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Signal transduction systems regulating fruit ripening

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Fruit ripening is a unique aspect of plant development with direct implications for a large component of the food supply and related areas of human health and nutrition. Recent advances in ripening research have given insights into the molecular basis of conserved developmental signals coordinating the ripening process and suggest that sequences related to floral development genes might be logical targets for additional discovery. Recent characterization of hormonal and environmental signal transduction components active in tomato fruit ripening (particularly ethylene and light) show conservation of signaling components yet novel gene family size and expression motifs that might facilitate complete and timely manifestation of ripening phenotypes. Emerging genomics tools and approaches are rapidly providing new clues and candidate genes that are expanding the known regulatory circuitry of ripening.

Fruit ripening is widely studied because of the specificity of this developmental process to plant biology and the practical importance of ripening to the human diet. Ripening can be generally defined as the summation of changes in tissue metabolism rendering the fruit organ attractive for consumption by organisms that assist in seed release and dispersal. Specific biochemical and physiological attributes of ripening fruits vary among species although generally include changes in color, texture, flavor, aroma, nutritional content and susceptibility to opportunistic pathogens (reviewed in Ref. [1]). Ripening is influenced by internal and external cues, including developmental gene regulation, hormones, light and temperature, but until recently, significant molecular understanding was limited primarily to the role and regulation of ethylene biosynthesis [2].

Ripening physiology has been classically defined as either 'climacteric' or 'non-climacteric'. Climacteric fruits show a sudden increase in respiration at the onset of ripening, usually in concert with increased production of the gaseous hormone ethylene. Whereas ethylene is typically necessary for climacteric ripening, non-climacteric fruits do not increase respiration at ripening and often have no requirement for ethylene to complete maturation. Earlier ripening research elucidated the role of ethylene synthesis and regulation in climacteric ripening (reviewed in Ref. [3]), which led to several new and important questions that have begun to be addressed in recent years and which are the subject of this review.

- Have modifications in the design or regulation of signal transduction systems evolved that are important for ripening compared with the model system (primarily *Arabidopsis thaliana*) in which they were defined?
- What regulates ethylene production in climacteric fruit and does this represent a conserved regulatory switch among climacteric and non-climacteric fruit species?
- In the absence of increased ethylene synthesis, do non-climacteric fruit still use the ethylene signaling pathway (possibly via altered ethylene sensitivity or cross-talk from other signal inputs) to regulate fruit ripening?

These questions represent a logical progression toward understanding early ripening regulatory events in addition to molecular details of those previously documented.

Fleshy and dry fruit

Fruit tissues are composed of enlarged floral components including one or more carpels and, in some cases (depending on species), include tissues derived from the calyx, receptacle, bracts or floral tube (the basal region of floral organ fusion). Mature fruits can be categorized generally as either fleshy or dry; fleshy fruits typically undergo ripening as defined above and dry fruits (e.g. *Arabidopsis*, cereals and legumes) mature in a process more akin to senescence and disperse their seeds via abscission-like programs, including dehiscence or shattering. *Arabidopsis* has proven an exceptional model for gaining insight into the molecular regulation of early steps in fruit formation and development [4,5] but does not develop fleshy ripe fruits. Nevertheless, ethylene and light signal transduction pathways defined primarily in *Arabidopsis* [6,7] have proven extremely useful in advancing ripening research in fleshy fruit species such as tomato. Tomato has emerged as the most tractable model to date for the analysis of fleshy fruit development and ripening, in part because of available mutants, excellent genetics, routine transformation and numerous molecular and genomics tools ([8,9], http://www.sgn.cornell.edu/). For these reasons, tomato is the system that has been used for many of the recent advances in ripening described here.

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Ethylene signaling pathway defined in *Arabidopsis*

Much of what is known regarding the steps involved in ethylene perception and signal transduction has been realized through studies of the model plant species *Arabidopsis* and it is therefore relevant to summarize this work here (reviewed in Refs [6,10–12]). In *Arabidopsis*, ethylene is perceived by a family of five ethylene receptors (*ETR1*, *ETR2*, *ERS1*, *ERS2* and *EIN4*), similar to bacterial two-component histidine kinase receptors (reviewed in Refs [13,14]). Whereas dominant gain-of-function mutations in single ethylene receptor genes confer ethylene insensitivity, double, triple and quadruple loss-of-function mutants result in constitutive ethylene response phenotypes indicating their activities as redundant and negative regulators of ethylene signaling [15–17]. Acting downstream of the receptors is a putative MAP-kinase kinase kinase (MAPKKK), termed *CONSTITUTIVE TRIPLE RESPONSE 1* (*CTR1*). CTR1 shares homology to members of the Raf family of Ser/Thr kinases and has been shown to possess intrinsic Ser/Thr protein kinase activity [18]. Loss-of-function mutations in *CTR1* result in constitutive activation of all the ethylene responses examined, supporting the role of *CTR1* as a negative regulator of ethylene response [19]. In addition, several lines of compelling evidence suggest CTR1 interacts directly with receptor molecules to form a signaling complex [20,21]. A MAP-kinase cascade has been implicated in the mediation of the ethylene response downstream of CTR1, whereby a MAPKK [stress-induced MAPKK (SIMKK)] activates an ethylene-inducible MAPK protein (MPK6) [22]. However, to date, direct association of CTR1 with SIMKK or any other MAPKK remains to be demonstrated. Epistasis analysis places *ETHYLENE INSENSITIVE 2* (*EIN2*) downstream of *CTR1* in the ethylene signaling pathway [23]. EIN2 also appears to act downstream or independently of MPK6 because *ein2* mutants exhibit wild-type activation of MPK6 activity upon treatment with and without ethylene [22]. Recent experiments imply that the entire continuum of ethylene phenotypes observed in receptor loss of function mutants could be attributed to the unregulated activity of EIN2 [16].

EIN2 encodes a protein with similarity to the Nramp family of metal ion carriers [24] and based on indirect evidence might represent a common convergence point through which multiple hormone signal transduction pathways, including abscisic acid [25,26], auxin [27], cytokinin [28] and jasmonate [29] might act. However, the mechanism by which EIN2 is activated remains unclear. Considering the similarity of the EIN2 N-terminus to the Nramp proteins, this domain might be important for sensing or transporting a divalent cation, although no metal-transporting capacity has been observed for EIN2 [24]. It is tempting to speculate that this cation might be $Ca^{2?+}$ given the role of this ion in ethylene-mediated pathogenesis response [30]. Based on epistasis, *EIN2* operates upstream of *EIN3* and the *EIL* (*EIN3-like*) family of nuclear localized *trans*-acting proteins [31,32]. EIN3 undergoes post-translational regulation by ethylene via ubiquitin or proteasome-dependent proteolysis mediated by two F-box proteins, EBF1 and EBF2 [33,34]. Homodimers of EIN3, EIL1 and EIL2 bind to a defined target site in the promoter region of the transcription factor, *ETHYLENE RESPONSE FACTOR 1* (*ERF1*) [32]. *ERF1* is part of a large multi-gene family of transcription factors and is important in the regulation of downstream ethylene responsive genes via binding to the 'GCC' box promoter element [35,36].

Tailoring ethylene signaling to the needs of a ripening fruit

Several ethylene signal transduction components homologous to those identified in *Arabidopsis* have been isolated from various plant species. Furthermore, sequence and functional analysis is beginning to reveal that although the basic machinery is apparently conserved, family composition and regulation of ethylene signal transduction genes in fruit species such as tomato can vary substantially (Figure 1).

Six ethylene receptors have been isolated in tomato, five of which have been shown to bind ethylene (reviewed in Refs [37,38]). Each tomato receptor gene has a distinct pattern of expression including a subset (*NEVER-RIPE* or *NR* and *LeETR4*) that is strongly induced during ripening [39–41]. Interestingly, although a dominant mutation in the ethylene binding site of *NR* confers ethylene insensitivity and results in fruits that do not fully ripen [42,43], analysis of transgenic loss-of-function mutations suggests that *NR* is not necessary for ripening to proceed [44,45]. The molecular explanation for this result proved to be compensatory up-regulation of another member of the tomato receptor family, *LeETR4,* as a response to reduced *NR* transcript. However, *NR* is not responsive to reductions in *LeETR4* mRNA, indeed transgene-mediated reduction in *LeETR4* expression resulted in constitutive ethylene responses including accelerated ripening [45]. Although reduced expression of the *LeETR4* receptor resulted in apparent increased ethylene sensitivity, overexpression of the wild-type *NR* receptor in tomato resulted in reduced sensitivity in seedlings and mature plants [46]. This is consistent with the model predicted in *Arabidopsis* where ethylene receptors are thought to act as negative regulators of ethylene signaling, thus reduced receptor expression increases sensitivity to ethylene whereas increased receptor expression decreases sensitivity [37,38]. However, neither the specificity of a single ethylene receptor to a specific biological function, nor compensatory regulation of receptor genes has been reported in *Arabidopsis* and might represent the result of selective pressures to insure maintenance of capacity to control ethylene responses in a tissue whose normal development is dependent on activity of this hormone.

Additional ethylene signaling components have been defined in tomato, including a *CTR1-like* gene (*LeCTR1*) that was shown through complementation of an *Arabidopsis ctr1* mutant to function in ethylene signaling [47]. Like *NR* and *LeETR4*, *LeCTR1* mRNA is also up-regulated during fruit ripening [47,48]. Only one *CTR1* gene has been identified in *Arabidopsis*. By contrast, additional *CTR* genes (*LeCTR2*, *LeCTR3* and *LeCTR4*) have been identified in tomato (Figure 1) [48]. Further mining of species-specific sequence databases indicates that a

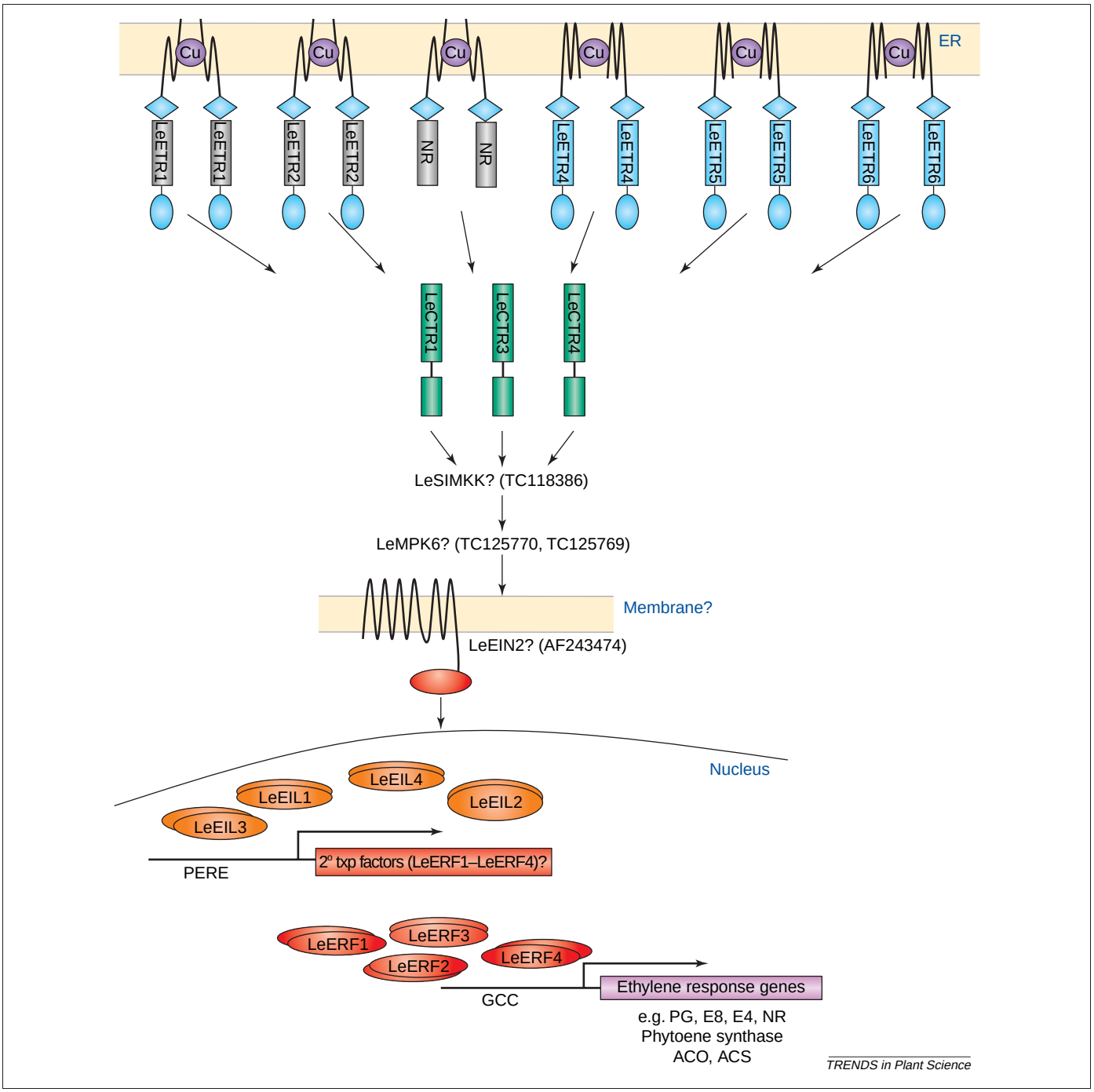

Figure 1. Ethylene perception and signal transduction in tomato. Binding of ethylene to members of the receptor family (here represented by LeETR1, LeETR2, NR, LeETR4, LeETR5, LeETR6) is mediated by a single copper ion (Cu), delivered by RAN1 (not shown). Ethylene negatively regulates the signal transduction pathway upon binding to the receptor, possibly through direct interaction with the tomato CTR1 proteins (LeCTR1, LeCTR3 and LeCTR4). Upon inactivation of LeCTR protein(s), a putative MAPK cascade (represented by LeSIMKK and LeMPK6 with candidate EST IDs shown in parentheses) is relieved from inhibition and activates ethylene signaling through a cascade to downstream components including LeEIN2 (probably membrane localized but the specific sub-cellular membrane is currently unknown) and EIN3-like proteins, LeEIL1–LeEIL4. LeEIL transcription factors probably initiate a transcription factor cascade through activation of secondary transcription (2° txp) factors (represented as LeERF1–LeERF4), which in turn activate ethylene-responsive target genes.

multi-gene family of likely *CTR1* genes is not limited to tomato [48].

Analysis of the *Arabidopsis* genome and extensive screening for constitutive triple response mutants resulting in multiple allelic mutations in *CTR1* suggests the existence of a single *CTR1* gene in *Arabidopsis*. Furthermore, *Arabidopsis CTR1* has been assigned to a subclass of MAPKKKs comprising six similar MAPKKK proteins related to the Raf kinases [49]. Phylogenetic analysis indicated that *Arabidopsis CTR1* is more similar to *LeCTR1*, *LeCTR3* and *LeCTR4* than to any of the other five members of the *Arabidopsis* MAPKKK subfamily, supporting the existence of a single *CTR* in *Arabidopsis* and multiple *CTRs* in tomato [48]. Based on phylogenetic analysis, *LeCTR2* (GenBank Accession number AJ005077) shares more similarity with *EDR1*

(ENHANCED DISEASE RESISTANCE 1) than with CTR1 [48,50]. In *Arabidopsis*, *EDR1* is involved in pathogen response but not in ethylene signaling [50].

Transient silencing of the *LeCTR1* gene resulted in plants with constitutive ethylene phenotypes, confirming the physiological role of *LeCTR1* in negatively regulating ethylene responses in tomato [51]. It is worth noting that the *LeCTR1* sequence used in these experiments has sufficient homology to, and thus could have silenced, the subsequently discovered *LeCTR3* and *LeCTR4* genes. In addition, although *LeCTR1* is induced during ripening, all three genes are known to be expressed at this stage of fruit development [48]. Individual silencing of each *LeCTR* gene will be necessary to assess individual gene function and to address the question of redundancy in tomato.

The presence of multiple *CTRs* in plants raises many questions about how signal outputs from individual receptors are transduced. Whether or not specific tomato *CTRs* interact with specific tomato receptors remains to be demonstrated. Assuming tomato ethylene receptors and *CTRs* interact, as in *Arabidopsis*, the interaction kinetics between the various *CTRs* and the receptors, in conjunction with the varying ratio of receptors and *CTRs* encoded by different family members (and for different tissues and responses), might represent a mechanism for optimizing fidelity of ethylene responses in tomato and other species with multiple *CTR* genes.

Lastly, homologs of *Arabidopsis EIN3*, *EIL* and *ERF* genes have also been identified and characterized in tomato. Three tomato *EIL* genes were isolated and shown to be functionally redundant, regulating multiple ethylene responses throughout plant development [52]. A fourth tomato *EIL* gene (*LeEIL4*) exhibiting ripening-induced expression has been recently cloned, although functional characterization has still to be completed [53]. Four members of the *ERF* family (*LeERF1–LeERF4*) have also been isolated in tomato and their levels of expression have been characterized in response to wound and ethylene treatments and in an assortment of developmental stages including ripening [54]. *LeERF2* exhibited ripening-associated expression and did not accumulate in several ripening mutants, suggesting a specific role in ripening. Proteins derived from all four *LeERFs* were capable of binding to a GCC-box containing *cis*-elements. Although the GCC-box has been shown to function in mediating ethylene-inducible expression in several systems, evidence of the involvement of this element in regulating ripening-related gene expression is lacking. Although functional characterization of the *ERF* gene family in tomato remains to be completed, ethylene-inducible ripening expression of genes encoding multiple steps in the tomato ethylene signal pathway strongly suggest a selective advantage for amplifying ethylene signaling machinery during climacteric fruit ripening.

The triple-response screen in combination with the powerful genetic tools derived from sequencing the *Arabidopsis* genome has enabled researchers to begin to unravel the intricacies of ethylene signaling in plants. Many of the loci characterized to date have encoded global regulators of ethylene responses in plants. Some tissue-specific ethylene response mutants have been identified also, for example, *ethylene insensitive root* (*eir1*), *hookless1* (*hls1*), and *weak ethylene-insensitive* (*wei2*, *wei3*) [23,55,56]. In tomato, the *epinastic* (*epi*) mutant displays a *ctr-like* seedling phenotype but none of the *LeCTR* loci identified to date map to the same chromosomal location as *epi* [48,57,58]. Further characterization of the *epi* mutant was carried out through double mutant analysis with the dominant ethylene insensitive receptor mutant, *Nr* [58]. Interestingly, in the *epi*/*epi* *Nr*/*Nr* double mutant, vegetative growth resembles that of *epi*, whereas petal senescence, pedicel abscission and fruit ripening are similar to *Nr* inhibition of these processes. This result suggests a role for *epi* in the regulation of a specific subset of ethylene responses controlling vegetative growth and development, or in an independent pathway that cross-talks with the ethylene signaling network [58]. A fruit-specific ethylene response mutant remains to be confirmed and reported in the literature.

ESTs provide candidates for filling in the gaps in fruit ethylene signal transduction

The generation of vast amounts of sequence information through EST and whole genome sequencing efforts is providing plant biologists with new tools to dissect development and response processes. Of particular interest for ripening are the fruit EST collections derived from tomato and grape (http://www.sgn.cornell.edu/, http://www.tigr.org). These collections provide resources for comparative studies of gene expression between non-climacteric (grape) and climacteric fruits (http://ted.bti.cornell.edu/).

In a recent report, Ashraf El-Kereamy and co-authors described enhanced anthocyanin accumulation in grape berries following ethylene treatment, suggesting ethylene-responsive ripening characteristics in non-climacteric fruit [59]. The responsiveness of at least some non-climacteric fruits to ethylene, particularly in the area of color development, is well documented and has commercial application (e.g. in promotion of color development in citrus peel). Examination of the grape EST collection indicates that genes homologous to *Arabidopsis ETR1*, *EIN2*, *SIMKK* and *MAPK6* are expressed in grape berries. This observation, combined with the results of El-Kereamy *et al*., support the intriguing hypothesis that although ethylene synthesis does not increase during the ripening of non-climacteric fruits, alterations in ethylene responsiveness might be able to mediate physiological changes associated with ripening.

ESTs representing candidate *EIN2*, *SIMKK* and *MAPK6* genes are also present in the tomato fruit EST collections (Figure 1). Furthermore, mining of microarray and EST prevalence data from the Tomato Expression Database (http://ted.bti.cornell.edu/) suggests that a MAPK6 homolog (TC125769) is up-regulated during ripening, consistent with other genes encoding ethylene signaling components in maturing tomato fruit. Expansion of EST resources from these and other fruit crops should reveal candidates for ethylene signal transduction from additional species but can also facilitate comparative analysis of family size and expression levels (this is true only when ESTs are derived from non-normalized,

non-subtracted cDNAs, as is the case for existing tomato and grape ESTs). In short, accumulating EST and associated expression data should enable more-accurate prediction of orthologs to known genes, in addition to facilitating identification of candidates for aspects of ethylene signaling that remain poorly defined (e.g. MAP-kinase cascade components downstream of *CTR1* and ultimate activators of ethylene-regulated gene expression during fruit ripening).

Light signal transduction impacts ripe fruit pigmentation and is a target for nutritional enhancement

In contrast to ethylene, which is required for completion of most, if not all, ripening processes in climacteric fruit, the impact of light during fruit ripening appears to be specific to regulation of pigment accumulation [60]. Tomato *high-pigment* mutations (*hp1*, *hp2*) result in elevated carotenoid and flavonoid accumulation because of increased sensitivity to light but have little impact on other ripening characteristics [61,62]. The genes responsible for both mutations have been cloned and represent tomato homologs of light signal transduction genes previously described in *Arabidopsis*. Specifically, *hp1* results from a lesion in a gene homologous to *UV-DAMAGED DNA BINDING PROTEIN 1* (*DDB1*) and *hp2* is mutated in the tomato *DE-ETIOLATED1* (*DET1*) ortholog [63,64]. The corresponding *Arabidopsis* proteins are capable of interaction [65] and analysis of single and *hp1 hp2* double mutants suggests that the same is likely to be true in tomato [63].

Ripe fruit pigments including carotenoids and flavonoids have antioxidant properties that assist in neutralizing the effects of photo-oxidation while also having nutritional significance to humans [66–68]. Because mutations in the light signaling pathway positively influence pigmentation of ripe fruit, targeting the light signaling pathway might be an effective means of engineering fruit nutritional quality. Although carotenoid accumulation in edible plant tissues has been manipulated by altering corresponding biosynthetic enzymes (e.g. Golden Rice, [68]), the outcome of such approaches has typically fallen short of expectations. This is probably because of a lack of understanding regarding endogenous mechanisms of regulation and accumulation of carotenoids and/or undesirable side effects on non-target metabolites derived from the altered pathway [68,69]. Engineering of an existing signal transduction network already capable of regulating flux through the carotenoid synthesis pathway in a biologically viable manner might represent a simplified alternative to optimizing carotenoid-associated nutritional benefit in plant tissues such as fruit. Indeed, recently it has been shown that manipulating tomato light signal transduction genes homologous to *HY5* and *COP1* from *Arabidopsis* can result in modified fruit carotenoid accumulation [63].

Developmental regulation of ripening: moving up the regulatory cascade

Insights into the molecular basis of ethylene synthesis and perception in climacteric fruit logically lead to questions concerning ripening regulation upstream of ethylene synthesis and response. As stated at the onset: what regulates ethylene during climacteric fruit ripening? Answers to this question could also conceivably lead to the discovery of conserved regulatory mechanisms shared by climacteric and non-climacteric species.

Three spontaneous tomato ripening mutations, *ripening-inhibitor* (*rin*), *non-ripening* (*nor*) and *Colorless non-ripening* (*Cnr*) are particularly interesting in this regard because their physiology is suggestive of roles in ripening regulation before ethylene synthesis. Fruit homozygous for either *rin* or *nor*, or carrying a dominant *Cnr* allele, undergo complete fruit expansion and yield mature seed, yet fail to proceed in any significant way to ripening. Mature and unripe *rin*, *nor* or *Cnr* fruit do not demonstrate climacteric respiration nor elevated ethylene synthesis [70,71]. However, both *rin* and *nor* are capable of ethylene synthesis in response to wounding [72], suggesting that the lack of ripening ethylene in these two mutants is because of a deficiency in appropriate developmental signals, as opposed to genetic lesions in ethylene biosynthetic genes. Data on *Cnr* wound ethylene has not been reported. Application of endogenous ethylene does not restore ripening to *rin*, *nor* or *Cnr* fruit, but does result in induction of ethylene-regulated genes [71,73]. This last observation is particularly intriguing because it suggests that *rin*, *nor* and *Cnr* have a broader influence on aspects of climacteric ripening than those aspects controlled solely by ethylene (Figure 2) and such mechanisms might be expected to be conserved between both climacteric and non-climacteric species [9].

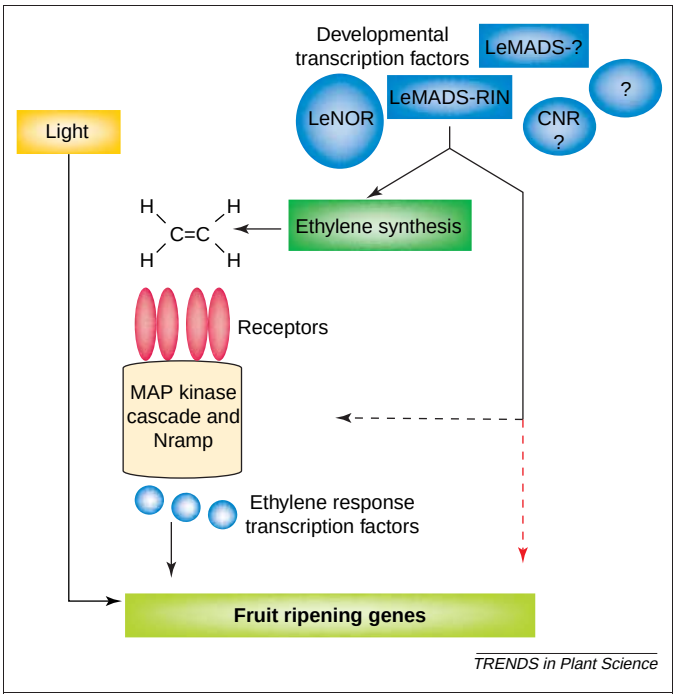

Figure 2. Model for regulation of climacteric ripening via coordinated signaling pathways. Transcription factors including LeMADS-RIN, LeNOR, likely additional MADS-box proteins, CNR and factors remaining to be discovered (?) represent the developmental signaling system that initiates ripening in climacteric fruit. Some components, such as those homologous to LeMADS-RIN can be used in non-climacteric species as well. The developmental signaling system regulates ethylene synthesis that is itself autocatalytic, in addition to non-ethylene-mediated ripening responses (represented by the red broken arrow). Light influences ripening, at least in tomato, only in relation to carotenoid accumulation and through activity of the *DET1* (*hp2*) and *DDB1* (*hp1*) gene products.

Positional cloning efforts have resulted in the isolation of both the *rin* and *nor* loci and molecular characterization of both mutations. *rin* results from deletion of the last exon of a tomato MADS-box transcription factor gene designated *LeMADS-RIN* [9,74]. The *nor* locus harbors a gene with structural features suggestive of a transcription factor, although not a member of the MADS-box family (J. Vrebalov and J. Giovannoni, unpublished).

MADS-box genes are ubiquitous among eukaryotes and are predominantly associated with floral determination and development in plants [75]. MADS-box proteins are capable of forming heterodimers and higher-order multimers, suggesting additional MADS-box genes might participate in ripening (Figure 2; [76]). Indeed, several MADS-box genes expressed in ripening tomato fruit have been identified and are logical candidates for functional analysis related to fruit ripening ([9], see also http://ted.bti.cornell.edu/ for expression data on tomato MADS-box genes). Even more intriguing is the use of the *LeMADS-RIN* cDNA to recover a similar sequence from strawberry, a non-climacteric fruit, suggesting a conserved link between climacteric and non-climacteric ripening control [74]. Orthologous genes from agriculturally important fruit species are now likely to be targeted as tools for engineering fruit quality and shelf-life.

Identification of two putative transcription factors regulating ripening in tomato through induction of climacteric ethylene biosynthesis and additional non-ethylene-regulated processes, represents a higher rung in the ladder of fruit ripening control as well as candidates for conserved molecular mechanisms governing climacteric and non-climacteric ripening. Isolation of the *Cnr* locus should contribute to a greater appreciation of the developmental component of ripening regulation. Understanding the relationships among the *Cnr*, *rin* and *nor* gene products represents a logical next target for understanding the molecular basis of ripening control.

Emerging genomics tools including ESTs and expression arrays are also likely to accelerate the discovery of homologous genes from additional species and the identification of additional novel ripening regulators, particularly when their evolutionary conservation is established via comparative genomics approaches. For example, a recent comparison of ripening-related gene expression in a non-climacteric fruit species (grape) versus a climacteric species (tomato) resulted in identification of ripening-related transcription factor sequences from families that previously had not been associated with ripening [77]. Specifically, EST abundance was used as a measure of gene expression in ripening tomatoes and grapes. Subsets of ripening-related genes from both species were compared at the level of predicted peptide homology to identify homologous genes with parallel expression patterns in maturing fruit tissues from both tomato and grape. Although ~20 ripening-related putative transcription factor sequences were identified in each species, three were highly homologous and thus represent candidates for conserved regulation of ripening in climacteric and non-climacteric species. The three common transcription factor sequences included members of the MADS-box, B-zip, and zinc-finger families; B-zip and zinc-finger have not been previously associated with ripening. Functional characterization of these genes, and additional regulatory candidates likely to result from continued genomics-based experiments should enable researchers studying ripening to identify broadly conserved and more-specific genetic regulators of ripening in the near future.

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