BMC Plant Biology

BioMed Central

Research article

Open Access

Developmental gene regulation during tomato fruit ripening and *in-vitro* sepal morphogenesis

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Published: 07 August 2003

BMC Plant Biology 2003, **3**:4

Received: 16 April 2003

Accepted: 07 August 2003

This article is available from: http://www.biomedcentral.com/1471-2229/3/4

Abstract

Background: Red ripe tomatoes are the result of numerous physiological changes controlled by hormonal and developmental signals, causing maturation or differentiation of various fruit tissues simultaneously. These physiological changes affect visual, textural, flavor, and aroma characteristics, making the fruit more appealing to potential consumers for seed dispersal. Developmental regulation of tomato fruit ripening has, until recently, been lacking in rigorous investigation. We previously indicated the presence of up-regulated transcription factors in ripening tomato fruit by data mining in TIGR Tomato Gene Index. In our in-vitro system, green tomato sepals cultured at 16 to 22°C turn red and swell like ripening tomato fruit while those at 28°C remain green.

Results: Here, we have further examined regulation of putative developmental genes possibly involved in tomato fruit ripening and development. Using molecular biological methods, we have determined the relative abundance of various transcripts of genes during *in vitro* sepal ripening and in tomato fruit pericarp at three stages of development. A number of transcripts show similar expression in fruits to *RIN* and *PSY1*, ripening-associated genes, and others show quite different expression.

Conclusions: Our investigation has resulted in confirmation of some of our previous database mining results and has revealed differences in gene expression that may be important for tomato cultivar variation. We present new and intriguing information on genes that should now be studied in a more focused fashion.

Background

Red ripe (RR) tomatoes, appealing to the eye as well as the palate, are the result of numerous physiological changes controlled by hormonal, environmental, and developmental signals, causing maturation or differentiation of various fruit tissues simultaneously. These physiological changes affect the visual, textural, flavor, and aroma characteristics to make fruit more appealing to potential consumers for dispersal of seed. One hormonal cue, ethylene evolution, active at the onset of the respiratory burst during ripening in this climacteric fruit, has been scrutinized in detail over the years [1,2]. Transgenic tomato plants, expressing antisense genes for ethylene biosynthesis enzymes, show that ethylene is necessary for tomato fruit ripening [3]. However, something must signal ethylene induction before the climacteric ethylene burst. Because 1-aminocyclopropane-1-carboxylic acid synthase (ACCS), an enzyme involved in ethylene biosynthesis, is induced before the onset of ethylene evolution, it seems reasonable to assume that other factors control early

developmental stages of ripening fruit [4,5]. *E8*, a gene of unknown function, is expressed in the *rin* mutant, which does not exhibit the climacteric burst of ethylene evolution [6]. Thus, at least two genes that are not controlled by ethylene are expressed during fruit ripening.

Developmental regulation of tomato fruit ripening has, until recently, been lacking rigorous investigation [1]. Transcription factors are crucial in many aspects of plant and animal development, as well as participating in plant responses to stress and environmental cues [7–9]. Transcriptional regulators have also been implicated as important elements of evolution and natural variation in plants [10,11]. Maize evolved from teosinte due at least in part to a mutation in the regulatory region of *teosinte branched1* (*tb1*), the gene responsible for branch length. A nucleotide polymorphism was found in the regulatory region of this gene, not in the coding region of the predicted protein [11]. Tomato fruit size variation is thought to result from changes in gene regulation involving a quantitative trait locus *fw2.2*, which contains an open reading frame *ORFX* [12]. *ORFX* is more abundant in smaller fruited tomato, suggesting *ORFX* may encode a negative regulator of fruit size [12]. Fruit shape is affected by a new type of regulatory gene, *OVATE*, which was found by chromosome walking to the *OVATE* quantitative trait locus (QTL)[13]. A single mutation in this gene causes a change in tomato fruit shape from round to pear-shaped [13]. One transcription factor, a MADS-Box gene *RIN*, is directly involved in tomato fruit ripening, and another, the tomato homolog of the *Arabidopsis* flower organ-identity gene *AGAMOUS*, *TAG1*, is up-regulated during fruit ripening and in-vitro sepal ripening at cool temperatures [14,15]. With the explosive increase in nucleotide sequence information in EST databases and new technologies such as microarray analysis, it should now be possible to delve more deeply into developmental processes of tomato fruit ripening. Sequence analysis of rice and *Arabidopsis thaliana* genomes indicates the number of putative transcription factors to be >1500 in *Arabidopsis* with similar numbers in rice and possibly tomato [16–19]. In fact, a recent survey of the TIGR tomato EST databases revealed a number of possible ripening-associated transcription factors [20].

A great deal of variation occurs among cultivars in the amount of lycopene accumulation in ripe tomato fruit. One survey of lycopene content reports a range from 0.21 to a very surprising 702.1 μg/g FW [21]. This surprisingly high content might have resulted from removal of inedible portions of fruit [22]. VFNT Cherry (VC), a small-fruited tomato, contains 200 μg/g FW lycopene in the ripe fruit, while Ailsa Craig (AC), a medium-fruited tomato, contains about 70.5 μg/g FW lycopene [23,24]. In our in-vitro system of VC sepal culture, green sepals kept between 16 and 22°C swell and ripen, producing tomato fruit volatiles and accumulating lycopene [23]. Sepals kept at 28°C remain green and do not accumulate lycopene. The carotenoid lycopene forms the red color of ripe tomato fruit and is also an antioxidant believed to help prevent some cancers including prostate cancer. In an effort to determine which of these transcription factors are important in tomato fruit ripening and in-vitro sepal ripening, we have characterized their regulation in ripening fruit of two different cultivars of tomato, VC and AC, and during in-vitro VC sepal culture at 16 and 28°C.

Results and Discussion

Gene Expression During Sepal Morphogenesis

VC tomato sepals cultured *in vitro* at 16 – 22°C switch their developmental program to that of ripening fruit [25]. They swell, decrease in chlorophyll content, evolve ethylene, accumulate lycopene, and give off fruit volatiles [23]. The RT-PCR results in Fig. 1 indicate occurrence of a number of patterns of gene expression during in-vitro cultured sepals at 16°C or 28°C. In this experiment green sepals at day 0 were removed from the plant and cultured at 16 or 28°C. After 2 days in culture, sepals were similar at both temperatures and remained green at 14 days but more swollen at both temperatures. At 24 days, sepals at 16°C started to accumulate lycopene and were yellowish orange, while the sepals at 28°C were still green. *PHYTOENE SYNTHASE 1* (*PSY1*), a carotenoid biosynthesis enzyme, is highly regulated during tomato fruit ripening [26,27] and was used here to indicate fruit ripening in cool temperature-treated sepals. Two *PHYTOENE SYNTHASE* genes are found in tomato, *PSY1* and *PSY2*; it is known that *PSY1* is the primary transcript in ripening tomato fruit [26]. We have also shown in similar experiments that *PSY1* is the primary transcript in ripened sepals (unpublished).

One of the more dramatic and dominant expression patterns to emerge is that of *PSY1*, *TAG1*, *TM4*, *TM6*, (AP2-like) TC85031, TC85646, (YABBY2-like) TC89502, and TC84976. Transcripts for these genes are all induced by day 14 or 24 at 16°C, while little or no change is seen in sepals cultured at 28°C. Of these transcripts, *TAG1* expression seemed the most dramatic with high expression at 16°C and almost none in sepals cultured at 28°C; other transcripts are induced at 16°C, but also have a low basal level of expression at 28°C with a slight increase at 24 days. *PSY1*, TC85031, TC89502, and TC85646 are all at least somewhat induced after 2 days at 16°C. Previously Ishida et al. [15] showed that *TAG1*, the tomato homolog of *AGAMOUS*, a MADS-Box gene involved in *Arabidopsis* flower development, was up-regulated during sepal morphogenesis and in ripening tomato fruit. Additionally, mRNA for POLYGALACTURONASE (PG) increased in cool temperature-treated sepals and ripening fruit. In fact,

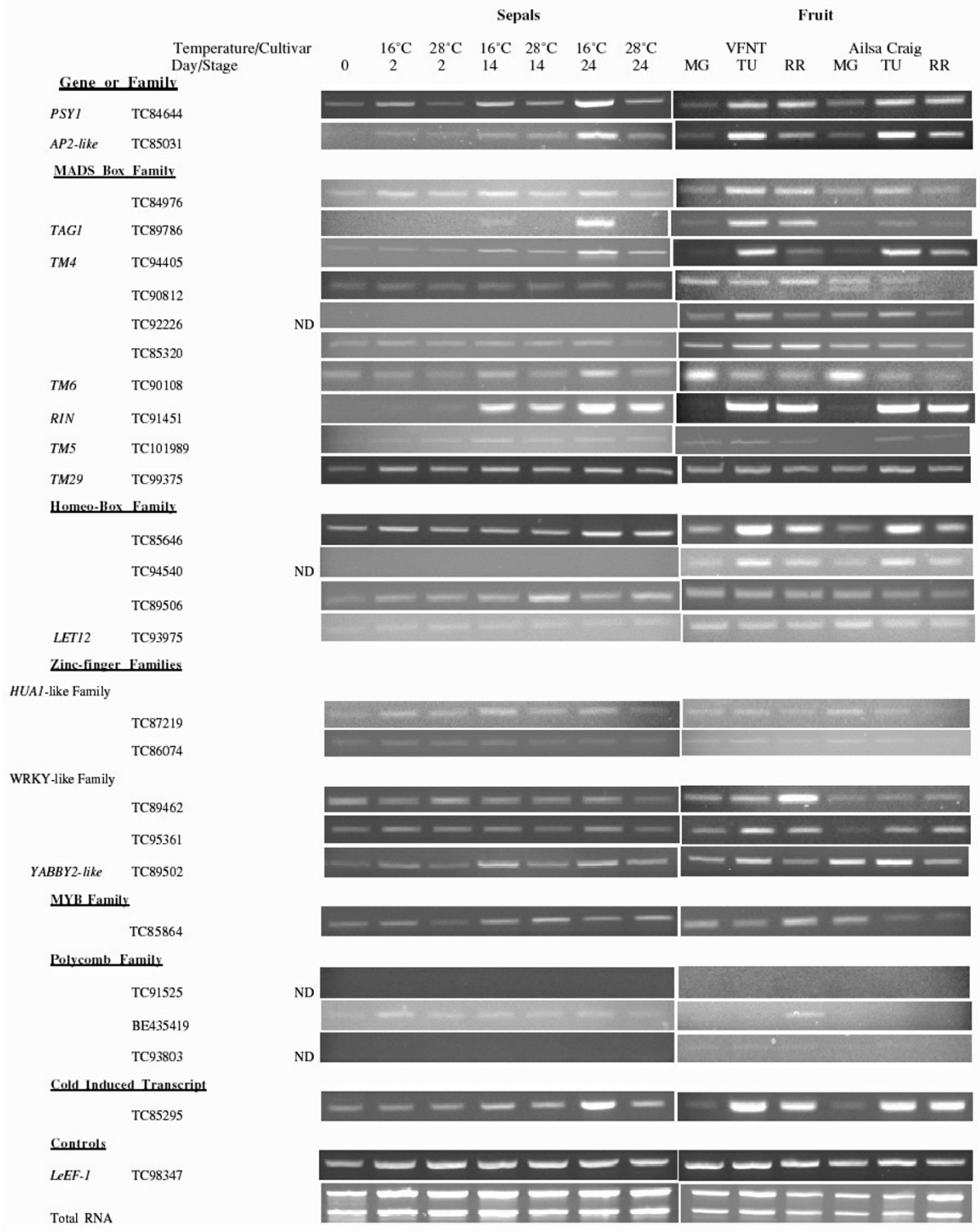

Figure 1

Images of RT-PCR reactions digitally captured using a BioRad gel doc system. The left column of images shows results from the cultured sepal experiment. Growth temperature and number of days in culture are at the top of the column. The right column shows images of results from fruit RT-PCR reactions. Cultivar and stage of the fruit are at the top of the column. Stages of the fruit are abbreviated: MG, mature green stage; TU, turning stage; RR, red ripe stage. *LeEF-1* control at the bottom is the tomato *ELONGATION FACTOR 1-*α..

ectopic expression of *AGAMOUS* in tomato caused sepals on greenhouse-grown plants to swell and lose chlorophyll, and, upon placing these plants at 16 – 18°C, the sepals accumulated lycopene [28,23]. The fact that ectopic expression alone of *AGAMOUS* did not suffice for significant lycopene accumulation indicates a requirement for additional factors for this ripening process to occur. Other MADS-Box genes are induced in tomato flowers of plants subjected to 7°C nights and 17°C days [29]. *TM4*, *TM5*, *TM6*, and *TAG1* are all induced in tomato flowers of cool temperature-treated plants. Flowers of these plants exhibited homeotic and meristic transformations such as petaloid sepals and carpelloid stamens [29]. These abnormalities could be related to the high expression of these genes [29]. We have investigated a number of putative transcription factors by their expression during ripening [20] to gain a more complete understanding of processes contributing to cool temperature sepal morphogenesis. Our RT-PCR results show a number of promising candidates for ripening-related developmental regulators (Fig. 1). *TM4*, *TAG1*, and TC84976 are all induced during cool temperature growth and indeed are also up-regulated during fruit ripening. Our results here confirm previous results showing *TM4* and *TAG1* up-regulation during cool temperature growth [15,29]. *TM6*, however, is induced by cool temperature, but down-regulated during fruit ripening. *TM5* showed only slight induction after 14 days at 16°C and did not show up in the TIGR database staged fruit collections.

A second pattern indicated by these results is that of *RIN*, a recently discovered MADS-Box gene required for ripening of tomato fruit [14], which has an extremely interesting expression pattern during cool temperature culturing. This gene is induced sometime before 14 days of culture at both 16 and 28°C (Fig. 1). While *RIN* is induced to a higher level at 16°C, induction at 28°C still seems to be significant and indicates perhaps the start of a developmental program induced by some other factor than cool temperature. However, this program at 28°C does not include a large lycopene accumulation or *TAG1* or *PG* up-regulation [25,15]. One putative MADS-Box gene, TC92226, is up-regulated at the breaker-turning stage of fruit, but not detected in sepals by RT-PCR at the number of cycles used in this experiment.

Homeobox genes encode transcription factors that contain a 60 amino acid motif, a DNA-binding structure called the homeodomain. Homeobox genes act in a number of developmental processes in plants [30]. *Bell1* (*BEL1*), a homeobox gene in *Arabidopsis*, affects ovule development [31]. The BEL1 protein can interact with other transcription factors, specifically KNOX TALE homeodomain proteins, through conserved protein motifs, and these factors together activate transcription [32]. TC85646 and TC89506 have 66 and 46 % similarity to *BEL1*at the amino acid level, respectively; however, their gene expression patterns differ (Fig. 1). TC85646 is induced at 16°C by 24 days of culture, but TC89506 is induced at 28°C by 14 days, continuing through 24 days. Whether these genes are suppressing or activating transcription of other genes needs further investigation.

Zinc finger motif-containing, nucleic acid-binding proteins affect plant reproductive development [9]. HUA1, a CCCH-type zinc-finger protein in *Arabidopsis*, regulates stamen and carpel identity and is an RNA-binding protein [33]. We previously identified five putative HUA1-like transcripts of tomato by sequence-similarity searches in EST databases [20]. Transcripts TC87219 and TC86074 were investigated in these experiments, and only one, TC87219, was slightly induced in the 16°C-cultured sepals. WRKY zinc-finger transcription factors, so named for the amino acid sequence WRKYGQK contained in the N-terminal region of their zinc finger motif, have been implicated primarily in defense responses of plants [9]. However, one WRKY transcription factor in *Arabidopsis*, *TRANSPARENT TESTA GLABRA 2* (*TTG2*), seems to be involved in trichome development [34,35]. In our sepal experiment, only TC95361 was slightly induced at 16°C after 24 days of culture.

TC89502 is most similar to YABBY2, an *Arabidopsis* zinc-finger protein that belongs to a family of transcription factor proteins that contain a zinc finger and a helix-loop-helix domain, the YABBY domain, and specify abaxial cell fate [36]. Expression of this transcript was induced in cool temperature growth with no corresponding increase at 28°C.

MYB genes contain DNA-binding, amino acid motifs similar to those found in *c-MYB*, the animal protooncogenic cellular counter part to *v-MYB*, the oncogenic component of avian myoblastoma virus [37]. In *Arabidopsis* more than 92 MYB genes have been described [38]. In plants, *MYB* genes regulate secondary metabolism, cell morphology, and signal transduction in plant growth and pathogen defense [39]. Fourteen *myb*-related cDNAs have been cloned and characterized from tomato by Lin et al. [39]. We previously identified more than 136 putative MYB transcripts in the TIGR tomato EST database [20]. We investigated one of these, TC85864, because of its expression in ripening tomato fruit. Our RT-PCR results in the sepal experiment indicate induction at 28°C after 14 days of culture and no induction at 16°C. The deduced protein for this transcript contains two MYB domains that closely resemble, 93 % similarity and 80 % identity, those of an *Arabidopsis* R2R3-MYB gene CAA74604. Further research is needed to determine the function of this transcription factor. However, in gene-disruption experiments,

Meissner et al. [38] found no obvious phenotypes in 32 homozygous insertion lines of 26 genes. Even more intensive greenhouse and plate-based screening failed to find a phenotype for most of these plants, possibly indicating redundancy [38].

Three putative polycomb genes identified in fruit collections of the TIGR database [20] were also investigated without much result in our sepal experiment (Fig. 1). Two transcripts were undetected at this number of PCR cycles, and the third showed little regulation. Polycomb proteins are thought to function by forming complexes with additional polycomb proteins to remodel chromatin and repress gene transcription [40,41].

One AP2 domain-containing gene previously identified as TC85031 was investigated with interesting results. The ABC model of floral development involves a system where class A gene expression specifies formation of sepals and in combination with a class B gene specifies petal formation. B and C gene expression together specifies stamen formation, and C expression alone specifies carpel identity [42,43]. In *Arabidopsis*, *APETALA2* (*AP2*), a B function gene according to the ABC model, is antagonistic to *AG* and negatively regulates *AG* expression in sepals and petals [44]. Our RT-PCR results show that TC85031 is highly induced at 16°C sometime before 24 days of growth. Expression of this *AP2*-like gene appears to mimic that of *TAG1*, and EST profiling indicates this gene is highly expressed in ripening tomato fruit [20]. *AP2* in *Arabidopsis* does not follow the same expression pattern of other floral organ identity genes as it is ubiquitously expressed in the floral organs of *Arabidopsis* [45]. This *AP2*-like gene may not have the same function as the *Arabidopsis* gene. The possible ortholog of *AP2* in petunia *PhAp2A* does complement the *Arabidopsis ap2-1* mutant, but expression of *Arabidopsis AP2* in petunia did not result in the expected phenotype [46,47]. The TIGR tomato database lists another *AP2*-like transcript TC100241, which is not highly expressed in the tomato fruit, but is expressed in the flower. Perhaps TC100241 is the *Arabidopsis* flower homolog, while TC85031 may have a different function in the fruit.

TC85295 is a transcript that codes for a protein very similar to WCOR413, a low temperature-induced protein in wheat (*Triticum aestivum*) [48]. This transcript in tomato is also induced in our system after 14 days of cool temperature growth. The function of this protein is unknown, but sequence analysis indicates several trans-membrane helices, suggesting that it stabilizes the plasma membrane during cold stress [48].

Over all, we have shown that cool temperature sepal morphogenesis is complex with induced gene expression of MADS-Box genes, *TAG1*, *TM4*, and *TM6*, and possible developmental regulation of *RIN*, as well as expression of other genes, TC85031, TC84976, TC85646, and TC85295 whose specific functions are unknown. Two genes of unknown regulatory function, TC89506 and TC85864, were up-regulated at 28°C. Further experiments are required to determine specific functions of these putative regulators.

RT-PCR of Genes Expressed During Fruit Development of Two Tomato Cultivars

Tomato fruit quality can be affected by many factors, genetic and environmental, pre- and post-harvest. Flavor and aroma volatiles differ from cultivar to cultivar and during ripening [49,50]. Environmental and cultural factors can also affect the flavor of tomatoes [51]. Harvesting, handling, and post-harvest treatment may also affect fruit quality [52]. A number of quantitative trait loci (QTLs) and genes are implicated in fruit size and shape of tomato [12,13]. Some of these loci and genes, i.e., the QTL *fw2.2*, which yielded *ORFX*, and the QTL *ovate* which yielded *OVATE*, appear to be novel negative regulators of fruit size and shape [12,13]. We are particularly interested in regulation of tomato ripening, a climacteric fruit. While ethylene evolution in the climacteric phase has been rigorously studied and manipulated in tomato, very little has been done in the investigation of developmental regulation of tomato fruit ripening [1]. As mentioned earlier, transcription factors are involved in many aspects of plant and animal development. Differences in gene regulation may account for physiological and morphological differences that developed during the evolution of organisms [10]. A change in the 5-prime untranslated sequence of a homeobox-containing gene *LeT6* caused over expression of the gene changing the phenotype of previously unpinnate leaves to pinnate [53]. This shows that simple differences in transcription factor abundance could be responsible for morphological and physiological variance in plants. At least one gene, a MADS-Box gene *RIN*, is required for developmental regulation of ripening, and another, *TAG1*, is implicated in fruit development [14,15,28]. Our results agree with previous *RIN* and *TAG1* results and suggest other developmental regulators may be involved in fruit development and/or ripening. Previously, we described gene expression profiles of a number of putative developmental regulators [20] at different stages in fruit development in the TIGR Tomato EST databases. Here we present further evidence that some of these genes may be involved in tomato fruit development.

The MADS Box gene family in *Arabidopsis* consists of more than 80 members, indicating the importance of these transcription factors in plants [18]. *RIN* and *TAG1* show similar regulation to that previously published. *RIN* has quite high expression in both cultivars at the turning and

red ripe stages. The similarity in expression in the two cultivars suggests that this gene is critical to the ripening process. *TAG1* expression differs considerably between VC and AC, and in fact TC84976, TC90812, and TC85320 all show differences in transcript abundance. Over-expression of *TAG1* in transgenic tomato plants caused sepals to become pericarpic [28]. This effect could reflect the fruit ripening association of *TAG1* in tomato or show that large amounts of TAG1 may partially mimic the action of another MADS-Box gene [1]. *TM4* was up-regulated at the turning stage and declined at the red ripe stage, showing little difference between cultivars. *TM4*, as mentioned earlier, is expressed early in flower development and up-regulated during cool temperature growth [29,54]. Seymour et al. [55] proposed *TM4 (TDR4)* involvement in fruit texture because of a lack of ripening-related induction of this gene in the *Cnr* mutant and similarity of the protein sequence to the *Arabidopsis* gene *FRUITFUL*. The *Cnr* mutant differs in fruit texture from wild type because of increased cell separation. These investigators have constructed transgenic plants to determine the relationship between *TM4* and fruit texture [55]. According to our results, *TM5* does not seem to be highly expressed in fruit, although it does show some induction at the turning stage. *TM6*, another tomato MADS Box gene induced by cool temperatures, has high expression during the mature green stage, but not in turning or red ripe stages. The absence of a difference in expression between the two cultivars might indicate that this transcript has a critical role just prior to the onset of the ripening process. In our previous paper we noted that *TM6* showed higher expression in the mature green stage than in the immature green, breaker, or red ripe stages according to the TIGR database [20]. *TM6* belongs to the *Antirrhinum DEF*/*Arabidopsis AP3* family of MADS Box genes that perform the B function in floral identity. Its expression pattern in tomato flowers, however, differs from that of *AP3* or *DEF*. *TM6* is expressed in the three inner whorls unlike the petal and anther expression of *AP3* and *DEF* [56]. *TM29* belongs to the *SEPALLATA* family of MADS Box genes that are involved in floral organ identity [57]. This gene is expressed in primordia of all four floral organs and in inflorescence and vegetative meristems [57]. Transgenic plants expressing the antisense gene develop ectopic shoots that emerge from parthenocarpic fruit, suggesting that *TM29* is a negative regulator of parthenocarpic fruit formation [57]. Our results suggest induction of *TM29* at cool temperatures and at the turning stage in both cultivar, as well as other functions of this gene. TC92226, which is most similar to the *Petunia AGAMOUS* gene *PAGL1* (GenBank Accession L33973), is up-regulated at the turning stage in both cultivars, but is not detected in sepals at the level of PCR we used in this experiment. This result may indicate incompleteness of the cool-temperature-ripening phenomenon of sepals cultured at 16°C. TC85320 and TC90812 are differentially expressed in the two cultivars and the TIGR database profile [20], but show little change in expression during cool temperature, in-vitro culture. A very interesting transcript, TC90812, shows a single band in VFNT that is up-regulated in RR, while in AC the PCR product appears as two bands that decrease in intensity in TU and RR fruits. Whether these two bands in AC fruit represent a gene family or splicing variants is unknown at this time, but, since the regulation of the two bands are similar, splicing could be a factor. TC90812 is very similar to MADS1 from pepper in primary amino acid sequence, but the pepper gene is highly expressed in flowers at fruit set and not in young fruit [58]. TC85320 is quite similar to a pepper MADS Box gene, *MADS6*, which has the same expression pattern as *MADS1* [58]. These two MADS Box genes may not be critical for ripening or fruit formation but might provide a source for fruit architecture or physiological variation.

Putative homeobox genes, TC85646, TC94540, are induced at turning stage with higher levels of expression of TC85646 in VC. Different levels of expression of the same gene can have phenotypic effects [10]. TC94540 was up-regulated during turning stage, but not detected in sepals during in-vitro ripening, thus suggesting the absence of some components of regulation.

Of the zinc-finger family of genes we investigated, only TC89462, TC95361, and TC89502 showed differential gene expression between VC and AC. Expression of TC89462 of the WRKY family of zinc-finger proteins increased in the red ripe stage of VC fruit, while in AC it remained low. In the cultivar TA496 used in TIGR databases, TC89462 expression was highest in the breaker stage. TC95361, also a WRKY type zinc-finger transcription factor, increased in the last two stages of ripening (TU and RR) in both cultivars, but to a greater extent in VC.

The only MYB-type transcription factor investigated did show differential expression in the two cultivars and TIGR database. TC85864 was more abundant in red ripe fruit of VC than at other stages and in was more abundant at the mature green stage of AC than at other stages. The TIGR database indicates higher expression of this gene in breaker stage fruit [20].

Of the three putative polycomb genes investigated in this experiment, only BE435419 expression was detectable in fruit tissue (Fig. 1). This gene was detected only in VC red ripe fruit and not in any fruit stages of AC examined. Again, polycomb genes are thought to affect gene expression through remodeling of chromatin [40,41].

Table 1: Oligonucleotide Sequences. Sequences of oligonucleotides used in these experiments not published elsewhere. Primer sets are given for each transcript with the upper sequence being the forward primer and the lower being the reverse primer.

#	Transcript	5' Sequence	3' Name
1	TC84976	GGCAAGTTGCGTTCTGTAAACGG	TC84976F1
2		TCGACGAATACGACGATAATCAC	TC84976R1
3	TC90812	TTTGCAGCACTTCAAGCATGGTG	TC90812F1
4		TTACCCAAAATGTGGAGAAGAAG	TC90812R1
5	TC92226	GAATTACTTGTCGATGAGTCGTC	TC922F1
6		AAATTCTTGAGGTCTCTAGGGC	TC922R1
7	TC85320	CATGAGATCTCTGTGCTTTGCG	TC853F1
8		CTTTCTCCCTTTCGTTAACCTGC	TC853R1
9	*TM6* (TC90108)	TCAGTATCAGAGTGCACTTGGAG	TM6F1
10		AACTGATATCTTCAGGAGAGACG	TM6R1
11	*RIN* (TC91451)	GGCAAGCTTTATGAATTTTGCAG	TC91451F1
12		GTAGCATCATGTGTTGATGGTGC	TC91451R1
13	*TM5* (TC101989)	GCATGCTAAAGACGTTGGAGAGG	TM5F1
14		TGATGATAGGAAAACCATTGAGC	TM5R1
15	*TM29* (TC99375)	AAAGTGCAGCTATGGAACATTGG	TM29F1
16		ATGCAAAGCTGAAGATAAAGGAC	TM29R1
17	TC85646	AAAGAGACGAATATAAGTGCTCC	TC85646F1
18		AGTTGGAAATCGCTTATTCCCAC	TC85646R1
19	TC94540	ATGGCTAAACATGATGGTGCAGC	TC94540F1
20		AAACTGTAAATCTCTCAATCCTC	TC94540R1
21	TC89506	CGGTTCGATGCCGGCTCAGACG	TC89506F1
22		TAATCTTGTAATAGTTGAGTCGC	TC89506R1
23	*LET12* (TC93975)	AGCACCAGGTGAAGGTACAGGAG	TC93975F1
24		TTACTGCTTGATTTCACCTGCAC	TC93975R1
25	TC87219	TCTTACCCGGTCCTTATGTACCT	TC87219F1
26		TCTAAGGGGCATTCGGTATATCAG	TC87219R1
27	TC86074	ATCTGTCTATGGGATGTCACAGC	TC86074F1
28		AGGAAGTATTAGGTTAACTGTACC	TC86074R1
29	TC89462	TAGGACAGTTAGAGAACCTAGAG	TC89462F1
30		AGTTTCAGCAAAGCAATGACTCC	TC89462R1
31	TC95361	GTCAGAAGAAACAGAAAGAGCCG	TC95361F1
32		TTGTAGTCATGTTTTAGCACCGC	TC95361R1
33	*YABBY2*-like(TC89502)	CTTCTGCAGCACAATTCTTGCGG	TC89502F1
34		ATAGAGACCAATTGTTTTCTGAGG	TC89502R1
35	TC85864	TCTGTTATGAATCCGGGTAGTCC	TC85864F1
36		GATTACTCAATCTTGCTGATGCC	TC85864R1
37	TC91525	ATTACATGGAGCTAGATATTCCG	TC91525F1
38		ATTTTGTAACAGGTTTCTTCAGG	TC91525R1
39	BE435419	CTATGGGACATGAAATACAAGGG	BE435F1
40		ATGGCTTTCTTTTGGTTTATTGC	BE435R1
41	TC93803	GGTTGCATAGATGTTGCATTCAG	TC93803F1
42		CCCATGAGCTTCATAAGCCTGAG	TC93803R1
43	TC85295	ATTGGAGAGAAGCATCAGTAGGC	TC85295F1
44		AAGTGGAGAATCAATGCCCAGAC	TC85295R1
45	*LeEF-1* (TC98347)	TGGCCCTACTGGTTTGACAACTG	alphaF1
46		CACAGTTCACTTCCCCTTCTTCTG	alphaR1

Sequences of oligonucleotides used in these experiments not published elsewhere. Primer sets are given for each transcript with the upper sequence being the forward primer and the lower being the reverse primer.

The *AP2*-like gene TC85031 revealed a similar pattern of expression in fruit as that of *TM4*, a MADS-Box gene. TC85031 was induced in turning fruits and was similarly expressed in VC and AC. Does this indicate its critical nature in some aspect of ripening?

We chose to investigate TC85295 because of its up-regulation during ripening, according to TIGR tomato fruit EST collections in which the relative abundance of this transcript increased from 0.2 ESTs per 1000 in mature green collection to 0.5 in breaker, and 1.0 in red ripe fruit. Our

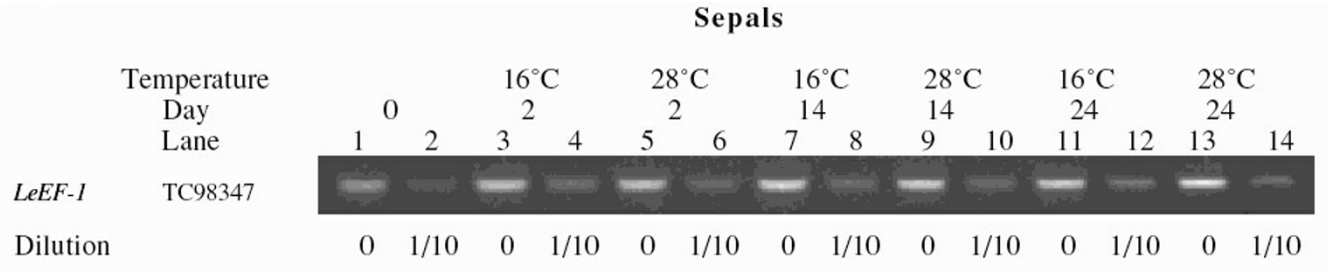

Figure 2
Control PCR of *LeEF-1* indicating relative abundance of RNAs. Odd numbered lanes were performed exactly as in Figure 1 (sepals), while the even numbered lanes were performed with a 1 in 10 dilution of the reverse transcription reaction.

results indicate high expression in turning and in red ripe fruit, but showed no difference between the two cultivars. These results might imply the importance of this gene to the ripening process. We believe this gene should be examined in further experiments.

Conclusion

Is cool temperature sepal morphogenesis the same process as tomato fruit ripening? While the expression of some genes such as *POLYGALACTURONASE*, *PHYTOENE SYNTHASE*, *TM4*, and *RIN* are similar, expression of other genes in ripening fruit and sepal morphogenesis differs. TC92226 and TC94540 are both induced in fruit, but their induction is not detectable during sepal ripening. On the other hand, *TM6* is induced in cool temperature-treated sepals, but not during fruit ripening. A number of other differences are also seen in gene expression in the two cultivars. We still must determine which of these putative regulators are critical to tomato fruit ripening and how they affect ripening and fruit development in general. We have revealed a number of very interesting genes to investigate further and have confirmed many of the results of our previous EST database mining. Hopefully, we have provided more interesting targets in the fruit development game.

Methods

Sepal cultures

Sepals from small green fruit 3- to 10-mm diameter were harvested from greenhouse-grown plants (Lycopersicon esculentum cv. VFNT Cherry). Sepals were disinfested, separated at the base, and cultured on a solidified medium as previously described [15] at 16 or 28°C. Samples of sepals cultured at both temperatures were subsequently harvested at various times, i.e., 0, 2, 14, and 24 days, and frozen immediately in liquid nitrogen.

Tomato Fruit

Mature green (MG), breaker to turning (TU), and red ripe fruit (RR) were harvested from greenhouse-grown VFNT Cherry LA1221 and Ailsa Craig varieties of tomato, both obtainable from the C. M. Rick Tomato Genetics Resource Center at the University of California, Davis [59]. Only pericarp and skin tissues were used for RNA extraction.

RNA Extraction and RT-PCR

Total RNA was extracted and purified and used in RT-PCR reactions according to Bartley and Ishida [20] with the following modifications: An initial denaturation at 94°C for 2 min and then 24 cycles of denaturation at 92°C for 30 s, annealing at 55°C for 3 min, and extension at 72°C for 7 min. A final extension program was performed at 72°C for 7 min. Oligonucleotide sequences for the AP2-like transcript (TC85031), TAG1 (TC89786), and TM4 (TC94405) can be found in Bartley and Ishida [20], and the sequence for the PSY primers SPS3 and PSY can be found in Bartley and Scolnik [26]. Oligonucleotides for other transcripts are shown in Table 1. The MADS-Box primers were compared to various available nucleotide sequences in the TIGR databases and GenBank to show lack of cross gene amplification among different MADS-Box genes at the conditions used in the PCR. Sequence comparison of primers designed on VFNT sequences such as: TM4, TM5, and TM6 to sequence in the TIGR database showed no differences among the cultivars and TIGR sequences. In fact very few differences between cultivars were found in overall sequence of these transcripts, approximately 2 to 3 nucleotides per gene except for TM4. A stretch of poor sequence in the TM4 entry has an additional 18 nucleotides alternately spaced with true sequence. PCR was performed on equivalent amounts of non–reverse transcribed total RNA of some of the transcripts to show lack of amplification of genomic DNA.

As a control, the tomato *ELONGATION FACTOR 1-α* gene, (*LeEF-1*, TC98347 and GenBank accession X14449)[60], was used because of its high and stable expression in mature tomato fruit [61,62]. However, the original paper involving cloning of this gene showed

some variability in expression even in mature fruit [60]. We therefore examined the expression profile of this gene in the TIGR database. We found fairly stable high expression in immature green (2.8 ESTs per 1000), mature green (3.4), and breaker stage fruit (2.8) with a slight decline in red ripe fruit (1.8). Leaf expression of TC98347, using the collection of leaf ESTs from the *Pseudomonas* susceptible library T1079, was less, 0.8 ESTs per 1000. This might account for the less intense band in 0 day sepals in the experiment if, sepals are indeed changing from leaf-like organs into fruit. However, this library was made from *Pseudomonas*-treated leaves. No normal leaf library with suitable numbers of ESTs was available for use at the time of writing of this paper. *LeEF-1* belongs to a gene family in tomato. We compared the sequences of the four most similar members of the family, TC98347, TC98345, TC98346, and TC98349 for primer design. The upstream primer, alphaF1, might possibly amplify other members because four nucleotide mismatches at most occur. The down stream primer alphaR1 should only amplify TC98347 as nucleotide triplets are missing and other mismatches in TC98346 and TC98345 occur, and six mismatched bases occur in TC98349. In the event that TC98349 was amplified, TIGR databases indicate expression at 0.2 ESTs per 1000 in mature green and breaker stages compared to 3.4 and 2.8, respectively, for TC98347 (our control). To show relative abundance differences in RNA, we made 10 fold dilutions of the reverse transcription reactions used for the sepal experiment and performed PCR using the same conditions and *LeEF-1* primers (Fig 2.). Loading of 2 μg of total RNA of each sepal sample or fruit stage in each lane (bottom of figure 1) was used as an additional control to compare overall amounts.

Abbreviations

Lycopersicon esculentum cv Ailsa Craig, AC; *Lycopersicon esculentum* cv VFNT Cherry, VC; expressed sequence tags, EST; reverse transcription-polymerase chain reaction, RT-PCR; The Institute for Genomic Research, TIGR; *Lycopersicon esculentum ELONGATION FACTOR 1-α*, LeEF-1; mature green stage, MG; breaker stage, BR; turning stage, TU; red ripe stage; RR

Authors' contributions

GB conceived of the study together with BI, carried out the gene expression analysis and drafted the manuscript. BI also edited the manuscript. All authors read and approved the manuscript.

Nomenclature

Tentative consensus sequences (TCs) are cDNA sequences assembled from overlapping ESTs common to that transcript. TC numbers contained in the TIGR Tomato Gene Index are continually changing as new sequences are added and more information developed. We have used the same numbers as we reported previously [20]; however, the numbers have changed in the database [63]. A history of TC numbers is included with each TC, and searches using previous numbers will still locate correct TCs.

Acknowledgements

The authors would like to thank Drs. Dominic Wong and Xiaohua He for critical reading of the manuscript.

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