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รายงานวิจัยฉบับสมบูรณ์

โครงการ Expression profiling of cassava genes during storage root initiation

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ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

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Project Title: Expression profiling of cassava genes during storage root initiation

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Understanding of molecular mechanism involved in storage root development at the early transition stage from fibrous to storage stage using oligo-microarray. A relatively high quality and large database were generated and the transcriptomic profiling in early storage root development were unraveled. A total of 1,259 genes were significantly expressed, of which only 919 genes were GO-term annotated. Transcription and gene expression were found to be the key regulating processes during the transition stage from fibrous to intermediate roots, while homeostasis and signal transduction influenced regulation from intermediate to storage roots. Clustering analysis of significant genes and transcription factors (TF) indicated that a number of phytohormone-related TF were differentially expressed; therefore, phytohormone-related genes were assembled into a network of correlative nodes. A model showed the relationships between KNOX1 and phytohormones during the storage root initiation. Exogeneous treatment of phyto- hormones N6-benzylaminopurine and 1-Naphthaleneacetic acid were used to induce in vitro storage root initiation stage and to investigate expression patterns of the genes involved in storage root initiation. The results support the hypothesis that phytohormones are acting in concert to regulate the onset of cassava storage root development. Moreover, MeKNOX1, MeCDPK and MeAGL20 are putative factors that might play important roles at the onset of storage root initiation when the root tip becomes swollen.

KEYWORDS: cassava / microarray / phytohormone / storage root initiation

<u>เอกสารแนบหมายเลข 2</u>

บทคัดย่อ

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โครงการ: การวิเคราะห์การแสดงออกของยีนในช่วงการกระตุ้นการสร้างและพัฒนารากสะสมแป้งในมันสำปะหลัง

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การศึกษากระบวนการพัฒนารากสะสมแป้งในมันสำปะหลัง ในระยะเริ่มต้นจากรากวิสามัญไปสู่รากสะสมโดยใช้เทคนิคไมโครอะเรย์พบว่า เมื่อนำกลุ่มของยีนที่มีการแสดงออกอย่างมีนัยสำคัญจำนวน 1,259 ยีน และทรานสคริปชันแฟคเตอร์ มาวิเคราะห์โดยการวิเคราะห์แบบจัดกลุ่ม ข้อมูลบ่งชี้ว่ากระบวนการหลักในขั้นตอนการกระตุ้นการเปลี่ยนแปลงจากรากวิสามัญไปสู่รากสะสม คือกระบวนการการถอดรหัส และการควบคุมการแสดงออกของยีน ในขณะที่การแสดงออกของยีนที่เกี่ยวข้องกับการรักษาสภาวะสมดุล และ การถ่ายโอนสัญญาณมีอิทธิพลต่อการควบคุมการพัฒนาจากรากสะสม จากการวิเคราะห์แบบจำลองเครือข่ายความสัมพันธ์ของการทำงานร่วมกันของยีนในระยะกระตุ้นการสร้างรากสะสม พบว่า ทรานสคริปชันแฟคเตอร์และการยีนที่เกี่ยวข้องกับการทำงานของฮอร์โมนพืชมีบทบาทสำคัญ ผลการทดลองบ่งบอกถึง ความสัมพันธ์ระหว่าง ยีน KNOX กับ ยีนที่เกี่ยวข้องหรือยีนที่ถูกควบคุมโดยฮอร์โมนพืช เมื่อทำการทดสอบแบบจำลองโดยการกระตุ้นรากวิสามัญด้วยฮอร์โมนไซโตไคนิน (BAP) และ ออกซิน (NAA) พบว่าสามารถกระตุ้นการพัฒนาของรากวิสามัญไปสู่รากสะสมอาหารได้ และ มีการเพิ่มขึ้นหรือลดลงของการแสดงออกของยีน ตามแบบจำลอง นอกจากนี้ยังพบว่า ยีน MeKNOX MeCDPK และ MeAGL20 มีบทบาทสำคัญในการกระตุ้นการพัฒนารากสะสมอาหารในมันสำปะหลัง

KEYWORDS: cassava / microarray / phytohormone / storage root initiation

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Introduction

Cassava is a shrubby, tropical, perennial, dicotyledonous plant belonging to family Euphorbiaceae. Cassava storage roots are used as an animal feed and as a human food in many tropical countries. Cassava storage roots are greatly used as a raw material for many industrial products. Cassava starch is used in pharmaceutical bases, in sizing paper and textiles, in coagulation of rubber latex, in bioethanol production, and in processed food such as glucose, high fructose syrup, dextrin, baby foods and sweeteners. Cassava is one of the most important economic crops in Thailand. Thailand exported most of cassava widely in the world market. At presence, the price for raw cassava is increase in relative to the high demand. With a growing demand for cassava used, cassava cultivation is expanded. However, the planting areas are limitted. Therefore, in response to expanded need, early storage root formation, high starch content and early harvesting root cultivars are in search. From this study, understanding of cassava storage root initiation and the genetic regulation involved in cassava storage root developmental process would be beneficial in improving cassava root production with early harvesting time and desired properties to fulfill a range of industrial demands. Starch with high quality and quantity would also enhance expansion in value and volume of industrial products. Consequence, this would augment the income of cassava farmers and job opportunity in industrial sector, which will lead to a better quality of life for farmers and industrial workers.

The knowledge of the genetic regulation of storage root initiation in cassava is still largely lacking. Most of the studies on the genetic control of storage organ initiation in plants have focused mainly on other genetically less complicated and better-characterized plants like potato, rice and cereals. Thus of the key genes, only a few have been studied in cassava. In 4 month-old storage root, rate-limiting enzymes were identified majority from glycolysis/gluconeogenesis pathway and starch metabolism pathway . The rapidly advancing knowledge in plant functional genomics could be the shining light to help overcoming this obstacle.

The rapid progress in plant functional genomic studies in recent years could be attributed, to most extent, to the development of several tools. It is now possible to obtain a comprehensive understanding of the complex orchestration of the transcriptome and its dynamic changes under various circumstances in a given plant cell with great speed and reliability. The microarray technique is one of such transcriptomic tools that have been successfully applied to plant functional genomic analysis. With prior knowledge of genome information, the oligomicroarray were established for genome wide analysis. The microarray technique allows the detailed characterization of gene expression in a wide range of biological processes. These applications of microarray to investigate plant transcriptomes provide reassurance for a successful outcome from the analysis of the cassava transcriptomes during early storage root development in this study. As previously mentioned, the storage root developmental process in cassava is only partially characterized in 4 month-old roots, which identified gluconeogenesis/glycolysis as main processes for storage root developmment [62]. The information indicated that it was a genetically complicated process and mainly involved some of the key enzymes committed to the gluconeogenesis/ glycolysis pathway and starch metabolism pathway. Since the cassava roots are developing into storage stage at around 7-8 weeks after planting [52], the gene expressions in root samples at 8-week-olds when the storage roots are at the early phase change from fibrous root into storage organ were examined using oligo-microarray With comparative analysis by displaying the corresponding differentially expressed genes during early storage root development allowed the visualization and the identification of the genes involved in the complex regulation of storage root initiation and early storage root development as well as lead to the understanding of the phytohormone as key regulators in storage root initiation.

Since phytohormone has been reported as important factors controlling storage organ development in other organisms [58]. In potato and sweet potato, phythormone play roles in tuber initiation. The cassava storage root development shared some general signaling pathways with potato, even though their storage organs are originated from a different organ [48]. According to the model that was generated from the microarray results, effect of phytohormone on storage root development were investigated.

The knowledge obtained from this project give us an overview on key regulations of metabolic pathways and phytohormone functions that associated with cassava storage root development and may provide a tool for cultivar selection from breeding program for early bulking cultivar. The other cultivars will be further investigated for their gene expressions and phytohormone responses after the affects of key genes and transcription factors as well as phytohormones are revealed from this study.

Materials and Methods

Sample Collection & Processing

Cassava cultivar KU 50 was planted from stem cuttings in the field at Rayong Field Crop Research Center, Rayong, Thailand. The roots were classified into fibrous (F), intermediate (I) and storage (S: layer 2-5) and used for both microarray analysis and quantitative RT-PCR (qRT-PCR). The root developmental stages were weekly investigated after planting. A fibrous root (F) was formed within 7 days after planting. Intermediate roots (I, 3-5 mm in diameter) were present at the 7th week, while storage roots (S, more than 10 mm in diameter) were present at the 8th week after planting (Fig. 1). In intermediate roots, early stage of secondary growth of phloem and xylem was initially observed (Fig. 2). Cell expansion and starch accumulation were also detected in I as observed in S [52]. The 8-week-old plantlets were chosen for investigation since the plants contained all 3 developing-root stages of interest: F8, I8 and S8 (Fig. 3). Fibrous root of 4-week-old plants (F4), without any evidence of secondary growth, was used as a control stage. Three independent biological replicates were harvested at 4 and 8 weeks after planting. The storage roots were washed thoroughly. The skin was peeled off and storage root tissues were grated into fine pieces, and quickly frozen in liquid nitrogen. The frozen tissues were stored at -80°C until use.

RNA Extraction

Total RNA from F, I and S were extracted using Concert™ Plant RNA Reagent (Invitrogen). Cassava storage root tissues contain large number of polysaccharides and polyphenolic compounds. These compounds have effect on quality of RNA. The RNA samples containing high amounts of polysaccharides and polyphenolic compounds are considered as a poor quality RNA [25, 45]. Therefore, additional step was used to reduce the co-precipitations. The 2.5M LiCl was used to re-precipitate RNA after Concert™ Plant RNA extraction. After re-precipitation, the total RNA was treated with DNA-Free (Ambion) to remove genomic DNA and RNA concentration was measured (Table 1). Since RNA is prone to enzymatic degradation by RNase, it is very important to assess the integrity of the cassava RNA extracted. The RNA samples obtained were run on 1.2% formaldehyde agarose gels to check for their integrity and presence of degradation, if any (Fig. 4). From the formaldehyde gel electrophoresis and Agilent 2100 Bioanalyzer, intact RNA with prominent bands of 28S and 18S rRNA without smear bands indicates high quality of RNA without nucleic acid degradation.

Microarray based gene expression analysis

Genome wide microarray analysis was performed to generate the expression profile of cassava genes (Fig. 5). The samples from 4 week-olds fibrous (F4), 8 week-olds fibrous, intermediate, and storage roots (F8, I8 and S8) were subjected to microarray analysis using Affymetrix Gene Chip cassava Genome Arrays version 1.0 [56]. In brief, total RNA was used for cRNA synthesis and labeled with cyanine-3 (Cy3) using the Quick Amp Labeling kit (Agilent Technologies). The Cy3-labelled cRNA was fragmented before being hybridized to the Agilent microarray (GPL14139). After hybridization, the microarrays were washed and scanned on the Agilent DNA Microarray Scanner (G2505B) using one color scan setting for 4x44k array slides. Signal intensities were detected from the obtained digital images using Feature Extraction software (Ver. 9.1; Agilent Technologies).

Microarray data analysis

The microarray data was normalized and transformed into log 2 ratios. Data was filtered to remove control probes and probe sets with an intensity value close to background levels. The remaining genes were filtered based on the deviation of the intensity (Fig. 6). Subsequently, the differentially expressed genes, hereafter called significant genes, were identified based upon t-tests with Benjamini-Hochberg correction (FDR) and fold changes (FC) of the expression level in eight-week-old roots with respect to that of four-week-old fibrous root.

GO enrichment analysis

The GO enrichment analysis of the significant genes was conducted using SEA approach (singular enrichment analysis), which was comprised of two steps: GO annotation and enriched GO determination. For the annotation step, the GO-based function of a gene were predicted through the Blast2GO® software [8], and the resulting annotated gene functions were employed in the later functional enrichment study using agriGO gene ontology analysis toolkit [10].

Clustering analysis

The 87 significant genes, consisting of 37 genes whose expression across the three developmental stages of root showed considerably high variation (standard deviation (sd) $\geq$ 15) and 50 significantly expressed TF genes, were clustered using a Genesis clustering-aided tool for microarray data [50]. The expression patterns were preliminarily clustered into ten cohorts based upon k-mean method, and then the cohorts with similar expression patterns were manually grouped together to minimize pattern redundancy.

Correlation analysis

The association of each gene pair was determined by correlation analysis, which statistically quantifies the relationship between genes based on their expression profiles. Pearson correlation coefficients (PCC) of each gene pair were calculated by Matlab software. Only highly correlated gene expression with $|\mathrm{PCC}| \geq 0.95$ and p-value $\leq$ 0.05 was subjected to network inference.

Upstream sequence analysis

Promoters of the 67 genes comprising the gene association network were explored, based on the 2-kb upstream sequence from the translation start site. Each upstream region was searched for the cis-acting regulatory elements or transcriptional factor biding sites (TFBS) through PlantPAN web tool (http://PlantPAN.mbc.nctu.edu.tw), using the default settings [5].

Quantitative RT-PCR

First-strand cDNA was synthesized by The SuperScript® III First-Strand Synthesis System according to the manufacturer's protocol (Invitrogen). After sequence analysis using full-length cDNA database [43] and Phytozome database [16], primers specific to each candidate gene were designed (Table 1). qRT-PCR was performed with SsoFast™ EvaGreen® Supermix (Bio-Rad). The amplified PCR products were quantified with CFX96 Touch™ (Bio-Rad). Cassava elongation factor 1α (MeEF1α) was used for normalization throughout the tests. The data were analyzed using CFX Manager™ software (Bio-Rad). Relative change in gene expression was calculated according to the ΔC_T method, using a reference gene.

In vitro storage root induction

KU50 was subcultured in MS media containing 1 μM CuSO4 (KU-media) for 45 days. Internode was sub-cultured in KU-media containing either 6 or 8 % sucrose with various combinations of NAA and BAP from 0.25–2.2 μM. After 4 weeks culturing at 25 °C, the roots were harvested for cross section and Iodine staining.

In vitro storage root initiation

The three independent 50 day-old KU50 roots were transferred into liquid KU-media containing 6 % sucrose as a control (non-hormonal treatment), 10 uM GA, 300 uM glucose, 300 uM sucrose or KU-media containing 6 % sucrose, 0.25 μM NAA and 0.44 μM BAP (KU-BN). The roots were cultured in a 120-rpm shaker at 25 °C. The triplicate samples of fibrous roots (FR) and swollen roots (SwR) were harvested at 24 h after treatment. 7 days after treatment, other triplicate samples of storage roots (7D-SR) were harvested. The expression levels of model genes were quantified by qRT-PCR. The results were expressed in mean ±SE of analyses. One-way analysis of variance (ANOVA) was used for statistical analysis with p value $\leq$0.05.

Amplification and sequence analysis of gene and promoter of interested

Interested gene sequences were retrieved from Phytozome database and used for primer design. All the primers contained restriction sites (underlined) for cloning facilitation. The PCR reactions were done using KAPA HiFi HotStart PCR Kit (Kapa Biosystems). The amplified genes were sub-cloned into pGEMT-Easy and at least 15 clones per construct were submitted for sequence analysis. The gene sequences and promoter sequences will be Blast against cassava genome database in Phytozome and NCBI genome database. The promoter analysis for gene regulation motif were analyzed with PLANT-care.

Fig.1 Cassava roots at 2-week-old to 12-week-old: Intermediate roots (I, 3-5 mm in diameter) were present at the 7th week, while storage roots (S, more than 10 mm in diameter) were present at the 8th week after planting

Fig.2 Cassava roots at 8-week-old: Intermediate roots showed early stage of secondary growth of phloem and xylem as well as starch accumulation as in storage root.

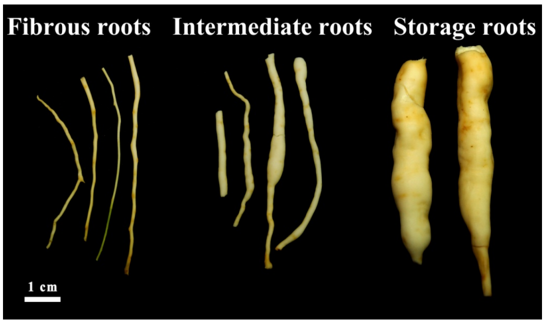


Fig.3 Cassava roots at 8-week-old: Fibrous roots showed diameter less than 1 mm. Intermediate roots showed diameter around 3-5 mm, while storage roots showed diameter more than 10 mm.

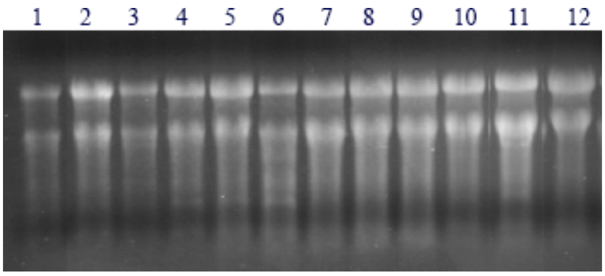


Fig. 4 Formaldehyde gel electrophoresis of the RNA samples to be subsequently used in the microarray analysis: 1 - KF4-1, 2 - KF4-5, 3 - KF4-9, 4 - KF8-1, 5 - KF8-5, 6 - KF8-9, 7 - KI8-1, 8 - KI8-5, 9 - KI8-9, 10 - KS8-1, 11 - KS8-5, and 12 - KS8-9

Results

Plant material for microarray analysis

The cassava roots were harvested every week after planting for 12 weeks. The root samples at different developing stages: fibrous, intermediate and storage root were obtained from 8-week-old samples since all stages were presented in the same plantlets (Fig. 1). The three independent plantlets were randomly selected and used for further experiments. High quality RNA was obtained from F4, F8, I8 and S8 samples. The RNA quality and quantity were determined using spectrophotometer, gel electrophoresis and Agilent 2100 Bioanalyzer. The results showed the high quality of RNA could be achieved (Fig. 4). The RNA was further analyzed for differentially gene expression by microarray. Fibrous root of 4-week-old plants (F4), without any evidence of secondary growth, was used as a control stage.

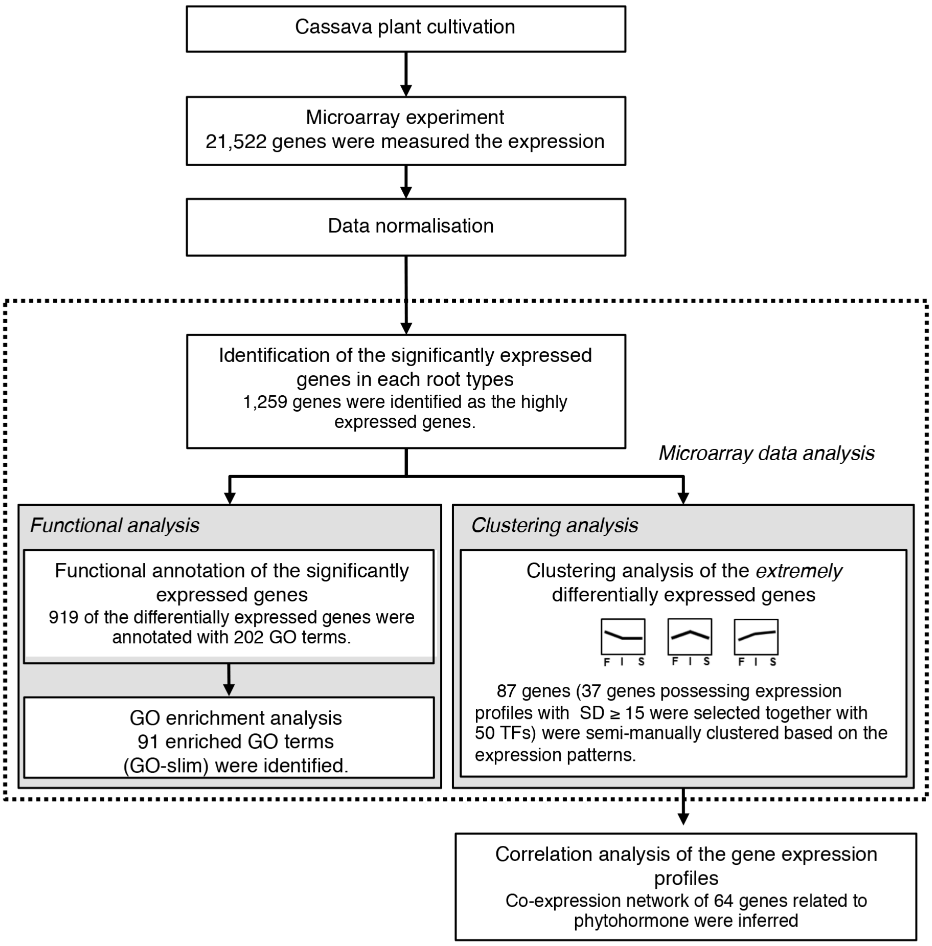


Fig. 5 Scheme of the overall methodology for microarray analysis

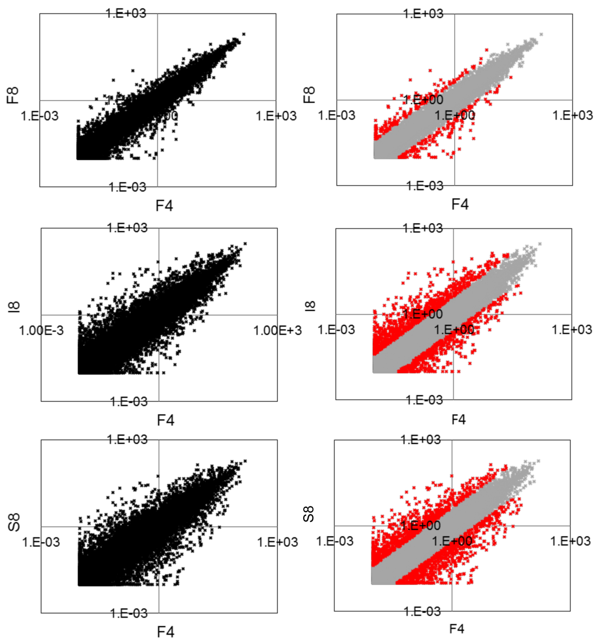


Fig. 6. Characteristics of the expression data in cassava roots. The left panel demonstrates the different expression of genes in root at 8 weeks old (F8, I8, and S8) with respect to that in fibrous root at 4 weeks old (F4). The right panel exhibits proportion of the significantly expressed genes identified in this work (red) according to following statistical criteria: 4-fold different expression from F4 with p-value (t-test) $\leq$ 0.05 and FDRF $\leq$ 0.72, FDRI $\leq$ 0.2, FDRS $\leq$ 0.25

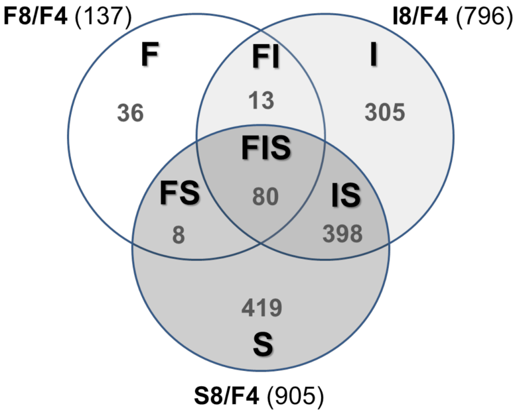


Fig. 7 The significantly expressed genes (FC ≥ 4 with p-value (t-test) ≤ 0.05 and FDRF ≤ 0.72, FDRI ≤ 0.2 and FDRS ≤ 0.25) in F8, I8 and S8 with respect to that in F4

Significant expressed genes during phase transition

The expression of 21,522 genes in F4, F8, I8 and S8 was simultaneously followed using microarray. Of these, 1,259 genes (6%) were significantly expressed with respect to control F4 (FC ≥ 4, p-value ≤ 0.05; and FDRF ≤ 0.72, FDRI ≤ 0.20, FDRS ≤ 0.25) (Fig. 6). These genes were classified into 7 subgroups according to the developmental stages at which the genes were significantly expressed: F –in fibrous, I –in intermediate, S –in storage, FI - in both F and I, IS - in I and S, FS - in F and S, and FIS - in all stages. The results showed 137, 796 and 905 genes were significantly expressed in F8, I8 and S8, respectively. Only 80 genes were expressed significantly in all stages (Fig. 7). The number of significant genes suggested that the differences in gene expression among the developmental stages (F8, I8 and S8) were more pronounced than those between F4 and F8 (137 genes). The distinctive gene expressions in each stage indicated that the transcriptional regulation associated with phenotypic changes. Moreover, the consensus significant genes during phase transition from I-stage to S-stage (398 genes) were higher than those from F-stage to I-stage (13 genes) suggested that the intermediate root represented the transition stage into storage root.

When an organism is exposed to certain conditions, the significant genes identified either at a specific time point or throughout the developmental stages reflect the biological regulation. The presence of genes whose expression was observed only at a particular stage of root development may imply that their roles were significant in retaining a certain developmental stage. For example, the 36 genes found to be significantly expressed only in F-stage may act as modulators to maintain the function of fibrous root (Fig. 7). On the other hand, the 13 genes co-significantly expressed in FI-stage may involve in the developmental regulation toward transformation into a storage organ (Fig. 7). According to these observations, study of the transcriptional behavior in these developing roots, especially those in the I-stage, is expected to reveal the process modulating storage root formation.

Functional analysis of significant genes during phase transition

The enriched GO terms and the degree of significance at certain root developmental stage was linked to biological processes activity (Fig. 8). Functional enrichment analysis was applied to all genes in a Venn diagram (Fig. 7). The ontology-based functions of 1,259 significant genes were re-annotated and 919 genes were annotated with 202 GO terms. The 91-enriched GO terms that determined from 7 subgroups (Fig. 1; F, FI, I, IS, S, FS and FIS) were identified with p-value ≤ 0.5; and FDR ≤ 0.1. The results suggest that the regulation of storage root development is highly dynamic. Some dominant biological processes functioned to retain their current stage, whereas others promoted a propagative stage (Fig. 8). The global view of the consecutively significant biological processes during the developmental stages suggested the regulation of storage root formation. The cellular processes and stress response were significant throughout F-I-S stages. In I-stage, macromolecule biosynthesis/metabolism, translation and protein modification such as starch synthase (SS, probeID: 391), geranylgeranyl reductase (2920), and ACC oxidase 1 (ACO1, 6029) became significant. In IS-stage, transcriptional regulation, signal transduction and enzyme regulation, such as Arabidopsis response regulator (ARR3, 157), geranylgeranyl pyrophosphate synthase1 (GGPS1, 1839, 1840), sucrose synthase 4 (SUS4, 10814), KNOX1 (10762), caffeoyl-CoA 3-O-methyltransferase (CCOMT, 11837), cinnamoyl-CoA reductase-related (12676) and AGAMOUS-like 20 (AGL20, 13975) played important roles. In the S-stage, cellular homeostasis became a dominant process, primary/secondary metabolic processes, as well as lipid and amino acid metabolic processes such as SS (389), cellulose synthase (6303), auxin-responsive protein IAA8 (11380), auxin transporter protein 1 (AUX1, 12207) and cinnamyl alcohol dehydrogenase (CAD, 2357) were active and maintained.

Clustering analysis of the 87 significant genes during phase transition

Among the 1,259 significant genes identified above, all TF and 37 significant genes with clear expression patterns were subjected to the clustering analysis. The clarity of the expression patterns was defined as a highly variant expression profile based on the standard deviation (sd) $\geq$ 15 of the gene expression across developmental stages (top 3 percentile of sd distribution curve), with the assumption of the relatively equal variation within a developmental stage. The expression profiles of these genes fell into one of the three patterns: cluster 1 – highly expressed in F, cluster 2 – highly expressed in I and cluster 3 – highly expressed in S (Fig. 9). The genes in cluster 1 were NAC, MYB, AGL20 and EREBP transcription factors that might be required for maintaining the F-stage. The downregulation or repression of the genes in cluster 1 might related to storage root induction. Genes in cluster 2 may involve in the transition from F-stage to I-stage or IS-stage, and might initiate storage root formation. The highest expression of metallothionein, auxin responsive factors 4 (ARF4) and pollen protein Ole e 1-like were found at the I-stage. Genes in cluster 3: KNOX1, ARF, dormancy/auxin associated proteins, aquaporin transporters, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) may involve in the transition from F-stage and progressively extend into S-stage for the long-term development. The most significant changes in the expression levels were observed in phytohormone biosynthesis and signaling related genes during early storage root development. Since KNOX1 was a TF regulating tuberization in potato and sweet potato [7, 51], the expression levels of TF genes, especially KNOX1, and other phytohormone-related genes were further examined using a correlation network.

Phytohormone biosynthesis and signaling involved in phase transition

The 67 phytohormone-related genes involved in phytohormone biosynthesis and signaling, were identified based on the GO and their original annotations from Phytozome database. These genes were then assembled into an association network showing negative correlation (red line) and positive correlation (blue line) (Fig. 11A). One of the subgroups within the network illustrated the relationship between KNOX1 and a set of genes involved in the GA metabolic pathway, auxin and cytokinin responsive genes, as well as genes involved in carbohydrate metabolism. In another subgroup, auxin transport carriers showed a link-relationship with auxin responsive genes, flowering, cell wall biosynthesis, cell expansion and differentiation. The gene expression levels in the KU50 sample set used for microarray and another field-grown KU50 sample set were measured by quantitative RT-PCR (qRT-PCR). The gene expression patterns were consistent with the microarray data (Fig. 10). From the correlative network and qRT-PCR results, we propose a model suggesting that phytohormone cross talk plays an important role in cassava storage root development (Fig. 11B).

KNOX1 might control GA level during the initiation stage. Upregulation of KNOX1 (10762) was correlated with downregulation of GA-related genes: kaurene synthase (KS, 8718), GGPS1 (1839) and AGL20 (13975). Compared with F4, KNOX1 was upregulated in I8 and S8 (Fig. 12A) while the GA-related biosynthesis genes (KS and GGPS1) were downregulated, which implied that GA level was reduced in the storage stage. In addition, expression of KNOX1 showed a positive correlation with ethylene biosynthesis gene ACO1 (6029) and ethylene signaling gene (ERF/CEJ1, 3227) (Fig. 12B). An increase in ethylene biosynthesis by ACO1 together with the reduction of the bioactive GA level may fine-tune AGL20 expression to co-regulate a phase transition into storage root.

The upregulation of genes downstream to CK responsive pathway suggests that cytokinin may play an important role during storage root development, which enhances cell differentiation. Expression of ARR3 (157) peaked at I8 and then slightly reduced in S8, whereas cytokine responsive factor 1 (CRF1, 11244) was upregulated and peaked at S8 (Fig. 12B). CK is commonly known to promote cell expansion, cell differentiation, radial cell growth, and nutrient mobilization. The genes involved in cell expansion such as expansin (15641), extension (14709) and spiral1 (13404) were upregulated in I8 and S8, whereas LPT (10934) and PIP1 (2883) expression was upregulated only in I8 and reduced in S8 (Fig. 12B) [9, 29, 59].

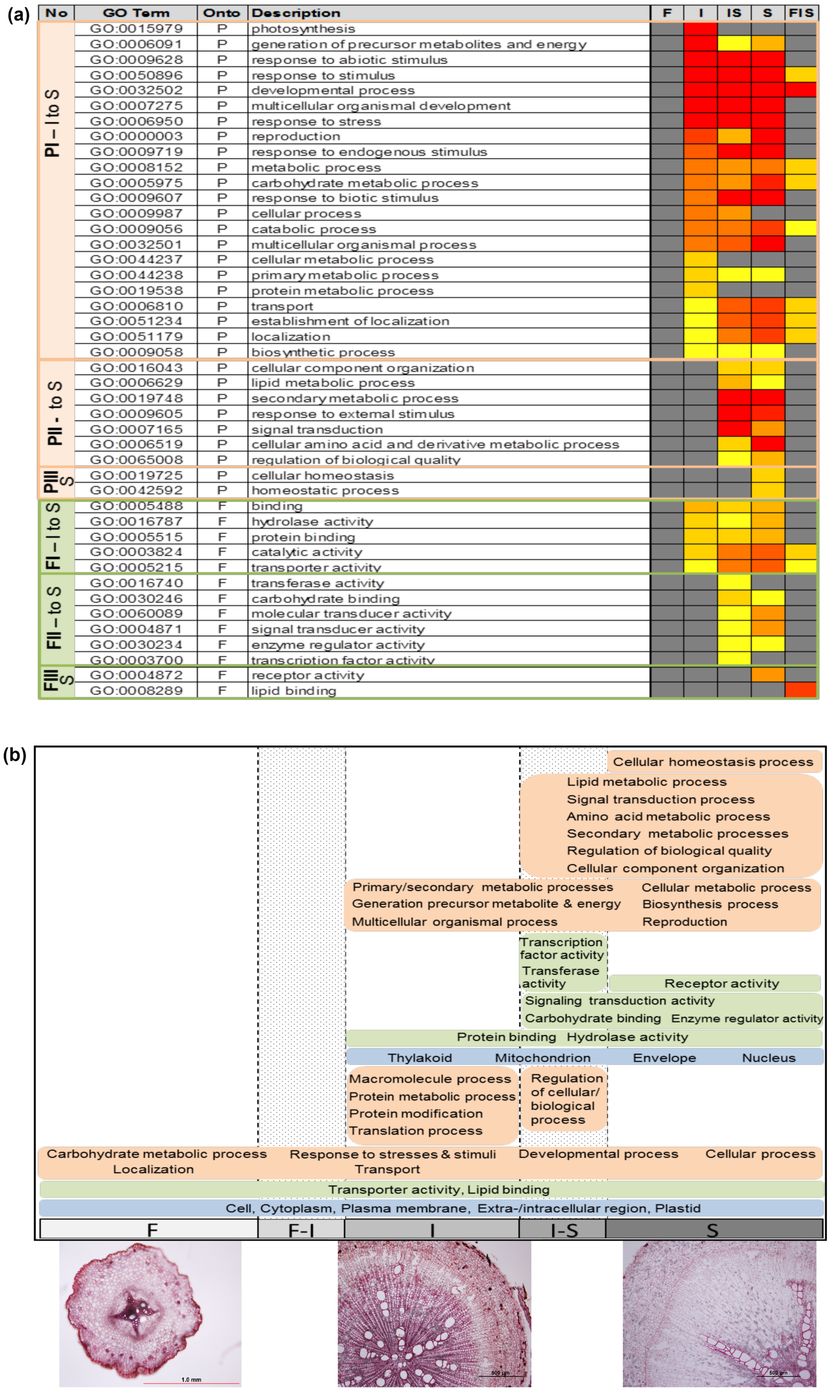


Fig. 8. Functional analysis of the significant genes at each stage of storage root development: F, I, S, FI, IS, FS, and FIS (a) The enriched GO-terms were determined based on Singular Enrichment Analysis SEA in agriGO (p-value $\leq$ 0.5, FDR $\leq$ 0.1). Two classes of the enriched GO-term were present: P – biological process and F – molecular function. The degree of enrichment for a GO-term (based on the magnitude of p-value) is represented in a color scale: from high in red to low in yellow, and not significant in grey. The patterns of enrichment degree of a GO-term were classified into 3 clusters depending on the stages at which the enriched GO terms were found to be significant: cluster I -I to S (significantly expressed in I-stage and continue to S-stage), cluster II -to S (significantly expressed during transition to S-stage) and cluster III -S (significantly expressed in S-stage). (b) The proposed scheme of active biological functions during the phase transition and root development. The active biological functions inferred by the GO enrichment analysis were aligned onto the stages of root development: F, I, S, FI, IS. The lower panel shows the cross-section of the 3 stages of cassava roots: left – fibrous root, middle – intermediate root, and right – storage root.

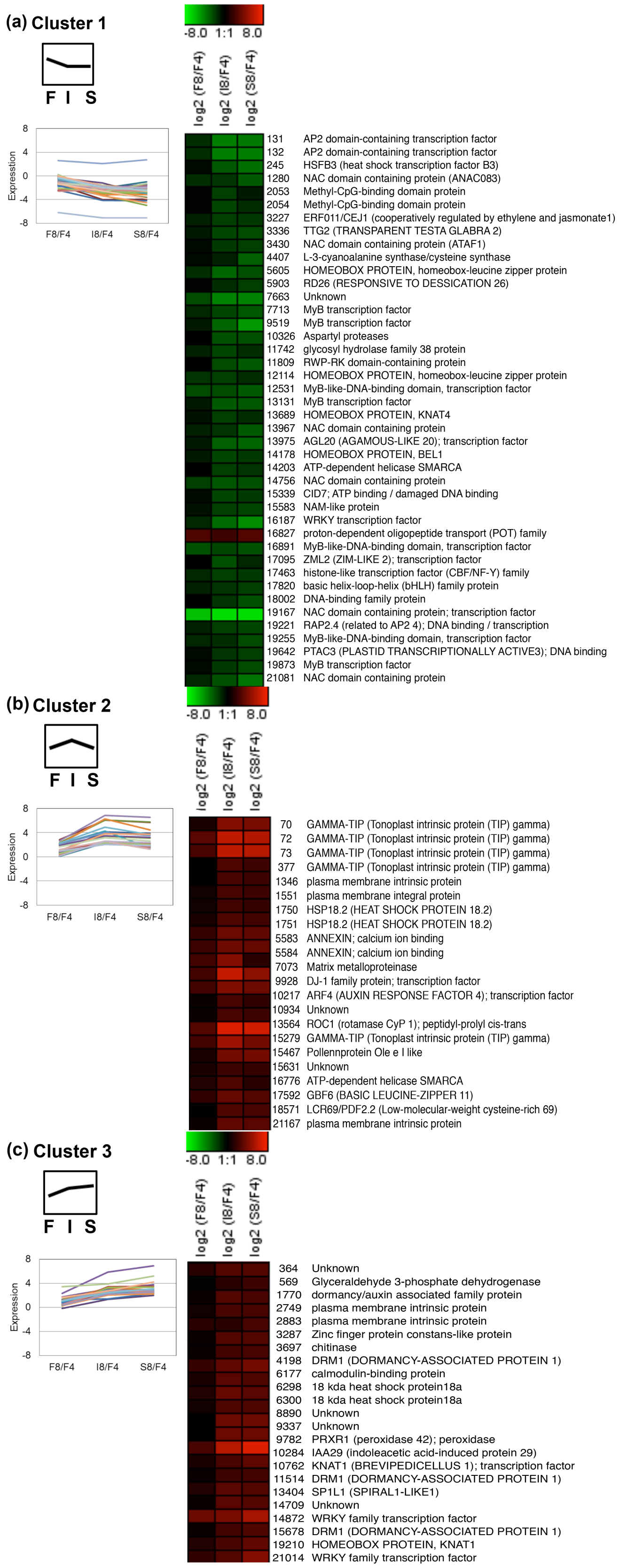


Fig. 9 Clustering analysis of the 87 significant genes during initiation stage The top 37 significant genes with sd ≥ 15 and 50 TF-encoding genes from 1,259 significant genes were investigated. The expression level is present in color scale: from low in green to high in red. (a) Cluster 1 showed highly expressed genes in fibrous root. (b) Cluster 2 showed highly expressed genes in intermediate root and (c) Cluster 3 showed highly expressed genes in storage root.

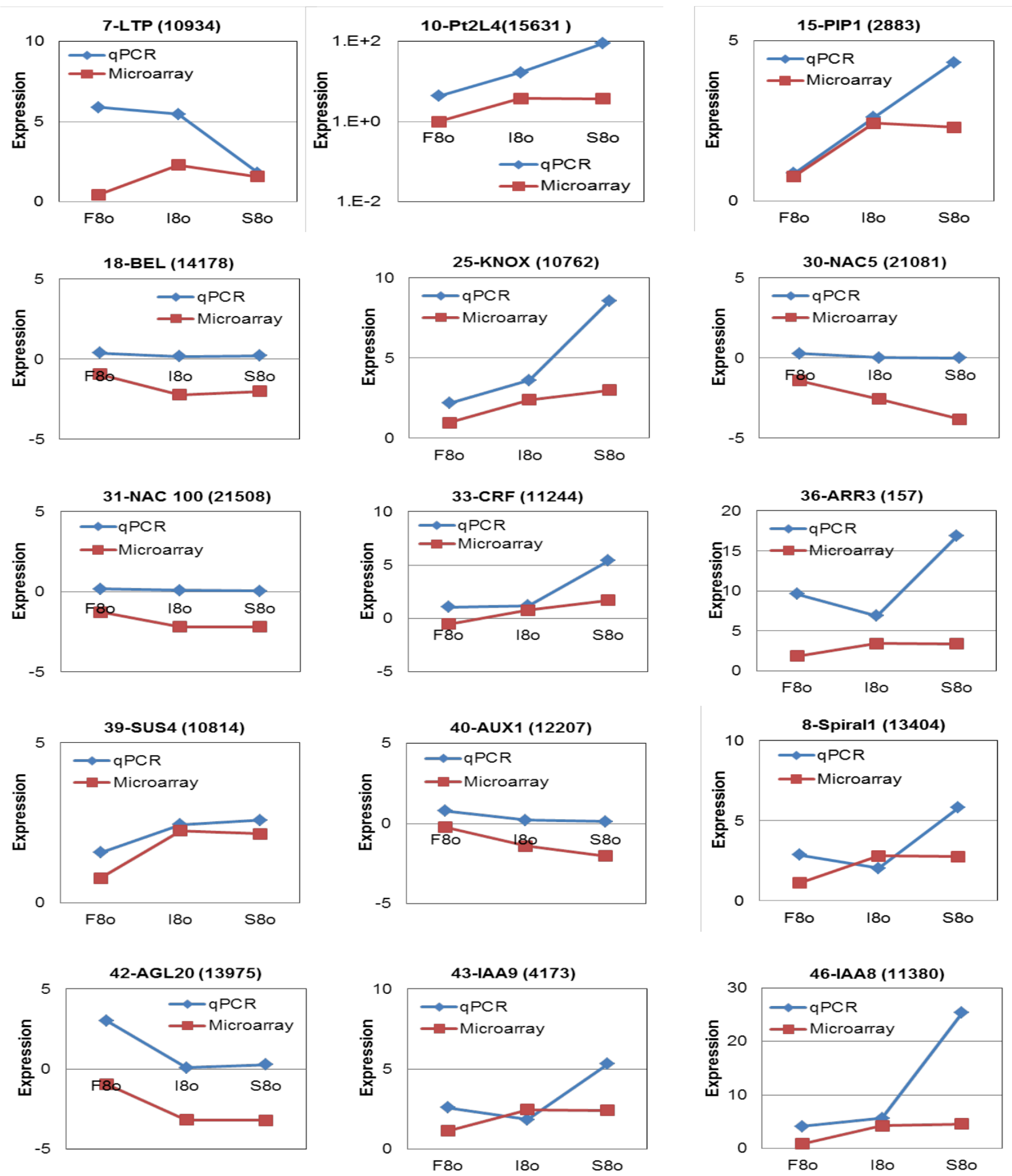


Fig. 10 The comparison of gene expression patterns derived from microarray data (red) and qRT-PCR data (blue) of the microarray sample set.

The model suggests that KNOX1 might regulate auxin levels during early storage root development through polar auxin transport (AUX1 and PIN1). Auxin level is controlled by auxin biosynthesis and auxin transport. However, our experiment showed that only polar auxin transporters (AUX1 and PIN1) were differentially expressed. The AUX1 (12207) was highly expressed in F8 but slightly reduced in I8 and drastically reduced upon reaching S8 (Fig. 12C). NAC100 (21508) and NAC5 (21081) also showed a similar expression pattern to AUX1 (Fig. 12C). Other auxin responsive genes, IAA (11380, 41731) and TPR1 (11849), were upregulated in I8 and S8, whereas the expression of ARF4 (10217) increased in I8 and then reduced in S8. The differential expression of these auxin-signaling genes implies that auxin plays an important role during the storage root's phase transition.

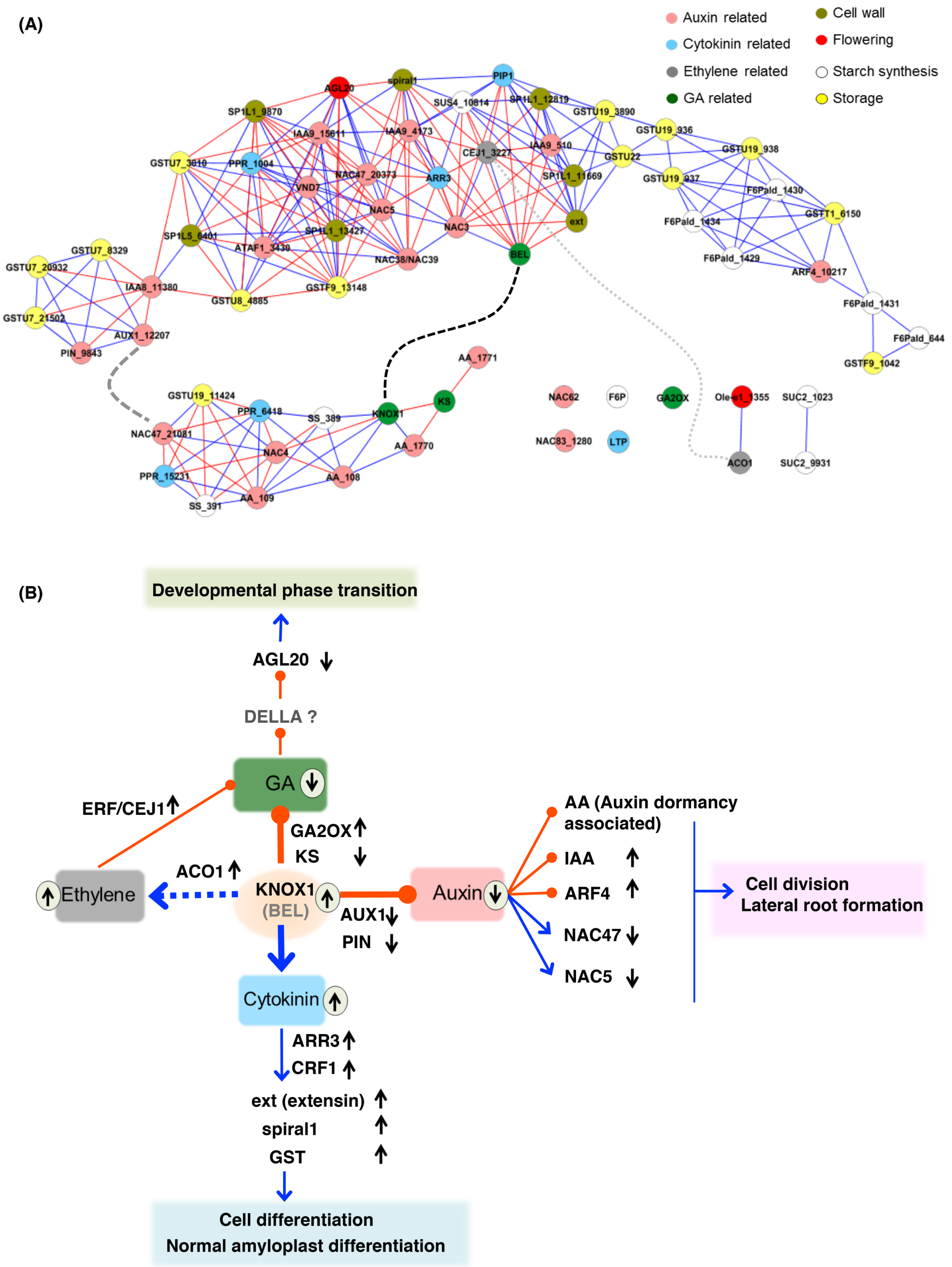


Fig. 11. The proposed genetic network and model involved in phase transition and development of storage root. The 67 genes of interest that were classified into auxin-related (pink), cytokinin-related (blue), ethylene-related (grey), GA-related (dark green), cell-wall-relevant (light green), flowering-relevant (red), starch-synthesis-relevant (white), and storage-relevant (yellow) genes were assembled into a co-expression network (a) The association network of 67 genes are shown by a red line for negative correlation and a blue line for positive correlation (b) The proposed model involved in phase transition during early development of storage root. The scheme describes the hypothetical scenario of KNOX modulating the phytohormone action in phase transition and development of storage root. The inhibitory and activating relationships of associated gene pairs are represented in circular and arrow headed lines, respectively.

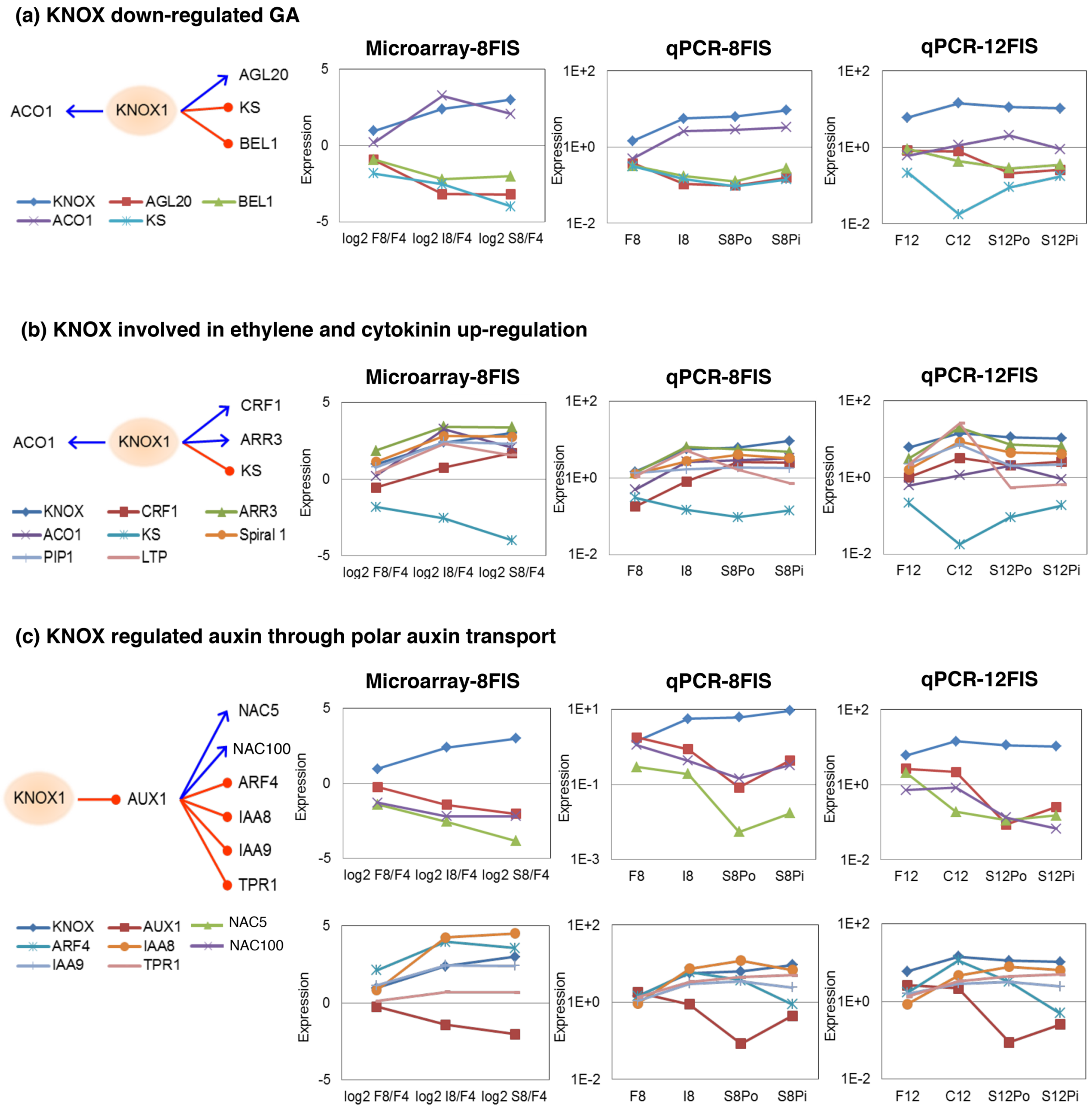


Fig. 12. The expression profiles of the modeled-genes in the field grown KU50 analyzed by microarray and qRT-PCR: The expression levels were calculated relative to the F4 control stage. (a) The set of genes relevant to the down-regulation of GA (b) The set of genes relevant to the up-regulation of ethylene and cytokinin (c) The set of genes relevant to the regulation of auxin via polar auxin transport. The roots of three independent plantlets at four, eight and 12-week-olds were classified into F, I, S and cortex (C; layer 1). Storage root was separated into outer parenchyma (SPo; layer 2-4) and inner parenchyma (SPi; layer 5). The qRT-PCR results (Ct) were averaged from three individual plants with the following maximum standard deviation (sd): KNOX = 2.43, ACO1 = 2.83, AGL20 = 1.08, KS = 3.17, BEL1 = 1.55, CRF1 = 3.20, ARR3 = 1.01, Spiral = 0.87, PIP1 = 1.33, LTP = 2.44, AUX1 = 2.60, NAC5 = 1.72, ARF4 = 2.62, IAA8 = 2.85, NAC100 = 2.88, IAA9 = 1.42, and TPR1 = 1.55

In 12-week-old storage root, KNOX1 was highly expressed, while BEL1 and AUX1 were downregulated and KS was diminished, suggesting that GA and auxin levels were reduced in the later stage of storage root development (Fig. 12). Upregulation of KNOX1, ARR3, CRF1 and spiral1 in C (layer 1) suggest that CK plays a major role in the cambium area, where cell division and differentiation take place, during the storage root developmental process.

Cytokinin and auxin played important roles in storage root initiation

The experiment showed that 6% sucrose with 0.44 μM BAP and 0.25 μM NAA (KU-BN media) could induce in vitro KU50 storage root (SR). In vitro SR with periderm tissue on the surface was observed within 2 weeks (Fig. 13). The cross-section and iodine staining of the 4-week-old in vitro SR with ≥1.5-2 mm in diameter revealed a secondary growth of vascular tissues and accumulated starch in parenchymatous tissue (Fig. 13f), which are characteristics of storage root. In contrast, control fibrous roots (FR) in KU-media had diameter ≤0.3 mm and showed only primary vascular growth, without starch accumulation (Fig. 13e).

In order to investigate gene expression at the storage root initiation stage, the condition that induce phase change from an adventitious root to a storage root was mimicked in *in vitro*, where cytokinin level was high but auxin level was low, using KU-BN media. At the initiation stage, swollen root tip with development of vascular tissue and starch accumulation was observed within 24 hours after hormone treatment, while non-hormonal treatment showed a normal fibrous root phenotype (Fig. 14a). In SwR, ARR3 and CRF1 were slightly up-regulated as a result of exogenous CK treatment. While BEL1 was slightly reduced, KNOX1 was up-regulated (Fig. 14b). However, their expression levels were not significantly different from those of FR; only PIN1 and AGL20 were significantly down-regulated in SwR (Fig. 14c and 14e). In 7D-SR, all of these genes were almost turned off. Since sucrose synthase expression is correlated with sink strength and storage organ development [24, 49], SUS3 and SUS4 expression levels have been measured. The SUS3 and SUS4 were significantly up-regulated in 7D-SR, suggesting that sucrose was mobilized into metabolic pathways and storage functions [2]. The results from in vitro storage root induction support our model that AGL20 and PIN1 are involved in storage root initiation and confirm that high levels of cytokinin and low levels of auxin play significant roles in storage root initiation.

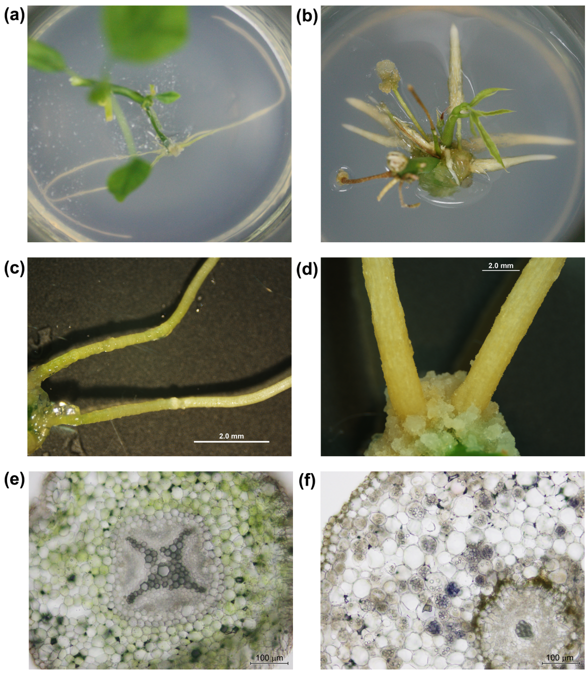


Fig. 13. Morphology and anatomy of in vitro KU50 storage root

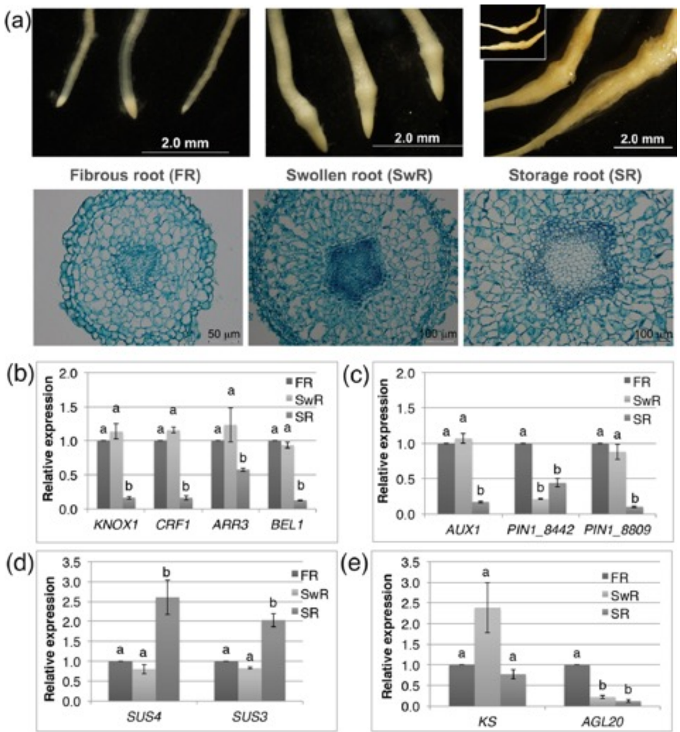


Fig. 14. The qRT-PCR expression profiles of the modeled key genes in in vitro KU50 storage root induction. (a) Fibrous root (FR) in non-hormonal treatment condition, swollen root (SwR) that were harvested at 24 hours after treatment in KU-BN and storage root (SR) that were harvested at 7 days after treatment in KU-BN. Cross-sections stained with fast green showed the development of vascular tissue and starch accumulation in SwR and SR. The bar shown are 2.0 mm in whole roots, 50 um in cross-section of fibrous root and 100 um in cross-sections of SwR and SR. (b) Expression level of KNOX1 and CK responsive genes (c) Expression level of polar auxin transport genes (d) Expression of sucrose synthase genes (e) Expression of GA responsive genes. Error bars correspond to the standard error. The a and b above the bar indicate statistically significant difference from FR with p-value $\leq$ 0.05

cDNA synthesis and sequence analysis of putative gene that involved in storage root initiation

From the results gathering from this project and the previous project “mRNA differentially display in leaf and storage root of *Manihot esculenta* Crantz (MRG5080119)”, 4 cadidate genes: MeKNOX I, MeAGL20, MeCDPK and MeHT that may play important roles in cassava storage root initiation were cloned for further investigation.

The cDNA sequence of putative MeKNOX 1, MeAGL20, MeCDPK, and Hexose transporter (MeHT154) were retrieved from Phytozome database. The gene specific primers for cDNA amplification were designed (Table 2). After cDNA amplification, at least 10 clones of each gene were submitted for sequencing analysis.

Seven clones of MeKNOX 1 cDNA from KU50 showed more than 99.7% sequence identity to Manes.05G184900.1 with 5 exons. MeKNOX 1 had C to A at position 77 causing amino acid change from P (CCC) to H (CAC), A to G at position 501 and C to T at position 1028 without amino acid change (Fig. 15). From BlastP of MeKNOX 1, the phylogenetic tree showed that at least 3 groups of Homeobox proteins: KNOTTED, BEL and Paired-like homeodomain proteins Phox2/Arix were found in cassava (Fig. 16a). MeKNOX 1 is evolutionary related to KNOTTED 1-like 2 from Hevea brasilensis, Jatropha curcas, Theobroma cacao, Herrania umbratica, Populus tomentosa with sequence identity more than 88% (Fig. 16b). There are 4 predicted domains in MeKNOX 1: KNOX1, KNOX2, ELK domain, and homeobox transcription factor KN domains (Fig. 16c). KNOX1 domain plays a role in suppressing target gene expression. KNOX2 is thought to be necessary for homo-dimerization, which is required for its function [28]. ELK domain is the nuclear localization signal[19]. homeobox transcription factor KN domain, conserved from fungi to human and plants, was first identified as TALE homeobox genes in eukaryotes, (including KNOX and MEIS genes) [19, 21, 23].

Table 1 Cassava gene locus and PCR primers for qRT-PCR

Gene Name	Phytozome locus	Arrray probe Name	Forward primer	Reverse Primer
ACO1	cassava4.1_012494m.g	R_Mes01_006029	GGAATTGATAAGAGTTTGATGGAG	TATGTACTCATCCATCCATTTGC
AdClc	cassava4.1_016571m	-	ACCCTTCACCAGCAAAAC	ACGGCTGACACCATTGAT
AGL20	scaffold03942	R_Mes01_013975	CACAAGCAGGCAAGTCACC	CTTCTTCACCATGTTCGTTGC
ARF4	cassava4.1_001979m.g	R_Mes01_010217	CTCCAGAAAGAAGATGTAGCGG	AACAATATCTTCATCCCACCTCAC
ARR3	cassava4.1_019933m.g	R_Mes01_000157	TGATCTTCTCATGGCTGTTAAGG	GCAAGACACCTGCTGATTCTC
AUX1	cassava4.1_006788m.g	R_Mes01_012207	CCTACACTGCCTGGTATTTGAC	ATGCATGATTTCCACAGTAACAG
BEL1	cassava4.1_006759m.g	R_Mes01_014179	AGCAGCCAATCTATCCTTCC	TTGTATCTTCTATCCACCTCATCC
CDPK	cassava4.1_004466m	-	GCAGTGGCAGTTCATGTT	GACTCCCAAAGAATGGCA
CEJ1	cassava4.1_029328m	-	TGTTCAAGTTCCAGCTCCAC	AGCCAAGCCAAATCCTAGAC
cINV	cassava4.1_004783m	-	AGCTGGTTGGAGAAATGC	CAGCCGTTAGCATCCATA
CRF1	cassava4.1_033707m.g	R_Mes01_011244	CGGAAGAAGTTCCGAGGTG	TCCGTCATAGCAGGATTTGG
cwINV	cassava4.1_004485m	-	GGCTTTTGTGGATGTGGA	AAGTGGGCAGCCTCATTT
DELLA 29701	cassava4.1_029701m	-	TGATTCTGTTGTGGCTGCAC	ATGAAACACCACTCCACGACCG
DELLA 33968	cassava4.1_033968m	-	ACAGTCCACTATAATCCCTCCG	TTGTTGGTGGGTATGTTCGG
EF1a	cassava4.1_007545m.g	-	AGCGTGTGATTGAGAGGT	TCGAACTTCCACAAGGCA
Extensin	cassava4.1_013581m.g	R_Mes01_014709	CCACCACCTCCCAAGAAACC	GGTGGTGGTGGTGATTTGTG
F16BP	cassava4.1_011197m.g	R_Mes01_005127	GCTCAATCATATTGTTCTTGGCTG	CAAGTATGCATGTTCGTCCAC
G3PD	cassava4.1_011366m.g	R_Mes01_000569	CTGCTATCAAGGAGGAATCAGAG	GATCAAGTCAACCACCCGAG
GBSS 3884	cassava4.1_003884m	-	TGGCACTTATGCTACCGCTG	TGCTGCTCAAGGCGTGG
GBSS 3916	cassava4.1_003916m	-	CCATTGCCACTTATACCACCAG	CACCTTCATCCTTGCTGGTTC
HT	cassava4.1_005379m	-	TCCTGCATAATGTTTGGTTACGAC	AGTAGTTGCTGTTGATTGTGGG
Hxk	cassava4.1_006138m	-	TGGAATCATGGGCATCCT	CCCAGCAATTCCTTGAGA
IAA8	cassava4.1_012069m.g	R_Mes01_011380	TTACAATTGGTCAGTGTGGCTC	AACAAGCATCCAATCACCGTC
IAA9	cassava4.1_010095m.g	R_Mes01_004173	CAAAGAACACGGAAGAAGTGG	CATATTGGCCTATGGTAAAGCAG
KNOX1	cassava4.1_010084m.g	R_Mes01_010762	AAGCCAAGATCATAGCTCATCC	CGCAATAAGCCTCCATAAACTG
KS	cassava4.1_001987m.g	R_Mes01_008718	TTGAGATATGGCTCATATTGCTGG	TGCACTCGACATATAATCATCCAC
LTP	cassava4.1_009864m.g	R_Mes01_010934	TACTATCCAAGTCCACCTGTAAC	GACGTCGACACATGCTC
NAC100	cassava4.1_010869m.g	R_Mes01_021508	AAACAGCAAAGAATGAATGGG	CCACATGAGCTTACTCTTCCC
NAC5	cassava4.1_010010m.g	R_Mes01_021081	AATGAGGCTTGATGATTGGG	CTTAATGGATTCTTCAAGCTCGG
PIN1-8442	cassava4.1_008442m.g	-	AGCACCACTCCATGAACAAG	GTTCCTTCCCCAAGTAATGTG
PIN1-8809	cassava4.1_008809m.g	R_Mes01_009843	CAACCTCATTCAAGGCTTAAAGTC	ACCAATATTCATGGCAGGTGG
PIP1-11898	cassava4.1_011898m.g	R_Mes01_002883	CAGACTGACAAAGACTACAAGGAG	TGATGTGTCCACCTGAGATACC
PPR1	cassava4.1_008103m.g	R_Mes01_006418	CTTTAGCACGCAAGATAATATGGG	CAAAGCATCATAGCTGAAAGGG
RasGp	cassava4.1_023622m	-	AGCTTGGAGATGACAGGA	CAGGCAAGAGGAACATGA
Spiral1	cassava4.1_019590m.g	R_Mes01_013404	GTTGAAGCGCCTCCTAAG	GCCATCAGCTCGGAAGTAG
SUS3	cassava4.1_001867m.g	-	GCCACAAGGGAAAGAACA	GGCCAATTTCCTGGAACT
SUS4	cassava4.1_001871m.g	R_Mes01_010814	CCCAATGGTGTTCAATGTTGTG	TCGAGTGATAATGAGAATGCGG
SUT	cassava4.1_004111m	-	CTGGTCAAGGACTTTCTTTGGG	TTGAGGAGATGGAAGTAAGGTGAG
TPR1	cassava4.1_000638m.g	R_Mes01_011849	ACATATCTTGCTTTCCACCCTC	GCTTGCTCTTAACCTCATCCAC

Table 2. Gene specific primers for cDNA amplification

Primer	Sequence
CDPKM300Bgl_F	5’ AAGATCTTGGAAATAATTGTGTTGGATCAAGGGG 3’
CDPKBstE_R	5’ TGGTCACCTCAACAAACTTGCAATGCCTCTCTAAAGCC 3'
AGL20Bgl_F	5’ AAGATCTTGTGAGAGGAAAGACTCAAATGAGG 3’
AGL20BstE_R	5’ AGGTCACCTCATTGGAGAAGGCGCTGGGTTCTG 3’
KNOXBgl_F	5’ TAGATCTTGAAGGCTATAATCATATAAATGATAACACAAATCC 3’
KNOXBstE_R	5’ TGGTCACCTCATGGCCCTAGACGATAAGG 3’
HT154Nco_F	5’ GAccATGGccATGCCTGCAGGAGGTTTCTC 3’
HT154Xma_R	5’ CACCCGGGAAACTGGGTAACAGGATCAAAACC 3’
pCDPKHind_F	5’ TAAGCTTGGTATGGTGGAGAATGCGGTG 3’
pCDPKNco_R	5’ ACCATGGCCTCATTTTCTCGCGTATTGG 3’
pHT154_F	5’ AGAGCTCCATGATGATAATGTGTCAG 3’
pHT154_R	5’ ACCATGGGATAACGTAGACAACAAG 3’

a) cDNA sequence of MeKNOX1

TGGAAGGCTATAATCATATAAATGATAACACAAATCCGAGGGGGAATTTCTTGTATGCTTCAGCACTTCTTGCACACAATTCTTCT
CCTTATGGTAGAACAAATAGTGGCTCTAACGTGAGCAATCAGCAGACCCAGATGCCTTTAAGTCCTTTCCATCTTCAATCAAGTG
AATGTTTCCAATCTGAAGCACATCCTATTGTGAAGACAGAAGCCAGCACTTCCCACCATGTTCAAAAATTTCACTACCCTTTATTA
AGAGGGCATCATCAAGCACTTAATCAACATCAAGGGAATGAAAGCTCCAGTGATGTGGAAGCTATCAAAGCCAAGATCATAGCTC
ATCCACAGTACTTTAAACTTTTGGAAGCATACATGGATTGCCAAAAGGTGGGAGCTCCGCCGGAAGTAGTAGCAAGGCTTGCCG
CTGCTCGCCAAGATTTCGAAGCTAAGCAACGATCTTCAGTCACTTCCAGGGATGCTTCGAAAGACCCAGAACTGGATCAGTTTAT
GGAGGCTTATTGCGATATGCTGGTGAAATACCGGGAAGAGCTTACAAGACCCATTCAAGAAGCCATGGATTTCATGCGAAGAAT
CGAAACACAACTAAATACGATCTGCAATGGCCCCTTGCGGATCTTCAACTCCGATGAGAAGTCTGAGGGTGTCGGGTCGTCCGA
GGAAGATCAGGAGAACAGTGGTGGGGAAACAGAACTACCGGAGATTGATCCCAGGGCTGAGGACCGAGAACTAAAGAACCACT
TGTTGAGGAAATATAGTGGATATTTGAGCAGTCTTAAGCAGGAGCTTTCTAAGAAGAAGAAGAAGGGAAAACTACCCAAAGAAGC
AAGACAGAAACTACTTAACTGGTGGGAGTTGCACTACAAATGGCCATATCCTTCGGAGACTGAGAAGGTGGCATTGGCTGAATC
AACGGGTTTGGACCAGAAACAAATAAATAACTGGTTCATAAATCAAAGGAAACGGCATTGGAAGCCGTCAGAAGATATGCAATTC
ATGGTGATGGATGGTCTCCATCCACAGAGTGCAGCACTCTACATGGATGGTCACTACATGGGTGATGGTCCTTATCGTCTAGGG
CCATGAGGTGACC

b) Predicted MeKNOX 1

MEGYNHINDNTNPRGNFLYASALLAHNSSPYGRTNSGSNVSNQQTQMPLSPFHLQSSECFQSEAHPIVKTEASTS
HHVQKFHYPLLRGHHQALNQHQGNESSSDVEAIKAKIIAHPQYFKLLEAYMDCQKVGAPPEVVARLAAARQDFEA
KQRSSVTSRDASKDPELDQFMEAYCDMLVKYREELTRPIQEAMDFMRRIETQLNTICNGPLRIFNSDEKSEGVGS
SEEDQENSGGETELPEIDPRAEDRELKNHLLRKYSGYLSSLKQELSKKKKKGKLPKEARQKLLNWWELHYKWPY
PSETEKVALAESTGLDQKQINNWFINQRKRHWKPSEDMQFMVMDGLHPQSAALYMDGHYMGDGPYRLGP*

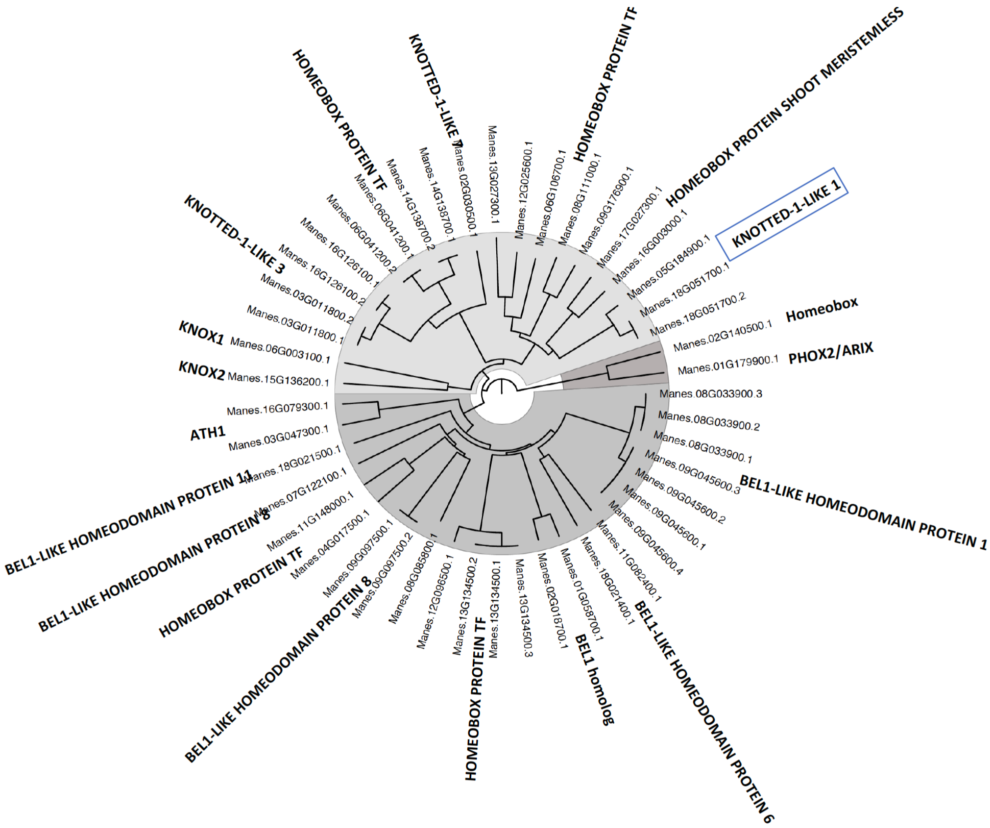
c) DNA sequence alignment of MeKNOX 1

```
Manes.05G184900.1       ATGGAAGGCTATAATCATATAAATGATAACACAAATCCGAGGGGGAATTTCTTGTATGCT   60
KNOX1                   ATGGAAGGCTATAATCATATAAATGATAACACAAATCCGAGGGGGAATTTCTTGTATGCT   60
                        ************************************************************

Manes.05G184900.1       TCAGCACTTCTTGCACCAATTCTTCTCCTTATGGTAGAACAAATAGTGGCTCTAACGTG   120
KNOX1                   TCAGCACTTCTTGCACACAATTCTTCTCCTTATGGTAGAACAAATAGTGGCTCTAACGTG  120
                        **************** *******************************************

Manes.05G184900.1       AGCAATCAGCAGACCCAGATGCCTTTAAGTCCTTTCCATCTTCAATCAAGTGAATGTTTC  180
KNOX1                   AGCAATCAGCAGACCCAGATGCCTTTAAGTCCTTTCCATCTTCAATCAAGTGAATGTTTC  180
                        ************************************************************

Manes.05G184900.1       CAATCTGAAGCACATCCTATTGTGAAGACAGAAGCCAGCACTTCCCACCATGTTCAAAAA  240
KNOX1                   CAATCTGAAGCACATCCTATTGTGAAGACAGAAGCCAGCACTTCCCACCATGTTCAAAAA  240
                        ************************************************************

Manes.05G184900.1       TTTCACTACCCTTTATTAAGAGGGCATCATCAAGCACTTAATCAACATCAAGGGAATGAA  300
KNOX1                   TTTCACTACCCTTTATTAAGAGGGCATCATCAAGCACTTAATCAACATCAAGGGAATGAA  300
                        ************************************************************

Manes.05G184900.1       AGCTCCAGTGATGTGGAAGCTATCAAAGCCAAGATCATAGCTCATCCACAGTACTTTAAA  360
KNOX1                   AGCTCCAGTGATGTGGAAGCTATCAAAGCCAAGATCATAGCTCATCCACAGTACTTTAAA  360
                        ************************************************************

Manes.05G184900.1       CTTTTGGAAGCATACATGGATTGCCAAAAGGTGGGAGCTCCGCCGGAAGTAGTAGCAAGG  420
KNOX1                   CTTTTGGAAGCATACATGGATTGCCAAAAGGTGGGAGCTCCGCCGGAAGTAGTAGCAAGG  420
                        ************************************************************

Manes.05G184900.1       CTTGCCGCTGCTCGCCAAGATTTCGAAGCTAAGCAACGATCTTCAGTCACTTCCAGGGAT  480
KNOX1                   CTTGCCGCTGCTCGCCAAGATTTCGAAGCTAAGCAACGATCTTCAGTCACTTCCAGGGAT  480
                        ************************************************************

Manes.05G184900.1       GCTTCGAAAGACCCAGAACTAGATCAGTTTATGGAGGCTTATTGCGATATGCTGGTGAAA  540
KNOX1                   GCTTCGAAAGACCCAGAACTGGATCAGTTTATGGAGGCTTATTGCGATATGCTGGTGAAA  540
                        ******************** ***************************************

Manes.05G184900.1       TACCGGGAAGAGCTTACAAGACCCATTCAAGAAGCCATGGATTTCATGCGAAGAATCGAA  600
KNOX1                   TACCGGGAAGAGCTTACAAGACCCATTCAAGAAGCCATGGATTTCATGCGAAGAATCGAA  600
                        ************************************************************

Manes.05G184900.1       ACACAACTAAATACGATCTGCAATGGCCCCTTGCGGATCTTCAACTCCGATGAGAAGTCT  660
KNOX1                   ACACAACTAAATACGATCTGCAATGGCCCCTTGCGGATCTTCAACTCCGATGAGAAGTCT  660
                        ************************************************************

Manes.05G184900.1       GAGGGTGTCGGGTCGTCCGAGGAAGATCAGGAGAACAGTGGTGGGGAAACAGAACTACCG  720
KNOX1                   GAGGGTGTCGGGTCGTCCGAGGAAGATCAGGAGAACAGTGGTGGGGAAACAGAACTACCG  720
                        ************************************************************

Manes.05G184900.1       GAGATTGATCCCAGGGCTGAGGACCGAGAACTAAAGAACCACTTGTTGAGGAAATATAGT  780
KNOX1                   GAGATTGATCCCAGGGCTGAGGACCGAGAACTAAAGAACCACTTGTTGAGGAAATATAGT  780
                        ************************************************************

Manes.05G184900.1       GGATATTTGAGCAGTCTTAAGCAGGAGCTTTCTAAGAAGAAGAAGAAGGGAAAACTACCC  840
KNOX1                   GGATATTTGAGCAGTCTTAAGCAGGAGCTTTCTAAGAAGAAGAAGAAGGGAAAACTACCC  840
                        ************************************************************

Manes.05G184900.1       AAAGAAGCAAGACAGAAACTACTTAACTGGTGGGAGTTGCACTACAAATGGCCATATCCT  900
KNOX1                   AAAGAagcaagacagaaactacttaactggtgggagttgcactacaaatggccatatcct  900
                        ************************************************************

Manes.05G184900.1       TCGGAGACTGAGAAGGTGGCATTGGCTGAATCAACGGGTTTGGACCAGAAACAAATAAAT  960
KNOX1                   tcggagactgagaaggtggcattggctgaatcaacgggtttggaccagaaacaaataaat  960
                        ************************************************************

Manes.05G184900.1       AACTGGTTCATAAATCAAAGGAAACGGCATTGGAAGCCGTCAGAAGATATGCAATTCATG  1020
KNOX1                   aactggttcataaatcaaaggaaacggcattggaagccgtcagaagatatgcaattcatg  1020
                        ************************************************************

Manes.05G184900.1       GTGATGGACGGTCTCCATCCACAGAGTGCAGCACTCTACATGGATGGTCACTACATGGGT  1080
KNOX1                   gtgatggatggtctccatccacagagtgcagcactctacatggatggtcactacatgggt  1080
                        ******** ***************************************************

Manes.05G184900.1       GATGGTCCTTATCGTCTAGGGCCATGA        1107
KNOX1                   gatggtccttatcgtctagggccatga        1115
                        ***************************
```

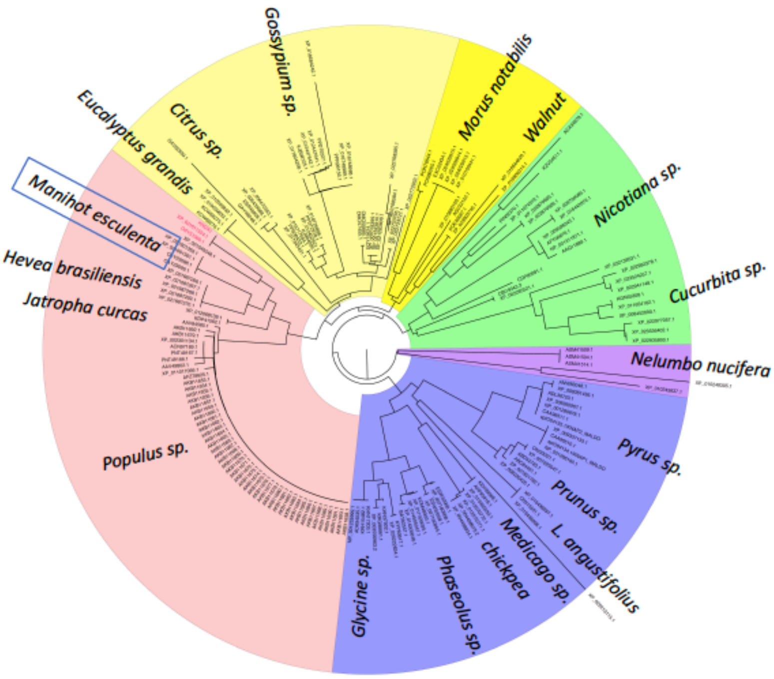
d) Protein sequence alignment of MeKNOX I

```
Manes.05G184900.1       MEGYNHINDNTNPRGNFLYASALLAPNSSPYGRTNSGSNVSNQQTQMPLSPFHLQSSECF  60
KNOX                    MEGYNHINDNTNPRGNFLYASALLAHNSSPYGRTNSGSNVSNQQTQMPLSPFHLQSSECF  60
                        ************************* **********************************

Manes.05G184900.1       QSEAHPIVKTEASTSHHVQKFHYPLLRGHHQALNQHQGNESSSDVEAIKAKIIAHPQYFK  120
KNOX                    QSEAHPIVKTEASTSHHVQKFHYPLLRGHHQALNQHQGNESSSDVEAIKAKIIAHPQYFK  120
                        ************************************************************

Manes.05G184900.1       LLEAYMDCQKVGAPPEVVARLAAARQDFEAKQRSSVTSRDASKDPELDQFMEAYCDMLVK  180
KNOX                    LLEAYMDCQKVGAPPEVVARLAAARQDFEAKQRSSVTSRDASKDPELDQFMEAYCDMLVK  180
                        ************************************************************

Manes.05G184900.1       YREELTRPIQEAMDFMRRIETQLNTICNGPLRIFNSDEKSEGVGSSEEDQENSGGETELP  240
KNOX                    YREELTRPIQEAMDFMRRIETQLNTICNGPLRIFNSDEKSEGVGSSEEDQENSGGETELP  240
                        ************************************************************

Manes.05G184900.1       EIDPRAEDRELKNHLLRKYSGYLSSLKQELSKKKKKGKLPKEARQKLLNWWELHYKWPYP  300
KNOX                    EIDPRAEDRELKNHLLRKYSGYLSSLKQELSKKKKKGKLPKEARQKLLNWWELHYKWPYP  300
                        ************************************************************

Manes.05G184900.1       SETEKVALAESTGLDQKQINNWFINQRKRHWKPSEDMQFMVMDGLHPQSAALYMDGHYMG  360
KNOX                    SETEKVALAESTGLDQKQINNWFINQRKRHWKPSEDMQFMVMDGLHPQSAALYMDGHYMG  360
                        ************************************************************

Manes.05G184900.1       DGPYRLGP        368
KNOX                    DGPYRLGP        368
                        ********
```

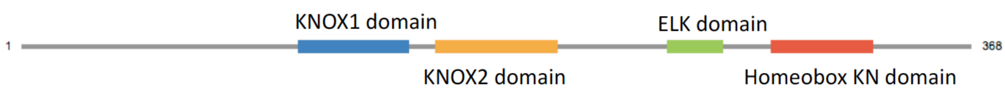
Fig. 15 The DNA sequence analysis of MeKNOX 1 a) cDNA sequence of MeKNOX 1 b) Predicted protein sequence of MeKNOX 1 c) DNA sequence alignment of MeKNOX 1 and Manes.05G184900.1 d) Protein squence alignment of MeKNOX 1 and Manes.05G184900.1



a)



b)



c)

Fig. 16 Phylogenetic tree and domain prediction of MeKNOX 1 a) Homeodomain proteins in cassava, b) Phylogenetic tree of MeKNOX 1 and c) domain prediction of MeKNOX 1

Three clones of AGL20 showed 100% sequence identity to Manes.05G041900.1 (Fig. 17) with 7 exons. From BlastP of MeAGL20, the phylogenetic tree showed that at least 4 groups of MADS-box proteins (Fig. 18a). MeAGL20 is evolutionary related to MADS-box protein SOC1 like of Hevea brasilensis, Ricinus communis, Jatropha curcas, Theobroma cacao, Populus sp. with sequence identity more than 75% (Fig. 18b) and 71% sequence identity to AtSOC1. There are 2 domains in MeAGL20: SRF-type transcription factor and K-box region domains (Fig. 18c) with Conserved C-terminal SOC1 motif: DVETELFIGPP. The K-box region is commonly found associated with SRF-type transcription factors. The K-box is a possible coiled-coil structure with possible role in multimer formation.

a) cDNA sequence of AGL20

ATGGTGAGAGGAAAGACTCAAATGAGGCGTATAGAGAACGCCACAAGCAGGCAAGTCACCTTCTCCAAGCG
GCGAAATGGGTTGCTGAAAAAGGCCTTTGAGCTTTCTGTTCTTTGCGATGCAGAGGTTGCCCTTATCATCTTT
TCTCCAAGAGGGAAGCTCTATGAATTTGCAAACTCCAGCATGCAGGAGACAATTGAACGTTATCGTAGACATG
TGAAAGACACCAAAATCAACAAGCAAACATCTGAAGAAGACATGCTGCTACTGAAGACGGAAGCAACGAACA
TGGTGAAGAAGATAGAGCTCCTTGAAATTGCAAAAAGGAAACTACTGGGAGAAGGTTTGGGTGCCTGCACAA
TTGAAGAACTACAGCAGATAGAACAACAGCTGGAGAGGAGCGTAAGCAACATCAGAGCTAGAAAGAATCAGG
TTTTTAAAGAACAGATTGAGCGACTAAAAGAAAAGGAGAAACAATTGGAAGATGAAAATGCAAAGTTGTCTGA
AAAGTGCGGTGCCCATAAATGGCAAGGCCTGAAAATGGTGGAAGAAAGTAGAGCCTGTGAAGAACGTAGCC
CAGTTTCAGATGTGGAAACTGAACTTTTCATTGGACCGCCAGAAACCAGAACCCAGCGCCTTCTCCAATGA

b) Predicted AGL20

MVRGKTQMRRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVALIIFSPRGKLYEFANSSMQETIERYRRHVKDT
KINKQTSEEDMLLLKTEATNMVKKIELLEIAKRKLLGEGLGACTIEELQQIEQQLERSVSNIRARKNQVFKEQIERLKE
KEKQLEDENAKLSEKCGAHKWQGLKMVEESRACEERSPVSDVETELFIGPPETRTQRLLQ

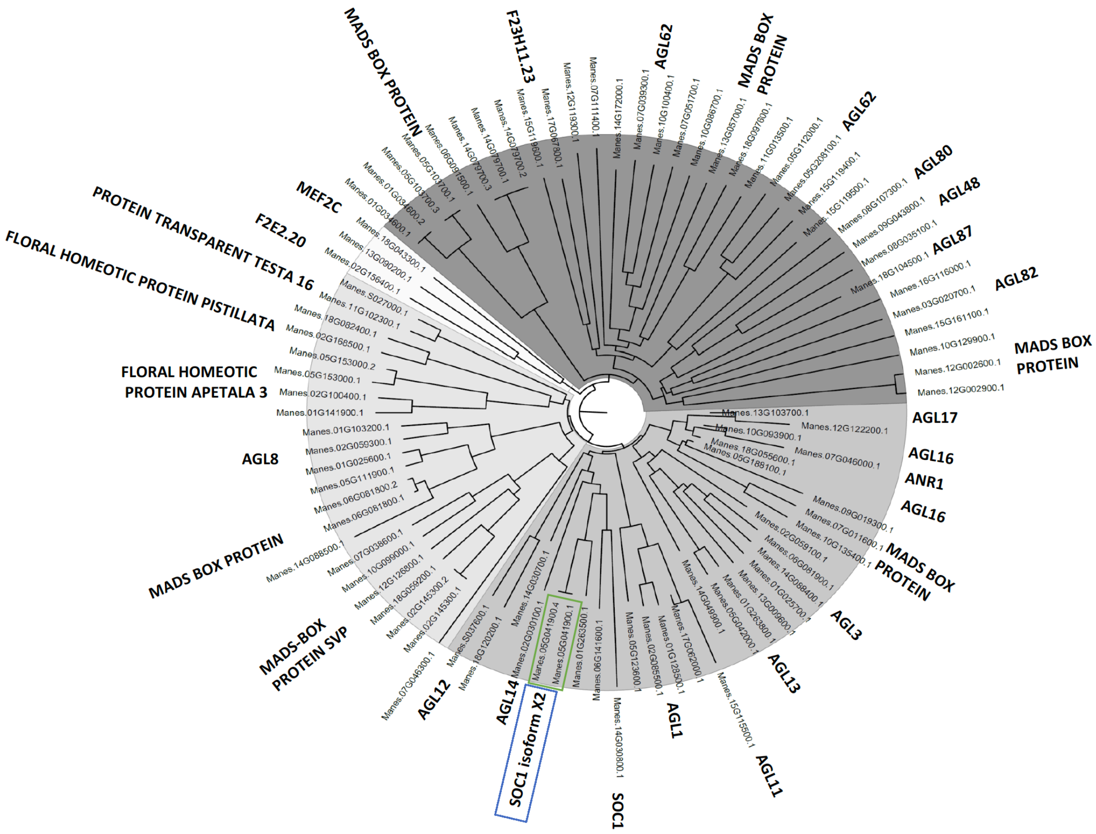
c) DNA sequence alignment of MeAGL20

```
AGL20              ATGGTGAGAGGAAAGACTCAAATGAGGCGTATAGAGAACGCCACAAGCAGGCAAGTCACC    60
Manes.05G041900    ATGGTGAGAGGAAAGACTCAAATGAGGCGTATAGAGAACGCCACAAGCAGGCAAGTCACC    60
                   ************************************************************

AGL20              TTCTCCAAGCGGCGAAATGGGTTGCTGAAAAAGGCCTTTGAGCTTTCTGTTCTTTGCGAT    120
Manes.05G041900    TTCTCCAAGCGGCGAAATGGGTTGCTGAAAAAGGCCTTTGAGCTTTCTGTTCTTTGCGAT    120
                   ************************************************************

AGL20              GCAGAGGTTGCCCTTATCATCTTTTCTCCAAGAGGGAAGCTCTATGAATTTGCAAACTCC    180
Manes.05G041900    GCAGAGGTTGCCCTTATCATCTTTTCTCCAAGAGGGAAGCTCTATGAATTTGCAAACTCC    180
                   ************************************************************

AGL20              AGCATGCAGGAGACAATTGAACGTTATCGTAGACATGTGAAAGACACCAAAATCAACAAG    240
Manes.05G041900    AGCATGCAGGAGACAATTGAACGTTATCGTAGACATGTGAAAGACACCAAAATCAACAAG    240
                   ************************************************************

AGL20              CAAACATCTGAAGAAGACATGCTGCTACTGAAGACGGAAGCAACGAACATGGTGAAGAAG    300
Manes.05G041900    CAAACATCTGAAGAAGACATGCTGCTACTGAAGACGGAAGCAACGAACATGGTGAAGAAG    300
                   ************************************************************

AGL20              ATAGAGCTCCTTGAAATTGCAAAAAGGAAACTACTGGGAGAAGGTTTGGGTGCCTGCACA    360
Manes.05G041900    ATAGAGCTCCTTGAAATTGCAAAAAGGAAACTACTGGGAGAAGGTTTGGGTGCCTGCACA    360
                   ************************************************************

AGL20              ATTGAAGAACTACAGCAGATAGAACAACAGCTGGAGAGGAGCGTAAGCAACATCAGAGCT    420
Manes.05G041900    ATTGAAGAACTACAGCAGATAGAACAACAGCTGGAGAGGAGCGTAAGCAACATCAGAGCT    420
                   ************************************************************

AGL20              AGAAAGAATCAGGTTTTTAAAGAACAGATTGAGCGACTAAAAGAAAAGGAGAAACAATTG    480
Manes.05G041900    AGAAAGAATCAGGTTTTTAAAGAACAGATTGAGCGACTAAAAGAAAAGGAGAAACAATTG    480
                   ************************************************************

AGL20              GAAGATGAAAATGCAAAGTTGTCTGAAAAGTGCGGTGCCCATAAATGGCAAGGCCTGAAA    540
Manes.05G041900    GAAGATGAAAATGCAAAGTTGTCTGAAAAGTGCGGTGCCCATAAATGGCAAGGCCTGAAA    540
                   ************************************************************

AGL20              ATGGTGGAAGAAAGTAGAGCCTGTGAAGAACGTAGCCCAGTTTCAGATGTGGAAACTGAA    600
Manes.05G041900    ATGGTGGAAGAAAGTAGAGCCTGTGAAGAACGTAGCCCAGTTTCAGATGTGGAAACTGAA    600
                   ************************************************************

AGL20              CTTTTCATTGGACCGCCAGAAACCAGAACCCAGCGCCTTCTCCAATGA        648
Manes.05G041900    CTTTTCATTGGACCGCCAGAAACCAGAACCCAGCGCCTTCTCCAATGA        648
                   ************************************************
```

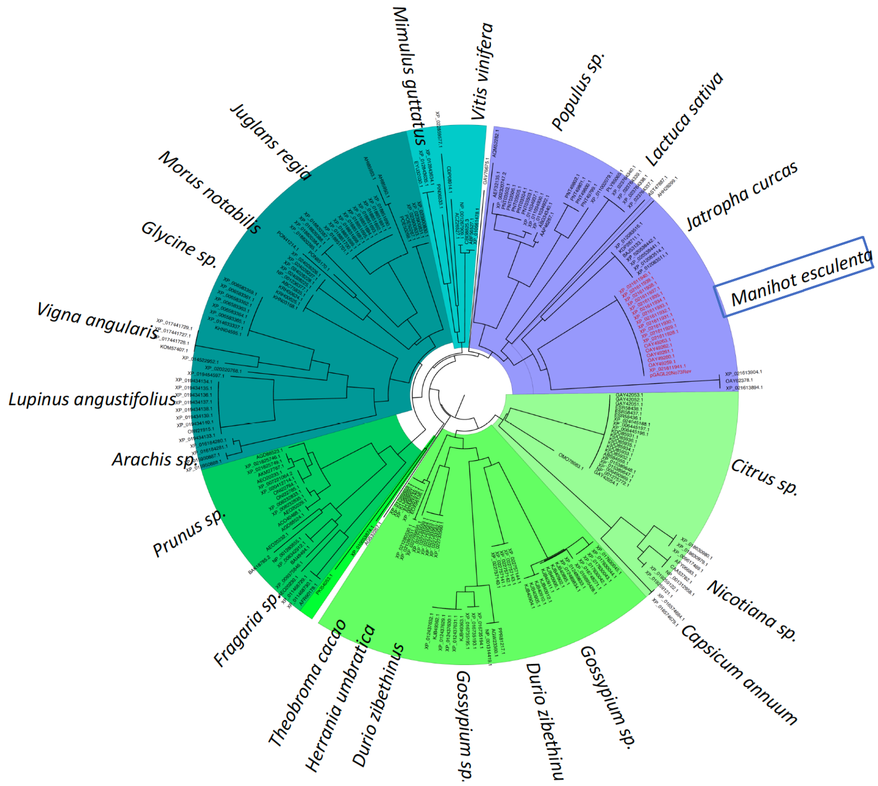
d) Amino acid sequence alignment of MeAGL20

```
AGL20               MVRGKTQMRRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVALIIFSPRGKLYEFANS      60
Manes.05G041900     MVRGKTQMRRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVALIIFSPRGKLYEFANS      60
                    ************************************************************

AGL20               SMQETIERYRRHVKDTKINKQTSEEDMLLLKTEATNMVKKIELLEIAKRKLLGEGLGACT      120
Manes.05G041900     SMQETIERYRRHVKDTKINKQTSEEDMLLLKTEATNMVKKIELLEIAKRKLLGEGLGACT      120
                    ************************************************************

AGL20               IEELQQIEQQLERSVSNIRARKNQVFKEQIERLKEKEKQLEDENAKLSEKCGAHKWQGLK      180
Manes.05G041900     IEELQQIEQQLERSVSNIRARKNQVFKEQIERLKEKEKQLEDENAKLSEKCGAHKWQGLK      180
                    ************************************************************

AGL20               MVEESRACEERSPVSDVETELFIGPPETRTQRLLQ 215
Manes.05G041900     MVEESRACEERSPVSDVETELFIGPPETRTQRLLQ 215
                    ***********************************
```

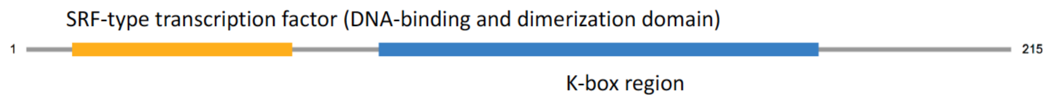
Fig. 17 The DNA sequence analysis of MeAGL20 a) cDNA sequence of MeAGL20 b) Predicted protein sequence of MeAGL20 c) DNA sequence alignment of MeAGL20 and Manes.05G041900.3 d) amino acid sequence alignment of MeAGL20 and Manes.05G041900.3



a)



b)



c)

Fig. 18 Phylogenetic tree and domain prediction of MeAGL20 a) MADS-box protein in cassava, b) Phylogenetic tree of MeAGL20 and c) domain prediction of MeAGL20

From eleven clones of CDPK cDNA, eight clones showed more than 99.8% DNA sequence identity to Manes.08G036300.1 (Fig. 19) with 7 exons. Two isoforms: CDPK54 and CDPK36 were amplified from KU50. CDPK54 have "CAGCCAGAAGCTACGCCTAAACAATCACCATTT" insertion at position 550 and mutation at position 609 from A to G without amino acid change. CDPK36 have nucleotide deletion at position 540-572 and mutation at position 615 from A to T with amino acid change from L to F (Fig 19). The insertion and deletion parts caused different in numbers of repeated amino acid sequence "SPFQPEATPKQ". There were 3 copies in Manes.08G036300, 4 copies in CDPK54 and 2 copies in CDPK36. From BlastP of MeCDPK, the phylogenetic tree showed that at least 4 groups of calcium dependent protein kinase (Fig. 20a). MeCDPK is evolutionary related to calcium dependent protein kinase of *Hevea brasilensis, Recinus communis, Jatropha curcas, Populus* sp. with sequence identity more than 80% (Fig. 20b). There are 2 domains in MeCDPK: protein tyrosine kinase and EF-hand domain pair (Fig. 20c). A tyrosine kinase functions as an "on" or "off" switch in many cellular functions by transferring a phosphate group from ATP to other proteins. Phosphorylation of proteins by kinases is an important mechanism in communicating signals within a cell (signal transduction) and regulating cellular activity, such as cell division. The EF hand is a helix-loop-helix structural domain or motif found in a large family of calcium-binding proteins. The EF-hand consists of two alpha helices linked by a short loop region (usually about 12 amino acids) that usually binds calcium ions. EF-hands also appear in each structural domain of the signaling protein calmodulin.

a) cDNA sequence of CDPK54

ATGGGAAATAATTGTGTTGGATCAAGGGGTTGGTGGATGCGACCAACGAATGGCCAGATTACTCATCCTACA
GGAGAAGGCATTGAAGGTGACCCTGTGAATAAAGAGCCACAAGATGTTTTACAAGCTCCACAAGAGCCCCCG
GAGCAAATGAAGATAACTAAGGAAGAGATTGTTGAGATTAAATCAGAACCATTGCCACCAGAACCCAAGGAA
GAGACTAAAGAAGAAGAGATTGTTGAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAA
GAAGAAGAGATTGTTGAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCA
TTACAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCATTACAGATTAAAT
CAGCACCACTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCATTACAGCCAGAAGCTACGCCTAAAC
AATCACCATTTCAGCCAGAAGCTACGCCTAAACAATCACCATTTCAGCCAGAAGCTACGCCTAAACAATCACC
ATTTCAGCCAGAAGCTACGCCTAAACAATCACCATTTCAGCCAGAAGCTACGCCTAAACAATCGCCATTACAG
CCAGAACACGAGGAGGAGACTGGACCAGCTAAGGAGGAGACTCATCAAGTACCATCACCCCCAAAGCCAAA
GGAAGAGACCGAATCATCAGAGGTCTCTTATCACAGGGAAGACAGTACACCAGCACAGCCAATAACACACAA
AGAAGAAGAAAAACCAGCAGTGTCCCCAGGACCAAGAAAACCCCATAATGTTAAGAGATTATCTAGTGCAGG
ACTTCAAGCAGAGTCGGTATTGAGAGTGAAAACAGGTAATTTGAAAGAGTACTACAGCTTGGGAAGGAAGCT
TGGACATGGGCAATTTGGAACAACTTTTCTTTGTGTGGAGAAAGGGACAGGGAAACAGTATGCTTGCAAATC
CATTGCCAAAAGGAAACTAACAACACCAGATGACGTTGAGGATGTGAGGAGAGAAATTCAGATAATGCATCA
CTTGTCAGGCCATCCTAATGTTGTATCGATCAAAGAGGCGTATGAAGATGCAGTGGCAGTTCATGTTGTGATG
GAATTGTGTGCTGGAGGTGAGCTCTTTGACAGGATTATTACGCGAGGTCATTACACGGAAAAAAAGGCAGCT
CAACTCACTAAGATTATAGTTGGTGTTATAGAAGCTTGCCATTCTTTGGGAGTCATGCATAGAGATTTGAAGC
CTGAGAATTTTCTCTTTGTCAATGAAGAGGAGGACTCACCTCTCAAAGCAATAGACTTTGGATTATCAGTGTTT
TTCAAGCCCGGGGAGACATTTACTGATGTGGTTGGAAGCCCATACTATGTGGCACCTGAAGTTCTGAAGAAA
TGGTATGGTCATGAAGCAGATGTTTGGAGTGCTGGAGTTATGGTTTACATTCTCTTAAGTGGGGTACCTCCAT
TCTGGGCTGAAACTGAGCAAGGCATATTTGACGAGGTACTGAATGGTGAGTTGGATTTCGTATCAGATCCAT
GGCCTAACATCTCTGAAAGTGCAAAGGATTTGGTTACGAGAATGCTTGTCAGGGACCCCAAGCAACGAATTA
CTGCACATGAAGTTCTTTGTCACCCTTGGGTTCGGGATGATGGGGTTGCTCCTGATAAGCCTCTTGATCCTGC
AGTTTTATCTCGCTTGAAGCAGTTTTCTGCAATGAACAAGCTTAAGAAAATGGCTCTTAGAATTATTGCTGAGA
ACCTCTCTGAAGAAGAAATTGCTGGGTTAACAGAAATGTTTAAGATGATAGACACAGATGGCAGTGGTCAAAT
TACTTTCGAAGAACTCAAAGTGGGACTAAAGAGATTTGGTGCTAATCTCAATGACTCTGAAATTTATGACCTAA
TGCAGGCGGCTGATATTGATAATAGTGGGACAATAGACTATGGGGAGTTCATAGCTGCCACATTGCATCTAAA
CAAAGTAGAAAAGGAAGATCATCTATTTGCAGCTTTCTCATACTTTGACAAAGATGGCAGTGGCTATATAACTC
TAGATGAACTGCAACAAGCTTGTAGCGAATTCGGCATGGAGGATGTTCAGTTGGAAGAAATGATAAGAGAAG
TTGATCAGGACAATGATGGTCGGATAGATTACAATGAGTTTGTGGACATGATGCAAATGGGTAAGAACGGAC
GACAACATAGAAGTTTCAGTGTTGGCTTTAGAGAGGCATTGCAAGTTTGTTGAGGTGA

b) Predicted CDPK54

MGNNCVGSRGWWMRPTNGQITHPTGEGIEGDPVNKEPQDVLQAPQEPPEQMKITKEEIVEIKSEPLPPEPKEETK
EEEIVEIKSAPLPPEPKEETKEEEIVEIKSAPLPPEPKEETKESPLQIKSAPLPPEPKEETKESPLQIKSAPLPPEPKEE
TKESPLQPEATPKQSPFQPEATPKQSPFQPEATPKQSPFQPEATPKQSPFQPEATPKQSPLQPEHEEETGPAKE
ETHQVPSPPKPKEETESSEVSYHREDSTPAQPITHKEEEKPAVSPGPRKPHNVKRLSSAGLQAESVLRVKTGNLK
EYYSLGRKLGHGQFGTTFLCVEKGTGKQYACKSIAKRKLTTPDDVEDVRREIQIMHHLSGHPNVVSIKEAYEDAVA
VHVVMELCAGGELFDRIITRGHYTEKKAAQLTKIIVGVIEACHSLGVMHRDLKPENFLFVNEEEDSPLKAIDFGLSVF

FKPGETFTDVVGSPYYVAPEVLKKWYGHEADVWSAGVMVYILLSGVPPFWAETEQGIFDEVLNGELDFVSDPWP
NISESAKDLVTRMLVRDPKQRITAHEVLCHPWVRDDGVAPDKPLDPAVLSRLKQFSAMNKLKKMALRIIAENLSEE
EIAGLTEMFKMIDTDGSGQITFEELKVGLKRFGANLNDSEIYDLMQAADIDNSGTIDYGEFIAATLHLNKVEKEDHLF
AAFSYFDKDGSGYITLDELQQACSEFGMEDVQLEEMIREVDQDNDGRIDYNEFVDMMQMGKNGRQHRSFSVGF
REALQVC

c) cDNA sequence of CDPK36

ATGGGAAATAATTGTGTTGGATCAAGGGGTTGGTGGATGCGACCAACGAATGGCCAGATTACTCATCCTACA
GGAGAAGGCATTGAAGGTGACCCTGTGAATAAAGAGCCACAAGATGTTTTACAAGCTCCACAAGAGCCCCCG
GAGCAAATGAAGATAACTAAGGAAGAGATTGTTGAGATTAAATCAGAACCATTGCCACCAGAACCCAAGGAA
GAGACTAAAGAAGAAGAGATTGTTGAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAA
GAAGAAGAGATTGTTGAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCA
TTACAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCATTACAGATTAAAT
CAGCACCACTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCATTACAGCCAGAAGCTACGCCTAAAC
AATCACCATTTCAGCCAGAAGCTACGCCTAAACAATCACCATTTCAGCCAGAAGCTACGCCTAAACAATCACC
ATTTCAGCCAGAACACGAGGAGGAGACTGGACCAGCTAAGGAGGAGACTCATCAAGTACCATCACCCCCAAA
GCCAAAGGAAGAGACCGAATCATCAGAGGTCTCTTATCACAGGGAAGACAGTACACCAGCACAGCCAATAAC
ACACAAAGAAGAAGAAAAACCAGCAGTGTCCCCAGGACCAAGAAAACCCCATAATGTTAAGAGATTATCTAGT
GCAGGACTTCAAGCAGAGTCGGTATTGAGAGTGAAAACAGGTAATTTGAAAGAGTACTACAGCTTGGGAAGG
AAGCTTGGACATGGGCAATTTGGAACAACTTTTCTTTGTGTGGAGAAAGGGACAGGGAAACAGTATGCTTGC
AAATCCATTGCCAAAAGGAAACTAACAACACCAGATGACGTTGAGGATGTGAGGAGAGAAATTCAGATAATGC
ATCACTTGTCAGGCCATCCTAATGTTGTATCGATCAAAGAGGCGTATGAAGATGCAGTGGCAGTTCATGTTGT
GATGGAATTGTGTGCTGGAGGTGAGCTCTTTGACAGGATTATTACGCGAGGTCATTACACGGAAAAAAAGGC
AGCTCAACTCACTAAGATTATAGTTGGTGTTATAGAAGCTTGCCATTCTTTGGGAGTCATGCATAGAGATTTGA
AGCCTGAGAATTTTCTCTTTGTCAATGAAGAGGAGGACTCACCTCTCAAAGCAATAGACTTTGGATTATCAGT
GTTTTTCAAGCCCGGGGAGACATTTACTGATGTGGTTGGAAGCCCATACTATGTGGCACCTGAAGTTCTGAA
GAAATGGTATGGTCATGAAGCAGATGTTTGGAGTGCTGGAGTTATGGTTTACATTCTCTTAAGTGGGGTACCT
CCATTCTGGGCTGAAACTGAGCAAGGCATATTTGACGAGGTACTGAATGGTGAGTTGGATTTCGTATCAGATC
CATGGCCTAACATCTCTGAAAGTGCAAAGGATTTGGTTACGAGAATGCTTGTCAGGGACCCCAAGCAACGAA
TTACTGCACATGAAGTTCTTTGTCACCCTTGGGTTCGGGATGATGGGGTTGCTCCTGATAAGCCTCTTGATCC
TGCAGTTTTATCTCGCTTGAAGCAGTTTTCTGCAATGAACAAGCTTAAGAAAATGGCTCTTAGAATTATTGCTG
AGAACCTCTCTGAAGAAGAAATTGCTGGGTTAACAGAAATGTTTAAGATGATAGACACAGATGGCAGTGGTCA
AATTACTTTCGAAGAACTCAAAGTGGGACTAAAGAGATTTGGTGCTAATCTCAATGACTCTGAAATTTATGACC
TAATGCAGGCGGCTGATATTGATAATAGTGGGACAATAGACTATGGGGAGTTCATAGCTGCCACATTGCATCT
AAACAAAGTAGAAAAGGAAGATCATCTATTTGCAGCTTTCTCATACTTTGACAAAGATGGCAGTGGCTATATAA
CTCTAGATGAACTGCAACAAGCTTGTAGCGAATTCGGCATGGAGGATGTTCAGTTGGAAGAAATGATAAGAG
AAGTTGATCAGGACAATGATGGTCGGATAGATTACAATGAGTTTGTGGACATGATGCAAATGGGTAAGAACG
GACGACAACATAGAAGTTTCAGTGTTGGCTTTAGAGAGGCATTGCAAGTTTGTTGA

d) Predicted CDPK36

MGNNCVGSRGWWMRPTNGQITHPTGEGIEGDPVNKEPQDVLQAPQEPPEQMKITKEEIVEIKSEPLPPEPKEETK
EEEIVEIKSAPLPPEPKEETKEEEIVEIKSAPLPPEPKEETKESPLQIKSAPLPPEPKEETKESPLQIKSAPLPPEPKEE
TKESPLQPEATPKQSPFQPEATPKQSPFQPEATPKQSPFQPEHEEETGPAKEETHQVPSPPKPKEETESSEVSYH
REDSTPAQPITHKEEEKPAVSPGPRKPHNVKRLSSAGLQAESVLRVKTGNLKEYYSLGRKLGHGQFGTTFLCVEK
GTGKQYACKSIAKRKLTTPDDVEDVRREIQIMHHLSGHPNVVSIKEAYEDAVAVHVVMELCAGGELFDRIITRGHYT

EKKAAQLTKIIVGVIEACHSLGVMHRDLKPENFLFVNEEEDSPLKAIDFGLSVFFKPGETFTDVVGSPYYVAPEVLK
KWYGHEADVWSAGVMVYILLSGVPPFWAETEQGIFDEVLNGELDFVSDPWPNISESAKDLVTRMLVRDPKQRITA
HEVLCHPWVRDDGVAPDKPLDPAVLSRLKQFSAMNKLKKMALRIIAENLSEEEIAGLTEMFKMIDTDGSGQITFEEL
KVGLKRFGANLNDSEIYDLMQAADIDNSGTIDYGEFIAATLHLNKVEKEDHLFAAFSYFDKDGSGYITLDELQQACS
EFGMEDVQLEEMIREVDQDNDGRIDYNEFVDMMQMGKNGRQHRSFSVGFREALQVC

e) DNA sequence alignment of MeCDPK

```
CDPK54                   ATGggaaataattgtgttggatcaaggggttggtggatgcgaccaacgaatggccagatt   60
Manes.08G036300.1        ATGGGAAATAATTGTGTTGGATCAAGGGGTTGGTGGATGCGACCAACGAATGGCCAGATT   60
CDPK36                   ATGGGAAATAATTGTGTTGGATCAAGGGGTTGGTGGATGCGACCAACGAATGGCCAGATT   60
                         ************************************************************

CDPK54                   actcatcctacaggagaaggcattgaaggtgaccctgtgaataaagagccacaagatgtt   120
Manes.08G036300.1        ACTCATCCTACAGGAGAAGGCATTGAAGGTGACCCTGTGAATAAAGAGCCACAAGATGTT   120
CDPK36                   ACTCATCCTACAGGAGAAGGCATTGAAGGTGACCCTGTGAATAAAGAGCCACAAGATGTT   120
                         ************************************************************

CDPK54                   ttacaagctccacaagagcccccggagcaaatgaagataactaaggaagagattgttgag   180
Manes.08G036300.1        TTACAAGCTCCACAAGAGCCCCCGGAGCAAATGAAGATAACTAAGGAAGAGATTGTTGAG   180
CDPK36                   TTACAAGCTCCACAAGAGCCCCCGGAGCAAATGAAGATAACTAAGGAAGAGATTGTTGAG   180
                         ************************************************************

CDPK54                   attaaatcagaaccattgccaccagaacccaaggaagagactaaagaagaagagattgtt   240
Manes.08G036300.1        ATTAAATCAGAACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAAGAAGAGATTGTT   240
CDPK36                   ATTAAATCAGAACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAAGAAGAGATTGTT   240
                         ************************************************************

CDPK54                   gagattaaatcagcaccattgccaccagaacccaaggaagagactaaagaagaagagatt   300
Manes.08G036300.1        GAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAAGAAGAGATT   300
CDPK36                   GAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAAGAAGAGATT   300
                         ************************************************************

CDPK54                   gttgagattaaatcagcaccattgccaccagaacccaaggaagagactaaagaatcgcca   360
Manes.08G036300.1        GTTGAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCA   360
CDPK36                   GTTGAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCA   360
                         ************************************************************

CDPK54                   ttacagattaaatcagcaccattgccaccagaacccaaggaagagactaaagaatcgcca   420
Manes.08G036300.1        TTACAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCA   420
CDPK36                   TTACAGATTAAATCAGCACCATTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCA   420
                         ************************************************************

CDPK54                   ttacagattaaatcagcaccactgccaccagaacccaaggaagagactaaagaatcgcca   480
Manes.08G036300.1        TTACAGATTAAATCAGCACCACTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCA   480
CDPK36                   TTACAGATTAAATCAGCACCACTGCCACCAGAACCCAAGGAAGAGACTAAAGAATCGCCA   480
                         ************************************************************

CDPK54                   ttacagccagaagctacgcctaaacaatcaccatttcagccagaagctacgcctaaacaa   540
Manes.08G036300.1        TTACAGCCAGAAGCTACGCCTAAACAATCACCATTTCAGCCAGAAGCTACGCCTAAACAA   540
CDPK36                   TTACAGCCAGAAGCTACGCCTAAACAATCACCATTTCAGCCAGAAGCTACGCCTAAACA-   539
                         ***********************************************************

CDPK54                   tcaccatttcagccagaagctacgcctaaacaatcaccatttcagccagaagctacgcct   600
Manes.08G036300.1        TCACCATTT---------------------------------CAGCCAGAAGCTACGCCT   567
CDPK36                   ------------------------------------------------------------   539

CDPK54                   aaacaatcaccatttcagccagaagctacgcctaaacaatcgccattacagccagaacac   660
Manes.08G036300.1        AAACAATCACCATTTCAGCCAGAAGCTACGCCTAAACAATCACCATTACAGCCAGAACAC   627
CDPK36                   -----ATCACCATTTCAGCCAGAAGCTACGCCTAAACAATCACCATTTCAGCCAGAACAC   594
                              ************************************ ***** ************

CDPK54                   gaggaggagactggaccagctaaggaggagactcatcaagtaccatcacccccaaagcca   720
Manes.08G036300.1        GAGGAGGAGACTGGACCAGCTAAGGAGGAGACTCATCAAGTACCATCACCCCCAAAGCCA   687
CDPK36                   GAGGAGGAGACTGGACCAGCTAAGGAGGAGACTCATCAAGTACCATCACCCCCAAAGCCA   654
                         ************************************************************

CDPK54                   aaggaagagaccgaatcatcagaggtctcttatcacagggaagacagtacaccagcacag   780
Manes.08G036300.1        AAGGAAGAGACCGAATCATCAGAGGTCTCTTATCACAGGGAAGACAGTACACCAGCACAG   747
CDPK36                   AAGGAAGAGACCGAATCATCAGAGGTCTCTTATCACAGGGAAGACAGTACACCAGCACAG   714
                         ************************************************************

CDPK54                   ccaataacacacaaagaagaagaaaaaccagcagtgtccccaggaccaagaaaaccccat   840
Manes.08G036300.1        CCAATAACACACAAAGAAGAAGAAAAACCAGCAGTGTCCCCAGGACCAAGAAAACCCCAT   807
CDPK36                   CCAATAACACACAAAGAAGAAGAAAAACCAGCAGTGTCCCCAGGACCAAGAAAACCCCAT   774
                         ************************************************************

CDPK54                   aatgttaagagattatctagtgcaggacttcaagcagagtcggtattgagagtgaaaaca   900
Manes.08G036300.1        AATGTTAAGAGATTATCTAGTGCAGGACTTCAAGCAGAGTCGGTATTGAGAGTGAAAACA   867
CDPK36                   AATGTTAAGAGATTATCTAGTGCAGGACTTCAAGCAGAGTCGGTATTGAGAGTGAAAACA   834
                         ************************************************************

CDPK54                   ggtaatttgaaagagtactacagcttgggaaggaagcttggacatgggcaatttggaaca   960
Manes.08G036300.1        GGTAATTTGAAAGAGTACTACAGCTTGGGAAGGAAGCTTGGACATGGGCAATTTGGAACA   927
CDPK36                   GGTAATTTGAAAGAGTACTACAGCTTGGGAAGGAAGCTTGGACATGGGCAATTTGGAACA   894
                         ************************************************************

CDPK54                   acttttctttgtgtggagaaagggacagggaaacagtatgcttgcaaatccattgccaaa   1020
Manes.08G036300.1        ACTTTTCTTTGTGTGGAGAAAGGGACAGGGAAACAGTATGCTTGCAAATCCATTGCCAAA   987
CDPK36                   ACTTTTCTTTGTGTGGAGAAAGGGACAGGGAAACAGTATGCTTGCAAATCCATTGCCAAA   954
                         ************************************************************

CDPK54                   aggaaactaacaacaccagatgacgttgaggatgTGAGGAGAGAAATTCAGATAATGCAT   1080
Manes.08G036300.1        AGGAAACTAACAACACCAGATGACGTTGAGGATGTGAGGAGAGAAATTCAGATAATGCAT   1047
CDPK36                   AGGAAACTAACAACACCAGATGACGTTGAGGATGTGAGGAGAGAAATTCAGATAATGCAT   1014
                         ************************************************************

CDPK54                   CACTTGTCAGGCCATCCTAATGTTGTATCGATCAAAGAGGCGTATGAAGATGCAGTGGCA   1140
Manes.08G036300.1        CACTTGTCAGGCCATCCTAATGTTGTATCGATCAAAGAGGCGTATGAAGATGCAGTGGCA   1107
CDPK36                   CACTTGTCAGGCCATCCTAATGTTGTATCGATCAAAGAGGCGTATGAAGATGCAGTGGCA   1074
                         ************************************************************

CDPK54                   GTTCATGTTGTGATGGAATTGTGTGCTGGAGGTGAGCTCTTTGACAGGATTATTACGCGA   1200
Manes.08G036300.1        GTTCATGTTGTGATGGAATTGTGTGCTGGAGGTGAGCTCTTTGACAGGATTATTACGCGA   1167
CDPK36                   GTTCATGTTGTGATGGAATTGTGTGCTGGAGGTGAGCTCTTTGACAGGATTATTACGCGA   1134
                         ************************************************************

CDPK54                   GGTCATTACACGGAAAAAAAGGCAGCTCAACTCACTAAGATTATAGTTGGTGTTATAGAA   1260
Manes.08G036300.1        GGTCATTACACGGAAAAAAAGGCAGCTCAACTCACTAAGATTATAGTTGGTGTTATAGAA   1227
CDPK36                   GGTCATTACACGGAAAAAAAGGCAGCTCAACTCACTAAGATTATAGTTGGTGTTATAGAA   1194
                         ************************************************************

CDPK54                   GCTTGCCATTCTTTGGGAGTCATGCATAGAGATTTGAAGCCTGAGAATTTTCTCTTTGTC   1320
Manes.08G036300.1        GCTTGCCATTCTTTGGGAGTCATGCATAGAGATTTGAAGCCTGAGAATTTTCTCTTTGTC   1287
CDPK36                   GCTTGCCATTCTTTGGGAGTCATGCATAGAGATTTGAAGCCTGAGAATTTTCTCTTTGTC   1254
                         ************************************************************
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CDPK54                  AATGAAGAGGAGGACTCACCTCTCAAAGCAATAGACTTTGGATTATCAGTGTTTTTCAAG   1380
Manes.08G036300.1       AATGAAGAGGAGGACTCACCTCTCAAAGCAATAGACTTTGGATTATCAGTGTTTTTCAAG   1347
CDPK36                  AATGAAGAGGAGGACTCACCTCTCAAAGCAATAGACTTTGGATTATCAGTGTTTTTCAAG   1314
                        ************************************************************

CDPK54                  CCCGGGGAGACATTTACTGATGTGGTTGGAAGCCCATACTATGTGGCACCTGAAGTTCTg   1440
Manes.08G036300.1       CCCGGGGAGACATTTACTGATGTGGTTGGAAGCCCATACTATGTGGCACCTGAAGTTCTG   1407
CDPK36                  CCCGGGGAGACATTTACTGATGTGGTTGGAAGCCCATACTATGTGGCACCTGAAGTTCTG   1374
                        ************************************************************

CDPK54                  aagaaatggtatggtcatgaagcagatgtttggagtgctggagttatggtttacattctc   1500
Manes.08G036300.1       AAGAAATGGTATGGTCATGAAGCAGATGTTTGGAGTGCTGGAGTTATGGTTTACATTCTC   1467
CDPK36                  AAGAAATGGTATGGTCATGAAGCAGATGTTTGGAGTGCTGGAGTTATGGTTTACATTCTC   1434
                        ************************************************************

CDPK54                  ttaagtggggtacctccattctgggctgaaactgagcaaggcatatttgacgaggtactg   1560
Manes.08G036300.1       TTAAGTGGGGTACCTCCATTCTGGGCTGAAACTGAGCAAGGCATATTTGACGAGGTACTG   1527
CDPK36                  TTAAGTGGGGTACCTCCATTCTGGGCTGAAACTGAGCAAGGCATATTTGACGAGGTACTG   1494
                        ************************************************************

CDPK54                  aatggtgagttggatttcgtatcagatccatggcctaacatctctgaaagtgcaaaggat   1620
Manes.08G036300.1       AATGGTGAGTTGGATTTCGTATCAGATCCATGGCCTAACATCTCTGAAAGTGCAAAGGAT   1587
CDPK36                  AATGGTGAGTTGGATTTCGTATCAGATCCATGGCCTAACATCTCTGAAAGTGCAAAGGAT   1554
                        ************************************************************

CDPK54                  ttggttacgagaatgcttgtcagggaccccaagcaacgaattactgcacatgaagttctt   1680
Manes.08G036300.1       TTGGTTACGAGAATGCTTGTCAGGGACCCCAAGCAACGAATTACTGCACATGAAGTTCTT   1647
CDPK36                  TTGGTTACGAGAATGCTTGTCAGGGACCCCAAGCAACGAATTACTGCACATGAAGTTCTT   1614
                        ************************************************************

CDPK54                  tgtcacccttgggttcgggatgatggggttgctcctgataagcctcttgatcctgcagtt   1740
Manes.08G036300.1       TGTCACCCTTGGGTTCGGGATGATGGGGTTGCTCCTGATAAGCCTCTTGATCCTGCAGTT   1707
CDPK36                  TGTCACCCTTGGGTTCGGGATGATGGGGTTGCTCCTGATAAGCCTCTTGATCCTGCAGTT   1674
                        ************************************************************

CDPK54                  ttatctcgcttgaagcagttttctgcaatgaacaagcttaagaaaatggctcttagaatt   1800
Manes.08G036300.1       TTATCTCGCTTGAAGCAGTTTTCTGCAATGAACAAGCTTAAGAAAATGGCTCTTAGAATT   1767
CDPK36                  TTATCTCGCTTGAAGCAGTTTTCTGCAATGAACAAGCTTAAGAAAATGGCTCTTAGAATT   1734
                        ************************************************************

CDPK54                  attgctgagaacctctctgaagaagaaattgctgggttaacagaaatgtttaagatgata   1860
Manes.08G036300.1       ATTGCTGAGAACCTCTCTGAAGAAGAAATTGCTGGGTTAACAGAAATGTTTAAGATGATA   1827
CDPK36                  ATTGCTGAGAACCTCTCTGAAGAAGAAATTGCTGGGTTAACAGAAATGTTTAAGATGATA   1794
                        ************************************************************

CDPK54                  gacacagatggcagtggtcaaattactttcgaagaactcaaagtgggactaaagagattt   1920
Manes.08G036300.1       GACACAGATGGCAGTGGTCAAATTACTTTCGAAGAACTCAAAGTGGGACTAAAGAGATTT   1887
CDPK36                  GACACAGATGGCAGTGGTCAAATTACTTTCGAAGAACTCAAAGTGGGACTAAAGAGATTT   1854
                        ************************************************************

CDPK54                  ggtgctaatctcaatgactctgaaatttatgacctaatgcaggcggctgatattgataat   1980
Manes.08G036300.1       GGTGCTAATCTCAATGACTCTGAAATTTATGACCTAATGCAGGCGGCTGATATTGATAAT   1947
CDPK36                  GGTGCTAATCTCAATGACTCTGAAATTTATGACCTAATGCAGGCGGCTGATATTGATAAT   1914
                        ************************************************************

CDPK54                  agtgggacaatagactatggggagttcatagctgccacattgcatctaaacaaagtagaa   2040
Manes.08G036300.1       AGTGGGACAATAGACTATGGGGAGTTCATAGCTGCCACATTGCATCTAAACAAAGTAGAA   2007
CDPK36                  AGTGGGACAATAGACTATGGGGAGTTCATAGCTGCCACATTGCATCTAAACAAAGTAGAA   1974
                        ************************************************************

CDPK54                  aaggaagatcatctatttgcagctttctcatactttgacaaagatggcagtggctatata   2100
Manes.08G036300.1       AAGGAAGATCATCTATTTGCAGCTTTCTCATACTTTGACAAAGATGGCAGTGGCTATATA   2067
CDPK36                  AAGGAAGATCATCTATTTGCAGCTTTCTCATACTTTGACAAAGATGGCAGTGGCTATATA   2034
                        ************************************************************

CDPK54                  actctagatgaactgcaacaagcttgtagcgaattcggcatggaggatgttcagttggaa   2160
Manes.08G036300.1       ACTCTAGATGAACTGCAACAAGCTTGTAGCGAATTCGGCATGGAGGATGTTCAGTTGGAA   2127
CDPK36                  ACTCTAGATGAACTGCAACAAGCTTGTAGCGAATTCGGCATGGAGGATGTTCAGTTGGAA   2094
                        ************************************************************

CDPK54                  gaaatgataagagaagttgatcaggacaatgatggtcggatagattacaatgagtttgtg   2220
Manes.08G036300.1       GAAATGATAAGAGAAGTTGATCAGGACAATGATGGTCGGATAGATTACAATGAGTTTGTG   2187
CDPK36                  GAAATGATAAGAGAAGTTGATCAGGACAATGATGGTCGGATAGATTACAATGAGTTTGTG   2154
                        ************************************************************

CDPK54                  gacatgatgcaaatgggtaagaacggacgacaacatagaagtttcagtgttggctttaga   2280
Manes.08G036300.1       GACATGATGCAAATGGGTAAGAACGGACGACAACATAGAAGTTTCAGTGTTGGCTTTAGA   2247
CDPK36                  GACATGATGCAAATGGGTAAGAACGGACGACAACATAGAAGTTTCAGTGTTGGCTTTAGA   2214
                        ************************************************************

CDPK54                  gaggcattgcaagtttgttgaggtgacca      2309
Manes.08G036300.1       GAGGCATTGCAAGTTTGTTGA--------      2268
CDPK36                  GAGGCATTGCAAGTTTGTTGA--------      2235
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f) Amino acid sequence alignment of MeCDPK

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MeCDPK54                MGNNCVGSRGWWMRPTNGQITHPTGEGIEGDPVNKEPQDVLQAPQEPPEQMKITKEEIVE   60
MeCDPK36                MGNNCVGSRGWWMRPTNGQITHPTGEGIEGDPVNKEPQDVLQAPQEPPEQMKITKEEIVE   60
Manes.08G036300.1       MGNNCVGSRGWWMRPTNGQITHPTGEGIEGDPVNKEPQDVLQAPQEPPEQMKITKEEIVE   60
                        ************************************************************

MeCDPK54                IKSEPLPPEPKEETKEEEIVEIKSAPLPPEPKEETKEEEIVEIKSAPLPPEPKEETKESP   120
MeCDPK36                IKSEPLPPEPKEETKEEEIVEIKSAPLPPEPKEETKEEEIVEIKSAPLPPEPKEETKESP   120
Manes.08G036300.1       IKSEPLPPEPKEETKEEEIVEIKSAPLPPEPKEETKEEEIVEIKSAPLPPEPKEETKESP   120
                        ************************************************************

MeCDPK54                LQIKSAPLPPEPKEETKESPLQIKSAPLPPEPKEETKESPLQPEATPKQSPFQPEATPKQ   180
MeCDPK36                LQIKSAPLPPEPKEETKESPLQIKSAPLPPEPKEETKESPLQPEATPK------------   168
Manes.08G036300.1       LQIKSAPLPPEPKEETKESPLQIKSAPLPPEPKEETKESPLQPEATPKQSPFQPEATPK-   173
                        ************************************************

MeCDPK54                SPFQPEATPKQSPFQPEATPKQSPFQPEATPKQSPLQPEHEEETGPAKEETHQVPSPPKP   240
MeCDPK36                ----------QSPFQPEATPKQSPFQPEATPKQSPFQPEHEEETGPAKEETHQVPSPPKP   218
Manes.08G036300.1       ----------QSPFQPEATPKQSPFQPEATPKQSPLQPEHEEETGPAKEETHQVPSPPKP   229
                                  ************************* ************************

MeCDPK54                KEETESSEVSYHREDSTPAQPITHKEEEKPAVSPGPRKPHNVKRLSSAGLQAESVLRVKT   300
MeCDPK36                KEETESSEVSYHREDSTPAQPITHKEEEKPAVSPGPRKPHNVKRLSSAGLQAESVLRVKT   278
Manes.08G036300.1       KEETESSEVSYHREDSTPAQPITHKEEEKPAVSPGPRKPHNVKRLSSAGLQAESVLRVKT   289
                        ************************************************************

MeCDPK54                GNLKEYYSLGRKLGHGQFGTTFLCVEKGTGKQYACKSIAKRKLTTPDDVEDVRREIQIMH   360
MeCDPK36                GNLKEYYSLGRKLGHGQFGTTFLCVEKGTGKQYACKSIAKRKLTTPDDVEDVRREIQIMH   338
Manes.08G036300.1       GNLKEYYSLGRKLGHGQFGTTFLCVEKGTGKQYACKSIAKRKLTTPDDVEDVRREIQIMH   349
                        ************************************************************

MeCDPK54                HLSGHPNVVSIKEAYEDAVAVHVVMELCAGGELFDRIITRGHYTEKKAAQLTKIIVGVIE   420
MeCDPK36                HLSGHPNVVSIKEAYEDAVAVHVVMELCAGGELFDRIITRGHYTEKKAAQLTKIIVGVIE   398
Manes.08G036300.1       HLSGHPNVVSIKEAYEDAVAVHVVMELCAGGELFDRIITRGHYTEKKAAQLTKIIVGVIE   409
                        ************************************************************

MeCDPK54                ACHSLGVMHRDLKPENFLFVNEEEDSPLKAIDFGLSVFFKPGETFTDVVGSPYYVAPEVL   480
MeCDPK36                ACHSLGVMHRDLKPENFLFVNEEEDSPLKAIDFGLSVFFKPGETFTDVVGSPYYVAPEVL   458
Manes.08G036300.1       ACHSLGVMHRDLKPENFLFVNEEEDSPLKAIDFGLSVFFKPGETFTDVVGSPYYVAPEVL   469
                        ************************************************************

MeCDPK54                KKWYGHEADVWSAGVMVYILLSGVPPFWAETEQGIFDEVLNGELDFVSDPWPNISESAKD   540
MeCDPK36                KKWYGHEADVWSAGVMVYILLSGVPPFWAETEQGIFDEVLNGELDFVSDPWPNISESAKD   518
Manes.08G036300.1       KKWYGHEADVWSAGVMVYILLSGVPPFWAETEQGIFDEVLNGELDFVSDPWPNISESAKD   529
                        ************************************************************

MeCDPK54                LVTRMLVRDPKQRITAHEVLCHPWVRDDGVAPDKPLDPAVLSRLKQFSAMNKLKKMALRI   600
MeCDPK36                LVTRMLVRDPKQRITAHEVLCHPWVRDDGVAPDKPLDPAVLSRLKQFSAMNKLKKMALRI   578
Manes.08G036300.1       LVTRMLVRDPKQRITAHEVLCHPWVRDDGVAPDKPLDPAVLSRLKQFSAMNKLKKMALRI   589
                        ************************************************************

MeCDPK54                IAENLSEEEIAGLTEMFKMIDTDGSGQITFEELKVGLKRFGANLNDSEIYDLMQAADIDN   660
MeCDPK36                IAENLSEEEIAGLTEMFKMIDTDGSGQITFEELKVGLKRFGANLNDSEIYDLMQAADIDN   638
Manes.08G036300.1       IAENLSEEEIAGLTEMFKMIDTDGSGQITFEELKVGLKRFGANLNDSEIYDLMQAADIDN   649
                        ************************************************************

MeCDPK54                SGTIDYGEFIAATLHLNKVEKEDHLFAAFSYFDKDGSGYITLDELQQACSEFGMEDVQLE   720
MeCDPK36                SGTIDYGEFIAATLHLNKVEKEDHLFAAFSYFDKDGSGYITLDELQQACSEFGMEDVQLE   698
Manes.08G036300.1       SGTIDYGEFIAATLHLNKVEKEDHLFAAFSYFDKDGSGYITLDELQQACSEFGMEDVQLE   709
                        ************************************************************

MeCDPK54                EMIREVDQDNDGRIDYNEFVDMMQMGKNGRQHRSFSVGFREALQVC        766
MeCDPK36                EMIREVDQDNDGRIDYNEFVDMMQMGKNGRQHRSFSVGFREALQVC        744
Manes.08G036300.1       EMIREVDQDNDGRIDYNEFVDMMQMGKNGRQHRSFSVGFREALQVC        755
                        **********************************************
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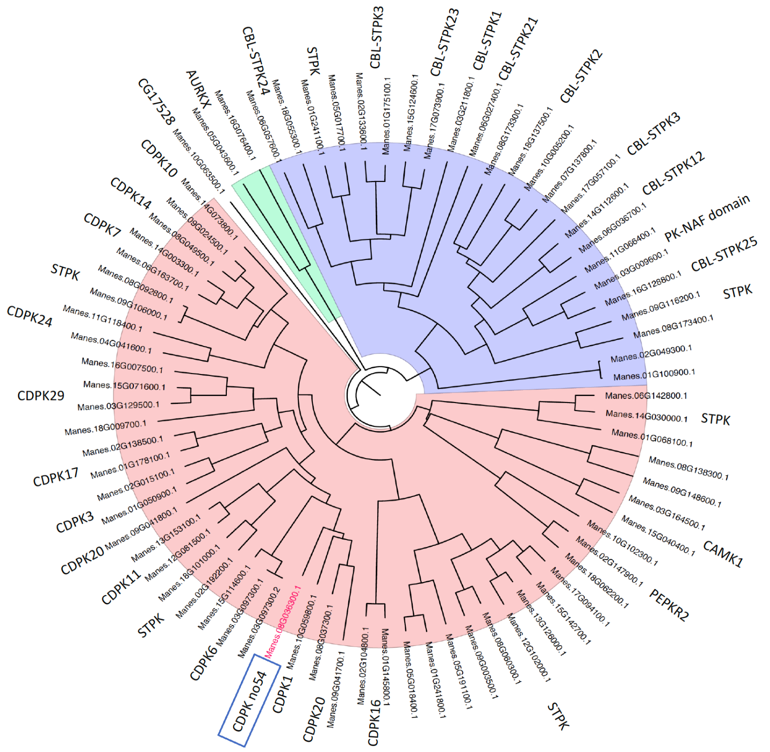
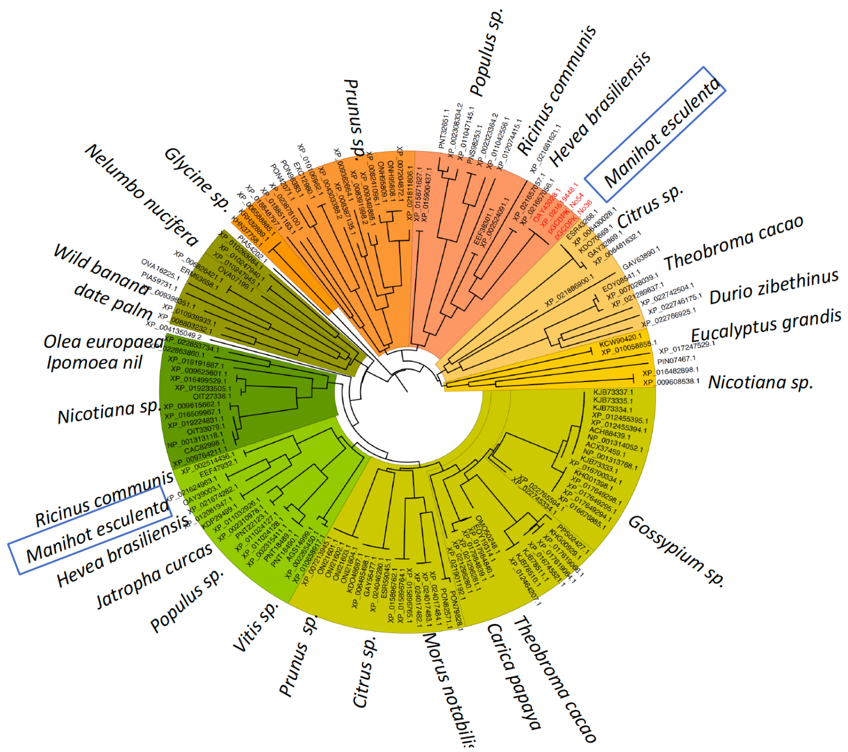
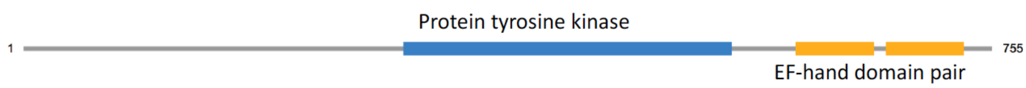
Fig. 19 The DNA sequence analysis of MeAGL20 a) cDNA sequence of MeCDPK54 b) Predicted protein sequence of MeCDPK54 c) cDNA sequence of MeCDPK36 d) Predicted protein sequence of MeCDPK36 e) DNA sequence alignment of MeAGL20 and Manes.08G036300.1 f) amino acid sequence alignment of MeAGL20 and Manes.08G036300.1



a)



b)



c)

Fig. 20 Phylogenetic tree and domain prediction of MeCDPK a) Calcium dependent protein kinase in cassava, b) Phylogenetic tree of MeCDPK and c) domain prediction of MeCDPK

For HT154, the full-length cDNA showed 100% sequence identity to Manes.03G180400.1 (Fig. 21) with 5 exons. From BlastP of MeHT154, the phylogenetic tree showed that at least 6 groups of sugar transport proteins (Fig. 22a). MeHT154 is evolutionary related to sugar transport protein 13 of *Hevea brasilensis, Jatropha curcas, Populus* sp. with sequence identity more than 85% (Fig. 22b). There is 1 domain in MeHT154: major facilitator superfamily, a superfamily of membrane transport proteins that facilitate movement of small solutes across cell membranes in response to chemiosmotic gradients (Fig. 22c).

a) cDNA sequence of HT154

ATGCCTGCAGGAGGTTTCTCTGCAGCTCCTGCCGGAGGAGTGGAATTCGAGGCGAAGATTACGCCGATCGT
CATCATTTCCTGCATAATGGCTGCCACCGGTGGCCTCATGTTTGGTTACGACGTCGGAGTTTCAGGGGGAGT
TACATCGATGCCCGATTTCTTGAAAAAATTCTTCCCAACTGTGTATGACAAGACTCAGGATCCCACAATCAACA
GCAACTACTGCAAGTATGATAATCAAGGCTTGCAATTATTCACCTCATCGCTGTACTTGGCTGGCTTAGTAGC
AACTTTCTTTGCTTCCTATACCACGAGAAAGCTTGGCAGGAGGCCAACCATGTTGATTGCTGGGATTTTCTTC
ATTATTGGTGTGGTGCTTAATACTGCAGCTCAAGACCTTGCCATGCTCATCATTGGCAGGATTTTACTCGGCT
GTGGAGTTGGTTTTGCTAATCAGGCGGTGCCTCTATTTCTATCTGAGATAGCACCCACAAGAATCCGTGGAG
GCCTAAACATACTCTTCCAGCTTAACGTCACCATCGGCATAGTTTTTGCTAACCTTGTCAATTACGGGACTGC
AAAAATTAAATCGGGATGGGGCTGGAGATTATCATTGGGATTGGCTGGCATTCCTGCACTTCTATTGACTTTC
GGGTCTCTCCTGGTGTCAGAAACTCCTAACAGCCTCATTGAGCGTGGCCGTTTGGAAGAAGGAAAAGCCATT
CTCAGAAAGATTCGGGGCACAGACAAGATTGAACCAGAGTTCCTGGAGCTTGTTGAGGCCAGCCGTATAGCA
AAAGAAGTGAAACATCCCTTCAGGAATCTCATGAAGAGGAGGAACCGGCCCCAGCTGGTTATATCAGTAGCC
TTGCAGATCTTCCAGCAGCTCACAGGCATCAATGCAATCATGTTCTACGCTCCTGTCTTGTTTGACACTCTAG
GATTTGGCAGCGATGCTTCCCTATATTCAGCTGTTATAACAGGTGCAGTGAATGTTATTTCTACTGTTGTATCC
ATCTACTCGGTGGACAGAGTAGGACGTCGAGTGCTGTTATTGGAAGCTGGAGTCCAAATGTTTGTCTCTCAA
GTAATAATAGCAATAATCCTAGGCATCAAGGTTAAAGACCATTCTGAAGACCTTCATCGTGGAATTGCAGTGT
TAGTGGTTATTATGATTTGCACATTTGTTTCTGGATTTGCCTGGTCATGGGGACCTCTTGGATGGCTAATCCC
CAGTGAAACATTCCCATTGGAGACCCGCTCAGCTGGTCAGAGTGTTACTGTTTGTGTGAACTTGCTCTTCACT
TTTGCTATAGCACAGGCCTTTCTCTCCATGCTCTGCCATTTCAAATATGGCATCTTCTTGTTCTTCTCTAGCTG
GGTCTTTGTCATGTCATTTTTCGTGTTTTTCCTCGTCCCGGAGACCAAAAATATACCCATTGAAGAGATGACG
GAGAGAGTGTGGAAGCAGCATTGGCTCTGGAAGAGATTTATGGATGACAATGAGGAGGGAGCAATTGAAATT
AATGGTCAGAAATCACAGAAAAAGGGGCATGCAAATGGTTTTGATCCTGTTACCCAGTTTTAA

b) Predicted HT154

MPAGGFSAAPAGGVEFEAKITPIVIISCIMAATGGLMFGYDVGVSGGVTSMPDFLKKFFPTVYDKTQDPTINSNYCK
YDNQGLQLFTSSLYLAGLVATFFASYTTRKLGRRPTMLIAGIFFIIGVVLNTAAQDLAMLIIGRILLGCGVGFANQAVP
LFLSEIAPTRIRGGLNILFQLNVTIGIVFANLVNYGTAKIKSGWGWRLSLGLAGIPALLLTFGSLLVSETPNSLIERGRL
EEGKAILRKIRGTDKIEPEFLELVEASRIAKEVKHPFRNLMKRRNRPQLVISVALQIFQQLTGINAIMFYAPVLFDTLG
FGSDASLYSAVITGAVNVISTVVSIYSVDRVGRRVLLLEAGVQMFVSQVIIAIILGIKVKDHSEDLHRGIAVLVVIMICTF
VSGFAWSWGPLGWLIPSETFPLETRSAGQSVTVCVNLLFTFAIAQAFLSMLCHFKYGIFLFFSSWVFVMSFFVFFL
VPETKNIPIEEMTERVWKQHWLWKRFMDDNEEGAIEINGQKSQKKGHANGFDPVTQF

c) DNA sequence alignment of MeHT154

```
Manes.03G180400.1      ATGCCTGCAGGAGGTTTCTCTGCAGCTCCTGCCGGAGGAGTGGAATTCGAGGCGAAGATT   60
HT154                  ATGCCTGCAGGAGGTTTCTCTGCAGCTCCTGCCGGAGGAGTGGAATTCGAGGCGAAGATT   60
                       ************************************************************

Manes.03G180400.1      ACGCCGATCGTCATCATTTCCTGCATAATGGCTGCCACCGGTGGCCTCATGTTTGGTTAC   120
HT154                  ACGCCGATCGTCATCATTTCCTGCATAATGGCTGCCACCGGTGGCCTCATGTTTGGTTAC   120
                       ************************************************************

Manes.03G180400.1      GACGTCGGAGTTTCAGGGGGAGTTACATCGATGCCCGATTTCTTGAAAAAATTCTTCCCA   180
HT154                  GACGTCGGAGTTTCAGGGGGAGTTACATCGATGCCCGATTTCTTGAAAAAATTCTTCCCA   180
                       ************************************************************

Manes.03G180400.1      ACTGTGTATGACAAGACTCAGGATCCCACAATCAACAGCAACTACTGCAAGTATGATAAT   240
HT154                  ACTGTGTATGACAAGACTCAGGATCCCACAATCAACAGCAACTACTGCAAGTATGATAAT   240
                       ************************************************************

Manes.03G180400.1      CAAGGCTTGCAATTATTCACCTCATCGCTGTACTTGGCTGGCTTAGTAGCAACTTTCTTT   300
HT154                  CAAGGCTTGCAATTATTCACCTCATCGCTGTACTTGGCTGGCTTAGTAGCAACTTTCTTT   300
                       ************************************************************

Manes.03G180400.1      GCTTCCTATACCACGAGAAAGCTTGGCAGGAGGCCAACCATGTTGATTGCTGGGATTTTC   360
HT154                  GCTTCCTATACCACGAGAAAGCTTGGCAGGAGGCCAACCATGTTGATTGCTGGGATTTTC   360
                       ************************************************************

Manes.03G180400.1      TTCATTATTGGTGTGGTGCTTAATACTGCAGCTCAAGACCTTGCCATGCTCATCATTGGC   420
HT154                  TTCATTATTGGTGTGGTGCTTAATACTGCAGCTCAAGACCTTGCCATGCTCATCATTGGC   420
                       ************************************************************

Manes.03G180400.1      AGGATTTTACTCGGCTGTGGAGTTGGTTTTGCTAATCAGGCGGTGCCTCTATTTCTATCT   480
HT154                  AGGATTTTACTCGGCTGTGGAGTTGGTTTTGCTAATCAGGCGGTGCCTCTATTTCTATCT   480
                       ************************************************************

Manes.03G180400.1      GAGATAGCACCCACAAGAATCCGTGGAGGCCTAAACATACTCTTCCAGCTTAACGTCACC   540
HT154                  GAGATAGCACCCACAAGAATCCGTGGAGGCCTAAACATACTCTTCCAGCTTAACGTCACC   540
                       ************************************************************

Manes.03G180400.1      ATCGGCATAGTTTTTGCTAACCTTGTCAATTACGGGACTGCAAAAATTAAATCGGGATGG   600
HT154                  ATCGGCATAGTTTTTGCTAACCTTGTCAATTACGGGACTGCAAAAATTAAATCGGGATGG   600
                       ************************************************************

Manes.03G180400.1      GGCTGGAGATTATCATTGGGATTGGCTGGCATTCCTGCACTTCTATTGACTTTCGGGTCT   660
HT154                  GGCTGGAGATTATCATTGGGATTGGCTGGCATTCCTGCACTTCTATTGACTTTCGGGTCT   660
                       ************************************************************

Manes.03G180400.1      CTCCTGGTGTCAGAAACTCCTAACAGCCTCATTGAGCGTGGCCGTTTGGAAGAAGGAAAA   720
HT154                  CTCCTGGTGTCAGAAACTCCTAACAGCCTCATTGAGCGTGGCCGTTTGGAAGAAGGAAAA   720
                       ************************************************************

Manes.03G180400.1      GCCATTCTCAGAAAGATTCGGGGCACAGACAAGATTGAACCAGAGTTCCTGGAGCTTGTT   780
HT154                  GCCATTCTCAGAAAGATTCGGGGCACAGACAAGATTGAACCAGAGTTCCTGGAGCTTGTT   780
                       ************************************************************

Manes.03G180400.1      GAGGCCAGCCGTATAGCAAAAGAAGTGAAACATCCCTTCAGGAATCTCATGAAGAGGAGG   840
HT154                  GAGGCCAGCCGTATAGCAAAAGAAGTGAAACATCCCTTCAGGAATCTCATGAAGAGGAGG   840
                       ************************************************************

Manes.03G180400.1      AACCGGCCCCAGCTGGTTATATCAGTAGCCTTGCAGATCTTCCAGCAGCTCACAGGCATC   900
HT154                  AACCGGCCCCAGCTGGTTATATCAGTAGCCTTGCAGATCTTCCAGCAGCTCACAGGCATC   900
                       ************************************************************

Manes.03G180400.1      AATGCAATCATGTTCTACGCTCCTGTCTTGTTTGACACTCTAGGATTTGGCAGCGATGCT   960
HT154                  AATGCAATCATGTTCTACGCTCCTGTCTTGTTTGACACTCTAGGATTTGGCAGCGATGCT   960
                       ************************************************************

Manes.03G180400.1      TCCCTATATTCAGCTGTTATAACAGGTGCAGTGAATGTTATTTCTACTGTTGTATCCATC   1020
HT154                  TCCCTATATTCAGCTGTTATAACAGGTGCAGTGAATGTTATTTCTACTGTTGTATCCATC   1020
                       ************************************************************

Manes.03G180400.1      TACTCGGTGGACAGAGTAGGACGTCGAGTGCTGTTATTGGAAGCTGGAGTCCAAATGTTT   1080
HT154                  TACTCGGTGGACAGAGTAGGACGTCGAGTGCTGTTATTGGAAGCTGGAGTCCAAATGTTT   1080
                       ************************************************************

Manes.03G180400.1      GTCTCTCAAGTAATAATAGCAATAATCCTAGGCATCAAGGTTAAAGACCATTCTGAAGAC   1140
HT154                  GTCTCTCAAGTAATAATAGCAATAATCCTAGGCATCAAGGTTAAAGACCATTCTGAAGAC   1140
                       ************************************************************

Manes.03G180400.1      CTTCATCGTGGAATTGCAGTGTTAGTGGTTATTATGATTTGCACATTTGTTTCTGGATTT   1200
HT154                  CTTCATCGTGGAATTGCAGTGTTAGTGGTTATTATGATTTGCACATTTGTTTCTGGATTT   1200
                       ************************************************************

Manes.03G180400.1      GCCTGGTCATGGGGACCTCTTGGATGGCTAATCCCCAGTGAAACATTCCCATTGGAGACC   1260
HT154                  GCCTGGTCATGGGGACCTCTTGGATGGCTAATCCCCAGTGAAACATTCCCATTGGAGACC   1260
                       ************************************************************

Manes.03G180400.1      CGCTCAGCTGGTCAGAGTGTTACTGTTTGTGTGAACTTGCTCTTCACTTTTGCTATAGCA   1320
HT154                  CGCTCAGCTGGTCAGAGTGTTACTGTTTGTGTGAACTTGCTCTTCACTTTTGCTATAGCA   1320
                       ************************************************************

Manes.03G180400.1      CAGGCCTTTCTCTCCATGCTCTGCCATTTCAAATATGGCATCTTCTTGTTCTTCTCTAGC   1380
HT154                  CAGGCCTTTCTCTCCATGCTCTGCCATTTCAAATATGGCATCTTCTTGTTCTTCTCTAGC   1380
                       ************************************************************

Manes.03G180400.1      TGGGTCTTTGTCATGTCATTTTTCGTGTTTTTCCTCGTCCCGGAGACCAAAAATATACCC   1440
HT154                  TGGGTCTTTGTCATGTCATTTTTCGTGTTTTTCCTCGTCCCGGAGACCAAAAATATACCC   1440
                       ************************************************************

Manes.03G180400.1      ATTGAAGAGATGACGGAGAGAGTGTGGAAGCAGCATTGGCTCTGGAAGAGATTTATGGAT   1500
HT154                  ATTGAAGAGATGACGGAGAGAGTGTGGAAGCAGCATTGGCTCTGGAAGAGATTTATGGAT   1500
                       ************************************************************

Manes.03G180400.1      GACAATGAGGAGGGAGCAATTGAAATTAATGGTCAGAAATCACAGAAAAAGGGGCATGCA   1560
HT154                  GACAATGAGGAGGGAGCAATTGAAATTAATGGTCAGAAATCACAGAAAAAGGGGCATGCA   1560
                       ************************************************************

Manes.03G180400.1      AATGGTTTTGATCCTGTTACCCAGTTTTAA    1590
HT154                  AATGGTTTTGATCCTGTTACCCAGTTTTAA    1590
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d) Amino acid sequence alignment of MeHT154

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Manes.03G180400.1      MPAGGFSAAPAGGVEFEAKITPIVIISCIMAATGGLMFGYDVGVSGGVTSMPDFLKKFFP   60
MeHT154                MPAGGFSAAPAGGVEFEAKITPIVIISCIMAATGGLMFGYDVGVSGGVTSMPDFLKKFFP   60
                       ************************************************************

Manes.03G180400.1      TVYDKTQDPTINSNYCKYDNQGLQLFTSSLYLAGLVATFFASYTTRKLGRRPTMLIAGIF   120
MeHT154                TVYDKTQDPTINSNYCKYDNQGLQLFTSSLYLAGLVATFFASYTTRKLGRRPTMLIAGIF   120
                       ************************************************************

Manes.03G180400.1      FIIGVVLNTAAQDLAMLIIGRILLGCGVGFANQAVPLFLSEIAPTRIRGGLNILFQLNVT   180
MeHT154                FIIGVVLNTAAQDLAMLIIGRILLGCGVGFANQAVPLFLSEIAPTRIRGGLNILFQLNVT   180
                       ************************************************************

Manes.03G180400.1      IGIVFANLVNYGTAKIKSGWGWRLSLGLAGIPALLLTFGSLLVSETPNSLIERGRLEEGK   240
MeHT154                IGIVFANLVNYGTAKIKSGWGWRLSLGLAGIPALLLTFGSLLVSETPNSLIERGRLEEGK   240
                       ************************************************************

Manes.03G180400.1      AILRKIRGTDKIEPEFLELVEASRIAKEVKHPFRNLMKRRNRPQLVISVALQIFQQLTGI   300
MeHT154                AILRKIRGTDKIEPEFLELVEASRIAKEVKHPFRNLMKRRNRPQLVISVALQIFQQLTGI   300
                       ************************************************************

Manes.03G180400.1      NAIMFYAPVLFDTLGFGSDASLYSAVITGAVNVISTVVSIYSVDRVGRRVLLLEAGVQMF   360
MeHT154                NAIMFYAPVLFDTLGFGSDASLYSAVITGAVNVISTVVSIYSVDRVGRRVLLLEAGVQMF   360
                       ************************************************************

Manes.03G180400.1      VSQVIIAIILGIKVKDHSEDLHRGIAVLVVIMICTFVSGFAWSWGPLGWLIPSETFPLET   420
MeHT154                VSQVIIAIILGIKVKDHSEDLHRGIAVLVVIMICTFVSGFAWSWGPLGWLIPSETFPLET   420
                       ************************************************************

Manes.03G180400.1      RSAGQSVTVCVNLLFTFAIAQAFLSMLCHFKYGIFLFFSSWVFVMSFFVFFLVPETKNIP   480
MeHT154                RSAGQSVTVCVNLLFTFAIAQAFLSMLCHFKYGIFLFFSSWVFVMSFFVFFLVPETKNIP   480
                       ************************************************************

Manes.03G180400.1      IEEMTERVWKQHWLWKRFMDDNEEGAIEINGQKSQKKGHANGFDPVTQF     529
MeHT154                IEEMTERVWKQHWLWKRFMDDNEEGAIEINGQKSQKKGHANGFDPVTQF     529
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Fig. 21 The DNA sequence analysis of MeHT154 a) cDNA sequence of MeHT154 b) Predicted protein sequence of MeHT154 c) DNA sequence alignment of MeHT154 and Manes.03G180400.1 d) amino acid sequence alignment of MeHT154 and Manes.03G180400.1

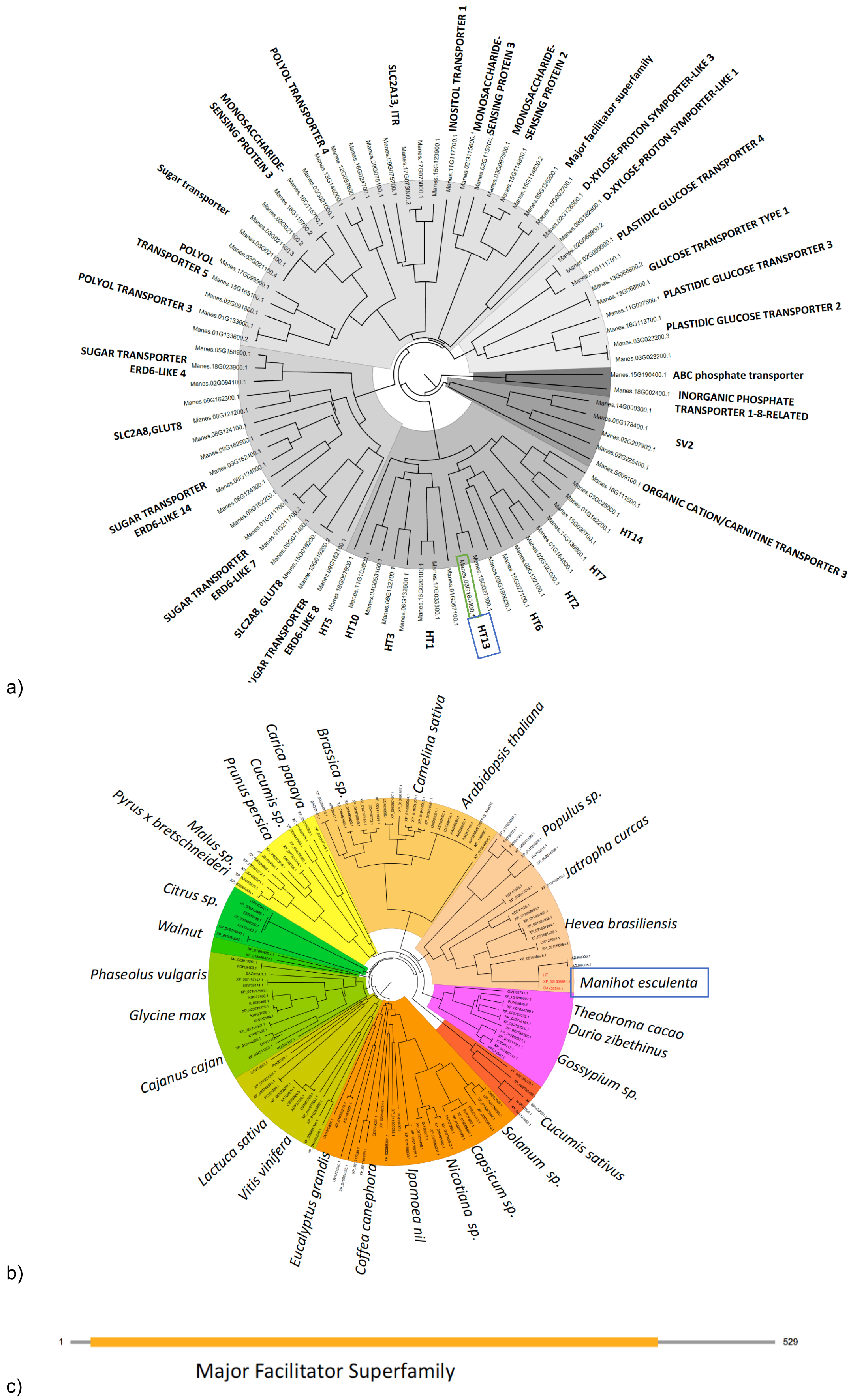

Fig. 22 Phylogenetic tree and domain prediction of MeHT154 a) Membrane transporter protein in cassava, b) Phylogenetic tree of MeHT154 and c) domain prediction of MeHT154

The effect of GA and sugar on MeCDPK, MeAGL20, MeHT154 and MecwINV were investigated in in vitro treatments. Unfortunately, the q-RTPCR results of MeAGL20 could not be quantify since the q-RTPCR showed non-specific amplification. The MeCDPK was upregulated in KU containing 10 uM GA, but not in storage root initiation media (KUBN). The 300 mM sucrose and 300 mM glucose inhibited CDPK expression in root (Fig. 23). When 10 uM GA and 300mM glucose or 10 uM GA and 300 mM sucrose were mixed in the KU media, the expression of CDPK was also suppressed (Fig. 23). Since cwINV hydrolyses sucrose to monosaccharide for subsequently importing into cells by HT, the expression of cwINV were co-investigated along with the expression of HT. The 10 uM GA transiently suppressed MeHT154 and MecwINV expressions. The 300 uM glucose and 300 uM sucrose induced MeHT154 and MecwINV expressions as well as in storage root initiation media (KUBN). When GA and glucose or GA and sucrose were mixed in the KU media, the expression of HT and cwINV were suppressed (Fig. 24, 25). The results supported that CDPK was downregulated, while cwINV and HT were upregulated by high concentration of sugar and cytokinin during storage root initiation.

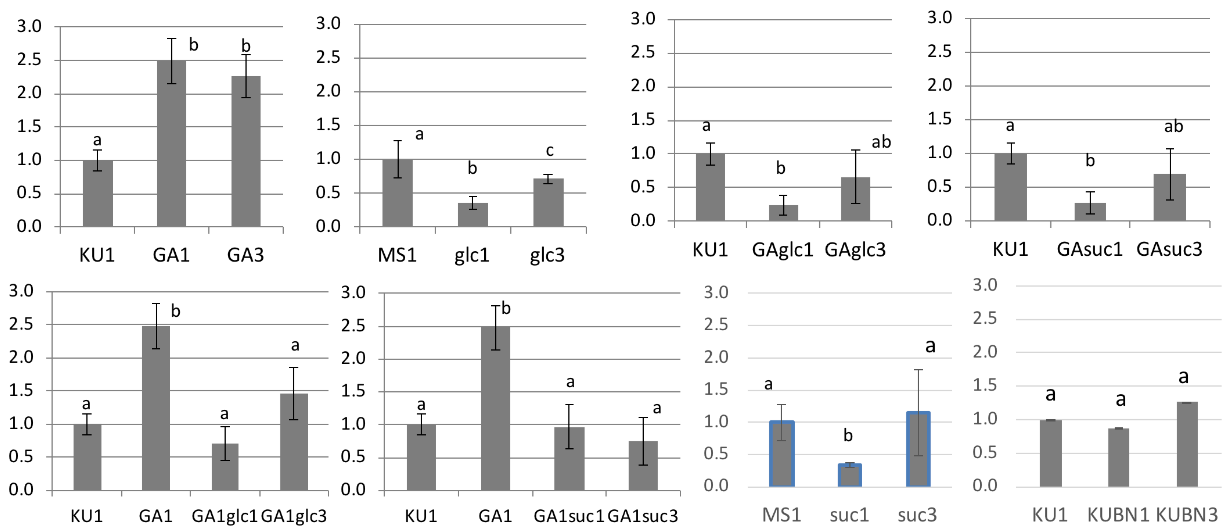

Fig. 23 The qRT-PCR expression profiles of CDPK after GA and sugar treatments by q-RTPCR KU1 was 45 day-olds KU50 root cultured in KU media for 1 day (negative control), The expression levels were calculated relative to the negative control. GA1 was 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day. GA3 was 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 3 days. Glc1 and glc3 were 45 day-olds KU50 root cultured in KU media containing 300 uM glucose for 1 and 3 days, respectively. GAglc1 and GAglc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA and 300 uM glucose for 1 and 3 days, respectively. GAsuc1 and GAsuc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA and 300 uM sucose for 1 and 3 days, respectively. GA1glc1 and GA1glc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day and then transfer to KU media containing 300 uM glucose for 1 and 3 days, respectively. GA1suc1 and GA1suc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day and then transfered to KU media containing 300 uM sucose for 1 and 3 days, respectively. Suc1 and suc3 were 45 day-olds KU50 root cultured in KU media containing 300 uM sucose for 1 and 3 days, respectively. KUBN1 and KUBN3 were 45 day-olds KU50 root cultured in KU media containing 6% sucrose with 0.44 uM BAP and 0.25 uM NAA for 1 and 3 days, respectively. Error bars correspond to the standard error. The a and b above the bar indicate statistically significant difference from FR with p-value $\leq$ 0.05. Unlable represented non-significant different.

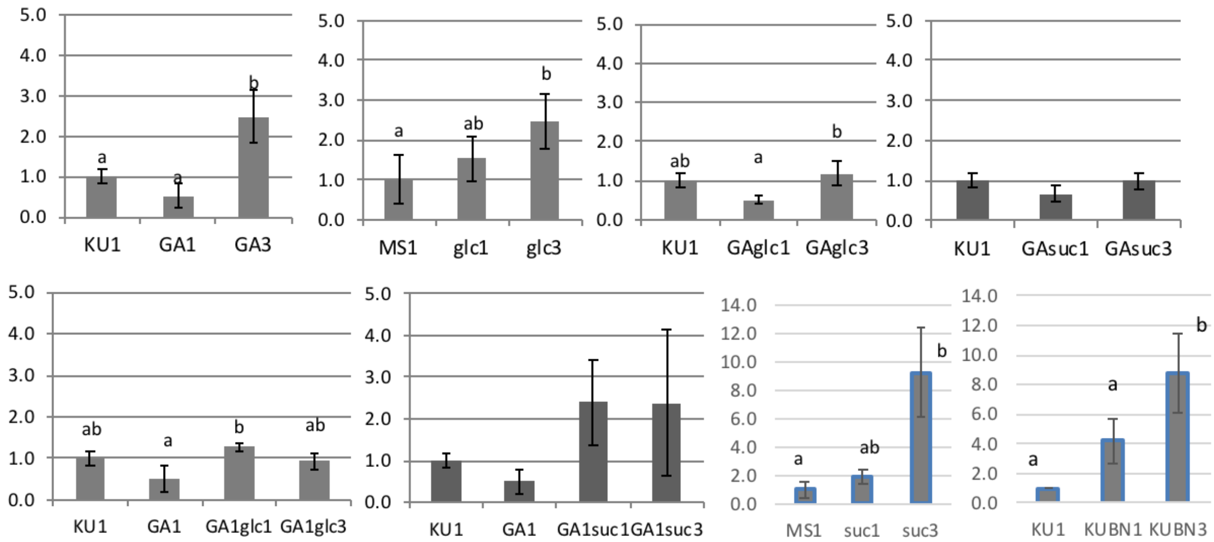

Fig. 24 The qRT-PCR expression profiles of HT154 after GA and sugar treatments by q-RTPCR KU1 was 45 day-olds KU50 root cultured in KU media for 1 day (negative control), The expression levels were calculated relative to the negative control. GA1 was 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day. GA3 was 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 3 days. Glc1 and glc3 were 45 day-olds KU50 root cultured in KU media containing 300 uM glucose for 1 and 3 days, respectively. GAglc1 and GAglc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA and 300 uM glucose for 1 and 3 days, respectively. GAsuc1 and GAsuc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA and 300 uM sucose for 1 and 3 days, respectively. GA1glc1 and GA1glc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day and then transfer to KU media containing 300 uM glucose for 1 and 3 days, respectively. GA1suc1 and GA1suc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day and then transfered to KU media containing 300 uM sucose for 1 and 3 days, respectively. Suc1 and suc3 were 45 day-olds KU50 root cultured in KU media containing 300 uM sucose for 1 and 3 days, respectively. KUBN1 and KUBN3 were 45 day-olds KU50 root cultured in KU media containing 6% sucrose with 0.44 uM BAP and 0.25 uM NAA for 1 and 3 days, respectively. Error bars correspond to the standard error. The a and b above the bar indicate statistically significant difference from FR with p-value $\leq$ 0.05. Unlable represented non-significant different.

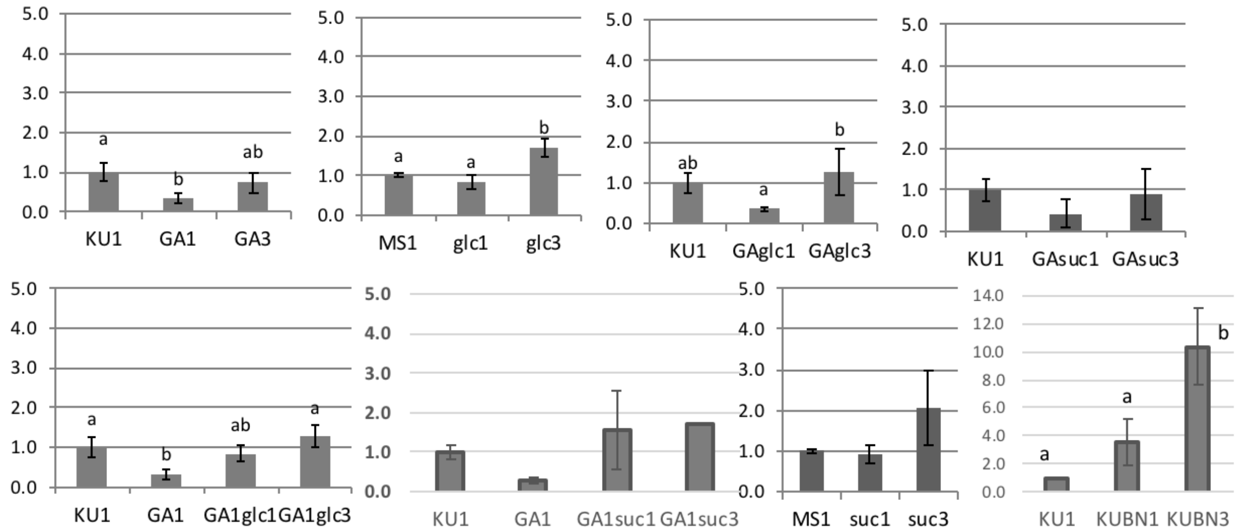

Fig. 25 The qRT-PCR expression profiles of cwINV after GA and sugar treatments by q-RTPCR KU1 was 45 day-olds KU50 root cultured in KU media for 1 day (negative control), The expression levels were calculated relative to the negative control. GA1 was 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day. GA3 was 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 3 days. Glc1 and glc3 were 45 day-olds KU50 root cultured in KU media containing 300 uM glucose for 1 and 3 days, respectively. GAglc1 and GAglc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA and 300 uM glucose for 1 and 3 days, respectively. GAsuc1 and GAsuc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA and 300 uM sucose for 1 and 3 days, respectively. GA1glc1 and GA1glc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day and then transfer to KU media containing 300 uM glucose for 1 and 3 days, respectively. GA1suc1 and GA1suc3 were 45 day-olds KU50 root cultured in KU media containing 10 uM GA for 1 day and then transfered to KU media containing 300 uM sucose for 1 and 3 days, respectively. Suc1 and suc3 were 45 day-olds KU50 root cultured in KU media containing 300 uM sucose for 1 and 3 days, respectively. KUBN1 and KUBN3 were 45 day-olds KU50 root cultured in KU media containing 6% sucrose with 0.44 uM BAP and 0.25 uM NAA for 1 and 3 days, respectively. Error bars correspond to the standard error. The a and b above the bar indicate statistically significant difference from FR with p-value $\leq$ 0.05. Unlable represented non-significant different.

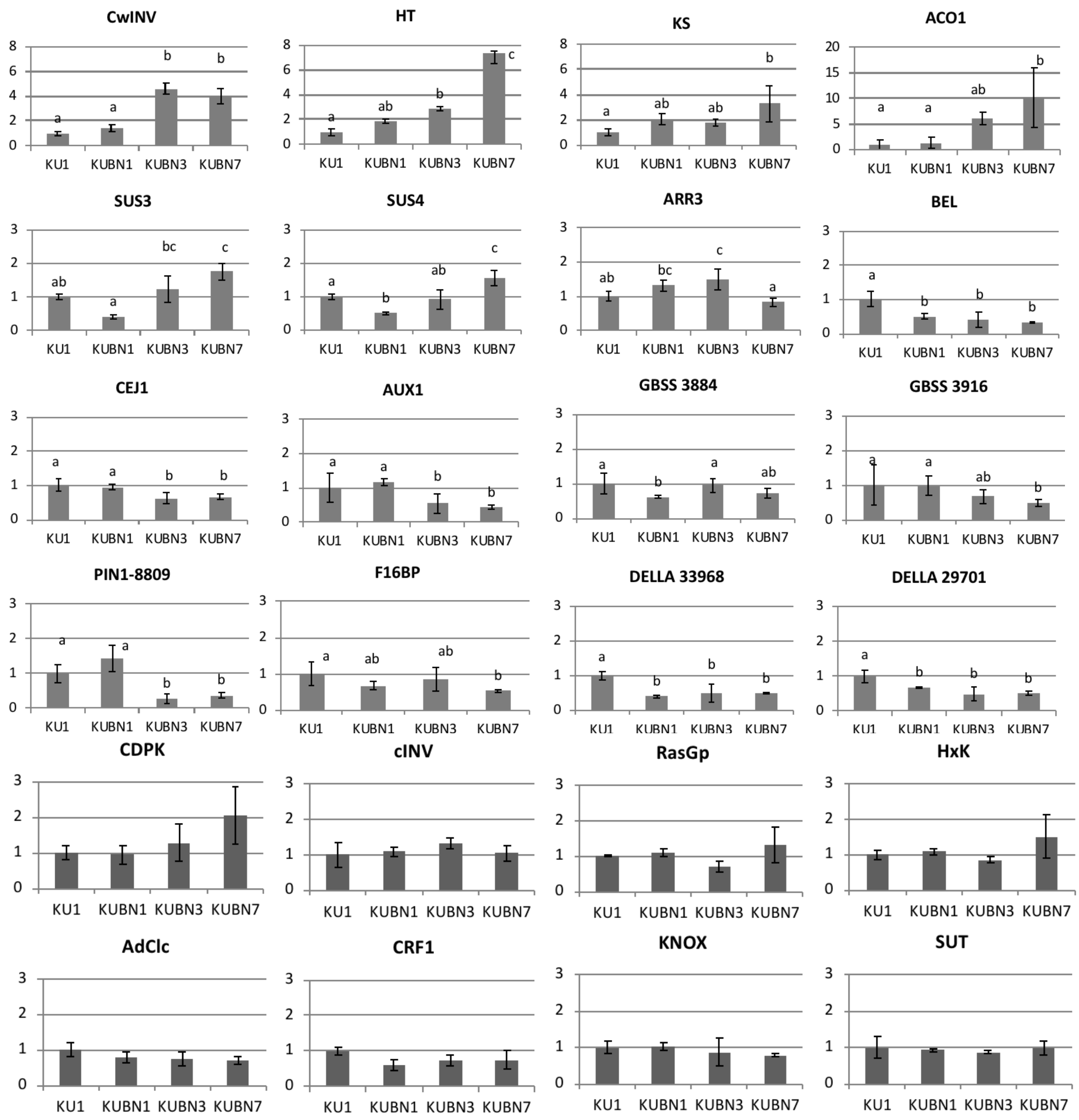

Fig. 26 The qRT-PCR expression profiles of the hormonal pathway and sugar sensing pathway genes in *in vitro* KU50 storage root induction KUBN1, KUBN3 and KUBN7 were 45 day-olds KU50 root cultured in KU media containing 6% sucrose, 0.44 uM BAP and 0.25 uM NAA for 1, 3 and 7 days, respectively. Error bars correspond to the standard error. The a and b above the bar indicate statistically significant difference from FR with p-value $\leq$ 0.05. Unlable represented non-significant different.

The gene expression profiles during storage root initiation were re-investigated with extended time points and also on other genes involved in sugar sensing. The genes involved in phloem unloading (cwINV, HT) and sugar catabolism (SUS3, SUS4) were upregulated during storage root initiatiom but the expression of cINV was not changed. The sugar sensing gene expressions (Hxk, AdClc, RasGp) were not change during storage root induction (Fig. 26).

Discussion

The experiments focused on the gene expression profiles at an early stage of storage root development. The profiles of fibrous, intermediate and storage roots at 8-weeks old were investigated and compared to those of the 4-week-old' fibrous roots. Root cells at I-stage underwent differentiation with secondary growth of vascular tissue and starch accumulation; while in S-stage, parenchymatous tissue was progressively enlarged and filled with starch granules, similar to that reported by Teerawanichpan, et al. [52]. From microarray analysis, the genes involved in glycolysis/gluconeogenesis were up-regulated, which was in accordance with a report by Yang, et al. [62]. The expression of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and fructose-1,6-bisphosphatase (FBP) was high in I- and S-stage, indicating an increase in glycolysis (cluster 3, Fig. 3). These results suggested that high starch catabolism rate might play a role in storage root development, supporting the bioinformatics model that the utilization of carbon substrates favored starch accumulation in storage roots [47].

Phytohormone involved in phase transition from fibrous to storage stage

Transcription factors that were differentially expressed in I8 and S8 have been reported to function in a hormonal regulating pathway in other organisms such as sweet potato [51]. MeKNOX1 was homologous to homeobox protein knotted-1-like 2. Since MeKNOX1 showed a negative correlation to KS and polar auxin transports, but a positive correlation to ACO1, ARR3 and CRF1 (Fig. 4 and 5), KNOX1 might be one of the phytohormone regulators during storage root initiation and development (Fig. 4c). MeKNOX1 has 60% sequence identity to OSH1, a trans-acting factor, that regulated the plant development through the hormone regulations by down regulated GA, but up-regulated ABA and CK [19, 21]. The results were correlated with our finding. In potato, overexpression of KNOX1 resulted in the reduction of GA and auxin levels, but the increase of CK levels [6, 7, 51, 61]. Therefore, the downregulation of KS and polar auxin transports in I-stage and S-stage might be regulated through KNOX1 and phytohormone cross talk. KNOX1 is commonly known as a CK biosynthesis activator during the early stage of tuber formation in both potato and sweet potato [7, 51]. The upregulations of CRF1 and ARR3 in I-stage and S-stage suggest the role of CK during storage root initiation and development, as CK signaling is mediated via ARR and CRF [35, 54]. The results give us an overview that the downregulation of GA and auxin together with the upregulation of CK in I-stage and S-stage might regulate the phase transition from fibrous to storage root. The effect of phytohormone on storage root initiation was then further investigated.

Role of cytokinin in suppression of AGL20 and GA biosynthesis

The downregulation of GGPS1, KS and AGL20 in I-stage and S-stage suggests that the bioactive GA level was reduced. Downregulation of GA might fine-tune AGL20 (MADS-box/SOC1-like) during phase transition (Fig. 5a and 7e). MeAGL20 was a MADS-box protein SOC1 isoform X2. SOC1 was a transcription factor that play role in flowering process in Arabidopsis through the photoperiodic module but regulate the duration of dormancy in kiwi [57]. In potato, overexpression of POTH1 and the reduction of bioactive GA level functioned as a tuberization switch [17, 39]. A high level of KNOX1 would lower the GA level, either via downregulation of GA biosynthesis genes or upregulation of GA deactivation genes during the phase transition from F-stage to I-stage [6]. In in vitro SR induction with exogenous BAP and NAA treatment, AGL20 expression was diminished after hormone treatment, but the KS expression was not significantly different (Fig. 7d). GA and AGL20 have been reported as bioactive molecules involved in the developmental phase transition, and that their expressions diminish after phase change [17, 42]. The diminishing of AGL20 might lead to inhibition of root elongation and induction of phase transition into storage stage [1, 12]. In potato, common regulatory pathways and homologous genes (CO, SOC1 and FT) were conserved between the flowering and tuberization processes [15, 36]. SOC1, acting downstream of FT21, is restricted to tuber sprouts [53]. However, overexpression of AtCO (CONSTANS) in potato inhibited potato tuber formation but did not affect the expression of an endogenous AGL20 gene. Therefore, AGL20 in potato may not being connected to the photoperiodic module [26]. IbMADS and AGL20-like (DV037991) were storage root initiation factors that responded to CK, were involved in auxin-mediated initial thickening of the storage root and were downregulated in storage root [22, 27, 30, 63]. Hence, these results suggest that, in cassava, CK and GA cross talk might control phase transition into the storage stage via MeAGL20.

Role of cytokinin and auxin in storage root initiation

In this study, the in vitro storage root was induced by 0.44 μM BAP and 0.25 μM NAA that were in the range of a considerable performance reported by Medina et al. (2007). Cytokinin alone could not induce KU50 storage root formation, which agreed with that observed by Medina et al. (2007). Also the overexpression of CK in potato could not induce tuberization in all events [46]. The combination of auxin and CK induced early tuber initiation and inhibited stolon elongation by counteracting the GA function, while CK and sucrose (but not auxin) induced secondary thickening growth of the tuber [11, 58, 61]. In I-stage, AUX1, PIN1 and KS were downregulated but CRF1 and ARR3 were upregulated. In in vitro storage root induction, PIN1 (8442) was significantly downregulated in SwR and 7D-SR (Fig. 4c). While AUX1 and PIN1 (8809) were significantly downregulated at the later stage of storage root development (7D-SR) (Fig. 4c), these results imply that repression of polar auxin transport and AGL20 during storage root initiation play roles in the phase transition from fibrous into storage stage. When auxin transport was impeded, the inhibitory effect of auxin was relieved and tuberization was promoted [40]. In addition, KNOX could inhibit polar auxin transport, as the ectopic expression of KNOX in knox regulator rs2 and semi mutant caused defective auxin transport [44, 55, 64]. GA is an auxin transport carrier regulator and stabilizer [60], while CK induces auxin transport carrier degradation [20]. The results suggest that at I-stage and in swollen root, phytohormones act in concert to regulate phase transition and primary thickening growth by inducing secondary cambium and metaxylem differentiation and nutrient mobilization (Fig. 7) [11, 20, 30-32, 41, 58].

Role of phytohormone on CDPK, cwINV and HT expressions

In potato, exogenous treatment of GA transiently induced stCDPK1 expression in swelling stolon [14, 33], which was correlated to our results that *in vitro* GA treatment induced MeCDPK expression (Fig. 23). Moreover, the result was consistent with that MeCDPK was highly expressed at the early initiation stage of storage root development and gradually down-regulated during storage root developmental stage [48]. With our tested condition, MeCDPK did not show any change in its expression during storage root induction (KUBN) but its expression was down regulated after sugar treatment either with GA pre-treatment or co-treatment. *St*CDPK1 was down-regulated in the differentiated storage tissue where starch granules were accumulated [34]. Antisense of *St*CDPK1 induced early tuberization when treated with 8% sucrose [14]. The results suggested that MeCDPK might also play a role in early stage of storage root induction as in potato.

Sugars play crucial roles as cellular carbon sources in energy metabolism and signaling molecules. Metabolic fluxes and sugar concentrations controled plant development and also responsed to environmental signals [3]. In plants, sucrose is generated by photosynthesis and carbon metabolism in source and sink tissues. Sucrose is transported between source and sink via phloem. In sink tissues, sucrose is imported into cells through plasmodesmata (symplastic transport) or cell wall (apoplastic transport). Sucrose are cleaved into monosaccharides by an extracellular invertase (cwINV) [37, 38]. Extracellular monosaccharides are taken up by hexose transporters that are plasma membrane carriers that function as proton (H^+) / hexose symporters [4]. MecwINV and MeHT was upregulated by sucrose with or without CK and NAA (Fig. 24). In grapevine (*Vitis vinifera*), 5 hexose transporters (VvHT) were functional characterized coordinately to cell wall invertase gene (VvcwINV) between source and sink organs [18]. VvHT1, VvHT2, and VvHT3 were highly expressed in berries. VvHT1 showed high expression in early berry development whereas VvHT2 and VvHT3 expression was elevated during sugar accumulation phase. In Arabidopsis, there are 14 genes encoding sugar transporters (AtSTPs). The AtSTPs gene showed coordinated expression with invertase gene [13]. These observations suggested that hexose transporters play important role in sink organ development and correlatly express to cell wall invertase. MeHT154 highly expressed at the initiation stage and early stage of storage root development [48], which conforms with the preferentially expression of this gene family in the sink organ. Thus, MeHT is involved in the monosaccharide upload needed for cell differentiation and starch accumulation at the early stage of storage root induction.

In conclusion, the results support that phytohormones are acting in concert to regulate the onset of cassava storage root development.

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<u>เอกสารแนบหมายเลข 3</u>

Output

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

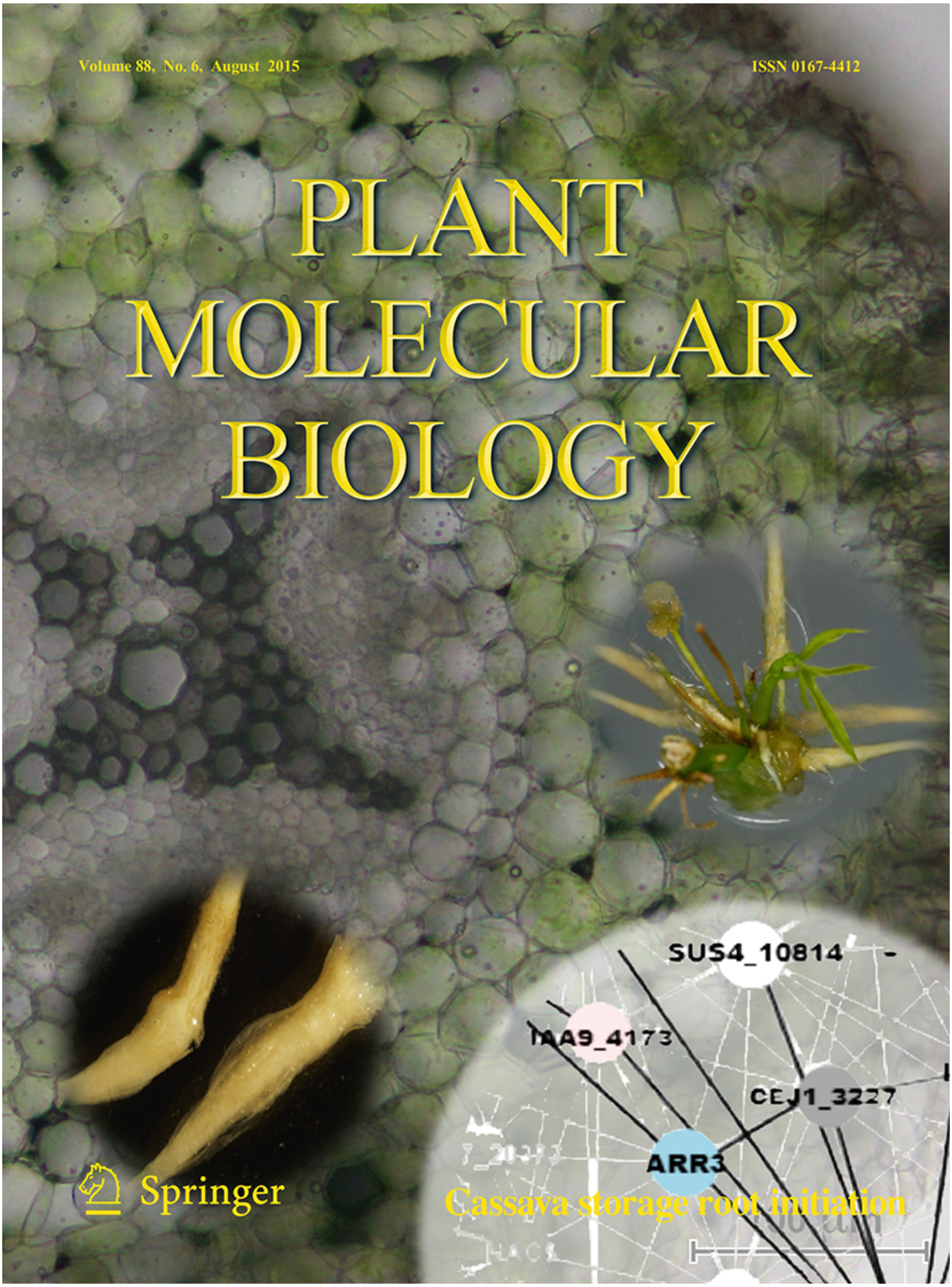
Sojikul, P., Saithong, T., Kalapanulak, S., Pisuttinusart, N., Limsirichaikul, S., Tanaka, M., Utsumi, Y., Sakurai, T., Seki, M. and Narangajavana, J. (2015) Genome-wide analysis reveals phytohormone action during cassava storage root initiation. Plant Mol. Biol. 88, 531-543.

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป) ไม่มี
   - เชิงนโยบาย(มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน) ไม่มี
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง) ไม่มี
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่) ไม่มี

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

Sojikul, P., Saithong, T., Kalapanulak, S., Pisuttinusart, N., Limsirichaikul, S., Tanaka, M., Utsumi, Y., Sakurai, T., Seki, M. and Narangajavana, J. (2016) Genome-wide analysis reveals phytohormone action during cassava storage root initiation, Global Cassava Partnership - GCP-III, January 18-22, 2015 Nanning, China

ภาคผนวก



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Genome-wide analysis reveals phytohormone action during cassava storage root initiation

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Abstract Development of storage roots is a process associated with a phase change from cell division and elongation to radial growth and accumulation of massive amounts of reserve substances such as starch. Knowledge of the regulation of cassava storage root formation has accumulated over time; however, gene regulation during the initiation and early stage of storage root development is still poorly understood. In this study, transcription profiling of fibrous, intermediate and storage roots at eight weeks old were investigated using a 60-mer-oligo microarray. Transcription and gene expression were found to be the key regulating processes during the transition stage from fibrous to intermediate roots, while homeostasis and signal transduction influenced regulation from intermediate roots to storage roots. Clustering analysis of significant genes and transcription factors (TF) indicated that a number of phytohormone-related TF were differentially expressed; therefore, phytohormone-related genes were assembled into a network of correlative nodes. We propose a model showing the relationship between *KNOX1* and phytohormones during storage root initiation. Exogeneous treatment of phytohormones N^6-benzylaminopurine and 1-Naphthaleneacetic acid were used to induce the storage root initiation stage and to investigate expression patterns of the genes involved in storage root initiation. The results support the hypothesis that phytohormones are acting in concert to regulate the onset of cassava storage root development. Moreover, Me*AGL20* is a factor that might play an important role at the onset of storage root initiation when the root tip becomes swollen.

Keywords *Manihot esculenta* · Me*AGL20* · *MeKNOX1* · Microarray · Phytohormones · In vitro storage root initiation

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Introduction

Cassava (*Manihot esculenta* Crantz) is one of the major starch crops that exhibit variation in storage root (SR) yield, starch content and starch composition. Although several factors, including environmental conditions and stage of harvest, have been illustrated to influence crop variation (Prammanee et al. 2010), the role of genetic modulation of SR initiation has not been clearly investigated. Understanding of gene regulation and molecular mechanisms involved in SR development at an early phase transition, from fibrous to storage, would be beneficial to the improvement of cassava cultivar production capacity.

Microarray studies have been conducted with cassava to explore its transcriptional response to cold stress (An et al. 2012), drought stress (Utsumi et al. 2012) and SR deterioration (Reilly et al. 2007). The gene expression profile of fibrous, developing storage and mature SRs at 16 weeks old have also been investigated (Yang et al. 2011). However, there is limited information on gene expression profiles at the early phase transition from fibrous to SR, especially at the initiation stage.

Phytohormone signaling, transcription factor (TF) and sugar sensing play significant roles in driving storage organ developmental processes (Li and Zhang 2003; Liu et al. 2008). In potato, gibberellin (GA) promotes stolon induction, while sugar, auxin, ethylene and ABA inhibit stolon but induce tuber formation (Xu et al. 1998). Low levels of GA and auxin, together with high levels of cytokinin (CK), are involved in storage organ formation and enhancement of enzyme activities in the starch biosynthesis pathway (Ravi et al. 2014; Sergeeva et al. 2000). In sweet potato, whose storage organ originates from root as in cassava, CK, GA, ethylene and auxin have also been found to influence SR development (Eguchi and Yoshida 2008; Ku et al. 2008; McGregor 2006; Noh et al. 2010; Wang et al. 2006).

In order to gain insights into the regulation of SR initiation, the transcriptional dynamics during early SR development was investigated by cDNA microarray (Online Resource 1). The experiment was performed using eight-week-old cassava roots, in which fibrous, intermediate and SRs were presented in the same plantlet. Our results uncovered a group of genes, as well as the active biological processes, which were found to be essential for SR transition. From a correlation network, relationships between the significantly expressed KNOX1, phytohormone biosynthesis and phytohormone signaling-related genes strongly support the hypothesis that phytohormones are acting in concert to regulate the onset of cassava SR development. To confirm this finding, hormone treatment was used to induce SR initiation stage where the variations of gene expressions were investigated.

Materials and methods

Plant materials and RNA extraction

Cassava cultivar KU50 was planted from stem cuttings in the field at Rayong Field Crop Research Center, Rayong, Thailand. Three independent biological replicates were harvested at four and eight weeks after planting. The roots were classified into fibrous (F), intermediate (I) and storage (S: layer 2–5) (Teerawanichpan et al. 2008) (Online Resource 2) and used for both microarray analysis and quantitative RT-PCR (qRT-PCR). In the second crop, planted for qRT-PCR, roots of three independent plantlets at four, eight and 12 weeks old were classified into F, I, S and cortex (C; layer 1). Layer 1 represented cortex, secondary phloem and cambium (Rouse-Miller et al. 2013). SR was further separated into outer parenchyma (SPo; layer 2–4) and inner parenchyma (SPi; layer 5). Total RNA was extracted using Concert™ Plant RNA Reagent (Invitrogen). Total RNA was treated with DNA-Free (Ambion) to remove genomic DNA.

Cassava oligonucleotide microarray

The hybridization experiment was carried out according to Utsumi et al. (2012). In brief, total RNA was used for cRNA synthesis and labeled with cyanine-3 (Cy3) using the Quick Amp Labeling kit (Agilent Technologies). The Cy3-labelled cRNA was fragmented before being hybridized to the Agilent microarray (GPL14139). After hybridization, the microarrays were washed and scanned on the Agilent DNA Microarray Scanner (G2505B) using one color scan setting for 4 × 44 k array slides. Signal intensities were detected from the obtained digital images using Feature Extraction software (Ver. 9.1; Agilent Technologies).

Microarray data analysis

The microarray data was normalized and transformed into log 2 ratio. Data was filtered to remove control probes and probe sets with an intensity value close to background levels. The remaining genes were filtered based on the deviation of the intensity. Subsequently, the differentially expressed genes, hereafter called significant genes, were identified based upon *t* tests with Benjamini–Hochberg correction (*FDR*) and fold changes (*FC*) of the expression level in 8-week-old roots with respect to that of four-week-old fibrous root.

GO enrichment analysis

The GO enrichment analysis of the significant genes was conducted using SEA approach (singular enrichment analysis), which was comprised of two steps: GO annotation and enriched GO determination. For the annotation step, the GO-based function of a gene were predicted through the Blast2GO® software (Conesa et al. 2005), and the resulting annotated gene functions were employed in the later functional enrichment study using agriGO gene ontology analysis toolkit (Du et al. 2010).

Clustering analysis

The 87 significant genes, consisting of 37 genes whose expression across the three developmental stages of root

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showed considerably high variation [standard deviation (sd) $\geq$ 15] and 50 significantly expressed TF genes, were clustered using a Genesis clustering-aided tool for microarray data (Sturn et al. 2002). The expression patterns were preliminarily clustered into ten cohorts based upon k-mean method, and then the cohorts with similar expression patterns were manually grouped together to minimize pattern redundancy.

Correlation analysis

The association of each gene pair was determined by correlation analysis, which statistically quantifies the relationship between genes based on their expression profiles. Pearson correlation coefficients (PCC) of each gene pair were calculated by Matlab software. Only highly correlated gene expression with $|PCC| \geq 0.95$ and p value ≤ 0.05 was subjected to network inference.

Upstream sequence analysis

Promoters of the 67 genes comprising the gene association network were explored, based on the 2-kb upstream sequence from the translation start site. Each upstream region was searched for the *cis*-acting regulatory elements or transcriptional factor biding sites through PlantPAN web tool (http://PlantPAN.mbc.nctu.edu.tw), using the default settings (Chang et al. 2008).

Quantitative RT-PCR

First-strand cDNA was synthesized by The SuperScript® III First-Strand Synthesis System according to the manufacturer's protocol (Invitrogen). After sequence analysis using full-length cDNA database (Sakurai et al. 2007) and Phytozome database (Goodstein et al. 2012), primers specific to each candidate gene were designed (Online Resource Table 1). qRT-PCR was performed with SsoFast™ EvaGreen® Supermix (Bio-Rad). The amplified PCR products were quantified with CFX96 Touch™ (Bio-Rad). Cassava elongation factor 1α (*MeEF1α*) was used for normalization throughout the tests. The data were analyzed using CFX Manager™ software (Bio-Rad). Relative change in gene expression was calculated according to the ΔC_T method, using a reference gene.

In vitro storage root induction

KU50 was sub-cultured in MS media containing 1 µM $CuSO_4$ (KU-media) for 45 days. Internode was sub-cultured in KU-media containing either 6 or 8 % sucrose with various combinations of NAA and BAP from 0.25–2.2 µM. After 4 weeks culturing at 25 °C, the roots were harvested for cross section and Iodine staining.

In vitro storage root initiation

The three independent 50 day-old KU50 roots were transferred into liquid KU-media containing 6 % sucrose as a control (non-hormonal treatment) or into KU-media containing 6 % sucrose, 0.25 µM NAA and 0.44 µM BAP (KU-BN). The roots were cultured in a 120 rpm shaker at 25 °C. Fibrous root (FR) and swollen root (SwR) were harvested at 24 h after treatment. 7 days after treatment, storage root (7D-SR) was harvested. The expression levels of model genes were quantified by qRT-PCR. The results were expressed in mean ±SE of analyses. One-way analysis of variance (ANOVA) was used for statistical analysis with p value ≤ 0.05.

Results

Plant material selection for microarray

Cassava cultivar KU50 was planted from stem cuttings. Fibrous roots (F) were formed within 7 days after planting. The intermediate root (I, 3–5 mm in diameter) was only observed at the seventh week after planting, followed by fully formed SRs (S, $\geq$10 mm in diameter) at eight weeks after planting (Online Resource 2). In I, early stage of secondary growth of phloem and xylem was initially observed. Cell expansion and starch accumulation were also detected in I as in S (Teerawanichpan et al. 2008). Eight-week-old plants were chosen for investigation, as they contained all three stages of interest: F8, I8 and S8. Fibrous root from four-week-old plants (F4), without any evidence of secondary growth, was used as a control stage.

Identification of significant genes during phase transition

Our experiment is one of the few studies attempting to focus on the characteristics of an individual plant rather than average profiles. Relatively loose statistical cut-off was applied in order to deal with the sample variation so interesting results could then be derived from the real characteristics of the individual plant sample. The gene expression profiles were then validated by qRT-PCR in two different KU50 sample sets. The expression levels of 21,522 genes in three individual eight-week-old samples (F8, I8 and S8) and in three individual four-week-old samples as a control (F4) were simultaneously followed using microarray. Their profiles were then compared with that of F4 in order to find genes with significant changes

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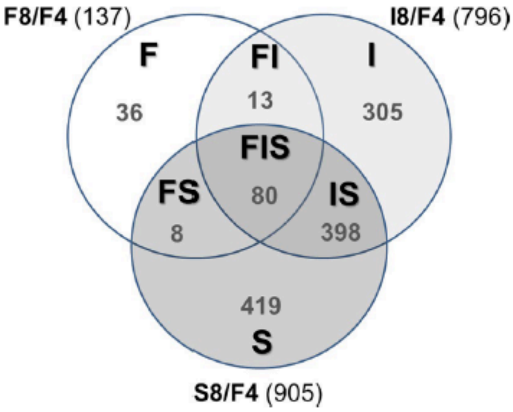

Fig. 1 Diagram of significant genes identified in F8, I8 and S8 with respect to that in F4 ($FC \geq 4$ with p value (t test) ≤ 0.05 and $FDR_F \leq 0.72$, $FDR_I \leq 0.2$ and $FDR_S \leq 0.25$). The significant genes were classified into 7 groups according to SR developmental stages: F—only in fibrous root, I—only in intermediate root, S—only in SR, FI—in both fibrous and intermediate roots, IS—in both intermediate and SRs, FS—in both fibrous and SRs, FIS—in all stages of roots

in their expression levels and genes that could be essential for root differentiation. The expression levels of 1259 genes (6 %) were significantly different from the control F4 (FC $\geq$ 4, p value $\leq$0.05; and FDRF $\leq$ 0.72, FDRI $\leq$ 0.20, FDRS $\leq$ 0.25) (Online Resource 3). These 1259 genes were classified into seven subgroups according to the developmental stages at which their expression levels were significantly altered: F—in fibrous, I—in intermediate, S—in storage, FI—in F and I, IS—in I and S, FS—in F and S and FIS—in all stages. The results showed that, compared to F4, the expression levels of 137, 796 and 905 genes were significantly changed in F8, I8 and S8, respectively. Only 80 genes have significant changes in their expressions in all stages (Fig. 1).

Functional analysis of significant genes during phase transition

The enriched GO terms and their degree of significance at a particular stage of root development can be linked to the activity of biological processes (Online Resource 4a). Therefore, functional enrichment analysis was applied to all classes of genes in a Venn diagram (Fig. 1). We hoped to determine the predominant gene functions for each developmental stage of the root, as well as during phase transition. Initially, the ontology-based functions of 1259 significant genes were re-annotated and 919 of them were annotated with 202 GO terms. The 91-enriched GO terms determined from 7 subgroups (Fig. 1; F, FI, I, IS, S, FS and FIS) were then identified (p value $\leq$0.5; and FDR $\leq$ 0.1). As observed, the enriched GO terms and their degree of significance at particular stages varied across all developmental stages. These results suggest that the regulation of SR development is highly dynamic. Some dominant biological processes functioned to retain their current stage, whereas others promoted a propagative stage (Online Resource 4). In addition, the global view of the consecutively significant biological processes during the developmental stages helped us to predict the regulation mandating SR formation. At F-stage and FI-stage, there was no dominant stage-specific process other than cellular processes and stress response, which were significant throughout F–I–S stages. When the root started to develop into I-stage, macromolecule biosynthesis/metabolism, translation and protein modification such as starch synthase (*SS*, probeID: 391), geranylgeranyl reductase (2920), and ACC oxidase 1 (*ACO1*, 6029) became significant. To differentiate into IS-stage, transcriptional regulation, signal transduction and enzyme regulation, such as Arabidopsis response regulator (*ARR3*, 157), geranylgeranyl pyrophosphate synthase1 (*GGPS1*, 1839, 1840), sucrose synthase 4 (*SUS4*, 10814), *KNOX1* (10762), caffeoyl-CoA 3-*O*-methyltransferase (*CCOMT*, 11837), cinnamoyl-CoA reductase-related (12676) and AGAMOUS-like 20 (*AGL20,* 13975) played important roles. Finally, when the root reached the S-stage, where cellular homeostasis also became a dominant process, primary/secondary metabolic processes, as well as lipid and amino acid metabolic processes such as *SS* (389), cellulose synthase (6303), auxin-responsive protein *IAA8* (11380), auxin transporter protein 1 (*AUX1*, 12207) and cinnamyl alcohol dehydrogenase (*CAD*, 2357) were active and maintained (Online Resource 4b).

Clustering analysis of the 87 significant genes during phase transition

In addition to the investigation at the level of biological processes, this section aims to provide a better understanding of the genetic regulation of root development through the analysis of gene expression patterns. Among the 1259 significant genes identified above, all TF and 37 significant genes with relatively clear expression patterns were subjected to the clustering analysis. The clarity of the expression patterns was defined, herein, as a highly variant expression profile based on the standard deviation (sd) $\geq$15 of the gene expression across developmental stages (top 3 percentile of sd distribution curve), with the assumption of the relatively equal variation within a developmental stage. This analysis showed that the expression profiles of these genes fell into one of the three patterns: cluster 1—highly expressed in F, cluster 2—highly expressed in I and cluster 3—highly expressed in S (Online Resource 5). The genes in cluster 1 mainly comprised of *NAC*, *MYB*, *AGL20* and *EREBP* TFs and they might be required for maintaining the F-stage. Therefore, the downregulation or repression of

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the genes in cluster 1 could lead to SR induction. Genes in cluster 2 might be involved in the transition from F-stage to I-stage or IS-stage, and might initiate SR formation. The expression levels of metallothionein, auxin responsive factors 4 (*ARF4*) and pollen protein Ole e 1-like became highest at the I-stage. Genes in cluster 3 were involved in the transition from F-stage and progressively extend into S-stage for the long-term development. These were genes associated with *KNOX1*, *ARF*, dormancy/auxin associated proteins, aquaporin transporters, and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*). In addition, the most significant changes in the expression levels were observed in phytohormone biosynthesis and signaling related genes during early SR development. Since KNOX1 was a TF regulating tuberization in potato and sweet potato (Chen et al. 2003; Tanaka et al. 2008), the expression levels of TF genes, especially KNOX1, and other phytohormone-related genes were further examined using a correlation network.

Phytohormone biosynthesis and signaling involved in phase transition

To closely investigate the phytohormone-relevant regulation during root phase transition, 67 phytohormone-related genes involved in phytohormone biosynthesis and signaling, were identified based on the GO and their original annotations from Phytozome database. These genes were then assembled into an association network showing negative correlation (red line) and positive correlation (blue line) (Fig. 2a). One of the subgroups within the network illustrated the relationship between *KNOX1* and a set of genes involved in the GA metabolic pathway, auxin and CK responsive genes, as well as genes involved in carbohydrate metabolism. In another subgroup, auxin transport carriers showed a link-relationship with auxin responsive genes, flowering, cell wall biosynthesis, cell expansion and differentiation. The correlation of the co-expressed genes in the network was further verified by the presence of the cis-element binding motif of a correlated TF on the target gene promoter. The confirmed results of TF and promoter analysis of the correlated gene were shown in black lines (Fig. 2b). To verify our findings, the gene expression levels in the KU50 sample set used for microarray and another field-grown KU50 sample set were measured by quantitative RT-PCR (qRT-PCR). The gene expression patterns were consistent with the microarray data (Fig. 3 and Online Resource 6). From the correlative network and qRT-PCR results, we propose a model suggesting that phytohormone cross talk plays an important role in cassava SR development (Fig. 2c).

KNOX1 might control GA level during the initiation stage. Upregulation of *KNOX1* (10762) was correlated with downregulation of GA-related genes: kaurene synthase (*KS*, 8718), GGPS1 (1839) and *AGL20* (13975). Compared with F4, *KNOX1* was upregulated in I8 and S8 (Fig. 3a) while the GA-related biosynthesis genes (*KS* and GGPS1) were downregulated, which implied that GA level was reduced in the storage stage. In addition, expression of *KNOX1* showed a positive correlation with ethylene biosynthesis gene *ACO1* (6029) and ethylene signaling gene (*ERF/CEJ1*, 3227) (Figs. 2, 3b). An increase in ethylene biosynthesis by *ACO1* together with the reduction of the bioactive GA level may fine-tune *AGL20* expression to co-regulate a phase transition into SR (Fig. 2).

The upregulation of genes downstream to CK responsive pathway suggests that CK may play an important role during SR development, which enhances cell differentiation. Expression of *ARR3* (157) peaked at I8 and then slightly reduced in S8, whereas cytokine responsive factor 1 (*CRF1*, 11244) was upregulated and peaked at S8 (Fig. 3b). CK is commonly known to promote cell expansion, cell differentiation, radial cell growth, and nutrient mobilization. The genes involved in cell expansion such as expansin (15641), extension (14709) and spiral1 (13404) were upregulated in I8 and S8, whereas *LPT* (10934) and *PIP1* (2883) expression was upregulated only in I8 and reduced in S8 (Fig. 3b) (Dello Ioio et al. 2007; Nakajima et al. 2004; Watillon et al. 1991).

Our model suggests that KNOX1 might regulate auxin levels during early SR development through polar auxin transport (*AUX1* and *PIN1*). Auxin level is controlled by auxin biosynthesis and auxin transport. However, our experiment showed that only polar auxin transporters (*AUX1* and *PIN1*) were differentially expressed. The *AUX1* (12207) was highly expressed in F8 but slightly reduced in I8 and drastically reduced upon reaching S8 (Fig. 3c). *NAC100* (21508) and *NAC5* (21081) also showed a similar expression pattern to *AUX1* (Fig. 3c). Other auxin responsive genes, *IAA* (11380, 41731) and *TPR1* (11849), were upregulated in I8 and S8, whereas the expression of *ARF4* (10217) increased in I8 and then reduced in S8. The differential expression of these auxin-signaling genes implies that auxin plays an important role during the SR's phase transition.

In 12-week-old SR, *KNOX1* was highly expressed, while *BEL1* and *AUX1* were downregulated and *KS* was diminished, suggesting that GA and auxin levels were reduced in the later stage of SR development (Fig. 3). Upregulation of *KNOX1*, *ARR3*, *CRF1* and spiral1 in C (layer 1) suggest that CK plays a major role in the cambium area, where cell division and differentiation take place, during the SR developmental process.

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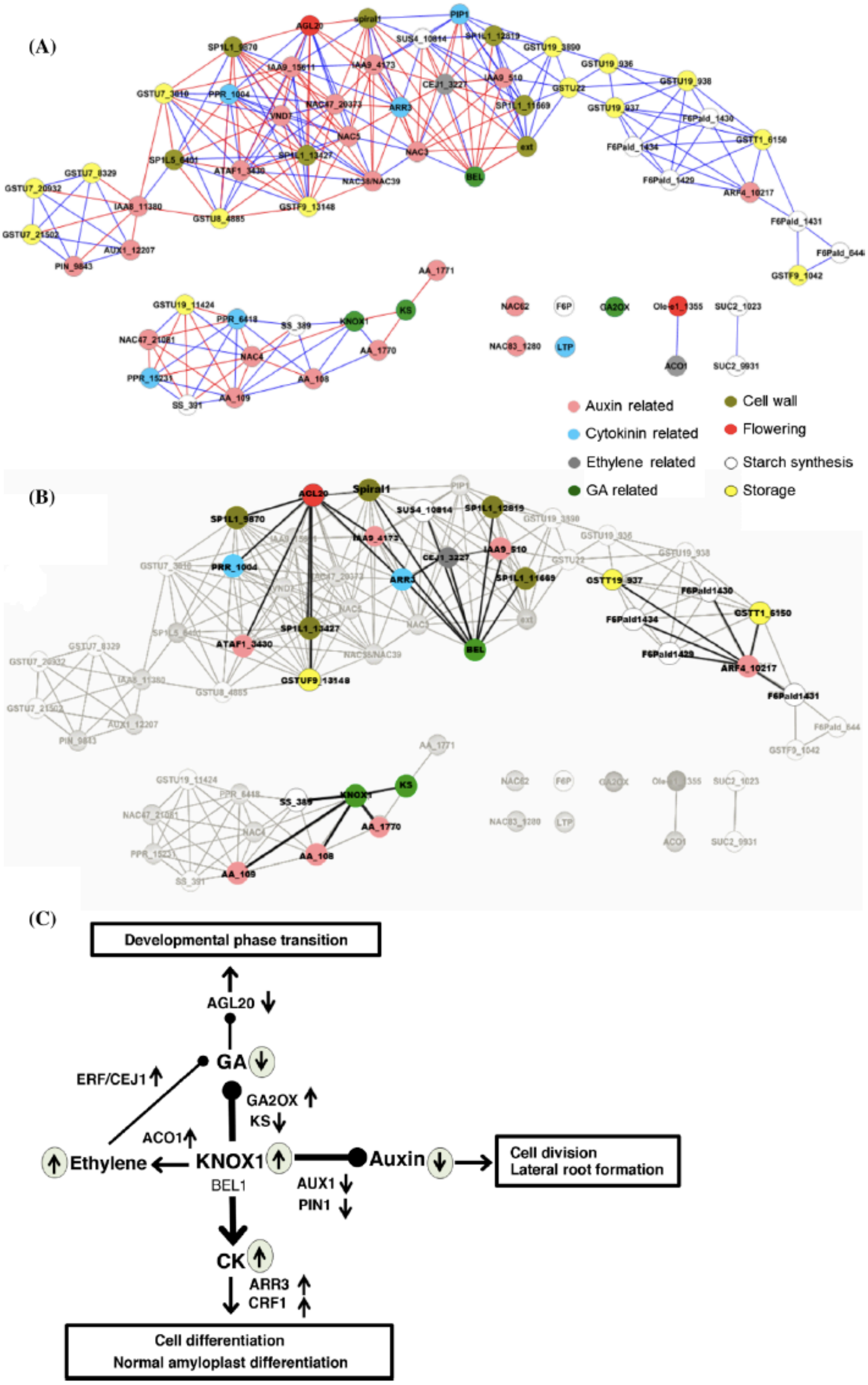

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◀**Fig. 2** The proposed genetic network and model involved in phase transition and development of SR. The 67 genes of interest that were classified into auxin-related (*pink*), CK-related (*blue*), ethylene-related (*grey*), GA-related (*dark green*), cell-wall-relevant (*light green*), flowering-relevant (*red*), starch-synthesis-relevant (*white*), and storage-relevant (*yellow*) genes were assembled into a co-expression network. **a** The association network of 67 genes are shown by a red line for negative correlation and a *blue line* for positive correlation. **b** The deduced correlations are based on the *cis*-element binding motif of a correlated TF on the target gene promoters. The results of the correlations are shown in *black lines*. **c** The proposed model involved in phase transition during early development of SR. The scheme describes the hypothetical scenario of KNOX modulating the phytohormone action in phase transition and development of SR. The inhibitory and activating relationships of associated gene pairs are represented in circular and *arrow headed lines*, respectively

Cytokinin and auxin played important roles in storage root initiation

A previous report (Medina et al. 2007) and our model showed that a combination of CK and auxin could induce SR formation. Our in vitro SR induction experiment showed that 6 % sucrose with 0.44 µM BAP and 0.25 µM NAA (KU-BN media) was an effective induction medium for KU50. In vitro SR with periderm tissue on the surface was observed within 2 weeks (Online Resource 7). The cross-section and iodine staining of the four week-old in vitro SR with diameter of ≥1.5–2 mm revealed a secondary growth of vascular tissues and accumulated starch in parenchymatous tissue (Online Resource 7f), which are characteristics of SR. In contrast, control FR in KU-media had diameter ≤0.3 mm and showed only primary vascular growth without starch accumulation (Online Resource 7e).

In order to investigate gene expression at the SR initiation stage, we mimicked the condition that induce phase change from an adventitious root to a SR, where CK level was high but auxin level was low, using KU-BN media. At the initiation stage, swollen root tip with development of vascular tissue and starch accumulation was observed within 24 h after hormone treatment, while non-hormonal treatment showed a normal fibrous root phenotype (Fig. 4a). In SwR, *ARR3* and *CRF1* were slightly upregulated as a result of exogenous CK treatment. While *BEL1* was slightly reduced, *KNOX1* was upregulated (Fig. 4b). However, their expression levels were not significantly different from those of FR; only *PIN1* and *AGL20* were significantly downregulated in SwR (Fig. 4c, e). In 7D-SR, all of these genes were almost turned off. Since sucrose synthase expression is correlated with sink strength and storage organ development (Li and Zhang 2003; Sturm and Tang 1999), *SUS3* and *SUS4* expression levels have been measured. The *SUS3* and *SUS4* were significantly upregulated in 7D-SR, suggesting that sucrose was mobilized into metabolic pathways and storage functions (Baud et al. 2004; Sturm and Tang 1999). The results from in vitro SR induction support our model that *AGL20* and *PIN1* are involved in SR initiation and confirm that high levels of CK and low levels of auxin play significant roles in SR initiation. Amounts of exogenous treatment of sucrose and hormones in this batch cultivation became limited, therefore this experiment could only show gene expression at the SR initiation stage, not for the prolonged SR development as is shown in field-grown conditions.

Discussion

Our transcriptomic study was performed on the individual plant basis under the possible experimental efforts. The combinatorial statistical criteria were used to analyze the data and to extract useful information. Pawitan et al. (2005) demonstrated that the extraordinarilly higher FDR than the typically acceptable cutoff (5–10 %) was inevitable for transcriptomic data measurement with low number of replicates ($n < 30$). The theoretical calculation of FDR for microarray study based on two-sample t statistics with pooled variance for $n = 5$ and p value $= 0.05$ was estimated to be greater than 60 % with about 35 % sensitivity. In the statistical adjustment for the false positive control, the sensitivity to acquire the real solution was dramatically reduced whereas FDR value tended to be persistently high. The rigorously working on the typically acceptable range of FDR was almost impossible, and it might result in sensitivity drop to a ground level. Therefore, relatively high fold change criteria ($|FC| \geq 4$) along with the calculated FDR statistics, which was suggested to improve the false positive control were used in our experiment (Pawitan et al. 2005). Moreover, with the concern of high FDR, the additional independent statistics with tight criteria (e.g. $sd \geq 15$) were applied for further analysis and then our inference was validated.

The gene expression profiles were obtained from samples at an early stage of SR development. The profiles of fibrous, intermediate and SRs at 8 weeks old were investigated, and compared to those of the four-week-olds' fibrous roots. Root cells at I-stage underwent differentiation with secondary growth of vascular tissue and starch accumulation; while in S-stage, parenchymatous tissue was progressively enlarged and filled with starch granules, similar to that reported by Teerawanichpan, et al. (2008) (Online Resources 2). When an organism is exposed to certain conditions, the significant changes in expression profiles, either at a specific time point or throughout the exposure period, reflect its biological regulation. Genes with significant changes in expression level at a particular root

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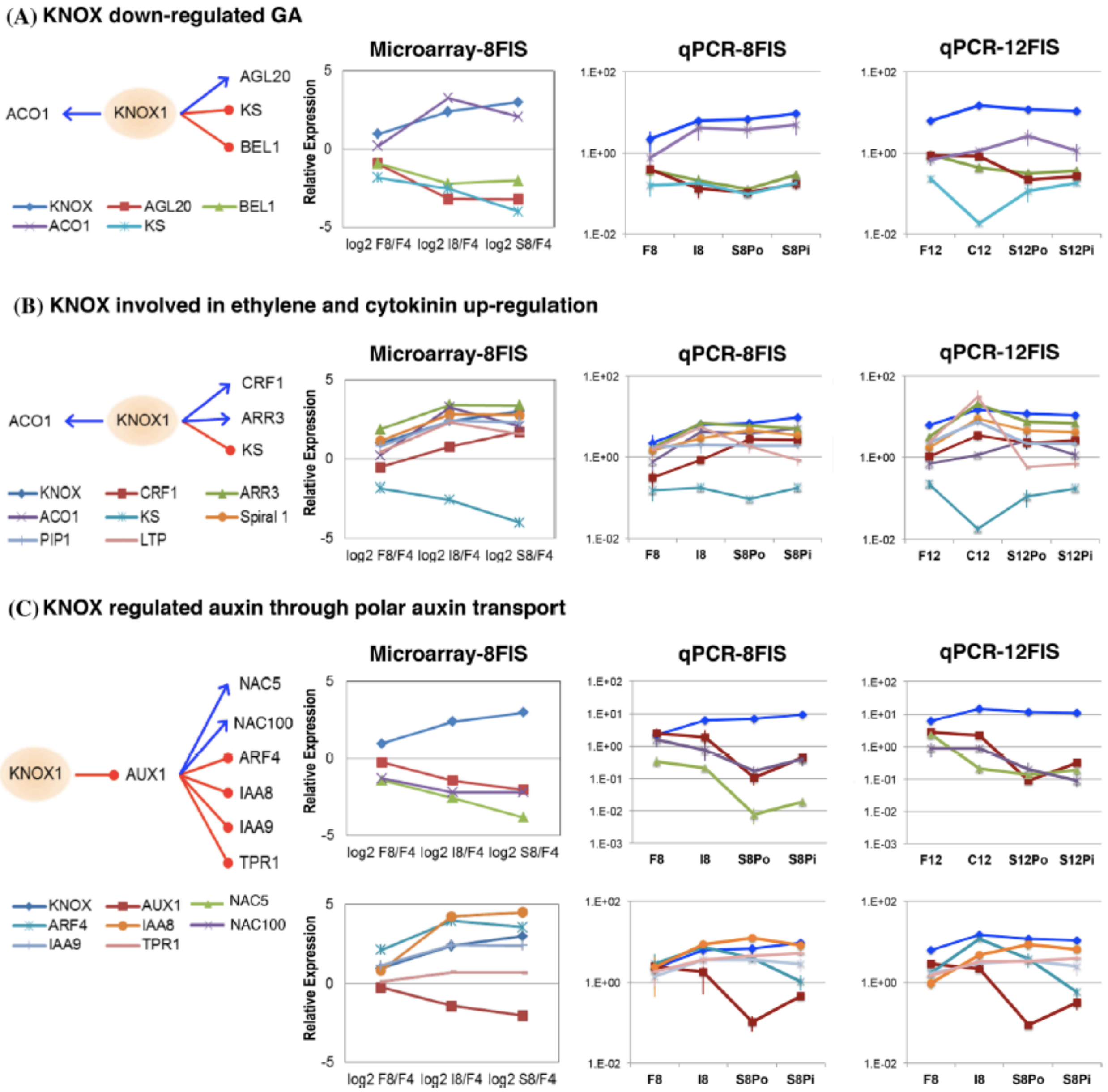

Fig. 3 The expression profiles of the modeled-genes in the field grown KU50 analyzed by microarray and qRT-PCR The first sample set for microarray that was harvested at four and 8 weeks old was classified into F, I, S. The next season's crop for qRT-PCR that was harvested at 4, 8 and 12 weeks old was classified into F, I, S and cortex (C; layer 1). SR was separated into outer parenchyma (SPo; layer 2–4) and inner parenchyma (SPi; layer 5). **a** The set of genes relevant to the downregulation of GA. **b** The set of genes relevant to the upregulation of ethylene and CK. **c** The set of genes relevant to the regulation of auxin via polar auxin transport. The relative expression levels were calculated in relation to the F4 control stage. The data are mean ± SE from three individual plants and the Y axis has a logarithmic scale

developmental stage may be essential for the retention of a certain developmental stage. The results illustrate that there are more genes involved in the development of I8 (796 genes) and S8 (905 genes) than those in F8 (137 genes) (Fig. 1). The distinctive gene expressions in each stage also indicated that transcriptional regulation was associated with phenotypic changes. The 36 genes found to be significantly expressed only in F-stage might act as important modulators to maintain the function and characteristics of fibrous root (Fig. 1). On the other hand, the 13 genes significantly

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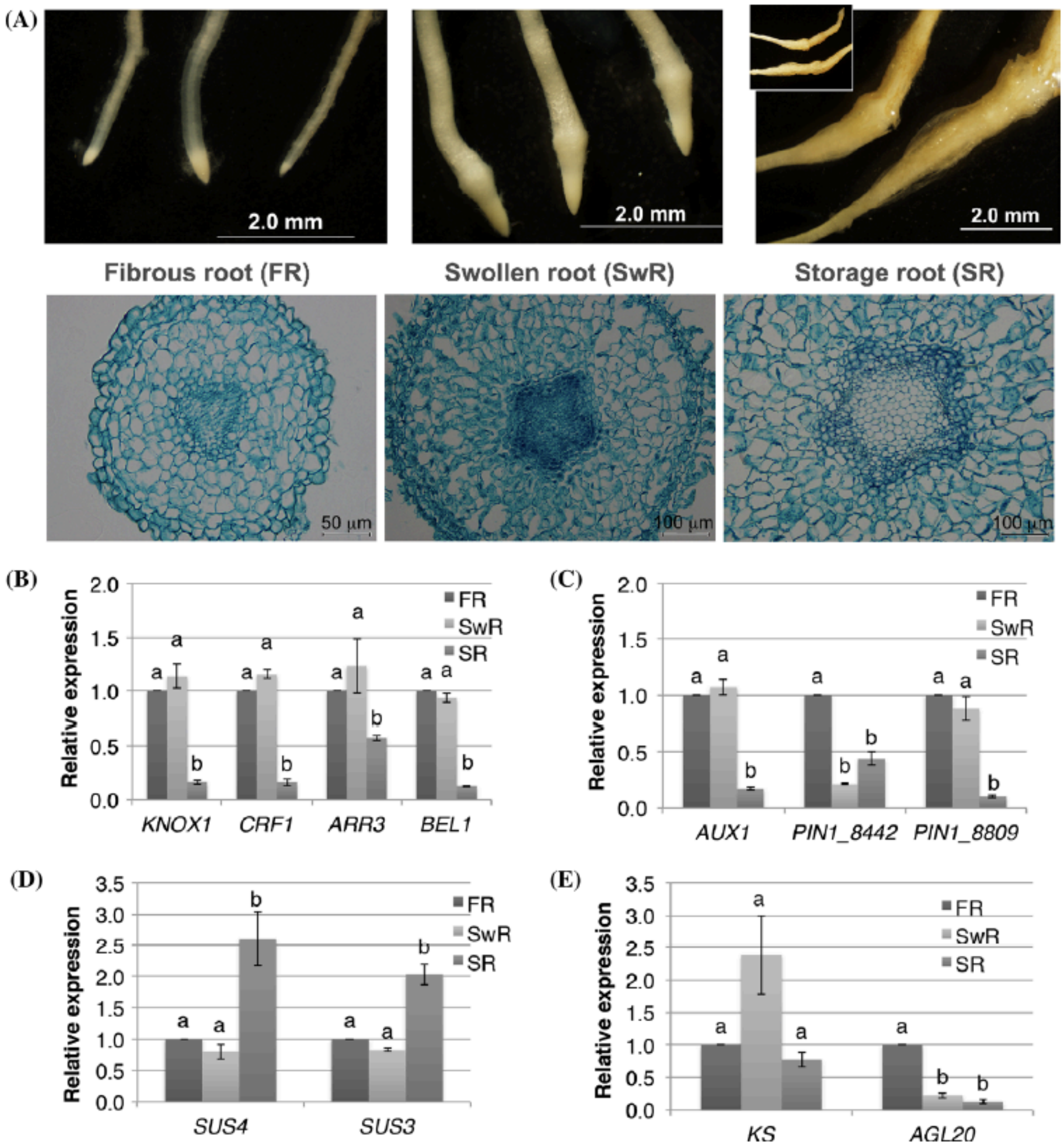

Fig. 4 The qRT-PCR expression profiles of the modeled key genes in in vitro KU50 SR induction. **a** FR in non-hormonal treatment condition, SwR that were harvested at 24 h after treatment in KU-BN and SR that were harvested at 7 days after treatment in KU-BN. Cross-sections stained with fast green showed the development of vascular tissue and starch accumulation in SwR and SR. The bar shown are 2.0 mm in whole roots, 50 µm in cross-section of fibrous root and 100 µm in cross-sections of SwR and SR. **b** Expression level of *KNOX1* and CK responsive genes, **c** expression level of polar auxin transport genes, **d** expression of sucrose synthase genes, **e** expression of GA responsive genes. The relative expression data represented an average of expression values that were normalized relative to the expression of EF1α from three biological replicates ±SE. The a and b above the *bar* indicate statistically significant difference from FR with *p* value $\leq$0.05

expressed in FI-stage might be involved in regulation of the transition into a storage organ. The significant genes in IS-stage (398 genes) might be involved in early development of SR. According to these observations, study of the transcriptional behavior in these developing roots, especially those in the I-stage and IS-stage, is expected to reveal the process modulating SR formation.

Functional analysis of significant genes during phase transition

The I8 sample represented a phase transition stage from fibrous root to SR. Microarray analysis showed that caffeoyl-coA-o-methyltransferase (CCOMT, 11837) and cinnamyl alcohol dehydrogenase (CAD, 2357) were downregulated in I8 and S8. The expression pattern of CCOMT and CAD indicated that lignin biosynthesis was reduced during the early stage of SR development. In addition, upregulation of *KNOX1* in I-stage and S-stage was negatively correlated with the expression of lignin biosynthesis key genes (*CCOMT* and *CAD*) (Guo et al. 2001; Townsley et al. 2013) suggesting that reduction of lignin biosynthesis corresponds to SR development (Firon et al. 2013; Testone et al. 2012).

The genes involved in glycolysis/gluconeogenesis were upregulated as the expression of glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) and fructose-1,6-bisphosphatase (*FBP*) increased in I-stage and S-stage (cluster 3, Online Resource 5). A similar pattern of G3PDH expression was also observed in 12-week-old cassava SRs (Sheffield et al. 2006). Our results suggest that high starch catabolism rate might play a role in the early stage of SR development, supporting the bioinformatics model in which the utilization of carbon substrates favored starch accumulation in SRs (Siriwat et al. 2012). However, our finding was in contrast to that of Yang et al. (2011). This could be due to the difference in time points for root sampling between these two experiments. Our experiment was designed to investigate an earlier state of SR development (8 weeks old) than in Yang et al. (2011) (16 week olds). We used cutting stem planting in the field, which mimicked the cassava planting conditions in Thailand, while Yang et al. (2011) cultivated plantlets that transferred from in vitro culture for four weeks in a pot and then transferred to the field. The gene expression profiles were investigated at 16 weeks old after planting in the field. In potato, reduction of cytosolic G3PDH had no effect on sugar metabolism in tuber, but the effects of other G3PDH isoforms could not be ruled out (Hajirezaei et al. 2006). In Arabidopsis, plastidial G3PDH plays roles in root development, as loss of G3PDH function results in root growth arrest (Muñoz-Bertomeu et al. 2009). Therefore the rate of glycolysis/gluconeogenesis during cassava SR development should be further investigated.

Functional analysis also showed that stress response processes were more active in all types of eight-week-old roots in comparison to the four-week-old fibrous roots. This observation was consistent with previous reports showing that the stress response-related genes were expressed at high levels relative to the plant's ability to grow in harsh environments (Li et al. 2010).

Phytohormone involved in phase transition from fibrous to storage stage

TFs that were differentially expressed in I8 and S8 have been reported to function in a hormonal regulating pathway in other organisms such as sweet potato (Tanaka et al. 2008). Since *KNOX1* showed a negative correlation to *KS* and polar auxin transports, but a positive correlation to *ACO1*, *ARR3* and *CRF1* (Figs. 2, 3), we propose that KNOX1 might be one of the phytohormone regulators during SR initiation and development (Fig. 2c). In potato, overexpression of *KNOX1* resulted in the reduction of GA and auxin levels, but the increase of CK levels (Chen et al. 2004, 2003; Tanaka et al. 2008; Xu et al. 1998). Therefore, the downregulation of *KS* and polar auxin transports in I-stage and S-stage might be regulated through KNOX1 and phytohormone cross talk. KNOX1 is commonly known as a CK biosynthesis activator during the early stage of tuber formation in both potato and sweet potato (Chen et al. 2003; Tanaka et al. 2008). The upregulations of *CRF1* and *ARR3* in I-stage and S-stage suggest the role of CK during SR initiation and development, as CK signaling is mediated via ARR and CRF (Rashotte et al. 2006; Tsai et al. 2012). Therefore, the results give us an overview that the downregulation of GA and auxin together with the upregulation of CK in I-stage and S-stage might function in concert to regulate the phase transition from fibrous to SR, in agreement with our preliminary experiments on hormone levels and their expression patterns in F, I and S (To be published elsewhere). The effect of phytohormone on SR initiation was then further investigated.

Role of cytokinin in suppression of AGL20 and GA biosynthesis

The downregulation of *GGPS1*, *KS* and *AGL20* in I-stage and S-stage suggests that the bioactive GA level was reduced. Downregulation of GA might fine-tune *AGL20* (MADS-box/SOC1-like) during phase transition (Figs. 3a, 4e). In potato, overexpression of POTH1 and the reduction of bioactive GA level functioned as a tuberization switch (Hannapel 2007; Rosin et al. 2003). A high level of KNOX1 would lower the GA level, either via downregulation of GA biosynthesis genes or upregulation of GA deactivation genes during the phase transition from F-stage to I-stage (Chen et al. 2004). In in vitro SR induction with exogenous BAP and NAA treatment, *AGL20* expression was diminished after hormone treatment, but the *KS* expression was not significantly different (Fig. 4d). GA and AGL20 have been reported as bioactive molecules involved in the developmental phase transition, and that their expressions diminish after phase change (Hannapel 2007; Sakamoto et al. 2001). The diminishing of *AGL20* might lead to inhibition of root elongation and induction of phase transition into storage stage (Achard et al. 2007; Fleet and Sun 2005). In potato, common regulatory pathways and homologous genes (*CO*, *SOC1* and *FT*) were conserved between the flowering and tuberization processes (González-Schain et al. 2012; Rodriguez-Falcon et al. 2006). *Ib*MADS and

AGL20-like (DV037991) were SR initiation factors that responded to CK, were involved in auxin-mediated initial thickening of the SR and were downregulated in SR (Ku et al. 2008; McGregor 2006; Noh et al. 2010; You et al. 2003). Hence, these results suggest that, in cassava, CK and GA cross talk might control phase transition into the storage stage via *AGL20*.

Role of cytokinin and auxin in storage root initiation

In this study, the in vitro SR was induced by 0.44 µM BAP and 0.25 µM NAA that were in the range of a considerable performance reported by Medina et al. (2007). We also found that CK alone could not induce KU50 SR formation (data not shown), which agreed with that observed by Medina et al. (2007). Also the overexpression of CK in potato could not induce tuberization in all events (Sergeeva et al. 2000). The combination of auxin and CK induced early tuber initiation and inhibited stolon elongation by counteracting the GA function, while CK and sucrose (but not auxin) induced secondary thickening growth of the tuber (Eguchi and Yoshida 2008; Wang et al. 2006; Xu et al. 1998). In I-stage, *AUX1*, *PIN1* and *KS* were downregulated but *CRF1* and *ARR3* were upregulated. In in vitro SR induction, *PIN1* (8442) was significantly downregulated in SwR and 7D-SR (Fig. 4c). While *AUX1* and *PIN1* (8809) were significantly downregulated at the later stage of storage root development (7D-SR) (Fig. 4c), these results imply that repression of polar auxin transport and *AGL20* during SR initiation play roles in the phase transition from fibrous into storage stage. When auxin transport was impeded, the inhibitory effect of auxin was relieved and tuberization was promoted (Roumeliotis et al. 2012a). In addition, KNOX could inhibit polar auxin transport, as the ectopic expression of KNOX in *knox* regulator rs2 and semi mutant caused defective auxin transport (Scanlon et al. 2002; Tsiantis et al. 1999; Zhang et al. 2011). GA is an auxin transport carrier regulator and stabilizer (Willige et al. 2011), while CK induces auxin transport carrier degradation (Ioio et al. 2008). Our results suggest that at I-stage and in swollen root, phytohormones act in concert to regulate phase transition and primary thickening growth by inducing secondary cambium and metaxylem differentiation and nutrient mobilization (Fig. 4) (Eguchi and Yoshida 2008; Ioio et al. 2008; Noh et al. 2010; Overvoorde et al. 2010; Perrot-Rechenmann 2010; Roumeliotis et al. 2012b; Wang et al. 2006).

Role of cytokinin on sucrose mobilization

In root, CK is a hormonal signal that regulates cell differentiation, radial cell growth, starch biosynthesis, nutrient mobilization, and tuber formation (Hwang et al. 2012; Smith and Palmer 1970). CK regulate starch biosynthesis and plastid differentiation from proplastids to amyloplasts (Enami et al. 2011; Miyazawa et al. 1999). In addition, sucrose synthase expression is correlated with sink strength and storage organ development (Li and Zhang 2003; Sturm and Tang 1999). The results showed upregulation of glucose-6-phosphate dehydrogenase, fructose-1,6-bisphophatase, starch branching enzyme and sucrose synthase during the I-stage to S-stage transition. *SUS3* and *SUS4* expression were included in the investigation as markers for sucrose mobilization and SR development. The upregulation of *SUS3* and *SUS4* by BAP treatment in in vitro SR induction suggests a role of CK on sucrose mobilization into metabolic pathways and storage functions.

In conclusion, our findings suggest a potential role of KNOX1 as a phytohormone linker in SR development. Moreover, AGL20 has a potential to play an important role in SR initiation. The phytohormone levels in each developmental stage are under investigation and the preliminary results support our model. Further investigation of the phase transition mechanism during SR initiation could unravel a complex metabolic pathway in SR initiation.

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Complinace with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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