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Genes induced by high concentration of salicylic acid in *Mitragyna speciosa*

Siti Sarah Jumali[1,2], Ikram M. Said[1], Ismanizan Ismail[1,2], Zamri Zainal[1,2]*

[1]Institute of Systems Biology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[2]Department of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

***Corresponding author: zz@ukm.my**

Abstract

Mitragyna speciosa is rich in secondary metabolites which are similar in effects as opium. Treatment with plant growth regulator such as salicylic acid (SA) is able to increase plant defense mechanism which later induces the expression of genes that encode secondary metabolite production. To identify genes that respond to elicitation of high concentration of SA, suppression subtractive hybridization (SSH) library was constructed using mRNA from SA-treated leaves and mRNA from non-SA-treated leaves. A total of 292 EST clones were randomly sequenced and all cleaned clone sequences (111) were analyzed using BLASTX against non-redundant NCBI databases. Results showed that most genes responding to acute SA treatment are related to stress and signaling pathways which eventually led to cell death. This include genes encoding chaperone, heatshock proteins (HSPs), antioxidants and genes involved in secondary metabolite biosynthesis, such as sinapyl alcohol dehydrogenase (SAD), cinnamyl alcohol dehydrogenase (CAD) and Cytochrome P450 (CYP 450). Further analysis was carried out using 8 out of 60 differentially expressed unique sequences through semi-quantitative RT-PCR on samples before and after treatment with 5 mM SA in 4 consecutive days. The result revealed that their expression changed over time when the plant was treated with 5 mM SA.

Keywords: Suppression subtractive hybridization (SSH), *Mitragyna speciosa*, salicylic acid (SA), heat stress.
Abbreviations: Suppression subtractive hybridization (SSH), *Mitragyna speciosa* (*M. speciosa*), salicylic acid (SA), jasmonic acid (JA)heat shock protein (HSP), late embryogenesis abundant (LEA), Mitogen activated protein kinase (MAP kinase).

Introduction

Mitragyna speciosa (*M. speciosa*), the plant from Rubiacea family is indigenous to South East Asia. Medicinal properties have been attributed to the plant because chewing the leaves or drinking the leaf extract has been a traditional means to cure fever and diarrhea. In addition, recently *M. speciosa* has been used to produce energy drinks (Boyer et al., 2008). The plant has also been used as a substitute for opium when it is unavailable or unaffordable (Jansen et al., 1988). There have been many studies conducted on *M. speciosa*, such as 7-hydroxyspeciociliatine compound identification (Kitajima et al., 2006), analgesic evaluation, mitragynaline and 9-methoxymitralactonine identification (Takayama et al., 2000). Most of the studies used gas chromatography and nuclear magnetic resonance (NMR) techniques to study its metabolites and compound structures. Furthermore, information regarding the genetic regulation of these metabolic processes is absent. Until now, only six sequences related to this organism are available in GenBank. Therefore, our work significantly contributes to the understanding of the molecular genetics of *M. speciosa*. Considering the appealing usage of *M. speciosa*, we are interested in generating a body of information by identifying and characterizing genes which are thought to be upregulated when *M. speciosa is* challenged by highly concentrated elicitors. It is also to determine the ability of high concentration of SA in eliciting the biosynthetic pathways of secondary metabolite production. Usually in plants, the defense system is activated through signaling pathways mediated by endogenous signaling molecules such as salicylic acid (SA), ethylene, and jasmonic acid (JA). These elicitors target secondary signals in the cell nucleus where they initiate the signal transduction pathways that lead to transcriptional activation of numerous genes and, consequently, induce the synthesis of a variety of defense proteins and secondary metabolites. SA was reported to induce a 5 fold increase of metabolite production in *Catharanthus roseus* tumor suspension cultures (Godoy-Hernández and Loyola-Vargas, 2005). However, thus far, studies mostly report on treatment with low concentration of elicitors. To identify the differentially expressed genes in high concentration SA-treated leaves, we constructed a subtractive cDNA library of *M. speciosa* using suppression subtractive hybridization (SSH) as its principle. SSH libraries have been successfully applied in identifying differentially expressed genes from numerous plants under different conditions. It is primarily based on suppression polymerase chain reaction (PCR) technique that combines normalization and subtraction in a single procedure. In the present study, we identified differentially expressed genes in *M. speciosa* leaves when the leaves were treated with concentrated elicitor (100 mM SA). The transcripts cloned were annotated using bioinformatics software BLAST2GO, InterProScan and KOBAS. Expression levels of these genes were further determined by semi-quantitative RT-PCR.

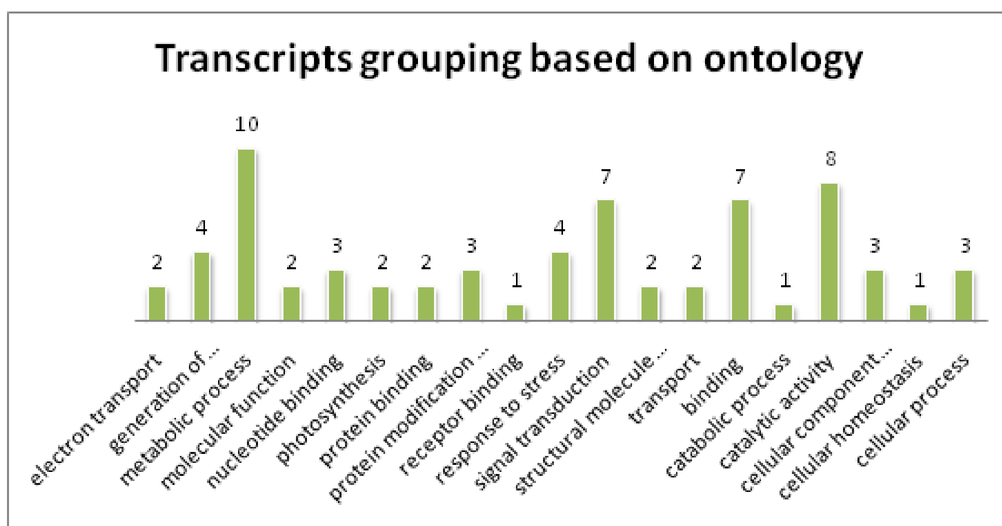

Fig 1. Grouping of transcript numbers according to gene ontology constructed using the Sequence Frontier software. The highest number of transcripts cloned belongs to metabolic process, followed by signal transduction and catalytic activity.

Materials and methods

Plant Material and SA Treatment

M. speciosa leaves from plants collected in Perlis, Malaysia were sprayed with 100 mM SA and collected after 2 hr. The untreated plant was used as control. An additional experiment for gene expression studies was conducted separately, in which the plant leaves were treated with 5 µM SA and collected after 24, 48, 72 and 96 hr.

Total RNA and mRNA Isolation

The leaves were ground with mortar and pestle in liquid nitrogen and homogenized with 20 ml of pre-chilled extraction buffer (Tris-HCL pH 8.5, 300 mM LiCl, 10 mM EDTA, SDS 1% w/v, 5 mM thiourea, and 1% β-mercaptoethanol) and 6 ml of 20% PVP40. Subsequently, the homogenate was spun at 10,000 x *g* for 15 min at 4°C. The supernatant was mixed with 3 M sodium acetate and ethanol and incubated at -20°C for at least 2 hr. Then, the samples were spun at 10,000 x g for 20 min at 4°C. The pellet was resuspended in 6 ml of extraction buffer and phenol: chloroform (1:1) and then vortexed and spun at 10,000 x g for 10 min at 4°C. The upper phase was incubated for 15 min at 65°C in a final concentration of 0.7 M NaCl and 2M CTAB. Next, an equal volume of chloroform: isoamyl alcohol (24:1) was added to the mixture followed by vortexing. The mixture was centrifuged at 10,000 x g for 10 min at 4°C. The upper phase was collected and incubated overnight with 3M LiCl. The mixture was spun, and the pellet was resuspended in 50 µl of DEPC water. The total RNA obtained was then pooled for mRNA isolation. Poly A^+ RNA was isolated using poly (A) tract (Promega).

Subtractive cDNA Library Construction

The library was constructed as instructed by manufacturer (Clontech PCR-Select™ kit). The PCR product was cloned into the pGEM-T Easy Vector (Promega). The ligated product was then transformed into DH5α competent cells and plated on Amp/IPTG/X-gal plates for blue-white screening. White colonies were cultured, and the plasmids were extracted using milipore plasmid miniprep (Montáge).

EST Sequencing and Analysis

In total, 292 cDNA clones were sent to 1stBASE Laboratories Pte Ltd, Malaysia for sequencing with the M13/pUC forward primer. Expressed sequence tags (ESTs) were cleaned and assembled using *seqclean* and CAP3 software respectively. The search for similarity was performed against available, non-redundant genes/transcripts in a public database (http.//www.ncbi.nlm.nih.gov/BlastT) using BLASTX and BLASTN. Similarity scores between the cDNA clones and known sequences were represented by the BLASTN probability E-value (e-value ≤ 10^{-5}).

Semi-quantitative RT-PCR Analysis

Eight genes were chosen, and their PCR primers were designed according to the received sequences (Table 1). PCR reactions for five different time courses were prepared. The optimized PCR reactions contained 3 µl cDNA (200 ng), 1x Green GoTaq® Flexi Buffer, 50 mM $MgCl_2$, 400 µM dNTPs, 50 mM $MgCl_2$, and 2.5 U of GoTaq® DNA polymerase (Promega). The amplification program was at a Tm of 66.5°C. The amplified products were analyzed through electrophoresis in 1% agarose gels and visualized by ethidium bromide staining.

Result and discussion

Leaf treatment

In order to test the effects of high concentration Salicylic Acid (SA) elicitor on gene expression of *M. speciosa*, plant leaves were treated with 100 mM SA and the results were analyzed. From our observation, the leaves exhibited distinctive phenotypic differences after SA treatment (data not shown). Morphological changes were observed within 2 hr after spraying with SA and included wrinkling, rolling, wilting and browning of the leaves, especially of the young shoots. These leaves and shoots later died when left for a longer time on the plant. These morphological changes indicated that the plant was adapting to stress. Adaptations to stress that took place are in order to reduce light harvesting and to reduce water loss. These are apart from the defense system when it is activated to maintain homeostasis (Oh et al., 2008). Meanwhile, we conducted a 5 mM SA treatment to

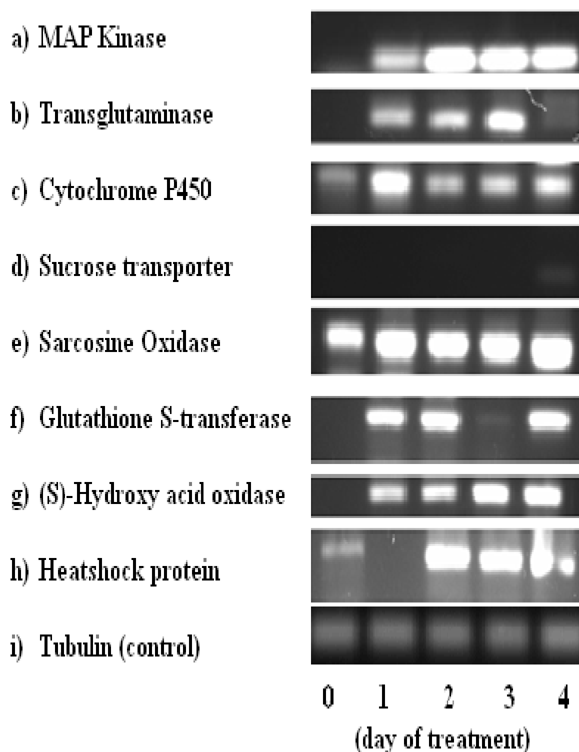

Fig 2. Gene expression of different transcripts from day 0 (positive control) through day 4, illustrated by semi-quantitative RT-PCR; a) MAP kinase; b) Transglutaminase; c) CYP450; d) Sucrose transporter; e) Sarcosine oxidase; f) GST; g) S-hydroxy acid oxidase (glycolate oxidase); h) HSP and i) Tubulin as the control

determine the survival period of the leaves. We observed that it took 4 days for the leaves to completely die on the plant after they started with wrinkling, followed by wilting and browning. At this stage, the leaves became easily detached from the stem.

Annotation of differentially expressed cDNAs

Sequence annotation was carried out after analyzing the sequences. From sequencing results, 292 sequences obtained were analyzed using the Sequence Frontier software. However, only 111 sequences were clean and qualified for analysis. The vector sequence was then trimmed followed by alignment. Based on the analysis using the Seqclean software, 43 transcripts were singletons, and 68 transcripts converged into 17 different contigs. BLASTX search was conducted with 60 of these singletons against the protein database in NCBI. The sequence homologies are listed in Table 2.

M. speciosa cDNA subtractive library analysis

Based on the obtained sequences, the transcripts were divided into few distinct groups in order to determine the genes' expression level. Semi-quantitative RT-PCR analysis was conducted on eight transcripts with known function to examine the correlation of these genes with the treatment of SA at a low concentration (5 mM). The results showed that the obtained cDNA clones represent upregulated stress-related genes in response to SA treatment. It was apparent that 5 mM SA treatment elicited abiotic stress on *M. speciosa.* Our result is in agreement with the previous finding of a study conducted by Rizhsky et al. (2004) in which transcript profiling was conducted to identify the differentially expressed genes in *Arabidopsis* when exposed to heat. The transcripts obtained from that study are also related to stress response and cell defense.

Cloning of transcripts involved in signal transduction

A MAP kinase gene was found to be overexpressed after the plants were treated with SA. MAP kinase is known to play significant roles in protein phosphorylation and dephosphorylation in signal transduction pathway (Montesano, 2002). Mitogen activated protein kinase (MAP kinase), a serine/ threonine-specific kinase, is responsive to exogenous stimuli such as mitogens, osmotic stress and heat stress through regulating multiple cellular activities, such as gene expression, mitosis, cell differentiation, cell division and apoptosis, cell growth and cell defense (Kant et al., 2006). Overexpression of the MAP kinase was identified through semi-quantitative RT-PCR (Figure 2a). The amplicon with the size of 373 bp was detected, where the expression increased after one day of treatment, and this increase persisted towards the last day of treatment (day 4). It was suggested that the overexpression of MAP kinase at the early stage can later induce the production of ethylene and JA followed by phenylpropanoid and secondary metabolite production (Zhao et al., 2005).

Sarcosine oxidase

Many genes are triggered in the cascading reactions following the activation of MAP kinase signaling pathway. One of the genes is a gene that encodes sarcosine oxidase which is involved in glycine, serine and threonine metabolism. It catalyzes the oxidative demethylation of sarcosine and generates equimolar amounts of formaldehyde, glycine, and hydrogen peroxide (Nishiya, 2000). Hydrogen peroxide production is important for plant cells because it serves as a substrate of peroxidase reactions, such as the crosslinking of cinnamyl alcohol during lignin biosynthesis,and crosslinking of cell wall extensin (Barceló, 1998) to impart strength on the cell wall. An EST clone (GT742115) encoding sarcosine oxidase was observed differentially expressed. The transcript's expression level of this gene was determined and it shows that the transcript was detected even in the untreated leaves (control) but at low level (Figure 2e). Nevertheless higher level of transcript expression was present at all time points inferring that SA has induced its expression.

Transglutaminase

Another EST clone which were observed to be differentially expressed are transglutaminase (GT742142, GT742144, and GT742145). Transglutaminase is a calcium-dependent transferase enzyme involved in many protein crosslinking reactions during plant and animal growth (Dondini et al., 2002). The reaction catalyzed by transglutaminase generates Nepsilon-(γ glutamyl) lysine between proteins. Transglutaminase catalyzes post-translational modification through transamidation of glutamine residues and produces inter- and intra-isopeptide bonds of numerous proteins by crosslinking the amino residue on lysine and the glutamine protein (Bönisch et al., 2007). Transglutaminase also plays an integral part in survival against osmotic and salt stress (Dondini et al., 2002). Crosslinks affect protein function

Table 1. Sequence of primers used for gene expression analysis by RT-PCR

Gene description	Primer pairs
Mitogen-activated protein kinase (MAP kinase)	5' CAGGTTCGTCCTCGTT 3' 5' AAGGGTCTTCCCACATA 3'
Sarcosine oxidase	5' GTATTGGTTGCTGGAGG 3' 5' GCCGAGGTACTTATTCTTA 3'
Transglutaminase	5' GTGCCCAACTTGAACTG 3' 5' GGTAAGGCTACTGGAACAT 3'
Alcohol dehydrogenase	5' CTTATGTGGCGGATTGG 3' 5' TGATGACCTTCCCTTGAGA 3'
Sucrose transporter	5' CTACGGTATCCTATTTCTTC 3' 5' GGGACCCTGAGTTGTG 3'
Heatshock protein	5' GGGTTATGCTACTTCTTCTA 3' 5' TTTCATTTCCTCCGTTT 3'
(S)-2-hydroxy-acid oxidase, putative glutathione s-transferase, putative	5' CCTCCCACTCAGTCATT 3' 5' TCCGTCTCCGTCTTG 3' 5' TGTCCAGTCCAGCAAAG 3' 5' AATCGGACCATCATCAGT 3'

because they promote stronger structure (Bönisch et al., 2007) such as in catalyzing cytoskeletal modification (Del Duca et al., 1997). By semi-quantitative RT-PCR, we observed that transglutaminase (GT742142) expression had increased upon SA treatment until day 3 before it started to decrease again (Figure 2b). Besides, in this study, the cytoskeletal actin gene (GT742111) was also cloned as a differentially expressed gene. Expression of the actin cytoskeleton is related to microtubule organization affected by the splitting or elongation of spindles, the formation of microtubule asters in mitotic cells, and the elongation of phragmoplast microtubules (Smertenko et al. 1997). However, the correlation between transglutaminase and the actin cytoskeleton in this study remains unknown.

Heat shock proteins (HSPs)

In many studies, identification of HSPs in important crops is crucial because HSPs enable the development of heat and drought resistance in plants. Several HSPs (GT735563, GT735560, GT735561, GT742121, GT735550, GT735551, and GW610913) and a chaperone (GT742123) were cloned in this study. HSPs are related to modification processes that allow proteins to avoid and/or to recover from aggregation during stress. Heat-shock response is an inducible molecular response to physiological, environmental, and biochemical stress conditions, resulting in the elevation of HSP expression. At the molecular level, abundant synthesis of HSPs is conserved in all organisms when exposed to elevated temperatures (Boyer et al. 2008). Meanwhile, molecular chaperones are often characterized by their ability to prevent aggregation of unfolded proteins formed in response to mild acid or heat treatment. When plants undergo heat stress, homeostasis is disturbed, resulting in protein destabilization. HSPs bind to these proteins to protect them from aggregation; meanwhile, they act as chaperone molecules that function in protein folding and biochemical reactions to ensure stable homeostasis in terms of pH and metabolites (Boyer et al. 2008). In order to understand their pattern of expression, semi-quantitative RT-PCR was carried out on one of the EST clones corresponding to HSPs; the band representing the HSPs level after treatment was more intense than that prior to the treatment (Figure 2h). Studies on HSPs have attracted significant attention in the field of transgenic research. A study conducted on transgenic tomatoes with HSPs overexpression showed that these tomatoes were more tolerant to heat. This observation is in agreement with another study conducted on rice in which the transgenic rice was observed to have less spots than the wild type when exposed to heat stress, indicating an increase in inducible heat tolerance (Wang et al. 2003) where under normal conditions; HSP is either not expressed or expressed but at low level.

Chlorophyll a-b binding protein

When plants are challenged with stress, many genes, either directly or indirectly related to stress become overexpressed in order to adapt to the stressful condition. Chlorophyll a-b binding protein (CAB) (GT735555, GT735556 and GT742124) is responsible for absorbing light during photosynthesis in photosystems I and II (Andersson et al., 2003). Meanwhile, the N-terminus that extends to the stroma is involved in binding to the grana membrane regulated by reversible phosphorylation of threonine residues, where both phosphorylation and dephosphorylation are considered to mediate the division of excitation energy between photosystems I and II (Liu and Shen, 2004). The accumulation of CAB and apoprotein was observed to increase as a means of photosystem stabilization. Therefore, CAB overexpression protects the photosystem by stabilizing light division between photosystems I and II (Andersson et al., 2003).

Secondary metabolite production as a result of Reactive Oxygen Species (ROS) induction

It was part of the objective of this study to identify that genes that encode secondary metabolite productions be cloned after treatment with SA. Abiotic stresses such as drought, salinity and flood can trigger ROS production that is toxic to plant cells. The affected cells rampage membranes, membrane-bound structures and other macromolecules such as protein and nucleic acid, especially in mitochondria and chloroplast. These events exert oxidative stress on the cells. During oxidative stress, superoxide dismutase is responsible for deionizing superoxides by changing it to hydrogen peroxide that will later be decomposed into water by catalase and peroxidase. According to Zhao et al., (2005), elicitors induce transduction of genes encoding ROS enzymes that are known to injure membranes, mitochondria and nuclei during its reaction to multiple stresses (Murakami et al., 1997, Rhoads et al., 2006). In this study, two oxidoreductase transcripts cloned were homolog of old yellow enzyme (GW610915) and adenylyl sulfate reductase (glutathione) (GT742127). Old yellow enzyme is involved in NADP/NADPH production, an oxidation and reduction agent, whereas adenylyl sulfate reductase functions in scavenging and detoxifying ROS. Although free radical Reactive Oxygen Intermediates (ROIs) are harmful to the cell, they enable the signal transduction that activates the defense system. Therefore, ROIs, such as $H_2O_2$and O_2^-, are indicators of cellular stress and a secondary signal in the reaction to the stress signal transduction pathway (Mittler, 2001).

Genes in the lignin biosynthetic pathway

Since cDNA hybridization and semi-quantitative RT-PCR imply that the sequenced clones are upregulated, it is also inferred that other transcripts such as Sinapyl alcohol dehydrogenase (SAD) and cinnamyl alcohol dehydrogenase (CAD) are upregulated. From previous observation, it is

understood that rolling of the leaves reduces water loss. Lignin formation also reduces water loss. SAD enzyme (GT742125) plays a role in catalyzing the last step in the biosynthesis of lignin, called monolignol (hydroxyl cinnamyl), whereas its ortholog, CAD (GT742138), is involved in the biosynthetic pathway of phenylpropanoid production to produce lignin (Li et al., 2001). Although CAD and SAD catalyze the same reaction because of their sequence similarities, the active site of SAD is different from the predicted active site of CAD. The pathway in which SAD and CAD are involved is the shikimate pathway generating phenylalanine, which is channeled into pathways producing secondary metabolites such as alkaloids, flavanoids, lignin and protein. Therefore, it is conceivable that treatment with 100 mM SA can induce secondary metabolite production.

Alcohol sugar production as a means of protection against osmotic stress

Other than secondary metabolites, alcohol sugars also play an important role in the protection against stress. Aldose reductase (GT742129) changes glucose to sorbitol, whereas mannitol dehydrogenase (GT735567) functions in converting D-mannitol and the NAD ion to D-mannose, NADH and H^+. The presence of alcohol sugar sorbitol is due to osmotic stress survival. This is consistent with the role of mannitol in protecting plants against salinity stress. The advantage for higher plants which metabolize mannitol is on sugar translocation whereby increased tolerance to salt and osmotic stress, other than protecting means during pathogenic invasionm (Stoop et al., 1995). The osmolite alcohol sugar is a compatible solute that increases the osmotic potential in plants where it plays a crucial role in maintaining cell turgidity by creating osmotic gradients to promote water uptake (Wang et al., 2003). Therefore, mannitol dehydrogenase also plays a crucial role in destroying ROS.

LEA (Late embryogenesis abundant) proteins

Since SA caused *M. speciosa* leaves to dry, cloning of the LEA transcript (GT735565) signifies the accumulation of LEA proteins in the treated leaves of *M. speciosa*. LEA proteins were first observed to increase during cotton (*Gossypium hirsutum*) maturation and embryogenesis. LEA proteins are present in plants; however, their exact role remains unknown. Nonetheless, many studies have suggested that LEA proteins play a role in protecting and stabilizing cell membrane. LEA proteins, therefore, are associated with the ability of retaining water and preventing crystallization of important cellular proteins and other molecules during desiccation. Because SA treatment induced the leaves to dry and wither, we suggested that LEA proteins might be expressed to stabilize cell membrane from damage caused by desiccation.

Transporter proteins

One of the mechanisms involved during heat stress include solute transport. From our SSH library, one EST clone shows homology to sucrose transporter (GT742140). By semi-quantitative RT-PCR, we observed the overexpression of this sucrose transporter on the last day of SA treatment. From previous studies, it was reported that the overexpression of a sucrose transporter was always observed during heat stress. The study by Qin (2008) also reported an increase in sucrose transporter expression by microarray. In that study it was discovered that the sugar content of *Triticum aestivum* tremendously increased under heat stress and it was probably due to the role of sugar in maintaining homeostasis under heat stress (Qin et al., 2008).

Detoxifying enzymes and secondary metabolites

CYP450 enzymes (GT742126) are a superfamily of heme-containing mono-oxygenases. They are important in the biosynthesis of several compounds, such as hormones, defensive compounds and fatty acids (Nebert et al., 1989). CYP450 expression is associated with *M. speciosa* secondary metabolism: during stress, one of the mechanisms is activating xenobiotics biosynthesis to survive. To overcome the oxidative stress, adenylyl sulfate reductase (GT742127) is expressed to catalyze the prime reaction of plant sulfate assimilation that produces cysteine and antioxidant glutathione (Bick et al., 2001). Oxidative stress caused by ROS production can be reduced by increasing the production of oxidized glutathione by producing glutathione S-transferases (GSTs) (GT742126) because GST is the largest detoxifying enzyme group aside from other ROS scavengers such as ascorbate peroxidase, superoxide dismutase, glutathione peroxidase, ferritin and thioredoxin (Qin et al., 2007). GSTs catalyze reduced glutathione conjugation through sulfhydryl groups to the hydrophilic center of multiple substrates, detoxify endogenous compounds such as lipid peroxide and promote xenobiotics degradation. In addition, H_2O_2 and ROS production also induces GST expression that plays a role in the defense against chemicals generated by electrophiles (Das et al., 2010).

Glycolate oxidase (S-hydroxy acid oxidase)

Other than disturbance in photosynthesis, stress also resulted in sugar accumulation. Sucrose accumulation during stress increases its impermeability. The key enzyme in photorespiratory pathway is suggested to play a part sucrose transport because glycolate oxidase is an intermediate in protein translocation. Therefore, the increase in expression of glycolate oxidase (GT742133) is suggested to play a role in sucrose transport during stress. Other than that, glycolate oxidase also plays a role in serine production to maintain a balanced cell homeostasis. However, this research contradicts the study on effects of drought and heat shock on the expression of genes (Rizhsky et al., 2002) where glycolate oxidase was suppressed. It is suggested that there are other mechanisms involved in this treatment that causes the disparity between results. Its overexpression was illustrated through semi-quantitative RT-PCR analysis (Figure 2g); the increase in glycolate oxidase expression was apparent after one day of treatment, and the increase persisted until the end of the treatment.

Conclusion

It is clear that acute treatment with 100 mM Salicylic Acid treatment is strong on *M. speciosa* because the plant underwent stressful condition and overexpressed stress related genes. Suppression subtractive hybridization method used in this experiment has been proven to be a powerful tool in demonstrating differential expression of genes when given abiotic stress. These findings either from them *M. speciosa* suppression subtractive library or the semi-quantitative RT-PCR agree with the heat stress mechanism from other previous studies, in which in order to cope with heat stress, plants implement various mechanisms, including maintenance of membrane stability, scavenging of ROS,

Table 2. Results for homology search for sequences' transcripts against non BLASTX on NCBI database.

No.	Size (bp)	GenBank accession #	Organism	E-value	Description
			Plastid related proteins		
1	587	GT742124	*Ricinus communis*	3.00E-39	Chlorophyll A/B binding protein, putative
2	700	GT735566	*Nicotiana tabacum*	3.00E-42	chloroplast ferredoxin I
3	671	GT7 42132	*Rheum australe*	2.00E-57	MAP kinase
			Enzymes		
4	631	GT742127	*Lycopersicon esculentum*	1.00E-48	adenylyl-sulfate reductase
5	460	GT742129	*Elaeis guineensis*	1.00E-14	aldose reductase
6	875	GT742133	*Ricinus communis*	2.00E-07	(S)-2-hydroxy-acid oxidase, putative
7	314	GT742115	*Populus trichocarpa*	2.00E-20	sarcosine oxidase
8	918	GT742113	*Zingiber officinale*	7.00E-10	Putative reverse transcriptase
9	505	GT742142	*Physarum polycephalum*	1.00E-10	transglutaminase
10	606	GT742144	*Physarum polycephalum*	7.00E-18	transglutaminase
11	638	GT742145	*Physarum polycephalum*	2.00E-61	transglutaminase
12	862	GT742120	*Populus trichocarpa*	1.00E-10	Aminotransferase family protein
13	638	GT742116	*Ricinus communis*	6.00E-15	glutathione s-transferase, putative
14	109	GW610915	*Catharanthus roseus*	1.00E-44	Old Yellow enzyme homolog
15	576	GT735567	-	5.00E-17	Mannitol dehydrogenase/*NAD-dependent mannitol dehydrogenase emb*
			General transcripts		
16	284	GT742114	*Vitis vinifera*	5.00E-20	General RNA transcription factor TAF7 (TBP-*Associated Factor* 7)
17	392	GT735565	*Nicotiana tabacum*	1.00E-16	late embryogenesisabundant protein 5 (LEA)
18	310	GT735570	*Zea mays*	2.00E-49	60S ribosomal protein L23
19	355	GW610911	*Hyacinthus orientalis*	7.00E-27	40S ribosomal protein S23
			Transcript encoding transporter protein		
20	1129	GT742140	*Hevea brasiliensis*	2.00E-27	Sucrose transporter 4
			Transcripts encoding proteins in secondary metabolite production		
21	369	GT742126	*Ricinus communis*	8.00E-31	cytochrome P450, putative
22	980	GT742125	*Populus tremuloides*	2.00E-100	sinapyl alcohol dehydrogenase
23	414	GT742138	*Ricinus communis*	3.00E-13	cinnamyl alcohol dehydrogenase, putative
			Transcripts related to cellular structure		
24	397	GT742111	*Vitis vinifera*	3.00E-45	Predicted: Actin cytoskeleton
			Heat shock proteins		
25	582	GT742121	*Vitis vinifera*	3.00E-22	Predicted: Similar to HSC70-1 (HSP cognate 70 kDa ATP-binding isoform 1)
26	720	GT735550	*Glycine max*	9.00E-58	HSP13 SOYBN 17.5 kDa Class 1 HSP
27	407	GT735563	*Nicotiana tabacum*	4.00E-56	Low-molecular-weight HSP
28	806	GT742123	*Agrostis stolonifera*	2.00E-14	HSP 16.5
29	316	GW610913	*Vitis vinifera*	7.00E-40	Predicted: Similar to HSC70-1 (HSP cognate 70 kDa ATP-binding isoform 2)
			Hypothetical proteins		
30	700	GT735564	*Vitis vinifera*	4.00E-48	Predicted: Hypothetical protein
31	607	GT735559	*Vitis vinifera*	9.00E-09	Predicted: Hypothetical protein
32	483	GT735568	*Vitis vinifera*	3.00E-43	Predicted: Hypothetical protein
33	605	GT735569	*Vitis vinifera*	7.00E-21	Predicted: Hypothetical protein
34	515	GT735573	*Vitis vinifera*	3.00E-13	Predicted: Hypothetical protein
35	353	GT735575	*Vitis vinifera*	1.00E-20	Predicted: Hypothetical protein
36	371	GT735571	*Vitis vinifera*	2.00E-26	Predicted: Hypothetical protein
37	328	GT742131	*Vitis vinifera*	7.00E-08	Predicted: Hypothetical protein
38	307	GW610918	*Vitis vinifera*	7.00E-14	Predicted: Hypothetical protein
39	350	GT742139	*Vitis vinifera*	4.00E-15	Predicted: Hypothetical protein
40	581	GT742135	*Vitis vinifera*	1.00E-25	Predicted: Hypothetical protein
41	603	GT742136	*Vitis vinifera*	8.00E-24	Predicted: Hypothetical protein
42	768	GT742137	*Vitis vinifera*	3.00E-17	Predicted: Hypothetical protein
43	465	GT742118	*Vitis vinifera*	4.00E-08	Predicted: Hypothetical protein
44	395	GT742117	*Vitis vinifera*	1.00E-13	Predicted: Hypothetical protein isoform 1
45	316	GW610910	*Vitis vinifera*	5.00E-14	Predicted: Hypothetical protein isoform 2

46	353	GW610919	*Vitis vinifera*	6.00E-18	Predicted: Hypothetical protein
47	57	GW610917	*Vitis vinifera*	5.00E-46	Predicted: Protein
48	562	GT742119	*Populus trichocarpa*	4.00E-33	Predicted: Protein
49	370	GW610912	*Populus trichocarpa*	2.00E-08	Predicted: Protein
50	509	GT735572	*Populus trichocarpa*	2.00E-11	Predicted: Protein
51	484	GT742130	*Populus trichocarpa*	2.00E-44	Predicted: Protein
52	613	GT735549	*Ricinus communis*	2.00E-43	Conserved hypothetical protein
53	488	GT742122	*Ricinus communis*	1.00E-18	Conserved hypothetical protein
54	739	GT742134	*Populus trichocarpa*	2.00E-13	Hypothetical protein
55	659	GT735562	*Vitis vinifera*	4.00E-29	Hypothetical protein
56	128	GW610914	*Vitis vinifera*	2.00E-54	Unnamed protein product
57	614	GT742112	*Vitis vinifera*	5.00E-15	Unnamed protein product
58	696	GT735552	*Vitis vinifera*	2.00E-13	Unnamed protein product
			Unknown transcripts		
59	472	GT742128	*Solanum tuberosum*	3.00E-29	Unknown
60	368	GW610916	*Glycine max*	1.00E-29	Unknown

production of antioxidants, accumulation and adjustment of compatible solutes, induction of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades, and, most importantly, chaperone signaling and transcriptional activation. In future, further analysis on the usage of heat-stress related protein can be made since the clones are already available.

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