Plant Science 294 (2020) 110436

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

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

Rufang Wang[a,b], Michiel Lammers[b], Yury Tikunov[c], Arnaud G. Bovy[c], Gerco C. Angenent[a,b], Ruud A. de Maagd[b,*]

[a] Laboratory of Molecular Biology, Wageningen University, the Netherlands
[b] Bioscience, Wageningen Plant Research, the Netherlands
[c] Plant Breeding, Wageningen University & Research, Wageningen, Netherlands

ARTICLE INFO

Keywords:
Tomato
Fruit ripening
Spontaneous mutation
ripening inhibitor
non-ripening
Colorless non-ripening

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.

1. 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 [1]. The process is also regulated by dynamic epigenetic modifications [2], 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*) [3], *non-ripening* (*nor*) [4] and *Colorless non-ripening* (*Cnr*) [5] are spontaneous mutants of the TF encoding genes *RIPENING INHIBITOR* (*MADS-RIN*) [6], *NON-RIPENING* (*NAC-NOR*) [7] and *COLORLESS NON-RIPENING* (*SPL-CNR*) [8], 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 [9], and this phenotype cannot be rescued by external ethylene treatment [10]. Another distinctive characteristic of *rin* fruits is their large leaf-like sepals or calyx [3]. The *rin* mutation comprises a deletion between *MADS-RIN* and its neighboring gene *MACROCALYX* (*MC*), another MADS-domain TF regulating sepal development [6]. 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 [4]. The *nor* mutation comprises a 2 bp deletion in the third exon of *NAC-NOR*, resulting in a truncated protein [11]. The mutation reduces both ethylene production and lycopene biosynthesis [12]. *Cnr* fruits have a mealy pericarp that turns pale yellow in later stages [5] since no lycopene is synthesized [13]. The *Cnr* mutation does not cause primary sequence changes, but is epigenetic: part of the upstream region of the gene is hypermethylated

* Corresponding author at: P.O. Box 16, 6700 AA Wageningen, the Netherlands.
E-mail address: ruud.demaagd@wur.nl (R.A. de Maagd).

https://doi.org/10.1016/j.plantsci.2020.110436
Received 9 October 2019; Received in revised form 31 January 2020; Accepted 2 February 2020
Available online 04 February 2020

compared to that in the wild-type and remains methylated during ripening [2,14], with a reduced expression of *SPL-CNR* [8]. There is no burst of ethylene during fruit development in this mutant, and its phenotype cannot be rescued by exogenous ethylene application [8]. *Rin* and *nor* were reported to be recessive [3,4], while *Cnr* is dominant since there was no significant difference between the phenotypes of the mutant and heterozygous lines reported [5].

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 [15,16]. 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 [9,17,18]. Additionally, a weaker allele of *nor*, *alcobaca* (*alc*) [19], which was also called Delayed Fruit Deterioration [20], was used for similar purposes [21]. 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) [22], FRUITFULL1 (FUL1) or FUL2 [23–25], 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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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 [26]. 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.

2.4. 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 [27]. As production levels were not distributed normally, a quasibinomial model was used to test the statistical significance of differences between genotypes.

2.5. 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 [28]. HPLC analysis was performed according to [29]. 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 [30] 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.

2.6. 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 [31]. All primers used for qRT-PCR are listed in Table S1. Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$ [32].

2.7. 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).

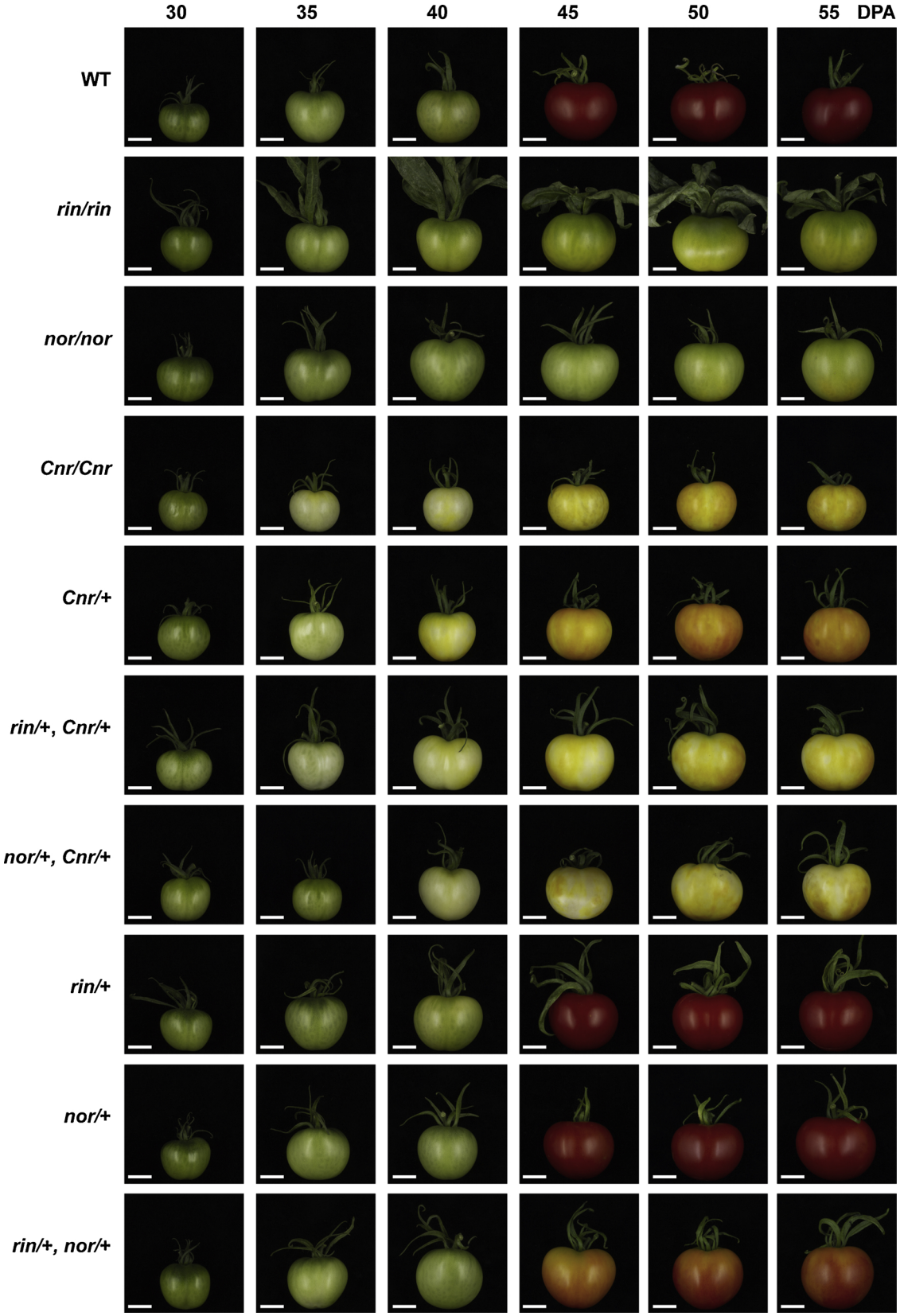

Fig. 1. Development and ripening of homozygous spontaneous mutants and their heterozygotes. Time-course images of representative fruits taken every five days from 30 DPA onwards showing differences in developmental and ripening processes. All lines are in the background of cv. Ailsa Craig. Scale bar,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

Fig. 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.

3. Results

3.1. 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).

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).

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 [3], which is caused by the loss of function of the adjoining MADS-box gene *MACROCALYX* [6]. 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.

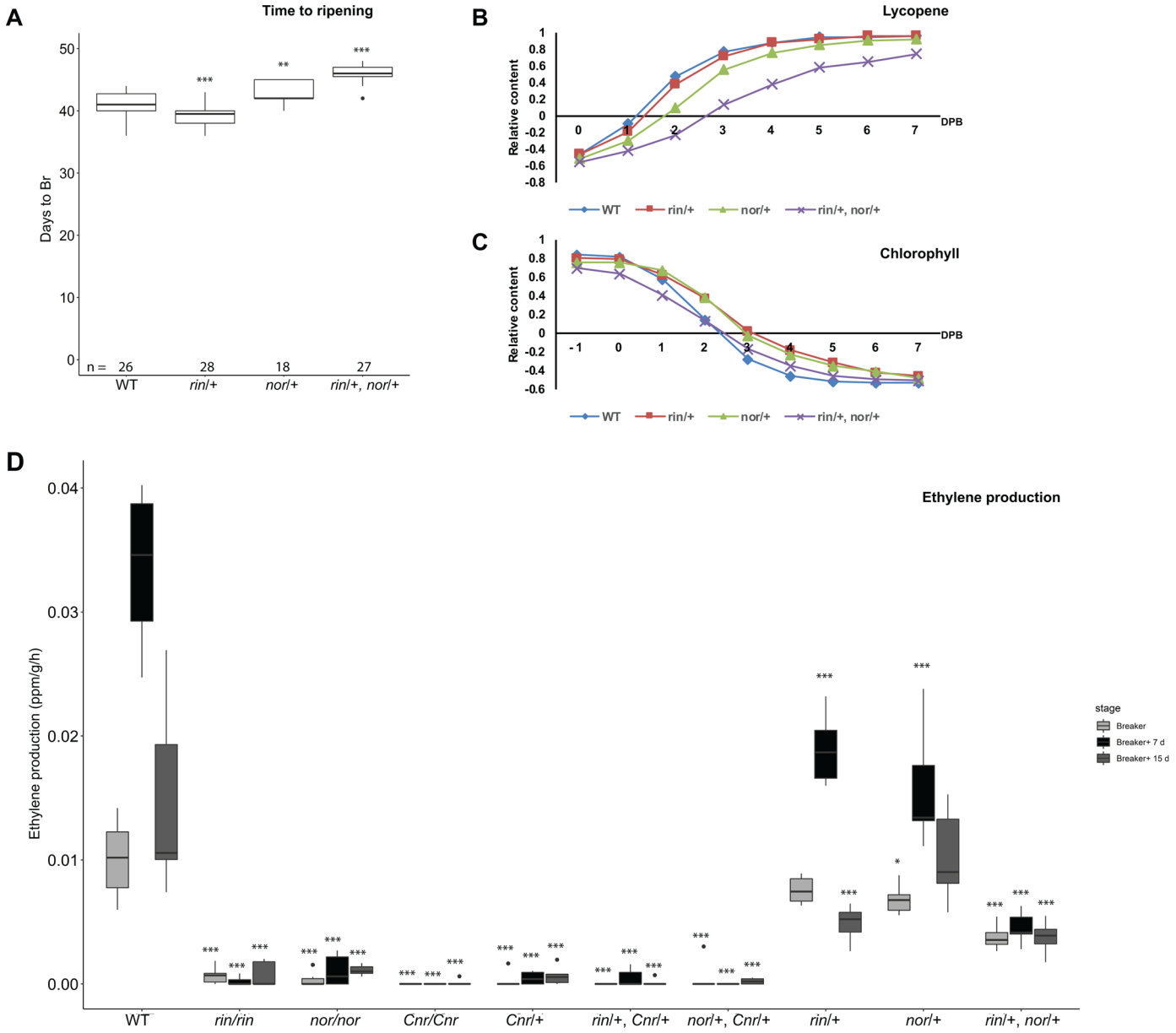

Fig. 3. Ripening traits of mutant fruits. (**A**) Time to the initiation of ripening (days to Br) of mutants with a discernible Br stage. (**B**) Lycopene and (**C**) chlorophyll change during ripening in mutants with a clear Br stage. The relative content of pigments of each fruit was measured every 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.

3.2. *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.

3.3. *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 ethylene 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). Thus, we conclude that *Cnr* is epistatic to *nor* and *rin* with regards to ethylene biosynthesis.

The loss of cell-to-cell adhesion in *Cnr* fruits resulted in 50 % more intercellular spaces in the pericarp [33] and reduced density, making the pericarp float in water [34]. 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*. Double heterozygous mutants of *Cnr* with *rin* or *nor* are pale yellow rather than green (*rin* or *nor* homozygous mutants) or red (*rin* or *nor* heterozygous mutants), indicating that also here, *Cnr* is epistatic to *rin* and *nor* (Figs. 1 and 2).

3.4. 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.

3.5. 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 [35] 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, Fig. S3A and Table S2). Fig. 4E-J and Table S2 show the content of six volatiles, representing groups distinguished by their biosynthetic origin [30].

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 [36]. Similarly, (E)-geranylacetone is representative of compounds produced from carotenoids, and both contribute to tomato flavor and quality [37]. Their production was almost undetectable in both the *Cnr* homozygote and its heterozygotes (Fig. 4E, F and Table S2) 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 [38]. 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 and Table S2). The phenolic volatiles eugenol and phenylacetaldehyde, derived from the phenylpropanoid pathway [35], 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, I and Table S2). 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 and Table S2). 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, S3A and Table S2). Thus, we conclude that also with regards to volatile profile, *Cnr* is epistatic to *nor* and *rin* in double heterozygous fruits.

3.6. 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 and Table S3). 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 [39]. 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 harbouring a single *Cnr* allele showed an as sharply reduced *ACS4* expression as in *Cnr/Cnr* (Fig. 5A and Table S3). The peaks in *ACS4* and *ACO1* expression (Fig. 5A and B) are associated with the transition from ethylene system 1 to system 2 [40]. 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*) [41], *PECTATE LYASE* (*PL*) [42] and *CELLULASE 2* (*CEL2*) [43] 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

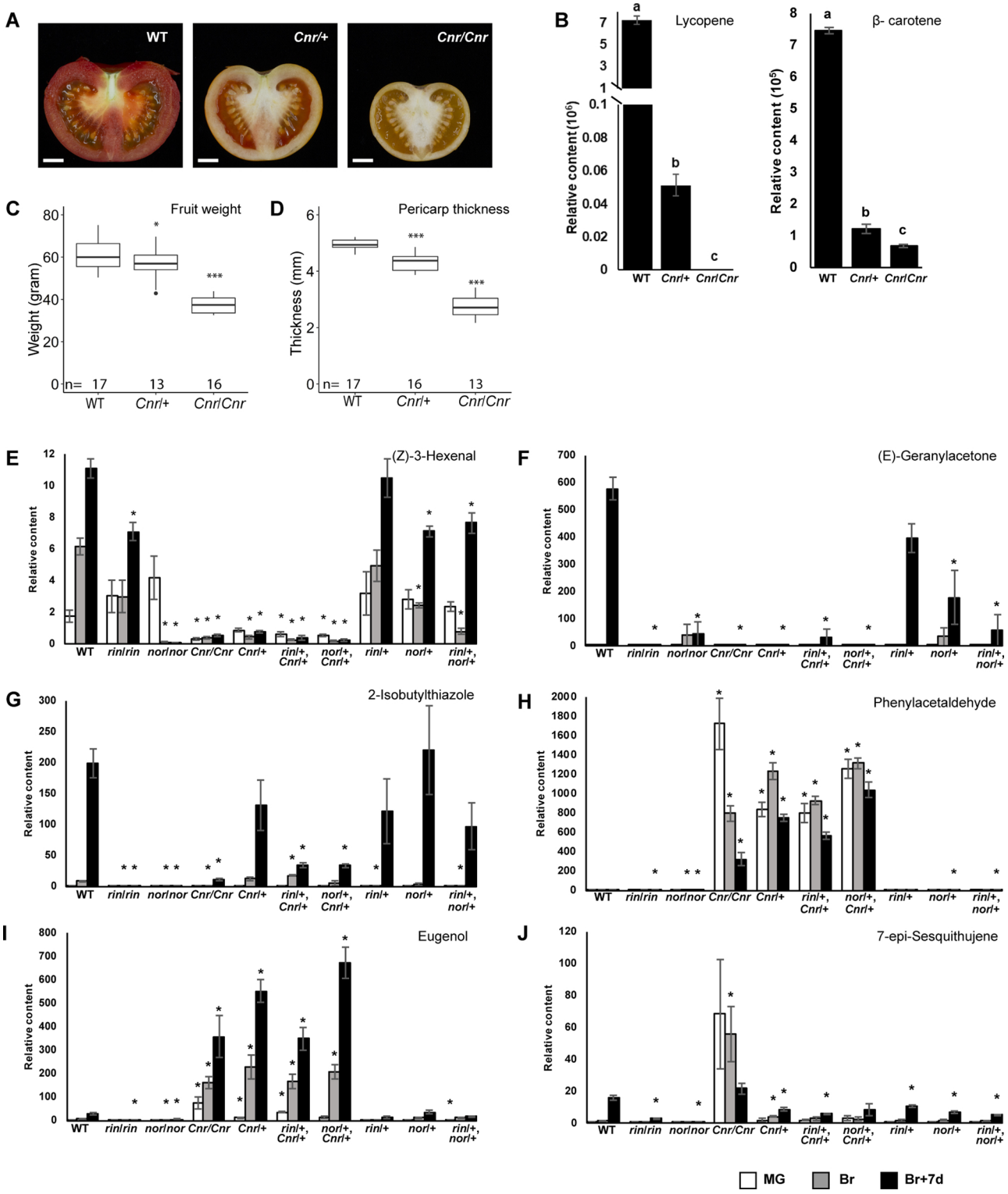

Fig. 4. Mutant allele effects on fruit developmental and ripening traits. (**A**) Longitudinally sectioned WT (left), *Cnr* single heterozygous (middle) and homozygous (right) fruits. Scale bar, 1 cm. (**B**) Carotenoid contents in wild-type, *Cnr* single heterozygous and homozygous fruits at ripe or 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 ($P < 0.05$).

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 and Table S3). *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 and Table

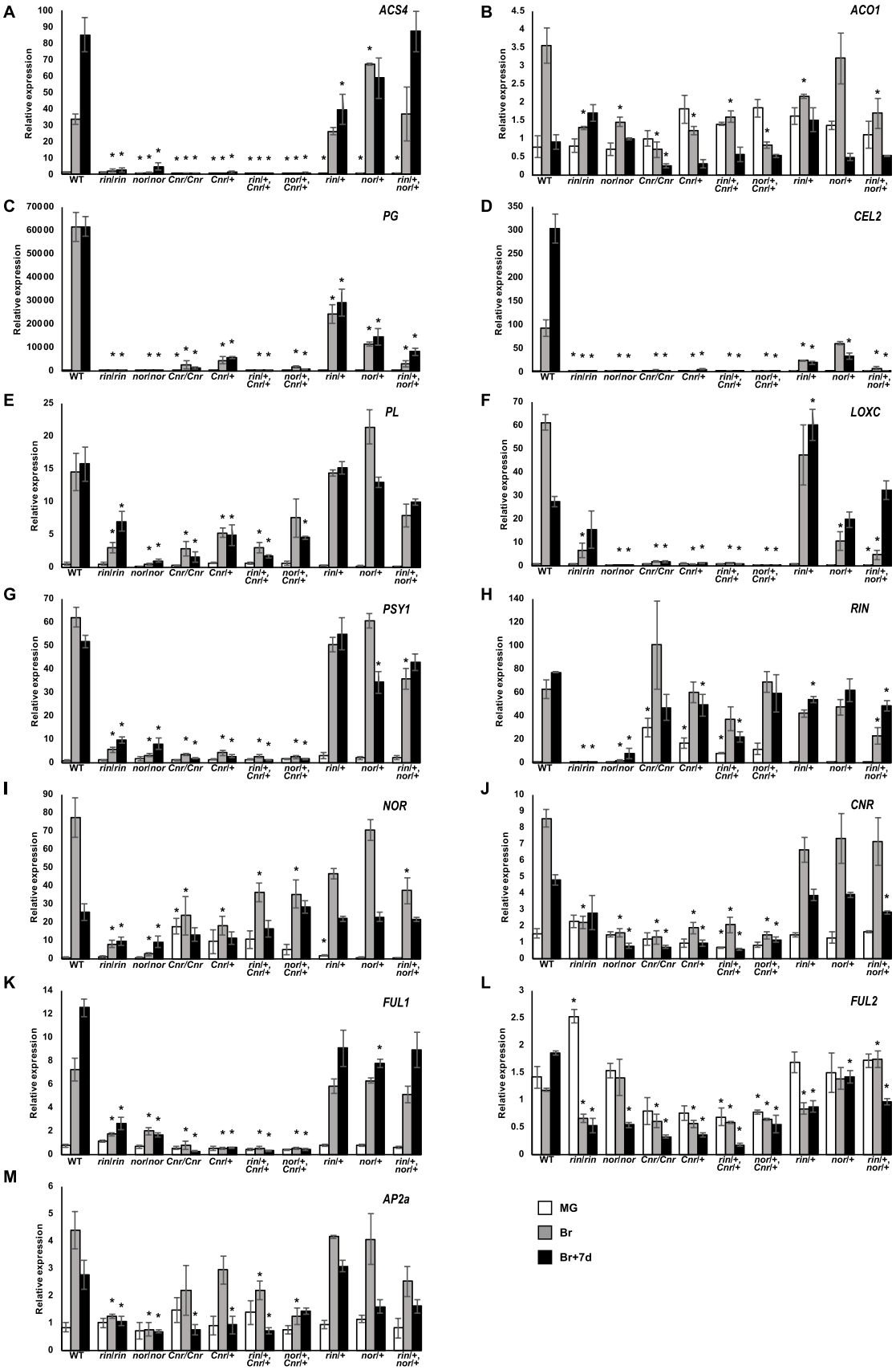

Fig. 5. Expression of ripening-related genes in all lines during fruit development and ripening. Three biological replicates of each were used, and error bars represent SE of means. Clear, grey and black blocks represent gene expression at MG, Br and Br+7d. Asterisks indicate significant differences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

S3). 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*) [44] and *PHYTOENE SYNTHASE1* (*PSY1*) [45] 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 and Table S3), 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.

3.7. 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 [46], 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*) [47] (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 [46,48], 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.

4. 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. Since *rin* and *nor* single homozygous mutants and *Cnr* mutants are already similarly, and completely blocked in all aspects of ripening studied here, we expected any double or triple combination of these mutants to give very little extra information. 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.

4.1. 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 [18].

On the other hand, other studies on *rin* or *nor* heterozygotes report a more distinct effect of heterozygosity on quality attributes, such as color [17]. 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 knockout (KO) mutants, where only 17 % remaining ethylene

production compared with wild-type fruits at Br stage initiated ripening [27]. 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 is 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* [49] 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* [46,48], All genes tested for expression in this study demonstrated *in vivo* binding of MADS-RIN as detected by ChIP, except *ACO1* and *PSY1* [46], and although similar data have not been published for NAC-NOR, inspection of the FruitEncode database [50] 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. This hypothesis is depicted in Fig. 6A. 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 [51].

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.

4.2. 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 [8]. 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 [35,52] and eugenol, which is associated with smoky off-flavor [53]. 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 [54].

4.3. The Cnr allele is epistatic to the rin and nor alleles

The *Cnr* allele is dominant [5], 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

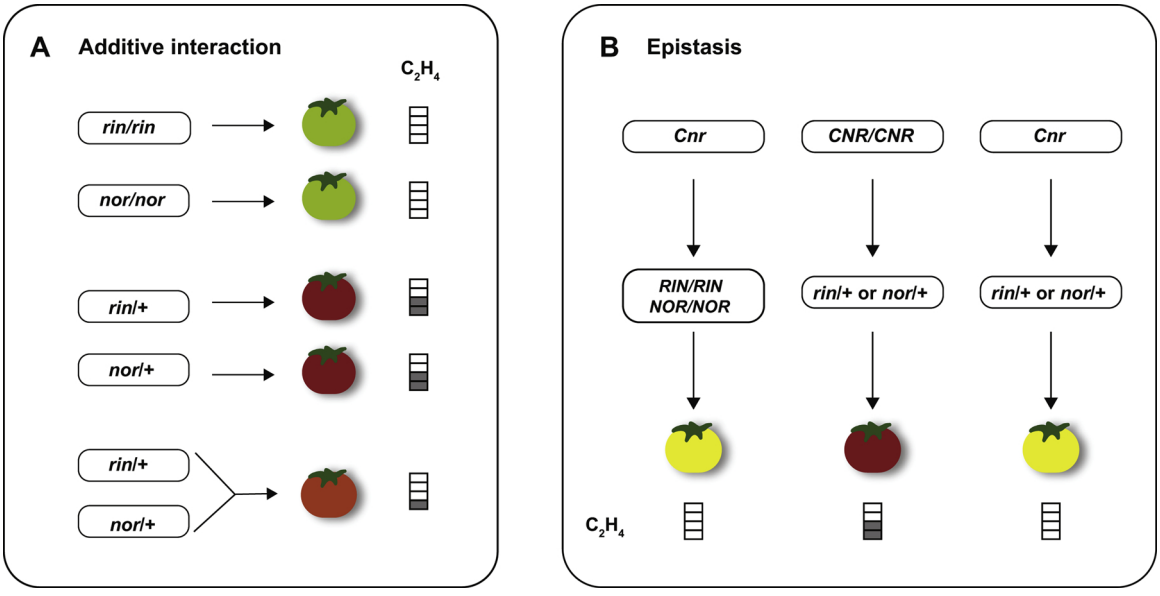

Fig. 6. Interactions between the three spontaneous alleles. (**A**) The additive effects of *rin* and *nor* on ripening. Ripening in homozygous *rin* and *nor* mutants is completely blocked with no ethylene produced. One dose of *rin* or *nor* has a mild effect on ripening with half the ethylene production (middle), but their combined doses make the ripening defects stronger and ethylene production is substantially lower (bottom). (**B**) The epistatic effect of *Cnr* on *rin* and *nor* in fruit ripening. *Cnr* fruits are pale yellow with no ethylene produced (left). Ripening is mildly affected when one dose of *rin* or *nor* in the absence of a *Cnr* mutant allele (middle), but fruits display the *Cnr* phenotype when the *Cnr* allele is present (right).

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 [55] or meristem establishment [56], but not yet in ripening. Here we show that *Cnr* is epistatic to *rin* and *nor* in ripening regulation. This is depicted in Fig. 6B.

4.4. 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. In contrast to the early reports, more recently *rin*, and *nor* were reported to produce dominant-negative proteins [27,57] and *Cnr* to likely be a gain-of-function mutation [54], whose associated severe ripening defects do not represent the phenotype of their *null* alleles. The spontaneous *nor* and *rin* 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 [58]. 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 [27,57]. 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 [59,60]. 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 [11]. 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 [27]. 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 [57]. Recently, Gao et al. showed that a CRISPR-derived *spl-cnr* KO mutant displayed a delayed but further normal progression of ripening as wild-type fruits, suggesting that *SPL-CNR* is not essential for ripening [54]. 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. Separate expression analysis of the knockout mutants of all three genes, which are available now, should tell us what the overlap between the two types of mutations (knockout vs. spontaneous) is. This will indicate which part of the phenotype in the spontaneous mutants is gained by the mutant transcription factors. A study on CRISPR-generated knockout mutants of *MADS-RIN* suggests that some of the genes affected by *rin* as shown here are similarly affected in the knockout mutants (*ACS4, CEL2*), while others (*PG2A, PL*) are only affected in *rin* [57].

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.

Author contributions

R.W. and R.A.d.M. designed research; R.W., M.L., Y.T. and A.G.B. performed research; R.W., Y.T. and A.G.B. analyzed data; R.W., G.C.A. and R.A.d.M. wrote the paper. All authors read and approved the manuscript.

CRediT authorship contribution statement

Rufang Wang: Validation, Formal analysis, Investigation, Writing - original draft. **Michiel Lammers:** Resources. **Yury Tikunov:** Formal analysis. **Arnaud G. Bovy:** Formal analysis. **Gerco C. Angenent:** Writing - review & editing, Supervision, Project administration. **Ruud A. de Maagd:** Writing - review & editing, Supervision, Project administration.

Declarations of Competing Interest

None.

Acknowledgements

We thank Professor Graham Seymour for supplying the *Cnr* seeds. Yi Wu and Arjen van de Peppel are acknowledged for the assistance with HPLC analysis and ethylene measurements, respectively. This work was supported by a fellowship from the China Scholarship Council (CSC) to R.W.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110436.

References

[1] R. Karlova, N. Chapman, K. David, G.C. Angenent, G.B. Seymour, R.A. de Maagd, Transcriptional control of fleshy fruit development and ripening, J. Exp. Bot. 65 (2014) 4527–4541.
[2] S. Zhong, Z. Fei, Y.R. Chen, Y. Zheng, M. Huang, J. Vrebalov, R. McQuinn, N. Gapper, B. Liu, J. Xiang, Y. Shao, J.J. Giovannoni, Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening, Nat. Biotechnol. 31 (2013) 154–159.
[3] R.W. Robinson, M.L. Tomes, Ripening inhibitor: a gene with multiple effect on ripening, Rep. Tomato Genet. Coop. 18 (1968) 36–37.
[4] E. Tigchelaar, M. Tomes, E. Kerr, R. Barman, A new fruit ripening mutant, *non-ripening* (*nor*), Rep. Tomato Genet. Coop. 23 (1973) 33–34.
[5] A.J. Thompson, M. Tor, C.S. Barry, J. Vrebalov, C. Orfila, M.C. Jarvis, J.J. Giovannoni, D. Grierson, G.B. Seymour, Molecular and genetic characterization of a novel pleiotropic tomato-ripening mutant, Plant Physiol. 120 (1999) 383–390.
[6] J. Vrebalov, D. Ruezinsky, V. Padmanabhan, R. White, D. Medrano, R. Drake, W. Schuch, J. Giovannoni, A MADS-box gene necessary for fruit ripening at the tomato *ripening-inhibitor* (*rin*) locus, Science. 296 (2002) 343–346.
[7] J. Giovannoni, S. Tanksley, J. Vrebalov, F. Noensie, NOR gene compositions and methods for use thereof, Patent US 6 (762) (2004) 347 B1.
[8] K. Manning, M. Tör, M. Poole, Y. Hong, A.J. Thompson, G.J. King, J.J. Giovannoni, G.B. Seymour, A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening, Nat. Genet. 38 (2006) 948–952.
[9] R.C. Herner, K.C. Sink, Ethylene production and respiratory behavior of the *rin* tomato mutant, Plant Physiol. 52 (1973) 38–42.
[10] J.E. Lincoln, R.L. Fischer, Regulation of gene expression by ethylene in wild-type and *rin* tomato (*Lycopersicon esculentum*) fruit, Plant Physiol. 88 (1988) 370–374.
[11] R. Kumar, V. Tamboli, R. Sharma, Y. Sreelakshmi, *NAC-NOR* mutations in tomato Penjar accessions attenuate multiple metabolic processes and prolong the fruit shelf life, Food Chem. 259 (2018) 234–244.
[12] J. Czapski, M. Saniewski, Stimulation of ethylene production and ethylene-forming

enzyme activity in fruits of the non-ripening nor and rin tomato mutants by methyl jasmonate, J. Plant Physiol. 139 (1992) 265–268.
[13] P. Fraser, P. Bramley, G. Seymour, Effect of the *Cnr* mutation on carotenoid formation during tomato fruit ripening, Phytochemistry 58 (2001) 75–79.
[14] R. Liu, A. How-Kit, L. Stammitti, E. Teyssier, D. Rolin, A. Mortain-Bertrand, S. Halle, M. Liu, J. Kong, C. Wu, C. Degraeve-Guibault, N.H. Chapman, M. Maucourt, T.C. Hodgman, J. Tost, M. Bouzayen, Y. Hong, G.B. Seymour, J.J. Giovannoni, P. Gallusci, A DEMETER-like DNA demethylase governs tomato fruit ripening, Proc. Natl. Acad. Sci. 112 (2015) 10804–10809.
[15] N. Garg, D.S. Cheema, D. Pathak, Heterosis breeding in tomato involving *rin*, *nor* and *alc* alleles: a review of literature, Adv. Hortic. Sci. 22 (2008) 54–62.
[16] E. Kopeliovitch, H.D. Rabinowitch, Y. Mizrahi, N. Kedar, The potential of ripening mutants for extending the storage life of the tomato fruit, Euphytica 28 (1979) 99–104.
[17] K.C. Sink Jr., R.C. Herner, L.L. Knowlton, Chlorophyll and carotenoids of the *rin* tomato mutant, Can. J. Bot. 52 (1974) 1657–1660.
[18] E. Tigchelaar, W. McGlasson, M. Franklin, Natural and ethephon-stimulated ripening of F_1 hybrids of the ripening inhibitor (*rin*) and non-ripening (*nor*) mutants of tomato (*Lycopevsicon esculentum* Mill.), Aust. J. Plant Physiol. 5 (1978) 449–456.
[19] N.R. Leal, Heranca Da Conservacao Natural Pos Colheita De Frutos Do Tomateiro (*Lycopersicon Esculentum* MILL) I - Conservacao De Frutos E Anatomia Do Pericarpo De Hibridos Entre a Introducao Alcobaca or Alguns Cultivares, MSc thesis Vicosa University, Minas Gerais, Brazil, 1973.
[20] M. Saladié, A.J. Matas, T. Isaacson, M.A. Jenks, S.M. Goodwin, K.J. Niklas, R. Xiaolin, J.M. Labavitch, K.A. Shackel, A.R. Fernie, A. Lytovchenko, M.A. O'Neill, C.B. Watkins, J.K.C.C. Rose, A reevaluation of the key factors that influence tomato fruit softening and integrity, Plant Physiol. 144 (2007) 1012–1028.
[21] J. Casals, L. Pascual, J. Cañizares, J. Cebolla-Cornejo, F. Casañas, F. Nuez, Genetic basis of long shelf life and variability into Penjar tomato, Genet. Resour. Crop Evol. 59 (2012) 219–229.
[22] J. Vrebalov, I.L. Pan, A.J.M. Arroyo, R. McQuinn, M. Chung, M. Poole, J. Rose, G. Seymour, S. Grandillo, J. Giovannoni, V.F. Irish, Fleshy fruit expansion and ripening are regulated by the tomato *SHATTERPROOF* gene *TAGL1*, Plant Cell 21 (2009) 3041–3062.
[23] Y. Shima, M. Kitagawa, M. Fujisawa, T. Nakano, H. Kato, J. Kimbara, T. Kasumi, Y. Ito, Tomato FRUITFULL homologues act in fruit ripening via forming MADS-box transcription factor complexes with RIN, Plant Mol. Biol. 82 (2013) 427–438.
[24] C.H. Leseberg, C.L. Eissler, X. Wang, M.A. Johns, M.R. Duvall, L. Mao, Interaction study of MADS-domain proteins in tomato, J. Exp. Bot. 59 (2008) 2253–2265.
[25] M. Bemer, R. Karlova, A.R. Ballester, Y.M. Tikunov, A.G. Bovy, M. Wolters-Arts, P. Rossetto, G.C. Angenent, R.A. de Maagd, The tomato FRUITFULL homologs TDR4/FUL1 and MBP7/FUL2 regulate ethylene-independent aspects of fruit ripening, Plant Cell 24 (2012) 4437–4451.
[26] R.E. Schouten, B. Farneti, L.M.M. Tijskens, A.A. Alarcón, E.J. Woltering, Quantifying lycopene synthesis and chlorophyll breakdown in tomato fruit using remittance VIS spectroscopy, Postharvest Biol. Technol. 96 (2014) 53–63.
[27] R. Wang, E.Cda R. Tavano, M. Lammers, A.P. Martinelli, G.C. Angenent, R.A. de Maagd, Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis, Sci. Rep. 9 (2019) 1696.
[28] Y. Wahyuni, A.-R. Ballester, E. Sudarmonowati, R.J. Bino, A.G. Bovy, Metabolite biodiversity in pepper (*Capsicum*) fruits of thirty-two diverse accessions: variation in health-related compounds and implications for breeding, Phytochemistry. 72 (2011) 1358–1370.
[29] R.J. Bino, C.H.R. De Vos, M. Lieberman, R.D. Hall, A. Bovy, H.H. Jonker, Y. Tikunov, A. Lommen, S. Moco, I. Levin, The light-hyperresponsive *high pigment-2*^{dg} mutation of tomato: alterations in the fruit metabolome, New Phytol. 166 (2005) 427–438.
[30] Y. Tikunov, A. Lommen, C.H.R. de Vos, H.A. Verhoeven, R.J. Bino, R.D. Hall, A.G. Bovy, A novel approach for nontargeted data analysis for metabolomics. Large-scale profiling of tomato fruit volatiles, Plant Physiol. 139 (2005) 1125–1137.
[31] M. Expósito-Rodríguez, A.A. Borges, A. Borges-Pérez, J.A. Pérez, Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process, BMC Plant Biol. 8 (2008) 131.
[32] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods. 25 (2001) 402–408.
[33] C. Orfila, G.B. Seymour, W.G.T. Willats, I.M. Huxham, M.C. Jarvis, C.J. Dover, A.J. Thompson, J.P. Knox, Altered middle lamella homogalacturonan and disrupted deposition of (1→5)-α-l-Arabinan in the pericarp of *Cnr*, a ripening mutant of tomato, Plant Physiol. 126 (2001) 210–221.
[34] G.B. Seymour, Genetic identification and genomic organization of factors affecting fruit texture, J. Exp. Bot. 53 (2002) 2065–2071.
[35] K. Kovács, R.G. Fray, Y. Tikunov, N. Graham, G. Bradley, G.B. Seymour, A.G. Bovy, D. Grierson, Effect of tomato pleiotropic ripening mutations on flavour volatile biosynthesis, Phytochemistry 70 (2009) 1003–1008.
[36] E.A. Baldwin, M.O. Nisperos-Carriedo, M.G. Moshonas, Quantitative analysis of flavor and other volatiles and for certain constituents of two tomato cultivars during ripening, J. Am. Soc. Hortic. Sci. 116 (1991) 265–269.
[37] R.G. Buttery, R.M. Seifert, D.G. Guadagni, L.C. Ling, Characterization of additional volatile components of tomato, J. Agric. Food Chem. 19 (1971) 524–529.
[38] Y. Zhu, H.J. Klee, P.J. Sarnoski, Development and characterization of a high quality plum tomato essence, Food Chem. 267 (2018) 337–343.
[39] L. Alexander, Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening, J. Exp. Bot. 53 (2002) 2039–2055.
[40] B. Cara, J.J. Giovannoni, Molecular biology of ethylene during tomato fruit development and maturation, Plant Sci. 175 (2008) 106–113.
[41] J.J. Giovannoni, D. DellaPenna, A.B. Bennett, R.L. Fischer, Expression of a chimeric polygalacturonase gene in transgenic *rin* (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening, Plant Cell 1 (1989) 53.
[42] S. Uluisik, N.H. Chapman, R. Smith, M. Poole, G. Adams, R.B. Gillis, T.M.D. Besong, J. Sheldon, S. Stiegelmeyer, L. Perez, N. Samsulrizal, D. Wang, I.D. Fisk, N. Yang, C. Baxter, D. Rickett, R. Fray, B. Blanco-Ulate, A.L.T. Powell, S.E. Harding, J. Craigon, J.K.C. Rose, E.A. Fich, L. Sun, D.S. Domozych, P.D. Fraser, G.A. Tucker, D. Grierson, G.B. Seymour, Genetic improvement of tomato by targeted control of fruit softening, Nat. Biotechnol. 34 (2016) 950–952.
[43] C.C. Lashbrook, C. Gonzalez-Bosch, A.B. Bennett, Two divergent endo-beta-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscising flowers, Plant Cell 6 (1994) 1485.
[44] G. Chen, R. Hackett, D. Walker, A. Taylor, Z. Lin, D. Grierson, Identification of a specific isoform of tomato lipoxygenase (TomloxC) involved in the generation of fatty acid-derived flavor compounds, Plant Physiol. 136 (2004) 2641–2651.
[45] G.E. Bartley, P.V. Viitanen, K.O. Bacot, P.A. Scolnik, A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway, J. Biol. Chem. 267 (1992) 5036–5039.
[46] M. Fujisawa, T. Nakano, Y. Shima, Y. Ito, A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening, Plant Cell 25 (2013) 371–386.
[47] R. Karlova, F.M. Rosin, J. Busscher-Lange, V. Parapunova, P.T. Do, A.R. Fernie, P.D. Fraser, C. Baxter, G.C. Angenent, R.A. de Maagd, Transcriptome and metabolite profiling show that APETALA2a is a major regulator of tomato fruit ripening, Plant Cell 23 (2011) 923–941.
[48] C. Martel, J. Vrebalov, P. Tafelmeyer, J.J. Giovannoni, The tomato MADS-Box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner, Plant Physiol. 157 (2011) 1568–1579.
[49] S. Li, H. Xu, Z. Ju, D. Cao, H. Zhu, D. Fu, D. Grierson, G. Qin, Y. Luo, B. Zhu, The *RIN-MC* fusion of MADS-box transcription factors has transcriptional activity and modulates expression of many ripening genes, Plant Physiol. 176 (2018) 891–909.
[50] P. Lü, S. Yu, N. Zhu, Y.-R. Chen, B. Zhou, Y. Pan, D. Tzeng, J.P. Fabi, J. Argyris, J. Garcia-Mas, N. Ye, J. Zhang, D. Grierson, J. Xiang, Z. Fei, J. Giovannoni, S. Zhong, Genome encode analyses reveal the basis of convergent evolution of fleshy fruit ripening, Nat. Plants 4 (2018) 784–791.
[51] S. Osorio, R.T. Carneiro, A. Lytovchenko, R. McQuinn, I. Sørensen, J.G. Vallarino, J.J. Giovannoni, A.R. Fernie, J.K. Rose, Genetic and metabolic effects of ripening nutations and vine detachment on tomato fruit quality, Plant Biotechnol. J. (2019) pbi.13176.
[52] Y. Tadmor, E. Fridman, A. Gur, O. Larkov, E. Lastochkin, U. Ravid, D. Zamir, E. Lewinsohn, Identification of *malodorous*, a wild species allele affecting tomato aroma that was selected against during domestication, J. Agric. Food Chem. 50 (2002) 2005–2009.
[53] Y.M. Tikunov, J. Molthoff, R.C.H. de Vos, J. Beekwilder, A. van Houwelingen, J.J.J. van der Hooft, M. Nijenhuis-de Vries, C.W. Labrie, W. Verkerke, H. van de Geest, M. Viquez Zamora, S. Presa, J.L. Rambla, A. Granell, R.D. Hall, A.G. Bovy, NON-SMOKY GLYCOSYLTRANSFERASE1 prevents the release of smoky aroma from tomato fruit, Plant Cell 25 (2013) 3067–3078.
[54] Y. Gao, N. Zhu, X. Zhu, M. Wu, C.-Z. Jiang, D. Grierson, Y. Luo, W. Shen, S. Zhong, D.-Q. Fu, G. Qu, Diversity and redundancy of the ripening regulatory networks revealed by the fruitENCODE and the new CRISPR/Cas9 *CNR* and *NOR* mutants, Hortic. Res. 6 (2019) 39.
[55] E. Van der Knaap, Z.B. Lippman, S.D. Tanksley, Extremely elongated tomato fruit controlled by four quantitative trait loci with epistatic interactions, Theor. Appl. Genet. 104 (2002) 241–247.
[56] H. Koltai, D.M.K. Bird, Epistatic repression of *PHANTASTICA* and class 1 *KNOTTED* genes is uncoupled in tomato, Plant J. 22 (2000) 455–459.
[57] Y. Ito, A. Nishizawa-Yokoi, M. Endo, M. Mikami, Y. Shima, N. Nakamura, E. Kotake-Nara, S. Kawasaki, S. Toki, Re-evaluation of the *rin* mutation and the role of *RIN* in the induction of tomato ripening, Nat. Plants 3 (2017) 866–874.
[58] R.A. Veitia, Exploring the molecular etiology of dominant-negative mutations, Plant Cell 19 (2007) 3843–3851.
[59] M. Duval, T.-F. Hsieh, S.Y. Kim, T.L. Thomas, Molecular characterization of *AtNAM*: a member of the *Arabidopsis* NAC domain superfamily, Plant Mol. Biol. 50 (2002) 237–248.
[60] Q. Xie, G. Frugis, D. Colgan, N.H. Chua, *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development, Genes Dev. 14 (2000) 3024–3036.