INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1779/2020/24–6–1520–1526
DOI: 10.17957/IJAB/15.1590
http://www.fspublishers.org

Full Length Article

Exogenous Nitrous Oxide Regulates Alkaloids Biosynthesis in *Catharanthus roseus*

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Received 20 November 2019; Accepted 21 July 2020; Published 10 October 2020

Abstract

Catharanthus roseus (L.) produces large quantities of Terpene Indole Alkaloids (TIAs) and is a good medicinal model plant. The present experiments evaluated the role of the exogenous NO on alkaloids content. The growth index, hormonal contents and alkaloids increased significantly under combined treatment; the expression of transcription factor *Myc1* and key genes were significantly up regulated. Correlation analysis showed that there were positively correlation between the 6-BA, ABA and *Myc1* expression ($P < 0.01$); *Myc1* expression positively correlated with the pathway genes (*Tdc*, *Str*, *Sgd*, and *Dat*) expression ($P < 0.01$). there was also a significant positive correlation between the pathway genes expression and alkaloid content ($P < 0.01$). As a signaling molecule, NO regulated the increase of hormones content and promoted the higher expression of transcription factors *Myc1*under light-shading stress, and activated the key genes expression in the alkaloids metabolic pathways increased the alkaloids (vinblastine and vincristine) content in *C. roseus*. © 2020 Friends Science Publishers

Key words: *Catharanthus roseus*; Terpenoid indole alkaloid; Plant hormones; *Myc1* transcription factor; The exogenous NO

Introduction

Many secondary metabolites synthesized by plants provide some valuable medicinal compounds. *Catharanthus roseus* (L.) belongs to the Apocynaceae family and produces more than 130 Terpene Indole Alkaloids (TIAs). These include vinblastine (VBL), vincristine (VCR), vindoline (VIN) and catharanthine (CAT), which have medicinal value. VBL and VCR can produce effective antitumor compounds used in the treatment of several types of cancer (Verma *et al.* 2007). These alkaloids are in low contents in leaves and other tissues (Mujib *et al.* 2014). Therefore, the alkaloids depend on a large amount of plant material, tissue cultures and chemical synthesis which are not economical or feasible to meet the commercial demand (El-Sayed and Verpoorte 2007). For this reason, field scale cultivation in *C. roseus* continues to be the only economic way to alkaloids source (Andrade *et al.* 2013).

Terpene Indole Alkaloids (TIAs) biosynthesis pathway has been widely studied and regulated by key enzymes (El-Sayed and Verpoorte 2007). A number of genes of TIAs metabolic pathway and transcriptional regulators have been cloned and characterized in *C. roseus* (Liu *et al.* 2017). The high expression of genes encoding some TIA pathway key enzymes cannot be completely increased alkaloids production (Pollier *et al.* 2014). A very promising method is to enhance the expression of several transcription factors (TFs) regulating the TIA pathway and enhance alkaloid production (Andrade *et al.* 2013). It has been reported that the expression of *Myc1* has positive regulation effect in secondary metabolic synthesis during environmental stress (Guo *et al.* 2016). *Myc1* is a basic transcription factor containing a conserved domain of the helix-loop-helix which belongs to the transcription enhancer.

Secondary metabolites are synthesized or induced by various developmental, hormonal and environmental factors (Liu *et al.* 2017). Evidence has been obtained that the TIAs accumulation in the plants of *C. roseus* was related to environmental factors (Xiao *et al.* 2013; Zhu *et al.* 2016). As a signal of plant morphogenesis, light strongly affects the secondary metabolism of plants. It has been reported that plant growth and the production of these metabolites compounds under light-shading environment can be significantly promoted (Lamattina *et al.* 2003). Panda *et al*.

(2011) have reported that exogenous nitric oxide (NO) can alleviate various stresses in plants. NO is a free radical and takes part in many plant physiological processes (Yu *et al.* 2005) including plant growth, development, and defense responses (Xiong *et al.* 2010). Recently, the relation of NO and plant growth hormones has been reported (Shen *et al.* 2013), but little information is available about the changes of plant hormones and TIAs biosynthesis accumulation under the light shading stress. The present study evaluated the relation of exogenous NO and the secondary metabolism in *C. roseus* under light-shading environment.

Materials and Methods

Plant materials and treatments

The study plant for experiment was *C. roseus*, and plants were exposed light-shading treatments at 45 days after sowing while the sun shading rate was 55–60%. A uniform concentration (0.01 mmol·L^{-1}) of sodium nitroprusside (SNP) was sprayed to soil surface; water spray of equal volume was used as a control. Treatments included the following: normal growth as control; application of SNP; light-shading; light-shading and with application of SNP, and the similar plants were treated by different conditions for 7 days. Three replicates were analyzed. Fresh leaves samples were frozen in liquid nitrogen and immediately kept at -80°C for further analyses.

Determination of plant growth and yield

The plants for growth assay were uprooted and carefully washed and adhered water particles were removed from roots. Then the fresh weight per plant, and length of shoot and leaf was measured. The plants were dried for 24 h with at 75°C) for dry weight. Leaf-area index (LAI) was measured by using the following formula as suggested by Watson (1947).

LAI =Leaf-area per plant/soil area occupied by plant

Determination of plant hormones content

The plant hormones including IAA, 6-BA and ABA contents were determined by high performance liquid chromatography (HPLC) method. The hormones from fresh leaves were extracted by methanol. The chromatographic conditions used were as follows: C18 silica gel column (250 mm × 4.6 mm, 7 μm), mobile phase: methanol-0.6% acetic acid (V:V=50:50), column temperature: 35°C, flow rate: 1 mL / min, volume flow rate: 10 μL, wavelength: 254 nm.

Estimation of alkaloids content

The analysis for alkaloids contents (Vindoline, VIN; Catharanthine, CAT; Vinblastine, VBL and Vincristine, VCR) in leaves, were performed by HPLC (Jasco, VG, England). The methanol extracts were dried at 50°C in a rotary evaporator. The chromatographic conditions were as follows: C18 silica gel column (250 mm × 4.6 mm, 7 μm), the mobile phase A was the mixture of deionized water and diethylamine (V_{H_2O}: $V_{diethylamine}$= 990:10, pH 7.3 with phosphoric acid); the mobile phase B was methanol, column temperature: 28°C, flow rate: 1.2 mL/min, volume flow rate 10 μL, wavelength: 220 nm.

RNA isolation and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from the leaf samples using TRIzol reagent (Sangon, Shang hai, China), and was stored at -80°C. The first-strand cDNA synthesis was performed by using the BeyoRT™ II cDNA (Beyotime, Nanjing, China), and was used in RT-qPCR reactions. Each reaction contained a mixture of 1 μL cDNA aliquots, 1.5 μL of mixed primers, 5 μL of BeyoFast™ SYBR Green qPCR Mix (Beyotime, Nanjing, China) and 11 μl nuclease-free water. The list of primer pairs used for the RT-qPCR is shown in Table 1. The RT-qPCR amplification was carried out in 96-well plates on a LightCycler® 480II System (Roche, Switzerland Roche Diagnostics, Indianapolis, I.N., U.S.A.). The cycling parameters consisted of a 95°C hold for 3 min, followed by 40 cycles of a 95° C denaturing step for 15 s and a 49–52.5°C annealing/extension step for 30 s, and PCR reactions with water were also carried out as negative controls. Each primer analyzed was performed in triplicate, the relative gene expression was quantified according to Schmittgen and Livak (2008) method. The primer of Ribosomal 40S protein S9 (RpS9) was used (Li *et al.* 2015) as internal control.

Statistical analysis

The data obtained were analyzed using Excel 2007 and S.P.S.S. 19, and the differences in the expression levels were tested by Duncan's multiple-range test. All the results were replicated three times, respectively.

Results

Growth and yield

There was significant increasing in growth traits in contrast to the CK, the change trend was light-shading and with application of SNP > light-shading > application of SNP > control; the change trend of node spacing, stem diameter and fresh weight was light-shading and with application of SNP > application of SNP > light-shading > control (Table 2). Compared with CK, the treatment of light-shading and with application of SNP significantly promoted the growth index of *C. roseus*, which indicated that exogenous NO significantly regulated the growth and development of *C. roseus* under shading stress.

Table 1: The primers were used in this work

Primer	Primer sequences (5'-3')	Amplified size (bp)	Gene ID
Myc1	CCT CAT TCA TGG CAT TGG C GTT TCC GAT GAA CAG CGC TAC	250	AF283506.2
40S	GGT TGT CAA TGT TCC TTC CTTC TCT TCA TCC TCT TCA TCT CCA TC	167	AJ749993.1
G10h	GTA CAG GAA CTA ATT GCG TAT TGC CGA CGT CAA CCG CTT CTC	106	AJ251269
Tdc	AAA ATG TTC GAA GAA TGG GTT AGA GTT TCT CGG TAC CAC AAT TTC G	109	X67662
Str	TGT GAG AAC AGC ACC GAT CC TTG TGG CTA GTTGTG TGG CA	157	X53602
Sgd	CAT TGG TGA ACC GTG CTA TG AGA TTG TAG AGT CCA GAT GGA ACA	121	EU072423
Dat	CAC GGT ATC AGG GAA ATC AG CTG GAA ATG GCA AAG ATT GG	142	AF053307

Table 2: Effects on growth morphological indexes of *C. roseus* seedlings

Treatments	Control	Application of SNP	Light-shading	Light-shading + SNP
Plant height (cm)	15.40 ± 1.33^{a}	16.80 ± 1.93^{a}	17.40 ± 1.29^{a}	20 ± 1.58^{b}
Leaf number per plant	6.67 ± 0.67^{a}	7.67 ± 1.45^{a}	7.33 ± 0.67^{a}	8.67 ± 1.76^{b}
Leaf length (cm)	6.56 ± 0.16^{ab}	5.78 ± 0.25^{b}	6.06 ± 0.43^{ab}	6.90 ± 0.41^{a}
Leaves wide (cm)	2.40 ± 0.19^{a}	2.32 ± 0.11^{a}	2.34 ± 0.14^{a}	2.50 ± 0.08^{a}
Radio of leaf length to width	2.79 ± 0.18^{a}	2.51 ± 0.16^{a}	2.60 ± 0.14^{a}	2.77 ± 0.19^{a}
Leaf area (cm^2)	9.20 ± 1.11^{a}	9.00 ± 0.45^{a}	8.80 ± 0.49^{a}	9.40 ± 0.6^{a}
Node spacing (cm)	0.92 ± 0.04^{c}	1.18 ± 0.07^{ab}	1.08 ± 0.04^{b}	1.30 ± 0.06^{a}
Stem diameter (cm)	0.25 ± 0.02^{c}	0.34 ± 0.02^{b}	0.30 ± 0.01^{bc}	0.52 ± 0.02^{a}
Whole plant fresh weight (g)	3.76 ± 0.07^{b}	3.38 ± 0.04^{bc}	2.96 ± 0.05^{d}	5.36 ± 0.26^{a}
Whole plant dry weight (g)	0.62 ± 0.03^{b}	0.60 ± 0.03^{b}	0.55 ± 0.04^{b}	0.80 ± 0.1^{a}

Different lower case letters indicate significant differences ($P < 0.05$) T1, application of SNP; T2, light-shading; T3, light-shading and with application of SNP. Same as below

Plant hormones content analyses

The IAA, 6-BA and ABA contents were increased significantly ($P < 0.01$) in comparison to CK that showed the exogenous NO increased hormone contents in the treatment of application of SNP and light-shading and with application of SNP. Under shading stress, the ABA content only increased significantly ($P < 0.05$) in comparison to CK. Plant hormone might improve plant metabolism, led to enhanced plant growth and production (Fig. 1).

Alkaloids content

Vindoline (VIN), Catharanthine (CAT), Vinblastine (VBL) and vincristine (VCR) contents in the leaves were slightly increased under application of SNP and light-shading treatment, but the VCR content significantly changed. The contents of VIN and CAT increased significantly ($P < 0.01$) under light-shading and with application of SNP treatment, which were 4.65 and 6.13 times compared to control, respectively. The contents of VBL and VCR also increased significantly ($P < 0.05$), which were 2.48 and 1.64 times compared to control, respectively (Fig. 2a–d).

TIA biosynthetic genes mRNA levels

The expression of transcription factor (Myc1) was down-regulated ($P < 0.05$) significantly under application of SNP and light-shading treatment (Fig. 3a) in comparison to control, and up-regulated ($P < 0.05$) significantly under light-shading and with application of SNP treatment (Fig. 3a). It showed that the expression of the transcription factor was activated under compound stress (light-shading and with application of SNP). In the treatment of light-shading and with application of SNP, the expression of G10h, Tdc and Str were significantly up-regulated ($P < 0.05$) (Fig. 3b–f). It indicated that compound treatment can promote the high expression of the upstream pathway genes during alkaloid synthesis. In the treatment of application of SNP, the expression of Sgd and Dat increased significantly ($P < 0.05$), suggesting that exogenous NO promoted the high expression of downstream pathway genes. In a word, the exogenous NO significantly regulated the expression of TF and key genes in the pathway under light-shading stress.

Correlation analysis

Correlation analysis between hormones content, alkaloids content and key genes expression in leaves of *C. roseus* seedlings showed that there was no correlation between Myc1 expression and plant hormone accumulation in the treatment of control (Table 3); There was a negative correlation between plant hormone accumulation and Myc1 expression, between Myc1 expression and Tdc, Str, Sgd and Dat in the treatment of application of SNP and light-shading (Table 3). But there was a significant positive

Table 3: Correlation coefficient (r) between accumulation of alkaloids, plant hormones and genes expression

Hormone	Control						Application of SNP						Light-shading						Light-shading + SNP					
	Myc1	*G10h*	*Tdc*	*Str*	*Sgd*	*Dat*	*Myc1*	*G10h*	*Tdc*	*Str*	*Sgd*	*Dat*	*Myc1*	*G10h*	*Tdc*	*Str*	*Sgd*	*Dat*	*Myc1*	*G10h*	*Tdc*	*Str*	*Sgd*	*Dat*
6-BA							-0.985**										0.813*		0.983**					
IAA													-0.996**											
ABA							-0.988**						-0.998**						0.977**					
CAT										0.854*	0.876*	0.861*				0.814*	0.901*	0.842*	0.992**	0.830*	0.986**	0.997**	0.999**	0.999**
VIN								-0.865*			0.864*	0.875*		-0.861*	-0.820*	0.850*	0.858*	0.883*	0.987**	0.845*	0.984**	0.994**	0.997**	0.999**
VBL			1.000**																0.975**	0.862*	0.991**	0.984**	0.993**	0.992**
VCR								-0.858*		0.874*	0.872*	0.875*							0.982**		0.955**	0.976**	0.975**	0.966**

**Significant correlation at $P < 0.01$, * Significant correlation at $P < 0.05$

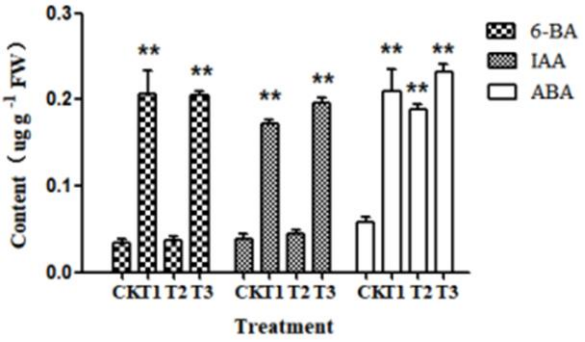

Fig. 1: Effects on the contents of 6-BA, IAA and ABA under four different treatments in *C. roseus* leaves (T1, application of SNP; T2, light-shading; T3, light-shading and with application of SNP. Same as below)
Data points are mean ± SD of three biologically independent experiments. ** Significant differences with $P < 0.01$, * Significant differences with $P < 0.05$, same as below

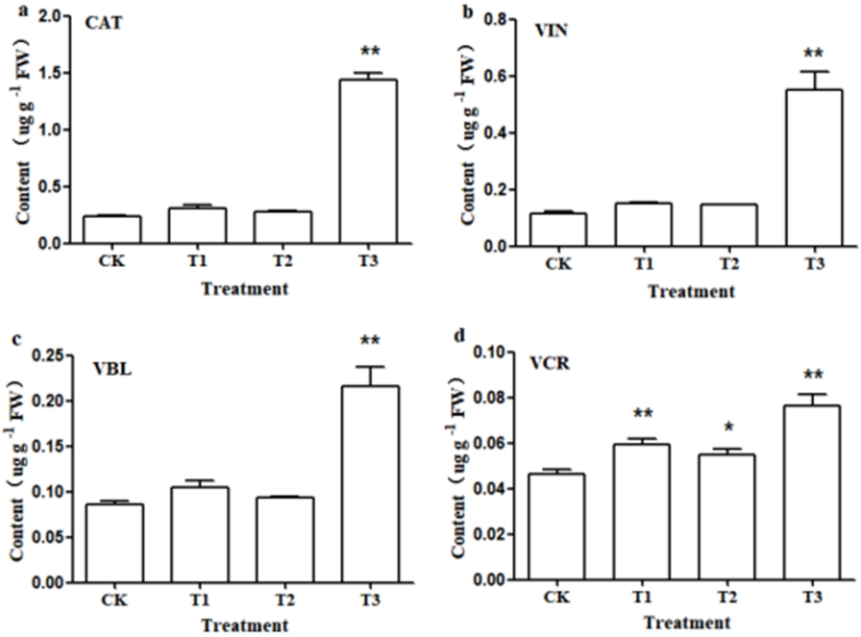

Fig. 2: Effects on alkaloids content in *C. roseus* leaves under four treatments

correlation between 6-BA and ABA content and Myc1 expression ($P < 0.01$) in the treatment of light-shading and with application of SNP (Table 3), and there was a significant positive correlation between Myc1 expression and the expression of Tdc, Str, Sgd and Dat ($P < 0.01$) (Fig. 4). It has been revealed that Myc1 were almost negative regulators in the treatment of application of SNP and light-shading, whereas Myc1 were positively regulators (marked in coarse arrow) in the treatment of light-shading and with application of SNP (Fig. 4). Furthermore, there was a significant positive correlation ($P < 0.01$) between the expression of Tdc, Str and the CAT content (Table 3) in the

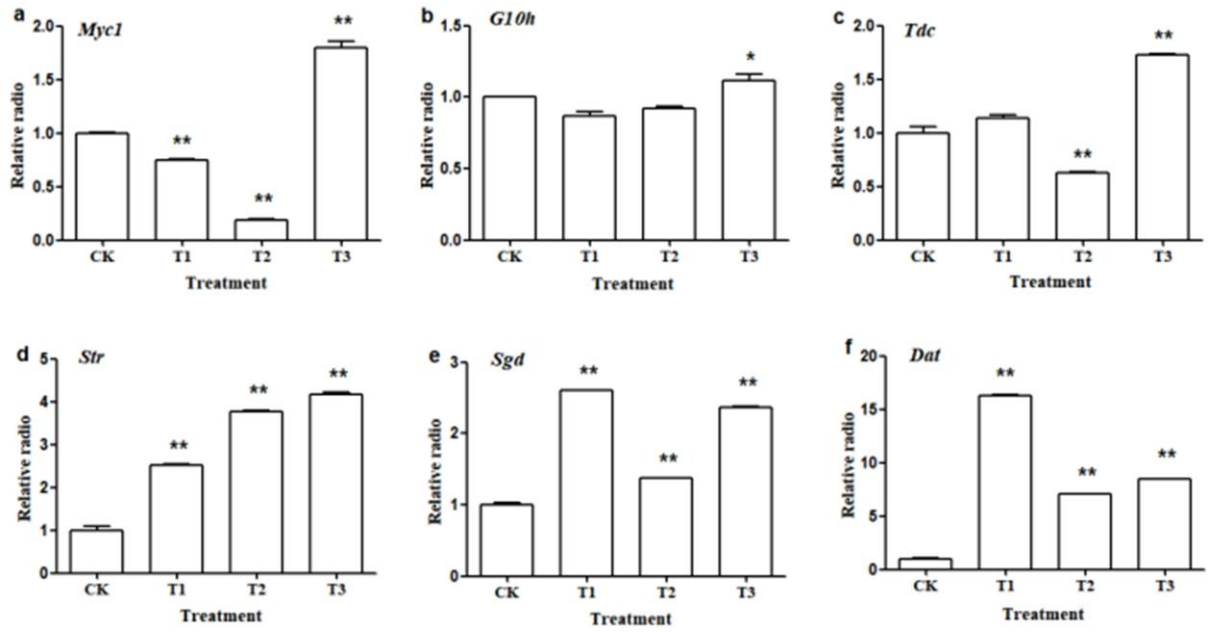

Fig. 3: The expression changes of *Myc1* and the TIA pathway genes were detected under the four treatments (40s as internal control, CK as control)

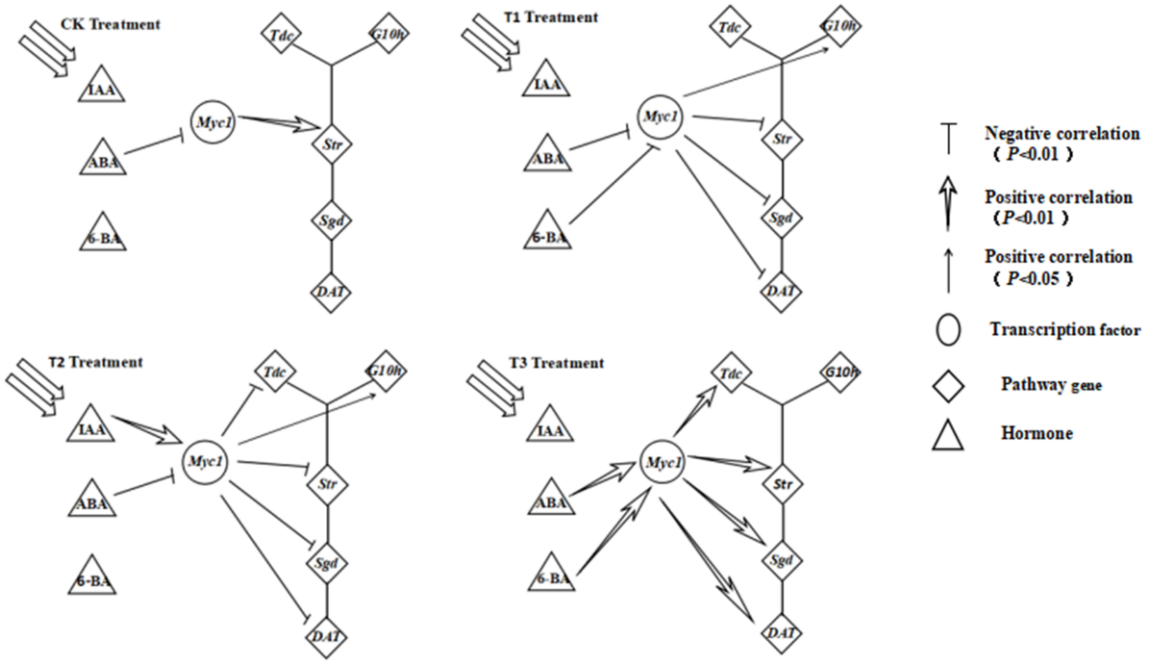

Fig. 4: Regulation of TIA biosynthetic pathway genes by transcription factor in *C. roseus*

treatment of light-shading and with application of SNP, between Dat expression and the VIN content, and between Sgd expression and the content of VBL and VCR. These results suggested that hormones, as endogenous signals, induced the expression of Myc1, regulated the high expression of key genes in the metabolic pathway of alkaloids, and promoted the synthesis of VBL and VCR (Fig. 2).

Discussion

As an internal factor, plant hormones play an important role in regulating plants growth and development. plant hormones regulated the genes expression through the modification of transcription and/or translation, and determined plant growth orientation, physiology, and productivity (Alam *et al.* 2012). Previous studies have

shown that 6-BA, as a cytokinin, promoted the differentiation and growth of multiple tissues, and had a synergistic effect with IAA (Chen *et al.* 2004). Similarly, Zhang *et al.* (2018) reported that ABA content could regulate the synthesis of indole alkaloids by promoting the expression of CrTdc, CrNmt and CrD4h in C. roseus. The plant hormones content changes manipulated secondary metabolism and affected alkaloids accumulation in the specific tissue (Srivastava and Srivastava 2007). Recently, many articles have reported that there is the relativity of exogenous NO treatment and the plant hormones content changes (Shen *et al.* 2013). In this study, the changes of the content of ABA, 6-BA and IAA were analyzed under the condition of light-shading + SNP which increased the ABA, 6-BA and IAA contents, as it was beneficial to increase the resistance of plants and regulate the growth, development and biomass (Table 2), and promote the synthesis of alkaloids.

The alkaloids biosynthesis is directly regulated at the transcriptional level. Evidence has been shown that transcription factors (TFs) play a critical role in regulating the synthesis of TIAs in C. roseus (Zeng *et al.* 2017). Myc1 expression could be up regulated by the induction of jasmonate (JA) or fungal elicitor and activate the transcription of Str and Tdc (Verma *et al.* 2012). There is little doubt that TIA biosynthesis does benefit from the key genes expression in biosynthesis pathway (Liu *et al.* 2011). Further studies have shown that that the Tdc expression was positively correlated with the content of CAT; Str expression was involved in VIN biosynthesis (Pandey *et al.* 2016). The mechanisms for promoting alkaloid synthesis include the combination of transcription factors and specific elements and/or regulation of the corresponding genes expression. This study reported that there was a transcriptional regulatory network of TFs in TIA biosynthetic pathways, and a significant enhancement in the alkaloid production under the compound treatment (light-shading and with application of SNP). It is a positive role for plant hormones (6-BA and ABA) in regulation of Myc1 expression, because TFs was usually regulated by signaling molecules or other elements (for example, plant hormones) (Gao *et al.* 2015), and then could activate the expression of Tdc and Str, there was a significantly positive correlation ($P < 0.01$) between the genes (Tdc and Str) expression and the alkaloids content (Table 3), then VBL and VCR content accumulation could be promoted.

Conclusion

This study evaluated that application of light-shading and SNP improved considerably alkaloids contents. The role of Myc1 transcription factor (TF) in plant hormones signaling and plant alkaloid biosynthesis had been systematically analyzed in C. roseus. Notably, there was a close correlation between NO and plant hormones by inducing high expression of Myc1 gene, then high expression of TIA synthetic genes, such as Tdc、Str、Sgd and Dat, promoted TIA biosynthesis.

Acknowledgements

The work was supported by the Foundamental Research Funds for the Central Universities (No. 2572019BU01, 2572017BA07); the National Key Research and Development Program of China (No. 2017YFD0600706) and Heilongjiang Province Natural Science Fund Project (No. QC2017009)

Author Contributions

Yu-jie Fu and Shu-ping Guo; methodology, Ying Liu; software, Chang Yang; validation, Yu-jie Fu, Hai-long Weng and Shu-ping Guo; formal analysis, De-wen Li; investigation, Ying Liu and Chao Yang; data curation, De-wen Li; writing—original draft preparation, Ying Liu; writing—review and editing, De-wen Li; supervision, Yu-jie Fu; project administration, Ying Liu; funding acquisition, De-wen Li and Ying Liu. All authors have seen the manuscript and approved to submit to your journal.

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