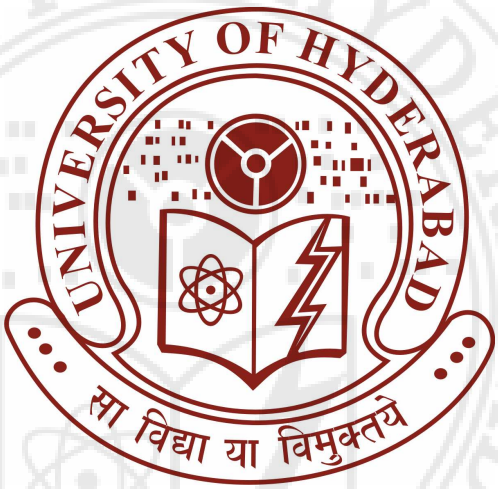
Induced Mutations As A Tool For Manipulating Tomato Fruit Ripening



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Induced Mutations As A Tool For Manipulating Tomato Fruit Ripening

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby declare that the work presented in this thesis entitled "**Induced mutations as a tool for manipulating tomato fruit ripening**" has been carried out by me under the supervision of Professor R.P. Sharma. Dept. of Plant Sciences, School of Life Sciences, University of Hyderabd, Hyderabad – 500 046, and this work has not been submitted for any degree or diploma of any other University.

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CERTIFICATE

This is to certify that the thesis entitled "**Induced mutations as a tool for manipulating tomato fruit ripening**" is based on the results of the work done by **Ms Santisree Parankusam** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any other University.

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To My Beloved Parents
&
Husband……

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----Santisree Parankusam

Abbreviations

A	Absorbance
Atr	Acetylene resistant mutant
adoMed	Adinosylmethionine
AC	Ailsa Craig
ACC	1-Aminocyclopropyl-1-carboxylic acid
ACS	ACC synthase
ACO	ACC oxidase
bp	Base pairs
B	Breaker
cm	Centimeter
DPA	Days post anthesis
dNTP	deoxy-nuclotide triphophate
CaC_2	Calcium carbide
EDTA	Ethylene diamine tetra acetic acid
EMS	Ethyl methane suphonate
EST	Expressed sequence tags
EtBr	Ethidium bromide
FW	Fresh weight
g	Gram
GC	Gas chromatograph
h	Hours
HPLC	High performance liquid chromatography
IAA	Indole acetic acid
Kb	kilo base pairs
Kin	Kinetin insensitive
L	Liter
µg	Microgram
µM	Micromolar
µl	Microliter
M_2	Second generation (of mutagenesis)

mRNA	Messenger RNA
MG	Mature green
mg	Milligram
ml	Milliliter
mM	Millimolar
min	Minutes
1-MCP	1-Methyl cyclo propene
cDNA	Complementary DNA
O	Orange
PCR	Polymerase chain reaction
PME	Pectin methyl esterase
PG	Polygalacturonase
RNA	Ribo nuclic acid
RT-PCR	Reverse transcriptase Polymerase chain reaction
R	Red ripe
rpm	Revolutions per minute
RT	Room temperature
ROS	Reactive oxygen species
SAM	S-adinosylmethionine
SAMDC	SAM decarboxylase
Sl	*Solanum lycopersicon*
TGRC	Tomato genetic research cooperative
T	Turning
WT	Wild type
v/v	volume in volume
w/v	Weight in volume
w/w	Weight per weight

Genes/Protein:

The symbol for genes is shown in italics and capitals (eg. *ETR*). The name of a mutation in a gene is italicized but not capitalized, eg. e*tr1-1*. the

symbol for the protein encoded by the gene uses the same letters and numbers, and is capitalized, but not italicized (eg. ETR1-1).

ACO	ACS oxidase
ACS	ACC Synthase
CTR	Constitutive triple response
EIL	EIN-Like
EIN	Ethylene insensitive
ERF	Ethylene response factor
ERS	Ethylene response sensor
ETR	Ethylene resistant

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Introduction

Tomato (*Solanum lycopersicum*) is the important system for genetic and molecular research of ethylene metabolism in solanaceae family. It has emerged as the primary model for climacteric fruit ripening for a combination of scientific and agricultural reasons. Simple diploid genetics, small genome size (0.9 pg per haploid genome)(Arumuganathan and Earle, 1991), short generation time, availability of genetic and genomic resources, extensive EST collection and a developing physical map render tomato among the most effective model crop system. In addition, numerous single gene mutations that regulate fruit size, shape, development, and readily quantifiable ripening phenotypes have enhanced the use of tomato fruit ripening studies. Economically tomatoes are valuable for their high nutritional value and large scale preparation of many processed items.

The ripening of a fruit represents the culmination of a series of biochemical processes that have evolved as a mechanism of seed dispersal. In the case of fleshy fruits, the changes that occur during ripening impart desirable characteristics to the fruit such as bright colors, softening, and sugar and volatile accumulations that attract animals and birds to aid dispersal. (Alexander and Grierson, 2002; Klee, 2002). Fleshy fruits are frequently harvested before ripening and ethylene burst. Following harvest they have a relatively short shelf life during which they undergo profound changes. Although ripening imparts desirable flavor, colour and texture, considerable expense and crop loss result from negative features of ripening caused by excessive softening and over-ripening. It has been estimated that post harvest losses in fresh fruit and vegetables is 5 to 25% in developed countries and 20 to 50% in developing countries. These negative attributes such as over ripening, senescence and physiological disorders like heat injury results in pathogen attach and post harvest loss of fruits and vegetables represents one greatest threat to a grower. Various facets of fruit ripening are stimulated by ethylene, though it certainly is not the only contributing component (Vrebalov *et al*., 2002). Modifications of ethylene synthesis and sensitivity to ethylene are promising methods to prevent spoilage of fruits. (Klee *et al*., 1991; Gray *et al*., 1994; Oeller *et al*., 1991; Ecker, 1995; Wilkinson *et al*., 1997; Knoester *et al*., 1998; Kopeliovitch *et al*., 1979). It is thought that knowledge of the identity and role of generic ripening-regulatory genes will enable the targeted manipulation of fruit ripening, fruit quality and shelf life.

In climacteric fruits like tomato, ethylene production is necessary for initiation and completion of the process of fruit ripening. The involvement of ethylene in regulating ripening has been defined by inhibitor studies, transgenic analysis through altered expression of genes involved in ethylene biosynthesis and signaling, and by characterization of mutants (Hobson *et al*., 1984; Lanahan *et al*., 1994; Wilkinson *et al*., 1995). Ethylene is a gaseous hormone has known to play a critical role in many diverse physiological processes, such as leaf and flower senescence, abscission of organs, flower initiation, fruit ripening, and seed germination (Abeles *et al*., 1992). The biosynthesis of ethylene is well established in higher plants (Yang and Hoffman, 1984) and exhibits a two step regulatory control. The first step catalyzed by the enzyme 1-aminocyclopropane-1-carboxylic acid (1-ACC) synthase (ACS), involves the conversion of S-adenosyl-L-methionine to 1-aminocyclopropane- 1-carboxylic acid (1-ACC) and the second step catalyzed by ACC oxidase (ACO), involves the conversion of 1-ACC to ethylene (Kende, 1993). The ethylene biosynthesis genes are coded by a multi-gene family whose expression is highly regulated in response to various internal and external stimuli mediated primarily through differential expression of ACC synthase genes (Nakatsaka *et al.*, 1998).In tomato, ethylene is perceived by ethylene receptors (ETR) that is encoded by a multi-gene family with at least six putative members (*ETR1-ETR6*). Ethylene is known to act as negative regulators of ethylene responses (Chang et. al., 1993; Ecker, 1995; Alexander and Grierson, 2002). Most of the mutants defective in ethylene perception or signal transduction were identified by exploiting the triple response of dark grown seedlings to ethylene or its precursor 1-aminocyclopropane-1-carboxylic acid (1-ACC). Although many steps of the ethylene signal transduction pathway have been identified, it is likely that others remain to be discovered. Identification of the components involved in signal transduction of ethylene or other factors that control or alter ethylene mediated responses will help us for improving the desired traits like fruit ripening and disease resistance.

The screening of seedlings for their ability to develop normally when exposed to exogenous ethylene or ethylene inducing hormones like cytokinins at levels that cause abnormal growth and morphogenesis in wild type has led to the identification of mutants with altered ethylene metabolism. In tomato very few

mutants have been identified that are defective in ethylene perception or action. The identified mutants such as *Nr* (*Never-ripe*) or *rin (Ripening-inhibitor)* show total block in ripening of tomato fruits, which limits their utility in tomato (Wilkinson *et al*., 1995; Lanahan *et al*., 1994; Vrebalov *et al*., 2002). The attempts to delay ripening of fruits using transgenic approach have demonstrated that specific silencing of genes leads to delay in ripening (Theologis *et al*., 1993; Picton *et al*., 1993a). These results have showed that in tomato like in Arabidopsis, additional loci can be identified by mutations which can lead to block in ethylene perception or action (Giovannoni, 2004). One advantage of having additional ethylene signal transduction mutants is few of them may have the phenotype related to fruit ripening. Screening and characterization of this kind of mutants may be important for the better understanding of the ethylene control of fruit ripening and to find out the additional signaling components.

In the present study we report the isolation and characterization of two new EMS induced mutants of tomato; *acetylene resistant mutant (atr-1)* and *kinetin insensitive (kin-1)* at the genetic, physiological, biochemical and molecular levels. Both of these mutants show pleiotropic affect of mutation at several stages of plant development including fruit ripening. Our study highlights that both loss of ethylene perception as well as reduction of ethylene production can improve the shelf life of tomato fruits. Identification of these novel loci responsible for delayed fruit ripening and enhanced shelf life of fruits may be useful to manipulate fruit ripening in other climacteric fruit as well. The stable mutant lines generated and characterized in this study can be useful to conventional breeding programs and further molecular and mapping studies.

Review of Literature

2.1 Tomato

Tomato is globally cultivated for its fleshy fruits and known as protective food because of its special nutritive value and its wide spread production. It is the world's largest vegetable crop after potato and it tops the list of canned vegetables. Tomatoes are eaten directly as raw vegetable or consumed in a variety of processed products like ketch-up, sauce, chutney, juice, diced, soup, paste, puree etc. It is a rich source of vitamin A and C, and also contains minerals like iron, phosphorus (Kalloo, 1991). Furthermore tomato is the richest source of nutrients, dietary fibers antioxidant like lycopene and beta-carotene, the compounds that protect cells from cancer (Hobson, 1993). Tomato has a short generation time of about three to four months. It is well fitted in different cropping systems of cereals grains, pulses and oilseeds. Hence, it is the most widely grown solanaceous vegetable crops grown worldwide under outdoor and indoor conditions.

2.1.1 Taxonomy of tomato

Tomato belongs to the family Solanaceae (also known as night shade family), genus *Solanum*, sub family Solanoidaeae and tribe solaneae (Taylor, 1986). The genus includes a small collection of cultivated and wild species like *S. lycopersicon Milli,* wild species like, *S. peruvianum, S. hirsutm, S. glandulosam, S .pimpinellifolium, S. cheesemannii.* Taxonomists recently changed the name as *Solanum lycopersicon.* The tomato is native to Central and South America. The tomato is a popular and versatile food. In tomato only fruits can be eaten since the leaves contain toxic alkaloids. The cultivated tomato, *Solanum* l*ycopersicon*, is the second most consumed vegetable worldwide and a well-studied crop species in terms of genetics, genomics, and breeding.

The tomato is perennial plant but usually grown as an annual plant. It is reported that the tomato plant can reach up to 3 meter. The stems are somewhat weak and often require staking or support such as a tomato cage. Branching at the base is monopodial, becomes sympodial higher up. The tomato leaves are

10 to 30 cm long and unevenly imparipinnate compound with variously indented or lobed margins. Both the stems and the leaves are slightly rough and fuzzy. The inflorescence of tomato bare small yellow flowers has five pointed lobes on the corolla. The tomato fruits are fleshy berries green when unripe and become deep red and shiny when ripe. The tomato cultivars differ a great deal in size, shape and colour. There are also yellow, orange, green and brown varieties of fruits. The shape can varies from small cherry tomatoes, pear shaped tomatoes to large irregular shaped beefy tomatoes. The shape, size and colour of tomato decide their market value. Number of processed items prepared on large scale for consumption as well as for export using different varieties of tomatoes.

2.1.2 Production of tomato

Previously tomatoes were grown only during favorable season, but now a days tomatoes are grown round the year. Because of its economic importance area under cultivation is increasing every year. The estimated area and production of tomato for India are about 3,50,000 hectares and 53,00,000 tons respectively. Worldwide tomato production in 2005 totaled 29.9 million metric tons and production for the 2005/2006 season in Europe totaled 10.6 million metric tons. Tomato is the second largest vegetable crop in India. The average productivity of tomato in our country is merely 158q/ha while its productivity in USA is 588q/ha, in Greece 498q/ha, in Italy 466q/ha and 465q/ha in Spain. Ten most promising states of India for tomato crop have been identified and utilized for further study on various aspects of tomato crop. Bihar State is at leading position followed by UP and Orissa in terms of area under tomato crop. The maximum production and productivity have been shown by UP followed by Karnataka, Punjab, West Bengal and Assam. India's export of value added tomato products was around 758.6 tons, which included 41 tons of canned tomato products, 38 tons tomato juice valued at and 595 tons ketchup in the year 2005-2006. This accounts for more than thousand crores to the Indian economy even with least price like three rupees per kilo. This significant achievement in tomato production is possible due to the development of high

yielding varieties/hybrids, breeding for biotic and abiotic stresses, resistance and heterosis breeding.

2.2 Classification of ripening fruits

Fruits are classically defined on the basis of the presence (climacteric) or absence (nonclimacteric) of synthesis of the gaseous hormone ethylene at the onset of ripening (Lelievre *et al*., 1997). Climacteric fruits, such as tomato, banana, mango, melon, apple, pear and peach are characterized by an extraordinary increment in ethylene production which accompanies the respiratory peak during ripening, called the 'climacteric crisis' (Abeles *et al*., 1992). Non-climacteric fruits are those whose maturation does not show increased respiration followed by ethylene burst, such as cherry, strawberry and pineapple. Interestingly, climacteric fruits span a wide range of angiosperm evolution, including both dicots (e.g., tomato) and monocots (e.g., banana). Nevertheless, members of the same (e.g., melon) or closely related (e.g., melon and watermelon) species are reported to include both climacteric and nonclimacteric varieties (Perin *et al*., 2002).

A great deal is known regarding specific downstream ripening processes in a several climacteric and nonclimacteric species, where as little is known about the regulation of ripening in nonclimacteric fruits or the upstream regulation of ethylene in their climacteric counterparts. Strawberry is the most widely studied system for nonclimacteric ripening while tomato is a model for climacteric fruit ripening, resulting in the identification and characterization of numerous ripening-related genes that affect cell wall metabolism, color, and aroma (Wilkinson *et al*., 1995). Although strawberry is readily transformed, the octaploid nature of cultivated strawberry limited genetic analysis in this species. Recent evidence of the MADS box regulation of ripening in both tomato and strawberry suggest common regulatory mechanisms operating early in both climacteric and non climacteric species (Vrebalov *et al*., 2002). The elucidation of the molecular basis of such early and common events represents an active frontier in fruit ripening research.

2.2.1 Tomato as an important model system for fleshy fruit ripening

Tomato is the centerpiece system for genetic and molecular research in the family Solanaceae. Tomato has emerged as a model for fleshy fruit ripening, in part due to its ease of use as a model system resulting from facilitating attributes including simple genetics, numerous characterized mutants, cross-fertile wild germplasm to promote genetic studies and routine transformation technology. Recently it has been taken for genome sequencing by an international consortium currently funded and supported by ten contributing countries (Giovannoni *et al*., 2006). From the stand point of view of genetic and molecular research, tomato has advantages such as ease of seed and clonal propagation, efficient cross and self pollination ability, short generation time (approximately 45-100 days) and year round growth potential in green house has made tomato as a plant of choice for fruit ripening studies as well.

The genetic map resulting from the relatively small genome (0.9pg/haploid genome, Arumuganathan and Earle, 1991) saturated with markers has been useful in identification quantitative trait loci (QTLs) regulating numerous fruit traits (Bucheli *et al*., 1999; Doganlar *et al*, 2000). High molecular weight insert genomic libraries are available in both yeast artificial chromosome and bacterial artificial chromosome vector systems to facilitate positional cloning and a limited number of characterized heterologous T-DNA insertion lines have been created. It is one of the earliest crop plants for which a genetic linkage map was constructed, and currently there are several molecular maps based on crosses between the cultivated and various wild species of tomato. The expanding tomato EST database, which currently includes 214,000 sequences, the new microarray DNA chips, and the ongoing sequencing project are expected to aid development of more practical markers (Van der Hoeven *et al*., 2002). Several BAC libraries have been developed that ease map-based cloning of genes and QTLs. Publicly available EST collections, microarrays and cDNA libraries of various tissues via the URL, http://www.tigr.org/tdb/Igi/index.html.

The high-density molecular map, developed based on an *S. lycopersicon* × *S. pennellii* cross, includes more than 2200 markers with an average marker distance of less than 1cM and an average of 750kbp per cM (Tanksley *et al*.,

1992). Different types of molecular markers such as RFLPs, AFLPs, SSRs, CAPS, RGAs, ESTs, and COSs have been developed and mapped onto the 12 tomato chromosomes. Efforts are being made to develop high-throughput markers with greater resolution, including SNPs. Markers have been used extensively for identification and mapping of genes and QTLs for many biologically and agriculturally important traits and occasionally for germplasm screening, fingerprinting, and marker-assisted breeding. Sequencing of the euchromatin portions of the tomato genome is paving the way for comparative and functional analysis of important genes and QTLs (Foolad *et al*., 2007). In addition, numerous single gene mutations that regulate fruit size, shape, development, and combined with dramatic and readily quantifiable ripening phenotypes (ethylene, color index, carotenoids, softening) have enhanced the use of tomato as a model for climacteric ripening.

2.2.3 Tomato ripening stages

Once the tomato fruit completes it development and attains final size then it is in MG stage. Then the fruit stop growing and starts ripening by sequential stage transition. Tomato ripening process sequentially passes through six stages (Fig.1), based on the percentage of the external colour: mature green (no external red coloration), Breaker (<10% red color at blossom end), Turning (10% to 30% of fruit surface having red color), Pink (30% to 60% of fruit surface having red shade), light red or Orange (60% to 90% of fruit surface having red colour), and Red (at least 90%-95% of fruit surface having red colour) (Fig. 1). The climacteric rise at ethylene observed in breaker stage which is the key regulator for all the changes during ripening. In developing countries usually fruit growers pick the fruits before breaker stage and apply exogenous ethylene to the fruits to induce ripening after reaching the

destination.

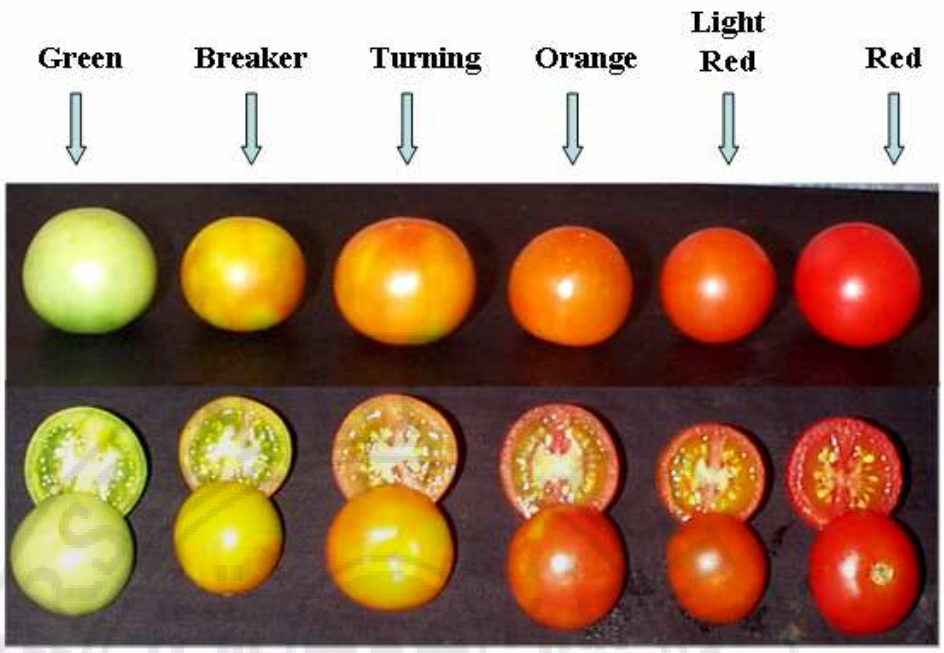


Figure 1: Fruit ripening stages of tomato

2.3 Fruit ripening

2.3.1 Fruit ripening

Fruit ripening is a developmental process that is exclusive to plants whereby mature seed-bearing organs undergo physiological and metabolic changes that promote seed dispersal (Seymour *et al*., 1993). Anatomically, fruits are swollen ovaries that may also contain associated flower parts. Their development follows fertilization, and occurs simultaneously with seed maturation. Initially, fruits enlarge through cell division and then by increasing cell volume. The embryo matures and the seed accumulates storage products, acquires desiccation tolerance, and loses water. The fruit then ripens (Fig.2). Fruit ripening is a highly coordinated, genetically programmed, and an irreversible phenomena involving a series of physiological, biochemical, and organoleptic changes, that finally leads to the development of a soft edible ripe fruit with desirable quality attributes (Seymour *et al.*, 2002).

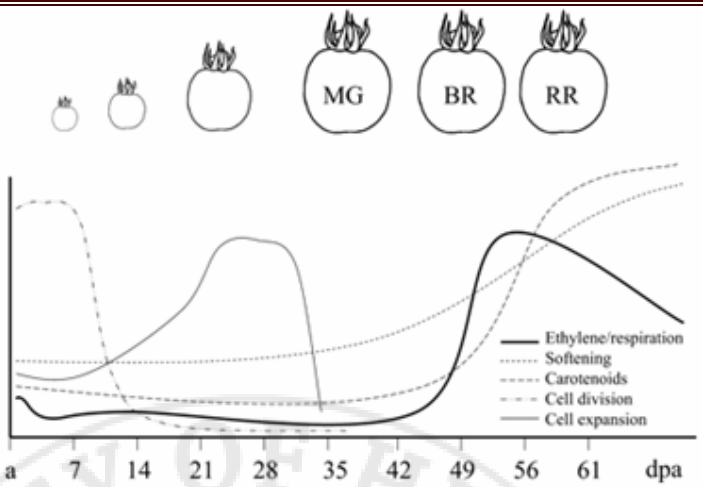


Figure 2. Major Developmental Changes during tomato Fruit Development and Ripening. Relative changes in cell division, cell expansion, respiration, ethylene synthesis, fruit softening, and carotenoid accumulation are shown over the course of fruit development. The time from anthesis (a) to mature green (MG; fully expanded unripe fruit with mature seed), breaker (BR; first visible carotenoid accumulation), and red ripe (RR) can vary substantially among cultivars. The time line shown would be for a medium-/large-fruit cultivar such as the breeding line MH1 (5 to 7 cm diameter mature fruit). (dpa, days after anthesis).(from Plant Cell (2004) Giovannoni, J. J. 16:S170-S180)

During maturation several structural and biochemical changes occur in fruit which confers on them specific organoleptic qualities, such as modifications in the external aspect, texture and flavor of the fruit. Although the specific biochemical programs resulting in ripening phenomena vary among species, changes typically include (1) modification of color through the alteration of chlorophyll, carotenoid, and/or flavonoid accumulation; (2) textural modification via alteration of cell turgor and cell wall structure and/or metabolism; (3) modification of sugars, acids, and volatile profiles that affect nutritional quality, flavor and aroma; and (4) generally enhanced susceptibility to opportunistic pathogens (likely associated with the loss of cell wall integrity). Fig. 2 explains the major changes that occur during fruit ripening. The series of cell divisions followed by a phase of cell expansion stops after reaching

maturity. The tomato maturation process is accompanied with alterations in the texture of the fruit, more specifically the loss of firmness, due to structural changes in the principle cell wall components (cellulose, hemicellulose and pectin). The change in the color of tomato fruit results from transformation of chloroplasts into chromoplasts and from the degradation of chlorophyll, as well from the accumulation of pigments such as carotenes and lycopenes, which are responsible for the orange and red color of the fruit (Gray *et al.*, 1992). Finally, the accumulation of sugars such as glucose and fructose and organic acids in vacuoles and the production of complex volatile compounds are responsible for the aroma and flavor of the fruit (Seymour *et al.*, 1993).

2.3.2 Importance of fruit ripening

The maturation and ripening of fleshy fruits contribute a major component of human diets, nutrition and agricultural activity. To date a large family of Arabidopsis MADS box genes influencing fruit development such as *AGAMOUS*, *SEPALLATA, APETALA*, *SHATTERPROOF* and *SEEDSTICK* were functionally defined (Alvarez-Buylla *et al*., 2000; Pelaz *et al*., 2000; Liljegren *et al*., 2000). In contrast to Arabidopsis, fleshy fruits such as tomato undergo a ripening process in which the biochemistry, physiology and structure of the organ are developmentally altered. There is much scientific interest in identifying the key regulatory mechanisms involved in fruit development and

ripening. Mutations in MADS box genes have been useful in defining its role in tomato pedicel abscission zone formation (Mao *et al*., 2000), fruit ripening (Vrebalov *et al*., 2002) and the functional basis of parthenocarpic (seedless) fruit development in apple (Yao *et al*., 2001). Several papers describe the role of ethylene in the ripening of climacteric fruit (Alexander and Grierson, 2002; Klee, 2002; Moore *et al*., 2002). They concentrate on the elucidation of biochemical and genetic signaling cascades that impact on the development and ripening of tomato fruit. Genomics tools have also been immensely useful in identifying and confirming the genes involved in fruit quality, and in defining the biochemical and molecular bases of texture, flavour, colour, and aroma.

Plants provide minerals and vitamins which humans can not produce including nonessential micronutrients that have been linked to the promotion of good health. Tomato fruits are a rich source of beta carotene, folate, potassium, vitamin C, vitamin E, flavonoids and lycopene. During the process of fruit ripening, changes in texture, color, flavor and aroma occur in addition to alteration in levels of vitamins and antioxidants (Jimenez *et al*., 2002; Ronen *et al*., 1999). In recent years, considerable attention has been placed on the enhancement of the nutritional value of crops as basic nutritional needs for much of the world's population remains unmet (DellaPenna, 1989).

Fruit biologists have studied numerous fruiting species with the intent of identifying strategies and technologies toward improving desirable ripening attributes while minimizing those with negative consequences. Key enzymes involved in fruit softening, and the genetic regulatory factors that influence fruit texture and shelf-life in tomato are being characterised (Seymour *et al*., 2002). Previous studies indicate that the differences in flavour between tomato varieties are due, at least in part, to variation in aroma volatile production (Brauss *et al*., 1998). Over 400 volatile compounds are detected in tomato fruit, although a group of seven including hexanal, hexenal, hexenol, 3-methylbutanal, 3-methylbutanol, methylnitrobutane, and isobutylthiazole are amongst the most important contributors to fruit aroma. In tomato fruit, linoleic and linolenic acid are the main LOX substrates, encoded by a family of at least five genes, *TomloxA* and *TomloxB* , *TomloxC* and *Tomlox D* and *TomloxE.* Analysis of the role that ethylene plays in the regulation of *TomloxA*, *TomloxB* and *TomloxC* during tomato ripening has shown that the individual isoforms are differentially regulated and may have different functions (Griffiths *et al*., 1999). Levels of *TomloxA* mRNA decrease as ripening progresses and *TomloxB* expression increases during ripening and is regulated by ethylene. *TomloxC* transcripts increase in response to ethylene. *TomloxC* and the mainly leaf expressed *TomloxD* differs from the other LOX enzymes in that they are chloroplast targeted. Molecular-markers for use in breeding programmes to improve the organoleptic qualities of tomato fruit have been developed using QTL approaches. Eventhough important regulatory points of ripening are known, lot many components are yet to be resolved. The ability to understand

key control points in global ripening regulation, such as carotenoid, flavonoid, vitamin and flavor volatiles, will allow manipulation of nutrition and quality characteristics associated with ripening.

2.3.3 Why to delay tomato fruit ripening

Ripening brings about highly desirable changes in tomato fruit character and chemistry in terms of flavor, appearance, texture and nutrition, the advanced stages of ripening lead to sub-optimal fruit quality and eventually post-harvest loss. Fleshy fruit like tomato is frequently harvested before ripening and ethylene burst, following harvest they have a relatively short shelf life during which they undergo profound changes. Excessive textural softening during ripening leads to adverse effects/spoilage on storage. From the agriculture stand point, ripening confers both positive and negative attributes to the resulting commodity. Due to improper storage, there is a loss in fresh weight of about 10-15%. This causes them to appear shriveled and stale, thus considerably lowering their market value and consumer acceptability.

It has been estimated that postharvest losses in fresh tomato is 5 to 25% in developed countries and 20 to 50% in developing countries. At the rate of rupees four per kg, this loss accounts to the loss of 800 crores per year for Indian farmers. These negative attributes such as over ripening, senescence and physiological disorders like heat injury results in pathogen attach and post harvest loss of fruits and vegetables, which represents one greatest threat to a grower. Hence the fruits with extended life would reduce the losses to farmers, distributors and consumers. The tomato fruits with delayed ripening or slow development would allow more time for shipment and storage.

2.3.4 Molecular biology of tomato fruit ripening

The physiological changes affect visual, textural, flavor, and aroma characteristics, making the fruit more appealing to potential consumers for seed dispersal. There is significant progress being made in identifying genes controlling the development of dry dehiscent fruits in the model plant species *Arabidopsis thaliana.* In plants with fleshy fruits, a major focus has been the

dissection of biochemical and genetic regulatory cascades controlling ripening, using tomato as a model species.

In plants, germination, growth, development, reproduction and environmental responses are all coordinated through hormones. Plants use hormones to communicate among tissues. In climacteric fruits like tomato, an increase in ethylene production is observed before the initiation of ripening, and ethylene is a trigger of the ripening process (Oeller *et al.*, 1991). Ripening usually begins in one region of a fruit, spreading to neighbouring regions as ethylene diffuses freely from cell to cell and integrates the ripening process throughout the fruit. However, not all the ripening-related events are dependent on ethylene; some are controlled by other hormonal and/or developmental factors. Both the ethylene-dependent and ethylene-independent pathways coexist to co-ordinate the ripening process (Lelièvre *et al.*, 1997; Alexander and Grierson, 2002). Although it has been suggested that both ethylene-dependent and ethylene-independent gene regulation pathways coexist to co-ordinate the process in climacteric and non-climacteric fruits, ethylene is the dominant trigger for ripening in climacteric fruit (Lelievre *et al*., 1997). Transgenic tomato fruits in which endogenous ethylene production was suppressed by the expression of an antisense ACC synthase, soften slowly. In these fruits, exogenous propylene or ethylene reversed the antisense phenotype, showing that softening was completely dependent on ethylene (Oeller *et al.*, 1991). Elucidating the mechanisms involved in ripening of climacteric fruit and the role that ethylene plays in the process is key to understand fruit production and quality.

2.3.5 Role of ethylene in plant development and post harvest management

Ethylene is a small, readily diffusible hormone that has an important role integrating developmental events with external stimuli. Several studies have demonstrated the intervention of this hormone in several phases of plant growth and development, such as fruit ripening (Abeles *et al.*, 1992), seed germination, leaf and flower senescence and abscission, root growth and development, somatic embryogenesis inhibition of stem and root elongation, leaf expansion, flower formation, root hair development and root nodulation, abscission, senescence and fruit ripening (Abeles *et al*., 1992; Mattoo and Suttle, 1991). It

is also known that ethylene is synthesized in response to different type of stress, such as wounding, very low and very high temperatures, flooding or drought, treatments with other hormones, heavy metals and attack by pathogens (Pech *et al.*, 1992).The ability of plants to perceive and respond to challenges in their environment is also critical to their survival. Ethylene synthesis can be induced by, and impact responses to, environmental stresses such as wounding, hypoxia and pathogen attack (Abeles *et al*., 1992).

In 1886 Neljubov discovered that ethylene was the biologically active component of illumination gas when he noticed that illumination gas was responsible for the horizontal growth of etiolated pea seedlings which he had been cultivating. In the 1930s most of the physiological effects of ethylene on plants had already been described (Pech *et al.*, 1992) and after this period ethylene became the object of numerous studies due to commercial interest related to its action on the ripening and conservation of fruit. The most widely documented example of the effect of ethylene on cell expansion is the triple-response phenotype exhibited by dicot seedlings grown in the dark in the presence of ethylene. With out ethylene, dark-grown seedlings have an etiolated morphology consisting of an elongated, slender root and hypocotyl with the development of a hypocotyl hook. In the presence of ethylene, seedlings develop a short, thickened root and hypocotyl with enhanced curvature of the apical hook due to ethylene inhibition of cell elongation (Guzman and Ecker, 1990). In contrast, in deepwater rice and other semiaquatic plants, ethylene acting with gibberellins has been shown to stimulate internode cell elongation (Kende *et al*., 1998).

From an agricultural perspective, not only is ethylene plays a role in disease resistance and stress tolerance, proper management of external ethylene plays a large role in postharvest handling procedures for a variety of fruits and vegetables. Just a few of the applications include: stimulation of flowering of pineapples and some flowering bulbs; promotion of fruit ripening; degreening of citrus; and defoliation of cotton (Reid, 2002). It has been estimated that post harvest losses in fresh fruit and vegetables is 5 to 25% in developed countries and 20 to 50% in developing countries (Kader, 2002). This is most certainly due

in part to various undesirable effects of ethylene such as: promotion of sprouting in potato; isocoumarin formation in carrots; abscission of leaves, flowers and fruits in ornamental plants; accelerated senescence through loss of chlorophyll in spinach, fresh herbs and broccoli; decreased shelf-life and over-ripening in numerous fruits (Reid, 2002). A greater understanding of the contributions of ethylene regarding fruit ripening, including a better understanding of interactions with other hormones and developmental factors, would facilitate the design of specific genetic tools to modify fruit and vegetable crops for enhanced quality, yield and nutritional value.

2.4 Ethylene and fruit ripening

2.4.1 Developmental and non-ethylene mediated control of fruit ripening

Naturally occurring mutants have been instrumental in dissecting ethylene and nonethylene mediated control of vegetative growth and fruit development (Gray *et al*., 1994). Evidence of the involvement of an ethylene-independent or developmental pathway that regulates ripening has come from the characterization of monogenic tomato mutants including *ripening-inhibitor* (*rin*), *non-ripening* (*nor*), and *Colorless non-ripening* (*Cnr*) in which ripening is severely impaired. These mutants fail to undergo an increase in ripening-related ethylene production and show inhibition of ripening-related gene expression, although gene expression but not ripening can be partly restored by ethylene treatment, indicating that they remain ethylene responsive (Tigchelaar *et al*., 1978; Della-Penna *et al*., 1989; Yen *et al*., 1995; Thompson *et al*., 1999). Although a complete molecular ripening pathway remains to be identified, an important first step toward this goal came from the discovery that the *rin* locus encoded a MADS-box transcription factor necessary for regulating ripening (Vrebalov *et al*., 2002).The *rin* mutant fruit does not undergo the usual increase in respiration and ethylene production during normal ripening, carotenoid accumulation is delayed and greatly reduced as the transition from chloroplasts to chromoplats is protracted and unsynchronized, and fruit softening is also inhibited (Tigchelaar *et al*., 1978). The *rin* mutant dos appear to be sensitive to ethylene in dark-grown seedlings (Lanahan *et al*., 1994), and during the processes of floral abscission, and petal and leaf senescence (Vrebalov *et al*.,

2002). Additionally, mutant fruit possesses the capability to produce ethylene similar to wild-type fruit in response to wounding or blossom end rot (Tigchelaar *et al*., 1978) indicating that *rin* represents a fruit specific ripening defect. While *rin* fruit do not ripen in response to exogenous ethylene, induction of some ethylene-responsive genes occurs (Lincoln and Fisher, 1988). Taken together, these results indicate that RIN is likely to act in ethylene-independent regulatory cascades during early stages of fruit ripening. *RIN* was eventually cloned and sequence identity reveals that it is a member of the MADSbox family of transcriptional regulators (Vrebalov *et al*., 2002). Plant MADS-box genes are usually associated with floral development in Arabidopsis and isolation of *RIN* has revealed a novel function for plant MADSbox genes in fruit development. Besides developmental factors, other hormones in addition to ethylene such as auxin, brassinosteroid, and cytokinin are likely to influence ripening though they are less-well characterized in this regard (Cohen, 1996; Martineau *et al*., 1994).

2.4.2 Role of ethylene in tomato fruit ripening

The involvement of ethylene in regulating ripening has been defined by inhibitor studies, transgenic analysis through altered expression of genes involved in ethylene biosynthesis and signaling, and by characterization of mutants (Hobson *et al*., 1984; Oeller *et al*., 1991; Picton *et al*., 1993b; Lanahan *et al*., 1994; Wilkinson *et al*., 1995; Hackett *et al*., 2000; Tieman *et al*., 2000). Furthermore, the expression of many genes encoding enzymes that cause to the ripe phenotype is known to be regulated by ethylene (Lincoln *et al*., 1987; Maunders *et al*., 1987; Zegzouti *et al*., 1999).

Fruits do not ripen uniformly. Ripening proceeds toward external tissue progressing from the blossom end toward the calyx. Ethylene is a readily diffusible gas within the confines of a fruit. To achieve full ripening, climacteric fruits, such as tomato require synthesis, perception and signal transduction of the plant hormone ethylene. Fruit ripening in tomato consists of two phases of ethylene production, that is system I and system II. In System I ethylene is auto-inhibitory, functions during normal vegetative growth, and is responsible

for basal levels of ethylene present in all tissues. In System 2, ethylene is auto-stimulatory and operates in climacteric fruit and during petal senescence.

Various facets of fruit ripening are stimulated by ethylene, though it certainly is not the only contributing component (Vrebalov *et al*., 2002) Fruits including tomato, banana, and apple undergo climacteric fruit ripening, characterized by a developmentally regulated, autocatalytic increase in ethylene production and associated rise in respiration. Non-climacteric fruits such as citrus, strawberry and grape, do not exhibit a dramatic change in respiration and ethylene remains at a very low level. Such fruits do not require ethylene for fruit ripening even though they may respond to ethylene. For instance, ethylene induces mRNA and pigment accumulation of flavor in orange and is used extensively in post-harvest practices in the de-greening of citrus.

The diversity and amplitude of fruit physiological and biochemical responses to ethylene suggested that this phytohormone controls the expression of many genes. Ethylene has indeed been shown to regulate expression of numerous genes related to ripening including: ACC synthases (Barry *et al*., 2000) and oxidases (Barry *et al*., 1996); *E4* (methionine sulphoxide reductase) and *E8* (dioxygenase) (Lincoln *et al*., 1987); *PSY* (phytoene synthase) (Bird *et al*., 1988); and *Tomlox A*, *B*, *C* (lipoxygenases), *PG* (polygalacturonse), and *SlEXP1* (expansin) (Alexander and Grierson. 2002; Zegzouti *et al*., 1999). The physiological importance of ethylene for fruit ripening has been demonstrated through analysis of tomato plants altered in their expression of genes involved in ethylene biosynthesis and perception, resulting in the inhibition of ripening and other ethylene associated responses (Klee, 1991; Lanahan *et al*., 1994; Oeller *et al*., 1991).

Using classical methods of differential screening of ripe fruit cDNA banks, several genes whose expression is regulated by ethylene or induced during ripening were isolated and cDNA clones corresponding to genes strongly expressed during tomato ripening were isolated (Mansson *et al*., 1985). To study ethylene-associated events during ripening, cDNAs corresponding to the mRNAs of ethylene-regulated genes have been isolated and characterized

(Mansson *et al.* 1985) and it has been demonstrated that the expression of some of these clones is regulated by ethylene (Pech *et al.*, 1992). Subsequently, other important genes expressed during ripening were isolated, identified and characterized, such as those encoding polygalacturonase (PG) and pectin methyltransferase (Smith *et al.*, 1990; Asif and Nath, 2005), heat shock proteins (Gray *et al.*, 1992 and 1994), histidine decarboxylase (Picton *et al.*, 1993b) as well the multigene families encoding the ethylene biosynthesis pathway enzymes ACC oxidase (Hamilton *et al.*, 1990; Flores *et al.*, 2002; Xiong *et al.*, 2005) and ACC synthase (Theologis, 1993; Hidalgo *et al.*, 2005). Other experimental methodologies like mutational studies also allowed the isolation of genes whose expression is regulated by ethylene and/or ripening.

The phenomenon of climacteric crisis is accompanied by important changes in gene expression and several cDNAs have been isolated corresponding with mRNAs that accumulate abundantly during the development and fruit ripening processes or in response to ethylene (Pech *et al.*, 1992). Other observations related to the action of ethylene on the physiology of fruit ripening reinforced this hypothesis. When submitted to either specific ethylene biosynthesis inhibitors or inhibitors which block the action of ethylene, tomato fruit shows strong inhibition of ripening but when fruits in the green-ripe stage were exposed to exogenous ethylene maturation could be activated (Gray *et al.*, 1992). All these show the role of ethylene as a modulator of fruit ripening and softness during fruit ripening (Brummell and Harpster, 2001).

2.4.3 Ethylene Biosynthesis

Ethylene biosynthesis is regulated by developmental processes as well as by numerous external stresses (Wang *et al.*, 2002). The ethylene biosynthesis pathway has now been completely elucidated due to advances in the techniques of biochemical analyses (Yang and Hoffman, 1984; Kende, 1993) The pathway of ethylene synthesis is well established in higher plants (Bleecker and Kende, 2000). Ethylene is formed from methionine via *S*-adenosyl-L-methionine (AdoMet) and the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is formed from AdoMet by the action of ACC synthase (ACS) and the conversion of ACC to ethylene is carried out by ACC

oxidase (ACO) (Kende, 1993). In addition to ACC, ACS produces 5'-methylthioadenosine, which is utilized for the synthesis of new methionine via a modified methionine cycle. This salvage pathway preserves the methylthio group through every revolution of the cycle at the cost of one molecule of ATP. Thus high rates of ethylene biosynthesis can be maintained even when the pool of free methionine is small. In this pathway it is well known that biosynthesis is subject to both positive and negative feedback regulation. Positive feedback regulation of ethylene biosynthesis is a characteristic feature of ripening fruits and senescing flowers in which exposure to exogenous ethylene or propylene results in a large increase in ethylene production due to the induction of ACS and ACO. The genes encoding these two enzymes have been cloned and characterized in several plant species and are known to belong to multigenic families whose members show strongly regulated expression (Zarembinski and Theologis 1994; Barry *et al*., 2000; Llop-Tous *et al*., 2000) and are differentially expressed in response to external stimuli including flooding, infection by pathogens and wounding as well as internal stimuli such as fruit ripening and senescence (Johnson and Ecker, 1998).

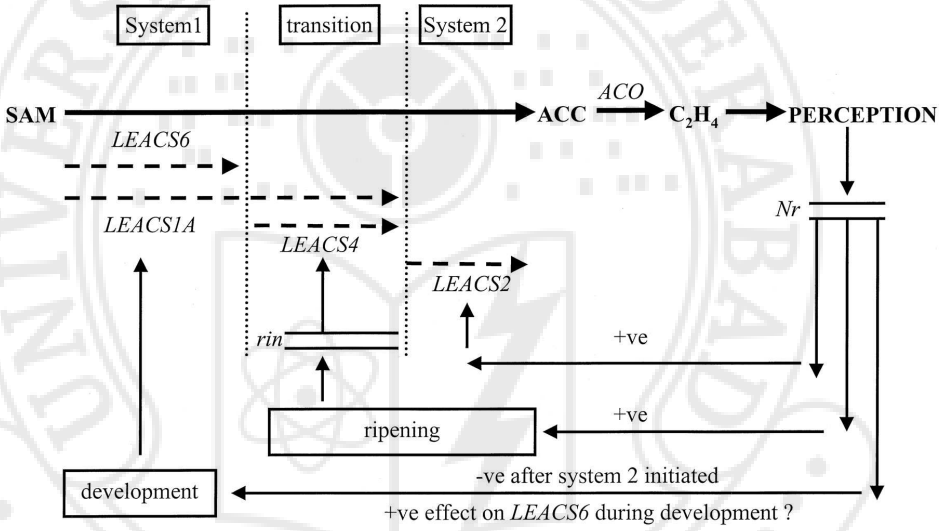
There are two pattern of ethylene synthesis in immature versus mature fruits. Immature fruits produce low levels of ethylene and exogenous ethylene treatment does not stimulate further synthesis (System 1). In contrast, the ethylene produced by ripening fruits is autocatalytic, stimulating its own synthesis (System 2) (Yang, 1987). The difference between the two systems can be explained at a molecular level by the lack of induction of *ACS* transcription, which is rate-limiting, during System 1 versus ethylene mediated induction during System 2 (Barry *et al*, 2000). Analysis of gene expression of members of the ACC synthase gene family in both the *rin* mutant and wild-type fruit has culminated in the model where System 1 ethylene is regulated by as yet unknown developmental pathway components through expression of *SlACS1A* and *SlACS6* (Barry *et al*., 2000). In fruit, transition from System 1 and System 2 is mediated by LeMADS-RIN which represents a key developmental signal indicating that the fruit has reached competency to ripen. During this transition period, combined induction of *SlACS1A* and *SlACS4* leads to the induction of *SlACS2* and autocatalytic ethylene production, defining System 2 (Fig. 3).

At least eight ACS genes have been identified in tomato (Sl*ACS1A*, *SlACS1B* and *SlACS2–7*), (Zarembinski and Theologis, 1994; Oetiker *et al*., 1997; Shiu *et al*., 1998). Recent studies of the ACS crystal structure and PLP co-factor binding (Huai *et al*., 2001) have confirmed similarity between the ACS catalytic binding site and those of other PLP-dependent aminotransferases. *SlACS1A* and *SlACS6* are involved in the production of system 1 ethylene in green fruit (Barry *et al*., 2000). During the transition period between system1 and system 2, *SlACS1A* expression increases and *SlACS4* is induced. During this transition autocatalytic ethylene production is initiated and maintained by ethylene-dependent induction of *SlACS2*. In addition, there is evidence to suggest regulation of these genes can occur beyond the level of gene expression. For example, the *SlACS2* protein from tomato is post-translationally modified through phosphorylation in response to wounding (Tatsuki and Mori, 2001). One model in Arabidopsis predicts the binding of a hypothetical inhibitor, possibly encoded by *ETO1* (ethylene overproducer), to *ACS5* (the Arabidopsis gene which corresponds to *SlACS2*) that could prevent activity of ACS5 until it is released through phosphorylation (Wang *et al*., 2002). It is likely, based on conservation of the phosphorylated serine residue, that other ACS genes undergo the same general form of negative regulation which would account for the rapid change (within seconds) in ACS activity in response to wounding, bypassing the requirement for ACS gene transcription (Wang *et al*., 2002).

It has recently been shown that *SlACS2* is phosphorylated in wounded tomato fruit and is not truncated (Tatsuki and Mori, 2001). It is shown that the role of phosphorylation is not to regulate the specific activity of the enzyme but to control the rate of enzyme turnover (Spanu *et al*., 1994). The phosphorylation of ACS protects the protein from degradation, which in turn could cause ACS to accumulate and ACS activity to increase, accounting for the burst of ethylene produced by ripening fruit. The rise in ACO activity precedes ACS activity in preclimacteric fruit in response to ethylene, indicating that ACO activity is important for controlling ethylene production (Lui *et al*., 1985). The first *ACO* gene was identified through antisense expression of a clone, pTOM13, then of unknown function (Holdsworth *et al*., 1987). A further three *ACO* genes have

been identified in tomato in response to wounding and during flower development, leaf and flower senescence and fruit ripening (Holdsworth *et al*., 1988; Barry *et al*., 1996; Blume and Grierson, 1997; Nakatsuka *et al*., 1998). *SlACO1* and, at a lower level *SlACO3*, are expressed at the onset of fruit ripening. *SlACO1* transcripts peak at breaker +3 and then fall back to levels observed at breaker, whereas *SlACO3* transcripts are only transiently expressed at breaker before disappearing.

Figure 3. Model proposing the regulation of ACS gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. (Barry, C. S., *et al*. Plant Physiol. 2000)



2.4.4 Ethylene Perception and Signal Transduction Pathway

Plants show a great diversity of physiological responses to ethylene depending on the stage of development and organ or tissue. The diversity and amplitude of these responses shows existence of several molecular mechanisms of regulation by ethylene. Most information regarding the steps involved in ethylene perception and signal transduction has been realized through studies of the model plant species *Arabidopsis thaliana*. One of the most valuable mutant screens in Arabidopsis for elucidating mechanisms of hormone signal transduction is based on alteration of the seedling triple response to ethylene.

This screen has been utilized to identify most of plant ethylene signal transduction mutants identified to date (Bleecker *et al*., 1988; Ecker, 1995; Kieber, 1997). The ethylene receptor ETR1 was the first protein to be unambiguously identified in Arabidopsis as a phytohormone receptor. ETR1 is a member of a family of five proteins (ETR1, ETR2, EIN4, ERS1, and ERS2) in Arabidopis. It was also the first protein with homology to His kinases to be identified in a higher eukaryote (Chang *et al*., 1993). Ethylene receptors have the homology to bacterial two-component receptors, which consist of a sensor protein, and a separate response regulator protein that functions together, allowing bacteria to respond to different environmental conditions (Chang and Stewart, 1998). All ethylene receptors have a sensor domain that can be subdivided into a transmembrane domain and a GAF domain (found in cGMP phosphodiesterases, adenylate cyclases and Fh1a transcription factors), a histidine kinase domain and a response domain. At the level of perception, sensitivity to hormones can be regulated both spatially and temporally during the life cycle. An example of spatial regulation is the differential response to a hormone that occurs during organ abscission. Temporally, sensitivity of an organ to a hormone may change during maturation, as occurs during fruit ripening (Klee and Tieman., 2002).

2.4.4.1 Ethylene receptors in tomato

Over the last few years, great progress has been made in elucidating the genes involved in ethylene action in tomato. Tomato has a family of at least six putative ethylene receptors, *SlETR1*, *SlETR2* (Zhou *et al*., 1996; Lashbrook *et al*., 1998), *NR/SlETR3* (Wilkinson *et al*., 1995; Payton *et al*., 1996), *SlERT4*, *SlETR5* (Tieman and Klee, 1999), and *SlETR6* (Ciardi and Klee, 2001). At molecular level tomato receptors are quite divergent, exhibiting less than 50% identity in primary sequence at the extremes. These receptors have a potential extra amino terminal membrane-spanning domain. Only one, *NR,* lacks the receiver domain. Three (*SlETR4–6*) are missing one or more conserved HK domains. Each ethylene receptor is expressed in different temporal and spatial patterns dependent on developmental stage and external stimuli (Klee *et al*., 1991; Klee *et al*., 2002; Tieman et. al., 2001; Solano *et al*., 1998; Deikman,

1997). *SlETR1* and *SlETR2* are expressed constitutively in all tissues throughout development with *SlETR1* expressed about 5-fold higher level than *SlETR2*. *NR* is up-regulated at anthesis and both *NR* and *SlETR4* are up-regulated during ripening, senescence, abscission (Tieman *et al*., 2000) and pathogen infection. The level of *NR* drops approximately 10-fold until the onset of ripening whereupon it rises about 20-fold. The ripening-associated rise in expression is an example of developmentally dependent ethylene inducibility. The pathogen inducibility of *SlETR4* is associated with increased ethylene synthesis following infection (Ciardi *et al*., 2001). In lines where *NR* expression has been reduced by 90%, *SlETR4* expression increases to compensate for the *NR* reduction. In antisense *SlETR4* lines, no such compensation occurs and overall receptor content is significantly reduced. Loss of *SlETR4* results in dramatic morphological changes associated with increased ethylene responsiveness. Strongly epinastic petiole, accelerated senescence of flower and delayed ripening of fruit were observed in the transgenic tomato mutant resulting from transcript reduction of *SlETR4* (Tieman *et al*., 2000; Knoester *et al*., 1998). *SlETR5* is also expressed in fruit, flowers and during pathogen infection (Tieman and Klee, 1999). The reduction in the levels of either of two family members, *SlETR4* or *SlETR6*, causes an early-ripening phenotype. These receptors are rapidly degraded in the presence of ethylene, and that degradation probably occurs through the 26S proteasome-dependent pathway. Ethylene exposure of immature fruits cause a reduction in the amount of receptor protein and earlier ripening. The fruit-specific suppression of the ethylene receptor *SlETR4* causes early ripening, whereas fruit size, yield and flavour-related chemical composition is largely unchanged (Kevany *et al*., 2007, 2008). Some expected and unexpected phenotypes related to ethylene response, such as shorter internodes and delayed abscission, resulted from the ethylene receptor gene *SlETR1* transgenic tomato (Whitelaw *et al*., 2002) and reduced expression of *SlETR1* in tomato. The antisense *SlETR1* displayed shorter length of seedling grew in the dark and adult plant in the light, severe epinastic petiole, and accelerated abscission of petiole explant and senescence of flower explant, compared with its wild type. The antisense *SlETR2* also exhibited shorter hypocotyls and slightly accelerated abscission (Wang *et al*., 2006).

It has been proposed that the response regulator of ETR1 forms a homodimer when unphosphorylated (Muller-Dieckmann *et al*., 1999) and that phosphorylation of the receptor in the presence of ethylene causes monomerization of the response regulator that may, in turn, inactivate CTR1. There are at least three genes encoding proteins with significant homology to CTR1 in tomato (Lin *et al*., 1998; Zegzouti *et al*., 1999). One of these, *LeCTR1*, has been shown to complement functionally the Arabidopsis *ctr1* mutation (Leclercq *et al*., 2002). The *CTR1* gene is also a negative regulator of the pathway acting downstream of the receptors and encodes a protein with similarity to the Raf family of Ser/Thr protein kinases (Kieber *et al*., 1993). *CTR1* may interact directly with the receptors (Clark *et al*., 1998) and has been proposed to represent the head of a putative mitogen-activated protein kinase cascade (Chang and Shockey 1999). The *CTR1* product acts downstream to ethylene receptors in the ethylene signal transduction pathway and corresponds to a negative regulator of other cascade components identified in *Arabidopsis*, including the *ethylene-insensitive* genes (*EIN2* and *EIN3*) and the *ethylene-response-factor* (*ERF1*) (Giovannoni, 2004; Stepanova and Alonso, 2005).

Studies shown that transcription factors are part of the ethylene signal transduction pathway, with the cloning of the *EIN3* gene encoding a nuclear protein providing the first direct evidence of nuclear regulation in this transduction pathway (Chao *et al*., 1997). The signaling events from CTR1 to the nucleus is unclear but appear to involve *EIN2*, a novel integral membrane protein with homology to mammalian natural resistance-associated macrophage protein metal ion transporters (Alonso *et al*., 1999) that acts downstream of the receptors and CTR1. *SlEIN2* is a transmembrane protein encoded by a two genes in tomato. Gene expression is unaltered during fruit development and is not ethylene inducible. Antisense reduction of expression delays ripening, as would be predicted on loss of function The biochemical function of *EIN2* remains unknown, but genetic studies have indicated that all ethylene responses described to date are transduced through this signaling intermediate. *SlEIN2* positively mediated ethylene signals during tomato development and *EIN2* might act as an important component for crosstalk between ethylene and auxin

hormones. The expression of *SlEIN2* was constant at different stages of fruit development, and was not regulated by ethylene.

A family of transcription factors encoded by *EIN3* and *EIL* (*EIN3-like*) act downstream of *EIN2*. In the case of EIN3, it consists of three genes in tomato (Tieman *et al*., 2001). Each of these genes was shown to complement functionally the Arabidopsis *ein3* mutation. The tomato EIN3 genes appear to be functionally redundant and expression of all three must be reduced before ethylene signaling is measurably affected. It is likely that the kinase cascades initiated by the *SlETR/SlCTR* complex eventually impact on the expression of the *SlEIL1–3* transcription factors (Alexander and Grierson, 2002). Ethylene signaling in the nucleus is mediated by the *EIN3* family of transcriptional regulators, which act directly on ethylene response factors to activate ethylene-inducible gene expression (Chao *et al*., 1997; Solano *et al*., 1998). There is little alteration in gene expression throughout growth and development and none of the genes are ethylene inducible. These are homologues of the Arabidopsis *EIN3* gene and have been shown to function as positive regulators of many ethylene responses, probably through the activation of *ERF1* transcription factors which upregulate the transcription of ethylene-responsive genes. The EIN3-like proteins (EILs, *e.g.* EIL1 and EIL2) also belongs to this family and are regulated by upstream cascade components (Bleecker and Kende, 2000; Giovannoni, 2004; Stepanova and Alonso, 2005).

The search for promoters for the *EIN3* gene family led to the identification of the *ERF1* gene (Solano *et al.*, 1998), a member of the large family of plant-specific transcription factors called *ethylene-response-element-binding-proteins* (EREBPs) originally identified as DNA-binding proteins which bind to promoter-specific elements in ethylene-inducible elements (Ohme-Takagi and Shinshi, 1995). Recently, four new members of the ERF family of plant-specific DNA-binding (GCC box) factors have been isolated from tomato fruit (SlERF1-4) and are being characterized (Tournier *et al.*, 2003). Homodimers of EIN3, EIL1, and EIL2 bind to a defined target in the promoter region of the transcription factor, *ETHYLENE RESPONSE FACTOR1* (*ERF-1*). *ERF1* is a member of a multigene family of transcription factors and is important in the

regulation of downstream ethylene responsive genes via binding to the "GCC" box promoter element. EIN3, and presumably the EIL proteins, is rapidly degraded by the ubiquitin/proteasome pathway without ethylene but accumulates to much higher levels in ethylene-treated plants. Ethylene responses are regulated at the level of EIN3 via ubiquitin/proteasome-dependent proteolysis mediated by the F-box proteins, EBF1 and EBF2. The F-box proteins that mediate this degradation is themselves positively transcriptionally regulated by ethylene (Fig. 4).

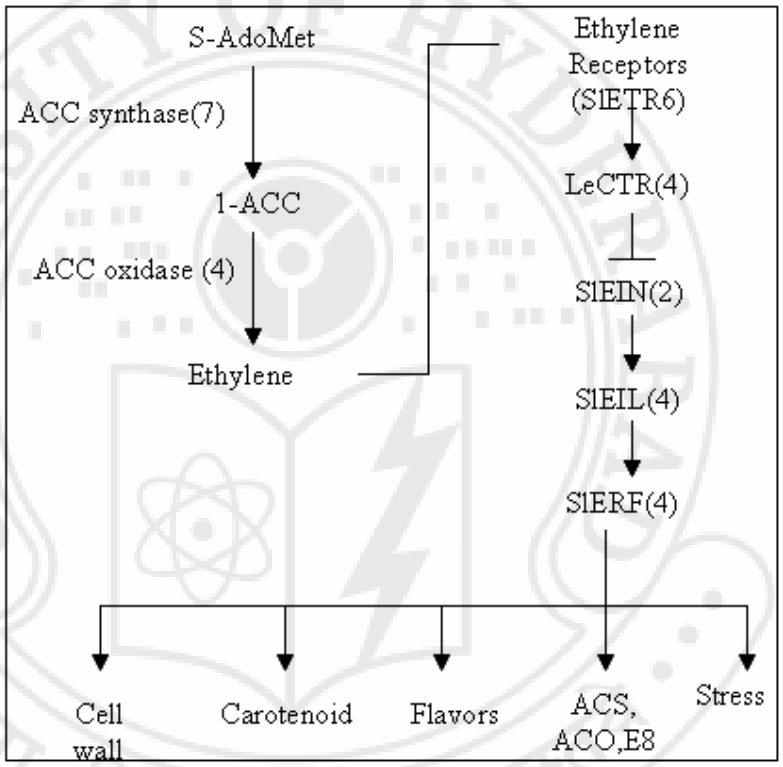


Figure 4. Schematic representation of ethylene biosynthesis and perception in tomato. The potential target processes of ethylene during ripening were also mentioned.

2.4.5 Cross-talk with other hormones and signaling molecules

One way in which multiple hormones interact to modulate plant development is through induction of biosynthesis of one hormone by another or through posttranscriptional/ translational modification of the genes involved in biosynthesis. This is most certainly the case regarding the regulation of ethylene biosynthesis as evidence for induction of ACC synthase gene expression by

application of another hormone is abundant in the literature. Several representative examples include: auxin regulation of ethylene biosynthesis through induction of *ACS4* in Arabidopsis and *ACS1* and *ACS2* in pea (Abel *et al*., 1995; Peck and Kende, 1998), cytokinin elevation of ethylene biosynthesis through post-transcriptional modification of ACS5 (Vogel *et al*., 1998), and brassinosteriod enhanced *ACS7* gene expression in mung bean (Yip *et al*., 1992).

Interactions between ethylene and other plant hormones are also being uncovered as mutations that were initially identified for alterations in response to one hormone often turn out to influence the sensitivity to another hormone or signaling molecule. For example, the *eir1* (*ethylene insensitive roots*) mutant that shows ethylene-insensitivity only in the roots (Roman *et al*., 1995) turned out to have a defect in an auxin transport protein. Likewise, the expression of the *HLS1* (*hookless1*) gene is regulated by ethylene but encodes a putative acetyltransferase that presumably controls auxin transport (Lehman *et al*., 1996). New *ein2* mutant alleles have turned up in screens for resistance to inhibition of auxin inhibitors (Fujita and Syono, 1996) and resistance to low levels of cytokinin (Cary *et al*., 1995). Additionally, new mutant *ctr1* and *ein2* alleles were recovered in screens for enhancers and suppressors, respectively, of the ABA-resistant seed germination mutant *abi1-1* (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). Unexpectedly, while *ein2* showed increased seed ABA responsiveness, it exhibited reduced ABA responsiveness in the roots (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). Screens for sucrose sensitivity resulted in identification of a sugar-insensitive mutant (*sis1)* which was found to be allelic to *ctr1* (Gibson *et al*., 2001) and characterization of the glucose-insensitive mutant *gin1* revealed that this mutant could be phenocopied in wild-type plants through application of exogenous ethylene (Zhou *et al*., 1998). It cannot be ruled out that abnormal ethylene sensitivity indirectly results in the phenomena observed in these hormones and sugar sensitivity assays. However, one example of how two separate linear signal transduction pathways could be communicating at the molecular level is illustrated below. Using *ein2* and *coi1* mutants deficient in ethylene and jasmonate responses, respectively, it was shown that activation of both ethylene and jasmonate pathways is required for

induction of the plant defensin gene *PDF1.2* in Arabidopsis and that these hormones act synergistically to induce *PDF1.2* expression (Penninckx, *et al*., 1998).

More recently, it has been shown that ERF1, a likely activator of PDF1.2, acts as a downstream component in both ethylene and jasmonate signaling pathways (Lorenzo *et al*., 2003). Not only is *ERF1* expression upregulated by both jasmonate and ethylene, ERF1 over-expression is sufficient to restore *PDF1.2* expression in *coi1* mutants. There appear to be a multitude of positive and negative interactions between different plant hormones and ethylene depending on the tissue and developmental stage of the plant. Thus, the type of response to a given stress or developmental event will likely depend on the positive or negative interaction that is established between ethylene and other hormonal signaling pathways. In establishing where "cross-talk" actually exists, it will be important to determine that the components of the two signaling pathways are expressed in the same cell and physically interact under normal physiological conditions (Wang *et al*., 2002).

2.5 Manipulation of tomato fruit ripening

Today, tomatoes are plucked from the vine early, when still green and firm, to ensure that they survive shipping without bruising and rotting. Picking tomatoes early means they have less chance to develop flavor, color, and nutrients naturally. To understand the function of specific genes and their role in ethylene metabolic pathways, as also to identify the key steps in their co regulation mechanisms of fruit ripening, several approaches have been exploited, including mutagenesis, genetic transformation, and transcriptome analysis. There are two major approaches followed to manipulate fruit ripening process in tomato. One is the transgenic approach and other is the non transgenic approach.

2.5.1 Manipulation of tomato fruit ripening by transgenic approach

The generation of targeted mutations using sense and antisense genes provides a means of manipulating endogenous gene expression, both for answering

fundamental questions and for crop improvement. The manipulation of tomato fruit quality through genetic engineering is reasonably well advanced. Many important processes in plants are regulated by ethylene, especially those involved with post-harvest processes. There are several methods available to manipulate ethylene responses. First, by blocking ethylene synthesis: with antisense RNA for *ACC synthase* or *ACC oxidase*, using gene silencing or co-suppression, by metabolic interference, diverting SAM or ACC away from ethylene synthesis. Secondly, by blocking the expression of genes that are induced in response to ethylene by antisense RNA for *PME* or *PG*. Third, by blocking the perception of ethylene by expression of a dominant mutant ethylene receptor.

The first genetically engineered fruits to achieve commercialization were marketed as improved-taste fresh tomatoes; this was accomplished by stopping or slowing the ripening process, allowing fruits to be picked later when they are more flavorful**.** More recent entries in the marketplaces are higher solids tomatoes, leading to better processing characteristics, and slower and more uniform ripening cherry tomatoes. These varieties and others in the research pipelines will provide farmers and processors with new ways to combat old problems. Several molecular approaches, including positional cloning, QTL mapping and genetic engineering, are helping to define the biochemical and molecular bases of texture, flavour, colour, and aroma. To identify the function of genes and their role in the ripening process, an antisense RNA strategy has been used by several research groups and several transgenic plants showing reduced expression of ripening related genes have been obtained (Gray *et al.*, 1994; Stearns and Glick, 2003). As the understanding of the biology of fruit ripening has improved, so the ability to improve the organoleptic and nutritional qualities of fruits through crop management, breeding or biotechnology.

2.5.1.1 Manipulation of fruit softening by transgenic approach

One important target for modification of tomato fruit ripening that is a is the softening of the fruit. There are two reasons why this is of interest. First, it is possible that fruit soften more slowly could be harvested after they have started to ripen on the vine, but still shipped and marketed before they have started to

deteriorate. This would allow the fruit to develop more flavor on the vine, an important trait that would improve consumer acceptance. Second, altering the softening of tomato fruit might alter the processing properties of the fruit to improve the efficiency and/or quality of the final processed product. During fruit ripening, various enzymes that degrade specific components of the cell walls are synthesized in the fruit. The events such as dissolution of the middle lamella, resulting in a reduction in intercellular adhesion, depolymerization, and solubilization of hemicellulosic and pectic cell wall polysaccharides are accompanied by the increased expression of numerous cell wall degrading enzymes, including polysaccharide hydrolases, transglycosylases, lyases, and other wall loosening proteins, such as expansin (Rose *et al*., 2003; Brummell, 2006). Among the enzymes that accumulate in the fruits are cellulases (to break down cellulose), and polygalacturonase (PG) and pectin methylesterase (PME), both of which are involved in breakdown of the pectin crosslinking molecules. These enzymes contribute to the softening of the fruit by reducing the rigidity of the cell wall structures. The expression of the genes encoding these enzymes are regulated by ethylene.

2.5.1.1.1 Polygalacturonase (PG)

Polygalacturonase or PG is an enzyme that degrades pectin in fruit cell walls and, together with other enzymes, causes the softening of fruits during fruit ripening. Antisense RNA techniques have been developed to generate novel mutant tomatoes in which the biochemical function of this enzyme and its involvement in fruit softening has been tested (Bird *et al*., 1988). The transgenic fruit with decreased levels of PG activity: 1) do not get overly soft when ripe, 2) show less damage due to fungal infection and 3) have elevated levels of soluble solids. The Calgene (Davis, CA) FlavrSavr^{TM} tomato based on antisense PG gene was commercialized in the U.S.market in 1995, marketed as a more flavorful fresh tomato. This tomato was modified by turning off PG synthesis using antisense technology (Redenbaugh *et al*., 1992). It was claimed that softening of the fruit was slowed in these fruit, allowing them to remain on the vine longer, with harvest later than the typical "mature green fruit" stage. The tomatoes could then be shipped and marketed before they spoil (Langley *et al*.,

1994). However, the product was not a commercial success and was withdrawn from the market after less than one year. Zeneca (U.K.), in collaboration with Peto Seeds (Wood- land, CA) and Hunt Wesson (Fullerton, CA), created a similar variety, which was also withdrawn from market in England.

2.5.1.1.2 Pectin methylesterase (PME)

PME is involved in metabolism of pectins in the cell wall. Pectins in mature green fruit are long polymers, and PME is expressed during fruit ripening to break these large polymers into shorter molecules. It is likely that PME is one of the first enzyme involved in the metabolism of pectins. Transgenic plants with low expression of pectin methylesterase (PME) in the fruits were developed again using antisense RNA approach (Tieman *et al*., 1992; Hall *et al*., 1993). The pectins in these fruit with reduced PME activity remain large as the fruit ripen. They are not broken down into shorter pectins because there is no PME activity. However, transgenic plants with reduced PME levels ripen normally as it does not interfere with ethylene production, the central regulator of fruit ripening. Suppression in transgenic fruit resulted in reduced pectin depolymerization, however there was no effect on firmness during ripening (Tieman and Handa, 1994). The outcome of this modification is that using this transgenic tomato fruit has more viscous juice as the starting material. In turn, it requires less processing to produce tomato paste of the desired consistency. These transgenic tomato potential to the cost of downstream processing during tomato product preparation, and perhaps improving the quality of their final product.

2.5.1.1.3 Other enzymes

Softening fallowing ripening proved to be significantly reduced in transgenic tomato fruit with suppressed β-galactosidase, an enzyme that is normally up-regulated during the early stages of ripening and serves to remove pectic galactan side chains (Smith *et al*., 2002). In addition, expansin proteins appear to play an integral role in fruit softening, probably by disrupting hydrogen bonding between cellulose microfibrils and matrix polysaccharides, resulting in loosening of the cell wall structure (Brummell and Harpster, 2001). In these

transgenic plants also, overall ripening process was not affected and fruit firmness was also not significantly differ from that of control plants except the improved juice viscosity.

2.5.1.1.3.1 LeRab11a

A cDNA clone from tomato fruit encodes a protein with strong homology with the *rab11/YPT3* class of small GTPases that is thought to be involved in the control of protein trafficking within cells. The corresponding mRNA *LeRab11a*, was developmentally regulated during fruit ripening, and its expression was inhibited in several ripening mutants. These antisense fruits changed color as expected but failed to soften normally. This was accompanied with reduced levels of two cell wall hydrolases, pectinesterase and polygalacturonase. There were other phenotypic effects in the plants, including determinate growth, reduced apical dominance, branched inflorescences, abnormal floral structure, and ectopic shoots on the leaves.

2.5.1.1.3.2 Phospholipase D

Phospholipase hydrolyze phospholipids, which are the backbones of the biological membranes. The transgenic tomato fruits (Solanum l*ycopersicon* cv. Celebrity) transformed with an antisense phospholipase D (PLD) cDNA construct, resulted in a 30-40% reduction of PLD activity in ripe fruits. The transgenic fruits were firmer, possessed better red colour, and flavour. The dry matter and ash contents, as well as the precipitate weight ratio (PPT) of the transgenic fruit products were significantly higher when compared to the products from the control fruits. The vitamin C content of the transgenic fruits was also higher compared to the control fruits. The results suggest that a reduction in PLD activity may lead to increased membrane stability and preservation of membrane compartmentalization (Pinhero *et al*., 2003).

2.5.1.1.3.3 Deoxyhypusine synthase

Regulation of expression of programmed cell death, including senescence, in plants is achieved by integration of a gene or gene fragment encoding

senescence-induced deoxyhypusine synthase, senescence-induced elF-5A or both into the plant genome in antisense orientation. DHS mediates the first of two sequential enzymatic reactions that activate eukaryotic translation initiation factor-5A (eIF-5A) by converting a conserved Lys to the unusual amino acid, deoxyhypusine. DHS levels were suppressed by anti sense approach under constitutive promoter in tomato (Wang *et al*., 2005). The suppression of *DHS* has pleiotropic effects on growth and development of tomato. Fruit from the transgenic plants ripened normally, but exhibited delayed postharvest softening and senescence that correlated with suppression of DHS protein levels. Transgenic plants in which *DHS* was more strongly suppressed were male sterile, did not produce fruit, and had larger, thicker leaves with enhanced levels of chlorophyll.

2.5.1.2 Manipulation of fruit pigments and flavors

Tomato fruits and vegetables contain an array of phytochemicals that are beneficial for human health and are often termed "functional foods". The manipulation of tomato fruit quality through genetic engineering is reasonably well advanced. The isoprenoid pathway has been well researched since its manipulation impacts not only on the organoleptic qualities of fruit but also their contribution to human health. Ripe tomato fruits accumulate large amounts of the red linear carotene, lycopene (a dietary antioxidant) and small amounts of its orange cyclisation product, β-carotene (pro-vitamin A). Lycopene is converted into β -carotene by the action of lycopene β -cyclase (β-Lcy), an enzyme introducing beta-ionone rings at both ends of the molecule (Cunningham *et al*., 1994). A competing epsilon cyclase (β-Lcy), which in tomato is encoded by the *Delta* gene, introduces a single epsilon-ionone ring (Ronen *et al*., 1999). The *Delta* gene is usually silent in fruits, but its de-repression in the *Delta* genotype results in the accumulation of compounds in the epsilon cyclisation branch (Ronen *et al*., 1999; Tomes, 1967). A second gene, the *B* gene (Tomes, 1967) resposible for the accumulation of beta-carotene in fruits. *B* does not encode β-Lcy (Pecker *et al*., 1996) nor does it increase expression of the *β-Lcy* gene. β -carotene is the major dietary precursor of vitamin A. Lycopene does not have pro-vitamin A activity, but it is a good

dietary antioxidant. High plasma lycopene levels have been associated with a decreased incidence of prostate cancer (Gann *et al*., 1999). Therefore, the elevation of carotenoid biosynthesis in plants, especially in tomato by genetic manipulation should increase the lycopene and β-carotene levels and hence improve the nutritional quality of the crop.

The genes controlling lycopene synthesis in tomato, such as *Psy1* and *Pds* (encoding, respectively, the fruit-specific phytoene synthase and the phytoene desaturase) are up-regulated during fruit ripening (Corona *et al*., 1996; Giuliano *et al*., 1993), while those controlling lycopene cyclisation, like *β-Lcy* or *ε-Lcy* are down-regulated (Pecker *et al*., 1996; Ronen *et al*., 1999). As a result, β-carotene in ripe tomato fruits does not exceed 15% of the total carotenoids. Lycopene accumulation arises during tomato fruit ripening because of reduced lycopene cyclization (and the presence of a ripening enhanced phytoene synthase 1 (PSY-1). A bacterial phytoene desaturase (crtI, able to transform phytoene into lycopene) was fused to a plastidic transit peptide and introduced in tomato plants under the control of the CaMV *35S* promoter (*35S/tp/crtI*). This experiment, aimed at increasing lycopene levels, has unexpectedly resulted in a threefold increase in β -carotene, but not in lycopene (Roemer *et al*., 2000). The total carotenoid levels are in fact decreased in the transformants, and the reasons for these results are still poorly understood The constitutive expression of *Psy*-1 in transgenic tomato resulted in a several pleiotrophic effects, including dwarfism caused by the diversion of GGPP from gibberellin formation. Most recently, the elevation of β-carotene content in tomato fruit has been achieved by the constitutive expression of phytoene desaturase from the bacterium *Erwinia uredovora* (*crt*I) and lycopene cyclase (*crt*L- β) from *Arabidopsis thaliana* . The promoter of the tomato phytoene desaturase (pDS) gene shows high levels of expression in tomato fruits and negligible levels of expression in both tobacco and tomato leaves (Corona *et al*., 1996). As expected, the overexpression increases the levels of β -carotene up to sevenfold. Total fruit carotenoid levels are slightly, but consistently, increased in most of the transformants.

2.5.1.3 Delayed fruit ripening by manipulating ethylene metabolism

Growers of tomatoes are concerned with fruit ripening since this affects harvesting and ultimately determines profits. A number of approaches have been used to develop tomatoes with reduced synthesis of ethylene, to alter the ripening of tomatoes, besides improved quality as fresh market tomatoes. Ethylene is responsible for inducing expression of genes that are involved in fruit ripening and fruit softening. In the genetically engineered varieties, ethylene production is blocked, so tomatoes can be picked later, at a more flavorful stage, and moreover the fruits do not over-ripen and rot. Once picked, fruits can be ripened when commercially appropriate by simple exposure to external ethylene. One version of this strategy was used by DNA Plant Technology (Oakland CA) to produce its 'Endless Summer' variety tomato; this strategy is also being used to control ripening in melon.

Modifying the amount of ethylene produced under ripening and stress conditions are the goal of a wide array of transgenic strategies. Expression of antisense versions of enzymes from the ethylene biosynthesis pathway should also allow for genetic control of ethylene levels. The important transgenic stratageis to lower the ethylene levels were as follow;

2.5.1.3.1 ACC synthase

ACC synthase is responsible for the conversion of SAM to 1-ACC. It is one of the rate limiting enzymes in ethylene biosynthesis and is the target for many strategies aimed at reducing ethylene. The existence of multigene family is the drawback for making specific construct in transgenic strategies. In order to inhibit a tomato ACC synthase (ACS2) mRNA, the entire gene including the untranslated regions was inserted in opposite orientation under the constititutive CaMV 35S promoter into plant genome. These transgenic tomatoes showed a 99.5% reduction in ethylene production and no ripening was observed and it can be reversed by exogenous ethylene treatment (Oeller *et al*., 1991). But these transgenic fruits showed abnormal pattern of ripening, reduced softening, reduced pigment accumulation and greatly reduced respiratory climacteric.

2.5.1.3.2 ACC oxidase

ACC oxidase is responsible for the conversion of 1-ACC to ethylene and it also encoded by amultigene family. Hamilton *et al*., (1990) over-expressed the antisense ACC oxidase gene in tomato. The transgenic plants appear to germinate, develop and grow normally but fruits did not over-ripen like controls. Eventhough the fruit ripening began at the same chronological stage to that of control but its subsequent progression is delayed. Transgenic fruits displayed reduced lycopene levels and reduced over ripening. Chlorophyll loss was delayed and accumulation of carotenoid pigments, particularly lycopene was sevearly inhibited in offvine ripening (Picton *et al*., 1993a). Previously, researchers have genetically engineered tomato with antisense ACC oxidase (Ye *et al*., 1996), the antisense ACC synthase (Yao *et al*., 1999), and the double-antisense ACC oxidase and ACC synthse fusion gene (Xiong *et al*., 2003). In another approach, RNAi-mediated gene silencing for the ACC oxidase gene was used for successful production of transgenic tomatoes with trace levels of ethylene and prolonged shelf life.

2.5.1.3.3 ACC deaminase

The enzyme ACC deaminase is shown to convert ACC to ammonia and α-ketobutyrate, both of them were further metabolized by microorganisms. This enzyme was first discovered in soil micro organisms (Honma and Shimomura, 1978). The transgenic tomato plants were produced by inserting the gene for ACC deaminase under the control of the CaMV 35S promoter (Klee *et al*., 1991). These transgenic plantsa showed 90-97% reduced ethylene production but they also had delayed pigment accumulation, altered ripening pattern and softening.

2.5.1.3.4 SAM decarboxylase

The enzyme SAM decarboxylase converts SAM to decarboxylated form, which can be used in the polyamine biosynthesis. The enhanced expression of expression of yeast SAM decarboxylase gene fused with ripening inducible E8 promotor, resulted in increased conversion of putrescine to spermindine and spermine (Matto, 2002). This led to an increase in lycopene, prolonged vine life and enhanced fruit juice quality.

2.5.1.3.5 SAM hydrolase

It is another SAM degrading enzyme found in bacteriophage T3 and converts SAM to 5V-methylthioadenosine (MTA) and homoserine (Good *et al*., 1994). The expression of these genes under constitutive promoter has no drastic effect on ethylene production in tomato where as the use of ripening specific E8 promoter reduced ethylene levels in transgenic fruit compared with control.

The genetic manipulation of alcohol dehydrogenase levels in ripening tomato fruit have been shown to affect the balance of some flavor aldehydes and alcohols (Speirs *et al.*, 2001).

2.5.1.4 Manipulation of ethylene perception

Another approach to manipulating plant responses to ethylene, including fruit ripening and flower senescence, is based on blocking the perception of the hormone. Arabidopsis mutants have been found that are unable to respond to ethylene, and one class of these ethylene-insensitive mutants have a defective ethylene receptor that is unable to transmit the signal to the nucleus when ethylene is present. This has led to another strategy to modify ethylene responses in transgenic plants, where the signal transduction pathway is blocked by expressing this dominant mutant ethylene receptor.

Transgenic plants (tomato and petunia) have been produced that express a dominant mutant ethylene receptor, either from Arabidopsis or tomato. Some of these transgenic plants have been shown to be insensitive to ethylene. As a result various ethylene responses are blocked: 1, fruit will not ripen, even if exposed to ethylene 2, flowers do not abscise 3, flowers do not senesce, even when pollinated. There is a lot of commercial interest in developing flowers with delayed senescence, both for the cut flower market and for ornamental plants.

Experiments designed to down-regulate specific tomato ethylene receptor isoforms using antisense suppression have been reported for *SlETR1*, *NR* and *SlETR4*. Down-regulation of *SlETR1* expression in transgenic plants did not

alter fruit ripening but resulted in plants with shorter internodes and reduced rates of floral abscission. Down-regulation of *NR* expression in a wild-type background did not result in any dramatic phenotypes but did result in subtle changes indicative of slightly delayed fruit ripening (Barry and Giovannoni, 2007). Reduction of *SlETR4* expression using an antisense transgene resulted in plants with enhanced ethylene sensitivity manifested through extreme epinasty, increased floral abscission, enhanced triple response and accelerated fruit ripening, confirming that *SlETR4* acts as a negative regulator of ethylene responses in tomato. Further, the severe ethylene hypersensitivity manifested in the *SlETR4* loss-of-function lines can be complemented by add-back of a 35S-*NR* transgene. Several reporter genes related to ethylene responses and fruit ripening, including *LeCTR1* and *SlEILs* genes, were also successfully silenced by Virus induced genesilencing (VIGS) method during fruit development (Fu *et al*., 2005). In addition, it was found that the silencing of the *SlEIN2* gene results in the suppression of tomato fruit ripening. SlEIN2-silenced tomato fruits were developed using a virus-induced gene silencing fruit system to study the role of *SlEIN2* in tomato fruit ripening. Silenced fruits had a delay in fruit development and ripening, related to greatly descended expression of ethylene-related and ripening-related genes in comparison with those of control fruits. These results suggested *SlEIN2* positively mediated ethylene signals during tomato development (Zhu *et al*., 2006). Antisense suppression of *E8* in transgenic tomato fruit results in increased ethylene production indicating that E8 participates in feedback regulation of ethylene during ripening (Kneissl and Deikman, 1996).

2.5.1.5 Draw backs of the transgenic strategies

Genetically manipulated products have more risks than traditional foods since genetic engineering processes can introduce new allergens in foods that previously were naturally safe. Moreover, some transgenic manipulations are either lethal or greatly affect the morphology of the plant. There are various risks in genetic engineering, for example the risk of unintentionally changing the genes of an organism, the risk of harming that organism, the risk of changing the ecosystem in which it was involved, and the risk of change, or

harm, to any other organism of that species or others, including human beings. The concept of risk in biotechnology involves both the potential to change something and the potential to harm. Gene pollution is very difficult to clean up, since new living organisms, bacteria, and viruses can be released into the environment to reproduce, migrate, and mutate. On the other hand, genes from bacteria, viruses, and insects, which have never been part of our human diet, could be spliced into food, and no one knows now if these foods are safe. Even if the gene itself is not dangerous or toxic it could alter complex biochemical systems and create new bioactive compounds or change the concentrations of those which are normally present. Transgenic crops constitute a threat to wild plants and rural varieties (traditional varieties of crops) that constitute a principle source of the genetic diversity of the crops.

The strategies in practice for preventing post harvest decay of fruits and vegetables are suffering from serious drawbacks. Explanations for the lack of progress in identifying the key individual causes of fruit softening include the possibility that important textural changes associated with wall disassembly is a consequence of numerous enzymes acting in concert on multiple wall structural components, or that the critical enzymatic activity or activities have not yet been identified. However, an alternative explanation is that polysaccharide degradation is not the sole determinant of fruit softening and that other ripening-related physiological processes also play critical roles. By suppressing cell wall modifying gene by antisense technology, although over all ripening was not effected, there is less significant difference in firmness of fruits of wild type and transgeneics. Since the fruit ripening and fruit softening are not a single gene trait but is governed by multigene families, inhibiting the expression of only one gne will not make much difference. The possible occurrence of an isoform of an enzyme of interst would block success through antisense approach as it would complement the suppressed isoform (Brummel and Harpster, 2001). The transgenics of ethylene biosynthesis results in delayed fruit ripening and extended shelf life but the fruits are short lived on vine fallowing ethylene induced ripening. These fruits exhibited inferior quality with respect to colour, aroma, and taste as all these characters are governed by gradual changes in metabolism by ethylene.

Thus, the transgenic strategies in practice are suffering from serious drawbacks and newer and much efficient strategies should be developed and targeted for successful achievement of the goal.

2.5.2 Manipulation of tomato fruit ripening by mutational approach

Tomato has proved to be an excellent model system for the analysis of fruit ripening and development, in part due to the availability of well characterized ripening mutants. The long-term solution to reduce over ripening probably be based on genetically modified plants with mutations that either suppress synthesis or reduce sensitivity to ethylene. Many natural tomato mutants affected at ripening have been used to study gene expression and regulation during fruit ripening. Two principal groups of mutants were used, *i.e.* those whose mutation affects only the color of the ripe fruit and those whose mutations determines the pleiotropic effects occurring during ripening.

Much of our knowledge concerning the mode of action of ethylene in plants has been generated from the use of the triple response screen in Arabidopsis to identify mutants that are either insensitive to ethylene or show enhanced ethylene responses in the absence of exogenous ethylene. Mutants displaying a constitutive response (*eto1*) were found to produce at least 40 times more ethylene than the wild type. Mutants that failed to display the apical hook without ethylene (*hls1*) exhibited reduced ethylene production. Mutants that were insensitive to ethylene (*ein1* and *ein2*) produced increased amounts of ethylene, displayed hormone insensitivity in both hypocotyl and root responses, and showed an apical hook. The power of Arabidopsis molecular genetics has facilitated the rapid identification of many components of the signalling pathway from an initial mutant phenotype. In tomato a small number of unique single gene mutations exist, such as *ripening-inhibitor* (*rin*), *non-ripening* (*nor*), and *Colorless non-ripening* (*Cnr*) which have pleiotropic effects resulting in the reduction or almost complete abolition of ripening. Evidence of the involvement of an ethylene-independent or developmental pathway that regulates ripening has come from the characterization of monogenic tomato mutants including *rin*, *Nor* and *Cnr* in which ripening is severely impaired. These mutants fail to undergo an increase in ripening-related ethylene

production and show inhibition of ripening-related gene expression, although gene expression but not ripening can be partly restored by ethylene treatment,showing that they remain ethylene responsive. The transcription factors such as those encoded by the *ripening-inhibitor* (*RIN*) MADS-box and *colourless non-ripening* (*CNR*) SPB-box genes, which are necessary for the progression of virtually all ripening processes also eased the elucidation of downstream signal transduction components that impact the hormonal and environmental stimuli that coordinate and modulate ripening phenotypes. Physiologically characterized single gene tomato ripening mutants that have some times been available for decades have recently become accessible at the molecular level as genomics infrastructure for tomato has expanded.

2.5.2.1 Spontaneous ripening mutants of tomato

2.5.2.1.1 *Never-ripe mutant*

The *Never-ripe* mutant of tomato is a semi-dominant, single gene muation. Utilization of the same triple response screen used in Arabidopsis led to the discovery that the tomato fruit-ripening mutant *Never-ripe* (*Nr*) was insensitive to ethylene (Lanahan *et al*., 1994). Wilkinson *et al*. (1995) showed that *Nr* was caused by a mutation in a member of the tomato ethylene receptor gene family (Wilkinson *et al*., 1995). *NR* was cloned and encodes a protein with homology to *ETR1* from Arabidopsis (Wilkinson *et al*., 1995; Yen *et al*., 1995). The *Nr* mutant contains a mutation in the ethylene binding site conferring ethylene insensitivity. Analysis of *Nr* showed a number of pleiotropic effects indicative of ethylene insensitivity throughout the plant (Lanahan *et al*., 1994). The mutant is greatly impaired in floral abscission and fruit ripening and exhibits significant delays in leaf and flower petal senescence due to ethylene insensitivity. The mutant only produces ~50% of the normal level of ethylene and ~20% of the normal level of lycopene (red carotenoid). The fruits are firmer than the fruit from the wild-type plants and are moderately pathogen resistant. *Nr* has subsequently proven to be an useful tool to assess the role of ethylene in a range of developmental (Clark *et al*., 1999; Hansen and Grossmann, 2000; Llop-Tous *et al*., 2000), gene expression (Nakatsuka *et al*.,

1998), and stress (Ciardi *et al*., 2000; O'Donnell *et al*., 2001; Diaz *et al*., 2002) processes.

2.5.2.1.2 *ripening-inhibitor mutant*

Evidence from biochemical and genetic studies infer that both ethylene-dependent and ethylene-independent regulatory cascades control the development of tomato fruit. Hence, although the non-ripening tomato mutants *ripening-inhibitor* (*rin*) and *non-ripening* (*nor*) do not produce autocatalytic ethylene nor ripen in the presence of exogenous ethylene, they do display signs of ethylene sensitivity and ethylene-inducible expression of several genes (Picton *et al*., 1993c). Thus, it is likely that *RIN* and *NOR* participate in ethylene-independent regulatory cascades during the early stages of fruit ripening. *LeMADS-RIN* encodes a member of the MADS-box family of transcription factor (Vrebalov *et al*., 2002). Interestingly, homologues of *LeMADS-RIN* are expressed during the ripening of other fruit including strawberry, which might indicate a common (ethylene-independent) function in the ripening of both climacteric and non-climacteric fruit. The tomato *ripening-inhibitor* mutant is a single locus mutation which arrests the normal ripening response to exogenous ethylene. Tomatoes homozygous for this allele yield fruits which remain firm and green for weeks after their normal counterparts mature and senesce. The *rin* mutant fruit fail to synthesize climacteric ethylene or accumulate lycopene (red carotenoid), in addition to being deficient in softening and remaining resistant to microbial infection. Both the *rin* and *nor* genes are cloned and sequences are available in pubmed. These mutations are already being used commercially to extend shelf-life in tomato. By manipulating the RIN gene, breeders are able to slow the ripening process, letting the tomato to develop on the vine for longer — but still keeping it firm enough to ship safely. The discovery that a homologue of the *RIN* gene is expressed in strawberry, a non-climacteric fruit, suggests that common regulatory cascades may operate in all fruits.

2.5.2.1.3 ***Colourless non-ripening mutant***

Seymour *et al*. (2002) have identified a rare dominant mutation in a tomato gene (*Cnr*, for colourless non-ripening) that results in a non-ripening phenotype with two distinct characteristics: (i) firm fruit with reduced cell adhesion and (ii) a complete absence of carotenoid biosynthesis in the pericarp. The *Cnr* mutation is the result of a lesion in a single gene that has pleiotropic effects on ripening, including a lack of pigmentation and minimal softening, indicating that the *Cnr* gene is required for normal ripening. Since the mealy phenotype of *Cnr* fruit is the opposite of the juicy phenotype desired by the consumer, this mutant may provide an insight to the molecular biology of juiciness (Thompson *et al*., 1999). Microarray analyses indicate that the expression of many genes impacting on many aspects of ripening is altered in the *Cnr* mutant, suggesting that *Cnr* may encode a regulatory factor. Several of these genes are themselves transcriptional regulators, including a MADS-box transcription factor (TDR4) homologous to the *Arabidopsis FUL* gene that may be linked to the mealy phenotype of *Cnr* fruit.

2.5.2.1.5 *epinastic (epi) mutant*

The *epinastic* (*epi*) mutant of tomato (*Lycopersicon esculentum*) has a dark-grown seedling phenotype similar to the triple response in the absence of ethylene. In addition, in adult plants both the leaves and the petioles display epinastic curvature and there is constitutive expression of an ethylene-inducible chitinase gene. Leaves also have a twisted epinastic morphology, and ethylene production is increased above the level of wild-type plants (Fujino *et al*., 1988, Barry et. al., 2001). However, petal senescence and abscission and fruit ripening are all normal in *epi*. The genetic mapping analysis revealed the location of the mutant to lie within a 12-cM region on chromosome 4 between CT133 and TG163.

2.5.2.1.6 *Green-ripe (Gr) mutant*

It is spontaneous and dominant mutant of tomato characterized based on the inhibition of fruit ripening. In addition, a subset of ethylene responses

associated with floral senescence, abscission, and root elongation are also impacted in mutant plants, but to a lesser extent (Barry *et al*., 2005). This is the result of reduced ethylene responsiveness in fruit tissues. However, ethylene-mediated inhibition of hypocotyl elongation and petiole epinasty remain normal, suggesting that these loci affect a subset of ethylene responses in tomato, with the strongest phenotypes observed in fruit. *Gr* encodes an evolutionary conserved protein of unknown biochemical function that may be associated with ethylene signaling (Barray and Giovannoni, 2006).

2.5.2.1.7 Tomato germplasm altered in fruit softening

Delayed Fruit Deterioration' (DFD) is a tomato cultivar that provides a unique opportunity to assess the contribution of wall metabolism to fruit firmness, since DFD fruits exhibit minimal softening but undergo otherwise normal ripening, unlike all known nonsoftening tomato mutants reported. However, ripening DFD fruit showed minimal transpirational water loss and substantially elevated cellular turgor (Saladie etal., 2007).

2.5.2.1.8 Tomato mutants altered in carotenoid biosynthesis

Genes encoding enzymes that catalyze carotenoid synthesis have been cloned from tomato and corresponds to a number of previously defined pigmentation mutants. Examples include the *yellow-flesh* (*r*) mutation, resulting in deletion of the ethylene-regulated phytoene synthase (*PSY*) gene (Fray and Grierson, 1993), loss of or reduced expression of the carotenoid isomerase gene, resulting in the prolycopene-accumulating orange fruit of the *tangerine* mutants (Isaacson *et al*., 2002), and overexpression and knockout mutations of the lycopene-ß-cyclase gene, resulting in high-ß-carotene *Beta* (*B*) and deep-red *crimson* fruit, respectively (Ronen *et al*., 1999, 2000).

Light has been shown to effect carotenoid accumulation in a number of species, including tomato. Tomato high-pigment (*hp1* and *hp2*) mutants, characterized by increased green fruit and leaf chlorophyll in addition to increased total ripe fruit carotenoids, have been shown to be hypersensitive to light (Peters *et al*., 1989). The *hp2* locus has been cloned and shown to harbor the tomato homolog

of the Arabidopsis *DE-ETIOLATED1* (*DET1*) negative regulator of light signal transduction, providing additional molecular evidence for the regulation of carotenoid synthesis via light signal transduction (Mustilli *et al*., 1999). Understanding the interaction of light with plant hormones may be an useful approach for optimizing fruit pigmentation and associated nutritional quality by fruit-specific manipulation of light-signaling genes.

2.6. Induced mutagenesis

A large collection of wild relatives and monogenic mutants effecting many aspects of plant development and responses, including disease resistance, are available for tomato, through such centers as the Tomato Genetics Resource Center (TGRC, http://tgrc.ucdavis.edu/index.cfm), which has one of the world's most comprehensive collection of genetic stocks and wild relatives for tomato. Recently, from the genetic background of the inbred variety M82, a comprehensive mutant population was generated and the mutant phenotypes in the population were classified for *in silico* searches (Menda *et al*. 2004). Over the past decade, the triple response phenotype has been used to screen for mutants that are defective in ethylene responses. "Triple response" refers to the morphological changes that seedlings undergo when they are grown in the dark in the presence of ethylene: exaggerated apical hook formation, inhibition of root and hypocotyl elongation, and swelling of the hypocotyl (Guzman and Ecker, 1990). Even though tomato has natural mutants that are affected in fruit ripening and ripening induced processes, these are very few mutants available to understand the complete mechanism and components involved in complex ripening process. There is a need to increase the number of mutants affected in fruit ripening by induced mutagenesis.

2.6.1 Screening in presence of ethylene or ethylene inhibitors by exploiting triple response

Over the past decade, the triple response phenotype has been used to screen for mutants that are defective in ethylene responses. The triple response phenotype has been used extremely successfully as a screen for the isolation of

components of the ethylene signal transduction pathway in Arabidopsis. Etiolated seedlings with minor or no phenotypic responses on ethylene application are termed ethyleneinsensitive (*ein*) or ethylene-resistant (*etr*) mutants. Mutants have also been identified that display a constitutive triple response in the absence of ethylene. This class can be divided into subgroups based on whether or not the constitutive triple response can be suppressed by inhibitors of ethylene perception and biosynthesis, such as silver thiosulphate and aminoethoxyvinyl glycine (AVG). Mutants that are unaffected by these inhibitors are termed constitutive triple response (*ctr*) mutants, whereas those whose phenotypes reverts to normal morphology are termed ethylene-overproducer (*eto*) mutants, which are defective in the regulation of hormone biosynthesis.

2.6.2 Screening in presence of ethylene inducing hormone

Cytokinins, N^6-substituted adenine derivatives, are one many factors that modulate the biosynthesis of the gaseous hormone ethylene (Yang and Hoffman 1984; Mattoo and Suttle 1991; Abeles *et al.,* 1992).The elevated ethylene biosynthesis by cytokinin results in triple response in etiolated seedlings. In Arabidopsis, the *cyr1* (cytokinin response) and *ckr1* (cytokinin resistant) mutants were identified by the ability to elongate their roots on inhibitory concentrations of cytokinin (Deikman and Ulrich 1995). *ckr1* has been found to be allelic to the ethylene-insensitive mutation *ein2*, and was identified because it was resistant to the ethylene produced in response to exogenous cytokinin (Cary *et al,*. 1995). The *cyr1* phenotype (pale green leaves, abbreviated shoot development and incomplete cotyledon and leaf expansion) is consistent with the predicted phenotype of a cytokinin-insensitive plant (Deikman and Ulrich 1995).*cyr1* plants are less responsive to exogenous cytokinin in root inhibition assays, anthocyanin accumulation assays and *in vitro* shoot initiation assays. *cyr1* plants are also affected in abscisic acid responsiveness in root inhibition assays. One other potential cytokinin response mutant from Arabidopsis is *stp1* (Baskin *et al.* 1995). The *stp1* mutant displayed decreased sensitivity to cytokinin in root inhibition assays, but had the normal sensitivity to cytokinin in callus and shoot initiation assays. *stp1* had wild-type sensitivity to auxin,

ethylene, gibberellin and abscisic acid in root inhibition assays. Recently, it was found that loss-of-function *acs5* mutations disrupt the induction of ethylene at low cytokinin concentrations (<10 µmol) but not at higher concentrations (vogel *et al.* 1998), indicating that this ACS isoform is responsible for the increase in ethylene biosynthesis observed in response to low levels of cytokinin. The dominant ethylene overproducing mutant *eto2* is the result of a perturbation of the 11 carboxy-terminal amino acids of ACS5 (Vogel *et al.* 1998), suggests that cytokinin acts by a post-transcriptional modification of ACS5. *cin4*, is allelic to the constitutive photomorphogenic mutant *fus9*/*cop10* which highlights the interaction between light and cytokinin in the regulation of ethylene biosynthesis.

The aim of my work is to gain a better understanding of how climacteric fruit use ethylene to regulate ripening. Specifically, examination of the function and regulation of key regulatory components in ethylene signal transduction pathway will ease our understanding of the basic biological foundation by which climacteric fruit perceives and transduce the ethylene signal. As our understanding of the overall biology of fruit ripening improves, so will the ability to improve the quality and nutritional value of fruit through traditional or non-traditional means.

Table 1: List of ripening mutants in tomato.

Genotype	Activity	Function	Reference
rin; ripening-inhibitor	MADS-box gene	Comprehensive ripening	Vrebalov et al., 2002
nor; non-ripening	Transcription factor	Comprehensive ripening	Wilkinson *et al*., 1995
Nr; Never-ripe	C2H4 receptor	Ethylene signaling	Mustilli *et al*., 1999
hp-2; high-pigment-2	DET1 homologue	Light signaling	Yen *et al*., 1997
hp-1; high-pigment-1	NA	Light signaling	Ronen *et al*., 2000
cr; crimson	Lycopene cyclase	Carotenoid metabolism	Fray and Grierson, 1993
B; Beta	Lycopene cyclase	Carotenoid metabolism	Isaacson *et al*., 2002
R; Phytoene Synthase	Phytoene synthase	Carotenoid metabolism	Kopeliovitch *et al*., 1981
t; tangerine	Carotenoid isomerase	Carotenoid metabolism	Barry *et al*.,2006
alc; alcobaca	NA	Comprehensive ripening	Thompson *et al*., 1999
Nr-2; Never-ripe-2	NA	Fruit softening	Akhtar *et al*., 1999
Cnr; Coulorless non-ripening	NA	Comprehensive ripening	Jenkins and Mackinney, 1955
gf; green-flesh	NA	Carotenoid metabolism	Rick and Butler, 1956
at;apricot	NA	Fruit pigmentation	
gs; green stripe	NA		

Table 2: List of transgenic lines modified in ethylene perception in tomato.

Gene/promoter	Consequence	Reference
At etr1-1 with CaMV 35S N-terminus of *etr1-1* fused to histidine kinase domain of tomato NR cDNAwith FMV 35S	Tomato: Delayed abscission of flowers and ripening of fruit	Wilkinson *et al*., 1997
Antisense *SlETR4* and antisense *NR*	Lowered level of SlETR4 mRNA, Severe epinasty, Enhanced flower senescence, Accelerating fruit ripening, Reduced level of *NR* mRNA,	Tieman *et al*., 2000
NR cDNA with FMV promoter	Normal ethylene sensitivity	Ciardi *et al*., 2000
NR antisense with CAMV promoter	Lower sensitivity to ethylene than *NR* mutant	Hackett *et al*., 2000
SlETR1 with antisense Receiver domaine	Fruit ripened normally; levels of *PSY1*, *ACO1* (ripening-related genes) and *E4* (ethylene responsive genes) were normal Delayed abscission reduced plant size Normal fruit ripening Normal triple response	Whitelaw *et al*, 2002

Materials & Methods

3.1 Plant materials and general growth conditions

Tomato (*Solanum* l*ycopersicon* cv. Ailsa Craig) seeds were surface sterilized with 20 % (v/v) sodium hypochlorite solution for 5-10 min at room temperature. Thereafter seeds were thoroughly washed with distilled water to remove hypochlorite. Seeds were then germinated on two layers of moist filter papers in transparent plastic boxes (9.5 cm *l* x 9.5 cm *b* x 5 cm *h*) in dark at 23±2°C. After emergence of radical the seeds were transferred to vermiculite (Vermiculite and peat mixture, Karnataka Explosive Ltd. Bangalore, India) in plastic trays or plastic glasses The seedlings were grown under continuous white light (100 $\mu molesm^2s^{-1}$) for two to three weeks. Seedlings were then transferred to pots filled with red loam soil and were grown in the green house under natural day night cycle till the end of life cycle. The age of the plant was counted from the time point of emergence of the radical. For all experiments where comparison of wild type and mutants were made, seed lots from the same year harvest was grown together under the same conditions.

3.2 Light sources

White light (100 $\mu mole\ m^{-2}s^{-1}$) was obtained by using four tube-lights (40 W each), three of which were white (Phillips no. 6500 K) and one was brown (Phillips no. 2700 K). The green safe light (<0.01 $\mu mole\ m^{-2}\ s^{-1}$) was obtained from a cool white fluorescent tube light wrapped in six layers of green cellophane paper (λ_{max} 530 nm). The screening of the dark grown seedlings was carried out under green safe light. The intensity of the light was measured with a Skye radiometer unit with SKP-215 and SKP-110 probes (Skye Instruments, Powys UK).

3.3 EMS-treatment

EMS treatment was carried out in the year 1996 as described in detail by Srinivas (2000). In brief, to induce mutations, the tomato cultivar Ailsa Craig seeds

[harvested in 1996] were imbibed in distilled water for 24 hours at 23 ± 2° C. The imbibed seeds were then submerged in freshly prepared unbuffered 60 mM ethyl methane sulphonate (EMS) solution for 24 hours in darkness at 23 ± 2° C (Koornneef *et al.,* 1990). Thereafter, the seeds were washed off with tap water for 8 hours to remove the traces of EMS. The mutageniged seeds were then sown in vermiculite (Vermiculite and peat mixture, Karnataka Explosive Limited, Bangalore, India) in plastic trays (40 cm *l* x 30 cm *b* x 7 cm *h*) and grown at 23 ± 2° C under continuous white light (100 µmoles m^{-2} s^{-1}) for 10-14 days. Seedlings were then transferred to red loam sandy soil in the open experimental field. Seeds of M_2 generation were harvested in bulk and stored in polythene bags with a desiccant at 23° C till further use.

3.4 Mutant Screening

3.4.1 Screening of acetylene resistant mutant

To screen for ethylene resistant mutants, we adopted a different strategy. For ease of handling and to maintain a steady source of ethylene gas supply, we replaced ethylene by its analogue acetylene. In literature it is reported that acetylene can induces triple response in plants about 10,000 fold higher concentration than ethylene (Abeles *et al*., 1992). Therefore acetylene can be used for screening ethylene mutants. To obtain acetylene 15 g (w/v) calcium carbide (CaC_2) pellets in polythene bag was placed directly on moist vermiculite. Surface sterilized M_2 seeds were spread on moist vermiculite at a density of 30 - 40 seeds per box. By absorbing atmospheric moisture, calcium carbide releases acetylene leaving residual calcium oxide (lime).

$$CaC_2 + H_2O \longrightarrow CaO\downarrow + C_2H_2\uparrow$$

Boxes were sealed tightly with parafilm or sealing tape to retain the released acetylene from CaC_2 incubated in dark at 25±2°C. The boxes were inspected daily under safe green light for seedling triple response. Seedlings not exhibiting the wild-type ethylene triple response was picked up as the putative acetylene insensitive mutant and hardened first in the culture room, transplanted to pots and grown to maturity in a greenhouse. (The acetylene resistant mutant was isolated by

Sagar Datir in the M_2 generation in 2000. It was later maintained in Net house by self pollination till M_4 generation by Dr. Sangeeta Negi).

3.4.1.1 Screening of 1-Methylcyclopropene (1-MCP) resistant mutant

An alternative approach to screen for ethylene receptor mutants we used 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action. For 1-MCP treatment the sterilized seeds were transferred to moist vermiculite contained in germination boxes (9.5 cm *l* x 9.5 cm *b* x 5 cm *h*). 1-MCP powder was dissolved in warm water in a plastic vial which was placed in the germination boxes to release the 1-MCP gas. The boxes were sealed properly to make air tight and kept under controlled condition in light.

3.4.2 Screening of kinetin resistant mutant

It is known that germination of plant seeds on medium supplemented with high concentration of plant hormones such as auxin or kinetin, impose severe stress on the germinating seedlings. In Arabidopsis, the attempts to select mutants in auxin and kinetin, several times resulted in mutant seedlings that were either under or over producers of ethylene. Therefore we screened for ethylene under producer mutants of tomato in presence of high concentration of kinetin. The M_2 seeds were germinated on 0.8% (w/v) water agar containing different concentrations of kinetin along with control. To optimize visualization of phenotype, screen was carried out in dark. In presence of kinetin, tomato seedlings undergo stress, what led to excess production of endogenous ethylene, causes triple response in seedlings. The seedling that did not show triple response on kinetin after two weeks of growth was selected as putative kinetin resistant mutant and multiplied in the net house. (From EMS mutagenized lines the kinetin resistant mutant was isolated by Sonal in the year 2001 and was later maintained till 2003 by Dr. Sarada).

3.5 Acetylene sensitivity assay of *kin-1* seedlings

To confirm that the kinetin insensitive mutants were not defective in the induction of ethylene biosynthesis by cytokinin, rather than ethylene perception, *kin-1*

mutant seedlings were exposed to acetylene right from germination. Surface sterilized seeds were spread on moist vermiculite containing 15g of CaC_2 in a polythene bag with holes. The untreated seedlings were kept as control. These boxes were sealed with parafilm and kept in darkness at 25±2°C. The hypocotyl lengths of seedlings were measured after two weeks of incubation (Vogel and Kieber, 1998).

3.6 Ethylene sensitivity assay of seedlings

Surface sterilized seeds of WT, *atr-1* and *kin-1* were germinated on moist filter papers till the emergence of radical. Thereafter seedlings were transferred to moist vermiculite in air tight boxes (9.5 cm *l* x 9.5 cm *b* x 5 cm *h*) and grown for three days in dark at 25±2°C. 20 µl l^{-1} of ethylene was injected into these boxes. The boxes were sealed with paraffin or white tape to make them air tight and observed for seedling triple response two days.

3.7 1-ACC sensitivity assay

For seedling dose response to exogenous 1-Aminocyclopropene1-carboxylic acid (Sigma; A-0430), seeds were germinated in water-agar supplemented with 1-ACC as described by Lanahan *et al*. (1994).

3.8 Dose response curves

For dose response, seedlings of *atr-1* and WT were grown right from germination on vermiculite with 0- 25 g of CaC_2 in dark at 25±2°C. The root and hypocotyls lengths were measured after 12 days. To determine the effect of cytokinin on hypocotyl elongation of WT and *kin-1* mutant, the seeds were surface sterilized and spread on the 0.6 % (w/v) agar medium containing increasing concentrations of kinetin ranging from 50 to 200 µM. These agar boxes were incubated in the dark room at 25 ± 2^0C for 12 days.

3.9 Crossing

Tomato is a self-pollinating species. For genetic crosses, young flowers were chosen in which sepals were still closed and anthers were green. To prevent selfing the flowers were emasculated by removing anther cone carefully with forceps. Care was taken not to damage the stigma of the flower. The emasculated flower was covered with cheesecloth or butter paper bags to protect from insect pollinator or cross-pollination due to wind. After 24 h of emasculation when stigma becomes receptive, the pollen collected from newly opened flowers of male parent was carefully applied to stigmas of female parent. The pollinated flowers were covered again with butter papers and the process of pollination was repeated 3-4 times for 2-3 consecutive days to ensure large number of seeds. Crossed flowers were labeled and seeds from crossed fruits were collected separately.

3.10 Fruit collection and seed harvest

Fruits were collected after complete ripening or at late orange stage of ripening. To harvest seeds, individual fruits were squashed and pulp was fermented for 24 hours to ease the removal of the mucilage from seeds. After that seeds were thoroughly washed with tap water and air dried for two to three days. Finally seeds were stored in labeled polythene bags until further use.

3.11 Phenotype characterization

3.11.1 Internodal length

The internodal lengths of three months old green house grown WT and mutant plants were recorded with a graduated ruler. The internodes lengths were noted for at least five to seven successive nodes from five plants of wild type and mutants.

3.11.2 Epinasty and abscission of cotyledons

Wild type and *atr-1* mutant seedlings were grown till five days on moist vermiculite in glass bottles under continuous white light at 25±2°C. Ethylene gas was injected into the bottles through the rubber stopper fitted in the lid. The effect

of exogenous ethylene on cotyledon epinasty and cotyledon abscission was observed during next two days.

3.11.3 In vitro floral abscission

Fresh and young inflorescences were cut from green house grown plants and the cut ends were immediately submerged in distilled water. The cut inflorescences were later treated with acetylene for 48 hours in a closed container in light. Floral abscission was recorded daily for next three days.

3.11.4 Leaf senescence

For leaf senescence studies, healthy whole leaflets were detached from 8th node of two months old green house grown *atr-1* mutant and WT plants by submerging the cut ends in water. These leaflets were placed on two layers of blotting paper in 14 cm Petri dishes wrapped with parafilm and incubated in dark for nine days at 25±2°C. The effect of ethylene was observed by placing the leaves in airtight containers with 2 ml l^{-1} ethylene.

3.11.5 Time of flowering

WT and mutant plants were grown in the green house with a maximal day temperature of 25-28ºC and night temperature of 18-20ºC with 50-80% humidity. Time of flowering can be recorded by counting the number of nodes before the first truss (inflorescence) of the green house grown plants.

3.11.6 Total fruit yield per plant

The total number of fruits harvested per plant was counted. The number of fruits harvested in each picking was added to sum up of all the fruits formed from single plant.

3.11.7 Chlorophyll estimation

For chlorophyll estimation, leaves were harvested from 8th node and fruits were harvested at breaker and red stage of ripening of two months old plants. Two grams of tissue was homogenized in precooled mortar with 5 ml hexane and acetone (60:40) and spin at 3000 *x g* for five minutes. The upper organic layer was transferred to a capped tube on ice. The remaining lower aqueous phase was re-extracted with 5 ml of the same solvent repeatedly and transfer the organic layer to the same tube until the aqueous layer becomes colorless. The concentration of chlorophyll was determined by measuring the absorbance at 645 nm (A_{645} and 663 nm (A_{663}) respectively in a spectrophotometer (Kirk, 1968; Davies, 1976). The amount of chlorophyll in one ml of sample was calculated using the following equation and finally expressed as amount of chlorophyll in gram fresh weight of tissue. (Porra *et al*., 1989).

$$\text{Chlorophyll } (\mu g/ml) = (20.2 \times OD_{645}) + (8.2 \times OD_{663})$$

3.12 Analysis of fruit development

3.12.1 Days to ripening

The fruit ripening in mutants and wild type plants grown in green house were characterized by recording the stages of the fruit development on a chronological scale from the date of flower anthesis (Virginia and Lomax, 2003). To ensure uniform growth conditions wild type and mutant plants were grown in the green house with a maximal day temperature of 25-28ºC and night temperature of 18-20ºC with 50-80% humidity. The first truss of plants was used for reading the fruit development stages. Individual flowers were tagged on the day of anthesis. The development of fruit was visually observed for each tagged flower right from anthesis to fruit abscission. Data for fruit stage transitions were recorded and compared for at least seven fruits from individual trusses of ten mutant and WT plants.

3.12.2 Lycopene content

Fruit pigments were analyzed by HPLC fallowing procedure described by Gangarao et. al., (2005) with slight modifications. In brief, five grams of fresh red fruit pericarp was homogenized in mortar and pestle with 50 mg of $MgCO_3$. The homogenate was suspended in 15 ml of acetone: methanol mixture (2:1) and 4 ml of hexane. All the solvents contained 0.1g/l of butylated hydroxyl toluene (BHT). Thereafter ten ml of cold saline was added and the mixture was shaken vigorously. The mixture was centrifuged at 5000 x *g* for two minutes and the hexane phase was collected for analysis. A 20 µl of the extract was injected to HPLC (model LC-20AT, Shimadzu, Kyoto, Japan) equipped with SPD-20A UV/VIS detector and C_{18} column. The peak for lycopene and β-carotene were detected at 504 nM and 450 nM wavelengths respectively. For HPLC run 85% of acetonitrile and 15% of methanol were used as mobile phase with flow rate of 2.5 ml/min. The peak was compared with the peak obtained from 10 µg/ml of standard lycopene from Sigma. The amount of lycopene was expressed as µg per gram fresh weight of tissue.

3.12.3 Average fruit weight, fruit size and number of seeds

For calculation of fruit weight and size, ten ripe fruits from mutants and WT were individually analyzed with respect to fruit weight, fruit diameter and number of seeds per fruit. Fruit diameter was measured with a thread twined horizontally at the equatorial (middle) region of the fully ripened fruit. Number of seeds per fruit was manually counted after extraction from the individual fruits.

3.12.4 Number of locules

Each fruit was sliced transversely to count the number of locules. An average of ten fruits from each mutant line and wild type was taken to determine the average number of locules per fruit.

3.12.5 Titrable acidity

Tomato juice was extracted from red ripened fruits of WT and mutants after removal of seeds using a juicer. Five ml of juice was titrated Sodium hydroxide (0.1 N) to pH 7.0 to calculate titrable acidity (Pinhero *et al*, 2003).

3.12.6 pH

The pH of the fruit juice of red ripe fruits was recorded with a pH meter.

3.13 Analysis of Post harvest quality traits

3.13.1 Shelf life

For observing shelf life, fruits at red ripe stage were harvested from green house grown plants. Harvested fruits were kept at room temperature (25±2°C) till eight weeks and observed visually for loss of firmness. Loss of firmness is characterized in three ways. Fruits that showed the sign of dehydration and wrinkling were recorded as 'wrinkled', fruits that began to rot or infected were referred as 'rotted' and the fruits that are totally lost integrity were termed as 'spoiled'.

3.13.2 Off vine ripening

For determination of off-vine ripening period, at least 15-20 fruits of wild type and mutant were harvested at mature green stage were harvested and kept at room temperature (25±2°C). The fruits were visually observed till they reach red stage and the time has taken were determined.

3.13.3 Ethylene induced fruit ripening

Early breaker fruits of wild type and mutants were harvested from green house and each fruit was kept in airtight boxes separately. Ethylene gas was injected into the boxes from the rubber stopper of the box. The induction of fruit ripening by ethylene was observed as the basis of red color development.

3.13.4 Determination of thermotolerance

The wild type as well as *atr-1* mutant fruits at red stage were harvested and kept at 60±2°C or 40±2°C for three days. The fruits were visually observed and photographed for the appearance of cracks and loss of firmness.

3.14 Measurement of ethylene evolution

For the determination of ethylene production, at least five fruits were harvested at different stages of ripening like Mature Green, Breaker, Turning, Orange and Red stage. Fruits were harvested, weighed and sealed individually in airtight boxes for six hours at room temperature (25±2°C). One ml of gas from headspace was injected into a gas chromatograph (model GC-17A, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a Porapak T column. The column temperatures were set at 110°C and detector temperatures were set at 135°C. Injection port temperature was set at 125°C. Using N_2 as a carrier gas (30 ml/min) and H_2 as fuel gas (30 ml/min) ethylene concentrations were determined using pure ethylene gas as standard. At least three readings were taken from each fruit. The total ethylene evolution was normalized to the fresh weight of the fruits, volume of the container and the time of incubation.

For measuring ethylene evolution from seedlings, seedlings were initially germinated on filter paper and after emergence of radical, transferred to moist vermiculite and grown under continuous light (100 µmole $m^{-2}s^{-1}$) for five days. The boxes were sealed for six hours. At least three readings were taken from each container daily. The total ethylene evolution was normalized to the number of seedlings and the time of incubation.

3.15 Gene expression analysis

3.15.1 RNA isolation

Total RNA was isolated from fruit pericarp at different stages of fruit ripening viz. mature Green (MG), breaker (B), turning (P), orange (O), red ripe (R). RNA isolated using RNeasy Plant mini kit (Qiagen, Germany) as per the manufactures

instructions. All the glassware, plasticware like mortars, pestles, tips, eppendorf tubes and MilliQ water used were treated overnight with 0.1% (v/v) diethyl pyrocarbonate (DEPC). Then they were autoclaved at 15 lb/inch^2 pressure at 121°C for 20 minutes. RNA samples were quantified by measuring the absorbance at 260 nm using spectophotometer. The amount of RNA was calculated according to the formula: 1 unit O.D$_{260}$ equivalent to 40 µg/ml of RNA.

3.15.2 Formaldehyde Agarose gel preparation

For checking the quality of the isolated RNA, sample volume equivalent to 5 µl was mixed with 5 µl of MiiliQ water and 2 µl of 6X loading dye (Appendix). The sample was resolved on 1.2% (w/v) agarose gel containing 4% (v/v) formaldehyde and autoclaved 1X MOPS buffer (Appendix). EtBr was added just before casting a gel. All the solvents and the gel apparatus used were pretreated with 0.1 % (v/v) DEPC. Electrophoresis of RNA was performed in 1X MOPS buffer at constant voltage (50 V) for 2h. The gel was then visualized using UV filter in a Gel documentation unit (Alpha Imager, India) for presence of two bands of 18S and 28S rRNA as a marker for RNA quality (Sambrook *et al*., 1989).

3.15.3 Primer designing and dissolution

Gene specific primers used for RT-PCR were designed using PRIMER3 software. The designed primers were analyzed for conditions like annealing temperatures, hairpin loop and dimer formation using IDT OLIGO ANALYZER software. The stock primers were dissolved in required amount of TE buffer (pH 7.4) (Appendix) as per the instructions of the supplier. Primers were thoroughly mixed by a brief spin and the stock was kept at 4°C overnight for proper dissolution. Next day stock solution was spinned again and aliquoted into fresh sterile tubes. Main primer stock was stored at -80°C and aliquots were stored at -20°C. Tubes were labeled with name of the primer, date of dissolution and concentration. Usually all primers were dissolved at 100 pmoles/µl concentration. Dilutions were made whenever is required from the single aliquot and dilutions were also kept at -20°C. The list of primers used was given in a separate table. (Table. 3)

Table 3: Sequence and annealing temperature of the various primers used in RT-PCR

Table 3A: Sequence and annealing temperatures of ethylene receptor gene primers

Target gene	Primer seq. (5'⟶ 3')	Length	Ann. Temp. (°C)	Product size (bp)
SlETR1	FP: 5'- GTG GAT TAT GGA TGC CAA CAG-3'	21mer	57	663
	RP: 5'- TCC AAG ACA TCG TTG ATG AGC-3'	21mer		
SlETR2	FP: 5'- AAG GCA GTG TGT CAG TTT CTG C-3'	22mer	57	485
	RP: 5'- ACA TCG CAC CCT AGA TGC AC-3'	20mer		
SlETR3	FP: 5'-TGA GGC TTC AGT TGC CAA AC-3'	20mer	55	500
	RP: 5'- CAT CCC ACC ATC ATC TCC AC-3'	20mer		
SlETR4	FP: 5'-ACC CCA ATG GAG GTC TTC TC-3'	20mer	57	680
	RP: 5'-CCT TGG AGG AGT GAG TGT GG-3'	20mer		
SlETR5	FP: 5'- TAA TCA GGT GAT GGG CGA TG-3'	20mer	55	467
	RP: 5'-GAA ATC GGT TGC TCC AAA GG-3'	20mer		

Table 3B: Sequence and annealing temperatures of ACS gene primers

Target gene	Primer seq. (5' ⟶ 3')	Length	Ann. Temp. (°C)	Product size (bp)
SlACS2	FP: 5'-CTA CGC AGC CAC TGT CTT TGA C-3'	22mer	53	150
	RP: 5'-TGA TTC CGA CTC TAA ATC CTG GTA A-3'	25mer		
SlACS4	FP: 5'-TTG CGA CGA AAT ATA TGC TGC T-3'	22mer	57	180
	RP: 5'-CAC TCG AAA TCC TGG AAA ACC T-3'	22mer		
SlACS6	FP: 5'-TAT GCA GCA ACC GCG TTT AGT-3'	21mer	57	150
	RP: 5'-TGT ACG AGT AAA TAA TCC CAA CCC TAA-3'	27mer		

Table 3C: Sequence and annealing temperatures of ethylene inducible gene primers

Target gene	Primer seq. (5' → 3')	Length	Ann. Temp. (°C)	Product size (bp)
SlPDS	FP: 5'-TTG TGT TTG CCG CTC CAG TGG ATA T-3'	25mer	67	500
	RP: 5'-GCG CCT TCC ATT GAA GCC AAG TAT-3'	24mer		
SlPSY	FP: 5'-GAG ATC TAC CAA TGA GTT AG-3'	20mer	49	717
	RP: 5'-TTG CCC TAT GTA TTT GTT TC-3'	20mer	50	671
SlPG	FP: 5'- GGA TCC TTA GAA GCA TCT AG-3'	20mer		563
	RP: 5'-TGT ATA CAT GGT TCA ACT CG-3'	20mer		
SlACT	FP: 5'-CCA AAA GCC AAT CGA GAG AA-3'	20mer	53	
	RP: 5'-GGT ACC ACC ACT GAG GAC GA-3'	20mer		

3.15.4 Reverse Transcribed-Polymerase chain reaction (RT-PCR)

Transcript levels of different genes were determined by RT-PCR carried out using either in one step or in two steps.

3.15.4.1 One step RT-PCR

One step RT-PCR reaction was carried out using Quiagen one step RT-PCR kit as per Manufacture's instructions. Total RNA at the concentration of 1.5 µg was used as a template and whole reaction was set up on ice. The reaction mixture was incubated at 52 °C for 30 min in a thermocycler (Eppendorf) with preheated lid for reverse transcription. The reaction mixture was heated to 95°C for 15 min to activate Hot Start Taq DNA polymerase and to inactivate the reverse transcriptase. 30 cycles of 1 min denaturation at 94°C, primer annealing at appropriate temperature for 1 min, extension at 72°C for 1 min and final extension for 10 min were used as initial cycling conditions for all the reaction. Later number of cycles and primer concentration were standardized depending on the gene copy number. PCR products were analyzed on 1.2 % (w/v) to 2% (w/v) agarose gel based on the product size.

3.15.4.2 Two step RT-PCR

In two step RT-PCR, synthesizing complementary DNA from total RNA is the first step and amplification of this cDNA by polymerase chain reaction is the second step. Invitrogen SuperScript™ First-Strand Synthesis System was used for cDNA synthesis from RNA isolated from different tissues as per the Manufactures instruction. Total RNA (2 µg) was mixed with dNTPs, Oligo (dT), and DEPC-treated water and incubated at 65°C for 5 min. At the end of incubation period, RT reaction mixture was added and incubated at 42°C for 2 min. It was fallowed by the addition of SuperScript™ II RT enzyme and the mixture was incubated at 42°C for 50 min. The reaction was terminated after 15 min by heating the mixture at 70°C. Thereafter RNase H was added and the reaction mixture was incubated for

20 min at 37°C. The cDNA concentration was measured and diluted to hundred folds. This cDNA was used as a template for amplification with any specific set of primers by regular PCR.

3.16 Treatment with ethylene action inhibitors

3.16.1 Treatment with 1-MCP

1-methylcyclopropene (1-MCP), inhibitor of ethylene action was dissolved in warm water in plastic vials to release the gas. The plastic vial was placed up right in germination boxes on vermiculite and boxes were tightly sealed immediately. Then the boxes were kept at 25±2°C under continuous white light (100 µmole $m^{-2}s^{-1}$). The length of roots and hypocotyls was measured at time points mentioned on tables and figures using a ruler. For experiments on agar, the surface sterilized seeds were first germinated on two layers of moist blotting paper in germination boxes in dark at 25±2°C. After radical emergence, the germinated seeds were transferred to 1.5 % (w/v) agar and seedlings were grown in light.

3.16.2 Treatment with silver nitrite ($AgNO_3$)

Silver nitrite was used at a final concentration of 10 µM. For silver nitrate treatment, vermiculite was moistened with 50 ml of double distilled water containing 10 µM $AgNO_3$. Wild type seeds were grown in presence of $AgNO_3$ right from germination under continuous white light (100 µmole $m^{-2}s^{-1}$). The length of roots and hypocotyls were measured five days after seedling emergence.

3.17 Time-lapse video imaging

Time lapse images were captured using a Quickcam Pro 4000 (**Logitech, USA**) as described by Roger Hangarter: (http://sunflower.bio.indiana.edu/hangart/quickcaminfo/quickcammod.html). For analysis of root growth, frames at defined time points were combined to produce a continuous movie of five days using MGI Video Wave4 PC video editing (USA).

3.18 Staining of root tips

3.18.1 Amyloplast staining

Control and 1-MCP treated seedlings were grown on vermiculite from germination to three days under continuous white light. Root tips of three days old light grown control and 1-MCP treated seedlings were stained for starch with (2 : 0.2 %(w/v) KI-I_2 for 2 minutes, washed with double distilled water for 30 s and observed under bright field for amyloplast in columella.

3.18.2 Detection of reactive oxygen species (ROS) formation in the root tips

ROS can be detected by oxidation of various derivatives of fluorescein. H_2DCFwas used in these experiments detect hydrogen peroxide (Hempel *et al.*, 1999). Control and 1-MCP treated seedlings were grown on vermiculite right from germination under continuous white light. The root tips from three days old seedlings were removed and roots were thoroughly washed with distilled water for few seconds to remove adhering vermiculite. Roots tips of one cm were fixed with methanol: acetic acid (1:3) for half an hour. Then the root tips were stained with 5 µM ROS dye (H_2DCF) in 10 mM MES-KCL buffer for 10-15 minutes in dark. After incubation the stained root tips were washed twice with MES-KCL buffer to remove excess stain. Root tips were observed first in bright field and then in florescence microscope with FITC filters.

3.19 Immunolocalization

3.19.1 Tissue fixation and embedding

Root tips of about one cm size were excised from 5 days old light grown seedlings and fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline pH 7.0 (PBS) containing 1.3 M NaCl, 70 mM Na_2HPO_4, 30 mM NaH_2PO_4 in eppendorf tubes under vacuum on ice for 30 min. The tips were allowed to settle down. The

root tips were washed twice with PBS for 5 minutes each on ice and then dehydrated by passing through an ethanol/water series of 15 % (v/v), 30% (v/v), 50% (v/v), 70% (v/v), 90% (v/v), 100% (v/v). The tips were finally placed in 0.1 % toulidine blue in 100% ethanol for one hour each on ice. There after tips were washed with 100% ethanol and left overnight in 100 % ethanol at 4°C.

3.19.2 Tissue clearing using xylene

After incubation the dehydrated tips were brought to room temperature for 30 min and then passed through an ethanol: xylene series (2:1, 1:1 and 1:2 respectively) for one hour each at room temperature. The tips were then incubated in 100 % xylene with three changes at interval of one hour. The tips was then embedded in 1:1 (xylene: paraffin) at 60°C over night. The tips were finally incubated in molten paraffin alone at 60°C, with changes of paraffin at 12 hours intervals for successive five days.

3.19.3 Block preparation and sectioning

After complete removal of xylene, the embedded tips were transferred into fresh wax maintained at 60°C. Embedding cassettes were overlaid with aluminum foil and preheated to 60-70ºC. Fresh molten wax was poured into the embedding cassettes just to cover the bottom of the cassette. Two root tips were carefully removed using a prewarmed forceps and aligned onto the cassette at 60-70ºC. The cassette was carefully placed on ice for a brief duration till the paraffin begins to solidify slightly. The remainder of cassette was then filled with the molten paraffin and then paraffin was allowed to solidify on ice. After solidification blocks were either used immediately for sectioning or stored at 4ºC until further use. Each block to be sectioned was carefully trimmed as wedge shape to about 5 mm from the margins of the embedded tissues. The blocks were sectioned at a thickness of 7 µ using a manual microtome (Leica, RM2125RT). Each block was sectioned into small ribbons shorter than the length of a standard microscope slide and the ribbons were floated onto a distilled water bath maintained at 30ºC until they have fully stretched. The individual ribbons were placed onto a clean microscopic slide, dried overnight at 42ºC and stored at 4ºC until used for immunolocalization.

3.19.4 Slide preparation

The sections were de-parafinised by incubating in 100% xylene thrice for 15 minutes each and then passed through a xylene-ethanol series of 2:1, 1:1, 1:2 respectively for 15 minutes each. The slides were then transferred to 100 % ethanol thrice for 15 minutes each. The sections were rehydrated in ethanol/ water series of 90%(v/v), 70%(v/v), 50%(v/v), 30%(v/v), 15%(v/v) for five minutes each and finally in distilled water for 10 minutes at room temperature. These rehydrated sections were then immersed in 1X Dako antigen retrieval solution (Code -Nr S2367) preheated to 90°C for 30 minutes.

3.19.5 Immunolocalization of PIN1-like proteins

The target retrieval solution was allowed to cool to room temperature and the sections were washed in PBS twice for 5 minutes each. The sections were blocked in blocking solution PBS containing 0.5 % (w/v) BSA for1 hr at room temperature and then washed with PBS for 5 minutes. Each slide was overlaid with 100 µl of *At*PIN1 antibody in blocking solution (1:20) and covered gently with parafilm through out the length of a standard microscopic slide. The slides were incubated overnight at 4ºC in humid chamber, with 4-5 layers of germination paper moistened with distilled water with out touching the moist paper lining. After 24 hours of incubation, slides were brought to room temperature and rinsed thrice with PBST (PBS+0.025% (v/v) Tween-20) for 5 minutes each. Each slide was then probed with 100 µl of secondary antibody solution containing fluorescent anti-mouse secondary antibody (Alexa Fluor-488 Cat No A11001) in blocking solution at 1:300 dilution for 2 hrs at 25±2ºC in a humid chamber as described previously. Then the sections were rinsed with PBST thrice for 5 minutes each. The slides were overlaid with 100µl of 1:1 (v/v) PBS: glycerol and covered with a cover slip and sealed with nail polish to prevent the sections from drying. These were then observed under confocal microscope (Leica) or stored at 4ºC in dark until visualized.

3.20 Data analysis

Statistical analysis was performed with **Sigmaplot 9**. All the experiments were repeated minimum of three times with replicates and the data presented are average with the standard error from all the experiments.

Results

Tomato has emerged as a model plant to study the ethylene regulated fruit ripening. Most information concerning the role of ethylene in tomato ripening has been derived from study with transgenics. Since tomato has very few mutants that are blocked in ethylene action, in this study we isolated and characterized two new mutants of tomato that are altered in ethylene action.

4.1 Isolation and characterization of *acetylene resistant (atr-1)* mutant

4.1.1 Mutant isolation

In Arabidopsis several ethylene insensitive mutants have been identified by selecting etiolated seedlings lacking triple response in ethylene rich environment (Bleecker *et al*., 1988). It is known that by virtue of its structural similarity acetylene can mimic ethylene responses in plants at higher concentration (Burg and Burg, 1962). Ethylene receptors in plants also sense acetylene and hence seedlings show triple response in presence of acetylene. Moreover acetylene can be easily produced compared to ethylene. Therefore ethylene can be used for large scale mutant screening. We used acetylene to identify tomato mutants defective in seedling triple response. A simple method was used to generate acetylene gas during seedling growth. A block of CaC_2 was placed on wet vermiculite after sowing seeds. The water present in vermiculite acts on blocks of CaC_2 releasing acetylene. The boxes sown with M_2 seeds were placed in dark and at regular intervals were observed for seedling triple response under safe green light.

In presence of acetylene etiolated tomato seedlings exhibited typical triple response with short and thick hypocotyls and tight hypocotyl hooks (Knight *et al*., 1910; Ecker, 1995). A population of 50,000 M_2 seedlings were screened, which descended from ethyl methane sulfonate (EMS) mutagenized seeds of tomato (*Solanum lycopersicon*, cv. Ailsa Craig) to isolate mutants with acetylene resistant phenotype. Seedlings with altered triple response were isolated from population after twelve days of germination under etiolation. Putative mutants were isolated in the initial screen, of which about two mutants were hookless in dark. All these mutant seedlings lacked triple response and were significantly taller in presence of

acetylene. Most of these isolated mutant plants perished after transfer to green house, except one that attained maturity and was named as acetylene resistant mutant (*atr-1*) (Fig. 5A) (Isolated by Sagar datir in 2001 and then maintained by Dr. Sangeeta Negi till 2003). The *atr-1* mutant plant produced few flowers and was rescued by crosses with wild type pollen. Subsequently, the F_1 plants were allowed to self pollinate to get homozygous seeds in F_2 generation. After multiplication of seeds the phenotype of *atr-1* mutant was compared with wild type under different concentrations of acetylene. Mutant seedlings showed the reduced inhibition of hypocotyls and root elongation in comparison to wild type under various concentrations of acetylene (Fig. 5B, C) (Dose response was carried out by Dr. Madusmita Panigrahy).

4.1.2 Genetic characterization

The genetic segregation of acetylene insensitivity was examined by crossing *atr-1* mutant plant (*atr-1/atr-1*) in M_4 generation with wild type plant (*ATR-1-1/ATR-1*). All F_1 plants of M_2 *atr-1* x WT exhibited normal acetylene sensitivity at the seedling stage and normal fruit ripening like wild type. For F_2 segregation analysis 12 days old etiolated F_2 seedlings were scored for the seedling triple response and segregation of the acetylene insensitivity was consistent with 3(sensitive):1(insensitive) Mendelian ratio, confirming the monogenic recessive nature of the mutation. These results indicate that the *atr-1* mutation is controlled by a single recessive nuclear gene (Fig. 6).

4.1.3 Phenotypic characterization of ethylene insensitivity

The effect of *atr-1* mutation was not restricted to reduced ethylene sensitivity in the seedlings alone. The *atr-1* mutation also exerted strong pleiotropic effect on all stages of plant development right from the germination to senescence. This required examination of morphology of *atr-1* mutant right from germination to flowering and fruit setting. In subsequent section specific variation in morphological features are described at different stages of *atr-1* mutant development.

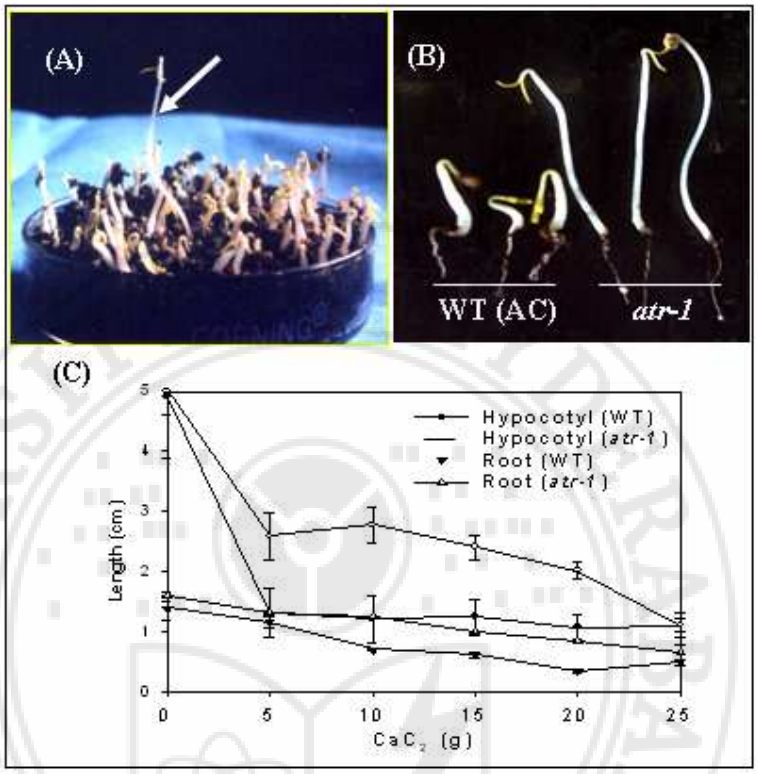


Figure 5. Seedling triple response and dose response to acetylene. **A,** Photograph showing selection of tomato *acetylene resistant* (*atr*-1) mutant seedling from EMS treated M_2 Population. Seeds were germinated on vermiculite in continuous presence of acetylene for nearly 12 days in total darkness in closed container. Thereafter containers were opened and putative mutant seedlings were selected based on elongated hypocotyl in presence of acetylene. The arrow show the acetylene resistant seedling that lacks triple response. **B,** The *atr-1* mutant (M_4) and WT seedlings were grown in darkness in presence of 15g of CaC_2 till 12 days. Note the reduced hypocotyl growth inhibition in *atr-1* (right) compared to wild type (left) seedlings. **C,** Tomato seedlings were grown in presence of different concentrations of CaC_2 in total darkness. At the end of 12 days length of hypocotyl and root was measured. The control seedlings were incubated similarly without calcium carbide. Vertical bars represent SE (n=21) (Data obtained by Dr. Madhusmita.Panigrahy).

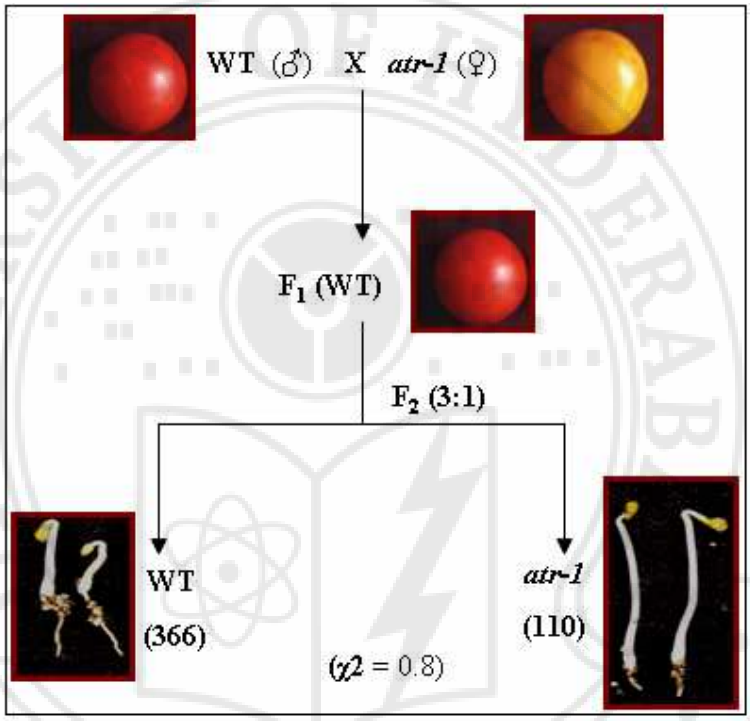


Figure 6. Genetic segregation of *atr-1* mutant. The F_2 seeds were germinated in presence of acetylene in dark and mutant seedlings were scored based on the resistant phenotype. The number in parenthesis indicate the number of F_2 seedlings showing triple response or resistant phenotype.

4.1.3.1 Ethylene responses in seedlings

4.1.3.1.1 The *atr-1* mutant show insensitivity to exogenous ethylene

It is known that CaC_2 on contact with water releases acetylene. Since acetylene is not the only end product of the reaction, it is likely that observed mutant phenotype can also result because of interaction with other components of the reaction. Therefore we ascertained whether *atr-1* mutant is also resistant to ethylene by growing seedlings in presence of ethylene and its precursor 1-ACC. Figure. 7A shows that etiolated wild type seedlings exhibited characteristic triple response to ethylene which is largely absent in *atr-1* mutant seedlings. Likewise the etiolated *atr-1* seedlings grew taller than the wild type control seedlings on agar medium containing 20 µM concentrations of 1-ACC, the precursor for ethylene biosynthesis (Fig. 7B). In presence of ethylene triple response is more exaggerated than 1-ACC, as 1-ACC is needed to be converted to ethylene in order to act.

4.1.3.1.2 Ethylene evolution is reduced in *atr-1* mutant seedlings

In view of ethylene/acetylene insensitivity of seedlings, we also examined whether the mutation had in any way effected ethylene biosynthesis too. The amount of ethylene evolution was estimated by gas chromatography at different days after seed germination at regular intervals. The *atr-1* seedlings released less amount of ethylene than the wild type seedlings during all time periods examined. The ten days old *atr-1* mutant seedlings released about 50% of ethylene compared to wild type control (Fig 7C).

4.1.3.2 Effect of ethylene on vegetative growth

4.1.3.2.1 The *atr-1* mutant plants has long internodes

The green house grown plants of *atr-1* mutant were taller than wild type. The length of internodes were recorded for at lest five wild type and mutant plants

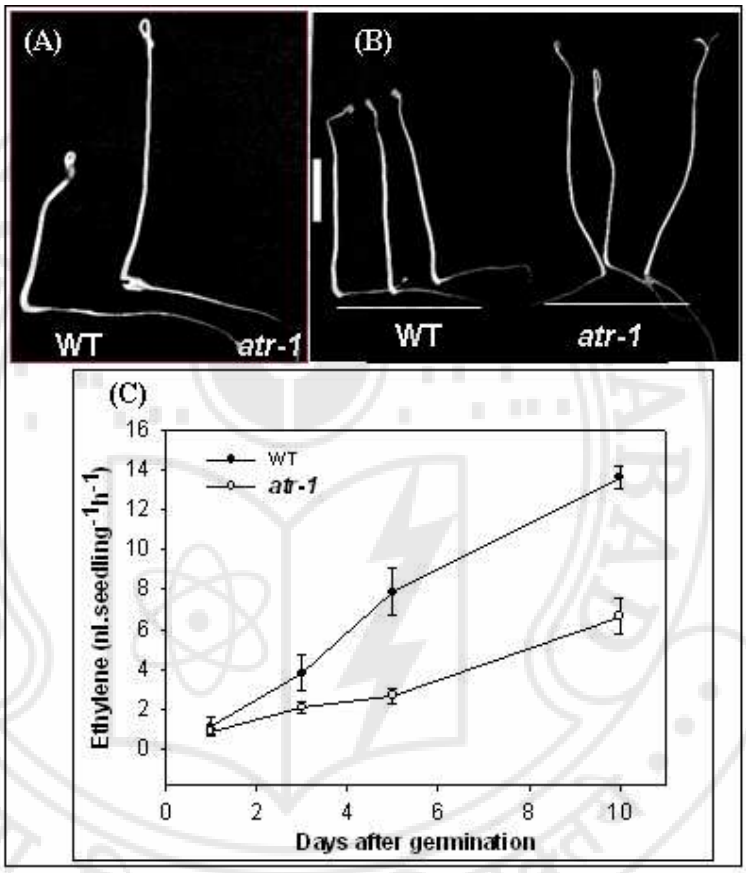


Figure 7. Ethylene induced triple response and ethylene evolution from seedlings. The triple response of 12 days old dark grown WT and *atr-1* in presence of 20 $\mu l.l^{-1}$ of ethylene **(A)** and 20 µM of 1-ACC **(B)**. **C,** Ethylene evolution in control wildtype and *atr*-1 seedlings at different days from germination. Vertical bars represent SE. (n=30-35).

starting from eighth node to five successive nodes. On examination it was found that though plants had same number of internodes, the mutant had elongated internodes. The internodes of three months old mutant plants were on average 3-4 cm longer than wild type plants (Fig. 8A).

4.1.3.2.2 Chlorophyll content is enhanced by *atr-1* mutation

The leaves of *atr-1* were greener than wild type leaves. Estimation of leaf chlorophyll content showed higher chlorophyll levels in mutant leaves compared to wild type (Fig. 8B).

4.1.3.2.3 The *atr-1* mutant show delayed leaf senescence

In plants ethylene promotes senescence of mature leaves both on plants and after detaching them. Since *atr-1* leaves showed more chlorophyll, we examined the senescence of detached leaves of *atr-1* mutant and wild type. Detached leaves of wild type and *atr-1* were incubated on two layers of moist blotting paper in dark for induction of senescence. The detached leaves of mutant were slower to senesce and appeared greener than wild type after nine days. (Fig. 9A,B). The loss of chlorophyll during senescence was visually and photographically monitored.

4.1.3.3 Ethylene responses in reproductive tissue

4.1.3.3.1 Quantitative traits

4.1.3.3.1.1 Inflorescence initiation is delayed in *atr-1* mutation

Inflorescence formation is slightly delayed in mutant plants compared to wild type plants. Under idle conditions wild type plants set inflorescence mostly from sixth node onwards whereas *atr-1* mutant plants set inflorescence from seventh or eighth node onwards.

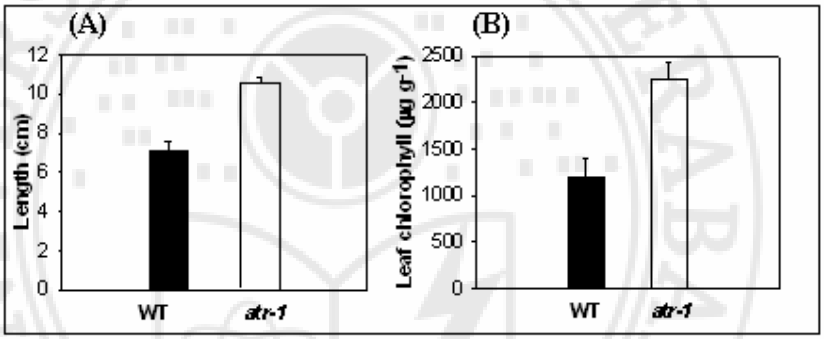


Figure 8. Effect of *atr-1* mutation on vegetative growth. **A,** Internodal length of *atr-1* mutant in comparison with wild type. **B,** Quantification of total leaf chlorophyll content from eighth node of wild type and mutant.

4.1.3.3.1.2 The *atr-1* mutant has enhanced fruit set

The *atr-1* mutation also influenced the number of flowers in inflorescence. Mutant had almost double the number of flowers in inflorescence compared to wild type (Fig. 9C). Most of these flowers set fruits after natural pollination. The increased number of trusses and increased number of flowers per truss lead to increased fruit set and yield in mutant plants (Fig. 9D).

4.1.3.3.1.3 Fruit characteristics are not altered by *atr-1* mutation

The *atr-1* mutation has no effect on placentation as both wild type and mutant fruits exhibit bilocular ovary. Fruits of mutant plants were similar in size to wild type fruits. The number of fruits in mutant plants were almost double than the control plants. In case of *atr-1* mutant lines, the fruit weight was slightly less than wild type. The number of seeds per fruit was also reduced by the mutation (Table 4).

4.1.3.3.2 Qualitative traits

4.1.3.3.2.1 On vine ripening period

The stage of on-vine ripening along with fruit development was chronologically recorded by visual examination of fruit development from time point of anthesis to final fruit drop. The interval to attain mature green stage and thereafter different stages of ripening from mature green to red stage were noted. Comparatively the fruits of *atr-1* mutant showed considerable delay in all stages of fruit development and ripening. Mutant fruits reached breaker stage about 44-46 days post anthesis (DPA) whereas wild type reached breaker stage 30-32 DPA. The delay of nearly 12-16 days in mutant plant is primarily due to slower fruit development. Moreover, the transition of fruit through different stages of ripening was also slower in the mutant than in wild type fruits. The wild type fruits took nearly five days from breaker stage to red ripe stage, while mutant fruits took nearly 15 days for this

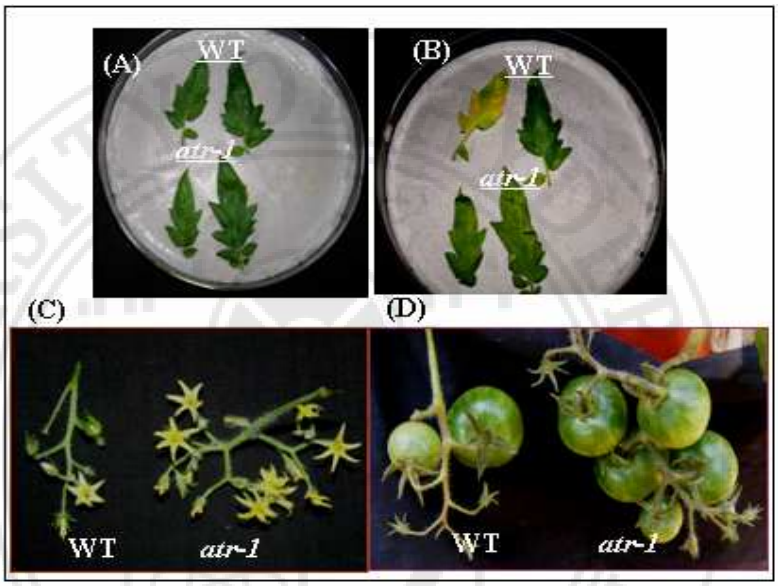


Figure 9. Effects of *atr-1* mutation on leaf senescence and reproductive tissues. The detached leaf lets from 8th node of two months old green house grown *atr-1* mutant and WT plants were incubated in dark to induce senescence. The leaflets were photographed at starting of the experiment **(A)** and at end of the experiment at nine days **(B)**. **C**, Increased number of flowers in inflorescence of *atr-1* in comparison with WT. **D,** The *atr-1* plants showing higher fruit set than wild type plants.

Name	Fruit weight (g)	Fruit diameter (cm)	No.of locules	No. of Seeds/fruit	Fruit pH
Wild Type	36.0 ± 0.9	14.2 ± 1.4	Bilocular	98 ± 3	7.2
atr-1	33.0 ±1.8	14.0 ± 1.8	Bilocular	50 ± 3	6.0

Table 4. Comparison of fruit characters of wild type and *atr*-1mutant. Fruits of wild type and mutant plants were harvested at red stage and fruit weight, fruit diameter and number of seeds per fruit were recorded (n=10).

transition. The *atr-1* mutant fruits also stayed on vine for a longer period after attaining red ripe stage. The fruit drop takes place after thirty days from breaker stage in mutant plants while in wild type fruit drop occurs within 15 days from breaker stage. Taken together the development and ripening, the *atr-1*mutant fruits showed nearly thirty days delay in fruit cycle compared to wild type (Fig. 10 A,B).

4.1.3.3.2.2 Ethylene evolution from fruits

To ascertain whether mutant fruits were compromised in ethylene synthesis, we examined the amount of ethylene generated in the head space of mutants and wild-type fruits by Gas Chromatography. The fruits were harvested at different stage of development such as Mature Green, Breaker, Turning, Orange and Red stage and enclosed in a closed container to collect ethylene. The total ethylene evolution was normalized to the fresh weight of the fruits and the time of incubation. Inspite of slow ripening of *atr-1* fruits, ethylene release from mutant fruits was nearly equal to wild type. In both cases, ethylene evolution was at the basal level at mature green stage, thereafter it increased and reached a peak at the orange stage, and declined at the red stage (Fig. 10C).

4.1.3.3.2.3 Fruit pigment content

The level of major fruit pigments β -carotene and lycopene during fruit ripening of wild type and *atr-1* was compared using HPLC. In wild type the carotenoid content was estimated at breaker stage (32 DPA) where fruit change colour due to onset of carotenoid synthesis and also at red ripe stage (46 DPA) where accumulation of carotenoids and lycopene is nearly complete. In view of chronological variation in mutant fruit development, we used physiologically similar stages for the mutant fruits. For mutant the fruits of 44 DPA and 65 DPA were used for estimation. In both mutant and wild type fruits the total carotenoid and lycopene content was nearly similar at breaker stage. At red ripe stage of wild type fruits have much higher levels of carotenoid and lycopene. Even though the mutant fruits appeared red, the pigment content was almost half to that of wild type of the same age (Fig. 11).

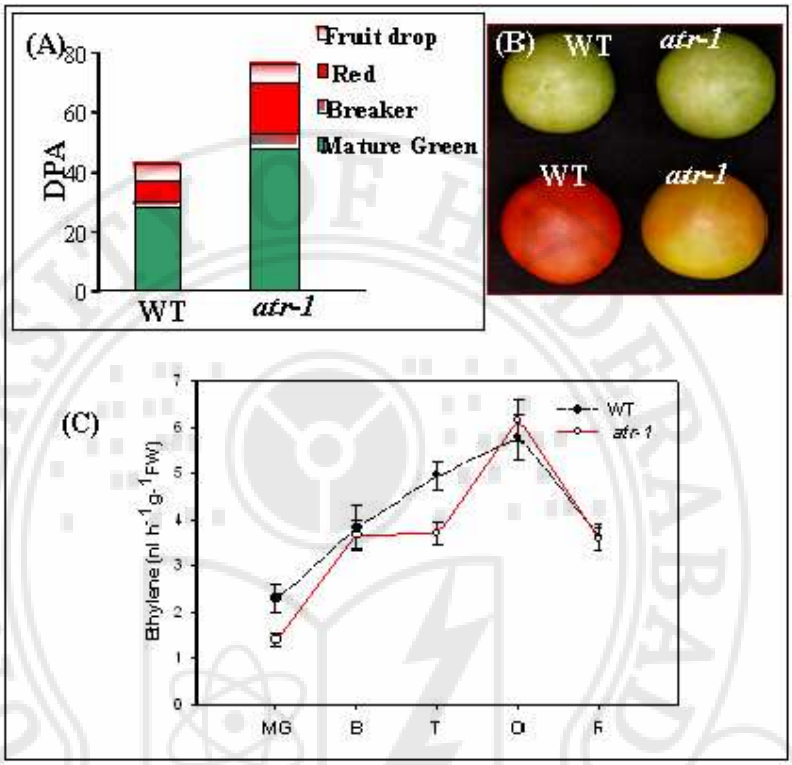


Figure 10. Effect of *atr-1* mutation on fruit ripening and ethylene evolution. **A,** Chronological development of tomato fruits. Time taken from day of anthesis to attain mature green, breaker, red stage of fruits and final fruit drop. Each stack of the bar in the graph corresponds to an average of seven fruits from first truss of wild type and *atr-1* mutant. **B,** Fruit phenotypes of wild type (WT) and *atr-1* mutant after 28 DPA and 44 DPA. Note the delay in ripening and ripening induced colour development in *atr-1* fruits compared to AC fruits. **C,** Ethylene production in control AC and *atr-1* during fruit ripening. Each fruit was sampled three times. Each data point in graph represents the average of at least ten individual fruits from each genotype.

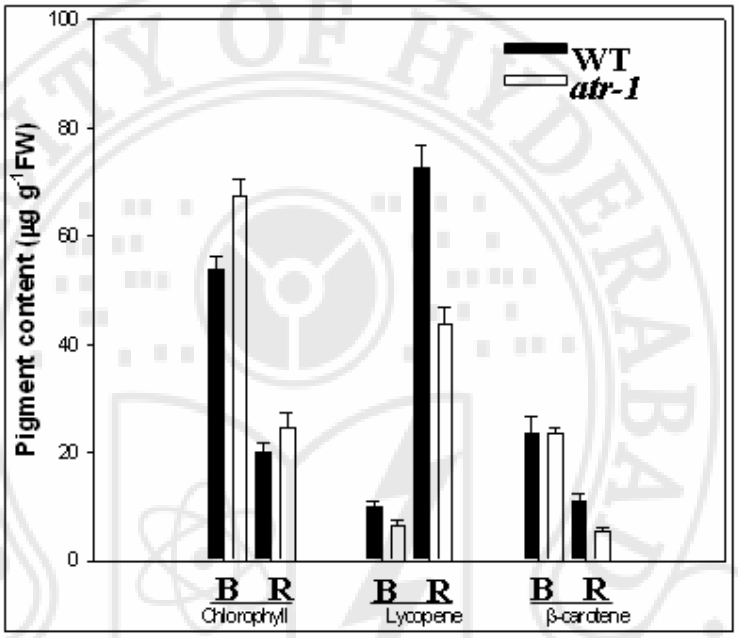


Figure 11. Chlorophyll and carotenoid content in fruits of *atr-1* mutant in comparison with wild type. The amount of total chlorophyll, β-carotene and lycopene present in breaker (B) and red fruits (R) of wild type and mutant were measured. The data represents the mean of ten fruits pooled from three individual experiments (n=10).

Fruit chlorophyll content was also estimated at breaker and red stages of ripening. The amount of chlorophyll was high at breaker stage and declined at red stage in wild type fruits. In wild type fruits chlorophyll reduction in red fruits was higher than mutant fruits. Fruits of *atr-1* mutant show delay in chlorophyll degradation and carotenoid accumulation compared to wild type (Fig. 11).

4.1.3.3.2.5 The fruits of *atr-1* were acidic

Fruit ripening process induces reduction in fruit acidity. The fruit juice from red ripe wild type and *atr-1* fruits titrated with 0.1 N NaOH to bring to neutral pH. The red ripe wild type fruits have almost neutral pH (7.2) whereas the red ripe mutant fruits have acidic pH (6.0). The titrable acidity of mutant fruit is almost three to four times to that of wild type (Table 4).

4.1.3.4 Responses of mutant to exogenous ethylene

4.1.3.4.1 The *atr-1* mutant show resistance to ethylene induced leaf senescence

The detached leaves of wild type and *atr-1* mutant were treated with 2 ml l^{-1} of ethylene in a closed chamber to trigger ethylene induced senescence. The wild type leaves began to lose visible green colour within 48 hours of treatment. By six days of treatment wild type turned yellow whereas the *atr-1* mutant retained visible green colour even after nine days (Fig. 12 A-D).

4.1.3.4.2 Cotyledon abscission is delayed but epinasty is normal in mutant seedlings

It is known that excess ethylene induces cotyledon abscission in tomato (Barry *et al*., 2005). To examine ethylene sensitivity, mutant and wild type light grown seedlings were exposed to ethylene. The *atr-1* mutant seedlings showed resistance to ethylene induced cotyledon abscission. However the mutant seedlings show epinasty. While nearly half of wild type cotyledons from five days old seedlings were abscised after 48 hours of ethylene treatment where as cotyledons of mutant seedlings did not show abscission (Fig. 13A).

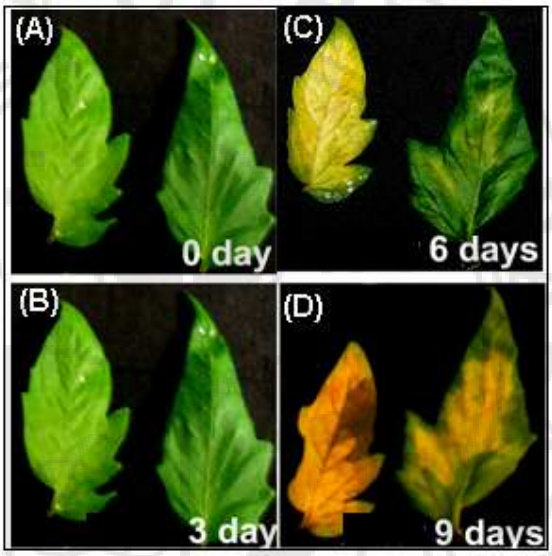


Figure 12. Ethylene induced leaf senescence. **A-D,** Senescence of wild type and *atr-1* mutant leaflets after exposure to ethylene. The leaflets from eighth node of wild type (left) and mutant (right) were treated with air or 2 mll^{-1} ethylene for nine days (n=5).

4.1.3.4.3 Floral abscission is delayed in *atr-1* mutant

It is known that application of ethylene to tomato plants strongly promotes floral abscission. The abscission of flowers in detached inflorescence in presence of acetylene is delayed in mutant compared to wild type. After 24 hours of acetylene treatment in wild type almost 40% of the flowers in detached inflorescence were abscised and 60% were wilted whereas in mutant only 5-10% abscised and 40% were wilted and remaining were just begun to senesce (Fig. 13B). After 48 hours nearly 93-95% of the wild type flowers were abscised while only 80-85% were abscised in mutant.

4.1.3.4.4 Induction of fruit ripening is slow in mutant fruits

The mutant fruits were also slow to ripen after exposure to exogenous ethylene. The mature green fruits of wild type and *atr-1* plants were detached based on external colour and treated with 2 ml l^{-1} of ethylene. While wild type fruits turned to red within 2-3 days, the mutant fruits turned orange after one week of treatment and stayed orange for 2-3 weeks before turning to light red colour (Fig. 13 C,D).

4.1.3.5 Post harvest quality traits

4.1.3.5.1 The *atr-1* mutant exhibit prolonged fruit shelf life

Red ripe fruits were detached from wild type and *atr-1* plants and examined for shelf-life at room temperature (25±2°C). After eight weeks of incubation fruits were examined (n=18-20). Nearly 60% of wild type fruits were wrinkled, 35% were rotten and 5% were totally degenerated. In case of *atr-1* nearly 70% of fruits were wrinkled, 21% began to rot and none of them were degenerated (Fig. 14A,B). Though *atr-1* fruits show wrinkling they can stay in this stage for three to four months compared to two months seen for wild type under controlled temperatures (23±2°C).

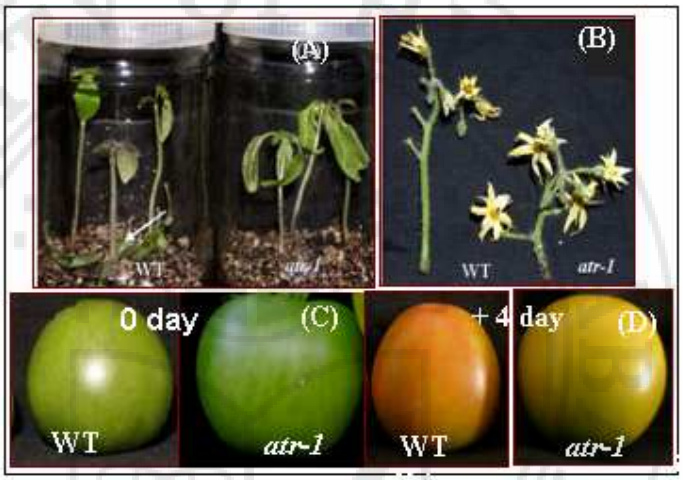


Figure 13. Ethylene responses in wild type and *atr-1* mutant. **A-B,** Effect of ethylene on organ abscission of tomato wild type and *atr-1* mutant. **A,** After 48 h of acetylene treatment most of the wild type seedling cotyledons abscised compared to mutant. The arrow points to abscised cotyledons lying on the vermiculite. **B,** After 24 h of acetylene treatment most of wild type flowers abscised compared to mutant. **C,** Mature green fruits of WT and *atr-1* before treating with ethylene. **D,** Fruits of WT and *atr-1* one week after treatment with 2 $ml.l^{-1}$ ethylene.

4.1.3.5.2 *atr-1* mutant show delayed off vine ripening

The fruits of *atr-1*harvested at mature green stage also showed slow ripening when stored at room temperature (25±2°C). Significant delay in transition of different stages was observed in mutant fruits compared to wild type fruits. Especially, the transition from orange to red stage was slow in mutant compared to wild type. Four weeks after harvest at mature green stage nearly 89% of wild type fruits attained red stage and only 11% were in orange stage, whereas after four weeks 98% of *atr-1* fruits were in orange stage and only 2%were in red stage (Fig. 14C,D).

4.1.3.5.3 The *atr-1* mutant fruits show enhanced thermotolerance

Ethylene sensitivity is also important for various abiotic stress responses such as high temperature. The red ripe fruits of wild type and *atr-1* were kept at 65°C for three days to check the thermotolerance of the fruits (Fig. 15A,B). Photographs were taken at regular intervals till three days. The *atr-1*fruits were found resistant to heat mediated deterioration. The reduced fruit deformation of mutant fruits compared to wild type fruits may be due to its insensitivity to ethylene or reduction in heat induced ethylene.

4.1.3.5.4 Wound induced ethylene production is reduced in mutant fruits

Red ripe fruits of wild type and *atr-1* were weighed and cut into four pieces and sealed in air tight container to estimate the wound induced ethylene production. The amount of ethylene evolved by these cut fruits was measured after 2 h, 4 h and 6 h. There is a burst of ethylene immediately after cutting and it is slowly reduced with time in wild type fruits. No such burst of ethylene was seen for cut *atr-1* fruits (Fig 15C).

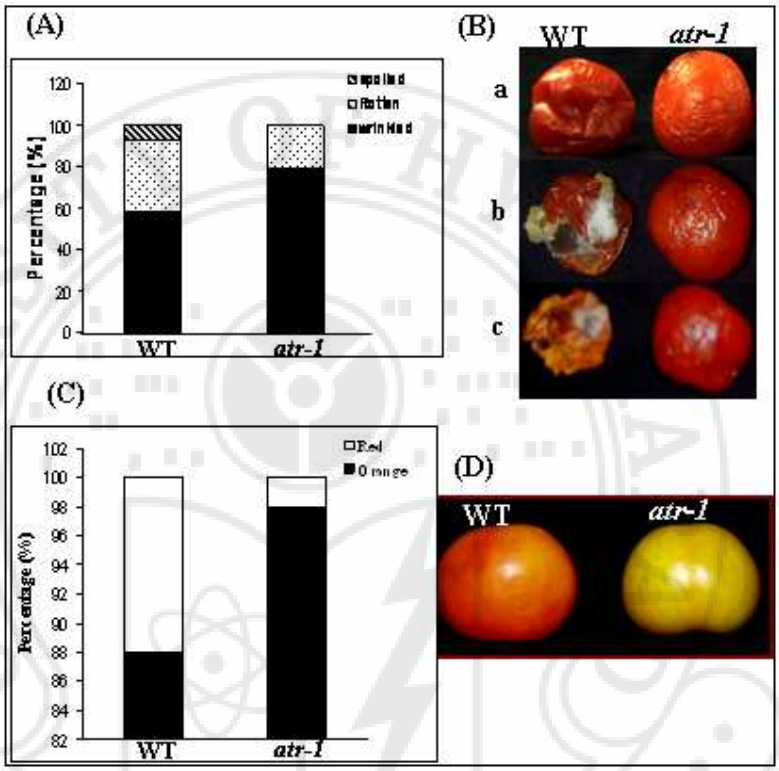


Figure 14: Shelf life and off vine ripening of WT and *atr-1* fruits. **A,** Graphical representation of offvine shelf life of WT and *atr-1* fruits after two months of harvest at red stage (n=15-20). **B,** Fruits were harvested at red ripe stage and observed for rot development at room temperature till 45 days. a, Fruits after 7 days of harvest at red stage. b, Fruits after 25 days of harvest at red stage. c, Fruits after 45 days of harvest at red stage. **C,** Graphical representation of off vine ripening of WT and *atr-1* fruits after one month of harvest at breaker stage (n=15-18). **D,** Fruit phenotypes of WT and *atr-1* ten days after harvest at mature green stage.

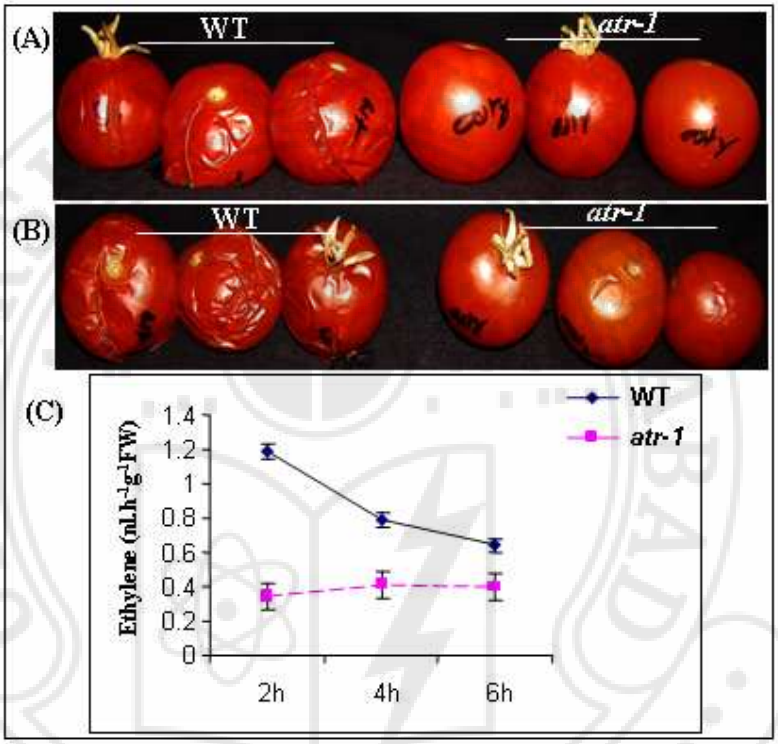


Figure 15: Heat and wound resistance in mutant fruits. **A,** Fruits of WT (left) and *atr-1* (right) one day after incubation at 65°C. **B,** Fruits of WT (left) and *atr-1* (right) three days after incubation at 65°C. **C,** Red ripe fruits of WT and *atr-1* were cut into four pieces and ethylene evolution was measured at regular intervals using gas chromatography (n=5).

4.1.4 Effect of *atr-1* mutation on gene expression

The onset of fruit ripening in tomato is characterized by rapid changes in gene expression that ultimately result in the fully ripened fruit (Giovannoni, 2001). The *Nr* mutant carries a mutation in a member of the tomato ethylene receptor gene family rendering tomato plants insensitive to ethylene in all tissues examined (Lanahan *et al*., 1994). The ethylene insensitivity of *atr-1* mutant prompted us to examine expression of genes regulating ethylene synthesis and perception in mutant fruits. The sequences of genes involved in ethylene perception and biosynthesis were aligned using EXPASY multiple alignment software for designing gene specific primers of tomato ETR-like genes from nonconserved regions and conditions were standardized. (Nakatsaka *et al*., 1998). RNA concentration was normalized by using actin as an internal control.

4.1.4.1 Ethylene receptor gene expression is altered in *atr-1* mutant

The expression of members of the tomato *ETR* gene family was analyzed during ripening of wild-type and *atr-1* fruits. Transcripts from Sl*ETR1*, Sl*ETR2*, Sl*ETR3*, Sl*ETR4* and Sl*ETR5* were detected in tomato fruit. The expression of *SlETR1* is enhanced in *atr-1* mutant particularly at orange and red stages of ripening than wild type. The *SlETR2* transcript levels were higher during middle of ripening at turning and orange stages and declined during red stage of ripening. The *SlETR2* gene expression is significantly lower at the breaker stage of mutant fruits. Since the overexpression of *SlETR3 (SlNR)* or *SlETR4* receptor gene expression eliminates the ethylene sensitivity of tomato seedlings we checked the expression levels of *SlETR4* and Sl*NR r*eceptor genes in *atr-1* (Klee**,** 2002). As ripening progressed the expression levels of *SlETR3* continued to increase in wild type and mutant. The rise was more in mutant fruits compared to control. However, the slight increase in expression of this gene in mutant may not account for the high degree of insensitivity to acetylene showed by the mutant seedlings. The expression of *SlETR4* gene was similar to wild type, but shows a decrease at red stage of fruits. The expression of *SlETR5* was also reduced in mutant fruits compared to wild type (Fig. 16).

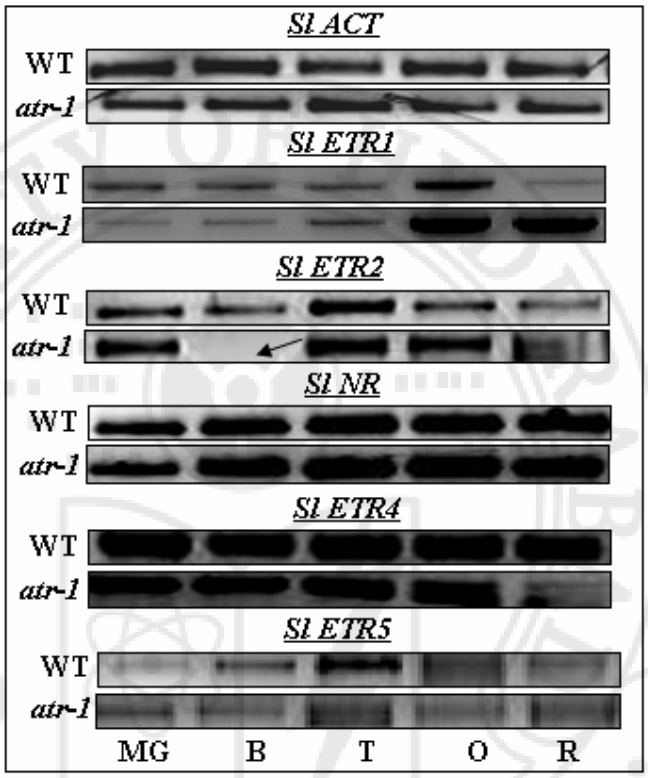


Figure 16. Relative transcript levels of ethylene receptor genes in wild type and *atr*-1 mutant. Total RNA isolated from pericarp of mature green (MG), breaker (B), turning (T), orange (O) and red (R) stages of WT and *atr-1* fruits were subjected to one step RT-PCR using ETR gene specific primers (as mentioned on top). Note the altered expression of ETR genes during ripening of mutant. Arrow points the striking reduction in ETR2 gene expression in mutant.

4.1.4.3 Expression of ACS genes in fruits

We observed similar ethylene levels in mutant fruits during ripening using gas chromatography. *SlACS6* involved in the production of system 1 ethylene in green tissue, *SlACS4* is induced during the transition between system1 and system 2, finally system2 ethylene production is maintained by ethylene-dependent induction of *SlACS2*(Barry et. al., 2000). We investigated the expression of three *SlACS* genes *SlACS2*, *SlACS4*, SlACS6 during various stages of ripening in both mutant and wild type fruits. The expression pattern of *SlACS6* gene is uniform, whereas wild type fruits showed progressive increase at different stages of ripening indicating the normal system 1 ethylene production in mutant fruits. The expression of *SlACS2* gene was higher till turning stage in *atr-1* fruits, there after its expression is comparable to wild type during later stages of ripening. The expression of *SlACS4* gene reduced during red stage of ripening in mutant fruit compared to wild type (Fig. 17A).

4.1.4.4 Ethylene regulated gene expression is altered in fruit

The regulation of ripening-related changes in gene expression is not fully understood, but in tomato, several genes are coregulated by ethylene-independent and ethylene-dependent signaling pathways (Giovannoni, 2004; Lincoln *et al*., 1987, 1988). We examined the accumulation of transcripts for the ripening-related, ethylene-regulated genes like polygalacturonase, phytoene synthase and phytoene desaturase in wild type and *atr-1* mutant fruits. *E8A* expression during ripening has been shown to be entirely due to increased ethylene biosynthesis. Its transcripts were reduced in mutant fruits during orange and red stages of ripening compared to the corresponding stages in wild type. However the expression of E8A was high at MG and B stages of mutant compared to wild type. The level of phytoene synthase (*PSY)* transcript was higher at MG stage compared to wild type. The expression of phytoene desaturase (*PDS*) and polygalacturonase (*PG*) expression were greatly reduced in red stage of mutant fruits (Fig. 17B).

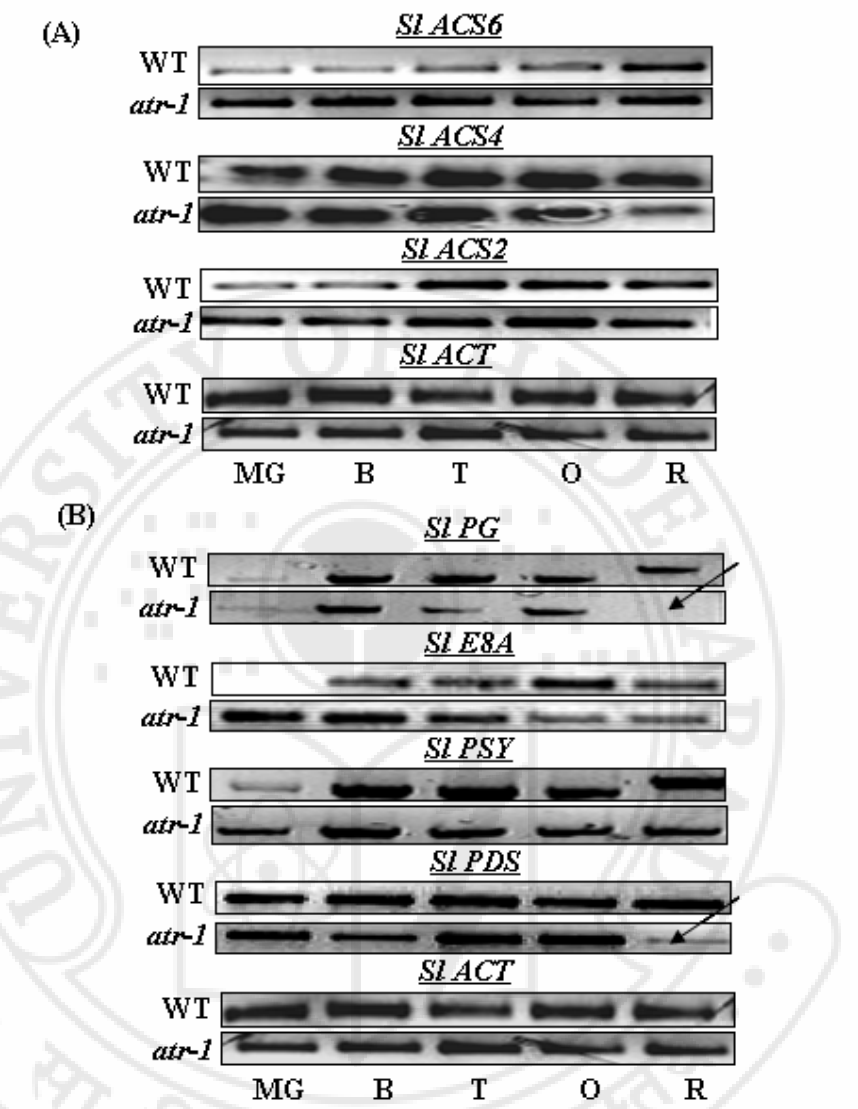


Figure 17. Expression of SlACC synthase 2,4,6 and other ethylene responsive genes during fruit ripening. Total RNA was isolated from mature green (MG), breaker (B), turning (T), orange (O) and red (R) stages of fruit ripening from WT and *atr-1*. 1.5 μg of total RNA was used as template for one step RT-PCR using *SlACC* synthase **(A)** and ethylene inducible **(B)** gene specific primers as indicated on the top of the figure. Expression of actin was used as internal control to ensure equal loading of the samples. Arrows points the striking reduction in expression.

4.2 Isolation and characterization of *kin-1* mutant

4.2.1 Isolation of *k*inetin *in*sensitive (*kin-1)* mutant

Kinetin is one of the many endogenous factors that can modulate ethylene biosynthesis (Vogel *et al*, 1998). The seedlings grown on higher concentrations of kinetin undergo stress and that result in stress induced synthesis of ethylene. Consequently, the seedlings grown on kinetin rich medium showed growth inhibition. The seedlings which fail to show inhibition of growth in presence of kinetin could be either a mutant defective in ethylene biosynthesis or insensitive to cytokinin. The M_2 population of tomato seedlings was screened for kinetin insensitive (*kin*) mutants. Seedlings were grown in darkness for two weeks after germination on kinetin rich media. In a population of 70,000 seedlings, thirty seedlings showing lack of growth inhibition in presence of kinetin were isolated (Fig. 18) (Screening was done by Ms. Sonal in 2001). The isolated putative mutant seedlings were grown to maturity in green house. Out of thirty, only ten plants survived till maturity and produced seeds. Out of ten plants, on examination of phenotype in subsequent generation, only four lines showed the kinetin insensitive (*kin)* phenotype. One of these lines with more promising phenotype was selected for further characterization and was named as *kin-1*. Fig 19 A shows dose response curve of hypocotyl lengths of wild type and *kin-1*. At 125 µM kinetin concentration tested *kin-1* mutant seedlings exhibited reduced growth inhibition compared to wild type (Fig. 19B).

4.2.1.1 Characterization of progressive fruit development

The kinetin insensitive mutants were observed for phenotypic changes during vegetative and reproductive growth. There were no obvious changes in phenotype during vegetative growth of the mutant lines. Since these mutants could be ethylene underproducer, the mutants were examined for changes in on vine fruit ripening (Virginia and Lomax, 2003). The precise sequence of fruit development was recorded on a chronological scale. Individual flowers were tagged on the day of anthesis and the development of fruits was visually recorded for each tagged

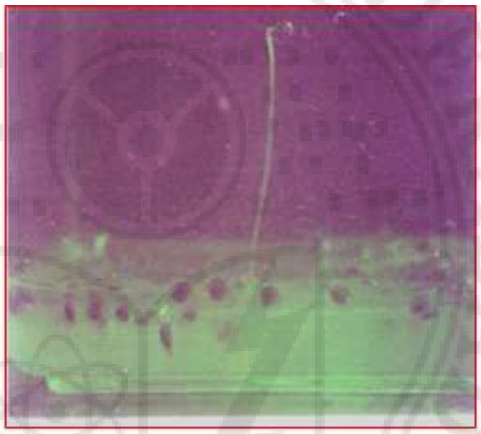


Figure 18. Isolation of kinetin insensitive mutant *(kin-1)* from EMS mutagenized M_2 population (Isolated by Sonal, 2001).

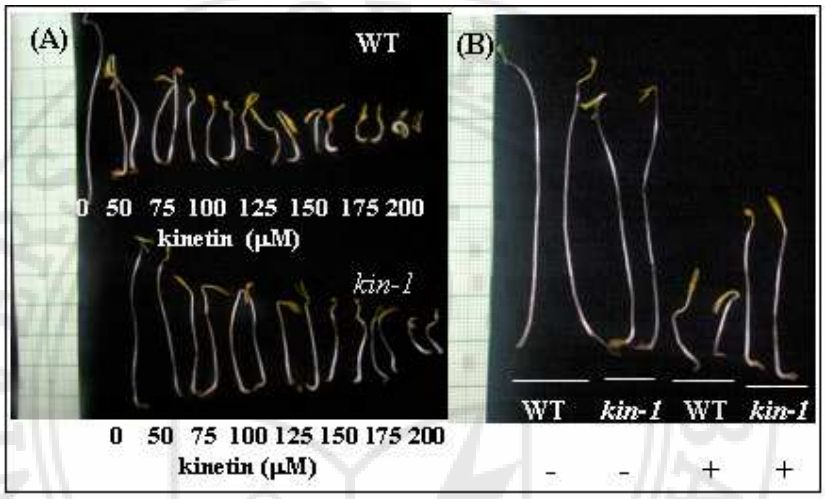


Figure 19. Dose response and seedling phenotype of WT and *kin-1*. **A,** WT and *kin-1* seedlings were grown from germination on water agar medium with increasing concentrations of kinetin in dark for two weeks. **B,** Phenotype of WT and *kin-1* seedlings in presence (+) and absence (-) of kinetin. Seeds of mutant and wild type were grown on agar supplemented with 125 µM kinetin in darkness. The seedlings were photographed after two weeks of growth. Note that *kin-1* mutant seedlings are more resistant to the growth inhibition of kinetin when compared to wild type seedlings.

flower right from anthesis to fruit drop along with wild type. The different stages of ripening were recorded for at least five fruits per plant from a total of seven plants. Even though all the putative mutants showed delay in fruit ripening, we selected *kin-1* mutant line that showed maximum delay of ripening as most promising line for further study.

4.2.2 Genetic characterization

4.2.2.1 Segregation analysis

Genetic crossing and segregation studies help to know the nature of the mutation, viz. dominant/recessive relationships, and number of genes contributing to the phenotype. The reciprocal crosses to wild type were made using the *kin-1* mutant line as female parent. The screening of two weeks old F_1 seedlings grown in darkness in presence of kinetin (125 µM) showed kinetin mediated growth inhibition similar to wild type control. The F_1 mutant plants also showed normal fruit ripening like wild type in green house. In F_2 generation examination of kinetin sensitivity showed segregation of seedlings into typical Mendelian 3:1 ratio which indicated monogenic recessive nature of *kin-1* mutation (Fig. 20).

4.2.3 Phenotypic characterization of kinetin insensitive (*kin-1)* mutant

The *kin-1* mutant plants were characterized at different stages of life cycle. The mutant plants were examined for various ethylene responses starting from germination to fruit ripening.

.2.3.1 Ethylene responses in the seedlings

4.2.3.1.1 *kin-1* seedlings exhibit normal acetylene sensitivity

The insensitivity of *kin-1* mutant to high concentration of kinetin can result either by underproduction of ethylene or mutant has lost its capacity to perceive ethylene.

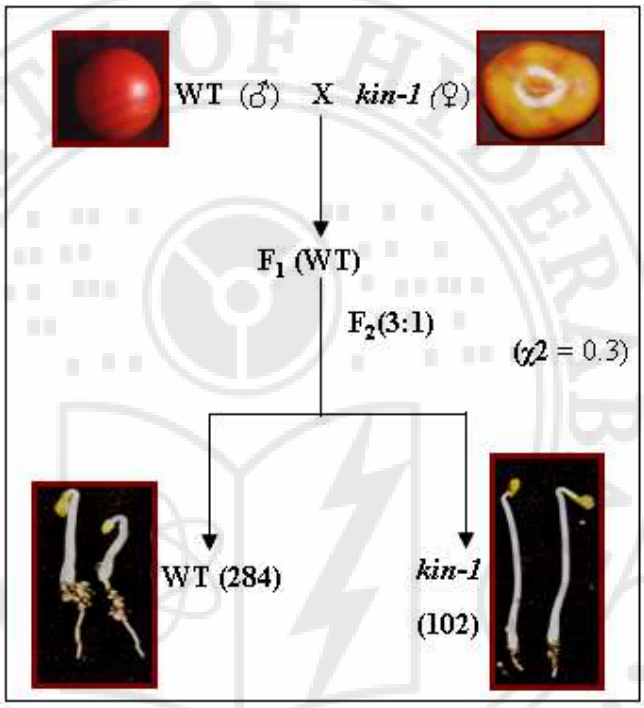


Figure 20. Genetic segregation of *kin-1* mutant. The F_2 seeds were germinated in presence of kinetin (125 µM) in dark and mutant seedlings were scored based on the resistant phenotype. The number in parenthesis indicate the number of F_2 seedlings observed for triple response or resistant phenotype.

4In view of this *kin-1* mutant seedlings were checked for the acetylene sensitivity by monitoring triple response. The *kin-1* seedlings were grown right from germination in presence of acetylene under continuous darkness. Fig. 21 A,B shows that in presence of acetylene inhibition of hypocotyls elongation in wild type and *kin-1* mutant seedlings were nearly similar.

4.2.3.1.2 Ethylene evolution is reduced in mutant seedlings

The ethylene evolution in light grown wild type and *kin-1* seedlings was examined from germination to ten days at regular intervals. During initial stages of germination both wild type and mutant seedlings showed similar level of ethylene evolution for one to three days from germination. After three days mutant showed no further increase in ethylene release compared to wild type. Ten days old light grown *kin-1* mutant seedlings released nearly 60% less ethylene than wild type (Fig. 21C).

4.2.3.2 Ethylene responses in vegetative tissue

4.2.3.2.1 *kin-1* mutant has reduced leaf chlorophyll content

The leaves of greenhouse grown *kin-1* plants were pale green in color compared to wild type (Fig. 22A). The chlorophyll content of leaves in wild type and *kin-1* mutant was measured. The mutant showed reduced chlorophyll content in leaves compared to wild type (Fig. 22B).

4.2.3.2.2 *kin-1* mutant exhibit reduced internodal elongation

Green house grown *kin-1* mutant plants appeared short and bushy compared to wild type (Fig. 22C). Internodes lengths of three months old wild type and mutant plants were measured. The internodal length in *kin-1* mutant was slightly shorter than wild type (Fig. 22D).

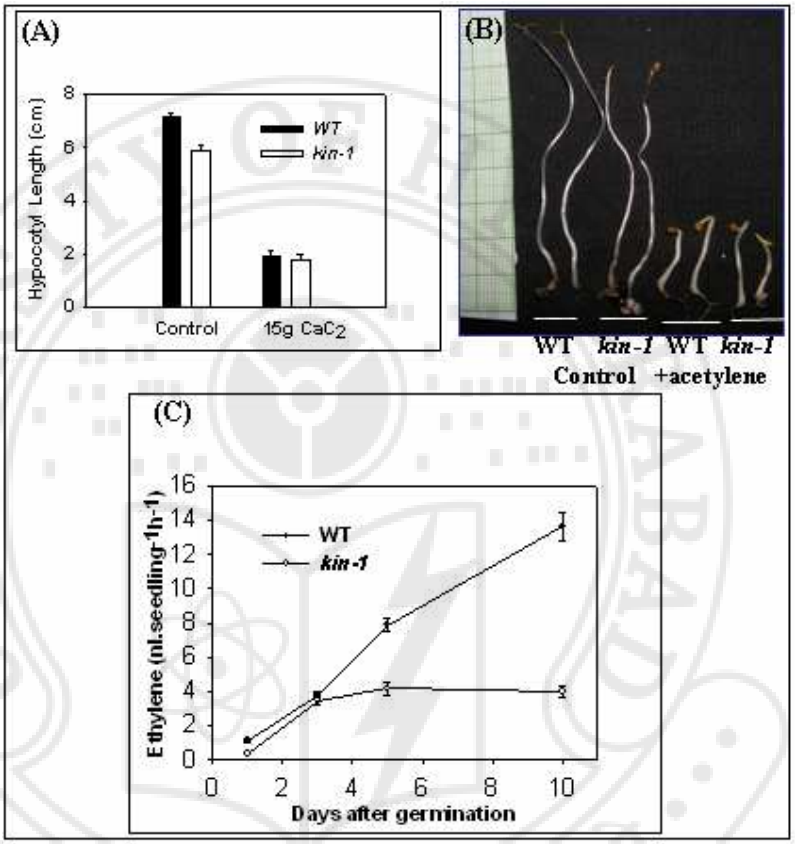


Figure 21. Ethylene sensitivity and ethylene production in wild type and *kin-1* seedlings. **A,** Graphical representation of hypocotyl lengths of two weeks old WT and *kin-1* seedlings grown in presence of 15g of CaC_2 in dark. **B,** Comparative seedling phenotype of WT and *kin-1* seedlings grown in presence and absence of CaC_2. **C,** Ethylene production in light grown seedlings of control and *kin-1* seedlings starting from germination to ten day (n=30).

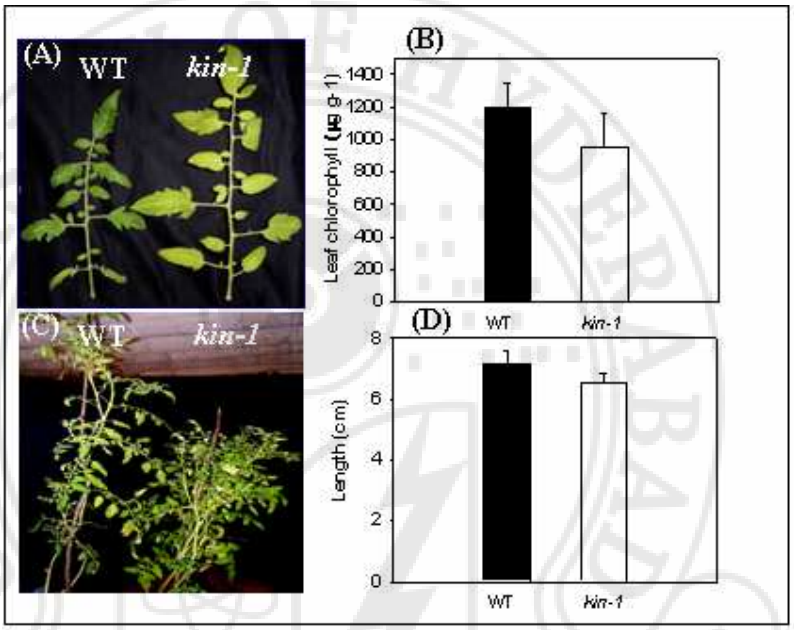


Figure 22. Effect of *kin-1* mutation on plant height and leaf chlorophyll content. **A,** Comparative leaf colour and morphology of WT and *kin-1* mutant. Note the pale green leaves in mutant compared to WT. **B,** Quantification of chlorophyll content of leaves harvested from eighth node of wild type and mutants. **C,** Phenotype of three months old WT and *kin-1* mutant. **D,** Internodal length of *kin-1* mutant and wild type (n=25).

4.2.3.2.3 *kin-1* mutation alters the inflorescence pattern

The *kin-1* mutation effected inflorescence placement and the number of flowers per inflorescence. In wild type plants the trusses were widely separated by at least by two to three nodes and each inflorescence bare 5-6 flowers out of which about three flowers set fruits. In *kin-1* mutant plants the trusses were parallel and appear at successive nodes to one another (Fig. 23A). The distance between two trusses is less in *kin-1* mutant having one truss on the main branch and another on the rachis of the earlier truss. Each inflorescence branch had only three flowers which in many instances lead to three fruits in *kin-1*.

4.2.3.2.4 Petal abscission is delayed by *kin-1* mutation

The *kin-1* mutant plants showed delayed petal abscission, one of the well characterized ethylene regulated trait in plants (Fig. 23B). The petals in wild type senesce and abscise after pollination. In mutant plants the petals remain attached to the blossom end of the developing fruits. The petals remained attached even after the fruits reached red ripe stage and harvested (Fig. 23C).

4.2.3.3 Analysis of fruit characteristics

4.2.3.3.1 Quantitative traits

4.2.3.3.1.1 *kin-1* mutant has altered placentation

The fruits of *kin-1* plants showed altered placentation from a regular bilocular to multilocular placentation. Because of the increase in locules the fruits of the mutant plants appear ridged unlike round wild type fruits. Compared to wild type fruit weight, size and number seeds per fruit were also higher in mutant (Table 5).

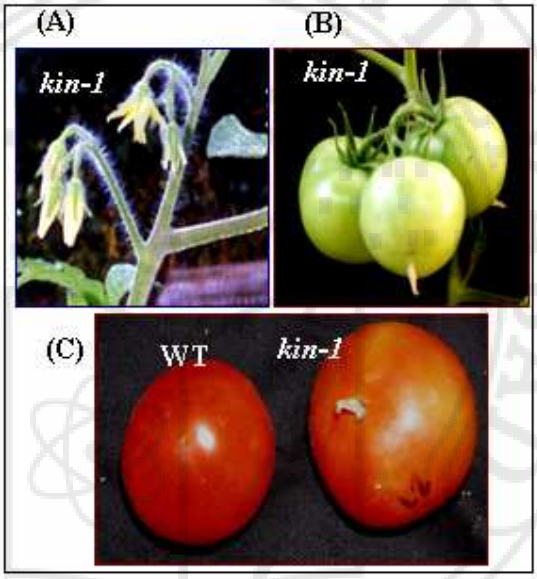


Figure 23. Effects of *kin-1* mutation on reproductive morphology. **A,** Altered inflorescence pattern in *kin-1* plants. **B,** Mature green fruits of *kin-1* showing petals even after fruit development. **C,** Ripened fruits of *kin-1* showing retention of petals. Note WT fruit has no attached petals.

Name	Fruit weight (g)	Fruit size (cm)	No.of locules	No. of Seeds/fruit	Fruit pH
Wild Type *kin-1*	36.0 ± 0.9 44.0 ± 0.7	14.2 ± 1.4 14.5 ± 1.9	Bilocular Multilocular (5-6)	98 ± 3 110 ± 4	7.2 6.3

Table 5. Effect of mutation on fruit characters of wild type and *kin-1* mutant. Fruits of wild type and mutant plants were harvested at red stage and fruit weight, fruit diameter and number of seeds per fruit were recorded (n=10).

4.2.3.3.2 Qualitative traits

4.2.3.3.2.1 *kin-1* mutants shows slow fruit development

Chronological fruit development from the time of anthesis to fruit drop was recorded for *kin*-1 mutant and wild type (Fig. 24A). The *kin*-1mutant fruits reached mature green stage about 38-40 DPA compared to wild type that reached mature green stage much earlier at 28-30 DPA. Above delay shows that there is a slower fruit development in *kin-1* mutant. Furthermore even after attaining MG stage, the transition to different stages of ripening is also delayed in *kin-1* mutant compared to wild type plants (Fig. 24B). On physiological scale after attainment of breaker stage, the *kin-1* mutant fruits were slow to progress through different stages of ripening. In wild type the complete fruit cycle from anthesis till fruit drop takes about 40-45 DPA whereas the mutant fruits complete this cycle in 58-62 DPA approximately.

4.2.3.3.2.2 Ethylene under production is observed in mutant fruits

To test whether above mutants are compromised in ethylene production, we examined the rate of ethylene evolution in *kin-1* mutants and wild-type fruits. The *kin-1* mutant fruits produced less ethylene at mature green stage than wild type. Subsequently, both in wild type and mutant ethylene production increased and peaked at orange stage. Thereafter the ethylene production declined in both genotypes. The *kin-1* mutant fruits were found to release less amount of ethylene than the wild type at all stages of ripening (Fig. 24C).

4.2.3.3.2.3 Fruit pigment content is reduced in *kin-1* mutant plants

The *kin-1* mutant not only showed delay in ripening but also showed the reduced pigment accumulation in fruits. The comparative analysis of carotenoid

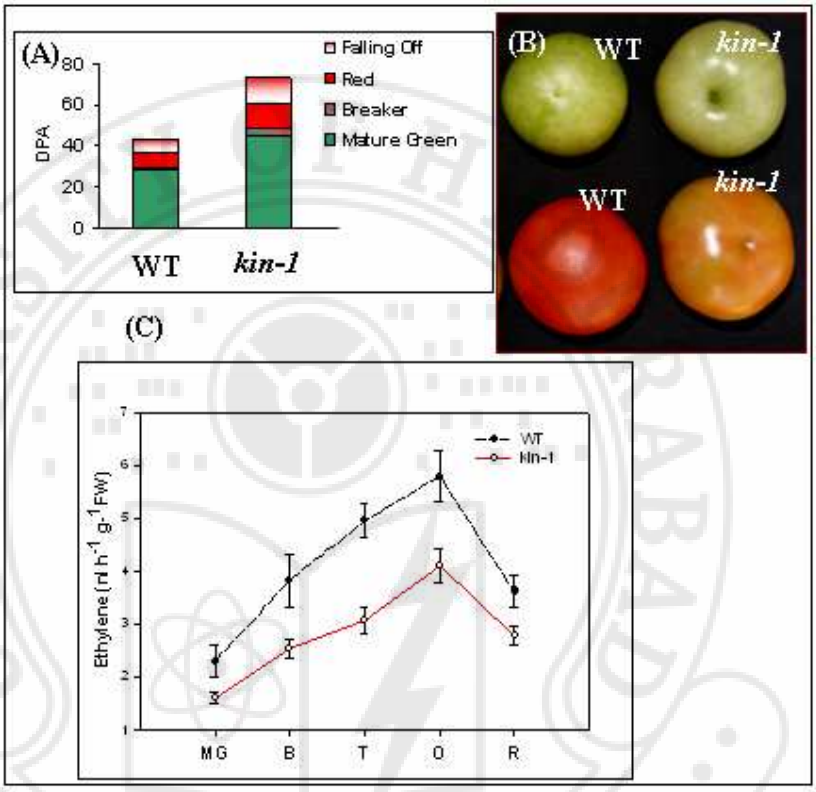


Figure 24. Effect of *kin-1* mutation on fruit ripening and ethylene evolution. **A,** The chronological development of tomato fruits in *kin-1* mutant and WT. The data represents the time taken to reach mature green, breaker, red stage of fruits and final fruit drop vine starting from day of anthesis. (n=8). **B,** Fruit phenotypes of WT (AC) and mutant after 28 DPA and 45 DPA. Note the delay in fruit development in *kin-1* fruits compared to WT fruits. **C,** Ethylene production in wild type and *kin-1* during fruit ripening. Each fruit was sampled three times (n=10).

composition of *kin-1* mutant fruit pericarp using HPLC indicated that the biosynthesis of these pigments is slower in mutant. At red stage, the lycopene content in mutant fruits is almost 10-15 % less than wild type and β–carotene content is about 50% less than wild type fruits (Fig. 25).

4.2.3.4 Post harvest quality traits

4.2.3.4.1 *kin-1* mutant fruits show enhanced shelf life

Fruits of wild type and *kin-1* mutant fruits were compared for off-vine shelf life at room temperature (25±2^{0}C). Fruits of wild type and *kin-1* harvested after they attained red stage and were kept in separate boxes for forty five days and visually observed for spoilage and loss of firmness (Fig 26A). In case of wild type approximately 60% of fruits were wrinkled, 35% were rotten and 5% were totally spoiled. In case of *kin-1* mutant 85% were wrinkled, 14% were rotten and 1% was spoiled. The results indicated the slower deterioration of mutant fruits compared to wild type fruits (Fig. 26B).

4.2.3.4.2 Off-vine ripening period is extended by *kin-1* mutation

The fruits of *kin-1* showed longer period to attain off vine ripening. Mature green fruits of wild type and *kin-1* mutant were harvested and stored for four weeks at room temperature (25±2^{0}C). Four weeks after harvest, approximately 89% of wild type fruits were in red stage and 11% were in orange stage where as 90% of *kin-1* fruits were in orange stage and 10% were in red stage (Fig. 26C).

4.2.3.4.3 Ripening can be induced in mutant fruits by ethylene

The induction of fruit ripening by ethylene was observed as the basis of red color development after treating with exogenous ethylene (Fig. 27A). Three days after

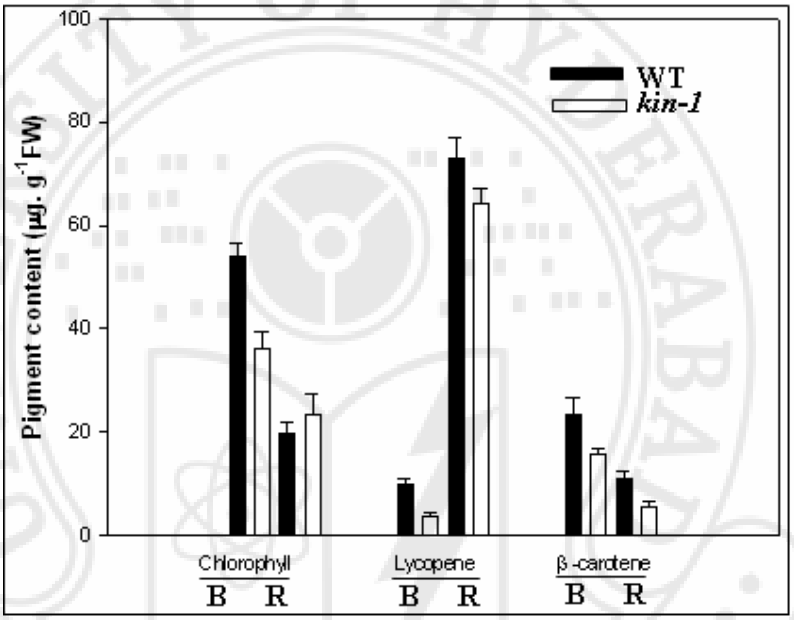


Figure 25. Pigment content from fruit pericarp of wild type and *kin-1* mutant. The amount of chlorophyll, carotenoid and lycopene content present in breaker and red fruits of wild type and mutant was estimated. The data represents the mean of ten fruits polled from three individual experiments (n=10).

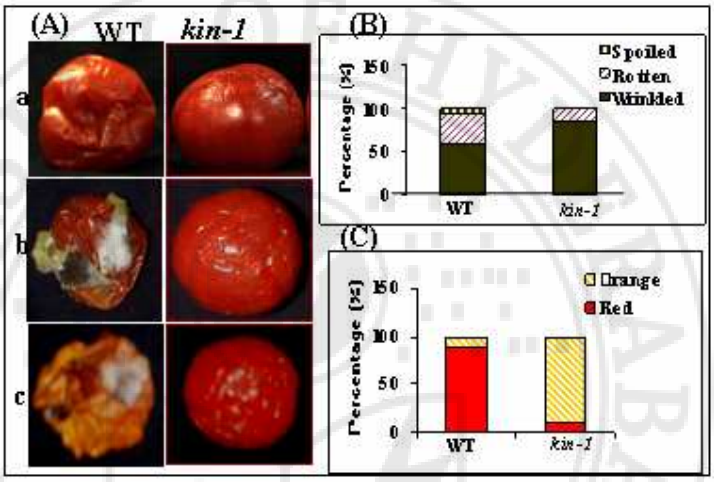


Figure 26: Shelf life and off vine ripening of WT and *kin-1* fruits. **A,** Fruits were harvested at red ripe stage and observed for spoilage at room temperature till 45 days. Photographs were taken at 7 days (a), 25 days (b) and 45 days (c) after harvest. **B,** Graphical representation of in vitro shelf life of WT and *kin-1* fruits after two months of harvest at red stage (n=15-20). **C,** Graphical representation of offvine ripening of WT and kin-*1* fruits after one month of harvest at breaker stage (n=15-18).

treatment, the mature green fruits of wild type have turned to complete red where as the fruits of *kin-1* also turned to red with in two days after treatment (Fig. 27B).

4.2.3.4.4 Wound induced ethylene production is enhanced in mutant fruits

The amount of ethylene evolved by the cut fruits was measured after 2 h, 4 h and 6 h after cutting. There is a burst of ethylene immediately after cutting and it slowly reduced eventually in wild type fruits. Two hours after cutting both wild type and mutant fruits showed similar level of ethylene, however after 4 h ethylene production dropped in wild type where as in *kin-1* mutant it stayed at same level. Thereafter it reached to similar level to wild type after six hours (Fig. 27C).

4.3 Screening for mutants in presence of ethylene action inhibitor

To screen for ethylene receptor mutants we studied the effect of ethylene action inhibitor 1-MCP on tomato seedling phenotype. When tomato seedlings were grown in presence of 1-MCP right from seed sowing, one of the striking effects was on the growth of roots, which in place of penetrating vermiculite grow in air. It appeared that exposure to 1-MCP blocked the penetration of root tip in substrate. The treated seedlings also exhibited increased root length and decreased hypocotyls length and prominent root growth in air in both light and dark. The agravitropic roots were more frequent and prominent in light grown treated seedlings (Fig. 28). To further support the role of ethylene in root penetration we treated the seedlings with 10 µM silver nitrate, another inhibitor of ethylene action. The effect of silver nitrite on root penetration and growth kinetics was similar to 1-MCP (Fig. 29A,B). We checked the effect of 1-MCP on root penetration by growing seedlings on soft agar (0.6%) medium and on vertical plates containing moist blotting paper. When ethylene action is blocked with 1-MCP, tomato roots

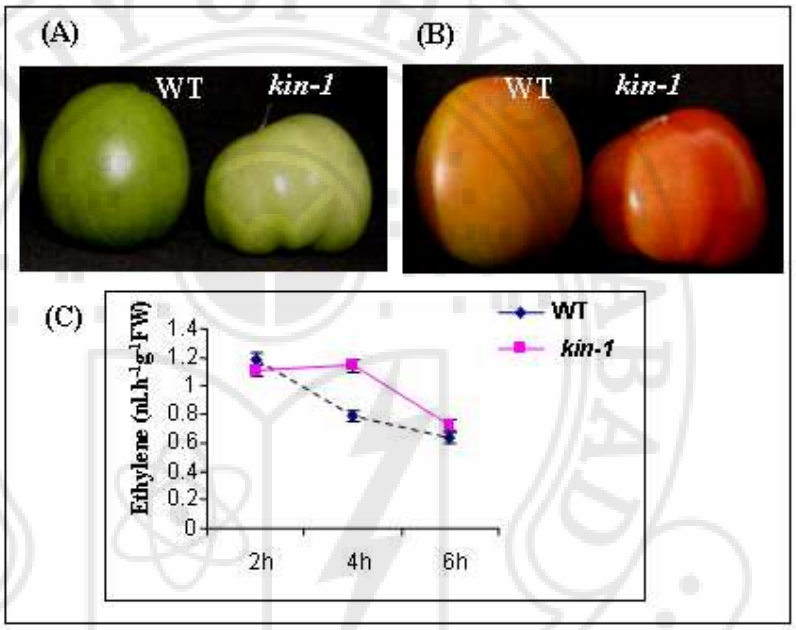


Figure 27. Ethylene induced ripening in WT and *kin-1* mutant fruits. **A,** Mature green fruits of WT and kin-*1* before treating with ethylene. **B,** Fruits of WT and *kin-1* one week after treatment with 2 $ml.l^{-1}$ ethylene. Note the sensitivity of *kin-1* mutant to exogenous ethylene. **C,** Wound induced ethylene production from the cut pieces of red ripe WT and *kin-1* fruits (n=5).

Figure 28. Effect of 1-MCP on root growth. Photograph showing the five days old light grown control (left) and 1-MCP treated (right) seedlings. Arrow points the roots growing in air with prominent loops in treated seedlings, compared to control, where roots are not visible as they grow in vermiculite.

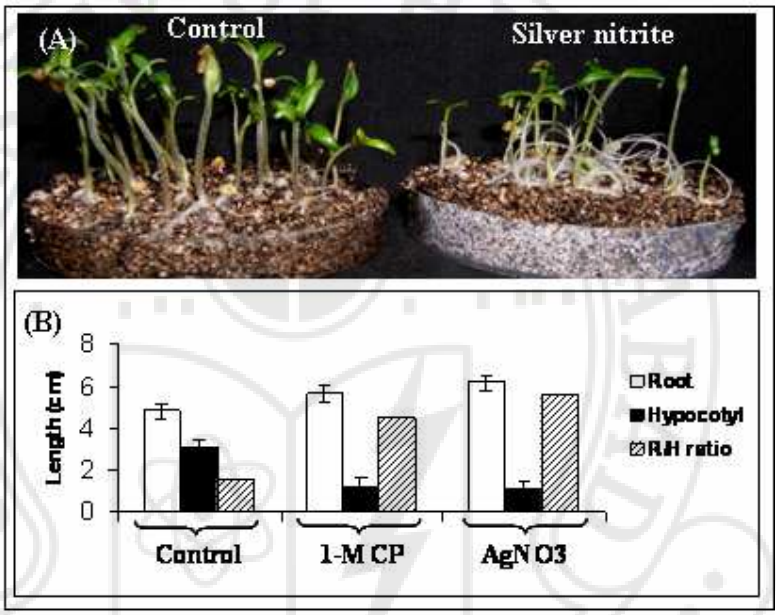


Figure 29. Effect of ethylene inhibitors on seedling growth. **A,** Photograph showing five days old light grown control (left) and 10 µM silver nitrite treated (right) tomato seedlings. **B,** Wild type seedlings were grown with 20 $\mu l.l^{-1}$ of 1-MCP or 10 µM silver nitrite right from seed sowing on vermiculite under continuous white light. The root and hypocotyls were measured after five days of germination (n=30).

were failed to penetrate such as soft agar (Fig. 30A), blotting paper (Fig. 30B) and vermiculite (Fig. 30C) as well. The roots formed loops in presence of 1-MCP while the roots of control seedlings penetrate and grow normally to wards gravity on all three substrates.

We also grew the seedlings of lettuce and tobacco along with tomato in presence of 1-MCP. Similar to tomato seedlings these seedlings also exhibited root coils in presence of 1-MCP due to lack of penetration into vermiculite. These coils can be seen even after removal from vermiculite (Fig. 31). The treated seedlings exhibited elongated roots and short hypocotyls compared to their respective control seedlings. The promoting effect of 1-MCP on aerial root growth was appeared as a common feature of treatment in all these seedlings. (Fig. 32).

4.3.1 1-MCP blocks root penetration but not root gravitropism

In presence of 1-MCP, roots of treated seedlings were unable to penetrate in vermiculite compared to controls that show normal root penetration. The roots of 1-MCP treated seedlings in vermiculite formed loops in the air, which could be due to either lack of root gravitropism or some other developmental response. To discriminate these two phenomenons, we made a time lapse video by taking pictures sequentially at every 150 seconds till three days after treatment with 1-MCP. In controls, the seedlings grew normally showing positive geotropism of roots and positive phototropism in the hypocotyls. In treated seedlings, while the root tips were positive to gravity but due to the lack of penetration of tip into vermiculite, root grew in air and forms loops. Even though the root tips of the

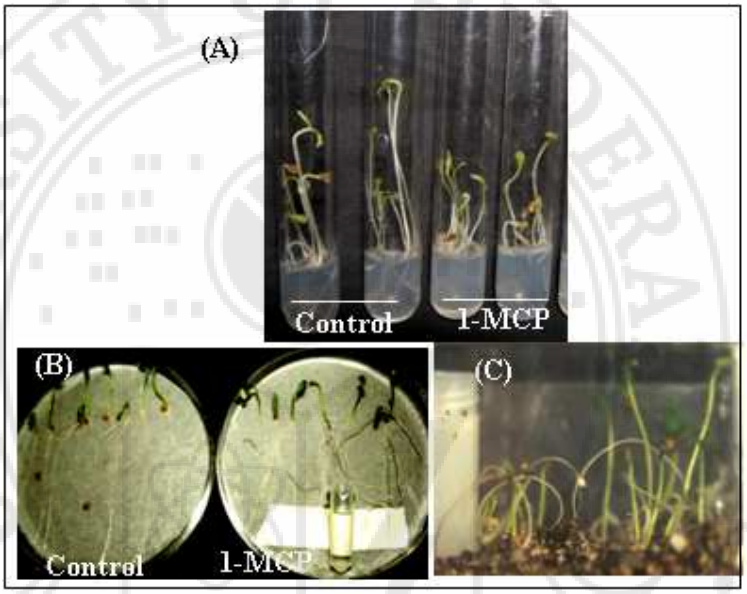


Figure 30. Effect of 1-MCP on root penetration. Tomato seedlings were grown on 0.6% agar **(A)**, blotting paper **(B)** and on vermiculite **(C)** in presence of light. Note the failure of root penetration in 1-MCP treated seedlings.

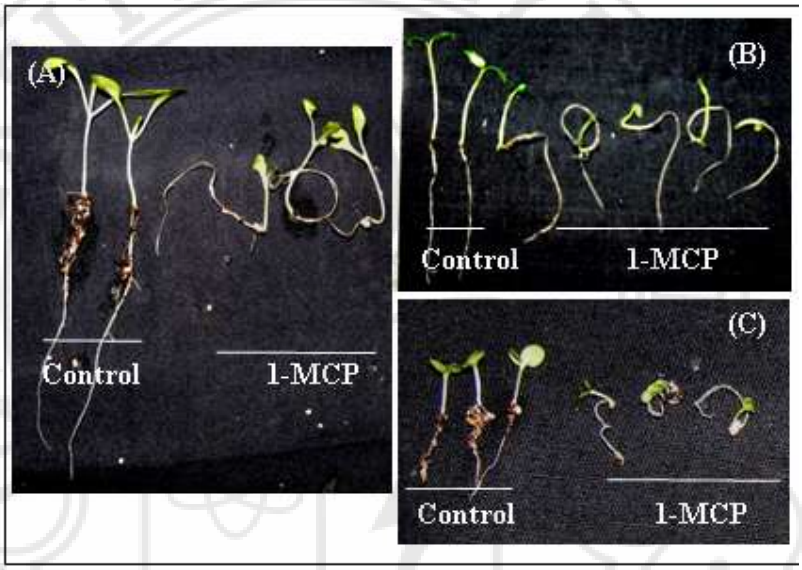


Figure 31. Root coiling in 1-MCP treated seedlings. The control and 1-MCP treated seedlings of tobacco **(A)**, tomato **(B)** and lettuce **(C)** after uprooting from vermiculite. Note the root coils in 1-MCP treated seedlings.

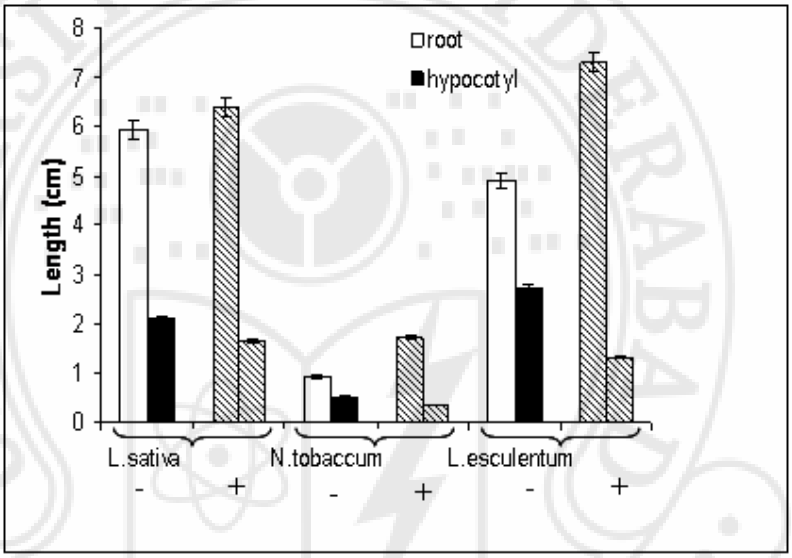


Figure 32. Effect of 1-MCP on seedling growth of lettuce, tobacco and tomato. Seedlings of lettuce, tobacco and tomato were grown on vermiculite in presence (+) and absence (-) of 20 $\mu l.l^{-1}$ of 1-MCP under continuous light. The root and hypocotyl lengths were taken after five days from incubation (n=15-20).

treated seedlings touched vermiculite, they were unable to penetrate vermiculite (Fig. 33). The size of root loop increased gradually till the removal of 1-MCP. At no stage the root tips of seedlings grown in presence of 1-MCP showed any penetration into vermiculite. Even after removal of 1-MCP a recovery period of 24 h was needed before roots could penetrate into vermiculite. However in presence of 1-MCP hypocotyls showed normal phototropic curvature to unilateral white light.

4.3.2 1-MCP blocks root penetration in an age dependent manner

To mark the precise developmental stage of seedlings at which the effect of 1-MCP on inhibition of root penetration was more prominent, tomato seeds were treated with 1-MCP at different days from germination. Table 6 shows that there is progressive decrease in the number of seedlings responding to 1-MCP as visualized by formation of loop with progression of germination. The data show that effect of 1-MCP on formation of aerial root loops are most prominent at 0-1 day that is even before emergence of root radical. Our data indicates that 1-MCP has no effect on root penetration when applied at three days after germination. Once the roots enter vermiculite 1-MCP appears to have no influence.

4.3.3 1-MCP reduces amyloplast number in root tips

The roots of seedlings grown in presence of 1-MCP were thinner compared to untreated seedlings. Apparently the ethylene released during penetration makes the roots thicker than controls. Since root tips in presence of 1-MCP lost capacity to penetrate, we examined the effect of 1-MCP on amyloplast number, a marker for

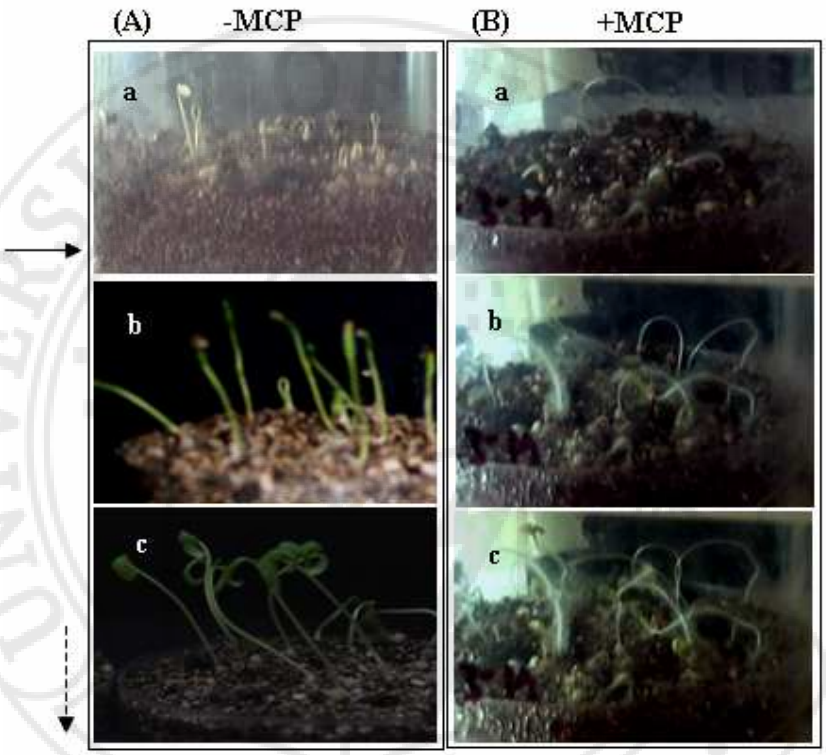


Figure 33. Illustration of root growth in 1-MCP treated tomato seedlings by time lapse video images. Tomato seedlings were grown in presence and absence of 20 $\mu l.l^{-1}$ of 1-MCP under continuous light. Time lapse images were captured to produce a continuous movie. Frames extracted at day 1 **(a)**, day 3 **(b)** and day 5 **(c)** from time lapse-video demonstrating wild type tomato root growth in absence of 1-MCP **(A)** and in presence of 1-MCP **(B)**. Note the prominent root loops in seedlings treated with 1-MCP in (b) and (c) compared to control The bold arrow indicate the direction of unilateral white light and the dashed arrow indicate the direction of gravity.

Days of 1-MCP treatment	Root Length (cm)	Hypocotyl Length (cm)	Percentage of seedlings forming loop (%)
Control	3.9	3.1	0.0
1-MCP treated: 0 day old	4.3	2.2	94
1 day old	5.3	2.6	84
2 days old	4.9	2.9	25
3 days old	5.4	3.0	2.0
4 days old	4.8	2.9	0.0
5 days old	4.8	4.1	0.0

Table 6. Effect of 1-MCP on root/hypocotyl lengths and loop formation at different days from germination. Seedlings were treated with 1-MCP at different days after germination from 0 days to five days under continuous white light. Root and hypocotyl length and percent of seedlings forming loops in air were examined after one week (n= 10-15).

geotropic responses in 1-MCP treated seedlings. The root tips of 1-MCP treated seedlings were stained with KI-I_2 to observe amyloplasts in root tips (Fig. 34). The starch staining showed the reduction in amyloplast number in the root tips of 1-MCP treated seedlings compared to control seedlings.

4.3.4 Immnolocalization of PIN1 protein shows the reduced expression in 1-MCP treated root tips

1-MCP treated seedlings showed enhanced root growth and reduced hypocotyls length compared to control seedlings. We therefore wanted to examine whether this increase in root growth was due to alteration in PIN1 expression, which are reported to be an efflux carriers of auxin. We examined the immunolocalization of PIN1 proteins which are responsible for the polar auxin transport. The PIN1-like proteins in both the wild type and treated seedlings were localized at the base of the cells which were similar to that reported from *Arabidopsis* PIN1 (Gälweiler *et al*., 1998). Immunolocalisation of root sections of control and 1-MCP treated seedlings probed with *At* anti-PIN1 showed reduced expression of PIN1-like proteins in root tips of treated seedlings (Fig 35C,D) as compared to that of control (Fig. 35A,B).

3.5 Transient changes in ROS may involved in ethylene mediated root penetration

ROS can serve as potential signaling molecules to mediate diverse biotic and abiotic stress responses. Root tips stained with 5 µM ROS dye (H_2DCF) in 10 mM MES-KCL buffer, shows the reduced ROS levels in1-MCP treated root tips compared to control (Fig. 36A,B).

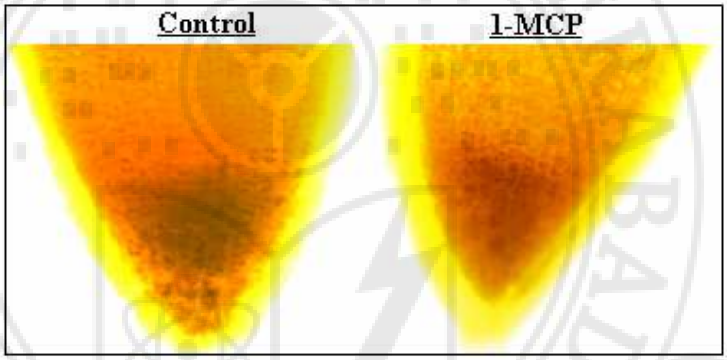


Figure 34. Amyloplast staining of control and 1-MCP treated root tips. Root tips of three days old control and 1-MCP treated seedlings stained with lugol's iodine and observed under microscope. Note the reduced amyloplst level in 1-MCP treated root tips compared to control.

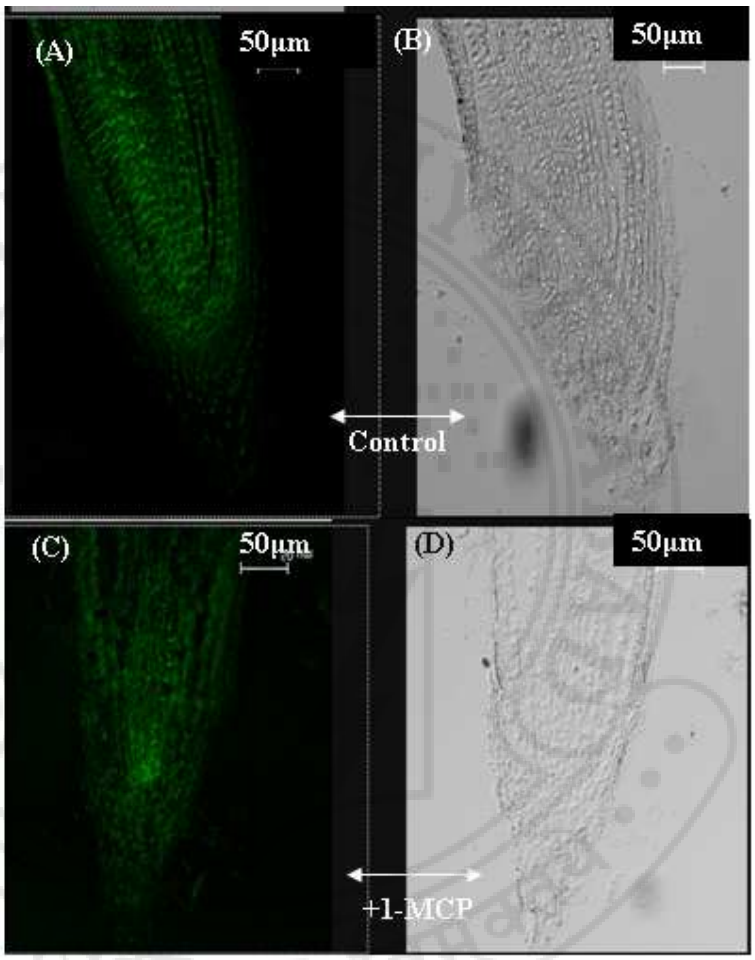


Figure 35. Immunolocalization of PIN1 protein in root tips. **A-D,** Confocal images of PIN1 protein localization in the root tips of three days old light grown control and 1-MCP treated **(A,C)** seedlings with bright field images **(B,D)** respectively.

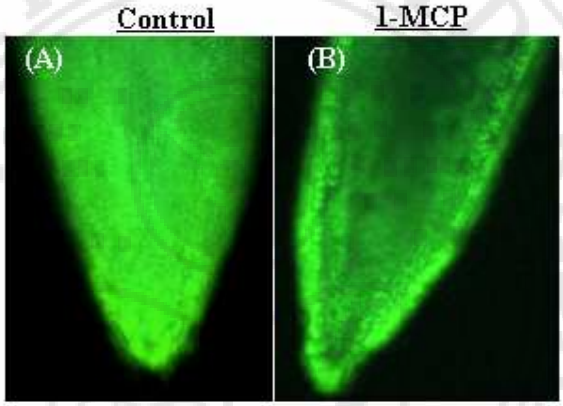


Figure 36. Levels of reactive oxygen species in 1-MCP treated root tips. Three days old control **(A)** and 1-MCP **(B)** treated root tips were stained with ROS dye (H2DCF). Note the reduced ROS levels in treated root tips.

Discussion

The constant natural selection of beneficial spontaneous mutations has led to the evolution of living organisms. One powerful approach to decipher plant hormone signaling pathways is isolation of mutants defective in hormone responsiveness. Mutants also serve as an important material to unravel the mechanisms governing various developmental processes. During last two decades molecular and genetic analysis of these developmental mutants has considerably advanced our understanding of the role and nature of the genes regulating various processes such as fruit ripening, disease resistance and plant organ development. An important contribution to our understanding of ethylene signal transduction has come from the studies of mutants with altered ethylene sensitivity in Arabidopsis (Chang and Shocky, 1999; Stepanova and Ecker, 2000). In the wild type population of tomato there are no induced mutants reported showing variation in ethylene sensitivity and production. In this study we describe genetic and physiological analyses of two mutants; the *atr-1* and *kin-1* mutant. Our results showed that these mutant loci effects ethylene mediated responses in tomato. Our studies further revealed that these loci play an important role during fruit ripening. In addition we also show that ethylene plays an important role during early root growth.

5.1 Acetylene resistant (*atr-1*) mutation in tomato confers ethylene insensitivity and delayed fruit ripening

Over the past decade, the seedling triple response phenotype has been used to screen for mutants that are defective in ethylene responses (Guzman and Ecker, 1990). This screen has been utilized to identify most of ethylene signal transduction mutants identified to date (Bleecker *et al*., 1988; Ecker, 1995; Kieber, 1997). Specifically, mutants have been isolated based on their sensitivity to the presence of ethylene and many of the corresponding genes have been cloned. By virtue of structural similarity some of the ethylene analogues can also induce triple response. In sweet pea seedlings besides ethylene, acetylene and propylene were also used to induce triple response (Crocker, 1948). It is observed that acetylene can provokes the similar effects as the phytohormone ethylene in plants when applied at concentrations approximately 1200 fold higher than ethylene (Abeles *et al*., 1992; Burg and Burg, 1962). In addition when hydrolyzed calcium carbide

along with acetylene also produces traces of ethylene and that too can contribute to triple response (Reid, 2002). There is not much information available for use of acetylene in tomato fruit ripening. Most information on usage of acetylene to induce ripening is obtained with banana. In banana ethylene induces ripening in a range of 1-1000 ppm (Inaba and Nakamura (1986), on the other hand acetylene stimulated ripening of banana at level of 0.5-1.0 ml/l (Smith and Thompson, 1987). It is therefore evident that acetylene at higher concentration that is about 1000 times higher than ethylene can replaces ethylene. Extending this observation we can assume that higher concentration of acetylene can be used for screening of mutants related to ethylene. In the current work, we have used acetylene to screen for tomato acetylene resistant mutant (*atr-1*) because of the ease in handling and low cost of acetylene compared to ethylene (Fig. 5A).

5.1.1 *atr-1* is a recessive nuclear mutation

The *atr-1* mutant was crossed with the parental wild type background and the resultant progeny was analyzed for the pattern of inheritance and segregation of the mutant phenotype that is absence of acetylene induced triple response. The segregation and inheritance analysis of *atr-1* mutant revealed that the mutated loci was functionally recessive to the wild type and followed Mendelian monogenic segregation pattern (Fig. 6). Based on *atr-1* insensitivity to acetylene, which is complementary to ethylene, it can be assumed that *atr-1* loci may represent either one of the ethylene receptor genes in tomato like *ETR* or *EIN* like genes. In Arabidopsis, the known *etr* mutants are dominant while *ein* mutants are recessive. Tomato has the six known ethylene receptor (*ETR1-ETR6*) genes, but so far only one receptor mutant *Never-ripe* (*Nr*) in tomato is reported so far which has a mutation in the ethylene receptors conferring insensitivity to ethylene (Lanahan *et al*., 1994: Wilkinson *et al*., 1995). The *NR* gene is cloned in tomato, though it is homologous to *ETR1* gene family it lacks response regulator domain and hence more similar to *ERS* gene family. The segregation pattern of *atr-1* mutant showed that it is a recessive gene, therefore it is more similar to *EIN* like genes.

5.1.2 The *atr-1* mutant exhibit reduced ethylene sensitivity

Our knowledge of the genes regulating ethylene signaling pathway and their mechanism of action in higher plants has come largely from studies on the model plant Arabidopsis (Guo and Ecker, 2004). These genes have been isolated using mutants showing altered triple response phenotype. Subsequent analysis revealed that the most of these mutant loci have pleiotropic effects on plant ethylene responses. The physiological studies of *atr*-1 mutant showed the reduced ethylene perception at all stages of plant development, right from germination to fruit development and senescence. The involvement of ethylene in determining the time to radical protrusion was investigated in ethylene-insensitive receptor mutants in tomato and Arabidopsis. Because ethylene evolution from seeds is coincident with radical protrusion, and the ability to convert 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene is diagnostic for seed vigor, it was hypothesized that ethylene-insensitive mutants would require more time to complete germination compared to wild-type seeds (Siriwitayawan *et al*., 2003). In accordance with this Arabidopsis seeds defective in ethylene receptors take longer time to complete germination, while *Nr* seeds were slow to germinate compared to wild type control plants supporting the role of ethylene perception. On examination *atr-1* seedlings displayed slower seed germination and required double the time to complete radical protrusion in 50% of mutant seeds.

The seedlings of *atr-1* mutant produces lower amount of ethylene compared to wild type seedlings. One may argue that due to less ethylene production, *atr-1* seedlings were taller. However exogenous ethylene treatment did not restore the seedling triple response indicates that observed phenotype may be related to loss of ethylene perception in *atr-1* seedlings. The insensitivity of *atr-1* seedlings to ethylene was also highlighted by dose response curve (Fig.5). The mutant seedlings require 2-to 3-fold higher levels of acetylene than wild type to cause 50% inhibition in root and hypocotyls lengths (Fig.5C). Insensitivity of mutant seedlings to ethylene was further conformed by lack of triple response in presence of exogenous ethylene and its precursor 1-ACC (Fig.7). The hypocotyls of etiolated mutant seedlings and also the green house plants of *atr-1*were taller then

WT, perhaps because of reduced ethylene perception. The recent observations support a role for ethylene in regulating a spectrum of developmental events associated with organ senescence and tissue necrosis (Yang *et al*., 2008). The flowers of ethylene insensitive *atr-1* mutant exhibited delayed floral abscission and leaf senescence in presence of ethylene compared to WT. Treatment of tomato flowers with exogenous ethylene resulted in abscission at the pedicel. This response is faster in wild-type and substantially delayed in a*tr-1* flowers (Fig. 13). Above observed phenotypes in response to exogenous ethylene of *atr-1* mutant indicate reduced ethylene responsiveness but on a relative scale they were weaker than the phenotypes typically associated with *Nr* flowers (Lanahan *et al*., 1994). Evidently a*tr*-1 mutation shows multiple pleiotropic effects on ethylene sensitivity at all stages of development.

5.1.3 Fruit development is slower in *atr-1* mutant

Tomato fruit development consists of growth, division and ripening. The development pattern of tomato fruit has been classified into four distinct phases that are 1, cell differentiation; 2, cell division; 3, cell expansion and 4, ripening (Gillapsy *et al*., 1993). Since phase 1 corresponds with flower development and pollination, we studied fruit development from anthesis onwards. We have taken phase two and three of fruit development together as MG stage. According to Seymour *et al* (1993) the phase 2 is about 11 days and phase 3 up to carotenoid visualization that is B stage 25 days followed by ripening of fruits. In *atr-1* mutant fruit development up to MG stage is extend by two weeks. On average, wild-type fruit reached the breaker stage of development at about 5 weeks postanthesis, whereas mutant fruits showed approximately two weeks delay to the onset of color change. The mutant fruits take 15-20 more days to develop than wild type fruits (Fig. 10A). It is possible that the lack of ethylene production could have delayed fruit development and ripening.

The importance of ethylene in regulating early stages of fruit ripening has only recently been observed (Nakatsuka *et al*., 1998; Barry *et al*., 2000). While the plants of *atr-1* showed delay in flowering time. The *atr-1* mutation does not effect the size and internal anatomy of tomato fruit but fruit weight was reduced in *atr-1*

plants. This decrease in weight was also associated with reduced number of seeds in mutant fruits compared to control. Most importantly the time necessary for fruits to progress from anthesis (A) to breaker (B) stage, that is the first appearance of orange color at the blossom end of fruit, is dramatically increased in a*tr-1* mutant under greenhouse conditions.

Previously described mutants such as *rin, Nr* that inhibit fruit ripening in tomato share two common phenotypic characteristics: an inability of ethylene to restore the ripening process and reduced expression of ripening-related genes (Lincoln and Fischer, 1988; Yen *et al*., 1995; Thompson *et al*., 1999). The delaying of fruit ripening is also observed in tomato plants with reduced expression of the tomato *EIN3*, like genes (*LeEIL1*, *LeEIL2*, and *LeEIL3*) (Fu *et al*. 2005).The *atr-1*mutant fruits showed slower ripening both on vine and off vine (Fig. 10A, 14C). The induction of ripening by the treatment of exogenous ethylene is also slow in *atr-1* mutant fruits than wild type fruits.

The *atr-1* mutation not only delays fruit ripening but also effects several other ethylene induced responses such as pigmentation in mutant plants. Tomato mutant analysis has provided a wealth of information on genes involved in carotenoid biosynthesis and sequestration. The color of tomato fruits begin to change from green to red at the breaker stage of ripening. Even though it coincides with climacteric ethylene production, ethylene regulation of carotenoid biosynthesis during fruit ripening is still poorly understood. Fruit softening and lycopene accumulation are slower in *atr-1* mutant fruits compared to wild type fruits. It is evident by the visual delay in colour development and the reduced carotenoid levels compared to wild type fruits of chronologically same age. At red stage the lycopene and carotinoid content in mutant fruits are almost half to that of wild type fruits (Fig.11). Increased leaf and fruit chlorophyll, slower degradation of chlorophyll and reduced carotenoid levels in fruits shows the pleiotropic effects of mutation on pigment synthesis in mutant plants. In tomato fruits, the induction of lycopene accumulation coincides with the increased expression of upstream genes of lycopene synthesis (*PSY* and *PDS* genes) (Giuliano *et al*., 1993; Pecker *et al*., 1996; Ronen *et al*., 1999). The previous results showed that phytoene synthase-1 and phytoene desaturase genes played key roles in carotenoid synthesis and in the

colour development of tomato fruits. The reduced carotenoid accumulation can be probably linked to reduced expression of phytoene desaturase gene at the red ripe stage of mutant fruits. Other reason could be the loss of perception at the breaker stage of mutant fruits may inhibit the accumulation of ripening induced pigments.

5.1.4 The *atr-1* mutant fruits show ethylene evolution similar to WT

Tomato being a climacteric fruit, its ripening is preceded by ethylene evolution after MG stage. One reason for slow ripening in mutant fruit could be reduction of ethylene biosynthesis. To test whether the *atr-1* mutation affects ethylene production during ripening stages, the rate of ethylene evolution was measured in mutant and wild-type fruits at several stages of development from MG state. Always ethylene production is low in preclimateric fruit up to MG stage and increases at the onset of ripening. A peak in ethylene production occurs at the orange (O) stage and thereafter declines. Although minor differences are observed at certain stages, there was not much difference in ethylene production between mutant and wild-type fruits at different stages of fruit ripening. The mutant fruits produce normal level of ethylene during ripening (Fig.10C). Hence the delay in ripening and other ripening related characteristics is probably due to the reduced perception of ethylene by *atr-1* fruits.

Though *atr-1* mutant fruits showed similar level of ethylene evolution, it could have affected differential expression of ethylene biosynthesis enzymes. Among eight ACS genes, we examined the expression level of selective genes, whose action is most important during phase 1 and phase 2 of fruit ripening. The ethylene synthesis during ripening phase of fruit development is normally contributed by *SlACS2* and SlACS4 enzymes. *SlACS6* is only expressed early in the development in both *atr-1* and wild-type fruits, a pattern that has been linked to the regulation of system 1 ethylene synthesis in tomato fruit (Nakatsuka *et al*., 1998; Barry *et al*., 2000). Though we did not directly estimated the activity of ACS and ACO enzymes in fruit extracts, we compared the gene expression levels for different members of ACS multigene family. Expression of *SlACS2* and *SlACS*4 was increased equally in both mutant and wild type with the onset of ripening (Fig. 17A), showing that system 2 is functional in mutant fruits. It also supported our

observations that ethylene biosynthesis is not effected by the *atr-1* mutation. The expression of these important ACS genes is normal in mutant fruits during ripening (Fig.17A).

5.1.5 The *atr-1* mutant fruits showed improved fruit shelf life

One advantage of tomato as an experimental model to study fleshy fruit softening is the availability of pleiotropic non-ripening mutants, such as *rin* (*ripening inhibitor*), *nor* (*non-ripening*), *alcobaça* and *Cnr* (*colorless non-ripening*), which are impaired in many ripening-related processes and exhibit delayed or impaired softening (Kopeliovitch *et al*., 1981; Thompson *et al*., 1999; Giovannoni, 2004). These mutants have provided insight into several specific aspects of ripening-related fruit softening (Rose *et al*., 2003; Ericksson *et al*., 2004), but their pleiotropic nature limits characterization of more complex physiological processes. *atr-1* mutant in consistent with previously reported tomato mutants loss of fruit firmness is largely coupled with other aspects of ripening indicating that fruit softening likely to be an integral and regulated part of ripening.

A decline in fruit firmness typically coincides with dissolution of the middle lamella, accompanied with the increased expression of numerous cell wall degrading enzymes. Polygalacturonase (PG)-catalyzed depolymerization of pectin in the wall and middle lamella was long believed to be the principle process underlying fruit softening (Bird *et al*., 1988; Grierson *et al*., 1993). Inhibition of cell wall degradation through genetic engineering has been used as a strategy to enhance the shelf life and firmness of tomato fruits and improve qualities of processed tomato products. PG had been reported to represent about 1% of ripening fruit mRNA and results in substantial pectinase activity to soften cell walls with the induction of ripening (DellaPenna *et al*. 1989). The PG promoter contained ethylene-inducible elements (Nicholass *et al*. 1995) and its mRNA had been induced at very low levels of ethylene (Sitrit and Bennett 1998). The reduced expression of PG at red ripe stage can also reasoned for delayed fruit spoilage in mutant fruits (Fig. 17B). The *atr-1* mutant fruits undergo normal pattern of ripening, but remain firm and show no infection for remarkably extended periods after reaching the fully ripe stage. Mutant fruits typically do not rot for at least 3

months after achieving a fully ripe stage under controlled conditions (25°C). Even two months after full ripening, the *atr-1* fruits showed slightly wrinkled appearance, with no signs of internal desiccation, tissue breakdown or other morphological changes like pigment photo bleaching (Fig. 14). During storage and over-ripening it was often observed that the WT fruits became infected by opportunistic fungal pathogens, while intact *atr-1* fruits never succumbed to infection, even following prolonged storage in high humidity conditions. The mutant fruits though get wrinkled but do not show infection for at least three months under controlled conditions (25 ± 2^0C). The *atr-1* mutant fruits also show more resistance to high temperatures. The ethylene insensitivity and delayed fruit development in mutant attributes positive effect on shelf life of mutant fruits. Therefore, the suppression of *PG* gene in mutant fruit suggested that *atr-1* played a positive role in the ethylene signaling transduction during fruit ripening.

Thus, the results presented here provide the evidence of the in vivo role of ethylene in fruit ripening and shelf life of fruits.

5.1.6 The ethylene insensitivity in *atr*-1 mutant is due to loss of ethylene perception

Ethylene insensitivity in *atr-1* mutant plants can be explained by two ways, either it is not able to perceive ethylene or it is an underproducer of ethylene. The exogenous ethylene does not restore normal phenotype in mutant plants, suggesting that either ethylene perception is lacking or it is likely that *atr-1* block ethylene response out of ethylene's influence. Several lines of evidence presented here suggest that the *atr-1* mutant of tomato is insensitive to ethylene mediated responses. The evidences are as follow: First, *atr-1* seedlings do not display the characteristic triple response to acetylene similar to ethylene insensitive *etr-1* seedlings of Arabidopsis (Bleecker *et al*., 1988; Ecker, 1995). The hypocotyls growth is more resistant to 1-ACC and ethylene induced inhibition (Fig. 7A,B). Second, even though the fruits of *atr-1* ripen fully, the process of fruit development and ripening is slower compared to WT on the vine. Off vine, the fruits of *atr-1* were slow to ripening when incubated with ethylene at MG stage (Fig.13C,D). Third, ethylene production is normal in fruits at all stages of ripening. All these strongly support the deficiency in perception. Even though the ethylene production

is lower in mutant seedlings compared to wild type the exogenous ethylene treatment does not induce the triple response in etiolated *atr-1* seedlings. Finally, Even though the fruits of *atr-1* turn red, the amount of carotenoid, lycopene were lesser than control WT fruits. It is interesting that resistant to ethylene does not alter the normal growth and development of the plant in addition it has the positive effect on yield traits like enhanced number of flowers and fruit set and delayed ripening.

5.1.7 The *atr-1* mutant retains minimal sensitivity to ethylene

Data from our experiments on *atr-1* seedlings suggests that *atr-1* is not totally impaired in ethylene sensitivity but retains minimal sensitivity. This is showed by the saturation of dose response of *atr-1* seedlings to increasing concentration of acetylene (Fig.5). Ethylene regulated responses such as epinasty, abscission and even fruit ripening are delayed in mutant but not totally inhibited. This is also evident by the normal ethylene biosynthesis during fruit ripening and activation of a subset of ethylene biosynthesis genes in *atr-1*. Fruits of mutant do perceive endogenous ethylene, as evident by the expression of a sub set of ethylene regulated genes like *PSY, E8A* (Fig. 17B). These results suggest that *atr-1* may have affected a subset of ethylene responses and can be described as a regulator of ethylene mediated responses. It may be a transcriptional factor cue leading to *atr-1* phenotype and has control on many other ethylene responsive genes. The ongoing gene mapping may help in resolving the position of *atr-1* in ethylene signal transduction.

5.1.8 Ethylene signaling is impaired in mutant

The pleiotropic effect of *atr-1* mutation shows that a variety of ethylene responses, occurring at different stages of life cycle and in different tissues of the wild type tomato plant are affected by mutation and therefore they may share some common element in their signal transduction pathway. It is also possible that the lesion produced by the *atr-1* mutation may exist in the early signal transduction pathway similar to *ein*. Evidences from Arabidopsis indicate that a single amino acid change in ethylene receptor can also cause ethylene insensitivity (Schaller and Bleecker,

1995; Hall *et al*., 1999). Ethylene insensitivity conferred by the *etr2-1* mutation is partly dependent on the functional *ETR1* (Cancel and Larsen, 2002; Tieman et. al., 2000). Ethylene insensitivity may be due to inability of receptor to bind to ethylene or by uncoupling of ethylene binding from the rest of the signal transduction pathway. To check whether *atr-1* mutation is due to the lesion in ethylene receptor gene we studied the expression of ethylene receptor genes in mutant. Previous reports confirm the fact that the change in the ethylene receptor levels can affects the fruit ripening and other ethylene mediated responses (Klee and Tieman, 2002). There is increase in expression of *NR* and *ETR4* genes during the ripening of *atr-1* fruits. In control fruits the gradual increase in the *NR* gene expression is responsible for the altered ethylene sensitivity during stage transition in fruit ripening of tomato. In case of mutant no such clear pattern is observed in *NR* gene expression. It is known that the over expression of *NR* or lowered *ETR4* gene expression eliminates the ethylene sensitive phenotype in tomato during ripening and infection (Klee *et al*., 1991; Ciardi et. al., 2001; Adams-Phillips *et al*.,2004). In off vine ripening the lack of ethylene perception at breaker stage is coinciding with the reduced expression of *ETR2* gene in *atr-1* mutant at breaker stage of ripening. Once fruits surpass this stage the stage transition is comparable to WT. The altered expression of members of ETR gene family in the *atr-1* mutant suggests a role of *atr-1* in regulation of these genes in tomato possibly as a down stream target of *atr-1*. Perhaps wild type *ATR* gene is necessary for the normal expression of ethylene receptor genes in tomato. It can be assumed that *atr*-1 encodes a mutant transcription factor which has a control over many other ethylene responsive genes like receptors. The pleiotropic effects of mutation shows the key regulatory role of mutation on tissue specific ethylene responses, but it is difficult to place it in the complex regulatory network with only physiological and biochemical assays.

5.1.9 The *atr-1* locus represents the unique mutant phenotype

Even though *atr-1* mutant show pleiotropic responses similar to mutants like *Cnr, Nr, Gr, Epi* mutants of tomato. The *atr-1* mutant is still distinct from these ripening mutants. We compared the features that differentiate the *atr-1* mutant from these earlier reported mutants. Similar to *atr-1* the delayed petal senescence, flower abscission, and fruit-ripening phenotypes of *Nr* were also shown to be the

result of ethylene insensitivity (Lanahan *et al*., 1994). However unlike *Nr* the ethylene production is normal in *atr-1* fruit tissue (Fig.10) and mutant is recessive in nature (Fig.6). The reduction of ethylene production by the seedlings but normal ethylene production in fruits indicates the tissue specific regulation of ethylene synsthesis by *atr-1* locus. *Green-ripe* (*Gr*) mutation controls a subset of ethylene responses regulating fruit ripening, abscission and root elongation but the mutant has no effect on hypocotyls and petiole epinasty (Barry *et al*.,2005). Unlike *Gr* mutant where only roots are insensitive to ethylene, in our mutant both root and hypocotyls lengths were insensitive to ethylene (Fig. 7A), while both of these mutants show delay in fruit ripening.

Epinastic (*Epi*) mutant controls cell growth and expansion and have no effect on ripening and abscission like *atr-1* mutant (Fujino et. al., 1988; Barry et. al., 2001). The effect of *atr-1*mutation on unripe and non fruit tissue (leaf) differentiates it from *Cnr* mutation (Seymour *et al*., 2002). Our results suggest that *atr-1* mutant falls to a different category controlling another subset of responses including root and hypocotyls length and fruit ripening. In *Epi* mutant the senescence and abscission and ripening are controlled together, in *atr-1* mutant they are independently regulated. Fruit ripening and triple response are controlled by same set of genes whereas abscission is under a separate set of genes like *Nr*, *Gr* (Barry and Giovannoni, 2007). The ethylene signal transduction is blocked somewhere at an unknown point by *atr-1* mutation.

Accumulated evidences suggested that the *atr-1* mutation produced pleiotropic effects on tomato responses to ethylene. Some of the responses like seedling triple response and ripening are strongly inhibited; some of the responses like seed germination, epinasty and abscission are slightly inhibited, some other ethylene mediated responses are not effected by the mutation. It seems that different subsets of ethylene responses may be regulated through different mechanisms in different mutants. Several subsets of ethylene response pathways might exist in tomato or the unknown connecting links of the pathway was missing in these mutants. The complete unraveling of this pathway(s) is possible only when we characterize the mutants completely and resolve their position with respect to one another in the signal transduction pathway.

5.2 A kinetin insensitive *(kin-1)* mutant of tomato confers ethylene underproduction

One powerful approach that has been employed in the analysis of plant hormone signaling pathways has been the isolation of mutants defective in hormone responsiveness. Growth of etiolated seedlings in the presence of higher concentrations of plant hormones like kinetin or auxin creates stress on growing seedlings. This stress causes an elevation of ethylene biosynthesis as a stress response. Hence the etiolated seedlings grown in presence of kinetin show triple response due to elevated endogenous ethylene. In Arabidopsis seedlings this appears to be due to induction of a single ACC synthase isoform (Vogel *et al*., 1998) by cytokinin. We assumed that similar to Arabidopsis, high concentration of kinetin would also cause stress to tomato seedlings. In such a case, a tomato mutant defective in ethylene production would fail to show triple response. Using this strategy we screened kinetin insensitive mutants in tomato that lacks the ethylene-mediated triple response in the presence of cytokinin. The *kinetin insensitive* (*kin-1)* mutant selected was characterized for ethylene evolution and other ethylene responses starting from seedling stage to fruit ripening.

5.2.1 *kin-1* is a recessive mutation

The *kin-1* mutant was crossed with the wild type and the resultant F_1 and F_2 progeny was analyzed for the pattern of inheritance and segregation of the mutant phenotype. The segregation and inheritance analysis of *kin-1* mutant seedlings in presence of kinetin revealed that the mutated gene was functionally recessive to the wild type. The kinetin resistance in mutant seedlings followed Mendelian segregation pattern indicating that it is a single gene (Fig. 20).

5.2.1 The *kin-1* seedlings exhibit ethylene underproduction

In Arabidopsis, many of the kinetin resistant mutants on detailed analysis were found to be ethylene biosynthesis mutants. The *cyr1* (cytokinin response) and *ckr1* (cytokinin resistant) mutants were identified by the ability to elongate their roots on

inhibitory concentrations of cytokinin (Su and Howell, 1992; Deikman and Ulrich, 1995). Several Arabidopsis *cytokinin insensitive (cin) mutants* that were isolated based on absence of triple response also exhibited alteration in ethylene production. Using the similar triple response assay for tomato, the seedlings of *kin-1* mutant were selected based on more elevated hypocotyls than wild type and absence of triple response in presence of kinetin (Fig. 18). The observed kinetin insensitivity can be either due to reduced production of endogenous ethylene in response to exogenous kinetin or due to reduced sensitivity to endogenous ethylene. The later probably is not true for *kin*-1 mutant as it retained normal ethylene responsiveness as seen by triple response. The *kin-1* mutant seedlings exhibit similar growth inhibition like wild type in presence of exogenous ethylene and also with acetylene indicates that mutant seedlings show normal ethylene responsiveness (Fig. 21A,B). On the other hand *kin-1* seedlings were found to be ethylene underproducer. The reduced ethylene production by *kin*-1 seedlings under normal growth condition as measured by the gas chromatography indicates the underproduction of ethylene by them (Fig.21C). Therefore, it is evident that the *kin-1* seedlings were not able to elevate endogenous ethylene levels in presence of kinetin and hence did not show triple response. Hence the reduced growth inhibition in presence of kinetin is due to ethylene underproduction but not due to loss of ethylene sensitivity.

5.2.3 The *kin-1* mutation shows pleiotropic effect

The faster seed germination is one early response to demonstrate ethylene under production in mutant seedlings (Siriwitayawan *et al*., 2003). The *kin-1* mutant shows reduced plant height due to reduced internodal length (Fig. 22C,D). In green house the *kin-1* mutant plants appear light green in colour due to reduced leaf and fruit chlorophyll (Fig. 22A,B). The pale green appearance of *kin*-27 mutant is similar to Arabidopsis *cyr1*mutant and is consistent with the observed phenotype of a cytokinin-insensitive plant (Deikman and Ulrich, 1995). The *kin-1*mutation also altered the inflorescence pattern and the reduced the number of flowers per inflorescence.

Floral abscission is one of the well characterized ethylene regulated trait in plants. In normal tomato flowers, petal abscission and senescence occur 4 to 5 days after the flower opens and precede fruit expansion. If fertilization does not occur, pedicel abscission occurs 5 to 8 days after petal senescence (Lanahan *et al*., 1994). In *kin-1* mutant plants petals does not wither after pollination. In fact they remained attached to the blossom end of the fruits even after the fruits are fully ripened and harvested (Fig. 23C). The mutation has no effect on fertility of plant as evident by the normal fruit set and fruit growth in *kin-1* plants similar to wild type.

5.2.4 The *kin-1* mutant show delayed fruit development and extended fruit shelf life

From agrobiotechnology perspective, there is much interest in identifying the key regulatory mechanisms involved in fruit development and ripening. Ethylene biosynthesis accelerates during onset of ripening from breaker stage onwards to stimulate climacteric fruit ripening (Oller *et al*., 1991; Moore *et al*., 2002). The ethylene evolution during ripening of *kin*-1 fruits follow a similar pattern to that of wild type control fruits but with much lower levels of ethylene then WT. The ethylene released by *kin*-1 fruits was much less then WT at all stages of ripening (Fig. 24C). The reduced ethylene levels can affect the auxin levels in the developing fruit, in turn could stimulates rapid cell divisions and cell elongation in fruits. Our study reveled that the fruits of *kin-1* mutant were slightly bigger in size than control plants (Table 5). Additionally the fruits of *kin*-1 mutant has multilocular ovary with 5-6 locules with higher seed set compared to WT with two locules. Locule number is positively correlated with final fruit weight in tomato (Houghtaling, 1935; Yeager, 1937; MacArthur and Butler, 1938; Lippman and Tanksley, 2001). The increased fruit size and fruit weight could also be due to multi locular ovary (Table 5).

One prominent effect of *kin*-1 mutation is increase in time taken by fruits to reach mature green stage. The mutant fruits reach breaker stage approximately fifteen days later than the corresponding wild type fruits indicating the delay in fruit development (Fig. 24A). Overall the mutant fruits exhibit at least one month delay from anthesis to ripening. The mutant fruits were also slow in off vine ripening

compared to WT. However, it remained possible that the reduced ethylene production could be the reason for delayed fruit development and ripening in *kin-1* mutant (Fig. 24C). The ripening of mature green fruits of *kin-1* mutant could be stimulated by exogenous treatment with ethylene strongly suggests the ethylene underproduction by mutant fruits (Fig. 27A,B).

Reducing the amount of ethylene produced during tomato fruit ripening is the goal of a wide array of transgenic strategies. The genetically engineered tomatoes with antisense ACC Synthase gene (Yao *et al*., 1999), and antisense ACC oxidase gene (Ye *et al*., 1996) and double antisense ACC oxidase and ACC synthase fusion gene (Xiong *et al*., 2003) showed decrease in ethylene production and delayed ripening. This phenotype was reversed by addition of exogenous ethylene (Oeller *et al*., 1991). These transgenic fruits have an extended shelf-life and reduced lycopene accumulation. The reduced ethylene levels in the *kin-1* mutant fruits appear to have enhanced the shelf life of ripe fruits potentially through reduced ethylene induced trigger to cell wall modifying enzymes. Such improved fruit shelf life is a major factor that determines the quality of processed tomato products.

Although significant advances have been made in understanding the molecular biology of carotenogenesis, the regulatory mechanisms that control carotenoid biosynthesis are still poorly understood. The *kin*-1 mutant fruits appear pale green during mature green stage. The pigment analysis of ripened mutant fruits showed the reduced level of chlorophyll and carotenoid content compared to wild type (Fig. 25). In previous reports, it has been demonstrated that ethylene, produced at the onset of ripening in climacteric fruits (Lelièvre *et al*., 1997), controlled ripening processes including colour changes. The reduced ethylene production may be the reason for reduced pigment content in mutant fruits. The Arabidopsis mutant *cin4*, is allelic to the constitutive photomorphogenic mutant *fus9*/*cop10*which highlights the interaction between light and cytokinin in the regulation of ethylene biosynthesis (Crowell and Amasino, 1994). The pale green leaves, plae green fruits and reduced fruit pigment levels in the mutant clearly demonstrates the role of ethylene in developing fruit pigmentation and its interaction with light and other plant hormones. The complete molecular characterization of the *kin-1* mutant locus may reveal the genetic and molecular control of fruit ripening and other ethylene

mediated responses. It is also useful to find out the interacting mechanisms between plant hormones and with light.

5.3 Ethylene action is required for root penetration under mechanical impedance

We attempted to screen for additional tomato ethylene receptor mutants using 1-methylcyclopropene (1-MCP), a gaseous inhibitor of ethylene receptors. During the course of standardization of screening conditions with 1-MCP, we observed that roots of light grown wild type seedlings germinated in presence of 1-MCP were unable to penetrate vermiculite (Fig. 28). The time lapse video showed the inability of 1-MCP treated root tips to enter vermiculite. The root tip though touches the vermiculite due to its inability to penetrate vermiculite root forms vertical loops above the soil as the roots continue to grow. In contrast to this, roots penetrated normally in vermiculite in the control seedlings grown without 1-MCP (Fig. 33). When we grew the seedlings on blotting paper, the control seedlings root grow normally attached to paper, where as 1-MCP treated seedlings showed roots with prominent loops in air (Fig. 30).

It is known that 1-MCP is a strong inhibitor of ethylene action in plants, where it blocks ethylene receptor by binding to it like ethylene. If 1-MCP acting via its action on ETR like ethylene receptors, it is reasonable to expect that blocking ethylene action by another inhibitor of ethylene should lead to response identical to 1-MCP. One of the effective inhibitor of ethylene receptor in plants is silver ions (Hobson *et al.,* 1984). Several ethylene mediated responses such as flower senescence can be effectively blocked by treating flowers with silver ions like silver nitrate. In our study to confirm the role of ethylene in root penetration we treated the light grown seedlings with silver ions right from germination. Blocking the ethylene action with silver ions also blocks the root penetration in vermiculite and forms similar kind of loops like with 1-MCP treatment (Fig. 29A). Only few studies have explained role of ethylene during root growth. Similar observation was made by Zacarias and Reid (1992) with silver thiosulfate and 2,4-norbromadiene. They showed that seedling roots germinated on 2% agar

containing above inhibitors failed to insert their radicals into the medium but did on 0.5% agar. There is one more report with ethylene insensitive *never ripe mutant* of tomato where some of the seedling roots failed to penetrate the medium when grown on sand (Clark *et al*., 1999). In Arabidopsis, it is shown that blocking ethylene action or synthesis cannot allow the seedlings to adapt to agar surface and hence show characteristic wavy nature (Buer *et al*., 2003).

There is increased production of ethylene at the site of touch stimulus during root penetration (Sarquis *et al*., 1991). This continuous production of ethylene in root maintains the growth in correct direction. Ethylene is needed for the continuous readaptation of growing roots to the surface they grow despite ongoing gravistimulation (Edelman *et al.,* 2006). Ethylene may also help the roots to adapt to the surface they are growing and help in root penetration by generating enough pressure to push the growing root tip into vermiculite. To test whether there is an universal requirement of ethylene during early stages of root growth, we also carried an experiment with Lettuce and Tobacco seeds in presence of 1-MCP. We observed the lack of root penetration in presence of 1-MCP even in these seeds similar to tomato seeds (Fig. 31).

5.3.1 1-MCP phenotype is age dependent

The inhibition of ethylene perception reduces ability of root tips to penetrate through vermiculite making the loops in air. The inhibition of root penetration by 1-MCP and silver ions demonstrates the significant role of the ethylene during root penetration. During normal course of plant growth, roots penetrate in soil and never come out, where as 1-MCP treated seedlings show the roots in air. In view of this we examined when exactly ethylene action is required for root penetration, by treating seedlings with 1-MCP at different days from germination. Our results showed that there is no effect of 1-MCP once the root tip enters the vermiculite. It is therefore understand that ethylene is required during very early stages of root growth to allow it to penetrate vermiculite. This is supported by observation that the effect of 1-MCP is most prominent if the seedlings are treated right from the germination or during early stages of germination. Nearly 98% of roots form loops when seeds were treated with 1-MCP right from germination (Table 6). This

percentage reduced to negligible level when 1-MCP treatment is given after three days of growth. Evidently once the roots penetrate into soil, there appears to be no effect of 1-MCP on root loop afterwards. This can be explained as once the roots of germinated seedlings penetrate in soil, it can continue to grow on soil. Our results suggest that the capacity for root penetration during very early stages of development requires ethylene mediated signaling events. If the ethylene action is blocked during these early stages of seedling development, the growing root tip does not penetrate and hence exhibit characteristic root loops (Okada and Shimura, 1990).

5.3.1 1-MCP blocks root thigmotropism but not gravitropism

In nature when the root tips encounter obstacles in soil, they avoid the obstacles by changing the direction of growth. To modulate their growth root use at least two sensory systems, gravitropism and thigmotropism, operating to guide roots around barriers. The extent to which these systems interact with one another is unclear. It is known that gravitropic response of Arabidopsis roots is delayed under conditions where significant mechanical perturbations accompany the gravistimulation. This suppression of gravitropism by mechanical stimulation is probably crucial to successful expression of obstacle avoidance response (Massa and Gilory, 2003). A physiological study in rye has demonstrated that the root capacity for gravitropic bending depends on ethylene (Kramer *et al*., 2003). Both gravitropism and thigmotropism might act in succession or in parallel. Both of these responses may also involve common signal transduction events. In case of seedlings grown on vertical plates in presence of 1-MCP, the root tips were able to grow downward following gravity stimulus (Fig. 30A). It shows that root retain normal gravitropism. The time lapse videos further confirms that the orientation of root tips remain in direction of gravity. In presence of 1-MCP roots follow gravitropism and touch vermiculite but do not penetrate into vermiculite. This clearly shows the mandatory role of ethylene action in thigmotropism of growing root. However 1-MCP appears to affect geotropism too, the reduced amyloplasts in 1-MCP treated root tips compared to control root tip shows the temporary compromise in gravitropic response (Fig. 34).

Our observation therefore points to a new role of ethylene during root growth. Ethylene action has been reported to be a fairly ubiquitous response in several environmental stresses, including mechanical stresses (Abeles *et al*., 1992; Bleecker and Kende, 2000). In response to mechanical stimuli such as wind or touch, plants undergo physiological and developmental changes that confer resistance to subsequent mechanical stress (Telewski, 2006, Tatsuki and Mori, 1999). Plants that are exposed to frequent touch stimulation are shorter, stockier and flexible. Exogenous application of ethylene to plants often results in developmental and morphological changes that are similar to those occurring during thigmomorphogenesis. Apart from block in root penetration, 1-MCP treated seedlings also showed growth changes like increased tap root length and reduced hypocotyls length (Fig. 29B). Apart from tomato, the other plant seedlings like lettuce and tobacco also exhibited similar growth changes. This is consistent with the results of Clark *et al*., (1999), where they showed that ethylene insensitivity in *Nr* mutant resulted in long tap roots, short hypocotyls and reduced root penetration capacity. Several results showed that differential growth caused by auxin during graviperception also involves ethylene (Edelman *et al*., 2006). Thigmotropism is complex multistep process which is influenced by factors like sensitivity to auxin and ethylene and also involves common events like changes in pH and Ca^{+2} levels (Philosph-Hadas *et al*., 1995, 1996; Legue *et al*., 1997)

5.3.3 Auxin transport is needed for ethylene mediated growth changes during root penetration

Several studies have provided information about of how ethylene regulates the growth and development of the Arabidopsis root by controlling auxin biosynthesis, transport and competence in distinct root apical tissues (Ruzcka *et al*., 2007; Stepanova *et al*., 2007; Swarup *et al*., 2007). In the presence of ethylene, auxin accumulation is induced in the root apex by the activation of *ASA1* and *ASB1* expression (Ljung *et al*., 2005; Stepanova *et al*., 2005; Gutjahr *et al*., 2005; Esmon *et al*., 2006). The auxin influx carrier AUX1 (Yang *et al*., 2006) and auxin efflux carrier PIN2 (Wisniewska *et al*., 2006) are essential for mobilizing and transporting auxin from the root apex to the elongation zone. Despite progress in understanding nature of hormone network controlling root growth and

development, little information is available about the function of these hormones in root penetration. Erner and Jaffe (1982) reported the accumulation of auxin-like substances and higher levels of abscisic acid (ABA) in response to mechanical bending. These authors hypothesized that the accumulation of these plant growth regulators resulted from ethylene production earlier in the thigmomorphogenetic response and was responsible for the reduction in internode (shoot) elongation.

In tomato seedlings treated with 1-MCP primary roots grown unimpeded were thinner and longer than grown under mechanical impedance. This indicated that in presence of applied pressure and ethylene action on elongation of primary root is stimulated. Since the action of ethylene is blocked by 1-MCP and there is no inhibition of growth in treated seedling roots. We observed that the rate of primary root elongation increased with increasing concentrations of 1-MCP in a dose dependent manner. The increased root growth in 1-MCP treated seedlings can be explained in two ways. First, 1-MCP can relive the roots from ethylene induced growth inhibition. Second is the reduction of ethylene-induced inhibition of auxin efflux by 1-MCP. In roots treated with exognous auxin, the root growth was inhibited even in presence of 1-MCP. Small amount of auxin can restore ethylene response in roots and intracellular level of auxin play crucial role in regulating growth changes during touch sensing of roots (Rahman *et al*., 2001). In our case shoots respond differently from roots to mechanical impedance. The root lengths of 1-MCP treated seedlings were more than control seedlings, Where as the hypocotyls length of treated seedlings is lesser than control seedlings (Fig. 29B). The hypocotyl length of treated seedlings is reduced in dose dependent manner.

The block of ethylene perception may prevent auxin transport or alter auxin levels in the root tip. The reduced ethylene action may be responsible to the Ca^{+2} transient which may lead to changes in membrane dynamics and polarity where the carriers of auxin lie. The observed reduction in PIN1 protein in 1-MCP treated root tips may lead to reduction in auxin transport (Fig. 35). This might affect the auxin transport and lead to differential growth observed in 1-MCP treated seedlings. Previous reports showed the need of ethylene responsiveness to have the normal auxin signaling (Ruzcka *et al*., 2007; Stepanova *et al*., 2007). Application of both exogenous auxin and ethylene commonly causes in inhibition of root growth. The

inhibitory effect of auxin on root growth may mediated by ethylene. In Arabidopsis isolation of several mutants explained this hormonal interaction. *aux1* mutant defective in auxin uptake carrier protein in roots and *eir1* mutant defective in auxin efflux were originally isolated based on their ethylene resistance root growth. Most interestingly, the mutant roots defective in auxin influx or efflux showed slightly agravitropic roots that confer resistance to ethylene in root elongation. For restoration of ethylene response certain level of auxin in root cells are required for sensing ethylene.

5.3.4 Reactive oxygen species (ROS) may play a role in ethylene mediated root penetration

Reactive oxygen species act as one of the immediate responsive elements for most of the tropic responses. In 1-MCP treated root tips the ROS levels were reduced compared to control root tips (Fig. 36). This indicates that ethylene perception is the prerequisite for ROS to accumulate during mechanical impedance. It is known that the reactive oxygen species (ROS) may activate the calcium channels under mechanoperception (Mori and Schroeder, 2004).There are reports suggesting the level of Ca^{+2} changes during touch and gravity sensing (Knight *et al*., 1992). Legue *et al*., (1997) showed changes in the cytoplasmic changes in the Ca^{+2} during touch and hypothesized the touch induced transient changes in Ca^{+2} levels may be the causative agent for altering membrane dynamics and auxin transport at the site of perception of signal. The partial rescue of root penetration by exogenous calcium in 1-MCP treated seedlings suggests the role of calcium in ethylene predominated root penetration and touch response.

It is still an open question whether the production of ethylene during root penetration is mediated by auxin or not. The evidences show that auxin is involved in changes in growth that occurs during resistance to mechanical force rather than in direct perception of signal. In our study, we propose that ethylene is mainly involved in sensing and the growth changes were mediated by auxin and other counter parts of the signal cascade. Neither Ca^{+2} nor auxin treatment can counteract the inability of the 1-MCP treated seedling to penetrate the soil.

Our possible hypothesis for the mechanism involved in touch sensitivity tries to correlate various events involved in the process of thigmotropism. When the root tip encounters the impeded soil or mechanical force there is increased production of ethylene, which may lead to the changes in cytosolic Ca^{+2} levels in the root tip. These altered Ca^{+2} levels changes membrane permeability and perhaps auxin transport. This will lead to further asymmetric production of ethylene across the root tip, leading to the differential root growth. During touch sensitivity ethylene trigger the accumulation of auxin. Treating with 1-MCP might prevent whole cascade of events by blocking ethylene perception and subsequent touch associated processes. However a detailed molecular analysis is needed to decipher the role of ethylene in early root development and root penetration in soil.

Summary

Development, maturation and ripening of fruits has received considerable experimental attention, primarily due to the uniqueness of such processes to plant species and the importance of fruits as a significant aspect of human dietary intake and nutrition. Tomato serves as a model for climacteric fruit ripening due to its simple diploid genetics, small genome size, short generation time, availability of genomic resources including mapping populations, mapped DNA markers and a developing physical map. The ripening of fleshy fruits correspond to a series of biochemical, physiological and structural changes that make the fruit attractive to the consumer. These changes, although variable among species, generally include modification of cell-wall ultrastructure and texture, conversion of starch to sugars, increased susceptibility to post-harvest pathogens, alteration in pigment accumulation, increased levels of flavor and aromatic volatiles. One of the limiting factors that influence their economic value is the relatively short ripening period and reduced post-harvest life. Significant efforts has been invested in recent years to understand the key control points of fleshy fruit ripening which will allows for manipulation of the shelf life and organoleptic qualities of fruits.

In climacteric fruits such as tomato, apple, melon and banana the ethylene burst is required for normal fruit ripening, as illustrated by the slowing or inhibition of ripening in ethylene-suppressed transgenic plants (Oeller *et al*., 1991; Theologis *et al*., 1993; Ayub *et al*., 1996). Using the tomato model system, investigators has long shown that ethylene biosynthesis and perception are necessary for the coordination and completion of fruit ripening (Yang, 1987; Tucker and Brady, 1987). To date, molecular factors influencing ethylene biosynthesis and perception have been described by mutant isolation, subsequent gene cloning and homology studies in Arabidopsis. The critical role of ethylene in coordinating climacteric fruit ripening at the molecular level was initially observed via analysis of ethylene-inducible, ripening related gene expression in tomato (Lincoln *et al*., 1987). Genetic mapping of putative tomato ethylene receptor loci employing the Arabidopsis ETR1 ethylene receptor as a probe suggested the presence of several tomato receptors (Yen *et al*., 1995). It is observed that manipulation of ethylene production or perception can substantially improve the shelf life of climacteric fruits. (Klee *et al*., 1991; Gray *et al*., 1994; Oeller *et al*., 1991; Ecker, 1995;

Wilkinson *et al*., 1997; Knoester *et al*., 1998; Kopeliovitch *et al*., 1979). Many natural tomato mutants like *Never-ripe(Nr), Colour less non ripening (Cnr), Green ripe (Gr)* affected at ripening have been used to study ethylene induced gene expression and regulation (Wilkinson *et al*., 1995; Wilkinson *et al*., 1997; Lanahan *et al*., 1994; Yen *et al*., 1995; Giovannoni *et al*., 1995; Thompson *et al*., 1999; Barry *et al*., 2005). Even though ethylene has predominant role in coordinating fruit ripening in fleshy fruits, it has been suggested that both ethylene-dependent and ethylene-independent gene regulation pathways coexist to co-ordinate the process in climacteric and non-climacteric fruits (Lelievre *et al*., 1997). The evidences for non-ethylene mediated ripening control come from the analysis of *ripening inhibitor* (rin), *non-ripening* (nor) mutants (Vrebalov *et al*., 2002; Lincoln and Fischer, 1988; Della-Penna *et al*., 1989). To further understand the ripening regulatory mechanisms that precede and fallow ethylene one should increase the number of mutants and further cloning of the novel loci.

Considering the importance of ethylene during fruit development and the importance of tomato as a model crop for fruit ripening, the present work was undertaken to gain insights into the participation of these novel loci in fruit ripening and other ethylene mediated responses in tomato. In this regard, first, the conditions were optimized for efficient screening procedures to screen for ethylene perception and biosynthesis mutants. Seedlings of tomato fruit ripening mutants can be screened at an early stage for their ability to respond to ethylene. Ethylene treated seedlings in dark display a characteristic 'triple response' with short root, an exaggerated apical hook, radial swelling and inhibition of hypocotyls elongation. Most of the mutants defective in ethylene perception or signal transduction were identified by exploiting the triple response of dark grown seedlings to ethylene or its precursor 1-aminocyclopropane-1-carboxylic acid (ACC). In this study, the seedlings were screened based on triple response in etiolated seedlings to exogenous and endogenous ethylene and ethylene action inhibitor. We have isolated and characterized two pleiotropic tomato ripening mutants *atr-1*(acetylene resistant-1) and *kin-1 (*kinetin insensitive*)* from EMS mutagenized M_2 population of tomato. These two mutants have been characterized as an ethylene underproducer (*kin-1)* and as ethylene-insensitive (*atr-1*) mutants respectively**.** These are the first induced mutants of tomato showing resistance to

seedling triple response assay and other ethylene related responses through out plant growth.

The *atr-1* mutant seedlings lacked triple response and were significantly taller in presence of acetylene. However, the insensitivity of etiolated atr-*1* seedlings was also observed in presence of exogenous ethylene and 1-ACC. Genetic segregation analysis showed the monogenic recessive nature of the mutation. The *atr-1* mutant also showed reduced sensitivity to ethylene during vegetative and reproductive phases of development. The mutant exhibited pleiotropic phenotype showing increased number of flowers in inflorescence, increased fruit set, delayed leaf senescence, reduced carotenoid accumulation in fruits and enhanced post harvest shelf life of fruits. In addition to these characters *atr-1* mutant shows increased thermotolerance, delayed leaf senescence and delayed cotyledon abscission. Physiological analysis suggested the reduced ethylene perception by the mutant plants through out life cycle. The *atr*-1 mutant exhibited differential ethylene production with reduced ethylene production at seedling stage and normal ethylene production in fruits. In spite of normal production of ethylene, mutant fruits showed significantly longer transition time from breaker to red ripe stage during both on vine and off vine. The mutant fruits can stay for at least three to four months at 23°C. Transcriptional profiling of ethylene related genes revealed altered expression of most of receptor genes with more striking reduction LeETR2 gene expression at breaker stage of in *atr-1* fruits.

Cytokinins are one of the many factors that modulate the biosynthesis of the gaseous hormone ethylene (Yang and Hoffman 1984, Mattoo and Suttle 1991). To characterize the induction of ethylene biosynthesis by cytokinin and to understand how these hormones interact to regulate plant development in tomato, in this study, we used the triple response to isolate *kin-1* mutant that is disrupted in the elevation of ethylene biosynthesis in response to cytokinin. The *kin-1* mutation is at a single nuclear locus. The *kin-1* mutant showed reduced ethylene production through out life cycle. The mutation exerts pleotropic effect on plant development including altered inflorescence pattern, reduced number fruits per truss, reduced leaf and fruit pigments and reduced plant height etc. The fruits of *kin-1* mutant show delay in both on vine and off vine ripening due to reduced ethylene production in the fruits

during ripening. The fruit size and number of seeds per fruit were increased by the mutation and ripening can be induced by exogenous ethylene. The mutants did not show any abnormalities in normal growth and fertility.

For many years a role for the phytohormone ethylene in thigmomorphogenesis and stress responses has been suspected (Braam and Davis, 1990). The mechanism by which roots penetrate impeded matrices has been studied extensively since the root penetration is importance factor to determine plant stability and nutrient uptake (Abdalla *et al*., 1969; Richards and Greacen, 1986). A role for ethylene has been proposed based on the observation that mechanostimulation of plants leads to ethylene evolution and exogenous ethylene leads to thigmomorphogenetic-like changes. Furthermore, blocking ethylene synthesis or signalling disrupts the ability of tomato roots to penetrate compact soil or even loose sand (Clark *et al*., 1999; Hussain *et al*., 1999). In this regard, we studied tomato root growth in presence of ethylene action inhibitor 1-MCP. Blocking ethylene action in the very early stages of seedling growth by 1-MCP has prevented the soil penetration of growing roots. The roots were not able to penetrate into vermiculite and formed loops in air which will stay till the removal of inhibitor. The time-lapse videos determine the positive geotropic growth of roots and loop formation due to enhanced root growth.

Even though the roots of 1-MCP treated seedlings were positively geotropic the decreased amyloplast number shows the temporary compromise of gravitropism by thigmotropism. Recent studies provide an initial model of how ethylene regulates the growth and development of the *Arabidopsis* root by controlling auxin biosynthesis, transport and competence in distinct root apical tissues (Ruzicka *et al*., 2007; Stepanova *et al*., 2007; Swarup *et al*., 2007). The reduced hypocotyls length and increased root length by 1-MCP treatment may indicate the involvement of auxin in ethylene mediated growth changes during mechanical impedance. The reduced PIN1 protein expression shows the reduced polar auxin transport in presence 1-MCP. The reactive oxygen species levels under mechanical impedance were blocked by 1-MCP. In summary, the work presented here indicates that a more direct mechanosignaling/transduction pathway must exist, perhaps involving the function of transmembrane proteins like ethylene receptors to perceive signal, oxidative burst (Yahraus *et al*., 1995) and involve auxin efflux lead to cell wall

changes (Bradley *et al*., 1992; Xu *et al*., 1995), and growth alterations (Jaffe and Forbes, 1993; Mitchell, 1996).

The present study emphasizes the role of ethylene in fruit ripening and root penetration under mechanical impedance. The study can be useful to understand the role of ethylene in various developmental processes and its interaction with other hormones. Furthermore, mutant lines generated during this study may provide a valuable resource for exploring how fruit ripening and senescence processes are regulated in plants. These delayed fruit ripening mutants may generate a convenient system for long distance transport and ripening can be induced by exogenous application of ethylene when needed. Due to their enhanced shelf life mutant fruits avoid losses during transport. The mutant germplasm can be used in breeding programs to develop tomatoes with more value-added traits. Being nontransgenic and ecofriendly nature, the mutant traits transferred to local cultivars can be easily acceptable by the public domain. It could be concluded that *atr-1* and *kin-1* plays a positive and essential components of ethylene-signaling during tomato fruit development and ripening. However, the mechanism of how these mutations affects the expression of ripening and ethylene related genes need further investigation. The identification of the downstream molecular targets and understanding its mode of action will provide new insights into the mode of ethylene regulation in plants.

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Appendix

dNTP Mix

Mixture of four dNTPS (dGTP, dATP, dTTP, dCTP) each at 2.5 mM. The dNTP mixture is made by adding equal volumes of a 10 mM solution of each of the four separate dNTPs together.

10X MOPS Buffer (FA gel Buffer)

Component	Stock	Working
MOPS	5 M	200 mM
Sodium Acetate	1 M	50 mM
EDTA	0.5 M	10 mM

Adjust pH to 7.0 with 1N NaOH

10 N NaOH

40g dissolved in 100 ml of double distilled water

50X TAE (1 Liter)

Triss Base	---------	24 g (w/v)
Glacial acetic acid	---------	57.1 ml (v/v)
0.5 M EDTA	---------	100 ml (v/v) pH adjusted to 8.0

Ethidium Bromide (EtBr)

Stock – 10 mg/ml Working – 10 µg/ml

Gel loading buffer (6X)

Bromophenol blue	-------	0.25% (w/v)
Xylene cynol	-------	0.25% (w/v)
Glycerol in water	-------	30% (w/v)

TE Buffer 10X

100 mM Tris-Cl, pH 7.4 or 7.6

10 mM EDTA, pH 8.0

Stocks

Component	Solvent	Stock	Working
Silver nitrite	water	0.1 mM	10 µM

MES Buffer (1 ml)

MES ----- 100 µl stock: 250 mM

Kcl ----- 100 µl stock: 100 mM

Dist.water ------ 800 µl

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