Summary and Conclusions
5. SUMMARY AND CONCLUSIONS

Tomato is a major vegetable crop and serves as an important source of nutrients for human consumption. Tomato fruit is perishable, with short shelf life. Thus, huge amount of crop loss due to over softening which influences shelf-life wastage and infection by post-harvest pathogens. To avoid such losses and to ensure that the fruits survive shipping without bruising and rotting, fleshy fruits like tomato are picked before ripening and ethylene burst. Picking tomatoes early means they have less chance to develop flavour, colour, and nutrients naturally. Fruits transported for long periods under refrigeration often lose their nutritional value. In this regard, it has always been a goal of plant biologists and of post-harvest physiologists, in particular, to be able to prevent or delay fruit ripening by slowing the process of ripening and senescence and also extending the storage and shelf life of fresh fruits. The ripening of fleshy fruits corresponds to a series of biochemical, physiological and structural changes that make the fruit attractive to the consumer. All these changes are driven by the cues from ethylene. Thus, while improving fruit texture and shelf life, one of the approaches involves manipulation of ethylene responses. The climacteric burst in ethylene production is the key event for the onset of ripening in climacteric fruits and so in tomatoes. Ethylene is involved in the initiation, modulation and co-ordination of expression of many ripening related genes that are responsible for a variety of processes, including enhancement in respiration rate, autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars, and increased activity of cell-wall degrading enzymes. Ethylene is thus held accountable for the tones of post-harvest losses due to over-ripening and subsequently resulting in fruit rotting. Synthesis of ethylene initiates with the conversion of S-adenosylmethionine (SAM) to 1-amino cyclo propane-1-carboxylic acid (ACC) catalysed by ACC synthase (ACS), ACC is finally oxidised to ethylene by action of ACC oxidase (ACO). Ethylene has been well established as an important cue for initiation of fruit ripening, thus delayed ripening can be achieved by modifying ethylene levels. Modifying the amount of ethylene produced or received during ripening have been the favourite aim of plant biotechnologist to achieve long lasting fruits. In this regard several attempts have been made. So far this has been achieved...
either by switching off or decreasing ethylene production: with antisense RNA for
\textit{ACS} or \textit{ACO}, using gene silencing or co-suppression, or by blocking the perception
of ethylene by expression of a dominant mutant ethylene receptor and also by
blocking the expression of genes that are induced in response to ethylene by antisense
RNA for cell wall modifying enzymes. However, such a strategy suffers due to the
pleiotropic effects of ethylene suppression on fruit ripening including loss of pigments
and reduced fruit quality in terms of nutrition. In many cases, the fruits even become
ethylene insensitive and fail to ripen. Several transgenic plants with reduced
expression of ripening related genes have thus been generated by employing various
molecular biology techniques. These strategies have also not been of much help since
fruit softening is governed by plethora of enzymes which act in conjunction. Thus the
inhibition of only one enzyme would have little effect on fruit softening and will not
make much difference.

However, ethylene, functionally and metabolically is inhibited by polyamines
(PAs), as they share a common precursor, SAM. PAs are ubiquitous polycationic
molecules which have been implicated in the regulation of growth and development in
all living organisms. PAs mainly comprise putrescine (Put), spermidine (Spd) and
spermine (Spm); however, other modified forms are also known in plants. Put is
synthesized from ornithine decarboxylation in a reaction catalyzed by ornithine
decarboxylase (ODC). An alternate polyamine biosynthesis pathway operates in plants,
bacteria and some of the fungi via arginine decarboxylation by arginine decarboxylsae
(ADC). Spd and Spm are synthesized from Put by subsequent addition of
aminopropyl groups from decarboxylated SAM, which is produced from SAM by the
action of S-adenosylmethionine decarboxylase (SAMDC). A number of physiological
effects of ethylene in plants seem to be antagonized by PAs treatment. PAs and
ethylene could therefore differently influence plant growth and senescence by sharing
SAM as intermediate. In contrast, mature non-dividing fruit tissues shift the
metabolism of SAM toward the formation of ACC and therefore ethylene, which is
necessary for the induction of fruit ripening. In this regard, it would appear that a
plant cell has the potential to commit the flux of SAM either into PA biosynthesis,
ethylene biosynthesis, or both. Thus, by harnessing the knowledge on competition
between PAs and ethylene, make it plausible to modulate postharvest fruit
development. By over-expressing PA biosynthesis genes, the more of SAM flux could be converted for the PA synthesis that in turn down-regulates the ethylene synthesis.

An alternative novel strategy is the application of RNA interference (RNAi), where targeted silencing of the ethylene biosynthesis genes (ACS and ACO) is possible, by expressing the same genes in a double-stranded RNA (dsRNA) form in the system of choice. Also, this circumvents the problem of complete inhibition which otherwise would put all the ripening processes on halt and thereby causing multifaceted ill effects. Rather such an approach will supposedly result in modulated/controlled levels of ethylene.

In the present study, the delayed ripening tomatoes were generated by silencing three homologs of ACC synthase gene in fruit-specific manner, using RNAi technology during the onset of fruit ripening, to decrease the climacteric effects of ethylene. The fruit specific homologs of ACS were considered in tandem for the RNAi-mediated down-regulation to eliminate any redundancy associated with gene function. Thus suppression of more than one homolog would be more effective over the shut-down of single homolog thereby, possibly resulting in the maximum suppression of autocatalytic burst in ethylene production. To alleviate the pleiotropic effects which could be damaging to plant growth, defense and development and might arise due to constitutive down-regulation of ethylene, fruit-specific regulation of RNAi-ACS was driven by Δ2A11 promoter. The ACS chimeric hpRNAi construct harbouring three fruit-specific homologues of ACS (ACS1A, ACS2 and ACS6) in sense and antisense orientation was prepared in pCAMBIA2300 under Δ2A11 promoter. The RNAi-ACS binary vector was mobilized into A. tumefaciens (LBA4404) and used for tomato transformation using the lab protocol.

The RNAi-ACS plants were raised with hpRNAi-ACS construct by Agrobacterium mediated transformation. The putative transformants were analyzed for integration of the introduced genes by PCR with NPT II gene specific primers. The RNAi-ACS tomato plants exhibited no morphological abnormalities and no alterations in flower development and fruit setting, when compared to wild-type (WT) plants. Red ripe fruits from RNAi-ACS as well as WT plants were evaluated for the ethylene levels. The fruits from RNAi-ACS tomato lines liberated reduced levels of
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Ethylene with varying degree of ethylene evolution in different RNAi-ACS lines. The ethylene evolution was found to be least in RNAi-ACS60 and RNAi-ACS81 lines (4-5%). To ascertain whether the observed decline in ethylene liberation in RNAi-ACS fruits is due to down-regulation of ACS gene expression (mRNA levels), the transcript levels of targeted homologs viz., ACS1A, ACS2 and ACS6, were studied and Northern hybridization was done to detect the ACS-specific siRNA formation. Fruits from RNAi-ACS60 line showed decline in ACS (all three) transcript levels, which correspond to the accumulation of the ACS-specific siRNAs in these fruits. Transcript levels of other genes involved in ethylene biosynthesis viz., ACS4, ACO1 and E8 showed decline, while that of TAGLI did not reveal any difference. Nevertheless, the decline caused ethylene suppression in RNAi-ACS fruits. These fruits also showed an enhanced accumulation of PA levels which correspond to an observed increase in SAMDC1 and SPDSYN transcripts over WT.

The fruits from RNAi-ACS tomato lines were further screened for the delayed ripening trait and other fruit quality parameters. Among the various lines screened for rate of respiration, RNAi-ACS 60 and RNAi-ACS 81 were found to be promising with respect to low respiratory activity of ~50% in harvested fruits in comparison to control fruits. Fruits from these lines also exhibited ~45 days delay in shift from BR to RR stage (on vine) and also exhibited prolonged shelf life of ~45 days (RR to shrivelling) in comparison to controls. A considerable reduction in mRNA levels of EXP1, TBG4, PG and XTH5 was also noted in the same fruits over WT. Such a reduction along with enhanced PA content in these tomatoes, explains the obtained extension in their shelf life. These RNAi-ACS fruits exhibited a very positive expression on post-harvest quality traits with an increase in total soluble solids (TSS) and titratable acids (TA) content over WT. Although the RNAi-ACS fruits showed up-regulated expression of GLDH1 and AO (ascorbic acid metabolism genes), no change in ascorbic acid content was found when compared to controls. Reduction in lycopene content in RNAi-ACS tomatoes was noted as compared to controls, which was found to be due to decrease in expression levels of lycopene biosynthesis genes viz., DXS1 and PSY1.
In the second part of thesis work, the evaluation of the of tomato transgenics (T₀ generation) over-expressing heterologous PA biosynthesis genes viz., LeADC, LeODC and LeSAMDC (selected promising lines) for fruit traits and expression pattern of few fruit-ripening specific genes was done. These transgenics were developed previously in our lab with over-expression of PA biosynthetic genes (oat-ADC, mouse-ODC and human-SAMDC) under the control of fruit-specific promoter (full-length 2A11). Earlier, these transgenics have shown to exhibit significant increase in PA levels and decrease/no change in ethylene production in fruits with a delay in fruit ripening, longer shelf-life of fruits, increased lycopene content and improved post-harvest qualities with respect to processing attributes. In the present work, few homozygous lines viz., LeODC23, LeODC27, LeADC38, LeADC51, LeSAMDC24 and LeSAMDC56, for each of these tomato transgenics has been identified in T₄ generation, via segregation analysis through PCR analysis. Further, stability of transgene expression and confirmation of improved fruit traits have been done in successive generations (T₂-T₄) of these tomato transgenics. The results showed the stable expression of improved fruit traits. The ethylene evolution rate was found to be the least in LeSAMDC53 line, LeADC93 and LeODC32. The lycopene content in transgenic tomatoes has been found to be significantly higher over the WT fruits. Lines ODC27, SAMDC24 and ADC93 were found to have significantly high levels of lycopene over control. Among the various lines analysed, LeADC93, LeADC25, LeADC30, LeODC27, LeODC32, LeSAMDC24, LeSAMDC16 were noted to be promised with respect to expression on post-harvest quality traits. These lines showed increase in TSS, TA, ascorbic acid (AsA), and total sugar content in comparison to WT fruits. Further to demonstrate the molecular basis of improved fruit traits, the expression profile of various fruit ripening genes was studied in transgenic and wild-type plants by semi-quantitative RT-PCR at different stages of fruit development. For some of the fruit ripening genes studied, an up-regulated expressions (native SAMDC, EXP1, TBG1, DXS1 and PSY1) was seen, while few other genes (viz. ethylene biosynthesis gene) showed down-regulation. Also, there were some other fruit ripening genes like native ODC, SPDSYN, E8and LES which showed no change in transcript level in transgenic tomato fruits over wild-type fruits.
In conclusion, the ACS chimeric RNAi construct designed to target the ripening-specific homologs of ACS effectively repressed the ethylene production in tomato fruits. These lines exhibited delayed ripening and extended shelf life of fruits for up to 45 days, with improved fruit juice quality. The ethylene suppression brought about compositional changes in these fruits by enhancing the PA levels. In addition, the inhibited ethylene has resulted in up-regulation of PA biosynthesis and ascorbic acid metabolism genes and down-regulation of cell wall hydrolysis and lycopene metabolism genes. The fusion of three ACS cDNA did not affect the non-target genes during fruit ripening. Our results thus suggest that the chimeric gene fusion can be used as an effective design for simultaneous silencing of more than one gene. It may also be concluded that by controlled suppression of ethylene, rate of ripening and over-ripening can be modulated, by simultaneous fortification of PAs. It is also suggested that PAs, may modulate the rate of ripening and over-ripening by counteracting the ripening promotion by ethylene. The observations made will be potentially useful in enhanced understanding of the ethylene and PA signalling during fruit ripening and thereby unrevealing the molecular mechanism underlying the role of these two molecules in effecting fruit quality traits.