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Alteration of Abiotic Stress Responsive Genes in *Polygonum minus* Roots by Jasmonic Acid Elicitation

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1. Introduction

Plants are continuously exposed to both biotic and abiotic stress in their natural environment. Unlike animals, plants are immobilized organisms which tend to be vulnerable to various environmental stresses. In order to survive, plants have evolved a wide range of defense mechanism to cope with these stresses. Both biotic and abiotic stresses might share some common signaling pathway in triggering the defense system in plants. Recent researches have revealed that phytohormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are intermediate molecules which play key roles in the crosstalk between biotic and abiotic signaling network (Fujita et al. 2006). In this chapter, we highlight the effects of exogenous applied jasmonic acid in triggering the synthesis of some molecules and activating their respective biosynthetic genes in plants as a response towards abiotic stresses.

Abiotic stress is defined as non-living external factors, usually environment conditions, which could reduce plant growth and cause huge devastation on agricultural productivity. Some of these major adverse environmental factors are drought, salinity, heavy metals, extreme temperatures, nutrient poor soils and other source of natural disasters. To our knowledge, these abiotic stresses have account for major crops lost worldwide where more than 50% of their average yields were decreased yearly (Rodriguez et al. 2005). However, not all effects are detrimental. Plants are able to exhibit various molecular mechanisms as a defense system and these responses could be generally divided into three main groups. Firstly, signalling of stress-activated molecules leading to changes of osmotic and ionic homeostasis as well as detoxification mechanism. Secondly, up-regulation of different gene expression leading to synthesis of specific proteins (e.g. heat-shock proteins and LEA proteins) and some protective molecules (e.g. sugars, polyalcohols and amino acids). Thirdly, generation of reactive oxygen species (ROS) and activation of antioxidant systems by synthesizing secondary metabolites such as flavonoids and phenolic compounds (Boscaiu et al. 2008). Among these changes, synthesis of secondary metabolites is at the highest interest because it has a wide range of functions, ranging from plant defense against abiotic stresses to human benefits.

Plants have the ability to produce vast variety of secondary metabolites naturally. Secondary metabolites have been defined as compounds that did not play a vital role in

plant growth and development but are important in the interaction between plant and its environment (Namdeo 2007). These molecules function primarily in plants adaptation towards their environment such as biotic and abiotic stress and also serve as a major source for pharmaceutical products (Ramachandra Rao & Ravishankar 2002). Secondary metabolites are usually release by plants as a type of defense system against insects feeding; herbivory effects and pathogens attack such as virus, bacteria and fungi. They also protect plants against abiotic stress such as draught, salinity, UV light, heavy metals, extreme temperatures, nutrient poor soils and other environmental factors. Several other functions of secondary metabolites include attracting pollinators for plants reproduction and serving as signaling molecules and hormones in plant cells secondary metabolism (Korkina 2007). Up to date, thousands of different secondary metabolites structures have been identified in plants. The bioactive compounds extracted from various plant parts were usually used in the pharmaceutical, agrochemical, cosmetic, perfumery, food flavouring and pesticide industries (Balandrin & Klocke 1988). For instance, morphine and codeine extracted from the latex of opium poppy are the commercial anesthesia available in market today whereas ginsenosides isolated from ginseng roots have been proven to be the stimulant for health and longetivity (Sticher 1998). Apart from that, alcohols, aldehydes, ester, free fatty acids, ketones and phenolic compounds purified from plants are also being used in the foods and beverages industries. The food flavouring that are succesfully marketed are apple (Drawert et al. 1984), cocoa (Townsley 1972), caryophylene (Longo & Sanroman 2006), flavanol (Nakao et al. 1999) and vanillin (Dornenburg & Knorr 1996). Many of these phytochemicals, especially the volatile compounds are secreted by plants as an indirect defense mechanism against herbivory and some other abiotic stress (Yuan et al. 2008).

The exploitation of novel secondary metabolites and their functions have gained the interest of many scientists worldwide and extensive studies have been done since the past 50 years. More than 80% of 30,000 known natural products were originated from plants (Fowler & Scragg 1988; Phillipson 1990). Although the advancement of computational biology has shedded light to medical field as new drugs could be designed base on predicted chemical structure, plants-derived compounds still serve as the model for drugs synthesis due to the complexity of their chemical structures (Pezzuto 1995). In fact, the world market for plant-derived drugs will be expected to achieve more than $26 billion in year 2011 (Saklani & Kutty 2008). However, there are some major drawbacks associated with phytochemicals production. Naturally these phytochemicals present at a much lower concentration compare to primary metabolites. The production of secondary metabolites is approximately 1% of the plant dry weight. Depending on the type of environmental stresses surrounding the plants, the type and level of secondary metabolites produce in plants changing from time to time, and from one place to another (Dixon 2001; Oksman-Caldenteyl & Inze 2004). Besides, the widespread of deforestation and instability of geopolitics make it difficult to extract pure secondary metabolites in whole plants (Shilpa et al. 2010). Fortunately, the advancement of biotechnology has made it possible to alter the production of secondary metabolites by means of plant cell cultures technology. The major advantages of plant cell cultures are that it could produce a continuous and more reliable source of plant pharmaceuticals (Vijaya et al. 2010). Though many efforts have been done since four decades ago, little success was achieved. Only the production of Shikonin from *Lithospermum erythrorhizon* cell culture and Taxol or Paclitaxel from *Taxus* cell cultures meeting the satisfactory yields for commercialization (Sekar & Kandavel 2010). Some other successful cases are such as rosmarinic acid production from *Anchusa officinallis*, indole alkaloids and catharantine

production from *Catharanthus roseus* and anthraquinone synthesize from *Morinda citrifolia* (Vanisree et al. 2004).

Up to now, many approaches have been done to increase the yield of secondary metabolites from plants. Strategies that have been accepted for used are manipulation of culturing media such as glucose, nitrate, phosphate and plant growth regulators concentration; screening and selection of cell lines which have the ability to produce better yield; optimization of culturing conditions such as temperature, light, pH and aeration; and addition of precursor or elicitor (Ramachandra Rao & Ravishankar 2002; Vanisree & Tsay 2004). Since the main roles of plant secondary metabolites are to increase plant adaptation to abiotic stresses and enhance plant defense system against pathogen attack, it is better to investigate some strategies to alter the production of metabolites based on this principle (Sekar & Kandavel 2010). In fact, elicitation by using molecules that involve in plant defense mechanism is the most efficient strategy to increase the productivity of secondary metabolites in plants (Roberts & Shuler 1997). Many biotic and abiotic elicitors have been employed to increase the production of desired compounds in plants (Barz et al. 1988), namely methyl jasmonate (MeJA), jasmonic acid (JA), salicylic acid (SA), fungus polysaccharide, yeast extract, heavy metal etc. Extensive researches have been done to study the roles of JA as the key signaling molecules in signal transduction system regulating the alteration of plant defense genes against environmental stresses. For instance, the expression of *pin* genes was activated by JA or its derivative, MeJA, in mechanically wounded tomato and potato (Farmer & Ryan 1992). The expression of *vsp* genes was also activated by MeJA as a defense mechanism towards wounded cells of soy bean (Creelman et al. 1992). Besides, exposure of *Hypericum perforatum* L. suspension culture to JA had activated the genes that expressed phenylalanine ammonia lyase (PAL) and chalcone isomerase (CHI) enzymes. Activation of these genes had increased the production of phenylpropanoids such as phenolic, flavanol and flavonol a 6-fold in cells treated with JA (Gadzovska et al. 2007). JA also altered the synthesis of ajmalicine and catharantine in *Catharanthus roseus* (Vazquez-Flota & De Luca 1998), rosmarinic acid and shikonin in *Lithospermum erythrorhizon* (Yazaki et al. 1997), scopoletin dan scopolin in *Nicotiana tabacum* (Sharon et al. 1998) and taxol dan paclitaxel in *Taxus* spp. (Palazon et al. 2003). Thus, jasmonate has been shown to be key molecules in the elicitation process leading to de novo transcription and translation that resulted in the enhancement of secondary metabolites biosynthesis in *in vitro* plants (Gundlach et al. 1992).

It is clear that harsh environmental conditions would activate the expression of certain abiotic stress related genes which involve in the biosynthesis pathway of secondary metabolites in plants. Therefore it becomes a crucial need to identify the stress responsive genes and study their signal transduction pathways not only for a better understanding of plants response and adaptation towards abiotic stress, but also for further development of strategies for commercial production of valuable compounds by manipulating certain metabolic pathways based on gene expression (Shilpa et al. 2010). A significant amount of studies have been done on application of elicitation to plant cultures, either to enhance secondary metabolites production or to discover novel compounds. Though many of these showed positive and encouraging results, the stress responsive genes which involved in the reactions of defense-related pathway and secondary metabolites biosynthesis pathway remain largely unexploited. Many molecular approaches such as mRNA differential display, representation difference analysis, RNA fingerprinting, cDNA microarray and suppression subtractive hybridization (SSH) technique have been applied to identify and characterize the

genes which were expressed differentially during stress condition. Of all, SSH is a more effective and efficient method compared to others especially when non-model organism is of concern because genome sequences information is not required (Huang et al. 2007). By combining normalization and suppression PCR in a single step, SSH technique could reduce the excessive target cDNA sequences; at the same time enriched the low amount differential expressed transcripts up to 1000 – 5000 times in the sample population (Diatchenko et al. 1996). Hence, it is rational to identify the JA responsive genes by SSH technique because these genes may be involved in the synthesis pathways of valuable metabolites and/or abiotic stress tolerance. In order to support this hypothesis, we had demonstrated the effects of jasmonic acid (JA) as the elicitor in altering secondary metabolites synthesis in a type of local herb called *Polygonum minus* and the expression of JA-responsive genes have been identified by subtractive cDNA library construction.

2. Elicitation

2.1 The concept of elicitation

In general, plants respond towards abiotic stress stimuli by regulating signaling cascade followed by modulating gene expression machinery which could lead to the synthesis of stress responsive protein or valuable bioactive compounds. When stress signal received by the receptor on plant membrane, the small endogenous signaling molecules such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) will regulate defense system, both synergistically and antagonistically. It has been proven scientifically that exogenous application of these signaling molecules on plant cultures could alter the expression of genes involved in biosynthesis of different classes of secondary metabolites. This approach is known as elicitation, a process that involves the application of chemicals or addition of physical stress to plant cultures or whole plants as a way to produce secondary metabolites which are normally absent in the plants (Bourgard et al. 2001; Roberts & Shuler 1997). Elicitors are defined as chemicals from various sources which could trigger the physiological and morphological changes or phytoalexin accumulation in plants as a defense mechanism against stresses (Sekar & Kandavel 2010). They could mimic the mode of action of natural stress stimuli and thus create a stress environment for plants growth and development. They are able to trigger the normal metabolism in plant cells to synthesize enzymes that catalyze the defense-related pathways which ultimately leading to secondary metabolites production.There are a few ways to classify type of elicitors. It could be categorized according to its nature, which is known as biotic and abiotic elicitors or based on its origin, which is known as exogenous and endogenous elicitors. Abiotic elicitors are substances that derived from non-living thing such as inorganic salts, heavy metal ion, UV radiation, high pH level and so on whereas biotic elicitors are derived from living organims, for instance, pectin and selulose from plant cell walls, and chitin and glucan from microorganism. On the other hand, as defined by the prefix “exo-” and “endo-”, exogenous elicitors are substances derived from outer cellular compartment such as polisaccharide, polyamine and fatty acids whereas endogenous elicitors are molecules present in the inner cellular compartment, such as glucuronide or hepta-β-glucoside (Namdeo 2007). Table 1 summarizes the classification of elicitors.

A handful of experiments have been done extensively to study the effects of abiotic elicitors in enhancing the production of secondary metabolites in whole plants or plant cell cultures. However, the mechanisms that actually take place in the elicited cells remain unclear. Till

now, a few hypotheses have been proposed as the biochemical responses of plants towards the challenges of elicitors. For examples, ion Ca2+ influx changes in the cytoplasm (Gelli et al. 1997) and significant changes in protein phosphorylation and kinase proteins activation (Romeis 2001). Besides, some scientists also hypothesized that deactivation of H^+-ATPase will result in acidic cytoplasm (Armero & Tena 2001). Generation of reactive oxygen species (ROS) such as superoxide anion and H_2O_2 may have direct antimicrobial effect against pathogen attack, in the case of biotic elicitors. These ROS could trigger the formation of bioactive fatty acids derivatives in plants (Apostol et al. 1989). Furthermore, ROS could act as secondary signals in the activation of plant defence genes expression (Low & Merida 1996). Thus, it could be concluded that the mechanism of elicitors is almost the same according to the origin, specificity, concentration, physio-chemical environment, stages in plant's life cycle and nutrient assimilation of plant (Namdeo 2007).

Nature-based elicitors		**Origin-based elicitors**	
Biotic elicitors	**Abiotic elicitors**	**Exogenous elicitors**	**Endogenous elicitors**
Enzyme, cell wall pieces, pectin, chitosan, glucan.	UV light, denatured protein, heavy metal, chemical.	Glucomannose, glucan, chitosan, poly-$_L$-lysin, polyamine, glycoprotein, polygalacturonase, selulase.	Dodeca-β-1,4-$_D$-galacturonide, hepta-β-glucoside, alginate oligomer

Table 1. Classification of elicitors according to their nature or origin

2.2 JA biosynthesis and its roles in plant secondary metabolism

Jasmonic acid (JA) or its methyl ester, Methyl jasmonate (MeJA) are endogenous signalling molecules derived from lipid and distributed widely in plants. They function primarily in plant response towards biotic and abiotic stress and a variety of plant growth and development processes, including flowering, fruit ripening, and root growth. It has also been recognised as promoter for senescence, growth inhibitor and elicitor for secondary metabolism in many plant species. It is synthesized from oxylipin by lipoxygenase pathway in plant cells. The signalling of oxilipin molecules which include JA, MeJA, JA and amino acid conjugate and other JA derivatives, are regulated by the fatty acid residues that form plant membranes (Wasternack 2007). Oxilipins are originated from either α-linolenic acid (α-LeA, 18:3) or linoleic acid (18:2) released by chloroplast membrane. JA biosynthesis is initiated with the formation of (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT) and (9S)-hydroperoxyoctadecatrienoic acid (9-HPOT) from α-LeA. Product formed from linolenic acid is (13S)-hydroperoxyoctadecadienoic acid (13-HPOD) whereas linoleic acid will produce (9S)-hydroperoxyoctadecadienoic acid (9-HPOD). This reaction is catalysed by lipoxygenase (LOX) enzyme (Feussner & Wasternack 2002). 13-HPOT will then be converted by allene oxide synthase (AOS) to 12-13-epoxy-octadecatrienoic acid (12, 13-EOT) which is very instable (Song et al. 1993). 12, 13-EOT will be converted to *cis*(+)-12-oxophytodienoic acid (OPDA) by allene oxide cyclase (AOC), and consequently to 3-oxo-2(2′-pentenyl)-cyclopentane-1-heptanoic acid (OPC) by 12-oxophytodienoic reductase acid (OPR). For the final step in JA biosynthesis, OPC will undergo three β-oxidation cycles in peroxysomes (Miersch & Wasternack 2000). Every gene that coded for JA biosynthesis enzyme could be

alter by JA itself (Wasternack 2006). Till now, many promoters have been tested and it was found that the activities of promoters have been increase upon exposure to JA (Kubigsteltig et al. 1999). This observation suggested that the biosynthesis of JA is regulated by positive feedback reaction.

JA is recognised as the signalling molecule in elicitation process that leads to *de novo* transcription and translation and eventually activates the secondary metabolites in plant cell cultures (Gundlach et al. 1992). It has also been known as molecule involves in the signal transduction pathway that leads to the activation of plant defense against pathogen, insect and herbivore attack (Menke et al. 1999). There are also some studies which shown that JA activities are not restricted to one type of secondary metabolite only but it is able to alter the production pathways of various classes of secondary metabolites such as phenylpropanoids, alkaloids and terpenoids (Zhao et al. 2005; Pauwels et al. 2009). Many studies were done using JA as the elicitor to induce the production of important metabolites. For example, the *Hypericum perforatum* (St. John's wort) cell cultures showed significant increase in the production of hypericin after treated with JA (Walker et al. 2002). This result was supported by another group of scientists where they have successfully increase 6-8 times the production of phenylpropanoids like phenolic compounds, flavanol, flavonol; and naphtodianthrones like hypericin in *H. perofatum* cell culture after elicited by JA (Gadzovska et al. 2007). Besides, the production of anthraquinone in *Morinda elliptica* cell cultures was also enhanced by JA treatment (Chong et al. 2005), so as to antioxidant like carotenoids, vitamin C and vitamin E (Chong et al. 2005). Furthermore, MeJA was showed to increase the accumulation of silymarin products in *Silybum marianum* (L.) Gaertn cell cultures (Sanchez-Sampedro et al. 2005) and shikonin in *Lithospermum* cell cultures (Yazaki et al. 1997).

In a recent study, we are interested in finding the genes involved in the biosynthesis of aromatic compounds or other valuable metabolites found in *P. minus* roots using the elicitation strategy. Since most of the secondary metabolites induced by elicitor present *de novo* in plant cells (Pare & Tumlinson 1997) and involved certain enzymes activities induction (Bouwmeester et al. 1999; Degenhardt & Gershenzon 2000), the production of volatile compounds triggered by JA in kesum roots will reflect the enzyme activities which catalysed the biosynthesis of those compounds. The enzyme activity is proportional with the mRNA transcripts related to the compounds production. By combining the analysis of metabolomics and differentially expressed genes dataset, we hope to have a better understanding in the correlation between plant defence system against abiotic stress and secondary metabolites production.

2.3 Case study: Elicitation of *Polygonum minus* roots with JA

We have recently conducted an experiment to evaluate the effect of JA elicitation on the production of volatile compounds in *Polygonum minus* roots by gas chromatography coupled with mass spectrometry, as well as using SSH technique to identify the transcripts responsive to JA stress. *Polygonum minus* Huds., or commonly known as kesum, is a type of local herbs rich in essential oils. It has been recognized by the Malaysian government in the Herbal Product Blueprint as an essential oil-producing crop (Wan Hassan 2007). Previous studies reported that the essential oil from kesum leaves was found to contain about 76.59% aliphatic aldehydes that consisted of two dominant compounds, decanal and dodecanal, and contained 0.18% b-caryophyllene, a sesquiterpene (Karim 1987). Other studies have successfully identified valuable compounds from *Polygonum* roots that possessed medicinal

properties. For example, researchers have found phytoestrogens from *P. cuspidatum* and *P. hydropiper* roots (Matsuda et al. 2001), phytohormones, and anthraquinones from *P. multiflorum* roots (Yu et al. 2006) and indigo from *P. tinctorium* roots (Chae et al. 2000). We decided to target roots as the organ for elicitation process because of their ability to import and export molecules between root cells and the rhizosphere (Gleba et al. 1999). In addition, roots also possess some advantages against other plant parts. As they are physically unprotected in the soil environment, they are surrounded by many types of microorganisms. Hence, they may produce a vast amount of metabolites that possess antimicrobial or aromatic properties to ensure plant survival (Poulev et al. 2003). The application of elicitors on plant shoots has serious limitations because the hydrophobic surfaces and impermeable characteristics of leaves result in low uptake of chemical elicitors. On the contrary, elicitors can be easily added into growth media and absorbed by roots and can easily harvest and screen for bioactive compounds. Roots also contain low levels of pigments and other compounds found in leaves, such as tannins, which may interfere in chemical screening and extraction (Gleba et al. 1999; Poulev et al.2003).

In our experiment, *P. minus* roots harvested from *in vitro* plantlets were used for elicitation process and subtractive cDNA library construction. *P. minus* were micropropagated by internode culture and subculture in MS solid medium at every 2 months interval. The cultures were incubated at 26 $\pm$ 2 ^{o}C with 16 hours photoperiod and 20 µM/m2/s light intensity. Prior to adding JA solution, *P. minus* were transferred to MS liquid medium supplemented with different concentrations of JA (50µM, 100 µM, 150 µM and 200 µM). The non-treated plantlets were used as control. *P. minus* roots were harvested from JA-treated plantlets at day-1, day-3 and day-6 for GC-MS analysis. Each experiment was performed in triplicate where each treatment contained 10 plantlets. The experimental designed was done based on factorial 5 x 3 (JA concentration x treatment period). The volatile compounds and other secondary metabolites induced by JA stress were extracted from 2g of roots by Solid Phase Micro Extraction (SPME). The extracted compounds were then purified and separated by gas chromatography in Shimadzu AQ5050P with HP-5MS non-polar column (30m x 0.25mm x 0.25µM) and detected by quadrupole mass spectrometry. The parameters for GC-MS analysis were as follow: injection temperature 220^{o}C; detector temperature 280^{o}C; column temperature 50^{o}C – 3 min, 20^{o}C/min - 100^{o}C, hold 3 min, 30^{o}C/min - 250^{o}C, hold 3 min; flow rate 1.3ml/min; injection volume 1µl; injection method – split ration and mass spectrometry was operated in scan mode. Finally the compounds detected by MS were compared against the GC-MS Nist. 147 library according to similarity index (SI) and retention time (RT). Only compounds with SI unit more than 80 and present consistently in two or more replicate were considered for further analysis. The results from qualitative analysis showed β-caryophyllene present abundantly and consistently in the sample. Hence, it was selected as a marker compounds which is known as single point external standard for quantitative analysis. The data obtained were analysed with SAS (Statistical Analysis Systems) statistic program version 9.0 at significant level $p < 0.05$. General Linear Modelling (GLM) and Duncan analysis were performed according to experimental design and the appropriate data obtained.

2.3.1 Phytochemical analysis of *P. minus* roots

The chemical compounds from some species in the Polygonum family have been studied decades ago, for example *P. minus* (Karim 1987) and *P. odoratum* (Dung et al. 1995; Hunter et al. 1997). From our experiment, 30 compounds were successfully detected by GC-MS from

the *P. minus* roots extract based on the comparison of similarity index (SI) and retention time (RT) with the database in NIST 147 library. The chromatogram profile for non-treated plant was shown in Figure 1.

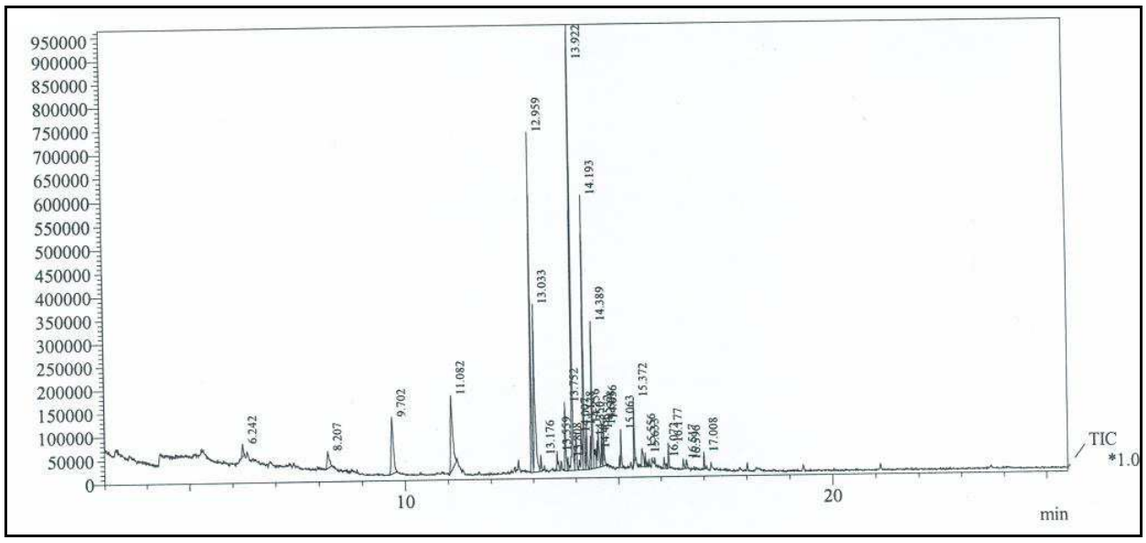


Fig. 1. Chromatogram profile for volatile compounds extracted from non-treated kesum roots extract using HP-5MS non-polar column (30m length x 0.25mm diameter x 0.25µM thickness). Injection temperature: 220°C; Detection temperature: 280 °C; Column temperature: 50 °C, 3 min; 20°C/min - 100°C, 3 min; 30°C/min - 250°C, 3 min. Flow rate: 1.3ml/min. Injection volume: 1µl. Injection method: split ratio. Type of detector: quadrupole. Mass spectrometry: scan mode. TIC: Total ion chromatogram.

Of 30 compounds detected, only 16 compounds were found to have SI value more than 80. These compounds were shown in Table 2 according to their own RT. The dominant compounds found in kesum roots extract are β-caryophyllene (17.57%), acetic acid (11.07%), α-caryophyllene (9.50%), octadecanal (3.08%), β-farnesene (2.84%), phenol (2.73%) and trans-α-bergamotene (2.13%). The volatile extracts consisted of 32.85% sesquiterpenes (β-caryophyllene, trans-α-bergamotene, β-farnesene, α-caryophyllene and α-panasinsene) and 5.58% alkanes (nonane, heptanes, octane and undecane). Only one aliphatic aldehyde was detected in kesum root extracts, which is known as octadecanal (3.07%).

2.3.2 The effect of JA elicitation on the production of volatile compounds in *P. minus* roots

We were looking into two factors that will affect JA elicitation on kesum, which is the JA concentration and treatment period. Four concentrations of JA (50µM, 100µM, 150µM and 200µM) and three treatment period (1, 3 and 6 days) were tested to ensure that the concentration of JA is not too high or the treatment period is not too long to cause plant death. This is because JA was proven to be the negative regulator for plant growth and development by creating a stress environmental condition to plants (Gadzovska et al. 2007). The optimum JA concentration and treatment period need to be carefully determined to ensure that the volatile compounds could be altered to the maximum level of production compared to control sample. Only the combination of JA concentration and treatment

period that could alter and increase the production of volatile compounds significantly, which is more than 2-fold compared to control will be chosen for subsequent subtractive screening. This step was to ensure that the differentially expressed genes are the genes coded for enzymes involved in volatile compounds induced by JA. We divided the compounds induced by JA into three categories. First group represents the compounds that increase in kesum roots treated with JA (nonane, heptane, β-caryophyllene, trans-α-bergamotene, β-farnesene, α-caryophyllene and pentanoic acid). Second group represents compounds that decrease or not detected in kesum roots after JA treatment (p-benzoquinone, phenol, α-panasinsen, octane, undecane and 1,2-benzenedicarboxylic acid) whereas the third group shows compounds that have slight increment after JA elicitation (octadecanal). The chromatogram profile for JA-induced compounds was shown in Figure 2.

Retention Time	Compounds	Total Peak Area (%)
8.027	Nonane	1.65
13.599	Heptane	1.11
13.752	Octadecanal	3.08
13.922	β-caryophyllene	17.57
14.007	Trans-α-bergamotene	2.13
14.128	β-farnesene	2.84
14.193	α-caryophyllene	9.50
14.256	p-benzoquinone	1.85
14.532	Phenol	2.73
14.656	α-panasinsen	1.82
15.063	Pentanoic acid	1.47
15.556	Octane	1.42
15.633	Heptane	0.44
16.072	Undecane	0.52
16.517	1,2-benzenedicarboxylic acid	0.52
16.596	Nonane	0.44

Table 2. Volatile compounds detected in kesum root extracts.

Among the elevated compounds, β-caryophyllene was found to be the most dominant sesquiterpene compound in kesum roots and it presents consistently in every experiment replicate. It has also been found in many other plant species such as *Elsholtzia argyi* flower (Peng & Yang 2004), *Salvia officinalis* flower (Dewick 2001), carrot seed oil (Ozcan & Chalchat 2007) and *Artemisia absinthium* essential oil (Judpentiene & Mockute 2004). This compound is the major ingredient for plant aroma (Peng & Yang 2004) and it is always used in the perfumery and aromatherapy industry (Dewick 2001). Therefore it was chosen as a marker compounds for further quantitative analysis where JA concentration and treatment period could be determined. For this purpose, pure β-caryophyllene was purchased from Sigma Ltd. as an external standard and diluted to 100ppm to obtain the standard peak area. The number of β-caryophyllene analytes from the JA-treated root samples was calculated by comparing their respective peak area with the standard peak area. All data collected were performed with ANOVA analysis. The increment of β-caryophyllene analytes was considered significant at the significant level of $p \leq 0.05$. Figure 3 below shows the effect of

JA elicitation on the production of β-caryophyllene analytes in both JA-treated and non-treated kesum roots.

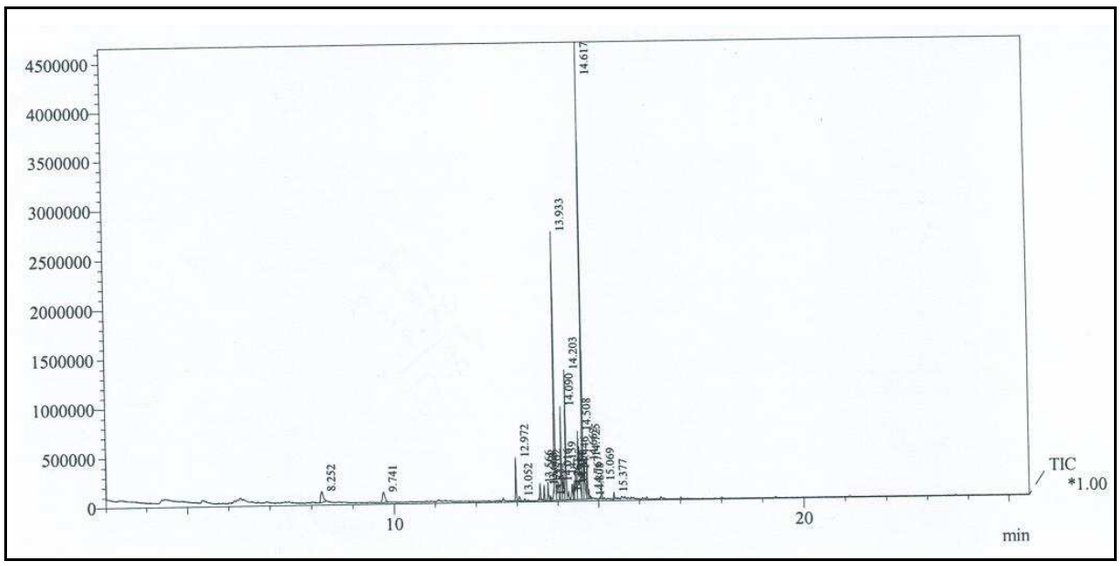


Fig. 2. Chromatogram profile for volatile compounds extracted from 150µM JA-treated kesum roots extract using HP-5MS non-polar column (30m length x 0.25mm diameter x 0.25µM thickness). Injection temperature: 220ºC; Detection temperature: 280 ºC; Column temperature: 50 ºC, 3 min; 20ºC/min - 100ºC, 3 min; 30ºC/min - 250ºC, 3 min. Flow rate: 1.3ml/min. Injection volume: 1µl. Injection method: split ratio. Type of detector: quadrupole. Mass spectrometry: scan mode. TIC: Total ion chromatogram.

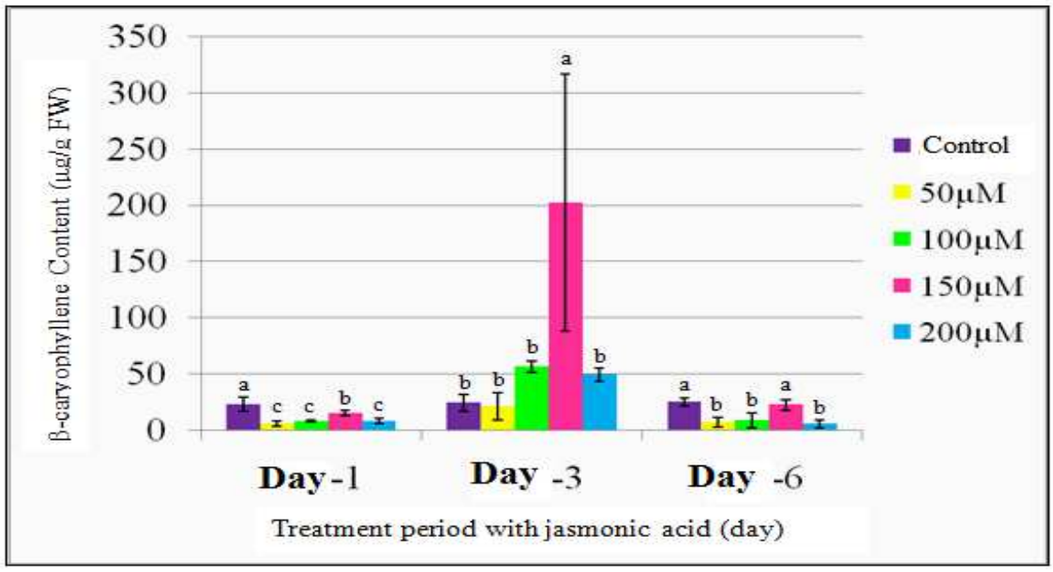


Fig. 3. The effect of JA treatment on β-caryophyllene in kesum roots. The values with different alphabet (a-c) are different significantly ($p \leq 0.05$). Data represent mean for triplicate with standard deviation.

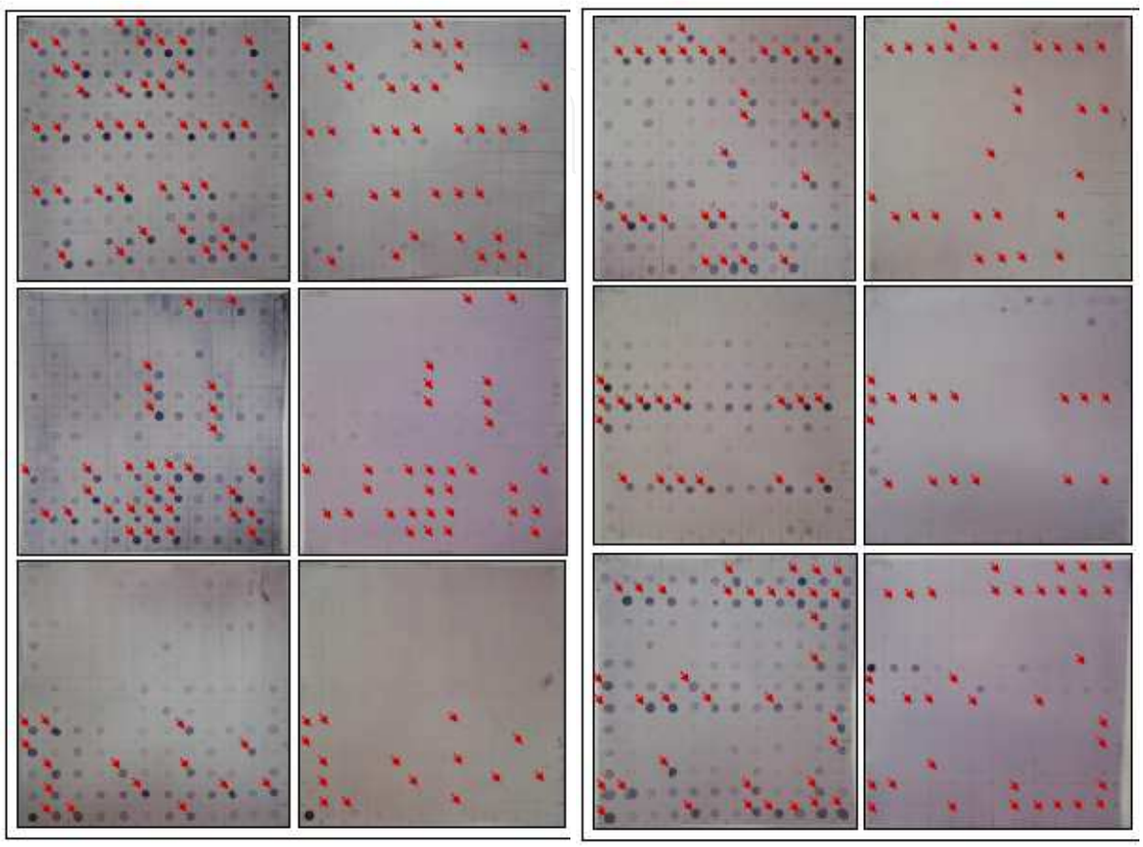
Day-1 observation indicate that the production of β-caryophyllene was occur at a much lower level and inconsistent because the standard deviation between replicates differ greatly (coefficient of variance = 27.86). The overall production of this sesquiterpene compound at all JA concentration was lower than control. The ANOVA analysis showed that no significant increase ($p > 0.5$) was observed in the production of β-caryophyllene in kesum roots treated with all four JA concentrations compared to control. This might be because of the kesum plantlets might need time to adapt to the stressful environment. The development of kesum root cells might be retarded after treated with JA which would suppress the primary metabolism and subsequently activated the secondary metabolism in root cells (Chen & Chen 2000; Wang et al. 2001). The level of production in day-3 was much higher and could be found in all three replicates for each JA concentration tested. The production of β-caryophyllene increased after treated with JA from 50μM to 150μM but decrease again at 200μM. Significant increase ($p \leq 0.05$) was seen when *P. minus* was treated with 150μM JA, which is 203.45 + 114.79μg/g compared to 25.59 + 8.96μg/g in control roots (coefficience of variance = 73.28). The production of this sesquiterpene compound increase about 1.2-fold, 2.1-fold and 8 fold in the roots treated with 50μM, 100μM and 150μM JA respectively compared to control sample when kesum plantlets established defense mechanism against abiotic stress created by JA elicitation. However, exposure of kesum to higher concentration of JA inhibited the production of β-caryophyllene where a decrease of 1.4-fold of this metabolite was observed in 200μM JA treatment. Necrosis was observed when the leaves and roots of kesum turned brownish as JA is an inhibitor to roots growth (Wasternack 2007). In day-6, β-caryophyllene present consistently in two or more replicates in every treatment. However, ANOVA analysis showed that the level of production decrease significantly ($p > 0.05$) compared to day-3 in all four treatments (coefficience of variance = 34.21). This situation might be caused by over exposure of kesum plantlets to JA stress. Our observation is similar to a study done by Sanchez-Sampedro et al. (2005) where they found that the production of silymarin in *Silybum marianum* increased to the maximum level after 3 days of MeJA treatment but decrease significantly after 7 days. Therefore, we concluded that kesum roots treated with 150μM JA for 3 days could produce β-caryophyllene at maximum level and we assume that the biosynthesis of other metabolites such as alkanes, aldehydes, alcohols and acids could also be enhance. Hence the RNA extracted from this treatment was subtracted against the non-treated kesum roots RNA to identify and clone the transcripts regulated by JA stress.

3. Differentially expressed genes induced by JA

3.1 Identification of JA-responsive genes

Subtractive screening is an efficient approach to clone the genes which are being expressed in one population but not being expressed or slightly expressed in another population. In this study, cDNA derived from kesum roots treated with 150μM JA for 3 days was served as tester whereas the cDNA derived from non-treated kesum roots was served as driver for subtracted cDNA library construction. Only forward subtraction was done as we were interested in identifying genes up-regulated by JA. A total of 1,344 white colonies were randomly picked from the subtracted cDNA library and screened by PCR using M13 forward and reverse universal primers to confirm the presence of cDNA inserts. PCR amplification revealed that 960 colonies were single stranded clones with the insert sizes ranging from 250bp to 1,200bp. These clones were subsequently hybridized against

unsubtracted tester cDNA and unsubtracted driver cDNA by Reverse Northern hybridization to reduce false positives. Our results showed that of these 960 clones, 195 clones hybridized strongly, 213 clones hybridized moderately, while 552 clones hybridized weakly with the unsubtracted tester cDNA whereas almost all of the clones showed weak or no signal when compared with the unsubtracted driver probe (Figure 4).



a) JA-treated sample b) Control sample a) JA-treated sample b) Control sample

a) JA-treated sample b) Control sample

Fig. 4. Reverse Northern analysis showing differential screening for putative cDNA clones altered by JA. (a) PCR products hybridized with DIG-labelled tester cDNA. (b) PCR products hybridized with DIG-labelled driver cDNA. Clones showing significant differential expression were pointed with red arrows. Negative control was pointed with blue arrow.

190 strongly hybridized clones were picked from Reverse Northern results and sent for sequencing. Of these, 174 clones were readable sequence in which 130 clones were unigenes that showed significant similarity to cDNA sequences in the NCBI database (E-value $\leq 10^{-5}$), 18 clones did not show any similarity to any known sequences and 26 clones had no significant results (Table 3). All of the 130 unigenes were deposited into NCBI and could be found in dbEST with accession numbers starting from GR505448 to GR505519. These clones were then classified into 11 categories according to their putative molecular functions (Figure 5). The largest set of genes was assigned to the stress-related genes (25%). This was followed by the following groups: other metabolism (20%), unknown genes (9%), transcription factors (8%), amino acid metabolism (6%), signal transduction and kinase (5%), carbohydrate metabolism (2%), transporter (2%), energy (2%), and regulation of gene expression (2%). Clones that showed similarity to the cDNA sequences from other plant species, but did not have any specific function, were assigned to the 'other' category (19%). The functional categories showed that these cDNAs might be involved in different biological processes. Clones that showed no similarity to any sequences in the GenBank were classified as hypothetical proteins and their putative structure and functions were predicted using bioinformatics software, which will be discussed later in this chapter. These clones may represent unique genes that were transcribed in response to the JA treatment that are involved in the metabolic pathway elicited by JA. We focused further analysis on clones that represent genes involved in biosynthesis of aromatic compounds in kesum, either under natural conditions or JA stress.

The largest group (25%) was assigned to stress-related genes. Exogenous JA application has been known as a stress treatment to plants and served as a stimulus to activate the expression of genes involved in the synthesis of plant secondary metabolites. As expected, a large number of clones encoded for stress-related genes were identified upon JA elicitation in this study. The complexity of kesum roots response towards JA elicitation suggested that many genes involved in plant defence mechanism against stress. A few clones were found to have homology with abiotic stress related genes as a defence response of kesum root cells towards JA treatment, including glutathione S-transferase from *Glycine max* (GR505459) and putative glutathione S-transferase T1 from *Lycopersicon esculentum* (GR505461), heat shock protein (GR505458), anionic peroxidise H from *Zea mays* (GR505463) and peroxidise 1 from *Scutellaria baicalensis* (GR505464), ELI3-1 (GR505453) and auxin induced protein (GR505454). Generally plant response towards pathogen or herbivore attack would activate a series of mechanism, including synthesizing anthocyanin and oligolignol, pathogenesis proteins (PR), generation of reactive oxygen species and formation of plant cell walls (Pauwels et al. 2009). The increase of gluthathione S-transferase (GST) was associated with hormone homeostasis or anthocyanin isolation in vacuole because GST played a role as auxin, cytokinin and anthocyanin transporter. When kesum roots were exposed to JA, excessive anthocyanin will be synthesized. The equilibrium of hormone in kesum root cells could be achieved by transporting the excessive anthocyanin into vacuole to be removed. This step was catalyzed by glutathione S-transferase enzyme (Moons 2003). On the other hand, peroxidase transcripts could be linked to generation of reactive oxygen species such as H_2O_2 and other metabolites like phenylpropanoids (Thimmaraju et al. 2006). Therefore we predicted that the peroxidase induced by JA was responsible for volatile compounds production such as sesquiterpenes in kesum roots. ELI3-1 gene is a type of elicitor activated gene and it responds to a wide range of elicitors (Ellard-Ivey & Douglas 1996). In this study, ELI3-1 was induced by JA, as well as heat shock protein which functions as a defence

Gene Bank Accession	Size (bp)	Similarity	Organism	Number of clones	E-Value
Transcription factor					
GR505449	265	F-box containing protein	*Populus tremula*	1	$3e^{-12}$
GR505460	595	Kelch repeat-containing F-box family protein	*Arabidopsis thaliana*	1	$1e^{-22}$
GR505470	423	GAMYB-binding protein (gbp5)	*Hordeum vulgare*	1	$2e^{-25}$
GR505481	298	ERF-like transcription factor	*Coffea canephora*	1	$2e^{-09}$
GR505492	583	BURP domain containing protein	*Solanum tuberosum*	2	$9e^{-05}$
GR505503	583	BURP domain containing protein	*Phaseolus vulgaris*	4	$8e^{-06}$
Signal transduction & kinase					
GR505514	285	Protein kinase	*Malus domestica*	2	$1e^{-55}$
GR505518	563	Multicopy supressor IRA1 (MSI1)	*Arabidopsis thaliana*	1	$3e^{-48}$
GR505519	712	Calmodulin-binding protein	*Arabidopsis thaliana*	2	$7e^{-116}$
Stress-related					
GR505451	666	MeJA-elicited root cell suspension culture	*Medicago trunculata*	1	$2e^{-18}$
GR505452	539	MeJA-elicited hairy roots culture	*Panax ginseng*	4	$3e^{-22}$
GR505453	503	ELI3-1 (ELICITOR ACTIVATED GENE 3)	*Arabidopsis thaliana*	1	$2e^{-29}$
GR505454	589	Auxin-induced protein	*Nicotina tabacum*	7	$6e^{-13}$
GR505455	269	EST from mild drought-stressed leaves	*Populus tremula*	1	$5e^{-16}$
GR505456	267	cDNA clone from senescing leaves	*Populus tremula*	1	$3e^{-05}$
GR505457	406	Cell cultures in osmotic stress	*Bouteloua gracilis*	3	0.0
GR505458	669	Gene for heat-shock protein	*Glycine max*	2	$3e^{-17}$
GR505459	656	Glutathione S-transferase (GST14)	*Glycine max*	7	$2e^{-21}$
GR505461	570	Putative glutathione S-transferase T1	*Lycopersicon esculentum*	3	$3e^{-10}$
GR505462	716	cDNA clones from water stress seedlings	*Zea mays*	1	$2e^{-07}$
GR505463	747	Anionic peroxidase H	*Zea mays*	1	$1e^{-09}$
GR505464	546	Peroxidase 1	*Scutellaria baicalensis*	1	$3e^{-48}$
Carbohydrate metabolism					
GR505465	684	Alcohol dehydrogenase	*Prunus armeniaca*	1	$3e^{-68}$
GR505448	603	Alcohol dehydrogenase 1 (adh1)	*Zea mays*	1	$1e^{-75}$
GR505466	436	β-fructofuranosidase	*Arabidopsis thaliana*	1	$2e^{-14}$

Acid amino metabolism					
GR505467	457	S-adenosyl-L-methionine synthetase	*Beta vulgaris*	3	$1e^{-135}$
GR505468	446	S-adenosyl-L-methionine synthetase	*Actinidia chinensis*	3	$2e^{-29}$
GR505469	443	S-adenosyl-L-methionine synthetase	*Elaeagnus umbrellata*	1	$6e^{-18}$
GR505471	741	S-adenosyl-L-homocystein hydrolase	*Mesembrayanthemum crystallinum*	1	0.0
Other metabolism					
GR505473	313	Glyoxal oxidase-related mRNA	*Arabidopsis thaliana*	2	$5e^{-06}$
GR505474	328	Dihydrolipoamide dehydrogenase 1	*Arabidopsis thaliana*	1	$3e^{-20}$
GR505475	460	Nodulin-35 (N-35) gene encoding a subunit of uricase II	*Glycine max*	1	$3e^{-09}$
GR505476	742	Nodulin family protein (NLP	*Gossypium hirsutum*	1	$4e^{-49}$
GR505477	520	Glycosyltransferase family protein 47	*Arabidopsis thaliana*	1	$2e^{-05}$
GR505478	524	Cytochrome oxidase subunit 1 (COI) gene	*Persicaria maculosa*	4	0.0
GR505479	391	Cytochrome oxidase subunit 1 (COI)	*Plumbago* sp.	3	$6e^{-163}$
GR505480	557	Cytochrome c oxidase	*Gossypium barbadense*	3	0.0
GR505482	401	NADH dehydrogenase	*Beta vulgaris*	7	0.0
GR505483	344	Urate oxidase	*Vitis vinifera*	1	$1e^{-20}$
GR505484	748	Glucan-endo-1,3-beta-glucosidase	*Nicotina tabacum*	1	$5e^{-29}$
GR505472	952	Lipoxigenase (lox gene)	*Capsicum annuum*	1	$7e^{-47}$
Energy					
GR505485	430	Type-AAA ATPase family protein	*Arabidopsis thaliana*	1	$1e^{-41}$
GR505486	393	Glyseraldehyde-3-phosphate dehydrogenase	*Zea mays*	2	$3e^{-11}$
Transporter					
GR505487	399	Root-specific metal transporter	*Lycopersicon esculentum*	1	$5e^{-12}$
GR505488	291	Auxin efflux carrier protein	*Zea mays*	2	$1e^{-11}$
Regulation of gene expression					
GR505489	377	Ribosomal protein S12	*Fagopyrum esculentum*	1	$8e^{-162}$
GR505490	675	18S rRNA gene	*Polygonum* sp. Soltis	2	0.0

Table 3. Putative JA-induced cDNA sequences in *P. minus* roots.

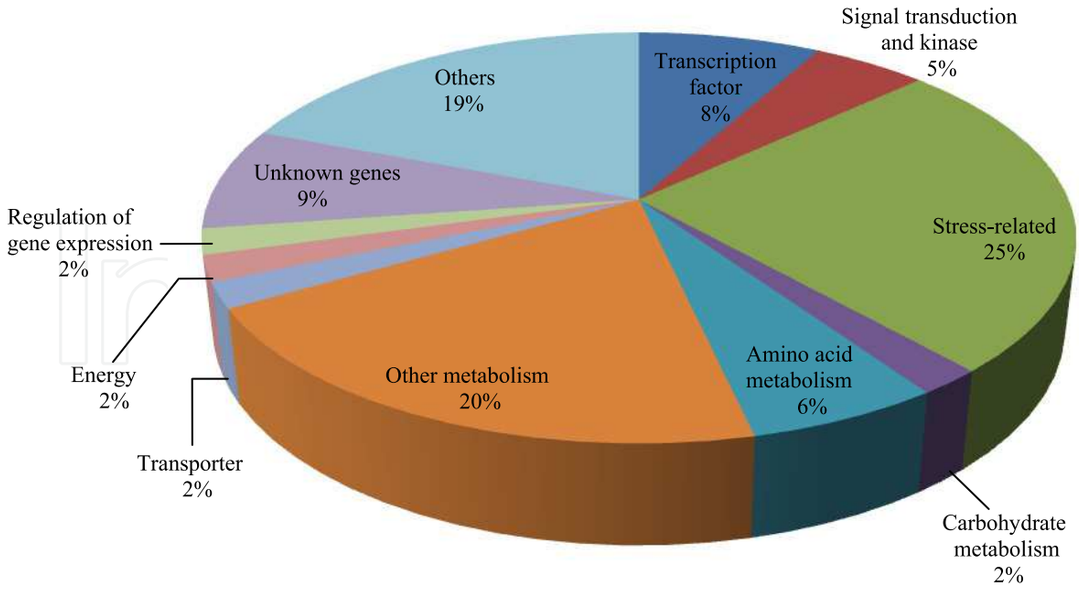


Fig. 5. Classification of clones based on their putative molecular functions.

response towards environmental stress. Besides, JA is known to be a phytohormone that regulates many plant physiological processes and it could interact with other hormone such as salicylic acid, absisic acid, auxin and giberellin in controlling plant growth and development (Creelman & Mulpuri 2002; Pauwels et al. 2009). Apart from that, some clones also showed homology to cDNA sequences in hairy roots of *Medicago trunculata* (GR505451) and *Panax ginseng* (GR505452) treated with MeJA. Other clones that showed homology to cDNA sequences of plants grown under drought stress (GR505455), osmotic stress (GR505457), water stress (GR505462) and leaves senescence (GR505456) were also identified. This result implies that many different stress factors will lead to the same gene expression (Sandermann et al. 1998). This result also suggests that JA, or its derivative MeJA, are signal molecules that regulate kesum defense responses in response to stressful environments, resulting in the activation of defense-related genes in plants.

The next group of transcripts which showed putative functions in plant growth and development were categorised under other metabolism (20%). Any changes in the primary metabolism will lead to plant defence response to stress (Ingram 7 Bartels 1996). Transcripts that were induced by JA in kesum roots include glyoxal oxidase (GR505473), dihydrolipoamide dehydrogenase (GR505474), Nodulin-35 gene (GR505475), nodulin family protein (NLP) (GR505476), cytochrome oxidase subunit I from *Persicaria maculosa* (GR505478) and *Plumbago sp.* (GR505479), cytochrome c oxidase from *Gossypium barbadense* (GR505480), urate oxidase (GR505483), glucan-endo-1,3-beta-glucoxidase (GR505484), glycosyltransferase family protein 47 (GR505477) and NADH dehydrogenase (GR505482). It was predicted that the Nodulin-35 and nodulin family protein were being expressed to ensure the normal development of root nodules as JA might inhibit roots elongation (Gadzovska et al. 2007). Glucan-endo-1,3-beta-glucosidase and glycosyltransferase family protein 47 were also being expressed to form kesum root cell walls. Besides, it was predicted that the generation of reactive oxygen species or secondary metabolites had affected the respiration process in kesum root cells and thus leading to the increase of cytochrome

oxidase transcripts. JA elicitation was also believed to induce the expression of urate oxidase and activates the production of H_2O_2 that resulted in hypersensitive cell death. The functions of glyoxal oxidase and dihydrolipoamide dehydrogenase in kesum roots under JA stress were yet to be discovered. Another unigene that encoded for lipoxygenase, the first enzyme in the oxylipin pathway for JA biosynthesis (Devitt et al. 2006) was also found in this study. It has been identified as the enzyme that involved in the production pathways of volatile compounds as the indirect plant defensive response to herbivory (Kessler and Baldwin 2001). Thus, it is believed that the lipoxygenase expression was associated with other volatile compounds detected by GC-MS, such as the alkanes, aldehydes and alcohols. This result suggests that there is a crosstalk between abiotic stress triggered by JA and other herbivory biotic stress.

Another group of cDNA sequences were associated with transcription factor (8%). For example, F-box containing TIR1 protein (GR505449), Kelch-repeat containing F-box family protein (GR505460), GAMYB-binding protein (gbp5) (GR505470), ERF-like transcription factor (GR505481) and BURP domain containing protein from *Solanum tuberosum* (GR505492) and *Phaseolus vulgaris* (GR505503). The F-box containing TIR1 protein (Parry & Estelle 2006) and Kelch-repeat containing F-box protein have been proven to be activated by JA in plant cells. Naturally F-box containing TIR 1 protein is a receptor to auxin. In plants, auxin is activated by auxin responsive factor (ARF) but inhibited by Aux/IAA protein. It was predicted that the expression of F-box containing TIR1 transcripts could activate the degradation of Aux/IAA protein so that auxin could be synthesized to equilibrate the hormones content in kesum roots. The Kelch-repeat containing F-box family protein was thought to be interacted with other proteins which involved in protein degradation process through ubiquitin-dependent pathway. Protein degradation is an important process in regulating cell cycle, transcription and signal transduction as a defence mechanism in kesum (Sun et al. 2007). The GAMYB-binding protein, BURP domain and ERF-like transcription factor induced by JA in this study were believed to be elements that regulate JA signalling in kesum roots.

Genes that were categorised into amino acid metabolism (6%) include cDNA clones coded for enzymes involved in phenylpropoanoids biosynthesis pathway, namely S-adenosyl-L-methionine sinthase from *Beta vulgaris* (GR505467), *Actinidia chinensis* (GR505468) and *Elaeagnus umbrellata* (GR505469), and S-adenosyl-L-homocystein hydrolase (GR505471) (Dewick 2001). The results of this study suggested that S-adenosyl methionine synthase and S-adenosyl homocystein hydrolase induced by JA could activate the production of aromatic compounds in kesum roots using aromatic amino acids as precursor. Carbohydrate metabolism (2%) or carbon-containing compounds covered alcohol dehydrogenase gene from *Prunus armeniaca* (GR505568), alcohol dehydrogenase 1 from *Zea mays* (GR505568) and β-fructofuranosidase (GR505466). The up-regulation of transcription of alcohol dehydrogenase may be associated with the biosynthesis of phenylpropenes, another important group of volatiles (Devitt et al. 2006). This result suggests that the genes involved in the biosynthesis of secondary metabolites can be induced by JA elicitation in kesum roots.

Genes categorized under signal transduction and kinase (5%) include kinase protein (GR505514), Multicopy suppressor IRA1 (MSI1) (GR505518) and calmodulin-binding protein (CaMBP) (GR505519). Kinase protein could modify other proteins or enzymes through phosphorylation of serine, threonine or tyrosine residues (Zheng et al. 2004). The kinase proteins found in this study might function in phosphotylation protein as a response

towards JA elicitation. Another plant response against stress is the increased of free calcium in cytosol as Ca^{2+} ion plays an important role in signal transduction that activated plant defence genes (Reddy et al. 2008). Ca2+ ion could activate calmodulin protein (CaM). The interaction between Ca2+/CaM and target molecules could lead to plant response towards environmental stress by activation of calmodulin-binding protein (CaMBPs) (Zielinski 1998). Besides, MSI1 has also been proven to function in signal transduction mechanism (Zheng et al. 2004). Therefore, the increase expression of these transcripts could possibly be linked to JA-induced signal transduction pathway.

AAA-type ATPase family protein mRNA (GR505485) and glyceraldehydes-3-phosphate dehydrogenase (GR505486) were categorized under energy group (2%). The increment of these transcripts was mainly due to energy consumption in regulation of plant metabolisms under JA stress. Root-specific metal transporter (GR505487) and auxin efflux carrier protein (GR505488) were classified into transporter group (2%). The discovery of root-specific metal transporter in JA-treated kesum roots may suggest that *P. minus* could be a suitable plant for phytoremediation of metal contamination in soil and further investigation need to be done by focusing to this aspect. The expression of auxin efflux carrier protein could be linked to auxin induced protein and F-box protein and it was predicted that the interaction of these three components could stabilize JA content in kesum roots by auxin synthesis. Next, S12 ribosomal protein (GR505489) and rRNA 18S gene (GR505490) were classified into regulation of gene expression group (2%). A few cDNA clones which represent ribosomal proteins such as 60S, 40S and 30S were known to be gene sequences respond towards stress. These ribosomal proteins play important roles in *de novo* protein synthesis (Machida et al. 2008).

Clones which have no significant similarity with any sequences in the databases were categorized as unknown genes (9%) and into others group (19%) that include cDNA clones that have no similarity with any nucleotide, mRNA or EST sequences in NCBI database (GR505498, GR505499, GR505500, GR505501, GR505502, GR505504, GR505505, GR505506, GR505507, GR505508, GR505509, GR505510, GR505511, GR505512, GR505513, GR505515, GR505516, GR505517), These sequences could be considered as novel genes induced by JA in kesum roots and further characterization must be done to identify their function in plant stress response, JA signalling and secondary metabolites production. The relationship between these clones and JA elicitation is yet to be identified and bioinformatics analysis has been carried out to investigate possible classification of these unknown sequences.

3.2 Discovery of unknown and novel cDNA sequences discovered during JA elicitation

A substantial fraction of the genes in the EST dataset encode for unknown proteins (also termed as hypothetical proteins) and about half the proteins in most genomes are candidates for hypothetical proteins (Minion et al. 2004). Hypothetical proteins are proteins that are predicted from nucleic acid sequences but have no corresponding experimental protein (Lubec et al. 2005). They are characterized by low identity to known annotated proteins. Thus, their functions remain unknown, and they pose a challenge to functional genomics and biology in general (Galperin 2001). Hypothetical proteins are utmost importance to complete genomic and proteomic information. Detailed knowledge on hypothetical proteins offers presentation of new structures and functions, contributing to the rising of new domains and motifs, revelation on a series of additional pathways hence completing fragmentary knowledge on the mosaic of proteins intrinsically.

The comparison of DNA or protein sequences from various organisms using computational methods is a powerful tool in protein study. By finding similarities between sequences, functional inference of newly sequenced genes can be achieved, new members of gene families can be predicted and evolutionary relationship can be explored. Computational analysis can quickly analyze and assign hypothetical proteins and able to generally predict their tentative biochemical functions (Lubec et al. 2005, Galperin 2001, Hoskeri 2010). Fundamentally, the prediction of functional inference is achievable by standard homology-based gene annotation complemented by genomic-context approaches (von Mering et al. 2003, Mellor et al. 2002, Marcotte et al. 1999) and, in some cases, requires structural intervention (Kolker et al. 2004). The combination of these approaches is intuitive and usually applies to various circumstances. Even though predictions can sometimes reliably infer the function of hypothetical proteins (Aravind and Koonin 1999), predictions do not provide necessary information regarding the exact biochemical function of a protein. Thus, predictions must still be validated through wet-lab experiments. However, computational analysis provides a faster and cheaper alternative to wet-lab experiments. Here, we cover the computational predictions of a set of hypothetical proteins obtained from the substracted cDNA library of a *P. minus* root that was treated with jasmonic acid (Gor et al. 2010).

3.2.1 Computational analysis of unknown genes

The unknown protein dataset discovered from a subtracted cDNA library of *P. minus* roots elicited with jasmonic acid were first translated to protein sequences for detailed bioinformatic analysis. The sequences were then examined for the existence of signal peptides using a signal peptide prediction tool. Knowledge of the existence of a signal peptide in a protein sequence is essential to defining and characterizing the protein. If there is a detectable signal peptide in a sequence, the signal peptide region must be cut off before the sequence can be used for further bioinformatics analysis. The sequences were compared to the databases of non-redundant proteins to detect any homologous sequences. The sequences with no significant outputs from the similarity search were further analyzed using preliminary structure-prediction analysis to identify a possible fold category. This information provided useful insights into the functional inference of these sequences. The analysis was performed using an in-house analysis portal called the Hypothetical Protein Analysis System (HPAS), which provided a systematic functional annotation procedure. The HPAS consisted of various tools for signal peptide prediction (SignalP 3.0) (Bendtsen et al. 2004), analysis of physicochemical properties (ProtParam (Gasteiger et al. 2003) and ProtScale (Yu et al. 2010)), topology analysis (Psortb (Bagos et al. 2008), SOSUI (Hirokawa et al. 1998), HMMTOP (Tusnady and Simon 2001), SignalP (Bendtsen et al. 2004), LipoP (Rahman et al. 2008)) and similarity search and annotation (NPSA@BLASTP (Altschul et al. 1990), NPSA@PSI-BLAST (Altschul et al. 1997), MPSrch (Agarwal et al. 1998), SSEARCH(Mazumder et al. 2008) and InterProScan (Zdobnov et al. 2001)). The HPAS covered all of the possible aspects of a protein sequence and, through a series of analytical tools, used all of the protein's characteristics to determine the protein's predicted functions.

3.2.2 Protein characterization by physicochemical properties

ProtParam was used to compute the physicochemical properties of these hypothetical proteins. Here, a few selected physicochemical properties were highlighted; molecular

weight, pI, instability index, aliphatic index and GRAVY (grand average of hydropathy). A GRAVY index greater than zero indicates a hydrophobic protein (Kyte and Doolittle 1982). Notably, only one sequence in this dataset (GR505502) had a GRAVY value (0.528) greater than zero. The other proteins were predicted to be hydrophilic. The aliphatic value refers to the relative volume occupied by aliphatic side chains (Ala, Val, Ile and Leu) and is considered to be a positive factor for increased thermal stability of globular proteins (Ikai 1980). Both GR505495 and GR505502 had the highest aliphatic indices (115.98 and 111.2, respectively). The stability index provides an estimate of the stability of a protein *in vitro*. An instability index higher than 40 indicates an unstable protein. Our results showed that five sequences were predicted to be unstable (GR505491, GR505497, GR505498, GR505499 and GR505502). Different protein localizations usually imply different biological functions. The prediction of subcellular localization is relevant to inferring possible functions, annotating genomes, designing proteomics experiments and characterizing pharmacological targets (Lubec et al. 2005). The prediction of the protein type from its primary sequence or the determination of whether an uncharacterized protein is a membrane protein is important in both bioinformatics and proteomics. For this purpose, a few programs were used (Psortb (Bagos et al. 2008), SOSUI (Hirokawa et al. 1998), HMMTOP (Tusnady and Simon 2001), SignalP (Bendtsen et al. 2004), LipoP (Rahman et al. 2008)) to predict the subcellular localizations of the hypothetical proteins. Two sequences were predicted to be membrane proteins (GR505495 and GR505450, with two and one transmembrane helices, respectively). A polypeptide can be a membrane protein if it contains at least one transmembrane helix. HMMTOP predicted transmembrane regions for both sequences, at residues 106-130 and 137-159 for GR505495 and residues 39-62 for GR505450. Table 4 shows the physicochemical analysis of the *P. minus* Huds hypothetical proteins achieved using various tools from HPAS and public databases. The consensus results were significant and were selected for further analysis (namely, the molecular responses of *P. minus* Huds roots to jasmonic acid induction).

3.2.3 Similarity search

Four programs are consecutively used for a similarity search analysis. Table 5 provides all results from the analysis. In the first round, BLAST was used to find sequences that were similar to the hypothetical proteins. If BLAST did not find any significant hits for the hypothetical sequences, then Psi-BLAST was used. MPSrch and SSearch were then used for the sequences that had no significant matches from the previous program. BLAST was able to reveal similarities to BURP-domain-containing protein 3 for GR505494, GR505505, GR505506, GR505507, GR505508, GR505509, GR505510, GR505512, GR505515 and GR505517. The sequence motif of the BURP-domain-containing protein family has been described previously (Hattori et al. 1998), and many plant species (but not other organisms) that contain this domain have been identified. The BURP-domain-containing protein consists of several modules, such as an N-terminal hydrophobic transit peptide, a short conserved segment, an optional segment consisting of repeating units that are unique to each protein and the BURP domain at the C-terminus. The BURP-domain-containing protein consists of four typical members, BNM2, USP, RD22 and PG1β. Thus far, this domain has been found only in plants, suggesting that its function may be plant-specific. The BURP-domain-containing protein family has been found in various plant species, but their specific functions are still being explored. Based on their existence in various plants at various stages and in various locations, many BURP family members are involved in maintaining normal plant metabolism and development. For example, in the oilseed rape (*Brassica napes* L.),

BNM2 is induced at the beginning of microspore embryogenesis, whereas the corresponding protein remains confined to the seed, where it is localized in the protein storage vacuoles (Zheng et al. 1992, Boutilier et al. 1994. Teerawanichpan et al. 2009, Treacy et 1997). USP from *Vicia faba* L., known as an abundant non-storage seed protein, is expressed during the early stage of zygotic embryogenesis (Bassuner et al. 1998) and at the very beginning of in vitro embryogenesis (21). However, PG1β is a non-catalytic β-subunit of the polygalacturonase isozyme from the ripening tomato and plays an important role in regulating pectin metabolism (Zheng et al. 1992, Watson et al. 1994). In contrast, RD22 is a drought-induced protein in Arabidopsis thaliana and is often used as a reference for drought-stress treatment in different plants (Yamaguchi-Shinozaki et al. 1993). To date, this is the first report of the prediction of the BURP-domain-containing protein from the hypothetical proteins of P. minus Huds.

Psi-BLAST was then used to identify remote homologs, especially for the sequences with no significant hits from the BLAST search output. A detailed sequence analysis of these sequences allowed us to provide a tentative characterization of GR505450. A distant homolog was identified as an elongation factor 1-gamma from *Danio rerio* (zebrafish) with an e-value of 2e-30. Unlike the BLAST search, Psi-BLAST identified the homolog for the hypothetical proteins GR505494, GR505505, GR505506, GR505507, GR505508, GR505509, GR505512, GR505515 and GR505517 as BURP-domain-containing protein 17 (B9G9L9). The homolog of GR505510 was BURP-domain-containing protein 5 (Q0JEP3), and the homolog of GR505511 was BURP-domain-containing protein 3 (Q942D4). Notably, for GR505511, the BLAST search identified a similarity to dehydration-responsive protein RD22 (Q08298). In this case, the BLAST output was favorable because the percentage of sequence identity between GR505511 and Q08298 is 39%. In general, Psi-BLAST was able to successfully identify distant homologs from the same protein family group and, hence, corroborate the output of the BLAST search.

Furthermore, there were a few more sequences (GR505491, GR505493, GR505495, GR505496, GR505497, GR505498, GR505499, GR505500, GR505502, GR50513 and GR505516) that had no significant hits from either the BLAST or Psi-BLAST runs. These sequences were then analyzed with two different similarity software packages (MPSrch and SSearch). The significance of the MPSrch results depends on the score value (i.e., a higher score implies more significant results). However, for the SSearch results, both the e-value and the SW score play important roles in choosing a significant hit. Both of these programs identified a putative uncharacterized protein from *Oryza sativa subsp. indica* (A2XQP1_ORYSI) and *Sorghum bicolor* (C5XJ38) for GR505494 and from *Oryza sativa subsp. indica* (A2XQP1_ORYSI) and *Glycine max* (C6TCE4) for GR505510. For GR505491, MPSrch identified a putative uncharacterized protein (D0ND35_PHYIN) from *Phytophthora infestans T30-4* as its homolog, while SSearch found no matches. GR505493 had no significant matches from either the BLAST or Psi-BLAST runs. Notably, MPSrch and SSearch detected N-acetylglucosaminyltransferase (Q70MW8) from *Bradyrhizobium sp. ISLU256* as similar to GR505493. N-acetylglucosaminyltransferase (EC.2.4.1.-) is a resident Golgi-enzyme that is essential for the processing of high mannose to hybrid and complex glycans (Strasser et al. 2005). For GR505500, the first two programs failed to detect any similarity matches, but MPSearch identified a match with a predicted protein (B7G4S6_PHATR) from *Phaeodactylum tricornutum CCAP 1055/1*, and SSearch identified galactoside-O-acetyltransferase (Q465V0) from *Methanosarcina barkeri*. This enzyme is usually found in bacteria, and it is interesting to experimentally investigate the existence of this protein in a plant. There were no significant

Peptide ID	ProtParam							
	Amino acid count	Molecular weight	pI value	Negative residues	Positive residues	Instability index	Aliphatic index	GRAVY
GR505450	121	14082.1	4.73	21	14	33.85 (Stable)	67.6	-0.269
GR505491	85	9656.2	6.39	7	6	69.9 (Unstable)	56.24	-0.5
GR505493	63	6679.5	9.52	4	7	23.04 (Stable)	76.19	-0.148
GR505494	201	22009.5	6.06	23	19	35.5 (Stable)	69.25	-0.277
GR505495	164	18007.6	6.98	13	13	23.11 (Stable)	115.98	0.346
GR505496	91	10096.5	5.45	13	12	36.17 (Stable)	83.41	-0.176
GR505497	151	16836.1	9.46	9	15	41.19 (Unstable)	66.56	-0.541
GR505498	43	4594	6.91	4	4	43.57 (Unstable)	43.02	-0.723
GR505499	87	9811.1	9	10	13	43.43 (Unstable)	50.46	-1.064
GR505500	164	18010.1	5.18	18	12	25.48 (Stable)	70.18	-0.367
GR505502	50	5511.6	4.46	6	4	46.33 (Unstable)	111.2	0.528
GR505505	174	19184.4	7.11	20	20	26.11 (Stable)	71.61	-0.29
GR505506	167	18241	6.89	16	16	31.5 (Stable)	73.47	-0.12
GR505507	166	18520.6	6.04	19	16	34.5 (Stable)	75.66	-0.245
GR505508	167	18383.3	5.83	20	16	32.49 (Stable)	74.01	-0.205
GR505509	159	17609.2	6.18	19	17	35.51 (Stable)	67.99	-0.348
GR505510	157	16928.4	6.83	16	16	27.45 (Stable)	69.55	-0.202
GR505511	135	14905.9	6.03	18	15	28.6 (Stable)	61.26	-0.485
GR505512	159	17609.2	6.18	19	17	35.51 (Stable)	67.99	-0.348
GR505513	126	13267.9	8.96	8	11	23.75 (Stable)	78.17	-0.06
GR505515	167	18256	7.58	16	17	33.9 (Stable)	71.14	-0.166
GR505516	127	13339	8.96	8	11	24.97 (Stable)	78.35	-0.045
GR505517	171	18937.5	7.9	18	19	26.83 (Stable)	81.4	-0.16

Table 4. Physicochemical properties of hypothetical proteins from the root of *P. minus*.

Peptide ID	BLAST Description	Psi-BLAST Description	MPSrch Description	SSearch Description
GR505450	No significant match	Elongation factor 1-gamma; Organism = *Danio rerio* (Zebrafish)	Putative uncharacterized protein; Organism = *Glycine max* (Soybean)	Probable glutathione S-transferase; Organism = *Nicotiana tabacum* (Common tobacco)
GR505491	No significant match	No sequence selected in the PSI-BLAST model	Putative uncharacterized protein; Organism = *Phytophthora infestans T30-4*	No significant match
GR505493	No significant match	No sequence found by PSI-BLAST	N-acetylglucosaminyltransferase; Organism = *Bradyrhizobium sp. ISLU256*	N-acetylglucosaminyl transferase; Organism = *Bradyrhizobium sp. ISLU256*
GR505494	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein Sb03g033760; Organism = *Sorghum bicolor* (Sorghum)
GR505495	No significant match	No sequence selected in the PSI-BLAST model	Predicted protein; Organism = *Nematostella vectensis* (Starlet sea anemone)	Predicted protein; Organism = *Nematostella vectensis* (Starlet sea anemone)
GR505496	No significant match	No sequence selected in the PSI-BLAST model	Putative uncharacterized protein; Organism = *marine gamma proteobacterium HTCC2148*	Putative uncharacterized protein; Organism = *marine gamma proteobacterium HTCC2148*
GR505497	No significant match	No sequence found by PSI-BLAST	Putative uncharacterized protein; Score = 93; Identity = 11.4; Identifier = A8URE8_9AQUI;	Putative uncharacterized protein; E-value = 0.45; SW Score = 127; Bit Score = 38.3; Identifier =

			Organism = *Hydrogenivirga sp. 128-5-R1-1*	A5Z3S2; Organism = *Eubacterium ventriosum ATCC 27560*
GR505498	No significant match	No sequence found by PSI-BLAST	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein; Organism = *Ruminococcus gnavus ATCC 29149*
GR505499	No significant match	No sequence selected in the PSI-BLAST model	Putative uncharacterized protein; Organism = *Sphingomonas wittichii* (strain RW1 / DSM 6014 / JCM 10273)	Putative uncharacterized protein; Organism = *Sphingomonas wittichii* (strain RW1 / DSM 6014 / JCM 10273)
GR505500	No significant match	No sequence selected in the PSI-BLAST model	Predicted protein; Organism = *Phaeodactylum tricornutum CCAP 1055/1*	Galactoside-O-acetyltransferase; Organism = *Methanosarcina barkeri* (strain Fusaro / DSM 804)
GR505502	No significant match	No sequence found by PSI-BLAST	Predicted protein; Organism = *Paracoccidioides brasiliensis (strain Pb03)*	No significant match
GR505505	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein Sb03g033760; Organism = *Sorghum bicolor* (Sorghum)
GR505506	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing; protein 17 Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	RD22-like protein; Organism = *Polygonum sibiricum*
GR505507	BURP-domain-containing protein 3;	BURP-domain-containing protein 17;	Putative uncharacterized protein;	RD22-like protein; Organism = *Polygonum*

	Organism = *Oryza sativa subsp. japonica*	Organism = *Oryza sativa subsp. japonica*	Organism = *Oryza sativa subsp. indica* (Rice)	*sibiricum*
GR505508	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein Sb03g033760; Organism = *Sorghum bicolor* (Sorghum)
GR505509	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	RD22-like protein; Organism = *Polygonum sibiricum*
GR505510	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 5; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein; Organism = *Glycine max* (Soybean)
GR505511	Dehydration-responsive protein RD22; Organism = *Arabidopsis thaliana*	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	BURP-domain-containing protein; Organism = *Brassica napus* (Rape)
GR505512	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	RD22-like protein; Organism = *Polygonum sibiricum*
GR505513	No significant match	No sequence selected in the PSI-BLAST model	Putative coat protein; Organism = *Elderberry latent virus*	Putative coat protein; Organism = *Elderberry latent virus*
GR505515	BURP-domain-containing protein	BURP-domain-containing protein 17;	Putative uncharacterized protein;	RD22-like protein; Organism = *Polygonum*

	Organism = *Oryza sativa subsp. japonica*	Organism = *Oryza sativa subsp. japonica*	Organism = *Oryza sativa subsp. indica* (Rice)	*sibiricum*
GR505516	No significant match	No sequence selected in the PSI-BLAST model	Putative coat protein; Organism = *Elderberry latent virus*	Putative coat protein; Organism = *Elderberry latent virus*
GR505517	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein Sb03g033760; Organism = *Sorghum bicolor* (Sorghum)

Table 5. Results of the similarity search using four similarity search programs (BLAST, Psi-BLAST, MPSrch and SSearch)

matches for GR505502, except for one match from MPSrch, but the score was low. Thus, thissequence is a good candidate for a structure-prediction approach for making a functional inference. Neither GR505513 nor GR505516 had matches from the BLAST or Psi-BLAST runs. Using MPSrch and SSearch, both sequences were predicted to be similar to a putative coat protein (Q911J7) from the elderberry latent virus. Even though the scores from both programs were reasonably low, at least the output can provide some insight into the functions of these hypothetical proteins and a basis for experimentation. A number of hypothetical proteins obtained from the roots of *P. minus* Huds were computationally identified as similar to at least one fully characterized domain. However, the functional interpretation of these proteins is limited. Notably, despite our best efforts, we were unable to provide functional annotations for GR505498 or GR505502. In addition, functional changes over evolutionary time (Devos and Valencia 2000, Todd et al. 2001) and database errors (Brenner 1999) confound the reliable computational predictions of the precise functions of these newly discovered genes. Further experimental evidence is required to successfully deduce their molecular roles.

3.3 Effect of JA elicitation on gene expression

RT-PCR analysis was performed to compare the transcripts expression between control root sample and JA-treated root samples. To verify whether the gene expression corresponding to the cDNA sequences generated by SSH were differentially expressed in *P. minus* under JA stress, four clones involved in the biosynthesis of aromatic compounds, three clones related to abiotic stress, and one clone representing a transcription factor were examined. Those clones were selected based on the nearest E-value to zero and molecular functions identified by BLAST. The clones that showed similarity to genes associated with aromatic compound biosynthesis were GR505472 (lipoxygenase, LOX), GR505465 (alcohol dehydrogenase, ADH), GR505467 (S-adenosyl-L-methionine synthetase, SAMS) and GR505471 (S-adenosyl-L-homocysteine hydrolase, SHH). The clones that were similar to abiotic stress response genes were GR505453 (ELI3-1), GR505459 (glutathione S-transferase, GST) and GR505464

(peroxidase, POD) and the clone involved in protein degradation pathway was GR505460, which had a cDNA sequence similar to the kelch-repeat containing F-box family protein (F-box). Ubiquitin 11, an endogenous gene expressed constitutively in plant was selected as internal control for normalization of gene expression. In general, the expression patterns were consistent with the results of the Reverse Northern analysis. The expression patterns can be divided into three types: (1) strong upregulation in JA-treated roots and slight up-regulation in normal roots, i.e., GR505453 and GR505459; (2) strong up-regulation in JA-treated roots but very little or no expression in normal roots, i.e., GR505465, GR505467, GR505471, GR505464, and GR505460; and (3) slight upregulation in JA-treated roots compared to non-treated roots, i.e., GR505472. The expression level of Ubiquitin 11 did not differ between samples (Fig. 6).

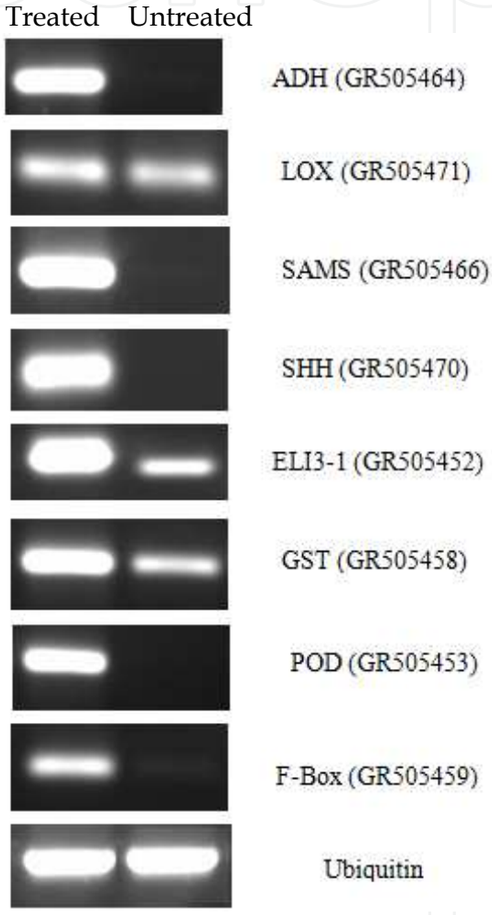


Fig. 6. Semi-quantitative RT-PCR analysis of expression patterns of genes responsive to jasmonic acid metabolism of JA-treated P. minus roots subtracted library. Expression pattern for each selected clone was examined in both JA-treated and non-treated roots' samples. Ubiquitin 11 was used as a control to demonstrate equal cDNA used as templates. Clones involved in aromatic compounds biosynthesis are GR505465 (ADH alcohol dehydrogenase); GR505472 (LOX lipoxygenase); GR505467 (SAMS S-adenosyl-L-methionine synthetase) and GR505471 (SHH S-adenosyl-L-homocysteine hydrolase). Clones related to abiotic stress are GR505453 (ELI3-1 ELI3-1 gene), GR505459 (GST glutathione S-transferase) and GR505464 (POD peroxidase). GR505460 is a clone similar to F-box protein (kelch repeat containing F-box family protein)

3.4 Correlation study between phytochemistry profiling and transcriptomic dataset

3.4.1 Genes involved in aromatic compounds production

A total of 11 cDNA sequences found in this study showed significant similarity with enzymes associated with biosynthesis of volatile compounds from plants. These sequences are found to be involved in acyl lipid catabolism pathway (2 ADH clones and 1 LOX clone) and shikimate pathway (7 SAMS clones and 1 SHH clone). GR505471 clone showed 81% identity similar to lipoxygenase gene isolated from *Capsicum annuum*. Lipoxygenase (LOX, EC 1.13.11.12) catalyzes deoxygenation of linoleic and linolenic acid to hydroperoxide in oxylipin pathway (Devitt 2006). Oxylipin is a common name for oxidized compounds derived from fatty acid though enzymatic reaction. Examples of oxidized compounds are hydroperoxide fatty acid, hydroxyl fatty acid, epoxy fatty acid, keto fatty acid, volatile aldehyde and cyclic compounds. This oxylipin pathway will affect plant aroma and taste (Yilmaz 2000). Lipoxygenase has been isolated from cucumber infected by spider mite and the expression of this gene was linked with the production of volatile compound named (Z)-3-hexynyl acetate (Mercke et al. 2004). It has also been isolated from papaya (Devitt et al. 2006) and apple (Dixon & Hewett 2000). Thus, we the expression of lipoxygenase was believed to be associated with the production of aldehyde in kesum, such as octadecanal which contribute to the aromatic flavour of kesum. Beside, GR505464 clone showed 72% identity similar to alcohol dehydrogenase from *Prunus armeniaca*. Alcohol dehydrogenase (ADH, EC 1.1.1.1) identified in this study might be involved in phenylpropanoids formation, another group of volatile aromatic compounds (Devitt et al. 2006). All the alkanes identified by GC-MS in kesum root extract could be oxidized into alcohols, aldehydes and acids homolog to the alkanes by using NAD and NADH as cofactor (Figure 7) (Dixon & Hewett 2000). ADH has also been identified in papaya (Devitt et al. 2006), apple (Dixon & Hewett 2000), corn (Walker et al. 1987) and grapevine (Torregrosa et al. 2008). It was believed that the expression of both LOX and ADH were involved in the oxidation of alkanes into alcohols, aldehydes and acids in kesum roots.

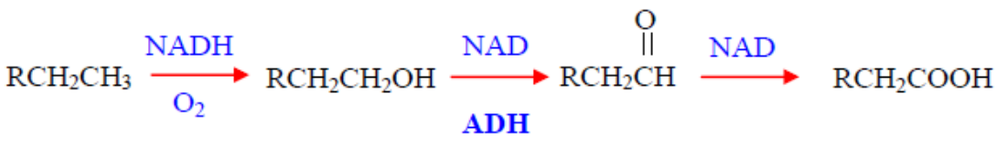


Fig. 7. Oxidation pathway of alkanes.

The identification of lipoxygenase and alcohol dehydrogenase in our study have further confirmed the results obtained by Karim (1987), who reported that approximately 76% of the essential oil in kesum leaves were comprised of aliphatic aldehydes, such as decanal and dodecanal (Karim 1987). Our results also strengthened the GC–MS data previously reported for *P. odoratum* leaves (Du˜ng et al. 1995; Hunter et al. 1997). Naturally these two genes may occur in kesum roots but are not routinely expressed. However, our study showed that their expression in roots could be up-regulated by exposure to JA. According to a model proposed by Yilmaz (2001) in a tomato-ripening study, lipoxygenase will catalyze the deoxygenation of linoleic and linolenic acid into hydroperoxides and subsequently to aldehydes and alcohols. Alcohol dehydrogenase will then catalyze the oxidation of aldehydes to the respective alcohols or vice versa (Figure 8) (Yilmaz 2001). In this study, JA elicitation may have caused the release of free fatty acids from the root cell membranes and

these fatty acids may have served as substrates for the production of volatile aromatic compounds in kesum.

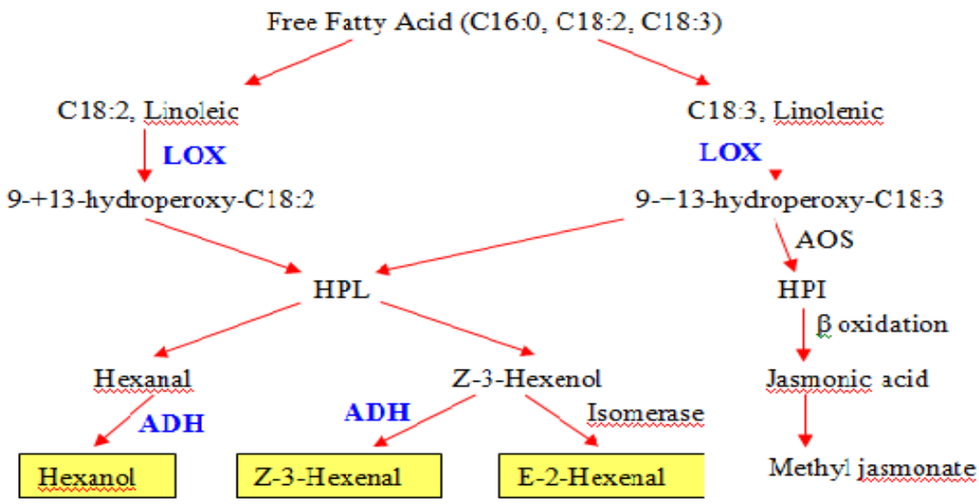


Fig. 8. Alcohol and aldehyde production by oxylipin pathway in tomato. (Source: Baldwin et al. 2000).

Apart from that, seven clones showed similarity to S-adenosyl-L-methionine synthetase (87% identity similar to *Beta vulgaris*) and one clone was similar to S-adenosyl-L-homocysteine hydrolase (87% identity similar to *Mesembrayanthemum crystallinum*). These clones were involved in the synthesis of aromatic shikimic acid through the shikimate pathway. Both of these clones demonstrated the type 2 expression pattern, where their expression in kesum roots was only up-regulated upon JA elicitation and no expression was observed under control conditions. Shikimic acid is the precursor for phenylpropanoid biosynthesis, another important group of secondary metabolites (Dewick 2001). Both S-adenosyl-L-methionine synthetase (SAMS, EC 2.5.1.6) and S-adenosyl-L-homocysteine hydrolase (SHH, EC 3.3.1.1) are enzymes that play a role in the synthesis of S-adenosyl-L-methionine (SAM), the main donor of the methyl group for many specific methyl transferase reactions, such as the transmethylation of alkaloids (Kutchan 1995). In plants, SAM is also associated with the production of phenylpropanoids (Kawalleck et al. 1992). Again, our data corroborate previous reports that identified flavonoids in leaves of the Polygonum family (including P. minus), which are synthesized by the phenylpropanoid metabolic pathway (Urones et al. 1990), P. stagninum (Datta et al. 2002) and P. hydropiper (Peng et al. 2003). The data suggest that the genes that are normally expressed in leaves could be triggered by JA in roots. On the other hand, SAM is also the intermediate molecule in ethylene and polyamine biosynthesis (Ravanel et al. 1998). Nevertheless, the expression of these two genes was also shown to be up-regulated in the petunia flower and they were linked to the production of benzenoid, a compound that contributes to the flower's scent (Schuurink et al. 2006). Hence, it was believed that the expression of the SAMS and SHH genes found in this study was related to the production of phenylpropanoids, alkaloids, ethylene or polyamine after JA elicitation. The activated-methyl cycle was predicted to be closely related to JA signaling and must be further investigated.

3.4.2 Genes related to abiotic stress

Clones encoding the genes related to abiotic stress,such as glutathione S-transferase (GST), ELI3-1 and peroxidase (POD) were evaluated to investigate the correlation between JA stress and other stress factors. Both GST and ELI3-1 demonstrated a type 1 expression pattern. Ten clones were similar to GST in this subtracted library. GST (EC 2.5.1.18) is a cytosolic enzyme found in all eukaryotes. This gene is always detected in stressed plants, such as heavy metal and salt-treated rice seedlings (Moons 2003), water-stressed maize seedlings (Zheng et al. 2004), drought-stressed horse gram (Chandra Obul Reddy et al. 2008) and fungus-elicited rice seedlings (Xiong et al. 2001). Therefore, and not surprisingly, the expression of GST was observed in normal kesum roots because the in vitro culture itself was a stress condition for kesum plantlets. It was strongly up-regulated in JAtreated roots as a defense mechanism, suggesting an overlap in the plant responses of plants to various stress factors. Also, GST catalyzes the conjugation between synthetic electrophilic compounds and the glutathione tripeptide (c-glutamyl-cysteinyl-glycine, GSH). The polar S-glutathionylated product will be actively transported into the vacuole by an ATP-binding cassette. Thus, GST is part of the detoxification mechanism in plants. In fact, it is the main ingredient for a variety of commercial herbicides (Andrews et al. 2005). Therefore, it is crucial to investigate kesum as a potential plant for phytoremediation purposes. ELI3-1 is another gene related to the abiotic stress caused by JA elicitation that will lead to phytoalexin and pathogenesis-related protein accumulation, phenolic compound production, and cell wall reconstruction. Seventeen of the ELI genes were identified in parsley cells cultured and treated with the Phytophthora megasperma fungus (Trezzini et al. 1993). In addition, MeJA elicitation has successfully activated ELI3, TyrDC, HRGP, and BMT genes in parsley (Ellard-Ivey and Douglas 1996). This observation proved that the expression of ELI3-1 could also be induced by JA elicitation. Peroxidase (POD, EC 1.11.1.7) plays an important role in the oxidation process, such as in peroxidative ooxidative oxidation and catalytic hydrosilylation (Umaya and Kobayashi 2003; Veitch 2004). It is an enzyme that catalyzes the oxidation of phenylpropanoids (Thimmaraju et al. 2006). It also functions in the plant-defense system, and it could be triggered by an elicitor (Go´mez-Va´squez et al. 2004; Perera and Jones 2004). The clones that were similar to POD in this study showed a type 2 expression pattern in the RT-PCR analysis. Its expression has been shown to induce the production of terpenoids in cucumbers infected by spider mites via oxidative degradation (Mercke et al. 2004). Our observation corroborates the results reported in kesum (Karim 1987) and *P. odoratum* (Du˜ng et al. 1995; Hunter et al. 1997), where various sesquiterpene hydrocarbons and their oxygenated compounds were identified. Therefore, it was predicted that stress stimuli, such as JA, could regulate the induction of important classes of plant secondary metabolites in kesum. In addition, its oxidative reaction showed that POD can be used as a component in the reagent for clinical diagnosis and various laboratory experiments (Thimmaraju et al. 2006) and thus increases the value of kesum.

3.4.3 Transcription factor activated by JA

Interestingly, the kelch-repeat containing F-box family protein and the TIR1 protein that is contained in the F-box found in this subtractive cDNA library are induced by JA (Craig and Tyers 1999; Parry and Estelle 2006). The kelchrepeat containing F-box family protein is involved in the protein–protein interaction in the ubiquitin protein degradation process via the ubiquitin-mediated pathway. The protein degradation process is important to regulate

the cell cycle, transcription, and signal transduction, as a mechanism for the root cells to adapt to JA elicitation stress (Sun et al. 2007). The functions of these proteins have been demonstrated in Arabidopsis and they may serve as transcription factors in genes expressed in response to JA treatment. While these proteins all play the same role in regulating JA, they also regulate species-specific secondary metabolite pathways (Pauwels et al. 2009). These proteins must be characterized and examined for the mechanism that drives secondary metabolites production in response to JA.

4. Conclusion

Our results showed that there is a close relationship between abiotic stress and the expression of genes involved in the biosynthesis of secondary metabolites. The subtractive cDNA library data set presented here provides the first collection of a set of JA responsive genes that may be involved in the secondary metabolite production in *P. minus* roots and also those participating in plant-defense mechanisms. These genes include dehydrogenase, lipoxygenase, S-adenosyl-L-methionine synthetase, S-adenosyl-L-homocysteine hydrolase, glutathione S-transferase, peroxidase, ELI3-1, and a transcription factor, F-box family protein. Identification of genes associated with flavour volatiles and the production of other aromatic compounds will provide a better understanding of the secondary metabolite biosynthetic pathways and their regulation in *P. minus*. Furthermore, the observed stress-related genes induced by JA elicitation indicate that plants respond to abiotic stresses in parallel with the biosynthesis of certain secondary metabolites. Characterization of these genes will be studied in details using *E. coli* expression system. Besides, their functions could be explored with GC or HPLC to confirm the synthesis of the corresponding compounds whereas their ability to cope with abiotic stress could be performed by culturing *P. minus* plantlets in various stress conditions. In this study, we concluded that the JA-responsive genes might be the genes associated with volatile compounds production as a defence response against abiotic stress.

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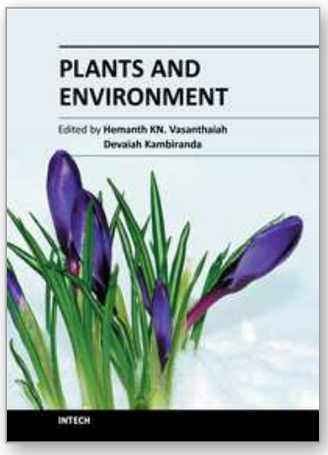

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Changing environmental condition and global population demands understanding the plant responses to hostile environment. Significant progress has been made over the past few decades through amalgamation of molecular breeding with non-conventional breeding. Understanding the cellular and molecular mechanisms to stress tolerance has received considerable scientific scrutiny because of the uniqueness of such processes to plant biology, and also its importance in the campaign â€œFreedom From Hungerâ€. The main intention of this publication is to provide a state-of-the-art and up-to-date knowledge of recent developments in understanding of plant responses to major abiotic stresses, limitations and the current status of crop improvement. A better insight will help in taking a multidisciplinary approach to address the issues affecting plant development and performance under adverse conditions. I trust this book will act as a platform to excel in the field of stress biology.

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