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Response of *Cayratia trifolia* towards Pb, NaCl, Diesel and Wounding Stresses through Expression of a *CtSRG1* Gene

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ABSTRACT

By-products of the petroleum industry contaminate the environment, hence decreasing the soil fertility. However, in some contaminated areas, plants such as *Cayratia trifolia* continue to survive despite the harsh environment. Therefore in this study we aim to investigate the survival of *C. trifolia* at a petroleum refinery, PETRONAS Penapisan Melaka Sdn. Bhd (PPMSB). The main objective of this study was to identify and characterize candidate gene involved in the stress response. Differential display approach was performed on *C. trifolia* grown on soil and sludge, to identify up-regulated and down-regulated partial cDNAs. Out of 23 cDNAs checked, 18 genes were up-regulated genes while five were down-regulated. A partial gene (244 bp) represented by DEG7 fragment has a high similarity to the translocon. Blast2GO analyses was performed and showed DEG7 as a gene responsible for stress responses. A fragment of 1371 bp named as the *CtSRG1* gene was succesfully amplified using the combination of RACE-PCR and degenerate PCR amplification. BlastN and BlastX analyses indicated that the *CtSRG1* gene had higher similarity to translocon. CtSRG1 protein consists of Rieske and SRPBCC ligand-binding domains. The expression profile of *CtSRG1* gene using qRT-PCR showed up-regulated expression when treated with Pb, NaCl and diesel but down-regulated with wounding treatment.

Keywords: Diesel, gene isolation, NaCl, Pb, stress-responsive gene, wounding

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INTRODUCTION

The root structure of a plant growing in normal and ideal environment tends to establish sufficient relationship with the soil matrix thus allowing an adequate supply of oxygen and water. However, if the roots grow in contaminated environments such as in petroleum sludge, the soil particles tend to be covered with a hydrophobic layer. This reduces the availability of water and requires high oxygen concentrations to survive hence creating a condition called water stress (Peña-Castro et al., 2006). Subsequently, the hydrophobic nature of the pollutant leads to various other abiotic stresses (Gogosz et al., 2010). Overall, the presence of environmental stresses in the sludge can interfere with the various systems in the plant, primarily preventing their absorption of water and nutrients (Kirchmann et al., 2017).

Petroleum sludge causes many adverse impacts on living organisms. Numerous studies have shown that plants grown around the area of petroleum sludge cannot thrive long. For example, germination and growth of *Vigna unguiculata* seedling was retarded in the area of high sludge concentrations (Sangeetha & Thangadurai 2014). In addition, Morales et al. (2012) showed that sludge also contained high concentrations of salt that consequently caused reduction of ion absorption including chloride (Cl^-) in rice. Sludges also contain heavy metals which can cause gradual decline in seedling height (Issoufi et al., 2006).

Interestingly, a liana plant known as *Cayratia trifolia* from the Vitaceae family was found to survive in areas contaminated with petroleum sludge (Singh et al., 2012). *Cayratia trifolia* is commonly known as 'bush grape', 'fox-grape', 'lakum' in Malaysia, 'kalit-kalit' in Philippines, 'galing-galing' in Indonesia, 'ta det' in Laos, 'thao kan khaao' in Vietnam and 'vualai' in Papua New Guinea (Gupta et al., 2012). Phytochemical investigation showed that the leaf of *C. trifolia* plants contained high levels of secondary metabolites such as kaempferol, myricetin, quercetin, triterpenes and epifriedelanol (Ragasa et al., 2014). The plant is widely used in traditional medicine for the treatment of wounding, anemia, stomachic diseases and diabetic. Additionally, the plant is reported to possess antibacterial, antiviral, antifungal, antiprotozoal, hypoglycemic, anticancer and diuretic activities (Kumar et al., 2011). Thus, *C. trifolia* was selected for this study because of its ability to defend itself when facing the stressful conditions created by the presence of petroleum sludge.

However, to date, there is still no conclusive molecular study to describe how the plant adapts in the harsh environment area. At a molecular level, stress defence in plants usually is influenced by a group of stress responsive genes (Agarwal et al., 2013). There are many stress responsive genes that function in controlling homeostasis when plants undergo stress involving ions shifts in the cell due to stress solute such as Pb, NaCl and diesel. When the plant is under environmental stresses, a polypeptide translocation across cell membrane occurs to balance the internal environmental condition of the plant cells (Pogson et al., 2015). These genes are also

needed to ensure balance and efficiency of protein importation during abiotic stress response (Sjuts et al., 2017). Therefore, this study was performed to investigate the molecular factors involved in the unique ability described.

MATERIALS AND METHOD

Plant Preparation

Cayratia trifolia plants growing in a petroleum refinery, PPMSB, Melaka, Malaysia were collected. Plants that were collected from sampling site were planted in greenhouse owned by Universiti Kebangsaan Malaysia, Selangor, Malaysia. The plants were placed in a white tank with a dimension of 2m x 1m x 0.3m. The plants were left to grow and reproduce for two months until the mother plants produce F1 generation was produced that was used for this study.

After two months of acclimatization process, *C. trifolia* was treated with Pb, NaCl, diesel and were also wounded. For each experiment, three replicates were undertaken for data accuracy. Plumbum (II) nitrate, $Pb(NO_3)_2$ was used throughout the experiments to induce Pb stress. Stock solutions of $Pb(NO_3)_2$ were prepared using distilled water, and diluted to Pb concentrations of 0.01, 0.3 and 0.6 g/kg. Whereas sodium chloride (NaCl) was used for salt treatment. NaCl solution with the concentration of 50, 100 and 300 mM were prepared by dissolving NaCl pellets with distilled water. As for diesel stress, fuel was obtained from a local gas station, PETRONAS. The diesel solution was mixed with acetone (1:1) as a solvent, and the final concentrations of 40, 70 and 100 g/kg were prepared by diluting the diesel solution with distilled water. Pb and NaCl treatments are carried out for 48 hours and the leaves were then harvested after 1, 24 and 48 hours of the treatments. Meanwhile, diesel treatment is carried out for 14 days where the *C. trifolia* were harvested after day 1, 7 and 14 of the treatments. Wounding stress of *C. trifolia* was carried out by crushing the leaves across the center of the vein using a sharp razor blade.

The leaves were then harvested after 1, 3, 6, 24 and 48 hours of the treatments. Experimental time intervals followed suggestion by Afzal et al. (2011), AL-Jobori et al. (2012), Park et al. (2011) and Shavrukov (2012) with some modifications. All these treatments are carried out separately on different plants.

Total RNA Isolation and Differential cDNA Display

Total RNA of *C. trifolia* was extracted using RNeasy Plant Mini Kit (Qiagen, Germany) according to the supplier's recommendation with some modification by (Yazid & Sidik, 2011). RNA extracted was used for first strand cDNA synthesis using SuperScript III FirstStrand Synthesis System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Invitrogen, USA) according to the manufacturer recommendations. Differential cDNA display was performed using GeneFishing™ DEG Premix Kit (Seegene) according to the instruction provided.

Cloning of Differentially-Displayed cDNA Fragments Into Cloning Vector

The differentially expressed (DEG) bands were individually excised out from the gel by using a QIAquick Gel Extraction Kit (QIAGEN, Germany), and were directly cloned into a yT&A cloning vector (Yeastern Biotech, Taiwan) according to manufacturer's protocol. Polymerase chain reaction (PCR) amplification was performed in a volume of 20 µl of 10× PCR Buffer (Promega) containing dNTP mix (10 mM), magnesium chloride (25 mM), *Taq* DNA polymerase (2.5 U), M13 forward primer (5'- GTTTTCCCAGTCACGAC-3') (10 pM) and M13 reverse primer (5'-GTCA TAGCTGTTTCCTGTGTGA) (10 pM). PCR was performed under the following conditions: preliminary denaturation (3 min, 95ºC), then 29 cycles of denaturing step (1 min, 95ºC), annealing step (1 min, 58ºC), extension (45 sec, 72ºC) and a final extension (10 min, 72ºC). As an additional screening step, the recombinant plasmids were double digested with *Eco*RI and *Bam*HI restriction enzymes (FastDigest®, Fermentas).

Functional Annotation and Classification

To provide insights into the putative function of the genes, all DEG sequences were compared with the non-redundant (NR) and UniProt databases (filter: E-value < 1e-5). The NR annotations of the resulting unigenes were searched with Blast2GO Gene Ontology (GO) functional classification algorithms. This software was used for GO functional classification for DEGs and to determine the distribution of the gene functions of species.

Stress-responsive Gene Isolation

The SMARTer™ RACE cDNA Amplification Kit (Clontech) was used in the amplification of 3' ends of *CtSRG1*. The experiments were conducted according to the recommendation protocols of the supplier using a set of primers, universal primer (UPM) '-AAGCAGTGGTATCAACGCAG AGT-3') and 17FGSP (5'-CGCTTCATGGGGGAGTG CGCTCGAGGTCG-3'). UPM primer was provided with the kit while the 17FGSP primer was designed from the original sequence of the DEG7 fragment. Meanwhile, a PCR-based approach was used to amplify the 5' region using the degenerated primers, PDGF1 (5'-GTKGTKTGGGAYAGGAAYGAR-3') and PDGR1 (5'-TTTGCXGCTT CAAXXTGGXTX-3'). The RACE-PCR cycles for amplification were as follows; 5 cycles of 94°C for 30 sec and 72°C for 3 min: followed by 5 cycles of 94°C for 30 ses, 70°C for 30 sec and 72°C for 3 min; then 25 cycles of 94°C for 30 sec and 68°C for 30 sec; followed by a 3 min final extension at 72°C. Meanwhile, the PCR degenerate amplification were; pre-denaturing at 95°C for 3 min, followed by 95°C for 30 sec, 55°C for 1 min and 72°C for 45 sec for 35 cycles, then followed by a final extension at 72°C for 10 min.

DNA Sequencing and Analyses

Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) and sent for sequencing. The sequences were edited with BIOEDIT software. Multiple sequence alignments were conducted by CLUSTALW (http://www.genome.jp/tools/clustalw/). The sequences were analysed with the Blast program and the NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/).

Quantitative Real-Time PCR (qRT-PCR) for Gene Expression

Total RNA was extracted from different treatment of *C. trifolia* leaves (Pb, NaCl, diesel and wounding) as described above. The qRT-PCR was done using iQ5 Real-time PCR System (BioRad, USA) and the amplifications were performed in a 25 µl reaction containing QuantiFast SYBR GreenPCR Master Mix (2X) (Qiagen, Germany), 10 µM of specific primers and 100 ng of cDNA. The amplification conditions were set as follows: 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 10 sec and 59°C for 30 sec. Dissociation curve was established at the end of each run to check the specificity of amplification. For each treatment of *C. trifolia*, three independent biological replicates were used for qRT-PCR analysis. Primer efficiencies were calculated using the standard curves generated by five different concentrations of cDNA prepared from four-fold serial dilution. The gene expression data was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The data obtained represented the fold change (increase or decrease) of the target gene in the treated sample relative to the control sample. The expression level of *CtSRG1* was then normalized against two housekeeping genes, including β-tubulin and elongation factor (EF1α).

RESULTS

Differential cDNA display approach was the first step used to identify differentially expressed genes in the samples of *C. trifolia* grown on two different conditions, on soil and on sludge. A total of 23 cDNA fragments were differentially expressed, where 18 of them were up-regulated whereas the other five were down-regulated (Table 1). The up-regulated gene fragments expressed in cDNA of *C. trifolia* from the sludge treatment were labeled as U, and the down-regulated gene fragments from the soil treatment were labeled as D. The specific DEG selection to the stress responsive genes is an important factor in the next analysis. During initial selection, BLASTX analysis was performed for all DEG sequences by comparing the highest homology matches with some other protein sequences in the NCBI database. All DEGs representing proteins from the BLASTX analysis were classified in the putative function of the gene based on the results of the literature. All analyzed DEG sequences have specific functions in the plant system (Table 2). Among all these, DEG7 indicates the function involved in the response to the stresses. DEG7 protein shows a high similarity to the translocon protein involved in the stress response.

All 14 DEG proteins that underwent classification BLASTX analysis were too general, based on the protein names and similarity percentage to the proteins in the NCBI database. Therefore, distinct protein classification analysis was performed to classify the proteins specifically, according to the Gene Ontology (GO) such as molecular function, biological processes and cell components. This approach was performed because the ontologies in GO are usually well controlled, structured and elucidated the function and products of the gene.

A total of nine sequence fragments was successfully annotated (sequence with annotation) while the other five sequence fragments were not identified (sequence without annotation) (Figure 1). Among the five fragments, two fragments (DEG3 and DEG5) showed sequence similarity with that of the NCBI database using a BlastX analysis (sequence with BLAST hits). On the other hand, three other fragments (DEG1, DEG2 and DEG4) did not present any similarity to the sequences from the database (sequence without BLAST hits).

Table 1
Total differential display cDNA fragments that were identified in samples of Cayratia trifolia *of soil and sludge. U: up-regulated fragment, D: down-regulated fragment*

Arbitrary primer (ACP)	Number of fragments	Name of fragments	Estimated size (bp)
ACP 1	1	DEG1 (U)	200
ACP 2	1	DEG2 (U)	300
ACP 3	3	DEG3 (U), DEG4 (U), DEG5 (U)	500 400 200
ACP 4	1	DEG6 (D)	700
ACP 5	1	DEG7 (U)	400
ACP 7	2	DEG8 (D), DEG9 (U)	400 500
ACP 8	2	DEG10 (U), DEG11(U)	700 300
ACP 9	3	DEG12 (U), DEG13 (U), DEG14 (U)	500 400 200
ACP 14	3	DEG15 (D), DEG 16 (U), DEG17 (U)	400 900 500
ACP 16	1	DEG18 (D)	600
ACP 17	2	DEG19 (D), DEG20 (U)	400 700
ACP 18	2	DEG21 (U), DEG22 (U)	800 300
ACP 19	1	DEG23 (U)	400

Table 2
BLASTX analyses of the DEGs amino acid sequence

Sequence name	Size (bp)	Similarity (%)	E-value	Description	Function	References	Organism	Accession number
DEG1	304	-	-	No similarity	-		-	-
DEG2	296	-	-	No similarity	-		-	-
DEG3	575	79	$4.76e^{-16}$	Broad-complex, Tramtrack and Bric-à-brac/poxvirus and zinc finger (BTB/POZ) domain-containing NPY5	Involved in plant organogenesis Play in root gravitropic responses	Cheng et al., 2008; Li et al., 2011	*Vitis vinifera*	XP_002284345.1
DEG4	393	-	-	No similarity	-		-	-
DEG5	274	83	$1.33e^{-10}$	Chlororespiratory reduction 42	Involved in respiration, cyclic electron transporters of Photosystem I (PSI) and carbon dioxide adoption	Munshi et al., 2005	*Pelargonium incrassatum*	AKH05251.1
DEG6	703	89	$2.9e^{-60}$	Metal transporter; natural resistance-associated macrophage protein (Nramp)	Involved in the uptake, translocation, intracellular transport, and detoxification of transition metals	Singh et al., 2016	*Methysia notabilis*	XP_010112784.1
DEG7	348	89	$1.71e^{-30}$	Protochlorophyllide-dependent translocon component 52	Involved in response to stresses	Sjuts et al., 2017	*Vitis vinifera*	XP_002283592.2
DEG8	368	93	$2.83e^{-37}$	Ribosomal L33 (chloroplast)	Involved in translation process	Ehrnthaler et al.,2014	*Tetrastigma hemsleyanum*	YP_009231268.1
DEG9	434	99	$6.76e^{-16}$	Haloacid dehalogenase-like hydrolase domain	Involved in various cellular processes	Koonin & Tatusov, 1994)	*Medicago truncatula*	XP_003625047.1
DEG13	324	83	$5.76e^{-26}$	SH3 domain-containing 2 isoform X1	Responsible for controlling protein-protein interactions in the signal transduction pathways	Zhuang & Jiang, 2015	*Vitis vinifera*	XP_002279562.1
DEG14	297	79	$9.7e^{-11}$	Suppressor of MAX2 1 isoform X2	Function in seed germination control	Stanga et al., 2013	*Vitis vinifera*	XP_010656698.1
DEG16	924	99	$7.15e^{-169}$	Aminoglycoside 3'-phosphotransferase	An antibiotic group used in the realm of genetic engineering	Nurizzo et al., 2003	*Enterobacter hormaechei*	WP_058675408.1
DEG17	382	93	$5.46e^{-14}$	Chalcone synthase	Work on the synthesis of secondary metabolite series in plants, fungi and bacteria	Han et al., 2016	*Lonicera japonica*	AFJ44312.1
DEG19	406	56	$3.25e^{-20}$	Glutaredoxin domain-containing cysteine-rich protein 1	Involved in electron carrier activity	Bick et al., 1998	*Jatropha curcas*	XP_012086699.1

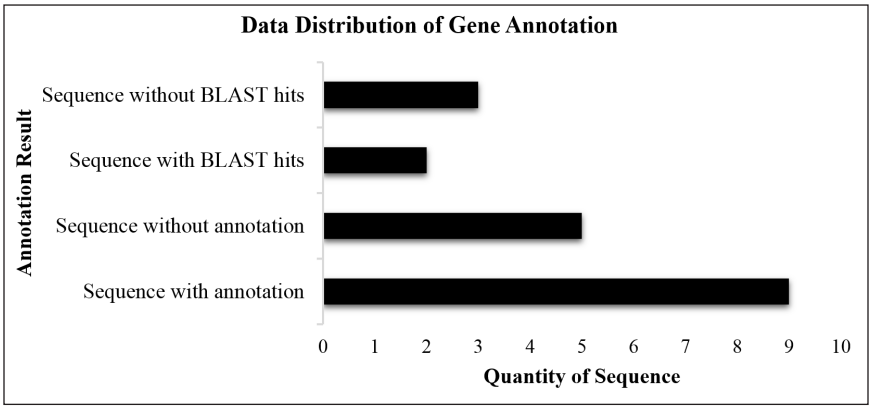

Figure 1. Distribution of the DEGs sequences annotation using Blast2GO

Subsequently, the protein sequences with predicted putative functions were classified according to the GO, which are molecular functions, biological processes and cellular components based on Blast2GO software. Molecular functions refer to the biochemical activities of the gene's product at a particular molecular level. While the biological processes indicate the biological objective of one or more of the said molecular functions. On the other hand, cellular components indicate the locations of the gene products before they become activated in the cell. The GO groups analyses have classified six protein sequences (DEG6, DEG7, DEG8, DEG9, DEG16 and DEG17) into the molecular functional group, while another six (DEG6, DEG7, DEG8, DEG14, DEG16 and DEG17) were classified into the biological process group and the others (DEG6, DEG7, DEG8 and DEG13) were in the cellular component group.

Our study prioritizes the GO distribution in the molecular functions category. It enables proteins functioning in response to environmental stress be identified, hence predict the genes that respond to the stresses. Four GO groups were identified based on their molecular functions such as catalytic activity (DEG7, DEG9, DEG16, DEG17), binding activity (DEG7, DEG 16), transporter activity (DEG6), and structural molecule activity (DEG8). The DEG7 sequence is classified both under the category of catalytic and binding activity. Both of these activities involved in plant stress response. Among these fragments, DEG7 fragment was identified and predicted as the cDNA encoding stress-responsive gene. This prediction was supported by the results from homology search using BlastX analyses and functional annotation and gene classification using Blast2GO.

Thus, DEG7 was selected for isolation and further classification. The information on DEG7 sequence was used for RACE-PCR and PCR analyses by using degenerated primer. Both the analyses resulted in the fragment with an estimated size of 1371 bp, named *CtSRG1* (*Cayratia trifolia Stress Responsive Gene*) gene (Accession no.: MG546210). BLAST was performed on

CtSRG1 and showed the highest similarity to the translocon (Table 3) that is important to the stress response. The presence of protein domain identified using Conserved Domain software NCBI revealed that CtSRG1 protein consisted of Rieske and START/RHO_alpha_C/PITP/Bet_v1/CoxG/CalC (SRPBCC) ligand-binding domain. Protein in SRPBCC domain has been reported to have an important role in biotic and abiotic stresses (Guo et al., 2017).

Quantitative real time PCR (qRT-PCR) analysis revealed that the expression of *CtSRG1* gene varied according to different treatment. Comparison of *CtSRG1* gene expression in *C. trifolia* leaves treated with Pb, diesel, NaCl, and wounding were relative to the expression of *CtSRG1* gene in control *C. trifolia* leaf (untreated). Relative expression for *CtSRG1* gene is up-regulated if the fold value is more than 1 (> 1). Whereas, the relative expression of the *CtSRG1* gene is down-regulated when the fold value is less than 1 (<1).

Relative expression for *CtSRG1* gene was significantly up-regulated ($p<0.05$) when treated with Pb at the concentration of 0.01 and 0.3 g/kg. However *CtSRG1* gene was down-regulated when *C. trifolia* treated with Pb at the concentration of 0.6 g/kg (Figure 2A). Figure 2B shows *CtSRG1* gene expression up-regulated significantly in leaves of *C. trifolia* when treated with 50 and 100 mM NaCl. However, *CtSRG1* gene was down-regulated under a higher concentration of NaCl of 300 mM. The results of the qRT-PCR analyses showed that the relative expression of *CtSRG1* gene was up-regulated when treated with 40g/kg of diesel. However, the results of the qRT-PCR analyses showed that the expression of *CtSRG1* gene was down-regulated under higher diesel treatment of 70 and 100 g/kg (Figure 2C). Overall, it is notable that the expression of *CtSRG1* gene is down-regulated when treated with wounding (Figure 2D).

DISCUSSION

In this study, DEG7 has the highest similarity to a stress responsive gene known as translocon. Translocon is a stress responsive protein complex that facilitate polypeptide translocation across cell membrane to balance cell homeostasis during environmental stress (Pogson et al., 2015; Sjuts et al., 2017; Yan et al., 2014). Plants such as *Arabidopsis thaliana* and *Solanum lycopersium* up-regulated their translocon gene expression during heat

Table 3
BLASTX analyses of the CtSRG1 amino acid sequence

Description	Organism	Accession No.	Similarity (%)	E-value
Translocon	*Vitis vinifera*	XP_002283592.2	67	$5e^{-173}$
ACD1-like protein	*Theobroma cacao*	EOY32295.1	54	$8e^{-148}$
Protein containing- Rieske domain (2Fe-2s)	*Populus trichocarpa*	XP_002299108.1	47	$3e^{-123}$
Pheophorbida a oxigenase	*Cynara cardunculus*	KVI08321.1	44	2^{e-117}

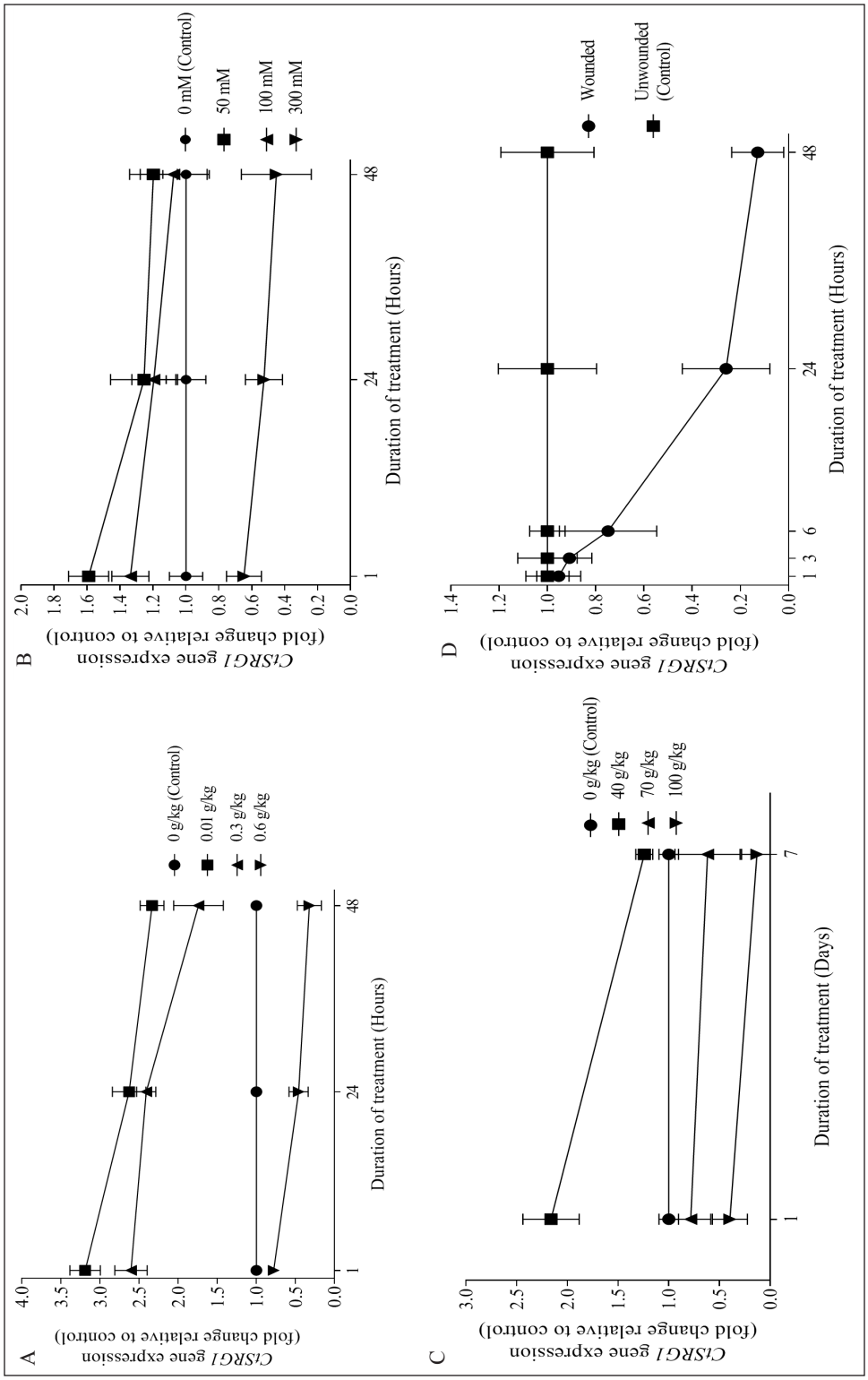

Figure 2. Changes in expression of the *CtSRG1* gene when treated with Pb (A), NaCl (B), diesel (C) and wounding (D). Vertical bars represent the means ± SD (n= 3)

(Ko et al., 2005) and NaCl stress (Yan et al., 2014), respectively. The DEG7 sequence is also classified under catalytic and binding activity category. Catalytic activity involving enzymes regulation is necessary for plant to maintain their cell homeostasis (Ryšlavá et al., 2013). Being a sessile organism, plant cannot avoid adverse effect of environmental stress. Thus, plant has to regulate specific enzyme activities to increase their survival rate (Doubnerova & Ryslava, 2013). On the other hand, binding activity also plays a role in plant survival against environmental stress. Protein binding is the center of the regulatory factors that control post-transcriptional RNA metabolism during the growth and development of plants as well as in response to stress (Lee & Kang, 2016).

CtSRG1 protein consists of two ligan-binding domains which are Rieske and START/RHO_alpha_C/PITP/Bet_v1/CoxG/CalC (SRPBCC). SRPBCC superfamily is known as Bet_v1 or steroidogenic acute regulatory protein (StAR)-related lipid transfer (START), and this superfamily consists of hydrophobic ligand-binding pocket (Schrick et al., 2014). Many studies have shown the involvement of START protein in generating response and tolerance towards environmental stresses. In *Arabidopsis thaliana*, transcription factors such as NO APICAL MERISTEM/ ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) family was identified to possess transmembrane domain and take part during stress response (Clercq et al., 2013). In addition, a novel *ITN1* (Sakamoto et al., 2008) and *NTHK1* gene (Zhou et al., 2005) which encoded for transmembrane protein were also involved in stress tolerance to salt in *A. thaliana*. In *Cicer arietinum* L., a gene known as *TM-START* was identified and characterised, in which the gene was up-regulated when the plant experienced water, salt, wounding and heat stresses (Satheesh et al., 2016). All of the studies show the activation of proteins when they bind to a ligand, followed by signal transduction towards stresses. Proteins act as a transcription factor which induces pathways or as an extensive component of a signal transduction pathway by taking part in protein-protein interactions responsible for environmental stress response. Therefore, the analysis of *CtSRG1* gene and CtSRG1 protein sequence support the suggestion that *CtSRG1* gene is a suitable stress response gene to be used in the next study.

The outcomes of this study showed that the response of *CtSRG1* gene towards Pb, NaCl, diesel and wounding stresses can be classified into two categories, i.e. positive and negative feedback response. *CtSRG1* gene exhibited positive feedback response, in which the gene was up-regulated when treated with Pb, NaCl and diesel. In contrast, *CtSRG1* gene was classified as a negative feedback response gene when the gene was down-regulated during wounding treatment. Both of these findings showed that *CtSRG1* gene was responsive to chemical stresses of Pb, NaCl and diesel and also wounding stress. Gene with positive feedback response, is an up-regulated gene that acts as defence

mechanism and crucial for plant survival during stress (Richter et al., 2010). One of the defence mechanism is the activation of stress response gene that acts as a positive regulator in the adaptation and tolerance of plant towards stress. Genes in which the expression are up-regulated during stress shows that they are ready to repair the stress condition in the plant system (de Nadal et al., 2011). Hence, as a stress response gene, *CtSRG1* might be involved in the positive feedback response towards Pb, NaCl and diesel stresses, and possibly act as an intermediate in defence signals of *C. trifolia* plant.

Meanwhile, this study discovered that *CtSRG1* gene was also involved in the negative feedback towards wounding stress, where the *CtSRG1* gene was being down-regulated during the wounding treatment period starting from the first hour until the 48th hour. Down regulation of *CtSRG1* gene shows the defence mechanism of *C. trifolia* may require other components such as lignin, callose and phenolic compound that are able to suppress the gene expression. For instance, when a plant is under wounding stress, the cell wall thickens due to lignin and callose deposition (Denness et al., 2011). Synthesis of phenolic compound also happens during localised response when the plant is wounded. These factors contribute to the suppression and the expression of a gene. Our results were in line with the findings observed by Li et al. (2016), where *HyPRP1* gene in tomato was down-regulated under heat, drought, wounding, NaCl and abscisic acid hormone (ABA) stresses. Instead, these stresses up regulate other antioxidant genes. Moreover, *HyPRP1* gene in *N. benthamiana* functions as a negative feedback mechanism towards pathogens invasion by negatively regulates the expression of other genes such as the defence and antioxidant genes (Yeom et al., 2012). This suggests that *CtSRG1* gene is responsive through negative feedback under wounding treatment, hence, helps *C. trifolia* to maintain its survival in PPMSB sludge contaminated area.

This study shows that *C. trifolia* plant is different from other plants since it possesses higher tolerance to Pb, NaCl and diesel concentrations. Thus, *C. trifolia* plant is potential to be used as a phytoremediation agent and can be applied directly to the environment in the future. However, when the plant were subjected to higher concentration of Pb (0.6 g/kg), NaCl (300 mM) and diesel (70 g/kg dan 100 g/kg), the *CtSRG1* gene was down-regulated. The concentration of 0.6 g/kg of Pb, 300 mM NaCl and 70-100 g/kg diesel are therefore toxic to *C. trifolia* plant. It can cause cell death and cell system failure due to the changes and damages of DNA and protein of the plant cells (Xu et al., 2017). Damaged DNA can disrupt genes transcription, protein translation and DNA replication (Xu et al., 2017) which correlate with down regulation of gene expression. Down regulation of stress response gene due to higher amount of Pb and NaCl were already reported in several plants such as *S. Lycopersicum* (Perez et al., 2013) and *Cuminum cyminum* L. (Soleimani et al., 2017), respectively. On the other hand, there is still no report

regarding the actual toxicity level for Pb, NaCl and diesel in *C. trifolia*. Therefore, the current study is the first to describe on *C. trifolia* limit against Pb, NaCl and diesel stresses.

CONCLUSION

We have isolated a new stress responsive gene from *C. trifolia* namely *CtSRG1*. *CtSRG1* expression was up-regulated by the Pb, NaCl and diesel but down-regulated by wounding, indicating the gene plays a very siginificant role towards stresses adaptation. This corresponds to the functionality of the gene that has a high similarity to translocon and possesses Rieske and SRPBCC domain involved in the response to biotic and abiotic stresses. In addition, *C. trifolia* plant has also been proven to have a high level of phytotoxic resistance to Pb, NaCl and diesel stress. Futher study needs to be conducted in the future to understand the specific manner of *CtSRG1* involvements in plant stress adaptation toward Pb, NaCl, diesel and wouding stresses.

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