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HIGH CONCENTRATION OF 2, 4-D TREATMENT MAY NOT RESULT IN MANTLED VARIATION DURING CALLUS INDUCTION OF OIL PALM

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ABSTRACT

Mantled is a kind of fruit abnormality in oil palm and can cause serious yield loss of palm oil. It was reported that mantled phenotype could be resulted from the application of plant growth regulators (e.g. 2, 4-D) during tissue culture. However, the effect of 2, 4-D treatment on the development of mantled variation is still very limited. In this study, based on our present oil palm tissue culture system, the inflorescence explants were treated with different concentrations of 2,4-D at callus induction stage to induce mantled. Along with a known mantled sample as control, all 2,4-D treated samples were detected with an available DNA marker (kDEF1) for mantled identification, the expression of EgDEF1 transcripts (kDEF1 and tDEF1) and the accumulation of mantled related metabolites (*deoxycytidine*) were then analyzed using transcriptomics and metabolomics analysis. Detection of mantled by kDEF1 marker showed that kDEF1 was detected only in control (mantled) sample whereas none was found in 2,4-D treated samples. Furthermore, transcriptome analysis confirmed that kDEF1 transcript was not presented at all in 2,4-D treated samples. In contrast with earlier reports in mantled samples, tDEF1 was down-regulated and the accumulation of deoxycytidine was increased during callus induction. Therefore, the mantled variation was not identified at DNA, RNA and metabolite levels in 2,4-D treated samples, suggesting that high concentration of 2,4-D may not result in mantled variation during callus induction. However, similar research at embryogenesis stage could be till needed to explore more possibilities.

Key words: *Mantled, 2,4-D, EgDEF1 transcripts, kDEF1, Deoxycytidine.*

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INTRODUCTION

Oil palm is considered as one of the most oil producing crops and that accounts for increasing interest to use in food industry and biofuels (Wang *et al*., 2019; Wang *et al*., 2018). Induction of somatic embryogenesis on hormone-supplemented media from high yielding plants can generate genetically identical plants as the parent plants and therefore enhance yield. However, the process often resulting in a somaclonal variant which affects flower development known as mantled. This causes detrimental effect on the yield or complete loss of the harvest of palm oil (Alwee *et al*., 2006; Shearman *et al*., 2013; Ong-Abdullah *et al*., 2015). Mantled somaclonal variation can be generated during tissue culture regeneration process, which may be consequences of the application of high concentration of plant hormones (eg. 2,4-D) (Eeuwens *et al*., 2002; Morcillo *et al*., 2006; Garcia *et al*., 2019). To the best of our knowledge, mantled phenotype cannot detect until it reaches sexual maturity because the phenotype becomes apparent only when flowering starts with the production of inflorescences where the staminodes of the female flower and the stamens of the male flower are transformed into carpel-like structures (Shearman *et al*., 2013; Ong-Abdullah *et al*., 2015).

Many studies have reported the association of DNA methylation and induction of mantled phenotype (Jaligot *et al*., 2000; Morcillo *et al*., 2006; Jaligot *et al*., 2014; Ong-Abdullah *et al*., 2015; Sarpan *et al*., 2020). The accumulation of nucleosides in leaves and callus tissues was investigated by Jaligot *et al*. (2000) to evaluate the methylation rates. Metabolites are the final products of various biological processes and metabolomics is a powerful tool that can reveal the changed functions exerted by the genes or proteins (Liu *et al*., 2016; Yang *et al*., 2016). Metabolomics approaches have identified potential markers involved in increased lipid production in oil palm (Teh *et al*., 2013).

Despite considerable research on the molecular mechanisms and DNA methylation underlying the mantled variation, very few reports examined the implication of 2, 4-D in the development of mantled phenotype. The present study thus aimed to determine

whether 2, 4-D is associated with mantling phenotype during callus induction by using DNA, RNA and metabolite analysis.

MATERIALS AND METHODS

Plant Materials and Tissue Culture Conditions: Female inflorescence explants from five trees were sampled from oil palm repository of Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, Wenchang, Hainan, China. Tissue culture media was prepared based on the Y3 medium formulation (Jain and Gupta, 2018) supplemented with 4 g/L Phytagel (Solarbio, P8170), 2.5 g/L activated charcoal (Solarbio, C7261) and 0, 60, 120, 180, 240, 300 mg/L of 2,4-dichlorophenoxyaceticacid (2,4-D). All chemicals were acquired from the Solarbio Science and Technology Co., China. Before autoclaving, the pH of the medium was adjusted to 5.8. Thereafter, the explants were cultured for 90 days under complete darkness at 28 ± 2 °C. All of these samples were collected at 90d and stored in freezer at -80˚C for further analysis. For RNA-seq and metabolites analysis, the explants and callus derived from the highest concentrations of 2,4-D (300 mg/L) treated samples from three trees were selected randomly for biological replicates.

DNA Extraction: The extraction of total genomic DNA from the samples was done by using the method reported by Ihase *et al*. (2016). NanoDrop 2000 spectrophotometer (Thermo Scientific) was used to quantify the extracted DNAs. The concentration (ng/µL) and purity of DNA was estimated by measuring the absorbance at 260 and 280 nm ($A_{260/280}$). Thereafter, the DNA was diluted to a working concentration of 50 ng/µL with sterile double-deionized water (ddH2O). A previous DNA from the fruit with mantled phenotypes were used as control for detection of mantled variation.

Primer Design and PCR Amplification: The kDEF1 primer (Forward: 5′-GCAGTGGCTTCAAAA TGGGG-3′ and Reverse: 5′-GTCATTCCTCAGTCGAGCCT-3′) were designed from kDEF1 sequence KR347486.1 (GenBank) using National Centre for Biotechnology Information (NCBI) Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/ primer-blast). Primer pairs were synthesized by BGI Tech (China).

The PCR amplification was performed in a TaKaRa Thermal Cycler Dice™ TP600 (TAKARA BIO INC, Japan). The PCR mixture contained 2 µL DNA (50 ng/µL), 0.2 µL deoxynucleoside triphosphate (dNTP) (10mM), 5 µL sterile ddH2O, 1 µL 10× RealTimes buffer containing MgCl2, 1µL RealTimes *Taq* DNA polymerase (5 U/µL), 0.4 µL of each forward and reverse primer (10 µM). The touchdown (Korbie and Mattick, 2008) PCR conditions was modified and used, which included an initial denaturation at 94 ◦C for 5 min, denaturation for 30 s, followed by decreasing the annealing temperature by 1 ◦C each cycle from 65 to 56 ◦C by running 10 cycles. Then, the annealing temperature was sustained at 55 ◦C for the next 25 cycles. The annealing time for each cycle was 45 s, elongation time was 1 min at 72 ◦C, and final elongation was at 72 ◦C for 10 min, after which the temperature was maintained at 4 ◦C for infinity.

Detection of Mantled Abnormality: To detect mantled abnormality, ten microliters of PCR products amplified by kDEF1 primer pairs were mixed with 2µL of 6X DNA loading buffer and 5µL of this mixture was loaded into well. Analysis was carried out on 1.5% agarose gel and gel image was captured using SYNGENE G: Box gel documentation system.

RNA-seq Analysis: Extraction of total RNA from the sample was done using Trizol Reagent (invitrogen, USA) according to the manufacturer's instruction. Extracted RNA were then treated with DNaseI (Takara, Japan) to degrade any possible DNA contamination. The concentration and purity of each RNA sample was estimated using ND-2000 spectrophotometer (NanoDrop, USA), and RNA integrity was examined by 1.5% agarose gel electrophoresis. RNA-seq analysis was performed using Illumina Hiseq 4000 by BGI (Shenzhen, China). For unigene expression analysis, the clean reads were mapped to unigenes using Bowtie2 software (version 2.2.5) (Langmead and Salzberg, 2012). For the calculation of gene expression level, RNAseq by expectation maximization (RSEM) (version 1.2.12) (Li and Dewey, 2011) was used. The fragments per kilobase of transcripts per million fragments mapped (FPKM) values were used for normalization of the expression level of each gene. Differentially expressed genes (DEGs) between two groups were identified using the PoissonDis method. Furthermore, false discovery rate (FDR) values were computed to adjust the resulting P values. The unigenes with FDR ≤ 0.001 and Fold Change ≥ 2.00 were used to determine significant DEGs.

Metabolome analysis: The freeze-dried samples were crushed by using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. Then, 100 mg powder was weighed and extracted overnight at 4°C with 1.0 mL 70% aqueous methanol containing 0.1 mg/L lidocaine for internal standard. After centrifugation at 10000 g for 10 min, the supernatants were absorbed and filtrated (SCAA-104, 0.22-µm pore size; ANPEL, Shanghai, China, www.anpel.com.cn/) before LC–MS/MS analysis.

The extracted compounds were then analyzed using an LC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM30A, MS/MS (Applied Biosystems 6500 QTRAP). The following analytical conditions were used: injection volume, 2 µL; column, Waters ACQUITY UPLC HSS T3 C18 column (2.1 mm*100 mm, 1.8 µm); solvent system, water (0.04 %

acetic acid) (Phase A) and acidified acetonitrile (0.04 % acetic acid) (Phase B); gradient program, 95:5 Phase A/Phase B at 0 min, 5:95 Phase A/Phase B at 11.0 min, 5:95 Phase A/Phase B at 12.0 min, 95:5 Phase A/Phase B at 12.1 min, 95:5 Phase A/Phase B at 15.0 min; flow rate, 0.4 mL/min; temperature, 40°C. The effluent was connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)–MS.

LIT and triple quadrupole (QQQ) scans were accessed on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), AB Sciex QTRAP6500 System, equipped with an ESI-Turbo Ion-Spray interface, operating in a positive ion mode and controlled by Analyst 1.6.1 software (AB Sciex). The following operation parameters were used: ESI source temperature 500°C; ion spray voltage (IS) 5500 V; curtain gas (CUR) 25psi; the collision-activated dissociation (CAD) was set highest. QQQ scans were obtained as MRM experiments with optimized declustering potential (DP) and collision energy (CE) for each individual MRM transitions. The m/z range was adjusted between 50 and 1000. Analyst 1.6.1 software were used to perform data filtering, peak detection, alignment, and calculations. Metabolite abundances were quantified using peak areas. Metabolites were identified using HMDB (Wishart *et al*., 2013), METLIN (Zhu *et al*., 2013) and other public databases according to standard metabolic operating procedures. The metabolites with a P value <0.05 and variable importance in projection VIP $\geq$ 1 were considered as differential metabolites between two groups.

RESULTS

Mantled Identification: To determine the effect of 2,4-D treatments on mantled variation, (control) mantled and 2,4-D treated samples were subjected to agarose gel analysis using kDEF1 marker. Results showed that kDEF1 was detected only in mantled sample whereas no band was amplified in 2,4-D treated samples (Fig. 1).

Analysis of EgDEF1 Transcripts: To further understand the effect of high exposure of 2,4-D on the accumulation of EgDEF1 transcripts, transcriptome sequencing (Data not shown) was performed. The results showed that only truncated DEF1 (tDEF1) was expressed during the transition from explant to callus. A BLAST search in the available oil palm database confirmed the occurred transcript was 99.81% similar (E value = 0) to tDEF1 (accession number: KF142649). The accumulation of tDEF1 transcript was found to decrease 0.2-fold during the transition (Fig. 2). Notably, kDEF1 was not expressed during these stages.

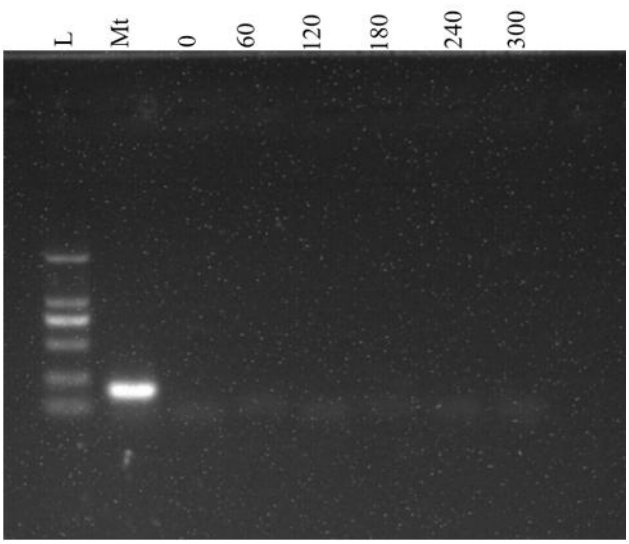

Fig. 1. Detection of mantled abnormality in Mantled (Mt) control sample and 2,4-D treated samples (0, 60, 120, 180, 240, 300 mg/L) using kDEF1 primer pairs. The kDEF1 was detected only in control sample whereas no band was amplified in 2,4-D treated samples.

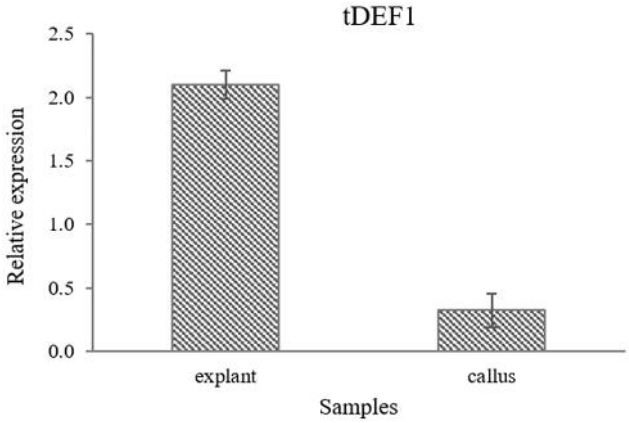

Fig. 2. Relative expression of tDEF1 transcripts from transcriptomics analysis (Data not shown). Error bars indicate the standard deviation between three replicates.

Analysis of Metabolites: Ultra-performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS) were performed to evaluate the changes of metabolites in explant and callus. The overlapping analysis of total ion chromatograms (TIC) in different QC samples can determine the repeatability of metabolite extraction and detection. Results showed that TIC curves were overlapped during the detection of metabolite. The retention time and peak intensities were compatible, indicating the stability of the signal when the same sample was detected at a different time and provided an assurance that our metabolomic data were reliable (Fig. 3).

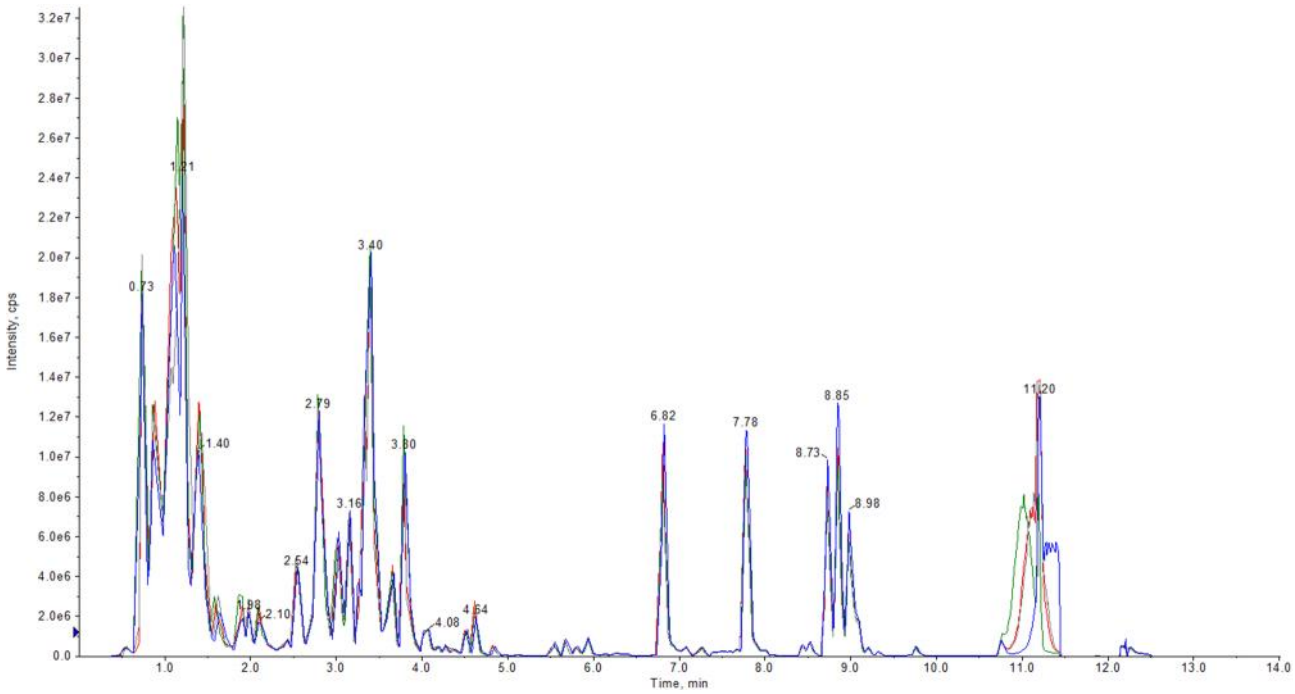

Fig. 3. Overlapping analysis of the total ion chromatograms (TICs) in different quality control (QC) samples. The horizontal axis represents the retention time (min) of metabolite detection and the vertical axis represents the intensity of the ion current (cps: count per second).

Totally, 43 metabolites were differentially expressed during the transition from explant to callus. The metabolite classes that changed between the group were shown in Fig. 4. Among DEMs, 34 metabolites showed increased accumulation and 9 metabolites showed decreased accumulation. The accumulation of all DEMs involved in nucleotides (Deoxycytidine, Uridine 5'-diphospho-D-glucose, UDP-α-D-glucose, Adenine, Guanosine and Deoxyadenosine) and carbohydrates (D-Fructose 6-phosphate, D-Glucose 6-phosphate and Glucose-1-phosphate) and lipids (13-HPODE and 4-Hydroxysphinganine) were increased during the transition from explant to callus. In amino acid and derivative class, 8 metabolites (L-Tryptophan, L-(-)-Tyrosine, L-Phenylalanine, L-Valine, DL-Methionine, L-Methionine, 5-Hydroxy-L-tryptophan, and D-erythro-sphinganine) showed increased accumulation whereas 5 metabolites (N-Acetylaspartate, L-Asparagine, L-Histidine, L(+)-Ornithine and N,N-Dimethylglycine) showed decreased accumulation. In organic acids and derivatives, 9 metabolites (Succinic acid, Citric acid, 2-Isopropylmalate, 5-Aminolevulinate, Phthalic acid, Kynurenic acid, 6-Aminocaproic acid, Adipic acid and Terephthalic acid) were increased and 1 metabolite (γ-aminobutyric acid) was decreased. Interestingly, the accumulation of deoxycytidines was found to increase 3-fold during the transition (Fig. 5).

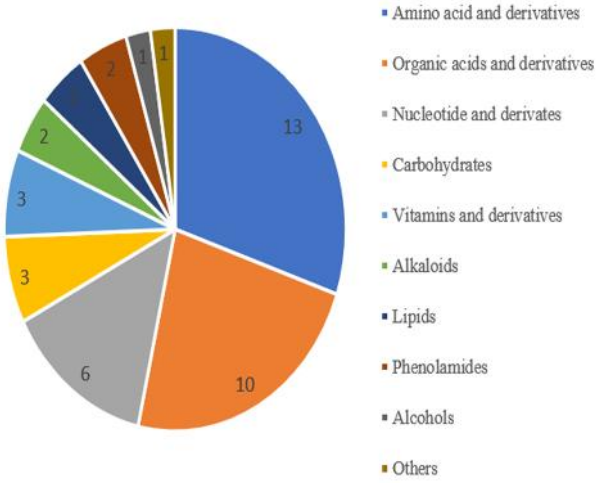

Fig. 4. Classes of metabolites that changed between explant and callus. Mostly changed classes are amino acid and derivatives; organic acids and derivatives; and nucleotide and derivatives.

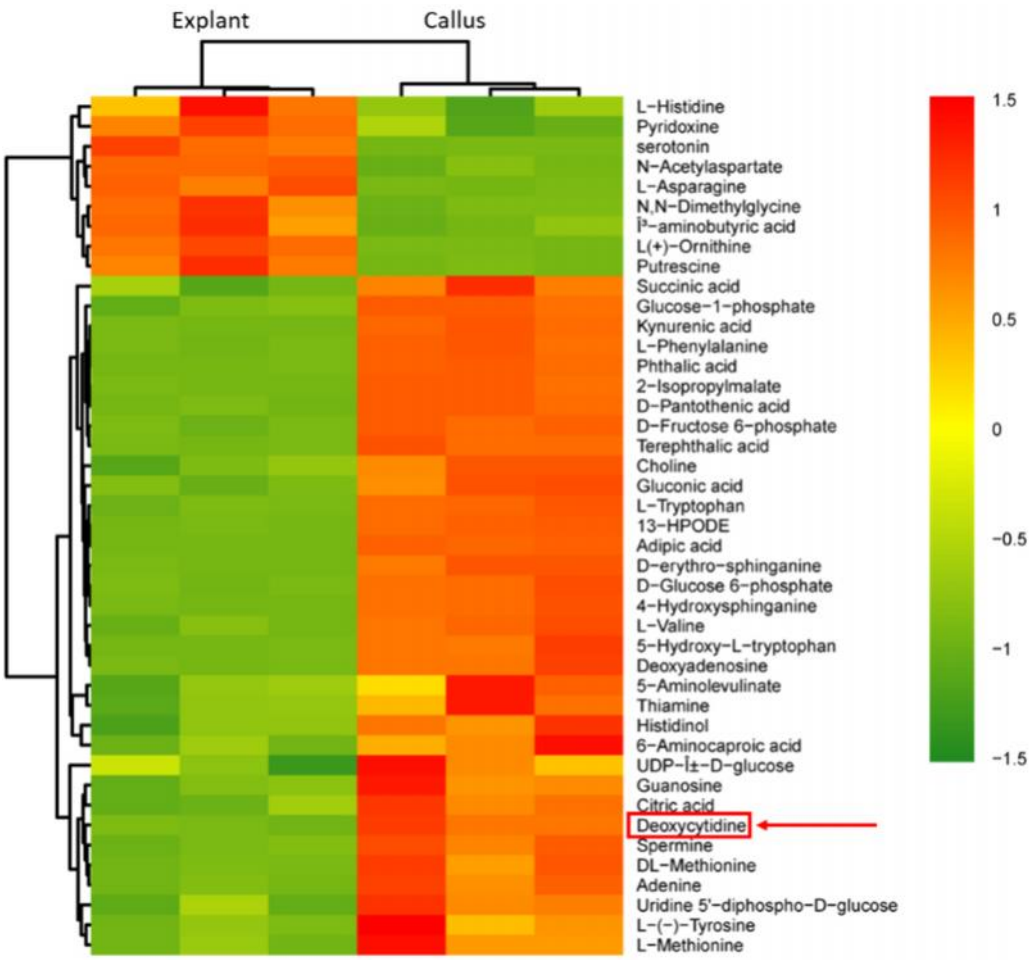

Fig. 5. Heatmap representing the metabolites that changed between explant and callus. The colors indicate relative metabolite concentration, red indicates increase accumulation while green indicates decrease accumulation. The arrow and metabolites in the red box indicate metabolite related to mantled.

DISCUSSION

The plant growth regulator 2,4-D may function as stress chemical to induce mantled somaclonal variation while other factors may also be contributed in the process (Morcillo *et al*., 2006). Several studies (Jaligot *et al*., 2000; Morcillo *et al*., 2006; Jaligot *et al*., 2014; Ong-Abdullah *et al*., 2015; Sarpan *et al*., 2020) have reported on the molecular mechanisms and DNA methylation underlying the mantled variation in oil palm. However, the effect of 2,4-D on the development of mantled phenotype is very limited. In this study, the inflorescence explants were treated with different concentrations of 2,4-D at callus induction stage trying to introduce mantled.

At DNA level, consistent with previous studies (Ong-Abdullah *et al*., 2015; Sarpan *et al*., 2020), identification of mantled abnormality by kDEF1 marker showed that kDEF1 was not detected in all 2,4-D treated samples, suggesting that the callus obtained from explants exposed to 2,4-D are not mantled.

The hypomethylation of a transposable element within EgDEF1 gene has been reported as a link to mantled abnormality. Two EgDEF1 transcripts (cDEF1 and tDEF1) were expressed in both normal and mantled tissues whereas lack of expression of a novel transcript kDEF1 was reported in normal clonal palms (Ong-Abdullah *et al*., 2015, Sarpan *et al*., 2020). In the present work, transcriptome analysis indicated that the expression of kDEF1 was not presented in 2,4-D treated samples, confirming that the deriving callus are not mantled and 2,4-D may not be implicated in the development of mantled during callus induction. As for tDEF1 transcript, its accumulation decreases 0.2-fold from explant to callus, while previous study (Ong-Abdullah *et al*., 2015) reported a stable accumulation of tDEF1 during different developmental stages in normal and mantled inflorescences. The decrease accumulation of tDEF1 in our study might be the consequences of high exposure to 2,4-D. The lack of expression of kDEF1 transcript and identification of mantled by kDEF1 primer pairs

suggested that the decreased accumulation of tDEF1 may not be related to mantled abnormality.

During tissue culture, the alterations in DNA methylation was occurred that is associated with the occurrence of mantled phenotype (Matthes *et al*., 2001; Eeuwens *et al*., 2002). DNA methylation on deoxycytidine residues were known to be involved in gene regulation at transcriptional level during the process of differentiation/dedifferentiation and as a response to various environmental stresses. Evaluation of methylation rates by quantification of deoxycytidines has shown that the contents of deoxycytidines were higher in normal regenerants than in abnormal ones (Jaligot *et al*., 2000). Previous study (Matthes *et al*., 2001) reported that the levels of 5-methylcytosine were significantly higher in abnormal regenerants than in normal ones, indicating that the accumulation of 5-methycytosine can result in mantled abnormality. Consistent with previous report of higher deoxycytidine content in normal regenerants, the accumulation of deoxycytidine was increased during the transition from explant to callus in this study. Furthermore, 5-methylcytosine was not accumulated during these stages. Thus, it was concluded that the high concentrations of 2,4-D treatment may not result in mantled-bearing callus.

Conclusions: The detection of mantled by kDEF1 marker, transcriptomes and metabolomes analyses enabled us to determine the possible relationship between high concentrations of 2,4-D treatment and mantled abnormality during callus induction. All these analyses consistently suggested that the plant growth regulator, 2,4-D may not be involved in the development of mantled somaclonal variation during callus induction stage. However, whether 2,4-D may induce mantled phenotype in late stages should be explored in future studies.

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