Gibberellins modulate auxin responses during tomato (*Solanum lycopersicum* L.) fruit development

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In tomato, auxin and gibberellins (GAs) interact with each other to drive fruit growth and development. While the role of auxin in directing GA biosynthesis and signal is already known, very little information has been obtained about GA-mediated control of auxin signalling and response. Interestingly, we show that GA_3 is able to modify the expression of several auxin signalling genes in the partial auxin-insensitive *diageotropica* (*dgt*) mutant, suggesting that GAs may override the control of DGT on auxin signal. *Procera* (*pro*) mutation, which confers a constitutively active GA signal, enhances the effects of exogenous auxin, indicating that PRO may act as a negative effector of auxin responses in fruits. Indeed, transcript modulation of some Aux/IAA and ARF genes in auxin-treated *dgt*/*pro* fruits, suggests that PRO controls their expression possibly bypassing DGT. It was also shown that GA biosynthesis, in response to auxin treatment, is largely controlled by DGT. It is therefore conceivable that the DGT-mediated increase of active GAs in auxin-treated or pollinated fruits, would promote PRO degradation, which in turn activates part of the auxin signalling cascade.

Abbreviations − Aux/IAA, auxin/indole acetic acid; ARF, auxin response factor; 4-CPA, 4-chlorophenoxyacetic acid; *Cyp1*, cyclophilin1; *dgt*, *diageotropica*; GAs, gibberellins; *pro, procera*.

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Introduction

Auxin is involved in every aspect of plant physiology including tropisms, lateral root formation, leaf abscission and response to pathogens (Sauer et al. 2013), and has a primary role during the conversion of the ovary into a growing fruit (reviewed by Pattison et al. 2014). In tomato (*Solanum lycopersicum*), the application of a synthetic auxin as well as the interruption of indoleacetic acid (IAA) export from unpollinated ovaries induces parthenocarpic fruit formation (Serrani et al. 2008, 2010). Similarly, enhancement of auxin sensitivity or increase of endogenous IAA biosynthesis spontaneously triggers tomato fruit growth (Carmi et al. 2003, Molesini et al. 2009). Using gene silencing and gene overexpression techniques, it has been possible to address functions related to tomato fruit-set and early development to several auxin signalling components. Members of the auxin signalling family Auxin/Indole Acetic Acid (Aux/IAAs) and Auxin Response Factors (ARFs) family are involved in the transition of flowers to fruits by regulating photoassimilate allocation to the ovary (SlIAA9, SlIAA27, SlARF4) (Wang et al. 2009, Bassa et al. 2012, Sagar et al. 2013), or by controlling cell divisions (SlARF9; de Jong et al. 2015) or cell expansions (SlARF7 and SlIAA17; de Jong et al. 2011, Su et al. 2014). A model was proposed for fruit-set, where heterodimers of specific Aux/IAAs and ARFs would repress transcription of auxin-related genes in pre-anthesis ovaries and, consequently, prevent ovaries from growing. Following pollination, a burst of endogenous auxin promotes the binding between the F-box auxin receptor proteins and Aux/IAAs leading to their degradation via the ubiquitin–proteasome pathway. Once freed from Aux/IAA repression, ARFs would subsequently regulate auxin-responsive genes (Sotelo-Silveira et al. 2014).

The *diageotropica* (*dgt*) mutant shows defects in some auxin-related phenotypical features such as gravitropism of shoots, lateral root formation and xylem development (Zobel 1973). These traits are not attributed to lower auxin content (Fujino et al. 1998) but rather to auxin insensitivity (Muday et al. 1995). Indeed, the *dgt* lesion disrupts part of the auxin signal transduction pathway (Nebenführ et al. 2000). Genetic studies revealed that DGT is a cyclophilin (Cyclophilin1, SlCyp1), a peptidyl-prolyl cis-trans isomerase (PPIase) (Oh et al. 2006). Interestingly, DGT mediates responses in target tissues by moving from the shoot to the roots via phloem as a mobile signal protein (Spiegelman et al. 2017). How cyclophilins-like DGT integrate auxin signalling is still unclear (Retzer and Luschnig 2015). However, recent findings demonstrated that the PPIase LATERAL ROOTLESS2 (LRT2) is responsible for the correct folding of OsIAA11 in rice. This conformational adjustment would enable OsIAA11 destabilization and the consequent derepression of auxin-regulated genes (Jing et al. 2015). Mutation at *DGT* locus reduces fruit size, number of seeds and number of locules as a result of auxin signalling alteration (Balbi and Lomax 2003).

Although auxin has a central role in controlling fruit-set and early growth, it is not the only hormone that takes part in these processes. Actually, various hormones were reported to be involved in complex networks during fruit development (Kumar et al. 2014). Gibberellins (GAs) represent a class of phytohormones that plays a fundamental role during fruit development. GAs are synthetized from geranylgeranyl diphosphate (GGDP), the precursor of diterpenoids. Through the action of *ent*-copalyl diphosphate synthase and *ent*-kaurene synthase, GGDP is converted to *ent*-Kaurene, which in turn is transformed into GA_{12} by *ent*-kaurene oxydase (KO) and *ent*-kaurenoic acid oxydase (KAO). In the 13-hydroxylated pathway, GA_{12} is oxidized on C_{13} by GA_{13} oxidase to form GA_{53}. The latter is converted to active GAs (GA_1 and GA_3) by sequential oxidation on C20 by GA 20-oxidases (GA20oxs) and on C3 by GA 3β-oxidases (GA3oxs). Inactivation of GAs is mainly catalysed by GA 2 β-oxidases (GA2oxs) that produce GA_{29}, GA_{34} and GA_8 from GA_{20}, GA_4 and GA_1, respectively (Yamaguchi et al. 2008).

It is widely known that in tomato and *Arabidopsis thaliana* (Arabidopsis), external application of GA_3 or genetically enhanced GA signal, triggers spontaneous fruit growth (Vivian-Smith and Koltunow 1999, Serrani et al. 2007a, Martí et al. 2007). On the other hand, block of GA biosynthesis in pollinated ovaries arrests fruit development (Serrani et al. 2007b) and active GAs (GA_1 and GA_3) accumulate in tomato ovaries following pollination due to upregulation of GA biosynthesis genes such as GA 20-oxidases (Rebers et al. 1999, Serrani et al. 2007b, Mariotti et al. 2011). Various lines of evidence indicate that auxin and GAs interact together during the first stages of fruit development. According to a hierarchical scheme, auxin induces GA biosynthesis and active GA accumulation which in turn promote destabilization of DELLA proteins, GA signalling repressors, triggering GA signal and fruit growth initiation (Tang et al. 2015). DELLA proteins have been shown to control fruit formation, since the accumulation of four *della* mutations in Arabidopsis and a silenced *SlDELLA* in tomato have led to parthenocarpic fruit formation (Dorcey et al. 2009, Martí et al. 2007). Similarly, the *procera* (*pro*) mutant of tomato, known for its GA-constitutive phenotype, displays spontaneous fruit growth due to a point mutation in the VHVID domain of the DELLA protein (Bassel et al. 2008, Jasinski et al. 2008, Carrera et al. 2012).

To date, very few studies have been carried out on GA-mediated auxin signalling regulation in fruits. In this context, it has been reported that *SlARF7* expression is modulated in GA-induced parthenocarpic tomato fruits (Carrera et al. 2012) and that cell divisions in tomato fruit pericarps are promoted by GAs that indirectly activate some ARF genes (Liu et al. 2016b).

Our study provides further evidence for GA modulation of the auxin signalling pathway during fruit development. In particular, using the *dgt* mutant, we found that GA_3 treatment stimulates fruit development by modifying the expression level of auxin signalling genes. Moreover, the *dgt/pro*

mutant allowed us to observe that, besides a direct effect, auxin treatment results in fruit development via a GA-mediated change in the expression of some key genes involved in auxin signalling. A possible mechanism of interaction between the auxin and GA pathways during tomato fruit growth and development is proposed.

Materials and methods

Plant material and hormonal treatments

Seeds of tomato (*Solanum lycopersicum* L.) cv. Ailsa Craig (AC, accession n. LA2838A) and *pro* mutant (accession n. LA3283, in AC background) were obtained from the Tomato Genetics Resource Center (University of California, Davis, CA). Near-isogenic line of *dgt*, repeatedly backcrossed in AC, was donated by Dr. C. Coenen (Allegheny College, Meadville, PA). *Dgt*/*pro* double mutant was obtained by screening F2 population for double recessive individuals. Typically, double mutant plants are smaller than the wild type, they show extremely slender and droopy growth habit, and have dark green lanceolate leaves with reduced leaf margin serrations (Fig. S1). Four-week-old plants were transplanted in 5-l pots with peat-based substrate pH 5.5-6.5 (Dynamics 2, Agriservice, Buenos Aires, Argentina) and grown under greenhouse conditions during autumn at the University of the Northeast (UNNE, Corrientes, Argentina). Plants were regularly watered and fertilized with complex NPK plus micro elements fertilizer (Blaukorn classic, Compo, Münster, Germany). Treatments to ovaries were carried out after emasculating the flowers at pre-anthesis stage (2 days before full bloom). Gibberellic acid (GA_3, Sigma-Aldrich, St Louis, MO; 2 µg per ovary), 4-chlorophenoxyacetic acid (4-CPA, Sigma-Aldrich, 100 ng per ovary) and the combination of the two hormones were applied as a 10 µl drop containing 1% ethanol and 0.01% Triton X (Mignolli et al. 2012). Equal volume of solvent was used as mock. Experiments with AC, *dgt*, *pro*, and *dgt*/*pro* were performed by collecting ovaries at pre-anthesis stage (0) and after 1, 4 and 8 days from mock and 4-CPA application, according what described above. Pollinated fruits (Figs S3 and S4) were obtained by manually pollinating with AC pollen, emasculated pre-anthesis flowers. Ovaries/fruits were then harvested a 0, 2, 4 and 6 days after pollination. In all cases, samples, were weighed and stored at -70°C up to analyses.

Expression analysis of auxin signalling genes

For total fruit RNA extraction, cDNA synthesis and qPCR reaction set up, we followed the method described by Mignolli et al. (2015). In brief, frozen fruit tissues of approximately 0.1 g were ground in mortar with the addition of 1 ml of TRI Reagent® (MRC, Cincinnati, OH). Samples were centrifuged (12 000 *g*, 10 min at 4°C) and the supernatant partitioned with chloroform. Precipitation

of RNA was accomplished by adding ice-cold isopropanol and high salt solution (0.8 M sodium citrate and 1.2 M sodium chloride, Sigma-Aldrich, St Louis, MO) to the aqueous fraction. RNA pellet was washed with 75% ethanol, dried, and solubilized in DEPC water. Contaminating DNA was removed by incubating RNA samples with DNAse TURBO DNA free kit (Ambion, Austin, TX) and successively 5 µg of purified RNA was reverse transcribed into cDNA with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Auxin signalling and GA biosynthesis gene transcripts were analysed by Real Time qPCR (ABI Prism 7500, Applied Biosystems). Fifty ng of cDNA were amplified with 7.5 µl of Master Mix (Mezcla Real, Biodynamics, Buenos Aires, Argentina), and 10 mM forward and reverse primers. Cycling stage was set to 40 cycles at 95°C for 15 sec and 60°C for 1 min. Expression was normalized with the transcript level of the housekeeping gene *LeEF1α*. Primer sequences and gene accessions are listed in supplemental material (Table S1).

Analysis of endogenous GAs in fruits

Endogenous GAs were determined in AC and *dgt* entire fruits following pollination and application of mock and 4-CPA according to the methodology described by Mignolli et al. (2015). In short, 1 g of frozen samples was ground in 80% methanol, centrifuged, and the supernatant was collected. Extraction procedure was repeated four times. Fifty ng of deuterated GAs were added to the extracts as internal standards. Extracts were first partitioned with ethyl acetate and then eluted in a methanol gradient by HPLC equipped with Hypersil ODS C18 column (Thermo Fisher Scientific, Waltham, MA). All fractions were dried and trimethylsilylated with N, O- bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (Pierce, Rockford, IL) at 70°C for 1 h. Endogenous GAs were finally detected and quantified 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).

Results

GA_3 enhances 4-CPA response in *dgt* fruits

We treated emasculated *dgt* and AC flowers at pre-anthesis stage with mock solution, 4-CPA, GA_3 and a combination of both hormones. In *dgt*, 4-CPA treatment produced parthenocarpic fruits that were 6-fold smaller than AC and presented an extremely reduced placental tissue (Fig. 1A, B; Mignolli et al. 2012). GA_3 induced fruit growth in both genotypes (Fig. 1). However, GA_3 treatment resulted in smaller fruits than 4-CPA in AC, while GA_3 fruits were two times bigger than fruits treated with auxin in *dgt* (Fig. 1A, B). In both genotypes, fruits obtained following GA_3 treatment

had thick pericarps with negligible development of placental tissue (Fig. 1A, B). Interestingly, when 4-CPA and GA_3 were simultaneously applied, no additive effect on fruit weight was observed in AC, whereas *dgt* ovaries produced parthenocarpic fruits that were more than 2.5 times heavier than fruits obtained with 4-CPA alone. In addition, 4-CPA+GA_3-treated *dgt* fruits had a well-developed placental tissue that filled all locular cavities (Fig. 1A).

4-CPA-induced fruit development is enhanced in *pro*/*dgt* mutant

With the aim to observe whether a constitutive GA signal was able to enhance auxin responsiveness in *dgt*, pre-anthesis ovaries of AC, *dgt*, *pro* and *dgt*/*pro* were treated with mock solution and 4-CPA. Growth and internal morphology of 30-day-old fruits were then observed. Mock-treated *pro* and *dgt*/*pro* fruits grew parthenocarpically showing thick pericarp and lack of placental tissue (Fig. 2A). Interestingly, although 4-CPA *dgt*/*pro* fruits attained approximately 7-fold higher fresh weight with respect to 4-CPA-treated *dgt* fruits, they were significantly smaller than 4-CPA-treated AC and *pro* fruits (Fig. 2B). Moreover, as evidenced by transversal cuts, placental tissue development was observed in 4-CPA-treated fruits in *dgt*/*pro* but not in *dgt* (Fig. 2A). While no statistical differences were observed between 4-CPA- and mock-treated *dgt* fruits, 4-CPA-treated *dgt*/*pro* fruits grew significantly more than its mock, indicating that 4-CPA had an additional effect on the double mutant fruit growth (Fig. 2B).

Pro mutation modulates some auxin signalling genes in dgt

In order to establish whether PRO (SlDELLA) is involved as modulator of auxin signalling, we performed gene transcript analysis of *SlIAA2*, *SlIAA14*, *SlARF7*, *SlARF8* and *SlARF9* in AC, *dgt*, *pro* and *dgt*/*pro* fruits after treatment with mock or 4-CPA (Fig. 3). *SlIAA2*, *SlIAA14* and *SlARF9* genes (Fig. 3A, B, and E) were up-regulated in AC after 4-CPA application showing a peak 4 days after the treatment. Lower induction of these genes was observed in *dgt* fruits at 4 and 8 days (Fig. 3A, B, and E). In 4-CPA-treated *pro* fruits, the expression levels of *SlIAA2* at 1, 4 and 8 days were respectively approximately 7-, 2.5-, and 1.5-fold higher than in AC fruits (Fig. 3A). A similar pattern of expression was observed also for *SlIAA14* in 4-CPA-treated *pro* fruits (Fig. 3B). Notably, *SlIAA2*, *SlIAA14* and *SlARF9* genes at 1, 4 and 8 days were more induced in *dgt*/*pro* than in *dgt* after treatment with 4-CPA (Fig. 3A, B and E).

4-CPA treatment reduced *SlARF7* transcript level in all four genotypes but the decrease was more pronounced in *pro* and *pro*/*dgt* at 1 and 8 days (Fig. 3C). Interestingly, a steady decline of *SlARF7* transcripts was observed in mock-treated *pro* and *pro*/*dgt* fruits (Fig. 3C). With a different trend, *SlARF8* was up-regulated only in *dgt* and *dgt*/*pro* fruits after 8 days from treatment with 4-CPA,

whereas its expression in 4-CPA-treated AC and *pro* fruits was maintained below mock levels (Fig. 3D).

GA_3 induces some auxin signalling genes independently from *dgt* mutation

We wanted to determine whether auxin signalling gene expression was modulated in response to GA_3 but not 4-CPA treatment in the *dgt* mutant. Therefore, we analysed the expression of several auxin signalling related genes in ovaries/fruits at 0, 1, 4 and 10 days treated with mock, 4-CPA and GA_3. In *dgt*, the expression of *SlIAA2* was much lower than in AC at 4 and 10 days after 4-CPA treatment, yet higher than in the mock. Interestingly, the application of GA_3 considerably raised *SlIAA2* expression, particularly in *dgt* (Fig. 4A). Although the treatment with GA_3 resulted in higher induction of *SlIAA14* in respect to 4-CPA in both genotypes, this was more evident in *dgt* (Fig. 4B). Conversely, *SlARF7* was similarly regulated in both genotypes, showing a sharp down-regulation in response to 4-CPA and GA_3 after 1 day (Fig. 4C). *SlARF9* was induced in response to 4-CPA after 1 day in both genotypes but its transcripts were relatively less abundant in *dgt* at 4 and 10 days. Similarly to *SlARF7, SlARF9* did not show any significant differences in terms of relative transcript content between AC and *dgt* GA_3-treated fruits at 1, 4 and 10 days after the hormone application. (Fig. 4D).

***Dgt* mutation reduces 4-CPA-induced GA biosynthesis**

We measured the content of GAs from the early 13-hydroxylation pathway (GA_{19}, GA_{20}, GA_1, GA_8, GA_5, GA_3 and GA_{29}) and the expression of some GA metabolism genes (*SlGA20ox1*, *SlGA3ox1*, *SlGA2ox1*) in AC and *dgt* fruits at 10 days from the application of 4-CPA (Table 1, Fig. 5). The content of endogenous GAs was significantly reduced in *dgt*. In particular, GA_3 content, one of the active GAs, was less than half of the level found in AC. Levels of GA_{19} and $GA_{20,}$ and of GA_8, the GA_1 catabolite, were also lower in the mutant (Table 1). The expression of *SlGA20ox1* was induced in response to 4-CPA application in both genotypes and a peak was produced after 4 days of treatment. However, transcript levels increased more steeply in AC showing 2.2-, 4.4- and 23-fold higher induction than in *dgt* at 1, 4 and 8 days after treatment, respectively (Fig. 5). In AC, *SlGA3ox1* was down-regulated following the application of the synthetic auxin. However, in *dgt*, the increase in *SlGA3ox1* expression one day after 4-CPA treatment was followed by an abrupt decrease 4 days after the treatment. *SlGA2ox2* was less induced in 4-CPA-treated AC and *dgt* fruits with respect to the mock, but no considerable differences in expression were observed in 4-CPA-treated AC and *dgt* fruits (Fig. 5).

Discussion

In tomato ovaries, full responsiveness to exogenous auxin depends on the activity of the cyclophilin DGT (Mignolli et al. 2012). However, when the synthetic auxin 4-CPA is applied in combination with GA_3 to *dgt* ovaries, an increase in fruit growth was observed with respect to treatment with 4-CPA or GA_3 alone (Fig. 1A, B). We suggest that GA_3 and 4-CPA may have an additive effect on the auxin signalling of fruits when the responsiveness to auxin treatment is hindered by the *dgt* mutation. In addition, 4-CPA+GA_3-treated *dgt* fruits presented conspicuous placental tissue if compared to that obtained by single hormone application (Fig. 1A). According to Lemaire-Chamley et al. (2005), the formation of the locular tissue in tomato requires the transduction of auxin signal that coordinates the enlargement of locular cells. Likewise, in cucumber fruits, CsGID1a (a GA receptor) would act as regulator of auxin synthesis and transport during the development of placental tissue (Liu et al. 2016a).

We also asked whether the lack of GA signal repression by PRO/DELLA dysfunction was able to rescue *dgt* responsiveness to exogenous auxin. For this purpose, we analysed the effect of 4-CPA in *pro* and in the double mutant *dgt*/*pro*. Mock-treated *pro*/*dgt* ovaries showed a certain level of spontaneous fruit growth, indicating that the *dgt* mutation does not block GA responses in fruits (Fig. 2A). Nevertheless, the presence of *dgt* seems to reduce the response to GAs (Fig. 1B) independently from DELLA protein (Fig 2B). Differently from 4-CPA-treated *dgt* fruits, 4-CPA-treated *dgt*/*pro* fruits grew significantly more than mock-treated ones (Fig. 2A, B) and their locular cavities were filled with placental tissue (Fig. 2A). These data suggest that GAs could partially overcome the *dgt* restriction on fruit development, through the release of PRO/DELLA constraint (Murase et al. 2008).

In order to determine whether PRO/DELLA integrates the DGT/Cyp1 route of auxin signal regulation, we analysed the expression levels of some auxin-related genes in 4-CPA-treated AC, *dgt*, *pro* and *dgt*/*pro* fruits (Fig. 3). In *dgt*/*pro* the expression levels of *SlIAA2*, *SlIAA14* and *SlARF9* genes were higher than in *dgt* after 1, 4 and 8 days, and exhibited a similar expression pattern in the *pro* mutant (Fig. 3A, B and E). These genes have been previously reported to be up-regulated following pollination (Vriezen et al. 2008) or 2,4-D treatments (Serrani et al. 2008). SlARF9 has been reported to be a repressor of fruit growth and its up-regulation in growing fruits should be considered part of a negative feedback mechanism (de Jong et al. 2015). SlARF7 is a negative modulator of auxin and GA response (de Jong et al. 2011) whose transcription is positively regulated by SlDELLA (Carrera et al. 2012). Our data showed that spontaneous parthenocarpy is probably associated with down-regulation of *SlARF7* in both mock-treated *pro* and *dgt*/*pro* but not in mock-treated AC or *dgt* (Fig. 3C), which suggests that the *SlARF7* expression is controlled by

PRO/DELLA but probably not dependent on DGT/Cyp1. Although gene induction was similar in *pro* and *dgt*/*pro* fruits after auxin treatment, *pro* fruits grew significantly more than *dgt*/*pro* fruits, which could indicate that *dgt* may reduce GA response downstream of DELLA. Taken together, these data suggest that the regulation of *SlIAA2*, *SlIAA14*, *SlARF9* and *SlARF7* gene expression by PRO/DELLA prevails over DGT/Cyp1-dependent signalling, possibly acting as downstream regulator in the same pathway. Conversely, *SlARF8*, which negatively affects fruit development (Goetz et al. 2007), was up-regulated only in 4-CPA-treated *dgt* and *dgt*/*pro* fruits, indicating that DGT/Cyp1 may directly control this gene without the participation of PRO/DELLA.

Following, we investigated whether GA_3 is able to modulate auxin responsive gene expression bypassing *dgt* constraint. Surprisingly, *SlIAA2* and *SlIAA14* were more up-regulated in GA_3- than in mock- and 4-CPA-treated *dgt* fruits (Fig. 4A and B). The fact that GA_3 induced the expression of auxin signalling genes in *dgt* could mean that GA-modulated auxin response in fruits overrides the control imposed by DGT. Liu et al. (2016b) reported that several ARFs in the tomato pericarp are targeted by miRNAs in response to GA treatment when the auxin signal is blocked. These data strengthen the idea that genes shared by auxin and GA signalling allows one hormone to induce growth and development when the other one is absent or deficient (Björklund et al. 2007). A shared signalling pathway between auxin and GAs could contribute to finely tune plant responses to changing environments (Gallego-Bartolomé et al. 2011).

The effect of auxin on raising GA biosynthesis in tomato fruits as well as in other species has been previously studied (Mariotti et al. 2011, Dorcey et al. 2009, Ozga et al. 2003). The content of GAs from the early 13-hydroxylation pathway, which is considered the most representative in tomato fruits (Fos et al. 2000), were lower in 4-CPA-treated *dgt* fruits (Table 1). In particular, levels of bioactive GA_3 were significantly below those of AC. Although the content of active GA_1 did not differ between AC and *dgt*, the lower amount of GA_{19}, GA_{20} and GA_8 in *dgt* suggests a reduced metabolic flux through GA_1 in the mutant. Interestingly, analysis of GA metabolism genes showed that *SlGA20ox1* transcripts were dramatically low in auxin-treated *dgt* fruits with respect to AC (Fig. 5). In tomato fruits, GA 20-oxidase activity is generally considered as a regulatory step for bioactive GAs production (Rebers et al. 1999, Olimpieri et al. 2007, Mariotti et al. 2011). It is then possible that higher induction of *SlGA3ox1*, which encodes the last step of active GA synthesis, in 4-CPA-treated *dgt* fruits (Fig. 5) is the result of up-regulation imposed by the reduction of the GA 20-oxidase activity. It is noteworthy that *SlGA20ox1* expression is likely to be controlled by auxin and by its signalling components (Martí et al. 2010, Mignolli et al. 2015). This supports the hypothesis that auxin-induced GA biosynthesis is largely, yet not completely, regulated by DGT/Cyp1 through the action of *SlGA20ox1*. According to a widely accepted model, active GAs

induce a conformational change in the GA receptor (GID1) allowing GID1-DELLA molecular interaction. GA-GID1-DELLA complex stimulates the degradation of DELLAs and release, in this way, the repressive effect of these proteins on GA-regulated gene expression (Davièr and Achard 2013). Transcription of *SlDELLA* was not affected in *dgt* ovaries (Fig. S2); therefore, DGT may indirectly control SlDELLA abundance by regulating GAs content which in turn would promote SlDELLA degradation (Fig. 6).

It is worth to note, that pollinated *dgt* fruits accumulate more GAs (GA_1 and GA_3 early after pollination) (Fig. S3) than AC and the expression of main GA biosynthesis genes is similar (*SlGA20ox1*) or higher (*SlGA3ox1*) than AC (Fig. S4). This strengthens the idea that signals other than auxin could be responsible for GA metabolism activation in tomato and they are able to circumvent the block imposed by *dgt*. In this respect, relatively recent publications have shown that hormones such as ethylene and cytokinins are able to control GA metabolism on its own (Ding et al. 2013, Shinozaki et al. 2015).

In conclusion, the increase of auxin (e.g. mainly IAA) content, that derived from pollination (Mariotti et al. 2011), would prompt the transduction of auxin signal through DGT/Cyp1 which regulates the expression of some auxin signalling genes (Fig. 6). These, in turn, would induce the expression of GA biosynthesis genes (e.g. *SlGA20ox1*) leading to an accumulation of active GA (mainly GA_1 and GA_3). The increase in active GAs promotes GA-dependent PRO/DELLA proteasomal degradation. The loss of PRO/DELLA constraint, besides promoting GA- responsive genes, alters the expression of a subset of auxin signalling genes modulating, in this way, the DGT/Cyp1-dependent auxin regulated route and resulting in fruit growth. We also hypothesized that DGT/Cyp1 could affect the response to GA downstream DELLA protein. This model would also explain previous findings which showed that in presence of mutated *dgt*, auxin signal is only activated in pollinated fruits but not after exogenous auxin treatment (Mignolli et al. 2012). Based on those and on present results, we suggest that after pollination auxin-independent GA biosynthesis could also contribute to the auxin signal activation.

Author contributions

M.F., M.L.V. conceived and designed the experiments; M.F. performed hormonal treatments; M.L.V. performed gene expression analysis; L.M. carried out endogenous GA analysis; M.F. wrote the manuscript; M.L.V., P.P. and L.M. critically reviewed and improved the manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1: Phenotypical characteristics of the *dgt*/*pro* double mutant and its monogenic mutant parents *dgt* and *pro*.

Fig. S2: Relative expression of *SlDELLA* in AC and *dgt* pre-anthesis ovaries.

Fig. S3: Endogenous GA levels of in pollinated AC and *dgt* ovaries/fruits.

Fig. S4: Relative expression levels of GA metabolism genes in AC and *dgt* pollinated ovaries/fruits.

Table S1: Gene accessions and sequences of primers used for quantitative PCR analysis.

Legends

Fig. 1. Sections of AC and *dgt* fruits after 10 days from mock, 4-CPA, GA_3 and 4-CPA+GA_3 treatment (A) Pt = placental tissue, P= pericarp. Bars indicate 2 mm. Fresh weights of 10 days old AC and *dgt* fruits after hormonal application (B). Values are the mean of 10-20 fruits ± SEM. Different letters indicate statistical differences between treatments within genotypes, ANOVA analysis of variance with Tuckey's post-test ($P < 0.05$). Asterisks indicate statistical differences between genotypes within treatments (Student's *t*-test).

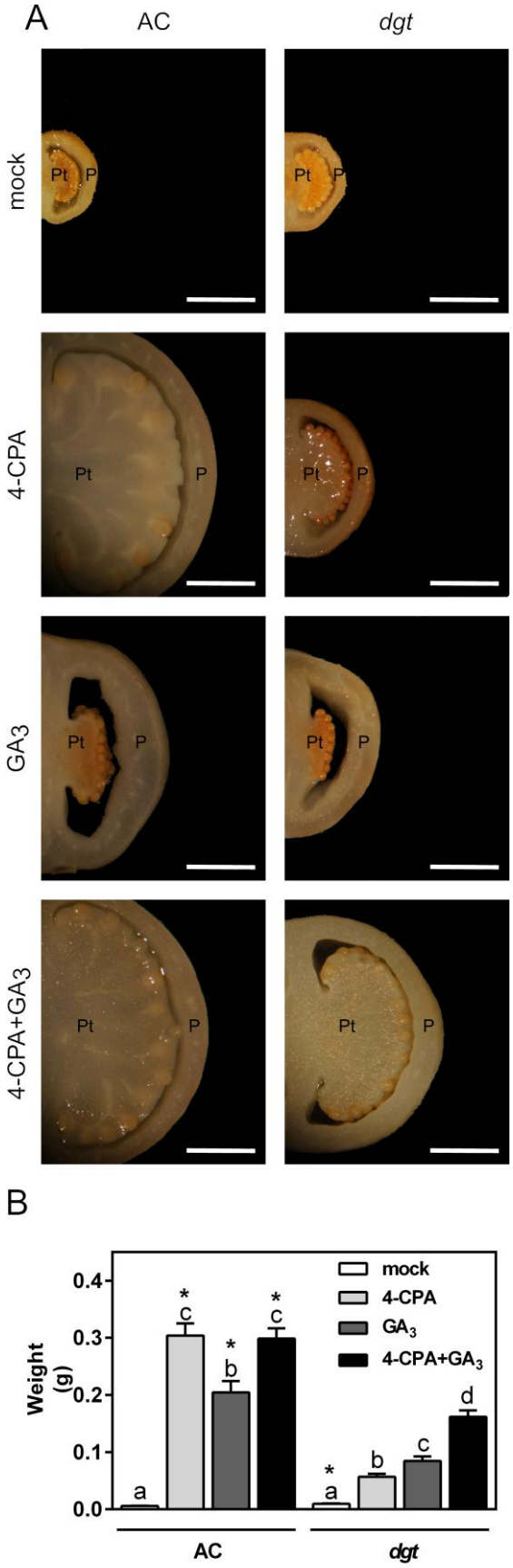
Fig. 2. Fruits of AC, *dgt*, *pro*, and *dgt/pro* after 30 days from mock and 4-CPA treatment (A), bars indicate 1 cm. Fresh weight of fruits after 30 days from mock and 4-CPA treatment (B). Values are the means of 10-20 fruits ± SEM. Different letters indicate statistical differences between genotypes within treatments, ANOVA analysis of variance with Tuckey's post-test ($P < 0.05$). Asterisks indicate statistical differences between treatments within genotypes (Student's *t*-test).

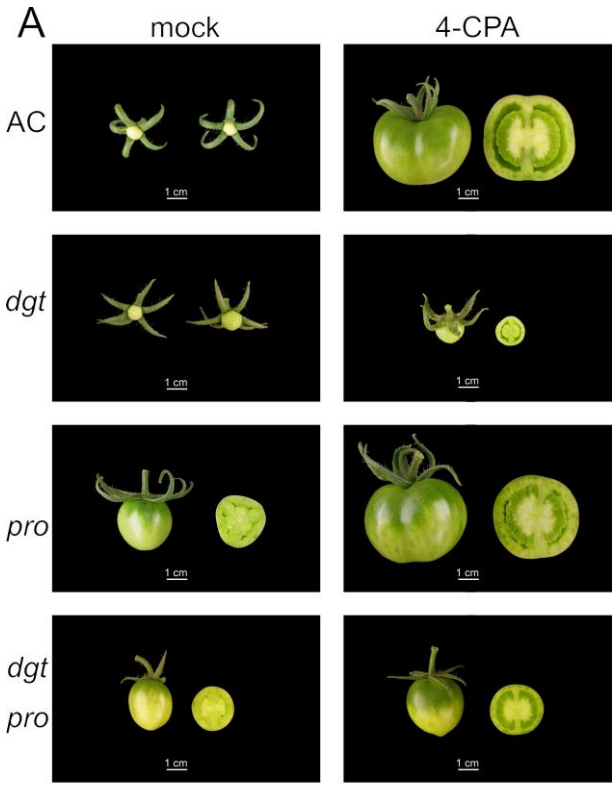
Fig. 3. Relative expression levels of *SlIAA2* (A), *SlIAA14* (B), *SlARF7* (C), *SlARF8* (D) and *SlARF9* (E) in AC, *dgt, pro* and *dgt/pro* fruits at the moment of treatment (0) and after 1, 4 and 8 days from mock and 4-CPA application. For each gene, expression of AC ovaries at pre-anthesis stage (0) was set to one. Values are the means of 3 replicates ± SD.

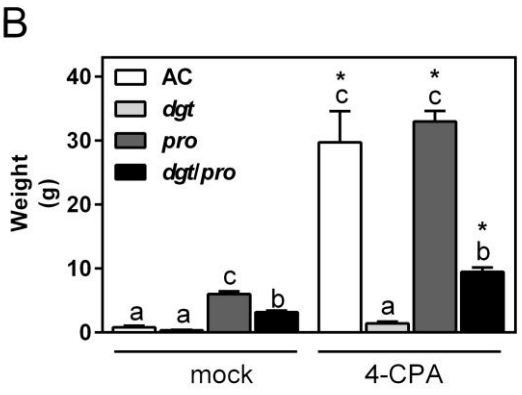
Fig. 4. Relative expression of *SlIAA2* (A), *SlIAA14* (B), *SlARF7* (C) and *SlARF9* (D) in AC and *dgt* fruits at 0, 1, 4 and 10 days from mock (white circles), 4-CPA (black circles) and GA_3 (grey circles) application. For each gene, expression of AC ovaries at pre-anthesis stage (0) was set to one. Each point represents means of 3 replicates ± SD.

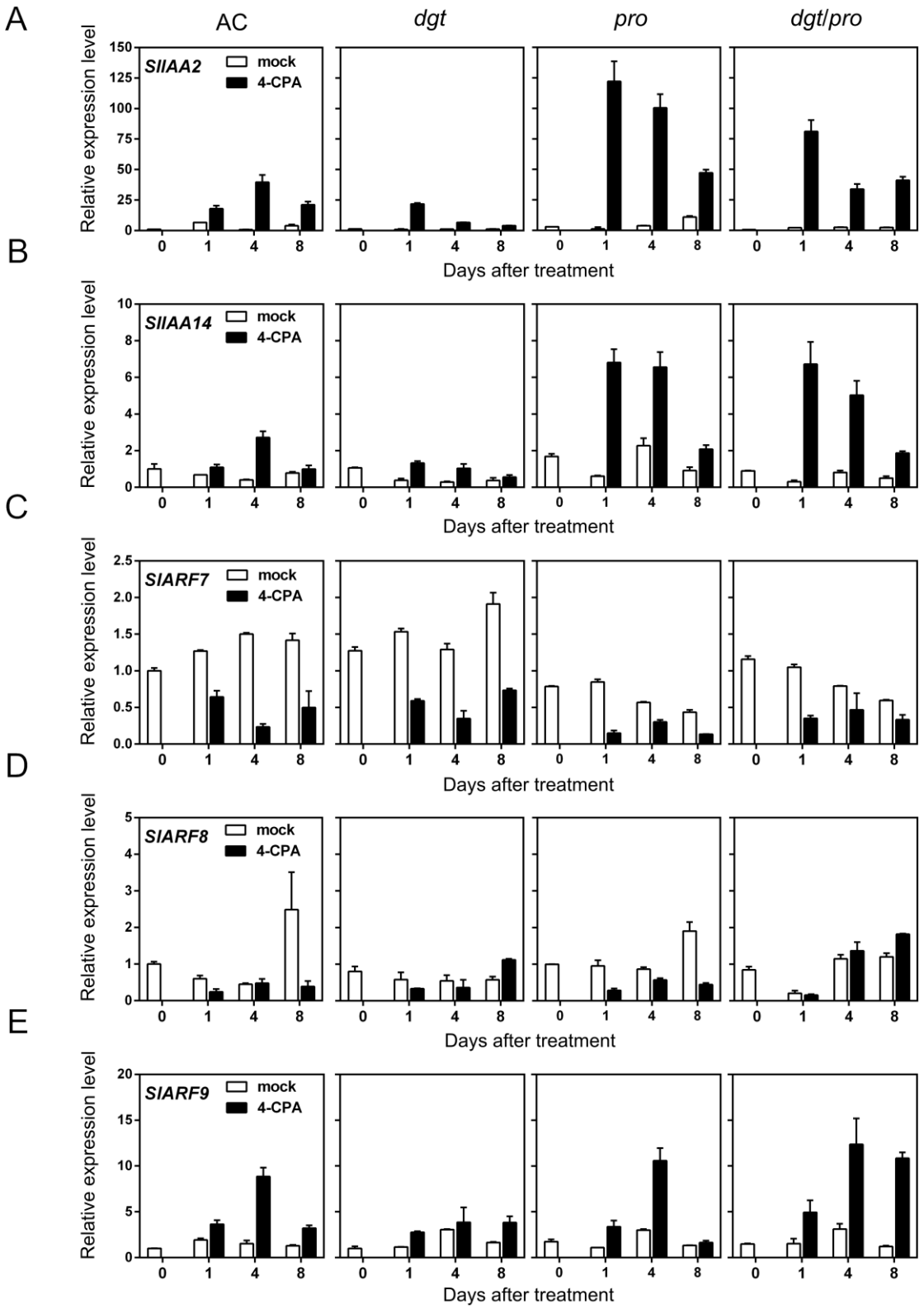
Fig. 5. Relative expression levels of GA biosynthesis (*SlGA20ox1* and *SlGA3ox1*, A and B respectively) and catabolism (*SlGA2ox2*, C) genes in AC and *dgt* fruits, at 0, 1, 4 and 8 days after the treatment with mock and 4-CPA. Values are the means of 3 replicates ± SD.

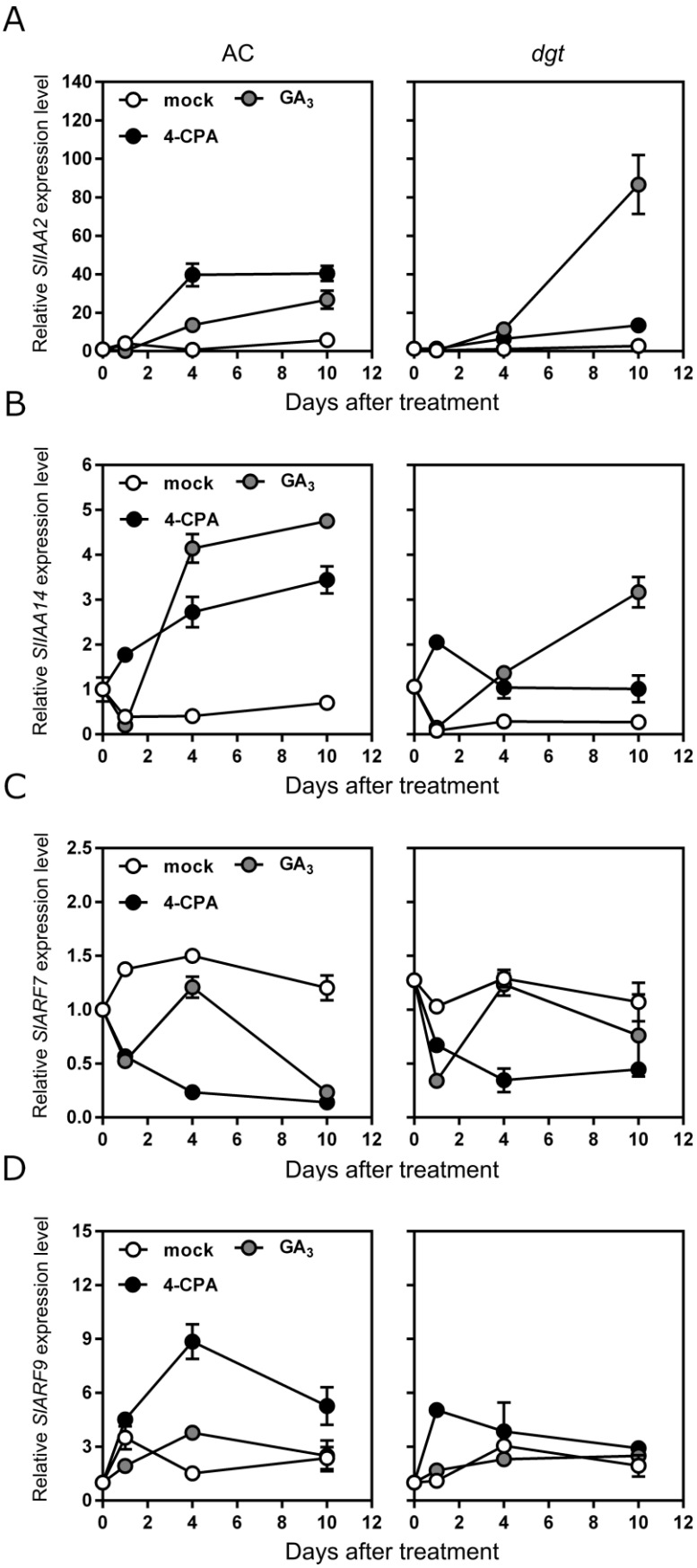
Fig. 6. Proposed model for auxin and GA crosstalk during tomato fruit development. The increase in auxin levels after pollination initiates the auxin signalling pathway via DGT/Cyp1, which results in the accumulation of active GAs and the consequent PRO/DELLA degradation. The removal of DELLA leads to GA response and a modulated auxin signalling, both of which induce fruit growth and development. The dashed line indicates a possible effect of DGT on GA responsive genes, whereas the dotted line indicates an alternative auxin signalling pathway not controlled by DGT.

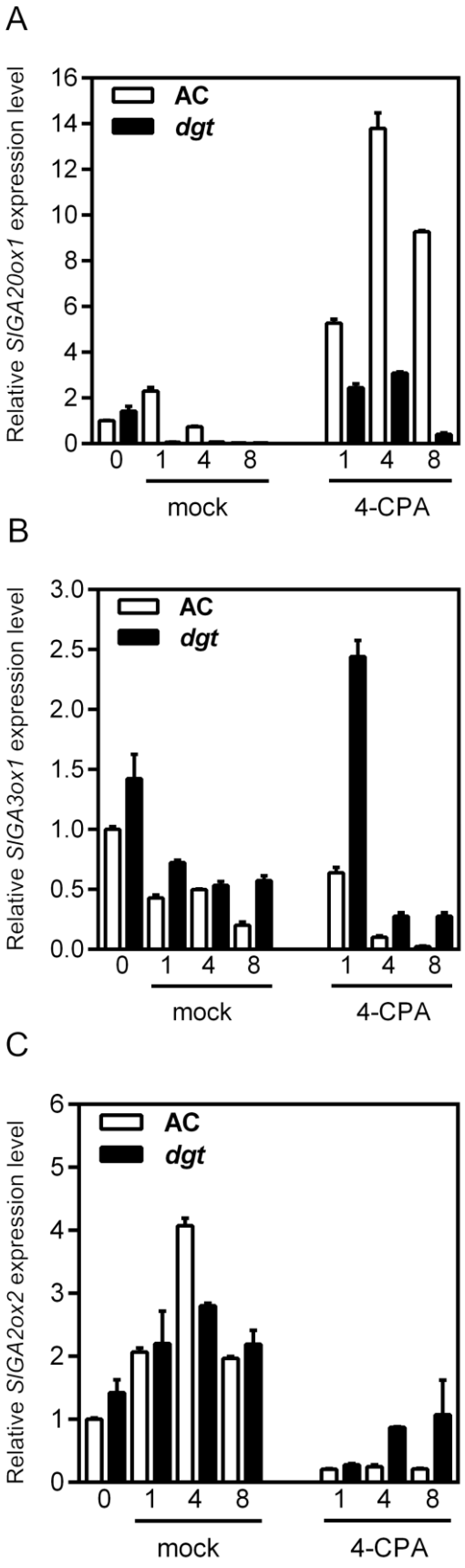












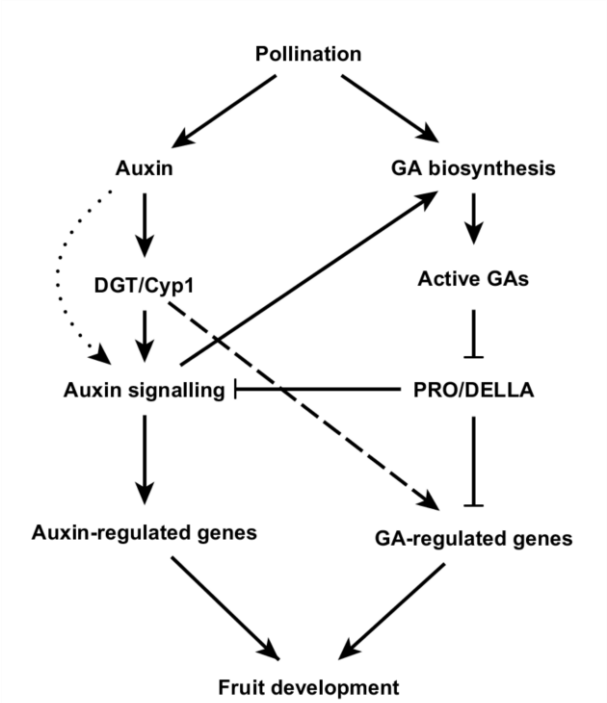


Table 1. Endogenous levels of the main GAs from the early 13-hydroxylation pathway in mock- and 4-CPA-treated fruits. Analysis was performed in AC and *dgt* fruits after 10 days from the treatments. Values are the mean of 3 replicates ± SEM. *indicate significant differences ($P < 0.05$, Student's t test) between AC and *dgt* for each GA within treatments.

Treatment	Genotype	GA_{19}	GA_{20}	GA_1	GA_8	GA_5	GA_3	GA_{29}
Mock	AC	nd	3.75 ± 0.3	nd	5.69 ± 0.1	nd	nd	nd
	dgt	nd	1.9 ± 0.2	nd	3.3± 0.1	nd	nd	nd
4-CPA	AC	12.2 ± 0.2	7.8 ± 0.3	1.9 ± 0.4	11.1 ± 0.1	0.6 ± 0.1	9.9 ± 0.6	4.5 ± 0.7
	dgt	6.4 ± 0.1*	2.7 ± 0.1*	1.1 ± 0.1	4.3 ± 0.1*	nd	4.5 ± 0.3*	2.7 ± 0.2*