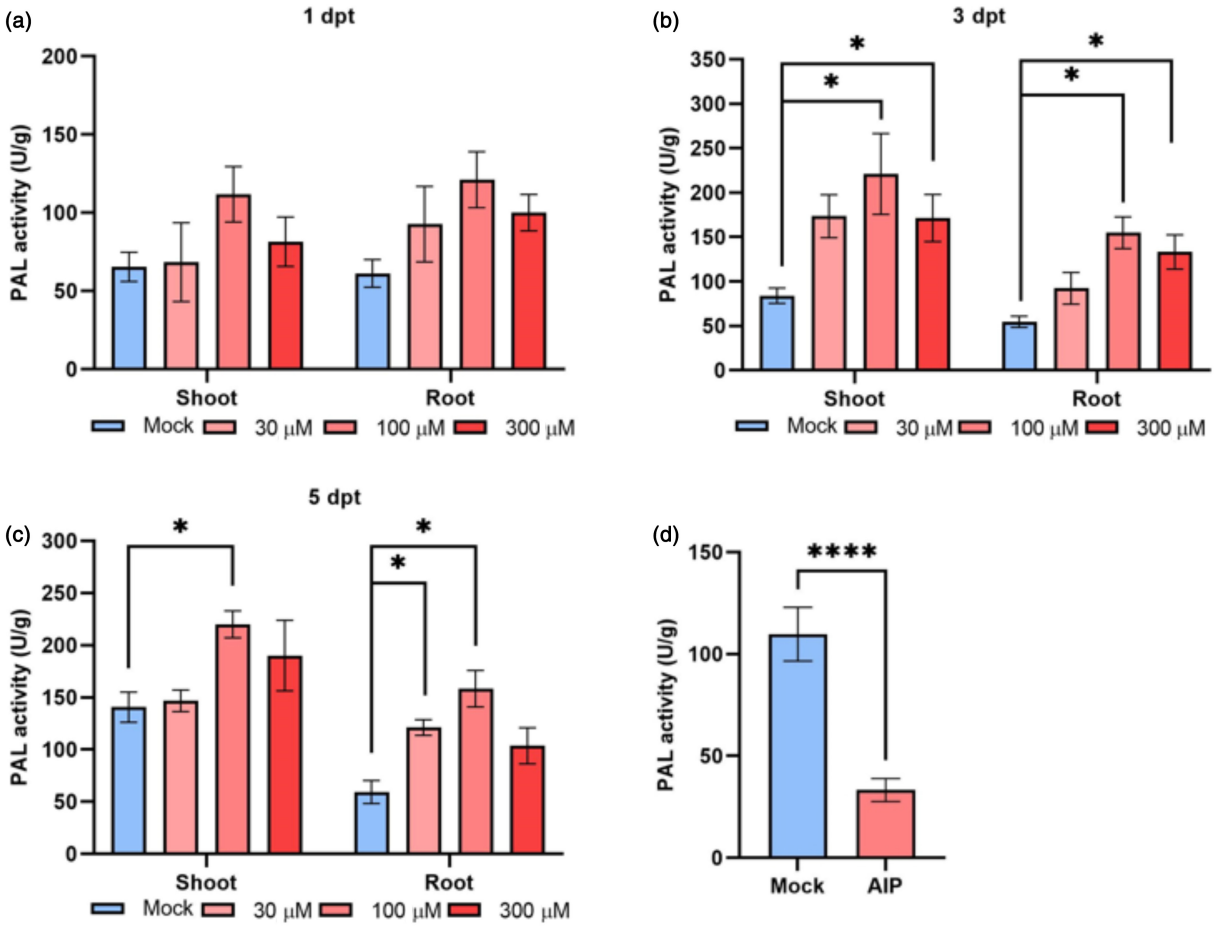

FIGURE 2 Phenylalanine ammonia-lyase (PAL) activity increased after the application of aminoindan-phosphonic acid (AIP) by root drenching. PAL activity was measured at 1 day post-treatment (dpt) (a), 3 dpt (b), 5 (dpt) (c) in rice shoots and roots. Bars represent means ± *SD* ($n = 4$). Asterisks shown in Figures (b) and (c) indicate the significant differences according to the Mann–Whitney *U* test (*$p < 0.05$). (d) PAL activity after adding 100 μM AIP directly to a crude rice protein extract. Bars represent means ± *SD* ($n = 8$). Asterisks indicate the significant differences according to Student's *t* test (****$p < 0.0001$). The data show one representative of three independent biological replicates.

conversion of phenylalanine to *t*-cinnamic acid, when PAL is inhibited by AIP, an increase in phenylalanine levels is expected. At 1 dpt, this is exactly what was seen both in shoots and roots (Figures 4 and S5). In roots, levels of tyrosine and tryptophan also increased at 1 dpt (Figure 4). The accumulation of aromatic amino acids increased to a greater magnitude at 3 dpt in both shoots and roots (Figures 4 and S5). Roots showed a phenylalanine level that was 30-fold higher, a tyrosine level that was more than 2-fold higher and a tryptophan level that was over threefold higher compared to mock-treated plants. Shoots showed similar significant increases, although not quite to the extent seen in roots (Figure S5). These results demonstrate that AIP might act as a PAL inhibitor in vivo.

PAL catalyses the first step of the phenylpropanoid pathway, which produces a wide array of compounds, many of which are related to plant defence. For example, PAL is involved in the biosynthesis of the defence hormone SA. In our analysis, both free SA and its inactive derivative SA-glucoside (SAG) were measured. At 1 dpt, no significant changes could be found in SA and SAG levels in both roots and shoots (Figures 5a and S6). However, at 3 dpt the SA level was significantly lower in roots while the content did not change in shoots (Figures 5a, S6, and S10). The content of SAG remained unchanged both in roots and shoots at 3 dpt (Figures 5a and S6).

Benzoic acid is considered a precursor for SA in rice. Despite the lower SA levels after AIP treatment, no difference in benzoic acid was observed in shoots or roots at both time points (Figures 5a and S6). *p*-Coumaric acid is a precursor for numerous metabolites derived from the phenylpropanoid pathway and is synthesized from *t*-cinnamic acid, the product of the PAL enzyme. PAL inhibition should logically cause a decrease in *p*-coumaric acid levels. While a significant decrease of *p*-coumaric acid levels at both time points was observed in roots (Figure 5b), this was not seen in shoots (Figure S7a). Ferulic acid, a downstream product of *p*-coumaric acid and an important intermediate for lignin biosynthesis, showed a similar decrease in roots (Figure 5b), but also in shoots at 3 dpt (Figure S7a). We then measured lignin levels using the acetyl bromide assay after AIP treatment at different time points (1, 3, 7 and 14 dpt). AIP treatment led to a significant decrease in the abundance of lignin in shoots at 7 dpt, but not in roots (Figure S8). The Folin–Ciocalteu assay was used to measure total phenolic compounds, including free phenolic compounds and cell wall-bound phenolic compounds. Relative levels of phenolics decreased

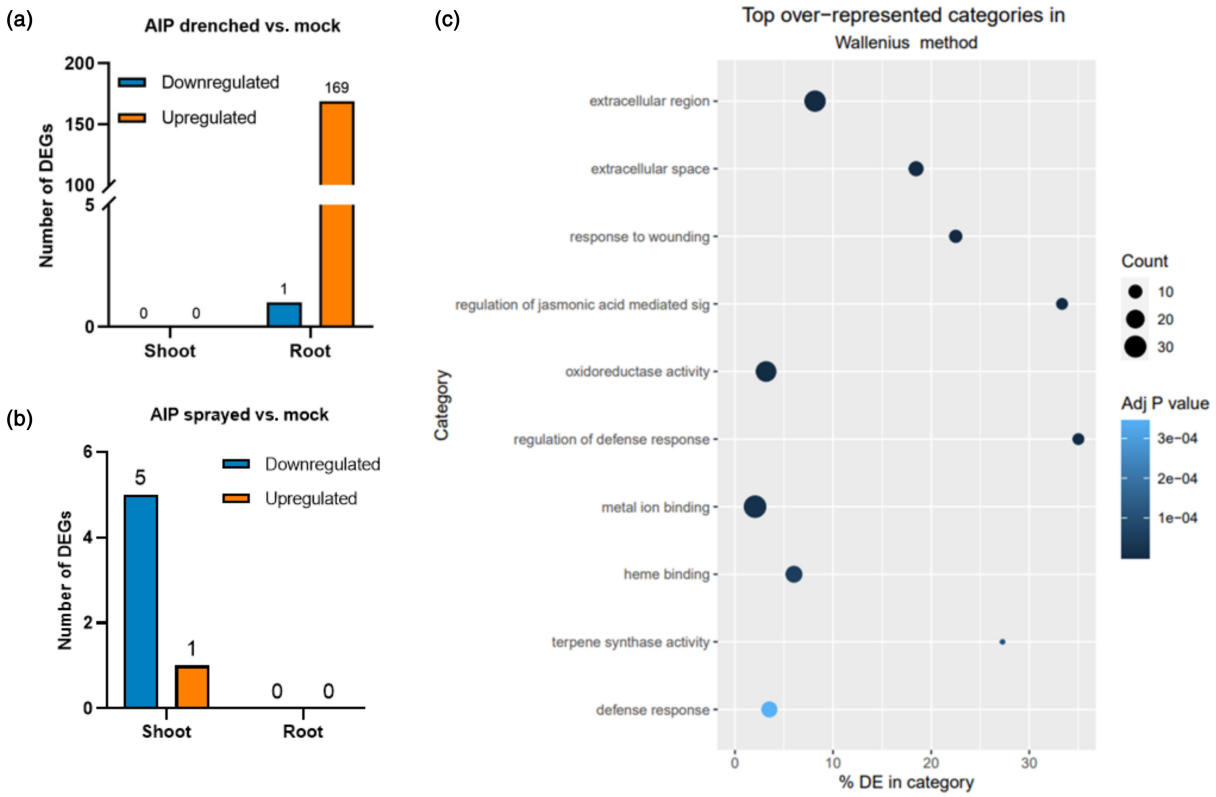

FIGURE 3 Aminoindan-phosphonic acid (AIP) treatment induced transcriptional changes in rice leaves and roots. (a) Bar chart represents the number of differentially expressed genes (DEGs) at 1 day post-treatment (dpt) in shoots and roots of rice treated with 100 μM AIP by root drenching. (b) Bar chart represents the number of DEGs at 1 dpt in shoots and roots of rice treated with 100 μM AIP by leaf spraying. (c) Top over-represented categories of DEGs in rice treated with 100 μM AIP by drenching at 1 dpt.

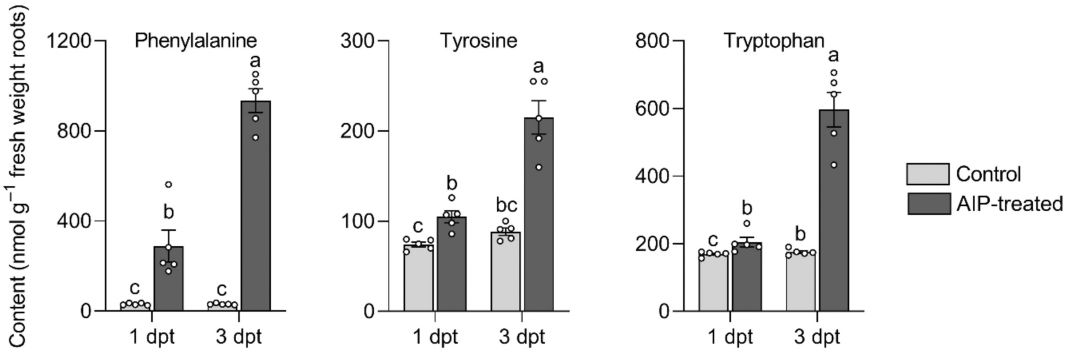

FIGURE 4 Levels of the aromatic amino acids increased in rice roots drenched with aminoindan-phosphonic acid (AIP). Two-week-old rice seedlings were drenched with 100 μM AIP, and root samples (each sample was a pool of five plants) were collected at 1 and 3 days post-treatment (dpt). Phenylalanine, tyrosine and tryptophan levels were quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Data were analysed using a two-way analysis of variance, followed by Tukey's multiple comparison test. Different letters above the bars indicate groups that are significantly different ($p < 0.05$). Data are presented as the mean ± *SE*, and all data points are shown on the graph as open circles ($n = 5$).

significantly in shoots at 14 dpt (Figure S9). We did not observe any significant change in total phenolic compounds in roots (Figure S9).

Downstream of the general phenylpropanoid pathway, several flavonoids act as phytoalexins in rice. To get an insight into the possible changes of the known flavonoid phytoalexins, we quantified the relative levels of naringenin, apigenin, luteolin and sakuranetin after AIP treatment. Naringenin acts as a precursor for a variety of flavonoids. Naringenin gets converted by a flavone synthase to apigenin, which can subsequently get hydroxylated to form luteolin or methylated to form sakuranetin. In roots, levels of naringenin, luteolin and sakuranetin remained unchanged and only the apigenin level decreased at 1 dpt (Figure 5c,d). At 3 dpt, a remarkable change was observed. Naringenin levels increased 10-fold, apigenin levels were 3.5-fold higher and sakuranetin levels were 2.5-fold higher; luteolin levels remained unchanged (Figure 5c,d). In shoots, naringenin and apigenin levels decreased at 1 dpt (Figure S7b), while at 3 dpt,

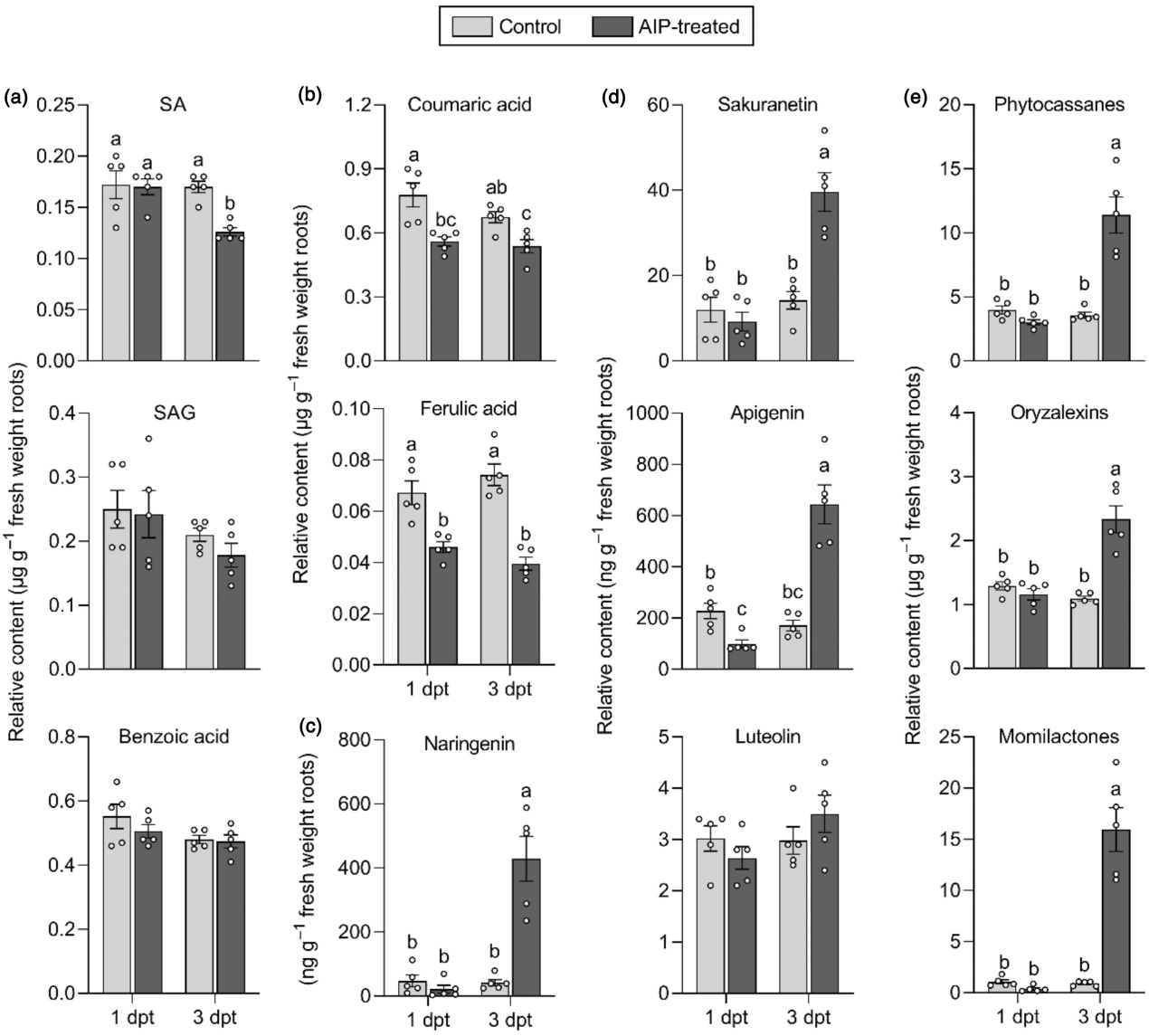

FIGURE 5 Flavonoid and diterpenoid phytoalexins accumulated in aminoindan-phosphonic acid (AIP)-treated rice roots. Two-week-old rice seedlings were drenched with 100 µM AIP, and root samples (each sample was a pool of five plants) were collected at 1 and 3 days post-treatment (dpt). Selected metabolites were quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Level of (a) salicylic acid and benzoic acid, (b) phenolic acids, (c,d) flavonoids and (e) diterpenoid phytoalexins. Metabolite data were analysed using a two-way analysis of variance, followed by Tukey's multiple comparison test. Different letters above the bars indicate groups that are significantly different ($p < 0.05$). Data are presented as the mean ± *SE*, and all data points are shown on the graph as open circles ($n = 5$). SA, salicylic acid; SAG, salicylic acid-glucoside.

naringenin was still reduced, while apigenin levels were unchanged and luteolin levels were reduced in response to AIP (Figure S7b).

Because the transcriptome analysis showed upregulation of genes involved in the biosynthesis of diterpenoid phytoalexins after root drenching with AIP, and these metabolites have been shown to be important for rice defence against *M. graminicola* (Desmedt et al., 2022), some members of this class of phytoalexins were quantified in root extracts using a triple quadrupole LC/MS system. At 1 dpt, levels of diterpenoid phytoalexins, including phytocassanes, oryzalexins and momilactones were unchanged. However, a remarkable increase in the concentrations of diterpenoid phytoalexins was observed in AIP-treated rice roots at 3 dpt (Figure 5e).

The plant hormone JA is known to play essential roles in regulating plant defence metabolites, including antimicrobial flavonoids and diterpenoid phytoalexins (Okada et al., 2015). The jasmonate signalling pathway in rice is considered vital to protect against infection by the root-knot nematode *M. graminicola* (Nahar et al., 2011). Because AIP-treated rice plants showed enhanced resistance to this nematode and increased flavonoid and diterpenoid phytoalexins in roots, we checked if jasmonate levels were also changed after AIP treatment. We performed an in-depth analysis of JA pathway metabolites, including 12-oxo-phytodienoic acid (OPDA, a JA-precursor oxylipin), JA-Ile (the most biologically active form of JA), and related products. In roots, the levels of OPDA did not change at 1 dpt but

increased by two-fold at 3 dpt (Figures 6 and S10). Similarly, the levels of JA, JA-Ile, COOH-JA-Ile and OH-JA-Ile increased by about two- to threefold in rice roots at 3 dpt. However, the level of jasmonic acid-glucoside (JAG, an inactive form of JA), was decreased almost by 50% at 1 dpt and then remained similar to the control at 3 dpt (Figure 6). In the systemic shoots, JA metabolites mostly remained unchanged (Figure S11).

Collectively, our metabolite analyses suggest that AIP treatment in rice through root-drenching triggered jasmonate biosynthesis and accumulation, and induced the accumulation of flavonoid and diterpenoid phytoalexins.

2.5 | AIP treatment primes defence against *M. graminicola* infection

Previously, it was shown that disturbance of the phenylpropanoid pathway can induce primed defence in tomato (Desmedt et al., 2021). Therefore, a combination experiment was set up to check the expression of specific defence genes after PAL inhibitor application plus nematode infection. Rice plants were drenched with AIP and treated plants were inoculated at 1 dpt with *M. graminicola*. Gene expression in these plants was analysed at 3 dpt or 2 dpi and compared with noninoculated plants after AIP treatment (3 dpt) and inoculated non-treated plants (2 dpi). RT-qPCR was performed on *PAL4*, *4CL5*, *JAmyb* and *PR10* in shoots and roots (Figure 7). No consistent changes were found in gene expression in the shoots between infected mock and infected treated plants. The phenylpropanoid genes *PAL4* and *4CL5* showed higher expression in roots after AIP drenching, but there was no significant difference after nematode infection. When AIP treatment was followed with nematode infection, the expression of the JA-responsive genes *JAMyb* and *PR10* appeared to be upregulated from the AIP-only treatment in the roots (Figure 7). These results suggest that AIP might stimulate the defence responses in rice roots to a greater magnitude under nematode attack.

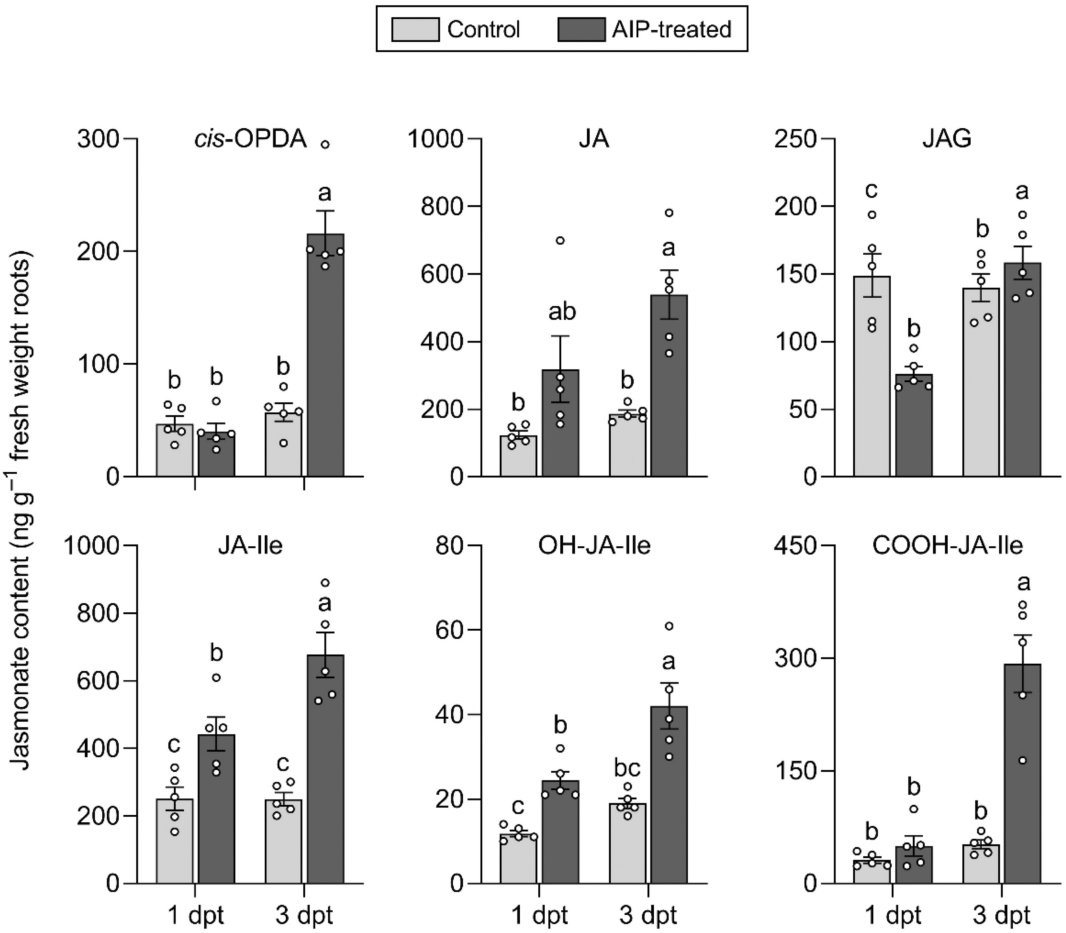

FIGURE 6 Jasmonate levels increased in rice roots drenched with aminoindan-phosphonic acid (AIP). Two-week-old rice seedlings were drenched with 100 μM AIP, and root samples (each sample was a pool of five plants) were collected at 1 and 3 days post-treatment (dpt). Jasmonate levels were quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Data were analysed using a two-way analysis of variance, followed by Tukey's multiple comparison test. Different letters above the bars indicate groups that are significantly different ($p < 0.05$). Data are presented as the mean ± *SE*, and all data points are shown on the graph as open circles ($n = 5$). *cis*-OPDA, *cis*-(+)-12-oxo-phytodienoic acid; COOH-JA-Ile, carboxy-JA-Ile; JA, jasmonic acid; JAG, jasmonic acid-glucoside; JA–Ile, jasmonoyl-L-isoleucine; OH-JA-Ile, hydroxy-JA-Ile.

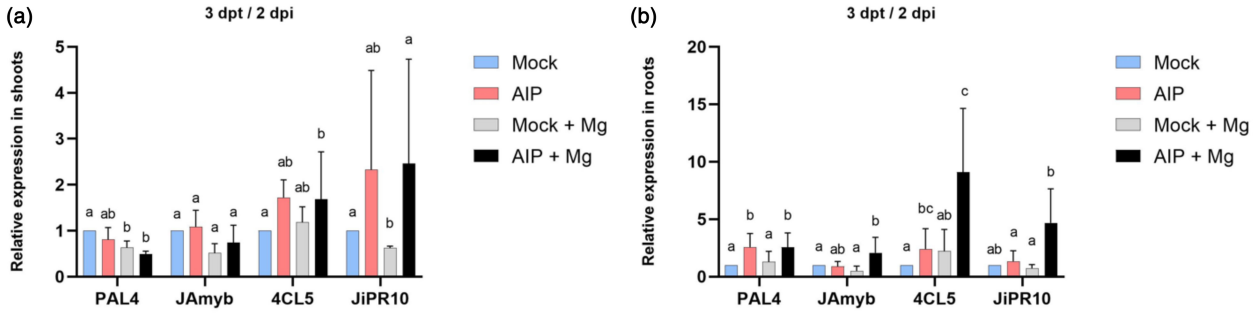

FIGURE 7 Reverse transcription-quantitative PCR (RT-qPCR) after root drenching with 100 μM aminoindan-phosphonic acid (AIP) and/or nematode infection. Relative gene expression in (a) shoots and (b) roots at 3 days post-treatment (dpt) and 2 days post-inoculation (dpi) of rice plants drenched with 100 μM AIP and/or infected with *Meloidogyne graminicola* (Mg). Bars represent means ± *SD* ($n = 3$, each replicate was a pool of three plants). Gene expression levels were obtained by RT-qPCR and normalized using three reference genes. Statistical analyses were done by REST 2009. Different letters indicate significant differential expression for that gene between conditions.

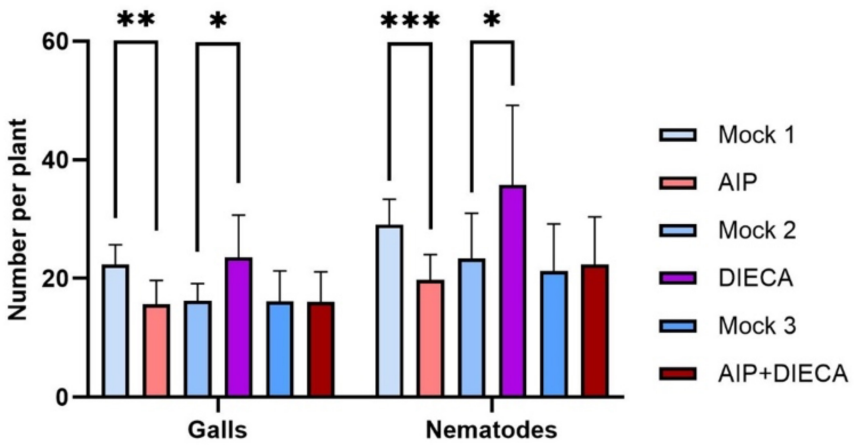

FIGURE 8 Aminoindan-phosphonic acid (AIP)-induced defence against *Meloidogyne graminicola* nematodes in rice via the jasmonate signalling pathway. Two-week-old rice seedlings were treated with 100 μM AIP (by root drenching), 100 μM diethyldithiocarbamic acid (DIECA) (by spraying) either alone or in combination, and with the respective solvent control, mock 1 (for AIP), mock 2 (for DIECA) and mock 3 (for AIP + DIECA). DIECA is an inhibitor of jasmonic acid (JA) biosynthesis. The plants were inoculated with *M. graminicola* at 24 h after treatment. Number of galls and nematodes were counted at 14 days post-inoculation. Bars represent means + *SD* ($n = 10$). Asterisks indicate the significant differences according to the Student's *t* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The data represent one of three independent biological replicates.

2.6 | AIP-induced defence against *M. graminicola* is mediated by the JA pathway

In rice, JA is the key hormone in defence against the root-knot nematode *M. graminicola* (Nahar et al., 2011). Our gene expression data from both RNA-seq and RT-qPCR and hormone quantification indicated that jasmonate signalling could play a role in AIP-induced rice defence. We hypothesized that AIP-induced rice resistance could be due to higher jasmonate accumulation as JA positively regulates defence metabolites including naringenin, sakuranetin, phytocassanes, oryzalexins and momilactones.

To test the role of the JA pathway in AIP-induced defence against *M. graminicola*, the JA biosynthesis inhibitor diethyldithiocarbamic acid (DIECA), combined with AIP was applied to plants 1 day before *M. graminicola* inoculation (Figures 8 and S12). A significant reduction in the number of galls and nematodes was observed in plants drenched with AIP alone, compared to control plants (Mock 1). The foliar application of DIECA increased (Figure 8, but not always, see Figure S12) the number of galls and nematodes when compared with control plants (Mock 2). However, the co-application of DIECA and AIP did not decrease the numbers of galls and nematodes compared with the control plants (Mock 3) (Figures 8 and S12). These data suggest that AIP-induced defence against *M. graminicola* in rice is dependent on JA biosynthesis. Because jasmonates and some phytoalexins (flavonoids and diterpenoids) are also involved in rice defence against the bacterial pathogen Xoo (De Vleesschauwer et al. 2013; Lu et al., 2018), we tested if AIP treatment also enhances defence against Xoo. We found that pretreatment of rice seedlings with the PAL inhibitor AIP significantly reduced the infection by Xoo (Figure S13). Taken together, AIP-induced rice defence might be dependent on the jasmonate pathway.

3 | DISCUSSION

The PAL enzyme catalyses the first reaction of the phenylpropanoid pathway, which produces a vast array of phenolic compounds,

including the phytohormone SA, hydroxyl-cinnamic acids and flavonoids. Many of these metabolites are known to be bioactive against pathogens. Studies have shown that knocking out of *PAL* genes causes an increase in susceptibility to several pathogens (Duan et al., 2014; Tonnessen et al., 2015). Thus, it is expected that inhibition of PAL by a competitive inhibitor like AIP (Zoń & Amrhein, 1992) would increase plant susceptibility to pathogens. To our surprise, treatment of rice with the PAL inhibitor AIP reduced susceptibility to the root-knot nematode *M. graminicola*. To explain these unexpected observations, in-depth transcriptome and metabolome analyses were performed. We found that AIP-drenching induced the accumulation of jasmonates and phytoalexins in rice roots.

When AIP was added to a rice crude protein extract, a drastic decrease in PAL activity was evident, verifying that AIP functions as an effective inhibitor against rice PAL in vitro. We also saw an increase in phenylalanine levels in both shoots and roots at 1 dpt and 3 dpt, which suggests that AIP acts as a PAL inhibitor in vivo as well. However, crude protein extracts obtained from roots and shoots of of AIP-treated rice plants exhibited increased PAL activity. A similar result was obtained earlier, when examining the effects of AIP treatment on PAL activity in *Hypericum canariense*, although a decrease in PAL activity was observed in *Hypericum perforatum* (Klejdus et al., 2013). An increase in PAL activity after AIP treatment was also measured in chamomile (Kováčik et al., 2011). So, the effects of whole-plant application of AIP on PAL activity might be species-dependent.

Treating rice plants with AIP caused a significant shift in their metabolic profiles. Some of these changes can be explained as a direct effect of reducing the PAL activity in vivo. For example, the total SA pool (free SA and its glucoside) was significantly reduced at 3 dpt in both shoots and roots. As SA is at least partly a downstream metabolite of the PAL product, *t*-cinnamic acid, a reduction of SA is expected when PAL is inhibited. *p*-Coumaric and ferulic acids, also downstream products of PAL, were also reduced in AIP-drenched roots.

To assess the downstream effects of PAL inhibition on the phenylpropanoid pathway, we analysed some antimicrobial rice flavonoids, including the flavanones naringenin and sakuranetin, and the flavones apigenin and luteolin. As might be expected, these compounds decreased at 1 dpt in both shoots and roots, and at 3 dpt in shoots. However, the levels of naringenin, apigenin and sakuranetin greatly increased in roots at 3 dpt. While this seems counter-intuitive, the transcript data offers a possible explanation for this phenomenon. At 1 dpt, we can see that several genes involved in the phenylpropanoid pathway (such as *PAL4*, *4CL5* and *C4H1*) are significantly upregulated, which could cause a delayed accumulation of naringenin and apigenin at 3 dpt. Increased flavonoid accumulation could also be ascribed to increased amounts of PAL produced to alleviate the effects of AIP inhibition. The build-up of phenylalanine after AIP treatment (Figure 4) could also increase metabolic flux through PAL because AIP is a reversible competitive inhibitor (Appert et al., 2003). Furthermore, the fact that only selected PAL genes, *PAL1* (3 dpt) and *PAL4* (1 dpt), were upregulated suggests that these could be committed to flavonoid biosynthesis and explains why we see a disproportionate increase in these compounds compared to other phenylalanine-derived metabolites.

The upregulation of phenylpropanoid pathway genes after AIP inhibition of PAL could result from a regulatory transcriptional feedback loop to counteract the decrease in phenylpropanoid metabolites. However, the general activation of defence genes by PAL inhibition suggests a somewhat different explanation. If invading pathogens cause inhibition of PAL (Bauters et al., 2021), plants might have evolved to activate JA biosynthesis and JA-induced defences upon detection of PAL inhibition. This would explain the induction of not only JA-induced defences here but also diterpene phytoalexin biosynthesis genes and the accumulation of the corresponding products after AIP treatment. Diterpenoids are a large class of defence compounds in plants that are particularly important in rice defence against *M. graminicola* (Desmedt et al., 2022). They are synthesized completely separately from the phenylpropanoid pathway, and upregulation of their biosynthesis was also seen after perturbation of the PAL pathway with the C4H inhibitor piperonylic acid (Desmedt et al., 2021, 2022).

The reduced susceptibility of AIP-treated plants to *M. graminicola* might thus be attributed to a general activation of plant defence caused by AIP treatment. We have also found the AIP-treated plants to be more susceptible to Xoo (Figure S13) but did not investigate this further. Among defence hormones, the level of free SA did not change after the AIP application. However, there was a strong induction of JA and other jasmonates in roots. JA-induced defence responses have previously been shown to play a key role in rice resistance to *M. graminicola* and Xoo (Nahar et al., 2011; Yamada et al., 2012). The role of JA and other jasmonates in our study was further reinforced by the fact that application of the JA biosynthetic inhibitor DIECA increased susceptibility to nematodes and, when applied together with AIP, negated the defensive effect of this PAL inhibitor. JA accumulation is known to cause a cascade of downstream defence responses, like the production of diterpenoids in roots, which are important for plant defence against nematodes (Desmedt et al., 2022). Moreover, AIP treatment increased the level of apigenin in roots, a compound that is directly toxic to nematodes (Bano et al., 2020). Taken together, these data give a convincing explanation of why AIP-treated plants show reduced susceptibility to the root-infesting nematode *M. graminicola*.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant material and growth conditions

All experiments were performed using the rice (*Oryza sativa*) cultivar Kitaake. Rice seeds were germinated on wet filter paper for 4 days in a growth chamber (30°C, 12/12 h light/dark) and were then transplanted to SAP tubes (sand + absorbent polymer; Reversat et al., 1999) in a growth room (28°C, 16/8 h light/dark). Plants were watered with Hoagland's solution. Two-week-old plants were used for nematode inoculation.

4.2 | Nematode and bacterial culture and infection

The root-knot nematode *M. graminicola* was cultured on the grass *Echinocloa crusgalli* in a growth room (28°C, 16/8 h light/dark). Two months after inoculation, the infected roots were cut into 1 cm pieces to extract nematodes using the modified Baermann method (Luc et al., 2005). About 250–300 second-stage juveniles (J2s) were inoculated per plant. At least three independent experiments were carried out, and each time using 10 plants per treatment. Fourteen days after inoculation, roots were stained using acid fuchsin to count galls, total nematodes and developmental stages of nematodes.

The Xoo strain PXO99 was used for rice leaf inoculation. The bacteria were grown on PYS (1 L: 8 g peptone, 2 g yeast extract, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 10% wt/vol glucose) for 2 days at 28°C. The bacterial cultures were collected and adjusted to 10^9, 10^8 or 10^7 cfu/mL in 0.9% NaCl solution. Six-week-old plants were used for infection tests by the clipping method (Ferluga et al., 2007). Three leaves were infected per plant. Ten plants were inoculated per treatment. Fourteen days after inoculation, the bacterial lesion length of the leaf was measured to evaluate the symptoms. The bacterial infection experiments were repeated three times.

4.3 | Chemical treatments

The PAL inhibitor aminoindan-phosphonic acid hydrochloride (AIP) was purchased from AA Blocks (AA001GNC). Diethyldithiocarbamic acid (DIECA) was purchased from Sigma (318116). AIP was dissolved in dimethyl sulphoxide (DMSO) to make a 400 mM stock solution. The final concentrations were 30, 100 and 300 μM AIP in distilled water, and for most experiments 100 μM AIP was used. For foliar application, 20 mL of AIP solution with 0.02% (vol/vol) of Tween 20 as a surfactant was sprayed on 10 plants. As a control, plants were sprayed with distilled water containing 0.025% (vol/vol) DMSO and Tween 20. For soil application, 5 mL of AIP solution was drenched on each plant, while the control was drenched with distilled water containing 0.025% (vol/vol) DMSO. Shoot and root lengths and weights were measured at several time points after treatment.

The JA biosynthesis inhibitor DIECA was dissolved in water to make a 100 mM stock solution, and the final used concentration was 100 μM. Ten plants were sprayed until run-off with 20 mL of 100 μM DIECA with 0.02% (vol/vol) of Tween 20. Subsequently, 24 h after treatment, those plants were inoculated with *M. graminicola* to analyse the infection levels. These treatments were all repeated three times.

For AIP and DIECA combined treatment, 100 μM AIP and the control mock 1 (0.025% DMSO in distilled water) were drenched on roots. DIECA and the control mock 2 (0.02% Tween 20 in distilled water) were sprayed on leaves. The combined treatment was applied by AIP drenching on roots and DIECA spraying on leaves, while the control mock 3 was drenching 0.025% DMSO in distilled water on roots and spraying 0.02% Tween 20 in distilled water on leaves.

4.4 | Biochemical analysis of PAL activity and phenylpropanoid pathway products

For the assay of PAL after AIP treatment of whole rice plants, shoots and roots were harvested at 3, 5, 7 and 14 dpt and the samples were ground by mortar and pestle in liquid nitrogen. The PAL activity assay was performed according to a previously described method with modifications (Camacho-Cristóbal et al., 2002). Approximately 100 mg of sample was added to 1 mL 50 mM Tris–HCl buffer (pH 8), containing 2% (wt/vol) polyvinylpolypyrrolidone (PVPP), 2 mM EDTA, 18 mM-mercaptoethanol and 0.1% (vol/vol) Triton X-100. After centrifugation at 10,000 *g* for 10 min at 4°C, 20 μL of the supernatant was added to 135 μL buffer (50 mM Tris–HCl, pH 8) and 50 μL of substrate, 20 mM L-phenylalanine. After mixing well *t*-cinnamic acid formation was measured by absorbance at 290 nm using a microplate reader (Tecan Infinite F200 Pro). Then, the sample was incubated at 37°C for 1 h, 10 μL of stop solution (5 M HCl) was added and the absorbance was measured again. One unit (U) of PAL activity was defined as the amount of the enzyme that produced 1 nmol *t*-cinnamic acid per hour. Control samples were included that had no L-phenylalanine. PAL activity was then calculated according to the weight of sample. The quantification was performed three times, using eight biological replicates (consisting of three pooled plants) per treatment.

For the assay of PAL, after AIP was added directly to the protein extract, 2-week-old rice shoots without any treatment were used. The crude protein was extracted for the PAL activity assay above. AIP (100 μM) was added to 20 μL supernatant, 135 μL buffer (50 mM Tris–HCl, pH 8) and 50 μL of the substrate 20 mM L-phenylalanine. As a control, 0.025% (vol/vol) DMSO was added. Then the assays were measured with a microplate reader as above. This assay was performed three times each on eight biological replicates (consisting of three pooled plants) per treatment.

Lignin content was measured using the acetyl bromide assay modified to a previously described method (Van Acker et al., 2013). Approximately 100 mg of plant material were incubated in distilled water, 100% ethanol (Sigma-Aldrich), chloroform (Sigma-Aldrich) and acetone (Sigma-Aldrich), sequentially. The residues were dried in a fume hood overnight. Dried samples were weighed to an accuracy of at least 0.1 mg. The pellet was dissolved in 200 μL 25% acetyl bromide (Sigma-Aldrich) and this was incubated for 2 h at 50°C. The blank sample consisted of 200 μL acetyl bromide solution without cell wall residue. After incubation, 1 mL of glacial acetic acid was added to the acetyl bromide solution. After centrifugation for 10 min at 13,000 *g*, the supernatant was mixed with equal volumes of 2 M NaOH and 0.5 M hydroxylamine and the absorbance of the supernatant was measured at 280 nm. The lignin content was calculated according to the weight of the sample (Vega-Sánchez et al., 2012). The measurements were performed three times each on five biological replicates (consisting of three pooled plants) per treatment.

The phenolic compounds were quantified using the Folin–Ciocalteu assay (Siranidou et al., 2002). Approximately 100 mg of plant material was added to 20 μL/mg cold methanol to extract free phenolics. After centrifugation, 125 μL supernatant was mixed

with 675 µL distilled water, 37.5 µL Folin–Ciocalteu reagent (Sigma-Aldrich) and 375 µL 20% (wt/vol) Na_2CO_3 in that order. After incubation for 30 min in the dark, the absorbance was measured at 765 nm. The concentration of phenolic compounds was calculated relative to a gallic acid standard curve. The residue left after ethanol extraction was used to measure the cell wall-bound phenolic compounds. The residue was extracted overnight with 20 µL/mg 1 M NaOH. After centrifugation, the supernatant was measured as for free phenolic compounds. These quantifications were performed three times each on five biological replicates (consisting of three pooled plants) per treatment.

4.5 | mRNA sequencing and RT-qPCR

Rice shoots and roots were harvested for mRNA sequencing at 1 day after AIP treatment. Four biological replicates, each consisting of three pooled plants, were used. Samples were ground by mortar and pestle in liquid nitrogen. RNA was extracted using the RNeasy Plant Mini kit (QIAGEN) with an additional DNase I (Thermo Fisher) treatment. The libraries were prepared using the QuantSeq 3′ mRNA-Seq library prep kit and were sequenced on an Illumina NextSeq 500 platform.

UMI-tools (v. 1.0.1) were used to extract unique molecular identifier (UMI) sequences from fastq reads. Reads were trimmed for bad quality base calling using a sliding window of four base pairs wide and a quality cut-off of 15 with Trimmomatic (v. 0.39). Trimmed reads were mapped to the *O. sativa* subsp. *japonica* reference genome (build IGRSP-1.0, release 51) using STAR (v. 2.7.2b). Subsequently, Samtools (v. 1.5) was used to index mapped sorted reads and UMI-tools were used to remove mapped duplicates based on the location of alignment and UMI sequences earlier extracted by UMI-tools. Finally, FeatureCounts (v. 2.0.0) was used to summarize count tables for each sample. Multiqc (v. 1.2) was used to construct an overview of the sequencing quality of raw reads and mapping statistics. To avoid bias of under-sequenced samples we removed samples whose numbers of aligned reads were one standard deviation below the average amount of aligned reads. The R package, biomaRt (v. 2.52.0) was used to obtain gene symbols, Entrez gene IDs, RefSeq peptide IDs and Uniprot gene symbols. Genes with very low expression (counts per million, cpm <2 in more than half of the samples in each comparison) were removed. For normalization and differential gene expression analysis was performed using the edgeR (v. 3.38.0). Default normalization was applied, using trimmed mean of M values (TMM). Quasi-likelihood test was performed to assess statistical significance. Benjamini–Hochberg correction was applied to correct for multiple testing. DEGs were assigned with a false discovery rate (FDR) <0.05.

For RT-qPCR, rice roots were treated with AIP or the control, and then shoots and roots were collected at 1 dpt and 3 dpt. For the AIP and *M. graminicola* combined experiment, 24 h after AIP treatment, *M. graminicola* was inoculated on the rice. The rice shoots and roots were collected at 3 dpt/2 dpi. Three biological replicates (each consisting of three pooled plants) were used. Then, RNA was extracted and converted to cDNA by a Tetro cDNA synthesis kit (Bioline). *EXP*, *EXP NARCAI* and *EIF5c* were used as reference genes. CFX Connect Real-Time PCR Detection System (BIO-RAD) and the SensiMix SYBR HI-ROX kit (Bioline) were used for RT-qPCR. Statistical significance was determined by REST2009 (Pfaffl et al., 2002). The primers are listed in Table S3.

4.6 | Extraction and metabolite analyses

Rice shoots and roots were harvested at 1 day and 3 days after AIP treatment (five biological replicates, each consisting of five pooled plants) and immediately frozen in liquid nitrogen. Shoot and root samples were ground in liquid nitrogen using mortar and pestle. Approximately 50 mg of finely ground fresh tissue was weighed in 2 mL Safe-Lock Eppendorf tubes. Metabolites were extracted by adding 1 mL methanol containing 40 ng of D_4-SA (Sigma-Aldrich), 40 ng of D_6-JA (HPC Standards GmbH), 8 ng D_6-JA-Ile (HPC Standards GmbH) and 40 ng D_6-ABA (Toronto Research Chemicals) as internal standards. The samples were then vortexed vigorously for a few seconds and incubated in a shaker at 20°C for 30 min, and subsequently centrifuged for 5 min at 4°C and 13,000*g*. Approximately 950 µL of the supernatant was transferred to a new 1.5 mL Safe-Lock Eppendorf tube and the samples were stored at −20°C until further use.

Hormones were analysed using a 1260 HPLC system (Agilent Technologies) coupled to a QTRAP 6500 tandem mass spectrometer (Sciex) equipped with a turbo-spray ion source operated in the negative ionization mode, as detailed previously (Ullah et al., 2022). Concentrations of SA, JA and JA-Ile were calculated relative to the internal standards of D_4-SA, D_6-JA and D_6-JA-Ile, respectively. Concentrations of SAG and JAG were calculated relative to D_4-SA and D_6-JA, respectively. Concentrations of OH-JA-Ile and COOH-JA-Ile were calculated relative to D_6-JA-Ile.

Benzoic acid, phenolic acids, apigenin and luteolin were quantified using a separate method described earlier (Ullah et al., 2022). The content of benzoic acid was calculated relative to D_4-SA. Other metabolites were calculated relative to D_6-JA-Ile. To quantify naringenin and sakuranetin, the 1260 HPLC-QTRAP 6500 mass spectrometer system equipped with a turbo-spray ion source was operated in positive ionization mode, as detailed in Lahari et al. (2024). The concentrations of naringenin and sakuranetin were determined by comparison to D_6-ABA, applying the experimental response factors. The same acquisition method was used to quantify the diterpenoid phytoalexins. Multiple reaction monitoring was used to monitor the parent ion → selected fragment ions of diterpenoid phytoalexins: momilactone A, 315 → 271; momilactone B, 331 → 269; oryzalexin E, 305 → 287; oryzalexin S, 287 → 105; phytocassane A, 317 → 299; phytocassane C, 319 → 301.

To quantify aromatic amino acids, the extracts were diluted to 1:10 in water containing a ^{15}N- or ^{13}C-labelled amino acid standard mix (Isotec) as internal standards, and diluted samples were analysed using an Agilent 1260 LC system coupled with a QTRAP 6500 tandem mass spectrometer (Ullah et al., 2022).

4.7 | Statistical analysis

The statistical analysis of the data was performed with the software program GraphPad Prism 10. First, normality was checked with the Shapiro–Wilk test and homoscedasticity was checked with Levene's test. If both conditions were met, a Student's *t* test was used to compare two samples, and for multiple comparisons analysis of variance was used with post hoc Tukey. In the other cases, a Mann–Whitney test was performed.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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