Identification of genes related to sugar content in *Carica papaya* L.: Differential expression and candidate marker development

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Abstract

High sugar content is a valued papaya fruit quality trait that is difficult to select due to a lack of knowledge regarding the major contributing genetic components and the potential for large environmental effects. The initial step towards better understanding this complex trait is to identify the functional genes involved in the sugar synthesis and accumulation pathways. This was achieved in the current study through differential expression analyses of a suit of sweetness-related genes among two phenotypically contrasting papaya cultivars.

The major sugar detected in leaf and ripening fruit tissues of two genotypes (120 days after anthesis to colour break 100%) was sucrose, which accounted for 40-60% of the total sugars detected. In general, genotype 'Sunrise Solo' had higher sugar content than genotype 'RB2' in all tissue types assessed. Subsequently, differential expression of 11 genes potentially associated with the main sugar synthesis and accumulation pathways were investigated. Of these, sucrose phosphate synthase (*cpSPS1*, *cpSPS2*, *cpSPS3* and *cpSPS4*) and invertase (*cpCWINV1* and *cpAVIN2*) gene family members were expressed significantly differently. In all tissue types, *cpSPS2* was between 0.5 to 3 times more highly expressed in 'Sunrise Solo' than 'RB2'. The maximum expression of *cpSPS2* in 'Sunrise Solo' was observed in mature fruit at 120 days after anthesis, while its expression remained constant in 'RB2'. Similarly, genes *cpCWINV1* and *cpAVIN2* were significantly more expressed in 'Sunrise Solo' compared to 'RB2' during ripening stages. Further validation and investigation of the expression of these gene sequences for association with the sweetness trait at specific fruit ripening stages and in different environments will aid in their identification as candidate sweetness markers for future selective breeding strategies.

Keywords: Papaya breeding, qPCR analysis, sugar-related genes and candidate gene marker selection

INTRODUCTION

Papaya (*Carica papaya* L.) is a commercial fruit crop that has been cultivated worldwide and is an important fruit crop in Australia. Global production of papaya was around 12 million tons per year in 2014 (FAOSTAT, 2017). Annual production of papaya in Australia in 2014 was approximately 6,097 tons (FAOSTAT, 2017) which is relatively small compared to other producers, despite the available suitable agro-geographical and climatic conditions. Therefore, to expand the Australian papaya industry, stable improvements in productivity and quality traits are required, in particular fruit flavour is a priority.

Sweetness or high sugar content is one of the key determinants of descriptive fruit flavour and quality, directly influencing papaya consumption (Del Carmen et al., 2013). There are three main sugars that contribute to papaya sweetness; glucose, fructose and sucrose (Gomez et al., 2002). Their accumulation in fruit flesh begins between 100 and 140 days after anthesis (DAA), and rapidly increases during fruit maturation (Zhou and Paull, 2001). Invertase, sucrose phosphate synthase and sucrose synthase are the main enzymes associated with papaya sugar content from early development to ripening stages (Zhou and Paull, 2001; Gomez et al., 2002). Sucrose phosphate synthase catalyses sucrose synthesis while invertase functions in the hydrolysis of sucrose to glucose and fructose. Sucrose synthase is involved in both sucrose synthesis and cleavage (McCollum et al., 1988; Miron and Schaffer, 1991). Genes that code for and govern the production of these key enzymes have been identified in various crops such as; tomato (Klann et al., 1993), grape (Davies and

Robinson, 1996), pineapple (Zhang et al., 2012) and apple (Li et al., 2012). However, these have not been identified in papaya and their expression in relation to fruit development and ripening is unknown.

In this study, we investigated putative sugar-related genes through differential expression between two papaya cultivars known to differ greatly in sweetness ('RB2' and 'Sunrise Solo') during fruit development.

MATERIALS AND METHODS

Plant materials

Two genotypes of papaya, 'RB2' and 'Sunrise Solo' representative of low and high sugar-content, or less-sweet and more-sweet, respectively (Kanchana-udomkan, 2015), were used in this study. They were grown at Lecker farming, Mareeba, Australia. Three plants of each genotype were selected and flowers were tagged at anthesis. Eight different sample types were harvested as follows (Figure 1): 1) mature leaves; 2) immature fruit at 30 DAA; 3) immature fruit at 60 DAA; 4) immature fruit at 90 DAA; 5) mature fruit at 120 DAA (mature green); 6) mature fruit at 25% colour break; 7) mature fruit at 50% colour break; 8) mature fruit at 75% colour break and; 9) fruit at full colour (fully ripe). All samples were harvested at the same time and immediately frozen in liquid nitrogen. The sample were stored at -80°C until analysis.

Figure1: Leaves and fruits of papaya genotypes 'Sunrise Solo' and 'RB2' sampled at different developmental stages (stage 2-5; 30-120 DAA) and ripening stages (stage 6-9; colour break 25-100%) for sugar content and molecular analyses.

Sugar level determination

Mature leaves were sampled from the plant apex and flesh samples were taken from the fruit equatorial regions. The experiment was performed in three replications (biological replications) with three technical replications per biological replication. The total sugar, glucose, fructose and sucrose concentrations were determined as mg/g fresh weight (FW) levels and were assessed using the microplate enzymatic assay previously described by Vermeir et al. (2007).

RNA extraction and cDNA synthesis from all tissues

Total RNA was isolated from all samples (Figure 1) using a modified cetyltrimethylammonium bromide based RNA extraction protocol (Fabi et al., 2009). RNA quality and quantity was measured with a ND-1000 spectrophotometer (NanoDrop, USA). Then, cDNA was synthesized using PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio Inc).

Sugar-related gene identification and gene expression profiling

To search for the homologs of sugar synthesis related gene sequences in papaya, known invertase (*INV*, *CWINV* and *AINV*), sucrose synthase (*SUS*) and sucrose phosphate synthase (*SPS*) genes from diverse plant species were searched for in the papaya whole-genome shotgun (WGS) database (<http://www.ncbi.nih.gov/>) using the BLASTN and BLASTX algorithms (identity $\geq 60\%$ and e-value $\leq 1e-30$ were used as cut-off values).

The expressions of selected sugar synthesis-related papaya genes were subsequently evaluated following Minimum Information for Publication of Quantitative Real-Time PCR (qPCR) Experiment (MIQE) guidelines (Bustin et al., 2009). Three biological replications of mature leaves and eight different stages of fruit development, as described in Figure 1, were used to perform qPCR analysis. Primer pairs were designed for optimum qPCR detection according to the product information of SYBR® *Premix Ex Taq*™ II [Tli RNaseH] Plus, Bulk (Takara Bio Inc) and 11 primer pairs were selected (Table 1).

Each qPCR reaction was performed in a final volume of 25 μ l using 1X SYBR® *Premix Ex Taq*™ II -Tli RNaseH Plus Bulk (contains 5 unit/ μ l of TaKaRa Ex Taq HS, 2.5 mM dNTPs, Tli RNase H stabilizers, Mg^{2+} , and SYBR Green I, Takara Bio Inc), 0.4 μ M of each primer (forward and reverse) and 1 μ L of 50-fold diluted cDNA template. Subsequently, qPCR reactions were performed in a CFX96 Real-Time PCR Detection System (Bio-Rad) using the following program: Initial denaturation at 95°C for 30 sec; followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec (data collection step), melt curve analysis at 95°C for 1 min, 55°C for 1 min and 80 cycles of 55°C for 10 sec, with a step increase of 0.5°C each cycle.

The expression value of each gene assessed under this study was normalized with three reference genes (*18S rRNA*, *CYPC* and *TBP2*). These reference genes were the three most stable targets previously determined across all samples (M values = 0.9215-1.1171). The mean expression values of each gene were expressed as an average from three technical replications.

The quantification was performed using the relative standard curve method (Pfaffl, 2001). Analysis of reference gene stability across developmental stages was carried out by geNorm analysis in qbase+ software (Biogazelle, Belgium) and the means of the Cq values were calculated for the analysis of relative expression. The expressions of target genes in leaves and fruit at different developmental stages were compared between 'RB2' and 'Sunrise Solo'. T-tests were performed for each gene to determine significant difference between the expressions between the two genotypes (categorical variable) using relative expression as the continuous variable.

RESULTS AND DISCUSSION

Fruit development and sugar content in papaya

Sugar analysis in leaf and fruit samples indicated similar overall patterns of sugar content in 'RB2' and 'Sunrise Solo' over the developmental stages (Figure 2). In particular, there was a rapid increase in sugar concentrations at 120 days after anthesis (DAA). Total sugar content was higher in 'Sunrise Solo' (47.38 - 134.89 mg/g FW) than in 'RB2' (27.87-103.84 mg/g FW) in all samples (Figure 2).

In general, leaf tissue had higher concentrations of sucrose than glucose and fructose (Figure 2). Leaf samples of 'Sunrise Solo' contained twice as much sucrose and glucose than in 'RB2'. During early stages of fruit development (30-90 DAA), glucose and fructose were the main sugars that accumulated in the flesh while sucrose remained constantly low. At 120 DAA, the sucrose concentration increased rapidly in both genotypes. After ripening commenced, glucose, fructose and sucrose levels continued to increase in 'Sunrise Solo', where sucrose remained the predominant sugar (~40% of total fruit sugar). This was a similar pattern in 'RB2', but with a decline in fructose concentration at the last stage of fruit ripening. This overall pattern of fruit sugar concentration was similar to previous observations in other cultivars (Chan et al., 1979; Nakasone, 1986; Zhou and Paull, 2001).

Table 1: Primers and target genes used for qPCR analysis

	Target gene	Primer sequence (5'→3')	A ^{1/} (°C)	E ^{2/} (bp)
Invertase	<i>cpCWINV1</i>	F: ATATCGAACGGCTCAGATGG R: CCAATACAACCCAAAGGGTG	60 60	120
	<i>cpCWINV2</i>	F: ACCGCAACCTTTACATTTCGC R: AGCTCGCTTTCAGAACGTGT	60.07 60.25	140
	<i>cpAINV1</i>	F: TGTAATGAATGCCAGGTGGA R: GATCTGCTACCATCCTCCCA	59.2 60	177
	<i>cpAINV2</i>	F: ATCACCGGTGTACAGCATCA R: CCGTATGGGGAAATATCACG	59.9 60	170
Sucrose synthase	<i>cpSUS3</i>	F: TGCTAGAGGGACAAAAGTGCA R: TGGGTAAATATCGAAACGGGA	58.9 56.8	175
	<i>cpSUS4</i>	F: TCGGAAGTAAGCATAACCCAA R: GGAGGCAATTGAATAAGACCCA	57.01 58.37	175
	<i>cpSUS5</i>	F: CTCTATGTGTAAGATGACCAGAAAAG R: CGTGAGCAGAATGATGAATACGT	57 59.2	95
Sucrose phosphate synthase	<i>cpSPS1</i>	F: GATAAGCAGCTTTGGGCAAG R: CCTCAAGGAATCACTAGGCG	59.98 59.83	186
	<i>cpSPS2</i>	F: GAACTCTCTCGCCTGGCTTT R: CAGATTCCCCTGGCAATTCC	60 58.5	157
	<i>cpSPS3</i>	F: GTTCGAGCGAGCCTCATTAC R: AAGCATTAAGATGTTGCCCG	59.8 60	178
	<i>cpSPS4</i>	F: AGCAAAACCGGAGACTCGAG R: CCACGTTGAGAAGGAGACGT	60 59.6	152

^{1/} Annealing temperature (°C)^{2/} Expected target size (bp) **Figure 2:** Sugar concentration of 'RB2' (solid line) and 'Sunrise Solo' (dot line) within tissues of 1 = leaf, 2-5 = fruit collected at 30, 60, 90 and 120 DAA and 6-9 = ripe fruit at colour break of 25%, 50%, 75% and 100%. Graphs represent the accumulation of total sugars (A), glucose (B), fructose (C) and sucrose (D), expressed in mg/g of fresh weight.

Table 2: Blast result of candidate genes related to sugar synthesis in the papaya genome

	Gene Position			Expected size		Homology				
	Code	Papaya WGS	contig	mRNA length	Exon	Organism	Protien /Gene	Acession number	E value	I ¹ /
Invertase	<i>cpCWINV1</i>	ABIM0101 7629.1	LG4, contig 17654	1969	-	<i>C. papaya</i>		AF420223_1	0	100%
				2048	5	<i>Arabidopsis thalia</i>	CWIN2	NM_115120.3	7e-97	70%
				2082	6	<i>A. thalia</i>	CWIN4	NM_129177.2	5e-80	68%
	<i>cpCWINV2</i>	ABIM0100 7212.1	LG2, contig 7220	1938	7	<i>Vitis vinifera</i>	CWINV	NM_001281279.1	4e-125	72%
				1953	7	<i>A. thalia</i>	CWIN1	NM_001202952.1	5e-92	69%
				1710	4	<i>A. thalia</i>	CWIN5	NM_112231.1	6e-65	67%
	<i>cpINV1</i>	ABIM0101 3721.1	LG1, contig 13738	2078	6	<i>A. thalia</i>	CINV1	NM_179419.3	8e-103	75%
				2032	6	<i>A. thalia</i>	CINV2	NM_117019.4	3e-107	76%
	<i>cpINV2</i>	ABIM0100 3134.1	LG5, contig 3137	2479	6	<i>Solanum lycopersicum</i>	LOC101 245982	XM_010320928.1	1e-113	78%
				2022	6	<i>V. vinifera</i>	NIN1	NM_001281047.1	6e-155	85%
				2049	6	<i>Malus domestica</i>	NI	NM_001294124.1	3e-152	84%
	<i>cpINV3</i>	ABIM0100 5774.1	LG9, contig 5781	2191	7	<i>A. thalia</i>	INV-E	NM_122156.3	1e-101	77%
Sucrose synthase	<i>cpAINV1</i>	ABIM0100 8002.1, ABIM0100 8006.1	LG2, contig 8011 and 8015	2461	-	<i>Cucumis melo</i>	INV1	FJ653659.1	2e-149	74%
				2274	-	<i>Pyrus pyrifolia</i>	PsS- AIV2	AB239590.1	0.0	77%
	<i>cpAINV2</i>	ABIM0100 1588.1	LG8, contig 1588	2173	7	<i>S. lycopersicum</i>	AI	NM_001247914.2	4e-164	75%
	<i>cpSUS1</i>	ABIM0101 5572.1	Un contig_1 5592	720	-	<i>C. papaya</i>		AF420224.1	3e-83	99%
				2835	15	<i>V.vinifera</i>	LOC100 249279	XM_010657782.1	7e-156	82%
	<i>cpSUS2</i>	ABIM0101 1863.1	LG6, contig 11878	2439	15	<i>M. domestica</i>	SS	NM_001293909.1	3e-58	83%
				2511	15	<i>A. thaliana</i>	SUS2	NM_124296.2	7e-73	80%
				2917	12	<i>A. thaliana</i>	SUS3	NM_116461.3	6e-49	80%
	<i>cpSUS3</i>	ABIM0102 9565.1	Un contig_2 9609	3053	12	<i>A. thaliana</i>	SUS6	NM_001198461.1	1e-95	76%
	<i>cpSUS4</i>	ABIM0102 2037.1	Un contig_2 2068	2511	13	<i>A. thaliana</i>	SUS5	NM_123077.2	3e-116	76%
	<i>cpSUS5</i>	ABIM0102 0824- ABIM0102 0825	LG5 contig_2 0853 and 20853	719	-	<i>C. papaya</i>		AB091538.1	4e-75	98%
Sucrose phosphate synthase	<i>cpSPS1</i>	ABIM0101 1235.1	LG7 contig_1 1250	3490	13	<i>S. lycopersicum</i>	sps	NM_001246991.2	0.0	82%
				1043	13	<i>A. thaliana</i>	SPS1F	NM_122035.2	0.0	81%
	<i>cpSPS2</i>	ABIM0102 3167.1	LG7 contig_2 3201	3576	13	<i>A. thaliana</i>	SPS2F	NM_121149.3	4e-173	80%
	<i>cpSPS3</i>	ABIM0102 0295.1	LG1 contig_2 0322	3394	14	<i>V. vinifera</i>	LOC100 232974	NM_001280928.1	3e-148	74%
				3469	15	<i>A. thaliana</i>	AtSPS4F	NM_001036532.2	8e-112	79%
	<i>cpSPS4</i>	ABIM0101 5610.1	LG6 contig_1 5630	3469	11	<i>A. thaliana</i>	SPS3F	NM_100370.2	5e-178	80%
	<i>cpSPP1</i>	ABIM0102 3303.1	LG4 contig_2 3337	1356	8	<i>A. thaliana</i>	SPP1	NM_104020.3	2e-45	78%
	<i>cpSPP2</i>	ABIM0100 9470.1	LG1 contig_9 483	1611	9	<i>S. lycopersicum</i>	LOC543 710	NM_001247754.1	8e-38	73%
				1844	10	<i>Vitis vinifera</i>	LOC100 258115	XM_010654940.1	9e-51	76%

¹/ Identity

Figure 3. Relative expressions of 11 sugar synthesis-related genes in leaf and fruit tissues of 'RB2' (dark bar) and 'Sunrise Solo' (light bar). The expression values of each gene were normalized with means of the Cq value of reference genes (*18s rRNA*, *CYPC* and *TBP2* genes). * indicates significant difference at 0.05 by T-test. The error bars represent mean value $\pm$ standard error.

Sugar-related gene identification and gene expression analysis

The comparison of sugar-related genes from various crops led to the identification of 18 candidate genes as shown in Table 2. The majority of the candidate genes were homologous to genes that code for several types of invertase enzymes including; two cell wall invertase genes (*cpCWINV1* and *cpCWINV2*), three neutral invertase genes (*cpINV1*, *cpINV2* and *cpINV3*) and two acid invertase genes (*cpAINV1* and *AINV2*). Also identified were five putative genes related to sucrose synthesis (*cpSUS1*, *cpSUS2*, *cpSUS3*, *cpSUS4* and *cpSUS5*) and six putatively involved in sucrose phosphate synthesis (*cpSPS1*, *cpSPS2*, *cpSPS3*, *cpSPS4*, *cpSPP1* and *cpSPP2*).

Preliminary PCR amplification was performed for all target sequences and 11 primers pairs amplified the target sequences in almost all of the tissue types assessed (Figure 2). These were then used for qPCR analysis. The sucrose phosphate synthase and sucrose synthase family genes were expressed in the leaf tissues of both genotypes, which agreed with observation of higher sucrose concentrations in leaf samples. In fruit tissues, the expression levels of sucrose phosphate synthase and invertase genes slightly increased during fruit development and ripening in contrast to the changes of sucrose synthase genes (Figure 3).

The relative expression of sucrose phosphate synthase genes in comparison to the steady expression of the three reference genes indicated there is a correlation with sugar content in papaya fruit. Higher expressions of *cpSPS1*, *cpSPS2*, *cpSPS3* and *cpSPS4* were observed in 'Sunrise Solo' than in 'RB2'. Among the sucrose phosphate synthase genes assessed, the differential expression of *cpSPS2* was found in all tissues with 0.5 to 3-fold higher expression in 'Sunrise Solo' than in 'RB2'. The maximum observed expression of *cpSPS2* was in mature 'Sunrise Solo' fruit (120 DAA).

Increases in the expressions of *cpCWINV1* and *cpAINV2* were observed in both genotypes during the fruit ripening stages. These were expressed 0.25 to 1-fold more in 'Sunrise Solo' than in 'RB2'. Differential expression of sucrose synthase genes between the two genotypes was also detected, predominantly in *cpSUS4* within leaf and fruit tissues at an early stage of development (30DAA), again with higher expression in 'Sunrise Solo' than 'RB2'. During the ripening stage (120 DAA to colour break 100%), the expression of *cpSUS4* reduced from 1.75 to 0.5 fold in 'Sunrise Solo' and from 1.25 to 0.5 fold in 'RB2'.

Large differential expression was detected for members of the sucrose phosphate synthase gene family with expression of *cpSPS1*, *cpSPS2*, *cpSPS3*, *cpSPS4* in 'Sunrise Solo' up to 0.5-3 fold more than in 'RB2' (Figure 3). The expression patterns of the sucrose phosphate synthase genes assessed were similar to those previously observed in pineapple (Zhang et al. 2012) and apple (Li et al., 2012). The expression patterns of invertase genes detected in this study during ripening stages is also similar to the patterns of invertase enzyme activity previously observed in papaya fruit (Zhou and Paull, 2001; Gomez et al., 2002).

CONCLUSION

Sugar content in papaya fruit was increased as fruit develop towards full ripening. The change of sugar composition was varied in different genotypes and tissue types. In this study, we observed higher sugar content, especially sucrose, in all tissue types of papaya genotype 'Sunrise Solo' compared to genotype 'RB2'. The differential expression was found in five candidate genes (*cpSPS1*, *cpSPS2*, *cpSPS3*, *cpSPS4*, *cpCWINV1* and *cpAINV2*) comparing between low and high sugar-content genotypes by the higher expression was detected in 'Sunrise Solo'. The expression of *cpSPS2*, *cpCWINV1* and *cpAINV2* indicated their major role related to sugar content in papaya. This indicates that sugar synthesis in papaya is complex, dependant on genotype and growth stage (Nakasone, 1986) and is likely controlled by multiple genes. In the future, molecular mapping and QTL analysis can be used to identify the loci of sugar synthesis-related genes and to further assessed their association with sweetness in papaya.

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