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MOLECULAR MARKERS ASSOCIATED WITH HIGH VITAMIN-C CONTENT IN GUAVA

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ABSTRACT

Vitamin-C content (VCC) was evaluated in 74 guava landraces using direct titration method with iodine during two seasons. Results showed that the highest value of VCC was 284.0±1.33, while the lowest VCC was 152.83±1.83 with an average of 221.26±3.17 mg/100g fresh weight. Analysis of variance showed the presence of highly significant differences among the tested landraces, as well as the interaction between landraces and seasons. Data of VCC showed normal distribution with high values of both broad sense heritability (0.97) and genetic advance (78.49) indicating high ability for selection. On the other hand, molecular analysis was performed using two molecular markers, i.e. sequence related amplified polymorphism (SRAP) and inter sequence simple repeats (ISSR) to determine unique and specific bands for high or low VCC. SRAP was more informative than ISSR and was able to generate 12 specific bands. Among these bands, 10 bands were specific for bulked DNA of landraces with high VCC, while the other two bands were specific for low VCC. However, ISSR only showed four bands where all of them were specific for low VCC. Results of this study gave good information for genotype selection for high VCC which could be used in guava breeding programs and/or biotechnological approaches. In addition, the specific bands generated by SRAP might assist in rapid screening for genotypes with high VCC, which could be identified in seedling or graft stage, therefore this would save time in a plant with long juvenile period like guava. Furthermore, these bands would be analyzed by sequencing in subsequent studies to locate related genome regions.

Keywords: *Psidium guajava*, Vitamin C content, SRAP, ISSR, Specific marker

INTRODUCTION

Guava (*Psidium guajava* L.) is the most important species in the family Myrtaceae, and is widely cultivated in many tropical and subtropical countries worldwide. Its fruit is rich in several important nutrients such as vitamins, calcium, phosphorus, iron as well as many antioxidants. Guava is a rich source of ascorbic acid (Vitamin C), which can reach three to six folds more than that of orange (Kwee and Chong 1990). However, vitamin C level in guava may vary depending on genotypic differences, pre-harvest climatic conditions, maturity, postharvest handling procedures (Chitravathi *et al.* 2014) and method of quantification (Raghu *et al.*, 2007).

Vitamin C must be obtained through the diet since human's body has no ability to synthesize it due to a mutation in the gene coding for L-gluconolactone oxidase (Ensminger *et al.*, 1994). Ascorbic acid involves in synthesis of lipids and protein, and metabolism of tyrosine, carbohydrate and iron, as well as its role in resistance to infections and cellular respiration (McEvoy 2000). In addition, vitamin C shows antioxidative effects which can protect against oxidativlely induced DNA damage (Sweetman *et al.*, 1997) and reduce the risk of chronic diseases such as cancer, cardiovascular disease, and cataracts (Carr and Frei 1999).

Characterization of guava genotypes by their vitamin C content can assist in selection of the proper plant for breeding and improvement programs. Furthermore, the association of phenotypic evaluation with molecular analysis considered as a very important task which provides an excellent tool for rapid screening, especially when specific markers are associated with the trait of interest. Several molecular markers can be used for this task however some markers showed their success over others. For instance, the sequence related amplified polymorphism (SRAP) that targets the open reading frames (ORFs) (Li and Quiros 2001) showed its efficiency with guava in several approaches including germplasm identification (Xiangyan *et al.,* 2011), genetic diversity (Youssef *et al.,* 2015a) and marker-based genetic map (Padmakar *et al.,* 2015). Similarly, inter simple sequence repeats (ISSR) have been proved as an effective tool for genetic fidelity assessment in guava plants derived by clonal propagation (Liu and Yang, 2012) and somatic embryogenesis (Rai *et al.*, 2012; Kamlea *et al.*, 2014) as well as in molecular characterization of guava landraces (Kidaha *et al.*, 2014).

Vitamin-C and other phenotypic traits in guava were evaluated in a study by Youssef *et al.* (2015a) who found significant variations in VCC among some guava landraces. For more focusing on this trait, the number of landraces was increased in the present study by collection from different locations, and the content of Vitamin-C was evaluated during two seasons followed by molecular analysis using SRAP and ISSR. Therefore, the main objective in this study was to determine specific markers associated with high or low content of Vitamin-C in guava using molecular markers.

MATERIALS AND METHODS

Plant materials

Seventy four guava landraces were used in this study to determine Vitamin-C content during the seasons 2012 and 2013 and to detect molecular markers associated with high Vitamin-C content. These landraces were collected from different locations in Egypt. Landraces grown in experimental farm of Genetics Department, Faculty of Agriculture, Assiut University were collected in a previous study by

Youssef *et al.* (2010), while the other were collected form experimental farm of Pomology Department and from local farms in Sahel Seleem.

Quantification of vitamin C content

Vitamin-C as the total ascorbic acid in the fresh fruit was determined using the direct titration method with iodine according to Suntornsuk *et al.* (2002). The experimental design was performed as randomized complete block with three replicates combined over seasons.

Statistical data analysis

Analysis of variance (ANOVA) was done using MSTAT-C statistical program (Nissen, 1984). Means were separated by least significant difference (LSD) test at 5% and 1% levels of probability. The heritability in broad sense was calculated according to Singh and Choudhury (1985). Genetic advance was also calculated for the studied traits by 5% selection intensity (Allard, 1964) and the genetic gain (GG) was calculated from the genetic advance as a percent of mean. Normal distribution of the averaged Vitamin-C content data was performed using SPSS-14 software.

Molecular analysis

DNA extraction and quantification

Total genomic DNA was extracted from five landraces of both highest and lowest vitamin C content, following the protocol of Youssef *et al.*, (2015b). DNA quality and concentration were determined using a spectrophotometer and Khirshyat 1.0 tools (Youssef 2012). DNA samples of each category were bulked to be used in molecular analysis.

SRAP and ISSR assays

SRAP was performed as described by Li and Quiros (2001) and ISSR was achieved according to Rai *et al.* (2012) and executed using Khirshyat 1.0 program (Youssef 2012). Ten SRAP primers and ten ISSR primers were selected and used for the analysis (Table 1). PCR products of SRAP and ISSR were separated on 2.5 and 1.5% agarose gel, respectively and visualized by staining with ethidium bromide.

Molecular data analysis

SRAP and ISSR profiles were converted to binary data matrices by detecting the presence (1) or the absence (0) of the strong, reproducible and clearly distinguished bands. The number of unique and specific bands for high and/or low Vitamin-C content was registered. The percentage of polymorphism was calculated for each primer by dividing the total number of polymorphic bands by the total number of bands.

Table 1. SRAP and ISSR primer sequences used for molecular analysis.

SRAP			ISSR		
No	Code	Sequence (5`-3`)	No	Codes	Sequence (5`-3`)
1	Me-01 Em-04	TGAGTCCAAACCGGATA GACTGCGTACGAATTTGA	1	UBC-807	AGAGAGAGAGAGAGAGT
2	Me-02 Em-02	TGAGTCCAAACCGGAGC GACTGCGTACGAATTTGC	2	UBC-808	AGAGAGAGAGAGAGAGC
3	Me-03 Em-03	TGAGTCCAAACCGGAAT GACTGCGTACGAATTGAC	3	UBC-810	GAGAGAGAGAGAGAGAT
4	Me-04 Em-01	TGAGTCCAAACCGGACC GACTGCGTACGAATTAAT	4	UBC-811	GAGAGAGAGAGAGAGAC
5	Me-04 Em-02	TGAGTCCAAACCGGACC GACTGCGTACGAATTTGC	5	UBC-812	GAGAGAGAGAGAGAGAA
6	Me-04 Em-03	TGAGTCCAAACCGGACC GACTGCGTACGAATTGAC	6	UBC-815	CTCTCTCTCTCTCTCTG
7	Me-04 Em-04	TGAGTCCAAACCGGACC GACTGCGTACGAATTTGA	7	UBC-826	ACACACACACACACACC
8	Me-04 Em-10	TGAGTCCAAACCGGACC GACTGCGTACGAATTTAG	8	UBC-834	GAGAGAGAGAGAGAGAGAGAT
9	Me-06 Em-03	TGAGTCCAAACCGGTAG GACTGCGTACGAATTGAC	9	UBC-840	GAGAGAGAGAGAGAGATT
10	Me-06 Em-10	TGAGTCCAAACCGGTAG GACTGCGTACGAATTTAG	10	UBC-846	CACACACACACACACAAT

RESULTS AND DISCUSSION

Genetic variability of Vitamin-C content (VCC) was evaluated among 74 guava landraces using molecular markers. Direct titration method showed large scale of variability in VCC among the tested landraces. Regarding, the averaged value of VCC over the two seasons was 221.26±3.17 mg/100g. The highest value of VCC was 284.0±1.33 mg/100g showed by landrace L10, while the lowest was 152.83±1.83 mg/100g showed by landrace L4 as an average of the two seasons (Table 2). Analysis of variance showed highly significant ($p<0.01$) differences among the tested landraces in Vitamin-C content as well as the interaction between landraces and seasons (Table 3). The content of ascorbic acid was found to be higher than other fruit crops commonly consumed in diet, such as mango (60.5 mg/100 g), kiwi fruits (29–80 mg/100g), papaya (92.9 mg/100 g), (Nishiyama *et al.*, 2004), or cherry (31–112 mg/100g) (Yilmaz *et al.*, 2009). The level of ascorbic acid can vary with genotypic differences, pre-harvest climatic conditions, maturity, postharvest handling

procedures (Chitravathi *et al.* 2014) and method of quantification (Raghu *et al.*, 2007). In this regard, the amount of ascorbic acid in the white and pink guava types was investigated by El-Faki and Saeed (1975) and Bashir and Abu-Goukh (2003), who found higher values in the white guava than pink types, while other investigators reported the reverse (Agnihortri *et al.*, 1962; El-Zorkani, 1968). In the present study, only two guava accessions with pink pulp (L3 and L71) were used and showed higher VCC similar to those of white pulp. In addition, Bashir and Abu-Goukh (2003) found that the peel exhibited much higher values of ascorbic acid than the pulp in both white and pink types.

Table 2. Averages of vitamin C content (mg/100g fresh weight) of the tested guava landraces.

Landrace	Mean ± SE	Observations	Landrace	Mean ± SE	Observations
L1	232.00 ± 0.67	A	L38	249.83 ± 1.83	A
L2	215.00 ± 1.33	A	L39	234.33 ± 1.00	A
L3	254.83 ± 0.50	A, Pink	L40	244.50 ± 20.17	A
L4	152.83 ± 1.83	A, Low	L41	222.17 ± 8.17	A
L5	174.17 ± 0.83	A, Low	L42	208.50 ± 16.17	A
L6	216.83 ± 0.83	A	L43	213.50 ± 0.50	A
L7	207.83 ± 0.83	A	L44	191.67 ± 1.67	A
L8	223.83 ± 0.50	A	L45	222.33 ± 10.00	A
L9	226.83 ± 1.17	A	L46	209.50 ± 16.17	A
L10	284.00 ± 1.33	A, High	L47	227.67 ± 1.00	A
L11	234.50 ± 0.17	A	L48	232.50 ± 7.83	A
L12	201.83 ± 0.50	A	L49	210.50 ± 14.17	B
L13	202.50 ± 0.83	A	L50	231.83 ± 0.17	B
L14	234.00 ± 0.67	A	L51	200.67 ± 3.00	B
L15	185.50 ± 1.83	A	L52	251.00 ± 0.67	B
L16	255.83 ± 1.83	A	L53	258.33 ± 1.33	B
L17	204.83 ± 0.83	A	L54	268.33 ± 0.33	B, High
L18	215.33 ± 0.00	A	L55	226.50 ± 1.50	B
L19	283.83 ± 0.50	A, High	L56	242.33 ± 0.00	B
L20	251.83 ± 0.50	A	L57	186.50 ± 2.50	B
L21	183.00 ± 1.33	A	L58	233.00 ± 2.00	B
L22	177.17 ± 1.83	A	L59	228.33 ± 1.33	B
L23	194.50 ± 1.17	A	L60	189.83 ± 0.17	B
L24	212.00± 1.33	A	L61	240.33 ± 0.00	B
L25	231.17 ± 1.17	A	L62	203.50 ± 1.83	B
L26	244.17 ± 1.50	A	L63	256.17 ± 0.17	B
L27	200.00 ± 1.67	A	L64	215.17 ± 0.17	B
L28	208.83 ± 1.17	A	L65	177.00 ± 2.00	B, Low
L29	187.50 ± 0.83	A	L66	231.00 ± 0.67	B
L30	212.00 ± 1.00	A	L67	242.50 ± 0.17	B
L31	224.33 ± 1.00	A	L68	231.00 ± 0.00	B
L32	276.17 ± 0.50	A, High	L69	243.50 ± 2.17	C
L33	236.17 ± 0.17	A	L70	220.50 ± 1.17	C
L34	175.50 ± 0.83	A, Low	L71	258.50 ± 0.17	C, High, Pink
L35	210.17 ± 2.50	A	L72	186.00 ± 1.33	C
L36	242.00 ± 0.33	A	L73	204.33 ± 3.00	C
L37	173.00 ± 0.67	A, Low	L74	234.00 ± 1.67	C
H^2	0.97		$LSD_{0.05}$	7.02	
GA	78.49		$LSD_{0.01}$	9.26	
GG	35.51		CV	2.80	

A: landraces collected from Genetics Dept. farm, B: landraces collected from Pomology Dept. farm, C: landraces collected from Sahel Seleem farms, Pink: fruit with pink pulp, High: selected landraces for high vitamin C content, Low: selected landraces for low vitamin C content, H^2: heritability in broad sense, GA: genetic advance, GG: genetic gain, CV: coefficient of variation.

The results in this study showed that the VCC in guava was higher than some reports (Suntornsuk *et al.*, 2002; Raghu *et al.*, 2007) and comparable to other (Jawaheer *et al.*, 2003) which indicate the effect of genotype. The stage of maturity as well was reported as a factor affecting the amount of vitamin C. The earlier study of Golberg and Levy (1941) reported that the VCC was 250-350 mg/100g for green and hard fruits, 300-450 mg/100g for ripe and firm fruits, while it was 50-100 mg/100g for over ripe and soft fruits. Furthermore, the mature green stage exposes the maximum level of ascorbic acid content in guava (Agnihortri *et al.*, 1962; El-Zorkani, 1968) due to the breakdown of starch to glucose which is used in the biosynthesis of ascorbic acid (Yan *et al.*, 2006) and then it starts to decline rapidly as the fruit ripens. On the

other hand, Raghu *et al.*, (2007) reported that the quantification method affected the Vitamin C content in guava. The same authors found that the spectrophotometric methods using 2,4-dinitrophenylhydrazine or indophenol–xylene provided an over-estimation of vitamin C content in guava fruits

The distribution of the 74 guava landraces for VCC is shown in Figure 1. The trait showed continuous variation and approached normality, indicating that it is under the control of polygenes. The broad sense heritability (H^2) was estimated for VCC which was significantly high (0.97). Moreover, H^2 was associated with high genetic advance (78.49) and genetic gain (35.51). High heritability may lead to increase genetic advance, when sufficient genetic variability existed in the germplasm (Sardana *et al.* 2007). The Vitamin C content would be controlled by additive gene action since it showed a highly heritability associated with a high genetic advance (Eid 2009).

Table 3. The combined analysis of variance of the tested guava landraces for vitamin C content over the two seasons.

Source of variance	Df	Means of Squares
Season (S)	1	42.89^{NS}
Error	4	178.80
Landrace (L)	73	4505.15**
S × L	73	147.38**
Error	292	38.25

NS: none significant, **: significant at 0.01 probability level.

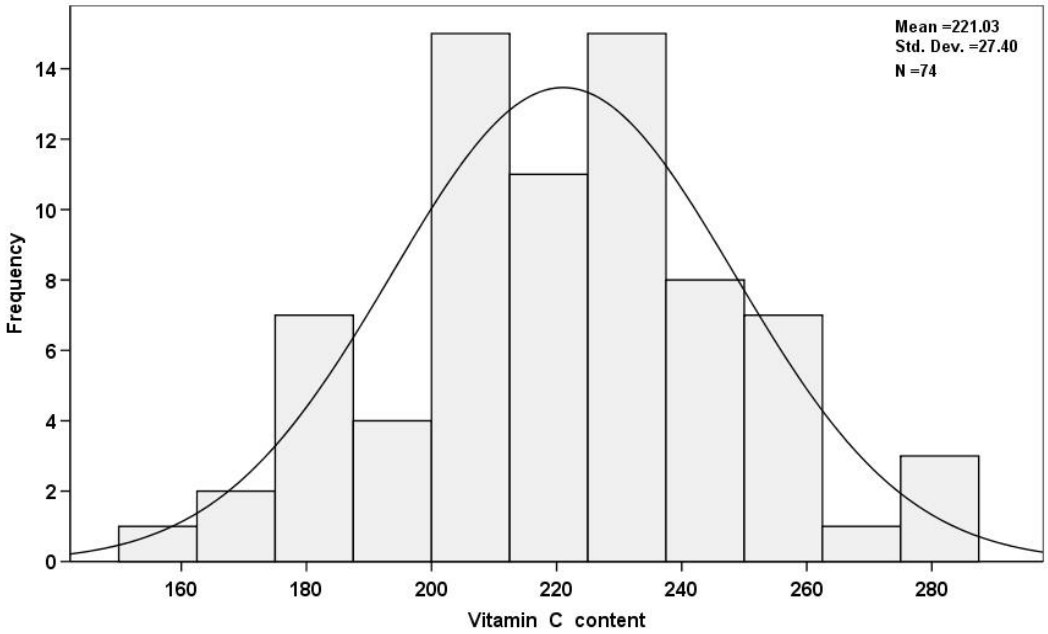

Figure 1. Distribution of Vitamin-C content in the tested 74 guava landraces.

Molecular analysis

The Sequence related amplified polymorphism (SRAP) vis-á-vis inter simple sequence repeats (ISSR) were used in this study for molecular analysis of guava landraces with high and low content of Vitamin-C. Five DNA samples of landraces exposing high or low content of vitamin C were used as a bulk for molecular analysis. Gathering the DNA samples of landraces with high or low content of Vitamin-C reduced the variations within each group and mainly focused on the difference between the high and low bulks of the trait of interest. Results showed that the ten primers of each SRAP and ISSR reproduced a total of 139 and 70 bands, respectively. The average of amplified number of bands per primer was 13.9 and 7 for SRAP and ISSR respectively. The averaged percentage of polymorphism (%P) between high and low bulks was 8.63 and 5.71% showed by SRAP and ISSR respectively. Figure 2 shows profiles of some primers of SRAP and ISSR.

SRAP showed its effectiveness by generating several specific bands for the high and low VCC bulked-landraces. Among the tested 10 primers of SRAP, six primers were able to generate unique and specific bands for high and/or low VCC. Regarding, a total of 10 bands were generated as specific for high VCC generated by five primers, i.e. three bands by Me2-Em2, four bands by Me3-Em3, one band by Me4-Em4 and one band by Me4-Em1. While, only two bands were generated as specific for low VCC generated by two primers, i.e. one band by both Me1-Em4 and Me3-Em3 as presented in Table 4. On the other hand, ISSR primers used in this study were less informative than SRAP in generating specific bands for the investigated trait. In this regard, ISSR generated four bands which were specific for low VCC generated by three primers, i.e. two bands by UBC-826, one band by UBC-834 and one band by UBC-846. However, no specific bands were generated by ISSR for high VCC. The generated bands could preliminarily serve as selectable markers for Vitamin-C content in guava; however purification, sequencing and analysis of these bands might be necessary in the proximate research work. The presence or absence of specific bands generated by SRAP and ISSR are shown in Table 5.

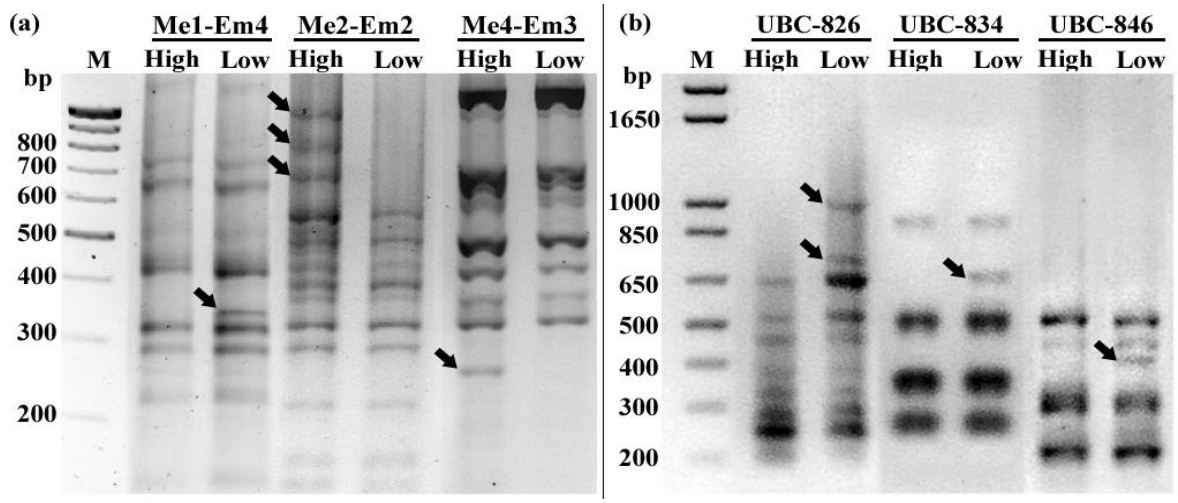

Figure 2. Profiles of some primers of (a) SRAP and (b) ISSR showing the difference between two bulked DNA of landraces with high and low Vitamin-C content. Arrows indicate specific bands.

Table 4. Level of polymorphism and number of specific bands for landraces with high and low vitamin C content generated by SRAP and ISSR primers.

Primer	TNB	%P	Specific bands		Primer	TNB	%P	Specific bands	
			High	Low				High	Low
Me1-Em4	13	7.69	0	1	UBC-807	6	0.00	0	0
Me2-Em2	13	23.08	3	0	UBC-808	5	0.00	0	0
Me3-Em3	21	23.81	4	1	UBC-810	9	0.00	0	0
Me4-Em1	15	6.67	1	0	UBC-811	10	0.00	0	0
Me4-Em2	9	0.00	0	0	UBC-812	8	0.00	0	0
Me4-Em3	14	7.14	1	0	UBC-815	11	0.00	0	0
Me4-Em4	8	12.50	1	0	UBC-826	9	22.22	0	2
Me4-Em10	21	0.00	0	0	UBC-834	5	20.00	0	1
Me6-Em3	16	0.00	0	0	UBC-840	2	0.00	0	0
Me6-Em10	9	0.00	0	0	UBC-846	5	20.00	0	1
Total	139	8.63	10	2	Total	70	5.71	0	4

TNB: total number of bands, %P: percentage of polymorphism.

Table 5. Survey of specific bands generated by SRAP and ISSR showing their size (bp) and presence or absence in high or low vitamin C content.

Marker	No.	Primer	Size (bp)	High	Low
SRAP	1	Me1-Em4	340	-	+
	2	Me2-Em2	960	+	-
	3	Me2-Em2	795	+	-
	4	Me2-Em2	675	+	-
	5	Me3-Em3	620	+	-
	6	Me3-Em3	510	+	-
	7	Me3-Em3	350	+	-
	8	Me3-Em3	200	+	-
	9	Me3-Em3	225	-	+
	10	Me4-Em3	255	+	-
	11	Me4-Em4	560	+	-
	12	Me4-Em1	350	+	-
ISSR	1	UBC-826	1005	-	+
	2	UBC-826	735	-	+
	3	UBC-834	660	-	+
	4	UBC-846	410	-	+

+,-: indicate presence or absence of specific bands in high and low Vitamin-C bulks

Both molecular markers used in this study have been reported as excellent tools for genome screening and plant diversity. For instance, the advantages of SRAP are reported to be simple, informative, reliable and easy to develop (Li and Quiros, 2001). On the other hand, ISSR proved to be effective and reproducible for

detecting genetic fidelity and variability (Martins *et al.*, 2004; Kumar *et al.*, 2009). SRAP was used with guava for the first time by Xiangyan *et al.* (2011) and it was reported as an effective tool in the germplasm identification and genetic diversity analysis in guava. Later, SRAP showed a high level of polymorphism in the assessment of genetic diversity in guava (Youssef *et al.*, 2015b). Recently, SRAP was used along with SSR for developing marker-based genetic map of guava, in which 20% of parental polymorphism was exhibited by SRAP (Padmakar *et al.*, 2015). On the other hand, ISSR has been proved as an effective tool for genetic fidelity assessment in guava plants derived by clonal propagation (Liu and Yang, 2012) and somatic embryogenesis (Rai *et al.*, 2012 and Kamlea *et al.*, 2014). Additionally, ISSR exposed a range of 51 to 85% polymorphism in molecular characterization of guava landraces in Kenya (Kidaha *et al.*, 2014). Comparing SRAP and ISSR data generated in the present study, SRAP was obviously more informative in generating specific bands for high and low content of vitamin C. These variations between the two molecular markers might be due to their different basis in analyzing genome regions.

In conclusion, the characterization of large number of guava landraces performed in this study provided good information for proper genotype selection with high vitamin C content which could be used in breeding programs and biotechnological approaches. In addition, molecular analysis of landraces with high and low vitamin C content was able to generate several specific bands for each category. These bands could assist in rapid screening of guava seedlings or scions with no need for mature plant evaluation. However, sequencing and analysis of these bands would be done in subsequent studies for more focus on genome related regions.

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الواسمات الجزيئية المصاحبة للمحتوى العالي لفيتامين ج في الجوافة

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تم في هذه الدراسة تقدير محتوى فيتامين ج في أربع وسبعين سلالة من الجوافة باستخدام طريقة المعايرة المباشرة في موسمين. أظهرت النتائج أن أعلى قيمة من محتوى فيتامين ج كانت 284.0±1.33 بينما كانت أقل قيمة 152.83±1.83 بمتوسط عام 221.26±3.17 مجم/100 جم وزن طازج. كما أظهر تحليل التباين إختلافات معنوية جداً بين السلالات المختبرة وأيضا التفاعل بين السلالات والموسمين في صفة محتوى فيتامين ج. أظهرت الصفة توزيعاً طبيعياً وقيمة عالية من معامل التوريث بالمعنى الواسع (0.97) وأيضاً قيمة عالية من درجة التقدم الوراثي (78.49). على الجانب الآخر، تم استخدام كلاً من الواسم الجزيئي SRAP و الواسم الجزيئي ISSR في التحليل الجزيئي بهدف تحديد حزم خاصة للتراكيب الوراثية ذات المحتوي العالي أو المنخفض من فيتامين ج. حيث كان الواسم الجزيئي SRAP أكثر فعالية من ISSR وتمكن من تخليق مجموع 12 حزمة متخصصة. كان من بين هذه الحزم عدد 10 حزم فريدة وخاصة بالمحتوي العالي من فيتامين ج بينما كانت الحزمتين الأخرتين خاصين بالمحتوي المنخفض من الفيتامين. في الوقت نفسه أظهر الواسم ISSR مجموع أربعة حزم فقط متخصصة للمحتوي المنخفض من فيتامين ج. تقدم النتائج المتحصل عليها في هذه الدراسة معلومات جيدة تساعد في انتخاب الطرز الوراثية ذات المحتوي العالي من فيتامين ج ليمكن استخدامها في برامج التربية التقليدية أو في مجال التقنية الحيوية. بالاضافة لذلك، يمكن أن تساعد الحزم الخاصة المتحصل عليها من الواسم الجزيئي SRAP في عمل حصر سريع للطرز الوراثية ذات المحتوي العالي من فيتامين ج والذي يمكن أن يتم في مرحلة البادرة أو الشتلات المطعمة مما يفيد في اختصار الوقت في نبات يستغرق فترة نمو طويلة للإثمار مثل الجوافة. هذا ويتعين تحليل هذه الحزم الخاصة المتحصل عليها في هذه الدراسة عن طريق تحديد تتابع النيوكليوتيدات الخاصة بها في دراسات لاحقة لمعرفة مناطق الجينوم الخاصة بهذه الحزم.