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Auxin is a central hormone that exerts pleiotropic effects on plant growth including the development of roots, shoots, flowers and fruit. The perception and signaling of the plant hormone auxin rely on the cooperative action of several components, among which auxin/indole-3-acetic acid (Aux/IAA) proteins play a pivotal role. In this study, we identified and comprehensively analyzed the entire *Aux/IAA* gene family in tomato (*Solanum lycopersicum*), a reference species for Solanaceae plants, and the model plant for fleshy fruit development. Functional characterization using a dedicated single cell system revealed that tomato Aux/IAA proteins function as active repressors of auxin-dependent gene transcription, with, however, different Aux/IAA members displaying varying levels of repression. Phylogenetic analysis indicated that the *Aux/IAA* gene family is slightly contracted in tomato compared with Arabidopsis, with a lower representation of non-canonical proteins. *Sl-IAA* genes display distinctive expression pattern in different tomato organs and tissues, and some of them display differential responses to auxin and ethylene, suggesting that Aux/IAAs may play a role in linking both hormone signaling pathways. The data presented here shed more light on *Sl-IAA* genes and provides new leads towards the elucidation of their function during plant development and in mediating hormone cross-talk.

Keywords: Auxin • Aux/IAA • Ethylene • Expression analysis • Tomato • Transcriptional repressor.

Abbreviations: AFB, auxin receptor F-box; ARF, auxin response factor; Aux/IAA, auxin/indole-3-acetic acid; AuxRE, auxin-responsive *cis*-element; CaMV, *Cauliflower mosaic virus*; EAR, ethylene-responsive element-binding factor-associated amphiphilic repression; EST, expressed sequence tag; GFP, green fluorescent protein; MS medium, Murashige and Skoog medium; NLS, nuclear localization signal; qRT–PCR, quantitative reverse transcription–PCR; *SAUR*, small auxin up RNA; SGN, Solanaceae Genomics Network; Sl-IAA, *Solanum lycopersicum* auxin/indole-3-acetic acid; TIR1, transport inhibitor response1; TPL, topless; YFP, yellow fluorescent protein.

The nucleotide sequence data from this article can be found in the Genbank/EMBL data libraries under the following accession numbers: JN379431 (*Sl-IAA1*), JN379432 (*Sl-IAA2*), JN379433 (*Sl-IAA3*), JN379434 (*Sl-IAA4*), JN379435 (*Sl-IAA7*), JN379436 (*Sl-IAA8*), JN379437 (*Sl-IAA9*), JN379438 (*Sl-IAA11*), JN379439 (*Sl-IAA12*), JN379440 (*Sl-IAA13*), JN379441 (*Sl-IAA14*), JN379442 (*Sl-IAA15*), JN379443 (*Sl-IAA16*), JN379444 (*Sl-IAA17*), JN379445 (*Sl-IAA19*), JN379446 (*Sl-IAA21*), JN379447 (*Sl-IAA22*), JN379448 (*Sl-IAA23*), JN379449 (*Sl-IAA26*), JN379450 (*Sl-IAA27*), JN379451 (*Sl-IAA29*), JN379452 (*Sl-IAA32*), JN379453 (*Sl-IAA33*), JN379454 (*Sl-IAA35*), JN379455 (*Sl-IAA36*).

Introduction

The perception and signaling of the plant hormone auxin involve the cooperative action of several components, among which auxin/indole-3-acetic acid (Aux/IAA) proteins play a pivotal role. Aux/IAA proteins were shown to be a direct target of the auxin transport inhibitor response1 (TIR1) and of its paralogs AUXIN RECEPTOR F-BOX/AFB1 and AFB3F-box receptors (AFBs) (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Tan et al. 2007). Binding of auxin to its receptors leads to the degradation of Aux/IAA proteins. This auxin-dependent proteolysis releases auxin response factors (ARFs) that otherwise remain trapped via their binding to Aux/IAA partners. The *Aux/IAA* genes represent a class of primary auxin-responsive genes which were shown to be, in the majority, rapidly induced by auxin (Theologis et al. 1985, Oeller et al. 1993, Yamamoto and Yamamoto 1998). Aux/IAAs are described as short-lived and nuclear-localized proteins (Hagen and Guilfoyle 2002, Liscum and Reed 2002), and biochemical and genetic studies indicated that they generally function as transcriptional repressors of auxin-regulated genes (Tiwari et al. 2001, Tiwari et al. 2004). Canonical Aux/IAA

proteins share four conserved amino acid sequence motifs known as domains I, II, III and IV, although several proteins lacking one or more of these domains are also included in the family (Reed 2001). Domain I is a repressor domain that contains a conserved leucine repeat motif (LxLxLx) similar to the so-called EAR (ethylene-responsive element-binding factor-associated amphiphilic repression) domain (Tiwari et al. 2004). Domain I is also required for the recruitment of the transcriptional co-repressor TOPLESS (Szemenyei et al. 2008). Domain II confers protein instability, leading to rapid degradation of Aux/IAA through the interaction with the F-box protein TIR1 (a component of the SCF^{TIR1} ubiquitin ligase complex) (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Tan et al. 2007). In fact, mutations in Aux/IAA domain II resulted in increased protein accumulation leading to auxin-related developmental phenotypes (Reed 2001, Liscum and Reed 2002, Uehara et al. 2008). The C-terminal domains III and IV are shared with ARF proteins, and are known to promote homo- and heterodimerization of Aux/IAA polypeptides, as well as interaction between Aux/IAAs and ARFs (Remington et al. 2004, Overvoorde et al. 2005). Aux/IAAs impact the transcriptional activity of target genes through the binding to their ARF partners. ARF proteins are capable of binding to the auxin-responsive *cis*-element (AuxRE) present upstream of the coding sequence of auxin-responsive genes (Ulmasov et al. 1997). Depending on the amino acid composition of their variable internal region, the ARF proteins can either activate or repress gene transcription (Ulmasov et al. 1999). Most of our understanding of the diverse roles of Aux/IAAs in planta is based on the characterization of gain-of-function mutants in the Arabidopsis model plant, whereas phenotypes associated with loss of function are scarce probably due to important functional redundancy among Aux/IAA family members (Overvoorde et al. 2005). In contrast, down-regulation of various *Aux/IAA* genes in the Solanaceae species results in visible and distinct phenotypes. Down-regulation of the tomato (*Solanum lycopersicum*) *Sl-IAA9* resulted in pleiotropic phenotypes, consistent with its ubiquitous expression pattern (Wang et al. 2005). *Sl-IAA9*-inhibited lines also displayed some specific phenotypes such as entire leaves and parthenocarpic fruit, indicating that Sl-IAA9 is a key regulator of fruit set and leaf morphogenesis (Wang et al. 2005, Wang et al. 2009). Down-regulation of another *Aux/IAA* gene in tomato, *Sl-IAA3*, results in both auxin- and ethylene-associated phenotypes including altered apical dominance, lower auxin sensitivity, exaggerated apical hook curvature in the dark and reduced petiole epinasty in the light, thus revealing new roles for *Aux/IAA* genes (Chaabouni et al. 2009a). These data position Sl-IAA3 at the crossroads of auxin and ethylene signaling in tomato (Chaabouni et al. 2009b). More recently, it was shown that Sl-IAA15 is involved in trichome development as *Sl-IAA15*-down-regulated lines display strong reduction of type I, V and VI trichomes (Deng et al. 2012). Likewise, suppression of St-IAA2 in *Solanum tuberosum* results in clear phenotypes including increased plant height, petiole hyponasty and curvature of growing leaf primordia in the shoot apex (Kloosterman et al. 2006). These data do not support the functional redundancy among *Aux/IAA* genes generally described in the plant model Arabidopsis and clearly emphasize the need to widen the functional characterization to other plant species in order to decipher thoroughly the physiological significance of different Aux/IAA family members. To lay the foundation for a better understanding of the Aux/IAA family in the Solanaceae family, the present study identified and comprehensively analysed the entire *Aux/IAA* gene family in tomato (*S. lycopersicum*), a reference species for Solanaceae plants. Phylogenetic analysis revealed that some Aux/IAA clades are either expanded or retracted in tomato compared with Arabidopsis. Expression studies revealed a distinctive spatio-temporal pattern of expression for tomato *Aux/IAA* genes, some of which display differential responsiveness to auxin and ethylene.

Results

Identification and sequence analysis of the tomato *Sl-IAA* gene family members

Aux/IAA genes belong to a large gene family found in all plant species ranging from 26 members in *Sorghum bicolor* (S. Wang et al. 2010) to 35 in poplar (Kalluri et al. 2007). In Arabidopsis, this gene family comprises 29 members (Liscum and Reed 2002) while it contains 31 in rice and maize (Jain et al. 2006, Y. Wang et al. 2010). To shed more light on this gene family, structural and functional characterizations of the tomato *Aux/IAA* genes were carried out. Both BLASTN and TBLASTN search were performed on the whole set of tomato unigenes in the SGN database (Solanaceae Genomics Network, http://www.sgn.cornell.edu/) using either partial tomato *Aux/IAA* clones (Nebenführ et al. 2000, Jones et al. 2002) or Aux/IAA Arabidopsis protein sequences. This search was further extended taking advantage of the recent sequence information generated by the tomato genome sequencing project (Solanaceae Genomics Network, http://www.sgn.cornell.edu/). In addition, the predicted proteome deduced from the tomato genome was searched against the pfam AUX_IAA hidden-Markov model (PF02309) recognizing both AUX-IAA and ARF protein sequences (Finn et al. 2010) using the HMMER3 software. This HMM-based search identified 24 *Aux//IAA* genes in the tomato genome annotation (ITAG Release 2.3 predicted CDS). With the exception of *Sl-IAA21*, all the *Aux/IAA* genes identified in this work are present in the tomato genome annotation file iTAG2.30. Overall, this in silico search resulted in the identification of 25 tomato genes displaying the conserved features of *Aux/IAA* (**Supplementary Table S1**). The coding sequences of these genes were submitted to GenBank/EMBL. The size of the deduced Aux/IAA proteins varies greatly, ranging from 147 amino acids (Sl-IAA33) to 349 amino acids (Sl-IAA9), and the corresponding molecular mass varies from 16 to 37 kDa (**Supplementary Table S2**). The predicted isoelectric point

also varies widely from 5.02 (Sl-IAA32) to 9.08 (Sl-IAA15) (**Supplementary Table S2**), suggesting that different Aux/IAA proteins might operate in different microenvironments. Pair-wise comparisons of these Sl-IAA protein sequences showed that the identity level ranges from as low as 19% (between Sl-IAA33 and Sl-IAA8/Sl-IAA27) to a highly identical level of 79% (Sl-IAA21 and Sl-IAA23) (**Supplementary Table S3**). The overall identity among the various proteins is low, even between members of the same phylogenetic branch (**Supplementary Fig. S1**). Alignment of amino acid sequences of tomato and Arabidopsis Aux/IAAs revealed the typical four highly conserved domains found in canonical Aux/IAA proteins (Reed 2001), with the exception of Sl-IAA32 which lacks domain II and Sl-IAA33 missing domains I and II and containing only a weakly conserved domain III (**Fig. 1**). Therefore, Sl-IAA32 and Sl-IAA33 can be considered as non-canonical Aux/IAA proteins like their putative orthologs in Arabidopsis (Dreher et al. 2006).

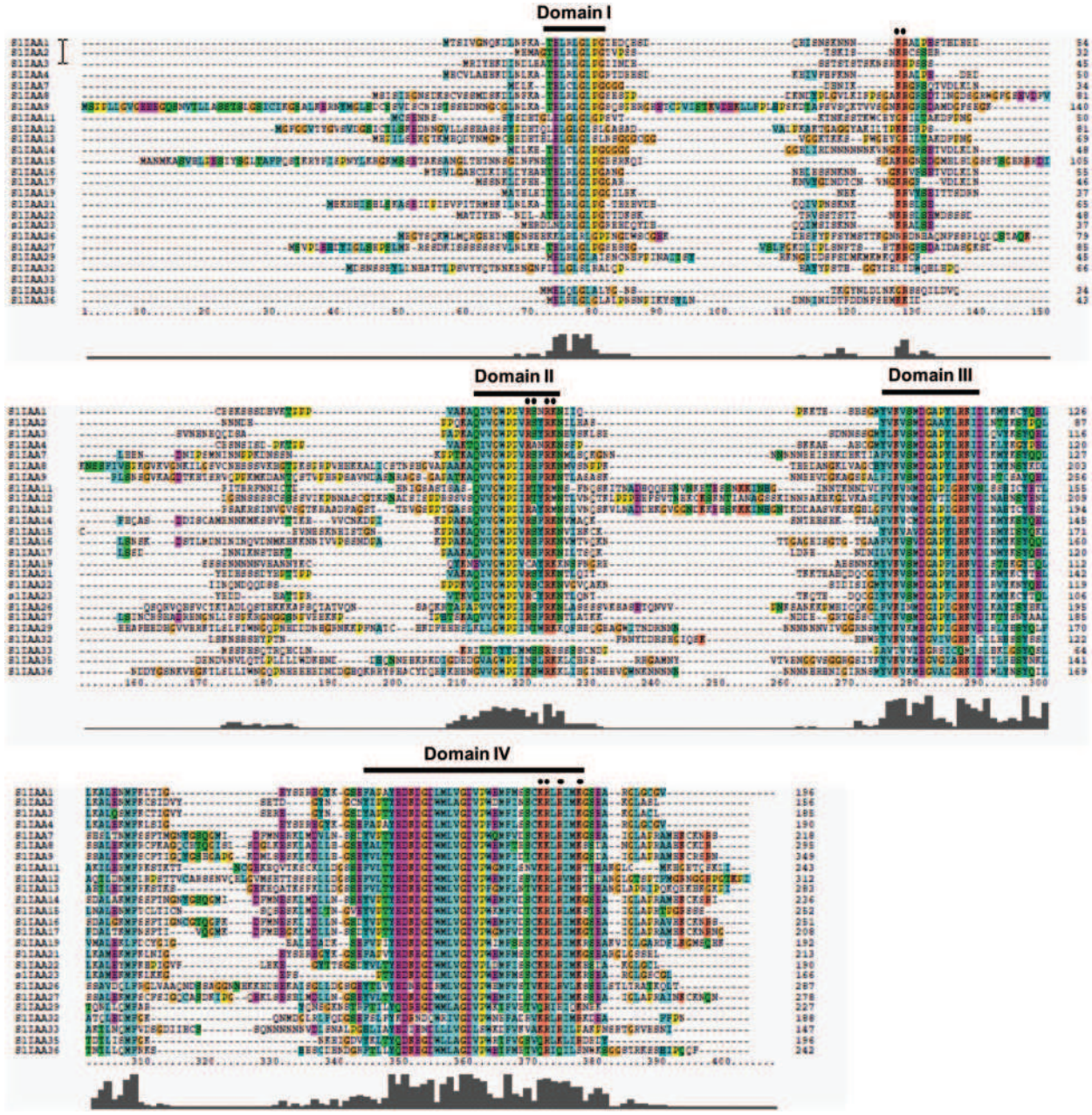

Fig. 1 Multiple sequence alignment of the full-length Sl-IAA proteins obtained with ClustalX and manual correction. Conserved domains of Aux/IAA proteins are underlined. Nuclear localization signals (NLSs) are indicated by filled circles. The amino acid position is given on the right of each sequence.

Phylogenetic analysis of Aux/IAAs

Phylogenetic analysis was conducted to assess the relationship between tomato and Arabidopsis Aux/IAAs. The tomato *Aux/IAA* genes were renamed to comply with the nomenclature of their closest Arabidopsis homologs. **Supplementary Fig. S1** shows that Aux/IAA proteins group into 11 distinct clades named here A–K. Overall, the tomato family is slightly contracted (25 members) compared with the size of that of Arabidopsis (29 members). With reference to Arabidopsis, four clades (D, F, G and I) are contracted in the tomato and two (A and J) are expanded. Clade A includes seven genes in tomato but only four members in Arabidopsis, while clade J is comprised of three genes in tomato and contains a single member in Arabidopsis. The non-canonical clade H lacking the conserved domains II contains three members (AtIAA20, AtIAA30 and AtIAA31) in Arabidopsis but is not represented in tomato. Clade I, which also gathers non-canonical Aux/IAAs lacking either one or two of the conserved domains, is represented by two Aux/IAAs in Arabidopsis (AtIAA32 and AtIAA34) but only by a single member in tomato (Sl-IAA32). Overall, the non-canonical Aux/IAAs are over-represented in Arabidopsis with six genes (*AtIAA20*, *AtIAA30*, *AtIAA31*, *AtIAA32*, *AtIAA33* and *AtIAA34*), while only two were found in tomato (*Sl-IAA32* and *Sl-IAA33*).

Chromosomal distribution of *Sl-IAA* genes

The *Sl-IAA* sequences were initially mapped on the tomato genome using the introgression line population obtained by crossing and successive back-crossing of cultivated *S. lycopersicum* with *Solanum pennelli* (Eshed and Zamir 1995), and the mapping was subsequently refined using the SGN Tomato Whole Genome Scaffolds data (2.40) (http://www.sgn.cornell.edu/tools/blast/; The International Tomato Genome Sequencing Consortium). The 25 tomato *Aux/IAA* genes are distributed among nine tomato chromosomes (**Supplementary Fig. S2**), with chromosomes 2, 10 and 11 being devoid of *Aux/IAA* genes. Six *Sl-IAA* genes are present on chromosome 6; five on chromosomes 3 and 9; two on chromosomes 4, 7 and 12; and one on chromosomes 1, 5 and 8. The *Aux/IAA* genes tend to be clustered in preferential genomic regions, with the presence of closely adjacent genes on chromosome 3 (*Sl-IAA19*, *Sl-IAA15*, *Sl-IAA27* and *Sl-IAA26*), chromosome 6 (*Sl-IAA22*, *Sl-IAA17* and *Sl-IAA7*, *Sl-IAA4*) and chromosome 9 (*Sl-IAA1* and *Sl-IAA14*). Remarkably, the four contiguous tomato *Aux/IAA* genes mapped on chromosome 3 are located in a region spanning <0.5 Mb. On chromosome 6, *Sl-IAA22* and *Sl-IAA17* display an inverted orientation and are separated by only 7.5 kb. Likewise, in another locus of chromosome 6, *Sl-IAA7* and *Sl-IAA4* show a similar situation, being 23.6 kb apart. The same situation prevails in chromosome 9 where *Sl-IAA1* and *Sl-IAA14* are 32 kb apart. These data suggest that the distribution of some *Sl-IAA* genes on the tomato genome probably results from either reverse or direct tandem duplication.

Aux/IAA proteins are nuclear localized

Two types of putative nuclear localization signals (NLSs) were detected in most of the Aux/IAA proteins. Generally, tomato Aux/IAA proteins display two conserved nuclear localization domains: (i) a bipartite structure comprising a conserved KR basic doublet between domains I and II associated with the presence of basic amino acids in domain II; and (ii) a basic residue-rich region located in domain IV that resembles the SV40-type NLS (**Fig. 1**). However, some Sl-IAAs display imperfect or weakly conserved nuclear targeting motifs. For instance, Sl-IAA35 lacks the two conserved NLSs, while Sl-IAA32 and Sl-IAA33 lack the bipartite structure and Sl-IAA29 and Sl-IAA36 contain a degenerated NLS. The ability of the degenerated NLS present in Sl-IAA29 and the absence of the bipartite structure in Sl-IAA32 to target the protein to the nucleus was assessed by transient expression assay. To this end, the coding sequence of the selected *Aux/IAA* genes was fused in-frame to either GFP (green fluorescent protein) or YFP (yellow fluorescent protein) coding sequences and expressed under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) in tobacco protoplasts. Two Aux/IAAs with a conserved NLS (Sl-IAA4 and Sl-IAA22) were used as reference proteins for nuclear targeting. Fluorescence microscopy analysis demonstrated that in contrast to control cells transformed with GFP alone where the fluorescence was found throughout the cell, the Sl-IAA4–GFP and Sl-IAA22–YFP fusion proteins were exclusively localized to the nucleus (**Fig. 2**). Likewise, Sl-IAA29–YFP was also strictly targeted to the nucleus, suggesting that the degenerated NLS was sufficient to drive the protein specifically to the nucleus. In contrast, though Sl-IAA32–YFP was localized in the nucleus, the accumulation of the protein was not restricted to this compartment (**Fig. 2**). The extension of the Sl-IAA32 localization to the extranuclear compartment was probably due to the lack of the bipartite NLS and/or the absence of domain II responsible for protein degradation. Taken together, the nuclear targeting of the tomato Aux/IAA proteins is consistent with a putative transcriptional regulatory function.

Tomato Aux/IAA proteins function as active repressors of auxin-dependent transcription

The ability of the tomato Aux/IAA proteins to regulate in vivo the activity of the synthetic DR5 auxin-responsive promoter fused to the GFP reporter gene (Ottenschlager et al. 2003) was investigated by transient expression experiments using tobacco BY-2 protoplasts. The DR5::GFP reporter construct was used to assess auxin-dependent transcriptional activity based on the presence in the DR5 promoter of several copies of the TGTCTC core motif that makes up the AuxRE (Ulmasov et al. 1997). In our system, DR5-driven GFP expression was enhanced up to 10-fold by auxin treatment, and co-transfection of the reporter construct with a mock effector plasmid containing the 35S promoter but lacking Sl-IAA coding sequence did not impact the auxin induction of the DR5 activity (**Fig. 3A**). While all Sl-IAA proteins were able to

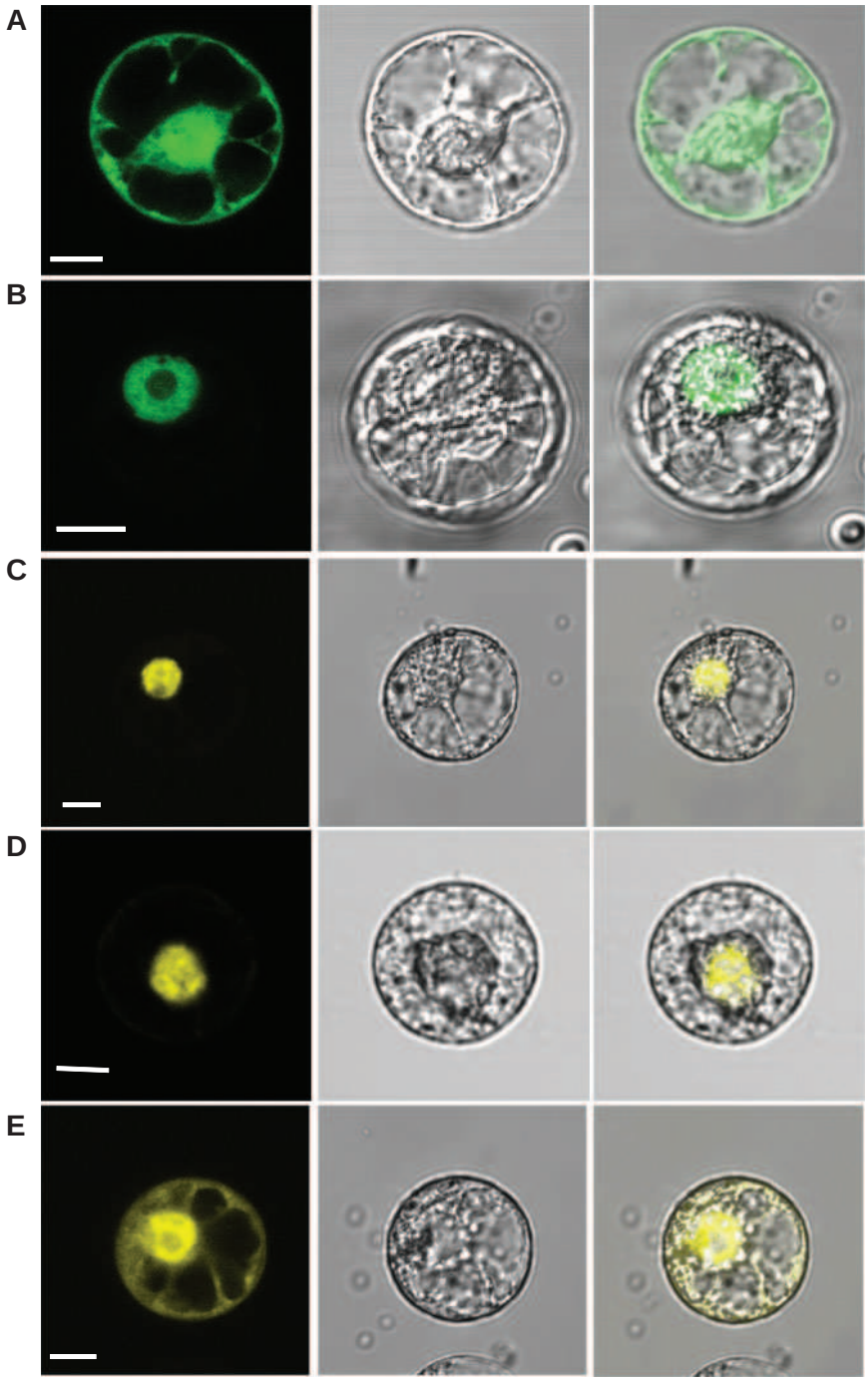

Fig. 2 Subcellular localization of Sl-IAA4, Sl-IAA22, Sl-IAA29 and Sl-IAA32 proteins. Sl-IAA4–GFP, Sl-IAA22–YFP, Sl-IAA29–YFP and Sl-IAA32–YFP fusion proteins were transiently expressed in BY-2 tobacco protoplasts, and their subcellular localization was analyzed by confocal laser scanning microscopy. The merged pictures of the green or yellow fluorescence channel (left panels) and the corresponding bright field (middle panels) are shown (right panels). (A) Control cells expressing GFP alone. (B) Cells expressing the Sl-IAA4–GFP fusion protein. (C) Cells expressing the Sl-IAA22–YFP fusion protein. (D) Cells expressing the Sl-IAA29–YFP fusion protein. (E) Cells expressing the Sl-IAA32–YFP fusion protein. The scale bar indicates 10 μm.

repress the auxin-induced expression of the DR5 promoter, the repression levels ranged from 23 to 87% (**Fig. 3A**), indicating that some proteins are strong repressors, e.g. Sl-IAA8, Sl-IAA9, Sl-IAA13 and Sl-IAA26, while others, e.g. Sl-IAA1, Sl-IAA11, Sl-IAA12 and Sl-IAA19, are weak repressors. The repression activity of Aux/IAA proteins is consistent with the presence of an LxLxL repression motif in domain I in all tomato Sl-IAA proteins tested, a motif that was shown to be important in

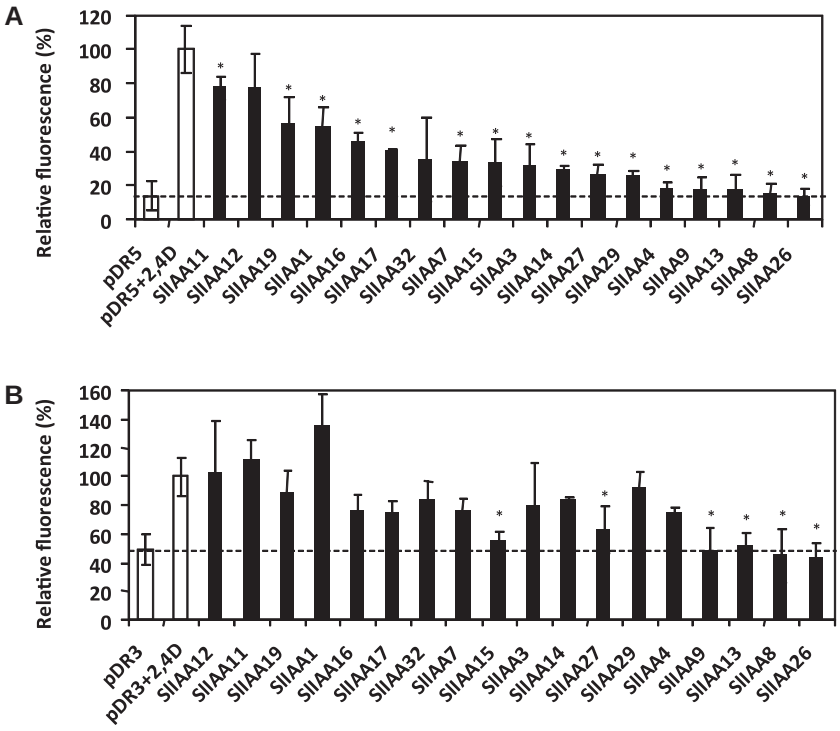

Fig. 3 Repressor activity of Aux/IAA proteins on a synthetic promoter and the native *Sl-IAA3* promoter. Transient expression in a single cell system has been used to assess the repression activity of Aux/IAA proteins on auxin-induced transcription of the GFP reporter gene driven by auxin-responsive promoters. The fluorescence of the reporter gene was measured by flow cytometry upon treatement with 50 μM 2,4-D and co-transfection with a reporter construct (DR5::GFP or Sl-IAA3promoter::GFP) and an effector construct (35S::SlIAA). The basal fluorescence obtained in the mock assay transfected with the reporter construct and an empty effector construct in the presence of auxin treatment was taken as reference (100% relative fluorescence). Biological triplicates were averaged and analyzed statistically using a Student *t*-test (*$P < 0.05$). Bars indicate the SEM. (A) Aux/IAA activity on a synthetic DR5 promoter gene containing nine TGTCTC boxes. (B) Aux/IAA activity on the auxin-inducible native *Sl-IAA3* promoter containing two TGTCCC boxes.

conferring repression activity in Arabidopsis Aux/IAAs (**Table 1**; Tiwari et al. 2004). No correlation was found between the level of repression and the amino acid environment surrounding the LxLxL motif present in domain I (**Fig. 3A**, **Table 1**). Among all the tomato Aux/IAA proteins, 12 contain the more representative domain I (TE**L**R**L**G**L**PG); however, these proteins displayed different levels of repression. For instance, Sl-IAA8 totally repressed the auxin-induced DR5 activity whereas Sl-IAA19 repressed only 50% of this activity. Moreover, Sl-IAA26 and Sl-IAA8 which contain the kk**L**e**L**r**L**gp and TE**L**R**L**G**L**PG type of domain I, respectively, were both capable of completely repressing DR5 activity (**Fig. 3A**, **Table 1**). Neither the length nor the number of repeats of this motif correlate with the level of transcriptional repression displayed by the tomato Aux/IAAs. Indeed, Sl-IAA12 has an expanded repression motif made up of five leucine repeats but only displayed a weak repression activity (**Fig. 3A**; **Supplementary Fig. S3A**). Likewise, the presence of two conserved repression motifs (LxLxLx and DLxLxL) in Sl-IAA16, Sl-IAA17 and Sl-IAA7 proteins did not result in stronger repression activity (**Fig. 3A**; **Supplementary Fig. S3B**). Overall, these results are consistent with tomato Aux/IAA proteins being transcriptional repressors on TGTCTC-containing promoters.

The repressor activity of the tomato Aux/IAAs was also tested with a native tomato auxin-responsive promoter, the *Sl-IAA3* promoter carrying degenerated AuxREs (TGTCCC). Among all the tomato Aux/IAAs tested, only six Sl-IAAs (Sl-IAA8, Sl-IAA9, Sl-IAA13, Sl-IAA15, Sl-IAA26 and Sl-IAA27) showed significant repression activity on the native *Sl-IAA3* promoter (**Fig. 3B**). All these repressors were even able to abolish totally the auxin-induced expression of the *Sl-IAA3* promoter-driven GFP (**Fig. 3B**). The remaining Aux/IAA proteins displayed no, or only partial, repression activity on the native auxin-responsive promoter. Moreover, the Aux/IAAs showing the strongest repression activity (Sl-IAA8, Sl-IAA9, Sl-IAA13 and Sl-IAA26) on the synthetic DR5 promoter were also those displaying the highest repression on the native *Sl-IAA3* promoter. Likewise, the Aux/IAAs showing weak repression activity on the synthetic promoter also displayed no repression on the native *Sl-IAA3* promoter (Sl-IAA1, Sl-IAA11, Sl-IAA12 and Sl-IAA19). The slight differences observed between the synthetic DR5 and the native *Sl-IAA3* promoter are likely to be due to the complexity of the latter promoter which contains several *cis*-regulatory elements, independent of auxin regulation (Chaabouni et al. 2009a).

Table 1 LxLxLx motifs in tomato Aux/IAA repression domain I

Protein name	Domain I
Sl-IAA1	TE**L**R**L**G**L**PG
Sl-IAA2	TE**L**R**L**G**L**PG
Sl-IAA3	TE**L**R**L**G**L**PG
Sl-IAA4	TE**L**R**L**G**L**PG
Sl-IAA9	TE**L**R**L**G**L**PG
Sl-IAA15	TE**L**R**L**G**L**PG
Sl-IAA16	TE**L**R**L**G**L**PG
Sl-IAA17	TE**L**R**L**G**L**PG
Sl-IAA19	TE**L**R**L**G**L**PG
Sl-IAA21	TE**L**R**L**G**L**PG
Sl-IAA22	TE**L**R**L**G**L**PG
Sl-IAA8	TE**L**R**L**G**L**PG
Sl-IAA14	TE**L**c**L**G**L**PG
Sl-IAA7	TE**L**c**L**G**L**PG
Sl-IAA27	TE**L**t**L**G**L**PG
Sl-IAA13	TE**L**e**L**G**L**gl
Sl-IAA23	ln**L**R**L**G**L**PG
Sl-IAA11	Tg**L**e**L**G**L**gl
Sl-IAA12	Tq**L**e**L**G**L**gl
Sl-IAA29	mE**L**e**L**G**L**ai
Sl-IAA36	mE**L**e**L**G**L**gl
Sl-IAA35	mE**L**q**L**G**L**al
Sl-IAA26	kk**L**e**L**r**L**gp
Sl-IAA32	id**L**g**L**s**L**ra

Conserved leucine residues in the LxLxLx motif are in bold. The most conserved amino acids in domain I of *A. thaliana* (Tiwari et al. 2004) and tomato Aux/IAA proteins are in uppercase. Sl-IAA33 which lacks a LxLxL motif is not shown.

Expression analysis of tomato Aux/IAA genes

Full-length cDNAs were amplified for 22 *Aux/IAA* genes attesting to their expression at least at the transcriptional level in different tomato plant tissues and organs. For the remaining three tomato *Aux/IAA* genes (*Sl-IAA21*, *Sl-IAA23* and *Sl-IAA33*), no corresponding cDNA could be isolated from the various plant tissues tested. In addition, with the exception of *Sl-IAA33* for which an expressed sequence tag (EST) was available from suspension cell culture, no sequence was identified for these genes in the available EST databases (Tomato gene index project: http://compbio.dfci.harvard.edu/tgi/cgi bin/tgi/gimain.pl?gudb=tomato; SGN: http://www.sgn.cornell.edu; KaFTom: http://www.pgb.kazusa.or.jp/kaftom/ ; MiBASE http://www.kazusa.or.jp/jsol/microtom/indexe.html). This supports the idea that these latter genes might be either preferentially expressed in small subsets of cells or not expressed at all.

To gain insight into the spatial pattern of expression of *Sl-IAA* genes, their transcript accumulation was assessed in

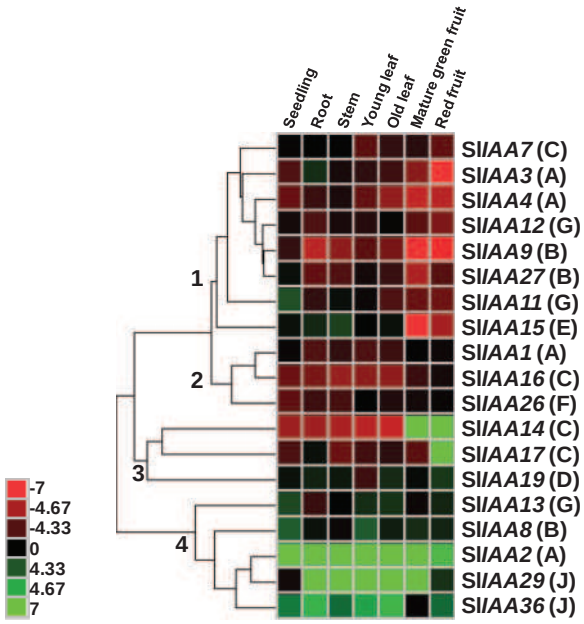

Fig. 4 Heatmap showing the expression of *Sl-IAA* genes in different tissues. Quantitative RT–PCR was used to assess *Sl-IAA* transcript accumulation in total RNA samples extracted from seedling, roots, stem, young leaf, old leaf, mature green fruit and red fruit. Values represent the best experiment among three independent biological replicates. Genes highly or weakly expressed in the tissues are colored red and green, respectively. The heat map was generated using cluster 3.0 software. The number in parentheses designates the phylogenetic clade of each *Aux/IAA* gene.

different plant tissues and organs. The expression pattern was studied by quantitative reverse transcription–PCR (qRT–PCR) for 19 out of the 22 expressed *Aux/IAA* genes. The Treeview presented in **Fig. 4** gathers the qRT–PCR data of 19 *Sl-IAA* genes using RNA samples corresponding to seven different plant tissues. The clustering revealed four main clades. *Aux/IAA* genes from clade 1 correspond to family members displaying the highest expression in fruit tissues. In contrast, genes in clade 2 and 3 displayed higher expression in vegetative tissues while clade 4 corresponded to *Aux/IAA* genes with a low level of expression in all tissues. No correlation was found between the clustering based on the expression pattern and that generated based on phylogenetic analysis (**Supplementary Fig. S1**; **Fig. 4**). For most *Aux/IAA* genes the highest expression level was found in young leaves and seedlings, two tissues where auxin is known to play an important role. Some *Aux/IAA* genes displayed clear preferential expression in a specific tissue, such as *Sl-IAA15* showing the highest expression in mature green fruit, *Sl-IAA7* and *Sl-IAA19* in young leaves and *Sl-IAA26* and *Sl-IAA29* in seedlings (**Fig. 5**). The expression of *Sl-IAA9*, *Sl-IAA13* and *Sl-IAA27* showed minimal variation between tissues, suggesting that the regulation of these genes might take place essentially at the post-translational level. Overall, the tissue-preferential expression displayed by some *Aux/IAA* genes could be indicative of their involvement in specific plant tissues and developmental processes.

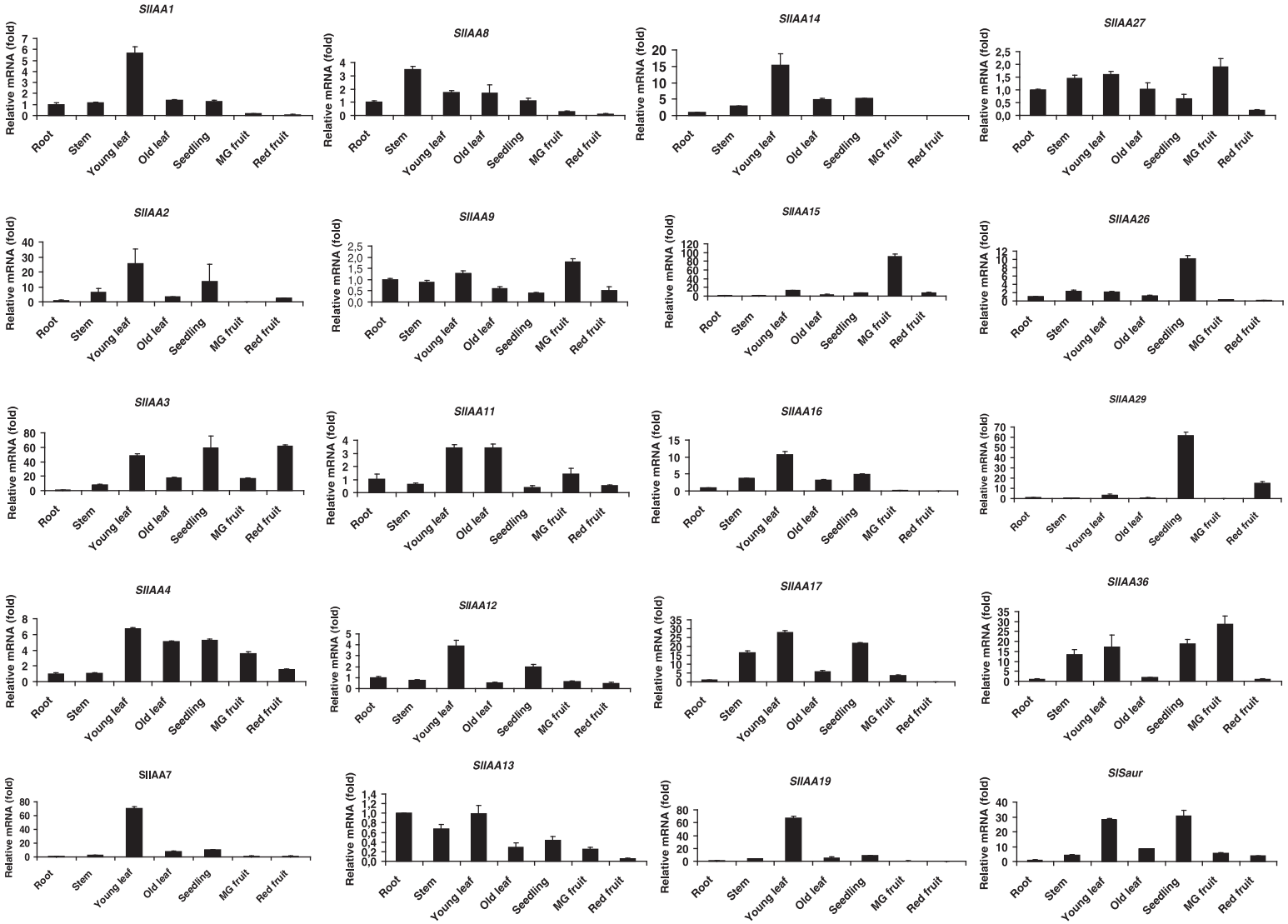

Fig. 5 Real-time PCR expression profiles of individual *Sl-IAA* genes. The relative mRNA level of individual *Sl-IAA* genes was normalized with respect to the housekeeping gene, actin, in different tissues. The results were expressed using the root as a reference for each gene (relative mRNA level 1). Values represent the best experiment among three independent biological repetitions. Bars indicate the SEM of three experimental repetitions.

Auxin and ethylene responsiveness of tomato *Aux/IAA* genes

The first *Aux/IAA* genes were isolated from various plant species based on their rapid induction in response to auxin. Screening for the presence of *cis*-acting elements within promoter regions (2 kb from the start codon) using the Place database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) revealed that the majority of the *Sl-IAA* promoters contain AuxREs as either a conserved (TGTCTC) or degenerate (TGT CCC) motif. In addition to the AuxREs, 16 out of the 25 *Sl-IAA* promoters contain conserved ethylene-response motifs, the so-called ERELEE4 motif found in the tomato E4 gene (AWTT CAAA) (**Supplementary Table S1**). The presence of these *cis*-regulatory elements suggests a potential regulation of the *Aux/IAA* genes by both auxin and ethylene. The ethylene and auxin responsiveness of the *Sl-IAA* genes was therefore investigated by qRT–PCR in seedling tissues. All of the *Aux/IAAs* tested, except two (*Sl-IAA8* and *Sl-IAA27*), displayed positive regulation of their transcript accumulation by auxin (**Fig. 6A**), with some genes being slightly up-regulated (*Sl-IAA9* and *Sl-IAA26*) and others strongly induced (*Sl-IAA2*, *Sl-IAA17* and *Sl-IAA19*). The analysis of ethylene regulation of tomato *Sl-IAA* genes in etiolated seedlings indicated that some genes were up-regulated while others were clearly down-regulated by ethylene (**Fig. 6B**). The data indicated that *Sl-IAA29* was strongly up-regulated, *Sl-IAA3* and *Sl-IAA36* were slightly up-regulated, and transcript accumulation of *Sl-IAA2*, *Sl-IAA11*, *Sl-IAA17* and *Sl-IAA19* genes was dramatically reduced upon ethylene treatment. These data suggest that in addition of being major molecular players in the auxin responses, some Aux/IAAs may also be potential components of the ethylene response.

Discussion

The comprehensive identification and subsequent characterization of the tomato *Aux/IAA* gene family members described here provide new insight regarding the potential role of some *Aux/IAA* genes in mediating plant responses to both auxin and ethylene. Moreover, by assessing the transcriptional repression

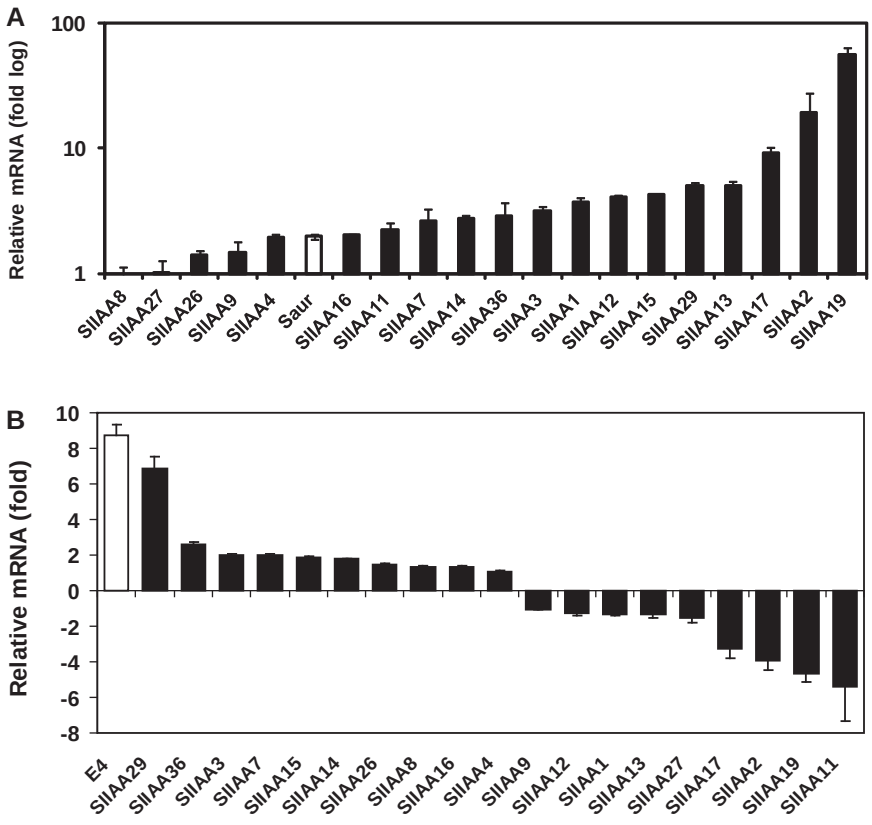

Fig. 6 Auxin and ethylene regulation of *Aux/IAA* genes in tomato. (A) Relative auxin induction of *Sl-IAA* genes in light-grown seedlings. Quantitative RT–PCR was used to assess *Sl-IAA* transcript accumulation in RNA samples extracted from 12-day-old tomato seedlings soaked in liquid MS medium with 10 μM IAA for 2 h. ΔΔCT refers to the fold difference in *IAA* expression compared with the untreated seedlings. The *SAUR* gene was used as a control to validate the auxin treatment. The vertical axis is displayed on a logarithmic scale to obtain a better comparison of transcript levels. (B) Ethylene regulation of *Sl-IAA* genes on dark-grown seedlings. Quantitative RT–PCR of *Sl-IAA* transcripts in RNA samples extracted from 5 d dark-grown tomato seedlings treated for 5 h with ethylene (50 $\mu l\, l^{-1}$). ΔΔCT refers to fold differences in *IAA* expression relative to untreated seedlings. The *E4* gene was used as control for efficient ethylene treatment.

capacity, the spatio-temporal expression patterns and the subcellular localization at the protein level, this study provides new leads towards addressing the putative function and mode of action of tomato *Aux/IAA* genes. The tomato *Aux/IAA* family is slightly contracted, with 25 members compared with Arabidopsis (29 genes) (Liscum and Reed 2002). However, while overall the tomato *Aux/IAA* gene family comprises a lower number of genes than in Arabidopsis, two clades are substantially expanded. Clades A and J contain seven and three genes in tomato, respectively, but only four and one in Arabidopsis. As an illustration of the wide diversification of Aux/IAA proteins in higher plants, the two clades are also expanded in *Populus trichocarpa*, with six members in clade A and three members in clade J (Kalluri et al. 2007). This diversification is also reflected by important structural variations found within Aux/IAA proteins. The accepted model for Aux/IAA function builds on auxin-mediated degradation of these short-lived proteins that typically have four conserved domains defining the gene family members. Notably, clade H comprising three non-canonical members (AtIAA20, AtIAA30 and AtIAA31) in Arabidopsis that lack the conserved domain II essential for protein degradation is not represented in tomato. In line with the absence or the alteration of domain II, AtIAA20 and AtIAA31 have been shown to be long-lived proteins compared with the canonical AtIAA17 (Dreher et al. 2006). The mechanism by which these non-canonical proteins impact auxin signaling remains unclear, even though the overexpression of *AtIAA20*, *AtIAA30* or *AtIAA31* results in aberrant auxin-related phenotypes in Arabidopsis (Sato and Yamamoto 2008). The tomato genome contains two non-canonical *Aux/IAA* genes (*Sl-IAA32* and *Sl-IAA33*), whereas up to six are found in Arabidopsis. Sl-IAA32 protein lacks domain II, whereas both domain I and domain II are missing in Sl-IAA33. The present study shows that Sl-IAA32 is a functional repressor of auxin signaling and its expression is limited to the breaker stage of fruit development (data not shown). A search in the SGN database identified an EST sequence from a cell culture suspension corresponding to *Sl-IAA33*, suggesting that the expression of this gene is highly constrained. Attempts to detect *Sl-IAA33* mRNA in the present study were unsuccessful in all tissues tested, further supporting the low level of expression of non-canonical *Aux/IAA* genes reported so far in Arabidopsis (Dreher et al. 2006). Considering their expression pattern apparently restricted to narrow developmental stages and their atypical long-lived feature due to the absence of domain II, the tomato non-canonical Aux/IAA proteins may have a specific function in mediating auxin responses during well-defined plant developmental events.

The expression patterns of *Sl-IAA* genes in various tissues and organs suggest that the encoded proteins may perform both specific and redundant functions. Nevertheless, no link was found between the clustering based on the expression pattern and the clustering obtained by phylogenetic analysis, with genes from the same clade, such as clade A, displaying either a high (*Sl-IAA3* and *Sl-IAA4*) or a low (*Sl-IAA2* and *Sl-IAA22*) level of expression. For the two remaining members of clade A (*Sl-IAA21* and *Sl-IAA23*) no corresponding EST was found in the databases, and attempts to detect the corresponding mRNAs failed in all tissues tested. The six members of the analog clade in *P. trichocarpa* (*PtIAA3* subgroup) are differentially transcribed (Kalluri et al. 2007) and, likewise, in Arabidopsis, gene expression patterns of Aux/IAA sister pairs are significantly different (Paponov et al. 2009). These data support the idea that the diversification of Aux/IAA family members in flowering plants has also been sustained by changes in their expression patterns. The majority of *Sl-IAA* genes identified are transcriptionally active as assessed by the isolation of the full-length open reading frame corresponding to 22 genes out of the 25 members present in the tomato genome. For most *Aux/IAA* genes, the highest expression level was found in young leaves and seedlings, two tissues known to accumulate a high amount of auxin. The transcript levels of 17 out of 19 *Sl-IAA* genes were up-regulated by auxin treatment in seedlings, though to varying degrees. Consistent with this high degree of regulation by auxin, promoter analysis revealed the presence of well-conserved AuxREs in the promoter region of the majority of *Sl-IAA* genes. Members of the Arabidopsis *Aux/IAA* gene family have also been shown to respond to exogenous IAA in a highly differential fashion with respect to dosage and time (Abel et al. 1994, Abel et al. 1995). A variety of factors may explain the differences observed in the response kinetics between individual *Aux/IAA* genes such as tissue-specific auxin perception, cell type dependence and differential regulation of free auxin concentrations, or different modes of auxin-dependent transcriptional and post-transcriptional regulation. It has been reported previously that down-regulation of *Sl-IAA3* results in auxin- and ethylene-related developmental defects including reduced apical dominance, reduced auxin response and an exaggerated apical hook in etiolated seedlings (Chaabouni et al. 2009a), supporting the hypothesis that *Sl-IAA3* represents a molecular link between ethylene and auxin signaling in tomato (Chaabouni et al. 2009b). Ethylene responsiveness of *Aux/IAA* genes was first described in late immature green tomato fruit (Jones et al. 2002). The present study provides a more comprehensive analysis of the ethylene regulation of *Sl-IAA* genes, revealing that the expression of some genes is clearly and rapidly induced by ethylene in etiolated seedlings, with *Sl-IAA29* transcript accumulation being the most strongly up-regulated. In contrast, ethylene treatment dramatically reduced transcript accumulation of *Sl-IAA2*, *Sl-IAA11*, *Sl-IAA17* and *Sl-IAA19* genes. Strikingly, none of these ethylene-regulated tomato *Aux/IAA* genes contains the conserved GCC-box motif, a *cis*-acting element present in the promoter regions of ethylene-responsive genes (Ohme-Takagi and Shinshi 1995). Notably, five out of seven ethylene-regulated *Sl-IAA* genes contain another ethylene-response motif, the so-called ERELEE4 motif (AWTTCAAA), found in the promoter of the tomato *E4* gene, a well-described ripening- and ethylene-regulated gene (Montgomery et al. 1993). The potential role of the ethylene-regulated *Aux/IAA* genes in mediating

the cross-talk between auxin and ethylene remains to be further investigated, in particular during developmental events such as apical hook formation or the transition from green to ripe fruit where ethylene is known to be a key player.

The nuclear targeting of tomato Aux/IAA proteins is consistent with a transcriptional regulatory function. Typical Aux/IAA proteins harbor two NLSs, one bipartite and one resembling an SV40-type NLS. In tomato, all the Sl-IAAs tested so far localize in the nuclear compartment (Wang et al. 2005, Chaabouni et al 2009a, Deng et al., 2012). While the present study confirms the nuclear targeting of some other members of the AUX/IAA proteins (Sl-IAA4, Sl-IAA22 and Sl-IAA29), it also reveals the presence of Sl-IAA32 protein, which lacks the bipartite NLS, in both the nucleus and the cytoplasm. The lack of a bipartite NLS in the native Sl-IAA32 protein is likely to be responsible for the targeting of this protein to the extranuclear compartment. These data suggest that some Aux/IAA proteins may have an extranuclear function that still remains to be elucidated. It is important to mention that, in addition to its cytoplasmic localization, Sl-IAA32 also lacks the conserved domain II required for the degradation of the protein mediated by the auxin–TIR1 complex, thus raising the hypothesis that this Aux/IAA may be involved in a mechanism independent from the conventional auxin signaling pathway.

In agreement with previous reports, all tomato Aux/IAAs displayed a repression activity of auxin-dependent transcription (Ulmasov et al. 1997, Tiwari et al. 2001, Bargmann and Birnbaum 2009). However, the repression levels vary widely (23–87%) among tomato Aux/IAA proteins when tested with the synthetic DR5 promoter. It has been previously described in Arabidopsis that domain I of Aux/IAA proteins is an active, portable repression domain containing the LxLxL motif (Tiwari et al. 2004) that interacts with the TOPLESS (TPL) co-repressor (Szemenyei et al. 2008). All the tomato Aux/IAAs tested in this study bear a conserved domain I, but no correlation was found between the level of repression and the amino acid environment surrounding the LxLxL repressor motif. Interestingly, Sl-IAA26, showing the strongest repression activity, contains, in addition to the LxLxL motif, a second LxLxPP motif, found in *Physcomitrella patens* and other flowering plants, that has been proposed to function as a putative repression domain (kk**Le**L**r**L**g****PP**) (Paponov et al. 2009). Sl-IAA26 belongs to clade F with three other Arabidopsis Aux/IAAs (AtIAA18, AtIAA26 and AtIAA28) also containing this overlapping LxLxLxPP motif. Yet, the potential of the LxLxPP motif to potentiate the repressor activity of Aux/IAA proteins is not supported by any direct experimental evidence. Recently, it has been reported that mutations in domain I of various Aux/IAA proteins can have profound, but different, consequences in terms of auxin responses in Arabidopsis plants, suggesting that some Aux/IAA proteins may have stronger or more complex repression domains than others (Li et al. 2011). However, in tomato, neither the length of the repression domains (e.g. an LxLxL vs. an LxLxLxLxL motif) nor the presence of two LxLxL motifs in the same Aux/IAA protein seems to correlate with the level of transcriptional repression of the synthetic DR5 or the native *Sl-IAA3* promoter. Dedicated tomato mutant resources are now needed to better understand the intrinsic differences in the repression domains of Sl-IAA proteins and to better clarify the functional significance of the diversification of Aux/IAA members between tomato and Arabidopsis. Moreover, to understand the functional differentiation among the Aux/IAA family in tomato will also require the determination of qualitative and quantitative interactions between Aux/IAAs and their ARF partners. It should also taken be into consideration in future studies that several lines of evidence in the literature support a model for EAR motif-mediated repression acting via epigenetic mechanisms resulting from chromatin modifications (Kagale and Rozwadowski 2011).

Materials and Methods

Plant material and growth conditions

Tomato seeds (*S. lycopersicum* cv. MicroTom or Ailsa Craig) were sterilized, rinsed in sterile water and sown in recipient Magenta vessels containing 50 ml of 50% Murashige and Skoog (MS) culture medium with added R3 vitamin (0.5 mg l^{-1} thiamine, 0.25 mg l^{-1} nicotinic acid and 0.5 mg l^{-1} pyridoxine), 1.5% (w/v) sucrose and 0.8% (w/v) agar, pH 5.9. Plants were grown under standard greenhouse conditions. The culture chamber rooms were set as follows: 14 h day/10 h night cycle, 25/20°C day/night temperature, 80% relative humidity and 250 µmol m^{-2} s^{-1} intense luminosity.

Transient expression using a single cell system

Protoplasts for transfection were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells according to the method described previously (Leclercq et al. 2005). Protoplasts were transfected by a modified polyethylene glycol method as described by Abel and Theologis (1994). For nuclear localization of the selected Aux/IAA fusion proteins, the coding sequences of genes were cloned as a C-terminal fusion in-frame with GFP or as an N-terminal fusion with YFP under the control of the 35S CaMV promoter. Transfected protoplasts were incubated for 16 h at 25°C and analyzed for GFP/YFP fluorescence by confocal microscopy. Confocal imaging was conducted on a Leica TCS SP2 confocal laser scanning microscope. Images were obtained with a ×40 1.25 numerical aperture water-immersion objective. GFP and YFP were excited at 488 nm, and the emitted light was captured at 505–535 nm and 530–570 nm, respectively. For co-transfection assays, aliquots of protoplasts (0.5×10^6) were transformed either with 10 µg of the reporter vector alone containing the promoter fused to the GFP reporter gene or in combination with 10 µg of Aux/IAA construct as the effector plasmid. Transformation assays were performed in three independent replicates. After 16 h, GFP expression was analyzed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences) on a flow cytometry platform

(IRF31). Data were analyzed using Cell Quest software. For each sample, 100–1,000 protoplasts were gated on forward light scatter and the GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the cell population after subtraction of autofluorescence determined with non-transformed BY-2 protoplasts. The data were normalized using an experiment, in the presence of 50 μM 2,4-D, with protoplasts transformed with the reporter vector in combination with the vector used as the effector plasmid but lacking the *Sl-IAA* coding region.

RNA isolation and qRT–PCR

Total RNA was extracted from fruit according to Hamilton et al. (1990). Total RNA from leaves and seedlings was extracted using a Plant RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated by DNase I to remove any genomic DNA contamination. First-strand cDNA was reverse transcribed from 2 μg of total RNA using an Omniscript kit (Qiagen) according to the manufacturer's instructions. qRT–PCR analyses were performed as previously described (Pirrello et al. 2006). The primer sequences are listed in **Supplementary Table S4**. Relative fold differences were calculated based on the comparative Ct method using Sl-Actin-51 as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct value of genes was normalized to the Ct value for Sl-Actin-51 (accession No. Q96483) and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. At least two to three independent RNA isolations were used for cDNA synthesis and each cDNA sample was subjected to real-time PCR analysis in triplicate. Heat map representation was performed using centring and the normalized ΔCt value, with Cluster 3.0 software and JavaTreeview to visualize the dendogram.

Hormone treatment

For auxin treatment on light-grown seedlings, 12-day-old Ailsa Craig seedlings (30 seedlings) were soaked in liquid MS medium with or without (mock treatment) 10 μM IAA for 2 h. The efficiency of the treatment was checked by measuring the induction of the tomato early auxin-responsive *SAUR* gene. For ethylene treatment on dark-grown seedlings, 5-day-old MicroTom seedlings (100 seedlings) were treated with air or ethylene gas (50 μl l^{-1}) for 5 h. The efficiency of the treatment was checked by measuring the induction of the tomato ethylene-responsive *E4* gene. The experiment was repeated with three biological replicates.

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: *AtIAA1* (AT4G14560), *AtIAA2* (AT3G23030), *AtIAA3* (AT1G04240), *AtIAA4* (AT5G43700), *AtIAA5* (AT1G15580), *AtIAA6* (AT1G52830), *AtIAA7* (AT3G23050), *AtIAA8* (AT2G22670), *AtIAA9* (AT5G65670), *AtIAA10* (AT1G04100), *AtIAA11* (AT4G28640), *AtIAA12* (AT1G04550), *AtIAA13* (AT2G33310), *AtIAA14* (AT4G14550), *AtIAA15* (AT1G80390), *AtIAA16* (AT3G04730), *AtIAA17* (AT1G04250), *AtIAA18* (AT1G51950), *AtIAA19* (AT3G15540), *AtIAA20* (AT2G46990), *AtIAA26* (AT3G16500), *AtIAA27* (AT4G29080), *AtIAA28* (AT5G25890), *AtIAA29* (AT4G32280), *AtIAA30* (AT3G62100), *AtIAA31* (AT3G17600), *AtIAA32* (AT2G01200), *AtIAA33* (AT5G57420), *AtIAA34* (AT1G15050).

Supplementary data

Supplementary data are available at PCP online

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