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Research

The tomato Ethylene Response Factor Sl-ERF.B3 integrates ethylene and auxin signaling via direct regulation of *Sl-Aux/IAA27*

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Summary

- Plant growth and development is coordinated by complex networks of interacting hormones, and cross-talk between ethylene and auxin signaling is essential for a wide range of plant developmental processes. Nevertheless, the molecular links underlying the interaction between the two hormones remain poorly understood.
- In order to decipher the cross-talk between the Ethylene Response Factor Sl-ERF.B3 and Sl-IAA27, mediating ethylene and auxin signaling, respectively, we combined reverse genetic approaches, physiological methods, transactivation experiments and electrophoretic mobility shift assays.
- *Sl-ERF.B3* is responsive to both ethylene and auxin and ectopic expression of its dominant repressor version (*ERF.B3-SRDX*) results in impaired sensitivity to auxin with phenotypes recalling those previously reported for *Sl-IAA27* downregulated tomato lines. The expression of *Sl-IAA27* is dramatically reduced in the *ERF.B3-SRDX* lines and Sl-ERF.B3 is shown to regulate the expression of *Sl-IAA27* via direct binding to its promoter.
- The data support a model in which the ethylene-responsive Sl-ERF.B3 integrates ethylene and auxin signaling via regulation of the expression of the auxin signaling component *Sl-IAA27*. The study uncovers a molecular mechanism that links ethylene and auxin signaling in tomato.

Introduction

Plant-coordinated growth and development rely largely on the intricate network of interactions between different plant hormones (Wolters & Jürgens, 2009). Interactions between ethylene and auxin have long been reported at both the physiological and molecular levels, and in recent years even more attention has been paid to the cross-talk between the two hormones (Muday *et al.*, 2012). Ethylene and auxin can interact synergistically or antagonistically to control a variety of plant development processes, including root formation and hypocotyl elongation (Swarup *et al.*, 2002; Růzicka *et al.*, 2007; Ivanchenko *et al.*, 2008). It has been shown that mutation affecting the synthesis, distribution or signaling of auxin results in abnormal responses to ethylene. This is the case of *AUX1* and *EIR1/AGR/PIN2* mutants deficient in auxin transport, *AXR2/IAA7* and *AXR3/IAA17* altered in auxin response, and *TIR1* impaired in auxin receptor (Pickett *et al.*, 1990; Luschnig *et al.*, 1998; Stepanova *et al.*, 2005; Ivanchenko *et al.*, 2008; Muday *et al.*, 2012). Strikingly, all of these mutants exhibit ethylene-insensitive root growth, suggesting interdependence of responses to both hormones. At the transcriptional level, ethylene and auxin have been shown to mutually regulate the transcriptional activity of key genes involved in the biosynthetic pathways of both hormones (Tsuchisaka & Theologis, 2004; Stepanova *et al.*, 2005, 2008). In addition, it has been reported that ethylene promotes auxin transport from the meristem to the root elongation zone where the resulting increase in auxin concentrations triggers the inhibition of the well-known ethylene-mediated root growth (Lewis *et al.*, 2011). Despite the growing number of studies on cross-talk between ethylene and auxin, little is known about the molecular factors involved in the interactions between the two hormones (Robles *et al.*, 2013).

The gaseous hormone, ethylene, is involved in many plant developmental processes and plays a critical role in a large panel of physiological responses (Lin *et al.*, 2009). Studies on the components of ethylene signaling have revealed a linear transduction pathway that ultimately leads to the activation of transcriptional regulators belonging to the Ethylene Response Factor (ERF)

family (Solano *et al.*, 1998; Benavente & Alonso, 2006; Pirrello *et al.*, 2012). ERFs belong to the AP2/ERF superfamily of transcription factors shown to regulate the expression of ethylene-responsive genes through direct binding to the multiple *cis*-acting elements found in the promoter regions of these ERF-target genes (Ohme-Takagi & Shinshi, 1995; Pirrello *et al.*, 2012). In different plant species, ERFs are involved in various processes such as hormonal signaling, responses to biotic and abiotic stresses, developmental processes, metabolic regulation, ethylene biosynthesis and fruit ripening (Ohme-Takagi & Shinshi, 1995; van der Fits & Memelink, 2000; Fujimoto *et al.*, 2000; Wu *et al.*, 2002; Dubouzet *et al.*, 2003; Zhang *et al.*, 2009; Lee *et al.*, 2012; Liu *et al.*, 2016). Interestingly, Arabidopsis ERF109 has recently been shown to regulate lateral root formation through direct binding to GCC-boxes in the promoters of *ASA1* and *YUC2* genes that encode two key enzymes in the auxin biosynthesis pathway (Cai *et al.*, 2014). More recently, ERF1 has been reported to mediate the cross-talk between ethylene and auxin during primary root elongation via the regulation of *ASA1* expression in *Arabidopsis* (Mao *et al.*, 2016). Although these studies reveal the existence of active cross-talk between the two hormones, direct evidence of the potential interaction between ERFs and Aux/IAAs, known to mediate transcriptional responses to ethylene and auxin, respectively, is still missing.

Auxin has long been recognized as a major regulator of plant growth and development, and auxin signaling is known to regulate the expression of target genes primarily through two types of transcriptional regulators: Aux/IAAs and Auxin Response Factors (ARF). ARFs bind to the Auxin-response elements of target genes to activate or repress their transcription (Guilfoyle & Hagen, 2007; Zouine *et al.*, 2014). The *Aux/IAA* genes encode short-lived proteins that typically share four conserved domains and display the ability to function as transcriptional repressors through interaction with ARF proteins (Reed, 2001; Tiwari *et al.*, 2004; Audran-Delalande *et al.*, 2012). The importance of Aux/IAAs in mediating auxin-related developmental processes has been revealed by both forward and reverse genetics approaches in different plant species. In *Arabidopsis*, *Aux/IAA* gain-of-function mutants exhibit a variety of auxin-related developmental phenotypes, including apical dominance, root formation, hypocotyl elongation, leaf expansion and phototropism/gravitropism (Kim *et al.*, 1996; Tian & Reed, 1999; Nagpal *et al.*, 2000; Rogg *et al.*, 2001; Fukaki *et al.*, 2002; Hamann *et al.*, 2002; Park *et al.*, 2002; Tatematsu *et al.*, 2004; Yang *et al.*, 2004). By contrast, in tomato, most auxin-related developmental phenotypes have been described in *Aux/IAA* downregulated lines, suggesting that members of the *Aux/IAA* gene family may have specific and overlapping functions (Wang *et al.*, 2005; Chaabouni *et al.*, 2009; Bassa *et al.*, 2012; Deng *et al.*, 2012; Su *et al.*, 2014). In tomato, silencing of *Sl-IAA27* results in impaired auxin sensitivity, reduced chlorophyll content in leaves and altered root development and arbuscular mycorrhization (Bassa *et al.*, 2012; Guillotin *et al.*, 2017). Interestingly, tomato lines underexpressing *Sl-IAA3* display both auxin- and ethylene-related phenotypes, including altered apical dominance, lower auxin sensitivity, exaggerated apical hook curvature in the dark and reduced petiole epinasty in the light, thus suggesting that Sl-IAA3 may act as a molecular link between ethylene and auxin signaling in tomato (Chaabouni *et al.*, 2009). Moreover, it has been recently shown that Sl-IAA27 positively regulates tomato root mycorrhization via the activation of the strigolactone biosynthesis pathway (Guillotin *et al.*, 2017). Although these studies support the idea that interplay between auxin and other hormones is instrumental to many plant developmental processes, little is known about the molecular mechanisms underlying the interaction between ethylene signaling and Aux/IAA.

Using a Chimeric Repressor Silencing Technology (CRES-T), we previously reported that Sl-ERF.B3, a member of the tomato ERF multi-gene family, plays an important role in controlling ethylene responses and fruit ripening (Liu *et al.*, 2013, 2014). In the present study, we show that cross-talk between ethylene and auxin involves an active interaction between Sl-ERF.B3 and Sl-IAA27. Overexpression of the dominant repressor version of *Sl-ERF.B3* (*ERF.B3-SRDX*) results in a change in auxin sensitivity, an altered root development and a decrease in chlorophyll accumulation that resembles the phenotypes of *SlIAA27* downregulation lines. In addition, the data reveal the ability of Sl-ERF.B3 to regulate the expression of *Sl-IAA27* by direct binding to its promoter, thus indicating that Sl-ERF.B3 and Sl-IAA27 are at the cross-roads of ethylene and auxin signaling.

Materials and Methods

Plant materials and growth conditions

Tomato (*Solanum lycopersicum* L. cv MicroTom) seeds were sterilized and sown in Magenta vessels containing 50 ml of 50% Murashige and Skoog (MS) medium with 0.8% (w/v) agar, pH 6.0. Wild-type (WT) and transgenic plants were then transferred to soil and grown under standard glasshouse conditions. The culture rooms were set as follows: 14 h 25°C : 10 h 20°C, day : night cycle, 80% relative humidity, 250 μmol m^{-2} s^{-1} intense luminosity.

Flower emasculation and cross fertilization assays

Flower emasculation and the crossing assays were performed as described by Wang *et al.* (2005). Flower buds of *Sl-ERF.B3-SRDX* or *Sl-IAA27-RNAi* lines were emasculated before dehiscence of anthers to avoid accidental self-pollination. Cross-pollination was then performed on emasculated flowers one day before anthesis.

Histochemical GUS analysis

For β-glucuronidase (GUS) histochemical analysis, *pSlERF.B3-GUS* lines containing the *Sl-ERF.B3* promoter fused with the *GUS* reporter gene were incubated in the presence of GUS staining solution at 37°C overnight as indicated by Wang *et al.* (2005). Following GUS staining, samples were then washed several times to extract chlorophyll using a gradual series of ethanol solutions.

Hormonal treatment

For auxin dose-response (0, 0.1, 1, 10, 100 μM 1-naphthaleneacetic acid, NAA) and NPA treatments, experiments were performed as described by Wang *et al.* (2005). For quantitative reverse transcription polymerase chain reaction (qRT-PCR) and GUS analysis, auxin, ethylene and 1-MCP treatments were carried out as described by Chaabouni *et al.* (2009).

RNA isolation and quantitative RT-PCR

Total RNA from the tissues analyzed in this study was extracted using a Plant RNA Purification Reagent (cat. no. 12322-012; Invitrogen) according to the manufacturer's instructions. Total RNA was then DNase-treated (cat. no. AM1906; Invitrogen) to remove contaminating genomic DNA. First-strand cDNA was obtained by reverse transcription using an Omniscript Reverse Transcription kit (cat. no. 74904; Qiagen) following the manufacturer's instructions. Quantitative RT-PCR analysis was carried out as described by Pirrello *et al.* (2006). The primer sequences used in this study are listed in Supporting Information Table S1.

Electrophoretic mobility shift assay

The full-length *Sl-ERF.B3* coding sequence was cloned into pGEX-4T-1 (Amersham Biosciences) to fuse in frame with GST and the construct was expressed in BM Rosetta (DE3). The electrophoretic mobility shift assay was performed using the electrophoretic mobility shift assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The 60-bp probe covering the DRE/CRT element (CCGAC) derived from *Sl-IAA27* promoter was labeled with biotin using DNA 3′ End Biotinylation Kit (Thermo Fisher Scientific). The same unlabeled DNA fragment was used as competitor. The binding reactions were performed at room temperature in binding buffer (10 mM Tris (pH7.5), 50 mM KCl, 1 mM DTT, 2.5% glycerol, 0.05% NP-40, 5 mM MgCl2, 0.5 mM EDTA, 50 ng ml^{-1} poly (dI-dC)) containing 1.5 μg purified GST-ERF.B3 fusion protein and 50 fmol probes. The reaction products were analyzed by 5% (w/v) native polyacrylamide gel electrophoresis and then transferred from the gel to a nitrocellulose membrane. After cross-linking, the membrane was detected by the chemiluminescence method according to the manufacturer's protocol.

Transient expression using a single cell system

Protoplasts used for transfection were isolated from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells according to the method described in Leclercq *et al.* (2005). To determine the regulation of *Sl-IAA27* promoter by SlERF.B3 and its dominant repression version (SlERF.B3-SRDX), the reporter construct (*pIAA27-GFP*) was generated by fusing *Sl-IAA27* promoter to the coding region of the green fluorescent protein (GFP). Co-transfection assays of the protoplast were performed using the reporter vector and effector vectors carrying 35S:ERF.B3 or 35S:ERF.B3-SRDX constructs. GFP quantification by flux cytometry were performed as described previously (Liu *et al.*, 2013).

Determination of chlorophyll content

Chlorophyll extraction and measurement were carried out as described by Bassa *et al.* (2012). Briefly, a 100 mg aliquot of leaves from WT or transgenic plants was ground with 1 ml of 80% acetone and the resulting liquid was then analyzed by spectrophotometry at two wavelengths, 645 and 663 nm, using 80% acetone as control. The chlorophyll *a* and *b* content was determined based on the following equations: $Chl a = 0.999A_{663} - 0.0989A_{645}$ and $Chl b = -0.328A_{663} + 1.77A_{645}$.

Results

Sl-ERF.B3 is positively regulated by both ethylene and auxin

Our previous studies demonstrated that *Sl-ERF.B3* plays an important role in mediating ethylene signaling and fruit ripening (Liu *et al.*, 2013, 2014). Interestingly, sequence analysis using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) database revealed the presence of two Ethylene Response (DRE; CCGAC) and two Auxin Response (AuxRE; TGTCTC) elements in the 2413 bp promoter region of *Sl-ERF.B3* (Fig. 1a). The presence of conserved DRE and AuxRE *cis*-regulatory elements in *Sl-ERF.B3* promoter prompted the investigation of the responsiveness of this gene to ethylene and auxin. Transcript accumulation assessed by qRT-PCR in 3-wk-old seedlings indicated that *Sl-ERF.B3* is responsive to both ethylene and auxin treatment (Fig. 1b,c); the effectiveness of the hormone treatments was validated by monitoring the expression of a set of reference genes known to be ethylene- (*E4*, *E8*) or auxin- (*GH3*, *SAUR*) responsive. Moreover, downregulation of *Sl-ERF.B3* in the tomato ethylene-insensitive *Nr* mutant further validated the responsiveness of this gene to ethylene (Fig. 1d). To further test the auxin-responsiveness of *Sl-ERF.B3*, we used a transient assay in a tobacco BY2 single cell system allowing assessment of the transcriptional activity of the *Sl-ERF.B3* promoter fused to the GFP coding sequence (Fig. 1e). Upon treatment with auxin (50 μM 2,4-D), the expression of the Sl-ERF.B3 promoter-driven GFP reporter increased two-fold. The effectiveness of the experimental system was confirmed using a reference construct consisting of the DR5 synthetic auxin-responsive promoter fused to GFP (Fig. 1e). To gain insight into the responsiveness of *Sl-ERF.B3* to exogenous ethylene and auxin *in planta*, the *Sl-ERF.B3* promoter (2413 bp fragment) was fused to the GUS reporter coding sequence and the construct obtained (*pERF.B3::GUS*) was used to stably transform tomato plants. Exogenous treatment with ethylene or auxin significantly induced the expression of the GUS reporter gene driven by the *Sl-ERF.B3* native promoter (Fig. 1f–h) indicating that *Sl-ERF.B3* is both ethylene- and auxin-inducible. Moreover, the stimulation of GUS expression by ethylene is repressed upon treatment with 1-MCP

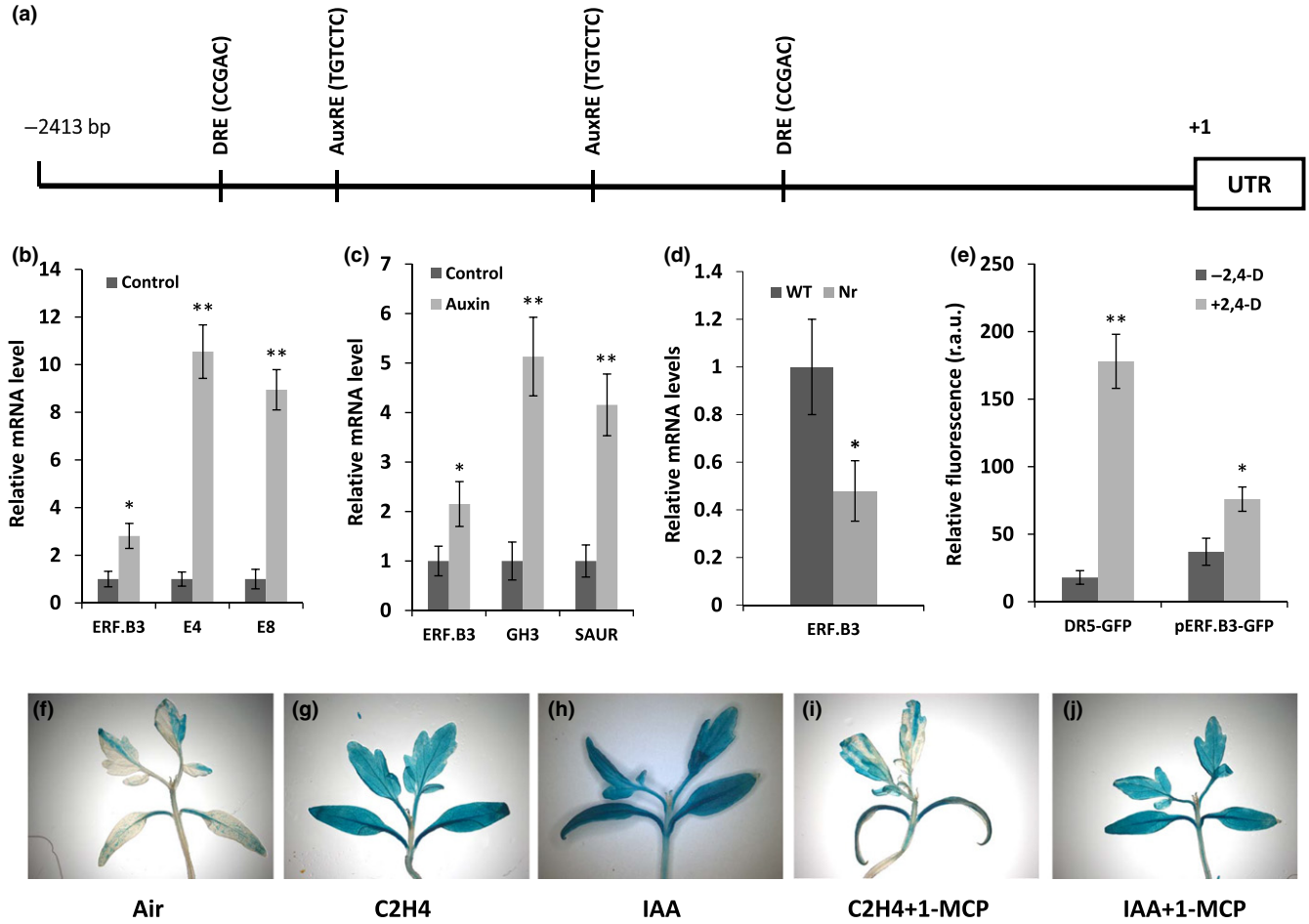

Fig. 1 Ethylene and auxin responsiveness of the *Sl-ERF.B3* gene. (a) The presence of putative ethylene and auxin response elements in the promoter of Sl-ERF.B3 gene. The *Cis*-acting elements identified are represented by black bars. (b) qRT-PCR analysis of Sl-ERF.B3 transcript in total RNA samples extracted from wild-type (WT) 3-wk-old seedlings treated with 50 μl l^{-1} ethylene for 6 h. (c) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of Sl-ERF.B3 transcript in total RNA samples extracted from 3-wk-old seedlings treated with 20 μM IAA for 2 h. (d) qRT-PCR analysis of Sl-ERF.B3 transcript levels in 3-wk-old seedlings of *Nr* mutant. (e) Auxin responsiveness of the Sl-ERF.B3 promoter. Tobacco protoplasts were transformed by pERF.B3-GFP and incubated in the presence or absence of 2,4-D (50 μM). (f–j) Expression of Sl-ERF.B3 assessed in transgenic tomato expressing GUS reporter gene driven by the Sl-ERF.B3 native promoter (pERF.B3-GUS). Plants are treated with air, ethylene (C_2H_4), auxin (IAA), ethylene+1-MCP (C_2H_4+1-MCP) or IAA+1-MCP (IAA+1-MCP). Error bars, mean ± SD of three biological replicates. Asterisks indicate the statistical significance using Student's *t*-test: *, 0.01 < *P*-value < 0.05; **, 0.001 < *P*-value < 0.01. *E4*, *E8*, ethylene response genes; *GH3-2*, *SAUR68*, auxin response genes; *DR5*, synthetic auxin-responsive promoter.

(Fig. 1i), an inhibitor of ethylene perception. By contrast, the auxin-inducible expression of *Sl-ERF.B3* cannot be repressed by 1-MCP (Fig. 1j), indicating that the auxin-responsiveness of this promoter is ethylene-independent.

ERF.B3-SRDX seedlings display altered root development and reduced auxin responsiveness

It has been shown previously that classical up- and downregulation approaches fail to provide clear clues on the functional significance of *Sl-ERF.B3*, likely due to functional redundancy among members of the *ERF* gene family (Liu *et al.*, 2013, 2014). This prompted us to generate a dominant repressor version of this transcription factor (*ERF.B3-SRDX*) using a Chimeric Repressor Silencing Technology (CRES-T). More than 10 transgenic *ERF.B3-SRDX* lines were obtained that display consistent phenotypes and three independent homozygous lines (*SR1*, *SR2* and *SR3*) with characteristic phenotypes were selected for further studies. Notably, *ERF.B3-SRDX* seedlings exhibited elongated primary root at 3-wk-old stage (Fig. 2a) with an average length being > 50% higher than in WT (Fig. 2b). In addition, *ERF.B3-SRDX* lines displayed a marked increase in lateral root formation compared to WT (Fig. 2a) with three times more lateral roots per centimeter of root length than WT (Fig. 2c).

Together the altered auxin responsiveness and root growth of *Sl-ERF.B3-SRDX* suggested a putative role for *Sl-ERF.B3* in mediating auxin sensitivity. To investigate the potential involvement of *Sl-ERF.B3* in auxin responses, we used hypocotyl elongation assays upon exogenous NAA treatment. Auxin-induced hypocotyl elongation is significantly reduced in *ERF.B3-SRDX* lines at the highest auxin concentrations used (Fig. 3a), consistent with a reduced auxin responsiveness in *Sl-ERF.B3-SRDX* hypocotyls. Further supporting this idea, treatment with N-1-naphthylphtalamic acid (NPA), the auxin transport inhibitor

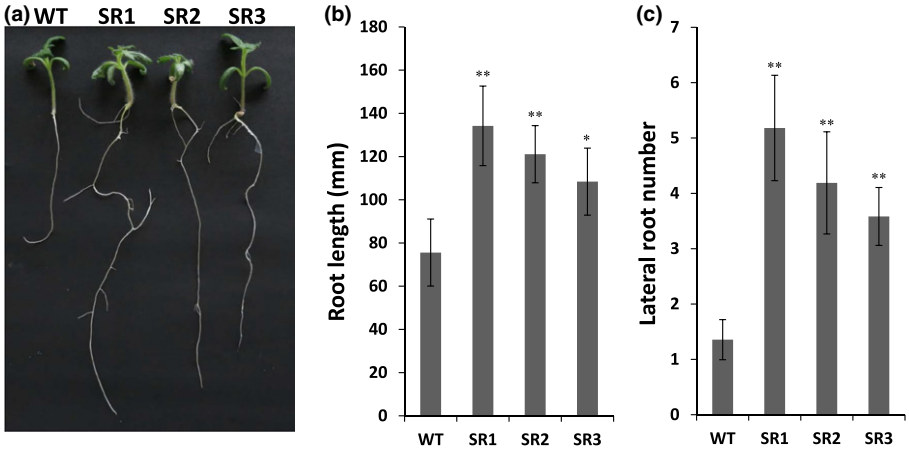

Fig. 2 Altered root development in *ERF.B3-SRDX* lines. (a) Primary root elongation and lateral root formation in wild-type (WT) and *ERF.B3-SRDX* lines assessed in 3-wk-old seedlings. (b) Length of primary root in WT and *Sl-ERF.B3-SRDX* seedlings. (c) Lateral root number in the WT and *Sl-ERF.B3-SRDX* lines. Values are means ± SD of three replicates. Asterisks indicate the statistical significance using Student's *t*-test: *, 0.01 < *P*-value < 0.05; **, 0.001 < *P*-value < 0.01. *SR1*, *SR2* and *SR3* are three independent tomato *ERF.B3-SRDX* lines.

known to alter the endogenous auxin gradients notably in roots, resulted in less inhibition of primary root growth in *ERF.B3-SRDX* lines than in WT (Fig. 3b,c). Considering the auxin-related phenotypes displayed by the *ERF.B3-SRDX* lines, and given the primary role established for Aux/indole acetic acid (IAA) in mediating auxin responses, we assessed the expression of all known members of the tomato *Aux/IAA* gene family in roots at the transcript level (Fig. 3d). The data corresponding to three independent transgenic lines clearly indicate that *Sl-IAA27* displays dramatically reduced expression in roots of *ERF.B3-SRDX* lines, whereas *Sl-IAA7* and *Sl-IAA14* only exhibit slight down- and upregulation, respectively. In addition, the expression of *IAA27* also is dramatically reduced in leaves (Fig. S1).

ERF.B3-SRDX lines show reduced chlorophyll content reminiscent of *Sl-IAA27* downregulated tomato lines

ERF.B3-SRDX plants exhibit a pale green leaf phenotype compared to the classical green color of WT leaves (Fig. 4a), suggesting an altered chlorophyll content or composition. Monitoring of chlorophyll *a* and *b* content revealed a decrease in their concentrations in the *ERF.B3-SRDX* leaves (Fig. 4b). Strikingly, the light green/yellowish leaf phenotype and the associated reduction in chlorophyll content resemble the previously described phenotypes of *IAA27 RNAi* lines (Bassa *et al.*, 2012). Moreover, similar to what has been observed in *IAA27 RNAi* plants (Bassa *et al.*, 2012), most genes involved in the key steps of chlorophyll biosynthesis are down-regulated in *ERF.B3-SRDX* lines (Fig. 4c). Transcript accumulation of genes such as HEMA1, protochlorophyllide reductase a, b, c (ProtoA, ProtoB, and ProtoC), chelatase subunit chli (CHLI) and chlh (CHLH), and also GUN4, a positive regulator of Chl biosynthesis, showed significantly lower concentrations compared to the WT (Fig. 4c). Of particular note, aminolevulinate dehydratase (Amino) which was not affected in *Sl-IAA27 RNAi* plants displayed a slight but significant downregulation in *ERF.B3-SRDX* lines (Fig. 4c).

Sl-IAA27 promoter is a target of Sl-ERF.B3

ERF.B3-SRDX and *Sl-IAA27 RNAi* lines exhibit striking similarities regarding various aspects of their phenotypes including altered auxin responsiveness, increased primary root growth and lateral root formation, as well as reduced chlorophyll content (Fig. 4a–c). This motivated the investigation of a possible link between Sl-ERF.B3 and Sl-IAA27. Interestingly, qPCR indicated that *Sl-IAA27* transcripts accumulate at higher concentrations in *ERF.B3* overexpressing lines than in WT. This clearly contrasts with the downregulation of *Sl-IAA27* in *ERF.B3-SRDX* plants (Fig. 5a) and would be rather consistent with Sl-ERF.B3 being previously assigned a transcriptional activator function (Pirrello *et al.*, 2012). Notably, the expression of *Sl-ERF.B3* is not affected in *IAA27 RNAi* lines (Fig. S2). Altogether, these data raise the hypothesis that Sl-ERF.B3 might activate the expression of *Sl-IAA27*. *In silico* search of typical regulatory motifs in the *Sl-IAA27* promoter sequence revealed the presence of conserved Ethylene Response Element (ERE) and DRE/CRT *cis*-elements known to be putative targets of the ERF type of transcription factors (Fig. S3). Although this suggested a direct regulation of *Sl-IAA27* by ERF.B3 protein, we set up an electrophoretic mobility shift assay (EMSA) to assess the ability of Sl-ERF.B3 to bind the *Sl-IAA27* promoter. As shown in Fig. 5(b), Sl-ERF.B3 can directly bind the DNA probe containing the DRE/CRT motif present in *Sl-IAA27* promoter, whereas the unlabeled promoter fragment displaced the binding of the labeled probe in a dose-dependent manner. These results reveal the ability of Sl-ERF.B3 to specifically bind a DNA fragment containing the DRE/CRT motif in the *Sl-IAA27*promoter, and suggest that *Sl-IAA27* might be a direct target for Sl-ERF.B3 *in planta*. To further investigate the putative regulation of *Sl-IAA27* gene by ERF.B3, we tested the ability of the native Sl-ERF.B3 and the chimeric ERF.B3-SRDX proteins to regulate the activity of *Sl-IAA27* promoter using a transient expression assay in a single cell system. Transactivation assays show that Sl-ERF.B3 promotes the activity of the *Sl-IAA27* promoter by inducing its activity up to three-fold,

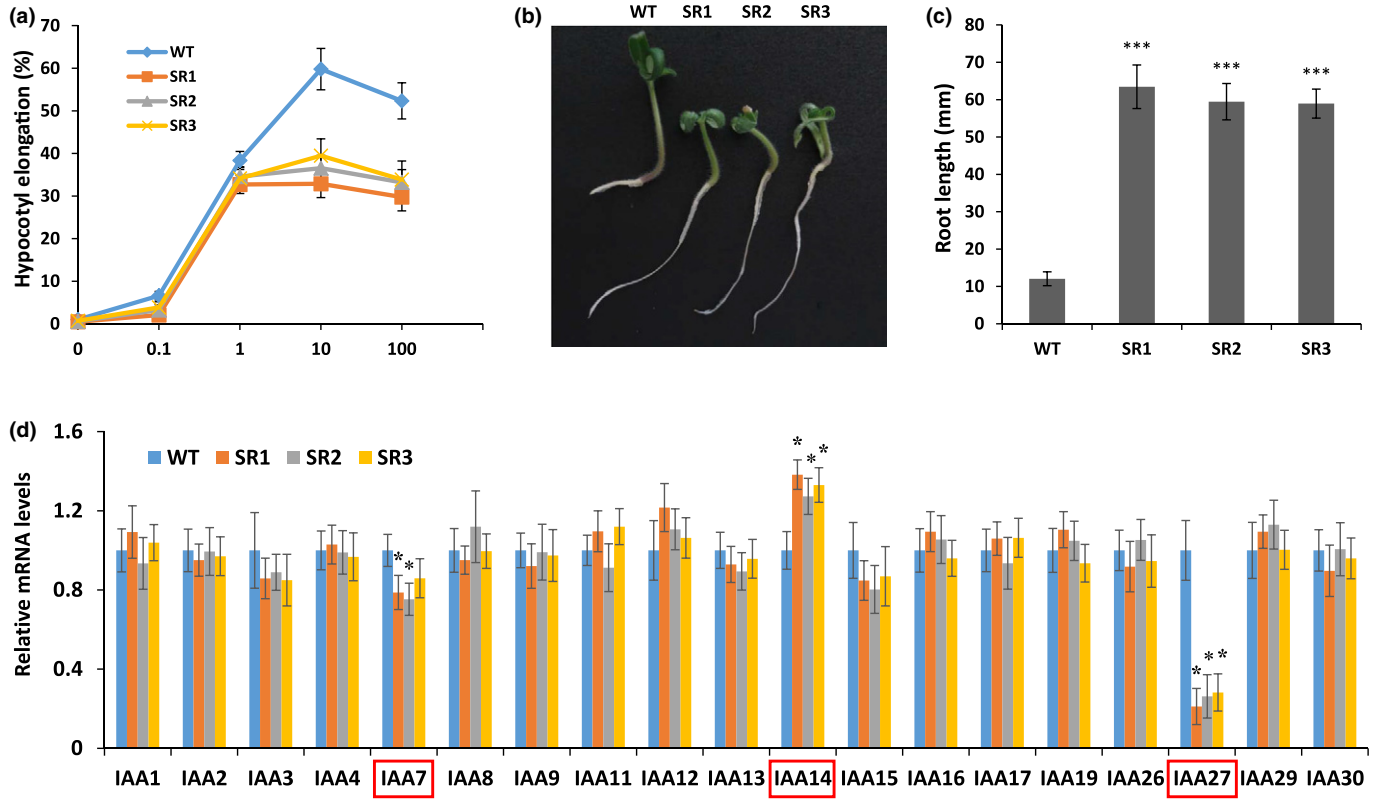

Fig. 3 Altered auxin responses in *ERF.B3-SRDX* lines. (a) Auxin dose response in hypocotyl segments. Hypocotyl fragments from 2-wk-old light-grown seedlings were incubated for 23 h in the presence of the indicated NAA concentration and hypocotyl elongation is given as percentage increase in final length over the initial length. Error bars represent mean ± SE ($n \geq 30$). (b) Effect of N-1-naphthylphtalamic acid (NPA) treatment on root development of light-grown wild-type (WT) and *ERF.B3-SRDX* lines. (c) Primary root length of WT and *ERF.B3-SRDX* lines treated with NPA. Error bars represent mean ± SE ($n \geq 30$). ***, *P*-value < 0.001 (Student's *t*-test). *SR1*, *SR2* and *SR3* are three independent *35S:ERF.B3-SRDX* lines. (d) Expression of *Aux/IAA* gene family members in roots of *ERF.B3-SRDX* lines. Values are means ± SD of three replicates. Stars indicate the statistical significance using Student's *t*-test: *, *P*-value < 0.05; *SR1*, *SR2* and *SR3* are three independent tomato *ERF.B3-SRDX* lines. Red boxed genes show significant change in their transcript levels.

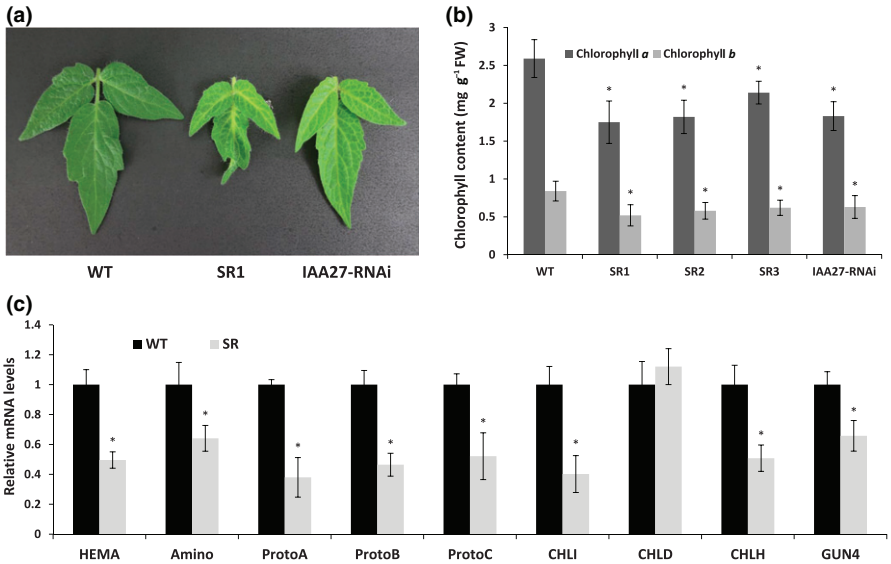

Fig. 4 Reduced chlorophyll content in *ERF.B3-SRDX* plants. (a) Light green leaf in *ERF.B3-SRDX* (SR1) and *IAA27-RNAi* lines compared to wild-type (WT). (b) Chl*a* and *b* content of WT, *ERF.B3-SRDX*, and *IAA27-RNAi* lines leaves. (c) Transcript accumulation corresponding to genes involved in photosynthesis and chlorophyll biosynthesis in leaves of WT and *ERF.B3-SRDX* lines. Values are means ± SD of three replicates. Stars indicate the statistical significance using Student's *t*-test: *, *P*-value < 0.05; *SR1*, *SR2* and *SR3* are three independent tomato *ERF.B3-SRDX* lines.

whereas ERF.B3-SRDX strongly suppresses the *Sl-IAA27* promoter activity (Fig. 5c). These data indicate that both Sl-ERF.B3 and its dominant repressor version (ERF.B3-SRDX) can regulate the expression of *Sl-IAA27* probably through direct binding to the typical DRE/CRT ethylene-responsive element present in its promoter region. This is consistent with the reduced accumulation of *Sl-IAA27* transcripts in *ERF.B3-SRDX* lines to levels that are similar to those found in *Sl-IAA27 RNAi* lines (Fig. 5d).

Cross-fertilization assay confirms the regulation of *SlIAA27* by Sl-ERF.B3

Both *ERF.B3-SRDX* and *IAA27* RNAi tomato lines show a remarkable dwarf phenotype, in contrast to tomato plants overexpressing *IAA27* driven by the 35S promoter which display a slight increase in size compared to WT (Fig. 6a,b). Assuming that ERF.B3 controls *Sl-IAA27* expression through direct binding to its promoter, we hypothesized that the expression of the 35S-driven *Sl-IAA27* should escape the ERF.B3 regulation. We then performed cross-fertilization assays to further confirm the requirement of a native *Sl-IAA27* promoter to enable the regulation of this gene by Sl-ERF.B3. Using *ERF.B3-SRDX* flowers as female recipient and *35S::IAA27* overexpressing as pollen donor fully rescued the dwarf phenotype of *ERF.B3-SRDX* plants in the F_1 progeny (Fig. 6a,b, CROSS-1). Likewise, using *ERF.B3-SRDX* as pollen donor to fertilize *35S::IAA27* overexpressing flowers did not result in any size reduction as the F_1 progeny plants maintained the tall phenotype of the maternal parent (Fig. 6a,b, CROSS-2). The effectiveness of the cross-fertilization was validated by checking the expression of ERF.B3-SRDX chimeric gene in the progeny plants obtained by the genetic cross of *ERF.B3-SRDX* as pollen donor and IAA27 overexpressing flowers as female recipient, thus validating (Fig. 6c). In these hemizygous lines, the expression of ERF.B3-SRDX, assessed by PCR was similar to that observed in ERF.B3-SRDX homozygous lines (Fig. 6c).

Discussion

It is now well recognized that interplay between multiple signaling is critical to driving the coordinated growth of plants and their adaptive processes towards a changing environment. In particular, hormones play a vital role in determining the most appropriate type of development for dealing with specific environmental conditions and for directing the adequate organ and tissue differentiation that is suitable to face a particular situation. In this regard, a better understanding of the mechanisms and molecular factors by which different hormone signaling intersect is essential to provide new leads for breeding superior crops.

The interaction between auxin and ethylene is required for a number of plant developmental processes; however, despite our growing knowledge of the cross-talk between the two hormones (Muday *et al.*, 2012; Kumar *et al.*, 2014), the precise actors and molecular mechanisms underpinning these interactions remain poorly understood. Tomato *Sl-ERF.B3* (Ethylene Response Factor, ERF), a downstream transcription factor in the ethylene

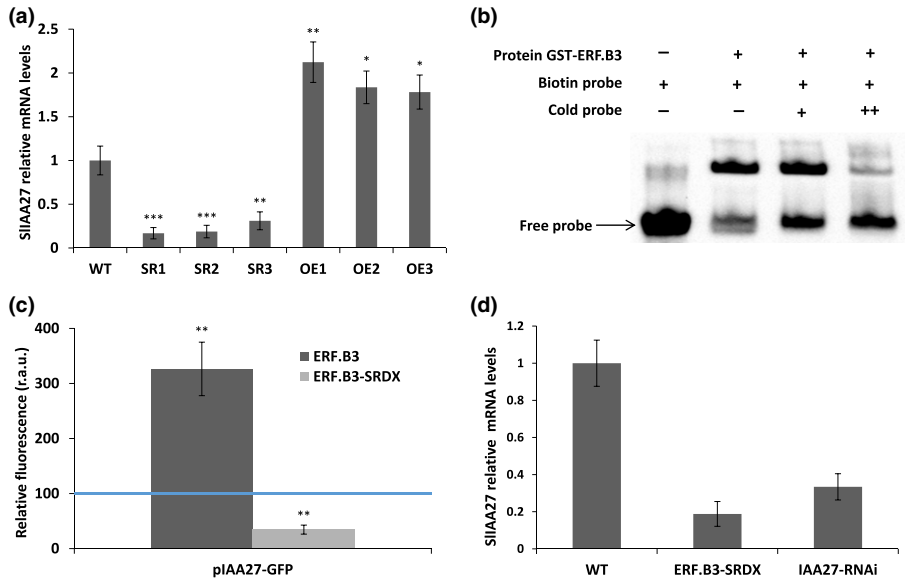

Fig. 5 *Sl-IAA27* is a direct target of Sl-ERF.B3 and ERF.B3-SRDX. (a) Accumulation of Sl-IAA27 transcript in leaves of wild-type (WT), *ERF.B3-SRDX* and *ERF.B3* overexpressing lines assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 4-wk-old plants. (b) ERF.B3 binding to the promoter of IAA27 containing a DRE/CRT element. The WT probe containing the DRE/CRT was biotin-labeled. Competition for ERF.B3 binding was performed with cold probes. The symbols − and + represent absence or presence of the probes and GST-tagged ERF.B3 protein, and ++ indicates enhanced amounts. (c) Transactivation of *Sl-IAA27* promoter by ERF.B3 and ERF.B3-SRDX. Protoplasts were co-transfected with GFP reporter fused to the *Sl-IAA27* promoter and the effector plasmid expressing ERF.B3 or ERF.B3-SRDX. (d) Expression of *Sl-IAA27* in WT, *ERF.B3-SRDX* and *IAA27-RNAi* lines. Values are means ± SD of three replicates. Stars indicate the statistical significance using student's *t*-test: *, 0.01 < *P*-value < 0.05; **, 0.001 < *P*-value < 0.01; ***, *P*-value < 0.001. SR1, SR2 and SR3 are three independent tomato ERF.B3-SRDX lines; *OE1*, *OE2* and *OE3* are three independent *ERF.B3* overexpressing lines.

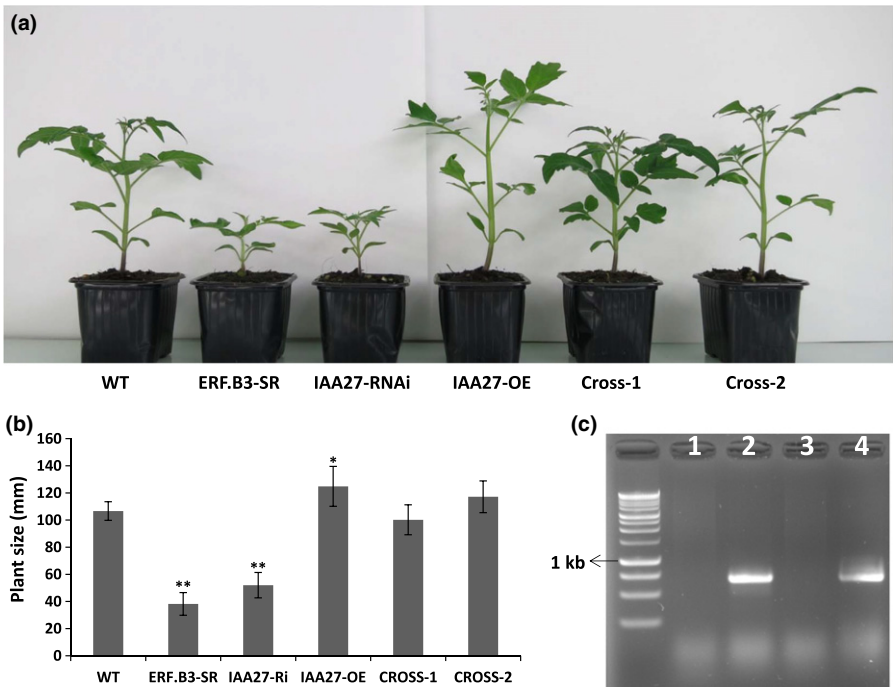

Fig. 6 Genetic crosses between *Sl-ERF.B3-SRDX* and *Sl-IAA27*-overexpressing lines to assess the Sl-ERF.B3-mediated regulation of *Sl-IAA27* in tomato. (a) *Sl-IAA27* overexpressing lines used as pollen donor were crossed with *Sl-ERF.B3-SRDX* lines resulting in the rescue of the dwarf phenotype of *ERF.B3-SRDX* plants. (b) Size of 4-wk-old plants corresponding to the progenies resulting from the genetic crosses. WT, wild type; ERF.B3-SR, *Sl-ERF.B3-SRDX* lines; IAA27-RNAi, *Sl-IAA27* downregulated lines; IAA27-OE, *Sl-IAA27* overexpression lines; Cross-1, *Sl-IAA27* overexpression plants as pollen donor crossed with *Sl-ERF.B3-SRDX* lines; Cross-2, *Sl-ERF.B3-SRDX* plants as pollen donor crossed with *Sl-IAA27* overexpression lines. Values are means ± SD of three replicates. Stars indicate the statistical significance using Student's *t*-test: *, $0.01 < P\text{-value} < 0.05$; **, $0.001 < P\text{-value} < 0.01$. (c) The effectiveness of the genetic crosses between *ERF.B3-SRDX* and *IAA27* overexpressing lines was confirmed by monitoring the expression of the ERF.B3-SRDX chimeric gene. (1) WT; (2) *ERF.B3-SRDX* line; (3) *IAA27* overexpressing line; (4) progeny resulting from genetic cross using ERF.B3-SRDX as pollen donor and *IAA27* overexpressing line as female recipient.

signaling pathway, already has been shown to play a role in mediating ethylene responses and fruit ripening (Liu *et al.*, 2013, 2014). The present study shows that Sl-ERF.B3 functions as an integrator in the cross-talk between ethylene and auxin through the regulation of *Sl-IAA27*, a member of the tomato *Aux/IAA* (indole acetic acid, IAA) family of transcriptional regulators. Indeed, the expression of *Sl-ERF.B3* is regulated by both ethylene and auxin, and the ectopic expression of the dominant repressor version of this gene (*Sl-ERF.B3-SRDX*) results in modification of auxin sensitivity, alteration of root development and decrease of chlorophyll accumulation reminiscent of the phenotypes of tomato lines underexpressing *Sl-IAA27* (Audran-Delalande *et al.*, 2012; Bassa *et al.*, 2012). In agreement with the similarity of the phenotypes, the expression of *Sl-IAA27* is significantly reduced in the *ERF.B3-SRDX* lines as a result of its downregulation by the repressor version of ERF.B3. The direct regulation of *Sl-IAA27* by ERF.B3 also is supported by Electromobility Shift Assay experiments, transactivation assays, as well as by genetic crosses. Altogether, the data concur in designating *Sl-IAA27* as a direct target of Sl-ERF.B3. Therefore, auxin-related phenotypes in *ERF.B3-SRDX* lines are likely due to the downregulation of *Sl-IAA27*.

The presence of ethylene and auxin responsive elements in the promoter regions of Sl-ERF.B3 and the regulation of its expression by the two hormones suggest an active role for Sl-ERF.B3 in mediating responses to both hormones. In this regard, it may explain why phenotypes related to both ethylene and auxin are observed in the dominant repressor lines. Indeed, it has been shown that *Sl-ERF.B3-SRDX* etiolated seedlings display a partial constitutive ethylene-response in the absence of ethylene and that the adult plants exhibit typical ethylene-associated alterations such as leaf epinasty, premature flower senescence and accelerated fruit abscission (Liu *et al.*, 2013). In addition to the modified ethylene sensitivity, we show here that *ERF.B3-SRDX* lines also exhibit impaired auxin sensitivity associated with enhanced primary root growth and lateral root formation. Previous studies have shown that downregulation of some members of the *Aux/IAA* gene family in tomato results in altered auxin sensitivity and root development (Wang *et al.*, 2005; Chaabouni *et al.*, 2009; Bassa *et al.*, 2012; Deng *et al.*, 2012).

Although it is already known that ethylene regulates root development through interaction with auxin (Růzicka *et al.*, 2007; Stepanova *et al.*, 2007; Muday *et al.*, 2012), ERF1 has been described as mediator between ethylene and auxin biosynthesis during primary root elongation through the regulation of *ASA1* expression in Arabidopsis (Mao *et al.*, 2016). Likewise, *Arabidopsis* ERF109 has been shown to impact lateral root formation through the regulation of auxin biosynthesis (Cai *et al.*, 2014). More recently, root slanting has been reported to be regulated by ERFVIIs and polar auxin transport in Arabidopsis, revealing a role for both ethylene and auxin in hypoxia

adaptation (Eysholdt-Derzsó and Sauter 2017). Our present study shows that, the down-regulation of *Sl-IAA27* in the *ERF.B3-SRDX* lines is likely to account for the auxin-related phenotypes and the observed decrease in chlorophyll content. A number of the phenotypes observed in *ERF.B3-SRDX* lines were similar to those seen the *Sl-IAA27* downregulated plants, such as reduction in plant size, change in auxin sensitivity, diminution of chlorophyll content, alteration of root development and modification of fruit shape (Bassa *et al.*, 2012; Liu *et al.*, 2013, 2014). Interestingly, *Sl-IAA27* transcript levels are as low in *ERF.B3-SRDX* plants as in the *IAA27* RNAi lines (Fig. 5d), which may account for the small size of *ERF.B3-SRDX* plants. Consistent with the idea that Sl-ERF.B3 regulates *in vivo* the expression of *Sl-IAA27*, this latter gene shows a marked upregulation in the *Sl-ERF.B3* overexpressing plants, in contrast to its downregulation in the *ERF.B3-SRDX* dominant repressor lines. The view that *Sl-IAA27* represents a direct target of Sl-ERF.B3 protein is supported by trans-activation assays showing that Sl-ERF.B3 can promote the *Sl-IAA27* promoter activity and by the EMSA showing the ability of Sl-ERF.B3 to bind directly to *Sl-IAA27* promoter. Moreover, the recovery from dwarf to normal phenotype of the progenies resulting from a cross between *ERF.B3-SRDX* and *35S:IAA27-OE* lines further indicates that ERF.B3 impacts *Sl-IAA27* through the regulation of its native promoter. The crossing experiments rule out the possibility that ERF.B3-SRDX can act downstream of Sl-IAA27 but rather suggest that ERF.B3 operates upstream of Sl-IAA27.

Overall, our results support a model in which the ERF Sl-ERF.B3 mediates cross-talk between ethylene and auxin via regulating the expression of *Sl-IAA27*, an important auxin signaling component. The dominant repressor version of Sl-ERF.B3 induces the downregulation of *Sl-IAA27*, which in turn has an impact on plant growth and root development. Considering that Sl-IAA27 has been shown to regulate strigolactone biosynthesis genes *Sl-D27* and *Sl-MAX1* in mycorrhized tomato (Guillotin *et al.*, 2017) via the regulation of the transcription factor NSP1 (Liu *et al.*, 2011), Sl-IAA27 emerges as an integrator of multiple signaling, connecting auxin to ethylene signaling on one side, and to strigolactone on the other side.

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Author contributions

M.L. planned and designed the research; M.L., Yao Chen, Ya Chen and I.M. performed experiments; J-H.S., C.A. and M.Z. analyzed data; and M.L., J.P. and M.B. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Expression of Aux/IAA gene family members in leaves of ERF.B3-SRDX lines.

Fig. S2 Relative expression levels of *Sl-ERF.B3* in *IAA27* down-regulated lines.

Fig. S3 The presence of putative ERF binding sites in the promoter of *Sl-IAA27* gene.

Table S1 List of primers used in the expression studies

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