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Ripening Inhibitor (RIN): A master switch in the molecular regulation of ethylene-dependent climacteric fruit ripening

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Abstract

Fruits are an essential source of vitamins and minerals in our diet and constitute a major part of essential plant commodities. Fruit ripening is a complex developmental process, responsible for the transformation of the seed-containing organ into a tissue, attractive to the agents causing seed dispersal as well as fruit consumers. The co-ordinated regulation of different biochemical pathways, involving the transcription of a wide array of genes, brings about the characteristic phenotype that define climacteric fruit ripening, viz., autocatalytic synthesis of the phytohormone ethylene, cell wall softening, cell expansion, colored pigment formation, sugar accumulation, and aroma and volatile production. Ripening Inhibitor (RIN), belonging to the family of SEPALLATA (SEP) group of MADS box transcription factor, is regarded as the nodal player and master switch in dictating the transition of fruit ripening from stage 1 to stage 2, accompanied with ethylene burst. This factor regulates the continuation of ripening process by binding to the CArG box, denoted by (T/A) (T/A) DCCA (A/T) (A/T) (A/T), present in the upstream region of different genes concerned with ethylene production, carotenoid biosynthesis, cell wall modification, volatile and flavor production and other ripening-related transcription factors like NAC-NOR, CNR, FUL1, etc. RIN can auto-regulate its synthesis by binding to the ethylene responsive element (ERE) of its own promoter as well as its target genes, thereby trans-activating the latter. *RIN* mutant, that constitute a fusion protein of the DNA-binding domain of MADS-box and repressor domain of the adjacent protein, MACROCALYX, behaves as a transcriptional repressor, instead of an activator. *RIN* mutants are overall not able to induce the autocatalytic system 2 of ethylene synthesis, causing a drop in the phytohormone level to be able to continue with the ripening process, thereby failing to produce completely ripened fruits. Therefore, silencing of *RIN* expression, via mutant generation or genetic engineering approaches like RNA interference and genome editing, holds promise in delaying the ripening process and enhancing the shelf life of fruits in order to avoid post-harvest losses. The present review highlights the regulatory

mechanism of climacteric fruit ripening, mediated by RIN and the activation of diverse groups of downstream ripening-associated genes.

Keywords: Climacteric fruit; Ethylene; Fruit ripening; MADS box factor; Ripening Inhibitor

Introduction

A fruit is actually a mature and ripened ovary that has important physiological functions in preventing the seeds from getting desiccated, and also helping in their dispersal. Fruits are specialized organs in higher plants with a high degree of versatility regarding their aroma, color, taste, season of maturation and ripening. A number of fruits we enjoy eating today have been around for millennia and even formed an integral part of the “prehistoric diet”, as indicated by evidences from archaeological and palaeontological studies. Being a direct source of vitamins, minerals, antioxidants, sugars and fibers, they constitute an invaluable component of the human as well as a number of animal diets. While we benefit greatly from this plant product, fruits are really an evolutionary adaptation of many angiosperms that help in the reproduction of plant species. Fruit development is a process unique to plants and requires the activation of and cross-talk among a number of signaling pathways. Three distinct stages constitute the process of fruit development in fleshy fruits, viz., fruit set, fruit development and fruit ripening. Of these, ripening has gained the most attention from geneticists and plant breeders, as the process involves the activation of a whole set of biochemical pathways, guided by various hormonal, developmental and environmental factors (Hayama et al. 2006). Ripening of fleshy fruits has evolved as a mechanism to aid seed dispersal. This adaptation is not characteristic of one particular group of plants, but is seen in taxonomically and anatomically different types of fruits, suggesting that it may have arisen independently several times during evolution. An intricate signaling mechanism, coordinated by the expression of a wide array of genes, governs the fruit ripening process. This is regulated by a number of phytohormones, metabolites and signaling molecules like ethylene, abscisic acid, sugars and reactive oxygen species, leading to changes in the fruit texture, color, flavor and aroma, thereby enhancing sensory qualities and making the fruit more attractive and edible for the consumers. Ripening is accompanied with cell enlargement, accumulation of reserves like sugars, acids, pigments and volatiles, destruction of toxic compounds like alkaloids, along with breakdown of starch and pectin by the enzymes like amylase and pectinase, respectively which enhances sweetening of the fruits and causes softening of the fruit wall (Osorio et al. 2011). Although such changes increase the palatability of the fruit, over-ripening due to certain senescence-related biochemical changes might make the fruit susceptible to pathogens as well as difficult to be handled during storage and marketing.

Ripening is connected with the disassembly of photosynthetic pigments in the thylakoid, resulting in chlorophyll degradation by the enzyme chlorophyllase, followed by development of yellow, pink or red coloration due to the activation of carotenoid pathway, and ultimate

conversion of chloroplast to chromoplast (Almeida et al. 2015). The degree of softening is regulated by (i) the structural integrity of primary cell wall and middle lamella, (ii) modifications (de-esterification and depolymerization) of cell wall polysaccharides and consequently, extensive loss of neutral sugars and galacturonic acid, followed by solubilization of oligosaccharides and remaining sugar residues, and (iii) turgor pressure generated within the cells by osmosis. Cell wall modifying enzymes, such as pectin methylesterase, polygalacturonase, β-galactosidase, endo-1, 4 β-D-glucanase and xyloglucan endotransglycosylase, are of primary interest in cell wall metabolism during fruit softening. The synthesis of certain volatile compounds, with a concomitant breakdown of certain metabolites like tannins and flavonoids together enhance the sensory qualities of a fruit. The significant aroma volatiles that increase during ripening are the esters, but certain aldehydes, alcohols, monoterpenes, sesquiterpenes, acids and carbonyls are also regarded as flavor compounds, generated from mevalonate/isoprene pathway and via fatty acid metabolism from substrates including amino acids, organic acids, sugars and lipids during ripening (Giovannoni et al. 2017). Several secondary metabolites like tannins are either broken down or polymerized into non astringent products during ripening. The starch to sugar conversions due to the breakdown of carbohydrate polymers has dual roles in altering the texture and taste, thereby increasing the sugar content of the fruit, rendering it much sweeter and therefore more acceptable. The ratio of sugar to acid is an important index in the flavor quality of many ripened fruits and their absolute amounts differ in different fruits.

Ethylene as major phytohormone in fruit ripening

Based on the pattern of ripening process, fruits can be categorized into: (i) climacteric –which continue to ripen after harvesting and there is a ‘respiratory burst’ with dramatic increase in biosynthesis and production of the phytohormone, ethylene, e.g., tomato, banana, mango, etc; (ii) non-climacteric-which do not ripen or do so slowly once harvested, producing basal levels of ethylene and without any increase in the rate of respiration, e.g., strawberry, grape, litchi, etc. The first evidence of the beginning of ethylene synthesis and initiation of ripening is the glistening locular gel surrounding the seeds. This can be seen before any changes in color. Ethylene and CO_2 production continues to increase over the next few days, which then initiates a cascade of changes, culminating into the transformation of the unpalatable, green, hard tomato into an eye-appealing, brightly colored, nutritious and succulent fruit. Therefore, ethylene appears to play a major role in the ripening and maturation of climacteric fruits, which simultaneously undergo other physiological and biochemical changes like cell wall softening, change in pigmentation and color, increase in specific volatiles and development of flavor and aroma, and alterations in the sugar/acid balance (Iqbal et al. 2017). In plants, methionine (Met) serves as the precursor of ethylene. Met is first converted to S-adenosylmethionine (SAM) by the enzyme SAM synthetase. SAM is subsequently metabolized to 1-aminocyclopropane-1-carboxylate (ACC) and 5′-methylthioadenosine by the enzyme ACC synthase (ACS). 5′-methylthioadenosine is then recycled back to Met by the Yang cycle for another round of ethylene biosynthesis, while the ACC is converted to

ethylene by ACC oxidase (ACO). Several isoforms of both ACS and ACO enzymes are known and they are encoded by multigene families. The unripe stage of a climacteric fruit is characterized by low or basal levels of ethylene, mostly regulated by feedback inhibition, under the control of ACS6 and ACS1A enzymes, when ethylene acts as auto-inhibitor of its own synthesis (System 1). However, as the fruit proceeds through the ripening stages towards senescence, unrestrained or autocatalytic ethylene production begins under the regulation of ACS4 and ACS2, so that there is a dramatic exponential increase in ethylene production, popularly known as 'climacteric burst', without any feedback inhibition (System 2). Application of exogenous ethylene to climacteric fruits further stimulates endogenous ethylene production, thereby quickening the ripening process. During System 1, *ACS1A* and *ACS3* are constitutively expressed at the basal levels, whereas *ACS6* shows a higher expression during the pre-climacteric stage. At the onset of System 2-mediated ripening, *ACS4* and *ACS1A* are the most active genes. As the fruit gradually progresses towards ripening, *ACS4* continues to express highly during climacteric phase, whereas *ACS1A* transcript levels decline. The rise in autocatalytic ethylene production results in the induction of *ACS2*, along with the inhibition of *ACS6* and *ACS1A* expression. Such fine tuning of the *ACS* genes is thought to be critical for the switch from pre-climacteric System 1 to climacteric System 2 for the ethylene-dependent ripening in climacteric fruits (Barry et al. 2000).

Ripening Inhibitor (RIN): MADS box-family of transcription factor

MADS box constitutes one of the most extensively studied transcription factor family, usually expressed in a tissue-specific manner, having roles in a number of physiological processes including vegetative growth, flowering, seed development, fruit ripening, abiotic stress response and organ abscission. The name MADS comes from the four letters, viz., M representing Minichromosome Maintenance 1 from *Saccharomyces cerevisiae*, A standing for AGAMOUS from *Arabidopis thaliana*, D representing Deficiens from *Antirrhinum majus*, and S stands for Serum Response Factor from *Homo sapiens*. The MIKC-Type MADS-box transcription factors were initially identified as proteins controlling floral development and leading to the establishment of the ABC Model which explains that dimers or tetramers in different combinations of the MADS-box proteins determine the identity of the four floral whorls, viz., sepals, petals, stamens and carpels (Honma and Goto 2001). This model proposed that SEPALLATA MADS-box proteins (SEPs) form tetramers with other MADS-box proteins and also confer trans-activation property to the complex. The MADS-box transcription factors usually work as multimers. They are characterized by four domains: (i) MADS domain at the N-terminal end (highly conserved DNA binding domain); (ii) Intervening (I) domain (responsible for specificity in the formation of DNA binding dimers); (iii) Keratin (K) Domain (promotes dimerization and other specific protein-protein interactions); and (iv) C-terminal domain (highly variable, responsible for transcriptional activation and assembly of higher order, multimeric protein complexes) (Ng and Yanofsky 2001).

Tomato has long being fostered as the standard model for ripening-related researches because of an efficient sexual hybridization, presence of diploid inheritance, a relatively short generation time and particularly due to the availability of a wide number of mutants. Years of extensive plant breeding studies on tomato have resulted in a valuable germplasm data resource, called the tomato expressed sequence tag (EST) database that houses information about a number of genes, functional during fruit development as well ripening. This led to the development of a range of pleiotropic mutants for ethylene hormone receptors (ETRs), MADS-box transcription factors, often called MADS-RIN, identified by cloning of the ripening inhibitor (rin), COLORLESS NONRIPENING (CNR) and NON-RIPENING (NOR). The discovery of the *rin* locus was an important breakthrough in the study of fruit ripening process. The MADS-RIN factor was found to be a tomato homolog of SEP. *In vitro* analysis showed that homodimer of MADS-RIN binds to MADS domain-specific consensus DNA sites, which is a typical CArG sequence, denoted by (T/A)(T/A)DCCA(A/T) (A/T)(A/T). RIN is the most extensively studied MADS-box factor in fruit ripening, since *rin* mutation has been bred into many commercial tomato varieties to delay ripening and extend the shelf life of the fruit (Martel et al. 2011). Subcellular localization indicated that RIN is a nuclear factor, forming a stable homodimer in a ripened fruit, necessary for its nuclear localization. Several investigations have shown that the *rin* mutation causes alterations of about 241 genes, resulting in a severely inhibited ripening phenotype, including loss of the characteristic burst of ethylene production and respiratory climacteric, normally associated with the onset of ripening, and a severe reduction in pigment accumulation, flavor production and softening (Ito et al. 2017). Fruits with another mutation (*rinG2)* that removed a region consisting of 43 amino acids and including a transcriptional activator domain from the C-terminal of the wild type RIN protein, producing a truncated RIN protein (tRIN), showed a reduced rate of softening and had an extended shelf life, than the wild type climacteric fruits. *RIN* mutants are overall not able to induce the autocatalytic system 2 of ethylene synthesis, causing a drop in the phytohormone level to be able to continue with the process, thereby failing to produce completely ripened fruits (Ito et al. 2020). Auxin has an inhibitory effect on ethylene, but if ethylene is provided at the appropriate time, it can interfere with auxin synthesis and signaling to assist in the process of ripening. In such cases, MADS-RIN can positively regulate the expression of *small auxin-up RNA69* (*Sl-SAUR69*), whose over expression has been seen to decrease the polar auxin transport. So, the MADS-RIN-mediated *SI-SAUR69* activation would initiate premature ripening (Shin et al. 2019).

Regulation of RIN during ripening: studies from mutant analysis

To understand the role played by RIN in the process of ripening, several studies were conducted in which the effect of different mutations of the *rin* locus of tomato on the process of ripening was monitored. Vrebalov et al. (2002) reported that the *rin* mutants never turned red and did not soften and this phenotype lasted for several months or longer. The *rin* mutants have a genomic deletion on chromosome 5 that includes the last exon of *RIN* and a part of the *cis*-acting regulatory region of the neighboring gene, *MACROCALYX* (*MC*), which encodes a

MADS-box transcription factor, regulating sepal size and development of pedicel abscission zones, but does not directly function as a ripening regulator. Transcription of the mutant locus produces a chimeric *RIN-MC* mRNA that lacks the last exon of *RIN* and the first exon of *MC*. The chimeric mRNAs are translated and the mutant proteins accumulate in the fruit cells, such that the ripening-inhibited phenotype is attributed to the lack of a functional *RIN* protein, even though the fusion protein retains its DNA-binding activity. The *RIN-MC* fusion in the *rin* mutant can act as an active transcription factor that has many gene targets and also has a repressor function in contrast to the *RIN* protein. Since *rin* mutation involved a deletion and a fusion of two adjacent genes, viz., *RIN* and *MC*, it was thought to be a loss of function mutation, proving that the wild type RIN regulates the ripening pathway, upstream of ethylene production (Li et al. 2018). RIN binds to more than a thousand genomic regions, including the promoters of diverse ripening-related genes, related to ethylene production, carotenoid biosynthesis, cell wall modification, and other ripening-related transcription factors. Later studies disproved the hypothesis that *rin* is a null mutation. It was proposed that *rin* mutation is a gain of function mutation which converted the protein produced by the *RIN* locus from an activator to a repressor. This is because the chimeric RIN fusion protein contained the DNA binding domain from RIN and a putative repressor motif from the adjacent gene *MC*. Studies conducted by Ito et al. (2017) and Li et al. (2018) showed that when the *rin* mutant allele was targeted by RNA interference or genome editing, the mutant protein got inactivated which partially restored ripening from the non-ripening *rin* phenotype. Such studies consolidated the fact that RIN is not required for the initiation of ripening, but necessary for the continuation of ripening. Initially, it was thought that RIN initiated the process of ripening which was further enhanced by the auto-catalytic up regulation of ethylene biosynthesis genes by ethylene itself. However, it was later observed that endogenous amount of ethylene produced by the plant and fruit was enough to induce ripening, even in the absence of RIN in *rin* mutants which were developed via Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 technology. The inhibition of the initiation of ripening on treatment of the *rin* mutant or *rin* deficient fruits with the ethylene inhibitor, 1-methyl cyclopropane (1-MCP), and the responsiveness of carotenoid biosynthesis genes such as *phytoene synthase 1* (*PSY1*) upon ethylene treatment proved that initiation of ripening was indeed a response to ethylene. However, no amount of ethylene treatment could fully restore ripening in the *RIN* mutant lines. Unlike wild type fruits with active RIN protein being produced, Stage 2 ethylene production could not be induced in the mutant lines (Li et al. 2020). Thus, the initiation of ripening occurs through RIN-independent pathway, but progression of ripening from Stage 1 to Stage 2 requires the expression of the *RIN* gene.

RIN as trans-activator of ripening-related target genes

RIN binds to the ethylene responsive element (ERE), viz., AGCCGCC (GCC-box), present in the upstream regions of ethylene biosynthetic genes like *ACS2*, *ACS4*, etc., and genes like *ETR6*, *EIN3*, *EBF1*, etc., encoding ethylene receptors, thereby up regulating their expression

which in turn leads to autocatalytic up regulation of ethylene, further enhancing the ripening phenomenon. *HB-1* is an HD-zip transcription factor that positively regulates the expression of *ACO1* during fruit development and ripening. *ACO1* is not directly targeted by RIN, but the promoter of the ACO regulator, *HB-1*, is a direct target of RIN. Hence, RIN can indirectly influence *ACO1* expression. Chromatin immunoprecipitation (ChIP) assays using antibodies specific for RIN first identified *ACS2*, the key enzyme for autocatalytic ethylene biosynthesis, whose expression is strictly up regulated in a RIN-dependent manner (Fujisawa et al. 2012). Subsequent ChIP assays also indicated direct RIN binding to the promoter region of the *E8* gene, which encodes the other rate limiting enzyme in ethylene biosynthesis, *ACO*. The regulatory effect of RIN on the expression of *ACS2* was earlier than that of *ACO1* (Li et al. 2011). Silencing of *RIN* gene expression in wild type fruit significantly reduced the transcripts of *ACO1*, *ACS2*, *ACS4* and *ETR3*. However, silencing of the *RIN-MC* fusion gene up regulated the expression of all the genes involved in ethylene perception such as *ERF6*, *ETR3* and *EBF*, indicating that the normal function of RIN is as an activator of ethylene synthesis and perception (Li et al. 2018).

The production of carotenoids increases significantly as ripening proceeds to later stages. Lycopene accumulation during ripening involves the RIN-mediated up regulation of genes encoding enzymes upstream of lycopene biosynthesis in the carotenoid biosynthesis pathway. This process also involves the down regulation of genes encoding enzymes that convert lycopene into other metabolites. Some of the genes which get up regulated during this stage are phytoene synthase 1 (*PSY1*), phytoene desaturase (*PDS*), carotene isomerase (*CRTISO*), lycopene-β cyclase (*LCY-b*), etc. ChIP and qRT-PCR studies revealed that the expression of *PSY1* increases dramatically in response to RIN. The influence of RIN over the regulation of *PSY1* is further consolidated by the fact that in tomato *rin* mutants, no such up regulation of *PSY1* could be detected. RIN-deficient mutants have very low levels of lycopene, and show low amounts of phytoene and alpha-carotene. This indicates how severely carotenoid accumulation is inhibited in the RIN-deficient mutants (Kitagawa et al. 2005). The key enzymes like phytoene synthase1 (PSY1), non-heme hydroxylases (CHY/SlBCH2) and carotene isomerase (*CRTISO*) were inhibited by 90%, 80% and 75%, respectively in RIN-deficient fruits, along with inhibition of other enzymes like 1-D-deoxy xylulose 5–phosphate synthase (DXS1), phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS). Surprisingly, geranylgeranyl pyrophosphate synthase (GGPS2) was not affected, while zeaxanthin epoxidase (ZEP) was found in higher concentration in RIN-deficient fruits. The *PNAE* gene, also regulated by RIN, encodes polyneuridine aldehyde esterase, an enzyme that is required for the formation of sarpagine-type alkaloids.

In case of tomato *rin* mutants, the volatiles derived from different pathways (as measured by high performance liquid chromatography and gas chromatography), contributing to the flavor and aroma, were found to be significantly reduced, as compared to the wild type. Such volatiles include 2-hydroxy-benzaldehyde, 2-isobutylthiazole, (E)-2-heptenal, hexanal, 6-methyl-5-hepten-2-one, (E)-3-Buten-2-one, etc (Klee and Tieman 2018; Zhang et al. 2016).

Genes like *TomLoxC* (encoding lipoxygenase C), *HPL* (encoding hydroperoxidelyase), *PAL3* (encoding L-phenylalanine ammonia lyase), *ADH2* (encoding alcohol dehydrogenase 2), etc., involved in the production of such volatile compounds were found at higher levels in the wild type fruits, but were observed at low levels in *rin* fruits. Electrophoretic mobility shift assay showed RIN binding to the promoter of *LoxC* and *ADH2* (Qin et al. 2012). ChIP analysis further strengthened the idea that the up regulation of such genes was due to increased expression of *RIN*.

Softening of fruits is also a major component of ripening and determines the texture of the fruit. RIN also directly interacts with the genes, *TBG4* and *MAN4* which are involved in cell wall softening, as confirmed by ChIP in vivo, as well as ubiquitin proteasome degradation genes, *SlUBC32* and *PSMD2*. Transcripts of genes related to cell wall metabolism like *CEL2* (encoding cellulase), *PG* (encoding polygalacturonase), *PL* (encoding pectate lyase), *PE1 AND PME9* (encoding pectin methyl esterase), *EXP1* (encoding expansin 1), *XYL1* (encoding β-D xylosidase) were found to be strongly increased with increasing levels of RIN in ripening fruits (Busi et al. 2003).

The *rin* mutants displayed highly reduced level of transcripts of all the aforementioned genes. Although application of exogenous ethylene enhanced the accumulation of these transcripts in the mutants, the fold-increase was much lower, as compared to the wild type. This indicated that RIN and ethylene, acting via ERFs and possibly other transcription factors, are both required for the maximum expression of all the cell wall-metabolic genes.

RIN also carries out the functional regulation of several carbohydrate-related genes, as found from their altered expression in the *rin* mutants. Analysis of the promoter sequences of TIV1 and cFBP, the two proteins involved in sucrose metabolism, showed the presence of one CArG box in the promoter of each gene. Furthermore, RIN can directly bind to the promoter of the *PGK* gene that catalyzes the transfer of a phosphate group from 1, 3-bisphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. The ATP produced could provide the energy required during fruit ripening (Qin et al. 2012). Sucrose metabolism genes, *SlVIF* and *SlVI* are also regulated by RIN.

Not only to the consensus sequence of the target genes, RIN was also found to bind *in vivo* to the CArG-box present in its own promoter, strongly suggesting that RIN autoregulates its own expression. The existence of this autoregulatory mechanism can explain how the rapid increase in the mRNA level of *RIN* at the onset of ripening is controlled (Fujisawa et al. 2012).

RIN controls the function of other transcription factors and MADS-box proteins during ripening

The MADS-RIN can directly or indirectly interact with a number of other MADS-box proteins that might play either positive or negative roles as activators or repressors,

respectively, controlling the expression of genes involved in ripening, either by interacting as heterodimers or tetramers with MADS-RIN, or competing with other MADS-box factors for MADS-RIN binding. RIN activates and regulates the expression of other genes like *NAC-NOR*, *SBP-CNR*, etc., which together serve to up regulate the expression of ethylene biosynthesis genes, along with other genes related to aroma and flavor development, color change, cell wall metabolism, etc. RIN has also been observed to form heterodimers or multimeric complexes with other transcription factors, involved in ripening like HD-ZIP HOMEOBOX PROTEIN 1 (LeHB-1), AGAMOUS LIKE 1 (TAGL1), APETALA2α (SlAP2 α), OPAQUE 2, NAC-NOR, FRUITFUL1 (FUL1), FRUITFUL2 (FUL2), etc. RIN, in association with such transcription factors, induce the expression of several secondary genes involved in fruit ripening. Thus, RIN can be aptly regarded as a master regulator of ripening that directly influences many ripening-associated processes (Lin et al. 2008).

Tomato MADS-RIN interacts with *FUL1/ FUL2*, *TAGL1*, *SlMBP18* and acts like a *SEP*-like protein bridging factor, generating higher order protein complexes with transcription factors including TM4 and SlMBP24 (Li et al. 2019). The *AtFRUITFULL* homologs in tomato, *FUL1* and *FUL2*, are two functionally redundant proteins. Knockdown of both the homologs negatively affected ripening, resulting in low levels of lycopene accumulation. Instead, suppression of either gene alone only caused a limited effect. These could form heterodimers with RIN. A ChIP assay with *FUL1*- and *FUL2*-specific antibodies was conducted, and the results revealed that *FUL* homologs bind to the promoter of *ACS2*, suggesting their roles in climacteric fruit ripening (Shima et al. 2013). The MADS-box protein, *TAGL1*, encodes a homolog of *Arabidopsis SHATTERPROOF* which regulates fruit dehiscence by specifying the identity of the valve margin cells. Tomato fruit, overexpressing *TAGL1*, accumulated more lycopene, whereas RNA interference (RNAi)-mediated reduction in *TAGL1* resulted in an incomplete ripening phenotype, and low ethylene and lycopene levels. This suggests the role of combined role of TAGL1 and MADS-RIN in controlling the carotenoid biosynthesis (Stanley and Yuan 2019).

SlCMB1, another homologue of SEP, is reported to interact with MADS-RIN, TAGL1, MADS1 and SlAP2a, suggesting that SlCMB1 could be a potential new regulator of ethylene and carotenoid biosynthesis during ripening (Li et al. 2020). Studies have also indicated the possible involvement of three other proteins, MADS1, SlMBP8 and SlFYFL in interaction with MADS-RIN that might affect ripening.

Conclusion

Climacteric fruit ripening is well orchestrated and synchronized through regulation of a wide variety of genes, among which RIN appears to play a pivotal role and master switch in ethylene-mediated signaling. Autocatalytic ethylene production and associated characteristics like softening, pigmentation and aroma development are abolished in the *rin* mutants. The reason why RIN is so important component in breeding programs of fruits like tomato is that manipulation of RIN provides an opportunity to enhance the shelf life of the fruits and deal

with the problems of post-harvest loss and spoilage, and improve storage. Although the sensory parameters like color, flavor, taste, texture and aroma altogether make a fruit attractive to the consumers, cell wall softening has an inevitable undesirable effect of making the fruit more susceptible to microbial attack, deterioration in quality and ultimately rottening. Much of the earlier researches were focused on creating transgenic fruits harboring antisense versions of *ACS2* and *ACO1*, silencing the expression of the genes, thereby inhibiting ethylene production and delaying ripening. The much discussed 'Flavr Savr' tomato was also generated in the same manner where antisense RNA technology was used to silence *PG*, to prevent cell wall softening. However, since RIN is a master regulator for most of the ripening-associated genes, fruit ripening can be delayed in a much better and holistic way, if *RIN* expression is silenced through genetic engineering approaches like RNA interference and CRISPR-Cas9 or through mutation breeding. The extended shelf life of *RIN*-mutated fruits, which accumulate substantial amounts of carotenoids, are promising phenotypes for use in breeding, while the excess flesh breakdown and easy peeling found in *RIN*-knockout fruits may have novel applications in the fruit processing industry. RIN mutants with distinct phenotypes could also be used to explore the transcriptional regulation of ripening. However, the number of target genes that were identified in most of the studies is limited, and therefore a large portion of the targets of RIN, and interactions among different ripening regulators remains to be identified. Hence, complete mechanism of fruit ripening has not been fully deciphered yet and demands further research in this area.

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