The tomato *SlIAA15* is involved in trichome formation and axillary shoot development

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Summary

- The *Aux/IAA* genes encode a large family of short-lived proteins known to regulate auxin signalling in plants. Functional characterization of *SlIAA15*, a member of the tomato (*Solanum lycopersicum*) *Aux/IAA* family, shows that the encoded protein acts as a strong repressor of auxin-dependent transcription. The physiological significance of *SlIAA15* was addressed by a reverse genetics approach, revealing that *SlIAA15* plays multiple roles in plant developmental processes.
- The *SlIAA15* down-regulated lines display lower trichome number, reduced apical dominance with associated modified pattern of axillary shoot development, increased lateral root formation and decreased fruit set. Moreover, the leaves of *SlIAA15*-inhibited plants are dark green and thick, with larger pavement cells, longer palisade cells and larger intercellular space of spongy mesophyll cells.
- The *SlIAA15*-suppressed plants exhibit a strong reduction in type I, V and VI trichome formation, suggesting that auxin-dependent transcriptional regulation is required for trichome initiation. Concomitant with reduced trichome formation, the expression of some R2R3 *MYB* genes, putatively involved in the control of trichome differentiation, is altered.
- These phenotypes uncover novel and 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.

Introduction

The phytohormone auxin controls many aspects of plant growth and development. These include cell division, apical dominance, lateral/adventitious root formation, shoot and root tropisms, fruit set and development, vascular differentiation and embryogenesis (Friml, 2003). Recent genetic and molecular studies in Arabidopsis have revealed a crucial intracellular auxin signalling pathway in which a ubiquitin-dependent proteolytic system plays a key role in sensing and transducing the hormone signal into transcriptional programmes (Dharmasiri & Estelle, 2004). At the centre of the signalling cascade is the ubiquitin-ligase complex, SCF^{TIR1}, which promotes the ubiquitin-dependent proteolysis of a family of transcriptional regulators known as Aux/IAAs in an auxin-dependent manner (Gray *et al.*, 2001). Aux/IAAs and auxin response factors (ARFs) are instrumental to auxin-dependent transcriptional regulation, and ARFs can be either transcriptional activators or repressors of primary/early auxin-responsive genes (Ulmasov *et al.*, 1997a; Ren *et al.*, 2011), among which *Aux/IAA*s are the best-known representatives (Abel *et al.*, 1995). *Aux/IAA* genes encode short-lived nuclear proteins comprising at least 29 members in Arabidopsis (Ulmasov *et al.*, 1997a; Remington *et al.*, 2004; Overvoorde *et al.*, 2005). Many Aux/IAA proteins function as transcriptional repressors through interactions with ARF proteins. Aux/IAA proteins share four highly conserved domains (domains I, II, III and IV), each contributing to the functional properties of the protein. Domain I is responsible for the repressing activity of the protein (Tiwari *et al.*, 2004), whereas domain II confers instability to the Aux/IAA proteins (Worley *et al.*, 2000; Ouellet *et al.*, 2001). Domains III and IV are involved in homo- and heterodimerization with other Aux/IAA proteins (Kim *et al.*, 1997; Ouellet *et al.*, 2001) and with ARFs (Ulmasov *et al.*, 1997b; Ouellet *et al.*, 2001).

Gain-of-function mutations in *Aux/IAA* genes have been identified in Arabidopsis, which provide insight into the role played by these proteins in the mediation of auxin responses and plant

developmental processes. Mutants in at least 10 different Arabidopsis *Aux/IAA* genes show altered auxin response or morphology: *IAA1/AXR5* (Park *et al.*, 2002; Yang *et al.*, 2004), *IAA3/SHY2* (Tian & Reed, 1999), *IAA6/SHY1* (Kim *et al.*, 1996), *IAA7/AXR2* (Nagpal *et al.*, 2000), *IAA12/BDL* (Hamann *et al.*, 2002), *IAA14/SLR* (Fukaki *et al.*, 2002), *IAA17/AXR3* (Rouse *et al.*, 1998), *IAA18* (Reed, 2001), *IAA19/MSG2* (Tatematsu *et al.*, 2004) and *IAA28* (Rogg *et al.*, 2001). Strikingly, all these mutations are found in the highly conserved domain II and stabilize the Aux/IAA proteins, resulting in gain-of-function phenotypes. These *Aux/IAA* mutants exhibit a variety of auxin-related developmental phenotypes, including altered phototropism/gravitropism, root formation, apical dominance, stem/hypocotyl elongation, leaf expansion and leaf formation in the dark. However, because the stabilization caused by these mutations may not mimic regulatory events actually occurring in wild-type plants, an accurate determination of the physiological significance of Aux/IAA proteins would benefit from the study of loss-of-function mutants. Unfortunately, in Arabidopsis, the null mutants fail to show visible phenotypes, probably as a result of extensive functional redundancy (Overvoorde *et al.*, 2005). In contrast with the absence of visible phenotypes associated with loss-of-function mutations in Arabidopsis, the down-regulation of several *Aux/IAA* genes in the Solanaceae results in clear and distinct phenotypes. In tomato, down-regulation of *SlIAA9* has been reported to have an impact on leaf morphology, fruit set and development, apical dominance and many other aspects of vegetative and reproductive growth (Wang *et al.*, 2005, 2009). The down-regulation of *SlIAA3*, another tomato *Aux/IAA* gene, results in auxin- and ethylene-related developmental defects, including reduced apical dominance, reduced auxin response and exaggerated apical hook in etiolated seedlings (Chaabouni *et al.*, 2009a), supporting the hypothesis that *SlIAA3* represents a molecular link between ethylene and auxin signalling in tomato (Chaabouni *et al.*, 2009b). Likewise, suppression of *StIAA2* in potato results in clear phenotypes, including increased plant height, petiole hyponasty and curvature of growing leaf primordia in the shoot apex (Kloosterman *et al.*, 2006). Although phenotypes associated with loss-of-function mutations in a single member of the Arabidopsis *Aux/IAA* gene family remain scarce, these data suggest that, in the Solanaceae, Aux/IAAs can have specialized functions, stressing the need to widen the functional characterization of *Aux/IAA* genes beyond the Arabidopsis plant model in order to gain more insight into their physiological significance.

Adding to the roles already reported for Aux/IAAs, the present study describes the involvement of *SlIAA15* in trichome formation, thus uncovering new roles for *Aux/IAA*s in tomato. The tomato *SlIAA15* was initially isolated following differential screening of gene expression during fruit development, and its expression was found to be positively regulated by exogenous auxin and negatively regulated by ethylene (Jones *et al.*, 2002). The phenotypes associated with the down-regulation of the *SlIAA15* gene described in the present study support the hypothesis that trichome formation requires a functional auxin signalling pathway, and uncover new functionalities for Aux/IAAs in developmental processes.

Materials and Methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum* L. cv Ailsa Craig) plants were grown under standard glasshouse conditions. The conditions for the culture chamber room were as follows: 14 h day : 10 h night cycle; 25 : 20°C day : night temperature; 80% hygrometry; 250 μmol m^{-2} s^{-1} intense luminosity.

Sequence analysis

The DNA sequences were analysed with DNAstar software. A BLAST search was performed at http://www.ncbi.nlm.nih.gov/blast/. Protein domains were searched for with the Pfam program (http://pfam.wustl.edu/) and the BLASTP program (Altschul *et al.*, 1997). Protein sequences were aligned with ClustalX version 2.0. Phylogenetic and molecular evolutionary analyses were constructed by the neighbour-joining (NJ) method using MEGA version 4 (Tamura *et al.*, 2007). The reliability of the tree was measured by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

Accession numbers

GenBank accession numbers for the sequences analysed in the alignment and phylogenetic analysis are as follows: AtIAA1 (P49677), AtIAA2 (P49678), AtIAA3 (Q38822), AtIAA4 (P33077), AtIAA5 (P33078), AtIAA6 (Q38824), AtIAA7 (Q38825), AtIAA8 (Q38826), AtIAA9 (Q38827), AtIAA10 (Q38828), AtIAA11 (Q38829), AtIAA12 (Q38830), AtIAA13 (Q38831), AtIAA14 (Q38832), AtIAA15 (Q9C966), AtIAA16 (O24407), AtIAA17 (P93830), AtIAA18 (O24408), AtIAA19 (O24409), AtIAA20 (O24410), AtIAA26 (Q8LAL2), AtIAA27 (Q9ZSY8), AtIAA28 (Q9XFM0), AtIAA29 (Q93WC4), AtIAA30 (Q9M1R4), AtIAA31 (Q8H174), AtIAA32 (Q8RYC6), AtIAA33 (Q9FKM7), AtIAA34 (Q9C5X0). Tomato Solanaceae Genomics Network (SGN) unigene accession numbers for the sequences analysed in the alignment and phylogenetic analysis are as follows: SlIAA1 (U579410), SlIAA2 (U599474), SlIAA3 (U577993), SlIAA4 (U579749), SlIAA9 (U568849), SlIAA12 (U579795), SlIAA14 (U579618), U581524, U580151, U579168, U577682, U581702, U579607, U573372, U577813, U579354, U586760, U568970, U563561, U603679.

Transient expression using a single-cell system

To assess the nuclear localization of the SlIAA15 protein, the green fluorescent protein (GFP) sequence was fused in frame with the C-terminus of the *SlIAA15* coding sequence, cloned into the pGreen vector (Hellens *et al.*, 2000) and expressed under the control of 35S CaMV, a cauliflower mosaic virus promoter. Transformation assays were performed as described previously (Chaabouni *et al.*, 2009a). To determine the ability of the SlIAA15 protein to regulate *in vivo* the activity of either the synthetic DR5 (Ottenschläger *et al.*, 2003) or the native *SlIAA3*

(Chaabouni *et al.*, 2009a) auxin-responsive promoter, BY-2 protoplasts were co-transformed with reporter and effector constructs as described previously (Chaabouni *et al.*, 2009a). GFP expression was then analysed and quantified by flow cytometry (FACS Calibur II, BD Biosciences, http://www.bdbiosciences.com) 16 h following protoplast transfection. For each sample, 100–1000 protoplasts were gated on forward light scatter, and the GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the population of cells above the background. The data were analysed using Cell Quest software.

Generation of transgenic lines

Forward primer P1 (5′-CGAGACTATCTAAAAAGGGGG-3′) and reverse primer P2 (5′-TGGTAACTGTCGTACATCTCC-3′) were used to amplify a partial *SlIAA15* clone. This fragment was then cloned into the pGA643 binary vector in the antisense orientation under the transcriptional control of the 35S CaMV promoter and the nopaline synthase (Nos) terminator. Transgenic plants were generated via *Agrobacterium tumefaciens*-mediated transformation according to Jones *et al.* (2002), and transformed lines were selected as in Wang *et al.* (2005). All experiments were carried out using homozygous lines from F2 or later generations.

RNA extraction and gene expression analysis by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For expression analysis of *Aux/IAA* genes in trichomes, trichome RNAs were obtained from tomato plants by scraping off stems of 3-month-old plants under liquid nitrogen. Total RNA was then extracted using a Plant RNeasy Mini kit (Qiagen, http://www.qiagen.com) 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 the Omniscript kit (Qiagen) according to the manufacturer's instructions. qRT-PCR analyses were performed as described previously (Pirrello *et al.*, 2006). Gene-specific primers were designed by Primer Express 1.0 software (PE-Applied Biosystems, Foster, CA, USA) and the primer sequences are listed in Supporting Information Table S1.

For the expression analysis of R2R3 *MYB* genes, RNAs were extracted from young expanding leaves using an RNA extraction kit (Promega, http://www.promega.com). DNase-treated RNA (2 μg) was then reverse transcribed in a total volume of 20 μl using a reverse transcription kit (TakaRa, http://www.takara-bio.com). Real-time qPCR was performed using cDNAs corresponding to 2.5 ng of total RNA in a reaction volume of 10 μl using SYBR Green PCR Master Mix (Toyobo, http://www.toyobo.co.jp/e) on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster, CA, USA). Primer 5 software was used to design gene-specific primers. Real-time PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and, finally, one cycle at 95°C for 15 s and 60°C for 15 s. For all real-time PCR experiments, three biological replicates were made and each reaction was run in triplicate. For each sample, a Ct (threshold constant) value was calculated from the amplification curves in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using *Ubiquitin* as internal standard. To determine the relative fold differences for each sample in each experiment, the Ct value for the *SlIAA15* gene was normalized to the Ct value for *Ubiquitin* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. The gene-specific primers used to assess transcript accumulation are listed in Table S1.

Anatomic characterization and scanning electron microscopy

Leaf segments were dissected from midway between the margin and midrib of fully expanded leaves. Leaf segments were fixed in formaldehyde, dehydrated in an ascending alcohol series and embedded in LR White resin. Transverse sections were sectioned at 2 μm with a rotary microtome. The sections were stained with toluidine blue, and investigated under a Motic-Optic B5 microscope (Bender Associates, Inc., Tempe, AZ, USA). For scanning electron microscopy, the leaf and shoot samples were prepared as described previously (Sun *et al.*, 2005). The samples were examined and photographed with a Hitachi S-3000 N scanning electron microscope (Hitachi, Ltd., Shin-Kawasaki, Japan).

Results

SlIAA15 belongs to a distinct clade of the *Aux/IAA* gene family

SlIAA15, formerly named *DR8*, was initially isolated from tomato fruit using gene family-specific degenerate primers designed to conserved sequences in *Aux/IAA*s from different plant species (Jones *et al.*, 2002). This clone contains an open reading frame of 756 bp encoding a putative protein of 252 amino acids. The derived protein comprises the four conserved domains characteristic of the *Aux/IAA* gene family, domains I–IV (Fig. 1), like the two other tomato Aux/IAA proteins identified so far (Wang *et al.*, 2005; Chaabouni *et al.*, 2009a). Phylogenetic analysis was conducted to assess the relationship between tomato *SlIAA15* and members of the Arabidopsis *Aux/IAA* family, indicating that this tomato gene belongs to a distinct clade which includes *AtIAA15*, its putative orthologue from Arabidopsis (Fig. 2). A more comprehensive phylogenetic analysis extended to all members of the tomato Aux/IAA family (Solanaceae Genomics Network, http://www.sgn.cornell.edu/) confirmed that *SlIAA15* is more closely related to *AtIAA15* than to any other tomato gene (Fig. 2).

SlIAA15, a nuclear localized protein acting as a repressor of auxin-responsive promoters

In silico analysis of the predicted protein encoded by *SlIAA15* indicated that, like most Aux/IAA proteins, SlIAA15 contains two types of putative nuclear localization signal: a bipartite

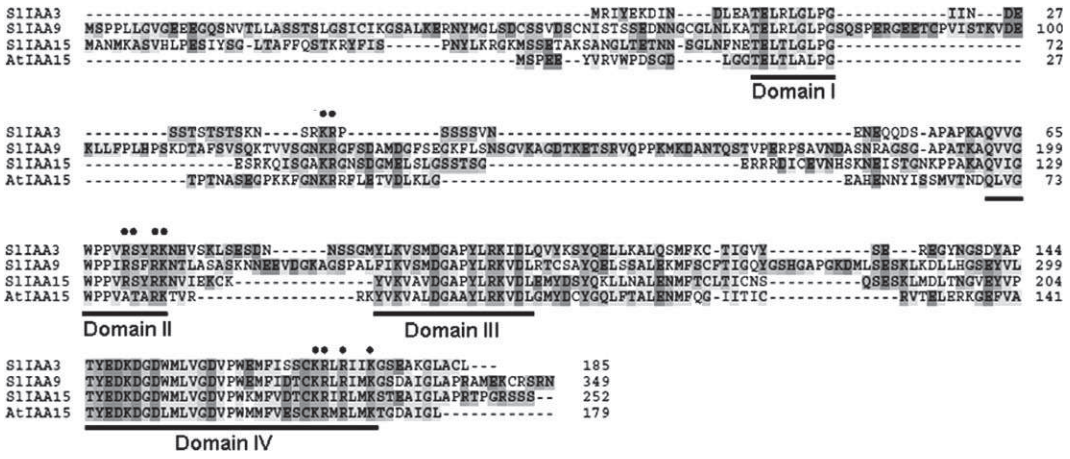

Fig. 1 Sequence comparison of SlIAA15 protein and its homologues in Arabidopsis and tomato (*Solanum lycopersicum*). Multiple alignments of SlIAA15, AtIAA15, SlIAA3 and SlIAA9 were obtained with ClustalX and manual correction. The dark and light shading indicate amino acids that are identical or similar, respectively. The four conserved domains I, II, III and IV are underlined. Conserved basic residues that putatively function as nuclear localization signals are indicated by closed circles.

structure with a conserved basic doublet KR between domains I and II and basic amino acids in domain II, as well as a region rich in basic residues located in domain IV that resembles SV40-type nuclear localization signals (NLS) (Fig. 1). The nuclear targeting of the SlIAA15 protein was experimentally validated by transient expression assay in tobacco protoplasts coupled to fluorescence microscopy analysis. The data in Fig. 3(a) indicate that, in contrast with control cells expressing GFP alone, where the fluorescence spreads throughout the cell, the SlIAA15-GFP fusion protein is exclusively targeted to the nucleus.

Transient expression in a single-cell system was also used to test the ability of the SlIAA15 protein to regulate, *in vivo*, the activity of the synthetic DR5 (Ottenschläger *et al.*, 2003) or native *SlIAA3* (Chaabouni *et al.*, 2009b) auxin-responsive promoters fused to the GFP reporter gene. SlIAA15 protein repressed the auxin-induced expression of DR5 and *SlIAA15* auxin-responsive promoters by 75% and 40%, respectively (Fig. 3b). This is consistent with the presence of an LxLxL motif present in domain I of the SlIAA15 protein (Fig. 1), shown to confer repression activity to Aux/IAAs in Arabidopsis (Tiwari *et al.*, 2004). This is also in line with the repressor activity reported so far for the Aux/IAA family members in various plant species.

SlIAA15 is involved in vegetative developmental growth

The physiological significance of *SlIAA15* was addressed *in planta* using an antisense strategy. Ten homozygous transgenic lines (AS-*IAA15*) corresponding to independent transformation events were generated. All the transgenic lines showed substantially lower accumulation of *SlIAA15* transcripts and common phenotypes related to vegetative growth. The phenotypes of two representative transgenic lines (lines 58 and 60) are shown in Figs 4, 5, including reduced plant height (Figs 4b, 5a) and weaker apical dominance associated with an altered pattern of axillary shoot development in all transgenic lines (Figs 4c, 5b). The number of lateral shoots is dramatically increased in *SlIAA15*-suppressed lines and these shoots develop from the first leaf node, whereas they develop only after floral transition in wild-type plants (Figs 4c, 5b). Root growth and architecture are also altered in *SlIAA15* down-regulated lines, with significantly enhanced lateral root development (Figs 4d, 5c). In addition, *SlIAA15* down-regulated lines display altered reproductive organs with decreased number of flowers and reduced fruit set efficiency (Table 1). The mean number of flowers decreased from 99 in wild-type plants to 52 and 20 in *SlIAA15*-suppressed lines 58 and 60, respectively. Moreover, the efficiency of fruit set decreased from 92% in wild-type plants to 46% and 30% in lines 58 and 60, respectively (Table 1).

To rule out any potential lack of specificity of the antisense strategy, the expression of tomato *Aux/IAA* genes (*SlIAA1*, *SlIAA2*, *SlIAA3*, *SlIAA4*, *SlIAA9* and *SlIAA14*) from the clades most closely related to *SlIAA15* in terms of sequence homology was performed. The expression of *SlIAA12*, which belongs to a more distant clade, was also assessed. Tomato *Aux/IAA* homologues were selected following BLASTN search of tomato unigenes (Solanaceae Genomics Network, http://www.sgn.cornell.edu/) using the corresponding Arabidopsis sequences. Real-time PCR experiments carried out using RNA samples from AS-*IAA15* and WT hypocotyls revealed no reduction in transcript level for any of these genes, indicating that the altered physiological processes observed in the transgenic lines are most probably a result of the down-regulation of *SlIAA15* (Fig. 6). Moreover, transcript accumulation of *SlIAA12* and *SlIAA9* was even higher in AS-*IAA15* lines than in wild-type plants (Fig. 6).

Down-regulation of *SlIAA15* affects leaf thickness

Although the leaf size was similar in wild-type and AS-*IAA15* plants, the transgenic lines displayed thicker leaves. The examination of leaf thickness in transverse sections of embedded leaves confirmed the increased thickness of the leaf blade in AS-*IAA15* relative to wild-type plants (Fig. 7a). Adaxial epidermal pavement cells of fully expanded fifth leaves, visualized by scanning electron microscopy, revealed a larger size of pavement cells in AS-*IAA15* relative to wild-type plants (Fig. 7b). Further

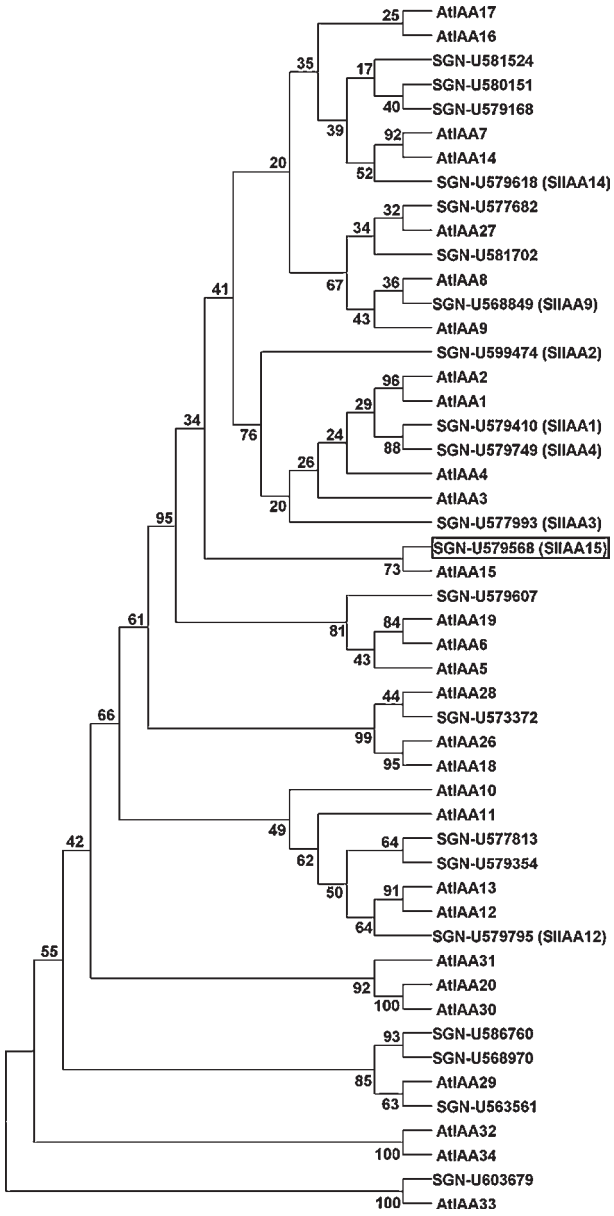

Fig. 2 SlIAA15 phylogenetic analysis. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007) to reveal the relationship between Arabidopsis Aux/IAA proteins and Aux/IAA proteins extracted from tomato (*Solanum lycopersicum*) unigenes available at the SGN database (Solanaceae Genomics Network; http://www.sgn.cornell.edu/). Names and numbering of the tomato Aux/IAAs (given in parentheses) refer to the putative Arabidopsis orthologues.

investigation indicated that the increase in leaf thickness resulted from an increase in the length of palisade cells and from a larger intercellular space of spongy mesophyll cells (Fig. 7c). The density of epidermal pavement cells in AS-*IAA15* lines was decreased by 53% relative to wild-type plants (Fig. 7d).

SlIAA15 down-regulated lines display reduced trichome number

Tomato trichomes are categorized into seven types, with types I, IV, VI and VII being glandular and types II, III and V being

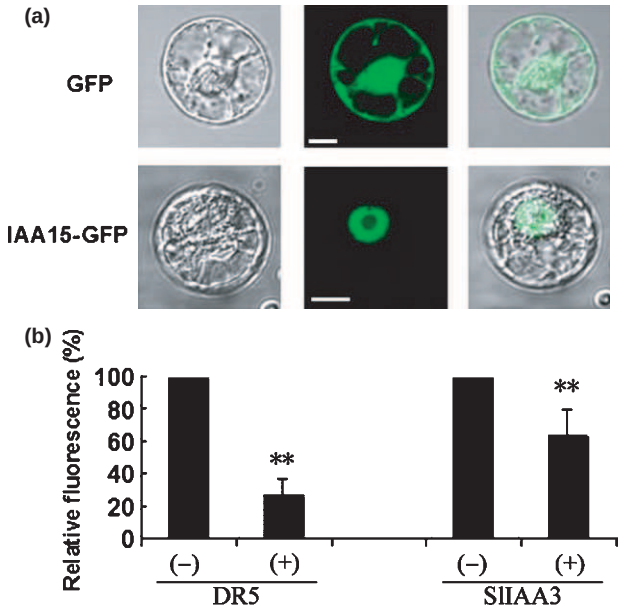

Fig. 3 (a) Subcellular localization of SlIAA15 protein. The SlIAA15::GFP (GFP, green fluorescent protein) fusion protein was transiently expressed in BY-2 tobacco protoplasts and the subcellular localization was analysed by confocal laser scanning microscopy. Merged pictures (right panels) of the green or yellow fluorescence channel (middle panels) and the corresponding bright field (left panels) are shown. Control cells expressing GFP alone are shown in the top panels and cells expressing the SlIAA15::GFP fusion protein are shown in the bottom panels. Bar, 10 μm. (b) Effect of SlIAA15 protein on auxin-dependent transcriptional regulation. Protoplasts were co-transfected with the effector construct giving constitutive 35S-driven SlIAA15 protein expression (+) or without cDNA as a control (–), and the reporter construct consisting of either the DR5 promoter::GFP fusion gene or the SlIAA3 promoter::GFP fusion. Protoplasts were treated with 50 μM 2,4-Dicholrophenoxyacetic acid, and GFP fluorescence was quantified by flow cytometry. Bars indicate + SE of the mean. **, Significant difference between transgenic and wild-type (WT) plants with $P < 0.05$, as determined by *t*-test.

nonglandular. Relative to wild-type plants, AS-*IAA15* showed dark green leaves and a dramatic reduction in trichome number in both leaves and shoots (Fig. 8). AS-*IAA15* lines displayed a strong reduction in the density of hair-like glandular type I and VI and nonglandular type V trichomes present in leaves and shoots (Fig. 8a–d). In the wild-type, the densities of types I, V and VI trichomes reached 22, 231 and 26 units per 2.5 mm^2, respectively, in 7-wk-old leaves, and 23, 383 and 52 units per 2.5 mm^2, respectively, in shoots. Comparable analysis performed with AS-*IAA15* plants showed that the densities of types I, V and VI trichomes were reduced to 14%, 27% and 12%, respectively, of their level in wild-type leaves, and 9%, 25% and 6%, respectively, of their density in wild-type shoots (Fig. 8e,f). Concomitantly, AS-*IAA15* lines displayed up to a 53% decrease in the density of epidermal cells relative to wild-type plants (Fig. 7d); however, the level of reduction in trichome density (up to 94%) is substantially higher than the decrease in epidermal cell density (53%).

Expression of *SlIAA15* in trichomes

Given the impact of the down-regulation of *SlIAA15* on trichome formation, the expression of *SlIAA15* and other tomato *Aux/IAA* genes was assessed in trichome tissues. The tomato

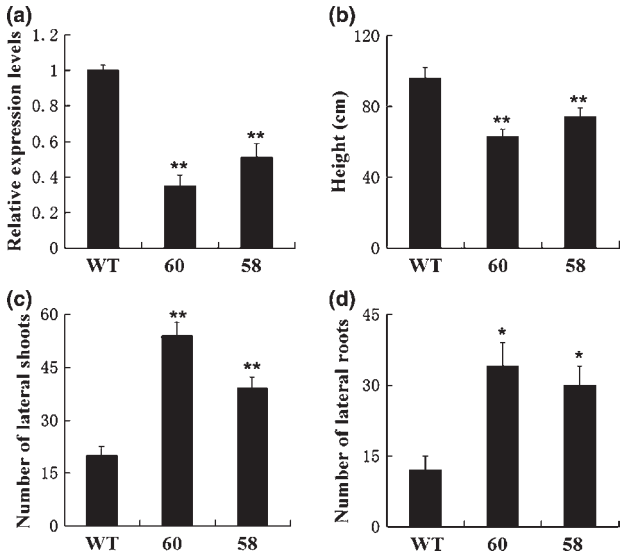

Fig. 4 Morphological alterations exhibited by *SlIAA15* down-regulated tomato (*Solanum lycopersicum*) lines. (a) Down-regulation of the *SlIAA15* gene in transgenic tomato plants. The level of *SlIAA15* transcripts in transgenic antisense lines was assessed by real-time PCR. The data are mean values corresponding to three independent experiments. (b) The height of transgenic plants. (c) The number of lateral shoots in *SlIAA15* down-regulated plants. (d) The number of lateral roots in *SlIAA15* down-regulated plants. WT, wild-type plants; 58 and 60, two representative *SlIAA15* down-regulated lines. Error bars, + SE. * and **, Significant differences between transgenic and WT plants with $P < 0.05$ and $P < 0.01$, respectively, as determined by *t*-test.

homologue of Arabidopsis *GASA4* was used as reference gene based on its preferential expression in trichomes (Kryvych *et al.*, 2008). To this end, the tomato orthologue (unigene U569289) of *GASA4* was isolated and its transcript level was assessed in shoot trichomes by qRT-PCR, together with that of eight tomato *Aux/IAA* genes. Although *SlIAA14* transcripts were the most highly abundant in trichome tissues, those corresponding to *SlIAA15* and *SlIAA1* also displayed significant accumulation relative to that of other *Aux/IAA*s, such as *SlIAA2* and *SlIAA12* (Fig. 9). Of particular interest, the transcript accumulation of *SlIAA14*, which showed the highest expression in trichomes, was not altered in AS-*IAA15* plants (Fig. 6), thus ruling out its direct role in causing the trichome phenotype displayed by the transgenic lines. To obtain further insight into what drives the expression of *SlIAA15* in trichome tissue, we performed *in silico* analysis of its promoter region using the PLACE program (http://www.dna.affrc.go.jp/PLACE/signalscan.html). A number of the *cis*-acting elements identified in the promoter region of the *SlIAA15* gene (Table 2) correspond to regulatory elements found in the promoter of Arabidopsis genes preferentially expressed in trichome initial cells (Kryvych *et al.*, 2008). Among these, various MYB-binding sites, regulatory elements involved in hormonal, metal, sulfur response and cell cycle regulation, are clearly present in the *SlIAA15* promoter.

Expression of selected R2R3 *MYB* genes is altered in *SlIAA15* down-regulated lines

To gain some clues into the mechanisms underlying the phenotypes observed in the AS-*IAA15* lines, we assessed the expression of genes known to be involved in the developmental processes that are altered in the transgenic lines. Notably, the accumulation of transcripts corresponding to *THM1* and *Anthocyanin1* (*Ant1*) genes from the R2R3 MYB family, known to play an important role in the regulation of trichome formation, was reduced significantly in *SlIAA15* down-regulated lines (Fig. 10). In addition, the expression of the gibberellin (GA) signalling genes, *GAMYB-like1* and *GAI*, was investigated because of the role reported for GA signalling during trichome formation. Assessment of the transcript levels of these genes in transgenic tomato plants indicated that the expression at the transcriptional level of *GAMYB-like1* was decreased strongly in AS-*IAA15* lines, whereas that of the *GAI* gene was not affected (Fig. 10). However, the expression of the *blind* gene, considered to be a general regulator of axillary shoot development in tomato, was increased in *SlIAA15* down-regulated lines (Fig. 10). This gene encodes another *MYB* transcription factor belonging to the R2R3 class, and its up-regulation could be correlated with the enhanced secondary shoot development in transgenic lines.

Discussion

Most of our understanding of the role of *Aux/IAA* genes in plant developmental processes has been achieved from the

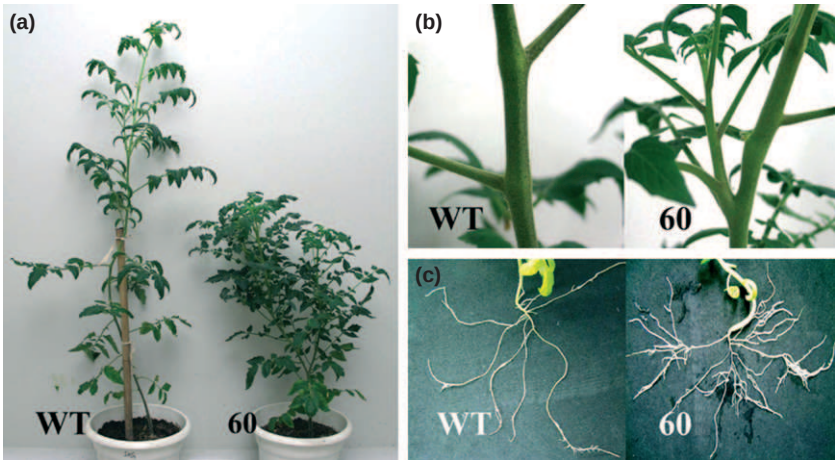

Fig. 5 Morphological alterations of *SlIAA15* down-regulated plants. (a) Down-regulation of *SlIAA15* in tomato (*Solanum lycopersicum*) results in reduced plant height and reduced apical dominance. (b) *SlAA15* down-regulated plants showing the development of axillary shoots from the first leaf node. (c) Down-regulation of *SlAA15* in tomato improves lateral root development. WT, wild-type plants. 60, one representative *SlIAA15* down-regulated line.

Table 1 Numbers of flowers and fruits in wild-type (WT) and AS-*SlIAA15* tomato (*Solanum lycopersicum*) plants

Plants	Anthotaxy number	Flower number	Fruit number	Set fruit rate (%)
WT	3 ± 1	25 ± 3	23 ± 3	92.05 ± 6.6
58#	2 ± 1	13 ± 2**	6 ± 2**	46.85 ± 12.54*
60#	3 ± 1	5 ± 2**	2 ± 1**	30.52 ± 9.98**

The data are mean values ± standard errors corresponding to four independent experiments. * and **, Significant differences between transgenic and WT plants with $P < 0.05$ and $P < 0.01$, respectively, as determined by *t*-test.

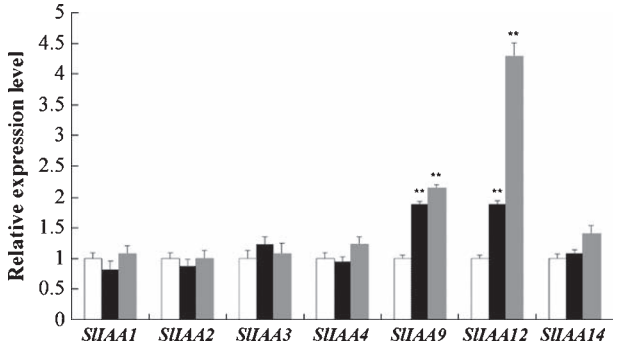

Fig. 6 Expression analysis of *Aux/IAA* genes in AS-*SlIAA15* by tomato (*Solanum lycopersicum*) plants real-time PCR. *SlIAA1* (U579410), *SlIAA2* (U599474), *SlIAA4* (U579749), *SlIAA12* (U579795) and *SlIAA14* (U579618) refer to unigene sequences identified in the Solanaceae Genomics Network (SGN) database. The relative mRNA level of individual *SlIAA* genes in hypocotyl tissues was normalized with respect to the *Ubiquitin* housekeeping gene. The results were expressed using wild-type plants as a reference expression level for each gene. WT (white bars), wild-type plants; 60 (grey bars) and 58 (black bars), two representative *SlIAA15* down-regulated lines. The data are mean (+ SE) values corresponding to three independent experiments. **, Significant difference between transgenic and WT plants with $P < 0.01$, as determined by *t*-test.

characterization of Arabidopsis gain-of-function mutations, as phenotypes associated with 'null mutants' in this plant species remain scarce, suggesting extensive functional redundancy among members of the Arabidopsis *Aux/IAA* gene family (Overvoorde *et al.*, 2005). Indeed, the Arabidopsis T-DNA insertional mutants characterized so far have not shown clear developmental defects, with the exception of the *shy2* mutant (Tian & Reed, 1999). Moreover, double or triple mutants of closely related *Aux/IAA* genes, such as *iaa8-1/iaa9-1* or *iaa5-1/iaa6-1/iaa19-1*, also have subtle or indiscernible phenotypes. In contrast with the situation prevailing in the Arabidopsis model species, an increasing number of reports have indicated that the down-regulation of a single *Aux/IAA* gene in solanaceous species can be sufficient to provoke visible and distinctive phenotypes. In tomato, under-expression of *SlIAA9* triggers pollination-independent fruit set, leading to parthenocarpy (Wang *et al.*, 2005, 2009), whereas down-regulation of *SlIAA3* results in auxin- and ethylene-related phenotypes, including exaggerated apical hook curvature and reduced petiole epinasty (Chaabouni *et al.*, 2009a). In potato, the suppression of *StIAA2* results in increased plant height, petiole hyponasty and curvature of growing leaf primordia in the shoot apex (Kloosterman *et al.*, 2006). The data described here establish that the normal expression of *SlIAA15* is critical for trichome formation in tomato, thus uncovering new roles for Aux/IAAs. Although phenotypes, such as reduced apical dominance, are more commonly associated with the *Aux/IAA* gene family, the altered trichome density phenotypes displayed by the down-regulated lines provide new insight on the physiological significance of the *Aux/IAA* genes. Taken together, these data indicate that members of the Aux/IAA family can have both specific and redundant functions.

Expression studies revealed no preferential accumulation of *SlIAA15* transcripts in all the tissues tested, and, in this regard, the ubiquitous expression in roots, stems, leaves, seedlings, flowers and fruit failed to provide clues into the potential role of the *SlIAA15* gene in particular developmental processes. In support of the idea that tomato *Aux/IAA* genes can play a common role in maintaining certain vegetative growth processes, the *SlIAA15* under-expressing lines display pleiotropic phenotypes, including reduced apical dominance, altered pattern of axillary shoot development and increased lateral root formation. Down-regulation of *SlIAA9*, a tomato gene from a distinct clade of the *Aux/IAA* gene family, has been similarly reported to result in pleiotropic phenotypes, including enhanced hypocotyl/stem elongation, increased leaf vascularization, reduced apical dominance and altered pattern of axillary shoot development (Wang *et al.*, 2005, 2009). Likewise, down-regulation of the tomato *SlIAA3* also induces multiple phenotypes related to vegetative growth, including a dramatically reduced apical dominance and altered pattern of axillary shoot development (Chaabouni *et al.*, 2009a). These phenotypes provide compelling evidence that the regulation of vegetative growth can be shared among different *Aux/IAA* genes in tomato. Nevertheless, the trichome phenotype of *SlIAA15* down-regulated lines also supports the idea that individual members of the Aux/IAA family can be involved in distinct developmental processes, consistent with both specific and overlapping functions of *Aux/IAA* genes. Moreover, the data highlight the importance of enlarging the functional characterization of candidate genes to species beyond the Arabidopsis model plant in order to gain new insight into the biological roles of *Aux/IAA* genes.

Tomato plants produce a variety of multicellular glandular and nonglandular trichomes that provide both physical and chemical barriers against insect invaders (Duffey, 1986; Kennedy, 2002). Tomato trichomes were first examined by Luckwill (1943) and categorized as types I–VII, with types I, IV, VI and VII being glandular and types II, III and V being nonglandular. In contrast with the detailed knowledge available on the molecular processes underlying the development of single-cell trichomes in Arabidopsis, relatively little is known about the genetic control of multicellular trichomes in tomato. Hormone signalling is likely to play an important role in trichome differentiation, and it has been reported that a tomato homologue of CORONATINE-INSENSITIVE1 (COI1), an F-box protein required for jasmonic acid (JA) signalled processes, is involved in the development of tomato glandular trichomes (Li *et al.*, 2004). The altered

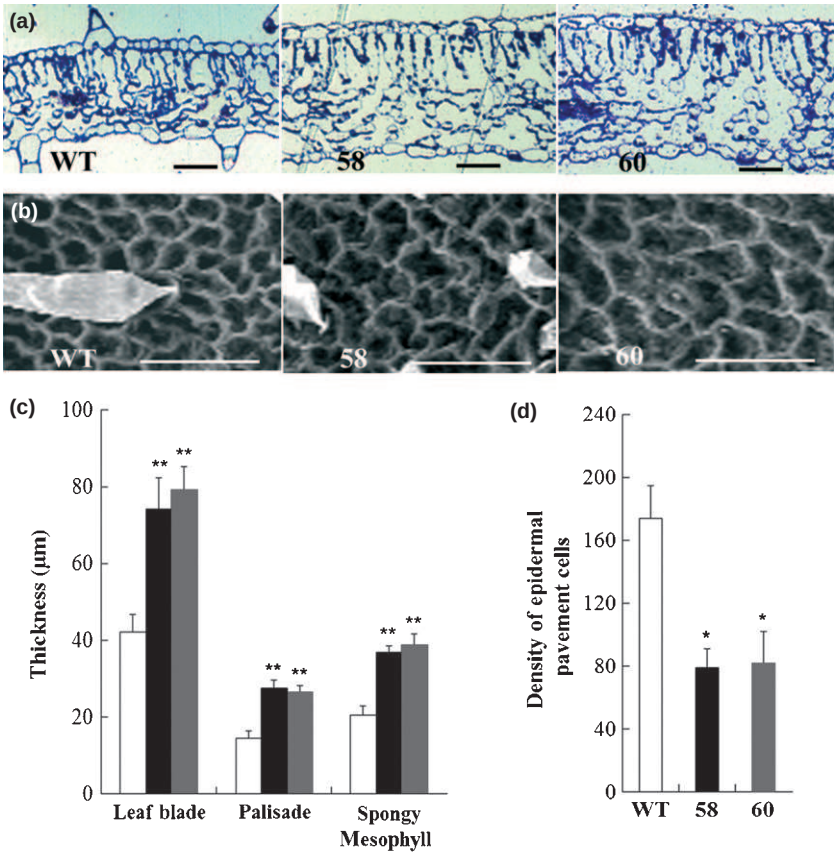

Fig. 7 Change in leaf anatomy in the AS-*SlIAA15* plants. (a) Transverse sections through the leaf of tomato (*Solanum lycopersicum*) wild-type and AS-*SlIAA15* plants. Semi-thin sections were stained with toluidine blue and viewed with a light microscope. (b) Adaxial epidermal pavement cells of fully expanded fifth leaves from wild-type and AS-*SlIAA15* plants. Bars, 20 µm. (c) Thickness of leaf blade, the palisade and the mesophyll. Four different measurements of the leaf blade, the palisade and the mesophyll thickness were taken into account. (d) Density of epidermal pavement cells of fully expanded fifth leaves. WT (white bars), wild-type plants; 60 (grey bars) and 58 (black bars), two representative *SlIAA15* down-regulated lines. The epidermal pavement cells in four replications of an area of 0.01 mm^2 were counted. Error bars, + SE. * and **, Significant differences between transgenic and WT plants with $P < 0.05$ and $P < 0.01$, respectively, as determined by *t*-test.

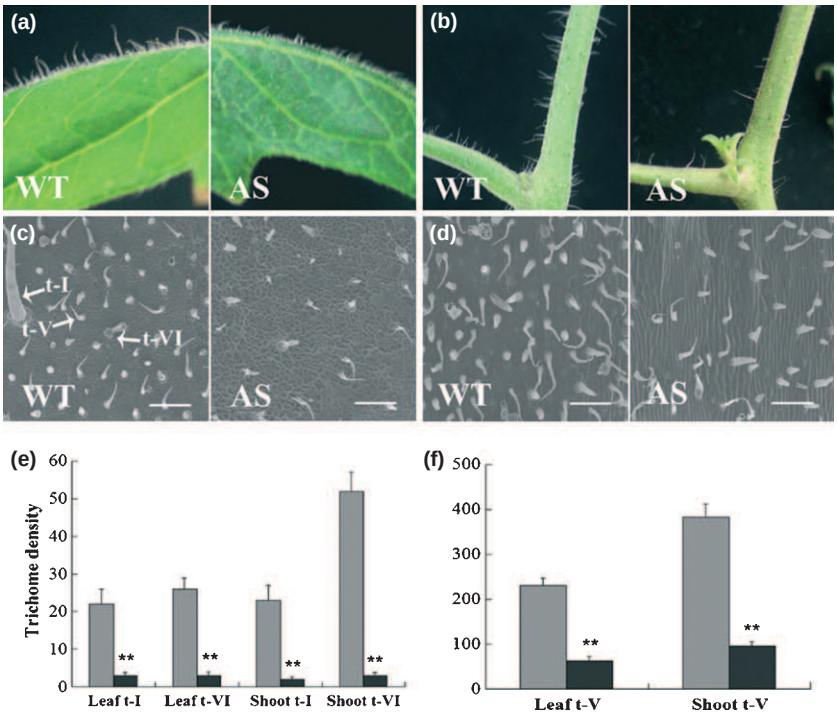

Fig. 8 Down-regulation of *SlIAA15* affects trichome development in tomato (*Solanum lycopersicum*) plants. (a) Leaf surface from wild-type and AS-*SlIAA15* plants. (b) Shoot surface from wild-type and AS-*SlIAA15* plants. (c) Scanning electron micrographs of the leaf surface from wild-type and AS-*SlIAA15* plants. (d) Scanning electron micrographs of the shoot surface from wild-type and AS-*SlIAA15* plants. Arrows denote type I (t-I), type V (t-V) and type VI (t-VI) trichomes. Bars, 100 µm. (e) Density of type I and type VI trichomes in leaves and shoots from wild-type (grey bars) and AS-*SlIAA15* (black bars) plants. (f) Density of type V trichomes in leaves and shoots from wild-type (grey bars) and AS-*SlIAA15* (black bars) plants. WT, wild-type plants. AS, *SlIAA15* down-regulated plants. The trichomes in four replications of an area of 2.5 mm^2 were counted. Error bars, + SE. **, Significant difference between transgenic and WT plants with $P < 0.01$, as determined by *t*-test.

trichome development in *SlIAA15* down-regulated lines uncovers new biological roles for *Aux/IAA*s that have not been reported for any of the *Aux/IAA* mutants. Notably, the level of reduction in trichome density (up to 94%) is substantially higher than the observed decrease in epidermal cell density (53%), which does not support the idea that the trichome phenotype is a simple consequence of the reduction in cell number, and clearly favours a direct effect of *SlIAA15* down-regulation on trichome formation.

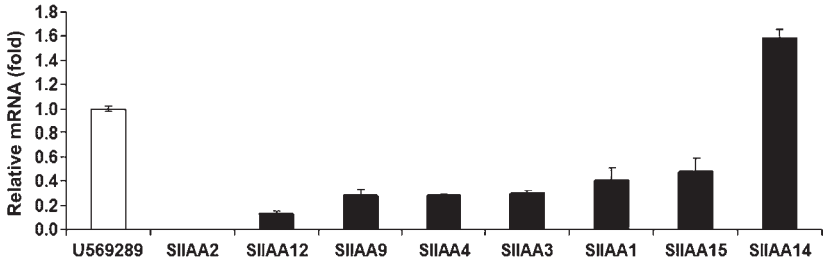

Fig. 9 Expression analysis of selected *Aux/IAA* genes in trichome tissues assessed by real-time PCR. *SlIAA1* (U579410), *SlIAA2* (U599474), *SlIAA4* (U579749), *SlIAA12* (U579795) and *SlIAA14* (U579618) refer to unigene sequences identified in the Solanaceae Genomics Network (SGN) database. The relative mRNA levels of individual *Aux/IAA* genes were normalized with respect to the housekeeping gene, *actin*. The tomato (*Solanum lycopersicum*) orthologue (unigene U569289) of the Arabidopsis *GASA4* gene, shown to be preferentially expressed in trichome tissues, was used as reference gene to estimate the relative levels of *Aux/IAA* transcripts. The data are mean (+ SE) values corresponding to three independent experiments.

Table 2 Distribution of *cis*-acting elements in *SlIAA15* promoter region (4.3 kb) compared with *cis*-acting elements found in the promoter regions of *Arabidopsis* genes preferentially expressed in trichome initial cells (including MYB element, sites involved in hormonal, metal, sulfur response and cell cycle regulation)

Site name	Number of sites	Description; organism
MYB1LEPR	1	Tomato Pti4 (ERF) regulates defence-related gene expression via GCC box and non-GCC box *cis* elements Myb1(GTTAGTT), G box; tomato, *A. thaliana*
MYB2CONSENSUSAT	1	MYB recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes; *A. thaliana*
MYBCORE	4	Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2; *A. thaliana*
MYBCOREATCYCB1	3	'Myb core' in the 18-bp sequence which is able to activate reporter gene without leading to M-phase-specific expression, found in the promoter of *A. thaliana* cyclin B1:1 gene; *A. thaliana*
MYBGAHV	4	Similar to c-myb and v-myb consensus binding site; barley, rice
MYBPLANT	1	Plant MYB-binding site; snapdragon, bean, petunia, *A. thaliana*, maize, parsley
MYBPZM	2	Core of consensus maize P (myb homologue)-binding site; maize
MYBST1	6	Core motif of MybSt1-binding site; potato
ARFAT	3	ARF (auxin response factor)-binding site found in the promoters of primary/early auxin response genes of *A. thaliana*; *A. thaliana*
ASF1MOTIFCAMV	4	Involved in transcriptional activation of several genes by auxin and/or salicylic acid; tobacco, *A. thaliana*
CATATGGMSAUR	4	Involved in auxin responsiveness; soybean
NTBBF1ARROLB	10	Required for tissue-specific expression and auxin induction; *Agrobacterium rhizogenes*
SEBFCONSSTPR10A	3	Similar to the auxin response element; potato
PYRIMIDINEBOXOSRAMY1A	4	Found in the promoter of barley α-amylase gene which is induced in the aleurone layers in response to GA; barley, rice
GAREAT	5	GA-responsive element; *A. thaliana*
TATCCAOSAMY	5	Mediates sugar and hormone regulation of α-amylase gene expression; rice
DPBFCOREDCDC3	6	bZIP transcription factors, abscisic acid response; carrot, *A. thaliana*
E2FCONSENSUS	1	'E2F consensus sequence' of all different E2F-DP-binding motifs in plants; *A. thaliana*, tobacco, rice, *N. benthamiana*
CURECORECR	20	Copper-response element; *Chlamydomonas reinhardtii*
SURECOREATSUL11	9	Core of sulfur-responsive element (SURE) found in the promoter of SULTR1; 1 high-affinity sulfate transporter gene in Arabidopsis; SURE contains auxin response factor (ARF)-binding sequence; *A. thaliana*

The *SlIAA15* under-expressing lines reveal that *SlIAA15* is instrumental to normal trichome formation in addition to being involved in the control mechanisms underlying leaf and shoot development in tomato.

Because both trichome and axillary shoot formation have been reported to involve MYB-mediated regulation, the expression of selected representatives of this gene family was assessed in the transgenic lines. The MYB supergene family comprises three subfamilies, MYB1R, R2R3 MYB and MYB3R, based on the number of adjacent repeats in the MYB domain. R2R3 MYB is the largest subfamily and functionally the most diverse. Members of this subfamily are known to play important roles in the regulation of processes as diverse as anthocyanin biosynthesis (Aharoni *et al.*, 2001; Nesi *et al.*, 2001), identity of cell shape and formation of plant organs (Schmitz *et al.*, 2002; Suo *et al.*, 2003) and responses to GA (Gubler *et al.*, 1995, 2002). In higher plants, including the Solanaceae, the development of trichome structures has been shown to be regulated by MYB transcription factors. In

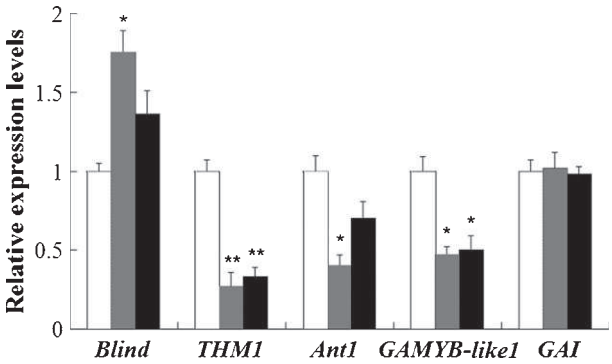

Fig. 10 Expression analysis of *Blind*, *THM1*, *Ant1*, *GAMBY-like1* and *GAI* genes assessed by real-time PCR. WT (white bars), wild-type tomato (*Solanum lycopersicum*) plants; 58 (black bars) and 60 (grey bars), two representative *SlIAA15* down-regulated lines. The data are mean (+ SE) values corresponding to three independent experiments. *, and **, Significant differences between transgenic and WT plants with $P < 0.05$ and $P < 0.01$, respectively, as determined by *t*-test.

Arabidopsis, several lines of evidence suggest that the duplication of a gene controlling anthocyanin production and subsequent divergence might be the major force driving trichome formation (Serna & Martin, 2006). Trichome initiation is regulated by the combinatorial action of the R2R3-MYB GLABRA1 (GL1) together with the bHLH GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) transcription factors (Serna & Martin, 2006). Although the identity of the R2R3 MYB factors controlling trichome formation in tomato remains unknown, the expression of *THM1* and *Ant1*, two tomato R2R3 *MYB* genes, displayed a marked decrease in *SlIAA15* under-expressing lines, suggesting their putative role in trichome formation. *THM1* is similar to the *GLABROUS1* (*GL1*) gene, which promotes trichome formation in Arabidopsis (Marks & Feldmann, 1989), and *Ant1* is a transcriptional regulator reported to be involved in anthocyanin biosynthesis, modification and transport (Mathews *et al.*, 2003). GAs are also known to influence trichome development (Perazza *et al.*, 1998), and it has been shown in Arabidopsis that the effect of GA is mediated by the GAMYB-like transcriptional regulators (Perazza *et al.*, 1998; Gocal *et al.*, 2001). The lower expression of the tomato *GAMYB-like1* gene in transgenic lines suggests its role as a positive regulator of trichome development. These data support the hypothesis that *SlIAA15* controls trichome formation and development in tomato via direct or indirect regulation of the MYB transcription factor.

Enhanced axillary shoot development is another remarkable phenotype exhibited by the *SlIAA15* down-regulated lines. The initiation of lateral meristems has been shown to be blocked during shoot and inflorescence development in tomato *blind* mutants, leading to a strong reduction in the number of lateral axes (Schmitz *et al.*, 2002). The tomato *blind* mutation resides in a R2R3 *MYB* gene considered to be a general regulator of shoot branching in tomato (Schmitz *et al.*, 2002). The *SlIAA15*-suppressed lines display enhanced axillary shoot proliferation and higher accumulation of the *blind* transcripts, suggesting that the expression of the *blind* gene is negatively regulated by SlIAA15, which may therefore act upstream of *blind*. This is consistent with the suppressor activity of auxin-dependent gene transcription shown here for SlIAA15. Moreover, because the expression of the early auxin-responsive genes *SlIAA9* and *SlIAA12* is increased significantly in the transgenic lines, it can be speculated that these mediators of auxin responses may also contribute to the phenotypes displayed by the *SlIAA15* down-regulated lines. SlIAA15 may therefore represent an important factor by which auxin impacts trichome formation in tomato. However, we cannot rule out the possibility that cumulative effects of the *SlIAA15* loss-of-function may lead to downstream effects underlying some of the phenotypes displayed by the transgenic lines.

Overall, the data described here add to the roles of *Aux/IAA* genes and provide new evidence supporting the hypothesis that different members of the Aux/IAA family can play distinct and specific roles, in addition to the shared redundant function. Further deciphering of the molecular mechanisms by which *SlIAA15* controls specific developmental processes will require the identification of its direct target genes. However, this task may prove to be difficult as Aux/IAA proteins have been shown not to bind directly to DNA but rather to interact with ARF transcription factors. Therefore, the search for the ARF protein partner(s) of SlIAA15 could be the initial step towards uncovering the primary target genes of this transcriptional regulator.

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Table S1 The accession numbers and primer sequences of the *Aux/IAA* genes and R2R3 *MYB* family genes described in this article