Genetic and epigenetic regulation of tomato fruit ripening

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Thesis

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Contents

Chapter 1

General Introduction

Fruits

Fleshy fruits, with their many appearances, flavours, and aromas are an enjoyable and healthy component of the human diet. Fruits are the mature reproductive organs of angiosperms, which develop from the ovaries of flowers. The ovules within the ovary develop to seeds after double fertilisation and the ovaries become fruits to protect seeds and aid dispersal. During evolution, plants have evolved flowers to attract insect or animal pollinators to help fertilize the ovules to increase the chances for successful pollination and fertilization. Although seeds can be expelled by wind or water, angiosperms also have evolved fruits with bright colours, fleshy textures, pleasant aromas and tastes to attract animals to help disseminate seeds over much longer distances, even to another continent.

Angiosperm fruits are mature ovaries of flowers. The ovary wall develops into the pericarp, usually being the major component of a fruit, which consists of three layers from the outside inwards: the exocarp, mesocarp and endocarp which can expand and become fleshy, or dry, and whose thickness varies in different fruits. Fertilized ovules develop into seeds within the protection of the pericarp (**Fig. 1A**).

Fruits can be categorized by many characteristics, for instance in the distinction of fleshy from dry fruits by the water content of the pericarp, climacteric from non-climacteric fruits by whether there is an ethylene burst at the onset of ripening, and others such as dehiscence or indehiscence. The silique of *Arabidopsis* is a typical dry and dehiscent fruit, while tomatoes and peaches are fleshy, non-dehiscent, and climacteric fruits.

Tomato domestication and fruit development

Tomato (*Solanum lycopersicum*) belongs to the Solanaceae family, comprising other common vegetables, like eggplants (*S. melongena*) and potatoes (*S. tuberosum*). *Lycopersicum*, meaning "wolf peach" in Greek, was classified as a distinct genus in 1694 by Tournefort.

The origin of tomatoes is the Andean region, but where they were domesticated is still unclear. There are two hypotheses for the location of tomato domestication, one is in Mexico, which was proposed by de Candolle in 1886 (de Candolle, 1886), and the other is in Peru, proposed by Jenkins in 1948 (Jenkins, 1948). Tomato fruits have changed a lot during domestication with improved resistance against biotic and abiotic stresses due to artificial selection, the increase in single fruit size and the number of fruits per plant which increase the yield, and higher sugar content together with more aromas that improve the taste and flavour. The wild ancestor *Solanum pimpinellifolium* fruits are quite small weighing only ~1 gram with two locules. However, cultivated tomato fruits can weight 500 gram with more than ten locules (Rodríguez-Leal et al., 2017). Several key genes have been identified as driving the domestication. *SUN*, a major gene controlling fruit elongation, is crucial to control fruit shape (round or

elongated) of tomato (Xiao et al., 2008). The cell cycle–control gene *fw2.2* negatively regulates cell division, and the selection of a weak allele during domestication increased fruit size (Frary et al., 2000). The increased locule number of *fasciated* (*fas*), an intrachromosomal inversion leading to deregulation of the tomato ortholog of CLV3 (Barrero and Tanksley, 2004), resulted in a 50% gain of fruit size (Cong et al., 2008). The classical CLAVATA-WUSCHEL (CLV-WUS) stem cell circuit is also an accelerator of domestication (Somssich et al., 2016). Mutations in *CLV3*, a signalling peptide gene in the CLV-WUS loop, led to much increased locule numbers that boosted tomato yield (Xu et al., 2015).

The domesticated tomato was imported to Europe in the 16th century by the Spanish and then was rapidly spread to the rest of the world. Now it is an important component of different cuisines and is one of the most produced crops in European greenhouses.

The successful pollination and fertilization of a flower is the signal to start growth of the fruit, which is characterised by fast size increase that results from cell divisions followed by cell expansion. There are three phases defined after the fruit set, the cell division phase, the cell expansion phase and the ripening phase (Gillaspy et al., 1993) (**Fig. 1B**). In the cell division phase of approximately 7~10 days (Mapelli and National, 1978), the final cell numbers are determined, resulting in some fruit size increase. The fruit size mostly increases during the cell expansion phase with an increase in cell volume with little additional cell division in the mesocarp, but continued division in the exocarp (resulting in smaller cells) until the mature green (MG) stage, and after that barely changes. The Breaker stage (Br) is defined as the initiation of ripening when the first colour change at the blossom end can be observed. Usually, it takes 35-50 days for a tomato to reach the ripening stage, and an additional 5 to 10 days to become fully red.

Tomato fruits, like those in other angiosperms, develop from ovaries, in which the ovary wall develops to become the fleshy thick pericarp. The mesocarp, containing vascular bundles and many layers of the highly vacuolated parenchymatous cells, is the major part of tomato pericarp (**Fig. 1C**). Seeds develop from ovules if fertilization is accomplished, but when seed set is impaired the fruits remain small, as there is a positive correlation between seed number and the final fruit size (Varga and Bruinsma, 1976). Divided by the septa and filled with gel, the locules are where the seeds grow, and their number is dependent on the number of carpels in a flower. The central parenchymatous axis of the fruit is called columella, which develops the placenta, and the peripheral part of the placenta develops into the gel.

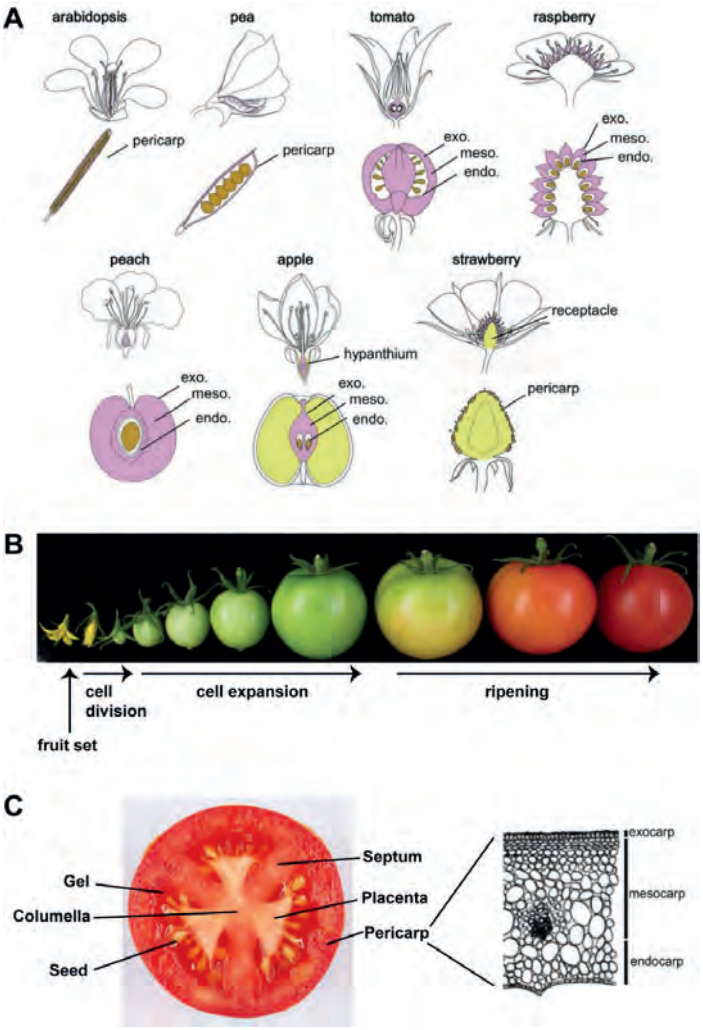

Figure 1. Fleshy fruit types and their morphology. **(A)** Floral tissue origin of the fruit. Ovary and ovary-derived tissue are represented in purple and accessory tissues in yellow. The pericarp, which originates from the ovary wall, can be divided into several layers: exocarp (exo.), mesocarp (meso.), and endocarp (endo.). Seeds are represented in brown. **(B)** A time series of tomato fruit development and ripening from flower to the red ripe stage. **(C)** Tomato fruit anatomy. The cross-section and pericarp layers of a ripe tomato. Completed from Karlova *et al*., Transcriptional control of fleshy fruit development and ripening, *Journal of Experimental Botany*, 2014, Vol. 65, No. 16, pp. 4527–4541, by permission of Oxford University Press.

The economic and scientific importance of tomato

With fleshy fruits containing nutrients, minerals and antioxidants that are important for the human diet, tomato ranks among the most consumed vegetables in the world. The scale of tomato business now is more than 50 billion US dollars (Vincent et al., 2013) with about 5 million hectares planted and a yearly

yield of 37.1 million metric tonnes. Tomato is a major crop in Dutch greenhouse horticulture and the Netherlands is the biggest tomato producer in Europe.

Tomato is also an excellent model plant for climacteric and fleshy fruit studies (Alexander, 2002; Giovannoni, 2004). Tomato is diploid and autogamous, with a relatively simple and small genome (approximately 950 Mb) that is relatively easy to study. Besides, the plenty and well-established genetic resources, like the bacterial artificial chromosome (BAC) libraries, expressed sequence tags (ESTs) and molecular markers of multiple wild species, introgression lines and mutants, are available and easy to access. Moreover, the recent developments in high-throughput sequencing make it possible to sequence many varieties (Lin et al., 2014). The tomato genome was first sequenced in 2012 (Sato et al., 2012) and improved assemblies and annotations have appeared regularly since then. Also, resequencing of genomes from a selection of 84 tomato accessions and related wild species has resulted in a wealth of information (Aflitos et al., 2014). In addition, the relative ease of stable plant transformation and distinct ripening phenotypes make it as an outstanding model for fruit ripening and gene function study.

Therefore, knowledge of tomato fruit ripening is vital for both commercial breeding and fruit ripening studies.

Changes during tomato fruit ripening

Fruit ripening involves changes in biochemical and physiological processes, resulting in changes in colour, flavour and texture (Karlova et al., 2014).

During ripening, with the accumulation of carotenoids and the degradation of chlorophyll (Cunningham and Gantt, 1998), when chloroplasts turn into chromoplasts, fruit colour changes from green to red, yellow or orange. The red colour of ripe fruits is caused by lycopene (Shneour and Zabin, 1959), a product of carotenoid biosynthesis, while other carotenoids in the pathway are orange, yellow, or colourless. Carotenoids in plants are synthesized from the 5-carbon compound isopentenyl diphosphate (IPP). The first rate-limiting step in carotenoid biosynthesis is the conversion from geranylgeranyl diphosphate (GGPP) to phytoene, requiring the catalysis by phytoene synthase (PSY) that is encoded by *PSY1* (chromoplast-specific) and *PSY2* (Fraser et al., 2002; Bartley et al., 1992). The naturally occurring mutant allele of *PSY1*, *r*, produces pale yellow flowers and yellow fruits (Ray et al., 1992; Fray and Grierson, 1993) since the mutation blocks the synthesis of lycopene. Other carotenoid pathway enzyme genes, like *PHYTOENE DESATURASE* (*PDS*), *Z-CAROTENE DESATURASE* (*ZDS*) and *LYCOPENE B-CYCLASE* (*LCYB*), are also major regulators of fruit colour change (Cunningham and Gantt, 1998).

Fruit texture changes from firm at the beginning of ripening (Br) to soft when fruits are completely ripe. Fruit firmness is an important trait in tomato production as firmer texture reduces the loss in long-distance transportation and extends shelf life. Cell wall modification, resulting from structural changes

in polysaccharides (Hyodo et al., 2013) that include pectin, cellulose and hemicelluloses degradation, is the main cause of fruit texture change. *POLYGALACTURONASE* (*PG*) (Giovannoni et al., 1989), *PECTATE LYASE* (*PL*) (Uluisik et al., 2016) and *CELLULASE 2* (*CEL2*) (Lashbrook et al., 1994) are major genes encoding enzymes that digest polysaccharides, although many more exist in the tomato genome. Expression of these genes goes up dramatically (more than 5,000 times) during ripening (**Chapter 2** of this thesis), consistent with the fruit texture change from firm to soft. However, attempts to improve fruit texture by silencing the polysaccharide degrading-enzyme genes so far has only been successful for *PL* (Uluisik et al., 2016), not for *PG* (Smith et al., 1990) or other similar genes (Brummell et al., 1999). This underlines our still relatively poor grasp of the essential processes and the roles of the respective enzymes during fruit softening.

Ripe fruits have more and better flavour than immature ones, which for us is "tastier". The flavour of the tomato fruit is the sum of volatiles, the balance of sugars and acids, which is determined by their integrations. There have been more than 400 volatiles compounds identified affecting tomato flavour (Baldwin et al., 1991), and some have been identified as major contributors, like (Z)-3-hexenal (Baldwin et al., 1991), (E)-geranylacetone (Buttery et al., 1971) and 2-isobutylthiazole (Zhu et al., 2018). *TOMATO LIPOXYGENASE C (TomloxC*; *LOXC*) (Chen et al., 2004) encodes an enzyme catalysing the production of polyunsaturated fatty acids and whose expression is considered to reflect tomato flavour. The substantial accumulation of sugar during ripening is also a key contributor to fruit flavour, which is regulated by, among others, the cell wall invertase gene *LIN5* (Godt and Roitsch, 1997), *ADP-GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 1* (*AGPL1*) (Preiss, 1982), and hexokinase and fructokinase encoding genes *HXK*s (Jang and Sheen, 1994) and *FRK*s (Odanaka et al., 2002).

Hormone regulation of tomato fruit ripening

Fruit development and ripening are associated with the regulation by plant hormones. Among the five classical plant hormones, auxin, abscisic acid (ABA) and ethylene play substantial roles in both climacteric and non-climacteric fruits.

Auxin is essential for fruit set and during fruit development for maintaining fruit growth, but a low level is required for the onset of ripening in both grapes and tomato (Bottcher et al., 2010; Gillaspy et al., 1993). Increased ethylene sensitivity caused by a reduction of auxin during fruit development (Liu et al., 2005) and ripening has been shown for tomato (Jones et al., 2002) and peach (Trainotti et al., 2007), indicating a cross-talk between hormones that regulate ripening. An increase in ABA production before the ethylene increase at the onset of ripening has been observed in both climacteric and non-climacteric fruits (Kondo and Inoue, 1997; Zhang et al., 2009), implying its importance in ripening. This role of ABA is further supported by the silencing of a gene *SlNCED1*, which encodes 9-cis-epoxycarotenoid dioxygenase (NCED), a key enzyme in ABA biosynthesis (Sun et al., 2012).

Tomato is categorised as climacteric, because of the burst in ethylene production accompanying ripening (Gray et al., 1994; Deikman, 1997). The application of exogenous ethylene at the mature stage of climacteric fruits results in fruit ripening, while applying the ethylene action inhibitor 1-methylcyclopropene (1-MCP) dramatically inhibits ripening in apple or markedly affects ripening by inhibiting ethylene production in tomato (Yokotani et al., 2009). Plant ethylene is synthesized from S-adenosyl-L-methionine (SAM) in two major steps: the conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) and the oxidation of ACC to ethylene, which are catalysed by ACC synthase (ACS) and ACC oxidase (ACO), respectively (Xu and Zhang, 2015; Kende, 1993; Alexander, 2002). *ACC OXIDASE 1* (*ACO1*), *ACC OXIDASE 6* (*ACO6*), *ACC SYNTHASE* 2 (*ACS2*) and *ACC SYNTHASE* 4 (*ACS4*) are the major genes regulating these steps by encoding the two key enzymes during tomato ripening. There are two systems in ethylene regulation of climacteric fruit ripening, Systems 1 and 2 (Barry et al., 2000; Lelievre et al., 1997). System 1 functions during early fruit development in both climacteric and non-climacteric fruits, where ethylene synthesis is inhibited by negative feedback, with only a low (basal) level of ethylene. System 2 starts and takes over the regulation during ripening in climacteric fruits, and is autocatalytic, with a sharp increase in ethylene produced in a short period from the onset of ripening. Ethylene is perceived by ethylene receptors encoded by *ETHYLENE RESPONSE* (*ETR*) genes, and their signal is translated into gene expression changes that regulate other developmental processes. Ethylene produced at the onset of ripening binds the receptor, which removes the receptor's suppression from CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) (Clark et al., 1998), then the signalling for stimulating all ripening actions proceeds. In tomato, some ETRs have been identified and their functions have been uncovered. LeETR3 is also called Never ripe (NR) after its spontaneous mutant *Never-ripe* (*Nr*) which has become ethylene-insensitive and in which normal ripening is blocked because of a mutation in the ethylene-binding domain of the NR ethylene receptor (Lanahan et al., 1994; Wilkinson et al., 1995; MacArthur, 1934). The ripening in the allelic dominant mutations *Nr-2* and *Green-ripe* (*Gr*) (Barry et al., 2005) fails because of mutations in a homolog of *Arabidopsis REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1)*, an ethylene receptor, which results in ethylene insensitivity that finally affects normal ripening (Barry and Giovannoni, 2006).

Genetic regulation of tomato fruit ripening

Transcription factors in ripening

All plant development processes happen under the control of the expression of effector genes that are regulated by upstream transcription factors (TFs). TFs are proteins that bind to specific regions of a gene, commonly the gene's promoter to activate or repress the transcription of that gene (Latchman, 1997). They are categorized in families according to their conserved domains, which are often involved in DNA binding. For example, the Minichromosome Maintenance (MCM1), AGAMOUS (AG), DEFICIENS (DEF) and Serum Response Element (SRF) (MADS) domain located at the N-terminus of a TF binds to

the 10 nt consensus sequence $CC(A/T)_6GG$, called the CArG box in a gene promoter (West et al., 1997), so those TFs harbouring this domain are categorized as MADS-domain TFs (Schwarz-Sommer et al., 1990).

In tomato fruit ripening, there are some TFs reported as major regulators according to the ripening defects in their spontaneous mutants. For instance, the severe non-ripe fruits of spontaneous mutants *ripening inhibitor* (*rin*) (Robinson and Tomes, 1968), *non-ripening* (*nor*) (Tigchelaar et al., 1973) and *Colourless non-ripening* (*Cnr*) (Thompson et al., 1999) suggest that TFs (MADS-RIN (Vrebalov et al., 2002), NAC-NOR (Giovannoni et al., 2004) and SPL-CNR (Manning et al., 2006), respectively), whose genes are linked to these mutations, are essential and activators of normal ripening. For TFs that do not have naturally occurring mutants, studies reported the role in fruit ripening through RNAi knock-down or virus-induced gene silencing (VIGS) experiments, like for APETALA2a (AP2a) (Karlova et al., 2011) and FRUITFULL1/2 (FUL1/2) (Leseberg et al., 2008; Bemer et al., 2012; Shima et al., 2013). As knock-down plants produced more than three times the normal amount of ethylene during ripening, *AP2a* is described as a repressor for ethylene biosynthesis (Karlova et al., 2011). The single knock-down plants of *FUL1* and *FUL2* only exhibited mild ripening changes, while the dual knock-down had more obvious defects, so both are documented as positive regulators and work redundantly in ripening (Bemer et al., 2012). Additional regulators include TOMATO AGAMOUS-LIKE1 (TAGL1) (Itkin et al., 2009; Vrebalov et al., 2009) and the HD-Zip homeobox protein LeHB-1 (Lin et al., 2008).

Transcriptional regulation is a complex network

Although several uses of the term exist in biology, here in the context of ripening regulation, we define developmental effector genes as those genes at the end of a transcriptional regulatory cascade or network, which themselves do not encode a TF or TF-interacting factor. This places them at the end of, but does not include them in so-called Gene Regulatory Networks (GRNs) (Wang et al., 2019a) as discussed below. The major regulators of tomato fruit ripening regulate the expression of downstream effector genes, directly through promoter binding to activate or inhibit a gene's transcription or indirectly, through the activation or inhibition of ethylene production with its own downstream regulatory circuit, or through regulating the expression of secondary transcription factors that in turn regulate effector genes. Then effector genes function, for example through their product's enzymatic activity, to regulate the processes that have become known to be characteristics of ripe fruit: colour development, softening, and flavour development. Mostly one-to-one regulatory relationships have been explored so far, however, instead of hierarchical linear regulatory interactions, TFs work in a network to control effector genes' and each other's expression– the so-called GRNs (Karlova et al., 2014).

On the one hand, multiple TFs have common targets, sometimes redundantly, which may act to buffer against disruption by single mutations. For example, the key genes in ethylene biosynthesis, *ACS2* and *ACS4*, are all direct targets of MADS-RIN (Fujisawa et al., 2011, 2013) and NOR-like1 (Gao et al.,

2018). TFs sometimes form a complex to regulate a gene. MADS-domain TFs usually form dimers and tetramers to bind to the promoters (Riechmann et al., 1996; Theißen and Saedler, 2001). TAGL1, MADS-RIN, FUL1 and FUL2 probably regulate downstream gene transcription by forming tetramers (Fujisawa et al., 2014). On the other hand, one TF can regulate multiple genes in different pathways. In addition to ethylene biosynthesis, MADS-RIN also directly regulates *PSY1* in lycopene biosynthesis and *CEL2* in cell wall degradation (Fujisawa et al., 2011, 2013), and their regulations were confirmed by the sharp decrease of their expression in the KO mutants of *MADS-RIN* (Ito et al., 2017).

Moreover, as in many GRN, TFs also regulate each other's expression, making the network complex. For instance, the negative feedback loop between *AP2a* and *SPL-CNR* (Karlova et al., 2011), and the positive regulation by MADS-RIN of *SPL-CNR* were illustrated by chromatin immunoprecipitation (ChIP) experiments (Fujisawa et al., 2011, 2013) together with the decreased *SPL-CNR* expression in *rin* KO mutants (Ito et al., 2017).

Cis-regulation in transcription

TFs regulate gene expression only when bound to the specific binding sites in a gene, often in its 5' promoter, so the presence of binding sites also determines the transcriptional regulation. TF binding sites belong to *cis*-regulatory elements (CREs) that are (often) non-coding DNA sequences and regulate the expression of nearby genes. *Cis*-regulation also comprises enhancers and silencers in other parts of the gene, e.g. introns, 3'-regions or even exons, or far away from the regulated gene. Since there are multiple CREs that regulate the same gene, including inhibitory ones, modifications of some may not cause a complete gene knock out but just alter gene expression, so their effects are not ON or OFF, but MORE or LESS. Mutations in CREs which are causing *cis*-regulatory variations were frequent and often essential during plant domestication, and their effect is milder than mutations in the coding sequence of a gene that result in protein structure change (Wittkopp and Kalay, 2011). A 3 bp InDel in the promoter of *Al-ACTIVATED MALATE TRANSPORTER9*, a key gene in malate synthesis and degradation, was selected during tomato domestication, which determines the fruit malate contents to improve the aluminium tolerance (Ye et al., 2017). Also, the already mentioned *fas* allele, as well as the *lc* (locule number) allele (Yeager, 1937) constitute deregulation of developmental genes, the latter through a 2 nt change, disrupting a negative-regulatory *cis*-element of the tomato *WUSCHEL (WUS)* ortholog (Muños et al., 2011).

Post-transcriptional regulation of tomato fruit ripening

Transcription is the first step between a gene and the functional protein. After transcription, the messenger RNA of a gene is still under regulation, post-transcriptionally, by non-coding RNAs and RNA binding proteins. In this thesis, we only focus on the effects of non-coding RNAs, more specifically of microRNAs.

microRNA regulation

In eukaryotes only 1~2% of the transcriptome encodes for proteins (Birney et al., 2007), suggesting a large potential of additional roles for non-coding RNAs. MicroRNAs are one category of non-coding RNAs of 20~22 nt in length and have been found in plants, animals and some viruses. They function post-transcriptionally by binding to complementary sequences of mRNA of their target genes and subsequently cleave the mRNA or remain bound and inhibit its translation.

Many microRNAs and their target genes have been identified in plants by BLAST with computational approaches (Adai, 2005; LI et al., 2005) and some of their functions have been well studied. In *Arabidopsis*, miR172 targets *APETALA2* (*AP2*) (Park et al., 2002) genes to regulate flowering time and floral patterning (Zhu and Helliwell, 2011), and miR156 targets *SPL* genes involved in flowering control, plastochron length and organ size regulation (Wang et al., 2009, 2008). In tomato, Moxon *et al.* identified microRNAs by deep sequencing and showed that miR156/157 regulates *SPL-CNR in vivo* (Moxon et al., 2008), and Silva *et al.* exhibited that miR156 controls ovary and fruit development by being involved in the maintenance of the ovary meristematic state (Silva et al., 2014). Karlova *et al.* identified by using high-throughput sequencing and degradome analysis 119 target genes of microRNAs in different stages of tomato fruit development (Karlova et al., 2013). In addition, it was also shown that the transcript cleavage of *SPL-CNR* by miR156/157 and *AP2a* by miR172 increased (30% for *SPL-CNR* and 48% for *AP2a*) at Br stage, indicating that they probably play roles in tomato fruit ripening regulation (Karlova et al., 2013).

Epigenetic regulation of tomato fruit ripening

Genetic regulation controls all plant developmental processes, but meanwhile, the role of epigenetic regulation cannot be neglected. Epigenetic modifications regulate the expression of genes without DNA sequence changes, comprising DNA or histone protein (de)methylation and (de)acetylation, which often are heritable and stable between generations. They alter the chromatin status to become more accessible or more restricted for regulatory (TF) protein binding, causing gene activation or silencing (Liu et al., 2010; Zhang et al., 2018, 2010).

DNA methylation during fruit ripening

The cytosines of DNA (Choi et al., 2002; Kawakatsu et al., 2016), adenosine of RNA (Zhong et al., 2008; Duan et al., 2017), and histone proteins can be methylated (Cartagena et al., 2008; de la Paz Sanchez and Gutierrez, 2009) to regulate plant developmental processes. Among epigenetic modifications, DNA methylation is the most well studied for tomato fruit ripening.

There is a global decrease of cytosine methylation (~30%) in the pericarp during tomato fruit ripening (Teyssier et al., 2008) and most of the demethylation happens in gene promoters (Lang et al., 2017).

More evidence that DNA demethylation plays an active role in tomato ripening (Liu et al., 2015), came from a study in which the inactivation of the DNA demethylase gene *SlDML2* resulted in a genome-wide DNA hypermethylation and an inhibition of fruit ripening (Lang et al., 2017). Tomato fruit ripening is accompanied by global DNA demethylation, however in contrast, in fruits of the orange (*Citrus sinensis*), there is a global DNA methylation increase during ripening (Huang et al., 2019). A very recent study reported that RNA methylation is also involved in tomato fruit ripening regulation, suggesting a new layer of epigenetic regulation on fruit ripening (Zhou et al., 2019).

Cnr, the spontaneous dominant mutation linked to the TF SPL-CNR, is a well-known tomato epi-mutant, which is caused by DNA methylation. *Cnr* was isolated from the F_1 hybrid cv. Liberto in 1993, and was named after its ripening defects as *colourless non-ripening* (Thompson et al., 1999). Fruits of *Cnr* become white, at the same stage where wild-type fruits change to red, and turns yellow later, but no lycopene is synthesised (Thompson et al., 1999). There is no ethylene production detected and the ripening defect cannot be reversed by exogenous ethylene treatment (Thompson et al., 1999). Based on the phenotype segregation ratio from a selfed F_1 generation, *Cnr* was documented as dominant (Thompson et al., 1999). Later in 2002, Manning *et al*., elucidated the nature of the *Cnr* locus by genetic mapping, being a 286 bp hypermethylated region upstream the *SPL-CNR* gene, thereby demonstrating that *Cnr* is an epi-mutant (Manning et al., 2006). Then with the help of whole-genome bisulfite sequencing, Zhong *et al*. exhibited that there is demethylation during wild-type fruit ripening including in the relevant hypermethylated region, while the *Cnr* epi-allele stayed hypermethylated (Zhong et al., 2013).

CRISPR revolution for tomato ripening studies

CRISPR/Cas9-mutagenesis

Clustered regularly interspaced short palindromic repeats (CRISPR) are DNA sequences in bacteria and Archaea, which are derived from DNA fragments (spacers) of bacteriophages that have previously infected them, interspersed with palindromic repeats. *CRISPR-associated* (*Cas*) genes encode DNA endonuclease enzymes and accessory proteins (CAS proteins) that cleave DNA based on recognition by the complementarity of guide RNAs, that are processed from the transcribed CRISPR arrays and bound by the CAS proteins (Jansen et al., 2002). Although CRISPR/Cas systems were discovered as an immune system in bacteria and Archaea (Bhaya et al., 2011), their potential value for gene editing was soon realized by scientists. Since then they have been developed rapidly and triggered a genome editing revolution. The CRISPR/Cas9 system from *Streptococcus pyogenes* was first adapted in 2012 (Jinek et al., 2012), and first applied in mammalian cells in 2013 (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013) and also in plants, such as *Arabidopsis*, rice and wheat in the same year (Shan et al., 2013; Li et al., 2013). At the same time, more and more online tools and databases combined with many available genome sequences make it easier to target a gene and avoid off-targeting. Due to the ease of use and

precise targeting, it took preference over zinc finger nucleases (ZFNs) (Smith et al., 2000) and TAL effector nucleases (TALENs) (Boch et al., 2009) and became the most popular gene-editing tool until now.

CAS9 protein targets a specific sequence in a genome (representing the protospacer derived from foreign DNA in the bacterial defence system) using the 5' 20 bases variable part of a guide RNA (gRNA) representing the spacer of the CRISPR array. Recognition of the target also depends on the presence of a protospacer adjacent motif (PAM) 3' of the targeted sequence, which is usually NGG (Jinek et al., 2012). Once the targeted site is located, the CAS9 protein unwinds the target DNA sequence using gRNA-DNA base pairing and cuts both DNA strands (3 bp upstream of the PAM) causing a blunt double-strand break (DSB) (**Fig. 2A**). Survival of the cell depends on naturally occurring DNA repair pathways. There are two main pathways for the repair, non-homologous end joining (NHEJ) (**Fig. 2B**) and homology-directed repair (HDR) (Symington and Gautier, 2011) (**Fig. 2C**). NHEJ is the predominant pathway for repairing the double-strand break in somatic plant cells. In the NHEJ mechanism, a DSB is repaired by re-joining the two ends with endogenous DNA ligase, which is usually precise but sometimes induces some small insertions or deletions (InDels) into the genome. If the InDel is in the coding sequence (CDS) of a gene it will most of the time result in a frameshift that causes a pre-mature stop in translation, resulting in a truncated protein, which commonly constitutes a gene knock-out. HDR is usually perfect as there is a template for the repair. In most of the cases, Cas9 only cuts one copy of a gene and the other copy in the sister chromatid, the sister chromosome, or an homologous sequence of another origin can function as a template during repair.

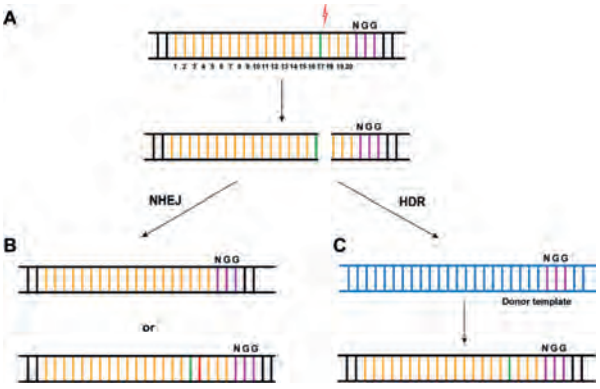

Figure 2. CRISPR/Cas9 as a genome-editing tool. **(A)** The cutting by CAS9 protein causes a blunt double-stranded break in the genome. The targeted 20 bp sequence and PAM site are in yellow and pink, respectively. The red flash and the base pair in green show the location which is the most frequently edited. **(B)** Non- homologous end joining (NHEJ) repair machinery introduces InDels in the genome. The DSB is repaired by re-joining the two ends, sometimes accompanied by a small deletion or insertion (error-prone repair). The red base pair represents an insertion. **(C)** Homology-directed repair (HDR) machinery uses an available DNA donor template (the same locus in the sister chromatid, sister chromosome, or exogenous) to repair the DSB and thereby replacing the original sequence by that of the template.

The application of CRISPR in tomato studies

CRISPR/Cas9-mutagenesis in tomato was used for the first time in 2014 to mutate *ARGONAUTE7* (*SlAGO7*), whose loss-of-function phenotype with wiry leaves was easily distinguished (Brooks et al., 2014). Since then it was used in studies related to domestication genes (Rodríguez-Leal et al., 2017; Xu et al., 2015; Soyk et al., 2017a, 2017b), biotic and abiotic stress studies (Chaudhary and Atamian, 2017; Wang et al., 2017; Ye et al., 2017) and fruit quality improvement (Yu et al., 2017; Uluisik et al., 2016). Researchers from the Lippman Lab simulated accelerated tomato domestication by knocking out *SELF PRUNING 5G* (Soyk et al., 2017b) or two MADS-box genes involved in inflorescence development (Soyk et al., 2017a), or by randomly mutating CREs in a gene promoter to create a domestication QTL (Rodríguez-Leal et al., 2017). Ye *et al*. observed a reduced malate content by introducing InDels through CRISPR mutagenesis in the promoter of *ALMT9*, which reduces aluminium tolerance of tomato, confirming the importance of these sequences to abiotic stress resistance (Ye et al., 2017). In 2015, CRISPR/Cas9-mutagenesis was used for the first time to study fruit ripening by targeting *MADS-RIN* (Ito et al., 2015), and the working mechanism was illustrated in detail in 2017 (Ito et al., 2017). We and another group created KO mutants of the ripening related TF genes *NAC-NOR* (Wang et al., 2019b) and *SPL-CNR* (Gao et al., 2019), which are also described in **Chapter 4** and **5** of this thesis.

Aim and outline of this thesis

As introduced above, tomato fruit ripening is a complex process regulated by plant hormones, TFs and their downstream effector genes, non-coding RNAs and the epigenome. The functions of ethylene and effector genes have been well studied for a long time, but the function of TFs involved in ripening was solely based on the phenotypes of their spontaneous mutants, which appeared now to be sometimes misleading. At the same time, more and more layers of regulation were reported, adding more complexity to the ripening process. Therefore, the aim of this thesis is to more precisely define the functions of what was traditionally considered as the "master regulators" (major TFs) due to the strong effects of their spontaneous mutations on ripening. The elucidation of the 'true' functions of these TFs in ripening is the first step in understanding the entire regulatory network, which also includes the action of microRNAs and the role of CREs and epigenetic DNA methylation on gene expression.

The spontaneous alleles *rin*, *nor* and *Cnr*, together with the phenotypes of their mutants have been reported and *rin* and *nor* have been applied in tomato breeding for a long time, but only individually. Very limited information is available about the interactions between these loci and their dose-dependency in the ripening regulatory network. This was studied in **Chapter 2** by using single and double heterozygous mutants, instead of homozygous mutants that usually completely block ripening. The ripening phenotypes, the effector gene expression, and the sensory and quality aspects of the fruits reveal the interaction and contribution of these loci to the tomato fruit ripening.

A recent study showed that the severe ripening phenotype in the *rin* mutant is caused by a gained function of the *rin* allele, not by a loss-of-function of the *MADS-RIN* gene. This triggered us to create true KO mutants by CRISPR/Cas9-mutagenesis in those TF genes that were previously considered as master regulators of tomato fruit ripening. In **Chapter 3** the function of TFs AP2a, NAC-NOR and FUL1 and 2 were re-evaluated with CRISPR/Cas9-mutagenesis and the phenotypes of their KO mutants confirmed the functions of *AP2a* and *FUL1/2*, which were based on knock-down lines. Surprisingly, the phenotype of the KO *NAC-NOR* lines was less severe than that of the spontaneous mutant, suggesting a dominant-negative action of the *nor-s* allele. These results demonstrate the need for a revisiting of the ripening regulatory network.

The function and mode of action of MADS-RIN and NAC-NOR were clarified recently, while SPL-CNR remains a mystery. In **Chapter 4** I evaluated *SPL-CNR*'s function by knocking-out the gene or deleting different parts of its promoter using CRISPR/Cas9-mutagenesis. The promoter mutants with low expression and KO mutants with no *SPL-CNR* function showed weaker and less obvious ripening defects than the spontaneous *Cnr* mutant, demonstrating that there may be another mechanism for SPL-CNR to regulate fruit ripening, which is different from or only a part of the function of the dominant *Cnr* mutation.

Chapter 5 focuses on the putative roles of three microRNAs in fruit ripening. The phenotypes of the CRISPR mutants of miR156, miR157 and miR172, which are targeting *SPL-CNR* and *AP2a*, respectively, provided insights into their functions in ripening.

With the help of CRISPR/Cas9-mutagenesis creating full KO mutants is much easier than it used to be, but with these advances also new phenomena were revealed. Several KO mutants appeared to have milder phenotypes than those previously found with RNAi, VIGS, or spontaneous mutants, which was in contrast to the expectation. **Chapter 6** is reviewing these studies and provides some possible mechanisms explaining these phenomena and giving some suggestions for future CRISPR work.

The concluding chapter discusses the results we obtained and how the research on the molecular regulation of tomato fruit ripening has evolved over the past few years. It appears a very dynamic field with new discoveries and research questions, which needs a revisiting of the current knowledge about the gene regulatory network of fruit ripening.

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Chapter 2

The *rin*, *nor* and *Cnr* spontaneous mutations inhibit tomato fruit ripening in additive and epistatic manners

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Abstract

Tomato fruit ripening is regulated by transcription factors (TFs), their downstream effector genes, and the ethylene biosynthesis and signalling pathway. Spontaneous non-ripening mutants *ripening inhibitor* (*rin)*, *non-ripening* (*nor)* and *Colorless non-ripening* (*Cnr*) correspond with mutations in or near the TF-encoding genes *MADS-RIN*, *NAC-NOR* and *SPL-CNR,* respectively. Here, we produced heterozygous single and double mutants of *rin, nor* and *Cnr* and evaluated their functions and genetic interactions in the same genetic background. We showed how these mutations interact at the level of phenotype, individual effector gene expression, and sensory and quality aspects, in a dose-dependent manner. *Rin* and *nor* have broadly similar quantitative effects on all aspects, demonstrating their additivity in fruit ripening regulation. We also found that the *Cnr* allele is epistatic to *rin* and *nor* and that its pleiotropic effects on fruit size and volatile production, in contrast to the well-known dominant effect on ripening, are incompletely dominant, or recessive.

Keywords: tomato; fruit ripening; spontaneous mutation; *ripening inhibitor*; *non-ripening*; *Colorless non-ripening*;

Introduction

Tomato (*Solanum lycopersicum*) has a diploid and high quality assembled reference genome, and the favorable biology and ease of transformation make it a model plant for fleshy fruit development and ripening studies. It is also ranked as the most consumed vegetable, and therefore, knowledge about tomato ripening regulation is vital for breeding.

Tomato fruit ripening is a complex process with physiological and biochemical changes, resulting in altered fruit color, texture and flavor. As in other climacteric fruits, there is a burst in ethylene production and respiration during ripening. Transcription factors (TFs) regulate the expression of downstream effector genes, together with ethylene, to coordinate these changes (Karlova et al., 2014). The process is also regulated by dynamic epigenetic modifications (Zhong et al., 2013), adding more complexity to this regulation.

As for many tomato genes, functions of ripening genes were usually discovered through forward genetics by selecting mutants with a ripening phenotype and subsequent mapping of the genes underlying these spontaneous mutations. Tomato *ripening inhibitor* (*rin)* (Robinson and Tomes, 1968), *non-ripening* (*nor)* (Tigchelaar et al., 1973) and *Colorless non-ripening* (*Cnr*) (Thompson et al., 1999) are spontaneous mutants of the TF encoding genes *RIPENING INHIBITOR* (*MADS-RIN*) (Vrebalov et al., 2002), *NON-RIPENING* (*NAC-NOR*) (Giovannoni et al., 2004) and *COLORLESS NON-RIPENING* (*SPL-CNR*) (Manning et al., 2006), which encode a Minichromosome Maintenance (MCM1), AGAMOUS (AG), DEFICIENS (DEF) and Serum Response Element (SRF) (MADS)-domain, a NAM, ATAF1/2 and CUC2 (NAC), and a SQUAMOSA promoter-binding protein-like (SPL) TF, respectively. The *rin* fruits remain green for a long time and eventually turn to a lemon color without lycopene or ethylene synthesized (Herner and Sink, 1973), and this phenotype cannot be rescued by external ethylene treatment (Lincoln and Fischer, 1988). Another distinctive characteristic of *rin* fruits is their large leaf-like sepals or calyx (Robinson and Tomes, 1968). The *rin* mutation comprises a deletion between *MADS-RIN* and its neighboring gene *MACROCALYX* (*MC*), another MADS-domain TF regulating sepal development (Vrebalov et al., 2002). A new fusion protein, RIN-MC is formed as a result of the deletion, containing most of MADS-RIN and MADS-MC, apparently affecting both MC (hence: *macrocalyx*) as well as MADS-RIN function. Similar to the *rin* mutant, *nor* fruits fail to ripen and have a green pericarp (Tigchelaar et al., 1973). The *nor* mutation comprises a 2 bp deletion in the third exon of *NAC-NOR*, resulting in a truncated protein (Kumar et al., 2018). The mutation reduces both ethylene production and lycopene biosynthesis (Czapski and Saniewski, 1992). *Cnr* fruits have a mealy pericarp that turns pale yellow in later stages (Thompson et al., 1999) since no lycopene is synthesized (Fraser et al., 2001). The *Cnr* mutation does not cause primary sequence changes, but is epigenetic: part of the upstream region of the gene is hypermethylated compared to that in the wild-type and remains methylated during ripening (Liu et al., 2015; Zhong et al., 2013), with a reduced expression of *SPL-CNR* (Manning et al., 2006).

There is no burst of ethylene during fruit development in this mutant, and its phenotype cannot be rescued by exogenous ethylene application (Manning et al., 2006). *Rin* and *nor* were reported to be recessive (Robinson and Tomes, 1968; Tigchelaar et al., 1973), while *Cnr* is dominant since there was no significant difference between the phenotypes of the mutant and heterozygous lines reported (Thompson et al., 1999).

While the respective homozygous mutations more or less completely block ripening, *rin* and *nor* have been used in a heterozygous state to slow down ripening and extend tomato shelf life with varying success (Garg et al., 2008; Kopeliovitch et al., 1979). Although this approach has the desired effect on shelf life, it may often be accompanied by a reduction in quality aspects such as color and taste (Herner and Sink, 1973; Sink Jr. et al., 1974; Tigchelaar et al., 1978). Additionally, a weaker allele of *nor*, *alcobaca* (*alc*) (Leal, 1973), which was also called Delayed Fruit Deterioration (Saladié et al., 2007), was used for similar purposes (Casals et al., 2012). TFs often act as a network rather than individually to regulate gene expression; however, how exactly is poorly understood. Although relevant for application in tomato breeding, neither do we know how natural or induced variation in the copy number of different regulatory mutant alleles, as in heterozygosity for mutations, can affect the progress of ripening at the molecular level. For example, MADS-RIN may regulate effector genes by forming a dimer with TOMATO AGAMOUS-LIKE 1 (TAGL1) (Vrebalov et al., 2009), FRUITFULL1 (FUL1) or FUL2 (Shima et al., 2013; Leseberg et al., 2008; Bemer et al., 2012), but how a double (homozygous) or single (heterozygous) copy of a mutant allele affects the activity of the network is still unknown, nor how different combinations of mutant alleles interact.

To study different and combined doses of *rin*, *nor* and *Cnr*, we produced single and double heterozygotes for all three mutations in an identical genetic background (cv. Ailsa Craig). In this study, we investigated their phenotypes and the expression of the underlying TF genes and downstream effector genes with one, or two allele dosages, or combinations thereof, during tomato ripening. We also evaluated several phenotypic and metabolic effects, including ones not previously reported, of the *Cnr* mutation, and observed different levels of the dominance or epistasis of *Cnr* therein.

Materials and Methods

Plants materials and growing conditions

Tomato cv. Ailsa Craig (AC), and *rin*, *nor* and *Cnr* mutants in this background were obtained from the Tomato Genetic Resource Centre (TGRC, Davis, CA, *rin* and *nor*) and from Professor Graham Seymour, Nottingham University, United Kingdom (*Cnr*). These genotypes were crossed with each other to obtain F_1 heterozygotes for each mutation alone as well as in each combination of two mutations. Genotypes of *rin* and *nor* heterozygotes were confirmed by sequencing (primers used are in Table S1**)** or for *Cnr* by phenotyping fruits. Plants growing under standard greenhouse conditions were used.

Fruit development phenotyping

Three plants per genotype were used for phenotyping. Flowers were labelled at anthesis and vibration was applied for pollination. Fruits at 35, 40, 45, 50 and 55 Days Post Anthesis (DPA) were collected for photography, and at least eighteen fruits per genotype were used to calculate the average time in days to the Breaker (Br) stage. At least thirteen wild-type AC, homozygous *Cnr* and heterozygous *Cnr* fruits collected at 55 DPA were used for the weight and pericarp thickness measurements. Student's *t*-test was used to detect significant differences between genotypes.

Fruit pigment measurements

Chlorophyll and lycopene contents during ripening were measured by remittance VIS spectroscopy with a hand-held photodiode array spectrophotometer (Pigment Analyzer PA1101, CP, Germany) and were calculated according to (Schouten et al., 2014). These measurements were performed in two years, in 2016, starting from the early immature stage (20 DPA), and in 2018 only for the ripening or the equivalent stages from one day before Br until 7 Days Post Br (DPB). The relative contents of chlorophyll and lycopene at the blossom end of fruits were measured every day. At least six fruits per genotype were used for pigment measurements, and their averages were used.

Ethylene production

Six fruits per genotype collected at Br, Br+7d and Br+15d were used for ethylene measurements. Ethylene production was measured and calculated according to (Wang et al., 2019). As production levels were not distributed normally, a quasibinomial model was used to test the statistical significance of differences between genotypes.

High-Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC/MS) analysis

Carotenoids from tomato pericarp at Br+7d fruits were extracted according to described methods (Wahyuni et al., 2011). HPLC analysis was performed according to (Bino et al., 2005). Chromatography was carried out on a Waters e2695 HPLC - 2996 PDA system and data were collected and analyzed using the Waters Empower software. The carotenoid contents were determined by computing the peak area at 478 nm.

Volatile compounds were analyzed by an SPME-GC-MS method as described (Tikunov et al., 2005) using the Thermo Fisher Trace GC-QDAII MS system. The chromatography and spectral data were evaluated by Xcalibur software (http://www.thermo.com) and processed using Metalign and MSCLust freeware packages (https://www.wur.nl/en/show/MetAlign-1.htm). Volatile compounds were identified using NIST MS Search mass spectral library software (https://chemdata.nist.gov/mass-spc/ms-search/) by matching mass spectra and by comparing retention indices.

Gene expression analysis

The pericarp of fruits at Mature Green (MG, 35 DPA), Br, and Br+7d or equivalent stages in mutants were collected in three pools of two fruits each for gene expression analysis. Total RNA was isolated by the Cetyl Trimethylammonium Bromide (CATB) method. Briefly, ground tomato pericarps were suspended and incubated in the CTAB buffer (2 % CTAB, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA and 2 M NaCl in Diethyl Pyrocarbonate (DEPC)-treated water with 2% β-mercaptoethanol) at 65 °C for 10 min. The RNA was separated to the water phase by mixing with chloroform and centrifugation for 10 min. 8 M LiCl was used to precipitate RNA for one hour at -20 °C followed by centrifugation for 30 min. After drying in a vacuum desiccator for 15 min, RNA was dissolved in DEPC-treated water. RNA was treated by the TURBO DNase (Thermo Fisher Scientific, Vilnius, Lithuania) to remove contaminating DNA. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA) and then was used for quantitative RT-PCR with iQ SYBR Green Supermix (Bio-Rad) in the iCycler iQ5 system (Bio-Rad). The expression of the tomato *CAC* gene was used as a reference for normalization (Expósito-Rodríguez et al., 2008). All primers used for qRT-PCR are listed in Table S1. Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Accession numbers

Genes in this study can be found in the Sol Genomics Network website (www.solgenomics.net) with the following accession numbers: *ACO1* (Solyc07g049530), *ACS4* (Solyc05g050010), *AP2a* (Solyc03g044300), *CAC* (Solyc08g006960), *CEL2* (Solyc09g010210), *FUL1* (Solyc06g069430), *FUL2* (Solyc03g114830), *LOXC* (Solyc01g006540), *MADS-RIN* (Solyc05g012020), *NAC-NOR* (Solyc10g006880), *PSY1* (Solyc03g031860), *PG* (Solyc10g080210), *PL* (Solyc03g111690) and *SPL-CNR* (Solyc02g077920).

Results

Cv. Ailsa Craig *rin* and *nor* single heterozygous fruits are only mildly affected in ripening

We monitored fruit development visually from 30 to 55 DPA at 5-day intervals for all genotypes (Fig. 1). Homozygous mutations completely blocked ripening, as described earlier. Most heterozygous mutant fruits showed visible phenotypes, which were intermediary between those of the wild-type and the corresponding homozygous mutants (Fig. 2), to varying extents, and for all changed very little after 45 DPA (Fig. 1). Exceptions were *rin* (Fig. 2H) and *nor* (Fig. 2I) single heterozygotes, which were visually indistinguishable from wild-type fruits at the Breaker (Br)+7d stage, with similar ripening progression as wild-type AC (Fig. 1). All double heterozygous lines were more affected in ripening than the respective single heterozygous mutants, suggesting an additive effect of the two mutant alleles. The Br stage, defined by the first color change at the blossom end of tomato fruits, marks the visible initiation of ripening. Homozygous mutants showed no distinct Br stage, so we only measured time to Br for wild-

type and heterozygous mutant fruits which had a clear Br stage (Fig. 3A). It took on average 39.4 days to reach this stage in *rin*/+ fruits, significantly less than in wild-type AC (41.1 days). In contrast, *nor*/+ was slightly delayed, taking on average 42.9 days to reach the Br stage (Fig. 3A).

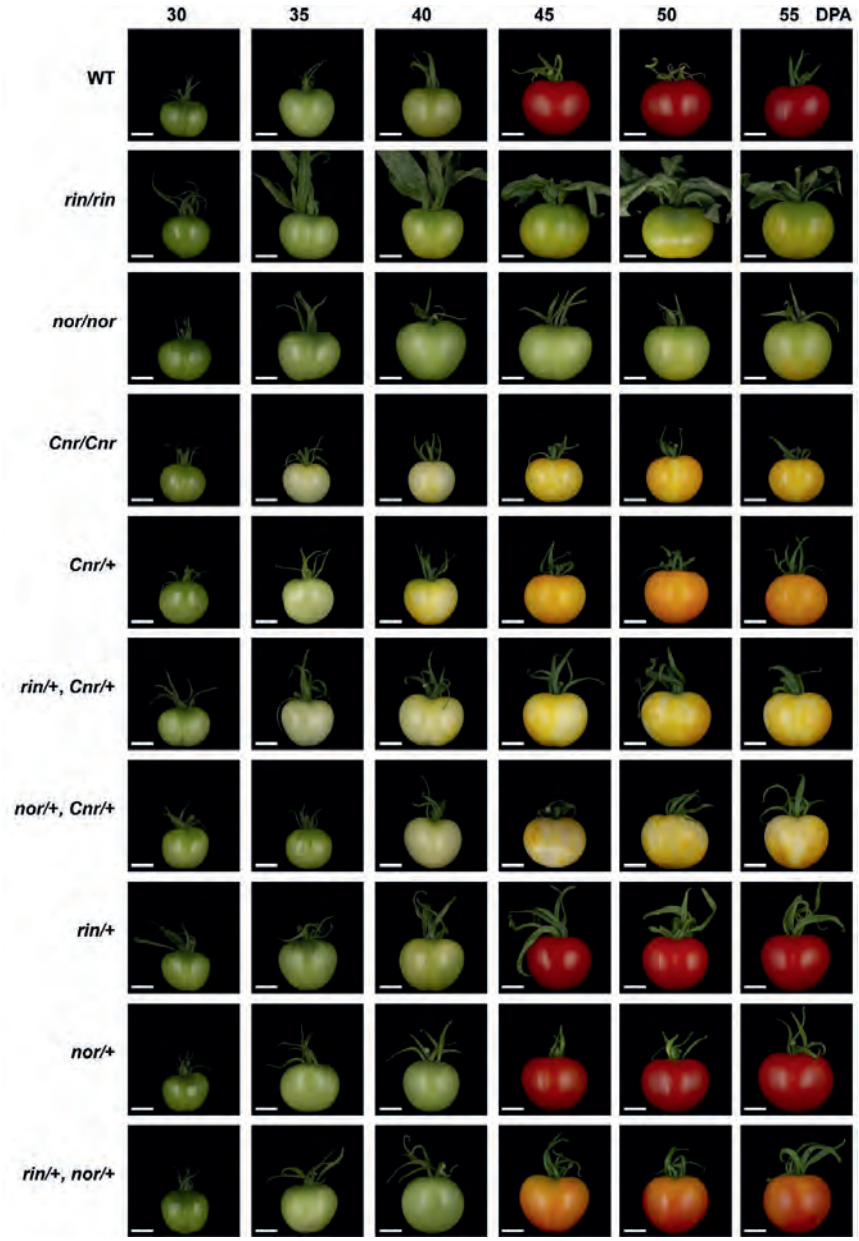

Figure 1. Development and ripening of homozygous spontaneous mutants and their heterozygotes. Time-course images of representative fruits taken every five days from 30 DPA onwards showing differences in developmental and ripening processes. All lines are in the background of cv. Ailsa Craig. Scale bar, 2 cm.

Chlorophyll degradation and lycopene accumulation were quantified by remittance spectroscopy. *nor*/+ and *rin*/+ fruits displayed an entirely red pericarp at 45 DPA (Fig. 1) and Br+ 7d (Fig. 2H and I), with similar lycopene content as wild-type fruits (Fig. 3B). The lycopene accumulation and chlorophyll degradation speed of *rin*/+ and *nor*/+ fruits were similar to that in the wild-type fruits when measured from one day before Br (Fig. 3B and C). We also monitored pigment changes in a second season, starting 20 DPA (immature green) and continuing until late-ripening (65 DPA) (Fig. S1 and S2). Data of chlorophyll changes confirmed the earlier and later initiation of ripening, the time at which the sharp decrease of chlorophyll started (Br), in *rin*/+ and *nor*/+ fruits, respectively (Fig. S1). The accumulation of lycopene confirmed the final full red color and a similar accumulation speed in *rin*/+ and *nor*/+ (Fig. S2).

Figure 2. Phenotypes of mutant fruits at the ripe or equivalent stage. Fruits (**A** and **H- J**) are at Br+7d or the equivalent stage (**B- G**) as wild-type Ailsa Craig fruits. Scale bar, 2 cm.

Tomato ripening is associated with climacteric ethylene production, so we measured ethylene production at Br, Br+7d and Br+15d for all genotypes (Fig. 3D). In wild-type fruits, production peaked around Br+7d and decreased towards Br+15d. As reported by others, all homozygous mutants showed a drastic decrease in ethylene production to nearly zero for *rin* and *nor*, or undetectable for *Cnr*. Although visually and quantitatively displaying normal ripening with regard to pigment development, there was a significant reduction of ethylene produced in both *rin*/+ and *nor*/+ to a level between that of wild-type and homozygous mutants. This decrease was especially substantial at Br+7d, with only 56% and 47% of the production of wild-type fruits, respectively, implying a dosage effect of the *rin* and *nor* alleles on ethylene production in cv. Ailsa Craig (Fig. 3D).

Another distinguishable characteristic in the *rin* mutant is its large, leaf-like sepals in the fruit calyx (Robinson and Tomes, 1968), which is caused by the loss of function of the adjoining MADS-box gene *MACROCALYX* (Vrebalov et al., 2002). We observed an intermediate sepal size between wild-type (Fig. 2A) and *rin*/*rin* (Fig. 2B) in all the heterozygotes containing one *rin* allele (Fig. 2F, H and J), indicating that the strength of this phenotype is also dependent on *rin* dosage.

Rin and *nor* alleles affect ripening quantitatively and additively

Both *rin* and *nor* single heterozygotes showed visibly normal ripening with mild defects, but we observed an apparent additive effect in the double heterozygote (*rin*/+, *nor*/+). The latter genotype required a significantly longer time to reach the Br stage, on average, five and three days longer than that in wild-type or *nor*/+ fruits, respectively (Fig. 3A). In contrast to the single heterozygous fruits, the double heterozygous fruits had only 76% of the lycopene content, significantly lower ($P<0.05$) than that of wild-type fruits at Br+7d (Fig. 3B), displaying an orange pericarp that remained unchanged up to 55 DPA (Fig. 1 and Fig. 2J). The accumulation rate of lycopene was also slower than that in wild-type and the single heterozygotes (Fig. 3B).

A significantly stronger reduction in ethylene production was observed in *nor*/+, *rin*/+, with only 37, 13 and 25% of the production in wild-type fruits at the same stages (Fig. 3D). Ethylene production at Br+7d was also significantly lower than that of the respective single heterozygous mutants ($P<10^{-4}$ for both *rin*/+ and *nor*/+), but still significantly higher than that of the respective homozygous mutants (Fig. 3D). These observations indicate that, as for pigment development, the single *rin* and *nor* alleles have a synergistic effect, on ethylene production during ripening.

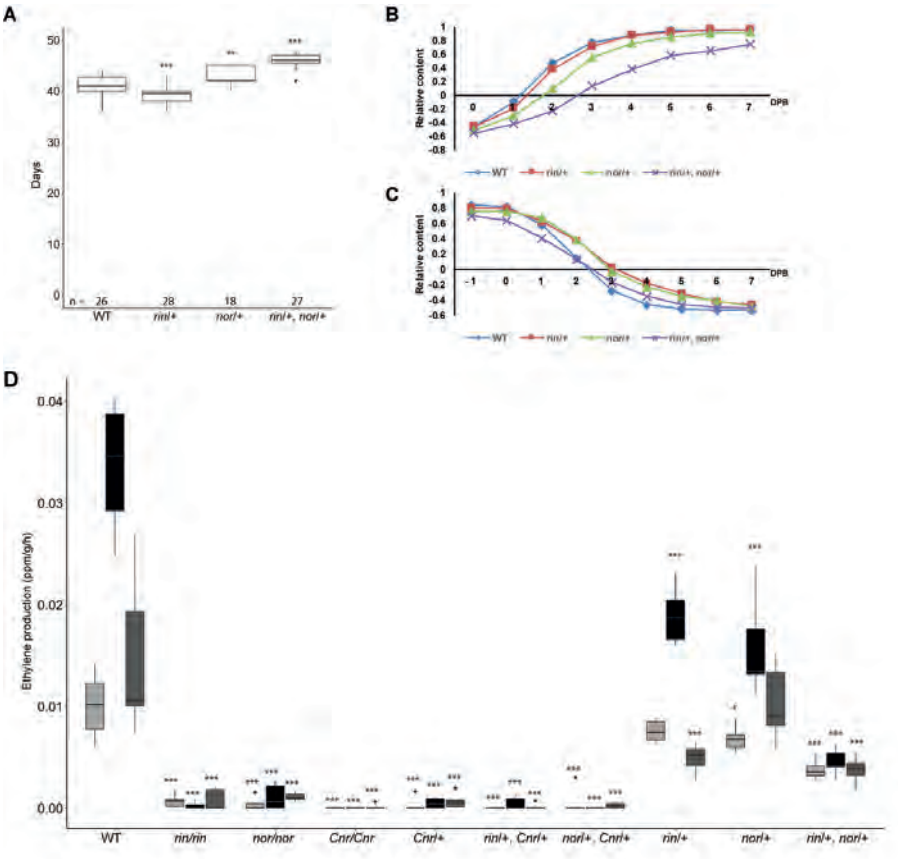

Figure 3. Ripening traits of mutant fruits. (**A**) Time to the initiation of ripening (days to Br) of mutants with a discernible Br stage. (**B**) Lycopene and (**C**) chlorophyll change during ripening in mutants with a clear Br stage. The relative content of pigments of each fruit was measured every day starting from Br for lycopene or one day before Br (-1 Days Post Breaker (DPB)) for chlorophyll until 7 DPB when all the fruits reached the final color stage. (**D**) Ethylene production (ppm/g/h) of all genotypes with normal ripening at Br, Br+7d and Br+15d, with *Cnr* heterozygotes and other spontaneous mutants at the equivalent stage. Grey, black and dark grey blocks represent ethylene production at Br, Br+7d and Br+15d, respectively. The average contents from nine fruits were used for (**B**) and (**C**). Data from six fruits of each genotype were used for (**D**). Asterisks indicate significant differences from the wild-type ($P<10^{-4}$ '***', $P<0.001$ '**', $P<0.01$ '*') from the equivalent stage in the wild-type.

Cnr is completely dominant for ethylene production and pericarp density but incompletely dominant for fruit pigmentation

Homozygous *rin*, *nor*, and *Cnr* mutations affected ripening as described earlier, with the *rin* and *nor* mutants remaining green and the *Cnr* mutant fruit turning pale yellow at the stage where the wild-type is entirely red. However, we observed that the onset of color change (Fig. 1) and chlorophyll degradation

(Fig. S1) in *Cnr* occurred at 30 DPA, approximately ten days earlier and progressing more gradual than in wild-type fruits (Fig. S1). We also measured ethylene production at the same stages as wild-type fruits at Br, Br+7d and Br+15d for the homozygous *Cnr* fruits and detected no ethylene (Fig. 3D). *Cnr*/+ fruits also did not produce at the same stages, confirming *Cnr*'s complete dominance in blocking ethylene biosynthesis. This dominance was also displayed in combinations of heterozygous *Cnr* with heterozygous *rin* and *nor* (Fig. 3D).

The loss of cell-to-cell adhesion in *Cnr* fruits resulted in 50% more intercellular spaces in the pericarp (Orfila et al., 2001) and reduced density, making the pericarp float in water (Seymour, 2002). We observed the same floating pericarp in all mutants containing at least one *Cnr* allele at the same mature stage, indicating that *Cnr* is dominant in this respect (Fig. S3B), although quantitative effects of *Cnr* on fruit density cannot be excluded by this simple experiment.

The same earlier color change as in *Cnr* homozygous fruits was observed in *Cnr* single heterozygous fruits, but, instead of pale yellow, pericarp color progressed to orange at 45 DPA and onwards (Fig. 1 and Fig. 2E). The spectroscopy analysis suggested that there was some carotenoid present (Fig. S2). To confirm this, we analyzed carotenoids of *Cnr/Cnr* and *Cnr*/+ together with wild-type fruits at Br+7d or the equivalent stage by HPLC. There was a minimal amount of lycopene detected in *Cnr/+* fruits, but more than twice the amount of β-carotene compared to that of *Cnr/Cnr* fruits (Fig. 4A and B). Thus, rather than completely, *Cnr* is incompletely dominant for inhibition of carotenoid biosynthesis during ripening, a novel dosage effect of *Cnr*.

Cnr negatively affects fruit size in a recessive manner

We noticed in several growing seasons that fruits of the *Cnr* mutant were consistently smaller than wild-type AC fruits, although this had not been reported before. To quantify this, we measured both the fruit weight and pericarp thickness of wild-type, *Cnr/Cnr* and *Cnr/+* (Fig. 4A, C and D). *Cnr/Cnr* fruits were significantly lighter, only 62% of wild-type fruit weight, confirming our observation, while *Cnr/+* fruits weighed almost as much (on average 92%) as wild-type without apparent differences in seed numbers (Fig. 4A and C). The thickness of *Cnr/Cnr* pericarp was half (56%) that of the wild-type, but this reduction was much less (to 87%), although still significant, in *Cnr/+* (Fig. 4A and D), implying a quantitative effect of *Cnr* in pericarp thickness as well as in fruit size.

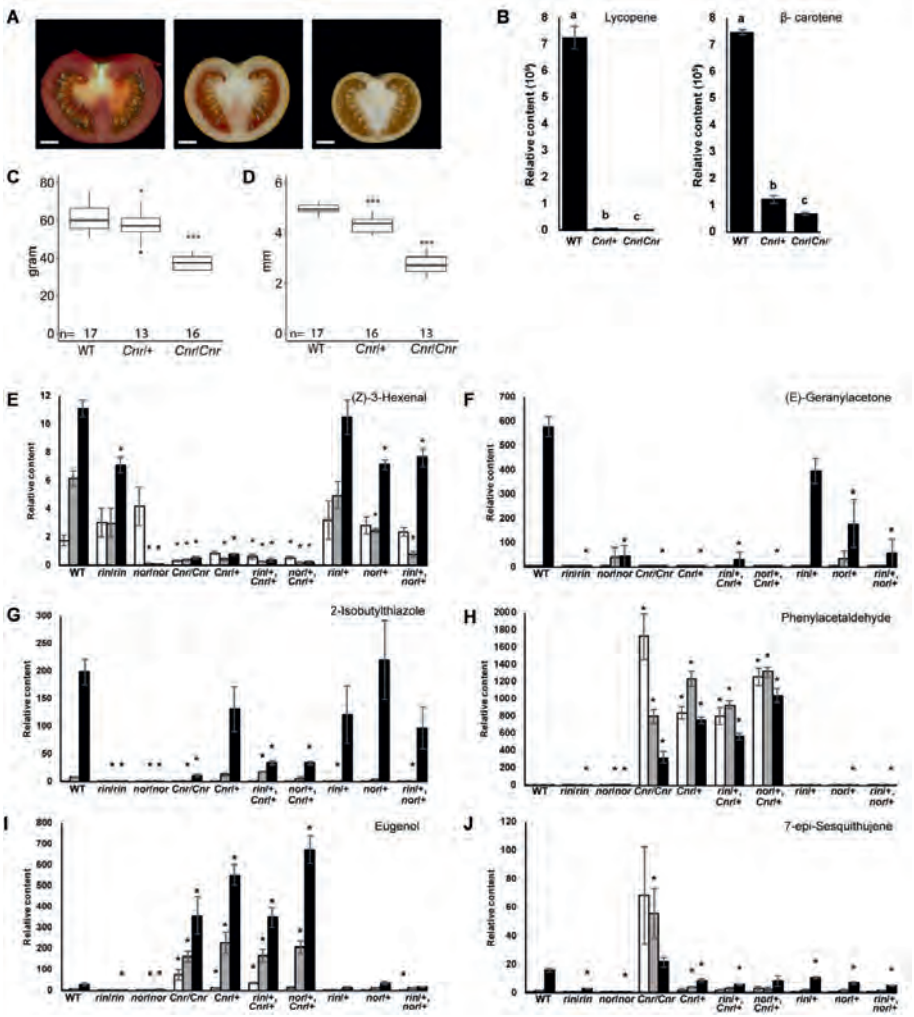

Figure 4. Mutant allele effects on fruit developmental and ripening traits. (**A**) Longitudinally sectioned WT (left), *Cnr* single heterozygous (middle) and homozygous (right) fruits. Scale bar, 1 cm. (**B**) Carotenoid contents in wild-type, *Cnr* single heterozygous and homozygous fruits at ripe or the equivalent stage. Letters a, b and c represent the significantly different classes (P<0.05). (**C**) Weight (gram) and (**D**) thickness (mm) of pericarp of wild-type, *Cnr* homozygous and heterozygous fruits at ripe or the equivalent stage. N is the number of fruits measured. Asterisks mark significant differences (P<10^{-4} '***', P<0.001 '**', P<0.01 '*'). (**E- J**) Volatile contents in the different genotypes. Clear, grey and black blocks represent relative contents at MG, Br and Br+7d. Asterisks indicate significant differences (P < 0.05).

Cnr causes changes in fruit volatiles that are distinct from those caused by *rin* and *nor*

It was reported that *Cnr* affects the production of fruit volatiles distinctly (Kovács et al., 2009) and here we investigated its effects in more detail while comparing it to normal ripening and ripening affected by

mutations. We detected 23 volatiles that are related to tomato flavor and quality and found that all mutant alleles have different effects on their production (Fig. 4E-J and Fig. S3A). Fig. 4E-J show the content of six volatiles, representing groups distinguished by their biosynthetic origin (Tikunov et al., 2005).

As one of the volatile compounds with the most accumulation during ripening, (Z)-3-hexenal is considered as the most representative compound derived from fatty acids (Baldwin et al., 1991). Similarly, (E)-geranylacetone is representative of compounds produced from carotenoids, and both contribute to tomato flavor and quality (Buttery et al., 1971). Their production was almost undetectable in both the *Cnr* homozygote and its heterozygotes (Fig. 4E and F,) while (Z)-3-hexenal was only mildly affected in *rin* and more strongly in *nor* single mutants (Fig. 4E). 2-Isobutylthiazole, a representative of volatiles derived from branched-chain and sulfurous amino acids, is associated with vine green flavor notes (Zhu et al., 2018). Its production was much lower in all homozygous mutants, but comparable to the wild-type in all single heterozygotes with no significant changes in most ripening stages. However, there were additive effects of *rin*, or *nor,* combined with *Cnr* (Fig. 4G). The phenolic volatiles eugenol and phenylacetaldehyde, derived from the phenylpropanoid pathway (Kovács et al., 2009), are characteristic volatiles, which are likely to be associated with unpleasant smell in tomato fruit, at low or high concentrations, respectively. They were not produced in the wild-type and *rin* or *nor*, but were induced dramatically by the *Cnr* allele. Their concentrations in all *Cnr* mutant fruits were more than 400 and 100 times higher than in the wild-type at Br and Br+7d, respectively, while hardly detected in lines without the allele (Fig. 4H and I). Although the concentration of these two compounds developed differently during ripening, *Cnr* is dominant for both. Another compound which is induced in the *Cnr* mutant, but in a clearly recessive manner, was putatively annotated as the sesquiterpenoid 7-epi-sesquithujene (Fig. 4J). In most of the cases, the *Cnr* allele leads to fruits with similar volatile contents as in *Cnr* homozygous fruits, obscuring the *rin* and *nor* effects, and distinct from compound profiles in the *rin* and *nor* mutants (Fig. 4E-J and Fig. S3A).

Changes at transcript level are associated with differences in ripening aspects

To study how *rin*, *nor,* and *Cnr* regulate different aspects of ripening at the gene expression level we measured the expression of several representative downstream genes involved in different aspects of ripening (Fig. 5A-G). As the essential plant hormone in climacteric ripening, ethylene is synthesized from its precursor 1-aminocyclopropane-1-carboxylic acid (ACC), which is produced from S-adenosyl-L-methionine (SAM), and these two steps are catalyzed by ACC oxidase (ACO) and ACC synthase (ACS), respectively (Alexander, 2002). We detected the expression of two representative genes, *ACO1* and *ACS4*, which partially explains the decreased ethylene production in the mutants. *ACS4* was not expressed in any of the homozygous mutants, but its expression was fully restored at Br stage in *rin* and *nor* single heterozygotes. However, there was no significant difference in their double heterozygote from wild-type fruits at the same stages. Genotypes harboring a single *Cnr* allele showed an as sharply

reduced *ACS4* expression as in *Cnr/Cnr* (Fig. 5A). The peaks in *ACS4* and *ACO1* expression (Fig. 5A and B) are associated with the transition from ethylene system 1 to system 2 (Cara and Giovannoni, 2008). Expression changes were much less for *ACO1,* but still mostly significantly lower in all mutant genotypes for the peak expression at Br stage (Fig. 5B). The *rin*/+ had lower *ACO1* expression at Br stage, but we did not see such a decrease in *nor*/+. The combined changes of *ACS4* and *ACO1* and possibly of their paralogs involved in ethylene biosynthesis during ripening affect ethylene production in *rin* and *nor* heterozygotes. *Cnr*'s effect on *ACS4* expression was dominant in all combinations, but its effect on *ACO1* expression was more similar to that of the other mutations.

Fruit firmness and pathogen susceptibility are important for tomato breeding as they contribute to shelf life. *POLYGALACTURONASE* (*PG*) (Giovannoni et al., 1989), *PECTATE LYASE* (*PL*) (Uluisik et al., 2016) and *CELLULASE 2* (*CEL2*) (Lashbrook et al., 1994) are critical genes involved in cell wall modifications that influence fruit texture and pathogen susceptibility during ripening. We observed that all three showed a sharp increase in expression from the beginning of ripening (Br) in wild-type fruits, while mutant alleles significantly repressed the expression individually and additively, but in different degrees for the three genes (Fig. 5C-E) Expression of *PG* increased approximately 60,000 times during ripening compared to the MG stage in wild-type fruits, while in all single and *rin*/+, *nor*/+ double heterozygotes that increase dropped to less than half (Fig. 5C). *PG* was not upregulated in any of the homozygotes or any *Cnr* heterozygotes, indicating the epistasis of *Cnr* over *nor* and *rin* for *PG* expression (Fig. 5C). *CEL2* showed a similar expression pattern as *PG* did with a strong (more than 90%) decrease in all mutants except in *rin*/+ and *nor*/+ in which the decrease was ~70% (Fig. 5D). The expression of *PL* was similar to wild-type in *rin* or *nor* single heterozygotes, revealing a recessive effect, but lower in homozygous mutants, *Cnr* heterozygotes, and (although not significant) *rin*/+, *nor*/+ double heterozygote (Fig. 5E). Thus, the relative effect of all mutations on the expression of *CEL2, PG* and *PL* is highest for *CEL2* and lowest for *PL,* while the *Cnr* allele affects the expression in a dominant manner and *rin* and *nor* alleles interact additively.

TOMATO LIPOXYGENASE C (TomloxC; *LOXC*) (Chen et al., 2004) and *PHYTOENE SYNTHETASE1* (*PSY1*) (Bartley et al., 1992) encode enzymes catalyzing the production of polyunsaturated fatty acids and lycopene precursors, respectively, which enhance flavor and pigmentation during ripening. The expression of *LOXC* reflected (Z)-3-hexenal content (Fig. 4E and 5F). As was predicted from the colorless pericarp in all *Cnr* mutants, we detected only 10% *PSY1* expression compared to wild-type fruits at the ripening stage (Fig. 5G), which was also consistent with the reduction in (E)-geranylacetone (Fig. 4F). *PSY1* expression was also low in *rin* and *nor* homozygotes, which concurs with their lack of lycopene production. A single *rin* or *nor* allele had little effect on *PSY1* expression (Fig. 5G), in line with the normal red pericarp in their single heterozygotes (Fig. 2H and I). At the same time, *rin* and *nor* showed additively negative effects on *PSY1*, with less expression in the double mutant than in single mutants, matching the orange fruit color.

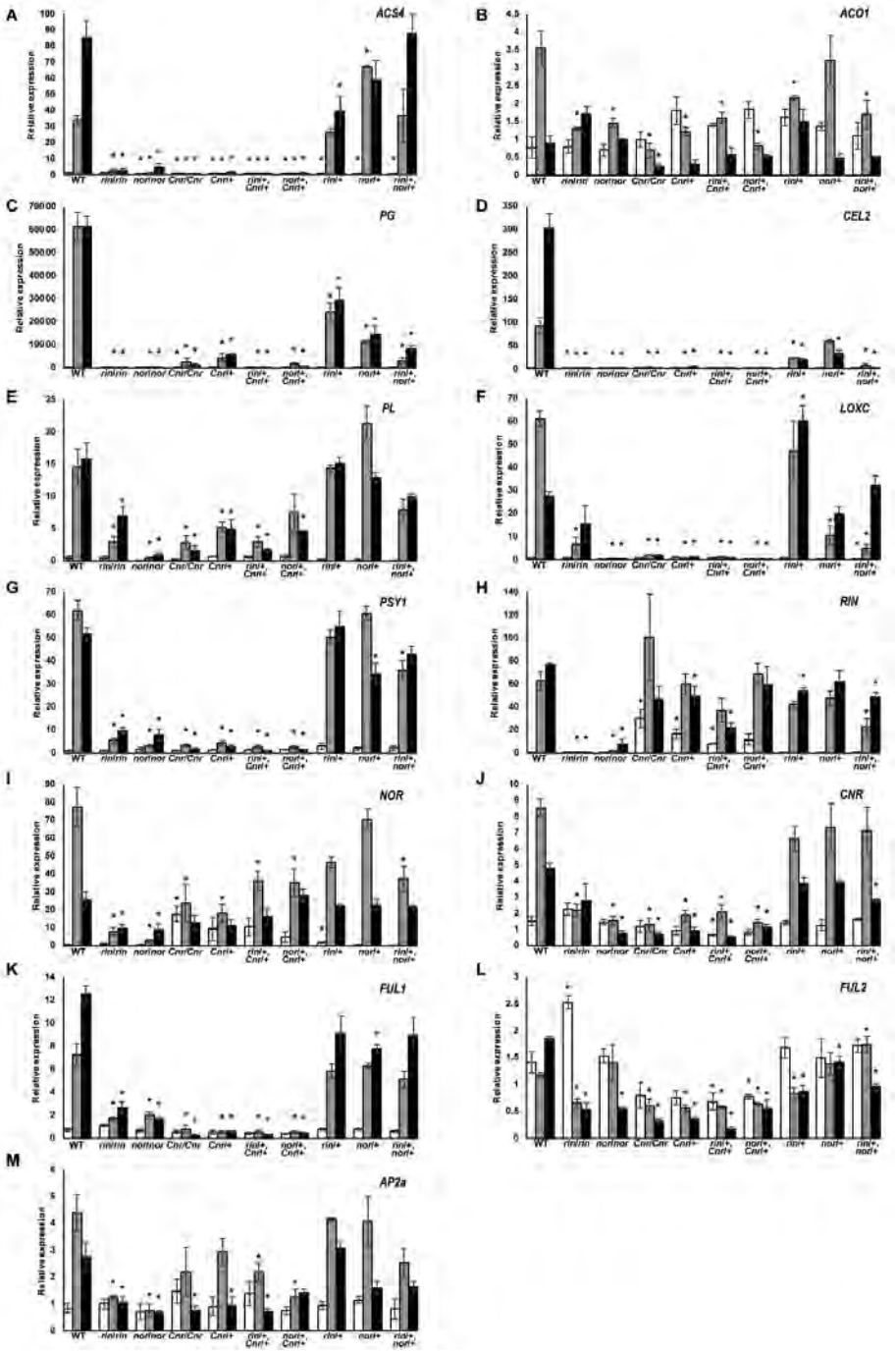

Figure 5. Expression of ripening-related genes in all lines during fruit development and ripening. Three biological replicates of each were used, and error bars represent SE of means. Clear, grey and black blocks represent gene expression at MG, Br and Br+7d. Asterisks indicate significant differences (P<0.05).

Transcript changes of TFs shed light on transcription regulatory network

To investigate the interactions of MADS-RIN, NAC-NOR, and SPL-CNR with each other's natural mutant alleles, we measured their expression in all genotypes (Fig. 5H-J). Expression of all three genes sharply increased during ripening in the wild-type (Br and Br+7d, Fig. 5H-J), although *CNR* did less so compared to expression at the MG stage. Mature green stage-expression of *CNR* was not affected by any of the three mutations. The three genes were all expressed lower in their mutant backgrounds as well as in the other two homozygous mutants except for *MADS-RIN* in *Cnr* (Fig. 5H). Since our qPCR primers are specific for the wild-type *RIN* allele, we detected no expression in the *rin/rin* background, but curiously *MADS-RIN* expression is more than 50% that of the wild type expression in *rin*/+, suggesting that a compensatory mechanism is upregulating the single wild-type allele in this genotype. *MADS-RIN* was only expressed after initiation of ripening in the wild-type but was upregulated in all *Cnr* containing genotypes at the MG stage, emphasizing the unique regulatory properties of this allele. Expression of *MADS-RIN* in *Cnr*/*Cnr* or *Cnr*/+ did not significantly change at Br and only mildly decreased at Br+7d compared to almost no expression in *nor* homozygous mutants (Fig. 5H). This suggests a more upstream position of *MADS-RIN* than the *Cnr* allele in ripening regulation, which also explains the only mild decrease of *MADS-RIN* in all *Cnr* heterozygotes. We noticed that *rin* and *nor* alleles seemed to act additively to regulate gene expression as genes always showed similar expression patterns in *rin*/+ and *nor*/+ and often a stronger decrease in *rin*/+, *nor*/+ (Fig. 5A-M). At the same time, the expression of *MADS-RIN* was down in the homozygous *nor* mutants, demonstrating that the *nor* allele directly or indirectly represses *MADS-RIN* gene expression (Fig. 5H). As our qPCR primers did not distinguish between wild-type and mutant *nor* alleles (which differ by just a 2 nt deletion in *nor*), Fig. 5I shows that the homozygous *nor* mutation also directly or indirectly represses its own expression, but not in the single heterozygous state. *SPL-CNR* is a direct target of MADS-RIN (Fujisawa et al., 2013), and its expression dropped during ripening in *rin*/*rin*. *SPL-CNR* showed similar lower expression in *nor*/*nor* and *rin/rin*, but unexpectedly, it was restored in both *rin*/+ or *nor*/+, as well as in their double heterozygote (Fig. 5J).

We also analyzed the effects of mutant alleles on the expression of other ripening-related TF genes: *FUL1*, *FUL2*, and *APETALA2a* (*AP2a*) (Karlova et al., 2011) (Fig. 5K-M). *FUL1* in wild-type was strongly up-regulated during ripening. As might be expected from it being a direct target of MADS-RIN (Fujisawa et al., 2013; Martel et al., 2011), the expression of *FUL1* was reduced in *rin* (Fig. 5K). *rin* and *nor* alleles functioned only partially additive for these TF encoding genes as expression changes were quite similar in both their single heterozygotes and not further reduced in the double heterozygote (Fig. 5K). *FUL2* was expressed in both mature green as well as in ripe fruit stages, and its expression only increased by approximately 50% late in ripening (Fig. 5L). Although only the *Cnr* allele decreased *FUL2* expression in the MG stage, all mutations affected the upregulation of *FUL2* during ripening, particularly at the Br+7d. Similar to *FUL1* (Fig. 5K), the expression of *AP2a* increased sharply during

ripening and was affected by all three mutations in their homozygous state, although less so by *Cnr* (Fig. 5M). Neither a single allele of *rin* or *nor*, nor their combination, had significant effects on *AP2a* expression.

Almost all genes in this study were similarly down-regulated by the three mutations in homozygous states (except *MADS-RIN* in *Cnr*, Fig. 5H). Also, in most cases *Cnr* has a dominant effect on expression in all combinations (except for *ACO1* and *NAC-NOR*, Fig. 5B and I), whereas *rin* and *nor* vary (as observed in their single heterozygous states) from recessive (*PL*, Fig. 5E, *PSY1*, Fig. 5G and *CNR*, Fig. 5J) through incompletely dominant (*ACS4*, Fig. 5A) to (almost) completely dominant (*PG*, Fig. 5C, and *CEL2*, Fig. 5D). The combination of *rin* and *nor* as single (heterozygous) alleles suggested that their effects on expression were mostly additive.

Discussion

In this study, we have brought together three spontaneous mutations affecting tomato ripening, as single homozygous mutations, single heterozygous mutations, and the latter in various combinations of two in a single common genetic background. Comparisons of our results with those of previous studies reveal sometimes conflicting results that may be due to the use of different genetic backgrounds, stressing the importance of using a common background.

Rin and *nor* function together to regulate downstream genes, and there is a positive feedback regulation between MADS-RIN and NAC-NOR

We observed very mild ripening changes from the wild-type in *rin* and *nor* single heterozygotes with only mild yet significant reductions in ethylene accumulation (Fig. 3D), while the pigmentation was not affected during ripening (Fig. 1 and Fig. 2H and I). The intermediate ethylene production suggests that *rin* and *nor* alleles negatively regulate ethylene biosynthesis but in a dosage-dependent way. At the same time, their additive effect on ethylene production was observed in the double heterozygote, (Fig. 3D), which is consistent with the study from Tigchelaar *et al*. in the background of cv. Rutgers, in which a 50% and 25% amount of ethylene was detected in their single and double heterozygotes, respectively (Tigchelaar et al., 1978).

On the other hand, other studies on *rin* or *nor* heterozygotes report a more distinct effect of heterozygosity on quality attributes, such as color (Sink Jr. et al., 1974). This suggests that such adverse effects on fruit quality that coincide with the desired positive effect on shelf life in these examples could be mitigated by proper choice of the genetic background or cultivar. The intermediate expression of *ACS4* and *ACO1* may well explain this dosage effect of *rin* and *nor* (Fig. 5A and B). The severely decreased ethylene levels indeed interfered with ripening progression in the double heterozygote whose initiation of ripening was significantly delayed (Fig. 3A). Apparently, the reduced climacteric ethylene

peaks in the single heterozygotes and even the severely reduced production in the double heterozygote are sufficient to initiate ripening processes. A similar phenomenon was found in the *ful1/ful2* double knock-out (KO) mutants, where only 17% remaining ethylene production compared with wild-type fruits at Br stage initiated ripening (Wang et al., 2019). These results suggest that there is a threshold for ethylene production to initiate ripening. Once the ethylene level meets the threshold the ripening progresses even if not completely in some TF mutants. However, what this threshold remains unclear, and it is essential to realize that not only ethylene but also its signal transduction plays a role. We observed that not only the ethylene production and pigmentation are similar in *rin* and *nor* single heterozygotes, but also the expression of all measured genes involved in different pathways of ripening are similarly affected. The expression of *FUL2* was quite stable in all lines at the MG stage, while there was a significant increase in *rin*/*rin* (Fig. 5L). We speculate that this upregulation is caused by the expression of *RIN-MC* as it goes up at the equivalent ripening stage in *rin*/*rin* (Li et al., 2018) and negatively regulates ripening. Alternatively, this may point to the repression of *FUL2* expression in the MG stage by MADS-MC, which is likewise affected in the *rin* mutant. Chromatin immunoprecipitation (ChIP) experiments have already shown that MADS-RIN directly targets *NAC-NOR* (Martel et al., 2011; Fujisawa et al., 2013), All genes tested for expression in this study demonstrated *in vivo* binding of MADS-RIN as detected by ChIP, except *ACO1* and *PSY1* (Fujisawa et al., 2013), and although similar data have not been published for NAC-NOR, inspection of the FruitEncode database (Lü et al., 2018) revealed that all but one (*ACO1*) had consensus sequence elements for NAC TF binding. We hypothesize that *rin* and *nor* operate in ripening regulation at the same level of target gene promoter binding, and that normal ripening is affected by combined doses when the two mutations are present at the same time. A recent study of Osorio *et al*. exhibited that *rin* and *nor* fruits displayed similar changes also in postharvest parameters, including water loss, firmness and color changes, which supports our hypothesis (Osorio et al., 2019).

Besides, we noticed a clear expression reduction of *MADS-RIN* in *nor* and *NAC-NOR* in *rin* homozygotes (Fig. 5H and I). The reduction of *NAC-NOR* makes sense as it is a direct target of MADS-RIN, but conversely, there are no data regarding direct targets of NAC-NOR. We assume that there is a feedback regulation between MADS-RIN and NAC-NOR and that they positively regulate each other, and one wild-type allele can rescue the expression of the other.

The *Cnr* mutation has multiple effects on ripening through different mechanisms

The *Cnr* mutation was reported to be repressing normal ripening in a genetically dominant fashion (Manning et al., 2006). We observed earlier reported defects and discovered novel effects caused by the *Cnr* allele, indicating its broader role in fruit development and ripening. Some changes are affected by the allele in a dominant manner, such as the lack of ethylene production during ripening and the lower pericarp density (Fig. 3D and Fig. S3B), while others are almost recessive, such as the thinner pericarp

and smaller fruit size (Fig. 4C and D). The *Cnr* allele affects fruit flavor and quality by regulating volatile biosynthesis in both recessive and dominant ways. *Cnr* fruits have a quite distinctive unpleasant smell. We detected possible candidates for causing this smell in phenylacetaldehyde, which is associated with the "malodorous" allele (Kovács et al., 2009; Tadmor et al., 2002) and eugenol, which is associated with smoky off-flavor (Tikunov et al., 2013). Both were produced only when a *Cnr* allele was present, suggesting its dominant role in conferring the unpleasant aroma. For other representative volatile compounds, the content of 2-isobutylthiazole is affected recessively (Fig. 4G), while others, like (Z)-3-hexenal are regulated dominantly (Fig. 4E).

Taking into account the pleiotropic effects of *Cnr* on fruit development as well as on ripening, we propose that its phenotypes are not just caused by the effect of the hypermethylated promoter region on *SPL-CNR* expression, but that also other, genome-wide effects of the mutation are responsible. This proposition is further supported by the recent observation that a knockout mutation in *SPL-CNR* has only a mild effect on ripening and none of the other visible effects on fruit development that the *Cnr* allele has (Gao et al., 2019).

The *Cnr* allele is epistatic to the *rin* and *nor* alleles

The *Cnr* allele is dominant (Thompson et al., 1999), but how it interacts with other natural mutations in affecting ripening was not yet reported. When comparing the ripening traits of mutants with combined doses of *rin*, *nor* and *Cnr* we observed that *Cnr* phenotypes always masked those caused by *rin* or *nor*. The normal pigmentation and initiation of ripening with ethylene synthesized in both *rin*/+ and *nor*/+ disappeared once a *Cnr* allele was introduced (Fig. 2F, G and Fig. 3D). These *Cnr* effects start from an earlier fruit developmental stage with much earlier chlorophyll degradation (Fig. 1 and Fig. S1), finally rendering fruits pale yellow (Fig. 1). At the stage equivalent to ripening, all fruits with a *Cnr* allele had low-lycopene yellow pericarp and no ethylene production (Fig. 2F, G and Fig. 3D). In addition, expression of TF genes and effector genes showed the same pattern in both single and double mutant backgrounds, not only mirroring the phenotypic changes caused by the *Cnr* allele but also illustrating its drastic effects on gene expression, leading us to conclude that *Cnr* is epistatic to *rin* and *nor*. There have been some epistatic interactions among quantitative trait loci reported in tomato fruit elongation (Van der Knaap et al., 2002) or meristem establishment (Koltai and Bird, 2000), but not yet in ripening. Here we show that *Cnr* is epistatic to *rin* and *nor* in ripening regulation.

The function of the underlying TF genes should be evaluated separately from the function of their spontaneous mutant alleles

Researchers often study gene function by analyzing the phenotype of its spontaneous mutant, but as several recent publications have revealed, this may not be the correct strategy for the three mutations studied here. *Rin*, *nor* and *Cnr* were reported to be dominant-negative (Ito et al., 2017; Wang et al., 2019) or gain-of-function (Gao et al., 2019) recently, whose severe ripening defects do not represent the

phenotype of their *null* alleles. These spontaneous alleles form modified TF proteins, which may still be able to interact with co-factors and bind promoters of (possibly additional) target genes, thereby competing with wild-type alleles and function dominantly (Veitia, 2007). CRISPR/Cas-mutagenesis enables to knock out a gene easier and more precise to get true *null* alleles. Using this approach, Ito *et al*. and we, created the *mads-rin* and *nac-nor* KO mutants, respectively (Ito et al., 2017; Wang et al., 2019). NAC TFs bind to the promoter of their effector genes and dimerize with themselves, other TFs or co-factors via the NAC domain at the N-terminus (Duval et al., 2002; Xie et al., 2000). The wild-type *NAC-NOR* allele also has the putative transcription regulatory region at the C-terminal end of the protein, while the *nor* allele produces a truncated protein lacking this region (Kumar et al., 2018). We created a true KO allele of *NAC-NOR* with a frame-shift close to the start codon, which eliminates the entire NAC domain in both wild-type and *nor* backgrounds (Wang et al., 2019). Ripening of the *null* mutant in the wild-type background was only partially affected, leading to orange pericarp, much milder than the spontaneous mutant showing severe non-ripening green fruits. In contrast, the CRISPR-derived *null* allele partially rescued the phenotype in the *nor* background, implying that the dominant-negative *nor* allele is the cause of the severe defects in ripening.

Similarly, the *rin* phenotype is specifically caused by the RIN-MC fusion protein rather than the loss-of-function of MADS-RIN (Ito et al., 2017). Recently, Gao *et al*. showed that a CRISPR-derived *spl-cnr* KO mutant displayed a delay but normal progression of ripening as wild-type fruits, suggesting that *SPL-CNR* is not essential for ripening (Gao et al., 2019). However, *Cnr* is more complicated as it is caused by an epi-mutation, with no sequence differences in the genome (no truncated proteins to compete with the wild-type one), resulting in 10~ 20% expression of *SPL-CNR* compared to wild-type fruits, while the ripening is entirely blocked. Thus, it is more likely that *Cnr* is also a gain-of-function mutation with a phenotype that does not just reflect the function of the SPL-CNR protein. Due to this unique character of the spontaneous mutants, the corresponding TF genes cannot be simply placed in a gene regulatory network model based on gene expression patterns in these mutants.

In conclusion, we showed the additive effect of *rin* and *nor* and the epistatic dominant role of the *Cnr* allele on tomato fruit ripening, improving our understanding of how these alleles interact to regulate the sensory and quality aspects of ripe fruits.

Acknowledgements

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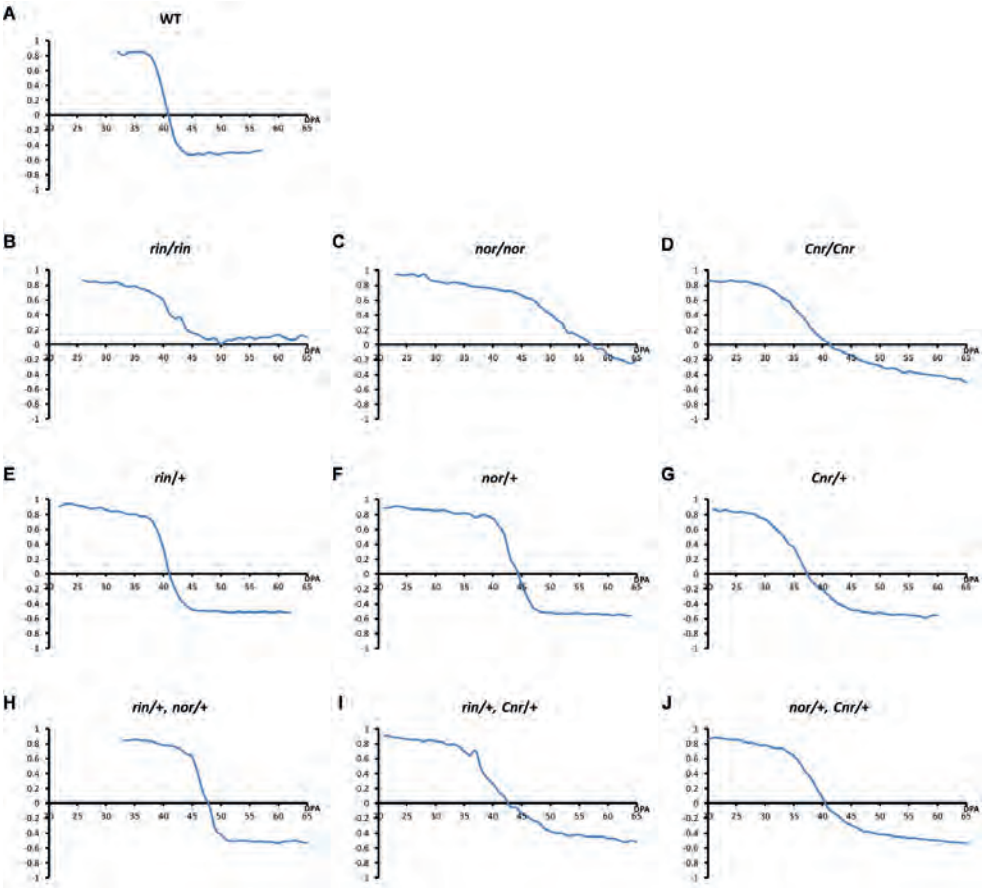

Figure S1. Chlorophyll degradation during fruit development and ripening. The relative content of chlorophyll at the bottom of at least six fruits was measured by a Pigment Analyzer every day and the average value was used to show the changes.

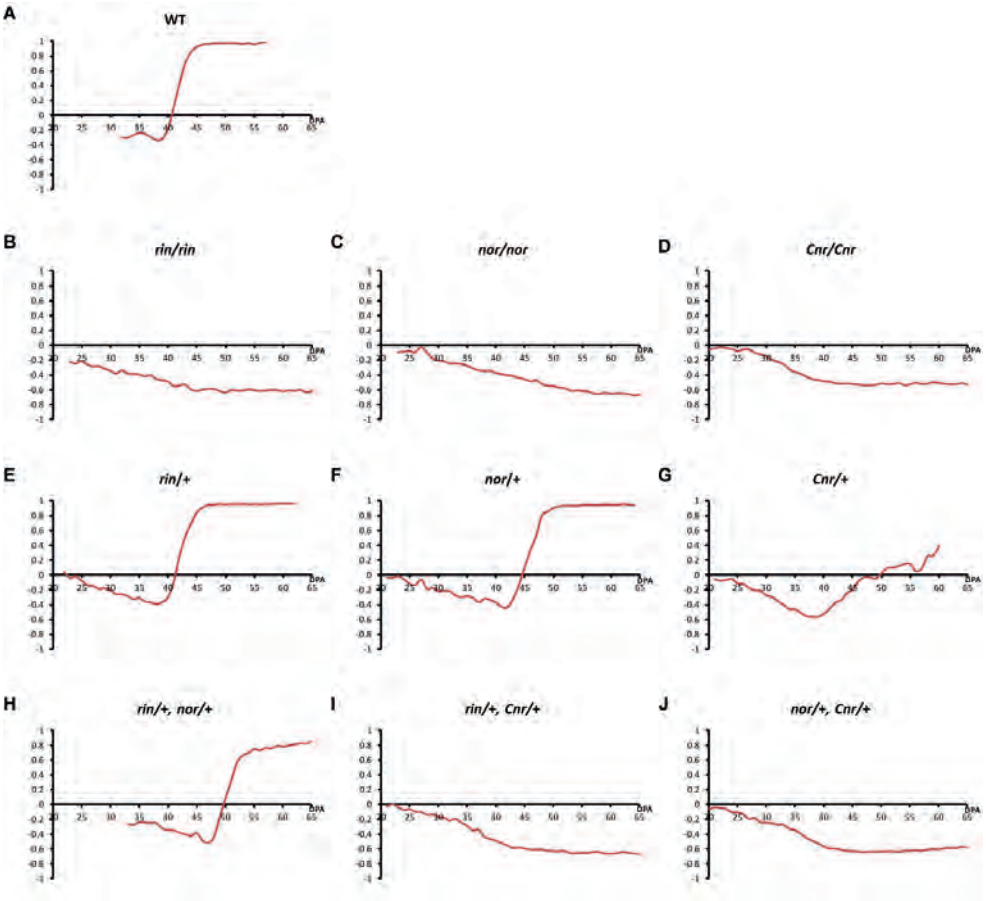

Figure S2. Lycopene accumulation during fruit development and ripening. The relative content of lycopene at the bottom of at least six fruits was measured by a Pigment Analyzer every day and the average value was used to show the changes.

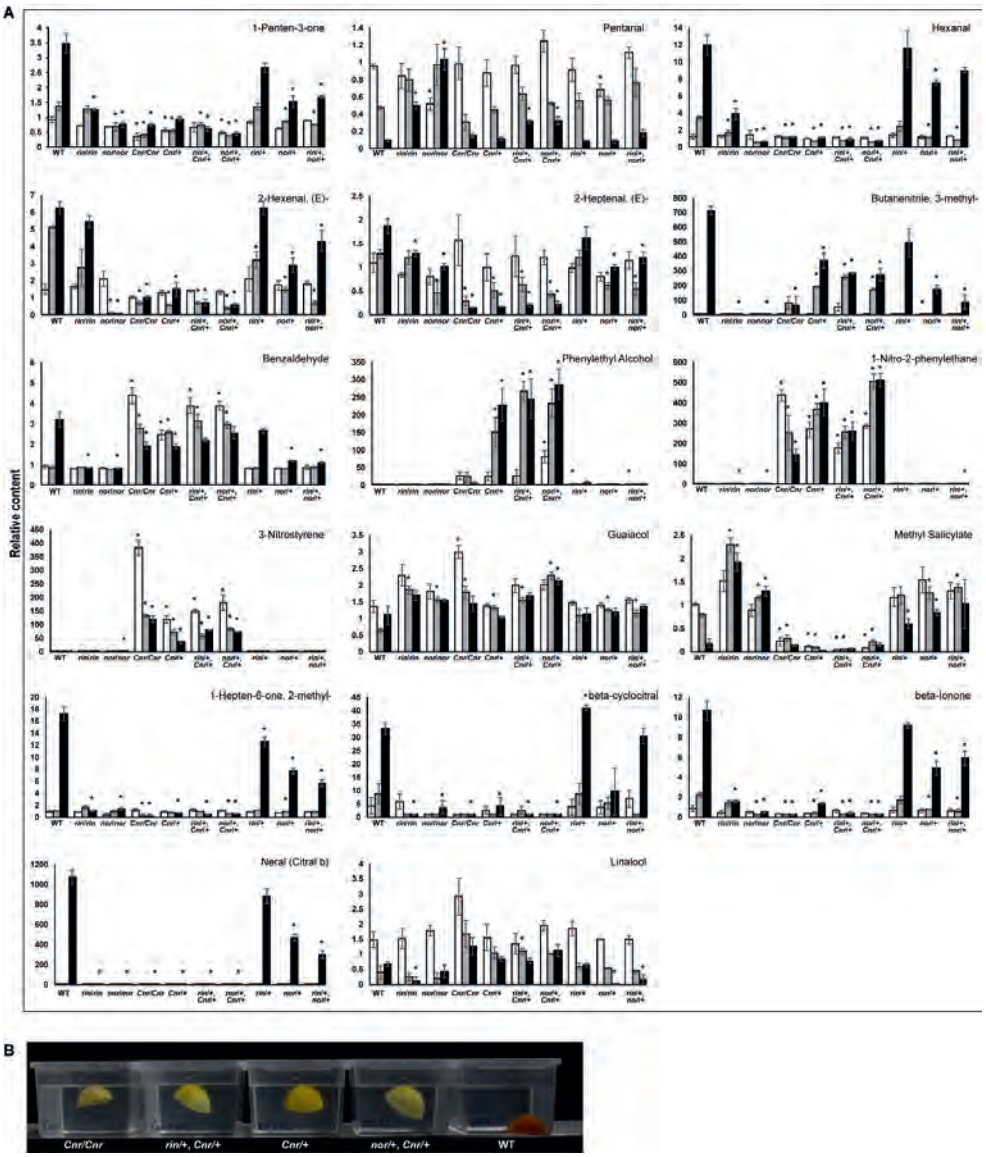

Figure S3. Mutant effects on volatile compounds and pericarp density change. (**A**) Volatile contents in all lines in this study. Clear, grey and black blocks represent the content at MG, Br and Br+7d. Asterisks show the significant difference (P<0.05). (**B**) Pericarp from fruits with different combinations of the *Cnr* allele have a lower density than that of the wild-type. The *Cnr* allele makes pericarp float in water. Pericarp from half fruits at the same stage were used.

Primer name	Sequence (5'-3')	Description
rin1- F	AGACATGAACAGCCTTCT	genotype *rin* hybrids
rin1- R	CCTGGATGGATGCTTTGA	genotype *rin* hybrids
rin2- F	GGGTTAGTGCGATGTATTTATT	genotype *rin* hybrids
rin2- R	AACGGAGGGAGAATAAGT	genotype *rin* hybrids
nor-F	ATGATTGGGTTTTATGTC	genotype *nor* hybrids
nor-R	GTTGTTGCTTCGTCGTTA	genotype *nor* hybrids
LOXC-F	GGAATGGGAGTGGTTCTTGA	qPCR for *LOXC*
LOXC-R	TGAATTTGTGAAGGGAAGCA	qPCR for *LOXC*
PSY1-F	TGAATTAGCACAGGCAGGTCT	qPCR for *PSY1*
PSY1-R	GGTCACCCTTCCAGCAAATA	qPCR for *PSY1*
AP2a-F	ATGGGATTGTGGGAAACAAG	qPCR for *AP2a*
AP2a-R	CTGCTGCATGTGCTGTATCA	qPCR for *AP2a*
PL-F	TACAAAGCACGAGGATGCAC	qPCR for *PL*
PL-R	CCTCCAATTCCAATTCTTCC	qPCR for *PL*
RIN-F	ATGCAGCACCATCAACACAT	qPCR for *RIN*
RIN-R	TCAAAGCATCCATCCAGGTA	qPCR for *RIN*
ACS4-F	GTTTGGCTGATCCTGGTGAT	qPCR for *ACS4*
ACS4-R	TCCTGGGTAATAGGGTGTGG	qPCR for *ACS4*
FUL1-F	TGCTGAGGTTGGTTTGATTG	qPCR for *FUL1*
FUL1-R	GCAGGAATCGTTGGCATATT	qPCR for *FUL1*
CEL2-F	ATCATGTTGTGCCATGTGGT	qPCR for *CEL2*
CEL2-R	TTGGCAATGTGTTTGAGGAG	qPCR for *CEL2*
ACO1-F	GGACTTGAGGCTGTTCAAGC	qPCR for *ACO1*
ACO1-R	AGTAGGAAGATGGCGCAAGA	qPCR for *ACO1*
PG2a-F	GGAGCTAAGGGTGATGGAAA	qPCR for *PG2a*
PG2a-R	CTTCATTCCATGCTTGCTCA	qPCR for *PG2a*
CNR-F	TTCCCGGATTTCTAAGCAAATTGT	qPCR for *CNR*
CNR-R	GTTGGAATGTCAACATGGATATGCA	qPCR for *CNR*
FUL2-F	AATGGAGAAGTAGAAGGATCATCG	qPCR for *FUL2*
FUL2-R	GATAACATAATATTGTCCGCTTGC	qPCR for *FUL2*
NOR-F	AGAGAACGATGCATGGAGGTTTGT	qPCR for *NOR*
NOR-R	ACTGGCTCAGGAAATTGGCAATGG	qPCR for *NOR*

Table S1. Primers used in this study.

Chapter 3

Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis

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Abstract

Tomato (*Solanum lycopersicum*) is a model for climacteric fleshy fruit ripening studies. Tomato ripening is regulated by multiple transcription factors together with the plant hormone ethylene and their downstream effector genes. Transcription Factors APETALA2a (AP2a), NON-RIPENING (NOR) and FRUITFULL (FUL1/TDR4 and FUL2/MBP7) were reported as master regulators controlling tomato fruit ripening. Their proposed functions were derived from studies of the phenotype of spontaneous mutants or RNAi knock-down lines rather than, as it appears now, actual *null* mutants. To study TF function in tomato fruit ripening in more detail, we used CRISPR/Cas9-mediated mutagenesis to knock out the encoding genes, and phenotypes of these mutants are reported for the first time. While the earlier ripening, orange-ripe phenotype of *ap2a* mutants was confirmed, the *nor null* mutant exhibited a much milder phenotype than the spontaneous *nor* mutant. Additional analyses revealed that the severe phenotype in the spontaneous mutant is caused by a dominant-negative allele. Our approach also provides new insight into the independent and overlapping functions of *FUL1* and *FUL2*. Single and combined *null* alleles of *FUL1* and *FUL2* illustrate that these two genes have partially redundant functions in fruit ripening, but also unveil an additional role for *FUL2* in early fruit development.

Introduction

Tomato (*Solanum lycopersicum*) produces fleshy fruits, which are climacteric, i.e. displaying a burst in ethylene production during ripening. Its diploid genome, available genome sequence and relative ease of transformation make it the ideal model for studying fleshy fruit development and ripening.

Biochemical and physiological processes during tomato fruit ripening result in changes in texture, colour and flavour. Together with ethylene, transcription factors (TFs) and their downstream effector genes regulate these changes (Karlova et al., 2014). RIPENING INHIBITOR (RIN), COLORLESS NON-RIPENING (CNR), TOMATO AGAMOUS-LIKE1 (TAGL1), APETALA2a (AP2a), NON-RIPENING (NOR) and FRUITFULL (FUL1 and FUL2) are major TFs regulating tomato fruit ripening, either by promoting or repressing this process (Karlova et al., 2014). RIN, TAGL1, and FRUITFULL1 and 2 are Minichromosome Maintenance (MCM1), AGAMOUS (AG), DEFICIENS (DEF) and Serum Response Element (SRF) (MADS) domain TFs (Robinson and Tomes, 1968; Busi et al., 2003), are highly expressed during the ripening stage and were reported as positive regulators of tomato fruit ripening (Vrebalov et al., 2009; Shima et al., 2013). MADS domain proteins often function as a dimer or tetramer for regulation (Smaczniak et al., 2012) and interaction between RIN and TAGL1 or FUL was shown by yeast-2-hybrid studies (Leseberg et al., 2008). Tomato SQUAMOSA promoter-binding protein-like (SPL) transcription factor CNR is also an activator of tomato fruit ripening, involving in ethylene and lycopene biosynthesis. The spontaneous *Cnr* mutant shows a colourless pericarp with strongly reduced ethylene production (Thompson et al., 1999; Manning et al., 2006).

The tomato transcription factor AP2a is a member of the APETALA2/ Ethylene Response Factor (AP2/ERF) family (Kim et al., 2006). Using RNAi Chung *et al.* and Karlova *et al.* showed that the down regulation of *AP2a* interfered with normal ripening in fruits, including decreased carotenoid production, but increased ethylene production resulting in early onset of fruit ripening and senescence (Chung et al., 2010; Karlova et al., 2011). Thus, AP2a is a negative regulator of ethylene production, but a positive regulator of other ripening aspects such as chlorophyll degradation and carotenoid biosynthesis. A negative feedback loop of *AP2a* and *CNR* during ripening was reported, in which *AP2a* was regulated by RIN, NOR and CNR, while AP2a itself negatively regulates *CNR* (Karlova et al., 2011). *AP2a* is also a target of post-transcriptional regulation by miR172 (Karlova et al., 2013). NAC-NOR is a NAM, ATAF1/2 and CUC2 (NAC) domain transcription factor, containing the conserved NAC domain that functions in DNA binding as well as in dimerization with other NAC proteins (Olsen et al., 2005; Xie et al., 2000). There are 101 *NAC* genes in tomato but, only three (*NAC1*, *NAC4* and *NAC-NOR*) were shown to be involved in regulation of fruit ripening so far (Zhu et al., 2014; Meng et al., 2016). NAC-NOR appeared to be the most strongly regulating activator based on the completely non-ripening phenotype of its spontaneous mutant (Tigchelaar E, Tomes M, Kerr E, 1973). In the spontaneous *nor* mutant, a 2 bp deletion in the third exon of *NAC-NOR* causes a frameshift, resulting in a truncated protein giving a strong non-ripening phenotype (Giovannoni et al., 2004). Tomato MADS domain transcription factors FUL1 and FUL2 are co-orthologs of *Arabidopsis* FRUITFULL (Bemer et al., 2012). *FUL2* is expressed in flowers and developing green fruits, and its expression increases during ripening. *FUL1* expression is detectable in flowers, but in fruits it is much higher and specific for the ripening stage (Bemer et al., 2012). Yeast-2-hybrid protein interaction experiments showed that both could interact with RIN, which is also expressed during ripening, while FUL2 interacts with other MADS domain proteins as well (Leseberg et al., 2008; Shima et al., 2013). RNAi experiments showed that *FUL1* and *FUL2* probably function redundantly in tomato fruit ripening (Bemer et al., 2012; Shima et al., 2014; Fujisawa et al., 2014). Phenotypes of *FUL1/2* RNAi fruits diverged between studies, showing an orange-ripe phenotype with reduced lycopene level and relatively normal ethylene production in one study (Bemer et al., 2012), and almost green fruits with strongly reduced ethylene production in another (Shima et al., 2014; Fujisawa et al., 2014).

In the absence of available spontaneous mutants Virus-induced Gene Silencing (VIGS) of gene expression and RNA interference (RNAi) have often been used for evaluating gene function. Both approaches however, may suffer from incomplete suppression of expression or lack of specificity for the targeted gene. Because RNAi silencing was the most popular tool in the past decades due to the relative ease of use (Saurabh et al., 2014), functional characterization of the gene of interest may have been imperfect in many cases. The action of Site-Specific Nucleases (SSN) allows targeted mutagenesis by utilizing the imperfect nature of double-strand DNA break (DSB) repair, creating mostly small INDELs which, when located in an open reading frame, can lead to frame-shifts resulting in loss-of-

function alleles. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/ CRISPR-associated protein 9 (Cas9) has rapidly gained popularity as the SSN of choice for mutagenesis due to its high efficiency and relative ease of use. It utilizes guideRNAs (gRNAs), which recognise the target sequence to direct the endonuclease Cas9 to cut there, causing a DSB (Jinek et al., 2012). Together with efficient modular cloning strategies such as Golden Gate cloning (Brooks et al., 2014) it allows multiple gRNAs targeting more than one gene at the same time with high efficiency (Li et al., 2013), and CRISPR/Cas9-mutagenesis has been successfully applied in many plant species, such as *Arabidopsis*, rice, maize and tomato (Ma et al., 2016).

Spontaneous mutants with fruit ripening phenotypes in tomato have been reported for decades and forward genetics studies have identified several of the underlying genes as encoding transcription factors, such as for the *rin* and *nor* mutants. These mutants have proven extremely valuable for both fundamental research as well as in applications. Yet, the availability of a larger set of alleles may improve our understanding of TF function further, as well as allow study of specific, true knock-out phenotypes where only RNAi studies were available before. Recently, by using CRISPR/Cas9-mediated mutagenesis *Ito et al.* illustrated that the *rin* phenotype is caused by the production of a fusion protein RIN-MC, rather than the mere loss of function of MADS-RIN (Ito et al., 2017). After knocking out *RIN* in a wild type background, fruit ripening was affected but not blocked as it was in the original *rin* mutant (Ito et al., 2015), and ripening was partially restored by knocking out *RIN-MC* in the *rin* background. There are so far no spontaneous mutants of *AP2a*, *FUL1* or *FUL2* reported, and RNAi or VIGS phenotypes may only partially reflect the functions of these genes.

In this study, by using CRISPR/Cas9-mutagenesis we generated genuine knock-out mutants of *AP2a*, *NAC-NOR*, *FUL1* and *FUL2* (as well as the latter two combined) to further study their function in tomato fruit ripening. In this way we confirm the previously found function of *AP2a*, but demonstrate that the spontaneous *nor* mutation represents a dominant-negative allele of *NAC-NOR* because a *null* allele has a milder phenotype. Moreover, true knock-out mutants of *FUL1* and *FUL2* allowed to differentiate between, shared functions during ripening and a specific *FUL2* function in early fruit development.

Results and Discussion

In order to obtain knock-out mutations in the selected transcription factor genes in tomato cv. Moneyberg, we have used binary vectors containing *SpCas9* combined with 2 guideRNA-encoding expression cassettes. Two guides were used to target a single gene or, combining two guides, one for *FUL1* and *FUL2* each, to produce *ful1/ful2* double mutants. In all cases, primary transformants were genotyped for targeted mutations, selected transformants were selfed and only homozygous or biallelic mutants were used for phenotyping. The locations of the targeted sites as well as the obtained (*-cr*) alleles are shown in Fig. 1 and Supplementary Fig. 1. As can be seen in the latter, and since mutant

alleles were derived from just one or two distinct guideRNAs, similar detrimental effects on the resulting protein functions were expected, and we selected those which were deemed representative for all similar mutations. Flowers were labelled at anthesis as 0 Days Post Anthesis (DPA) to record the time required to reach the Breaker (Br) stage, and ethylene production of fruits was measured at Breaker and Breaker + 5 days stages.

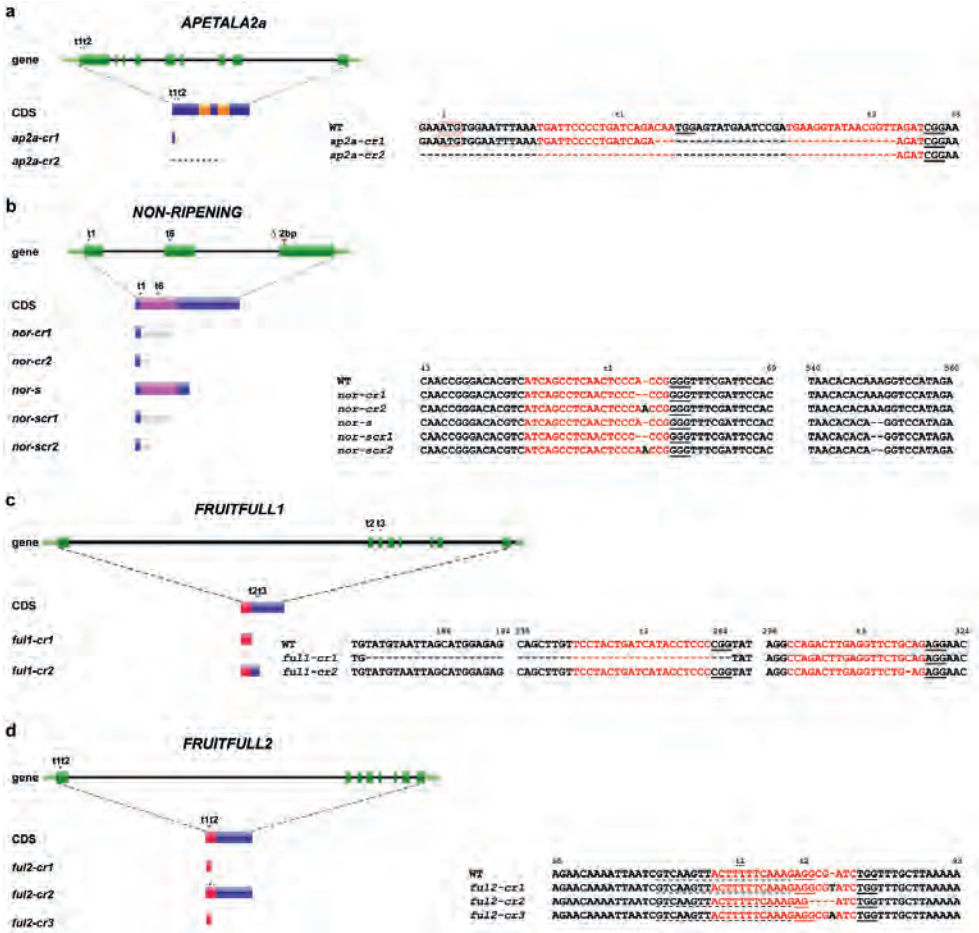

Figure 1. Targets for CRISPR/CAS9 mutagenesis of tomato transcription factor genes, and resulting mutant alleles. (**a**) Mutations in *AP2a*. Overview of the *AP2a* gene and protein changes in knock-out mutants. sgRNA AP2a-t1 and AP2a-t2 located in the first exon were used. (**b**) Mutations in *NAC-NOR*. Overview of the *NAC-NOR* gene and protein changes in the spontaneous *nor* (*nor-s*) and CRISPR alleles. sgRNA NOR-t1 and NOR-t6 were designed at the start and middle of the NAC domain for all mutagenesis experiments. (**c**) Mutations in *FUL1*. *FUL1* gene and protein changes in knock-out alleles. (**d**) Mutations in *FUL2*. Overview of the *FUL2* gene and protein changes in CRISPR alleles. Regions in orange, pink and red represent the AP2, NAC and MADS domain, respectively. Letters in red indicate spacer sequences and underlined are protospacer adjacent motifs (PAM). The start codon is indicated with red boxes. Numbers represent the location of the nucleotide in the coding sequence. A black diamond shows the single amino acid deletion in *ful2-cr2*.

ap2a mutants initiate fruit ripening earlier, but do not fully ripen

AP2a (Solyc03g044300) was reported to be a negative regulator of tomato fruit ripening initiation based on RNAi suppression of expression (Karlova et al., 2011), but true knock-out mutants were not available so far. The encoded protein of 401 amino acids contains two AP2 domains (amino acids 135 to 201 and 227 to 294), presumably involved in DNA binding (Jofuku, 1994). By using two gRNAs, four alleles *ap2a-cr1- 4* were obtained with deletions in the first of 9 exons (Fig. 1a and Supplementary Fig. 1a). We selected two lines with deletions most probably resulting in *null* alleles. In *ap2a-cr1* a 35 bp deletion joining the two gRNA-target sites is predicted to produce a peptide of 27 aa with no AP2 domains, while the 133 bp deletion in allele *ap2a-cr2* extends to 67 nucleotides upstream of the start codon, and therefore no AP2a protein is expected to be produced (Fig. 1a). The 133 bp deletion in *ap2a-cr2* extends into the 5' UTR of *AP2a*, which does not necessarily affect its transcription. This mutation deletes the first start codon, as well as an alternative start codon at amino acid position 12. The next in-frame start codon at amino acid position 204 is located in exon 5 and 3' of the first of two conserved AP2 domain-encoding regions and therefore even if used, unlikely to result in a functional protein.

When compared to wild type fruits (Fig. 2a) pericarp of the two lines remained orange/brown (Fig. 2b and 2c) 20 days after Breaker stage and did not become fully red (Supplementary Fig. 2a). Faster ripening was accompanied by earlier senescence: *ap2a-cr1* and *ap2a-cr2* fruits started to crack before 60 DPA while fruits from other mutants were still intact (Supplementary Fig. 2a). Fruits of the *ap2a* mutants took only 39 to 41 days to reach Br stage in *ap2a-cr1* and *ap2a-cr2*, respectively, significantly less than in wild type fruits (47 days) (Fig. 3a). This is consistent with the observed 7 days earlier colour change in RNAi fruits (Chung et al., 2010), confirming AP2a's negative regulatory role in the initiation of tomato fruit ripening. Fruits of both *ap2* knock-out mutants produced at least twice the wild type amount of ethylene at Br stage (Fig. 3b).

The AP2 protein in *Arabidopsis* is known to negatively regulate its own transcription by a feedback regulation (Schwab et al., 2005). While RNAi knock-down of expression usually reduces mRNA levels of the target gene with varying efficacy, remaining mRNA can still produce functional protein. Mutants producing no functional proteins, apart from giving a stronger phenotype also allow the exploration of positive or negative feedback autoregulation of the protein on its own expression. This is exemplified by expression analysis of the tomato *AP2a* mRNA in wild type and *ap2a-cr1* and *-cr2* fruits. As the mutations result in no functional protein production, mutant fruits display higher levels of *AP2a* mRNA, indicating that similar to *Arabidopsis* AP2, tomato AP2a protein negatively regulates its own transcription (Fig. 3c). The much higher ethylene production and faster ripening in *ap2a-cr1* and *ap2a-cr2* confirm AP2a's negative role in both ethylene production as well as in initiation of tomato fruit ripening. Its positive regulatory role in ripening is shown by the orange/brown fruit colour resulting

from a lack of lycopene production and a defect in chlorophyll degradation, consistent with the RNAi phenotype from Karlova *et al*. (Karlova et al., 2011).

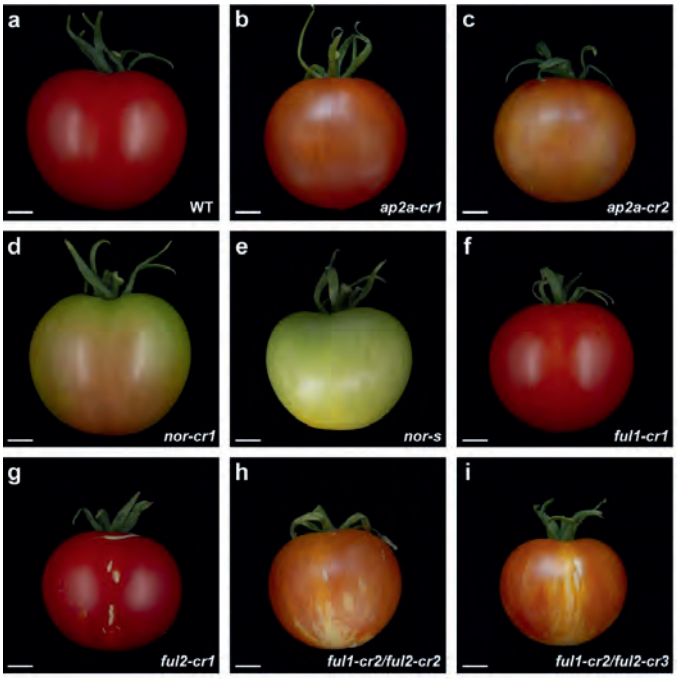

Figure 2. Phenotypes of mutant fruits. Fruits of homozygous mutants at 55 DPA. All mutants are in cv. Moneyberg except **(e)** which shows cv. Ailsa Craig *nor-s* for comparison at an equivalent stage. Scale bar, 1 cm.

A *nor null* mutant has a milder phenotype than the spontaneous *nor* mutant

Ripening defects in the spontaneous *nor* mutant are likely due to a 2 bp deletion in the third exon of *NAC-NOR* (Solyc10g006880), leading to a frame shift and a truncated protein of 186 amino acid (aa) versus 355 aa for the intact gene (Kumar et al., 2018). This truncation is located after the NAC domain, leaving the possibility that the truncated protein retains its dimerization and DNA-binding capacity. To knock out *NOR* in cv. Moneyberg we designed two gRNAs for *NAC-NOR* but found no edits at position t6. Two alleles at position t1 were obtained, *nor-cr1* with a 1 bp deletion and *nor-cr3* with a 2 bp deletion 5' of the NAC-domain coding sequence (Fig. 1b and Supplementary Fig. 1b), both resulting in a frameshift and a protein predicted to contain only 17 aa of NAC-NOR and no conserved NAC domain (Fig. 1b).

Homozygous *nor-cr1* fruits initiated ripening later than wild type fruits (Fig. 2d) by 3 days on average (time to Br stage, Fig. 3a), but surprisingly progress of ripening was only partially affected, in contrast

to being totally blocked as it is in the spontaneous mutant (Fig. 2e). Homozygous *nor-cr1* exhibited an orange pericarp at 60 DPA, indicating that lycopene biosynthesis was affected. Colour change after Breaker in *nor-cr1* was much slower than in wild type fruits and the pericarp remained orange until 70 DPA and beyond (Supplementary Fig. 2b). Ethylene production in *nor-cr1* fruits at both Br and Br+ 5 d stages was significantly lower than in wild type fruits, possibly explaining the delayed initiation of ripening, but clearly higher than in spontaneous *nor* mutant fruits, where no ethylene production was detectable in the time frame where normally ripening occurs (Fig. 3b).

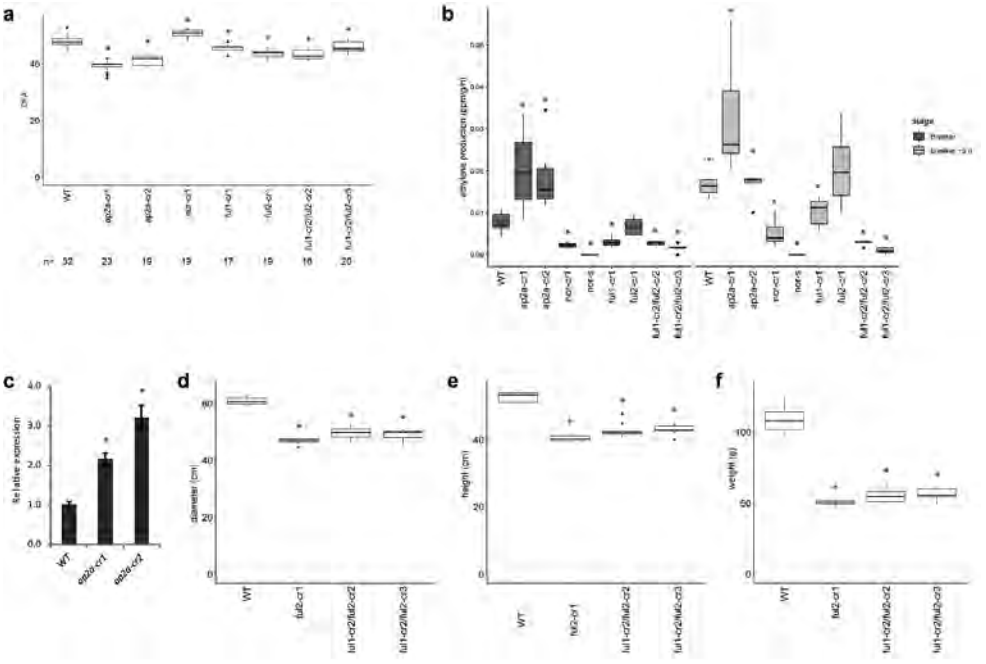

Figure 3. Differences in developmental and ripening processes of mutants compared to wild type. (**a**) Time to initiation of ripening (Days Post Anthesis (DPA) to Breaker) of wild type and homozygous mutants. (**b**) Ethylene production (ppm/g/h) for wild type and homozygous mutants at Br and Br+ 5 d stages and of the spontaneous *nor* mutant at the equivalent stage. Values of five or six fruits were used. (**c**) Relative *AP2a* expression in two *ap2a* knock-out lines. Error bars represent SE of means. (**d**) Diameter (cm), (**e**) Height (cm) and (**f**) Weight (g) of wild type and homozygous *ful2* mutant fruits. Values of eleven fruits for each genotype were used for (**d**)- (**f**). Asterisks show significant differences ($P < 0.05$).

The *alcobaca* (*alc*) mutation, encoding a deleterious V106D substitution in the NAC domain is allelic to *nor* and displays a weaker effect on ripening (Casals et al., 2012). By CRISPR/Cas9-induced gene replacement Yu *et al.* replaced thymine to adenine at position 317 of the *NAC-NOR* coding sequencing, creating an *alc* allele and confirming the long-shelf life character of *alc* (Yu et al., 2017). Three Penjar accessions contain the *alc* allele, while a fourth contains an early frame-shift and a truncated protein of

6 aa, which is similar to the *nor-cr1* allele described here. Our *nor-cr1* fruits displayed delayed ripening and an orange-ripe phenotype similar to that of *alc* fruits. As the truncated protein in *nor-cr1* contains no NAC domain this likely makes *nor-cr1* a true *null* allele. A transcriptional activation region located in the C-terminal region of NAC proteins is essential for activating transcription (Puranik et al., 2011). Candidates for interaction with NAC-NOR would be NAC-NOR itself forming homodimers or the two other tomato NAC proteins involved in ripening, NAC1 and NAC4, and such an interaction was demonstrated for NAC4 (Zhu et al., 2014). The decreased ethylene production in *nor-cr1* confirms that NAC-NOR, as a master regulator, is located upstream of ethylene biosynthesis and has a positive regulatory role.

The spontaneous *nor* mutation produces a dominant-negative protein

Ripening in the spontaneous *nor* mutant, whose allele we shall from here on call *nor-s* for convenience, is totally blocked but only partially so in *nor-cr1* and *alc* fruits. Therefore we hypothesize that the non-ripening phenotype in *nor-s* is caused by the truncated protein functioning in a dominant-negative manner, where the protein product is able to interact with other NAC proteins and to bind DNA without transcriptionally activating its targets. This is reminiscent of the NAC TF SND1 in poplar, where an alternative splice variant retains the last intron and due to a premature stop forms a protein without an activation domain but with an almost intact NAC domain. This protein acts as a dominant-negative repressor of its downstream targets as well as of its own and family members' expression (Li et al., 2012). This is also reminiscent of the situation with the *mads- rin* allele blocking ripening, although there the newly formed RIN-MC fusion protein has a novel combination of expression and transcriptional activation not seen in a *rin* knock-out line (Ito et al., 2017; Li et al., 2017). To further study this we introduced mutations 5' of the location of the spontaneous mutation in *NOR* in both the spontaneous *nor* mutant as well as in the wild type Ailsa Craig background. Two alleles, *nor-scr1* and *nor-scr2* with a 1 bp deletion and insertion, respectively, were obtained in the spontaneous *nor-s* mutant background, and one allele (*nor-cr2*) with the same 1 bp insertion as *nor-scr2* was obtained in wild type Ailsa Craig (Fig. 1b). Homozygous *nor-cr2* caused an orange-ripe pericarp in Ailsa Craig fruits, and ripening in homozygous *nor-scr2* and biallelic *nor-scr1*/*nor-scr2* mutants was similar to this (Fig. 4). Therefore we can conclude that the frameshift upstream of the spontaneous mutation precludes translation of the NAC domain and negates the dominant-negative function of the spontaneous mutant, which retained an intact NAC domain.

A classical dominant- negative TF would still interact with the same regulatory DNA elements or form the same dimers as the wild type protein, but the activity of the dimer in a heterozygote would only be 25% compared to homozygous wild type thus giving a phenotype more similar to that of the homozygous mutant (Veitia, 2007). Heterozygous *nor-s* mutants in cv. Rutgers have an intermediate phenotype, both in timing of ripening, carotene production, as well as in ethylene production during

ripening (Tigchelaar et al., 1978). This indicates that *nor-s*' negative effect is dose-dependent. This suggests that *nor-s* is a so-called *trans*-acting dominant-negative allele having *inter*locus interactions, rather than the classical *intra*locus interaction (Veitia, 2007). The tomato NAC1 and NAC4 TFs (see previous section) would be obvious candidates for such intralocus interactions.

Figure 4. Phenotypes of homozygous mutant fruits. (**a- e**) Fruits at Br + 7 d and (**f- j**) Br + 15 d of wild type Ailsa Craig, *nor-cr2*, *nor-scr2*, *nor-scr1/nor-scr2* and the spontaneous *nor* mutant (all in cv. Ailsa Craig background) at equivalent stages. Scale bar, 1 cm.

FUL1 and *FUL2* have overlapping functions during fruit ripening, but *FUL2* has an additional role in fruit development

Since tomato FUL1 (Solyc06g069430) and FUL2 (Solyc03g114830) are close paralogs, it was particularly challenging to achieve RNAi-mediated knock-down of expression in a specific as well as effective manner (Bemer et al., 2012). Use of a less specific RNAi construct might have led to knock-down of multiple homologous genes. Both studies reporting knock-down of tomato *FUL* genes achieved knock-down of both concomitantly (Wang et al., 2014; Shima et al., 2014), but only relatively weak and not completely specific knock-down of each gene individually in another study (Bemer et al., 2012). These results suggest that *FUL1* and *FUL2* were functioning at least partially redundant in tomato fruit ripening, but were inconclusive about the relative roles of the two genes. We therefore generated both *ful1* and *ful2* single mutants as well as double mutants using CRISPR/Cas9. We obtained multiple knock-out alleles in both *FUL1* and *FUL2*, alone or in combination. Double mutants were obtained with constructs containing sgRNAs for both genes. A *ful1* single mutant line containing the *ful1-cr1* allele with a 91 bp deletion, presumably caused by microhomology-directed repair of the double strand break, which completely deleted the second exon , produces a truncated protein of 62 aa consisting of the MADS domain only (Fig. 1c). The 1 bp deletion in mutant allele *ful1-cr2* leads to a truncation at amino acid position 105 (Fig. 1c). Both alleles disable FUL1 function. In the *ful2* single mutant, the *ful2-cr1* allele has a 1 bp insertion in the middle of the MADS domain-encoding region resulting in a truncated

protein with only 29 aa (Fig. 1d). A 3 bp deletion in *FUL2* in the *ful2-cr2* allele allows production of the entire protein minus one amino acid, arginine 25 in the middle of the extremely conserved MADS domain. This mutation is very likely to be deleterious to protein function, as further supported by analysis in the Provean protein website (Provean score: -12.037) (Choi et al., 2012). Other obtained alleles are shown in Supplementary Fig. 1c and 1d. We also checked the sequences of the corresponding parts in the non-target paralog for *FUL1* and *FUL2* (Supplementary Fig. 1c and 1d) reciprocally but found no mutations there, demonstrating the high specificity of sgRNAs.

There were no apparent differences in final overall fruit colour between *ful1-cr1*, *ful2-cr1* and wild type fruits at 55 DPA (Fig. 2a, 2f and 2g). However, in both double mutants (Fig. 2h and 2i) the pericarp stayed orange until 60 DPA (Supplementary Fig. 2a) and did not reach a red ripe colour as in the wild type fruits (Fig. 2a and Supplementary Fig. 2a), which is similar to the phenotype observed for the RNAi *FUL1*/*FUL2* silenced lines in the study of Bemer *et al*. (Bemer et al., 2012).

Interestingly, we observed a phenotype in *ful2-cr* mutants that had not been described before in the RNAi knock-down lines. In early stages of fruit development, superficial stripes lighter than the surrounding pericarp were visible at the bottom of homozygous *ful2-cr1* fruits, but not in homozygous *ful1-cr1* fruits, which became less distinguishable as fruits ripened (Fig. 2g, 5a and Supplementary Fig. 2c). Significantly lighter-pigmented stripes were also found at the bottom of all fruits in the two double mutants *ful1-cr2/ful2-cr2* and *ful1-cr2/ful2-cr3*, which only changed from white to yellow around the time when wild type fruits are fully ripe (Fig. 2h, 2i and 5a). These stripes were not only visible superficially, but also in the mesocarp of sections of both homozygous *ful1/ful2* double mutants, making the pericarp at the bottom region and septum much lighter coloured than the rest of the fruit (Fig. 5b). Moreover, vertical, possibly suberized cracks in the surface of all *ful2* mutant lines, including double mutant lines, were visible from the early stages of fruit development (Fig. 2g- 2i, 5c and 5d). Additionally, the columella and placenta of *ful2* and *ful1/ful2* fruits remained white when fruits were fully ripe (Fig. 5b, 5d). Besides, we noticed that fruits of all the mutants with a *ful2 null* allele were more than 10 mm smaller than wild type fruits, both in diameter and in height (Fig. 3d and 3e) and almost half the weight of wild type fruits (Fig. 3f). Also there was a significant effect of *ful2* on time to onset of ripening (Br stage). It took 46 days in *ful1-cr1* and only 43 days from anthesis to Breaker in *ful2-cr1* fruits, significantly less than in wild type fruits (Fig. 3a and Supplementary Fig. 2a). The time to ripening was also significantly shorter than in wild type fruits in both double mutant lines (Fig. 3a), but the pericarp stayed orange until 60 DPA and did not reach a red ripe colour as in the wild type fruits. We speculate that the smaller fruit size of *ful2* mutants may have a causal relation to the earlier ripening phenotype, but this will require more study.

We also measured ethylene production in all mutants, and found significantly lower production in the *ful1-cr1* single mutants and the double mutants, but not so in the *ful2-cr1* mutants (Fig. 3b). The strong

reduction in the *ful1-cr* mutant indicates that *FUL1* is an important regulator of ethylene biosynthesis in the ripening phase, although its reduction in the single mutant has little effect on visible ripening aspects. However, *FUL2* also contributes, because ethylene production is even more reduced in the *ful1-cr2/ful2-cr3* double mutants. Compared to wild type fruits, ethylene production decreased to 32% and 17% in two double mutant lines at Breaker stage, and to only 15% and 5% at Br+ 5 d (Fig. 3b). Thus, although *FUL1* and *FUL2* were reported to act redundantly during fruit ripening, *ful2* mutants affect unique aspects of fruit development, both time to ripening and as well as fruit skin integrity, which are similarly affected in double mutants. By contrast, *ful1-cr1* showed a very mild difference from WT without cracks or stripes in the pericarp, nor a difference in time to ripening, but a stronger effect on ethylene production. This is consistent with the strong increase in *FUL1* expression during ripening, while *FUL2* is expressed in developing green fruits as well. Some of the differences in the functions of *FUL1* and *FUL2* that have become apparent in this study may also reflect tissue-specific and temporal differences in expression. According to the Tomato Expression Atlas (TEA) (Shinozaki et al., 2018) *FUL2* expression is especially high in internal fruit tissues (columella, placenta, locule tissues) while *FUL1* expression, during ripening, is more evenly distributed over pericarp and internal tissues (Supplementary Fig. 3). *FUL2* expression however is higher than *FUL1* expression in all fruit tissues up to the mature green stage.

Phenotypes of *ful1* or *ful2 null* alleles are reported for the first time in our study and although their combined mutations had a severe ripening phenotype, they are very different from *FUL1/FUL2* double knock-down lines reported by Shima *et al.* or Wang *et al.*, in which they showed a totally blocked ripening phenotype (Shima et al., 2014) or bright yellow fruits (Wang et al., 2014) when silencing both *FUL1* and *FUL2*. The orange pericarp of *ful1/ful2* true knock-out mutants is similar to what Bemer *et al*. presented in their RNAi experiment (Bemer et al., 2012), while single mutants exhibited normal red-ripe pericarp, confirming their redundant function in fruit ripening. Light coloured stripes at the bottom region and vertical (possibly suberized) cracks in the surface of all lines containing homozygous *ful2* alleles have never been reported before. They are visible from the green fruit stage, illustrating additional roles of *FUL2* in early fruit development and in carotenoid biosynthesis. Fujisawa *et al*. showed that the promoter of *PHYTOENE SYNTHASE 1* (*PSY1*) was a direct target of RIN and FUL1, but not of FUL2 (Fujisawa et al., 2014), suggesting that another mechanism is responsible for FUL2 regulating fruit pigmentation. Possibly FUL2 is involved in chloroplast formation, stability, or function during early fruit development, which would account for the lighter green areas. The cracks in the surface of all *ful2* (both single and double mutant) lines are a unique phenotype of *ful2* mutants that is not shared with *ful1* single mutants. The aforementioned RNAi studies of combined *FUL1*/*FUL2*-function all reported thinner-cuticles, leading to faster water loss in ripe fruits (Bemer et al., 2012; Wang et al., 2014). The observed cracks in the *ful2* CRISPR mutants indicate that this may well represent a specific *FUL2* function in cutin formation or epidermal development in tomato fruit.

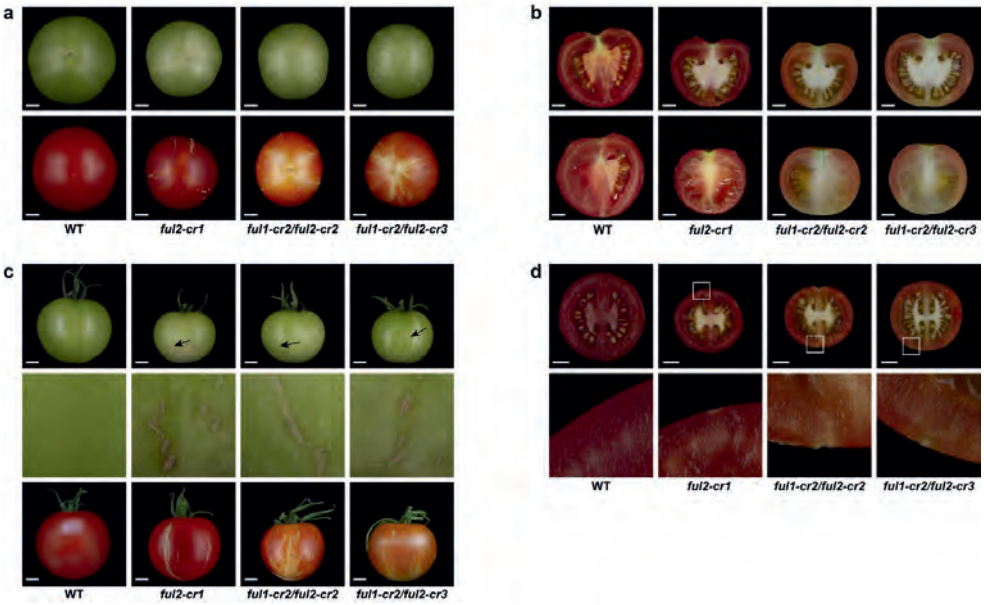

Figure 5. Details of fruit phenotypes and differences in *ful2* mutants compared to wild type. (**a**) Bottom view of *ful2* single and double mutants. Light coloured stripes only at the bottom of *ful2* mutant fruits appear from early green fruit stage. (**b**) Longitudinally sliced fruits of *ful2 null* mutants with light coloured-pericarp at the bottom of fruits. (**c**) Details of cracks in *ful2* mutants. Cracks in all *ful2* mutants at unripe (top), including details (middle) and ripe (bottom) stage. Black arrows indicate cracks in unripe fruits. (**d**) Latitudinally sliced fruits of *ful2* mutants. Boxed regions are enlarged in the lower row. Scale bar, 1 cm.

The additive effect in ethylene production among single and double mutants proves the partially redundant functions of *FUL1* and *FUL2* in ethylene biosynthesis and fruit ripening. In contrast to the unchanged ethylene production in knock-down lines shown by Bemer *et al.* (Bemer et al., 2012), the strongly decreased ethylene level in *ful1*/*2* double knock-out mutants in our study illustrates that *FUL1* and *FUL2* regulate tomato fruit ripening via ethylene biosynthesis, consistent with other studies (Shima et al., 2014; Wang et al., 2014). These discrepancies between studies are possibly caused by the use of different genotypes (MicroTom, Ailsa Craig or Moneyberg), and by the limited downregulation in the RNAi lines previously analysed (Karlova et al., 2011). Here it is shown that only one third or less of wild type ethylene production in *ful1/ful2* double mutants could still support some ripening progression, as it does in the *nor-cr* mutants indicating that the initiation of ripening may only require a limited amount of ethylene, and once it reaches a threshold, ripening starts even if not progressing to its full extent.

In conclusion, we have demonstrated the utility of CRISPR/Cas9-mutagenesis in tomato for reassessing transcription factor gene functions. Some phenotypes closely resembled those that were previously

reported, but in addition allow the study of regulatory features such as an auto feedback regulation of transcription (*ap2a*) or complementation by retransformation. For others, studying alternative alleles gives more insight into gene function by revealing a distinction between *null* and dominant-negative alleles (*nor-s*), respectively. Finally, gene-specific mutations allow the separation of functions (or demonstrate redundancy) of pairs of very similar paralogs (*ful1* and *ful2*), which are difficult to separate by older methods such as RNAi or VIGS.

Materials and Methods

Plant materials and growing conditions

Tomato cv. Moneyberg, Ailsa Craig (AC) and the AC *nor* mutant (the latter two obtained from the Tomato Genetics Resource Centre, TGRC) were used for the *Agrobacterium tumefaciens*-mediated transformation experiments (van Roekel et al., 1993). Tissue culture was done in a growth chamber with 16 h light and 8 h dark at 25 °C. Larger plants before flowering were moved to the greenhouse facilities of Unifarm, Wageningen University & Research and grown and phenotyped under standard greenhouse conditions.

gRNA design and mutagenesis constructs

Online programs CRISPR-P 1.0 (http://crispr.hzau.edu.cn/CRISPR/) (Lei et al., 2014) and CRISPOR (http://crispor.tefor.net/crispor.py) (Haeussler et al., 2016) were used for designing gRNAs and for excluding off-targets.

The MoClo Toolkit (Weber et al., 2011) was used to assemble constructs with gRNAs targeting each gene and the Golden Gate cloning strategy was used as described earlier to assemble binary vectors for tomato mutagenesis (Engler et al., 2008). Briefly, each gRNA fused to the synthetic Arabidopsis U6 promoter as AtU6p::gRNA was ligated in a Level 1 vector. Level 1 constructs *pICH47732-NOSpro::NPTII::OCST*, *pICH47742-35S::Cas9::NOST*, *pICH47732*-gRNA1, *pICH47742*-gRNA2 and the linker *pICH41780* were cut/ligated into the Level 2 vector *pICSL4723* as described (Werner et al., 2012). All primers used for amplifying gRNAs with backbones are listed in Supplementary Table 1.

Transgenic plant genotyping

Genomic DNA from young leaves was isolated using the CTAB method (Porebski et al., 1997). PCRs for *Cas9* and *NPT2* were performed for all regenerated plants and only from the *Cas9/NPT2* positive plants target regions were sequenced. Heterozygous and biallelic mutants were selfed and T_1 seedlings were screened for the absence of *Cas9* and the presences of homozygous mutations. Homozygous mutants without *Cas9* after segregation were used for further study. All primers used for genotyping are listed in Supplementary Table 1.

Fruit development phenotyping

Two plants per genotype were used for phenotyping. Tomato flowers of all the mutant lines and WT were vibrated and labelled at the day when they were first fully open as 0 Days Post Anthesis (DPA). Data from at least fifteen flowers/fruits per genotype was used for calculating the time taken to reach Breaker stage. Fruits at 35, 40, 45, 50, 55 and 60 DPA were collected for photography. Ten or eleven fruits at 60 DPA were collected for size and weight measurement. Normal distribution of data was confirmed with the R package version 3.5.0 and ANOVA was used to test for the significance of differences between individual mutants and wild type, respectively.

Ethylene measurements

Tomato fruits at Breaker stage and Breaker +5 d were harvested. After being in open air for 30 min at room temperature fruits were placed in sealed jars for 3 h. Ethylene concentration was measured when immediately after sealing and after 3 h by injecting 1.5 mL gas to a Focus GC gas chromatograph. Ethylene production was calculated as ppm per gram of fruit per hour (ppm/g/h). Values of five or six fruits per genotype were used for analysis. Since ethylene data did not display a normal distribution, generalized linear model regression with a quasibinomial model was used to determine the significance of differences between genotypes.

Real-time PCR gene expression analysis

RNA was isolated from pericarp of fruits at Br +5 d stage by using the InviTrap Spin Plant RNA kit (Stratec) and cDNA was synthesized by the iScript cDNA synthesis kit (Bio-Rad). Two replicates were analysed per plant containing one fruit each from two plants per line. Primers used for qRT-PCR are listed in Supplementary Table 1. iQ SYBR Green Supermix (Bio-Rad) and iCycler iQ5 system (Bio-Rad) were used for quantitative RT PCR. *Actin* was used as a reference and relative expression changes of *AP2a* were calculated according to $2^{-\Delta\Delta Ct}$ method as described (Livak and Schmittgen, 2001). Student's t-test was performed to detect significant differences.

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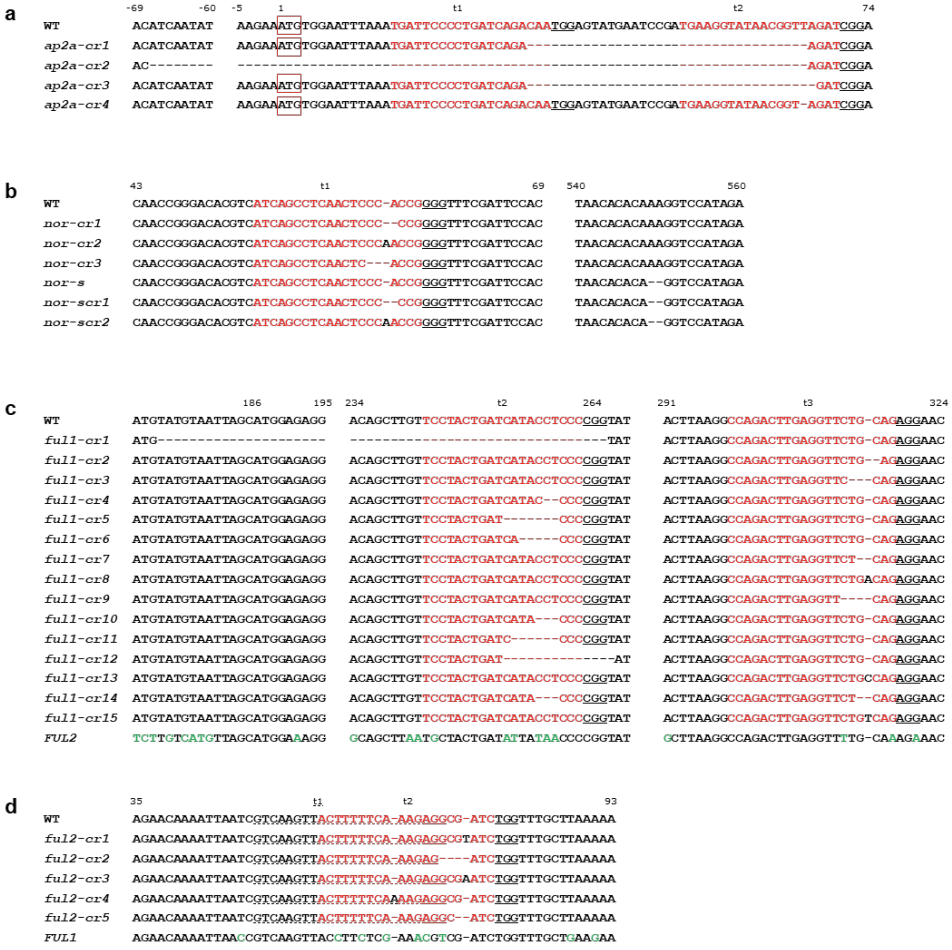
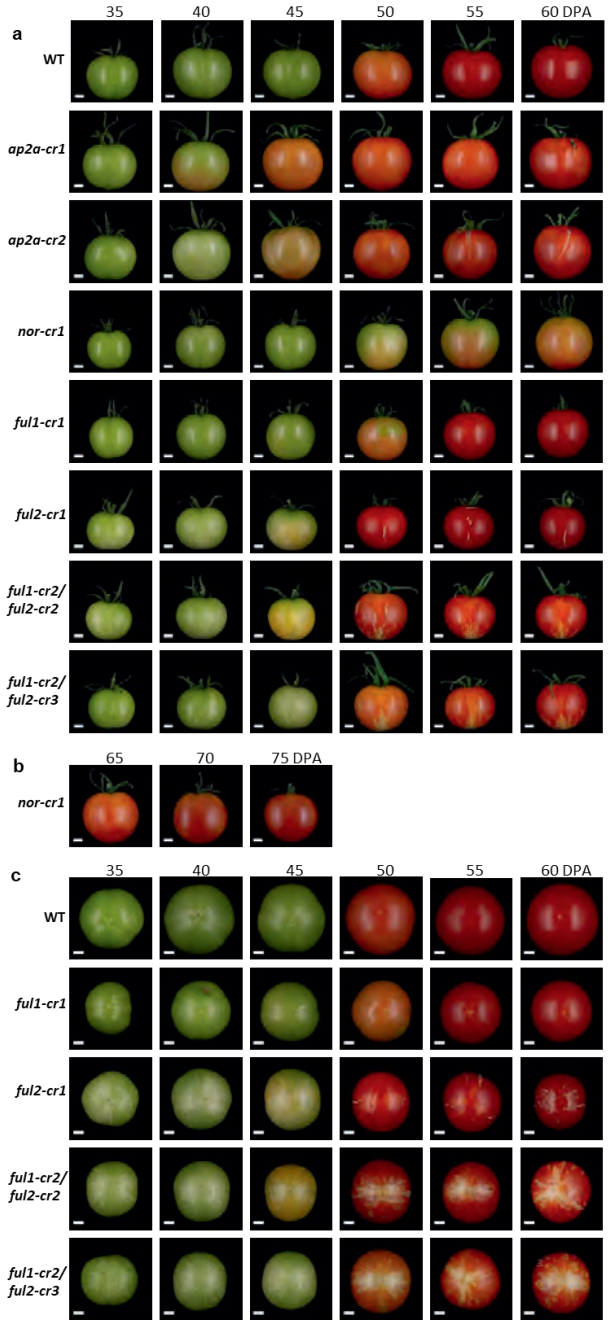
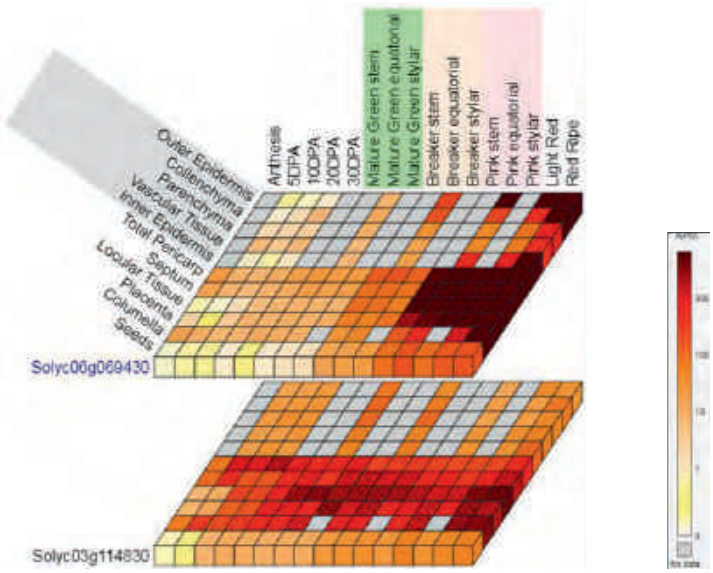


Supplementary Figure 1. All alleles obtained by using CRISPR/CAS9 mutagenesis. (**a**) All mutant alleles in *AP2a*. (**b**) All alleles obtained in *NAC-NOR*. (**c**) 15 alleles obtained in *FUL1*. Alignment differences between *FUL1* and the corresponding region in *FUL2* are shown in green. (**d**) Mutant alleles in *FUL2*. Alignment differences between *FUL2* and the corresponding region in *FUL1* are shown in green. Letters in red indicate spacer sequences and underlined are protospacer adjacent motifs (PAM). The start codon is indicated with red boxes. Numbers represent the location of the nucleotide in coding sequence.



Supplementary Figure 2. Development and ripening of homozygous mutants in the T_1 generation. (**a**) Pictures taken every 5 days from 35 DPA show the difference in developmental and ripening processes among all lines in this study. (**b**) Ripening in fruits of *nor-cr1* is slower than other mutants and its pericarp still stays in dark orange until 75 DPA. (**c**) Development of stripes at the bottom of all *ful* mutants. Light coloured-stripes are visible from the early stages of fruit development and only in mutants with *ful2* alleles. Scale bar, 1 cm.



Supplementary Figure 3. Expression of *FUL1* (Solyc06g069430) and *FUL2* (Solyc03g114830) in different tissues and stages of tomato fruit development and ripening from the TEA.

Primer name	Sequence (5'-3')	Description
AP2a-t1-F	TGTGGTCTCAATTGGATTCCCCTGATCAGACAAGTTTTAGA GCTAGAAATAGCAAG	Forward primer for target 1 in *AP2a*
AP2a-t2-F	TGTGGTCTCAATTGGAAGGTATAACGGTTAGATGTTTTAGA GCTAGAAATAGCAAG	Forward primer for target 2 in *AP2a*
NOR-t1-F	TGTGGTCTCAATTGATCAGCCTCAACTCCCACCGGTTTTAG AGCTAGAAATAGCAAG	Forward primer for target 1 in *NOR*
NOR-t6-F	TGTGGTCTCAATTGGAAAATATCCTAACGGGGCGGTTTTAG AGCTAGAAATAGCAAG	Forward primer for target 6 in *NOR*
FUL1-t2-F	TGTGGTCTCAATTGCCTACTGATCATACCTCCCGTTTTAGAG CTAGAAATAGCAAG	Forward primer for target 2 in *FUL1*
FUL1-t3-F	TGTGGTCTCAATTGCAGACTTGAGGTTCTGCAGGTTTTAGA GCTAGAAATAGCAAG	Forward primer for target 3 in *FUL1*
FUL2-t1-F	TGTGGTCTCAATTGTCAAGTTACTTTTTCAAAGGTTTTAGAG CTAGAAATAGCAAG	Forward primer for target 1 in *FUL2*
FUL2-t2-F	TGTGGTCTCAATTGCTTTTTCAAAGAGGCGATCGTTTTAGA GCTAGAAATAGCAAG	Forward primer for target 2 in *FUL2*
CRISPR Universal R	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC	Universal Reverse primer for all gRNAs
NPT2-F	AGACAATCGGCTGCTCTGAT	Genotyping transgenic plants for NPT2
NPT2-R	AGCCAACGCTATGTCCTGAT	Genotyping transgenic plants for NPT2
CAS9-F	CTTTGGCAATATCGTGGACG	Genotyping transgenic plants for CAS9
CAS9-R	CGTTCTTCTTCTCCCCAGGG	Genotyping transgenic plants for CAS9
AP2a-F	CGTGGGGGTGTATTTAACG	Genotyping *ap2a* mutants
AP2a-R	GGCGATTCCAAATTGTGG	Genotyping *ap2a* mutants
NOR-F	CGAATTTATCAACCTCGTA	Genotyping *nor* mutants
NOR-R	TTATCACAACCAAGTGGC	Genotyping *nor* mutants
FUL1-F	GACCTTCGCTTATAGCTCTATCCC	Genotyping *ful1* mutants
FUL1-R	CTTCTCCCACATAATGCCTGC	Genotyping *ful1* mutants
FUL2IN1-F	TTTCTTCCGTCTGTCTCCA	Check FUL2 targets region in *ful1* mutants
FUL2IN1-R	GAATCAGGGCGGCTCAATA	Check FUL2 targets region in *ful1* mutants
FUL2-F	CTGGGGAGATCCTTCC	Genotyping *ful2* mutants
FUL2-R	TGAGTCCAACTTCAGCATCG	Genotyping *ful2* mutants
FUL1IN2-F	CGCTGGCTTAGTGCAAGTA	Check FUL1 targets region in *ful2* mutants
FUL1IN2-R	AGCAGAATCGAGCTGGTGT	Check FUL1 targets region in *ful2* mutants
Actin-F	TGAGAGGTGCCTGATGCATTGC	qPCR for *Actin*
Actin-R	ACGCTTCGACCAAGGGATGG	qPCR for *Actin*
qAP2a-F	AACGGACCACAATCTTGAC	qPCR for *AP2a*
qAP2a-R	CTGCTCGGAGTCTGAACC	qPCR for *AP2a*

Supplementary Table 1. Primers used in this study.

Chapter 4

SPL-CNR is not essential for tomato fruit ripening

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Abstract

COLORLESS NON-RIPENING (SPL-CNR) was documented as a transcription factor involved in tomato fruit ripening due to the ripening defects in the spontaneous epi-mutant *Cnr*, which down-regulates *SPL-CNR* expression. In this study, with the use of CRISPR/Cas9-mutagenesis, we created *SPL-CNR* knock-out mutants as well as promoter deletion mutants with decreased *SPL-CNR* expression. We observed that in these mutants ripening was only attenuated but not interrupted, demonstrating that *SPL-CNR* is involved but not essential for normal fruit ripening as was assumed based on the non-ripening *Cnr* mutant. Pericarp firmness of the mutants decreased more slowly during ripening, which could be a valuable trait for commercial breeding. Mutants with deletions of the previously reported hypermethylated region upstream of *SPL-CNR* underlying the *Cnr* mutation ripened as wild-type. This region is therefore not required for normal ripening or expression of *SPL-CNR*. Gene expression measurements of ripening related genes did not yet reveal in what way CRISPR mutations in *SPL-CNR* affect ripening. Except for down-regulation of *SPL-CNR* in promoter deletion mutants, expression changes observed earlier in *Cnr* mutants were not found here, indicating that the strong ripening defects in *Cnr* are not caused by down-regulation of *SPL-CNR*.

Introduction

Tomato (*Solanum lycopersicum*) fruit ripening is a complex process with physiological and biochemical changes (Seymour et al., 2013), like the softening during ripening caused by cell wall degradation and colour change from green to red due to chlorophyll degradation and lycopene accumulation. All these changes are regulated by endogenous ethylene and effector genes, which are regulated by upstream transcription factors (TFs) (Karlova et al., 2014). Instead of hierarchical, linear regulatory interactions, TFs work as a network to control effector genes and regulate each other's expression (Giovannoni, 2004), making the ripening regulation complex. For example, chromatin immunoprecipitation (ChIP) experiments showed that *NON-RIPENING* (*NAC-NOR*) (Giovannoni et al., 2004) is a direct target of RIPENING INHIBITOR (MADS-RIN) (Fujisawa et al., 2013; Vrebalov et al., 2002). Besides, epigenetic modifications, like cytosine methylation and histone (de)acetylation and methylation, can also affect fruit ripening by changing the accessibility status of chromatin, resulting in modulation of gene expression (Liu et al., 2015; Guo et al., 2017). Moreover, more and more studies have shown that non-coding RNAs, such as microRNAs or long non-coding RNAs, also play roles in post-transcriptional regulation of tomato fruit ripening (Itaya et al., 2008; Zhu et al., 2015). For instance, miR172 has been demonstrated to cleave the messenger RNA of *APETALA2a* (*AP2a*), in this way, possibly regulating fruit ripening (Karlova et al., 2011, 2013).

COLORLESS NON-RIPENING (SPL-CNR) is a member of the SQUAMOSA promoter-binding (SBP) protein-like (SPL) transcription factor family, which regulates its target genes by binding *cis*-regulatory

elements through their SBP domain (Manning et al., 2006). SPL-CNR was identified as a significant positive regulator in tomato fruit ripening due to the severe ripening defects in its spontaneous mutant *Cnr* (Thompson et al., 1999). There is no lycopene synthesised in *Cnr* fruits, while chlorophyll is degraded, so the fruit turns a pale yellow without further changes (Fraser et al., 2001). Besides, no ethylene is produced in *Cnr* fruits, and their ripening cannot be restored to normal by external ethylene treatment (Manning et al., 2006). There are no sequence differences in the *Cnr* genome compared to the wild-type, but through positional cloning, the mutant allele was mapped to a 286 bp region upstream of the *SPL-CNR* gene. This region is hypermethylated in *Cnr* compared to wild-type, remains methylated throughout *Cnr* fruit development and is correlated with 10~ 20% *SPL-CNR* expression of that in wild-type fruits. Thus *Cnr* is an epi-mutant, and it was generally assumed that its phenotype was due to down-regulation of the TF SPL-CNR, which itself is upregulated during normal ripening (Manning et al., 2006).

In this study, we created both *SPL-CNR* knockout (KO) mutants as well as promoter mutants with deletions in a region up to 2.2 kb upstream of the start codon, including the relevant region that is hypermethylated in *Cnr*, by using CRISPR/Cas-mutagenesis. We observed a slower but apparently complete ripening in both the KO and large promoter deletion mutants, of which the fruit firmness was obviously improved. However, the deletion of the region that is hypermethylated in *Cnr* did not affect the normal speed and progress of ripening. Finally, we measured the expression of some ripening related genes, but their expression was not or only slightly changed in all mutants, despite the strong effect on *SPL-CNR* function or expression. We speculate that there might be other genes involved in fruit texture and carotenoids biosynthesis, which yet have to be identified as being affected in the *Cnr* mutant.

Materials and Methods

Plants materials and growing conditions

Tomato cv. Moneyberg (MB) was used for all the *Agrobacterium tumefaciens*-mediated tomato transformations (van Roekel et al., 1993) in this study. Regenerated shoots were initially grown in a growth chamber with 16-hour light at 21 ºC. Then plants were transferred to the greenhouse, growing at ambient temperatures under natural light with supplementation from bulbs to make a 16-h light photoperiod.

gRNA designing and construct assembling

Online programs CRISPR-P 1.0 (http://crispr.hzau.edu.cn/CRISPR/) (Lei et al., 2014) and CRISPOR (http://crispor.tefor.net/crispor.py) (Haeussler et al., 2016) were used for gRNA design and avoiding off-targets.

Plasmids for gene targeting were assembled with the MoClo Toolkit (Weber et al., 2011) by using the Golden Gate cloning strategy. There were two, four or twelve gRNAs, with a synthetic *Arabidopsis* U6 promoter followed by each as AtU6::gRNA, in each construct. The constructs with two and four gRNAs were for the coding sequence to knock out the *SPL-CNR* gene and for deleting the region in the wild-type MB that corresponded to the hypermethylated region in the *Cnr* spontaneous mutant, respectively. The construct containing twelve gRNAs was used for creating deletions in the -2.2 kb promoter region of the *SPL-CNR* gene. For constructs with two and four AtU6::gRNAs, each was cloned to a Level 1 vector and then assembled with *pICH47732-NOSpro::NPTII::OCST* and *pICH47742-35S::Cas9::NOST* from other Level 1 vectors to the same Level 2 vector *pICSL4723*. To assemble twelve AtU6::gRNAs modules in the same Level 2 construct, we assembled three modules in each of four Level 1 vectors by using 4 nt overhangs that determine their order in the Level 1 vector (Table S1). Next, all the four Level 1 vectors were assembled together with *pICH47732-NOSpro::NPTII::OCST* and *pICH47742-35S::Cas9::NOST* to the same Level 2 vector as described above. All three Level 2 vectors were sequenced before the tomato plant transformation to ensure all modules were present in the right order with correct sequences. Primers used are listed in Table S1.

DNA isolation and genotyping

Genomic DNA from young leaves of regenerated plants was isolated with the Phire Plant Direct PCR Kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to its manual and only those T_0 plants containing *Cas9* and *NPT2* were used for further genotyping. The targeted regions were amplified by the Phire DNA Polymerase and then sequenced for mutations. Plants segregated in the T_1 generation with the homozygous mutation, but without *Cas9* (presumably after segregating out the transgene) were grown for further study. Primers used in genotyping are listed in Table S1.

Fruit phenotyping and photography

Tomato flowers were labelled on the day when they were completely open and the time to the initiation of ripening (Breaker (Br)) was recorded as Days Post Anthesis (DPA). The average value of more than 22 fruits per line was used to compare for differences in time to ripening. Fruits at Br+7d and Br+15d were collected for photography to show the differences at the same ripening stage.

Ethylene measurement

Ethylene production of fruits at Br, Br+3d and Br+7d was detected as earlier described (Wang et al., 2019). Six fruits per line were used for the analysis, and their averages were used to show differences. As data were not normally distributed the quasibinomial model was used for statistical tests with the R package 3.5.0.

Firmness measurements

Fruits at Br+7d and Br+15d were longitudinally sliced to give four equal quarters, and only the pericarp was used for the firmness measurement. The average value of the four quarters was used as the firmness value of that fruit. A Fruit Texture Analyser (GÜSS, Cape Town, South Africa) was used to measure the force (Newton) required to penetrate pericarps to the same depth (2 mm) with the same setting. More than eight fruits per line at two time points were used for this measurement, and their average value was used to show the firmness of that line. Student's *t*-test was used to identify significant differences among genotypes.

Carotene extraction and high-performance liquid chromatography (HPLC) analysis

The relative contents of lycopene and lutein of fruits at Br+7d were measured by HPLC. Pericarps of two fruits were mixed and frozen as a pool, and there were three pools of each line. Carotenoids were extracted with a modified method described earlier (Wahyuni et al., 2011). After the extraction, the dried residues were dissolved in ethyl acetate containing 0.1% butylated hydroxytoluene for the HPLC analysis, which was performed according to described (Bino et al., 2005). Carotenoids were detected by setting the photodiode array detector to scan from 220 to 700 nm. All carotenoids were quantified at 478 nm.

RNA isolation and gene expression analysis

Three pools of pericarps, each from two fruits at Br, Br+7d and Br+15d were collected for each line. RNA from each pool was isolated by the cetyl trimethylammonium bromide (CTAB) method. Around 300 mg ground pericarps were incubated in the 2% CTAB buffer at 65 °C for 10 min, then mixed with chloroform and centrifuged to separate RNA to the upper water phase. The supernatant was mixed with 8 M LiCl to a final concentration of 2-3 M and held for one hour at -20 °C following by centrifugation at 4 °C for 30 min at full speed to precipitate RNA, and 70% ethanol was used to wash RNA. The isolated RNA was dried in a vacuum desiccator and then dissolved in DEPC-treated water. The iScript cDNA synthesis kit (Bio-Rad, Hercules, USA) was used for reverse transcription and the iCycler iQ5 system (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad) were used for the quantitative RT-PCR. The tomato *CAC* gene (Expósito-Rodríguez et al., 2008) was used as a reference to normalise all the gene expressions with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers used in this study are listed in Table S1.

Accession numbers

Genes in this study can be found by the following accession numbers in the Sol Genomics Network (www.solgenomics.net): *CAC* (Solyc08g006960), *CEL2* (Solyc09g010210), *FUL1* (Solyc06g069430),

MADS-RIN (Solyc05g012020), *NAC-NOR* (Solyc10g006880), *PSY1* (Solyc03g031860), *PG* (Solyc10g080210), *PL* (Solyc03g111690), *SPL3* (Solyc10g009080) and *SPL-CNR* (Solyc02g077920).

Results

SPL-CNR KO and truncated promoter mutants obtained from CRISPR/Cas9-mediated mutagenesis

To knock out *SPL-CNR,* we designed one gRNA to cut at position 136/137 of its coding sequence in front of the SBP domain and obtained two primary transformants with mutations. One had a 1 bp deletion, this allele was named *cnr-cr1*, and the other, *cnr-cr2* had a 5 bp deletion (Fig. 1A). Both mutations caused frameshifts leading to a premature stop in translation resulting in C-terminally truncated proteins containing 44 or 46 amino acids, respectively, out of the original 136 of SPL-CNR, and lacking the SBP domain (AA 52-125) (Fig. 1A).

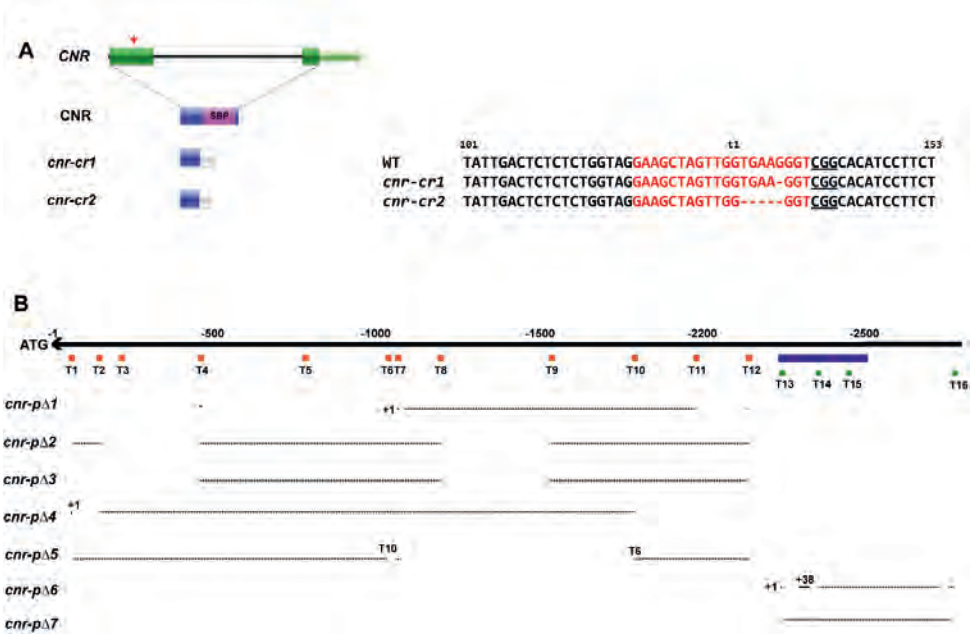

Figure 1. *SPL-CNR* knock-out and promoter deletion mutants obtained through CRISPR/Cas-mutagenesis. (**A**) KO mutations in *SPL-CNR*. The pink and light grey regions represent the SBP domain and differences in the truncated protein of the KO mutants, respectively. The red arrow shows the location of the gRNA t1 cloned twice in the same construct. Letters in red or underlined indicate spacer sequences and protospacer adjacent motifs (PAM), respectively, with numbers showing the location of the nucleotide in the coding sequence. (**B**) Deletions in the promoter of *SPL-CNR*. Orange squares depict the position of the twelve gRNAs designed for the -2.2 kb region. The blue rectangle indicates the position of the hypermethylated region in the *Cnr* mutant background and the four gRNAs (green blocks) targeting it. The sizes and locations of deletions are indicated by dashed lines. Note that the *cnr-pΔ5* mutant retains the promoter fragment from T6 to T10 in reverse orientation.

We also mutated the promoter (-2.2 kb) of *SPL-CNR* to obtain mutants with variations in *SPL-CNR* expression. We designed twelve gRNAs for the -2.2 kb region upstream of *SPL-CNR* (Fig. 1B and Table 1) and screened for lines with large deletions visible by gel electrophoresis. We obtained five lines with extensive deletions, especially the deletion in *cnr-pΔ4*, deleting most of the promoter (Fig. 1B and Table 3). The ripening defects of the spontaneous *Cnr* mutant were caused by a 286 bp hypermethylated region upstream (-2269-> -2554) of the *SPL-CNR* gene (Manning et al., 2006). To study the function of this region in normal ripening, we applied CRISPR/Cas9-mutagenesis with four gRNAs spanning this region in the wild-type Moneyberg (MB) background (Fig. 1B and Table 2). Two mutant lines with different sizes of the deletions were obtained, *cnr-pΔ6* with a 420 bp deletion (368+ 38+ 14 bp) and a fragment (14 bp) out of the area was inserted as filler DNA into the deletion, and *cnr-pΔ7* with a 553 bp deletion, completely removing the equivalent region (Fig. 1B and Table 3).

For the -2.2 kb promoter region												
gRNA	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Cutting	-69	-152	-207	-453	-790	-1029	-1074	-1191	-1546	-1802	-1976	-2137

Table 1. Details of gRNAs for targeting the -2.2 kb region upstream *SPL-CNR*. Twelve gRNAs in the same construct and locations of their expected cutting (3 bp from the PAM). The expected cutting sites are shown as the relative distance from ATG of *SPL-CNR*.

For the hypermethylated region				
gRNA	T13	T14	T15	T16
Cutting	-2277	-2406	-2502	2832

Table 2. Details of gRNAs for targeting the hypermethylated region in *Cnr* upstream of *SPL-CNR*. Four gRNAs in the same construct and locations of their expected cutting (3 bp from the PAM). The expected cutting sites are shown as the relative distance from ATG of *SPL-CNR*.

cnr-pΔ1	size	Δ9	+1	Δ882	Δ2
	location	-450-> -458	-1074	-1098-> -1979	-2139-> -2140
cnr-pΔ2	size	Δ89	Δ738	Δ597	
	location	-62-> -150	-454-> -1191	-1547-> -2143	
cnr-pΔ3	size	Δ738	Δ597		
	location	-454-> -1191	-1547-> -2143		
cnr-pΔ4	size	+1	Δ1650	Δ5	
	location	-23	-152-> -1801	-2138-> -2142	
cnr-pΔ5	size	Δ973	Δ19	Δ372	
	location	-68-> -1030	-1055-> -1073	-1802-> -2173	
cnr-pΔ6	size	+1	Δ368	Δ14	
	location	-2285	-2406-> -2773	-2817-> -2830	
cnr-pΔ7	size	Δ553			
	location	-2278-> -2830			

Table 3. Details of mutations obtained in each line. Triangles and plus signs represent deletions and insertions, respectively, and the numbers following show the size of mutation. Numbers in the locations indicate the relative distance from ATG of *SPL-CNR*.

Ripening of the *SPL-CNR* KO mutants initiated normally

The Breaker (Br) stage is the day of the first colour change at the blossom end of fruits, which can be observed by eye, and which marks the initiation of ripening. Fruits of both *cnr-cr1* and *cnr-cr2* showed a distinct Br stage, indicating that their ripening can start normally. The initiation of ripening was slightly delayed in the *cnr-cr* genotypes (Fig. 2A). It took on average 58 d for wild-type MB fruits to reach the Br stage, but for *cnr-cr1* and *cnr-cr2*, 61 and 60 d, respectively, and thus slightly but significantly longer than in the wild-type (Fig. 2B).

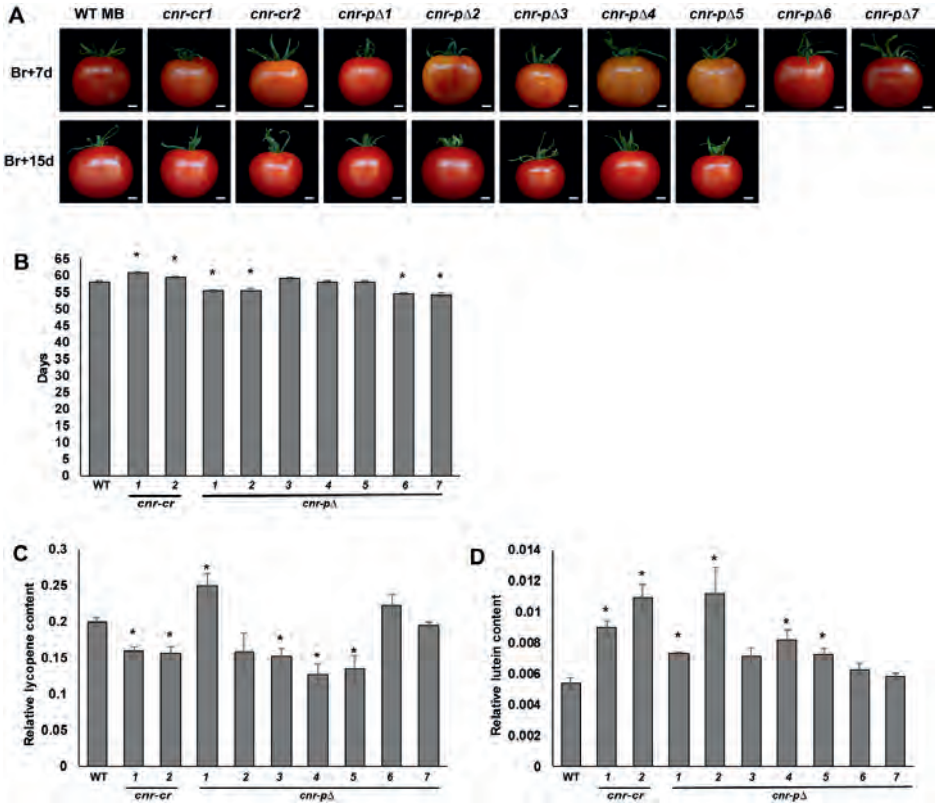

Figure 2. Phenotypes of *SPL-CNR* mutants. (**A**) Ripening phenotype of mutants at Br+7d and Br+15d. Scale bar, 1 cm. (**B**) Time to the initiation of ripening (days from anthesis, DPA, to Breaker). (**C**) Relative lycopene content of fruits at Br+7d. (**D**) Relative lutein content of fruits at Br+7d. Asterisks show significant differences ($P < 0.05$).

Colour of wild-type fruits started to change from Br stage until the red ripe stage, which was reached at Br+7d, with completely red pericarp. This colour change can be used as a means to evaluate the speed of ripening in tomato. We observed dark orange fruits at Br+7d for both *cnr-cr1* and *cnr-cr2*, and fully red fruits at Br+15 (Fig. 2A), indicating a slightly decreased speed of ripening caused by knocking out

SPL-CNR. To quantify the colour differences, we measured the carotenoid contents of the fruits at Br+7d. There was significantly less of the red carotenoid lycopene in *cnr-cr1* and *cnr-cr2* at Br+7 (Fig. 2C), while the content of lutein, a yellow carotenoid, was obviously elevated at Bt+7d (Fig. 2D), probably explaining their orange fruit colour.

A peak in ethylene production is associated with climacteric fruit ripening such as in tomato, playing a vital role in stimulating and controlling ripening. Ethylene production increased during ripening from Br towards Br+7d in wild-type fruits (Fig. 3A). The ethylene production in *cnr-cr1* at all time points was similar to those in the wild-type without significant differences, while *cnr-cr2* fruits produced slightly less (78%) the amount of that in wild-type fruits at Br+7d (Fig. 3A). This indicates that in contrast to what was suggested by the phenotype of the *Cnr* mutation, SPL-CNR function is not required for ethylene production.

Ripening is slower in the *SPL-CNR* promoter mutants

Most mutants with large deletions in the -2.2 kb region of the *SPL-CNR* promoter showed distinguishably slower ripening with orange fruits at Br+7d (Fig. 2A), while they differed in the time to the ripening stage (Fig. 2B). *cnr-pΔ1* and *cnr-pΔ2* needed significantly fewer days (3 and 2d, respectively) to Br, while for *cnr-pΔ3* - *cnr-pΔ5* it took longer, although not significantly (59, 58 and 58 d), to reach Br stage (Fig. 2B). Fruits of four out of five lines with promoter deletions in the upstream 2.2 kb region (*pΔ2, 3, 4,* and *5*) were only light orange at Br+7d, but had also turned red at Br+15d, indicating slower ripening than that in the wild-type fruits (Fig. 2A). There was significantly less lycopene in *cnr-pΔ2-5* as expected, as their fruits were still light orange at Br+7d. In contrast, in *cnr-pΔ1*, whose fruits turned red as wild-type did at Br+7d (Fig. 2C), its high lycopene content mirrors the normal red pericarp (Fig. 2A). At the same time, we noticed the lutein content was altered in the opposite way, with less lycopene, correlating with relatively more lutein (Fig. 2D). Surprisingly, *cnr-pΔ1-5* fruits also produced similar amounts of ethylene until Br+7d as wild type fruits did, even if they showed quite orange fruits and slower ripening (Fig. 3A). There was less ethylene produced in *cnr-pΔ1* and *cnr-pΔ2* at the initiation of ripening at Br stage but the significant differences disappeared later (Fig. 3A). Overall, slower ripening in these mutants was not correlated with lower ethylene production.

Mutations in the promoter region that is hypermethylated in *Cnr* did not affect ripening in wild type fruits

Both *cnr-pΔ6* and *cnr-pΔ7* lines, with deletions in (most of) the relevant hypermethylated region that is implicated in the spontaneous *Cnr* mutant, needed 54 d on average to initiate ripening, slightly shorter than in wild-type fruits (Fig. 2B). Unlike the other promoter mutants, which showed slower ripening with orange pericarp, both *cnr-pΔ6* and *cnr-pΔ7* had the same red pericarp as wild-type fruits at Br+7d (Fig. 2A), which was mirrored by a similar level of lycopene (Fig. 2C) and of lutein (Fig. 2D). This

indicates that, despite its role in the *Cnr* mutation, this region is not required for normal ripening in a wild-type background.

Truncated *SPL-CNR* proteins and promoters improved fruit firmness

We noticed that the orange fruits of *SPL-CNR* KO and promoter mutants had a firmer texture than wild-type fruits at the same stage, so we measured the firmness of fruits of four promoter mutants together with two KO lines at Br+7d and Br+15d to quantify the texture differences (Fig. 3B and C). The average firmness of wild-type MB fruits at Br+7d was 3.1 N, and significantly higher at 3.8 and 3.7 N for the two KO genotypes (Fig. 3B). All four promoter mutants also had firmer fruits, especially the *cnr-pΔ4* fruits with firmness 4.5 N, which is approximately 50% more than the wild-type fruits (Fig. 3B). Fruit firmness decreased with progressing ripening, from 3.1 N at Br+7 to 1.9 N at Br+15 in wild-type, but the decreases in *SPL-CNR* KO and promoter mutant fruits were smaller, which led to significantly firmer texture of all mutant lines at Br+15d (Fig. 3C). Overall, *cnr* mutants at Br+15 had an average firmness ranging from 2.5 to 3.2 N, which was not significantly different from wild type at Br+7, indicating that both colour development and softening are slower in the mutants.

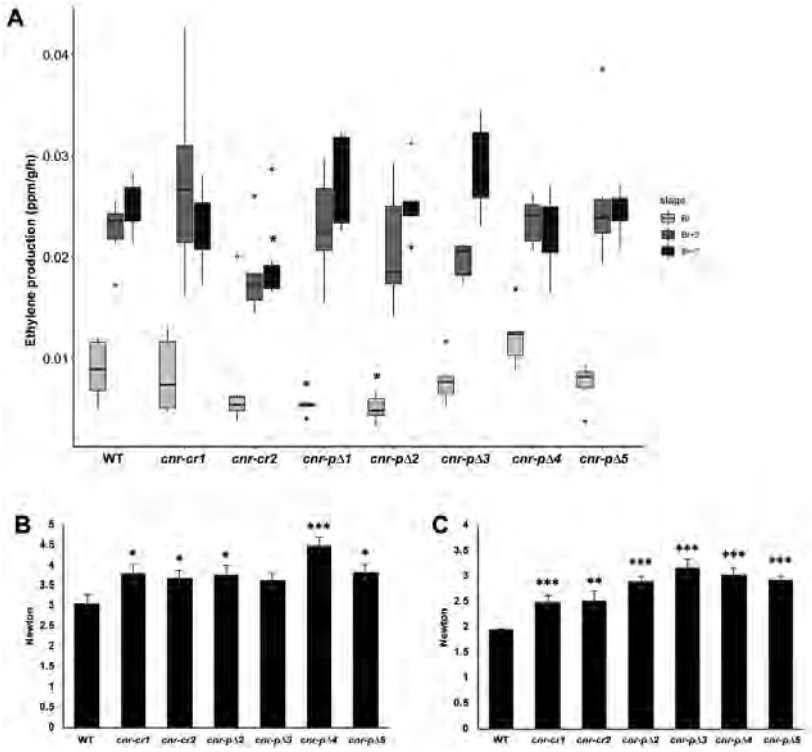

Figure 3. Fruit traits of mutants. (**A**) Ethylene production (ppm/g/h) of fruits at Br, Br+3d and Br+7d. (**B**) Firmness (Newton) of fruits at Br+7d. (**C**) Firmness (Newton) of fruits at Br+15d. Asterisks show significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Changes at the transcript level of genes involved in fruit ripening

TF binding sites in a gene's promoter are essential for its transcription and altering them could affect the expression of the gene, so we first detected the transcript level of *SPL-CNR* in all promoter mutants in this study (Fig. 4A). In wild-type fruits, *SPL-CNR* expression was low at the mature green (MG) stage and peaked at Br, then dropped during ripening (Fig. 4A). The dramatic leap of *SPL-CNR* expression disappeared in *cnr-pΔ1-5* mutants, with almost no expression at the Br stage (Fig. 4A). Moreover, the expression also decreased significantly at Br+7d, to much lower than that at Br in all the five lines, making of *SPL-CNR* expression dramatically lower than that in wild-type fruits in all the stages that we studied (Fig. 4A). Differing from the very low expression in *cnr-pΔ1-5*, *SPL-CNR* expression was not affected in *cnr-pΔ6* or *cnr-pΔ7* deleting the region that is hypermethylated in *Cnr* (Fig. 4A), indicating that despite its importance in the mutant, this region is not essential for normal *SPL-CNR* expression. In theory, a KO mutation only changes the sequence of the gene's transcript, which later results in truncated protein due to the earlier stop in translation, but not the expression of the transcript. We saw similar to wild type expressions in both the KO lines, with a clear peak at the Br stage (Fig. 4A). Thus, there is no evidence for positive autoregulation or nonsense-mediated decay (NMD) of the messenger RNA, which might have been caused by the premature stop codons in both KO lines, although it also cannot be excluded that such decay was masked by the release of negative feedback on expression by the lack of a functional protein.

Phenotypic changes are caused by expression changes of effector genes involved in the ripening process. We saw the fruit colour and texture differences in the *SPL-CNR* KO and the large promoter deletion mutants, and therefore we checked the expression of some representative ripening-related genes in mutant lines (Fig. 4B-E). Expression of these genes was shown to be severely negatively affected by the spontaneous *Cnr* mutation (**Chapter 2** of this thesis). *PHYTOENE SYNTHASE1* (*PSY1*) (Bartley et al., 1992) encodes the enzyme PHYTOENE SYNTHASE1 catalysing the conversion of GGPP to phytoene, which is the first committed step in lycopene biosynthesis. The expression of *PSY1* increased sharply from Br stage along with the start of lycopene biosynthesis in the wild-type fruits and remained high until at least Br+7d (Fig. 4B). Despite the orange pericarp of KO mutants, we did not see a significant reduction of *PSY1* expression at Br+7d, only at Br in *cnr-cr1*, and the expression pattern was the same in the two promoter mutants (Fig. 4B). *POLYGALACTURONASE* (*PG*) (Giovannoni et al., 1989), *PECTATE LYASE* (*PL*) (Uluisik et al., 2016) and *CELLULASE 2* (*CEL2*) (Lashbrook et al., 1994) are representative genes involved in cell wall degradation, which influences fruit texure (Wang et al., 2018). The expression of all three was up-regulated during ripening, but was not or only slightly changed in all mutants comparing to the wild-type at the same stages (Fig. 4C-E), despite the strong effect on *SPL-CNR* expression and the strong effect of the *Cnr* mutation. This indicates that in contrast to the impact of the *Cnr* mutation, SPL-CNR does not directly or indirectly control the expression of these genes.

We also detected the expression of TF MADS-RIN, NAC-NOR and FRUITFULL1 (FUL1) (Leseberg et al., 2008; Bemer et al., 2012) encoding genes, and all three increased sharply during ripening in all lines (Fig. 4F-H). Their expression was not affected in KO lines, indicating that SPL-CNR does not regulate their expression, in contrast to the *Cnr* mutation (Fig. 4F-H and **Chapter 2** of this thesis). Taking the recently reported gene compensation theory in KO mutants into account we also checked the expression of *SPL3*, the closest paralog gene of *SPL-CNR*, in both KO and promoter mutants (Fig. 4I). We did not see an upregulation of *SPL3* in KO mutants, indicating that there is no extra expression of *SPL3* compensating for *SPL-CNR* loss of expression or function. Not surprisingly, the same expression level was also found in two promoter mutants with low *SPL-CNR* expression (Fig. 4I).

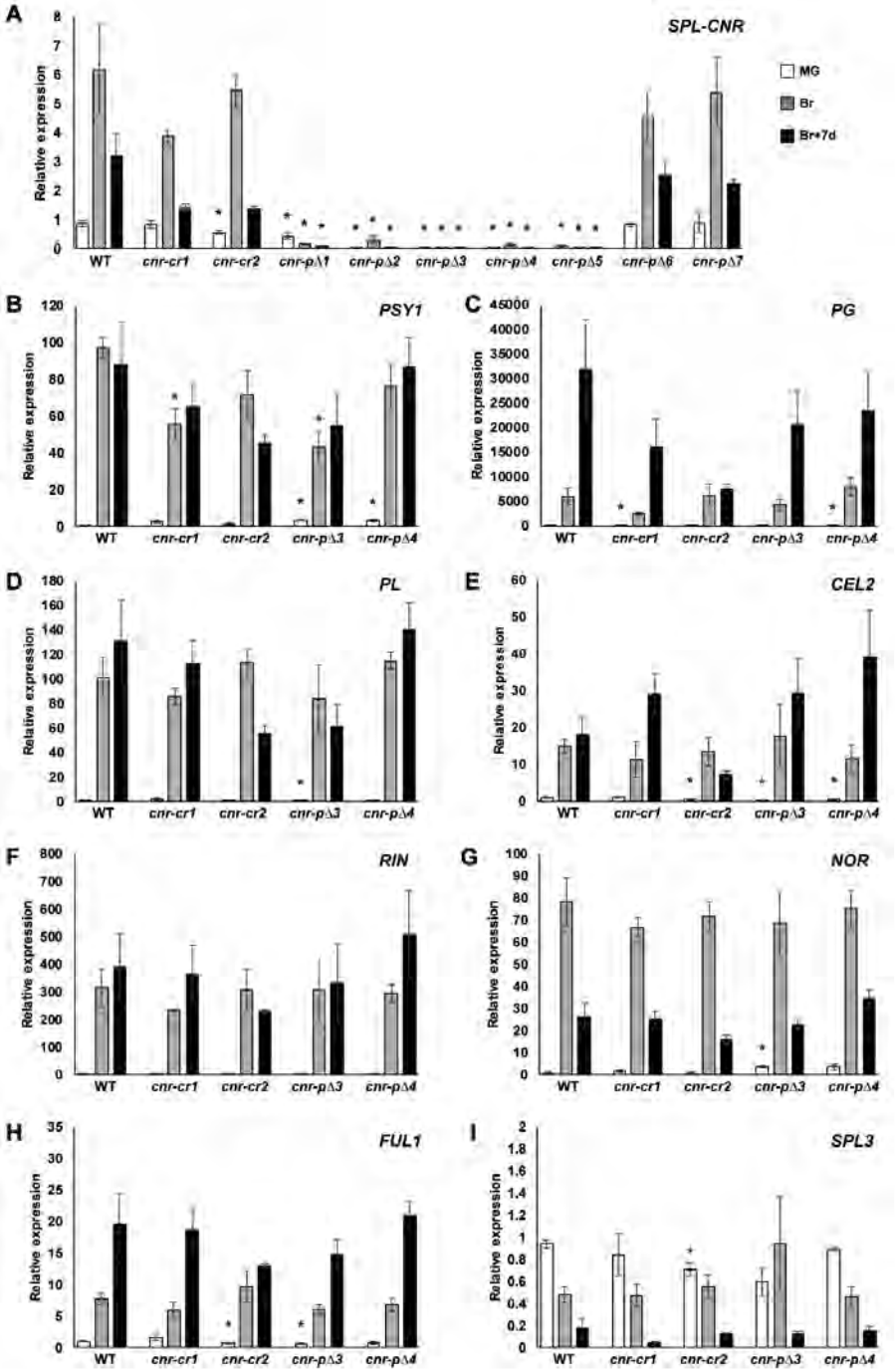

Figure 4. Expression of ripening related genes in mutants during fruit development and ripening. (**A**) expression of *SPL-CNR* in all lines. (**B**)- (**I**) Gene expression in KO mutants and representative promoter mutants. The clear, grey and filled blocks show the expression of genes at MG, Br and Br+7d, respectively. Asterisks show significant differences ($P < 0.05$).

Discussion

SPL-CNR is not essential for tomato fruit ripening

In this study, we have created KO mutants of *SPL-CNR*, and demonstrate the utility of an alternative strategy leading to extreme, marked knock-down of expression by the production of promoter deletions of the same gene. This allows comparisons of effects of the two types of mutations and the identification of discrepancies, if any, between the two types that would point to additional regulatory mechanisms of gene activity or genetic compensation effects triggered by degradation of mRNA.

It took significantly longer for our *cnr-cr* mutants to reach the initiation of ripening (Br) (Fig. 2B), which is consistent with the study of Gao *at al*. who also created *SPL-CNR* KO mutants by using CRISPR/Cas-mutagenesis and saw a delayed ripening in the background cv. Ailsa Craig (Gao et al., 2019). Our *cnr-cr* mutants showed dark orange pericarp at Br+7d when the wild-type fruits were completely red (Fig. 2A), indicating slower ripening, which was not observed in the other study (Gao et al., 2019). We and Gao *et al*. propose that *SPL-CNR* is not essential for tomato fruit ripening since ripening could be initiated normally without a functional gene copy. Studies for MADS-RIN and NAC-NOR have already uncovered that the severe fruit ripening blockade of their spontaneous mutants are actually caused by the repressive gain-of-function or dominant-negative characteristics of these alleles, respectively, and not by a loss of function (Ito et al., 2017; Wang et al., 2019). The situation for *Cnr* appears to be even more complex as it is an epi-mutant, but considering the contrast between the severe pleiotropic effects of *Cnr* with ~20% of *SPL-CNR* expression and much milder ripening delay caused by a lack of functional *SPL-CNR*, we propose that *SPL-CNR* is not essential for tomato fruit ripening and that the *Cnr* phenotype is caused by other, probably genome-wide effects.

The decreased *SPL-CNR* expression slows ripening but improves fruit firmness

The large promoter deletions not only decreased *SPL-CNR* expression but also resulted in slower ripening. The more or less unaltered ethylene production in all lines implies that *SPL-CNR* may function downstream of ethylene biosynthesis or work independently from ethylene, which is a novel finding here. Fruits of the -2.2 kb promoter mutants and KO lines were orange at Br+7d, when the wild-type fruits were already red, but turned to normal red at Br+15d (Fig. 2A), so their ripening was merely slowed down. The slower but finally complete ripening contributed to firmer than wild-type fruit texture at the same post-harvest stages (Fig. 3B and C), which could be a valuable trait for commercial breeding, as higher fruit firmness helps reducing losses in long-distance transportation.

The orange fruits with firmer fruit texture caused by no or low *SPL-CNR* expression demonstrate that this gene regulates effector genes involved in lycopene biosynthesis and softening, but the unchanged expression of related gene *PSY1*, *PG*, *PL* and *CEL2* during ripening did not reflect those changes.

Besides, with lower content of lycopene, most of the KO and large promoter mutants had significantly more lutein. Lutein is a yellow carotenoid downstream in the carotenoid biosynthesis pathway, synthesised from lycopene, but *PSY1* is only functioning at the beginning of the carotenoid pathway. The reduced lycopene content with increased lutein may also be due to increased activity of the pathway downstream of lycopene. Although from these results it is clear that SPL-CNR does not regulate the genes that were shown to be down-regulated in the *Cnr* mutation (**Chapter 2** of this thesis), it remains to be determined which gene expression changes are responsible for the *SPL-CNR* knock-out/knock-down phenotypes.

The region upstream of *SPL-CNR*, which is hypermethylated in *Cnr*, is not required for normal ripening

The hypermethylated region in the *Cnr* spontaneous mutant was causing 10~20% of wild-type *SPL-CNR* expression, which was assumed to result in the severe ripening defects of that mutant (Manning et al., 2006) and other phenotypic changes related to fruit development (**Chapter 2** of this thesis). Therefore, it was hypothesized that there are binding sites for essential positive regulators of *SPL-CNR* expression in that region in the wild-type which are blocked by methylation in *Cnr* fruits, causing the low *SPL-CNR* expression. Our results clearly reject this hypothesis, as the deletion of the region has no effect on expression or ripening phenotype. Alternative explanations, such as the specific binding of negative regulators to only the hypermethylated region in *Cnr*, are still possible and there are examples in mammalian cells where some TF binding relies on DNA methylation (Prokhortchouk et al., 2001; Zhu et al., 2016) and in rice that for some proteins binding to gene promoters depend on cytosine methylation (He et al., 2001). In such a scenario, deleting the region in the wild-type background would not affect the normal expression and ripening. Further experiments are needed to test this option. Nonetheless, the phenotypic effects of the *Cnr* mutation are so much more severe than those of *SPL-CNR* knockouts and knock-downs, that it is more likely that the *Cnr* mutation affects genome-wide gene expression through a yet unknown mechanism and not only affects fruit development and ripening through modulation of *SPL-CNR* expression.

How *SPL-CNR* regulates fruit ripening is still unclear

We saw changes in pigmentation and firmness during fruit ripening in *cnr-cr* and *cnr-p*Δ mutants, but the expression of representative genes was not or only slightly altered in all mutants.

The putative function of SPL-CNR has been evaluated according to the severe ripening defects in *Cnr* for a long time. Besides, ripening defects from a previous virus-induced gene silencing (VIGS) experiment implied that SPL-CNR played a role in tomato fruit ripening (Manning et al., 2006). Nowadays, with the help of CRISPR/Cas-mutagenesis, it is easier to more precisely mutate a gene for studying its function. Recently, *spl-cnr* KO mutants in the Ailsa Craig background were produced in

which ripening was only slightly delayed, implying that it is not as important as it was thought until now (Gao et al., 2019). At the same time, several studies indicated that knock-out mutants showed much milder phenotypes than those previously observed for knock-down plants produced by RNAi (see for review, **Chapter 6**). Recently, it has been found in mammals that small insertions and deletions (InDels), resulting in premature termination codons in KO mutants caused non-sense mediated decay (NMD). This activates the complex of proteins associated with Set1 (COMPASS) that can induce the expression of the paralogs of the knocked-out gene, resulting in compensation, which could buffer the defects caused by the KO of the gene (Ma et al., 2019; El-Brolosy et al., 2019). A very recent study found a similar, if not the same, compensation mechanism in tomato stem cell proliferation (Rodriguez-Leal et al., 2019), partially explaining the milder phenotype in CRISPR KO mutants than those from RNAi knock-down. We speculated that the mild ripening defects in our *cnr-cr* mutants could also be explained by the compensation through increased expression of other *SPL* genes, but unfortunately, we did not see apparent upregulation of *SPL-CNR*'s closest paralog *SPL3*, nor did we see a large phenotypic difference between knock-out mutants and knock-down/promoter-deletion mutants (Fig. 2A and Fig. 4I). However, compensation from other *SPL*s cannot be ruled out, and requires more genome-wide gene expression studies, such as by RNA-seq.

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Primer name	Sequence (5'-3')	Description
T1-F	TGTGGTCTCATACTGAGAGTATGAAACTTGAACAAG TTTTAGAGCTAGAAATAGCAAG	Forward primer of T1 in P1
T2-F	TGTGGTCTCAAATGAAGTCTAGTAAGTGTCTAAGGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T2 in P2
T3-F	TGTGGTCTCAGCTTGTTTCCTGGCCCACTTCTGTAGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T3 in P3
T4-F	TGTGGTCTCATACTGAGTGAATACTGAACGCTGAGG TTTTAGAGCTAGAAATAGCAAG	Forward primer of T4 in P1
T5-F	TGTGGTCTCAAATGTTTATGTAGTAGATTCCGAAGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T5 in P2
T6-F	TGTGGTCTCAGCTTGCTGCAGTGACTACCAAGTAGG TTTTAGAGCTAGAAATAGCAAG	Forward primer of T6 in P3
T7-F	TGTGGTCTCATACTGTTGGTAGTCCTTTTCAAGCAGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T7 in P1
T8-F	TGTGGTCTCAAATGTCCATATCACAAAAATTAGAGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T8 in P2
T9-F	TGTGGTCTCAGCTTGCTATCGAGATAGGCAAGTAGG TTTTAGAGCTAGAAATAGCAAG	Forward primer of T9 in P3
T10-F	TGTGGTCTCATACTGGATAATTATTAGTGAAGGGGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T10 in P1
T11-F	TGTGGTCTCAAATGAGAATATCCTGACAAATTAAGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T11 in P2
T12-F	TGTGGTCTCAGCTTGTATGAGATGACATATTTATGG TTTTAGAGCTAGAAATAGCAAG	Forward primer of T12 in P3
T13-F	TGTGGTCTCAATTGGTGTTTTAATCTGACACTTCGTT TTAGAGCTAGAAATAGCAAG	Forward primer of T13 for the hypermethylated region
T14-F	TGTGGTCTCAATTGGTAATATAGCTAGGCAGAGGGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T14 for the hypermethylated region
T15-F	TGTGGTCTCAATTGGATTAACACGATACAAGATGGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T15 for the hypermethylated region
T16-F	TGTGGTCTCAATTGAAATTAACATGGTATCAGAGGT TTTAGAGCTAGAAATAGCAAG	Forward primer of T16 for the hypermethylated region
P1-R	TGTGGTCTCAATGGTAATGCCAACTTTGTAC	Reverse primer of gRNA in P1
P2-R	TGTGGTCTCACGAATAATGCCAACTTTGTAC	Reverse primer of gRNA in P2
P3-R	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC	Reverse primer of gRNA in P3 or the only gRNA in the Level 1 vector
GM-F	GTATCAACCTCAAGAAGA	Forward primer for genotyping mutants in the hypermethylated region
GM-R	TTTCCCATTACGCTTCTA	Reverse primer for genotyping mutants in the hypermethylated region
GP-F	AAAATAGAGTGAGGAAGCCAGATAA	Forward primer for genotyping mutants in *CNR* promoter
GP-R	GAGGCTCTAAACAGGAAA	Reverse primer for genotyping mutants in *CNR* promoter
CNR-F	TTCCCGGATTTCTAAGCAAATTGT	qPCR for *CNR*
CNR-R	GTTGGAATGTCAACATGGATATGCA	qPCR for *CNR*
PSY1-F	TGAATTAGCACAGGCAGGTCT	qPCR for *PSY1*
PSY1-R	GGTCACCCTTCCAGCAAATA	qPCR for *PSY1*
PG-F	GGAGCTAAGGGTGATGGAAA	qPCR for *PG*
PG-R	CTTCATTCCATGCTTGCTCA	qPCR for *PG*
PL-F	TACAAAGCACGAGGATGCAC	qPCR for *PL*
PL-R	CCTCCAATTCCAATTCTTCC	qPCR for *PL*
CEL2-F	ATCATGTTGTGCCATGTGGT	qPCR for *CEL2*
CEL2-R	TTGGCAATGTGTTTGAGGAG	qPCR for *CEL2*
RIN-F	ATGCAGCACCATCAACACAT	qPCR for *RIN*
RIN-R	TCAAAGCATCCATCCAGGTA	qPCR for *RIN*

NOR-F	AGAGAACGATGCATGGAGGTTTGT	qPCR for *NOR*
NOR-R	ACTGGCTCAGGAAATTGGCAATGG	qPCR for *NOR*
FUL1-F	TGCTGAGGTTGGTTTGATTG	qPCR for *FUL1*
FUL1-R	GCAGGAATCGTTGGCATATT	qPCR for *FUL1*

Table S1. Primers used in this study. The 4 nt overhangs that determine the order of gRNAs in Level 1 vectors are coloured as red, blue and orange to indicate the first, second and third position, respectively.

Chapter 5

Exploring microRNA function in tomato fruit development and ripening using CRISPR/Cas9-mediated deletions

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Abstract

Central to the control of two-phase transitions in the plant life cycle is a regulatory circuit involving temporal changes in expression of members of the *SQUAMOSA BINDING PROTEIN-LIKE* (*SPL*) and *APETALA2* (*AP2*)-like transcription factor (TF) gene families, and of their regulatory microRNAs, miR156/157 and miR172. In tomato, a member of each of these transcription families, *COLORLESS NON-RIPENING* (*CNR*) and *AP2a*, respectively are expressed and required for normal ripening, and both are actively targeted by their cognate microRNAs. In this study, we have explored the role of these miRNAs in fruit development and ripening. The *MIR* gene copy with the highest expression in ripening fruit was identified for each of the three mature miRNAs and subsequently mutated with CRISPR/Cas9. We obtained deletion mutants for all three and assessed the effect on mature miRNA levels and on the fruit phenotype. The deletion in a complex polycistronic *MIR156* gene caused a significant decrease in the mature miRNA156 level, while the other two mutations had no detectable effect on their respective miRNA levels. The *MIR156* deletion mutant was also the only one with a visible phenotype, i.e. smaller fruit size and oblong shape, and earlier initiation of ripening.

Introduction

Fruit ripening is a complex developmental process that requires the coordination of transcriptional and metabolic networks (Klee and Giovannoni, 2011; Karlova et al., 2014). The fleshy fruit of tomato is a widely-used model for studying this process (Klee and Giovannoni, 2011; Karlova et al., 2014). Tomato ripening involves a switch from ethylene synthesis system 1 (autoinhibitory) to the autocatalytic system 2 (Alexander, 2002), the breakdown of chlorophyll and a massive change in the production of metabolites, e.g. lycopene and other carotenoids (Carrari et al., 2006; Lytovchenko et al., 2011). Tomato fruit ripening requires ethylene production, but ethylene cannot initiate ripening in immature fruits (Klee and Giovannoni, 2011). Therefore, it has been suggested that other developmental signals are needed for acquiring the competence to respond to ethylene and to initiate fruit ripening. A number of transcription factors (TFs) that regulate ripening independently or in conjunction with ethylene have been described (Klee and Giovannoni, 2011; Karlova et al., 2014). For example, COLORLESS NON-RIPENING (SPL-CNR), belonging to the SQUAMOSA BINDING PROTEIN-LIKE (SPL) TF family, was presumed to be an early activator of fruit ripening-related processes (Manning et al., 2006). *Cnr* mutant fruits produce less ethylene compared to wild type fruits (**Chapter 2**), while the exogenous application of ethylene could not rescue the mutant phenotype (Thompson et al., 1999). We (**Chapter 4**) and others (Gao et al., 2019) have recently shown that the *Cnr* mutation does not reflect the function of the underlying transcription factor gene, as knockouts of this gene showed a much milder phenotype, with no change in ethylene production or in the expression of the genes that are severely down-regulated in *Cnr*. Nonetheless, CRISPR/Cas9-generated *SPL-CNR* knock-out mutants show a slower progression of ripening, indicating the function of this gene (**Chapter 4**). Similarly, we and others have shown that

SlAP2a, a member of the AP2 TF family, also plays an important role in ripening control, and acts to negatively regulate ethylene biosynthesis and signalling (Karlova et al., 2011; Chung et al., 2010). Both mRNAs, of *SPL-CNR* as well as of *SlAP2a*, are actively cleaved by miRNA156 or 157 and miR172, respectively, at the onset of ripening (Karlova et al., 2013). These miRNAs and their *SPL* and *AP2* targets are known to control the transition from the vegetative to the reproductive phase of development in Angiosperms (Wu et al., 2009; Yant et al., 2010; Chuck et al., 2007a, 2007b). These data indicate that the regulation of tomato ripening has features that are similar to the regulation of other developmental switches, warranting a study of the mentioned microRNAs' function in tomato fruit development and ripening.

By gene expression analysis in ripening fruits, we identified the highest expressed precursor genes for each of the three *MIR* gene families. Subsequently, we used a CRISPR-CAS9 deletion approach to perturb their functions and studied the fruit phenotypes of the respective mutants. We found that partial deletion of *MIR R156e-h* accelerates the time to ripening in tomato fruits.

Materials and Methods

Plant material and growth conditions

Tomato plants were grown in the greenhouse at ambient temperatures (>20 ^{0}C) under natural light supplemented with artificial sodium lights, according to 16 hrs light/8 hrs dark cycle.

RNA and DNA isolation

Genomic DNA was isolated from young leaves according to the CTAB protocol. Four pools, each with 2 fruits collected at 20 and 40 DPA, were used for RNA isolation. Total RNA including miRNAs were isolated by ReliaPrep™ miRNA Cell and Tissue Miniprep System (Promega), followed by using the Xpose (Trinean) to detect the concentration and quality. Only RNAs with $OD_{260/230} \geq 1.9$ were used for the next step.

Real-time RT-qPCR analysis

RNA isolation from fruits was performed with the InviTrap Spin Plant RNA kit (Invitek, Germany), according to the manufacture protocol. DNaseI treated RNA was reverse-transcribed with the iScript™ cDNA synthesis kit (BioRad). Real-time RT-PCR was performed with the iQ™ SYBR® Green Supermix from BioRad and 1µl cDNA was used for the real-time PCR analysis. Real-time qPCR was performed using the iCycler iQ5 system (Bio-Rad) as described before (Karlova et al., 2011) and gene-specific primers (Table S1). Two independent biological replicates were analyzed per sample (or 2-3 fruits were mixed for each replicate). Relative quantification of specific mRNA levels was performed

using the comparative $2^{-\Delta\Delta Ct}$ method as described before (Karlova et al., 2011). Expression values were normalized using the *Actin* gene.

Detection of mature miRNA expression by Northern blotting

For the detection of mature miRNA by Northern blotting, total RNA was isolated from 1 gram frozen and ground fruit samples (3-4 fruits pooled per sample) following the method of (Rosas-Cárdenas et al., 2011). RNA samples were treated with DNAse I (Life Technologies, Fisher Scientific, Landsmeer, NL) before measuring their concentrations on a NanoDrop 1000 spectrophotometer. 15 µg RNA per sample was loaded and separated on a 15% (acrylamide: bisacrylamide 29:1) polyacrylamide gel by electrophoresis, blotted and cross-linked to Amersham Hybond-NX membranes (GE Healthcare, Little Chalfont, UK) as described by (Pall et al., 2007).

For hybridization and detection, LNA (Locked Nucleic Acid, Eurogentec, Maastricht, NL) probes complementary to the miRNA (*miR156*: 5'-GTGCTCTCTATCTTCTGTCAA-3, *miR172*: 5'-ATGCAGCATCATCAAGATTCT-3') or a deoxyribonucleotide probe complementary to the U6 small nucleolar RNA (5'- CGCAGGGGCCATGCTAATCT-3') were end labelled with [γ-^{32}P]ATP (Perkin-Elmer, Groningen, NL) using USB OptiKinase (Affymetrix, Santa Clara, CA), according to the manufacturers manual. (Pre-) Hybridization was performed as described by (Pall et al., 2007).

MicroRNA precursor discovery, alignment, and synteny analysis

Genomic locations of putative miR156, miR157, and miR172 precursors (stem-loop or hairpins) were determined by BlastN searches using published precursors of tomato and potato miR156 or miR172, collected in miRBase (Kozomara and Griffiths-Jones, 2014), as queries. BlastN searches and mapping for tomato was performed on the Sol Genomics Network (SGN) website using version SL2.40 chromosome sequences and iTAG release 2.3 genomic annotations. For poplar and *Arabidopsis*, *Populus trichocarpa* - JGI v3.0, and *Arabidopsis thaliana* Columbia- TAIR 10 on Phytozome v9.1 were used, respectively. For alignment, sequences were downloaded from miRBase (Kozomara and Griffiths-Jones, 2014) or, for tomato, extracted from the SL2.40 chromosome sequences. The alignment was performed with MUSCLE (Edgar, 2004). For synteny analysis, syntenic genome blocks were first identified by searching with the loci on both sides of the respective MIR genes (which are not annotated, except in *Arabidopsis*) in the Plant Genome Duplication Database (Lee et al., 2013). This approach was supplemented by searching for orthologous gene families containing genes from the depicted blocks in at least two species, in PLAZA Dicots v3 (Proost et al., 2015).

gRNAs design and construct assembly

Online programs sgRNA Scorer 1.0 (https://crispr.med.harvard.edu/sgRNAScorerV1/), CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/) and Cas-OFFinder (http://www.rgenome.net/cas-offinder) were

used to design gRNAs targeting *MIR156e-h*, *MIR157ch3-1* and *MIR172ch5-2* and primers for all the gRNAs are in Table S1.

Plant transformation

Tomato cv. Moneyberg was used for all the *Agrobacterium tumefaciens*-mediated transformations according to described (van Roekel et al., 1993).

Plant genotyping

PCRs for *NPT II* and *Cas9* were performed for all the regenerated plats and the ones which were both *NPTII* and *Cas9*-positive were selected for amplifying and sequencing the target regions to detect mutations. The program SeqMan pro from DNASTAR Navigator 12 was used for alignment. Homozygous progenies without *Cas9* could be segregated in the T_1 generation and were selected for all the studies. All primers used for amplifying the target regions are in Table S1.

Plant phenotyping

Tomato flowers of all the mutant lines (three plants per line) and WT were labelled on the day when the flower is fully opened as 0 days after anthesis (DPA). The day when the first carotenoid accumulation is visible is defined as Breaker (Br) which is the start of ripening. Chlorophyll and lycopene content of nine fruits (three per plant) was measured from 1 day before Br (-1 DPB) until 7 days after Br (7 DPB) using Pigment Analyzer PA1101 and the average value of nine fruits was used to show the pigment change during tomato fruit ripening.

Stem-loop PCR and quantitative Real-Time PCR for mature miRNA expression

All the primers used are listed in Table S1. SuperScript™ II Reverse Transcriptase kit (Thermo Fisher) was used for stem-loop PCR. iQ SYBR Green Supermix (Bio-Rad) and iCycler iQ5 system(Bio-Rad) were used for quantitative RT PCR. Stem-loop PCR for reverse transcription and quantitative RT-PCR for mature miRNAs were performed as described (Varkonyi-Gasic et al., 2007). Relative expression changes of mature miRNAs were calculated according to $2^{-\Delta\Delta Ct}$ method as described (Livak and Schmittgen, 2001). The average value of changing folds from four pools was used for Fig. 4E-G and student's *t*-test was performed to detect the significant difference between samples. The gene *Actin* was used as a reference.

The MoClo Toolkit was used for Golden Gate-cloning to assemble constructs with two or four gRNAs. Merged by PCR with the *Arabidopsis* U6 promoter, every gRNA was cloned to a Level 1 vector as AtU6p::gRNA. Level 1 construct *pICH47732-NOSpro::NPTII*, *pICH47742-35S:Cas9*, *pICH47732*-gRNA1, *pICH47742*-gRNA2, *pICH47751*-gRNA3, *pICH47761*-gRNA4 and the linker *pICH41822* to

Level 2 vector *pICSL4723* as described (Werner et al., 2012). The linker *pICH41780* was used for assembling two gRNAs in Level 2 vector *pICSL4723*.

Results

SPL-CNR and AP2a regulate the expression of miR172 and miR156

Previously, we have shown that *SPL-CNR* and *AP2a* are actively cleaved at the Breaker (Br) fruit stage by miR156/157 and miR172, respectively (Karlova et al., 2013). To determine which *MIR156/157* and *MIR172* genes are expressed during ripening stages we used the frequency of degradome tags uniquely mapping to their mRNAs, which we showed previously can be used as a proxy for their expression levels (Karlova et al., 2013). We used these data to predict the expression levels of 15 putative miR156/157 and 6 miR172 precursors, as their expression could not be inferred from microarray or RNAseq experimental data. We identified that five *MIR156/157* and three *MIR172* putative genes were expressed in developing or ripening fruits (Table S2). PCR on fruits of cv. Moneyberg subsequently confirmed this observation, with *MIR156e-h* and *MIR172ch5-2* being most highly expressed in fruits (Fig. 1A and B). A single *MIR* gene on chromosome 3, which was originally annotated as *MIR156_Ch3* (Table S2) and selected for qPCR based on its degradome counts, upon alignment with other MIR156 and MIR157 stemloop regions (Fig. S1, sly-Ch03-1) appeared to encode a mature miRNA that was clustering with miR157s and therefore was renamed *MIR157ch03-1* (Fig. 1A). *MIR157ch03-1* was much lower expressed than *MIR156e-h* and *MIR172ch5-2* in cv. Moneyberg, contrary to a lesser difference in degradome read counts in cv. Ailsa Craig, with which the latter experiment was performed. Confirming the preliminary observations with degradome read counts in cv. Ailsa Craig, in cv. Moneyberg fruits *MIR156e-h* and *MIR172ch5-2* expression increased and decreased, respectively, with progression of ripening (Fig. 1).

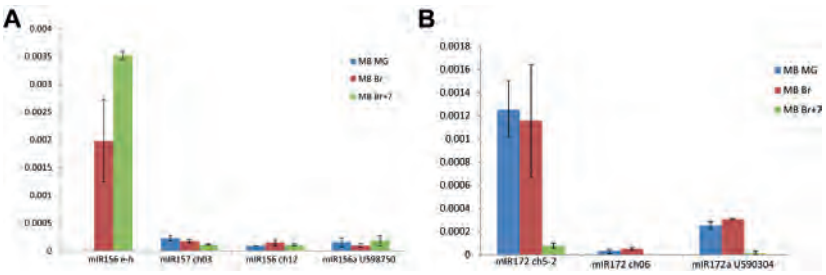

Figure 1. Detection and expression of tomato *MIR* genes. qRT-PCR analysis of the precursors of (**A**) miR156 and miR157 and (**B**) miR172 in fruits of cv. Moneyberg at MG, Br and Br+7d. Error bars show SE of means from three biological replicates. (*, $P < 0.05$).

Both phylogenetic analysis of aligned stem-loops (Fig. S1), as well as analysis of synteny of their genomic regions with those of *Arabidopsis* and other Eudicots (Fig. S2) suggest that *SlMIR156e-h* and

SlMIR172ch5-2 are orthologs of *Arabidopsis MIR156e* and *MIR172b*, respectively. Interestingly, the single *MIR156e-h* gene appears to be the result of multiple duplications resulting in a total of 7 or more stem-loop sequences with homology to *Arabidopsis MIR156e* (Fig. S2). It also appeared to have two residual homologous stem-loop sequences nearby (depicted as "v" in Fig. 3A).

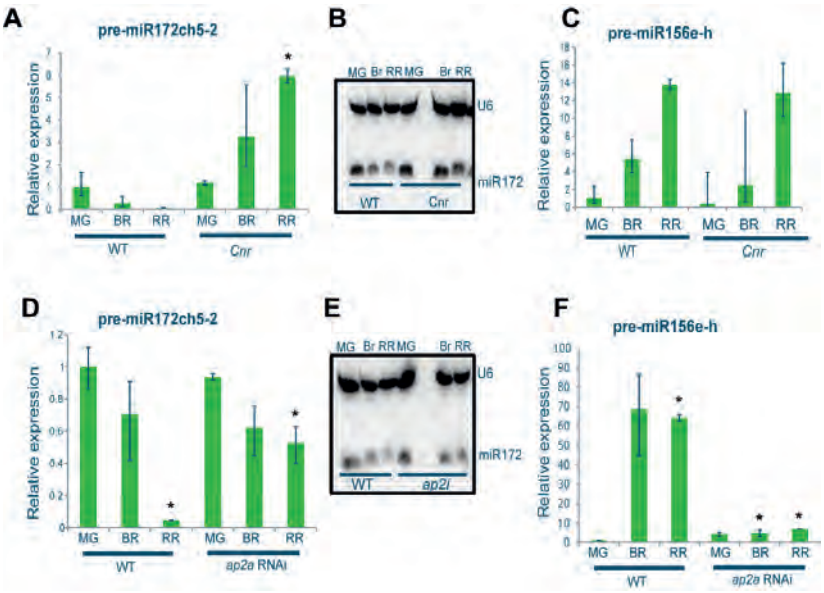

Figure 2. Effects of *Cnr* and *ap2a* RNAi on (**A**) and (**C**) Relative expression profiles of *MIR172ch5-2* and *MIR156e-h*, in wild-type (WT) cv. Ailsa Craig at different fruit stages: MG, Br and red ripe (RR). (**B**) Small RNA gel blotting to detect the expression levels of mature miR172 in WT and *Cnr* mutant fruits. (**D**) and (**F**) expression profile of *MIR172ch5-2* and *MIR156e-h* in WT and *ap2a* RNAi fruits. (**E**) Small RNA gel blotting to detect the expression level of mature miR172 in wild type and *ap2a* RNAi fruits. Asterisks indicate significant difference to the MG stage or in the *Cnr* and *ap2a* RNAi fruits to the corresponding WT stage, Error bars show SE of means. (*, $P < 0.05$).

To investigate whether *Cnr* or *AP2a* regulates *MIR156e-h* and *MIR172ch5-2* in a *MIR* gene-target feedback interaction we performed expression analysis in *Cnr* and *ap2a* RNAi fruits. Our data showed that in WT cv. Ailsa Craig fruits both the primary transcript of *MIR172ch5* and mature miR172 expression decreased during normal ripening and markedly increased in *Cnr* mutant fruits (Fig. 2A and B). Interestingly, this contrasts with observations in *Arabidopsis* where SPLs promote the expression of miR172 (Wu et al., 2009) and miR172 levels increase as plants age (Aukerman and Sakai, 2003). In *Arabidopsis*, miR156 has an opposite expression pattern compared to miR172 and its level decreases as the plant ages. We observed that in tomato, *MIR156e-h* levels increase during ripening consistent with its expression regulation being opposite to that of miR172 in the fruit (Fig. 2). No significant change of the precursor *MIR156e-h* expression was observed in the *Cnr* mutant fruits when compared with wild type, indicating that *Cnr* does not influence the expression of *MIR156e-h* (Fig. 2C). In *Arabidopsis*

miR156 and miR172 are positively and negatively regulated, respectively, by AP2 TFs (Yant et al., 2010). As shown in Fig. 2D-F and in agreement with the *Arabidopsis* model SlAP2a in tomato negatively regulates both the precursor *MIR172ch5-2* (Fig. 2D), as well as mature miR172 expression (Fig. 2E) at the red ripe stage and positively regulates *MIR156e-h* (Fig. 2F) at the Breaker and red ripe fruit stages.

MiR156 regulates the transition to fruit ripening

To unravel the function of miR156 we created a deletion mutant in *MIR156e-h* using CRISPR/Cas9 mutagenesis (Fig. 3A), by targeting the locus with 4 gRNAs and obtained a mutant with three deletions totalling 883 base pairs. Similarly, we targeted *MIR157ch3-1* with 2 gRNA's and obtained a deletion of 115 base pair (bp) (Fig. 3B) and targeted *MIR172ch5-2* with 4 gRNAs and obtained a 689 bp deletion (Fig. 3C).

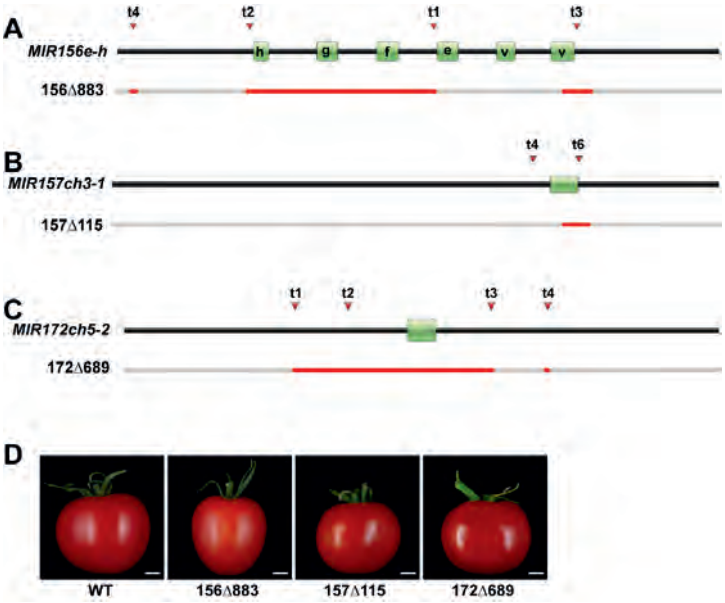

Figure 3. CRISPR mutants of *MIR* genes. (**A**) miR156, (**B**) miR157 and (**C**) miR172 encoding gene schemes (5' to 3') and the CRISPR mutants deleting their stem-loops. Green blocks represent the stem-loop structures in the genes and red arrows indicate the location of guide RNAs used. The red lines underneath show the size and location of deletions. (**D**) CRISPR fruits of homozygous T_1 plants at Br+7d. Scale bars, 1 cm.

All three mutations were propagated as T_1 generation and homozygous plants were selected. None of the mutants had obvious ripening defects at the Br+7d, but fruits of 156Δ883 appeared smaller and oblong-shaped compared to the round wild type fruits (Fig. 3D). Additionally, we measured time to ripening for all mutants and observed that the fruits from the mutant line 156Δ883 reached Br stage on

average 5 days earlier than the WT fruits, proving that miR156's action in WT fruits may increase the time to ripening during tomato fruit development (Fig. 4A). This also indicates that the fruits of the 156Δ883 mutant were smaller than the WT fruits, possibly due to the significantly shorter development time to ripening (Fig. 4B). No other ripening phenotypes were observed as the progress of ripening, measured as the rates of chlorophyll degradation (Fig. 4C) and lycopene accumulation (Fig. 4D) showed no significant differences from the wild type.

Considering the relatively small effect of the *MIR* gene deletions on fruit ripening progression, we employed stem-loop PCR to determine mature microRNA expression for all three miRNAs 20 DPA, 40 DPA (Fig. 4E-G) and MG, Br, and Br+7d (Fig. S3). Surprisingly, only mature miR156 expression at 20 DPA, and less so at 40 DPA was negatively affected by the 156Δ883 mutation, in line with the observation of an effect only in that line (Fig. 4E), while mature miR157 (Fig. 4F) and mature miR172 (Fig. 4G) levels were not affected by their respective mutations. At a later stage (MG, 42 DPA), the expression of miR172 in the deletion mutant dropped to significantly lower than that in WT until the Br, the initiation of ripening (Fig. S3B), but this reduction did not cause visible phenotypic changes in mutant fruit of 172Δ689 (Fig. 3D and 4A-D).

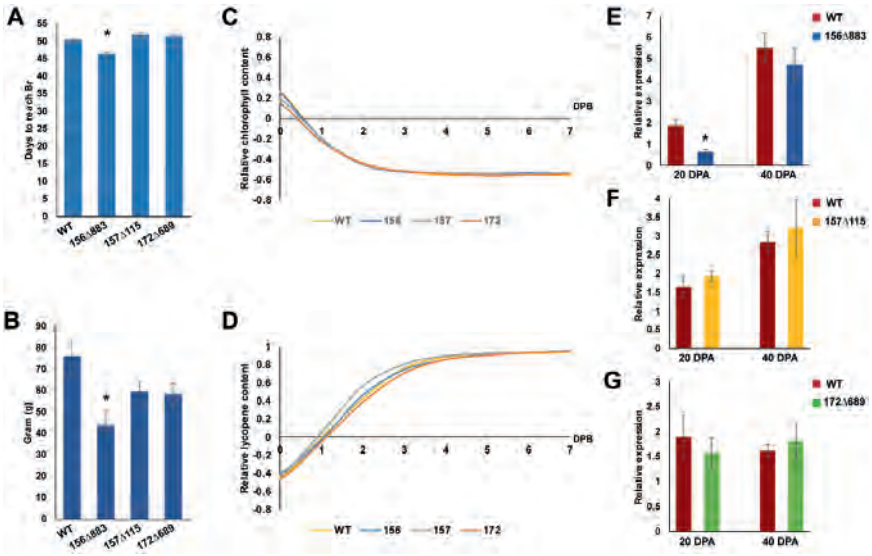

Figure 4. miR156 regulates the initiation of ripening. (**A**) Time to the initiation of ripening in mutants. (**B**) Fruit weight of mutants at Br+7d. (**C**) Chlorophyll decrease during ripening. (**D**) Lycopene accumulation during ripening. (**E, F, G**) The expression of mature miRNAs in WT and their respective mutants (**E**) *MIR156e-h*, (**F**) *MIR157ch3-1* and (**G**) *MIR172ch5-2* at 20 and 40 DPA. Error bars represent SE of means. *, $P < 0.05$.

Discussion

Plants undergo several developmental phase transitions during their life cycle (Huijser and Schmid, 2011; Boehm and Slack, 2005). In flowering plants, these include major morphological and physiological changes in shoots and leaves when switching from the "juvenile" to the "adult" vegetative phase or when obtaining competence for flowering (Kaufmann et al., 2010; Rubio-Somoza and Weigel, 2011). Central to the control of these two-phase transitions in the plant life cycle is a regulatory circuit involving temporal changes in expression of members of the *SQUAMOSA BINDING PROTEIN-LIKE* (*SPL*) and *APETALA2* (*AP2*)-like transcription factor (TF) gene families, and of their regulatory microRNAs, miR156/157 and miR172, respectively. In tomato, a member of each of these transcription families, *SPL-COLORLESS NON-RIPENING* (*SPL-CNR*) and *AP2a*, respectively are highly expressed in ripening fruits and required for normal ripening (Manning et al., 2006; Karlova et al., 2011; Chung et al., 2010), although the role of *SPL-CNR* in ripening remains elusive (**Chapter 4**). Both are actively targeted by their cognate microRNAs as demonstrated by the fact that during ripening their miRNA action-derived products, sliced at the predicted target site can be readily detected (Karlova et al., 2013).

It was proposed that a developmental signal is needed to develop competency for fruit ripening in tomato, as immature fruit does not respond to exogenous ethylene by initiating ripening (Klee and Giovannoni, 2011), and secondly, artificially reducing DNA cytosine methylation can induce early ripening (Zhong et al., 2013). Here, we show that components of an evolutionary conserved network regulating phase transition in plants are expressed in ripening fruit. In flowering plants, it has been shown that miR156 expression is temporally regulated, with a progressive reduction as the plant ages. In contrast to the accumulation of its target genes, *SPL*s increase with age (Huijser and Schmid, 2011). Our data shows that in tomato fruits miR156 is developmentally regulated and its expression increases with fruit ripening progression. This observation is in agreement with published data from expression profiling of short RNAs during fruit development (Mohorianu et al., 2011). Furthermore, we showed that the downregulation of miR156 accelerates the time to ripening in tomato fruits.

Another study suggests that *SPL-CNR* is a target of miR157 rather than miR156 due to better complementarity, and supported by ectopic expression of a miR157 that causes an orange ripe fruit phenotype, suggesting that this is due to increased down-regulation of *SPL-CNR* (Chen et al., 2015). However, no clear correlation between native miR157 expression, *SPL-CNR* down-regulation, and inhibition of ripening was shown. MiR156, even with imperfect complementarity to the target may still be able to inhibit *SPL-CNR* expression by translational inhibition, as shown for miR156 in *Arabidopsis* (Wu and Poethig, 2006). Moreover, although miR157 may well have the ability to target *SPL-CNR*, its ectopic expression is not conclusive proof of such a function in the wild-type. Although the low expression of *MIR157* genes suggested by our results is in itself not proof of lack of function, this leaves the question: miR156 or miR157 unresolved.

Curiously, the (partial) deletion of three *MIR* genes that are expressed during fruit ripening had little effect on the levels of the corresponding mature miRNAs at the same stages with the notable exception of miR172, and only decreased the miR156 level in immature fruit stages. This suggests that there may be a compensatory response from other, homologous *MIR* genes to keep mature miRNA levels stable, which precluded the manipulation of the miRNA/target-interaction. This could be checked in the future by studying the expression of these additional genes in the *MIR* deletion mutants, and if such compensation exists, several or all genes would need to be modified to achieve a real effect. For now, the conservation of several of the vegetative phase transition components as fruit-expressed paralogs in tomato remains an intriguing phenomenon without a known function.

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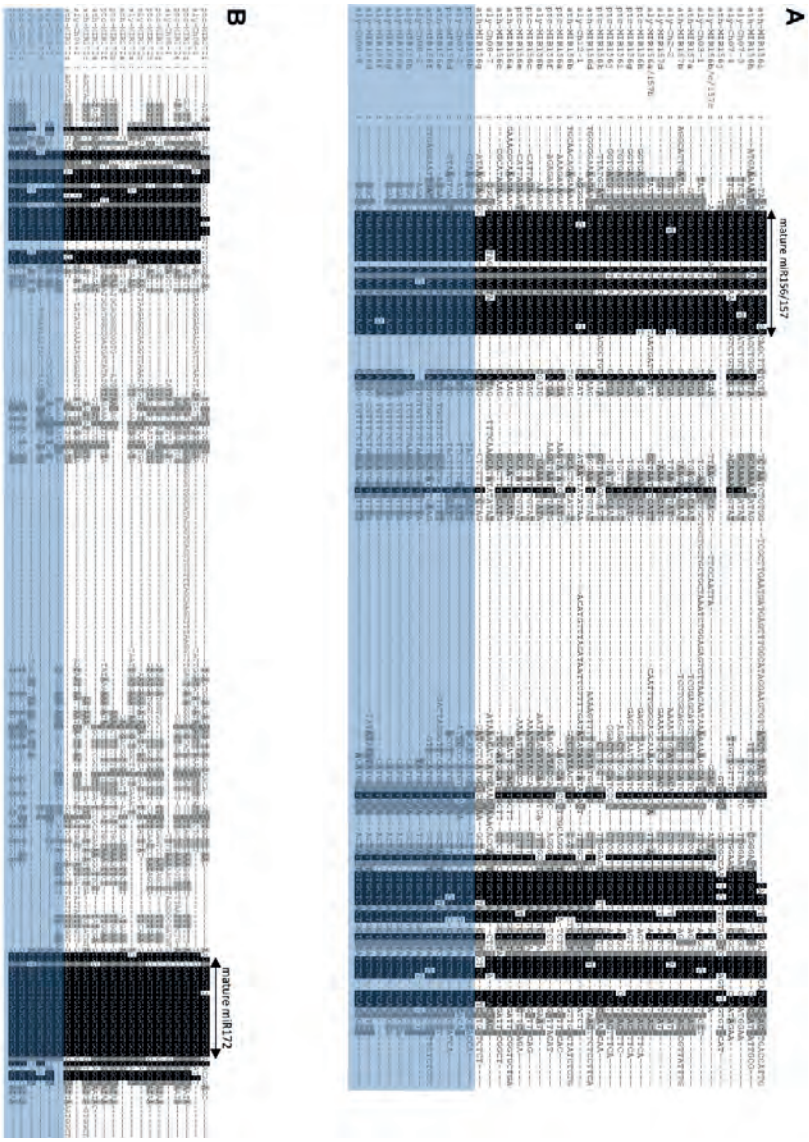

Figure S1. Alignment of stem-loop sequences of *MIR156/157* and *MIR172* genes from tomato, *Arabidopsis* and poplar. Tomato (sly-), *Arabidopsis* (ath-), and poplar (ptc-) stem-loop sequences were extracted from miRBase and named accordingly, with the exception of *MIR156b*, *d-h*, and *157d*, which were named according to (Salinas et al., 2012). All other putative stem-loops are named by the chromosomal position according to Table S2. (**A**) *MIR156/157*. The double arrow highlights the position of the mature miRNA in the sequences. *Sly-Ch08-7* is unlikely to encode a mature miR156, but was included for its overall homology with other stem-loops. The horizontal rectangle highlights the homologous cluster of tomato chromosome 8 stem-loops, and of *Arabidopsis MIR156e* and *MIR156f*, and of poplar *MIR156b* and *MIR156d*, used for synteny analysis as shown in Fig. S2A. (**B**) *MIR172*. The double arrow highlights the position of the mature miRNA in the sequences. The horizontal rectangle highlights the homologous cluster of tomato *MIR172-Ch05-2* and *MIR172-Ch11-01* stem-loops, *Arabidopsis MIR172b*, and of poplar *MIR172a* and *MIR172c*, used for synteny analysis as shown in Fig. S2B.

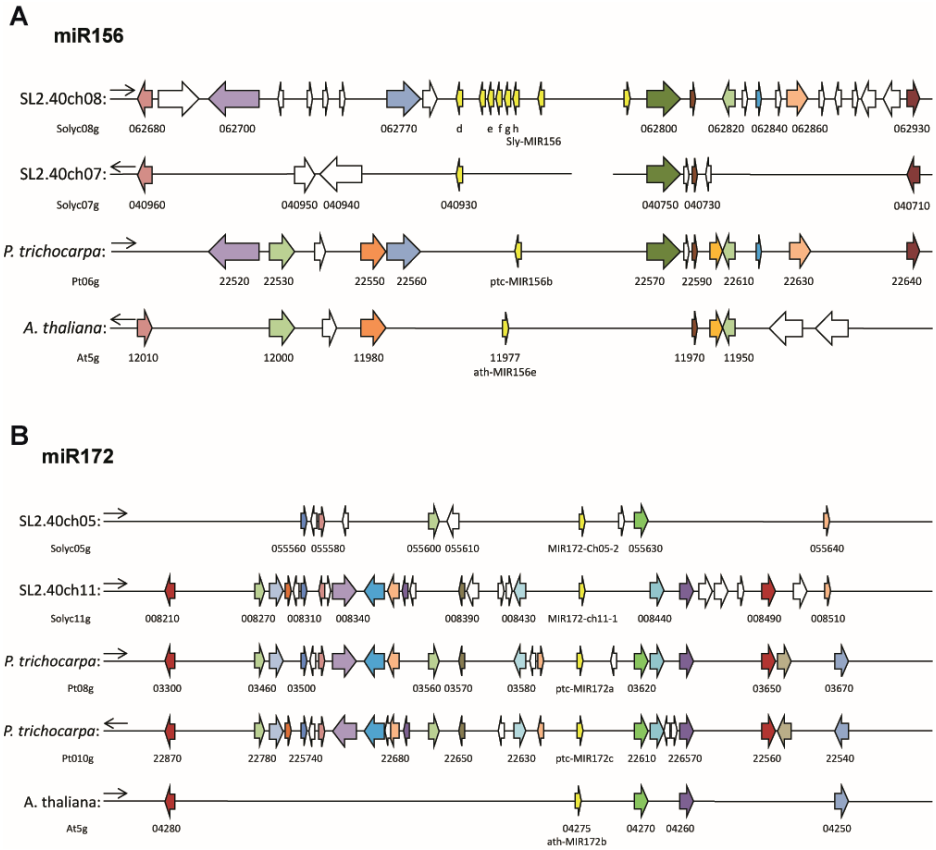

Figure S2. Synteny between genomic regions containing putatively orthologous miRNA genes in tomato, poplar, and *Arabidopsis*. Vertically aligned genes in the same color are putative orthologs as determined by synteny analysis in the Plant Genome Duplication Database, supplemented with ortholog groups from PLAZA v3 (Dicots). Putative MIR stem-loops are depicted in yellow. Gene sizes and intergene distances in the figure are not depicted to scale. (**A**). Tomato *MIR156e-h* and additional putative MIR156 stem-loops clustered on chromosome 8, and synteny of this region with tomato chromosome 7 (a putative miR156 stem-loop is located in the intron of gene model Solyc07g040930), poplar chromosome 6 containing *ptc-MIR156b*, and *Arabidopsis* chromosome 5 containing *ath-MIR156e*. (**B**) Tomato *MIR172-Ch05-2* on chromosome 5 and its synteny with putative *MIR172-Ch11-1* on chromosome 11, poplar chromosomes 8 and 10, containing *ptc-MIR172a* and MIR172c, respectively, and *Arabidopsis* chromosome 5 containing *ath-MIR172b*.

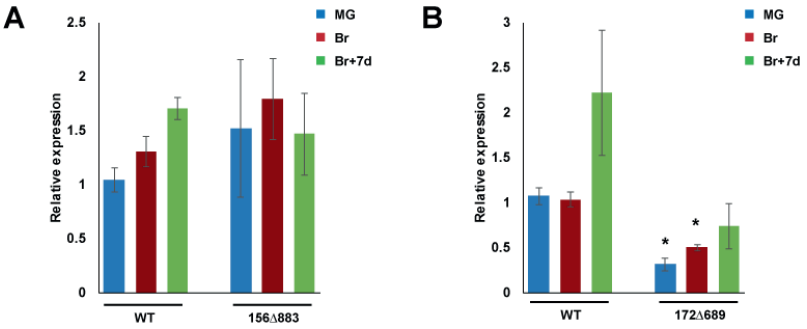

Figure S3. Expression changes of mature (**A**) miR156 and (**B**) miR172 in CRISPR mutants. Error bars represent SE of means. *, $P < 0.05$.

Primer name	Sequence (5'-3')	Description
MIR172 ch05-F	AGAATCTTGATGATGCTGCATTGG	qRT-PCR FW
MIR172 ch05-R	AATTGCCCAAATTTTCTTGTGGAA	qRT-PCR RV
MIR156 h-e-F	CTGTCATCCCTACCATTTTCTCCG	qRT-PCR FW
MIR156 h-e-R	GCACGAAAATAGAGGCAAGCAG	qRT-PCR RV
SlAP2-F	AGACCACAATTTGGAATCGCCG	qRT-PCR FW
SlAP2-R	GCCCTTGGAAAATTGGTAGACTCG	qRT-PCR RV
SlCNR-F	TTCCCGGATTTCTAAGCAAATTGT	qRT-PCR FW
SlCNR-R	GTTGGAATGTCAACATGGATATGCA	qRT-PCR RV
MIR157 ch03-F	TTCCGTCATCCCTTTCATCCTCTT	qRT-PCR FW
MIR157 ch03-R	CAGAAATTGGAAGAAATTTCCCCA	qRT-PCR RV
MIR156 ch12-F	TGCGTGTGCTCACTTCTCTTTCTG	qRT-PCR FW
MIR156 ch12-R	AATCTCGCGGTACAATCTAAATCTGG	qRT-PCR RV
MIR156a U598750-F	TGCTTCTGTCATCACCTTCACCTC	qRT-PCR FW
MIR156a U598750-R	GGAAAACCTATAGGGCGCAACC	qRT-PCR RV
MIR172 ch06-F	TCTCTCTTTGGGAATCTTGATGATGC	qRT-PCR FW
MIR172 ch06-R	CCACCATGACTTTTTGTTTACAAGGA	qRT-PCR RV
MIR172a U590304-F	GGCTGGCTATTTGAAACTCACGAG	qRT-PCR FW
MIR172a U590304-R	TCGTTGCAATGCTCTAATTGCAAT	qRT-PCR RV
NPT2-F	AGACAATCGGCTGCTCTGAT	genotyping NPT2
NPT2-R	AGCCAACGCTATGTCCTGAT	genotyping NPT3
CAS9-F	CTTTGGCAATATCGTGGACG	genotyping CAS9
CAS9-R	CGTTCTTCTTCTCCCCAGGG	genotyping CAS9
miR156 SLR	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACGTGCTC	stemloop PCR reverse transcription miR156
miR172 SLR	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACATGCAG	stemloop PCR reverse transcription miR172
miR157 SLR	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACGTGCTC	stemloop PCR reverse transcription miR157
Actin-F	TGAGAGGTGCCTGATGCATTGC	qPCR for Actin
Actin-R	ACGCTTCGACCAAGGGATGG	qPCR for Actin
miR156-F	GCGGCGGTGACAGAAGAGAGT	qPCR for miR156
miR172-F	GCGGCGGAGAATCTTGATGATG	qPCR for miR172
miR157-F	GCGGCGGTTGACAGAAGATAGA	qPCR for miR157
miR universal R	GTGCAGGGTCCGAGGT	universal primer for stemloop PCR
MIR156-F	CAGCAAATGGAGGAAGTA	genotyping miR156 mutant
MIR156-R	CAGAAGAAAGCCCTAACC	genotyping miR156 mutant
MIR172-F	GGTTCTTTAAACTCGTGAATAATAC	genotyping miR172 mutant
MIR172-R	AATCGAATTTTACTTCTCCACA	genotyping miR172 mutant
MIR157-F	GGTAACCCACCCACACTAAATAAATC	genotyping miR157 mutant
MIR157-R	CAGAAATTGGAAGAAATTTCCCCA	genotyping miR157 mutant
MIR156h-e t1	TGTGGTCTCAATTGTGGGATGTAAAACAAAAGGGG TTTTAGAGCTAGAAATAGCAAG	forward primer for t1 in *MIR156*
MIR156h-e t2	TGTGGTCTCAATTGTTTTGTGAAGGGTAAAAGGGGT TTTAGAGCTAGAAATAGCAAG	forward primer for t2 in *MIR156*
MIR156h-e t3	TGTGGTCTCAATTGGAGAGAGAGGAAAAGCGATGG TTTTAGAGCTAGAAATAGCAAG	forward primer for t3 in *MIR156*
MIR156h-e t4	TGTGGTCTCAATTGGTTTATCTAATAGTACCATAGT TTTAGAGCTAGAAATAGCAAG	forward primer for t4 in *MIR156*

MIR172ch5-02 t1	TGTGGTCTCAATTGAAAAAATCAAGCAGACAAAAG TTTTAGAGCTAGAAATAGCAAG	forward primer for t1 in *MIR172*
MIR172ch5-02 t2	TGTGGTCTCAATTGAACGAAAAAATTCATTAAAAGT TTTAGAGCTAGAAATAGCAAG	forward primer for t2 in *MIR172*
MIR172ch5-02 t3	TGTGGTCTCAATTGTGCAGAGGATGAAGAATTCGGT TTTAGAGCTAGAAATAGCAAG	forward primer for t3 in *MIR172*
MIR172ch5-02 t4	TGTGGTCTCAATTGAACGCGTCAATAATCAATGGGT TTTAGAGCTAGAAATAGCAAG	forward primer for t4 in *MIR172*
MIR157ch3-01 t4	TGTGGTCTCAATTGAGTGTTAAATTAATACACAGGT TTTAGAGCTAGAAATAGCAAG	forward primer for t4 in *MIR157*
MIR157ch3-01 t6	TGTGGTCTCAATTGGAAGAGGATGAAAGGGATGAG TTTTAGAGCTAGAAATAGCAAG	forward primer for t6 in *MIR157*
Universal R	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC	universal primer for gRNAs

Table S1. Oligonucleotides used in this study.

								Mapped counts, normalized (per million)					Uniquely mapped counts, normalized				
Chromosome	**Start**	**End**	**Strand**	**Name**	**Reference**	**SGN Unigene**	**Potato homolog (miRbase)**	**SDAP**	**MG**	**Br**	**RR**	**Total**	**SDAP**	**MG**	**Br**	**RR**	**Total**
miR156/157																	
SL2.40ch02	29897678	29897771	+	MIR157d	Salinas et al.		stu-mir156a	0	0	0	0	0	0	0	0	0	0
SL2.40ch02	29898721	29898814	+				stu-mir156b	0	0	0	0	0	0	0	0	0	0
				sly-MIR156b/c;													
SL2.40ch02	47055512	47055595	+	MIR157c	miRBase/Salinas et al.		stu-mir156c	0	0	0	0	0	0	0	0	0	0
SL2.40ch03	61720034	61720161	+	**MIR156_Ch3**				11	3	12	4	30	11	3	12	4	30
SL2.40ch06	39241672	39241780	-	**sly-MIR156a; 157b**	miRBase/Salinas et al.	U598750	stu-mir156d	0	2	0	1	2	0	2	0	1	2
SL2.40ch07	324082	324177	+	MIR156b	Salinas et al.			6	6	0	1	13	6	6	0	1	13
SL2.40ch07	48503777	48503871	+				stu-mir156e	0	0	0	0	0	0	0	0	0	0
SL2.40ch07	58834541	58834633	-					1	1	2	3	7	1	1	2	3	7
SL2.40ch07	64534504	64534598	-					1	0	3	1	5	1	0	3	1	5
SL2.40ch08	49122870	49122973	-	MIR156d	Salinas et al.			6	4	1	3	14	4	2	0	2	8
SL2.40ch08	49142584	49142678	-	**MIR156_Ch8-2**			stu-mir156h,i,j,k	1	1	3	2	6	1	1	3	2	6
SL2.40ch08	49143049	49143144	-	**MIR156e**	Salinas et al.	Presumed part of a polycistronic messenger	stu-mir156h,i,j,k	0	3	2	4	9	0	1	1	1	3
SL2.40ch08			-	MIR156f	Salinas et al.		stu-mir156h,i,j,k										0
SL2.40ch08	49143533	49143628	-	MIR156g	Salinas et al.		stu-mir156h,i,j,k	0	3	3	5	11	0	1	1	3	5
SL2.40ch08	49143788	49143883	-	MIR156h	Salinas et al.		stu-mir156h,i,j,k	2	10	27	4	44	2	10	27	4	44
SL2.40ch08	49154386	49154488	-					2	4	0	0	6	0	3	0	0	3
SL2.40ch08	49276195	49276291	+					0	1	1	1	2	0	0	0	0	0
SL2.40ch12	3275345	3275461	-	**MIR156_Ch12**			stu-mir156f	6	5	16	4	31	6	5	16	4	31
miR172																	0
SL2.40ch04	4093787	4093941	-				stu-mir172c	20	23	7	6	55	19	23	7	5	55
SL2.40ch05	62347695	62347798	+	MIR172_Ch5-1			stu-mir172d	2	2	0	0	4	2	1	0	0	3
SL2.40ch05	64339675	64339770	+	**MIR172_Ch5-2**			stu-mir172b	108	76	10	2	197	108	76	10	2	196
SL2.40ch06	654159	654300	+	**MIR172_Ch6_1**				4	3	1	2	9	4	3	0	2	9
SL2.40ch06	42918731	42918846	+	**sly-MIR172a**	miRBase	U590304	stu-mir172a	3	28	2	5	38	1	12	2	3	18
SL2.40ch10	63061186	63061270	+					1	0	0	0	2	1	0	0	0	1

Table S2. Numbers of degradome reads mapping to tomato miR156/157 and miR172 stem-loops locations for fruits of 5 DPA (days post anthesis), mature green (MG), Breaker (Br), and Red Ripe (RR) stages.

Chapter 6

Revisiting the role of master regulators in tomato ripening

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Abstract

The study of transcriptional regulation of tomato ripening has been led by spontaneous mutations in transcription factor genes that completely inhibit normal ripening, suggesting that they are "master regulators". Studies using CRISPR/Cas9-mutagenesis to produce knockouts of the underlying genes indicate a different picture, suggesting that the regulation is more robust than previously thought. This requires us to revisit our model of regulation of ripening and replace it with one involving a network of partially redundant components. At the same time, the fast rise of CRISPR/Cas-mutagenesis, resulting in unexpectedly weak phenotypes, compared to knockdown technology, suggest that compensatory mechanisms may obscure protein functions. This emphasises the need for assessment of these mechanisms in plants and for careful design of mutagenesis experiments.

Keywords

Tomato; ripening; transcription factors; mutants; gain-of-function; CRISPR- mutagenesis

Highlights

A string of recent publications has put into question the function of transcription factors, which were previously considered "master regulators" in control of tomato fruit ripening.

RNA interference and CRISPR/Cas-mutagenesis have enabled a re-evaluation of their proposed functions, which until that time were mostly derived from the phenotype of spontaneous ripening mutations.

Three spontaneous mutations in TF genes all appear to be gain-of-function mutations and interestingly, probably represent three different mechanisms of this phenomenon.

These observations raise interesting fundamental and practical questions related to the manipulation of fruit ripening and other processes.

CRISPR/Cas-mutagenesis has dramatically improved our options to address gene function, but at the same time some recent results suggest that compensatory mechanisms in CRISPR mutants may also hide true gene functions.

Tomato as a model for fleshy fruit ripening

Fleshy fruits ripen in order to become attractive to **frugivores** (see Glossary) that can act as seed dispersers, or when not eaten, to soften and decay, allowing the release of seeds. In line with these functions, during ripening fruits undergo many physiological and metabolic changes accompanying pervasive alterations in gene expression, which lead to the improved flavour and softer flesh that render them edible. This process follows a period of growth starting from the ovary after pollination and fertilization, during which seeds develop and mature to become ready for dispersal. Thus the timing of onset of ripening needs to be coordinated with seed maturity, and this has led to the evolution of developmental processes involving control of transcriptional regulation and signalling pathways of plant hormones such as ethylene, gibberellin, abscisic acid, and auxin (Seymour et al., 2013b). Tomato (*Solanum lycopersicum*) has become a model for fleshy fruit development due to several practical advantages (relatively short life cycle, **autogamy**, genetic transformability, availability of mutants) as well as a high-quality reference genome. Ripening of tomato (Figure 1A) is of the **climacteric** type. In this type a burst in ethylene production occurs through the activation of an autocatalytic biosynthesis circuit ("system 2") unique to the ripening stage. This replaces the "system 1" circuit that slowly increases ethylene production as a function of fruit age until a threshold is reached at which "system 2" is induced (Mcmurchie et al., 1972). Both ethylene signalling and transcriptional regulation play a key role in this switch (Liu et al., 2015a).

Transcriptional regulation of tomato ripening

Much of the research on transcriptional regulation of tomato fruit ripening has relied on the study of spontaneous mutants. Three of those are spontaneous mutations discovered in breeding programs or a commercial orchard and appeared in the past 50-60 years (Robinson and Tomes, 1968; Tigchelaar et al., 1973; Thompson et al., 1999). Since then they have been instrumental in the study of transcriptional regulation of fruit ripening. These studies have identified, by positional cloning of the underlying genes, which encode transcription factors (TF). These TFs were long considered "master regulators" of tomato fruit ripening due to the dramatic lack of ripening initiation in the respective mutants: *ripening inhibitor* (*rin*) (Robinson and Tomes, 1968), *non-ripening* (*nor*) (Tigchelaar et al., 1973), and more recently, *Colorless non-ripening* (*Cnr*) (Thompson et al., 1999). The expression of all three TF genes is sharply upregulated at the onset of ripening, further supporting the assumption that they must be important in the transcriptional regulation of ripening. In *rin* mutants a chimaeric messenger and protein are produced, due to a deletion between two **MADS-domain** transcription factor-encoding genes, which in the wild-type genome are positioned in tandem: *MADS-RIN* (so named because it complemented the *rin* ripening phenotype) and *MADS-MC (MC=MACROCALYX)* (Vrebalov et al., 2002). The resulting fusion protein contains the first 215 of 242 amino acids (AA) from the MADS-RIN protein followed by all but the first 62 of 219 AA from MADS-MC (Figure 1B). The *nor* mutation consists of a two-base pair (bp) deletion

in the open reading frame of the **NAC** TF-encoding gene *NAC-NOR*, resulting in a frame-shift and production of a protein that is truncated at 186 AA of the original 355 AA of the full-length protein (Figure 1B) (Kumar et al., 2018). The *Cnr* mutation maps to a 286 bp hypermethylated region ~2300 bp upstream of the **SPB-domain** transcription factor encoding gene ***SPL-CNR***. This stable epigenetic mutation is associated with a substantial decrease in the expression of *SPL-CNR* (Manning et al., 2006). All three mutants lack the characteristic burst of ethylene production at the onset of ripening, and do not undergo softening of the **pericarp**, nor do they show the characteristic change in colour from green to the deep red (although *rin* and *nor* fruits remain mostly green, *Cnr* fruits lose their chlorophyll and turn a pale yellow).

Apart from ethylene production and regulation by transcription factors, DNA methylation and demethylation is a crucial third component of ripening regulation. Overall, DNA methylation decreases during tomato fruit ripening and induced genome-wide cytosine demethylation leads to premature ripening (Zhong et al., 2013). Cytosine demethylation during tomato ripening requires and is mediated by a cytosine demethylase, DNA DEMETHYLASE 2 (DML2) (Liu et al., 2015b; Lang et al., 2017). Although the overall decrease in methylation is suggestive of a function, it remains to be determined which of these changes are merely passive, and which are essential for driving proper gene expression during ripening (Gao et al., 2019). The hypermethylated promoter region of the *Cnr* mutant allele, which is demethylated during ripening in wild-type tomato fruits, remains methylated in *Cnr* fruits. Tomato CHROMOMETHYLASE3 (CMT3) activity, involved in methylation maintenance at CHG sites, is required for sustaining the *Cnr* allele, as knocking down its activity reverts *Cnr* fruits to ripening (Chen et al., 2015). Since the hypermethylated region is situated close to two MADS-RIN binding sites in the *CNR* promoter, one hypothesis suggests that the hypermethylation somehow interferes with MADS-RIN binding and subsequent activation of *CNR* expression (Zhong et al., 2013). Although this remains a possible scenario, it is unlikely to be able to explain the entire phenotype of *Cnr*, which in part occurs earlier than the onset of *MADS-RIN* expression at ripening (Eriksson et al., 2004).

"Master regulators"?

These observations led to the designation of the underlying transcription factor-encoding genes (*MADS-RIN*, *NAC-NOR*, and *SPL-CNR*) as "master regulators" of fruit ripening, based on their phenotypes reflecting a loss of function (*NAC-NOR* and *MADS-RIN*), or of expression (*SPL-CNR*) (Karlova et al., 2014; Seymour et al., 2013a; Lü et al., 2018). Some support for this assumption came from partially mimicking the mutant phenotype by knockdown of the respective gene's expression using **antisense suppression** (*rin*) (Vrebalov et al., 2002), **VIGS** (*Cnr*) (Manning et al., 2006), or observations on a (weaker) allele of *nor*, *alcobaca* (Leal, 1973). Only after the introduction of CRISPR/Cas9-mutagenesis in tomato (Brooks et al., 2014) (Figure 1B), we and others re-investigated the function of these

transcription factors with surprising results, which in our opinion should make us revisit our current model of the role of these regulators and other transcription factors in the ripening regulatory network.

First, CRISPR/Cas9-induced mutations in *MADS-RIN*, considered to be real knockouts (*null*), revealed a much milder effect on ripening than the corresponding *rin* mutation: ripe mutant fruits were orange rather than green and many ripening-related genes that were repressed in *rin* mutants were not or only partially so in CRISPR mutants (Ito et al., 2017) (Figure 1B). Creation of similar CRISPR mutations in the chimaeric *rin-mc* gene of the *rin* background, partially reverted the strong ripening phenotype to one similar to that of the same mutations in the wild-type background, as did knockdown of the *mads-rin* allele by RNAi (Li et al., 2018) (Fig. 1B). Moreover, this difference in phenotype could be attributed to the MC-portion of the fusion protein, which was shown to have an inhibiting effect on the expression of many ripening genes, while it is transcriptionally regulated as wild-type *RIN* was (Li et al., 2018). This evidence points to the spontaneous *rin* mutation being a gain-of-function mutation, more specifically a dominant repressor, rather than a knockout mutation. Second, CRISPR mutations in *NAC-NOR* had a much milder phenotype than the spontaneous *nor* mutation (Wang et al., 2019; Gao et al., 2019). Also here, phenotypes of CRISPR-induced frame-shift mutations in the open reading frame upstream of the location of the *nor* mutation were much milder (orange fruit vs green fruit in the spontaneous *nor* mutant) than that of the *nor* mutation itself (Wang et al., 2019; Gao et al., 2019), and comparable to that of the *alcobaca* mutant (Kumar et al., 2018). Also here, the introduction of an upstream CRISPR mutation in the *nor* background converted the strong phenotype into the milder one of the CRISPR mutation (Wang et al., 2019) (Figure 1B). In this case, the *nor* mutation produces a truncated protein that retains most of the conserved DNA-binding NAC-domain. It was hypothesised that this protein, which has lost its transcriptional activation domain, might be interfering with normal transcriptional activation of NAC-NOR targets by binding to their promoters without activating them and preventing binding of other activators to that site, or by sequestering other NAC TFs with similar functions and thus inactivating them, or both. Also here the *nor* mutation in *NAC-NOR* can be considered as a gain-of-function or dominant-negative mutation, rather than a loss-of-function mutation. Dominant-negative transcription factor mutants are common in plants and can act through various mechanisms (Box 1).

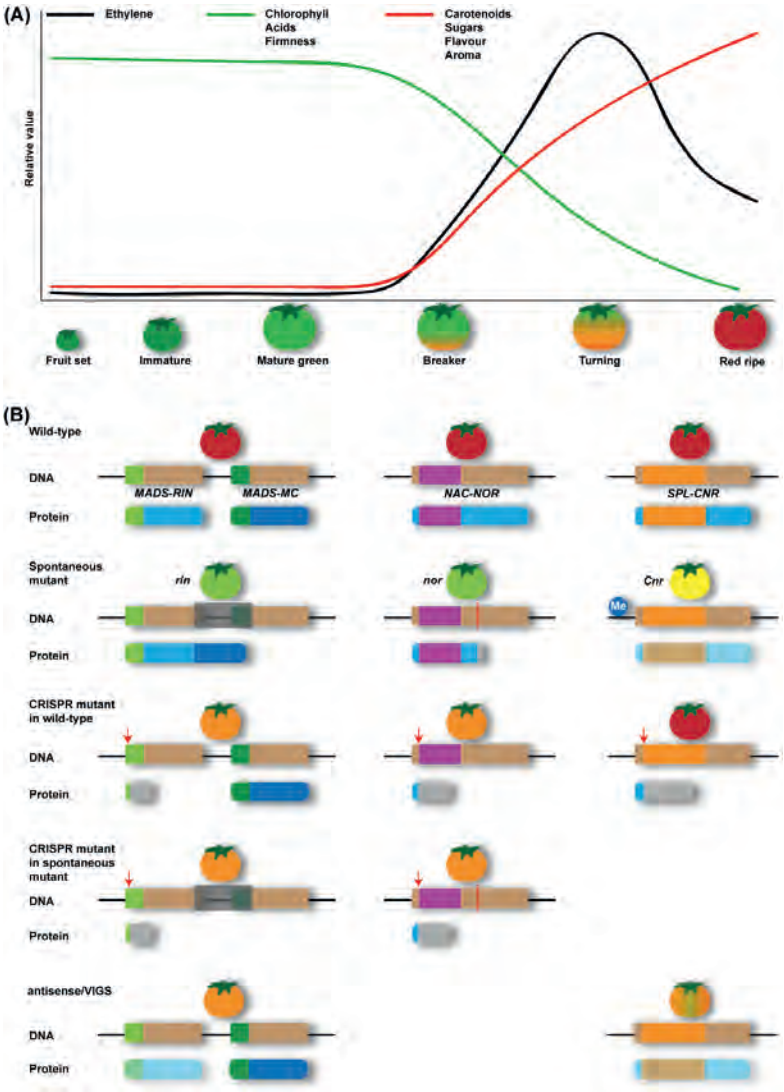

Figure 1. Tomato fruit ripening and the effects of mutations in three transcription factor genes. (**A**) Summary of the significant physiological and metabolic changes in normal fruit ripening. (**B**) Overview of various types of mutations in three transcription factor genes (left: *MADS-RIN*, centre: *NAC-NOR*, right: *SPL-CNR*) and their phenotypes. Each row depicts the phenotype at the ripening or comparable stage (top), the genes (with introns omitted) and resulting proteins. From the top downwards: wild-type, spontaneous mutants, CRISPR mutants in the wild-type background, CRISPR mutants in the spontaneous mutant background, and knock-down by VIGS or RNAi. Coloured boxes in both DNA and protein depict the location of the conserved DNA-binding (encoding-) domains (MADS-domain, NAC-domain, and SPB-domain, respectively). The dark grey box in *rin* DNA depicts the genomic deletion producing a chimaeric protein, the red vertical line in *nor* depicts a 2 nucleotide deletion causing a frameshift, and the circle with "Me" in *Cnr* depicts the upstream DNA-methylation causing down-regulation of expression, respectively. Red arrows indicate the relative positions of CRISPR mutations, causing a frameshift. Lighter-coloured proteins in *Cnr*, antisense suppression of MADS-RIN or VIGS of SPL-CNR indicate down-regulation of expression.

Third, a CRISPR mutation in the open reading frame of *SPL-CNR* also has only a very mild phenotype as compared to the pleiotropic *Cnr* mutation (Gao et al., 2019). It was claimed, though not shown, that *CNR* CRISPR lines merely show delayed ripening as opposed to partial non-ripening in *NOR* CRISPR lines. Moreover, many ripening-related genes that were differentially expressed in *Cnr* mutants were much less so, or even oppositely regulated in CNR CRISPR lines (Gao et al., 2019). These findings await a more elaborate analysis in order to be able to pinpoint the real function of the SPL-CNR protein in tomato fruit ripening, but also here it is now likely that this protein is also not the "master regulator" it was previously thought to be. There is strong evidence for the link between the epimark and the non-ripening phenotype based on positional cloning, *CMT3* silencing and *SPL-CNR* promoter methylation experiments (Kanazawa et al., 2011; Manning et al., 2006; Chen et al., 2015). However, the mechanism involved is unclear. In our opinion, this makes this particular mutant the most enigmatic of the three.

Use CRISPR mutants with caution

As argued above, CRISPR/Cas-mutagenesis is an excellent and precise tool for generating new alleles, including for genes in which spontaneous mutations were already available, to study the regulation of ripening in tomato. However, recent results suggest that creating such alleles can add another layer of complexity to this regulation. Studies, some so far exclusively in animals, but other in plants as well, suggest that CRISPR-mutant phenotypes may be obfuscated in various ways that need considering when designing experiments or drawing conclusions from results. Above all, it is essential to realize that it is tempting to promptly assume that, as for the three TF genes described here, the function of the locus is in the encoded protein. However, functional non-coding transcripts such as lncRNAs, antisense RNAs, as well as undetected **uORF**s may be not or much less affected by small indels generated by CRISPR mutagenesis. Even when the predicted protein is the functional product of a locus, deleterious mutations can be compensated for in various ways, which are shown in Figure 2.

1. **Indels** causing a frameshift in the first exon of a gene, or mutations that destroy the original start codon may cause translation from the next, alternative start codon.

2. A premature stop codon may be overridden by alternative splicing that skips the stop codon and produces a (partially) functional protein, a process called "nonsense-associated alternative splicing (**NAS**)".

3. In a process called "transcriptional adaptation" or "genetic compensation response" (GCR), nonsense-mediated decay (**NMD**) induced by the introduction of a premature termination codon (**PTC**) causes upregulation of transcription of one or more homologous genes with a similar function, which partially or entirely hides the phenotype.

Naturally, these phenomena can occur with any frameshift mutation, not necessarily just ones produced by CRISPR mutagenesis. However, in our opinion, the growing use of CRISPR mutagenesis should make us think carefully about our mutagenesis strategies before we start experimenting. Unlike in that other model, Arabidopsis, transformation and regeneration of tomato for CRISPR mutagenesis is still a labour-intensive and lengthy process.

How can CRISPR mutations obscure protein functions?

The alternative start codon use through leaky ribosome scanning or reinitiation after a stop codon certainly has examples in plants (Merchante et al., 2017), although it is too early to tell to what extent this occurs with premature termination codons (PTCs) produced by CRISPR mutations. It is estimated that up to 60% of all intron-containing plant genes display alternative splicing (Syed et al., 2012), so this is a process to reckon with. In mammals, it has been shown that CRISPR mutations can induce skipping of (part of) the exon containing the PTC, one of the modes of alternative splicing (Mou et al., 2017) and that it may partially restore protein function (Rodriguez-Rodriguez et al., 2018). It is not clear yet whether this phenomenon is common or limited to cases where a *null* mutation would be lethal, such as in the example of *Bub1* (Meraldi, 2019; Rodriguez-Rodriguez et al., 2018). Finally, "transcriptional adaptation" or "genetic compensation response" (**GCR**) are relatively new names for a phenomenon observed with mutations producing premature termination codons (PTC). Research into mechanisms causing this were stimulated by several observations of discrepancies between previous knock-down phenotypes and more recent CRISPR- (and other-) mutations thought to produce real *null* mutants. This phenomenon is distinctly different from 'classic' redundancy, where expression of another gene with the same function is sufficient or becomes high enough to be sufficient through the release of repression by the now mutated gene (see next sections and Figure 3). Studies in zebrafish *null* mutants showed that, *in contrast to knockdowns of the same gene*, mutant alleles were compensated by the upregulation of expression of homologous genes, apparently taking over its function through a yet unknown mechanism (Rossi et al., 2015; Zhu et al., 2017). Recently the mechanism of this compensation was shown to be dependent on the presence of a PTC in the mutant gene, as well as on components of the NMD pathway, and on components of the COMPASS (complex of proteins associated with Set1) complex. According to the model, PTC-containing mRNA recruits not only the NMD components but also the COMPASS components and then guide it to implement expression-permissive **H3K4-trimethylation** at Transcription Start Sites of the homologous genes, whose expression is subsequently upregulated (Ma et al., 2019; El-Brolosy et al., 2019). The COMPASS components are conserved in plants, and in Arabidopsis were shown to mediate H3K4-trimethylation and activate gene expression as well (Jiang et al., 2011). How exactly sequence homology directs the choice of genes to be upregulated, and how much homology is required for that, is not clear yet. While this particular compensatory mechanism as such has not yet been demonstrated to occur in plants, a recent example in tomato suggests

that we may just have seen the tip of the iceberg. CRISPR mutations in tomato CLV3, encoding a stem cell-regulating peptide, resulted in simultaneous upregulation of expression of *CLV3*, as well as of its closest paralog *CLV9*. However, *clv9* mutations had little effect on the phenotype, but *clv3 clv9* double mutants had an enhanced phenotype compared to *clv3* single mutants, indicating that increased *CLV9* expression partially compensates for *clv3* loss in the latter (Rodriguez-Leal et al., 2019).

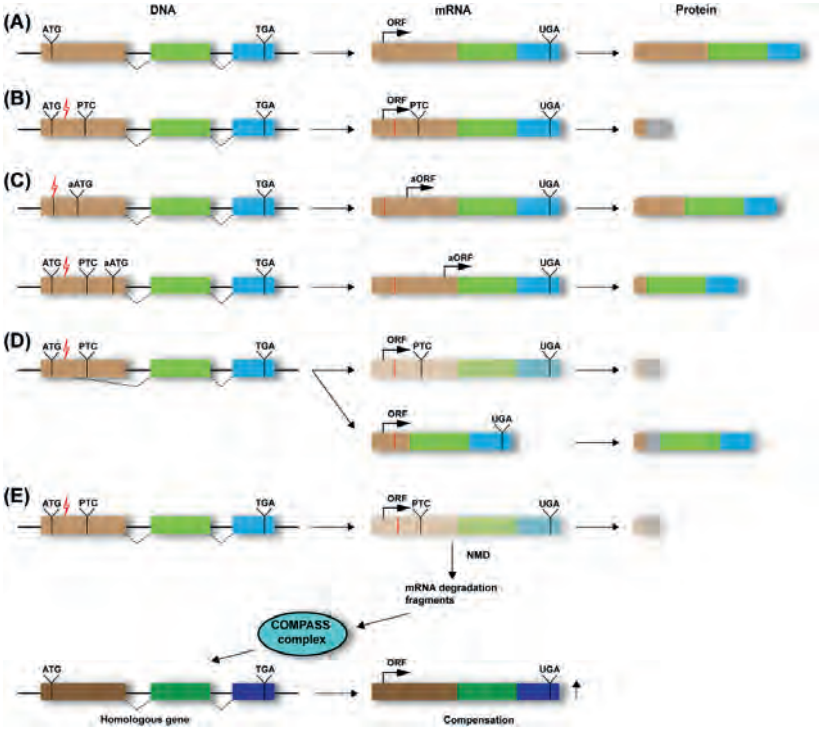

Figure 2. Models of compensation mechanisms that may occur in CRISPR mutants. All show gene models on the left with exon/intron-structure and the position of the start codon (ATG), stop codon (TGA), PTC, and alternative start codons (aATG). Lightning bolts mark the position of mutations causing a frame-shift. Mature mRNA structure with the positions of the start of the open reading frame (ORF), PTC, and a termination codon (UGA) is depicted in the centre. Hypothetical protein products, with truncations where applicable, are shown on the right. (**A**) Wild-type. A full-length protein is produced. (**B**) A knockout mutation is causing a frameshift with associated PTC, producing a non-functional truncated protein. (**C**) Translation from an alternative start codon when either the original start codon is removed (top) or an alternative ATG downstream of a PTC is used for translation. In both cases, an N-terminally truncated protein is produced, which may be functional. (**D**) NAS: a mutation producing a PTC when normally spliced, would produce a truncated protein, but an alternative splicing event that results in the exclusion of the PTC from the mature mRNA may result in a truncated, yet functional protein. (**E**) GCR: a mutation causing a frameshift with an associated PTC causes NMD of the mRNA, resulting in little if any (truncated) protein. mRNA fragments resulting from NMD are picked up by components of the COMPASS complex and transported to the nucleus where they activate expression of homologous genes, which can compensate for the loss of function of the mutated gene.

Lessons for CRISPR mutagenesis design

Ironically, none of the above pitfalls of CRISPR mutagenesis of open reading frames for gene function studies seem to apply to knockdown of gene expression by RNAi or VIGS. Without, or with a diminished amount of normal mRNA, there is no alternative start codon available, alternative splice forms are equally degraded, and there is no PTC to induce transcriptional adaptation or genetic compensation response that depends on NMD. Thus it would seem that even when CRISPR mutants helped to expose spontaneous mutants as gain-of-function mutants, we may be introducing new problems for functional genomics with CRISPR/Cas that in our opinion, require attention. The way forward, without losing the advantages of the ease and precision of this technology, may be to apply it in a way that reduces the chances of producing aberrant transcripts or inducing NMD of the gene that one would like to knock out. This could be achieved by deleting an entire transcript, or by focusing on promoter deletions that severely reduce or block the expression of a transcript. Alternatively, instead of aiming at INDELs resulting in PTCs (nonsense mutations), one might aim at producing deleterious missense mutations by **base-editing**, or screen for those in mutant populations by **TILLING**. The latter alternatives obviously (so far) lack the predictability that CRISPR-mutagenesis usually has: one would have to know or predict which mutations will be deleterious and in the case of TILLING, the mutations have to be present in the population.

From “Master regulators” to a model of redundancy in a network

Transcriptional regulation of tomato fruit ripening seems to be more robust than previously imagined as it is only partially inhibited by knocking out any of the transcription factors known to be involved so far. The latter include the TFs that were once thought to be “master regulators” because their spontaneous mutations completely inhibited ripening. The most likely explanations for this are functional redundancy or additivity when more genes contribute to a trait quantitatively (Figure 3). Redundancy and quantitative interaction would be most likely occurring among members of the same transcription factor family as they are more likely to share binding sites in target gene promoters, as well as share protein interaction partners and transcription regulatory domains. For NAC-NOR, likely candidates have been identified, as mutations in *NOR-like 1,* VIGS of *NAC9* or *NAC4*, and RNAi of *NAC4* delay or cause incomplete fruit ripening (Gao et al., 2018; Zhu et al., 2014; Kou et al., 2016). MADS-domain TFs throughout the plant world show redundancy in function, particularly among more recently diverged paralogs (Moore and Purugganan, 2005). Partial redundancy of tomato FUL1 and FUL2 in ripening is one such example (Wang et al., 2019). RIN is a member of the so-called SEPALLATA (SEP) subclade of MADS-domain TFs, a group that is believed to be part of many functional heteromeric MADS-domain protein complexes (Immink et al., 2009). Looking in this way at protein interactions, other SEP-clade TFs than RIN, which show similar interactions with other MADS-domain proteins involved in ripening (FUL1, FUL2, TAGL1), would be apparent candidates (Leseberg et al., 2008). Alternatively,

other complexes that are independent of RIN function may perform some of the same functions. Possible scenarios are elaborated elsewhere (Li et al., 2019). Work on a wide range of species indicates that orthologs of *RIN* and *NOR* along with a significant number of other transcription factors play an important role in the control of ripening in fleshy fruits (Li et al., 2019; Lü et al., 2018)). The role of *SBP/SPL* genes other than *SPL-CNR* in ripening remains relatively unexplored.

Besides transcriptional regulation by TFs, many other regulatory layers are likely to be involved in fruit ripening, and their role has been established to varying degrees (box 2).

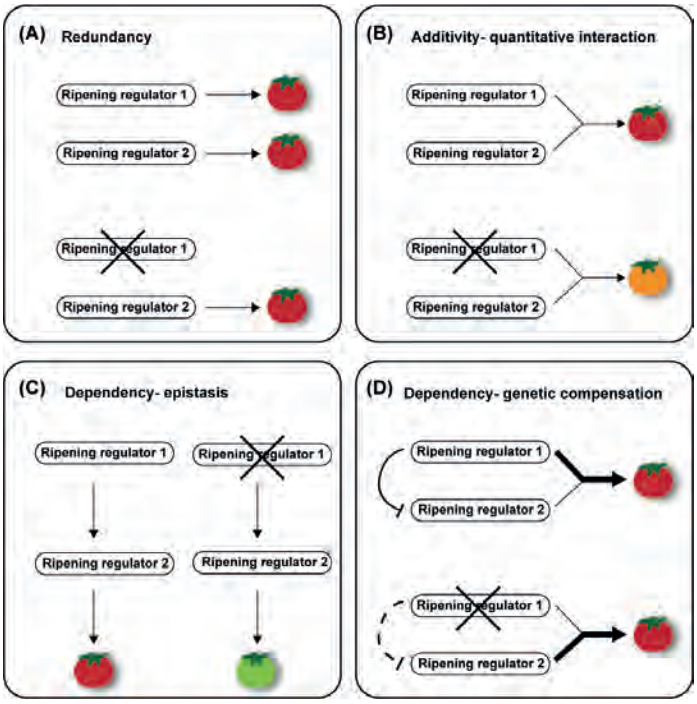

Figure 3. Models of genetic interactions of transcription factors in tomato ripening. (**A**) Redundancy. Two or more regulatory transcription factors may have identical functions. Knocking out one leaves the other(s) to allow fruit ripening to progress without a visible phenotype. Partial redundancy would allow some, but not all, of the ripening processes to proceed as in wild-type. (**B**) Additivity or quantitative interactions. Two or more ripening regulators contribute to the same processes in normal ripening in an additive manner. A knockout of one of them cannot be adequately compensated by the other, so a more or less severe phenotype occurs without complete inhibition of ripening. (**C**) Dependency with epistasis. Two or more regulators act in the same regulatory pathway, with one being dependent on the activity of the other. Knockout of either causes a ripening phenotype. A severe phenotype as a result of knockout of the top regulator may obscure a milder phenotype of a knockout mutation in the dependent one (epistasis). (**D**) Dependency with genetic compensation. Two regulators can regulate the same processes in ripening, but the expression of one is suppressed by the other (top). A mutation in the suppressing regulator will release the expression of the other, which takes over the function with no resulting phenotype.

Concluding remarks and future perspectives

Until relatively recent, studies on the molecular control of ripening have been limited to a few genes encoding specific enzymes or transcription factors, and in the latter case, these have often been the result of identification of genes underlying non-ripening mutations. The advent of next-generation sequencing has revealed that thousands of genes and hundreds of transcription factors show altered expression in the ripening of fleshy fruit species. Genome sequences have enabled ripening traits to be linked directly with genetic loci. These data indicate that ripening is much more complex than previously imagined, but the overall control mechanisms are still a matter of debate and especially with new information from CRISPR mutations that have recently been published.

The lessons from mutant studies described here suggest that there are more dominant-negative or gain-of-function mutations in tomato fruit ripening (by comparison to true null alleles) than had been expected and this information opens the way to an improved understanding of its transcriptional regulation. Producing multiple alleles of ripening-related TF genes with CRISPR/Cas provides a tractable and rapid way forward.

At the same time, these results suggest that the genes above act not as a "master regulators" in the pure sense of a few major genes controlling the ripening process, but each as part of a group (two or more) of redundantly acting homologous genes. This should be studied by assessing the effect of combined mutations. This approach is substantially easier nowadays, with the availability of (multiplexed) CRISPR/Cas-mutagenesis (see Outstanding Questions).

Outstanding Questions

- There are still important unanswered questions about the *Cnr* mutant. *Cnr* hypermethylation >2kb upstream of the coding sequence is correlated with reduced mRNA levels. Yet, a CRISPR-generated *null* mutation of *SPL-CNR* has a very mild phenotype. Thus either reduced expression is not the cause of the *Cnr* phenotype, or some other mechanism reduces the effects of the knockout mutation.

- Our understanding of many developmental processes, including fruit ripening, is based on spontaneous or (chemically) induced mutants, assuming that they were loss-of-function mutants. It is the question to what extent these developmental processes and the function of regulators should be revisited with the current knowledge about master regulators involved in fruit ripening.

- CRISPR/Cas mutants containing Indels in the ORF causing a premature termination codon are considered as real knock-out mutants. However, novel results indicate that this is not the case

for all CRISPR-derived mutants, where alternative mechanisms compensate for the loss-of-function of the ORF- encoded protein. How frequent this occurs and which mechanisms are involved needs further attention.

Box 1. Dominant-negative transcription factors

A dominant-negative mutation is generally defined as a mutation whose protein product can interfere with the function of the natural product in the same cell (Herskowitz, 1987). This can occur with proteins that usually function as homodimers, where the presence of one or two mutant copies would "poison" the complex to render it inactive. This so-called "intralocus" interaction predicts that in the heterozygous state, only 25% active complex is formed, often not enough for a wild-type phenotype (Figure IA, B). It is now also accepted that "interlocus" or trans-interactions exist, where the interaction of the dominant-negative protein with products of other loci can result in a strong phenotypic effect in the homozygous state, but intermediary effects in the heterozygous state (Veitia, 2007, 2009). Both *rin* and *nor* mutations have a strong inhibitory effect on ripening in the homozygous state, but a milder yet distinct effect in the heterozygous state, which is utilized in breeding to extend fruit shelf life (Garg et al., 2008), and therefore may qualify as trans-acting dominant-negative mutations, as was proposed earlier for *nor* (Wang et al., 2019). Figure I. depicts some of the possible ways in which a dominant-negative transcription factor may affect target gene expression directly or through interference with the function of other TFs with which it interacts or competes with the same DNA binding site.

Dominant-negative alleles are useful for studying gene functions in situations where multiple paralogs display redundancy in function, requiring the knockout of several genes before a phenotype becomes apparent. For this purpose, dominant-negative alleles can be produced artificially by fusion of a TF with a universal repressor domain (Hiratsu et al., 2003; Chandler and Werr, 2003). Alternatively, with the availability of CRISPR/Cas-mutagenesis, it now becomes possible to design and test dominant-negative versions of TFs in a rational way, guided by the example of the *nor* mutation.

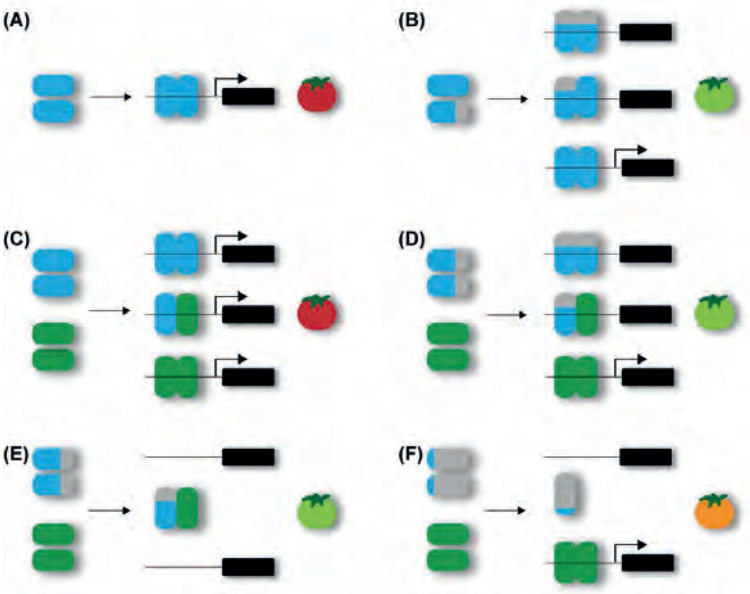

Figure I (box 1). Simplified models for dominant-negative effects of a dimeric TF. (**A**) wild type, where a single TF acts as an activator. (**B**) Intralocus dominant-negative. The homodimer of the truncated protein has lost its activation. The dimer of mutant and wild type protein is inactive, and the residual 25% wild-type dimer has insufficient activity. (**C**) Two TF's regulate the same target gene in an additive manner, as in Figure 2B, by forming obligate or facultative heterodimers. (**D**) Interlocus dominant-negative. A homozygous mutation in one of the two partners renders 75% of the dimers inactive (or 100% in obligate heterodimers), insufficient for a response. (**E**) as in (**D**) but instead of binding to the target gene, the mutant protein sequesters interaction partners. (**F**) In contrast, a knockout mutation produces no protein that can interact or bind its target. In the case of additivity, this produces an intermediate phenotype.

Box 2. More regulatory layers for fruit ripening

Besides the transcriptional regulation and hormonal control, other molecules and mechanisms are involved in fruit ripening. The most substantial evidence available is for DNA methylation and demethylation in epigenetic regulation of *CNR* and the DNA demethylase *DML2* (Manning et al., 2006; Zhong et al., 2013; Liu et al., 2015b). However, the control of ripening involves many additional layers of regulation and these likely include RNA regulatory networks. In 2012 the international ENCODE project established that in humans 98% of RNA transcripts are not translated into proteins and there is growing evidence that the vast non-coding portions of eukaryotic genomes are critical in the regulation of development (Vandevenne et al., 2019).

The best-studied non-coding RNAs in plants are those that are processed into microRNAs of 21 to 22 nucleotides, which recognise and bind to mRNAs of members of specific gene families, in which TF genes are overrepresented (Fahlgren et al., 2007). mRNAs of tomato TFs AP2a and CNR, both upregulated during and (putatively) involved in the regulation of ripening, are targets of miR172 and miR156 or 157, respectively, and actively cleaved *in vivo* (Karlova et al., 2013). However, the role and importance of this regulatory interaction in ripening remain to be established. Regulatory RNAs also include long non-coding RNAs (lncRNAs) from intergenic sequences, those from intronic sequences and natural antisense transcripts (Chekanova, 2015). LncRNAs may play roles in determining colour development and other aspects of ripening in fruit species (Bai et al., 2019; Zuo et al., 2019). Important functional roles in plants and animals have also been attributed to circular RNAs (Chu et al., 2018).

The role of the epitranscriptome (RNA modifications) is yet to be explored with respect to ripening as well as the role of histone modifications. H3K27me3 histone methylation is associated with gene silencing and has been linked to the control of gene expression during ripening (Lü et al., 2018). New models of the regulation of ripening can now build on our understanding based on ripening mutants and CRISPR knockouts, but will need to consider all of these various layers of control, and we are only just beginning to grasp the level of complexity that is involved.

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Glossary

Antisense suppression: expression of (part of) an mRNA in reverse orientation in a transgenic plant can cause antisense RNA (asRNA) to hybridise with its corresponding sense mRNA, which is processed to short interfering siRNAs that recognises and cleaves the original mRNA.

Autogamy: the phenomenon of self-fertilisation, in general fusion of gametes from the same individual and more, precisely, from the same flower.

Base-editing: conversion of single base pairs to another (for example A-T to C-G) by combining enzymes that modify DNA with a Cas9-mutant that causes no double-strand breaks, but is still able to guide the enzyme to the correct position base on an RNA guide.

Climacteric: a stage in fruit ripening associated with rising respiration and increased ethylene synthesis.

Frugivores: Fruit (and nuts or seeds) -consuming animals.

GCR: genetic compensation response. Changes in the expression of a gene(s), which can compensate for the loss of function of another gene.

H3K4-trimethylation: an epigenetic modification involved in regulation of gene expression, in this case, trimethylation of lysine 4 of histone H3 protein, associated with transcription activation

Indel: insertion or deletion of bases in a genome.

MADS-domain: The common DNA-binding domain of 55-60 amino acids in transcription factors encoded by the MADS-box gene family. MADS is an acronym made out of the first four reported genes: MCM1, AGAMOUS, DEFICIENS, and SRF.

NAC: a family of plant-specific transcription factors containing a conserved 160 amino acid DNA-binding and dimerisation domain. NAC is an acronym of the NAM, ATAF1/2, and CUC2 TF names.

NAS: nonsense-associated alternative splicing, a putative corrective response that increases the production of alternative transcripts, which have skipped a premature PTC.

NMD: nonsense-mediated decay. Coupled with the translation of the mRNA, this mechanism eliminates mRNAs containing PTCs.

Pericarp: the outer layer of a fleshy fruit, like tomato, which is usually -but not always- the edible part and is botanically derived from the ovary wall.

PTC: premature termination codon. A stop codon (UAA, UAG, or UGA) in the messenger 5’ of the original termination codon, which becomes functional after a mutation causes a frameshift in the open reading frame.

SPB-box: Squamosa-promoter binding-domain encoding plant-specific TF gene family, with a conserved, approximately 80 amino acid DNA-binding domain.

SPL: SQUAMOSA Promoter Binding Protein-like. An alternative name for SPB TFs.

TILLING: Targeting Induced Local Lesions in Genomes. A method to detect or identify induced mutations in specific genes or genomic regions in individuals of a mutant population.

uORF: upstream open reading frame an ORF within the 5' untranslated region of an mRNA.

VIGS: Virus-Induced Gene Silencing. A specific application of RNA interference, like antisense suppression, where the expression of an antisense RNA from a plant virus vector after transfection of a plant, rather than stable transformation, is used to inhibit gene expression.

Chapter 7

General Discussion and Perspective

Plant developmental processes are controlled by expression changes of genes that are regulated by upstream transcription factors (TFs). Fruit ripening is a complex process involving biochemical and physiological changes, which eventually result in changes in colour, flavour, and texture. In tomato, TFs MADS-RIN, NAC-NOR, and SPL-CNR, which were discovered through spontaneous mutations (*rin*, *nor*, and *Cnr*, respectively) and then were mapped to their respective genomic locations, are reported as major regulators of ripening based on the phenotype of their spontaneous mutations. Additional components of the controlling network include AP2a, FUL1/2, TAGL1, and LeHB-1 (Karlova et al., 2014). Most of these TFs directly regulate genes in different biosynthesis and signalling pathways as activators or repressors. However, the connections between components of this highly interlinked regulatory network and its downstream effectors modulating different aspects of ripening are still relatively poorly understood.

Besides transcriptional regulation, researchers have found other layers of regulation, such as the strong inhibiting effect on ripening by high global DNA methylation (Zhong et al., 2013) or the regulation by miRNAs, adding more complexity to fruit ripening regulation. Recently, other newly discovered types of regulation, like m^6A RNA methylation, are also part of the whole ripening regulation. The continuous new discoveries make it more complex, but at the same time, make us understand the ripening regulation better and deeper.

Severe versus mild ripening defects in spontaneous and KO mutants

Naturally occurring mutants are often discovered through their distinctive phenotypes. Likewise, in tomato, spontaneous fruit-ripening mutants with defects in colour, fruit texture, and ripening were reported and named according to their severe and distinctive ripening phenotype. After fine mapping by forward genetics, the physical location in the genome and the gene harbouring the causal mutation were discovered. Among the many spontaneous fruit mutants in tomato, *rin*, *nor* and *Cnr* are the best studied, and the *rin* and *nor* alleles are also applied in tomato breeding to improve fruit shelf life. These mutants have strong non-ripening defects with green or yellow fruits and undetectable ethylene production, hence the mutated genes have been documented as activators or even master regulators of fruit ripening.

The use of spontaneous mutations for gene function evaluation had a long and useful history until the gene-editing revolution came along, which allowed us to obtain, in a relatively simple way, (presumably) complete KO mutants. However, this also brought unexpected results. The KO mutants of *MADS-RIN*, *NAC-NOR,* and *SPL-CNR* showed (much) weaker ripening defects than their respective spontaneous mutants with orange or red fruits as shown in **Chapters 3** and **4,** for *NAC-NOR* and *SPL-CNR*, respectively. These results made us realize that the earlier function annotation for these genes was incomplete, if not to say misleading. Therefore, in **Chapter 3**, in addition to only knocking out the *NAC-NOR* in the wild-type background, we also knocked out the *nor-s* allele in the mutant background. Mutants from both experiments showed the same orange-ripe fruits, which proves that the severe non-

ripening phenotype in *nor* is caused by the protein coded by the *nor-s* allele, not the loss-of-function of *NAC-NOR*. The 2 nt deletion in *nor* results in a truncated protein NOR-S that still harbours the NAC domain, probably allowing it to bind to the same promoters of effector genes or to bind and sequester interaction partners forming an inactive transcription factor complex or both, and thus negatively regulates ripening. This suggests that *nor-s* is a so-called *trans*-acting dominant-negative allele having *inter*locus interactions, rather than the classical *intra*locus interaction of *cis*-dominant negative mutations (Veitia, 2007). Besides, the *nor-s*' negative effect is dose-dependent and in the heterozygous state it is only co-dominant.

Similar mild ripening changes were also observed in the KO mutants of *MADS-RIN* (Ito et al., 2017) and *SPL-CNR* (Gao et al., 2019) (also in **Chapter 4**). In the *rin* mutant, a chimeric protein, RIN-MC, is produced due to a deletion between *MADS-RIN* and another MADS-domain TF gene *MACROCALYX* (*MC*), which results in a new fusion protein being produced containing the first part of the MADS-RIN protein followed by a C-terminal sequence from MADS-MC (Ito et al., 2017). The new protein RIN-MC functions as a repressor for ripening since the inhibition disappeared after *RIN-MC* was knocked out by CRISPR mutagenesis (Ito et al., 2017) or silenced by RNAi (Li et al., 2018), and the lack of MADS-RIN only causes orange fruits. Both we (**Chapter 4**) and Gao *et al*. (Gao et al., 2019) created CRISPR-generated KO mutants of *SPL-CNR* and we showed that these, although with delay, eventually achieved full ripening (at least visually), suggesting that *SPL-CNR* is not essential for ripening. We observed a slower ripening, but not a delay in its initiation, while Gao *et al*. reported a delayed ripening without further qualification (Gao et al., 2019). The epi-allele *Cnr* results in 10~ 20% expression of *SPL-CNR* compared to wild-type fruits, but blocks ripening almost entirely (with the exception of chlorophyll degradation). It was long assumed by many that this phenotype reflected the decreased expression of the transcription factor because the region (presumed promoter) that was hypermethylated in *Cnr* was apparently required in wild-type fruits for proper expression of the gene. In such a scenario, cytosine methylation in *cis*-elements in that region might interfere with the binding of regulatory TFs that are required for *SPL-CNR* expression. However, in our study, we have shown by deletions that this area is not required for normal expression, although we cannot exclude an inhibitory *cis*-regulatory effect specifically for the hypermethylated version of the region. However, even further decreasing expression in the other promoter deletion mutants of our study clearly show that down-regulating *SPL-CNR*, as for knocking out the gene, is in itself not enough to result in the *Cnr* phenotype. Thus, and further underscored by its pleiotropic effects (**Chapter 2**), it is more likely that *Cnr* is also a gain-of-function mutant whose phenotype does not reflect the function of the SPL-CNR protein. Unlike for the examples of *nor* and *rin*, this cannot be simply proven by knocking-out the dominant-negative protein product of the mutant: there is none in *Cnr*. Understanding the mechanism behind *Cnr*'s phenotype is therefore a challenge for the future. One classical approach is to look for and characterize suppressor mutants of *Cnr* and identify the underlying gene(s).

These studies suggest that these three genes act not as "master regulators" controlling the ripening process, but each as part of a group (two or more) acting redundantly. NOR-like1, another NAC transcription factor, is also involved in fruit ripening (Gao et al., 2018). The ripening in *NOR-like1* KO mutants was partially suppressed, which is similar to what we observed when knocking out *NAC-NOR* (Wang et al., 2019). In addition, both the suppression of *SlNAC1* and the silencing of *SlNAC4* delayed fruit ripening (Meng et al., 2016; Zhu et al., 2014). So the partially affected ripening may be caused by the redundancy of NAC-NOR, NOR-like1, and other NAC TFs for the same function. Similarly, other fruit-expressed MADS-domain TFs such as LeMADS1 and TAGL2, both members of the same SEPALLATA subclade may be functionally redundant with MADS-RIN. This points the way to future experiments using combinations of multiple CRISPR mutations to further identify (partially) redundant groups of TFs and their relative contributions.

The phenotype of spontaneous mutant alleles does not necessarily reflect the normal function of the TF

We evaluated the interactions between *rin*, *nor* and *Cnr* with different and combined mutant allele doses in **Chapter 2** and found that *rin* and *nor* alleles work additively to negatively regulate fruit ripening, and *Cnr* is epistatic to the other two when present. All three mutations act as inhibitors for ripening. Given the results in **Chapters 3, 4** and **5**, and studies from others, as well as the unique character of the spontaneous mutants, we suggest to carefully evaluate the function of spontaneous mutant alleles and the function of the genes. Due to the strong defects of the natural mutants, the corresponding TF genes cannot be simply placed in a gene regulatory network model based on genome-wide expression pattern changes in these mutants. It also taught us that we should be careful to not too quickly assume that the phenotype of any natural mutation completely reflects the function of the underlying gene and makes one wonder how common this phenomenon is. It is obviously not limited to TF genes, and one does not have to look further than the tomato fruit for examples. The spontaneous mutation *Never-ripe* is an example of a dominant-negative mutation of an ethylene receptor that blocks ethylene perception and ripening (Lanahan et al., 1994).

Alternative hypotheses for mechanisms causing mild phenotypes in KO mutants

With more studies reporting that KO mutants obtained through CRISPR/Cas-mutagenesis have weaker phenotypes than those from knockdowns such as by RNAi or VIGS, researchers started to investigate the likely mechanisms to explain that. We reviewed three possible mechanisms in **Chapter 6**, comprising two where still functional truncated proteins are produced and one involving genetic compensation.

To completely knock out the function of a gene we initially designed guide RNAs (gRNAs) targeting at the 5' end, usually in the first exon of the coding sequence (CDS). Small insertions and deletions (InDels)

introduced by non-homologous end joining (NHEJ) cause frameshifts in the first exon of a gene or mutations that destroy the original start codon. Potentially, alternative translation starts can be used from the next start codon in the original reading frame or the premature termination codon (PTC) can be overridden by alternative splicing, both of which could produce a (partially) functional protein. The third one is called "transcriptional adaptation" or "genetic compensation response" (GCR), in which a PTC causes upregulation of transcription of one or more homologous genes with a similar function, which partially or entirely hides the phenotype (Zhu et al., 2017; Rossi et al., 2015).

Among the alternatives, genetic compensation is considered to happen more frequently and recently a similar genetic compensation was reported in tomato, although there the mechanism has not been elucidated yet (Rodriguez-Leal et al., 2019). Since these observations are rather new and have only few examples, it remains to be determined how common such manners of compensation of KO mutants are in plants. In **Chapter 4,** we examined the expression of *SPL3*, the closest homolog of *SPL-CNR*, in the *cnr* KO mutants which had much milder ripening defects than *Cnr*, but did not observe its upregulation. The defects could still be buffered by compensation from other homologous genes. In another study where Gao *et al*. also presented *cnr* KO mutants with an RNA-seq experiment (Gao et al., 2019), we did not find in their data that other *SPL* genes were strongly upregulated, contrary to the more than a hundred times increase in another study (Rodriguez-Leal et al., 2019). On the other hand, there may be other mechanisms for compensation that could be discovered and reported in the future.

Use CRISPR/Cas with caution and choose an appropriate strategy

As discussed above, there have been several mechanisms found to buffer the negative effects of knocking out a gene, which remind us to think of better or more appropriate strategies for CRISPR/Cas-mutagenesis in our studies. Firstly, considering the alternative start codons and alternative splicing of the PTCs that result in truncated, yet functional proteins, or mutations located after the binding domain of a TF (or after any functional domain, for that matter) resulting in a dominant-negative protein, we suggest and have already started to use multiple gRNAs to delete the whole transcript to avoid such effects. Deletions between gRNAs have been created numerous times in our experiments and they can be very large (> 1.6 kb, **Chapter 4**), making the deletion of the entire coding sequence feasible. Secondly, it was shown in **Chapter 4** that we were able to suppress gene expression to a very low level by deleting (parts of) the promoter, which is another way to bypass genetic compensation as no PTCs will occur. Deleting a promoter causes a similar knock-down effect as RNAi, but more precisely and efficiently. This knocking-down is stably heritable among generations once the homozygous mutant is selected in the T_1 progeny and does not require extra checks for down-regulation. In addition, as there are no PTCs caused by frameshift, in theory, there should be no other genes to compensate and affect the phenotype, although a negative transcriptional feedback regulation on homologous genes remains possible. Thirdly, taking the redundancy of homologous genes into account we suggested to knock out two or more

paralogs with suspected redundancy at the same time to assess the effect of combined mutations. In **Chapter 5**, we chose the microRNA encoding genes with the highest expression for miR156/7 and miR172 to knock out, but it turned out that the level of the mature microRNAs was not affected. This could be caused by the compensation from other precursor genes as there are multiple (five for miR156/7 and three for miR172). So last but not least, we advise to knock out genes encoding the same components simultaneously to avoid another type of redundancy or "gene compensation".

Experiments aiming at creating multiple deletions in the same region, such as mutating CREs in a gene promoter, require many gRNAs. In **Chapter 4**, we tried to use many (twelve) gRNA modules in the same construct for editing. It turned out that more gRNAs increase the chance of causing larger deletions between targets at the distal ends of the targeted region (and thereby deleting all targets in between), rather than single InDels or a collection of small guide-to-guide deletions as originally was the goal. For this aim, we now suggest the following two alternative approaches instead. The first one is to put one gRNA gene per construct and use mixed *Agrobacterium* cultures for plant transformation as previously demonstrated (Jacobs et al., 2017). With all gRNAs targeting the same promoter and under the right conditions, there will be cells undergoing transformation with different combinations of two or more gRNA modules, allowing distinct guide-guide deletions in each such cells. The other approach is to put only a few, for instance, four gRNA modules in a construct for targeting a more distinct region of the promoter and use these for separate transformations. This will result in mutants with smaller deletions in only the targeted region, which makes the study of their function more precise, and still allows for producing larger deletions by crossing plants with mutations in different fragments.

Importance of spontaneous alleles and CRISPR/Cas-mutagenesis in tomato breeding and some concerns

Tomato breeding is a huge industry nowadays with more than 50 billion US dollars business (Vincent et al., 2013). Breeders perform many crosses to breed new cultivars with desired traits like higher yields, better resistance to biotic and abiotic stresses, or to meet various preferences from customers, like the sweeter cherry tomatoes. To identify the genetic variation for these desired traits spontaneous mutants or wild relatives of tomato can be used. To introduce these desired traits in their breeding material, breeders perform multiple backcrossings to create introgression lines, which is quite time and labour consuming. Though natural alleles are valuable genetic resources for breeding, now with the various new demands from customers and society (sustainability), breeders are facing the challenge to speed-up their breeding program. Only using the established strategies may not be sufficient. Among the modern techniques, CRISPR/Cas-mutagenesis is definitely one of the most powerful tools that could accelerate breeding. For instance, CRISPR-induced mutations of *ZmDMP* enhance doubled-haploid induction in maize, which shortens the time to obtain homozygosity in parent breeding lines from six or seven generations to only two, largely decreasing the breeding time (Zhong et al., 2019). CRISPR-induced

mutations that increase the frequency of meiotic crossovers may also contribute to faster breeding (de Maagd et al., 2019). However, the most straightforward way is to introduce an advantageous fruit trait directly by CRISPR mutagenesis, as exemplified by studies that mimic, in an extremely fast pace, domestication mutations or variants (Soyk et al., 2017; Rodríguez-Leal et al., 2017).

In some countries, like the USA and Japan, plants obtained from CRISPR/Cas-mutagenesis after segregating out the Cas9 transgene (null segregants) are considered and regulated as NON-GMO. For instance, the gene-edited mushrooms obtained through CRISPR/Cas-mutagenesis targeting one of the six genes encoding polyphenol oxidase, an enzyme that causes browning, were approved by the US Department of Agriculture (USDA) and will not be regulated as GMO, because they do not contain foreign DNA anymore. However, in the European Union, unfortunately, they are still regulated as GMOs. The Court of Justice of the European Union ruled in 2018 that all plants obtained by using CRISPR/Cas-mutagenesis should be considered and regulated as GMO, in contrast to exempted techniques such as induced mutagenesis, because the safety of the new mutagenesis technique has not yet been established (Gelinsky and Hilbeck, 2018). This causes concerns among breeding companies and researchers and some companies have moved their research activities outside Europe, which will affect the progress of innovation in Europe.

Conclusion and perspective

Fruit ripening regulation at the molecular level has been studied for a long time and some major regulatory genes were identified by classical forward and reverse genetics methods. With the advances in genome sequencing techniques and various experimental and bioinformatic tools to study gene function and regulation, recent studies have increased our knowledge about fruit ripening substantially. Now researches have been accelerated even more by CRISPR/Cas-mutagenesis. Some spontaneous ripening mutants had been studied extensively, while only recently their nature has been elucidated. Despite that the nature of these mutants is different from what we thought, they are still valuable genetic resources for tomato breeding. The interactions of three spontaneous alleles, the role of multiple TFs, CREs in gene promoters and microRNAs in ripening are evaluated in this thesis, taking us a step further in understanding the entire regulatory network. However, the regulation of ripening, consisting of many factors and different layers of regulation at genetic and epigenetic, transcriptional and post-transcriptional, and *trans* and *cis* levels, is still very complex. This thesis opens new avenues for ripening regulation studies, but also illustrates that a complete understanding of this regulation still requires a lot of work and remains a challenge.

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Summary

Tomato is one of the top consumed vegetables and has a crucial role in the human diet and food industry. Additionally, its value as an excellent model for fleshy fruits makes tomato fruit ripening studies important. In tomato, ripening is a complex process regulated by genetic and epigenetic components together with plant hormones. So far mostly one-to-one regulatory relationships between regulators and effectors were explored, but the true function of some major regulators is still not clear, and so are the interactions of their spontaneous mutant alleles in ripening regulation.

The aim of this thesis is to study and extend our knowledge of tomato fruit ripening regulation at the molecular level. I focussed on evaluating the true function of the "master regulators" (major TFs), the interaction of their spontaneous mutant alleles and the effects of non-coding elements. To achieve that, I used CRISPR/Cas9-mutagenesis to obtain real knock-out mutants as well as crossings to produce genotypes with different and combined doses of spontaneous mutant alleles, followed by phenotyping and gene expression analysis to evaluate their functions in ripening.

Chapter 2 focuses on the interactions between the spontaneous mutant alleles *rin*, *nor* and *Cnr* in the ripening regulatory network. Instead of using only homozygous mutants that usually completely block ripening, we investigated the interactions through the introduction of different regulator levels by using heterozygous lines in an identical genetic background (cv. Ailsa Craig). The heterozygous single and double mutants of *rin*, *nor* and *Cnr* were obtained by crossing them with wild-type or by crossing them with each other. We evaluated several phenotypic and metabolic effects in these genotypes, showing how these mutations interact at the level of visible phenotype, effector gene expression, and sensory and quality aspects, in a dose-dependent manner. *Rin* and *nor* had similar quantitative effects on all investigated aspects of ripening, and the effects of combined allele doses were additive. We also found that *Cnr* is epistatic to *rin* and *nor* as their respective phenotypes are obscured when *Cnr* is present. Finally, we found that in contrast to the well-known dominant effect on ripening, the *Cnr* allele is incompletely dominant, or recessive in fruit size reduction (a phenotype first described here) and in altered fruit volatile production.

The reported characterization of the *rin* mutation as a gain-of-function mutant, due to the production of a novel chimeric protein, reminds us that the function of the established "master regulators" maybe not always be as what was assumed and that their function might have to be re-evaluated. Thus, in **Chapter 3** we re-visited the functions of TFs AP2a, NOR and FUL1/2 with CRISPR/Cas9-mutagenesis and reported the phenotypes of their KO mutants for the first time. The earlier ripening, orange-ripe phenotype with higher ethylene production of earlier RNAi plants were confirmed with full *ap2a* KO mutants. *FUL1* and *FUL2* were assumed to be downstream of ethylene biosynthesis as ethylene

production was not affected when both were (partially) silenced by RNAi. However, we saw less ethylene produced in the single, and much less in double KO mutants, correcting their location in ethylene regulation and confirming their partial redundancy in fruit ripening. We also reported a novel fruit skin phenotype, which suggests a novel function of *FUL2* in early fruit development. Finally, KO mutants of *NAC-NOR* in the wild-type background exhibited much milder ripening defects than the natural mutant *nor* (here called *nor-s*), while knocking out the function of *nor-s* with an upstream mutation partially restored ripening. This illustrates that the non-ripening phenotype in *nor* is caused by the dominant-negative function of the spontaneous *nor-s* allele, and not the loss-of-function of *NAC-NOR*.

The discrepancies between the functions of MADS-RIN and NAC-NOR and the phenotypes caused by their spontaneous mutants were clarified recently (also in **Chapter 3**), but the role of SPL-CNR remained a mystery. Also here, the decreased expression of the transcription factor in the spontaneous *Cnr* mutant was believed to underlie its ripening phenotype, but both the mutant's dominance, as well as its effects on early fruit development, suggested that the situation is more complex. In **Chapter 4**, in addition to creating KO mutants, we also generated large promoter deletion mutants with decreased *SPL-CNR* expression by deleting promoter fragments between gRNA-target sites. Surprisingly, both promoter and KO mutants showed only slower ripening and much weaker defects than *Cnr*. Fruit firmness decreased slower in these CRISPR mutants, which may be useful in long-distance transportation of fruits and for elongating fruit shelf life. In line with these observations, most of the genes, which are representative of different pathways in ripening, and which were all strongly affected in *Cnr*, showed no altered expression in those mutants. We speculate that there might be other genes involved in fruit texture and carotenoid biosynthesis, which have not yet been measured or even discovered, but are influenced by the epi-mutation in *Cnr*.

Chapter 5 studies the post-transcriptional effects of three microRNAs on fruit ripening. With more and more non-coding RNAs being reported their roles in plant development are getting more attention. Three miRNAs that were previously shown to function in the phase transition from vegetative to reproductive phases in plant's life cycle are also active in tomato fruit. The targeting of *SPL-CNR* by miR156/7 and *AP2a* by miR172 had been proved in tomato, but their functions have not yet been elucidated by mutant analysis. In **Chapter 5** we created CRISPR mutants of *MIR156, MIR157*, and *MIR172* genes, which are expressed in the fruit. The *MIR156* KO mutant produced smaller fruits and took less time to reach the ripening stage, revealing its repressive role in fruit ripening regulation. The other MIR-mutants did not show a mutant phenotype in the fruit, either because other genes producing the same miRNAs are still active or their role in fruit development and ripening is doubtful.

The genome-editing revolution of CRISPR/Cas9-mutagenesis enabled us to knock out genes much more effectively for function studies, but a string of recent publications showed that some KO mutants,

contrary to expectation, had milder phenotypes than those from RNAi or VIGS. **Chapter 6** reviews some possible mechanisms explaining the milder phenotypes in KO mutants, suggesting that a genetic compensation mechanism could hide true gene functions. Other possible mechanisms that could lead to incomplete gene function knockouts are also described, as well as the possibility that there are more dominant-negative or gain-of-function mutants in tomato fruit ripening and in plants as a whole. For regulation of ripening, we speculate that there is greater redundancy for regulatory function than previously thought and that related TFs and possibly even less-related ones (MADS-RIN and NAC-NOR) function as a group, making the regulation more robust and less sensitive to single-gene mutations. Therefore, to test this hypothesis, the function of such groups should be studied by assessing the effect of combined mutations.

Finally, in **Chapter 7** I discussed the genetic regulation of tomato fruit ripening. This includes all the results we obtained in this thesis in relation to other ripening regulation studies. Moreover, I discussed what we have learned from using CRISPR/Cas9-mutagenesis and recommended some strategies for future experiment design to avoid some of the pitfalls found by us and others. In conclusion, the thesis illustrates the function of some major TFs and interactions of their spontaneous mutant alleles in tomato ripening regulation, which enhances our knowledge, and points the way to future work on fruit ripening regulation.

Samenvatting

Tomaat is een van de meest gegeten groenten en speelt een cruciale rol in de menselijke voeding en in de voedingsindustrie. Bovendien maakt zijn waarde als model bij uitstek voor vlezige vruchten het bestuderen van tomaatrijping belangrijk. Rijping in tomaat is een complex proces dat wordt gereguleerd door genetische en epigenetische factoren, samen met plantenhormonen. Tot dusverre werden meestal één-op-één relaties tussen regulatoren en effectoren onderzocht, maar de echte functie van sommige belangrijke regulatoren is nog steeds niet duidelijk. Dit geldt ook voor de interacties van spontane mutantallelen van die regulatoren in tijdens rijping.

Het doel van dit proefschrift was om de rijping van tomaten op moleculair niveau te bestuderen en onze kennis ervan uit te breiden. Ik heb me gericht op het bepalen van de ware functie van de 'master regulators' (belangrijke transcriptiefactoren), op de interactie tussen hun spontane mutantallelen, en op de rol van niet-coderende elementen. Om dat te bereiken, gebruikte ik CRISPR/Cas9-mutagenese om echte knock-outmutanten te verkrijgen en kruisingen om genotypen met verschillende en gecombineerde doses van spontane mutantallelen te produceren, gevolgd door fenotypering en genexpressieanalyse om hun functies bij rijping te evalueren.

Hoofdstuk 2 richt zich op de interacties tussen de spontane mutantallelen *rin*, *nor* en *Cnr* in het regulatienetwerk van rijping. In plaats van alleen homozygote mutanten te gebruiken, waarin de rijping meestal volledig geblokkeerd is, hebben we hun interacties onderzocht door het maken en bestuderen van verschillende regulatorniveaus in heterozygote lijnen in een verder identieke genetische achtergrond (cv. Ailsa Craig). De heterozygote enkele en dubbele mutanten met *rin*, *nor* en *Cnr* werden verkregen door ze te kruisen met wildtype planten of door ze met elkaar te kruisen. We hebben verschillende fenotypische en metabole effecten in deze genotypen onderzocht en laten zien hoe deze mutaties op het niveau van het zichtbare fenotype, effectorgenexpressie en sensorische en kwaliteitsaspecten op een dosisafhankelijke manier interacteren. *rin* en *nor* hadden vergelijkbare kwantitatieve effecten op alle onderzochte aspecten van rijping, en de effecten van gecombineerde alleldoses waren additief. We hebben ook vastgesteld dat *Cnr* epistatisch is voor *rin* en *nor* omdat hun respectievelijke fenotypes onzichtbaar zijn wanneer *Cnr* aanwezig is. Ten slotte vonden we dat, in tegenstelling tot het bekende dominante effect op rijping, het *Cnr* allel onvolledig dominant is, of recessief, voor de afname van vruchtgrootte (een fenotype dat hier voor het eerst wordt beschreven), en voor veranderde productie van vluchtige verbindingen door de vrucht.

De door anderen gerapporteerde karakterisering van de *rin* mutatie als een ‘gain-of-function'-mutant, als gevolg van de productie van een nieuw, chimeer eiwit, herinnert ons eraan dat de functie van de gevestigde ‘hoofdregulatoren’ misschien niet altijd is wat werd verondersteld en dat hun functie

mogelijk opnieuw moet worden geëvalueerd. Daarom hebben we in hoofdstuk 3 de functies van de transcriptiefactoren AP2a, NOR en FUL1 en 2 met CRISPR/Cas9-mutagenese opnieuw onderzocht en voor het eerst de fenotypes van hun knockout (KO)-mutanten gerapporteerd. Het vroeg rijpende, oranje-rijpe fenotype met hogere ethyleenproductie van eerder gemaakte RNAi-planten werd bevestigd met volledige *ap2a* KO-mutanten. *FUL1* en *FUL2* werden verondersteld zich stroomafwaarts van ethyleenbiosynthese te bevinden omdat de ethyleenproductie niet werd beïnvloed wanneer beide (gedeeltelijk) werden geïnactiveerd met RNAi. We zagen echter lagere ethyleenproductie in de enkele, en veel lagere in dubbele KO-mutanten, waardoor hun functie in de regulatie van ethyleenproductie werd gecorrigeerd en hun gedeeltelijke redundantie bij het rijpen van vruchten werd bevestigd. We rapporteerden ook een nieuw fenotype in de schil van de vrucht, wat een nieuwe functie van *FUL2* in de vroege vruchtontwikkeling suggereert. Ten slotte vertoonden KO-mutanten van *NAC-NOR* in de wildtype achtergrond veel mildere rijpingsdefecten dan de natuurlijke mutant *nor* (hier *nor-s* genoemd), terwijl de uitschakeling van de functie van *nor-s* met een 5’ mutatie de rijping gedeeltelijk herstelde. Dit toont aan dat het niet-rijpende fenotype in *nor* wordt veroorzaakt door de dominant-negatieve functie van het spontane *nor-s* allel, en niet door het verlies van functie van *NAC-NOR*.

De discrepanties tussen de functies van MADS-RIN en NAC-NOR en de fenotypes veroorzaakt door hun spontane mutanten werden onlangs opgehelderd (ook in hoofdstuk 3), maar de rol van SPL-CNR bleef een mysterie. Ook hier werd aangenomen dat de verminderde expressie van de transcriptiefactor in de spontane *Cnr* mutant ten grondslag lag aan het rijpingsfenotype ervan, maar zowel de dominantie van de mutant als de effecten op de vroege vruchtontwikkeling suggereerden dat de situatie complexer is. In hoofdstuk 4 hebben we, naast het creëren van KO-mutanten, ook grote promotor-deletiemutanten met verminderde *SPL-CNR*-expressie gemaakt door promotorfragmenten tussen gRNA-doellocaties te verwijderen. Verrassenderwijs vertoonden zowel promotor- als KO-mutanten slechts langzamere rijping en veel zwakkere defecten dan *Cnr*. Vruchtstevigheid nam langzamer af bij deze CRISPR-mutanten, wat nuttig kan zijn bij het transport van vruchten over lange afstand en voor het verlengen van de houdbaarheid van fruit. In overeenstemming met deze waarnemingen vertoonden de meeste genen, die representatief zijn voor verschillende rijpingsroutes, en die alle sterk werden beïnvloed in *Cnr*, geen veranderde expressie in die mutanten. We speculeren dat er mogelijk andere genen betrokken zijn bij vruchttextuur en carotenoïde biosynthese, die hier nog niet zijn gemeten of zelfs nog niet ontdekt, maar die worden beïnvloed door de epi-mutatie in *Cnr*.

Hoofdstuk 5 bestudeert de post-transcriptionele effecten van drie microRNA's op het rijpen van tomaat. Nu steeds meer niet-coderende RNA's worden gerapporteerd, krijgt hun rol in de ontwikkeling van planten meer aandacht. Drie miRNA's waarvan eerder is aangetoond dat ze werken in de overgang van de vegetatieve naar de reproductieve fase in de levenscyclus van planten, zijn ook actief in tomatenvruchten. De regulatie van *SPL-CNR* door miR156/7 en *AP2a* door miR172 was bewezen in tomaat, maar hun functies zijn nog niet opgehelderd door mutantanalyse. In hoofdstuk 5 hebben we

CRISPR-mutanten gemaakt van *MIR156*-, *MIR157*- en *MIR172*-genen, die tot expressie komen in de vrucht. De *MIR156* KO-mutant produceerde kleinere vruchten en had minder tijd nodig om het rijpingsstadium te bereiken, waardoor zijn repressieve rol in de regulatie van vruchtrijping werd onthuld. De andere MIR-mutanten vertoonden geen mutant fenotype in de vrucht, hetzij omdat andere genen die dezelfde miRNA's produceren nog steeds actief zijn of omdat hun rol in de vruchtontwikkeling en rijping twijfelachtig is.

De genoommodificatierevolutie van CRISPR/Cas9-mutagenese stelde ons in staat voor functiestudies genen veel effectiever te elimineren, maar een reeks recente publicaties toonde aan dat sommige KO-mutanten, in tegenstelling tot de verwachting, mildere fenotypes hadden dan die van RNAi of VIGS. Hoofdstuk 6 bespreekt enkele mogelijke mechanismen die de mildere fenotypes in KO-mutanten verklaren, suggererend dat een genetisch compensatiemechanisme echte genfuncties zou kunnen verhullen. Andere mogelijke mechanismen die kunnen leiden tot onvolledige knock-out van de genfunctie worden ook beschreven, evenals de mogelijkheid dat er meer dominant-negatieve of 'gain-of-function'-mutanten zijn in de rijping van tomaat en in planten in het algemeen. Over de regulering van rijping speculeren we dat er een grotere redundantie is voor de regulerende functies dan eerder gedacht en dat gerelateerde transcriptiefactoren en mogelijk zelfs minder gerelateerde (MADS-RIN en NAC-NOR) als een groep functioneren, waardoor de regulatie robuuster en minder gevoelig wordt voor mutaties in één gen. Om deze hypothese te testen, moet daarom de functie van dergelijke groepen worden bestudeerd door het effect van gecombineerde mutaties te beoordelen.

Tot slot heb ik in hoofdstuk 7 de genetische regulatie van tomaatrijping besproken. Dit omvat alle resultaten die we in dit proefschrift hebben verkregen en de relatie tot andere studies naar regulatie van rijping. Bovendien heb ik besproken wat we hebben geleerd van het gebruik van CRISPR/Cas9-mutagenese en heb ik enkele strategieën aanbevolen voor het ontwerpen van toekomstige experimenten teneinde enkele van de valkuilen die wij en anderen hebben gevonden te ontwijken. Concluderend illustreert het proefschrift de functie van enkele belangrijke transcriptiefactoren en interacties van hun spontane mutantallelen in de regulering van de rijping van tomaten, die onze kennis verbetert, en de weg wijst naar toekomstig werk aan de regulering van de vruchtrijping.

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The tomato group was quite small when I started in 2015 and I was the only PhD student. Then it keeps growing with Vera, Ellen, Jin and Julia joining, but also with someone leaving. **Rumyana**, you are a nice scientist and I am happy for your permanent position. **Eveline**, my dear friend, you helped so much when I started and whenever I asked for a favour your answer is always yes. I am so lucky to have your help and really appreciate it a lot.

Ellen, it is only your second year here but I feel I have known you for a long time. Thanks for the walk we had together and the talks that helped me relax when I struggled with writing. I see your passion for science and you are doing excellently, so relax and be proud of yourself. **Julia**, you are so energetic and excited about your project every time I see you and I enjoy working with people like you with enthusiasm. Thanks for showing me your exciting results before the tomato meeting and I am sure you will achieve more in the future. **Jin**, you are quite busy but always willing to give a hand. Thanks for the help in vertical farming things and the super delicious cookies form Japan. **Julia** (student) and **Remi**, thanks for the happy memory in the lab outing.

My students **Marlot** and **Jurian**, thanks for choosing me as your supervisor, wish you all the best in the future.

Special thanks to my paranymphs. Michiel and Vera, your help through my PhD means a lot. **Michiel**, I still remember the first time we met when Ruud introduced you as "the most important person in tomato group", and yes you indeed are. You are always ready to help and it seems that you know the answers to all kinds of problems in the lab. I was frustrated at the beginning as things failed but you told and showed me how to be positive, be positive and be positive. Thank you, Michiel! **Vera**, you will be a leader someday and I never doubt it. You are my "statistics expert" offering lots of help in my data analysis in R. You are keen and willing to try new methods and techniques to be more efficient in both your work and life, which helps change my mind on many things, of course in a good way. Although I never mention it, you truly give positive influences, so thank you Vera.

Tomato people, thanks for all the discussions, help and the unforgettable group conference in Serbia!

PDS people, you guys are the best! My wonderful and unforgettable PhD journey consists of every one of you.

Mengfan and **Baojian**, we started our PhD at the same time and I am so happy to have your company. There are a lot of fun to go to the PhD school in Retzbach and the trip to Würzburg with you. Thank you for all the help and the hotpot we had together. As you are also finishing soon I wish you both have a brand new life as you wish. **Manjunath**, my neighbour in the open office, you are always so nice and never complain, like the BFG. Thanks for the help in R and the good memory in Keukenhof and Doorwerth, hope you all the best in India. **Lena**, thank you for those so nice calendars and always taking over the "too sweet" things from me, and of course also the amazing trip in Serbia together with Vera, Ellen and Mengfan. It is not only a trip but also an adventure I never had in my life that I will never forget, so thank you girls! **Charlotte**, you are so considerate and always think for other people. I really like talking with you and thanks for the patient explanation to all my questions and the nice talks during lunch. **Judit**, my new deskmate, thanks for all the "professional suggestions" for the colour of my tomatoes and the happy mood you bring every day. **Francesca**, thank you for the help for the gifts and I'm sure the people get them will be happy. **Xiaobing**, thanks for the help in RNA-seq library. **Suze**, thank you for organizing the Efteling day. Although I cannot enjoy roller coasters I indeed enjoy the happy time that everybody takes one day off for excitements.

Suraj, I miss so much the time that we played our music, of course mainly yours, after five o'clock in the lab, and it is a pity nobody does it anymore. You told me to enjoy my PhD and you showed a perfect example how to do it. Thanks for all the help in PDS and also the advice for my future career. **Tati** and **Mark**, thanks for the pool after work and the tennis course you gave. **Alice**, **Sam**, **Hilda**, **Suzanne**, **Leonie**, **Patricia**, **Sylvia**, **Miao Bai** and **William**, I enjoyed the time with all of you, thank you!

Kim, you are the first and the only PDS person I met when I arrived in Wageningen as all others went for the cluster outing. Thanks for picking me up from the train station and helping settle me down.

Steven, thanks for the photography course from which I learn how to take good pictures. **Marian**, I am happy that your group is growing so fast and I still remember our discussion in the growth chamber downstairs and I said that you will be a professor leading a big group. Thanks for all the suggestions for my study and the dinner after Veluwe Loop. **Hana**, thanks for helping the visa stuff and the plums from your garden. **Froukje**, your magic banana cake really helped accelerate my writing, thank you! **Annemarie**, thanks for organising the nice visit to your company. **Richard** and **Wilma**, thanks for the original idea and the feedback for the TIPS paper. **Martijn**, the snack manager and the best actor in PDS, thanks for the email every Friday and sorry I ordered rarely. **Marco** and **Jacqueline**, thanks for the help in the lab. **Jan**, thanks for helping phenotype mutants with the Videometer. **Tjitske** and **Mieke**, thanks for the amazing lab outings and the Sinterklaas borrels. To all who joined PDS recently, **Iris**, **Amit** and **Amalia**, I am sure you will enjoy your time in PDS.

Thank you all for the lunch club, talks during lunch, Sinterklaas, cluster outing and all other happy times, which are the unique PDS atmosphere that I will definitely miss.

Also many thanks to **Yury** and **Arnaud** for offering kind help to my thesis, **Geurt** for taking care of my plants in the past four years and **Alice** (plant physiology) for the help in AI to make my tomatoes look much better. Besides, I would like to acknowledge my **thesis committee** for the opportunity to defend my thesis.

Yanyan and **Zhijiao**, you are not only my roommates in my master but also dear friends in my life. Yanyan and I came to Europe for our PhD and Zhijiao stayed in Beijing and we have seven hours' time difference, but the distance doesn't stop our friendship. Thank you so much for listening to my complaints and the trip to Paris at almost the end of my writing, which helped me to relax a lot.

I could not imagine in the past that I would make friends online with someone, but the chatting group really surprises me. All the eleven people come to Europe for our PhD and we do our best to help each other. **Lingping Zhu** and **Ran Lu**, thanks for all the help and happy memory. We three talked and discussed so much in the past five years, and travelled together, even we are in three countries. We support each other in both our PhD and life so thank you! **Zhaoju Deng**, we came to the Netherlands by the same flight and start our PhD on the same day. Thanks for the help in R, all the talks and nice meals and hope you can come back soon for your defence. It is so nice that another one in our group also came to Wageningen, **Mengjing Sun**, thanks for all the help and sharing the experience in finding jobs. **Lidong Mo**, your tips for my travel in Germany and Austria are quite useful, thank you.

Many thanks to the Chinese community who offer all the help: **Yanting Wang**, **Xu Cheng**, **Yi Wu**, **Kaile Sun**, **Hui Tian**, **Huchen Li**, **Shuang Song**, **Jinbin Wu**, **Lu Luo**, **Jieyu Liu**, **Xinping Yang**, **Tian Yu, Libin Zhou** and **Siye Chen**.

最后，我要感谢一群和这本论文无关，但是没有他们我甚至无法开始这段旅程的人，我的父母和家人。但也恰恰是他们，让我不知道用什么词汇去感谢。我的父母总是在背后默默理解和支持，除了希望我开心和健康外对我没有任何要求，但是他们需要我的时候我却不能在他们身边。写这里时坐在办公室哭成狗，吓到同事了，写不下去也写不出来了。我的词汇量表达不出对他们的感谢，只希望他们身体健康，做个快乐的小老太太和小老头。我一定会尽快回去，陪着他们就好。我庆幸我有一群有爱的家人，毫无条件地相互帮助相互支持，在我不在的时候帮我尽到一个子女的义务。希望你们一切都好。

Thank you so much! (衷心感谢)

Rufang Wang (王茹芳)

Dec 2019

About the author

Rufang Wang (王茹芳) was born on June 20th 1989, in Nanyang, China. She went to Gansu Agricultural University, China to study Grassland Science in 2009. After receiving her Bachelor degree in 2013 she started her Master study in Department of Vegetable Science in China Agricultural University. Since then she has worked with tomato for more than six years.

In 2015, after finishing her Master study, she got the opportunity to start her Ph.D. at the Laboratory of Molecular Biology, Wageningen University & Research, under the supervision of Prof. Gerco Angenent and Dr. Ruud de Maagd. She studied the genetic and epigenetic regulation of tomato fruit ripening as her project and especially focused on the transcriptional regulation. The work performed during her Ph.D. is described in this thesis.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Rufang Wang
Date: 18 Feb 2020
Group: Laboratory of Molecular Biology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
Epigenetic and transcriptional regulation of tomato ripening	24 Mar 2016	1.5
► **Writing or rewriting a project proposal**		
Epigenetic and transcriptional regulation of tomato ripening	Feb 2016	2.5
► **MSc courses**		
Subtotal Start-Up Phase		4.0

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
EPS PhD Student Days "Get2Gether 2016", 2 days, Soest, NL	28-29 Jan 2016	0.6
EPS PhD Student Days "Get2Gether 2017", 2 days, Soest, NL	9-10 Feb 2017	0.6
EPS PhD Student Days "Get2Gether 2019", 2 days, Soest, NL	11-12 Feb 2019	0.6
► **EPS theme symposia**		
EPS Theme 4 Symposia 'Genome Biology', Amsterdam, NL	15 Dec 2015	0.3
EPS Theme 1 Symposia 'Developmental Biology of Plants', Wageningen, NL	21 Jan 2016	0.3
EPS Theme 1 Symposia 'Developmental Biology of Plants', Leiden, NL	28 Feb 2017	0.3
EPS Theme 1 Symposia 'Developmental Biology of Plants', Wageningen, NL	30 Jan 2018	0.3
EPS Theme 1 Symposia 'Developmental Biology of Plants', Leiden, NL	31 Jan 2019	0.3
► **Lunteren Days and other national platforms**		
Annual Experimental Plant Sciences Meeting, Lunteren, NL	11-12 Apr 2016	0.6
Annual Experimental Plant Sciences Meeting, Lunteren, NL	10-11 Apr 2017	0.6
Annual Experimental Plant Sciences Meeting, Lunteren, NL	9-10 Apr 2018	0.6
Annual Experimental Plant Sciences Meeting, Lunteren, NL	8-9 Apr 2019	0.6
► **Seminars (series), workshops and symposia**		
1st Symposium 'WURomics: Technology- Driven Innovation for Plant Breeding', Wageningen,NL	15 Dec 2016	0.3
KEYS symposium 'Boosting Crop Productivity', KeyGene, Wageningen,NL	14 Jun 2017	0.2
Seminar of Sanjay Kapoor 'Regulators of Reproductive Development in Rice', Wageningen, NL	29 Aug 2017	0.1
Workshop 'Functional Plant Bioinformatics - PLAZA', Ghent, Belguim	14-15 Sep 2017	0.6
Seminar of Annette Becker 'A phylogenetic framework for carpel development regulation: mixing and matching old with new', Wageningen, NL	20 Nov 2017	0.1
Seminar of Claire Périlleux 'Flowering in tomato, Arabidopsis and Sinapis', Wageningen, NL	9 Feb 2018	0.1
KNAW Symposia 'CRISPR-Cas - From Evolution to Revolution', Wageningen, NL	8 Mar 2018	0.2
Seminar of Bob Schmitz 'Epigenomic Studies of Natural and Induced Epialleles in Plants', NIOO-KNAW, Wageningen, NL	6 Jun 2018	0.1
100 years WUR symposium 'Food for Future', Wageningen, NL	22 Jun 2018	0.2
Seminar of Lucia Strader 'Condensation of the ARF19 transcription factor regulates its activity', Wageningen, NL	10 Sep 2018	0.1
Seminar of Rosanna Petrella 'Transcriptional and epigenetic regulation of STK during flower development in Arabidopsis', Wageningen, NL	13 Nov 2018	0.1
Seminar of Emanuela Franchini 'The role of ALOG family genes in inflorescence patterning in rice and Arabidopsis', Wageningen, NL	13 Nov 2018	0.1
► **Seminar plus**		
► **International symposia and congresses**		
10th International PhD School Plant Development, Retzbach, Germany	4-6 Oct 2017	0.8
The 15th Solanaceae Conference, Chiang Mai, Thailand	30 Sep- 4 Oct 2018	1.1
1st PlantEd conferencePlant Genome Editing -State of the Art, Novi Sad, Serbia	5-7 Nov 2019	0.8
► **Presentations**		
Poster: Epigenetic and transcriptional regulation of tomato fruit ripening, Wageningen, NL	12-14 Dec 2016	1.0
Poster: CRISPR/Cas9-mediated mutagenesis in tomato, Lunteren, NL	10-11 Apr 2017	1.0
Talk: Thematic Meeting Bioscience, Wageningen, NL	25 Apr 2017	1.0
Poster: Regulatory network interactions in tomato fruit ripening, Retzbach, Germany	4-6 Oct 2017	1.0
Talk: Thematic Meeting Bioscience, Wageningen, NL	5 Dec 2017	1.0
Talk: CRISPR/Cas9-mediated mutagenesis for understanding fruit ripening in tomato, EPS Theme 1 Symposium, Wageningen, NL	30 Jan 2018	1.0
Poster: Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis, The 15th Solanaceae Conference, Chiang Mai, Thailand	30 Sep- 4 Oct 2018	1.0
Talk: Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis, The 15th Solanaceae Conference, Chiang Mai, Thailand	1 Oct 2018	1.0
Talk: Thematic Meeting Bioscience, Wageningen, NL	4 Dec 2018	1.0
Talk: Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis, EPS PhD Student Days "Get2Gether 2019", Soest, NL	12 Feb 2019	1.0
Poster: Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis, Lunteren, NL	8-9 Apr 2019	1.0
Talk: CRISPR/Cas9-mutagenesis for revisiting the role of master regulators in tomato ripening, 1st PlantEd conferencePlant Genome Editing -State of the Art, Novi Sad, Serbia	5-7 Nov 2019	1.0

CONTINUED ON NEXT PAGE

►	**3rd year interview**		
►	**Excursions**		
	Tomato world	14 Oct 2016	0.2
	Dekker Chrysanthemum	5 Mar 2019	0.2
	Subtotal Scientific Exposure		23.0

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
EPS PhD course 'Transcription Factors and Transcriptional Regulation', Wageningen, NL	12-14 Dec 2016	1.0
Introduction to R for Statistical Analysis, Wageningen, NL	17-18 May 2018	0.6
Workshop Linux Shell, Wageningen, NL	16 Aug 2018	0.1
► **Journal club**		
Literature discussion group at Bioscience-Plant Developmental Systems	Sep 2015- Oct 2019	3.0
► **Individual research training**		
Subtotal In-Depth Studies		4.7

4) Personal Development	*date*	*cp*
► **General skill training courses**		
Information Literacy for PhD including EndNote introduction, Wageningen, NL	1-2 Dec 2015	0.6
EPS introduction course, Wageningen, NL	11 Feb 2016	0.3
Brain Training, Wageningen, NL	8 Nov 2017	0.3
Scientific Writing, Wageningen, NL	13 Nov 2017- 23 Jan 2018	1.8
► **Organisation of meetings, PhD courses or outreach activities**		
► **Membership of EPS PhD Council**		
Subtotal Personal Development		3.0

5) Teaching & Supervision Duties	*date*	*cp*
► **Courses**		
► **Supervision of BSc/MSc students**		
Supervision of BSc student Marlot Westera	4 Sep- 24 Dec 2017	0.7
Supervision of BSc student Jurian de Graaf	19 Feb- 2 Jun 2018	0.7
Subtotal Teaching & Supervision Duties		1.4

TOTAL NUMBER OF CREDIT POINTS*	**36.1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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Propositions

1. Tomato fruit ripening is a complex process with different layers of regulation.

   (this thesis)

2. The phenotype of spontaneous mutant alleles does not necessarily reflect the normal function of the underlying gene.

   (this thesis)

3. *P* value is over-rated as a measure of significance, instead one should analyse data from multiple angles to decide on its certainty.

   (Wasserstein *et al*., 2019. The American Statistician 73, 1-19)

4. The trend of recent scientific programmes to work in multi- and interdisciplinary teams will come at the expense of quality and breakthroughs in the different scientific fields.

5. Baking a cake is more difficult than doing a qPCR.

6. The strict regulation of crops from CRISPR/Cas-mutagenesis as GMO in Europe slows down the innovation in breeding and agriculture.

Propositions belong to the thesis, entitled:

Genetic and epigenetic regulation of tomato fruit ripening

Rufang Wang

Wageningen, 18 February 2020