A TILLING allele of the tomato Aux/IAA9 gene offers new insights into fruit set mechanisms and perspectives for breeding seedless tomatoes

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A TILLING allele of the tomato *Aux/IAA9* gene offers new insights into fruit set mechanisms and perspectives for breeding seedless tomatoes

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Abstract Parthenocarpy is a desired trait in fruit crops; it enables fruit set under environmental conditions suboptimal for pollination, and seedless fruits represent a valuable consumer product. We employed TILLING-based screening of a mutant tomato population to find genetic lesions in *Aux/IAA9*, a negative regulator of the auxin response involved in the control of fruit set. We identified three mutations located in the coding region of this gene, including two single-base substitutions and one single-base deletion, which leads to a frame shift and premature stop codon. The transcription of *IAA9* was strongly reduced in the frame-shift mutant, and partial loss of mutated protein activity was evidenced by an in vitro transactivation assay. Whereas missense mutations were predicted to be tolerated and did not cause mutant phenotypes, the frame-shift mutation-induced phenotypes expected for a loss of IAA9 function, including altered axillary shoot growth, reduced leaf compoundness and a strong tendency to produce parthenocarpic fruits. Mutant flowers showed pleiotropic anther cone defects, a phenotype frequently associated with parthenocarpy in tomato and other species. Mutant fruits were larger than those of the seeded control, with higher brix

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values and similar firmness. Fruit set was higher in the mutant than in wild type in the greenhouse, but lower in the open field. Facultative expression of parthenocarpy indicated that the mutant is suitable for hybrid seed production and for increasing seeds of parental lines. The results highlight the utility of this novel *IAA9* allele for exploiting parthenocarpy by breeding tomato adapted to pollination-limiting growth conditions.

Keywords Aux/IAA transcription factors · Fruit set · Parthenocarpy · *Solanum lycopersicum* · TILLING · Tomato

Introduction

The parthenocarpic growth of the ovary into a seedless fruit without pollination and/or fertilisation is a very attractive trait for breeders and has been extensively studied in tomato, where natural or induced facultative parthenocarpic mutants are known to occur (Gorguet et al. 2005; Srivastava and Handa 2005). Notwithstanding the potential interest of these mutations, their adoption in tomato breeding has been hindered by the facultative expression, negative pleiotropic traits and lack of markers to accelerate selection schemes. The *parthenocarpic fruit* (*pat*) mutation, the first gene for parthenocarpy discovered in tomato (Bianchi and Soressi 1969), is characterised by a phenotype with high penetrance and expressivity that also entails earlier ripening and enhanced fruit quality (Falavigna et al. 1978). At maturity, the seedless *pat* fruits are typically smaller than seeded fruits produced by wild-type plants (Bianchi and Soressi 1969; Falavigna et al. 1978). Moreover, such a genotype presents floral defects that make the production of seeds difficult, even under conditions favourable for pollination (Mazzucato et al. 1998, 2003). The *pat-2* mutant, an alternative parthenocarpic mutant non-allelic to *pat*, yields fruits of the same shape and weight as the corresponding wild-type fruits (Philouze et al. 1988). However, *pat-2* is more facultative than *pat* (Lin et al. 1984). While facultativeness simplifies increasing seeds of parthenocarpic stocks, it hinders selection in breeding schemes. A lack of information about the map position of the *Pat-2* locus has prevented the development of molecular markers for assisted selection. A third parthenocarpic system, *pat-3*/*pat-4*, presents the problem of polygenic inheritance as well as smaller fruits (Nuez et al. 1986). In addition, all of the described parthenocarpic genotypes exhibit reduced firmness of the seedless fruits (Philouze et al. 1988; Santangelo et al. 1990; Gorguet et al. 2005), which further limits the adoption of genetic parthenocarpy in tomato breeding.

The recent elucidation of the molecular genetic mechanisms controlling fruit set in tomato has paved the way for harnessing new mutations for parthenocarpy. The flower-to-fruit transition is under complex multihormonal regulation, and the molecular mechanisms underlying this process continue to be intensively investigated (reviewed by Ruan et al. 2012). The molecular characterisation of mutants (Fos et al. 2000, 2001; Olimpieri et al. 2007; Mazzucato et al. 2008) and extensive reverse genetic studies (Ampomah-Dwamena et al. 2002; Wang et al. 2005; Goetz et al. 2007; Martí et al. 2007; De Jong et al. 2009; Molesini et al. 2009; Ren et al. 2011) have indicated that a network of repressors is established in the ovary of a mature tomato flower to control its growth until fertilisation has taken place.

IAA9, a member of the Aux/IAA family of transcription factors (TFs) in tomato, plays a major role in fruit set, as plants silenced by antisense technology (AS-IAA9) show several IAA-related developmental defects, including strong parthenocarpic behaviour (Wang et al. 2005). AS-IAA9 plants exhibit a dramatic reduction in leaf compoundness, enhanced hypocotyl/stem elongation, increased leaf vascularisation and reduced apical dominance (Wang et al. 2005). At flowering, downregulation of *IAA9* results in a precocious set prior to anthesis of seedless fruits, which are similar in appearance to wild-type tomato fruits in terms of size, skin colour and flesh consistency (Wang et al. 2005). RT-PCR and in situ hybridisation have shown that a tissue-specific gradient of *IAA9* expression is established during flower development, the release of which, upon pollination and fertilisation, triggers the initiation of fruit development (Wang et al. 2009).

A single-base deletion of the *IAA9* gene, resulting in a frame shift and a truncated protein, underlies the *entire* (*e*) tomato mutant, whose simple leaves phenocopy those of AS-IAA9 plants (Zhang et al. 2007). However, the reproductive phenotypes of this mutant have not been described.

Studies in Arabidopsis have unravelled several insights into the structure and function of the Aux/IAA proteins. Aux/IAA are short-lived TFs that regulate downstream auxin responses (Guilfoyle 1998; Reed 2001). These proteins share four highly conserved domains: domain I contains a functionally characterised transcriptional repressor motif, domain II interacts with a component of the ubiquitin–proteasome protein degradation pathway that is essential for auxin signalling, and domains III and IV act as C-terminal dimerisation domains mediating homodimerisation and heterodimerisation among Aux/IAA family members, and dimerisation with similar domains found in Auxin Response Factor (ARF) proteins (Kim et al. 1997; Ulmasov et al. 1997). The presence of IAA promotes Aux/IAA protein ubiquitination and its degradation by the 26S proteasome (Gray et al. 2001). ARFs are thus released from the repressive effect, and auxin response genes are finally activated (Dharmasiri et al. 2005; Tan et al. 2007).

In contrast to Arabidopsis, where loss-of-function mutations fail to provide clues to the physiological significance of Aux/IAA proteins likely due to functional redundancy (Overvoorde et al. 2005), downregulation of *Aux/IAA* genes in tomato frequently results in strong and differentiated phenotypes (Wang et al. 2005; Chaabouni et al. 2009; Bassa et al. 2012; Deng et al. 2012). This has provided an efficient means for deciphering the specific roles of various members of the *Aux/IAA* family, comprising 25 genes in this *Solanaceae* species (Audran-Delalande et al. 2012).

Recently, the Targeting-Induced Local Lesions IN Genomes (TILLING) strategy was developed to investigate the functions of specific genes and to provide a non-transgenic tool for improving domesticated crops by identifying novel genetic variations in genes that affect key traits (Slade et al. 2005). The screening of a TILLING resource in the tomato Micro-Tom genetic background yielded three *IAA9* mutant alleles showing vegetative and reproductive phenotypes comparable to those of AS-IAA9 (Saito et al. 2011). Genotypes carrying these alleles show variable rates of parthenocarpic fruit development from emasculated flowers (Saito et al. 2011).

In the current study, to identify novel loss-of-function alleles affecting the *IAA9* coding sequence, we performed screening using a TILLING platform developed in the genetic background of Red Setter, a processing cultivar with a determinate habit (Minoia et al. 2010). Due to their expected parthenocarpic phenotype, *IAA9*-mutated lines can be valuable for breeding seedless tomato varieties and for further elucidating the role of *IAA9* in the machinery controlling fruit set.

Materials and methods

TILLING screening

To search for induced point mutations in the *IAA9* gene, the Red Setter TILLING platform described by Minoia et al. (2010) was employed. Red Setter is a processing tomato variety with a determinate growing habit, which is suitable for mechanical harvesting, showing a very early and concentrated set of elongated fruits with remarkable firmness. The molecular analysis was based on nested-PCR and was carried out using two pairs of gene-specific primers designed based on the deposited sequence for *IAA9* [GenBank: AJ937282 and Solyc04g076850]. These primers amplify the first four exons including the first three conserved domains (Fig. S1). In the first PCR, 4 ng of eightfold pooled genomic DNA was used as a substrate for amplification with the external primers 5′-TGGGTCTATCTGATTGTTCGTC-3′ (F1, forward) and 5′-GAGCAGAAGATAGCTCCTGGTA TG-3′ (R1, reverse). Subsequently, 1 µl of the first PCR reaction served as a template for the second amplification using the internal primers 5′-TCCAC CTCATCAGAGGACAA-3′ (F2, forward) and 5′-TCAAATAGGGAGCACCATCC-3′ (R2, reverse). The internal primers were 5′-end labelled with IRDye 700 and IRDye 800 dye (LI-COR®, Lincoln, NE, USA), respectively. The PCR amplifications were carried out according to the experimental conditions described in Dalmais et al. (2008).

Mutation detection was performed with the mismatch-specific endonuclease *ENDO I* (Triques et al. 2007) and a LI-COR 4300 DNA analyser (LI-COR®). Gel images were analysed using Adobe Photoshop software (Adobe Systems Inc., San José, CA, USA). After discovery, mutations were validated by Sanger sequencing. Sequence alignment was performed using the ClustalW (version 1.82) multiple sequence alignment program (Thompson et al. 1994). SIFT software (Ng and Henikoff 2003) was used to predict the effect of amino acid changes on protein activity.

Genotyping of mutant plants

M_3 seeds of the families containing mutations were recovered, and 15 M_3 plants per family were grown in a greenhouse and genotyped by Sanger sequence analysis. Plants homozygous for the mutations C274T and C512T and for the single-base deletion T618 (hereafter referred to as T618*) were identified and used in a first backcross to Red Setter (hereafter referred to as WT). BC_1F_1 plants were selfed, and BC_1F_2 progenies were genotyped for the respective mutations as described below. From homozygous BC_1F_2 mutant plants, a further selfing was adopted to obtain BC_1F_3 seed stocks fixed for the mutations (hereafter referred to as *iaa9-274*, *iaa9-512* and *iaa9-618* mutant lines for the C274T, C512T and T618* alleles, respectively).

To genotype the C274T and C512T mutations, codominant cleaved amplified polymorphic sequence (CAPS) markers were developed. Genomic DNA extracted according to Doyle and Doyle (1987) was PCR-amplified using the external primers employed for the TILLING screening, and the 820-bp-long PCR fragment was digested with *Bp1I* and *RsaI* for the assessment of each mutation, respectively. The homozygous allelic condition of the C274T mutation was revealed by the presence of two *Bp1I*-digested DNA bands (687 and 133 bp), while the WT allele showed the uncut DNA band. For the C512T mutation, the 587 and 233 bp *RsaI* digestion products enabled plants homozygous for the mutation to be distinguished from the WT allelic condition, which presented three digested DNA bands (464, 233 and 123 bp). Progeny segregating T618* were genotyped by sequencing.

Real-time PCR

Young leaves from 30-day-old WT plants and BC_1F_2 plants homozygous for the three mutations were collected and quick-frozen in liquid nitrogen. From the BC_1F_2 population segregating T618*, heterozygous and wild-type sibling plants were also sampled. In addition, *IAA9* expression analysis was performed using ovaries dissected from WT and *iaa9-618* flowers 2 d before and 2 d after anthesis. Plant tissues were ground to a powder with a mortar and pestle, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The yield and purity of the RNA samples were assessed by spectrophotometry, and RNA integrity was confirmed by 1 % (w/v) agarose gel electrophoresis.

Next, cDNA was synthesised from 3 μg of three independent RNA preparations using ThermoScript RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Tenfold dilutions of first-strand cDNA were used for real-time PCR (qRT-PCR) amplification experiments. Duplicate quantitative assays for each sample were performed with a SensiMix Capillary Kit (Bioline, Luckenwalde, Germany) in a LightCycler 2.0 system (Roche Applied Science) according to the manufacturer's instructions. Primers used to analyse the expression of *IAA9* were 5′-TAGATGCTTTACCTGATTACGACA-3′ (forward) and 5′-TGCAGACAAACTCCAATATCAAAC-3′ (reverse). Reactions were performed via an initial incubation at 95 °C for 10 min followed by 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s for 40 cycles. Relative expression was calculated with the comparative Ct method using CAC (Solyc08g006960) as a housekeeping gene (Expósito-Rodríguez et al. 2008). Relative expression data were reported as the percentage of the value estimated in the WT control.

Transactivation assay between DR5 auxin response promoter and proteins encoded by three IAA9 mutant alleles

Total RNA was extracted with a Totally RNA™ Kit (Ambion, Inc., TX, USA) from leaf tissue of plants homozygous for the C274T, C512T and T618* mutations, and an aliquot was reverse transcribed with oligo $(dT)_{20}$ using a ThermoScript™ RT-PCR System (Invitrogen, Carlsbad, CA).

The cDNA was solubilised in 18 μl of water, and a PCR was run on the cDNA with primers 5′-ATGTCTCCGCCGCTCCTTGGTGTTG-3′ (forward) and 5′-GCTGGATGGATAGCTTTAATCA-3′ (reverse) using high-fidelity polymerase (Phusion, Finnzymes, Helsinki, Finland) to amplify the complete cds. Then, the PCR products were cloned into the pGMNT vector (Promega, Wisconsin, USA), and the sequences were checked by sequencing. A second PCR was performed on two positive pGMNT clones for each cDNA, and the PCR product was transferred to a pEntry vector (pDonor207, Gateway, Invitrogen) and an expression vector (pEarleyGate 201).

The ability of the IAA9 mutant proteins to in vivo regulate the activity of the synthetic DR5 auxin-responsive promoter fused to the GFP reporter gene (Ottenschlager et al. 2003) was investigated by transient expression experiments using BY-2 protoplasts (Audran-Delalande et al. 2012). The DR5::GFP construct acts as an auxin response reporting element containing AuxREs, which indirectly reflects the auxin response (Ulmasov et al. 1997).

Protoplasts for transfection were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells according to a previously described method (Leclercq et al. 2005). Protoplasts were transfected by a modified polyethylene glycol method as described (Abel and Theologis 1994). For co-transfection assays, aliquots of protoplasts (0.2×10^6) were transformed with either 10 μg of the reporter vector alone (containing the DR5 promoter fused to the GFP reporter gene) or in combination with 10 μg of the *IAA9* mutant constructs as the effector plasmid. A control with the DR5 promoter co-transformed with the WT *IAA9* construct was also performed. Then, the protoplasts were treated (or not) with auxin (50 μM 2,4-D). Transformation assays were performed in three independent replicates. After 16 h, GFP expression was analysed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences, San Jose, CA). For each sample, between 1,000 and 3,000 protoplasts were gated on forward light scatter. The GFP fluorescence per population of cells corresponded to the average fluorescence intensity of the population of cells above the background threshold (set arbitrarily based on a zero DNA transformed control). Data were analysed using Cell Quest software (BD Biosciences, Palo Alto, CA). The activity of the DR5 promoter was measured in the presence of the empty vector as a control (pEarleyGate), the IAA9-WT or the three *IAA9* mutant alleles.

Phenotyping of the iaa9-618 mutant plants

Data on vegetative and reproductive phenotypes were obtained from BC_1F_3 plants of the *iaa9-618* line compared with plants of the WT control.

A trial with BC_1F_3 plants in a protected environment was carried out in a tunnel-shaped, ventilated greenhouse under ambient light conditions in Viterbo (42°26′N, 12°04′E), Italy, in late spring–summer. During the flowering period (month of May), the mean maximum temperature was 29.3 °C and the mean minimum temperature was 12.2 °C, while the mean natural photoperiod was 14.5 h. Twenty-four plantlets per genotype (divided in two replicates) were planted in soil and grown under standard cultural practices.

The number of seedling showing aberrations in cotyledon phenotype was counted and reported as the percentage over the total number of seedlings. On a single plant basis, leaf compoundness (number of simple and compound leaflets of the fourth leaf) and apical dominance (length of the axillary shoot at the second, fourth and last node before the first inflorescence at the time the second inflorescence was opening) were measured.

The time to flowering was estimated by counting the number of leaves produced before the first inflorescence. On the second inflorescence, the type of inflorescence (simple, double or multiple) was scored and the number of flowers counted. A total of 24 flowers, harvested at anthesis from the second inflorescence of each plant, were used to examine the morphology of floral organs, to count their number and to weigh the ovaries. A second flower from the same inflorescence was emasculated 2 d before anthesis and kept unpollinated. The weight of ovaries/fruitlets from unpollinated flowers was measured after 15 d.

The fertility of *iaa9-618* mutant plants was assessed by counting the seeds produced in controlled crosses. Ovule viability was estimated using the mutant as the seed parent and WT plants as pollen donors. Conversely, pollen fertility was estimated by pollinating WT flowers with pollen collected from the *iaa9-618* line.

At maturity, the productive performance was estimated by observing the percentage of fruit set on the first four trusses (taking into account that two flowers from the second truss had been detached for earlier analysis). On the red ripe fruits harvested, the fruit weight, polar diameter and equatorial diameter were measured. Fruits were bulked on a single plant basis yielding 24 data points involving a total of about 120 fruits per genotype. The fruit shape index was calculated as the ratio between polar and equatorial diameter. The potential yield of the first four trusses was calculated considering the total number of fruits (red and green) multiplied by the mean weight of red ripe fruits.

The first two fruits that ripened on each plant were cut, and the soluble solids content (brix) was measured

in the juice obtained after extracting the seeds using a digital refractometer (MA871, Milwaukee, Milwaukee Instruments, Inc., NC, USA) on a single fruit basis. The number of seeds was also counted on a single fruit basis. For the remaining fruits, brix values were measured in the juice extracted in bulk, bulked seeds were counted and the unitary number of seeds per fruit was calculated. Fruit firmness was measured on a representative red ripe fruit per plant with an Instron Universal Testing Machine (Instron Ltd., High Wycombe, UK) at 10 N and 25 m/s of speed. On the *iaa9-618* line, trusses of orders higher than four were also harvested to extract the seeds.

The open-field trial was set up with BC_1F_3 plants in the summer in Lavello (41°03′N, 15°48′E; Potenza province), Italy, a location and growing season particularly favourable for processing tomato. During the flowering period (15th June–15th July), the mean maximum temperature was 30.2 °C and the mean minimum temperature was 16.5 °C, while the mean natural photoperiod was 15.0 h. A randomised block design with three replicates and 30 plants per replicate for the WT and *iaa9-618* line, respectively, was adopted. At maturity, the five best performing plants per replicate were sampled, and on a single plant basis, red ripe fruits were harvested and counted together with green and rotten fruits. Using ten representative fruits per replicate, the productive and qualitative traits were measured (fruit weight, fruit diameters, number of seeds, brix and firmness) or calculated (shape index) as described above. The potential yield was calculated considering the total number of fruits (red, green and rotten) multiplied by the mean weight of red ripe fruits.

Statistical analysis

The significance of differences in expression data and DR5 promoter activity was tested on raw data by analysis of variance adopting the General Linear Model (GLM) using SAS software (SAS 2004). Data were graphically reported as a percentage of the value detected in the WT or under control conditions. Significance between mean values was estimated by *Tukey*'s HSD test carried out on raw data. The significance of differences between genotypes for all other phenotypic traits was assessed by Student's *t* test using Microsoft Excel. However, to compare estimates made as percentages, homogeneity χ^2 analysis was adopted, where expected values were determined on the basis of totals in lines and columns in a 2×2 table. The regression between fruit weight and number of seeds was calculated for WT and *iaa9-618* fruits by SAS (PROC REG).

Results

Identification of new allelic variants of the IAA9 gene

To identify novel mutations in the *IAA9* coding sequence, we employed TILLING as a reverse genetic approach (McCallum et al. 2000) using the ethyl methane sulphonate (EMS) mutant collection generated in the genetic background of the processing tomato cultivar Red Setter (Minoia et al. 2010). Specific primers were designed based on the *IAA9* cDNA sequence to amplify the first four exons of the gene including the first three conserved domains (Fig. S1).

The screening of 5,200 M_3 families allowed the identification of three mutations located in the *IAA9* coding region. Two mutations, C274T and C512T, consisted of a single-base substitution (transition) while the third, T618*, was a single-base deletion. At the protein level, C274T and C512T caused amino acid changes leading to the substitution of proline with serine at position 92 (P92S) and threonine with isoleucine at position 171 (T171I), respectively (Fig. 1). The deletion of thymine at position 618 of the *IAA9* coding region led to a frame shift and the formation of a premature stop codon, generating a predicted truncated protein of 210 amino acids in comparison with the 349 residues of the WT protein. The first 205 amino acids of the truncated and WT IAA9 proteins were identical, while the frame shift caused five amino acid substitutions (Fig. 1).

By considering the position of the mutations with reference to the four functional domains of the IAA9 protein, the P92S and T171I amino acid substitutions fell within the protein region between domain I and II (Fig. 1). These substitutions were both considered to be functionally tolerated according to SIFT analysis (data not shown). The premature stop codon caused by T618* was localised at the end of domain II, leading to an IAA9 protein devoid of domain III and IV (Fig. 1).

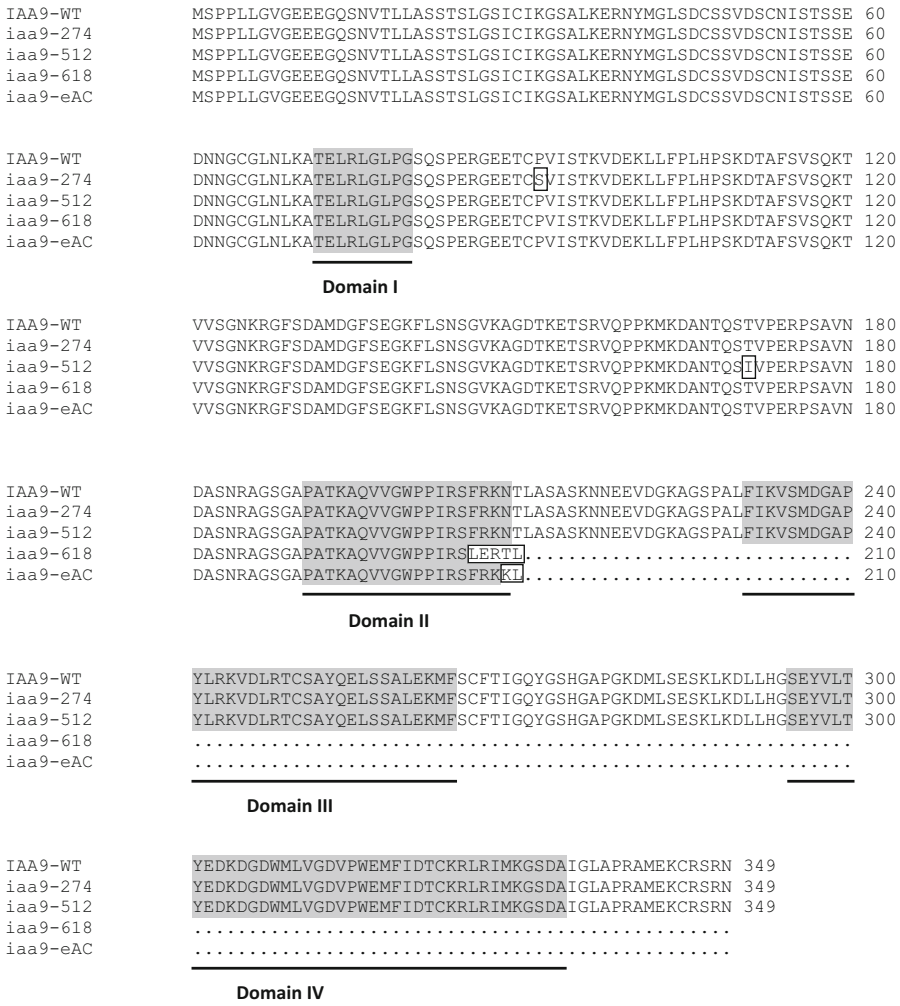

Fig. 1 ClustalW protein alignment of the IAA9 wild-type (IAA9-WT) and three mutant proteins (iaa9-274, iaa9-512 and iaa9-618) identified by TILLING screenings. The IAA9 amino acid sequence of the natural mutant *entire* AC (iaa9-eAC), having a single-base deletion at the nucleotide position 626 of the *IAA9* mRNA (Zhang et al. 2007), has also been included in the comparison. The amino acid changes are *boxed*, while the conserved functional domains of the IAA9 protein are highlighted in *grey*

Impact of the three mutations on IAA9 mRNA steady state levels and the functionality of the IAA9 protein

Since auxin signalling is characterised by high self-regulating dynamics, we set out to measure the expression of *IAA9* in leaves from the WT and the three mutants. The two missense mutations showed a transcription of the *IAA9* gene that was not different from that of the WT; conversely, the *iaa9-618* leaves showed a more than fourfold reduction in *IAA9* transcript levels (Fig. 2a). The association of these low *IAA9* mRNA levels with the homozygous state of the mutation was confirmed by the WT-like gene expression levels of siblings homozygous for the WT allele or heterozygous for T618* (Fig. 2a). In addition, the reduction in *IAA9* transcripts in the *iaa9-618* mutant was detected in ovaries at the fruit set stage, where the mRNA levels of the gene were barely 10 % of those found in WT organs (not shown).

To investigate the impact of the mutations on the ability of the corresponding Aux/IAA proteins to in vivo regulate the activity of the synthetic DR5 auxin-responsive promoter, we carried out transient expression experiments using BY-2 protoplasts. DR5-driven GFP expression was enhanced up to 25-fold by auxin treatment, and the presence of WT IAA9 proteins strongly repressed this auxin-induced activity of the DR5 promoter (83 % repression; Fig. 2b). The iaa9-274 and iaa9-512 mutant forms of the protein retained the same repression activity as the WT protein. Confirming the sequence-based hypothesis

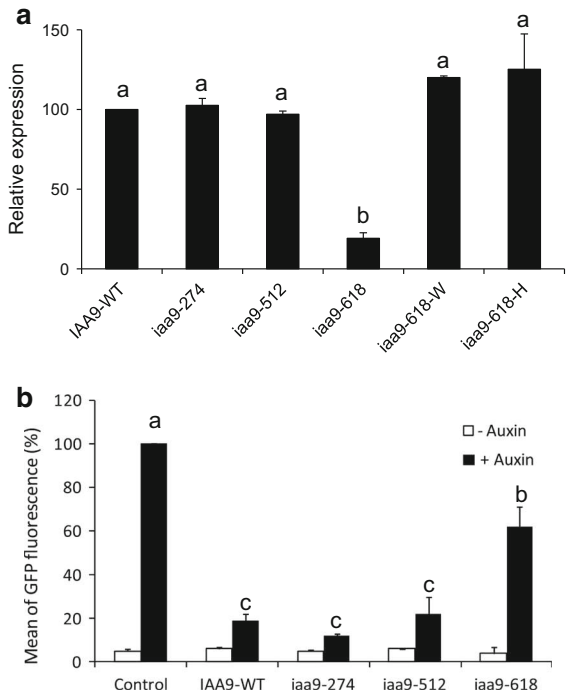

Fig. 2 Transcript levels of *IAA9* gene and transcriptional repression activity of wild-type and mutated IAA9 proteins. **a** Transcript levels of *IAA9* measured by qRT-PCR in young leaves of wild-type plants (IAA9-WT), plants homozygous for the three *IAA9* mutations (iaa9-274, iaa9-512 and iaa9-618) and siblings homozygous for the WT allele (iaa9-618-W) and heterozygous for T618* (iaa9-618-H). **b** Activity of the DR5 promoter measured in the presence of the empty effector vector used as a control (Control), vector containing the wild-type *IAA9* allele (IAA9-WT) or the three *IAA9* mutant alleles (iaa9-274, iaa9-512 and iaa9-618) without (*white bars*) or with treatment with 50 µM auxin (*black bars*). In both histograms, *bars* represent the mean ± SEM of independent experiments ($n = 3$) expressed as a percentage of the mean WT or control value. Means indicated by the same lowercase letter are not statistically different at $P \leq 0.001$ according to *Tukey*'s HSD test carried out on raw data

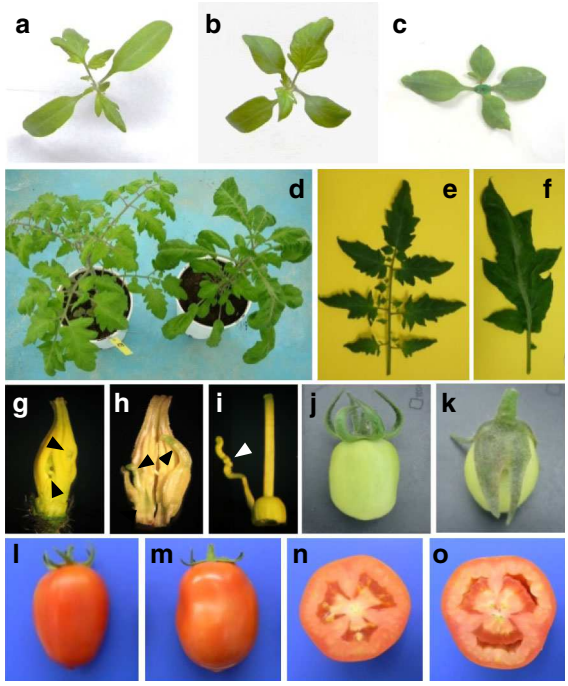

Fig. 3 Vegetative and reproductive phenotypes of the *iaa9-618* mutant line compared with the Red Setter cultivar (WT). **a** WT seedling and **b, c** *iaa9-618* mutant seedlings showing cotyledonary defects. **d** WT (*left*) and *iaa9-618* (*right*) six-week-old plants. **e**, **f** Representative leaf morphology in the WT and *iaa9-618* mutant, respectively. **g–i** Details of carpelloid structures (*arrowheads*) in the anther cones of *iaa9-618* flowers. **j** WT and **k** *iaa9-618* developing fruits 3 weeks post-anthesis. **l** WT and **m** *iaa9-618* whole mature fruits and **n, o** in cross section, respectively

of IAA9 loss-of-function, the truncated iaa9-618 form lost a significant amount of its capacity to repress DR5-driven GFP expression (Fig. 2b).

Phenotyping of the iaa9-618 mutant line

In addition to the targeted mutations, EMS-mutated plants carry a large number of background mutations. To help confirm that the observed phenotype was due to the targeted mutations, we carried out a first backcross to the WT to halve the genetic load of the mutagenised lines. Moreover, in the BC_1F_2 segregant population, we examined plants homozygous for the WT allele and heterozygotes for the expected phenotypes in parallel with homozygous mutant plants. Comparisons between WT and mutant lines were finally carried out using the Red Setter cultivar as a control in all experiments.

As suggested by the functional characterisation, the two missense mutant lines showed no clear phenotypic difference compared with the WT for the traits expected as a consequence of *IAA9* silencing (Wang et al. 2005). Therefore, we carried out a thorough phenotypic characterisation only for the *iaa9-618* line. With respect to the phenotypic effects described below, the *IAA9* T618* mutation behaved as a monogenic recessive.

Vegetative development

Aberrations in cotyledon morphology were never found in the WT (Fig. 3a) or in BC_1F_2 siblings

homozygous for the WT allele. Conversely, these aberrations were evident in plants homozygous for the *IAA9* T618* mutation, with an overall penetrance of 47.1 % (Table 1). Cotyledon aberrations consisted of "leafy" cotyledons and tricots (Fig. 3b). Plants carrying the T618* mutation showed additional cotyledonary defects, such as a wider lamina (Fig. 3c), often with varying degrees of distortion (not shown).

Compared to the WT, the *iaa9-618* mutant line exhibited leaves with a significant reduction in compoundness and a minimally lobed leaf margin (Fig. 3d–f; Table 1). The association of the leaf phenotype with the lesion in the *IAA9* gene in this line was confirmed by the phenotype of siblings heterozygous for T618* or homozygous for the WT allele, which exhibited leaf compoundness comparable to that of the WT (not shown).

Although the pattern of axillary shoot growth was variable in the analysed plants, *iaa9-618* mutants showed significantly longer axillary shoots at two out of three examined nodes compared to the WT (Table 1).

Reproductive development

The *iaa9-618* mutant line did not differ from the WT in terms of flowering time, number of flowers per inflorescence or inflorescence type, maintaining the simple inflorescence typical of Red Setter (not shown). The floral organ number was also not different in the mutant. The most relevant difference in floral morphology was a homeotic transformation of stamens, which showed carpelloid features; this trait was 41 % penetrant in the *iaa9-618* line (Fig. 3g–i; Table 1). Moreover, the mutant flowers exhibited fusion of sepals at the insertion in the receptacle, a phenotype that was remarkably evident during early fruit growth (Fig. 3k).

We first evaluated the parthenocarpic capacity of the mutant plants by measuring the fresh ovary weight at anthesis and the capability of emasculated flowers to develop in the absence of fertilisation. As happens in other parthenocarpic systems (Mapelli et al. 1978; Fos and Nuez 1991; Carmi et al. 2003), the ovary weight at anthesis was significantly higher in the *iaa9-618* mutant line than in the WT (Table 1). The weight of fruitlets that developed after emasculation was also higher in the mutant, although this value was highly variable because only approximately 40 % of the emasculated flowers showed parthenocarpic development of unpollinated ovaries (not shown).

Production data

For plants grown in a protected environment, the fruit set was higher in the *iaa9-618* line than in the WT (Table 2). Compared to the WT (Fig. 3l), *iaa9-618* fruits (Fig. 3m) had a higher mean weight, a more elongated shape and a similar potential yield (Table 2). As expected, the frequency of seedless fruits was very low in the WT (Fig. 3n) and high in the mutant (Fig. 3o). In addition, the seeded mutant fruits carried, on average, approximately half the number of seeds present in WT fruits under the same growth conditions (Table 2). The internal fruit structure was similar in WT and mutant fruits; the increase in

Table 1 Vegetative and reproductive traits in the cultivar Red Setter (WT) and the *iaa9-618* mutant line

Trait category	Trait	WT	*iaa9-618*	*P* value[a]
Vegetative traits	Seedlings with aberrant cotyledon morphology (%)	0	47.1	≤0.01
	No. of simple leaflets	9.2	1.5	≤0.01
	No. of compound leaflets	5.3	0	≤0.01
	Length of axillary shoot at 2nd node (cm)	11.5	25.9	≤0.01
	Length of axillary shoot at 4th node (cm)	9.5	20.3	≤0.01
	Length of axillary shoot at last node (cm)	16.0	21.9	0.06
Reproductive traits	Carpelloid stamens (%)	6.3	41.0	≤0.01
	Ovary weight at anthesis (mg)	5.9	7.5	≤0.01
	Ovary/fruitlet weight from unpollinated flowers 15 DAE (mg)[b]	8.5	53.2	0.03

[a] According to Student's *t* test or, for estimates made as percentages, to homogeneity χ^2 analysis

[b] DAE, d after emasculation

Table 2 Productive traits in the cultivar Red Setter (WT) and the BC_1F_3 *iaa9-618* mutant plants grown under greenhouse and open-field conditions

Trait	Greenhouse			Field		
	WT	*iaa9-618*	*P* value	WT	*iaa9-618*	*P* value[a]
Fruit set (%)	44.6	55.4	0.05	–[b]	–	–
Fruit weight (g)[c]	58.5	72.6	0.02	65.5	76.6	≤0.01
Fruit shape index	1.25	1.42	≤0.01	1.21	1.20	0.21
Potential yield (g plant^{-1})	742[d]	857[d]	0.34	3661[e]	1907[e]	0.02
Seedless fruits (%)	3.5	68.1	≤0.01	0	93.3	≤0.01
No. of seeds per fruit	20.5	11.8[f]	0.03	59.3	19.0[f]	≤0.01
Brix value	4.23	5.08	≤0.01	4.02	4.84	≤0.01
Firmness	2.53	2.37	0.27	2.26	2.38	0.28

[a] According to Student's *t* test or, for estimates made as percentages, to homogeneity χ^2 analysis

[b] Not determined

[c] Calculated on about 120 and 30 fruits harvested from plants grown in greenhouse and open-field conditions respectively

[d] Calculated for the first four trusses

[e] Calculated for the whole plant at maturity

[f] Calculated only for fruits with seeds

pericarp thickness described by Saito et al. (2011) in *iaa9* mutants in the Micro-Tom background was not observed in *iaa9-618*. Finally, *iaa9-618* fruits showed a significantly higher brix value and similar firmness compared to seeded fruits produced by the WT (Table 2).

To determine whether the quality traits of the mutant fruits were related to their seedless or seeded condition, we measured the fruit weight and shape, brix value and seedlessness on a single fruit basis in a sub-sample of fruits. For all traits, seedless and seeded *iaa9-618* fruits did not show significantly different values (Table S1).

We also compared the productive performance of the *iaa9-618* line to that of the WT in the open field. In the field, the fruit weight and brix value were higher in the mutant than in the WT, but the fruit shape index was similar between the two genotypes (Table 2). The mutant line exhibited a high degree of parthenocarpy (approximately 93 % seedless fruits and one-third the number of seeds in mutant seeded fruits compared to WT), although the potential yield under open-field conditions was lower in the *iaa9-618* plants (Table 2). As observed under protected cultivation, the two genotypes produced fruits with comparable firmness (Table 2).

The fertility of the *iaa9-618* mutant plants was indirectly assessed in controlled crosses. When used as the seed parent and pollinated with WT pollen, *iaa9-618* plants produced approximately 60 seeds per fruit, whereas WT plants pollinated with *iaa9-618* pollen produced approximately 50 seeds per fruit. These values, which are comparable to the values for seed production in the WT under open (Table 2) or hand pollination in the field (not shown), indicate that the *iaa9-618* plants did not exhibit a major decline in fertility.

The capacity for seed production in the mutant line was influenced by inflorescence order; more than 80 % of the seeds obtained from *iaa9-618* plants in the BC_1F_3 generation under greenhouse cultivation were produced by trusses of order higher than four, whereas fruits of the first four trusses were either seedless or contained very few seeds (not shown).

Discussion

To identify novel sources of parthenocarpy to be used for breeding, we harnessed mutant alleles of the *IAA9* gene by TILLING using a platform developed in a processing tomato genetic background. It was previously reported that silencing of this gene specifically induces parthenocarpy in tomato (Wang et al. 2005; Saito et al. 2011). The screening of 5,200 M_3 families yielded three mutant tomato lines with genetic lesions

in the *IAA9* coding sequence. Of these, two carried a point mutation leading to amino acid substitutions and the third carried a single-base deletion leading to a frame shift and a premature stop codon. This stop codon was coincident with that predicted in the *e* mutant (Zhang et al. 2007; Fig. 1).

The iaa9-618 mutation affects both the transcript level of the gene and the function of the protein

In accordance with the predicted tolerability of the relative mutations, the *iaa9-274* and *iaa9-512* mutant lines were not different from the WT in terms of gene transcription or the capacity of the respective proteins to repress the activation of the auxin-inducible DR5 promoter. Conversely, plants carrying the frame-shift mutation showed remarkable phenotypes when analysed at the transcriptional or protein activity level. Compared with the WT, the missense mutant lines and siblings heterozygous for the *IAA9* T618* mutation or homozygous for the WT allele, *iaa9-618* mutants showed a strong reduction in the transcription level of the *IAA9* gene. Accordingly, low levels of *IAA9* expression were also found in the *e* mutant (Wang et al. 2005; Zhang et al. 2007). This reduction was even more dramatic in ovaries, suggesting the occurrence of feed-forward regulation or nonsense-mediated mRNA decay (NMD) mechanisms. NMD functions as a quality control mechanism to eliminate abnormal transcripts (Lejeune and Maquat 2005).

Moreover, analysis of protein functionality showed that the truncated protein lost approximately 68 % of its repressing ability. Thus, even if the iaa9-618 protein lacks functional domains III and IV, part of its activity is maintained in the absence of dimerisation or alternatively, the protein can still form functional complexes in its truncated form.

Taken together, the data indicate that among the three mutated versions of the IAA9 protein, only the truncated form resulted in a partial loss of IAA9 function due to a combination of reduced gene transcription and reduced protein functionality.

The IAA9 T618* mutation causes IAA-related vegetative and reproductive defects

Observation of plants carrying the missense mutations in the homozygous state did not reveal the phenotypes expected from the analysis of AS-IAA9 tomato plants (Wang et al. 2005). Conversely, characterisation of plants carrying the frame-shift mutation T618* revealed severe phenotypes, which were in agreement with the expected phenotypes.

Vegetative phenotypes such as those affecting cotyledon number and morphology were previously observed in AS-IAA9 (Wang et al. 2005) and in other tomato parthenocarpic backgrounds such as *TPRP-F1::rolB* (Carmi et al. 2003) and the *pat* mutant (Olimpieri et al. 2007). Thus, regulatory elements controlling ovary growth also play a role in the control of cotyledon patterning during embryogenesis. Other vegetative phenotypes in agreement with *IAA9* silencing (Wang et al. 2005; Zhang et al. 2007; Berger et al. 2009; Saito et al. 2011) were observed in the *iaa9-618* line, such as defects in apical dominance and a reduction in leaf compoundness. These phenotypes support the important role played by auxin in patterning leaf morphogenesis and dissection (Molesini et al. 2009; Ren et al. 2011).

In parallel with other IAA9 knock-out genotypes (Wang et al. 2005, 2009; Saito et al. 2011), *iaa9-618* mutant plants showed a strong tendency to develop parthenocarpic fruits. Ovaries of mutated plants at anthesis weighed more than WT ovaries and showed the capacity to develop in emasculated, unpollinated flowers. As the *iaa9-618* and *e* mutations produce very similar truncated proteins, it will be interesting to investigate the reproductive behaviour of the *e* mutant.

Notably, *iaa9-618* flowers exhibited carpelloid stamens, a phenotype not described in AS-IAA9 (Wang et al. 2009) or *IAA9* mutants (Zhang et al. 2007; Saito et al. 2011). This novel phenotype co-segregated with the *IAA9* T618* mutation. Although it cannot be excluded that the carpelloidy of stamens was driven by a linked mutation, several lines of evidence suggest that this phenotype is a pleiotropic effect of IAA9 loss-of-function. Carpelloidy in *iaa9-618* parallels the association between parthenocarpy and stamen defects found in parthenocarpic (Mazzucato et al. 1998) and male sterile (Gomez et al. 1999; Olimpieri and Mazzucato 2008) tomato mutants and in plants downregulated for stamen identity genes (Lifschitz et al. 1993; Ampomah-Dwamena et al. 2002; Geuten and Irish 2010). A similar, partially penetrant phenotype was also observed in the flowers of the tomato *pat-2* mutant (A. Mazzucato and F. Ruiu, unpublished data). Accordingly, the expression of floral identity genes is modulated by pollination in

wild-type flowers, and this modulation is altered in flowers of parthenocarpic mutants (Mazzucato et al. 2008) and of plants engineered for parthenocarpy (Wang et al. 2009). *DEFICIENS* (*DEF*), a class B MADS-box gene that is responsible for the identity of the second and third floral whorl (Weigel and Meyerowitz 1994), is also expressed and modulated in the ovary at fruit set (Mazzucato et al. 2008; Vriezen et al. 2008). The expression of *DEF* is also significantly lower in *iaa9-618* ovaries than in WT ovaries at the same developmental stage (A. Mazzucato and F. Ruiu, unpublished data). Thus, *DEF* shows additional functions in the fourth whorl, taking part in mechanisms mediating the ovary-to-fruit transition. Accordingly, downregulation of genes orthologue to *GLOBOSA* (*GLO*) that encodes the functional partner of *DEF*, is correlated with the post-pollination development of the ovary in *Phalaenopsis* (Tsai et al. 2005) and with parthenocarpy in apple (Yao et al. 2001). In this context, carpelloidy in *iaa9-618* stamens may be related to a deregulation of *DEF*, which in turn is also involved in the parthenocarpic behaviour of the ovary.

The iaa9-618 line may be useful for breeding parthenocarpic tomatoes

Genetic parthenocarpy represents an interesting breeding goal because it offers the opportunity for higher yields under conditions that are suboptimal for pollination, as well as the opportunity for higher fruit quality in terms of soluble solids content. However, parthenocarpic mutants have not been widely adopted in tomato breeding because they often exhibit fruit defects (smaller size, misshapen aspect and lower firmness) or difficulties in seed production (Table S2). Since known mutations for parthenocarpy in tomato are recessive, they must be introgressed in both parental lines in hybrid breeding; this requires simple genetic control of parthenocarpy and mapping information for assisted selection. None of the previously described sources of parthenocarpy in tomato fulfil all of these requirements.

Plants carrying the T618* frame-shift mutation described here showed fruits the same size as, or larger than, those of the WT, with normal shapes, similar firmness and higher brix values. As reported in other parthenocarpic systems (Santangelo et al. 1990; Carmi et al. 2003), the correlation between fruit weight and seed content found in wild-type tomatoes was not observed in fruits of the *iaa9-618* line (Fig. S2). Interestingly, characteristic traits distinguishing *iaa9-618* from WT fruits (higher weight and brix values and a more elongated shape) were similar in seeded and seedless *iaa9-618* fruits.

Few reports have compared the yield potential of parthenocarpic lines to their corresponding, near isogenic, non-parthenocarpic counterparts. Under standard growth conditions, parthenocarpy is thought to enable yields similar to those of wild-type tomatoes, whereas it represents an advantage under conditions limiting pollen production and fertilisation. It is generally recognised that the maximum expressivity of parthenocarpy occurs under environmental conditions with low night temperatures and high light intensity (Santangelo et al. 1990; George et al. 1984). The greenhouse cultivation performed in the current experiment represents a pollination-limiting environment, at least for the night temperature, which averaged below 15 °C, a lower limit for good pollen production and pollination in tomato (George et al. 1984). Indeed, the unitary seed production in WT fruits was one-third that recorded under open-field conditions or in the greenhouse after hand pollination. In controlled environments, fruit set is also hampered by a reduction in wind and insects, which shake the flowers, greatly facilitating pollination and increasing fruit setting in tomato. Under these conditions, the *iaa9-618* line set 25 % more fruits and was estimated to have 15 % higher potential productivity than the WT, although the latter difference was not significant. Finally, the data show that seedlessness in *iaa9-618* is essentially due to the precocious growth of the ovary. Mutant flowers are fertile and seeds may easily be produced by hand pollination or by open pollination at higher plant trusses. Seed increase could be further facilitated if more permissive conditions for seed production are identified, as was reported for various *pat* tomato mutants (Philouze et al. 1988; Mazzucato et al. 1998; Fos et al. 2001).

Conclusion

The data presented here describe T618*, a novel *IAA9* allele that may contribute to our understanding of fruit set and parthenocarpy mechanisms and may facilitate the use of genetic parthenocarpy in tomato breeding. As *iaa9-618* plants set more and larger fruits than the

WT, with higher brix values and comparable firmness, they present the potential for improving yields under pollination-limiting conditions and for breaking the negative correlation between seedlessness and firmness reported in other parthenocarpic mutants (Gorguet et al. 2005). The *iaa9-618* mutant condition is easily selectable by leaf morphology or molecular analysis. In addition, the *iaa9-618* mutant line did not show any major fertility problems and is thus suitable for hybrid seed production and seed increase in the parental lines. Although wider experiments, including growth under harsher growth conditions, are needed to better define the potential of using *IAA9* mutations in breeding, previous (Wang et al. 2005; Saito et al. 2011) and present data suggest that this gene is a promising target for harnessing parthenocarpy in tomato.

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