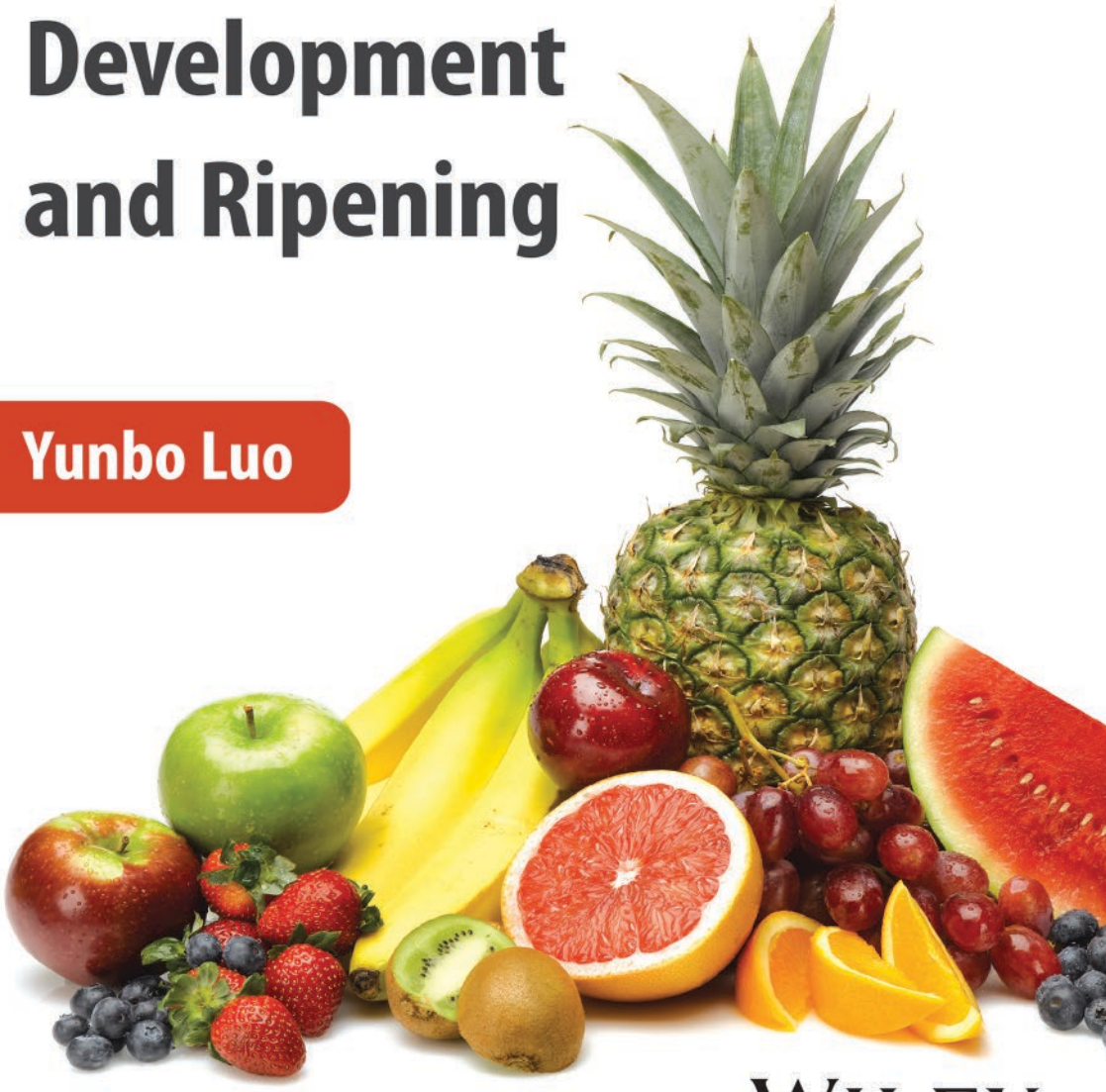
Transcriptional Regulation of Flesh Fruit Development and Ripening

Yunbo Luo



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Preface

Since the 1990s, I have led the laboratory to engage in the research of postharvest biotechnology and molecular biology of fruits and vegetables in China for the first time. Aiming at the major industrial needs of postharvest quality preservation of fruits, significant advances and contribution have been achieved focusing on the important scientific issue of fruit ripening and senescence regulation, provide a new theoretical basis and way for regulating fruit ripening and senescence and improving fruit quality. With years of research accumulation, we have made many achievements in this field worthy of exchange. We have kept close exchanges with many well-known universities and research institutes at home and abroad through academic exchanges, publishing papers and other ways. Our research has driven the development of this discipline in China like a spark, and we are pleased to see that many laboratories have developed, making China an important force in this field.

The process of fruit development and ripening is a critical stage for metabolism and nutrient formation. Fruit development, ripening, and quality formation are the result of a comprehensive regulation of many transcription factors (TFs). Nearly 60 TFs have been reported to play roles in fruit development, ripening, and quality formation in many plant species, including tomato, apple, pear, peach, grape, banana, strawberry, pepper, and Arabidopsis. According to the different transcription factor families that regulate fruit development and ripening, this monograph is comprised of the following eight parts: Overview of the transcriptional regulation of flesh fruit development and ripening (Chap. 1), Screening method for the identification and characterization of transcription factors regulating flesh fruit development and ripening (Chap. 2), MADS-box transcription factors necessary for flesh fruit development and ripening (Chap. 3), NAC transcription factor family regulation of flesh fruit development and ripening (Chap.4), Role of ERF transcription factors in flesh fruit development and ripening (Chap.5), The ARF side of the fruit tuning of flesh fruit development and ripening (Chap.6), HD-ZIPs are involved

in flesh fruit development and ripening (Chap.7), SBP-box transcription factors and flesh fruit development and ripening (Chap.8). Each chapter explains comprehensively analyzing the mechanism of transcriptional regulation of fruit ripening and senescence and investigating the functions and relationships of these TFs have great value in delaying the process of fruit ripening and senescence, effectively maintaining fruit postharvest quality, and extending the storage period.

This book is the first monograph on transcriptional regulation of fruit, fleshy fruit development, ripening and maturity. It embodies the research accumulation and efforts of our research team in the past 30 years and also introduced the globe research progress and achievements in this field in recent years. The content classification of this monograph is very easy for readers in different fields to read. The completion of this book should be grateful to all participants in our group for their hard work on organizing documents, drawings, proofreading, and modification. The book was fulfilled through the joint efforts of all contributors: Daqi Fu, Benzhong Zhu, Hongliang Zhu, Guiqin Qu, Huiqin Tian, Jinlan Xuhui, Shan Li, Yin Gao, Dongyan Cao, Yongfang Yang, Tian Wang, Tongtong Yu, Lanhuan Meng, Yiyang Chu, Cuicui Wang, Ran Li, Xindi Li, Ke Cheng, Peiwen Wu, Lanting Xiang, Shuai Zhang, Liqun Ma. I am very grateful for their suggestions and kind help in pre-paring the manuscripts. Of course, shortcomings in the writing process are unavoidable, and we are eager for reader's criticism to make the book better. I believe that a continuous process of understanding nature from mature aging control to quality formation after harvest. However, the research work is endless, and colleagues have a long way to go.

December 2022

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Beijing, China

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Overview of the Transcriptional Regulation of Flesh Fruit Development and Ripening

1.1 Introduction

As an indispensable part of the human diet, fruit has become increasingly important in daily life. The process of fruit development and ripening is a critical stage for metabolism and nutrient formation. As a result, fruit development, ripening, and the regulation of fruit quality have always been a research hotspot. Fruit development and ripening is a complex but highly regulated physiological and biochemical process; it involves changes in fruit quality such as color, flavor, fragrance, and texture, all of which directly affect the commercial value, postharvest shelf life, and market competitiveness. Fruit development, ripening, and quality formation are the result of a comprehensive regulation of many transcription factors (TFs). Nearly 60 TFs have been reported to play roles in fruit development, ripening, and quality formation in many plant species, including tomato, apple, pear, peach, grape, banana, strawberry, pepper, and *Arabidopsis*. This book focuses on the major transcription factor families that regulate fruit development and ripening, including the MADS-box family, NAC family, ethylene response factor (ERF) family, ARF family, SBP family, and HD-Zip family. In addition, this book introduces methods for the screening and identification of fruit-ripening-related TFs in order to provide theoretical references for the genetic improvement of fruit quality.

1.2 TFs Regulate Fruit Development and Ripening

1.2.1 Overview

The earliest evidence of TFs being involved in fruit ripening and senescence was the discovery of tomato mutants *ripening inhibitor* (*rin*), *nonripening* (*nor*), and *colorless nonripening* (*cnr*). The fruit of these mutants fail to ripen normally;

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during the fruit-ripening process, they can neither synthesize large amounts of ethylene nor respond to external ethylene signal (Vrebalov et al. 2002; Manning et al. 2006; Giovannoni 2007; Gapper et al. 2013; Seymour et al. 2013b; Pesaresi et al. 2014). Studies have further shown that the *rin*, *cnr*, and *nor* mutants arose from mutations in MADS-box, SBP-box, and NAC TFs, respectively (Vrebalov et al. 2002; Manning et al. 2006; Giovannoni 2007). In recent years, with the development of genomics, proteomics, transcriptomics, and metabolomics, as well as technologies in DNA–protein and protein–protein interactions, many TFs related to ripening and senescence have been subsequently isolated and identified in tomato, kiwifruit, strawberry, grape, peach, banana, and longan, including EIN3/EIL, AP2/ERF, MADS-box, NAC, and SBP/SPL. Thus, comprehensively analyzing the mechanism of transcriptional regulation of fruit ripening and senescence and investigating the functions and relationships of these TFs have great value in delaying the process of fruit ripening and senescence, effectively maintaining fruit postharvest quality, and extending the storage period.

1.2.2 Model Plant Species for Studying the Transcriptional Regulation of Fruit Development and Ripening

With a long history of cultivation, tomato (*Solanum lycopersicum*) has a small genome (950 Mb) and a short growth cycle (approximately 40 days from fruit set to ripening). Additionally, tomato is distantly related to model plant species such as *Arabidopsis*, rice, and maize. As a result, it has become a model plant species for studying fruit development and ripening in fleshy fruit. A large number of tomato germplasm resources and mutant libraries are currently available (https://solgenomics.net/projects/tomato100), as are high-density genetic map and expressed sequence tag (EST) resources (https://solgenomics.net). Moreover, transient expression and stable transformation technologies are mature in tomato. In addition, the high-quality whole-genome sequence analysis of domesticated tomato was completed in May 2012 (The Tomato Genome Consortium 2012). This information has tremendously promoted the research on tomato functional genomics and molecular genetics. Fruit ripening involves massive regulation of metabolic pathways and significant changes in physiological and biochemical properties. The ripening process requires precise transcriptional regulation of the temporal expression of a series of genes. Thus, studying the functions of ripening-related TFs in tomato has become a research hotspot in plant biology. Bioinformatic analysis has predicted that there are at least 62 TF families in tomato and that they play important regulatory roles in plant hormone biosynthesis, cell expansion, cell wall metabolism, regulation of fruit ripening, and pigment accumulation (Zhang et al. 2011). Investigating the functions of these TFs and their relationships is the foundation of systemically studying tomato fruit ripening and senescence

and its regulatory mechanism; this information will provide theoretical reference for targeted tomato fruit quality improvement.

During the early developmental stages of tomato (from fruit set to breaker stage), 11 TF families, including AP2-EREBP, AUX/IAA, GBP, bHLH, HB, ARF, LIM, SPB, HSF, FHA, and PBF-2-Like, are active. At the fruit-ripening stage (breaker stage to fully ripened), seven TF families, including VBI3-VP1, MADS, C2H2, CCAAT, HMG, G2-Like, and MYB-related, have high expression levels. During the process of fruit development and ripening, five TF families, including GRAS, MBF1, MYB, bZIP, and WRKY, have stable expression levels. Among them, the WRKY family exhibited two distinct expression patterns: some WRKY family members exhibited significantly increased expression after the breaker stage, while some other WRKY family members gradually decreased before the breaker stage and gradually increased during fruit ripening (Rohrmann et al. 2011, 2012; Mori et al. 2013). TF families such as C3H, PHD, SET, and Trihelix exhibited very low expression in tomato fruit and may not significantly affect fruit development and ripening. Additionally, many TF families, including ABI3-VP1, bZIP, bHLH, and C2C2-Dof, are expressed in non-fruit tissues, such as leaves, stems, and flowers (Rohrmann et al. 2012).

1.2.3 TF Families that Regulate Fruit Development and Ripening

This book mainly introduces the transcriptional regulation of fruit development and ripening by major TF families, including MADS-box, NAC, ERF, ARF, SBP, and HD-ZIP.

1.2.3.1 MADS-box Family Regulates Fruit Ripening

Members of the MADS-box family are the most extensively studied TFs that play important roles in regulating fruit ripening and senescence. In 2002, Vrebalov et al. found that the tomato *rin* mutants could not ripen normally due to a mutation in a gene encoding a MADS-box TF. Positional cloning revealed that the genomic DNA of the *rin* mutants had a 3 kb deletion, which led to a fusion of two neighboring MADS-box TF genes (*SlMADS-RIN* and *SlMADS-MC*), thereby losing its function (Vrebalov et al. 2002). A subsequent study showed that SlMADS-RIN could bind to the promoter of the ethylene biosynthetic gene *SlACS2* (Ito et al. 2008), indicating that the SlMADS-RIN TF is upstream of ethylene signaling transduction and regulates ethylene biosynthesis. However, the regulatory role of SlMADS-RIN in fruit ripening and senescence is not only through its effect on ethylene signaling, it also acts on other downstream genes. A large number of comparative studies on SlMADS-RIN downstream target genes using proteomics, chromatin immunoprecipitation (ChIP), and electrophoretic mobility shift assay (EMSA) further revealed the transcriptional regulatory network of SlMADS-RIN.

To date, it has been found that SlMADS-RIN downstream target genes include two main categories: ripening-related genes (a, ethylene biosynthetic and responsive genes, such as *SlACS2*, *SlACS4*, *NR*, and *E8*; b, genes encoding cell wall-modifying enzymes, such as *PG*, *TBG4*, and *SlEXP1*; c, genes encoding enzymes for carotenoids metabolism, such as *PSY*; d, genes related to the metabolism of aromatic substances, such as *LOX*, *ADH2*, and *HPL*; e, glycolysis-related genes, such as *PGK*) and TFs (including *RIN* itself, *CNR*, and *SlHB1*) (Fujisawa et al. 2011; Martel et al. 2011; Qin et al. 2012). Fujisawa et al. identified a large number of RIN downstream target genes using transcriptomics and ChIP-chip technologies and found them to be involved in more than 60 biological pathways related to fruit ripening (Fujisawa et al. 2012, 2013). Recently, Wang et al. combined quantitative nuclear proteome analysis with ChIP and EMSA and confirmed that RIN could directly bind to the promoter regions of *SlUBC32* and *PSMB2A*, two genes encoding ubiquitin E2 enzymes, to regulate their expression. Moreover, RIN could affect the level of ubiquitination of many nuclear proteins in the fruit (Wang et al. 2014). RIN can also directly bind to the promoter of *MIR172a* to regulate microRNA accumulation in tomato fruit, thereby further affecting ripening (Gao et al. 2015). In addition to SlMADS-RIN, other tomato MADS TFs, such as TAGL1 (Itkin et al. 2009; Vrebalov et al. 2009), TDR4/FUL1 (Bemer et al. 2012), and MBP7/FUL2 (Bemer et al. 2012; Shima et al. 2013), also play roles in fruit ripening, where TAGL1 is required for normal tomato fruit ripening (Itkin et al. 2009; Vrebalov et al. 2009). Fujisawa et al. identified 860 FUL1 and FUL2 target genes by ChIP-chip, among which 262 were also the target genes of RIN (Fujisawa et al. 2014). It is worth noting that tomato SlMADS1 is a negative regulator of fruit ripening; inhibiting *SlMADS1* expression reduced the ripening time by 2–4 days, significantly increased the carotenoid content in tomato fruit, and significantly upregulated the expression of ethylene biosynthesis-related genes *ACS1A*, *ACS2*, *ACS6*, and *ACO1*. Additionally, the expression of ethylene-responsive genes E4 and E8 was significantly increased, and there was a protein interaction between SlMADS1 and RIN (Dong et al. 2013). Another MADS-box TF SlFYFL is also a negative regulator of ripening. Overexpressing *SlFYFL* resulted in delayed fruit ripening, decreased carotenoid levels, inhibited ethylene biosynthesis, and repressed expression of genes related to ethylene biosynthesis. Furthermore, SlFYFL interacts with SlMADS1 and RIN (Xie et al. 2014). The results of these studies greatly enriched the transcriptional regulatory network of tomato fruit ripening and senescence. Moreover, other studies have shown that the MADS-box gene family is also involved in regulating various important physiological processes, including regulation of seed formation, vegetative organ development, fruit ripening, flower and fruit abscission, root development, and seed embryonic development (Kitazawa Y et al. 2022; Zhang et al. 2022). In addition, MADS-box TFs have been found to be related to fruit ripening and senescence in other plant species, such as grape (Boss et al. 2001), strawberry (Seymour et al. 2011), banana

(Liu et al. 2009a; Elitzur et al. 2010), peach (Tadiello et al. 2009), and bilberry (Jaakola et al. 2010). It is worth noting that in banana, MADS-box genes have been confirmed to play roles in fruit ripening (Liu et al. 2009a; Elitzur et al. 2010). Among these genes, MuMADS1 interacts with ubiquitin-activating enzyme E1 MuUBA (Liu et al. 2013a). MA-MADS5 could also directly regulate ripening-related genes, such as *MA-SPS*, *MA-ACS1*, *MA-ACO1*, *MA-EXP1*, and *MA-lec* (Roy et al. 2012), suggesting that the function of MADS-box TFs in regulating fruit ripening in other plant species (both dicot and monocot) is conserved. Interestingly, although *MaMADS2* is highly homologous to the tomato *RIN* gene, overexpressing *MaMADS2* in tomato *rin* mutants could not restore mutant phenotypes (Elitzur et al. 2010), indicating that differences exist in the regulation mechanism of fruit ripening by MADS-box TFs in different plant species.

1.2.3.2 NAC Family Regulates Fruit Ripening

NAC TFs are plant-specific TFs that have highly conserved sequences consisting of 160-amino acid at the N-terminus, which can be divided into five subdomains (A–E) and may be responsible for the binding of DNA and other proteins (Ernst et al. 2004). The C-terminus exhibits transcriptional activity and is responsible for the activation or repression of their target genes (Ernst et al. 2004). Through binding to the core CGT[G/A] or GCTT on the NAC recognition sequence (NACRS) or NAC-binding sequence (NACBS) in promoters, NAC TFs transcriptionally regulate their target genes and further regulate various biological processes (Lindemose et al. 2014), including fruit ripening and senescence, as well as changes in fruit color, aroma, and texture during this process. The tomato *nonripening* (*nor*) mutants fail to ripen due to a mutation in a NAC domain gene (Martel et al. 2011). Two other NAC TFs related to fruit ripening have been discovered recently in tomato: SlNAC1 (Ma et al. 2014) and SlNAC4 (Ma et al. 2014). In tomato fruit overexpressing *SlNAC1*, the expression of carotenoids and ethylene biosynthetic genes was repressed, causing fruit ripening to be inhibited. However, the pericarp of the fruit was thinner and the level of abscisic acid (ABA) was increased, leading to earlier fruit softening. This result indicates that SlNAC1 affects fruit ripening through an ethylene and ABA-dependent signaling pathway (Ma et al. 2014). In contrast, SlNAC4 is a positive regulator of fruit ripening; inhibiting *SlNAC4* expression resulted in delayed fruit ripening, reduced synthesis of ethylene and carotenoids, and repressed expression of genes related to chlorophyll degradation and fruit ripening. However, the target genes directly regulated by SlNAC4 are still unknown (Zhu et al. 2014). In *Citrus sinensis*, *CitNAC* expression was detected in the fruit during fruit ripening and senescence, suggesting that fruit ripening and senescence in *Citrus sinensis* may be regulated by CitNAC (Liu et al. 2009b). Recently, Gao et al. reassessed the function of the nor natural mutants. The fact is that NOR is a key transcription factor regulating tomato fruit ripening, involving in the regulation of ethylene biosynthesis, carotenoid accumulation, fruit softening and flavor

volatiles metabolism of tomato fruit (Gao et al. 2020, 2022). In addition, NOR could bind to and activate the promoter of SlDML2 (DNA demethylase), affect the promoter methylation level of flavor volatiles biosynthesis genes, and regulate tomato fruit flavor volatiles accumulation via interacting with SlDML2 (Gao et al. 2022). Other transcription factors of NAC family in tomato, SlNAM1/2/3, also participate in tomato fruit ripening. As positive regulators of tomato fruit ripening, they could directly bind to and activate the promoters of SlACS2 and SlACS4, as a result, regulate ethylene production (Gao et al. 2021; Lin et al. 2022). In the fruit of *Actinidia arguta*, AaNAC1/2/3/4 could directly bind to the promoter of *TERPENE SYNTHASE 1* (*AaTPS1*), whereas it could not bind to the promoter of *AcTPS1* in *Actinidia chinensis* fruit (a mutation was present in the NAC-binding element of its promoter). As a result, the aroma profiles of these two types of kiwifruit were different (Nieuwenhuizen et al. 2015). In peach, a NAC TF, Designated Blood (BL), is tightly associated with fruit color (Zhou et al. 2015). DkNAC1/3/5/6 in persimmon is also related to fruit deastringency (Min et al. 2015). EjNAC1 directly regulates the expression of EjPAL1 and Ej4CL1 and functions in the lignification of loquat fruit (Xu et al. 2015). Among the six NAC TFs in banana, MaNAC1–MaNAC6, the expression of *MaNAC1* and *MaNAC2* was significantly induced during the fruit-ripening process and the promoter of *MaNAC2* is activated by external ethylene (Shan et al. 2012). Further studies revealed that MaNAC1 and MaNAC2 can directly bind to the promoter of *MaGRL*, a negative regulator of ethylene signaling, to inhibit its activity. These results indicate that NAC TFs are involved in the regulation of fruit ripening and senescence by influencing ethylene signaling. Studies in rose flowers have found that RhNAC2 could bind to the promoter of a cell wall-modifying gene *RhEXP4A*; RhNAC3 could bind to the promoters of five stress-related genes including *SnRK* (RU09156), *PP2C* (RU23063), *TIP* (RU01501), *GST* (RU07111), and *P5CS* (RU20896); RhNAC100 could bind to the promoter of one cellulose synthase gene, *RhCesA2*, as well as two aquaporin genes, *RhPIP1.1* and *RhPIP2.1*, to regulate postharvest dehydration and senescence in rose flower (Dai et al. 2012; Pei et al. 2013; Jiang et al. 2014a, 2014b). Thus, further investigating and identifying downstream target genes of fruit-ripening-related NAC TFs will deepen the understanding of the regulatory mechanism of NAC TFs in fruit ripening and senescence.

1.2.3.3 ERF Family Regulates Fruit Ripening

The ERF subfamily mainly includes two classes—DREB and ERF—among which ERFs are involved in fruit ripening and senescence and have been in the spotlight. In the earliest report, there were five ERF members in tomato: SlERF1–SlERF4 and SlERF3b. SlERF1 and SlERF4 are transcriptional activators, while SlERF3 is a transcriptional inhibitor (Tournier et al. 2003). *SlERF1* overexpression led to ethylene triple response in etiolated seedlings of transgenic tomatoes, while antisense *SlERF1* resulted in longer shelf life (Li et al. 2007). The expression level

of *SlERF2* increases during fruit ripening and senescence (Tournier et al. 2003). *SlERF2* expression is also induced by ethylene; it binds to the DRE element on the *SlACO3* promoter to transcriptionally regulate the feedback loop of ethylene production (Zhang et al. 2009). Using RNAi to inhibit the expression of *SlERF6* could increase the content of carotenoids in tomato fruit and induce the expression of ethylene biosynthesis-related genes *ACS2*, *ACO1*, and *ACO3*. This finding indicates that SlERF6, similar to SlAP2a, could directly or indirectly inhibit the accumulation of carotenoids and ethylene biosynthesis and is a negative regulator of fruit ripening (Li et al. 2012). Recent studies have found that tomato *SlERF.B3* is a multifunctional regulatory gene (Liu et al. 2013b, 2014); the fruit phenotype of the transgenic tomato with the chimeric dominant repressor version (ERF.B3-SRDX) exhibited many changes, including smaller fruit, delayed ripening, enhanced ethylene production, accelerated softening, decreased level of lycopene, and increased level of β-carotene, which resulted in an orange color of the fruit. Correspondingly, fruit of the transgenic tomato with ERF.B3-SRDX had higher expression levels of ethylene biosynthetic genes and genes related to fruit ripening and softening, while genes related to carotenoid biosynthesis were downregulated. In addition, other ripening regulators exhibited significantly different expression levels, including *RIN*, *NOR*, *CNR*, *HB-1*, and *ERF* members (Liu et al. 2014). Deng et al. (2022) demonstrated that SlERF.F12-mediated transcriptional repression of key ripening-related genes ACS2, ACS4, PL, is dependent on the presence of its C-terminal EAR motif (Deng et al. 2022). ERF TFs have been reported to play roles in fruit ripening and senescence in apple, plum, kiwifruit, papaya, persimmon, and banana (Wang et al. 2007; El-Sharkawy et al. 2009; Yin et al. 2010, 2012; Fabi et al. 2012; Min et al. 2012, 2014; Li et al. 2013; Xiao et al. 2013; An et al. 2020). Kiwifruit ERF TFs can directly bind to the promoters of fruit-ripening-related genes to transcriptionally regulate ripening and senescence. AdERF9, which contains the EAR repressor domain, can bind to the promoter of cell wall metabolism-related gene *AdXET5*, which does not contain a GCC box, and inhibit *AdXET5* transcription. This activity suggests that ERFs can also bind to certain unknown *cis*-acting elements (Yin et al. 2010). There are at least 20 deastringency-responsive *DkERF* genes in persimmon fruit, including *DkERF1*, *6*, *9*, *10*, *19*, and *22* (Min et al. 2012, 2014; Yin et al. 2012). Further study has found that DkERF10 and DkERF22 could regulate the promoters of deastringency-related genes *DkADH1* and *DkPDC2*, respectively, while DkERF9 and DkERF10 recognize the promoter of *DkPDC2*, indicating differences among the target genes transcriptionally regulated by DkERF during the process of deastringency (Min et al. 2012, 2014). Two ERF genes in banana (MaERF9 and MaERF11) are closely associated with fruit ripening. MaERF9, as a transcriptional activator, can activate the promoter of ethylene biosynthesis-related gene *MaACO1*, while MaERF11, as a transcriptional repressor, can inhibit the promoter activity of ethylene biosynthesis-related genes *MaACO1* and *MaACS1*. These results showed that ERF TFs

control ethylene production via feedback regulation on ethylene biosynthesis-related gene expression, thereby playing a role in regulating fruit ripening (Xiao et al. 2013). The above results indicate that different ERF members may have different regulatory mechanisms during fruit ripening and senescence.

1.2.3.4 ARF Family Regulates Fruit Ripening

Auxin is an important plant hormone involved in plant growth and development. The auxin response factor (ARF) family participates in auxin signal transduction.

ARF proteins consist of three functional domains: a conserved N-terminal DNA-binding domain, a nonconserved middle region with transcriptional activator or repressor activity, and a conserved C-terminal dimerization domain. The expression pattern of ARFs has shown that ARFs are expressed in different organs and different developmental stages in various species. Research in the past 20 years has revealed that ARFs function throughout plant growth and development and are involved in the transcriptional regulation of numerous biological processes related to growth and development. These processes include embryo development, leaf expansion and senescence, lateral root growth, floral organ development and senescence, fruit development, seed development, and responses to external stresses. Many studies have investigated the function of the ARF family in fruit ripening. For example, SlARF4 negatively regulates starch content during tomato fruit ripening (Sagar et al. 2013). SlARF7 negatively regulates the process of fruit set in tomato (De Jong et al. 2011). SlARF9 regulates early fruit development in tomato by negatively regulating cell division (De Jong et al. 2015). SlARF4 participates in the molecular mechanism of auxin and abscisic acid antagonistic regulation of ascorbic acid (AsA) production in tomato (Xu et al. 2022).

1.2.3.5 SBP Family Regulates Fruit Ripening

The SBP gene family is a TF family unique to plants. TFs from the SBP gene family all contain a conserved SBP domain consisting of 76 amino acid residues (SBP-DBD). SBP-DBD has three antiparallel β-sheets at the N-terminus and one or two short helices at the C-terminus. The SBP gene family is broadly involved in the regulation of plant metabolism, growth, and development as well as responses to environmental stresses, such as hormonal responses, the development of fruit, flower, and spores, and defense against pathogen infections. One SBP gene, *LeSPL-CNR*, encodes a key TF that regulates tomato fruit ripening; mutants from DNA methylation in its promoter region significantly inhibited fruit ripening. The fruit of the tomato *cnr* mutants failed to ripen; the pericarp was yellow, and the fruit retained a hard texture even at later stages. The expression of genes encoding many ripening-related enzymes was affected. For example, the synthesis of carotenoids in the pericarp was decreased (Fraser et al. 2001). Compared to wild-type fruit at the same stage, the mutants exhibited a lower level and lower activity of polygalacturonase and pectinesterase (Eriksson et al. 2004).To further identify the

CNR function with CRISPR/Cas 9 in tomato fruit ripening, Gao et al. got the homozygous CRISPR/Cas9-*cnr* mutants with Cas9-free after three generation planting. The phenotype of *CNR* knockout fruits is completely different from that of *cnr* mutants. CRISPR-cnr fruits can turn red, while Cnr mutant ripening fruits appear yellow. Ripening-related genes expression profile seems also different in Cnr mutant and CRISPR-cnr ripen fruits by RNA-Seq. The above results from CRISPR-cnr fruit indicated that the function of CNR in tomato fruit-ripening control still need to be further validated (Gao et al. 2019).

1.2.3.6 HD-ZIP Family Regulates Fruit Ripening

Homeodomain leucine zipper (HD-ZIP) TFs are unique to plants and widely present in most plant species, from moss to higher plants. Based on sequence homology, gene structure, physiological function, and other characteristics, the HD-ZIP TF family can be divided into four classes (I, II, III, IV) (Hu et al. 2012). HD- ZIP Class I proteins are involved in regulating fruit ripening. Class II HD-ZIP proteins are involved in regulating flowering time. Class III HD-ZIP members mainly regulate the development of apical meristems, vascular tissue, and adaxial portions of lateral organs. Class IV HD-ZIP genes are expressed specifically in plant epidermal cells and mainly regulate epidermal cell differentiation, root growth, anthocyanin accumulation, and trichome formation (Fang et al. 2021; Zhang et al. 2021) For example, LeHB-1, a member of the HD-ZIP family, can interact with the promoter of *LeACO1* (an ACC oxidase gene expressed during ripening). Reduced expression of *LeHB-1* led to inhibited ripening, while increasing its expression resulted in altered floral organ morphology in tomato, including the production of multiple flowers within one sepal whorl, fusion of sepals and petals, and the conversion of sepals into carpel-like structures.

1.2.4 Relationships among TF Families

A series of precise transcriptional regulations are present during the tomato fruit-ripening process. Many genes with important biological functions are usually regulated by multiple TFs or regulated at different levels. Furthermore, TFs can self-regulate or regulate each other at the transcriptional level. Rohrmann et al. conducted a correlation analysis of the expression of 134 TFs in fruit (tomato-*Arabidopsis* cross-species coexpression analysis) using qRT-PCR. Through Pearson's correlation analysis, 91 TFs were found to be present in a complex correlation network (Rohrmann et al. 2012). Martel et al. found that binding of SlMADS-RIN to its target loci required the presence of SlSPL-CNR or the gene product regulated by it (Martel et al. 2011). Karlova et al. showed that SlAP2a was downstream of SlNAC-NOR, SlMADS-RIN, and SlSPL-CNR; SlSPL-CNR could directly bind to the promoter of *SlAP2a*, while SlAP2a could negatively regulate the expression of *SlSPL-CNR*. The interactions among MADS TFs are

crucial for their functions (Karlova et al. 2011). Leserberg et al. (2008) and Dong et al. (2013) used yeast two-hybrid experiments and found that SlFUL2 interacted with SlMADS-RIN, while SlMADS1 could interact with SlMADS-RIN. Bemer et al. (2012) and Shima et al. (2013) used bimolecular fluorescence complementation (BiFC) and yeast two-hybrid screening, respectively, and confirmed that both SlFUL1 and SlFUL2 could interact with SlMADS-RIN, although SlFUL1 and SlFUL2 did not interact with each other. Fujisawa et al. (2014) conducted a gel retardation assay and found that SlFUL2, SlMADS-RIN, and SlTAGL1 could form tetramers, while SlFUL1 and SlFUL2 could form complexes with SlTAGL1 separately, with SlFUL2-TAGL1 as the predominating form. The direct evidence described above has provided opportunities for revealing the interacting network of tomato fruit-ripening-related TFs and elucidating their regulation of downstream genes, including ripening-related enzymes and receptor proteins at the level of transcription, translation, protein structure, and functional correlation.

1.3 Methods of Screening and Identifying Ripening-related TFs

The methods of screening and identifying ripening-related TFs mainly involve using bioinformatics to screen candidate genes and identifying gene functions by virus-induced gene silencing (VIGS), generating transgenic plants, and gene editing. VIGS uses virus-carrying target gene fragments to infect plants and induce plant endogenous gene silencing and phenotypic changes. In this way, the function of the target gene can be studied based on phenotypic variations. The advantages of using VIGS include short period, rapid results, and high throughput, while the disadvantages are limited vectors, presence of off-target genes, and difficult-to-control environmental conditions. Plant transgenic technology takes external genes originated from animals, plants, microbes, or any other organism, even artificially synthesized genes and then transforms them into the genome of the receiving plant via biological, physical, or chemical approaches that provide genetic stability in the receiving plant and achieve a stable expression and genetic traits against the new genetic background. This approach has the advantage of obtaining research materials with genetic stability but is limited by the long period required to complete the process. Gene editing refers to the technology that utilizes external DNA elements to perform genome modification at specific loci on the chromosome to obtain organisms that contain genetic changes in the desired genomic sequences. This technology can be highly specific to a certain locus in the genome by using certain nucleases. It has advantages of simple design, fast construction, and low cost, but it has the limitation of including possible off-target genes.