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4 **Running title:** Regulation of *SlGA20ox1* expression

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**Hormonal regulation of tomato *gibberellin 20-oxidase1* expressed in
*Arabidopsis***

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Summary

Gibberellin 20-oxidases, enzymes of gibberellin (GA) biosynthesis, play an important role in (GA) homeostasis. To investigate the regulation of tomato *SlGA20ox1* expression a genomic clone was isolated, its promoter transcriptionally fused to the GUS reporter gene and the construct used to transform Arabidopsis. Expression was found in diverse vegetative (leaves and roots) and reproductive (flowers) organs. GUS staining was also localized in the columella of secondary roots. GA negative feed-back regulation of *SlGA20ox1:GUS* was shown to be active both in tomato and in transformed Arabidopsis. Auxin (indol-3-acetic acid, 2,4-dichlorophenoxyacetic acid and naphthaleneacetic acid), triiodobenzoic acid (an inhibitor of auxin transport) and benzyladenine (a cytokinin) treatment induced *SlGA20ox1:GUS* expression associated with increased auxin content and/or signalling, detected using *DR5:GUS* expression as a marker. Interestingly, *SlGA20ox:GUS* expression was induced by auxin and root excision in the hypocotyl, an organ not showing GUS staining in control seedlings. In etiolated seedlings, *SlGA20ox1:GUS* expression occurred in the elongating hypocotyl region of etiolated seedlings and was down-regulated upon transfer to light associated with decrease of growth rate elongation. Our results show that feed-back, auxin and light regulation of *SlGA20ox1* expression depends on DNA elements contained within the first 834 bp of the 5' upstream promoter region. Putative DNA regulatory sequences involved in negative feed-back regulation and auxin response were identified in that promoter.

Keywords: Arabidopsis; Auxin; Gibberellin 20-oxidase; Gibberellins; Gene promoter; *Solanum lycopersicum*; Tomato

1 **Abbreviations:** d, day; LD, long day; NAA, 1-naphthalenacetic acid; PAC,
2 paclobutrazol; TIBA, 2,3,5-triiodobenzoic acid.

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1 **Introduction**

2 The gibberellins (GA) constitute a group of plant hormones which regulate
3 diverse developmental processes such as germination, stem elongation, flowering and
4 fruit development. GAs are synthesized using three kinds of enzymes (Yamaguchi,
5 2008). The first GA biosynthetic reactions (catalyzed by cyclases) produce *ent*-kaurene
6 in the plastids. *Ent*-kaurene is then metabolized by membrane-associated P450-
7 dependent monooxygenases to GA₁₂, which is C-13-hydroxylated to produce GA₅₃.
8 GA₁₂ and GA₅₃ are converted by cytoplasmic dioxygenases to active GAs following
9 two parallel pathways: the early-13-hydroxylation pathway (leading to GA₁), and the
10 non-13-hydroxylation pathway (leading to GA₄). The last reactions are catalyzed by GA
11 20- and GA 3-oxidases. Active GAs and their precursors can be inactivated by GA 2-
12 oxidases and other catabolic enzymes. Most of the genes encoding the enzymes
13 catalyzing the diverse GA metabolic steps have been cloned and characterized, and it
14 has been found that the three groups of dioxygenases are encoded by small multigene
15 families which are expressed differentially in diverse organs (Hedden and Phillips,
16 2000). The overexpression and downregulation of *GA20ox* in diverse species modified
17 the levels of active GAs associated with increase or reduction of plant height (e.g. Coles
18 et al., 1999; Carrera et al., 2000; Vidal et al., 2001; Fagoaga et al., 2007). This shows
19 that the regulation of *GA20ox* expression plays an important role in GA homeostasis.

20 The genes of GA metabolism are regulated through development (Phillips et al.,
21 1995; Garcia-Martinez et al., 1997; Silverstone et al., 1997; Rebers et al., 1999; Ayele
22 et al., 2006; Mitchum et al., 2006) and by environmental factors (Kamiya and Garcia-
23 Martinez, 1999; Vidal et al., 2003; Stavang et al., 2005). Transcript levels of many GA-
24 dioxygenases are also subjected to negative (*GA20ox* and *GA3ox*) and positive (*GA2ox*)
25 feed-back regulation by the GA signalling pathway (Yamaguchi, 2008). In addition to

GA other hormones, mainly auxins, also affect GA biosynthesis and catabolism (Ross et al., 202; Frigerio et al., 2006; Weiss et al., 2007; Desgagné-Penix and Sponsel, 2008; Serrani et al., 2008).

It has been shown that fruit set and growth in tomato depend on GAs (Fos et al., 2000; Serrani et al., 2007). The tomato parthenocarpic mutants *pat-2* and *pat* accumulate GA₂₀ (the immediate precursor of the active GA₁) (Fos et al., 2000; Olimpieri et al., 2007), due to higher GA20ox activity, at least in the case of *pat* (Olimpieri et al., 2007). The importance of GA20ox activity in tomato fruit-set is also shown by the significant increase of *SIGA20ox1* transcript levels upon pollination (Serrani et al., 2007) and auxin-induced fruit-set (Serrani et al., 2008). Therefore, the availability of transgenic plants of tomato expressing *SIGA20ox1::GUS* would be of great interest to investigate the role of different factors in relation to GA metabolism and fruit-set and growth. However, given the relative long time and effort to produce those plants, it may be convenient to test first the construct in a species easier to transform and manipulate such Arabidopsis. This could also unveil some aspects of *SIGA20ox1* regulation.

In this work we have isolated a genomic clone of *SIGA20ox1* from tomato and the regulation of its expression was investigated using Arabidopsis plants transformed with a *SIGA20ox1::GUS* construct. The results show that *SIGA20ox1::GUS* was actively expressed in diverse vegetative and reproductive organs and that negative feed-back (as also occurs in tomato), as well as auxin, cytokinin and light regulation of *SIGA20ox1::GUS* were operative. Unexpected expression in the columella of secondary roots, and in the hypocotyls upon auxin application was also found. Putative DNA regulatory sequences involved in negative feed-back regulation and auxin response were identified in the proximal region of the *SIGA20ox1* promoter gene.

Materials and methods

Plant material and hormone application

Sterilized Arabidopsis seeds (Columbia ecotype Col-0 and transgenic *DR5:GUS* (obtained from Dr. T. Guilfoyle, University of Missouri, USA) plated in Petri dishes containing 4.3 g L⁻¹ Murashige and Skoog (MS) salts, 1 g L⁻¹ MES, 1% sucrose and 1% agar 1%, pH 5.7 were cultured at 4 °C in the dark for 3 d, then placed on horizontal or vertical position for 7 to 21 d under long day (LD) conditions (16 h light/ 8 h dark at 25 °C). Arabidopsis plants were also grown in the greenhouse, under long day (LD) conditions, in pots with a mixture of peat:vermiculite:perlite (3:3:1).

Different plant growth substances [GA₃ (Sigma), Paclobutrazol (Duchefa), indole-3-acetic acid (IAA) (Duchefa), 1-naphthaleneacetic acid (NAA) (Duchefa), 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma), 2,3,5-triiodobenzoic acid (TIBA) (Sigma) and benzyl adenine (BA) (Duchefa)] were added to the autoclaved medium using 70% ethanol stock solutions before pouring into the Petri dishes. Equal volume of ethanol was added to control plates.

DNA and RNA extraction

For isolation of the *SlGA20ox1* gene, genomic DNA was extracted from 0.5-1 g of young leaves of tomato (*Solanum lycopersicum* L. cv Madrigal) as described by Dellaporta et al. (1983). Total RNA from 100 mg material of tomato seedlings was extracted using the “RNeasy[®] Plant MiniKit” (Qiagen).

Semiquantitative RT-PCR

Three µg of total tomato RNA were subjected to reverse transcription using the “First-Strand cDNA Synthesis Kit” (Amersham Biosciences), according to manufacturer’s instructions. PCR reactions were carried out in total 50 µL volume

containing 1xPCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 1 µM primers, 10 ng cDNA, and 2.6 U of “Expand High Fidelity” DNA polymerase (Roche) using the following thermocycling conditions: 94°C/5 min, 30 cycles of 94 °C/45 s, 58 °C/45 s and 72 °C/1 min, and 72 °C for 7 min. RT-PCR products were separated on 1% agarose gels and stained with ethidium bromide. Primers used to amplify *SIGA20ox1* (AF049898) were 5'-GGAGCTCGCCTTAGGAACG-3' (forward) and 5'-GTAGAAGCTAAGAGAACGTGTACACG-3' (reverse) [designed to prevent the amplification of other highly similar *LeGA20ox* genes; Rebers et al. (1999)]. Primers for *Actin* (U60482) (internal control) amplification were 5'-ATGTATGTTGCCATCCAGGCTG-3' (forward) and 5'-CCTTGCTCATCCTATCAGCAATACC-3' (reverse). The experiments were carried out using three biological replicates.

Isolation of a genomic *SIGA20ox1* clone and its promoter

A *SIGA20ox1* genomic clone corresponding to the coding sequence was isolated by PCR using a 50 µL volume reaction containing 1xPCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 1 µM primers, 0.1 µg genomic DNA, and 2.6 U of “Expand High Fidelity” DNA polymerase (Roche). The thermocycling conditions used were an initial denaturation at 94°C/2 min followed by 40 cycles of 94 °C/1 min, 55 °C/1.5 min and 72 °C/1 min, and a final extension at 72 °C for 10 min. The primers used to amplify the *SIGA20ox1* genomic sequence were 5'-ATGGCTATTGATTGTATGATCAC-3' (forward) and 5'-AGCTTGTGTAGTAGTGTGTG-3' (reverse) (using published information for cDNA sequence of *SIGA20ox1*; AF049898). PCR fragments were separated on 1% agarose/EtBr gel electrophoresis, cloned into pGEM-T Easy vector system I (Promega) and sequenced. Sequence data of the *SIGA20ox1* genomic clone have been deposited at the GenBank under the accession number EU043161.

A tomato *SlGA20ox1* promoter fragment of 834 bp upstream of the first coding ATG was isolated from genomic DNA using the “Universal GenomeWalker™ Kit” (Clontech), following manufacturer’s instructions. Briefly, after DNA digestion and ligation to adaptors, two rounds of PCR were carried out in 50 µl volume reaction containing 1xPCR reaction buffer, 1.1 mM magnesium acetate, 0.2 mM dNTPs, 0.2 µM primers, 1 µL of digested or amplified DNA, and 1x of “Advantage Genomic polymerase mix” (Clontech). The primers used were adaptor-related primers (provided by the manufacturer) and *SlGA20ox1* specific primers (5’-TAAGGCACAAGGCTTCTCGTGGTCAG-3’ and 5’-CCATTGTTGGGATCATACAATCAATAGCC-3’ for the first and second rounds, respectively). Thermocycling conditions used during the first PCR reaction were: 7 cycles of 94 °C/2 s and 72 °C/3 min, 37 cycles of 94 °C/2 s and 67 °C/3 min, and a final extension of 7 min at 67 °C. For the second PCR similar conditions were used, but in this case the number of cycles was 5 and 25 for the first and second temperature profiles, respectively. The PCR fragments were separated on 0.8 % agarose/EtBr gel electrophoresis, cloned into pGEM-T Easy vector system I (Promega) and sequenced.

***SlGA20ox1*:GUS construct preparation and isolation of transgenic Arabidopsis**

The 834 bp fragment of promoter cloned into pGEM-T Easy vector was amplified by PCR using the primers 5’-GGATCCCGACGGCCCGGGCTGG-3’ (forward) and 5’- CTGCAGATTATAATTGCATGCAAAGAC-3’ (reverse) (underlined sequences correspond to the *Bam*HI and *Pst*I restriction sites, respectively), and directionally cloned into the *Bam*HI/*Pst*I sites of the pCAMBIA 1381Z (Cambia, Canberra) binary vector to produce the *SlGA20ox1*(834pb):GUS fusion reporter, containing the promoter (834 bp) plus the first AT of the *SlGA20ox1* coding region (Fig. 1B).

The *SlGA20ox1* promoter construct was used to transform Arabidopsis Col-0 using *Agrobacterium tumefaciens* strain C58C1:pGV3101 and the dipping method (Clough and Bent, 1998). Transgenic seedlings were identified by their resistance to hygromycin (20 $\mu\text{g mL}^{-1}$). Homozygous *SlGA20ox1(834bp):GUS* lines with a single insertion (3:1 segregation of hygromycin resistance: hygromycin sensitive seedlings in T_2) were isolated.

GUS staining and GUS activity

Histochemical GUS assays were performed as described by Jefferson et al. (1987) with minor modifications. Tissues were prefixed in 90% acetone for 20 min, washed in water and vacuum infiltrated for 15 min in staining solution: 50 mM sodium phosphate buffer pH 7.2, 10 mM ferricyanide, 10 mM ferrocyanide, 0.2% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronidase (Duchefa). Incubation was carried out at 37 °C until blue coloration appeared (usually between 16-24 h). Chlorophyll in green tissues was cleared by series of 20-35-50-70% (v/v) ethanol solutions. Images were taken under a dissecting microscope (Nikon SMZ800) or under optic microscope (Nikon Eclipse E600).

For GUS activity determination, 50 mg of entire seedlings were ground in 150 mL extraction solution (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 10 mM β -mercaptoethanol, 0.1% Triton X-100, and 0.1% [w/v] sarcosyl) in a microcentrifuge tube. Cell debris was removed from the homogenate by centrifugation at 12,000 rpm for 10 min at 4 °C, and 5 μL of the supernatant were mixed with 500 μL of GUS assay buffer (1 mM 4-methylumbelliferyl β -D-glucuronide (Duchefa) in extraction solution) and incubated at 37 °C for 40 min. Aliquots of 100 μL were taken at different times and the reaction was stopped by adding 0.9 mL of 0.2 M Na_2CO_3 . Fluorescence

was determined with a fluorometer (Perkin Elmer LS50B) using 365 nm (excitation) and 455 nm (emission) wavelengths.

Results

Isolation of a *SIGA20ox1* genomic clone and expression of *SIGA20ox1:GUS* in *Arabidopsis*

A genomic gene clone containing the *SIGA20ox1* gene was isolated from tomato. It was composed of three exons (E1, 536 bp; E2, 322 bp; and E3, 279 bp) and two introns (I1, 155 bp; and I2, 168 bp) (Fig. 1A). The proximal 834 bp promoter region immediately upstream of the start codon ATG (Fig. 1C) was fused to the GUS gene, and the construct *SIGA20ox1(834bp):GUS* (Fig. 1B) used to obtain two homozygous transgenic lines (lines A4 and L2).

GUS expression in 7 and 21 d-old seedlings of transgenic lines A4 and L2 (*SIGA20ox1:GUS*) was observed in cotyledons (vascular vessels and stomata) (Fig. 2A, 2B, and 2D), young and expanded rosette leaves (vascular vessels, stomata, trichomes and hydrotodes) (Fig. 2A, 2C, 2H, and 2K), and roots (vascular vessels) (Fig. 2A and 2F). No expression was detected in the hypocotyl (Fig. 2A and 2E) or in the apex of the primary root (Fig. 2G). GUS expression was observed in the columella cells of the secondary roots from early after emergence (Fig. 2O, 2P and 2Q) until they were almost 40 mm long (Fig. 2R). Since the columella is claimed to be the site of gravity detection, experiments were carried out to see whether growing seedlings with the primary root in horizontal direction (as occurs during the early stages of secondary root development) would induce GUS expression in the columella. However, no effect of root position on GUS expression in the apex of the primary root was observed (data not presented). This was in contrast with the lateral distribution of GUS staining in the root apex of

DR5:GUS seedlings, used as a positive control for IAA redistribution, following gravitropic stimulus (data not presented).

GUS expression was quite intensive in flowers at anthesis, localized in sepal and petal veins (Fig. 3A), stamen filaments (Fig. 3B), style and stigma of the ovary (Fig. 3C), and apical part of the flower peduncle (Fig. 3A and 3C). No GUS staining was observed in pollinated siliques 1 d (Fig. 3D), 3 d (Fig. 3E) and 5 d (Fig. 3F) after anthesis.

Negative feed-back regulation of *SIGA20ox1:GUS* expression

No effect of GA₃ was seen on *SIGA20ox1(834bp):GUS* expression in Arabidopsis seedlings determined by GUS activity (Fig. 4A) (although some seedlings had apparently less GUS staining at the base of the cotyledons; Fig. 4B), probably because they contained already relatively high endogenous GA levels. The addition of paclobutrazol (PAC; an inhibitor of GA biosynthesis) enhanced *SIGA20ox1* expression (an effect particularly apparent in the root), and GA₃ application negated the effect of PAC (Fig. 4A and 4B). This means that the 834 bp region of the promoter contains the cis-element(s) responsible for the negative feed-back regulation of *SIGA20ox1:GUS* expression in Arabidopsis. A strong negative feed-back regulation of *SIGA20ox1* expression, analyzed by RT-PCR, was also seen in tomato using seedlings cultured in Petri dishes with GA₃ and PAC (Fig. 4C).

Auxin and cytokinin regulation of *SIGA20ox1:GUS* expression

The addition of 0.1 μM and 1 μM IAA, NAA and 2,4-D to the culture medium reduced root growth and enhanced GUS staining in *SIGA20ox1:GUS* seedlings proportionally to auxin dose (Fig. 5A to 5G). Interestingly, while *SIGA20ox1* expression was never found in hypocotyls of control, it was seen in auxin treated seedlings (at all doses of NAA and 2,4-D, and at 1 μM IAA) (Figs 5C, 5E and 5G), associated with

enhanced level of auxin, detected by *DR5:GUS* expression in that organ (Fig. 5H and 5I).

In the presence of TIBA (an inhibitor of auxin transport), intensive staining of cotyledons and hypocotyls in *SIGA20ox1:GUS* seedlings was observed (Fig. 6B). In *DR5:GUS* seedlings GUS staining was also detected in cotyledons and apical part of the hypocotyl, and in the root apex (Fig 6G). Therefore, TIBA-induced *SIGA20ox1:GUS* expression in the hypocotyl seems to be the result of IAA transported from the cotyledons and accumulated in that organ. Similar results as those obtained with TIBA application were obtained after excision of the main root (Figs 6C and 6H), purported sink of IAA transported from the aerial parts.

Cytokinins are synthesized in the roots and transported to the aerial parts in the transpiration stream. Since cytokinins have been reported to interact with auxin synthesis (Bangerth et al., 2000, Kakani et al., 2009), and with GA in many developmental processes (Weiss and Ori, 2007), we also investigated whether benzyladenine (BA; a synthetic cytokinin) affected GUS expression. We found that in both *SIGA20ox1:GUS* and *DR5:GUS* intact seedlings, BA enhanced GUS staining in cotyledons, hypocotyls and roots (Figs 6D and 6I). Also, GUS staining in seedlings with excised roots was not prevented by BA application (Figs 6E and 6J).

Light regulation of *SIGA20ox1:GUS* expression

6-d-old *Arabidopsis SIGA20ox1(834):GUS* seedlings grown under LD conditions expressed GUS in the cotyledons, leaves and root, but not in the hypocotyl, as shown above. When these seedlings were transferred to darkness, their elongation was enhanced but no GUS staining was found in the hypocotyls, at least up to 48 h later (data not presented).

1 In contrast to these results, in seedlings grown under continuous darkness
2 (etiolated) GUS expression was found in the upper part of the hypocotyl (the elongating
3 region), in addition to the hook (unopened cotyledons) and roots (Fig. 7, T₀, upper
4 panel). When etiolated seedlings were transferred to LD conditions cotyledons
5 expanded, hypocotyl elongation was reduced and GUS expression in the hypocotyl
6 disappeared (Fig. 7, T₂₄ and T₄₈, upper panel). In the case of *DR5:GUS* seedlings GUS
7 staining was limited to the cotyledons in the hook (Fig. 7, T₀, lower panel), and no
8 effect on its expression was observed upon de-etiolation (Fig. 7, T₂₄ and T₄₈, lower
9 panel).

11 **Discussion**

12 **The *SIGA20ox1* promoter is functional in Arabidopsis**

13 The ectopic expression of *SIGA20ox1* in Arabidopsis, using a 834 bp promoter
14 fragment fused to the GUS reporter gene, produced high expression in the roots, leaves,
15 cotyledons and flowers, but not in the hypocotyls nor in unpollinated or pollinated pistil
16 (Figs 2 and 3). Similar expression pattern was obtained with plants transformed using a
17 longer construct (1391 bp promoter) (provided by Drs JM Davière, A Phillips and P
18 Hedden; data not presented).

19 Columella cells are target cells of gravitropic stimulus, perceived by
20 sedimentation of starch containing organelles (statoliths), and auxin has been shown to
21 act as a mediator of that stimulus (Morris et al., 2004). The *SIGA20ox1:GUS* expression
22 in these cells of secondary roots, that grow in horizontal direction during the period of
23 time at which this localized expression was observed (Fig. 2O to 2R), suggested that
24 GAs might also been involved in the gravitropic response. However, experiments
25 modifying the direction of this stimulus in the primary root (by growing them in

horizontal position) and so trying to also induce expression in its columella cells were carried out without success. Also, diverse factors such as low temperature (4 °C) and GA₃ and PAC application had no effect on GUS expression. Thus, the possible physiological meaning of the specific localization of *SIGA20ox1* in the columella cells of the secondary roots remains an unanswered question.

Negative feed-back regulation of *SIGA20ox1*

Negative feed-back regulation of many *GA20ox* occurs in diverse species such as *Arabidopsis* (Phillips et al., 1995), rice (Toyomasu et al., 1997) and potato (Carrera et al., 2000). The fact that *SIGA20ox1* negative feed-back regulation was also found both in *Arabidopsis* (Figs 4A and 4B) and in tomato (Fig. 4C) means that the 834 bp 5' upper region used to transform *Arabidopsis* contains cis-elements necessary for that kind of regulation. This opens the possibility of using our transgenic plants to localize sequence(s) involved in *SIGA20ox1* feed-back regulation. A cis-acting sequence responsible for negative feed-back regulation of *AtGA3ox1* (composed of six repeated AA(A/T)T sequences), as well as an AT-hook protein binding to that DNA sequence (although only the two central DNA repeats are important and the adjacent ones are dispensable for binding) have been identified (Matsushita et al., 2007). Interestingly, a DNA motif (composed of six AAAT direct and complementary sequences very close located; Fig. 1C) similar to that found in *AtGA3ox1* was also present in the *SIGA20ox1* promoter, suggesting that this cis-region may also be involved in feed-back regulation of the gene. To further substantiate this hypothesis we analyzed the *GA20ox* promoters of genes from *Arabidopsis* (Phillips et al., 1995), tobacco (Kusaba et al., 1998), rice (Toyomasu et al., 1997), pea (Martin et al., 1996) and aspen (Eriksson and Moritz, 2002) reported to be under negative feed-back regulation. The presence of AA(A/T)A rich sequences within the upper 800 bp region, and a bit further up in the case of rice,

was identified in all of them (Supplementary Fig. 1). In pea, many AA(A/T)T sequences are present even upstream the first 800 bp (Supplementary Fig. 1). Promoter analysis of *AtGA20ox1* carried out by Meier et al. (2001) showed that the cis-elements for negative feed-back regulation of that gene should be located within the first 500 bp from the transcription start. Some AA(A/T)T scattered sequences were also found in this part of the promoter (Supplementary Fig. 1). Certainly, comprehensive promoter deletion analysis and mutagenesis experiments should be done to support our hypothesis.

Expression of *SIGA20ox1* is regulated by auxin

Auxin (IAA, 2,4-D and NAA) application upregulated *SIGA20ox1* expression in all organs of Arabidopsis (cotyledons, hypocotyls and roots), associated with a reduction of hypocotyl and root development (Fig. 5). This effect was dose dependent and also observed at low auxin concentrations (0.1 μ M), when root growth alteration was relatively little affected. However, it is important to note that since the treatments were applied throughout the growth of the seedlings, shorter treatment applications might have produced less developmental changes (particularly in the roots) thus indicating more direct effects. Enhancement of Arabidopsis *AtGA20ox1* expression in the shoot, but not in the roots, upon auxin and auxin transport inhibitor application has been reported (Desgagné-Penix and Sponsel, 2008). Interestingly, in our case *SIGA20ox1:GUS* expression was also induced by auxin in the hypocotyl, an organ where GUS staining was never detected under normal culture conditions. This was associated with accumulation of exogenous auxin as shown by enhanced *DR5:GUS* expression in the hypocotyl (particularly in the lower part) (Fig. 5I). Up-regulation of *SIGA20ox1:GUS* was certainly not a consequence of reduced hypocotyl growth because no GUS staining was seen in PAC treated seedlings (Fig. 4B), nor in seedlings cultured at 4 °C vs 22 °C (data not presented), which had shorter hypocotyls. This agrees with the

1 absence of induced up-regulation of Arabidopsis *AtGA20ox1* in stunted seedlings
2 (Desgagné-Penix and Sponsel, 2008). *SIGA20ox1:GUS* expression in the hypocotyl,
3 associated with higher IAA content, was also found after TIBA treatment. Cotyledons,
4 young leaves and roots of Arabidopsis seedlings have the capacity to synthesize IAA,
5 thus potentially contributing to the auxin needed for growth and development (Ljung et
6 al., 2001). In the case of cotyledons, IAA is produced in localized sites (e.g.
7 hydathodes) (Aloni, 2004; see also Fig 5H and 6F in this paper). IAA from the aerial
8 part is also known to be actively transported through the vascular parenchyma to the
9 roots (Teale et al., 2006). Therefore, endogenous auxin accumulated in the upper part of
10 the hypocotyl in TIBA-treated seedlings may be due to blockage of auxin basal
11 transport.

12 Enhanced GUS staining in hypocotyls of *SIGA20ox1:GUS* and *DR5:GUS*
13 seedlings was also observed upon root excision (Fig. 5). This could be due to removal
14 of a possible sink for IAA transported from the aerial parts, an effect similar to that
15 found upon auxin transport inhibitor application. Cytokinins are transported from the
16 roots and have been shown to inhibit *GA20ox* and *GA3ox* expression in Arabidopsis
17 (Brenner et al., 2005). Therefore, an alternative possibility to explain *SIGA20ox1:GUS*
18 expression in the hypocotyl upon root excision is the absence of cytokinin transport
19 from the roots to the aerial part. However, this hypothesis was not substantiated by two
20 kinds of observations: a) BA induced GUS expression in the hypocotyls of intact
21 *DR5:GUS* seedlings (Fig. 6I), in agreement with Bai and DeMason (2008); and b)
22 application of BA to seedlings with excised roots did not prevent GUS staining in
23 *SIGA20ox1:GUS* hypocotyls (Fig. 6J).

24 All these results support the conclusion that auxin induces *SIGA20ox1*
25 expression. Enhancement of diverse endogenous *GA20ox* by auxin in Arabidopsis

seedlings (Desgagné-Penix et al., 2005; Frigerio et al., 2006; Desgagné-Penix and Sponsel, 2008), pea internodes (Ross et al., 2002), and pea (Ozga et al., 2009) and tomato (Serrani et al., 2008) fruit has also been reported. In the case of pea internodes and tomato ovaries, auxin-induction of *SIGA20ox* expression is associated with an increase of GA content. Frigerio et al. (2005) suggested that auxin has a direct effect on *AtGA20ox1* and -2 upregulation because it occurs very rapidly and also in the presence of cycloheximide, probably through Aux/IAA and ARF proteins. Desgagné-Penix and Sponsel (2008) did not find evidence of auxin promoting RGA (a GA repressor protein) degradation in any Arabidopsis tissue accumulating auxin, in contrast to the results of Fu and Harberd (2003) in the root tip. Therefore, those authors concluded that auxin-enhanced expression of *AtGA20ox1* is not due to increased flux through the GA metabolic pathway (which would increase endogenous GA content), but rather to metabolic (feed-back) regulation, which would override auxin regulation. Our observation that auxin application reduces hypocotyl length (which depends on GA) while increasing *SIGA20ox1* in that organ agrees with that hypothesis. However, quantification of endogenous GA is certainly needed to further support this conclusion.

A corollary of our results is that the observed auxin regulation of ectopic *SIGA20ox1* expression in Arabidopsis resides, at least partially, in the 834 bp 5'upper region of the tomato promoter. This promoter contains the sequences CATATG, present in one of the regions (NDE) of *SAUR* genes promoters which are rapidly inducible by auxins (McClure et al., 1989, Xu et al., 1997), and the sequence TGTCCA, quite similar to a pea auxin-responsive element (TGTCAC; Ballas et al., 1995) (Fig. 1C). Functional analysis of several auxin-specific promoters has revealed the importance of combined utilization of both conserved and variable elements for this kind of regulation (Abel et al., 1996).

***SIGA20ox1* expression is regulated by light in etiolated seedlings**

The expression of *SIGA20ox1:GUS* in etiolated Arabidopsis was also light regulated because GUS staining was detected in the upper part of hypocotyls from seedlings grown in the dark (etiolated), and the staining disappeared after transfer to light (de-etiolation) associated with a reduction of hypocotyl elongation (Fig. 7, upper panel). Since GUS staining was not detected in hypocotyls of light-grown seedlings after transfer to continuous dark, which induced hypocotyl elongation (results not presented), it means that the absence of light is not sufficient per se to induce the expression of *SIGA20ox1*. Interestingly, *SIGA20ox1* expression in the upper region of etiolated hypocotyls was not associated with *DR5:GUS* expression (Fig. 7, lower panel). Therefore, in contrast to the clear effect of auxin on *SIGA20ox1* regulation in plants grown under light described above, the expression of this gene in etiolated seedling is not mediated by auxin. The 834 bp 5' upper region of the *SIGA20ox1* tomato promoter should thus contain sequences, still non-identified, involved in this kind of gene regulation. Decrease of *PsGA3ox1* expression upon de-etiolation in pea epicotyls, associated with rapid reduction of GA₁ content and elongation has been reported (Gil and García-Martínez, 2000; Reid et al., 2002).

In summary, our results show that the promoter of *SIGA20ox1* (a gene encoding an enzyme of GA biosynthesis from tomato) can be expressed in diverse vegetative and reproductive organs of Arabidopsis using the construct *SIGA20ox1:GUS*. The results revealed new aspects of *GA20ox* regulation (e.g. localized expression in the columella, and auxin-induced expression in the hypocotyl). Negative feed-back regulation (as also occurs in tomato), in addition to auxin, cytokinin and light regulation of that promoter, was also demonstrated. Element(s) involved in feed-back regulation of *SIGA20ox1* is (are) located within the 834 bp of the 5' promoter region used for Arabidopsis

transformation, which contains AA(A/A)T sequences very similar to those described for feed-back regulation of Arabidopsis *AtGA3ox1*. The promoter also contains sequence(s) putatively responsible of the observed upregulation of *SIGA20ox1:GUS* by auxin, as well as non-identified sequence(s) responsible of its expression upon de-etiolation. Our results suggest that Arabidopsis transgenic plants bearing *SIGA20ox1:GUS* constructs with specific promoter-deleted regions may be a convenient system to identify DNA elements involved in *SIGA20ox1* feed-back, auxin and light regulation.

Acknowledgments

We thank Dr. T. Guilfoyle (University of Missouri, Columbia, USA) for *DR5:GUS* seeds of Arabidopsis, and Drs J. M. Davière, A. Phillips and P. Hedden (Rothamsted Research, Harpenden, UK) for the *SIGA20ox1(1391):GUS* construct. This work was supported by Ministerio de Educación y Ciencia of Spain (grants BIO2003-00151 and BIO2006-13437 to J.L.G.-M.) and Consellería de Agricultura de la Generalitat Valenciana (fellowship to E. M.)

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Figure legends

Figure 1. Structure of tomato *SlGA20ox1* gene (A), *SlGA20ox1(834bp):GUS* construct used for Arabidopsis transformation (B), and DNA sequence of the 834 bp 5' upstream region of the *SlGA20ox1* promoter (C). Putative sequences responsible of *SlGA20ox1* feed-back regulation are in bold letters and underlined, and those corresponding to auxin regulation double underlined.

Figure 2. *GUS* expression in different vegetative tissues of transgenic *SlGA20ox1(834bp):GUS* Arabidopsis. A, 7-d-old entire seedling; B, cotyledon; C, first developing leaves; the arrow indicates the trichomes; D, veins and stomata of cotyledons; E, hypocotyl and root transition; F, vascular vessels of the main root; G, primary root apex; H, entire 21 d-old seedling; I, rosette leaf; the arrow indicates a hydrotide; J, veins and stomata of rosette leaf; K, trichomes of rosette leaf; L, M, N, early growing stages of emerging secondary root; the arrow indicates the secondary root; O, P, Q, secondary root with GUS staining in the columella; the arrows indicate the stained columella cells; R, % of secondary roots displaying columella staining as a function of root length. CA, root cap; QC, quiescent center.

Figure 3. *GUS* expression in different reproductive tissues of transgenic *SlGA20ox1(834bp):GUS* Arabidopsis. A, flower at anthesis; B, anther at anthesis; C, pistil at anthesis; D, E and F, pollinated (P) and unpollinated (UP) siliques 1 d, 3 d and 5 d after anthesis.

Figure 4. Effect of GA₃ and PAC on *SlGA20ox1(834bp):GUS* expression in Arabidopsis and *SlGA20ox1* in tomato. A. Mean values $\pm$ SE (n = 5) of GUS activity, determined by fluorometry, of 4-d-old *SlGA20ox1:GUS* seedlings of Arabidopsis control or grown with GA₃, PAC or PAC + GA₃. B. Representative histochemical GUS expression of 4-d-old Arabidopsis seedlings control or grown with GA₃, PAC or PAC +

GA₃. C. Transcript levels of *SlGA20ox1* in 8-d-old tomato seedlings control and grown with GA₃, PAC or GA₃+PAC determined by semiquantitative RT-PCR. GA₃ was applied at 50 µM and PAC at 1 µM in the medium. Results of three biological replicates are presented.

Figure 5. Effect of auxins on GUS expression in *SlGA20ox1:GUS* and *DR5:GUS* Arabidopsis seedlings. *SlGA20ox1:GUS* seedlings: (A) control; (B, C) IAA 0.1 µM and 1 µM respectively; (D, E) 2,4-D 0.1 µM and 1 µM respectively; (F, G) NAA 0.1 µM and 1 µM respectively. *DR5:GUS* seedlings: (H) control; (I) 1 µM IAA.

Figure 6. Effect of TIBA (12.5 µM) and BA (1 µM) application and root excision on GUS expression in *SlGA20ox1:GUS* (upper panel) and *DR5:GUS* (lower panel) Arabidopsis seedlings. (A, F) Control; (B, G) TIBA; (C, H) excised root; (D, I) BA; (E, J) excised root + BA. The arrows indicate the secondary root developed after excision of the main root.

Figure 7. Effect of de-etiolation on GUS expression in Arabidopsis *SlGA20ox1:GUS* (upper panel) and *DR5:GUS* (lower panel) seedlings. Seedlings were grown for 4 d in the dark (etiolated), and then maintained in the dark or transferred to LD conditions for additional 48 h. D, dark; LD, long-day. The point arrows indicate transition between hypocotyl and root.

Supplementary Figure 1. Promoter sequences of *AtGA20ox1* (At4g25420), *AtGA20ox2* (At5g51810), *AtGA20ox3* (At5g07200), *NtGA20ox1* (AB012856), *OsGA20ox1* (OsJNBa0059G06.22), *PsGA20ox1* (AF138704) and *PtGA20ox1* (AARH01006569.1). AA(A/T)T sequences are indicated in bold letters and underlined (other scattered AA(A/T)T sequences present in the promoters are not marked). In *AtGA20ox1*, AA(A/T)T sequences within the first 500 bp are double underlined. The

- 1 numbers below the name of each gene indicate position of the base relative to the first
- 2 coding ATG.
- 3

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Fig. 1

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Fig. 2

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Fig. 3

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Fig. 4

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Fig.5

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Fig. 6

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Fig. 7

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Supplementary Figure 1

AtGA20ox1

-800

GATAAAATATGGATAAATTACATATTTAGTCAGAAATTATTAAATTTATCATTTGATATAAT
TATTTAATTAATTAAATTATTTTGATTATGTAAATGATTTAATATGCAACTACGTAATTTTA
TTTATTATAAATCTTTAATTTCAATTCAAGAAATATTTTATATGAATTAATTTTGTG
CCAGTTTTGAATGAATGGTTAGAGCTATAATCTTTAAGGTATCCTTATGTATGGAGAAATAG
AAGGGAGTAAATTCAAATAAAATCAACCACCTATGACTAGTAAGCTATACATTATTAGTGG
AATAGTGGTAAATGTCCATGTTGCCACACAACAAACATGTAGGGAATCGATAAAAGACAGT
TTTCAACATTTTCACCATTTTCAGTTAAAAAAAATAGCTTTGCGTACAAAAAAAAGA
AAGACAAAAGACAAAAAAAATAATCAATATGTTAATTAGATTTATAATATAAAAT
TTAGGAATGATTCCACCACTGTTTTGTCAACATCATTCCACCACTGTTTTAGCGAATTTAA
CTCCATTACAGTGAATAATCTAAAAATCCTTTACTCTTTGGATTATATATCACTCGTGGCAA
AAGTATTGATAACTCCATTACAGACTATAGTATTGTACTACTAGAAAACAAAACAAA
AAAAGAAGTGGACAACACTATACGATCGACTTAAATGCTTGCTTATATAAAGACTAAAAGG
ACCATTGGTTCCTGATCTCCTCGCAATACTACTACTACTTTACTATAATCTCTCAAAAtg

AtGA20ox2

-322

ACGCAATTTAATTTAGGGATTAAATGTCGTTTAGTTTTTCTTGTCCTTTAGATTGAGACCAA
GATTTTGTTCAATATACACAATTTTGACACTATTATATTCACCTTATAAAGAGATTAGTATG
AAATCCCATTGTGGCAAAAAAAGAAGAAATTATTATAAGAAAGATTAAAAAACCAACAA
ATGTACACTAACATGACTTGAAGCTTGCTTATATAAAGACTTAAAGGACCCTTTGTTCCCC
ATCTCCTCAACAACACTCACTCAGAACAAGACAAAACAAAACCCCAAACTCTCAAGAAA
AAAAAAGAAAAGAAAtg

AtGA20ox3

-796

TATATAAATAATTTGTCATTTTAGCAAACATAATTTATCACAGAAAACCTATTAAATTC
ATATTAATTTTTTAAATATGATTGCATATGTTTTAAACATGTTTACATAAATTATAGATATAG
ATTTTAAGAAATATTGGTTTATTCGTTTGGATGGGTTTATACTTAAATATCAATATGCTTC
GGTTCAATATTTTCGGTTTGTGATTAATGGCAATATTCAATATTAATGTATTTACAAATAAT
AATAAATACATTTATAACTGAACTATACTTGAATTTATCCCTAATTTCAAAGAAAGATT
AATTATTCAATAGTTTATTATGTTTATAAACGGTTTAATACTCGACTGGCAATTTTAAAAA
TAAAAATGAAAAGTATCATGATAATTTTCGCTAATATATATGTTGTAAAGAAAAACAAGAA
TTTAAACATGATTTTTCATCTGTTTGTCTTATCTTCGAATTGCAGCCCATTCAAAAATCAA
TATAGTCGATACAGCCAGTTTACGCCACTAAATAAATTAATAGATTAAAAACTACAGCCAAA
ATTCATCTAGAAATATGTTACCATTTACAGCCAAAAAAGAAAGAAACACCAACCTCCCAA
ATGCCGCTTACGTACTATTCCTGCACCTAAGTTCCCTCTCGCACCTATATATACCACTCCT
TTCCTCTCCCACTTACCGACCACTGTACTGAATCTTTAAGCCTCTCAACGTGTTTTTATATAT
ATTTTGAAAATCTTTTACGCCTTAAAGGATCTACGATAATTAAAAAAAAtg

NtGA20ox1

-445

ATTATATTTGTCTTAAAAAATTCATTAAATTATATATAAATTATTAATTTAAAAATTCATAAC
TTAAATATGCTTAAATTTCTGAATCTGCTGCACCAACCGACCAAAGGAAACTCCAAATACCAT
TGGGAAACGCTTTTCTGTCAACAATATAAAAGACAAATAACATATGGACCTCTATCCCAATT
TTAAGCATTATTAAGCCCTTCTCAGCAAAGTGTTTATTTTGGAGCTCCTGTTTTCTGTCTTAG
GGACACCACAAAGGAAGACCTATAAGATTCCAATTTTGTTCACCTATTTACCCAATGAACG
GCCTTGGCTTTACCTTTATCTCAACTCTCTAACTCCATTCTTCCCACTCGCTATCTTTTGCTC
TTAAATTTTCTTCCCTTTGGCTCAATTAACACTCTTTCTTCTGTTAAAAAGAAAACAAAATC
GCATGCAAtg

OsGA20ox1

-1216

AATCCATTTTTCGCAAAATTTGTGTGAAGCTGGATTTAAAAATATATAATTCTTGAATTAAA
TATAAACAACTCTCTTATTTCTATTAGCCTGTTATAATTTATATACAACTATATCTATTAATT
TATAATCTAGACCAACCACAATCTAGGAATGTAAATCTTTCTGTTTTCTTATTAATGTAGTA
ATAATTTCTAGGTTTTTGTGAACGCGCAATAATTTCTAGGTCTCTCTAGAGTAACATGACCTT

1 ATTGCGGAGTTGTTTGTCCAGTCATCAATCTGGTGGTGGCACAGGCGCTGGGGTGTGTTTGT
2 GTTTCTACTTGAGTAAATTGCATAAACGTGAATTTACAGTTTTAAAAATCAAACATGTGAAG
3 TGGTTGTCTAAATCTCGACATAGCGTCATGTGCGAGTACAACAACACTTCTGCAACGCCAGC
4 CAAAGAAAAAACTTGCATTTAGATAAACCTTGCATGTCCAAGATAAAATTGGCCCAAG
5 TGAAAAACTCAGAACTACATAAATAATTTTCGCAAATGGCCAAAACACTCATTTTTGGATGTGG
6 TATTTTGACGTGTGGGCATCACAGGTGCACGTGAAAACCCATTTTCACATATGGATCTGTTG
7 AGGAGGCTGCTTGCAAACTGATTTTTGCAGGCAGGCAAGTAAGGACTTTTCGACTAGAACG
8 ATTTTTTTTTTAAAAAAGAATAAACACTACAGATCCAAGACCCTATAGCTAGGGTAAGC
9 CGCATGTGGCCGCGGCCGTGTGGTCCGTTGGCTGGGTACAAAGAGCAGAGTGGGCTAAGCA
10 AATACCGGTTGTGGCACCATCCCTTTCACATTCACCTCGCTCTTGATATCTTCTCTCATG
11 GAAAAGAAGAGATAAGTAATTTAATTGATGCCGGGATAGAGAGAGAGAGAGAGTGAAGA
12 AGGTAGCTAGGGGAGAGAGCGAGGTTGATGCCGTGATCGATCGATCGATCTGTTGGCGCAG
13 CGTGTATATAAGGGCGGAAGGGAGTGAGAGAGCAGCAGCTAGCTAGCCGCGGTCCGT
14 CGATCCAGCTGCTGGGGATGAGTACTTAGTTAGCTCGGAGCTAGCTACTAATGGATGATATA
15 CTTATGCTAGTTAGTTAAATACAGTTATTAGTTAGTTGTAGGTTGCATCTATCATATCTCCAT
16 CGGTTAATTAATTGATTGATAGCTAGATTATCAACAATTAatg

PsGA20ox1

-1204

20 TAATTATTTTAATTTTCCTTAATGAAAATATACAAAATTTATTATTTAATTTTGTTCATTGATATATG
21 TTGAAATATAAGTGTTTTTAGACAGTATTTATTATATTTAATATGGTCACTATTATAAAATA
22 AAAGAGTAAATTATAATTTNATNATGAGAAAAATTCTATTTTCTCATATGTGAGAAATCAAA
23 AATGTTGTTGTTGTCTTGAGTATATTATTTGATATTATATAAATGTTATTGTAGTAAAATAT
24 ATACACATGGTAAAAGATTATCGCATACACTATTATATTTTTAAGATCAACTATGAGCGAGTT
25 ATCTTTAACATTAAAAATTAAAAATATTTGTTTCAAGTATTATTTTATTACTATAATTTTTAAGA
26 AAATAAAAAATATTTTTATTGTGTTTGGATTCTCATATCTCTTTTAATTTTAAAAATTTTAAAA
27 TAATATTTTTAGTTGAAGTAACTAGGGTAATATACATAAAATTAGAGTAATAAAATTGAATAGTTA
28 GAGTAAATTGTGCGTTTGTAGACCATTTTTATCTAAAAATGAGATTTTTTTTTTCGTCTGTAGAG
29 AGTTCGAACTCGTGACCTTCAATAAAATTAAAAATTATTGTATTTAGTCTTTTGAATAGTAAAA
30 ATGAGTTTTGATAAATGTACCTAGAAGTATGAGATTATGACATAAATGAAAATAGAAGTGT
31 TAAAAATGAAAATTATGTGATTCTGTTTTAATATATTATAATAGATAAAATTAATATAACTTTATT
32 TTTATCTTTGCTTTTAATGAGGTTTTAAAGGCATAAATGTAATGGTAATTATTTAAAGGCTAA
33 CTTGATTAATAACTCTTTTAAGAAAATTATCCTTTAAAAAGAGATAAACTTTTTTCAATTTG
34 TCTTTGTTGCTTGAAAGGTTATATATCTTCCCTCAAGATATATTTTACTCTATATTTTTTTTTT
35 GTCTTTTATCAAGGGTAAGTTAATTATATATATTTTAAATCCAAATCTTTATGATTTTTTTAA
36 TTAATAATTATATATTTTTTTTCTTTTCTATTTTTGTTTTTATATGTATCATCAATAACTTTT
37 TTTCCATGTTGGAGAGAAATAGAATTCTTTTCTCTCTATATATAATACAAGTTTTTCTCTCA
38 TTGTACCAATAACTAGCCTTTGCTTTTCTTACATCTCTCTCACACACACACTTTTTTATTATAGT
39 TAAGGCAatg

PtGA20ox1

-709

43 AAGAGAAATGAGTAGAGAGAGAGAGAGATTATTTAGTGGAGAAATCTGTTTTACAGGGTA
44 ATTAGGCCCTCAAAACAGATTTCTTCTTCCTTCATTATTTTTTTGTAACTTAGTGTTGCTC
45 CCAAATAAGAAAGCAGAGAAGACTATAGAGGGAGGACCTATGTCAGGTGGGGGCTTCGGC
46 ATTTTAAAAACAGAACAGGAAGTTATGGGTTTAAATACAGGTCTAAGCACACTCTTGATACAA
47 GACAAAATAAAGTGCCCTGCTGACACTAGCATAGAGCATCCAACAAAAAACTCCATTGGG
48 AAGAGCTTTCCTGGCAGCTATAAAAAGGACAGAACTCCAACAAATTGGTCTATCACCTAATT
49 TTCTCATTAAGCAACCCCTTGCGCTGCAAATCCCCTCCTCTCTCAAGCCCTTCCTTGCAAAA
50 ATAAATCCACACAGCATATAAGAGTGAGGGAAAGATAGCTAGCTACAAGATTCCAACATT
51 CGGGCTCACCGATTTCCCAATGAGAGCGGTCTTGCTTTTCTTTTATCATCATCGGTACACT
52 TCTTTCTTTTATCTTAACTAGGAGCTAAGTACCAACTCTCCCTCCCTGCCTCTCAAACCTCAC
53 ACGATCACAATCACATACATACATACATACATACATATATATATATATACACACAGAC
54 CGTGCCCTGCCACAAAATTTGTAATGCAatg