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Fruit-set and Early Fruit Growth in Tomato are Associated with Increases in Indoleacetic Acid, Cytokinin, and Bioactive Gibberellin Contents

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Abstract Fruit-set, defined as the activation of a developmental program which converts the ovary into a developing fruit, depends on the crosstalk among plant hormones. Here we show that in pollinated ovaries fruit-set is associated with an increase in indole-3-acetic acid (IAA) content and high transcript levels of *ToFZY*, a gene involved in the tryptophan-dependent auxin biosynthesis pathway. In unpollinated ovaries developed parthenocarpically in response to the synthetic auxin chlorophenoxyacetic acid (4-CPA), *ToFZY* mRNA levels and IAA content slightly increase. The most likely sequence of events after fertilization involves auxin-mediated activation of gibberellin (GA) synthesis. Fertilization events not only strongly increase *SlGA20ox1* and *SlGA20ox3* message levels but also increase *SlGA2ox2*, *SlGA2ox4*, and *SlGA2ox5* mRNA levels, suggesting a concerted regulation to modulate the level of bioactive GAs, GA_1 and GA_3. 4-chlorophenoxyacetic acid was found to mimic the fertilization events in the stimulation of *SlGA20ox1* and *SlGA20ox3* mRNA levels, which were also enhanced and increased earlier, but in contrast with pollinated ovaries, *SlGA2ox2*, *SlGA2ox4*, and *SlGA2ox5* mRNA levels were repressed leading to higher levels of bioactive GAs. We have also analyzed the content of abscisic acid (ABA) and its metabolites dihydrophaseic acid, phaseic acid, and ABA-glucosyl ester and the level of cytokinins (CKs) (free bases and their corresponding ribosides and ribotides) in pollinated and auxin-treated tomato fruits. We show that ABA levels decrease whereas the levels of free CKs increase immediately after pollination or auxin treatment.

Keywords ABA · Cytokinins · Fruit-set · Gibberellins · IAA · Ovary · Parthenocarpy · Tomato

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Introduction

Fruit-set is the most critical phase of fruit production and is of vital importance in agriculture. Despite this enormous economic impact, the molecular mechanisms underlying fruit-set remain poorly defined (Wang and others 2009).

It is currently accepted that fruit-set and development are regulated by the coordinated action of hormones produced in the ovary after pollination and/or fertilization (Srivastava and Handa 2005). In tomato, the effect of pollination can be mimicked by exogenous application of hormones, mainly auxins and gibberellins (GAs), which induce parthenocarpic fruit growth. Several genotypes carrying genes inducing parthenocarpy have also been described, and it has long been assumed that natural parthenocarpy was the result of elevated hormone levels in the ovaries in the absence of pollination and fertilization (Gorguet and others 2005; Srivastava and Handa 2005).

Strong support for the role of auxins in fruit-set and growth in tomato and other species has been obtained from work with loss-of-function mutants IAA9, ARF7, and ARF8 (de Jong and others 2009). Recent transcriptome analyses also suggest that genes involved in the synthesis, transport, and response of auxin have a critical role in

tomato fruit-set (de Jong and others 2009). In addition, it has been demonstrated that overexpression of genes involved in auxin biosynthesis or response in ovaries is associated with parthenocarpy in different species (Rotino and others 1997).

Support for the role of GAs during early events leading to fruit-set and development in tomato has been obtained using a variety of experimental approaches: GA application, quantitation of GA levels, and transcript abundance analysis of GA metabolism genes (Srivastava and Handa 2005). The application of GA biosynthesis inhibitors reduces tomato fruit-set in both pollinated wild-type ovaries and parthenocarpic mutants, and the effect is reversed by the addition of GA_3 (Olimpieri and others 2007 and literature cited therein). Endogenous GAs increase in tomato ovaries following pollination (Koshioka and others 1994; Serrani and others 2007b) and higher GA levels have been found in the fruits of the parthenocarpic tomato *pat*2, *pat3/pat4*, and *pat* mutants (Olimpieri and others 2007 and literature cited therein). Upregulation of *CPS* and *GA20ox* genes, but not of *GA3ox* genes, in tomato ovaries after pollination has been reported (Rebers and others 1999; Olimpieri and others 2007). All these findings strongly suggest that the regulation of the GA signal is part of the mechanism underlying fruit-set in tomato, whether this is induced via pollination or parthenocarpy.

Besides GAs and auxins, abscisic acid (ABA) and cytokinins (CKs) are generally believed to be involved in fruit growth and development. Abscisic acid levels have been reported to decrease in tomato pistils after pollination (Kojima and others 1993) and higher levels were observed in pollinated than parthenocarpic fruits (Srivastava and Handa 2005). The role of ABA in relation to tomato fruit-set has been recently examined (Vriezen and others 2008; Nitsch and others 2009). Although CKs are generally considered to play a critical role in the stimulation of cell division during fruit development (Srivastava and Handa 2005), very few experimental data support the involvement of these hormones in the initial cell-division phase of fruit growth (Mapelli 1981; Bohner and Bangerth 1988; Martineau and others 1995). Cytokinin-related gene expression during tomato fruit-set was recently investigated (Vriezen and others 2008; Pascual and others 2009).

Although the fundamental role of auxins and GAs in fruit-set and growth has been demonstrated, the molecular mechanisms by which hormones interact in the regulation of these processes remain to be elucidated. Among them, the hierarchy of auxin and GA interaction during the initial phase of ovary growth characterized by cell division remains to be elucidated, although most recent models of tomato fruit-set point to auxins as the critical signal, acting in part throughout the induction of GA biosynthesis (Wang and others 2009). Issues such as what are the active GAs and what are the fruit tissues where hormones are synthesized are still poorly known.

During recent years, the role of GAs in the regulation of fruit development following pollination or auxin treatment has been investigated using the tomato cv. Micro-Tom (Serrani and others 2007a, b, 2008). Although this cultivar is a convenient experimental material, the presence of several mutations suggests that the data obtained with this material must be considered with some caution, as advised by the same authors (Serrani and others 2010).

Lastly, experimental evidence concerning the role played by other hormones during the early development of tomato fruit characterized by cell division is quite limited.

In this study we have investigated the interaction between GAs and auxin in wild-type tomato plants by comparing the dynamic of endogenous GAs and IAA in tomato ovaries during the initial growth phase induced by pollination or auxin treatment. The profile of endogenous hormones has been compared with the temporal and spatial regulation of key genes of GA (*GA20ox*, *GA3ox*, *GA2ox*) and indole-3-acetic acid (IAA) (*ToFZY*) biosynthesis. Moreover, the endogenous levels of ABA and CKs have also been analyzed.

Materials and Methods

Plant Material and Treatments

Tomato plants (*Solanum lycopersicum* L.) cv. Pearson were grown in the field in Pisa, Italy, during spring–summer with standard plant culture practices. Flowers were emasculated 1 day before anthesis to prevent self-pollination (Jacobsen and Olszewski 1991) and then pollinated manually or dipped in a solution of the synthetic auxin chlorophenoxyacetic acid (4-CPA, 15 mg l^{-1}) with Tween 20 (0.01% v/v) (Koshioka and others 1994).

At −1, 2, 5, and 8 days post anthesis (DPA) the ovaries were collected and when necessary separated under a microscope into pericarp and locular tissue (including ovules), frozen in liquid nitrogen, and stored at −80°C until analysis. Unpollinated ovaries were analyzed only at 2 DPA.

Hormone Analysis

Gibberellin and IAA analysis was performed as previously described (Olimpieri and others 2007). Deuterated GAs ([17,17-$^2H_2$]-GA_{19}, [17,17-$^2H_2$]-GA_{20}, [17,17-$^2H_2$]-GA_{29}, [17,17-$^2H_2$]-GA_1, [17,17-$^2H_2$]-GA_8, [17,17-$^2H_2$]-GA_3, [17,17-$^2H_2$]-GA_5, obtained from L. N. Mander, Australian National University, Canberra, Australia) and [$^{13}C_6$]-IAA (Cambridge Isotopes Laboratories Inc., Andover, MA,

USA), 50 ng each, were added as internal standards. High-performance liquid chromatography (HPLC) was performed with a Kontron instrument (Munich, Germany) equipped with a UV absorbance detector operating at 210 nm. The samples were applied to a 250-mm × 4.6-mm i.d. column, packed with Nucleosil 100-5 $N(CH_3)_2$, particle size 5 µm (Macherey-Nagel, Düren, Germany), eluted isocratically with 100% methanol containing 0.01% acetic acid at a flow rate of 1 ml min^{-1}. The fractions corresponding to the elution volumes of standard GAs and IAA were dried, trimethylsilylated with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (Pierce, Rockford, IL, USA) at 70°C for 1 h, and analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS).

Abscisic acid and its metabolites were analyzed following the procedure described by Sorce and others (1996). [$^2H_6$]-ABA (100 ng, OlChemlm Ltd, Olomouc, Czech Republic) was added as an internal standard. High-performance liquid chromatography analysis was performed with an instrument (Kontron) equipped with a UV absorbance detector operating at 254 nm. Samples were applied to a 150-mm × 4.5-mm i.d. column, packed with Hypersil ODS, particle size 5 µm (Thermo Fisher Scientific, Waltham, MA, USA). The column was eluted with a linear gradient from 20 to 100% methanol in 20 min at a flow rate of 1 ml min^{-1}. The fractions corresponding to the elution volume of ABA, phaseic acid, and dihydrophaseic acid standards were dried under vacuum, methylated with ethereal diazomethane, and analyzed by GC-MS/MS. High-performance liquid chromatography fractions containing ABA-GE were resuspended in 10 ml of water, adjusted to pH 11 with 1 N KOH, and hydrolyzed at 65°C for 2 h under helium flow. Hydrolysates were adjusted to pH 3, supplemented with 100 ng of [$^2H_6$]-ABA, and partitioned against ethyl acetate. The organic phase was dried under vacuum, methylated with ethereal diazomethane, and analyzed by GC-MS/MS.

Cytokinins were extracted with cold Bieleski solvent (12 MeOH:5 chloroform:2 water:1 formic acid v/v) (1:6 w/v). Deuterated CKs ([$^2H_3$]-DHZ, [$^2H_3$]-DHZR, [$^2H_5$]-Z, [$^2H_5$]-ZR, [$^2H_6$]-iP, [$^2H_6$]-iPR, and [$^2H_6$]-iPMP (OlChemlm Ltd., Olomouc, Czech Republic), 300 ng each, were added as internal standards. Extracts were passed through an ion-exchange DEAE column, which was eluted with water at pH 7 to collect CK free bases and ribosides, followed by elution with NH_4HCO_3 (1 M) to collect CK ribotides. The ribotide fraction was treated with bacterial alkaline phosphatase (1 U) at 50°C for 1 h to remove the phosphoryl groups. Both fractions were then partitioned three times against equal volumes of butanol at pH 8.2 and further analyzed by HPLC. The HPLC instrument (Kontron) was equipped with a UV absorbance detector operating at 254 nm. Samples were applied to a 150-mm × 4.6-mm i.d. column packed with Kromasil ODS, particle size 5 µm (EKA Nobel, Bohus, Sweden). The column was eluted with 10% MeOH in water for 4 min, followed by a double-gradient elution from 10 to 30% and from 30 to 100% over 20 min with a flow rate of 1 ml min^{-1}. The fractions corresponding to the elution volumes of Z, DHZ, ZR, DHZR, iP, and iPR were dried, trimethylsilylated with BSTFA containing 1% trimethylchlorosilane at 70°C for 1 h, and analyzed by GC-MS/MS.

Final quantification of hormones was accomplished by GC-MS/MS. This was performed with a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) as previously described (Olimpieri and others 2007), with the exception that a Mega 1MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) (Mega, Milano, Italy) was used. Plant hormones were identified by comparison of full mass spectra with those of authentic compounds. Quantification was carried out by reference to standard plots of concentration ratios versus ion ratios, obtained by analyzing known mixtures of unlabeled and labeled compounds. Phaseic acid and dihydrophaseic acid quantification was obtained by analyzing samples supplemented with 20,000 dpm of [^{14}C]-phaseic acid and 20,000 dpm of [^{14}C]-dihydrophaseic acid to account for purification losses, as described by Lorenzi and Ceccarelli (1994).

Quantitative RT-PCR Analysis of Gene Expression

RNA was extracted from pericarp and locular tissues using the RNAqueous kit (Ambion®, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was subjected to DNase treatment using a TURBO DNA free kit (Ambion). Five micrograms of each sample was reverse transcribed into cDNA with a high-capacity cDNA archive kit (Applied Biosystems®, Invitrogen).

Quantitative RT-PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems) (http://www.appliedbiosystems.com/) and specific probes for each gene analyzed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Elongation factor (*SlEF1α*) was used as an endogenous control (Anjanasree and others 2005) and primer and probe sequences for amplification are reported in Supplementary Table 1. In a single experiment, each sample was assayed in triplicate. Relative quantification of gene expression was performed using the comparative C_t method as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems). Efficiency of each primer pair on each target sequence was measured. Values of efficiency among the different genes were similar. Transcript levels of genes

were compared across developmental stages and tissues using *SlGA20ox1* from the 2 DPA pericarp sample ($C_t = 34.9$) as a reference sample. All experiments were repeated twice with similar results.

Results

The fresh weight of parthenocarpic tomato fruits induced by 4-CPA was always higher than that of pollinated fruits throughout the whole development. Unpollinated mock-treated ovaries did not show appreciable growth (data not shown).

To assess the effect of pollination or exogenously applied 4-CPA on endogenous GA content, GAs from the early 13-hydroxylation pathways (GA_{19}, GA_{20}, GA_{29}, GA_1, GA_8, GA_5, and GA_3) were quantified in pollinated and parthenocarpic tomato ovaries at several time points before and after anthesis. At anthesis (−1 DPA) the most abundant GA was GA_{19} (Fig. 1). In the absence of pollination (2 DPA), the level of most GAs dropped and the active GAs, GA_1–GA_3, were not detected (Fig. 1). In the same period (from −1 to 2 DPA), the concentrations of all GAs remained quite similar in pollinated ovaries, whereas the level of GA_{19} decreased (from 45 to 12 ng g^{-1} FW) and those of GA_8 and of the bioactive GA_3 significantly increased in 4-CPA-treated ovaries (GA_8: from 7 to 21 ng g^{-1} FW; GA_3: from undetectable levels to 18 ng g^{-1} FW). At 5 and 8 DPA the GA content of pollinated and parthenocarpic ovaries was analyzed in pericarp and locular tissue. The level of GAs in the locular tissue was in most cases higher than in the pericarp in both types of ovaries (Fig. 1). At 5 DPA the locular tissue of parthenocarpic ovaries contained higher concentrations of bioactive GAs ($GA_1 + GA_3$) compared with pollinated ovaries: 15 versus 7 ng g^{-1} FW. The locular tissue content of GA_8, a direct GA_1 metabolite, was also higher in 4-CPA-treated than in pollinated ovaries. In contrast, the levels of GA_{19} and GA_{29} (a GA_{20} metabolite) were clearly higher in the locular tissue of pollinated versus treated ovaries. At 8 DPA the content of bioactive GAs in the locular tissue was similar in both types of ovaries. In contrast, the concentrations of GA precursors (GA_{19} and GA_{20}) and GA_{29} and GA_8 were much higher in pollinated compared with treated ovaries.

The level of free IAA in pollinated ovaries dramatically increased from 42 ng g^{-1} FW at anthesis (−1 DPA) to 247 ng g^{-1} FW at 2 DPA (Fig. 2). In contrast, in 4-CPA-treated ovaries during the same period, the free-IAA level slightly increased to 61 ng g^{-1} FW. In the absence of pollination, the endogenous level of free IAA dropped to quite low values (Fig. 2). Free-IAA content declined at 5 DPA in both pollinated and 4-CPA-treated ovaries and was minimally detectable by 8 DPA. At 5 DPA the level of free IAA was greater in locular than in pericarp tissue.

Endogenous levels of free ABA were highest at anthesis and started to decrease soon after pollination or 4-CPA treatment (Fig. 3). A more abrupt drop of free ABA was observed in unpollinated ovaries at 2 DPA (Fig. 3). At 5 and 8 DPA the ABA concentration was determined in both pericarp and locular tissue and appreciable differences between the two types of ovaries were not found. The main ABA metabolite was found to be ABAGE in both pollinated and 4-CPA-treated ovaries, its concentration increasing from −1 to 2 DPA and decreasing thereafter, whereas phaseic acid was under detection limits and the levels of dihydrophaseic acid were undetectable or quite low at these stages, rising at 5 and 8 DPA. Dihydrophaseic acid and ABAGE were undetectable in the absence of pollination at 2 DPA (Fig. 3).

Free CKs and their corresponding ribosides and ribotides were analyzed in whole ovaries during development induced by pollination or 4-CPA application and in unpollinated ovaries at 2 DPA. The most abundant CK forms in all types of ovaries were the free bases Z, DHZ, and iP, whereas the ribotide level was constantly quite low (data not reported). In unpollinated ovaries only traces of Z and DHZ could be detected at 2 DPA (Fig. 4). In contrast, a clear peak in endogenous free-CK levels was observed at the same stage following pollination or 4-CPA treatment (Fig. 4). Although parthenocarpic fruits did show a similar change in CK concentration, the increase at 2 DPA appeared much less pronounced.

To investigate whether changes in GA levels were associated with changes in the activity of GA metabolism genes, transcript levels of key genes involved in GA metabolism (*SlGA20ox1*, *-2*, and *-3*; *SlGA3ox1* and *-2*; *SlGA2ox1*, *-2*, *-3*, *-4*, and *-5*) were analyzed by quantitative real-time PCR (qRT-PCR). *SlGA20ox3* transcript levels were minimal in both pericarp and locular tissues at anthesis (−1 DPA), whereas the expression of *SlGA20ox1* was undetectable (Fig. 5). Both genes were upregulated upon pollination or 4-CPA treatment, but induction occurred almost exclusively in the locular tissue. In this tissue, transcript levels of both *SlGA20ox1* and *SlGA20ox3* were at least twofold higher in 4-CPA-treated than in pollinated ovaries. A low expression of *SlGA20ox2* was detected in every ovary tissue at any developmental stage between 2 and 8 DPA (Fig. 5). In the absence of pollination, only a very low expression of *SlGA20ox3* was detected in ovaries at 2 DPA (Fig. 5).

Expression of *SlGA3ox1* was detected in ovaries before anthesis and dropped to undetectable or very low levels by 2 DPA in both pollinated and unpollinated ovaries (Fig. 5). In contrast, only a slight decrease in *SlGA3ox1* transcripts was observed in both tissues of 4-CPA-treated ovaries at

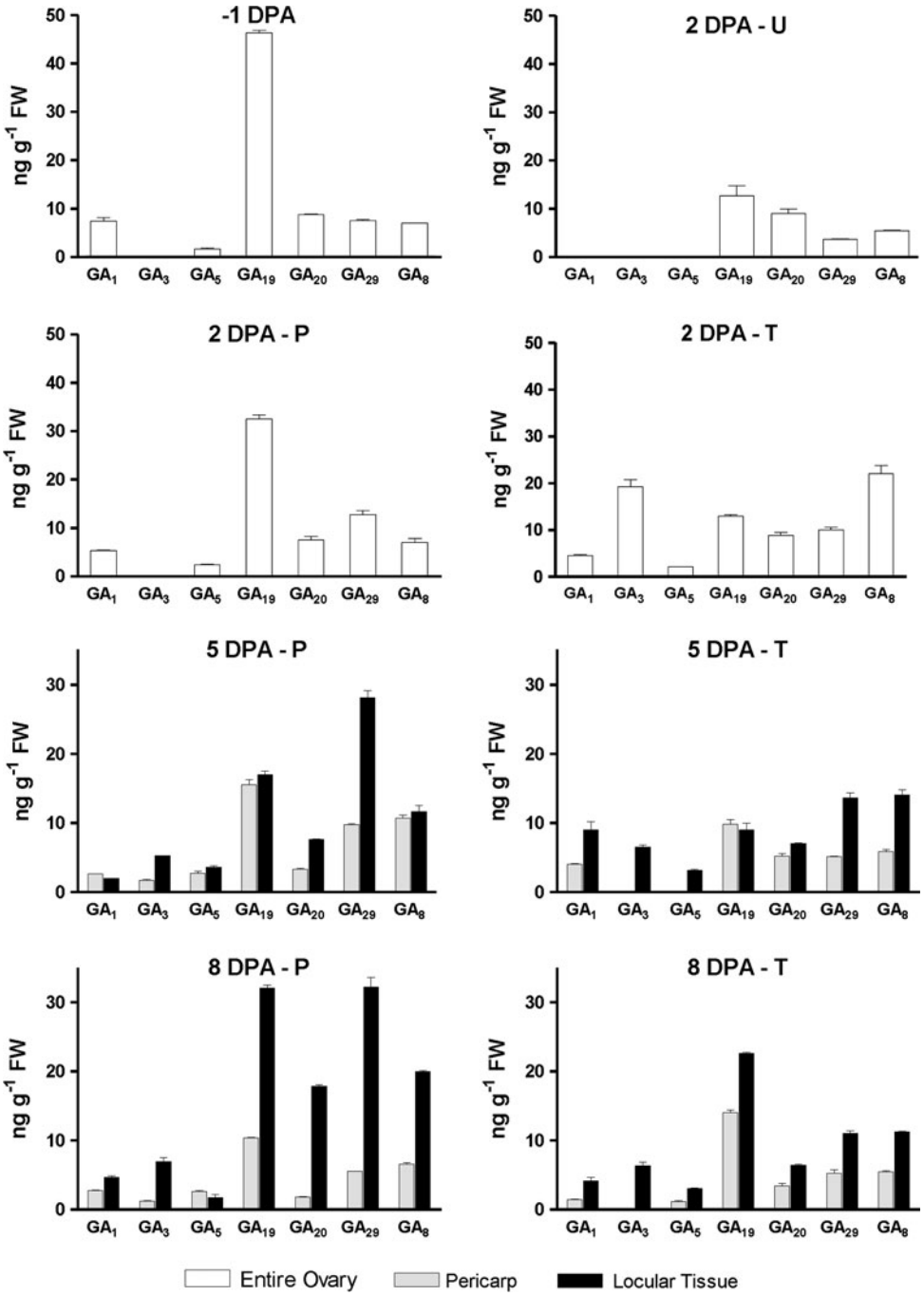

Fig. 1 Endogenous level of GAs in pollinated, 4-CPA treated, and unpollinated ovaries. The analysis was performed on entire ovaries at −1 and 2 DPA and on locular tissue and pericarp at 5 and 8 DPA. Analyses were carried out by GC-MS/MS as described in Materials and Methods. Data are means ± SE ($n = 3$). *P* pollinated, *T* treated with 4-CPA, *U* unpollinated, *FW* fresh weight

2 DPA. At 5 and 8 DPA transcript levels of *SlGA3ox1* dropped to low levels in the pericarp of both ovaries. Transcripts of *SlGA3ox2* were barely detected in ovaries before and after anthesis in unpollinated, pollinated, and 4-CPA-treated ovaries (data not shown).

Tomato ovaries contained transcripts for all *SlGA2ox* genes except *SlGA2ox3*. At anthesis (−1 DPA), *SlGA2ox4* was highly expressed in pericarp and locular tissue. In contrast, *SlGA2ox2* and *-5* were expressed mostly in the pericarp (Fig. 5). At 2 DPA, transcript levels of *SlGA2ox2*, *SlGA2ox4* and *SlGA2ox5* were unchanged or decreased in unpollinated ovaries (Fig. 5). At 2 DPA, *SlGA2ox1* and *SlGA2ox2* were upregulated in the locular tissue of pollinated ovaries, whereas the other GA inactivation genes (*SlGA2ox4* and *SlGA2ox5*) were upregulated later (5 DPA). In pericarp of pollinated ovaries, transcript levels of *SlGA2ox2* and *-5* were not significantly affected, except *SlGA2ox4* for which transcript levels were very low at 2 and 8 DPA and upregulated at 5 DPA. In 4-CPA-treated ovaries, the expression of *SlGA2ox4* was lower than in pollinated

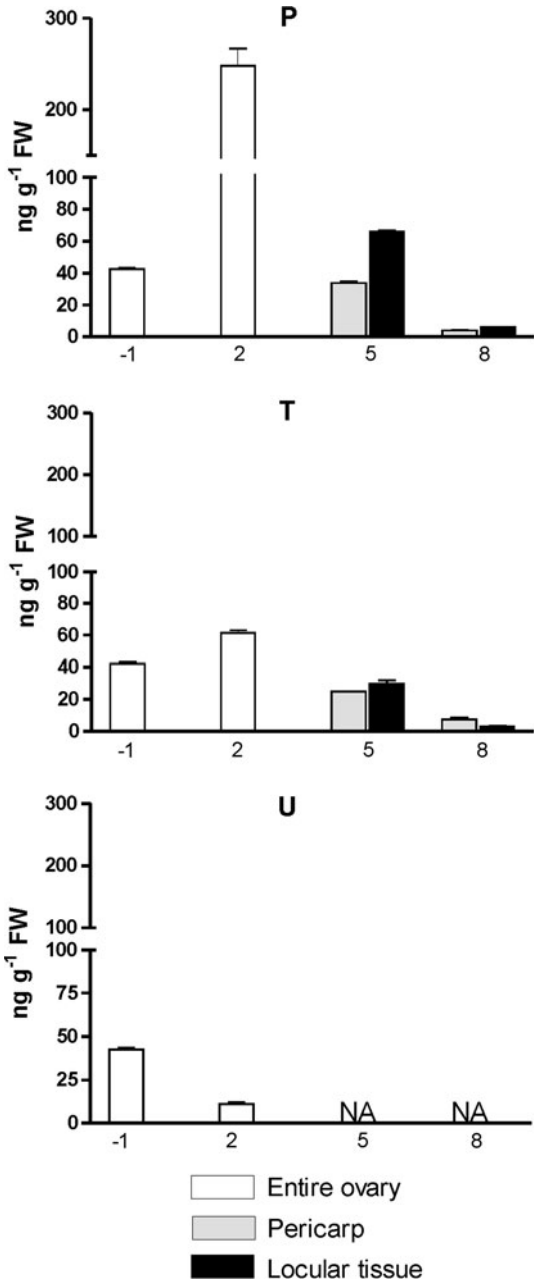

Fig. 2 Endogenous level of IAA in pollinated, 4-CPA-treated, and unpollinated ovaries. The analysis was performed on entire ovaries at −1 and 2 DPA and on locular tissue and pericarp at 5 and 8 DPA. Analyses were carried out by GC-MS/MS as described in Materials and Methods. Data are means ± SE ($n = 3$). *P* pollinated, *T* treated with 4-CPA, *U* unpollinated, *FW* fresh weight, *NA* not analyzed

ovaries between 2 and 8 DPA in both tissues. In the case of *SlGA2ox5*, the transcript level always remained low or undetectable. *SlGA2ox1* and *SlGA2ox2* transcript levels in locular tissue of 4-CPA-treated ovaries were similar to those of pollinated ovaries. In contrast, in the pericarp of 4-CPA-treated ovaries, transcript levels of *SlGA2ox2* were lower than in pollinated ovaries at 2 and 5 DPA.

To test whether the different IAA levels in pollinated and 4-CPA-treated ovaries were caused by altered IAA biosynthesis, we analyzed the expression of the *ToFZY* gene by qRT-PCR. In tomato the *ToFZY* gene encodes a flavin monooxygenase involved in a tryptophan-dependent auxin biosynthesis pathway (Expósito-Rodríguez and others 2007). At anthesis (−1 DPA), transcripts of *ToFZY* were undetectable in the ovaries and no change was observed in the absence of pollination at 2 DPA (Fig. 5). Two days after anthesis, *ToFZY* was transiently upregulated only in the pericarp in both pollinated and 4-CPA-treated ovaries. In this tissue, *ToFZY* transcript levels in pollinated ovaries were about twice those detected in treated ovaries. Very low expression of *ToFZY* was detected in the locular tissue between 5 and 8 DPA.

Discussion

Our aim was to investigate the role of several plant hormones (GAs, auxins, CKs, and ABA) during tomato fruit-set by analyzing the differences between pollinated and parthenocarpic fruits induced by auxin treatment. We focused our study on the early stages of fruit development in a wild-type tomato plant because the precise actions and interactions of these hormones in regulating fruit initiation and growth are only partly understood.

Endogenous Levels of Hormones

The dynamics of endogenous GAs during the first developmental phase of the tomato fruit indicates that pollination induces a significant increase in endogenous levels of active GAs in the ovary. This induction is enhanced and occurs earlier in the ovaries, which were stimulated to parthenocarpic growth through application of exogenous auxin. Moreover, we show that the higher endogenous levels of GAs are found in locular tissues in both pollinated and parthenocarpic fruits.

It has been previously suggested that GA_1 is the biologically active GA during tomato fruit-set (Koshioka and others 1994; Serrani and others 2007b). However, our data show for the first time that GA_5 and GA_3 also accumulate in tomato ovaries. These GAs are also produced by the early-13-hydroxylated pathway, as is GA_1, and we found that the concentration of GA_3 is even higher than that of GA_1. Therefore, presumably GA_1 is not the only GA active during tomato fruit development. In their work with the tomato Micro-Tom mutant, Serrani and others (2008) reported a higher level of GA_1 in 2,4-D-treated fruits compared to pollinated fruits at 10 DPA. We did not detect a significantly higher level of GA_1 in auxin-induced compared to pollinated ovaries, but we did demonstrate that a

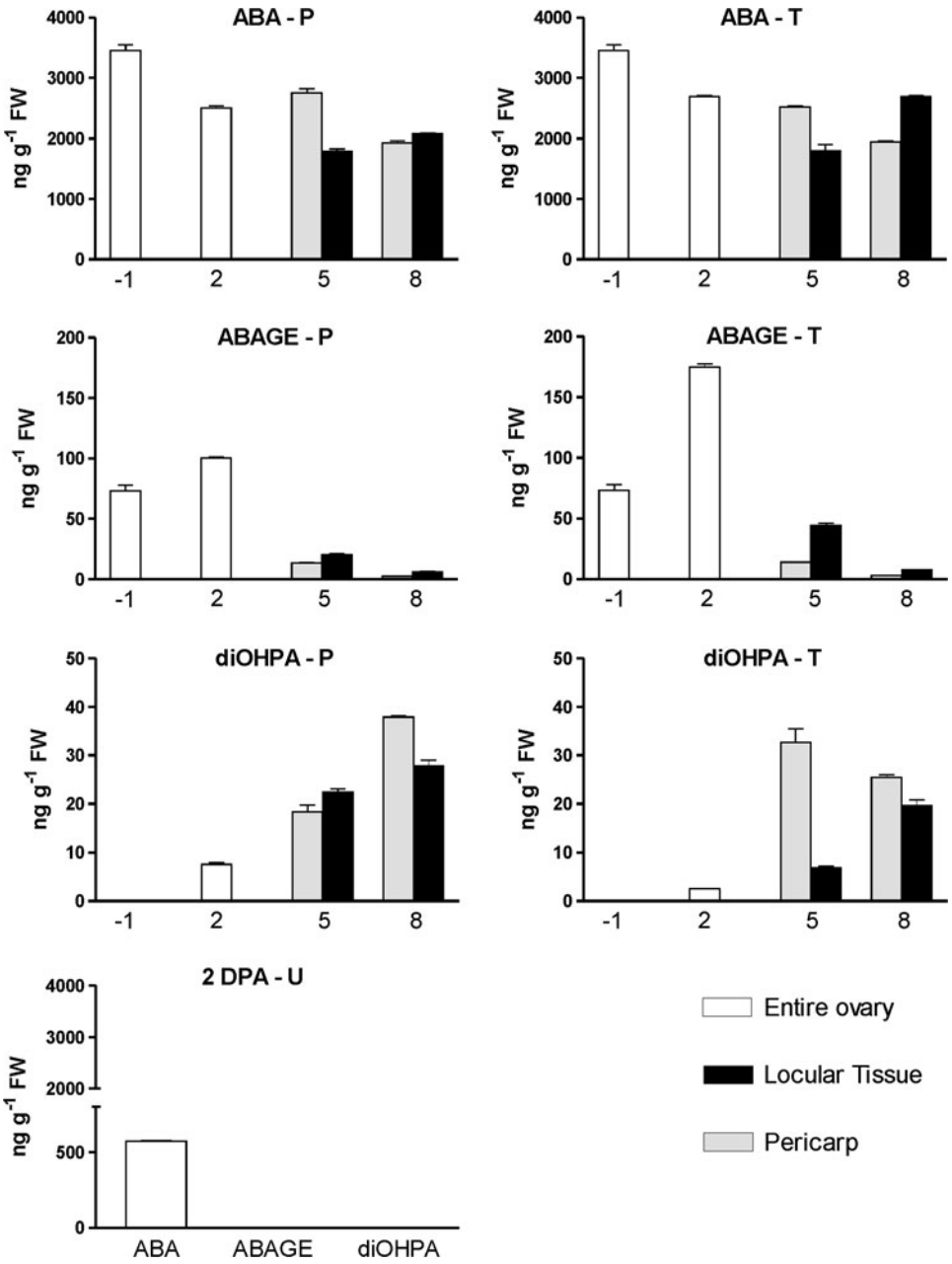

Fig. 3 Endogenous level of ABA and its catabolites in pollinated, 4-CPA treated, and unpollinated ovaries. The analysis was performed on entire ovaries at −1 and 2 DPA and on locular tissue and pericarp at 5 and 8 DPA. Analyses were carried out by GC-MS/MS as described in Materials and Methods. Data are means ± SE ($n = 3$). *P* pollinated, *T* treated with 4-CPA, *U* unpollinated, *FW* fresh weight

peak of GA_3 is observed at 2 DPA in parthenocarpic ovaries but not in pollinated and unpollinated ovaries.

We showed that a peak in the endogenous level of IAA occurs as early as 2 DPA in fruits developing after either pollination or 4-CPA application, whereas the level of IAA declines to very low levels in unpollinated fruits. The increase at 2 DPA reaches a much higher level in pollinated than in parthenocarpic fruits.

Indole-3-acetic acid distribution within the tomato fruit during development was investigated by Kojima and others (1994) who reported a much higher concentration in locular tissue and axis than in pericarp of young fruits. Our data are consistent with these observations, and, in addition, we found that a higher concentration in the locular tissues is already detectable at 5 DPA.

We found that free-ABA levels were high in the ovaries at anthesis and declined after pollination/fertilization. A more dramatic decline was found in unpollinated ovaries at 2 DPA. These results are consistent with the findings of Kojima and others (1993). Recently, ABA response genes were reported to be highly expressed in unpollinated ovaries and downregulated after pollination (Vriezen and others 2008). Moreover, Nitsch and others (2009) reported that ABA content decreased during the 3 days following pollination through downregulation of biosynthetic genes and upregulation of catabolic genes. However, whereas

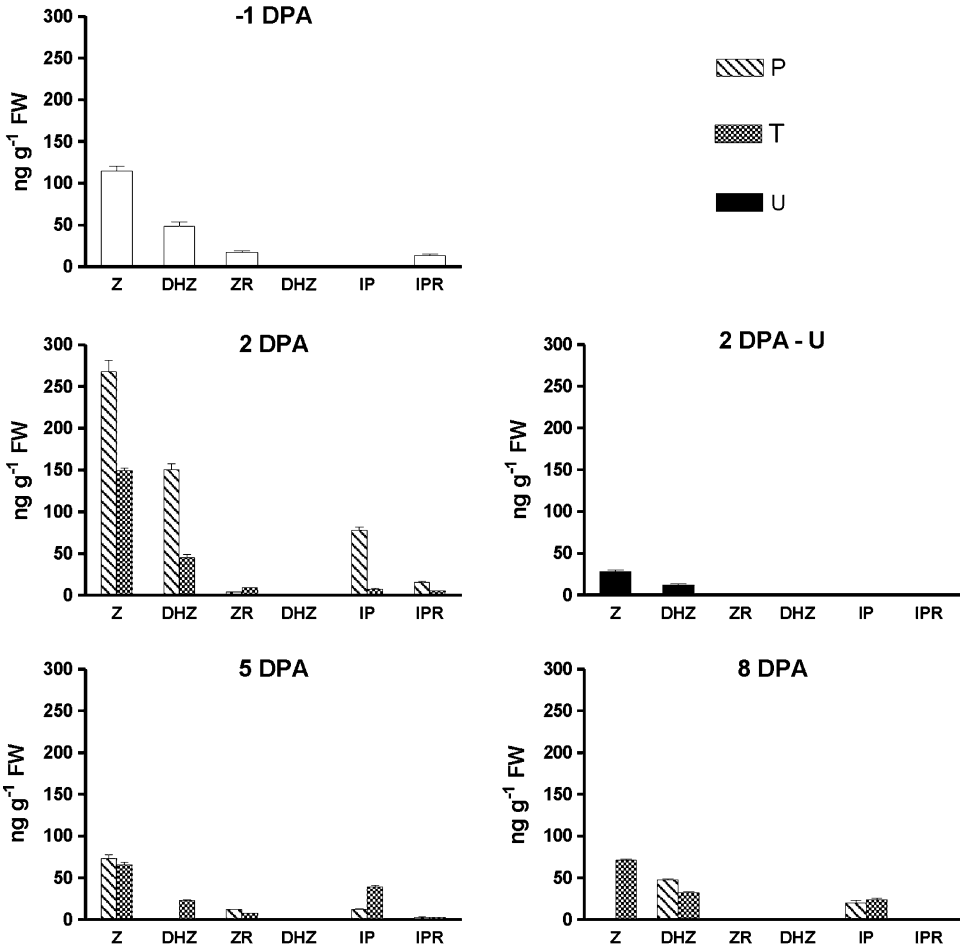

Fig. 4 Endogenous level of CKs in pollinated, 4-CPA treated, and unpollinated ovaries. Analyses were carried out by GC-MS/MS on entire ovaries as described in Materials and Methods. Data are means ± SE ($n = 3$). *P* pollinated, *T* treated with 4-CPA, *U* unpollinated, *FW* fresh weight

these last authors reported an increase in dihydrophaseic acid and a decrease in ABAGE after pollination, we observed a significant ABAGE increase at 2 DPA in both in pollinated and 4-CPA-treated ovaries, suggesting that conjugation may also play a role in the early decrease of endogenous ABA.

A distinct peak in CK concentration can be observed as early as 2 DPA. The increase essentially affects the free bases, whereas the ribosylated forms do not show significant changes and ribotides are undetectable. Furthermore, this early peak of endogenous CKs is much more pronounced in pollinated compared to parthenocarpic fruits. Surprisingly, in contrast with the general belief that CKs have a key function in controlling cell division during the first growth phase in tomato fruit, the few data previously available on endogenous CKs were partial or obtained with unreliable methods (Mapelli 1981; Bohner and Bangerth 1988; Kojima and others 2003). Our data provide significant experimental evidence to support the idea that CKs play a critical role in fruit-set and early growth of tomato fruits. Recently, transcriptome analyses reported expression of CK-related genes in developing tomato fruits (Vriezen and others 2008; de Jong and others 2009; Pascual and others 2009).

Transcript Levels of Genes Encoding Enzymes of GA Metabolism and IAA Synthesis

The GA biosynthetic pathway in tomato ovaries was previously reported to be downregulated at anthesis and reactivated following pollination/fertilization (Rebers and others 1999; Olimpieri and others 2007). Recently, Serrani and others (2007b, 2008) analyzed the transcript accumulation of GA metabolism genes in the tomato Micro-Tom fruits after pollination or 2,4-D application. However, no information on transcript accumulation of GA metabolism genes before 5 DPA and in the different fruit parts earlier than 10 DPA was provided.

We have found that transcript accumulation of *SlGA20ox1* and *SlGA20ox3* genes occurs earlier and is significantly enhanced in parthenocarpic compared with pollinated ovaries and that it is mostly localized in the locular tissues in both types of ovaries. The genes encoding the enzymes that potentially catalyze the conversion of

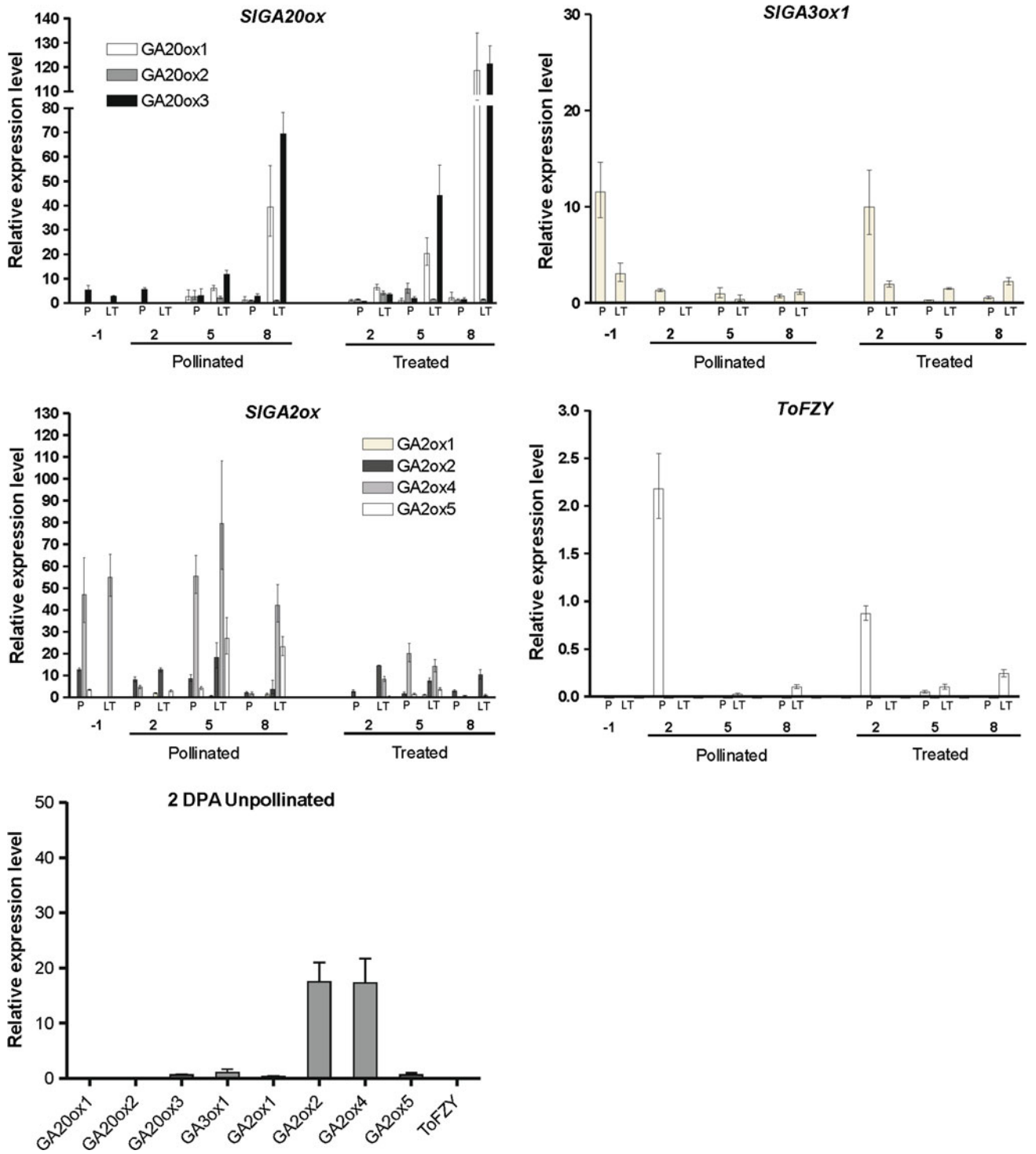

Fig. 5 Effect of pollination or 4-CPA treatment on *SlGA20ox1*, *-2*, *-3*, *SlGA3ox1*, *SlGA2ox1*, *-2*, *-4*, -5, and *ToFZY* mRNA levels in locular tissue (LT) and pericarp (P) from −1 to 8 DPA. Entire unpollinated ovaries were analyzed at 2 DPA. Transcript analysis was carried out by quantitative RT-PCR as described in Materials and Methods. Data are means ± SE ($n = 3$)

GA_{20} to GA_1 and GA_3, *SlGA3ox1* and *SlGA3ox2*, were previously reported to be transcribed at low levels and to be not induced in the early stages of growth after pollination (Rebers and others 1999; Olimpieri and others 2007). Consistent with these studies, our data show that transcript accumulation of these genes declines in the ovaries after pollination. However, *SlGA3ox1* transcripts, which are barely detected in pollinated ovaries, were found to

accumulate in the pericarp of parthenocarpic ovaries at 2 DPA. This is in accord with the peak of endogenous GA_3 found at the same growth stage in the auxin-treated ovaries. Induction of *SlGA3ox1* in entire ovaries of the tomato Micro-Tom was previously reported to occur 5 days after 2,4-D application (Serrani and others 2008).

After pollination, expression levels of all *GA2ox* genes are upregulated to a variable extent, whereas a general downregulation of *GA2ox* is observed in auxin-induced ovaries. A lower transcript level of at least one *GA2ox* gene (*SlGA2ox2*), compared to levels in the unpollinated ovaries, was also found in the Micro-Tom parthenocarpic tomato fruit induced by the synthetic auxin 2,4-D (Serrani and others 2008). Therefore, the higher levels of bioactive GAs (GA_1 and, as demonstrated here, GA_3) in parthenocarpic fruits may be the result of the concerted action of auxin on biosynthetic and catabolic enzymes.

It has been proposed that the seeds and the tissues in direct contact with the seeds are the source of auxins involved in fruit initiation and the early growth phase (Mapelli and others 1978; Kojima and others 1994), and auxin synthesis in the developing tomato fruit was reported by Epstein and others (2002). We showed that in the early stages of development (2 DPA), induced by either pollination or exogenous auxin application, *ToFZY* transcripts accumulate in pericarp cells of tomato ovaries, whereas in the absence of pollination, gene expression is undetectable. *ToFZY* is involved in tryptophan-dependent auxin biosynthesis (Expósito-Rodríguez and others 2007), and evidence has been reported that auxin synthesis in developing tomato occurs via the Trp-dependent pathway (de Jong and others 2009). This result is fully consistent with the fact that the expression of genes for the synthesis of IAA in tomato ovaries stimulates the development of parthenocarpic fruit (Rotino and others 1997). Additionally, our results show that a peak in *ToFZY* transcripts occurs at 2 DPA, coinciding with the peak of endogenous IAA. At this stage, the transcript level is three times higher in pollinated compared to parthenocarpic fruit, with a sharp decrease thereafter in both types of fruit. On the other hand, poor IAA transport into tomato ovary from the pedicel has been reported (Homan 1964; Serrani and others 2010). Therefore, our data suggest that auxin biosynthesis in the ovary is necessary to promote fruit growth.

In conclusion, our data show that auxin is present in unfertilized ovaries but after fertilization auxin biosynthesis is activated in the ovaries. *ToFZY*, a gene involved in the early steps of the Trp-dependent auxin biosynthesis pathway, is strongly upregulated during early fruit development. We suggest that elevated auxin levels in the ovaries initiate a cascade of events that finally promote fruit growth. These events would include crosstalk with other hormones, especially the activation of GA metabolism, the increase in CK, and the decrease in ABA levels.

The activation of GA metabolism is characterized by elevated mRNA levels of *SlGA20ox1* and *SlGA20ox3* and increased levels of most *SlGA2ox* genes, resulting in a higher production of bioactive GAs. The higher *GA2ox* expression may be the result of positive feedback regulation in response to increased GA levels (Hedden and Phillips 2000). Besides GA_1, GA_3 also plays a role as an active GA during tomato fruit-set and growth.

In comparison with pollinated ovaries, the fruits induced to develop by the exogenous application of auxin show an altered GA metabolism, characterized by the earlier upregulation of *SlGA20ox1* and *SlGA20ox3* gene transcription, the prevention of *SlGA3ox1* gene downregulation, and a reduced GA inactivation activity (*SlGA2ox* genes).

The increase in CK content after pollination suggests that this hormone also contributes to the regulatory network that is required for the tight coordination of fruit growth.

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