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Research Article

Transcriptional analysis of *Rhazya stricta* in response to jasmonic acid

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ABSTRACT

Background: Jasmonic acid (JA) is a signal transducer molecule that plays an important role in plant development and stress response; it can also efficiently stimulate secondary metabolism in plant cells.
Results: RNA-Seq technology was applied to identify differentially expressed genes and study the time course of gene expression in *Rhazya stricta* in response to JA. Of more than 288 million total reads, approximately 27% were mapped to genes in the reference genome. Genes involved during the secondary metabolite pathways were up- or downregulated when treated with JA in *R. stricta*. Functional annotation and pathway analysis of all up- and downregulated genes identified many biological processes and molecular functions. Jasmonic acid biosynthetic, cell wall organization, and chlorophyll metabolic processes were upregulated at days 2, 6, and 12, respectively. Similarly, the molecular functions of calcium-transporting ATPase activity, ADP binding, and protein kinase activity were also upregulated at days 2, 6, and 12, respectively. Time-dependent transcriptional gene expression analysis showed that JA can induce signaling in the phenylpropanoid and aromatic acid pathways. These pathways are responsible for the production of secondary metabolites, which are essential for the development and environmental defense mechanism of *R. stricta* during stress conditions.
Conclusions: Our results suggested that genes involved in flavonoid biosynthesis and aromatic acid synthesis pathways were upregulated during JA stress. However, monoterpenoid indole alkaloid (MIA) was unaffected by JA treatment. Hence, we can postulate that JA plays an important role in *R. stricta* during plant development and environmental stress conditions.

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1. Introduction

Rhazya stricta is an evergreen woody shrub that belongs to the *Apocynaceae* family; it is mostly found in the Middle East and South Asia. *R. stricta* produces many bioactive compounds, and in the Arabian Peninsula, this shrub is used as traditional medicine. More than 100 alkaloids have been isolated from *R. stricta* plants [1,2]. Among them, indole alkaloids, I6R-19,20-E-isositsirikine acetate, leepacine, and dihydroeburnamenine have been isolated from the roots and leaves of *R. stricta* [3].

Jasmonic acid (JA) is a signaling molecule that plays an important role in plant development and stress response. JA and its derivatives are known as jasmonates (JAs) and are lipid-derived

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compounds belonging to the oxylipin family, which are formed through oxidation of α-linolenic acid [4,5,6]. The importance of JA in the plant stress response has been recognized for a long time. JA was implicated in senescence promotion and growth inhibition in many plants many years ago [7,8]. The basal levels of JA are found to increase rapidly upon wounding or other environmental stresses [9,10]. Damage elicits a rapid JA burst in leaves, where JA signals the pathways that lead to the accumulation of various secondary metabolites that protect the plant against biotic and abiotic stresses, such as pathogens, herbivores or drought [11,12,13].

In response to jasmonic acid stress, the genes involved in the JA pathway are activated by the MYC2 transcription factor. However, under normal conditions, MYC2 is bound by a family of inhibitory proteins called JAZ repressors [6]. JA binds the COL-1 protein (an F-Box component of a SKIP-CULLEN-F-BOX complex) to the JAZ repressor proteins and targets them for proteasome degradation [4]. During abiotic stress, JA signaling pathways control several transcription factors and genes. The JAZ-MYC module plays a central role in JA signaling by combining regulatory transcription factors and associated genes [14].

JA induces a wide variety of secondary metabolites that are bioactive and, therefore, a rich source of potential medicines. Using three-month-old seedlings of *Lycoris chinensis*, Mu et al. [15] demonstrated that the addition of JA can promote galanthamine accumulation. Liu et al. [16] demonstrated that the combined action of ethylene and JA in *Catharanthus roseus* promoted the production of phenolic compounds, especially salicylic acid, benzoic acid, and cinnamic acid. They were also able to correlate gene networks with metabolite accumulation. Vázquez-Flota et al. [17] showed that induction with methyl-jasmonate increases the accumulation of alkaloids in *Catharansus roseus*, and Góngora-Castillo et al. [18] demonstrated that JA specifically induces the transcription of numerous metabolic pathways in a variety of medicinal plants.

Monoterpenoid indole alkaloids (MIAs) are a diverse group of plant products containing tryptamine and a terpenoid unit. The genome of R. stricta has been sequenced, and the MIA pathway genes have been characterized. The R. stricta lineage is believed to have undergone an expansion of genes in this pathway, leading to the diversification of MIA products. Sabir et al. showed that the gene coding for strictosidine ß-D-glucosidase (SGD) was missing in the genome of *R. stricta*, and SGD could potentially be replaced by a related gene, raucaffricine-O-ß-D-glucosidase (RG), leading to alternative end products [19].

An important step in secondary metabolic pathways in plants is the synthesis of the aromatic amino acids tryptophan, tyrosine, and phenylalanine [20]. These amino acids act on chorismate that is generated by the shikimate pathway. Tryptophan is required to produce indole alkaloids, while tyrosine or phenylalanine is the required substrate for the production of phenylpropanoids [21]. In *Arabidopsis thaliana*, knockout experiments suggest that the regulation of these pathways is coordinated with the downstream processes leading to secondary metabolite production [22].

We used transcriptomic analysis to study the stress response in *R. stricta* with a specific focus on secondary metabolite production. Our results suggest that JA treatment of *R. stricta* seedlings with JA upregulates genes involved in the stress response and downregulates genes involved in cell division and other housekeeping functions. A detailed analysis of secondary metabolite pathways showed that genes involved in the MIA pathway and aromatic amino acid synthesis are not upregulated when plants are treated with JA. However, there are strong signals of upregulation of the polypropanoid pathway enzymes leading to the synthesis of anthocyanins, flavones, and flavonoids.

2. Material and methods

2.1. Sampling

Seeds collected from three *R. stricta* plants were germinated in the greenhouse, and leaf samples of 30-day-old seedlings untreated (control) and treated with JA (6 μM) were collected across different time intervals (0, 2, 6, and 12 d). Seedlings were grown in 7 pots (3 seedlings/pot). Three pots were treated with JA at 6 μM for 2, 6, and 12 d, while four pots were used as controls, in which leaf samples were collected after 0, 2, 6, and 12 d.

2.2. RNA extraction

For RNA extraction, 100 mg of frozen leaf material from all treated and untreated plants was collected and crushed into a fine powder in a microfuge tube using a sterilized rod. RNA was extracted using an RNeasy Plant Mini extraction kit according to the manufacturer's instructions. Additionally, B-mercaptoethanol was added to the RLT lysis buffer. RNA was then treated with DNase using the Ambion TURBO DNA-Free kit (cat no. AM1907). RNA ampure beads were used for cleaning the RNA. To check the purity and integrity of the RNA, an Agilent Bioanalyzer was used, and the RNA concentration was measured. An Illumina RIbozero Plant leaf kit was used with 2 μg of RNA, and the RNA was further eluted in 15 μl of RNase-free water. The purity of Ribozero-depleted RNA was checked by an Agilent Bioanalyzer using an RNA pico chip. The purified RNA was used for the preparation of the Illumina ScriptSeq library. All libraries were run on the Fragment Analyzer using the NGS High Sensitivity kit.

2.3. RNA sequencing of the leaf samples at different time points

In total, 48 RNA leaf samples from *R. stricta* plants were sequenced with HISEQ 2500. The raw files of Fastq sequences were trimmed for the presence of Illumina adapter sequences by Cutadapt version 1.2 [23]. The criterion for the trimming of sequences was set at option -O 3 so that the 3′ ends of any reads matching the adapter sequence for 3 bp were trimmed off. The reads were then trimmed using Sickle version 1.200 with a minimum window quality score of 20 [24]. Reads shorter than 10 bp were removed after the trimming of sequences.

2.4. Expression analysis

For the gene expression studies, all sequencing reads obtained after trimming were aligned individually with the reference genome of *R. stricta* using the HISAT2 program [25], which aligns RNA-seq reads to reference sequences and identifies splice junctions. Uniquely mapped reads were selected, and duplicate reads were filtered out using picard tools. The program StringTie was implemented after read alignment to assemble transcripts and to estimate their abundances for each sample. The transcript assemblies or gene structure annotations or functions were collated across the samples, guided by the read alignments to the reference genomes, to form an analysis-specific gene annotation summary. Gene function was assigned by BLASTX analysis with reference genomes from the NCBI database, and GO terms were assigned using BLAST2GO. The principal component analysis was performed in R using the genecount matrix generated by DEseq. Heatmaps were generated using a heatmapper on the average FPKM values for each gene and clustered using average linkage and Euclidian distances [26]. StringTie was then used to calculate gene and transcript abundances for each sample across the analysis-specific

annotated genes. DE-Seq [27] was used to identify differentially expressed genes.

3. Results and discussion

The 30-day-old *R. stricta* plants were treated with 6 μM JA at different time intervals (0, 2, 6, and 12 days). The RNA samples were collected from the leaf extract of *R. stricta* (0, 2, 6, and 12 days) under controlled conditions. High-throughput RNA-Seq was applied to investigate the gene expression profiles of *R. stricta* across a time course of 12 days with and without JA treatment. RNA-Seq reads generated in this study were approximately 328 million for control samples and 238 million for the treated samples. The raw sequencing reads were submitted to the European Nucleotide Archive data accession number PRJEB30669.

Approximately 27% of the control and treated sample reads, with at least one reported alignment, were mapped to the reference genome (Table S1). Many genome-wide high-throughput sequencing studies have been applied to study the functional role of various genes involved in *R. stricta* during stress conditions [19,23,28]. Furthermore, principal component analysis (PCA) of the samples demonstrates the clustering of replicates and separation of treated and untreated samples along PC1. The PCA plot shows that 12-day-old seedlings separate over PC1 in both the control and treated samples. This result suggests that the gene expression profile changes during plant development from day 1 to day 12; however, the treated samples separate from the untreated samples across PC2, reflecting the changes caused by the response to JA (Fig. 1). PCA was used to reduce the dimensionality of our RNA sequencing datasets.

3.1. Differentially expressed genes in R. stricta in response to JA treatment

After mapping the sequencing reads with the reference genome, sequence transcripts were assembled, and their relative expression levels were computed. In response to JA, there was a significant change in the gene expression of *R. stricta.* Many genes were up- and downregulated; the genes were identified using DE-seq with a p value cutoff of 0.05. The upregulation of genes was highest in the treated samples at day 6; similarly, the downregulation of genes was higher on day 2, as shown in Fig. 2. Based on the DESeq-DataSet analysis, the average expression between the treated and untreated data was compared by MA plot for the normalization counts of the samples (Fig. S1).

Interestingly, very few upregulated genes were common among different time points. Our results indicate that only 3 genes were upregulated during the time-course study. Among them, 2 genes were conserved hypothetical proteins, which are not homologous to other genes in the NCBI Genbank. The only single annotated gene upregulated across all time points was the MYC-2 transcription factor known as Jasmonate Insensitive 1 (JAI1). This MYC-2 transcription factor in *A. thaliana* and other plants is upregulated in response to the jasmonate signaling pathway and is involved in the regulation of downstream genes [29]. Our results follow a previous study in which *MYC-2* expression is rapidly induced by both jasmonic acid and abscisic acid in *A. thaliana.* It represses genes involved in pathogen defense and induces genes involved in wounding [30]. The upregulation of this gene here strongly indicates that similar pathways will be followed in *R. stricta* as well. This is the first time that the role of the MYC-2 transcription factor has been shown in *R. stricta* when treated with JA.

Furthermore, it is also clear that many genes with unknown function were downregulated during the time-course study when treated with JA. All downregulated genes were almost 3-fold downregulated during embryo sac developmental arrest, and 3 genes were highly downregulated in all samples. One of these genes encodes a heat-shock protein involved in the development of female gametophytes and is required for embryo sac development [31]. The reason for its downregulation may be in response to growth inhibition. Another gene, flagellin sensitive 2 (FLS2), a homolog of the leucine-rich repeat protein, was also downregulated by 1.5-fold. The main function of FLS2 is microbe recognition, and it acts independently of JA [32]. However, jasmonate can act antagonistically to biotic stress signaling pathways, and hence, genes involved in these processes could be downregulated in response to JA treatment. The 5 most up- and downregulated genes that show higher fold differences are listed in Table 1 and Table 2.

Previously, our group reported that severe salt stress could upregulate many transcripts, including genes encoding tetrapyrroles and pentatricopeptide repeat (PPR) proteins. Many genes,

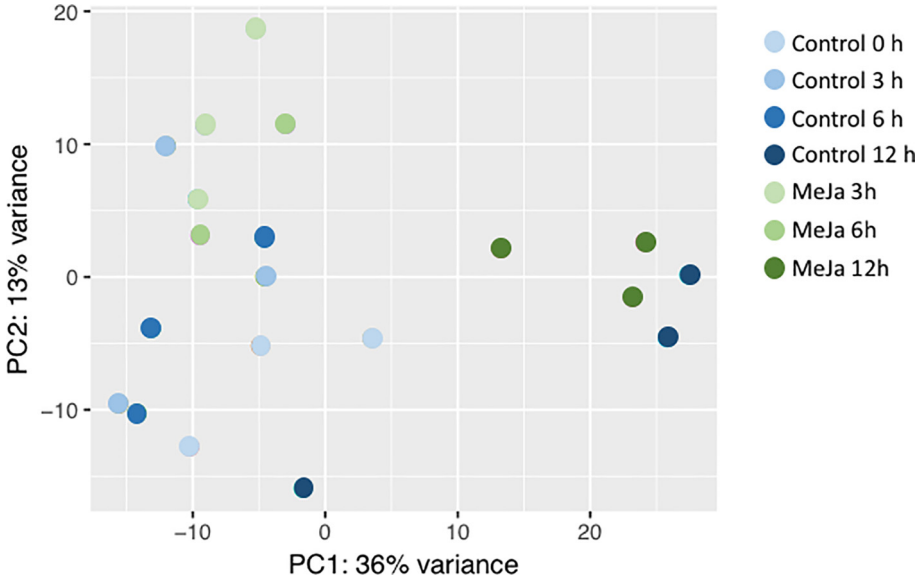

Fig. 1. PCA of samples with the outlier sample removed. Untreated samples are labeled 0 h, 3 h, 6 h and 12 h. The treated samples are labeled MeJa 3 h, 6 h, and 12 h. Each sample is coded with a different color.

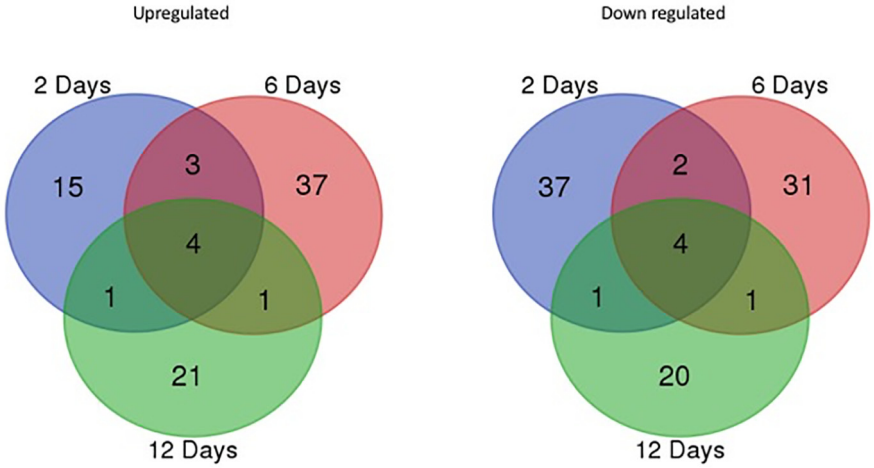

Fig. 2. Venn diagrams of differentially expressed genes at different time points after JA treatment. The blue, purple, and green circles represent 2, 6, and 12 days differentially expressed genes, respectively.

Table 1
The 5 most upregulated genes that show higher fold differences at different time intervals.

Gene ID	Fold change	Accession number	Blast annotation
Top five upregulated genes at day 2			
MSTRG.11763	2.79	NP_001312938.1	Transcription factor MYC2-like [Nicotiana tabacum]
MSTRG.16469	2.12	XP_013463072.1	Cytochrome P450 family ent-kaurenoic acid oxidase [Medicago truncatula]
MSTRG.9949	2.0	KVI06624.1	Homeodomain-like protein [Cynara cardunculus var. scolymus]
MSTRG.20927	1.95	KCW78768.1	Hypothetical protein EUGRSUZ_C00202 [Eucalyptus grandis]
MSTRG.847	1.92	XP_007131974.1	Hypothetical protein PHAVU_011G056100g [Phaseolus vulgaris]
Top five upregulated genes at day 6			
MSTRG.11763	2.44	NP_001312938.1	Transcription factor MYC2-like [Nicotiana tabacum]
MSTRG.1266	2.13	XP_002281574.1	Xyloglucan endotransglucosylase/ hydrolase protein 9 [Vitis vinifera]
MSTRG.3421	1.98	KVH97678.1	Pectinesterase inhibitor [Cynara cardunculus var. scolymus]
MSTRG.16469	1.96	XP_013463072.1	Cytochrome P450 family ent-kaurenoic acid oxidase [Medicago truncatula]
MSTRG.21317	1.81	XP_004240117.1	pectinesterase/pectinesterase inhibitor 12 [Solanum lycopersicum]
Top five upregulated genes at day 12			
MSTRG.17867	3.2	XP_006429290.1	Hypothetical protein CICLE_v10013166mg [Citrus clementina]
MSTRG.5930	1.98	XP_016672452.1	COBRA-like protein 4 [Gossypium hirsutum]
MSTRG.11763	1.95	NP_001312938.1	Transcription factor MYC2-like [Nicotiana tabacum]
MSTRG.1107	1.93	EOY05359.1	Transmembrane amino acid transporter family protein [Theobroma cacao]
MSTRG.983	1.75	XP_012831306.1	Xyloglucan endotransglucosylase/ hydrolase protein

Table 2
The 5 most highly downregulated genes that show higher fold differences at different time intervals.

Gene ID	Fold change	Accession number	Blast annotation
Top five downregulated genes at day 2			
MSTRG.4003	2.93	KDO62934.1	Hypothetical protein CISIN [Citrus sinensis]
MSTRG.10194	1.88	XP_007160935.1	Hypothetical protein PHAVU [Phaseolus vulgaris]
MSTRG.20608	1.61	XP_006442975.1	Hypothetical protein CICLE [Citrus clementina]
MSTRG.6212	1.1	XP_010048302.1	Thymidylate kinase isoform X1 [Eucalyptus grandis]
MSTRG.3120	0.89	KRH15761.1	Hypothetical protein GLYMA_14G109400 [Glycine max]
Top five downregulated genes at day 6			
MSTRG.17867	3.9	XP_006429290.1	Hypothetical protein CICLE [Citrus clementina]
MSTRG.20832	1.83	EOY31300.1	Molybdenum cofactor sulfurase family protein isoform 2 [Theobroma cacao]
MSTRG.4003	1.79	KDO62934.1	Hypothetical protein CISIN [Citrus sinensis]
MSTRG.20239	1.79	XP_006452002.1	Hypothetical protein CICLE [Citrus clementina]
MSTRG.11048	1.73	KJB56214.1	Hypothetical protein B456_009G110500 [Gossypium raimondii]
Top five downregulated genes at day 12			
MSTRG.14717	1.76	XP_010091051.1	UDP-glycosyltransferase [Morus notabilis]
MSTRG.7933	1.75	XP_006343127.1	Peroxisomal (S)-2-hydroxy-acid oxidase GLO4-like [Solanum tuberosum]
MSTRG.12548	1.72	XP_010035185.1	S-type anion channel SLAH2 isoform X3 [Eucalyptus grandis]
MSTRG.4003	1.72	KDO62934.1	Hypothetical protein CISIN [Citrus sinensis]
MSTRG.12836	1.70	CDP08216.1	Unnamed protein product [Coffea canephora]

such as chaperone protein Dnaj6, UDP-glucosyl transferase 85a2, protein transparent testa 12, and respiratory burst oxidase homolog protein b, were upregulated. Interestingly, genes involved in the flavonoid pathway were also upregulated [23]. Our RNA-Seq analysis revealed that genes involved in the flavonoid pathway

were highly upregulated when plants were treated with 6 μM JA. Thus, our results confirm that both salt stress and JA treatment of R. stricta can upregulate genes involved in the flavonoid pathway.

Functional analysis of differentially expressed genes was performed with blast2GO to identify biological pathways and molecular processes that respond to JA treatment at different time points. The selection of functional groups with the highest number of transcripts at 2, 6, and 12 days, either upregulated or downregulated, was categorized into two main molecular functions and biological processes. Many different biological processes and molecular functions were upregulated and downregulated during the time-course study with JA (Table 3).

On day 2, gene ontology results showed that most of the upregulated genes were associated with many important biological processes, including the oxylipin biosynthetic process and JA biosynthetic process. Jasmonate is an oxylipin, and the results suggest that jasmonate induces its production. Positive feedback of jasmonate synthesis genes in response to jasmonate has been previously described in Arabidopsis [33]. Moreover, the upregulation of salicylic acid biosynthesis and alkaloid biosynthesis processes was also observed. Our results agree with the plant sensing JA and the upregulation of genes associated with transcriptional changes, signaling, and protein turnover, suggesting that the cells have sensed the signaling molecule and have begun to respond.

On day 6, gene ontology results revealed the upregulation of brassinosteroid biosynthesis. Brassinosteroids are critical molecules in regulating plant growth and development, and the relationship between brassinosteroid-regulated pathways and the jasmonate pathway is not well understood. In rice, these brassinosteroids have been shown to act antagonistically, affecting both leaf angle development and JA and inhibiting lamina joint inclination by downregulating brassinosteroid biosynthesis and signaling pathways [34]. Sucrose phosphate synthase was also upregulated on day 6. It has been reported that this enzyme was upregulated during osmotic stress, which in turn upregulated amino acid permease family proteins that may enable the accumulation of amino acids within cells to further resist osmotic pressure. It has previously been shown that amino acid accumulation may play an important role in the osmotic stress response [35,36,37]. Moreover,

Table 3
Biological process and molecular functions up- and downregulated during the treatment of jasmonic acid during the time course study.

Biological process	Molecular function	Biological process	Molecular function
Upregulated Day 2		*Downregulated Day 2*	
Oxylipin biosynthetic process	Secondary active sulfate transmembrane	Defense response by callose deposition process	Ribonucleotide binding
Regulation of salicylic acid biosynthetic process	Calcium-transporting ATPase activity	Megagametogenesis process	Heat shock protein binding
Alkaloid biosynthetic process	Triglyceride lipase activity	Translational initiation process	Thymidylate kinase activity
Jasmonic acid biosynthetic process	FMN binding	Proteasome-mediated ubiquitin-dependent process	Ubiquitin binding
Calcium ion transmembrane transport process	Zinc ion binding	Response to biotic stimulus process	ATP binding
Gibberellin biosynthetic process	Oxidoreductase activity	Defense response to bacterium process	Zinc ion binding
RNA phosphodiester bond hydrolysis process	Sequence-specific DNA binding	Intracellular signal transduction process	Protein dimerization activity
Sulfate transmembrane transport process	Metalloendopeptidase activity	Defense response process	Protein kinase activity
Brassinosteroid homeostasis process	Calmodulin binding	Regulation of transcription process	Calmodulin binding
Brassinosteroid biosynthetic process	Protein dimerization activity	Nucleobase-containing compound process	Translation initiation factor activity
Upregulated Day 6		*Downregulated Day 6*	
Cell wall organization process	Xyloglucan:xyloglucosyl transferase activity	Defense response by callose deposition process	Protein dimerization activity
Cell wall biogenesis process	Pectinesterase activity	Pollen wall assembly process	ADP binding
Brassinosteroid biosynthetic process	Polyamine transmembrane transporter	Megagametogenesis process	Amino acid binding
Xyloglucan metabolic process	Channel regulator activity	Sucrose metabolic process	O-acyltransferase activity
Xenobiotic transport process	ADP binding	Oxidation-reduction process	Calmodulin binding
Drug transmembrane transport process	Xenobiotic-transporting ATPase activity	Translational initiation process	Translation initiation factor
DNA integration process	Aspartyl esterase activity	Root development process	Ion channel activity
Regulation of cell size process	Serine-type endopeptidase activity	Cell proliferation process	Pyridoxal phosphate binding
Formation of organ boundary process	Auxin efflux transmembrane transporter	Shoot system morphogenesis process	GTPase activity
Negative regulation of GTPase activity process	Protein dimerization activity	Negative regulation of transcription process	Kinase activity
Upregulated Day 12		*Downregulated Day 12*	
Protein phosphorylation process	Sulfite oxidase activity	Flavonoid biosynthetic process	Phosphatase activity
Cell wall organization process	Galactolipase activity	Oxidation-reduction process	Quercetin 7-O-glucosyltransferase activity
Chlorophyll metabolic process	Actin filament binding	Cellular ion homeostasis process	Voltage-gated anion channel activity
Signal transduction Process	Amino acid transmembrane transporter	Steroid biosynthetic process	Unfolded protein binding
Actin filament organization process	Damaged DNA binding	Protein oligomerization process	Pyridoxal phosphate binding
Secondary cell wall biogenesis process	Protein dimerization activity	Tricarboxylic acid cycle process	NAD binding
DNA integration process	Xyloglucan:xyloglucosyl transferase activity	Iron-sulfur cluster assembly process	Xenobiotic-transporting ATPase activity
Cell wall biogenesis process	Molybdenum ion binding	Pectin biosynthetic process	Heat shock protein binding
Xyloglucan metabolic process	Calmodulin binding	Megagametogenesis process	Quercetin 3-O-glucosyltransferase
Abscisic acid-activated signaling pathway process	Protein kinase activity	Root cap development process	Chaperone binding

it was noted that the number of pectinesterase inhibitors and xyloglucan endotransglucosylase/hydrolase proteins were upregulated. The xyloglucan genes are involved in cell wall biosynthesis and cell elongation; thus, an increase in cellulose synthesis could enable the growth of cells by maintaining cell wall integrity [38]. Pectinesterases are involved in cell wall modification and affect cell wall rigidity; hence, the upregulation of inhibitors would suggest changes in cell wall physiology.

Similarly, gene ontology results on day 12 showed that the transcriptional profile of the treated plants was most distinct from that of the control plants. The gene homolog of auxin-binding protein was upregulated. In maize, this gene is localized within the cell wall and participates in signal transduction during abiotic stresses [39]. Many biological processes, such as cell wall organization, chlorophyll metabolism, and signal transduction, were upregulated on day 12. Jasmonic acid (JA) is an essential molecule in regulating many physiological processes in plant growth and development. MeJA-treated Arabidopsis showed upregulation of genes involved in signal transduction [40]. Among the molecular functions, actin filament binding, sulfite oxidase, and protein dimerization activity were also upregulated after JA treatment on day 12. Interestingly, flavonoid biosynthetic, steroid biosynthetic and pectin biosynthetic pathways were downregulated on day 12 after JA treatment.

3.2. Gene expression study of genes involved in the metabolism of secondary metabolites in response to JA in R. stricta

The effect on the gene expression profile in response to JA in *R. stricta* confirmed that many genes are upregulated in different metabolic pathways responsible for the production of secondary metabolites. These secondary metabolites play an important role in the defense mechanism and protect plants from various adverse conditions. However, many genes were downregulated when treated with JA. To visualize common gene expression profiles between treated and control samples at different time intervals, heatmap analysis was carried out. Heatmap analysis of genes involved in indole alkaloid biosynthesis pathways shows very similar gene expression profiles during the time-course study (Fig. S2). However, hydroxylase, loganate O-methyltransferase, and polyneuridine aldehyde esterase showed upregulation toward the end of the experiment. From the results, genes involved in indole alkaloid biosynthesis pathways were unaffected before and after JA treatment. Compounds involved in the monoterpene indole alkaloid (MIA) pathway are only produced in Gentianales. Rauvolfioideae, a subfamily of Apocynaceae, appears to have the greatest diversity of enzymes and can generate thousands of unique molecules. Previously, it was reported that JA can modulate monoterpenoid indole alkaloid biosynthesis in *Catharanthus roseus* [41]. Our results indicate that JA is not responsible for the upregulation of genes involved in the MIA pathway.

We have also shown that genes involved in the aromatic amino acid biosynthesis pathway showed both upregulation and downregulation during treatment. Treated plants appear to downregulate the majority of genes responsible for the production of phenylalanine (Phe) and tyrosine (Tyr). However, the genes involved in tryptophan (Trp) biosynthesis pathways were upregulated. Furthermore, it was noted that the gene coding for chorismate mutase was not affected by JA treatment during the time-course study (Fig. 3). Further gene expression in the aromatic amino acid biosynthesis pathway at different time points showed varying fold differences (Fig. 4). Amino acids such as Phe, Tyr, and Trp play an important role in plant metabolism. They act as precursors for a wide range of secondary metabolites and serve as precursors for a variety of plant hormones, such as auxin and salicylate [42].

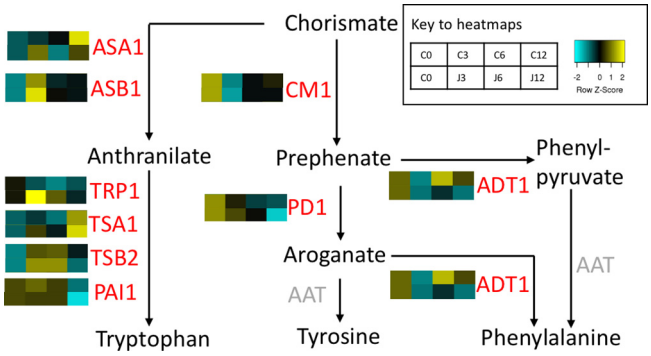

Fig. 3. Aromatic amino acid biosynthesis pathway showing gene expression changes. **TRP1:** Phosphoribosylanthranilate transferase, **PAI:** Phosphoribosylanthranilate isomerase, **TSA:** Tryptophan synthase, **CM1**: Chorismate mutase 1, **ASA1** Anthranilate synthase alpha subunit, **GAT:** GABA Transporter, **ASB1** Anthranilate synthase beta subunit 1. **PDH**/**PDH1**: Proline dehydrogenase. **PD1:** Prephenate dehydratase, **AAT**: Aspartate amino transferase, **PDT:** Prephenate dehydratase, **ADT1:** Arogenate dehydratase-1, **ADT-6:** Arogenate dehydratase-6. C0, C3, C6, and C12 are control samples, whereas J3, J6 and J12 are treated samples at day 3, day 6 and day12, respectively.

3.3. The flavonoid biosynthesis pathway is upregulated on day 6 postinduction of JA

Flavonoids are widely distributed in plants and have six major subgroups: chalcones, flavones, flavonols, flavonoids, anthocyanins, and proanthocyanidins [43]. In a few specialized plants, there are additional groups of flavonoids, such as aurones (heterocyclic chemical) and isoflavonoids, and other plants produce phlobaphenes [44,45]. On day 6 of JA induction, the gene expression profile showed upregulation of genes involved in the phenylpropanoid pathway compared with that in control plants (Fig. 5). Gene expression studies showed that many genes involved in the phenylpropanoid pathway were highly upregulated, such as FLS1 and FNS, which showed an almost 3-fold increase compared to the gene expression of control plants (Fig. 6). Surprisingly, there is no concurrent upregulation of the phenylalanine biosynthesis genes to generate these compounds' precursors. However, this may be due to other factors regulating phenylalanine flux in the treated plants. Many marker genes for the phenylpropanoid pathway, such as phenylalanine ammonia lyase, cinnamate 4-hydroxylase (C4H), chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase, showed gene expression changes with the treatment. Different flavonoids, such as quercetin, hesperetin, kaempferol, quercetin-3-rhamnaside, isoquercitrin, rutin, apigenin, luteolin, luteolin-7-glucoside, acacetin, and apigenin-8-Cglucoside, were isolated from *Rhazya stricta* [46]. Flavonoids play an important role in plant growth, development, and responses to environmental stresses and can significantly impact agricultural productivity. Different plant-based medicines containing flavonoids have long been used by humans [47]. Our gene expression results showed upregulation of genes involved in the phenylpropanoid pathway, including genes involved in the flavonoid pathway, when plants were treated with JA compared to control plants with no treatment. Flavonol synthase (FLS1), a marker protein involved in flavonoid biosynthesis, showed an almost 6-fold increase after plants were treated with JA. Anthocyanidins (or glycosidic forms, anthocyanins) are part of the secondary metabolite flavonoid class and are incredibly essential water-soluble pigments in plants [48]. Our results did not show a change in the gene expression profile of the anthocyanidin pathway when treated with JA at all time points.

Transcriptome studies of *R. stricta* suggest that there is not a well-known pathway to produce flavonoids; moreover, we failed

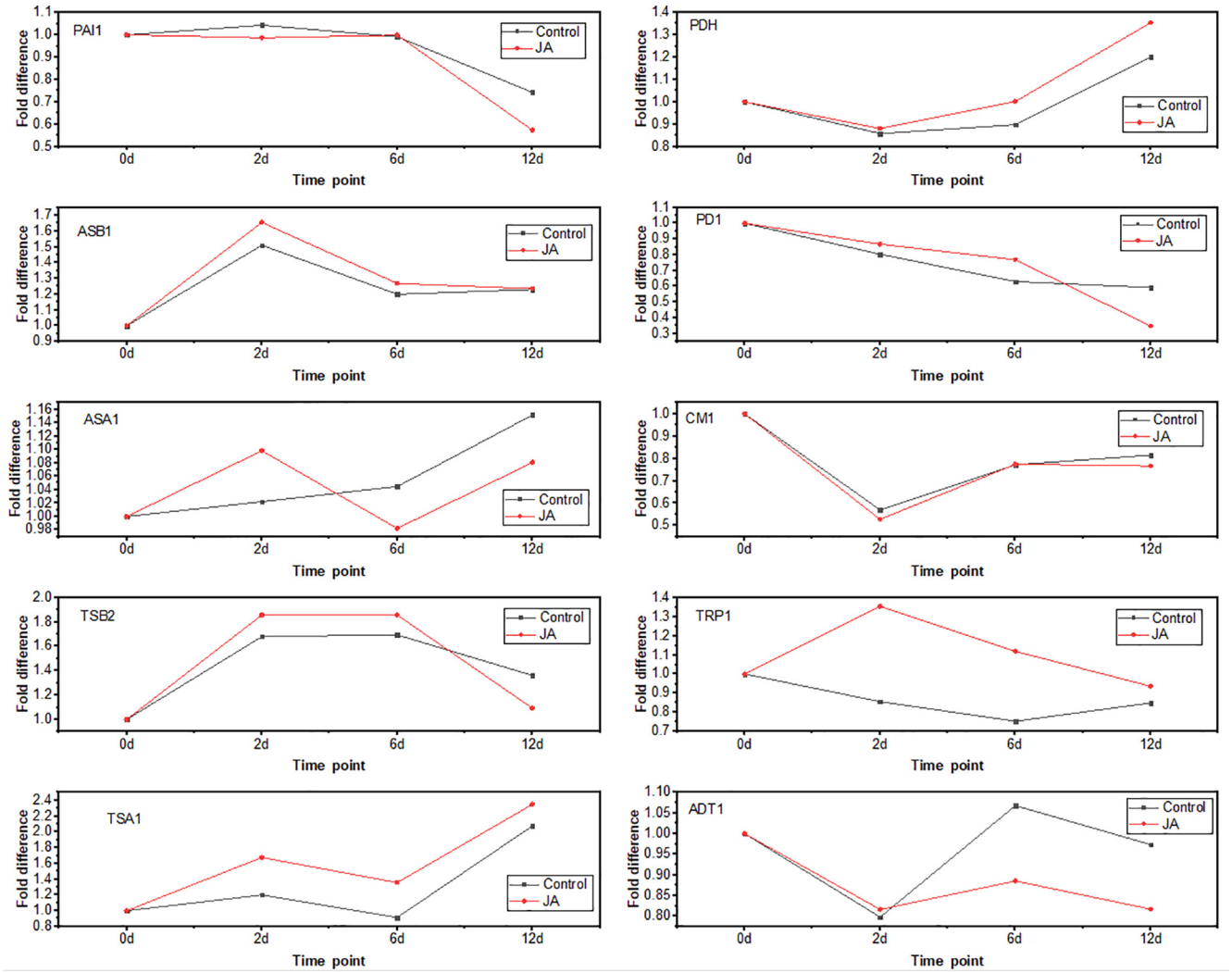

Fig. 4. Fold change in gene expression in the aromatic amino acid biosynthesis pathway at different time points.

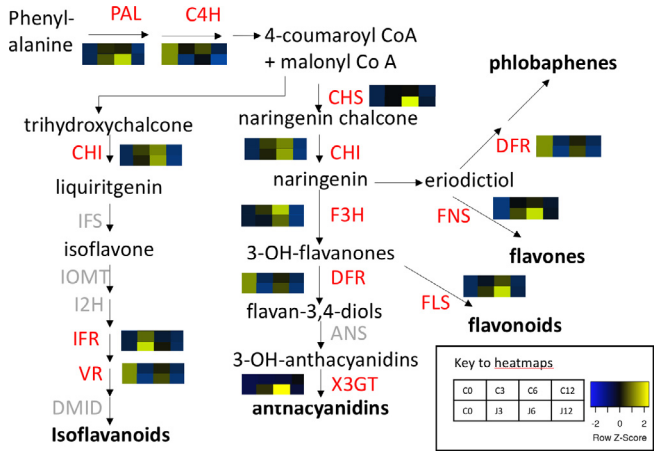

Fig. 5. Phenylpropanoid pathway showing gene expression changes. PAL: phenylalanine ammonia lyase, C4H: cinnamate 4-hydroxylase, CHS: chalcone synthase, CHI: chalcone isomerase F3H: flavonoid 3′-hydroxylase, IFS: isoflavone synthase, IOMT: isoflavone-7-O-methyltransferase, IFR: isoflavone reductase, VR: Vestitone reductase, DMID: 7,2′-dihydroxy, 4′-methoxyisoflavanol dehydratase, DFR: dihydroflavonol-4-reductase, ANS, Anthocyanin synthase, X3GT: flavonoid-3-O-glucosyltransferase, FLS: Flavonol synthase, FNS: Flavone synthase. C0, C3, C6, and C12 are control samples, whereas J3, J6 and J12 are treated samples on days 3, 6, and 12, respectively.

to identify a convincing candidate for ANS, one of the terminal genes in the anthocyanin pathway. The role of flavonoids has been well studied in *R. stricta* and *R. orientalis* [49]. Several plant species that do not produce isoflavonoids have genes with high sequence homology to legume IFR. The IFR genes isolated from nonlegume species with significant homology to IFR are collectively called IFR-like genes. Similarly, in tobacco, the vestitone reductase-like gene is believed to play a key role in the production of nicotine-like alkaloids and is stress responsive [50].

4. Conclusions

We undertook a targeted analysis of gene expression in secondary metabolite pathways during JA treatment in *R. stricta*. Our results showed that JA treatment can alter gene expression in *R. stricta*, which was sufficient to induce signaling in indole alkaloid, phenylpropanoid biosynthesis, flavonoid, phenylalanine, tyrosine, and tryptophan biosynthesis pathways. Our analysis suggests that the induction of the jasmonate pathway in *R. stricta* using JA induces flavonoid pathways that continue through a series of enzymatic modifications to yield flavones, flavonoids and anthocyanins. However, the response in the MIA pathways is more complex, and there is no clear upregulation of genes involved in this pathway.

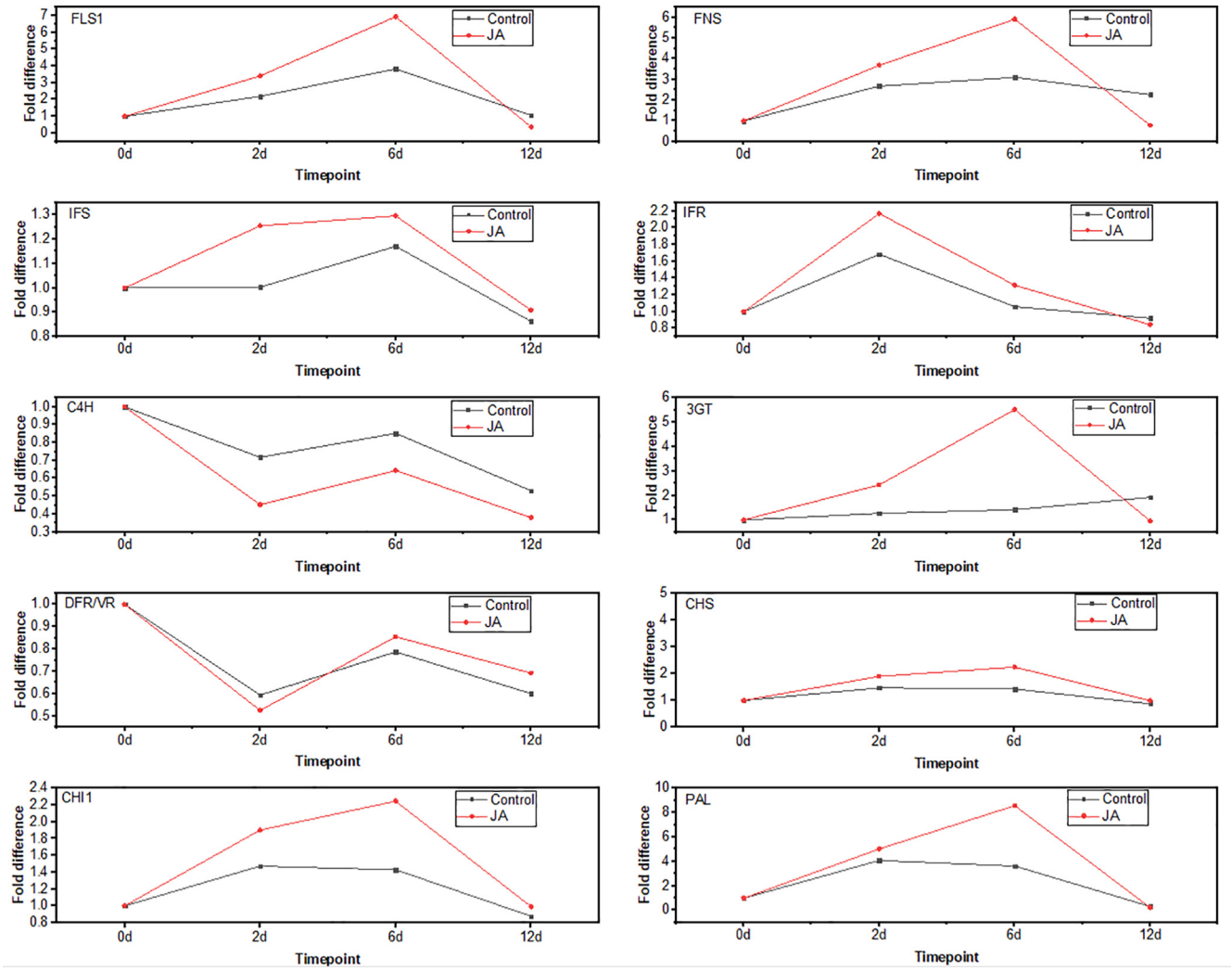

Fig. 6. Fold change in gene expression in the phenylpropanoid pathway at different time points.

Conflict of interest

Authors have no conflict of interest.

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Supplementary material

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