รายงานวิจัยฉบับสมบูรณ์

การศึกษาการแสดงออกของยีนและการประเมินเครื่องหมายโมเลกุลที่มีผลต่อปริมาณสารปลดปล่อยไซยาไนด์ในรากมันสำปะหลัง

Expression of genes and validation of molecular markers underlying cyanogen content in cassava root

โดย นางสาวสุขุมาล หวานแก้ว

มิถุนายน พ.ศ.2557

สัญญาเลขที่ TRG5580002

รายงานวิจัยฉบับสมบูรณ์

การศึกษาการแสดงออกของยีนและการประเมินเครื่องหมายโมเลกุลที่มีผลต่อปริมาณสารปลดปล่อยไซยาไนด์ในรากมันสำปะหลัง

Expression of genes and validation of molecular markers underlying cyanogen content in cassava root

นางสาวสุขุมาล หวานแก้ว มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

งานวิจัยฉบับนี้สำเร็จลุล่วงไปได้ด้วยดีด้วยความช่วยเหลือ ให้คำปรึกษา และความเอาใจใส่ตลอดการดำเนินงาน ของรองศาสตราจารย์กนกพร ไตรวิทยากร อาจารย์ประจำสถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล ซึ่งเป็นอาจารย์ที่ปรึกษาของโครงการวิจัยนี้ เพื่อให้ผู้วิจัยสามารถปฏิบัติได้อย่างราบรื่นจนบรรลุผลสำเร็จ ผู้วิจัยจึงขอกราบขอบพระคุณเป็นอย่างสูงไว้ ณ ที่นี้

นอกจากนี้ ผู้วิจัยขอขอบพระคุณ ดร.โอภาษ บุญเส็ง นักวิชาการเกษตรของ ศูนย์วิจัยพืชไร่ จ. ระยอง ที่ทำให้สามารถดำเนินงานในศูนย์วิจัยพืชไร่ได้ และช่วยเหลือในด้านการจัดการพืชสำหรับงานวิจัยในครั้งนี้ รวมทั้งสถาบันชีววิทยาศาสตร์โมเลกุล ที่ให้การสนับสนุนทั้งด้านสถานที่และผู้วิจัย และผู้ร่วมงานวิจัยที่มิได้เอ่ยนามไว้ ณ ที่นี้ ที่มีน้ำใจให้ความช่วยเหลือเป็นอย่างดี

สุดท้ายนี้ ผู้วิจัยขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยมหิดลที่ให้ทุนสนับสนุนการวิจัยในครั้งนี้

สุขุมาล หวานแก้ว

Abstract

Project Code: TRG5580002

Project Title: Expression of genes and validation of molecular markers underlying cyanogen content in cassava root

Investigator: Miss Sukhuman Whankaew Mahidol University

E-mail Address: sukhuman.wha@mahidol.ac.th

Project Period: 2nd July 2012 – 1st July 2014

Cyanogen content (CN) in cassava causes concerning on health and environment. In order to obtain better understanding on genes affecting CN, this project therefore aims to study the expression of candidate genes related to CN using real-time PCR. For accuracy of real-time PCR analysis, validation of appropriate reference genes is required. The stability analyses of six common reference genes therefore have been investigated at 6, 9 and 12 months after planting (MAP) in both leaf and root of low and high CN varieties revealed that TATA box binding protein (TBP) was the best reference gene. Transcription profiles of two characterized genes in cyanogenesis pathway (linamarase and α-hydroxynitrile lyase) and eight annotate genes from previously identified QTL were analyzed in low and high CN groups. In leaf at 6 MAP, the transcription profiles of linamarase and PheRS showed significant increase in high CN group. The expression of PheRS, PEX, PD I and PD II increased at 9 MAP, whereas α-hydroxynitrile lyase increased at 12 MAP. In root, DSP, PheRS and PD II were significantly increased in high CN group at 9 MAP, but SPDS was significantly decreased. As the results, markers for selection of low CN were identified which will be useful.

Keywords: real-time PCR, cyanogenic potential, reference gene validation

บทคัดย่อ

รหัสโครงการ: TRG5580002

ชื่อโครงการ: การศึกษาการแสดงออกของยีนและการประเมินเครื่องหมายโมเลกุลที่มีผลต่อปริมาณสารปลดปล่อยไซยาไนด์ในรากมันสำปะหลัง

ชื่อนักวิจัย: นางสาวสุขุมาล หวานแก้ว มหาวิทยาลัยมหิดล

E-mail Address : sukhuman.wha@mahidol.ac.th

ระยะเวลาโครงการ: 2 กรกฎาคม 2555 – 1 กรกฎาคม 2557

มันสำปะหลังสามารถปลดปล่อยสารพิษไซยาไนด์ได้ ส่งผลให้เกิดความกังวลถึงผลกระทบต่อสุขภาพและสิ่งแวดล้อม ดังนั้นงานวิจัยนี้จึงมุ่งเน้นศึกษาการแสดงออกของยีนที่คาดว่าจะมีผลต่อปริมาณสารปลดปล่อยไซยาไนด์ในหัวมันสำปะหลัง ด้วยเทคนิค real-time PCR เพื่อให้มีความเข้าใจเกี่ยวกับยีนเหล่านี้มากขึ้น การศึกษาการแสดงออกของยีนด้วยเทคนิคดังกล่าว จำเป็นต้องมี reference gene ที่เหมาะสมเพื่อความแม่นยำของผลที่ได้ จึงได้มีการทดสอบ reference gene ที่เหมาะสมก่อน โดยใช้ตัวอย่างพืชที่มีปริมาณสารปลดปล่อยไซยาไนด์แตกต่างกันที่เก็บจากช่วงอายุ 6 9 และ 12 เดือนหลังปลูก พบว่า TATA box binding protein (TBP) เป็น reference gene ที่ดีที่สุดที่ได้จากการทดลองนี้ จึงได้นำมาใช้ในการศึกษาการแสดงออกของยีน จากการศึกษาการแสดงออกของยีนที่อยู่ในกระบวนการปลดปล่อยไซยาไนด์ (linamarase และ α-hydroxynitrile lyase) และยีนที่ได้จาก QTL ที่มีความสัมพันธ์กับปริมาณสารปลดปล่อยไซยาไนด์ ในมันสำปะหลังกลุ่มสารปลดปล่อยไซยาไนด์สูงและต่ำ พบว่า ที่อายุ 6 เดือนหลังปลูก ยีน linamarase และ PheRS ในใบของกลุ่มที่มีสารปลดปล่อยไซยาไนด์สูงมีการแสดงออกมากกว่ากลุ่มต่ำ อย่างมีนัยสำคัญทางสถิติ และที่อายุ 9 เดือนหลังปลูก การแสดงออกของยีน PheRS ยังคงสูงกว่ากลุ่มต่ำ นอกจากนี้ ยีน PEX, PD I และ PD II ในกลุ่มสูง ก็เพิ่มสูงกว่าในกลุ่มต่ำอีกด้วย และที่ 12 เดือนหลังปลูก พบว่า α-hydroxynitrile lyase ซึ่งเป็นยีนที่ปลดปล่อยไซยาไนด์ มีการแสดงออกในกลุ่มสูงมากกว่าในกลุ่มต่ำ สำหรับการแสดงออกของยีนในหัว พบว่า ที่ 9 เดือนหลังปลูก DSP, PheRS และ PD II ในกลุ่มสูงแสดงออกมากกว่าในกลุ่มต่ำ ในขณะที่ SPDS ให้ผลตรงกันข้าม ผลที่ได้จากการศึกษาการแสดงออกของยีน ทำให้ได้เครื่องหมายโมเลกุลที่สามารถใช้คัดเลือกมันสำปะหลังไซยาไนด์ต่ำในลูกผสมที่ศึกษาได้ ซึ่งจะเป็นประโยชน์ในการคัดเลือกมันสำปะหลังไซยาไนด์ต่ำต่อไปในอนาคต

คำหลัก : ไซยาไนด์; การแสดงออกของยีน; มันสำปะหลัง;

Introduction

Cassava (*Manihot esculenta* Crantz) is an economically important crop of the world (1). In 2010, international prices of cassava products increased from last year, particularly, Thai cassava flour and starch (1). However, the production of cassava products yields unavoidable toxic compound, hydrogen cyanide (HCN). This appearance is normally occurred in cyanogenic plants (2). The release of HCN causes health problem such as acute in toxicity, manifested as vomiting, dizziness, etc (3) and environmental toxicity (4, 5). Although, HCN can be reduced to the safe levels by proper processing, but it requires labor intensive and time, leading to economic and nutrient losses. In such case, released HCN still remains in environment (5). Additionally, shortcut processing can yield toxic products (3). These cause a serious problem to environment and quality of life. Therefore, more understanding of genes affecting the amount of released HCN will be helpful to improve safety cassava varieties.

Three key enzymes involving in the release of HCN, including cytochrome P450 (CYP), linamarase (LNM) and hydroxynitrile lyase (HNL) have been cloned and sequenced (2, 6-9). Even these enzymes have been well characterized however, how they relate to the amount of released HCN has not yet been well studied. In 2011, QTL affecting cyanogen content in cassava have been identified and five QTL were detected (10). Blast results revealed that these QTL were not located on the same scaffolds containing genes involved in the release of HCN as mentioned earlier.

In this study, three key enzymes involve in the releasing of HCN and identified genes from QTL affecting cyanogen content were evaluated by gene expression in order to investigate correlation to the amount of cyanogen content. In conclusion, better understanding of genes affected cyanogen content in cassava were obtained and potential DNA markers were identified from QTL map based on gene expression result. These founding will be useful for the researches and breeding program.

Methodology

Material preparation

Cassava varieties 'Hanatee', 'Huay Bong 60', and five of each low and high cyanogen content (CN) of their progenies were grown at Rayong Field Crops Research Center, Rayong province. Cassava leaves and roots were collected at 6, 9, and 12 month after planting (MAP), then snapped freeze in liquid nitrogen for RNA isolation. The CN in root of all samples were evaluate at 6, 9, and 12 MAP using picrate paper kit method (11).

Identification of candidate genes from QTL analysis

Nucleotide sequences of each QTL reported by Whankaew et al (10) were analyzed in order to construct physical map using cassava genome database on Phytozome. Based on the genome database, fine mapping of each QTL region was performed and QTL was re-analyzed using JoinMap 3 and MapQTL 4. Then, candidate genes at each QTL were annotated and selected for gene expression analysis.

RNA isolation and cDNA synthesis

Frozen samples were ground into fine powder using liquid nitrogen. Fruit-mate™ for RNA Purification (Takara, Japan) was applied in order to get rid of polysaccharides and polyphenol. Total RNA was then extracted using TriReagent® (Molecular Research Center, USA) according to supplier's instruction. The RNA was treated with DNA *free* kit (Ambion, USA) to remove DNA contamination. The concentration of each RNA sample was measured by a NanoDrop 1000 Spectrophotometer (Thermoscientific, USA). First-strand cDNA was synthesized using ImProm-II Reverse Transcription System (Promega, USA), according to the manufacturer's instructions.

Primer design and amplification efficiency

Specific primers of reference genes (40S ribosomal protein (40S), actin (ACT), cyclophilin C (CYCC), EF-1 alpha (EF1), TATA box binding protein (TBP), and Polyubiquitin (UBI)), candidate genes and cloned genes (CYP, LNM and HNL) were designed from available sequences on phytozome.

Each primer was assessed for efficiency in a 2 fold-serially diluted mixture of Hanatee cDNA with technical triplicates using Real-time PCR. Specific amplification was identified by melting curve analysis. The primer efficiency (E) was calculated from $\%E = (10^{(-1/slope)}-1)x100$ (12).

Determination of reference genes stability

Real-time PCR of 6 reference genes (40S, ACT, CYCC, EF1, TBP, and UBI) were evaluated using cDNA of 'Huay Bong 60' and 'Hanatee' in both leaf and root samples at 6, 9 and 12 MAP as template with technical triplicates. The most stable reference gene was established by NormFinder program (13).

Quantification of candidate genes affecting cyanogen content

Real-time PCR of 6 low CN clones and 6 high CN clones were performed with technical duplicates. The ΔCT method using the best reference gene was used for relative quantification. The expression level of low and high CN groups was compared by PASW Statistics 18 (14).

Validation of marker for Marker assisted selection

The location of genes which showed the level of expression related to the level of CN were indicated by Phytozome database. The closet SSR markers to the genes were analyzed for their ability to select low CN in 200 progenies of 'Hanatee' x 'Huay Bong 60', using average CN.

Result and discussion

Identification of candidate genes for transcript analysis

In 2011, QTL affecting CNP have been reported on linkage map of 100 F1 created by a cross between 'Hanatee' and 'Huay Bong 60', consisting of 303 SSR markers. Recently, additional markers have been developed (15) and therefore, the map was re-constructed by including more markers and number of samples to 200. The new linkage map increased the number of markers to 489 loci, relied on 23 linkage groups, and covered 1846.6 cM with an average interval distance of 6.1 cM. QTL affecting CN evaluated at Rayong and Lop Buri provinces in 2009 and at Rayong province in 2010 were detected as shown in Table 1.

Table 1. Description of the QTL for cyanogen content in cassava.

Years	Locations	QTL	LG	α_g	α_c	Loci	LOD	%PVE
2009	Rayong	CN09R1	5	4.5	2.8	NS308	7.4	12.5
		CN09R2	5		2.8	ESSRY54-CA141	7.2	13.4
		CN09R3	6		2.9	EME502	3.8	3.1
		CN09R4	11		3.2	SSRY219	5.8	9.5
		CN09R5	16		3.2	CA76	4.5	7.5
		CN09R6	16		3.2	SSRY103	5.5	9.0
	Lop Buri	CN09L1	6	4.5	2.9	CA46	4.4	12.5
		CN09L2	11		3.2	SSRY219	3.2	7.8
2010	Rayong	CN10R1	4	4.2	2.7	EME303-CA591	2.9	8.9

*LG = Linkage group

α_g = Genome wide significance threshold

α_c = Chromosome wide significance threshold

LOD = Logarithm of odds

%PVE = Percentage of phenotypic variation explained.

With the established results, CN09R1, CN09R4/CN09L2, and CN09R6 were considered for fine mapping based on LOD score, percentage of phenotypic variation explained and significance of single marker analysis (Fig. 1.).

Fine mapping was done at scaffold 00368, 05214, 07933 and 10689 (scaffold at interested QTL), and s08265, s12341 and s07743 (scaffolds containing genes involving in CNP: CYP79D2, LNM and HNL). After re-QTL analysis, the high density of QTL map was generated (Fig 2). The fine map could narrow down the QTL regions. The rearrangement of marker loci resulted in changing of QTL on linkage group 5 from 2 to 1 QTL, as well as on linkage group 16 which pointed out the better loci to be focused on. Mapping scaffolds containing genes involving in CNP was successful, but no QTL was detected on those scaffolds.

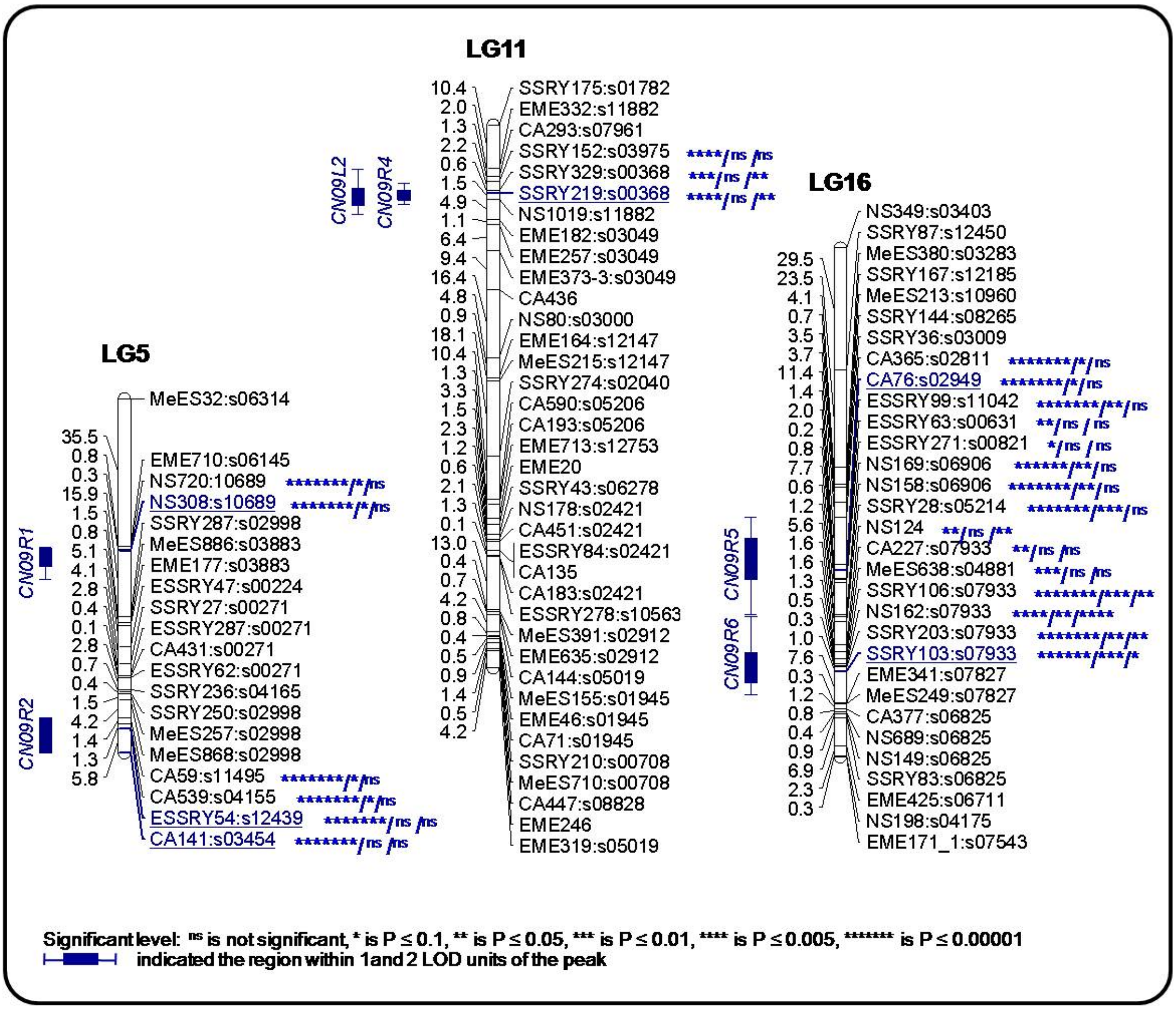

Fig. 1 The positions of selected QTL underlying cyanogen content for fine mapping. The significance of single marker analyzed in 2009 at Rayong and Lop Buri provinces and in 2010 at Rayong province are shown on the left side of the linkage group, separated by "/".

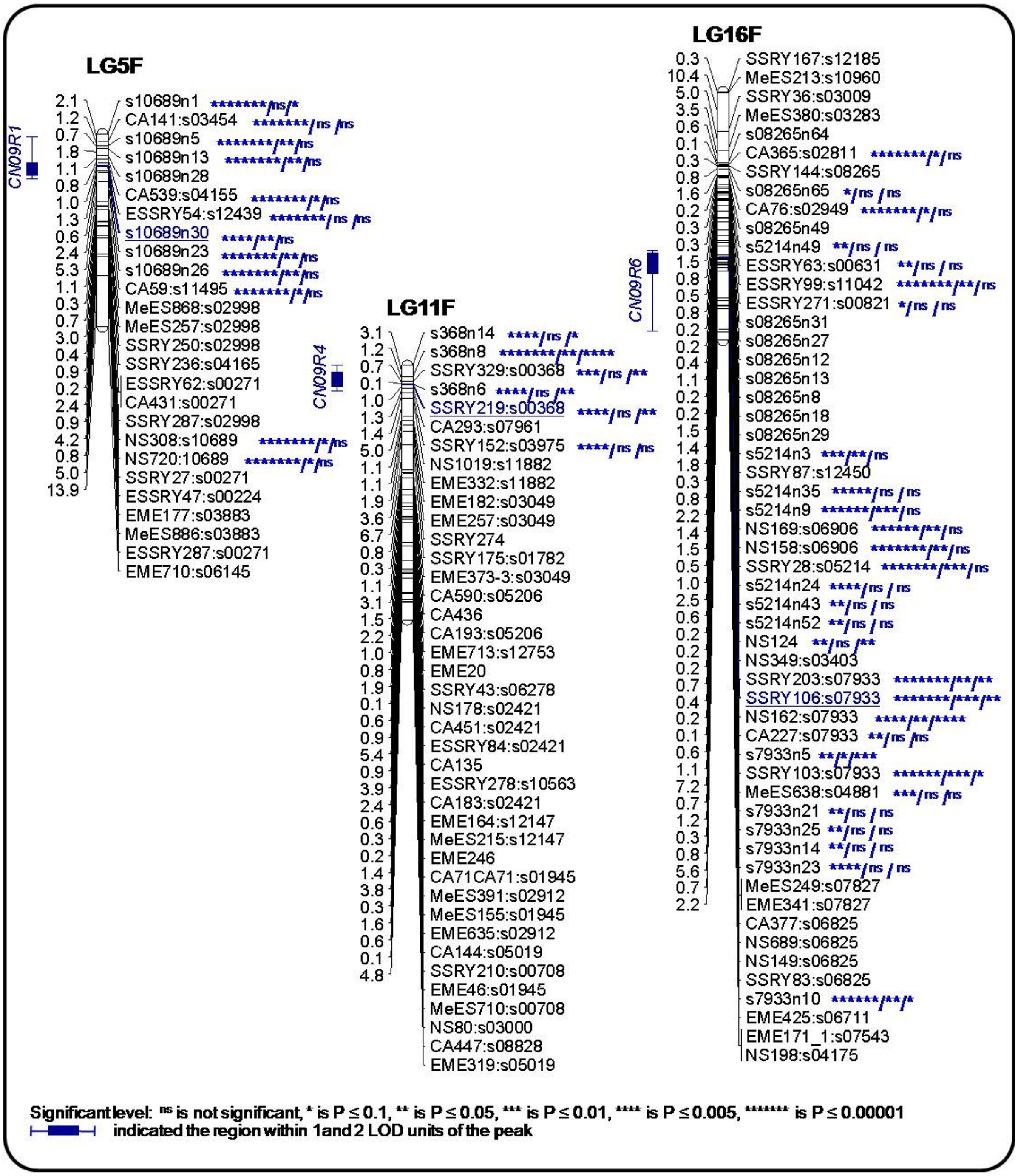

Fig. 2 The positions of QTL underlying cyanogen content on the fine map. The significance of single marker analyzed in 2009 at Rayong and Lop Buri provinces and in 2010 at Rayong province are shown on the left side of the linkage group, separated by "/".

Scaffold 00368, 05214 and 07933 were considered for functional gene annotation as they found highly significant markers. The interested genes are shown in Table 2.

Table 2 Annotate genes for gene expression study

Scaffold	Name	Annotate function	Possible pathway related to CN
00368	PEX	Peroxisomal membrane	Transport and catabolism
	SPDS	Spermidinesynthase	Arginine and proline metabolism
			B-alanine metabolism
			Cysteine and methionine metabolism
05214	NIR	Multicopperoxidases	Nitrogen metabolism
	PD I	Prephenatedehydratase	Biosynthesis of amino acid
	PD II	Prephenatedehydratase	Biosynthesis of amino acid
07933	DSP	Dual specificity phosphatase	Signal transduction
	EMP70	Endomembrane protein 70	Transport
	PheRS	Phenylalanyl-tRNAsynthetase	Aminoacyl-tRNA biosynthesis

Validation of reference genes

Six commonly used reference genes (40S, ACT, CYCC, EF1, TBP, and UBI) were designed and test for efficiency. The gene names, primer sequences and amplicon length are provided in Table 1. The efficiency of all primers ranged from 95-105% and r^2 >0.99, which are in the criteria for real-time PCR. In order to validate the most stable reference genes, expression level of these 6 reference genes was determined in ‘Huay Bong 60’ and ‘Hanatee’ which significantly different in CN at 3 different growth stages (6, 9, 12 MAP). Based on NormFinder, stability value of reference genes are shown in Fig 3. NormFinder calculated stability value based on variance estimation for identifying suitable normalization gene among a set of candidate. The lower the stability value, the higher the expression stability. (13). As a result, TBP was the most stable reference gene with the stability value of 0.01 across variation samples. The presented reference gene was not only able to apply in gene expression study of CN, but also in the other traits that have phenotypically different between ‘Huay Bong 60’ and ‘Hanatee’ varieties. This allowed more reliable normalization of quantification data in real-time RT-PCR.

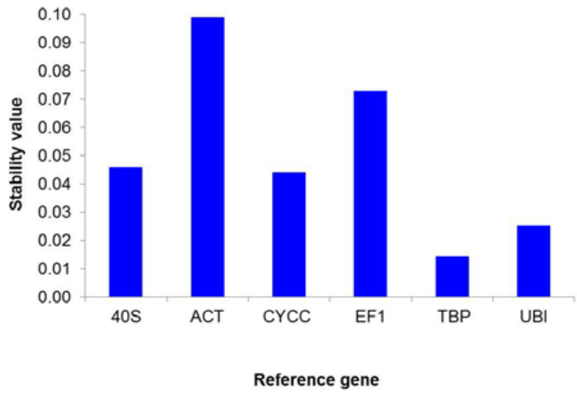

Fig. 3 Stability of 6 reference genes for cyanogenic potential study from leaf and root at 6, 9, 12 MAP calculated using NormFinder. Lower stability value corresponds to higher gene stability.

Quantification of genes candidate genes affecting cyanogen content

The expression profiles of three major genes involving in CN (CYP, LNM and HNL) and eight annotated genes from QTL affecting CN as shown in Table 2, have been analyzed.

The level of CN within the low CN group at each stage was not different, whereas the high CN group showed increasing of CN after 6 MAP (Fig. 4A). As a result, the gene encoded for CN production might be increased. Accordingly expression level of LNM (Fig 4B) in high CN group was significantly increased, and probably led to the accumulation of acetone cyanohydrin, a substrate to produce hydrogen cyanide, and glucose. At 9 and 12 MAP, the expression of LNM was reduced to the same level of low CN group. It indicated that the accumulation of acetone cyanohydrin and glucose were probably high in young cassava which according to the level of CN in in root at 6 MAP (Fig 4A). Even though LNM were highly expressed at 6 MAP of high CN group, but HNL transcript was not different (Fig 4C). This indicated that acetone cyanohydrin was accumulated in high CN group more than the low one which related to the CN level in root. However at 12 MAP, HNL highly transcript in both groups, related to the reduction of CN from 9 MAP in both groups. This founding suggested that harvesting cassava at 9 MAP might obtain higher CN than at 12 MAP. The level of HNL transcript of the low group was also lower than that of the high CN group, this maybe because the high level of acetone cyanohydrin in high CN group in early stage.

The expression of CYP was very low, therefore with the detection limit, the expression level is not reported. Similarly, the expressions of LNM and HNL in root were also very low which according to the reports that indicated the lack of LNM and HNL in root (2, 16).

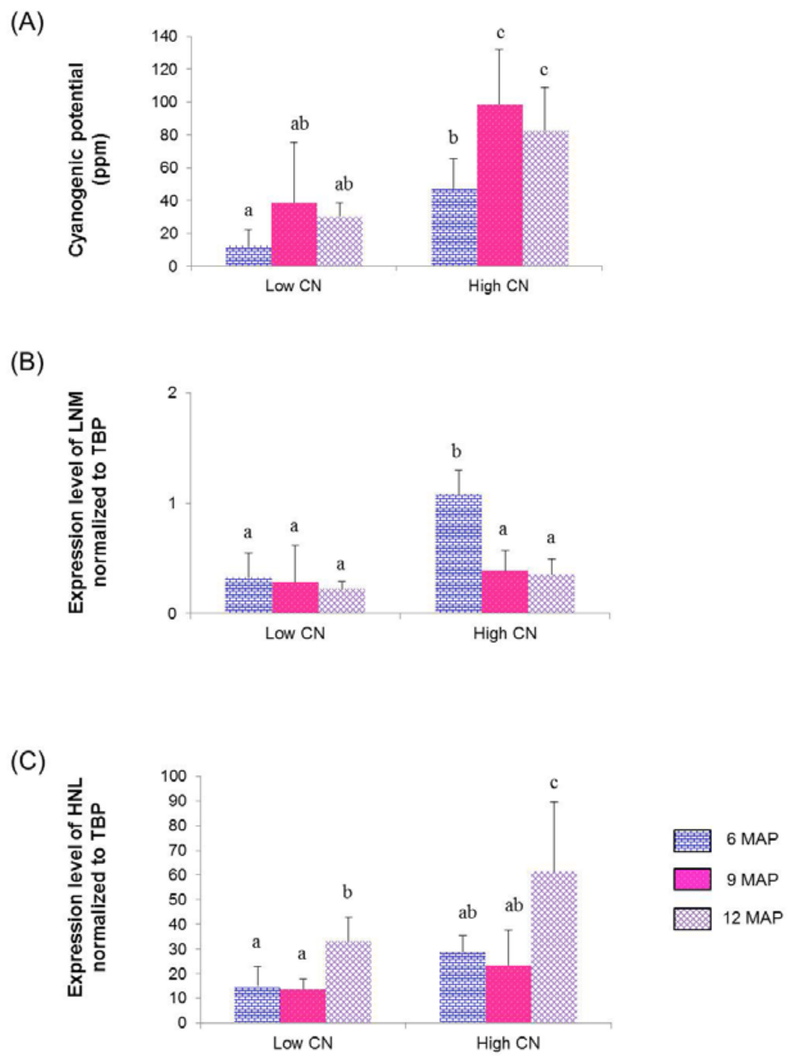

Fig 4. Expression level of linamarase (LNM) and hydroxynitrile lyase (HNL) in cassava leaf. Cyanogen content, LNM transcript, and HNL transcript are shown in Panel A, B and C, respectively. Mean values + standard deviation are represented. The significant different at *P*<0.05 are indicated as a, b and c.

Focused on the expression of annotate genes, the result showed that at early stage (6 MAP), PheRS in leaf of high CN group was increased (Fig. 5). While the expression of PheRS in low CN group was not change across the stages, the expression of high CN group at 9 MAP was increased from 6 MAP. These indicated that some aminoacyl-tRNA biosynthesis in high CN might increased at 6 and 9 MAP which related to the CN level. However, at 12 MAP, PheRS expression dropped to the same level of low CN group which maybe because aminoacyl-tRNA biosynthesis was not quite necessary for old plant. The significant different at 9 MAP of PheRS in root also detected, but the expression level was dramatically decreased.

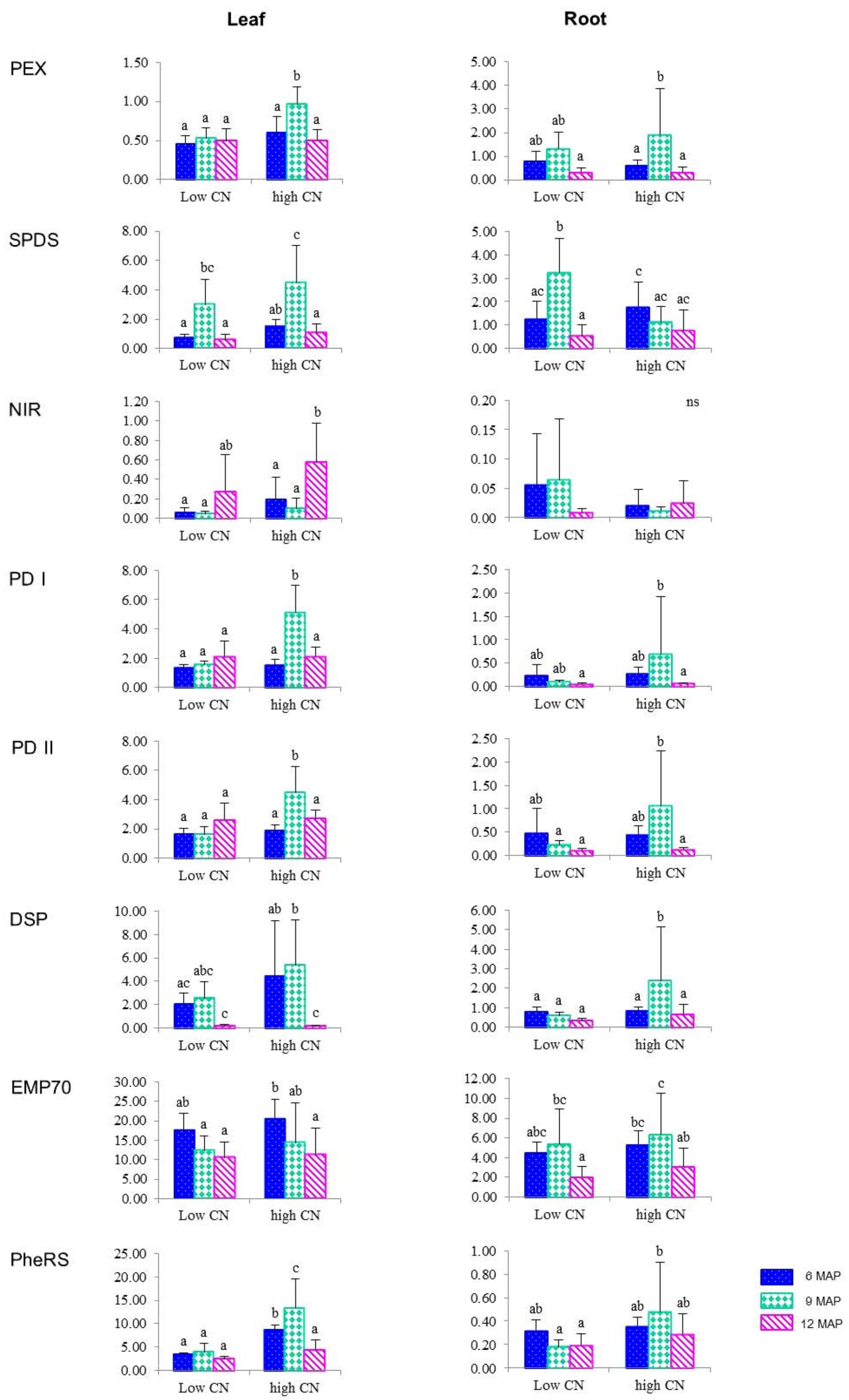

Fig 5. Expression level of annotate genes from QTL affecting cyanogen content. Mean values + standard deviation are represented. The significant different at *P*<0.05 are indicated as a, b and c.

At 9 MAP, not only PheRS was up-expressed in both leaf and root, but PEX, PD I and PD II also up-expressed in leaf, and DSP and PD II also up-expressed in root (Fig. 5). Even through PD I which quite similar to PD II was not significantly increased in root, but its trend seemed to increased. Interestingly, SPDS which involved in β-alanine metabolism was significantly down-expression in root at 9 MAP. β-alanine metabolism is associated to the utilization of cyanogenic glycoside, a substrate of hydrogen cyanide, therefore the reduction of SPDS expression may cause the remaining of high amount of cyanogenic glycoside, subsequently lead to high CN.

Validation of marker for MAS

Based on the gene expression result, PheRS, SPDS, PD I and PD II were interested. The location of these genes and selected markers are shown in Table 3. The result indicated that genotype pattern “ab”, “ad” and “ab” of s00368n8, SSRY28 and SSRY106 can select low cyanogen content, significantly (Fig. 6).

Table 3 Selected candidate genes and markers with their position.

Candidate gene	Gene position	Selected marker	Marker position
SPDS	scaffold00368:110307..112609	s00368n8	scaffold00368:102,643..102,662
PD I	scaffold05214:155928..157768	SSRY28	scaffold05214:157,470..157,494
PD II	scaffold05214:163754..165622	SSRY28	scaffold05214:163754..165622
PheRS	scaffold07933:137377..142320	SSRY106	scaffold07933:46,097..46,117

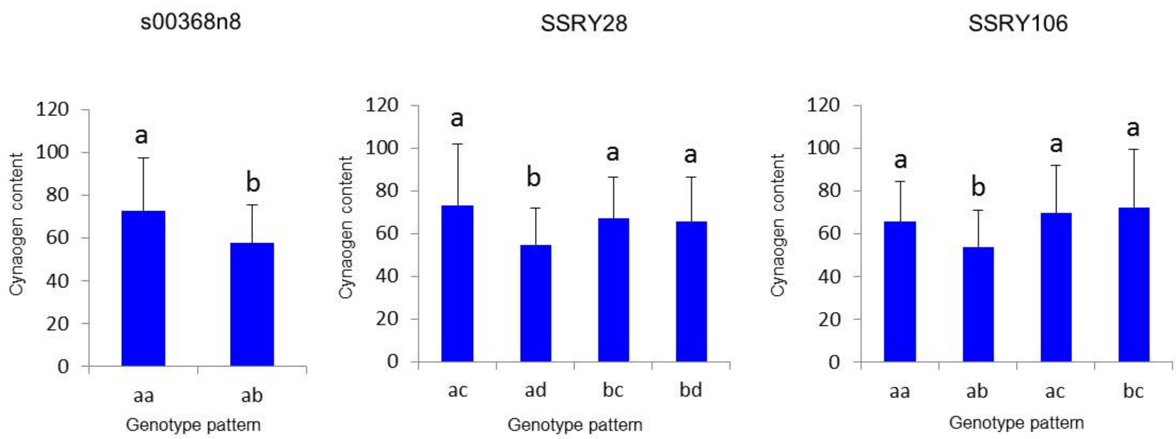

Fig. 6 Average of cyanogen content of each genotype pattern. Mean values + standard deviation are represented. The significant different at *P*<0.05 are indicated as a and b.

In summary, for reliable relative quantification by real time PCR, validation for the best stability reference gene is known as the important step. From the evaluation in 2 phenotypically different cassava at 6, 9 and 12 MAP in both leaf and root, TBP was found to be the most stable reference gene among all evaluated reference genes. Gene expression study normalized to TBP indicated that at early stage (6 MAP), LNM expression in high CN group expressed higher than that in low CN group, whereas higher expressed HNL was showed at late stage (12 MAP). For annotate genes, compared their expression between low and high CN groups in leaf, PheRS transcription level was increased in high CN group at 6 MAP and continuously to 9 MAP. At 9 MAP, PD I and PD II transcription level trended to up-express in both leaf and root. Interestingly, the expression of SPDS which involve in detoxification of cyanide, was significantly decreased in root, comparing with low CN group. The validation of markers to select low CN indicated that genotype pattern “ab”, “ad” and “ab” of s00368n8, SSRY28 and SSRY106 can select low cyanogen content in the evaluated population, significantly.

References

1. FAO. Food outlook: Global market analysis Rome, Italy: Food and Agriculture Organization of the United Nations. 2010.
2. White WLB, Arias-Garzon DI, McMahon JM, Sayre RT. Cyanogenesis in Cassava. The Role of Hydroxynitrile Lyase in Root Cyanide Production. Plant Physiol. 1998;116(4):1219-25.
3. Bokanga M, Ekanayake I, Dixon A, Porto M. Genotype-environment interactions for cyanogenic potential in cassava. Acta Hort. 1994;375:131- 40.
4. Dixon AGO, Asiedu R, Bokanga M. Breeding of cassava for low cyanogenic potential: problems, progress and perspectives. Acta Hort. 1994;375:153-61.
5. Balagopalan C, Rajalakshmy L. Cyanogen Accumulation in Environment During Processing of Cassava (*Manihot Esculenta* Crantz) for Starch and Sago. Water, Air, & Soil Pollution. 1998;102(3):407-13.
6. Jorgensen K, Morant AV, Morant M, Jensen NB, Olsen CE, Kannangara R, et al. Biosynthesis of the Cyanogenic Glucosides Linamarin and Lotaustralin in Cassava: Isolation, Biochemical Characterization, and Expression Pattern of CYP71E7, the Oxime-Metabolizing Cytochrome P450 Enzyme. Plant Physiol. 2011;155(1):282-92.
7. Andersen MD, Busk PK, Svendsen I, Møller BL. Cytochromes P-450 from Cassava (*Manihot esculenta* Crantz) Catalyzing the First Steps in the Biosynthesis of the Cyanogenic Glucosides Linamarin and Lotaustralin: cloning, functional expression in pichia pastoris, and substrate specificity of the isolated recombinant enzymes. J Biol Chem. 2000;275(3):1966-75.
8. Hughes MA, Brown K, Pancoro A, Murray BS, Oxtoby E, Hughes J. A molecular and biochemical analysis of the structure of the cyanogenic beta-glucosidase (linamarase) from cassava (*Manihot esculenta* Cranz). Arch Biochem Biophys. 1992;295(2):273-9.
9. Hughes J, Carvalho FJ, Hughes MA. Purification, characterization, and cloning of alpha-hydroxynitrile lyase from cassava (*Manihot esculenta* Crantz). Arch Biochem Biophys. 1994;311(2):496-502.
10. Whankaew S, Poopear S, Kanjanawattanawong S, Tangphatsornruang S, Boonseng O, Lightfoot DA, et al. A genome scan for quantitative trait loci affecting cyanogenic potential of cassava root in an outbred population. BMC Genomics. 2011;12:266.
11. Bradbury M, Egan S, Bradbury J. Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. J Sci Food Agri. 1999;79:593 - 601.

12. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem. 2009;55(4):611-22.

13. Andersen CL, Jensen JL, Ørntoft TF. Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. Cancer Res. 2004;64(15):5245-50.

14. SPSS Inc. PASW Statistics 18, Release version 18.0.0 SPSS Inc., Chicaco, IL. www.spss.com2009.

15. Sraphet S, Boonchanawiwat A, Tangphatsornroung S, Boonseng O, Tabata S, Lightfoot D, et al. Development of simple sequence repeat markers and construction of genetic linkage map of cassava (*Manihot esculenta* Crantz). Theor Appl Genet. 2011;122:1161 - 70.

16. Santana MA, Vásquez V, Matehus J, Aldao RR. Linamarase Expression in Cassava Cultivars with Roots of Low- and High-Cyanide Content. Plant Physiol. 2002;129(4):1686-94.

Research output

1. Whankaew S, Sraphet S, Boonseng O, and Triwitayakorn K. Fine mapping of QTL for cyanogenic potential in cassava storage root. Proceeding of The 5th ASIAHORCs Joint Symposium on Food Sciences, Bali, 27 November 2013. In press.

2. Whankaew S, Sraphet S, Thaikert R, Smith D and Triwitayakorn K. Validation of reference genes for transcript analysis in cassava (*Manihot esculenta* Crantz) and its application in linamarase and α-hydroxynitrile lyase at different growing stage. (under preparation for submit)

ภาคผนวก

Full Paper

Fine mapping of QTL for cyanogenic potential in cassava storage root

Sukhuman Whankaew [1*)], Supajit Sraphet [2)] Opas Boonseng [3] and Kanokporn Triwitayakorn [2)]

1) Institute of Molecular Biosciences, Mahidol University,
25/25 Phutthamonthon 4 Rd., Salaya, Nakhon Pathom 73170 Thailand
e-mail: sukhuman.wha@mahidol.ac.th
2) Institute of Molecular Biosciences, Mahidol University
3)Rayong Field Crops Research Center, Ministry of Agriculture and Cooperatives

** Corresponding author*

Abstract

Cassava is a cyanogenic crop which causes a serious problem to quality of life and environment. To gain knowledge on genes affecting cyanogenic potential (CNP) based on identified QTL underlying the trait and to validate potential molecular markers that are applicable in marker-assisted selection (MAS), this study aims to develop more tightly linked-markers to the QTL by fine mapping in order to increase power of QTL detection. With available cassava genome database, SSR markers were developed based on four scaffolds within the detected QTL and three interesting scaffolds containing genes in cyanogenesis pathway and genotyped with the mapping population. The QTL responded to CNP were analyzed with the CNP value, collected at Rayong and Lop Buri provinces in 2009, and at Rayong province in 2010. The results revealed the high density of QTL map which are effective for prediction of candidate genes and identification of trait-linked markers. The output of this study provides the opportunities to validate potential markers for selection of new cassava lines with low CNP in the breeding program.

Keywords: Cassava, Cyanogen content, High resolution mapping, Microsatellites.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) has been cultivated as an important food source, animal feed and industrial feedstock. World cassava production is expected to increase in order to sustain the industrial demand (Food and Agriculture Organization 2012). In Asia, the utilization of cassava in industrial as biofuel has been the main driver of the 80 percent expansion in the cultivation. In Thailand, cassava is the world's leading exporter (Food and Agriculture Organization 2012). The incresing of the output demand lead to increasing of production. However, cassava is a cyanogenic crop, which can release hydrogen cyanide (HCN) (White et al. 1998). The presence of HCN during cell damage causes concerns about health effects such as acute intoxication, manifested as vomiting, dizziness, etc. (Bokanga et al. 1994) and environmental toxicity (Dixon et al. 1994). Although, HCN can be reduced to the safe levels by proper processing, but it requires labor intensive and time, leading to economic and nutrient losses. In such case, released HCN still remains in environment (Dixon et al. 1994). Additionally, shortcut processing can yield toxic products (Bokanga et al. 1994). These cause a serious problem to environment and quality of life, especially in rural area. The level of cyanogenic potential (CNP) varies depended on variety and environment (Bokanga et al. 1994). Therefore, improved cassava cultivars for low CNP would be helpful.

CNP is known as quantitative trait (Dixon et al. 1994). In such case, conventional breeding is not effective, but molecular breeding is expected to be more efficient, reliable and cost effective breeding approach (Xinyou et al. 2003; Collard et al. 2005). For molecular breeding, QTL mapping has been applies to identify trait-linked markers which can be used as marker-assisted selection (MAS). Moreover, it also provides better understanding on information of genes underlying the trait (Xinyou et al. 2003; Collard et al. 2005).

Three key enzymes involved in the releasing of HCN, including cytochrome P450, linamarase and hydroxynitrile lyase have been characterized (Hughes, M. A. et al. 1992; Hughes, J. et al. 1994; White et al. 1998; Andersen et al. 2000). Even these enzymes have been well characterized to be involved in the releasing of HCN, they might not effect to the amount CNP, but the regulatory genes. Recently, five QTL of CNP has been identified from an SSR based linkage map (Whankaew et al. 2011). With available of cassava genome database, the information revealed that the cloned genes were not found in the QTL.

In this study, fine mapping of the QTL regions was accomplished in order to narrow down the regions containing the potential genes and pinpoint closely linked-markers in order to select potential markers for safety cassava varieties selection. Nevertheless, fine mapping of regions containing genes involved in the releasing of HCN will also be fulfilled in order to provide more information about these genes.

2. MATERIALS AND METHODS

Plant material and CNP evaluation

Cassava variety 'Hanatee' (Thai local variety), exhibited low CNP and 'Huay Bong 60' (commercial variety), displayed high CNP were used as the parents to generate 200 of progenies used for the mapping population. For CNP evaluation, the storage roots of the population and its parents were measured at Rayong province in 2010 using picrate paper kit (Bradbury et al. 1999) as described in Whankaew et al. (2011).

Fine mapping

QTL analysis has been updated from previous report (Whankaew et al. 2011) by increasing number of population from 100 to 200, and adding phenotypic data of evaluation at Rayong in 2010. The QTL mapping were done by JoinMap® 3.0 (van Ooijen & Voorrips 2001) and MapQTL® 4.0 (Van Ooijen et al. 2002) with phenotypic data evaluated in this study (at Rayong province in 2010) and in previous study (at Rayong and Lop Buri provinces in 2009). The identified QTL were blasted with cassava genome database "Phytozome". Nucleotide sequences of scaffolds containing interesting QTL as well as genes involved in HCN production were used for SSR primer designed by "WebSat". The SSR primers were synthesized and tested for amplification and polymorphism. Polymorphic primers were genotyped with the population as described in Whankaew et al. (2011). Finally, fine map was constructed using JoinMap® 3.0.

Identification of tightly linked markers

Tightly linked markers were identified on fine map using three sets of phenotypic data evaluated in this study (at Rayong province in 2010) and in previous study (at Rayong and Lop Buri provinces in 2009) by MapQTL® 4.0.

3. RESULTS AND DISCUSSION

In 2011, QTL affecting CNP have been reported on linkage map of 100 F1 created by a cross between ‘Hanatee’ and ‘Huay Bong 60’, consisting of 303 SSR markers. Recently, additional markers have been developed (Sraphet et al. 2011) and therefore, the map was re-constructed by including more markers and number of samples to 200. The new linkage map increased the number of markers to 489 loci, relied on 23 linkage groups, and cover 1846.6 cM with an average interval distance of 6.1 cM. QTL affecting CNP evaluated at Rayong and Lop Buri provinces in 2009 and at Rayong province in 2010 were detected as shown in Table 1.

Table 1. Description of the QTL for cyanogenic potential in cassava.

Years	Locations	QTL	LG	αg	αc	Loci	LOD	%PVE
2009	Rayong	CN09R1	5	4.5	2.8	NS308	7.4	12.5
		CN09R2	5		2.8	ESSRY54-CA141	7.2	13.4
		CN09R3	6		2.9	EME502	3.8	3.1
		CN09R4	11		3.2	SSRY219	5.8	9.5
		CN09R5	16		3.2	CA76	4.5	7.5
		CN09R6	16		3.2	SSRY103	5.5	9.0
	Lop Buri	CN09L1	6	4.5	2.9	CA46	4.4	12.5
		CN09L2	11		3.2	SSRY219	3.2	7.8
2010	Rayong	CN10R1	4	4.2	2.7	EME303-CA591	2.9	8.9

*LG = Linkage group
αg = Genome wide significance threshold
αc = Chromosome wide significance threshold
LOD = Logarithm of odds
%PVE = Percentage of phenotypic variation explained.

With the established results, the significant loci for fine mapping were considered based on LOD score, percentage of phenotypic variation explained and significance of single marker analysis. The selected loci indicated the significance of single marker analysis are shown in Fig.1. Each locus was blasted with cassava genome database and given the name of their scaffolds followed the name of markers. Four scaffolds relied on the peaks of CN09R1 (s10689), CN09R4 and CN09L2 (s00368), and CN09R6 (s07933) and within 2-LOD support (s05214) were selected for fine mapping. In addition, three scaffolds containing genes involving in CNP were also mapped in fine map that include s08265, s12341 and s07743 in which CYP79D2, linamarase and hydroxynitrile lyase, respectively were found.

After fine mapping and QTL analysis, the high density of QTL map was generated (Fig 2). The fine map could narrow down the QTL regions. The rearrangement of marker loci resulted in changing of QTL on linkage group 5 from 2 to 1 QTL, as well as on linkage group 16 which pointed out the better loci to be focused on. For linkage group 11, the QTL peak was still relied on the same locus, made scaffold s00368 become more interested. Mapping scaffolds containing genes involving in CNP was successful, but no QTL was detected on those scaffolds, indicating that these genes might not directly affect to the amount of released HCN as the trait is controlled by multiple genes. The prediction of candidate genes on scaffolds s00368, s07933 and s10689, gene characterization, and validation of tightly linked markers responded to CNP is in the progress of investigation.

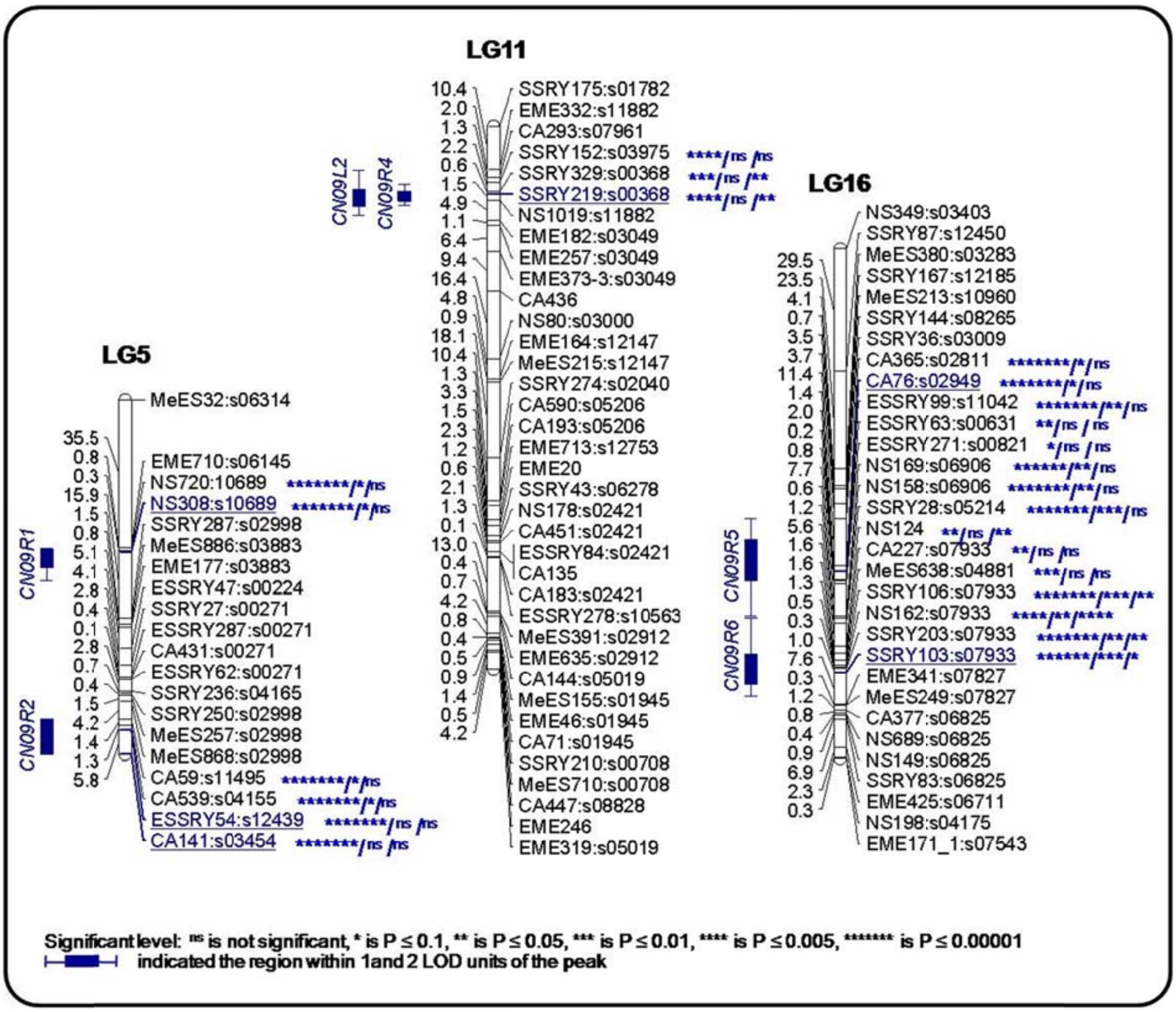

Fig. 1 The positions of selected QTL underlying cyanogenic potential for fine mapping. The significance of single marker analyzed in 2009 at Rayong and Lop Buri provinces and in 2010 at Rayong province are shown on the left side of the linkage group, separated by "/".

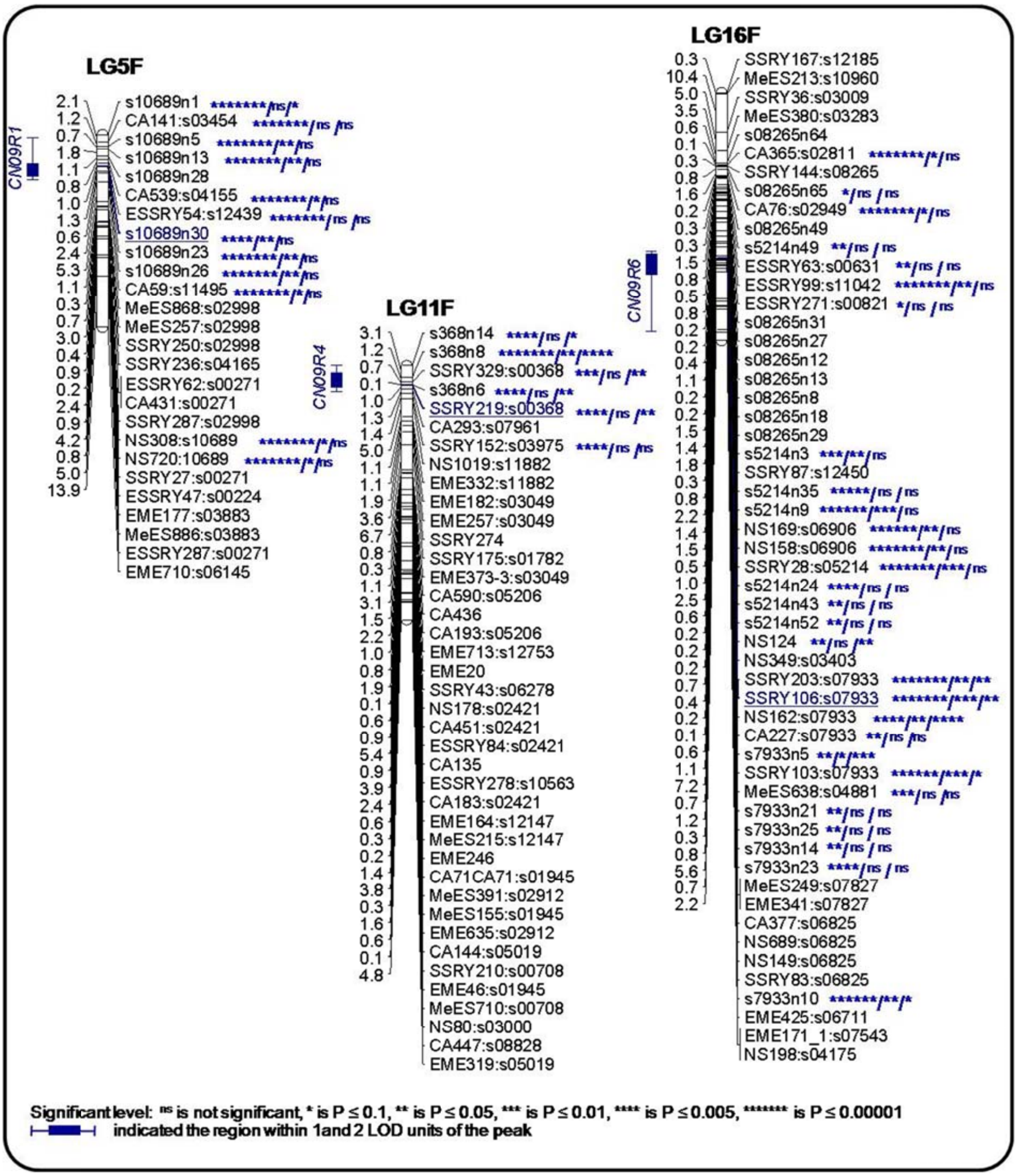

Fig. 2 The positions of QTL underlying cyanogenic potential on the fine map. The significance of single marker analyzed in 2009 at Rayong and Lop Buri provinces and in 2010 at Rayong province are shown on the left side of the linkage group, separated by "/".

4. CONCLUSIONS

Fine mapping of relevance QTL regions is importance in the identification of tightly linked markers to the target genes. In this study, the high density QTL map of CNP provides better tool for identification of the tightly linked markers which are useful for marker assisted selection, and also offers the opportunities to predict candidate genes affecting CNP which will give better understanding of genes and gene network involved in CNP. The information will also produces a huge benefit on selection of new cassava lines with low CNP in the breeding program.

5. ACKNOWLEDGEMENTS

This research was supported by National Research Council of Thailand in collaborated with National Science Technology Development Agency (P-12-00849), and Thailand Research Fund (TRG5580002). Mahidol University and Rayong Field Crops Research Center are also acknowledged.

6. REFERENCES

Andersen, M. D., Busk, P. K., Svendsen, I. & Møller, B. L. (2000). Cytochromes P-450 from Cassava (*Manihot esculenta* Crantz) Catalyzing the First Steps in the Biosynthesis of the Cyanogenic Glucosides Linamarin and Lotaustralin: cloning, functional expression in pichia pastoris, and substrate specificity of the isolated recombinant enzymes. *Journal of Biological Chemistry,* (275), 1966-1975.

Bokanga, M., Ekanayake, I., Dixon, A. & Porto, M. (1994). Genotype-environment interactions for cyanogenic potential in cassava. *Acta Horticulturae,* (375), 131 - 140.

Bradbury, M., Egan, S. & Bradbury, J. (1999). Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. *Journal of the Science of Food and Agriculture,* (79), 593 - 601.

Collard, B., Jahufer, M., Brouwer, J. & Pang, E. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica,* (142), 169 - 196.

Dixon, A., Asiedu, R. & Bokanga, M. (1994). Breeding of cassava for low cyanogenic potential: problems, progress and perspectives. *Acta Horticulturae,* (375), 153 - 161.

Food and Agriculture Organization. (2012). Food outlook. In. Global information and early warning system on food and agriculture.

Hughes, J., Carvalho, F. J. & Hughes, M. A. (1994). Purification, characterization, and cloning of alpha-hydroxynitrile lyase from cassava (*Manihot esculenta* Crantz). *Archives of Biochemistry and Biophysics,* (311), 496-502.

Hughes, M. A., Brown, K., Pancoro, A., Murray, B. S., Oxtoby, E. & Hughes, J. (1992). A molecular and biochemical analysis of the structure of the cyanogenic beta-glucosidase (linamarase) from cassava (*Manihot esculenta* Cranz). *Archives of Biochemistry and Biophysics,* (295), 273-279.

Sraphet, S., Boonchanawiwat, A., Tangphatsornroung, S., Boonseng, O., Tabata, S., Lightfoot, D., *et al.* (2011). Development of simple sequence repeat markers and construction of genetic linkage map of cassava (*Manihot esculenta* Crantz). *Theoritical and Applied Genetics,* (122), 1161 - 1170.

Van Ooijen, J. W., Boer, M. P., Jansen, R. C. & Maliepaard, C. (2002). MapQTL 4.0, Software for the calculation of QTL position on genetic maps. *Plant Research International*, Wageningen.

van Ooijen, J. W. & Voorrips, R. E. (2001). JoinMap 3.0. Software for the calculation of genetic linkage maps. *Plant Research International*, Wageningen.

Whankaew, S., Poopear, S., Kanjanawattanawong, S., Tangphatsornruang, S., Boonseng, O., Lightfoot, D.*, et al.* (2011). A genome scan for quantitative trait loci affecting cyanogenic potential of cassava root in an outbred population. *BMC Genomics,* (12), 266.

White, W., Arias-Garzon, D., McMahon, J. & Sayre, R. (1998). Cyanogenesis in Cassava. The Role of Hydroxynitrile Lyase in Root Cyanide Production. *Plant Physiology,* (116), 1219 - 1225.

Xinyou, Y., Stam, P., Kropff, J. & Schapendonk, H. (2003). Crop modeling, QTL mapping, and their complementary role in plant breeding. *Agronomy Journal,* (95), 90 - 98.