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 \( \textit{atr-1} \) and \( \textit{kin-1} \) 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 (\( \textit{atr-1} \)) 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 \textit{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 \textit{et al}., 1992; Burg and Burg, 1962). In addition when hydrolyzed calcium carbide
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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 atr-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 atr-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 atr-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
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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 SlACS4 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°C). 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,
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 synthesis 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-1mutation 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 F₁ and F₂ 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
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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 cyrl 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
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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
Discussion

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 (Philosh-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 relieve 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 exogenous 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 be mediated by ethylene. In Arabidopsis isolation of several mutants explained this hormonal interaction. \textit{aux1} mutant defective in auxin uptake carrier protein in roots and \textit{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 \textbf{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 \textit{et al.}, 1992). Legue \textit{et al.}, (1997) showed changes in the cytoplasmic changes in the 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.