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Changing light promotes isoflavone biosynthesis in soybean pods and enhances their resistance to mildew infection

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Abstract

Mildew severely reduces soybean yield and quality, and pods are the first line of defence against pathogens. Maize-soybean intercropping (MSI) reduces mildew incidence on soybean pods; however, the mechanism remains unclear. Changing light (CL) from maize shading is the most important environmental feature in MSI. We hypothesized that CL affects isoflavone accumulation in soybean pods, affecting their disease resistance. In the present study, shading treatments were applied to soybean plants during different developmental stages according to various CL environments under MSI. Chlorophyll fluorescence imaging (CFI) and classical evaluation methods confirmed that CL, especially vegetative stage shading (VS), enhanced pod resistance to mildew. Further metabolomic analyses and exogenous jasmonic acid (JA) and biosynthesis inhibitor experiments revealed the important relationship between JA and isoflavone biosynthesis, which had a synergistic effect on the enhanced resistance of CL-treated pods to mildew. VS promoted the biosynthesis and accumulation of constitutive isoflavones upstream of the isoflavone pathway, such as aglycones and glycosides, in soybean pods. When mildew infects pods, endogenous JA signalling stimulated the biosynthesis of downstream inducible malonyl isoflavone (MIF) and glyceollin to improve pod resistance.

KEYWORDS
changing light, intercropping, isoflavone, metabolomic, mildew, soybean pod

1 | INTRODUCTION

Intercropping (IT) involves the growth of two or more crops on the same piece of land, and the period of symbiosis among different crops may be long or short. As an important agronomic strategy, IT has many benefits, including highly efficient utilization of resources, weeds, pests and disease control and soil fertility improvement. Although IT has been used for several centuries in traditional

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agriculture, it is still common globally, especially in developing countries (Iqbal et al., 2019). The maize-soybean intercropping (MSI) system is a successful case that provides massive advantages in low-input and high-risk environments. As explained by biodiversity theory, MSI systems are an ecological strategy to control or relieve diseases and insect pests; optimized field allocation in MSI can reduce pests and diseases (Du et al., 2018).

As a serious plant disease, field mildew (FM) is caused by the infection of *Fusarium verticillioides* during the wet season before soybean harvest and results in significant soybean yield losses (Liu et al., 2017). Our multiyear survey of disease dynamics confirmed the FM rate of soybean plants growing under the MSI system was significantly lower than that in soybean plants growing alone (Chang et al., 2020). From our field survey in recent years, the mildew rate of soybean pods harvested from the maize-soybean relay strip IT system was less than a quarter of sole cropping soybean pod (Supplemental Figure S1). Multiple aspects underlie these findings. One important point is that the IT environment, especially light conditions, including light quality and intensity (Liu, Yang, et al., 2016), affects soybean plant chemicals. The soybean pod is the first line of defence for this leguminous seed against pests and pathogens, and pod chemicals play an important role in this process (Deng et al., 2017). Previous studies have focused more on the resistance of soybean seeds and less on that of bean pods (Deng et al., 2017). Our recent studies indicated the physical barrier effect of the soybean seed pod, which reduced the damage to soybean seeds from FM and revealed a protective effect against mold infection (Liu et al., 2017).

In agricultural practice, MSI is employed in two ways: IT and relay intercropping (RIT); their marked difference is the shading periods from the maize (Supplemental Figure S2) (Wang et al., 2016). In the IT system, maize and soybean are sown at the same time, and there is no shading from maize on the soybean plants in the vegetative stage. With the development of maize and soybean, when soybeans enter the reproductive stage, the lush leaves of maize result in shading of the soybean plant. Inversely, in the RIT system, the main shading period is during the vegetative stage of the soybean; when soybean plants enter the reproductive stage, the maize is harvested, which increases the light in the soybean canopy. These different symbiotic periods cause a changing light environment (CLE) for soybean plants in the IT and RIT systems: from bright to weak with IT and from weak to bright with RIT (Yang et al., 2014). Our initial research found that isoflavone content in soybean seeds increased as the PAR transmittance decreased (i.e., shade deepening) in different treatments of RIT systems (Liu, Yang, et al., 2016). However, this information has never been studied in soybean pods, while research has confirmed the antifungal activity of soy isoflavones (Naim, Gestetner, Zilkah, Birk, & Bondi, 1974). Thus, we hypothesized that the CLE is one reason why intercropped soybeans have higher FM resistance and that the CLE elevates the resistance of soybean pods via secondary metabolism. Many studies have shown that different light qualities or light intensities could affect the secondary metabolism of crops (Rechner, Neugart, Schreiner, Wu, & Poehling, 2016). However, the evidence and mechanisms revealed so far are limited. Although too many studies have focused on regulating crop secondary metabolism by uniform light, CLEs have rarely been studied.

The current study aimed to evaluate whether changing light, especially vegetative stage shading (VS), enhances isoflavone accumulation in soybean pods, impacting their disease resistance and to explore the potential metabolic regulatory mechanism in cross-resistance caused by sequential stresses of shading and fungal infection. For this purpose, in this current research, we conducted a field shade experiment to simulate CLEs under different IT systems. In vitro infection and exogenous jasmonic acid methyl ester (MeJA) and its biosynthetic inhibitor tests were performed to reveal the effect of the CLE on soybean pod resistance to mildew infection. The untargeted metabolomics workflow involving UPLC-Q-TOF/MS, targeted quantitation of isoflavones using UPLC-MS/MS, and real-time quantitative PCR for genes expression analysis were used to explore the mechanism of improved mildew resistance in soybean pods growing under a CLE that was simulated from an MSI system.

2 | MATERIALS AND METHODS

2.1 | Plant materials and experimental design

A conventional soybean cultivar C103, which was provided by a specialist company in the region and was used for this study, was grown at the farm of Sichuan Agricultural University in Ya'an, Sichuan Province, China (29°59′N, 103°00′E), in 2018 and 2019. A single-factor random block design was applied to soybean plants in the wide-narrow row planting mode (Figure 1), which was the same as the maize-soybean strip IT system without maize culture (Liu, Yang, et al., 2016). The experimental design comprised three replicates, and each block comprised four shading treatments, including whole growth stage shading (WS), vegetative stage shading (VS), reproductive stage shading (RS) and whole growth stage normal light (WL), which were applied over 12 plots (Figure 1). The shading treatments were conducted using a net (black, high-density, polyethylene tape four-pin net) to control the transmittance to approximately 30%; all

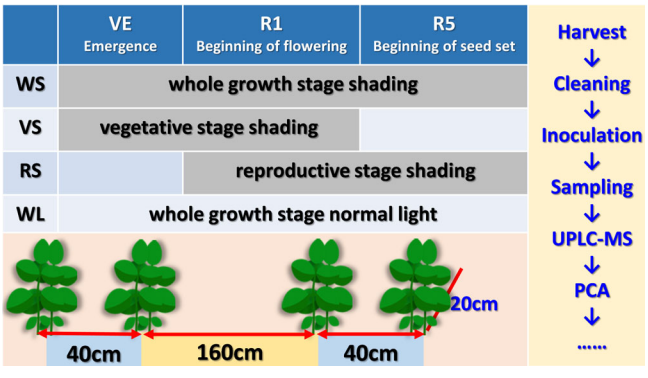

FIGURE 1 Experimental design and workflow

nets were installed 2 m above the ground surface (Supplemental Figure S3). The term WS treatment means that the plants were covered by the net during the entire growth period as the negative control; WL treatment means that the soybean plants were grown with no covering and served as the non-shaded positive control. Soybean pods were harvested, cleaned and inoculated at the R5 (beginning seed) stage, including uninoculated (CK) and *Fusarium verticillioides* (Fv)-inoculated pods, and each treatment was repeated for five independent biological replicates from different plants, which were collected from the randomized blocks. CFI parameters and the mildew index were used to test Fv-resistance. From seven DAI (days after inoculation), soybean pods were frozen in liquid nitrogen and stored at −80°C. Metabolites were qualitatively and quantitatively analysed by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS); data were analysed by multivariate statistics, such as principal component analysis (PCA). The main workflow is shown in Figure 1.

2.2 | Fungal inoculation

The *F. verticillioides* strain was isolated and identified from field-grown soybean pods in our previous study (Liu et al., 2017). The strain was cultured at 30°C overnight in PDA medium on a rotary shaker. Then, the culture medium was centrifuged for 10 min at 3000 rpm, and the supernatant was discarded. The conidia were washed 3 times, counted with a haemocytometer, and adjusted to a density of 10^6 cfu·ml^{-1}.

Full, healthy soybean pods were washed 2–3 times with sterile water, and the surface was wiped clean. To facilitate fungal infection, the surface of each soybean pod was punctured into three portions using a sterile needle, and each surface-damaged portion was inoculated with a conidial suspension of *F. verticillioides* (5 μL, 10^6 cfu·ml^{-1}) or sterile distilled water (as a control). The inoculated pods were placed in an incubator at 30°C for 7 d.

2.3 | Mildew survey and resistance evaluation

Soybean pods were used to test the resistance to fungal infection with the mildew index, which classifies infections into five different levels: 0 level (no lesions), first level (the inoculated area shows a few white hyphal strands), second level (the lesioned area ≤ 1/4 of a single pod's total surface area), third level (1/4 of a single pod's total surface area < lesioned area ≤ 1/2 of a single pod's total surface area), fourth level (1/2 of a single pod's total surface area < lesioned area ≤ 3/4 of a single pod's total surface area) and fifth level (lesioned area > 3/4 of a single pod's total surface area). The lesioned area was measured by visual inspection. The mildew index and mildew rate were calculated as described in our previous study: Mildew index $= \frac{\sum p_i c_i}{n c_{max}} \times 100$; Mildew rate $= \frac{f}{n} \times 100$ (p_i: number of molded pods of various grades; c_i: corresponding infection grades; n: number of pods surveyed; c_{max}: top infection grade; f: the sum of p_i;).

The maximum quantum yield of photosystem II (*Fv/Fm*) and photochemical quenching (*qP*) in soybean pods were detected by a chlorophyll fluorescence imager (British Technologica company) at 1, 2, 3, 4, 5, 6, and 7 DAI. *Fv/Fm* reflects the potential maximum light energy conversion rate in plants and reveals the adaptability of plants to light intensity in the environment. *qP* is the photochemical quenching caused by increasing photosynthesis and reflects the efficiency of light energy conversion to photosynthetic assimilates (Rousseau et al., 2013).

2.4 | Exogenous jasmonic acid (JA) and its biosynthesis inhibitor treatments

Exogenous JA experiments included three treatments: sterile water sprayed on pods harvested from soybean plants growing under the VS shading environment (VS-W); 100μM MeJA sprayed on pods harvested from soybean plants growing under the WL environment (WL-J); and sterile water sprayed on pods harvested from soybean plants growing under the WL environment (WL-W).

Exogenous inhibitor experimental treatments, including fungal inoculation (Fv), spraying with the lipoxygenase inhibitor phenidone (PHD) before inoculation (PF), and water applied as a control (CK), were applied in this section. In brief, three groups of healthy uniform soybean pods were selected for the test; 100 μM PHD was sprayed on the pod surfaces of the PF group, and the pods of the other two groups, Fv and CK, were sprayed with the same amount of sterile water. After 30 min, all the groups were inoculated with 10 μL of 10^6 cfu·ml^{-1} spore solution (containing 0.01% Tween solution). Treated soybean pods were collected after 0 h, 12 h, 24 h, 48 h and 84 h and frozen in liquid nitrogen immediately. All the samples were stored at −80°C for further analysis.

2.5 | Real-time quantitative PCR

Total RNA from soybean pods was extracted using a TIANGEN RNAprep Pure Plant Kit (TIANGEN Biotech [Beijing] Co., Ltd.) according to the manufacturer's instructions. A NanoVue plus instrument determined the concentration and purity of total RNA from the samples. Complementary DNA (cDNA) was synthesized using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) following the manufacturer's (Vazyme Biotech Co., Ltd.) protocol. RT-qPCR of diluted template cDNA was carried out using ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme Biotech Co., Ltd.) as described the manufacturer's instructions. The reaction system (10 μL) comprised diluted template cDNA (1 μL), ddH_2O (3.6 μL), forward and reverse primers (0.2 μL, respectively) and 2 × ChamQ Universal SYBR qPCR Master Mix (5 μL). RT-qPCR was performed on cDNA from three biological replicates by a fluorescence quantitative PCR instrument (Life Technologies, QuantStudio6 Flax) using *GmACTIN1* as the reference gene; the primers used in this study are listed in Supplemental Table S1.

2.6 | Metabolomics analysis

2.6.1 | Sample extraction and preparation

The 7 DAI soybean pods were ground with liquid nitrogen and lyophilized for 48 h using a vacuum freeze dryer. Approximately 20 mg pod powder from each replicate was transferred into a 2 mL precooled centrifuge tube, and 1 mL 80% methanol extraction solution was added. The samples were mixed for 10 s and extracted at 4°C for 1 h in an ultrasonic water bath (40 kHz, 300 W). The samples were centrifuged at 11,000 g and 4°C for 10 min, and then the supernatant fluid (approximately 800 μL) was filtered into a sample bottle through a syringe filter (0.22 μm) and injected directly into a UPLC-MS system.

2.6.2 | Untargeted metabolomics analysis by UPLC-Q-TOF/MS

Untargeted metabolomics analysis was carried out by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS); the UPLC system was a 1,290 Infinity from Agilent Technologies, and the MS was a 6,545 Q-TOFMS. Approximately 1 μL of each separated sample was separated with an Agilent Eclipse Plus-C18 column (100 × 2.1 mm, 1.8 μm), and the column temperature was 35°C. The mobile phase was composed of eluent A (0.1% formic acid aqueous solution) and eluent B (0.1% formic acid acetonitrile solution), and the flow rate was set as 0.300 mL·min^{-1}. Gradient elution was as follows: 0 to 4 min, 85 to 78% eluent A; 4 to 10.5 min, 78 to 61% eluent A; 10.5 to 13 min, 61 to 56% eluent A; 13 to 17.5 min, 56 to 0% eluent A; 17.5 to 20 min, 0 to 85% eluent A. For mass spectral conditions, positive ion mode was used. Nitrogen was used as the collision gas; the ESI sprayer pressure was 20 psig, and the MS-TOF fragment pressure was 140 V. The desolvation gas flow rate and temperature were 10 L·min^{-1} and 325°C, respectively. The autosampler temperature was maintained at 4°C. In addition, full scan mode was used for mass spectral acquisition, and the scanning frequency was 1 s/spectra.

2.6.3 | Targeted quantitation of isoflavones

The isoflavones in soybean pods were quantified using ultra-performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-MS/MS, Waters Xevo TQ-Smicro). The main chromatographic conditions were as follows: chromatographic column, ACQUIY UPLC BEH-C18 column (50 mm × 2.1, 1.7 μm); column temperature, 35°C; mobile phase, A (0.1% formic acid aqueous solution) and B (0.1% formic acid acetonitrile solution); gradient elution, 85–75% A (0–4.8 min), 75–60% A (4.8–7.2 min), 60–85% A (7.2–7.23 min), 85% A (7.23–9 min); injection volume, 1 μL; flow rate, 0.350 mL·min^{-1}. The mass spectra were measured under the following conditions: electrospray ionization (ESI); positive ion mode; desolvation temperature, 350°C; desolvation volume, 10 L·min^{-1}; cone voltage, 35 kV. Nitrogen was used in the ion source and as the collision gas, and the collision voltage was 3 kV. The main isoflavones in soybean pods were quantified in absolute terms via linear regression of their corresponding standards.

2.7 | Statistical analyses

The raw chromatography peaks obtained from UPLC-QTOF-MS were uploaded to XCMS Online (https://xcmsonline.scripps.edu/) and subsequently deconvolved, which included peak recognition, peak filtering, peak alignment, etc. The eventual results included a two-dimensional data matrix after deconvolution (m/z, retention time, *P*-value, *q* value, peak area), differential feature ion compound matching, and metabolic pathway annotation. MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/) was used for PCA, heatmap and pathway enrichment analysis. Quantitative data were subjected to correlation, regression and variance analyses using SPSS.

3 | RESULTS

3.1 | Mildew resistance evaluation

To assess the fungal resistance of soybean pods grown under a CLE, *F. verticillioides* was inoculated onto the pod surface; chlorophyll fluorescence parameters and mildew indexes were monitored over 7 d in an incubator (Figure 2). As shown in Figure 2(a), chlorophyll fluorescence imaging of soybean pod lesions was significantly affected during fungal infection. After inoculation, the fluorescence colour of the inoculated portion of the soybean pods changed from red to green to dark blue, which showed that the infection degree increased (Figure 2(a)). The changed portions of some pods appeared blue at three to four DAI in the WL-Fv and WS-Fv groups. At five DAI, the inoculated spots of some pods in the RS-Fv group also became blue. At seven DAI, the inoculated spots of all pods in WS-Fv turned dark blue, which showed tissue necrosis, while the inoculated spots of only a few of the pods appeared dark blue in the VS-Fv group.

The chlorophyll fluorescence parameters also changed, as shown by the fluorescence imaging in inoculated soybean pods. The *Fv/Fm* (optimal photochemical efficiency of PSII in the dark) ratio of all pods decreased with the DAI (Figure 2(b)), and the *Fv/Fm* ratios of pods in WS-Fv significantly declined (by 0.5387 from 1 d to 7 d). However, the *Fv/Fm* ratio of all pods in the VS-Fv group merely decreased by 0.1888. Another important fluorescence parameter, the *qP* (photochemical quenching) value of pods, was enhanced with DAI but not significantly, except in the WS-Fv group (Figure 2(c)); however, the *qP* value of the WS-Fv group was significantly higher than that of the other groups at seven DAI, and the VS-Fv group had the lowest qP value. Compared with those at 1 d, the *qP* values of infected pods at 7 d were increased by 0.8202, 0.2312, 0.1841 and 0.1047 for the WS-Fv, WL-Fv, RS-Fv and VS-Fv groups, respectively (Figure 2(c)).

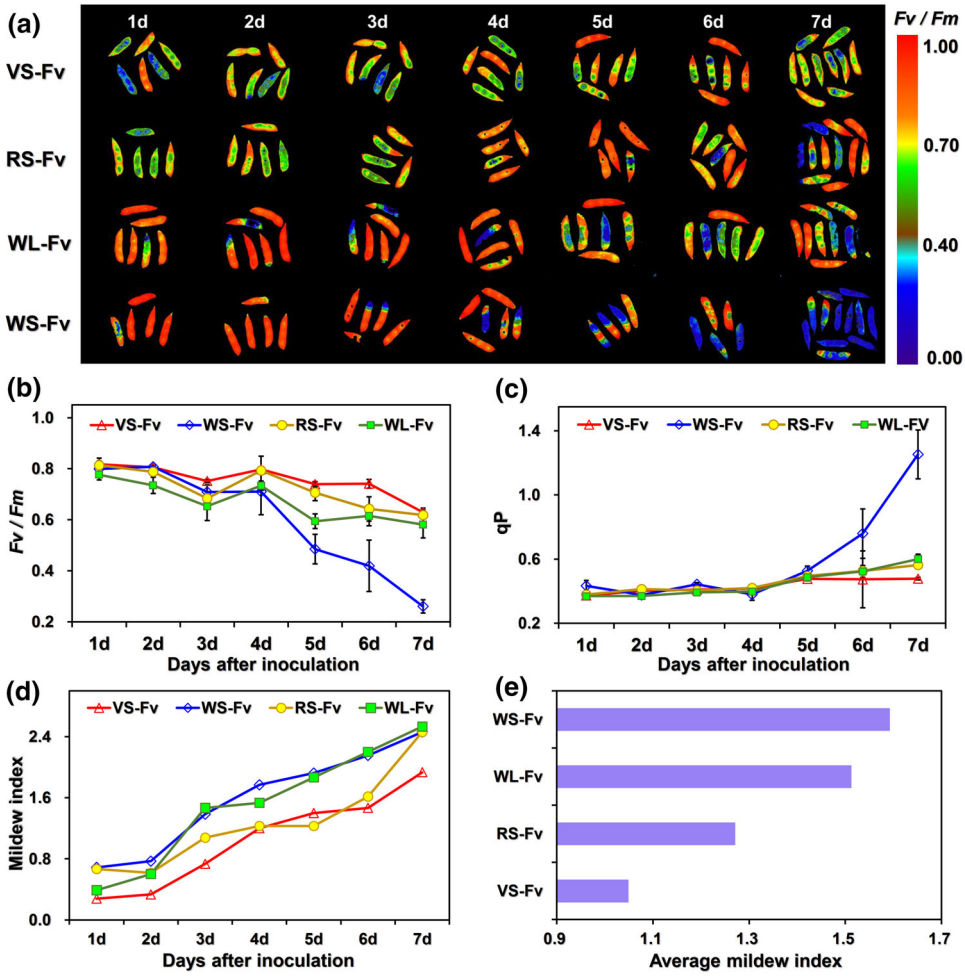

FIGURE 2 Comparison of mildew resistance of soybean pods. Chlorophyll fluorescence imaging of pods showing a mildew gradient based on *Fv/Fm* (a), the maximum quantum yield of photosystem II (b), photochemical quenching (c), mildew index (d), average mildew index (e) [Colour figure can be viewed at wileyonlinelibrary.com]

Similar to the changes in the *qP* values, the mildew index of soybean pods rose with the DAI. Although the macroscopic mildew index was not more accurate than the chlorophyll fluorescence parameters, the mildew indexes of the VS-Fv and RS-Fv groups were lower than those of the other two groups (Figure 2(d)). As shown in Figure 2(e), the average mildew indexes of distinct groups at 7 d were sorted: VS-Fv (20.98) < RS-Fv (25.42) < WL-Fv (30.25) < WS-Fv (31.85). Combined with the CFI and mildew index, these results suggest that the mildew resistance of pods growing under changing light conditions (VS-Fv and RS-Fv) was higher than that of pods growing under uniform light conditions (WL-Fv and WS-Fv), especially for pods subjected to shading at the vegetative stage (VS-Fv).

3.2 | Untargeted metabolomics analysis

3.2.1 | Metabolic profiling and clustering

According to the CFI and mildew index analyses, the mildew resistance of soybean pods growing under different light environments exhibited significant disparities. To further reveal the important metabolites in soybean pods that contribute to mildew resistance, a comparative metabolomic analysis among all treatments was conducted by UPLC-Q/TOF-MS. The PCA results showed that the metabolic profiling of soybean pods was affected by light conditions and fungi (Figure 3(a)). In particular, as shown in Figure 3(a), the Fv-inoculated (right side) and uninoculated groups (left side) were considerably separated by PC1 (50.9%), which showed that mold dramatically affected the pod metabolites. Although all *Fv*-inoculated groups were clustered together, the VS-Fv group (blue area) was separated from the other groups, and it was especially separated from the WS-Fv group (orange area) and WL-Fv group (yellow area). This regular phenotype was confirmed from the cluster analysis; as shown in the heat map of Figure 3(b), the RS-Fv and WL-Fv groups were clustered together and close to the VS-Fv group. A red area in the top clustering of the VS-Fv group is quite prominent, demonstrating that the contents of some metabolites were increased significantly compared to those of other groups; these up-regulated compounds in VS-Fv pods might play an important role in pathogen resistance.

3.2.2 | Pathway enrichment analysis

To explore the potential resistance mechanism in soybean pods, the different metabolites screened by VS-Fv and WL-Fv (as controls)

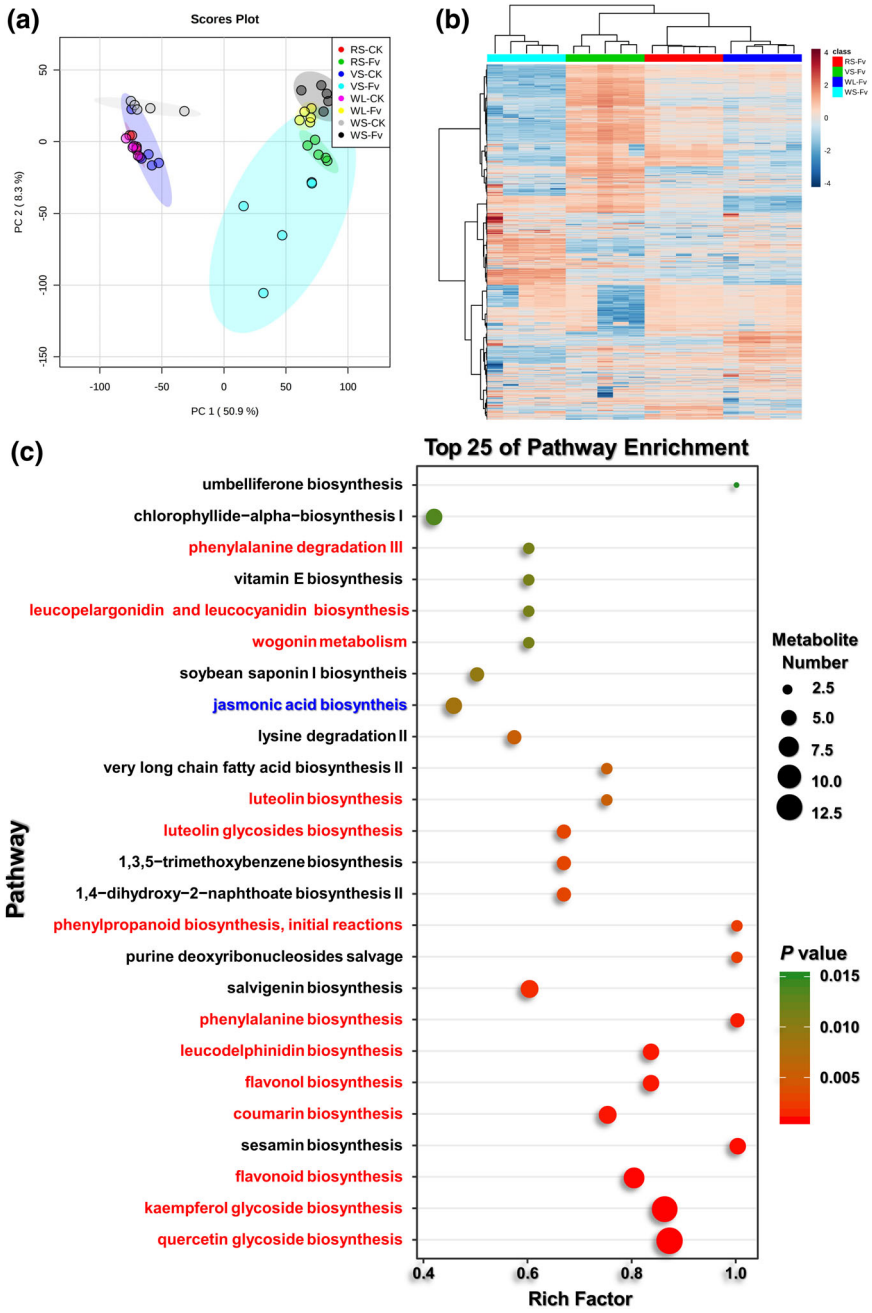

FIGURE 3 PCA score plots (a) and clustering heatmap (b) of mold-infected soybean pods growing under different light conditions. Pathway analysis of mold-infected pods growing under different light conditions of VS and WL (c). The colour and size of pathway symbols represent the significance level in the enrichment analysis and the impact factor, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

were imported into the KEGG pathway database for pathway annotations. The pathway enrichment analysis showed that there were significant differences in phenylpropanoid and flavone metabolic pathways, including kaempferol glycoside biosynthesis, quercetin glycoside biosynthesis, flavonoid biosynthesis, coumarin biosynthesis, flavonol biosynthesis, leucodelphinidin biosynthesis, phenylalanine biosynthesis, luteolin biosynthesis and wogonin metabolism (Figure 3(c)). In particular, as shown in Figure 3(c), quercetin glycoside biosynthesis, kaempferol glycoside biosynthesis, flavonoid biosynthesis, and flavonol biosynthesis were significantly up-regulated in the VS-Fv group compared with the WL-Fv group, in which 13, 12, 8 and 5 metabolites were annotated, respectively. The significant increase in flavonoid and flavonol contents in soybean pods of the VS-Fv group suggested that these metabolites might play a key potential resistance function.

Moreover, the JA biosynthesis pathway was also associated with the VS-Fv group (as shown in Figure 3(c) in blue). Abundant evidence has confirmed the antifungal activities of flavonoids in plants (Weston & Mathesius, 2013); it is well known that JA is frequently induced by biological stress and plays an important role in plant immune and defence responses (Jang, Yoon, & Choi, 2020). Hence, according to the pathway enrichment analysis, JA and flavonoids could be involved in the VS-treated pod defence response and may enhance mildew resistance.

3.3 | Targeted analysis of isoflavones

Isoflavones are the major flavonoid metabolites in soybean plants. Our previous research confirmed the significance of isoflavones to the mold resistance of soybean pods (Liu, Deng, et al., 2016). Combined with the above metabolomics analysis, which indicated that the flavonoid synthesis pathway was significantly enriched in phenylpropane metabolism (Figure 4), we hypothesized that isoflavone and downstream glyceollin biosynthesis was a key response to mold infection (Sukumaran, McDowell, Chen, Renaud, & Dhaubhadel, 2018). Hence, the isoflavones of soybean pods were quantitated by UPLC-MS/MS and annotated into metabolic pathways by using heat maps (Figure 4). As shown in Figure 4 and Supplemental Table S2, almost all of the flavonoid contents in the inoculated pods were higher than those in non-inoculated pods. The contents of all flavonoids in soybean pods growing under CLE (VS-CK and RS-CK) were higher than those in the WL-CK group (Figure 4). Detailed comparisons of the fold-changes of flavonoid profiles indicated that Fv inoculations and CLE mainly induced upstream flavanones and aglycones. In addition, malonyl-glucosides in VS and *Fv*-inoculation soybean pods were also increased more than 1.5-fold (Supplemental Figure S4(a), (b)). On the other hand, upstream flavanones and aglycones were significantly downregulated in the VS-Fv group compared with the WL-Fv group (Figure S4(c), Table S2). The contents of isoflavone glycosides and glyceollin, which are located downstream of the isoflavone biosynthesis pathway, were significantly higher in VS-Fv than in WL-Fv, especially malonyl-glucoside and glyceollin (Figure S4(c), Table S2). CLE, especially of VS, had a significant positive effect on the increase in mold-induced isoflavone content in soybean pods.

3.4 | Gene expression analysis

Moreover, we focused on comparing the quantified expression patterns of key genes involve in isoflavone biosynthesis in the soybean pods of the VS and WL groups (Figure 5). Fv inoculation significantly

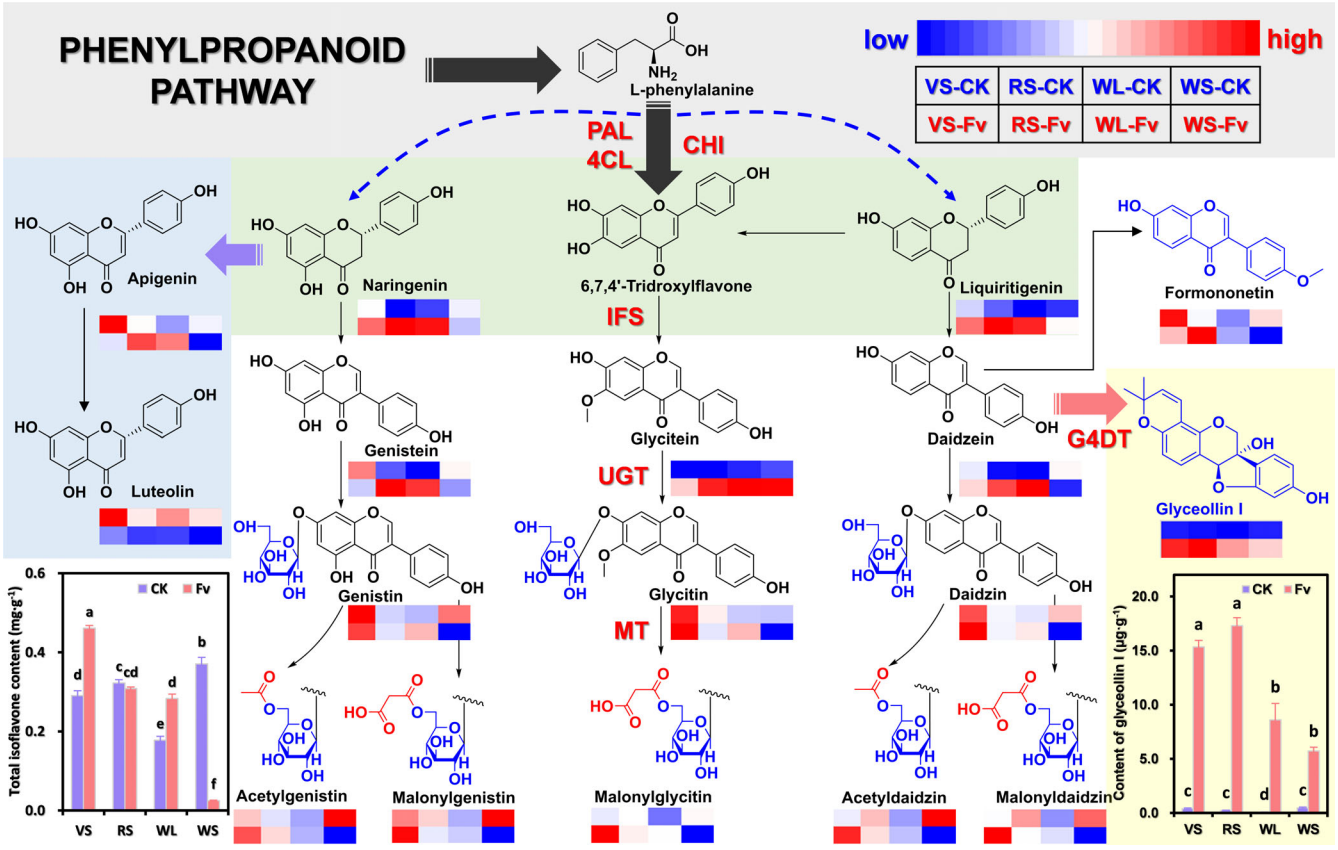

FIGURE 4 Integrated effects of shading and mold infection on isoflavone biosynthesis in soybean pods. The isoflavone contents in soybean pods under different treatments are represented by colours ranging from blue (low) to red (high) for each compound. The grey background is the primary metabolic pathway; the blue background is the branch pathway of flavones (apigenin and luteolin); the green background is the pathway of flavanones (naringenin and liquiritigenin); the white background is the pathway of soy isoflavones, including aglycones (genistein, glycitein, daidzein), β-glucosides (genistin, glycitin, daidzin), malonylglucosides (malonylgenistin, malonylglycitin, malonyldaidzin) and acetylglucosides (acetylgenistin, acetyldaidzin); the yellow background is the pathway of glyceollin. PAL: phenylalanine ammonialyase; 4CL: 4-coumarate-CoA-ligase; CHI: chalcone isomerase; IFS: 2-hydroxyisoflavanone synthase; UGT: glycosyl-transferase; MT: malonyl-transferase; G4DT: glycinol 4-dimethylallyltransferase. The two histograms are shown as the mean value ± standard error; values marked by the same letters are not significantly different ($p \geq 0.05$); CK: sterile water inoculation; Fv: fungal inoculation [Colour figure can be viewed at wileyonlinelibrary.com]

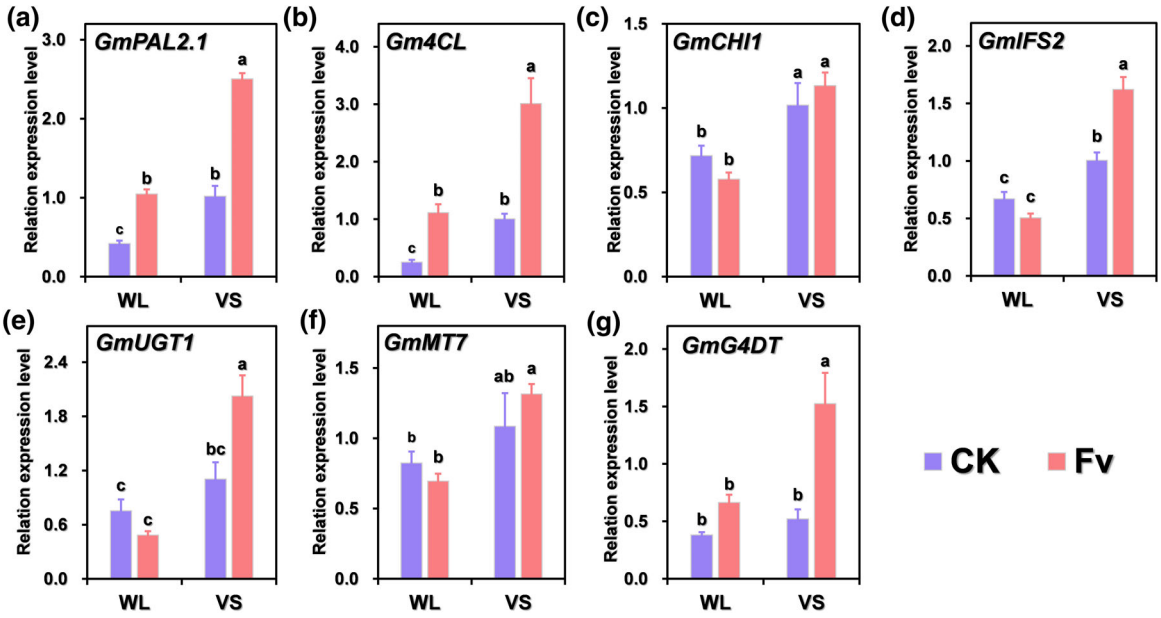

FIGURE 5 Quantitative expression of key genes involves in isoflavone biosynthesis. The histograms are shown as the mean value ± standard error; values marked by the same letter are not significantly different ($p \geq 0.05$); CK: sterile water inoculation; Fv: fungal inoculation [Colour figure can be viewed at wileyonlinelibrary.com]

up-regulated the key genes of *PAL* and *4CL* upstream of the phenylpropane synthesis pathway in soybean pods harvested from plants growing in the WL environment (Figure 5(a), (b)). Fv induced the up-regulation of these two genes by 2.49 and 4.41 times, respectively (Supplemental Figure S5(a)). However, Fv had no significant effect on downstream secondary metabolic regulatory genes (Figure 5 (c)–(g)). On the other hand, for uninoculated pods, VS treatment also significantly up-regulated the expression of key genes upstream of isoflavone biosynthesis, such as *PAL*, *4CL*, *CHI* and *IFS* (Figure 5(a)–(d)). In particular, *PAL* and *4CL* were up-regulated by 2.42 and 3.99 times, respectively (Figure S5(b)); however, there was no significant effect on downstream modifying genes, such as *UGT*, *MT* and *G4DT* (Figure 5(e)–(g)). Interestingly, for the VS groups, Fv inoculation not only significantly up-regulated the expression of upstream synthetic genes, but also the downstream modifying genes (Figure S5(c)). Moreover, the expression levels of the above key genes were the highest in VS-Fv successive stress pods (Figure S5(d)), which was consistent with the results of targeted metabolomics analysis (Figure 4).

In conclusion, single mold inoculation or CLE treatment can up-regulate key genes involve in the upstream flavonoid biosynthesis pathway and promote aglycone accumulation in soybean pods, while sequential stresses of VS and Fv mainly increased the contents of MIFs and glyceollin in soybean pods. The enhancing effect of CLE on the upstream aglycones provides enough precursors for the synthesis of induced resistance compounds, including MIF and glyceollin, eventually leading to an increase in the resistance of pods growing under a CLE. In addition, the fold increase of MIF content in the soybean pods of VS-Fv groups also implied that MIF could have both the characteristics of constitutive and induced resistance components (Figure S4).

3.5 | Correlation analysis of JA and isoflavones

According to the published literature, fungus-induced resistance components have three important characteristics: firstly, the content increases significantly after fungal infection (Jahan, Harris, Lowery, & Infante, 2020); second, it has potent antifungal activity (Lozovaya et al., 2004); and third, it is closely related to the JA signal (Jeong et al., 2018). The targeted metabolome and quantitative PCR results suggested that the contents of MIFs in the VS-Fv group increased markedly after Fv-induction (Figures 4 and 5). Enrichment analysis of different metabolites also showed that both JA and flavonoid biosynthesis in soybean pods were significantly affected by VS-Fv (Figure 3 (c)). To reveal the relationship between JA and MIFs, the metabolomics data of inoculated pods (all Fv-marked groups) were used for correlation analysis. According to the exact mass of ion features and retention times, the ionic strength of JA and MIFs were extracted from the metabolomic data matrix. Two ionic derivatives of JA, which were elucidated as JA + H^+ and JA-H_2O + H^+, were used for correlation analysis (Supplemental Figure S6).

The linear regression analysis of the ionic strength of JA derivatives and MIFs (MG, MGL and MD) indicated that MIFs increased with the JA content (Figure 6). Here, the ionic strengths of JA + H^+ and JA-H_2O + H^+ were both significantly positively related to the contents of MG, MGL and MD with high correlation coefficients. Simultaneously, the ionic strength of JA and three MIFs in the VS-Fv group were the highest, followed by those of the RS-Fv and WL-Fv groups, while those of the WS-Fv group were the lowest. This phenotype tends toward Fv resistance in seed pods harvested from soybean plants growing under a CLE. The above results suggested that JA and MIFs are synergistically vital during resistance to Fv-infection in

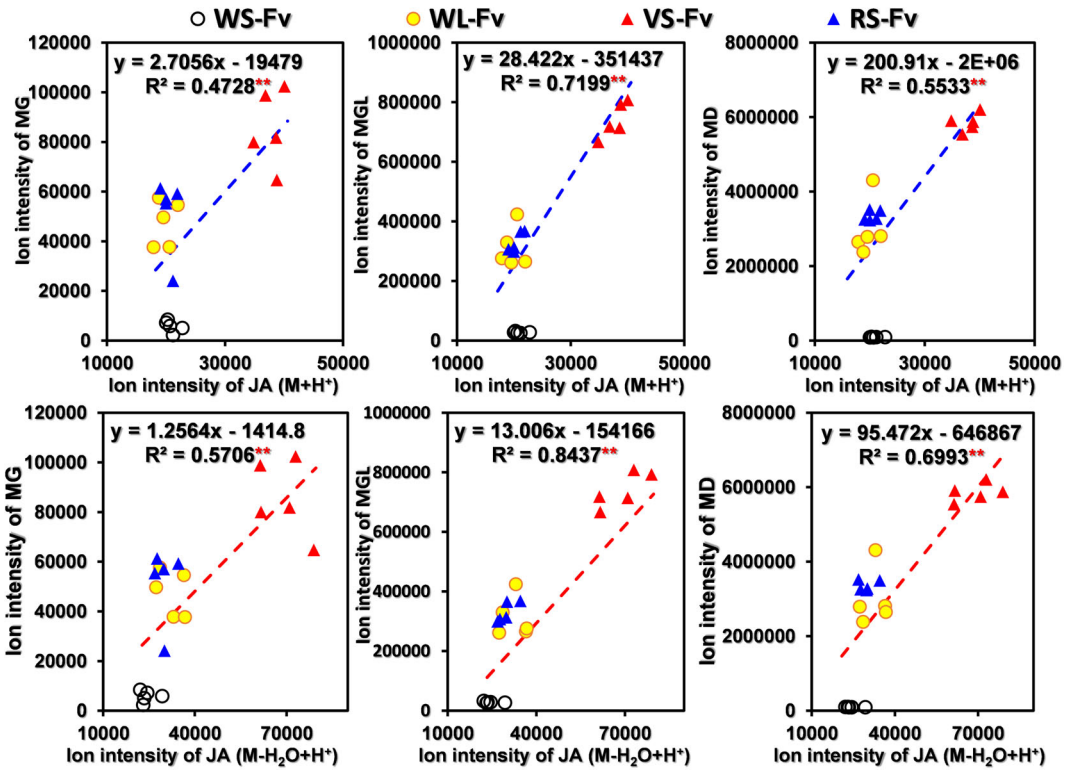

FIGURE 6 Correlation analysis of JA and isoflavone content in soybean pods. R^2: The determination coefficient (squared Pearson correlation coefficient) represents the imitative effect of the linear regression equation; **: Correlation is significant at the 0.01 level (2-tailed). MG: malonylgenistin, MGL: malonylglycitin, MD: malonyldaidzin [Colour figure can be viewed at wileyonlinelibrary.com]

soybean pods. The highest contents of JA and MIFs were induced by a fungal infection in VS treatment pods.

3.6 | Effect of JA on mildew resistance and flavonoid biosynthesis in soybean pods

3.6.1 | Exogenous application of MeJA

To further confirm the effects of JA on isoflavone biosynthesis in soybean pods, an exogenous MeJA application test was conducted in vitro. As shown in Figure 7(a), chlorophyll fluorescence imaging of soybean pod lesions was significantly affected at 7 DAI. The inoculated spots of the WL-W pods (harvest from WL-growing plants without MeJA treatment) turned dark blue, which indicated tissue necrosis, while the inoculated spots of only a few of the pods appeared dark blue in the groups of VS-W and WL-J. The *Fv/Fm* ratios and NPQ of pods in the WL-W were significantly lower than that of the VS-W groups, while MeJA treatment (WL-J) dramatically enhanced these two parameters (Figure 7(b)). In contrast to the changes in the *Fv/Fm* ratios and NPQ values, the mildew index of soybean pods of the VS-W groups was lower than those of the other two groups; MeJA treatment significantly inhibited pod mildew (Figure 7 (b)). Corresponding to the above mildew resistance phenotypes, all flavonoid contents in the pods of WL-J groups were higher than those

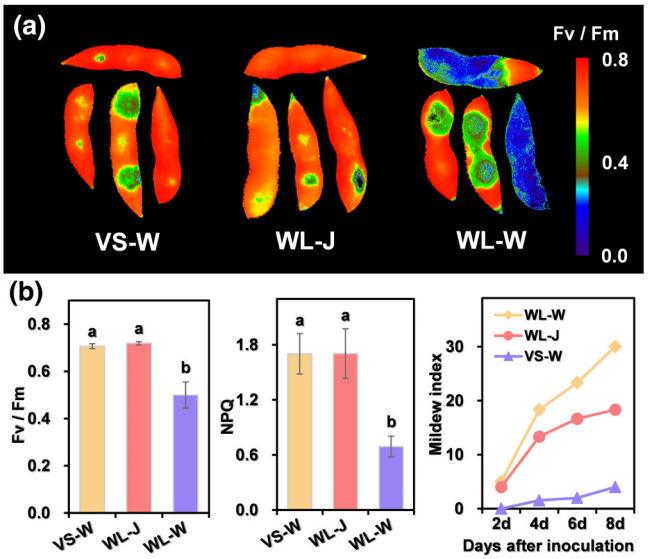

FIGURE 7 Comparison of mildew resistance of JA-treatment soybean pods. Chlorophyll fluorescence imaging of pods showing mildew difference based on *Fv/Fm* (a); parameters of *Fv/Fm*, NPQ and mildew index (b). The histograms are shown as the mean value ± standard error; values marked by the same letters are not significantly different ($p \geq 0.05$); *indicates significant differences ($p \leq 0.05$) between different treatments of the same point in time; VS-W: sterile water sprayed on the VS-pods; WL-J: MeJA sprayed on WL-pods; WL-W: sterile water sprayed on WL-pods [Colour figure can be viewed at wileyonlinelibrary.com]

in the WL-W groups, especially MIF and glyceollin, which were increased the maximum of 1.6-fold and 3.3-fold, respectively (Supplemental Figure S7(a)). The expression levels of all key genes involved in isoflavone biosynthesis, including *GmPAL2.1*, *Gm4CL*, *GmCHI1*, *GmIFS2*, *GmUGT1*, *GmUGT1*, *GmMT7* and *GmG4DT*, were significantly up-regulated with a 12-h inoculation, especially *GmUGT1*, which was up-regulated the maximum 9.0-fold at 12 h (Figure S7(b)). Hence, JA can promote the biosynthesis and accumulation of isoflavone in soybean pods, especially to MIF and glyceollin and improve their mildew resistance.

3.6.2 | Exogenous application of JA inhibitor

PHD is an inhibitor of JA biosynthesis that blocks endogenous JA synthesis by inhibiting lipoxygenases (Patkar et al., 2015). The mildew resistance phenotypes of soybean pods are shown in (Supplemental Figure S8). Fungal inoculation induced taupe fungal plaques covered with white hyphae, which could reflect the mildew degree. There were significantly more plaques on PHD-treated pods (PF) than on non-PHD-treated pods (Fv) (Figure S8(a)). The mildew index and mildew rate of the PF pods were significantly higher than those of pods without PHD application (Figure S8(a)). At the same time, PHD treatment indeed significantly decreased the JA content in soybean pods (-Figure S8(b)). This result indicated that PHD-treated soybean pods were more susceptible to fungal infection and that JA had a positive impact on the mildew resistance of pods.

According to the time sequence monitoring of major flavone constitutions in soybean pods with different treatments, all flavonoid contents in the inoculated pods were significantly higher than those in the control groups (CK) within 12–84 h of infection (Figure 8). However, PHD application led to a decrease in flavonoid levels in soybean pods, which were almost the same as those in the CK groups (Figure 8). Detailed quantitative analysis showed that flavones, flavanones and aglycones in the upstream pathway responded to mildew early; the changing curves of their contents were immediately different from those of the control groups within 12 h after inoculation and showed a peak value within 24 h (Figure 8(a)–(c)). In contrast, other isoflavones glycosides (β-glycoside, acetylglucoside and malonylglucoside) and glyceollin in the downstream pathway responded later, within 24 h after inoculation and their accumulation continued to increase with the infection duration (Figure 8(d)–(h)). However, all flavonoid contents decreased drastically when PHD was applied. In particular, the contents of MIFs and glyceollins were substantially inhibited by PHD; their accumulation was decreased by up to 2.9-times and 3.3-times (Figure 8(f), (g)), respectively, compared with that in pods receiving inoculation only (Fv). In contrast, flavanones and aglycones were less affected by PHD application (Figure 8 (b), (c)).

Moreover, the expression patterns of key genes involved in isoflavone biosynthesis in pods after 5 h of treatment were quantified by qPCR (Figure S8(c)). *GmUGT1* and *GmMT7* are the key enzyme genes closely related to downstream isoflavone glycosylation and malonylation; *GmG4DT* is the key gene of glyceollin biosynthesis

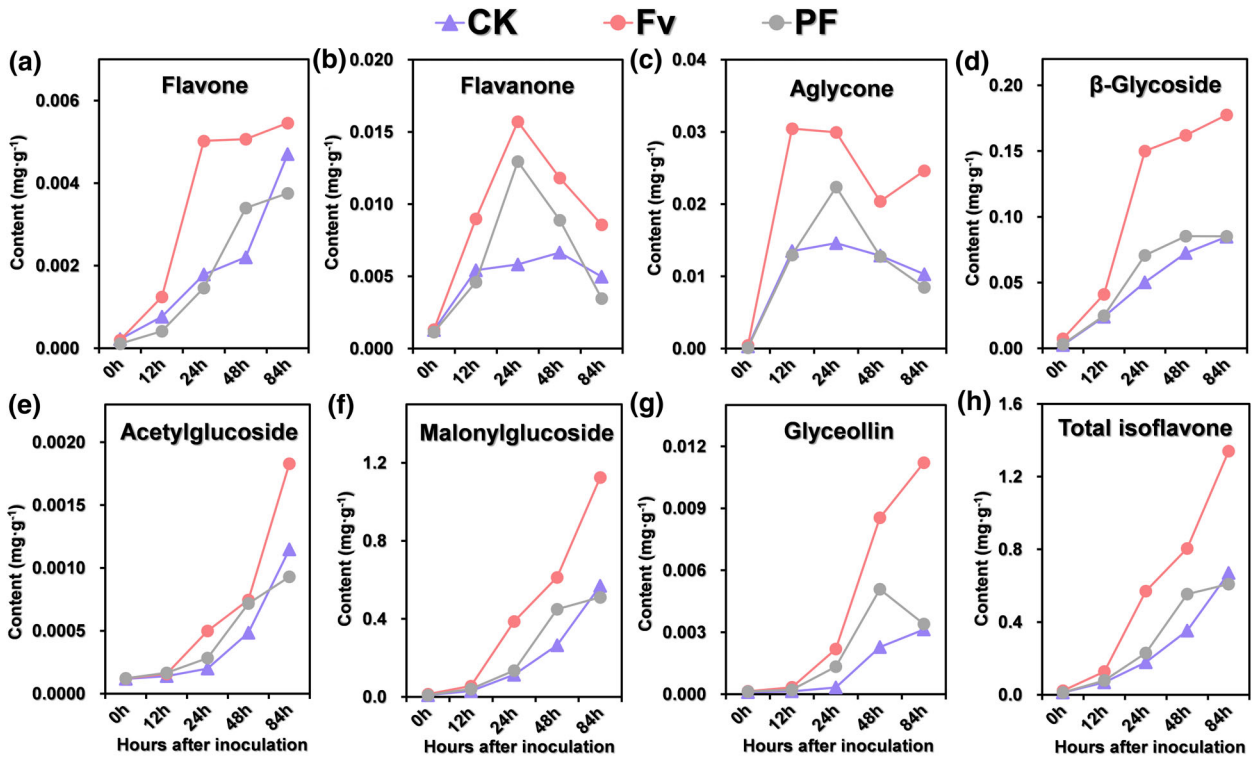

FIGURE 8 Effect of phenidone on the dynamic changes of flavonoid accumulation in soybean pods. CK: sterile water control; Fv: inoculation with *F. verticillioides*; PF: PHD pretreatment + fungal inoculation [Colour figure can be viewed at wileyonlinelibrary.com]

regulation. As shown in Figure S8(c), the expression levels of *GmUGT1*, *GmMT7* and *GmG4DT* were significantly higher in the inoculation-only group (Fv) than in the CK group, while the upstream *GmIFS* expression was not significantly different. However, after the spray application of PHD, the expression levels of the above key genes in the PF groups were downregulated significantly compared with those in the Fv group, in which *GmUGT1*, *GmMT7* and *GmG4DT* were downregulated by 64.95%, 66.74% and 97.18%, respectively (-Figure S8(c)). This finding suggests that PHD could inhibit the expression of pod isoflavone-related genes by repressing JA signalling in response to *F. verticillioides*. In addition, the above results also reveal that the JA signalling pathway mainly affects special MIFs and glyceollins, which are downstream of the flavonoid biosynthesis pathway.

4 | DISCUSSION

4.1 | Promotion of isoflavone synthesis in the seed pod by changing light partially unveils the mystery of higher resistance of intercropped soybean seeds

Intercropping is an effective measure to improve land utilization; reasonable IT can partially protect crops from pathogens and herbivore attacks (Li et al., 2020). MSI is an ecological, efficient and sustainable cultivation mode that is widely used in developing countries and has recently attracted much attention in developed countries (Gao et al., 2014). MSI has multiple ecological effects, such as reducing pests and diseases; elucidation of the mechanism of action will provide an important theoretical basis for further optimizing cultivation measures and improving crop yield and quality. However, we still know little about this so far. Although most of the previous studies focused on the inhibitory effect of biodiversity on pests and diseases, few studies have examined the mechanism of crop resistance enhancement mediated by the abiotic environment, especially the light environment (Brooker et al., 2015). The novelty of this study came from the examination of an actual field MSI system, in which the maize's higher height resulted in shading for the soybean plant (Wang et al., 2016).

In legumes, flavonoids are known to play pivotal roles in response to biotic and abiotic factors. The current results indicated that light conditions and mold inoculation both affect metabolic profiling. The PCA score plots and clustering analyses suggested that fungal infection had a greater effect on the pod metabolism than light treatment (Figure 3(a)). Plant disease resistance can be divided into two types: constitutive resistance (CR) and induced resistance (IR). CR is an inherent characteristic of plants before they are harmed by stresses; it is produced from phyletic evolution over a long time and determined by plant genotype. Plant CR is also affected by environmental factors. IR refers to the resistance components or reactions induced by the pathogen or herbivore. The effect of abiotic light conditions on the plant metabolism is sustained for a long time and is mainly reflected in the accumulation of constituent metabolites. However, the response of plants to fungal infection is usually quick, and some reactions are even instantaneous (Kempel, Schädler, Chrobock, Fischer, & Van Kleunen, 2011). The reaction during this stage is mainly reflected in the accumulation of inductive metabolites. Additional studies also confirmed that there are trade-offs between CR and IR (Kempel, Schädler, Chrobock, Fischer, & Van Kleunen, 2011). In this study, there was a trade-off in the metabolic flow conversion among isoflavones with different structural types as their core. The synergy of the CR isoflavone metabolite aglycone and the IR isoflavone metabolites MIF and glyceollin improved the resistance of soybean pods.

Many studies have shown that flavonoid biosynthesis is a light-dependent carbon fixation process (Nam, Lim, & Eom, 2018). Better light conditions are beneficial to the synthesis and accumulation of flavonoids in plant tissue, especially high-intensity and short-wavelength light irradiation (such as ultraviolet and blue light), which can improve flavonoid biosynthesis (Siipola et al., 2015). Soybean isoflavone, as an important type of plant flavones, is no exception; many research studies have shown that high latitude and long days are beneficial to the synthesis and accumulation of isoflavone in soybean seeds (Wu et al., 2017). In the field MSI system, maize leaves do not always shade soybean plants. In particular, soybean is shaded by maize, mainly during the vegetative stage (VS) in the relay IT system. After maize harvest, the light conditions of the soybean canopy are improved. Therefore, the light condition in this MSI system has the characteristic of "changing from weak to bright" with spatiotemporal heterogeneity (Dennis et al., 2020).

Previous studies have focused on the homogeneous light environment, namely, the influence of continuous, stable light conditions; however, less attention has been given to the heterogeneous light environment. Yushan Wu et al. researched the physiological responses to shade and subsequent recovery of soybean in an MSI system and indicated that the luminance of the soybean canopy was recovered after maize harvest; soybean leaf area and leaf mass increased even more than those of monocropping soybean (Wu et al., 2016). The compensatory growth of intercropped soybean plants in the reproductive stage is important and provides carbon sources for the growth of reproductive structures and the biosynthesis of secondary metabolites.

Our recent study showed that the isoflavone contents of soybean seeds were significantly higher in MSI than in monocropping systems (Liu, Yang, et al., 2016). We, therefore, speculated that vegetative stage shading (VS) could also improve isoflavone accumulation in soybean seedpods and enhance tolerance to pathogens. The enrichment analysis of different metabolites (Figure 3(c)) and targeted flavonoid quantification (Figure 4) indicated that phenylpropanoid metabolism in pods, especially flavonoid biosynthesis, was significantly upregulated by VS. These current results confirmed that CL conditions, especially VS, could improve the resistance of soybean seedpods to *F. verticillioides*. Hence, the increase in isoflavone contents in soybean pods under a CLE was one mechanism of the enhancement of soybean resistance by IT.

4.2 | JA-induced isoflavone biosynthesis mediated responses to the combined stress of shading and mildew triggered potential cross-resistance in soybean seedpods

In nature or agricultural practice, plants are often affected by multiple stresses, which is defined as combined stress. The effects of combined stress on crops are not always disadvantageous and are determined by the interaction mechanisms between stress factors. More studies have shown that abiotic stresses such as drought, high/low temperature and salt stress can affect the occurrence and spread of pathogenic bacteria, insects and weeds. These stresses directly or indirectly regulate plant-pest/disease interactions by altering plant physiology and related physicochemical defence responses. Multiple individual stresses are special combined stresses that refer to two or more stresses that do not occur in the same period, namely, one stress after another with no overlapping effect on plants (Pandey, Irulappan, Bagavathiannan & Senthil-Kumar, 2017). This is also called sequential stresses. Under the MSI pattern, the soybean plants experienced maize shading in the vegetative stage and fungal infection in the late reproductive stage, which is similar to an abiotic-biotic sequential stress. Our results confirmed the beneficial regulatory effects on soybean pods from the above-mentioned sequential stresses. This advantage effect can be called "cross-resistance" (Foyer, Rasool, Davey, & Hancock, 2016). Phytohormone signalling networks are actively stimulated by diverse environmental factors (Yang et al., 2019). The synergistic effect of endogenous hormones and secondary metabolites is an important mechanism of cross-resistance in plants under combined stress. Prior stress may lead either to priming or predisposition for plant metabolite biosynthesis during a subsequent stress (Prachi Pandey, Ramegowda, & Senthil-Kumar, 2015).

Recent research on pod resistance to insects indicated that herbivory- and solar UV-B radiation-induced increases in the level of defensive isoflavones in pods against stink bugs were mediated by ethylene signalling (Dillon, Chludil, Mithöfer, & Zavala, 2020). Another well-known factor, JA, is a typical biological stress hormone that plays a key role in plant disease resistance (Jang et al., 2020). Fungal inoculation increased the JA content and significantly induced isoflavone synthesis, which was consistent with existing research results (Jeong et al., 2018; Lozovaya et al., 2004). However, even more importantly, JA biosynthesis was significantly increased by mold infection in the VS group compared to the WL group growing under normal light conditions (Figure 6). Namely, JA could defend against mold infection by positively regulating the accumulation of isoflavone in soybean pods, which can be promoted by VS.

4.3 | Malonyl isoflavones are cross-resistance chemicals induced by the combined stresses of shading and fungal infection

Several studies have confirmed that the antifungal-activated soy isoflavones are aglycone (Naim et al., 1974) and the induced glyceollin downstream of isoflavone (Jahan et al., 2020). Glycosylation generally reduces their biological activity; however, the antifungal activity of MIFs remains unclear. Despite the highly unstable nature of MIF, the majority of soybean isoflavones accumulate in the form of their malonyl derivatives. There is an unknown mechanism that confers stability to MIF conjugates *in planta* (Dastmalchi & Dhaubhadel, 2014). Thus, it is difficult to purify MIF in nature, and there is no accurate report on the antifungal activity of MIFs in vitro to date.

However, in combination with our study, especially the fold change in flavonoid profiles of different shading treatment and Fv-inoculated pods, indicated that MIFs have the characteristics of induced resistance chemicals as a high-activity fungistat. As shown in Figure S4(a), the flavonoid contents in pods increased significantly after mold infection, in which the contents of flavanones (naringenin and liquiritigenin) and isoflavone aglycones (genistein, glycitein and daidzein) showed the strongest increase. Especially, the content of MIF in the VS-treatment pods increased by 1.64-fold after mold infection (Figure S4(a)). Compared with WL groups, shading significantly increased the flavonoid content of uninoculated pods. Besides flavanone and isoflavone aglycones, the content of MIF in VS treatment pods was also increased by 1.68-fold (Figure S4(b)). Similarly, VS treatment also significantly increased the flavonoid content in inoculated pods; the increased dominant type was MIF, which increased by 1.89-fold (Figure S4(c)). Therefore, we believe that MIF with the induced resistance characteristic also has strong antifungal activity.

To verify the potential induced antifungal activity of MIF and explore its functional mechanism, molecular docking analysis was performed on typical isoflavones (Figure S9). Phytopathogenic fungi produce several cell wall-degrading enzymes to invade plant tissue; among them, endopolygalacturonase (PG) catalyzes the fragmentation and solubilization of homogalacturonan. PG-inhibiting factors counteract fungal PGs by forming specific complexes with them (Federici et al., 2001). Thus, the binding affinities of molecular docking between the compounds and PG can indicate their antifungal activity potential. The theoretical binding mode of malonyldaidzin (MD) in the binding site of *Fm*PG is indicated in Figure S9. MD adopted a conformation to bind in the parallel helix of the *Fm*PG (Figure S9(a)). Importantly, a hydrogen bond interaction (bond length, 2.5 Å) and two polarity contacts (bond lengths, 3.3 Å and 3.4 Å) were observed between the carboxyl group of MD and the residues ASP-66 and SER-93, which were the main interactions between MD and *Fm*PG (Figure S9(b)). These interactions helped MD to anchor in the binding site of the *Fm*PG. We compared the *Fm*PG affinities with the isoflavone aglycone genistein, MD and the high antifungal activity chemical glyceollin I (induced isoflavone) (Kalli, Araya-Cloutier, Bruijn, Chapman, & Vincken, 2020). The affinity ranking was glyceollin I (−4.9) > MD (−4.7) > daidzin (−4.5) > daidzein (−3.7) (Figure S9(c)). The above molecular simulations and affinity analysis suggest a rational explanation for the interactions between isoflavone and *Fm*PG, especially the MD malonyl group, and provide valuable information for the further development of the *Fm*PG inhibitors.

In conclusion, this study shows that both shading and fungal infection could induce flavonoid biosynthesis in soybean pods.

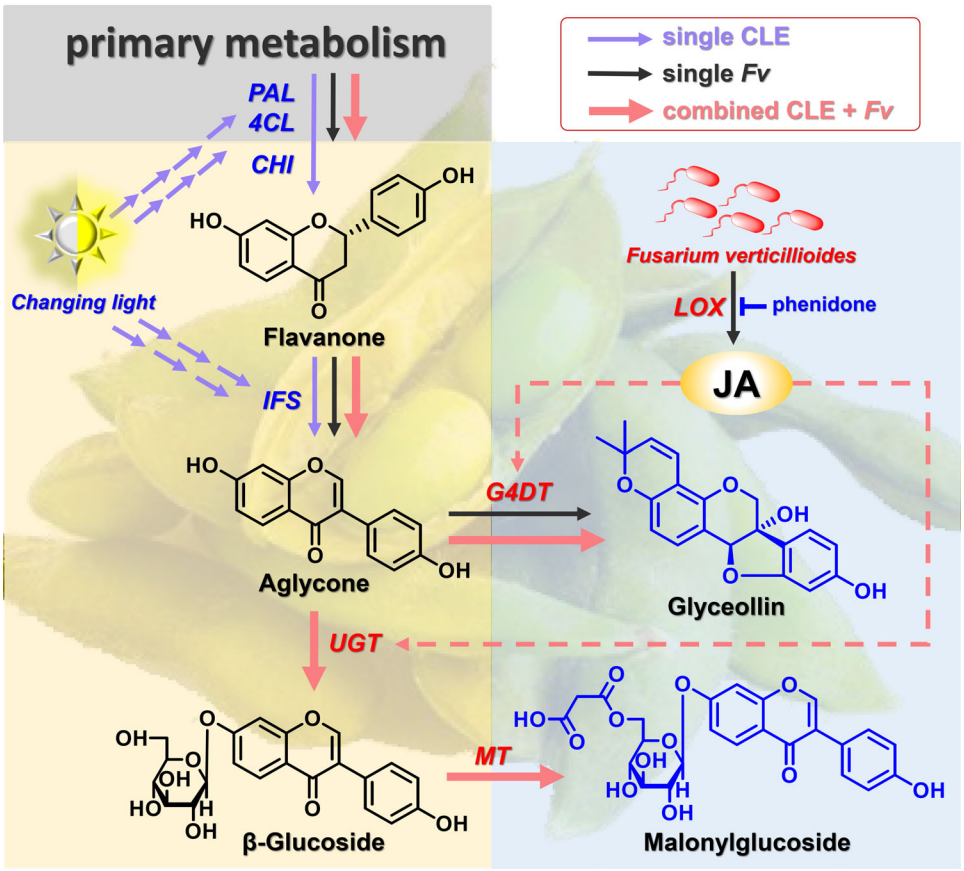

FIGURE 9 A proposed model for the mechanism by which the combination of changing light and endogenous JA enhances mildew resistance in soybean pods. CLE (VS) increases the level of carbon assimilation, providing an adequate carbon source for the biosynthesis of upstream aglycones. In response to fungal infection, the JA signalling pathway of soybean pods was activated and induced the biosynthesis of downstream flavonoids and further enhanced the mildew resistance of soybean pods. The grey background is the primary metabolic pathway; the yellow background is the pathway of constitutive flavonoids; the blue background is the pathway of inducible isoflavonoids. The blue and black arrows respectively show the steps activated by single CLE and Fv-inoculation; the red thick arrows show the steps activated by combined stresses of CLE and Fv; the dotted red arrows show potential transcriptional control mediated by the JA signal [Colour figure can be viewed at wileyonlinelibrary.com]

Aglycones are the main components induced by single shading or Fv infection, and MIFs were the main increased forms induced by the combined stresses of shading and fungal infection. MIFs are induced resistance chemicals and have potential activity in cross-resistance caused by combined stresses. This study advanced our understanding of IT in relation to disease resistance. We can summarize the mechanism by which soybean pod resistance improves mildew infection under a CLE or relay IT systems as follows (Figure 9). Fungal infection activates JA signalling in soybean pods and induces the biosynthesis of MIF and glyceollin. Beforehand, the photosynthetic carbon fixation capacity of the pods was enhanced by a CLE, which provided sufficient precursors for the biosynthesis and accumulation of downstream isoflavones and then improved the defence function of seed pods. To our knowledge, this study is the first to show that CLE can promote isoflavone biosynthesis in soybean pods, which has potential to suppress fungal infection. This study has opened up a new direction for further research on the strengthening of soybean stress tolerance. It is expected that through the further optimization of IT cultivation practice and the quality breeding for the improvement of isoflavones in seedpod, the FM of soybean can be effectively controlled, and the seed yield and quality will be improved.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Jiang Liu and Wenyu Yang designed the research. Xiaoman Li, Caiqiong Yang, Jianhua Chen, Yuan yuan He, Juncai Deng, Congwei Xie, Xinli Xiao and Xiyang Long performed the experiments. Xiaoling

Wu, Weiguo Liu, Junbo Du, Feng Yang, Xiaochun Wang, Taiwen Yong, Jing Zhang and YuShan Wu assisted the experimental design, analysed and discussed the data. Xiaoman Li and Jiang Liu wrote the manuscript with contributions from all the co-authors. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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SUPPORTING INFORMATION

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

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