

RESEARCH PAPER

# SI-IAA3, a tomato Aux/IAA at the crossroads of auxin and ethylene signalling involved in differential growth

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## Abstract

Whereas the interplay of multiple hormones is essential for most plant developmental processes, the key integrating molecular players remain largely undiscovered or uncharacterized. It is shown here that a member of the tomato auxin/indole-3-acetic acid (*Aux/IAA*) gene family, *SI-IAA3*, intersects the auxin and ethylene signal transduction pathways. *Aux/IAA* genes encode short-lived transcriptional regulators central to the control of auxin responses. Their functions have been defined primarily by dominant, gain-of-function mutant alleles in *Arabidopsis*. The *SI-IAA3* gene encodes a nuclear-targeted protein that can repress transcription from auxin-responsive promoters. *SI-IAA3* expression is auxin and ethylene dependent, is regulated on a tight tissue-specific basis, and is associated with tissues undergoing differential growth such as in epinastic petioles and apical hook. Antisense down-regulation of *SI-IAA3* results in auxin and ethylene-related phenotypes, including altered apical dominance, lower auxin sensitivity, exaggerated apical hook curvature in the dark and reduced petiole epinasty in the light. The results provide novel insights into the roles of *Aux/IAAs* and position the *SI-IAA3* protein at the crossroads of auxin and ethylene signalling in tomato.

**Key words:** Auxin, differential growth, ethylene, hormone cross-talk, tomato.

## Introduction

Development in multicellular organisms is a highly complex process that requires the precise coordination of inter- and intracellular signalling and responses. Before the molecular era, the regulation of plant developmental processes was most often described as modifications in the hormonal balance, rather than as changes in the level of a single hormone. Subsequently, genetic screens led to tremendous advances in our understanding of the key components of the individual hormone metabolism and response pathways. As the understanding of these mechanisms grew, it became more apparent that the growth of plant organs is dependent on an intricate orchestration of hormonal and non-

hormonal signals (Stepanova *et al.*, 2007; Swarup *et al.*, 2007). Identifying the central players in the interplay between different signalling pathways is critical to unravelling the complex mechanisms underlying the control of plant growth and development. Despite interactions between ethylene and auxin being among the most frequently addressed in hormonal cross-talk studies, little is known about the main actors that take part in this dialogue (Chae *et al.*, 2000; Stepanova *et al.*, 2005, 2007).

The plant hormone auxin, indole-3-acetic acid (IAA), has long been recognized as being a major regulator of plant growth and developmental processes. It exerts its effects by

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modulating the expression of downstream genes that encode proteins involved in a vast array of physiological processes. Recent genetic and molecular studies in *Arabidopsis* have revealed that auxin regulates gene expression through an ubiquitin-dependent proteolytic signal transduction system (Dharmasiri and Estelle, 2004). At the centre of the signaling cascade is the ubiquitin–ligase complex; auxin binding to Transport Inhibitor Response1/TIR1 (or its paralogues, the F-box protein AUXIN RECEPTOR F-BOX/AFB1 and AFB3) promotes the ubiquitin-dependent proteolysis of a family of transcriptional regulators known as Aux/IAAs in an auxin-dependent manner (Gray *et al.*, 2001, Dharmasiri *et al.*, 2005a, b; Kepinski and Leyser, 2005). Aux/IAA proteins inhibit the activity of the DNA-binding auxin response factors (ARF) whereas their degradation leads to the activation of ARFs and to subsequent auxin-responsive gene expression (Reed, 2001; Tiwari *et al.*, 2001; Zenser *et al.*, 2001; Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). Aux/IAAs are therefore central to the regulation of auxin-mediated processes. The *Arabidopsis* genome encodes 29 Aux/IAA proteins (Remington *et al.*, 2004; Overvoorde *et al.*, 2005). Biochemical and genetic studies indicate that they generally function as transcriptional repressors of auxin-regulated genes (Ulmasov *et al.*, 1997; Tiwari *et al.*, 2004; Woodward and Bartel, 2005).

Gain-of-function mutations in several *Aux/IAA* genes have pleiotropic effects on plant growth, including altered root formation, apical dominance, stem/hypocotyl elongation, leaf expansion, and phototropism/gravitropism. These mutants have been identified in a variety of developmental and auxin-specific genetic screens. Each of these mutants is caused by a single mutation in domain II that results in the stabilization of the Aux/IAA. Strikingly, with the exception of the *shy2* mutant that displays subtle modifications (Tian and Reed, 1999), none of the *Arabidopsis* ‘null mutants’ show obvious visible phenotypes, suggesting considerable functional redundancy among *Aux/IAA* family members (Overvoorde *et al.*, 2005). The wide diversity of auxin responses and the tissue-specific expression of gene family members suggest, however, that individual Aux/IAAs have precise and distinct functions during normal plant growth and development. In both *Arabidopsis* and tomato, *Aux/IAAs* are themselves auxin responsive. Moreover, it has been reported previously that tomato *Aux/IAA* gene family

members can be regulated by ethylene (Jones *et al.*, 2002). Here, it is shown that SI-IAA3, a tomato Aux/IAA, is critical to both auxin and ethylene signalling and is a key molecular link between ethylene and auxin responses in tomato plants.

## Materials and methods

### Plant material and growth conditions

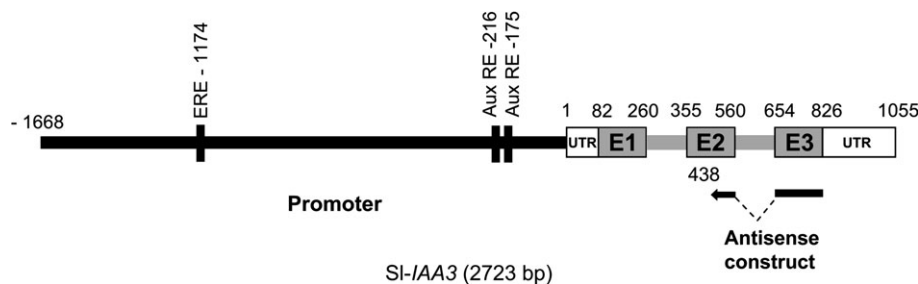
Tomato [*Solanum lycopersicum* cv. MicroTom] plants were grown under standard greenhouse conditions. The culture chamber room was set as follows: 14-h-day/10-h-night cycle, 25/20 °C day/night temperature, 80% relative humidity, 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  intense light. Seeds were sterilized, rinsed in sterile water, and sown in recipient Magenta vessels containing 50 ml of 50% Murashige and Skoog (MS) culture medium to which was added R3 vitamin (0.5 mg l<sup>-1</sup> thiamine, 0.25 mg l<sup>-1</sup> nicotinic acid, and 0.5 mg l<sup>-1</sup> pyridoxine), 1.5% (w/v) sucrose, and 0.8% (w/v) agar, pH 5.9.

### Plant transformation

To generate *AS-IAA3* transgenic plants, the forward 5'-ACAAGACTCAGCTCCTGCACC-3' and reverse 5'-CATCAACAACAAGCATCCAATC-3' primers were used to amplify a partial SI-*IAA3* clone (antisense construct in Fig. 1). The percentage sequence identity of the amplified fragment relative to the other members of the tomato *Aux/IAAs* family was checked (see Table S1 in Supplementary data available at *JXB* online) in order to validate its use in the antisense strategy. This 297 bp 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 according to Wang *et al.* (2005) and all experiments were carried out using homozygous lines from F<sub>3</sub> or later generations.

### Isolation of the SI-IAA3 genomic clone

SI-*IAA3* genomic clone was isolated by PCR amplification on genomic DNA template using primers encompassing the



**Fig. 1.** Genomic structure of the tomato SI-*IAA3* gene. The black portion represents the promoter region, the grey lines the introns, the grey boxes the exons, and the white boxes the untranslated regions (UTR). The putative auxin and ethylene *cis*-acting elements are indicated by black bars. The black arrow represents the antisense construct used to generate the silenced lines.

coding sequence. The Universal Genome Walker Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) was used to isolate the *Sl-IAA3* gene promoter region. The *Sl-IAA3* promoter was then fused to the  $\beta$ -glucuronidase (*GUS*) reporter gene in the pIp100 binary vector (Szabados *et al.*, 1995) and used for stable tomato transformation. DNA sequences were analysed with BLAST network services at the National Center for Biotechnology Information (Altschul *et al.*, 1997) and by PlantCARE (Lescot *et al.*, 2002).

#### *Transient expression using a single cell system*

For nuclear localization of the *Sl-IAA3* fusion protein, the coding sequence of *Sl-IAA3* was cloned as a C-terminal fusion in frame with green fluorescent protein (GFP) into the pGreen vector (Hellens *et al.*, 2000) and expressed under the control of the 35S CaMV, a cauliflower mosaic virus promoter. Protoplasts were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells and transfected according to the method described previously (Leclercq *et al.*, 2005). Transfected protoplasts were incubated for 16 h at 25 °C and analysed for GFP fluorescence by confocal microscopy. For co-transfection assays, the coding sequence of *Sl-IAA3* was cloned into the pGreen vector and expressed under the control of the 35S CaMV promoter. Aliquots of protoplasts ( $0.5 \times 10^6$ ) were transformed either with 10  $\mu$ g of the reporter vector alone containing the *DR5* synthetic auxin-response element fused to the *GFP* reporter gene (gift from Prof. K Palme, Freiburg, Germany) or in combination with 10  $\mu$ g of the effector plasmid, allowing the constitutive expression of the *Sl-IAA3* protein. Transformation assays were performed in three independent replicates. After 16 h of incubation in the presence or absence of 2,4-D (50  $\mu$ M), GFP expression was analysed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA, USA) as indicated in Hagenbeek and Rock (2001). All transient expression assays were repeated at least three times with similar results.

#### *Auxin and ethylene treatment*

For auxin dose-response (0, 1, 10, 100  $\mu$ M NAA) and NPA treatment, experiments were carried out as described by Wang *et al.* (2005). For quantitative real-time PCR (qRT-PCR) studies, 21-d-old seedlings were treated for 16 h with 1  $\mu$ l l<sup>-1</sup> 1-methyl cyclopropene (1-MCP), the ethylene perception inhibitor (Agrofresh, USA) and then incubated in presence or absence of 20  $\mu$ M IAA. For *GUS* analysis, 21-d-old tomato seedlings and sections of mature green (MG) fruit (Vibratom, Leica VT 1000 S, Vetzlar, Germany) were incubated for 2 h with or without 20  $\mu$ M IAA. MG and breaker (Br) fruit were treated for 5 h with 50  $\mu$ l l<sup>-1</sup> ethylene and 1-MCP (1  $\mu$ l l<sup>-1</sup>) for 16 h, respectively. Ethylene treatment (10  $\mu$ l l<sup>-1</sup>) was performed on 5-d-old etiolated *P<sub>IAA3</sub>::GUS*, *DR5::GUS* transformed seedlings. For the epinastic response, light-grown plants were treated with ethylene (50  $\mu$ l l<sup>-1</sup>) for 16 h.

For histochemical *GUS* analysis, *P<sub>IAA3</sub>::GUS* or *DR5::GUS* transgenic lines were incubated at 37 °C for 5–15 h with *GUS*-staining solution as indicated by Wang *et al.* (2005)

#### *qRT-PCR*

RNAs extraction and qRT-PCR analyses were performed as described previously (Pirrello *et al.*, 2006). The primer sequences are listed in Table S2 in Supplementary data available at *JXB* online.

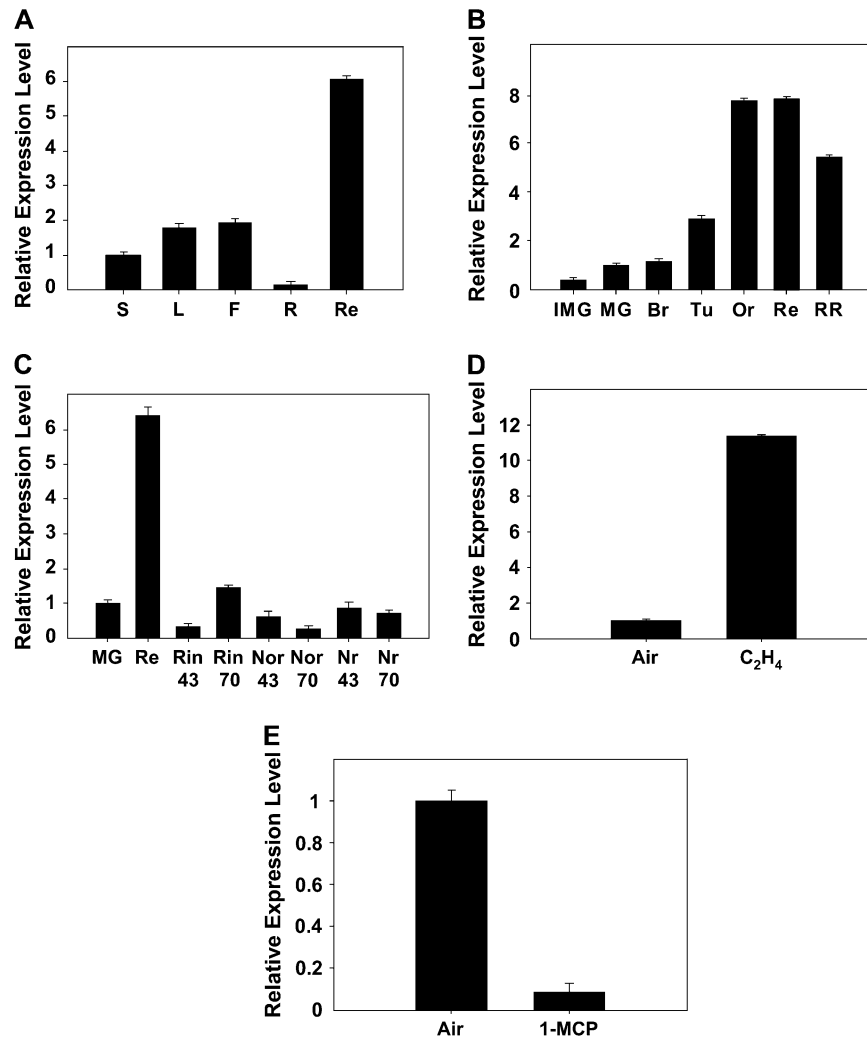
## Results

### *Isolation and structure of the Sl-IAA3 gene*

It has previously been shown that *Sl-IAA3* (formerly named *DR3*) is ethylene inducible and differentially expressed during tomato fruit ripening (Jones *et al.*, 2002). Subsequently the full-length *Sl-IAA3* cDNA (U 320812, now available from the Solanaceae Genome Network Database, <http://www.sgn.cornell.edu>) has been isolated and the transcription start site determined by 5' RACE-PCR. The 558 bp cDNA encoded a predicted *Sl-IAA3* protein of 185 amino acids comprising the four conserved domains (I–IV) characteristic of Aux/IAA proteins. *Sl-IAA3* falls into sub-family I of the four Aux/IAA sub-families (Wang *et al.*, 2005). A genomic fragment of 2723 bp was also isolated comprising 1668 bp of upstream sequence containing promoter and 1055 bp of gene sequence composed of three exons and two introns (Fig. 1) matching that of its closest *Arabidopsis* homologues, *At-IAA3* (AT1G04240) and *At-IAA4* (AT5G43700). The *Sl-IAA3* nucleotide coding and predicted amino acid sequences displayed 65.8% and 56% identity, respectively, with *At-IAA3* and 65.4% and 56.3% identity, respectively, with *At-IAA4*. Analysis of the 1668 bp promoter fragment with the PlantCare software (Lescot *et al.*, 2002) identified two degenerate auxin-response elements (TGTCNC) at positions –216 and –175, and an ethylene-response element ERE (ATTTCAA) at position –1174 (Fig. 1).

### *Sl-IAA3 transcripts are ubiquitous in all plant tissues but show higher accumulation during fruit ripening*

qRT-PCR showed that *Sl-IAA3* transcripts were present in all tissues tested (Fig. 2A), with the highest levels in red fruit, where they were 6-fold higher than in the reference (stem) tissue. In wild-type fruit, *Sl-IAA3* transcript levels increased commensurate with endogenous ethylene production levels throughout the ripening process (Fig. 2B). In the ripening and ethylene response-impaired monogenic tomato mutants, *rin* (*ripening inhibitor*), *nor* (*non-ripening*), and *Nr* (*Never-ripe*), *Sl-IAA3* transcript levels were substantially lower than in the wild-type at the equivalent to ripening stages (Fig. 2C), indicating that *Sl-IAA3* is integral to normal ethylene-responsive fruit-ripening processes. To verify that the ripening-associated *Sl-IAA3* transcript accumulation was ethylene-dependent, the effect of exogenous

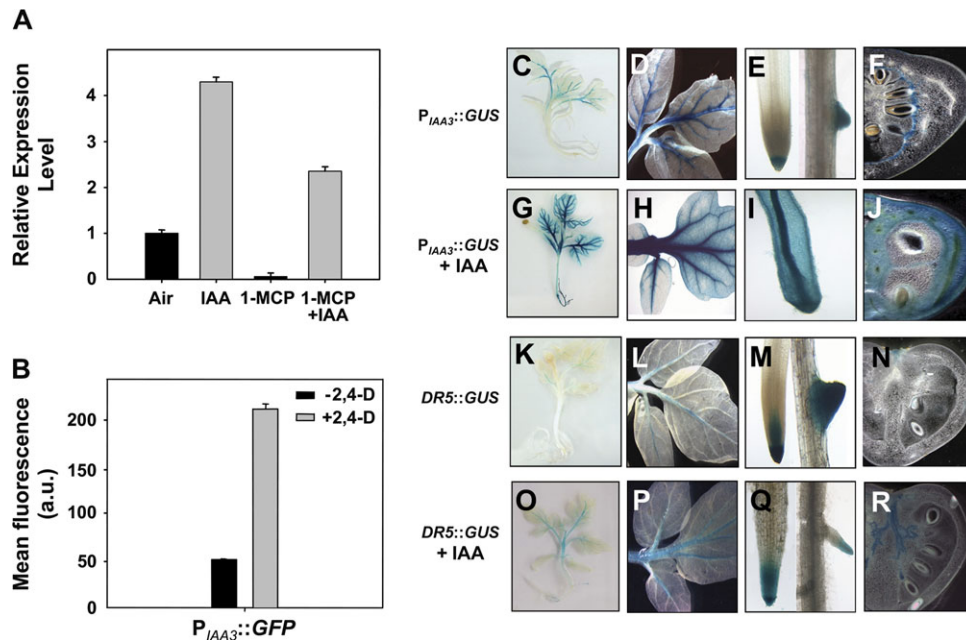


**Fig. 2.** Tissue-specific and ethylene-dependent expression of *SI-IAA3*. The expression analyses were carried out by qRT-PCR using RNA samples extracted from various tomato tissues. (A) Analysis of *SI-IAA3* transcript levels in different organs. *SI-IAA3* mRNA accumulation was monitored in stem (S), leaf (L), flower (F), root (R), and red fruit (Re). (B) Expression pattern of *SI-IAA3* during the late stages of fruit development: immature green fruit, IMG; mature green, MG; breaker, Br; turning, Tu; orange, Or; red, Re; red-ripe, RR. (C) Expression pattern of *SI-IAA3* in wild type (WT) and *rin*, *nor*, and *Nr* ripening mutants. RNA samples were extracted from fruit collected 43 d and 70 d after anthesis, corresponding in the WT to MG and Re stages, respectively. (D) Ethylene responsiveness of the *SI-IAA3* gene. RNA samples were extracted from MG fruit treated for 5 h with air or with 50  $\mu\text{l l}^{-1}$  ethylene. (E) Br fruit treated with 1  $\mu\text{l l}^{-1}$  of 1-MCP for 16 h. Relative expression level on the y-axis refers to the fold difference in *SI-IAA3* expression relative to stem in (A), MG stage in (B, C), and untreated control fruit in (D, E). The expression data are means of three replicates  $\pm$  standard error.

ethylene was assessed on MG fruit that are responsive to exogenous ethylene but not yet producing elevated levels of ripening-associated ethylene, and, conversely, the effect of 1-MCP, a potent inhibitor of ethylene perception, on Br fruit producing elevated endogenous ethylene. Five hours of ethylene treatment of MG fruit (50  $\mu\text{l l}^{-1}$ ) resulted in an almost 11-fold increase in *SI-IAA3* transcript accumulation (Fig. 2D). Conversely, in Br-stage fruit, an overnight treatment with 1-MCP (1  $\mu\text{l l}^{-1}$ ) led to a 10-fold reduction in *SI-IAA3* transcripts (Fig. 2E). Given that *SI-IAA3* is a presumptive auxin response regulator, these results reveal that one of the roles for ethylene during climacteric fruit ripening is the modification of auxin responsiveness in ripening fruit.

#### *SI-IAA3* transcript accumulation is positively regulated by auxin and ethylene in tomato seedlings

In dark-grown seedlings, qRT-PCR analysis revealed that ethylene induction of *SI-IAA3* transcript accumulation mimicked both the dose-response and the time-course gradient of the well-characterized ethylene-responsive gene, *E8* (see Fig. S1 in Supplementary data available at *JXB* online). *SI-IAA3* transcript levels also increased 4-fold in light-grown tomato seedlings after 2 h of auxin (20  $\mu\text{M}$  IAA) treatment (Fig. 3A). In tobacco BY2 protoplasts transfection assays, *SI-IAA3* promoter (1668 bp)-driven GFP levels increased 4-fold after auxin treatment (50  $\mu\text{M}$  2,4-D) (Fig. 3B). As auxin is known to stimulate ethylene



**Fig. 3.** Auxin responsiveness of the SI-*IAA3* gene. (A) qRT-PCR analysis of SI-*IAA3* transcript levels in 3-week-old light-grown control and auxin-treated (20  $\mu$ M IAA for 2 h) seedlings in presence or absence of 1  $\mu$ l l<sup>-1</sup> 1-MCP applied 16 h prior to auxin treatment. Relative expression level on the y-axis refers to the fold difference in SI-*IAA3* transcript levels relative to the non-treated plantlets. (B) Auxin responsiveness of the SI-*IAA3* promoter. Tobacco protoplasts were transformed by P<sub>IAA3</sub>::GFP and incubated in the presence or absence of 2,4-D (50  $\mu$ M). Transformation was performed in triplicate and, in each experiment, GFP fluorescence was measured by flow cytometry 16 h after transfection. Values are expressed in arbitrary units (a.u.)  $\pm$  standard error. (C–F) Tissue-specific expression of SI-*IAA3* assessed in transgenic tomato expressing *GUS* reporter gene driven by the SI-*IAA3* promoter (P<sub>IAA3</sub>::*GUS*). The expression pattern was analysed in 3-week-old seedlings (C), leaves (D), roots (E), and MG fruit (F). (G–J) These images correspond to the same tissues treated for 2 h with 20  $\mu$ M IAA. (K–N) These images correspond to the same tissues expressing the *DR5* auxin-responsive promoter fused to the *GUS* reporter gene (*DR5*::*GUS*) and those in (O–R) to *DR5*::*GUS* treated with 20  $\mu$ M IAA. The data are representative of at least three independent experiments with  $n > 20$  seedlings examined per experiment.

production (Abel *et al.*, 1995), it was decided to determine whether this auxin-responsiveness resulted from an increase in ethylene production. Light-grown tomato seedlings were treated overnight with 1-MCP (1  $\mu$ l l<sup>-1</sup>) and then incubated in presence or absence of auxin. Similarly to the observation in fruit, 1-MCP almost completely abolished SI-*IAA3* transcripts in untreated tomato seedlings (Fig. 3A). In the presence of both 1-MCP and auxin, however, SI-*IAA3* transcript levels were only partially reduced (Fig. 3A), indicating that in light-grown tomato seedlings SI-*IAA3* is both auxin and ethylene-inducible and that the auxin-responsiveness is partially mediated by ethylene.

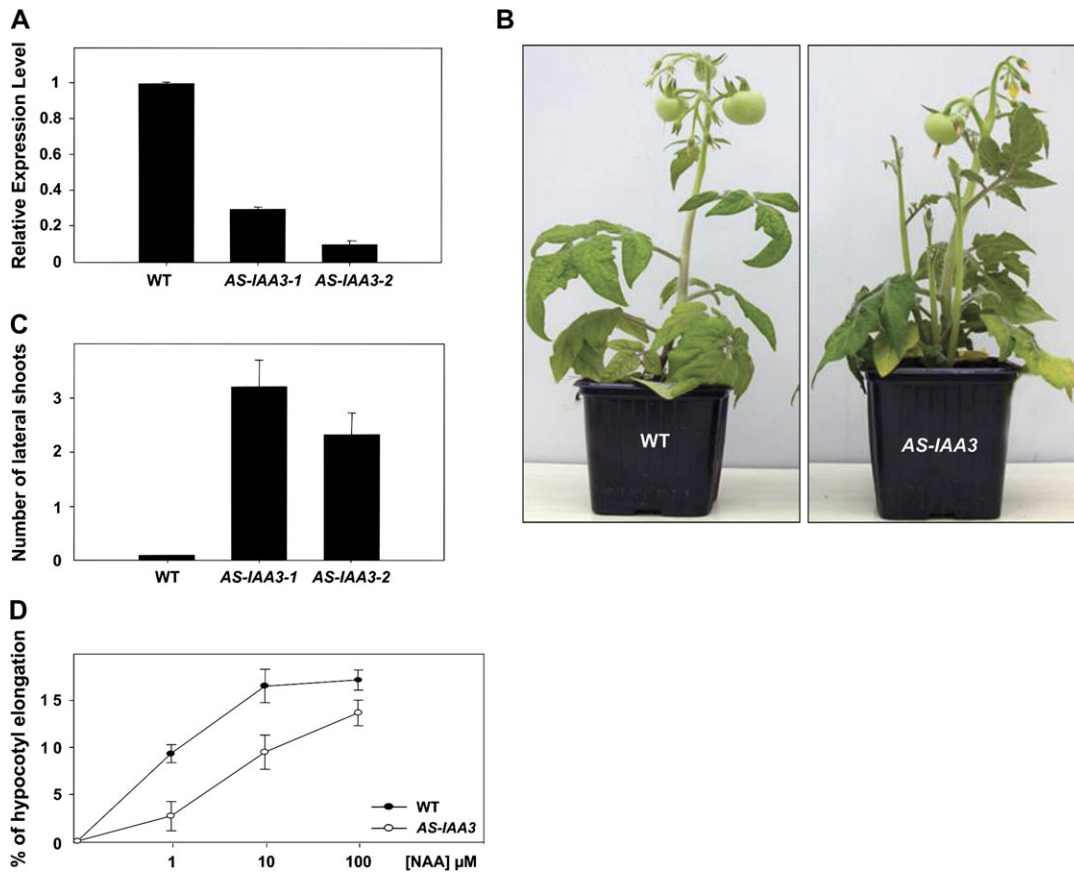
#### SI-*IAA3* displays tightly regulated tissue-specific expression

To gain further insight into SI-*IAA3* expression, the SI-*IAA3* promoter was fused to the *GUS* reporter gene (P<sub>IAA3</sub>::*GUS*) and this construct stably introduced into tomato plants. In untreated vegetative tissues, the SI-*IAA3* promoter drove *GUS* expression predominantly in the leaf vasculature, root cap, and developing lateral roots (Fig. 3C–E). A brief auxin treatment (20  $\mu$ M for 2 h) of light-grown seedlings led to a dramatic increase in *GUS* expression throughout the roots and shoots (Fig. 3G–I). In MG fruit, *GUS* staining was restricted to a narrow band in

the placental exo-layer at the junction between the placenta and pericarp tissues (Fig. 3F). Auxin treatment, led to *GUS* staining throughout the pericarp and columella tissues, while it remained excluded from placental tissues (Fig. 3J). As a control for auxin responsiveness, *GUS* expression driven by the synthetic auxin-responsive promoter, *DR5*, was also assessed. Interestingly, in the absence of exogenous auxin, *DR5* drove *GUS* expression in the leaf midrib and root tips (Fig. 3K–M), but not in the fruit (Fig. 3N). Exogenous auxin treatment resulted in enhanced staining in vegetative tissues but the fruit expression remained restricted to the vascular tissues (Fig. 3O–R), providing evidence that, although SI-*IAA3* is auxin responsive, its transcriptional control is more complex than that of *DR5*.

#### SI-*IAA3* down-regulation results in vegetative growth phenotypes

Several independent homozygous SI-*IAA3*-suppressed antisense lines (*AS-IAA3*) were generated and two representative lines (1 and 2) with 3.5-fold and 10-fold reductions, respectively, in SI-*IAA3* transcript levels were selected for further study (Fig. 4A). Down-regulation of SI-*IAA3* resulted in a variety of vegetative growth phenotypes (Figs 4, 5). In determinate wild-type tomato plants, lateral shoots develop only after floral transition, and their growth is initiated in an



**Fig. 4.** Altered vegetative growth phenotypes in antisense SI-IAA3 plants. (A) Down-regulation of SI-IAA3 in transgenic tomato plants. The level of SI-IAA3 transcripts in antisense lines (1 and 2) was assessed by qRT-PCR. Relative expression level refers to the fold difference in SI-IAA3 transcript levels relative to the wild type (WT). (B) Reduced apical dominance in 7-week-old AS-IAA3 plants compared with WT. (C) The number of lateral shoots branching from the first leaf node in WT and AS-IAA3 plants. The data are the mean  $\pm$  standard error of 30 plants and are representative of three independent experiments. (D) Auxin dose-response in hypocotyl segments. Hypocotyl fragments (8 mm long) from 3-week-old light-grown seedlings were incubated for 2 h in the presence of the indicated concentration of NAA. Elongation is given as percentage increase in final length over the initial length. The results are representative of data obtained with two independent AS-IAA3 lines and with two replicates for each line. Standard errors are indicated ( $n \geq 25$ ).

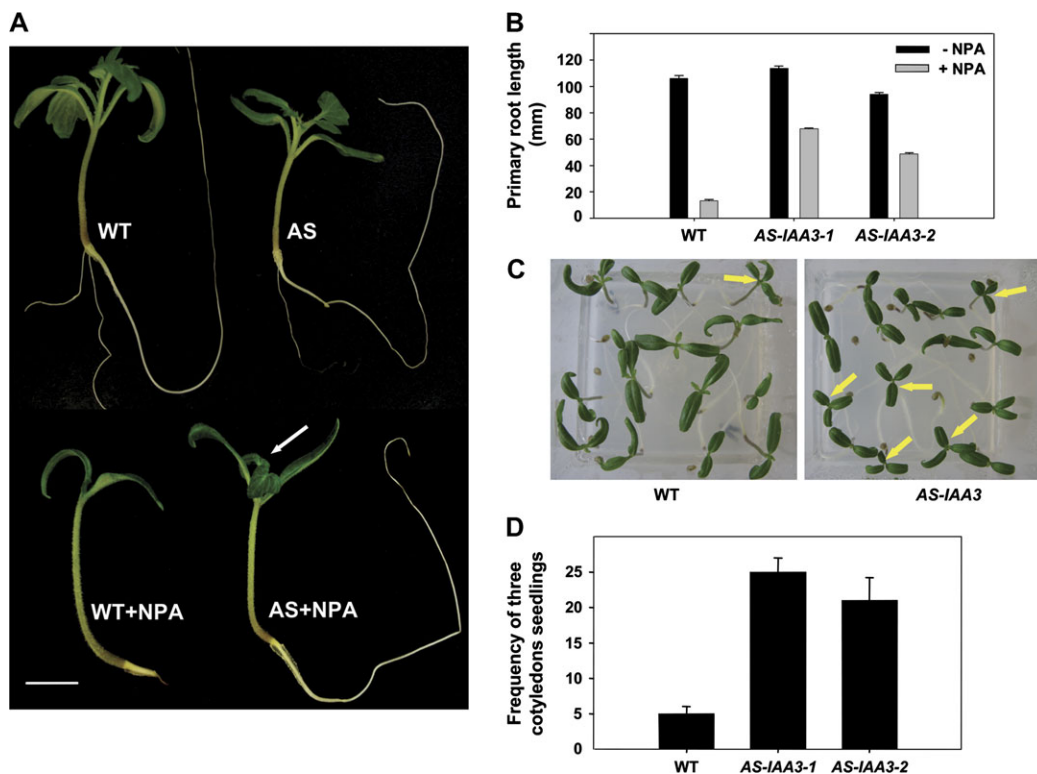
apical–basal sequence along the primary shoot axis. In the AS-IAA3 plants, by contrast, axillary shoot development began in the lowest leaf node (Fig. 4B) and the number of lateral shoots was greater in the transgenic lines (Fig. 4C). This loss of apical dominance suggests a reduced response to endogenous auxin in the transgenic lines. Similarly, auxin-induced hypocotyl elongation was reduced in AS-IAA3 hypocotyls compared with the wild type (Fig. 4D), further indicating a reduction in auxin responsiveness in the transgenic lines. To investigate this apparent reduction in auxin responsiveness, the effects of the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) on the growth of wild-type and AS-IAA3 seedlings were examined. Wild-type seedlings grown in the presence of 1  $\mu\text{M}$  NPA showed a marked reduction in primary root elongation and a complete suppression of lateral root formation (Fig. 5A, B). By contrast, NPA only weakly affected primary and lateral root growth in the AS-IAA3 plants (Fig. 5A, B). Also, leaf emergence was strongly inhibited in NPA-treated wild-type seedlings, but not in the AS-IAA3 plants (arrow in Fig. 5A). The AS-IAA3

lines also had a higher frequency of ectopic cotyledons than the wild type (Fig. 5C, D). The frequency of poly-cotyledons was 25% and 20% in AS-IAA3-1 and AS-IAA3-2 lines, respectively, compared with only 5% in the wild type (Fig. 5D).

#### SI-IAA3 suppression results in modified ethylene sensitivity

The ethylene responsiveness of SI-IAA3 prompted the examination of the role of the encoded protein in two classical ethylene response processes, epinastic petiole curvature in light-grown plants and the formation of an apical hook in etiolated seedlings. Tomato leaf petioles typically curve downwards in response to exogenous ethylene (Kazemi and Kefford, 1974). To investigate the impact of the down-regulation of SI-IAA3 on this epinastic response, light-grown wild-type and AS-IAA3 tomato plantlets were treated with exogenous ethylene (50  $\mu\text{l l}^{-1}$ ) for 16 h. The subsequent angles of the petioles to the main stem were





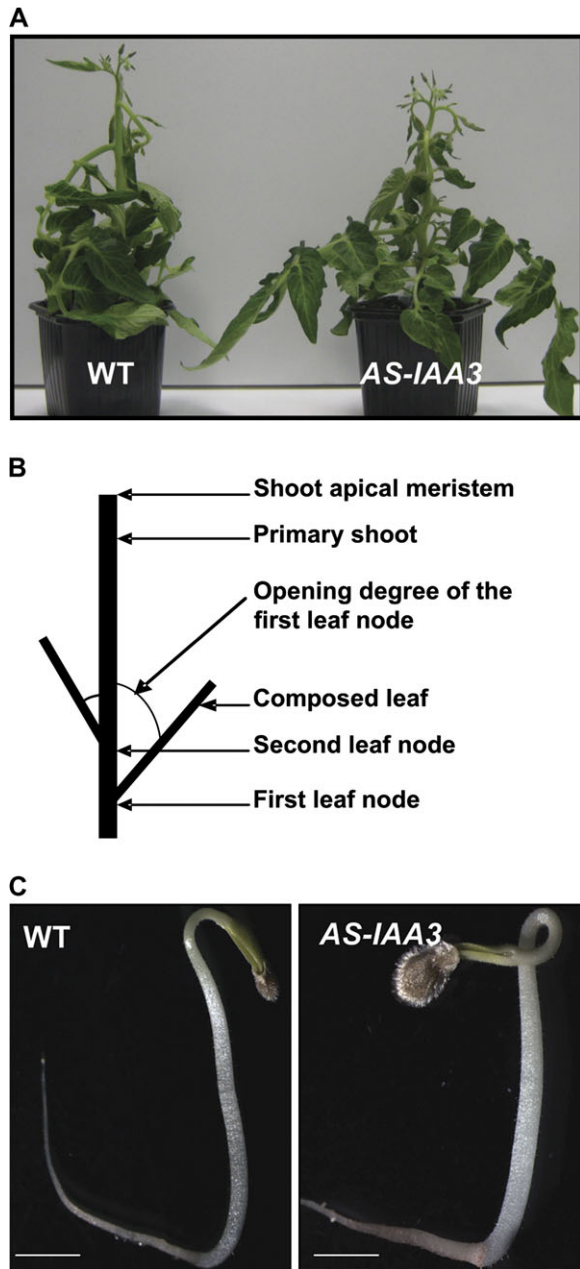
**Fig. 5.** Auxin-associated phenotypes of *SI-IAA3* down-regulated lines. (A) Effect of NPA treatment on the development of light-grown wild-type (WT) and *AS-IAA3* seedlings. WT and *AS-IAA3* tomato seedlings (19-d-old) were grown in the presence or absence of 1  $\mu$ M NPA. Leaf emergence is inhibited in WT but not in *AS-IAA3* lines (white arrow). The scale bar indicates 10 mm. (B) Primary root length upon NPA treatment of light-grown WT and *AS-IAA3* lines. Error bars represent mean  $\pm$  standard error ( $n \geq 60$ ). (C) Triple cotyledon phenotype occurring at higher frequency in *AS-IAA3* lines compared with WT. Three cotyledon structures are indicated by arrows in 7-d-old light-grown plantlets. (D) Frequency of triplicate cotyledons occurring in *AS-IAA3* and WT seedlings expressed as a percentage of the total population. Error bars represent mean  $\pm$  standard error of 40 plants.

measured for leaves 1 and 2 (Fig. 6B). In both *AS-IAA3* lines 1 and 2, the leaf angle after ethylene treatment was 87° and 75°, respectively (Fig. 6A, Table 1). In the wild type, the leaf angle was 100° (Fig. 6A, Table 1), indicating a reduced epinastic response in the transgenic lines.

The exaggeration of the apical hook is one of the hallmarks of the classical ethylene triple response, although the process is known to involve changes in both ethylene and auxin signalling (Ecker, 1995). One of the most striking phenotypes in the *AS-IAA3* seedlings was the exaggerated apical hook formation in dark-grown seedlings in the absence of exogenous ethylene (Fig. 6C). To characterize this phenotype better, different grades of hook formation (Fig. 7A) were defined ranging from stage 1, corresponding to minimal exaggerated hook with a curvature angle lower than 180°, to stage 4, corresponding to a maximal exaggerated hook with a curvature angle higher than 360°. Sixty percent of air-grown *AS-IAA3* seedlings displayed hook curvatures corresponding to stage 3 and 35% corresponded to stage 2. In the same growth conditions, most wild-type seedlings had hook curvatures of either stage 1 (60% of seedlings) or stage 2 (37% of seedlings) (Fig. 7C). A low level of exogenous ethylene (0.1  $\mu$ l l<sup>-1</sup>) shifted hook curvature to stage 2 (63% of seedlings) and stage 3 (25% of seedlings) in the wild-type and to stage 3 (90% of seedlings)

in the antisense plants (Fig. 7D). Increasing the exogenous ethylene to 1  $\mu$ l l<sup>-1</sup> shifted hook curvature to stages 4 (50% of seedlings) and 3 (45% of seedlings) in the wild-type and to stages 4 (80% of seedlings) and stage 3 (20% of seedlings) in the transgenic seedlings (Fig. 7E). Treatment with 1-MCP (Fig. 7B) strongly reduced the difference between wild type (98% of seedlings at stage 1) and antisense (90% of seedlings at stage 1), suggesting that the exaggerated apical hook curvature phenotype of the *AS-IAA3* plants requires active ethylene signalling.

To get more insight on the role of *SI-IAA3* in apical hook formation and epinastic response, the expression pattern of this gene was analysed in tomato lines expressing the *P<sub>IAA3</sub>::GUS* construct. In the absence of exogenous ethylene treatment there was minimal GUS staining associated with the apical hook in dark-grown wild-type *P<sub>IAA3</sub>::GUS* lines. By contrast, after 48 h ethylene treatment (10  $\mu$ l l<sup>-1</sup>), a strong band of GUS staining was observed on the inner surface of the apical hook (Fig. 8A). The same ethylene treatment did not result in detectable *DR5*-driven GUS staining in the hook. The putative role of auxin in mediating the ethylene-associated expression of *SI-IAA3* was then investigated by performing the ethylene treatment in the presence of NPA, a known inhibitor of auxin transport. NPA completely prevented ethylene-induced apical



**Fig. 6.** Ethylene-associated phenotypes of *AS-IAA3* lines. (A) Petiole epinasty in wild-type (WT) and *AS-IAA3* plants in response to ethylene. Five-week-old light-grown plants were treated by  $50 \mu\text{l l}^{-1}$  ethylene for 16 h. (B) Diagram depicting the position of the first and second leaf node in tomato plants. (C) Hook curvature in 5-d-old WT (left panel) and *AS-IAA3* (right panel) etiolated seedlings. The scale bar indicates 5 mm.

hook formation and simultaneously suppressed *SI-IAA3* expression, suggesting that auxin is required for apical hook formation and for the expression of *IAA3* in the inner side of the hook. Noteworthy, upon ethylene treatment, intense staining was present in the root tips of both transgenic lines, attesting that *DR5* and *IAA3* promoters exhibit similar capacity to drive *GUS* activity in tissues accumulating high amounts of auxin. Taken together these data suggest that

**Table 1.** Altered petiole epinastic response in *AS-IAA3* plants

Petiole opening degree of the first and the second leaf node was measured before and after ethylene treatment in wild-type and *AS-IAA3* plants. The data are means  $\pm$  standard error of at least 36 plants and are representative of three independent experiments.

	Petiole opening degree	
	Air	C <sub>2</sub> H <sub>4</sub>
WT	70.8 $\pm$ 2.8	100 $\pm$ 4.46
<i>AS-IAA3-1</i>	70.1 $\pm$ 3.5	87 $\pm$ 4.31
<i>AS-IAA3-2</i>	72.2 $\pm$ 1.8	75 $\pm$ 2.87

the higher ethylene-induced expression of *SI-IAA3* in the inner side of the apical hook could not be ascribed only to increased auxin levels (Fig. 8A).

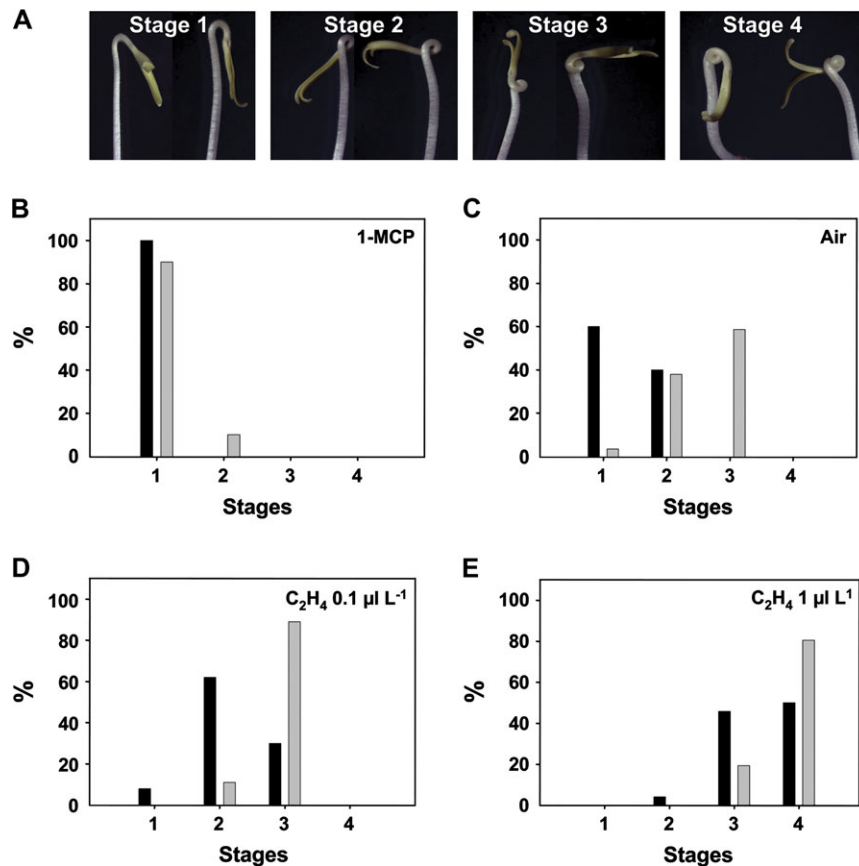
The role of *SI-IAA3* in ethylene-induced differential growth was further investigated by assessing the expression of *SI-IAA3* in light-grown epinastic tissues. Ethylene treatment of epinastic petioles led to *P<sub>IAA3</sub>::GUS* expression in restricted zones on the upper side of the leaf nodes (Fig. 8B) whereas no expression was detected in untreated non-epinastic petioles (Fig. 8B). These data indicate that *SI-IAA3* expression is associated with tissues undergoing differential growth, albeit in opposite directions relative to the ethylene-induced expression in the two tissues.

#### *Down-regulation of SI-IAA3 specifically impacts on the expression of selected auxin and ethylene transcription factors*

An *SI-IAA3::GFP* fusion protein localized exclusively to the nucleus in transient expression assays in tobacco protoplasts (see Fig. S2 in Supplementary data available at *JXB* online) consistent with the native *SI-IAA3* being a transcriptional regulator. To address the ability of the *SI-IAA3* protein to regulate the activity of auxin-responsive promoters, a *DR5*-driven GFP reporter construct was used (Ottenschlager *et al.*, 2003) in a protoplast transient expression assay. In the absence of effector construct, *DR5*-driven GFP expression was enhanced up to 10-fold by the auxin (2,4-D) treatment (see Fig. S3 in Supplementary data) whereas the presence of 35S-driven *SI-IAA3* in co-transfection assays, strongly reduced this auxin induction. These data indicate that *SI-IAA3* acts in protoplast as a repressor of auxin-dependent transcription and is consistent with *SI-IAA3* being a member of the Aux/IAA family.

To provide mechanistic insight into how *SI-IAA3* functions to bring about the observed phenotypes in the transgenic lines, the expression of transcription factors known to mediate auxin and ethylene responses, including 14 *Aux/IAA*, 10 *ARF*, and 12 *ERF* (*Ethylene Response Factor*) genes was analysed (Fig. 9). While most of the genes showed similar expression in 5-d-old wild-type and transgenic line seedlings, there was a clear down-regulation of the tomato homologue of *Arabidopsis ARF2* (SGN-U314233) and conversely a significant up-regulation of transcript levels for the tomato homologue of *ARF8* (SGN-U327976) (Fig. 9A). The expression of *IAA29* (SGN-U320261) and *Pti4*





**Fig. 7.** Hook formation in *AS-IAA3* lines upon ethylene treatment. (A) Assessment of different grades of hook formation in etiolated tomato seedlings treated with different concentrations of ethylene (0–1  $\mu\text{l l}^{-1}$ ). Four stages have been defined corresponding to minimal exaggerated hook with a curvature angle lower than  $180^\circ$  (stage 1) to a maximal exaggerated hook with a curvature angle higher than  $360^\circ$  (stage 4). (B–E) Proportion of wild-type (black columns) and *AS-IAA3* (grey columns) plants corresponding to the four stages of hook formation upon treatment with 1  $\mu\text{l l}^{-1}$  1-MCP for 16 h (B), air (C), or 0.1 (D) and 1  $\mu\text{l l}^{-1}$  exogenous ethylene (E).

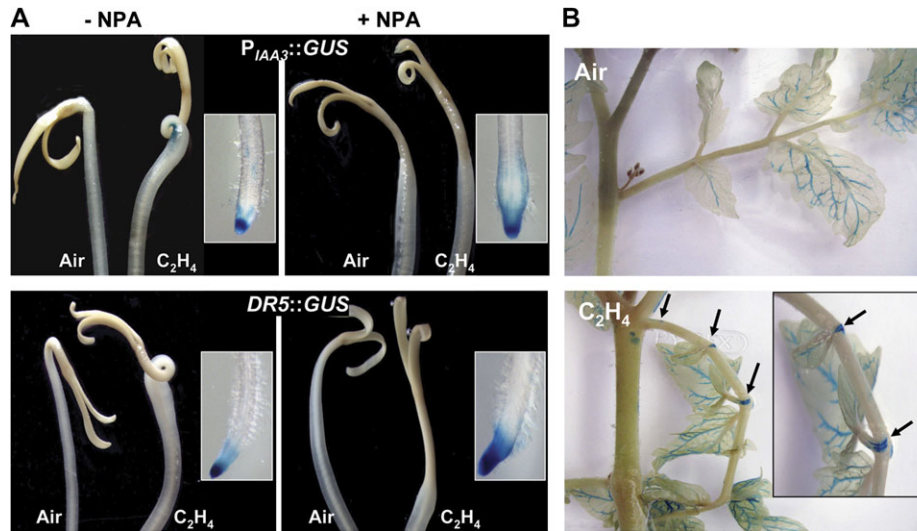
(SGN-U317071), a tomato *ERF* gene, were also significantly up-regulated in the transgenic lines (Fig. 9B, C), indicating that down-regulation of *Sl-IAA3* alters the expression of specific auxin and ethylene transcriptional mediators. In *Arabidopsis*, *Hookless1* (*At-HLS1*) is a key regulator of apical hook formation and the *hls1* mutant showed no differential growth in the apical region of the hypocotyl even after ethylene treatment (Lehman *et al.*, 1996). Notably, accumulation of transcripts of the tomato *Hookless* gene (*Sl-HLS*) was not altered in antisense lines (Fig. 9D).

## Discussion

Aux/IAA proteins are critical components of the auxin response. In *Arabidopsis*, dominant gain-of-function mutations in individual *Aux/IAAs* have provided telling insights into the roles played by the various family members in eliciting specific auxin responses. It is shown here that *Sl-IAA3*, a tomato Aux/IAA, is an integral component of both auxin and ethylene response pathways. Indeed, transcripts for the gene accumulate in response to both hormones, and its down-regulation results in auxin- and ethylene-related

phenotypes. Phenotypic responses to *Sl-IAA3* down-regulation include alterations to the classical auxin-regulated processes of apical dominance and hypocotyl elongation, and to typical ethylene responses such as apical hook formation in etiolated seedlings and leaf epinasty in light-grown plants.

*Sl-IAA3* and a number of other partial tomato *Aux/IAA* clones were initially isolated from fruit tissues. The *Sl-IAA3* gene has strong sequence and structural similarities with its putative *Arabidopsis* orthologues, *At-IAA4* and *At-IAA3*. An *Arabidopsis At-IAA4* mutant with an insertion in the first exon shows no obvious growth phenotype (Overvoorde *et al.*, 2005). In fact, although loss-of-function mutations have been identified in *Arabidopsis* for several *Aux/IAA* genes, the only phenotypes reported are subtle changes in plants mutated in one of the putative orthologues of tomato *Sl-IAA3*, *SHY2/IAA3* (Tian and Reed, 1999). Double or triple mutants of closely related *Aux/IAA* genes, such as *iaa8-1/iaa9-1* or *iaa5-1/iaa6-1/iaa19-1* also exhibit wild-type phenotypes, indicating extensive functional redundancy among *Arabidopsis* *Aux/IAA* family members (Overvoorde *et al.*, 2005). It has previously been shown that down-regulation of a tomato *Aux/IAA* gene, *Sl-IAA9*, resulted in altered leaf architecture and parthenocarpic fruit, consistent



**Fig. 8.** Expression of  $P_{IAA3}::GUS$  is associated with differential growth during hook formation and leaf epinastic response. (A) Tissue-specific expression of  $P_{IAA3}::GUS$  and  $DR5::GUS$  in etiolated seedlings.  $P_{IAA3}::GUS$  and  $DR5::GUS$  seedlings were dark-grown for 5 d and then treated for 48 h with air or  $10 \mu\text{l l}^{-1}$  of ethylene in absence (left panel) or presence of NPA (right panel). The upper-panel shows the ethylene-dependent GUS staining in the apical hook of  $P_{IAA3}::GUS$  tomato plants. The lower-panel shows GUS staining in the  $DR5::GUS$ -transformed plants used for detection of active auxin signalling in the hook. Inserts correspond to the expression of  $P_{IAA3}::GUS$  and  $DR5::GUS$  in the root caps following ethylene treatment. (B) Expression of  $P_{IAA3}::GUS$  in epinastic petioles. Six-week-old light-grown plants were placed in airtight chambers for 16 h in the absence (upper-panel) or presence (lower-panel) of  $50 \mu\text{l l}^{-1}$  of ethylene. The arrows indicate the expression of GUS in the leaf nodes of the petiole. The images are representative of at least three independent experiments with  $n > 30$  seedlings per experiment.

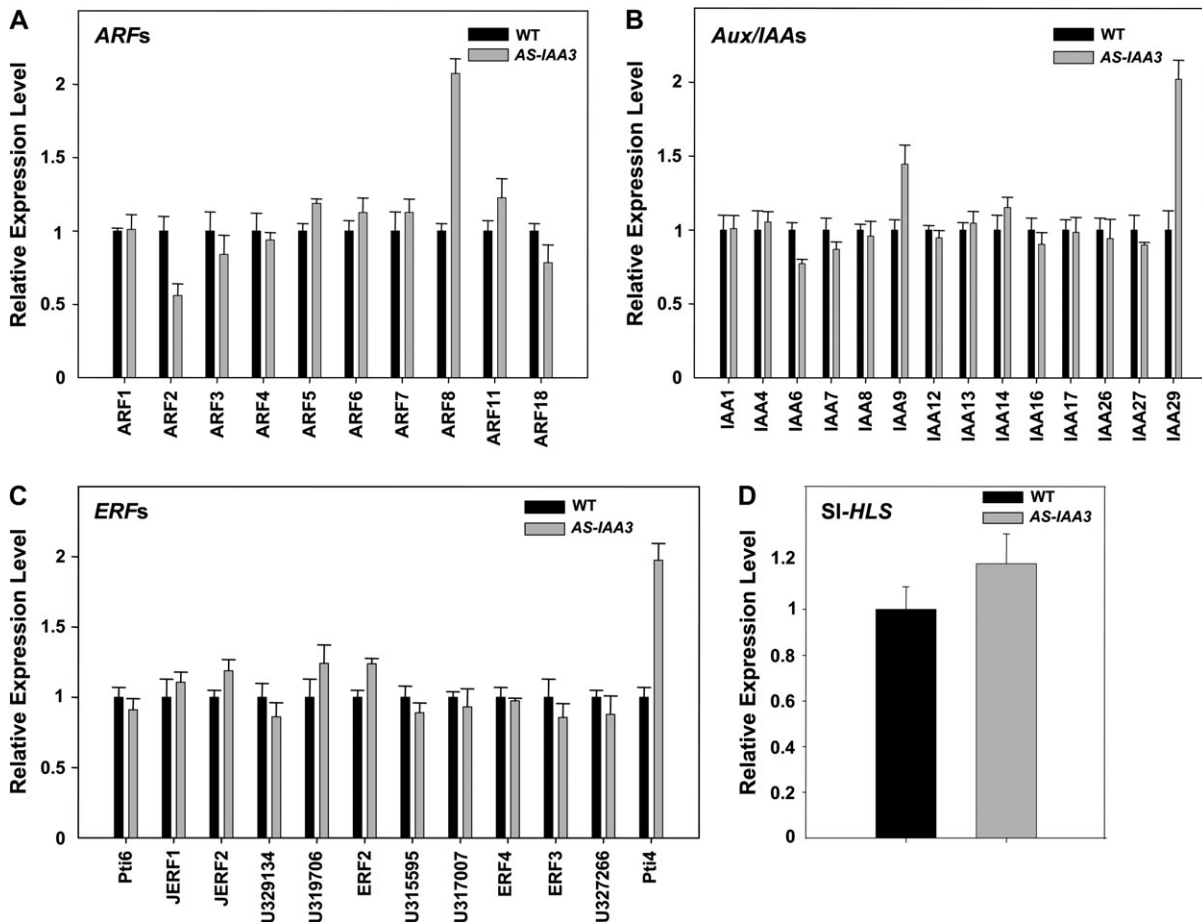
with a pivotal role for auxin in tomato fruit set and leaf morphogenesis (Wang *et al.*, 2005). In the present study, it is shown that the down-regulation of *Sl-IAA3* (*AS-IAA3*) also leads to well-defined phenotypes in transgenic tomato lines. The possibility that the observed changes might result from a lack of specificity of the antisense strategy was ruled out by verifying that the expression of closely related *Aux/IAA* genes was not altered in the *AS-IAA3* transgenic lines. The sequence homology rule predicts that *IAA3* antisense would primarily target *IAA1*, *IAA4*, and *IAA17* among all members of the *Aux/IAA* gene family. However, none of the best potential *Aux/IAA* targets displayed detectable change in transcript accumulation in the *AS-IAA3* lines (Fig. 9). Moreover, *ARF2* which showed down-regulation in the antisense lines displayed an extremely poor sequence match with *IAA3*. The present data strongly support the hypothesis that different members of the *Aux/IAA* family are involved in distinct developmental processes. This is also supported by the work of Kloosterman *et al.* (2006) who showed that suppression of *St-IAA2* in potato results in distinctive phenotypes, including increased plant height, petiole hyponasty, and curvature of growing leaf primordia in the shoot apex.

#### *Sl-IAA3* mediates auxin-dependent gene transcription and auxin-associated phenotypes

*Aux/IAA* genes were originally identified based on their rapid induction by auxin in etiolated soybean (*Glycine max*)

and pea (*Pisum sativum*) tissues (Walker and Key, 1982; Theologis *et al.*, 1985). Many *Arabidopsis* auxin-responsive genes contain the canonical auxin response elements (*AuxRE*), TGTCTC or GAGACA, in their promoters (Guilfoyle and Hagen, 2007). The present *in silico* search led to the identification of two degenerate *AuxRE* elements in the *Sl-IAA3* promoter that may be responsible for the auxin responsiveness observed in this study (Figs 1, 3).

*Sl-IAA3* transcript levels varied dramatically among the different tomato tissues, and analyses of tomato  $P_{IAA3}::GUS$  lines revealed that basal levels of expression were spatially restricted within organs. In the root, *Sl-IAA3*-driven *GUS* expression was restricted to the root cap and lateral root meristems, in the leaves to the vasculature, and in the fruit to a narrow band defining the junction between placenta and pericarp. This well-defined tissue-specific expression pattern was abolished by exogenous auxin treatment leading to GUS staining throughout the whole fruit pericarp and leaf and root tissues. While the auxin responsiveness is in agreement with previous data (Jones *et al.*, 2002), the expression pattern of *Sl-IAA3* in the hook differed from that of the artificial auxin-responsive promoter, *DR5*, suggesting that a combination of promoter elements contributes to the precise tissue-specific pattern of *Sl-IAA3* expression. Because the expression of  $P_{IAA3}::GUS$  and  $DR5::GUS$  gave similar staining in the root tips but not in the apical hook, the ethylene-induced expression of *Sl-IAA3* in the inner side of the apical hook cannot be ascribed to increased levels of auxin only. Nevertheless, auxin is also



**Fig. 9.** Impact of SI-*IAA3* down-regulation on the expression of auxin and ethylene response genes. The expression of members of the *ARF* (A), *Aux/IAA* (B), and *ERF* (C) gene families of transcription factors as well as the *SI-HLS* gene (D) was assessed by qRT-PCR in 5-d-old dark-grown wild-type (WT) and *AS-IAA3* etiolated seedlings. Primers used are listed in Table S2 in Supplementary data available at *JXB* online. Relative expression level on the y-axis refers to the fold difference in expression of each gene relative to that in WT seedlings taken as reference tissues. The data correspond to mean values of three replicates  $\pm$  standard error.

contributing to both the apical hook formation and the associated *SI-IAA3* expression as suggested by the abolished hook and *SI-IAA3* expression in NPA-treated seedlings (Fig. 8A).

In *Arabidopsis*, *Aux/IAA* gain-of-function mutations that stabilize the *Aux/IAA* proteins (Reed, 2001) are, in most cases, associated with phenotypes reminiscent of reduced auxin responsiveness (Nagpal *et al.*, 2000; Rogg *et al.*, 2001; Tian *et al.*, 2002). Since *Arabidopsis* *Aux/IAAs* have been shown to repress *DR5*-driven transcription (Ulmasov *et al.*, 1997; Tiwari *et al.*, 2001), it was hypothesized that the down-regulation of *SI-IAA3* would lead to enhanced auxin responses. Unexpectedly, the *AS-IAA3* lines have many phenotypes consistent with reduced auxin sensitivity. This suggests that, even though *SI-IAA3* has the capacity to repress auxin-responsive gene expression in protoplasts (see Fig. S2 in Supplementary data available at *JXB* online), *in planta* the protein seems to act as a positive regulator of auxin responses. One possible explanation for this apparent discrepancy is that *in planta* *SI-IAA3* may repress the expression of negative regulators of auxin responses. Two *ARFs* (*ARF2* and *ARF8*) and one *Aux/IAA* (*IAA29*) that

were differentially regulated in the *AS-IAA3* lines, may contribute to the reduced auxin-responsiveness in *AS-IAA3*.

#### *Ethylene-related expression and phenotypes*

It has been shown previously that the accumulation of *SI-IAA3* transcripts is enhanced by ethylene treatment in MG fruit (Jones *et al.*, 2002). In the present work, it was shown that *SI-IAA3* transcript accumulation mimicked both the dose-response and the time-course gradient of the well-characterized ethylene-responsive gene, *E8* (Lincoln *et al.*, 1987). Importantly, *SI-IAA3* had an ethylene-dependent, ripening-associated expression pattern that was revealed by a sharp reduction in *SI-IAA3* transcripts when Br fruit were treated with the ethylene inhibitor, 1-MCP. Moreover, accumulation of *SI-IAA3* transcripts was dramatically reduced in the tomato ripening mutants (*rin*, *nor*, and *Nr*) that lack the capacity to respond to autocatalytic ethylene and to undergo normal ethylene-regulated ripening processes (Giovannoni, 2007). Given that *SI-IAA3* is a presumptive auxin response regulator, these results strongly suggest that one of the roles for ethylene during climacteric fruit

ripening is the modification of auxin responsiveness in the ripening fruit. Whereas these observations suggested that down-regulation of SI-*IAA3* in transgenic lines may have resulted in a fruit ripening phenotype, none of the ripening features examined in the present study differed between antisense and wild-type lines (timing of the onset of ripening, levels of climacteric ethylene production, and pigment accumulation). Though it cannot be excluded that other ripening aspects may have been altered, the present data suggest that either the SI-*IAA3* is functionally redundant in fruit tissues or that residual levels of SI-*IAA3* were sufficient to drive the ripening processes that rely on the *IAA3* protein.

Two other phenotypes in the *AS-IAA3* lines, the exaggerated apical hook formation and reduced epinasty, indicated that SI-*IAA3* is important for physiological responses involving ethylene. Apical hook formation in etiolated seedlings forms the classical ethylene triple response together with reduced hypocotyl and root elongation (Bleecker *et al.*, 1988; Ecker, 1995). The involvement of both ethylene and auxin in this differential cell elongation has been demonstrated through the analysis of ethylene- and auxin-signalling mutants that are altered in the process of hook formation. In *Arabidopsis*, mutants that are defective in ethylene perception and signalling, such as *etr1-1*, *ein2*, and *ein3*, do not form an exaggerated hook in response to ethylene treatment. By contrast, the constitutive ethylene response mutant, *ctr1*, develops an exaggerated hook in the absence of ethylene (Guzman and Ecker, 1990; Kieber *et al.*, 1993). Auxin promotes hypocotyl cell elongation and is unequally distributed in the apical hook (Schwark and Schierle, 1992). The *axr1* mutant, which is altered in auxin responses, lacks a normal apical hook and the inhibition of auxin transport disrupts formation of the hook (Lincoln *et al.*, 1990). Clearly, the apical hook is established and maintained by interplay between ethylene and auxin. The exaggerated apical hook phenotype in the *AS-IAA3* lines provides direct evidence that SI-*IAA3* is important in physiological processes that rely on both auxin and ethylene. Active ethylene signalling is essential for the appearance of the exaggerated hook phenotype since blocking ethylene perception with 1-MCP prevents hook formation in the *AS-IAA3* plants. The other aspects of the triple response, namely exaggerated hypocotyl elongation and the thickening and shortening of roots, were not altered in the *AS-IAA3* lines, indicating that SI-*IAA3* is specifically involved in differential growth processes. Ethylene treatment of etiolated seedlings increased the P<sub>IAA3::GUS</sub> expression in the inner surface of the apical hook (Fig. 8). Likewise, P<sub>IAA3::GUS</sub> staining was also clearly delimited in epinastic petioles, suggesting that the ethylene-induced gradient of SI-*IAA3* expression is involved in the differential growth associated with both apical hook formation and the petiole epinastic response. However, whereas down-regulation of SI-*IAA3* resulted in an exaggerated ethylene-response of etiolated seedlings, it conferred reduced ethylene sensitivity in light-grown plants. The ability of ethylene to induce opposite growth responses in the dark and in the light have

been described previously (Smalle *et al.*, 1997) and could explain the seemingly contradictory phenotypes displayed by *AS-IAA3* plants in the seedlings and petioles. In keeping with this complex regulation of SI-*IAA3*, the ethylene-induced expression of this gene in light-grown plants was found in the upper side of epinastic petioles, opposite to the pattern observed in the hook of etiolated seedlings.

*Arabidopsis* plants with a loss-of-function mutation in *HLS1* are unable to form an apical hook even in the presence of ethylene (Lehman *et al.*, 1996). A mutation that reverses the *hls1* phenotype has been identified and was found to encode the auxin-response factor, *ARF2* (Li *et al.*, 2004). Interestingly, the putative tomato orthologue of *ARF2* is also down-regulated in the *AS-IAA3* lines, suggesting that the process of hook formation may require an interplay between *HLS1*, *IAA3*, and *ARF2*. The previous model proposed by Li *et al.* (2004) postulates that *ARF2* acts downstream of *HLS1*. It was shown here that the expression of SI-*HLS* is not altered in the *AS-IAA3* plants, suggesting that SI-*IAA3* and SI-*HLS* may act in parallel pathways both of them involving *ARF2* as a downstream component. On the other hand, it cannot be ruled out that SI-*HLS* may also act upstream of SI-*IAA3*.

The altered apical dominance found in the *AS-IAA3* lines was also observed in the previously described antisense SI-*IAA9* plants (Wang *et al.*, 2005). Unlike SI-*IAA9*, however, SI-*IAA3* has distinct roles in ethylene-related responses. By revealing that a number of transcription factors from the ARF (SI-*ARF2* and SI-*ARF8*), Aux/IAA (SI-*IAA29*), and ERF (*Ethylene Response Factor Pti4*) families are under direct or indirect regulation by SI-*IAA3*, the present study provides insights into how SI-*IAA3* functions to bring about some of the observed phenotypes. While continued effort is required to gain a more complete understanding of the hormonal dialogue mediated by SI-*IAA3*, the data described here confirm that Aux/IAA proteins have both distinct and overlapping roles and reveal that these proteins can be integral auxin as well as ethylene response regulators.

## Supplementary data

**Table S1.** Percentage identity of the antisense region relative to the other members of tomato *Aux/IAAs* family.

**Table S2.** Auxin- and ethylene-response genes.

**Fig. S1.** Subcellular localization of SI-*IAA3* protein.

**Fig. S2.** SI-*IAA3* protein represses the *in vivo* activity of *DR5*.

**Fig. S3.** Ethylene regulation of SI-*IAA3*.

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