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Engineering Papaya Plants with Improved Fruit Shelf Life by Ripening related genes through TILLING Approach

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Abstract:
Fruit ripening and softening are key traits that have an effect on food supply, fruit nutritional value and consequently, human health. Since ethylene induces ripening of climacteric fruit, it is one of the main targets to control fruit over ripening that leads to fruit softening and deterioration. The characterization of the ethylene pathway in Arabidopsis and tomato identified key genes that control fruit ripening.

Introduction

Papaya is an important fruit crop in the India and in other ASEAN countries. Its greater production and export potential are hampered by the papaya ring spot virus (PRSV) disease and relatively short life. This research finding addresses the latter problem with the objective of developing papaya mutant varieties with delayed fruit ripening trait, thus, improved shelf life. In climacteric fruits such as papaya, ethylene controls the rate of ripening (Mardas, *et al*. 2016). One approach to delay fruit ripening which has been employed in other fruits with success is through the TILLING Technology in which genetic modifications like point mutations or INDELS (Insertions and Deletions) of plant gene ACC Synthase and ACC-Oxidase involved in ethylene production during the ripening process. Altered the ripening-related ACC synthase genes from papaya cv Arka Prabhath gynodioecious variety developed by IIHR. The development of delayed ripening phenotypes via TILLING (Reverse genetics) technology will produce papaya mutant lines with better postharvest and transport characteristics that will be reflected in fruits of consistent superior quality and therefore better market value. The DNA Sequencing was done to know the exact position of the gene mutation in some mutant lines (Ramesh, A.N.*et al.* 2019)

Methodology/ Principle finding

An experiment on mutation was carried out using gamma radiations of different doses *viz*., 50, 100, 250, 500 and 750 Gy mainly to induce variability in mutant progenies of Cv. Arka Prabhath. The LD_{50} was found to be 500 Gy for gamma radiations, above which there was a maximum lethality in Cv. Arka Prabhath after mutation induction(Ramesh, A.N.*et al.* 2021). M_1 populations of papaya were selected and forwarded to M_2 based on particularly outstanding in vigor with medium dwarf stature, bearing the first flower at a height of 50-60 cm from the ground, improved fruit quality and disease

resistance when compared to control plants, African type, Arka Prabhath type, highly terratogenic type, Orange red group mutants were selected and assigned into families. Molecular characterization of M_2 mutant plants was performed by using freshly matured leaves, free from disease and developmental deformities were used for DNA extraction and TILLING work was initiated by using ACC-Synthase gene specific HRM (High resolution melt curve) primers to know the extent of variation between the mutant lines and shelf life studies by down regulating the expression of ACC-Synthase or by analyzing point mutations like insertions and deletions by sequencing for the some of the mutant lines(Li, et al. 2016)

Exon sequences of ACC-Synthase gene

>Exon1
ATGGTGCTAATGTTGAGAAATCAAG
AGCTGTTGTCCAAGATTGCAACCAG
CA
ACGGACATGGCGAGGACTCTCCCTA
CTTTGATGGGTGGAAAGCATACGAC
AG
TGACCCTTTTCATCCTACACAGAAT
CCAGAAGGAGTTATACAGATGGGC
CTTG
CAGAGAATCAG
>Exon2
CTTTGCTTTAATTTAATTCACGAGTG
GCTGCTGAAAAACCCAGAAGCCTCC
AT
TTGTACAGCACAAGGAGCAGCTGA
ATTCAGAGATATAGCTATCTTTCAA
GAT
TATCATGGCTTGGCTGAATTCAGAG
AGG
>Exon3
CTGTTGCAAAGTTTATGGGGAAAGT
GAGAAGAAACAGAGCTTCATTTGAC
CC
TGATCGGATTGTTATGAGTGGAGGA
GCAACTGGAGCTCATGAAATGATTG
CT
TTCTGTTTGGCTGATCCTGGCGATG
CATTCTTGGTTCCAACTCCTTATTAT
CCA
GGGT
>Exon 4
TTGATAGAGATTTGAGATGGAGAAC
GGGAGTCAAACTCATTCCAGTTGTC
TG
TGAAAGCTCAAACGATTACCAGATC
ACCATAGAAGCCCTGGAAGCTGCTT
AT
GAAACCGCACAAGAAGCTGACATC
AAGGTAAAGGGTTTGCTCATAACCA
ACC
CATCAAACCACTGGGAACAATTATT
ACCAAGGACACATTAGAAGCTCTAG
TC
ACCTTTCACCAACCACAAGAACATT
CATCTGGTGTGTGATGAGATATATG
CTG
CTACCGTCTTCAGCCAGCCCGAATT
CACCAGCATAGCCGAGATAATTGAA
GA
AGATAAAATTTGTTGCAATCGTGAT
CTCATCCACATCATTTACAGTTTATC
CA
AAGACATGGGATTCCCTGGATTTAG
AGTTGGCATTGTGTATTCATACAAT
GAT
GCAGTGGTGAGTTGTGCTCGTAAGA
TGTCGAGCTTCGGCCTAGTATCTTC
GCA
AACCCAGTATCTGATTGCATCCATG
TTAGCAGACGATGAATTTGTAGACA
AA
TTTATTGTAGAGAGCAGAAAGAGGC
TGGCAATGAGACATAGTTTTTTCAC
AC
AAAGACTTGCTCAAGTAGGCATTAA
CTGTTTAAAAAGCAATGCTGGTCTT
TTT
GTGTGGATGGATTTGCGTAGACTGC
TGAAAGAACAGACATTTGAAGCAG
AAA
TGGTGTTATGGAGAGTAATTATAAA
CGAAATGAAACTCAATGTATCTCCT
GG

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TTCGTCTTTCCACTGCTCAGAACCTG
GCTGGTTCAGGGTTTGCTTTGCAAA
CA
TGGACGATAAGACAACGGAAATTG
CACTGTCAAGAATCAAAACCTTCAT
GCT
TCAACATAAGGAAGCAATGGTGCCT
AAAAAGAAACTTTGCTGGCAAACTA
GT
CTTAGACTCAGCTTCTCCTCTCGCTA
TGAGGATATCATGAAGACACCGGGT
TC
GTTCATGTCTCCTCACTCGCCTATAC
CTCAATCACCTCTTGTTCGAGCCAA
GG
CATAG

HRM master mix reaction mixture of ACC-synthase gene

Reagent	Volume
Water	3µl (up to 16 µl)
2X Sensi FAST HRM mix	10 µl
10 µM Forward primer	1.0 µl
10 µM Reverse primer	1.0 µl
Template	5 µl
Final volume	20 µl

Aliquot to 96-Well Plate for HRM (High Resolution Melt Curve)

Procedure

1. Add 2 µL of the control template dilutions, the unknown library dilutions, or water to each well in a 96-well plate. Take care to pipette accurately into the wells as small variations will affect the assay.
2. Dispense 18 µL of the master mix into each well of the 96-well plates using a multichannel pipette. Take care to pipette accurately into the wells as variations in volume will affect the assay. Change tips for each new column.
3. Place the optical strip lids on the wells, taking care to avoid cross contamination and to
Avoid smudging the surface of the lids
4. Centrifuge the 96-well plate to 280 x g for 1 minute.(Liu, *et al* 2015).

Quantify by qPCR (Quantitative PCR)

Procedure

1. Place the 96-well plate in the qPCR machine in the correct orientation and clean the
optical lids with lens tissue to remove any dust before closing the qPCR machine lid.
2. Use the following thermal profile programme for HRM (Li, *et al.* 2013)

q PCR working condition for HRM.

Cycles	Temperature	Time	Notes
1	95	5 Min	Polymerase activation
40	95	10 Sec	Denaturation
	58	10 Sec	Annealing
	72	**15** Sec	Extension
	Acquisition 25 ^{0}C		

Results and Discussion:

Out of 315 mutant individuals finally 11 mutants were identified (R5P6, R13P10, R6P18, R3P14, R9P13, R8P18, R7P18, R18P19, R6P8, R7P4 and R4P16) to study quality and shelf life parameters like respiration and ethylene content by down regulating ripening related genes ACC synthase and
ACC oxidase using gene specific primers (Kushwah, *et al* 2010). The length of the ACC synthase and ACC oxidase are 1.6 kb and 1.4 kb. Out of 315 DNA samples majority samples were amplified and some were not amplified. Amplified samples were pooled in5X manner and HRM work was performed through qPCR. Some reports say ACC synthase is present only in matured fruits whereas ACC oxidase

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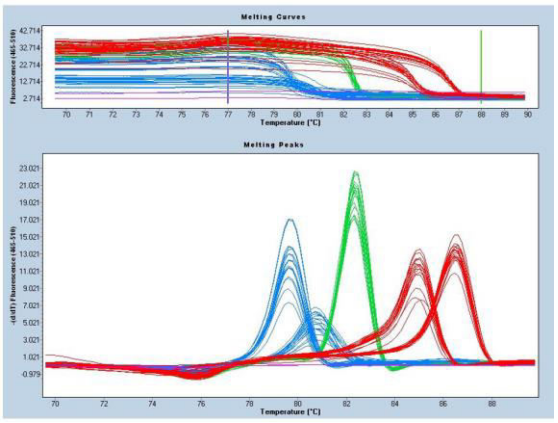
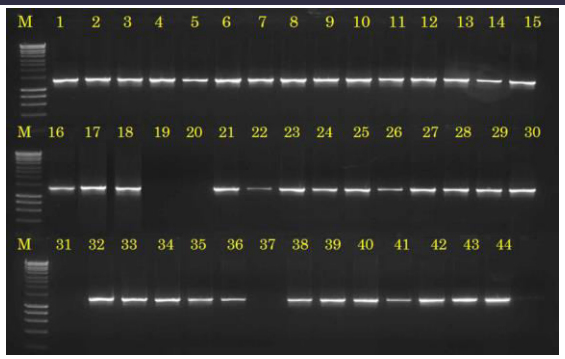
present in all stages of the fruit. Melting curves and melting peaks were obtained for all the amplified samples and the extent of variation was analyzed between the samples by comparing with control and non template control(Li, *et al* 2010).

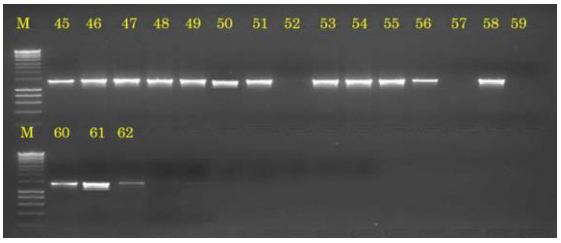


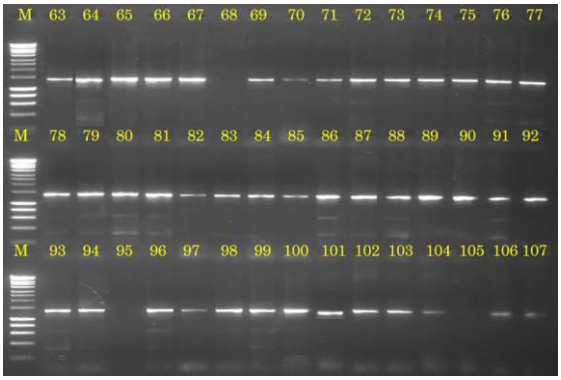
Graph depicting HRM Melting curves and peaks of all the samples with five ACC –synthase Primers.

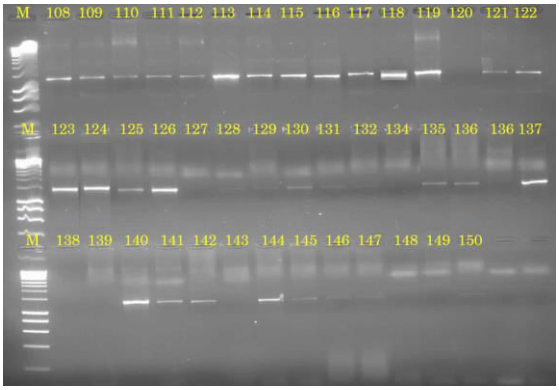
Sequencing of the PCR product or Mutant individuals

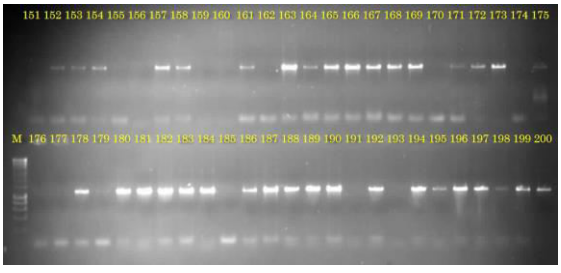
The DNA sequencing was done to know the exact position of the gene mutated in the sample and
information regarding the type of mutation either it may be SNP (Single nucleotide polymorphism), or INDELS(Insertions or deletions). Here 4 mutants sequence data are presented along with the control or non treated papaya. The HRM melting curves and melting peaks of ACC-Synthase were performed in qPCR.The data showed that, control sample or untreated (Gamma rays) DNA got dissociated fast and showed maximum melting peak or melting curve compared to all the mutant samples for all gene specific primers. Higher the Cp values lower the target sequence and lower the Cp values higher the target sequence of the genes(Laurena, *et al* 2002).











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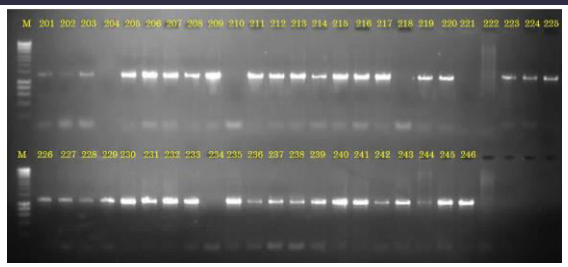

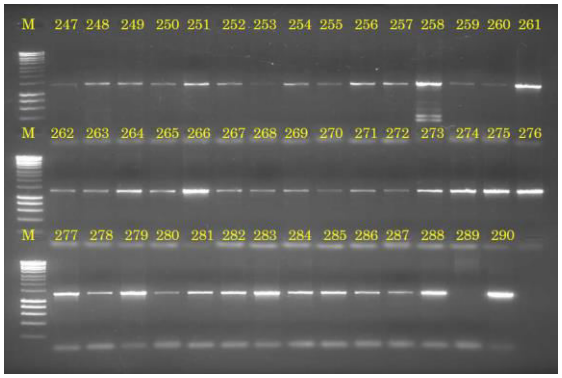

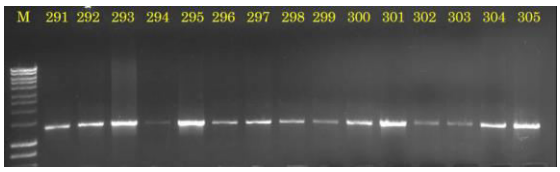

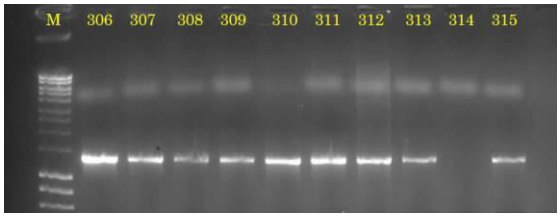

Gel pictures depicting DNA amplification of M_2 populations using ACC synthase HRM primers.

Sequence data of the castor leaf mutant: (R_7P_{18})

The castor leaf mutant had broad leaves and moderately resistant to PRSV and having good qualitative and quantitative characters. The HRM data on castor leaf mutant for ACC- Synthase showed that melting temperature of both mutant and control were 77$_0$C and 83$_0$C but fluorescence level detected was 11.881ct for the mutant sample and nearly21.881 for the control sample. The HRM data for ACC-oxidase showed that melting temperature for both the control and mutant were 77$_0$C and 83$_0$C as like ACC-synthase but fluorescence level detected was6.891ct for mutant and 21.891ct for control sample. The changes in fluorescence along with the melting of the ds DNA was measured using saturated binding dye and high accurate detection system (Plate1).

The results of the DNA sequencing indicate that the ACC synthase gene get mutated at 469 th position of the control and castor leaf mutant. SNP were observed by T/G transversions in control and castor leaf mutant. The DNA sequencing indicated that ACC oxidase gene gets mutated at 868 th position and884 th position through C/T and C/A nucleotides changes in ACC-oxidase of the control and castor leaf mutant. This mutation may be the one of the reasons for broader leaves and thicker cuticle and which prevented the aphids to pierce the viral particles.

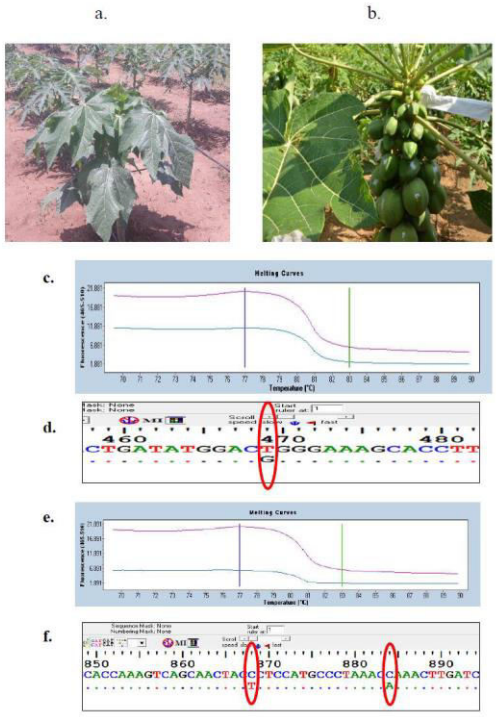

Plate 1: a) Castor leaf like mutant (R_7 P_{18}), b) Castor leaf fruits, c) ACC synthase gene melting curve of castor mutant (R_7 P_{18}), d) DNA sequence variation of ACC synthase gene in Castor leaf mutant (R_7 P_{18}), e) ACC-

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oxidase gene melting curve of castor mutant (R_7 P_{18}), f) DNA sequence variation of ACC-oxidase gene in Castor leaf like mutant (R_7 P_{18}).

Sequencing data of African type mutant (R_1P_2)

This line is having uneven fruit surface and a high yielder. The HRM data on African type mutant for ACC- Synthase showed that melting temperature of both mutant and control were 83^{0}C and 87^{0}C but fluorescence level detected was 32.633 for the mutant sample and nearly 37.633 for the control sample.

The HRM data for ACC-oxidase showed that melting temperature for both the control and mutant were 78^{0}C and 86^{0}C, but fluorescence level detected was 25.817ct for mutant and 35.817ct for control sample. The changes in fluorescence along with the melting of the ds DNA was measured using saturated binding dye and high accurate detection system.

The results of the DNA sequencing indicated that the gene got mutated at 324 th position of the control and African type mutant. SNP were observed by T/G in control and African type mutant for ACC-Synthase. The DNA sequencing indicated that the gene got mutated at 504 th position by inserting nucleotides by -/G for ACC-Oxidase of the control and African type mutant. (Plate 2)

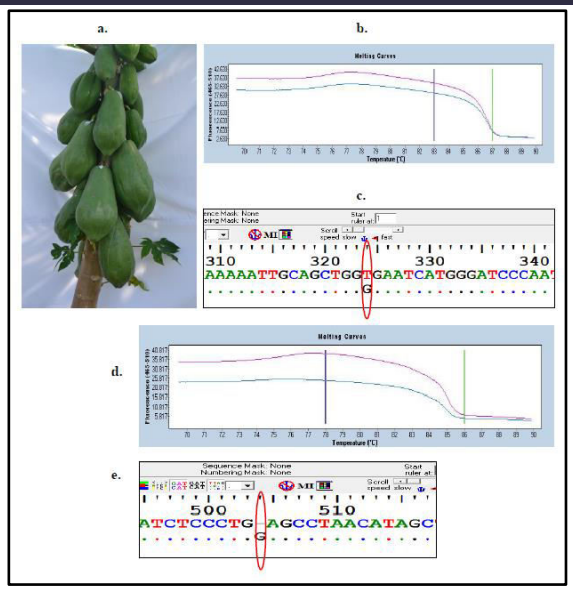

Plate 2: a) African type mutant (R_1 P_2), b) ACC-synthase gene melting curve of African type mutant (R_1 P_2) c) DNA sequence variation of ACC synthase gene in African type mutant (R_1 P_2), d) ACC-oxidase gene melting curve of African type mutant (R_1 P_2), e) DNA sequence variation of ACC-oxidase gene in African type mutant (R_1 P_2)

Sequencing data of female dwarf mutant ($R_{13}P_{10}$)

Female dwarf plant is highly robust and it bears round shape fruits and moderately resistant to PRSV. The HRM data on female dwarf type mutant for ACC-Synthase showed that melting temperature of both mutant and control were 77^{0}C and 83^{0}C but fluorescence level detected was 6.890 for the mutant sample and nearly 21.880 for the control sample.

The HRM data for ACC-oxidase showed that melting peaks temperature for both the control and mutant were 77^{0}C and 83^{0}C, but fluorescence level detected was 1.291ct for mutant and 6.891ct for control sample. The results of the DNA sequencing indicated that the gene got

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mutated at 469 th position of the control and female dwarf mutant. SNP were observed by T/G in control and female dwarf mutant for ACC-synthase. The DNA sequencing indicated that the gene got mutated at 324 th position by changing nucleotides by T/G for ACC-Oxidase of the control and female dwarf mutant. (Plate 3).

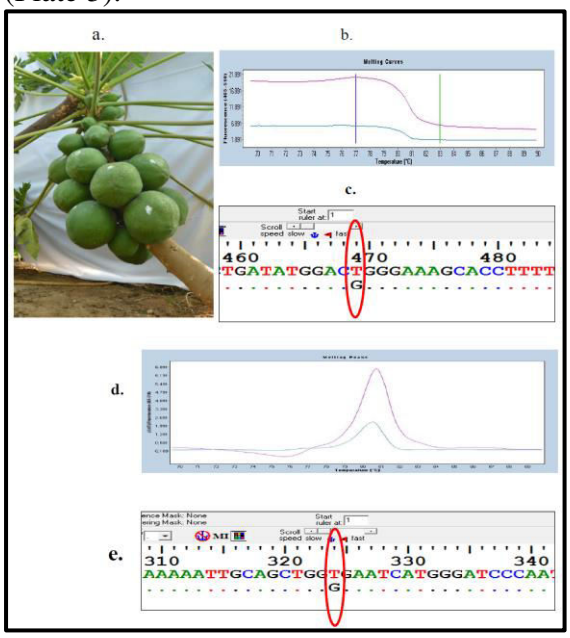

Plate 3: a) Female Dwarf mutant (R_{13} P_{10}), b) ACC-synthase gene melting curve of Female Dwarf mutant (R_{13} P_{10}), c) DNA sequence variation of ACC synthase gene in Female Dwarf mutant (R_{13} P_{10}), d) ACC-oxidase gene melting curve of Female Dwarf mutant (R_{13} P_{10}), e) DNA sequence variation of ACC-oxidase gene in Female Dwarf mutant ($R_{13}P_{10}$)

Sequencing data of ultra dwarf mutant (R_8P_{20})

The ultra dwarf mutant bears the fruits from 50-60 cm above from ground level and it bears perfect hermaphroditic fruits. The fruits have attractive pulp colour, sweet in taste and having good shelf life, moderately resistant to biotic and abiotic stresses. The HRM results of ultra dwarf mutant showed that melting temperature of both mutant and control were 760C and 830C but fluorescence level detected was 19.77ct for the mutant sample and 29.77ct for the control sample.

The HRM data for ACC-oxidase showed that melting peaks temperature for both the control and mutant were 750C and 870C, but fluorescence level detected was 43.731ct for mutant and 53.731ct for control sample. The results of the DNA sequencing indicated that the gene got mutated at323 th position by T/G, 330 th by T/G and 335th by T/C of the control and ultra dwarf mutant for ACC-synthase.

The DNA sequencing data indicates that the ACC-oxidase gene got mutated at 395 th position by T/G transversions,402 th and 404th positions by G/A transitions for control and ultra dwarf mutant. In this mutant both transition, transversion and multiple mutations were observed. So this may be one of the reason for the ultra dwarf stature of the plant. (Plate 4).

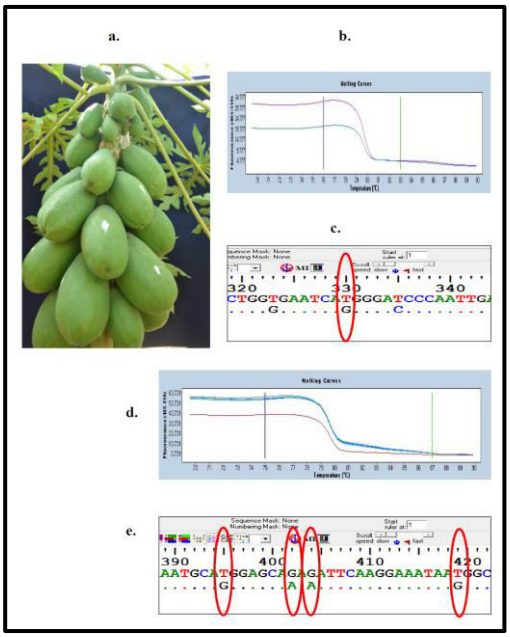

Plate 4: a) Ultra Dwarf mutant (R_8P_{20}), b) ACC-synthase gene melting curve of Ultra Dwarf mutant (R_8P_{20}), c) DNA sequence variation of ACC synthase gene

in Ultra Dwarf mutant (R_8P_{20}), d) ACC-oxidase gene melting curve of Ultra Dwarf mutant (R_8P_{20}), e) DNA sequence variation of ACC-oxidase gene in Ultra Dwarf mutant (R_8P_{20}).

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