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FUNCTIONAL ANALYSIS OF THE *PERSICARIA MINOR* SESQUITERPENE SYNTHASE GENE PROMOTER IN TRANSGENIC *ARABIDOPSIS THALIANA*

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Abstract

Sesquiterpene synthase is an enzyme involved in sesquiterpene biosynthesis which catalyzed sesquiterpene formation from farnesyl diphosphate (FDP). In this research, the sesquiterpene synthase promoter (*PmSS*) was isolated from *Persicaria minor (P.minor)* to identify the functional region of the promoter and possible *cis* - regulatory element involved in the regulation of sesquiterpene synthase gene. Various putative *cis* - regulatory element involved in environmental stress and hormones were identified on *PmSS* promoter. The *PmSS* promoter and three series of deletion promoter were fused to β-glucuronidase (*gus*) gene and transformed into *Arabidopsis thaliana*. This study showed *PmSS* promoter was regulated in a developmental - specific manner and response to wounding, drought, heat, abscisic acid (ABA) and methyl jasmonate (MeJa) treatment. The results revealed the existence of *cis* regulatory elements that control the regulation of promoter activity in a developmental specific manner at -1758 to -1078 promoter sequences. The presence of *cis*-element acting as a repressor is expected to be present at the promoter between -1540 to -1078 bp. The region from -1078 to -855 was critical for maximal *PmSS* promoter activity. Deletion of promoter region from -1758 to -855 induced regulation of promoter in an organ-specific manner. Drought stress treatment did not induced GUS activity in deleted ABRE motif construct, suggested that ABRE motif is essential *cis* element during drought stress.

Keywords: Promoter, *PmSS, cis*-regulatory element, β-glucuronidase, GUS activity

Abstrak

Sesquiterpene sintase merupakan enzim yang terlibat biosintesis sesquiterpene di mana ia memangkinkan pembentukan sesquiterpene dari farnesil difosfat (FDP). Dalam kajian ini, promoter sesquiterpene sintase (*PmSS*) dipencilkan dari *Persicaria minor (P.minor*) untuk mengenal pasti bahagian promoter yang berfungsi dan elemen tindakan *cis* yang mungkin terlibat dalam pengawalaturan gen sesquiterpene sintase. Pelbagai elemen pengawalaturan *cis* putatif yang terlibat dalam tekanan persekitaran dan hormone dikenal pasti. Promoter *PmSS* serta tiga siri delesi promoter digabungkan dengan gen β-glucuronidase (gus) dan ditransformasikan ke dalam *Arabidopsis thaliana*. Kajian ini menunjukkan promoter *PmSS* dikawal atur mengikut tahap perkembangan dan bertindak balas terhadap rawatan tekanan perlukaan, kemarau, haba, asid absisik (ABA) dan metil jasmonat (MeJa). Hasil kajian mendedahkan kewujudan elemen pengawalaturan *cis* yang mengawal aktiviti promoter mengikut tahap perkembangan pada jujukan promoter di kedudukan -1758 ke-1078. Kehadiran elemen cis yang bertindak sebagai penindas dijangkakan hadir pada jujukan promoter di antara -1540 ke -1078 pb. Kawasan jujukan -1078 ke-855 adalah penting untuk aktiviti *PmSS* promoter yang maksimum. Delesi kawasan promoter dari jujukan -1758 ke -855 mengaruh pengawalaturan promoter secara spesifik organ. Rawatan tekanan kemarau tidak mengaruh aktiviti GUS dalam konstruk delesi motif ABRE, bermakna motif ABRE merupakan elemen *cis* penting semasa tekanan kemarau.

Kata kunci: Promoter, PmSS, elemen pengawalaturan *cis,* β-glucuronidase, aktiviti GUS

1.0 INTRODUCTION

Plants are sessile organisms which produce a variety of secondary metabolites as their mechanism in response to environmental stress. The secondary metabolite that is produce by plants are consist of terpenoids, amino acid, fatty acid derivatives and phenylpropanoid (1).Terpenes are the largest class of secondary metabolite most commonly found in plants and act as a defense mechanism from various biotic and abiotic stress. Volatile terpenoid emitted from plants plays an important roles in plants defense, attractant for pollinator and interaction with surrounding environment [2]. Terpene comprised of isoprene molecules [3] and classified into five class based on the number of isoprene unit present in their basic skeleton such as monoterpene,sesquiterpene, diterpene, triterpene dan polyterpene [4].

Sesquiterpenes are C15 terpenoid made up from three isoprene units and generally found in higher plants,marine organisms and fungi [5]. Sesquiterpene is a volatile compound and is known to contribute to the production of aromatic essential oils [6]. Due to its unique nature, essential oils from sesquiterpene are often used in the production of food, beverages, medicines and cosmetics[7]. Sesquiterpene also plays a vital role in direct and indirect plant defense systems such as protection against herbivorous attacks, thus generating chemical signals interactions between plants and insects [8]. In some wild relatives maize species, sesquiterpenes were emitted when attacked by herbivore[9]. Besides that, sesquiterpene (E)- β-caryophyllene was emitted by Z. *mays* roots as a response to lepidopteran larva attacks indirectly attracts their natural enemies [10]. Sesquiterpene (E)-β-caryophyllene is also functioning as a defense against a bacterial pathogen, the bacterial growth on the stigma of flower lacking (E)-β-caryophyllene emission were greater than wildtype flower [11]. Recent studies have found that sesquiterpene acts as a chemo-preventing agent in colon and skin cancer [12]. Because of its commercial value and its vital role in plant defense systems , various studies have been conducted in studying the mechanism of sesquiterpene biosynthesis.

Sesquiterpene was synthesized in two distinct pathways including cytosolic mevalonic-acid (MVA) pathway and deoxyxylulose phosphate (1-deoxy-D-xylulose5-phosphate) pathway [13]. The major step in sesquiterpene biosynthesis involves a group of enzymes known as sesquiterpene synthase. Sesquiterpene synthase is a group of proteins that converts farnesil diphosphate (FPP) to over 300 sesquiterpenes [12]. Since sesquiterpene synthase is directly involved in sesquiterpene production, it is one of the critical vital enzymes that need to be studied extensively. Research at the molecular level is crucial for better understanding of biosynthesis sesquiterpene pathways in plants. For instance studies of promoter improved fundamental knowledge of gene regulation, since its control the regulation of gene expression at the transcriptional level [14]. Expression of the gene during developmental stages, in different tissues or in response to environmental stress-mediated at the transcriptional level. Promoters and their *cis*-acting elements control the transcriptional regulation which is critical for activation and suppression of gene expression [15]. Hence, the promoters studies are crucial because they are basic to understanding the regulation of gene expression in plants.

To date, sesquiterpene synthase promoter has been studied in various plants species. In *Elaies oleifera*, the activity of sesquiterpene synthase promoter was induced by exogenous elicitation of Jasmonic acid (JA) [16]. Whereas, in *Artemisia annua*, the four sesquiterpene synthases β-caryophyllene (CPS), β-farnesene (FS), epi-cedrol (ECS), and amorpha-4,11-diene synthase (ADS) promoter were activated by wounding and methyl jasmonate treatment in a time-dependent manner [17]. However, there are limited studies of Sesquiterpene synthase promoter in response to various environmental stress based on *cis*-acting elements present on the promoter.

Previous studies have proven that abundance of terpenoids particularly sesquiterpenes were found in the essential oils of *P.minor*, suggesting *P.minor* as a good source for sesquiterpenes study [18]. *P.minor* usually known as kesum in Malaysia is an aromatic herbaceous plant that contains high secondary metabolic compounds [19] Hence,widely used as a flavouring agent, food additive and to treat body and stomach aches [20]. In *P.minor*, a cDNA sequence of (PmSS) gene was successfully cloned and characterized. Prior research has shown that under normal condition the expression of *PmSS* was regulated differently in different organs and was regulated by the jasmonic acid signalling pathway [21]. Thus, this research aimed to elucidate the functional region of the PmSS promoter and essential *cis* element that involved in regulation of sesquiterpene synthase gene expression. In the recent study the PmSS gene promoter was isolated and analyzed by PLACE and PLANTCARE database. A series of promoter deletion was created to identify the functional region of PmSS promoter. Later, PmSS promoter and its three deletion version were combined to the GUS gene and transformed into *A.thaliana*. GUS histochemical and fluorometric analysis were conducted to investigate the expression of GUS gene in transgenic *A.thaliana* in response to various stress treatment.

2.0 METHODOLOGY

2.1 Materials

P.minor was obtained from in vitro cultures that were maintained on solid Murashige and Skoog medium. The mother plants were originated from Ulu Yam, Selangor, Malaysia. In vitro culture around 7 to 8 weeks has been used in this experiment. Seeds of *A. thaliana* Col-0 were obtained from Plant Biotechnology Laboratory, UKM. *A. thaliana* Col-0 was grown in a growth chamber maintained at a temperature of 22◦C day/ 20◦C night and relative humidity of 50–70%. The photoperiod was set at 16 hday/8 h night, with a light intensity of 100–150 µmoles m^{-2} s^{-1} using fluorescent bulbs.

2.2 Cloning and *in silico* Analysis of *PmSS* Promoter

The full-length sequence of *PmSS* gene information was taken from NCBI (GenBank accession no.KT192706). The *PmSS* promoter region was isolated using Genome Walker Universal Kit (Clonetech) from genomic DNA of *Persicaria minor* leaves. Then, *PmSS* promoter was cloned into a pGEM-T easy vector and subsequently sequenced. Putative *cis*-acting elements of *PmSS* promoter was analyzed using PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)[22] and PLACE database (http://www.dna.affrc.go.jp/PLACE/signalup.html) [23]. The

2.3 Generation of Promoter Deletion GUS Constructs

Three promoter deletion were conducted by PCR amplification using three forward primers and a universal reverse primer as listed in Table 1. The full length of *PmSS* promoter and amplified deletion promoter fragments *PmSSD1*(-1540/+66), *PmSSD2*(-1078/+66) and *PmSSD3* (-855+66) were fused with GUS gene using Gateway Cloning Kit from Invitrogen to assess the promoter activity. The PCR products of *PmSS, PmSSD1, PmSSD2* and *PmSSD3* were cloned into the pDONRTM221 using Gateway BP recombination reaction and transformed into *E.coli* strain Top10. Then, all the constructs were extracted using GeneJET Plasmid Miniprep from ThermoFisher Scientific and sequenced for verification. Using Gateway LR recombination reaction, all constructs were inserted into a destination vector pBGWFS7.0 which contains GUS gene. These recombinant vectors and a pBGWFS7 .0 vector without promoter (control) were transferred into *Agrobacterium tumefaciens* strain GV3101 by using the freeze-thaw method.

Table 1 Primer sequences used in promoter deletion

Primer	Primer sequence (5'-3')
PmSSD1_F	5'- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG GGT TCA AAT CTC A -3'
PmSSD2_F	5'- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC GCC ACT GTT CGT GTC TC GTG TAT GT -3'
PmSSD3_F	5'- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CCC AAC CAA AGT TGA CTA -3'
PmSS_R	5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CGA CGA TCG AGC GAT GTG GTC AAT -3'

2.4 Transformation of *A. thaliana* Expressing GUS Reporter Fusions

The *A.tumefaciens* carrying *PmSS, PmSSD1, PmSSD2, PmSSD3* and pBGWFS7.0 were transferred into *A.thaliana* by floral dip method [24]. Transformed *A.thaliana* were directly screened by spraying with the herbicide Basta (120 µg/ml) on 10th, 12th, 15th and 17th day after germination (DAG). Resistance *A.thaliana* expected to be transgenic and confirmed by PCR using the same specific primers as listed in Table 1. Segregation test was conducted on T2 seeds to get single copy number line and was kept grow to obtain T3 seeds. Homozygosity test was performed on T3 seeds for each construct to obtain homozygous seeds and used for promoter functional analysis.

2.5 Hormone and Abiotic Stress Treatment

Homozygous transgenic *A.thaliana* were grown on MS medium for functional analysis. 2 weeks old seedlings of transgenic *A.thaliana* were treated with hormone and abiotic stress treatment. For wound treatment, *A.thaliana* leaves were crushed several times with laminated forcep which effectively wounded around 40% of leaf area. Plants were incubated for 1 hour before proceeding with GUS histochemical and fluorometric test. For drought stress treatment, *A.thaliana* seedlings were put on the surface of dry filter paper and incubated in a growth chamber at 22 ℃ for 6 hours [25]. Heat stress treatment was conducted as described by Eun *et al*. [25] with slight modification. *A.thaliana* seedlings on MS plate were incubated at 37°C for 6 hours in the dark instead of transfer to filter paper saturated with MS medium. ABA and MeJa treatments were conducted as described by Kim *et al.* [26] with slight modification. *A.thaliana* seedlings were transferred to filter paper dampened with 100 µM ABA and filter paper dampened with 100 µM MeJa instead of filter paper saturated with MS medium and incubated for 24h at 22 °C in the dark.

2.6 GUS Histochemical Staining

Histochemical staining for GUS activity was performed on treated *A.thaliana* seedlings and at different developmental stages of *A.thaliana* according to the method reported by Jefferson *et al*. [27]. Transgenic *A.thaliana* seedlings were incubated in 1 mL X-Gluc solution for 24h at 37°C. Then, for destaining samples were soaked in 50% alcohol solution for 24 h solution.

2.7 GUS Fluorometric Analysis

For the fluorometric assay, FluoroAce β-glucuronidase Reporter Assay (BioRad) was used and conducted according to manufacturer' settings. Transgenic *A.thaliana* seedlings were homogenized in GUS extraction buffer. Specific volume (30 µg) of *A.thaliana* extract were added to 500 µL assay buffer (1.2 mM MUG, 10 mM β-mercaptoethanol and 1X reaction buffer). The mixture was vortex and incubated for 30 minutes at 37°C. I mL stop buffer was added to the incubated sample. Then, relative fluorescence units (RFU) were measured by VersaFluor Fluorometer 100 (Bio Rad) according to FluoroAce β-glucuronidase Reporter Assay (Bio-Rad 2000 instruction's manual.

3.0 RESULTS AND DISCUSSION

3.1 Cloning of the *PmSS* Promoter Region and its Deletion Derivatives

The full length of *PmSS* promoter region from *P. minor* was cloned and sequenced in order to identify the putative *cis* –elements exist in *PmSS* promoter. The 1758 bp promoter region and 66-bp 5'UTR of *PmSS* promoter was obtained from the *P.minor* genomic DNA (GenBank accession no. MH423578). The transcription start site located 75 bp upstream of the ATG translational codon (GenBank accession no.KT192706) was determined by 5'RACE. *PmSS* putative promoter sequences was evaluated in silico by using PLACE and PlantCare database to identified *cis*-regulatory elements. The core promoter containing putative CAAT and TATA boxes were detected at -52 bp and -18 bp, respectively. In contrast, the CAAT and TATA boxes in *Elaeis oleifera* sesquiterpene synthase promoter were observed at -352 and-25 bp [16]. Numerous potential putative *cis* elements associated with hormone responsive and environmental stress were discovered in the *PmSS* promoter region as shown in Table 2.

A few putative *cis* elements that are involved in ABA responsiveness were detected including ABRE motif which is responsive towards ABA [28-29] and MYCATRD22 motif which is a binding site for MYC protein when induced by ABA [29]. Furthermore, *cis* elements that responsive toward dehydration also have been found in *PmSS* promoter including MYCCONSENSUSAT and MYCATERD1which are MYC recognition site is found in the promoters of the dehydration-responsive gene rd22 and necessary for expression of erd1 in dehydrated Arabidopsis [29], whereas MYB2CONSENSUSAT and MYB1AT were MYB recognition site which is responsive toward dehydration. MYC and MYB transcription factor are synthesized when the accumulation endogenous ABA occured [30]. Other important motifs that were found in *PmSS* promoter are WUN motif which is *cis* elements that responsive towards wounding, TC, the *cis* element that are responsive in defense and stress responsiveness, GT1GMSCAM4 motif which is induced by pathogen and salt [31] and GATABOX, *cis* elements involved in light regulation. Prior study of sesquiterpene synthase promoter in *Artemisia annua* also have identified the WUN, HSE, TC, ABRE and GATABOX motifs [17] on the promoter. This indicates, the sesquiterpene synthase expression was regulated by the hormone and the environmental factors.

Several significant *cis* regulatory elements were selected for promoter deletion study to understand the regulation of *PmSS* promoter. These including *cis* regulatory element involved in ABA responsiveness (ABRE) located at -922 bp; *cis*- regulatory element involved in heat stress responsiveness (HSE) located at -1116 and -1232 bp; *cis*-acting element involved in wound responsiveness (WUN) located at -1598 bp, and *cis* – acting element involved in defense and stress responsiveness (TC) located at- 1529,-1333 and -12 bp. Previous studies reported that herbivore attacks in the plants resulted in sesquiterpene emission from the plants to attracts enemies of the herbivore.

In response to herbivore attack to attract natural enemies of the herbivore, sesquiterpene act as indirect defense system in several plants species including *Z. mays, Oryza sativa* and *A.thaliana,* [10] [32-33]. Based on, *in silico* results, several factors that might control the regulation of *PmSS* gene during plant stress can be identified.

Table 2 Putative *cis*-regulatory elements identified in the *PmSS* promoter based on PlantCARE and PLACE database

Regulatory element	Sequence	Location	Function
WUN	TCATTACGAA	1598 (-)	involved in wound responsiveness
TC	ATTTTCTCCA, ATTTTCTTCA	1529 (+), 1333 (-)	involved in defense and stress responsiveness
HSE	AAAAAATTTC	1116 (+), 1232 (+)	involved in heat stress responsiveness
ABRE	AGTACGTGGC	922 (+)	involved in ABA responsiveness
MYBCORE	CNGTTR	1745(-),	MYB binding

Regulatory element	Sequence	Location	Function
		1738(-)	site. Involved in drought tolerance
MYB2CONSENSUSAT	YAACKG	1738(+), 29 (-)	MYB recogntion site. Involved in drought tolerance
GT1GMSCAM4	GAAAAA	1560 (-), 1239 (+), 481 (+)	Involved in salt and pathogen induction
GATABOX	GATA	1518(-), 1320 (+), 1195(-), 881 (-), 502 (+)	Light responsive element
MYCCONSENSUSAT	CANNTG	1472(+), 1323(-), 895(+), 757(-), 51(-), 723 (-)	MYC recognition site. Involved in cold and drought tolerance
MYB1AT	WAACCA	1381 (+)	MYB recognition site. Involved in drought tolerance
WBOXNTERF3	TGACY	840(+)	Involved in wounding stress
MYCATRD22	CACATG	723(-)	MYC binding site, ABA responsive element
MYCATERD1	CATGTG	723(+)	Involved in drought tolerance

*(+): sense strand ; (-):antisense strand

To identify the significant regulatory region of *PmSS* promoter three series of deletion were generated to obtain three truncated fragments which are *PmSSD1*, 1606 -bp fragment without WUN motif, *PmSSD2* 1144 – bp fragment without WUN, TC and HSE motifs, and *PmSSD3* 921-bp fragment without WUN, TC, HSE and ABRE motifs. The full length of *PmSS* promoter and its three 5' deletion fragments were inserted into pBGWFS7.0 vector with GUS reporter gene to analyze the promoter activity. Figure 1 show schematic diagram of the *PmSS* promoter. Transgenic *A.thaliana* carrying all constructs were created using floral dip method [25]. The single copy gene was detected by performed segregation test on T2 transgenic lines. The T3 homozygous and single copy transformants were used for promoter functional analysis

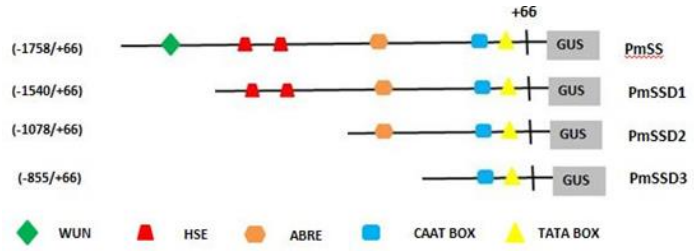

Figure 1 A schematic diagram of *PmSS, PmSSD1, PmSSD2,* and *PmSSD3* promoter fused with GUS reporter gene in the pBGWFS7.0 vector. The putative *cis* regulatory elements are exhibited by different symbols. The numbers show the nucleotide position of each *PmSS* promoter fragment

3.2 Developmental-specific Activity of the *PmSS* Promoter in Transgenic Arabidopsis

In order to evaluate the expression pattern of the *PmSS* gene at different developmental stages, the GUS expression pattern of transgenic *A.thaliana* carrying all the promoter constructs were analyzed. Different developmental stages of *A.thaliana* transformants expressing *PmSS, PmSSD1, PmSSD2, PmSSD3* and control (pBGWFS7.0) plants were subjected to histochemical staining to observed GUS expression pattern. The GUS expression pattern was observed at 3 days after germination (DAG), 7 DAG and 14 DAG seedlings. Figure 2 shows the results of GUS histochemical staining in *PmSS, PmSSD1, PmSSD2, PmSSD3* and control plants in different developmental stages. In the *PmSS* plants, GUS expression was detected only in leaf, hypocotyl and a small area of the root. However, in the 7 DAG and 14 DAG seedlings GUS activity was observed in the entire transgenic Arabidopsis seedlings tissue. Whereas in the *PmSSD1* plant, GUS expression level was lowest compared to other constructs. GUS expression was detected only at the root tip in 3 DAG seedlings. In the 7 DAG seedlings, GUS expression was located at the tip of leaf apex and root tip. Strong GUS expression was observed at leaf vein and lower GUS expression at the root tip in the 14 DAG seedlings compared to 3 DAG and 7 DAG seedlings. This result indicate that *PmSS* and *PmSSD1* promoter activity regulated developmental specific gene expression. However, strong GUS expression was observed in the entire *PmSSD2* seedlings tissue of the 3 DAG, 7 DAG and 14 DAG seedlings. GUS expression was observed only at the stem of *PmSSD3* seedlings of the 3 DAG, 7 DAG and 14 DAG. The GUS expression of *PmSSD2* and *PmSSD3* transgenic Arabidopsis seedlings were consistent from 3 DAG till 14 DAG. This revealed that *PmSSD2* and *PmSSD3* promoter construct do not direct plant developmental specific gene expression. Whereas no GUS expression was detected in control plants, suggested there is no endogenous GUS activity in *A.thaliana*. Based on the developmental-specific activity of all the constructs, revealed that

unknown regulatory elements that control plant developmental specific gene expression might exist between - 1758 to -1078 bp on *PmSS* promoter.

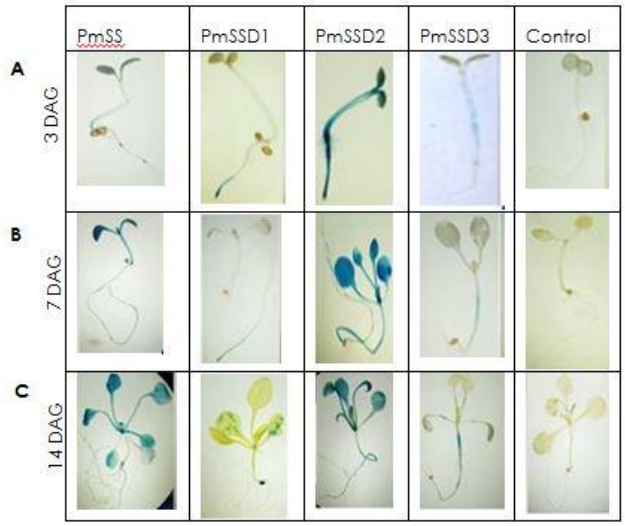

Figure 2 GUS histochemical staining and qualitative GUS expression in transgenic *PmSS* , *PmSSD1, PmSSD2, PmSSD3* promoter and control (pBGWFS7.0) plants at (A) 3 DAG seedlings, (B) 7 DAG seedlings and (C) 3 DAG seedlings

The fluometric analysis reveals that the *PmSSD2* plants GUS activity was the highest, whereas GUS activity in the *PmSS*, *PmSSD1* and *PmSSD3* were 1.1, 41.6 and 14 fold lower compared to *PmSSD2* plants (Figure 2). These data are consistent with GUS histochemical staining results. Deletion of a *PmSS* promoter region from -1758 to -1540 bp induced a sharp decrease in GUS activity by 37 fold(*PmSSD1*). However, further deletion from -1758 to -1078 bp in *PmSSD2* construct strongly induced GUS activity by 41 fold. This result shows that the promoter region from-1758 to-1078 was critical in maintaining maximal promoter activity and have vital regulatory elements. Besides that, it also illustrates that there might be enhancer at region -1758 to -1540 that activated *PmSS* promoter activity and unknown regulatory *cis* element that act as a repressor at -1540 to -1078 region. Prior studies confirmed that GUS expression of the of *CRTISO promoter, a Citrus unshiu* carotenoid isomerase only observed in shortest construct compared to the long promoter constructs when treated with ethylene, indicated that putative repressor element(s) might present in the promoter region between -2325 and -861 bp [25]. Deletion of 218 bp promoter fragment might activate specific repressor that inhibits GUS gene expression at *PmSSD1* plants. The loss of a region from-1758 to -855 in *PmSSD3* constructs cause the GUS activity only expressed at the stem of *PmSSD3* plants. These data indicate that, *PmSSD3* constructs is a tissue-specific promoter. Combination of promoter *cis*- regulatory elements (CREs) aid the binding of several transcription factors , resulted in the change of the gene expression pattern (34].

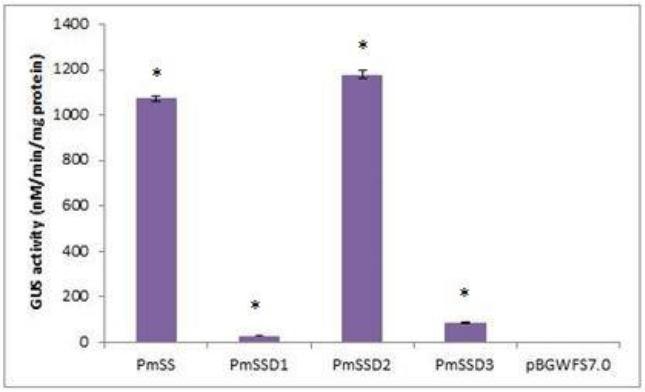

Figure 2 Activity of GUS in 14 DAG *PmSS* (-1758/66), *PmSSD1* (-1540/+66), *PmSSD2* (-1078/+66), and *PmSSD3* (-855/+66) transgenic Arabidopsis seedlings analyzed fluorometrically. Significant differences were determined by Fisher's least significant difference test ($P < 0.01$)

3.3 Functional Analysis of *cis*-regulatory Elements in the *PmSS* Promoter

Several *cis* regulatory elements were selected for promoter deletion study to examine the regulation and vital regulatory region pf *PmSS* promoter including WUN, TC, HSE, and ABRE motifs. To evaluate whether these selected *cis*-elements were functional, 2 - weeks old seedlings of transgenic Arabidopsis with all constructs were treated with hormone and environmental stress. Histochemical GUS staining and fluorometric test of GUS expression were conducted to these treated transgenic *A.thaliana.* GUS histochemical staining result has shown that there were no differences of GUS expression pattern between treated and non-treated transgenic *PmSS* promoter-GUS line. However, there was a difference in concentration of GUS expression in fluorometric analysis results between treated and non-treated transgenic *A.thaliana.*

Based on GUS fluorometric analysis result have shown GUS activity was higher in transgenic *A.thaliana PmSS* promoter-GUS line that received wounding, heat and ABA treatment compared to non-treated transgenic *A.thaliana. The PmSS, PmSSD1, PmSSD2* and *PmSSD3* plants treated with wounding treatment showed a 1.3-, 5.3-, 1.1 and 1.5-fold increase in GUS activity, respectively (Figure 3). In *A.annua*, similar to *PmSS* promoter, the four sesquiterpene synthase promoters which are β-caryophyllene (CPS), β-farnesene (FS), epi-cedrol (ECS) and amorpha-4,11-diene synthase (ADS) were induced by wounding treatment[17]. These findings conclude that, *PmSS* genes might be induced in response to herbivory attacks as a defense mechanism.

Heat stress treatment induced 1.5-, 2.8-,1.9- and 1.6-fold increases in GUS activity in the *PmSS*, *PmSSD1*, *PmSSD2* and *PmSSD3* respectively (Figure 3). The *PmSS*, *PmSSD1*, *PmSSD2* and *PmSSD3* plants treated with ABA increased GUS activity 2.9-, 2-, 1.8- and 1.2 fold respectively (Figure 3) Deletion of WUN, HSE and ABRE motifs has no significant impact on *PmSS* promoter activity. These results suggest that activity of *PmSS* promoter does not depend on WUN, HSE and ABRE motifs under wounding, heat and ABA treatment. Furthermore, WBOXNTERF3 motif which is involved in wounding stress and MYCATRD22 motif which is ABA responsive element [35] exists in all promoter constructs.

Drought treatment induced GUS activity in all construct except *PmSSD3* construct promoter. The *PmSS*, *PmSSD1* and *PmSSD2* plants were responsive to the drought treatment, resulting in 1.5-, 4.5- and 2.6-fold increase of GUS activity, respectively. However, the GUS activity was decrease by 2.3 fold in *PmSSD3* plants. Deletion of ABRE motif at -922 bp in *PmSSD3* construct decreased promoter activity after received drought treatment. The previous study has found that, drought stress increased the production of ABA resulted in activation of MYC and MYB transcription factors, thereby induced the expression of the gene. [30]. The absence of ABRE motifs on *PmSSD3* promoters might not activate the MYC and MYB transcription factors which explain why the activity of *PmSSD3* promoter not induced by drought stress treatment. This deduced ABRE play a critical role in *PmSS* promoter regulation under drought stress.

MeJa treatment induced GUS activity in *PmSS* and *PmSSD2* constructs. GUS activity in *PmSS* and *PmSSD2* construct increased by 3 and 1.8 fold after treated with MeJa. The previous study has reported that the expression of *PmSS* gene was responsive towards the elicitation of jasmonic acid in all organs of the *P. minor*. Furthermore, application of Jasmonic acid (JA) towards in vitro culture of *P.minor* significantly increased the production of sesquiterpene [19] The transcript level of four sesquiterpene synthase promoter in *A.annua* increased after the Meja treatment [17]. However GUS activity was decreased 4 - and 2.7- fold in *PmSSD1* and *PmSSD3* constructs. The deletion of promoter region from -1758 bp to -1540 bp induced a sharp decrease in GUS activity which is 3.9 fold when treated with MeJa. These results suggest there might have unknown regulatory elements presents in the *PmSS* promoter region from -1758 to -1540 bp that responsive towards MeJa. However, there is no GUS activity was detected in treated control plants, indicated the absence of endogenous GUS activity.

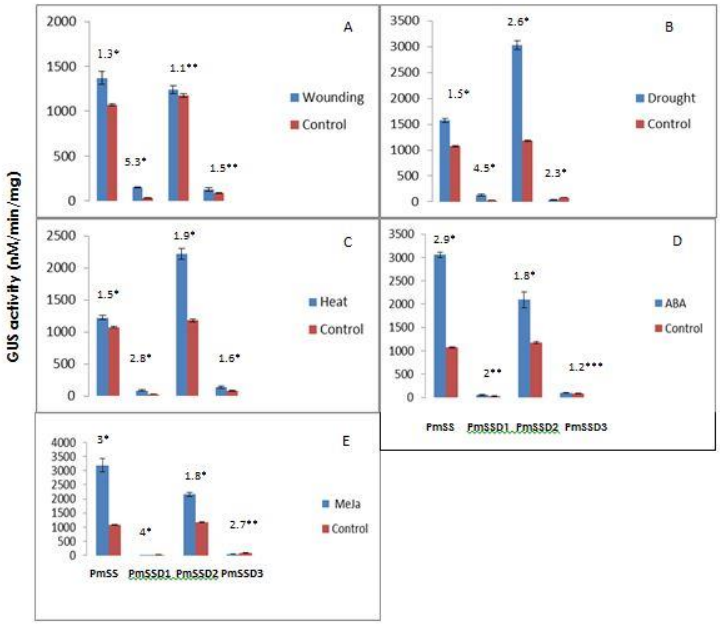

Figure 3 Activity of GUS in 14 DAG *PmSS* (-1758/+66), *PmSSD1* (-1540/+130), *PmSSD2 (-1078/66) and PmSSD3* (-855/+66) transgenic *Arabidopsis* seedlings treated with with wounding(A), drought(B), heat stress (C), 100 µM Abscisic acid (ABA)(D) *and*100 µM methyl jasmonate (MeJA) (*E*),The numbers over the bars represent GUS activity fold increase in response to the stress treatment versus the control. Significant differences between the treatment and control were determine by the two-tailed unpaired t-test (*P < 0.01, **P < 0.05, P > 0.05)

4.0 CONCLUSION

PmSS promoter possessed several *cis*-regulatory elements that are responsive to hormone and environmental stress. The functional analysis of the *PmSS* gene has proven that *PmSS* promoter directs developmentally specific gene expression. *PmSS* promoter was induced by wounding, drought, heat, ABA and MeJa treatment. Deletion of WUN, HSE and ABRE did not affect GUS activity in *PmSSD1, PmSSD2* and *PmSSD3* plants when treated with wounding, heat stress and ABA treatment. These indicate, WUN, HSE and ABRE motif might not have functional activity in *PmSS* promoter. Wounding, heat stress and ABA treatment did not affect GUS activity in three truncated *PmSS*-GUS constructs, indicate WUN. HSE and ABRE motif might not have functional activity in *PmSS* promoter. The highest GUS activity was found in *PmSSD2* plants (-1078/+66), therefore the region from -1078 to -855 bp presents in *PmSS* promoter was important for maximal activity of the promoter. In contrast, GUS activity in *PmSSD1* plants (-1540/+66) was the lowest, demonstrated there are unknown *cis* element that act as repressor between region -1540 to-1078 bp. The deletion of -1540 to -1078 bp region in *PmSS* promoter highly increased the promoter

activity. *PmSSD3* plants were expressed in tissue specific manner, GUS activity only observed at the plant's stem. *PmSSD3* construct might have an unknown *cis* elements that control specific tissue gene expression in *PmSS* promoter located at -855 to +1 bp. Further analysis need to be performed to identify unknown *cis*-regulatory elements that control the regulation of *PmSS* promoter in the truncated promoter. In conclusion, this research has provided a new insight to understand the critical region in *PmSS* promoter and in plant metabolism.

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