Differential expression of tomato ACC oxidase gene family in relation to fruit ripening

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The enzyme ACC oxidase (ACO) catalyses the terminal step in the ethylene biosynthesis pathway and is encoded by a small multigene family in tomato. We studied the expression of the ACO gene family during various stages of fruit ripening using the multiplex RT–PCR approach in three tomato cultivars differing in their rate of ripening. The ACO1 gene expression, compared to other isoforms of the ACO gene family, showed a strong correlation with the rate of fruit ripening and ethylene evolution. The results revealed LEACO1 as the predominant ripening-regulated isoform, and demonstrated the potential of the multiplex RT–PCR technique to analyse the rate of ripening in diverse tomato genotypes.

Keywords: ACC oxidase, fruit ripening, multiplex RT–PCR, tomato.

The gaseous phytohormone ethylene orchestrates many aspects of plant growth and development, including fruit ripening, leaf and flower senescence and abscission, seed germination and plant responses to environmental stimuli²,³. Fruit ripening is a genetically programmed specialized phase of plant senescence and requires differential gene expression that is triggered, modulated and coordinated by ethylene. The terminal ethylene-forming enzyme (EFE), now confirmed as ACC oxidase, is encoded by at least four members of a multigene family in tomato² and appears to be primarily located in the cell wall of ripening fruits⁴. The enzyme apparently is a dioxygenase belonging to the superfamily of iron-ascorbate oxidases, expressed constitutively in most vegetative tissues¹ and found to be induced during fruit ripening⁵. In view of the mounting evidence for major changes in ACC oxidase transcript abundance in the ripening tomato fruit, the present study was undertaken to investigate the differential expression pattern of ACC oxidase gene family during fruit ripening in three selected Indian tomato cultivars, which differed in their rate of ripening.

The level of ACC oxidase gene expression during fruit ripening was monitored by multiplex RT–PCR⁶ analysis in three commercially important Indian tomato cultivars, Pusa Ruby, Pusa Sheetal and Pusa Uphaar, selected on the basis

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of their contrasting morphological features, as described in Table 1.

The plants were grown from seeds in a greenhouse under natural environmental conditions (10–14 h day length and 28/22°C temperature). Fruits from selected stages, i.e. mature-green (pre-climacteric, no lycopene in locular gel), breaker (early climacteric, slight colour blush at blossom end), turning pink and ripe (red)\(^7\), were harvested and frozen immediately. The frozen fruit tissue was ground to a powder in liquid nitrogen, homogenized and total RNA was isolated\(^a\) using Trizol reagent followed by RNase-free DNAse-I treatment. Reverse transcription of RNA was performed in 20 µl reaction mixture containing 500 ng of total RNA, 2.5 mM MgCl\(_2\), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 200 µM dNTP, 0.5 U/µl of recombinant RNAse inhibitor (GIBCO-BRL), 10 µM DTT, 100 ng of random hexamers\(^5\) and 200 U/µl SUPERSCRIPT II reverse transcriptase (GIBCO-BRL). The samples were incubated at 42°C for 45 min; thereafter, the reverse transcriptase was inactivated by heating at 97°C for 5 min. To detect the possibility of genomic or plasmid DNA contamination, reverse transcriptase was omitted from the reaction control tube.

For designing primers to be used in the multiplex RT-PCR, analysis of the ACC oxidase cDNA sequences was carried out, which revealed a high degree of conservation at the nucleotide level in the exonic sequences among various members. However, the 5′- and 3′-UTRs were found unique to each member. Exonic regions of all the four ACC oxidase genes (ACO1: X58273; ACO2: Y00478; ACO3: Z54199; ACO4: AB013101) were analysed by multiple sequence alignment using the software CLUSTAL version 1.8. A common region of 22 bp was identified, which was conserved completely except for two nucleotides in all the four isoforms of the ACO gene family. Based on this alignment, a common reverse primer of 22 bases was designed with degeneracy at the two unmatched nucleotide locations (Table 2). The gene-specific forward primer for each member was designed based on its 5′-UTR (Table 2). Primers of the ubiquitously expressed housekeeping gene, elongation factor-1-alpha (EF-1α), were also included in the multiplex primer mix to correct for the variations arising out of unequal loading and/or degradation of mRNA. The primers for EF-1α gene were designed from the exonic region corresponding to a 450 bp fragment (Table 2). It has already been reported that EF-1α mRNA expression is insensitive to several types of acute hormonal stimuli\(^9\). The primers were designed using PRIMER 3.0 software.

PCR amplification of genomic DNA of cultivar Pusa Ruby was performed using gene-specific primers. RT-PCR reactions were carried out using individual gene-specific primers along with the common reverse primer in separate reactions to confirm the size of the amplified product of each gene member. The multiplex RT-PCR reactions were carried out in a total volume of 50 µl containing 48 µl of PCR master mix [4 mM MgCl\(_2\), 10 mM KCl, 10 mM Tris-HCl pH 8.3, 50 pmol each of the gene-specific

<table>
<thead>
<tr>
<th>Feature</th>
<th>Pusa Ruby</th>
<th>Pusa Sheetal</th>
<th>Pusa Uphar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant type</td>
<td>Indeterminate</td>
<td>Determine</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>Foliage colour</td>
<td>Light green</td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td>Fruit shape</td>
<td>Flat, slightly furrowed</td>
<td>Flat, round</td>
<td>Round</td>
</tr>
<tr>
<td>Average fruit weight (g)</td>
<td>60</td>
<td>80</td>
<td>70</td>
</tr>
<tr>
<td>Pericarp thickness</td>
<td>Thin</td>
<td>Intermediate</td>
<td>Thick</td>
</tr>
<tr>
<td>Rate of ripening</td>
<td>Fast</td>
<td>Medium</td>
<td>Slow</td>
</tr>
<tr>
<td>Days post breaker to red ripe</td>
<td>9</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 2. Nucleotide sequence of various oligonucleotide primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Temperature (°C)</th>
<th>Size of amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common reverse primer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>5′-CCACTSACYTGTGCATCTTGGAG-3′</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Gene-specific forward primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACO1</td>
<td>5′-GGAAAGACACCTTACGACAAATAAG-3′</td>
<td>71</td>
<td>606</td>
</tr>
<tr>
<td>ACO2</td>
<td>5′-CACCAACACAAACCAACAAATGTCAC-3′</td>
<td>76</td>
<td>698</td>
</tr>
<tr>
<td>ACO3</td>
<td>5′-CATCTCTTCAATCTCTTGTATAATCTC-3′</td>
<td>71</td>
<td>640</td>
</tr>
<tr>
<td>ACO4</td>
<td>5′-CAAATCTGAGAAAGCGCCTGA-3′</td>
<td>72</td>
<td>544</td>
</tr>
<tr>
<td>Elongation factor primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EFAF</td>
<td>5′-CTTGACCCAGATTAACCAGGC-3′</td>
<td>68</td>
<td>450</td>
</tr>
<tr>
<td>EFAAR</td>
<td>5′-GCTGTCAATCTTGTCGAAG-3′</td>
<td>68</td>
<td></td>
</tr>
</tbody>
</table>
primers, 250 pmol of the common primer, and 5U of Stoffel fragment (Perkin Elmer), and 2 μl of reverse-transcriptase reaction mix in 0.2 ml thin-walled tubes, using a thermal cycler (Perkin Elmer). The PCR cycling profile included denaturation at 92°C for 1 min, annealing at 60°C for 1 min and an extension at 72°C for 1.5 min for 25 cycles. The amplification products were separated by electrophoresis on 2% agarose gels containing ethidium bromide and photographed using Alpha Imagertm 1220 Documentation and Analysis System. The intensity of bands was determined using 'Line Densitometry' software package of the Alpha Imagertm.

For ethylene measurements, tomato fruits of selected varieties were harvested at mature-green stage and incubated at 25°C for uniform ripening. Ethylene production from whole fruits at different stages of development was measured by enclosing samples in an airtight chamber of capacity 500 ml for 12 h at 25°C and injecting 1ml of headspace gas into a gas chromatograph (Model Shimadzu-ER4A) fitted with a flame-ionization detector and a porapak N column for ethylene quantification. For measurement of polygalacturonase (PG) activity, fruit pericarp tissue was homogenized thoroughly in the presence of two volumes of 0.2 M sodium acetate buffer (pH 6.0), a

Figure 1. a, RT–PCR analysis of individual isoforms of ACC oxidase multigene family in fruits of tomato cv. Pusa Ruby at the turning stage. Lane 1, 100 bp marker (Promega); lane 2, Negative control (RT-minus); lane 3, ACCO1 (size, 600 bp); lane 4, ACCO2 (not expressed); lane 5, ACCO3 (size, 640 bp); Lane 6, ACCO4 (size, 544 bp); lane 7, EF-1α (size, 450 bp), a constitutive gene, used as an internal control; lane 8, Multiplex RT–PCR of all isoforms of ACC oxidase with EF-1α as control. b-d, Multiplex RT–PCR analysis of ACC oxidase gene family at different stages of fruit ripening in the selected cultivars. b, Pusa Ruby, c, Pusa Sheetal, d, Pusa Uphar. Lane M, 100 bp DNA ladder (Promega). Different ripening stages: Mature green (MG), breaker (BR), turning (TU), pink (PI), and red ripe (RR).
pinch of sodium thiosulphate and PVP, and the supernatant recovered after centrifugation (15000 g, 20 min) for the enzyme assay.

Individual RT–PCR assays using mRNA from turning stage fruits confirmed the expression of the LEAC01, LEAC03 and LEAC04 isoforms that are implicated in fruit ripening, whereas no expression was observed in case of LEAC02 (Figure 1a). Although LEAC02 transcription is known to occur in tissues associated with another cone in tomato, we did not detect any expression in the examined fruits. To ascertain this, we tested the efficacy of the LEAC02 gene-specific primers by amplifying a genomic region of about 1.8 kb using genomic DNA as template (Figure 2). This clearly demonstrated that LEAC02 isoform is not expressed in fruits. On the contrary, expression of LEAC01 mRNA was up-regulated in ripening fruits at different stages (Figure 1 b–d). Very low basal level of the LEAC01 transcript was detectable in mature green fruits, but the level substantially increased at the breaker stage, when the fruit begins to ripen in different cultivars (Figure 1 b–d). Subsequently, the LEAC01 message reached a maximum at the turning stage before declining at the fully ripe stage in all cultivars studied. Expression of AC03 isoform was detected in fruits, but only transiently at the turning stage of development. The expression pattern of AC04 isoform at different stages was also similar to that of the AC01 isoform, but less in abundance. At the turning stage, the level of AC01 transcript was highest for the cultivar Pusa Ruby, which was almost three times more compared to the other two cultivars, after normalizing the transcript abundance based on the internal control EF-1α expression levels (Figure 3).

The activity of the major cell-wall hydrolysing PG enzyme was negligible in mature green fruit, became detectable as the fruits initiated colour change and increased progressively as the ripening proceeded, with peak activity at the red ripe stage (Figure 4). There was on an average 2.5 to three-fold increase in enzyme activity presumably due to de novo synthesis, but with no significant difference among the cultivars. The rate of ethylene evolution from intact tomato fruits was critical in the present investigation.

![Figure 2. PCR amplification of different ACO isoforms using tomato (cultivar Pusa Ruby) genomic DNA as template and different gene-specific primers. Lane 1, 1 kb DNA ladder (MBI Fermentas); lane 2, ACO1 (965 bp); lane 3, ACO2 (1858 bp); lane 4, ACO3 (972 bp); lane 5, ACO4 (size not known, approximately 900 bp); lane 6, EF-1α (450 bp).](image)

![Figure 3. Transcript accumulation of ACO1 mRNA at different stages of fruit ripening in the three cultivars that differ in the rate of ripening.](image)

![Figure 4. Polysaccharidase enzyme (PG) activity in pericarp of three tomato cultivars at various stages of fruit ripening. Fruits were harvested at five different stages: Mature green, breaker, turning, pink and red ripe. Bars represent standard error of four replications.](image)

![Figure 5. Changes in the rate of ethylene production from intact tomato fruits during different stages of fruit ripening in the three cultivars. Fruits were harvested at five different stages: Mature green, breaker, turning, pink and red ripe. Bars represent standard error of four replications.](image)
since ACC oxidase enzyme catalyses the key terminal step in the biosynthetic pathway of this unique biomolecule. Ethylene generated during tomato fruit ripening also showed a characteristic pattern with a very low basal level at the mature green stage, exponentially increasing till pink/light red stage, and declining thereafter at the red ripe stage in all the cultivars studied (Figure 5). The cultivar Pusa Ruby showed a significantly higher ethylene production at breaker and turning stages compared to the other two cultivars (Figure 5).

The results revealed that the three tomato cultivars used in the present study also exhibit substantial accumulation of ACC oxidase transcripts during ripening, as has been reported previously. The results were also in general agreement with the observation that LEACO1 is the predominant member of the ACO gene family expressing in tomato fruits, along with ripening-related expression of LEACO3 and LEACO4 isoforms. Our results revealed a strong correlation of LEACO1 transcript abundance with the rate of ripening in the three cultivars studied. In addition, expression of the ACO3 isoform was observed at the turning stage in indigenous cultivars as against the earlier report of its expression specific to the breaker stage in exotic cultivars.

PG (EC 3.2.1.1.5) catalyses the hydrolytic cleavage of α(1–4) galacturonic linkages and is a key enzyme involved in changes in pectin structure that accompany the ripening of many fruits. The tomato fruit PG gene has been shown to be a single-copy gene belonging to the family of PG genes present in tomato, whose expression is confined to fruits and is transcriptionally activated during ripening. Our results showed that PG activity remains more or less the same in all the three cultivars, indicating thereby that it is not a crucial parameter associated with the rate of ripening. However, it is possible that complete solubilization of the enzyme must not have occurred as no detergent or high salt solution was used to extract the enzyme.

It has been demonstrated earlier that the variation in ACC oxidase enzyme activity as well as protein abundance at different stages of fruit ripening correlates well with the pattern of accumulation of ACO1 transcripts. This proves that the dramatic increase in ACO transcripts (especially ACO1 isoform) is directly related to the ACC oxidase activity in determining ethylene production. The fact that the rate of ripening of tomato fruits is dependent on ethylene production has already been established. Our results on ethylene evolution from intact fruits showed a strong correlation with ACO1 expression level till the ‘turning’ stage, whereas activity of PG enzyme is independent of ethylene level or ACO1 transcript abundance. However, differences in the amount of ethylene production are not as striking as those in the ACO1 transcript abundance in the three cultivars. It may thus be possible as suggested that ACO1 alone or in combination with ACO3 and ACO4 may be responsible for the regulation of autocatalytic ethylene biosynthesis during fruit ripening in tomato.

In conclusion, it may be mentioned that the LEACO1 gene expression compared to other members of the ACO gene family during different stages of ripening, is closely related with the rate of fruit ripening and ethylene evolution in tomato. The ACO1 isoform may, therefore, be considered as a reliable marker to analyse and compare any two varieties for their ripening behaviour or rate of ripening. The analysis of upstream regions of the individual ACO gene promoters for different cis-acting elements regulating the expression level, ripening-specific pattern, and ethylene-inducibility would provide conclusive evidence for the differential expression of this gene family.

Anomalous fluctuation of radon, gamma dose and helium emanating from a thermal spring prior to an earthquake

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Anomalous large-scale radon emission, striking high gamma dose rate and significant increase in He/CH₄ ratio have been observed in thermal spring emanations at Bakreswar, West Bengal prior to the recent major earthquake near the island of Sumatra, Indonesia. An attempt has been made to correlate the field observations as the precursory phenomena to the quake.

Keywords. Earthquake, gamma, helium, radon, thermal springs.

RESEARCH dealing with earthquake prediction has drawn serious attention of geophysicists as well as investigators in different branches of natural sciences in many countries across the globe for over several decades. It is a problem that is as yet unresolved, only because of its sheer complexity. Although no reliable method to accurately foretell the time, place and magnitude of an impending earthquake has as yet been established, it has been reported that several earthquakes are heralded by the occurrence of foreshocks that can be detected by various dense local monitoring networks. A seismic event might start with a pre-slip stage, hours to days before the earthquake¹. This pre-slip nucleation phase would be silent and too slow to be picked up with conventional seismic instrumentation. There are other instruments that can measure seismic-induced deviations, such as changes in the level of radon and helium gas¹⁷,¹⁸, electric³¹⁸ and magnet properties¹⁹, velocity changes of seismic waves, high-energy charged particle flux variations in space²⁰ and even abnormal animal behaviour²¹-²⁴. We do not have direct access to observing deep earth phenomena, especially those relating to the curious behaviour of heterogeneous rock structures during the build-up of an earthquake. However, crustal changes that occur during the process find expression in changes in gas geochemical features in spring emissions and hydrothermal activity. Changes in the concentration of subsurface volatile components discharged from spring vents across time may be exploited to extract useful information related with deep earth phenomena that result in changes in the contour and content of gas emission at surface level.

Studies relating geochemical changes to seismic occurrences based on phenomenological approach and short-term precursory observations are available²⁵,²⁶,²². Some of these are found to coincide with the subsequent occurrence of tectonic activities.

A strong earthquake or large-scale disturbance of the ocean floor, such as landslides or volcanic eruptions can generate a potentially dangerous tsunami²⁵. Its threat lies in the great speed at which it travels (as much as 950 km/h), its long wavelength (up to 200 km), its low observable height in the open ocean and its ability to pile up rapidly to heights of 30 m or more as it moves into shallow water along an exposed coast. The suddenness of the arrival of a tsunami and consequent lack of warning time results in numerous casualties and catastrophic devastation, when the tsunami moves into populated areas, as we have recently seen in South East Asian countries.

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Figure 1. Block diagram of integrated experimental arrangement.

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