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RESEARCH PAPER

# Down-regulation of a single auxin efflux transport protein in tomato induces precocious fruit development

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## Abstract

**The PIN-FORMED (PIN) auxin efflux transport protein family has been well characterized in the model plant *Arabidopsis thaliana*, where these proteins are crucial for auxin regulation of various aspects of plant development. Recent evidence indicates that PIN proteins may play a role in fruit set and early fruit development in tomato (*Solanum lycopersicum*), but functional analyses of *PIN*-silenced plants failed to corroborate this hypothesis. Here it is demonstrated that silencing specifically the tomato *SlPIN4* gene, which is predominantly expressed in tomato flower bud and young developing fruit, leads to parthenocarpic fruits due to precocious fruit development before fertilization. This phenotype was associated with only slight modifications of auxin homeostasis at early stages of flower bud development and with minor alterations of *ARF* and *Aux/IAA* gene expression. However, microarray transcriptome analysis and large-scale quantitative RT-PCR profiling of transcription factors in developing flower bud and fruit highlighted differentially expressed regulatory genes, which are potential targets for auxin control of fruit set and development in tomato. In conclusion, this work provides clear evidence that the tomato PIN protein SlPIN4 plays a major role in auxin regulation of tomato fruit set, possibly by preventing precocious fruit development in the absence of pollination, and further gives new insights into the target genes involved in fruit set.**

**Key words:** Auxin efflux transport protein (PIN), CRABS-CLAW, fruit set, MADS-BOX, parthenocarpy, tomato (Solanum lycopersicum), transcription factor.

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All plant material described in the manuscript can be made available upon request, either as seeds from homozygous plants or as explants (for the obligatory parthenocarpic plants), so that the transgenic plants and their phenotypes can be tested in independent conditions.

## Introduction

The formation of seedless or parthenocarpic fruit is a desired trait in several crop species producing hard seeds (e.g. banana and grape) as well as in species where the pollination/fertilization process is strongly dependent on environmental conditions (tomato). Seventy years ago, it was demonstrated that exogenous application of auxins, cytokinins, and gibberellins (GAs) to tomato flowers leads to the formation of parthenocarpic fruits. It was further suggested that this particular fruit development is due to the deregulation of the hormonal balance within the ovary, which substitutes for pollination/fertilization (Schwabe and Mills, 1981; Gorguet *et al.*, 2005). The manipulation of auxin synthesis/signalling within tomato ovaries later emphasized the crucial role played by auxin in fruit set and parthenocarpy (Ficcadenti *et al.*, 1999; Carmi *et al.*, 2003; Pandolfini *et al.*, 2007). More recently, the fundamental discoveries on auxin signalling in *Arabidopsis thaliana* (reviewed in Chapman and Estelle, 2009; Kieffer *et al.*, 2009) paved the way for deciphering the mechanisms involved in auxin regulation of fruit set (Wang *et al.*, 2005; Goetz *et al.*, 2007; de Jong *et al.*, 2009*a*; Wang *et al.*, 2009; Ren *et al.*, 2011).

The current scheme of ovary growth regulation involves an Aux/IAA (auxin/indole-3-acetic acid) complex which represses downstream auxin-responsive genes up to fertilization (Swain and Koltunov, 2006). Upon pollination and fertilization, an increase in auxin levels in the fertilized ovules/ovary would activate Aux/IAA degradation via the ubiquitin–proteasome pathway (Dharmasiri *et al.*, 2005; Mockaitis and Estelle, 2008). Consequently, free auxin response factor (ARF) proteins would regulate auxin-responsive genes, thus triggering fruit set (Swain and Koltunov, 2006; de Jong *et al.*, 2009*a*). According to this model, alterations at any step of the auxin signalling cascade, either by changing the auxin level in the ovule area, by affecting the Aux/IAA–ARF repressive complex, or by modifying the expression of target genes may dissociate fruit development from ovule fertilization and trigger parthenocarpic fruit development. Although this scheme remains to be fully validated, it is consistent with the development of parthenocarpic fruit when the auxin level in the ovules/ovary is increased by the overexpression of genes of auxin biosynthesis (Ficcadenti *et al.*, 1999; Pandolfini *et al.*, 2002; Carmi *et al.*, 2003) or by application of auxin or auxin efflux transport inhibitors (Beyer and Quebedeaux, 1974; Serrani *et al.*, 2010). A recent work using a *DR5rev::mRFPer* synthetic reporter described the distribution of auxin in ovary and fruit tissues and showed that the auxin signal was localized in the ovule before anthesis and close to the funiculus after anthesis (Pattison and Catala, 2012). Experimental data confimed the participation of several Aux/IAA and ARF proteins in fruit set in tomato, including SlAux/IAA9 (Wang *et al.*, 2005), SlARF7 (de Jong *et al.*, 2009*b*), and an orthologue of AtARF8 (Goetz *et al.*, 2007). Besides recent data indicate that the *Aucsia* silencing-induced parthenocarpy, which is connected to auxin accumulation in reproductive organs, is not linked to the down-regulation of SlAux/IAA9 and SlARF8 (Molesini *et al.*, 2009*a*). Up to now, few auxin-regulated target genes responsible for the initiation of fruit development have been identified. Profound changes in the expression of auxin, GA, and ethylene signalling-related genes have, been described in the ovaries developing into parthenocarpic fruits (Vriezen *et al.*, 2008; Wang *et al.*, 2009; de Jong *et al.*, 2011). In addition, three MADS-BOX genes have been identified as potential candidate genes in the control of fruit set in tomato (Ampomah-Dwamena *et al.*, 2002; Wang *et al.*, 2009).

In *Arabidopsis*, polar auxin transport plays a crucial role in auxin signalling (Zazimalova *et al.*, 2007). It is responsible for the local distribution of auxin in plant tissues, due to the asymmetric location of auxin influx and efflux transporters (Kleine-Vehn *et al.*, 2006; Petrásek and Friml, 2009). Auxin efflux transport is achieved by the PIN-FORMED (PIN) protein family made up of eight members in *Arabidopsis* (Paponov *et al.*, 2005). PIN proteins are involved in various developmental processes including embryogenesis, shoot and root morphogenesis, gravitropism, and phototropism (Vieten *et al.*, 2007; Petrásek and Friml, 2009). In tomato, the application of auxin efflux transport inhibitors leads to parthenocarpic fruit development (Serrani *et al.*, 2010) while fruit parthenocarpy observed in the *Aucsia*-silenced plants is associated with an alteration of polar auxin transport (Molesini *et al.*, 2009*a*), therefore suggesting the implication of auxin transport in fruit set.

Despite the crucial role of auxin signalling during fruit development in tomato and the likely implication of auxin efflux transport in this process, scarce data were available until very recently concerning the implication of PIN in fruit set and development in tomato (Lemaire-Chamley *et al.*, 2005; Kharshiing *et al.*, 2010; Nishio *et al.*, 2010; Pattison and Catala, 2012). The aim of the present study was to unravel the role of the SlPIN4 protein, which is highly expressed in developing ovary and fruit in tomato. Towards this end, RNA interference (RNAi) transgenic lines specifically silenced for the *SlPIN4* gene were generated. Partial to full parthenocarpic fruit phenotypes were observed, thereby suggesting the involvement of the *SlPIN4* gene in tomato fruit set. Transcriptome analyses of wild-type (WT) and transgenic ovaries by microarray and targeted quantitative RT-PCR (qRT-PCR) transcription factor (TF) profiling allowed the identification of downstream genes involved in auxin-dependent fruit set regulation in tomato and provided new insights into the fruit set process.

## Materials and methods

### *Plant material*

Tomato plants used for expression profile analyses (*Solanum lycopersicum* Ailsa Craig) were grown in a growth chamber as previously described (Mounet *et al.*, 2009). Transgenic plants and the corresponding WT [*Solanum lycopersicum* var. *cerasiformae* 'West Virginia 106' (WVA 106)] were grown in a greenhouse (Alhagdow *et al.*, 2007). The RNAi-mediated silencing of the tomato *SlPIN4* gene (AB508932/HQ127078) was performed by stable transformation (Alhagdow *et al.*, 2007) using a 475bp DNA fragment located in the 3'-untranslated region of the cDNA amplified by the specific primers attB1SlPIN4 (AAAAAGCAGGCTGCCAAGTGAAGAAAGGAGGA) and attB2SlPIN4 (AGAAAGCTGGGTGGAAGTAAACAACTAGCAAACC), introduced into the destination vector pK7GWIWG2(1) as an inverted repeat under the control of the 35S promoter (Karimi *et al.*, 2007). Primary transformants were further checked for ploidy level by flow cytometry. Ten independent diploid

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primary transformants ($T_0$) with single-copy T-DNA insertion were selected and grown in a greenhouse. Obligate parthenocarpic lines were maintained as cuttings, and homozygous lines were generated for two facultative parthenocarpic primary transformants (L-2 and L-21). Seeds of homozygous lines L-2 and L-21 were selected by segregation test in the presence of kanamycin (300 μg $l^{-1}$).

### *Phenotypic analyses of $P_{35S}$:SlPIN4$^{RNAi}$ lines*

Flower development in WT and transgenic plants was investigated by measuring flower length every day from 1 mm bud to anthesis stage. Flower emasculation was performed on 10 mm closed flowers. For cytological analyses of flower buds, samples were harvested at 4 mm, 8 mm, and anthesis stages from $T_0$ plants, and processed as previously described (Bereterbide *et al*., 2002). Mean carpel area was measured by using ImagePro-Plus software (Media Cybernetics) on at least 10 carpel transverse sections. The effects of IAA on seedling germination was tested on WT, and L-2 and L-21 homozygous lines grown *in vitro*. Sterilized seeds were sown on MS (1.1 g $l^{-1}$) agar (5 g $l^{-1}$) medium containing 7.5 g $l^{-1}$ sucrose (w/v), pH 5.8 ± 0.5 μM IAA (Sigma). Phenotypes were observed on fifty 12-day-old seedlings.

### *Analyses of auxin content*

Auxin analyses were performed on three biological replicates of flower buds (4 mm and 8 mm) and ovaries at anthesis harvested on $T_0$ ($P_{35S}$:*SlPIN4*$^{RNAi}$ L-14) and homozygous $T_2$ plants ($P_{35S}$:*SlPIN4*$^{RNAi}$ L-2 and L-21). A 25 mg aliquot of fresh samples was extracted, purified, and analysed by gas chromatography–selected reaction monitoring–mass spectrometry as described previously (Edlund *et al*., 1995). Calculation of the isotopic dilution factors was based on the addition of 50 pg of [6-$^{13}$C]IAA $mg^{-1}$ tissue.

### *qRT-PCR analyses*

Expression profiles of *PIN* genes in WT tomato fruit (entire and separated tissues) and vegetative organs were performed on Ailsa Craig tomato plants. Samples collected from 15 plants were pooled and divided into three subsamples (technical replicates). Expression profiles in flower buds and ovaries of WT and $P_{35S}$:*SlPIN4*$^{RNAi}$ transgenic lines were performed on three biological replicates collected from three WVA 106 tomato plants ($T_0$ plants maintained as cuttings for $P_{35S}$:*SlPIN4*$^{RNAi}$ L-14 and homozygous $T_2$ plants for $P_{35S}$:*SlPIN4*$^{RNAi}$ L-2 and L-21). RNA extraction was performed on each subsample and each biological replicate, treated with DNase, and reverse transcribed as previously described (Mounet *et al*., 2009). The reverse transcription product was diluted 10-fold in water, and 2 μl were used in qRT-PCR (25 μl final volume) in the presence of 0.2 μM specific primers using the iQ-SYBR Green Supermix (Bio-Rad, Marne La Coquette, France) according to the manufacturer's instructions. qRT-PCR was performed on a CFX-96 (Bio-Rad). Data acquisition and analysis was done using Bio-Rad CFX manager software (version 1.1) using ΔΔCT normalization. The specific primers used are indicated in Supplementary Table S1 available at *JXB* online.

### *Microarray analysis*

The transcriptome of $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (L-2, L-21, and L-14) was compared with that of WT plants. The microarray experimental design consisted of two biological replicates with one dye swap for a total of four slides per $P_{35S}$:*SlPIN4*$^{RNAi}$ line/WT comparison. A 1 μg aliquot of DNA-free total RNA extracted from 6 mm flower buds was used to prepare the labelled probes as already described (Mounet *et al*., 2009). Hybridization on TOM2 oligonucleotide microarrays and scanning parameters were as described in Prudent *et al*. (2010).

Statistical analysis of microarray data was performed using the Bioconductor LIMMA package v2.15.16 (Smyth, 2005*a*). After background correction using the Normexp method (Ritchie *et al*., 2007), the data were normalized using the print-tip lowess (within-array normalization) and A-quantile (between-arrays normalization) functions (default parameters). Flagged spots were given a weight of 0.1 using the weight function. For the statistical analysis, a reference design analysis was performed (Smyth, 2005*b*) and a linear model with a coefficient for each of the three factors (L-2, L-21, and L-14) was fitted. The *P*-values resulting from the moderated *t*-test were corrected for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) adjustment. Genes were considered to be significantly differentially expressed if adjusted *P*-values were $<5\times10^{-2}$. Spots with low expression (A <9) were eliminated from further analysis, resulting in 1460 spots with a significant variation of expression. Genes that changed >1.5-fold in at least one $P_{35S}$:*SlPIN4*$^{RNAi}$ line when compared with the WT were considered as differentially expressed [Log2($P_{35S}$:*SlPIN4*$^{RNAi}$/WT] < –0.58 or > 0.58; Supplementary Table S3 at *JBX* online).

### *TF profiling*

TF profiling was performed on three biological replicates of 4 mm flower buds and 0 days post-anthesis (DPA) ovaries harvested from three plants of each genotype (WT, $T_0$ plants maintained by cutting in the case of $P_{35S}$:*SlPIN4*$^{RNAi}$ L-14, and homozygous $T_2$ plants in the case of $P_{35S}$:*SlPIN4*$^{RNAi}$ L-2). RNA extraction and DNase treatement were as previously described (Mounet *et al*., 2009). Control of RNA, reverse transcription, qRT-PCR, and collection of the raw data were performed as described in Rohrmann *et al.* (2011). TFs with a threshold cycle (CT) value <35 in two of the three replicates were considered as expressed in the sample considered. TF normalized expression ($\Delta CT=CT_{TF}-CT_{Control}$) was calculated as previously described, using for each independent run the mean value of the two replicates of the ubiquitin gene as a $CT_{Control}$ (Rohrmann *et al*., 2011). TFs displaying statistically altered expression in flower buds or in ovary were found via the use of analysis of variance (ANOVA) testing with FDR-corrected *P*-values (10%) implemented with the corresponding data (three genotypes×three biological replicates). The relative expression of the transformant lines was calculated as $2^{-\Delta\Delta CT}=2^{-(\text{mean}\Delta CTRNAi-\text{mean}\Delta CTWT)}$. TFs that changed >1.5-fold in at least one $P_{35S}$:*SlPIN4*$^{RNAi}$ line when compared with the WT were considered to be differentially expressed [Log2($P_{35S}$:*SlPIN4*$^{RNAi}$/WT] < –0.58 or > 0.58; Supplementary Table S4 at *JBX* online).

## Results

### *Characterization of the tomato PIN auxin efflux transport protein family*

Phylogenetic analysis of tomato (HQ127074–HQ127083, Supplementary Table S2 at *JXB* online) and *Arabidopsis* PIN proteins highlighted likely tomato orthologues for AtPIN1, AtPIN2, AtPIN6, and AtPIN8 (Fig. 1A). In agreement with the very recent study of Pattison and Catala (2012), six tomato PIN sequences were clustered with the group of *Arabidopsis* plasma membrane PIN proteins, composed of AtPIN1, AtPIN2, AtPIN3, AtPIN4, and AtPIN7 (Petrásek and Friml, 2009). Expression analysis revealed that only five of these putative plasma membrane *PIN* genes were expressed in tomato fruit, with *SlPIN4* being the most highly expressed (Fig. 1B). The SlPIN4 protein is close to AtPIN3, AtPIN7, and AtPIN4 (76, 75, and 73% amino acid sequence identity, respectively). The relative expression of *SlPIN4* increased durng flower development up to the anthesis stage and decreased during fruit development (Fig. 1C), and suggests its potential role in fruit set.

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The *SlPIN3* gene displayed a very similar pattern of expression during flower and fruit development but its expression was one-tenth that of *SlPIN4* in fruit at 0 DPA (Fig. 1B, 1C). Other *PIN* genes were characterized by little variation (*SlPIN1* and *SlPIN7*) or even a decline (*SlPIN9*) in expression during flower development. In agreement with published data (Nishio *et al*., 2010; Pattison and Catala, 2012), the expression of all *PIN* genes decreased during fruit development, except for *SlPIN7* whose expression increased at the onset of fruit ripening (Fig. 1C). The *SlPIN4* gene was expressed in all plant organs, with a higher expression in sepals, ovary, young leaves, and petals (Fig. 2). The other *PIN* genes were also expressed in the various plant organs analysed, except *SlPIN3*, which was mainly expressed in petals. As previously shown (Lemaire-Chamley *et al*., 2005; Pattison and Catala, 2012), *SlPIN4* gene expression increased gradually from the outer part of the fruit (exocarp) to the central part of the fruit (columella, locular tissue, Fig. 2).

### *Silencing of* SlPIN4 *leads to precocious ovary development and to parthenocarpic fruit*

Since the *SlPIN4* gene was the major *PIN* gene expressed in tomato ovary and young fruit, its role in fruit set was characterized. Recent attempts to alter fruit set or development by silencing *SlPIN4* were unsuccessfull (Pattison and Catala, 2012). However, in contrast to the study presented herein, which targeted specifically *SlPIN4*, at least two genes (*SlPIN3* and *SlPIN4*) and up to five *SlPIN* genes were significantly silenced in the lines studied by Pattison and Catala (2012). Considering the complexity of the regulation of fruit set through auxin transport in the ovary and from the apical shoot (Serrani *et al.*, 2010), it is likely that co-silencing multiple *SlPIN* genes in both ovary and stem may lead to complex and, possibly, opposing effects on fruit set. Therefore, in the study described herein, transgenic lines were generated in which *SlPIN4* was specifically RNAi silenced by targeting its 3'-untranslated region

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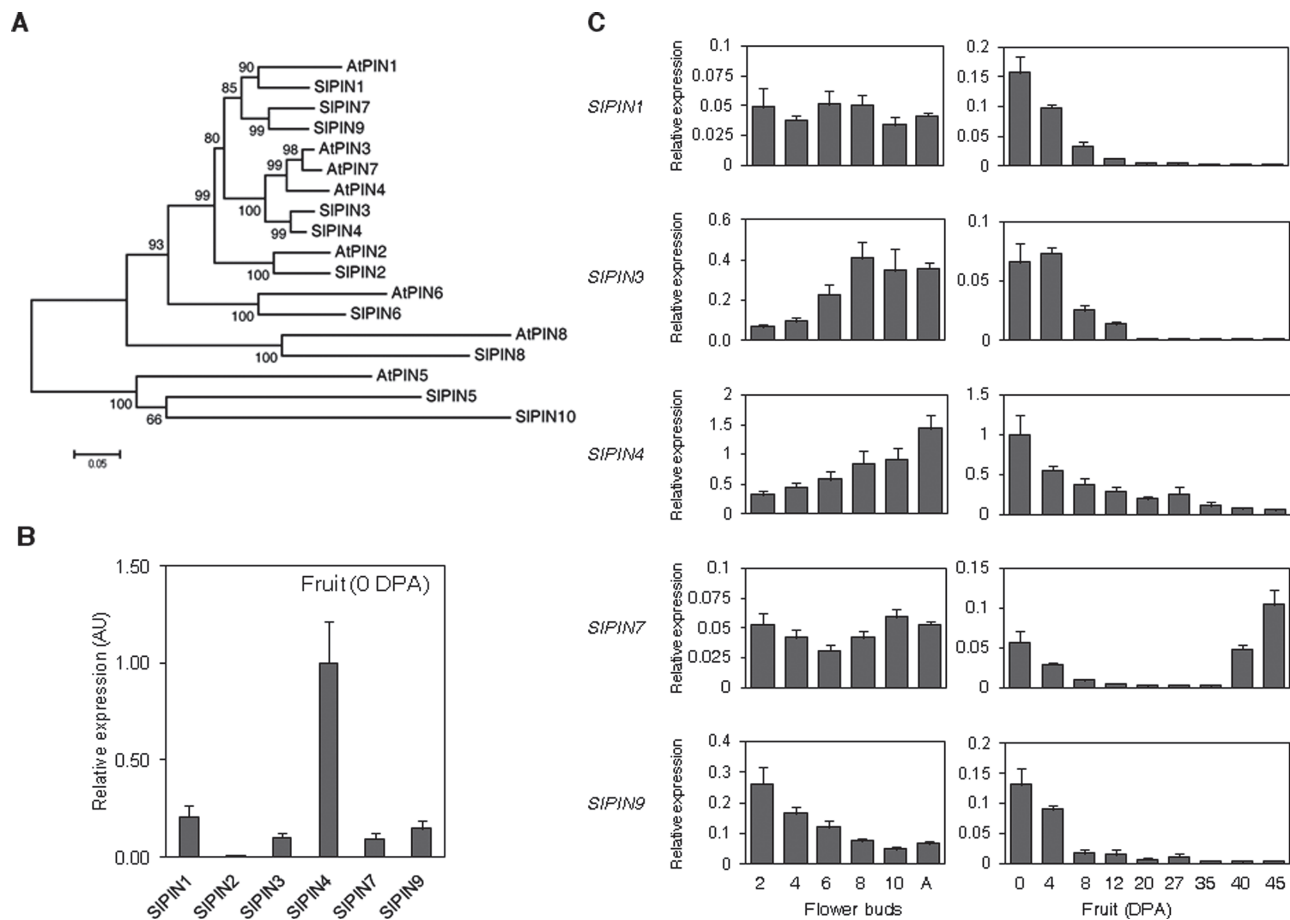


**Fig. 1.** *PIN* homologues in tomato. (A) Phylogenetic tree of *Arabidopsis* PIN proteins and of tomato putative PIN proteins. The phylogenetic tree was constructed based on a complete protein sequence alignement of PINs by the Neighbor–Joining method with bootstrapping analysis (5000 replicates) using MEGA 4.0 software (http://www.megasoftware.net/) after sequence alignment using ClustalW. (B) Expression analysis of tomato plasma membrane *PIN* genes in 0 DPA fruit by qRT-PCR. The relative expression of each gene (arbitrary units) corresponds to gene expression normalized with the expression of actin, β-tubulin, and EiF4a. Bars represent the standard deviation (*n*=3). (C) Expression profile of *PIN* genes during tomato reproductive development. Relative expression of *PIN* genes was measured by qRT-PCR in 2–10 mm flower bud, flower at anthesis (A), and in 0–45 DPA fruit. The relative expression of each gene (arbitrary units) corresponds to gene expression normalized with the expression of β-tubulin and EiF4a. Error bars represent the standard deviation (*n*=3).

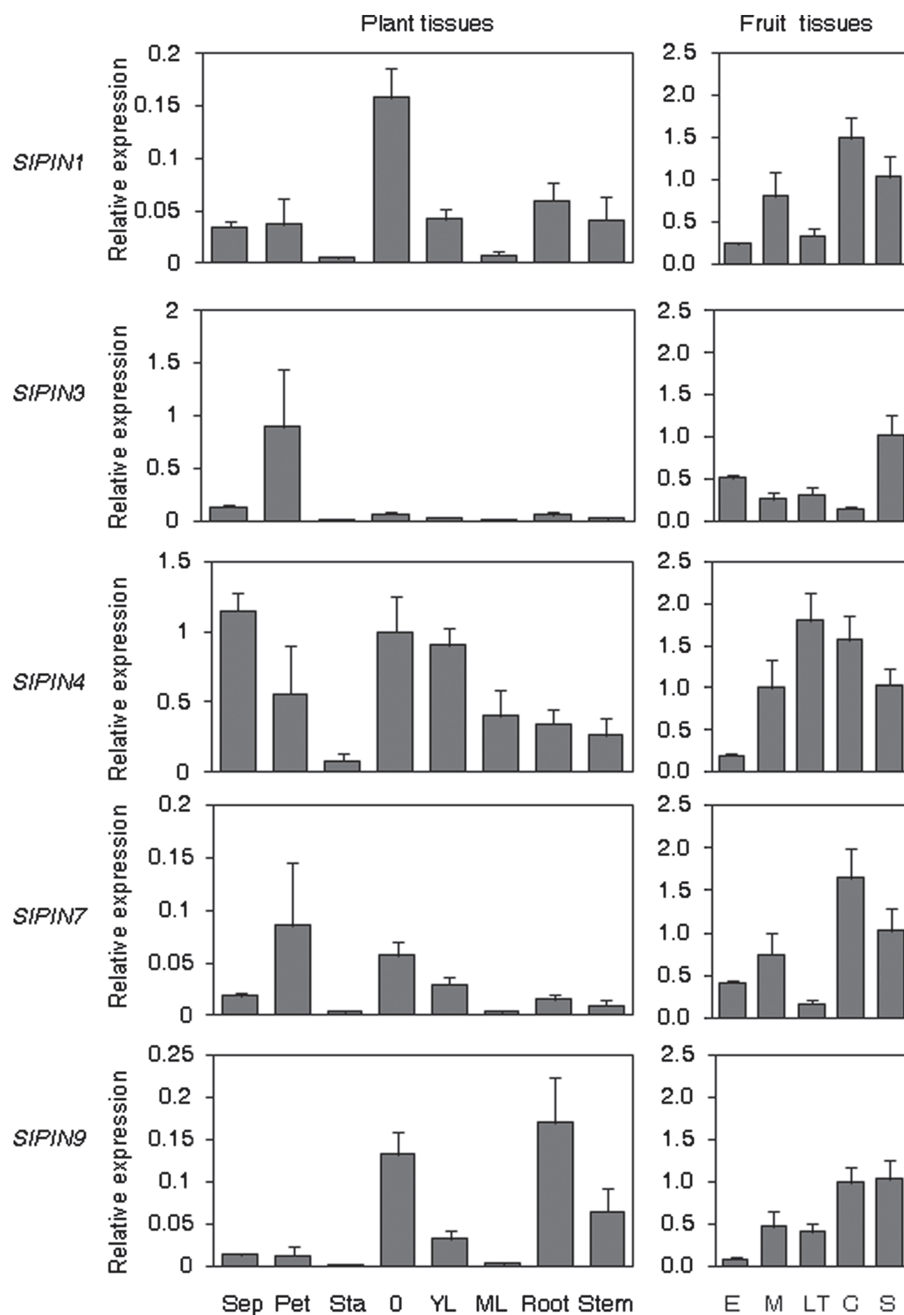


**Fig. 2.** Expression profile of *PIN* genes in tomato tissues. The relative expression of *PIN* genes was measured by qRT-PCR. The relative expression of each gene (arbitrary units) corresponds to gene expression normalized with the expression of actin, β-tubulin, and EiF4a. Bars represent the standard deviation ($n$=3). Sep, sepal; Pet, petal; Sta, stamen; 0, fruit at anthesis; YL, young leaf; ML, mature leaf. E, exocarp; M, mesocarp; LT, locular tissue; C, columella; S, seed.

under the control of the 35S promoter. The risk of silencing potential off-targets was checked beforehand (Xu *et al.*, 2006). Screening the tomato genome for sequences sharing at least 21 nucleotide identity with the *SlPIN4*-targeted region revealed the absence of such potential off-targets. Ten independent primary ($T_0$) transgenic lines were generated that presented a range of 2- to 4.3-fold reduction in *SlPIN4* expression (Supplementary Fig. S1 at *JBX* online). Among these plants, the severely affected line L-8 displayed wiry leaves and a low number of flowers, which generally aborted. The nine other $T_0$ plants did not show any obvious phenotype at the vegetative level and were able to develop flowers and fruits. Among these $T_0$ plants, four were sterile, their flowers giving rise to the development of seedless (parthenocarpic) fruits (Fig. 3, L-7, L-9, L-13, and L-14) similar to the previously described transgenic plants in which auxin signalling genes were down-regulated (Wang *et al.*, 2005; de Jong *et al.*, 2009*a*). The other $T_0$ plants (L-2, L-3, L-4, L-5, and L-21) showed facultative

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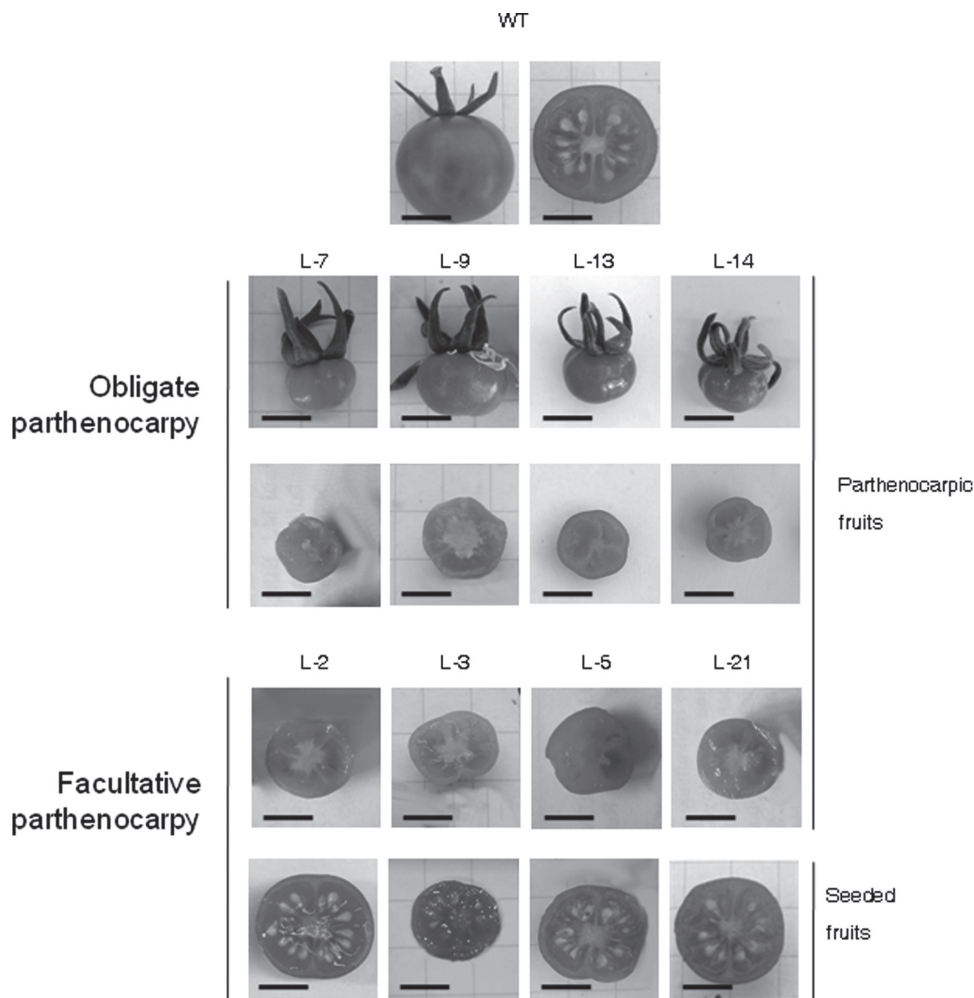


**Fig. 3.** Down-regulation of *SlPIN4* in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines triggers the development of parthenocarpic fruits. Fruit and fruit transverse sections of WT and $P_{35S}$:*SlPIN4*$^{RNAi}$ lines at the red ripe stage. The bar represents 1 cm.

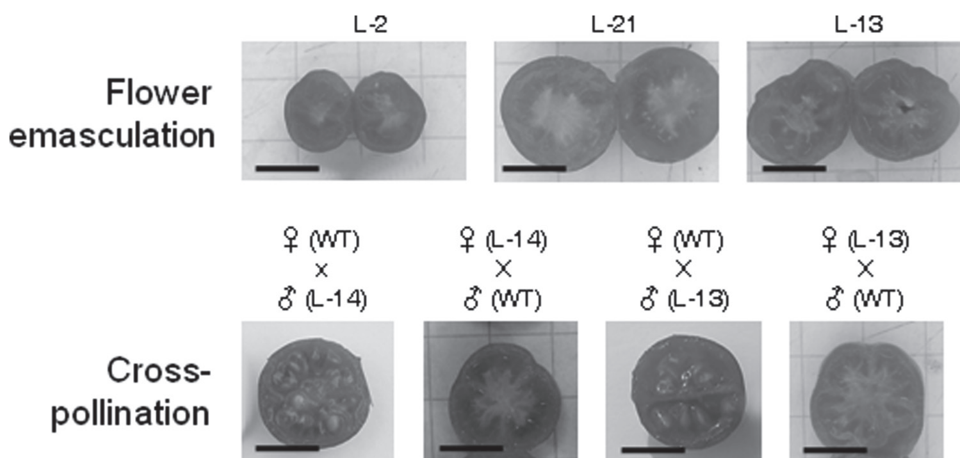


**Fig. 4.** Parthenocarpic fruit development in $P_{35S}$:*SlPIN4*$^{RNAi}$ transgenic lines is due to the uncoupling of fruit development from ovule fertilization. Emasculation was performed by removal of stamens on flowers from lines L-2, L-21, and L-13. Pollen from parthenocarpic lines L-13 and L-14 was used to fertilize carpel on WT plants. WT pollen was used to fertilize carpel from lines L13 and L-14. The bar represents 1 cm.

parthenocarpy, giving rise to parthenocarpic and seeded fruits (Fig. 3), with a normal or reduced number of seeds (data not shown). Emasculation of flowers before anthesis in obligate and facultative parthenocarpic $P_{35S}$:*SlPIN4*$^{RNAi}$ lines also led to the development of seedless fruits (Fig. 4), whereas in WVA 106 WT plants emasculation of flowers always led to flower

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abortion within a week after anthesis. These results indicate that in the transgenic lines fruit set was independent of ovule fertilization and was due to the precocious development of the ovary into a fruit. Failure to set seeds was not associated with male fertility defects since no significant difference in pollen germination rate was detected between WT and $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (data not shown). In addition, cross-pollination experiments using obligate parthenocarpic $P_{35S}$:*SlPIN4*$^{RNAi}$ pollen on WT ovaries triggered seed formation, whereas cross-pollination using WT pollen on obligate parthenocarpic $P_{35S}$:*SlPIN4*$^{RNAi}$ ovaries led to seedless fruits (Fig. 4).

Down-regulation of the *SlPIN4* gene resulted in visible morphological modifications of flowers in obligate parthenocarpic $P_{35S}$:*SlPIN4*$^{RNAi}$ lines and, to a lesser extent, in the facultative parthenocarpic lines (Fig. 5A). Sepals were longer than in WT plants and had a yellowish colour. The fused stamen cones were pushed away by the developing carpels, and flowers presented a protruding style. In the most severe cases, multiple carpels were fused within one flower (data not shown). According to the lines, flowers with altered morphology represented 0% (L-5 and L-21) to 100% of the flowers (obligate parthenocarpic lines L-7, L-9, L-13, and L-14), the facultative parthenocarpic lines L-2, L-3, and L-4 presenting an intermediate proportion of altered flowers (60, 85, and 10% respectively). Although the duration of flower development was not modified in the transgenic lines (data not shown), carpel size at anthesis was 1.5–3 times that of the WT (Fig. 5B). Histological analysis of the three $P_{35S}$:*SlPIN4*$^{RNAi}$ lines selected for in-depth analysis (facultative parthenocarpic line L-2 and L-21; obligate parthenocarpic line L-14) showed an increased development of vascular tissues in the flower buds and revealed that carpel size was larger than those of the control WT plants as early as the 4 mm stage in the $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (Fig. 5C).

As root development is affected in the young seedlings from the *Arabidopsis Atpin3*, *Atpin4*, and *Atpin7* mutants (Blilou *et al*., 2005; Petrásek and Friml, 2009), experiments were also carried out to examine whether root development was similarly modified in the $P_{35S}$:*SlPIN4*$^{RNAi}$ lines. Seeds from facultative parthenocarpic lines L-2 and L-21 where sown *in vitro* in the presence and absence of IAA. In the absence of IAA, $P_{35S}$:*SlPIN4*$^{RNAi}$ lines showed no significant differences in root growth when compared with the WT (Table 1). In the presence of IAA, primary root length was shortened in the WT and in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (data not shown). In addition, L-2 and L-21 $P_{35S}$:*SlPIN4*$^{RNAi}$ lines presented a significant reduction in the number of lateral roots when compared with the WT (Table 1). This phenotype is similar to the inhibition of lateral root initiation following naphthylphthalamic acid (NPA) treatments in tomato and in *Arabidopsis* (Muday and Haworth, 1994; Casimiro *et al*., 2001) and suggests that auxin transport could be impaired in the roots of $P_{35S}$:*SlPIN4*$^{RNAi}$ lines.

**Table 1.** Number of lateral roots developing on 12-day-old seedlings germinated in the presence/absence of 0.5 μM IAA

± represents the standard deviation (*n*=50).
* Significant variation of transgenic lines compared with the WT (*t*-test, *P*=0.001).

| | WT | L-2 | L-21 |
|---|---|---|---|
| – IAA | 8.62 ± 1.71 | 8.32 ± 1.96 | 8.22 ± 1.97 |
| + IAA | 7.56 ± 1.63 | 6.00 ± 1.92* | 6.15 ± 1.40* |

### *Auxin metabolism is slightly affected in the $P_{35S}$:SlPIN4$^{RNAi}$ lines*

Expression analysis of *PIN* genes during flower bud and early fruit development (Fig. 6) confirmed that *SlPIN4* expression was strongly down-regulated in lines L-2, L-21, and L-14 at each stage of floral and fruit development studied. In addition, it revealed that the expression of other *PIN* genes was slightly modified in the transgenic lines during flower bud development. The *SlPIN1* and *SlPIN7* transcript levels were significantly higher at the 4 mm and 8 mm stages in the obligate parthenocarpic line L-14, while *SlPIN3*, the closest homologue of *SlPIN4*, displayed an opposite behaviour in the obligate parthenocarpic line L-14 and in the facultative parthenocarpic lines L-2 and L-21.

According to the severe down-regulation of *SlPIN4* in the three transgenic lines studied (Fig. 6), studies were conducted to determine whether auxin levels were affected in flower buds and ovaries from these plants. In 0 DPA ovaries, no significant differences in free IAA, oxindole-3-acetic acid (OxIAA), or auxin–aspartate conjugate (IAAasp) levels were observed between transgenic and WT plants (Fig. 7). In 8 mm flower buds, only the IAAasp level was affected in the obligate parthenocarpic line L-14. The 4 mm flower buds displayed more contrasted changes, depending on the auxin compound considered. The free IAA level was not significantly affected whatever the transgenic line considered, though an increase was clearly perceptible in the obligate parthenocarpic line L-14. The OxIAA level increased only in the obligate parthenocarpic line L-14. In contrast, the IAAasp level increased considerably in all three transgenic lines (Fig. 7C), clearly indicating a modification of auxin metabolism in the very young flower buds of the $P_{35S}$:*SlPIN4*$^{RNAi}$ lines.

### *Several transcription factors are misregulated in the $P_{35S}$:SlPIN4$^{RNAi}$ lines*

In order to identify auxin targets involved in the regulation of precocious ovary development in the *SlPIN4*-silenced lines, the transcriptome of flower buds (6 mm stage) of WT and transgenic lines L-2, L-21, and L-14 was analysed using TOM2 microarrays. Although they do not represent the whole genome, TOM2 microarrays are enriched in fruit-expressed genes, in pre- and post-anthesis ovary-expressed genes, and in TFs (Alba *et al*., 2004; Fei *et al*., 2004). Among the 1460 genes significantly expressed in the flower buds of either transgenic lines or the WT, 167 genes displayed a variation of expression >1.5-fold in at least one $P_{35S}$:*SlPIN4*$^{RNAi}$ line when compared with the WT (Supplementary Table S3 at *JBX* online). Some genes previously highlighted in other parthenocarpic lines (Vriezen *et al*., 2008; Martinelli *et al*., 2009; Wang *et al*., 2009; de Jong *et al*., 2011) were misregulated in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines, such

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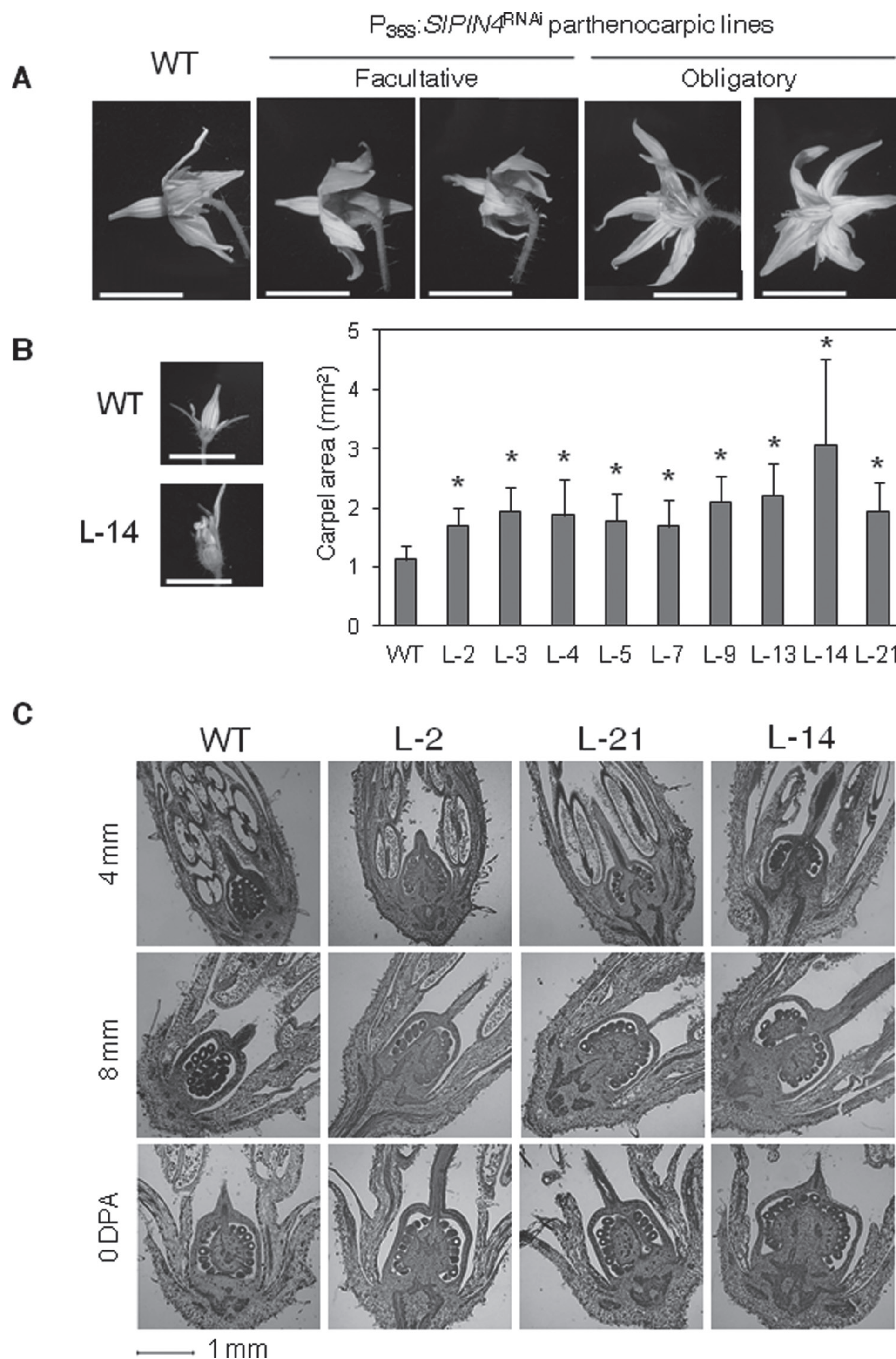


**Fig. 5.** Modification of flowers in $P_{35S}$:*SlPIN4*$^{RNAi}$ plants. (A) Flower phenotype with visible alterations of sepals and stamens. The white bar represent 1 cm. (B) Mean carpel area of flowers at anthesis in the WT and in transgenic lines. Vertical bars represent the standard deviation and an asterisk indicates a significant difference from the WT (*t*-test, $n$=10, $P < 0.001$). (C) Cytological analyses on 4 mm and 8 mm flower buds and 0 DPA ovaries.

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as chlorophyll *a*/*b*-binding proteins, lipid transfer protein, and expansin (Supplementary Table S3). As previously shown (Molesini *et al*., 2009*b*; Wang *et al*., 2009), polyamine metabolism was particulary impaired in $P_{35S}$:*SlPIN4*$^{RNAi}$ parthenocarpic lines (Table 2). Genes involved in abscisic acid or brassinosteroid metabolism were up-regulated in the three $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (zeaxanthin epoxidase and sterol methyltransferase), whereas one gene coding for IAA-amido synthase GH3.1, involved in auxin conjugation, was down-regulated in these lines. Because the parthenocarpic fruit set resulting from inhibition of auxin transport is mediated by GA (Serrani *et al*., 2010), the expression of three genes involved in GA biosynthesis (*SlGA20ox-1*,

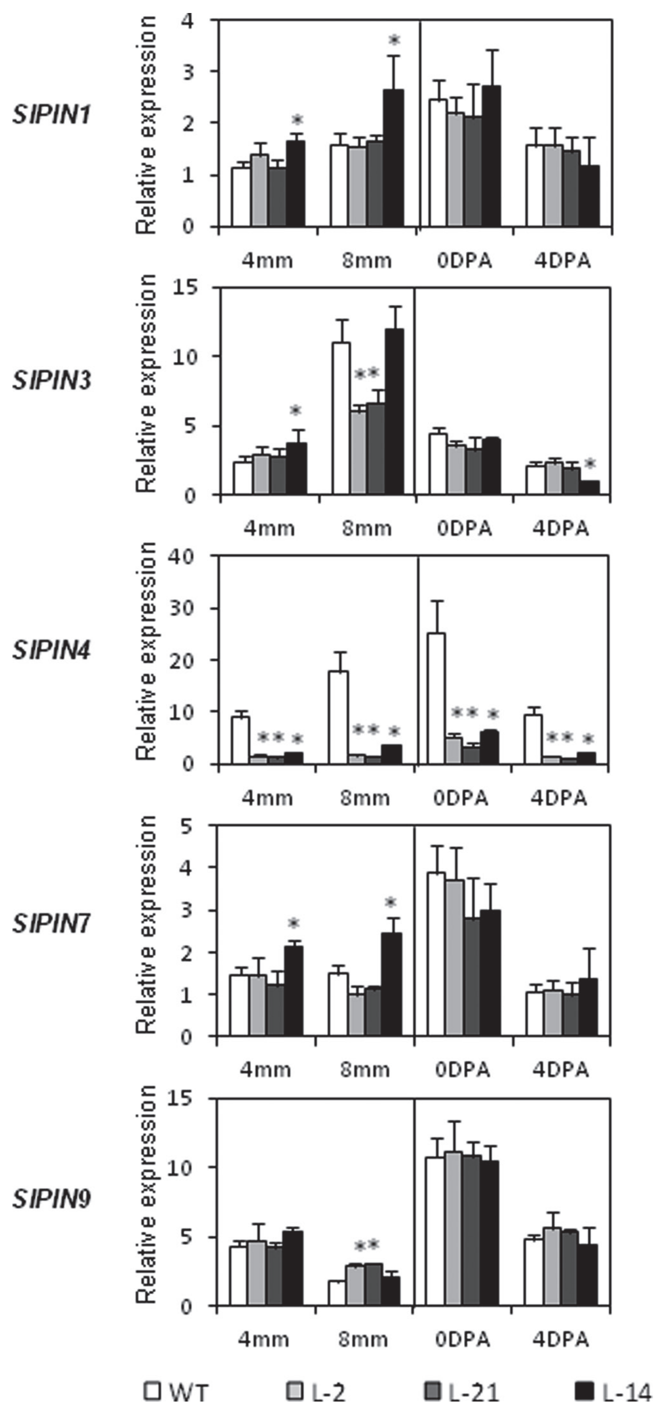


**Fig. 6.** Expression profile of *PIN* genes in the WT and $P_{35S}$:*SlPIN4*$^{RNAi}$ lines. Gene expression was measured by qRT-PCR in flower buds (4 mm and 8 mm) and in ovary at anthesis and 4 DPA. The relative expression of each gene (arbitrary units) corresponds to gene expression normalized with the expression of β-tubulin and EiF4a. Vertical bars represent the standard deviation and an asterisk indicates a significant difference from the WT (*t*-test, *n*=3, $P < 0.05$).

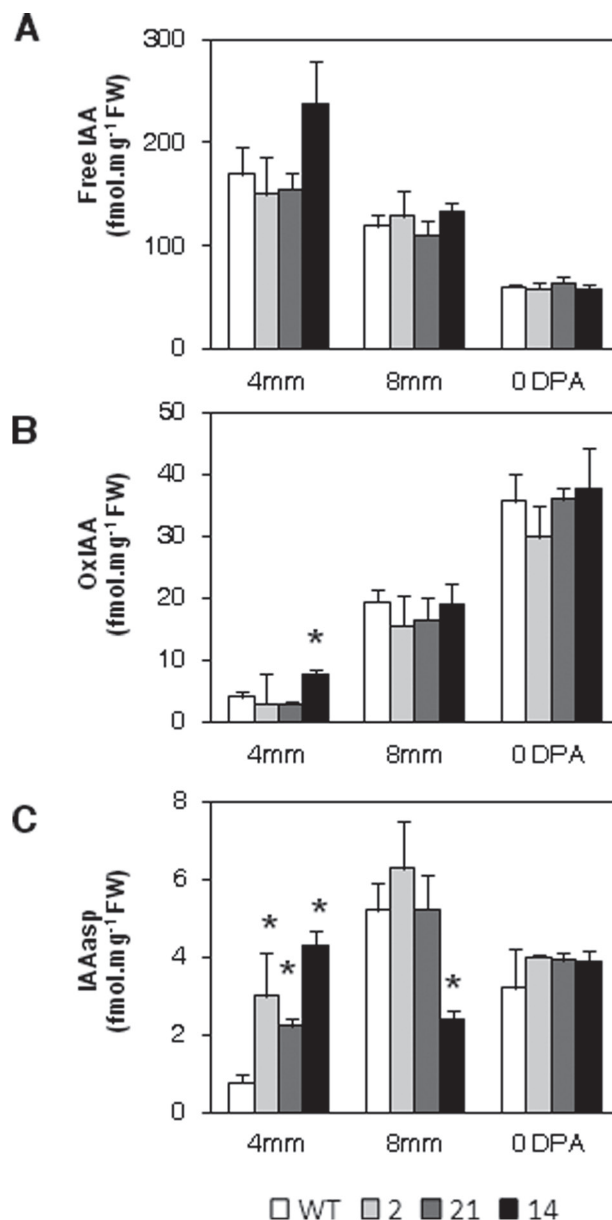


**Fig. 7.** Quantification of free auxin and related compounds in WT and in $P_{35S}$:*SlPIN4*$^{RNAi}$ flower buds (4 mm and 8 mm) and in ovary at anthesis (0 DPA). (A) Free IAA levels. (B) OxIAA levels. (C) IAA–aspartate conjugate levels. Vertical bars represent the standard deviation and an asterisk indicates significant differences (*t*-test, *n*=3, $P < 0.05$).

*SlGA20ox-2*, and *SlGA3ox-1*) and one GA-responsive gene (*SlGAST*) was further analysed by the more sensitive qRT-PCR. As shown in Supplementary Fig. S2 at *JBX* online, *SlGA20ox-1*, *SlGA20ox-2*, and *SlGAST* were significantly down-regulated in 8 mm flower bud and/or in developing fruit, while *SlGA3ox-1* was significantly up-regulated in 8 mm flower bud in the most severely affected line L-14. For the GA biosynthetic genes, these results are consistent with those observed in the parthenocarpic transgenic lines silenced for *SlARF7*, an ARF acting as a negative regulator of fruit set (de Jong *et al.*, 2011). However, cross-talk between auxin and GA in the regulation of fruit set remains complex. Indeed, at the opposite, Wang *et al*. (2009) did not detect any variation of GA-related genes in the tomato parthenocarpic lines silenced for *SlAux/IAA9*.

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**Table 2.** Genes involved in hormone metabolism and transcription factors differentially expressed between $P_{35S}$:SlPIN4$^{RNAi}$ lines and the WT in flower buds revealed by TOM2 microarray expression analysis

| Spot ID | Unigene ID | Annotation | *Arabidopsis* gene | Fold change $P_{35S}$:*SlPIN4*$^{RNAi}$/WT | | |
|---|---|---|---|---|---|---|
| | | | | L-2 | L-21 | L-14 |
| Hormone and polyamine synthesis | | | | | | |
| LE13G23 | U573534 | IAA-amido synthase GH3.1 | AT2G14960 | 0.76 | 0.72 | 0.64 |
| LE15N13 | U578173 | Sterol methyltransferase SMT2 | AT1G20330 | 1.29 | 1.44 | 1.86 |
| LE21D21 | U590155 | Squalene epoxidase XF1 | AT1G58440 | 0.95 | 0.89 | 1.51 |
| LE15J06 | U569421 | Zeaxanthin epoxidase ABA1 | AT5G67030 | 1.39 | 1.52 | 1.33 |
| LE5C02 | U593751 | Spermidine synthase SPMS, SPDS3 | AT5G53120 | 1.46 | 1.20 | 1.58 |
| LE3O19 | U569175 | Spermidine synthase ACL5 | AT5G19530 | 0.63 | 0.59 | 0.44 |
| LE16K13 | U577418 | Adenosylmethionine decarboxylase | AT3G25570 | 0.76 | 0.50 | 1.42 |
| Transcription factors | | | | | | |
| LE28D08 | U573372 | | AT3G16500 | 0.98 | 1.01 | 0.62 |
| LE23I19 | U594329 | bHLP transcription factor | AT1G01260 | 0.79 | 0.55 | 0.97 |
| LE14K15 | U578320 | bZIP transcription factor AtBZIP44 | AT1G75390 | 0.74 | 0.81 | 0.61 |
| LE18G14 | U573993 | Homeobox protein AtHB23 | AT5G39760 | 1.15 | 0.97 | 0.57 |
| LE16K18 | U577422 | Cycling DOF factor CDF3 | AT3G47500 | 1.56 | 1.81 | 1.63 |
| LE1F12 | U569398 | MADS-box PI (PISTILLATA) | AT5G20240 | 1.24 | 1.56 | 0.87 |
| LE33O18 | U573183 | MADS-box TPI | AT5G20240 | 1.55 | 2.12 | 0.22 |
| LE21H22 | U576253 | MYB transcription factor AtMYB21 | AT3G27810 | 1.42 | 1.79 | 1.49 |
| LE32N01 | U602726 | MYB transcription factor MYB24 | AT5G40350 | 1.36 | 2.07 | 1.38 |
| LE28D07 | U569474 | MYB transcription factor AtMYB44 | AT5G67300 | 0.65 | 0.57 | 1.18 |
| LE8A24 | U566039 | NAC transcription factor AtNAP | AT1G69490 | 1.06 | 1.50 | 1.04 |
| LE11K21 | U568609 | NAC transcription factor AtNAC2 | AT3G15510 | 0.86 | 1.03 | 0.65 |

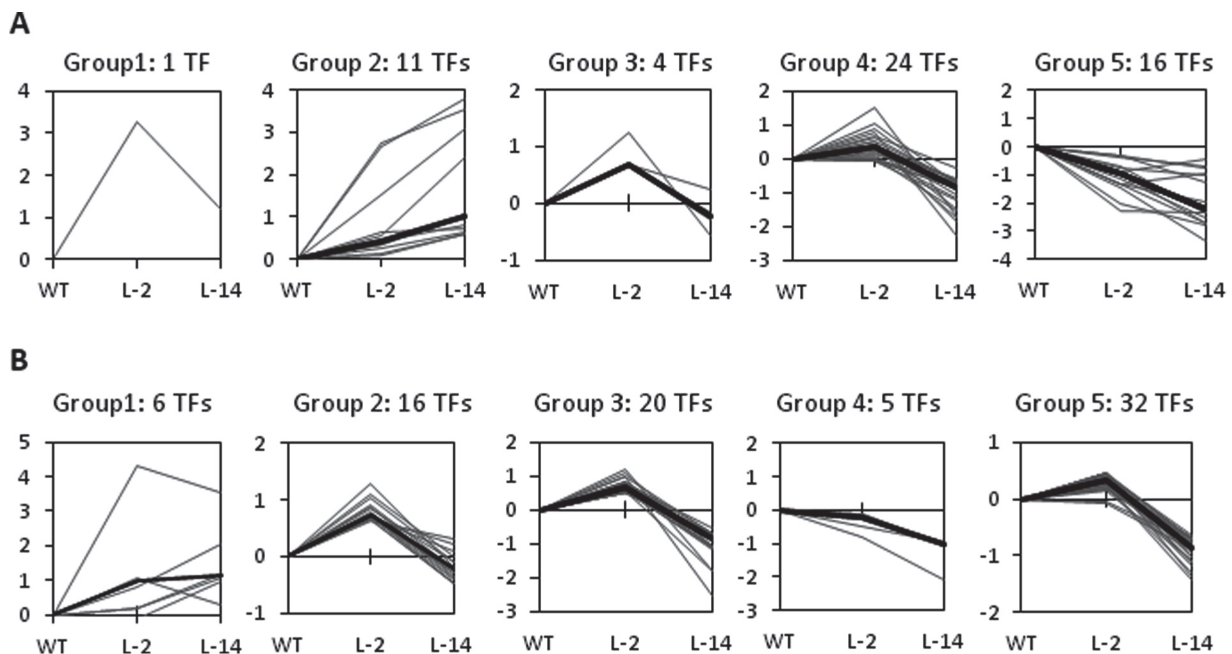


**Fig. 8.** Groups of transcription factors differentially expressed in a global expression analysis by qRT-PCR using a TF profiling platform. (A) Clustering of the 56 TFs differnentially expressed in 4 mm flower buds in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines compared with the WT. (B) Clustering of the 79 TFs differentially expressed in 0 DPA ovaries in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines compared with the WT. TFs were manually assigned to an expression group according to the values of log2(L-2/WT) and log2(L-14/WT). The relative expression of each gene (arbitrary units) corresponds to log2 (normalized gene expression WT/normalized gene expression transgenic line). The bold line corresponds to the mean of the values in the group.

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In agreement with results from Wang *et al*. (2009), the TF category was one of the main categories presenting a differential expression in the $P_{35S}$:*SlPIN4*$^{RNAi}$ lines. The 12 TFs significantly misregulated in the transgenic lines (Table 2) belong to different classes, the major ones being the MYB, the MADS-box, and the NAC classes. Three genes (one *DOF* and two *MYB*) were up-regulated and one *bZIP* was down-regulated in all three transgenic lines. Three additional genes (*Aux/IAA26* and homologues of *AtHB23* and *AtNAC2*) were specifically down-regulated in the most severely affected obligate parthenocarpic line L-14. Other genes displayed dissimilar or even opposite patterns (TPI) of expression in the facultative and obligate parthenocarpic lines.

These results and the reported importance of the coordinated expression of TFs in the regulation of the transition from ovary to fruit development (Wang *et al*., 2009) and in auxin signalling (reviewed in Chapman and Estelle, 2009; Kieffer *et al*., 2009) prompted an extension of the investigations on the role of TFs. To this end, a qRT-PCR platform, more sensitive than microarrays and able to profile a large set (1090) of putative tomato TFs (Rohrmann *et al*., 2011), was used. Flower bud (4 mm) and ovary at anthesis (0 DPA) RNAs from one facultative parthenocarpic line (L-2) and one obligate parthenocarpic line (L-14) were compared with WT RNA. The 4 mm flower bud was chosen because auxin metabolism is altered in the transgenic lines at this stage of flower bud development (Fig. 7). Previous reports also indicated that ovary at anthesis is the developmental stage at which the largest differences in gene expression can be observed between parthenocarpic and WT ovary/fruit (Pascual *et al*., 2009; Wang *et al*., 2009). Among the TFs significantly expressed in flower buds (1015) and in ovary (1005), 172 TFs were differentially expressed between $P_{35S}$:*SlPIN4*$^{RNAi}$ and the WT in flower buds and/or in ovary. Particular attention was drawn to the TFs with a ≥1.5-fold change in gene expression in $P_{35S}$:*SlPIN4*$^{RNAi}$ when compared with the WT (Supplementary Table S4 at *JBX* online).

In flower buds, 56 TFs were differentially expressed in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (Fig. 8A; Supplementary Table S4), 12 of them being up-regulated in both transgenic lines (Group 1 and 2, Table 3). Among these genes, two homologues of *AtMYB21*, a GATA zinc finger, and a homologue of *AtERF110*, presented the highest variation in expression (8- to 13-fold). Only two *Aux/IAA* TFs were up-regulated, corresponding to the homologues of *AtAux/IAA16* and *AtAux/IAA19*. Interestingly, *CRABS-CLAW*, a C2C2-YABBY TF, was up-regulated in this analysis (Table 3). Sixteen genes were down-regulated in both transgenic lines, the main TF classes represented being the MADS-box (four TFs) and the MYB/MYB-related TFs (three TFs, Group 5, Table 3). A homologue of *AtWRK16* was the most affected TF, with a 10-fold reduction of expression in the obligate parthenocarpic line (Table 3). Additional differentially regulated genes showed either a specific up-regulation in the facultative parthenocarpic line (Group 3, four TFs), or a specific down-regulation in the obligate parthenocarpic line (Group 4, 24 TFs). The main TF classes represented in the latter group are MADS-box TFs, including *TPI* and *Jointless*, and MYB/MYB-related TFs (Supplementary Table S4 at *JBX* online).

In ovary at anthesis, 79 TFs were differentially expressed in the $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (Fig. 8B; Supplementary Table S4). In these samples, the majority of the genes were differently regulated in the facultative and in the obligate parthenocarpic lines. This may reflect both the differences in ovary development at anthesis between the various transgenic lines and the absence of fertilization in the obligate parthenocarpic line. In this regard, 16 TFs were up-regulated only in L-2 (Group 2; Supplementary Table S4), including four MADS-box and four C3H zinc finger TFs. Twenty TFs corresponding mostly to MYB/MYB-related and MADS-box TFs (Group 3, Supplementary Table S4) were up-regulated in L-2 and down-regulated in L-14. However, six TFs were up-regulated in both transgenic lines (Group 1, Table 4). Among these genes, one bZIP (homologue of *AtTGA10*) presented the highest variation in expression (19-fold in line L-2). In addition, two ERFs (ethylene-responsive factors) were present in this group. Conversely, five TFs were down-regulated in both transgenic lines, among which were two heat shock factors (homologues of *AtHSFA6B* and *AtHSF4*) and the MADS-box *TAGL11* (Group 4, Table 4). Finally, a large number of TFs were down-regulated only in the obligate parthenocarpic line (Group 5, 32 TFs). The main TF classes represented in these groups correspond to MYB/MYB-related, zinc finger, and MADS-box TFs including *TAG1* and *TDR3* (Supplementary Table S4 at *JBX* online). *CRABS-CLAW* was also included in this group of TFs repressed only in the obligate parthenocarpic line.

## Discussion

### *Parthenocarpic fruit development in* SlPIN4*-silenced lines*

Among the 10 homologues of *Arabidopsis* PIN proteins found in tomato (Fig. 1A; Supplementary Table S2 at *JBX* online; Pattison and Catala, 2012), *SlPIN4* displays remarkable features regarding flower and fruit development in tomato. *SlPIN4* is much more highly expressed than its close homologue *SlPIN3* or any other *PIN* gene in tomato ovary and fruit (Fig. 1B). Its transcript abundance peaks at anthesis, when the ovary develops into a fruit following fertilization (Fig. 1C). The *SlPIN4* gene therefore appears to be a likely candidate for controlling auxin efflux in the ovary and early developing fruit of tomato.

*AtPIN1* and *AtPIN3* are the most highly expressed *PIN* genes in *Arabidopsis* ovaries (Paponov *et al*., 2005 and http://jsp.weigelworld.org/expviz/expviz.jsp). However, inactivation of either *AtPIN1* or *AtPIN3* in *Arabidopsis* and of *SlPIN4* in tomato leads to different phenotypic alterations in reproductive organs. Floral traits are strongly affected in the *Arabidopsis pin1* and *pin3* homozygous mutants, which are characterized by a drastic reduction in flower numbers and by modifications of the number and structure of floral components (Goto *et al*., 1991; Bennett *et al*., 1995). In addition, the *pin1* homozygous mutant is sterile because of the homeotic transformations of floral organs, for example the presence of petaloid sepals or stamenoid petals (Goto *et al*., 1991). Silencing of *SlPIN4* in tomato only resulted in modifications of the shape of the floral components and, in a few cases, in increased carpel numbers (Fig. 4). The most striking feature in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines was the development of parthenocarpic fruits, a phenotypic alteration which was not

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**Table 3** TFs up-regulated or down-regulated in the flower buds of the facultative and the obligate parthenocarpic $P_{35S}$:SlPIN4$^{RNAi}$ lines revealed by qRT-PCR

[a] Clustering group according to Fig. 8A.

| ID | Unigene ID | Annotation | Arabidopsis homologue | Group[a] | Fold-change $P_{35S}$:*SlPIN4*$^{RNAi}$/WT | |
|---|---|---|---|---|---|---|
| | | | | | L-2 | L-14 |
| J0905 | U573092 | GATA type zinc finger TF | AT1G01780 | 1 | 9.68 | 2.26 |
| J0021 | U584903 | AP2/B3-like TF | AT3G06160 | 2 | 1.36 | 1.72 |
| J0074 | U577088 | Ethylene response TF, ERF110 | AT5G50080 | 2 | 2.89 | 8.43 |
| J0125 | U579607 | IAA19, MSG2 | AT3G15540 | 2 | 1.26 | 2.06 |
| J0141 | U580151 | IAA16 | AT3G04730 | 2 | 1.18 | 1.57 |
| J0166 | U575251 | ILR3, bHLH105 | AT5G54680 | 2 | 1.10 | 1.53 |
| J0240 | U576188 | AtBZIP53 | AT3G62420 | 2 | 1.54 | 1.65 |
| J0306 | U572646 | CRABS-CLAW, CRC | AT1G69180 | 2 | 1.26 | 1.99 |
| J0349 | U576689 | AtZFP2 | AT5G57520 | 2 | 1.45 | 5.28 |
| J0385 | U585955 | Zinc finger (CCCH-type)TF | AT3G51950 | 2 | 1.07 | 1.52 |
| J0712 | U576253 | AtMYB21 | AT3G27810 | 2 | 6.30 | 13.84 |
| J0746 | U602726 | AtMYB21 | AT3G27810 | 2 | 6.65 | 11.62 |
| J0261 | U593980 | TGA10, bZIP65 | AT5G06839 | 5 | 0.81 | 0.42 |
| J0355 | U575936 | C2H2-type zinc finger TF | AT2G28200 | 5 | 0.39 | 0.51 |
| J0407 | U586223 | Zinc finger (CCCH type) TF | AT5G06770 | 5 | 0.78 | 0.62 |
| J0453 | U564651 | NF-YB5 | AT2G47810 | 5 | 0.51 | 0.15 |
| J0498 | U571553 | DNA-binding storekeeper protein | AT4G00390 | 5 | 0.79 | 0.59 |
| J0632 | U597825 | MADS-box TF | AT1G17310 | 5 | 0.40 | 0.26 |
| J0637 | U572195 | AGAMOUS-like 2AGL20, SOC1 | AT2G45660 | 5 | 0.59 | 0.50 |
| J0660 | U585967 | AGAMOUS-like 66, AGL66 | AT1G77980 | 5 | 0.55 | 0.74 |
| J0681 | U583379 | MADS-BOX AP1, AGL7 | AT1G69120 | 5 | 0.60 | 0.19 |
| J0742 | U563618 | AtMYB62 | AT1G68320 | 5 | 0.45 | 0.17 |
| J0750 | U600259 | AtMYB80 | AT5G56110 | 5 | 0.20 | 0.20 |
| J0761 | U604577 | Myb-like TF | AT5G61620 | 5 | 0.41 | 0.15 |
| J0803 | U603443 | NAC TF, NAC2, anac078 | AT5G04410 | 5 | 0.25 | 0.15 |
| J0858 | U600327 | Calmodulin binding TF | AT4G16150 | 5 | 0.51 | 0.20 |
| J1031 | U600423 | AtWRKY16 | AT5G45050 | 5 | 0.36 | 0.10 |
| J1087 | U576656 | Mini zinc finger, MIF3 | AT1G18835 | 5 | 0.51 | 0.22 |

**Table 4.** TFs up-regulated or down-regulated in the ovary of the facultative and the obligate parthenocarpic $P_{35S}$:SlPIN4$^{RNAi}$ lines revealed by qRT-PCR

[a] Clustering group according to Fig. 8B.

| ID | Unigene ID | Annotation | Arabidopsis homologue | Group[a] | Fold-change $P_{35S}$:*SlPIN4*$^{RNAi}$/WT | |
|---|---|---|---|---|---|---|
| | | | | | L-2 | L-14 |
| J0087 | U577090 | ERF | AT5G25190 | 1 | 1.72 | 4.09 |
| J0107 | U565667 | ERF, SHN3 | AT5G25390 | 1 | 1.12 | 2.06 |
| J0182 | U571566 | bHLH TF | AT5G57150 | 1 | 0.92 | 1.96 |
| J0208 | U581766 | bHLH TF | AT1G72210 | 1 | 1.13 | 2.28 |
| J0261 | U593980 | TGA10, bZIP65 | AT5G06839 | 1 | 19.64 | 11.57 |
| J0301 | U563209 | GATA type zinc finger TF, GATA21 | AT5G56860 | 1 | 2.09 | 1.21 |
| J0050 | U591075 | RAP2.7, TOE1 | AT2G28550 | 4 | 0.86 | 0.52 |
| J0603 | U590552 | AtHSFA6B | AT3G22830 | 4 | 0.72 | 0.51 |
| J0607 | U566892 | AtHSF4 | AT4G36990 | 4 | 0.92 | 0.49 |
| J0672 | U585390 | MADS-box TF, STK, AGL11 | AT4G09960 | 4 | 0.92 | 0.47 |
| J1067 | U580500 | AtWRKY75 | AT5G13080 | 4 | 0.57 | 0.23 |

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observed the *Arabidopsis pin* mutants already described. A possible explanation is that large alterations of flower development (Goto *et al*., 1991) and lack of emasculation experiments in the *Arabidopsis pin1* mutant hindered further observations of silique parthenocarpy. Another likely explanation is that in tomato the *SlPIN4* gene has evolved to fulfil specialized functions related to the control of fruit set by auxin. The strong expression of *SlPIN4* in the ovaries (Fig. 1), which has recently been shown to be restricted to the ovules (Nishio *et al*., 2010), as well as the lack of compensation of *SlPIN4* silencing by other *PIN* genes expressed in flower buds (Fig. 6), support this hypothesis.

Indeed, specific silencing of *SlPIN4* had profound effects on fruit set in tomato, and led to the development of parthenocarpic fruits in the transgenic lines displaying phenotypic alterations. In these lines, two types of parthenocarpy were observed. The most severely affected lines such as L-14, called obligate parthenocarpic lines, were unable to produce seeds. The moderately affected lines such as L-2 and L-21, called facultative parthenocarpic lines (Fig. 3), could produce seeds. However, seed production was irregular: it was not observed in all fruits, and seed number was highly variable. It is important to mention that the 'WVA 106' cultivar has long been used for studying tomato flower and fruit development (Joubès *et al*., 1999; Gilbert *et al*., 2009; Mathieu-Rivet *et al*., 2010) and that in normal growth conditions the development of parthenocarpic fruits has never been observed in this cultivar. In the obligate parthenocarpic lines, the onset of fruit development is very precocious and takes place as early as the 4 mm flower bud stage, thus leading to the development of seedless fruits (Figs 3, 5C). In the facultative parthenocarpic lines, the onset of fruit development also takes place before anthesis, but later than in the obligate parthenocarpic lines (Fig. 5C). In contrast to obligate parthenocarpic lines, seeds can be produced after manual pollination, indicating that precocious fruit development does not hamper fertilization. The irregular production of seeds in these lines probably stems from the morphological alterations of the flowers, in which protruding styles were often observed. In that case, self-pollination was no longer possible due to the exsertion of the stigmas from the anther cone (Fig. 5A), unlike WT flowers in which the cone of anthers encloses the ovary, style, and stigmas (Chen *et al*., 2007). Cross-pollination is unlikely in the insect-proof greenhouse and culture conditions used in these experiments and, indeed, has never been observed. The combination of precocious fruit development and lack of fertilization therefore provides a likely explanation for the occurrence of unseeded or few-seeded fruits in the facultative parthenocarpic lines.

The findings of this study do not support the research of Pattison and Catala (2012) who observed strong phenotypic alterations at the vegetative level when silencing *SlPIN4*. One likely explanation is that the simultaneous silencing of multiple PIN genes, including not only *SlPIN4* but also the closely related *SlPIN3*, and, in one line, also the *SlPIN1*, *SlPIN7*, and *SlPIN9* genes, led to the pleiotropic plant phenotypes observed at the vegetative level in Pattison and Catala (2012). Such strong alterations were absent in the lines used here, which were specifically silenced for *SlPIN4* (Fig. 6). More surprising is the lack of ovary/fruit phenotype in the study of Pattison and Catala (2012). In the present study, all the lines displaying comparable down-regulation of *SlPIN4* exhibited fruit parthenocarpy (Figs 3, 6). This phenotype is strongly reminiscent of the effect of the polar auxin transport inhibitor NPA, a well known inducer of fruit parthenocarpy in tomato (Serrani *et al*., 2010; Pattison and Catala, 2012). Again, the combination of multiple *PIN* gene silencing in Pattison and Catala (2012) may bias the results observed, for example by redirecting the auxin fluxes in the plant, ovary, and fruit, therefore counter-balancing the effect of *SlPIN4* silencing. An alternative explanation is that the *SlPIN4* silencing effect is genotype dependent. The Pattison and Catala (2012) study used 'Ailsa Craig', an *S. lycopersicum* variety commonly used in laboratory studies. The present study used the cherry tomato cultivar 'WVA 106', a tomato variety used for years in early fruit development studies (Joubès *et al*., 1999; Gilbert *et al*., 2009; Mathieu-Rivet *et al*., 2010). As recently shown (Ranc *et al*., 2008), cherry tomatoes can be considered as hybrids between *S. lycopersicum* and *Solanum pimpinellifolium*, the 'WVA 106' cultivar being closer to *S. pimpinellifolium.* Whether these different genetic origins may explain why the 'Ailsa Craig' cultivar is less sensitive than the 'WVA 106' cultivar to modifications of ovule to carpel auxin fluxes in the ovary before anthesis remains an open question.

### *Regulation of precocious fruit development in* SlPIN4*-silenced lines*

How modulation of auxin transport and responses in tomato ovary via *SlPIN4* silencing affects fruit set remains an open question. In *Arabidopsis*, the genetic manipulation of auxin efflux carriers of the AtPIN1, AtPIN3, AtPIN4, and AtPIN7 subfamily revealed how modifications of auxin distribution in plant tissues may affect a wide range of developmental processes such as response to gravity (Blilou *et al*., 2005; Petrásek and Friml, 2009; Zadnikova *et al*., 2010). Such a detailed analysis of the auxin fluxes between ovules, placenta, and carpel walls of the tomato ovary was beyond the scope of this study. Recent studies, however, suggested that the patterns of auxin distribution in tomato ovary and fruit were dependent on polar auxin transport (Nishio *et al*., 2010; Pattison and Catala, 2012). Using the *SlPIN4*-silenced lines, it was shown that the induction of precocious fruit development in tomato can be achieved by modifying polar auxin transport, without the large increases in ovary auxin content induced by ectopic expression of genes encoding enzymes of auxin biosynthesis (Pandolfini *et al*., 2002; Molesini *et al*., 2009*b*). Indeed, the phenotypes observed in *SlPIN4*-silenced lines are fully consistent with the effects of polar auxin transport inhibitors in tomato (Serrani *et al*., 2010). It was further shown that redirection of the ovary developmental programme probably occurs very prematurely since alterations in auxin metabolism were detected at very early stages of flower bud development (Fig. 7). These results and the temporal and spatial distribution of *SlPIN4* in tomato ovary (Fig. 1 and Nishio *et al*., 2010) suggest that *SlPIN4* interacts with the ovary to fruit transition by controlling local distribution of auxin in ovules and nearby tissues.

In turn, this modification of auxin local distribution in the ovaries of $P_{35S}$:*SlPIN4*$^{RNAi}$ lines may affect GA biosynthesis and response. Indeed, GA has been shown to be involved in tomato fruit set (reviewed in Schwabe and Mills, 1981; Gorguet *et al*.,

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2005; deJong *et al*., 2009*a*). Serrani *et al*. (2010) recently proposed that the fruit parthenocarpy induced by inhibition of auxin transport is mediated by GAs (Serrani *et al.*, 2010). In this study, it was shown that the expression profiles of GA biosynthetic and response genes were significantly altered in the ovary buds from transgenic lines, as early as the 4 mm stage, and later on during early fruit development (Supplementary Fig. S2 at *JBX* online). These results are consistent with those obtained in the *SlARF7*-silenced lines (deJong *et al*., 2010), but not with those from Serrani *et al.* (2008, 2010) who observed the up-regulation of GA biosynthetic genes following 2,4-D or NPA treatments. These apparent discrepancies reflect the complexity of fruit set regulation and highlight the need for additional studies, using various approaches (e.g. tissue- or cell-specific promoters with various target genes) to further our understanding of the hormonal regulation of fruit set.

Whatever the mode of action of *SlPIN4*, modification of auxin fluxes in the ovary from *SlPIN4*-silenced lines must trigger the precocious deregulation of genes associated with the parthenocarpic development of the ovary into a fruit. Various transcriptomic approaches have been undertaken to unravel changes in gene expression during fruit set (Pascual *et al*., 2007; Serrani *et al*., 2008; Vriezen *et al*., 2008) or to characterize parthenocarpic fruits (Martinelli *et al*., 2009; Molesini *et al*., 2009*b*; Pascual *et al*., 2009; Wang *et al*., 2009; de Jong *et al*., 2011). The differentially expressed genes mainly detected were involved in hormonal signalling or in fruit growth processes such as the cell cycle and cell wall modification. Here, attention was focused on TFs, which are likely targets of auxin signalling with possible roles in fruit set.

In flower buds, a number of differentially expressed genes implicated in flower development such as *AtMYB21* and *AtMYB24* (Cheng *et al*., 2009), *TGA10* (Murmu *et al*., 2010), and *Aux/IAA19* (Tashiro *et al*., 2009) were identified (Table 2; Supplementary Table S3 at *JBX* online). These results are consistent with the role of auxin in flower development (Aloni *et al*., 2006) and with the visible flower morphological alterations observed in the $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (Fig. 5A). However, several of these genes, such as the homologues of *AtMYB21* and *TGA10*, were misregulated in both flower buds and ovaries (Supplementary Table S3), suggesting that their role in tomato is not restricted to the three first flower whorls but is extended to ovary/fruit development.

In the ovary at anthesis, TF profiling allowed the identification of several categories of TFs with possible roles in the ovary to fruit transition (Table 3; Supplementary Table S4 at *JBX* online). Though auxin signalling responsible for the onset of fruit development is transmitted through a specific Aux/IAA–ARF complex (Goetz *et al*., 2006; Swain and Koltunow, 2006; Pandolfini *et al*., 2007; Wang *et al*., 2009, de Jong *et al*., 2011), only minor changes in *SlAux/IAA* expression were detected in flower buds from $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (Tables 2, 3). Molesini *et al.* (2009*a*) reported results similar to those in the present study in the *SlAucsia*-silenced plants displaying auxin transport deficiency. These authors therefore proposed that auxin transport-related parthenocarpy could be independent of *SlAux/IAA9* and *SlARF8*, two genes implicated in auxin-dependent parthenocarpic fruit development (Goetz *et al*., 2007; Wang *et al*., 2009). A recent study also suggested that induction of fruit set in unpollinated tomato ovary treated with NPA, an auxin efflux transport inhibitor, may be mediated through changes in GA metabolism (Serrani *et al*., 2010). Actually, most of the differentially expressed TFs identified through $P_{35S}$:*SlPIN4*$^{RNAi}$ profiling are likely to be implicated in the regulation of developmental processes. These include the MADS-box genes, which have a wide implication in the regulation of flower and fruit development (Dornelas *et al*., 2011; Klee and Giovannoni, 2011). Indeed, a large number of MADS-box TFs were down-regulated in the ovaries of the facultative and/or obligate parthenocarpic $P_{35S}$:*SlPIN4*$^{RNAi}$ lines, including *TAGL11*, *Fruitfull 1/TDR4*, *TDR3*, *TAG1*, *PI*, homologues of *AGL22*, *AGL42*, and *AGL68* (Table 4; Supplementary Table S4 at *JBX* online). These results are in agreement with previous studies showing the misregulation of *Fruitfull 1/TDR4* (Vriezen *et al*., 2008), *TAG1*, and *TAGL6* (Wang *et al*., 2009) in tomato parthenocarpic fruits. Previous functional analysis of *TAG1* did not reveal any function for this gene in fruit set, probably because of the dramatic phenotypic changes linked to its homeotic function (Pnueli *et al*., 1994). However, the up-regulation of *Fruitfull 1/TDR4*, *TAG1*, and *TAGL11* in tomato carpel at anthesis (Busi *et al*., 2003) is in agreement with their possible implication in the regulation of fruit set. Interestingly, MADS-box TFs are active as multimers, and numerous interactions have been identified between the MADS-box TFs identified in the present study and TM29 (Busi *et al*., 2003; Leseberg *et al*., 2008), a MADS-box implicated in the regulation of fruit set (Ampomah-Dwamena *et al*., 2002). It has been recently proposed that the MADS-box network controls the identity of floral organs and the growth of floral components and fruit by targeting genes associated with cell proliferation and growth (Dornelas *et al*., 2011). In *Arabidopsis*, one of the targets of the MADS-box *AGAMOUS* is *CRABS-CLAW*, a YABBY TF implicated in the control of carpel development (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Gómez-Mena *et al*., 2005; Lee *et al*., 2005). Similarly, the co-regulation of *CRABS-CLAW* and *TAG1* in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines (Supplementary Table S4 at *JBX* online) may suggest the implication of both genes in tomato fruit set.

In conclusion, this work provides clear evidence that the tomato PIN protein *SlPIN4* plays a major role in the auxin control of tomato fruit set, possibly by preventing precocious fruit development in the absence of pollination. It further sheds new light on the regulation of fruit set and onset of parthenocarpic fruit development in tomato by highlighting the TFs involved in the regulation of fruit set and ovary/fruit development, which are likely targets of auxin signalling.

## Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Expression profile of the *SlPIN4* gene in leaves of $T_0$ $P_{35S}$:*SlPIN4*$^{RNAi}$ lines.

Figure S2. Expression profile of genes involved in gibberellin signalling.

Table S1. List of the specific primers used for qRT-PCR analysis.

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Table S2. Accession numbers of tomato PIN genes and mRNA sequences.

Table S3. List of the 1460 genes with significant expression in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines in TOM2 microarray analysis flower buds at the 6 mm stage.

Table S4. List of the TFs with a differential expression in $P_{35S}$:*SlPIN4*$^{RNAi}$ lines compared with the WT in qRT-PCR profiling analysis in flower buds at the 4 mm stage and in ovaries at 0 DPA.

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