Medicarpin confers powdery mildew resistance in *Medicago truncatula* and activates the salicylic acid signalling pathway

Arunima Gupta1 | Pallavi Awasthi2,3 | Neha Sharma4 | Sajiya Parveen2,3 | Ravi P. Vats2,3 | Nirpendra Singh4 | Yashwant Kumar5 | Atul Goel2,3 | Divya Chandran1

**Abstract**

Powdery mildew (PM) caused by the obligate biotrophic fungal pathogen *Erysiphe pisi* is an economically important disease of legumes. Legumes are rich in isoflavonoids, a class of secondary metabolites whose role in PM resistance is ambiguous. Here we show that the pterocarpan medicarpin accumulates at fungal infection sites, as analysed by fluorescein-tagged medicarpin, and provides penetration and post-penetration resistance against *E. pisi* in *Medicago truncatula* in part through the activation of the salicylic acid (SA) signalling pathway. Comparative gene expression and metabolite analyses revealed an early induction of isoflavonoid biosynthesis and accumulation of the defence phytohormones SA and jasmonic acid (JA) in the highly resistant *M. truncatula* genotype A17 but not in moderately susceptible R108 in response to PM infection. Pretreatment of R108 leaves with medicarpin increased SA levels, SA-associated gene expression, and accumulation of hydrogen peroxide at PM infection sites, and reduced fungal penetration and colony formation. Strong parallels in the levels of medicarpin and SA, but not JA, were observed on medicarpin/SA treatment pre- or post-PM infection. Collectively, our results suggest that medicarpin and SA may act in concert to restrict *E. pisi* growth, providing new insights into the metabolic and signalling pathways required for PM resistance in legumes.

**KEYWORDS**

fluorescein-tagged medicarpin, isoflavone reductase, isoflavonoids, penetration resistance, phytoalexin
INTRODUCTION

Powdery mildew (PM) is one of the most widespread plant diseases, featuring among the top 10 fungal diseases based on economic importance (Dean et al., 2012). It is caused by obligate biotrophic ascomycete fungi, which require a continuous supply of nutrients from living host plants for propagation (Glawe, 2008). PM fungi cause significant yield and quality losses in several agriculturally important crops, including legumes such as pea and mung bean (Fondevilla & Rubiales, 2012; Pandey et al., 2018). Current chemical methods used to control this disease are not ecofriendly, and the development of fungal resistance to these chemicals is of concern (Vielba-Fernández et al., 2020). Thus, a desirable strategy is to genetically engineer PM resistance in legumes, which requires an in-depth understanding of the molecular mechanisms underlying legume–PM interactions.

Medicago truncatula has emerged as an attractive model to investigate disease resistance in legumes due to its small diploid genome (c.450 Mb), short generation time, and transformation potential (Rispail et al., 2008). Furthermore, the availability of M. truncatula accessions exhibiting varying degrees of resistance against the adapted PM Erysiphe pisi enables the discovery of genetic and molecular determinants of resistance. Genetic studies have previously identified three distinct PM resistance loci (Ameline-Torregrosa et al., 2008) and a resistance gene, MtREP1, that controls PM resistance in the highly resistant M. truncatula genotype Jemalong A17 (Yang et al., 2013). Pathogen growth on A17 is arrested after appressorium development and is associated with the accumulation of hydrogen peroxide and fluorescent compounds at fungal penetration sites, consistent with a hypersensitive response (HR) (Foster-Hartnett et al., 2007; Gupta et al., 2020). However, to date, the composition of the fluorescent compound(s) and underlying molecular mechanisms of resistance are not known.

Transcriptomics studies have hinted at a significant role for the isoflavonoid subclass of flavonoid secondary metabolites in M. truncatula–E. pisi interactions; genes encoding enzymes involved in isoflavonoid biosynthesis are differentially regulated in response to PM infection in resistant and susceptible M. truncatula genotypes (Foster-Hartnett et al., 2007; Gupta et al., 2020). Isoflavonoids are almost exclusively synthesized by the plant family Fabaceae (Du et al., 2010) via a branch of the phenylpropanoid pathway, which produces flavanones, isoflavones, isoflavonanes, and pterocarpsans (Dixon et al., 2002). Medicarpin is the major pterocarpan end product of the 5-deoxyisoflavonoid subbranch in M. truncatula that is synthesized from the flavanone liquiritigenin through its enzymatic conversion into a series of intermediates, including the isoflavones daidzein and formononetin, and the precursor isoflavonane 2′-hydroxyformononetin (Figure 1a; Farag et al., 2008; Naoumkina et al., 2007). Another subbranch uses the same set of enzymatic reactions to synthesize the 5-hydroxyisoflavones genistin and biochanin A from the precursor naringenin. The key genes involved in the synthesis of isoflavonoid compounds from liquiritigenin and naringenin include isoflavone synthase (IFS), isoflavone 4′-O-methyltransferase (IOMT), 2′-hydroxyisoflavone dehydratase (2HID), isoflavone 2′-hydroxylase (I2H), isoflavone reductase (IFR), and vestitone reductase (VR) (Naoumkina et al., 2007).

Few isoflavonoids are reported to function as phytoalexins, antimicrobial compounds that are rapidly synthesized in response to biotic/abiotic elicitors (Jiménez-González et al., 2008; Samac & Graham, 2007). For example, isoflavonoid pathway intermediates and the pterocarpan medicarpin were shown to accumulate in M. truncatula on infection with the nonadapted Asian soybean rust Phakopsora pachyrhizi (Ishiga et al., 2015) and the root-infecting necrotroph Rhizoctonia solani (Liu et al., 2017). These studies also demonstrated the antifungal nature of medicarpin against these pathogens through in vitro experiments or exogenous tissue supplementation assays. Legume plants engineered to accumulate higher levels of isoflavonoids also often exhibit higher pathogen resistance (Cheng et al., 2015; He et al., 2000; Liu et al., 2017), highlighting the protective role of these metabolites against plant diseases.

(Iso)flavonoid biosynthesis is also reported to be induced on treatment with defence phytohormones. Methyl jasmonate treatment induced the accumulation of medicarpin in M. truncatula cell suspensions (Naoumkina et al., 2007) whereas salicylic acid (SA), which is required for both basal and resistance (R) gene-mediated resistance against PM (Dewdney et al., 2000; Xiao et al., 2005), activated the biosynthesis of flavanols in poplar during rust infection (Ullah et al., 2019). SA is also known to induce the expression of flavonoid biosynthetic genes and accumulation of flavonoids in other plant species (e.g., Ahn et al., 2014; Gondor et al., 2016; Thiruvengadam et al., 2015), suggesting a role in the regulation of flavonoid biosynthesis.

Despite extensive studies on isoflavonoid biosynthesis, very little is known about their regulatory role in response to pathogen attack and their antifungal potential against PM. Here, we used the M. truncatula–E. pisi pathosystem to investigate the role of the isoflavonoid pathway in PM resistance. Comparative gene expression and metabolite analysis were performed to understand the kinetics of medicarpin biosynthesis in resistant and moderately susceptible M. truncatula genotypes during early PM infection stages. Furthermore, the protective role of medicarpin against E. pisi was demonstrated, and the involvement of the phytohormones SA and jasmonic acid (JA) in medicarpin-induced PM resistance was investigated.

RESULTS

2.1 Isoflavonoid biosynthetic genes are induced earlier in the resistant genotype A17 compared to the moderately susceptible R108 during PM infection

In a previous genome-wide transcriptomics study, we found that a number of isoflavonoid biosynthetic genes were differentially regulated (by >2-fold) between leaves of resistant and susceptible M. truncatula genotypes at 24 h postinoculation (hpi) with the
FIGURE 1  Relative expression of isoflavonoid biosynthetic genes in *Medicago truncatula* at early time points of powdery mildew (PM) infection. (a) Biosynthetic pathway leading to medicarpin synthesis in *M. truncatula* (adapted from Farag et al., 2008; Naoumkina et al., 2007). Genes are represented in italics: PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; IOMT, isoflavone 4′-O-methyltransferase; 2HID, 2′-hydroxyisoflavanone dehydratase; I2H, isoflavone 2′-hydroxylase; IFR, isoflavone reductase; VR, vestitone reductase. Upstream pathway genes are depicted in maroon, central pathway genes in teal and downstream pathway genes in blue.  
(b–p) Relative expression of isoflavonoid biosynthetic genes in A17 and R108 leaves. The x axes represent hours postinoculation (hpi) with *Erisyphe pisi*. Data represent the mean ± SEM of three independent biological replicates. Significant differences between infection time points compared to 0 hpi were computed using one-way analysis of variance with Dunnett’s multiple comparisons test and between genotypes by unpaired *t* test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001); N.D., not detected
Only in A17 (resistant, R) and R108 (moderately susceptible, S) M. truncatula genotypes at 0, 6, 9, 12, and 24 hpi with E. pisi via reverse transcription quantitative PCR (RT-qPCR) (Figure 1). By 24 hpi, E. pisi conidia typically penetrate host cells, form a primary haustorium, and develop a small hyphal peg on R108 whereas they are mostly arrested at the appressorial stage on A17 (Figure S1).

We found that the basal expression levels (0 hpi) of the majority of the upstream and central pathway genes (Figure 1a) were similar in the two genotypes. Exceptions include PAL2, which exhibited >5-fold higher basal expression in A17 (Figure 1c), and IOMT1 and 2HID, which exhibited 3- to 4-fold higher basal expression in R108 (Figure 1k). In contrast, basal expression of downstream genes showed significant differences between genotypes. IFR exhibited >5-fold higher basal expression in A17 (Figure 1o), I2H1 and VR exhibited 1.5 to c.6- fold higher basal expression in R108 (Figure 1l,p), and transcripts of two I2H homologs (I2H2 and I2H3) were detected only in A17 (Figure 1m,n).

In response to E. pisi infection, isoflavonoid pathway genes were significantly induced in both genotypes but marked differences in the temporal kinetics and magnitude of expression were observed. In A17 (R), the expression of all genes, except I2H and IOMT2, was significantly induced between 6 and 12 or 9 and 12 hpi, time points at which the pathogen attempts to penetrate the host cell wall via the appressorium. I2H2 displayed significant induction at 24 hpi (Figure 1m) whereas IOMT2 was repressed at all infection time points (Figure 1l). By contrast, the infection-dependent induction of the majority of the isoflavonoid pathway genes was slightly delayed in R108 (S), occurring mainly between 12 and 24 hpi, time points at which the fungal infection peg develops into a primary haustorium after successful penetration. Only PAL1, CHS2, and IFR were induced early (6–9 hpi) in R108 but, with the exception of PAL2, quickly repressed to basal levels (Figure 1b,c,e,o).

In terms of magnitude, the genes involved in the first (PAL1) and downstream steps of the pathway leading to medicarpin synthesis (I2H1, I2H3, and IFR) exhibited significantly higher expression in A17 compared to R108 at almost all infection time points (Figure 1b,l,n,o). On the contrary, pathway genes involved in the conversion of flavanones to isoflavones (IFS and IOMT1) showed significantly higher expression in R108 than in A17, particularly at 12 and/or 24 hpi (Figure 1g–i).

2.2 | PM infection induces differential folar accumulation of isoflavonoids in A17 and R108

To ascertain whether infection-dependent transcriptional activation of the isoflavonoid pathway translates into metabolite accumulation, we quantified isoflavonoids in leaf tissues of A17 (R) and R108 (S) at 0, 6, 9, 12, and 24 hpi via liquid chromatography-mass spectrometry (LC-MS) analysis (Figure 2). Basal levels (0 hpi) of most compounds were similar in A17 and R108, except for daidzein, formononetin, and 2′-hydroxyformononetin (2HF), which were c.4- to 9-fold higher in R108 (Figure 2d,f,g). In response to PM infection, levels of all pathway metabolites, except 2′-methoxyformononetin (2MF), increased significantly in both A17 and R108 but differences in the timing and/or magnitude of accumulation were observed between genotypes (Figure 2), correlating with the transcriptional activation of pathway genes (Figure 1). The flavonones naringenin and liquiritigenin, and the isoflavone genistein accumulated between 12 and 24 hpi in both genotypes but their levels were significantly higher in A17 than in R108 (Figure 2a–c). The isoflavones daidzein, biochanin, and formononetin, and the isoflavone 2HF accumulated earlier (12 hpi) and to higher levels in R108 (Figure 2d–g); significant accumulation of these compounds was observed only at 24 hpi in A17. The opposite pattern was observed for the pterocarpan end product medicarpin and its precursor 2MF. Medicarpin accumulated earlier in A17 with a 2.6-fold higher concentration than R108 at 6 hpi (Figure 2i) and 2MF levels increased progressively from 6 to 24 hpi in A17 but remained unaltered in R108 (Figure 2h).

2.3 | Pretreatment with medicarpin or select isoflavonoids enhances PM resistance of R108

To test whether exogenously applied isoflavonoids confer PM resistance in M. truncatula, we treated mature leaves of R108 (S) with 10 µM genistein, daidzein, biochanin A, formononetin, 2HF, 2MF, medicarpin (Figure 3a) or 0.2% dimethyl sulphoxide (DMSO) (mock) for 3 h followed by PM inoculation. At 48 hpi with E. pisi, the time when the fungus develops ≥3 hyphae, the pathogen load was c.40%–60% lower in daidzein-, formononetin-, 2MF-, and medicarpin-treated leaves than in mock-treated leaves (Figure 3b). Additionally, treatment with 2.5, 5, and 10 µM medicarpin reduced PM biomass in a concentration-dependent manner (Figure 3c). At these medicarpin concentrations, no visible signs of cytotoxicity (as evidenced by trypan blue and 3,3′-diaminobenzidine [DAB] staining) were observed in noninoculated R108 leaves up to 48 h after medicarpin treatment (Figure S2).

To determine which PM infection stage was primarily inhibited by medicarpin, E. pisi growth was quantified. At 48 hpi, 10 µM medicarpin-treated leaves contained 75% appressorial-stage conidia as compared to 44% in mock-treated leaves (Figure 3d,e). Conversely, only 12% of E. pisi conidia formed colonies (conidia with ≥3 hyphae) in medicarpin-treated leaves as opposed to 34% in mock-treated controls (Figure 3d,e). These results indicate that medicarpin inhibits PM growth early in the infection process.

2.4 | Medicarpin localizes at PM penetration sites in R108

The inhibitory effect of medicarpin on E. pisi growth prompted us to investigate its spatial dynamics in response to PM infection. To visualize medicarpin in plant tissues, we chemically synthesized medicarpin tagged with the organic fluorophore fluorescein (Figure 4a). The excitation and fluorescence spectra of 2-[(6-(4-[[19}
methoxy-6a,11a-dihydro-6H-benzofuro[3,2-c]chromen-3-yl)oxy)butoxy)-3-oxo-3H-xanthen-9-yl]benzoic acid (8) showed absorption maxima at 525 nm and emission maxima at 545 nm (Figure 4a).

To examine the localization of medicarpin during PM infection, R108 (S) leaves were infiltrated with fluorescein-medecarpin and then inoculated with *E. pisi*. We observed that 3 h after infiltration (preinoculation) with fluorescein-medecarpin, fluorescence was observed throughout the leaf epidermal cells (Figure 4b). At 24 hpi with *E. pisi*, fluorescence was visible in a concentrated ring beneath the appressorium at 84% of the infection sites (Figure 4b,c) and at a few infection sites surrounding the haustorial complex (HC) (Figure 4b) in fluorescein-medecarpin-treated leaves. In contrast, only 20% of the infection sites showed this fluorescence pattern in fluorescein-treated control leaves (Figure 4b,c).

### 2.5 Medicarpin pretreatment enhances focal accumulation of hydrogen peroxide at fungal penetration sites in R108

Accumulation of reactive oxygen species, such as hydrogen peroxide (H$_2$O$_2$), is one of the early host responses to pathogen attack that eventually leads to HR and pathogen resistance (Huckelhoven & Kogel, 2003). To determine whether medicarpin pretreatment affects H$_2$O$_2$ accumulation in response to PM infection, we detected H$_2$O$_2$ at the PM infection site in 10 µM medicarpin and mock-treated R108 (S) leaves at 24 hpi through DAB staining, which produces a brown precipitate. Regardless of treatment type, DAB staining revealed two patterns of H$_2$O$_2$ accumulation at the PM infection site: (a) restricted to a small area beneath the appressorium (Figure 5a,d) and (b) in the entire HC-containing epidermal cell (Figure 5e,f). The percentage of infection sites showing appressorium-localized H$_2$O$_2$ accumulation was similar in medicarpin- and mock-treated leaves at 24 hpi (Figure 5g). However, 43% of the infection sites showed H$_2$O$_2$ accumulation in the entire infected cell in medicarpin-treated leaves as opposed to only 2% in mock-treated leaves. Moreover, at a few infection sites, H$_2$O$_2$ accumulation was clearly visible around the HC in medicarpin-treated leaves (Figure 5f) but not in mock-treated leaves. Conversely, the mock-treated leaves contained a greater percentage of infection sites in which H$_2$O$_2$ was not detected than in medicarpin-treated leaves (Figure 5c,g). We verified the DAB staining results by quantifying H$_2$O$_2$ levels using the potassium iodide colourimetric assay. Higher H$_2$O$_2$ content was observed in medicarpin-treated leaves than in mock-treated leaves.
FIGURE 3 Evaluation of powdery mildew (PM) growth after exogenous foliar application of isoflavonoids in R108. (a) Structure of isoflavonoids. (b) Relative expression of Ep18S rRNA in mock and 10 µM isoflavonoid-treated leaves at 48 h postinoculation (hpi) with Erysiphe pisi. Data represent the mean ± SEM of eight biological replicates from two or three independent experiments. (c) Relative expression of Ep18S rRNA in mock and medicarpin-treated leaves at 48 hpi. Data represent the mean ± SEM of five biological replicates from two independent experiments. Significant differences in (b) and (c) were computed using one-way analysis of variance with Dunnett's multiple comparisons test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001) (d) Representative images showing E. pisi growth on mock and 10 µM medicarpin-treated leaves at 48 hpi. Scale bar, 50 µm. c, conidium; a, appressorium (e) The mean percentage (± SEM) of E. pisi conidia that reached different infection stages on mock and 10 µM medicarpin-treated leaves at 48 hpi assessed from c.2000 conidia on a total of eight leaflets from three independent experiments. Significant differences were computed using multiple unpaired t test (**p ≤ 0.01, ***p ≤ 0.001)
FIGURE 4  Chemical synthesis and localization of fluorescein-medicarpin at powdery mildew (PM) infection-sites in R108 leaves. (a) Schematic for the chemical synthesis of 2MF (4), 2HF (5), medicarpin (6), and fluorescein-medicarpin (8), and excitation/emission fluorescence spectra of fluorescein-medicarpin. AlCl₃, aluminium chloride; ACN, acetonitrile; BF₃·OEt₂, boron trifluoride etherate; MeSO₂Cl, methanesulfonyl chloride; DMF, dimethylformamide; RT, room temperature. (b) Representative images showing localization of fluorescein-medicarpin or fluorescein (mock) 3 h after medicarpin treatment (preinoculation) and at 24 h postinoculation (hpi) after Erysiphe pisi inoculation. Fluorescein-medicarpin is visible as green fluorescence (arrows) beneath the appressorium (middle panel) and in the primary haustorial complex (HC; bottom panel). c, conidium; a, appressorium, h, hypha. PI, propidium iodide; DAPI, 4',6-diamidino-2-phenylindole; Scale bar, 20 µm. (c) Percentage of E. pisi conidia showing PM infection site-localized fluorescence in medicarpin- and mock-treated leaves. A total of 50 infection sites were assessed from five leaflets/treatment.
at 24 hpi (Figure 5h). In addition, fewer conidia developed primary haustoria in medicarpin-treated leaves than in mock-treated controls (Figure 5i).

2.6 | PM infection induces differential foliar accumulation of major defence phytohormones in A17 and R108

To determine whether the phytohormones SA and JA differentially accumulate during PM infection, we measured SA (total) and JA levels in A17 (R) and R108 (S) leaves at 0, 6, 9, 12, and 24 hpi. Similar to medicarpin, SA levels increased early in A17, accumulating at 6 and 9 hpi (Figure 6a). By contrast, SA levels were unaltered at 6 hpi and decreased between 9 and 24 hpi in R108. Consequently, pathogen-induced SA levels were between 2- to 5-fold higher in A17 compared to R108 at all infection time points. On the other hand, JA levels increased at 9 and 24 hpi in A17 whereas a slight increase was observed in R108 at 12 hpi (Figure 6b). JA levels were c.3-fold higher at 9 and 24 hpi and c.1.7-fold lower at 12 hpi in A17 than in R108.

2.7 | Pretreatment with medicarpin enhances SA accumulation in R108

Because SA, JA, and medicarpin accumulate early in response to PM infection in A17, we investigated whether medicarpin treatment affects SA and/or JA accumulation. We first measured total SA and
JA levels in R108 (S) leaves 3 h after 10 µM medicarpin treatment (preinoculation; Figure 6c). Leaf medicarpin content increased by c.5-fold 3 h after medicarpin infiltration (Figure 6d). Furthermore, as compared to the mock-treated controls, a 1.8-fold increase in total SA content was detected 3 h after medicarpin treatment whereas JA levels remained unaltered (Figure 6d). We also measured SA (total) and JA levels in mock- and medicarpin-treated leaves after PM inoculation. For this, we inoculated R108 leaves with PM 3 h after medicarpin treatment and harvested samples at 48 hpi (post-PM inoculation; Figure 6c), the time point at which quantifiable differences in PM growth were observed. Medicarpin, SA, and JA levels increased by c.2.5-fold in mock-treated leaves at 48 hpi with E. pisi (Figure 6d), indicating a delayed induction of these metabolites in response to PM infection in R108. Compared to the mock-treated
leaves, medicarpin-treated leaves contained significantly higher levels of medicarpin but not JA at 48 hpi (Figure 6d). Total SA levels were higher but the difference was not statistically significant (Figure 6d).

We also investigated the effect of medicarpin pretreatment on the content of 5-deoxyisoflavonoids and their precursor liquiritigenin in R108 leaves 3 h after 10 µM medicarpin treatment and at 48 hpi with E. pisi (Figure 6e). At 3 h after medicarpin treatment, only formononetin levels increased significantly compared to the mock treatment. However, compared to the 3 h time point, levels of all 5-deoxyisoflavonoid intermediates and their precursor liquiritigenin increased in both mock- and medicarpin-treated leaves at 48 hpi with E. pisi, with the increase consistently higher in the medicarpin-treated leaves (Figure 6e).

2.8 | Medicarpin pretreatment affects the expression of SA biosynthesis and signalling marker genes in R108

To determine whether the medicarpin-induced increase in SA levels (Figure 6d) results from induced biosynthesis, we analysed the expression of the SA biosynthetic genes ICS1 and PAL1 (Dempsey et al., 2011) in 3 h (preinoculation) and 48 hpi (post-PM inoculation) medicarpin- and mock-treated R108 (S) leaves (Figure 6c). Transcript levels of PAL1 and ICS1 remained unaltered at 3 h, but at 48 hpi with E. pisi, the expression of both genes was significantly induced in medicarpin-treated leaves as compared to mock-treated leaves (Figure 7a). We also examined the expression of these genes 1 h (noninoculated) after medicarpin treatment to determine whether transcriptional induction of these biosynthetic genes precedes metabolite accumulation. PAL1 expression was significantly induced 1 h after medicarpin treatment whereas the expression of ICS1 remained unaltered (Figure 7d). This suggests that medicarpin treatment alone, in the absence of PM challenge, may induce SA biosynthesis through the PAL1 pathway whereas in response to PM infection, the ICS1 pathway may also be involved, as ICS1 transcript levels were significantly induced at 48 hpi in both mock- and medicarpin-treated leaves (Figure 7a).

To determine whether elevated SA levels in medicarpin-treated leaves lead to enhanced defence signalling, we quantified the expression of known markers of the SA signalling pathway, that is, PR1, PR2, PR5, and PR10. We found that the expression of PR1 was significantly induced at 3 h and 48 hpi in medicarpin-treated leaves compared to mock-treated controls, whereas the expression of PR2 and PR5 was significantly induced at 3 h and/or 48 hpi, respectively (Figure 7a), suggesting that medicarpin treatment enhances SA signalling. We also examined the effect of medicarpin treatment on the expression of JA biosynthetic genes (LOX3, LOX4, AOS1, and AOC) and a JA signalling marker (VSP1). With the exception of AOS1 and LOX3, the expression of JA biosynthetic genes remained unaltered in 1 h, 3 h, and 48 hpi medicarpin-treated leaves compared to mock-treated controls (Figure 7b,d). Expression of the JA signalling marker VSP1 also did not change after medicarpin treatment at 3 h and 48 hpi (Figure 7b).

We also examined the impact of medicarpin treatment on the expression of the isoflavonoid biosynthetic genes IFS1 and IFR. IFS1 expression was significantly induced in 1 h (noninoculated) and 48 hpi medicarpin-treated leaves but remained unaltered in 3 h medicarpin-treated leaves (preinoculation) compared to the mock-treated controls (Figure 7c,d). In contrast, IFR expression did not change significantly at any time point after medicarpin treatment (Figure 7c,d).

2.9 | SA treatment induces the expression of key isoflavonoid biosynthesis genes and accumulation of PM-induced medicarpin levels in R108

Because SA is known to induce the expression of phenylpropanoid pathway genes, we checked whether SA treatment can also induce the expression of isoflavonoid pathway genes in M. truncatula. Promoter analysis revealed the presence of SA-responsive TCA elements within the 2-kb upstream regions of IFS1 and IFR genes in A17 and R108 (Figure 8a and Table S1). To verify whether SA can induce the expression of these genes, we quantified IFS1 and IFR transcript levels after SA treatment in R108 (S) leaves. An SA concentration of 1 mM was chosen on the basis of preliminary experiments that measured the ability of two different SA concentrations (0.5 and 1.0 mM) to induce the expression of these genes at 3 h after treatment. As compared to the mock treatment, 3 h of SA treatment significantly induced the expression of IFS1 and IFR by 3.0- and 2.3-fold, respectively (Figure 8b). We also quantified levels of medicarpin, JA, the 5-deoxyisoflavonoid intermediates daidzein, formononetin, 2HF, and 2MF, and their precursor liquiritigenin in SA- and mock-treated leaves pre- (3 h) and post-PM (48 hpi) inoculation. Pretreatment of R108 leaves with SA increased the SA content by c.18- and 16-fold at 3 h and 48 hpi, respectively (Figure 8c). Medicarpin levels did not differ significantly between SA- and mock-treated leaves at 3 h; however, at 48 hpi, a significant increase (c.2-fold) in medicarpin levels was observed in SA-treated leaves compared to mock-treated controls (Figure 8c). JA levels were significantly lower in SA-treated leaves compared to mock-treated controls at 3 h but not at 48 hpi (Figure 8c). In the case of the isoflavonoids, daidzein levels decreased significantly at 3 h whereas 2MF levels increased significantly at 48 hpi in SA-treated leaves compared to mock-treated controls (Figure 8d). Levels of medicarpin, JA, and 5-deoxyisoflavonoids increased in both mock- and SA-treated leaves in response to PM infection (Figure 8c,d).

3 | DISCUSSION

3.1 | Expression of isoflavonoid biosynthetic genes and accumulation of specific isoflavonoids occurs early in the resistant M. truncatula genotype in response to PM infection

Previous transcriptomic studies revealed that isoflavonoid biosynthetic genes are differentially regulated in resistant and susceptible M. truncatula genotypes following E. pisi inoculation (Foster-Hartnett et al., 2007; Gupta et al., 2020). Here, we show
Figure 7  Effect of medicarpin pretreatment on the expression of salicylic acid (SA) and jasmonic acid (JA) pathway genes and select isoflavonoid biosynthetic genes in R108. Relative expression of (a) SA biosynthesis and signalling markers, (b) JA biosynthesis and signalling markers, and (c) IFS1 and IFR 3 h (preinoculation) and 48 h postinoculation (hpi) (post-powdery mildew [PM] inoculation) in medicarpin- and mock-treated R108 leaves. (d) Relative expression of SA biosynthesis and signalling markers (left), JA biosynthesis and signalling markers (middle), and IFS1 and IFR (right) in 1 h (noninoculated) medicarpin- and mock-treated R108 leaves. Data represent the mean ± SEM of six biological replicates from two independent experiments. Significant differences between treatments and between time points were computed by unpaired t test (*p < 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). PAL, phenylalanine ammonia-lyase; ICS1, isochorismate synthase 1; PR1, pathogenesis-related 1; PR2, pathogenesis-related 2; PR5, pathogenesis-related 5; PR10, pathogenesis-related 10; LOX3, lipoxygenase 3; LOX4, lipoxygenase 4; AOS1, allene oxide synthase 1; AOC, allene oxide cyclase; VSP1, vegetative storage protein 1; IFS1, isoflavone synthase 1; IFR, isoflavone reductase.
**FIGURE 8** Effect of salicylic acid (SA) pretreatment on isoflavonoid biosynthesis in R108. (a) Location of SA-responsive cis-acting TCA element in the 2-kb upstream promoter region of \( IFS1 \) and \( IFR \) genes in A17 (black triangles) and R108 (striped maroon circles); \( IFS1 \), isoflavone synthase 1; \( IFR \), isoflavone reductase. (b) Relative expression of \( IFS1 \) and \( IFR \) genes in R108 leaves 3 h after mock or 1 mM SA treatment. Data represent mean ± SEM of six biological replicates from two independent experiments. Significant differences were computed by unpaired t test (* \( p < 0.05 \), ** \( p \leq 0.01 \)) (c, d) Levels of total SA, medicarpin, jasmonic acid (JA), the 5-deoxyisoflavonoid intermediates, and their precursor liquiritigenin in 3 h (preinoculation) and 48 h postinoculation (hpi) (after powdery mildew [PM]-inoculation) SA- or mock-treated R108 leaves. Data represent the mean ± SEM of 10 biological replicates from three independent experiments. Significant differences between mock and SA treatment and between time points were computed by unpaired t test (* \( p < 0.05 \), ** \( p \leq 0.01 \), *** \( p \leq 0.001 \), **** \( p \leq 0.0001 \)). (e) Proposed model showing the contribution of medicarpin and SA in PM resistance in *Medicago truncatula*. PM infection stimulates biosynthesis of medicarpin, SA, and JA. Medicarpin and SA act in concert, perhaps in a regulatory loop, triggering the localized accumulation of medicarpin and \( \mathrm{H}_2\mathrm{O}_2 \) at fungal penetration sites that leads to hypersensitive response and PM resistance.
through comparative and targeted temporal gene expression and metabolite profiling that isoflavonoid biosynthetic gene expression and accumulation of specific isoflavonoids are induced earlier in the highly resistant M. truncatula genotype A17 compared to the susceptible genotype R108 during the initial penetration phase of PM infection.

The majority of the isoflavonoid pathway genes were induced between 6 and 12 hpi in A17 (R) whereas the PM-responsive genes in R108 (S) were mainly induced between 12 and 24 hpi (Figure 1). Such genotypic differences in expression kinetics of isoflavonoid biosynthetic genes was previously observed in M. truncatula in response to the Asian soybean rust (Mortel et al., 2007). In addition, although the transcriptional activation of isoflavonoid biosynthetic genes in response to PM infection correlated with metabolite accumulation in both genotypes (Figure 2), accumulation of the pterocarpan end product of the 5-deoxyisoflavonoid subbranch, medicarpin, occurred earlier in the R genotype. In A17, medicarpin levels increased early (6 hpi) and remained elevated until 24 hpi. Such genotypic differences in expression kinetics of isoflavonoid biosynthetic genes was previously observed in M. truncatula in response to the Asian soybean rust (Mortel et al., 2007). In addition, although the transcriptional activation of isoflavonoid biosynthetic genes in response to PM infection correlated with metabolite accumulation in both genotypes (Figure 2), accumulation of the pterocarpan end product of the 5-deoxyisoflavonoid subbranch, medicarpin, occurred earlier in the R genotype. In A17, medicarpin levels increased early (6 hpi) and remained elevated until 24 hpi, whereas significant accumulation of the isoflavone intermediate and the isoflavonone precursor 2HF was observed only at 24 hpi. This early increase in medicarpin concentration without a concomitant increase in levels of the intermediate and precursor metabolites indicates a rapid conversion of the intermediate metabolites to medicarpin. By contrast, intermediate and precursor metabolites accumulated at 12 hpi in R108 (S) but a significant increase in medicarpin levels was observed only at 24 hpi. This supports the idea that a rapid conversion of precursors to medicarpin during PM infection is critical for effective penetration resistance. A similar pattern was previously observed in soybean-rust pathogen interactions where higher concentrations of precursor isoflavones were detected in rust-susceptible genotypes whereas higher levels of the pterocarpan glyceollin were detected in tolerant genotypes (Lygin et al., 2009). We speculate that the differential transcriptional regulation of the final steps in the pathway, from formononetin to medicarpin, may be principally responsible for the rapid accumulation of medicarpin in A17 during PM infection as higher basal and/or pathogen-induced expression of I2H and IFR was observed in this genotype (Figure 1).

Isoflavonoids are generally stored as stable glucoside conjugates in the cell's vacuole and studies have shown that in response to certain elicitors, such as MeJA (Naoumkina et al., 2007), or conditions in which the activity of isoflavonoid biosynthetic enzymes is inhibited (Mackenbrock & Barz, 1991), the vacuolar pools of isoflavonoid glucoside conjugates are remobilized and used as precursors for medicarpin biosynthesis as an alternative to de novo synthesis. Because we measured total (aglycone plus glucoside conjugates) metabolite levels in this study, we cannot exclude the possibility that a similar mechanism may operate in response to PM infection where remobilization of the conjugated forms may lead to early accumulation of medicarpin in A17 (R), ahead of the synthesis of its precursor metabolites. Consistent with this hypothesis, a number of β-glucosidase genes were found to be specifically induced in response to PM infection in A17 (Gupta et al., 2020).

3.2 Medicarpin functions as a phytoalexin against PM

Elevated isoflavonoid phytoalexin content in plants has often been correlated with pathogen resistance (Cheng et al., 2015; He et al., 2000; Liu et al., 2017) but direct evidence for their antifungal potential has primarily relied on in vitro studies (Adesanya et al., 1986; Blount et al., 1992; Ishiga et al., 2015; VanEtten, 1976; Weidenbörner & Jha, 1994). For example, medicarpin and its precursor 2HF inhibited the radial growth of Colletotrichum spp. and Phoma medicaginis in agar plate assays (Blount et al., 1992). Daidzein and formononetin also display antifungal activity against a few pathogens; however, their inhibitory effect varies depending on the metabolite concentration and/or fungal species targeted (Blount et al., 1992). Few studies also reported the inhibitory activity of isoflavonoids on the in planta growth of fungal pathogens such as Fusarium oxysporum (Stevenson et al., 1997), Heminthosporium carbonum (Duczek & Higgins, 1976), and Rhizoctonia solani (Liu et al., 2017). However, similar studies were not available for PMs. In this study, we show that treatment with medicarpin, its putative precursor 2MF, or the isoflavones daidzein and formononetin significantly inhibited E. pisi growth on R108 (S) leaves (Figure 3), demonstrating their antifungal role against PM. Notable among these is 2MF, which showed markedly higher accumulation in A17 compared to R108 (Figure 2h). This compound, which had not been detected in M. truncatula until now, was previously identified in a methanol extract of the heartwood of Dalbergia parviflora and shown to exert cell proliferation stimulatory activity against two human breast cancer cell lines (Umehara et al., 2009). Structurally, 2MF differs from 2HF by the presence of a methoxy group instead of a hydroxy group at the 2′ position (Figure 3a). Although previous structure–activity-related studies have indicated that an extra methoxy group does not always correlate with higher antifungal activity (Adesanya et al., 1986; Weidenbörner et al., 1990), sativan, a 7-hydroxy-2′,4′-dimethoxy-isoflavon, was found to be more active than the corresponding 7,2′-dihydroxy-4′-methoxy-isoflavan vestitol (VanEtten, 1976), suggesting that such substitutions may enhance the antifungal potential of isoflavonones.

In addition to a direct effect, the 5-deoxyisoflavonoids daidzein, formononetin, and 2MF may negatively impact PM growth indirectly through their conversion into medicarpin, which also exhibits antifungal activity against E. pisi. A greater number of apressorial stage E. pisi conidia were visible on medicarpin-treated leaves than in controls (Figure 3), indicating that medicarpin contributes to PM resistance in M. truncatula. Although naringenin and the 5-hydroxyisoflavones genistein and biochanin A were also induced on PM infection, and in some cases to greater levels in R108 (S) (Figure 2), these metabolites did not exhibit antifungal activity against E. pisi at the concentration tested (Figure 3). This finding further supports our conclusion that the PM infection-dependent induction of the isoflavonoid subbranch leading to medicarpin synthesis primarily contributes to PM resistance.

For further insights into its antifungal role, we developed fluorescein-medicarpin and examined its spatial accumulation in...
planta during PM infection. Fluorescent probes have emerged as versatile analytical and imaging tools for the visualization and tracing of small molecule dynamics within plant tissues (Devree et al., 2021; Lace & Prandi, 2016). We observed that exogenously applied fluorescein-medicarpin localized to PM penetration sites at 24 hpi (Figure 4), suggesting that the translocation of the phytoalexin to the site of fungal attack, after its synthesis, may be an integral part of the host plant’s resistance response. It will be interesting to test whether members of the plant ABC transporter family are involved in this translocation, as secondary metabolites, including isoflavonoids, are known to be substrates of these transporters (Biala et al., 2017; Biala & Jasiński, 2018). The common induction pattern of MtABCG10, PAL, and IFS genes following treatment with a fungal elicitor (Banaśiak et al., 2013) further supports this idea.

We found that medicarpin treatment also enhanced H2O2 accumulation at fungal penetration sites in R108 (S) leaves (Figure 5). Studies on barley–PM interactions indicate that strong H2O2 accumulation in cell wall appositions produced around attempted sites of penetration is generally associated with host cell inaccessibility whereas H2O2 accumulation in the entire attacked cell is linked to HR-associated cell death and pathogen arrest (Hückelhoven & Kogel, 2003). The focal accumulation of H2O2 underneath the E. pisi appressorium and in some cases in the entire attacked epidermal cell in response to medicarpin treatment suggests that medicarpin may contribute to penetration resistance and postpenetration HR in M. truncatula. In addition, the similar localization patterns of H2O2 and fluorescein-medicarpin (Figures 4 and 5) suggests that accumulation of medicarpin at PM infection sites may be the trigger for H2O2 production and HR. Such a link between isoflavonoids and HR was previously demonstrated in soybean cultivars with race-specific resistance against the oomycete Phytophthora sojae, where silencing of IFS and chalcone reductase (CHR) genes in roots suppressed daidzein levels, infection-localized H2O2 accumulation and HR, and enhanced root rot development (Graham et al., 2007).

3.3 Medicarpin and SA may act synergistically to restrict PM growth in M. truncatula

SA is known to play a fundamental role in plant defence against PM in Arabidopsis (e.g., Chandran et al., 2009; Dewdney et al., 2000; Xiao et al., 2005). A recent study showed that free SA levels were unaltered in the highly susceptible M. truncatula genotype DZA315.16 and susceptible Pisum sativum (pea) cultivar at 1 day postinoculation with E. pisi but pre-established nodulation enhanced free SA levels and PM resistance of these plants (Smigielski et al., 2019), implying a defensive role for SA against PM in legumes as well. Here, we show that total SA levels were induced early during PM infection in A17 (R) but not in R108 (S), and that pretreatment with medicarpin enhanced SA biosynthesis (Figure 6) and PM resistance of R108. SA is synthesized in plants via the ICS and PAL pathways (Dempsey et al., 2011; the ICS pathway predominates in Arabidopsis (Wildermuth et al., 2001), whereas an equal contribution of both ICS and PAL pathways was reported in the legume soybean (Shine et al., 2016). Our data suggests that medicarpin treatment positively regulates SA biosynthesis in M. truncatula primarily through transcriptional activation of the PAL pathway and that both ICS and PAL pathways may contribute to SA accumulation during PM infection (Figures 6 and 7). Medicarpin treatment also induced the expression of IFS and enhanced accumulation of the 5-deoxyisoflavonoid formononetin and its precursor liquiritigenin, pre- and/or post-PM inoculation (Figures 6 and 7), indicating that it may also regulate its own synthesis through a positive feedback mechanism. Reports on the nuclear localization of flavonoids within plant cells (Naoumkina & Dixon, 2008; Saslowsky et al., 2005) support a role for these metabolites in transcriptional regulation but this requires further experimental validation.

SA biosynthetic and signalling components are known to be essential for R gene-mediated H2O2 accumulation and HR at PM infection sites in Arabidopsis (Wang et al., 2009; Xiao et al., 2003). It is therefore possible that the enhanced PM resistance phenotype of medicarpin-treated R108 leaves is a result of elevated SA-mediated defence priming, which leads to enhanced accumulation of infection site-localized H2O2 and expression of SA signalling markers (PRs). Furthermore, a rapid and coordinated accumulation of medicarpin and SA in response to PM infection appears to be required for this early resistance response as mock-treated R108 leaves with low medicarpin content exhibited moderate susceptibility to PM despite having elevated levels of SA and isoflavonoid intermediates at 48 hpi (Figure 6d,e). The observation that medicarpin treatment alone did not cause H2O2 accumulation in R108 leaves (Figure S2) further supports this idea. This may explain why A17, which accumulates both medicarpin and SA at early infection time points, is highly resistant to PM, whereas R108, which shows delayed accumulation of medicarpin and SA, is moderately susceptible. Consistent with this notion, a recent study showed that the coordinated accumulation of specific phenylpropanoids, SA, and jasmonate in susceptible grape berries during PM infection was not sufficient to restrict fungal infection, perhaps due to delayed induction of these compounds (Pimentel et al., 2021). While our data shows that medicarpin treatment did not significantly affect JA accumulation or the expression of JA biosynthetic and signalling marker genes, investigations into its role in the accumulation of other active forms of jasmonate, such as the JA-isoleucine conjugate, will help ascertain its full impact on the JA pathway.

SA is known to positively regulate (iso)flavonoid biosynthesis in plants (Durango et al., 2014; Gondor et al., 2016; Katoch et al., 2005; Ullah et al., 2019). To determine whether SA regulates medicarpin synthesis in M. truncatula in the context of PM infection, we performed exogenous SA treatment assays in R108 leaves pre- and post-PM inoculation. We found that 3 h of SA treatment induced the expression of IFS1 and IFR genes in R108 but a concomitant increase in medicarpin levels or its isoflavonoid precursors was not observed (Figure 8b,c), suggesting that the isoflavonoid biosynthesis pathway may also be regulated at the posttranscriptional level. However, at
48 hpi with *E. pisi*, a significant increase in medicarpin content and its precursor 2MF was observed in SA-treated leaves compared to mock-treated controls (Figure 8c,d). This implies that SA has the potential to induce medicarpin synthesis in *M. truncatula* and this function is exhibited during PM infection, potentially as a way to regulate infection-induced medicarpin levels. In the absence of PM infection, SA treatment significantly reduced JA levels, supporting the known antagonism between the two phytohormones (Thaler et al., 2012). However, at 48 hpi with *E. pisi*, JA levels were not significantly different between SA- and mock-treated leaves, indicating that PM-induced JA accumulation is not affected by SA treatment.

In conclusion, we propose that rapid and coordinated accumulation of medicarpin and SA in response to PM infection triggers H$_2$O$_2$ production of medicarpin and SA in response to PM infection triggers H$_2$O$_2$ accumulation and HR at fungal penetration sites, providing early resistance against PM in the highly resistant *M. truncatula* genotype A17 (Figure 8e). Furthermore, we demonstrate that medicarpin treatment enhances the accumulation of SA, and SA treatment in turn increases medicarpin levels in *M. truncatula* only in the presence of infection, indicating a synergism between the isoflavonoid and SA biosynthetic pathways that may be crucial for early PM resistance (Figure 8e). Future studies with *M. truncatula* isoflavonoid/SA biosynthetic mutants will reveal whether endogenously produced medicarpin can induce SA biosynthesis and vice versa during PM infection. Our study has important implications for agriculture, as the use of medicarpin can enhance crop protection against PM and potentially reduce the use of harmful pesticides.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Plant growth and pathogen inoculation

*M. truncatula* seeds were scarified using concentrated sulphuric acid and germinated on moist filter paper in the dark at 4°C for 2 days followed by 1 day at 22°C. Seedlings were grown and inoculated with *E. pisi* (Palampur-1 isolate) as per Gupta et al. (2021).

### 4.2 | Synthesis of medicarpin and its fluorescent derivative

Medicarpin was prepared according to the efficient synthetic route shown in Figure 4a (Goel et al., 2012). Briefly, the reaction was initiated by stirring a mixture of resorcinol (1) and 2,4-dimethoxyphenyl acetic acid (2) in the presence of Lewis acid BF$_3$·OEt$_2$ to produce the deoxybenzoyl derivative (3). This derivative, on treatment with mesyl chloride in dimethylformamide (DMF), produced the isoflavone 3-(2,4-dimethoxyphenyl)-7-hydroxy-4H-chromen-4-one [2′-methoxyformononetin (2MF), (4)] in 70% yield. Furthermore, the selective demethylation of 2MF was carried out in the presence of AlCl$_3$ and acetonitrile at refluxing temperature to furnish 7-hydroxy-3′-(2-hydroxy-4-methoxyphenyl)-4H-chromen-4-one [2′-hydroxyformononetin (2HF); (5)] in 60% yield. Treatment of 2HF with sodium borohydride in absolute ethanol yielded medicarpin (6) in 65% yield. Fluorescent medicarpin was synthesized by attaching fluorescein (TCI Chemicals) via a linker. For this, medicarpin was treated with 1,4-dibromobutane in the presence of potassium carbonate in DMF. Furthermore, 3-(4-bromobutoxy)-9-methoxy-6a,1 1a-dihydro-6H-benzofuro[3,2-c]chromene (7) was reacted in DMF with fluorescein in the presence of potassium carbonate to synthesize fluorescent medicarpin (8). All compounds were characterized by mass spectrometry-electrospray ionization (MS-ESI), $^1$H and $^{13}$C nuclear magnetic resonance (NMR), and melting point analysis. The photophysical properties of the fluorescein-medicarpin were examined by UV-vis and fluorescence spectroscopic techniques using DMSO as the solvent at a concentration of $10^{-5}$ M. Details of synthesis and analyses are provided in Methods S1.

### 4.3 | RNA extraction, cDNA synthesis, and RT-qPCR

Total RNA was extracted from c.50 mg *M. truncatula* leaves using the Nucleospin RNA Plant kit (Macherey-Nagel) with on-column DNase treatment. First-strand cDNA was synthesized from 1 μg total RNA using the iScript cDNA synthesis kit (Bio-Rad) or PrimeScript cDNA synthesis kit (Takara-Bio Inc.). Four-fold diluted cDNA was used for RT-qPCR using the 5x HOT FIREPol EvaGreen qPCR mix (Solis BioDyne) or TB Green Premix qPCR mix (Takara-Bio Inc.) in a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). Three independent biological replicates were processed. *MtUbiquitin* (UBQ; Medtr3g092130) was used as the internal control due to its invariant expression across infection time points and genotypes (Figure S3). Relative expression was calculated using LinRegPCR v. 2015.1 (Ruijter et al., 2009). Primers were designed using NCBI Primer-Blast (Table S2) and product specificity was confirmed by melt curve analysis.

### 4.4 | Metabolite extraction and LC-MS

Standards for naringenin, liquiritigenin, genistein, daidzein, formononetin, biochanin A, SA, and JA were obtained from Sigma Aldrich. Standards for 2HF, 2MF, and medicarpin were synthesized as described above. Metabolite extraction was performed according to Liu et al. (2017) with modifications. Approximately 100 mg of leaf tissue was ground in liquid nitrogen and extracted three times in 1 ml of 80% methanol. The mixture was vortexed and sonicated for 10–15 min each followed by centrifugation for 15 min at 15,000 × g. The collective supernatants were vacuum-dried and treated with 25 U β-glucosidase (Sigma Aldrich) per reaction in citrate-phosphate buffer (pH 5.2) at 37°C for 10–12 h in the dark and vacuum-dried again. The dried samples were dissolved in 100 μl of 20% methanol, diluted 10-fold, filtered through 0.2 μm polytetrafluoroethylene filters (Millipore), and analysed by LC-ESI-MS/MS using a QTRAP 6500+ mass spectrometer (Sciex) according to Bisht et al. (2021).
4.5 | Isoflavonoid/SA treatment and PM growth assessment

Mature leaves of 3-week-old R108 plants were individually infiltrated with 10 µM of each isoflavonoid precursor, 2.5, 5 or 10 µM medicarpin, or 0.2% DMSO (mock) using a needleless syringe. At 3 h postinfiltration, leaves were inoculated with PM as described above. Fungal load was quantified via RT-qPCR amplification of Ep18S rRNA. E. pisi growth stages were quantified by staining infected leaves with trypan blue (Vogel & Somerville, 2000) and visualizing under a 20x objective in bright-field using a PALM MicroBeam microscope (Carl Zeiss). Gene expression analysis was performed at 1 h (noninoculated) and 3 h (preinoculation) after 10 µM medicarpin or 0.2% DMSO (mock) treatment and at 48 hpi (post-PM inoculation) in 3 h medicarpin- or mock-treated leaves. For the SA treatment, 3-week-old R108 leaves were infiltrated with 1 mM SA or 0.1% ethanol (mock) and harvested after 3 h for expression analysis and at 3 h and 48 hpi for metabolite analysis. Each experiment was repeated two or three times with at least four biological replicates/experiment for expression analysis and 10 biological replicates/experiment for metabolite analysis.

4.6 | Visualization of fluorescein-medicarpin

Fluorescein-medicarpin and fluorescein (Sigma) were dissolved in DMSO to prepare 1 mM stock solutions. R108 leaves were infiltrated with 10 µM fluorescein-medicarpin or fluorescein (control) and inoculated with PM as described above. Leaf discs were harvested at 24 hpi and stained with propidium iodide (5 µg/ml; Sigma) and 4’,6-diamidino-2-phenylindole (DAPI) (4 µg/ml; Sigma) in a vacuum desiccator. Stained samples were visualized in an SP8 confocal microscope (Leica) with z-stack projections acquired using a 63x oil objective.

4.7 | H₂O₂ detection

The accumulation of H₂O₂ at PM infection sites was detected by DAB (Sigma) staining (Thordal-Christensen et al., 1997). Leaves were stained with 1 mg/ml DAB for approximately 10–12 h in the dark, and stained with trypan blue as described above. Fungal structures were visualized under a 60x objective bright-field using an eclipse Ti microscope (Nikon). H₂O₂ quantification in whole leaves was performed using potassium iodide (Velikova et al., 2000). Experiments were repeated three times.

4.8 | Promoter analysis

Upstream gene sequences (2 kb) obtained from PhytoMine (phytozome.jgi.doe.gov/phytomine/begin.do) and the M. truncatula Hapmap Project (medicagohapmap.org) were analysed using PlantCARE (Lescot, 2002).

ACKNOWLEDGEMENTS

We thank Dr Banyal for the E. pisi isolate, Dr Samac for M. truncatula seeds, and S. Tewari for assistance with confocal microscopy. This work was supported by the Innovative Young Biotechnologist Award from DBT, the Government of India (BT/09/1YBA/2015/12) and intramural funds from R.C.B. to D.C. The chemical synthesis work under project MLP2028 and research fellowships were supported by CSIR. The CDRI communication number is 10352.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

D.C. and A. Gupta conceived the overall study and designed the experiments involving M. truncatula–PM interactions, and A. Gupta performed all related experiments. A. Goel and P.A. designed the experiments involving the chemical synthesis and spectroscopic analysis of 2MF, 2HF, medicarpin, and its fluorophore-tagged version, and P.A. performed related experiments. S.P. and R.P.V. purified the compounds and performed photophysical studies under the supervision of A. Goel and P.A. N. Sharma generated the LC-MS data under the supervision of N. Singh. Y.K. provided valuable scientific inputs and assisted with sample preparation for the LC-MS experiment. D.C. and A. Gupta analysed the data and wrote the manuscript with contributions from A. Goel, P.A., and N. Singh. All authors have read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supporting information material for this article.

ORCID

Atul Goel https://orcid.org/0000-0003-2758-2461
Divya Chandran https://orcid.org/0000-0001-6206-8826

REFERENCES

Adesanya, S.A., O’Neill, M.J. & Roberts, M.F. (1986) Structure-related fungitoxicity of isoflavonoids. Physiological and Molecular Plant Pathology, 29, 95–103.
Ahn, S.Y., Kim, S.A., Cho, K.S. & Yun, H.K. (2014) Expression of genes related to flavonoid and stilbene synthesis as affected by signalling chemicals and Botrytis cinerea in grapevines. Biologia Plantarum, 58, 758–767.
Ameline-Torregrosa, C., Cazaux, M., Danesh, D., Chardon, F., Cannon, S.B., Esquerre-Tugayé, M.T. et al. (2008) Genetic dissection of resistance to anthracnose and powdery mildew in Medicago truncatula. Molecular Plant-Microbe Interactions, 21, 61–69.
Banasiak, J., Biała, W., Staszkow, A., Swarczewicz, B., Kępczyńska, E., Figlerowicz, M. et al. (2013) A Medicago truncatula ABC transporter belonging to subgroup G modulates the level of isoflavonoids. Journal of Experimental Botany, 64, 1005–1015.
Biała, W., Banasiak, J., Jarzynka, K., Pawela, A. & Jasiński, M. (2017) Medicago truncatula ABCG10 is a transporter of 4-coumarate and liriquiritigenin in the medicarpin biosynthetic pathway. Journal of Experimental Botany, 68, 3231–3241.
Biała, W. & Jasieński, M. (2018) The phenylpropanoid case – it is transport that matters. *Frontiers in Plant Science*, 9, 1610.

Bisht, N., Gupta, A., Awasthi, P., Goel, A., Chandran, D., Sharma, N. et al. (2021) Development of a rapid LC-MS/MS method for the simultaneous quantification of various flavonoids and phytohormones extracted from *Medicago truncatula* leaves. bioRxiv. https://doi.org/10.1101/2021.04.14.439919

Blount, J.W., Dixon, R.A. & Paiva, N.L. (1992) Stress responses in alfalfa (*Medicago sativa L.*) XVI. Antifungal activity of medicarpin and its biosynthetic precursors: implications for the genetic manipulation of stress metabolites. *Physiological and Molecular Plant Pathology*, 41, 333–349.

Chandran, D., Tai, Y.C., Hather, G., Dewdney, J., Denoux, C., Burgess, D.G. et al. (2009) Temporal global expression data reveal known and novel salicylate-impacted processes and regulators mediating powdery mildew growth and reproduction on Arabidopsis. *Plant Physiology*, 149, 1435–1451.

Cheng, Q., Li, N., Dong, L., Zhang, D., Fan, S., Jiang, L. et al. (2015) Overexpression of soybean isoflavone reductase (GmFLR) enhances resistance to *Phytophthora sojae* in soybean. *Frontiers in Plant Science*, 6, 1024.

Dean, R., Van Kan, J.A.L., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D. et al. (2012) The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13, 414–430.

Dempsey, D.A., Vlot, A.C., Wildermuth, M.C. & Klessig, D.F. (2011) Salicylic acid biosynthesis and metabolism. *The Arabidopsis Book*, 9, e0156.

Devree, B.T., Steiner, L.M., Glazowska, S., Ruhnow, F., Herburger, K., Persson, S. et al. (2021) Current and future advances in fluorescence-based visualization of plant cell wall components and cell wall biosynthetic machineries. *Biotechnology for Biofuels*, 14, 78.

Dewdney, J., Reuber, T.L., Wildermuth, M.C., Devoto, A., Cui, J., Stutius, L.M. et al. (2000) Three unique mutants of Arabidopsis identify eds loci required for limiting growth of a biotrophic fungal pathogen. *The Plant Journal*, 24, 205–218.

Dixon, R.A., Achnine, L., Kota, P., Liu, C.J., Reddy, M.S.S. & Wang, L. (2014) Differential accumulation of defense-related isoflavonoids and disease resistance in alfalfa. *Molecular Plant-Microbe Interactions*, 6, 650–667.

Duczek, L.J. & Higgins, V.J. (1976) Effect of treatment with the phytoalexins medicarpin and maackiaiin on fungal growth in vitro and in vivo. *Canadian Journal of Botany*, 54, 2620–2629.

Durango, D., Pulgarin, N., Gil, J., Escobar, G., Echeverri, F. & Quinones, W. (2014) Differential accumulation of defense-related isoflavonoids in hypocotyls/roots of common bean (*Phaseolus vulgaris L.*) cultivars treated with salicylic acid and structurally related compounds. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*, 13, 381–405.

Farag, M.A., Huhman, D.V., Dixon, R.A. & Sumner, L.W. (2008) Metabolomics reveals novel pathways and differential mechanistic and elicitor-specific responses in phenylpropanoid and isoflavonoid biosynthesis in *Medicago truncatula* cell cultures. *Plant Physiology*, 146, 387–402.

Fondellvila, S. & Rubiales, D. (2012) Powdery mildew control in pea. A review. *Agronomy for Sustainable Development*, 32, 401–409.

Foster-hartnett, D., Danesh, D., Peñuela, S., Sharopova, N., Endre, G., Vandenbosch, K.A. et al. (2007) Molecular and cytological responses of *Medicago truncatula* to *Erysiphe pisi*. *Molecular Plant Pathology*, 8, 307–319.

Glawe, D.A. (2008) The powdery mildews: a review of the world’s most familiar (yet poorly known) plant pathogens. *Annual Review of Phytopathology*, 46, 27–51.

Goel, A., Kumar, A., Hemberger, Y., Raghuvanshi, A., Jeet, R., Tiwari, G. et al. (2012) Synthesis, optical resolution, absolute configuration, and osteogenic activity of cis-petrocarpans. *Organic & Biomolecular Chemistry*, 10, 9583–9592.

Gondor, O.K., Janda, T., Soós, V., Pál, M., Majláth, I., Adak, M.K. et al. (2016) Salicylic acid induction of flavonoid biosynthesis pathways in wheat varies by treatment. *Frontiers in Plant Science*, 7, 1447.

Graham, T.L., Graham, M.Y., Subramanian, S. & Yu, O. (2007) RNAi silencing of genes for elicitation or biosynthesis of 5-deoxyisoflavonoids suppresses race-specific resistance and hypersensitive cell death in *Phytophthora sojae* infected tissues. *Plant Physiology*, 144, 728–740.

Gupta, M., Dubey, S., Jain, D. & Chandran, D. (2021) The *Medicago truncatula* sugar transport protein 13 and its Lr6767s-like variant confer powdery mildew resistance in legumes via defense modulation. *Plant and Cell Physiology*, 62, 650–667.

Gupta, M., Sharma, G., Saxena, D., Budhwar, R., Vasudevan, M., Gupta, V. et al. (2020) Dual RNA-Seq analysis of *Medicago truncatula* and the pea powdery mildew *Erysiphe pisi* uncovers distinct host transcriptional signatures during incompatible and compatible interactions and pathogen effector candidates. *Genomics*, 112, 2130–2145.

He, X., Dixon, R.A., Division, P.B., Roberts, S., Foundation, N. & Parkway, S.N. (2000) Genetic manipulation of isoflavone 7-O-methyltransferase enhances biosynthesis of 4‘-O-methylated isoflavonoid phytoalexins and disease resistance in alfalfa. *The Plant Cell*, 12, 1689–1702.

Hückelhoven, R. & Kogl, K.H. (2003) Reactive oxygen intermediates in plan-microbe interactions: who is who in powdery mildew resistance? *Planta*, 216, 891–902.

Ishiga, Y., Rao Uppalapati, S., Gill, U.S., Huhman, D., Tang, Y. & Mysore, K.S. (2015) Transcriptomic and metabolomic analyses identify a role for chlorophyll catabolism and phytoalexin during *Medicago non* host resistance against Asian soybean rust. *Scientific Reports*, 5, 13061.

Jiménez-González, L., Álvarez-Corral, M., Muñoz-Dorado, M. & Rodríguez-García, I. (2008) Petrocarpans: interesting natural products with antifungal activity and other biological properties. *Phytochemistry Reviews*, 7, 125–154.

Katoch, R., Mann, A.P.S., Kohl, B.S., Munshi, D., Pradesh, H., Vishvavidhalya, K. et al. (2005) Induction of pisatin synthesis in pea with salicylic acid or inoculation with powdery mildew. *Journal of Vegetable Science*, 11, 85–96.

Lace, B. & Prandi, C. (2016) Shaping small bioactive molecules to untangle their biological function: a focus on fluorescent plant hormones. *Molecular Plant*, 9, 1099–1118.

Lescot, M. (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Research*, 30, 325–327.

Liu, Y., Hassan, S., Kidd, B.N., Garg, G., Mathesius, U., Singh, K.B. et al. (2017) Ethylene signalling is important for isoflavonoid-mediated resistance to *Rhizoctonia solani* in roots of *Medicago truncatula*. *Molecular Plant-Microbe Interactions*, 30, 691–700.

Lygin, A.V., Li, S., Vittal, R., Widholm, J.M., Hartman, G.L. & Lozovaya, V.V. (2009) The importance of phenolic metabolism to limit the growth of *Phakopsora pachyrhizi*. *Phytopathology*, 99, 1412–1420.

Mackenbrock, U. & Barz, W. (1991) Elicitor-induced formation of ptero- carpan phytoalexins in chickpea (*Cicer arietinum L.*) cell suspension cultures from constitutive isoflavone conjugates upon inhibition of phenylalanine ammonia lyase. *Zeitschrift für Naturforschung C*, 46, 43–50.

Naoumkina, M. & Dixon, R.A. (2008) Subcellular localization of flavonoid natural products: a signaling function? *Plant Signaling & Behavior*, 3, 573–575.

Naoumkina, M., Farag, M.A., Sumner, L.W., Tang, Y., Liu, C.J. & Dixon, R.A. (2007) Different mechanisms for phytoalexin induction by pathogen and wound signals in *Medicago truncatula*. *Proceedings of
the National Academy of Sciences of the United States of America, 104, 17909–17915.

Pandey, A.K., Burlakoti, R.R., Kenyon, L. & Nair, R.M. (2018) Perspectives and challenges for sustainable management of fungal diseases of mungbean [Vigna radiata (L.) R. Wilczek var. radiata]: a review. Frontiers in Environmental Science, 6, 53.

Pimentel, D., Amaro, R., Erban, A., Mauri, N., Soares, F., Rego, C. et al. (2021) Transcriptional, hormonal, and metabolic changes in susceptible grape berries under powdery mildew infection. Journal of Experimental Botany, 72, 6544–6569.

Rispail, N., Prats, E. & Rubiales, D. (2019) Medicago truncatula as a model to study powdery mildew resistance. In: de Brujin, F. (Ed.) The model legume. Medicago truncatula. New Jersey: John Wiley & Sons Inc, pp. 390–397.

Rujiter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., van den Hoff, M.J.B. et al. (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Research, 37, e45.

Samac, D.A. & Graham, M.A. (2007) Recent advances in legume–microbe interactions: recognition, defense response, and symbiosis from a genomic perspective. Plant Physiology, 144, 582–587.

Saslowsky, D.E., Warek, U. & Winkel, B.S. (2005) Nuclear localization of Smigielski, L., Laubach, E.M., Pesch, L., Glock, J.M.L., Albrecht, F., Thordal-Christensen, H., Zhang, Z., Wei, Y. & Collinge, D.B. (1997) Phytoalexin activation and isochorismate synthase activity contribute to salicylic acid biosynthesis in soybean. New Phytologist, 212, 627–636.

Smigielski, L., Laubach, E.M., Pesch, L., Glock, J.M.L., Albrecht, F., Slusarekono, A. et al. (2019) Nodulation induces systemic resistance of Medicago truncatula and Pisum sativum against Erysiphe pisi and primes for powdery mildew-triggered salicylic acid accumulation. Molecular Plant-Microbe Interactions, 32, 1243–1255.

Steinbock, P.C., Turner, H.C. & Haware, M.P. (1997) Phytalexin accumulation in the roots of chickpea (Cicer arietinum L.) seedlings associated with resistance to fusarium wilt (Fusarium oxysporum f.sp. ciceri). Physiological and Molecular Plant Pathology, 50, 167–178.

Thaler, J.S., Humphrey, P.T. & Whiteman, N.K. (2012) Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science, 17, 260–270.

Thiruvengadam, M., Kim, S.H. & Chung, I.M. (2015) Exogenous phytohormones increase the accumulation of health-promoting metabolites, and influence the expression patterns of biosynthesis related genes and biological activity in Chinese cabbage (Brassica rapa spp. pekinensis). Scientia Horticulturae, 193, 136–146.

Thordal-Christensen, H., Zhang, Z., Wei, Y. & Collinge, D.B. (1997) Subcellular localization of H$_2$O$_2$ in plants. H$_2$O$_2$ accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. The Plant Journal, 11, 1187–1194.

Ullah, C., Tsai, C.J., Uniscker, S.B., Xue, L., Reichelt, M., Gershenson, J. et al. (2019) Salicylic acid activates poplar defense against the biotrophic rust fungus Melampsora larici-populina via increased biosynthesis of catechin and proanthocyanidins. New Phytopathology, 221, 960–975.

Umehara, K., Nemoto, K., Matsuhashi, A., Terada, E., Monthakanirat, O., De-Eknamkul, W. et al. (2009) Flavonoids from the heartwood of the Thai medicinal plant Dalbergia parviflora and their effects on estrogenic-responsive human breast cancer cells. Journal of Natural Products, 72, 2163–2168.

van de Mortel, M., Recknor, J.C., Graham, M.A., Nettleton, D., Dittman, J.D., Nelson, R.T. et al. (2007) Distinct biphasic mRNA changes in response to Asian soybean rust infection. Molecular Plant-Microbe Interactions, 20, 887–899.

VanEtten, H.D. (1976) Antifungal activity of pterocarpans and other selected isoflavonoids. Phytochemistry, 15, 655–659.

Velikova, V., Yordanov, I. & Edreva, A. (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants protective role of exogenous polyanimes. Plant Science, 151, 59–66.

Vieiba-Fernández, A., Polonio, Á., Ruiz-Jiménez, L., de Vicente, A., Pérez-García, A. & Fernández-Ortuño, D. (2020) Fungicide resistance in powdery mildew fungi. Microorganisms, 8, 1431.

Vogel, J. & Somerville, S. (2000) Isolation and characterization of powdery mildew-resistant Arabidopsis mutants. Proceedings of the National Academy of Sciences of the United States of America, 97, 1987–1992.

Wang, W., Wen, Y., Berkey, R. & Xiao, S. (2009) Specific targeting of the Arabidopsis resistance protein RPW8.2 to the interfacial membrane encasing the fungal haustorium renders broad-spectrum resistance to powdery mildew. The Plant Cell, 21, 2898–2913.

Weidenbörner, M., Hindorf, H., Chandra Jha, H., Tsotsosonos, P. & Egge, H. (1990) Antifungal activity of isoflavonoids in different reduced stages on Rhizoctonia solani and Sclerotium rolfsii. Phytochemistry, 29, 801–803.

Weidenbörner, M. & Jha, H.C. (1994) Structure–activity relationships among isoflavonoids with regard to their antifungal properties. Mycological Research, 98, 1376–1378.

Wildermuth, M.C., Dewdney, J., Wu, G. & Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature, 414, 562–565.

Xiao, S., Brown, S., Patrick, E., Brearley, C. & Turner, J.G. (2003) Enhanced transcription of the Arabidopsis disease resistance genes RPW8.1 and RPW8.2 via a salicylic acid-dependent amplification circuit is required for hypersensitive cell death. The Plant Cell, 15, 33–45.

Xiao, S., Calis, O., Patrick, E., Zhang, G., Charoenwattana, P., Muskett, P. et al. (2005) The atypical resistance gene, RPW8, recruits components of basal defence for powdery mildew resistance in Arabidopsis. The Plant Journal, 42, 95–110.

Yang, S., Tang, F., Caixeta, E.T. & Zhu, H. (2013) Epigenetic regulation of a powdery mildew resistance gene in Medicago truncatula. Molecular Plant, 6, 2000–2003.

### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

---

**How to cite this article:** Gupta, A., Awasthi, P., Sharma, N., Parveen, S., Vats, R.P., Singh, N., et al (2022) Medicarpin confers powdery mildew resistance in Medicago truncatula and activates the salicylic acid signalling pathway. Molecular Plant Pathology, 23, 966–983. [https://doi.org/10.1111/mpp.13202](https://doi.org/10.1111/mpp.13202)