

1 **Auxin-induced fruit-set in tomato is mediated in part by gibberellins**

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Summary

Tomato (*Solanum lycopersicum* L.) fruit-set and growth depend on gibberellins (GA). Auxins, another kind of hormone, can also induce parthenocarpic fruit growth in tomato, although their possible interaction with GA is unknown. We showed that fruit development induced by the auxins indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid (2,4-D) were significantly reduced by simultaneous application of inhibitors of GA biosynthesis (Paclobutrazol and LAB 198999), and that this effect was reversed by applied GA₃. This suggested that the effect of auxin was mediated by GA. Parthenocarpic fruits induced by 2,4-D had higher contents of the active GA₁, its precursors and metabolite, than unpollinated non-treated ovaries, but similar to pollinated ovaries. Application experiments of radioactive-labelled GAs to unpollinated ovaries showed that 2,4-D altered in vivo GA metabolism (both biosynthesis and catabolism). Transcript levels of genes encoding copalyldiphosphate synthase (SlCPS), SlGA20ox1, -2 and -3, and SlGA3ox1 were higher in unpollinated ovaries treated with 2,4-D. In contrast, transcript levels of SlGA2ox2 (out of the five SlGA2ox genes known to encode this kind of GA inactivating enzymes) were lower in 2,4-D treated ovaries. Our results support the idea that auxins induce fruit-set and growth in tomato, at least partially, by enhancing GA biosynthesis (GA 20-oxidase, GA 3-oxidase and CPS), and probably decreasing GA inactivation (GA2ox2) activity, leading to higher GA₁ content. The expression of diverse Aux/IAA and auxin response factors, which may be involved in this effect of auxin, was also altered in 2,4-D-induced ovaries.

1 **Introduction**

2 Tomato (*Solanum lycopersicum* L.) is one of the most studied fleshy fruits due to its
3 great commercial interest. In this species fruit development occurs normally after fruit-
4 set (changeover from the static condition of the flower ovary to the rapidly growing
5 condition of the young fruit) induced by fertilization, in two consecutive phases: an
6 active division, lasting about 7-10 d post-anthesis, and a cell expansion phase (Gillaspy
7 *et al.*, 1993). The ovary wall develops during fruit growth into a pericarp, while the
8 placental parenchyma fills the locular cavities with a jelly-like homogenous tissue
9 (locular tissue) enclosing the developing seeds (Gillaspy *et al.*, 1993; Ho and Hewitt,
10 1986).

11 Parthenocarpic fruit-set and growth can be induced by application of diverse
12 plant growth substances to unpollinated ovaries, mainly auxins and gibberellins (GAs)
13 (García-Martínez and Hedden, 1997; Gorquet *et al.*, 2005; Srivastava and Handa, 2005).
14 GA metabolism in plants initiates from geranylgeranyl diphosphate, which is converted
15 to *ent*-kaurene by the action of two consecutive cyclases (copalyldiphosphate synthase,
16 CPS, and *ent*-kaurene synthase, KS), followed by the action of P450 monooxygenases
17 (*ent*-kaurene oxidase, KO, and *ent*-kaurenoic acid oxidase, KAO), and of three kinds of
18 Fe²⁺- and 2-oxoglutarate-dependent dioxygenases (the biosynthetic enzymes GA 20-
19 oxidases and GA 3-oxidases, and the inactivating enzymes GA 2-oxidases), which are
20 encoded by small multigenic families (Sponsel and Hedden, 2004) (Supplementary Fig.
21 1). GA biosynthesis can occur through two parallel pathways: the non-13-
22 hydroxylation, leading to GA₄ as the active GA, and the early-13-hydroxylation
23 pathway, leading to the active GA₁ (Supplementary Fig. 1). The latter is the main
24 metabolic pathway in tomato, although GAs from the non-13-hydroxylation pathway
25 have also been identified in tomato fruit (Fos *et al.*, 2000). Fruit-set in tomato depends

1 on gibberellins (GAs), as shown by application of GA biosynthesis inhibitors to
2 pollinated ovaries (Fos *et al.*, 2000, 2001; Serrani *et al.*, 2007b), and of GAs to
3 unpollinated ovaries (Alabadí and Carbonell, 1998; Fos *et al.*, 2000, 2001; Serrani *et al.*,
4 2007a; Sjut and Bangerth, 1982/83), and the active form is GA₁ (Serrani *et al.*, 2007b).
5 The increase of GA content in the ovary upon pollination (Bohner *et al.*, 1988;
6 Koshioka *et al.*, 1994; Serrani *et al.*, 2007b) is associated with upregulation of
7 *SIGA20ox1*, -2 and -3 genes, which encode GA 20-oxidase biosynthetic enzymes, but
8 not of those encoding *SIGA3ox*, nor with downregulation of genes encoding *SIGA2ox*
9 (inactivating enzymes) (Serrani *et al.*, 2007b). Increase of *SIGA20ox1* gene expression
10 24 h after pollination has also been reported (Olimpieri *et al.*, 2007). On the other hand,
11 post-transcriptional antisense silencing of *SIDELLA* gene, encoding a nuclear repressor
12 of GA mode of action (Sun and Gubler, 2004; Schwechheimer, 2008), induces the
13 production of parthenocarpic fruits in the absence of pollination (Martí *et al.*, 2007),
14 further supporting a role for GA on tomato fruit-set and growth.

15 Auxin application (review of Abad and Monteiro, 1989; Koshioka *et al.*, 1994;
16 Serrani *et al.*, 2007a) and overexpression of genes of indole-3-acetic acid (IAA)
17 biosynthesis (Pandolfini *et al.*, 2002), induce fruit-set and growth in tomato, generally
18 more efficiently than GAs. Moreover, transcriptome analysis of expanding locular cells
19 from pollinated fruits shows preferential expression of genes involved in synthesis,
20 transport and response to auxins in this tissue (Lemaire-Chamley *et al.*, 2005). It is
21 known that auxin signal transduction depends on the degradation of the transcriptional
22 regulators Aux/IAAs (Tiwari *et al.*, 2001), which participate in complex dimerization
23 networks modulating the effect of auxin response factors (ARFs) that bind to auxin
24 response elements in promoter regions of auxin-regulated genes (Leyser, 2002;
25 Guilfoyle and Hagen, 2007). Partial tomato clones of members of the Aux/IAA family

1 have been reported (Nebenführ *et al.*, 2000; Vriezen *et al.*, 2008), although the function
2 of most of them is not clear. Transgenic tomato lines displaying downregulation of
3 *SlIAA9* (before *IAA4*) present parthenocarpic fruit development capability (Wang *et al.*,
4 2005), showing that the product of this gene has parthenocarpic repressor capacity.
5 Interestingly, mutations in the *AUXIN RESPONSE FACTOR8* (*ARF8*) gene induces
6 parthenocarpic development in Arabidopsis (Goetz *et al.*, 2006) and tomato (Goetz *et al.*,
7 2007), indicating that ARF8 also acts as an inhibitor in the absence of fertilization.
8 Previous results indicate that unpollinated ovaries are certainly auxin deficient (Varga
9 and Bruinsma, 1976). All these observations, together with the increase of auxin-like
10 substances (Mapelli *et al.*, 1978) and IAA (Sjut and Bangerth, 1981) content found
11 early after anthesis indicate that these hormones are also involved in tomato fruit-set
12 and development. However, GA and auxin application induce different morphological
13 and histological development of tissue ovaries. For instance, while parthenocarpic
14 growth induced by auxin is associated with more cell divisions in the mesocarp, GA-
15 induced fruits have much larger mesocarp cells (Serrani *et al.*, 2007a). Also, the
16 presence of pseudoembryos with unknown function in auxin- but not in GA-induced
17 fruits has been reported (Kataoka *et al.*, 2003; Serrani *et al.*, 2007a).

18 Auxins have been shown to interact with GAs in diverse physiological GA-
19 dependent processes by altering GA metabolism or mode of action. For instance, IAA
20 transported from the apical shoot induces the synthesis of GA₁ in elongating internodes
21 of pea and tobacco by upregulating the expression of GA biosynthetic genes, and
22 downregulating the expression of a *GA2ox* gene in the case of pea (O'Neil and Ross,
23 2002; Ross *et al.*, 2002). Auxins also control the expression of genes encoding enzymes
24 involved in GA metabolism in Arabidopsis seedlings (Frigerio *et al.*, 2006; Desgagné-
25 Penix and Sponsel, 2008). In pea, fruit-set and growth depend on GAs (Rodrigo *et al.*,

1 1997), and 4-Cl-IAA, presumably synthesized in fertilized ovules, enhances transcript
2 levels encoding GA20ox (Ngo *et al.*, 2002) and GA3ox (Ozga *et al.*, 2003) in the
3 pericarp. On the other hand, Arabidopsis root elongation is controlled by GAs through
4 degradation of DELLA proteins. In this case, the existence of cross-talk between auxin
5 and GAs has been demonstrated by showing that RGA (a DELLA protein) degradation
6 is IAA dependent (Fu and Harberd, 2003).

7 In this study we have investigated the interaction between auxin and GA content
8 during tomato fruit-set using the dwarf cv Micro-Tom, a brassinosteroid-deficient
9 mutant (Meissner *et al.*, 1997; Scott and Harbaugh, 1989). The phenotype of this
10 cultivar is the result of several point mutations (*D*, *SP* and *Ilr*), but not of GA deficiency
11 (Martí *et al.*, 2006), and has been shown to constitute a good experimental system to
12 investigate the hormone regulation of fruit-set and growth in tomato (Serrani *et al.*,
13 2007a, b). We have found, using inhibitors of GA biosynthesis, that induction of fruit-
14 set induced by auxins is mediated by GAs. Auxin alters GA metabolism and increases
15 active GA₁ level in unpollinated ovaries through upregulation of genes encoding
16 enzymes of GA biosynthesis (*CPS*, *GA20ox* and *GA3ox*) and downregulating one gene
17 encoding a GA2ox enzyme. The effect of 2,4-D- and GA₃-induced fruit-set on
18 expression of diverse genes involved in auxin and GA signalling was also investigated.

19

20 **Results**

21 *Inhibitors of GA biosynthesis reduce auxin-induced parthenocarpic fruit-set and growth*

22 To investigate whether the development of auxin-induced fruits depends on GAs,
23 unpollinated tomato ovaries were treated with 2,4-dichlorophenoxyacetic acid (2,4-D)
24 the day equivalent to anthesis, in the absence or presence of two different kinds of
25 inhibitors of GA biosynthesis: LAB 198999, an acylcyclohexanedione derivative which

1 inhibits 2-oxoglutarate-dependent dioxygenases (Santes and García-Martínez, 1995),
2 and Paclobutrazol (PCB), an inhibitor of P450-dependent monooxygenases (Hedden
3 and Graebe, 1985). LAB 198999 was applied directly to ovaries and PCB to the roots.
4 Inhibitors were also applied to unpollinated and pollinated ovaries not treated with 2,4-
5 D, as controls.

6 In pollinated ovaries, tomato fruit-set was totally inhibited by PCB application,
7 while LAB 198999 slightly reduced final fruit weight (Figs 1A, B). Fruit-set was fully
8 reversed by GA₃ in the case of PCB application, but fruit-size only partially. We
9 confirmed that PCB was efficient at molecular level by quantifying transcript levels of
10 several GA metabolism genes in pollinated ovaries (Serrani *et al.*, 2007b): those of
11 *SIGA20ox1* and *SIGA3ox1* increased, whereas those of *SIGA2ox2* decreased with PCB
12 application (Fig. 1C), in agreement with their expected negative and positive feed-back
13 regulation, respectively (Sponsel and Hedden, 2004). PCB application also reduced the
14 expression of *GAST1* (Fig. 1C), a tomato GA-responsive gene (Shi *et al.*, 1992),
15 supporting that PCB decreased GA content. These results agree with the hypothesis that
16 GAs are necessary for fruit-set and growth in tomato (Fos *et al.*, 2000; Serrani *et al.*,
17 2007b). In unpollinated ovaries treated with two different doses of 2,4-D (6 and 20 ng),
18 PCB reduced fruit-set and weight, an effect that was reversed by exogenous GA₃ (Fig.
19 1A). In the case of LAB 198999, both fruit-set and final fruit size decreased with 6 ng
20 2,4-D, and only fruit weight with 20 ng 2,4-D. This inhibition was fully reversed by
21 GA₃ application (Fig. 1B). As shown in Fig. 1D, 2,4-D-induced fruits treated with PCB
22 were morphologically similar to fruits non-treated with PCB, but smaller, and the
23 locular cavities were filled with locular gel. The application of GA₃ reversed the effect
24 of PCB on fruit size reduction (Fig. 1D).

1 We wanted to know whether the results obtained with the synthetic auxin 2,4-D
2 were also valid for indole-3-acetic acid (IAA), an endogenous auxin in tomato (Kojima
3 *et al.*, 2002; Varga and Bruinsma, 1976). IAA was less efficient than 2,4-D because
4 poorer locular tissue development occurred in the first case (compare Figs 1 and 2). We
5 used a dose of PCB (10^{-5} M) (the most efficient GA biosynthesis inhibitor; Fig. 1) that
6 did not affect the response of unpollinated ovaries to GA₃ (Fig. 2A). PCB application
7 reduced both fruit-set and fruit weight of IAA-induced parthenocarpic ovaries, and its
8 effect was reversed by GA₃ application (Figs 2A, B). Simultaneous application of IAA
9 plus GA₃ had an additive effect on fruit growth (Figs 2A and B).

10 Auxins have been reported to induce formation of pseudoembryos (embryo-like
11 or embryoid structures, originating from the division of the innermost integument cells
12 and formed in the ovule cavity) (Kataoka *et al.*, 2003; Serrani *et al.*, 2007a). Application
13 of GA biosynthesis inhibitors to auxin-induced ovaries did not affect pseudoembryo
14 development (data not presented).

16 *Parthenocarpic fruits induced by 2,4-D contain high GA levels*

17 The concentration of GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₂₉, GA₁ and GA₈, GAs from the
18 early-13-hydroxylation pathway (Supplementary Fig. 1) was quantified in 10-d-old
19 pollinated, and in unpollinated ovaries treated with 2,4-D or not treated (control).

20 Unpollinated non-treated ovaries contained much lower concentrations of all
21 GAs compared to 2,4-D-treated and to pollinated ovaries (Table 1). This agrees with a
22 qualitative report by Koshioka *et al.* (1994) indicating that pollination increases GA
23 content in the ovary. We found that the concentration of active GA₁ in pollinated and
24 2,4-D-induced ovaries was about 5 and 25 times higher, respectively, than in
25 unpollinated ovaries. The concentration of all GA₁ precursors (GA₅₃, GA₄₄, GA₁₉ and

1 GA₂₀) and GA₈ (a GA₁ metabolite) was also much lower in unpollinated non-treated
2 ovaries (only traces of GA₅₃, GA₄₄ and GA₂₉, and 1/20th of GA₁₉, almost 1/100th of
3 GA₂₀ and 1/50th of GA₈ compared to 2,4-D-treated ovaries). It is interesting to point out
4 that the different concentration of some GAs found between the pollinated ovaries
5 analyzed this time (Table 1) and previously (see Table I of Serrani et al., 2007b) (1.1 vs
6 2.7 ng g⁻¹ for GA₁, 9.7 vs 18.5 ng g⁻¹ for GA₂₉, and 0.6 vs <0.1 ng g⁻¹ for GA₅₃) was
7 probably due to the slightly different developmental stage of both samples (1.26 g fruit⁻¹
8 now vs 1.04 g fruit⁻¹ in the case of Serrani et al., 2007b), which in pea is known to affect
9 dramatically the content of GAs, at least GA₁ and GA₂₉ (Rodrigo *et al.*, 1997).

10

11 *Effect of 2,4-D on in vivo metabolism of GAs in unpollinated ovaries*

12 [17-¹⁴C]GA₁₂ and [17-¹⁴C]GA₅₃, substrates of GA 20-oxidases, [17-¹⁴C]GA₂₀, substrate
13 of GA 3-oxidase, and [17-¹⁴C]GA₂₀ and [17-¹⁴C]GA₁ (both from the early-13-
14 hydroxylation pathway), purported substrates of GA 2-oxidases (see Supplementary Fig.
15 1), were applied to unpollinated ovaries from emasculated flowers to examine the
16 possible effect of 2,4-D on *in vivo* GA metabolism. For comparison purposes,
17 metabolism of all these GA substrates was also investigated in pollinated ovaries. GA₁₂
18 was not metabolized in untreated ovaries whereas it was converted to compounds with
19 the same retention time as GA₉ and GA₄ in 2,4-D-treated ovaries and in pollinated
20 ovaries, respectively (Fig. 3). GA₅₃ was not metabolized in untreated or 2,4-D-treated
21 ovaries but, interestingly, in the case of pollinated ovaries a peak with the same
22 retention time as GA₁₉/GA₄₄ was detected (Fig. 3). GA₂₀ was metabolized to a
23 compound with the same retention time as GA₁ in untreated, 2,4-D-treated and
24 pollinated ovaries, but this metabolite was more abundant in the case of 2,4-D-induced
25 ovaries (Fig. 3). In addition, a large peak with the same retention time as GA₂₉ and GA₈

1 (GA_{2ox} metabolites of GA₂₀ and GA₁, respectively) was present in untreated but not in
2 2,4-D-treated or pollinated ovaries (Fig. 3). Finally, GA₁ was metabolized to a
3 compound with the same retention time as GA₈ in untreated, treated and pollinated
4 ovaries, but much less in the latter two cases (Fig. 3). These results show that the high
5 content of GA₁ and its precursors in parthenocarpic 2,4-D-induced ovaries is a
6 consequence of an alteration of GA metabolism, mainly of an increase of GA
7 biosynthesis (GA_{20ox} and GA_{3ox} activity) and reduction of GA inactivation (GA_{2ox}
8 activity).

9

10 *Effect of 2,4-D on transcript levels of genes encoding enzymes of GA biosynthesis*

11 To investigate whether the altered *in vivo* GA metabolism in 2,4-D-induced fruits was
12 the result of a modification of transcript activity of GA metabolism genes, we compared
13 transcript levels of *SICPS*, *SIGA20ox1*, -2 and -3, and *SIGA3ox1* and -2, which encode
14 three kinds of GA biosynthesis enzymes (Rebers *et al.*, 1999), in unpollinated ovaries
15 untreated or treated with 2,4-D, by quantitative RT-PCR. Transcript levels of *SIGPS*
16 (encoding a geranyl diphosphate synthase reported to control GA biosynthesis; van
17 Schie *et al.*, 2007) and *SIGA20ox4*, a new *GA20ox* of tomato not previously described
18 (Accession number EU652334), whose expression product was shown to metabolize
19 GA₁₂ and GA₅₃ to putative GA₉ and GA₂₀, respectively (data not presented), was also
20 investigated.

21 Expression of *SIGPS* was relatively high and constant in unpollinated ovaries at
22 all stages, and it was not affected by 2,4-D application (Fig. 4A). Expression of *SICPS*
23 was detected in non-treated ovaries before anthesis (d-3) but it was very low later on
24 (from 0 to 20 days post anthesis, dpa). In contrast, *SICPS* transcript levels in 2,4-D
25 treated fruits did not decrease after the time equivalent to anthesis (d0) and therefore

1 were much higher than in non-treated ovaries between d5 and d20. *SICPS* transcripts
2 were present both in pericarp and locular gel of 10- and 20-d-old 2,4-D-induced fruits
3 (Fig. 4A).

4 Some expression of *SIGA20ox1*, -2, -3 and -4 was detected in ovaries before
5 anthesis (d-3), but transcripts were essentially undetected in unpollinated non-treated
6 ovaries between d0 and d20 (Fig. 4B). In contrast, *SIGA20ox1*, -2 and -3 transcript
7 levels were high (particularly those of *SIGA20ox1*) in the entire fruit as well as in the
8 pericarp and locular gel of 2,4-D-induced ovaries 5 and 10 d after hormone application
9 (Fig. 4B). At 20 d after 2,4-D application transcript contents were also high in locular
10 gel. Very low expression of *SIGA20ox4* was detected in any ovary tissue at any
11 developmental stage between d5 and d20 (Fig. 4B).

12 *SIGA3ox1* transcripts were at a relatively high level in unpollinated ovaries
13 before anthesis (d-3), decreased at anthesis, and were very low between d5 and d20
14 (Fig. 4D). The expression of *SIGA3ox1* was much higher in 2,4-D-induced than in non-
15 induced ovaries between d5 and d20, transcripts being concentrated mostly in the
16 locular gel (Fig. 4D). In the case of *SIGA3ox2*, expression was detected before anthesis
17 (at a relatively high level) and at d0, but not in untreated ovaries afterwards. Expression
18 of *SIGA3ox2* was detected in 2,4-D-treated ovaries at d5, but much less at later stages
19 (Fig. 4D).

20

21 *Effect of 2,4-D on transcript levels of genes encoding enzymes of GA inactivation*

22 To determine whether the high GA content in 2,4-D-induced ovaries was also due to
23 reduced GA-inactivation activity, transcript levels of *SIGA2ox1*, -2, -3, -4 and -5, genes
24 encoding GA 2-oxidases (considered as the main GA catabolic enzymes) in tomato
25 (Serrani *et al.*, 2007b) were also quantified by RT-PCR.

1 Transcript content of *GA2ox* genes was relatively elevated in d-3 and d0 ovaries
2 (with the exception of *SIGA2ox5* at d-3 and of *SIGA2ox3* at d0) (Figs 4C). In
3 unpollinated non-treated ovaries the content of all of them decreased at d5 and d10, with
4 the exception of *SIGA2ox2* that remained relatively high up to d20. It increased again at
5 d20 for *SIGA2ox1*, -4 and -5, associated to starting of ovary senescence. In unpollinated
6 ovaries treated with 2,4-D, only the expression of *SIGA2ox2* decreased between 5 and
7 20 d after 2,4-D application (at least 4-fold) compared to non-treated ovaries (Fig. 4C).
8 In the case of *SIGA2ox1* transcript content was very low at d5 but increased to a very
9 high level 10 d after treatment (Fig. 4C). *SIGA2ox3* transcript content was always low,
10 as in untreated ovaries (Fig. 4C). *SIGA2ox4* transcript contents in 2,4-D induced fruits
11 were similar to those of untreated ovaries of a similar age (Fig. 4C). In the case of
12 *SIGA2ox5* transcript levels were even higher in treated than in non-treated ovaries at d5
13 and d10 (Fig. 4C).

14

15 *Effect of 2,4-D and GA₃ on expression of auxin- and GA-responsive genes, and of genes*
16 *encoding GA signal transduction factors*

17 To further characterize the cross-talk between auxin and GA during fruit-set, transcript
18 levels of diverse genes involved in auxin response previously reported to be expressed
19 in ovary and/or developing fruit (encoding the tomato Aux/IAA IAA1, IAA2, IAA3,
20 IAA8, IAA9, IAA14, IAA16 and IAA18, and ARFs ARF1, ARF8 and ARF9) (Balbi
21 and Lomax, 2003; Goetz *et al.*, 2007; Vriezen *et al.*, 2008; Wang *et al.*, 2005) were
22 determined by quantitative RT-PCR in unpollinated ovaries untreated or treated with
23 2,4-D or GA₃ (Fig. 5A, B and C). The effect of 2,4-D and GA₃ on expression of several
24 genes encoding tomato homologs of components of the GA signal transduction pathway

1 (*GIDI* and *DELLA*; Sun and Gubler, 2004) and GA response (*GASTI*; Shi *et al.*, 1992)
2 was also investigated.

3 As seen in Fig. 5A, transcripts of genes encoding the Aux/IAA repressors IAA1,
4 IAA2, IAA8 and IAA14 were undetected or at a very low level in unpollinated non-
5 treated, but were at a high level in 2,4-D-treated ovaries, as expected. In the case of
6 *IAA3*, *IAA9*, *IAA16* and *IAA18*, transcripts were already present in unpollinated ovaries,
7 mostly those of *IAA9* and *IAA16* which were the only genes whose expression increased
8 upon 2,4-D application. Expression of *IAA1* and *IAA8* was also clearly induced in GA₃-
9 treated ovaries, although less than in 2,4-D-induced ovaries. With regard to genes
10 encoding ARFs, transcripts of *ARF8* and *ARF9* were at a very low level in unpollinated
11 untreated ovaries and significantly induced by 2,4-D, but not by GA₃. Those of *ARF1*
12 were at a very high level in unpollinated untreated ovaries and decreased slightly by
13 2,4-D and GA₃ (Fig. 5B and C).

14 Expression of *SIGIDI*, encoding a putative GA receptor (Ueguchi-Tanaka *et al.*,
15 2007), was clearly induced in ovaries by GA₃ application compared to untreated and
16 2,4-D-treated ovaries, where transcripts were not detected. *SIDELLA* expression,
17 encoding a tomato GA repressor (Martí *et al.*, 2007) was induced by GA₃ and 2,4-D,
18 although more by the former. Expression of the tomato GA-inducible gene *SIGASTI*
19 was similar in unpollinated ovaries untreated and treated with 2,4-D, but was enhanced
20 by GA₃, as expected (Fig. 5D).

21

22 **Discussion**

23 The results presented here show that parthenocarpic growth in tomato induced by auxin
24 application is mediated by GAs. This conclusion was supported by the observation that
25 fruit-set and growth of unpollinated ovaries induced by 2,4-D or IAA was negated or

1 significantly reduced in the presence of inhibitors of GA biosynthesis (mainly PCB),
2 and that the effect of these inhibitors was reversed by GA₃ application (Figs 1 and 2).
3 The absence of complete negation of fruit-set by PCB in the case of 2,4-D, compared to
4 the almost 100% efficiency in the case of IAA, was probably due to the much higher
5 activity of the synthetic auxin, and to the relatively low concentration of PCB used in
6 the experiments to prevent negative side effects of the inhibitor. In the case of pollinated
7 ovaries, containing only endogenous IAA, PCB was fully efficient probably because
8 IAA concentration was lower than that present in IAA treated ovaries. Parthenocarpic
9 fruit-set and growth in tomato can be induced by GA or auxin treatment to unpollinated
10 ovaries (Serrani *et al.*, 2007a, and references there in). Application of GA biosynthesis
11 inhibitors to pollinated ovaries supports the hypothesis that tomato fruit-set and growth
12 depend on GAs synthesized after pollination and fertilization (Fos *et al.*, 2000; Serrani
13 *et al.*, 2007b). The absence of inhibitors of auxin biosynthesis has not allowed us to
14 carry out auxin experiments with this kind of hormone, similar to those performed with
15 GA and PCB, to get direct evidence on their role in tomato fruit-set.

16 The concentration of GAs in unpollinated non-induced ovaries was very low or
17 undetected compared to pollinated ovaries (Table 1), in agreement with the qualitative
18 results described by Koshioka *et al.* (1994). In contrast, the application of 2,4-D induced
19 parthenocarpic fruit-set and growth was associated with high content of GA₁ (the active
20 GA in tomato fruit-set; Serrani *et al.*, 2007b) in the ovary, as well as of its precursors
21 (GA₅₃, GA₄₄, GA₁₉ and GA₂₀) and GA₈ (a GA₂ox metabolite) (Table 1). The levels of
22 GA₁ precursors were similar in 2,4-D-induced and in pollinated fruits, and those of GA₁
23 and GA₈ were even higher in the former (Table 1), in agreement with our hypothesis
24 that the effect of 2,4-D on tomato fruit-set is mediated by GAs through enhancement of
25 GA biosynthesis.

1 *In vivo* metabolism analysis showed that 2,4-D altered several aspects of GA
2 metabolism in unpollinated ovaries. It was quite clear that 2,4-D induced the conversion
3 of GA₁₂ to putative GA₉, probably due to higher GA20ox activity (Fig. 3), indicating
4 that the non-13-hydroxylation pathway was active. In the case of pollinated ovaries
5 GA₁₂ was further metabolized to putative GA₄. Curiously, GA₅₃ metabolism was not
6 induced by 2,4-D in spite of the fact that the early-13-hydroxylation-pathway is
7 considered to be the main one in tomato (Fos *et al.*, 2000), but it was metabolized to
8 putative GA_{44/19} in pollinated ovaries (Fig. 3). This suggests that the enhanced GA20ox
9 activity in pollinated ovaries may be different to that in auxin-induced ovaries (where
10 no developing seeds are present). Thus, the possible importance of the non-13-
11 hydroxylation pathway should be further investigated, both in pollinated and in auxin-
12 induced fruit-set. The use of GA₂₀ and GA₁ as substrates of *in vivo* metabolism also
13 showed that 2,4-D decreased GA2ox activity in both cases (Fig. 3). In addition, the
14 higher production of GA₁ from GA₂₀ as a substrate in 2,4-D-induced ovaries suggests
15 that auxin may enhance GA3ox activity. This hypothesis was further supported by the
16 observation that 2,4-D increased transcript levels of *SIGA3ox1* in unpollinated-treated
17 ovaries (Fig. 4D).

18 The increase of GA content in 2,4-D-induced growing fruits was probably the
19 result of the effect of this hormone on higher transcription of genes encoding CPS,
20 GA20ox1, -2 and -3, in addition to GA3ox1 (GA biosynthetic enzymes) (Fig. 4). CPS
21 (formerly *ent*-kaurene synthase A) activity has been shown to be present in extracts of
22 tomato fruits (Bensen and Zeevaart, 1990), and the higher transcript levels of *SICPS*
23 suggests that early biosynthetic enzymes may contribute to the increase of GA content.
24 2,4-D application seems to prevent downregulation of this gene in unpollinated ovaries,
25 whose transcript level is relatively high before anthesis. With regard to the expression

1 of the *SIGA2ox* multigene family, only that of *SIGA2ox2* showed early downregulation
2 by 2,4-D (Fig. 4). Nevertheless, the possible effect of 2,4-D on GA inactivation through
3 *GA2ox* is not clear because it has been found that the protein encoded by *SIGA2ox2* can
4 be inactive (Serrani *et al.*, 2007b). Indeed, our results do not discard the possibility that
5 2,4-D may increase GA₁ content by also downregulating other kinds of GA inactivating
6 genes, shown to regulate GA homeostasis through 16 α ,17-epoxidation (Zhu *et al.*,
7 2006) and methylation (Varbanova *et al.*, 2007). Interestingly, genes encoding SICPS
8 and *SIGA20ox1*, -2 and -3 were also found to be upregulated in young pollinated
9 ovaries associated with GA content increase (Serrani *et al.*, 2007b). However, in
10 pollinated ovaries no upregulation of genes encoding *SIGA3ox* was observed (Serrani *et*
11 *al.*, 2007b). These results suggest that pollination and/or fertilization alters GA
12 metabolism through auxin, probably synthesized in the fertilized ovules (Varga and
13 Bruinsma, 1986). But in contrast to 2,4-D-induced fruits (where *SIGA3ox1* transcript
14 levels increased and those of *SIGA2ox2* decreased) no early *GA3ox* upregulation or
15 *GA2ox* downregulation was observed in pollinated ones (Serrani *et al.*, 2007) (see
16 proposed scheme on auxin mode of action in Fig. 6). Our results in tomato are
17 comparable to the promotion of GA biosynthesis in pea by 4-Cl-IAA, which increases
18 transcription of both *GA20ox* (Ngo *et al.*, 2002) and *GA3ox* (Ozga *et al.*, 2003) genes in
19 deseeded pods. Downregulation of *PsGA2ox1* and upregulation of *PsGA2ox2* by 4-Cl-
20 IAA in pea pericarp has been reported recently (Ozga *et al.*, 2007).

21 Regulation of GA metabolism by auxins has also been found in vegetative
22 tissues. For instance, internode elongation depends on GAs, and it has been shown that
23 the level of GA₁ (the active form) is regulated by IAA transported basipetally from the
24 apical shoot through upregulation of *GA20ox* in tobacco (Wolbang and Ross, 2001), and
25 of *GA3ox* in pea (O'Neill and Ross, 2002) and barley (Wolbang *et al.*, 2004). In pea

1 downregulation of *GA2ox* genes has been also detected. Application of a synthetic auxin
2 (naphthaleneacetic acid) to *Arabidopsis* seedlings regulates differentially the expression
3 of several *GA20ox* and *GA2ox* too (Frigerio *et al.*, 2006). Interestingly, auxin transport
4 inhibitors enhance *AtGA20ox1* expression in the shoot but not in the roots of
5 *Arabidopsis* (Desgagné-Penix and Sponsel, 2008). All these results show that the
6 mechanism regulating GA metabolism by auxin may vary with the species (and maybe
7 tissue), either enhancing the expression of different genes encoding enzymes of GA
8 biosynthesis in some cases, and/or downregulating the expression of GA inactivation
9 genes in others.

10 The expression of early auxin-responsive genes is induced rapidly by auxin,
11 although responsiveness varies from gene to gene and with the dose of applied hormone
12 (Abel and Theologis, 1996). We have found that transcript levels of five tomato
13 *Aux/IAA* (*IAA1*, *IAA2*, *IAA8*, *IAA9* and *IAA14*) genes were clearly enhanced in 2,4-D-
14 treated ovaries 5 d after hormone application, indicating that unpollinated tomato
15 ovaries responded efficiently to that auxin. However, the possible role of those genes on
16 tomato fruit-set is not known. Antisense tomato plants with downregulated *IAA9*
17 produce parthenocarpic fruits (Wang *et al.*, 2005), leading to the conclusion that *IAA9*
18 is a negative regulator of fruit-set. However, the enhanced expression of *IAA9* in
19 parthenocarpic 2,4-D-induced fruits is in apparent contradiction with the results of
20 Wang *et al.* (2005), suggesting that *IAA9* transcript level may not be correlated with
21 protein level or, alternatively, that auxin parthenocarpic induction is not mediated by
22 *IAA9* downregulation. This last hypothesis is in agreement with the observation that
23 transcript level of *IAA3* was not altered in 2,4-D-induced ovaries, in contrast to the
24 higher level found in antisense *IAA9* tomato with parthenocarpic fruit (Wang *et al.*,
25 2005). 2,4-D also enhanced the expression of *ARF8* and *ARF9*, but not of *ARF1*, further

1 supporting the efficiency of 2,4-D application at the molecular level. ARF8 loss of
2 function produces parthenocarpic fruits in Arabidopsis (Goetz *et al.*, 2006) and tomato
3 (Goetz *et al.*, 2007), and it has been suggested that it interacts with the tomato IAA9,
4 and other unknown proteins, to prevent fruit-set before fertilization (Goetz *et al.*, 2006).
5 However, since *ARF8* expression was enhanced in 2,4-D-induced ovaries, this suggests,
6 as occurred with *IAA9*, either an absence of correlation between transcript and protein
7 level or that auxin parthenocarpic induction does not involve *ARF8* downregulation.

8 Elucidating which are the target genes of specific ARFs and the interactions
9 among ARFs and Aux/IAA repressors is a major challenge (Guilfoyle and Hagen,
10 2007). The observation that transcript levels of some of the *Aux/IAA* and *ARF* genes
11 analyzed were enhanced by 2,4-D (*IAA2*, *IAA14*, *ARF8* and *ARF9*) but not, or very
12 little, by GA₃ indicates that these genes act as specific mediators of auxin action on fruit
13 growth (see “?” in Fig. 6). It is also tempting to speculate that some of them might be
14 involved in the regulation of the expression of GA metabolic genes by auxin shown in
15 this study. This agrees with the proposal of Frigerio *et al* (2006) after investigating the
16 expression of GA metabolism genes in several *aux/iaa* and *arf* mutants in response to
17 auxin application in Arabidopsis seedlings. Some auxin-responsive genes (*IAA1* and
18 *IAA8*) seem to be up-regulated both by 2,4-D and GA. The regulation of the expression
19 of these genes by GA is of interest, and it is possible that in this case the effect of auxin
20 is indirect, and mediated by the increase of GA content induced by auxin.

21 Transcript levels of *SIDELLA* (encoding a repressor of GA mode of action;
22 Martí *et al.*, 2007) increased upon GA₃ and 2,4-D application, suggesting that it is also
23 regulated by auxin through GA. Interestingly, the effect of GA₃ on transcript levels
24 encoding this factor was opposite to that expected, supporting the contention that their
25 transcription is subjected to negative feed-back regulation by their protein products.

1 Availability of appropriate tomato antibodies would be necessary to clarify this issue. A
2 role for *SIDELLA* as a repressor of fruit-set is supported by the observations that
3 antisense *SIDELLA* (Martí et al., 2007) and *procera* (reported to be a putative inactive
4 *DELLA* mutant; Bassel *et al.*, 2008) plants produce parthenocarpic fruits (Dr Lazaro
5 Peres, personal communication). On the other hand, *GAST1*, a tomato GA-responsive
6 gene of unknown function (whose expression is known to be partially inhibited by
7 ABA; Shi *et al.*, 1992) acts specifically through GA since its expression was enhanced
8 in GA₃- but not in 2,4-D-induced ovaries. Also *SIGIDI*, a gene encoding a putative GA
9 receptor (homolog to the rice GA receptor; Ueguchi-Tanaka *et al.*, 2007) responds
10 specifically to GA. Our *SIGIDI* results in ovaries are in contrast with those described
11 previously for *AtGIDI* genes in Arabidopsis seedlings, where they are down-regulated
12 by GA application (Hirano *et al.*, 2008), suggesting that this GA response may be tissue
13 dependent.

14 In summary, we have shown that the effect of auxin on parthenocarpic tomato
15 fruit-set is mediated, at least partially, by GAs. Auxins, probably synthesized in the
16 developing seeds following pollination and ovule fertilization, increase active GA
17 content in the fruit by upregulating genes encoding enzymes of GA biosynthesis (CPS,
18 GA20ox and GA3ox) and down-regulating at least one gene (GA2ox2) encoding GA
19 inactivating enzymes, and thus inducing fruit-set (Fig. 6). This effect of auxin may be
20 carried out through *Aux/IAA* and/or *ARF* genes whose expression was altered by auxin
21 application to unpollinated ovaries. Auxins have probably other additional effects on
22 tomato fruit growth, independent of GAs (Fig. 6). This may explain why the
23 morphology of GA-induced fruit (with poor locular tissue development) is different to
24 those induced by pollination and auxin (Serrani *et al.*, 2007a). This hypothesis is also

1 supported by the observation that simultaneous application of GA₃ and IAA had an
2 additive effect on fruit growth (Fig. 2).

3

4 **Experimental procedures**

5 *Plant material and growth conditions*

6 Plants of tomato (*Solanum lycopersicum* L.) cv Micro-Tom (seeds obtained originally
7 from Dr A Levy) were used in the experiments. Plants (one per pot) were grown in 1 L
8 pots with a mixture of peat:vermiculite (1:1), cultured in a greenhouse under 24°C
9 (day)/ 20°C (night) conditions, and irrigated daily with Hoagland's solution. Natural
10 light was supplemented with Osram lamps (Powerstar HQI-BT, 400W) to get a 16 h
11 light photoperiod.

12 Only one flower per truss and the first two trusses were left per plant for the
13 experiments, as described previously (Serrani *et al.*, 2007a), unless otherwise stated. All
14 non-selected flowers were removed 2 d before anthesis. Entire unpollinated non-treated
15 ovaries of different ages were collected for qRT-PCR analysis. In the case of 10- and
16 20-d-old unpollinated hormone-induced ovaries, pericarp and locular gel plus placenta
17 tissues (including unfertilized ovules) were collected separately.

18

19 *Plant hormone applications*

20 Application of 2,4-D (Duchefa, Haarlem, The Netherlands), IAA (Duchefa), and GA₃
21 (gift of Dr Michel Beale, Rothamsted Research, UK) was carried out to unpollinated
22 ovaries the day equivalent to anthesis, in 10 µl of 5% ethanol, 0.1% Tween 80 solution.
23 Flower emasculation was carried out 2 d before anthesis to prevent self-pollination. 0.1
24 M LAB 198999 (3,5-dioxo-4-butyryl-cyclohexane carboxylic acid ethyl ester) (BASF,
25 Limbergerhof, Germany) was applied in 5% ethanol, 0.1% Tween solution, 10 µl per

1 ovary the day equivalent to anthesis. In the case of pollinated ovaries, LAB 198999 was
2 applied 2 d after anthesis, after removal of petals and stamens, to ascertain that
3 pollination was not affected by the inhibitor solution. PCB (Duchefa) was applied to the
4 roots in the nutrient solution at 10^{-5} M every two days, starting when flowers on which
5 the effect of the inhibitor was going to be determined were about 7 d before anthesis
6 (estimated by flower bud size) so it would be transported in time to the ovary. Equal
7 volume of solvent solution was applied to control ovaries (in the case of LAB 198999)
8 and to the culture medium (case of PCB).

9

10 *Quantification of gibberellins*

11 GAs were quantified following the protocol described in Fos *et al.* (2000). Briefly,
12 aliquots (1 to 5 g fresh weight) of frozen material were extracted with 80% methanol
13 and, after removing the organic phase, the water fraction was partitioned against ethyl
14 acetate and purified by QAE-Sephadex chromatography and C_{18} cartridges. The GAs
15 were then separated by reverse phase HPLC chromatography (4- μ m C_{18} column, 15
16 cm long, 3.9 mm i.d.; NovaPak, Millipore, Milford, MA), and appropriate fractions
17 grouped for GC-SIM analysis after methylation and trimethylsilylation. [$^{17,17-2}H$]GA₁,
18 [$^{17,17-2}H$]GA₃, [$^{17,17-2}H$]GA₈, [$^{17,17-2}H$]GA₁₉, [$^{17,17-2}H$]GA₂₀, [$^{17,17-2}H$]GA₂₉,
19 [$^{17,17-2}H$]GA₄₄ and [$^{17,17-2}H$]GA₅₃ (purchased from Prof. L Mander, Australian
20 National University, Canberra) were added to the extracts as internal standards for
21 quantification, and [3H]GA₂₀ and [3H]GA₉ (purchased from Prof. L. Mander) to monitor
22 the separation of GAs after HPLC using a 10 to 100% methanol gradient containing 50
23 μ l acetic acid per litre and taking 1 ml fractions. Quantification was carried out by GC-
24 SIM using a gas chromatograph (model 5890, Hewlett-Packard, Palo Alto, CA) coupled

1 to a mass-selective detector (model 5971A, Hewlett-Packard). The concentrations of
2 GAs in the extracts were determined using the calibration curves methodology.

3

4 *In vivo gibberellin metabolism*

5 Unpollinated ovaries from emasculated flowers were treated the day equivalent to
6 anthesis (d0) with [17-¹⁴C]GA₁, [17-¹⁴C]GA₂₀, [17-¹⁴C]GA₅₃ and [17-¹⁴C]GA₁₂
7 solutions (purchased from Prof. L. Mander; 34-55 μCi μmol⁻¹) (10 000 dpm ovary⁻¹ in
8 10 μl of 10% methanol, two replicates of 12 ovaries per treatment) without or with 2,4-
9 D (200 ng ovary⁻¹). A similar experiment was carried out using self-pollinated ovaries,
10 also treated with labelled GAs at d0. Fruits (ovaries) were harvested 48 h after
11 treatment, frozen in N₂ and kept at -80 °C until analysis. Ground ovaries (12 per
12 replicate and treatment) were extracted overnight at 4 °C in 80% methanol with
13 agitation, centrifuged at 13000 rpm and re-extracted twice for 20 min in 100%
14 methanol. The joined supernatants were taken to dryness, the residue dissolved in 10%
15 methanol and the metabolic products separated by HPLC, as described before for
16 quantification of GAs. Metabolites were detected using an on-line radioactive monitor
17 (Radioflow Detector LB 508, Berthold Technologies) and identified by their retention
18 times compared to pure GAs. Only data from one replicate, out of two with similar
19 results, are given in Results.

20

21 *Quantitative RT-PCR*

22 Total RNA was isolated from ovaries using the RNAqueous-4PCR kit and Plant RNA
23 Isolation Aid, according to manufacturer's recommendations (all reagents used for
24 quantitative real time PCR, qRT-PCR, were from Ambion, Applied Biosystems, unless
25 otherwise stated). First-strand cDNA was synthesized in 50 μl total volume reaction

1 using 1 µg of total RNA, random hexamers and a TaqMan reverse transcription kit, with
2 the following thermocycling conditions: 95 °C 10 min + [95 °C 15 s + 60 °C 1 min] x 40
3 cycles + 95 °C 15 s + 60 °C 1 min + 95 °C 15 s. 2 µl aliquots of diluted (1/400) cDNA
4 solution were used for qRT-PCR in 20 µl volume reaction using specific primers
5 (Supplementary Table 1), *Power* SYBR Green PCR master mix and a 7500 Fast Real-
6 Time PCR System (Applied Biosystems). Absolute quantification was carried out using
7 external standard curves, as described elsewhere (Olmos *et al.*, 2005), with minor
8 modifications. Briefly, short PCR fragments (80 to 200 bp) of the sequence of interest
9 were obtained using the specific primers indicated in Supplementary Table 1 (in this
10 case each forward primer also contained the T7 promoter sequence 5'-
11 TAATACGACTCACTATAGGG-3') and cDNA from specific clones for each analyzed
12 gene. These PCR fragments, containing the T7 promoter, were purified from a 3%
13 agarose gel using a QIAquick gel extraction kit (QIAGEN), and transcribed *in vitro*
14 with the Megashortscript kit. 2.5 ng of positive sense single strand RNA (ssRNA)
15 transcripts were treated with TURBO DNase to remove the DNA fragment used as
16 template, purified by a glass filter-based system (MEGAClear kit), and used to
17 synthesize first-strand cDNA in 50 µl total volume reaction, as described before. Serial
18 dilutions of cDNA solution corresponding to about 10⁵ to 10⁸ molecules of ssRNA were
19 used to set up external standard curves under identical amplification conditions to those
20 used to amplify RNA targets from samples. Moles of ssRNA template were calculated
21 taking into account average ribonucleotide mass (340 g mol⁻¹) and transcript base
22 number (Nb), according to the equation: pmol ssRNA = X pg ssRNA x (1 pmol/340 pg)
23 x (1/Nb). Molecules of ssRNA were estimated using the Avogadro's constant (6.023 x
24 10²³ molecules mol⁻¹). Absolute amounts of mRNA in samples were quantified in

1 triplicate, using two biological independent experiments. Only results from a
2 representative experiment are given in Results.

3

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9

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- 20
21

1 **Table 1.** Endogenous GA content (ng g fresh weight⁻¹) of unpollinated non treated
 2 ovaries and of 2,4-D-induced and pollinated fruits. Unpollinated ovaries were collected
 3 10 d after the day equivalent to anthesis, and fruits were collected 10 d after pollination
 4 or 2,4-D (200 ng) application. Weight data are means of eight fruits (and more than
 5 1,000 ovaries in the case of unpollinated non-treated), and GA data come from three
 6 biological replicates (aliquots of 1-5 g each) ± SE.

7
 8

	Weight (mg fruit ⁻¹)	GA ₅₃	GA ₄₄	GA ₁₉	GA ₂₀	GA ₂₉	GA ₁	GA ₈
Unpollinated	2	< 0.01	< 0.01	0.5 ± 0.1	0.3 ± 0.05	< 0.1	0.2 ± 0.1	0.6 ± 0.05
Unpollinated + 2,4-D	1,510 ± 70	0.4 -	2.3 ± 0.3	10.0 ± 0.9	22.2 ± 0.2	5.7 ± 0.6	5.3 ± 0.4	31.5 ± 2.1
Pollinated	1,260 ± 60	0.6 ± 0.1	2.3 ± 0.4	8.7 ± 1.1	26.6 ± 1.9	9.7 ± 0.6	1.1 ± 0.3	22.9 ± 2.4

9
 10
 11

1 **Supplementary Table 1.** Primer sequences used for PCR amplification and quantitative
2 RT-PCR analysis of genes from GA metabolism, and auxin- and GA-response of
3 tomato. *SIGPS* (DQ286930), *SICPS* (AB015675), *SIGA20ox1* (AF049898), *SIGA20ox2*
4 (AF049899), *SIGA20ox3* (AF049900), *SIGA20ox4* (EU652334), *SIGA3ox1*
5 (AB010991), *SIGA3ox2* (AB010992), *SIGA2ox1*(EF441351), *SIGA2ox2* (EF441352),
6 *SIGA2ox3* (EF441353), *SIGA2ox4* (EF441354), *SIGA2ox5* (EF441355), *SIIAA1*
7 (AF022012), *SIIAA2* (AF022013), *SIIAA3* (AF022014), *SIIAA8* (AF022019), *SIIAA9*
8 (AJ937282), *SIIAA14* (BE462113), *SIIAA16* (AF022020), *SIIAA18* (AI485829),
9 *SIARF1*(BT013711), *SIARF8* (EF664362), *SIARF9* (BT013639), *SIGAST1* (X63093),
10 *SIGIDI* (BN001197), and *SIDELLA* (AY269087).

11

Gene	Sense	Antisense
<i>SIGPS</i>	5'-TAT GCAGAAAACATATTACAAGA-3'	5'-ATCAAGAACATCATCTATTAATTG-3'
<i>SICPS</i>	5'-ATACCTAGAGCTAGCGAAATC-3'	5'-ACTGCCTAAATAGTACGTAACC-3'
<i>SIGA20ox1</i>	5'-CTCATTCTAATGCTCATCGT-3'	5'-TGCAGATGATTCTTTCTTA GCG-3'
<i>SIGA20ox2</i>	5'-TTTCCATATTCTACCCTACAAG-3'	5'-TCATCGCATTACAATACTCTT-3'
<i>SIGA20ox3</i>	5'-AGCCAAATTATGCTAGTGTTAC-3'	5'-TTTTATGAGATTTGTGTCAACC-3'
<i>SIGA20ox4</i>	5'-GATGATAAATGGCACTCTATTC-3'	5'-TGACTTCCTTGTTCTTCTACAG-3'
<i>SIGA3ox1</i>	5'-GGCATTAGTAGTTAATATAGGTGA-3'	5'-AAATAAGCTACAGAAAGTCGATA-3'
<i>SIGA3ox2</i>	5'-GATCATAAATTTGTCATGGATAC-3'	5'-TGTTTTCCATATGGTTAAGTAATC-3'
<i>SIGA2ox1</i>	5'-GGCATGTAAGATATTAGAATTGA-3'	5'-TTAATCCGTAGTAGAGAATCAGA-3'
<i>SIGA2ox2</i>	5'-ATTAAGATCCAATAACACTTCG-3'	5'-TCTTGATTTCACACTATTTGC-3'
<i>SIGA2ox3</i>	5'-GACCCTTCTACTTTCAGCTC-3'	5'-AAATTGAATTGTCTTCTATCCA-3'
<i>SIGA2ox4</i>	5'-ATGGAAGGAAAAGACAGTTTA-3'	5'-CTTTTCTCAAATAGGACCAAC-3'
<i>SIGA2ox5</i>	5'-GATCACTTACCAATAATCAACAG-3'	5'-CGTCATGGTTTACGACTTTA-3'

<i>IAA1</i>	5'-GAAAATGTTCAAGCTGAGTATC-3'	5'-CTGATCCTTTCATTATCCTTAG-3'
<i>IAA2</i>	5'-TACAAAAGTTATCCACAATTAATC-3'	5'-GGTATATAATTACATCCGTTGTATC-3'
<i>IAA3</i>	5'-CTCAGGAATGTATTTAAAAGTTAG-3'	5'-TCCTTCTCTTTCTGAATACACT-3'
<i>IAA8</i>	5'-CTTGCCTAACAACTGTGAATTC-3'	5'-TGTTCTTGGAGCTAATCCTATA-3'
<i>IAA9</i>	5'-TCTACTGGCTTCTTCAACTTC-3'	5'-CAGATAGACCCATATAGTTTCG-3'
<i>IAA14</i>	5'-AGATGTTTAGCTCCTTTACTAATG-3'	5'-GTTGGTACATATTCAGAACTGTTA-3'
<i>IAA16</i>	5'-ACTGGAATCGAGTAATAAGAAC-3'	5'-TATTCTTCTTCTCCTTCATGTTA-3'
<i>IAA18</i>	5'-TATATGAGGATAATGAAGGTGAC-3'	5'-TTAGTTGCACGAGTAAGTGTAG-3'
<i>ARF1</i>	5'-TTAGATAGTTATGAAGATCTGCTTA-3'	5'-CTGTATAGACGTAAATTTTTCTAAC-3'
<i>ARF8</i>	5'-AGGAAGTAATAATTCATTGAATATC-3'	5'-TTAGTTGTGACTCTGTAAATTTTG-3'
<i>ARF9</i>	5'-ACAAATACTTAGAGGCTCTTAAAC-3'	5'-ATAGTGCCCATAAATCTTCTATC-3'
<i>GAST1</i>	5'-CAACAACAGAGAAATAACCAAC-3'	5'-TTATACGATGTCTTTGAACACC-3'
<i>GID1</i>	5'-GATCTTGATACACCTCTCAGTACTA-3'	5'-ACAGCCTTACATATACTAACAAGAC-3'
<i>DELLA</i>	5'-TGATGCGACTATACTTGATATAAG-3'	5'-GGGTAAATCTGTTTAATAGAGTTC-3'

1

2

1 **Figure legends**

2 **Figure 1.** Inhibition by PCB (A) and LAB 198999 (B) of fruit-set and growth of
3 pollinated and parthenocarpic fruits induced by 2,4-D (6 and 20 ng), and reversion by
4 GA₃. (C) Effect of PCB on selected genes of GA-metabolism (*SIGA20ox1*, *SIGA3ox1*
5 and *SIGA2ox2*) and GA-response (*GAST1*) in pollinated ovaries. (D) Photography of
6 representative parthenocarpic fruits induced by 2,4-D (6 ng), alone or plus PCB without
7 or with GA₃. Fruits were collected 20 d after anthesis or hormone treatment, except in
8 the case of (C), which were collected 10 d after anthesis. Values are means of eight
9 fruits ± SE, except when otherwise specified. Values in brackets indicate the number of
10 fruits set when less than eight. PCB was applied at 10⁻⁵ M to the pots, and LAB 198999
11 (0.1 M) and GA₃ (2000 ng) to the ovaries.

12 **Figure 2.** (A) Effect of PCB on parthenocarpic fruit induction by GA₃ and IAA, and
13 reversion by GA₃. (B) Photography of representative parthenocarpic fruits induced by
14 IAA, alone or plus PCB without or with GA₃. Fruits were collected 20 d after hormone
15 application. Values are means of eight fruits ± SE, except when otherwise specified.
16 Values in brackets indicate the number of fruits set when less than eight. PCB was
17 applied at 10⁻⁵ M to the pots, and LAB 198999 (0.1 M), GA₃ (2000 ng) and IAA (2000
18 ng) to the ovaries.

19 **Figure 3.** Radioactive HPLC traces of metabolites of [17-¹⁴C]GA₁₂, [17-¹⁴C]GA₅₃, [17-
20 ¹⁴C]GA₂₀ and [17-¹⁴C]GA₁ applied to unpollinated untreated and treated with 2,4-D
21 (200 ng) and pollinated ovaries. Labelled GAs were applied at d0 and ovaries collected
22 2 days after application. See more details in Experimental procedures.

23 **Figure 4.** Effect of 2,4-D application to unpollinated ovaries on transcript levels of
24 *SIGPS* and *SICPS* (A), *SIGA20ox1*, -2, -3 and -4 (B), *SIGA3ox1* and -2 (D), and
25 *SIGA2ox1*, -2, -3, -4 and -5 (C) genes. Transcript analysis was carried out by

1 quantitative RT-PCR, as described in Experimental procedures, using poli(A⁺) RNA
2 from unpollinated d0, d5, d10 and d20 ovaries untreated or treated with 2,4-D (200 ng).
3 E, entire ovaries; P, pericarp; LG, locular gel, including unfertilized ovules. Data are
4 means ± SE of three replicates from a representative experiment.

5 **Figure 5.** Effect of 2,4-D and GA₃ on transcript levels of IAA/Aux (*IAA1*, *IAA2*, *IAA3*,
6 *IAA7*, *IAA8*, *IAA9*, *IAA18*), ARF (*ARF1*, *ARF8* and *ARF9*) (B, C), and GA signal-
7 transduction (*SIGIDI1* and *SIDELLA* and *SIGASTI*) (D) genes in 5-d-old unpollinated
8 ovaries untreated or treated with 2,4-D (200 ng) and GA₃ (2000 ng). Data are means ±
9 SE of three replicates from a representative experiment.

10 **Figure 6.** Scheme of proposed interaction of auxin and GAs during tomato fruit-set and
11 growth. Auxin, either applied or synthesized in the pollinated ovary, increases the
12 content of active GA₁ in the ovary by upregulating transcription of genes encoding
13 enzymes of GA biosynthesis (CPS, GA20ox1, -2 and -3 and GA3ox1) and
14 downregulating that of a gene encoding an enzyme of GA inactivation (GA2ox2). This
15 effect may be mediated by Aux/IAA factors whose expression is modified upon auxin
16 and GA application to the ovary. ?, specific effect of auxin on fruit growth through
17 other Aux/IAA and ARF factors (see Fig. 5A, B, C and Discussion for more details).

18

19 **Supplementary Figure 1.** Scheme of GA metabolism including the non-13-
20 hydroxylation and the early-13-hydroxylation pathways. CPS, copalyldiphosphate
21 synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid
22 oxidase.

23

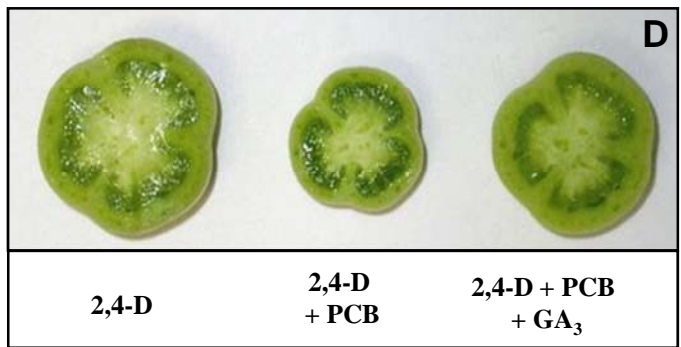
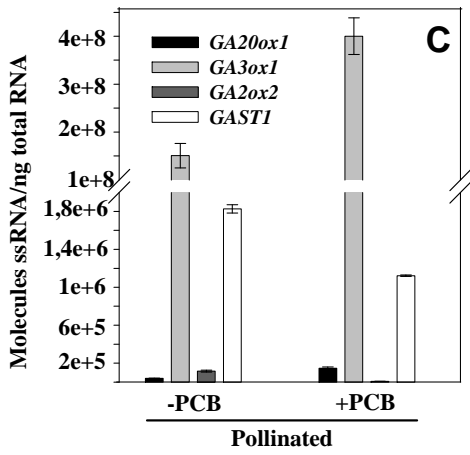
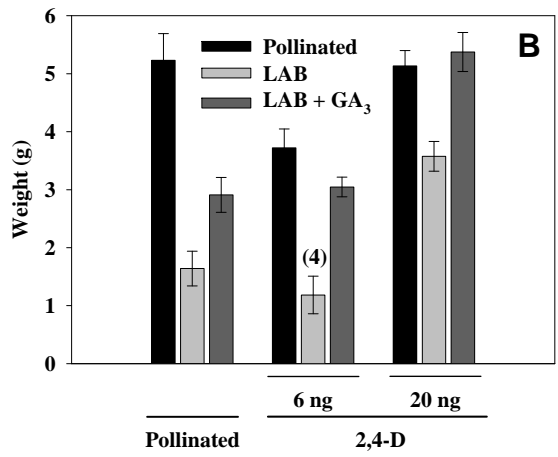
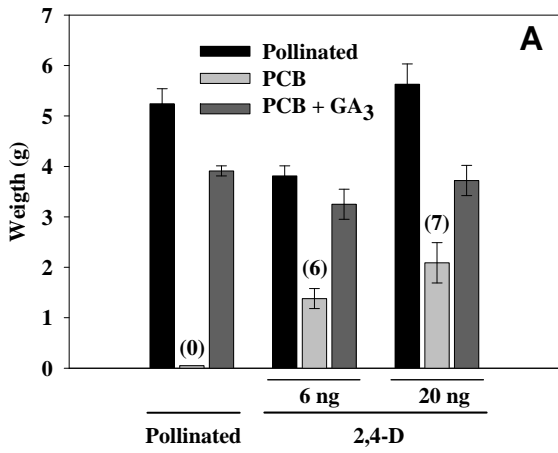


Fig.1

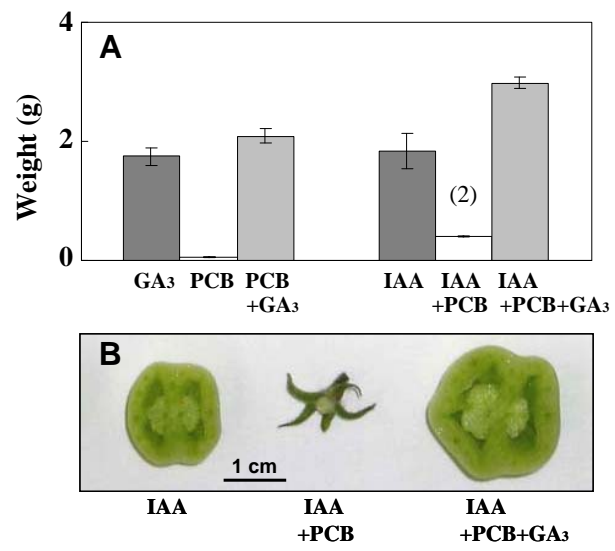


Fig. 2

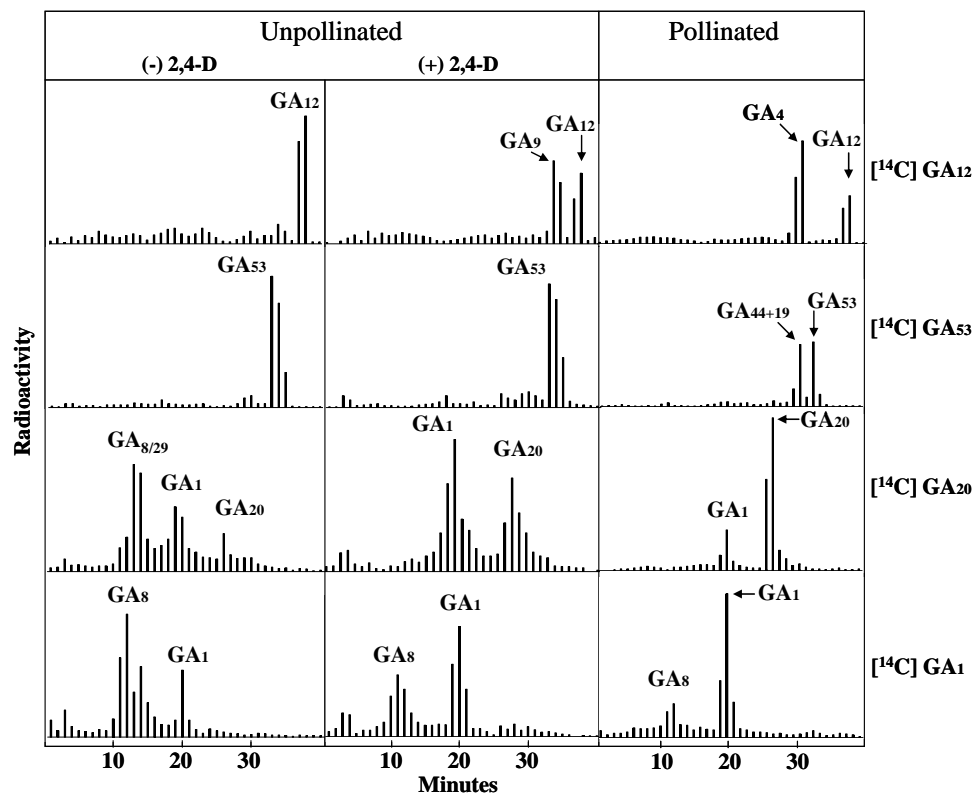


Fig 3

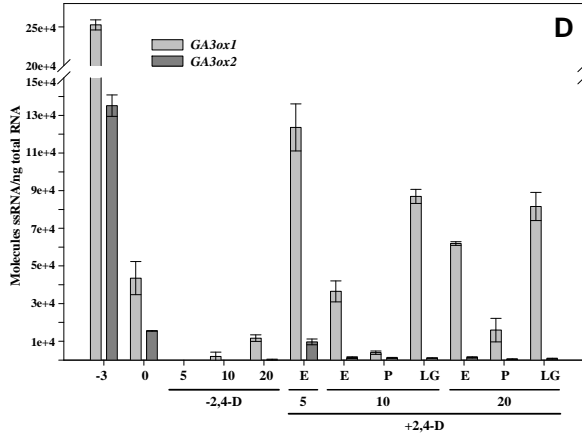
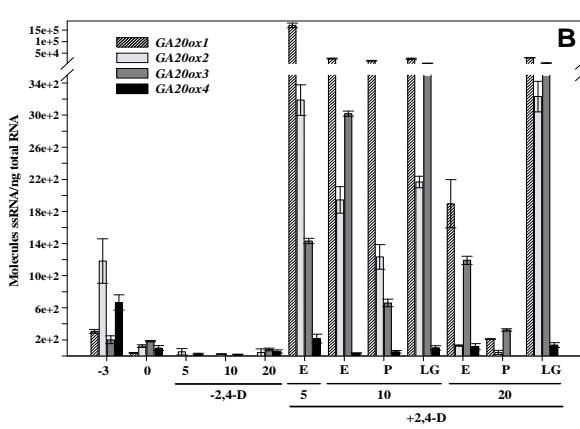
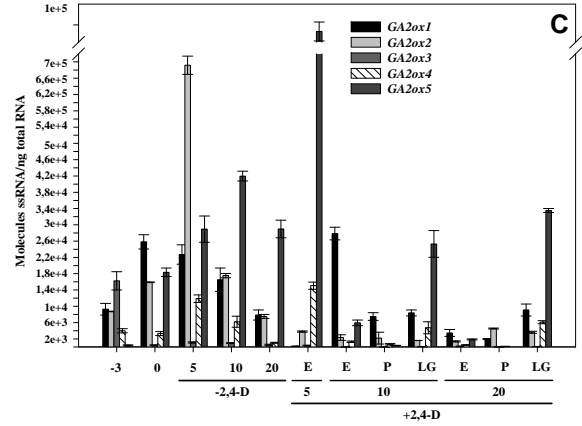
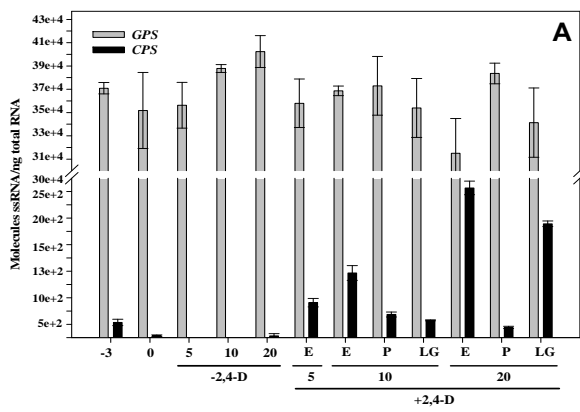


Fig. 4

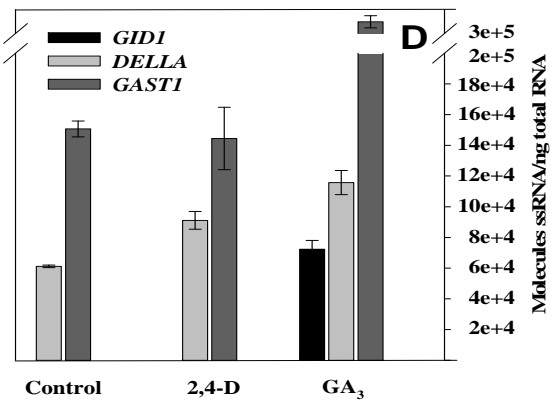
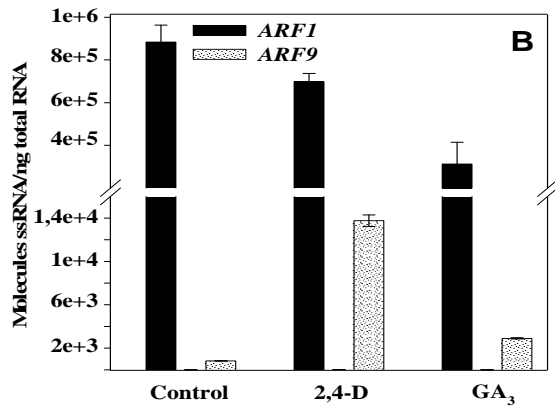
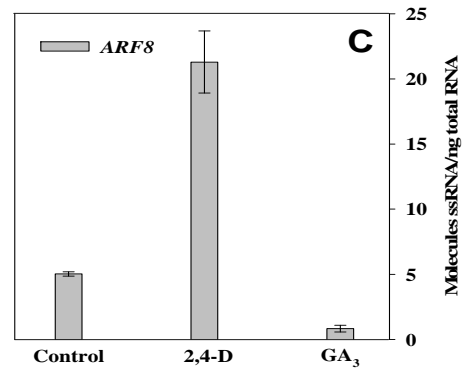
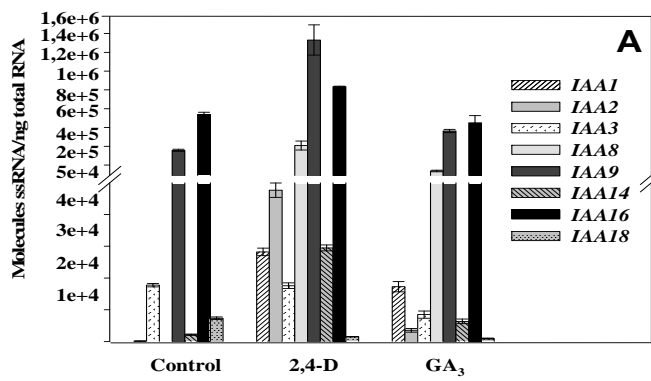


Fig. 5

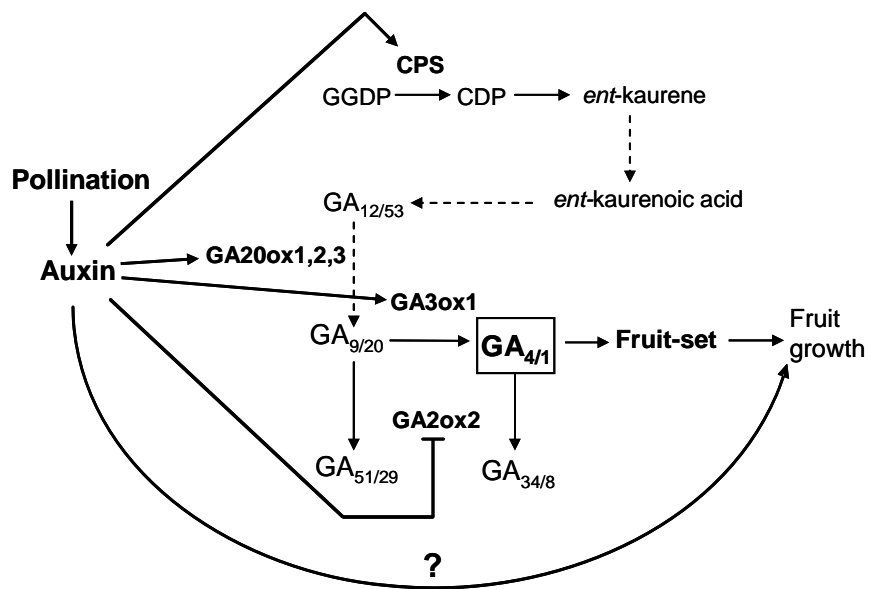


Fig. 6

Supplementary 1

