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CANDIDATE HORMONE-RESPONSIVE MARKERS FOR CALLOGENESIS OF OIL PALM (*Elaeis guineensis* Jacq.)

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ABSTRACT

The in vitro *propagation of the oil palm involves an indirect somatic embryogenesis process through an intervening callogenesis phase. Both processes are determining factors in the success of clonal propagation. This study aims to discover expression markers associated with hormone response as a means to measure the favourable response of oil palms to callogenesis. Expression levels of hormone responsive genes in cultured and uncultured leaf explants were measured by using quantitative real time PCR, followed by statistical analysis to determine whether a relationship to callogenesis existed. The potential for callogenesis in cultured leaf explants of oil palms was significantly correlated to the expression changes of a putative brassinosteroid leucine-rich repeat (LRR) receptor kinase (*EgBrRK*), a putative cytokinin dehydrogenase (*EgCKX*) and a putative response regulator type A gene (*EgRR1*). A regression model for callogenesis incorporating these three genes indicated a predicted R_2 value of 67.89%. The larger reduction in the expression of* EgRR1 *and another cytokinin responsive gene,* EgCK REGULATED KINASE*, in cultures exhibiting higher callogenesis rates suggested an increase in cytokinin signalling output and cytokinin levels. This inference was supported by a slight decrease in the expression of* EgCKX*, suggesting a mild reduction in cytokinin degradation in these cultures. The use of these markers for the prediction of callogenesis rate in uncultured and one-day cultured leaf explants, may provide an early assessment of the callogenesis potential of oil palms.*

Keywords: hormone, callogenesis, oil palm, cytokinin, brassinosteroid.

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INTRODUCTION

Clonal propagation is an attractive option to increase yield per hectare, as new farming land diminishes over time. The 30% yield increase projected of clonal materials over conventional seedlings has been achieved (Corley *et al*., 1982; Hardon *et al*., 1987; Corley and Law, 1997; Duval *et al*., 1997). In Malaysia, most tissue culture laboratories use young spear leaves, as the explant to initiate somatic embryogenesis via an intervening callogenesis phase. Callogenesis rates can range from 11%-20%, depending on genotypes (Ho *et al*., 2009). To improve the efficiency of the tissue culture process, one approach would be to screen palms for their amenability to tissue culture using molecular markers. Only palms that are predicted to be amenable to cloning would be subjected to the culturing process and this would improve the overall tissue culture efficiency.

In oil palm tissue culture, auxin is the primary plant growth regulator used at the early culturing stages (Duval *et al*., 1995; Rohani *et al*., 2003; Roowi *et al*., 2010). Plant growth regulators or plant hormones play a key role in inducing somatic embryogenesis

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as a whole, due to the interactions of the associated signal transduction pathways (Jimenez, 2001). Auxins and cytokinins are the main plant hormones that trigger embryogenic responses in the majority of plant species (Jimenez, 2005).

Numerous studies have found that plant hormone signalling pathways are highly interactive. Exogenous auxin application could transcriptionally regulate genes associated with various hormone responses (Goda *et al*., 2004; Nemhauser *et al*., 2006; Pischke *et al*., 2006; Paponov *et al*., 2008). Auxin for instance, increases transcription of brassinosteroid signalling and biosynthetic genes (Goda *et al*., 2004; Nakamura *et al*., 2006; Nemhauser *et al*., 2006; Paponov *et al*., 2008). Brassinosteroid induced effects were proposed to be mediated via auxin, with brassinosteroid treatment possibly altering endogenous auxin levels or enhancing the sensitivity to auxin (Sasse, 1999). An addition of brassinosteroid to tissue culture media has also been shown to improve somatic embryogenic initiation from conifers and rice (Pullman *et al*., 2003). The brassinolide biosynthesis genes, *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* gene (CPD) and *DWARF4* (DWF4), are markers for determining the strength of brassinosteroid signalling outputs (Wang and Chory, 2006; Zhang *et al*., 2009). Auxin could also induce a cytokinin dehydrogenase gene, while down-regulating a number of type A *RESPONSE REGULATOR* genes, which are mostly negative regulators of cytokinin signalling and are commonly used as markers of cytokinin signalling (Muller and Sheen, 2008; Paponov *et al*., 2008).

Previous transcriptome profiling of hormone-associated genes in plants indicated a host of intricate interactions occur among the different hormone signalling pathways (Goda *et al*., 2004; Nemhauser *et al*., 2006; Pischke *et al*., 2006; Paponov *et al*., 2008). Auxin responsive genes of the Aux/IAA, ARF, GH3-SAUR and PIN families showed rapid expression changes to the auxins indole acetic acid (IAA) and naphthalene acetic acid (NAA) (Paponov *et al*., 2008). Several auxin-sensitive marker genes were up-regulated under a wide range of conditions, including IAA, ARF and SAUR genes, a cytokinin dehydrogenase gene and an ethylene biosynthesis ACC synthase gene (ACS). The expression of a cytokinin dehydrogenase CKX6, was exclusively induced by auxin while a number of *RESPONSE REGULATOR TYPE A* genes were down-regulated by auxin. Several ACS and ACC oxidase genes that are involved in the final steps of ethylene biosynthesis as well as ethylene receptor genes were also differentially regulated by auxin treatment (Goda *et al*., 2004; Paponov *et al*., 2008). The expression of a few gibberellin oxidases involved in gibberellin biosynthesis were also regulated by auxin (Goda *et al*., 2004). Synergism between the auxin and brassinosteroid signaling pathways have been reported and auxin also increases the transcription of brassinosteroid signaling and biosynthetic genes including *BRI1, BIN2, BAS1, DWF4* (Goda *et al*., 2004; Nakamura *et al*., 2006; Nemhauser *et al*., 2006; Paponov *et al*., 2008).

Due to the association of plant hormones to callogenesis and the rapid response in their gene expression changes to exogenously applied hormones (Goda *et al*., 2004; Nemhauser *et al*., 2006; Pischke *et al*., 2006; Paponov *et al*., 2008), this study attempts to find whether there is an association between the expression changes of hormone responsive genes to the callogenesis capability of cultured oil palms (ortets). As auxin is the main hormone used for oil palm callus and embryo induction, the response of these genes to the auxin 2,4-dichlorophenoxyacetic acid was also conducted.

MATERIALS AND METHODS

Plant Materials

The selection of ortets for this study was based on past tissue culture results, mainly their callogenesis and embryogenesis rates. Therefore, 12 ortets (designated as AM3-14) of the *tenera* variety were selected for resampling and clonal propagation. The AM3-8 and AM12-14 were of *Dumpy* x *Avros* genetic background; AM9, 10 and 11 were of *Dumpy* x *Yangambi* x *Avros* background. A total of 2500 pieces of young leaf explants from each ortet were sampled for tissue culture and subcultured at 12-weekly intervals. Random sampling of the explants for molecular analysis was conducted at several time points of the explant culture stages: before induction and one-day culture. Nine of the 12 ortets were sampled at extended time points that were 12-weeks (before first subculture) and 12-weeks and one-day (one-day after first subculture). The leaf explant cultures were subcultured at 12-weekly intervals. Tissue culture data was tabulated after one year of recording and used for statistical analysis together with the gene expression data. The callogenesis rate was calculated as the ratio of the number of callus lines formed, to the number of cultured leaf explants.

Ribonucleic Acid (RNA) Extraction

Total RNA extraction protocol (Rochester *et al*., 1986) was used with the following minor modifications. MaXtract High Density Tubes (Qiagen, Germany) was incorporated to shorten the duration of the protocol and improve

nucleic acid yield, as losses were reduced by less phenol:chloroform:isoamyl alcohol (25:24:1) extractions. The gel in the MaXtract tubes trapped both the organic phase and interphase from the aqueous phase, allowing an easier and relatively cleaner transfer of the aqueous phase to a new tube. The phenol:chloroform:isoamyl alcohol extractions were reduced from four to five extractions to one to two extractions using the MaXtract tubes. RNA quantitation was conducted with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The genes for this study were selected based on their transcriptional regulation by auxin treatments in other plant species (Goda *et al*., 2004; Nemhauser *et al*., 2006; Paponov *et al*., 2008) and on the availability of putative oil palm orthologues in the GenBank database. However, a few genes that were considered important from the literature review but were not found in oil palm EST databases (in-house or in GenBank), included the *CPD* gene and *RESPONSE REGULATOR TYPE A* genes.

Degenerate primers were manually designed to isolate the oil palm gene orthologues of a *RESPONSE REGULATOR TYPE A* (cytokinin-responsive) and *CPD* (brassinolide synthesis) genes (*Table 1*). For the former, the primers were designed to conserved regions in the receiver domain of the *RESPONSE REGULATOR TYPE A* genes (Imamura *et al*., 1999). *CPD* primers were designed to the region located after the proline-rich domain till the steroid binding domain (nucleotides 258-1063) of *CPD* (Accession No. X87367) (Szekeres *et al*., 1996). Reverse transcription was carried out using the High Capacity Archive kit (Applied Biosystems, USA) from the total RNA of embryogenic suspension cultures, according to the manufacturer's instructions. PCR was conducted in 1x PCR buffer (Invitrogen, USA), 2 mM $MgCl_2$, 250 nM each primer, 250 uM dNTP mix, 1 U *Taq* polymerase (Invitrogen, USA) and first strand cDNA of embryogenic suspension cultures. PCR programme was carried out at 95°C for 2 min; 35 cycles of 95°C for 30 s and 60°C for 32 s.

Sequence Analysis

The ClustalW (Thompson *et al*., 1994) alignment of multiple sequences was done using BioEdit (Hall, 1999). The alignment of cDNA sequences to the corresponding genomic sequence was conducted using the Spidey tool available on the NCBI website (http://www.ncbi.nlm.nih.gov/spidey/index.html) (Wheelan *et al*., 2001).

Quantitative Real Time PCR

As 21 genes were selected for this study, quantitative real time PCR (qPCR) with SYBR® Green dye approach was selected. These hormone-responsive genes were identified from oil palm EST and partial genome in-house databases available then. Primers for qPCR were designed for these genes with the aid of Primer3 (version 0.4.0) tool available online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) (Rozen and Skaletsky, 2000) (*Table 2*). The primers were designed based on unique regions identified from sequence alignment with their respective gene family members. The same set of primers were used in a similar study to discover gene expression patterns associated with embryogenesis (Ooi *et al*., 2012).

The first strand cDNA was synthesised from total RNA by using Quantitect cDNA synthesis kit (Qiagen, Germany). Five microlitres of cDNA (1:50 dilution) were used for real-time PCR. Reactions of 20 µl were carried out with SYBR® Premix Ex Taq™ (Takara Bio Inc., Japan) according to the manufacturer's instructions. Real Time PCR was conducted on an ABI Prism Sequence Detection System 7000 (Applied Biosystems, USA) according to the PCR programme of 10 s at 95°C; and 40 cycles of 95°C for 5 s and 60°C for 32 s with an additional dissociation cycling stage at the end. Each PCR reaction was conducted in duplicate and no template controls were included.

The selection of endogenous reference genes for qPCR data normalisation was conducted using GeNorm (Vandesompele *et al*., 2002) on uninduced and induced leaf explants (Ooi *et al*., 2012). Relative transcript levels of genes of interest (GOI) were normalised to the geometric mean levels of three endogenous reference genes according to the delta Ct approach (Livak and Schmittgen 2001; Vandesompele *et al*., 2002).

Exogenous Auxin Treatment

As auxin is the main hormone used during the initiation of oil palm callogenesis and embryogenesis in commercial *in vitro* propagation, the genes' response to auxin was tested by analysing their expression patterns in increasing concentrations of exogenous 2,4-D. Leaf explants were sampled from three clonal ortets (palms of the same clone). Leaf explants were induced and maintained on Murashige and Skoog (MS) basal media containing increasing concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D): 0, 25, 50 and 100 mg $litre^{-1}$. Sampling of the leaf explants for RNA extraction was conducted at these time points in culture: one-day, seven-days, four-weeks and 12-weeks.

TABLE 1. DEGENERATE PRIMER SEQUENCES FOR THE CORRESPONDING TARGET GENES

Primer	Sequence (5′→3′)	Target gene
CPDF1 CPDR1	TTCACSACSCACGTYTTCGGCGA GTCTCATTTATCACAGATTG	CPD
RRAF1 RRAR2	ATCACBGACTACTGSATG GGCACRTTCTCCGACGACATGA	RESPONSE REGULATOR TYPE A

TABLE 2. PRIMER SEQUENCES AND THEIR PUTATIVE GENE IDENTITIES

Primer sequence (5′ → 3′)	Category	Putative gene identity	EST (Accession No.)
ATCCTGTTGTTTGCGGTTTC AACGGTGACCAAACCATAGC	Auxin	IAA9	EL689638.1
ACCCGTCTGATCCTCATCAT CCGGCGTCGGATAGTATAGA	Auxin	PIN	EL689701.1
GCATCGACACTGACAAGACC TTTTGAACTCCCAGAAGAGCA	Auxin	GH3-SAUR	EL689939.1
AAGATGAAGCCCAAGAGCAA CCCTCCCAAAATGAAGACAA	Auxin	ARF9	EL684802.1
GCAAGCCTTGAGAGGAGATG CCCAAGAAAGGGGTTCTAGC	Auxin	IAA13	EY407776.1
CACTGTCGATCCTGCTTGAA CTCATCGGACTTTGGGGATA	Brassinosteroid	BRI1	EY411667.1
TCCTCACTGACAACCCTCCT ATCCGTCACCGCTCTTCTAA	Brassinosteroid	EgCPD	HQ832481
GTGTGTCCAAGGAGCCAAAT GCTATGCTTGCTTGCAACTG	Brassinosteroid	Br LRR receptor kinase	EY407055.1
CCAATCTGGATGTGTCCTCA CTGCATACTGTGGCTGGAAA	Brassinosteroid / sterol	DWF1	EL685251.1
GCGCTTTTAGCTTCCGATAA GGTCCGCATCTGTTCACTCT	Brassinosteroid / sterol	Sterol-4α-methyl oxidase (SMO)	EY406295.1
CTCTCCATCCTCTCCCCTTC CTAACAGCACGACCCCAAAG	Cytokinin	Cytokinin regulated kinase 1	EY411273.1
ACCGAAGGACTGAAGCATTG CCTAAATACGCCCTCATGGA	Cytokinin	Cytokinin dehydrogenase	EL692728.1
GCCTGAAATGACTGGCTATGA CCTCATAAGCCCTTTTTATTGC	Cytokinin	EgRR1	HQ832480
GCCCTCGTACATCCTTGAGT TGGAGTTTGGGTGGAGAAAG	Ethylene	EIN2	EL689442.1
CATCATCATCATCATGGCATC GGGGTGACCTGGAGGTAGTC	Ethylene	EREBP-4	EL681773.1
CGGACAAGGCGAACCAACACCACCT ATCGAAAGGCAGCATCTTGGGCGGC	Ethylene	ERF3b	EL688481.1
AGGTGAACTTCCCTGATGAGGTGCG AGCTTGCTTGATGGGCTGCTTCTCC	Ethylene	ERF2a	EL681772.1
GTCCCCAAGACAAAGCACAT CAACCCTTACCAGCACCTTC	Ethylene	ERS (Nurniwalis, 2006)	Unavailable
TGGAAAAGGTCAAGGGACTG GTTGGATTCTGGCTGATGCT	Ethylene	ACC oxidase (OPSC10)	AY254310
AGTGTAAGGAGCCCCCAACT GGCTTGCAAGGGTTTGTATC	Gibberellin	SCR	EY410843.1
GGGTTCTTCAAGGTCACCAA CCAGCCCACATCTCCAGTAT	Gibberellin	GA-2-oxidase	EY410726.1
CCAGGCCTACCACAACATCT GGGGATTGGTGCTCCTTTA	Endogenous reference	EA1332; Unknown protein (rice)	EY406625.1
GGATTGAGGGGGTCTGTTCT CACTTCCTGACAGAGCATCAAG	Endogenous reference	PD380; No significant similarity	EY403346.1
AGAGCGCCATCAAGTTCAAT CTTATCCAGAGCAAGCCACAC	Endogenous reference	PD569; Manganese superoxide dismutase	EL682210.1

Statistical Analysis

All statistical analyses were conducted using Minitab® Release 14.13. The Anderson-Darling test (Anderson and Darling, 1952) was used to determine the normality of the gene expression and callogenesis rate data. Partial least squares regression (PLS) was conducted as the number of predictors (genes) outnumbered the number of observations (ortets/samples) (Wold, 1975; Abdi, 2007). The results from this indicated the number of components required in the optimal regression model for callogenesis. In addition, Pearson correlation analysis was done to identify gene expression patterns in one-day cultured explants that were associated with callogenesis ($P < 0.05$). Strongly related gene expression patterns to callogenesis were indicated by Pearson correlation coefficients of ≥ 0.7. Both PLS regression and Pearson correlation coefficients were analysed to select several predictors (genes) for regular regression analysis. The subsequent regression model with high R^2 values for the prediction of callogenesis rate was deemed the most appropriate model. The VIF (variance inflation factors) for the predictors in the regression model were also checked to be close to the value of one to ensure low multicollinearity among the predictors in the model.

RESULTS

Isolation of *EgCPD* and *EgRR1*

RT-PCR using degenerate primers was conducted to amplify the cDNA fragments of *CPD* and a *RESPONSE REGULATOR TYPE A* gene from oil palm embryogenic suspension cultures. The partial putative oil palm *CPD* homologue of 746 nucleotides in size was designated as *EgCPD*. BLASTX (Altschul *et al*., 1997) results of *EgCPD* showed that the translated polypeptide was 70% identical to the *CPD* brassinosteroid C-23 hydroxylase from barley in a 239-amino acid region. The *EgCPD* shows high identity to other *CPD* homologues in the polypeptide sequence (*Figure 1a*).

The 246 nucleotides of a putative *RESPONSE REGULATOR TYPE A* partial cDNA, designated as *EgRR1*, showed conservation in the receiver domain of the protein at only the 5′ and 3′-regions of the fragment (*Figure 1b*). The oil palm fragment revealed a unique 44 amino acid region (nucleotides 132-175) that was not present in *RESPONSE REGULATOR TYPE A* genes from other plants. For verification, the genomic sequence of *EgRR1* was identified through *in silico* analysis of the in-house oil palm genome database that was recently established. Four mRNA sequences were also identified through BLASTN (Zhang *et al*., 2000) query of *EgRR1* to an oil palm transcriptome database established in-house. BLASTX (Altschul *et al*., 1997) search of these sequences to GenBank confirmed that they were similar to *RESPONSE REGULATOR TYPE A* genes. Subsequently, the sequence alignment of these mRNA sequences and the *EgRR1* transcript sequence to the corresponding genomic sequence suggested alternative splicing locations in this gene (*Figure 2*). The *EgRR1* sequence isolated from embryogenic suspension cultures was most similar to part of the AASpikelet_isotig20566 sequence isolated from spikelet. The latter appeared to be a primary transcript, or a spliced variant that includes transcribed intron regions. In *Arabidopsis*, splice variants of *ARR22*, a type A response regulator, had been spatially detected (Gattolin *et al*., 2006). In elongating *Arabidopsis* pods, the predominant form was the fully processed transcript, while a combination of the splice variants was detected in open flowers and small pods. This calls for future investigations into the characterisation of *EgRR1* and its splice variants in oil palm. For the subsequent qPCR experiments in this study however, specific primers for both *EgRR1* and *EgCPD* were designed based on their identified cDNA sequences isolated from embryogenic suspensions.

EgBrRK, EgCKX and *EgRR1* as Predictors for Callogenesis

The expression of hormone-responsive genes was analysed by qPCR in 0-day and one-day cultured leaf explants on auxin supplemented media. The leaf explants were derived from 12 ortets of callogenesis rates ranging from 2.45% to 53.56%. The qPCR products were sequenced and verified to encode the expected regions flanked by the respective primers. Three endogenous reference genes, *EA1332, PD380* and *PD569*, were determined to be the most stably expressed in uncultured and cultured leaf explants (Ooi *et al*., 2012). All expression data were normalised to the geometric mean of these three endogenous references, using the delta Ct approach. Normalised expression values were rescaled to the expression values in Day 0 explants (T0), according to calculations recommended in the GeNorm manual (Vandesompele *et al*., 2002).

Log_2 transformed expression data in one-day cultured explants and untransformed callogenesis data were normally distributed according to the Anderson-Darling test. Hence, subsequent statistical analysis was conducted with log_2 transformed expression data and linear callogenesis rates. Genes with expression levels in one-day cultured explants that were highly correlated to callogenesis rate with Pearson correlation coefficients of higher than 0.7 ($P < 0.05$) were identified. PLS regression was conducted as the number of predictors outnumbered the number of observations and PLS may overcome the collinearity problem associated with linear

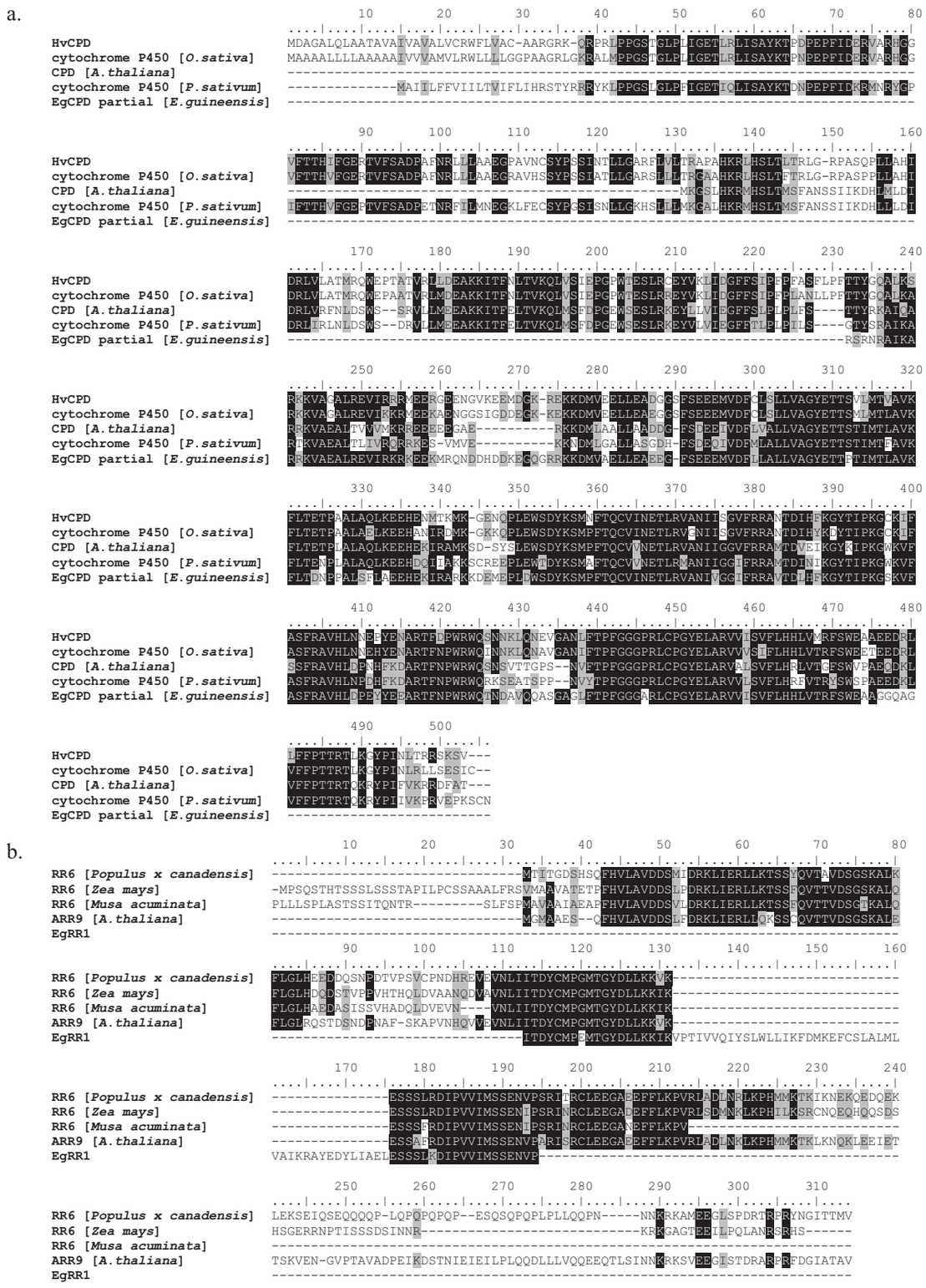

Figure 1. Amino acid sequence alignments of the oil palm partial EgCPD *and* EgRR1 *to the respective homologues from other plants. (a) Sequences from top to bottom:* HvCPD, CPD *brassinosteroid C-23 hydroxylase [*Hordeum vulgare subsp. vulgare*] (ACA83752.1),* Oryza sativa *cytochrome P450 (BAD90973.1),* Arabidopsis thaliana *CPD (NP_001078535),* P. sativum *cytochrome P450 enzyme (BAF56238.1),* E. guineensis *putative CPD, partial (EgCPD; Accession No. HQ832481). (b) Sequences from top to bottom:* Populus *x* canadensis *putative type A response regulator 6 (CBX43988.1),* Zea mays *response regulator 6 (BAB20581.1),* Musa acuminata *response regulator 6 (ABG33766.1),* Arabidopsis thaliana *ARR9 (NP_191263.1) and* Elaeis guineensis *putative type A response regulator, partial (EgRR1; Accession No. HQ832480). Identical residues are shaded in black and similar residues are shaded in grey, based on BLOSUM62 scoring matrix.*

regression (Wold, 1975; Yeniay and Goktas, 2002). PLS regression results from the 21 genes (predictors) suggested that two components could explain 86% of the variance in the optimal model for callogenesis. Each PLS component is computed as a linear combination of the predictors (Yeniay and Goktas, 2002). High regression coefficients (>2) of the predictors computed from PLS regression were attributed to *EgBrRK*, *EgCPD*, *EgEIN2*, *EgCKX* and *EgRR1* predictor genes. However, some of these predictor genes exhibited multicollinearity with other genes as shown by their Pearson correlation coefficients with other genes and are thus not suitable for normal regression analysis (Simon, 2004). Hence, taking into account the PLS regression coefficients, Pearson correlation coefficients and VIF generated from multiple attempts in building the normal regression model, the best regression model was obtained with *EgBrRK* (Accession No. EY407055.1), *EgCKX* (Accession No. EL692728.1) and *EgRR1* (Accession No. HQ832480) (*Figure 3a*). The expression levels of each gene in one-day cultured explants (relative to levels at 0-day) do not correlate well to callogenesis rates as observed by the low R^2 values, but incorporation of all three into a regression model improved the correlation to callogenesis rate. The *EgBrRK* is similar to a putative brassinosteroid LRR receptor kinase gene while *EgCKX* is a putative cytokinin dehydrogenase. This model was significant (P=0.001) with a regression equation of: callogenesis rate (%) = 44.6 + 10.1 EgBrRK + 14.5 EgCKX – 8.81 EgRR1. The predicted R^2 value of

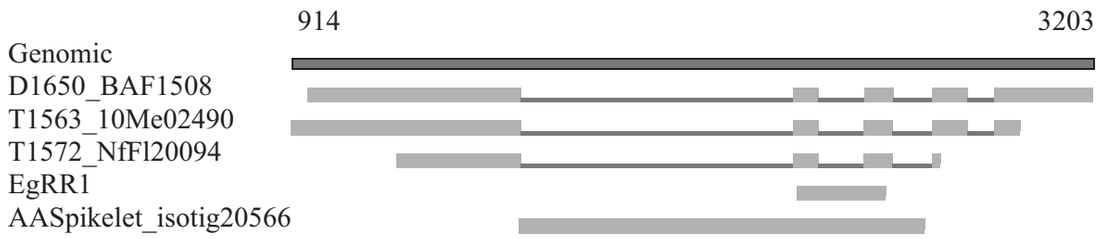

Figure 2. Sequence alignment representation of five transcript sequences to the corresponding genomic sequence. Transcript sequences D1650_BAF1508 is isolated from floret before anthesis, T1563_10Me02490 from mesocarp, T1572_NfFl20094 from young female flowers, EgRR1 from embryogenic suspension cultures and AASpikelet_isotig20566 from spikelet. For the transcript sequences, exons are indicated as rectangles and spliced introns are indicated as bridging lines.

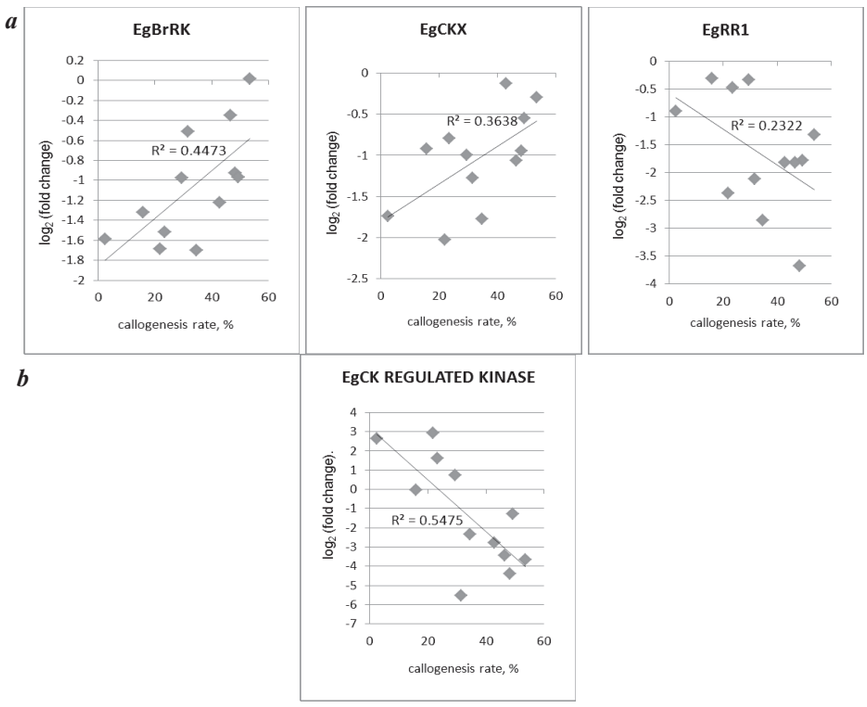

Figure 3. Scatter plots of gene expression profiles correlated with callogenesis rates of ortets (AM3-14). A. EgBrRK, EgCKX *and* EgRR1*; b.* EgCK REGULATED KINASE. *Expression fold changes (at log_2 scale) represent expression levels in one-day explant cultures relative to their respective levels in uninduced explants (T0). Values below y=0 indicate down-regulation in expression and* vice versa.

67.89% is considerably close to the adjusted R^2 value of 79.9%, indicating that the model is not overfit, and therefore has a predictive ability of 67.89%.

Another cytokinin associated gene, *EgCK REGULATED KINASE*, also correlated with callogenesis performance with a Pearson correlation coefficient of -0.740 (P<0.05) (*Figure 3b*). The *EgCK REGULATED KINASE* is 68% similar in protein sequence to a cytokinin regulated kinase of *Nicotiana tabacum* (E-value: 5e-57). However, its Pearson correlation coefficients with other genes and the resulting VIF from incorporation of *EgCK REGULATED KINASE* into the regression model suggested high multicollinearity with gene expression profiles of six other genes, including *EgBrRK*. Hence, *EgCK REGULATED KINASE* expression values were not used in the regression model for callogenesis. However, the role of *EgCK REGULATED KINASE* orthologues in other plants lends support to the inference of cytokinin levels in relations to callogenesis.

EgRR1 Demonstrated Clustered Expression Profiles in Callogenesis Groups up to 12-week in Culture

The nine ortets that were sampled up to 12-week in culture were divided into two groups; high (40%-55%) and low (16%-35%) callogenesis groups. Only *EgRR1* exhibited clustering in its expression profile between the two callogenesis groups across time (*Figure 4*). The *EgRR1* expression fold changes were significantly lower (P<0.05) in the high callogenesis group relative to the low callogenesis group at the 12-week time point before subculture (T12w).

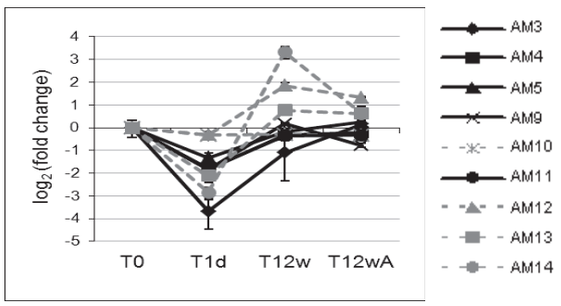

Figure 4. The EgRR1 expression pattern of cultured explants from nine ortets across time. Ortets AM3, AM4, AM5, AM9, AM11 (bold lines) have high callogenesis rates (40%-55%) while ortets AM10, AM12, AM13, AM14 (dotted lines) have low callogenesis rates (16%-35%). Expression levels were normalised to the geometric mean expression levels of three endogenous reference genes (EA1332, PD380 and PD569) and rescaled to expression levels in the respective T0 explants. Expression fold changes are depicted at a $\log_2$ scale. Time series: T0 – uninduced explants; T1d – one-day explant culture; T12w – 12-weeks explant culture before subculture; T12wA – 12-week explant culture after subculture.

EgBrRK and *EgCPD* Expression Levels are Suppressed by Increasing Exogenous 2,4-D

RNA could not be obtained from 4- and 12-week old explants cultured on 100 mg litre^{-1} 2,4-D, even after several attempts, possibly due to toxicity from the high dose of 2,4-D. Therefore, the time points analysed for 100 mg litre^{-1} 2,4-D treatment was from 0-day up to the seven-day time points. The three ortets (clonal ortets) sampled for the 2,4-D experiment were of identical genotype, as these were palms previously cloned from a single individual palm. Even then, only two of these ortets responded similarly to the 2,4-D treatments, based on the similarity in their gene expression patterns exhibited at early time points. Therefore, the analysis was conducted on data from these two ortets.

The expression patterns of *EgBrRK* and *EgCPD* in 2,4-D treated explants suggested that both genes were down-regulated at one-day relative to 0-day and after that, transcript levels increased to the levels observed at seven-day (*Figure 5*). An inverse correlation between expression fold change and 2,4-D concentration was observed at one-day for *EgBrRK* and at seven-day for *EgCPD*. The increasing 2,4-D was observed to exert a selective repression on the expression of these two genes, as seen by their gradation of decreasing expression fold changes at these time points. As the concentration of supplemented 2,4-D increased from 25 mg litre^{-1} to 100 mg litre^{-1}, the relative transcript levels of *EgBrRK* at one-day decreased, compared to its levels at 0-day. Similarly, *EgCPD* expression fold change also decreased with the increasing 2,4-D at seven-day, relative to its expression levels at 0-day. The *EgRR1* and *EgCKX* were not responsive to 2,4-D as their expression changes did not correlate with the increasing concentrations of 2,4-D. The expression of *EgRR1* and *EgCKX* was generally down-regulated after one-day exposure to 2,4-D.

DISCUSSION

Callogenesis is an unpredictable event though most ortets can generate callus at different degrees (Ho *et al*., 2009). The primary focus of this study is on the identification of expression markers for callogenesis. In this study, gene expression patterns correlated with callogenesis performance of oil palm ortets were associated with two types of hormones, cytokinin and brassinosteroid. The *EgRR1* and *EgCKX* are putatively associated with cytokinin response and *EgBrRK* is putatively associated with brassinosteroid signalling. These three genes were identified to correlate to callogenesis rates based on their expression in one-day cultured explants, even though callogenesis visibly manifests at

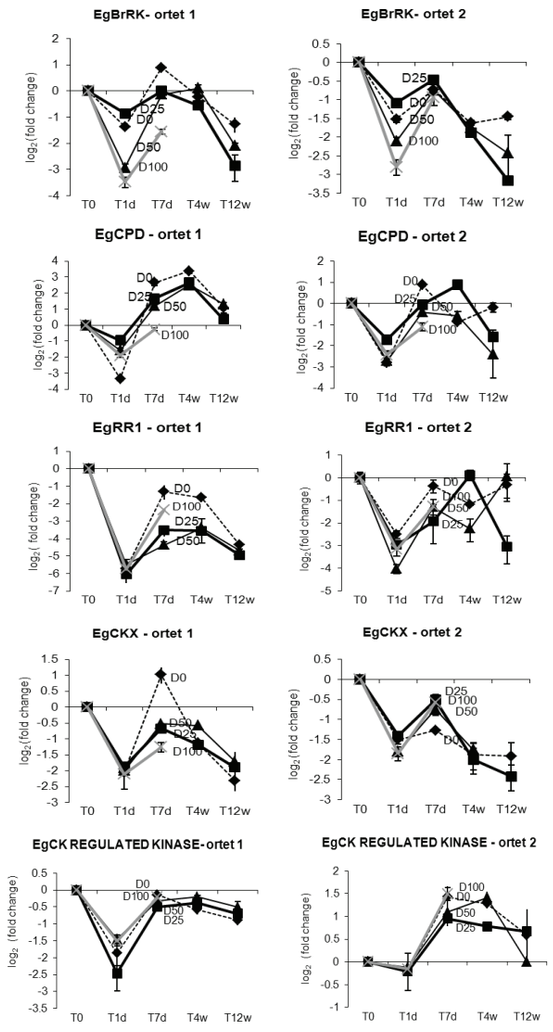

Figure 5. Expression profiles of EgBrRK, EgCPD, EgRR1, EgCKX *and* EgCK REGULATED KINASE *in leaf explants from three clonal ortets cultured on increasing 2,4-D concentrations over time. The line graphs represent the different 2,4-D concentrations used (--♦-- D0 (0 mg litre^{-1} 2,4-D); —■— D25 (25 mg litre^{-1} 2,4-D); —▲— D50 (50 mg litre^{-1} 2,4-D); —X— D100 (100 mg litre^{-1} 2,4-D)). Expression levels were normalised to the geometric mean expression levels of three endogenous reference genes (EA1332, PD380 and PD569) and rescaled to the expression levels in the respective T0 explants. Expression fold changes are depicted at log_2 scale. Explant culture time series: T0 - uninduced explants; T1d - one-day; T7d - seven-day; T4w - four-week; T12w - 12-week.*

an average of three months after tissue culture initiation. However, histological analysis detected perivascular division within the leaf explants after six weeks in culture, which is indicative of callus initiation (Nur Fatihah *et al*., 2012). Hence, gene expression changes taking place prior to perivascular division are likely.

Callogenesis, an event that involves dedifferentiation and division usually requires both cytokinin and auxin. Cytokinins are known to induce cell division in tissue culture in the presence of auxin (Roef and Van Onckelen, 2007). For example, auxin was needed to initiate callus in potato while cytokinin was necessary to maintain its growth (Okazawa *et al*., 1967). The candidate markers for oil palm callogenesis found in this study were not directly associated to auxin, though several auxin responsive genes from the ARF, Aux/IAA and GH3-SAUR gene families were included. However, as these gene families are large, the auxin-responsive genes selected for this study were not exhaustive, and future studies may incorporate more genes from these families.

In this study, the expression levels of the cytokinin response genes, *EgCK REGULATED KINASE* and *EgRR1*, were inversely correlated with oil palm callogenesis rates. Transcript levels of the tobacco *CRK1* (Accession No. AF302082), a putative homologue of *EgCK REGULATED KINASE*, decreased rapidly after cytokinin induction, suggesting a negative regulation by cytokinin (Schafer and Schmulling, 2002). Thus, the reduction in levels of *EgCK REGULATED KINASE* in explants of high callogenic ortets suggested that cytokinin levels increased in these cultured explants relative to uninoculated explants. As most *RESPONSE REGULATOR TYPE A* genes are negative regulators in cytokinin signalling (Kiba *et al*., 2003; To *et al*., 2004; Lee *et al*., 2007), the decrease in *EgRR1* expression in one-day cultured explants of high callogenic ortets suggested an increase in cytokinin output signal. This may lead to increased cytokinin synthesis. In addition, the loss of several type A response regulator gene function in *Arabidopsis* also strongly stimulates callus development (Buechel *et al*., 2010).

In the case of the low callogenic ortets, the increase in fold-expression of *EgCK REGULATED KINASE* and the slight decline of *EgRR1* expression levels from 0-day to one-day in culture suggested low cytokinin levels or a low cytokinin output signal. In conjunction with this, expression of the putative cytokinin dehydrogenase *EgCKX*, decreased after one-day on callus initiation medium, shown by the log_2 transformed expression values of below zero. The CKX catalyse the irreversible degradation of cytokinins (Houba-Herin *et al*., 1999; Morris *et al*., 1999). The decline in *EgCKX* levels was more pronounced in one-day cultured explants of low callogenic ortets. This suggests that cytokinin degradation activity was higher in low callogenic ortets before inoculation, which would therefore reduce cytokinin levels. This hypothesis can be verified with further experiments to quantify the endogenous cytokinin levels in cultured explants. The identification of other members of the *CKX* and *RESPONSE REGULATORS TYPE A* gene families in the oil palm and the analysis of their expression patterns in these explants will also provide additional information. This is because different

CKX family members are known to be differentially regulated and may have different functions, as shown in *Arabidopsis* and rice (Werner *et al*., 2003; Ashikari *et al*., 2005; Galuszka *et al*., 2007).

Due to auxin's role in callogenesis and embryogenesis, the candidate markers were tested for their response to auxin in the media. In *Arabidopsis*, a few *RESPONSE REGULATOR TYPE A* genes were down-regulated in roots by auxin (Paponov *et al*., 2008). The *CRK1* was suppressed by the auxin NAA after 45 min in culture (Schafer and Schmulling, 2002). However, in the present study, it appeared that both *EgRR1* and *EgCK REGULATED KINASE* were not affected by exogenous 2,4-D. The down-regulation in expression of both genes was also observed on hormone-free media (D0), suggesting that *in vitro* culture conditions, rather than 2,4-D, suppressed their transcription once inoculated *in vitro*. One of these *in vitro* factors may be the sugar content in the culture media, as sugar sensing has been linked to cytokinin signalling (Rolland *et al*., 2002). Sucrose is required for cell division and appears to synergistically enhance the effect of cytokinin on cyclin δ3 expression (Hemerly *et al*., 1993; John *et al*., 1993; Soni *et al*., 1995; Coenen and Lomax, 1997).

Auxin has been suggested to act synergistically with brassinosteroid response (Arteca *et al*., 1983; Schlagnhaufer *et al*., 1984; Hardtke, 2007). Brassinosteroid can substitute cytokinin in callus and suspension cultures of *Arabidopsis* to promote cell division (Hu *et al*., 2000). In 2,4-D treated oil palm leaf explants, *EgBrRK* and *EgCPD* were generally down-regulated at one-day in culture and then up-regulated on seven-day. The EgIAA9, a putative Aux/IAA gene, was up-regulated in 2,4-D treated oil palm leaf explants within one day (Ooi *et al*., 2012). Hence, both *EgBrRK* and the brassinosteroid biosynthesis gene, *EgCPD*, were up-regulated later compared to *EgIAA9*. Nakamura *et al*. (2006) proposed that Aux/IAA genes share signalling components of both auxin and brassinosteroid. Auxin also increases brassinosteroid levels through indirect induction of the brassinosteroid biosynthesis gene *CPD* and auxin signalling may directly control brassinosteroid biosynthesis (Mouchel *et al*., 2006; Chung *et al*., 2011).

CONCLUSION

The association of genes linked to cytokinin and brassinosteroid responses to callogenesis in oil palm is supported by the roles of these two phytohormones in cell division. The identified candidate markers for callogenesis can be evaluated on 0-day and one-day cultured leaf explants, even though callogenesis visibly manifests at an average of three months after tissue culture initiation. As callogenesis rate can be predicted using this model only to 67.89% accuracy, an improvement to this prediction model is necessary through a larger sampling population which thus requires the support of a labourious tissue culture programme. Consequently, oil palm tissue culture laboratories may be able to estimate the callogenesis rate of their ortets and thus design their tissue culture programmes more effectively.

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