Fruit-specific suppression of the ethylene receptor LeETR4 results in early-ripening tomato fruit

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Summary
Tomato is an economically important crop and a significant dietary source of important phytochemicals, such as carotenoids and flavonoids. Although it has been known for many years that the plant hormone ethylene is essential for the ripening of climacteric fruits, its role in fruit growth and maturation is much less well understood. In this study, data are presented which indicate that fruit-specific suppression of the ethylene receptor LeETR4 causes early ripening, whereas fruit size, yield and flavour-related chemical composition are largely unchanged. Early fruit ripening is a highly desirable and valuable trait, and the approach demonstrated here should be applicable to any fruit species requiring ethylene to ripen. These results demonstrate that ethylene receptors probably act as biological clocks regulating the onset of tomato fruit ripening.

Keywords: climacteric ripening, fruit development, hormone, protein degradation.

Introduction
Tomato is the most economically important vegetable crop grown in the USA. World-wide, ~70 million metric tonnes are produced each year. Short growing seasons in higher latitudes often reduce the number of cultivars that a grower can use in outdoor cultivation. One mechanism to circumvent climate-related limitations is to grow early maturing varieties. These varieties also offer a distinct advantage to growers, because the first fruit to market in a season can fetch a higher price. As our knowledge of the molecular control of fruit ripening expands, biotechnology could provide useful tools for the generation of early-ripening cultivars.

Ethylene is a phytohormone that controls or influences many aspects of plant growth and development (Abeles et al., 1992). Many of the developmental processes controlled by ethylene, such as senescence, organ abscission and fruit ripening, are critically important to agriculture. For example, climacteric fruits, such as tomato, banana and apple, require an increase in ethylene biosynthesis at maturity in order to ripen. Transgenic plants that are reduced in either the synthesis or perception of ethylene exhibit delayed ripening (Hamilton et al., 1990; Klee et al., 1991; Oeller et al., 1991; Wilkinson et al., 1995). Conversely, it should be possible to speed up fruit maturation by increasing the synthesis or perception of ethylene. Indeed, it has been known for many years that ethylene application to immature tomato fruits causes an earlier onset of ripening (Yang, 1987). Because of the pleiotropic negative effects of excessive ethylene exposure on plant growth, simply increasing ethylene synthesis is not practical. Receptors function as negative regulators of the ethylene response pathway (Hua and Meyerowitz, 1998; Tieman et al., 2000). In the absence of the hormone, the receptor actively suppresses ethylene responses and ethylene binding removes this suppression. In practical terms, this means that ethylene sensitivity is inversely correlated with receptor levels; depletion of receptors effectively increases ethylene sensitivity because there are fewer receptors to inactivate. Recent work on the tomato ethylene receptor family has demonstrated that receptor levels during fruit development determine the timing of ripening (Kevany et al., 2007). Protein levels are at their highest during immature fruit development and decrease significantly at the onset of ripening, facilitating ethylene-mediated ripening processes. Ethylene treatment of immature fruits causes receptor degradation and earlier fruit ripening (Kevany et al., 2007). Although much effort has been focused on delayed ripening, particularly as it relates to ethylene, opportunities to hasten fruit development have been relatively neglected. Therefore, in this study, a tissue-specific approach has been developed to enhance ethylene responses in tomato
fruits by depletion of an ethylene receptor. Transgenic fruits matured earlier than controls, with no deleterious effects on yield, fruit size or quality. This technology should be applicable to any fruit whose ripening is dependent on ethylene.

Results

LeETR4 RNAi transgenic plants produce early-ripening fruit

The antisense-mediated decrease in either of two tomato ethylene receptors, LeETR4 or LeETR6, results in premature ripening (Tieman et al., 2000; Kevany et al., 2007). However, these plants are severely affected with regard to many aspects of growth, and it is not clear whether the early ripening is a direct effect of transgene expression. It was postulated that fruit-specific suppression of the LeETR4 receptor would result in early ripening without undesirable ethylene-related effects. In order to test the hypothesis, a strategy was developed to specifically reduce LeETR4 expression throughout fruit development. To achieve this goal, a construct was generated consisting of an LeETR4 RNAi inverted repeat sequence fused to the transcriptional promoter of Tfm7, a gene that is expressed specifically in immature fruits (Santino et al., 1997). Transgenic plants were generated by Agrobacterium-mediated transformation into the tomato cultivar Flora-Dade, a large-fruited variety developed for Florida fresh tomato production. Transgenic lines that showed no vegetative expression of the silencing construct were identified and assayed in a glasshouse for time from anthesis to breaker stage (the first visible signs of ripening). Six T0 lines with significantly earlier ripening were identified in initial screening. Of these, three T1 lines that exhibited both a decrease in time from anthesis to breaker stage and a reduction in LeETR4 transcript throughout fruit development were chosen for further characterization. Transgenic lines began ripening between 5 and 7 days earlier than controls (Figure 1). No significant effects were observed on the time from breaker stage to fully ripe, nor were there differences in the colour of ripe fruits (data not shown). As expected, LeETR4 transcript levels were reduced by as much as 73% in immature fruit and 95% in ripening fruit (Figure 2a). Although Tfm7 expression has been reported to be specific to immature fruit, the RNAi effect persisted into ripening fruit (Figure 2a). This gene-specific decrease in expression was not seen in non-target tissues, such as leaves (Figure 2a). Expression analysis of the other family members showed no decrease in transcript levels in transgenic plants (data not shown). Protein blot analysis confirmed that LeETR4 protein levels were correspondingly decreased at all stages of development.

Figure 1  Fruit-specific LeETR4 RNAi transgenic lines produce early-ripening fruit. (a) The number of days from anthesis to breaker stage was measured by tagging open flowers on T1 plants and recording the number of days until the first signs of color development. Values represent the means of at least 25 fruits per line. (b) Fruit from transgenic lines are similar in shape and colour to control fruit.

Figure 2  Suppression of LeETR4 is fruit specific. (a) Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of LeETR4 transcript levels in leaf tissue and throughout fruit development in control and RNAi transgenic lines. Each value is the average of three biological replicates. (b) Protein blot analysis of ETR4 protein levels in control and transgenic lines. IMG, immature green; Breaker, first external colour change; Turning, ~30% red colour. Each lane contained 20 μg of protein prepared as described in ‘Experimental procedures’.
Ethylene receptors and early fruit ripening

The performance of the transgenic plants was also assessed in the field using standard commercial practices. Harvests were conducted on a weekly basis in which all fruit that had begun to show external colour development were picked and staged for their degree of ripeness. Transgenic plants showed more ripening fruit in the first harvest than control plants, and transgenic lines were stripped of between 77% and 86% of their fruit within the first two harvests (Figure 3).

Transgenic fruits are indistinguishable from wild-type fruits with regard to horticultural traits

Early maturing varieties of fruits frequently lack the quality of slower ripening varieties. To achieve maximum value, it would be advantageous if early-ripening fruits maintained the size, yield and flavour qualities of later ripening cultivars. Altering the time to maturation could potentially have an impact on the synthesis of sugars, acids and volatile compounds associated with flavour. In addition, fruit size and yield could potentially be negatively affected by earlier maturation and harvest. To address these questions, tests were performed to assess the quality and yield attributes.

Analyses of yield and fruit size were conducted in both glasshouse- and field-grown plants. To assess the yield, fruits were harvested at the onset of ripening and individually weighed. The average fruit size for two of the transgenic lines was slightly smaller than that of control fruit, but this difference was not statistically significant (Tables 1 and 2). The total yield and the number of fruit per plant were not affected by the presence of the transgene (Table 1).

Tomato flavour is the sum of a complex interaction between taste and olfaction. Sugars and organic acids stimulate taste receptors, whereas a number of volatile organic compounds (VOCs) stimulate olfactory receptors (Buttery, 1993; Buttery and Ling, 1993). In order to assess the potential effects on flavour, total soluble solids, citric acid, malic acid and the 16 most important VOCs were measured (Table 1; see also Tables S1 and S2 in ‘Supplementary material’). Similar results were obtained for both field- and glasshouse-grown samples. Although there were a few statistically significant differences in citric acid and some VOCs, they were not repeated from season to season. All of these differences were well within the range of our observed environmental variations (see, for example, http://tomet.bti.cornell.edu/). Therefore, it was concluded that transgenic and control fruits are essentially equivalent.

Discussion

Although the essential role of ethylene in mediating climacteric fruit ripening has been known for many years, its role during immature fruit development is only now being elucidated.

Table 1 Fruit weight, yield, soluble solids (°Brix), citric acid and malic acid from field-grown T1 plants

<table>
<thead>
<tr>
<th>Line</th>
<th>Weight (g)</th>
<th>Yield per plant (kg)</th>
<th>Soluble solids °Brix</th>
<th>Citric acid (mg/g fresh weight)</th>
<th>Malic acid (mg/g fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>135.5 ± 3.1</td>
<td>218</td>
<td>4.51 ± 0.4</td>
<td>9.1 ± 0.2</td>
<td>2.76 ± 0.04</td>
</tr>
<tr>
<td>RNAI-1</td>
<td>130.7 ± 3.9</td>
<td>185</td>
<td>4.53 ± 0.6</td>
<td>3.9 ± 0.1</td>
<td>2.63 ± 0.06</td>
</tr>
<tr>
<td>RNAI-2</td>
<td>131.4 ± 3.1</td>
<td>262</td>
<td>4.49 ± 0.4</td>
<td>3.8 ± 0.1</td>
<td>2.69 ± 0.14</td>
</tr>
<tr>
<td>RNAI-3</td>
<td>142.9 ± 4.1</td>
<td>137</td>
<td>4.56 ± 0.3</td>
<td>4.0 ± 0.1</td>
<td>2.57 ± 0.16</td>
</tr>
</tbody>
</table>

Mean ± standard error; n, number of fruit examined, or plants in the case of the yield study.

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Previous work has shown that ethylene treatment of immature tomatoes or bananas quantitatively reduces the time to the onset of ripening (Burg and Burg, 1962; Lyons and Pratt, 1964; McGlasson et al., 1975; Yang, 1987), but the mechanism by which fruits measure cumulative ethylene exposure has remained unknown until now. A potential mechanism has been identified by which plants use ethylene receptor levels to measure cumulative ethylene exposure (Kevany et al., 2007). Ethylene binding triggers ubiquitin-dependent receptor protein degradation. If receptors are not replaced after ethylene-mediated degradation, as occurs in immature fruit (Kevany et al., 2007), the fruit will become more sensitive to subsequent ethylene exposure and ripen earlier. The precise, fruit-specific targeting of *LeETR4*, described here, validates the model. These results define a critical role for *LeETR4* in mediating ethylene responses. The special importance of this and another subfamily 2 receptor, *LeETR6*, to ethylene responses (Kevany et al., 2007) contrasts markedly with what is known about ethylene perception in *Arabidopsis*. In *Arabidopsis*, no single loss-of-function receptor mutant has an obvious effect on ethylene responses, and the subfamily 1 receptors seem to have a more important role in ethylene signal transduction (Wang et al., 2003). These results, taken together with the observations described in Kevany et al. (2007), more broadly demonstrate that plants have the capacity to regulate hormone responses by modulating receptor levels.

It is generally accepted that ethylene receptors act as negative regulators of ethylene responses; reduced levels of receptors increase signal output, whereas higher levels decrease signal output (Hua and Meyerowitz, 1998; Tieman et al., 2000). The results presented here are consistent with this model. In this context, it seems paradoxical that the transcription of receptor RNAs increases substantially at the onset of ripening. Ripening is entirely dependent on ethylene, and increased transcription of receptors would be expected to increase receptor protein content and suppress ethylene responses. Although it has been shown that the modulation of *LeETR4* transcript levels has a significant impact on immature fruit development, this depletion of RNA does not influence post-breaker stage ripening (data not shown).

Recently, it has been shown that ethylene binding results in rapid receptor protein degradation (Kevany et al., 2007). Despite a large increase in receptor RNA levels, the overall receptor content decreases significantly during ripening. These results suggest that the large increase in ethylene synthesis associated with climacteric fruit ripening is more than sufficient to deplete the receptor pool to a point at which ripening can proceed.

The tissue-specific modulation of ethylene sensitivity in transgenic plants has resulted in fruits with altered ripening without an agronomic penalty. A similar approach for the precise separation of an advantageous trait from pleiotropic negative effects was employed by Davuluri et al. (2005), who used fruit-specific suppression of *DET1*, a photomorphogenesis regulatory gene, to increase both carotenoid and flavonoid content in transgenic tomatoes. Previous work on *DET1* has reported increases in these phytochemicals in loss-of-function mutants, but global suppression of *DET1* leads to a number of serious developmental defects that prevent these plants from being used commercially.

In this study, a crop improvement has been presented that should provide significant value to producers. Early season harvests of tomatoes and many other horticultural crops usually constitute a substantial percentage of a season’s profits. The first fruit picked can be sold at a premium because supply is generally low and demand is high. In this study, the transgenic lines in an elite background ripened up to 1 week earlier than controls (Figure 1). These lines have none of the developmental defects associated with global receptor suppression (Tieman et al., 2000; Kevany et al., 2007) because of fruit-specific suppression of the gene (Figure 2a). This approach for the engineering of early ripening should be applicable to any climacteric fruit species.
Experimental procedures

Plant materials and growth conditions

*Solanum lycopersicum* cv. Flora-Dade and TFM7-LeETR4-RNAi lines were grown in a glasshouse maintained at approximately 27 °C. Individual plants were grown in 11.35-liter pots that were watered twice daily and supplemented with slow-release fertilizer. Time to ripening data were collected by tagging open flowers and recording the number of days from anthesis to breaker stage (first signs of external colour development). T1 generation transgenic and azygous control field plants were grown in randomized, replicated plots in Live Oak, FL, USA. Plants were grown using standard commercial practices in raised plastic mulched beds with drip irrigation. For the T1 generation, plants that inherited the transgene were identified using neomycin phosphotransferase type II (NPTII)-specific polymerase chain reaction (PCR) primers. Homozygous T2 plants were identified from the T1 populations for each independent transgenic event.

Development of transgenic plants

A TFM7-LeETR4 fruit-specific RNAi construct was generated using the method outlined by Dexter et al. (2007). Briefly, two overlapping fragments of the coding region were PCR amplified from tomato fruit cDNAs, one 400 bp and one 200 bp in length (Table S1, see ‘Supplementary material’). The two PCR products were ligated in inverse orientation to one another, and subsequently ligated into a ‘Supplementary material’). The two PCR products were ligated in inverse orientation to one another, and subsequently ligated into a vector containing ~2.3 kb of the TFM7 fruit-specific promoter. The cassette containing the promoter, RNAi fragment and nopaline synthase terminator were excised from the vector and ligated into the pHK1001 plant expression vector. The transgene was introduced into cv. Flora-Dade by Agrobacterium-mediated transformation, according to McCormick et al. (1986), using kanamycin selection. The specificity of the transgene was determined by quantification of other receptor mRNAs from fruit tissue (data not shown). In each case, there was no effect on the RNA levels of other receptors.

RNA expression analysis

Total RNA extractions were performed using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA), with subsequent DNase treatment to remove any contaminating DNA. RNA was quantified by spectrophotometry and visually analysed on ethidium bromide-stained gels to ensure equal concentrations of all RNAs. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assays were performed using the Applied Biosystems Taqman One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) in a BioRad MyiQ Single-Colour Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The PCR conditions were as follows: step 1, 48 °C for 30 min; step 2, 95 °C for 10 min; step 3, 95 °C for 15 s and 60 °C for 1 min (40 times). The primer and probe pairs for each gene assayed are given in Table S3 (see ‘Supplementary material’). Levels of LeETR RNAs were quantified using RNAs synthesized by *in vitro* transcription from plasmids containing the coding region of each gene using a Maxiscript In Vitro Transcription Kit (Ambion, Austin, TX, USA). The total amount (micrograms) of *in vitro*-transcribed RNA was determined and the *in vitro* transcription product was used to construct a standard curve in real-time RT-PCR analysis. The results are reported as the percentage of LeETR RNA in total RNA.

Microsomal membrane isolation and protein blot analysis

Microsomal membrane fractions were isolated from fruit with a homogenization buffer containing 30 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8.2), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) and 20% (v/v) glycerol with protease inhibitors [1 mM phenylmethylsulphonylfluoride (PMSF), 10 µg/mL aprotinin, 1 µg/mL leupeptin and 1 µg/mL chymostatin], as described in Schaller et al. 1995. The membrane pellet was resuspended in 10 mM Tris (pH 7.5), 5 mM EDTA and 10% (w/w) sucrose with protease inhibitors, and stored at ~80 °C. Protein blots were probed with a custom anti-ETR4 (1 : 2000) obtained from Cocalico Biologicals (Reamstown, PA, USA), as described previously (Kevany et al., 2007).

Acid and soluble solids analysis

Individual tomato fruit were homogenized in a blender for 30 s and frozen at ~80 °C prior to analysis. Samples were thawed and centrifuged at 16 000 *g* for 5 min. The supernatant was analysed for citric and malic acid content using citric acid and malic acid analysis kits (R-Biopharm, Marshall, MI, USA), according to the manufacturer’s instructions. Soluble solids were expressed as Brix, which is a measurement of the mass ratio of dissolved sucrose to water in a liquid. Individual fruits were homogenized in a blender for 30 s; 1 mL of the homogenate was centrifuged at 16 000 *g* for 2 min; ~75 µL of supernatant was applied to a handheld refractometer.

Volatil analysis

Ripe tomato fruit from each line and its corresponding control collected from the field were harvested, and volatiles from pooled fruits were collected on the day after harvest. Fruits collected from plants grown in the glasshouse were analysed for fruit volatiles immediately after harvest. Tomato fruit volatiles were collected from chopped fruit with nonyl acetate as an internal standard, as described previously (Tieman et al., 2006). Chopped fruit was enclosed in glass tubes and the air was filtered through a hydrocarbon trap (Agilent, Palo Alto, CA, USA) for 1 h for the collection of the volatile compounds on a Super Q column. Volatiles collected on the Super Q column were eluted with methylene chloride after the addition of nonyl acetate as an internal standard. Volatiles were separated on an Agilent DB-5 column and analysed on an Agilent 6890N gas chromatograph, with retention times compared with known standards (Sigma Aldrich, St Louis, MO, USA). Volatile levels were calculated as the amount in nanograms per gram fresh weight per hour of collection. The identities of the volatile peaks were confirmed by gas chromatography-mass spectrometry (GC-MS), as described previously (Tieman et al., 2006).

References


Supplementary material

The following supplementary material is available for this article:

Table S1 Volatile organic compounds from fruits of field-grown T1 plants

Table S2 Volatile organic compounds from fruits of glasshouse-grown T1 plants

Table S3 Oligonucleotide primers and probes

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1467-7652.2007.00319.x

(This link will take you to the article abstract).

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