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The oil palm stearoyl-acyl-carrier-protein desaturase (*Des*) promoter drives transient gene expression in tomato fruits and is affected by gibberellic acid

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Abstract

Stearoyl-acyl-carrier-protein (ACP) desaturase gene plays an important role in oil palm mesocarp where main substrate stearic acid is converted to oleic acid. In this study, *β-glucuronidase* (GUS) activity regulated by the oil palm stearoyl-ACP desaturase promoter (*Des*) and the effects of gibberellic acid (GA_3) treatment on promoter activation were evaluated in transgenic tomato. A high level of GUS activity in transformed tomato (*Solanum lycopersicum*) revealed that *Des* promoter is active in heterologous system. With histochemical assays, we detected GUS activity in all of the agroinfiltrated tomato tissues. Fluorometric GUS assays indicated that the GUS activity was markedly higher when regulated by the *Des* promoter constructs in comparison with the CaMV 35S promoter. The analysis of 5'-deletion of the *Des* promoter indicated that the GUS activity driven by a 590-bp region were approximately 1.2-fold stronger than that of the full-length *Des* promoter. Semi-quantitative reverse transcriptase PCR results also showed that the 590-bp region of *Des* promoter directed the highest level of *uidA* expression. The GUS activity of *Des1*-transformed tomato mesocarp was induced by 1.7-fold, whereas the expression driven by the shortest promoter fragment of *Des4* was reduced by 1.9-fold following incubation with 10 µM GA_3. These results suggest that gibberellic acid-responsive elements reside within the region between nt -590 and nt -306 of the *Des* promoter, and based on our 5'-deletion analysis, the *cis*-element(s) necessary for strong promoter activity is also located within the region from -590 to -306 from the transcriptional start site. The oil palm *Des* promoter was activated by GA_3 and is potentially useful for engineering fruit-specific gene expression to enhance quality of fruits.

Keywords: Agroinfiltration, Oil palm, Stearoyl-ACP desaturase promoter, Cherry tomato, Gibberellic acid.
Abbreviations: ACP_Acyl-carrier-protein; CaMV_Cauliflower Mosaic Virus; *Des*_Stearoyl-acyl-carrier-protein desaturase; GUS_ *β-glucuronidase*; RT_Reverse transcription; Tub_α-tubulin; DMRT_Duncan's Multiple Range Test.

Introduction

Oil palm (*Elaeis guineensis*) is an important crop of the Aracaceae family and has been extensively cultivated in Southeast Asia for the production of palm oil. Malaysia and Indonesia are the leading palm oil-producing countries in the world, and each country contributes 45.18% and 45.26%, respectively, to the global palm oil production (MPOB, 2009). Over the past few decades, the rising economic importance of oil palm has led to increased efforts through the use of biotechnology for the genetic improvement of this crop. The genetic modifications in oil palm have been aimed at producing new cultivars that carry elite traits, such as an increased oil yield, enhanced carotenoid synthesis, and increased vitamin E (Wahid et al., 2005) and oleic acid (Parveez et al., 2004) contents. To date, these modifications have been conducted through conventional breeding methods (Hansen and Wright 1999; Piffanelli et al., 2002), recombinant gene technology (Chowhudry et al., 1996; Purba et al., 2000) and marker-assisted selection (Billotte et al., 2001). Oil palm is the only plant that produces two types of oil, namely palm oil, which is extracted from the mesocarp, and palm kernel oil, which is obtained from the kernel of the oil palm fruits (Vaughan and Geissler, 1997). Palm oil is enriched in nutrients and minerals, such as tocotreinol, carotenoids, fatty acids and micronutrients, which play essential roles in both growth and developmental processes. The major fatty acids in palm oil are oleic acid (40%; mono-unsaturated fatty acid), linoleic acid (10%; polyunsaturated fatty acid), and the saturated fatty acids, palmitic acid (45%) and stearic acid (5%). Clinical studies and health organizations indicated that palmitic acid raises the blood cholesterol level and increases the risk of cardiovascular disease (Clarke et al., 1997; World health Organization, 2003). High palmitic acid content in palm oil has rendered it as oil that is unhealthy for consumption. This issue has drawn considerable attention among consumers to the nutritional impact of various fats and oils. The growing consumer recognition that high levels of cholesterol and saturated fatty acids in the daily diet tends to increase the risk of cardiovascular disease has prompted scientists to improve palm oil quality by increasing the content of such unsaturated fatty acids as monounsaturated oleic acid (C18:1) (Cheah et al., 1995; Kadir and Parveez, 2000) at the expense of the saturated fatty acid content. The oil palm mesocarp contains an active stearoyl-acyl-carrier-protein (ACP) desaturase gene

that is responsible for the desaturation of stearic acid (C18:0) to oleic acid (C18:1) (Cheah et al., 1995). Increasing stearoyl-ACP desaturase (*Des*) activity and the stearoyl-ACP pool may enhance the conversion of stearoyl-ACP to oleoyl-ACP, subsequently increase oleic acid content (Cheah et al., 1995). *Des* which acts mainly on stearic acid, can also use palmitic acid as a substrate to produce palmitoleic acid (C16:1) (Kadir and Parveez, 2000). Therefore, induction of *Des* promoter and subsequently *Des* activity may increase oleic acid and palmitoleic acid content to produce more desirable palm oil. A thorough understanding of the *Des* promoter strength and identification of regulatory regions are necessary when developing a strategy for the genetic improvement of the palm oil quality. Promoters can be studied either in transgenic plants or through transient expression systems. The latter method, however, is often favored, as it does not involve the regeneration of the transformed cells into a transgenic plant. Transient expression is a relatively simple, rapid and efficient method to analyze the functionality of a gene of interest. Several methods, including protoplast fusion (Bilang et al., 1994), biolistic (Christou et al., 1994) and the use of *Agrobacterium* transformation, have been widely adopted for plant transformation in transient expression studies. Among these, *Agrobacterium*-mediated transformation is most frequently employed for the introduction and subsequent integration of a transgene into the genome of targeted plants (Hansen and Wright, 1999). To overcome the limitation of traditional oil palm breeding which includes a long regeneration time, between 2 to 5 years, the study of the *Des* promoter through transient expression in a heterologous system is essential. In recent years, agroinfiltration has become the method of choice for transient assay systems to study gene function, promoter elements analysis and inducible genes (Lee and Yang, 2006). Moreover, this technique has been applied to several different types of fruits, in which it was used to assay reporter activity (Spolaore et al., 2001). In this study, we assessed the expression of the *Escherichia coli uidA* reporter gene driven the by full-length *Des* promoter and four deletion derivatives through the transient expression of *β-glucuronidase* (GUS) in cherry tomato. Tomato (*Lycopersicum esculentum*) is one of the most important herbaceous crops and serves as a genetic model for the improvement of other dicotyledonous plants (McCormick et al., 1986). The oil palm *Des* promoter can be studied by investigating putative *cis*-elements present within the promoter or by analyzing the functions of these elements through mutagenesis. However, the effect of phytohormones treatment on the activation of the *Des* promoter has not been reported to date. The exogenous application of phytohormones which serve as elicitors is an efficient approach and had been widely adopted to understand role of hormones in the regulation of differential expression of genes in plants. The effects of elicitors vary according to the plant species, physiochemical environment (Namdeo, 2007), concentration, stage of their growth cycle and contact time of elicitation (Bhagwath and Hjorts, 2000). An in silico analysis of the *Des* promoter indicated the presence of a GAmyb transcription factor binding site in the region -313/-306 (Rima et al., 2012). As GAmyb is responsive to gibberellins, we speculated that the exogenous application of gibberellic acid may exert an effect on the *Des* promoter activity. In the present study, we evaluate *Des* promoter activity by monitoring *uidA* expression in cherry tomato fruits transiently regulated by the *Des* promoter or progressively smaller promoter reporter constructs. The activity of the *Des* promoter constructs in the presence of gibberellic acid was evaluated in heterologous system cherry tomato (*Solanum lycopersicum* var. cerasiforme). The transformation of 5'-deletion series of this promoter into cherry tomato through agroinfiltration and the subsequent treatment with gibberellic acid will validate the role of this phytohormone in the activation of the *Des* promoter.

Results and discussions

Histochemical analysis of uidA expression driven by stearoyl-ACP desaturase promoter deletions

Oil palm *Des* promoter was isolated and further investigations of the *Des* gene expression pattern in transgenic tomato plants showed that it was functionally active in fruits but not in other organs (Rima et al., 2012). The histochemical GUS staining experiments were performed to analyze the *uidA* gene expression pattern driven by *Des* promoter deletions and CaMV 35S promoter qualitatively in the transformed tomato. In general, both the CaMV35S and *Des* promoter constructs were able to drive *uidA* gene expression in the tomato mesocarp with the latter showing a considerably higher GUS activity, as deduced from the intensity of the blue color (Fig 1.). As expected, no GUS activity was detected in the non-transformed tomato (Fig 1f.). The histochemical staining was the greatest in the transformed tomato slices carrying the *Des3* construct, whereas a low level of GUS activity was observed with the *Des4* construct and the CaMV 35S promoter. We suggest that the various *uidA* expression levels are attributed to the removal of important *cis*-elements which are essential for the expression of *uidA* gene in the *Des* promoter deletion constructs. CAAT box element is common in eukaryote promoter and it plays essential role to initiate transcription and control promoter activity especially in promoter lacking of TATA box (Mantovani, 1999). They are nine CAAT box in oil palm *Des* promoter. The presence of one CAAT box in *Des4* as relative to eight CAAT box in *Des1*, *Des2* and *Des3* might contribute to the low amount of GUS staining in *Des4*-transformed tomato. The reported versatility of agroinfiltration in tomato (*Solanum lycopersicum* cv. Micro Tom) (Orzaez et al., 2006) prompted us to use cherry tomato (*Solanum lycopersicum* var. cerasiforme) as a heterologous system to study the oil palm *Des* promoter via a transient expression system. The staining of the transformed cherry tomato slices revealed that the GUS activity was usually observed in the pericarp and septum. In the agroinfiltration system, the pattern of *uidA* expression pattern is due to both the way in which the suspension is injected and the anatomy of the fruit tissues, which can cause a preferential penetration of the *Agrobacterium* culture due to the spongy structure of the tissue (Spolaore et al., 2001). However, a previous study demonstrated that the susceptibility of the tissue to *Agrobacterium* infection plays an important role in the efficiency of the agroinfiltration approach (Orzaez et al., 2006). It is proposed that the spatial *uidA* expression pattern, as observed in Fig 1. might be partially governed by constraints imposed by the fruit architecture and the ability of the bacterial culture to penetrate the different tissues in the fruit. However, it should not be particularly relevant in this study, which was aimed at understanding whether a fruit-specific *Des* promoter from oil palm is also active in the heterologous dicot system of tomato. Promoters that are capable of inducing high constitutive expression are valuable in plant genetic engineering. The CaMV 35S is among the most widely used promoter to drive constitutive expression in dicot plants. In a study conducted by McCabe et al. (1999) to

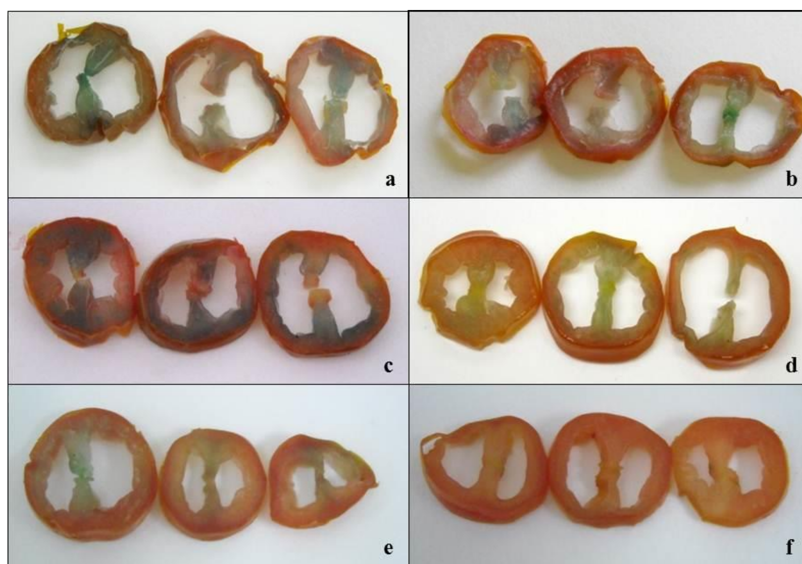

Fig 1. Representative images from analyses of GUS activity in transformed cherry tomatoes, as driven by the following: (a) : *Des1*, (b) : *Des2*, (c) : *Des3*, (d) : *Des4*, (e) : CaMV 35S and (f) Negative control (non-transformed).

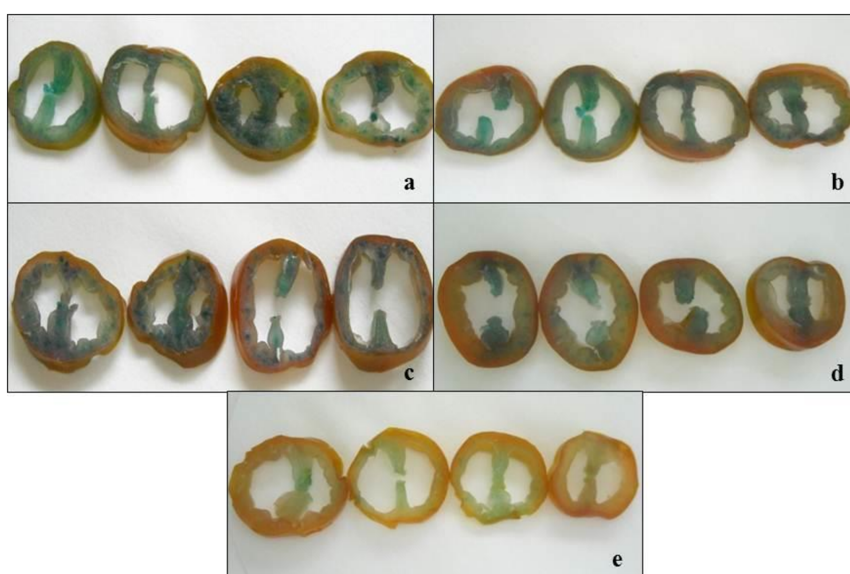

Fig 2. Representative images of GUS staining in mesocarp slices of transformed cherry tomatoes after 10 µM gibberellic acid (GA_3) treatment for one day. (a) : *Des1*, (b) : *Des2*, (c) : *Des3*, (d) : *Des4* and (e) : CaMV 35S promoter.

investigate inheritance of herbicide resistance in transgenic lettuce carrying a *pet*E promoter-*bar* gene compared with a CaMV 35S-*bar* gene, the latter showed a considerably higher frequency of bar gene transmitted herbicide resistance in transgenic lettuce. The high amount of methylation sites in CaMV 35S promoter as relative to *pet*E promoter may render transgene silencing and susceptibility of the promoter to inactivation. Thus, novel plant promoters that are as strong as the CaMV 35S are valuable for the spatial expression of genes of interest in plants.

Induction effects of GA_3 on the activity of the stearoyl-ACP desaturase promoter deletion

In this study, both qualitative and quantitative analyses were performed to assess the effects of gibberellic acid (GA_3) treatment on the *Des* promoter activation. A qualitative histochemical GUS assay was performed to examine the differential expression of *uidA* gene due to GA_3 treatment by comparing untreated tomatoes (Fig 1.) with those treated with GA_3 for 1 day (Fig 2.). The histochemical staining results shown in Fig 2 are representative of the staining pattern analyzed in the tomato mesocarp transformed with the Des-*uidA* and 35S-*uidA* constructs after GA_3 treatment for 1 day. From our observations, it appeared that the tomato tissue transformed with the *Des* promoter constructs generally showed a higher expression of GUS after GA_3 treatment, compared to the non-treated samples. A higher GUS activity was observed in the tomatoes transformed with *Des1*, *Des2* and *Des3* compared to *Des4*, which lacks the gibberellin-responsive element. This suggests that the -1022 to -306 region contains potential *cis*-regulatory element that is responsive to GA_3. Consistent with the histochemical assays, sequence analysis of the *Des* promoter detected the presence of a gibberellins responsive element, at position -313/-306 (Rima et al., 2012). Nevertheless, it is difficult to quantify the *uidA* gene expression of transformed samples using a histochemical GUS assay.

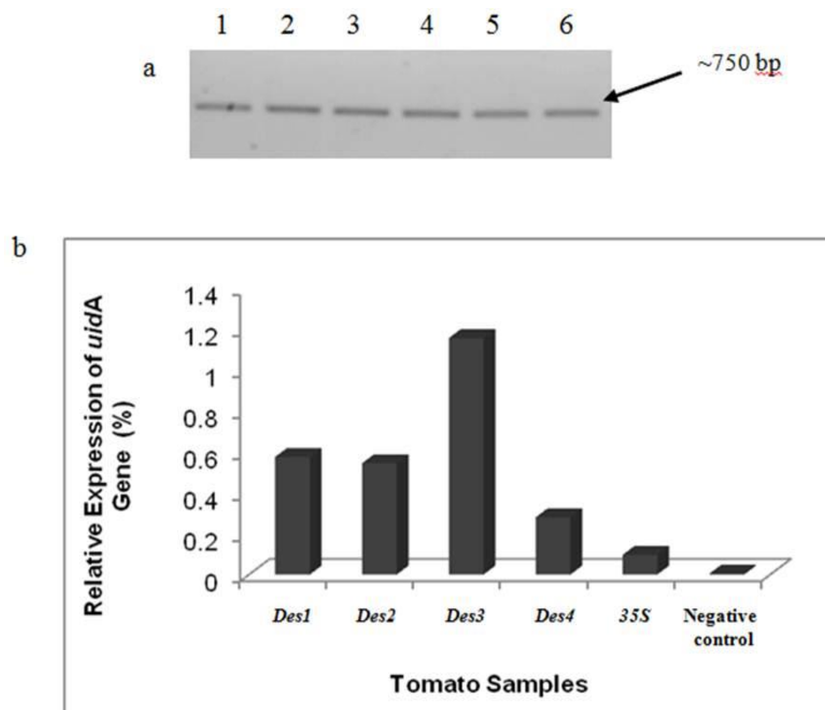

Fig 3. Semi-quantitative RT-PCR analysis of the uidA expression pattern driven by the Des promoter deletions and CaMV 35S promoter. (a) Equal loading of cDNA during the RT-PCR analysis was verified with the cDNA of the gene encoding α-tubulin. Lanes 1-4: Des1, Des2, Des3, Des4; lane 5: CaMV 35S; lane 6: Negative control (non-transformed tomato). (b) Graphical representation of the expression of the uidA cDNA in mesocarp of the transformed and non-transformed cherry tomatoes.

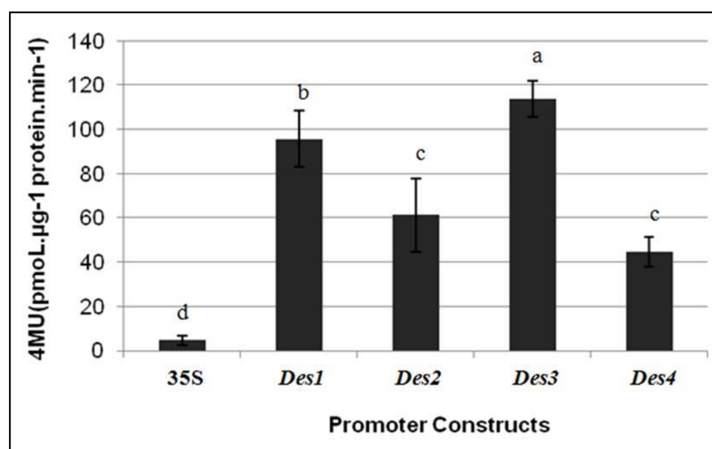

Fig 4. The GUS activity in the mesocarp of tomato transformed with Des promoter deletions and the CaMV 35S promoter. 4MU readings represented by different letters ($^{a\text{-}d}$) are significantly different ($P \leq 0.05$) according to DMRT. Data are means ± standard deviation of two independent experiments.

Semi-quantitative reverse transcription PCR analysis

Semi-quantitative RT-PCR analysis was performed on the transformed tomatoes to compare the *uidA* expression driven by the *Des* promoter deletions. A constitutively expressed housekeeping gene in plants, as that coding for α-tubulin, was chosen as the internal control to normalize the *uidA* mRNA levels. The amplification of α-tubulin (Tub) cDNA from the transformed tomato was performed with α-tubulin-specific primers, producing an amplicon of approximately 750 bp, and the expression level did not differ among the samples (Fig 3a.). Total RNA (1.6 µg) isolated from tomato transformed with the CaMV35S, *Des* constructs and the non-transformed tomato was used for the reverse transcription reaction. Transcripts of the *uidA* gene, with a size of approximately 250 bp, were detected in the RT-PCR assays on all of the transformed tomatoes, indicating that *uidA* gene was being activated at variable degrees when driven by the promoter deletions. The highest activation was detected in tomato transformed with the *Des3* construct, whereas GUS activity of *Des1* and *Des2* were almost similar. The lowest activation was observed with the *Des4* construct, but it was relatively higher than that of the CaMV 35S promoter. In contrast, no amplicon was amplified in the non-transformed tomato, as we expected. RT-PCR analysis is better presented in the form of relative expression fold to show the different levels of expression regulated by *Des* constructs. Thus, a bar chart was generated which is representative of the relative *uidA* cDNA expression levels in *Des*-transformed tomatoes by using ImageJ 1.42q analysis program (National Institute of

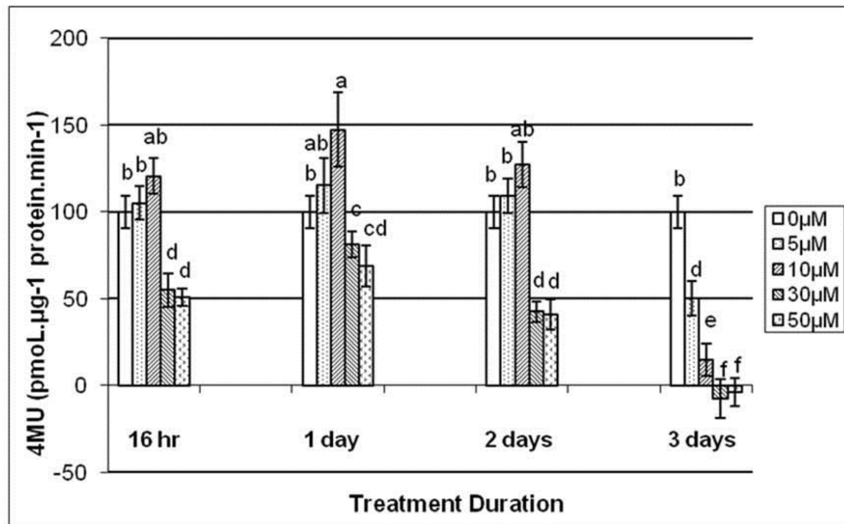

Fig 5. Fluorometric analyses of GUS activity regulated by the full length *Des* promoter (*Des1*) in transformed tomato after gibberellic acid (GA_3) treatment. 4MU readings represented by different letters ($^{a-d}$) are significantly different ($P \leq 0.05$) according to DMRT. Data are means ± standard deviation of two independent experiments.

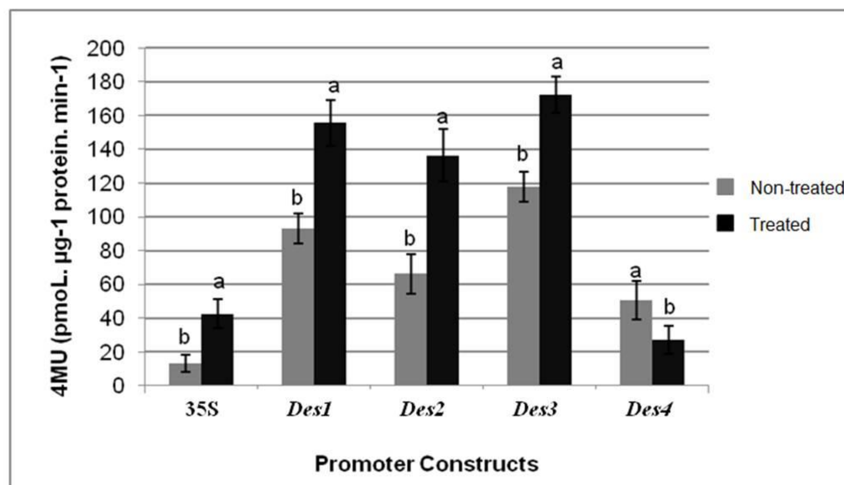

Fig 6. GUS activity induced by 10 µM GA_3 in the mesocarp slices of tomato transformed with the *Des* promoter deletions and the CaMV 35S promoter. 4MU readings represented by different letters ($^{a-d}$) are significantly different ($P \leq 0.05$) according to DMRT. Data are means ± standard deviation of two independent experiments.

Health, Maryland, US) (Fig 3b.). Highest activation was detected in *Des3* construct.

Fluorometric analysis

A fluorometric analysis was performed to quantify the GUS activity in the transformed tomatoes (Fig 4.) and was conducted in two repeated experiments. Despite *uidA* gene was detected in all of the transformants, a varying degree of GUS activity was observed. Based on the fluorometric assays shown in Fig 4., the GUS activity regulated by the *Des* promoter constructs was higher than that regulated by the CaMV 35S promoter. The *Des3* construct (600 bp) conferred a higher level of GUS activity in comparison to the other three deletion derivatives. The GUS activity in the tomato carrying the *Des1* (1022 bp) construct showed a slight decrease compared to *Des3*, whereas the GUS activity of the *Des2* construct was drastically reduced by 1.9-fold, compared to *Des3*. The lower amount of GUS activity with the *Des1* and *Des2* constructs compared to *Des3* might be due to the presence of negative regulatory elements located between -1022 and -591 of the *Des* promoter. The drastic reduction of GUS activity with *Des4*, suggests the presence of *cis*-elements in *Des3* that are crucial to drive *uidA* gene expression. Thus, the removal of the *cis*-element led to a reduction of the GUS activity regulated by the *Des4*. This result was identical to that reported by Xie et al. (2003) using the *cotton curl Multan virus* (CLCuMV) promoter in transgenic plants. It was reported that a 5'-deletion of the full length CLCuMV promoter (-407) to nucleotides -257 and -241 from the transcription initiation site resulted in a 5-fold and 2-fold higher promoter activity respectively. This result indicated that the deletions of the CLCuMV promoter conferred a higher activity, suggesting the presence of negative regulatory elements in those deleted fragments (Brand et al., 1985). Activity of the truncated CLCuMV promoter (-146) was identical to the full length promoter. Nevertheless, further deletion of the CLCuMV promoter to nucleotides -112 abolished the promoter activity in transgenic plants. It might be attributed to the presence of enhancer elements located between -146 to -112. In general, the expression patterns observed in our fluorometric analysis was consistent with the results of the RT-PCR analysis (Fig 3b.). Surprisingly, the GUS activity regulated by *Des1* was 20-fold

higher than CaMV 35S in the fluorometric analysis, whereas it appeared to be only a few fold higher than CaMV35S in the histochemical GUS assay (Fig 1.). This variable result by different analyses could be attributed to the possibility that the different samples used for analysis may exhibit variable GUS expression levels. The structure of the fruits and the infiltration methods might affect the transformation efficiency or gene expression, thus contributing to various degrees of transformation in tomato (Almeida and Huber, 1999; Vancanneyt et al., 1990).

Stearoyl-ACP desaturase promoter activity in response to gibberellic acid (GA_3) treatment

Several important motifs were detected in the *Des* promoter, including, gibberellins-responsive elements (GAmyb), EMHVCHORD (Endosperm box), Dof binding factors sites, CIRCADIANLELHC and MYB elements (Rima et al., 2012). The presence of a binding site of the GAmyb transcription factor, a DNA sequence element putatively responsive to gibberellins, located at -313/-306 of the promoter had prompted us to investigate the effects of GA_3 in regulating the *Des* promoter activity. Study conducted by Gubler and Jacobsen (1992) showed gibberellins up-regulated expression of barley transcription factor, GAMyb gene, resulting activation of α-amylase promoter activity in barley aleurone. In this study, we treated *Des*-transformed tomatoes with exogenous GA_3. *Des1*-transformed tomato was submerged in MS medium plus GA_3 elicitor at various concentrations (5 µM, 10 µM, 30 µM or 50 µM) and was incubated for different duration (16 hour, 1 day, 2 days or 3 days). After the incubation period, the tomatoes were randomly sampled from each treatment, and a fluorometric analysis was used to identify the treatment that had the most significant effect on promoter activation. Gibberellin is a naturally occurring diterpene hormone that has both stimulatory and inhibitory effects on morphological or physiological mechanisms in plants. It has been shown that, gibberellic acid is able to mitigate inhibitory effects resulting from environmental stress during seed germination and seedling growth (Alonso-Ramírez et al., 2009). In addition, gibberellins are also involved in regulating gene expression in the presence of other hormones (Loreti et al., 2008) and induce plant promoter activity (Blázquez et al., 1998). Activity of LFY promoter was markedly increased upon exogenous application of GA_3 which in turn accelerates flowering in short-day plants (Wilson et al., 1992; Blázquez et al., 1997). Both endogenous gibberellic acid (GA_3), ubiquitously present in plants, and the exogenous application of GA_3 stimulate growth and developmental processes in plants. Gibberellins are known to promote plant growth, seed germination (Loreti et al., 2008), flowering and fruit development (Sun and Gubler, 2004), and enhance gene expression through modulation of promoter activities (Wang et al., 2005). The promoter activity of *Arabidopsis* ACC synthase gene, *AtACS7*, increased approximately 80% upon GA_3 treatment on 2-week-old *Arabidopsis* seedlings (Wang et al., 2005). Based on the study conducted by Miyoshi and Sato (1997), the optimum GA_3 concentration to induce rice seedlings germination is 10 mM. However, it was reported that the exogenous application of 10 µM GA_3 successfully increased the GUS activity of transgenic tobacco by 31.7% compared to non-treated plants (Wu et al., 2007). Thus, it was hypothesized that the optimum GA_3 concentration might differ depending on the plant species, culture environment and different desired end result of the GA_3 application. Referring to the GUS activity of *Des1* after GA_3 incubation, the effects were both inhibitory and stimulatory (Fig 5.). The GUS activity of *Des1* decreased abruptly after the GA_3 treatment at 30 µM and 50 µM for all four of the incubation periods tested relative to the control samples. This might be due to a high concentration of GA_3 induced stress in the tomato cells that suppressed the GUS activity. While it was reported that GUS activity regulated by *Gossypium hirsutum* cellulose synthase catalytic subunit 4 (*GhCeA4*) was significantly increased after a 1-day incubation with 5 µM GA_3 in transgenic *Arabidopsis* (Kim et al., 2011), exogenous application of 20 µM GA_3 on transgenic *Arabidopsis* seedlings for 1 day induced the promoter activity of *AtACS7* (Wang et al., 2005). In our study, the quantitative fluorometric assay of the *Des1*-transformed tomato under the various concentrations of gibberellic acid (GA_3) treatment revealed that the mean GUS activity was strongly up-regulated after the exposure to 10 µM GA_3 for 1 day. This observation indicates that GA_3 is able to enhance the *Des* promoter activity. As illustrated in Fig 5, the promoter activity did not show significant difference to 5 µM and 10 µM GA_3 treatment after a 16 hour incubation period. *Des1* promoter might be less responsive to the short incubation period and low concentration of GA_3. However, activity of *Des1* promoter after 2-day incubation showed slight decrement as compared to 1-day incubation. Promoter activity was the lowest after 3 days treatment at all concentration level of GA_3. The long period of abiotic stress treatment may suppress activity of *Des* promoter contributes to the low GUS activity. Based on study conducted by Wu et al. (2007) on the cotton glucuronosyltransferase promoter in transgenic tobaccos, the promoter was induced by 10 µM GA_3, 24 hour incubation duration, which was consistent to the findings in this experiment. Tomatoes transformed with *Des* promoter constructs were then individually immersed in MS medium plus 10 µM GA_3 and incubated in the dark for 1 day. A fluorometric analysis was performed immediately after the incubation period to determine which *Des* promoter sequences are responsible for the induction of the GUS activity by exogenous GA_3. All of the promoter constructs were tested with regard to the inducibility of the GUS expression in the transformed tomato mesocarp after GA_3 treatment. The ANOVA results showed that the GUS activity under the control of the CaMV 35S, *Des1*, *Des2* and *Des3* sequences increased significantly after gibberellic acid treatment. In contrast, the GUS activity under the control of *Des4* showed a reduction after the 10 µM GA_3 treatment (Fig 6.). Upon GA_3 induction, the GUS activity of *Des4* decreased substantially to 27.15 ± 8.22 pmoL.µg-1.min-1 compared with 172.3 ± 10.95 pmoL.µg-1.min-1 for *Des3*. These results suggest that a gibberellic acid-responsive element resides within the region between -590 and -306 (*Des3*) of the *Des* promoter. The removal of this element abolished the induction by GA_3 of *Des4*. Surprisingly, 35S-transformed tomato showed an increase of GUS activity after GA_3 treatment. To date, no study had reported the presence of gibberellins-responsive element in CaMV 35S promoter. Further investigations need to be carried out to understand the underlying mechanism which led to this observation. The fluorometric analysis results further validate the prediction of PlantCARE analysis, which detected the presence of a binding site of the GAmyb transcription factor at position -313/-306 that is responsive to gibberellins. As discussed earlier, the presence of a GAmyb binding site in the *Des* promoter might contribute to its responsiveness to GA_3 induction. The rapid decrease in the GUS activity with the *Des4* construct was due to the loss of the GAmyb element. In conclusion, the present study clearly shows that investigation

of the oil palm *Des* promoter can be performed using a heterologous dicot system of cherry tomato through a transient expression system. One upstream region (-590/-306) of the *Des* promoter was involved in activating promoter activity in cherry tomato fruits. The shorter promoter segment (*Des3*) was the strongest to direct *uidA* gene expression. Furthermore, we demonstrated that effects of gibberellic acid treatment on the *Des* promoter in transformed tomato were various among different concentrations and treatment duration. The *Des* promoter showed a significant induction in response to exogenous 10 μM GA_3 after 1 day incubation. The findings reported in this study may be helpful in facilitating future experiments on the modification of fatty acid biosynthesis in oil palm fruits through genetic engineering.

Materials and Methods

Bacterial strain and plasmids

The promoter region of the stearoyl-ACP desaturase (*Des*) gene isolated from oil palm (GenBank Accession No. JQ348912) was obtained from Universiti Putra Malaysia, Selangor, Malaysia. Four constructs comprising of the regions of the *Des* promoter were generated and designated as *Des1* to *Des4* (Rima et al., 2012). The binary vector, pCAMBIA 1301 (CSIRO, Melbourne, Australia) contains a reporter gene, *uidA*, which encodes *β-glucuronidase* and a selectable marker gene, *hpt*, which encodes a hygromycin-resistance gene. This plasmid also harbors a kanamycin-resistance gene. These three genes are driven by the CaMV 35S promoter. The modified *uidA* reporter gene used for *Agrobacterium* transformation was interrupted by a plant intron (Vancanneyt et al., 1990) that prevents the expression of the *uidA* gene in the prokaryote, *Agrobacterium*.

Preparation of Agrobacterium for fruit injection

The *A. tumefaciens* culture strain LBA 4404 was grown for 24 hours in Luria Bertani-Glucose (5 mM) broth supplemented with kanamycin (50 mg L^{-1}) and streptomycin (100 mg L^{-1}). *Agrobacterium* harboring the *Des* promoter constructs (*Des1-Des4*) were individually prepared by incubation at 28°C in an orbital shaking incubator at 200 rpm until the cultures reached an OD_{600} of 0.4 to 0.6.

Infiltration of fruits

Commercially ripe cherry tomatoes (*Solanum lycopersicum* var. cerasiforme) were purchased at a market (Selangor, Malaysia). The fruits were rinsed thoroughly in water plus Clorox (20%) and then rinsed twice with distilled water under a laminar-flow hood. The fruits were surface-sterilized with 100% ethanol prior to injection of the *Agrobacterium* suspension. *Agrobacterium* suspension was then evenly injected throughout the entire fruits tissue using a sterile syringe. The needle was introduced 3 to 4 mm depth into the fruit tissue, and the infiltration solution was introduced gently into the fruit by avoiding the locules where most of the bacteria may concentrate, thus, reducing the efficiency of the injection. The infiltrated fruits were surface-dried on filter paper to remove the excess bacteria, and the fruits were then incubated in sterile conical flasks at 28°C for 2 days in the dark. After incubation period and prior to assaying the reporter gene activity, the infiltrated fruits were cut into thin slices and washed with distilled water plus 300 mg L^{-1} of cefotaxime to kill the remaining bacteria. The slices were then rinsed with distilled water three times and surface-dried on sterile tissue under a laminar-flow hood. One half of the samples was immediately used for the histochemical assay, whereas the other half was frozen in liquid nitrogen and stored at -80°C for subsequent use in a quantitative assay. Both the locules and the seeds were discarded when sampling the tissues for the transient *uidA* expression.

Histochemical assay of GUS activity

Histochemical GUS assays (Jefferson, 1987) were carried out in triplicates (six cherry tomatoes per replication) for both treated and non-treated samples. Injected tomatoes were sampled and immersed into X-Gluc solution consisting of 1 mmol L^{-1} of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) (Duchefa Biochemie B.V., The Netherlands), 100 mmol L^{-1} phosphate buffer pH 7.0, 0.1% Triton X-100 (Sigma Chemical, USA), 0.5 mmol L^{-1} $K_3Fe(CN)_6$ (Sigma Chemical, USA), 0.5 mmol L^{-1} $K_4Fe(CN)_6$ (Sigma Chemical, USA), 10 mmol L^{-1} EDTA (Fluka Chemical, Switzerland) and 20% methanol (Merck, Darmstadt, Germany). The immersed tissues were incubated for 3 hours in the dark at 37°C. Non-transformed cherry tomatoes were used as negative control.

Expression patterns of 5'-deletions of the Des promoter in transgenic tomato

The mRNA expression patterns of the *uidA* reporter gene driven by the various constructs of the *Des* promoter (*Des1* to *Des4*) were examined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The amplification of α-tubulin (Tub) cDNA from the transformed tomatoes was used as an internal control. The cDNA synthesized from the tomatoes transformed with the *Des* promoter deletion derivatives was used as the template for the PCR amplification. The PCR reaction was composed of 15.3 µL distilled water, 1x PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.02 µM forward primer (5'-CAACGGGGAAACTCAGCAAG-3'), 0.02 µM reverse primer (5'-AGCGTCGCAGAACATTACAT-3'), 1.6 µg cDNA and 0.04 U *Taq* Polymerase (Promega, USA). For the amplification, the samples were pre-denatured at 95ºC for 5 min, followed by 35 cycles of 1 min at 94ºC, 30 s at 60ºC, 2 min at 72ºC and a final extension at 72ºC for 10 min. cDNA isolated from non-transformed tomato was used as negative control. The results of RT-PCR analysis were converted into bar chart by using ImageJ 1.42q analysis program (National Institute of Health, Maryland, US) for relative *uidA* expression levels as regulated by *Des* constructs.

Quantitative assay of GUS activity

For the fluorometric assay, FluoroAce (BioRad, Hercules, California, USA) was used. Quantitative assay of the GUS activity in transiently transformed tomato was carried out as described by Jefferson (1987). The crude protein extract of transformed tomato was recovered by centrifugation at 16,200xg for 5 min at 4°C. Fluorescence, 4-methylumbelliferone (4MU), was measured using a VersaFluor™ Fluorometer (BioRad, USA) according to the manufacturer's instructions. The fluorometric GUS activity was expressed in terms of pmoL 4MU.μg^{-1} protein.min^{-1} (Jefferson 1987). All data represent mean value of two independent experiments (three samples per replicate).

Exogenous phytohormonal treatment

Immediately after the co-cultivation, the transformed cherry tomatoes were cut into thin slices and were exposed to gibberellic acid (GA_3) treatment. GA_3 (Duchefa, The Netherlands) was first dissolved in 100% ethanol (EtOH) and then sterilized by filtration. The transformed tomato slices were then transferred to sterile flasks containing 30 mL of MS liquid medium supplemented with various concentration of GA_3 (5 µM, 10 µM, 30 µM or 50 µM) and incubated for 1, 2 or 3 days.

Statistical analysis

Statistical analysis for the quantification of GUS was carried out using the analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT). To analyze the data, SAS software version 9.0 was used. Significance was declared at $P \leq 0.05$.

Conclusion

This study showed that investigation of the oil palm *Des* promoter can be performed using a heterologous dicot system of cherry tomato through a transient expression system. The agroinfiltration transformation method described here is an inexpensive and efficient tool to study promoter activity. The knowledge of promoter strength and its inducibility by abiotic elicitors is particularly important. In summary, we demonstrated that one upstream region (-590/-306) of the *Des* promoter was involved in up-regulation of promoter activity in cherry tomato fruits. The shorter promoter region (*Des3*) was the strongest to direct *uidA* gene expression. We showed that effects of gibberellic acid elicitation on the *Des* promoter in transformed tomato were various among different concentrations and treatment duration. The *Des* promoter showed a significant induction in response to exogenous 10 µM GA_3 after 1 day incubation. The findings reported in this study will facilitate future experiments on the modification of fatty acid biosynthesis in oil palm fruits through genetic engineering.

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