



ELSEVIER

Contents lists available at ScienceDirect

## Comptes Rendus Biologies

www.sciencedirect.com



Molecular biology and genetics/Biologie et génétique moléculaires

Stepwise response of MeJA-induced genes and pathways in leaves of *C. roseus*

Ahmed Bahieldin <sup>a,\*</sup>, Ahmed Atef <sup>a</sup>, Sherif Edris <sup>a,b,c</sup>, Nour O. Gadalla <sup>d,e</sup>, Mohammed Al-matary <sup>a</sup>, Magdy A. Al-Kordy <sup>e</sup>, Ahmed M. Ramadan <sup>a,f</sup>, Sameera Bafeel <sup>a</sup>, Mona G. Alharbi <sup>a</sup>, Diana A.H. Al-Quwaie <sup>g</sup>, Jamal S.M. Sabir <sup>a</sup>, Hassan S. Al-Zahrani <sup>a</sup>, Mahmoud E. Nasr <sup>h</sup>, Fotouh M. El-Domyati <sup>b</sup>

<sup>a</sup> Department of Biological Sciences, Faculty of Science, King Abdulaziz University (KAU), P.O. Box 80141, 21589 Jeddah, Saudi Arabia

<sup>b</sup> Department of Genetics, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

<sup>c</sup> Princess Al-Jawhara Al-Brahim Centre of Excellence in Research of Hereditary Disorders (PACER-HD), Faculty of Medicine, King Abdulaziz University (KAU), Jeddah, Saudi Arabia

<sup>d</sup> Department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah, Saudi Arabia

<sup>e</sup> Genetics and Cytology Department, Genetic Engineering and Biotechnology Division, National Research Center, Dokki, Egypt

<sup>f</sup> Agricultural Genetic Engineering Research Institute (AGERI), Agriculture Research Center (ARC), Giza, Egypt

<sup>g</sup> Department of Biological Sciences, Rabigh College of Science and Arts, King Abdulaziz University (KAU), Rabigh, Saudi Arabia

<sup>h</sup> Faculty of Agriculture, Menofia University, Shebeen Elkom, Egypt

## ARTICLE INFO

## Article history:

Received 18 May 2018

Accepted after revision 1st October 2018

Available online 26 October 2018

## Keywords:

RNA-Seq

TIA pathway

BHLH

Vindoline

## ABSTRACT

*Catharanthus roseus* is a perennial herb known for the production of important terpenoid indole alkaloids (TIAs) in addition to a variety of phenolic compounds. The goal of the present work was to detect the prolonged effects of MeJA (6  $\mu$ M) treatment across time (up to 24 days) in order to detect the stepwise response of MeJA-induced genes and pathways in leaves of *C. roseus*. Prolonged exposure of plants to MeJA (6  $\mu$ M) treatment for different time points (6, 12 and 24 days) indicated that genes in the indole alkaloid biosynthesis pathway and upstream pathways were triggered earlier (e.g., 6 days) than those in the anthocyanin biosynthesis pathway and its upstream pathways (e.g., 12 days). Three enzymes, e.g., T16H, OMT, and D4H, in the six-step vindoline biosynthesis and two enzymes, e.g., TDC and STR, acting consecutively in the conversion of tryptophan to strictosidine, were activated after 6 days of MeJA treatment. Two other key enzymes, e.g., TRP and CYP72A1, acting concurrently upstream of the TIA biosynthesis pathway were upregulated after 6 days. The genes encoding TDC and STR might concurrently act as a master switch of the TIA pathway towards the production of the indole alkaloids. On the other hand, we speculate that the gene encoding PAL enzyme also acts as the master switch of phenylpropanoid biosynthesis and the downstream flavonoid biosynthesis and anthocyanin biosynthesis pathways towards the production of several phenolic compounds. PAL and the downstream enzymes were activated 12 days after treatment. Cluster analysis confirmed the concordant activities of the flower- and silique-specific BHLH25 transcription factor and the key enzyme in the TIA biosynthesis pathway, e.g., STR.

\* Corresponding author.

E-mail addresses: [abmahmed@kau.edu.sa](mailto:abmahmed@kau.edu.sa), [bahieldin55@gmail.com](mailto:bahieldin55@gmail.com) (A. Bahieldin).

Due to the stepwise response of the two sets of pathways, we speculate that enzymes activated earlier likely make TIA biosynthesis pathway a more favourable target in *C. roseus* than anthocyanin biosynthesis pathway.

© 2018 Published by Elsevier Masson SAS on behalf of Académie des sciences.

## 1. Introduction

*Catharanthus roseus* (Madagascar periwinkle) is a perennial herb or ornamental of the Apocynaceae producing over 130 terpenoid indole alkaloids (TIAs) of which the metabolic compounds vinblastine and vincristine are used as anticancer agents [1]. These two compounds are synthesized in the indole alkaloid biosynthesis pathway. *C. roseus* also harbours a large number of phenolic compounds via the phenylpropanoid biosynthesis pathway [2–4] that induce several defence mechanisms against biotic and abiotic stresses [5,6].

Large efforts have been made for improving the biosynthesis of the two anticancer compounds of *Catharanthus roseus* (L.) G. Don. However, these efforts are yet to be potentially successful at the commercial-scale in reducing the cost of the therapy. Therefore, it is a demand to detect the mechanisms of metabolic regulation by which the indole alkaloids biosynthesis pathway takes the priority over other competing mechanisms. Eliciting core enzymes in the TIA pathway and acquiring more information on this obscure pathway likely raise the chance to enhance the production of several other valuable compounds. Alkaloids in the pathway such as ajmalicine and serpentine are commercially used against hypertension [1] and circulatory disorders [7]. The two bisindoles vinblastine and vincristine are derived from coupling vindoline and catharanthine in the TIA pathway [8]. The pathway involves three main stages towards the biosynthesis of the two bisindole alkaloids. The first is the formation of tryptamine and secologanin from the shikimate and monoterpenoid biosynthesis pathways, respectively. The first compound is driven by tryptophan synthase, while the second is driven by secologanin synthase. The second stage includes the core reaction in the TIA pathway, which is the formation of monomeric alkaloid strictosidine that is eventually converted to either vindoline or catharanthine [9]. Formation of strictosidine requires the action of strictosidine synthase [10]. Towards the formation of vindoline from tabersonine, six steps are required, of which five were deciphered up to date. They involve the enzymes tabersonine 16-hydroxylase, *O*-methyltransferase, *N*-methyltransferase, desacetoxylvindoline-4-hydroxylase and deacetylvindoline-4-*O*-acetyltransferase [11]. The third stage is the formation of the two bisindole alkaloids after the eventual condensation of catharanthine and vindoline [12,13]. Compound produced instantly from the condensation is  $\alpha$ -3',4'-anhydrovinblastine, which is converted into vinblastine that is further converted into vincristine. The vacuolar class-III peroxidase acts directly upstream the step of biosynthesis of the two bisindoles in *Catharanthus roseus* [8].

Methyl jasmonate (MeJA) was reported to elicit secondary metabolites in plant, such as alkaloids and phenolic compounds, to regulate plant growth and adaptation [5,14–17]. Rijhwani and Shanks [18] indicated that MeJA increases the yield of alkaloids of the TIA pathway in *C. roseus* hairy root cultures, while Cocetta et al. [16] indicated that MeJA modulates phenolic metabolism in blueberry. More recently, Liu et al. [19] studied the combinational effects of ethylene and MeJA on indole alkaloid and phenolic compounds profiles in *C. roseus* and provided new insights on metabolisms governing the production of the two types of compounds in leaves of *C. roseus*. Jasmonic acid (JA) was also reported to play a major role in the biosynthesis of toxic secondary metabolites [19] and as an elicitor towards enhancing several steps in the indole alkaloid pathway [20].

In the present study, we aim to detect the effects of MeJA (6  $\mu$ M) treatment across time, e.g., 2, 6, 12 and 24 days, in a trial to detect the stepwise response of MeJA-induced genes and pathways in leaves of *C. roseus*.

## 2. Methods

### 2.1. Materials

Seeds of *C. roseus* were left to germinate in the greenhouse of King Abdulaziz University, Jeddah, KSA complying with the institutional, national and international guidelines. No ethics approval was required for any aspect of this study. Plantlets were grown in pots containing sterilized soil: vermiculate at 1:1 and watered with sterile ddH<sub>2</sub>O for 4 weeks. Seedlings were grown at 25 °C with 16-h light periods,  $\sim 125 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  light intensity and  $\sim 70\%$  humidity. Evaporated water was replaced daily. Then, 4-week-old seedlings were watered with MeJA (6  $\mu$ M) and leaf samples were collected after 0, 6, 12, and 24 days. Concurrently, control seedlings were watered with the solvent DMSO at equivalent volume. Leaf samples of the two growth conditions were collected in two replicates at the different time points.

### 2.2. RNA-Seq analysis

Flash-frozen similar-sized leaf samples were crushed into a fine powder in microcentrifuge tubes and total RNAs were extracted, quantified and checked for presence of DNA contamination as previously described [21]. Then, RNAs were shipped to BGI, China, for deep sequencing and raw data were submitted to the National Centre for Biotechnology Information (NCBI) (submission ID: SUB2671065, BioProject ID: PRJNA386850). Filtered reads were *de novo* assembled and quantified as previously

described [22]. Raw reads were mapped to the recovered *de novo* assembled contigs (transcripts). Differentially expressed (DE) transcripts were annotated and KEGG pathway analyses were performed using blast-2-GO software (version 2.3.5, <http://www.blast2go.com/>) and GO terms were obtained with the default parameters. Clusters with functional enrichment were identified via significant Pearson correlation and permutation analysis [23]. Subsequent clustering was done as previously described [22]. RPKM-derived read counts ( $\text{RPKM} \geq 2$ ) were compared using a likelihood ratio test [24]. To determine DE transcripts,  $\geq 2$ -fold change in expression and false discovery rate (FDR) of  $\leq 10^{-3}$  was used. Resulted data were subjected to hierarchical clustering using the Pearson correlation as the distance metric.

### 2.3. Semi-quantitative RT-PCR

RNA-Seq data was further validated via semi-quantitative RT-PCR for transcripts upregulated at 6-day-time point. The *actin* gene of *C. roseus* was used as the house-keeping gene. Primers (Table S1) were designed using the Netprimer software (<http://www.premierbiosoft.com/netprimer/index.html>) with the criteria previously described [25].

## 3. Results and discussion

### 3.1. Statistics of the sequencing datasets

Sequencing of RNA samples of *C. roseus* leaves under the two growth conditions across time yielded a total of ~255 million reads corresponding to an average of ~21 billion nucleotides of cDNA per sample (Table 1). Around 72% of the total reads mapped to the *de novo* assembled contigs, ~60% of the total reads showed perfect matches, while ~12% showed mismatches. Around 45 and 27% of the total reads showed unique and multi-position matches, respectively. Around 28% of the total reads unmapped to the *de novo* assembled contigs. This indicates that the unmapped reads were not incorporated in the originally *de novo* assembled contigs.

### 3.2. Cluster analysis of DE transcripts due to MeJA treatment

Globally, a number of 9456 DE transcripts was recovered (Table S2) and distributed in 294 expression profiles or clusters (Fig. S1). Hierarchical cluster analysis of gene expression under either condition across different time points of MeJA treatment indicated the high quality of sampling and RNA-Seq analysis as replicates of each treatment at a given time point were clustered together (Fig. 1). Two-dimensional hierarchical clustering classified DE profiles according to the similarity of their expression profiles of which three (Nos. 13, 142 and 216) and one (No. 165) clusters fitted within the required expression profiles of upregulation after 6 and 12 days of MeJA treatment, respectively (Fig. 2 and Table S2). There is only one cluster (No. 12) that showed upregulation after both 12 and 24 days of MeJA treatment and one cluster (No. 79) for

upregulation after 24 days of MeJA treatment (Table S2 and Fig. S1). A number of 11 clusters (No. 5, 26, 33, 62, 67, 71, 121, 130, 154, 182, and 284) showed upregulation after MeJA treatment across all time points. Semi-quantitative RT-PCR (sqRT-PCR) of six upregulated transcripts at the TIA pathway was performed to validate the RNA-Seq data (Fig. S2). The analysis of sqRT-PCR indicated that the selected genes were upregulated only after 6 days of MeJA treatment supporting the results of RNA-Seq analysis.

The most relevant upregulated genes in terms of their involvement in the TIA and upstream pathways as well as in those related to phenolic compound biosynthesis were further analysed. The regulated pathways after 6 days of MeJA treatment are “indole alkaloid biosynthesis or TIA”, “phenylalanine, tyrosine and tryptophan biosynthesis or PTT” and “monoterpenoid biosynthesis or MT” (Fig. 3 and S3–S4, respectively). While, the major regulated pathways after 12 days of MeJA treatment are “phenylpropanoid biosynthesis or PP”, “anthocyanin biosynthesis or AC” and “ubiquinone and other terpenoid-quinone biosynthesis or UTQ” (Figs. S5–S7, respectively).

Six enzymes encoded by genes in the TIA pathway were activated after 6 days of MeJA treatment (Fig. 3 and Table S2). Three of these genes encode tabersonine 16-hydroxylase (EC: 1.14.13.73, T16H, clusters 13 and 216), tabersonine 16-O-methyltransferase (EC: 2.1.1.94, OMT, cluster 216) and deacetoxyvindoline-4-hydroxylase (EC: 1.14.11.20, D4H, clusters 13 and 216) towards the biosynthesis of vindoline. Two other genes encode enzymes in two consecutive steps in the TIA pathway. These enzymes are tryptophan decarboxylase (EC: 4.1.1.28, TDC, cluster 216) and strictosidine synthase (EC: 4.3.3.2, STR, cluster 142) that altogether contribute to the conversion of tryptophan to strictosidine (Fig. 3). Otherwise, this amino acid likely act as a substrate for two other pathways (Fig. 4). The first is “tryptophan metabolism” pathway of which tryptophan is depleted towards the production of indole acetic acid, which in turn contribute to plant growth and hormone signal transduction pathway. The other pathway is “staurosporine biosynthesis” towards the production of several valuable drugs. Several routes in the TIA pathway can be followed due to the action of the core gene *TDC* such as biosyntheses of catharanthine, ajmalicine, serpentine and/or ajmaline. Interestingly, the sixth regulated gene, encoding polynneuridine-aldehyde esterase (EC: 3.1.1.78, PNAE, cluster 13), acts in other route in the TIA pathway towards the possible production of ajmaline, raucaffricine, and/or sarpagine.

Genes encoding two other enzymes acting upstream of the six enzymes of the TIA pathway were also regulated after 6 h of MeJA treatment. They are tryptophan synthase (EC: 4.2.1.20, TRP, clusters 13 and 142) and scologanin synthase (EC: 1.3.3.9, CYP72A1, cluster 13) in the PTT (Fig. S3) and MT (Fig. S4) pathways, respectively. There are several other enzymes in the MT pathway that were activated due to MeJA treatment but none of them contributes in the promotion of TIA pathway.

The other three pathways are PP (Fig. S5), AC (Fig. S6) and UTQ (Fig. S7). The first two pathways mostly contribute to the biosynthesis of phenolic compounds,

**Table 1**  
Statistics RNA-Seq numerical data analysis of *C. roseus* leaves.

Sample ID	Total No. reads <sup>a</sup>	No. mapped reads <sup>b</sup>	(%) <sup>c</sup>	No. reads with perfect match (%) <sup>d</sup>	No. reads with mismatches (%) <sup>e</sup>	No. of reads with unique matches (%) <sup>f</sup>	No. reads with multi-position matches (%) <sup>g</sup>	No. unmapped reads (%) <sup>h</sup>
C-W (1)	24,122,605	17,469,363	72.42	14,696,064 (60.9)	2,773,299 (11.5)	10,330,698 (42.8)	7,138,665 (29.5)	6,653,241 (27.6)
C-W (2)	24,120,444	17,589,969	72.93	14,481,605 (60.0)	3,108,364 (12.8)	10,361,874 (43.0)	7,228,095 (29.9)	6,530,474 (27.1)
6D-W	24,121,216	18,088,051	74.99	15,201,880 (63.0)	2,886,171 (11.9)	11,299,801 (46.8)	6,788,250 (28.1)	6,033,164 (25.0)
12D-W (1)	15,023,698	10,996,216	73.19	9,176,725 (61.08)	1,819,491 (12.1)	6,893,581 (45.9)	4,102,635 (27.3)	4,027,481 (26.8)
12D-W (2)	24,117,952	17,799,779	73.80	14,772,072 (61.2)	3,027,707 (12.5)	11,246,111 (46.6)	6,553,668 (27.2)	6,318,172 (26.2)
24D-W (1)	24,110,564	16,642,629	69.03	13,733,007 (57.0)	2,909,622 (12.0)	10,224,138 (42.4)	6,418,491 (26.6)	7,467,934 (31.0)
24D-W (2)	24,120,004	16,917,271	70.14	13,990,560 (58.0)	2,926,711 (12.1)	10,449,089 (43.3)	6,468,182 (26.8)	7,202,732 (29.9)
6D-MJ	23,230,225	16,690,402	71.85	14,099,088 (60.7)	2,591,314 (11.1)	10,020,620 (43.1)	6,669,782 (28.7)	6,539,822 (28.2)
12D-MJ (1)	24,117,745	17,822,229	73.90	14,653,856 (60.8)	3,168,373 (13.1)	11,409,242 (47.3)	6,412,987 (26.5)	6,295,515 (26.1)
12D-MJ (2)	24,121,278	17,797,991	73.79	14,979,278 (62.1)	2,818,713 (11.7)	11,438,173 (47.4)	6,359,818 (26.3)	6,323,286 (26.2)
24D-MJ (1)	24,076,941	17,238,193	71.60	14,452,921 (60.0)	2,785,272 (11.5)	10,690,263 (44.4)	6,547,930 (27.2)	6,838,747 (28.4)
24D-MJ (2)	24,040,120	16,990,589	70.68	14,249,950 (59.2)	2,740,639 (11.4)	10,459,786 (43.5)	6,530,803 (27.1)	7,049,530 (29.3)

C-W (1,2) (control), 6D-W (watered for 6 days), 12D-W (1,2) (watered for 12 days), 24D-W (watered for 24 days), 6D-MJ (MeJA treated at 6  $\mu$ M for 6 days), 12D-MJ (1,2) (MeJA treated at 6  $\mu$ M for 12 days) and 24D-MJ (MeJA treated at 6  $\mu$ M for 24 days).

<sup>a</sup> Total number of reads recovered from RNA-Seq.

<sup>b</sup> Number of mapped reads to the de novo assembled contigs.

<sup>c</sup> Percentage of mapped reads over total No. of reads.

<sup>d</sup> Number and percentage of reads with perfect match over total No. of reads.

<sup>e</sup> Number and percentage of mismatch reads over total No. of reads.

<sup>f</sup> Number and percentage of reads with unique match over total No. of reads.

<sup>g</sup> Number and percentage of reads with multi-position match over total No. of reads.

<sup>h</sup> Number and percentage of reads unmapped to the assembled contigs over total No. of reads.

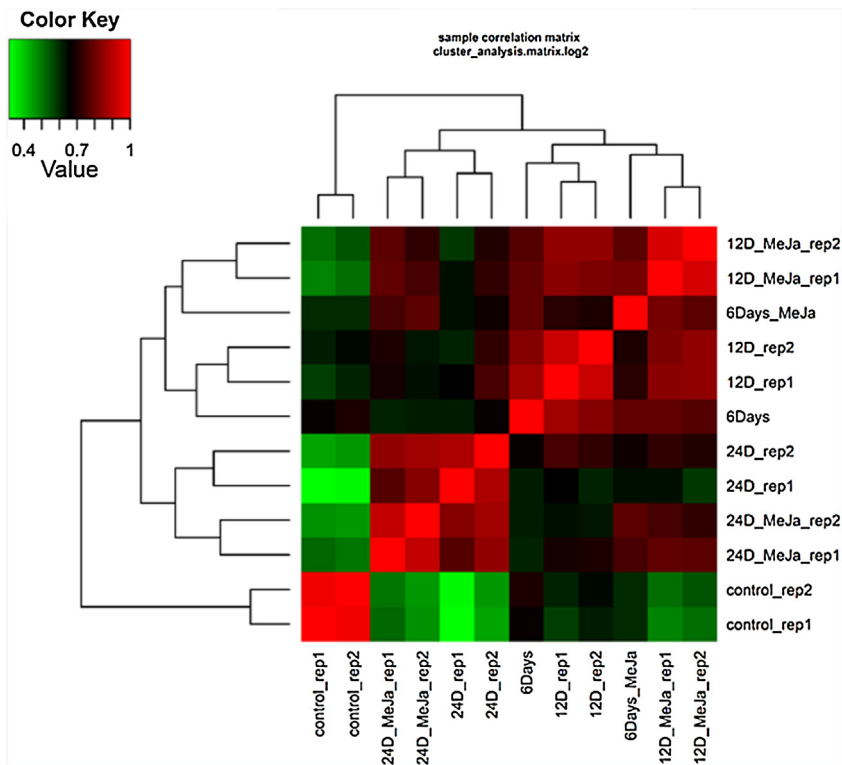


Fig. 1. Hierarchical cluster analysis of gene expression based on log ratio RPKM data for *C. roseus* leaf transcriptome under water and MeJA (6 uM) treatment for 0, 6, 12 and 24 days. Rate of gene expression is referred to by a 0 (green) to 1 (red) scale. C.

while the third is not normally activated in plant. A pathway that connect PP and AC pathways is the flavonoid biosynthesis (FN) pathway. Interestingly, the FN pathway is not regulated under MeJA treatment, but it looks that this negative response made no barrier to the flow of information from PP to AC pathways. The main concern towards the activation of enzymes in the pathways contributing to the phenolic compound biosynthesis is that they might compete with enzymes in the TIA, PTT and MT pathways that contribute to the indole alkaloid biosynthesis. This concern is high in case the enzymes of the two sets of crosstalking pathways are concurrently activated under MeJA treatment.

The PP pathway that starts with the conversion of phenylalanine to cinnamic acid by phenylalanine ammonia lyase (EC: 4.3.1.24, PAL, cluster 165) and the downstream AC pathway (Fig. S6) that starts with the conversion of pelargonidin to several phenolic compounds by anthocyanidin 3-*O*-glucosyltransferase (EC: 2.4.1.115, A3GT, cluster 165) compete with the TIA pathway for the precursor chorismate [4]. The latter enzyme was triggered in the present study by MeJA treatment at all time points (Fig. S1 and Table S2). In addition, the activation of chorismate mutase (EC: 5.4.99.5, CM, cluster 165) of the PTT pathway (Fig. S8) and isochorismate synthase (EC: 5.4.4.2, ICS, cluster 165) of the UTQ pathway (Fig. S7) after 12 h of MeJA treatment promote the latter two pathways. No explanation for the latter pathway being activated as

this is unlikely in plant. However, we speculate that earlier activation (e.g., 6 h after MeJA treatment) of tryptophan synthase (cluster 13, Fig. S1) likely makes TIA pathway a more favourable target as this enzyme as well as secologanin synthase might act as a master switch towards the promotion of the TIA pathway.

Anthocyanins are synthesized due to the action of the gene encoding anthocyanidin 3-*O*-glucosyltransferase (*a3gt*). The enzyme acts downstream of the FN biosynthesis pathway. The latter pathway occurs downstream the PP biosynthesis pathway. The enzyme that triggers these consecutive reactions is phenylalanine ammonia-lyase (PAL) of the PP biosynthesis pathway. Then, we speculate that *PAL* gene acts as the master switch of several gene expression in the leaves treated with MeJA in the PP pathway and the downstream FN, then, AC pathways towards the production of several phenolic compounds. Flow of information starts with triggering expression of the gene (*c4h*) encoding trans-cinnamate 4-monooxygenase (EC: 1.14.13.11, C4H, cluster 165) in the PP biosynthesis pathway and ends with the expression of anthocyanidin 3-*O*-glucosyltransferase (*a3gt*) in the AC biosynthesis pathway. This type of crosstalking of pathways has been argued in earlier reports [4]. MeJA was reported earlier to accelerate fruit ripening in *Fragaria* by increasing soluble solid content and anthocyanin accumulation [26]. This indicates that the PP and AC pathways are favoured at later stages of plant development.

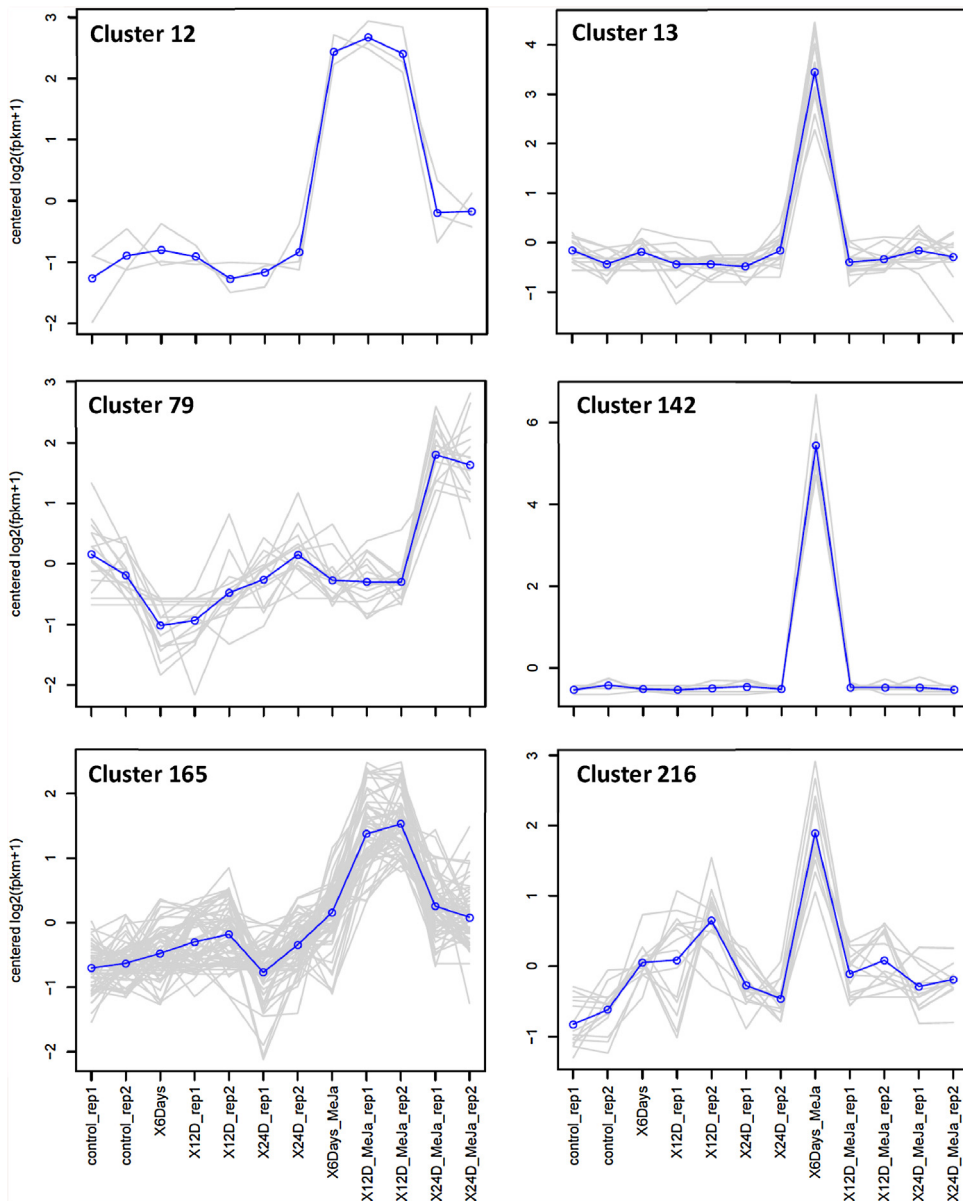
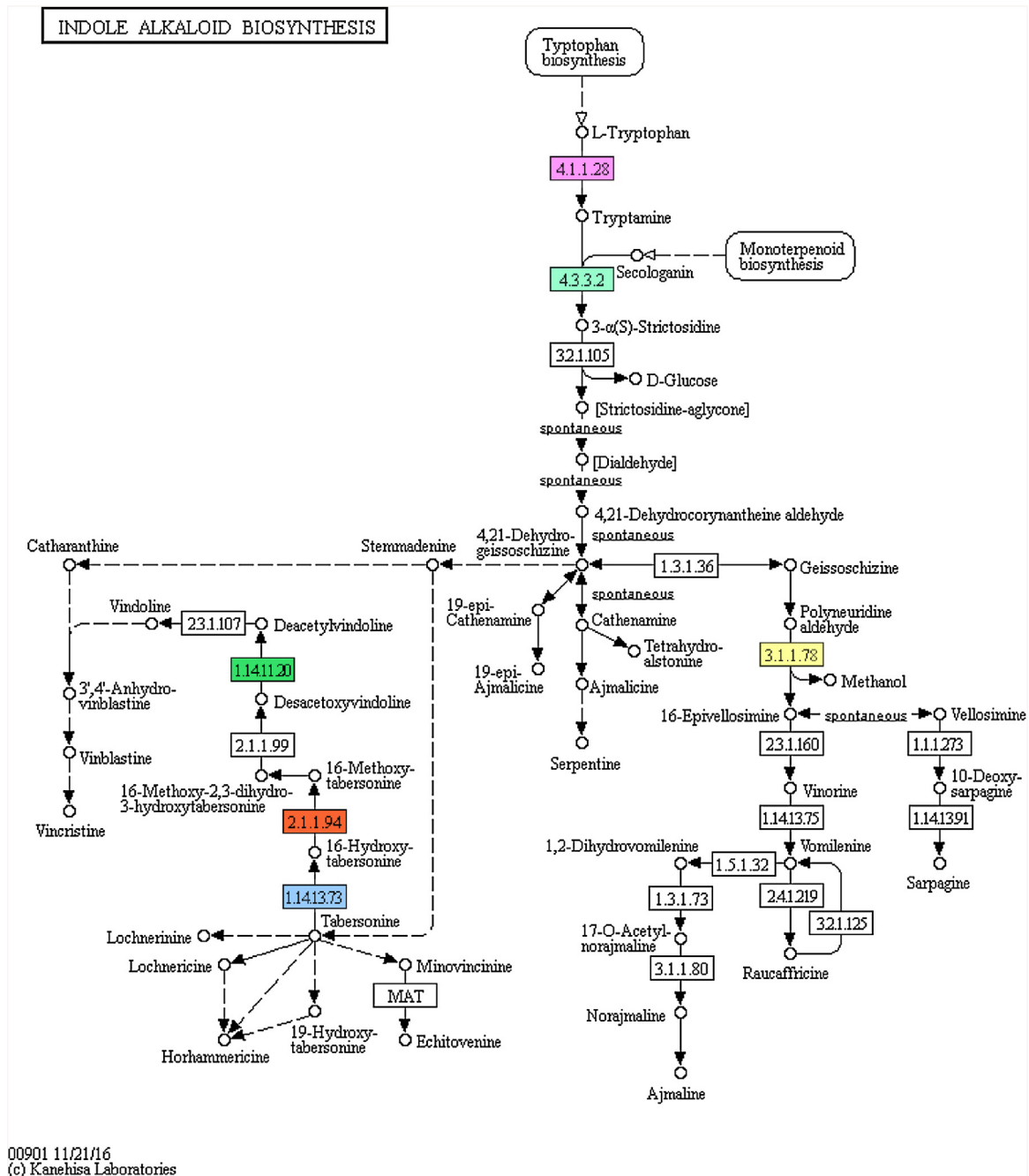


Fig. 2. Selected clusters of upregulated genes of *C. roseus* leaves after 6 (clusters 13, 142 and 216), 12 (cluster 165), 24 (cluster 79) h and both 6 and 12 days (cluster 12) of MeJA (6  $\mu$ M) treatment. Blue lines indicate overall expression pattern across different transcripts of a given cluster.

Light is thought to significantly influence the biosynthesis of vindoline of which three genes, e.g., *tdc*, *d4h* and *dat* were proven to be light-inducible [27]. The first two genes were upregulated in the present study after 6 days of MeJA treatment, while the third was not. The two regulated genes might harbour elements upstream the promoter sequences that positively respond to either light or MeJA signals, or otherwise, light might induce biosynthesis of indigenous MeJA in the plant cells. The latter speculation indicates that light influences the biosynthesis of vindoline indirectly. Interestingly, one light-responsive enzyme namely zeaxanthin epoxidase (EC: 1.14.15.21), in the carotenoid

biosynthesis (or CT) pathway for the production of the photoprotective violaxanthin [28], was activated (Fig. S9) after 12 days of MeJA treatment (cluster 165). Under high light condition, violaxanthin deepoxidates to zeaxanthin to facilitate safe removal of excess excitation energy. Additionally, a gene encoding short-chain alcohol dehydrogenase basically acts in MeJA biosynthesis [29]. This gene is upregulated after 12 days of MeJA treatment (cluster 165). This indicates that MeJA might stimulate the expression of the genes synthesizing it under harsh condition. In turn, this action promotes ABA biosynthesis in plants leading to reduced grain yield.

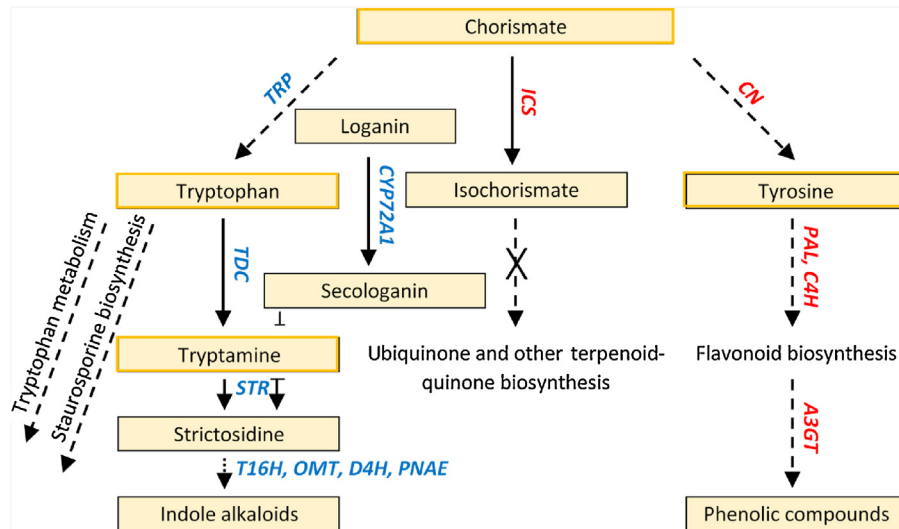


**Fig. 3.** Enzymes in the “indole alkaloid biosynthesis or TIA” pathway in *C. roseus* leaves activated under MeJA (6  $\mu$ M) treatment for 6 days. Activated enzymes are shown in coloured boxes, while the enzymes with unchanged activation rates are shown in uncoloured boxes. Different box colours in the pathway indicates different activated enzymes. EC: 1.14.13.73 = tabersonine 16-hydroxylase or T16H (blue box), EC: 2.1.1.94 = tabersonine 16-O-methyltransferase or OMT (red box), EC: 1.14.11.20 = deacetoxyvindoline-4-hydroxylase or D4H (green box), EC: 4.1.1.28 = tryptophan decarboxylase or TDC (violate box), EC: 4.3.3.2 = strictosidine synthase or STR (light green box), EC: 3.1.1.78 = polyneuridine-aldehyde esterase or PNAE (yellow box).

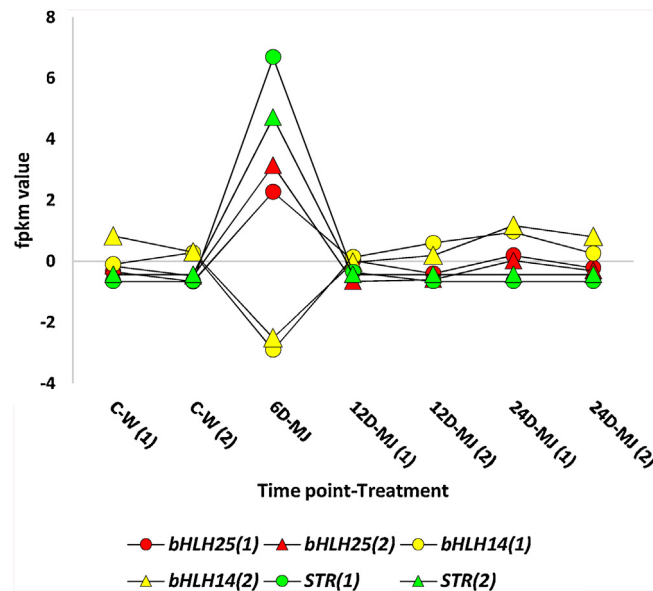
### 3.3. bHLH transcription factors (TFs) triggered by MeJA treatment

The basic helix-loop-helix (bHLH) represents one of the largest families of dimerizing transcription factors [30]. In

the present study, two TFs were regulated after 6 days of MeJA treatment (Fig. 5). Of which, one (e.g., bHLH25) was upregulated (cluster 13), while the other (e.g., bHLH14) was downregulated (cluster 224). Cluster analysis indicated the coexpression of *bHLH25* and *STR* genes (clusters



**Fig. 4.** Activated steps in the pathways of “indole alkaloid biosynthesis”, “phenylalanine, tyrosine and tryptophan biosynthesis”, “monoterpenoid biosynthesis”, “phenylpropanoid biosynthesis”, “anthocyanin biosynthesis” and “ubiquinone and other terpenoid-quinone biosynthesis” due to MeJA treatment. Genes indicated in blue are upregulated after 6 days of treatment, while those indicated in red are upregulated after 12 days of the treatment. Boxes for compounds act upstream master switches are orange-lined. Solid arrows indicate single-step reactions and dashed arrows indicate multi-step reactions. “X” on arrow indicates the unlikely occurrence of this step in the pathway.



**Fig. 5.** Regulation of *STR* (1 & 2), *bHLH25* (1 & 2) and *bHLH14* (1 & 2) transcripts of clusters 142, 13 and 224, respectively, at 6-d time point of MeJA (6 uM) treatment in *C. roseus* leaves. Two versions of transcripts *STR* (green round and rectangle shapes) as well as *bHLH25* (red round and rectangle shapes), on the one hand, and two other versions of *bHLH14* (yellow round and rectangle shapes), on the other hand, were upregulated and downregulated, respectively. Values of fpkm are shown in Table S2. The figure indicates MeJA treatment time points of 0 (C-W), 6, 12 and 24 days, only.

13 and 142, respectively; Fig. 5) in alignment with our previous findings [22] of which knockdown of the TF proved its role in driving *STR* gene via virus-induced gene silencing (VIGS). *bHLH25* is mainly expressed in flower and silique of *Arabidopsis* under MeJA treatment [22,31]. The previous results also indicated coexpression of *t16h* gene with three TFs, two of which harbour the B3 domain, while the third is a *bHLH135*. Although *t16h* gene was

upregulated after 6 days of MeJA treatment, none of the three TFs are regulated under MeJA treatment. This indicates that *t16h* gene might either be triggered by the action of one or more TFs or by MeJA treatment, while the TF genes are not definitely triggered by MeJA treatment. Otherwise, coexpression of the TFs and *t16h* genes is arbitrary. The other *bHLH*, e.g., *bHLH14* is part of the *bHLH* subgroup IIIId TFs acting as transcription repressors of



jasmonic acid (JA) responses, such as the induction of anthocyanin accumulation as well as the block of plant defence against pathogens and insects [32]. This transcription repressor competes with the transcription activators, e.g., MYC2/MYC3/MYC4 and WD-repeat/bHLH/MYB complex, in binding their target sequences. Thus, downregulation of bHLH14 results in the induction of MeJA towards the accumulation of anthocyanin in accordance with our results. The coordinated regulation of JA responses acts as an attenuator of plant defence and development as well as JA-induced leaf senescence [33] that is induced to relocate nutrients and energy from old leaves to newly developing leaves and organs to eventually promote plant growth, reproduction and defence.

In earlier reports, auxins were reported to downregulate the expression of the genes encoding core enzymes in the bisindole alkaloid biosynthesis in *C. roseus* [34] such as *tdc*, while cytokinins (CKs) are proven to upregulate this gene [35]. Interestingly, treatment with exogenous MeJA stimulates the production of CKs, thus might indirectly upregulate *tdc* gene in *C. roseus* [36]. We speculate that the aforementioned stimulation might be triggered by MeJA-induced CK rather than being directly triggered by MeJA. Several genes in the TIA pathway responded differently to MeJA treatment in terms of expression rate and timing of which *d4h* gene followed by *STR* then *TDC* genes showed the strongest response [37,38]. Vázquez-Flota et al. [39] also indicated that MeJA induces the biosynthesis of several alkaloids in the TIA pathway in *C. roseus* such as ajmaline, ajmalicine, vindoline and catharanthine. Other reports indicated that the biosyntheses of catharanthine, vindoline, and vinblastine in *C. roseus* cultured cells were accompanied by upregulation of *tdc*, *t16h*, and *d4h* [40,41]. Based on the results of the present study with regard to the latter three genes, MeJA was proven to contribute to the biosynthesis of the three important indole alkaloids catharanthine, vindoline, and vinblastine of the TIA pathway.

#### 4. Conclusion

The present work aimed at detecting the prolonged effects of MeJA across time (up to 24 days) in order to detect the stepwise response of MeJA-induced genes and pathways in leaves of *C. roseus*. The genes encoding TDC and STR might concurrently act as a master switch of the TIA pathway towards the production of the indole alkaloids. While the gene encoding PAL enzyme might act as the master switch of phenylpropanoid biosynthesis and the downstream flavonoid biosynthesis and anthocyanin biosynthesis pathways towards the production of several phenolic compounds. Concordant activities of the flower- and silique-specific bHLH25 transcription factor and the key enzyme in the TIA biosynthesis pathway namely STR were suggested. We speculate that enzymes activated earlier might make TIA biosynthesis pathway more favourable in *C. roseus* than anthocyanin biosynthesis pathway. Transcriptomic data of the present study can provide resources for future improvement of this important medicinal wild plant via metabolic engineering

towards the overproduction of the two important bisindoles at the commercial scale.

#### Authors' contributions

All authors have read and approved this manuscript. Conceived and designed the experiments: AB AA SE NOG MA AMR MGA FE. Performed the experiments: AA AA MAA SB JSMS. Analyzed the data: AA HSA FME. Wrote the paper: AB SB MGA JSMS MEN FME.

#### Disclosure of interest

The authors declare that they have no competing interest.

#### Acknowledgements

This project was funded by the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, the Kingdom of Saudi Arabia—award No. 12-BIO3064-03. The authors also acknowledge with thanks Science and Technology Unit, King Abdulaziz University, for technical support.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.crvi.2018.10.001>.

#### References

- [1] R. van der Heijden, D.I. Jacobs, W. Snoeijer, D. Hallard, R. Verpoorte, et al., The Catharanthus alkaloids: Pharmacognosy and biotechnology, *Curr. Med. Chem.* 11 (2004) 607–628.
- [2] O. van Tellingen, J.H. Sips, J.H. Beijnen, A. Bult, W.J. Nooijen, Pharmacology, bio-analysis and pharmacokinetics of the vinca alkaloids and semi-synthetic derivatives, *Anticancer Res.* 12 (1992) 1699–1715.
- [3] R. Filippini, R. Caniato, A. Piovano, E.M. Cappelletti, Production of anthocyanins by *Catharanthus roseus*, *Fitoterapia* 74 (2003) 62–67.
- [4] N. Mustafa, R. Verpoorte, Phenolic compounds in *Catharanthus roseus*, *Phytochem. Rev.* 6 (2007) 243–258.
- [5] F. Ferreres, D.M. Pereira, P. Valentao, P.B. Andrade, R.M. Seabra, et al., New phenolic compounds and antioxidant potential of *Catharanthus roseus*, *J. Agric. Food Chem.* 56 (2008) 9967–9974.
- [6] P.V. Demkura, G. Abdala, I.T. Baldwin, C.L. Ballare, Jasmonate-dependent and -independent pathways mediate specific effects of solar ultraviolet B radiation on leaf phenolics and antiherbivore defense, *Plant Physiol.* 152 (2010) 1084–1095.
- [7] M. Garcia-Calderon, P. Pons-Ferrer, A. Mrazova, P. Pal'ove-Balang, M. Vilkova, et al., Modulation of phenolic metabolism under stress conditions in a *Lotus japonicus* mutant lacking plastidic glutamine synthetase, *Front. Plant Sci.* 6 (2015) 760.
- [8] M.M. Costa, F. Hilliou, P. Duarte, L.G. Pereira, I. Almeida, et al., Molecular cloning and characterization of a vacuolar class III peroxidase involved in the metabolism of anticancer alkaloids in *Catharanthus roseus*, *Plant Physiol.* 146 (2008) 403–417.
- [9] H. Rischer, M. Oresic, T. Seppänen-Laakso, M. Katajamaa, F. Lammertyn, et al., Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells, *Proc. Natl. Acad. Sci. USA* 103 (2006) 5614–5619.
- [10] J.F. Treimer, M.H. Zenk, Purification and properties of strictosidine synthase, the key enzyme in indole alkaloid formation, *Eur. J. Biochem.* 101 (1979) 225–233.

- [11] D.K. Liscombe, S.E. O'Connor, A virus-induced gene silencing approach to understanding alkaloid metabolism in *Catharanthus roseus*, *Phytochem.* 72 (2011) 1969–1977.
- [12] M. El-Sayed, R. Verpoorte, *Catharanthus* terpenoid indole alkaloids: Biosynthesis and regulation, *Phytochem. Rev.* 6 (2007) 277–305.
- [13] D. Liu, H. Jin, Y. Chen, L. Cui, W. Ren, et al., Terpenoid indole alkaloids biosynthesis and metabolic engineering in *Catharanthus roseus*, *J. Integr. Plant Biol.* 49 (2007) 961–974.
- [14] M. Vanstraelen, E. Benkova, Hormonal interactions in the regulation of plant development, *Annu. Rev. Cell Dev. Biol.* 28 (2012) 463–487.
- [15] C. Wasternack, B. Hause, Jasmonates: Biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in *Annals of Botany*, *Ann. Bot.* 111 (2013) 1021–1058.
- [16] G. Cocetta, M. Rossoni, C. Gardana, I. Mignani, A. Ferrante, et al., Methyl jasmonate affects phenolic metabolism and gene expression in blueberry (*Vaccinium corymbosum*), *Physiol. Plant.* 153 (2015) 269–283.
- [17] M.J. Pozo, J.A. López-Ráez, C. Azcón-Aguilar, J.M. García-Garrido, Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses, *New Phytol.* 205 (2015) 1431–1436.
- [18] S.K. Rijhwani, J.V. Shanks, Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures, *Biotechnol. Prog.* 14 (1998) 442–449.
- [19] J. Liu, Y. Liu, Y. Wang, Z.-H. Zhang, Y.-G. Zu, et al., The combined effects of ethylene and MeJA on metabolic profiling of phenolic compounds in *Catharanthus roseus* revealed by metabolomics analysis, *Front. Physiol.* 7 (2016) 217.
- [20] G. Montiel, A. Zarei, A.P. Körbes, J. Memelink, The Jasmonate-responsive element from the ORCA3 promoter from *Catharanthus roseus* is active in *Arabidopsis* and is controlled by the transcription factor AtMYC2, *Plant Cell Physiol.* 52 (2011) 578–587.
- [21] N.H. Hajrah, A.Y. Obaid, A. Atef, A.M. Ramadan, D. Arasappan, et al., Transcriptomic analysis of salt stress responsive genes in *Rhazya stricta*, *PLoS ONE* 12 (2017) e0177589.
- [22] F.M. El-Domyati, A. Atef, A.K.M. Al-Ghamdi, T.K. Khan, S. Edris, et al., Identification of organ-specific regulatory frameworks of *C. roseus* with emphasis to the TIA biosynthetic pathway, *Pakistan J. Bot.* 49 (2017) 2243–2254.
- [23] J.A. Brown, G. Sherlock, C.L. Myers, N.M. Burrows, C. Deng, et al., Global analysis of gene function in yeast by quantitative phenotypic profiling, *Mol. Systems Biol.* 2 (2006), 2006.0001.
- [24] J.C. Marioni, C.E. Mason, S.M. Mane, M. Stephens, Y. Gilad, RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays, *Genome Res.* 18 (2008) 1509–1517.
- [25] A. Bahieldin, D.A.M. Alqarni, A. Atef, N.O. Gadalla, M. Al-matary, et al., Suppression of PCD-related genes affects salt tolerance in *Arabidopsis*, *C.R. Biologies* 339 (2016) 105–114.
- [26] C.M. Concha, N.E. Figueroa, L.A. Poblete, F.A. Oñate, W. Schwab, et al., Methyl jasmonate treatment induces changes in fruit ripening by modifying the expression of several ripening genes in *Fragaria chiloensis* fruit, *Plant Physiol. Biochem.* 70 (2013) 433–444.
- [27] Y. Liu, D. Zhao, Y. Zu, Z. Tang, Z. Zhang, et al., Effects of low light on terpenoid indole alkaloid accumulation and related biosynthetic pathway gene expression in leaves of *Catharanthus roseus* seedlings, *Bot. Stud.* 52 (2011) 191–196.
- [28] E.E. Pfündel, M. Renganathan, A.M. Gilmore, H.Y. Yamamoto, R.A. Dille, Intrathylakoid pH in isolated pea chloroplasts as probed by violaxanthin deepoxidation, *Plant Physiol.* 106 (1994) 1647–1658.
- [29] E.H. Kim, Y.S. Kim, S.-H. Park, Y.J. Koo, Y.D. Choi, Methyl jasmonate reduces grain yield by mediating stress signals to alter spikelet development in rice, *Plant Physiol.* 149 (2009) 1751–1760.
- [30] C. Murre, G. Bain, M.A. van Dijk, I. Engel, B.A. Furnari, et al., Structure and function of helix-loop-helix proteins, *Biochim. Biophys. Acta.* 1218 (1995) 129–135.
- [31] M.A. Heim, M. Jakoby, M. Werber, C. Martin, B. Weisshaar, et al., The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity, *Mol. Biol. Evol.* 20 (2003) 735–747.
- [32] S. Song, T. Qi, M. Fan, X. Zhang, H. Gao, et al., The bHLH subgroup IIIId factors negatively regulate jasmonate-mediated plant defense and development, *PLoS Genet.* 9 (2013) e1003653.
- [33] T. Qi, J. Wang, H. Huang, B. Liu, H. Gao, et al., Regulation of jasmonate-induced leaf senescence by antagonism between bHLH subgroup IIIe and IIIId factors in *Arabidopsis*, *The Plant Cell* 27 (2015) 1634–1649.
- [34] G. Pasquali, O.J. Goddijn, A. de Waal, R. Verpoorte, R.A. Schilperoort, et al., Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors, *Plant Mol. Biol.* 18 (1992) 1121–1131.
- [35] N. Papon, J. Bremer, A. Vansiri, F. Andreu, M. Rideau, et al., Cytokinin and ethylene control indole alkaloid production at the level of the MEP/terpenoid pathway in *Catharanthus roseus* suspension cells, *Planta Med.* 71 (2005) 572–574.
- [36] A. Avalbaev, R. Yuldashev, K. Fedorova, K. Somov, L. Vysotskaya, et al., Exogenous methyl jasmonate regulates cytokinin content by modulating cytokinin oxidase activity in wheat seedlings under salinity, *J. Plant Physiol.* 191 (2016) 101–110.
- [37] S. Wei, Methyl jasmonic acid induced expression pattern of terpenoid indole alkaloid pathway genes in *Catharanthus roseus* seedlings, *Plant Growth Regul.* 61 (2010) 243–251.
- [38] S. Goklany, N.F. Rizvi, R.H. Loring, E.J. Cram, C.W. Lee-Parsons, Jasmonate-dependent alkaloid biosynthesis in *Catharanthus roseus* hairy root cultures is correlated with the relative expression of Orca and Zct transcription factors, *Biotechnol. Prog.* 29 (2013) 1367–1376.
- [39] F. Vázquez-Flota, E. Hernández-Domínguez, M. de Lourdes Miranda-Ham, M. Monforte-González, A differential response to chemical elicitors in *Catharanthus roseus* in vitro cultures, *Biotechnol. Lett.* 31 (2009) 591–595.
- [40] W. Wen, J. Zhu, J. Liu, R.M. Yu, Impact of artemisinic acid on the growth and catharanthine production in *Catharanthus roseus* cultured cells, *J. Med. Plants Res.* 6 (2012) 2019–2028.
- [41] J. Liu, J. Zhu, L. Tang, W. Wen, S. Lv, et al., Enhancement of vindoline and vinblastine production in suspension-cultured cells of *Catharanthus roseus* by artemisinic acid elicitation, *World J. Microbiol. Biotechnol.* 30 (2014) 175–180.