Fruits form an indispensable part of the human diet. A complex genetically programmed event regulated by ethylene provides the flavor, color, taste and aroma to the fruit. Tomato being the model plant for fleshy fruit development and ripening has been used in the present study. Till date tomato development has been extensively examined at the level of transcriptome (Alba et al., 2005; Lemaire-Chamley et al., 2005; Carrari et al., 2006; Vriezen et al., 2008; Wang et al., 2009), proteome (Saravanan and Rose, 2004; Faurobert et al., 2007) and metabolome (Roessner-Tunali et al., 2003; Carrari et al., 2006; Fraser et al., 2007; Moco et al., 2007; Osorio et al., 2011). Sequencing the tomato genome has revealed various genes essential for the fruit ripening. Further task is to annotate these genes to elucidate the function and analyse their promoters to gain better knowledge about the regulatory aspects.

Spatio-temporal regulation of these genes occurs at the transcriptional as well as post-transcriptional levels and is dependent on cis regulatory elements present in the promoter regions. Regulation of the expression pattern of a particular gene involves the specific binding of trans-acting factors to the cis regulatory elements present in the promoter. Various fruit specific promoters have been characterized in tomato till date. One of the most important application of fruits-specific promoter is to delay the ripening, which facilitates the ease of transportation. But most of them characterized so far have one or the other limitations. Some of these fruit-specific promoters represent narrow range of expression being active only during early fruit development, still others possess leaky expression in vegetative tissue (e.g. p119) thereby limiting their use in driving gene expression in a fruit specific manner. Using promoter-reporter gene fusion studies and progressive 5’ deletion analysis, the organ specificity of promoter and the cis-acting regulatory elements necessary for its activity can be delineated (Tyagi, 2001). The immediate challenge ahead is to isolate and functionally characterize fruit-specific promoters for delaying the ripening process and to investigate mechanisms of developmental regulation.

RNAi is a potent tool for shutting down gene expression in sequence specific manner. It has been widely used as a silencing tool for the improvement of various crop plants (Isshiki and Kodama, 2010; Jagtap et al., 2011). To alleviate the pleiotropic effects which could be damaging for plant growth, defense and development due to constitutive down regulation of genes, fruit specific regulation of RNAi needs to be achieved.
Discussion

In the present work, first part of the study demonstrates the activity of 5’ upstream regulatory region (URR) of an uncharacterized fruit-specific gene RIP1. The activity of the RIP1 URR has been investigated in homologous and heterologous model systems, namely tomato and Arabidopsis thaliana, respectively, by raising transgenics. In the second part, RNAi of transcription factor LeEIL1, LeEIL3 and LeEIL1All(all the LeEIL genes) were made under the control of RIP1 as well as with E8 promoter and tomato transgenics were raised. E8 promoter shows low level of expression throughout the fruit development but, expression increases at the onset of ripening.

Role of RIP1 gene

RIP1 is an uncharacterized fruit-specific gene isolated from tomato and showed maximum transcript accumulation during red ripe fruit stage present in the library of TIGR database. Amino acid sequence of this particular gene showed homology to zinc finger motif, CCHC type. It codes for a zinc knuckle protein. Zinc knuckle domain is important for protein-protein interactions and for binding single stranded DNA (Vo et al., 2001). The nucleotide sequence of RIP1 was used for homology search with Arabidopsis database TAIR and it showed similarity to protein kinase superfamily. This suggests that RIP1 gene might play an important role in cell growth and development in the tomato fruit.

RIP1 promoter has potential fruit-specific elements

Using PLACE and PlantCARE database the probable minimum regulatory region of 1.45 kb conferring fruit-specific expression was observed. Transcription start site was predicted using Softberry online tool. The analysis of the sequence revealed the URR to contain potential regulatory motifs corresponding to known cis-regulatory sequences. The fruit-specific motif TGTCACA was observed in negative strand in reverse orientation at -636 bp position with respect to transcription start site. Another fruit-specific element TCCAAAA was found at position -827 bp. Single putative fruit-specific element TCTTCACA was present at -1350 bp. Two E box elements were found at -1195 bp and -511 bp position. Single ethylene biosynthesis element TAAAATAT was located at -706 bp position. TGTCACA motif is a novel cis-regulatory enhancer element involved in fruit-specific expression of the Cucumisin gene of Cucumis melo. (Yamagata et al., 2002) TCCAAAA motif functions as a fruit-specific element by inhibiting gene expression in leaves of watermelon (Yin et al.,
An 8 bp motif of TAAAATAT in the LeACS2 gene plays an important role in ethylene biosynthesis of tomato (Matarasso et al 2005). The E8 promoter includes two copies of this motif (Zhao et al., 2009). So RIP1 promoter includes ethylene responsive as well as biosynthesis elements.

**RIP1 promoter is fruit-specific**

In order to study the activity of RIP1 promoter, 1450 bp promoter fragment was cloned upstream of GUS reporter gene and transformed into tomato. The transformants obtained were evaluated by GUS histochemical staining. The blue color was observed in the fruit tissues. The activity of promoter began at breaker stage peaked during red ripe stage and then declined. No expression was observed in the vegetative tissues. GUS fluorometric analysis showed the highest level of GUS activity in red ripe stage followed by breaker stage fruits and negligible GUS activity in mature green fruit and flowers. From these analyses it can be concluded that RIP1 can be classified as ripening-specific gene and has a role during breaker and red ripe stages of fruit development. Various other promoters of tomato genes have also been found to give the fruit-specific expression like PG (Montgomery et al., 1993), E4 (Xu et al., 1996), E8 (Deikman et al., 1998; Coupe and Deikman, 1997), 2A11 (Van Haaren and Houck, 1993), and ACO1 (Moon and Callahan, 2004) etc.

**Full length RIP1 promoter is required for maximum fruit-specific expression**

In order to delineate the minimal promoter region of RIP1, four truncated fragments along with full length were used for transforming tomato. Varied levels of GUS activity was observed in different tomato transformants. Maximum gus expression was observed in red ripe fruits of RIP1_Del1 but comparatively less activity than full length promoter. The GUS expression was not observed in transgenic fruits of RIP1_Del2, RIP1_Del3 and RIP1_Del4 transformants. Quantitative analysis of GUS activity also gave similar results i.e., highest GUS activity was only observed in the red ripe fruit of RIP1_Del1 followed by little activity in breaker stage fruits. It might be because fruit-specific elements were dispersed along the whole length of the promoter and only the cumulative effect of all the elements could result in full activity of the promoter. The wheat endosperm-specific activity of storage protein gene promoter in wheat requires the cumulative effect of the regulatory sequences to deliver the maximum expression (Lamacchia et al., 2001).
From deletion analysis it can be inferred that cis regulatory elements important for maximum fruit-specific expression reside in -1450 bp region. Decrease in gus expression of RIP1_Del1 could be attributed to the absence of E box element and putative fruit-specific element from the fragment. In other truncated transformants, deletion of the fruit-specific motif and enhancer element might have resulted in loss of activity from the fruit tissue. Similar decline in the specific GUS activity was observed when the rice arabinogalactan (OSIAGP) promoter region was deleted from -1000 bp to -300 bp, and a further deletion to -100 bp resulted in the complete loss of GUS activity (Anand and Tyagi 2010). Deletion analysis of the potato SBgLR gene promoter also revealed that several cis-regulatory motifs are responsible for the activity of the promoter (Zhou et al., 2010).

**TCCAAAA motif functions as fruit-specific element by inhibiting gene expression in tomato leaves**

RIP1_Del2 and RIP_Del3 showed slight GUS expression in leaves which was absent in plants harboring RIP1_FL, RIP1_Del1 and RIP1_Del4. The full length promoter RIP1_FL and RIP1_Del1 did not show any activity in leaf, which could be because of the presence of negative element i.e. TCCAAAA along with the fruit-specific element thereby conferring only fruit-specific expression and inhibiting the expression in leaves. The smallest deletion fragment RIP1_Del4 did not show any GUS activity due to absence of all the motifs which resulted in complete loss of the activity. These results are in accordance with the report in which TCCAAAA motif was suggested to functions as a fruit-specific element by inhibiting gene expression in leaves of watermelon (Yin et al., 2009).

**RIP1 promoter is also active in heterologus system Arabidopsis**

To establish the regulatory function of RIP1 promoter in heterologous system promoter-reporter gene fusion (full length and deletion) constructs were used to transform Arabidopsis. Histochemical GUS staining of various tissues (root, stem, leaf, flower, anther and silique) of transgenic Arabidopsis plants harboring RIP1_FL and all the deletion constructs showed variable expression in different tissues. RIP1_FL activity was observed in flowers, anther and siliques and similar but, weaker expression was observed in RIP1_Del1 transgenic plants. Interestingly, in Arabidopsis
also, TCCAAAA motif behaved in same manner by inhibiting the expression in leaf. In RIP1_Del2 and RIP1_Del3 transgenic plants, due to the absence of negative and fruit-specific element GUS expression was observed only in leaf and not in siliques. Since RIP1_Del4 did not have any element, the expression was observed in none of the tissues.

Similar expression in both Arabidopsis and tomato transgenic plants demonstrated that RIP1 regulation is mediated, at least in part, at the transcriptional level by a mechanism which is conserved between the two different species. Developmental and pathogen-induced activation of the Arabidopsis acidic chitinase promoter behaved in a similar manner (Samac and Shah, 1991). Expression analysis of the BFN1 nuclease gene promoter during senescence, abscission, and programmed cell death-related processes revealed similar pattern in tomato and Arabidopsis (Sarit et al., 2008). The spatial and temporal expression of potato ci21A promoter in tomato and Arabidopsis also exhibited the similar expression pattern (Chian and Wang, 2009).

**Formation of an effective hpRNAi construct**

To form the constructs for effective RNAi of LeEIL1, 3 and of all the LeEILs, 300 bp to 350 bp length of sense and antisense strand was interspaced by catalase intron of 192 bp in the present study. Fragment of 250-350 bp is optimum as a trigger for hpRNAi vectors, since it has been demonstrated that the chances of off-target silencing increase with greater length of the initial dsRNA sequence (Senthil-Kumar and Mysore, 2011). Intron is preferred for increasing the silencing efficiency in plants (Smith et al., 2000, Helliwell and Waterhouse, 2003). The size of the spacer sequence also matters when it comes to efficacy of RNAi which has been well demonstrated by Xiong et al. (2005).

**Similar expression profile of promoter and targeted genes in fruit tissues**

E8 expression is induced at breaker stage and gets amplified at red ripe stage and then activity diminishes. RIP1 promoter also follows the similar pattern but it shows 25% expression as compared to E8. So, silencing under the control of E8 would be more severe as compared to RIP1. Expression of both the promoters and
LeEIL transcription factors follow the same expression pattern i.e. both get induced during the onset of ripening.

**Nuclear localization of LeEIL1 and LeEIL3**

Both the proteins were found to have putative nuclear localization signal (NLS) i.e. KRLK. Subcellular localization of both the transcription factors was checked by particle bombardment and it was found that both are nuclear localized. It confirmed their site of action is in the nucleus only. Identification of NLS is important because a number of proteins have short, very basic sequences, but are unlikely to be nuclear proteins (Boulikas 1994). Hence, the presence of a sequence similar to known NLSs, does not establish that particular protein will move to the nucleus. Chen et al., (2004) also showed the nuclear localization of LeEIL1, though they detected low levels of this protein in cytoplasm also which might be due to diffusion. VrEIL1 from mung bean also has been effectively targeted in the nucleus (Lee and Kim, 2003).

**Phenotypic changes in RNAi lines in terms of color development**

All the transgenic lines obtained did not show any phenotypic change during vegetative stages. Large difference in color development were observed between the transgenic and wild type fruit during the red ripe stage. RIP1 and E8 express in flower but phenotypically flowers were found to be identical to the wild type. In case of fruits, change was observed in the color of pericarp. Chronologically, at B+7 stage all the fruits of transgenic lines were not red in color as was observed in wild-type. Fruits of LeEILAll were green in color at B+7 stage while LeEIL1 and LeEIL3 fruits were orange in color when expressed under the control of both the promoters. In case of plants harboring LeEIL1 and LeEIL3 under the control of RIP1 promoter, fruits showed the nearred ripe stage after B+35 days with LeEIL3 attaining more red color. With E8 promoter, even after B+55 days fruits were red orange in color, though the intensity of orange was less in LeEIL3 than LeEIL1.

These results suggest that the activity of enzymes involved in the process is somehow associated with ethylene action. The slowdown of color development is indicative of general halt in overall ripening process. The retardation of color development in transgenic tomato fruit could be attributed to the low ethylene production and delayed increment in ethylene production to reach the threshold concentration for color development as well as inability of the ethylene to activate downstream genes of ethylene signaling pathaway. With antisense suppression line
of ACC deaminase, red color was delayed by 6-10 days (Klee, 1993). With hpRNAi ACO construct also color development was found to be delayed (Behboodian et al., 2012).

**Low levels of ethylene and CO$_2$ production in transgenic plants**

Ethylene production and respiration rates of tomato transgenic fruits were affected by _LeEIL_ suppression. Transgenic fruits had a significantly lower rate of ethylene production than wild-type fruits during storage period. A rapid increase of ethylene in wild-type fruits occurred during first 7 days after harvest and followed by decline in the subsequent phase of ripening. In transgenic RNAi fruits of _LeEIL1_ and _LeEIL3_, no major burst of ethylene could be detected till B+35 days, though till breaker stage ethylene level was comparable to that of wild-type fruits. In comparison to wild-type, the peak was observed at B+35 days in RNAi fruits but ethylene levels were less (10-20%). In _LeEIL1_-RNAi transgenic fruits, ethylene was present at residual level throughout the fruit development.

The rate of CO$_2$ production during ripening of wild-type fruits reached a peak at seventh day after the harvest and then declined, while in RNAi fruits, respiratory peak was delayed to 35 to 40 days. Significant differences were found between wild-type and transgenic fruit in the temporal aspect of CO$_2$ accumulation. It can be concluded that silencing of _LeEIL_ decreases and delays the ethylene production, which in turn resulted in reduced fruit metabolism in transgenic fruits, and hence less and delayed CO$_2$ production during postharvest. Rate of respiration is often a good index for the storage life of fresh fruits and vegetables; that is, the higher the rate, the shorter the life, and the lower the rate, the longer the life. Accordingly, in this study, tomato transgenic fruits did not ripe early since ethylene and CO$_2$ production was less.

Ethylene is known to play an utmost important role in regulating fruit development and ripening, especially in climacteric fruits by coordinately inducing the expression of large numbers of genes which finally contribute to ripening (Dolan, 2001). There are various reports in which inhibition of ripening has been achieved in fruits by reducing ethylene production. One approach involves the downregulation of genes encoding key enzymes in the ethylene biosynthetic pathway. By antisense inhibition of ACC oxidase (Picton et al., 1993, Schaffer et al., 2007), or RNAi inhibition of ACO (Xiong et al., 2005) or ACC synthase antisense (Oeller et al., 1991). Transgenic tomato plants were also produced with antisense apple fruit ACC-oxidase...
RNA (Bolitho et al., 1997), in which 95% reduction was observed. Silencing of *LeEIL* in transgenic fruits exhibited a slightly low but consistent increase in ethylene production. So, in the present study less compromised fruits were obtained without much reduction in ethylene level. So it does not result in other pleiotropic effects. This result also shows good correlation to the work of Yokotani et al. (2009) in which they suggested that ripening associated ethylene biosynthesis is autocatalytically and developmentally regulated which leads to fruit ripening. Even by microarray analysis expression of any of the ethylene signaling component as well as the regulators were not found to be altered.

**Percentage of weight loss in transgenic fruits**

The weight loss increased in both wild-type and transgenic fruits during storage time. After 28 days post harvesting, the wild-type fruits lost about 30% to 40% of the weight while, the transgenic RNAi lines with *LeEIL1* and *LeEIL3* under *RIP1* promoter lost only 20% to 25% of the weight. Under the control of *E8* promoter, weight loss was around 15% to 20%. Interestingly, when all the *LeEILs* were silenced, after 28 days weight loss was only around 5%. The postharvest weight loss of fruit is mainly the loss of water due to environmental conditions. But the environmental conditions were same for all the cases. So weight loss might be related to the presence of extra cuticular waxes present on the fruit which generally prevent the transpiration during storage. By microarray analysis expression of β-Ketoacyl-CoA Synthase was found to be more in the transgenic fruits, which catalyses the formation of waxes. In this study, the weight loss was significantly higher in the wild-type fruits at every stage of ripening.

**Transgenic fruits with higher total titratable acidity**

In general, organic acid levels can be used as an indicator for postharvest quality. Titratable acids (TA) are the major factors determining the fruit taste (Ferguson and Boyd, 2002). Results obtained from our study show that TA contents were more in the RNAi lines as compared to wild-type. RNAi fruits when analysed for total acid content were found to have ~1.5-2 fold increase in total acids as compared to controls. Though the content decreases with increase in number of days but less reduction was observed in the case of RNAi lines. Maximum reduction was obtained in *LeEIL3* RNAi under the control of *RIP1* promoter. Similar observations were made by Opiyo and Ying (2005). Martinez et al., (2002) reported higher levels of fruit TA in
antisense ACC oxidase melon. The lower rate of respiration in RNAi fruits explains higher accumulation of TA in these fruits, since organic acids have been established as substrates of respiration, and in an ethylene dependent manner (Defilippi et al., 2005). The higher levels of TA thus, maintain the characteristic flavor of the RNAi fruits.

**Reduction in the level of ascorbic acid in RNAi fruits**

Tomato is an important source of vitamin C or ascorbic acid (AsA) which has antioxidant properties and provides health benefits for human. It also acts as a cofactor for various enzymes and contributes to the regulation of cell division and expansion (Smirnoff and Wheeler, 2000). AsA and its oxidized form dehydroascorbic acid (DHA) function as signaling agents (Pastori et al., 2003; Fotopoulos et al., 2008). AsA is an indicator of the vitamins in any processed product since the retention of AsA signifies the retention of majority of the phytonutrients in a processed product. In plant cells AsA is continuously oxidized and reduced and produce ascorbic acid free radicals and DHA as the oxidation products, which can be reconverted back to AsA. AsA has been established as an important co-factor for the *in-vitro* activity of 1-aminocyclopropane-1-carboxylate oxidase (Smith et al., 1992). In general, the AsA levels decline during ripening and senescence and has been correlated with its getting used up in ethylene biosynthesis pathway. L-galactono-1, 4-lactone dehydrogenase (GLDH1) oxidizes L-galactono-1,4-lactone to AsA while DHAR1 (Dehydroascorbatereductase), DHAR2, MDHAR and AO are involved in AsA oxidation and recycling (Stevens et al., 2007).

In the present study less but comparable level of AsA was recorded in all the RNAi lines screened with reference to wild type morphologically. Maximum reduction was observed when all the *LeEILs* were silenced followed by *LeEIL1* and then *LeEIL3* RNAi lines. Under *E8* promoter reduction was found to be more in comparison to the *RIP1*. Microarray analysis and QPCR revealed that genes involved in AsA biosynthesis GLDH1 and AO showed an enhanced expression at B stage while the other genes viz., DHAR1, DHAR2, MDHAR were unaltered in their expression pattern. The upregulated expression of both the biosynthetic as well as oxidation genes maintained the AsA pools in the RNAi lines of tomato.

**Less lycopene content in RNAi lines of tomato fruits**
Lycopene is a carotenoid pigment imparting sensory and nutritional quality to both fresh ripe and processed tomato products (Pirello et al., 2009). An increase in lycopene is a highly desirable quality in tomato fruits and processed products because of its nutritive value and effectiveness in preventing certain types of chronic diseases including cancer (Mein et al., 2008; Palozza et al., 2011). Lycopene fractions of the RR fruits were quantified using spectrophotometric method. There was a significant reduction in lycopene levels in RNAi RR fruits ranging from 10-40% reduction compared to control. The fruits from the RNAi lines with reduced ethylene levels could only develop light red color even after ~60 days post harvest.

Expression of lycopene biosynthesis gene, PSY1 (Bartley and Scolnik, 1993) was significantly reduced in Band RR stages of fruit ripening in these RNAi lines, while DXS1 (1-deoxy-d-xylulose-5-phosphate-synthase, (Lange et al., 1998; Romer et al., 2000) and LES (which is involved in catabolism of lycopene, Ronen et al., 1999) showed no significant difference from that of WT in terms of their mRNA levels. Reduction in lycopene content in RNAi fruits over controls can be attributed to reduced transcripts of lycopene biosynthetic genes which is possibly a manifestation of dramatic reduction in ethylene release shown by these RNAi lines. Itkin et al. (2009) have reported TAGL1 to be the factor controlling lycopene biosynthesis. However, in present study transcript profile for TAGL1 revealed no significant change in its mRNA levels in RNAi fruits. Moreover, this transcription factor acts upstream of ethylene and controls ethylene biosynthesis. Thus the observed effects (reduced lycopene) seems to be the manifestation of ethylene action and points towards ethylene mediated regulation of lycopene biosynthesis.

**Higher firmness in transgenic fruits**

Compression measurements indicated that there was no statistical difference in firmness of a fully ripe fruit, but 14 d after harvest, transgenic fruit were significantly firmer than control fruit. The transgenic fruit usually showed no signs of senescence for at least 6-7 weeks, even after attaining the red color. In the case of LeEILAIiRNAi under the control E8 promoter, fruits remained firm even after three months. Firmness decreased with increased storage period at a slower rate in RNAi fruit. In the beginning of storage, all the fruits were very firm, but for wild-type fruit, a large reduction in firmness occurred between day 2 and day 8. The wild-type fruits lost their textural integrity faster than the transgenic fruits. The maintenance of firmness in
transgenic fruits is suggested to be due to the reduced respiration and other ripening processes during storage.

Due to the economic importance of fruit softening, expression of important hydrolases that are implicated in tomato cell wall degradation were checked by microarray analysis and QPCR. The activity of polygalacturonase was found to be very low in transgenic fruits. The enzyme activity of PME was more in the wild-type fruits than in transgenic fruits. In fruits of wild-type tomato β-gal activity was higher as compared to transgenic fruits. There is a correlation between firmness loss and PG and PME activity. In transgenic fruits, slow increase in the activity of enzyme resulted in slow softening process of the fruits in comparison with wild-type.

**Increased shelf life of transgenic fruits**

The time taken for fruit at B to reach RR was noted for all the RNAi lines as well as control. The tomato fruits were plucked from WT and the RNAi lines at the BR stage and were stored at 28°C under humidity chamber. WT fruits kept at 28°C changed from green to red within 10-12 days and rotted by another 6-7 days, whereas, the RNAi fruits changed to orange red color after 40-45 days and had a prolonged shelf life of about another 30-32 days.

The delay in ripening period and prolonged shelf life of the transgenic tomato can be ascribed to traces of ethylene produced by these tomatoes and the reduced rate of respiration. Cell wall components including cellulose, hemicelluloses and pectin are the major contributors for flesh firmness. During ripening, cell wall undergoes substantial disassembly caused by increased expression of various cell wall degrading enzymes like polysaccharide hydrolase/glycoside hydrolase, transglycolases, lyases and expansions (Brummell, 2006). EXP1, TBG4, XTH5 (xyloglucanendotransglycosylase/hydrolases), and PG (Pirrello et al., 2009) have been shown to be specifically expressed during fruit ripening and play major role in fruit softening. EXP1 acts by increasing accessibility of other cell wall modifying proteins such as PG to cell wall polymers (Rose and Bennett, 1999). TBG4 encodes β-galactosidase II which catalyses the removal of galactosyl residues from cell wall polymers. Here, comparative expression analyses of genes involved in cell wall metabolism indicated no apparent change in LOX C expression levels, while considerable reduction in mRNA levels of EXP1, TBG4, PG and XTH5 were noted at
Discussion

RR stages of fruit development in RNAi fruits over WT fruits, thereby contributing to the longer shelf life of fruits.

Our results showing decreased levels of PG transcript are in agreement with those of Sirit and Bennett (1998) who demonstrated that PG is ethylene sensitive and low levels of ethylene are sufficient for PG induction and mRNA accumulation. The results showed that in RNAi tomato lines the inhibition of EXP1, TBG, PG, and XTH5 expression is consistent with the delay of fruit ripening and prolonged shelf life of fruits influenced by reduction in ethylene levels. Thus, the decline in respiratory activity and lower transcript abundance of cell wall degrading genes has led to the delayed ripening and enhanced shelf life in RNAi tomato lines.