รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษาการแสดงออกของยีนที่ควบคุมผลผลิตและปริมาณแป้งในมันสำปะหลัง

Identification and functional analysis of candidate genes within QTL controlling yield and starch content in cassava (*Manihot esculenta* Crantz)

โดย นางสาวศุภจิต สระเพชร

กรกฎาคม พ.ศ. 2557

สัญญาเลขที่ TRG5580003

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นางสาวศุภจิต สระเพชร มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุการวิจัยและมหาวิทยาลัยมหิดล

กิตติกรรมประกาศ

รายงานวิจัยฉบับนี้ ได้รับทุนสนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล ผู้วิจัยจึงขอขอบพระคุณเป็นอย่างสูงต่อการสนับสนุนโครงการวิจัยนี้

งานวิจัยนี้สำเร็จลุล่วงไปด้วยดีเนื่องจากได้รับความกรุณาอย่างสูงจาก รองศาสตราจารย์ ดร. กนกพร ไตรวิทยากร สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล อาจารย์ที่ปรึกษางานวิจัย ซึ่งท่านได้ให้คำแนะนำและข้อคิดเห็นต่างๆ อันเป็นประโยชน์อย่างยิ่งในการทำวิจัย

ขอขอบพระคุณ ดร.โอภาษ บุญเส็ง ศูนย์วิจัยพืชไร่ระยอง กรมวิชาการเกษตร ซึ่งเป็นผู้ทรงคุณวุฒิให้ความอนุเคราะห์สถานที่ในการปลูกพืชวิจัย รวมถึงขอบคุณเจ้าหน้าที่บุคลากร เพื่อนและน้องทุกท่าน จากสถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล ซึ่งให้ความช่วยเหลือเป็นอย่างดี

อนึ่ง ผู้วิจัยหวังว่า งานวิจัยฉบับนี้จะมีประโยชน์ในการพัฒนางานวิจัยด้านมันสำปะหลังต่อไป

ศุภจิต สระเพชร

Abstract

Project Code : TRG5580003

Project Title : Identification and functional analysis of candidate genes within QTL controlling yield and starch content in cassava (*Manihot esculenta* Crantz)

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Project Period : 2 years

The aim of this study was to identify and validate quantitative trait loci (QTL) underlying fresh root and starch content in cassava roots. In this study, seven QTL associated with fresh root yield with 7.6-17.3% of the phenotypic variation (PVE), and 11 QTL for fresh starch content with 11.3% to 27.3% of PVE were identified from four different environments. Cluster of major QTL controlling fresh root yield (Yr10_1 with 17.3% PVE and Yr09_3 with 9.7% PVE) and starch content (Sr10_3 with 14.7% PVE) was identified on LG16. In addition, another major QTL Sr10_1 (PVE= 21.5%) was identified on LG6. Candidate genes within the consistent QTL across environments and selected QTL were identified based on cassava genome sequences. The candidate genes at peak QTL at periphery regions were selected to evaluate their expression among the F_1 lines with high (H) and low starch content (L) including parental lines at 6, 9 and 12 MAP (Month after planting). The RPM1-interacting protein 4, homeobox domain, auxin/cyclin G-associated kinase, endomembrane protein 70 and zing finger showed similar gene expression profiles in both groups while phenylalanyl-tRNA synthetase showed gene expression profile.

The QTL controlling fresh weight root yield and starch content in this study will be useful for molecular breeding of cassava through marker-assisted selection (MAS). The information in this study could be useful for further study of cassava gene expression.

Keywords : QTL, yield, starch content, cassava

บทคัดย่อ

รหัสโครงการ: TRG5580003

ชื่อโครงการ: การศึกษาการแสดงออกของยีนที่ควบคุมผลผลิตและปริมาณแป้งในมันสำปะหลัง

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ระยะเวลาโครงการ: 2 ปี

จากการวิเคราะห์หาตำแหน่งของยีนที่ควบคุมปริมาณผลผลิตและปริมาณแป้งในมันสำปะหลัง พบว่ามี 7 ตำแหน่งซึ่งมีความสัมพันธ์กับปริมาณผลผลิต โดยมีความผันแปรทางลักษณะที่แสดงออกตั้งแต่ 7.6% ถึง 17.3% และ 11 ตำแหน่งที่สัมพันธ์กับปริมาณแป้งและสามารถอธิบายความแปรผันของลักษณะปริมาณแป้งที่แสดงออกได้ตั้งแต่ 11.3% ถึง 27.3%

จากการวิเคราะห์หน้าที่ของยีนบริเวณตำแหน่งยีนควบคุมปริมาณผลผลิต (Yr10_1 และ Yr09_3) และควบคุมปริมาณแป้ง (Sr10_3) ซึ่งอยู่บริเวณเดียวกันบนกลุ่มเครื่องหมายพันธุกรรมที่ 16 และการวิเคราะห์หน้าที่ของยีนที่ควบคุมปริมาณผลผลิตและปริมาณแป้งของมันสำปะหลังในรากมันสำปะหลังสายพันธุ์ห้วยบง 60 สายพันธุ์ห้านาที และลูกผสมรุ่นที่ 1 ที่มีค่าปริมาณแป้งสูงและต่ำ ที่มีอายุ 6 เดือน 9 เดือน และ 12 เดือนหลังการปลูก พบว่าพบว่ายีน RPM1-interacting protein 4 ยีน homeobox domain ยีน auxin/cyclin G-associated kinase ยีนendomembrane protein 70 และยีน zing finger มีรูปแบบการแสดงออกเหมือนกันในกลุ่มที่มีปริมาณแป้งสูงและต่ำ ส่วนยีน Phenylalanyl-tRNAsynthetase มีรูปแบบการแสดงออกเหมือนต่างกันในกลุ่มที่มีปริมาณแป้งสูงและต่ำ อย่างไรก็ตามระดับการแสดงของของยีนไม่มีความแตกต่างกันอย่างมีนัยสำคัญระหว่างกลุ่มแป้งสูงและต่ำ

ผลการศึกษานี้เป็นข้อมูลพื้นฐาน เพื่อนำไปประยุกต์ใช้ในการศึกษาการแสดงออกของยีนซึ่งน่าจะมีความเกี่ยวข้องกับลักษณะปริมาณแป้ง รวมถึงลักษณะผลผลิตในมันสำปะหลัง และสามารถนำไประยุกต์ใช้ในการคัดเลือกและพัฒนาสายพันธุ์มันสำปะหลังที่มีปริมาณผลผลิตและแป้งที่สูงต่อไป

คำหลัก : QTL, yield, ลักษณะผลผลิต ปริมาณแป้ง มันสำปะหลัง

Introduction

Cassava is one of the most important staple food crops (El-Sharkawy et al. 2004). It is widely grown in Africa, Latin America and Asia (Raji et al. 2009). Recently, Thailand is the world second ranking cassava producer after Nigeria (FAOSTAT 2010). Most of cassava roots are processed to cassava starch, flour, chips and pellets for export, leading Thailand to be the largest exporter of cassava products (Onwueme et al. 2002). Moreover, cassava is an alternative crop for ethanol production (Ziska et al. 2009). Therefore, the goal of cassava breeding in Thailand is to increase cassava yield and starch content to support the demands of cassava in variance industries as well as in ethanol production (Sriroth et al. 2005).

Most of agricultural traits including yield and starch content are controlled by multigenic genes known as quantitative trait loci (QTL), and environmental factors and show continuous variation within population (Asíns et al. 2002). Therefore, QTL analysis is a powerful tool for identification of the location of genomic regions controlling the quantitative traits (Kearsey et al. 1998). To identify QTL positions, genetic linkage map is constructed from segregation population using DNA markers such as simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and express sequence tags-SSR (EST-SSR) (Collard et al. 2005).

The genetic linkage maps of cassava were constructed using different DNA marker such as RFLPs, RAPDs and isoenzymes (Fregene et al. 1997), SSRs (Fregene et al. 1997, Mba et al. 2001, Okogbenin et al. 2006, Boonchanawiwat et al. 201, Chen et al. 2010), and EST-SSRs (Kunkeaw et al. 2010). The most recent map encompassed around 88% of cassava was developed using SSR and EST-SSR markers (Sraphet et al. 2011).

QTL associated with yield production in cassava have been reported in several studies. QTL effecting early root bulking was identified (Okogbenin et al. 2002). In 2003, Okogbenin and Fregene reported QTL controlling productivity and plant architecture. Moreover, an F2 population was used to identify QTL for components of early yield by Okogbenin et al. (2008). Kizito et al. (2007) revealed QTL for dry matter in root and for cyanogen content of cassava that were also identified in F1 population. Other QTL for plant and first branch height which associated with yield and for yield-related traits were identified by Boonchanawiwat et al. (2011) and Chen et al. (2012), respectively. Recently, Supajit et al. (2014, accepted) reported QTL controlling fresh root yield and starch content

The information derived from genetic linkage maps and QTL analysis can be applied in marker assisted selection (MAS) programs which are a powerful tool in plant breeding to

developed new variety of plants with desirable traits and also applicable for study functions of genes controlling the traits of interest (Collard et al. 2005, Mohan et al. 1997).

Therefore in the present study, potential QTL associated with starch content will be subjected to gene annotation in order to identify potential genes controlling starch content of cassava for functional analysis.

Objective

1. To identify genes controlling QTL for yield and starch content in cassava
2. To evaluate expression of candidate genes associated with yield and starch content in cassava between parental lines and F_1 progenies at 6, 9 and 12 months after planting (MAP)

Methodology

Plant materials

F_1 cassava population was developed from a cross between Huay Bong 60 and Hanatee at Rayong Field Crops Research Center, Rayong. Each individual line was planted by stem cutting based on 10 plants per line. At 6, 9 and 12 months after planting (MAP), cassava roots of parental lines were collected from one plant. For F_1 progenies, six lines with extremely high and low starch content (%) were selected. Cassava root was sliced as small pieces and immediately frozen into liquid nitrogen and stored at -80 ºC.

Potential QTL selection controlling starch content

From previous study of QTL associated with starch content in cassava by Sraphet et al. (2011, accepted), potential QTL will be selected based on QTL across years and locations, major QTL showing the phenotypic variance explained (PVE) greater than 10% (Collard et al. 2005) and co-localization of QTL from different traits.

Functional gene annotation

To annotate gene function of candidate QTL region, primer sequence of all makers within 2-LOD support interval of the QTL will be subjected to blast against cassava genome database using blast function available at Phytozome (http://www.phytozome.net/). The sequences of scaffolds will be used to predict protein-coding genes.

RNA isolation

Total RNA was extracted from grinded storage root using Fruit -mate™ for RNA purification (Takara) and TRIzol reagent (Invitrogen) according to manufacturer's instructions with some modification.Total extracted RNA was treatedwith DNA-free™ DNA removal Kit (Applied Biosystems/Ambion). The concentration of RNA was determined by Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Welmington, DE, USA). The ratio of absorbance at 260/280 between 1.9-2.1 and 260/230 ration greater than 2.0 were used to access the purity of RNA.

Primer designing and efficiency of PCR reaction

Primers of candidate gene were designed using IDT integrated DNA technologies website (http://sg.idtdna.com/primerquest/Home/Index) under default parameters. Primer

BLAST was performed for checking specificity using NCBI database (http://www.ncbi.nlm.nih.gov/nuccore).

Two-fold serial dilutions of cDNA from HB60 or HT were performed for calculation PCR efficiency according to the following equation.

$$\text{Amplification efficiency} = [10^{(-1/\text{slope})} - 1] \times 100$$

The primer showingthe efficiency of PCR between 90-110% ($-3.6 \geq \text{slope} \geq -3.1$) with R^2 above 0.98 were used forrelative gene expression analysis.

Relative quantitative gene expression PCR analysis

First strand cDNA was synthesized from 1 µg of total RNA and Oligo-(dT)17 primers using ImProm-II™ reverse transcriptase (Promega) in final reaction volume of 20 µl according to manufacturer's instructions. Quantitative real time PCR reaction contained 2 µl of diluted cDNA, 10 µl of 2x KAPA SYBR® FAST qPCR Master Mix ABI Prism™ (KAPABIOSYSTEM) and 0.2 µM each of gene-specific primer in a final volume of 20 µl.Triplicate of each cDNA sample were performed in twin.tec PCR 96-well plate (Eppendorf).The real-time PCR was performed using Mastercycler® eprealplex (Eppendorf) with 2 step cycles of PCR condition consisted of enzyme activation at 95ºC for 3 min, followed by 40 cycles of denaturation at 95ºC for 5 sec, annealing/extension at 60ºC for 30 sec. The melting curves 60 to 95 ºC was performedafter 40 cycles for checking specificity of amplicons. The relative fold change expression of target gene was determined as $2^{-(\Delta Ct)}$, where ΔCt was Ct (target gene) – Ct (housekeeping gene). The housekeeping gene TATA was selected in this study.

Results

Plant materials

At 6, 9 and 12 months after planting (MAP), cassava root of parental line and six lines of F1 cassava population with extremely high ranging from 23.4-26.0% and extremely low ranging from 14.7-17.7% were selected for real time PCR analysis (Table 1).

Potential QTL selection

Seven QTL effecting fresh root yield were detected on seven linkage groups from four different environments. These QTL explained phenotypic variance that ranged from 7.6-17.3%. A total of 11 QTL influenced fresh starch content with explaining 11.3-27.3% of phenotypic variation were identified on 10 linkage groups.

Major QTL underlying traits at the same region across years and locations were considered for identification of potential candidate genes. Sr10_3 for fresh starch content (PVE=14.7%) was closely located with Yr10_1 for fresh root yield (PVE=17.3%) on Lg16. In addition, Yr09_3 accounting for 9.7% of PVE was located nearby Sr10_3 and Yr10_1. The confidence interval of these QTL was found on scaffold07933 and scaffold07827. Another interesting major QTL of Sr10_1 for fresh starch content belongs to scaffold10963 accounting for 13.5% of PVE was found on Lg6 (Figure 1). All of these selected QTL were further gene annotation analysis.

Functional gene annotation

The QTL region on scaffold07933 (Sr10_3 and Yr09_3), scaffold07827 (Yr10_1), scaffold10963 (Sr10_1) examined to identify potential candidate genes using blast function available at Phytozome. From GBrowse view of Phytozome database, the length of scaffolds07827 was 62.9 kbp consisting of 9 transcripts with 8 known gene functions. The scaffold07933 and scaffold10963 found 16 transcripts with 9 protein of known function and 107 transcripts with 79 protein of known function, respectively.

Genes that were found near the highest LOD peak included those encoding; RPM1-interacting protein 4 (scaffold07827), homeobox domain (scaffold07933), zinc finger (scaffold 10963). The candidate genes of at peak QTL and at periphery regions were selected for primer design (Table 2). In addition, some interested gene which involve in traits were selected and used for primer design.

Primer designing and efficiency of PCR reaction

A total of 18 primers were designed from candidate genes at QTL peak and periphery regions (Table 3). The PCR efficiency was performed using diluted DNA of parents. The standard curve was generated, and the slope derived standard curve was used to estimate the amplification efficiency of primers based on the serial dilution of standard sample representing as a semi-log regression line plot of Cq value and log of serial dilutions. The PCR efficiency between 90-110% (-3.6 $\geq$ slope $\geq$-3.1) with R2 above 0.98 is considered acceptable for RT-PCR analysis. There were 8 primers showed the expected PCR efficiency as shown in Table 8 ranging from 91.4 to 105.8% with the slope of standard curve of -3.1 to -3.4 and R2 of 0.98 to 0.99. The dissociation curve of all genes of interest and reference gene (TATA) showed a single peak of the PCR product (Figure 2).

Relative quantitative gene expression PCR analysis

There were 7 annotated candidate genes which were RPM1-interacting protein 4, PPR repeat, homeobox domain, auxin/cyclin G-associated kinase, phenylalanyl-tRNA synthetase, endomembrane protein 70 and zinc finger were showed the expected PCR efficiency. All of these genes were used to evaluate their expressions among the F1 lines with high and low starch content including parental lines at 6, 9 and 12 MAP using real time PCR. The relative expression levels were calculated using the ΔCq method. The target samples were normalized with the samples amplified by TATA gene (Figure 3).

The relative expression levels of all candidate genes between high starch content (H) and low starch content (L) groups showed no significant difference between the groups of high and low starch content.

There were two groups of gene expression which were same and different pattern between H and L group. The RPM1-interacting protein 4 and homeobox domain showed decreasing trend in expression at 9 and 12 MAP in both H and L groups. In contrast, the auxin/cyclin G-associated kinase, endomembrane protein 70 and PPR repeat showed increasing at 9MAP and decreasing at 12 MAP of both groups. The zinc finger expression of H and L groups decreased at 9 MAP to slightly increase at 12 MAP. Another group of gene, the expression PheRS gene showed decreasing at 9MAP and increasing at 12 MAP in H group while decreasing trend at 9 and 12 MAP.

Discussion

Among candidate genes identified within the QTL intervals underlying starch content, the glycosyl hydrolases; the UDP-glucuronosyl; and the UDP-glucosyltransferases were predicted to be involved in carbohydrate metabolism in casssava. Glycosyl hydrolases family 15 (GH15) belong among the glycosyl hydrolase (GH) enzymes which are classified into EC 3.2.1- by CAZy (Cantarel et al. 2009). Enzymes in this group were involved inreactions in starch synthesis (Keeling and Myers 2010). UDP-glucuronosyl (EC 2.4.1.17) and UDP-glucosyltransferase from scaffold09732 is classified among the glycosyltransferases (GTs) (EC 2.4.x.y) that catalyze the transfers of the glycosyl group from a UTP-sugar, forming small hydrophobic molecule. Similarly, candidate genes underlying QTL for starch pasting temperature in cassava were glucosyltransferases and glycosyl hydrolases (Thanyasiriwat et al. 2014).

The candidate genes underlying the QTL for fresh root yield included those encoding a transcription factor, phosphatase activity, response signaling pathway and thiolester hydrolase activity. As reported in a meta-analysis of yield QTL (Swamy and Sarla 2011) and dissection yield (Fu et al. 2010), the zinc finger, auxin/cyclin G-associated kinase, Ubiquitin-associated UBA/UBX domian-cointaning and pentatricopeptide repeat (PPR) were also identified in this study. Other candidate genes within QTL associated with fresh root yield and starch content may influence with these traits in cassava.

Amplification efficiency is an important consideration for relative gene expression quantitation. Ideally, the PCR amplification is 100% efficiency (Wong et al.2005). The primers used in this study showed amplification efficiency close to 100% indicating a PCR amplicon is generated doubling in quantity during every cycle within exponential phase of PCR reaction. From dissociation curve, the PCR product amplified by those candidate genes showed a single peak indicates that there were not primer-dimer and non-specific products.

Real-time RT-PCR analysis demonstrated that candidate genes which identified at peak QTL and at periphery of QTL showed no significant differences in gene expression between H and L group of F_1 lines. This suggesting that yield and starch content which are the complex quantitative trait were control by several genes (Ding et al. 2012, Wilson et al. 20014)

Normally, the phenotype F_1 hybrid is outside the parental range and the gene expression in hybrid show more variable than their parent (Renaut et al. 2009). The F_1 lines

might have the pleiotropic effects on the activities of other isoforms of each gene causing the difference in gene expression level, as described by Craig et al. (1998).

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Table 1 Phenotype data of F_1 selected lines and parental lines

Group	Line	Fresh starch content (%)							
		Rayong						Lop Buri	Average
		2008	2009	2010	2011	2012	2013	2009	
Parents	HB	20.4	19.8	21.4	24.2	24.1	24.1	22.0	22.7
	HT	23.0	15.3	15.8	21.2	17.8	17.8	18.5	17.6
High starch	A48	27.1	20.2	20.7	27.1	24.7	24.7	23.3	23.5
	A94	24.8	25.0	20.9	28.7	27.7	27.7	20.6	26.0
	B4	28.1	21.9	23.4	22.5	24.6	24.6	24.6	23.4
	B23	29.4	21.4	22.3	27.2	26.4	26.4	*	24.7
	B35	28.0	19.7	22.5	26.4	29.4	29.4	16.5	25.5
	B42	29.1	22.2	22.0	27.1	27.6	27.6	20.3	25.3
Low starch	A66	19.4	12.9	10.9	21.2	14.3	14.3	24.9	14.7
	A67	21.1	13.2	15.3	21.4	19.4	19.4	19.1	17.7
	A80	22.8	14.2	16.4	18.6	15.6	15.6	*	16.1
	B44	23.7	17.3	13.8	21.1	*	*	*	17.4
	B58	22.5	16.8	13.1	21.8	17.7	17.7	*	17.4
	B83	19.1	16.8	13.3	20.7	17.1	17.1	23.2	17.0

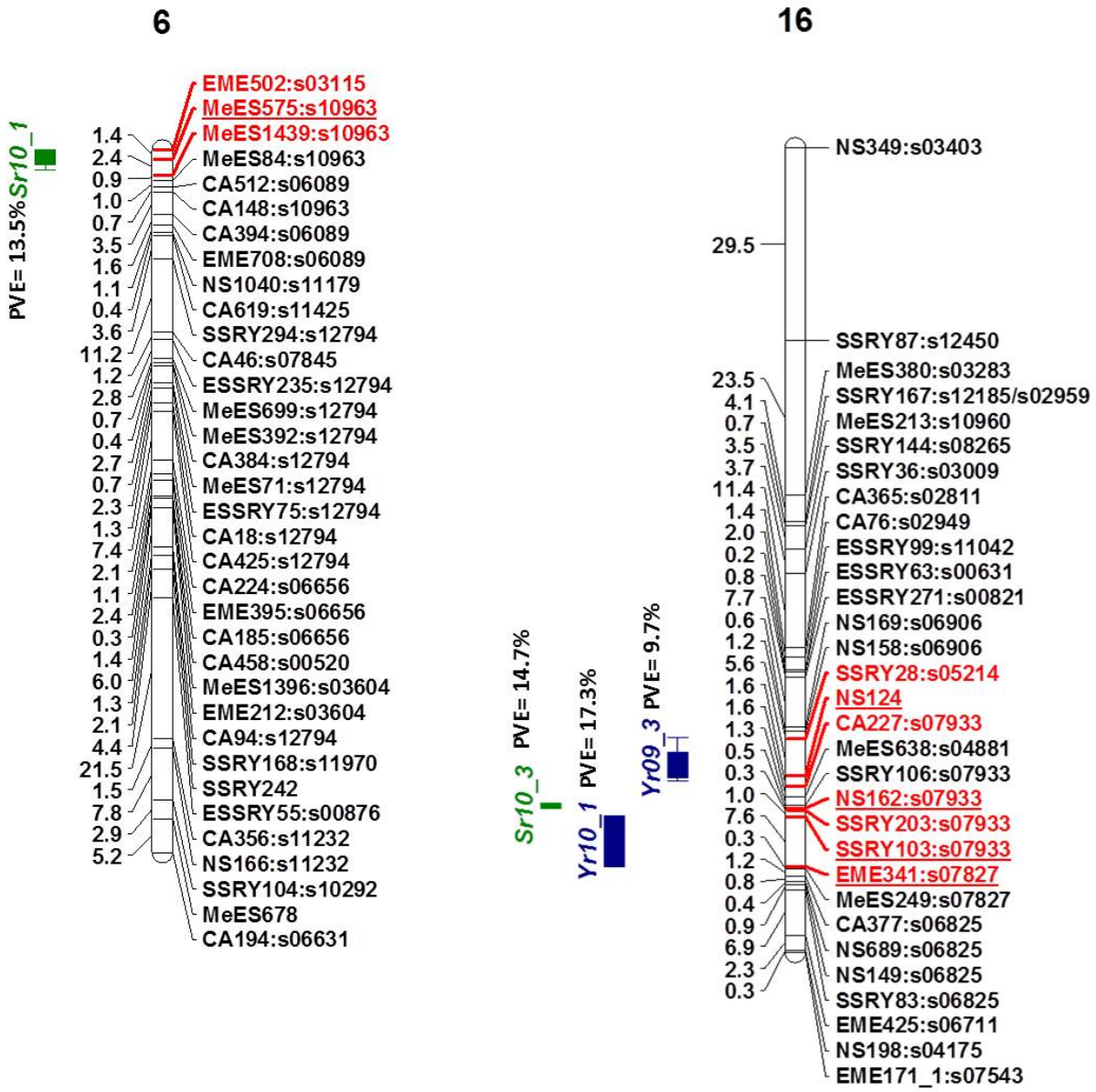

Figure 1 Position of selected QTL associated with fresh starch content and root yield

The green and blue bars represent QTL controlling fresh starch content and root yield. The underlined markers are the closet marker at the highest LOD score peak.

Table 2 Number of transcript, annotated protein functions assembled into scaffolds assigning onto the QTL linked markers and selected candidate gene for primer design

QTL	Marker at QTL peak:scaffold	Length (kbp.)	Transcripts	Genes function		Position of genes	Locus name	Selected candidate genes
				Known	Unknown			
Yr10_1	EME341/s07827	62.9	9	8	1	at QTL peak	cassava4.1_013976m.g	RPM1-interacting protein 4 (RIN4)
						at QTL periphery	cassava4.1_023165m.g	Ubiquitin-associated UBA/UBX domian-cointaning
							cassava4.1_003618m.g	Pentatricopeptide repeat (PPR)
							cassava4.1_025029m.g	Acyl-ACP thioesterase
							cassava4.1_013976m.g	Cleavage site for pathogenic type III effector avirulence factor Avr
							cassava4.1_016656m.g	Zinc finger
Sr10_3	SSRY103:s07933	200.4	16	9	7	at QTL peak	cassava4.1_000562m.g	Homeobox domain
Yr09_3	NS162:s07933					at QTL periphery	cassava4.1_001004m.g	Auxin/cyclin G-associated kinase
							cassava4.1_013810m.g	Dual specificity phosphatase, catalytic domain
							cassava4.1_008276m.g	Phenylalanyl-tRNA synthetase
							cassava4.1_003219m.g	EMP70
Sr10_1	MeES575:s10963	838.1	107	79	28	at QTL peak	cassava4.1_012053m.g	Zinc finger
						at QTL periphery	cassava4.1_020961m.g	Galactosyltransferases
							cassava4.1_024488m.g	No apical meristem (NAM) protein
							cassava4.1_029907m.g	PPR repeat
							cassava4.1_017669m.g	AN1-like Zinc finger
							cassava4.1_034196m.g	UDP-glucuronosyl and UDP-glucosyl transferase
							cassava4.1_002976m.g	Myb-like DNA-binding domain
							cassava4.1_005907m.g	Hexokinase
							cassava4.1_022645m.g	Acylglycerol-3-phosphate acyltransferase
							cassava4.1_022953m.g	Glycosyl hydrolase

Table 3 The designed primers for RT-PCR analysis

Gene name	Primer name		Sequence (5'-3')	Tm (°C)	GC content (%)	Product size (bp.)
RPM1-interacting protein 4 (RIN4)	RIN	F:	CCAGCACGCCATGACAACATGAAT	60.3	50	172
		R:	TCGCTTTATGATGCACGGGAGAGT	60.3	50	
Ubiquitin-associated UBA/UBX domian-cointaning	UBA	F:	AGGAAGAGAAGAGGGCAAGG	56.4	55	170
		R:	AAAGGCCCTCTTGACTGTAGC	57.1	52	
PPR repeat	PPR	F:	ATGTGTTTGTTGCGTCATCC	54.0	45	195
		R:	CTTCAAAACCCACCTTCTGC	54.3	50	
Acyl-ACP thioesterase	Acyl-ACP	F:	AACAGGCCTCATTTCACTCG	55.2	50	171
		R:	ACCATCCTCCGTCAAGTTCC	56.8	55	
Cleavage site for pathogenic type III effector avirulence factor Avr	Avr	F:	GTTAAGGGTGCTGCTGTTCC	56.3	55	152
		R:	CCCATTGATGACTCGGTAGG	54.9	55	
Homeobox domain	HD	F:	GTGATGTCGTGCATGATTCC	54.1	50	185
		R:	TTGCTTGCCTGTACATTTGG	53.8	45	
Auxin/cyclin G-associated kinase	Aux	F:	GGGAAAGAGGGGAATCTACG	54.6	55	178
		R:	GATTGGCACCTTTTTGTTGG	52.8	45	
Dual specificity phosphatase	DUSPs	F:	TAA CGC CTC TCG CTC TGA GC	59.2	60	176
		R:	CCT TAT CCT TCT CAC ACT GCC C	57.3	54	
Phenylalanyl-tRNAsynthetase	PheRs	F:	CGT TTC TGT GCT CGA ACT TGG	56.5	52	134
		R:	GTG GGT GTT GAT TCC TCC TAT G	55.2	50	
Endomembrane protein 70	EMP70	F:	TGG AGG TGT GTT TCC TGG GC	60.0	60	170
		R:	AGG GTG AGT GGC ACT GAAATA C	57.0	50	
Zinc finger	Zinc	F:	AACTGCAGCAGAAATGGACGATGC	60.3	50	166
		R:	ACAGCCTGCTTGTGTCCTTATCCT	60.3	50	
AN1-like Zinc finger	AN1	F:	GCTTGCTGCATCATCTGCTGGAAA	60.4	50	127
		R:	TGGCTGCACAGAGATGGTCTTTGA	60.6	50	
Galactosyltransferases	GAL	F:	GAGCTGGCTATGCTTTGAGC	56.4	55	217
		R:	AGAGGAGATTGTGGGTGAGC	56.6	55	
No apical meristem (NAM) protein	NAM	F:	CGTCTGCTTCCACTTCTTCC	55.7	55	153
		R:	TGTTGCTGGCCTTGAATAGG	55.4	50	
UDP-glucuronosyl and UDP-glucosyltransferase	UDP	F:	AGGCCATATCATCCCTTTCC	54.3	50	197
		R:	CATCGGTGTTTTCAGTGTGG	54.2	50	
Myb-like DNA-binding domain	MyB	F:	ATTGGTCCCACAATGGTACG	55.1	50	195
		R:	TTTGCACGTCACTGTTTTGC	54.8	45	
Hexokinase	Hex	F:	TGATGTGGTAACCCGTAGAGC	56.5	52	200
		R:	AGGCTTCGTGCAAGTACTCC	57.2	55	
Acylglycerol-3-phosphate acyltransferase	AGPA	F:	ATTCCGATGGTTGTCATTCC	52.8	45	174
		R:	TTCAGAATAAGCCTCTCCTTCG	54.2	45	

Table 4 Amplification efficiency of primers

Gene name	Primer name	Efficiency (%)	R2	Slope
RPM1-interacting protein 4 (RIN4)	RIN	98.4	0.99	-3.4
PPR repeat	PPR	105.8	0.98	-3.1
Homeobox domain	HD	94.2	0.99	-3.4
Auxin/cyclin G-associated kinase	Aux	94.1	0.99	-3.4
Phenylalanyl-tRNAsynthetase	PheRs	91.4	0.99	-3.5
Endomembrane protein 70	EMP70	98.3	0.99	-3.3
Zinc finger	Zinc	95.6	0.99	-3.4

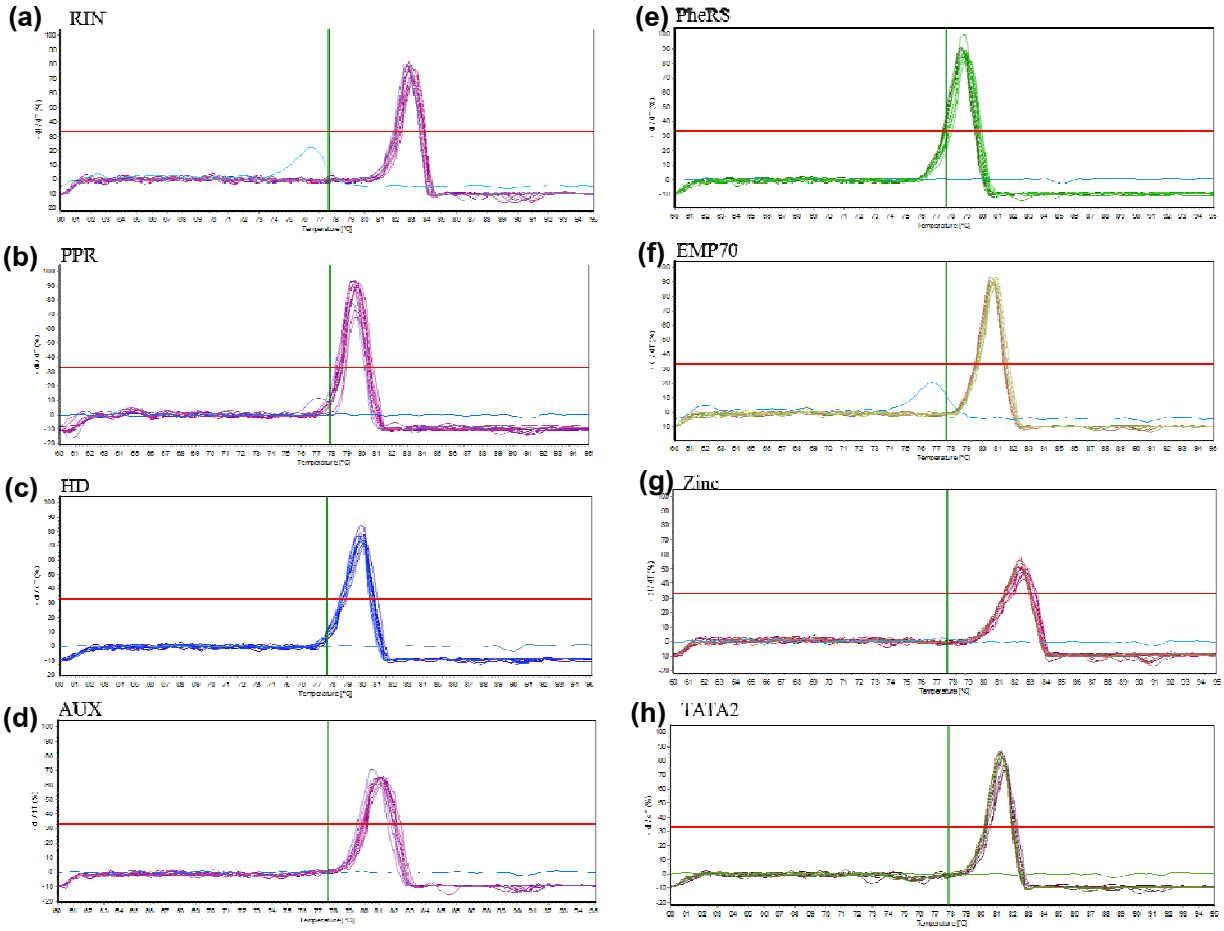

Figure 2 Dissociation curve analysis of primers

This analysis showed a single peak indicating specific product amplified by the primers for (a) RPM1-interacting protein 4 (RIN4), (b) PPR repeat (PPR), (c) Homeobox domain (HD), (d) Auxin/cyclin G-associated kinase (AUX), (e) Phenylalanyl-tRNAsynthetase (PheRS), (f) EMP70, (g) Zinc finger and (h) TATA. The line indicates the cycle threshold (33%).

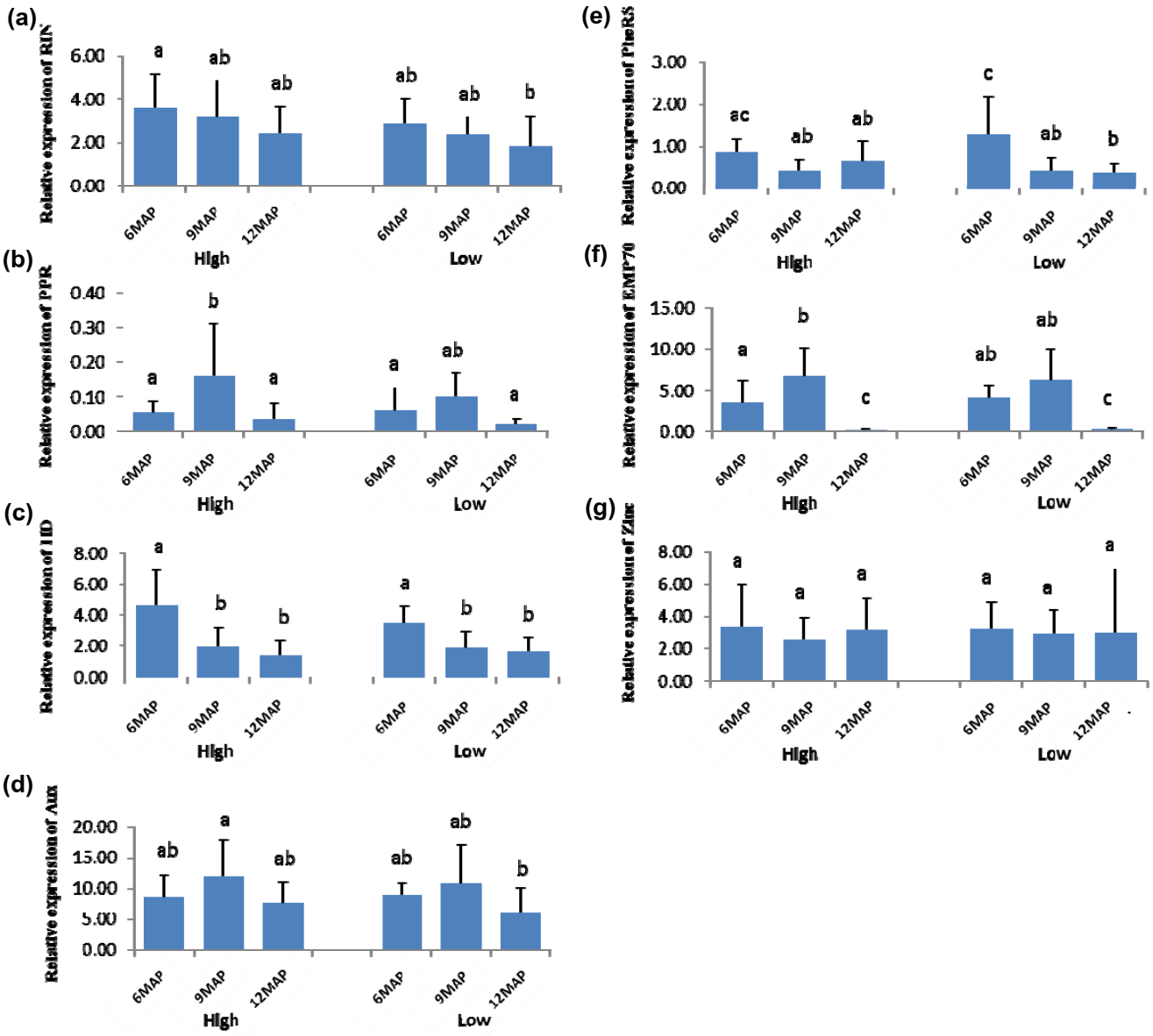

Figure 3: Expression levels of candidate genes at 6, 9 and 12 MAP

The relative expression between high and low starch content groups of gene RIN4 (a), PPR (b), HD (c), Aux (d), PheRS (e), EMP70 (f) and Zinc (g).

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)

   Sraphet S., Boonchanawiwat A., Thanyasiriwat T., Thaikert R., Whankaew S., TappibanP., Boonseng O., Lightfoot D. A. and Triwitayakorn K. QTL underlying root yield and starch content fresh weights in an F_1 derived cassava population (*Manihot esculenta* Crantz). Euphytica (2014) (Accepted)

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
     ไม่มี
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
     ไม่มี
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     ไม่มี
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     ไม่มี

3. อื่นๆ (เช่น หนังสือ การจดสิทธิบัตร)
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ภาคผนวก

QTL underlying root yield and starch content fresh weights in an F_1 derived cassava population (*Manihot esculenta* Crantz)

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Abstract

Cassava (*Manihot esculenta* Crantz) yields measured as fresh weight are declining in much of Asia and Africa. The aim of this study was to identify quantitative trait loci (QTL) underlying root and starch fresh weights in cassava roots. In this study, eight QTL associated with fresh root yield with 12.9-40.0 % of the phenotypic variation (PVE), and nine QTL for fresh starch content with 11.3% to 27.3% of PVE were identified from four different environments. Consistent QTL for the fresh root yield, *YLD5_R11* and *YLD8_L09* on linkage group (LG) 16, were detected across years and locations. QTL for the fresh starch content, *ST3_R09, ST6_R10* and *ST7_R*11 on LG 11, were found across three years. Co-localization of QTL for both traits with positive correlation was detected on *YLD3_R10* and *ST5_R10* on LG 9. Candidate genes within the consistent QTL across environments were identified based on cassava genome sequences. Glycosyl hydrolases, UDP-glucuronosyl and UDP-glucosyl transferase genes were found to be located within the region containing the QTL controlling fresh starch content while other genes were possibly involved fresh root yield. The QTL controlling fresh root yield and starch content in this study will be useful for molecular breeding of cassava through marker-assisted selection (MAS). The identification of candidate genes underlying both traits will be useful both as markers and for gene expression studies.

Keywords: Cassava, Quantitative trait loci (QTL), Fresh root yield, Fresh starch content, Gene annotation

Introduction

Cassava (*Manihot esculenta* Crantz) is the one of the most important crops in the world including Thailand (FAOSTAT, 2013). Thailand is ranked fourth in the world cassava producers after Nigeria, Brazil and Indonesia. Cassava yield in Thailand increased from 1999 to 2009 but the yield was slightly decreased in 2010 (FAOSTAT, 2013). In Thailand, cassava is not used as a food staple. In consequence most of cassava roots are used to produce cassava starch, flour, chips and pellets for export. That enables Thailand to be the largest exporter of cassava products (Onwueme 2002) and leading income about $2,300 million US (Office of Agricultural Economics, 2013) .Therefore, fresh root yield and starch content are the most important traits for cassava breeding in Thailand.

QTL analysis is a powerful tool for identification of the location of genomic regions controlling quantitative traits based on an association between marker genotypes and trait values (Kearsey 1998). QTL associated with yield-related production in cassava have been reported in several studies. QTL effecting early root growth were identified from an F_1 population (Okogbenin and Fregene 2002) and QTL controlling productivity and plant architecture were reported in a F_1 full-sib cross (Okogbenin and Fregene 2003). Moreover, an F_2 population was used to identify QTL for components of early yield (Okogbenin et al. 2008) while another F_1 population was used to study QTL controlling root dry matter and cyanogen content (Balyejusa Kizito et al. 2007). In addition, QTL controlling plant and first branch height which was associated with yield was reported by Boonchanawiwat et al. (2011). Recently, QTL controlling fresh weight root yield, root dry matter content, root starch content (Chen et al. 2012), and starch pasting viscosity (Thanyasiriwat et al. 2014) were identified from F_1 cassava population.

QTL controlling traits of interest will be useful for molecular breeding in cassava through Marker assisted selection (MAS) for developing new cassava varieties with high yield and starch content and for identification of candidate genes controlling the traits of interest. This study, therefore aimed to identify QTL controlling fresh root yield and fresh starch content from F_1 mapping population derived from a cross between 'Huay Bong 60' and 'Hanatee' and to annotate putative candidate genes based on QTL regions using cassava database from phytozome genome browser (http://www.phytozome.net/cassava).

Materials and methods

Plant materials

The F_1 mapping population of 100 individuals was developed by a cross of Huay Bong 60 (HB60, as female parent) and Hanatee (HN, as male parent) in 2006 as described

by Sraphet et al. (2011). Each individual line was planted by stem cutting at the Rayong Field Crops Research Center, Department of Agriculture, Rayong, Thailand in 2008, 2009 and 2010, and at Lop Buri Crops and Production Resources Technical Service Center, Department of Agriculture, Lop Buri, Thailand in 2008 with two replications of each location. Plot size was arranged at 4x10 m with 1 m between the rows and 1 m between individual plants in the rows. Fertilizer (N:P:K; 15:15:15) 312.5 kg/Hectare and chicken manure 3,100 kg/ hectare were applied at one month after planting (MAP). Pest management was applied as necessary.

Phenotypic evaluation

The phenotypes of fresh root yield and starch content were evaluated at 12 MAP based on 10 plants per line in 2009, 2010 and 2011 from Rayong Field Crops Research Center, and in 2009 from Lop Buri Crops and Production Resources Technical Service Center. Fresh root yield data were measured by collecting cassava roots from 10 plants in the plot. All root samples of each line were shaken to remove soil and debris, and weighed using a balance. The fresh root yield data (kg) were converted into tonnes/hectare. Starch content (%) of cassava was measured using a Rieman™ balance (Bainbridge et al. 1996). Five kg of clean cassava roots from several plants per plot and weight in air and in water. Percentages of starch contents were recorded directly from the Rieman balance. The average value within two replications of the trait at each location was calculated.

Statistical analysis

The statistical analyses including the analysis of variance (ANOVA) of fresh root yield and starch content were carried out using SPSS version 17.0 (SPSS, 2008). Correlations of both traits were tested in four different environments with Pearson correlation test. Broad-sense heritability (h_b^2) was computed using the following formula;

$$h^2 = \frac{\sigma_\alpha^2}{\sigma^2 + \sigma_\alpha^2}$$

Where σ_α^2 and σ^2 were the estimates of genetic and residual variance, respectively. Moreover, σ_α^2 and σ^2 values were obtained from analysis of variance result.

QTL analysis

The F_1 cassava genetic linkage map constructed by Sraphet et al. (2011) was integrated with phenotypic data in order to identify and locate QTL positions on linkage map using MapQTL version 4.0 software (Van Ooijen et al. 2002). The significant LOD score threshold appropriate to declare a QTL significant was calculated by tests with 1,000

permutations, which corresponded to a chromosome-wide (αc=0.05) and genome-wide LOD significance threshold of 5% (αg=0.05). Interval mapping (IM) was performed and genome-wide LOD significance threshold was used to declare a putative QTL. If there was no peak LOD score that exceeded the genome-wide LOD significance threshold, chromosome wide significance thresholds were used to identify putative QTL. The closest markers at the highest LOD peak of each putative QTL were used as cofactors for the automatic cofactor selection option at P<0.02 and significant markers cofactors were used in the restricted MQM (rMQM) analysis. The closest marker to a new peak of a putative QTL was set as a new cofactor and automatic cofactor selection was reanalyzed. If the LOD score of QTL with a cofactor dropped under the chromosome-wide LOD significance threshold, the dropped cofactor was removed from the list of cofactors. The rMQM was calculated until no new putative QTL was detected. Thereafter, the multiple-QTL model (MQM) analysis was executed to detect significant QTL using the stable set of cofactors from rMQM. The confidence region of the QTL position was drawn by Map Chart version 2.2 program (Voorrips 2002) using 2-LOD support interval (Van Ooijen 1992). A Kruskal-Wallis (KW) analysis, non-parametric test, was performed to the correlation between marker genotype data and significantly phenotype trait and to confirm significant QTL detected by MQM.

Gene annotation

The gene annotations within the identified QTL intervals were derived from BlastN searches against the cassava genome database in Phytozome v 9.1 (http://phytozome.net/search.php?show=blast&method=Org_Mesculenta) using the markers at the highest LOD peak of QTL as references. Functional annotations were made based on PFAM, Panther, KOG, EC, GO and KEGG.

Results

Phenotypic evaluations

The descriptive statistic data of fresh root yield and starch content are shown in Table1. Both traits of the F_1 population showed normal distribution, except for starch content at Rayong in 2009. In addition, frequency distributions of the population at each environment showed skewness to either HB60 or HN and transgressive segregation of the F_1 population was also observed in all years (Figure 1). Broad-sense heritability of fresh root yield and starch content ranged between 0.52 to 0.57, and 0.37 to 0.79, respectively. The correlations between and within fresh root yield and starch content traits were given in Table

2 and the ANOVA the results of both traits from four different environments are shown in Table 3.

QTL underlying fresh root yield

Eight QTL effecting fresh root yield were detected on seven linkage groups (Figure 2). These QTL explained phenotypic variance that ranged from 12.9-40.0% with LOD values of 3.16-5.07. Of these, six QTL (*YLD1_R09*, *YLD2_R10*, *YLD3_R10*, *YLD4_R11*, *YLD6_L09* and *YLD7_L09*) were identified at specific environment and two QTL (*YLD5_R11* and *YLD8_L09*) were found across two environments on linkage group 16 with no significant correlation between environments. Marker CA373 at QTL *YLD5_R11* showed significance at $P<0.01$ by KW.

QTL underlying fresh starch content

A total of nine QTL influenced fresh starch content with LOD values ranging from 2.85-4.42, explaining 11.3-27.3% of phenotypic variation were identified on seven linkage groups. Among these, five QTL (*ST1_R09*, *ST2_R09*, *ST4_R10*, *ST5_R10* and *ST8_R11*) were location specific and three QTL (*ST3_R09, ST6_R10* and *ST7_R*11) were detected across three environments on linkage group 11. Of these QTL, the two closest markers, NS1021 (*ST6_R10*) and MeES769 (*ST7_R*11), were significant at $P\leq0.05$ and $P\leq0.01$, respectively. In addition, positive correlations among environments and traits was observed as shown in Table 2.

Co-localization of QTL between traits

Co-localization of QTL controlling both traits with positive correlation (r=0.334**) between traits was found on linkage group 9 between *YLD3_R10* (PVE=16.4%) for fresh root yield and *ST5_R10* (PVE=11.3%) for starch content. The closest markers MeES249 and SSRY106 showed significance for *YLD3_R10* ($P\leq0.05$) and for *ST5_R10* ($P\leq0.0001$), respectively. In addition, co-localization between *YLD8_LP09* and *ST8_R11*, and *YLD7_L09* and *ST9_L09* was observed, but with no correlation.

Gene annotation

QTL underlying traits at the same region across years and locations were considered for identification of potential candidate genes. The consistent QTL on LG11 for fresh starch content showed positive correlation between *ST3_R09* and *S6T_R10* (r=0.657**), *S6T_R10* and *ST7_R11* (r=0.666**), and *ST3_R09* and *ST7_R11* (r=0.585**).

The highest LOD peaks of these QTL were located on scaffold09372 (*ST3_R09*), scaffold12091 (*S6T_R10*) and scaffold02717 (*ST7_R11*). Interestingly, confidence intervals of QTL controlling fresh root yield (*YLD3_R10*) and fresh starch content (*ST5_R10*) with a positive correlation (r=0.334**) was found on scaffold07933 and scaffold07827. Identified candidate genes at the highest LOD peaks and their peripheries are shown in Table 4. A total of 53 candidate genes were analyzed from five consistent QTL underlying both traits. Of these, 17 genes were identified from co-localized QTL underlying fresh root yield and starch content. Genes that were found near the highest LOD peak included those encoding; cleavage site enzyme for pathogenic type III effector avirulence factor Avr; (scaffold07827); endomembrane protein 70 (scaffold07933); phosphor adenosine phosphosulfate reductase family; EC Number: 1.8.4.9 (scaffold02717); and D-isomer specific 2-hydroxyacid dehydrogenase NAD binding domain; EC:1.2.1.2 (scaffold09372).

Discussion

F_1 cassava population

In this study, a cassava F_1 mapping population was used for QTL mapping although, F_1 population was not common for QTL mapping due to limitation for detection recessive genes and epistatic interaction (Paterson et al. 1991; Okogbenin et al. 2006). Limitations of developing populations of cassava include its out crossing nature, time consuming propagation and the high cost for measuring breeding traits and small number of seed produced (Kunkeaw et al. 2010). However, transgressive segregation was observed among the progeny for both traits indicating that the variation in the population was sufficient for QTL analysis. Alleles from both parents contributed positively to both the traits which were therefore inferred to be controlled by multiple genes (Wang and Goldman 1997). Moreover, the moderate broad sense heritabilities of fresh root yield and starch content inferred that these traits were strongly influenced by the environment (Song et al. 2010). Such traits were expected to be difficult to handle by direct selection (Ntawuruhunga and Dixon 2010).

QTL analysis

In this study, large numbers of identified QTL controlling both traits were identified indicating that these traits were controlled by multiple genes (Okogbenin and Fregene 2002). In addition, most QTL were major QTL which explained >10% of phenotypic variance (Collard et al. 2005).

The consistency of QTL for fresh root yield (*YLD5_R11* and *YLD8_L09*) and starch content (*ST3_R09*, *ST6_R10* and *ST7_R11*) across years indicated the stability of QTL across years and locations. QTL with large effects and consistency in different environments

exhibited genotype x environment interaction (GxE) (Malosetti et al. 2004; Zhang et al. 2010) were also preferable in MAS (Okogbenin et al. 2008).

The co-localization of QTL with positive correlation for fresh root yield (*YLD3_R10*) and starch content (*ST5_R10*) was identified on linkage group 9. Interestingly, the QTL for plant and first branch height (Boonchanawiwat et al. 2011) and pasting time of starch pasting viscosity (Thanyasiriwat et al. 2014) deriving from the same F_1 population were also reported on the same region. Generally, QTL underlying related traits tend to map in the same genomic regions or adjacent regions in the same linkage group (Sun et al. 2009). Therefore, QTL for yield and yield associated traits such as yield components (e.g. starch content, dry-matter, seed number and seed weight) and yield-related traits (e.g. plant architecture, biomass and harvesting index) appear to be clustered in the genome (Shi et al. 2009). The QTL effecting different traits within the same genomic regions could be explained by pleiotropic effects or the close linkage of multiple genes (Okogbenin and Fregene 2003). The major indicators for pleiotropic QTL were overlapped confidence interval regions of separate QTL, trait correlations, and environmental correlations (Timmerman-Vaughan et al. 2005; Lou et al. 2007). The consistent QTL and the coincident QTL controlling different traits should be useful for MAS (Xiao et al. 1996; Redoña and Mackill 1998).

Gene annotation

Among candidate genes identified within the QTL intervals underlying starch content, the glycosyl hydrolases family 15; the UDP-glucuronosyl; and the UDP-glucosyl transferases were predicted to be involved in carbohydrate metabolism in casssava. Glycosyl hydrolases family 15 (GH15) belong among the glycosyl hydrolase (GH) enzymes which are classified into EC 3.2.1- by CAZy (Cantarel et al. 2009). Enzymes in this group were involved in reactions in starch synthesis (Keeling and Myers 2010). GH15 included glucoamylase (EC 3.2.1.3), α, α-trehalase (EC 3.2.1.28) and glucodextranase (EC 3.2.1.70). UDP-glucuronosyl (EC 2.4.1.17) and UDP-glucosyl transferase from scaffold09732 is classified among the glycosyl transferases (GTs) (EC 2.4.x.y) that catalyze the transfers of the glycosyl group from a UTP-sugar, forming small hydrophobic molecule. Similarly, candidate genes underlying QTL for starch pasting temperature in cassava were glucosyl transferases and glycosyl hydrolases (Thanyasiriwat et al. 2014).

The candidate genes underlying the QTL for fresh root yield included those encoding a transcription factor, phosphatase activity, response signaling pathway and thiolester hydrolase activity. As reported in a meta-analysis of yield QTL in (Swamy and

Sarla 2011), the zinc finger and pentatricopeptide repeat (PPR) were also identified in this study.

Other candidate genes within QTL associated with fresh root yield and starch content may influence with these traits in cassava. Therefore, some candidate genes may be useful for further gene validation.

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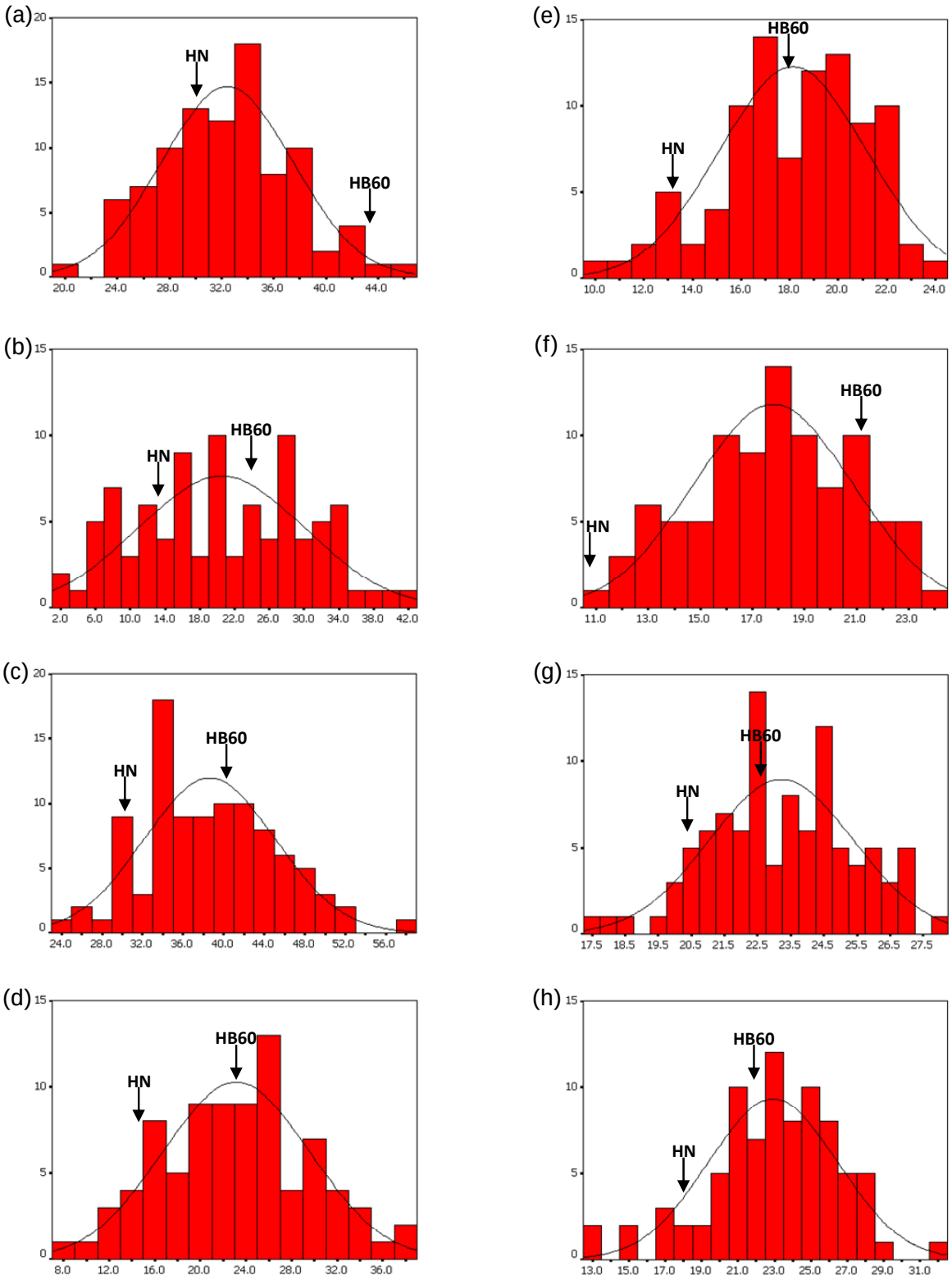

Figure 1. Frequency distribution in F_1 mapping population of fresh weight root yield (YLD) at Rayong (a) 2009, (b) 2010 and (c) 2011 , and Lop Buri 2009 (d) and fresh weight starch content (ST) at Rayong (e) 2009, (f) 2010 and (g) 2011 , and Lop Buri 2009 (h)

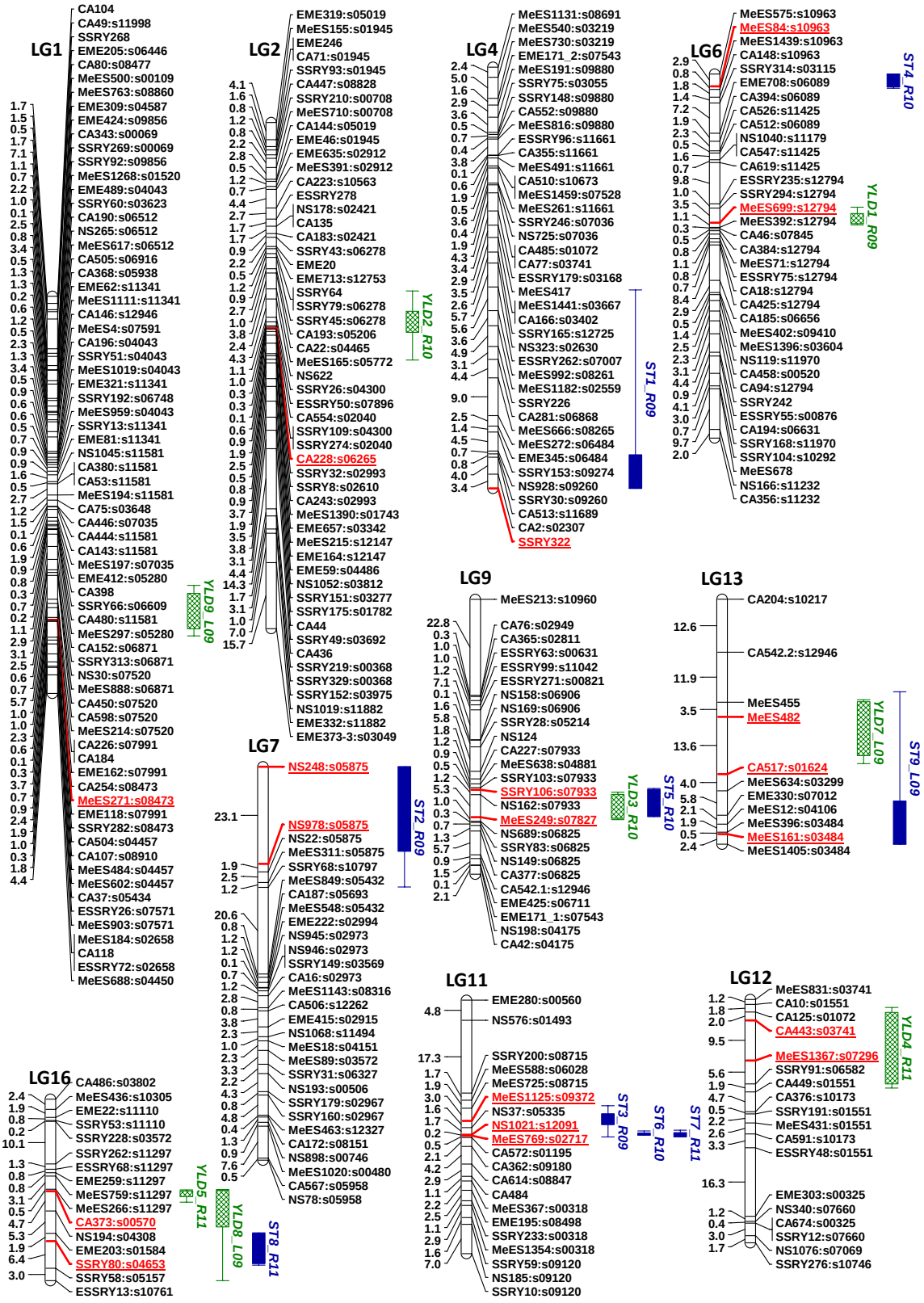

Figure 2. QTL specific to root yield and starch content fresh weights in F_1 cassava population

Table 1: Phenotypic value of root yield and starch content fresh weights of F_1 mapping population

Trait	Location	Year	Parent		F_1 population							
			HB60	HN	Maximum	Minimum	Mean±SE	SD	Skewness	Kurtosis	N	h^2 [a]
Fresh root yield	Rayong	2009	43.5	30.2	46.8	19.5	32.44±0.52	5.04	0.16	0.09	93	0.57
(tonne/hectare)		2010	24.1	13.4	41.1	2.6	20.49±1.00	9.60	0.04	-0.88	93	0.00[n]
		2011	40.3	30.3	57.2	23.9	38.62±0.66	6.48	0.25	-0.22	97	0.55
	Lop Buri	2009	23.6	14.8	38.7	8.8	23.14±0.71	6.43	0.10	-0.31	83	0.52
Starch content	Rayong	2009	18.0	13.2	24.1	10.2	18.13±0.31	3.02	-0.48	-0.22	93	0.59
(%)		2010	21.2	10.4	24.2	11.2	17.80±0.32	3.07	-0.13	-0.67	91	0.79
		2011	22.7	20.3	28.2	17.7	23.22±0.22	2.18	-0.08	-0.28	98	0.56
	Lop Buri	2009	22.0	18.5	31.5	12.6	22.92±0.39	3.54	-0.59	0.66	83	0.37

[a] Broad-sense heritability

n: showed negative broad sense heritability estimates.

Table 2: Correlation analyses between and within root yield and starch content fresh weights traits

Trait	Location	Year	Fresh root yield (tonne/hectare)				Starch content (%)			
			Rayong			Lop Buri	Rayong			Lop Buri
			2009	2010	2011	2009	2009	2010	2011	2009
Fresh root yield	Rayong	2009	1.000							
(tonne/hectare)		2010	0.151	1.000						
		2011	0.445**	0.262*	1.000					
	Lop Buri	2009	-0.033	-0.129	-0.097	1.000				
Starch content	Rayong	2009	-0.073	0.234*	-0.144	0.012	1.000			
(%)		2010	-0.271**	0.334**	-0.134	-0.092	0.657**	1.000		
		2011	-0.346**	0.181	-0.197	-0.122	0.585**	0.666**	1.000	
	Lop Buri	2009	-0.048	-0.022	-0.063	0.161	-0.058	-0.057	0.103	1.000

* $P \leq 0.05$; ** $P \leq 0.01$

Table 3. Analysis variance of root yield and starch content fresh weights in four environments

Source	df	Mean squares	
		Fresh root yield	Starch content
Genotype (G)	99	139.57***	27.14***
Environment (E)	3	12565***	1619***
G x E	264	90.79^{ns}	12.88***
Error	364	76.11	7.20

*, ** and ***= Significant at *P*< 0.05, 0.01 and 0.001 ; ns= not significant

Table 4: QTL controlling root yield and starch content fresh weights in F_1 population of cassava

Traits	Location	Year	QTL[a]	LG	LOD	%PVE[b]	Marker at peak[c]	Position of marker at peak (cM)	KW[d]	Scaffold on physical map	α_c[e]	α_g[f]
Fresh root yield	Rayong	2009	*YLD1_R09*	6	4.67	21.5	MeES699	35.397	**	12749	3.1	4.4
		2010	*YLD2_R10*	2	3.34	13.1	CA228	48.962	**	06265	3.3	4.6
			YLD3_R10	9	3.99	16.4	MeES249	51.751	**	07827	2.8	
		2011	*YLD4_R11*	12	3.66	17.9	CA443-MeES1367	4.978, 14.524	***,ns	03741, 07296	3	4.5
			YLD5_R11	16	3.98	20.3	CA373	21.854	***	00570	2.6	
	Lop Buri	2009	*YLD6_L09*	1	3.48	14.1	MeES271	76.952	ns	08473	3.3	4.4
			YLD7_L09	13	5.07	40.0	MeES482	28.047	ns	-	2.7	
			YLD8_L09	16	3.16	12.9	CA373	21.854	ns	00570	2.6	
Starch content	Rayong	2009	*ST1_R09*	4	3.34	15.6	SSRY322	100.216	**	-	3.0	4.4
			ST2_R09	7	3.87	23.9	NS248	0.00	****	05875	3.2	
			ST3_R09	11	3.21	27.3	MeES1125	28.698	ns	09372	3.1	
		2010	*ST4_R10*	6	3.56	12.3	MeES84	2.913	*******	10963	3.1	4.7
			ST5_R10	9	3.85	11.3	SSRY106	45.267	*******	07933	2.7	
			ST6_R10	11	4.09	11.7	NS1021	32.045	**	12091	2.9	
		2011	*ST7_R11*	11	3.45	13.7	MeES769	32.205	***	02717	3.0	4.5
			ST8_R11	16	4.42	18.2	SSRY80	33.826	ns	04653	2.8	
	Lop Buri	2009	*ST9_L09*	13	2.85	15.0	MeES161	55.927	**	03484	2.7	4.4

[a] R: Rayong province; L: Lop Buri province; 09: 2009; 10: 2010; 11: 2011

[b] The percentage of phenotypic variation explained by the QTL

[c] The marker at highest LOD score peak

[d] Significant level of Kruskal-Wallis analysis : *:0.1, **:0.05, ***:0.01, ****:0.005, *****:0.001, ******:0.0005, *******:0.0001 and ns: not significant

[e] Chromosome-wild significant LOD threshold

[f] Genome-wild significant LOD threshold

ns: not significant

Table 5: Candidate genes within QTL for root yield and starch content fresh weights

QTL	Trait	Scaffold	Size (kb)	Locus name	Candidate genes
YLD6_R10	Fresh root yield	07827	62.94	cassava4.1_013976m.g	Cleavage site for pathogenic type III effector avirulence factor Avr (at LOD peak)
				cassava4.1_016005m.g	ER lumen protein retaining receptor
				cassava4.1_023165m.g	PUB domain
				cassava4.1_016305m.g	SAM domain (Sterile alpha motif)
				cassava4.1_003618m.g	Pentatricopeptide repeat (PPR)
				cassava4.1_015899m.g	B-cell receptor-associated protein
				cassava4.1_016656m.g	Zinc finger, C3HC4 type (RING finger)
				cassava4.1_025029m.g	Acyl-ACP thioesterase
ST5_R10	Fresh root yield	07933	200.4	cassava4.1_003219m.g	Endomembrane protein 70 (at LOD peak)
	starch content			cassava4.1_001004m.g	Auxin/cyclin G-Associated kinase-related
				cassava4.1_000562m.g	Homobox protein
				cassava4.1_002144m.g	Centromere/kinetochore Zw10
				cassava4.1_001951m.g	PUB domain
				cassava4.1_013810m.g	Dual specificity phosphatase, catalytic domain
				cassava4.1_008276m.g	tRNA synthetases class II core domain (F)
				cassava4.1_033427m.g	LBP / BPI / CETP family, C-terminal domain
ST7_R11	Starch content	02717	315.4	cassava4.1_007015m.g	Phosphoadenosine phosphosulfate reductase family (at LOD peak)
				cassava4.1_015720m.g	Cation efflux family
				cassava4.1_019182m.g	Microtubule associated protein 1A/1B, light chain 3
				cassava4.1_031667m.g	Prephenate dehydratase (P protein)
				cassava4.1_013158m.g	Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily
				cassava4.1_021465m.g	Glycosyl hydrolases family 17
				cassava4.1_021436m.g	Myb-like DNA-binding domain
				cassava4.1_008834m.g	Glycine cleavage T-protein domain (Aminomethyltransferase)

QTL	Trait	Scaffold	Size (kb)	Locus name	Candidate genes
YLD6_R10	Fresh root yield	07827	62.94	cassava4.1_013976m.g	Cleavage site for pathogenic type III effector avirulence factor Avr (at LOD peak)
				cassava4.1_016005m.g	ER lumen protein retaining receptor
				cassava4.1_023165m.g	PUB domain
				cassava4.1_016305m.g	SAM domain (Sterile alpha motif)
				cassava4.1_003618m.g	Pentatricopeptide repeat (PPR)
				cassava4.1_015899m.g	B-cell receptor-associated protein
				cassava4.1_016656m.g	Zinc finger, C3HC4 type (RING finger)
				cassava4.1_025029m.g	Acyl-ACP thioesterase
ST5_R10	Fresh root yield	07933	200.4	cassava4.1_003219m.g	Endomembrane protein 70 (at LOD peak)
	starch content			cassava4.1_001004m.g	Auxin/cyclin G-Associated kinase-related
				cassava4.1_000562m.g	Homobox protein
				cassava4.1_002144m.g	Centromere/kinetochore Zw10
				cassava4.1_001951m.g	PUB domain
				cassava4.1_013810m.g	Dual specificity phosphatase, catalytic domain
				cassava4.1_008276m.g	tRNA synthetases class II core domain (F)
				cassava4.1_033427m.g	LBP / BPI / CETP family, C-terminal domain
ST7_R11	Starch content	02717	315.4	cassava4.1_007015m.g	Phosphoadenosine phosphosulfate reductase family (at LOD peak)
				cassava4.1_015720m.g	Cation efflux family
				cassava4.1_019182m.g	Microtubule associated protein 1A/1B, light chain 3
				cassava4.1_031667m.g	Prephenate dehydratase (P protein)
				cassava4.1_013158m.g	Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily
				cassava4.1_021465m.g	Glycosyl hydrolases family 17
				cassava4.1_021436m.g	Myb-like DNA-binding domain
				cassava4.1_008834m.g	Glycine cleavage T-protein domain (Aminomethyltransferase)

QTL	Trait	Scaffold	Size (kb)	Locus name	Candidate genes
ST6_R10	Starch content	12091	213.3	cassava4.1_005933m.g	MatE (Multidrug and Toxic Compound Extrusion)
				cassava4.1_031352m.g	AT hook motif
				cassava4.1_007820m.g	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)
				cassava4.1_003612m.g	MYND zinc finger
				cassava4.1_018977m.g	Hsp20/alpha crystallin family
				cassava4.1_006131m.g	IQ calmodulin-binding motif
				cassava4.1_014891m.g	Der1-like family
				cassava4.1_011055m.g	CDP-alcohol phosphatidyltransferase
ST3_R09	Starch content	09372	400.1	cassava4.1_008265m.g	D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain (at LOD peak)
				cassava4.1_003341m.g	FAD binding domain
				cassava4.1_005174m.g	Tetratricopeptide repeat (TTR)
				cassava4.1_021733m.g	Exonuclease
				cassava4.1_011998m.g	Zinc-binding dehydrogenase
				cassava4.1_017988m.g	Prenyltransferase and squalene oxidase repeat
				cassava4.1_033416m.g	Ankyrin repeat
				cassava4.1_026242m.g	Cupin domain
				cassava4.1_013902m.g	Prenyltransferase and squalene oxidase repeat
				cassava4.1_024984m.g	Prenyltransferase and squalene oxidase repeat
				cassava4.1_009908m.g	Protein tyrosine kinase
				cassava4.1_013480m.g	Homeobox associated leucine zipper
				cassava4.1_011616m.g	CLIP-associated proteins N terminal
				cassava4.1_022533m.g	UDP-glucuronosyl and UDP-glucosyl transferase
				cassava4.1_033134m.g	Protein kinase domain
				cassava4.1_026719m.g	Aldehyde dehydrogenase family
				cassava4.1_030492m.g	Cyclic nucleotide-binding domain
				cassava4.1_002050m.g	K^+ potassium transporter
				cassava4.1_005667m.g	NHL repeat
				cassava4.1_009886m.g	Zinc finger, C3HC4 type (RING finger)