Studies on Factors Responsible for Early Ripening of Tomatoes: Towards Development of Strategies for Mitigation of Post Harvest Losses

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Abstract

The aim of this study was to isolate and characterize the genes responsible for early ripening in tomatoes so as to establish future strategies on how this could be stemmed biotechnologically. About 30 tomato cultivars were collected as seedlings from the Nigerian - Green Agro-Allied Industrial Zone (GAAIZ), Kaduna and cultivated in a nursery bed for four weeks before transferring to a greenhouse. Fruit ripening stages were divided into five stages and total genomic DNA were extracted from these different growth stages using a DNA Plant extraction Kit (Qiagen). Fourteen SSR primers designed for tomato DNA fingerprinting and further molecular analysis were then carried out. For salt stress treatment, the roots of tomato seedlings were submerged in a solution containing 250 mM NaCl for 0, 1, 2, 4, 8, 12 and 24 hours. These were done to know the type of volatile oils expressed from the plant due to environmental factors. Based on the data obtained after the molecular analysis, nine MTases were identified in tomato through Blastp alignment. The open reading frame (ORF) length of these genes varied from 1.1 kb to 4.6 kb and their protein length ranged from 381 to 1559 amino acids. All the deduced polypeptides are hydrophilic. Salt tolerant stress test gave 5 different types of volatile oil. This study shows that series of modifications that transform a mature green fruit into a ripe fruit occur during the ripening stages and involve many different metabolic pathways. The implications of these findings are discussed here.

Keywords: Tomato, Molecular Characterization, Genes, Voltile Oils, Blastp, Salt Tolerance.

1. INTRODUCTION

Early ripening of fruits is a complex process influenced by various genetic factors. Several genes have been identified that play crucial roles in this process. Fruit ripening is a developmental process characterized by changes in color, flavor, aroma, texture, and nutritional content, making the fruit attractive and palatable for seed dispersal (Ahmad and Munawar, 2003). Early ripening can be advantageous for both producers and consumers, as it allows for quicker harvesting and delivery of ripe fruits to the market. However, in perishable fruits like those of tomatoes, it may not be advantageous due to colossal waste of their post-harvest commercial value. In climacteric fruit, ethylene is necessary for the initiation of fruit ripening and senescence because it drives the majority of the ripening processes such as fruit softening and cell wall disassembly. The effects of ethylene on fruits mainly depend on its biosynthesis and signal transduction during fruit development (Arruabarrena *et al.*, 2023). The synthesis of ethylene begins with the production of S-adenosylmethionine (SAM) which is catalyzed by S-adenosylmethionine synthetase (SAM synthetase) from methionine. SAM is then metabolized to 5-methylthioadenine (MTA), which is incorporated into the methionine cycle to recover the sulfur atom and 1-aminocyclopropane-1-carboxylic acid (ACC); this reaction is catalyzed by ACC synthase (ACS). Finally, in the presence of oxygen, ACC is oxidized by ACC oxidase (ACO) to yield ethylene and CO_2 (Chen *et al.*, 2023).

The ripening processes of many kinds of fruits involve all of the cell compartments including the nucleus (expression of new ripening genes), chloroplast (loss of chlorophyll, dismantling of thylakoids, synthesis of carotenoids and conversion to a chromoplast), mitochondria (respiratory climacteric), endoplasmic reticulum (ethylene perception and enzyme secretion), cytosol (synthesis of many new proteins and pathway changes), vacuole (storage of metabolites and anthocyanins biosynthesis in some fruits) and the cell wall (partial dissolution and solubilization). During this process, fruits also undergo biochemical and physiological changes in flesh texture, pigmentation, sugar contents, aroma, and nutritional qualities. All these changes occur in a coordinated manner over a period of a few days and make fruits attractive for the human diet. According to whether or not they show a rapid rise in respiration and a burst of ethylene production at the onset of ripening, fleshy fruits have been classified into climacteric and non-climacteric types. Gene expression changes are necessary for ripening in climacteric and non-climacteric fruits. The genetic relationship between the two ripening types is intriguing and it is still possible that ethylene plays a role in non-climacteric types (Bianchetti *et al.*, 2022). Understanding the mechanisms that regulate fruit ripening can help to improve fruit quality, storage life, and the wastage of many fresh plant products worldwide.

The study was carried out in order to unravel the complexities of early ripening processes, so that researchers and growers can work towards a more sustainable, resilient, and productive agricultural system. The focus is to study factors that could lead to delayed ripening especially in fruits like tomato. It is a well-known fact that tomatoes are grown largely in the Northern part of Nigeria but mostly transported down south when ripe. This leads to a lot of waste and economic loss as most of these products perish even on transit due to incidences of early and over ripening. Therefore, finding ways to mitigate these occurrences would lead to so many economic gains. The aim of this current study was to isolate and characterize the genes responsible for early ripening in fruits like tomatoes so as to establish future strategies on how this could be stemmed biotechnologically. The work will specifically focus on how to; identify and isolate genes responsible for fruit ripening in tomatoes at different growth stage., understand the role of ethylene response factors (*ERF)* gene family members in fruit development and ripening and determine the role of other ripening factors like volatile oils exuded from the fruit (Cai *et al.*, 2021).

2. MATERIALS AND METHODS

Materials Used

1. Tomato Descriptor (IPGRI) 1999 program
2. Tomato Analyzer software program Version 3
3. DNA Plant extraction Kit (Qiagen, China)
4. ND-1000 spectrophotometer (Genway)
5. Trizol reagent
6. Incubator
7. Mortar and pestle
8. Plant DNA Extraction kit (Qiagen)
9. AccuPower 2X GreenStar qPCR Master Mix, etc.
10. Gel Documentation machine and Sequencer, etc.

All other reagents used were of analytical grade.

Sample Collection and Preparation

This research work was carried out at the National Geosciences Agency Research Laboratory (NGSA). Kaduna, Nigeria. The tomato cultivars, Chikun Local (JM94/46) and Chikun cultivars were collected as seedlings from the Nigerian - Green Agro-Allied Industrial Zone (GAAIZ), Zaria-Kaduna and cultivated in a nursery bed for four weeks before transferring them to a greenhouse according to Chen *et al.,* (2023) method.

Plant Material

Tomato (Solanum lycopersicum Mill. cv. Ailsa Craig) seedlings from the nursery were then grown under greenhouse conditions (16h in the days at 27 °C and 8 h nights at 19 °C) for organ-specific expression profiling of genes by harvesting tomato fruit pericarp tissues. Other parts of the plants were also harvested to conduct some pilot study on plant growth and development (Arruabarrena *et al.*, 2023). As such, leaves were taken from three different parts of 6-5-day-old tomato plant, namely young leaves (3 leaves of new growth), mature leaves (5 to 7 leaves from top to bottom) and senescent leaves (8 to 10 leaves from top to bottom). Sepals and petals were also collected at the same time. Flowers were marked as anthesis and fruit development was recorded as days post-anthesis (DPA). Fruits ripening stages were divided into five stages, namely, IMG (immature green, 28 DPA), MG (mature green, 35 DPA, full fruit expansion but no obvious color change), B (breaker, fruit showing the first signs of ripening-associated color change from green to yellow), B4 (4 days after breaker) and B7 (7 days after breaker) (Ding *et al.*, 2023).

DNA Extraction and Simple Sequence Repeat (SSR) Assays

Total genomic DNA was extracted from early ripening stages of the fruit for five different plants per line using a DNA Plant extraction Kit (Qiagen). DNA quantification was performed with an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The DNA quality was assessed using the absorbance ratio at 260 to that at 280 nm wavelengths (A260/A280). DNA quantity was calculated as DNA (µg/µL) = A_{260} × 50, where A_{260} is the absorbance at 260 nm. Thus, the concentration of DNA in µg/mL was calculated as DNA (µg/mL) = [A260 × 50] × DF where DF is the dilution factor. Fourteen SSR primers designed for tomato DNA fingerprinting were used. Six primers were selected for the next analysis to determine genetic diversity in tomato collection (LEat018, LEct004, LEta014, LEta020, CT114, and Asr2) based on the screening of fourteen SSR primers (Bianchetti *et al.*, 2023).

Stress Treatments

Potted 35 days-old tomato seedlings which were chosen based on their uniformity were used for all stress treatments. For salt stress treatment, the roots of tomato seedlings were submerged in a solution containing 250 mM NaCl for 0, 1, 2, 4, 8, 12 and 24 hours, and the young leaves of the treated seedlings and controls were collected. For low temperature stress treatment, the whole potted tomato seedlings were incubated at 4 °C for 0, 1, 2, 4, 8, 12 and 24 hours, after which the leaves were collected (Zhu et al. 2014). All stress treatments were performed with three biological replicates. These were carried out to know the type of volatile oils expressed from the plant due to environmental factors (Diretto *et al.*, 2020).

3. RESULT AND DISCUSSION

Identification of Tomato DNA Methyl Transferase (MTases) and Sequence Analysis

Based on the data obtained after the molecular analysis and Blastp search on the NCBI database, nine MTases were identified in tomato via alignment from the sequence submitted (Table 1). The open reading

frame (ORF) length of these genes varied from 1.1 kb to 4.6 kb and their protein length ranged from 381 to 1559 amino acids. All the deduced polypeptides are hydrophilic. In addition, the intron-exon organization (numbers of intron and exon) of nine MTases in tomato are also shown. The coding regions of CMT subfamily genes were interrupted by 14-21 introns, MET gene (SlMET1) length was approximately 4.6 kb in tomato harboring 12 exons. The length of the DRM subfamily genes in tomato varied from 1.8-2.1 kb with 9 exons. DNMT2 gene (SlMETL) was smallest in length (1.1 kb) harboring 9 exons. Genomic distribution of these tomato MTase genes was also analyzed. Eight tomato MTases genes were dispersedly located on chromosomes, so one MTase variant was mostly located on a single chromosome (Table 1) suggesting at least partial influencing of the gene in the diversification of MTases family in tomato rather than gene duplication.

Table 1: CArG-Box Sequences Found in the Promoters of Tomoato Ripening-Genes From Blastp

Site	CArG-Box and its Flanking Sequences (5' to 3')[1)]	Motif [2)]	CArG-Box Position (bps)[3)]
ACS4-a	ATCAAACA-CAAATATAAG-TTTGGAAC[5])	Atypical	M88487 (567 - 576) (+)
ACO1	GGTTGAAT-CTATAAAAAG-AAAAATAT	Possible	X58273 (1,285 - 1,294) (+)
ETR3-a	GGAGAAAT-CCTATAATAG-GGCAAACA	Intermediate	AY600437 (3,121 - 3,130) (+)
PG-b	CTTAAAAT-CTATAAATAG-ACAAACCC	MEF2-like	scaffold01076 (1,533,632 - 1,533,641) (-)
TBG4-a	TATATGCT-CTATTTTTGG-ACGGCAGG[5])	Possible	scaffold00061 (457,025 - 457,034) (+)
EXP1-a	TTATTTTA-CATTTATATG-TTATTATT	Atypical	scaffold00114 (3,118,161 - 3,118,170) (-)
RIN-a	GTTGCACT-CTAAAAAAAG-TTAAAAGG[5])	Possible	scaffold00243 (210,835 - 210,844) (-)
RIN-b	ACAAAGAA-CCATTAAAAG-GTTAAAAA[5])	Intermediate	scaffold00243 (210,262 - 210,271) (-)

Volatile Organic Compounds

Fruits like those of tomato normally contain a large amount of different volatile compounds that are responsible for the aroma and flavor of the fruit. Thus, a large amount of the different volatiles are synthesized during the ripening phase of the fruit and the content and composition of the volatiles are known to change with maturity stages of the fruit (Ernesto *et al.*, 2018). Volatile compounds are known to be part of the defence mechanisms against herbivores in tomatoes. Furthermore, damages by insects or by mechanical wounding produced changes in the composition and content of volatile organic compounds. (Gao *et al.*, 2020). Also, plants under attack of insects have been found to show higher concentration of volatile compounds compared with controls. Thus, a tomato plant attacked by *Tuta absoluta* for instance may expel an increased amount of volatile compounds (76% to 86%), e.g. β-caryophyllene, α-pinene and α-phellandrene, and these compounds are found to attract *Macrolophus pygmaeus*, a natural enemy of the attacking insects. Table 2 shows the result of volatile compounds from 3 cultivars (Chikun 1 ,2, and 3) of the tomatoes used for this work. The results are in agreement with Chen *et al.*, 2023 Arruabarrena *et al.*, 2023.

Table 2 Volatile Compounds Quantified in Three Tomato Varieties

Volatile Compound	Chi 1 (Average)	Chi 1 (%SD)	Chi 2 (Average)	Chi 2 (%SD)	Chi 3 (Average)	Chi 3 (%SD)
3-methyl-butanal	24.6	26.6	92	16.8	179.2	14.2
2-methyl-butanal	10.9	47.7	65.8	5.5	108	16.1
pent-1-en-3-one	155.7	17.8	295.3	24.1	202.6	4.5
pentanal	47.8	21	52.4	14.3	38.1	4
pentan-3-one	33.9	6.5	48	10.8	33.8	8.6
2-methylbut-2-enal	44.9	64.1	246.1	12.3	349.8	7.6

Values are The Average of Three Replicates in µg/kg Fruit and The Standard Deviation in Percentage

Ethylene (ET) is the simplest unsaturated hydrocarbon with the formula C_2H_2. It acts as a global regulator of developmental processes and defense in plants. The ethylene biosynthetic pathway includes three steps: S-adenosylmethionine synthetase (SAMS) modifies methionine to form S-adenosylmethionine (SAM), SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by synthase (ACS), and in the last step ACC converts ACC oxidase (ACO) with the formation of ethylene. In this work, ethylene was seen as a participant in the signaling cascades, including during the ripening process as shown by the genes encoding different proteins obtained from the sequence alignment (Table 1). Ethylene normally accelerates fruit ripening with simultaneous repression of auxin signaling (Wang *et al.*, 2023). It has been established that its synthesis during ripening is presumably regulated by FER receptor kinases (FERONIA). FERL6 and FERL1 were found to interact physically with the SAMS promoter. Expression of FER genes in tomatoes showed negative regulation of ethylene accumulation at the initial stages of fruit development and, as a consequence, delayed fruit ripening. This could serve as a potential target in developing strategies for mitigating post-harvest loses (Yan *et al.*, 2023). The promoter of the transcription factor EIN3 (ethylene insensitive) gene has been shown to contain several motifs associated with hormones influencing fruit development and ripening which agrees with; Imran *et al.*, 2019). Overexpression of EIN3 in tomatoes resulted in the activation of the expression of ethylene biosynthesis genes ACO1, ACS1, and SAMS1, which promoted early fruit ripening. Alternatively, EIN3 silencing will show the opposite effect. An EIN3-like gene causes premature onset of ovule senescence. Ethylene is bound by a family of ETR (ethylene receptor) proteins located in the membrane of the endoplasmic reticulum. ETRs have functional redundancy. ETR3-mediated signaling inhibits pollen tube growth without sufficient ethylene. ETR3 promotes the activation of cell wall remodeling genes and Ca2+ transporters—overexpression of ETR7 results in earlier flowering, short plants, and small fruits. Targeted base substitution in ETR1/2 causes a delay in ripening and ensures prolonged storage of fruits. Ethylene response factors (ERFs) are signaling components involved in ethylenedependent developmental processes. They can perform both the positive and negative regulation of target genes. Their number is large, as is the specificity of the reactions of tomato genes to ethylene: the regulation of fruit ripening processes, control of aging, participation in the activation of protective reactions, growth, accumulation of chlorophyll and formation of chloroplasts, and regulation of other signaling pathways (Li *et al.*, 2022).

Our data strongly indicate that RIN is a positive regulator of the carotenoid biosynthesis pathway along with the ripening process and ethylene burst. In addition, RIN and TDR4 cooperatively affect ripening, suggesting the regulatory effects of these TFs on fruit ripening involve ethylene as well as abscisic acid (ABA) or other hormones (Kamiyoshihara *et al.*, 2022). The relationship of early ripening among different

fruits is multifaceted, involving a combination of genetic, hormonal, and environmental factors. While some fruits like bananas and tomatoes are known for their ethylene-driven early ripening, others like apples and citrus fruits may have ripening processes influenced by different genetic and environmental factors. Understanding these dynamics is essential for agriculture, storage, and ensuring a consistent supply of ripe fruits to consumers. The volatile oils were studied in order to determine the effect of various environmental conditions on the ripening mechanism. In this study, eight Ps-ERFs were isolated. The difference in the *Ps-ERF*s accumulation profile throughout flower development suggested the contribution of different plant hormones, strongly occurring during this stage and in the regulation of the various *Ps-ERF*s. This work has shown that favorable conditions for growth also promote the release of different volatile compounds from tomatoes with their own individual contributions to ripening processes.

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