Protoplasma
DOI 10.1007/s00709-014-0718-9

ORIGINAL ARTICLE

Gene transcript profiles of the TIA biosynthetic pathway in response to ethylene and copper reveal their interactive role in modulating TIA biosynthesis in *Catharanthus roseus*

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Received: 10 July 2014 / Accepted: 11 October 2014

Abstract Research on transcriptional regulation of terpenoid indole alkaloid (TIA) biosynthesis of the medicinal plant, *Catharanthus roseus*, has largely been focused on gene function and not clustering analysis of multiple genes at the transcript level. Here, more than ten key genes encoding key enzyme of alkaloid synthesis in TIA biosynthetic pathways were chosen to investigate the integrative responses to exogenous elicitor ethylene and copper (Cu) at both transcriptional and metabolic levels. The ethylene-induced gene transcripts in leaves and roots, respectively, were subjected to principal component analysis (PCA) and the results showed the overall expression of TIA pathway genes indicated as the *Q* value followed a standard normal distribution after ethylene treatments. Peak gene expression was at 15–30 μM of ethephon, and the pre-mature leaf had a higher *Q* value than the immature or mature leaf and root. Treatment with elicitor Cu found that Cu up-regulated overall TIA gene expression more in roots than in leaves. The combined effects of Cu and ethephon on TIA gene expression were stronger than their separate effects. It has been documented that TIA gene expression is tightly regulated by the transcriptional factor (TF) ethylene responsive factor (ERF) and mitogen-activated protein kinase (MAPK) cascade. The loading plot combination with correlation analysis for the genes of *C. roseus* showed that expression of the *MPK* gene correlated with strictosidine synthase (*STR*) and strictosidine b-D-glucosidase(*SGD*). In addition, *ERF* expression correlated with expression of secologanin synthase (*SLS*) and tryptophan decarboxylase (*TDC*), specifically in roots, whereas *MPK* and myelocytomatosis oncogene (*MYC*) correlated with *STR* and *SGD* genes. In conclusion, the ERF regulates the upstream pathway genes in response to heavy metal Cu mainly in *C. roseus* roots, while the MPK mainly participates in regulating the *STR* gene in response to ethylene in pre-mature leaf. Interestingly, the change in TIA accumulation does not correlate with expression of the associated genes. Our previous research found significant accumulation of vinblastine in response to high concentration of ethylene and Cu suggesting the involvement of posttranscriptional and posttranslational mechanisms in a spatial and temporal manner. In this study, meta-analysis reveals *ERF* and *MPK* form a positive feedback loop connecting two pathways actively involved in response of TIA pathway genes to ethylene and copper in *C. roseus*.

Keywords *Catharanthus roseus* · Copper · Ethylene · Terpenoid indole alkaloids · Transcript clustering

Abbreviations

AS	Anthranilate synthase
DAT	Acetyl-CoA: 4-*O*-deacetylvindoline4-*O*-acetyltransferase
D4H	Desacetoxyvindoline-4-hydroxylase
ERF	Ethylene responsive factor
G10H	Geraniol 10-hydroxylase
MAPK	Mitogen-activated protein kinase
MeJA	Methyl-jasmonate
MYC	Myelocytomatosis oncogen
ORCA	Octadecanoid-responsive Catharanthus AP2-domain
PCA	Principal component analysis
qRT-PCR	Quantitative real-time reverse transcription
RPS	Ribosomal protein subunit
SGD	Strictosidine b-D-glucosidase

Handling Editor: Peter Nick

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Published online: 26 October 2014

SLS	Secologanin synthase
STR	Strictosidine synthase
TDC	Tryptophan decarboxylase
TF	Transcriptional factor
TIA	Terpenoid indole alkaloid
T16H	Tabersonine-16-hydroxylase

Introduction

Terpenoid indole alkaloids (TIAs) have a high medical value, however, the TIA biosynthetic pathways are found in only a few plant species. *Madagascar periwinkle*, or *Catharanthus roseus*, have been extensively studied due to their economic and pharmaceutical value as a source for useful TIAs, including ajmalicine, catharanthine, serpentine, vindoline, vinblastine, and vincristine (Verma et al. 2013; Pan et al. 2012; Zhou et al. 2011; Favali et al. 2004). Parts of them are efficient inhibitors of microtubule polymerization and used to treat certain cancers. In addition, more than 100 *C. roseus* alkaloids that have been identified share many common biosynthetic steps (Fig. 1). The early stages of alkaloid biosynthesis in *C. roseus* involve the shikimate pathway. Chorismate is converted to anthranilate by anthranilate synthase (AS) and then tryptophan is converted to tryptamine by tryptophan decarboxylase (TDC). Tryptamine is condensed with secologanin derived from 10-hydroxygeraniol (itself formed from geraniol by the action of G10H) in the terpenoid pathway, to produce the intermediate strictosidine, the common precursor for monoterpenoid indole alkaloid synthesis. The conversion of 10-hydroxygeraniol to secaloganin occurs via loganin which is the substrate for secologanin synthase (SLS). Strictosidine is formed by the coupling of tryptamine and secologanin by strictosidine synthase (STR).and then converted to tabersonine by strictosidine β-glucosidase (SGD). Strictosidine is also the common precursor of catharanthine and ajmalicine. After tabersonine is converted into 16-methoxytabersonine by tabersonine-16-hydroxylase (T16H), hydroxylation by deacetoxyvindoline4-hydroxylase (D4H) and acetylation by deacetylvindoline acetyl CoA acetyltransferase (DAT) yield vindoline. Finally, through the activity of peroxidase 1 (PRX1), catharanthine and vindoline together form the MIAs (Suttipanta et al. 2011), which are effective against some forms of cancer.

C. roseus is the sole commercial source of TIAs, producing the anticancer drugs vinblastine and vincristine used to treat a range of human cancers (Wang et al. 2010). However,

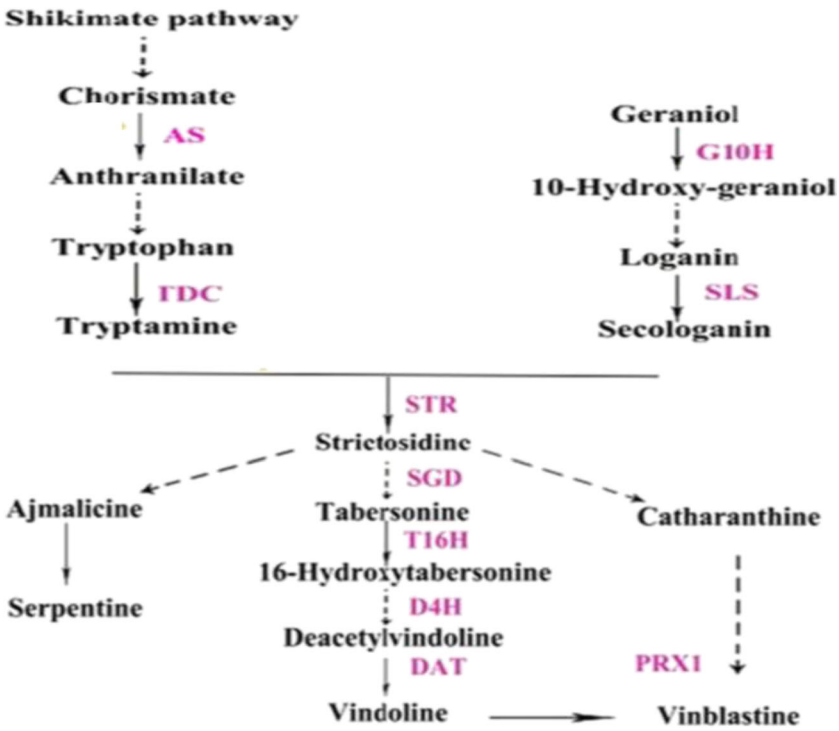

Fig. 1 Biosynthetic pathway for TIAs in *C. roseus*. *Solid arrows* indicate single enzymatic conversions, *dashed arrows* indicate multiple enzymatic conversions. The 10-hydroxygeraniol required for biosynthesis of secologanin is made via the terpenoid pathway and *G10H*, loganic acid is converted to secologanin via secologanin synthase (*SLS*), tryptophan is decarboxylated to tryptamine by tryptophan decarboxylase (*TDC*) and anthranilate synthase (*AS*), strictosidine is formed by the coupling of tryptamine and secologanin by strictosidine synthase (*STR*). Strictosidine β-glucosidase (*SGD*), a common intermediate is converted to catharanthine or to tabersonine. After, tabersonine is converted into 16-methoxytabersonine by tabersonine-16-hydroxylase (*T16H*), hydroxylation (*D4H*), and O-acetylation (*DAT*) to yield vindoline. Finally, through peroxidase 1 (*PRX1*), catharanthine and vindoline together to formation of dimeric anticancer MIAs

extremely low yields have motivated studies to elucidate the molecular mechanisms of TIA pathways. TIAs are synthesized in different organs or tissues at particular developmental stages, and in response to various biotic and abiotic stimuli (Yang et al. 2012). Phytohormones regulate plant growth and stress responses and also affect secondary metabolite production. Controlled transcription of biosynthetic genes is one major mechanism regulating secondary metabolite production in plants by phytohormones (Rischer et al. 2006). For example, auxins negatively influence transcriptional level of the TIA pathway genes. Jasmonate and salicylate act as signaling molecules for gene expression (Reymond and Farmer 1998; Rijhwani and Shanks 1998).

The spatio-temporal transcriptional regulation of metabolic pathways is controlled by a complex network involving many TFs (Patra et al. 2013). Recently, APETALA2/ethylene response factor (AP2/ERF) family proteins have been reported to participate in regulation of secondary metabolism in many plant species (Yang et al. 2012). $ORCA_3$ is a jasmonate-responsive APETALA2 (AP2)-domain TF from *C. roseus* (Li et al. 2013; Peebles et al. 2009; Memelink et al. 2001). *$ORCA_3$* overexpression results in enhanced expression of several TIA biosynthetic genes, resulting in increased accumulation of TIAs (van der Fits and Memelink 2000). Moreover, in tobacco, an ethylene-responsive AP2/ERF protein, Tsi1, is shown to bind the GCC box and DRE/CRT *cis* elements. Overexpression of *Tsi1*, simultaneously, improved resistance to *P. S. tabaci* and increased tolerance to salt stress (Quan et al. 2010; Memelink et al. 2001).

ERF is a member of the AP2 / ERF TF family (Yang et al. 2012; Agarwal et al. 2006; Memelink et al. 2001; van der Fits and Memelink 2000). Numerous studies have demonstrated that ERF is closely related to both biotic and abiotic stress responses, mainly through different signaling pathways, and regulate the plant responses to adverse conditions. Ethylene is a versatile plant hormone that regulates, through ERF, the expression of pathogen-related (*PR*) genes, of which the promoter often contain the GCC box (TAGCCGCC) motif (van Loon et al. 2006; Memelink et al. 2001). Although the ethylene signal transduction pathway in many plant species has been well-studied, its precise role of TIA biosynthesis remains poorly investigated so far. We have demonstrated that an *ERF* gene is involved in the regulation of TIA genes in response to ethylene (Sears et al. 2014).

The mitogen-activated protein kinase (MAPK) cascade is important for production of TIAs under several abiotic stress conditions (Raina et al. 2012). MPK3 activates the TIA pathway genes as well as the *ERF* gene. It has been found that overexpression of *CrMPK3* in *C. roseus* leaves results in up-regulation of the TIA pathway genes and increased specific alkaloid accumulation in response to wounding and MeJA. Future studies with stable transgenic of CrMPK3 and identification of interacting partner of CrMPK3 in the form of the already identified MIA pathway regulators could shed more light on the signal transduction network (Raina et al. 2012).

The heavy metal Cu is an essential micro element for plant growth and development and is actively involved in redox reaction, photosynthesis, and nitrogen fixation (Ouzounidou 1995; Lolkema and Vooijs 1986; Clijsters and Assche 1985). Because of its function in enzymes, this element serves as catalysts of oxygen chemistry and redox reactions (Zulfiqar and Shakoori 2012). Furthermore, Cu may interfere with the biosynthesis of chlorophyll or cause lipid peroxidation processes in the photosynthetic membranes. Our previous work found that Cu and ethephon combination treatment significantly enhanced gene expression and alkaloid accumulation.

In the process of scientific research, there are a lot of factors influencing the characteristics and the law of development, which need to make comprehensive analysis and evaluation. Principal component analysis (PCA) is based on the idea of dimensional reduction and it is a method of multivariate statistical analysis which reduces multiple related variables to a handful of mutually independent variables to reflect most of the information about the original data. It has obvious advantages on dealing with many variables, large sample statistical problems, and it is popular with the academic scholars widely (Wang et al. 2013). In this study, we selected the two factors, Cu and ethephon, to stress *C. roseus*, and used qRT-PCR followed by PCA to obtain comprehensive profiling of the expression of several TIA pathway genes in response to these factors. Based on our findings, we propose a model for the effects of ERF and MPK in transcriptional regulation of the TIA pathway genes in response to Cu and ethephon in *C. roseus*. This study provides a foundation for a better understanding of the regulation and accumulation of major TIAs.

Materials and methods

Plant materials and growth conditions

C. roseus plants were grown in a greenhouse (ZPW-400, China), under a 12/12 light/dark photoperiod at a temperature of 28 (day) / 25 °C (night) and 80 % relative humidity as described previously (Chang et al. 2013). *C. roseus* seeds were planted in pots using perlite as culture substrate and kept moistened until the seeds had germinated, and then irrigated with 1/2 strength Hoagland's solution. Young leaves from the 3-month-old were harvested, three fully expanded leaves of seedlings were randomly selected and subjected to hydroponic treatment for 4, 24 h.

Chemical treatments

Ethephon (ETH) was used to release ethylene, and $CuSO_4$ was used as an inhibitor of ethylene biosynthesis. We treated

3-month-old *C. roseus* seedlings with five concentrations of ethephon (15, 30, 45, 60, 90 μM), and one concentration of $CuSO_4$ (300 μM), $CuSO_4$ and ethephon were dissolved in Hoagland's solution, to study their effects on TIA accumulation. When three pairs of leaves had appeared, at the top of the young leaves called immature leaves, the middle part of leaves called pre-mature leaves, and the base of mature leaves called mature leaves. For this study, RNA was isolated from immature leaves, pre-mature leaves, mature leaves and roots, respectively.

Individual plants were harvested for morphological and physiological determinations 1 day after the treatments were applied. For the biomass measurements, three plants from each treatment were harvested after washing the sand from the roots. Morphological measurements, height, and root length were also measured (Table 4).

Determination of alkaloid contents

Fresh plant tissues were freeze-dried (1.5 g) in 20 mL absolute methanol (analytical grade) for extraction of serpentine, vinblastine, catharanthine, loganin, tabersonine, and vindoline. Low-frequency ultrasonication (250 W, 40 kHz) for 30 min was used to extract the alkaloids. The methanol extract was centrifuged at 8000 rpm for 10 min, concentrated to 1 mL, and the amount of alkaloids were determined by reversed phase HPLC using COSMOSIL Packed Column C18 (5 μm, 4.6× 250 mm) in an oven at 30 °C,with CH_3CN / H_2O and 0.05 moL/L ammonium acetate standard solvent system, respectively. Absorption at 220, 240, 290, 220, 220, and 220 nm was monitored. Retention times of the three alkaloids were serpentine (4.95 min), tabersonine (6.19 min), vindoline (6.91 min), vinblastine (7.40 min), catharanthine (8.60 min), and loganin (10.11 min). Sample injection volume was 10 μL at a flow rate of 1 mL / min.

RNA isolation and quantitative real time PCR analysis

After a 4-h treatment, seeds were collected and ground in liquid nitrogen. Total RNA was extracted by TRIZOL reagent (Invitrogen). DNA contamination was removed using Dnase I following the instructions provided by the manufacturer (TaKaRa, Japan). DNA and RNA purity were observed using 1 % agarose gel electrophoresis and RNA concentration was determined using a Nanodrap spectrophotometer (Thermo). cDNA was synthesized from total RNA (2 μg) using ReverTra Ace QPCR RT Kit (TOYOBO, Japan) according to the manufacturer's instructions, using oligo (dT) as the primer. qRT-PCR analysis using cDNA as template and gene-specific primers was performed using a SYBR Premix Ex Taq (TaKaRa, Japan) with an initial denaturation at 95 C for 30 s, followed by 35 cycles at 94 C for 30 s, 56 C for 30 s, and 72 C for 30 s. These were repeated three times for each sample to ensure the reproducibility of results. Gene-specific primers used are listed in Table 1 (from ExPlant Technologies B.V.). Ribosomal protein subunit 9 (*Rsp9*) was used as an internal control to evaluate all *C. roseus* plants. The relative expression value was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

PCA, the multivariate analyze tool, is used to reduce a set of original variables and to extract a small number of latent factors (principal components [PCs]) for analyzing relationships among the observed variables. PCA was performed in order to investigate the distribution pattern of TIA pathway gene expression quantity in response to ethephon and Cu in *C.roseus*. Variations in the composition of the TIA pathway gene expression were evaluated by PCA using SPSS version 17.0. Generally, there are three approaches used: Catell scree test, Kaiser Criterion and variance explained criteria. We used the scree plot method to assess the number of PCs to be retained. Loading plot, the two-dimensional plane formed by the two first principal components is the most informative in PCA. The loading plots display the relationships among the detected compounds. The loading of PC1 and PC2 against

Table 1 Primers for qRT-PCR analyses of *C. roseus* reference and TIA pathway genes

C. roseus gene	Primer sequences	Tm, °C
TDC	ATCCGATCAAACCCATACCA CGTCATCCTCGACCATTTTT	50
G10H	TTATTCGGATTCTGCCAAGG TCCCCAAAGTGAATCGTCAT	50
SLS	GTTCCTTCTCACCGGAGTTG CCCATTTGGTCAACATGTCA	58
STR	ACCATTGTGTGGGAGGACAT ATTTGAATGGCACTCCTTGC	56
SGD	GGAGGCTTCTTGAGTGATCG GCAAATTCACCAGTGGCATA	50
D4H	GACTTGAACTTTCATGCTGCTACAC TCTCATCAAAAGCCTTCAATTCC	58
DAT	AATCCCTCAGCCGCTATAACC ACGGATACGCACGTTTGGTAT	58
T16H	GCTTCATCCACCAGTTCCAT CCGGACATATCCTTCTTCCA	58
RPS	GAGGGCCAAAACAAACTTGA CCCTTATGTGCCTTTGCCTA	58
AS	GCGAACATTTGCAGATCCAT GGCCGATTTGTTATTGTTCC	58
MPK3	ACGAAATGAGGATGCAAAAAGATAC TGCTAACTGCTGACGAGGGAAT	57.5
MYC2	TTTGGCAGTCGTCTGTTGTC TCGGTATCGGTCACCTCTTC	57.5
ERF	ATCCGAGAGAAAACAAACCGA CATATTGCCACCTTAAAACCA	58

each other shows the summary of the relationship among variables. In addition, the principal component scores could explain how much the two components can explain the variation, while the *Q* value is a little different. That is using the variance contribution rates of the principal component of weights and calculated under various processing, composite scores of gene expression. The score of principal component "Q" is an indicator of a comprehensive analysis, scientific evaluation of objective phenomenon, which has no practical significance, and negative just means below the average (Thavamani et al. 2012).

$$F1 = a_{11}X_{11} + a_{21}X_{21} + \ldots\ldots + a_{p1}X_p$$
$$F2 = a_{12}X_{12} + a_{22}X_{22} + \ldots\ldots + a_{p2}X_p$$
$$\ldots\ldots$$
$$Fp = a_{1m}X_{11} + a_{2m}X_{22} + \ldots\ldots + a_{pm}X_p$$
$$Q = \left(\lambda_1 F1 + \lambda_2 F2 + \ldots\ldots + \lambda_p Fp\right) \Big/ \left(\lambda_1 + \lambda_2 + \ldots\ldots + \lambda_p\right)$$

F_p is the value of the principal components. $a_{1i}a_{2i,}\ldots$, a_{PI} (I=1,…, m) is corresponding eigenvectors of the eigenvalues of the X covariance matrix Σ, $X1$, $X2$,…Xp is the value of the original variables through standardized processing because in practice, indicators of dimension are often different, so the calculation must be to eliminate the influence of this to standardize original data.

Results

TIA pathway gene expression in response to ethephon treatment

PCA '*Q*' value and loading plot were determined based on the expression data of TIA pathway genes and the *ERF* gene (Fig. 2). The result revealed the overall expression of TIA pathway genes in *C. roseus* followed a stable normal distribution after ethylene treatments [Fig. 2(A)]. Low concentration of ethephon significantly promoted gene expression in roots. Increasing concentrations of ethephon, and up to 30 μM ethephon concentration, resulted in enhanced gene expression that peaked in leaves. On the contrary, root gene transcripts were suppressed. As the concentration of ethephon increased, there were fewer organ differences, and gene expression in the leaf and root tended to be consistent. Therefore, it appears TIA pathway genes respond specifically to a certain concentration range of ethephon in leaves.

As indicated by loading plot [Fig. 2(B)], *C. roseus* TIA pathway genes were divided into upstream pathway genes (*SLS*, *TDC* and *STR*) and downstream pathway genes (*D4H*, *DAT* and *SGD*). *ERF* presented a significant level of activity in TIA pathway gene expression during ethephon treatments. In

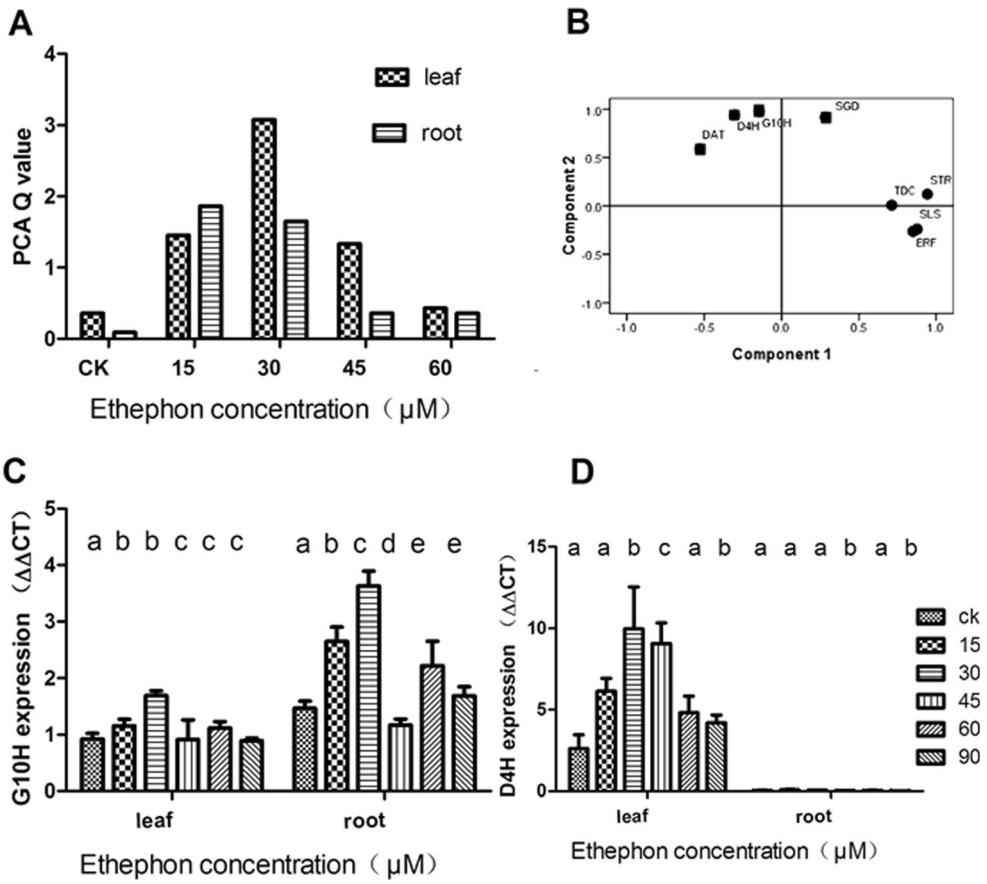

Fig. 2 Relative expression levels by qPCR of selected TIA pathway genes following ethylene application in *C. roseus* leaf and root. The results were analyzed using the comparative Ct method (The *Rps9* gene was used as an internal control) followed by PCA. (*A*) Score plot of PCA; (*B*) loading plot of PCA. The first principal component (PC1) was the expression of genes *TDC*, *SLS*, *STR* and *ERF*, which were shown as *solid circles*. The second principal component (PC2) was the expression of genes *G10H*, *SGD*, *D4H*, and *DAT*, which were shown as *solid squares*. Data presented here are the mean of three replicates. (C) Relative quantification expression of G10H in leaf and root between treatment and control in respond to ET concentration. (*D*) Relative quantification expression of D4H in leaf and root between treatment and control in respond to ET concentration

addition, expression of the upstream gene, *G10H*, was significantly related to expression of vindoline pathway genes, *D4H* and *DAT*. In addition, *G10H*, was specifically expressed in roots [Fig. 2(C)]. *D4H* expression was extremely low in roots but at least 10-fold higher in leaves [Fig. 2(D)].

TIA pathway genes expression in response to Cu and ethephon combination treatment in different development stages

Compared to the control, no major changes in transcript levels of TIA pathway genes in *C. roseus* leaves were observed when treated with ethephon alone [Fig. 3(C)]. However, gene expression in roots decreased markedly following ethephon treatment. On the contrary, treatment with Cu alone specifically enhanced gene expression in roots compared to that in leaves [Fig. 3(C)]. Treatment with ethephon and Cu together, increased TIA pathway gene expression about 3-fold in both leaves and roots of *C. roseus* seedlings, compared with the control. Treatment with Cu alone induced gene expression in roots, about 7-fold of that in the control group [Fig. 3(B)]. In contrast, as the leaf matured, TIA gene expression in leaves was gradually suppressed. Ethephon alone

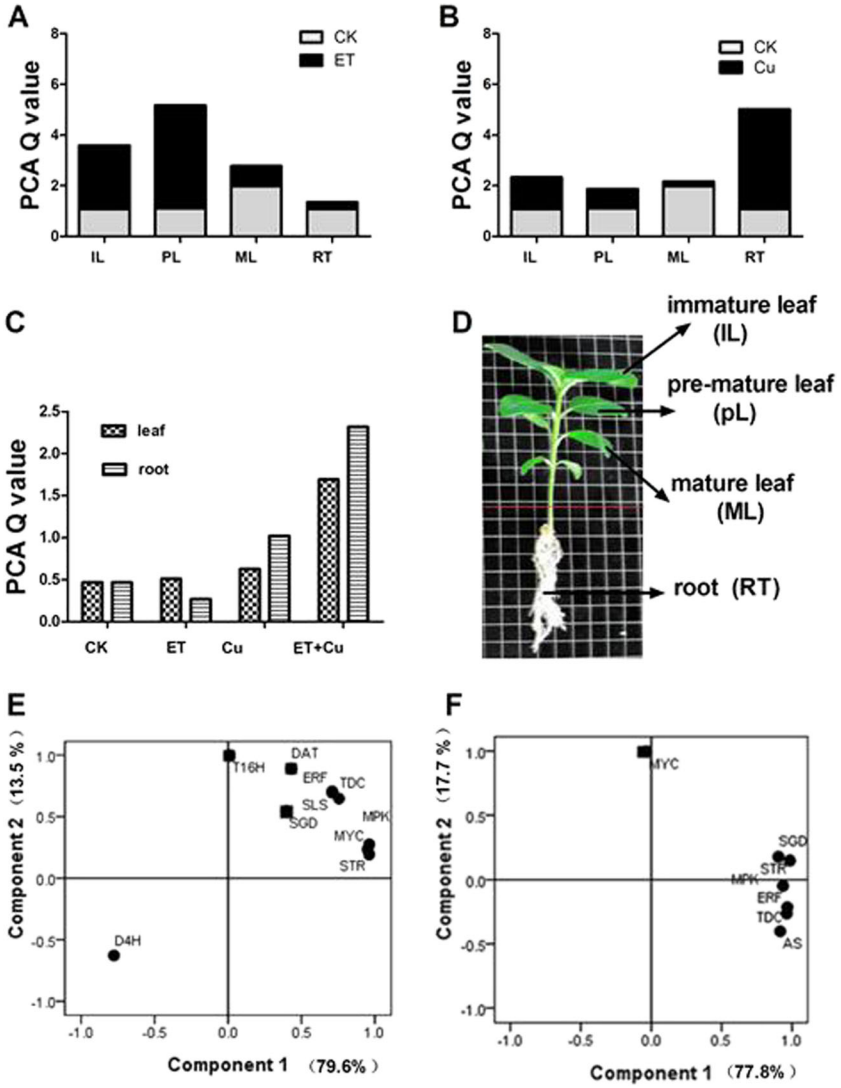

Fig. 3 Relative expression levels of selected TIA pathway genes following ethylene and Cu application. The experiments were measured by qPCR. The results were analyzed using the comparative Ct method (The *Rps9* gene was used as an internal control) followed by PCA. (*A*) Score plot of PCA of the ethylene in *C. roseus* different developmental lever (immature leaf, pre-mature leaf, mature leaf, root). (*B*) Score plot of PCA of the Cu in *C. roseus* different developmental stages. (*C*) Score plot of PCA in different tissues. (*D*) Three-month-old *C. roseus* without treatment. (*E*) Loading plot of PCA of the *C. roseus* leaves. The first principal component (PC1) was the expression of genes *TDC*, *SLS*, *STR*, *MPK*, *ERF*, *MYC*, and *D4H*, which were shown as *solid circles*. The second principal component (PC2) was the expression of genes *SGD*, *DAT and T16H*, which were shown as *solid squares*. (*F*) Loading plot of PCA of the *C. roseus* root. The first principal component (PC1) was the expression of genes *TDC*, *AS*, *STR*, *SGD*, *MPK*, and *ERF*, which were shown as *solid circles*. The second principal component (PC2) was the expression of gene *MYC*, which was shown as *solid squares*. Data presented here are the mean of three replicates

specifically promoted expression of leaf genes with particular effects in the pre-mature leaf, while it inhibited gene expression in roots [Fig. 3(A)]. In addition, the loading plot combination with correlation analysis [Fig. 3(E) and Tables 2 and 3] for the genes of *C. roseus* leaf, showed that expression of the *SLS* gene correlated with *TDC*, *SGD*, *MPK*, *MYC* genes. In addition, *ERF* expression correlated with expression of *SGD* and *AS*, specifically in roots, whereas *MPK* and *MYC* correlated with *STR* and *SGD* [Fig. 3(F)]. Together, these results indicate high correlation between TFs.

MPK and ***ERF*** gene expression in response to Cu and ethephon treatment

qRT-PCR was used to study *ERF* transcript in response to Cu and ethephon. Our results showed that ethephon induced *ERF* expression significantly in roots [Fig. 4(A)] and was correlated markedly to the *SLS* gene expression. Previous experiments demonstrated significant expression of TIA pathway genes in leaves when treated with ethephon alone [Fig. 2(A)]. However, in our experiment, almost no expression of *ERF* was induced by ethephon in leaves [Fig. 4(A)]. Moreover, at 60 μM ethephon, *ERF* expression was significantly reduced compared to that at 30 μM. In addition, while *ERF* preferentially expressed in the root and was not induced by treatment with Cu alone, its expression level increased significantly under Cu and ethephon treatments combined [Fig. 4(B)].

MPK expression followed a similar trend to that of TIA pathway genes [Fig. 3(C)], it specifically responded to exogenous ethephon in leaves, and was further enhanced by combined treatment with both Cu and ethephon [Fig. 4(C)]. At different developmental stages, Cu treatment induced *MPK* expression in the root. In control plants, *MPK* expression increased as the leaves matured; however, MPK expression was induced by Cu treatment in immature leaves but severely repressed in pre-matured and matured leaves [Fig. 4(D)].

Table 3 Pearson correlation matrix of seven genes in the root of *C. roseus*

	TDC	STR	SGD	ERF	MPK	MYC	AS
TDC	1	0.829	0.841	0.826	0.363	−0.436	0.854
STR		1	0.442	0.401	0.814	−0.054	0.421
SGD			1	0.997**	−0.161	−0.410	0.977*
ERF				1	−.0203	−0.468	0.987*
MPK					1	0.230	−0.174
MYC						1	−0.594
AS							1

*$p<0.05$; **$p<0.01$

C. roseus alkaloid synthesis in response to Cu and ethylene treatment

At the transcriptional level, the detected TIA pathway genes respond positively to abiotic stresses. In contrast, at the metabolite level, alkaloid synthesis does not coincide with the overall gene expression profile. TIA are synthesized in different organs or tissues at particular developmental stages, for example, accumulation of serpentine was found to be 3-fold more in roots [Fig. 5(C)], while catharanthine was 2-fold less in roots than in leaves [Fig. 5(D)]. TIAs were significantly accumulated just in response to Cu and ethephon treatment together, especially, vindoline and vinblastine, in leaves [Fig. 5(A)], while, the tabersonine and serpentine accumulated in minute amounts. After 24 h of treatment, as was shown [Fig. 5(E)], a series of physiological indicators were measured (Table 4). Plants height ($p<0.01$) and root length ($p<0.05$) were all significantly reduced with the ethephon or Cu concentration decreasing compared with the control group (CK). In contrast, when Cu and ethephon are given together, the inhibition of plant height and root length were slightly alleviated.

Table 2 Pearson correlation matrix of ten genes in the leaves of *C. roseus*

	TDC	STR	SGD	ERF	MPK	MYC	DAT	SLS	D4H	T16H
TDC	1	0.877	0.990*	−0.119	0.987*	0.979*	−0.814	0.998**	−0.493	0.262
STR		1	0.812	0.343	0.798	0.770	−0.529	0.903	−0.813	0.661
SGD			1	−0.255	1.000***	0.998**	−0.886	0.983*	−0.366	0.125
ERF				1	−0.274	−0.318	0.606	−0.073	−0.803	0.927
MPK					1	0.999***	−0.890	0.979*	−0.349	0.106
MYC						1	−0.906	0.969*	−0.306	0.060
DAT							1	−0.799	−0.059	0.286
SLS								1	−0.530	0.305
D4H									1	−0.968
T16H										1

*$p<0.05$; **$p<0.01$; ***$p<0.001$

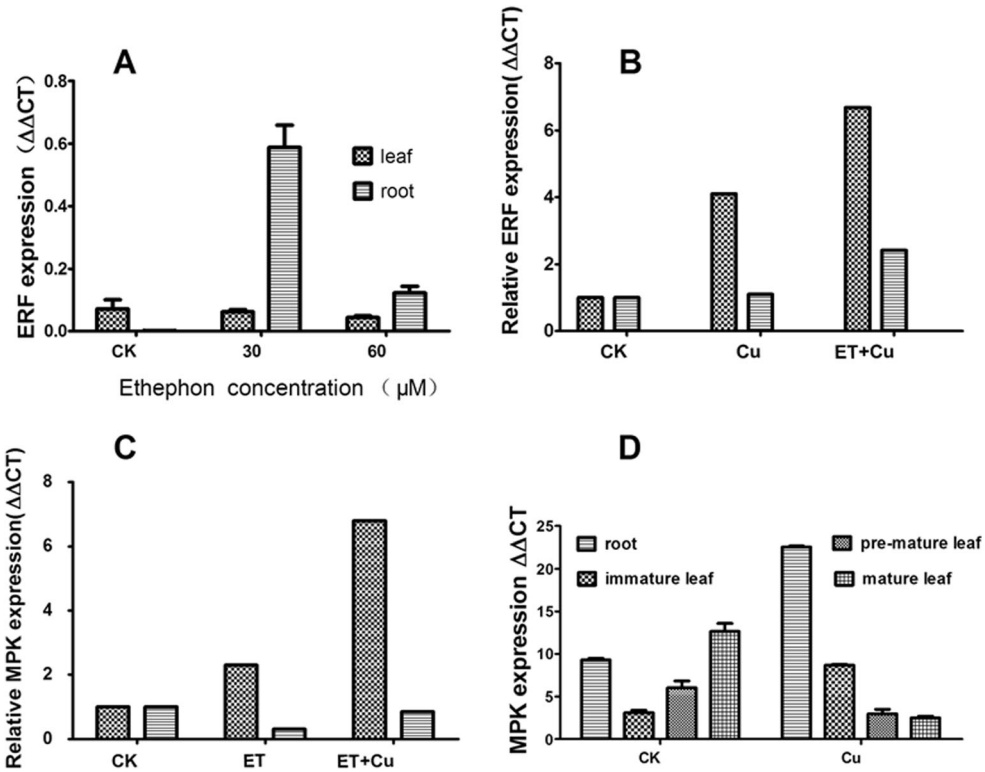

Fig. 4 Expression levels of selected *ERF* and *MPK* upon induction by heavy metal Cu and ethylene. The experiments were measured by qPCR. The results were analyzed using the comparative Ct method (The *Rps9* gene was used as an internal control). (*A*) Relative expression of *ERF* in *C.roseus* leaf and root upon control, 30 μM ET, 60 μM ET for 4 h. (*B*) Expression of *ERF* in *C. roseus* leaf and root in control, 300 μM $CuSO_4$, 300 μM $CuSO_4$, and 30 μM ET combination treatment. (*C*) relative expression of *MPK* in *C. roseus* leaf and root in control, 30 μM ET, 300 μM $CuSO_4$, and 30 μM ET combination treatment. (*D*) Expression of *MPK* in *C. roseus* different developmental stage in control, 300 μM $CuSO_4$. Each relative gene expression represents the average of three measurements, with *error bars* representing standard deviation

Discussion

Biosynthesis of TIAs is achieved by coordinated transcription of TIA pathway genes through transcriptional activators and repressors. Research on transcriptional regulation of TIA biosynthesis of the medicinal plant, *C. roseus*, has largely been focused on gene function and not clustering analysis of multiple genes at the transcript level (Patra et al. 2013; Kumar et al. 2012). Several recent reports have provided insightful information about the regulation of TFs by internal or external signals leading to controlled responses (Li et al. 2013). Our work investigates the roles of *ERF* and *MPK* genes on TIA pathway gene expression in response to heavy metal Cu and ethephon. Though pathway gene expression studies are available, we have used integrated elicitation and gene expression analysis using PCA approach to analyze regulation of pathway in differential manner and also its possible controls. The approach used is quite productive and illustrates the linkage and regulation of TIA under the influence of Cu and ethephon. In addition, PCA loading plot is a general statistical property of environmental variables that characterizes the correlation between signals at spatially nearby locations. The principal component scores is a measure of standard deviation that verifies the statistical significance of the PCA. The *Q* value is the significance level of the probability of completely random distribution (Papaioannou et al. 2012). In our research, the *Q* value described the overall expression of the TIA pathway genes for each treatment. Through the comparison of the *Q* value, we can draw that different treatment has the influence on the level of gene expression. Thus we can provide useful addition to the data and knowledge about alkaloid biosynthesis in Catharanthus.

The results of our previous studies have shown that ethylene is involved in TIA pathway gene expression (Chang et al. 2013). Here, we further investigated the detailed role of ethylene in the TIAs biosynthesis. The plants with *G10H* overexpression were observed to significantly increase the accumulation of strictosidine, vindoline, catharanthine, and ajmalicine (Pan et al. 2012), indicating that G10H actively participates the synthesis of alkaloids. D4H was another key enzyme in the vindoline biosynthetic steps. As shown in Fig. 2(C, D), we found that the expression levels of *G10H* and *D4H* were significantly improved after treatment with exogenous ethephon. So at least in part, we deduce that *G10H* and *D4H* expressions are induced in response to ethephon, actively participating in the biosynthesis of TIAs synthesis.

Research on heavy metal toxicity to plants is extensive, but there is limited research at the genetic level. So we specifically introduced heavy metal Cu to further study the effect of heavy metal Cu on TIA pathway genes expression and accumulation of TIAs in *C. roseus*. And we found that TIAs are synthesized in different tissues in response to Cu and ethephon treatment.

Under the stress of Cu and ethephon, expression of *C. roseus* TIA pathway genes showed spatial–temporal specificity. We demonstrated that Cu induces *C. roseus* root–

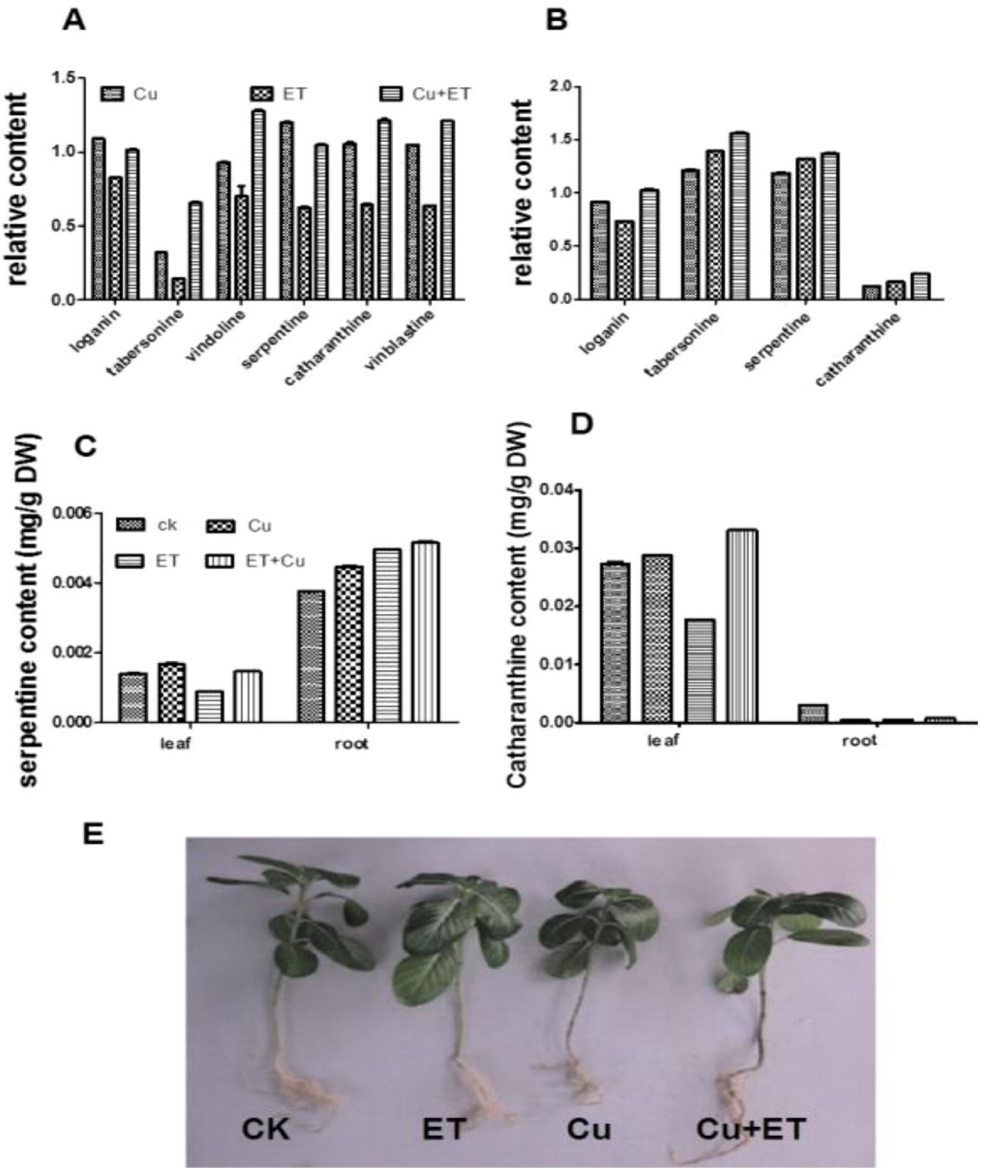

Fig. 5 Effect of heavy metal Cu and ethylene on TIAs biosynthesis in 3-month-old *C. roseus* seedlings. (*A*) TIAs, including loganin, tabersonine, vindoline, serpentine, catharanthine, and vinblastine content in leaves. (*B*) TIAs, including loganin, tabersonine, serpentine and catharanthine content in root. (*C*) Serpentine contents in the leaf and root. (*D*) Catharanthine content in leaf and root. (*E*) Three-month-old *C. roseus* with control, 300 μM $CuSO_4$, 300 μM $CuSO_4$, and 30 μM ET combination treatment. The average of three measurements, with *error bars* representing standard deviation. Different *letters* indicate significant difference ($p<0.05$ by ANOVA)

branch TIA gene expression but limited impact on leaf–branch TIA gene expression [Fig. 3(B). Cu appears to play a more dominant role in the regulation of gene expression in roots, which was significantly higher than in leaves. Ethephon is a plant ripening hormone (Yahia et al. 1998). Thus, ethephon can promote pre-mature leaf maturity by promoting gene expression in leaves. After leaves matured, gene expression in the leaf is not actively induced in response to ethephon both in leaves and roots [Fig. 3(A)]. In addition, we have found that when exposed to Cu and ethephon together, TIA gene expression in both leaves and roots are about 3-fold more than either treatment alone. From these studies we infer that exogenous ethephon and Cu may form an important component of the signal transduction pathway leading to stress induced accumulation of TIAs in *C. roseus*.

Table 4 Biomass accumulation and morphological changes in *C. roseus*

Treatment	Root length (cm)	Height (cm)
Control	7.86±0.1	6.74±0.14
30 (μM)	7.36±0.32	6.17±0.28
300 (μM) Cu	6.36±0.1	5.39±0.21
30 (μM)ET + 300 (μM)Cu	7.41±0.21	7.04±0.1
ANOVA	**	**

The asterisk indicates significant effects of ETH and Cu on observed parameters (**$p<0.001$)

The above studies, together with the loading plot [Fig. 3(e, f)], suggest that there may be cross-talk between the two upstream pathways that suppress or promote each other in order to combat exogenous stresses. The cus genetic determinants in *Klebsiella pneumoniae* are comprised of a structural

component (cus*CFBA*) and a regulatory component (cus*RS*), which show several fold increased expression following copper induction under both aerobic and anaerobic conditions (Zulfiqar and Shakoori 2012). Whether *C. roseus* also has operons as well as *RS* and *CFBA* in response to heavy metal Cu is to be established. We hypothesize that the two pathways are not independently expressed in specific cells; rather, a central hub or signal system connecting the pathways exists. In this process, *ERF* is significantly related to *SLS* and *TDC* genes. This suggests that *ERF* may be the central hub of the two upstream pathways, and thus, actively involved in gene expression of the two pathways specifically in response to heavy metal Cu stress. Numerous studies have shown that *STR* is a key enzyme of the *C. roseus* TIA pathway (Li et al. 2013), and its synthesis of strictosidine plays an irreplaceable role in the synthesis of TIAs. We can further study how *MPK* influences *STR* gene expression, and thereby affect the biosynthesis of TIAs. In addition, we found that *ERF* and *MPK* genes are correlated to each other, specifically in root, and TF and MAPK may also have mutual regulation function between each other jointly promote the expression of the TIA pathway genes to resist abiotic stress. The expression of TF was not obvious in this study, it is hypothesized that these two TFs expression mechanisms are induced by abiotic stresses that act on TIA pathway gene expression in a different manner.

This work presents a study of the effect of the *ERF* and *MPK* genes on TIA pathway gene expression. An extremely significant amount of activation of *ERF* transcription occurs in roots in response to abiotic stress, especially exposure to heavy metal Cu and ethephon together [Fig. 4(A, B)]. For this purpose, the model of the regulation of TIA gene expression by *ERF* in *C. roseus*, seems to be a good model for studying a new level of complexity in plant responses to abiotic stress. In addition, we found a significant level of *MPK* expression activity during ethylene treatments [Fig. 4(C)], suggesting *MPK* is required for high level TIA pathway gene expression in *C. roseus* leaves. We observed an active response of *MPK* to heavy metal Cu stress in roots. The gene expression showed obvious developmental differences in response to Cu [Fig. 4(D]. Gene expression was suppressed upon heavy metal Cu stress at leaf maturity, suggesting a defense mechanism against abiotic stress in mature leaves (Raina et al. 2012).

We found that the expression of TIA pathway genes is highly regulated through a combination of heavy metal Cu and ethephon treatments and depends on tissue-specific and developmental-specific factors, as well as environmental signals. Ethephon mainly promoted expression in the pre-mature leaf, while Cu mainly promoted expression of root genes. We describe a simplified picture of our results of the ethephon and heavy metal Cu induced responses in *C. roseus* (Fig. 6). Ethylene specifically recognizes the receptor and Cu attaches to Cu channel protein, which is then transferred to the nucleus.

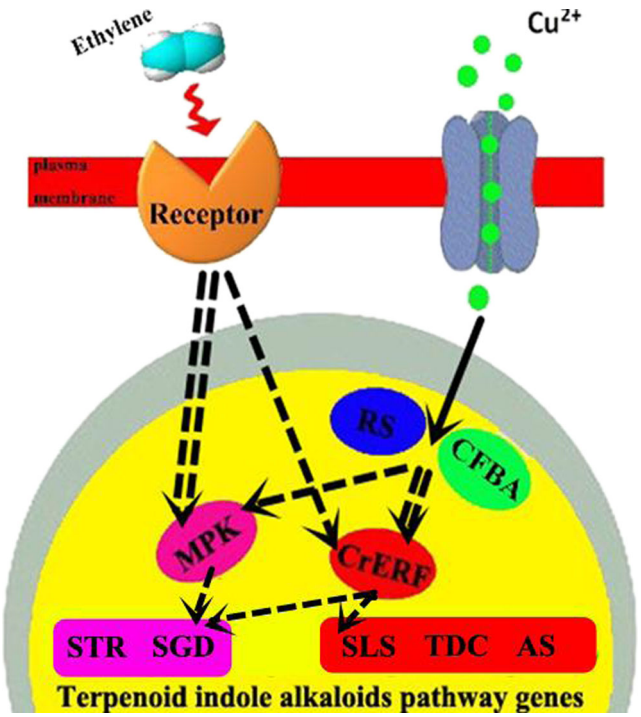

Fig. 6 Model for ERF and MPK regulated TIA pathway genes expression responding to heavy metal Cu and ethylene signal in *C. roseus*. *Unbroken* and *broken lines* indicate proven and hypothetical (yet to be experimentally established) links, respectively, that can be either direct or indirect. *Single arrows* indicate a linder role. *Double arrows* indicate certain role in promoting. Ethylene can activate TIA gene expression via *MPK*. Also transcription factor *ERF* interacts with the PR genes in a Cu-dependent signal transduction pathway. The TIA pathway genes expression exclusively regulated by ERF and MPK are shown with *red* and *purple box*, respectively

TF and MAPK interact with each other and play an irreplaceable and specific role in response to abiotic stress. For instance, *ERF* and *MPK* positively respond to these signals, indicating specific recognition. *ERF* and *MPK* activity followed a similar pattern to that of *ORCA* and exhibited its maximal activity in the expression of TIA pathway genes (Pre et al. 2008). In addition, the *ERF* mainly respond to Cu stress principal regulating the two upstream pathway gene expression and the MPK mainly participates in the expression of the key enzyme gene *STR* in response to ethephon treatments.

At the transcriptional level, TIA pathway genes actively respond to combined stress of Cu and ethephon. We expected that although a small step towards unraveling the complex signal transduction mechanism in *C. roseus*, our results would help explain more clear on the signal transduction network. However, the change of alkaloid accumulation was inconsistent with the expression of associated genes. Our previous research indicated that there is significant accumulation of vinblastine through high concentrations of ethephon and Cu. This study shows that metabolic pathways are also controlled by posttranscriptional and posttranslational mechanisms. Recent research has revealed that the synthesis and proper

accumulation of TIAs are strictly controlled in a spatial and temporal manner. Assembly of TIAs in *C. roseus* is remarkably dynamic, involving at least three cell types within leaves. Rates of biosynthesis, as well as oriented transport mechanisms, precisely regulate where, when, and how, different MIAs accumulate during plant growth and development (Roepke et al. 2010). The regulation of alkaloid biosynthesis is complex and still poorly understood. Lack of genetic tools is a major bottle-neck in identifying potential regulators involved in pathway regulation (Patra et al. 2013). It should be noted that this study was an investigation at the gene level only and does not provide enough data to understand the processes in considerable detail. The posttranscriptional and posttranslational mechanisms must be studied in order to clarify these issues in more detail.

Acknowledgments We sincerely thank Matthew Paul from Rothamsted Research and Ling Yuan from University of Kentucky for their constructive and critical comments on this manuscript. This study was financially supported by the Fundamental Research Funds for the Central Universities (2572014DA01) and National Natural Science Foundation of China (31400337 and 31370007).

References

Agarwal P, Agarwal P, Reddy MK, Sopory S (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep 25:1263–1274

Chang B-W, Cong W-W, Chen Q, Y-G Z, Tang Z-H (2013) The influence of different forms and concentrations of potassium nutrition on growth and alkaloid metabolism in *Catharanthus roseus* seedlings. J Plant Interact 9:370–377

Clijsters H, Assche F (1985) Inhibition of photosynthesis by heavy metals. Photosynth Res 7:31–40

Favali MA, Musetti R et al (2004) *Catharanthus roseus L.* plants and explants infected with phytoplasmas: alkaloid production and structural observations. Protoplasma 223(1):45–51

Kumar S, Jaggi M et al (2012) Ectopic overexpression of vacuolar and apoplastic *Catharanthus roseus* peroxidases confers differential tolerance to salt and dehydration stress in transgenic tobacco. Protoplasma 249(2):423–432

Li C, Leopold A, Sander G, Shanks J, Zhao L, Gibson S (2013) The ORCA2 transcription factor plays a key role in regulation of the terpenoid indole alkaloid pathway. BMC Plant Biol 13:155

Lolkema PC, Vooijs R (1986) Copper tolerance in *Silene cucubalus*. Planta 167:30–36

Memelink J, Verpoorte R, Kijne JW (2001) ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 6:212–219

Ouzounidou G (1995) Cu-ions mediated changes in growth, chlorophyll and other ion contents in a Cu-tolerant *Koeleria splendens*. Biol Plant 37:71–78

Pan Q, Wang Q, Yuan F, Xing S, Zhao J, Choi YH, Verpoorte R, Tian Y, Wang G, Tang K (2012) Overexpression of ORCA3 and G10H in *Catharanthus roseus* plants regulated alkaloid biosynthesis and metabolism revealed by NMR-metabolomics. PLoS One 7:e43038

Papaioannou VA, Kranioti EF, Joveneaux P, Nathena D, Michalodimitrakis M (2012) Sexual dimorphism of the scapula and the clavicle in a contemporary Greek population: applications in forensic identification. Forensic Sci Int 217(231):e231–e237

Patra B, Schluttenhofer C, Wu Y, Pattanaik S, Yuan L (2013) Transcriptional regulation of secondary metabolite biosynthesis in plants. Biochim Biophys Acta 1829:1236–1247

Peebles CAM, Hughes EH, Shanks JV, San K-Y (2009) Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. Metab Eng 11:76–86

Pre M, Atallah M, Champion A, De Vos M, Pieterse CM, Memelink J (2008) The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol 147:1347–1357

Quan R, Hu S, Zhang Z, Zhang H, Zhang Z, Huang R (2010) Overexpression of an ERF transcription factor TSRF1 improves rice drought tolerance. Plant Biotechnol J 8:476–488

Raina SK, Wankhede DP, Jaggi M, Singh P, Jalmi SK, Raghuram B, Sheikh AH, Sinha AK (2012) CrMPK3, a mitogen activated protein kinase from *Catharanthus roseus* and its possible role in stress induced biosynthesis of monoterpenoid indole alkaloids. BMC Plant Biol 12:134

Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. Curr Opin Plant Biol 1: 404–411

Rijhwani SK, Shanks JV (1998) Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol Prog 14:442–449

Rischer H, Oresic M et al (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci U S A 103(14):5614–5619

Roepke J, Salim V, Wu M, Thamm AM, Murata J, Ploss K, Boland W, De Luca V (2010) Vinca drug components accumulate exclusively in leaf exudates of *Madagascar periwinkle*. Proc Natl Acad Sci U S A 107:15287–15292

Sears MT, Zhang H, Rushton PJ, Wu M, Han S, Spano AJ, Timko MP (2014) NtERF32: a non-NIC2 locus AP2/ERF transcription factor required in jasmonate-inducible nicotine biosynthesis in tobacco. Plant Mol Biol 84:49–66

Suttipanta N, Pattanaik S, Kulshrestha M, Patra B, Singh SK, Yuan L (2011) The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in Catharanthus roseus. Plant Physiol 157(4):2081–2093

Thavamani P, Megharaj M, Naidu R (2012) Multivariate analysis of mixed contaminants (PAHs and heavy metals) at manufactured gas plant site soils. Environ Monit Assess 184:3875–3885

van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289:295–297

van Loon LC, Geraats BPJ, Linthorst HJM (2006) Ethylene as a modulator of disease resistance in plants. Trends Plant Sci 11:184–191

Verma P, Mathur AK, Masood N, Luqman S, Shanker K (2013) Tryptophan over-producing cell suspensions of *Catharanthus roseus* (L) G. Don and their up-scaling in stirred tank bioreactor: detection of a phenolic compound with antioxidant potential. Protoplasma 250:371–380

Wang CT, Liu H, Gao XS, Zhang HX (2010) Overexpression of G10H and ORCA3 in the hairy roots of *Catharanthus roseus* improves catharanthine production. Plant Cell Rep 29:887–894

Wang Z, Ma P, Xu L, He C, Peng Y, Xiao P (2013) Evaluation of the content variation of anthraquinone glycosides in rhubarb by UPLC-PDA. Chem Cent J 7:170

Yahia A, Kevers C, Gaspar T, Chénieux J-C, Rideau M, Crèche J (1998) Cytokinins and ethylene stimulate indole alkaloid accumulation in cell suspension cultures of *Catharanthus roseus* by two distinct mechanisms. Plant Sci 133:9–15

Yang C-Q, Fang X, Wu X-M, Mao Y-B, Wang L-J, Chen X-Y (2012) Transcriptional regulation of plant secondary metabolismF. J Integr Plant Biol 54:703–712

Zhou M-L, Hou H-L, Zhu X-M, Shao J-R, Wu Y-M, Tang Y-X (2011) Molecular regulation of terpenoid indole alkaloids pathway in the medicinal plant, *Catharanthus roseus*. J Med Plants Res 5:663–676

Zulfiqar S, Shakoori AR (2012) Molecular characterization, metal uptake and copper induced transcriptional activation of efflux determinants in copper resistant isolates of *Klebsiella pneumoniae*. Gene 510:32–38