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 GA_3. This suggested that the effect of auxin was mediated by GA. Parthenocarpic fruits induced by 2,4-D had higher contents of the active GA_1, its precursors and metabolite, than unpollinated non-treated ovaries, but similar to pollinated ovaries. Application experiments of radioactive-labelled GAs to unpollinated ovaries showed than 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 GA_1 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.

Introduction

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

Parthenocarpic fruit-set and growth can be induced by application of diverse plant growth substances to unpollinated ovaries, mainly auxins and gibberellins (GAs) (García-Martínez and Hedden, 1997; Gorquet *et al.,* 2005; Srivastava and Handa, 2005). GA metabolism in plants initiates from geranylgeranyl diphosphate, which is converted to *ent*-kaurene by the action of two consecutive cyclases (copalyldiphosphate synthase, CPS, and *ent*-kaurene synthase, KS), followed by the action of P450 monooxygenases (*ent*-kaurene oxidase, KO, and *ent*-kaurenoic acid oxidase, KAO), and of three kinds of Fe^{2+}- and 2-oxoglutarate-dependent dioxygenases (the biosynthetic enzymes GA 20-oxidases and GA 3-oxidases, and the inactivating enzymes GA 2-oxidases), which are encoded by small multigenic families (Sponsel and Hedden, 2004) (Supplementary Fig. 1). GA biosynthesis can occur through two parallel pathways: the non-13-hydroxylation, leading to GA_4 as the active GA, and the early-13-hydroxylation pathway, leading to the active GA_1 (Supplementary Fig. 1). The latter is the main metabolic pathway in tomato, although GAs from the non-13-hydroxylation pathway have also been identified in tomato fruit (Fos *et al.,* 2000). Fruit-set in tomato depends

on gibberellins (GAs), as shown by application of GA biosynthesis inhibitors to pollinated ovaries (Fos *et al.,* 2000, 2001; Serrani *et al.,* 2007b), and of GAs to unpollinated ovaries (Alabadí and Carbonell, 1998; Fos *et al.,* 2000, 2001; Serrani *et al.,* 2007a; Sjut and Bangerth, 1982/83), and the active form is GA_1 (Serrani *et al.,* 2007b). The increase of GA content in the ovary upon pollination (Bohner *et al*., 1988; Koshioka *et al*., 1994; Serrani *et al*., 2007b) is associated with upregulation of *SlGA20ox1, -2* and *-3* genes, which encode GA 20-oxidase biosynthetic enzymes, but not of those encoding SlGA3ox, nor with downregulation of genes encoding SlGA2ox (inactivating enzymes) (Serrani *et al.,* 2007b). Increase of *SlGA20ox1* gene expression 24 h after pollination has also been reported (Olimpieri *et al.,* 2007). On the other hand, post-transcriptional antisense silencing of *SlDELLA* gene, encoding a nuclear repressor of GA mode of action (Sun and Gubler, 2004; Schwechheimer, 2008), induces the production of parthenocarpic fruits in the absence of pollination (Martí *et al*., 2007), further supporting a role for GA on tomato fruit-set and growth.

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

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

Auxins have been shown to interact with GAs in diverse physiological GA-dependent processes by altering GA metabolism or mode of action. For instance, IAA transported from the apical shoot induces the synthesis of GA_1 in elongating internodes of pea and tobacco by upregulating the expression of GA biosynthetic genes, and downregulating the expression of a *GA2ox* gene in the case of pea (O´Neil and Ross, 2002; Ross *et al.,* 2002). Auxins also control the expression of genes encoding enzymes involved in GA metabolism in Arabidopsis seedlings (Frigerio *et al.,* 2006; Desgagné-Penix and Sponsel, 2008). In pea, fruit-set and growth depend on GAs (Rodrigo *et al.,*

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

In this study we have investigated the interaction between auxin and GA content during tomato fruit-set using the dwarf cv Micro-Tom, a brassinosteroid-deficient mutant (Meissner *et al.,* 1997; Scott and Harbaugh, 1989). The phenotype of this cultivar is the result of several point mutations (*D, SP* and *Ilr*), but not of GA deficiency (Martí *et al.,* 2006), and has been shown to constitute a good experimental system to investigate the hormone regulation of fruit-set and growth in tomato (Serrani *et al.,* 2007a, b). We have found, using inhibitors of GA biosynthesis, that induction of fruit-set induced by auxins is mediated by GAs. Auxin alters GA metabolism and increases active GA_1 level in unpollinated ovaries through upregulation of genes encoding enzymes of GA biosynthesis (*CPS*, *GA20ox* and *GA3ox*) and downregulating one gene encoding a GA2ox enzyme. The effect of 2,4-D- and GA_3-induced fruit-set on expression of diverse genes involved in auxin and GA signalling was also investigated.

Results

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

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

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

In pollinated ovaries, tomato fruit-set was totally inhibited by PCB application, while LAB 198999 slightly reduced final fruit weight (Figs 1A, B). Fruit-set was fully reversed by GA_3 in the case of PCB application, but fruit-size only partially. We confirmed that PCB was efficient at molecular level by quantifying transcript levels of several GA metabolism genes in pollinated ovaries (Serrani *et al*., 2007b): those of *SlGA20ox1* and *SlGA3ox1* increased, whereas those of *SlGA2ox2* decreased with PCB application (Fig. 1C), in agreement with their expected negative and positive feed-back regulation, respectively (Sponsel and Hedden, 2004). PCB application also reduced the expression of *GAST1* (Fig. 1C), a tomato GA-responsive gene (Shi *et al*., 1992), supporting that PCB decreased GA content. These results agree with the hypothesis that GAs are necessary for fruit-set and growth in tomato (Fos *et al.,* 2000; Serrani *et al.,* 2007b). In unpollinated ovaries treated with two different doses of 2,4-D (6 and 20 ng), PCB reduced fruit-set and weight, an effect that was reversed by exogenous GA_3 (Fig. 1A). In the case of LAB 198999, both fruit-set and final fruit size decreased with 6 ng 2,4-D, and only fruit weight with 20 ng 2,4-D. This inhibition was fully reversed by GA_3 application (Fig. 1B). As shown in Fig. 1D, 2,4-D-induced fruits treated with PCB were morphologically similar to fruits non-treated with PCB, but smaller, and the locular cavities were filled with locular gel. The application of GA_3 reversed the effect of PCB on fruit size reduction (Fig. 1D).

We wanted to know whether the results obtained with the synthetic auxin 2,4-D were also valid for indole-3-acetic acid (IAA), an endogenous auxin in tomato (Kojima *et al.,* 2002; Varga and Bruinsma, 1976). IAA was less efficient than 2,4-D because poorer locular tissue development occurred in the first case (compare Figs 1 and 2). We used a dose of PCB (10^{-5} M) (the most efficient GA biosynthesis inhibitor; Fig. 1) that did not affect the response of unpollinated ovaries to GA_3 (Fig. 2A). PCB application reduced both fruit-set and fruit weight of IAA-induced parthenocarpic ovaries, and its effect was reversed by GA_3 application (Figs 2A, B). Simultaneous application of IAA plus GA_3 had an additive effect on fruit growth (Figs 2A and B).

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

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

The concentration of GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_{29}, GA_1 and GA_8, GAs from the early-13-hydroxylation pathway (Supplementary Fig. 1) was quantified in 10-d-old pollinated, and in unpollinated ovaries treated with 2,4-D or not treated (control).

Unpollinated non-treated ovaries contained much lower concentrations of all GAs compared to 2,4-D-treated and to pollinated ovaries (Table 1). This agrees with a qualitative report by Koshioka *et al*. (1994) indicating that pollination increases GA content in the ovary. We found that the concentration of active GA_1 in pollinated and 2,4-D-induced ovaries was about 5 and 25 times higher, respectively, than in unpollinated ovaries. The concentration of all GA_1 precursors (GA_{53}, GA_{44}, GA_{19} and

GA_{20}) and GA_8 (a GA_1 metabolite) was also much lower in unpollinated non-treated ovaries (only traces of GA_{53}, GA_{44} and GA_{29}, and $1/20^{th}$ of GA_{19}, almost $1/100^{th}$ of GA_{20} and $1/50^{th}$ of GA_8 compared to 2,4-D-treated ovaries). It is interesting to point out that the different concentration of some GAs found between the pollinated ovaries analyzed this time (Table 1) and previously (see Table I of Serrani et al., 2007b) (1.1 vs 2.7 ng g^{-1} for GA_1, 9.7 vs 18.5 ng g^{-1} for GA_{29}, and 0.6 vs <0.1 ng g^{-1} for GA_{53}) was probably due to the slightly different developmental stage of both samples (1.26 g $fruit^{-1}$ now vs 1.04 g $fruit^{-1}$ in the case of Serrani et al., 2007b), which in pea is known to affect dramatically the content of GAs, at least GA_1 and GA_{29} (Rodrigo *et al*., 1997).

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

[17-^{14}C]GA_{12} and [17-^{14}C]GA_{53}, substrates of GA 20-oxidases, [17-^{14}C]GA_{20}, substrate of GA 3-oxidase, and [17-^{14}C]GA_{20} and [17-^{14}C]GA_1 (both from the early-13-hydroxylation pathway), purported substrates of GA 2-oxidases (see Supplementary Fig. 1), were applied to unpollinated ovaries from emasculated flowers to examine the possible effect of 2,4-D on *in vivo* GA metabolism. For comparison purposes, metabolism of all these GA substrates was also investigated in pollinated ovaries. GA_{12} was not metabolized in untreated ovaries whereas it was converted to compounds with the same retention time as GA_9 and GA_4 in 2,4-D-treated ovaries and in pollinated ovaries, respectively (Fig. 3). GA_{53} was not metabolized in untreated or 2,4-D-treated ovaries but, interestingly, in the case of pollinated ovaries a peak with the same retention time as GA_{19}/GA_{44} was detected (Fig. 3). GA_{20} was metabolized to a compound with the same retention time as GA_1 in untreated, 2,4-D-treated and pollinated ovaries, but this metabolite was more abundant in the case of 2,4-D-induced ovaries (Fig. 3). In addition, a large peak with the same retention time as GA_{29} and GA_8

(GA2ox metabolites of GA_{20} and GA_1, respectively) was present in untreated but not in 2,4-D-treated or pollinated ovaries (Fig. 3). Finally, GA_1 was metabolized to a compound with the same retention time as GA_8 in untreated, treated and pollinated ovaries, but much less in the latter two cases (Fig. 3). These results show that the high content of GA_1 and its precursors in parthenocarpic 2,4-D-induced ovaries is a consequence of an alteration of GA metabolism, mainly of an increase of GA biosynthesis (GA20ox and GA3ox activity) and reduction of GA inactivation (GA2ox activity).

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

To investigate whether the altered *in vivo* GA metabolism in 2,4-D-induced fruits was the result of a modification of transcript activity of GA metabolism genes, we compared transcript levels of *SlCPS*, *SlGA20ox1*, *-2* and *-3*, and *SlGA3ox1* and *-2*, which encode three kinds of GA biosynthesis enzymes (Rebers *et al.,* 1999), in unpollinated ovaries untreated or treated with 2,4-D, by quantitative RT-PCR. Transcript levels of *SlGPS* (encoding a geranyl diphosphate synthase reported to control GA biosynthesis; van Schie *et al*., 2007) and *SlGA20ox4*, a new *GA20ox* of tomato not previously described (Accession number EU652334), whose expression product was shown to metabolize GA_{12} and GA_{53} to putative GA_9 and GA_{20}, respectively (data not presented), was also investigated.

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

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

Some expression of *SlGA20ox1*, *-2*, *-3* and *-4* was detected in ovaries before anthesis (d-3), but transcripts were essentially undetected in unpollinated non-treated ovaries between d0 and d20 (Fig. 4B). In contrast, *SlGA20ox1*, *-2* and *-3* transcript levels were high (particularly those of *SlGA20ox1*) in the entire fruit as well as in the pericarp and locular gel of 2,4-D-induced ovaries 5 and 10 d after hormone application (Fig. 4B). At 20 d after 2,4-D application transcript contents were also high in locular gel. Very low expression of *SlGA20ox4* was detected in any ovary tissue at any developmental stage between d5 and d20 (Fig. 4B).

SlGA3ox1 transcripts were at a relatively high level in unpollinated ovaries before anthesis (d-3), decreased at anthesis, and were very low between d5 and d20 (Fig. 4D). The expression of *SlGA3ox1* was much higher in 2,4-D-induced than in non-induced ovaries between d5 and d20, transcripts being concentrated mostly in the locular gel (Fig. 4D). In the case of *SlGA3ox2,* expression was detected before anthesis (at a relatively high level) and at d0, but not in untreated ovaries afterwards. Expression of *SlGA3ox2* was detected in 2,4-D-treated ovaries at d5, but much less at later stages (Fig. 4D).

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

To determine whether the high GA content in 2,4-D-induced ovaries was also due to reduced GA-inactivation activity, transcript levels of *SlGA2ox1*, *-2*, *-3*, *-4* and *-5*, genes encoding GA 2-oxidases (considered as the main GA catabolic enzymes) in tomato (Serrani *et al.*, 2007b) were also quantified by RT-PCR.

Transcript content of *GA2ox* genes was relatively elevated in d-3 and d0 ovaries (with the exception of *SlGA2ox5* at d-3 and of *SlGA2ox3* at d0) (Figs 4C). In unpollinated non-treated ovaries the content of all of them decreased at d5 and d10, with the exception of *SlGA2ox2* that remained relatively high up to d20. It increased again at d20 for *SlGA2ox1*, *-4* and *-5,* associated to starting of ovary senescence. In unpollinated ovaries treated with 2,4-D, only the expression of *SlGA2ox2* decreased between 5 and 20 d after 2,4-D application (at least 4-fold) compared to non-treated ovaries (Fig. 4C). In the case of *SlGA2ox1* transcript content was very low at d5 but increased to a very high level 10 d after treatment (Fig. 4C). *SlGA2ox3* transcript content was always low, as in untreated ovaries (Fig. 4C). *SlGA2ox4* transcript contents in 2,4-D induced fruits were similar to those of untreated ovaries of a similar age (Fig. 4C). In the case of *SlGA2ox5* transcript levels were even higher in treated than in non-treated ovaries at d5 and d10 (Fig. 4C).

Effect of 2,4-D and GA_3 on expression of auxin- and GA-responsive genes, and of genes encoding GA signal transduction factors

To further characterize the cross-talk between auxin and GA during fruit-set, transcript levels of diverse genes involved in auxin response previously reported to be expressed in ovary and/or developing fruit (encoding the tomato Aux/IAA IAA1, IAA2, IAA3, IAA8, IAA9, IAA14, IAA16 and IAA18, and ARFs ARF1, ARF8 and ARF9) (Balbi and Lomax, 2003; Goetz *et al*., 2007; Vriezen *et al*., 2008; Wang *et al*., 2005) were determined by quantitative RT-PCR in unpollinated ovaries untreated or treated with 2,4-D or GA_3 (Fig. 5A, B and C). The effect of 2,4-D and GA_3 on expression of several genes encoding tomato homologs of components of the GA signal transduction pathway

(*GID1* and *DELLA*; Sun and Gubler, 2004) and GA response (*GAST1*; Shi *et al.*, 1992) was also investigated.

As seen in Fig. 5A, transcripts of genes encoding the Aux/IAA repressors IAA1, IAA2, IAA8 and IAA14 were undetected or at a very low level in unpollinated non-treated, but were at a high level in 2,4-D-treated ovaries, as expected. In the case of *IAA3*, *IAA9*, *IAA16* and *IAA18*, transcripts were already present in unpollinated ovaries, mostly those of *IAA9* and *IAA16* which were the only genes whose expression increased upon 2,4-D application. Expression of *IAA1* and *IAA8* was also clearly induced in GA_3-treated ovaries, although less than in 2,4-D-induced ovaries. With regard to genes encoding ARFs, transcripts of *ARF8* and *ARF9* were at a very low level in unpollinated untreated ovaries and significantly induced by 2,4-D, but not by GA_3. Those of *ARF1* were at a very high level in unpollinated untreated ovaries and decreased slightly by 2,4-D and GA_3 (Fig. 5B and C).

Expression of *SlGID1*, encoding a putative GA receptor (Ueguchi-Tanaka *et al.*, 2007), was clearly induced in ovaries by GA_3 application compared to untreated and 2,4-D-treated ovaries, where transcripts were not detected. *SlDELLA* expression, encoding a tomato GA repressor (Martí *et al.*, 2007) was induced by GA_3 and 2,4-D, although more by the former. Expression of the tomato GA-inducible gene *SlGAST1* was similar in unpollinated ovaries untreated and treated with 2,4-D, but was enhanced by GA_3, as expected (Fig. 5D).

Discussion

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

significantly reduced in the presence of inhibitors of GA biosynthesis (mainly PCB), and that the effect of these inhibitors was reversed by GA_3 application (Figs 1 and 2). The absence of complete negation of fruit-set by PCB in the case of 2,4-D, compared to the almost 100% efficiency in the case of IAA, was probably due to the much higher activity of the synthetic auxin, and to the relatively low concentration of PCB used in the experiments to prevent negative side effects of the inhibitor. In the case of pollinated ovaries, containing only endogenous IAA, PCB was fully efficient probably because IAA concentration was lower than that present in IAA treated ovaries. Parthenocarpic fruit-set and growth in tomato can be induced by GA or auxin treatment to unpollinated ovaries (Serrani *et al.,* 2007a, and references there in). Application of GA biosynthesis inhibitors to pollinated ovaries supports the hypothesis that tomato fruit-set and growth depend on GAs synthesized after pollination and fertilization (Fos *et al.,* 2000; Serrani *et al.,* 2007b). The absence of inhibitors of auxin biosynthesis has not allowed us to carry out auxin experiments with this kind of hormone, similar to those performed with GA and PCB, to get direct evidence on their role in tomato fruit-set.

The concentration of GAs in unpollinated non-induced ovaries was very low or undetected compared to pollinated ovaries (Table 1), in agreement with the qualitative results described by Koshioka *et al.* (1994). In contrast, the application of 2,4-D induced parthenocarpic fruit-set and growth was associated with high content of GA_1 (the active GA in tomato fruit-set; Serrani *et al.,* 2007b) in the ovary, as well as of its precursors (GA_{53}, GA_{44}, GA_{19} and GA_{20}) and GA_8 (a GA2ox metabolite) (Table 1). The levels of GA_1 precursors were similar in 2,4-D-induced and in pollinated fruits, and those of GA_1 and GA_8 were even higher in the former (Table 1), in agreement with our hypothesis that the effect of 2,4-D on tomato fruit-set is mediated by GAs through enhancement of GA biosynthesis.

In vivo metabolism analysis showed that 2,4-D altered several aspects of GA metabolism in unpollinated ovaries. It was quite clear that 2,4-D induced the conversion of GA_{12} to putative GA_9, probably due to higher GA20ox activity (Fig. 3), indicating that the non-13-hydroxylation pathway was active. In the case of pollinated ovaries GA_{12} was further metabolized to putative GA_4. Curiously, GA_{53} metabolism was not induced by 2,4-D in spite of the fact that the early-13-hydroxylation-pathway is considered to be the main one in tomato (Fos *et al*., 2000), but it was metabolized to putative $GA_{44/19}$ in pollinated ovaries (Fig. 3). This suggests that the enhanced GA20ox activity in pollinated ovaries may be different to that in auxin-induced ovaries (where no developing seeds are present). Thus, the possible importance of the non-13-hydroxylation pathway should be further investigated, both in pollinated and in auxin-induced fruit-set. The use of GA_{20} and GA_1 as substrates of *in vivo* metabolism also showed that 2,4-D decreased GA2ox activity in both cases (Fig. 3). In addition, the higher production of GA_1 from GA_{20} as a substrate in 2,4-D-induced ovaries suggests that auxin may enhance GA3ox activity. This hypothesis was further supported by the observation that 2,4-D increased transcript levels of *SlGA3ox1* in unpollinated-treated ovaries (Fig. 4D).

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

of the *SlGA2ox* multigene family, only that of *SlGA2ox2* showed early downregulation by 2,4-D (Fig. 4). Nevertheless, the possible effect of 2,4-D on GA inactivation through GA2ox is not clear because it has been found that the protein encoded by *SlGA2ox2* can be inactive (Serrani *et al.,* 2007b). Indeed, our results do not discard the possibility that 2,4-D may increase GA_1 content by also downregulating other kinds of GA inactivating genes, shown to regulate GA homeostasis through 16α,17-epoxidation (Zhu *et al.,* 2006) and methylation (Varbanova *et al.,* 2007). Interestingly, genes encoding SlCPS and SlGA20ox1, -2 and -3 were also found to be upregulated in young pollinated ovaries associated with GA content increase (Serrani *et al.,* 2007b). However, in pollinated ovaries no upregulation of genes encoding SlGA3ox was observed (Serrani *et al.,* 2007b). These results suggest that pollination and/or fertilization alters GA metabolism through auxin, probably synthesized in the fertilized ovules (Varga and Bruinsma, 1986). But in contrast to 2,4-D-induced fruits (where *SlGA3ox1* transcript levels increased and those of *SlGA2ox2* decreased) no early *GA3ox* upregulation or *GA2ox* downregulation was observed in pollinated ones (Serrani *et al*., 2007) (see proposed scheme on auxin mode of action in Fig. 6). Our results in tomato are comparable to the promotion of GA biosynthesis in pea by 4-Cl-IAA, which increases transcription of both *GA20ox* (Ngo *et al.,* 2002) and *GA3ox* (Ozga *et al.,* 2003) genes in deseeded pods. Downregulation of *PsGA2ox1* and upregulation of *PsGA2ox2* by 4-Cl-IAA in pea pericarp has been reported recently (Ozga *et al*., 2007).

Regulation of GA metabolism by auxins has also been found in vegetative tissues. For instance, internode elongation depends on GAs, and it has been shown that the level of GA_1 (the active form) is regulated by IAA transported basipetally from the apical shoot through upregulation of *GA20ox* in tobacco (Wolbang and Ross, 2001), and of *GA3ox* in pea (O´Neill and Ross, 2002) and barley (Wolbang *et al.,* 2004). In pea

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

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

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

Elucidating which are the target genes of specific ARFs and the interactions among ARFs and Aux/IAA repressors is a major challenge (Guilfoyle and Hagen, 2007). The observation that transcript levels of some of the *Aux/IAA* and *ARF* genes analyzed were enhanced by 2,4-D (*IAA2*, *IAA14*, *ARF8* and *ARF9*) but not, or very little, by GA_3 indicates that these genes act as specific mediators of auxin action on fruit growth (see "?" in Fig. 6). It is also tempting to speculate that some of them might be involved in the regulation of the expression of GA metabolic genes by auxin shown in this study. This agrees with the proposal of Frigerio et al (2006) after investigating the expression of GA metabolism genes in several *aux/iaa* and *arf* mutants in response to auxin application in Arabidopsis seedlings. Some auxin-responsive genes (*IAA1* and *IAA8*) seem to be up-regulated both by 2,4-D and GA. The regulation of the expression of these genes by GA is of interest, and it is possible that in this case the effect of auxin is indirect, and mediated by the increase of GA content induced by auxin.

Transcript levels of *SlDELLA* (encoding a repressor of GA mode of action; Martí *et al*., 2007) increased upon GA_3 and 2,4-D application, suggesting that it is also regulated by auxin through GA. Interestingly, the effect of GA_3 on transcript levels encoding this factorwas opposite to that expected, supporting the contention that their transcription is subjected to negative feed-back regulation by their protein products.

Availability of appropriate tomato antibodies would be necessary to clarify this issue. A role for SlDELLA as a repressor of fruit-set is supported by the observations that antisense *SlDELLA* (Martí et al., 2007) and *procera* (reported to be a putative inactive DELLA mutant; Bassel *et al*., 2008) plants produce parthenocarpic fruits (Dr Lazaro Peres, personal communication). On the other hand, *GAST1*, a tomato GA-responsive gene of unknown function (whose expression is known to be partially inhibited by ABA; Shi *et al*., 1992) acts specifically through GA since its expression was enhanced in GA_3- but not in 2,4-D-induced ovaries. Also *SlGID1*, a gene encoding a putative GA receptor (homolog to the rice GA receptor; Ueguchi-Tanaka *et al*., 2007) responds specifically to GA. Our *SlGID1* results in ovaries are in contrast with those described previously for *AtGID1* genes in Arabidopsis seedlings, where they are down-regulated by GA application (Hirano *et al*., 2008), suggesting that this GA response may be tissue dependent.

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

supported by the observation that simultaneous application of GA_3 and IAA had an additive effect on fruit growth (Fig. 2).

Experimental procedures

Plant material and growth conditions

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

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

Plant hormone applications

Application of 2,4-D (Duchefa, Haarlem, The Netherlands), IAA (Duchefa), and GA_3 (gift of Dr Michel Beale, Rothamsted Research, UK) was carried out to unpollinated ovaries the day equivalent to anthesis, in 10 µl of 5% ethanol, 0.1% Tween 80 solution. Flower emasculation was carried out 2 d before anthesis to prevent self-pollination. 0.1 M LAB 198999 (3,5-dioxo-4-butyryl-cyclohexane carboxylic acid ethyl ester) (BASF, Limbergerhof, Germany) was applied in 5% ethanol, 0.1% Tween solution, 10 µl per

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

Quantification of gibberellins

GAs were quantified following the protocol described in Fos *et al*. (2000). Briefly, aliquots (1 to 5 g fresh weight) of frozen material were extracted with 80% methanol and, after removing the organic phase, the water fraction was partitioned against ethyl acetate and purified by QAE-Sephadex chromatography and C_{18} cartridges. The GAs where then separated by reverse phase HPLC chromatography (4-µm C_{18} column, 15 cm long, 3.9 mm i.d.; NovaPak, Millipore, Milford, MA), and appropriate fractions grouped for GC-SIM analysis after methylation and trimethylsililation. [17,17-^{2}H]GA_1, [17,17-^{2}H]GA_3, [17,17-^{2}H]GA_8, [17,17-^{2}H]GA_{19}, [17,17-^{2}H]GA_{20}, [17,17-^{2}H]GA_{29}, [17,17-^{2}H]GA_{44} and [17,17-^{2}H]GA_{53} (purchased from Prof. L Mander, Australian National University, Canberra) were added to the extracts as internal standards for quantification, and [^{3}H]GA_{20} and [^{3}H]GA_9 (purchased from Prof. L. Mander) to monitor the separation of GAs after HPLC using a 10 to 100% methanol gradient containing 50 µl acetic acid per litre and taking 1 ml fractions. Quantification was carried out by GC-SIM using a gas chromatograph (model 5890, Hewlett-Packard, Palo Alto, CA) coupled

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

In vivo gibberellin metabolism

Unpollinated ovaries from emasculated flowers were treated the day equivalent to anthesis (d0) with [17-^{14}C]GA_1, [17-^{14}C]GA_{20}, [17-^{14}C]GA_{53} and [17-^{14}C]GA_{12} solutions (purchased from Prof. L. Mander; 34-55 μCi μmol^{-1}) (10 000 dpm ovary^{-1} in 10 μl of 10% methanol, two replicates of 12 ovaries per treatment) without or with 2,4-D (200 ng ovary^{-1}). A similar experiment was carried out using self-pollinated ovaries, also treated with labelled GAs at d0. Fruits (ovaries) were harvested 48 h after treatment, frozen in N_2 and kept at -80 ºC until analysis. Ground ovaries (12 per replicate and treatment) were extracted overnight at 4 ºC in 80% methanol with agitation, centrifuged at 13000 rpm and re-extracted twice for 20 min in 100% methanol. The joined supernatants were taken to dryness, the residue dissolved in 10% methanol and the metabolic products separated by HPLC, as described before for quantification of GAs. Metabolites were detected using an on-line radioactive monitor (Radioflow Detector LB 508, Berthold Technologies) and identified by their retention times compared to pure GAs. Only data from one replicate, out of two with similar results, are given in Results.

Quantitative RT-PCR

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

using 1 µg of total RNA, random hexamers and a TaqMan reverse transcription kit, with the following thermocycling conditions: 95 ºC 10 min + [95 ºC 15 s + 60 ºC 1 min] x 40 cycles + 95 ºC 15 s + 60 ºC 1 min + 95 ºC 15 s. 2 µl aliquots of diluted (1/400) cDNA solution were used for qRT-PCR in 20 µl volume reaction using specific primers (Supplementary Table 1), *Power* SYBR Green PCR master mix and a 7500 Fast Real-Time PCR System (Applied Biosystems). Absolute quantification was carried out using external standard curves, as described elsewhere (Olmos *et al.*, 2005), with minor modifications. Briefly, short PCR fragments (80 to 200 bp) of the sequence of interest were obtained using the specific primers indicated in Supplementary Table 1 (in this case each forward primer also contained the T7 promoter sequence 5'-TAATACGACTCACTATAGGG-3') and cDNA from specific clones for each analyzed gene. These PCR fragments, containing the T7 promoter, were purified from a 3% agarose gel using a QIAquick gel extraction kit (QIAGEN), and transcribed *in vitro* with the Megashortscript kit. 2.5 ng of positive sense single strand RNA (ssRNA) transcripts were treated with TURBO DNase to remove the DNA fragment used as template, purified by a glass filter-based system (MEGAClear kit), and used to synthesize first-strand cDNA in 50 µl total volume reaction, as described before. Serial dilutions of cDNA solution corresponding to about 10^5 to10^8 molecules of ssRNA were used to set up external standard curves under identical amplification conditions to those used to amplify RNA targets from samples. Moles of ssRNA template were calculated taking into account average ribonucleotide mass (340 g mol^{-1}) and transcript base number (Nb), according to the equation: pmol ssRNA = X pg ssRNA x (1 pmol/340 pg) x (1/Nb). Molecules of ssRNA were estimated using the Avogadro's constant (6.023 x 10^{23} molecules mol^{-1}). Absolute amounts of mRNA in samples were quantified in

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

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

	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

Supplementary Table 1. Primer sequences used for PCR amplification and quantitative RT-PCR analysis of genes from GA metabolism, and auxin- and GA-response of tomato. *SlGPS* (DQ286930), *SlCPS* (AB015675), *SlGA20ox1* (AF049898), *SlGA20ox2* (AF049899), *SlGA20ox3* (AF049900), *SlGA20ox4* (EU652334), *SlGA3ox1* (AB010991), *SlGA3ox2* (AB010992), *SlGA2ox1*(EF441351), *SlGA2ox2* (EF441352), *SlGA2ox3* (EF441353), *SlGA2ox4* (EF441354), *SlGA2ox5* (EF441355), *SlIAA1* (AF022012), *SlIAA2* (AF022013), *SlIAA3* (AF022014), *SlIAA8* (AF022019), *SlIAA9* (AJ937282), *SlIAA14* (BE462113), *SlIAA16* (AF022020), *SlIAA18* (AI485829), *SlARF1*(BT013711), *SlARF8* (EF664362), *SlARF9* (BT013639), *SlGAST1* (X63093), *SlGID1* (BN001197), and *SlDELLA* (AY269087).

Gene	Sense	Antisense
SlGPS	5'-TAT GCAGAAAACATATTACAAGA-3'	5'-ATCAAGAACATCATCTATTAATTG-3'
SlCPS	5'-ATACCTAGAGCTAGCGAAATC-3'	5'-ACTGCCTAAATAGTACGTAACC-3'
SlGA20ox1	5'-CTCATTTCTAATGCTCATCGT-3'	5'-TGCAGATGATTCTTTCTTA GCG-3'
SlGA20ox2	5'-TTTCCATATTCTACCCTACAAG-3'	5'-TCATCGCATTACAATACTCTT-3'
SlGA20ox3	5'-AGCCAAATTATGCTAGTGTTAC-3'	5'-TTTTATGAGATTTGTGTCAACC-3'
SlGA20ox4	5'GATGATAAATGGCACTCTATTC-3'	5'-TGACTTCCTTGTTCTTCTACAG-3'
SlGA3ox1	5'-GGCATTAGTAGTTAATATAGGTGA-3'	5'-AAATAAGCTACAGAAAGTCGATA-3'
SlGA3ox2	5'-GATCATAAATTTGTCATGGATAC-3'	5'-TGTTTCCATATGGTTAAGTAATC-3'
SlGA2ox1	5'-GGCATGTAAGATATTAGAATTGA-3'	5'-TTAATCCGTAGTAGAGAATCAGA-3'
SlGA2ox2	5'-ATTAAGATCCAATAACACTTCG-3'	5'-TCTTGATTTCACACTATTTGC-3'
SlGA2ox3	5'-GACCCTTCTACTTTCAGCTC-3'	5'-AAATTGAATTGTCTTCTATCCA-3'
SlGA2ox4	5'-ATGGAAGGAAAAGACAGTTTA-3'	5'-CTTTTCTCAAATAGGACCAAC-3'
SlGA2ox5	5'-GATCACTTACCAATAATCAACAG-3'	5'-CGTCATGGTTTACGACTTTA-3'

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

1

2

Figure legends

Figure 1. Inhibition by PCB (A) and LAB 198999 (B) of fruit-set and growth of pollinated and parthenocarpic fruits induced by 2,4-D (6 and 20 ng), and reversion by GA_3. (C) Effect of PCB on selected genes of GA-metabolism (*SlGA20ox1*, *SlGA3ox1* and *SlGA2ox2*) and GA-response (*GAST1*) in pollinated ovaries. (D) Photography of representative parthenocarpic fruits induced by 2,4-D (6 ng), alone or plus PCB without or with GA_3. Fruits were collected 20 d after anthesis or hormone treatment, except in the case of (C), which were collected 10 d after anthesis. Values are means of eight fruits ± SE, except when otherwise specified. Values in brackets indicate the number of fruits set when less than eight. PCB was applied at 10^{-5} M to the pots, and LAB 198999 (0.1 M) and GA_3 (2000 ng) to the ovaries.

Figure 2. (A) Effect of PCB on parthenocarpic fruit induction by GA_3 and IAA , and reversion by GA_3. (B) Photography of representative parthenocarpic fruits induced by IAA, alone or plus PCB without or with GA_3. Fruits were collected 20 d after hormone application. Values are means of eight fruits ± SE, except when otherwise specified. Values in brackets indicate the number of fruits set when less than eight. PCB was applied at 10^{-5} M to the pots, and LAB 198999 (0.1 M), GA_3 (2000 ng) and IAA (2000 ng) to the ovaries.

Figure 3. Radioactive HPLC traces of metabolites of [17-^{14}C]GA_{12}, [17-^{14}C]GA_{53}, [17-^{14}C]GA_{20} and [17-^{14}C]GA_1 applied to unpollinated untreated and treated with 2,4-D (200 ng) and pollinated ovaries. Labelled GAs were applied at d0 and ovaries collected 2 days after application. See more details in Experimental procedures.

Figure 4. Effect of 2,4-D application to unpollinated ovaries on transcript levels of *SlGPS* and *SlCPS* (A), *SlGA20ox1, -2*, *-3* and *-4* (B), *SlGA3ox1* and *-2* (D), and *SlGA2ox1, -2, -3, -4* and *-5* (C) genes. Transcript analysis was carried out by

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

Figure 5. Effect of 2,4-D and GA_3 on transcript levels of IAA/Aux (*IAA1*, *IAA2*, *IAA3*, *IAA7*, *IAA8*, *IAA9*, *IAA18*), ARF (*ARF1*, *ARF8* and *ARF9*) (B, C), and GA signal-transduction (*SlGID1* and *SlDELLA* and *SlGAST1*) (D) genes in 5-d-old unpollinated ovaries untreated or treated with 2,4-D (200 ng) and GA_3 (2000 ng). Data are means ± SE of three replicates from a representative experiment.

Figure 6. Scheme of proposed interaction of auxin and GAs during tomato fruit-set and growth. Auxin, either applied or synthesized in the pollinated ovary, increases the content of active GA_1 in the ovary by upregulating transcription of genes encoding enzymes of GA biosynthesis (CPS, GA20ox1, -2 and -3 and GA3ox1) and downregulating that of a gene encoding an enzyme of GA inactivation (GA2ox2). This effect may be mediated by Aux/IAA factors whose expression is modified upon auxin and GA application to the ovary. ?, specific effect of auxin on fruit growth through other Aux/IAA and ARF factors (see Fig. 5A, B, C and Discussion for more details).

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

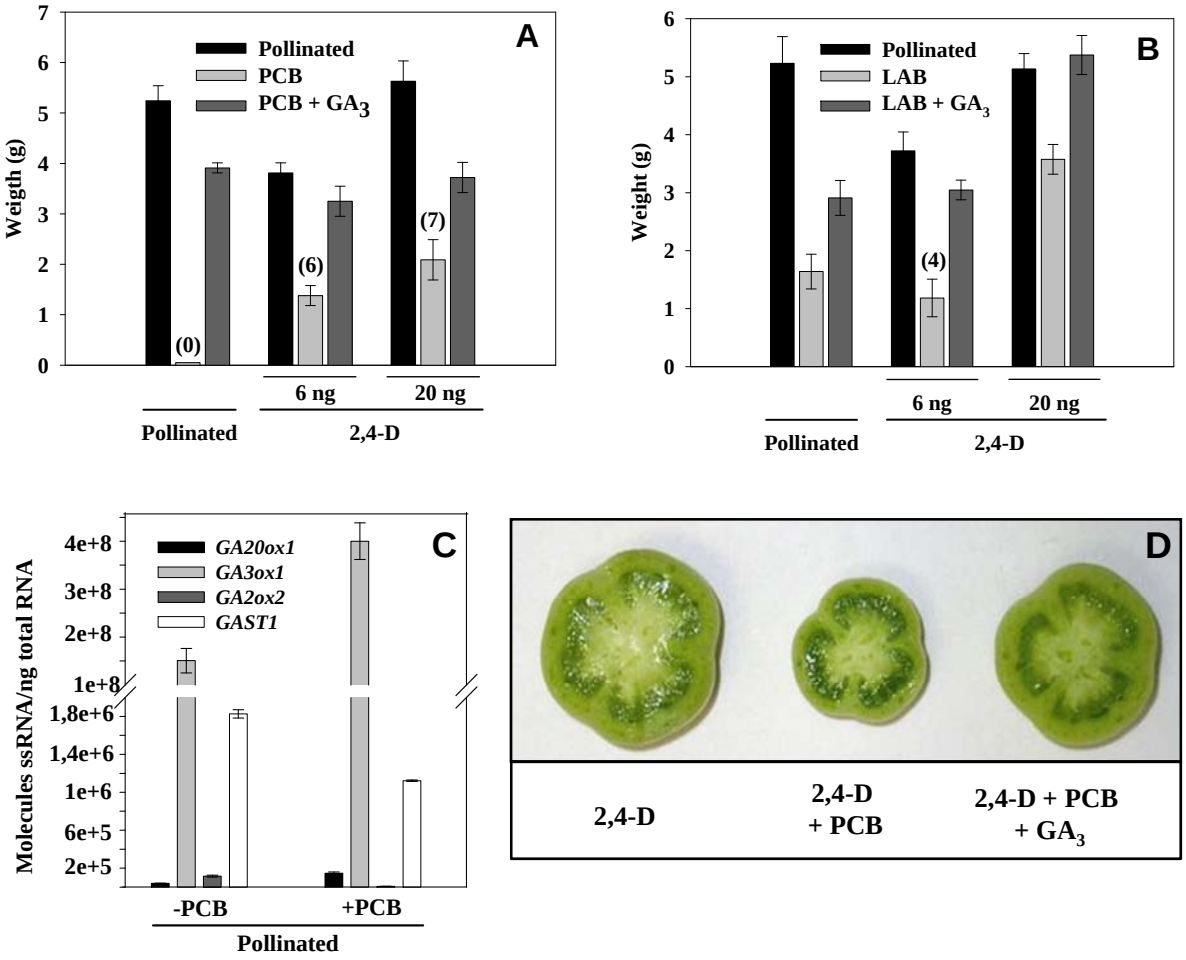
A
Pollinated
PCB
PCB + GA3
Weigth (g)
(0)
(6)
(7)
6 ng
20 ng
Pollinated
2,4-D
B
Pollinated
LAB
LAB + GA3
Weight (g)
(4)
6 ng
20 ng
Pollinated
2,4-D
C
GA20ox1
GA3ox1
GA2ox2
GAST1
Molecules ssRNA/ng total RNA
-PCB
+PCB
Pollinated
D
2,4-D
2,4-D + PCB
2,4-D + PCB + GA3

Fig.1

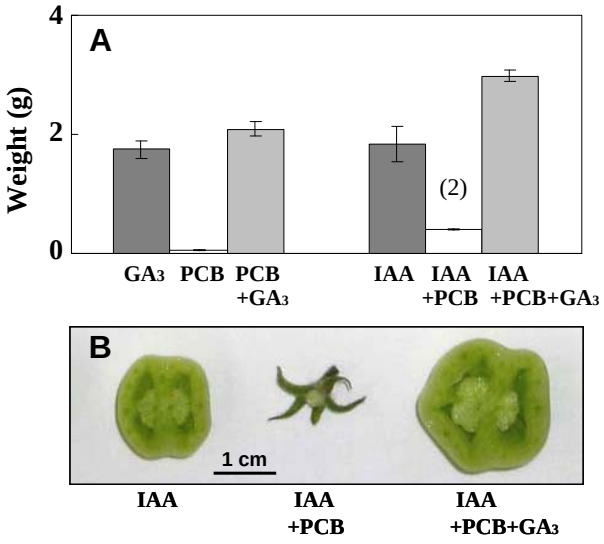
A
Weight (g)
4
2
0
(2)
GA3
PCB
PCB +GA3
IAA
IAA +PCB
IAA +PCB+GA3
B
1 cm
IAA
IAA +PCB
IAA +PCB+GA3

Fig. 2

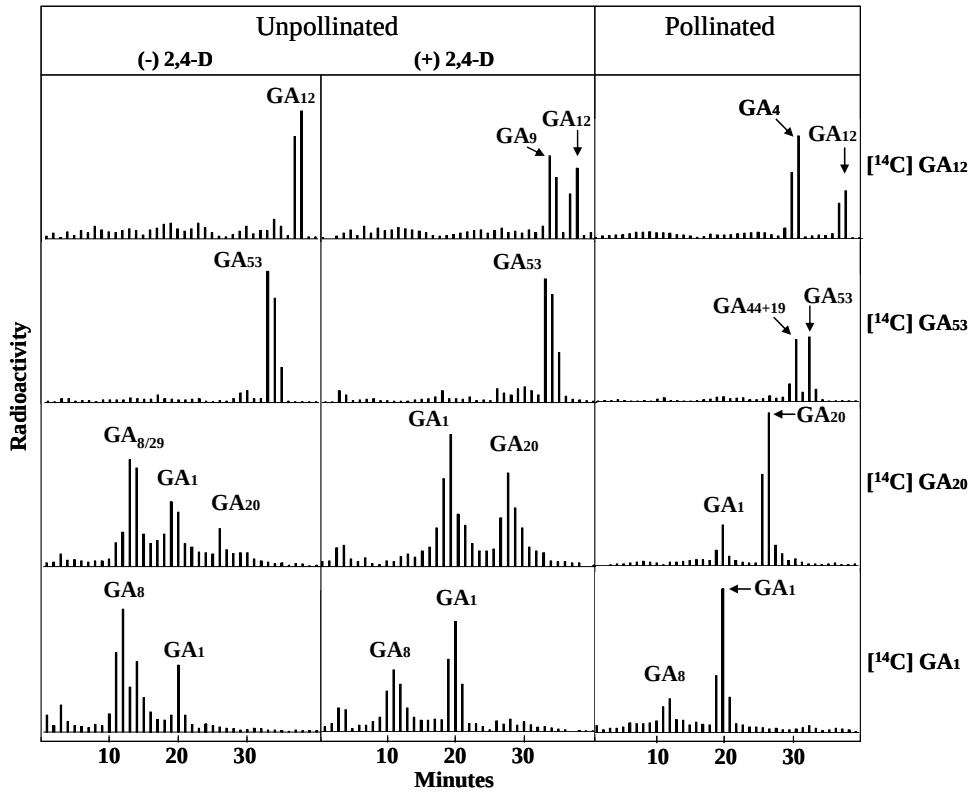
Unpollinated
Pollinated
(-) 2,4-D
(+) 2,4-D
GA12
GA9
GA12
GA4
GA12
[14C] GA12
GA53
GA53
GA44+19
GA53
[14C] GA53
GA8/29
GA1
GA20
GA1
GA20
GA1
GA20
[14C] GA20
GA8
GA1
GA8
GA1
GA1
GA8
[14C] GA1
Radioactivity
10
20
30
10
20
30
10
20
30
Minutes

Fig 3

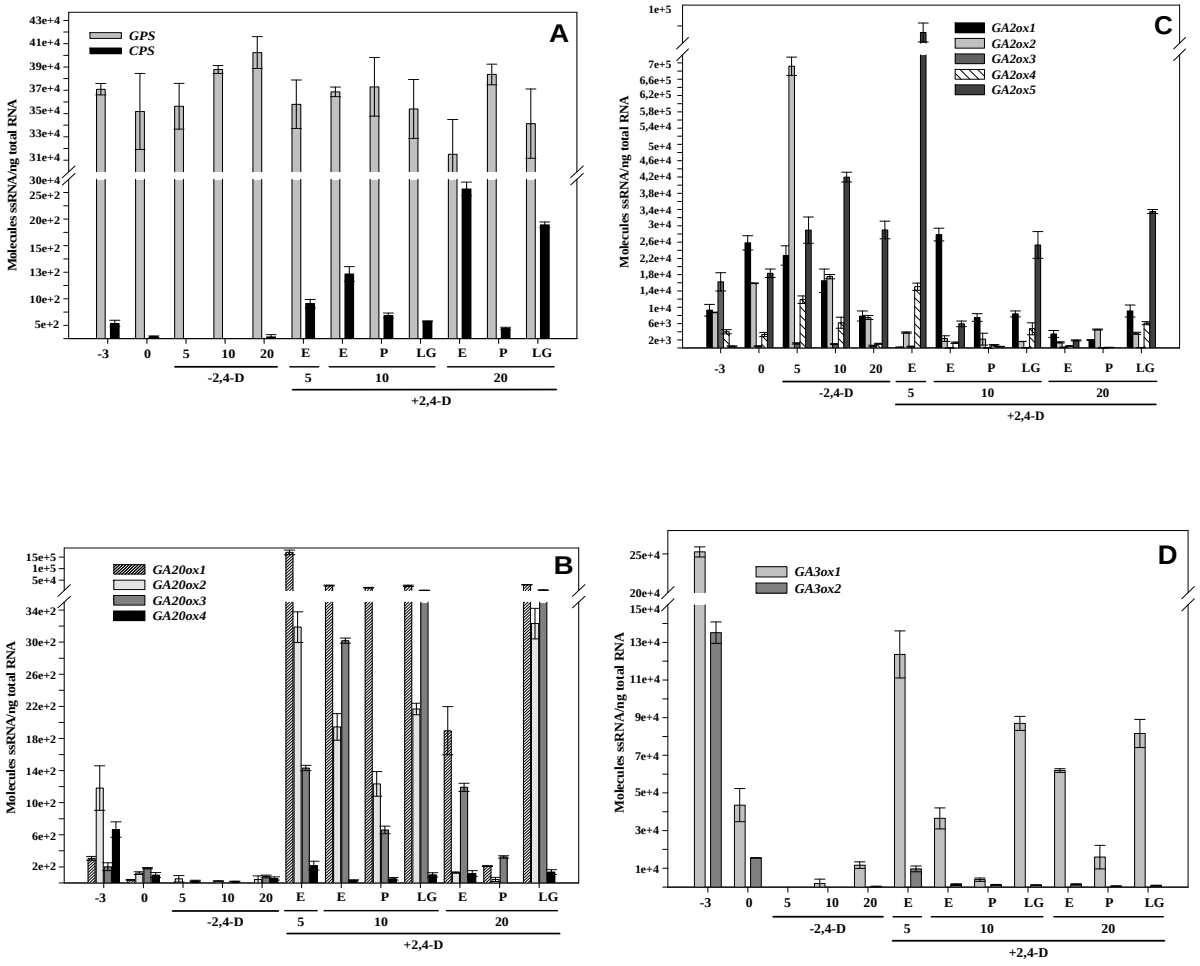
A
GPS
CPS
Molecules ssRNA/ng total RNA
B
GA20ox1
GA20ox2
GA20ox3
GA20ox4
C
GA2ox1
GA2ox2
GA2ox3
GA2ox4
GA2ox5
D
GA3ox1
GA3ox2
-3
0
5
10
20
E
P
LG
-2,4-D
+2,4-D

Fig. 4

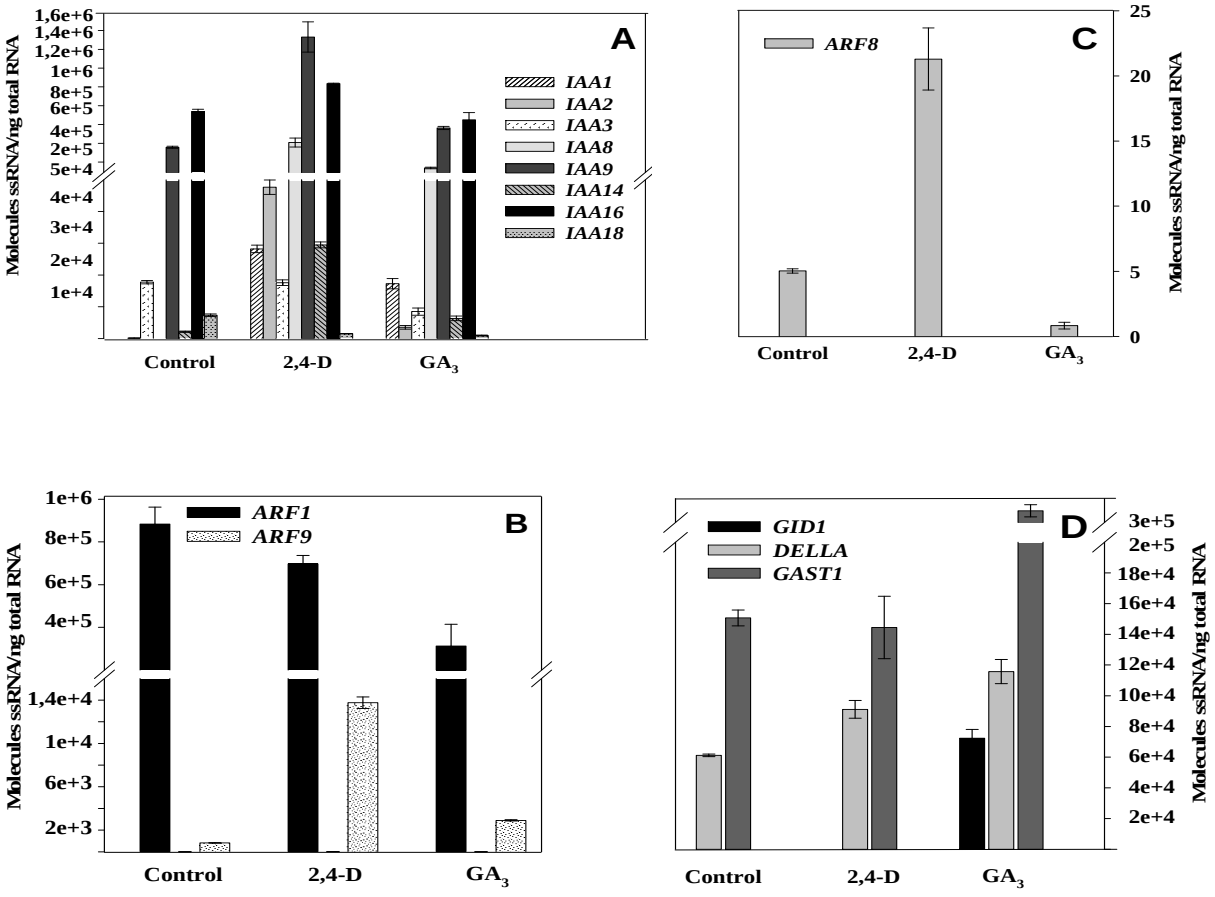
A
IAA1
IAA2
IAA3
IAA8
IAA9
IAA14
IAA16
IAA18
Molecules ssRNA/ng total RNA
1,6e+6
1,4e+6
1,2e+6
1e+6
8e+5
6e+5
4e+5
2e+5
5e+4
4e+4
3e+4
2e+4
1e+4
Control
2,4-D
GA3
C
ARF8
25
20
15
10
5
0
B
ARF1
ARF9
1e+6
8e+5
6e+5
4e+5
1,4e+4
1e+4
6e+3
2e+3
D
GID1
DELLA
GAST1
3e+5
2e+5
18e+4
16e+4
14e+4
12e+4
10e+4
8e+4
6e+4
4e+4
2e+4

Fig. 5

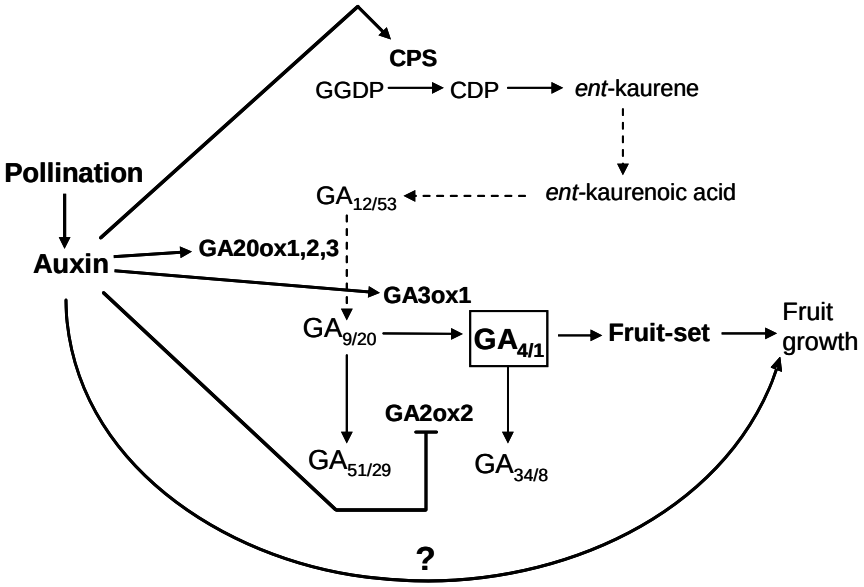
CPS
GGDP
CDP
ent-kaurene
Pollination
GA12/53
ent-kaurenoic acid
Auxin
GA20ox1,2,3
GA3ox1
GA9/20
GA4/1
Fruit-set
Fruit growth
GA2ox2
GA51/29
GA34/8
?

Fig. 6

Supplementary 1