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Trails to Explain the Variations in Fruits Pulp Color of *Citrus unshiu* Based on SSR Marker Associated to Proton Pump

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Abstract: At the cell level, anthocyanins are synthesized in the cytoplasm and accumulated into the vacuole. Hitherto, poor knowledge of these compounds limited factors for transportation through the tonoplast. Recently, next-generation sequencing of the citrus genome allowed us to identify adaptive mechanism genes due to expression with a high sequencing of genes encoding proteins Cs7g7300, Cs7g07300.1 and Cs7g07300.2. One of the adaptive mechanism expression genes for H+ATPases controlling acidity resulting colour expression was selected. In the present study, we tend to focus to study the identification and validation of DNA based marker was carried out for proton pump in *Citrus unshiu* based on nine SSR primers which were specific to evaluate the ability of the initial material for Mandarin fruits to distinguish whether acid-base proton pump expressed normal fruits and red pulp fruits using a total of 18 DNA samples divided into two groups, each group contain nine samples from normal Satsuma fruits and the second group contains nine samples from red pulp. The result presented 12 polymorphic loci and 19 alleles, with 1.87 alleles per locus with genetic diversity of 0.38. The genetic coefficient between these two groups (normal pulp and red pulp) was 0.36 on average, ranging from 0.22 to 0.80. According to cluster formation, these samples could be divided into four sub-groups. Furthermore, the locus association will be more beneficial to select the excellent plant materials through the marker-assisted selection and for further genes research in the future. Generally, the variations in pulp colour from yellow to dark yellow or red due to the level of acidity which controlling by the proton pump.

Key words: Satsuma Mandarin · Proton Pumps · Red Pulp · DNA Markers · SSR

INTRODUCTION

Citrus cultivated in Egypt since ancient time. Citrus cultivation area has increased rapidly, this area reached to (204095 ha) representing about 29% of the total fruit area (700854 ha), while, the total produced area reached about 175734 ha, produce 4.27 million metric tons approximately, from which around 1.34 million tons are exported. Therefore, Egypt ranking as the sixth most significant producer of orange throughout the world after Brazil, China, US, EU and Mexico [1, 2].

Mandarins group: Mandarins group is consisting of several intergeneric species and interspecific hybrids such as mandarin, Satsuma, Clementine, willowleaf, tangors and tangelos. Mandarins group is the second larger cultivated group after sweet orange. There are different mandarins varieties cultivated in Egypt and the total cultivated area reach to 47646 ha.

Meanwhile, the fruitful area about (44553 ha) produced 982790 t and there are three main areas for mandarin cultivation in Egypt; the first one is Nubaria district (20363 ha), then North Sinai (3386 ha) and Ismailia (3006 ha), the fruit mature from December to March approximately. Satsuma mandarin (*Citrus unshiu*): Satsuma fruit is seedless medium to small sized fruits and easily peel with loose skin and has a moderately sweet flavor, rind and flesh are orange. It is one of early ripen fruits about November and December [3].

In a few citrus cultivars, the high acid content characteristic of most citrus fruit is absent. In such varieties, the lower acid content is related to a higher vacuolar pH. As an example, the acid less sweet lime

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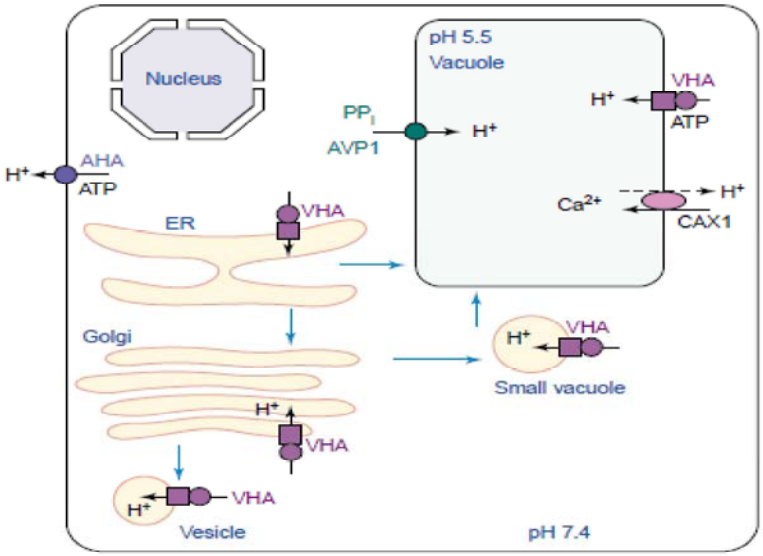

Fig. A: The proton pump mechanism, especially a pump with various functions. Vacuolar-type H+-ATPase (VHA) has been localized to vacuoles, endoplasmic reticulum (ER), Golgi, little vacuoles, vesicles and therefore the plasma membrane in plants. Acidification of intracellular compartments provides energy for the release or uptake of many ions (e.g., Ca2+) and metabolites. The ensuing changes in pH, ion gradient or activities and osmotic potential of these compartments conceivably affect diverse cellular functions **[5]**, including protein sorting, growth and guard cell signaling. Abbreviations: AHA, Arabidopsis plasma membrane H+-pumping ATPase; AVP1, vacuolar H+-pumping PPase; CAX1, Ca2+/H+ antiporter.

(*Citrus limmetioides*) accumulates a fraction of the citric acid present in its close relatives Persian and Key limes (*Citrus aurantifolia*) and lemons solely and is characterized by a vacuolar pH of ≈5.0 [4]. The vacuolar H+-ATPase is so subjected to distinct regulative surroundings in sweet limes than in acidic limes and lemons.

V-ATPase is rising as a pump with various and V-ATPase has been localized to vacuoles and other membranes of the secretory system, together with the endoplasmic reticulum (ER), Golgi and small vesicles, besides, the plasma membrane as shown in Fig. A [5, 6].

The proton gradients are significant for the transport of various ions and solutes across the various membranes in plant cells. Many developmental processes need a tightly controlled necessary proton gradient across the cellular membranes. The discovery of acid solutions stimulates and elongation of cells leads to the hypothesis that auxins may act by acidifying the cell wall. This acidification might be due to direct or indirect stimulation of the electrogenic proton pump outwardly directed, which are present in the plasma membrane of plant cells. The H+ -ATPases belong to a large family of pumps type plasma membrane, P-type ATPases, all of that energized by supplement of ATPs and kind a phosphorylated aspartyl transformation product throughout the reaction cycle. P-type H+-ATPases are kind of active transporters that utilize ATP to transport H+ across the plasma membrane. As a result, which generates an electrochemical gradient that creates energizes channels along with co-transporters [7-9].

Many substances i.e., transcription factors, plant hormones and microRNA are reported to involve within the regulation of fruit ripening method. Recently, a study of tomato fruit ripening known a fruit explicitly expressed enzyme b-D-N-acetylhexosaminidase (b-Hex) and verified that RIN might directly or indirectly regulate the transcription of b-Hex through recent development for understanding the molecular mechanism and therefore the proton pumps structure of action

In this publication we draw upon validation of molecular markers developed from one of three genes encoded Cs7g07300, might code a plasma membrane H+ATPase which has two transcripts as atrial to explain the variations in fruit pulp colour of *Citrus Unshiu* and the relationship between acidity and proton pump which controlling the fruit pulp colour.

MATERIALS AND METHODS

Plant Material: *Citrus unshiu* (Satsuma Mandarin) experimental field on the experimental station of Agriculture College, Benha University, Egypt (30° 27' 38" N/31° 11' 15" E), using total 18 samples divided to two groups, the first group contains nine samples from normal *Satsuma* fruits and the second group provide nine samples from red pulp which were collected at mature stage through three mature fruits per each tree, three trees on each group and two groups with different pulp colours were collected after dissection for peel and pulp. Pulp was ground on three replicates into fine powders using liquid nitrogen and stored at -80°C until use.

Determination of Titratable Acidity: Total titratable acidity was determined according to the conventional method of Sanchez-Moreno *et al.* [10]. Ten ml of fruit juice was diluted with 50 ml of distilled water and titrated against 0.1M NaOH and repeated twice for each replication and recorded the titre value as an average for each replicate. Total titrable acidity was calculated using the formula:

$$\text{Grams/ litre acid} = \frac{\text{Titre x acid factor* x 100 x 10}}{\text{10 (ml juice)}}$$

*The acid factor is 0.0064 for citric acid, which is the dominant acid in citrus fruits [11].

Total Nucleic acids Extraction: Total genomic DNA was extracted from the pulp of 18 samples nine from each type normal Satsuma fruits and red pulp fruits according to LIU Yong Zhong *et al*. [12], an efficient protocol for genomic DNA extraction from Citrus species as follow:

- Add 10 mL of extraction buffer and 10 mL of acid phenol in a clean, autoclaved 50mL centrifuge tube. Each sample with two tubes and place them into ice.
- Transfer 2~ 3 g ground powder s into tube prepared before and mix violently at least 2 min,

Then add 10 mL chloroform-isoamyl alcohol (24: 1) and vortex for 5 min.

- Let the sample stand at 4 for 5 min and then centrifuge e for 20~ 30 min at 5 000 r/ min at 4°C.
- Collect the upper supernatant of two tubes to a DEPC treated 50 mL tube and then re-extract the supernatant with an equal volume of chloroform-isoamyl alcohol (24:1).
- Add 1/ 3 volume of 8 mol/ L LiCl to the supernatant, mix well by inversion and store it at 20°C over three h to overnight.
- Centrifuge for 15 min at 16 000 r/ min at 4°C. The RNA pellet s are washed one time with an appropriate volume of 75% d ethanol and then dried in the ventilated cabinet for 5 min.
- Dissolve the RNA pellet in 800 µL of TESAR. Then add 800 µL of aq/ CTAB and 800 µL of bu/CTAB. Vortex for 2 min.
- Transfer the recovery RNA solution into two DEPC-treated 1. 5mL microfuge tubes (1. 2 mL per tube). Then spin in a microfuge at maximum speed at room temperature for 5 min to resolve the phases.
- Collect the upper butanol phase of two tubes to a new one. 5mL microfuge tube and then add 300 µL 0. 2 mol/ L NaCl to the tube. Vortex for 30 s and spin at maximum speed for 5 min at room temperature.
- Carefully transfer the lower aqueous phase to a new microfuge tube. Re-extract the upper layer with 300 µL of 0. 2 mol/ L NaCl and combine the lower aqueous phase with the first aqueous phase. The RNA is now sodium salt and is soluble in the aqueous phase.
- Add the same volume of chloroform-isoamyl alcohol (24:1) to the combined aqueous phases, vortex for 30 s and then centrifuge at maximum speed at 4 for 5 min.
- Transfer the upper aqueous phase to a new tube. Add 1/ 10 volume of 3 mol/ L sodium acetate and 2. 5 volume of cold ethanol to the aqueous phase. Mix gently well and store it at - 20°C for at least one h.
- Precipitate the RNA through centrifugation at maximum speed for 20 min at 4°C.
- Dry the pellet in a super clean bench for 10 min and resuspend in 100 µL of DEPC-treated water H2O.
- RNA quantification was performed spectrophotometrically at wavelengths of 230, 260 and 280 nm on ND1000 UV-spectrophotometer (Thermofisher Scientific Pvt. Ltd.). To confirm the RNA quality, the RNA was electrophoresed on a 1. 0% agarose gel.

Then reverse Tran-scripted procedure was carried out according to the protocols of Fermentas, to the gDNAs 1and gDNAs2 for each sample (18 samples nine samples from the yellow pulp fruits and other nine samples from red pulp fruits) against Nine SSR primers which were designed to test the genes related to protein pump according to Chandrika and Richard [13] (Table 1) on the studied samples.

Table 1: Nine SSRs primers related to acid-base proton pump expressed normal fruits and red pulp fruits [13].

Primer no.		5 to 3 end sequence	Annealing TM°C
Sh1	F	TGACTGTTGACCGAAACCTG	60°C
	R	CTCCTGCAGCCTCTTAACAA	60°C
Sh2	F	GATGCATATGGTCCCATCAGT	51°C
	R	GCGAAAAAGGGAGCAAAAAC	58°C
Sh3	F	GCAATGCTTACAGGTGAGTT	55°C
	R	AGTTGCGTGGTCTGGTTTTC	60°C
Sh4	F	AAAGATGGCGCAAAACCAG	56°C
	R	TCTTCCACCAGAAACCAAGC	60°C
Sh5	F	CGTGAAATGGCATCAAGGTA	58°C
	R	ATTGCCGCACAGTTCTTCA	56°C
Sh6	F	TCTTGCTGTTGTCCCCAAGT	60°C
	R	TGGCAAGATCTTCTCCACAA	58°C
Sh7	F	CAGAGATGGCACGCAAGTTA	60°C
	R	GGGTAGGTGTCACGCACTTT	62°C
Sh8	F	TTTGAGGCAAGCTGTTGTTG	58°C
	R	ATGGACAGGTTCATAGACGAC	58°C
Sh9	F	AGGCTGGTGGCTTTCTTGTA	60°C
	R	ACAAGTAGTGTCCCGGCATC	62°C

DL2000 plus Marker and Primer Design using Software: DL2000 plus marker was conducted according to the manual of DL2000 plus marker analysis. Oligonucleotide primers designed using Primer-5 software (Table 1) and synthesized by AnyGene (Paris, France). Genome sequences were obtained from Nucleotide databases at NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm [14].

PCR Conditions: PCR amplifications were carried out in 20iL final volume, using thermal cycler (PERKIN ELMER MODEL 480). The PCR reaction mixture contained 0.5-il template DNA concentration (20 ng), double distilled water 11il, 2 X master mix 12.5 il (Ready Times), Primers forward and reverse 0.5il. The PCR conditions were repeated on 35 cycles with preheating for 5 min at 94°C, Denaturing for 30sec at 94°C, annealing for 45sec at 58°C and extension for 2 min at 72°C and final extension was done for 4 min at 72°C. The confirmation of all amplifications was performed by running PCR product (6iL) on agarose gels (3%).

Data Analysis: Bands were binary scored presence (1) or absence (0) characters to assemble the matrix of the DL2000 plus marker and the number of total bands (TB), the number of specific polymorphic bands (PB) and also the percentage of specific polymorphic bands (PB%) were calculated [15, 16]. The parameters of genetic diversity were calculated using POPGENE 3.2 software.

RESULTS

Analysis of Titratable Acidity: The total titratable acid content of mature fruits presented in Fig. (1). the harvested fruits, which have red pulp (abnormal), showed consistently higher acid content than normal (yellow) fruits. The TTA content varied between 0.74 to 1.09% acid and 0.82 to 1.22% acid in the red pulp fruits and yellow pulp fruits, respectively. Significant differences ($P<0.05$) existed in the TTA content between abnormal and normal fruits start from yellow to dark yellow (red) pulp fruits; it means the fruits divided into two types.

DL2000 plus Marker Profiling: Three DL2000 plus markers were used and exhibited polymorphic in nine fruit samples. All of these loci were successfully amplified. The nine specific SSR primer pairs generated 12 alleles in all individuals (Fig. 4). Distribution of alleles in all fruit samples for all normal and abnormal fruit colour was normal distributed; suggesting that dark yellow (abnormal) is stepwise in the fruit samples. The number of alleles produced by different primers was one; except in Primer sh1, sh2, sh3, sh4 and sh9, which produced two alleles with an average of 1.9 alleles per primer.

Genetic Analysis: Comparative analysis of 18 fruit samples revealed moderate variation. A pair-wise similarity among the fruit colour ranged from 0.16 to 0.75, with an average of 0.45 based on band matrix data. Maximum similarity (0.62) was observed between the

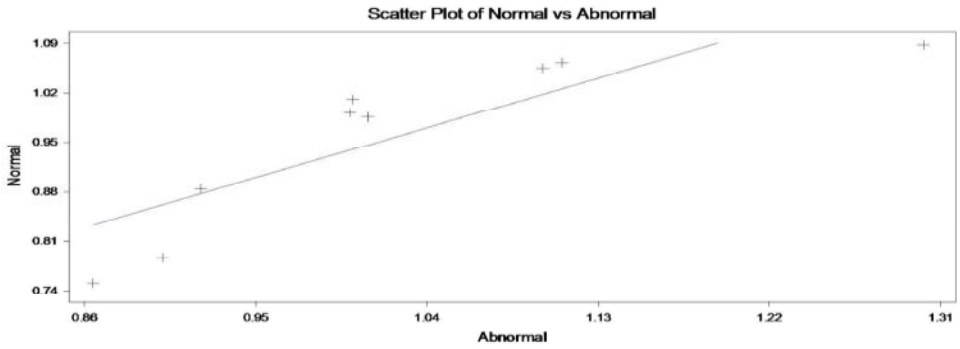

Fig. 1: Scattered plot showing titratable acidity level of two types (normal and abnormal)

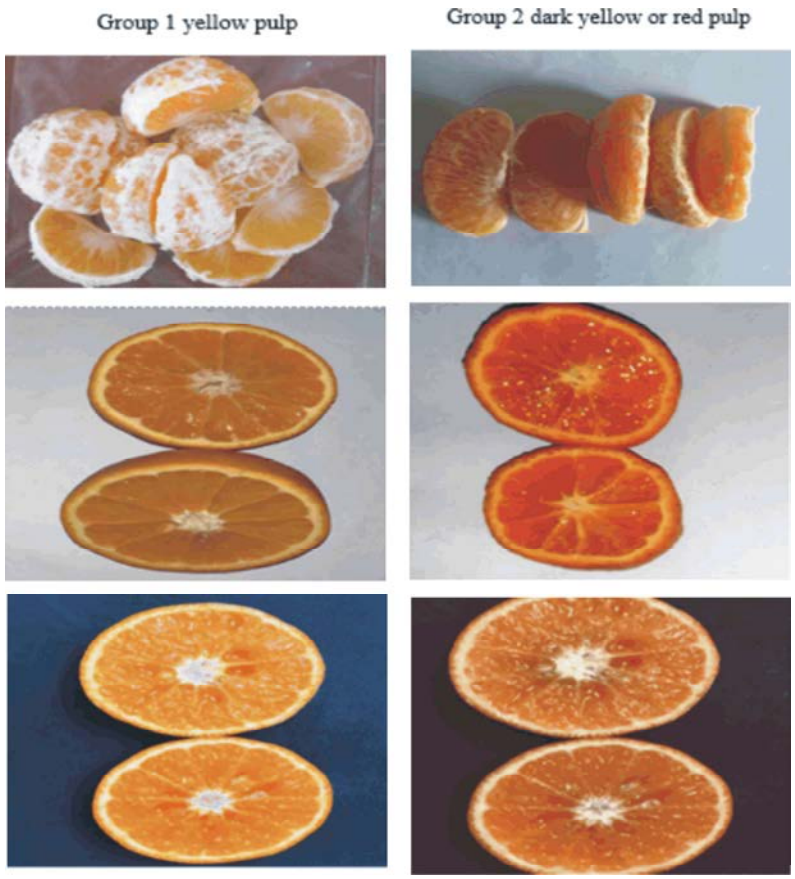

Fig. 2: Different fruit pulp colour from different samples during the same mature stage at harvest time (group 1 fruits, which have yellow pulp and group 2 fruits, which have dark yellow or red pulp).

normal fruit colour cultivars, while minimum (0.25) was observed between fruit cultivars (Fig. 2). A dendrogram was generated based on bands Matrix data of PCR amplification, which separated into four major clusters, which were created according to 18 fruit samples under investigation. The clustering data divided into two main clusters and the first cluster divided into two sub-clusters. The first central cluster gathered 12 samples together into two sub-clusters. The first sub-cluster consisted of six samples of normal and abnormal cultivars and presented sample no (10) alone and far from the other five samples (11, 12, 1, 2 and 3) on the same sub-cluster. In addition, the second sub-cluster contained six samples of normal and abnormal fruits and it showed samples four and six together while samples (5, 13, 14 and 15) were picked together.

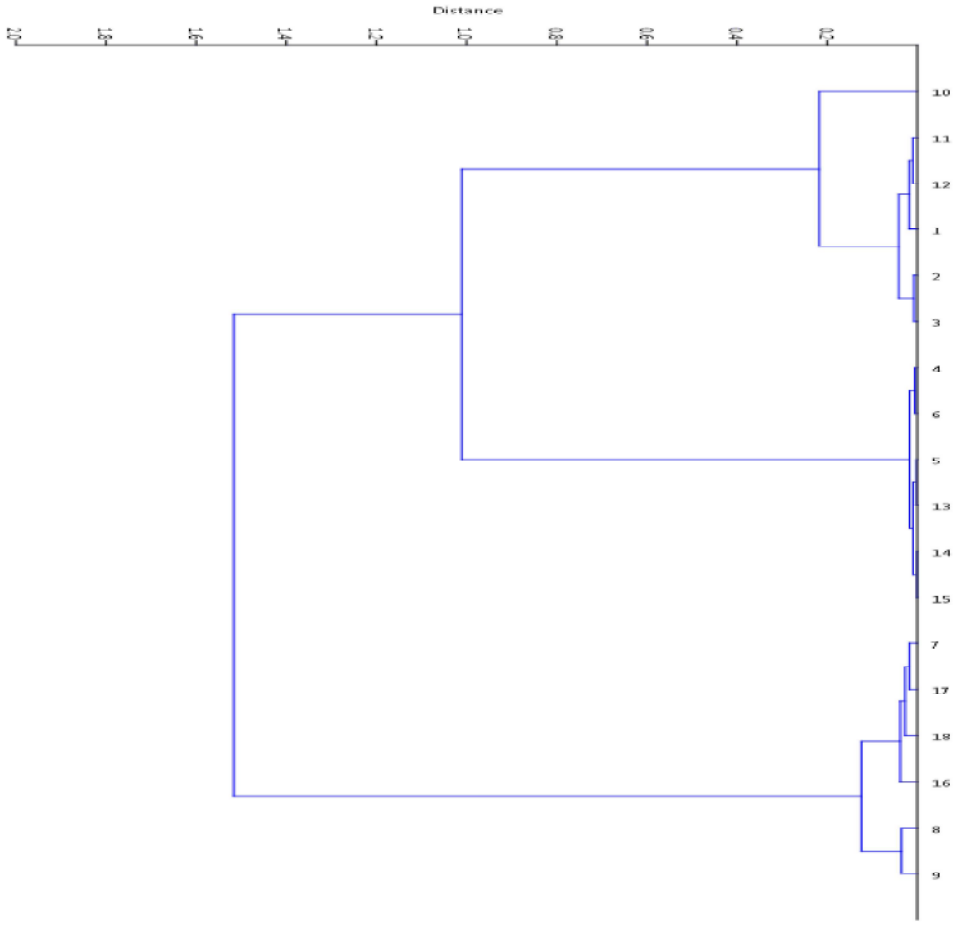

Fig. 3: Cluster analyses of 18 samples (nine normal yellow pulp gDNAs and nine abnormal red pulp gDNAs samples)

(biplot) (Screen plot)

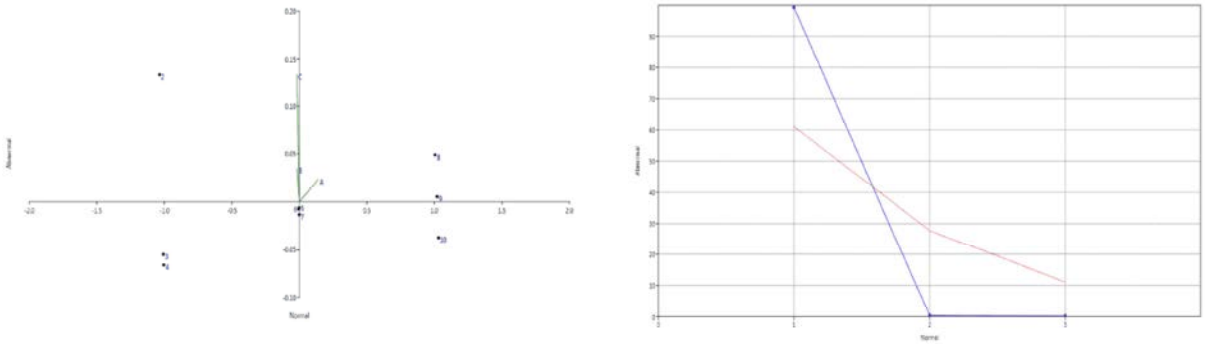

Fig. 4: Bi-plot and scree plot showing sample distribution for acidity patron

On the other hand, the second main cluster consisted of the remaining six samples; fruits No. 7, 17, 18 and 16 appeared on one sub-cluster and the fruits No. nine and eight visualized on one group together. As a conclusion, the clustering data presented both nine normal yellow pulp gDNAs and nine abnormal red pulp gDNAs samples from different fruits having the same colour on one main group. The abnormal fruits (with red pulp) were the most distinct cultivar between two cultivars separated with a value of 0.19. The second cluster was abnormal cultivar highly similar, showing similarity of 0.09.

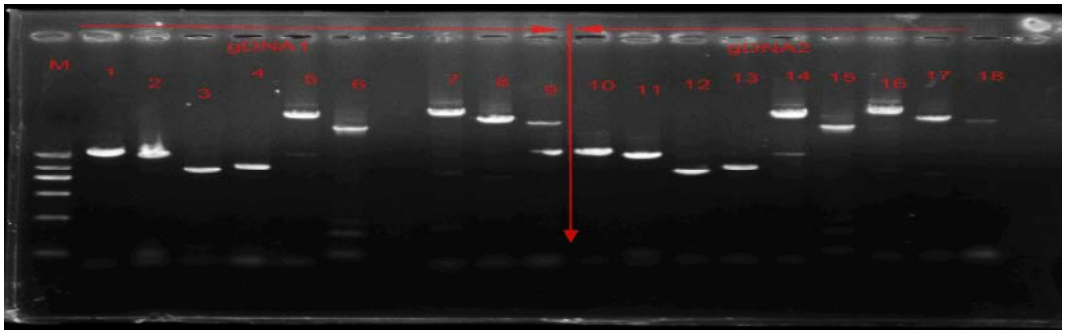

Fig. 5: Agarose gel pattern showing different bands for a total of 18 samples (nine normal yellow pulp gDNAs1 and nine abnormal red pulp gDNAs2 samples)

Plot Approach (biplot and Scree plot): Clustering approached also showed the two groups, which were found almost dissimilar to the other, but the most similar among colour groups as in clustering pattern of POGEN dendrogram (Fig. 2). The cultivar normal group constituted one group and abnormal another.

DISCUSSION

The results obtained from the different analysis are highly promising and demonstrated the viability of using DNA markers to inspect the fruit colour at the maturity stage in normal and abnormal fruit colours of *citrus unshui*. Most of the normal fruit samples are differentiated from the abnormal samples selected based on their acidity character, which indicated pale colour Satsuma of the pulp. This variation no double pointed the proton pumps in vacuolar cavity fruit developed the Satsuma colour of the fruit. The literature also supported our findings that fruit colour is a very complex trait which is controlled by chemical, biochemical and physicochemical mechanisms [17]. In general, the vacuolar H+ATPase (V-ATPase) and H+pyrophosphatase (V-PPase) maintain the pH acidity of vacuoles [18]. Additionally, the plasma membrane H+ATPase (PM-ATPase) may be a vital regulator of the cytosol pH [19].

Regulatory gene expression contributes to several physiological changes that provide a lot to fruit colour development [20]. Fukada-Tanaka [21] reported that the involvement of the gene for a Na+/H+ exchanger (InNHX1) [21-23]. The H1-ATPase has also been proposed to play a direct role in the regulation of growth and development. Citrate accumulation in the vacuole of plant cells is determined primarily by the vacuolar ΔμH+ [24-26]. In citrus juice cells, although a ΔμH+-independent transports a portion of the accumulated citrate, ATP-dependent uptake mechanism [24, 27].

In agreement with the previous characterization of lemon, fruit V-ATPase [28], formation and maintenance of the high ΔpH across the tonoplast of highly acidic citrus juice cells are made possible by the presence of a specialized VATPase and a tonoplast impermeable to H+. The low vacuolar pH of ≈2.0 and a trans-tonoplast ΔpH of concerning five units are sufficient to drive the uptake of 325 mM citric acid from a cytosolic citrate concentration of <10 nM [24]. This capacity for vacuolar hyper acidification is absent from citrus fruit and different vegetative cells with a higher vacuolar pH. Therefore, the novel vanadate-sensitive V-ATPase described for lemons and acid limes appear to be a characteristic of hyper acidifying tissues and not a property of citrus juice cells generally. The study can facilitate the progressive farmers to confirm consistent quality in term of pulp and flesh colour whereas packing the fruits in batches. Also, will facilitate the fruit breeders to select the early genotypes supported their colour and will these genotypes be helpful within the citrus crop improvement program.

CONCLUSION

The nine expressing SSR primers enabled us to discriminate all the two groups for studying the acid-base proton pump expression among the improved groups, SSR patterns illustrated that there are bands appeared in the normal group samples Satsuma fruits. However, other bands were present in abnormal red pulp group samples indicated to the direct relationship with acid-base proton pump expression, which reflects the genetic background of gene expression. On the other hand, our studies showed that the vacuolar H+-ATPase metabolism linked with molecular mechanism likely to drive them. It conjointly showed that H+-ATPase metabolism factors have an effect on the acidity of fruit by functioning on varied cellular devices. This molecular

data, as a tool for a model as the parameter, might advance our understanding of the response of acid accumulation to genetic control, which explained the variations in pulp colour of *Citrus unshiu*.

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