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• Fruit ripening involves a complex interplay between ethylene and ripening-associated transcriptional regulators. Ethylene Response Factors (*ERF*s) are downstream components of ethylene signaling, known to regulate the expression of ethylene-responsive genes. Although fruit ripening is an ethylene-regulated process, the role of ERFs remains poorly understood.

• The role of Sl-ERF.B3 in tomato (*Solanum lycopersicum*) fruit maturation and ripening is addressed here using a chimeric dominant repressor version (ERF.B3-SRDX).

• Over-expression of *ERF.B3-SRDX* results in a dramatic delay of the onset of ripening, enhanced climacteric ethylene production and fruit softening, and reduced pigment accumulation. Consistently, genes involved in ethylene biosynthesis and in softening are up-regulated and those of carotenoid biosynthesis are down-regulated. Moreover, the expression of ripening regulators, such as RIN, NOR, CNR and HB-1, is stimulated in ERF.B3-SRDX dominant repressor fruits and the expression pattern of a number of ERFs is severely altered.

• The data suggest the existence of a complex network enabling interconnection between ERF genes which may account for the pleiotropic alterations in fruit maturation and ripening. Overall, the study sheds new light on the role of Sl-ERF.B3 in the transcriptional network controlling the ripening process and uncovers a means towards uncoupling some of the main ripening-associated processes.

The maturation and ripening of fleshy fruits are developmental processes unique to plants. Although specific fruit ripening characteristics vary among species, fruit ripening can be generally described as a complex, genetically programmed process that culminates in dramatic changes in color, texture, flavor, aroma and nutritional characteristics (Carrari & Fernie, 2006). In the case of fleshy fruits, these changes not only make fruit attractive for seed dispersal organisms, but also provide essential vitamins, minerals and antioxidants (phenolics, folate, lycopene and β-carotene) for human diet (Seymour *et al.*, 1993; Fraser *et al.*, 2009; Chung *et al.*, 2010).

Fruits have been classically categorized into climacteric and non-climacteric based on increased ethylene synthesis and a concomitant rise in the rate of respiration during ripening. Climacteric fruits display a burst in respiration at the onset of ripening, in contrast with non-climacteric fruits. Climacteric fruits, such as tomatoes, bananas and apples, also show increased biosynthesis of the gaseous hormone ethylene, which is a fundamental signal in climacteric fruit ripening (Vrebalov *et al.*, 2002; Alba *et al.*, 2005). In the ethylene biosynthetic pathway, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) catalyze the conversion of *S*-adenosyl-L-methionine (SAM) to ACC and of ACC to ethylene, respectively (Adams & Yang, 1979; Bleecker & Kende, 2000). Autocatalytic ethylene synthesis at the onset of tomato fruit ripening is mainly mediated through ethylene-stimulated expression of *ACS2*, *ACS4*, *ACO1* and *ACO4* (Barry & Giovannoni, 2007). Unraveling the regulation of the ethylene signaling pathway is important to understanding the processes of fruit ripening.

Tomato possesses many favorable genetic characteristics, such as simple diploid genetics, relatively small genome size, short generation time, efficient genetic transformation and distinct ripening phenotypes, making it a primary model system for the study of the molecular basis of fleshy fruit development and the role of ethylene in climacteric fruit ripening. The adaptation of a range of technological tools and the generation of new biological

resources on tomato (e.g. expressed sequence tag (EST) databases, TILLING (targeting-induced local lesions in genomes) resources, genetic and physical maps) have led to significant progress in our understanding of the molecular mechanisms underlying the ripening process through the identification of the associated key regulatory genes (Pirrello *et al.*, 2009). Indeed, tomato fruit ripening has been thoroughly characterized with regard to metabolic changes impacting softening, the accumulation of sugars, acids and lycopene, chlorophyll degradation and dramatic increases in ethylene and flavor volatiles (Chung *et al.*, 2010).

The investigation of tomato mutants affected in fruit development and ripening (mainly ripening-deficient mutants), such as *ripening inhibitor* (*rin*; Vrebalov *et al.*, 2002), *Colorless non-ripening* (*Cnr*; Manning *et al.*, 2006), *non-ripening* (*nor*; Giovannoni, 2004), *Green-ripe* (*Gr*; Barry & Giovannoni, 2006) and *Never-ripe* (*Nr*; Wilkinson *et al.*, 1995), led to the isolation of genes acting upstream of ethylene in the ripening cascade, and involved in determining the attainment of competence to ripen (Barry & Giovannoni, 2007). The *RIN*, *CNR* and *NOR* genes encode transcription factors regulating the expression of genes responsible for various fruit ripening processes, including ethylene and carotenoid biosynthesis (Vrebalov *et al.*, 2002; Giovannoni, 2004; Manning *et al.*, 2006; Martel *et al.*, 2011; Fujisawa *et al.*, 2013). The *Gr* gene encodes a still poorly defined component of ethylene signal transduction, whereas *Nr* encodes an ethylene receptor important for both fruit and non-fruit ethylene responses (Lanahan *et al.*, 1994; Barry & Giovannoni, 2006). Other ripening transcriptional regulators have recently been characterized via functional studies in transgenic plants, including *LeHB-1*, which regulates directly ACC oxidase expression (Lin *et al.*, 2008), and *TAGL1*, a MADS box transcription factor, which links early fruit fleshy expansion with downstream ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009). The putative transcription factor, *SlAP2a*, a member of the APETALA2/Ethylene Response Factor (AP2/ERF) superfamily, has also been described recently as a negative regulator of fruit ripening and of ethylene production (Chung *et al.*, 2010; Karlova *et al.*, 2011). Unraveling the transcriptional networks that regulate fruit ripening is crucial for the understanding of this complex process.

*ERF*s are plant-specific transcription factors, belonging to the large AP2/ERF superfamily (Riechmann *et al.*, 2000). Proteins encoded by this family have a highly conserved DNA-binding domain, known as the AP2 domain, containing 58–59 amino acids involved in the high-affinity binding to target DNA sequences (Allen *et al.*, 1998). A growing number of investigations have suggested that, through interactions with multiple *cis*-acting elements found in the promoter regions of ethylene-responsive genes, including the GCC box and dehydration-responsive element/C-repeat (DRE/CRT), ERF proteins play a critical role during plant development and adaptation to stress conditions (Ohme-Takagi & Shinshi, 1995; Wu *et al.*, 2002; Wan *et al.*, 2011). In different plant species, ERFs have been shown to be involved in hormonal signaling, responses to biotic and abiotic stresses, developmental processes, metabolic regulation, ethylene biosynthesis and fruit ripening (Ohme-Takagi & Shinshi, 1995; Fujimoto *et al.*, 2000; Van der Fits & Memelink, 2000; Wu *et al.*, 2002; Dubouzet *et al.*, 2003; Zhang *et al.*, 2009; Lee *et al.*, 2012; Pirrello *et al.*, 2012; Zhao *et al.*, 2012). In tomato, although *SlERF6*, which corresponds to *Sl-ERF.E4* in the new unified nomenclature by Pirrello *et al.* (2012), has been reported to play an important role in fruit ripening by integrating the ethylene and carotenoid pathways (Lee *et al.*, 2012), the role of most ERF proteins in the ripening process awaits elucidation. The present study describes the critical role of *Sl-ERF.B3*, a member of the tomato *ERF* multi-family genes, in fruit ripening.

To date, no *ERF-like* mutants have been identified in tomato and, as reported previously (Liu *et al.*, 2013), a classical reverse genetics approach based on the down- and up-regulation of *ERF* genes failed to provide sufficient clues regarding their functional significance. In an attempt to overcome the experimental limitations caused by the functional redundancy among members of the *ERF* gene family, we generated a dominant repressor version of *ERF.B3* (*ERF.B3-SRDX*) using Chimeric Repressor Silencing Technology (CRES-T). *Sl-ERF.B3* was selected as a target ERF in the present study because it has been shown previously to be an activator of GCC box-containing promoters and its transcripts accumulate on ethylene treatment, suggesting its putative involvement in ethylene-regulated processes (Tournier *et al.*, 2003; Pirrello *et al.*, 2012; Liu *et al.*, 2013). This gene has been described previously as an important regulator of ethylene response and plant development (Liu *et al.*, 2013). Using the CRES-T strategy, we show here that *Sl-ERF.B3* plays a critical role in fruit development and ripening. Moreover, the altered expression of major regulators of fruit ripening, including *RIN*, *CNR*, *NOR* and *HB-1*, in *ERF.B3-SRDX* lines reveals that *Sl-ERF.B3* is a new regulator involved in the regulatory network controlling the ripening process.

Materials and Methods

Plant materials and growth conditions

Tomato (*Solanum lycopersicum* L. cv MicroTom) plants were transferred to soil and grown under standard glasshouse conditions. Conditions in the culture chamber room were set as follows: 14 h day : 10 h night cycle, 25 : 20°C day : night temperature, 80% hygrometry, 250 μmol m^{-2} s^{-1} light intensity. For the measurement of time to ripening, flowers were tagged at anthesis and the number of days from anthesis to the breaker (Br) stage was counted. More than 15 fruits of each genotype were used for this analysis.

Plant transformation

To generate the *ERF.B3-SRDX* transgenic plants, the coding sequence of *Sl-ERF.B3* missing the stop codon was amplified by PCR from a tomato fruit cDNA library. This coding region was cloned via blunt-end ligation into the *Sma*I site of p35SSRDXG in frame to the region that encodes the SRDX repression domain (LDLDLELRLGFA) from SUPERMAN (Hiratsu *et al.*, 2003; Mitsuda *et al.*, 2006). The transgene cassette was transferred into the destination vector pBCKH, which was derived from the plant

transformation vector pBIG-HYG (Becker, 1990) using the gateway LR reaction (Invitrogen Corp.) *Agrobacterium tumefaciens*-mediated transformation of tomato plants was carried out according to Wang *et al.* (2005), and transformed lines were selected on a hygromycin-containing medium. All experiments were carried out using homozygous lines from F3 or later generations.

RNA isolation and quantitative real-time-polymerase chain reaction (qRT-PCR)

Fruits were harvested, frozen in liquid nitrogen and stored at −80°C. Total RNA from the pericarp of at least five individual fruits at each developmental stage analyzed in this article was extracted using a Plant RNA Purification Reagent (Invitrogen, Cat. No. 12322-012) according to the manufacturer's instructions. Total RNA was then DNase-treated (Invitrogen, Cat. No. AM1906) to remove contaminating genomic DNA. First-strand cDNA was reverse transcribed from 2 μg of total RNA using an Omniscript Reverse Transcription kit (Qiagen, Cat. No. 74904) following the manufacturer's instructions. Gene-specific primers were designed by Primer Express software (PE-Applied Biosystems, Foster City, CA, USA) and were further checked using BLAST against all tomato unigenes (Tomato unigene database). Quantitative real-time PCR analyses were performed as described previously (Pirrello *et al.*, 2006). The primer sequences used in this study are listed in Supporting Information Table S1.

LC-MS analysis of fruit carotenoids

Carotenoid extractions were performed as described previously (Fantini *et al.*, 2013). Briefly, 5 mg of ground lyophilized tomato fruit powder were extracted with chloroform (spiked with 50 mg l^{-1} DL-α-tocopherol acetate as internal standard) and methanol (2 : 1 by volume); subsequently, 1 volume of 50 mM Tris buffer (pH 7.5, containing 1 M NaCl) was added and the samples were kept for 20 min on ice. After centrifugation (15 000 ***g*** for 10 min at 4°C), the organic hypophase was collected and the aqueous phase was re-extracted with the same amount of spiked chloroform. The combined organic phases were then dried by speedvac and resuspended in 100 μl of ethyl acetate. For each genotype, at least five independent extractions were performed. LC-MS analyses were carried out using a Discovery LTQ-Orbitrap mass spectrometry system operating in atmospheric pressure chemical ionization (APCI) and in positive mode, coupled to an Accela U-HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). LC separations were performed using a C30 reverse-phase column (100 × 3.0 mm) from YMC (YMC Europe GmbH, Schermbeck, Germany). The mobile phases used were methanol (A), water–methanol (20 : 80 by volume) containing 0.2% ammonium acetate (B) and *tert*-methyl butyl ether (C). The gradient was 95% A : 5% B for 1.3 min, followed by 80% A : 5% B : 15% C for 2.0 min and by a linear gradient to 30% A : 5% B : 65% C over 9.2 min. UV–visible detection was performed continuously from 220 to 700 nm with an online Accela Surveyor photodiode array detector (PDA; Thermo Fisher Scientific). All solvents used were LC-MS grade quality (CHROMASOLV® from Sigma-Aldrich). Carotenoids were quantified on the basis of the internal standard amounts, obtained through comparison with peak areas of known amounts of external standard LC-MS runs and by extinction coefficient correction. For APCI-MS ionization of xanthophylls (0–6 min of LC-MS run), nitrogen was used as sheath and auxiliary gas, set to 40 and 20 units, respectively, the vaporizer temperature and capillary temperature were 300 and 250°C, respectively, the discharge current was set to 4.0 μA, and the capillary voltage and tube lens settings were 27 V and 90 V, respectively. APCI-MS ionization of carotenes (6–14 min of LC-MS runs) was performed with the following parameters: 30 and 10 units of nitrogen sheath and auxiliary gas, respectively; vaporizer and capillary temperatures of 300 and 250°C, respectively; discharge current of 5.0 μA; capillary voltage and tube lens settings of 0 and 95 V, respectively. Identification was performed as reported previously (Fantini *et al.*, 2013), and on the basis of the *m/z* accurate masses, as reported on Pubchem (http://pubchem.ncbi.nlm.nih.gov/) or Chemspider (http://www.chemspider.com) for the identification of monoisotopic masses, or on Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/) for adduct ion detection.

Color measurement

The *L*, *a* and *b* values were measured with a Minolta chromameter (CR-200, 78903131; Ramsey, NJ, USA) on fruit at different stages during fruit ripening. The chromameter was calibrated against a standard white tile. Hue angle values were calculated according to the following equation: Hue angle $= \tan^{-1}(b/a)$ if $a > 0$ or $180 + \tan^{-1}(b/a)$ if $a < 0$.

Fruit firmness

Fifteen fruits from each line were harvested at the Br stage and the firmness was assessed using Harpenden calipers (British Indicators Ltd, Burgess Hill, UK) as described by Ecarnot *et al.* (2013).

Ethylene measurement

Fruits at each developmental stage were harvested and placed in open 120-ml jars for 2 h to minimize the effect of wound ethylene caused by picking. Jars were then sealed and incubated at room temperature for 35 min, and 1 ml of headspace gas was injected into an Agilent 7820A gas chromatograph equipped with a flame ionization detector (Agilent, Santa Clara, CA, USA). Samples were compared with reagent-grade ethylene standards of known concentration and normalized for fruit weight.

Accession numbers

Gene ID data for the genes described in this article are listed in Table S2.

Results

Expression patterns of tomato *ERF* genes during fruit ripening

Ethylene is known to play a critical role in fruit development and ripening, and ERFs are considered to be the primary actors in mediating responses to this hormone. To gain further insight into the expression of members of the tomato ERF gene family during the ripening process, the accumulation of *Sl-ERF* transcripts was assessed by quantitative RT-PCR at different stages of fruit ripening. Although the expression dynamics of most *ERF* genes suggest their involvement in the ripening process, no clear link could be established between their repressor or activator function and their pattern of expression. The data indicated (Fig. 1) that, among the 25 *ERFs* tested, transcript accumulation of five genes (*Sl-ERF.B3*, *C2*, *E2*, *E4* and *F2*) peaked at the Br stage and then decreased at later ripening stages. The transcript levels of 11 genes (*Sl-ERF.A2*, *A3*, *B1*, *B2*, *C1*, *C3*, *D1*, *D2*, *F3*, *F5* and *G2*) showed an increase, peaking at 3 d post-Br (Br + 3), and then declined. A distinct expression pattern was displayed by a group of *ERF* genes (*Sl-ERF.A1*, *C6*, *D3*, *D4*, *E1*, *F1*, *F4* and *H1*) that underwent a steady increase in transcript accumulation throughout ripening.

Sl-ERF.B3 shows a fruit development- and ripening-related expression pattern

Of particular interest, *Sl-ERF.B3* transcript accumulation showed a dramatic increase at the onset of ripening and maintained high levels at subsequent post-Br stages, suggesting that its expression may be continuously required throughout the ripening process. This observation motivated the further assessment of *Sl-ERF.B3* transcript accumulation in vegetative and reproductive tissues by quantitative RT-PCR (Fig. 2a). Expression analysis in various plant tissues and organs, including stem, root, leaf, flower and a

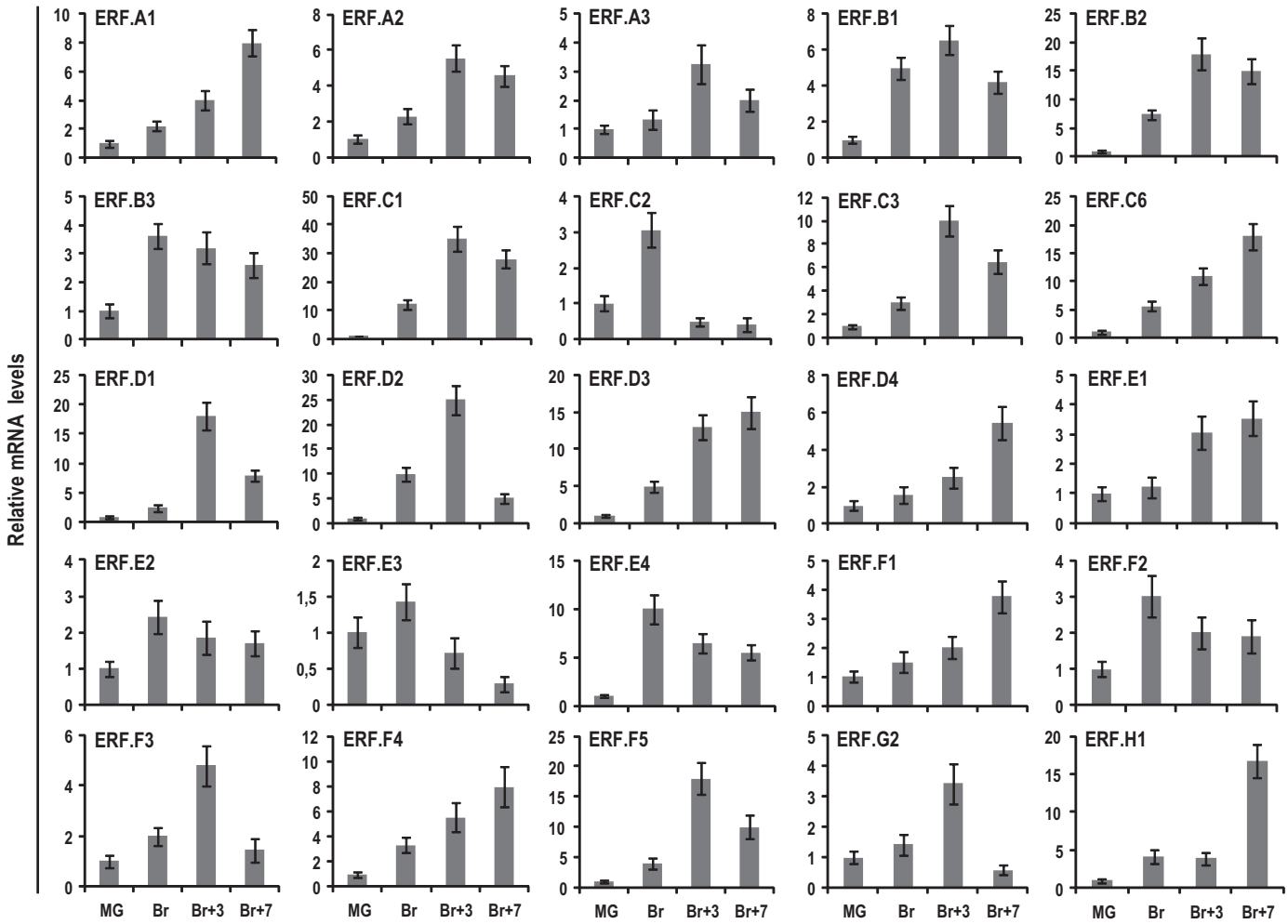

Fig. 1 Ripening-associated pattern of expression of tomato (*Solanum lycopersicum*) *Ethylene Response Factor* (*ERF*) genes. Transcript accumulation of *ERF* genes was assessed by quantitative real-time-polymerase chain reaction (qRT-PCR) at different ripening stages. MG, mature green fruit; Br, breaker stage fruit; Br+3, 3 d post-breaker; Br+7, 7 d post-breaker. The relative mRNA levels of each *ERF* gene at the mature green (MG) stage were standardized to 1.0, referring to the *Sl-Actin* gene as an internal control. Values represent the means of three biological replicates, and vertical bars represent ± SD of the means.

series of fruit developmental stages, indicated that the accumulation of *Sl-ERF.B3* transcripts was relatively high in both vegetative and reproductive tissues (Fig. 2a).

The functional significance of this tomato ERF family member was first addressed by attempting to alter its expression using antisense or over-expression strategies. However, both approaches failed to provide significant clues on the physiological role of *Sl-ERF.B3*, which prompted the use of a dominant repressor version of the gene (*ERF.B3-SRDX*), relying on CRES-T. This technology has been developed to study the consequences of silencing of the target genes of single transcription factors, and has also been used to overcome the experimental limitations caused by functional redundancy of transcription factor families (Hiratsu *et al.*, 2003). Ten transgenic *ERF.B3-SRDX* lines showed a characteristic phenotype with different expressivity, and three (*SR1*, *SR2* and *SR3*) were selected for further molecular and physiological studies. The relative expression levels of the *ERF.B3-SRDX* transcript in fruit tissues of the three independent lines was assessed by quantitative RT-PCR using primers specific for the transgene (Fig. 2b).

Altered fruit development in *ERF.B3-SRDX* dominant repressor lines

One of the most evident phenotypes displayed by *ERF.B3-SRDX* transgenic lines was the altered fruit shape and reduced size (Fig. 3a). Wild-type tomato fruits (Fig. 3a) were round in shape, in contrast with the *ERF.B3-SRDX* fruits, which were heart shaped with bumpy areas present intermittently on the surface of the fruit (Fig. 3a). Changes in fruit anatomy also included a thicker pericarp and decreased jelly formation, with enhanced pericarp to fruit radius ratio when compared with the wild-type (Figs 3a, S1a,b). As a consequence of the smaller size, the mean weight of *ERF.B3-SRDX* fruits was significantly reduced (Fig. 3b). The number of seeds was also dramatically decreased in *ERF.B3-SRDX* fruits compared with the wild-type, and the average seed number decreased from 25 per fruit in the wild-type to six in *ERF.B3-SRDX* lines (Fig. S2a). In addition, the seeds showed reduced size (Fig. S2b) and, in the *ERF.B3-SRDX* line showing the strongest phenotype, seed weight was less than half that of the wild-type (Fig. S2c). Interestingly, although *ERF.B3-SRDX* lines yielded hairless seeds with altered morphology (Fig. S2b), the seeds produced viable plants.

ERF.B3-SRDX fruits fail to display a red-ripe phenotype

In addition to the altered fruit shape and size, *ERF.B3-SRDX* lines exhibited distinct ripening features. The ripening-related phenotypes were investigated in wild-type and *ERF.B3-SRDX* lines using fruits at different developmental stages sampled from the same truss. Dramatic changes were revealed with regard to both the time at which the ripening started and the speed at which it proceeded in the dominant repressor lines. The onset of ripening occurred with at least 2 wk delay in *ERF.B3-SRDX* lines (57 d post-anthesis) compared with the wild-type (41 d post-anthesis), suggesting that the attainment of competence to ripen was dramatically delayed in the transgenic lines (Table 1). Moreover, once the ripening process started at the Br stage, the color change proceeded much more slowly in *ERF.B3-SRDX* fruits compared with the wild-type (Fig. 4a). Indeed, in contrast with wild-type fruit, which reached the red-ripe stage 5 d post-Br (Br + 5), *ERF.B3-SRDX* fruits remained orange at the Br + 10 stage (Fig. 4a). The assessment of color change via measurement of the evolution of hue angle values, indicative of color saturation, further emphasized the difference between the wild-type and dominant repressor lines throughout the ripening process (Fig. 4b). The value of the hue angle was even higher for *ERF.B3-SRDX* fruit at Br + 10 than for the wild-type at Br + 5, thus confirming the orange-ripe phenotype observed visually (Fig. 4b).

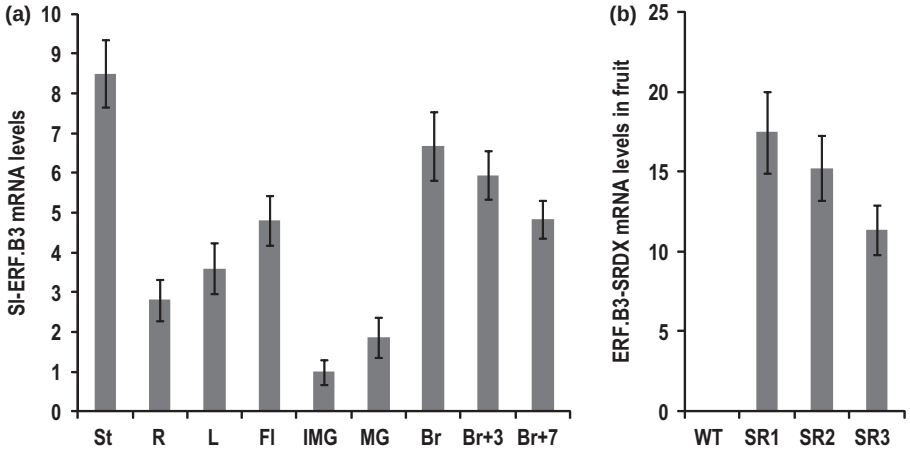

Fig. 2 *Sl-ERF.B3* gene expression in vegetative and reproductive tomato (*Solanum lycopersicum*) tissues. (a) Accumulation of *Sl-ERF.B3* transcripts was assessed by quantitative real-time-polymerase chain reaction (qRT-PCR) in stem (St), root (R), leaf (L), flower (Fl), immature fruit (IMG), mature green fruit (MG), breaker fruit (Br), 3 d post-breaker fruit (Br+3) and 7 d post-breaker fruit (Br+7). The relative mRNA levels of *Sl-ERF.B3* at the immature green stage were standardized to 1.0, referring to the *Sl-Actin* gene as an internal control. (b) Transcript accumulation corresponding to the chimeric *ERF.B3-SRDX* gene in three independent *ERF.B3-SRDX* dominant repressor lines (*SR1*, *SR2* and *SR3*) and control wild-type (WT) fruit at the 3 d post-breaker stage. Values are means ± SD of three biological replicates.

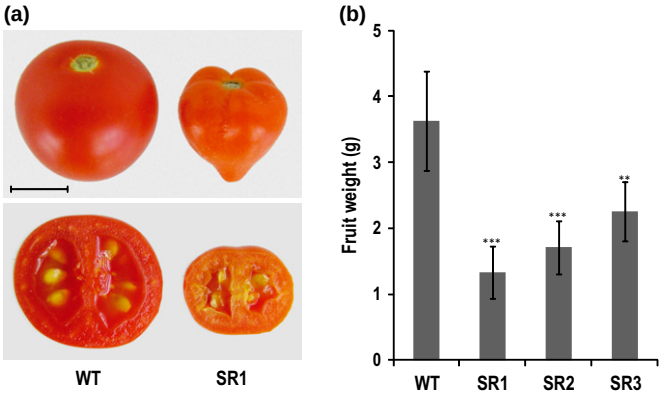

Fig. 3 Fruit morphology in wild-type (WT) and *ERF.B3-SRDX* tomato (*Solanum lycopersicum*) lines. (a) Altered fruit shape and size in *ERF.B3-SRDX* fruits. Bar, 1.0 cm. (b) Fruit weight is significantly reduced in *ERF.B3-SRDX* lines compared with the wild-type. A total of 50 fruits was used for each measurement and the values shown are the means ± SD. **, $0.001 < P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). *SR1–SR3* are three independent *ERF.B3-SRDX* lines.

ERF.B3-SRDX fruits show fast softening and elevated ethylene production

To uncover whether the failure to reach the red-ripe stage in dominant repressor fruits resulted from the incapacity to enter a ripening process, other major ripening-associated features, such as softening and climacteric increase in ethylene production, were investigated. The evolution of firmness determined from the Br stage to 20 d post-Br (Br + 20) clearly showed that *ERF.B3-SRDX* transgenic fruits underwent significantly faster softening than control fruits (Fig. 4c, Table S3). Given that fruit softening is highly regulated by ethylene, the production of ripening-associated ethylene was assessed in *ERF.B3-SRDX* fruits. As shown in Fig. 4(d), the accelerated softening observed in transgenic fruits was associated with a dramatic increase in climacteric ethylene production (Fig. 4d), which reached a maximum of three to four times higher than that in wild-type fruit. Altogether, these data indicate that, once the ripening process is triggered, it proceeds more rapidly in the *ERF.B3-SRDX* repressor than in wild-type fruit.

Decreased lycopene and increased β-carotene levels are responsible for the orange-ripe phenotype in *ERF.B3-SRDX* fruits

To investigate the cause of the altered pigmentation in *ERF.B3-SRDX* fruits, LC-PDA-MS analysis of carotenoid levels was performed on wild-type and *ERF.B3-SRDX* fruits at both the Br and Br + 7 stages. Total carotenoid content was reduced by 38–51% in dominant repressor lines at Br and post-Br stages (Fig. 5, Table S4). Notably, levels of lycopene and its precursors, phytoene, phytofluene, ζ-carotene and neurosporene, were decreased significantly in *ERF.B3-SRDX* fruits at the ripe stage (Br + 7) and, concomitantly, a sharp increase in β-carotene content was observed (Fig. 5, Table S4), in keeping with the orange-ripe phenotype. Moreover, dominant repressor fruits accumulated higher levels of α-carotene than did wild-type fruits (Fig. 5, Table S4).

Table 1 Time period from anthesis to the breaker stage in wild-type and *ERF.B3-SRDX* tomato (*Solanum lycopersicum*) lines

Lines	Days
Wild-type	41.49 ± 2.49
SR1	59.34 ± 3.27 ***
SR2	56.18 ± 2.19 **
SR3	54.82 ± 3.46 **

Values represent means ± SD of at least 15 fruits for each line. **, $0.001 < P < 0.01$; ***, $P < 0.001$ (Student's *t*-test).

To uncover the molecular basis of the altered carotenoid composition in *ERF.B3-SRDX* lines, we examined the transcript levels of genes involved in the carotenoid biosynthesis pathway at different stages of fruit ripening by quantitative RT-PCR (Fig. 6). Although the phytoene synthase (*PSY1*) transcript showed, in *ERF.B3-SRDX* fruits, a ripening-regulated pattern similar to that of the wild-type, its levels were dramatically reduced at all ripening stages (Fig. 6). *PSY1* is a key regulator of flux through the carotenoid pathway and its repression is consistent with the reduction in lycopene and total carotenoids in Br stage fruits (Fig. 5). A decrease in phytoene desaturase (*PDS*) expression levels was also observed in *ERF.B3-SRDX* fruits (Fig. 6). By contrast, transcript accumulation of all three lycopene β-cyclases (β-*LCY1*, β-*LCY2*, *CYC-β*) was markedly elevated in *ERF.B3-SRDX* fruits compared with the wild-type (Fig. 6), probably accounting for the increased α- and β-carotene content in *ERF.B3-SRDX* lines (Fig. 5). The data indicate that the dominant repressor version of *ERF.B3* leads to decreased expression of *PSY1* and *PDS*, and increased expression of lycopene β-cyclases, thus resulting in a modified lycopene to β-carotene ratio.

Ethylene- and ripening-related genes are highly induced in *ERF.B3-SRDX*-expressing fruits

To gain some insight at the molecular level into the ripening of *ERF.B3-SRDX* fruits, we examined the transcript accumulation of a set of ripening-related genes. Once the ripening process had started, the expression of ethylene biosynthesis genes, such as *ACS2*, *ACS4* and *ACO1*, was significantly higher in *ERF.B3-SRDX*-expressing fruits than in the wild-type (Fig. 7). Transcript accumulation of these genes was similarly low in transgenic and control fruit at the mature green stage, but was more strongly induced after the Br stage in the dominant repressor lines, concomitant with the rise in ethylene production. In addition, mRNA accumulation of ethylene-inducible genes, such as *E4* and *E8*, was also increased in *ERF.B3-SRDX* lines (Fig. 7), consistent with the elevated ethylene production. The transcript accumulation of a major fruit polygalacturonase gene, *PG2A*, involved in ripening-related cell wall metabolism, was significantly induced in *ERF.B3-SRDX* fruits (Fig. 7), in line with the enhanced softening phenotype. Similarly, the

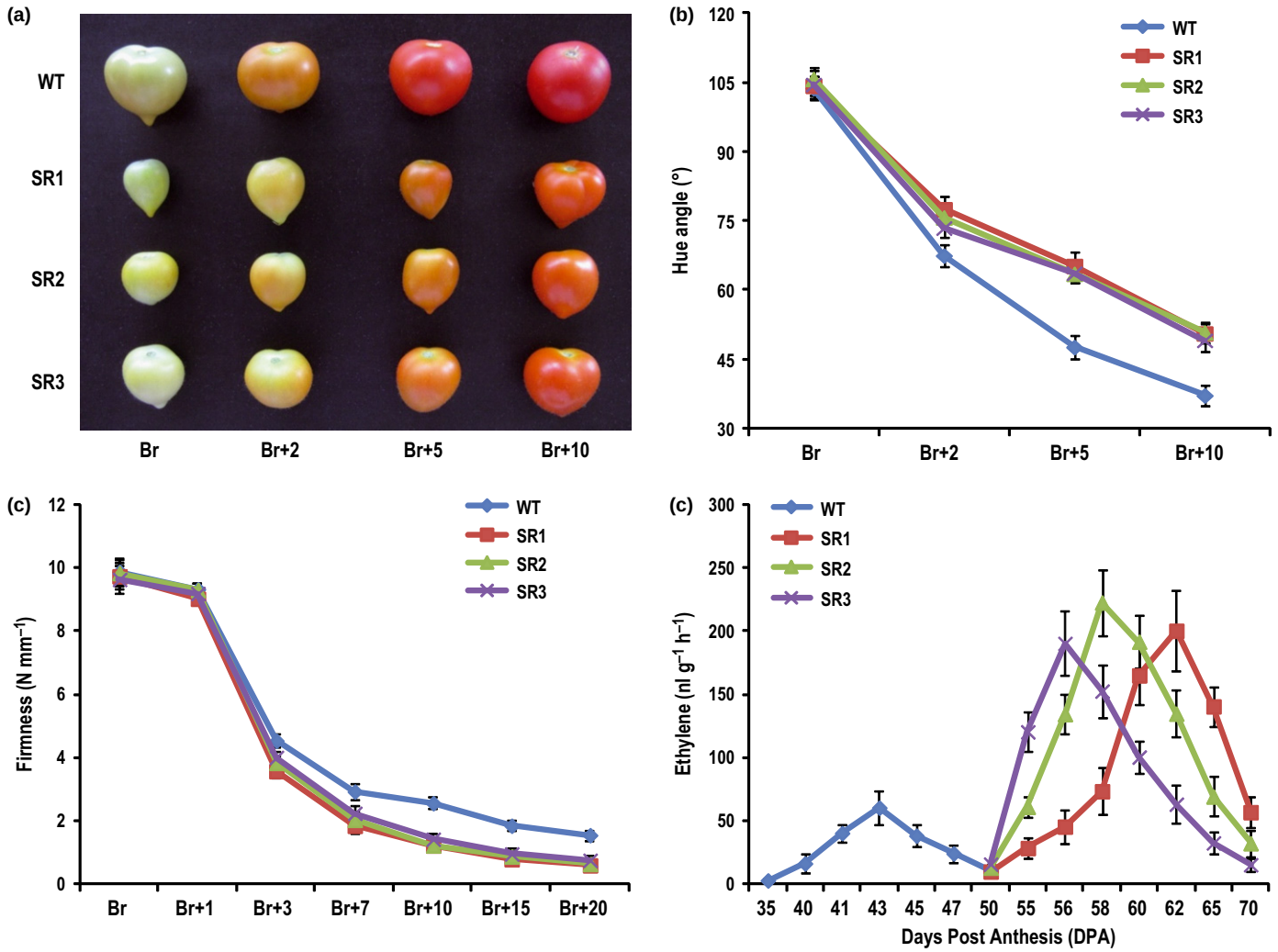

Fig. 4 Altered ripening features of *ERF.B3-SRDX* tomato (*Solanum lycopersicum*) fruits. (a) Different stages of fruit ripening of wild-type (WT) and *ERF.B3-SRDX* lines. Fruits from three independent transgenic lines show delayed color development, never reaching a full red color. Br, breaker stage; Br+2, 2 d post-breaker stage; Br+5, 5 d post-breaker stage; Br+10, 10 d post-breaker stage. (b) Changes in hue angle in WT and *ERF.B3-SRDX* lines during different ripening stages. (c) Fruit firmness of wild-type and *ERF.B3-SRDX* fruits. Fruits were harvested at the breaker stage, kept at room temperate and the firmness was measured at different stages. A total of 15 fruits was used for each measurement and the values shown are the means ± SD. (d) Ethylene production of wild-type and *ERF.B3-SRDX* fruits was assessed at different ripening stages indicated as days post-anthesis (DPA). Values represent means of at least 10 individual fruits. Vertical bars represent SD. In the wild-type, 35 DPA corresponds to the mature green (MG) stage and 40 DPA to the breaker (Br) stage. In *ERF.B3-SRDX* lines, 50 DPA corresponds to the mature green (MG) stage and 55 DPA to the breaker (Br) stage. *SR1–SR3* are three independent *ERF.B3-SRDX* lines.

expression of key regulatory genes of the ripening process, such as *RIN*, *NOR* and *CNR*, was increased at post-Br stages compared with the wild-type, although their induction took place later than in control fruit (Fig. 8). The altered expression pattern of these genes in the *ERF.B3-SRDX* fruits is consistent with the dramatically delayed onset of ripening in the transgenic fruits. Moreover, the mRNA levels of *LeHB-1*, another ripening regulator gene, were higher in *ERF.B3-SRDX* lines at all ripening stages (Fig. 8). By contrast, the expression of *TAGL1*, a tomato *SHATTERPROOF* gene, and *AP2a*, an *AP2/ERF* family gene acting as a negative regulator of fruit ripening, did not display significant changes in *ERF.B3-SRDX* dominant repressor fruits compared with the wild-type (Fig. 8).

A number of *ERF* gene family members show altered expression in the *ERF.B3-SRDX* lines

Considering the putative role of ERFs in mediating ethylene responses, and given the major role devoted to ethylene in regulating the ripening process, we examined the transcript levels of 25 *Sl-ERF* genes in both wild-type and *ERF.B3-SRDX* fruits. A dramatic change in the transcript levels for a number of *ERF* genes was revealed in the dominant repressor lines (Fig. 9). That is, among the 25 *Sl-ERF*s that showed detectable transcript accumulation, 10 were significantly down-regulated in the *ERF.B3-SRDX* dominant repressor lines, and eight *Sl-ERF*s displayed up-regulation in the transgenic lines (Fig. 9). It is noteworthy that the accumulation of transcripts corresponding to *Sl-*

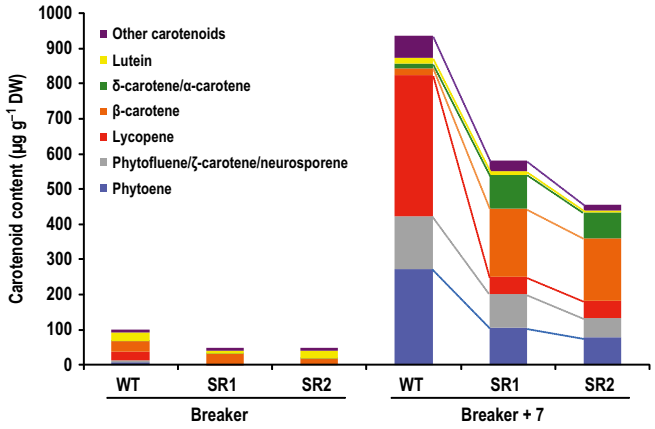

Fig. 5 Carotenoid composition of wild-type (WT) and *ERF.B3-SRDX* tomato (*Solanum lycopersicum*) fruits at the breaker and breaker + 7 stages. Amounts of the different carotenoid species in wild-type and *ERF.B3-SRDX* fruits, plotted as stacked bars. *SR1* and *SR2* are two independent *ERF.B3-SRDX* lines. Detailed data are shown in Supporting Information Table S4.

ERF.A1, whose expression was strongly induced during ripening (Fig. 1), was dramatically enhanced in the *ERF.B3-SRDX*-expressing lines.

Discussion

So far, only a limited number of studies have reported the direct involvement of ERF genes in fleshy fruit development and ripening (Li *et al.*, 2007; Lee *et al.*, 2012). This is rather striking given the well-accepted role of ERFs in mediating ethylene responses and the major role played by this phytohormone during the ripening of climacteric fruit. In this regard, the present study, by shedding new light onto the physiological significance of *Sl-ERF.B3*, supports the idea that members of the tomato ERF family of transcription factors are active players in the control of fruit ripening in tomato. *Sl-ERF.B3* has been shown recently to play an important role in controlling pleiotropic ethylene responses via the feedback regulation of genes encoding components of ethylene signaling and other ERFs (Liu *et al.*, 2013). Here, we show that the expression of this dominant repression version of *Sl-ERF.B3* (*ERF.B3-SRDX*) broadly impacts tomato fruit development and ripening. The ripening-related phenotypes displayed by the *ERF.B3-SRDX* lines are supportive of a prominent role for *Sl-ERF.B3* in regulating important aspects of fruit ripening in tomato, including the attainment of competence to ripen and subsequent changes in color and texture. Although *Sl-ERF.B3* expression is high in stem tissue, the induced accumulation of its transcripts at the Br stage is indicative of an active role for this *ERF* gene in triggering the ripening process and, in this regard, it is not surprising that the onset of ripening is delayed in the dominant repressor lines. Compared with the wild-type, the time period from anthesis to Br was extended by *c.* 2 wk in the transgenic lines, indicating that the dominant repression activity of *Sl-ERF.B3* impacts tomato early fruit development and, particularly, the attainment of competence to ripen. Another obvious effect of *Sl-ERF.B3-SRDX* on fruit development is the reduced fruit size and bumpy shape, as a result of a reduction in epidermal cell size and a defect in the normal coordinated expansion of the pericarp (Fig. S3). This is illustrated by a thicker pericarp, smaller volume of jelly and dry/crumbly appearance of the pericarp, suggesting a defect in the expansion or elasticity of the epidermis.

The difference in color development between wild-type and *ERF.B3-SRDX* fruits is obvious at the post-Br stages, and *ERF.B3-SRDX* fruits fail to turn red, retaining an orange color at late ripening. However, the incapacity to reach a red-ripe color is not caused by an inability to undergo ripening. Indeed, once ripening is triggered, the *ERF.B3-SRDX* fruits display faster softening and higher climacteric ethylene production, indicating that these aspects of ripening are disconnected from red pigment accumulation. The dramatic development of red pigmentation of ripening fruits is one of the most notable features of tomato, mainly as a result of an accumulation of the red carotene, lycopene. Lycopene accumulation during ripening is caused by the

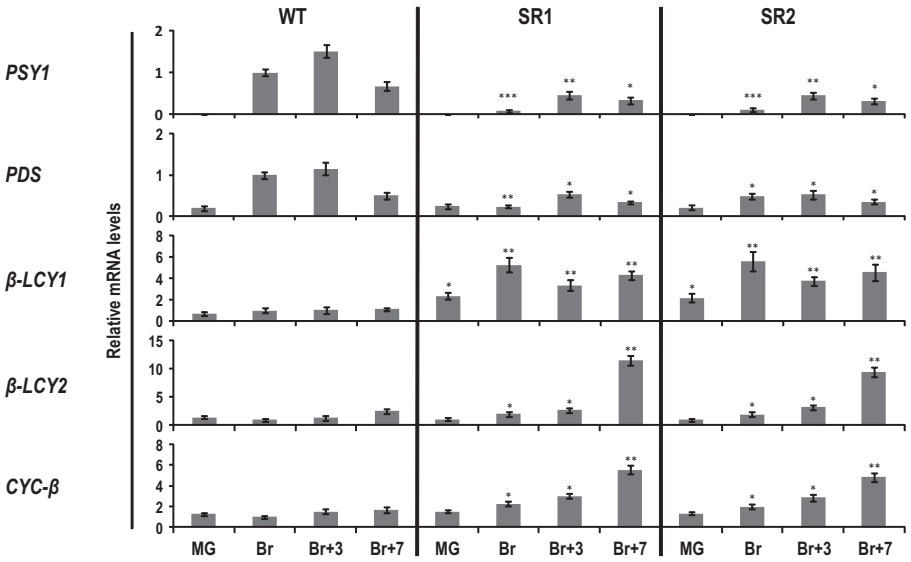

Fig. 6 Expression of carotenoid biosynthesis genes in wild-type (WT) and *ERF.B3-SRDX* (*Solanum lycopersicum*) tomato lines. Total RNA was extracted from the indicated developmental stages of fruit (MG, mature green; Br, breaker; Br+3, 3 d post-breaker; Br+7, 7 d post-breaker). The relative mRNA levels of each gene in WT at the breaker (Br) stage were standardized to 1.0, referring to the *Sl-Actin* gene as an internal control. Values are means ± SD of three biological replicates. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). *SR1* and *SR2* are two independent *ERF.B3-SRDX* lines. *PSY1*, phytoene synthase; *PDS*, phytoene desaturase; β-*LCY1*, β-*LCY2*, *CYC*-β, lycopene β-cyclases.

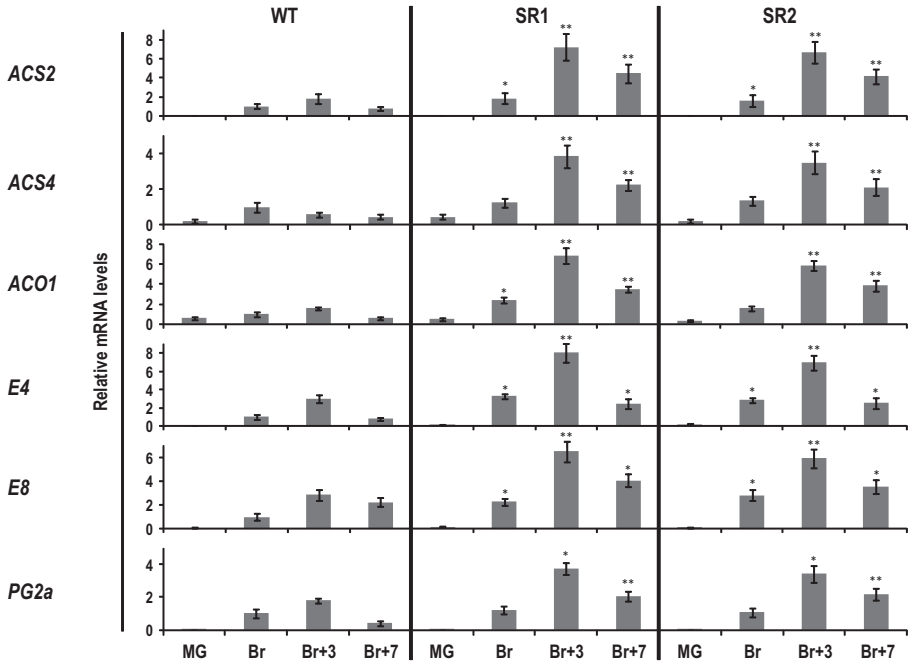

Fig. 7 Ripening-related gene expression in wild-type (WT) and *ERF.B3-SRDX* tomato (*Solanum lycopersicum*) lines during fruit ripening. Total RNA was extracted from the indicated developmental stages of fruit (MG, mature green; Br, breaker; Br+3, 3 d post-breaker; Br+7, 7 d post breaker). The relative mRNA levels of each gene in WT at the breaker (Br) stage were standardized to 1.0, referring to the *Sl-Actin* gene as an internal control. Values are means ± SD of three biological replicates. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$ (Student's *t*-test). *SR1* and *SR2* are two independent *ERF.B3-SRDX* lines. ACO1, aminocyclopropane-1-carboxylic acid oxidase; ACS2, ACS4, aminocyclopropane-1-carboxylic acid synthases; E4, E8, ethylene response genes; PG2a, polygalacturonase.

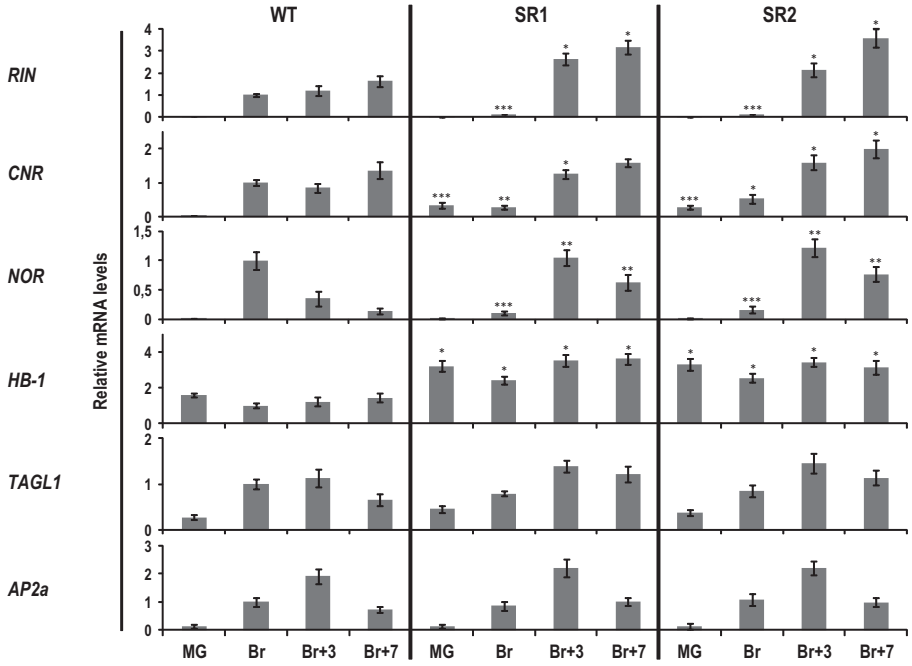

Fig. 8 Expression of ripening regulator genes in wild-type (WT) and *ERF.B3-SRDX* lines during tomato (*Solanum lycopersicum*) fruit ripening. Total RNA was extracted from the indicated developmental stages of fruit (MG, mature green; Br, breaker; Br+3, 3 d post-breaker; Br+7, 7 d post breaker) as defined in the Materials and Methods section. The relative mRNA levels of each gene in WT at the breaker (Br) stage were standardized to 1.0, referring to the *Sl-Actin* gene as an internal control. Values are means ± SD of three biological replicates. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). *SR1* and *SR2* are two independent *ERF.B3-SRDX* lines. AP2a, APETALA2/ERF gene; *CNR*, colorless non-ripening; *HB-1*, HD-Zip homeobox; *NOR*, non-ripening; *RIN*, ripening inhibitor; *TAGL1*, tomato AGAMOUS-LIKE 1.

up-regulation of genes for lycopene biosynthesis, including *PSY1* and *PDS* (Giuliano *et al.*, 1993), and the down-regulation of lycopene cyclases, which convert lycopene into downstream compounds (Pecker *et al.*, 1996; Ronen *et al.*, 1999, 2000). Accordingly, the silencing of *PSY1* or *PDS* results in a reduction in fruit lycopene (Fray & Grierson, 1993; Fantini *et al.*, 2013), whereas over-expression of β-*LCY* or *CYC*-β cyclases results in a conversion of lycopene into β-carotene, which turns the fruit orange (Ronen *et al.*, 2000; Rosati *et al.*, 2000). In addition, a positive link between β-*LCY* and *CYC*-β expression and β-carotene levels was revealed by correlation analysis of fruit metabolome and transcriptome data from *S. pennellii* × *S lycopersicum* introgression lines (Lee *et al.*, 2012). Thus, the changes in fruit carotenoid content and pigmentation observed in *ERF.B3-SRDX* fruits are perfectly compatible with the changes in expression of the *PSY1*, β-*LCY* and *CYC*-β genes observed in such fruits. It should be noted that this effect contrasts with that exerted on other ripening-regulated genes. That is, although the majority of ripening-regulated genes show an exaggerated response in *ERF.B3-SRDX* fruits, carotenoid genes show an opposite trend, with *PSY1* and

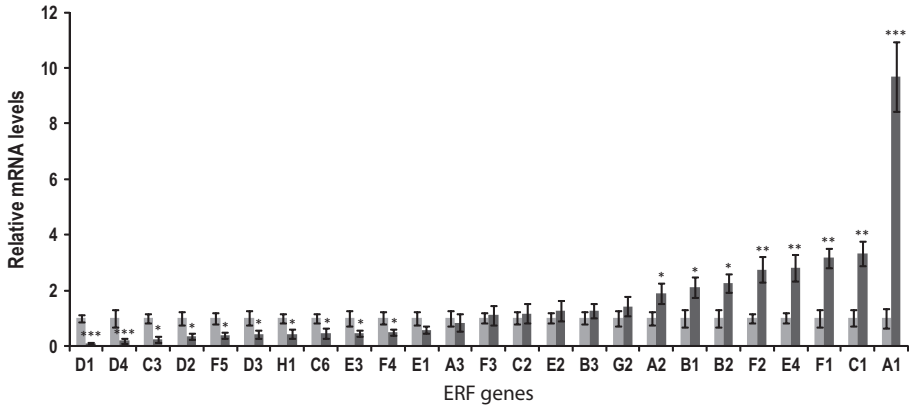

Fig. 9 Accumulation of *Sl-ERF* transcripts in wild-type (WT) and *ERF.B3-SRDX* lines assessed by quantitative real-time-polymerase chain reaction (qRT-PCR) in tomato (*Solanum lycopersicum*) fruits at Br + 3 (3 d post-breaker) stage. The relative mRNA level of each gene in the WT was standardized to 1.0, referring to *Sl-Actin* as an internal control. Values are means ± SD of three biological replicates. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). *SR* is representative of data from three independent *ERF.B3-SRDX* lines (*SR1*–*SR3*). (▒) WT; (■) SR.

PDS induction during ripening being repressed and lycopene cyclases being up-regulated, contrasting with their behavior in wild-type fruits. These contrasting effects of *ERF.B3-SRDX* on carotenoid biosynthesis genes, in comparison with other ripening-inducible genes, result in the contrasting phenotypes displayed by *ERF.B3-SRDX* fruits: exaggerated softening vs reduced lycopene accumulation.

The effects of *ERF.B3-SRDX* over-expression on carotenoid gene expression are not necessarily mediated by ethylene: first, because *PSY1* expression seems to be controlled by upstream ripening regulators, such as NOR, rather than by ethylene itself (Alba *et al.*, 2005; Osorio *et al.*, 2011), and, second, because the expression of carotenoid genes in *ERF.B3-SRDX* fruits shows a contrasting behavior with other ethylene responses and with ethylene itself.

The decreased total carotenoid levels and elevated ethylene production in *Sl-ERF.B3* fruits at least partially resemble the phenotype of lines repressed in the *SlAP2a* regulatory gene, in which significantly elevated ethylene levels are associated with altered total carotenoids and a shift to β-carotene rather than lycopene (Chung *et al.*, 2010; Karlova *et al.*, 2011). Moreover, the phenotype also recalls that of the tomato *Nr* mutant, in which lycopene biosynthesis and *PDS* gene expression are repressed and ethylene production is increased (Alba *et al.*, 2005). The assessment of the relative mRNA accumulation of *SlAP2a* failed to reveal a significant difference in its levels between *ERF.B3-SRDX* and wild-type fruits, suggesting that the regulation of *Sl-ERF.B3* dominant repression activity in fruit ripening is probably independent of *SlAP2a*. It is also possible that dominant repression of *Sl-ERF.B3* in tomato leads to complex alterations in the carotenoid accumulation network and impacts carotenoid biosynthesis genes through mechanisms beyond the influence of ethylene. Transcription factors impacting carotenoid accumulation in tomato include *RIN* (Vrebalov *et al.*, 2002), *CNR* (Manning *et al.*, 2006), *HB-1* (Lin *et al.*, 2008), *TAGL1* (Vrebalov *et al.*, 2009), *SlAP2a* (Chung *et al.*, 2010; Karlova *et al.*, 2011), *Sl-ERF.E4*, formerly *SlERF6* (Lee *et al.*, 2012), and *SlMADS1* (Dong *et al.*, 2013). The expression data revealed altered transcript accumulation for *RIN*, *CNR*, *HB-1* and *Sl-ERF.E4* in ripening *ERF.B3-SRDX* fruit, suggesting that these transcription factors may be involved in the regulation networks of carotenoid accumulation in the *ERF.B3-SRDX* lines. Interestingly, an *Arabidopsis* ERF transcription factor, RAP2.2, has been reported to regulate the expression of carotenoid biosynthesis genes via binding to the ATCTA *cis*-element in the promoter regions of *PSY* and *PDS* (Welsch *et al.*, 2007). The presence of the ATCTA motif in the promoter regions of both tomato *PSY1* and *PDS* genes, together with the suppression mediated by ERF.B3-SRDX on its target genes, support the hypothesis that the chimeric ERF.B3-SRDX protein represses the expression of *PSY1* and *PDS* in *ERF.B3-SRDX* lines by binding directly to their promoters.

A hallmark of climacteric fruit ripening, such as tomato, is the dramatic induction of respiration and ethylene production at the onset of ripening. The dominant repressor version of *Sl-ERF.B3* in tomato resulted in substantially elevated levels of ethylene production (Fig. 4d), although the onset of ripening was dramatically delayed. This indicates that the altered fruit ripening phenotype in *ERF.B3-SRDX* lines occurs at least partly through an influence on ethylene synthesis. *ERF.B3-SRDX* fruits produced up to four-fold more ripening ethylene than the wild-type and, accordingly, displayed elevated transcript accumulation of ethylene biosynthesis genes, including *ACS2*, *ACS4* and *ACO1* (Fig. 7). Ethylene biosynthesis is tightly controlled by *ACS* and *ACO* multigene families during fruit development and ripening (Nakatsuka *et al.*, 1998; Barry *et al.*, 2000; Barry & Giovannoni, 2007). Two systems of ethylene regulation have been proposed (McMurchie *et al.*, 1972), with System 1 representing the basal level of ethylene in immature fruit and vegetative tissues, and System 2 corresponding to high levels of ethylene production associated with fruit ripening (Oetiker & Yang, 1995). In contrast with System 1, where ethylene autoinhibits its own biosynthesis, System 2 ethylene has a stimulatory effect on its own synthesis, the so-called autocatalytic ethylene production. Tomato *ACS1* and *ACS6* have been shown to mediate System 1 ethylene production in immature fruit in tomato, whereas autocatalytic ethylene biosynthesis in System 2

is mediated through ethylene-stimulated expression of *ACS2*, *ACS4*, *ACO1* and *ACO4* genes (Nakatsuka *et al.*, 1998; Barry *et al.*, 2000; Barry & Giovannoni, 2007). Tomato *ACS2* and *ACS4* are the predominant *ACS* mRNAs in ripening fruit (Barry *et al.*, 2000; Yokotani *et al.*, 2004). The accumulation of *ACS2* mRNA is induced at the onset of ripening in an ethylene-dependent, but RIN-independent, manner (Nakatsuka *et al.*, 1998; Barry *et al.*, 2000), and repression of the *ACS2* gene blocks fruit ripening in tomato (Oeller *et al.*, 1991). Likewise, *ACS4* is also up-regulated during ripening, but this induction is *RIN* dependent. The accumulation of *ACO1* transcripts increases at the onset of ripening and is sustained at a high level during subsequent tomato fruit ripening (Nakatsuka *et al.*, 1998), suggesting the critical role of this gene in controlling ripening-associated ethylene synthesis. As *ACS* and *ACO* catalyze the rate-limiting and final steps in ethylene biosynthesis, the significantly high mRNA levels of *ACS2*, *ACS4* and *ACO1* are probably responsible for the elevated ethylene levels in the *ERF.B3-SRDX* lines. It is noteworthy that expression of the repressor version of *Sl-ERF.B3* leads to reduced ethylene production in dark-grown seedlings (Liu *et al.*, 2013), whereas it results in elevated ethylene production in ripening fruit, in keeping with the contrasting behavior of vegetative and ripening fruit tissues, in which ethylene production is controlled by System 1 and System 2, respectively.

The expression dynamics of most *Sl-ERF* genes during fruit ripening supports their putative involvement in the ripening process. Interestingly, the expression pattern of a large number of *Sl-ERF* genes was found to be markedly altered in *ERF.B3-SRDX* fruits at the post-Br stage, which may account for the altered ripening phenotype displayed by the dominant repressor fruits. Of particular note, the transcript accumulation of *Sl-ERF.A1*, whose expression is strongly induced during ripening (Fig. 1), was dramatically enhanced in the *ERF.B3-SRDX*-expressing lines. Although the dramatic change in the expression of *Sl-ERF.A1* suggests a particular role for this gene in the ripening process, its up-regulation in the dominant repressor lines seems to rule out the possibility that it may be a direct target of Sl-ERF.B3. By contrast, *Sl-ERF.C3*, *Sl-ERF.D2*, *Sl-ERF.F5* and *Sl-ERF.F4* genes, shown to be putative targets of Sl-ERF.B3 (Liu *et al.*, 2013), are all down-regulated in *ERF.B3-SRDX* fruits, further supporting the model that a single ERF can impact the expression of other members of the gene family. This interconnected regulation among *ERF* genes may account for the complexity of the mechanisms controlling the ripening-associated processes, and therefore for the pleiotropic alterations displayed by the *ERF.B3-SRDX* lines.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Enhanced pericarp thickness in *ERF.B3-SRDX* dominant repressor tomato fruits.

Fig. S2 Reduced seed production and altered seed morphology in *ERF.B3-SRDX* dominant repressor tomato lines.

Fig. S3 Reduced cell size in *ERF.B3-SRDX* tomato fruits.

Table S1 List of primers used in the expression studies

Table S2 Gene names used in the study and corresponding gene ID

Table S3 Fruit firmness of wild-type and *ERF.B3-SRDX* tomato fruits

Table S4 Carotenoid content of *ERF.B3-SRDX* and wild-type tomato fruits at breaker and breaker + 7 stages

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