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Genetic Regulation of Fruit Development and Ripening

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INTRODUCTION

Fruit development and ripening are unique to plants and represent an important component of human and animal diets. Recent discoveries have shed light on the molecular basis of developmental ripening control, suggested common regulators of climacteric and nonclimacteric ripening physiology, and defined a new role for MADS box genes in this late stage of floral development. Analyses of fruit-ripening mutants and ripening-related gene expression suggest higher levels of a developmental regulatory cascade that remain to be defined. Examination of the molecular basis of ethylene signaling in tomato has demonstrated conservation of the basic model defined in Arabidopsis, yet with modifications in gene family composition and expression that may represent adaptations to promote successful fruit development and seed dispersal. The role of light signaling in fruit carotenoid accumulation is being examined and may represent a target for practical manipulation of fruit pigmentation and nutrient content. The continuing development of genomics tools, including ESTs and cDNA microarrays, for important fruit crops should foster accelerated discovery in fruit development and ripening research.

THE FRUIT ORGAN: DIVERSE FORMS AND FUNCTIONS

By anatomical definition, the fruit is a mature ovary and therefore typically includes carpel tissues in part or in whole. Many fleshy fruit species important to humans additionally develop mature fruit tissues, including extracarpellary floral components. Examples include strawberry, pineapple, mulberry, and pome fruit (apple, pear), in which the receptacle, bracts, calyx, and floral tube (the fused base of floral organs), respectively, constitute the majority of mature fruit tissue. Even species with fruit derived from carpel tissue exclusively can display a range of developmental programs, spanning the relatively uniform single expanded carpel or drupe of stone fruit to the differentiated carpel tissues giving rise to the peel (flavedo) and multicarpel flesh of citrus and banana.

Evolutionary pressures have resulted in a variety of developmental manifestations of fruit tissues, resulting in structures that range in design and function from hardened fruit capsules or pods that forcefully expel seeds at maturation, to forms optimized for seed movement by wind, water, animal fur, or gravity, to those implementing developmental programs that yield succulent and flavorful tissues for organisms that consume and disperse the associated seed. Tanksley (this issue) discusses the impact of domestication on selection for fruit genes that influence size and shape early in fruit development in addition to discoveries regarding their underlying molecular functions. The focus here will be on recent advances in our understanding of developmental and signaling pathways that affect later fruit maturation and ripening.

Although dehiscent and dry fruit types (e.g., cereals) represent the majority of plant species, fruit developmental studies to date have focused primarily on fleshy species because of their importance in the human diet. Particular emphasis has been placed on tomato as an especially tractable system for molecular genetic analysis of fleshy fruit development and ripening (Giovannoni, 2001). Arabidopsis also has proven exceptionally informative as a model system for floral development in general (Lohmann and Weigel, 2002) and gene identification and the subsequent functional analysis of carpel identity–, development-, and maturation-associated genes (Pinyopich et al., 2003, and references therein). In addition, the Arabidopsis silique is a dehiscent fruit characteristic of the legumes and thus represents another exceptionally important fruit type in terms of human and animal food. Other systems will be mentioned where appropriate, but the majority of this review will focus on Arabidopsis and tomato as the major systems underlying many recent discoveries in fruit development and ripening.

ARABIDOPSIS: A MODEL SYSTEM FOR GENETIC REGULATION OF FRUIT DEVELOPMENT

Since the initial description of a requirement for the AGAMOUS (AG) protein for carpel and stamen determination (Bowman et al., 1989), a large family of Arabidopsis MADS box genes has been reported and in many cases functionally defined (Alvarez-Buylla et al., 2000, and references therein). For example, the redundant *SEPALLATA* genes (*SEP1*, *SEP2*, and *SEP3*) can be eliminated via mutation individually with minimal impact on floral development, yet the triple mutant results in the conversion of all floral organs to sepals, indicating roles in normal petal, stamen, and carpel development (Pelaz et al., 2000). Spatial constraint of *AG* expression via negative regulation by the APETALA2 (AP2) EREBP-like protein provided early evidence that additional transcription factors also play important roles in floral and carpel development, in part via the regulation of MADS box genes (Drews et al., 1991).

Additional insight into the molecular basis of carpel determination in the developing flower came through the recent discovery that two previously described MADS box *SHATTERPROOF* genes (*SHP1* and *SHP2*; Liljegren et al., 2000), originally

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associated with carpel dehiscence and residing in the same phylogenetic clade as *AG*, provide functional redundancy to *AG* in carpel determination (Pinyopich et al., 2003). *AG*, *SHP1*, and *SHP2* also are functionally redundant with the *SEEDSTICK* MADS box gene as determinants of ovule identity. Normal ovule development in turn influences later carpel expansion as a response to successful fertilization (Ferrandiz et al., 2000). *SHP1* and *SHP2* in particular are capable of functioning in carpel and ovule determination, carpel expansion, and dehiscence of the mature fruit. This level of functional redundancy suggests that the main determinant of the primary in vivo developmental roles of members of this group of related MADS box genes is through differential gene expression (Pinyopich et al., 2003). Given the number and similarity of plant MADS box genes, this paradigm may be repeated, possibly for functions that are unique to fruit development of specific or related sets of plant species, suggesting that it may be useful to identify and characterize MADS box genes expressed in developing fruit.

Given the diversity of fruit development programs across the plant kingdom and molecular insights developed in Arabidopsis (especially with respect to MADS box genes), it will be important to determine how this family has evolved in number, function, and target genes to facilitate fruit form and development in diverse plant species. Antisense repression of the tomato *AG* homolog *TAG1* caused homeotic conversion of inner floral whorls similar to that observed in Arabidopsis *ag* mutants, whereas ectopic expression resulted in the development of red fleshy sepals, suggestive of ripe fruit tissues and consistent with a role in carpel determination (Pnueli et al., 1994b). Independent antisense repression of two tomato *SEP* homologs, *TM5* (Pnueli et al., 1994a) and *TM29* (Apomah-Dwamena et al., 2002), resulted in a range of anticipated and unanticipated phenotypes (based on the Arabidopsis model) suggestive of less functional redundancy than was seen in Arabidopsis. Specifically, *TM5* repression resulted in partial conversions of carpels, stamens, and petals to less specialized structures and resulted in additional organ whorls, whereas *TM29* repression yielded green stamens and petals in addition to parthenocarpic fruit from which additional shoots emerged. Although mutations in *SEP* genes indicate redundant functions in the determination and development of the three inner floral whorls of Arabidopsis, the use of full-length cDNAs for antisense of both *TM5* and *TM29* (and the high degree of sequence similarity in the MADS box domain) limits our ability to define specific roles for these genes in tomato in the absence of additional characterization of transgene effects at the molecular level. Nevertheless, mutations in crop MADS box genes have been useful in defining a MADS box role in tomato pedicel abscission zone formation (Mao et al., 2000) and the functional basis of parthenocarpic (seedless) fruit development in apple (Yao et al., 2001). An additional tomato MADS box gene regulating fruit ripening (Vrebalov et al., 2002) is described below.

PROGRAMS FOR MATURATION, RIPENING, AND SEED DISPERSAL: NONCLIMACTERIC FRUIT OR ETHYLENE MUTANT?

The ripening of fruit organs represents the terminal stage of development in which the matured seeds are released. In the dehiscent fruit of the Arabidopsis silique, this process is facilitated by senescence of the mature carpel tissue followed by separation of the valves at an abscission cell layer (termed the dehiscence zone) that is formed between the valve-replum boundary. The MADS box *SHP1* and *SHP2* genes were shown originally to regulate the formation of the dehiscence zone (Liljegren et al., 2000) under the negative regulation of the FRUITFUL (FUL) and REPLUMLESS gene products, which together limit *SHP* expression to the dehiscence zone (Ferrandiz et al., 2000; Roeder et al., 2003). The *SEEDSTICK* MADS box gene was demonstrated recently to be required for the formation of the funiculus/seed abscission zone that allows separation of the seed from the carpel to facilitate seed dispersal at dehiscence (Pinyopich et al., 2003).

In contrast to Arabidopsis, fleshy fruits such as tomato undergo a ripening process in which the biochemistry, physiology, and structure of the organ are developmentally altered to influence appearance, texture, flavor, and aroma in ways designed to attract seed-dispersing organisms (Figure 1) (Seymour et al., 1993). Although the specific biochemical programs resulting in ripening phenomena vary among species, changes typically include (1) modification of color through the alteration of chlorophyll, carotenoid, and/or flavonoid accumulation; (2) textural modification via alteration of cell turgor and cell wall structure and/or metabolism; (3) modification of sugars, acids, and volatile profiles that affect nutritional quality, flavor, and aroma; and (4) generally enhanced susceptibility to opportunistic pathogens (likely associated with the loss of cell wall integrity). Although fruit species are classically defined physiologically on the basis of the presence (climacteric) or absence (nonclimacteric) of increased respiration and synthesis of the gaseous hormone ethylene at the onset of ripening (Lelievre et al., 1997), fruit displaying both ripening programs typically follow the general developmental changes described above.

Examples of common climacteric fruits that require ethylene for ripening include tomato, apple, banana, and most stone fruits, whereas nonclimacteric fruits, including grape, citrus, and strawberry, are capable of ripening in the absence of increased ethylene synthesis. Interestingly, climacteric fruit span a wide range of angiosperm evolution, including both dicots (e.g., tomato) and monocots (e.g., banana). Nevertheless, members of the same (e.g., melon) or closely related (e.g., melon and watermelon) species are reported to include both climacteric and nonclimacteric varieties. The molecular distinctions underlying climacteric versus nonclimacteric ripening are poorly understood. Nevertheless, it seems likely that at least in instances of the same or closely related species with examples of both climacteric and nonclimacteric types, that nonclimacteric phenotypes may represent mutations in ethylene synthesis or signaling as opposed to more complex distinctions. Indeed, nonclimacteric melons are notoriously difficult to harvest compared with their climacteric counterparts because of reduced abscission, suggesting a defect in ethylene synthesis or response and a mature phenotype consistent with incomplete ripening (Perin et al., 2002). In this regard, it is especially important when selecting a system for the analysis of nonclimacteric ripening to be certain that the ripening physiology of the candidate species is well characterized and consistent with

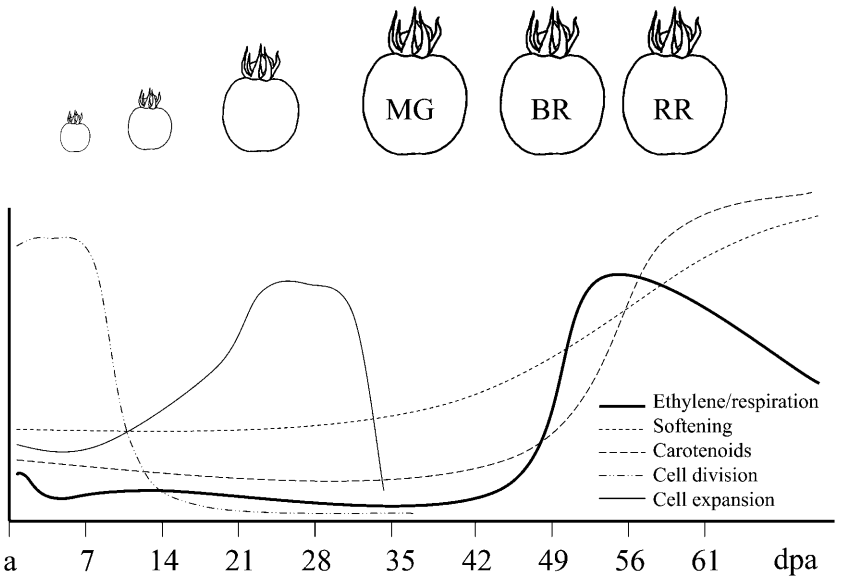

Figure 1. Major Developmental Changes during Tomato Fruit Development and Ripening.

Relative changes in cell division, cell expansion, respiration, ethylene synthesis, fruit softening, and carotenoid accumulation are shown over the course of fruit development. The time from anthesis (a) to mature green (MG; fully expanded unripe fruit with mature seed), breaker (BR; first visible carotenoid accumulation), and red ripe (RR) can vary substantially among cultivars. The time line shown would be for a medium-/large-fruit cultivar such as the breeding line MH1 (5 to 7 cm diameter mature fruit). dpa, days after anthesis.

nonclimacteric ripening as opposed to inhibited ripening resulting from reduced ethylene synthesis or response.

Although the specific role of climacteric respiration in fruit ripening remains unclear, the recruitment of ethylene as a coordinator of ripening in climacteric species likely serves to facilitate rapid and coordinated ripening. A great deal is known regarding specific downstream ripening processes in a number of climacteric and nonclimacteric species, yet little is known about the regulation of ripening in nonclimacteric fruit or the upstream regulation of ethylene in their climacteric counterparts. Recent evidence of the MADS box regulation of ripening in both tomato and strawberry suggests common regulatory mechanisms operating early in both climacteric and nonclimacteric species (Vrebalov et al., 2002). The elucidation of the molecular basis of such early and common events represents an active frontier in fruit ripening research.

MODELS SYSTEMS FOR FRUIT RIPENING

Tomato has emerged as the primary model for climacteric fruit ripening for a combination of scientific and agricultural reasons. The importance of tomato as an agricultural commodity has resulted in decades of public and private breeding efforts that have yielded numerous spontaneous and induced mutations, including many that affect fruit development and ripening (tomato germplasm can be viewed and ordered at the following World Wide Web sites: Tomato Genetic Resource Center [http://tgrc.ucdavis.edu/] and Hebrew University [http://zamir.sgn.cornell.edu/mutants/]). Simple diploid genetics, small genome size (0.9 pg per haploid genome [Arumuganathan and Earle, 1991]), short generation time, routine transformation technology, and availability of genetic and genomic resources, including mapping populations, mapped DNA markers (Tanksley et al., 1992), extensive EST collections (Van der Hoeven et al., 2002) (Table 1), publicly available microarrays, and a developing physical map, render tomato among the most effective model crop systems (http://www.sgn.cornell.edu/index.html). In addition, numerous single gene mutations that regulate fruit size, shape, development, and ripening (described below and by Tanksley in this issue) combined with dramatic and readily quantifiable ripening phenotypes (ethylene, color index, carotenoids, softening) have enhanced the use of tomato as a model for climacteric ripening (Figure 1).

Strawberry is the most widely studied system for nonclimacteric ripening, resulting in the identification and characterization of numerous ripening-related genes that affect cell wall metabolism, color, and aroma (Wilkinson et al., 1995b; Manning, 1998; Aharoni and O'Connell, 2002). The octaploid nature of cultivated strawberry has limited genetic analysis in this species, although strawberry is readily transformed (Woolley et al., 2001) and diploid varieties are available. Recent and extensive EST sequencing of grape and to a lesser degree *Citrus* species (Table 1) suggests the possibility of their greater roles as models for nonclimacteric ripening, although the seasonal nature of these crops will limit their ultimate utilization as basic research systems.

REGULATION OF ETHYLENE SYNTHESIS DURING CLIMACTERIC RIPENING

Ethylene production in plant tissues results from Met metabolism (Yang, 1985). The rate-limiting steps in fruit ethylene synthesis

Table 1. Total and Fruit ESTs Available from Crop Species (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/dbEST/index.html, October 2003)

Species	Common Name	Total ESTs	Fruit ESTs
Ananas comosus	Pineapple	0	0
Capsicum annuum	Pepper	22,433	8,580
Citrus × *paradisi*	Grapefruit	312	312
Citrus sinensis	Orange	16,180	5,623
Citrus unshiu satsuma	Orange	2,561	2,561
Citrullus lanatus	Watermelon	593	593
Coffea arabica	Coffee	453	0
Cucumis melo	Melon	50	50
Cucumis sativus	Cucumber	358	0
Fragaria × *ananassa*	Strawberry	58	0
Lycopersicon esculentum	Tomato	150,228	41,186
Malus × *domestica*	Apple	945	903
Musa acuminata	Banana	27	27
Persea americana	Avocado	0	0
Prunus armeniaca	Apricot	4,535	4,535
Prunus avium	Sweet cherry	21	21
Prunus domestica	Plum	0	0
Prunus dulcis	Almond	3,845	0
Prunus persica	Peach	10,185	10,185
Pyrus communis	Pear	212	212
Vitis spp	Grape	144,483	59,735
Total		357,483	134,565
Total excluding tomato and grape		62,726	33,644

include the conversion of *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) via ACC synthase (ACS) and the subsequent metabolism of ACC to ethylene by ACC oxidase (ACO). In tomato and most characterized plants, both steps are encoded by multigene families. At least four *ACS* genes are expressed in tomato fruit (Rottmann et al., 1991; Barry et al., 2000). *LeACS1A* and *LeACS4* are under developmental control and are responsible for the initiation of ripening ethylene. Both are induced at the onset of ripening, and this induction is impaired by mutation at the *ripening-inhibitor* (*rin*) locus (Barry et al., 2000). Fruit homozygous for the *rin* mutation fail to exhibit the typical ripening-associated increase in ethylene production and do not ripen. Furthermore, although *rin* fruit are capable of responding to exogenous ethylene, as shown by the induction of ethylene-regulated gene expression, they do not ripen (Lincoln and Fischer, 1988). The *rin* locus encodes a MADS box transcription factor termed *LeMADS-RIN*, and the combination of mutant phenotypes described above has been interpreted to reflect a function in ripening control over climacteric ethylene synthesis (presumably via the control of *LeACS1A* and *LeACS4*) in addition to a regulatory process operating outside the sphere of ethylene influence (Vrebalov et al., 2002). *LeACS4* is under ethylene control and thus facilitates autocatalytic ethylene production (characteristic of climacteric fruits) in response to ethylene resulting from LeACS1A and LeACS2 activity. The fourth tomato fruit ACS gene, *LeACS6*, is responsible for preripening ethylene synthesis and is repressed in response to ripening ethylene (Barry et al., 2000). Although most plant tissues harbor an excess of ACO activity, two tomato fruit *ACO* genes also are induced during ripening in response to ethylene and thus contribute to autocatalytic ethylene synthesis (Barry et al., 1996).

A presumed dioxygenase encoded by the *E8* gene is upregulated during ripening and is related to members of the *ACO* family, yet it does not catalyze the conversion of ACC to ethylene (Deikman et al., 1992). Antisense repression of *E8* resulted in the unusual combination of increased ethylene evolution and delayed ripening (Penarrubia et al., 1992), whereas overexpression facilitated a corresponding reduction in ethylene synthesis (Kneissl and Deikman, 1996). Although the molecular mechanism of *E8* function remains unclear, experiments in which *E8* expression is altered in tomato highlight a role in the negative regulation of ethylene synthesis, apparently through repression of ethylene signal transduction. A model summarizing the developmental, hormonal, and environmental regulation of ripening control in tomato is depicted in Figure 2.

ETHYLENE SIGNALING IN TOMATO: MAINTENANCE OF DEFINED COMPONENTS WITH MODULATION OF FAMILY SIZE AND EXPRESSION

Characterization of Arabidopsis ethylene response mutants, tests for epistatic interactions, and isolation of their corresponding genes have resulted in the development of an extensive network of ethylene signal transduction components (reviewed by Bleecker and Kende, 2000; Stepanova and Ecker, 2000). Several groups have isolated and characterized homologous genes from tomato in an effort to assess the degree of conservation of the basic signaling structure defined in Arabidopsis and to ascertain any variation (especially related to fruit development, ripening, and senescence) in crop species. The creation of ethylene-insensitive tomato and petunia plants via the introduction of dominant Arabidopsis ethylene receptor alleles demonstrated the functional conservation for this component of ethylene signaling (Wilkinson et al., 1997).

The first ethylene receptor identified in tomato was revealed through the isolation of the *Never-ripe* (*Nr*) fruit-ripening locus (Wilkinson et al., 1995a). Observation of global and dominant ethylene insensitivity in the *Nr* mutant (Lanahan et al., 1994) led to a candidate approach in which tomato homologs of Arabidopsis ethylene receptor genes were isolated and tested for linkage to *Nr* (Yen et al., 1995). *Nr* proved to be a tomato gene structurally similar to the Arabidopsis *ETHYLENE RESPONSE SENSOR* receptor (Hua et al., 1995) encoding a mutation that impairs ethylene binding capability (Wilkinson et al., 1995b). Gene expression analysis of *Nr* and additional tomato receptor homologs indicated that *Nr* and *LeETR4* transcripts are most abundant in ripening fruit tissues (Payton et al., 1996; Zhou et al., 1996; Lashbrook et al., 1998a). Repression of each gene using gene-specific antisense constructs suggested functional redundancy similar to that reported for the Arabidopsis receptor family, although novel compensatory gene expression was observed, resulting in unique phenotypic manifestations (Tieman et al., 2000). Specifically, lines deficient in *Nr* expression displayed normal phenotypes as a result of the compensatory upregulation of *LeETR4*. However, *LeETR4* repression was not compensated for by the altered expression of *Nr*, nor was any

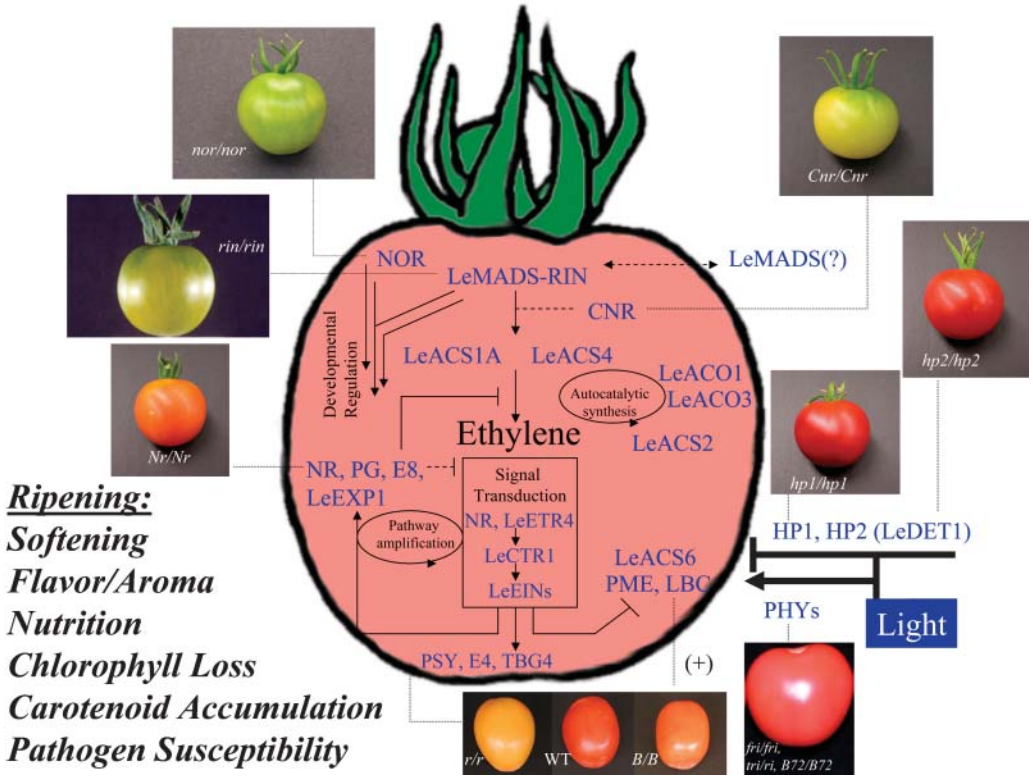

Figure 2. Model for the Molecular Regulation of Tomato Fruit Ripening.

Fruit harboring homozygous mutations for the indicated genes or loci are shown. The *nor*, *rin*, *Nr*, *Cnr*, *hp1*, and *hp2* mutants are all nearly isogenic with cv Ailsa Craig. The *r* and *B* mutants are from reported introgression lines (Eshed and Zamir, 1994) nearly isogenic with cv M82 (WT). A triple phytochrome (PHY) mutant deficient in *PHYA* (*fri*), *PHYB1* (*tri*), and *PHYB2* (*B72*) with associated modification of carotenoid accumulation also is shown.

other tomato receptor gene, resulting in constitutive global ethylene response (leaf epinasty, premature petal senescence, and accelerated ripening) recovered via the ectopic expression of *Nr*. The phenotypic impact of *LeETR4* repression was particularly intriguing in that it suggested that in tomato a single receptor gene, *LeETR4*, plays a prominent role in ethylene signaling, in contrast to Arabidopsis, in which multiple receptor genes must be mutated to reveal constitutive ethylene phenotypes (Hua and Meyerowitz, 1998). The increased *Nr* and *LeETR4* expression during ripening also suggests a tissue-specific response to a heightened need for receptor molecules to facilitate the continued modulation of ethylene responsiveness in ripening fruit tissues. Although five ethylene receptor genes have been identified in Arabidopsis, six have been identified to date in tomato (Tieman and Klee, 1999).

Ethylene receptors in Arabidopsis have been shown to interact with the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) mitogen-activated protein kinase kinase kinase (Clark et al., 1998; Gao et al., 2003), and mutation in *CTR1* results in constitutive activation of all measured ethylene responses, indicating a negative regulatory role in ethylene signaling (Kieber et al., 1993). A tomato *CTR1* homolog (*LeCTR1*) was isolated from ripening fruit and shown to be capable of functioning in Arabidopsis ethylene signal transduction via complementation of the *ctr1-1* mutation (Leclercq et al., 2002). *LeCTR1* also was shown to be upregulated during ripening and in response to ethylene, indicating amplification of the components of ethylene signaling in addition to receptors during fruit ripening. Furthermore, mining of the tomato EST collection yielded an additional *LeCTR1*-like gene, and a third was recovered from a library screen using *LeCTR1* cDNA as a probe. Only one ethylene-signaling *CTR1* gene has been reported to date in Arabidopsis. Both of the additional tomato *CTR1* genes are significantly more similar at the DNA and predicted peptide sequence levels to *CTR1* than to any other gene in the Arabidopsis genome, suggesting the possibility of additional genes encoding CTR1 function in tomato (L. Adams and J. Giovannoni, unpublished data).

The Arabidopsis *ETHYLENE INSENSITIVE3* (*EIN3*) and related *EIN3-like* (*EIL*) genes encode transcription factors that represent downstream components of ethylene signaling (Chao et al., 1997). Three tomato *EIL* (*LeEIL*) genes capable of restoring the Arabidopsis *ein3* ethylene insensitivity phenotype were isolated, although none showed induction during ripening or by ethylene (Tieman et al., 2001). Repression of each gene via gene-specific antisense resulted in no measurable phenotype, whereas simultaneous repression of all three genes resulted in constitutive ethylene responses for all monitored phenotypes. Although this collection of *LeEIL* genes is not suggestive of modulation in

tomato *EIN/EIL* family size or responsiveness, it remains possible that additional *LeEIL* genes could be discovered.

Analysis of ethylene signaling components in tomato suggests that at minimum, the early steps of the pathway demonstrate induction during ripening. Because ethylene receptors exhibit exceptionally strong ligand binding (Rodriguez et al., 1999) and CTR1 may participate in the formation of a complex with receptor molecules (Clark et al., 1998; Gao et al., 2003), species whose tissues are exposed to high concentrations of ethylene as part of normal developmental or response processes may have altered gene family size and/or expression to allow continued ethylene responsiveness and signal modulation during periods of increased ethylene synthesis. A model in which the E8 dioxygenase contributes to the maintenance of ethylene receptor activity (Theologis, 1992) is consistent with a negative feedback regulatory loop that may serve to damp ethylene signaling during climacteric ripening (Figure 2).

THE MOLECULAR BASIS OF THE DEVELOPMENTAL REGULATION OF FRUIT RIPENING

The molecular basis of ethylene synthesis and regulation has been a focal point of ripening analysis in the past, with more recent emphasis shifting to characterization of ethylene signal transduction (see above). Antisense repression of tomato *ACS* (Oeller et al., 1991) and *ACO* (Hamilton et al., 1990) genes and similar experiments in melon (Ben-Amor et al., 1999) clarified the role of these genes in regulating climacteric ethylene synthesis. It is well known, however, that ethylene alone is not sufficient for ripening and that a developmental ''competence'' to respond to ethylene must be achieved (Wilkinson et al., 1995a; Lelievre et al., 1997; Giovannoni, 2001). Consequently, immature fruit typically do not ripen in response to exogenous ethylene. Careful examination of multiple ripening phenotypes, including the production of volatile aroma compounds in ethylene-repressed transgenic melons, demonstrated that aspects of climacteric fruit ripening are regulated by developmental factors that must be properly coordinated with ethylene synthesis (Bauchot et al., 1998).

Developmental mutations that affect all aspects of the tomato fruit-ripening process have been available for decades and include the *rin* mutation (described above). A second mutation, termed *non-ripening* (*nor*), is phenotypically similar to *rin* in that *nor* fruit fail to produce climacteric ethylene or ripen yet show responsiveness to ethylene at the molecular level while similarly failing to ripen in response to ethylene (Lincoln and Fischer, 1988). Both the *rin* and *nor* loci were positioned on the tomato genetic map as first steps in positional cloning (Giovannoni et al., 1995).

Isolation of the *rin* locus revealed tandem MADS box genes separated by 2.6 kb of intervening genomic DNA (Vrebalov et al., 2002). The *rin* lesion resulted in a deletion starting in the last intron of *LeMADS-RIN*, removing the final exon, and extending into the region separating *LeMADS-RIN* from the adjacent MADS box gene (termed *LeMADS-MC*) (Figure 3). *LeMADS-MC* was shown to be the tomato ortholog of the Arabidopsis *AP1* gene and to be responsible for the *macrocalyx* (*mc*) large-sepal phenotype of *rin* (Vrebalov et al., 2002). Interestingly, the *LeMADS-MC* transcribed region is not mutated in *rin*, suggesting that the deleted sequences include *cis*-acting regulatory regions necessary for *LeMADS-MC* expression (J. Vrebalov and J. Giovannoni, unpublished data). Recovery and sequencing of cDNA derived from *rin* fruit mRNA indicate a perfect fusion of *LeMADS-RIN* to *LeMADS-MC* coding sequence, excluding both the last intron of the former and the first intron of the latter gene (Vrebalov et al., 2002). The sequence of the chimeric transcript combined with the genome sequence information described here indicates that transcription of *LeMADS-RIN* proceeds past the deletion and terminates normally at the end of *LeMADS-MC*. mRNA processing of the chimeric transcript apparently results in treatment of the sequence spanning from the remnant 5′ splice site of the last intron of *LeMADS-RIN* to the 3′ splice site of the first intron of *LeMADS-MC* as a single intron, yielding the in-frame mutant chimeric mRNA (Figure 3). The largely recessive nature of both the ripening and sepal phenotypes, in conjunction with the ability to duplicate each phenotype separately via antisense of *LeMADS-RIN* and *LeMADS-MC*, respectively (Vrebalov et al., 2002), indicate that any chimeric MADS box protein produced in *rin* has little if any function.

Elucidation of the tomato *rin* locus provided the first molecular insight into the developmental regulation of climacteric ethylene

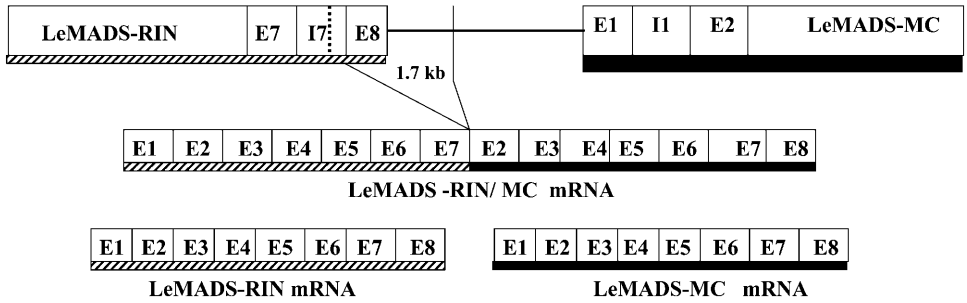

Figure 3. The *rin* Mutation Affects Adjacent MADS Box Genes.

The region of tomato chromosome 5 altered in the *rin* mutant is shown graphically with the 1.7-kb deletion region indicated. The deletion begins in the last intron (I7) of *LeMADS-RIN* and removes the final exon of this gene (E8) in addition to sequences separating the *LeMADS-RIN* and *LeMADS-MC* transcription units. A diagram of the exons that constitute the chimeric *LeMADS-RIN/MC* transcript of the *rin* mutant is shown in the center with representations of the normal *LeMADS-RIN* and *LeMADS-MC* transcripts shown below (Vrebalov et al., 2002).

synthesis and fruit ripening. The identification of a fruit-specific strawberry MADS box cDNA homologous with *LeMADS-RIN* suggests the tantalizing possibility that MADS box proteins may represent a conserved function in the regulation of ripening in both climacteric and nonclimacteric species (Vrebalov et al., 2002). Functional analysis of this gene through antisense repression in strawberry is in progress (K. Manning, G. Seymour, and J. Giovannoni, unpublished data). Finally, because MADS box genes have been shown to act as dimers or higher order heterogeneous multimers (Favaro et al., 2003), it is plausible that additional tomato MADS box genes may participate in ripening. More than 30 different members of the tomato MADS box family are available as ESTs (for tomato EST resources, go to http://www.tigr.org/tdb/tgi/lgi/ and http://www.sgn.cornell.edu/), and corresponding cDNA library representation indicates that transcripts for at least six of these genes are expressed in early ripening (breaker) or fully ripe fruit (for tomato gene expression based on EST prevalence, go to http://ted.bti.cornell.edu/digital/). *LeMADS-MC*, which was shown previously to be expressed in immature and ripe fruit (Vrebalov et al., 2002), in addition to these six genes (*TDR4*, *TDR6*, *TAG1*, *TC125359*/*TM29*, *TC117868*, and *TC124330*) are thus candidates for genes encoding MADS box proteins that may interact with LeMADS-RIN. *TAG1* and *SEP29* have been repressed in transgenic tomatoes (Pnueli et al., 1994b; Ampomah-Dwamena et al., 2002), and although it is unclear if additional MADS box genes may have been targeted in these lines, in both instances, ripening of mature carpel tissues did occur.

Phylogenetic analysis of available tomato and Arabidopsis MADS box genes indicates that *LeMADS-RIN* is most similar to the *AGL3* and *SEP* genes of Arabidopsis (Figure 4) (Vrebalov et al., 2002). Several additional tomato ESTs representing putative orthologs of the Arabidopsis *SEP* genes are available and include three of the ripening fruit MADS box genes (*TC125359*, *TC117868*, and *TC124330*/*TM29*) in addition to AI486089, which is represented by a single EST (Figure 4). The existence of multiple ripe-fruit-expressed MADS box genes in this clade and the presence of at least one additional gene in tomato compared with Arabidopsis could represent duplication and/or retention of MADS box genes in tomato that influence ripening. The redundant functions of Arabidopsis *SEP* genes suggest the need for specific repression of each tomato gene followed by the development of pyramidal repression lines to accurately elucidate possible ripening functions.

The *LeMADS-RIN* gene itself is induced at the onset of ripening without substantial influence by ethylene, indicating higher order regulatory control (Vrebalov et al., 2002). Comparative gene expression analysis in *rin* and *nor* fruit suggests an interesting subclass of ethylene-responsive genes, including *E8*, that respond to developmental signals and ethylene in *rin* but not *nor* fruit (DellaPenna et al., 1989). *E8* expression has been characterized extensively and is induced to ~30% of maximal ripening levels in mature green *rin* fruit at a time consistent with the onset of ripening and attains normal expression in response to exogenous ethylene (DellaPenna et al., 1989; Giovannoni et al., 1989). The bimodal regulation of *E8* expression in *rin* and the absence of expression in *nor*, combined with the inability to induce ripening in either mutant via exogenous ethylene, defines

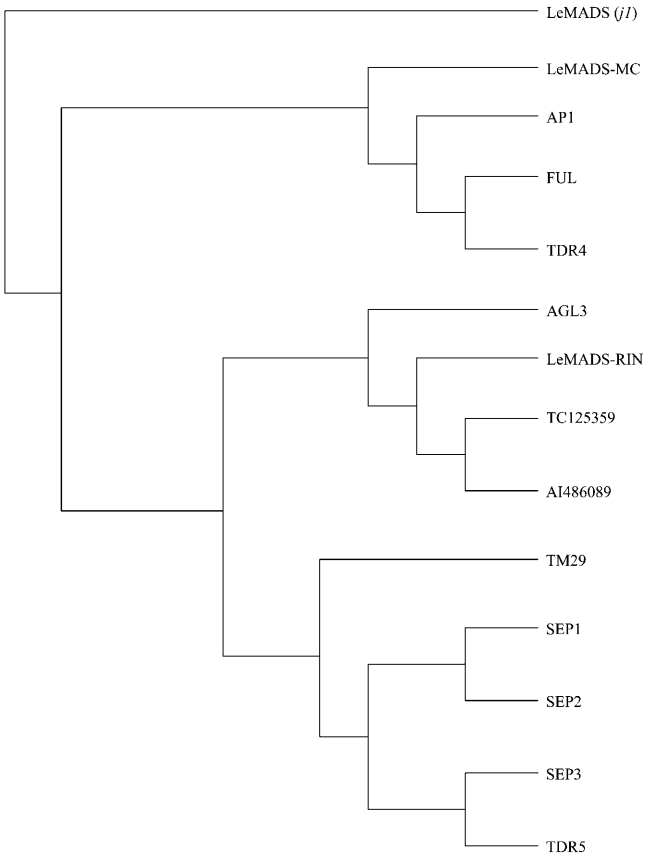

Figure 4. Maximum Parsimony Map of MADS Box Genes.

Comparisons of MIK (MADS box, I, and K domain) amino acid sequences of each MADS box gene were used in maximum parsimony analysis using the Phylogeny Inference Package (PYLIP) version 3.5c (http://evolution.genetics.washington.edu/phylip.html). LeMADS (j1), LeMADS-MC, TDR4, LeMADS-RIN, TC125359, AI486089, TM29, and TDR5 are tomato genes, and AP1, FUL, AGL3, SEP1, SEP2, and SEP3 are Arabidopsis genes.

a minimal regulatory network that operates during fruit ripening. In this network, ethylene regulates a subset of ripening genes either directly or in concert with developmental signals influenced by *LeMADS-RIN* and/or the *nor* gene product. *E8* represents a regulatory motif in which *nor* but not *LeMADS-RIN* provides developmental control. In this instance, *LeMADS-RIN* affects *E8* gene expression mainly via the activation of autocatalytic ethylene synthesis. Although numerous tomato ripening genes have been assessed for expression changes in *rin*, relatively few have been characterized in *nor*. Future characterization of ripening gene expression in *nor* will facilitate the further definition of developmental regulation during ripening, which is clearly affected by both mutations in ways that do not overlap. The *nor* locus has been cloned and encodes a putative transcription factor with no relationship to MADS box gene sequences (J. Vrebalov and J. Giovannoni, unpublished data). The availability of this sequence also should promote the

refinement of the developmental regulatory network governing fruit ripening (Figure 2).

Additional tomato ripening mutants are available, and the cloning of their corresponding loci should further assist in the expansion of our understanding of ripening (Giovannoni, 2001). For example, the *Colorless non-ripening* (*Cnr*) mutation also results in comprehensive ripening inhibition (Thompson et al., 1999). *E8* can be induced by ethylene in *Cnr*, and the mutation does not affect the expression of *LeMADS-RIN* (Seymour et al., 2002), suggesting a function either downstream of *LeMADS-RIN* or in a separate regulatory network.

CELL WALLS AND SOFTENING

Ripening-related cell wall metabolism and associated textural changes have been a major focus of ripening research since the isolation of the tomato fruit *POLYGALACTURONASE* (*PG*) gene. *PG* has been reported to represent ~1% of ripening fruit mRNA and results in substantial cell wall pectinase activity, in concert with the induction of ripening and softening (DellaPenna et al., 1989, and references therein). *PG* expression is inhibited substantially by both the *rin* and *nor* mutations, with additional influence by ethylene (DellaPenna et al., 1989). Both antisense repression (Smith et al., 1988) and ectopic expression in unripe fruit (Giovannoni et al., 1989) indicated that PG alone is not sufficient for softening. Nevertheless, a reduction in ripe fruit susceptibility to postharvest pathogenesis in antisense *PG* fruit led to the commercialization of *PG* antisense tomatoes.

The collapse of the hypothesis that PG represented the primary determinant of tomato fruit softening caused attention to turn to the isolation and functional analysis of alternative cell wall–associated and/or metabolizing proteins (reviewed by Brummell and Harpster, 2001; Orfila et al., 2001). Removal of pectin methylester groups from fruit cell walls before ripening by pectin methylesterase (PME) facilitates access of PG to its substrate. PME is expressed before ripening and is down-regulated by ethylene as ripening begins. Although repression of tomato fruit *PME* via antisense resulted in increased juice viscosity attributable to the retention of preripening pectin chain length, softening was not altered measurably (Tieman et al., 1992).

In addition to pectin-modifying enzymes, several hemicellulose-metabolizing enzymes have been characterized in ripening fruit. Repression of the ripening-related endo-β-1,4-glucanases (also known as EGases or cellulases) *CEL1* and *CEL2* altered pedicel and fruit abscission, respectively, but did not influence fruit softening (Lashbrook et al., 1998; Brummell et al., 1999a). However, a ripening-related and ethylene-inducible tomato β-galactosidase gene, *TBG4*, did have a modest impact on fruit softening when repressed via antisense (Smith et al., 2002), as did repression of the ripening expansin *LeExp1* (Rose et al., 1997; Brummell et al., 1999b).

The complexity of cell wall ultrastructure is matched by an equally complex repertoire of cell wall–metabolizing and structural activities, many of which are encoded by multigene families that likely contribute to the difficulty of determining the molecular basis of fruit cell wall metabolism. Although considerable progress has been made in determining the biochemical contribution of specific cell wall proteins during fruit ripening, the molecular basis of fruit softening is still poorly understood and remains an active area of investigation.

THE ROLE OF LIGHT IN FRUIT RIPENING

Carotenoids, particularly lycopene and β-carotene, represent the primary components of ripe fruit pigmentation in tomato. Genes encoding enzymes that catalyze carotenoid synthesis have been cloned from tomato and correspond to a number of previously defined pigmentation mutants (Bramley, 2002; Isaacson et al., 2002, and references therein). Examples include the *yellow-flesh* (*r*) mutation, resulting in deletion of the ethylene-regulated phytoene synthase (*PSY*) gene (Fray and Grierson, 1993), loss of or reduced expression of the carotenoid isomerase gene, resulting in the prolycopene-accumulating orange fruit of the *tangerine* mutants (Isaacson et al., 2002), and overexpression and knockout mutations of the lycopene-β-cyclase gene, resulting in high-β-carotene *Beta* (*B*) and deep-red *crimson* fruit, respectively (Ronen et al., 1999, 2000). Although a great deal has been learned about the structural components of the carotenoid synthesis pathway in recent years, regulation of flux through the pathway is largely a mystery. It is known that *PSY* is strongly induced by ethylene during ripening, indicating a major control point for total fruit carotenoid accumulation (Lois et al., 2000). In addition, analysis of quantitative trait loci associated with tomato fruit carotenoid metabolism indicates that multiple loci, in addition to known structural components, contribute to carotenoid flux (Liu et al., 2003).

Light has been shown to affect carotenoid accumulation in a number of species, including tomato. Alba et al. (2000) showed that phytochrome-mediated light signal transduction was required for normal ripe fruit pigmentation but did not affect other ripening attributes. Tomato high-pigment (*hp1* and *hp2*) mutants, characterized by increased green fruit and leaf chlorophyll in addition to increased total ripe fruit carotenoids, have been shown to be hypersensitive to light (Peters et al., 1989). Ectopic expression of an oat phytochrome in tomato resulted in phenotypes similar to those exhibited by *hp1* and *hp2*, further emphasizing the role of light in fruit carotenoid accumulation (Boylan and Quail, 1989). The *hp2* locus has been cloned and shown to harbor the tomato homolog of the Arabidopsis *DE-ETIOLATED1* (*DET1*) negative regulator of light signal transduction, providing additional molecular evidence for the regulation of carotenoid synthesis via light signal transduction (Mustilli et al., 1999). Indeed, this result, combined with the effect of phytochromes on fruit pigmentation, suggests that fruit-specific manipulation of light-signaling genes may be a useful approach for optimizing fruit pigmentation and associated nutritional quality.

GENOMICS IN FRUIT SPECIES: PROMISING SIGNS OF DEVELOPMENT

The emergence of genomics technologies holds the promise of more rapid and comprehensive strides in elucidating ripening phenomena in coming years. Using a strawberry cDNA microarray, Aharoni et al. (2000) identified an acyl transferase that contributes to flavor development in one of the first

demonstrations of large-scale expression analysis in fruit. Although the genome of a major fruit crop remains to be sequenced, review of the public EST collections is an indicator of where developments are likely to occur in the near future. More than 150,000 tomato ESTs have been developed and deposited in public databases, making this the largest EST collection of any fruit crop species (Table 1). Represented are 27 different cDNA libraries defining at least eight tissue types and including >40,000 ESTs from fruit at various stages of development (Van der Hoeven et al., 2002). A similar number of total ESTs are available from grape. In fact, grape is currently the species for which the greatest number of fruit ESTs are publicly available (nearly 50% more fruit ESTs than for tomato). Interestingly, public EST resources are sparse to nonexistent for many of the most important fruit species in terms of worldwide consumption (Table 1).

ESTs derived from nonnormalized and nonsubtracted libraries can be informative in their own right in that the numbers and origins of ESTs within a contig can serve as a reflection of relative gene expression (Ewing et al., 1999; Ogihara et al., 2003). Because the majority of existing tomato and grape ESTs are derived from such libraries, so-called digital expression analysis should be feasible and indeed has been developed for tomato (http://ted.bti.cornell.edu/digital/). As such data become available for additional fruit species, it will become possible to compare the expression of homologous genes across species boundaries on a genomics scale, facilitating, for example, comparisons of genome activity between climacteric and nonclimacteric species to identify both conserved and unique ripening functions.

Although digital expression analysis can be useful for characterizing expression in tissues from which ESTs have been developed, this is not a feasible approach for targeted gene expression analysis. A publicly available tomato cDNA array has been developed with National Science Foundation funding that contains >13,000 elements representing 8,700 independent gene sequences (http://bti.cornell.edu/CGEP/CGEP.html). A companion database (http://ted.bti.cornell.edu/) has been developed to allow public deposition and retrieval of raw microarray data resulting from the use of the public tomato array according to MIAME guidelines (Brazma et al., 2001). We and others are beginning to use these resources for comprehensive analysis of fruit development and ripening (http://ted.bti.cornell.edu/). The recent development of public genomics resources for tomato will promote continued and expanded discovery in this model of fruit development and ripening.

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