Phenotypes Associated with Down-Regulation of *Sl-IAA27* Support Functional Diversity Among Aux/IAA Family Members in Tomato

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The phytohormone auxin is known to regulate several aspects of plant development, and Aux/IAA transcription factors play a pivotal role in auxin signaling. To extend our understanding of the multiple functions of Aux/IAAs further, the present study describes the functional characterization of *Sl-IAA27*, a member of the tomato *Aux/IAA* gene family. *Sl-IAA27* displays a distinct behavior compared with most Aux/IAA genes regarding the regulation of its expression by auxin, and the *Sl-IAA27*-encoded protein harbors a unique motif of unknown function also present in Sl-IAA9 and remarkably conserved in monocot and dicot species. Tomato transgenic plants underexpressing the *Sl-IAA27* gene revealed multiple phenotypes related to vegetative and reproductive growth. Silencing of *Sl-IAA27* results in higher auxin sensitivity, altered root development and reduced Chl content in leaves. Both ovule and pollen display a dramatic loss of fertility in *Sl-IAA27* down-regulated lines, and the internal anatomy of the flower and the fruit are modified, with an enlarged placenta in smaller fruits. In line with the reduced Chl content in *Sl-IAA27* RNA interference (RNAi) leaves, genes involved in Chl synthesis display lower expression at the level of transcript accumulation. Even though *Sl-IAA27* is closely related to *Sl-IAA9* in terms of sequence homology and the encoded proteins share common structural features, the data indicate that the two genes regulate tomato fruit initiation and development in a distinct manner.

Keywords: Auxin • Aux/IAA • Chl • Fruit • Tomato.

Abbreviations: AFB, auxin receptor F-box; ARF, auxin response factor; Aux/IAA, auxin/IAA; AuxRE, auxin responsive *cis*-element; CaMV, *Cauliflower mosaic virus*; Chl, chlorophyll; EAR, ethylene-responsive element-binding factor-associated amphiphilic repression; GFP, green fluorescent protein; GH3, Gretchen Hagen 3, GUS, β-glucuronidase; MS medium, Murashige and Skoog medium; NAA, naphthalene-1-acetic acid; NLS, nuclear localization signal; NPA, *N*-1-naphthylphtalamic acid; qRT–PCR, quantitative reverse transcription–PCR; RNAi, RNA interference; SAUR, small auxin up RNA; SCF, SKP1-Cullin-F-box; SGN, Solanaceae Genomics Network; Sl-IAA, *Solanum lycopersicum* auxin/IAA; TIR1, transport inhibitor response1; TPL, topless; YFP, yellow fluorescent protein.

Introduction

The phytohormone auxin (IAA) plays a determinant role in plant development by notably controlling cell division, expansion and differentiation and by regulating organ initiation, embryogenesis and root development (Vanneste and Friml 2009). Auxin is also fundamental for successful fertilization of the flower and for fruit initiation and subsequent growth (Gillaspy et al. 1993, Vivian-Smith and Koltunow 1999, Carmi et al. 2003, Rotino et al. 2005). Components of the auxin signaling pathway have been shown to be involved in repressing fruit initiation until the fertilization cue (Vivian-Smith et al. 2001, Wang et al. 2005, Goetz et al. 2006, Pandolfini et al. 2007). Pioneering studies on Arabidopsis have identified different components of auxin signaling, among which the *Aux/IAA* family of transcriptional regulators (Abel et al. 1995). *Aux/IAA* genes constitute one of the three major groups of primary auxin-responsive genes which also include *Small Auxin Up RNA* (*SAUR*) and *Gretchen Hagen 3* (*GH3*) (Theologis et al. 1985, Oeller et al. 1993). *Aux/IAA* genes encode short-lived proteins that typically share four conserved domains (Reed 2001) and display the ability to function as transcriptional repressors due to a conserved leucine motif (LxLxLx) located in domain I (Tiwari et al. 2004) similar to the so-called EAR (ethylene-responsive element binding factor-associated amphiphilic repression) repression domain (Kagale et al. 2010, Kagale and Rozwadowski 2010, Kagale and Rozwadowski 2011). It is well acknowledged now that *Aux/IAA* genes have a dual activity, one as a transcriptional regulator and another as

a component of the auxin receptor complex. That is, in the absence of auxin, Aux/IAAs can bind ARFs (auxin response factors) through domains III and IV present in the C-terminal part of both proteins and recruit the TOPLESS (TPL) co-repressors, thus preventing ARFs from activating the transcription of their target genes (Guilfoyle and Hagen 2007, Szemenyei et al. 2008).

The presence of auxin promotes the association of Aux/IAAs with the SKP1-Cullin-F-box (SCF) complex through binding to the auxin transport inhibitor response1 (TIR1) or to its paralogs AUXIN RECEPTOR F-BOX (AFB) proteins. The SCF complex targets Aux/IAAs to the proteasome, leading to their rapid degradation (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Leyser 2006, Tan et al. 2007, Chapman and Estelle 2009). The degradation of Aux/IAAs results in the release of ARFs which can then activate the transcription of target genes via binding to the auxin responsive elements (AuxREs) present in the promoter regions of auxin-regulated genes (Hagen et al. 1991, Ulmasov et al. 1997, Hagen and Guilfoyle 2002). In Arabidopsis, the unraveling of Aux/IAA functions in planta was almost exclusively achieved from the characterization of gain-of-function mutants. Indeed, phenotypes associated with loss-of-function mutations show an important functional redundancy among Aux/IAA family members (Rouse et al. 1998, Tian and Reed 1999, Nagpal et al. 2000, Rogg et al. 2001, Fukaki et al. 2002, Tatematsu et al. 2004, Overvoorde et al. 2005, Uehara et al. 2008). In contrast, down-regulation of *Aux/IAA* genes in tomato proved to be, so far, quite efficient in revealing the physiological significance of a number of Aux/IAAs (Wang et al. 2005, Chaabouni et al. 2009a, Deng et al. 2012). Therefore, this Solanaceae reference species is a suitable system for further deciphering the specific roles of various members of the *Aux/IAA* gene family made up of 25 genes (Audran-Delalande et al. 2012). Using a reverse genetic approach, aiming to down-regulate the expression of *Sl-IAA9* in tomato, revealed the specific role of this Aux/IAA as a switch of the fruit set process and regulator of leaf morphogenesis (Wang et al. 2005). Likewise, the underexpression of the tomato *Sl-IAA3* gene resulted in both auxin- and ethylene-related developmental defects including reduced apical dominance and an exaggerated apical hook in dark-grown seedlings in the absence of ethylene, thus supporting the hypothesis that *Sl-IAA3* may act as a molecular link between ethylene and auxin signaling (Chaabouni et al. 2009a, Chaabouni et al. 2009b). More recently, it was reported that *Sl-IAA15* down-regulated lines display a lower trichome number, reduced apical dominance, increased lateral root formation and dark green leaves (Deng et al. 2012). Taken together, these phenotypes uncover specialized roles for Aux/IAAs in plant developmental processes, clearly indicating that members of the *Aux/IAA* gene family in tomato perform both overlapping and specific functions.

To gain further insight into the physiological significance and diversity of roles associated with Aux/IAAs in tomato, the present study addresses the function of the *Sl-IAA27* gene in planta. The expression pattern of *Sl-IAA27* displays an atypical auxin regulation compared with classical *Aux/IAA* genes, and the down-regulation of this *Aux/IAA* gene leads to Chl underaccumulation in the leaves and to altered fruit growth.

Results

Sl-IAA27 is a canonical *Aux/IAA* gene closely related to *Sl-IAA9*

Aux/IAA genes belong to a large family found in many species and comprises 25 members in tomato (Audran-Delalande et al. 2012). Sl-IAA27 is closely related to Sl-IAA9 and Sl-IAA8, these three proteins forming a distinct clade, named clade B, in the tomato Aux/IAA protein family (Audran-Delalande et al. 2012). To shed more light on this clade B, members were compared with those from the same clade in potato (SGN database: Solanaceae Genomics Network, http://www.sgn.cornell), poplar (Kalluri et al. 2007), rice (Jain et al. 2006) (DRTB database http://drtf.cbi.pku.edu.cn/index.php) and maize (Wang et al. 2010b). Alignment of amino acid sequences of clade B Aux/IAAs revealed the presence in IAA9 and IAA27 of a conserved motif (YxGLS) before domain I in all monocot and dicot species investigated. Notably, the YxGLS motif was also found in Arabidopsis At-IAA8 while it is absent in IAA8 proteins from all other species (**Fig. 1A**). Because this motif is not found in any other Aux/IAA proteins, it provides a specific signature of clade B.

The *Sl-IAA27* coding sequence is 837 bp long and made up of five exons like its closest Arabidopsis homolog (**Fig. 1B**). The derived protein contains 278 amino acids. At the nucleotide and amino acid sequence levels, Sl-IAA27 displays 67 and 52% identity, respectively, with its putative Arabidopsis ortholog At-IAA27. The four conserved domains (I–IV) characteristic of the Aux/IAA family (Abel et al. 1995) are present in Sl-IAA27 protein as well as putative nuclear localization signals (NLSs) with both the bipartite structure of a conserved basic doublet KR between domains I and II and basic amino acids in domain II, and the SV40-type NLS located in domain IV (**Fig. 1B**). The subcellular localization of the Sl-IAA27 protein was assessed by transient expression assay in tobacco protoplasts using a translational fusion between Sl-IAA27 and yellow fluorescent protein (YFP) under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV). Microscopy analysis showed that in contrast to control protoplasts transformed with YFP alone which displayed fluorescence throughout the cell, the YFP–Sl-IAA27 fusion was only localized in the nucleus (**Fig. 2A**). These data shows that Sl-IAA27 was able to direct the fusion protein to the nucleus, consistent with its putative transcriptional regulatory function. To gain insight into the spatial pattern of expression of the *Sl-IAA27* gene, accumulation of its transcript was assessed in different plant tissues and organs by quantitative reverse transcription–PCR (qRT-PCR) and by analysis of expression of the β-glucuronidase (GUS) reporter gene driven by the *Sl-IAA27* promoter. These data revealed that

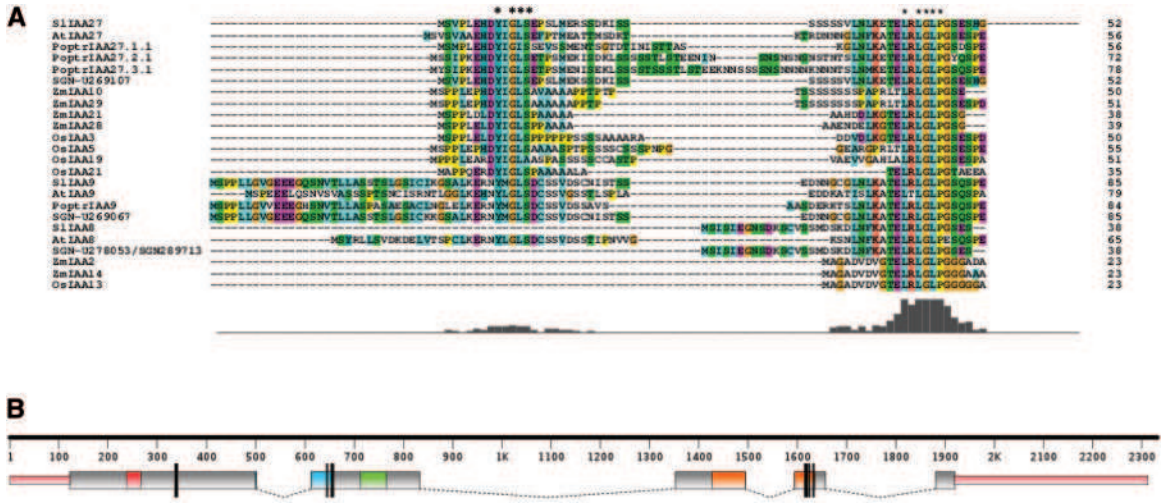

Fig. 1 Structural features of the *Sl-IAA27* gene and derived protein. (A) Multiple sequence alignment of the N-terminal part of Aux/IAA proteins belonging to clade B from tomato, Arabidopsis, maize, rice, potato and poplar obtained with ClustalX and manual correction. Conserved amino acid residues within domain I are shown by stars, as are the conserved YxGLs motif located upstream of domain I and found only in clade B Aux/IAA members. The amino acid position is given on the right of each sequence. (B) Structure of the *Sl-IAA27* gene designed with fancyGENE software (http://host13.bioinfo3.ifom-ieo-campus.it/fancygene/). Exons are represented by gray boxes, introns by hats, untranslated regions by pink boxes, domain I by a red box, domain II by a blue box, domain III by a green box and domain IV by an amber box. Nuclear localization signals are represented by black vertical straight lines.

Sl-IAA27 was expressed in all organs analyzed, with the lowest expression found in leaves (**Fig. 2B**; **Supplementary Fig. S1**). In the flower, *Sl-IAA27* was expressed in sepals, anthers and in the top of the stamen, but not in petals (**Fig. 2B**; **Supplementary Fig. S1D**). The expression was high in the ovary at the anthesis stage (**Supplementary Fig. S1F**). In mature green fruit, the expression was mainly observed in pericarp, endocarp and columella (**Supplementary Fig. S1G**), whereas it was down-regulated during ripening with strongly reduced expression in red fruits (**Fig. 2B**; **Supplementary Fig. S1G**, **H**). In hypocotyls and roots, *Sl-IAA27* is expressed in vascular tissues (**Supplementary Fig. S1A–C**).

Sl-IAA27 transgenic plants showed altered root development and modified auxin sensitivity

Both RNA interference (RNAi) and overexpression approaches were used to address the physiological significance of Sl-IAA27 protein. Several independent homozygous lines were generated; three *Sl-IAA27*-overexpressing lines and three *Sl-IAA27* RNAi lines were selected for further studies. The *Sl-IAA27*-overexpressing lines were named Sline1, Sline2 and Sline3, and the *Sl-IAA27* RNAi lines were named Rline1, Rline2 and Rline3. Analysis by real time-PCR showed that *Sl-IAA27* is overexpressed in the overexpressing transgenic lines with transcript levels 120 times higher than the wild type in Sline1, and up to 50 times higher in Sline2 and Sline3 (**Supplementary Fig. S2**). In the three RNAi lines, real-time PCR experiments showed substantially lower accumulation of *Sl-IAA27* transcripts compared with the wild type. With less than one-third of the mRNA level displayed in the wild type, Rline1 showed the highest level of repression, whereas Rline2 and Rline3 retained half of the normal mRNA levels (**Fig. 2C**). To check the specificity of the RNAi strategy and rule out any potential interference with other *Aux/IAA* genes, the expression of clade B members *Sl-IAA8* and *Sl-IAA9* was assessed. No reduction in the transcript levels of *Sl-IAA8* and *Sl-IAA9* genes was detected, indicating that the altered physiological processes observed in the transgenic lines are primarily due to the down-regulation of *Sl-IAA27*. Moreover, transcript accumulation of *Sl-IAA9*, the most closely related *Aux/IAA* in terms of sequence homology, was even higher in *Sl-IAA27* RNAi lines than in wild-type plants (**Fig. 2C**).

Primary roots were 23–43% more elongated in *Sl-IAA27* RNAi lines than in wild-type plants, and the increase in root length correlated with the level of transcript reduction in the three lines (**Fig. 3A, B**). In addition, *Sl-IAA27* RNAi transgenic lines displayed increased lateral root formation (**Fig. 3A, C**). In contrast, *Sl-IAA27*-overexpressing lines presented a reduced root length, the main root being up to 50% less elongated than in the wild type and producing no lateral root (**Supplementary Fig. S3A**, **B**).

The altered root growth in the transgenic plants suggested that the modification of *Sl-IAA27* expression may result in altered auxin sensitivity. The physiological auxin response of *Sl-IAA27* RNAi and overexpressing plants was assessed using the auxin transport inhibitor *N*-1-naphthylphtalamic acid (NPA) known to alter the endogenous auxin gradients notably in roots. Lateral root formation was inhibited in both wild-type and RNAi plants. Primary root growth was reduced in wild-type, *Sl-IAA27*-overexpressing and RNAi plants with, however, a much lower reduction in the *Sl-IAA27* RNAi lines (**Fig. 4A, B**;

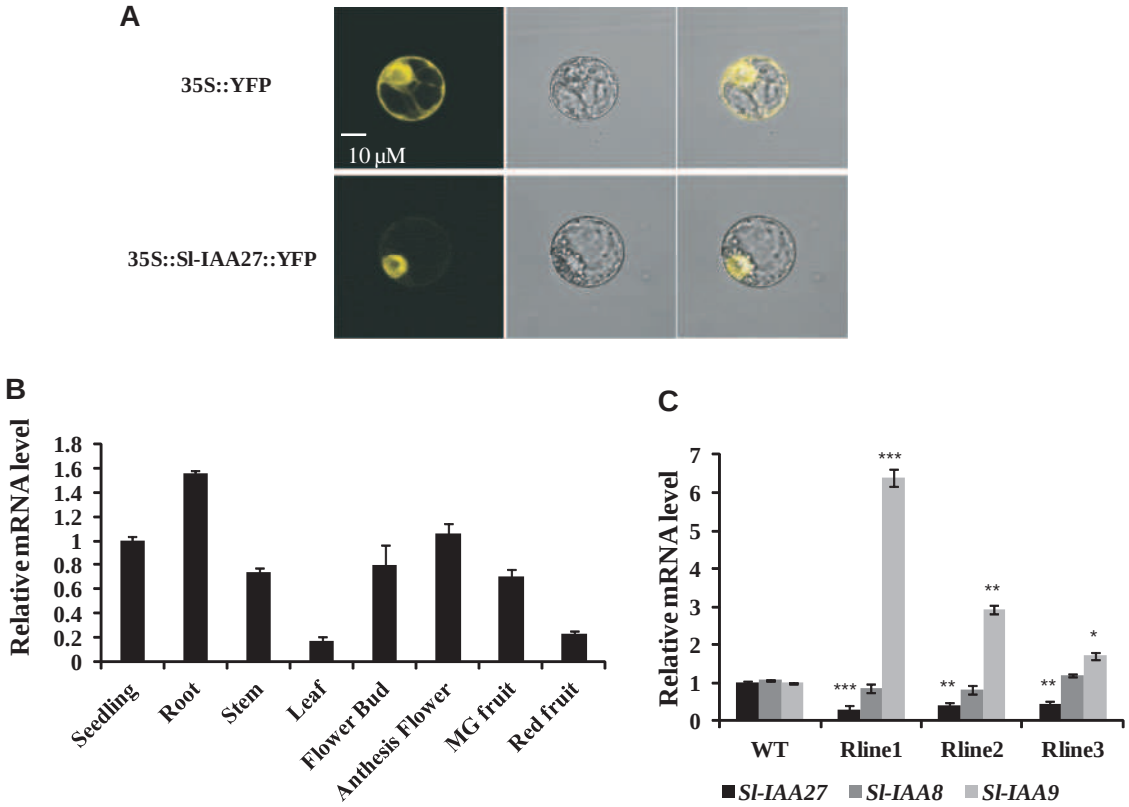

Fig. 2 Subcellular localization of Sl-IAA27 protein and the expression pattern of the *Sl-IAA27* gene at the transcriptional level. (A) Transient transformation in tobacco protoplasts showing the nuclear targeting of the Sl-IAA27 protein fused to YFP and expressed under the control of the 35S promoter. (B) Profiling of *Sl-IAA27* transcript accumulation in various tomato plant tissues monitored by qRT–PCR. Seedlings were used as reference (MG = mature green). (C) Transcript levels of *Sl-IAA27*, *Sl-IAA8* and *Sl-IAA9* genes in three independent *Sl-IAA27* RNAi lines (Rline1, Rline2 and Rline3) assessed in young fruits (10 d after anthesis) by qRT–PCR.

Supplementary Fig. S3C). Due to the difference in NPA sensitivity of *Sl-IAA27* RNAi roots, the auxin sensitivity of *Sl-IAA27* RNAi plants was further explored by determining the auxin dose–response on elongation of hypocotyl segments. **Fig. 4C** shows that independently of the auxin concentration used, the *Sl-IAA27* RNAi hypocotyls were always more elongated than wild-type hypocotyls. After 24 h of auxin treatment, maximum hypocotyl elongation was obtained with a 10^{-5} M NAA (naphthalene-1-acetic acid) concentration in the transgenic lines; while this was within the range of auxin concentrations used in the experiment, it was not reached for the wild type. These data indicate that down-regulation of *Sl-IAA27* confers higher auxin sensitivity to the transgenic plants at the level of hypocotyl elongation.

Because *Aux/IAA* genes have been reported to be up-regulated by auxin (Abel et al. 1994), the auxin responsiveness of the *Sl-IAA27* gene was investigated by qRT–PCR in seedling tissues. The data in **Fig. 4D** clearly indicate that *Sl-IAA27* was down-regulated by auxin treatment. Moreover, using a transient expression experiment in the tobacco protoplast system, the activity of the *Sl-IAA27* promoter fused to green fluorescent protein (GFP) showed no auxin-induced regulation compared with the DR5 synthetic promoter (**Fig. 4E**). In silico analysis of 2,000 bp of the *Sl-IAA27* promoter sequence using PLACE software (http://www.dna.affrc.go.jp/PLACE/index.html) revealed the presence of a variety of conserved motifs, but none of these putative *cis*-elements corresponded to the AuxREs known to drive auxin responsiveness. Strikingly, up to 41 conserved motifs putatively involved in light regulation were found, including a GT1 motif (S000198 GRWAAW), one GT1CORE motif (S000125 GGTTAA) and 17 GATA motifs (S000039 GATA).

Sl-IAA27 RNAi plants showed a lower Chl content

Sl-IAA27 RNAi leaves showed a pale green color more dramatically visible during the first weeks of plant development. In contrast to the green leaves in wild-type plants, transgenic lines presented yellow to light green leaves depending on the level of *Sl-IAA27* down-regulation, with a more marked phenotype in Rline1 (**Fig. 5A**). Chl *a* and *b* content measured by spectrophotometry showed a significant reduction in leaves of the transgenic plants compared with wild-type plants, in line with their light green color (**Fig. 5C**). Notably, the lower level of Chl observed in the RNAi leaves is not related to a diminution of chloroplast number. Indeed, microscopic observation of leaves from the wild type and Rline1 showed that

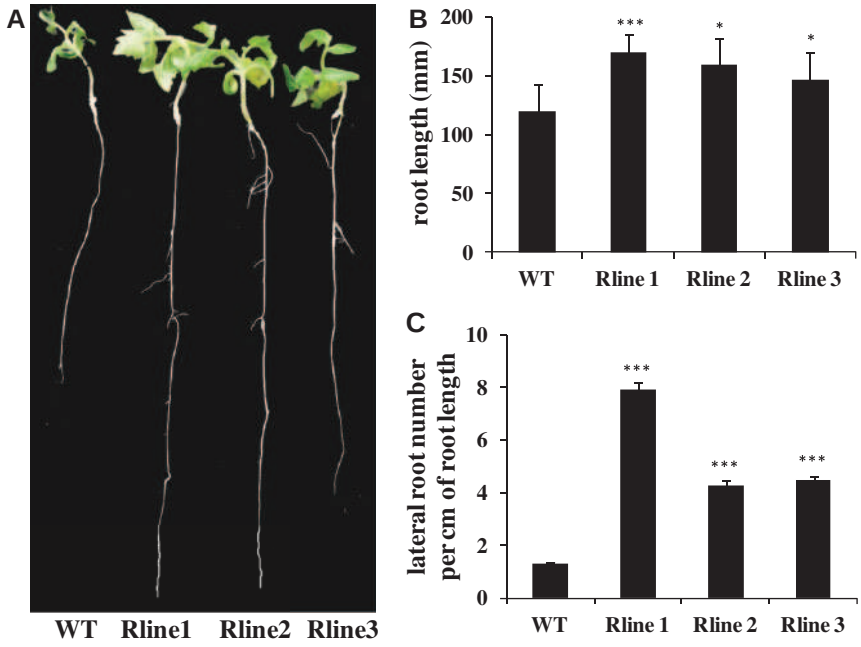

Fig. 3 Altered root growth in *Sl-IAA27* RNAi lines. (A) Root development in the wild type and *Sl-IAA27* RNAi lines assessed in 3-week-old seedlings grown on MS/2 medium. (B) The mean primary root length in the wild type and *Sl-IAA27* down-regulated lines. (C) Lateral root number per cm of root length in the wild type and *Sl-IAA27* RNAi lines. Statistical analysis were realized using the Student's test, ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

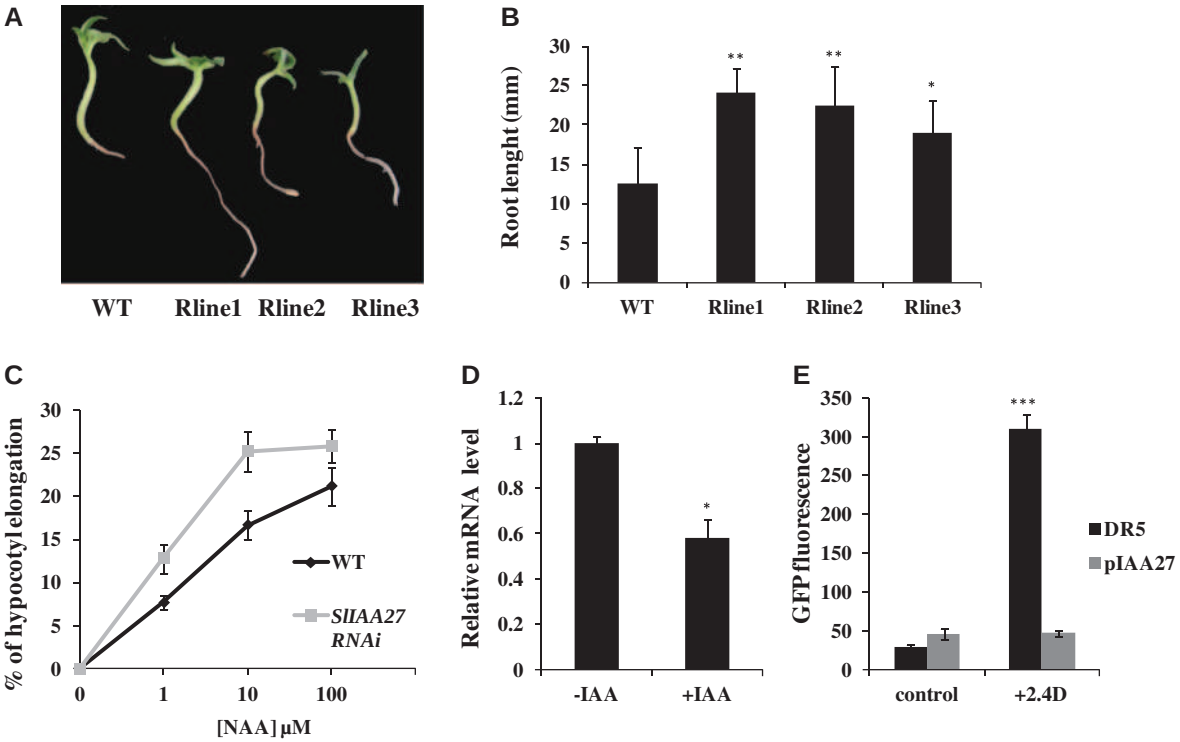

Fig. 4 Altered auxin response in *Sl-IAA27* down-regulated lines. (A) Effect of NPA (5 µM) treatment on root development of 3-week-old wild type (WT) and RNAi tomato lines grown on MS/2 medium. (B) Primary root length of WT and RNAi lines assessed on seedlings treated with NPA. (C) Auxin dose–response of elongation of hypocotyl segments treated with NAA. Hypocotyl segments 9 mm long were cut from 8-day-old seedlings and treated for 24 h with the indicated concentration of NAA. Data represent mean values obtained with three replicates for each of the three *Sl-IAA27* RNAi lines. (D) Auxin regulation of *Sl-IAA27* expression. *Sl-IAA27* transcript accumulation was monitored by qRT–PCR in seedlings treated for 2 h with 20 µM IAA. (E) Auxin responsiveness of the *Sl-IAA27* promoter. GFP fluorescence of protoplasts transformed either with the synthetic promoter DR5 or the *Sl-IAA27* promoter fused to GFP and treated or not with 2,4-D (50 µM). Statistical analyses were realized using the Student's test, ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

chloroplast number was not modified but that Chl fluorescence was dramatically reduced as assessed by fluorescence emission (**Fig. 5B**). In *Sl-IAA27*-overexpressing plants, no visible difference was observed in leaf color compared with the wild type. Analysis of Chl *a* and *b* content revealed that the Chl content is not modified by the overexpression of *Sl-IAA27* in leaves (**Supplementary Fig. S4A**).

The lower Chl content in *Sl-IAA27* RNAi leaves was correlated with the down-regulation of genes involved in key steps of Chl biosynthesis. In particular, transcript accumulation of *HEMA1*, *protochlorophyllide reductase a*, *b*, *c* and chelatase subunit *chlh* and *chli* genes displayed significantly lower levels than the wild type, while those corresponding to *aminolevulinate dehydratase* and chelatase subunit *chld* were not affected. Moreover, transcript accumulation of *GUN4*, a positive regulator of Chl biosynthesis (Davison et al. 2005, Peter and Grimm 2009, Adhikari et al. 2011), was also significantly reduced in the transgenic lines (**Fig. 5D**; **Supplementary Fig. S5**, **Supplementary Table S1**). Down-regulation of genes related to Chl biosynthesis was still observed in *Sl-IAA27* RNAi plants in flowers at the anthesis stage and in young fruits (7 d after anthesis) (**Fig. 6A, B**). Analysis of the 2,000 bp promoter sequence of the Chl biosynthesis genes analyzed above showed the presence of AuxREs in all corresponding promoters except for *aminolevulinate dehydratase*, chelatase subunit *chli* and *GUN4*, thus suggesting a putative auxin regulation of most of these genes.

To check whether the down-regulation of Chl biosynthesis-related genes results from the higher sensitivity to auxin of *Sl-IAA27* RNAi plants, the auxin regulation of these genes was assessed at the transcriptional level in wild-type plants. None of these genes showed down-regulation by auxin and, in fact, *HEMA1* and *protochlorophyllide a* genes rather were up-regulated (**Supplementary Fig. S6**). These data suggest that down-regulation of Chl biosynthesis-related genes in the *Sl-IAA27* RNAi lines is not directly induced by higher auxin responsiveness of the plants.

Sl-IAA27 silencing resulted in altered fruit development and reduced fertilization

The effect of *Sl-IAA27* silencing on fruit development was investigated in the three Sl-IAA27 RNAi lines. While the leaves of *Sl-IAA27* RNAi plants display a pale green color compared with the wild type, the fruit color is not modified at any stage of fruit development and ripening (**Supplementary Fig. S7**). However, down-regulation of *Sl-IAA27* resulted in alteration of fruit morphology, with the formation of fruits with a modified shape and reduced size. Indeed, *Sl-IAA27* RNAi fruits displayed a lower fruit width but normal fruit length (**Fig. 6C**). Fruit volume was dramatically reduced in *Sl-IAA27* RNAi lines compared with wild-type plants (**Fig. 6D**). Modification of *Sl-IAA27* RNAi fruit morphology was correlated with altered flower anatomy. Observation of an *Sl-IAA27* RNAi ovary by microscopy

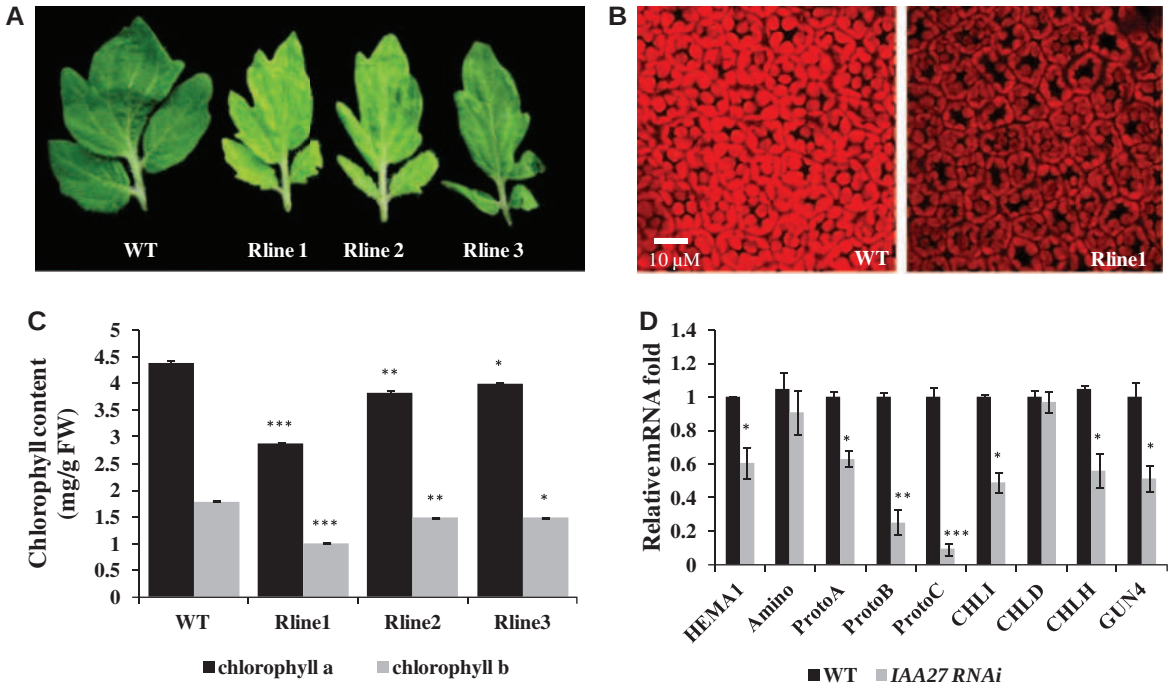

Fig. 5 Alteration of Chl content in *Sl-IAA27* down-regulated lines. (A) Young leaves of *Sl-IAA27* RNAi lines displaying light green color compared with the wild type (WT). (B) Leaf chloroplasts of WT and *Sl-IAA27* Rline1 observed by confocal microscopy. (C) Chl *a* and *b* leaf content (mg g FW^{-1}) in *Sl-IAA27* RNAi and WT lines assessed by spectrophotometry. (D) Transcript accumulation corresponding to genes involved in photosynthesis and Chl biosynthesis monitored by qRT–PCR in leaf tissues of RNAi and WT lines. Statistical analyses were realized using the Student's test, $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

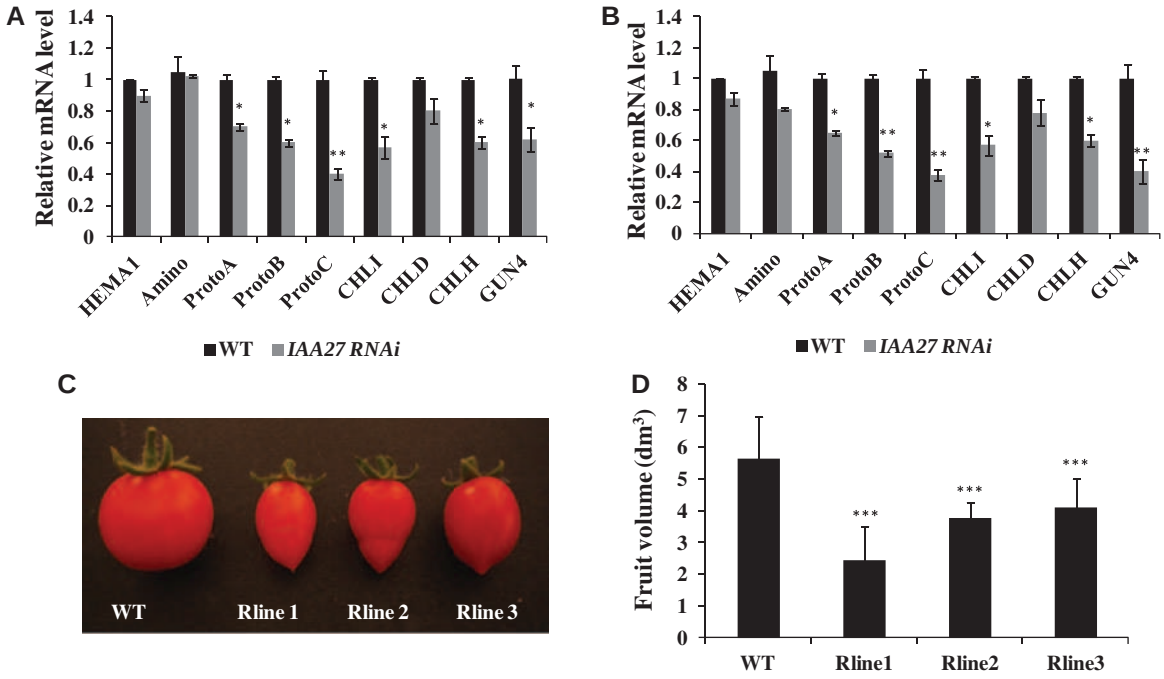

Fig. 6 Altered Chl biosynthesis-related gene regulation and reduced fruit size in *Sl-IAA27* RNAi lines. (A) and (B) Transcript levels of genes involved in photosynthesis and Chl biosynthesis monitored by qRT–PCR in mature flowers at the anthesis stage (A) and in young fruits (10 d after anthesis) (B). (C) Altered fruit shape and volume observed at the full-ripe stage (BK + 10) stage. (D) Fruit volume determined at BK + 10. The data represent mean values obtained with three replicates for each RNAi line. Statistical analyses were realized using the Student's test, $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

revealed a modified shape with enlarged placenta and lower number of ovules compared with the wild type (**Fig. 7A**). This altered anatomy structure was maintained in the developed fruits (**Fig. 7B**). Ovule numbers showed a dramatic reduction in Rline1, with an average of 15 ovules per ovary compared with 24 in the wild type (**Fig. 7C**). A decrease in seed number was also observed in *Sl-IAA27* RNAi lines that was, however, more severe than the reduction in ovule number. Indeed, only 27% of Rline1 ovules developed seeds (**Fig. 7C, D**). To check whether in addition to the reduction in number, the ovules also have altered fertility in the transgenic lines, cross-fertilization was performed using transgenic lines as the female recipient and the wild type as the pollen donor. The data presented in the upper part of **Table 1** clearly show that wild-type pollen is unable to increase the seed number, suggesting that ovule fertility is reduced in *Sl-IAA27* RNAi. Moreover, reciprocal crossing using the wild type as the female recipient also revealed a loss of pollen in the transgenic lines (**Table 1**). Overall, these data indicated that both ovules and pollen display reduced fertility in *Sl-IAA27* RNAi. Fruits of *Sl-IAA27*-overexpressing plants displayed no modification in fruit shape or volume, and presented no reduction in seed number (**Supplementary Fig. S4B, C**).

Discussion

Most of our knowledge of the physiological significance of *Aux/IAA* genes comes from the characterization of gain-of-function mutants in Arabidopsis (Rouse et al. 1998, Tian and Reed 1999, Nagpal et al. 2000, Rogg et al. 2001, Fukaki et al. 2002, Tatematsu et al. 2004, Uehara et al. 2008). In Solanaceae species and particularly in tomato, a wide range of phenotypes associated with down-regulation of *Aux/IAA* genes have been reported (Wang et al. 2005, Kloosterman et al. 2006, Chaabouni et al. 2009a, Deng et al. 2012), but the role of most tomato *Aux/IAA* genes still remains to be elucidated. While aiming to illuminate further the multiple functions of *Aux/IAA* genes, the present study more specifically deals with the functional characterization of *Sl-IAA27* for which information regarding the putative function is lacking, in the Solanaceae as well as for its ortholog in Arabidopsis. Silencing of *Sl-IAA27* results in pleiotropic morphological and developmental phenotypes including altered fruit development and Chl accumulation. In these *Sl-IAA27* RNAi plants, both ovule and pollen fertility are reduced, indicating that normal expression of *Sl-IAA27* is required for flower fertility. Furthermore, *Sl-IAA27* down-regulated lines showed a dramatic reduction in ovule formation which, associated with reduced fertility, leads to the production of seedless fruits. Compared with the wild type, the fruit shape and internal fruit structure are also altered, with an enlarged placenta and smaller final fruit size. In *Sl-IAA27*-overexpressing plants, no phenotypes were observed regarding the Chl content in leaves or fruit development, suggesting that even if the *Sl-IAA27* mRNA level is high, its protein level is probably regulated by auxin-mediated degradation, restoring its level to that in the wild type.

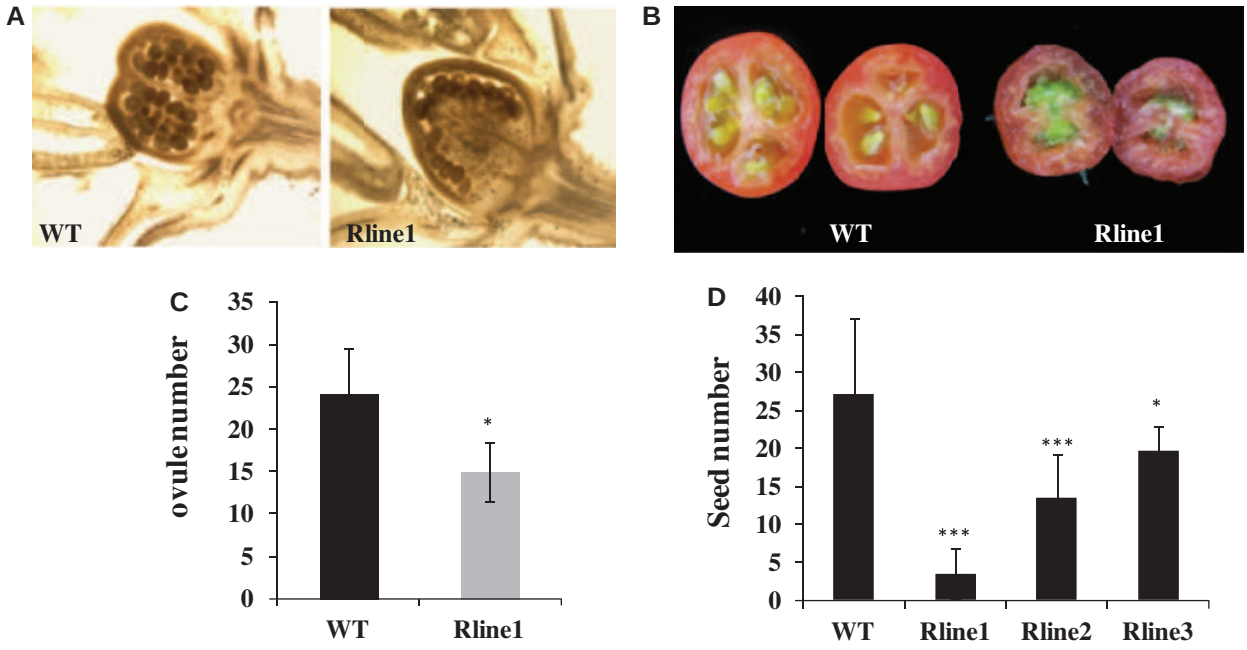

Fig. 7 Altered ovule, seed and fruit development in *Sl-IAA27* down-regulated lines. (A) Ovary shape in mature flowers at the anthesis stage in the wild type (WT) and *Sl-IAA27* Rline1. (B) Fruit shape in WT and *Sl-IAA27* Rline1 displaying smaller size and lower seed content. (C) Average ovule number per flower at the anthesis stage in WT and *Sl-IAA27* Rline1. (D) Seed number in WT and *Sl-IAA27* RNAi mature fruits. Statistical analyses were realized using the Student's test, $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

Table 1 Fruit set and seed number in cross-pollinated flowers

Female recipient	Pollen donor	Fruit set/crossed flowers	Fruit set (%)	Average seed number per fruit
Rline1	WT	6/30	20	9
Rline2	WT	11/30	37	12
Rline3	WT	11/30	37	21
WT	Rline1	12/30	40	7
WT	Rline2	14/30	47	14
WT	Rline3	13/30	43	18
WT	WT	28/30	93	28

Percentage of fruit set and seed number determined following cross-fertilization of emasculated flowers [wild type (WT) and RNAi lines] with pollen from WT or *Sl-IAA27* down-regulated lines (Rline1, Rline2 and Rline3).

Previous studies showed that down-regulation of either *Sl-IAA3*, *Sl-IAA9* or *Sl-IAA15* results in modified auxin sensitivity associated with altered leaf structure and modified plant architecture (Wang et al. 2005, Chaabouni et al. 2009a, Deng et al. 2012). In contrast, the higher auxin sensitivity of *Sl-IAA27* down-regulated plants did not result in visible alteration of the plant architecture. The transgenic lines display altered primary root growth and lateral root formation. Interestingly, overexpressing and RNAi plants displayed opposite phenotypes, with reduced primary root growth and no lateral root formation in overexpressing lines and increased primary root length with more lateral roots in RNAi lines. This suggests that in roots of overexpressing plants, auxin-dependent degradation of Sl-IAA27 is probably not able to restore protein levels similar to the wild type.

In the presence of the auxin transport inhibitor NPA, *Sl-IAA27* RNAi roots displayed no lateral roots, suggesting that NPA is still able to block transverse divisions as in the wild type (Casimiro et al. 2001). Nevertheless, though root elongation was reduced upon NPA treatment in *Sl-IAA27* RNAi lines, the root length remains more important than in the wild type subjected to the same treatment. Root elongation is controlled by a specific auxin concentration balance, and auxin is known to play a predominant role in root development mainly through controlling cell division (Friml et al. 2002, Ullah et al. 2003, Ding and Friml 2010, Dubrovsky et al. 2011). In Arabidopsis, characterization of several *Aux/IAA* mutants (CRANE/IAA18, SHY2/IAA3, AXR2/IAA7 and SLR/IAA14) revealed the function of these genes in regulating the root architecture (Nagpal et al. 2000, Vanneste et al. 2005, Uehara et al. 2008). NPA application was reported to lead to IAA accumulation in the root tip by blocking auxin basipetal transport in roots (Casimiro et al. 2001). The more elongated primary root in *Sl-IAA27* RNAi plants upon NPA treatment may result from the difference either in auxin concentration or in auxin sensitivity of transgenic lines compared with the wild type. Analysis of the auxin dose–response of hypocotyl elongation where endogenous auxin was removed showed that transgenic plants were more sensitive to NAA. These data indicate that for a specific auxin concentration, *Sl-IAA27* RNAi plants present higher auxin sensitivity, suggesting that Sl-IAA27 protein acts as a repressor of auxin responses. In line with this hypothesis,

previous data showed that Sl-IAA27 represses the auxin-dependent transcription of both synthetic and native auxin-responsive promoters (Audran-Delalande et al. 2012).

In contrast to classical *Aux/IAA* genes characterized by a rapid induction of their expression in response to auxin (Walker and Key 1982, Theologis et al. 1985), *Sl-IAA27* expression is down-regulated by exogenous auxin treatment. *At-IAA27* and *Sb-IAA21*, the *Sl-IAA27* homologs from Arabidopsis and Sorghum, respectively, are also down-regulated by auxin (Paponov et al. 2008, Wang et al. 2010a), suggesting that this feature might be conserved across plant species. Previous analysis of the auxin regulation of tomato *Aux/IAA* genes with short-term treatment has shown that *Sl-IAA8* is also down-regulated by auxin and that *Sl-IAA9* expression is not significantly responsive to auxin (Audran-Delalande et al. 2012). These data indicate that members of clade B of the *Aux/IAA* gene family (*Sl-IAA8*, *Sl-IAA9* and *Sl-IAA27*) behave differently from other members regarding their regulation by auxin. Recently, phylogenetic analysis of the tomato Aux/IAA family revealed that Sl-IAA9 is closely related to Sl-IAA27 (Audran-Delalande et al. 2012), raising the hypothesis that protein structure similarity may result in functional redundancy. Beside domains I–IV which are characteristic of the Aux/IAA family (Abel et al. 1995), our study identified another conserved motif (YxGLS) located before domain I in the N-terminal part. It is noteworthy that this motif, present in both Sl-IAA27 and Sl-IAA9, is not found in any other Sl-IAA and is remarkably conserved in monocot and dicot species, including Arabidopsis, potato, poplar, maize and rice. Whether the conserved YxGLS motif determines some of the functional roles of Sl-IAA27 and Sl-IAA9 remains to be elucidated in planta. Despite their common structural features, the two genes seem to have distinct functions in plant developmental processes, as indicated by the contrasting phenotypes resulting from the down-regulation of *Sl-IAA27* and *Sl-IAA9*. Indeed, down-regulation of *Sl-IAA9* leads to altered leaf morphogenesis (Wang et al. 2005) while such a phenotype is absent in *Sl-IAA27*-underexpressing lines. Also, down-regulation of *Sl-IAA27* results in lower fertility of both ovule and pollen, while reduced expression of *Sl-IAA9* did not affect this (Wang et al. 2005). Moreover, in contrast to *Sl-IAA9* down-regulated plants where fruits display no morphological or size alteration, *Sl-IAA27* RNAi fruits present a reduced size and altered shape. Taken together, these data suggest that normal expression of *Sl-IAA27* is required for the fertilization process and for fruit development, whereas *Sl-IAA9* is essential for triggering fruit initiation (Wang et al. 2005). Even though both *Sl-IAA27* and *Sl-IAA9* down-regulated lines yield seedless fruit, two different mechanisms seem to underlie this phenotype. This is further supported by the observed up-regulation of *Sl-IAA9* expression in *Sl-IAA27* down-regulated plants shown in **Fig. 2C**. Nevertheless, our data suggest a link between the two genes while revealing that the expression of *Sl-IAA9* is regulated by *Sl-IAA27*, either directly or indirectly.

Even though the level of *Sl-IAA27* expression is lowest in wild-type leaves, the phenotypes displayed by the RNAi lines indicate that a threshold level of *Sl-IAA27* expression is necessary to promote Chl biosynthesis in leaves. On the other hand, the overexpression of *Sl-IAA27* does not result in any increase in Chl content in leaves, suggesting that the basal Sl-IAA27 protein level in the wild type is sufficient to sustain maximum activity of Chl biosynthesis genes. This may explain the lack of effect on Chl content in the overexpressing lines. The reduced Chl content of *Sl-IAA27* RNAi plants correlates with the down-regulation of many genes involved in Chl biosynthesis in leaves. Moreover, *Sl-IAA27* seems to regulate at the transcriptional level the expression of the *GUN4* gene which encodes a transcription factor known to activate the Chl biosynthesis pathway. The presence of AuxREs in the promoter of most of these genes suggests that they may potentially undergo direct regulation by auxin signaling. In wild-type plants, the expression of *HEMA1* and *ProtoA* genes is up-regulated upon auxin treatment, while in *Sl-IAA27* RNAi plants the expression of these two genes is down-regulated. To explain this apparent contradiction, it is possible to consider that these two genes are under competitive regulation by activator and repressor ARFs. Taking into account that auxin accumulation is known to lead to the degradation of the majority of Aux/IAA proteins which frees both activator and repressor ARFs (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Leyser 2006, Tan et al. 2007, Chapman and Estelle 2009), it can be hypothesized that in normal situations the expression of genes related to Chl biosynthesis is down-regulated by one or more repressor ARFs that are trapped by Sl-IAA27 protein. These repressor ARFs are released in the absence of Sl-IAA27 in RNAi plants or upon auxin treatment. On the other hand, the release of activator ARFs in the presence of auxin may result in the up-regulation of *HEMA1* and *ProtoA* genes observed in wild-type plants. Taken together, the data suggest a complex and finely tuned mechanism of regulation of auxin responses involving Aux/IAAs and ARFs.

The smaller size of the *Sl-IAA27* down-regulated fruits may result from the reduced photosynthetic activity in the leaves. This hypothesis is supported by the prevailing opinion that fruit growth is predominantly supported by photoassimilate supply from source tissues (Lytovchenko et al. 2011). In keeping with the prominent role of photosynthetic activity in the leaves, down-regulation of Chl biosynthesis in a fruit-specific manner indicated that photosynthesis was essential for seed set but did not affect fruit growth (Lytovchenko et al. 2011). It is important to mention, however, that cells in developing fruit contain photosynthetically active chloroplasts and express both nuclear-encoded and plastid-encoded genes for photosynthetic proteins (Piechulla et al. 1987). It has been reported that during early fruit development, photosynthesis itself may provide a significant contribution to sustain the growth of the organ (Obiadalla-Ali et al. 2004). More recently, global transcriptomic profiling of fruit set and early fruit development also revealed a strong activation of photosynthesis-related genes

(Wang et al. 2009). Interestingly, our present data show that the expression of a number of Chl biosynthesis genes is down-regulated during the initial phase of fruit development in *Sl-IAA27* RNAi lines, which may impact final fruit size. In line with this hypothesis, previous studies indicated that the induction of genes related to photosynthesis and chloroplast biogenesis in tomato pericarp cells is positively correlated with fruit size (Kolotilin et al. 2007). While taking these data together supports the idea that both leaf and fruit photosynthetic activities may contribute to sustain fruit growth, the relative contribution of the two source tissues to the needs of the fruit in terms of photoassimilate supply remains unclear.

Materials and Methods

Plant materials and growth conditions

Tomato plants (*Solanum lycopersicum* cv. MicroTom) were grown under standard greenhouse conditions. The conditions were as follows: 14 h day/10 h night cycle, 25/20°C day/night temperature, 80% relative humidity, 250 μmol m^{-2} s^{-1} light intensity (intense luminosity). Tomato seeds were first surface sterilized for 5 min in bleach and water, rinsed five times in sterile water and sown in recipient Magenta vessels containing 50 ml of 50% Murashige and Skoog (MS) culture medium and 0.8% (w/v) agar, pH 5.9.

Plant transformation

To generate *Sl-IAA27* RNAi plants, the forward 5′-atgtctgtaccattag-3′ and reverse 5′-gttctgagtctcatgg-3′ primers were used to amplify the 155 bp *Sl-IAA27* RNAi sequence. This fragment was cloned into the pHellsgate12 vector using Gateway cloning. To generate *Sl-IAA27*-overexpressing plants, the forward 5′-atgtctgtaccattag-3′ and reverse 5′-ctaattttggttcttgc-3′ primers were used to amplify the 837 bp of *Sl-IAA27* coding sequence. This fragment was cloned into the pMDC32 vector using Gateway cloning. To generate *pIAA27*–GUS (the promoter of the *Sl-IAA27* gene fused to the GUS reporter gene) plants, the forward 5′-catacaggagatatggattg-3′ and reverse 5′-tgctcaactttcc-3′ primers were used to amplify the 2,000 bp *SlIAA27* promoter sequence. This fragment was cloned into the pMDC162 expression vector by Gateway cloning. Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation according to Wang et al. (2005). All experiments were carried out using homozygous lines from the F_2 and F_3 generation selected on kanamycin resistance.

Histochemical GUS analysis

For histochemical GUS analysis, tissues of *pIAA27*–GUS lines were put in GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Triton X-100, 0.3 mg ml^{-1} X-Gluc); a vacuum was made for 15 min twice. Tissues were then incubated in GUS staning solution at 37°C overnight. Samples were then decolorated using several washes of graded ethanol series as indicated by Wang et al. (2005).

Transient expression using a single cell system

For nuclear localization of the Sl-IAA27 protein, the *Sl-IAA27* CDS sequence was cloned by Gateway technology (forward 5′-atgtctgtaccattag-3′ and reverse 5′-gcaagaaccaaaattag-3′ primers) in-frame with YFP into the pEarlyGate104 vector, and expressed under the control of the 35S CaMV promoter. The empty vector pEarleyGate104 was used as control. For the analysis of *Sl-IAA27* promoter regulation by auxin, the 2,000 bp of the *Sl-IAA27* promoter sequence was fused to the GFP reporter gene by Gateway technology, into the pMDC107 vector. Protoplasts were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells and transfected according to the method described previously (Leclercq et al. 2005). YFP localization by confocal microscopy and GFP measurement by flux cytometry were performed as described previously (Audran-Delalande et al. 2012).

Hormonal treatment

For hypocotyl auxin dose–response (0, 1, 10 and 100 μM NAA) and NPA treatment, experiments were carried out as described by Chaabouni et al. (2009a). Auxin treatment of seedlings was performed as previously described by Audran-Delalande et al. (2012).

RNA isolation and real-time PCR analysis

RNA extraction, cDNA synthesis and qRT–PCR analyses were performed as previously described (Pirrello et al. 2006). The primer sequences are listed in **Supplementary Table S2**.

Determination of Chl content

A 100 mg aliquot of leaves from 3-week-old *Sl-IAA27* RNAi and wild-type plants was weighed and ground with 1 ml of 80% acetone. The liquid obtained was centrifuged for 1 min at 10,000 r.p.m. to remove any remaining solid tissue. Samples were analyzed by spectrophotometry at two wavelengths, 645 and 663 nm, using 80% acetone as the blank. The Chl *a* and *b* content was determined using the following equations: Chl $a = 0.999A_{663} - 0.0989A_{645}$ and Chl $b = -0.328A_{663} + 1.77A_{645}$

Microscopy analysis

For chloroplast observation, air was removed from leaves which were then analyzed with a Leica TCS SP2 confocal laser scanning microscope. Images were obtained with a ×40, 1.25 numerical aperture water-immersion objective. Excitation was at 488 nm and the emitted light was captured at 505–535 nm. For flower observation, flowers at the anthesis stage were fixed in ethanol and included in 5% agarose. Flowers were cut every 80 μm using a vibratome (vibratome Leica VT 100S) and then observed with a Leica DM IRBE inverted microscope.

Phenotypic analysis

The phenotypic analyses were performed on fruits of 10 plants from each transgenic line and from the wild-type plants cultivated in the same conditions. The inflorescence formation occurred at the same time in transgenic plants and in the wild type. Phenotypic analyses were performed on the six first fruits developed on each plant, which were similar with regard to their stage of development in transgenic plants and in the wild type (data not shown). In total, 60 fruits of the transgenic lines and the wild type were studied.

The experiment was done twice. Fruit color (Hue angle) was determined using a Chroma Meter CR-310 Minolta. Cross-pollination between *Sl-IAA27* RNAi and WT flowers was performed as described in Wang et al. (2005).

Sequence data

To identify homologs of tomato clade B, phylogenetic analysis were performed with all Aux/IAA members in maize, rice and poplar genomes, available, respectively, in the Maize GDB genome browser tool (http://gbrowse./maizegdb.org/gbrowse/cgi-bin/gbrowse/maize) and KOME database (http://www.cdna.01.dna.affrc.go.jp/cDNA/), and *Populus trichocarpa* Genome database (http://www.plantgdb.org/PtGDB/). Both BLASTN and TBLASTN search were performed on the whole set of potato unigenes in the SGN database (Solanaceae Genomics Network, http://www.sgn.cornell) using Aux/IAA tomato clade B sequences.

Sequence data for the Arabidopsis genes used in this article can be found in the Arabidopsis Genome Initiative data library under the following accession numbers: *AtIAA8* (AT2G22670), *AtIAA9* (AT5G65670) and *AtIAA27* (AT4G29080).

Sequence data for maize genes used in this article can be found in Maize GDB genome browser tool (http://gbrowse./maizegdb.org/gbrowse/cgi-bin/gbrowse/maize) under the following accession numbers: *ZmIAA2* (GRMZM2G077356_PO1), *ZmIAA10* (GRMZM2G037369_PO1), *ZmIAA14* (GRMZM2G077356_PO1), *ZmIAA21* (GRMZM2G147243_PO2), *ZmIAA28* (GRMZM2G035465_PO3) and *ZmIAA29* (GRMZM2G163848_PO5).

Sequence data for the rice genes used in this article can be found in the KOME database (http://www.cdna.01.dna.affrc.go.jp/cDNA/) under the following accession numbers: *OsIAA3* (AK104654), *OsIAA5* (AK106121), *OsIAA13* (AK059838), *OsIAA19* (AK109363) and *OsIAA21* (AK121989).

Sequence data for the poplar genes used in this article can be found in the *P. trichocarpa* genome database under the following accession numbers: *PoptrIAA27.1.1* (POPTR_0001s18680.1), *PoptrIAA27.2.1* (POPTR_0003s04980.1), *PoptrIAA27.3.1* (POPTR_0006s16640.1) and *PoptrIAA9* (POPTR_0002s10880.1).

Sequence data for the tomato genes used in this article can be found in Genbank/EMBL data libraries under the following accession numbers: *Sl-IAA8* (JN379436), *Sl-IAA9* (JN379437) and *Sl-IAA27* (JN379450).

Supplementary data

Supplementary data are available at PCP online

Funding

This work was supported by the Laboratoire d' Excellence (LABEX) [TULIP (ANR-10-LABX-41)]; the French government [grant for the degree of PhD of C.B.].

Acknowledgments

We thank O. Berseille and D. Saint-Martin for tomato culture, and the 'plateforme de microscopie TRI' (Fédération de Recherches FR3450 Castanet-Tolosan) for microscopy analyses. The authors thank C. Pecher for his technical assistance and expertise in flow cytometry (FacsCalibur, RIO Imaging platform, INSERM UPS UMR 1048, Toulouse). The authors thank F.Regad for his help regarding experimental design and analysis.

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