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Tomato fruit development in the auxin-resistant *dgt* mutant is induced by pollination but not by auxin treatment

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ABSTRACT

In tomato (*Solanum lycopersicum* Mill.), auxin is believed to play a pivotal role in controlling fruit-set and early ovary growth. In this paper we investigated the effect of the reduced auxin sensitivity exhibited by the *diageotropica* (*dgt*) tomato mutant on ovary growth during early stage of fruit development. Here we show that in hand-pollinated ovaries fruit-set was not affected by the *dgt* lesion while fruit growth was reduced. This reduction was associated with a smaller cell size of mesocarp cells, with a lower mean C values and with a lower gene expression of the expansin gene *LeExp2*. When a synthetic auxin (4-CPA, chlorophenoxyacetic acid) was applied to the flowers of wild type plants, parthenocarpic ovary growth was induced. On the contrary, auxin application to the flowers of *dgt* plants failed to induce parthenocarpy. Hand-pollinated ovaries of *dgt* contained higher levels of IAA compared to wild type and this was not associated with high transcript levels of genes encoding a key regulatory enzyme of IAA biosynthesis (*ToFZYs*) but with lower expression levels of *GH3*, a gene involved in the conjugation of IAA to amino acids. The expression of diverse *Aux/IAA* genes and *SAUR* (small *a*uxin *u*p-regulated *R*NA) was also altered in the *dgt* ovaries. The *dgt* lesion does not seem to affect specific *Aux/IAA* genes in terms of transcript occurrence but rather in terms of relative levels of expression. Transcript levels of *Aux/IAA* genes were up regulated in auxin-treated ovaries of wild-type but not in *dgt*.

Together, our results suggest that *dgt* ovary cells are not able to sense and/or transduce the external auxin signal, whereas pollinated *dgt* ovary cells are able to detect the IAA present in fertilized ovules promoting fruit development.

Introduction

In tomato (*Solanum lycopersicum* Mill.), successful completion of pollination and fertilization, determines the development of the ovary into a fruit (Gillaspy et al., 1993). Basically, fruit organogenesis is considered as the result of a fine balance between cell division and cell expansion (Bohner and Bangerth, 1988). After an initial phase of cell division, which lasts 7–10 d, the fruit grows mainly by cell expansion from 10 d after anthesis to the onset of maturation (Gillaspy et al., 1993; Joubès et al., 1999). During fruit growth, the carpel wall develops into the pericarp while the placenta develops into a jelly-like matter called locular tissue. The beginning of cell expansion is characterized by a progressive arrest of mitotic activity concomitant with the endopolyploidy (Joubès et al., 1999). The positive correlation between the mean ploidy level and pericarp cell dimensions and between ploidy level and the final fruit size has been well established (Chevalier et al., 2011).

Control of fruit set and early growth process is dependent on one or more positive cues generated during pollination, and likely after fertilization (Gillaspy et al., 1993). Positive signals for inducing fruit development are believed to be originated from pollen, ovules or vegetative parts of the plant (Vivian-Smith et al., 2001). Possibly, fruit formation may be triggered after the relief of some repressor elements (Olimpieri et al., 2007). It is known that transition from flower to growing fruit can be effectively obtained by exogenous hormones application. In particular, auxin, gibberellins and cytokinins are shown to be able to efficiently trigger fruit growth, leading to the idea that hormones may act as post-fertilization signals coordinating the normal fruit development in time and space (Gorguet et al., 2005; Srivastava and Handa, 2005). Increasing body of evidence indicates that auxin might represent one of the primary signal molecules capable of driving fruit-set (Vivian-Smith et al., 2001; Dorcey et al., 2009). Auxin content is found to increase in tomato ovaries and rice caryopsis once fertilization is

Abbreviations: 4-CPA, 4-chlorophenoxyacetic acid; AC, Ailsa Craig; DAP, days after pollination; *dgt*, diageotropica; GC–MS/MS, chromatography–tandem mass spectrometry; MCV, mean C value; *SAUR*, small auxin up-regulated RNA; *ToFZY*, tomato floozy gene; IPA, indole-3-pyruvic acid.

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completed indicating its strong connection with ovary development (Uchiumi and Okamoto, 2010; Mariotti et al., 2011). Two main routes of indoleacetic acid (IAA) biosynthesis have been proposed: the tryptophan (Trp) dependent and Trp-independent (Benjamins and Scheres, 2008). It is suggested that while the tryptophan-independent pathway controls constitutive levels of IAA, the Trp-dependent pathways is implicated when higher levels are required (Zazimalova and Napier, 2003). Although in tomato both pathways are active, the Trp-dependent one is proposed to be the main IAA source during fruit development (Epstein et al., 2002; Ehlert et al., 2008). The flavin monooxygenases (FMO) encoded by tomato floozy gene (*ToFZY*) gene family are believed to catalyze a rate-limiting step in the tryptamine pathway for indole-3-acetic acid biosynthesis (Expósito-Rodríguez et al., 2007, 2011). Beside new biosynthesis, IAA content is effectively controlled by IAA conjugation and the auxin-inducible GH3 proteins seem to provide a regulatory point to control auxin homoeostasis (Staswick et al., 2005).

It is well known that auxin exerts a rapid and specific regulation on the expression of auxin-inducible genes at transcriptional level (Abel and Theologis, 1996; Hagen and Guilfoyle, 2002). Among these, *Aux/IAA* gene family is considered the most representative class (Ulmasov et al., 1997; Hagen and Guilfoyle, 2002). Since most Aux/IAA proteins function as auxin signal repressors, auxin-mediated Aux/IAA degradation relieves the repression over auxin responsive genes (Tiwari et al., 2001). The balance between *Aux/IAA* gene expression and Aux/IAA protein degradation provides a regulatory loop controlling the steady-state levels of Aux/IAAs and the degree of auxin response (Benjamins and Scheres, 2008).

In tomato, the central role of auxin during the first stages of fruit developmental program has been further supported by the identification of genes associated with auxin perception and signal transduction (Pandolfini et al., 2007). Down-regulation of the tomato *IAA9* gene leads to parthenocarpic fruit formation, suggesting that this transcription element might act as a key repressor of fruit development (Wang et al., 2005). Goetz et al. (2007) have shown that expression of an aberrant form of *Arabidopsis* ARF8 in tomato resulted in parthenocarpic fruit growth, supporting a role for ARF8 in regulating fruit initiation.

Although the *DIAGEOTROPICA (DGT)* gene is known to be one of the first genes associated with auxin perception and signalling in tomato, its role remains to be fully elucidated. The *dgt* mutant exhibits an auxin-resistant phenotype that includes agravitropic bending of shoots, reduced apical dominance, lack of lateral roots, impaired auxin-induced proton secretion and ethylene production (Zobel, 1973; Kelly and Bradford, 1986; Coenen et al., 2002). Balbi and Lomax (2003) documented that the *DGT* gene also affects the early stages of fruit development at least in part by auxin- and ethylene-mediated gene expression. Genetic characterization of the mutation revealed that the *DGT* locus encodes a cyclophilin (LeCYP1; Oh et al., 2006). Cyclophilins, FK506 binding proteins (FKBPs) and parvulins are distinct families of peptydil-prolyl *cis-trans* isomerases (PPIases) that catalyze the rapid isomerization of prolyl bonds from *cis* to *trans* configurations during protein folding (Romano et al., 2005; Wang and Heitman, 2005). Interestingly, the auxin-dependent interaction between Aux/IAAs and the SCF^{TIR1} complex has been proposed to require prolyl isomerization within domain II of Aux/IAA proteins (Dharmasiri et al., 2003). Since LeCyP1 is required for the expression of a subset of Aux/*IAAs*, it is possible that LeCyP1 is localized in the cell nucleus where it may interact with other proteins to regulate the expression of early auxin-response genes (Oh et al., 2006).

In this study we have investigated the effects of the *dgt* mutation on the initial growth phase of ovary development induced by hand-pollination or auxin treatment. We have carried out a comparative characterization, at histological level, of ovary development in *dgt* versus wild-type plants. Endogenous indole-3 acetic acid (IAA) content and the expression of key genes involved in its synthesis and response have been also compared. Moreover, the effect of the *dgt* lesion on the expression of several genes involved in auxin signalling was investigated.

Materials and methods

Plant material

Seeds of tomato (*Solanum lycopersicum* Mill.) cv. Ailsa Craig (AC) were obtained from the Tomato Genetic Resources Center (University of California, Davis, CA, USA) and *diageotropica* (*dgt*) mutant (backcrossed into AC background) were kindly provided by Dr. C. Coenen (Allegheny College, Meadville, PA, USA). Four-week old plants were transplanted in 5 L pots with a mixture of peat:pumice stones (3:1 v/v) and grown in the greenhouse during spring–summer at the University of Pisa Dept. of Biology (Pisa, Italy). Plants were regularly irrigated with a nutrient solution composed of 12 mM N—NO_3, 0.5 mM N—NH_4, 1.30 mM P; 8 mM K; 4 mM Ca; 1.19 mM Mg; 9 mM Na; 1.59 mM S—SO_4; 9.87 mM Cl; 19.5 mM Fe; 28.6 mM B; 3.6 mM Cu; 4.5 mM Zn; 10.9 mM Mn and 0.2 mM Mo. Only four flowers per trusses were left in order to limit fruit competition. Flowers were emasculated one d before anthesis to prevent self-pollination and manually pollinated or treated with synthetic auxin 4-chlorophenoxyacetic acid (4-CPA, Sigma–Aldrich, St. Louis, MO, USA) (Mariotti et al., 2011). Ovary treatments were performed by applying 2 or 10 μL of chlorophenoxyacetic acid (4-CPA) (10 ng μL^{-1} dissolved in 1% of ethanol and 0.1% of Tween 20 solution). Equal volume of solvent was used as mock. Ovaries collected at different times after pollination/treatment, were immediately frozen in liquid nitrogen and stored at −80 °C up to analyses.

Quantification of endogenous IAA

Endogenous IAA were identified and quantified following the procedure described in Mariotti et al. (2011) with minor changes. In summary, 0.5–1 g of frozen fruits were extracted with 80% methanol adding a known amount of deuterated [$^{13}C_6$]-IAA (Cambridge Isotopes Laboratories Inc., Andover, MA, USA) as internal standard. After removing the organic phase, the water fraction was acidified to pH 2.8–3.0 and partitioned with ethyl acetate. Extracts were then purified by an HPLC chromatography apparatus (Kontron, Munich, Germany) equipped with a C18 column (150 mm long, 4.6 i.d., particle size 5 μm, Hypersil ODS, Thermo Fisher Scientific, Waltham, MA, USA). The column was eluted with a linear gradient of methanol and water (0.01% of acetic acid) from 10% to 100% of methanol at the flow rate of 1 mL min^{-1} and fractions corresponding IAA standard retention time were collected. All fractions were dried and trimethylsilylated with N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (Pierce, Rockford, IL, USA) at 70 °C for 1 h.

Finally, IAA were identified and quantified through chromatography–tandem mass spectrometry (GC–MS/MS). Analysis were carried out with a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) using a 1MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Mega, Milan, Italy). The concentration of IAA in the original extracts was determined from the peak area ratio of labelled and non-labelled ions of internal standard and endogenous hormone respectively.

Table 1
Morphological parameters of AC and *dgt* 10 d-old fruit pericarp. Measurements were performed on three representative biological replicates ± SEM.

	Thickness (μm)	No. of cell layers	Cell area (μm²)	
			EM	IM
AC	1988 ± 79*	27.3 ± 0.8 ns	5376 ± 544*	13633 ± 1605*
dgt	1113 ± 31	27.6 ± 0.8 ns	2159 ± 287	3911 ± 339

* Significant differences between AC and *dgt* (Student's *t*-test, $P < 0.05$).

RNA extraction and quantitative Real Time-PCR

Total RNA was isolated from approximately 200 mg of entire fruits using the TRI Reagent® (Sigma–Aldrich) according to the manufacturer's procedures. In brief, after initial homogenization of samples with 2 mL of TRI Reagent®, the supernatant was cleaned by chloroform partitioning. RNA was then precipitated by adding isopropyl alcohol followed by 75% ethanol rinsing steps and finally resuspended in DEPC water.

RNA was then subjected to purification from contaminating DNA with the TURBO DNA free kit (Applied Biosystems/Ambion, Austin, TX, USA) and 5 μg of each samples were reverse transcribed into cDNA with the High-Capacity cDNA Archive Kit (Applied Biosystems). Real Time-PCR was performed with an ABI PRISM 7700 Sequence Detection Systems (Applied Biosystems). Analysis of expression *LeExp2*, *ToFZYs* (*ToFZY1*, *-2*, *-3*, *-4*, *-5*, *-6*) *GH3*, *Aux/IAAs* (*IAA1*, *-2*, *-3*, *-8*, *-9*, *-10*, *-11*, *-16*) and small auxin up-regulated RNA (*SAUR*) genes were measured using 50 ng of cDNA and Power SYBR® Green PCR master mix and specific primers pairs (See Supplementary material) according to the manufacturer's instructions. Transcript levels of all genes were normalized with the expression of *SlEF1α* used as internal control. Each samples derived from a pool and all analyses were repeated twice with similar results.

Microscopy and determination of cellular parameters

Fresh 10 days after pollination (DAP) fruit halves (three representative biological replicates) were fixed in FAA (4% formaldehyde: 5% acetic acid: 50% ethanol v/v) applying vacuum for 1 h and left overnight at 4 °C. After dehydration in ethanol series (50, 70, 90, and 100%) samples were immersed for at least 4 h in xylene:ethanol solution series (1:3, 1:1, 3:1 and finally 100% xylene). Subsequently, tissues were embedded in paraffin at 60 °C for at least 48 h rinsing periodically with fresh paraffin. Sections of 10 μm were stained with haematoxylin and eosin and observed with optical microscope Axio Observer. Z1® (Carl Zeiss MicroImaging GmbH, Jena, Germany). Digital pictures of sections were acquired with AxioCam® (Carl Zeiss MicroImaging GmbH) and analyzed for morphometric measurements with the open source software ImageJ® (http://rsb.info.nih.gov/ij/). According to Serrani et al. (2007), the number of pericarp cell layers was estimated by counting the number of cells along a perpendicular line between epidermis and endocarp. For cell size measurement, pericarp was delimited in external mesocarp (EM, between vascular bundles and epidermis) and internal mesocarp (IM, between vascular bundles and endocarp) (Serrani et al., 2007). Mean cell area was calculated by measuring 90–100 cell in three different sections for each biological replicate.

Nuclear content determination

Nuclear DNA content or ploidy level, was determined in pericarp and locular gel of 10 DAP fruits. 1–2 g of fresh pericarp or locular gel were sampled from a pool of 5 fruits. Tissues were gently chopped with a sharp razor blade in a Petri dish adding 0.5 mL of a nuclei isolation buffer (CyStain PI Absolute P®, solution A: Nuclei Extraction Buffer, Partec GmbH, Münster, Germany). After 90 s of incubation, the extract was filtered with 50 μm nylon mesh (CellTrics®, Partec GmbH) and mixed with 2 mL of staining buffer (CyStain PI Absolute P®, solution B: Staining Buffer, Propidium Iodide solution, RNAse A). More than 5000 nuclei were detected using a flow cytometer (FACSCanto™, Becton Dickinson, Franklin Lakes, New Jersey, USA) equipped with a 488 nm laser. Propidium iodide fluorescence was plotted on a semi-logarithmic scale. Extracts from etiolated hypocotyls of AC seedlings were employed as reference standard for 2 and 4C value. Nuclei counting with different ploidy were performed with the software FacsDiva (Becton Dickinson) and the mean of C value (MCV) corresponds to the sum of the number of nuclei for each ploidy level multiplied by its endoreduplication cycle, divided by the total number of nuclei. Analysis was performed twice.

Results

Effect of dgt mutation on fruit development

We investigated the effects of the *diageotropica* (*dgt*) mutation on fruit set and early fruit growth in hand-pollinated and auxin-treated ovaries. Hand-pollination and auxin treatment were done on emasculated flowers the same day that was equivalent to one day before anthesis.

In hand-pollinated ovaries the *dgt* mutation did not affect fruit-set but size and fruit weight appeared slightly reduced at 10 days after pollination (DAP) (Fig. 1A). 4-CPA application at the dose of 20 ng per ovary was insufficient to produce an effect on ovary growth (data not shown), whereas treatments with 100 ng effectively promoted parthenocarpic fruit development in AC (Fig. 1A).

Although in *dgt* auxin-treated ovaries fruit-set was not affected, the exogenous hormone had only a slight effect on fruit growth induction, almost 8-fold less than in the wild-type after 10 d from the treatment (Fig. 1A). The *dgt* mutation also affects the internal anatomy of hand-pollinated tomato ovaries. Pericarp of *dgt* fruits was significantly thinner in comparison with the pericarp of AC (Fig. 1B). In order to discriminate if this was a consequence of differences in cell number or cell size, the number of cells across the pericarp and the average cell area were measured. Although no significant differences were observed in the number of cell layers, cell area of external and internal mesocarp were strongly reduced in *dgt* (more than 2 and 3-fold respectively, Fig. 1B and Table 1). To ascertain if auxin insensitivity in the *dgt* mutant negatively affected cell wall-modifying proteins, transcript levels of *LeExp2*, a gene reported to be auxin regulated and preferentially induced during early tomato fruit cell expansion (Catalá et al., 2000), were quantified by Real Time-PCR in AC and *dgt* fruits. Both in AC and *dgt*, *LeExp2* expression was high at pollination (0 DAP) but rapidly decreased at 2 DAP. In both genotypes transcript levels peaked at 6 DAP but *dgt* exhibited a lower induction and showed almost no change later on (Fig. 1C).

Cell endoreduplication, leading to an increase of nuclear DNA content, is considered a major determinant for the final cell size (Chevalier et al., 2011). In order to assess if the difference in cell expansion between AC and *dgt* fruit tissues reflected an altered ploidy level, flow cytometry analysis of pericarp and locular tissue

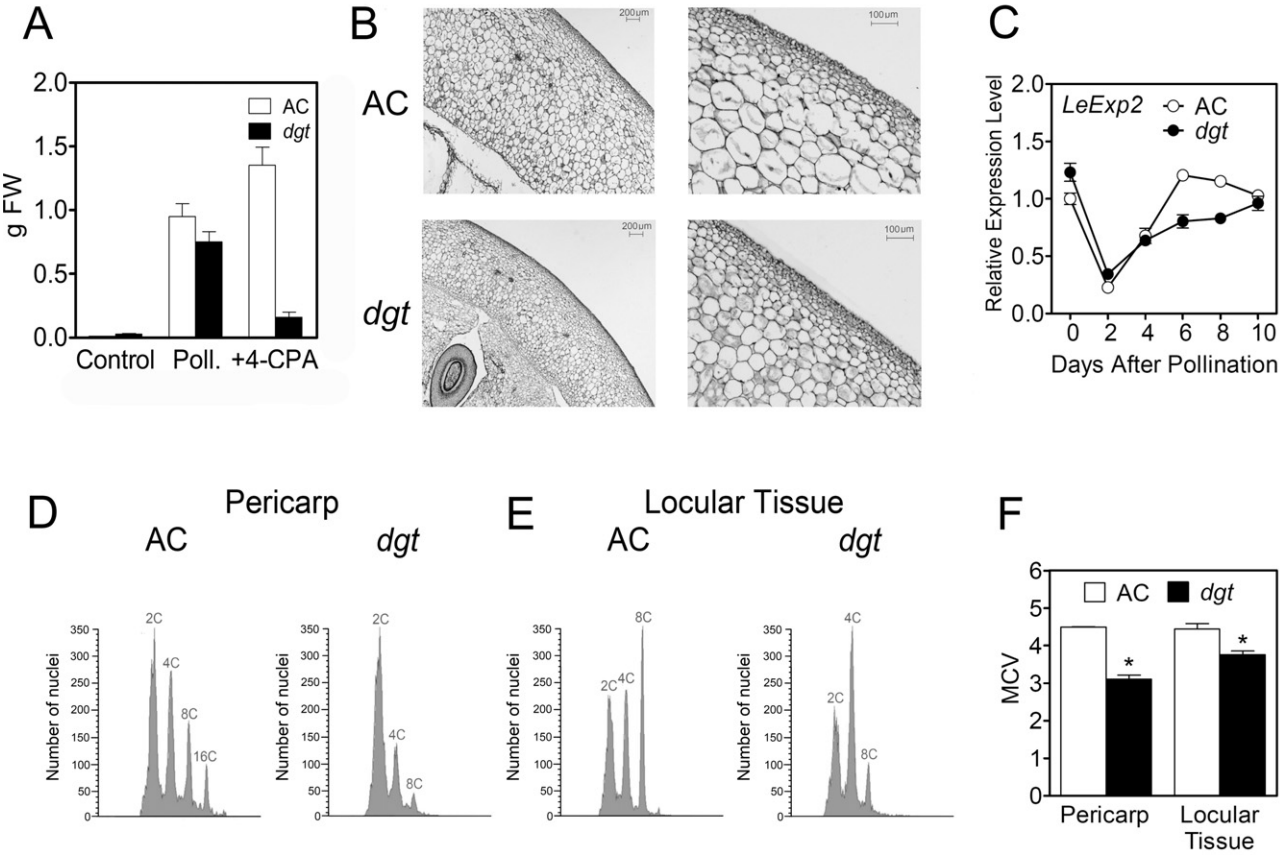

Fig. 1. Effect of *dgt* mutation on fruit development. (A) Mean fruit weight after 10 d in AC and *dgt* hand-pollinated mock treated fruits and in unpollinated and treated with 4-chlorophenoxyacetic acid (4-CPA, 100 ng ovary^{-1}). Unpollinated mock treated ovaries were taken as control. Each point represents mean ± SEM of 20–90 ovaries/fruits. (B) Anatomical features of *dgt* pollinated fruits. Pericarp sections at 10 DAP stage of AC and *dgt* fruits at 10× and 40× of magnification scale. (C) Relative expression levels of *LeExp2* gene in *dgt* and AC pollinated fruits. Analysis were performed by Real Time-PCR as described in Material and Methods. Data correspond to mean and SD ($n = 3$) from a representative experiment. (D and E) Ploidy level in tomato fruit. Nuclear DNA content was analyzed by flow cytometry in AC and *dgt* fruit after 10 d from pollination either in pericarp, D, or in locular tissue, E. (F) Mean of C value (MCV) in pericarp and locular tissue of AC and *dgt*. Data were calculated from measurement performed on a pool of 5 fruit in two independent experiments. Asterisks indicate significant differences between AC and *dgt* (Student's *t*-test, $P < 0.05$).

in AC and *dgt* fruits was performed. As shown in Fig. 1D and E, in AC fruits the pericarp exhibited higher C value heterogeneity than locular tissue. However, there were no visible differences in terms mean of C value (MCV) (Fig. 1F). Approximately 65% of nuclei in the pericarp remained at 2C and 4C, whereas all other nuclei were subjected to other rounds of endocycling reaching 8C and 16C. By contrast, the proportion of 2C and 4C nuclei in *dgt* was noticeably increased up to 85% together with a strong reduction of 8C nuclei and the disappearance of the 16C peak. MCV value was also reduced in *dgt* both in pericarp (3.1 versus 4.5 in AC) and in locular tissue (3.7 versus 4.4 in AC).

IAA metabolism in AC and dgt fruits

To test whether the *dgt* mutation affects auxin metabolism, endogenous levels of free IAA and transcript levels of IAA metabolism genes were investigated in *dgt* and AC hand-pollinated ovaries (Fig. 2).

The level of IAA decreased quite fast from 0 DAP to 2 d after pollination. While a clear peak of IAA concentration was observed in *dgt* ovaries at 4 DAP, this increase was lower in AC. At 6 DAP IAA content declined drastically (from 24.4 to 12.9 ng g^{-1} FW in AC and 32.9 to 13.8 ng g^{-1} FW in *dgt*) reaching the lowest value at 10 DAP (5.9 ng g^{-1} FW in AC and 7.5 ng g^{-1} FW in *dgt*).

To determine whether the slight higher IAA level found in *dgt* ovaries was due to a more active biosynthetic pathway, we performed quantitative analysis of six tomato floozy gene (*ToFZY*) genes (*ToFZY1, -2, -3, -4, -5, and -6)* transcripts throughout the first ten d after pollination both in AC and *dgt* (Fig. 3A).

The highest expression level was that of *ToFZY6* at 0 DAP in both genotypes. *ToFZY6* transcripts dropped drastically in AC ovaries

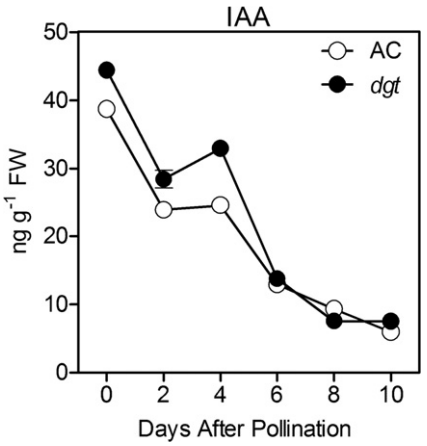

Fig. 2. Endogenous indole-3 acetic acid (IAA) content in pollinated AC and *dgt* fruits. Samples (approx. 1 g of fresh ovaries or fruits) were collected from 1 d before anthesis (0 DAP) to 10 d after pollination. Analyses were carried out with through GC–MS/MS as reported in Materials and Methods. Data are means ± SEM ($n = 3$) from a representative experiment.

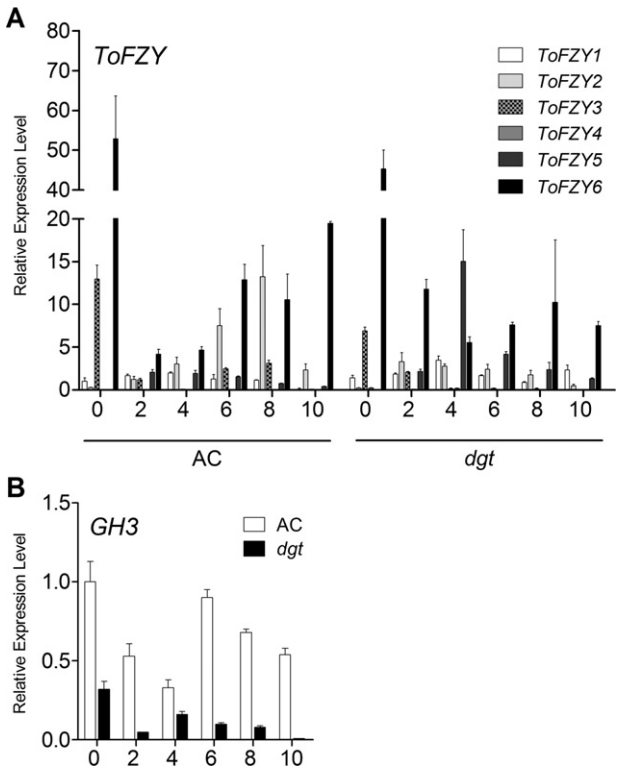

Fig. 3. IAA biosynthetic genes regulation in developing AC and *dgt* fruits. (A) Relative expression levels of *ToFZY* gene family and (B) tomato IAA–amino synthetase *GH3* gene in ovaries and fruits during the first 10 d after pollination. Transcript levels were normalized to the *SlEF1α* expression. Value at 0 DAP of *ToFZY1* (A) and *GH3* gene (B) in AC was set to 1. Data are mean ± SD ($n = 3$) from a representative experiment.

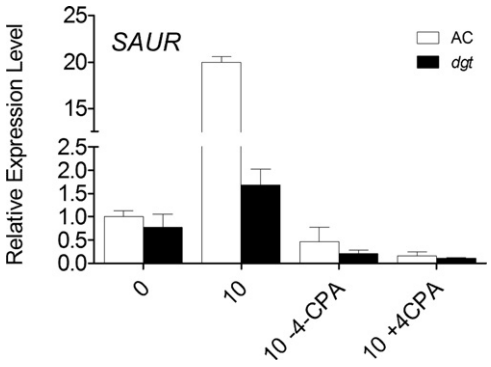

Fig. 5. Effect of pollination and exogenous auxin treatment on tomato *Small Auxin Up-Regulated* (*SAUR*) gene transcript in AC and *dgt* fruit. 4-CPA (100 ng ovary^{-1}) was applied directly to ovaries at one d after anthesis (0 DAP). Samples were collected at the moment of pollination/treatment and 10 d later. Unpollinated mock treated (4-CPA) ovaries were considered as control. Transcript levels were normalized to the *SlEF1α* expression. Transcript levels in wild-type AC (0) sample were set to 1 for each gene, and the levels in *dgt* samples were calculated relative to this. Data are mean ± SD ($n = 3$) from a representative experiment.

at 2 and 4 DAP, while it decreased more gradually in *dgt* ovary (Fig. 3A). In contrast, from 6 to 10 DAP, transcript levels of *ToFZY6* were upregulated in AC but remained more or less constant in *dgt* ovaries. In both genotypes the maximum expression level of *ToFZY3* was detected at 0 DAP but declined from 2 to 10 DAP. This was more evident in *dgt* where undetectable levels were found by the forth d. *ToFZY2* was transiently upregulated at 6 and 8 DAP in AC, while in *dgt* it was not significantly affected. *ToFZY5* showed a slight transient increase at 4 DAP only in *dgt*. Finally, *ToFZY1* and *ToFZY4* were transcribed marginally in developing ovaries of both genotypes.

Staswick et al. (2005) provided evidence that the synthesis of IAA-amino acid conjugates plays an important role in IAA homeostasis and determined that several *Arabidopsis GH3s* genes encode IAA-amido synthetases. *GH3* is the tomato homologue of the *Arabidopsis GH3.6* encoding an IAA-amido synthetase that conjugates amino acids to IAA (Staswick et al., 2005). Fig. 3B shows that the tomato *GH3* mRNA level was quite lower in pollinated *dgt* ovaries than in AC ovaries at all stages of fruit development.

Auxin-response genes expression in AC and dgt fruits

In order to further characterize the role of *dgt* mutation in mediating the auxin response, the expression of various early auxin-responsive genes was investigated in hand-pollinated, in auxin-treated and in unpollinated non-treated AC and *dgt* ovaries. The expression of tomato *Aux/IAAs* (*IAA1, IAA2, IAA3*, *IAA8*, *IAA9*, *IAA10*, *IAA11*, *IAA16*) and small auxin up-regulated RNA (*SAUR*) genes was determined by quantitative PCR at anthesis (0 DAP) and at 10 DAP in *dgt* and AC ovaries (Figs. 4 and 5).

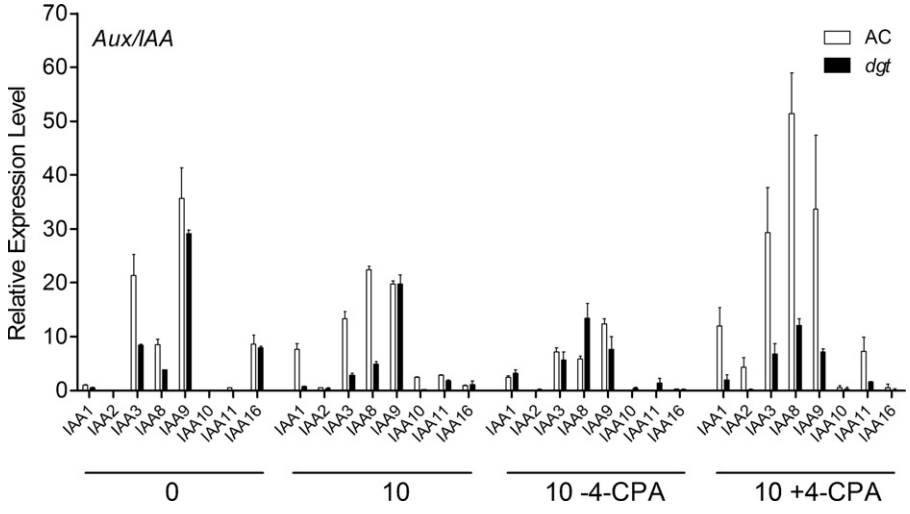

Fig. 4. Effect of pollination and applications of synthetic auxin 4-CPA on transcript levels of *Aux/IAA* genes (*IAA1*, *IAA2*, *IAA3*, *IAA8*, *IAA9*, *IAA10*, *IAA11*, and *IAA16*) in AC and *dgt* fruits. Samples were collected at the moment of pollination/treatment and 10 d later. Expression levels in unpollinated mock treated ovaries (4-CPA) were chosen as control. Transcript levels were normalized to the *SlEF1α* expression. Expression at time 0 of *IAA1* gene in AC was set to 1 and the other levels were calculated relative to this. Data are mean ± SD ($n = 3$) from a representative experiment.

As shown in Fig. 4, *IAA1*, *IAA2*, *IAA10* and *IAA11* gene transcripts were undetected or were at a very low level at anthesis in both AC and *dgt* ovaries but were clearly induced (with the exception of *IAA2*) at 10 DAP in hand-pollinated ovaries of AC plants. In contrast, only *IAA11* gene in *dgt* fruits appeared slightly upregulated.

In the case of *IAA3*, *IAA8*, *IAA9* and *IAA16* transcripts were already present at anthesis but only *IAA8* was significantly induced in hand-pollinated AC ovaries at 10 DAP. The relative expression levels of most *Aux/IAAs* revealed that wild-type ovaries contained a higher level of transcripts compared with *dgt* ovaries at both evaluated stages. Interestingly, auxin treatment caused a strong activation of most *Aux/IAA* genes (*IAA1*, *IAA2*, *IAA3*, *IAA8*, *IAA9* and *IAA11*) in AC ovaries but not in *dgt*, in which the relative expression levels were basically similar to the unpollinated non-treated ovaries (Fig. 4).

SAUR genes are a class of primary auxin response genes first isolated in soybean (McClure and Guilfoyle, 1987) and then in several plants included tomato (Mito and Bennett, 1995). The accumulation of *SAUR* mRNAs has become a molecular assay for rapid auxin action. *SAUR* transcripts were absent at anthesis in both ovaries and were clearly induced at 10 DAP in AC but not in *dgt* hand-pollinated ovaries (Fig. 5). Transcript content of *SAUR* was lower in unpollinated non-treated and treated ovaries of AC and *dgt* at 10 DAP.

Discussion

The auxin-resistant *diageotropica* mutant of tomato provides a tool to further investigate the nature of signalling events during reproductive development.

Balbi and Lomax (2003) reported that fruit-set and early fruit development were dramatically altered in the *dgt* tomato mutant. They argued that the lower fruit-set may be a result of an effect of the *dgt* mutation on pollen release or on fruit-set directly. In our experiments the flowers were hand-pollinated and lower fruit set was not observed suggesting that the reduced auxin responsiveness in *dgt* has no effect on fruit-set. When auxin was applied to the flowers of *dgt* plants, fruit-set was induced but ovary growth was greatly reduced compared with hand-pollinated *dgt* plants. On the contrary, application of auxin to flowers of AC normally induced parthenocarpic growth. Our data suggest that fruit growth in response to either exogenous or internal auxin proceed by different pathways. Rice and Lomax (2000) also found that hypocotyls of the *dgt* mutant do not elongate in response to exogenous auxin but can respond to gravity. Based on these results they suggested the participation of two auxin-induced signal transduction pathways in the gravitropic response mechanism. A slight reduction of fruit growth in hand-pollinated *dgt* ovaries was also observed and it was associated with a reduction in the cell size of mesocarp cells while no difference in the number of cell layers was observed. Characterization of the effects of the *dgt* mutation on auxin and cytokinin responses during *in vitro* plant regeneration, revealed that a DGT-independent auxin response pathway might be involved in the induction of cell division in hypocotyl explants (Coenen and Lomax, 1998). Thus, *dgt* fruits may represent an interesting material to better understand the relative importance of cell division and cell expansion as determinants of final fruit size, as outlined by Balbi and Lomax (2003).

Increase in plant cell size usually results from the combination of two processes: the increase in cell ploidy level by endoreduplication, and cell expansion driven by internal turgor pressure (Perrot-Rechenmann, 2010). During tomato fruit development, strong positive correlations have been established between the mean cell size within the pericarp and the mean ploidy level of various tomato genotypes. Final fruit weight appears also significantly correlated with cell size and ploidy (Cheniclet et al., 2005). Although in *dgt* pericarp and locular tissue the mechanism of endoreduplication appeared not completely impaired, the number of endoreduplicating nuclei and mean C value (MCV) were significantly reduced (in agreement with the difference in pericarp cell size). Cell expansion requires uptake of water and irreversible extension of cell wall which involves several cell wall loosening enzymes (Rose et al., 2002). We found that reduction of mesocarp cell size in *dgt* fruits was accompanied by a lower expression of the expansin gene *LeExp2*. The patterns of expression suggest a specific role for this gene in the regulation of cell wall loosening during the period of rapid tomato fruit growth. Moreover, *LeExp2* transcript levels were reported to be auxin-regulated (Catalá et al., 2000).

Auxin is also known to induce rapid cell expansion, by activating ATPase-driven H^+ secretion across the plasma membrane. Extracellular acidification is believed to activate cell wall loosening enzymes. Coenen et al. (2002) showed that in the hypocotyl of *dgt* mutant, auxin-induced H^+ secretion is impaired without detectable reduction in transcript levels of two ATPase isoforms (LHA2 and LHA4). They suggested that growth is controlled by at least two auxin signalling processes that are dependent on H^+ secretion: a DGT-dependent and a DGT-independent pathway. In addition, Christian et al. (2003) demonstrated that the *dgt* mutation abolishes auxin-induced protoplast swelling and proposed that *dgt* is a signal-transduction mutation interfering with an auxin-signalling pathway that uses ABP1 as a receptor.

Measurement of auxin content in hand-pollinated fruits revealed that *dgt* ovaries contain slightly increased auxin levels compared to wild-type ovaries. The amount of active IAA in specific tissues is determined by several metabolic processes, including regulation of its synthesis, transport to or from specific cells or tissues, IAA inactivation and reactivation, and degradation. To investigate whether auxin biosynthesis is altered by *dgt* mutation we measured the expression levels of all known *ToFZY* gene family members. Recently Mashiguchi et al. (2011) showed that YUC proteins catalyze a rate-limiting step of the indole-3-pyruvic acid (IPA) pathway, which is the main IAA biosynthesis pathway in *Arabidopsis*. We found that transcript levels of *ToFZY* genes are lower in *dgt* versus wild-type ovaries at most developmental stages evaluated, with the exception at 2 and 4 DAP. At these stages, *ToFZY5* and *-6* genes are more expressed in *dgt* than in AC ovaries.

Fujino et al. (1988) investigated the levels of IAA in shoot apices of wild-type (VFN8) and its single-gene *dgt* mutant. IAA levels were $89 \pm 9\,ng\,g^{-1}$ DW in VFN8 and $134 \pm 22\,ng\,g^{-1}$ DW in *dgt*. Ivanchenko et al. (2006) also showed that the level of free IAA in dgt^{1-1} root samples was three to five times higher than in corresponding wild-type root samples. Taken together, these data show that *dgt* is abnormal in auxin content in every part of the plant. On the other hand, measurements of auxin polar transport and uptake demonstrated no significant differences between *dgt* and wild-type (Rice and Lomax, 2000).

In addition to the free acid, IAA occurs in a variety of forms, such as glycosyl esters and amide-linked conjugates with various amino acids. Several evidences indicate that IAA conjugate compounds help to maintain IAA homeostasis, both by inactivating IAA and by serving as a reservoir of IAA that can be released upon hydrolysis and any of these mechanisms could account for the increased auxin level in *dgt*. Staswick et al. (2005) demonstrated that several *Arabidopsis GH3s* encode IAA-amido synthetases that conjugate amino acids to IAA. Most *GH3* genes are also induced by auxins and this provides a mechanism for plants to cope with the presence of excess auxin. We found that the tomato *GH3* gene, which is homologue to the *Arabidopsis GH3.6*, is transcribed at low levels in hand-pollinated *dgt* ovaries at all stages of fruit development. Therefore, our results suggest that the higher level of IAA in *dgt* ovaries depends not only on auxin biosynthesis but also on

conjugation. Presumably, the low expression level of *GH3* is a consequence of the reduced auxin responsiveness in the mutant.

It has been reported that the *dgt* lesion specifically disrupts the expression of a subset of *Aux/IAA* gene family members in fruit (Balbi and Lomax, 2003) and hypocotyls (Nebenführ et al., 2000). Since the expression of *Aux/IAA* members was conducted late in fruit development (Balbi and Lomax, 2003), we decided to investigate gene expression in AC and *dgt* ovaries at pre-anthesis (0 DAP) and at 10 d after pollination or auxin treatment. Our results show that, in hand-pollinated ovaries, the *dgt* lesion reduces the transcript levels of most *Aux/IAA* genes at early stages of fruit development compared with AC fruit. Therefore we suggest that the *dgt* mutation does not affect specific *Aux/IAA* genes in terms of transcript occurrence but rather in terms of relative levels of expression.

We have found that the transcript levels of six *Aux/IAA* genes (*IAA1*, *IAA2*, *IAA3*, *IAA8*, *IAA9*, *IAA11*) were clearly enhanced in 4-CPA treated ovaries of AC but not in *dgt* 10 d after the application of the hormone. A higher transcript level of several *Aux/IAA* genes (including *IAA9*) was also observed by Serrani et al. (2008) in 2,4-D-treated Micro-Tom tomato ovaries 5 d after auxin treatment.

Down-regulation of *IAA9* gene in tomato plants was reported to give rise to parthenocarpic fruit (Wang et al., 2005) and it was suggested that IAA9 protein acted as a negative regulator of fruit initiation. We found that *IAA9* expression is unaffected by hand-pollination in AC and *dgt* ovaries while the expression is enhanced in auxin-induced AC fruits. Similar results were observed by Serrani et al. (2008) in Micro-Tom ovaries suggesting that fruit-set after pollination or auxin treatment is not mediated by *IAA9* down-regulation.

Our Real Time-PCR analysis demonstrated that the auxin-induced accumulation of *SAUR* transcripts was reduced in *dgt* versus AC ovaries. These results are in agreement with previous reports on *dgt* hypocotyl segments (Mito and Bennett, 1995; Coenen et al., 2003).

In tomato the effect of pollination and fertilization in stimulating fruit set and growth can be mimicked by application of exogenous hormones, such as auxin and gibberellins (de Jong et al., 2009). As reported above, hand-pollination/fertilization in the *dgt* ovary initiates a cascade of events that finally promote fruit growth, while auxin treatment does not induce parthenocarpic fruit growth. These data support the hypothesis that *dgt* ovary cells are not able to sense and/or transduce the external auxin signal, whereas pollinated *dgt* ovary cells are able, to some extent, to detect the IAA present in fertilized ovules promoting fruit development.

To date, three different auxin receptors have been discovered: ABP1, the $SCF^{TIR1/AFB}$ family, and very recently the SCF^{SKP2A} (Sauer and Kleine-Vehn, 2011; Scherer, 2011). Auxin signalling is largely governed by TIR1/AFBs. However, several auxin responses such as the rapid responses at the plasma membrane do not use this pathway. ABP1, one of the first characterized proteins that bind auxin, has been implied as a receptor for a number of rapid auxin responses. SKP2A and TIR1/AFBs are predominantly localized in the nucleus, while ABP1 is an ER-resident protein and some ABP1 proteins are secreted to the plasma membrane and/or the extracellular matrix.

Early studies showed that the *dgt* mutation impaired auxin signalling and suggested the existence of several auxin-signalling pathways, not all being DGT-dependent (Coenen et al., 2002). Later, Christian et al. (2003), demonstrated that the *dgt* mutation blocked both auxin-induced growth of tomato hypocotyls and auxin-induced swelling of tomato hypocotyls protoplast. Protoplast swelling is a well-established ABP1-dependent auxin response. Therefore, they suggested that DGT is involved in a signalling chain originating at ABP1. Successively Oh et al. (2006) discovered that the *DGT* gene encoded a cyclophilin (CYP), a new component in some mediated auxin responses.

Taken together, these and our results, suggest that in the vegetative and reproductive parts of tomato plants a DGT-dependent (ABP1?) pathway may be involved in the perception of apoplastic auxin while TIR1 may perceive only cytosolic auxin. The reduced growth response in hand-pollinated *dgt* ovaries is compatible with the idea of two signalling pathways, originating from different receptors, but synergistically controlling growth.

Our data on early auxin-induced gene regulation show that all tested *Aux/IAA* genes were disregulated in the *dgt* ovaries compared to wild-type, including *SAUR* and *GH3* genes. These results suggest a link between a DGT-dependent pathway and the TIR1 auxin signalling pathway because it is known that the early auxin-regulated genes are associated with the TIR1-dependent IAA ubiquitation pathway. Our findings are consistent with the results of Braun et al. (2008) and Effendi et al. (2011). Both groups tested the transcription of early auxin-regulated genes in *Arabidopsis* plants where ABP1 was functionally down-regulated (Braun et al., 2008) and in heterozygous abp1/ABP1 insertional mutant (Effendi et al., 2011) reporting that all *Aux/IAA* tested were down-regulated. In conclusion, they suggest that in planta both pathways may interact for the fine-tuning of early auxin-responsive gene regulation. Understanding the nature of this interaction will be an important challenge on the coming years.

After fertilization, fruit-set and growth in tomato depend on the cross-talk among plant hormones, especially auxins and gibberellins (de Jong et al., 2009). Several findings have highlighted a hierarchy in the action of these hormones, according to which fruit development would be initiated by the sequential action of auxins and GAs (Serrani et al., 2008, 2010; Mariotti et al., 2011).

If the reduced auxin-responsiveness in hand-pollinated *dgt* ovary affects GA biosynthesis and signalling and concurs to the reduced fruit development is currently under investigation.

In this study we observed that the *dgt* mutant did not develop parthenocarpic fruit growth after auxin treatment so studying the effect of GA application in this mutant may represent a valuable tool to investigate the specific independent roles of auxin and GAs in the promotion of fruit development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2012.04.005.

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