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Analysis of gene expression in *Kalanchoe daigremontiana* leaves during plantlet formation under drought stress

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Abstract

Background: *Kalanchoe daigremontiana* is an attractive model system for the study of the molecular mechanisms of somatic embryogenesis and organogenesis competence due to its formation of plantlets with adventitious roots on the leaf margins that are derived from somatic embryos. The suppression subtractive hybridization technique was used to investigate gene expression during asexual reproduction. Leaves from plants subjected to drought stress provided the source of 'Tester' DNA, and leaves from plants grown under normal conditions provided the 'Driver' DNA for subtractive hybridization.
Results: A total of 481 high quality ESTs were generated, which clustered into 390 unigenes. Of these unigenes, 132 grouped into 12 functional categories, suggesting that asexual reproduction is a complicated process involving a large number of genes. The expression characteristics of selected genes from the SSH library were determined by real-time PCR and were classified into five groups, suggesting that gene expression patterns during asexual reproduction are complex. Up-regulation of S-adenosylhomocysteine hydrolase suggested that a decrease in cytokinin levels promotes the initiation of plantlet formation. Many other genes, such as inorganic pyrophosphatase and glutamate decarboxylase, play important roles in gene regulation during asexual reproduction.
Conclusion: Our results provide a framework and unified platform on which future research on asexual reproduction in *K*. *daigremontiana* can be based. This represents the first genome-wide study of asexual reproduction in *K. daigremontiana*.

Keywords: bioinformatics analysis; real time PCR; suppression subtractive hybridization (SSH).

INTRODUCTION

Kalanchoe daigremontiana originated in Madagascar and became fashionable during the last century, with numerous horticultural forms, cultivars, and hybrids brought under cultivation (Garces and Sinha, 2009b). *K*. *daigremontiana* remains an important horticultural plant that produces plantlets at the edges of the thick and fleshy leaves. In China, *K*. *daigremontiana* is a significant Chinese herbal medicine that has been used for hundreds of years both orally and externally to treat conditions such as vomiting blood (Wu, 1848), scalding (Zhao, 1765), and cough with lung-heat (Nanning Traditional Chinese Medicine Institute, 1960). Recently, *K*. *daigremontiana* has become a source of new compounds for the treatment of tumors and inflammatory and allergic diseases (Supratman et al. 2001; Maharani et al. 2008). Research on asexual reproduction in *K*. *daigremontiana* has become a topic of increasing interest in recent years.

Morphological studies have documented that plantlets develop from the row of lamina mother-cells located at the bottoms of parent leaf notches, and are about 2.5 to 3.0 mm in length. In addition, the swollen base on the lower plantlet serves as a storage organ for water and nutrients and produces roots (Johnson, 1934). Upon the formation of roots and six leaves, a plantlet detaches and grows as a new, separate plant. Anatomical studies demonstrated that plantlets proceed through globular, heart-shaped, and torpedo-shaped developmental stages, followed by the development of starch-containing cotyledons with a differentiated vascular system (Batygina et al. 1996) indicating that plantlet formation shares many features with embryo development. The absence of a main root apical meristem and the formation of an adventitious root system suggest that plantlet formation is similar to shoot formation (Garces et al. 2007). The *SHOOT MERISTEMLESS (STM)* gene, which is expressed during organogenesis and is required for shoot apical meristem (SAM) formation, and the *LEAFY COTYLEDON1 (LEC1)* gene, which is expressed during embryogenesis, are both expressed during plantlet development, indicating that plantlet development proceeds through both organogenesis- and embryogenesis-like stages (Garces et al. 2007). *K. daigremontiana* is an attractive model system for the study of the molecular mechanisms of somatic embryogenesis and organogenesis competence because of its ability to form embryos and to acquire organogenic competence, and because *Kalanchoe* is the genus most closely related to *Arabidopsis thaliana* (Garces and Sinha, 2009b).

In this paper, we used suppression subtractive hybridization (SSH) to provide insights into plantlet formation, which is part of the unique asexual reproduction of *K. daigremontiana*. SSH is a well-known technique for the isolation of differentially expressed genes. Attempts have been made to identify embryogenesis-related genes in cotton (Zeng et al. 2006) and organogenesis-related genes in tobacco by SSH (Shary and Mukherjee, 2004). To our knowledge, gene expression involved in asexual reproduction in *K. daigremontiana* has not been studied on a genome-wide basis. In this experiment, we used drought stress to deal with plant to get coincident plantlet material. Therefore, the present work was carried out with the aim of analysis gene expression of asexual reproduction under drought stress in *K. daigremontiana* using PCR-based SSH technology.

MATERIALS AND METHODS

Plant materials and growth condition

Plantlets from the same individual 'Mother of Thousands' *Kalanchoe daigremontiana* (*K. daigremontiana*) plant were cultivated in a mixture of peat moss, vermiculite, and perlite (4:2:1, v/v) in pots 150 mm tall and 150 mm in diameter at 30 ± 3ºC with 16 hrs/8hrs 2000Lx light. After plantlets reached 30 cm in height, they were subjected to drought stress by withholding irrigation. Control plants received 300-mL water daily. Plantlets were present on the leaves of parent plants after ~20 days under drought treatment. Plantlets were collected from the leaves of the drought-treated parent plants and control plants separately each day thereafter. All plant samples were immediately frozen in liquid nitrogen and stored at -80ºC as required.

RNA extraction

Total RNA was isolated from the leaves of both the control and drought-treated plants and processed simultaneously for mRNA purification and cDNA preparation. RNA was extracted according to Garces and Sinha (2009a) with some modifications. Briefly, 200 mg of *K. daigremontiana* leaf were ground into powder in liquid nitrogen in a chilled mortar and pestle. 'Tester' represented leaves from plants subjected to drought stress including leaves with plantlets, and only leaves from plants growing under normal conditions provided the 'Driver' RNA. The powder was transferred into a pre-chilled 1.5-mL centrifuge tube. Pre-heated extraction ZT buffer (1 mL) was added to the centrifuge tube and the remaining steps of the published protocol were followed. RNA was resuspended in 10-µL RNAse-free H_2O.

Isolation of mRNA and cDNA preparation

Messenger RNA (mRNA) was isolated from total RNA using an Oligotex™-dt30<super> mRNA purification kit (Takara, Japan). Double-stranded cDNAs was prepared by reverse transcription of 300 ng of the purified mRNA following the steps outlined in the cDNAs preparation kit (SMARTer PCR cDNA Synthesis Kit, Clontech, Palo Alto, USA).

SSH library construction (PCR-selected cDNA subtraction)

Forward and reverse SSH libraries were constructed to enrich for genes that are up-regulated and down-regulated during asexual reproduction. Prepared double stranded cDNAs of 'Tester' and 'Driver' were digested with the enzyme *Rsa*I for 1.5 hr and subsequently divided into four equal parts to add different adaptors respectively (adaptor 1 and adaptor 2R). The ligation of adaptor was carried out overnight at 16ºC. The cDNAs with adaptors were subjected to two rounds of hybridization and PCR following the manufacturer's protocol for the PCR-select™ cDNA subtraction kit (Clontech, Palo Alto, USA). PCR products were ligated into PMD 19-T vector (Takara, Japan) and transformed into *Escherichia coli* Top 10 (Tiangen, China). After blue/white colour screening, positive white clones were picked and cultured in liquid LB medium, and then was amplified using M13 forward and reverse primers to identify the length of inserts. Approximately 250 colonies which were longer than 700 bp from both the forward and the reverse SSH libraries were obtained.

Sequencing and bioinformatics analysis of the cloned ESTs

The plasmid inserts were amplified by PCR using the MR47 and MR48 primer pair for sequencing. Only the plasmids with PCR products greater than 700 bp in length were sequenced by the 5' single-pass method (Huada gene center: BGI, China). The original sequences were analyzed using Cross_match (Jeffries et al. 2007; Wheeler et al. 2008) software to remove vector sequences. PolyA, repeat, and adaptor sequences were also removed to produce clean sequences. Expressed sequence tags (ESTs) were assembled using the Phrap software (Jeffries et al. 2007; Parida et al. 2009) and were designated as unigenes. The unigenes were divided into singletons and contigs and were analyzed for homology using BLAST at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and then grouped into functional categories using the Cluster of Orthologous Groups of Proteins (COG) database (ftp://ftp.ncbi.nih.gov/pub/COG/). Unigenes that did not have a homology match were analyzed using the InterProScan software (Hunter et al. 2009; Ye et al. 2013) to identify homologous protein structural domains for the purpose of determining potential functions of novel genes.

Expression validation by real-time PCR (qRT-PCR)

PCR primers (Table 1) were designed with primer premier 5.0 software based on the EST sequences obtained from the asexual reproduction SSH libraries. RNA extraction and cDNA preparation were performed using the procedure described above. The qRT-PCR reactions were conducted using KAPA SYBR FAST Master Mix Universal (KAPA, USA) and a Roche LightCycler480 real-time PCR system (Roche, Switzerland). Each qRT-PCR reaction mix contained 8.2-µL water (sterile and deionized), 0.4-µL primers (50 µM each), 1-µL cDNA, and 10-µL KAPA mix. After completion of the reactions, the threshold cycle (C_T) value of actin was used as an internal control and the fold change in the transcript level of each gene was calculated based on the method of (Livak and Schmittgen, 2001).

RESULTS

Construction and sequence analysis of the *K. daigremontiana* SSH cDNA libraries

To identify differentially expressed genes, sample tissues were collected at various time points from day 20 to 35 and pooled. One thousand recombinant clones were recovered on agarose plates for each SSH cDNA library, of which ~630 clones were found to be of a quality suitable for sequencing and analysis. After vector, repeat, and useless sequences were removed, 481 high-quality ESTs remained (GenBank accession numbers are listed in Supplementary Table 1). Of these ESTs, 143 sequences clustered into 52 contigs with most containing two sequences (Table 2 and Table 3). In addition, 338 singletons constituted 390 unigenes. Thus, the redundancy of the EST collection was roughly 19% (Table 2). Non-overlapping sequences of the same gene may have caused additional redundancy. Almost all of the unigenes from the SSH cDNA libraries were annotated for putative functions based on sequence similarity to genes or proteins with known functions included in GenBank. More than 70% of the unigenes had matches in Swissprot and approximately 76% of the unigenes had matches in the Nt database (nucleotide sequence database), while about 92% of the unigenes had matches in the Nr database (non-redundant protein sequence database) (Table 2). However, 29

unigenes had no matches in any of the four databases and were considered to be candidate novel genes. Further analysis using InterProScan software eliminated 21 unigenes as potential novel genes because of homology with known protein structural domains. Four of the remaining eight unigenes had open reading frames (ORFs) using ORF Finder (Open Reading Frame Finder) at the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) that longer than 290 bp; we confirmed these four unigenes as new ESTs. The new EST sequences are available at NCBI [GenBank: JK340183 and JK340282 (contig41), JK340351 (singleton14), JK317242 (singleton 1007), and JK340114 (singleton 1577)].

Table 1. Real-time PCR primer sequences of selected unigenes.

Gene Name	Forward Primer	Reverse Primer
Contig 14	5':TGCACATCCAAAGGAAGATCC	5':CAAGGTTGGGAGAGCAGGTG
Contig 29	5':GCCTTTTCCCCCTCACCA	5':CTGCCGGAGTTATAAGCCTT
Contig 32	5':ATTGACCCTATCTGTGCCCTTC	5':CACTAACCATAATGATGTCCTTGC
Contig 37	5':GATGAGCCTGAACAGAAACGC	5':GATGGGGGTGGATGGGGT
Contig 43	5':GATGAGGTTGGAGAAGTGTTGG	5':GCCTTTGTCCATTTTACCTTAC
Contig 46	5':CCTATTTGGGGCTTTTGTGAG	5':CCATGATACCTGGGATGTCGTT
Contig 48	5':ATCGAAACATCATGGAGAACTGC	5':AACTCATTGTGGCGACTGCTG
Contig 52	5':TCTCCACAGCACATTCTCTCAAC	5':AGCTGCCAGACCAATCACTATG
Singleton 177	5':ACCCTCTCTTCAGGCTTTTGC	5':TCCAAACTTGTAGCATTTCTCG
Singleton 506	5':ACTATGCCGATGTCGTAACCAC	5':CGGTGATGGTGTGATTGTGTGG
Singleton 627	5':CACTGCGTCTGTCGTCCTTGC	5':TCTTACGAGCCTCCTCCAATG
Singleton 974	5':CGACAAGAGCAAGTCAATCAAG	5':CAAGTCCGTAAAGCCCATCC
Singleton 1038	5':CCAGGAGCCCAACCAACAAG	5':CGCCCATACCATTTTCTTCTC
Singleton 1040	5':GGAGACGCAATCTGACCTGAG	5':AACTTATGACTTCTCTACCAACTG
Singleton 1129	5':TCTTCTCCCTCCATTTTCCAT	5':ACACTTTACCCCGTCCTCTCA
Singleton 1236	5':TCAGGTTTGGTAGGGAGGATG	5':AGACGGATCAGCCATTGGAG
Singleton 1266	5':GCTTTGTTCACCTCCACCATC	5':ATCACGTTCGGGTCCATGC
Singleton 1314	5':GGAGGGTTGGTGGAAGAGTCT	5':AGTGCGTCATCATCTTCAAACC
Singleton 1357	5':GAGTTGTTTCGTGGTATTGAGC	5':TGAGCATTAGTGAGCTTGGTTC
Singleton 1393	5':TAGGTCATAGCGGGGGTTTG	5':CAACACGGCAGAAACACGAAT
Singleton 1452	5':GGTCAGAGACTGGTGTTTATGGC	5':TTGTTGAACGCAGCACGAAG
Singleton 1591	5':CTGCTAACTGCCGCTGTGTG	5':AGGAGCCTTGATGTCATACCAG
Singleton 1634	5':TAGATGGTGTGGGTGCTGAGG	5':TGTTTCTCTACATTTCGCTTGG
Singleton 1642	5':CGGATACTGGAGAATAGCAAAC	5':CCTTTTCATAATGAGTTGGCAG
Actin[a]	5':GACTATGAGGCTGAGTTGGAGAC	5':TCAATGAAGGCTGGAAAAGG

[a] Actin is a housekeeping gene and was used as a control.

Table 2. Summary of ESTs from the *K. daigremontiana* SSH cDNA library.

	Values	Percentage
Total ESTs sequenced	630	
Number of EST sequences with readable sequence	586	
Repeat sequences	102	
Number of high quality ESTs	481	
Number of unigenes	390	
Mean unigene length (bp)	740	
Number of singleton sequences	338	70.27
Number of contig sequences	52	10.81
Redundancy	91	18.92
ESTs with significant matches against Nr	359	92.05
ESTs with significant matches against Nt	296	75.90
ESTs with significant matches against Swissprot	273	70.00
ESTs with significant matches against KEGG	360	92.31

The SSH cDNA library, including genes up-regulated and down-regulated during asexual reproduction under drought stress, was constructed using mRNA isolated from the leaves of drought-treated plants (with plantlets) as 'Tester' and from the leaves of the control plants (no plantlets) as 'Driver'.

Functional classification of the unigenes

The functional classification of the unigenes was based on the COG functional catalogue. One hundred and thirty-two unigenes that were classified into 12 functional categories (listed as A-L in Figure 1), While 258 unigenes had no matching proteins in the database. Almost half of the sequences (40%) were related to transport and metabolism (category A). This largest category encoded proteins with functions related to carbohydrates (12 unigenes), lipids (9 unigenes), amino acids (9 unigenes), inorganic ions (8 unigenes), coenzymes (6 unigenes), nucleotides (5 unigenes), secondary metabolites (2 unigenes), and intracellular trafficking/secretion/ vesicular transport (2 unigenes). These unigenes contained 36 singletons and 17 contigs. Another 13% (category B) of the unigenes, including 16 singletons and 1 contig, were predicted to have only general function that was not clearly proved but can be predicted by software. Twelve percent of the unigenes were in category C, including 13 singletons and 3 contigs, and were homologous to proteins involved in posttranslational modification and another 12% of the unigenes had functions related to translation (category D). The energy production and conversion category E (8% of unigenes) included 10 singletons and 1 contig, indicating that asexual reproduction requires a large number of genes involved in energy flow. Five percent of the unigenes encoded proteins involved in cell-related process (category F), including four sequences related to cell wall/membrane/envelope biogenesis, one sequence functionally related to the cytoskeleton, and one sequence associated with cell cycle control/cell division/chromosome partitioning. Among the remaining categories, genes encoding proteins responsible for defence mechanisms (category G) and signal-transduction mechanisms (category H) contributed 2% of the unigenes. Replication/recombination/repair functions (category I) were represented by two sequences, as were RNA processing/modification (category J) and transcription (category K) functions. Only one unigene was of unknown function (Supplementary Table 2).

Real-time PCR of selected *K. daigremontiana* genes

Genes were selected for real-time PCR analysis based on their putative functions to validate the SSH cDNA libraries and to quantify differences in expression. Five groups of genes were selected based on differential expression in drought-treated and control plants. The maximum level of differential expression was over fourfold for three unigenes (Figure 2a). A second group of seven unigenes were differentially expressed with an approximately twofold increase in expression (Figure 2b). Five genes were expressed approximately 1.5-fold higher in drought-treated plants than in control plants (no plantlets) (Figure 2c). Only three genes were down-regulated under drought-stress (with plantlets) (Figure 2d), while six genes had no differential expression (Figure 2e). Although some false positives were identified in the SSH libraries, it remains an effective technique for enriching differentially expressed genes. Hypothetical protein LOC (singleton 1129), whose function was related to inorganic ion transport and metabolism, represented a large portion of category A (Supplementary Table 3). Real-time PCR analysis showed that the expression of singleton 1129 was almost sevenfold greater in drought-treated plants than in the control (no plantlets) (Figure 2a). The rest unigene C32 S-adenosylhomocysteine hydrolase (SAHH), which is related to coenzyme transport and metabolism (Supplementary Table 3), exhibited roughly fourfold higher expression in treated plants than in the control plants; moreover, malic enzyme (singleton 627) was up-regulated during plantlet formation. The expression levels of two contigs, C37 (category A) and C43 (category D), and the four singletons 506 (category A), 1040 (category A), 1634 (category B), and 1642 (category J) were ~twofold higher in treated plants than in the control (Supplementary Table 3). Slight up-regulation was confirmed for the RPE1 protein (C14, category A), a gene with a putative role in RNA-binding (singleton 177, category B), a subunit of a general transcription factor (singleton 1357, category K), an exosome complex component (singleton 1236, category D), and a ribosomal protein (singleton 1591, category D) (Supplementary Table 3). These five genes exhibited approximately 1.5-fold higher expression in the treated plants than in the control. Only three unigenes were down-regulated during plantlet formation; these grouped into three different functional categories. Cellulose synthase (singleton 1393, category F) expression was lowest, suggesting that cell division and differentiation were active during plantlet formation (Supplementary Table 3). Six genes showed no significant difference between the two treatments, including four (C29, S974, S1266, and S1344) in category A, one gene (S1033) in category I, and S1452 in category C (Supplementary Table 3).

Table 3. Selected contigs from the SSH libraries.

Contigs	Number of clones	Putative function/ closest homolog	Length (bp)	Species
C52	3	phosphoribulose kinase, putative	836	*Ricinus communis*
C51	3	major latex protein, putative	736	*Ricinus communis*
C50	3	major latex protein, putative	790	*Ricinus communis*
C44	2	unnamed protein product	620	*Vitis vinifera*
C43	2	hypothetical protein	754	*Vitis vinifera*
C42	2	hypothetical protein	961	*Vitis vinifera*
C41	2	no hits	604	-
C40	2	unknown	727	*Picea sitchensis*
C39	2	unnamed protein product	944	*Vitis vinifera*
C38	2	unnamed protein product	922	*Vitis vinifera*
C32	2	adenosylhomocysteinase	771	*Medicago truncatula*
C3	1	hypothetical protein	865	*Vitis vinifera*
C2	1	friedelin synthase	759	*Kalanchoe daigremontiana*
C1	1	friedelin synthase	639	*Kalanchoe daigremontiana*

DISCUSSION

The over-expression of as many as 390 unigenes suggests the involvement of a large number of genes in the asexual reproduction of *K*. *daigremontiana.* Redundancy of approximately 19% in the EST collection indicated that novel genes, particularly low abundance sequences, could be discovered. More than 66% of the unigenes could not be classified into functional categories. Four novel genes were identified, suggesting that SSH can be used to identify novel genes differentially expressed in various tissues. Although SSH is a powerful tool to enrich differentially expressed and new genes, it is not perfect-as shown by the isolation of several genes whose expression did not differ between the two treatments according to real-time PCR. Even though no clear expression difference or clear function was found for some of these genes, their importance in plantlet formation should not be ignored. Based on putative functions derived from the COG database, we assigned 132 unigenes to 12 functional categories; this demonstrates the level of gene diversity in the SSH libraries.

Adventitious root development in *K*. *daigremontiana* plantlets represents organogenesis (Garces et al. 2007). In this study, histidinol dehydrogenase, the key enzyme acting as the final step in the catalytic pathway of histidine (His) biosynthesis, exhibited twofold higher expression during plantlet formation (singleton 1040, Figure 2b). Mo et al. (2006) observed that, in an *Arabidopsis* His biosynthesis pathway mutant with reduced His content; defective root growth was linked to impaired mechanisms of root meristem maintenance. Thus, an increase in His levels via increased histidinol dehydrogenase expression may be part of the mechanism that regulates asexual reproduction in *K*. *daigremontiana*. In addition, glutathione S-transferase (Che et al. 2006) was one of the three most highly up-regulated genes in the *Arabidopsis*, and was also found in our libraries (singleton 1634, contig 15, and contig 44). The F-box family protein, reported to be one of 20 genes specifically down-regulated during root development (Che et al. 2006), was also identified in our libraries as singleton 1208. These results indicate that the SSH libraries in this study facilitated identification of differentially expressed genes. Type-A response regulators, such as RR9 (singleton 1380), which are not significantly induced by cytokine (D'Agostino et al. 2000), but are expressed strongly throughout the root and weakly in the seedling vasculature (To et al. 2004), could be used as markers of root development.

A consensus has not been reached, based on previous studies, on the effect of cytokinin in terms of promoting or inhibiting the initiation of plantlet formation. (Catarino, 1965) reported that cytokinin activated bud development in *K*. *pinnata (Bryophyllum calcynum)*. In contrast, cytokinin was found to play a central role in the suppression of plantlet primordium development in the leaf (Kulka, 2006). Our study complements the results of Kulka in that the expression of the SAHH gene (contig 32) under drought stress (with plantlets) was more than threefold greater than in the control (no plantlets). (Masuta et al. 1995) reported that antisense RNA for tobacco SAHH resulted in cytokinin levels threefold higher in transgenic tobacco plants than in non-transgenic plants. Therefore, it seems likely that increased SAHH triggers a decrease in the number of free active cytokinins, resulting in the

release of plantlet primordium dormancy. Moreover, a gene encoding inorganic pyrophosphatase protein (contig 46) that was expressed under the control of the tuber-specific patatin promoter, significantly accelerated sprouting by 6 or 7 weeks compared to wild-type potato tubers (Farré et al. 2001). Glutamate decarboxylase (contig 48), which plays an important role in glutamate catalysis, was closely linked to germination in developing soybean seeds (Matsuyama et al. 2009). However, both inorganic pyrophosphatase (C46) and glutamate decarboxylase (C48) were down-regulated during plantlet formation. These results indicate that the complexity of the regulatory pathway of asexual reproduction needs to be explored in depth

The SAHH gene was identified as a novel protein response to drought stress. It is not surprising that additional genes are associated with drought stress. Catalase, which protects the plant from oxidative damage caused by the accumulation of H_2O_2, was represented three times in our library (contig 36, contig 37, and contig 47). Heat shock protein, a chaperonin known to respond to drought stress, was identified as singleton 434 in our SSH library. We speculate that drought stress plays a stimulatory role in asexual reproduction; however, further evidence is needed to demonstrate this.

In this study, we performed the first enrichment of differentially expressed genes of *K*. *daigremontiana.* The large number of unigenes involved in asexual reproduction suggests that plantlet formation is a concerted process involving multiple cellular pathways controlled by a complex gene regulatory system. The initiation of plantlet formation and subsequent developmental programs is accompanied by dynamic changes in gene expression.

Plantlet formation is a process involving cell division and differentiation. It is surprising that only one clone (singleton 928, JK340319, cyclin dependent kinase, Supplementary Table 1) was functionally related to cell cycle control. SSH library genes included 3' untranslated region sequences that were difficult to annotate. Therefore, it is likely that additional genes related to plantlet formation are differentially expressed but were not identified as such. Our results suggest that transcription factors are important in asexual reproduction and that regulatory genes in *K*. *daigremontiana* may resemble those of other species. Our results provide a framework and unified platform on which future research on asexual reproduction in *K*. *daigremontiana* can be based. Once the full-length cDNAs of genes of interest have been obtained, their specific functions in plantlet formation can be further investigated in suitable model systems using molecular biological techniques. Conclusively determining the functions of these genes will extend our understanding of asexual reproduction in *K*. *daigremontiana.*

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Figures

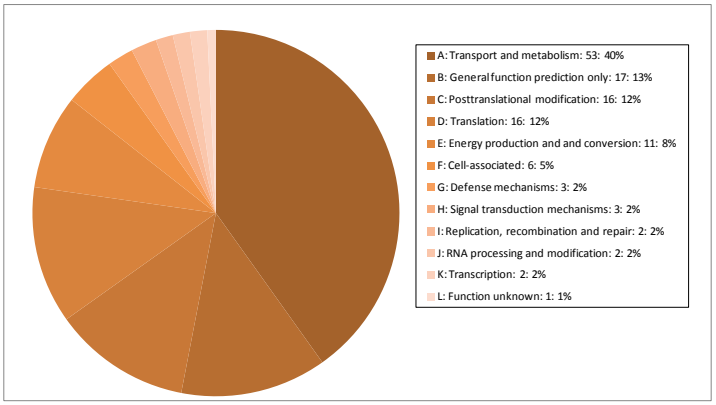

Fig. 1 Functional classification of the unigenes. Functional classes were based on COG database annotations. The 132 unigenes were grouped into 12 functional categories (A-L). The largest category comprised genes related to transport and metabolism. The category of genes with unknown functions was the smallest. Letters correspond to the functional categories. The numbers of unigenes in each category and percentages of the total number of unigenes are listed.

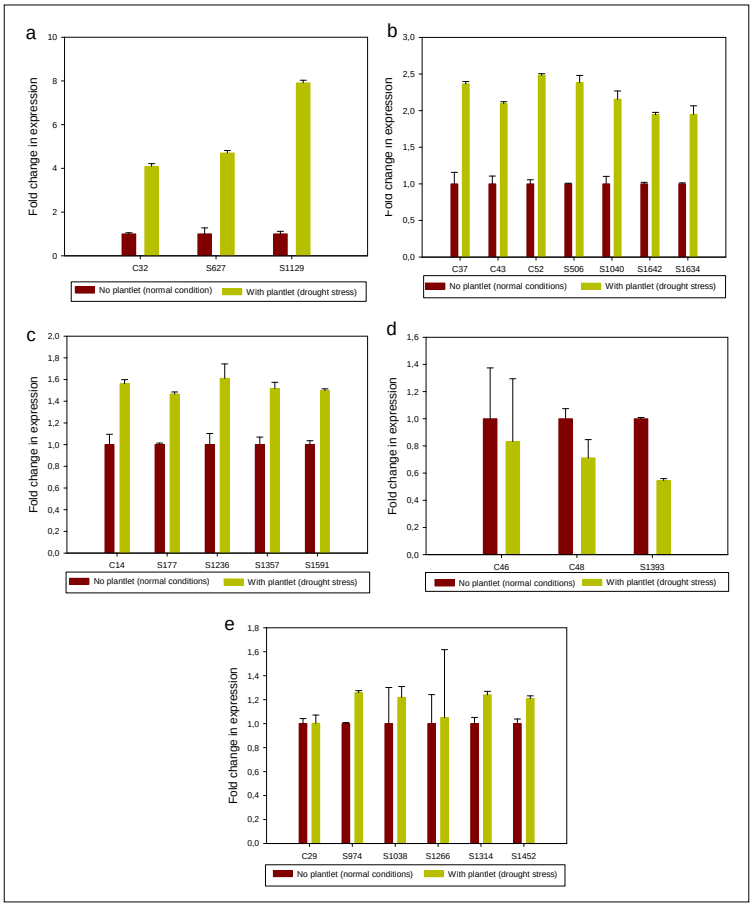

Fig. 2 Differential expression of selected genes by real-time PCR analysis. (a) Genes with threefold or greater differential expression. (b) Genes with ~onefold greater differential expression. (c) Genes with ~0.5-fold greater differential expression. (d) Down-regulated genes. (e) Genes with no differential expression between drought-stressed plants (with plantlets) and control plants (no plantlets). The expression levels of genes under normal conditions (no plantlets) were set to a value of 1. Data represent the means of three biological replicates for each treatment.

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