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INFLUENCE OF YEAST EXTRACT ON GENE EXPRESSION OF *CRWRKY1* AS A REGULATORY GENE IN ALKALOIDS PATHWAY IN *CATHARANTHUS ROSEUS* (L.) G. DON

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ABSTRACT

Catharanthus roseus (L.) G. Don is a medicinal plant rich in many alkaloids that are used in treatment of many diseases. It is unique in the production of vinblastine and vincristine compounds, which are used in treatment of several cancers. There are many genes in the biosynthesis pathway that produce these anti-cancer compounds, including the gene *Crwrky1* which is a regulatory gene that codes for the transcription factor in the monoterpene indole alkaloid (MIA) pathway. In this study, the effect of yeast extract on *crwrky1* gene expression levels from treated *calli* was studied. Quantitative RT-PCR (QRT-PCR) using SYBR Green I/ROX was used to analyze the changes in the expression level of this gene in response to different treatments of yeast extract. *Crwrky1* expression increased to 3.6 folds in treated callus obtained under 0.4 mg/l yeast elicitor for 4 hours (YE2) treatment to compare with the control (untreated) callus.

INTRODUCTION

Catharanthus roseus (L.) G. Don belongs to the family *Apocynaceae*, and is a solo source of vinblastine (VBL) and vincristine (VCR), mainly present in the aerial parts. All terpenoid indole alkaloids (TIAs) compounds originate from the central compound strictosidine, a condensation product of the monoterpenoid alkaloid (MIA) compound, secologanin and tryptamine compounds, due to the action of strictosidine synthase encoded by *str* gene. In the end of biosynthetic pathway, combinate between two monoterpenoid to get dimeric compounds (VBL and VCR). These compounds have a high economic value in cancer drugs **(Singh & Jagdev, 1996 and Facchini & De Luca, 2008).**

Plant produces these component as a response to pathogen attack by induction of various defense responses for these biotic or abiotic stresses, including the biosynthesis of protective secondary metabolites. In *C. roseus*, the fungal elicitor-induced (like yeast extract) expression of the TIAs biosynthetic gene. A principal elicitor of TIA production in *C. roseus*, as well as natural products in many other medicinal species, is the phytohormone jasmonate, which functions in plant defense signaling to protect the plant from biotic stresses (**Roepke et al 2010 and Jaleel et al 2007).**

Yeast extract (YE) activates the production of Reactive oxygen species (ROS) which is dependent on protein phosphorylation and calcium influx, these proteins are necessary for the activation of the octadecanoid pathway and TIA biosynthetic genes by YE **(Menke et al 1999a and van der Fits et al 2000).** Overproduction of ROS caused by stress conditions in plant cells is highly reactive and toxic to proteins, lipids, and nucleic acid which ultimately results in cellular damage and death **(Gill and Tuteja, 2010).** that are significantly accumulated under stress conditions, which cause oxidative damage and eventually resulting in cell death.

In *C. roseus*, *Crwrky1* gene has been demonstrated to respond to jasmonate, ethylene, and gibberellin signaling to regulate TIA production **(Suttipantaa et al 2011).** In plants, WRKY proteins are critical regulators that are implicated in various

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molecular events in plants, such as seed development, senescence, dormancy and germination, and abiotic and biotic stresses among others **(Rushton et al 2010).** Many members of the WRKY family are related to pathogen infection and thus are crucial factors for plant immunity. Some WRKY protein partners (like another protein, such as sigma factor) have already been identified, and the interactions between WRKY and its binding partners may play roles in signaling, transcription, chromatin remodeling, and other cellular processes **(Chi et al 2013).** For example, The AtWRKY33 protein in *Arabidopsis* plays a significant role during infection by *necrotrophic* pathogens and is a part of the group I WRKY family. AtWRKY33 interacts with the proteins sigma factor-interacting protein 1 and 2 **(SIB1 and SIB2) (Zheng et al 2006; Lai et al 2011).**

Over-expression of *Crwrky1* gene increased the production of serpentine, while simultaneously decreased catharanthine accumulation, suggesting, that this *Crwrky1* gene may function in governing gene expression that specifically directs the flow of metabolites to synthesize TIAs in *Catharanthus* roots **(Schluttenhofer et al 2014).**

The production of secondary metabolites by tissue culture systems has become an active field of study because of its potential as a source of valuable pharmaceutical compounds to produce large-scale of these **(Tikhomiroff & Jolicoeur, 2002 and Mulabagal & Tsay, 2004). Pauw et al 2004** found in *Catharanthus*, the yeast extract induced a 6-fold increase in the production of ROS compared to untreated cells. However, in this research aims to study the influence of yeast extract (0.4mg/l) to increase of another gene in Catharanthus such as *Crwrky1* gene in terms of expression levels through three periods treatment (2, 4 and 6h).

MATERIALS AND MATHODS

Callus initiation and treatments

The Egyptian *C. roseus* var. roseus that has a red purple flower was used in this study as a source of explants for callus initiation. Mature leaves collected from the herbal garden, Faculty of Agriculture, Ain Shams Univ., were used leaves were surface sterilized under aseptic conditions of laminar flow hood through were washed under running tap water for 30 min and then treated with 12 % H_2O_2 for 5 min, then washed with sterile dd H_2O. The callus initiation treatment with in medium was contained (1-mg/l 2, 4D with 0.1-mg/l Kinetin and 30-g/l sucrose) and transversal leaf sections proved to be the best explant, which produced healthy callus on MS medium (Murashige and Skoog,1962), average of pieces of each jar is around 3-4. the jar was contented 20 ml according to **Moghazee et al 2014.**

After initiation, callus was transferred into treatment medium supplemented with 0.4-mg/l of YE modified and three treatment periods (2, 4 and 6 hours) were applied to determine their influence on alkaloid formation in *C. roseus* callus cultures **(Zhao et al 2001).** These treatments were designated: W1 (YE of 0.4 mg/l at 2h), W2 (YE of 0.4 mg/l at 4h) and W3 (YE of 0.4 mg/l at 6 h) as shown in **Figure (1).**

Fig. 1. Callus was produced, then callus was transferred into the liquid medium treatment 0.4 mg/l of YE

RNA isolation and quantitative real time QRT-PCR

RNA extraction to need 0.1- 0.5 g callus, total RNA from the three treated and the untreated *C. roseus* calli were DNase-treated prior to cDNA synthesis (Chomczynski, 1993). Synthesis of first strand cDNA from total RNA was isolated from plant tissue and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed as previously reported **(Suttipantaa et al 2007)** Superscript III 1st Strand Synthesis Kit (Invitrogen, Cat #18080-051) was typically used 1-5 µg RNA to synthesize 20 µl cDNA per reaction, Real-time PCR was carried out using the Agilent Mx3000P QPCR systems (Agilent technology, USA). PCR products were separated on 2% agarose gel and visualized after staining with ethidium bromide **(Liu et al 2011 and Moghazee et al 2014)**

Primer design for real time PCR analysis

Both forward and reverse primers, were designed based on the known nucleotide sequences obtained from the GenBank database for this regulatory gene *Crwrky1* gene (GenBank Acc. HQ646368.1) of *C. roseus*. One of the most important criteria to take into account is when designing a primer, is the targeting of relatively small amplicon size of not more than 250 bp. Primerquest software was used to design the primers (http://eu.idtdna.com/Primerquest/Home/Index). This experiment was repeated 3 times for each treatment. Primer pairs of the endogenous reference gene, actin (*CrActin*) gene of *C. roseus* (GenBank Acc. No. DQ117850.1) shown in **Table (1)**. The designed primers were synthesized by Metabion, Germany. PCR products were separated on 2% agarose gel and visualized after staining with ethidium bromide as described by **Liu et al (2011).**

The ΔΔCT calculation for the relative quantification of target gene was used as follows; ΔΔCT = (CT, target gene - CT, *CrActin*) χ - (CT, target gene - CT, control) y, where χ = treated sample and y = control sample. After validation of the method, results for each sample were expressed in N-fold changes in χ target gene copies, normalized to *CrActin* relative to the copy number of the target gene in control, according to the following equation: amount of target = $2^{-\Delta\Delta Ct}$ (**Livak and Schmittgen, 2001)**

Table 1. Endogenous gene - primer sequences of *Crwrky1* and *CrActin* for Real time PCR.

Gene	*Primer*	
wrky1	F:	GGATTCTTGCCTCCTCAGTTT
	R:	TCCTTTGTTACGGCCACTATTAC
CrActin	F:	GTTCCCAGGTATTGCAGATAGAA
	R:	GCCTCCAATCCACACACTATAC

***F= Forward, *R= Reverse Primer**

RESULTS AND DISCUSSION

Establishment of tissue culture system for Egyptian *C. roseus*

Plant hormones, like animal hormones, are relatively small molecules that are effective at low tissue concentrations. The two types of plant hormones used in this experiment are cytokinins and auxins. cytokinins are derived from adenine and produce two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increased cell division. Auxins are indole or indole-like compounds that stimulate cell expansion, particularly cell elongation **(Ting, 1988).**

The best callus produced of *C. roseus* for the biotic induction study was obtained at 1 mg/l 2, 4-D with Kin at 0.1mg/l. The healthy callus with appropriate size, shape and his color is off white callus with friable texture in all replicates was found to be the best when this callus transferred into the liquid medium treatment supplement with YE, it starts disintegrating easily making good suspension culture

Transcriptional profiling of *Crwrky1* gene as a regulatory gene under different periods of YE treatment

In other plant species a network of transcription factors (TFs) is involved in the regulation of this pathway, including members of the AP2, AP2/ERF, bZIP, MYB, MYC, NAC, WRKY, and YABBY families (**De Geyter et al 2012; Patra et al 2013; Nieuwenhuizen et al 2015 and Wang et al 2016).**

The jasmonate responsive *Crwrkys* are potential candidate Transcription Factors (TFs) for playing key roles in modulating jasmonate signaling and regulating TIA biosynthesis. Information on how *Arabidopsis WRKY (Atwrky)* gene responds to various phytohormones and stresses may also apply to Catharanthus. This information may be useful for understanding how other phytohormones also contribute to the regulation of TIA production. Moreover, elucidation of *Crwrky* functions may provide valuable insights into the regulation of natural products biosynthesis in other medicinal plants **(Schluttenhofer et al 2014).** MeJA was shown to stimulate ROS production in tomato leaves. In contrast, in C. roseus cells MeJA treatment did not stimulate ROS production, suggesting that the induction of ROS production by Yeast extract (YE) would be upstream or independent of the induction of the octadecanoid pathway by YE **(Orozco-Ca´rdenas and Ryan, 1999).**

Wang et al (2015) reported a multiple stress-responsive WRKY gene, *G. Max* WRKY27 (GmWRKY27), reduces ROS level and enhances salt and drought tolerance in transgenic soybean hairy roots. The conserved WRKY domain plays significant roles in various physiological processes by linked to the W-box in the promoter regions of target genes **(Ulker and Somssich, 2004 and Rushton et al 2010).**

YE was shown to induce an increase in cytosolic calcium levels, which was necessary for the induction of Jasmonate acid (JA) accumulation and Strictosidine synthase *(str)* and tryptophan decarboxylase (*tdc)* gene expression **Menke et al 1999a and Memelink et al 2001.** Menke and Memelink reported YE was shown a transient increase in calcium levels in *C. roseus* cells. The JA responsive *Crwrky*1 gene may be important for regulating the production of valuable TIAs **(Schluttenhofer et al 2014).**

The PCR amplification results are shown in **Fig. (2)** with a fragment size 115 bp for both *Crwrky1* and *Cr-Actin,* as expected from the designed primers. Thermo Scientific GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bp was used

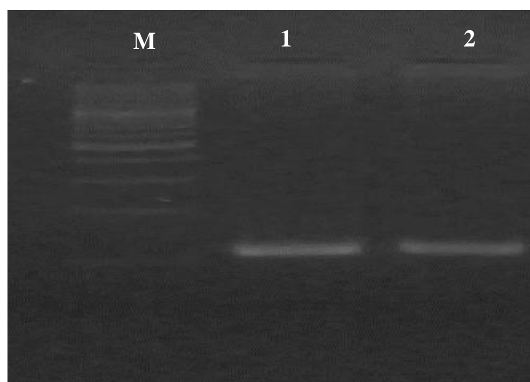

Fig.2. PCR reaction to confirm primers for the target genes; No.1 = *wrky1* and No.2 = *Cr-Actin.*

Quantitative RT-PCR (QRT-PCR) using SYBR green I/ROX was used to analyze the changes in the gene expression level of *Crwrky1* in response to different treatments of YE. YE was shown to induce high levels of *Crwrky1* gene expression a 3.6-fold increase in the production *Crwrky1* between treated and untreated (control) of *C. Roseus*, which was determined by the comparative ΔΔCT method. Positive values of ΔΔCT were detected in two cases only among the three treatments; W1 and W2 indicating up-regulation of this gene under these two treatments. Gene expression of *Crwrky1* increased when *C. roseus* callus was treated with 0.4 mg/l YE for 4 h (W2) to 3.6-fold, while it was only to1.6x folds when callus was treated with 0.4 mg/l YE for 2 h (W1). Despite, W3 indicating down-regulation of this gene under treatment by 0.4 mg/l YE for 6h, which agreed with **Schluttenhofer et al (2014)** who suggested that this *Crwrky1* gene may function in governing gene expression that specifically directs the flow of metabolites to synthesize TIAs in *Catharanthus*, as shown in **Table (2)** and **Figure (3)**.

Once the cell death in at the later stage of the infection process, the cell will eventually lead have an increased catabolism capacity and may lose the ability to produce secondary metabolites. This component plays important roles in plant to fungal pathogens induction. Mutation of key enzymes in the secondary metabolites biosynthetic pathway compromises resistance, especially *wrky* gene is important to the activation of genes in secondary metabolites (like VBL & VCR) biosynthetic pathway, this speech is compatible with **(Glazebrook et al 1997; Thomma et al 1999; Zhou et al 1999; Ferrari et al 2003, 2007 and Nafisi et al 2007).** Combined with the genetic evidence that the Mitogen activated protein kinases (MAPKs) phosphorylation sites are required for the complementation of the wrky33 mutant phenotype, we can conclude that MPK3/MPK6 phosphorylation of WRKY33 is important to the activation of camalexin biosynthetic pathway **(Kim and Zhang, 2004; Liu & Zhang, 2004 and Ren et al 2008).**

Finally, knowledge of the regulation via elucidation of *Crwrky1* gene regulating specialized metabolite production could be a valuable approach for future metabolic engineering of terpenoid indole alkaloid productivity projects to increase production of pharmaceutically valuable TIAs

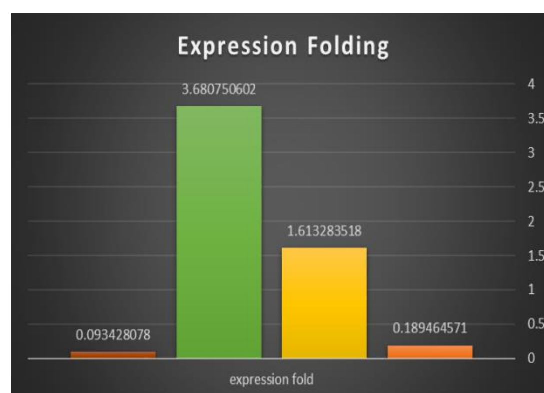

Fig. 3. The folding levels of *Crwrky1* gene expression in *C. roseus*. 2h,4h and 6h from 0.4mg/l of YE treatments

Table 2. The level of *wrky1* gene expression between treated and untreated (control) of *C. roseus* determined by the comparative $\Delta\Delta C_T$ method

Genes	Treat.	C_t	ΔCt	ΔΔCt	Folding
wrky1	Control	27.54	-2.4	0	0.189464571
	W1= YE of 0.4 mg/L at 2h	25.05	-0.69	1.8	1.613283518
	W2= YE of 0.4 mg/L at 4h	25.63	-1.88	0.61	3.680750602
	W3= YE of 0.4 mg/L at 6h	27.07	3.42	-5.91	0.093428078

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