Identification of QTLs Associated with Callogenesis and Embryogenesis in Oil Palm Using Genetic Linkage Maps Improved with SSR Markers

Ngoot-Chin Ting¹, Johannes Jansen², Jayanthi Nagappan¹, Zamzuri Ishak¹, Cheuk-Weng Chin³, Soon-Guan Tan⁴, Suan-Choo Cheah¹**, Rajinder Singh¹*

¹ Advanced Biotechnology and Breeding Centre, Malaysian Palm Oil Board, Kajang, Selangor, Malaysia, ² Biometris, Wageningen University and Research Centre, AC Wageningen, The Netherlands, ³ Federal Land Development Authority Malaysia Biotechnology Centre, Federal Land Development Authority Malaysia Agriculture Services Sdn. Bhd., Kuala Lumpur, Malaysia, ⁴ Department of Cell and Molecular Biology, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Abstract

Clonal reproduction of oil palm by means of tissue culture is a very inefficient process. Tissue culturability is known to be genotype dependent with some genotypes being more amenable to tissue culture than others. In this study, genetic linkage maps enriched with simple sequence repeat (SSR) markers were developed for dura (ENL48) and pisifera (ML161), the two fruit forms of oil palm, Elaeis guineensis. The SSR markers were mapped onto earlier reported parental maps based on amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers. The new linkage map of ENL48 contains 148 markers (33 AFLPs, 38 RFLPs and 77 SRSs) in 23 linkage groups (LGs), covering a total map length of 798.0 cM. The ML161 map contains 240 markers (50 AFLPs, 71 RFLPs and 119 SSRs) in 24 LGs covering a total of 1,328.1 cM. Using the improved maps, two quantitative trait loci (QTLs) associated with tissue culturability were identified each for callusing rate and embryogenesis rate. A QTL for callogenesis was identified in LGD4b of ENL48 and explained 17.5% of the phenotypic variation. For embryogenesis rate, a QTL was detected on LGP16b in ML161 and explained 20.1% of the variation. This study is the first attempt to identify QTL associated with tissue culture amenity in oil palm which is an important step towards understanding the molecular processes underlying clonal regeneration of oil palm.

Introduction

Tissue-cultured oil palm clones are in high demand because of their greater uniformity and higher yields compared to conventional seedling material [1]. Current commercial seedling material consists of hybrids, referred to as tenera, that result from crossing dura and pisifera palms, the two fruit forms of the African oil palm (Elaeis guineensis, Jacq.). A large range of variation of up to 30.0% from the mean yield can be observed in tenera [2]. On the other hand, the best clones have been reported to yield at least 30.0% more than seedling populations [3], although admittedly there is a problem choosing representative seedlings standard for clonal trials [4]. Although the projected yield increases of up to 30.0% for clonal palms have met with some skepticism, the oil palm industry is confident that eventually the use of clonal planting material will lead to the “next wave” of yield improvement. For this reason in Malaysia twelve oil palm tissue culture laboratories produce annually over two million clonal palms (or ramets), mostly for evaluation within their own organizations [5].

However, clonal reproduction of oil palm is beset by a host of challenges and thus requires further improvements to cope with an ever increasing demand. Too long a period in culture can give rise to abnormal ramets, the causes of which are still being investigated. This per se can be overcome by merely culturing more palms with more lines making up for the shorter runs. But, herein lies the second, perhaps more insidious problem – oil palm tissue culture is a very inefficient process with, on average, over 80.0% of the cultures failing to generate plants [6]. The reasons for this are not known but similar results are obtained with the tissue culture of other major economic crops, like rice, tobacco, potato and tomato [7]. Hence, the efficiency of tissue culture has to be

Citation: Ting N-C, Jansen J, Nagappan J, Ishak Z, Chin C-W, et al. (2013) Identification of QTLs Associated with Callogenesis and Embryogenesis in Oil Palm Using Genetic Linkage Maps Improved with SSR Markers. PLoS ONE 8(1): e53076. doi:10.1371/journal.pone.0053076

Editor: Edward J. Louis, University of Nottingham, United Kingdom

Received July 11, 2012; Accepted November 23, 2012; Published January 29, 2013

Competing Interests: The authors have the following interests. Cheuk-Weng Chin is employed by FELDA Agricultural Services Sdn. Bhd and Choo Cheah by ACCT Sdn. Bhd. Support in tissue culture of the palms was provided by: Dr. Hamidah Musa (and her predecessor, Dr. Zaleha Mohd. Mydin) and Ms. Halina Mohd Ramly of Guthrie Biotech Laboratory Sdn Bhd; Ms. Ho Yuk Wah formerly from United Plantations Bhd; Dr. Azahar Mohd Yusoff and Ms. Halilah Khafidz of Golden Hope Plantations Bhd; Ms. Suzaini Yahya of Ebor Laboratories; and Ms. Girlie Wong and Ms. Joyce Chong of Applied Agriculture Research Sdn Bhd. Mr. Suhaimi Shamsuddin, oil palm breeder of the Federal Land Development Authority Malaysia Biotechnology Centre, Federal Land Development Authority Malaysia Agricultural Services Sdn. Bhd. Support in tissue culture of the palms was provided by: Dr. Hamidah Musa (and her predecessor, Dr. Zaleha Mohd. Mydin) and Ms. Halina Mohd Ramly of Guthrie Biotech Laboratory Sdn Bhd; Ms. Ho Yuk Wah formerly from United Plantations Bhd; Dr. Azahar Mohd Yusoff and Ms. Halilah Khafidz of Golden Hope Plantations Bhd; Ms. Suzaini Yahya of Ebor Laboratories; and Ms. Girlie Wong and Ms. Joyce Chong of Applied Agriculture Research Sdn Bhd.

Copyright: © 2013 Ting et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This project is funded by the Ministry of Science, Technology and Innovation (MOSTI), Malaysia under the project code MM8PP 04-03-T0045-TC3.2. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

* E-mail: rajinder@mpob.gov.my
** Current address: ACCT Sdn. Bhd., Bukit Jall, Kuala Lumpur, Malaysia
improved if the Malaysian oil palm industry wants to realize its target of producing 40 million ramets by 2017 [8].

Evidence exist that tissue culturability of oil palm has a genetic basis with some genotypes being more amenable to tissue culture than others [9]. The question is whether genotypes with improved tissue culturability can be identified. In several plant species, the genomic loci affecting tissue culturability have been mapped as quantitative trait loci (QTL) on genetic linkage maps. QTL responsible for tissue culture amenity have been identified in rice [10], wheat [11] and barley [12]. This demonstrates the potential of this approach for identifying markers associated with tissue culture response. However, to date no QTL for tissue culturability has been reported for oil palm.

In this study, both genomic and EST-SSR markers were generated and mapped to the Ulu Remis Deli dura (ENL48) and Yangambi pisifera (ML161) genetic maps reported earlier [13]. The use of common SSR markers allowed the present linkage maps to be linked to the oil palm reference map [14]. This also allowed standardized labeling and orientation of linkage groups with the reference map. This study is the first attempt to identify QTL associated with tissue culture amenity in oil palm. This report also discusses the potential application of the markers linked to the QTL for tissue culturability to improve the efficiency of clonal propagation in oil palm.

### Materials and Methods

#### Mapping Population

The mapping population (P2) consisted of 97 F1 palms obtained from a cross between Ulu Remis Deli dura (ENL48) and Yangambi pisifera (ML161) [13] grown at Kota Gelanggi, Malaysia. The two parental palms were not cultured due to the long recovery period anticipated after tissue culture, which would have interfered with the on-going breeding program.

#### Initiation of Calli and Embryoids

The general flow of the tissue culture process is described in Figure 1. Tissue culture was carried out by the Malaysian Palm Oil Board (MPOB) and seven collaborating laboratories (listed in the Acknowledgements) with each culturing a number of palms to its capacity using standardized procedures. Each palm in the mapping population was sampled for tissue culture by carefully harvesting the unopened spear leaves (leaf cabbage) as shown in Figure S1.

Both ends of the cabbage and its outer layers were removed except the petioles of frond number 0. All the surfaces were swabbed with absolute alcohol. This was followed by a longitudinal cut to disclose the internal fronds (fronds from to or lower) comprising stacks of young leaflets. In order to avoid contamination, about 10 cm at the distal ends of leaflets were discarded and

![Figure 1. General workflow of oil palm tissue culture. Explant (E0) is cultured to form callus (C) which is transferred to a new medium (C1) to form embryoids. Cultures not forming callus (NC) are transferred to a fresh medium (E1–E3) and undergo the same process again. Embryoids (EC) generated from C1 proceed to polyembryoid culture (PE1–PE15) for the regeneration of plantlets. Callus cultures that fail to generate embryoids (NEC) are transferred to a fresh medium (C2–C4) and undergo the same process again. doi:10.1371/journal.pone.0053076.g001](https://www.plosone.org/doi/10.1371/journal.pone.0053076.g001)
the remaining leaflets were cut into 12 segments of approximately 1.5 cm width. The explants were sterilized in the following steps: i. immersion in a freshly prepared calcium hypochlorite solution (45 g/l) at room temperature for 20 min, ii. rinsing with sterile-distilled water for 10 sec and, iii. dipping in 30 g/l sterile glucose solution before culturing on the modified medium of Murashige and Skoog [15] containing one of two concentrations of alpha-naphthalene acetic acid (NAA), hereinafter referred to as Treatments 1 and 2.

In Treatment 1, explants were inoculated at 28±2°C under continuous darkness for three months in 5 mg/l NAA (E0). Explants that did not form callus (Xc) were transferred to a fresh medium similar to E0 and undergo the same process at E1, E2 and E3. The resulting calli were transferred to a new medium containing 5 mg/l NAA (C1) to form embryos (EC). This process was followed by polyembryoid cultures (PE1-PE15) with 0.1 mg/l NAA. Each PE subculture took two months under 12 hr photoperiod. Callus culture that failed to generate embryoid (MEC) was transferred to a fresh medium to undergo the same process again (C2-C4). For Treatment 2, 10 mg/l NAA was used for the cultures of explants and calli (E0 E3 and C3-C4), followed by 0.5 mg/l NAA in PE1-PE3 and 0.1 mg/l NAA in PE4-PE15.

Over a period of two years, the callusing rate (CR) and embryogenesis rate (ER) were determined. CR and ER were measured as: CR = (total number of calli formed from E0 to E3/total number of clean cultures)×100.0%; ER = (total number of embryoid lines formed from C1 to C4/total number of calli formed)×100.0%. The measurements were labeled as CR1 and ER1 and, CR2 and ER2, for Treatment 1 and 2, respectively.

DNA Extraction

Genomic DNA was extracted from the spear leaves (stored at −80°C) of the 87 progenies and the two parental palms using the modified CTAB method [16]. The DNA concentration and purity were measured using a UV/VIS Spectrometer (Perkin-Elmer Lambda Bio 22). For SSR analysis, DNA was prepared at 30 ng/ul in TE (pH 8.0) buffer.

SSR Analysis

In this study, SSR primers were mainly obtained from the oil palm SSR collection of MPOB. Additional genomic SSR primer sequences were downloaded from the TropGENE database (http://tropgenedb.cirad.fr/html/oilpalm_Marker.html) and labeled as mEqCIR. MPOB in-house SSRs were developed from the oil palm ESTs and genomic sequences reported by [17–20]. SSRs developed from E. guineensis expressed sequence tags (ESTs), E. guineensis genomic sequences, E. oleifera genomic sequences and interspecific hybrid (E. guineensis×E. oleifera) genomic sequences were labeled sEg, sMg, sMo and sMh, respectively.

Screening and genotyping of polymorphic SSRs were carried out as described by [19]. In addition, an ABI3100 genetic analyzer (Applied Biosystems, UK) was used to accelerate the genotyping process using M13-tailed primers as described by [21]. A 19-bp M13 sequence (CAGCAGCTTTGTTAATAAAGCAG) was attached to each of the forward primers (Fwd 5'-M13) and the fluorescent dye (HEX-/-6-FAM-/-NED-M13). PCR was carried out in a 10.0 ul volume containing 100 ng DNA, 1×PCR buffer (NEB, USA), 2 mM of each dNTP (NEB, USA), 2.5 μM of each primer (Fwd 5'-M13, reverse unlabelled primer and dye-M13 primer) and 0.5 U Taq DNA polymerase (NEB, USA). PCR was carried out as described by [19]. A maximum of three PCR products were each labeled with HEX, 6-FAM and NED and multiplexed at a ratio of 1:1:2. Two-ul of the multiplexed mix was denatured in 7.84 ul Hi-Di™ Formamide (Applied Biosystems, UK) and 0.16 ul GeneScan™ 400HD ROX® Size Standard (Applied Biosystems, UK). The denatured sample was then fragmented and size-called on the ABI3100 genetic analyzer.

Genotype data generated from the SSR analysis were scored based on the segregation profiles 1, 5, 8 and 9 in [14] which are illustrated in Table S1. In profile 1, polymorphism of the locus was observed in either one of the parents. The heterozygous and homozygous genotypes were scored as ln and H for allele segregating in the ENL48 parent and, np and nn for the pisifera parent. The ratio for genotypes lnHL and nnHn are expected to follow the 1:1 segregation ratio. In profile 5, the Mendelian segregation ratio of 1:2:1 (for combination hh/hc/hc) is expected when two common alleles (h and c) are segregating in both parents. Profile 8 shows three co-segregating alleles with one common allele (scored as e) in both the parents and two different alleles (scored as f and g). The segregation of these alleles ecfg/ecfg is expected to be in 1:1:1:1 ratio. In the configuration where there are four co-segregating alleles (profile 9), two different alleles are segregating in each parent and they were scored as a and b in parent ENL48 and c and d in parent ML161. The genotypes achcadcbd are also expected to segregate according to a 1:1:1:1 ratio.

RFLP Analysis

RFLP analysis was carried out according to [22] with most of the marker data generated by [13,23]. The markers were named after different tissue types from which the cDNA probes were obtained. The nomenclatures used were: CA/CB (non-embryogenic callus), E/O (embryogenic callus), EA/EO (proliferating embryoid), FDA/FDB/SFB (inflorescence), G/GT (young etiolated seedling), K/KD/KT (kernel), M/ME/MET/MT (mesocarp) and RD (root). Scoring of the RFLP marker was similar to SSR.

AFLP Analysis

AFLP markers were generated using three restriction enzyme-combinations: EcoRI/MseI, PsAl/MseI and TaqI/HindIII as described by [13,23]. The marker nomenclature represents the selective primer-pair followed by size of the observed fragment. Data was scored for polymorphic fragments as in profile 1 (Table S1).

Genotypic Data Analysis and Construction of the Parental Linkage Maps

The SSR data were incorporated into the previous parental data sets consisting of RFLP and AFLP markers. Chi-square analysis was performed to determine markers with distorted segregation at several levels from P<0.0001 to <0.1. Markers showing distorted segregation and missing data were excluded as per the criteria of [24]. In this study, the mapping strategy was to examine marker data in a systematic manner, thereby removing problematic markers at every step of the map construction process.

Linkage analysis was carried out separately for ENL48 and ML161 using JoinMap® 4.0 [25]. All markers (except those with segregation type <hhkhk>) were re-coded to the double-haploid (DH1) format as described by [26], which is equivalent to the double pseudo-testcross approach [27]. Subsequently, the re-coded markers were grouped using a recombination frequency threshold of 0.2 and the linkage phases of the markers were determined. For each parent, a basic map was constructed using the maximum likelihood method. Markers with a nearest neighbor stress (N.N. Stress) value greater than 4 (cM) were discarded from further analysis.

Markers of segregation type <hhkhk> were subsequently included with those mapped in the basic maps. The dataset
(now including the <hkxhk> markers) was re-analyzed using the regression mapping function in JoinMap® 4.0. Markers were grouped using a recombination frequency threshold of 0.2. The recombination frequencies between markers were transformed into map distances in centiMorgan (cM) using the Haldane mapping function. On each linkage group, the contribution of each marker to the average goodness-of-fit (mean Chi-square) and nearest neighbor fit (N.N. Fit) was inspected to confirm its most likely position in order to get the best possible map. In addition, stability of the marker order on every linkage group was checked by comparing with the parental maps (generated earlier using DH1 format) using MapChart 2.2 [28]. The <hkxhk> type markers that caused a change in order were discarded.

Statistical Analysis

The CR was transformed using a log-transformation \( \log(CR + 0.2) \), subsequently denoted as LnCR. Approximately half of the observed ER were equal to zero. Therefore, two transformations were used: (1) a transformation into a binary variable, denoted as binER, with values: 0 if \( ER = 0 \) and 1 if \( ER > 0 \); (2) a transformation into an ordinal variable, denoted as ordER, with values: 0 if \( ER = 0 \), 1 if \( 0 < ER \leq 1 \) and 2 if \( ER > 1 \).

The following analysis was made based on the fact that palms were assigned randomly to eight laboratories. Differences between laboratories and treatments were removed by using a mixed model, \( y_{ijk} = \mu + l_i + p_{ik} + e_{ijk} \), in which \( y_{ijk} \) is the observation on palm \( k \) (= 1…\( n_j \)) assigned to laboratory \( i \) (= 1…8) with treatment \( j \) (= 1, 2), \( l_i \) is the fixed effect of laboratory \( i \), \( p_{ik} \) is the random effect of palm \( k \) within laboratory \( i \) (with zero mean and variance \( \sigma_p^2 \)) and \( e_{ijk} \) is a residual effect (with zero mean and variance \( \sigma_e^2 \)). Parameter estimation was carried out using the REML facilities in GenStat 14 [29]. Predictions of the random effects \( p_{ik} \), denoted as PLnCR, PbinER and PordER, respectively, were subjected to QTL analysis. The coefficient of determination was calculated as \( \sigma_p^2 / (\sigma_p^2 + \sigma_e^2) \), with parameters replaced with estimates; the coefficient of determination is a measure of resemblance of the observations under Treatments 1 and 2.

Detection of Quantitative Trait Loci (QTL)

Detection of QTL was carried out using the GenStat QTL library [29]. The traits PLnCR and PbinER, and PLnCR and PordER, were subjected to a two-trait QTL analysis. Tests for significance of QTL were only carried out at marker positions. For determining the significance threshold, the method of Li and Ji [30] was used with a genome wide significance level of 95.0%. The final selection of QTL was obtained after MQM mapping and backward elimination of putative QTL.

| Random effects: | Component  | S.E  | Component  | S.E  | Component  | S.E  |
|-----------------|------------|------|------------|------|------------|------|
| Labs and palms  | 0.963      | 0.160| 0.108      | 0.028| 0.382      | 0.096|
| Residuals       | 0.060      | 0.009| 0.118      | 0.018| 0.369      | 0.057|
| Fixed effects:  | Wald statistic | p-value | Wald statistic | p-value | Wald statistic | p-value |
| Labs            | 35.41      | <0.001| 17.45      | 0.023| 18.01      | 0.019|
| Treatments      | 0.06       | 0.800| 0.80       | 0.374| 1.29       | 0.259|

Table 1. Estimates of variation components and effects of laboratories and treatments on LnCR, binER and ordER.
The ENL48 and ML161 parental Maps

With the addition of SSR markers, the number of markers in ENL48 increased to 425 (152 AFLPs, 102 RFLPs and 171 SSRs) and 702 (272 AFLPs, 165 RFLPs and 265 SSRs) in ML161. In ENL48, 55 markers (49 AFLPs and 6 RFLPs) with ≥10.0% missing data and 12 markers (9 AFLPs, 1 RFLP and 2 SSRs) with severe segregation distortion (p < 0.0001) were excluded from further analysis. A majority of the remaining skewed markers showed distorted segregations at p < 0.05–0.1 and less than 10.0% at p < 0.0005–0.01. Similar criteria were also used to examine the ML161 data set, where 94 markers were excluded (83 AFLPs, 6 RFLPs and 5 SSRs). After removing the severely distorted markers at p < 0.0001, the percentage of distortion observed in ML161 was about 2.0%, considerably lower than that (18.0%) in ENL48.

Finally, the data set used for construction of the ENL48 linkage map consisted of 94 AFLPs, 95 RFLPs and 169 SSRs. Of the 358 markers analyzed, 330 were assembled into 23 groups. In order to determine the best position for every marker in a linkage group, the markers contributing to insufficient linkages were also determined and removed. The final map consisted of 148 markers (33 AFLPs, 38 RFLPs and 77 SSRs) in 23 groups (Figure 2). The sequences of the SSR primers and the RFLP clones mapped in this study have been deposited into public databases with their accession numbers shown in Tables 3 and 4.

The individual linkage groups were linked to the map published by [14] using mEgCIR markers. For example, mEgCIR0268, mEgCIR3813 and mEgCIR3809 from LG1 in [14] were also mapped in ENL48 and the linkage group thus labeled LGD1 (where ‘L’ represents the linkage group and ‘D’ dura). In some cases, markers reported by [14] to be in one linkage group were separated in ENL48. For example, markers mEgCIR3693, mEgCIR3477 and mEgCIR3557 were mapped in LG4 [14] however, in this study marker mEgCIR3693 was in a separate group from mEgCIR3477 and mEgCIR3557. In this scenario, the two linkage groups were considered as two sub-groups for LGD4 and labeled LGD4a and LGD4b. The resulting framework map covered a total genetic distance of 798.0 cM with an average of 5.4 cM between markers.

As for ML161, of the 608 markers analyzed, 27 were ungrouped and 341 could not be positioned confidently on the map. The remaining 50 AFLPs, 71 RFLPs and 119 SSRs were assigned to 24 groups. In comparison with ENL48, a denser map was constructed for ML161 with 240 markers spanning a total map length of 1,320.6 cM at an average density of 5.3 cM between markers. Similar to ENL48, the linkage groups were labeled accordingly with ‘P’ representing pisifera. Stability of the marker order was shown by the co-linearity of the mEgCIR markers compared to those of [14]. Sixteen linkage groups, which also represent the basic numbers of chromosome pairs in oil palm, were identified and labeled LGP1 to LGP16.

The resulting ML161 map was used as second reference map for the ENL48 map by using the co-segregating SSR (from MPOB database) and RFLP markers. This was particularly useful for linking groups between the two parental maps, especially for those that did not have any or with only one mEgCIR marker, such as LGD5, D1a, D3, D7, D10a, D10b, D11a, D11c, D12b, D13, D15 and D16. Using this approach, the alignment of linkage groups between the two parental maps was determined and presented in Figure 2, making comparisons of the positions of markers on the corresponding linkage groups in ENL48 and ML161 much easier.

A total of 53 co-segregating markers (16 RFLPs and 37 SSRs) were mapped on both the ENL48 and ML161 maps. Theoretically, map integration is possible with at least 2 common co-segregating markers in a group. This would indicate that most of the groups in the two parental maps (D1/P1, D2/P2a, D3/P3, D4a/P4a, D4b/P4b, D5/P5a, D7/P7, D8b/P8b, D10a/P10, D11a/P11a, D11c/P11b, D12b/P12b, D13/P13, D15/P15 and D16/P16b) could be integrated. However, our experience in this study was that the numbers of co-segregating markers were not sufficient to accurately combine the two parental maps. It was observed that in almost all the integrated groups (data not shown), the differences in recombination frequencies between the parents were high (0.3–0.5). This could be due to the markers being sparse in one of the parental linkage groups (in this case, mostly on the ENL48 map).

### QTL Associated with Tissue Culture Response

Two QTLs were detected: each for calllogenesis (PLnCR) and embryogenesis (PordER). As shown in Figure 3, a QTL for PLnCR was detected in LGD1b of ENL48 at position zero. The marker pointing to the QTL was mEgCIR3477 and explained 17.5% of the variance; the allele substitution effect was 0.048 (S.E. = 0.012). The marker was also found to have a minor effect (0.014 ± 0.006) on PordER explaining 5.7% of the variance. A much more important QTL effect for PordER was detected in LGP16b in ML161. The QTL was located at 26.34 cM (marker sMo00109) and explained 20.1% of the variance; the allele substitution effect was 0.027 (S.E. = 0.006).

### Discussion

#### Mapping Population

Crosses involving dura and pisifera palms produce the hybrid teneras which are currently used as commercial planting materials. Therefore, they are of utmost importance to the oil palm industry. The mapping population used in this study was an already existing population involving a cross between Ulu Remis Deli dura (ENL48) and Yangambi pisifera (ML161). Deli dura palms with Ulu Remis genetic background are known to combine well with Yangambi pisifera producing high yielding tenera progenies [31]. A few palms from this cross had been tissue cultured before and showed variation in response to tissue culture. As such, this cross of

| Table 2. Means of callusing (LnCR) and embryogenesis (binER and ordER) observed for samples tissue cultured by the different laboratories. |
|--------------------------------------------------|
| Labs                  | No. of palms cultured | LnCR ± S.E | binER ± S.E | ordER ± S.E |
|-----------------------|------------------------|------------|------------|------------|
| 1                      | 33                     | 1.032±0.137 | 0.318±0.062 | 0.546±0.113 |
| 2                      | 8                      | 2.426±0.336 | 0.375±0.140 | 0.563±0.258 |
| 3                      | 8                      | 1.617±0.325 | 0.125±0.140 | 0.250±0.250 |
| 4                      | 2                      | 2.409±0.697 | 0.250±0.287 | 0.500±0.528 |
| 5                      | 8                      | 1.541±0.336 | 0.813±0.140 | 1.563±0.258 |
| 6                      | 8                      | 3.017±0.336 | 0.625±0.140 | 1.063±0.258 |
| 7                      | 11                     | 1.732±0.281 | 0.500±0.118 | 0.864±0.217 |
| 8                      | 7                      | 2.113±0.361 | 0.571±0.150 | 0.929±0.276 |

DOI:10.1371/journal.pone.0053076.t002
Figure 2. Alignment of the ENL48 (left) and ML161 (right) maps using co-segregating markers. Markers showing distorted segregation are indicated by asterisk (*) representing significance at $p < 0.1$; (**) $p < 0.05$; (***) $p < 0.01$; (****) $p < 0.005$ and; (******) $p < 0.0005$. doi:10.1371/journal.pone.0053076.g002
| No. | SSR locus | Linkage group | TA (°C) | SSR motif | Accession no. | Putative ID [organism] | Blast search was carried out on 12th Oct 2012 |
|-----|-----------|---------------|---------|-----------|---------------|------------------------|-----------------------------------------------|
| 1.  | sEg00025  | LGD4a         | 53      | (TTA)$_4$ | EY397492      | No significant similarity |
| 2.  | sEg00038  | LGD1          | 52      | (AAT)$_3$ | Pr009947960   | No significant similarity |
| 3.  | sEg00047  | LGD12b        | 56      | (AT)$_{12}$ | EY400727      | Predicted: uncharacterized protein LOC100243686 [Vitis vinifera] |
| 4.  | sEg00066  | LGD5          | 52      | (AT)$_4$  | EY403542      | No significant similarity |
| 5.  | sEg00067  | LGD5          | 52      | (TGA)$_3$ | EY404537      | No significant similarity |
| 6.  | sEg00068  | LGD5          | 53      | (AT)$_4$  | EY404017      | No significant similarity |
| 7.  | sEg00086  | LGP5          | 57      | (ATAC)$_{10}$ | EY407048      | Predicted: uncharacterized protein LOC100243686 [Vitis vinifera] |
| 8.  | sEg00092  | LGD9          | 52      | (TATG)$_3$ | EY407741      | No significant similarity |
| 9.  | sEg00095  | LGP9a         | 52      | (TATG)$_3$ | EY405343      | No significant similarity |
| 10. | sEg00098  | LGP4a         | 52      | (GTT)$_2$ | EY405527      | Developmentally regulated GTP-binding protein, putative [Ricinus communis] |
| 11. | sEg01008  | LGP1          | 57      | (CGG)$_{3}$ | EY408074      | Histone deacetylase [Papulcus trichocarpa] |
| 12. | sEg0151   | LGD13         | 57      | (CAAT)$_3$ | EY411661      | Transcription factor [Lycoris longituba] |
| 13. | sEg0154   | LGP6          | 57      | (CAG)$_{3}$ | EY410356      | Predicted: Transcription factor bHLH96-like [Vitis vinifera] |
| 14. | sEg0159   | LGP12a        | 57      | (AT)$_{3}$ | EY408671      | TGA transcription factor [Medicago truncatula] |
| 15. | sEg0161   | LGP12b        | 57      | (AT)$_{15}$ | EY401342      | Cytosolic aldehyde dehydrogenase RF2C [Ze a mays] |
| 16. | sEg0175   | LGP1          | 57      | (CT)$_{10}$ | EY413618      | Uncharacterized protein LOC10052350 precursor [Zea mays] |
| 17. | sEg0197   | LGD13         | 59      | (GA)$_{10}$ | EL684358      | Predicted: Uncharacterized protein LOC100828466 [Brachypodium distachyon] |
| 18. | sEg0203   | LGD10a        | 58      | (CT)$_{2}$ |ELS95153      | Hypothetical protein SORBIDRAFT__07g019420 [Sorghum bicolor] |
| 19. | sEg0235   | LGP10         | 51      | (CT)$_{3}$ | EY409185      | Putative oxalyl-CoA decarboxylase [Vitis vinifera] |
| 20. | sEg0236   | LGP1          | 55      | (CT)$_{2}$ | EY413618      | Hypothetical protein SORBIDRAFT__10g003220 [Sorghum bicolor] |
| 21. | sMg00009  | LGD2          | 52      | (AT)$_{3}$ | Pr010615860   | No significant similarity |
| 22. | sMg00016  | LGP9b         | 52      | (GA)$_{13}$ | Pr010615861   | No significant similarity |
| 23. | sMg00025  | LGD5          | 52      | (TC)$_{11}$ | Pr010615864   | No significant similarity |
| 24. | sMg00050  | LGP5a         | 50      | (TA)$_{17}$ | Pr010615868   | No significant similarity |
| 25. | sMg00051  | LGD6a         | 52      | (CT)$_{4}$ | Pr010615869   | No significant similarity |
| 26. | sMg00056  | LGD11a        | 53      | (CT)$_{18}$ | Pr010615871   | No significant similarity |
| No. | SSR locus | Linkage group | TA (°C) | SSR motif | Accession no. | Putative ID [organism] Blast search was carried out on 12th Oct 2012 |
|-----|-----------|---------------|---------|-----------|--------------|---------------------------------------------------------------|
| 27. | sMg00064  | LGD11b        | 52      | (GA)$_3$  | Pr01061584   | No significant similarity                                      |
| 28. | sMg00071  | LGP6          | 54      | (GA)$_2$GGAG(GCT)$_3$ | Pr01061587 | No significant similarity                                      |
| 29. | sMg00074  | LGD10a, D14b  | 52      | (AGG)$_4$AGCCCAGCCCT-CGTCACCCCTT(GCC)$_5$ | Pr01061587 | Predicted: *Vitis vinifera* uncharacterized LOC100260255 (LOC100260255), mRNA. *[Vitis vinifera]* |
| 30. | sMg00079  | LGD14a        | 54      | (TG)$_2$(AG)$_3$_1 | Pr01061587 | No significant similarity                                      |
| 31. | sMg00122  | LGD6b         | 54      | (AT)$_3$  | Pr01061588   | No significant similarity                                      |
| 32. | sMg00130  | LGD11a        | 52      | (TA)$_3$_4 | Pr01061588 | No significant similarity                                      |
| 33. | sMg00136  | LGP16b        | 56      | (AG)$_3$_1 | Pr01061588 | No significant similarity                                      |
| 34. | sMg00147  | LGP2b         | 56      | (AT)$_3$_1 | Pr01061586 | No significant similarity                                      |
| 35. | sMg00152  | LGD13         | 54      | (AT)$_3$_2 | Pr01061587 | No significant similarity                                      |
| 36. | sMg00164  | LGD10a        | 55      | (TA)$_3$_2 | Pr01061589 | No significant similarity                                      |
| 37. | sMg00168  | LGD11a        | 55      | (CT)$_3$_4 | Pr01061590 | No significant similarity                                      |
| 38. | sMg00172  | LGP15         | 56      | (CT)$_3$_4 | Pr01061589 | Predicted protein *[Populus trichocarpa]*                      |
| 39. | sMg00175  | LGD7          | 54      | (CGG)$_3$_0 | Pr01061589 | *Camellia sinensis* clone U10BCDNA13045S5 5S ribosomal protein L29 mRNA, complete cds *[Camellia sinensis]* |
| 40. | sMg00188  | LGP13         | 52      | (ACCG)$_3$_8 | Pr01061589 | No significant similarity                                      |
| 41. | sMg00194  | LGP2a         | 54      | (TA)$_2$_6 | Pr01061587 | No significant similarity                                      |
| 42. | sMg00197  | LGP1          | 56      | (AG)$_3$_3 | Pr01061589 | No significant similarity                                      |
| 43. | sMg00198  | LGD15         | 56      | (AG)$_3$_4 | Pr01061588 | No significant similarity                                      |
| 44. | sMg00200  | LGD8b         | 60      | (CT)$_3$_8 | Pr01061590 | No significant similarity                                      |
| 45. | sMg00209  | LGP4a         | 54      | (GA)$_3$_3 | Pr01061590 | No significant similarity                                      |
| 46. | sMg00214  | LGP12c        | 52      | (AT)$_3$_4 | Pr01061590 | No significant similarity                                      |
| 47. | sMg00217  | LGD3          | 54      | (AG)$_3$_4 | Pr01061590 | No significant similarity                                      |
| 48. | sMg00220  | LGP13         | 52      | (AT)$_2$_9 | Pr01061590 | No significant similarity                                      |
| 49. | sMg00222  | LGP7          | 50      | (AG)$_2$_2 | Pr01061591 | No significant similarity                                      |
| 50. | sMg00223  | LGD8b         | 54      | (GA)$_3$_1 | Pr01061591 | No significant similarity                                      |
| 51. | sMg00225  | LGP8b         | 56      | (TG)$_3$_4 | Pr01061591 | No significant similarity                                      |
| 52. | sMg00228  | LGP8b         | 54      | (AT)$_2$_5 | Pr01061591 | No significant similarity                                      |
| 53. | sMg00232  | LGP12c        | 54      | (GA)$_3$_3 | Pr01061591 | No significant similarity                                      |
| No. | SSR locus | Linkage group | TA (°C) | SSR motif | Accession no. | Putative ID [organism] Blitz search was carried out on 12th Oct 2012 |
|-----|-----------|----------------|--------|-----------|---------------|--------------------------------------------------|
| 54. | sMg00235  | LGD2           | 58     | (GA)$_{15}$ | Pr010615916   | Predicted: Vitis vinifera hydroxysteroid 11-beta-dehydrogenase 1-like protein (LOC100260124), mRNA [Vitis vinifera] |
| 55. | sMg00236  | LGD8a          | 56     | (TC)$_{13}$ | Pr010615917   | No significant similarity |
| 56. | sMg00259  | LGP16a         | 57     | (C)$_{13}$  | Pr010615923   | No significant similarity |
| 57. | sMg00260  | LGP8a          | 57     | (CTG)$_{3}$ | Pr010615924   | Predicted: Glycine max DELLA protein DWARF8-like (LOC100805996), mRNA [Glycine max] |
| 58. | sMo00007  | LGP2a          | 50     | (TA)$_{12}$ | Pr010615926   | No significant similarity |
| 59. | sMo00020  | LGP2a          | 58     | (AG)$_{15}$ | Pr009947964   | No significant similarity |
| 60. | sMo00027  | LGP15          | 50     | (TA)$_{14}$ | Pr009947965   | No significant similarity |
| 61. | sMo00043  | LGP5b          | 50     | (AG)$_{24}$ | Pr010615928   | No significant similarity |
| 62. | sMo00051  | LGP3           | 54     | (TA)$_{20}$ | Pr010615929   | No significant similarity |
| 63. | sMo00054  | LGP1           | 54     | (TA)$_{12}$ | Pr010615930   | No significant similarity |
| 64. | sMo00056  | LGP12a         | 54     | (CT)$_{11}$ | Pr010615931   | No significant similarity |
| 65. | sMo00061  | LGP12a         | 56     | (CT)$_{12}$ | Pr010615932   | No significant similarity |
| 66. | sMo00063  | LGD14b         | 54     | (GA)$_{12}$ | Pr010615933   | No significant similarity |
| 67. | sMo00071  | LGP1           | 56     | (AG)$_{22}$ | Pr010615934   | No significant similarity |
| 68. | sMo00085  | LGP13          | 56     | (TC)$_{12}$ | Pr010882585   | cDNA clone:OSIGRA119H18, full insert sequence [Oryza sativa indica cultivar-group] |
| 69. | sMo00102  | LGD7           | 53     | (AG)$_{11}$ | Pr010615939   | No significant similarity |
| 70. | sMo00106  | LGP8a          | 52     | (CT)$_{20}$ | Pr010615940   | No significant similarity |
| 71. | sMo00108  | LGP15          | 53     | (AT)$_{19}$ | Pr010882586   | Predicted: Vitis vinifera uncharacterized LOC100263245 (LOC100263245), mRNA [Vitis vinifera] |
| 72. | sMo00109  | LGP16b         | 56     | (TA)$_{23}$ | Pr010882587   | No significant similarity |
| 73. | sMo00117  | LGP5b          | 54     | (AG)$_{24}$ | Pr010615941   | No significant similarity |
| 74. | sMo00123  | LGP13          | 54     | (TC)$_{12}$ | Pr010882588   | No significant similarity |
| 75. | sMo00131  | LGD16          | 54     | (TTA)$_{9}$ | Pr010615943   | No significant similarity |
| 76. | sMo00151  | LGP7           | 50     | (TG)$_{10}$ | Pr010615945   | No significant similarity |
| 77. | sMo00161  | LGP12a         | 54     | (TG)$_{1}$(AG)$_{6}$ | Pr010317032 | No significant similarity |
| 78. | sMo00170  | LGP7           | 53     | (GA)$_{17}$ | Pr010615948   | No significant similarity |
| 79. | sMo00182  | LGP1           | 58     | (CTC)$_{1}$ | Pr010615949   | No significant similarity |
| 80. | sMo00196  | LGD13          | 56     | (ACAA)$_{12}$(ACAT)$_{10}$(AT)$_{10}$ | Pr010615950 | No significant similarity |
| No.  | SSR locus | Linkage group | TA (°C) | SSR motif      | Accession no. | Putative ID [organism] Blast search was carried out on 12th Oct 2012 |
|------|-----------|---------------|---------|----------------|---------------|---------------------------------------------------------------|
| 81.  | sMo00200  | LGD1          | 57      | (ATAC)₃d(AT)₃a| Pr010615951d  | No significant similarity                                      |
| 82.  | sMo00208  | LGD15         | 58      | (TC)₁₀        | Pr010615952d  | No significant similarity                                      |
| 83.  | sMo00211  | LGP1          | 57      | (AC)₇         | Pr010615953d  | No significant similarity                                      |
| 84.  | sMo00222  | LGD2          | 57      | (CT)₉         | Pr010615956d  | Predicted: *Vitis vinifera* hydroxysteroid 11-beta-dehydrogenase 1-like protein (LOC100260124), mRNA [*Vitis vinifera*] |
| 85.  | sMo00234  | LGD8b         | 57      | (TC)₉         | Pr010615957d  | *Phoenix dactylifera* mitochondrion, complete genome [*Phoenix dactylifera*] |
| 86.  | sMo00240  | LGP11a        | 57      | (GA)₈         | Pr010615958d  | Predicted: *Vitis vinifera* dynamin-related protein 1E-like, transcript variant 1 (LOC100266825), mRNA [*Vitis vinifera*] |
| 87.  | sMo00242  | LGD13b        | 57      | (TC)₁₁        | Pr010615959d  | No significant similarity                                      |
| 88.  | sMo00259  | LGD3          | 56      | (AGA)₈       | Pr010615961d  | No significant similarity                                      |
| 89.  | sMo00270  | LGD7          | 57      | (TTC)₉       | Pr010615963d  | No significant similarity                                      |
| 90.  | sMo00274  | LGP2a         | 58      | (AGA)₉       | Pr010882589d  | No significant similarity                                      |
| 91.  | sMo00285  | LGD10b        | 56      | (ACCl₈        | Pr010615964d  | No significant similarity                                      |
| 92.  | sMo00286  | LGP8b         | 57      | (CGG)₉       | Pr010615965d  | No significant similarity                                      |
| 93.  | sMo00289  | LGD14a        | 58      | (TGT)₉       | Pr010615966d  | No significant similarity                                      |
| 94.  | sMo00294  | LGD8b         | 57      | (ACAT)₈      | Pr010615968d  | No significant similarity                                      |
| 95.  | sMo00302  | LGP6          | 56      | (AG)₁₂       | Pr010615970d  | No significant similarity                                      |

Putative IDs were deduced for the SSR-containing sequences by comparing to the non-redundant protein database (Blastx for EST sequences) and nucleotide database of GenBank (tBlastx for genomic sequences). A threshold score of >80 was used to assign significant similarity.

1Two SSR markers were mapped.
2SSRs developed from oil palm sequences from NCBI GenBank.
3Accession numbers of NCBI GenBank.
4Probe Unique Identifiers (PUIs) of NCBI Probe Database.

DOI: 10.1371/journal.pone.0053076.t003
Table 4. RFLP markers mapped on both the ENL48 and ML161 parental maps with their GenBank accession numbers.

| No. | RFLP locus | Linkage group | Accession no. | Putative ID [organism] | Blast search was carried out on 12th Oct 2012 |
|-----|------------|---------------|---------------|------------------------|---------------------------------------------|
| ENL48 | ML161 |
| 1 | CA00026B | LGP16b | EY396203 | Aquaporin [Elaeis guineensis] |
| 2 | CA00077 | LGP16a | JK629436 | Hox12, partial [Oryza sativa Indica Group] |
| 3 | CA00095 | LGP4b | JK629437 | Ubiquitin carrier protein [Elaeis guineensis] |
| 4 | CA00184 | LGD8a | LGP8b | Cyclin d, putative [Ricinus communis] |
| 5 | CA00197 | LGP4a | EY396360 | Predicted: uncharacterized protein LOC100249262 [Vitis vinifera] |
| 6 | CB00001F | LGP11b | EY396521 | Predicted: heat shock cognate 70 kDa protein-like [Brachypodium distachyon] |
| 7 | CB00006F | LGP10 | EY396591 | Predicted: phosphoenolpyruvate/phosphate translocator 2, chloroplastic [Vitis vinifera] |
| 8 | CB00055F | LGD10b | LGP10 | GST6 protein [Elaeis guineensis] |
| 9 | CB00142 | LGD3 | JK629438 | Pathogenesis-related protein 10c [Elaeis guineensis] |
| 10 | CB00145 | LGD8b | JK629439 | Hypersensitive-induced response protein [Carica papaya] |
| 11 | CED02026 | LGP12c | EY398261 | Hypothetical protein SORBIDRAFT_09g002030 [Sorghum bicolor] |
| 12 | CED02683 | LGD9 | EY397095 | Sucrose synthase1 [Elaeis guineensis] |
| 13 | EO02487 | LGP10 | EY408525 | Pathogenesis-related protein [Elaeis guineensis] |
| 14 | EO02817 | LGP8b | EY410649 | Serine/threonine protein phosphatase PP1 [Medicago truncatula] |
| 15 | FDA000089 | LGD11c | LGP11b | JK629440 | No significant similarity |
| 16 | FDB00046 | LGD14a | Failed to sequence | – |
| 17 | FDB00074 | LGP6 | JK629441 | No significant similarity |
| 18 | FDB00086 | LGP3 | JK629442 | No significant similarity |
| 19 | FDB00120 | LGP1 | JK629443 | No significant similarity |
| 20 | G00016 | LGP6 | JK629444 | Ribosomal protein L32 [Elaeis guineensis] |
| 21 | G00037 | LGD8b | GH159168 | No significant similarity |
| 22 | G00057 | LGP2b | JK629445 | Glyceraldehyde 3-phosphate dehydrogenase [Elaeis guineensis] |
| 23 | G00058 | LGP13 | JK629446 | Predicted: probable polygalacturonase-like [Vitis vinifera] |
| 24 | G00069 | LGD12a | LGP12a | JK629447 | Os01g0300200 [Oryza sativa Japonica Group] |
| 25 | G00080 | LGP10 | JK629448 | Beta-mannosidase 1 [Oncidium Gower Ramsey] |
| 26 | G00122 | LGD11b | JK629449 | Hypothetical protein SORBIDRAFT_01g017570 [Sorghum bicolor] |
| 27 | G00132 | LGD13 | LGP13 | JK629450 | No significant similarity |
| 28 | G00138A | LGP11a | JK629451 | Ubiquitin-conjugating enzyme E2, putative [Ricinus communis] |
| 29 | G00142 | LGD12b | LGP12b | GH159171 | No significant similarity |
| 30 | G00146 | LGP11b | JK629452 | Putative DMT-like protein [Glycine max] |
| 31 | G00152 | LGP4a | JK629453 | OMT4 [Vanilla planifolia] |
| 32 | G00158 | LGP6 | JK629454 | Hypothetical protein VITISV_030281 [Vitis vinifera] |
| 33 | G00163 | LGD16 | LGP16b | JK629455 | 40S ribosomal protein S23 [Elaeis guineensis] |
| 34 | G00170 | LGD4b | JK629456 | S-adenosylmethionine synthetase 1 [Oryza sativa Indica Group] |
| 35 | G00200 | LGP12a | JK629457 | Translationally controlled tumor protein [Elaeis guineensis] |
| 36 | G00233 | LGP4b | JK629458 | Chain A, crystal structure of highly glycosylated peroxidase from royal palm [Raysonnea regina] |
| 37 | G00246 | LGP8b | JK629459 | Ubiquitin conjugating enzyme [Cicer arctium] |
| 38 | GT00008 | LGD12b | LGP12b | GH159173 | No significant similarity |
| 39 | K00007 | LGD10a | LGP10 | JK629460 | Ras-related protein RIC1 [Elaeis guineensis] |
| 40 | K00032A | LGP6 | JK629461 | Predicted: Low quality protein:polyadenylate-binding protein 3 [Vitis vinifera] |
| 41 | KT00015 | LGD14b | JK629462 | Hypothetical protein SORBIDRAFT_02g028940 [Sorghum bicolor] |
| 42 | KT00029 | LGP8b | JK629463 | Predicted: universal stress protein A-like protein [Vitis vinifera] |
| 43 | KT00040 | LGD11c | LGP11b | JK629464 | Endochitinase precursor (EC 3.2.1.14) [Nicotiana tabacum] |
| 44 | M00113A | LGD2 | JK629465 | No significant similarity |
| 45 | M0020A | LGP14 | JK629466 | No significant similarity |
| 46 | ME00051 | LGP10 | JK629467 | No significant similarity |
| 47 | MET00004 | LGP8b | JK629468 | Metallothionein-like protein [Elaeis guineensis] |
| No. | RFLP locus | Linkage group | Accession no. | Putative ID [organism] | Blast search was carried out on 12th Oct 2012 |
|-----|------------|---------------|---------------|------------------------|---------------------------------------------|
| 48  | MT00002    | LGP2b         | JK629469      | Putative cytochrome c oxidase subunit 6b-1 [Oryza sativa Japonica Group] |
| 49  | MT00030    | LGP5a         | JK629470      | No significant similarity |
| 50  | MT00045    | LGP15         | JK629471      | No significant similarity |
| 51  | MT00060    | LGD14a        | JK629472      | Predicted: Uncharacterized protein LOC100253066 isoform 2 [Vitis vinifera] |
| 52  | MT00137    | LGD13         | JK629473      | Predicted: Histone H2A-like [Glycine max] |
| 53  | MT00142    | LGP8b         | JK629474      | No significant similarity |
| 54  | RD00049    | LGP3          | JK629475      | Pathogenesis-related protein 10c [Elaeis guineensis] |
| 55  | SFB00003   | LGD4b         | JK629476      | No significant similarity |
| 56  | SFB00012   | LGP5b         | JK629477      | No significant similarity |
| 57  | SFB00015   | LGP12a        | JK629478      | Translationally controlled tumor protein [Elaeis guineensis] |
| 58  | SFB00016   | LGD8b         | JK629479      | No significant similarity |
| 59  | SFB00021   | LGP5b         | GH159184      | No significant similarity |
| 60  | SFB00022   | LGP12a        | JK629480      | No significant similarity |
| 61  | SFB00031   | LGP8b         | GH159186      | Profilin 2 [Elaeis guineensis] |
| 62  | SFB00039   | LGP5b         | GH159189      | No significant similarity |
| 63  | SFB00041   | LGD1          | GH159190      | No significant similarity |
| 64  | SFB00042   | LGD11c        | JK629481      | SK3-type dehydrin [Musa ABB Group] |
| 65  | SFB00043   | LGP6          | JK629482      | No significant similarity |
| 66  | SFB00047   | LGP15         | JK629483      | Cationic peroxidase 2 [Glycine max] |
| 67  | SFB00054   | LGD12b        | JK629484      | Metallothionein type 2a-FL [Elaeis guineensis] |
| 68  | SFB00062   | LGP2a         | GH159193      | Hypothetical protein ARALYDRAFT_899257 [Arabidopsis lyrata subsp. lyrata] |
| 69  | SFB00063   | LGD11a        | JK629484      | Predicted: 60S ribosomal protein L8 [Vitis vinifera] |
| 70  | SFB00066   | LGP12a        | JK629485      | Predicted: 60S ribosomal protein L8 [Vitis vinifera] |
| 71  | SFB00072   | LGP16b        | JK629486      | No significant similarity |
| 72  | SFB00073   | LGP11a        | JK629487      | Hypothetical protein SORBIDRAFT_06g018700 [Sorghum bicolor] |
| 73  | SFB00082   | LGP4a         | JK629488      | Ribosomal protein S27 [Arabidopsis lyrata subsp. lyrata] |
| 74  | SFB00088   | LGP12c        | JK629489      | Metallothionein type 2a-FL [Elaeis guineensis] |
| 75  | SFB00093   | LGD15         | JK629490      | Hypothetical protein SORBIDRAFT_10g028130 [Sorghum bicolor] |
| 76  | SFB00097   | LGP11a        | JK629491      | Hypothetical protein SORBIDRAFT_06g018700 [Sorghum bicolor] |
| 77  | SFB00109   | LGD14b        | JK629492      | No significant similarity |
| 78  | SFB00111   | LGP2a         | JK629493      | No significant similarity |
| 79  | SFB00118   | LGP3          | JK629494      | Histone H4 [Zea mays] |
| 80  | SFB00120   | LGD2          | JK629495      | Predicted: pectinesterase inhibitor [Vitis vinifera] |
| 81  | SFB00130   | LGP3          | GH159198      | No significant similarity |
| 82  | SFB00131   | LGD4b         | JK629496      | Ubiquitin [Morus bombycis] |
| 83  | SFB00141   | LGD15         | JK629497      | No significant similarity |
| 84  | SFB00144   | LGP11b        | JK629498      | Putative DIM-like protein [Glycine max] |
| 85  | SFB00145   | LGD10b        | JK629499      | No significant similarity |
| 86  | SFB00152   | LGP3          | JK629500      | Metallothionein-like protein [Typha latifolia] |
| 87  | SFB00154   | LGD14a        | JK629501      | Ubiquitin extension protein-like protein [Elaeis guineensis] |
| 88  | SFB00157   | LGD15         | JK629502      | Histone H2B [Arabidopsis thaliana] |
| 89  | SFB00167   | LGP12c        | JK629503      | Metallothionein-like protein [Typha latifolia] |
| 90  | SFB00219   | LGD8b         | JK629504      | Ribosomal protein L35A [Elaeis guineensis] |
| 91  | SFB00241   | LGP2a         | JK629505      | Histone H4 [Arabidopsis thaliana] |
| 92  | SFB00243   | LGP12c        | JK629506      | No significant similarity |
| 93  | SFB00246   | LGD12b        | JK629507      | Histone H2A [Camellia sinensis] |

Putative IDs were deduced for the SSR-containing sequences by comparing to the non-redundant protein database of GenBank (Blastx). A threshold score of >80 was used to assign significant similarity.

Two RFLP markers were mapped.

doi:10.1371/journal.pone.0053076.t004
high yielding *tenera* was deemed appropriate for detecting the QTL for tissue culture amenity.

**Tissue Culture of the Mapping Population**

The height of oil palm makes sampling of its young leaves for culture a challenging task. The process requires skilled workers to climb the palm and harvest the very young spear leaves, which have not yet even emerged, without damaging the apical growing point. Because of the sustained damage, repeat sampling of a palm is only possible after three to five years [32]. Thus, re-sampling of palms was not possible within the time frame of the research project. The parental palms were not sampled as they were being actively used in the breeding program and it was not practical to wait for at least three years for the palms to recover. Furthermore, there was a desire to avoid risk of any permanent damage to the palms.

Most of the palms were recalcitrant to tissue culture as was to be expected from previous experience on oil palm worldwide. Significant deviation of tissue culture amenity data from normal distribution had also been frequently reported for other crops,

![Figure 3. QTLs detected for PLnCR and PordER using Multi-trait QTL analysis, GenStat 14. Upper panel shows the QTL profiles at –log10 (P-value) which resulted from interval mapping scanning. The horizontal line shows the genome-wide significant threshold determined by Li and Ji (P = 3.5). Lower panel shows the QTL effects (green square) resulting from multi-trait interactions: QTL on LGD4b was affected by PLnCR (dark blue square) and PordER (light blue square) while; QTL on LGP16b only contains effect from PordER (brown square).](image-url)

doi:10.1371/journal.pone.0053076.g003
such as red clover [7], wheat [11], rice [10], barley [12] and loblolly pine [33], and the data had to be transformed for normality. Indeed, normality may not be obtained even after transformation, such as in the case of the data on shoot differentiation rate in barley [34], callus formation in maize [35] and callus induction and somatic embryogenesis in rye [36]. As such, in these studies, the non-normal data were used for QTL analysis. In the current study, we improved the normality of CR and ER in two stages (as described in Materials and Methods) involving transformations and correction of experimental variables prior to QTL analysis.

Development of Additional SSR Markers

The SSRs were developed from both ESTs and genomic libraries of oil palm. Mining of these SSRs was previously reported by [17–20,37]. The authors (except [17]) selected some SSRs for genetic diversity studies and this study reports on their applicability to genetic mapping and QTL analysis. Although a large number of SSRs have been reported from the existing oil palm sequence collections, this number is expected to increase rapidly from the genome sequencing project being carried out for oil palm [38]. There is also no doubt that with time, a large number of single nucleotide polymorphism (SNP) markers will also become available for oil palm.

The additional co-segregating SSR markers used in this study are crucial for further saturating and integrating both parental maps. The approach taken was to focus on SSRs rather than AFLPs which are known to be of low throughput and costly. EST-derived SSRs are essentially similar to cDNA AFLP-probes as they are also from the genic regions. The approach was thus appropriate as the EST-SSRs revealed more co-segregating markers (about 38.0%) than the 24.4% obtained by using AFLPs. Previously [14] had shown the potential use of genomic-SSR markers for integrating the *tenera* and *dura* maps in this study. Genomic SSRs were also used and contributed a reasonable number of co-segregating markers - about 35.0% of the total genomic SSRs genotyped. Therefore, there is potential in using both EST- and genomic-derived SSRs for map saturation and integration as observed in this study.

Oil Palm Genetic Linkage Maps

The current maps were constructed using very stringent parameters (as described in Materials and Methods). Markers (mostly AFLPs) as reported in [13] that failed to meet the criteria were excluded from analysis. Removing them resulted in some groups reported earlier, such as group 3 in ML161, to be separated into two sub-groups now labeled as P4a and P4b. Similar changes were observed on groups 7 (now labeled as sub-groups P2a and P2b), 10 (sub-groups P5a and P5b) and 15 (sub-groups P11a and P11b). Significant changes were also observed in groups 1 and 2 of [13] which were separated into 3–4 sub-groups. However, most of the groups - 4, 5, 6, 8, 9, 11, 12, 13 and 16 - remained intact and were renamed LGP1, P10, P13, P7, P15, P12b, P16a, P14 and P16b, respectively, in line with [14]. Changes were also obvious on the ENL48 map. Although the current ENL48 and ML161 maps have more groups, they are greatly improved in accuracy of marker order.

The mapping of published SSR markers (mEgCIR) allowed comparison with a published oil palm genetic map. This, in turn, allowed labeling of linkage groups in the current map to match those by [14]. More importantly, by comparing with the 16 linkage groups reported by [14], linkage groups belonging to the same chromosome could be identified. The orders of common markers were also compared and found to be consistent, boosting confidence in the genetic maps constructed in this study. This also allowed for standardized labeling of every linkage group in both ENL48 and ML161 which also made comparison between the two parental maps much easier.

The genome size for *E. guineensis* is estimated to be 2C = 3.86 ± 0.26 pg [39] which is equivalent to 1,807.54 ± 127 Mbp (number of base pairs = mass in pg × 9.970 × 10⁶, where 1.0 pg = 978 Mbp [40]). Considering the estimates as reference, the ML161 map (1,328.6 cM) has 70.4% genome coverage and ENL48 (798 cM) 42.3%. The estimated genome coverage appears consistent with the marker density observed in the two parental maps. Gaps of > 20.0 cM were still observed between markers in the same chromosome. Additional SSR markers (and perhaps SNPs) are needed to saturate the two parental maps. This is also useful to further reduce the number of linkage groups to the basic chromosome number of 16. This would be particularly challenging for ENL48 because its genome appears more homoyzogous than that of ML161. In fact, the *dura* parent was about 28.0% less polymorphic than the *pisifera*. This is probably due to the narrow genetic background of ENL48 which is a Deli *dura*. In general, the Deli *dura* materials are known to demonstrate less diversity compared to other sources of *E. guineensis* [19]. Furthermore, in oil palm breeding programs, the maternal *dura* lines undergo both selfing and sibbing to increase homozygosity before being crossed with the paternal *pisifera* palm. As such, it is not surprising that ENL48 was more homoyzogous than the paternal palm (ML161). Therefore, a larger number of SSRs and possibly SNPs have to be screened to saturate the ENL48 map.

QTLs Associated with Callusing and Embryogenesis Rates

In this study, the numbers of QTLs detected for tissue culture response are within the range reported for rice, barley, wheat, maize, sunflower, Arabidopsis, broccoli, poplar and tomato [41]. The type and size of the mapping population are among the factors believed to influence the numbers and effects of the detected QTLs. Ideally, a cross between two palms showing extreme differences in tissue culturability would be more effective for detection of QTL related to tissue culture response. However, issues, particularly regarding the availability of palm materials, limited our options in selecting the mapping population to study. With respect to the size of the mapping population, the difficulty in tissue culturing oil palm would not have allowed for too many palms to be used. The 87 palms used in this study already tested the limits of the tissue culture laboratories involved.

The existing tissue culture laboratories in Malaysia do routinely culture oil palm. The numbers of palms and the different genotypes cultured may allow for association analysis of markers to tissue culturability. This may allow for validation of existing markers linked to the QTLs for CR and ER and/or allow detection of additional QTLs. However, the standardization of phenotype data collection and effect of the different media used by the various laboratories on CR and ER will have to be sorted out before this is possible.

It has been suggested that only a few simply inherited genes are of major importance in the genetics of embryogenesis [33]. In oil palm, research had also been carried out on gene expression during embryogenesis. In fact, some interesting genes, such as lipid transfer proteins, were found to be highly expressed in oil palm embryogenic tissues [17]. In other crops, auxin- and wound-responsive genes, such as DNA-binding proteins, calcium-modulated proteins, cell cycle-associated genes, cell wall proteins and glutathione-S-transferase, have also been associated with tissue culture [42]. Therefore, it will be worthwhile to explore some of the identified candidate genes for mapping on the current maps. It
will be interesting to see if the candidate markers can be mapped closer to the existing QTLs or can detect additional QTLs.

**Application of QTL in Improving Oil Palm Tissue Culture**

Ideally the marker-QTL should be evaluated in other independent crosses of oil palm. This has been done for barley with common QTLs associated with callus growth detected across four populations by [12]. Although it will be a challenging endeavor, the markers linked to QTLs in this study can be used to determine if they reveal the same QTLs in other oil palm populations.

Subject to confirmation of the QTLs in other mapping populations or genotypes, they could be important for selecting ortets to be cultured. Unlike expressed traits (e.g. yield and height), tissue culture amenity remains unknown until the palms are actually cloned. Furthermore, some high yielding palms have at times failed to be cultured. The availability of markers linked to tissue culturability can facilitate the cloning of such palms where, the favorable alleles can be incorporated into the progenies of these palms through marker assisted selection (MAS), and the progenies then cloned. As the markers for yield are becoming available for oil palm [43], it is possible to select palms that are not only high yielding but amenable to tissue culture as well. In fact, the large MPOB oil palm germplasm could be screened for favorable alleles for yield and tissue culture before any palms are included in the breeding program. [6] opined that the biggest advantage of a clone is the early exploitation of new genetic materials produced by introgressing useful gene from wide crosses, which would also help to broaden the genetic base of the current planting materials.

Although the production of oil palm clones has increased, there has more to do with more laboratories entering the fray than a real improvement of the tissue culture process [6]. As such, there remains the need to improve the process at least allow more of the demand to be satisfied. The markers linked to QTLs for tissue culturability may be helpful in this effort. Palms identified for cloning (based on favorable traits, like high yield or disease resistance), could first be screened with markers to find out whether they have the favorable alleles for tissue culturability. This could help to reduce the time and other resources wasted on tissue culturing recalcitrant palms.

**Supporting Information**

**Figure S1** Sampling of unopened spear leaves and the explants used for tissue culture in oil palm. Figures A & B show the skilled workers climbing the palm to cut the unopened spear leaves from the apical growing point; C: Outer layers of the leaf cabbage are removed except the petioles of frond number 0. This is followed by a longitudinal cut to disclose the internal fronds (fronds ~3 to ~7 or lower) comprising stacks of young leaflets. D. The leaflets are cut into 12 segments, each having a width of 1.5 cm and sterilized before being cultured on the modified Murashige and Skoog media [15].

**Table S1** The profiles of alleles segregating in the P2 mapping population.

**Table S2** Data obtained from the various markers tested and mapped in the P2 parental linkage maps.

**Acknowledgments**

The authors would like to thank the Director-General of MPOB for the permission to publish this paper. We would also like to thank the tissue culturists from the various oil palm agencies, namely, Dr. Hamidah Musa (and her predecessor, Dr. Zaleha Mohd Mydin) and Ms. Halina Mohd Randly of Guthrie Biotech Laboratory Sdn Bhd; Dr. Maharan Abu Bakar and Mr. Aw Khoo Teng of FELDA Agricultural Services Sdn Bhd; Dr. Lim Loon Lui of IOI Corporation Bhd; Ms. Ho Yuk Wah formerly from United Plantations Bhd; Dr. Aziah Mohd Yusoff and Ms. Haliah Khafidz of Golden Hope Plantations Bhd; Ms. Suzaini Yahya of Ebor Laboratories; and Ms. Girli Wong and Ms. Joyce Chong of Applied Agriculture Research Sdn Bhd, for their support in tissue culturing the palms. Guthrie Biotech Laboratory Sdn Bhd, Golden Hope Plantations Bhd and Ebor Laboratories are now part of Sime Darby Berhad. We would also like to extend our appreciation to Mr. Suhaime Shamsuddin, oil palm breeder at FELDA, for his assistance in maintaining the cross and sampling the palms. Part of the work leading to this paper was carried out at Biometris, Wageningen University and Research Centre, the Netherlands. We would like to thank Dr. Azhar Mohammad for his kindness in allowing us to use the ABI3100 genetic analyzer (Applied Biosystems, UK) at the Malaysian Nuclear Agency. We would also like to extend our appreciation to Mr. Andy Kwong Choong Chang for his valuable comments on this manuscript.

**Author Contributions**

Conceived and designed the experiments: NCT JJ ZI CWC SGT RS. Performed the experiments: NCT JJ. Analyzed the data: NCT JJ RS. Contributed reagents/materials/analysis tools: ZI CWC SCC SGT RS. Wrote the paper: NCT JJ SGT RS.

**References**

1. Ho YW, Tan CC, Soh AC, Wong G, Wong SP, et al. (2009) Oil palm for farmers’ prosperity and edible oil security. Proc National Conference On Oil Palm. India: 86–93.
2. Hardon JJ, Corley RHV, Lee CH (1987) Breeding and selecting the oil palm. In: Abbot AJ, Akin KK, editors. Improving vegetatively propagated crops. London: Academic Press. 63–81.
3. Corley RHV, Law HI (1997) The future for oil palm clones. Proc Int. Planters Conf. Incorp. Soc. Kuala Lumpur: 279–289.
4. Corley RHV, Stratford R (1998) Biotechnology and oil palm: opportunities and future impact. Proc Int. Oil Palm Conf. Bali: 1–19.
5. Tarmizi AH, Zamzuri I, Ooi SE, Samsul KR, Chan PL, et al. (2010) Forging ahead with clones. In: Basri MW, Choo YM, Chan KW, editors. Further development towards realization. Journal of Oil Palm Research 23: 935–952.
6. Keyes GJ, Collins GB, Taylor NL (1980) Genetic variation in tissue culture of red clover. Theor Appl Genet 50: 263–271.
7. Kushairi A, Tarmizi AH, Zamzuri I, Ong-Abdullah M, Samsul KR, et al. (2010) Production, performance and advances in oil palm tissue culture. Paper presented at the International Seminar On Advances In Oil Palm Tissue Culture. Yogyakarta.
9. Wooi KC (1995) Oil palm tissue culture: current practice and constraints. Proc The 1995 International Symposium On Recent Developments In Oil Palm Tissue Culture And Biotechnology. Bangi: 21–32.

10. Taguchi-Shiobara F, Lin SY, Tanmo K, Komatsuda T, Yano M, et al. (1997) Mapping quantitative trait loci associated with regeneration ability of seed callus in rice. *Oryza sativa* L. Theor Appl Genet 95: 829–833.

11. Ben Amer IM, Korzun V, Worland AJ, Bomer A (1997) Genetic mapping of QTLs controlling tissue-culture response on chromosome 2B of wheat (*Triticum aestivum* L.) in relation to major genes and AFLP markers. Theor Appl Genet 94: 1047–1052.

12. Mano Y, Komatsuda T (2002) Identification of QTLs controlling tissue-culture traits in barley (* Hordeum vulgare* L.). Theor Appl Genet 103: 708–715.

13. Ting NC, Cheah SC, Zamzuri I, Tan SG, Faridah QZ, et al. (2006) Statistical mapping of quantitative trait loci controlling the time to first callusing in oil palm (*Elaeis guineensis* Jacq.) tissue culture. Pertanika J Trop Agric Sci 29(1&2): 35–45.

14. Billotte N, Marsilli R, Risterucci AM, Azon B, Brotmer P, et al. (2005) Microsatellite-based high density linkage map in oil palm (*Elaeis guineensis* Jacq.). Theor Appl Genet 110: 734–765.

15. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473–497.

16. Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. FOCUS 12: 13–15.

17. Low ETL, Halimah A, Boun SH, Elayana MS, Tan CY, et al. (2008) Oil palm (*Elaeis guineensis* Jacq.) tissue culture ESTs: identifying genes associated with callogenesis and embryogenesis. BMC Plant Biology 8: 62.

18. Singh R, Noorhariza MZ, Ting NC, Rosana R, Tan SG, et al. (2008) Exploiting an oil palm EST database for the development of gene-derived SSR markers and their exploitation for assessment of genetic diversity. Biologia 63(2): 1–9.

19. Ting NC, Noorhariza MZ, Rosana R, Low ETL, Maiariza I, et al. (2010). SSR mining in oil palm EST database: application in oil palm germplasm diversity studies. Journal of Genetics 89: 135–143.

20. Noorhariza MZ, Imanizan I, Rosana R, Ting NC, Singh R (2010) Development and characterization of *Elaeis oleifera* microsatellite markers. Sains Malaysia 39(6): 909–912.

21. Boutin-Ganache I, Kapoor M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. BioTechniques 31: 24–28.

22. Cheah SC, Siri Nor Akmar A, Ooi LCL, Rahimah AR, Maria M (1993) Detection of DNA variability in the oil palm using RFLP probes. Proc The 1991 PORIM International Palm Oil Conference-Agriculture. Bangi: 144–150.

23. Chua KL (2006) Construction of AFLP and AFLP genetic linkage maps for oil palm (*Elaeis guineensis* Jacq.) using a Deli duon X Yangambi pisifera cross. Thesis.

24. Singh R, Tan SG, Panandam JM, Rahimah AR, Ooi LCL, et al. (2009) Mapping quantitative trait loci (QTLs) for fatty acid composition in an intraspecific cross of oil palm. BMC Plant Biology 9: 114.

25. Van Ooijen JW (2006) JoinMap®4.0: software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen, Netherlands.

26. Jansen J (2005) Construction of linkage maps in full-sib families of diploid outbreeding species by minimizing the number of recombinations in hidden inheritance vectors. Genetics 170(4): 2013–2025.

27. Grattapaglia D, Sederoff RR (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using commercial-testcross mapping strategy and RAPD markers. Genetics 137: 1121–1137.

28. Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J. Hered 93(3): 77–78.

29. VSN International (2011) GenStat for windows 14th edition. VSN International, Hemel Hempstead, UK. Web page: GenStat.co.uk.

30. Li J, Ji L (2005) Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. Heredity 95: 221–227.

31. Chua CW, Sulaiman S (1996) Flemia oil palm planting materials. Proc Oil Palm Planting Materials For Local And Overseas Ventures. Bangi: 71–90.

32. Syed Ahwa SSR, Roowi SH, Aw KT, Othman AZ (2010) Progress of oil palm tissue culture in Felda and its challenges. Proc Advances In Oil Palm Tissue Culture. Yogyakarta: 45–52.

33. MacKay JJ, Becwar MR, Park Y-S, Corderro JP, Pullman GS (2006) Genetic control of somatic embryogenesis initiation in lobolly pine and implications for breeding. Tree Genetics & Genomes 2: 1–9.

34. Komatsuda T, Asumak T, Oka S (1993) Genetic mapping of a quantitative trait locus (QTL) that enhances the shoot differentiation rate in *Hordeum vulgare* L. Theor Appl Genet 86: 713–720.

35. Krakowsky MD, Lee M, Garay I, Woodman-Clikeman W, Long MJ, et al. (2006) Quantitative trait loci for callus initiation and totipotency in maize (*Zea mays* L.). Theor Appl Genet 113: 821–830.

36. Bobbok H, Grusczynska A, Hromada-Judycka A, Rakocy-Trojanowska M (2007) The identification of QTLs associated with the in vitro response of *Secale cereale* L. Cellular & Molecular Biology Letters 12: 523–535.

37. Singh R, Maria M, Low ETL, Paul-Kei GL, Chan PL, et al. (2011) Oil palm genomics: a foundation for improved agricultural productivity. In: Basri MW, Choo YM, Chan KW, editors. Further advances in oil palm research (2000–2010). Bangi: Malaysian Palm Oil Board. 202–251.

38. Mohd Basri W (2009) Sequencing the oil palm genome: the beginning. Paper presented at the International Oil Palm Congress (PIPOC). Kuala Lumpur.

39. Madon M, Phocon LQ, Clyde MM, Mohd Din A (2008) Application of flow cytometry for estimation of nuclear DNA content in *Elaeis*. J. Oil Palm Research 20: 447–452.

40. Doelez J, Bartos H, Voqlmavny J, Greilhuber J (2003) Nuclear DNA content and genome size of trout and human. Cytometry 51A(2): 127–128.

41. Bobbok H, Rakocy-Trojanowska M (2006) Genetic mapping of QTLs for tissue-culture response in plants. *Euphytica* 149: 73–83.

42. Ong-Abdullah M, Ooi SE (2006) Biomarkers: finding a niche in oil palm tissue culture. Part 1- laying the foundation. Oil Palm Bulletin 53: 36–48.

43. Billotte N, Jourjon MF, Marseillac N, Berger A, Flori A, et al. (2010) QTL for Tissue Culturability in Oil Palm. Thoir Appl Genet 120: 1673–1677.