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ORIGINAL PAPER

Induction of gibberellin 20-oxidases and repression of gibberellin 2β-oxidases in unfertilized ovaries of *entire* tomato mutant, leads to accumulation of active gibberellins and parthenocarpic fruit formation

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Abstract In tomato (*Solanum lycopersicum* L.), auxin and gibberellins (GAs) cross-talk plays an important role during fruit-set. The *entire* tomato mutant has been previously reported to carry a deletion in the coding region of the *SlIAA9* gene, a member of the auxin signal repressor family Aux/IAA. In this paper, we examined the role of *ENTIRE* gene in controlling GAs metabolism and directing spontaneous fruit initiation and early ovary growth. It was shown that, similarly to pollinated fruits, facultative parthenocarpy in *entire* depends on active GA metabolism, since fruit growth is suppressed when GA biosynthesis is blocked. Analysis of endogenous GAs during the first 10 days after flower emasculation revealed that *entire* fruits accumulated higher amounts of active GAs (GA_1 and GA_3) in comparison to wild type pollinated fruits, suggesting that a different GA homeostasis regulation occurs. Transcript analysis of the main GA biosynthesis genes showed that differently from unpollinated and non parthenocarpic wild type ovaries, in *entire* active GA flux modulation is regulated by the activation of *SlGA20ox1* and *SlGA20ox2* and also by a marked reduction of GA catabolism (reduced transcription of GA 2β-oxidase genes) during the early fruit expansion phase.

Keywords *Entire* mutant · SlIAA9 · Fruit development · Parthenocarpy · Gibberellin metabolism · Tomato

Abbreviations

AC	Ailsa Craig
GA	Gibberellin
GC–MS/MS	Gas chromatography-tandem mass spectrometry
Aux/IAA	Auxin/indoleacetic acid
GA20ox	GA 20-oxidase
GA3ox	GA 3β-oxidase
GA2ox	GA 2β-oxidase

Introduction

Fruit-set is defined as the start of a developmental program that leads the conversion of a static ovary into an actively growing fruit and depends on a successful pollination and ovule fertilization. In tomato, *Solanum lycopersicum* L., early fruit development is characterized by an intense mitotic activity in the pericarp and placenta during the first 7–14 days after fertilization. At the end of this stage, cell divisions stop and fruit growth proceeds mostly by cell expansion for 6 or 7 weeks up to its final size (Gillaspy et al. 1993).

Coordination of fruit development relies on a complicate network of hormonal, metabolic and environmental signals that has only recently started to be untangled (Ruan et al.

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2012). Among hormones, auxin and GAs are known to have a central role during fruit development. This is sustained by the fact that both hormones promote parthenocarpic fruit growth when applied to ovaries (Vivian-Smith and Koltunow 1999; Bünger-Kibler and Bangerth 1982).

Auxin- and GA-mediated fruit growth takes place through different morphological and cellular processes and their simultaneous application to ovaries is required to obtain fruits that resemble pollinated ones in their size and shape (Serrani et al. 2007a; Ozga et al. 2002). Auxin signal has been recognized as one of the main hormonal cues responsible for driving flower and fruit development (Sundberg and Østergaard 2009). Indeed, auxin content has been shown to increase in tomato fruits after pollination and its distribution across the growing fruits suggests that seeds are most likely the site of auxin biosynthesis (Mariotti et al. 2011; Pattison and Catalá 2012).

The involvement of GAs in growing tomato fruits has been extensively reported (Mapelli et al. 1978; Bohner et al. 1988; Mariotti et al. 2011). Parthenocarpic fruit growth in *pat* and *pat-2* tomato mutants is related to a precocious activation of GA biosynthesis (Fos et al. 2000; Olimpieri et al. 2007). Moreover, the pollination stimulus is able to activate GA metabolism while application of GA biosynthesis inhibitors determines a strong reduction of fruit growth and seed number (Koshioka et al. 1994; Serrani et al. 2007b). In tomato, the increase of endogenous GA content upon pollination is associated with the upregulation of biosynthetic genes such as *SlCPS* (copalyldiphosphate synthase), *SlGA20ox1*, *SlGA20ox2* and *SlGA20ox3* encoding GA 20-oxidases enzymes, whereas gene expression of *SlGA3ox1* and *SlGA3ox2* appear basically unaffected (Rebers et al. 1999; Serrani et al. 2007b). Moreover, GA accumulation does not seem to be determined by a reduction of GA inactivation due to GA 2β-oxidases activity (Serrani et al. 2007b). The importance of GAs in fruit formation was further confirmed by silencing the GA signalling repressor, *SlDELLA* gene, which is known to be a repressor of fruit development in tomato (Martí et al. 2007).

It is widely documented that GAs influence their own regulatory system by negative feedback regulation of GA biosynthesis and positive feedforward regulation of GA catabolism (Hedden and Thomas 2012). However, other hormones like auxin also affect GA biosynthesis, catabolism and signalling (Weiss and Ori 2007). In Arabidopsis siliques, auxin exerts control on GA metabolism by the upregulation of *AtGA20ox1*, *AtGA20ox2* and *AtGA3ox1* without affecting GA 2β-oxidase genes expression (Dorcey et al. 2009). Similarly, in tomato ovaries, auxin treatment induces fruit-set by enhancing GA biosynthesis through GA 20-oxidases and GA 3β-oxidases gene upregulation, and by reducing GA inactivation; this supports the assumption that auxin acts prior to GAs as the early post-fertilization signal (Serrani et al. 2008). Further evidence for auxin and GA interplay is provided by a reduction of the *Auxin Response Factor 7* (*SlARF7*) gene transcripts in transgenic tomato lines in which fertilization–independent fruit growth depends on the partial activation of the auxin and GA signalling pathways (de Jong et al. 2011).

Among the auxin signalling components, the gene family *Auxin/INDOLE-3-ACETIC ACID* (*Aux/IAA*) encodes short-lived, nuclear-localized proteins with a central role in the control of auxin response (Hagen and Guilfoyle 2002). It has been demonstrated that auxin is required to facilitate the interaction between AUX/IAAs and the F-box protein TIR1 which is part of the ubiquitin ligase complex SCF^{TIR1} (Dharmasiri et al. 2005). Ubiquitination of AUX/IAAs and successive proteolysis via 26S proteasome are necessary to release the AUX/IAA transcription constraint of auxin responsive genes (Worley et al. 2000). In tomato, at least 26 different *Aux/IAA* genes have been identified up to now (Wu et al. 2012). The specific spatio-temporal expression pattern of several tomato *Aux/IAA* genes is consistent with the idea that the encoded proteins may perform specific plant developmental functions (Audran-Delalande et al. 2012).

Molecular characterization of the *entire* mutant of tomato revealed a recessive point mutation within the coding sequence of the *SlIAA9* gene (a member of *Aux/IAA* family). This mutation determines a premature stop in the sequence reading frame that would convert the IAA9 protein in a functionally defective peptide (Zhang et al. 2007). The most striking phenotype of *entire* is the extreme reduction in leaf complexity. Reproductive organ aberrations such as asymmetrical growth of flowers and fused sepals are also common features in this mutant. Little is known about the facultative parthenocarpic nature of *entire* mutant. Wang et al. (2005) suggests that IAA9 would act as an auxin signalling repressor that prevents ovary development before fertilization takes place since antisense downregulation of *SlIAA9* gene in the tomato cv. Micro-Tom induces parthenocarpy. However it is not known whether this precocious fruit set is mediated by a modified GA metabolism and/or response.

In this paper we report the role of *ENTIRE* (*SlIAA9*) gene as regulator of gibberellin metabolism in tomato fruit. Indeed, parthenocarpic fruit formation in *entire* depends on an alternative GAs metabolism regulation that leads to a high accumulation of active gibberellins.

Materials and methods

Plant material

Seeds of *entire* tomato (*S. lycopersicum* L.) mutant (accession n. LA2922) and its genetic background cv. Ailsa

Craig (AC, accession n. LA2838A) were obtained from the Tomato Genetic Resources Center (University of California, Davis, CA, USA). Plants were grown under standard greenhouse conditions as described by Mignolli et al. (2012). Six flowers for truss were left in order to limit fruit competition. To prevent self-pollination, flowers were emasculated at pre-anthesis stage (PA) and then manually pollinated with wild type (AC) pollen or left unpollinated. Flowers were tagged and harvested at different times. In the case of *entire*, only unpollinated fruits that showed parthenocarpic growth were selected for analysis.

Uniconazole treatments

Ten μL of a solution composed of 0.2 mM uniconazole (Uni) [(E)-(RS)-1-(4-Chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pent-1-en-3-ol; Santa Cruz Biotechnology, Dallas, TX, USA], 1 % ethanol and 0.1 % Tween 20 were applied on emasculated pollinated and unpollinated flowers at PA stage. Equal volume of solvent was used as mock. In order to revert the effect of uniconazole, gibberellic acid (GA_3 2 μg ovary^{-1}, Sigma-Aldrich, St. Louis, MO, USA) was applied to emasculated flowers simultaneously with the inhibitor. Fruits were collected and weighted 10 days later.

Quantification of endogenous GAs

Endogenous GAs were determined in ovaries at PA, and during the first 10 days in pollinated AC and parthenocarpic *entire* fruits. Extraction, purification and GAs determination were performed according to Mariotti et al. (2011). In brief, 0.5–1 g of frozen ovaries/fruits were ground in mortar adding 5 mL of 80 % methanol, centrifuged for 10 min at 4,000 rpm and supernatant was collected. Extraction procedure was repeated four times. A known amount of 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, purchased from Dr. L.N. Mander, Australian National University, Canberra, Australia) were added to the extracts, as internal standards. Extracts were first partitioned with ethyl acetate and successively purified by HPLC (Kontron, Munich, Germany) equipped with Hypersil ODS C18 column 150 mm long and 4.6 mm i.d. A linear elution gradient from 10 to 100 % methanol with a rate of 1 mL min^{-1} was applied. 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. Endogenous GAs quantification was accomplished by GC–MS/MS equipment Saturn 2200 quadrupole ion trap mass spectrometer coupled with a CP-3800 gas chromatograph (Varian analytical Instrument, Walnut Creek, CA, USA) according to the methodology described by Mariotti et al. (2011).

RNA extraction and gene expression analysis

According to Mignolli et al. (2012), total RNA was extracted from approximately 200 mg of fruits using the TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's procedures. Samples were initially homogenated with 2 mL of TRI Reagent and the supernatant was cleaned by chloroform partitioning. RNA was then precipitated by adding isopropyl alcohol followed by the addition of 75 % ethanol. RNA was finally resuspended in DEPC water. RNA was subjected to purification from contaminating DNA with the TURBO DNA free kit (Applied Biosystems/Ambion, Austin, TX, USA) and 5 μg of each sample was reverse transcribed into cDNA with the High-Capacity cDNA Archive Kit (Applied Biosystems). Real Time RT-PCR was performed with an ABI PRISM 7700 Sequence Detection Systems (Applied Biosystems).

Expression analysis of GA metabolism genes and *ToFZY* gene was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems) in presence of 200 ng of cDNA and specific probes as reported by Mariotti et al. (2011). Transcripts analysis of *LeGAST1* was performed using 50 ng of cDNA and Power SYBR® Green PCR master mix. Primer pairs used for *LeGAST1* were: Fw 5′-ACAATGGCTGGGAAAATGAG-3′ and Rv 5′-CTCTG TTGTTGCTGCTGCTC-3′.

Transcript levels of all genes were normalized with the expression of *LeEF1α* (Fw 5′-TGCTTGCTTTCACCC TTGGT-3′, Rv 5′-CGATTTCATCATACCTAGCCTTG GA-3 TaqMan probe 5′-CTGCTGTAACAAGATGGATGC 3′ for TaqMan amplification method or Fw 5′-GCTGCTGT AACAAGATGGATGC-3′, Rv 5′-GGGGATTTTGTCAGG GTTGTAA-3′ for SYBR® Green amplification method) used as internal control. Each sample was composed of a pool of five or ten fruits and all analyses were repeated twice (Fig. 1).

Statistical methods

One-way ANOVA analysis of variance with Tuckey's post test was performed for the experiments in Fig. 2B, C using Infostat version 2012 (di Rienzo et al. 2012).

Gene accession numbers

SlGA20ox1 no. AF049898; *SlGA20ox2* no. AF049899; *SlGA20ox3* no. AF04900; *SlGA3ox1* no. AB010991; *SlGA3ox2* no. AB010992; *SlGA2ox1* no. EF441351; *SlGA2ox2* no. EF441352; *SlGA2ox3* no. EF441353; *SlGA2ox4* no.

EF441354; *SlGA2ox5* no. EF441355; *LeGAST1* no. X63093; *LeEF1α* no. X53043.

Results

Spontaneous parthenocarpy in entire mutant

While no fruit-set was observed in AC unpollinated pistils, around 25 % of unfertilized *entire* ovaries showed spontaneous and facultative parthenocarpic growth (Table 1) with the development of a normal locular tissue (Fig. 1). Although no differences in size were observed between pollinated AC and *entire* fruits at 10 and 65 days after pollination, unpollinated parthenocarpic fruits of *entire* were significantly smaller than pollinated ones. Indeed, parthenocarpic fruits gained weight at a slower rate than pollinated ovaries and after 10 days from emasculation their weight was 1/5 of AC pollinated ones at the corresponding stage (Fig. 2A). At full ripe stage this difference was reduced and parthenocarpic fruits were 31.5 % smaller than wild type pollinated ones (Fig. 2B).

Table 1 Fruit-set percentage in pollinated and unpollinated ovaries of AC and *entire* in three independent experiments

		AC	*Entire*
Pollinated	Exp. I	27/27 (100 %)	16/16 (100 %)
	Exp. II	14/14 (100 %)	18/18 (100 %)
Unpollinated	Exp. I	0/69 (0 %)	24/115 (20.9 %)
	Exp. II	0/38 (0 %)	9/32 (28.1 %)

Inhibition of GA biosynthesis in pollinated and unpollinated fruits

In order to ascertain whether fruit development in the *entire* mutant is mediated by GA biosynthesis, we performed treatments with uniconazole (Uni), an inhibitor of the ent-kaurene oxidase (Rademacher 2000) (Fig. 2C). Application of Uni to pollinated ovaries significantly reduced fruit growth in AC and *entire*, although the effect was less severe in the mutant. In both genotypes,

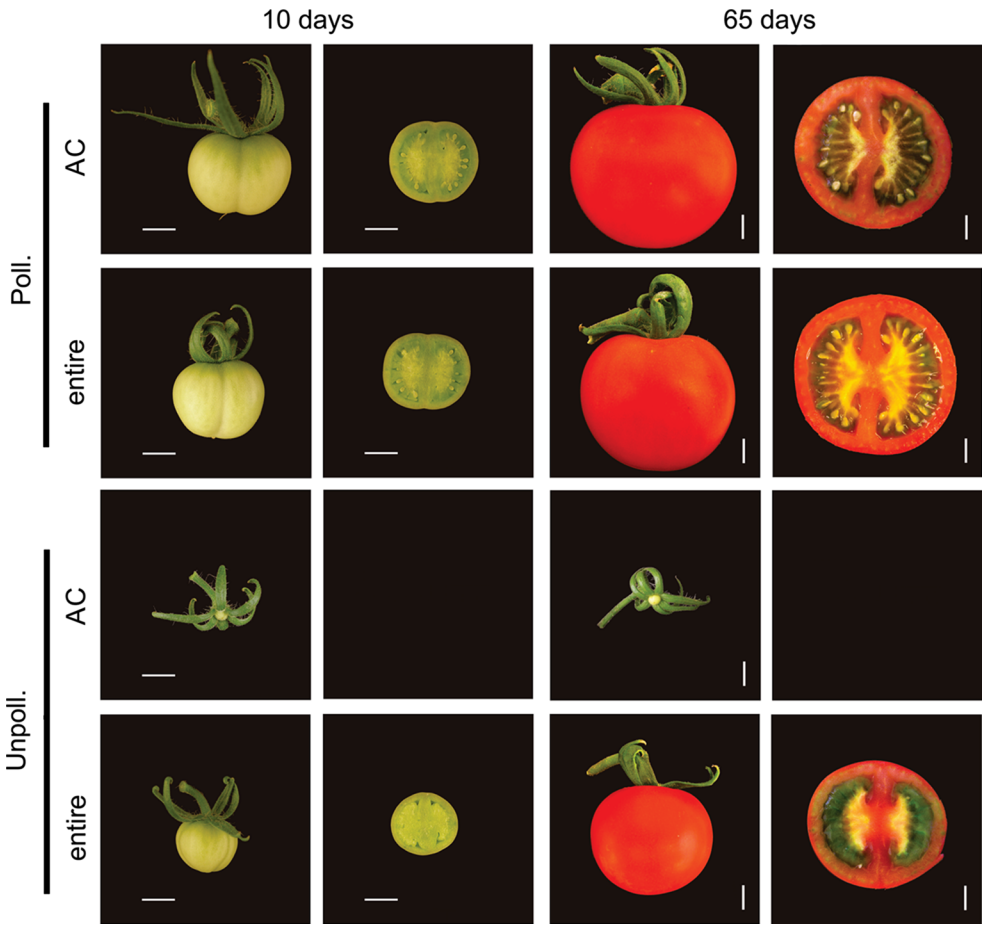

Fig. 1 Overview of pollinated and unpollinated fruits of *entire* and AC at 10 and 65 days (full ripe stages). Cross sections of AC and *entire* growing fruits are shown. White bars indicate 1 cm

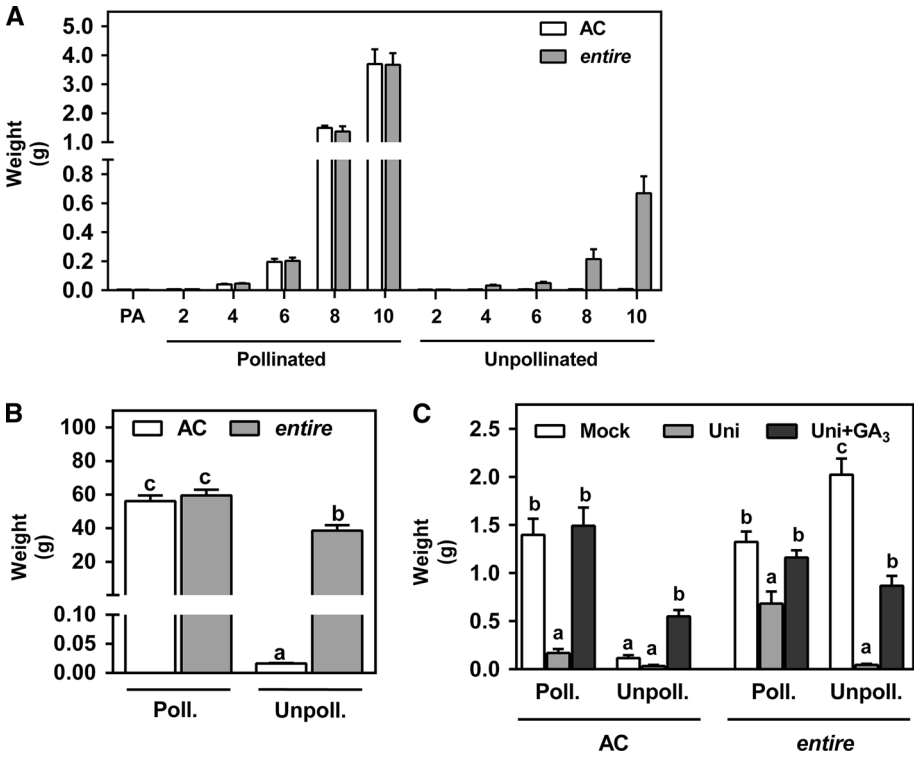

Fig. 2 Growth of pollinated and unpollinated AC and *entire* fruits during the first 10 days (**A**). In *entire* we selected only those fruits whose weight was higher than the maximum registered in AC unpollinated ovaries at the same stage. **B** Weight of 65-day-old fruits corresponding to full-ripe stage. **C** Inhibition of GA biosynthesis by uniconazole (Uni) in pollinated and unpollinated ovaries of AC and *entire* mutant. Fruits and ovaries were collected after 10 days from the treatment and weighed. Statistical analysis was performed in pollinated and unpollinated fruits for each genotype separately. Data are the means of 15–20 fruits. Different letters indicate statistical difference ($P < 0.05$)

application of GA_3 counteracted the effect of the inhibitor. Notably, mock-treated *entire* ovaries grew parthenocarpically but application of Uni strongly prevented fruits from setting. Simultaneous application of Uni and GA_3 was able to revert, at least in part, the effect of the inhibitor on *entire* fruit growth. The difference in fruit weight observed between 10-day-old fruits in Fig. 2A and C is most likely due to the presence of ethanol in the mock solution used to treat ovaries in this experiment. Indeed, even the lowest possible ethanol concentration reduced growth in pollinated fruits, possibly due to toxic effects during fertilization, but significantly enhanced unpollinated ovary growth (data not shown). In this respect, it has been reported that alcohols exert some positive effect on vegetative growth although the precise mechanism remains controversial (Rowe et al. 1994; McGiffen and Manthey 1996).

Endogenous GA levels in parthenocarpic fruits of entire

To determine whether parthenocarpic fruit development in *entire* was associated to GAs accumulation, we measured the endogenous content of some GAs from the early 13-hydroxylation pathways (GA_{19}, GA_{20}, GA_1, GA_3 GA_{29} and GA_8) during the first 10 days from emasculation (Fig. 3). At pre-anthesis stage GA levels did not differ substantially between AC and *entire*. Similarly to pollinated ovaries, concentration of GA_{19} (the precursor of GA_{20}) progressively decreased in *entire* fruits, indicating that conversion into GA_{20} took place. In fact, the conversion rate of GA_{19} into GA_{20} was clearly reduced in *entire* between 4 and 10 days from emasculation as indicated by the higher GA_{19} to $GA_{20} + GA_{29}$ ratio (O'Neill et al. 2010, see supplementary data S1).

The amount of biologically active GA_1 was reduced to less than a half 2 days after pollination in AC and slowly increased during the following stages. On the contrary, *entire* accumulated a five-fold higher level of GA_1 2 days after emasculation and this high level was maintained throughout the first 10 days. GA_3 (other biologically active GA) was also detected in tomato fruits and, similarly to GA_1, its content was generally higher in *entire,* with the highest peak at 6 days from emasculation. The product of 2β-hydroxylation of GA_1 leads to its inactive form GA_8. In wild type fruits, pollination determined a marked increase of GA_8 levels at 8 and 10 days from pollination. With an opposite trend, GA_8 accumulated during the first 4 days from emasculation in *entire* fruits, to decrease afterwards down to levels that are five-fold lower than in AC. The inactive form of GA_{20}, GA_{29}, is constantly lower in *entire* parthenocarpic fruits from two to 10 days and peaked in AC fruits at 4 days.

Expression of GA metabolism genes and a LeGAST1, a GA responsive gene

To evaluate whether *ENTIRE* gene plays a role in regulating the expression of GA biosynthesis genes, relative

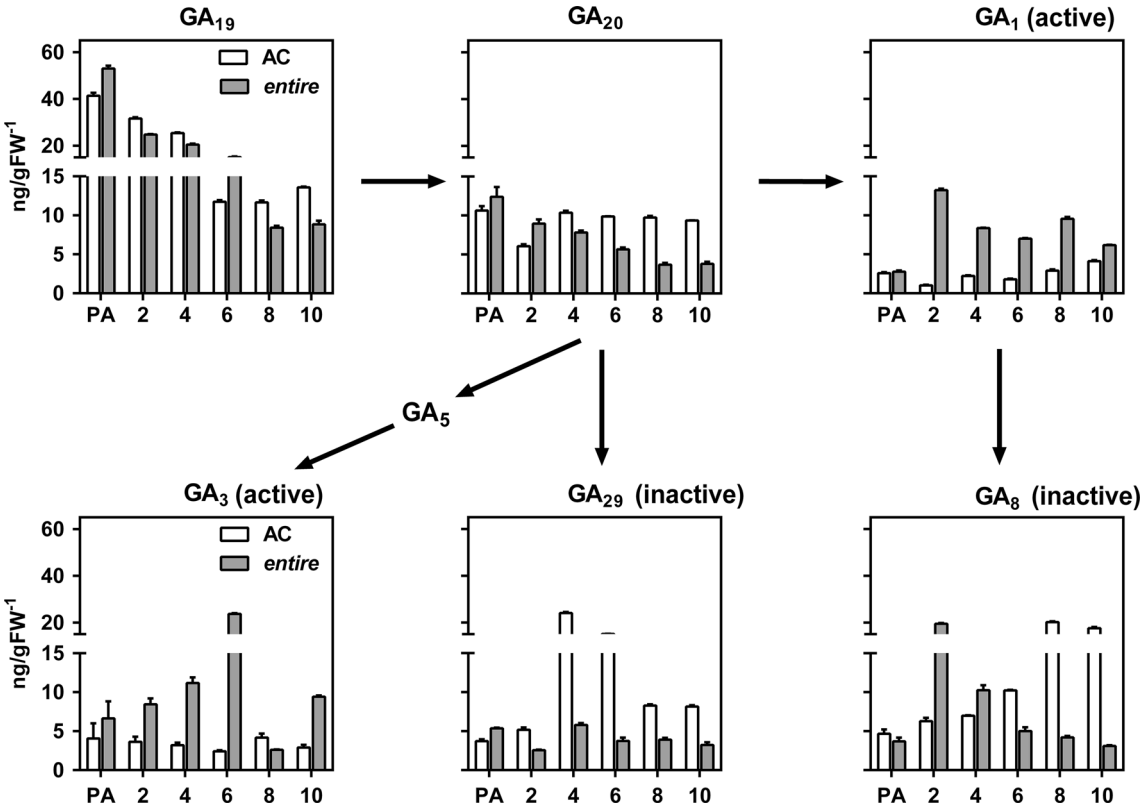

Fig. 3 Concentration of GA_{19}, GA_{20}, GA_{29}, GA_3, GA_1 and GA_8 in pollinated AC and parthenocarpic *entire* fruits at pre-anthesis stage (PA) and 2, 4, 6, 8 or 10 days after pollination (AC) or emasculation (*entire*). Data are mean ± SE (n = 3)

transcript abundance of *SlGA20ox1*, *SlGA20ox2*, *SlGA20ox3*, *SlGA3ox1* and *SlGA3ox2* was analysed in pollinated and unpollinated AC and *entire* unpollinated ovaries/fruits (Fig. 4).

Among members of the GA 20-oxidase gene family, *SlGA20ox1* was the most highly upregulated. Its expression in AC and *entire* fruits sharply increased from the time of pollination to 8 days later. While in AC unpollinated flowers the gene was practically unexpressed, in *entire*, *SlGA20ox1* was induced also in absence of pollination although at a lower extent with respect to pollinated fruits. *SlGA20ox2* was induced in pollinated fruits and in *entire* unpollinated ovaries, while very low expression was observed in AC unpollinated ovaries. Transcripts levels of *SlGA20ox3* peaked in AC and *entire* at 8 days after pollination and in AC emasculated flowers after 6 days.

In the case of AC and *entire* pollinated fruits, downregulation of *SlGA3ox1* occurred. Conversely, in unpollinated ovaries of *entire* and AC the gene reached its maximum expression at 2 days from emasculation and was maintained at higher levels than in pollinated fruits for up to 8 days from emasculation. *SlGA3ox2* was mainly expressed at PA but its transcripts were extremely low in both genotypes during all developing stages of pollinated and unpollinated fruits.

Active GAs homeostasis was also maintained by a fine modulation of GA inactivating enzymes (Hedden and Thomas 2012). In order to determine whether *entire* parthenocarpic fruit growth was also associated with an altered GA catabolism, we studied relative transcript levels of genes encoding GA 2β-oxidases (the main GA catabolic enzymes in tomato) *SlGA2ox1*, *SlGA2ox2*, *SlGA2ox3*, *SlGA2ox4* and *SlGA2ox5* (Serrani et al. 2007b) (Fig. 5). In AC and *entire*, with the exception of *SlGA2ox1* and *SlGA2ox5,* all *SlGA2oxs* transcripts dropped after pollination but upregulation of *SlGA2ox4* and *-5* was observed at later stages. With an opposite trend, expression level of *SlGA2ox2 and -4* increased in *entire* during the first 4 days that followed emasculation whereas *SlGA2ox3*, *-4* and *-5* genes appeared strongly downregulated from the forth day on. Conversely, in AC unpollinated and non parthenocarpic ovaries with the only exception of *SlGA2ox1* all GA2oxs were highly induced.

With the aim to evaluate whether the higher content of active GAs correlated with the activation of GA signal in *entire* fruit, the expression of the tomato GA-inducible gene *LeGAST1* (Shi et al. 1992) was analysed. As shown in Fig. 6, *LeGAST1* transcript accumulation was significantly higher at two, 6 and 8 days after emasculation in the mutant fruits.

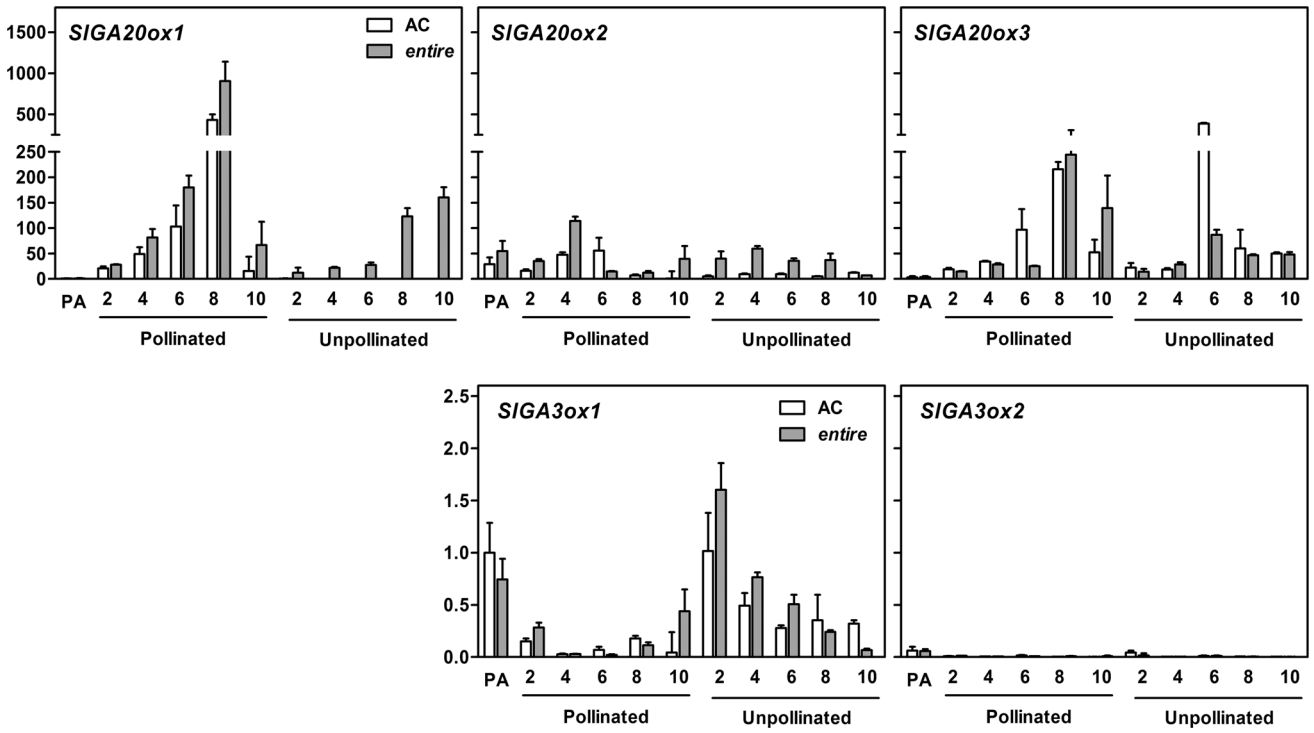

Fig. 4 Transcript levels of GA metabolism genes in pollinated AC and unpollinated parthenocarpic *entire* fruits. Relative expression level of *SlGA20ox1*, *-2*, and *-3*, *SlGA3ox1*, and *-2* was measured during the fist 10 days from pre-anthesis stage (PA), which corresponds to the moment of emasculation and pollination (AC) or simple emasculation (*entire*). Expression of *SlGA20ox1* and *SlGA3ox1* at PA in AC was set to one respectively for *GA20ox* and *GA3ox* gene family. Data are mean ± SE (n = 2)

Discussion

In *entire* tomato mutant, the lack of functionality of the auxin signalling repressor *SlIAA9* (Zhang et al. 2007) leads to a strong reduction of leaf complexity as a consequence of the abnormal activation of auxin signalling in leaf margins (Koenig et al. 2009). Although this mutation also affects fruit formation, very little is known about this aspect and up to date there are no reports explaining the involvement in parthenocarpic fruit growth. Working with *SlIAA9* antisense lines in Micro-Tom tomato, Wang et al. (2005) have suggested that the SlIAA9 protein would act as a negative regulator of the transition from flower to fruit in tomato. The *entire* mutant also showed parthenocarpy (Table 1; Fig. 1) but, differently from *SlIAA9* antisense plants (Wang et al. 2009), its fruit growth rate was slower during early stages, and full ripe fruits did not reach the same size as the pollinated ones (Fig. 2A, B). In the case of *entire*, it is possible that these differences depend on the genetic background or on the silencing technique used by Wang et al. (2009). In this context, it should be noted that antisense *SlIAA9* lines showed high variability in the level of parthenocarpy as a consequence of the degree of *SlIAA9* silencing (Wang et al. 2005). Alternatively, the degree of parthenocarpy that we observed in *entire* together with the loss of domains III and IV (Zhang et al. 2007) may reveal that for SlIAA9, an independent pathway for homo- and heterodimerisation with Aux/IAA and ARF proteins (Tiwari et al. 2003) is also functioning to prevent fruit from setting. When exogenous auxin is applied to *entire* ovaries, a dose-dependent growth is induced (see supplementary data S2) suggesting that auxin response in fruits is not saturated by the abolishment of *ENTIRE* gene, and that other auxin signalling elements might be implicated to induce a full development of the fruits. Moreover, the dominance of earlier developed flowers over later developed ones in the same truss (Bangerth 1989) may play a significant role in *entire* parthenocarpy induction. This hypothesis is supported by the fact that when only the first flower of a truss was left in *entire*, the level of parthenocarpy can be raised up to 70 % (data not shown) and that the highest frequency of parthenocarpic fruit growth was observed in flowers that occupy the first positions in the truss (Fig. S3).

Although auxin dynamics in the ovary have a crucial role in controlling tomato fruit development (Pattison and Catalá 2012), auxin does not act alone, as this process is largely mediated by GAs whose metabolism increases in response to auxin (Serrani et al. 2008; Ozga et al. 2009). Uniconazole application to PA flowers almost completely

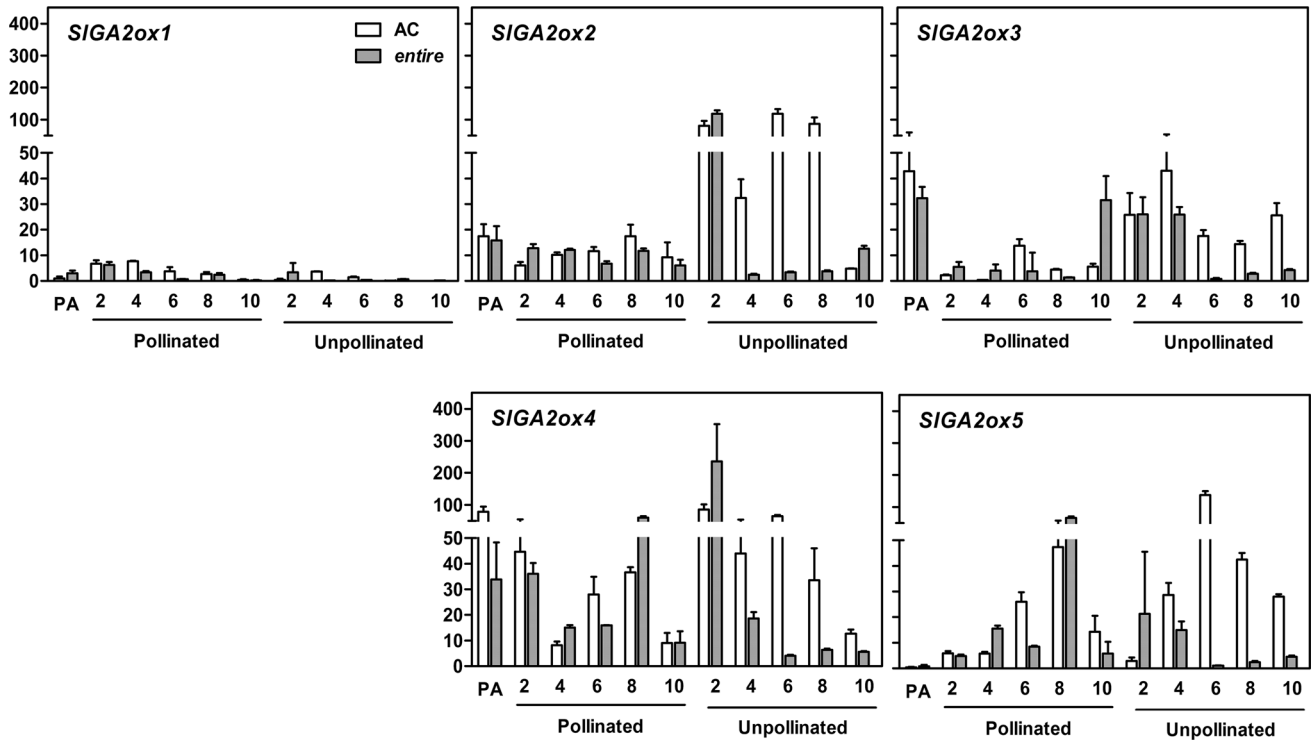

Fig. 5 Transcript level of GA catabolism genes in pollinated AC and unpollinated parthenocarpic *entire* fruits. Relative expression level of *SlGA2ox1*, *-2*, *-3*, *-4* and *-5* was studied during the first 10 days from pre-anthesis stage (PA). Expression of *SlGA2ox1* at PA in AC was set to one. Data are mean ± SE (n = 2)

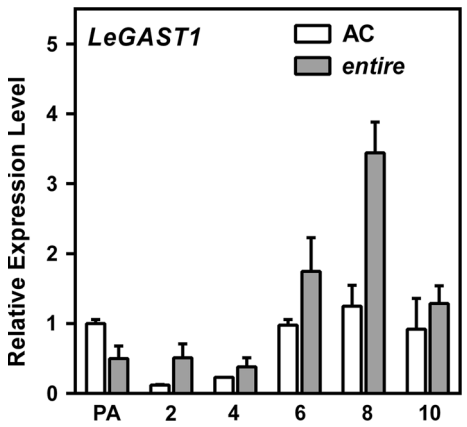

Fig. 6 GA response gene *LeGAST1* in AC and *entire* fruits during the first 10 days from pollination (AC) or emasculation (*entire*). Expression at PA in AC was set to one. Data are mean ± SE (n = 2)

blocked parthenocarpic fruit growth in *entire* and its effect was at least partially reverted by simultaneous application of GA_3 (Fig. 2C). This indicates that, similarly to pollinated fruits, fruit-set and growth of *entire* unfertilized ovaries depends on active metabolism of GAs.

Analysis of the endogenous level of the early 13-hydroxylation (early 13-OH) GA biosynthetic pathway, the most representative in tomato fruit (Fos et al. 2000), was performed in order to assess whether GA metabolism is affected by the mutation. In many cases, parthenocarpic fruit development was associated with accumulation of active GAs very early in ovary development (Olimpieri et al. 2007; Fos et al. 2000, 2001). Conversely, *entire* did not accumulate significantly higher amounts of GAs at PA in comparison to non-parthenocarpic AC ovaries (Fig. 3). Although evidently essentials for fruit growth (Fig. 2C), it is unlikely that GAs can constitute the first signal responsible for priming parthenocarpy in *entire*. Wang et al. (2009) have proposed that SlIAA9 would control fruit initiation by regulating photoassimilate loading into the ovary along with auxin and ethylene signalling activation. However, the participation of other hormones cannot be excluded in consideration of a possible modified auxin/GA interaction when auxin signal repression imposed by SlIAA9 is abolished (Wang et al. 2005).

The endogenous GA pattern indicates that an altered GA homeostatic regulation takes place during fruit-set and early growth in absence of a functional *ENTIRE* gene in unpollinated ovaries. Indeed, *entire* fruits exhibit a higher content of active GAs (GA_1 and GA_3) than pollinated wild type fruits (Fig. 3). Since GA_8 is the direct catabolite of GA_1, its content is considered the record of the amount of GA_1 that was formerly present (Coles et al. 1999). Thus, the decrease of GA_8 that is registered during the early

expansion phase in *entire* could be due to a slower conversion of GA_1 into GA_8. It should be noted that higher levels of active GAs could also be the result of the lower conversion from GA_{20} to GA_{29} which results in a higher availability of active GAs precursor (Fig. 3).

The *GAST* gene family is known to be rapidly induced by GAs in different plant species (Aubert et al. 1998; Ben-Nissan and Weiss 1996; Zimmermann et al. 2010). In tomato, transcription of the *LeGAST1* is positively regulated by GA_3 (Shi et al. 1992; Serrani et al. 2008). The high *LeGAST1* expression level found in *entire* provided further evidence that the elevated level of active GAs triggers the GA signal transduction (Fig. 6).

The accordance between endogenous GA levels and transcript abundance of GA biosynthesis and catabolism genes supports the general use of gene expression analysis to infer protein levels and enzymatic activity (Nadeau et al. 2011). In our work, we analysed the expression of GA 20-oxidases and GA 3β-oxidases genes in both pollinated AC and parthenocarpic *entire* fruits. Gibberellin C-20 oxidation is considered a rate-limiting step for the regulation of active GAs flux in fruits (Rebers et al. 1999; Serrani et al. 2007b). In this sense, parthenocarpy in *pat* tomato mutant is associated with higher accumulation of GAs as a consequence of high *SlGA20ox1* induction (Olimpieri et al. 2007). While *SlGA20ox1* was not expressed in AC in absence of pollination, it was induced in *entire* unpollinated fruits at a lower extent than in pollinated fruits (Fig. 4). It is then conceivable that the partial activation of auxin signalling in *entire* would be sufficient to induce *SlGA20ox1* in growing fruits. It is known that *SlGA20ox1* is induced by auxin probably due to the presence of auxin responsive elements in its promoter region (Martí et al. 2010). These findings are supported by the role of auxin in enhancing GA 20-oxidases gene expression as previously reported in Arabidopsis siliques (Dorcey et al. 2009), pea pericarps (Ngo et al. 2002) and tomato fruits (Serrani et al. 2008). Since *SlGA20ox1* transcript levels was higher in pollinated *entire* fruits than in parthenocarpic fruits, it suggests that other fertilization-linked signals could exert a supplemental inductive effects on *SlGA20ox1* gene.

In the last step, active GAs are produced by the action of a class of 2-oxoglutarate dependent dioxygenases, GA 3β-oxidases, that convert GA_{20} and GA_9 to GA_1 and GA_4, respectively. GA_3 is also synthesized from GA_{20} through GA_5 as intermediate (Hedden and Thomas 2012). In tomato, *SlGA3ox1* was barely transcribed early after pollination in both genotypes. Nevertheless, high transcript levels of *SlGA3ox1* in *entire* parthenocarpic fruits during the first 6 days from emasculation (Fig. 4) are probably associated with the lack of a fertilization signal, since the gene is also expressed in AC unpollinated and non parthenocarpic ovaries.

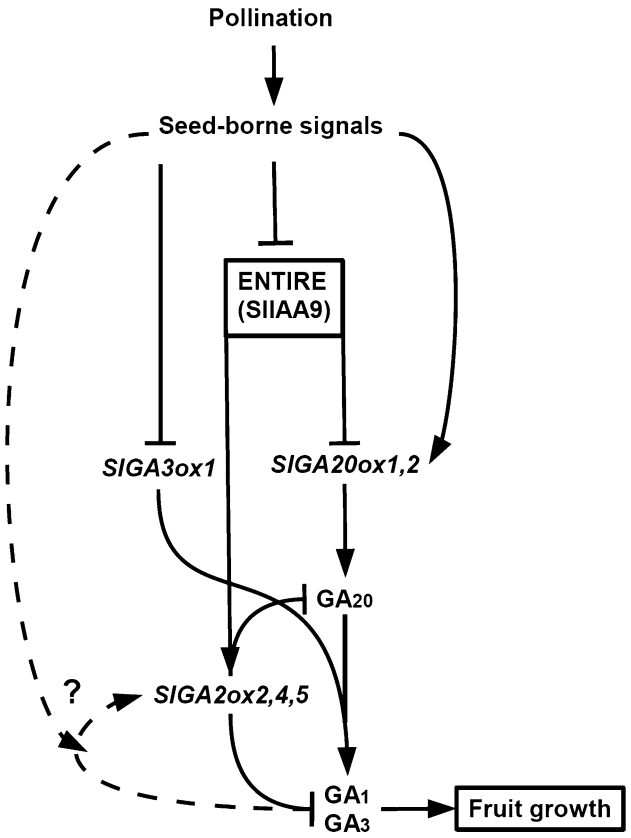

Fig. 7 Schematic model for the proposed role of ENTIRE (SlIAA9) protein in the regulation of gibberellin metabolism during fruit development. When pollination takes place, seed-specific signals degrade ENTIRE (IAA9) protein as reported by Wang et al. (2009). Release of IAA9 repression plus other seed-borne signals induces GA biosynthesis by strong activation of GA 20-oxidases genes (mostly *SlGA20ox1*). Homeostatic levels of active GAs are in turn controlled by a pollination-dependent feed-forward regulation (?) of GA2oxs (segmented lines) that cause the inactivation of GA_{20} and GA_1. In absence of fertilized ovules, impaired function of the *ENTIRE* gene results in the accumulation of active gibberellins (GA_1 and GA_3) through the combined action of GA 20-oxidases (*GA20ox1, -2*) and by downregulation of *GA2oxs* genes (*GA2ox2*, *-4*, *-5*) that reduces GA catabolism

Endogenous GAs homeostasis not only depends on biosynthetic processes but also on reactions that lead to GA inactivation (Thomas et al. 1999). It is well documented that negative feedback regulation of GA biosynthesis and positive feed-forward control of GA catabolism enzymes (particularly GA 2β-oxidases) are the two mechanisms that maintain active GAs within physiological limits (Hedden and Thomas 2012). Consistent with this, high content of GA_8 and strong upregulation of *SlGA2ox4* and *-5* genes (Fig. 5) were observed in pollinated fruits in both genotypes and paralleled with the induction of *SlGA20ox1*. Interestingly, in unpollinated wild type ovaries, where it is known that both metabolism and accumulation of GAs are extremely reduced (Koshioka et al. 1994; Serrani et al.

2008; Mariotti et al. 2011), the high induction of most *GA2ox*s seems independent from the feed-forward regulation. In e*ntire* unpollinated fruits that showed high active GAs content, most of *GA2ox*s were found downregulated after 6 days from emasculation but were upregulated in *entire* pollinated fruits (Fig. 5), suggesting that a positive feed-forward regulation is only active in response to pollination.

In summary, mutation of *ENTIRE* (*SlIAA9*) gene determines the release of at least part of the auxin signal that was sufficient to circumvent the pollination stimulus and initiate fruit growth in a facultative way. Our results suggest that the ENTIRE protein could participate in GAs metabolism by inhibiting *SlGA20ox1* and *SlGA20ox2* but activating most of GA 2β-oxidases in absence of pollination (see scheme in Fig. 7). Indeed, knock out of *ENTIRE* gene leads to high accumulation of active GAs as response to high activity of *SlGA3ox1* in presence of a basal induction of *SlGA20ox1*. At later stages the concerted action of *SlGA20ox1* upregulation and the strong reduction of GA catabolism would contribute to maintain the higher levels of active GAs at the start of expansion phase in *entire* parthenocarpic fruits. In presence of fertilization stimulus the inactivation of ENTIRE protein (Wang et al. 2009) takes place. This, that along with other signals, produces a high induction of *SlGA20ox1* concomitantly with the increase of active GAs flux at the onset of rapid fruit growth (from 6 to 10 days). GA 3β-oxidation shows a significant reduction but does not appear to be limiting, as active GAs were still synthesized. In addition, high levels of GA_{29} and GA_8 indicate that active GAs are subjected to a fast turnover in growing fruits following a feed-forward regulation in the pollinated wild type fruit probably as a consequence of *SlGA2ox4* and *SlGA2ox5* upregulation.

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