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Transcriptome analysis of normal and mantled developing oil palm flower and fruit

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ABSTRACT

Elaeis guineensis (oil palm) accounts for a large and increasing proportion of the world's cooking oil production. Cloning via somatic embryogenesis results in a somaclonal variant known as mantled which produce fruit with little to no oil yield. The mantled phenotype is believed to be epigenetic in nature. We performed RNA-Seq on developing flower and fruit samples of normal and mantled oil palm to characterize their transcriptomes. We present expression data for all transcripts in normal and mantled flower and fruit samples. Many genes are differentially expressed, including several from pathways that may be the cause of the mantled phenotype if disrupted, such as genes involved in primary hormone responses, DNA replication and repair, chromatin remodeling and a gene involved in RNA mediated DNA methylation. In addition, the gene expression data for developing flower and fruit will serve as a valuable resource for oil palm genetics and genomic studies.

1. Introduction

Elaeis guineensis (oil palm) is a member of the Arecaceae family that accounts for a large and increasing proportion of the world's cooking oil production. In addition to this it is gaining interest for use in bio diesel production. Oil palm is monoecious and produces a mature inflorescence roughly every 1–2 months after reaching sexual maturity. Each inflorescence is sexually determined early during development and commercial strains have been selected to produce a large proportion of female inflorescences. The proportion of male to female inflorescences can be affected by environmental factors such as water availability and defoliation, with drought or leaf removal resulting in more male than female [1]. The male inflorescence consists of many rachillae each bearing 400–1500 staminate flowers and the female inflorescence consists of many rachillae with 5–30 floral triads consisting of a pistillate flower flanked by two abortive staminate flowers [1]. The inflorescence develops enclosed by a fibrous, lignified prophyll and peduncular bract which dies off to reveal the developed inflorescence [1].

Current oil palm crops are often seed derived from high yielding parents, but can result in offspring with less favorable yields. To overcome this problem, a lot of attention has been given to developing a somatic embryogenesis protocol to allow crops to be generated from the highest producing parental plant(s). Somatic embryogenesis is performed by harvesting cells from a donor plant and growing those cells on media. The cells undergo reprogramming of gene expression and return to a totipotent state where they either replicate in an unorganized manner producing a callus or in a polarized fashion leading to somatic embryogenesis. The type of growth can be controlled by adding growth hormones to the media with auxin being the main hormone used [2,3]. High auxin levels promote callus growth while removing or reducing the level of auxins induces the callus to form plantlets which can then be planted in the field. There are two main forms of callus, nodular compact callus and fast growing callus. Fast-growing callus is associated with a high auxin to cytokinin ratio [4], and is often associated with the addition of the synthetic auxin 2,4-dichlorophenoxyacetic acid to the media [2]. Fast-growing callus can also spontaneously occur from nodular compact callus in the absence of hormones, but at a lower rate [2]. Nodular compact callus typically has higher embryogenic capability than fast-growing callus but with the disadvantage of a slower growth rate.

The primary advantage of somatic embryogenesis is the production of genetically identical plants that will have the same phenotype as the parent plant. However, in many different species the process results in abnormal or variant plantlets being generated, these variants are termed somaclonal variants. Somaclonal variants result from a variety of causes ranging from gross chromosomal rearrangements to single base variants to changes in methylation patterns [5]. A study where

Abbreviations: DE, differentially expressed; GO, gene ontology; qPCR, quantitative PCR; RdDM, RNA-directed DNA methylation pathway.

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somatic embryogenesis of oil palm was carried out for 20 years without the use of growth hormones shows that an increasing number of defects arise with increasing culture age [2]. A somaclonal variant that affects oil palm flower development, known as mantled, is observed in oil palm derived from somatic embryogenesis in approximately 5% of plants grown from nodular compact callus and higher in plants from fast-growing callus [6–8]. The mantled phenotype is only observed in plants derived from somatic embryogenesis. Currently there is no way to detect a mantled oil palm until it reaches sexual maturity because the phenotype appears normal, however, once flowering starts the phenotype becomes apparent with the production of inflorescences where the staminodes of the female flower and the stamens of the male flower are transformed into carpel-like structures [1,8]. The extent of this transformation is quite variable between different plants, with a mild phenotype resulting in almost normal looking fruit ranging to an extreme phenotype where no fruit body is produced at all. The mantled phenotype is not specific to any genotype, but occurs at slightly different frequencies between different genotypes and between somatic embryogenesis culture conditions [9].

Genome-wide hypomethylation has been found in oil palm with the mantled phenotype [10] and confirmed multiple times [11–13]. The current theory is that the phenotype is epigenetic in nature. Mantled oil palm has also been observed to revert back to the normal phenotype in approximately half of severely affected and all mildly affected mantled plants by around nine years of age consistent with an epigenetic cause [6]. No gross genetic abnormalities have been found in mantled oil palm or between clonal offspring and the mother oil palm [14,15]. In addition, several groups have looked for methylation sensitive amplification polymorphisms and found many such loci at which mantled oil palm exhibit a reduced level of methylation [11–13]. However, another study found a mixture of reduced methylation at some loci and increased methylation at others compared to normal oil palm suggesting that the phenotype is a deregulation of methylation patterns [16]. Hypomethylation is associated with developmental disorders in flowering time [5] and structure in plants with mutations in methylation pathway genes, suggesting that the observed methylation changes in mantled oil palm could be causative. Indeed, mantled oil palm from fast growing callus show a reduced level of methylation compared to mantled plants from nodular compact callus cells consistent with methylation changes being causative [10].

Several candidate genes have been investigated in mantled oil palm, including DNA methyltransferases and genes from the ABC flower development pathway. Three primary DNA methyltransferases have been isolated from oil palm and *DNA (cytosine-5)-methyltransferase 1* was found to be up-regulated in mantled developing inflorescence [17]. Expression analysis of oil palm MADS box genes from the ABC flower development pathway revealed differential expressions of *EgAGL2-1*, *EgDEF1*, *EgGLO2*, and *EgAG2* and showed that the mantled phenotype most closely resembles mutants of B function genes [18]. One hypothesis for what causes the mantled phenotype is an altered response to one or more hormones based on observed differential expression in auxin response genes [19]. In addition, there is a growing body of evidence that plants respond to hormones through chromatin remodeling [20]. Each of these proposed mechanisms would be expected to change the expression pattern of a number of genes responsible for early flower and fruit development.

A promising method that has recently become mainstream is high throughput RNA-sequencing to identify differential expression [21]. In organisms with a published genome, sequence reads are mapped against the genome and gene expression is given as a count of the number of reads that map to each exon [22]. Differential expression in organisms without a genome is done by first generating a *de-novo* assembly and then calculating expression based on the number of reads that map back to the assembled sequences. This makes RNA-sequencing desirable for organisms that do not have an available reference genome, such as the oil palm, because the data can also be used to generate a transcriptome database. The process can be likened to large scale qPCR with the added benefit of generating a transcriptome library [21].

With the aim of characterizing the expression profile of late developing female flower, we have sequenced the transcriptome of flowers from two mantled and two normal plants. We have combined this sequence data with a previously sequenced transcriptome of fruit samples from the same plants [23]. The resulting transcriptome assembly represents both sample types and gives insight into expression patterns between normal and mantled flower and fruit.

2. Results and discussion

2.1. RNA sequencing and sequence annotation

Total sequence output for the normal plants was 195,315 reads totaling 60.1 Mb and 213,340 reads totaling 71.6 Mb and for the mantled plants was 197,556 reads totaling 62.5 Mb and 212,915 reads totaling 71.6 Mb. The samples were assembled using Newbler v2.6 and produced 16,986 contigs that formed 13,788 isotigs (representative of mRNAs) from 10,659 isogroups (groups of isotigs produced from a subset of contigs and representative of genes or gene families). This data was also assembled with data from previously sequenced fruit samples [23] producing 26,478 contigs that formed 21,179 isotigs from 16,045 isogroups (Supplementary file 1). The N50 contig size in the combined assembly was 983 bp, larger than the N50 contig size of 874 bp when only the flower samples were used, suggesting an improvement in assembly quality [24]. The large number of additional isotigs likely reflects the difference in expression patterns between developing flowers and maturing fruit.

We compared the assembly of the developing flower and the previously published fruit assembly [23] to the larger combined assembly. The assembly from the developing flower samples had 98% of sequences that matched to sequences in the combined assembly and the fruit-only assembly had 96% similarity. A blastx of the unmatched sequences reveals that approximately a third from both assemblies does not match any sequences in the NCBI non redundant protein database and the rest receives an annotation that is also present in the combined assembly.

The isotigs from the combined assembly were annotated using Blast2GO against the plant non redundant database which assigned a sequence description to 88.8% of the sequences (Fig. 1). An additional blast against the TAIR Arabidopsis database was also performed because Arabidopsis has the highest amount of genetic information among the plant species and thus can give the most useful information about potential gene function. There were several instances where isotigs from multiple isogroups matched the same Arabidopsis gene suggesting additional diversity of gene function in oil palm.

We used the sequences from the combined assembly to perform a blastx against the plant transcription factor database and identified 1050 isotigs from 761 isogroups that matched a transcription factor with a stringent E value threshold of 1E-60 (Supplementary file 2).

We looked for MADS box genes because these have previously been implicated as playing a role in the mantled phenotype. There were 12 genes that completely matched previously identified MADS box genes from oil palm [18,25]. In addition to these genes there were 12 genes not previously described in oil palm. We also found an isotig somewhat similar to Arabidopsis AGL26 (which has a MADS domain and a methylase domain) containing the methylase domain but lacking the MADS box domain (Supplementary file 2).

In addition we identified 195 isotigs from 156 isogroups with methyltransferase activity (Supplementary file 2). These genes are of interest because the mantled phenotype occurs with large changes in methylation patterns and is currently considered to be the result of

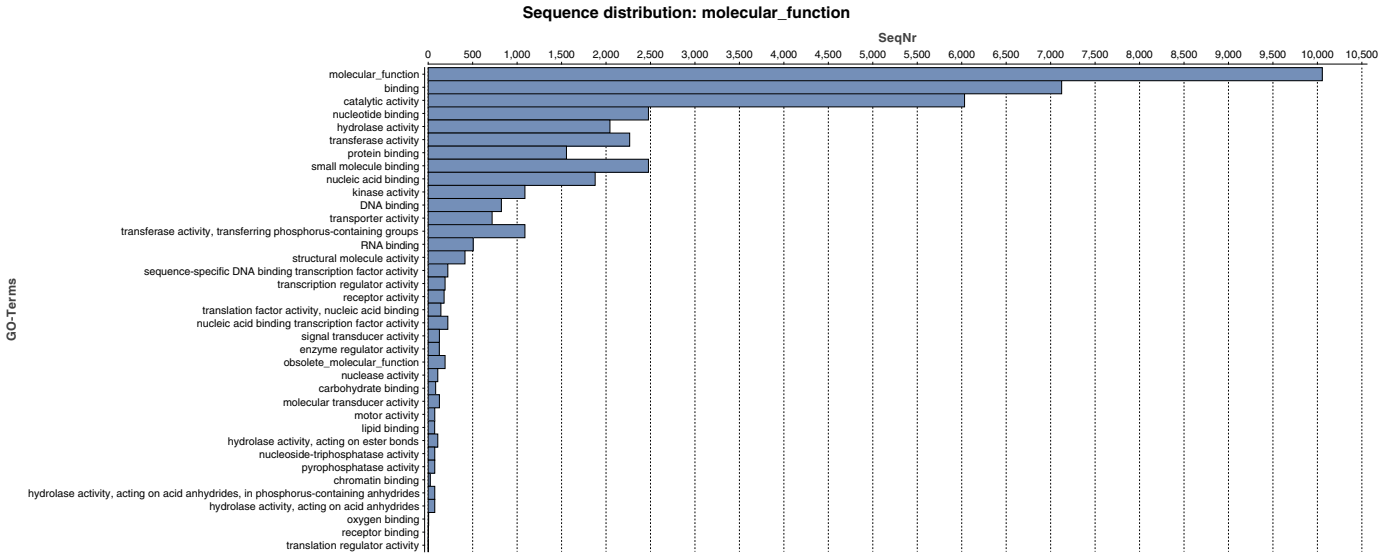

Fig. 1. GO term distribution of transcriptome assembled from oil palm flower and fruit samples.

methylation polymorphisms affecting the expression of one or more key genes.

2.2. Differential expression

Expression is represented as normalized read count per isotig (Supplementary files 3 and 4) and was checked by qPCR for 12 genes that showed consistent relative expression values (Fig. S1). Both the isotigs and isogroups of the developing flower and fruit samples from the combined assembly were tested for differential expression using the DESeq (v1.4.1; CRAN: DESeq) and NOISeq (v1.1.0; CRAN: NOISeq) packages in R. The developing flower data and the fruit data represent two common RNA-Seq data types, with and without biological replicates (Supplementary file 3). For this reason we tested how well the programs function with each data type. To do this we summed the read counts for each biological replicate to produce a data set with a higher read count but no information on biological variation for each gene.

In the developing flower samples using biological replicates NOISeq identified 12 isotigs from 12 isogroups as differentially expressed between mantled and normal samples (Supplementary file 3). Using the summed flower sample read counts NOISeq identified 4568 isotigs and 955 isogroups as differentially expressed (DE) (Supplementary file 3). Interestingly, there were still over 4000 isotigs DE when we took the average read count as opposed to the sum indicating that the increased read number has little effect on the number of DE sequences. DESeq identified 467 isotigs from 438 isogroups as DE using biological replicates and 673 isotigs from 604 isogroups when the biological replicates were combined (Supplementary file 3). To account for the differences in number of DE genes between the two data set types we looked at the biological variation within sample groups and found that the variation within groups often exceeded the variation between groups, this vital information was lost when biological replicates were not used. In many cases, the large difference in expression between groups was due to a single outlier, subsequently we called these genes as not DE. There was no difference in the total variation observed in the mantled flower samples compared to the normal flower samples. In addition, we performed a t-test on each gene which identified 325 isotigs from 299 isogroups as DE (Supplementary file 3). For this dataset it appears that that DESeq performs better than NOISeq since DESeq gave a number of DE genes closest to the t-test results and a t-test has a low false positive rate when sample sizes are small [26]. Out of these tests 159 isotigs were DE according to both DESeq and t-test, 308 isotigs according to DESeq only and 166 isotigs according to t-test only (Supplementary file 3).

This number of DE genes is somewhat consistent with the number found by Beule et al. using a suppression subtractive hybridization approach, which identified 1350 DE sequences [27]. The lower number presented in our dataset is likely because we used biological replicates allowing for outliers to be removed. Comparison of the DE sequences from Beule et al. [27] to our list identified 963 isotigs that matched a sequence from Beule et al. [27]. From these sequences there were 23 that were DE, but 12 of them were DE in the opposite direction to that identified by Beule et al. [27] (Supplementary file 5). The lack of overlap in DE genes is not overly surprising considering that Beule et al. [27] used male inflorescence samples where we used female samples.

The DE isotigs had a GO term distribution similar to the entire isotig set (Fig. 2). GO term enrichment analysis of the DE genes identified some over-represented GO terms including 'regulation of gene expression' and 'regulation of gene expression, epigenetic', however none were significant after multiple testing correction. Similarly, we saw no direct interactions among the DE genes from the Arabidopsis interactions viewer. This shows that the DE genes are from many pathways and suggests that the mantled phenotype is a large non-specific disruption to gene expression. Inspection of the DE genes yield several genes with a function relevant to the mantled phenotype including many genes involved in the response to auxin (Supplementary file 3) and many involved in development. Most notably of these is the up-regulation of *TIC* (isotig01111) which is a member of the circadian rhythm pathway that regulates flowering time [28] and up-regulation of *CNOT1* (isotig16091), which is highly conserved in both plants and animals and functions in human and mouse to maintain stem cells in an undifferentiated state [29].

In addition we see DE genes involved in DNA repair and replication, but perhaps the most interesting DE genes are the ones involved in chromatin remodeling. We see two chromatin remodeling genes that are up-regulated. They are *SYD* (isotig16132) which is a SWI2/SNF2-like protein and is a co-activator of floral homeotic genes. *SYD* is a master regulator of several developmental processes including the maintenance of the stem cell pool [30], suggesting that up-regulation of this gene could be blocking differentiation of cells into floral organs. The other gene is *CHR1* (isotig19129), also up-regulated, which is involved in DNA

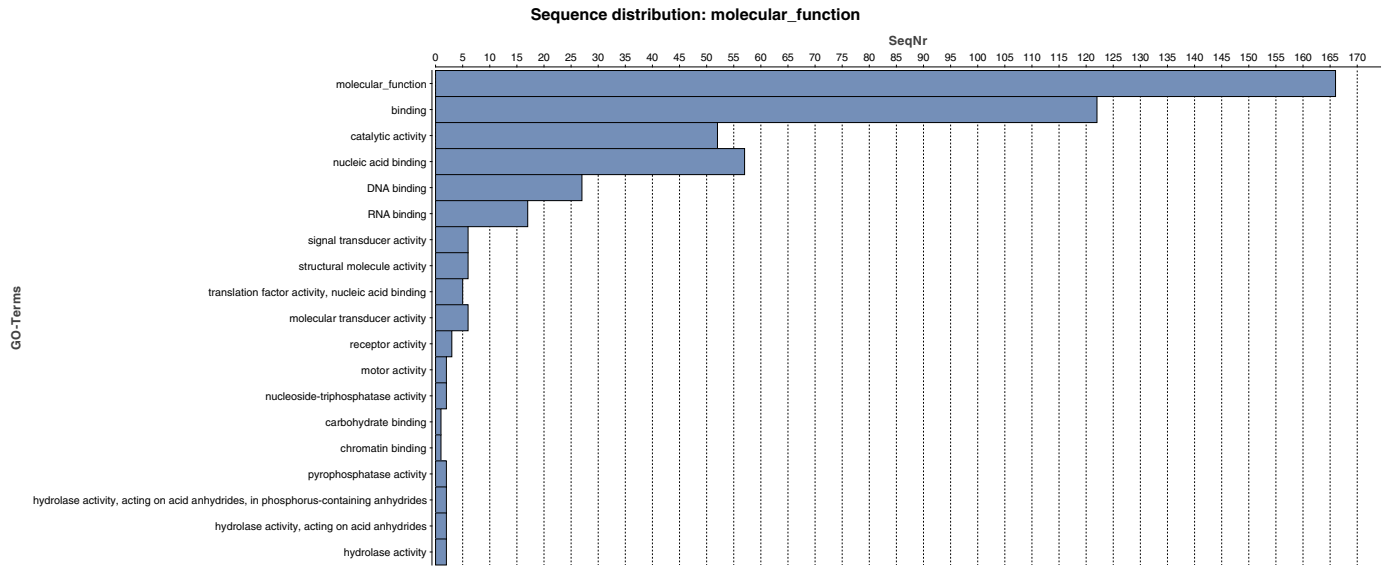

Fig. 2. GO term distribution of transcripts differentially expressed between normal and mantled developing flower samples.

methylation and chromatin remodeling [31]. Up-regulation of this gene may explain the observations of some loci having increased methylation despite global hypomethylation [16].

We see down-regulation of a histone acetyletransferase *HAC1* (isotig07668 and isotig07669), a histone gene *H2AZ* (isotig17428) and a histone methyl transferase *PRMT6* (isotig15007). Mutations to *HAC1* result in late flowering in Arabidopsis [32]. RNAi induced knock-down of *H2AZ* also results in an early flowering phenotype [33]. In addition, a study that looked at gene expression in a histone methyltransferase mutant of Arabidopsis, resulting in pleiotropic defects including floral abnormalities, found that 1910 genes were differentially expressed in inflorescence, including many genes involved in flower development [34], showing that disruption to histone function can result in floral abnormalities.

Changes in methylation patterns have been observed so often in the mantled phenotype that it is considered a part of the phenotype. Down regulation of an isotig (isotig15872) similar to Arabidopsis *KTF1* is observed in mantled flower. This gene is expressed in inflorescence, colocalizes with *AGO4* and is involved in the RNA-directed DNA methylation pathway (RdDM) [35]. Loss-of-function Arabidopsis mutants for *KTF1* show a reduced level of DNA methylation and reduced silencing of RdDM targeted loci [35]. Additionally *KTF1* is required for repressive histone modifications [36]. This suggests that down regulation of this gene may be responsible for disrupted methylation patterns.

2.3. Fruit samples

We looked at the overlap of expression in each sample (Fig. 3A). Common to all sample types were 11,940 isotigs from 8525 isogroups, and 896 isotigs from 821 isogroups were found in fruit samples and not in flower samples and 2290 isotigs from 2015 isogroups were found in flower samples but not in fruit. The isotigs expressed only in flower were enriched for functions consistent

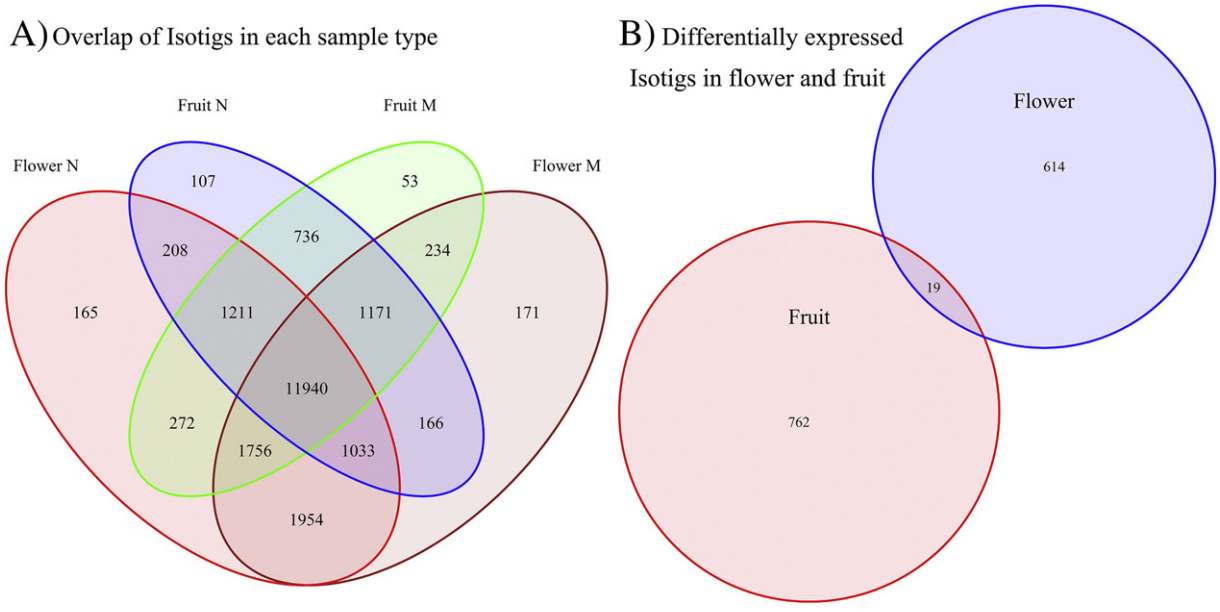

Fig. 3. A. Overlap of isotigs expressed in each sample type. B. Overlap of differentially expressed isotigs in flower and fruit samples.

with a developing organ (Fig. 4). Isotigs expressed only in fruit were enriched for death and cell death function from Blast2GO, but when the Arabidopsis matches were used enrichment was observed for carbohydrate transport. Over representation of the functions shown in Fig. 2 and those listed for fruit makes sense since the flowers were undergoing significant development and the fruit samples were at roughly the age when fatty acids begin to build up [37].

For the 90 day fruit NOISeq identified 3855 isotigs from 3033 isogroups as differentially expressed and DESeq identified 781 isotigs from 636 isogroups as differentially expressed (Supplementary file 4). The results from the fruit samples are likely to contain many false positives based on the results from combining the flower samples. One possible way to extract true positive results is to compare to the flower samples, if genes are up or down regulated in both age groups then they are more likely to represent true positive results. 19 of the isotigs from 17 isogroups were differentially expressed in both the developing flowers and fruit (Fig. 3B). 3 isotigs from 3 isogroups were down regulated in both, 9 isotigs from 7 isogroups were up-regulated in both, 4 isotigs from 4 isogroups were down regulated in flowers but up-regulated in fruit and 3 isotigs from 3 isogroups were up-regulated in flowers but down regulated in fruit.

One interesting gene (isotig10881) that is up-regulated in both flower and fruit samples is a gene that has previously been called EgNAC1 (GenBank: DQ267440.1) and is most similar to Arabidopsis ATAF2. ATAF2 is a transcription factor that is induced by dehydration, salicylic acid and jasmonic acid, it shows highest expression in root and leaves and lowest expression in flower buds [38]. What makes this gene interesting is that it is up-regulated in callus cells that are embryogenic compared to callus cells that are non-embryogenic and has been identified as a potential marker gene for successful somatic embryogenesis [39]. This suggests that this gene may represent a link between somatic embryogenesis and the mantled phenotype.

One of the isotigs down regulated in both flower and fruit was a gene similar to Arabidopsis *AT1G21780* (isotig05542) which is involved in flower morphogenesis and floral organ abscission. This gene contains similarity to human SPOP which binds to CUL3 and is involved in transcription, chromatin remodeling and apoptosis repression [40]. While AT1G21780 and SPOP are quite different, they both bind to CUL3 and both have the BTB domain suggesting functional similarity. Interestingly, overexpression of a *BTB/POZ* domain containing gene has been linked to human tumor progression [41], perhaps furthering the analogy between cancer and the mantled phenotype made previously [17].

3. Conclusion

We have shown that the mantled phenotype involves a complex and seemingly unrelated set of genes being differentially expressed. We have confirmed previous suggestions that the mantled phenotype is more complex than interruption to a single pathway and have also confirmed previous findings of no differential expression to any of the MADS box genes. We have found a large number of genes differentially expressed between normal and mantled samples. Among these genes are several good candidates for explaining the phenotype including *KTF1*, the chromatin remodeling genes and *AT1G21780*. The most interesting find is differential expression in chromatin remodeling genes and histone methylation genes consistent with the hypothesis that somatic embryogenesis is disrupting the methylation pathway in a non-specific manner. Furthermore, non-specific disruption to the methylation pathway provides a mechanism for why different studies have found different patterns of methylation and different patterns of gene expression. This finding also suggests that the mantled phenotype is not a single phenotype, but rather a collection of phenotypes that include disruptions to other organs such as leaf and root but undergo selection at the plantlet stage to exclude any disruptions resulting in an apparent phenotype. In addition to this we have shown that the potential marker for embryogenic callus, *EgNAC1*, is also a potential marker for the mantled phenotype supporting the hypothesis that the mantled phenotype is caused by the somatic embryogenesis process.

4. Methods

4.1. Samples

Inflorescence and fruit samples were collected from two normal plants and two mantled plants, produced via somatic embryogenesis from the same clonal background, from the same field in Krabi, Thailand as previously described in [23]. RNA was extracted from individual flowers of the samples of prophyll and peduncular bract enclosed inflorescence (leaf stage +15 to +17 as described by Adam et al. [1]), and sequenced using the method previously described in [23].

4.2. Transcriptome assembly and annotation

We produced two transcriptome assemblies using Newbler v2.6 (Roche), one from the prophyll and peduncular bract enclosed

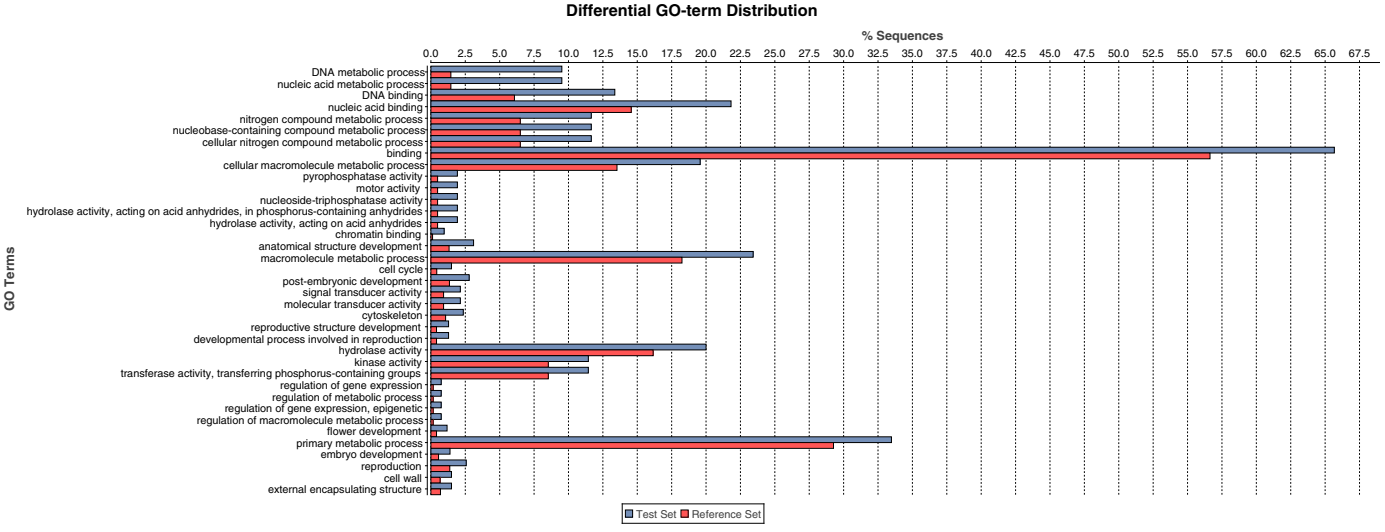

Fig. 4. GO term enrichment for isotigs unique to flower. Test set consists of the isotigs found only in flower, the reference set contains all of the isotigs.

inflorescence flower samples and a second assembly in combination with sequence data previously obtained from oil palm fruit aged 90 days after pollination, as described in [23]. The assembly from the flower samples was compared against the combined assembly. The combined assembly was annotated using Blast2GO against the plant non-redundant database and a blastx against the TAIR Arabidopsis protein database using a stringent e-value cut-off of 1E-30. Isotigs that matched genes of interest that had other isotigs with the same or a similar annotation were checked by blastx and sequence alignment to see if they were alleles or portions of the same gene.

4.3. Differential expression

Differential expression was calculated from the raw read counts using the DESeq [42] and NOISeq [43] packages in R and by t-test. The t-test was applied after first normalizing via the method utilized by DESeq, all read counts presented are the normalized reads resulting from this normalization. Read counts per contig were summed from the output file from the assembly program Newbler, which lists the alignment of the 3′ and 5′ ends of each read. Thus each read was counted twice, which takes advantage of the long read lengths obtained by the 454 platform. Read counts per isotig were calculated by summing the reads from constituent contigs. To this output we applied selection criteria: a fold change of at least two and a minimum combined read count of 10 for either the normal or mantled samples. In addition, genes that had higher variation within biological replicates than between phenotypes were listed as not DE. A blastx was performed for all DE isotigs discussed in the results (Supplementary file 6).

Cases of false positive differentially expressed genes were controlled for by performing a blast and sequence alignment of any differentially expressed sequence with all sequences that received the same annotation or Arabidopsis match.

4.4. GO term enrichment

GO term enrichment was tested both by using the Blast2GO enrichment test (which uses a Fisher's exact test with Benjamini–Hochberg correction for multiple testing) and by using the AmiGO website with the closest Arabidopsis gene match for each isotig [44]. The Arabidopsis match for each differentially expressed isotig was also used as input for the Arabidopsis interactions viewer.

4.5. qPCR

Primers (Supplementary file 7) were designed using Primer3 [45]. PCR products were confirmed by Sanger sequencing. qPCR was performed for each sample in triplicate for each of the six genes on a Corbett Rotor Gene 3000 (Corbett Life Science) using Faststart SYBR green master mix according to manufacturer instructions (Roche Diagnostics). Relative concentrations of each gene were calculated using the delta-delta-ct method in the flower and fruit samples and relative expression levels were consistent with those identified in the RNA-Seq data.

All raw data used in the transcriptome assembly has been deposited in the NCBI Short Read Archive (accessions: SRR618511, SRR618512, SRR618513, SRR618514, SRR618515, SRR618516). Supplementary data to this article can be found online at doi: http://dx.doi.org/10.1016/j.ygeno.2013.02.012.

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