The Expression of Terpenoid Indole Alkaloid (TIAs) Pathway Genes in *Catharanthus roseus* in Response to Salicylic Acid treatment

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Abbreviations

AS: Anthranilate Synthase
CM: Chorismate Mutase
DAT: Deacetylvindoline-4-O-acetyltransferase
D4H: Desacetoxyvindoline-4-hydroxylase
G10H: Geraniol-10-hydroxylase
RSP9: 40S ribosomal protein S9
SA: Salicylic Acid
SLS: Secologanin Synthase
STR: Strictosidine Synthase
TDC: Tryptophan Decarboxylase
TIAs: Terpenoid Indole Alkaloids

Abstract

Vinblastine and vincristine are two important anti-cancer drugs that are synthesized by the Terpenoid Indole Alkaloids (TIAs) pathway in periwinkle (*Catharanthus roseus*). The major challenge in the pharmaceutical industry is the low production rate of these alkaloids. TIA pathway is affected by elicitors, such as salicylic acid (SA). TIA pathway is elevated and is affected by elicitors, such as Salicylic Acid (SA). This study aimed to investigate the expression pattern of some key genes in TIAs pathway under SA treatment. Foliar application of SA (0.01 and 0.1 mM) was used and leaves samples were taken at 0, 12, 18, 24 and 48 hours after the treatment. qRT-PCR was used to investigate the expression pattern of Chorismate mutase (*Cm*), Tryptophan decarboxylase (*Tdc*), Geraniol-10-hydroxylase (*G10h*), Secologanin synthase (*Sls*), Strictosidine synthase (*Str*), Desacetoxyvindoline-4-hydroxylase (*D4h*) and Deacetylvindoline-4-O-acetyltransferase (*Dat*) genes, following the SA treatment. The results of this experiment showed that transcript levels of *Tdc*, *G10h*, *Sls*, *Str*, *D4h* and *Dat* genes were significantly up-regulated in both SA concentration treatments. Furthermore, the highest transcript levels of *Dat* was observed after 48 hours of the SA treatments. qRT-PCR results suggests that SA induces transcription of major genes involved in alkaloids biosynthesis in *Catharanthus roseus*. It can be concluded that up-regulation of *Tdc*, *G10h*, *Sls*, *Str*, *D4h* and *Dat* genes can result in a higher production rate of vinblastine and vincristine alkaloids.

Keywords

Elicitor, Gene Expression, Salicylic Acid, Terpenoid Indole Alkaloids, TIAs pathway, qRT-PCR

Introduction

Madagascar Periwinkle (*Catharanthus roseus*), a member of Apocynaceae family, is an important medicinal plant native to Madagascar [1,2]. It is also known as *Cape periwinkle*, *Vinca rosea*, *Rosy periwinkle*, *Rose periwinkle*, *Ammocallis rosea*, and *Lochnera rosea* [3]. *C. roseus* is an excellent model system plant to study the plants' secondary metabolome [4] due to its small genome size (1500 Mbp), herbaceous perennial habit, short life cycle, and sexual and vegetative modes of propagation, [5,6,4]. Periwinkle is rich in alkaloids, phenols, steroids, anthocyanins, saponins, and carbohydrate compounds [1,5], with over 400 alkaloids [2] and more than 130 Terpenoid Indole Alkaloids (TIAs) [7-9]. Periwinkle possesses anti-oxidant, anti-bacterial, anti-ulcer, anti-diarrheal activities, and it is used to treat cancer, diabetes, blood pressure, asthma, mouth ulcers, and constipation [1,3]. Vincristine and vinblastine, as two important alkaloids used in the treatment of cancer [3,8] are solely synthesized by the TIAs pathway in the leaves and

stems of the *C. roseus* [1,3,8]. Despite the huge interest and demand by the pharmaceutical industry regarding the production of these vital alkaloids, they are naturally produced in a very low amount in the *C. roseus* [10,11,8,12]. Furthermore, their laboratory synthesis by the chemical methods faces some obstacles due to their complex chemical structures [13,8]. The TIAs biosynthesis pathway leading to the production of pharmaceutical valuable compounds seems to be highly complex involving at least 35 intermediates and 30 enzymes from indole pathway, methylerythritol 4-phosphate (ME), terpenoid, and alkaloid pathways [9].

Tryptamine is produced from the tryptophan through the activity of tryptophan decarboxylase (TDC) [14] and the indole pathway that consequently serves as the indole moiety for production of the TIAs [15,16,8]. Secologanin synthase (SLS) is a member of P450 enzymes that catalyzes an unusual ring-opening reaction of loganin leading to the secologanin production [8]. In the alkaloids pathway, tabersonine is converted into different compounds, especially vindoline, a chemical precursor to vinblastine by deacetylvindoline-4-O-acetyltransferase (DAT) [13]. Finally, vinblastine and vincristine are synthesized by the compression of catharanthine and vindoline metabolites [11]. Production of diverse types of TIAs pathway metabolites is controlled at transcriptional, translational, and post-translational levels [8] and is fluctuated by many internal and external elicitors. For instance, phytohormones including Salicylic Acid (SA) and Ethylene (ET) stimulate the production rate of catharanthine, vindoline, and vinblastine in the *C. roseus*, whereas Abscisic Acid (ABA) and Gibberellic Acid (GA) tend to decrease the production rate of TIAs [13]. However, the signal transduction pathway mechanisms explaining how these growth hormones regulate TIAs biosynthesis are largely unknown. Many factors such as SA have been reported to regulate TIAs pathway [17,8]. SA is involved in many physiological processes such as plant growth and development, flowering, ion uptake, stomatal closure, disease resistance, and heat production [18,19]. SA, as a plant signaling molecule improves the anti-oxidant defense system of the *C. roseus*, and plays a pivotal role in combating the abiotic stresses such as drought, salinity, cold and restraining nickel toxicity as well as increasing the amount of vincristine and vinblastine alkaloids [13,20,21,17,11]. For instance, Pan et al. [13] have shown that spraying the SA at 0.1 mM concentration on the *C. roseus* leaves significantly increased the accumulation of catharanthine, vinblastine, and vindoline alkaloids. It has also been reported that spraying 0.01 mM of SA under water [22] and salinity [20] stresses reduces the harmful effects of abiotic stresses eventually leading to an increase in the total amount of alkaloids, vincristine, and vinblastine in the *C. roseus*. Despite SA benefits, the negative effects of surplus application of SA have also been reported and its efficiency depends on the age of plant and concentration of SA [18].

In the present study, the expression level seven major genes associated with indole, terpenoid and alkaloids pathways was evaluated by the quantitative Real Time Polymerase Chain Reaction (qRT-PCR) analysis after application of the SA (0.01 and 0.1 mM).

Materials and Methods

Plant Material and Treatments

The Periwinkle seeds were obtained from Pan American Seed Company (USA) and were cultivated in the pots (10 cm diameter × 10 cm height) and then, were maintained in a growth chamber (25°C, 16 h photoperiod, and light intensity of 70% µmol $m^{-2}s^{-1}$). SA was sprayed at concentrations of 0.01 and 0.1 mM [23] at the beginning of flowering stage [24,5]. Samples were taken from the third and fourth leaves as described by Roepke et al. [25]. The sampling was carried out at 12, 18, 24, and 48 hours after the treatment (3 replicates each), and the samples were immediately frozen in the liquid nitrogen and were stored at -80°C for RNA extraction.

RNA Extraction, cDNA Synthesis, and Primer Designing

Total RNA was extracted from 100 mg of leaves using the Monarch® Total RNA Miniprep Kit (NEB Company, UK. Cat# T2010) according to the manufacturer's instructions. RNA concentration was quantified using the NanoDrop 2000c Spectrophotometer (Thermo Scientific NanoDrop 2000, USA) and were qualified by 1% agarose gel electrophoresis. The first strand cDNA was synthesized from 500 ng of total RNA using the Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase according to the manufacturer's instructions in the final volume of 20µL (RNase H−) Kit (NEB Company, UK. Cat# E6300S). The cDNA was diluted to 100 ng/µL as the template for the real-time PCR analysis.

The sequences of target genes (Cm, *Tdc*, *G10h*, *Sls*, *Str*, *D4h* and *Dat*) and the 40S ribosomal protein S9 (*Rsp9*) reference gene were downloaded from the Genbank database. Primers were designed (Table 1) by the Oligo7 and Primer3Plus softwares except *Cm* primers, which had been previously designed and validated by Sun et al., (2016).

Gene Expression Profiling and Analysis

The qRT-PCR analysis (Step One Plus Real-time PCR System, Applied Biosystems, USA) was performed using the specific primers to ensure amplification of the target genes. The expression level of the target and reference genes was

determined using the SYBR Green PCR Master Mix (NEB Company, UK. Cat# M3003S). Each reaction consisted of a mixture containing~~Each reaction was consisted of a mixture containing~~ 16 ng/µL of cDNA (2 µL of initial concentration in 100 ng/µL), 0.4 µM of each of the forward and reverse primers, and SYBR Green PCR master mix (1X). The qRT-PCR was performed in three biological and three technical replicates. The thermal cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 30s. After each cycle, melting curve analysis was conducted by increasing the temperature from 60 to 95°C along with continuous monitoring of the SYBR Green fluorescence. The relative gene transcription was quantified using the comparative threshold cycle (CT) method and the data were analyzed using the REST® software [26] according to the ΔΔCT method [27].

Results

The elicitor effect of SA was investigated on gene expression of the TIAs pathway. Since *Cm* and Anthranilate synthase (*As*) genes compete for the same precursor (Chorismate); the expression pattern of *Cm* was monitored at two different concentrations of SA. Although, the transcript level of the *Cm* gene showed a down-regulation at C2 (0.01 mM of SA) and up-regulation at C1 (0.1 mM of SA) (Fig. 1A), the higher amount of the SA led to significant up-regulation of *Cm* only at 24 h after the treatment. No significant (P>0.5) differences were found between different time points after the SA application at lower concentrations.

In the indole pathway, the transcripts level ~~the mRNA transcripts~~ of the *Tdc* gene were elevated following the application of both concentrations of SA (Fig. 1B). The expression of the *Tdc* ~~gene~~ was up-regulated at C2 concentration, and the transcription level was slowly increased from 12h to 24 h. The highest level of transcription was observed at 48h. Similarly, the ~~mRNA~~ transcript level of *Tdc* was up-regulated at C1 concentration, and the highest level was observed at 48h. The transcript level showed a significant up-regulation trend in both treatments except at 24h at C1 concentration and reached a peak value at 48h (3.76 and 3.82 fold changes, respectively).

The transcript level of the ~~The transcript mRNA level of the~~ *G10h* ~~gene~~ in the terpenoid pathway was significantly up-regulated at 24 and 48 h following the application of the C1 concentration of SA (Fig. 2A). The expression of the *G10h* gene was slowly increased, and the highest ~~mRNA~~ transcript level of *G10h* was observed at 48h. Interestingly, the ~~mRNA transcript level~~ expression of *G10h* gene was significantly increased during 12h-24h. The expression of the *G10h* gene was increased by 2.82 and 7.27 folds at 48 h, respectively.

~~The *Sls* mRNA transcript~~ Transcript level of *Sls* was significantly increased over time with the highest level observed at 48h post SA treatment at C1 concentration (Fig. 2B), which was similar to that of the *G10h* gene in terms of changes over time points and at various SA concentrations. The expression of *Sls* gene was significantly increased at C2 concentration, at 12 and 48h but it was decreased at 24h.

Strictosidine synthase (*Str*) activity play a major role at the beginning of the alkaloid pathway. The ~~mRNA transcripts level~~ expression of *Str* gene was increased after SA treatment (Fig 3 A). The expression of *Str* was significantly increased by time and reached the highest level 48 hours after SA treatment. The same trend was observed for desacetoxyvindoline-4-hydroxylase (*D4h*) gene (Fig 3B). However, the expression level of *D4h* was significantly lower than *Str* gene.

The expression of the *Dat* gene showed a significant up-regulati

on following both treatments over time except at C2 concentration after 18h (Fig. 3C). However, increase in the transcription level at 18h was lower than expression level at 12h. The highest expression was observed at both concentrations at 48h and reached 7.98 and 8.2 fold changes at C1 and C2 concentrations, respectively.

Discussion

Plant Growth Regulators (PGRs) significantly affect the synthesis of secondary metabolites [23]. The mechanism of stress defense in plants is mediated through various signaling pathways and many defensive proteins and non-protein compounds. Abscisic Acid, Jasmonic Acid, Ethylene and Salicylic Acid (SA) involved in plant defense are important components of different signaling pathways. SA also play a major role as an antioxidative compound in plants. Besides association in cross-talking with other pathways mediating plant resistance, SA regulates the components of its own signaling pathway. It also plays an important role in increasing metabolic rates, which contributes to the plant's energy-saving through alternative pathways accompanied by a change in the level of amino acids within the plant (War et al., 2011; Hassoon and Abduljabbar, 2019). For instance, the effect of SA on the alkaloid accumulation and enhancement of the plant defense system in *C. roseus* has been documented [13,22,20,21,23]. It has been shown that foliar application of SA at two concentrations (0.01 mM and 0.1 mM) significantly increased the amount of catharanthine, vindoline, vinblastine, and vincristine alkaloids in the *C. roseus* [23]. Since many alkaloids in the *C. roseus* are synthesized in the TIAs pathway, it has been shown that the changes in the transcription level of upstream genes affect the expression of downstream genes and consequently the synthesis of downstream alkaloids [28,8].

As and *Cm*, the first genes involved in the indole and flavonoid pathways, respectively, compete for the same substrate, the chorismate precursor. As a result of such a competition, the activities of As and Cm enzymes influence the final product as well as the number of intermediate compounds in both pathways [29,8]. If a higher amount of chorismate enters the flavonoid and phenylpropanoid pathways, the reaction shifts to the synthesis of phenylalanine, tyrosine, flavonoids, and phenylpropanoids instead and production of indole terpenoid alkaloids [29]. Higher activity of As in the TIAs pathway leads to the fluctuation of downstream genes and downstream alkaloids [29,15,8]. Overexpression of the *As gene* in the transgenic *C. roseus* hairy root lines has been reported to be driven by an inducible promoter [8]. In line with these findings, in the present study, the foliar application of SA did not show a constant trend (Fig. 1A).

Tdc gene encodes the final enzyme in the TIAs indole pathway and converts tryptophan to tryptamine. Later, tryptamine enters the TIAs alkaloid pathway through the activity of strictosidine synthase (STR) enzyme [15,11]. *Tdc* and *As* genes are both active in the indole pathway. Overexpression of the *As* gene in the hairy roots of *C. roseus* has been found to result in the induction of *As* gene expression and subsequently the transcription level of the *Tdc* gene. The highest *Tdc* gene transcription rate was observed at 12h after induction of *As* expression [8]. Results of a study on the *Tdc* gene transcription level and vindoline content following the treatment with the two fungal endophytes (Curvularia sp. CATDLF5 and Choanephora infundibulifera CATDLF6) in the *C. roseus* showed that the symbiosis significantly increased the transcript levels of the *Tdc*, *As*, *G10h*, *Str*, *D4h*, and *Dat* genes as well as the alkaloid content of vindoline [30]. Interestingly, in another study, the ~~mRNA~~ transcript level of *Tdc* was increased in the *C. roseus* seedlings following treatment with methyl jasmonate (MeJA). MeJA treatment led to an increase in the expression level of *Tdc* over time and the highest expression was observed at 0.2 mM and 2 mM concentrations after 48h [31]. Changes in the transcript levels of any of the upstream genes can also lead to changes in the transcript levels of the downstream genes depending on the position of genes in the TIAs cascade pathway. An increase in the *Tdc* transcript level and other TIAs indole pathway genes has been found to increase the production of the alkaloids [8,30,32]. In this study, the expression level of *Tdc* gene was up-regulated following the SA foliar application regardless of the SA concentration used. Despite the differences between the treatments applied in the present study and reported studies, an increase in the *Tdc* gene transcription level over time can lead to the induction of downstream genes in the TIAs cascade pathway and consequently accumulation of the alkaloids.

In this study, the expression ~~level of mRNA transcripts~~ of *G10h* and *Sls* genes belonging to the terpenoid pathway was investigated. G10H converts geraniol into 10-hydroxygeraniol, and ~~SLS enzyme finally converts 10-hydroxygeraniol into secologanin~~ SLS converts loganin into secologanin. Later, secologanin enters the

Commented [HP1]: دکتر در این بخش منظور این بوده که 10-هیدروکسی ژرانیول طی چندین مرحله واکنش در آخرین مرحله با فعالیت انزیم اس.ال.اس. به سکولوگانین تبدیل می‌شود. که داور محترم جمله را به این صورت تغیر داده‌اند. بر اساس نظر ایشان این جمله جایگزین شده.

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alkaloid pathway through the activity of the STR enzyme [15,8,6]. Studies have shown that the *G10h* gene plays an important role in the TIAs pathway [33] and any fluctuation in the *G10h* gene transcript level influences the alkaloid pathway and consequently the synthesis of commercial *C. roseus* alkaloids. In this regard, overexpression of the *G10h* and ORCA3 (Octadecanoid-derivative Responsive Catharanthus AP2-domain transcription factor) genes has been shown to increase the transcript level of the genes involved in the TIAs pathway including *G10h*, *Tdc*, *As*, *Str*, and *D4h* as well as an increase in the accumulation of strictosidine, vindoline, catharanthine, ajmalicine, and vinblastine alkaloids in the transgenic plants [28]. Furthermore, Papon et al., have shown that the combination of Cytokinin and Ethylene increased the accumulation of alkaloids and expression of *G10h* and MEP pathway genes but did not affect *Tdc* and Mevalonate (MVA) pathway genes [33]. In line with other reports [31,30], herein, an increase was observed in the *G10h* gene expression following SA application, suggesting that up-regulation of the genes involved in terpenoid pathway induces the transcription of associated genes and as a result an increase in the TIAs pathway end products [28,8,30].

SLS is the final enzyme in the TIAs terpenoid pathway and converts loganin to ~~secologenin~~secologanin. Secologanin and tryptamine enter the TIAs alkaloid pathway through the activity of the STR enzyme [15,32,34]. Report shows that the transcript levels of the *Sls* did not change during the treatment with symbiotic fungal endophytes [30]. A significant increase was found in the transcript level of the *Sls* mRNA gene in the *C. roseus* cell cultures after treatment with Cyclodextrins alone and combined co-incubation with MeJA and Cyclodextrins. Similar to the *G10h* gene, the expression of the *Sls* gene was elevated after treatment with MeJA and Cyclodextrins leading to a significant increase in the amount of ajmalicine and catharanthine alkaloids [32]. Although, our results were consistent with the findings of the studies by Almagro et al., (2014) and Pandey et al., (2016), no significant changes were found in the transcript level of the *Sls* gene after inoculation with two symbiotic fungal endophytes.

Tryptophan and secologanin are converted to the strictosidine by enzymatic activity of strictosidine synthase [30]. Change in expression level of *Str* gene can have a significant impact on expression of downstream genes in TIAs pathway [8]. Strictosidine, the product of strictosidine synthase activity, is a precursor important major genes of the alkaloid pathway, such as *D4h*, *Dat*, and *Prx 1* [6, 15]. Desacetoxyvindoline-4-hydroxylase (*D4h*) gene is one of the final genes in the alkaloid pathway that turns desacetoxyvindoline into deacetylvindoline and this product is one of the main precursors for making vindoline [15, 30]. There is a direct link between an increase in vincristine accumulation and expression level of *Dat* and *D4h* genes [11, 30]. DAT converts deacetylvindoline into vindoline in

the TIAs alkaloid pathway [15,6,8]. An increase in the expression level of *Dat* has been found to result in the accumulation of vinblastine and vincristine alkaloids [12,35]. Inoculation of *C. roseus* with fungal endophytes led to an increase in the expression of *Str*, *D4h* and *Dat* genes and consequently vindoline content *C. roseus* increased [30]. Consistent with these findings, an increase has been reported in the accumulation of vindoline in the transgenic *Dat*-expressing plants [12]. Furthermore, MeJA and putrescine treatments have been shown to lead to the up-regulation of *Str*, *Prx1*, *Dat* genes and consequently the production of vincristine and vinblastine alkaloids [35]. In another study, foliar application of SA on the leaves of *C. roseus* seedlings exposed to drought and salinity stresses increased the transcript level of the *Dat* and *D4h* genes [11].

Conclusion

The results of this study showed a change in the expression of some TIAs pathway genes following the foliar application of SA. It is expected that the changes in the expression of the upstream genes will consequently influence the ~~mRNA~~ transcript level of the downstream genes and production of the final products. Foliar application of SA up-regulated the transcript level of *G10h* and *Sls* genes. The treatments also increased the expression level of the *Dat* gene, which synthesizes the vindoline. It can be concluded that up-regulation of TIAs pathway genes can result in a higher production rate of vinblastine and vincristine alkaloids in the *C. roseus*.

Compliance with ethical standards

Conflict of interest Authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Legend to the Figures

Figure 1. qRT-PCR expression analysis ~~mRNA transcript fold changes~~ of *Cm* (A), and *Tdc* (B) genes following treatment of *Catharanthus roseus* leaves with salicylic acid at two different concentrations (C_1: 0.1 mM and C2: 0.01 mM) at 12, 18, 24 and 48 hours post treatment.

Figure 2. qRT-PCR expression analysis of *G10h* (A), and *Sls* (B) genes following treatment of *Catharanthus roseus* leaves with salicylic acid at two different concentrations (C_1: 0.1 mM and C2: 0.01 mM) at 12, 18, 24 and 48 hours post treatment.

Figure 3. qRT-PCR expression analysis of *Str* (A), *D4h* (B) and *Dat* (C) after treatment of *Catharanthus roseus* leaves with salicylic acid at two different concentrations (C1: 0.1 mM and C2: 0.01 mM) at 12, 18, 24 and 48 hours post treatment.