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Quantification of the gene expression of bell peppers (*Capsicum annuum*) ripening gene(s) using real -time PCR

Osama S. Hassan, Fatma Badie and Gehan Safwat*

Faculty of Biotechnology, October University for Modern Science and Arts (MSA), Cairo, Egypt.

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Fruits can be divided into two groups according to the regulatory mechanisms underlying their ripening process. The two ripening processes are climacteric and non-climacteric process; bell peppers are part of the non-climacteric plant groups. Bell peppers are members of the *Solanacaea* family. The *Solanacaea* family is best known for its fruits around the world. Today's main focus is targeted to fruit ripening, in an attempt to increase the fruit's shelf life. Many genes have been linked to the maturation of the fruit such as in Arabidopsis, the genes found were elongation factor-1α (LeEF-1A), expansin protein (leEXP1) and ripening inhibitor (RIN). This research focused on discovering similar genes that may play an important role in the ripening of peppers. Real-time PCR was performed on the cDNA of the green bell pepper fruit during its stage of development in order to detect and identify the expression pattern of the gene. Through the comparison of the gene expression found in bell pepper and the pods of Arabidopsis as model to dicotyledonous plant, some variations have been detected.

Key words: Bell pepper (*Capsicum annuum*), arabidopsis (*Lycoperiscum esceluntum*), fruit ripening, expansin gene (EXP1), elongation factor alpha gene (EF-1a), ripening inhibitor (RIN), MADs box, complementary DNA (cDNA), gene expression profiling, real -time PCR.

INTRODUCTION

Pepper (*Capsicum spp.*) is one of the most important cultivated vegetables around the world. It is a member of the *Solanaceae* family that contains almost 2500 species; most of them are edible which gives it a countable economic value (Wien, 1999). One of these species is the bell pepper (*Capsicum annuum*). A group of genes belonging to the pepper plant had been published by Wang and Bosland (2006). Those genes control the qualities, the hereditary foundation of the mutants/lines, activity mechanisms of genes, gene linkage, molecular markers, and chromosome localization when accessible. The edible part of the pepper plant is the fruit. Therefore, studying its ripening process of fruiting is very important. The bio-chemical components help in the variability development among species that include: adjustment of colors (of chlorophyll, carotenoid, or flavonoid aggregation), textural alteration through variation of cell turgor and cell wall structure and/or synthesis, alterations of sugars, acids and

*Corresponding author. E-mail: gehan.safwat@hotmail.co.uk.

unpredictable profiles that influence nutritious quality, flavor and smell, in addition it improves weaknesses to pathogens. Distinctive types of foods grown from the ground are physiologically characterized by the vicinity (climacteric) or nonattendance (non-climacteric) of expanded breath and amalgamation of the vaporous hormone ethylene at the start of maturation (Lelievre et al., 1997). Therefore, studying this process on a molecular basis may provide better understanding for the fruiting development stages. Constrained data have been published on the likenesses and contrasts between climacteric and non-climacteric fruits on transcriptional and expression levels of the responsible genes. Fruit ripening process is closely similar in both types. The bell pepper is a non-climacteric fruit. The discovery of these genes aided in pointing out the purpose of the maturing components preservation during fruit development. The majority of developmental genes that encode for the ripening process are just regulated in different stages of ripening (Lee et al., 2010). Regardless of the advancement made in Arabidopsis as a model dicot plant, late discoveries demonstrated that flowering and fruit (silique) formation in Arabidopsis are close to those of other plant species (Quinet et al., 2006; Carrari et al., 2007).

The outflow profiles accessed 1100 novel Arabidopsis genes coding for known and putative transcription elements (Tfs) throughout silique improvement through utilizing microarray hybridization. Various leveled bunch investigates uncovered unique expression profiles for the diverse silique developmental stages (De Folter et al., 2004). Many genes discovered that are linked to the fruit ripening in the tomato included the elongation factor-1 alpha (EF-1a), ripening inhibitor (RIN) and expansin proteins (EXP1). These genes are expected to play a major role in most of fruit ripening processes with the majority of the flowering plants (Powell et al., 2003).

MATERIALS AND METHODS

Total RNA extraction

According to Cathala et al. (1983), first mature bell pepper fruit and Arapidoposis samples were homogenized with ice cold extraction buffer [2% hexadecyltrimethyl ammonium bromide (CTAB), 2% polyvinyl pyrrolidinone K 30 (PVP), 100 mMTris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, and 0.5 g/L spermidine] and ß-mercaptoetanol. The samples were incubated at -20°C for 30 min then centrifuged at 8000 round per min (rpm) in 4°C to obtain the supernatant of the mixture that contained the nucleic acids (both DNA and RNA). To make sure that all residues were removed from the supernatant except of the nucleic acids, phenol, chloroform and proteinase K were added. The supernatant was shook briefly to allow the extraction through the supernatant and then incubated at room temperature for 30 min. The supernatant was spun at 8500 rpm for 20 min at 4°C and the aqueous layer was removed to other clean tubes by pasture pipette. After that the precipitation buffer (4 M lithium chloride, 100 mM Tris-HCl, 10 mM EDTA) was added and incubated for 3 h at -70°C, where then the supernatant was centrifuged at 12000 rpm for 30 min in 4°C. Finally, the supernatant was discarded and the RNA pellet was dissolved in 100 ul TE buffer. The RNA quality was displayed on 1.2% agarose form-aldehyde gel electrophoresis at 65 V for 50 min. The rest of the RNA samples were stored at -70°C. In spite of the low content of the mRNA (1% of the total RNA), identification of the transcript of any gene could be done using reverse transcriptase to synthesis a cDNA of the targeted genes. Double stranded *cDNAs* were synthesized using SuperScript® III Reverse Transcriptase kit (Life technology™). Choosing the correct reverse transcriptase (RT) for cDNA synthesis is critical for obtaining high yields of quality full-length cDNA that accurately represents the input RNA of the bell pepper fruits and Arabidopsis siliques. The primers used for the three ripening-regulated cDNAs were EF-1a with the forward as 'CAGAACGTGAGCGTGGTATCA' and the reverse as 'CAGTTGGGTCCTTCTTGTCAA'; EXP1 with the forward as 'ATGGGTATCATAATTTTCAT' and the reverse as 'AGGTAGAAGATCGATGGTCA'; and RIN gene with the forward as 'GTGGAAATGTTACAACAG' and the reverse as 'TAGGCAATGTATTATTGC'. The cDNA was then amplified exponentially by polymerase chain reaction (PCR).

The amplified transcripts had been cloned using the pGEM®-T vector. A partial-length sequence of the candidate fragments was achieved using an automated DNA sequencer (ABI 377, Perkin Elmer®/ABI, Foster City, CA) using the T7 universal primer. The partial-length sequences were analyzed using the National Center for Biotechnology Information (NCBI), followed by pairwise alignment with the Arabidopsis transcripts using the online CLUSTAL W2 software to compare both sequences: www.ebi.ac.uk/Tools/msa/clustalw2/.

Quantification of the targeted genes using the real-time PCR

Several variables need to be controlled for gene-expression analysis, such as the amount of initial material, enzymatic efficiencies, and the differences between tissues or cells in overall transcriptional activity. For the conduction of the Real-time PCR, this analysis was done using a kinetic PCR instrument (ABI PRISM® 7900 Sequence Detection System "SDS"); The SYBR® Green PCR master Mix (PE Applied Biosystems®, Foster City, CA, USA). The reaction contained cDNA, 10 nM of the specific primers of the investigated genes, SYBR® Green PCR Master Mix, and deionized water. The results were analyzed using ABI PRISM® SDS software (version 2.0), and using the comparative threshold cycle (CT) method ($\Delta\Delta$CT) for calculations. For data normalization, 18S rRNA gene was used as internal control by the following primers; forward (AGTCATCAGCTCGCGTTGACT) and the reverse 'ACGGGCGGTGTGTACAAAG'. The target amount was normalized to an endogenous reference and relative to a calibrator (fold differences), is given by $2^{-\Delta\Delta CT}$. Using the expression level of target genes in the control tissue as a base line, the expression folds have been detected in the treated ones assuming that both standard and target have same efficiencies (Molestina and Sinai, 2005). Threshold cycle values (Ct) were used, as Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold in order to convert threshold cycles in copy numbers.

RESULTS

The total RNA of the bell pepper fruits and Arabidopsis siliques were isolated, and visualized on 1.2% form-aldehyde agarose gel electrophoresis (Figure 1a). RT-PCR was applied in order to determine expression of the genes under investigation. The RT-PCR products were displayed on 1.2% formaldehyde agarose gel electrophoresis

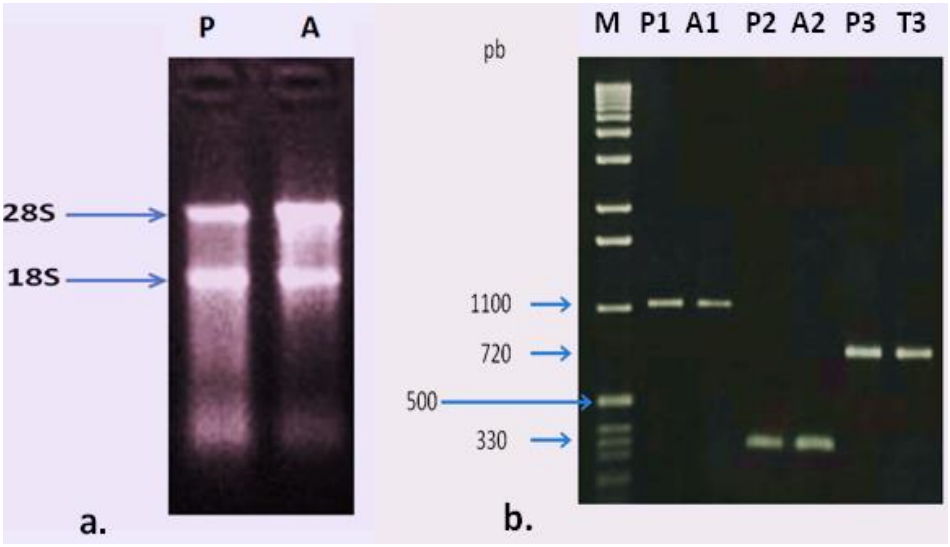

Figure 1. a. The 1.2% formaldehyde agarose gel electrophoresis analysis of total RNA of bell pepper and Arabidopsis silique: Lane (P) represents the total RNA of bell pepper and lane (A) represents the total RNA of Arabidopsis. **b.** 1.2% agarose gel electrophoresis analysis for bell pepper fruits and Arabidopsis silique ripening genes (EF-1a, RIN& EXP1). Lane M represents the 100 bp DNA marker which indicates the length of the amplified fragments, lane (P1) shows the expression of EF-1a in bell pepper, lane A1displays the EF-1a expression in Arabidopsis, lane P2 shows the expression of RIN in bell pepper, lane A2 illustrates the expression of RIN in Arabidopsis, while lane P3 and A3 display the expression of Exp1 in bell pepper and Arabidopsis respectively.

(Figure 1b). The amplification of the three genes fragments were accurate, distinguishable and in the expected size of the designed primers. The elongation factor -1a gene (EF-1a) in both the bell pepper and Arabidopsis siliques were ≈1,100 bp, the RIN fragments ≈330 bp, and the EXP1 fragments ≈ 720 bp. RNA with high quality had been obtained (Figure 1a) and underwent RT-PCR which displayed on 1.2% formaldehyde formaldehyde agarose gel. It was clear that the targeted DNA visible fragments were of the size expected on 1.2% agarose gel (Figure 1b). P1 represents the lane of the DNA fragment of the Elongation factor -1a gene (EF-1a) in the bell pepper and A1, that of the Arabidopsis. Both have the exact size of ≈1100 bp when juxtaposed with the DNA ladder; P2 lane represents the DNA fragment of the RIN gene which has 330 bp and A2 of the Arabidopsis. In addition to that are P3 A3 lanes: where P3 lane is the DNA fragment of the Expansin gene (EXP1) with a size of roughly 720 bp and A3 of the Arabidopsis.

Partial sequencing of expected differential fragments

Partial-length sequences of each of the positive cDNA clones of the three candidate fragments (EF-1a, RIN and EXP1) had been sequenced using the dideoxynucleotide method (Sanger et al., 1977) as shown in Figure 2. The obtained sequences had been aligned with each other to detect homology between the genes in both plants (Figures 3, 4 and 5). The alignment results show an 88.59% similarity between the sequences of the cDNA fragment of the bell pepper and the Arabidopsis' EL-1a gene and 100% similarity between the cDNA fragment of the bell pepper and the Arabidopsis' RIN gene. Finally, there was an 82.73% similarity between the cDNA fragment of the bell pepper and the Arabidopsis' EXP1 gene.

Quantification of the genes' expression

In order to evaluate the gene expression fold of the targeted genes, real-time PCR was performed to measure the CT value of each gene in the bell pepper to be compared with the Arabidopsis CT value, in order to measure the expression in both and for the assessment. Slight differences were observed between the CT of both the bell pepper and Arabidopsis; the bell pepper exhibited higher CT value in both the RIN and EXP1 genes along with a higher standard deviation than Arabidopsis. The EF-1 gene exhibited a lower CT value in the bell pepper than the Arabidopsis (Table 1). Gene expression and folds of the genes were measured using RT-qPCR to compare between Arabidopsis and the bell pepper. The Total RNA and rRNA levels are not proper references, because of the observed imbalance between rRNA and mRNA fractions. Accurate normalization of gene-expression

Figure 2. The cDNA fragments' sequences of the bell pepper fruits.

levels is an absolute prerequisite for reliable results, especially when the biological significance of subtle gene-expression differences is studied. Still, little attention has been paid to the systematic study of normalization procedures and the impact on the conclusions. The expression folds of the candidate genes were calculated and represented in Figure 6. The used formula for copy number detection was:

Y molecules μL^{-1} = (Xg μL^{-1} DNA/ [Length of PCR product in base pairs x 660]) x 6.022 x 1023)

which is a simple method for determining the variable values in experimental biology (Reed and Muench, 1938).

DISCUSSION

There have been impressive advances in understanding the progressions connected with fruit development at the physiological, biochemical and molecular levels. These advances incorporate revelations on the components and indicate pathways involved and linked with the methodology of products of the fruit maturation. Late research has uncovered that the few genes that control fruit development and ripening have been conserved throughout the course of advancement. The image formulating indicates plainly the conjunction of ethylene-dependent and ethylene-independent pathways in both categories. It has been indicated unmistakably that numerous controllers of fruit development and maturing are normal

Figure 3. The EF-1a alignment between bell pepper and Arabidopsis sequences.

Figure 4. The RIN alignment between bell pepper and Arabidopsis sequences.

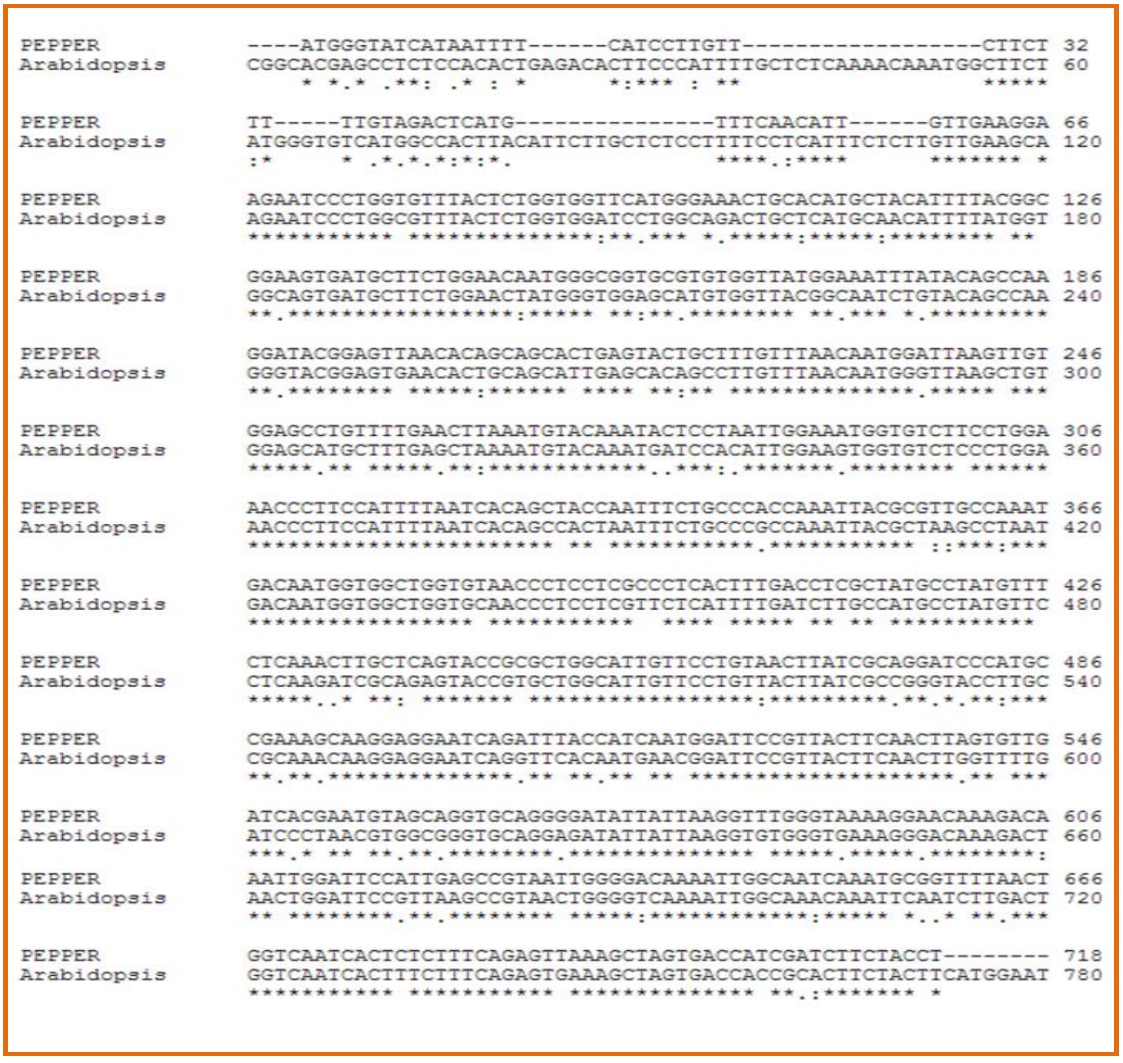

Figure 5. The EXP1 alignment between bell pepper and Arabidopsis sequences.

Table 1. CT values and standard deviation of the fruit ripening genes in mature bell pepper fruits.

Genes	Pepper		Arabidopsis	
	C_T value	Std. deviation	C_T value	Std. deviation
EF-1	7.11	± 0.026	8.53	± 0.016
RIN	12.28	± 0.017	11.92	± 0.031
EXP1	9.67	± 0.021	8.95	± 0.008

Ct, Threshold cycle values.

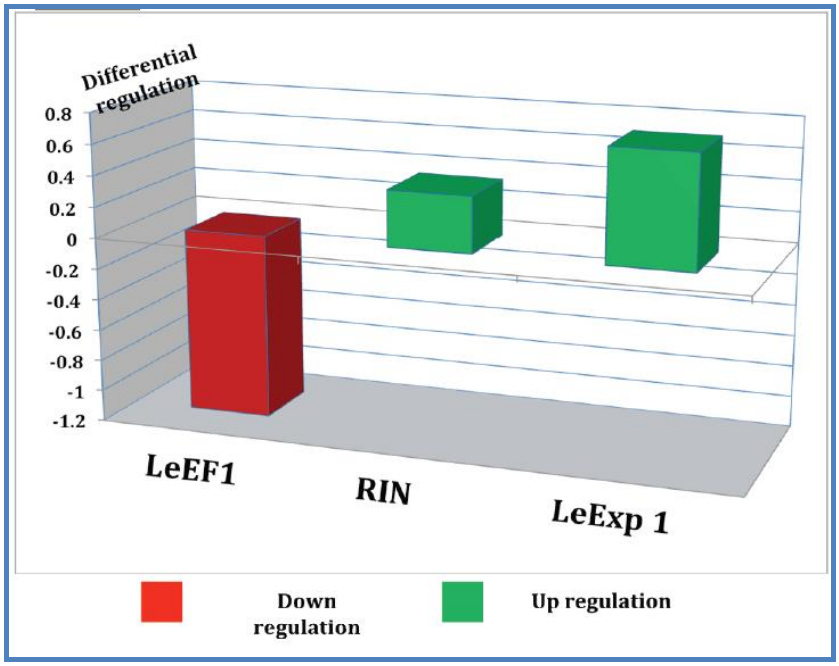

Figure 6. Normalized differential expression of the *LeEF-1, rin* and *LeExp1* between the bell pepper and Arabidopsis.

to both climacteric and non-climacteric types (Paul et al., 2012). Partial sequencing of the genes under investigation revealed high similarity among the genes in Arabidopsis and bell pepper fruits. The differential display reverse transcriptase-PCR was used to extract EF-1a which is an ethylene-inducible gene (Kendrick and Chang, 2008). However, an 88.59% similarity has been detected between the partial sequence of EF-1a in bell pepper and Arabidopsis (1, 100 bp). Moreover, the expression analysis of EF-1a in the bell pepper was minor in comparison to its expression in the Arabidopsis. The expression studies indicated that the EF-1a gene is ripening-regulated, and illustrated changes in transcript accumulation at different fruit developmental stages. The analysis of transcript accumulation in different organs indicated a compelling bias towards expression in the fruit (Yokotani et al., 2009). However, the RIN fragment sequence had a complete similarity of a 100% to the RIN of the Arabidopsis. The increase of the expression of RIN gene induces the ripening process of the fruits (Tieman et al., 2012). In the case of the EXP1 gene of the bell pepper, it had an 82.73% similarity to the Arabidopsis'. In addition, the gene expression of the bell pepper EXP1 was higher than the gene expression in the Arabidopsis fruit. Regarding the RT-PCR, there is a general consensus on using a single control gene for normalization purposes. Accurate normalization of gene-expression levels is an absolute prerequisite for reliable results, especially when the biological significance of subtle gene-expression differences is studied. Still, little attention has been paid to the systematic study of normalization procedures and the impact on the conclusions (Vandesompele et al., 2002). The Real-time PCR differential regulation results illustrated that LeEF-1a gene was significantly down-regulated in bell pepper in comparison to Arabidopsis. Low expression levels of the EF-1a decreased during fruit ripening process

(Pokalsky et al., 1989). Some studies showed high degree of conservation between other previously isolated EF-1a genes which suggested that a fungal EF-1a gene might serve as an appropriate probe for a plant EF-1a cDNA. The study also suggested that EF-1a is a multigene family due to the occurrence of six bands on hybridization with EF-1a probe in southern blot analysis.

The result of the RIN expression levels appeared adjacent in bell pepper and Arabidopsis. However, the level of the expression of RIN in the bell pepper was higher than the Arabidopsis. The expression of RIN increases dramatically during Arabidopsis ripening regardless of the temperature. However, it is influenced by both a developmental and ethylene factors (Bartley and Ishida, 2007). In addition to the expression level of the EXP1 in the bell pepper showed a higher score than Arabidopsis. Over-expression of the LeEXP1 gene resulted in enhanced fruit softening which resulted in an improved texture of the fruit, which may be useful for the tomato processing industry (Kaur et al., 2010). Another study by Fujisawa et al. (2011) found a link between the presence of LeEXP1 and CArG boxes in the promoters of several genes involved in fruit ripening.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Bartley GE, Ishida BK (2007) Ethylene sensitive and insensitive regulation of transcription factor expression during *in vitro* tomato sepal ripening. Exp. Bot. 58(8):2043-2051.

Carrari F, Asis R, Fernie AR (2007). The metabolic shifts underlying tomato fruit development. Plant Biotechnol. 24:45-55 .

Cathala G, Savouret J, Mendez B, West BL, Karin M, Martial JA, Baxter JD (1983). A Method for Isolation of Intact, Translationally Active Ribonucleic Acid. DNA 2:329-335.

De Folter S, Busscher J, Colombo L, Losa A, Angenent GC (2004) Transcript profiling of transcription factor genes during silique development in Arabidopsis. Plant Mol. Biol. 56(3):351-366.

Fujisawa M, Nakano T, Ito Y (2011). Identification of potential target genes for the tomato fruit-ripening regulator RIN by chromatin immunoprecipitation. BMC Plant Boil. 11:26-31.

Kaur P, Samuel DVK, Bansal KC (2010). Fruit-specific over expression of *LeEXP1* gene in tomato alters fruit texture. Plant Biochem. Biotechnol. 19(2):177-183.

Kendrick MD, Chang C (2008) Ethylene signaling: new levels of complexity and regulation. Curr. Opin. Plant Biol. 11:479-485.

Lee S, Eun-Joo C, Young-Hee J, Doil C (2010). Non-climacteric fruit ripening in pepper: increased transcription of EIL-like genes normally regulated by ethylene. Funct. Integr. Genomics 10(1):135-146.

Lelievre, Jean-Marc Lelièvre, Alain Latchè, Brian Jones, Mondher Bouzayen, Jean-Claude Pech (1997). Ethylene and fruit ripening. Physiol. Plant. 101-4:727-739.

Molestina RE, Sinai AP (2005) Host and parasite-derived IKK activities direct distinct temporal phases of NF-kappaB activation and target gene expression following *Toxoplasma gondii* infection. J. Cell Sci. 15-118:5785-96.

Paul V, Pandey R, Srivastava GC (2012). The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene. J. Food Sci. Technol. 49-1:1-21.

Pokalsky AR, Hiatt WR, Ridge N, Rasmussen R, Houck CM, Shewmaker CK (1989). Structure and expression of elongation factor 1 alpha in tomato. Nucleic Acid Res. 17(12):4661-4673.

Powell ALT, Kalamaki MS, Kurien PA, Gurrieri S, Bennett AB (2003). Simultaneous transgenic suppression of LePG and LeExp1 influences fruit texture and juice viscosity in a fresh market tomato variety. J. Agric. Food Chem. 51:7450-7455.

Quinet M, Dubois C, Goffin MC, Chao J, Dielen V, Batoko H, Boutry M, Kinet JM (2006) Characterization of tomato (*Solanum lycopersicum* L.) mutants affected in their fl owering time and in the morphogenesis of their reproductive structure. J. Exp. Bot. 57:1381-1390.

Reed LJ, Muench H (1938). A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493-497.

Sanger F, Nicklen S, Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74-12:5463-7.

Tieman DM, McIntyre L, Blandon-Ubeda A, Bies D, Odabasi A, Rodriguez G, van der Knaap E, Taylor M, Goulet C, Mageroy MH, Snyder D, Colquoun T, Moskowitz H, Sims C, Clark D, Bartoshuk L, Klee H (2012) The chemical interactions underlying tomato flavor preferences. Curr. Biol. 22:1-5.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3(7):RESEARCH0034.

Wang D, Bosland PW (2006). The Genes of Capsicum. Hortic. Sci. 41-5:1169-1187.

Wien HC (1997). The Physiology of Vegetable Crops. Plant Growth Regul. 27- 2:137-138.

Yokotani N, Nakano R, Imanishi S, Nagata, M, Inaba A, Kubo Y (2009). Ripening-associated ethylene biosynthesis in tomato fruit is autocatalytically and developmentally regulated. J. Exp. Bot. 60-12: 3433-3442.