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, Sl-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 Sl-IAA3 gene encodes a nuclear-targeted protein that can repress transcription from auxin-responsive promoters. Sl-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 Sl-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 Sl-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 unraveling 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
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 auxin-dependent proteolytic signal transduction system (Dharmasiri and Estelle, 2004). At the centre of the signalling cascade is the ubiquitin–ligase complex; auxin binding to Transport Inhibitor Response1/TIR1 (or its paralogues, 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 family members can be regulated by ethylene (Jones et al., 2002). Here, it is shown that Sl-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 μmol m⁻² 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⁻¹ thiamine, 0.25 mg l⁻¹ nicotinic acid, and 0.5 mg l⁻¹ pyridoxine), 1.5% (w/v) sucrose, and 0.8% (w/v) agar, pH 5.9.

Plant transformation

To generate AS-IAA3 transgenic plants, the forward 5'-AACAAAGCTCGCTTGCCCACC-3’ and reverse 5’-CATCCAAGGATCCATCAATC-3’ primers were used to amplify a partial Sl-IAA3 clone (antisense construct in Fig. 1). The percentage sequence identity of the amplified fragment relative to the other members of the tomato Aux/IAA 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 F₃ or later generations.

Isolation of the Sl-IAA3 genomic clone

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

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**Fig. 1.** Genomic structure of the tomato Sl-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 β-glucuronidase (GUS) reporter gene in the pLP100 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 (0.5×10⁶) were transfected either with 10 μg 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 μg 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 μM), GFP expression was analysed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA, USA) as indicated by 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 μM 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 μl 1⁻¹ 1-methyl cyclopentene (1-MCP), the ethylene perception inhibitor (Agrofresh, USA) and then incubated in presence or absence of 20 μM IAA. For GUS analysis, 21-d-old tomato seedlings and sections of mature green (MG) fruit (Vibratrom, Leica VT 1000 S, Vetzlar, Germany) were incubated for 2 h with or without 20 μM IAA. MG and breaker (Br) fruit were treated for 5 h with 50 μl 1⁻¹ ethylene and 1-MCP (1 μl 1⁻¹) for 16 h, respectively. Ethylene treatment (10 μl 1⁻¹) was performed on 5-d-old etiolated PIAA5::GUS, DR5::GUS transformed seedlings. For the epinastic response, light-grown plants were treated with ethylene (50 μl 1⁻¹) for 16 h.

For histochemical GUS analysis, PIAA5::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 (TGTTCNC) at positions –216 and –175, and an ethylene-response element ERE (ATTTCAGA) 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
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 μl l⁻¹) resulted in an almost 11-fold increase in Sl-IAA3 transcript accumulation (Fig. 2D). Conversely, in Br-stage fruit, an overnight treatment with 1-MCP (1 μl l⁻¹) led to a 10-fold reduction in Sl-IAA3 transcripts (Fig. 2E). Given that Sl-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 μM IAA) treatment (Fig. 3A). In tobacco BY2 protoplast transfection assays, SI-IAA3 promoter (1668 bp)-driven GFP levels increased 4-fold after auxin treatment (50 μM 2,4-D) (Fig. 3B). As auxin is known to stimulate ethylene
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 μl l⁻¹ 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_{IAA3}::GFP and incubated in the presence or absence of 2,4-D (50 μM). 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.) ± 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_{IAA3}::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 μM 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 μM IAA. The data are representative of at least three independent experiments with n > 20 seedlings examined per experiment.

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 μM IAA for 2 h) seedlings in presence or absence of 1 μl l⁻¹ 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_{IAA3}::GFP and incubated in the presence or absence of 2,4-D (50 μM). 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.) ± 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_{IAA3}::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 μM 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 μM IAA. The data are representative of at least three independent experiments with n > 20 seedlings examined per experiment.

To gain further insight into SI-IAA3 expression, the SI-IAA3 promoter was fused to the GUS reporter gene (P_{IAA3}::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 μM 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
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 μM 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 polycotyledons was 25% and 20% in AS-IAA3-1 and AS-IAA3-2 lines, respectively, compared with only 5% in the wild type (Fig. 5D).

Sl-IAA3 suppression results in modified ethylene sensitivity

The ethylene responsiveness of Sl-AA3 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 Sl-IAA3 on this epinastic response, light-grown wild-type and AS-IAA3 tomato plantlets were treated with exogenous ethylene (50 μl l⁻¹) for 16 h. The subsequent angles of the petioles to the main stem were...
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 μl l⁻¹) 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 μl l⁻¹ 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 Sl-IAA3 in apical hook formation and epinastic response, the expression pattern of this gene was analysed in tomato lines expressing the PIAA3::GUS construct. In the absence of exogenous ethylene treatment, there was minimal GUS staining associated with the apical hook in dark-grown wild-type PIAA3 lines. By contrast, after 48 h ethylene treatment (10 μl l⁻¹), 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 Sl-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. 5. Auxin-associated phenotypes of Sl-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 μM 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 ± standard error (n > 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 ± standard error of 40 plants.
hook formation and simultaneously suppressed Sl-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 the higher ethylene-induced expression of Sl-IAA3 in the inner side of the apical hook could not be ascribed only to increased auxin levels (Fig. 8A).

The role of Sl-IAA3 in ethylene-induced differential growth was further investigated by assessing the expression of Sl-IAA3 in light-grown epinastic tissues. Ethylene treatment of epinastic petioles led to PIAA3::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 Sl-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 Sl-IAA3 specifically impacts on the expression of selected auxin and ethylene transcription factors**

An Sl-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 Sl-IAA3 being a transcriptional regulator. To address the ability of the Sl-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 Sl-IAA3 in co-transfection assays, strongly reduced this auxin induction. These data indicate that Sl-IAA3 acts in protoplast as a repressor of auxin-dependent transcription and is consistent with Sl-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...
(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
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 Sl-IAA2 in potato results in distinctive phenotypes, including increased plant height, petiole hyponasty, and curvature of growing leaf primordia in the shoot apex.

**SI-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).

SI-IAA3 transcript levels varied dramatically among the different tomato tissues, and analyses of tomato PI4A3::GUS lines revealed that basal levels of expression were spatially restricted within organs. In the root, SI-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 SI-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 SI-IAA3 expression. Because the expression of PI4A3::GUS and *DR5::GUS* gave similar staining in the root tips but not in the apical hook, the ethylene-induced expression of SI-IAA3 in the inner side of the apical hook cannot be ascribed to increased levels of auxin only. Nevertheless, auxin is also
contributing to both the apical hook formation and the associated Sl-IAA3 expression as suggested by the abolished hook and Sl-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 Sl-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 Sl-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 Sl-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 Sl-IAA3 transcripts is enhanced by ethylene treatment in MG fruit (Jones et al., 2002). In the present work, it was shown that Sl-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, Sl-IAA3 had an ethylene-dependent, ripening-associated expression pattern that was revealed by a sharp reduction in Sl-IAA3 transcripts when Br fruit were treated with the ethylene inhibitor, 1-MCP. Moreover, accumulation of Sl-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 Sl-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 Sl-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 Sl-IAA3 is functionally redundant in fruit tissues or that residual levels of Sl-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 Sl-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 ctr1-1, 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 Sl-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 Sl-IAA3 is specifically involved in differential growth processes. Ethylene treatment of etiolated seedlings increased the P_{IAA3::GUS} expression in the inner surface of the apical hook (Fig. 8). Likewise, P_{IAA3::GUS} staining was also clearly delimited in epinastic petioles, suggesting that the ethylene-induced gradient of Sl-IAA3 expression is involved in the differential growth associated with both apical hook formation and the petiole epinastic response. However, whereas down-regulation of Sl-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 Sl-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 Sl-HLS is not altered in the AS-IAA3 plants, suggesting that Sl-IAA3 and Sl-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 Sl-HLS may also act upstream of Sl-IAA3.

The altered apical dominance found in the AS-IAA3 lines was also observed in the previously described antisense Sl-IAA9 plants (Wang et al., 2005). Unlike Sl-IAA9, however, Sl-IAA3 has distinct roles in ethylene-related responses. By revealing that a number of transcription factors from the ARF (Sl-ARF2 and Sl-ARF8), Aux/IAA (Sl-IAA29), and ERF (Ethylene Response Factor Pot4) families are under direct or indirect regulation by Sl-IAA3, the present study provides insights into how Sl-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 Sl-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/IAA family.

Table S2. Auxin- and ethylene-response genes.

Fig. S1. Subcellular localization of Sl-IAA3 protein.

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

Fig. S3. Ethylene regulation of Sl-IAA3.

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