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Gibberellin Regulation of Fruit-Set and Growth in Tomato[1]

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ABSTRACT

The role of gibberellins (GAs) in tomato (*Solanum lycopersicum* L.) fruit development was investigated. Two different inhibitors of GA biosynthesis (LAB 198999 and paclobutrazol) decreased fruit growth and fruit-set, an effect reversed by GA_3 application. LAB 198999 reduced GA_1 and GA_8 content, but increased that of their precursors GA_{53}, GA_{44}, GA_{19} and GA_{20} in pollinated fruits. This supports the hypothesis that GA_1 is the active GA for tomato fruit growth. Unpollinated ovaries developed parthenocarpically in response to $GA_3 > GA_1 = GA_4 > GA_{20}$, but not to GA_{19}, suggesting that GA 20-oxidase activity was limiting in unpollinated ovaries. This was confirmed by analyzing the effect of pollination on transcript levels of *SlCPS*, *SlGA20ox1*, *-2* and *-3,* and *SlGA3ox1* and *-2*, encoding enzymes of GA biosynthesis. Pollination increased transcript content of *SlGA20ox1*, *-2*, and *-3*, and *SlCPS*, but not of *SlGA3ox1* and *-2.* To investigate whether pollination also altered GA inactivation, full length cDNA clones of genes encoding enzymes catalyzing GA 2-oxidases (*SlGA2ox1*, *-2*, *-3*-, *-4* and *-5*) were isolated and characterized. Transcript levels of these genes did not decrease early after pollination (5-d-old fruits), but transcript content reduction of all of them, mainly of *SlGA2ox2*, was found later (from 10 d after anthesis). We conclude that pollination mediates fruit-set by activating GA biosynthesis mainly through up regulation of *GA20ox.* Finally, the phylogenetic reconstruction of the *GA2ox* familiy clearly showed the existence of three gene subfamilies, and the phylogenetic position of *SlGA2ox1*, *-2*, *-3*, *-4* and *-5* was established.

INTRODUCTION

Fruit-set has been defined as the changeover from the static condition of the flower ovary to the rapidly growing condition of the young fruit following ovary fertilization. In the case of tomato (*Solanum lycopersicum* L.), one of the most studied fleshy fruits, fruit growth takes place after fruit-set in two consecutive phases: an active division, lasting about 7-10 d post-anthesis, and a cell expansion phase (Gillaspy et al, 1993). During the growth process the ovary wall develops into a pericarp composed of exocarp, mesocarp and endocarp, while the placental parenchyma, supported by the columella, grows by division and expansion, enclosing the developing seeds and filling the locular cavities with a jelly-like homogenous tissue (locular tissue) (Ho and Hewitt, 1986; Gillaspy et al, 1993).

Gibberellins (GA) constitute a group of plant hormones which control developmental processes such as germination, shoot elongation, tuber formation, flowering, and fruit-set and growth in diverse species (Hedden and Kamiya, 1997; Olszewski et al, 2002). The metabolism of GA has been deeply investigated and is quite well understood (Sponsel and Hedden, 2004). In summary, *ent*-kaurene, synthesized from geranylgeranyl diphosphate by the action of two cyclases, is metabolized by the action of P450-dependent monoxygenases to GA_{12} and/or GA_{53}, which in turn are metabolized by GA 20-oxidases and GA 3-oxidases, acting consecutively, to active GAs through two parallel pathways: the non-13-hydroxylation (leading to GA_4) and the early-13-hydroxylation one (leading to GA_1, and GA_3 in some cases) (Supplementary Fig. 1). Active GAs and their precursors can be irreversibly inactivated by GA 2-oxidases introducing a hydroxyl at the 2ß position (Sponsel and Hedden, 2004). The existence of genes encoding GA deactivating enzymes catalyzing 16α,17-epoxidation in rice (Zhu et al, 2006) and formation of GA methyl esters in Arabidopsis (Varbanova et al, 2007) has been reported, although the importance of these reactions for GA homeostasis in other species is unknown. Most of the genes encoding all those enzymes have been cloned in many plant species (Hedden and Kamiya, 1997; Hedden and Phillips, 2000; Sponsel and Hedden, 2004), and their expression is regulated by endogenous and environmental factors (Yamaguchi and Kamiya, 2000; García-Martínez and Gil, 2002). GA 20-oxidases, GA 3-oxidases and GA 2-oxidases are 2-cetoglutarate-dependent dioxygenases which have been found to be encoded by small gene families (e.g., in the case of Arabidopis 5 *GA20ox*, 4 *GA3ox* and 7 *GA2ox*), whose expression is temporarily and developmentally regulated (Hedden and Phillips, 2000). The *GA2ox* family is particularly complex since it is composed of two classes differing in their substrate specificity, C_{19}-GAs and C_{20}-GAs, respectively (Schomburg et al, 2003). In addition, some GA2ox enzymes using C_{19}-

GAs as substrates have multicatalytic activity, converting the GAs successively to 2ß-hydroxylated metabolites and to GA catabolites (Supplementary Fig. 1) (Thomas et al, 1999; Ubeda-Tomás et al, 2006).

Analysis of gibberellins (GAs) has shown that seeded fruits of tomato contain mainly GAs from the early-13-hydroxylation biosynthetic pathway (Bohner et al, 1988; Fos et al, 2000), and that pollination induces an increase of GA content in the ovary (Mapelli et al, 1978; Koshioka et al, 1994), suggesting that these hormones are involved in fruit-set and growth of tomato. This hypothesis is supported by results of GA application experiments to unpollinated ovaries (Sjut and Bangerth, 1982/83; Alabadí and Carbonell, 1998; Fos et al, 2000, 2001), and of inhibitors of GA biosynthesis to pollinated ovaries (Fos et al, 2000, 2001). There is however no demonstration on the nature of the active GA, nor on the possible changes in GA metabolism affected by pollination in relation to fruit-set and early fruit growth in tomato.

The tomato cultivar Micro-Tom (Scott and Harbaught, 1989) has been proposed as a convenient model system to carry out research on the hormonal regulation of berry fruit development due to its small size, rapid growth, and easy transformation (Meissner et al, 1997; Eyal and Levy, 2002; Dan et al, 2006). The phenotype of this cultivar is the result of mutations in the genes *Dwarf* (*D*) (encoding 6-deoxocatasterone dehydrogenase, of the brassinosteroid biosynthesis pathway), *Self-Pruning* (*SP*) (which controls the determinate/indeterminate phenotype), and *Internode length reduction* (*Ilr*) (probably similar to *Miniature, Mnt*, still uncharacterized) (Martí et al, 2006). The dwarf phenotype of Micro-Tom is not the result of GA deficiency (Martí et al, 2006). It has been found that pollinated ovaries of Micro-Tom develop into normal fruits, and that unpollinated ovaries respond to GA_3 and auxin (but not to brassinosteroid) application (Serrani et al, 2007), showing that Micro-Tom constitutes a good experimental system to investigate the role of hormones in fruit development.

In this work, using the tomato cv Micro-Tom, we have shown by application of different GAs and inhibitors of GA biosynthesis that tomato fruit-set after pollination depends on GAs, and that GA_1 is the active form to induce fruit development. Pollination increased the expression of genes encoding GA20ox, but not of those encoding GA3ox, supporting the hypothesis that GA 20-oxidase activity is limiting in unpollinated ovaries. Five members of the *SlGA2ox* family have also been isolated to investigate the effect of pollination on expression of genes of GA catabolism. No decrease in transcript levels was found for any of these genes early after pollination (at d5 after anthesis), indicating that fruit-set may not be induced by regulation of GA inactivation. Phylogenetic analysis of genes encoding GA2ox indicates the existence of

three subfamilies denoted I, II, and III, the new five *SlGA2ox* being clustered within groups I and II, constituted by enzymes using C_{19}-GAs as substrates.

RESULTS

Effect of Inhibitors of GA Biosynthesis on Growth of Pollinated Fruits

To investigate whether the development of pollinated fruits depends on GAs, two different kinds of inhibitors of GA biosynthesis were used: LAB 198999, an acylcyclohexanedione derivative which inhibits 2-oxoglutarate-dependent dioxygenases (Santes and García-Martínez, 1995), was applied to pollinated ovaries, and paclobutrazol, an inhibitor of P450-dependent monooxygenases (Hedden and Graebe, 1985), to the roots in the nutrient solution. In the case of LAB 198999, direct application to the ovaries was carried out 2 d after pollination, after removing stamen and petals, to facilitate absorption. This inhibitor was applied at that time because earlier application might prevent pollen germination or fertilization. It was shown previously that removal of those organs 2 d after pollination did not reduce the number of seeds per fruit nor the final fruit weight (Fig. 1A). Paclobutrazol was applied to the roots because direct treatment of pollinated ovaries the day equivalent to anthesis or later was not efficient. Paclobutrazol application was started 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) to ascertain that it was transported in time to the pollinated ovary.

LAB 198999 application (0.3 to 10 mM) reduced the weight of the fruit, effect which was reversed by exogenous GA_3. At the highest doses of inhibitor (3 and 10 mM) fruit-set was also reduced, but could not be recovered by GA_3 (Fig. 1B), probably due to non-specific toxic effect of the inhibitor (necrotic spots appeared on the surface of the ovary) at those doses. In the case of paclobutrazol application, both fruit-set and final fruit size decreased proportionally to the dose of inhibitor, and at 10^{-2} M fruit-set was 0% (Fig. 1C). This inhibition was fully reverted with GA_3 application (Fig. 1C). Vegetative growth of plants treated with LAB 198999 was not affected (due probably to direct ovary application), and in the case of paclobutrazol the apical shoot length was only slightly reduced (due probably to application after flowering time, when most vegetative growth had already occurred). Interestingly, both kinds of inhibitors did not prevent the development of seeds in developed fruits (data not presented).

Effect of Inhibitors of GA Biosynthesis on GA content of Pollinated Fruits

In order to assess the effect of modification of endogenous GA content in relation to early fruit development, GAs from the early-13-hydroxylation pathway were quantified in 10-d-old pollinated ovaries control or treated with 1 mM LAB 198999 (dose of inhibitor at which the effects are fully reverted by applied GA_3; Fig. 1B). At that time, the weight of LAB 198999 treated ovaries was about half of control (Table 1). This weight reduction was associated with significantly lower concentration (about half) of GA_1 (the active GA), of its metabolite GA_8 (about one tenth), and of GA_{29} (a metabolite of GA_{20}, more than half) (Table 1). In contrast, LAB 198999 produced accumulation of all precursors of GA_1 (GA_{53}, GA_{44}, GA_{19} and GA_{20}) (Table 1). These results strongly support that fruit development in tomato depends on GAs, and specifically on GA_1.

Response of Unpollinated Ovaries to Application of Different Kinds of Gibberellins

Diverse GAs from the early-13-hydroxylation pathway (GA_1, GA_3, GA_{19} and GA_{20}) and GA_4 (from the non-13-hydroxylation pathway) were tested for their activity to induce fruit-set and growth of unpollinated ovaries. As in many other systems, GA_3 was the most active followed by GA_1 and GA_4 (equally active), and GA_{20}. Interestingly, GA_{19} (the immediate metabolic precursor of GA_{20}) was completely inactive (Fig. 2). These results suggested that GA 20-oxidase activity is limiting in unpollinated ovaries.

Effect of pollination on transcript levels of genes encoding enzymes of GA biosynthesis

To test the last hypothesis we compared in unpollinated and pollinated ovaries transcript levels of *SlCPS*, *SlGA20ox1*, *-2* and *-3* and *SlGA3ox1* and *-2*, genes previously cloned by Rebers et al (1999) which encode three kinds of GA biosynthesis enzymes. The expression of those genes in diverse tomato organs is given in Fig. 3. All the genes were expressed in aerial vegetative (leaves and internodes) and reproductive (flowers and their diverse parts) tissues. In roots we could only detect transcripts of *SlGA20ox3* and *SlGA3ox1*. Transcripts of *SlCPS*, *GA20ox3*, and *SlGA3ox1* and *-2* were detected in ovaries of flowers at anthesis, and transcripts of all the analyzed genes ,except of *SlGA3ox2* also in pollinated 20-d-old fruits.

Expression of *SlCPS* was detected in unpollinated ovaries before anthesis (d-3) but decreased later on (from d0 to 20 d post-anthesis, dpa) (Fig. 4). In contrast, in entire (E) pollinated ovaries *SlCPS* transcript levels did not decrease and remained similar or higher than

unpollinated ovaries before anthesis. Transcripts were present both in pericarp and developing seeds, more in the latter than in the former (Fig. 4).

Almost undetectable expression of *SlGA20ox1* was found in unpollinated ovaries (between -3 and 20 dpa). In the case of *SlGA20ox2*, high expression was detected before anthesis (d-3), but dropped to undetected or very low in unpollinated ovaries between d0 and d20 (Fig. 4). Interestingly, transcript levels of both *SlGA20ox1* and *-2* were very high in entire pollinated ovaries (5 to 20 dpa) (at least ten-fold those of unpollinated ovaries). Transcript content could also be analyzed separately in pericarp and seeds of 10- and 20-old-fruits. Transcripts were equally distributed in the pericarp and seeds at d10, but were much more concentrated in seeds at d20 (Fig. 4). *SlGA20ox3* transcripts could be clearly detected and their levels did not vary in unpollinated ovaries (from d-3 to d20). Interestingly, they increased also (about twice) in pollinated ovaries, particularly in developing seeds at d20 (Fig. 4).

SlGA3ox1 transcript content was high in unpollinated ovaries before anthesis (d-3), and decreased from anthesis until d20. Similar levels were found in unpollinated and pollinated ovaries until d20 (Fig. 4). At d10 and d20 transcripts were concentrated in developing seeds (Fig. 4). In contrast, transcripts of *SlGA3ox2,* detected in ovaries before anthesis, were at very low level or not detected in unpollinated ovaries after anthesis. In d10 and d20 pollinated ovaries *SlGA3ox2* transcripts were barely detected, and present mainly in the seeds (Fig. 4).

Cloning and characterization of genes encoding enzymes of GA inactivation in tomato

At the time of starting this work no GA 2-oxidase had been cloned in tomato. Therefore, in order to know whether pollination increased active GA content by also altering GA inactivation, we isolated genes encoding GA2ox. Using RT-PCR and degenerated primers, followed by 5´ and 3´ RACE only one full length cDNA clone could be isolated (*SlGA2ox1;* EF441351) (see Materials and Methods). This cDNA was 1281 bp long (including 88 and 143 bp in the 5´and 3´ untranslated regions, respectively) and encoded a protein of 349 aminoacids.

Using BLAST search of EST data bases we identified 18 sequences with high similarity to *SlGA2ox1* and *GA2ox* from other species, which corresponded apparently to four additional different incomplete genes (gene 2, AW930043, BI935635, AW222239, BE434782, BE433301, BE435345; gene 3, AW030357, AI777086, BI921857, AW031637; gene 4, BI208568, AW931003, AW030225; and gene 5, AI899222, AI487548, AI488712, AW650238, AW650160). Full length clones of these genes were isolated by 5´ and 3´ RACE, amplified and named accordingly *SlGA2ox2* (EF441352; 322 aminoacids long), *SlGA2ox3* (EF441353; 344

aminoacids long), *SlGA2ox4* (EF441354; 341 aminoacids long) and *SlGA2ox5* (EF441355; 346 aminoacids long). Recently, the sequence of a clone similar to our *SlGA2ox2* (EF017805) was also submitted to GeneBank.

A phylogenetic analysis was carried out with the sequences of all published GA dioxygenase genes from tomato and those of the Arabidopsis genome, including the five putative GA2ox genes isolated in this work, previously published sequences of tomato GA20ox (3 genes) and GA3ox (2 genes), plus all sequences encoding GA dioxygenases (5 *GA20ox*, 4 *GA3ox*, and 7 *GA2ox*) in Arabidopis. Four groups corresponding to GA20ox, GA3ox, GA2ox using C_{19}-GAs as substrate, and GA2ox using C_{20}-GAs as substrate were found. The five *SlGA2ox* genes from tomato clustered with the group of GA2ox of Arabidopsis using C_{19}-GAs as substrate, suggesting that all of them encode this kind of enzymes (Supplementary Fig. 2).

After subcloning the five *SlGA2ox* genes in the expression vector pET45b, the activity of the expressed proteins was analyzed using [^{14}C]GA_1, [^{14}C]GA_4, [^{14}C]GA_9, [^{14}C]GA_{12}, [^{14}C]GA_{20} and [^{14}C]GA_{53} as substrates. Separation of radioactive metabolites by HPLC showed that extracts from *SlGA2ox1* metabolized [^{14}C]GA_1 and [^{14}C]GA_4 to compounds with the same retention times as [^{14}C]GA_8 and [^{14}C]GA_{34}, respectively; those from *SlGA2ox3* metabolized [^{14}C]GA_1, [^{14}C]GA_4 and [^{14}C]GA_9 to compounds with the same retention times as [^{14}C]GA_8, [^{14}C]GA_{34} and [^{14}C]GA_{51}, respectively; and those from *SlGA2ox4* metabolized completely [^{14}C]GA_9 to a compound with the same retention time as [^{14}C]GA_{51} (Supplementary Fig. 3). Activity of *SlGA2ox5* extracts was very poor, and only small peaks corresponding to putative [^{14}C]GA_{34} and [^{14}C]GA_{51} were found using [^{14}C]GA_4 and [^{14}C]GA_9 substrates, respectively (Supplementary Fig. 3). [^{14}C]GA_{12} and [^{14}C]GA_{53} were not metabolized in any case (Supplementary Fig. 3), confirming that *SlGA2ox1*, *-3*, *-4* and *-5* encoded C_{19} GA 2-oxidases. Expressed extracts from *SlGA2ox2* did not metabolize any of the six labelled GAs used as substrates (data not presented), suggesting that the corresponding protein was probably inactive.

Phylogenetic analysis of GA 2-oxidases

To better locate the new *SlGA2ox* genes within the large *GA2ox* family, a phylogenetic analysis was performed with all of the full-length *GA2ox* genes found in the data bases, using the outgroup sequence AtGA20ox1 to position the root of the tree. The analysis showed the existence of three large subfamilies of GA2ox (Fig. 5): groups I and II correspond to GA2ox using C_{19}-GAs as substrate (the occurrence of these two groups was pointed out earlier by Elliott et al, 2001), and group III corresponds to GA2ox using C_{20}-GAs as substrate. According to this

phylogenetic tree, OsGA2ox5 and -6, and NsGA2ox1, for which catalytic properties have not been reported yet, would use C_{20}-GAs as substrates. These subfamilies are similar to those described by Lee and Zeevaart (2005) in a previous analysis carried out with a selected number of sequences (20 versus 44 in this work). The topology of the root tree indicates that groups I and II are more closely related each other than to group III. In other words, these data suggest that group III diverged from all other GA 2-dioxygenase genes before the split between groups I and II. Both monocot and dicot genes are present in each of the three groups, indicating that the gene duplication events that gave rise to these three subfamilies occurred before the split between monocots and dicots.

Interestingly, the seven GA2ox reported in the literature as having multicatalytic activity were located in group I (underlined in Fig. 5; see also appropriate references in Fig. 5 legend). Certainly, not all GA2ox present in this group have been shown to be multicatalytic. Absence of annotation of this biochemical property in enzymes of group I may be due to: a) the catalytic properties have not been investigated in these enzymes; b) catabolite formation may have not been detected since it depends strongly on enzyme concentration and is adversely affected by dilution (Martin et al, 1999).

Aminoacid sequence comparison of all GA2ox enzymes used to construct the phylogenetic tree of Fig. 5 is given in Supplementary Fig. 4. Interestingly, groups I and II differ in at least two specific aminoacids at conserved regions which might be related to their possible different catalytic properties. For instance, within the sequence (N/T/S)GDXG(W/R/E/D/H)X(L/V/I)E(Y/H)(L/I)L (located between positions 90 and 100 of AtGA2ox1) the W present in all the sequences of group I (except in SlGA2ox2 which has an R) is substituted by a D/E in all the sequences of group II (except in VaGA2oxB3 which has an H). Also, within the sequence (Y/F)XX(F/L)(T/K)(W/R)X(E/D/Q)(Y/F)K (located between positions 294 and 303 of AtGA2ox1), the E present in all the sequences of group I (at position 296 of AtGA2ox1) is substituted by diverse non-acidic aminoacids in all the enzymes of group II. According to these predictions (see Fig. 5 and Supplementary Fig. 4), of the five genes isolated in this work, SlGA2ox1 and -3 would be monocatalytic (confirmed in this work) and SlGA2ox2, -4 and -5 would be multicatalytic (a prediction that we were unable to confirm; possible reasons for the absence of this kind of activity are given in Discussion).

Effect of pollination of transcript levels of genes encoding enzymes of GA inactivation in tomato

Distribution of *SlGA2ox1* to *-5* transcripts in diverse tomato organs is presented in Fig. 6. *SlGA2ox1* was expressed only in ovaries at anthesis and developing pollinated fruits. The other four genes were expressed to different extents in leaves (young and old), internodes (young and adult) and flowers at anthesis. In the roots we could only detect transcripts of *SlGA2ox3*, *-4* and *-5*. In flowers at anthesis, *SlGA2ox2* transcripts were present in all the organs (ovary, stamens, petals and sepals), *SlGA2ox3* mainly in petals and sepals, *SlGA2ox4* in ovary, petals and sepals, and those of *SlGA2ox5* only in ovaries. Developing 20-d-old fruits contained transcripts of all *GA2ox* genes, except *SlGA2ox3*.

The effect of pollination on expression of *SlGA2ox1* to *-5* is shown in Fig. 7. In unpollinated ovaries transcripts of all genes were present before or at the time of anthesis (d-3 and d0). In unpollinated ovaries expression of all *SlGA2ox* remained high later on, except for *SlGA2ox3* whose transcripts were at very low level or undetected between d0 and d20 (in agreement with results presented in Fig. 6). In 5-d-old pollinated ovaries (a time at which fruit-set and some growth had occurred already) transcript levels of the five *SlGA2ox* genes were similar to those of unpollinated ovaries. In contrast, in 10- and 20-d-old pollinated ovaries transcript levels of all *SlGA2ox* were lower than in unpollinated ovaries, particularly in the case of *SlGA2ox2* and -3 (in the latter case transcripts were bearly detected). An exception was *SlGA2ox1* at d10 where transcript levels were not reduced. Pericarp and seeds could be separated in 10- and 20-d-old fruits and therefore *GA2ox* transcript content were also analyzed in both organs at those times. *SlGA2ox1* was always highly expressed in the pericarp, and in seeds at d10. In contrast, *SlGA2ox4* and *-5* were expressed mostly in the developing seeds and therefore they may not contribute to GA homeostasis in the pericarp.

DISCUSSION

Fruit-set and fruit growth of pollinated Micro-Tom ovaries was reduced significantly, on a dose-effect response, by application of paclobutrazol, an inhibitor of GA biosynthesis that inhibits P450-dependent dioxygenases. The effect of paclobutrazol was fully counteracted by applied GA_3 (Fig. 1C). LAB 198999, another inhibitor of GA biosynthesis that inhibits 2-oxoglutarate-dependent dioxygenases, also reduced fruit-set and fruit growth, but the former effect could not be reverted by GA application (Fig. 1B), probably due to non-specific toxic effect. These results support the hypothesis that tomato fruit development depends on GAs, as suggested previously (Fos et al, 2000, 2001).

The reduction of fruit growth (about 50%) by LAB 198999 was associated with a reduction of GA_1 content to about 50% whereas GA_8 content was reduced to 10% (Table I). At the same time, in LAB 198999 treated fruits there was accumulation of GA_{53}, GA_{44}, GA_{19} and GA_{20} (Table I) Since the early-13-hydroxylation is the main GA metabolic pathway in tomato (Bohner et al, 1988; Koshioka et al, 1994; Fos et al, 2000, 2001) this means: a) that GA_1 is the main active GA in tomato fruit development; b) that the precursors of GA_1 are not active per se but only after conversion to this active hormone. GA_1 has been shown to be the active GA in shoot growth of many species such as pea (Ingram et al, 1984), lettuce (Waycott et al, 1991), rice (Fujioka et al, 1988), spinach (Zeevaart et al, 1993) and *Salix* (Olsen et al, 1995). In contrast, GA_4 is the main active hormone in others species like cucumber (Nakayama et al, 1991) and Arabidopsis (Cowling et al, 1998). Application of GA_4 is certainly capable of inducing tomato fruit development also (Fig. 2), but this hormone may have a minor physiological role because the non-13-hydroxylation pathway seems to be minor in this species. GA_{20} and GA_1 were almost equally active to induce parthenocarpic fruit growth in tomato, while GA_{19} was completely inactive (Fig. 2). This suggests that unpollinated ovaries are capable of metabolizing GA_{20} but not GA_{19} to GA_1 and, therefore, that the activity of GA 20-oxidase (that metabolizes GA_{19} to GA_{20}) but not that of GA 3-oxidase (that metabolizes GA_{20} to GA_1) is limiting in unpollinated tomato ovaries. Interestingly, in *pat-2*, a facultative parthenocarpic mutant of tomato, parthenocarpy is associated with a dramatic increase of GA_{20} and more GA_1 and GA_8 contents (Fos et al, 2000), due probably to enhanced activity of GA 20-oxidase.

The above mentioned hypothesis was supported by results of comparing the effect of pollination on transcript levels of diverse *SlGA20ox* and *SlGA3ox* genes of tomato previously isolated by Rebers et al (1999). *SlGA3ox2* transcripts were almost undetected in unpollinated and pollinated ovaries, whereas *SlGA3ox1* transcripts were present in unpollinated ovaries at d0 and remained essentially constant in both unpollinated and pollinated ovaries at least until d20 (Fig. 4). This supports the idea that GA 3-oxidase activity (encoded from *SlGA3ox1*) is present in ovaries before pollination, and that pollination does not alter that activity. In contrast, *SlGA20ox1* and *-2* transcripts were at very low levels or undetected at d0 and in 5 to 20-d-old unpollinated ovaries, but at high levels in 5 to 20-d-old pollinated ovaries. Transcript levels of *SlGA20ox3*, which were present in unpollinated ovaries, also increased upon pollination (Fig. 4). This suggests that GA 20-oxidase activity increases upon pollination, as indicated by previous GA application experiments (Fig. 2). However, we can not decide, based on our data, whether the three *SlGA20ox* are or not equally important for fruit-development regulation because transcripts of all of them were similarly distributed in the pericarp and seeds, at least until d10

(Fig. 4). In any case, our results do not support a role for GA 3-oxidase activity for fruit development, and are in contrast with the suggestions of Bohner et al (1988) and Koshioka et al (1994), based on endogenous GA content analyses, that 3ß-hydroxylation of GA_{20} is a rate limiting step in GA_1 biosynthesis after pollination in tomato.

Since transcript levels of *SlCPS* were higher in pollinated than in unpollinated ovaries, activity of earlier biosynthetic enzymes (e.g. CPS) might also contribute to the increase of GA content after pollination. CPS (formerly *ent*-kaurene synthetase A) activity is certainly present in extracts of tomato fruits (Bensen and Zeevaart, 1990). Arabidopsis CPS transcripts occurs in actively growing tissues, particularly in developing flowers and seeds (Silverstone et al, 1997), and expression of *PsCPS* (locus *LS*) seems to play an important role on the regulation of GA biosynthesis in relation to seed development in pea (Ait-Ali et al, 1997). In contrast, overexpression of *AtCPS* in Arabidopsis, although increasing *ent*-kaurene production did not result in increase of active GAs (Fleet et al, 2003). Rebers et al (1999) found that the expression of all the GA biosynthetic genes analyzed in this work (*SlCPS*, *SlGA20ox* and *SlGA3ox*) change during flower bud development in tomato, with different patterns of mRNA accumulation, indicating a complex regulatory mechanism for controlling GA biosynthesis during flower development. However, no comparison of transcript levels in unpollinated and pollinated tomato ovaries was carried out. GA metabolism during fruit-set and growth has also been investigated in pea. In this case, the increase of GA content upon pollination (Rodrigo et al, 1997) is also associated with an increase of *PsGA20ox1* expression (van Huizen et al, 1997). But in contrast to tomato, the presence of seeds seems also to up-regulate the expression of a *GA3ox* (*PsGA3ox1*; Ozga and Reinecke, 2003).

GA levels are a result of GA biosynthesis and inactivation (Hedden and Phillips, 2000). Therefore, modification of active GA levels may be due to simultaneous transcription alteration of genes encoding GA biosynthesis (e. g. GA20ox and/or GA3ox) and GA inactivating enzymes (GA2ox, GA epoxidases and GA methyltransferases, GAMT). For instance, GA_1 content decrease in the shoot during deetiolation in pea is due to down-regulation of *PsGA3ox1*, which controls the conversion of GA_{20} to GA_1, and by up-regulation of *PsGA2ox2*, encoding a GA2ox that converts GA_1 to inactive GA_8 (Symons and Reid, 2003). Developing siliques of null mutants of *GAMT1* and *GAMT2* have higher GA_1 and GA_4 contents and their seeds are more resistant to ancymidol, suggesting that they also contain more active GAs (Varbanova et al, 2007). Since GA2ox are generally considered the main GA inactivating enzymes, in order to know whether the increase of GA_1 upon pollination in tomato ovary is not only due to enhanced GA biosynthesis (through increase of *GA20ox* transcript levels, and may be *SlCPS*, as shown

before), but also to reduction of catabolic activity, five cDNA clones encoding putative GA 2-oxidases from tomato (*SlGA2ox1* to -*5*) were isolated. *SlGA2ox1*, *-3* and -*4,* and -*5* to a lesser extent were shown to encode active C_{19} GA2ox using different kinds of GAs as substrates (Supplementary Fig. 3).. Expressed *SlGA2ox2* extracts did not show activity with any of the six GAs used as substrate, suggesting that the corresponding protein was inactive in spite of carrying the purported amino acids binding Fe^{2+} and 2-cetoglutarate, and essentially all the amino acids conserved in GA2ox (Supplementary Fig. 4). A reason for SlGA2ox2 inactivity might be the presence of a mutation leading to the change of W (conserved in all GA2ox from group I) by an R at position 92 (Supplementary Fig. 4). This observation points out the possible importance this W residue for GA2ox activity. Additionally, SlGA2ox2, -4 and -5 have a D at a site (position 44 of SlGA2ox2) where most GA2ox have a conserved G (Supplementary Fig. 4), which might also affect their activity.

Transcripts of the five *SlGA2ox* genes were detected in different tissues (Fig. 6), suggesting that their expression is developmentally regulated. All of them were expressed in unpollinated ovaries before and/or at the time of anthesis and also up to d20 in unpollinated ovaries, at more or less extent. However, no decrease of expression was observed in any of the *SlGA2ox* genes in pollinated ovaries 5 d after anthesis, a time at which fruit-set has already been established, as shown by the observation that a significant growth had occurred. This means that the effect of pollination on early fruit development may not be mediated by an effect on GA inactivation through GA2ox. However, we can not discard a possible effect of GA2ox on later growth of tomato fruit (because transcripts of all *SlGA2ox* genes were lower in pollinated than in pollinated ovaries at d10 and/or d20), nor a possible role of other GA catabolic enzymes (e. g. GA epoxidases and GAMT) in GA homeostasis during fruit-set and growth.

The phylogenetic analysis of GA2ox, using all the sequences available in data bank and AtGA20ox1 as outgroup (Fig. 5), indicates that a first split occurred between enzymes using C_{20}-GAs as substrate (group III) and those using C_{19}-GAs, and that divergence between groups I and II occurred more recently. The five *SlGA2ox* genes isolated in this work were distributed between groups I and II, and therefore, according to this prediction, should differ in their catalytic properties. While SlGA2ox1 and -3 presented monocatalytic activity, as expected, no multicatalytic activity could be demonstrated for SlGA2ox2, -4 and -5 (expressed *SlGA2ox2* was completely inactive). Therefore, our results do not support the proposed hypothesis. However, since the three translated sequences of SlGA2ox2, -4 and -5 present changes in specific conserved amino acids which might affect activity, and it has been reported that detection of GA catabolites may be difficult and dependent on enzyme concentration (Martin et al, 1999), it may

not be possible to completely discard that hypothesis before carrying out more biochemical work to substantiate it. Monocot and dicot genes are both present in each of the three groups, indicating that gene subfamilies I, II, and III were originated from gene duplications early in evolution. Finally, additional gene duplications occurred within each of the groups I and II as indicated by te presence of several duplicates of Arabidopsis and other species in those groups, whereas no further duplication seem to have occurred within the more ancestral group III (Fig 5). Altogether, the data support the general hypothesis that acquisition of evolutionarily novel functions among GA-dioxygenases is associated with gene duplication events, as previously shown for other gene families (Sanjuan and Marin, 2001).

The results of experiments of GA and inhibitors of GA biosynthesis application presented here, as well as of GA quantification analysis support the hypothesis that fruit-set and early growth in tomato depend on GAs, and that GA_1 is the active hormone involved in these processes. Pollination increases the content of GAs in the ovary by increasing GA biosynthesis (through up-regulating *GA20ox* and *SlCPS*, but not *GA3ox* expression), not by reducing GA catabolic inactivation through GA2ox, at least in the cv Micro-Tom used in this work.

MATERIALS AND METHODS

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 to prevent interaction between fruits at the same truss (Serrani et al, 2007).

Plant Hormone Applications

Application of GAs (GA_1, GA_4, GA_{19} and GA_{20}, obtained from Prof. L Mander, Australian National University, Canberra, Australia) and GA_3 (Duchefa) was carried out to unpollinated ovaries in 10 µl of 5% ethanol, 0.1% Tween 80 solution. Flower emasculation was carried out two days before anthesis to prevent self-pollination. LAB 198999 (3,5-dioxo-4-

butyryl-cyclohexane carboxylic acid ethyl ester) (BASF, Limbergerhof, Germany) was applied in 10 µl of 5% ethanol, 0.1% Tween solution to pollinated ovaries, at different times after pollination, after removal of petals and stamens. Equal volumen of solvent solution was applied to control ovaries. Paclobutrazol (Duchefa, Haarlem, The Netherlands) was applied to the roots in the nutrient solution.

Quantification of Gibberellins

GAs were quantified following the protocol described in Fos et al (2000). In summary, aliquots (about 3 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_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) were added to the extracts as internal standards for quantification, and [^{3}H]GA_{20} and [^{3}H]GA_9 to monitor the separation of GAs after HPLC using a 10 to 100% methanol gradient. 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.

Isolation of cDNA Clones of *GA2ox* from Tomato

Total RNA was isolated from 20-d-old pollinated fruits using a phenol-chloroform method (Barttels and Thompson, 1983). Clones of *SlGA2ox* were isolated by RT-PCR using degenerated oligonucleotides. 2 µg of total RNA were reverse transcribed with a First-strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK) in 33 µl total volume reaction. PCR was performed taking 1 µl aliquot of cDNA solution in a 50 µl total volume reaction containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1x reaction buffer, 1 U of NETZYME® DNA Polymerase (Fermentas Gmbh, Germany), and 1 µM of degenerated primers A [5'-(GA)TXGGXTT(CT)GGXGA(AG)(CA)(CA)(AT)-3´] and B [5'-X(GC)CX(GC)(AC)(AG)AA(AG)TAXATCAT-3']. Thermocycling conditions for amplification

consisted of initial denaturation at 94ºC for 2 min, followed by 40 cycles of 94ºC/ 30 sec, 45ºC/ 60 sec and 72ºC/ 60 sec, and finally 10 min extension at 72ºC. The products of an amplified band of about 250 bp, separated on 1% agarose gel electrophoresis, were purified (CONCERT Rapid Gel Extraction System, GIBCO-BRL), cloned in the pGEM-T Easy Vector (Promega) and sequenced. Six of these clones (out of 11 sequenced) were identical and homologous to *GA2ox* previously cloned from diverse species. Sequences of the 5´ and 3' regions were obtained by RACE (RACE cDNA amplification kit, Clontech) using appropriate primers (Supplemental Table I) and the following conditions for amplification: 95ºC/ 5 min followed by 5 cycles of 94ºC/ 30 sec and 72ºC/ 2.5 min, 5 cycles of 94ºC/ 30 sec and 70ºC/ 2,5 min, and 30 cycles of 94ºC/ 30 sec and 68ºC/ 2,5 min , and finally 10 min extension at 72ºC. A full length cDNA clone, named *SlGA2ox1*, was obtained by RT-PCR using appropriate primers(Supplemental Table I), and the following thermocycling conditions: 94ºC/ 2 min, followed by 40 cycles of 94ºC/ 1 min, 57ºC/ 2 min and 72ºC/ 3 min, and 10 min extension at 72ºC, cloned in the pGEM-T Easy Vector and sequenced.

Additional *GA2ox* clones of tomato were identified by searching for tomato sequences homologous to *GA2ox* from diverse species (including Arabidopsis and *SlGA2ox1*, previously cloned) in Genebank EST data bases. Four groups coming from 18 EST corresponding to genes different to *SlGA2ox1* were identified. Using this sequence information 5´ and 3´ regions were obtained by RACE, when necessary, as described before. Full length cDNA clones (named *SlGA2ox2*, *SlGA2ox3*, *SlGA2ox4*, and *SlGA2ox5*) were amplified by RT-PCR using RNA from pollinated fruits (*SlGA2ox2*, *-4* and *-5*) and mature leaves (*SlGA2ox4*), the primers given in Supplemental Table I, and the thermocycling conditions described previously for *SlGA2ox1* (but using as annealing temperatures of 50ºC for *SlGA2ox3* and *-4*, and 54ºC for *SlGA2ox2* and *-5*). Amplified products were cloned in pGEM-T Easy Vector and sequenced.

Heterologous Expression of cDNA Clones and Determination of Enzyme Activities

Coding cDNA sequences of *SlGA2ox1, -2, -3, -4* and *-5* were amplified by PCR, cloned using a Zero Blunt TOPO Cloning kit (Invitrogen) and inserted as a translational fusion into the pET45b prokariote expression vector (Novagen) using *Bam*HI-*Hin*dIII (*SlGA2ox1*, *-3* and *-4*) and *Not*I-*Xho*I (*SlGA2ox2* and *-5*) sites. Recombinant clones were sequenced and expressed in BL21 (pLysS) D3 *E. coli* cells (Novagen) following manufacture instructions. Activity of expressed proteins from at least two PCR independent clones of each gene was determined enzymatically using appropriate cofactors, [17-^{14}C]GA_1, [17-^{14}C]GA_4, [17-^{14}C]GA_9, [17-

^{14}C]GA_{12}, [17-^{14}C]GA_{20} and [17-^{14}C]GA_{53} (333 Bq, 100-150 pmol; purchased from Dr L. Mander, Australian National University, Canberra) as substrates, and 93 µl aliquots of cell lysates in a total 100 µl reaction volume, as described elsewhere (García-Martínez et al, 1997). Metabolic products were separated by HPLC, detected using an on-line radioactive monitor (Radioflow Detector LB 508, Berthold Technologies), and identified by their retention times compared to pure GAs.

Semiquantitative RT-PCR

Total RNA was isolated from different tomato organs: roots, young and old leaves, young and old internodes, flowers, and separated flower organs at anthesis. Unpollinated and pollinated ovaries at 0, 5, 10 and 20 days post anthesis (dpa) were also collected, and pericarp and seeds of 10- and 20-d-old pollinated ovaries separated for RNA extraction. RNA was treated with DNAse, according to manufacturer's protocol using an RNAeasy Plant Mini Kit (Quiagen, Courtaboeuf Cedex, France). Then, 2 µg of total RNA were reverse transcribed with a First-strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK) in 33 µl total volume reaction. PCRs were performed taking 1 µl aliquots of cDNA solution in a 50 µl total volume reaction containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1x reaction buffer, 1 U of NETZYME® DNA Polymerase (Fermentas Gmbh, Germany), and 1 µM of the appropriate pair of primers (Supplementary Table II. PCR conditions for amplification of *SlCPS*, *SlGA20ox1, -2* and *-3* and *SlGA3ox1* and *-2* consisted of initial denaturation at 94ºC for 2 min, followed by 32 cycles of 94ºC/ 30 sec, 57ºC/ 60 sec and 72ºC/ 60 sec, and finally 10 min extension at 72ºC. For amplification of *SlGA2ox1, -2, -3, 4* and *-5*, 31 cycles were used with annealing temperatures of 60ºC (*SlGA2ox1*) or 62ºC (*SlGA2ox2*, *-3*, *-4* and *-5*), and for *SlCPS* 33 cycles and 61ºC. In the case of *Actin* annealing temperature of 60ºC and 24 cycles were used. In all cases, the number of cycles was chosen to give amplified products within the linear synthesis reaction. 15 µl aliquots of PCR products were separated on 1% agarose gel electrophoresis. The spots were stained with ethidium bromide, visualized under UV using a GeneGenius Bio Imaging System (Syngene), captured with the GeneSnap program (Syngene) and quantified with the GeneTools software (Syngene). Expression was normalized using Actin as internal control, by comparing expression ratios to that of the specific tissues indicated in the Figure legends (set to 1.0).

The analyses were carried out in duplicate using biologically independent material, with similar results. Only data from one representative replicate are given under Results.

Phylogenetic analyses

Nucleotide sequences were translated into protein sequences using GeneDoc software (available at http://www.psc.edu/biomed/genedoc), and aligned with *MUSCLE* algorithm (freely available at http://www.drive5.com/muscle), using default parameters. Sequences were highly divergent, which led us to pursue the phylogenetic reconstruction using aminoacid rather than nucleotide sequences. The best model of protein evolution was selected based on the Akaike criterion (AIC) with the ProtTest online server (http://darwin.uvigo.es/software/prottest_server.html). The Jones-Taylor-Thornton evolutionary model (Jones et al, 1992) with evolution rates varying according to a Gamma distribution plus a class of invariant sites was judged optima in both phylogenetic analyses. In the 44 *GA2ox* dataset, the inferred parameters were α = 1.20 for the shape of the Gamma and p = 0.04 for the fraction of invariant sites, whereas for the 26-sequence dataset containing only tomato and Arabidopsis *GA2ox*, *GA3ox*, and *GA20ox* genes, the estimated values were α = 1.36 and p = 0.01. A maximum-likelihood tree was obtained with the *proml* implementation of the *PHYLIP* package version 3.66 (freely available at http://evolution.genetics.washington.edu/phylip.html), using the Hidden Markov Model method of inferring different rates of evolution at different amino acid positions (Felsenstein and Churchill, 1996), with six discrete classes for the rates and prior probabilities chosen according to the above estimated parameters. To identify ancestral and derivate clusters in the 44 *GA2ox* dataset analysis, the outgroup AtGA20ox1 was used to root the tree, whereas in the other analysis, the tree was left unrooted.

To assess the statistical significance of each internal branch, 1000 bootstrap pseudo-replicates of the protein alignments were generated using the *seqboot* implementation of the *PHYLIP* package version 3.66. The maximum-likelihood procedure was repeated for 100 of the pseudo-replicates (doing more pseudo-replicates would be computationally too intensive) and a consensus tree was obtained using the *consense* implementation of the same package, setting all parameters at their default values. The branch lengths of the tree were then estimated using the same maximum likelihood method. A node is judged statistically significant if it is supported by a high bootstrap proportion, though the appropriate threshold value depends on many factors (Hillis and Bull, 1993). To have an additional criterion for clade selection, we performed a weighted least-squares likelihood ratio test (Sanjuan and Wrobel, 2005) on each node using the *WeightLESS* implementation (freely available at http://www.iopan.gda.pl/~wrobel). To do that, we used the 1000 pseudo-replicates to estimate the involved parameters, the distance matrix

derived from the above Jones-Taylor-Thornton plus Gamma plus invariant class evolutionary model, and the above consensus tree.

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FIGURE LEGENDS

Figure 1. Fruit-set and growth inhibition of pollinated ovaries with inhibitors of GA biosynthesis and its reversal by GA_3 application. A) Effect of time of emasculation and removal of petals, anthers and style on number of seeds and fruit growth of pollinated ovaries (at d0). B) Effect of different doses of LAB 198999. C) Effect of different doses of paclobutrazol (PCB). Pollination was carried out at d0. LAB 198999 was applied directly to the ovary in 10 µl solution, two days after anthesis, after emasculation and petal removal. Paclobutrazol was applied to the roots in the nutrient solution, every two days, from 7 d before anthesis to 15 d after anthesis. GA_3 (2000 ng) was applied to the ovary in 10 µl solution at anthesis. Fruits were collected 20 d after treatment. Values are data from eight fruits ± SE. 100% of fruits developed in all treatments, except those marked with figures in brackets (number of fruits developed over eight treated).

Figure 2. Response of unpollinated tomato ovaries to GA_1, GA_3, GA_4, GA_{19} and GA_{20} (2000 ng per ovary) application. Fruits were collected 20 d after treatment, and values are means of eight fruits ± SE. Values of pollinated ovaries are also included as control. Poll., pollinated.

Figure 3. Distribution of transcript levels of *SlCPS*, *SlGA20ox1, -2* and *-3,* and *SlGA3ox1* and *-2* in different organs of tomato. Semiquantitative transcript analysis was carried out by RT-PCR, as described in Materials and Methods, using total RNA from roots (R), young leaves before flowering (YLp), young and old leaves from plants at flowering (YL, OL), young and old internodes (YI, OI), flowers (Fl), ovary at anthesis (O), stamens (St), sepals (Se), petals (Pe), and 20-d-old fruit (Fr). For each gene, figures below the blots mean normalized values of gene expression versus that of *Actin* (used as an internal control) (flower expression set at 1.0). Data come from a representative experiment out of two biological replicates with similar results.

Figure 4. Effect of pollination on transcript levels of *SlCPS*, *SlGA20ox1, -2* and *-3,* and *SlGA3ox1* and *-2* genes. Semiquantitative transcript analysis was carried out by RT-PCR, as described in Materials and Methods, using total RNA from unpollinated (d0, d5, d10 and d20) and pollinated (d5, d10 and d20) ovaries. E, entire ovary; P, pericarp; S, seeds. For each gene, figures below the blots mean normalized values of gene expression versus that of *Actin* (used as an internal control) (expression of entire 20-d-old pollinated fruits set at 1.0 for all the genes but for *SlGA3ox2*, where expression of d0 unpollinated ovaries was used as reference). Data come from a representative experiment out of two biological replicates with similar results.

Figure 5. Maximum likelihood phylogenetic tree based on comparison of GA2ox protein sequences from different species. The tree was rooted using AtGA20ox1 as outgroup and branch lengths are proportional to the estimated sequence divergence. Bootstrap values above 50% are

shown, whereas asterisks indicate statistical significance according to the weighted least-squares likelihood ration test (**, $P < 0.01$; *, $P < 0.05$). The three *GA2ox* subfamilies I, II, and II are indicated, and genes that have been shown to codify for multicatalytic enzymes are underlined. The five genes characterized in this study are shown in bold type. Accession numbers corresponding to the sequences in the tree are the following: AtGA20ox1, X83379; AtGA2ox1, AJ132435; AtGA2ox2, AJ132436, Thomas et al, 1999; AtGA2ox3, AJ322437, Thomas et al, 1999; AtGA2ox4, NM103695; AtGA2ox6, NM100121; AtGA2ox7, AC079284; AtGA2ox8, AL021960; CmGA2ox1, AJ315663; HvGA2ox4, AY551432; HvGA2ox5, AY551433; Ls2ox1, AB031206; Ls2ox2, AB031207; LtGA2ox1, DQ324114; NoGA2ox1, AY594291; NoGA2ox2, AY594292; NoGA2ox3, AY588978, Ubeda-Tomás et al, 2006; NsGA2ox1, Ay242858; NtGA2ox1, AB125232; NtGA2ox2, AB125233; OsGA2ox1, AB059416; OsGA2ox2, AB092484; OsGA2ox3, AB092485, Sakai et al, 2003; OsGA2ox4, AC132485; OsGA2ox5, BAC10398; OsGA2ox6, AL662958; PcGA2ox1, AJ132438, Thomas et al, 1999; PsGA2ox1, AF056935, Martin et al, 1999; PsGA2ox2, AF100954; PttGA2ox1, AY392094; RpGA2ox1, DQ641499; SlGA2ox1, EF441351; SlGA2ox2, EF441352; SlGA2ox3, EF441353; SlGA2ox4, EF441354; SlGA2ox5, EF441355; SoGA2ox1, AF506281, Lee and Zeevaart, 2002; SoGA2ox2, AF506282; SoGA2ox3, AY935713; VaGA2oxA1, AB181372; VaGA2oxA2, AB181373; VaGA2oxB1, AB181374. VaGA2oxB2, AB181375; VaGA2oxB3, AB181376; VaGA2oxC1, AB181377.

Figure 6. Distribution of transcript levels of *SlGA2ox1, -2, -3,-4* and *-5* in different organs of tomato. Semiquantitative transcript analysis was carried out by RT-PCR, as described in Materials and Methods, using total RNA from roots (R), young leaves before flowering (YLp), young and old leaves from flowering plants (YL, OL), young and old internodes (YI, OI), flowers (Fl), ovary at anthesis (O), stamens (St), sepals (Se), petals (Pe), and 20-d-old fruit (Fr). For each gene, figures below the blots mean normalized values of gene expression versus that of *Actin* (used as an internal control) (flower expression set at 1.0 for all the genes, except for *SlGA2ox5*, where expression of YI was used as reference). Data come from a representative experiment out of two biological replicates with similar results.

Figure 7. Effect of pollination on transcript levels of *SlGA2ox1, -2, -3,-4* and *-5* genes. Semiquantitative transcript analysis was carried out by RT-PCR, as described in Materials and Methods, using total RNA from unpollinated (d0, d5, d10 and d20) and pollinated (d5, d10 and d20) ovaries. E, entire ovary; P, pericarp; S, seeds. For each gene, figures below the blots mean normalized values of gene expression versus that of *Actin* (used as an internal control) (expression of unpollinated d5 and d10 ovaries set at 1.0 for *SlGA2ox1*, *-3* and *-4*, of pollinated

d5 ovaries for *SlGA2ox2*, and seeds from d10 pollinated ovaries for *SlGA2ox5*). Data come from a representative experiment out of two biological replicates with similar results.

Supplementary Fig. 1. Scheme of GA metabolic pathways.

Supplementary Fig. 2. Maximum likelihood phylogenetic tree based on comparison of *GA20ox*, *GA3ox* and *GA2ox* protein sequences from Arabidopsis and tomato. Branch lengths are proportional to sequence divergence. Bootstrap values above 50% are shown, whereas asterisks indicate statistical significance according to the weighted least-squares likelihood ration test (**, $P < 0.01$; *, $P < 0.05$). The five genes characterized in this study are shown in bold type. Accession numbers corresponding to the sequences in the tree are the following: AtGA20ox1, X83379; AtGA20ox2, X83380; AtGA20ox3, X83381; AtGA20ox4, NM104778; AtGA20ox5, DQ056484; AtGA3ox1, L37126; AtGA3ox2, AF070937; AtGA3ox3, NM118289; AtGA3ox4, NM106682; AtGA2ox1, AJ132435; AtGA2ox2, AJ132436; AtGA2ox3, AJ322437; AtGA2ox4, NM103695; AtGA2ox6, NM100121; AtGA2ox7, AC079284; AtGA2ox8, AL021960; SlGA20ox1, AF049898; SlGA20ox2, AF049899; SlGA20ox3, AF049900; SlGA3ox1, AB010991; SlGA3ox2, AB010992; SlGA2ox1, EF441351; SlGA2ox2, EF441352; SlGA2ox3, EF441353; SlGA2ox4, EF441354; SlGA2ox5, EF441355**.**

Supplementary Fig. 3. HPLC radioactivity traces of products of [^{14}C]GA_{12}, [^{14}C]GA_9, [^{14}C]GA_4, [^{14}C]GA_{53}, [^{14}C]GA_{20}, and [^{14}C]GA_1, incubated with heterologous expression products of *SlGA2ox1*, *-3*, *-4* and *-5* after 2 h incubation at 30ºC.

Supplementary Fig. 4. Alignment of amino acid sequences corresponding to GA 2-oxidases from groups I, II and III used to construct the phylogenetic tree of Fig. 5. ○, Fe^{2+} binding residues; ↑, 2-cetoglutarate binding residues; ●, amino acids conserved in groups I and II.

Table I. Effect of LAB 198999 on weight and endogenous GA content (ng g fresh weight^{-1}) of pollinated fruits. Fruits were collected 10 d after pollination (8 and a half days after 1 mM LAB 198999 application). Fruit weight data are means of 26 (-LAB) and 31 (+LAB) fruits, and GA data from three biological replicates (aliquots of about 5 g each) ± SE.

	Weight (g fruit^{-1})	GA_1	GA_8	GA_{19}	GA_{20}	GA_{29}	GA_{44}	GA_{53}
- LAB	1.04 ± 0.06	2.7 ± 0.8	31.4 ± 0.3	8.7 ± 0.4	23.5 ± 0.6	18.5 ± 2.6	2.7 ± 0.1	< 0.1
+ LAB	0.47 ± 0.04	1.2 ± 0.0	3.3 ± 0.9	30.5 ± 0.9	50.5 ± 6.1	7.0 ± 1.2	3.4 ± 0.2	3.1 ± 1.6

Supplementary Table I. Primer sequences used to amplify full-length cDNA clones of *SlGA2ox1*, *-2*, *-3*, *-4* and *-5*.

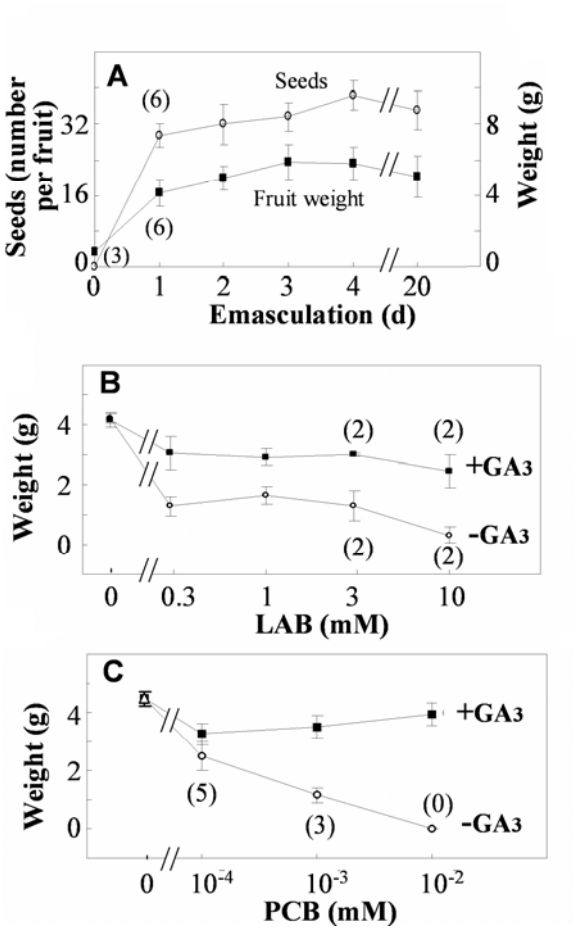
Gene	Sense primer	Antisense primer
SlGA2ox1	5'- CCTCAACTTCCAACATGGTTTCTG -3'	*Not* I-d(T)$_{18}$
SlGA2ox2	5'- CACTTACCAAAATCAACCATGGTG -3'	5'- CCCACAATGAGCATCTTGACAACC -3'
SlGA2ox3	5'- CATTCGATTAATTATGGTAGTAGC -3'	*Not* I-d(T)$_{18}$
SlGA2ox4	5'- ACAAACAACAATTTCTACCAAAGT -3'	*Not* I-d(T)$_{18}$
SlGA2ox5	5'-CACCAGCAACAGTTGTAACAAGA-3'	5'- GATCCAAACATGGTATATTTGCGGAGG -3'

Not I-d(T)$_{18}$ (from Amersham Biosciences)

Supplementary Table II. Primer sequences used for semiquantitative RT-PCR analysis of diverse GA metabolism genes of tomato. *SlCPS* (AB015675), *SlGA20ox1* (AF049898), *SlGA20ox2* (AF049899), *SlGA20ox3* (AF049900), *SlGA3ox1* (AB010991), *SlGA3ox2* (AB010992), *SlGA2ox1, -2, -3, -4* and -5, and *SlACT (Actin*) (AB199316).

Gene	Sense	Antisense
SlCPS	5'-GGAAAATTGGCTACTGACGGTAGG-3'	5'-GGCATCCAATTCGGAAGCA-3'
SlGA20ox1	5'-GGAGCTCGCCTTAGGAACG-3'	5'-GTAGAAGCTAAGAGAACGTGTACACG-3'
SlGA20ox2	5'-CAACGTCTCAGGACTACAAGTTTTC-3'	5'-AGGCTAAGGTCTTGATCTACATTGG-3'
SlGA20ox3	5'-ACACCATCACTCCAAATTTCAAC-3'	5'-CCATGAGGTTCCATTTCTATGTC-3'
SlGA3ox1	5'-GTGAAACCAAAGAAGGATGTG-3'	5'-GCATCAGTAAATCCATTTAAAGGGA-3'
SlGA3ox2	5'-GTAACGGTTCCTCTCCTTCGC-3'	5'-ACCTACTTGGACGCCACTTTG-3'
SlGA2ox1	5'-ACCCCACATCTTCTCCATCAT G-3'	5'-ACATGTTTCATCAAGGGTTCGAT-3'
SlGA2ox2	5'-GCCATGCTCAGAGATTGAACGATTG-3'	5'-CCCACAATGAGCATCTTGACAACC-3'
SlGA2ox3	5'-GCTAACAATCCTTCGATCAAATGACG-3'	5'-GCATAATGCATACACCTCCAAGGCC-3'
SlGA2ox4	5'-GTCGATTTTAAGATCCAACAACACTTCCGGT-3'	5'-CATCATTTTCAACATAACGAGTCCTTCC-3'
SlGA2ox5	5'-ATATCGGTATTAAGATCCAACAACACATCC-3'	5'-GATCCAAACATGGTATATTTGCGGAGG-3'
SlACT	5'-ATGTATGTTGCCATCCAGGCTG-3'	5'-CCTTGCTCATCCTATCAGCAGCAATACC-3'

1 Fig. 1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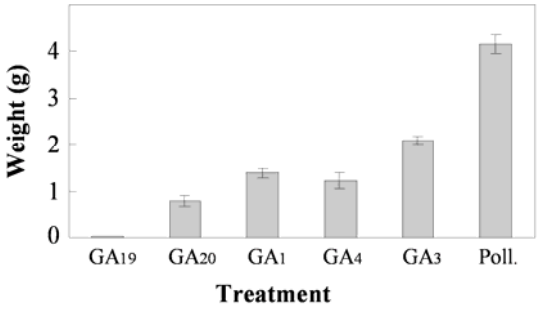
Seeds
Fruit weight
Seeds (number per fruit)
Weight (g)
Emasculation (d)
B
+GA3
-GA3
Weight (g)
LAB (mM)
C
+GA3
-GA3
Weight (g)
PCB (mM)

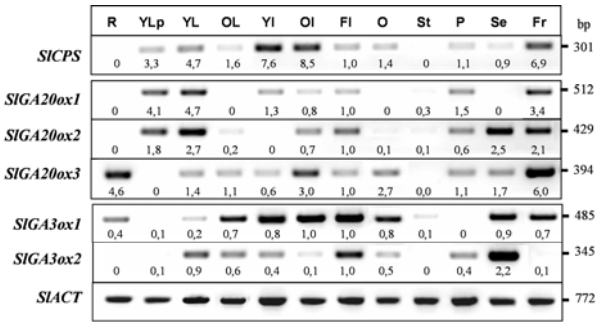
2
3

1 Fig. 2



2
3
4

Fig. 3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301
SlGA20ox1
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512
SlGA20ox2
0 1,8 2,7 0,2 0 0,7 1,0 0,1 0,1 0,6 2,5 2,1
429
SlGA20ox3
4,6 0 1,4 1,1 0,6 3,0 1,0 2,7 0,0 1,1 1,7 6,0
394
SlGA3ox1
0,4 0,1 0,2 0,7 0,8 1,0 1,0 0,8 0,1 0 0,9 0,7
485
SlGA3ox2
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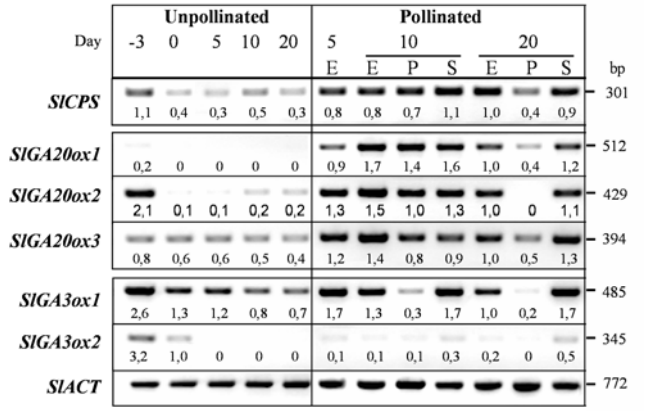
345
SlACT
772

1 Fig. 4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Pollinated
Day
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E E P S E P S
bp
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301
SlGA20ox1
0,2 0 0 0 0 0,9 1,7 1,4 1,6 1,0 0,4 1,2
512
SlGA20ox2
2,1 0,1 0,1 0,2 0,2 1,3 1,5 1,0 1,3 1,0 0 1,1
429
SlGA20ox3
0,8 0,6 0,6 0,5 0,4 1,2 1,4 0,8 0,9 1,0 0,5 1,3
394
SlGA3ox1
2,6 1,3 1,2 0,8 0,7 1,7 1,3 0,3 1,7 1,0 0,2 1,7
485
SlGA3ox2
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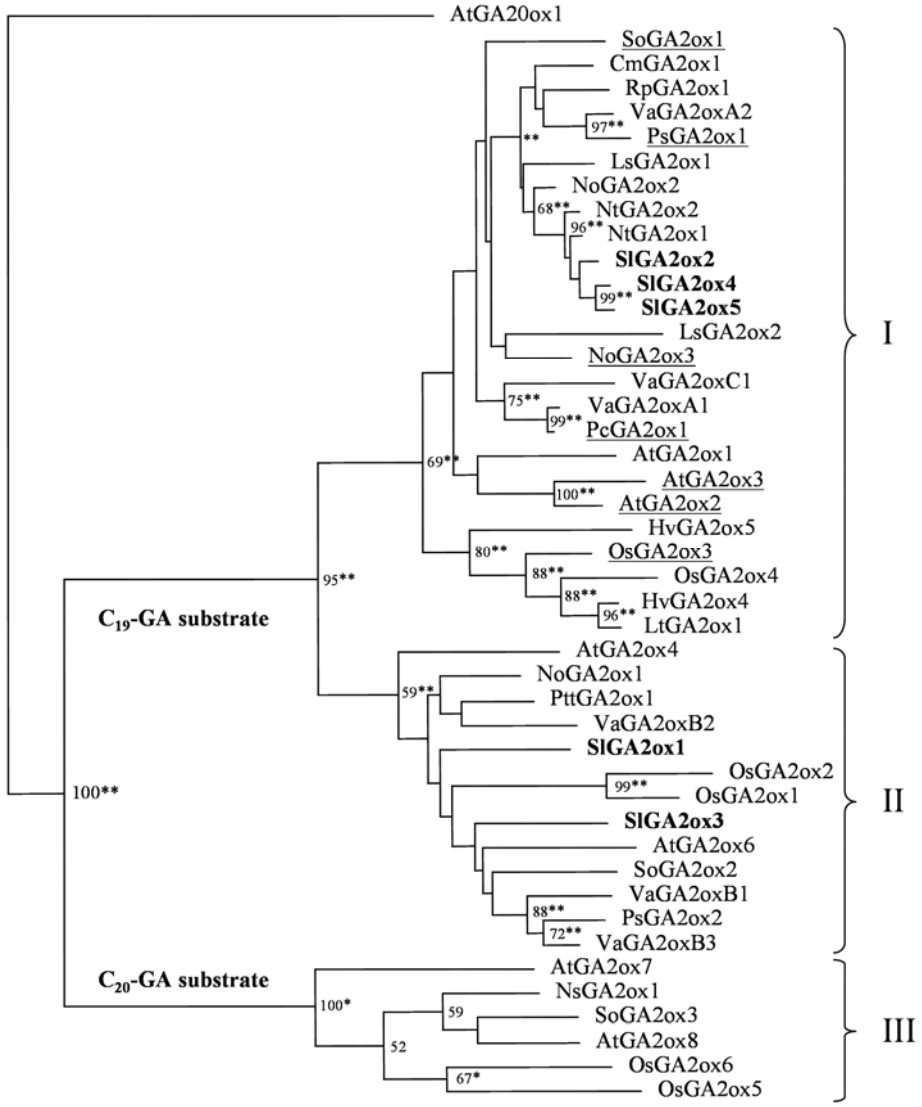
345
SlACT
772

2
3

1 Fig. 5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SoGA2ox1
CmGA2ox1
RpGA2ox1
VaGA2oxA2
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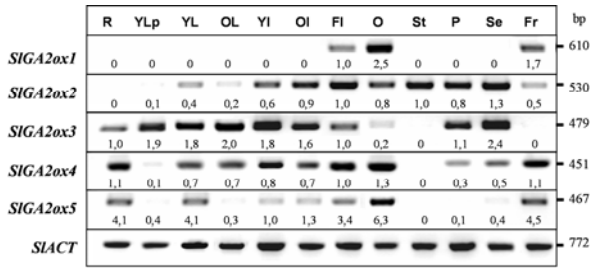
PsGA2ox1
**
LsGA2ox1
NoGA2ox2
68**
NtGA2ox2
96**
NtGA2ox1
SlGA2ox2
99**
SlGA2ox4
SlGA2ox5
LsGA2ox2
NoGA2ox3
VaGA2oxC1
75**
VaGA2oxA1
99**
PcGA2ox1
69**
AtGA2ox1
100**
AtGA2ox3
AtGA2ox2
HvGA2ox5
80**
OsGA2ox3
88**
OsGA2ox4
88**
HvGA2ox4
96**
LtGA2ox1
95**
C19-GA substrate
AtGA2ox4
NoGA2ox1
59**
PttGA2ox1
VaGA2oxB2
SlGA2ox1
OsGA2ox2
99**
OsGA2ox1
100**
SlGA2ox3
AtGA2ox6
SoGA2ox2
VaGA2oxB1
88**
PsGA2ox2
72**
VaGA2oxB3
AtGA2ox7
C20-GA substrate
NsGA2ox1
100*
59
SoGA2ox3
AtGA2ox8
52
OsGA2ox6
67*
OsGA2ox5
I
II
III

2
3

Fig. 6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530
SlGA2ox3
1,0 1,9 1,8 2,0 1,8 1,6 1,0 0,2 0 1,1 2,4 0
479
SlGA2ox4
1,1 0,1 0,7 0,7 0,8 0,7 1,0 1,3 0 0,3 0,5 1,1
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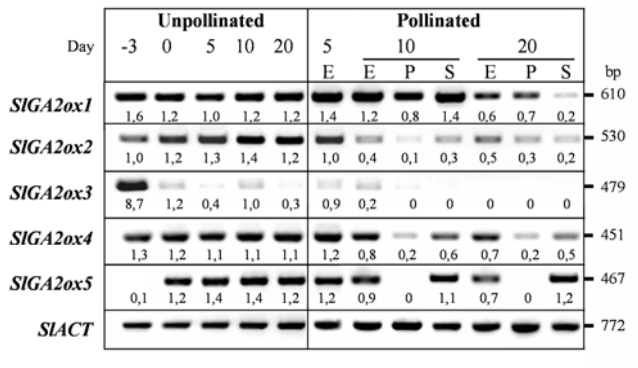
467
SlACT
772

Fig. 7


Unpollinated
Pollinated
Day
-3
0
5
10
20
5
10
20
E
E
P
S
E
P
S
bp
SlGA2ox1
1,6 1,2 1,0 1,2 1,2 1,4 1,2 0,8 1,4 0,6 0,7 0,2
610
SlGA2ox2
1,0 1,2 1,3 1,4 1,2 1,0 0,4 0,1 0,3 0,5 0,3 0,2
530
SlGA2ox3
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479
SlGA2ox4
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451
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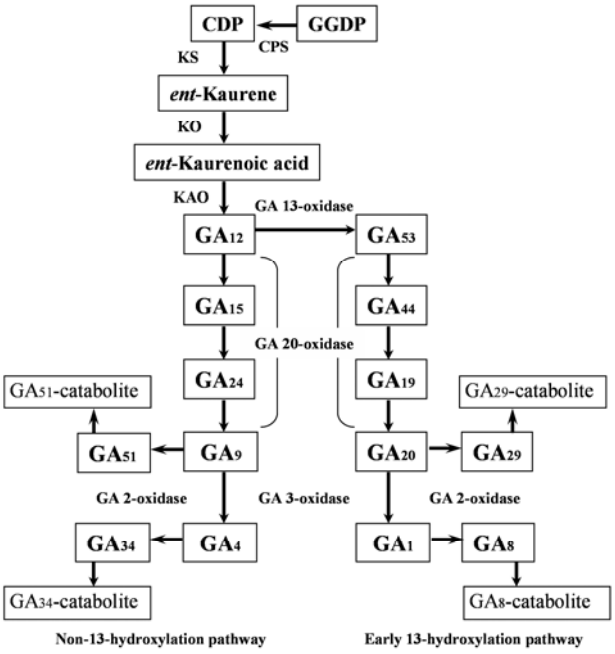
467
SlACT
772

Suplem. Fig. 1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CPS
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KO
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KAO
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GA 20-oxidase
GA24
GA19
GA51-catabolite
GA29-catabolite
GA51
GA9
GA20
GA29
GA 2-oxidase
GA 3-oxidase
GA 2-oxidase
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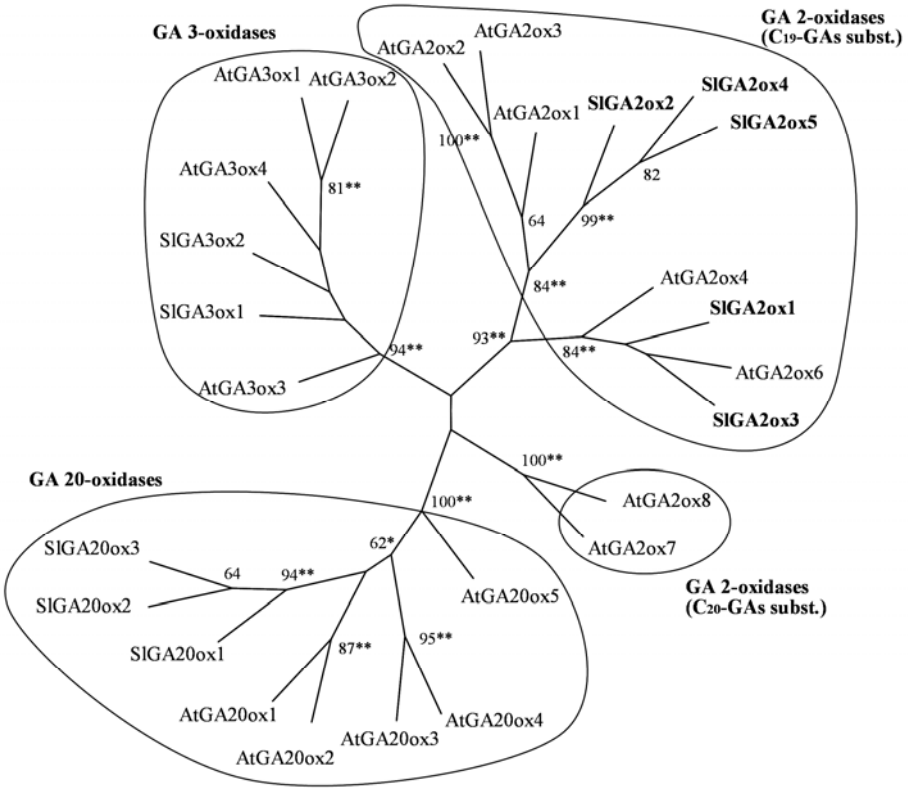
GA8-catabolite
Non-13-hydroxylation pathway
Early 13-hydroxylation pathway

Suplem. Fig. 2


GA 3-oxidases
GA 2-oxidases
(C19-GAs subst.)
AtGA3ox1
AtGA3ox2
AtGA3ox4
SlGA3ox2
SlGA3ox1
AtGA3ox3
AtGA2ox2
AtGA2ox3
AtGA2ox1
SlGA2ox2
SlGA2ox4
SlGA2ox5
AtGA2ox4
SlGA2ox1
AtGA2ox6
SlGA2ox3
100**
81**
64
99**
82
84**
93**
84**
94**
100**
GA 20-oxidases
100**
AtGA2ox8
AtGA2ox7
62*
SlGA20ox3
64
94**
SlGA20ox2
SlGA20ox1
AtGA20ox5
87**
95**
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AtGA20ox3
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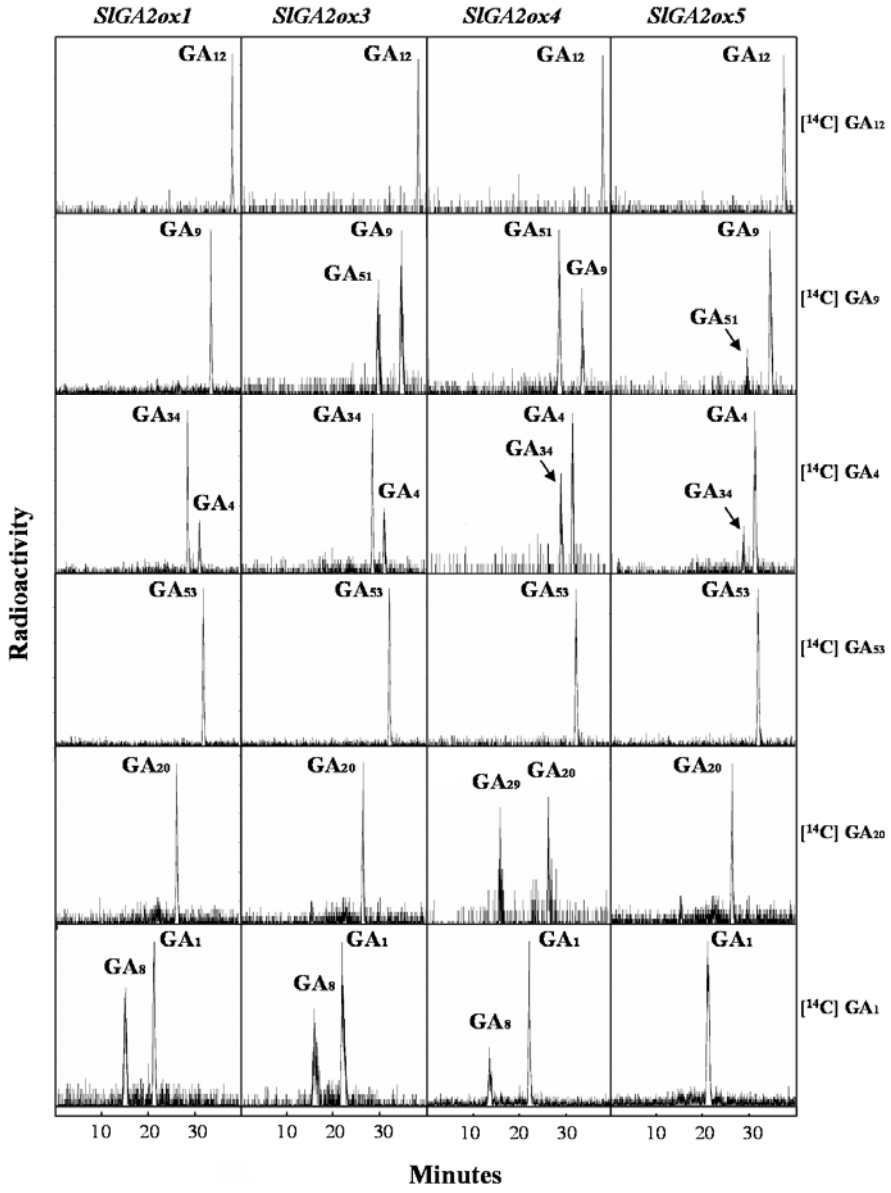
GA 2-oxidases
(C20-GAs subst.)

Suplem. Fig. 3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SlGA2ox3
SlGA2ox4
SlGA2ox5
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[14C] GA9
[14C] GA4
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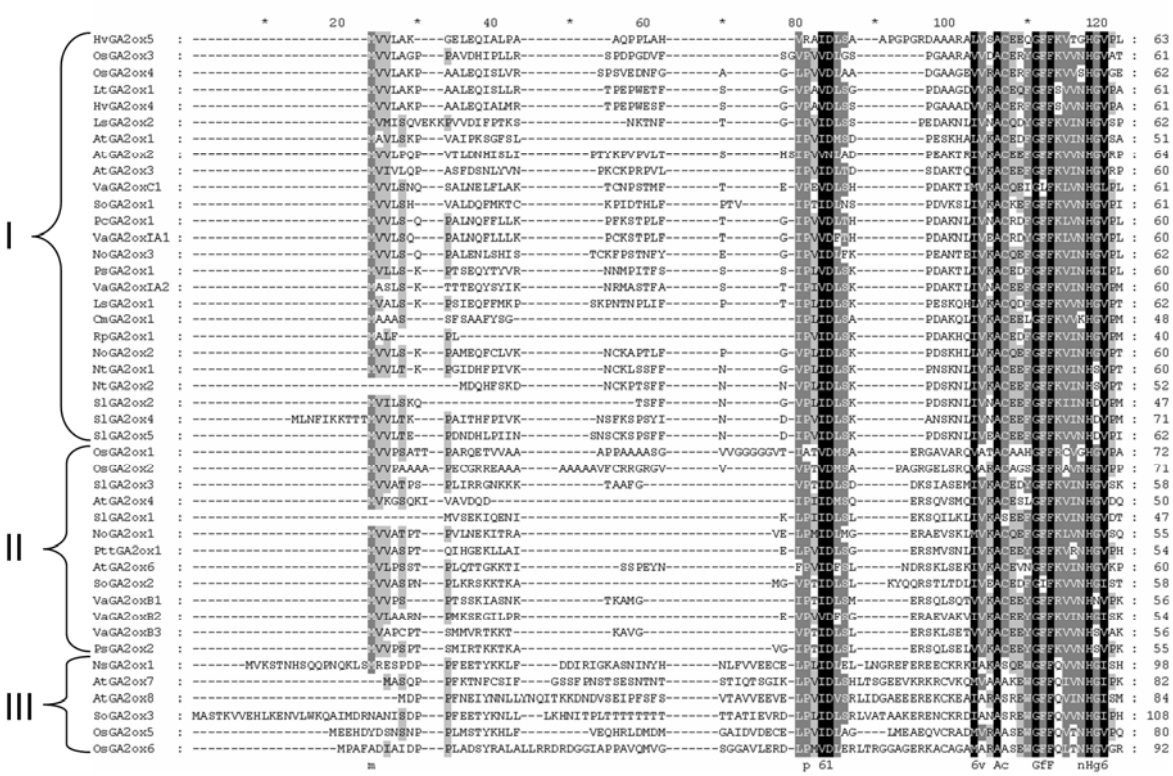
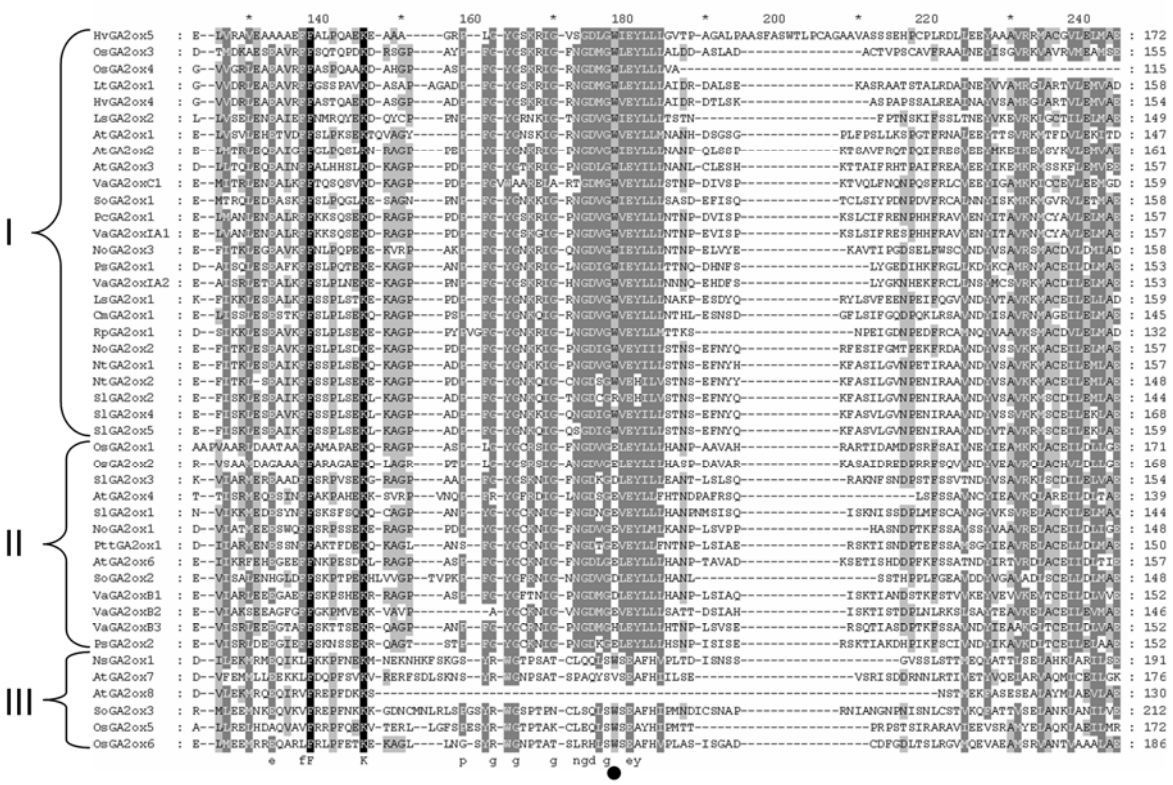
[14C] GA1
Radioactivity
Minutes
10
20
30

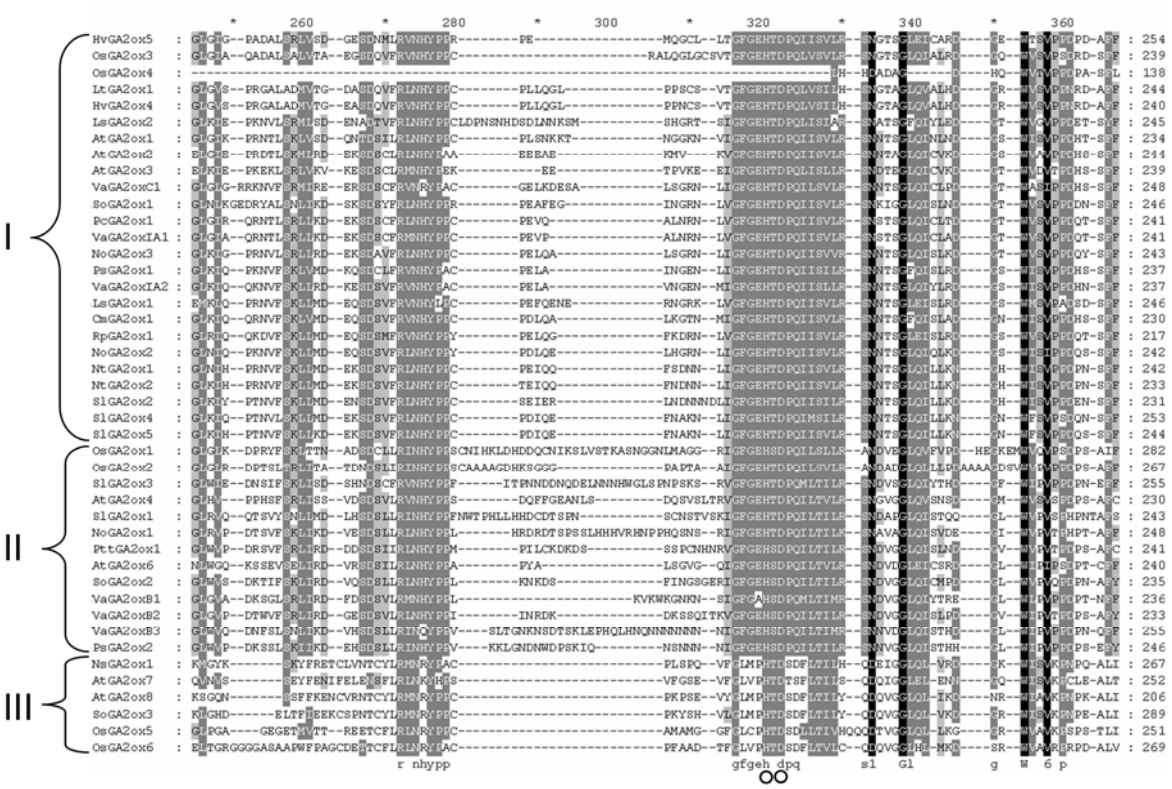
Suplem. Fig. 4[a]



Suplem. Fig. 4B



Suplem. Fig. 4C



Suplem. Fig. 4D