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 \textit{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 \textit{SlGA20ox1:GUS} was shown to be active both in tomato and in transformed Arabidopsis. Auxin (indol-3-acetic acid, 2,4-dichlorophenoxyacetic acid and naphtaleneacetic acid), triiodobenzoic acid (an inhibitor of auxin transport) and benzyladenine (a cytokinin) treatment induced \textit{SlGA20ox1:GUS} expression associated with increased auxin content and/or signalling, detected using \textit{DR5:GUS} expression as a marker. Interestingly, \textit{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, \textit{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 \textit{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; \textit{Solanum lycopersicum}; Tomato
Abbreviations: d, day; LD, long day; NAA, 1-naphthalenacetic acid; PAC, paclobutrazol; TIBA, 2,3,5-triiodobenzoic acid.
Introduction

The gibberellins (GA) constitute a group of plant hormones which regulate diverse developmental processes such as germination, stem elongation, flowering and fruit development. GAs are synthesized using three kinds of enzymes (Yamaguchi, 2008). The first GA biosynthetic reactions (catalyzed by cyclases) produce ent-kaurene in the plastids. Ent-kaurene is then metabolized by membrane-associated P450-dependent monoxygenases to GA12, which is C-13-hydroxylated to produce GA53. GA12 and GA53 are converted by cytoplasmic dioxygenases to active GAs following two parallel pathways: the early-13-hydroxylation pathway (leading to GA1), and the non-13-hydroxylation pathway (leading to GA4). The last reactions are catalyzed by GA20- and GA3-oxidases. Active GAs and their precursors can be inactivated by GA2-oxidases and other catabolic enzymes. Most of the genes encoding the enzymes catalyzing the diverse GA metabolic steps have been cloned and characterized, and it has been found that the three groups of dioxygenases are encoded by small multigene families which are expressed differentially in diverse organs (Hedden and Phillips, 2000). The overexpression and downregulation of GA20ox in diverse species modified the levels of active GAs associated with increase or reduction of plant height (e.g. Coles et al., 1999; Carrera et al., 2000; Vidal et al., 2001; Fagoaga et al., 2007). This shows that the regulation of GA20ox expression plays an important role in GA homeostasis.

The genes of GA metabolism are regulated through development (Phillips et al., 1995; Garcia-Martinez et al., 1997; Silverstone et al., 1997; Rebers et al., 1999; Ayele et al., 2006; Mitchum et al., 2006) and by environmental factors (Kamiya and Garcia-Martinez, 1999; Vidal et al., 2003; Stavang et al., 2005). Transcript levels of many GA-dioxygenases are also subjected to negative (GA20ox and GA3ox) and positive (GA2ox) 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 GA$_{20}$ (the immediate precursor of the active GA$_1$) (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 *SlGA20ox1* 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 *SlGA20ox1: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 *SlGA20ox1* regulation.

In this work we have isolated a genomic clone of *SlGA20ox1* from tomato and the regulation of its expression was investigated using Arabidopsis plants transformed with a *SlGA20ox1:GUS* construct. The results show that *SlGA20ox1:GUS* was actively expressed in diverse vegetative and reproductive organs and that negative feedback (as also occurs in tomato), as well as auxin, cytokinin and light regulation of *SlGA20ox1: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 feedback regulation and auxin response were identified in the proximal region of the *SlGA20ox1* 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 ºC 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 ºC). 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-diclorophenoxyacetic 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 SlGA20ox1 (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 SlGA20ox1 clone and its promoter**

A SlGA20ox1 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 SlGA20ox1 genomic sequence were 5’-ATGGCTATTGATTGTATGATCAC-3’ (forward) and 5’-AGCTTGTGTAGTAGTGTTGTG-3’ (reverse) (using published information for cDNA sequence of SlGA20ox1; 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 SlGA20ox1 genomic clone have been deposited at the GenBank under the accession number EU043161.
A tomato $\textit{SI\!GA20ox1}$ 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 $\textit{SI\!GA20ox1}$ specific primers (5’-TAAGGCACAAGGCTTCTCGTGGTCAG-3’ and 5’-CCATTGGGATCATAATCAATAGCC-3’ for the first and second rounds, respectively). Thermocycling conditions used during the first PCR reaction were: 7 cycles of 94 ºC/2 s and 72 ºC/3 min, 37 cycles of 94 ºC/2 s and 67 ºC/3 min, and a final extension of 7 min at 67 ºC. 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.

$\textit{SI\!GA20ox1}:\text{GUS}$ 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’-GGATCCCGACGGCCGCGCTGG-3’ (forward) and 5’-CTGCAGATTATAATTGCATGCAAAGAC-3’ (reverse) (underlined sequences correspond to the BamHI and PstI restriction sites, respectively), and directionally cloned into the BamHI/PstI sites of the pCAMBIA 1381Z (Cambia, Canberra) binary vector to produce the $\textit{SI\!GA20ox1}(834pb):\text{GUS}$ fusion reporter, containing the promoter (834 bp) plus the first AT of the $\textit{SI\!GA20ox1}$ 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 μg mL⁻¹). Homozygous *SLGA20ox1*(834bp):GUS lines with a single insertion (3:1 segregation of hygromycin resistance: hygromycin sensitive seedlings in T₂) 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 ºC 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 ºC, 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 ºC 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 Na₂CO₃. Fluorescence
was determined with a fluorometer (Perkin Elmer LS50B) using 365 nm (excitation) and 455 nm (emission) wavelengths.

Results

Isolation of a \textit{SIGA20ox1} genomic clone and expression of \textit{SIGA20ox1:GUS} in Arabidopsis

A genomic gene clone containing the \textit{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 \textit{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 (\textit{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 hydatodes) (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 SlGA20ox1:GUS expression

No effect of GA3 was seen on SlGA20ox1(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 SlGA20ox1 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 SlGA20ox1:GUS expression in Arabidopsis. A strong negative feed-back regulation of SlGA20ox1 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 SlGA20ox1: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 SlGA20ox1:GUS seedlings proportionally to auxin dose (Fig. 5A to 5G). Interestingly, while SlGA20ox1 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 \textit{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 \textit{SlGA20ox1:GUS} seedlings was observed (Fig. 6B). In \textit{DR5:GUS} seedlings GUS staining was also detected in cotyledons and apical part of the hypocotyl, an in the root apex (Fig 6G). Therefore, TIBA-induced \textit{SlGA20ox1: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 \textit{SlGA20ox1:GUS} and \textit{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 \textit{SlGA20ox1:GUS} expression**

6-d-old Arabidopsis \textit{SlGA20ox1(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).
In contrast to these results, in seedlings grown under continuous darkness (etiolated) GUS expression was found in the upper part of the hypocotyl (the elongating region), in addition to the hook (unopened cotyledons) and roots (Fig. 7, T₀, upper panel). When etiolated seedlings were transferred to LD conditions cotyledons expanded, hypocotyl elongation was reduced and GUS expression in the hypocotyl disappeared (Fig. 7, T₂₄ and T₄₈, upper panel). In the case of DR5:GUS seedlings GUS staining was limited to the cotyledons in the hook (Fig. 7, T₀, lower panel), and no effect on its expression was observed upon de-etiolation (Fig. 7, T₂₄ and T₄₈, lower panel).

Discussion

The SlGA20ox1 promoter is functional in Arabidopsis

The ectopic expression of SlGA20ox1 in Arabidopsis, using a 834 bp promoter fragment fused to the GUS reporter gene, produced high expression in the roots, leaves, cotyledons and flowers, but not in the hypocotyls nor in unpollinated or pollinated pistil (Figs 2 and 3). Similar expression pattern was obtained with plants transformed using a longer construct (1391 bp promoter) (provided by Drs JM Davière, A Phillips and P Hedden; data not presented).

Columella cells are target cells of gravitropic stimulus, perceived by sedimentation of starch containing organelles (statoliths), and auxin has been shown to act as a mediator of that stimulus (Morris et al., 2004). The SlGA20ox1:GUS expression in these cells of secondary roots, that grow in horizontal direction during the period of time at which this localized expression was observed (Fig. 2O to 2R), suggested that GAs might also been involved in the gravitropic response. However, experiments 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 \textit{SlGA20ox1} in the columella cells
of the secondary roots remains an unanswered question.

**Negative feed-back regulation of \textit{SlGA20ox1}**

Negative feed-back regulation of many \textit{GA20ox} occurs in diverse species such
Arabidopsis (Phillips et al., 1995), rice (Toyomasu et al., 1997) and potato (Carrera et
al., 2000). The fact that \textit{SlGA20ox1} 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 \textit{SlGA20ox1} feed-back regulation. A cis-acting sequence
responsible for negative feed-back regulation of \textit{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 \textit{AtGA3ox1} was also present in the \textit{SlGA20ox1}
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 \textit{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 \textit{AtGA2ox1} 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 \textit{SlGA20ox1} is regulated by auxin**

Auxin (IAA, 2,4-D and NAA) application upregulated \textit{SlGA20ox1} 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 µM), 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 \textit{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 \textit{SlGA20ox1}:\textit{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 \textit{DR5:GUS} expression in the hypocotyl (particularly in the lower part) (Fig. 5I). Up-regulation of \textit{SlGA20ox1}:\textit{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 ºC vs 22 ºC (data not presented), which had shorter hypocotyls. This agrees with the
absence of induced up-regulation of Arabidopsis *AtGA20ox1* in stunted seedlings (Desgagné-Penix and Sponsel, 2008). *SIGA20ox1:GUS* expression in the hypocotyl, associated with higher IAA content, was also found after TIBA treatment. Cotyledons, young leaves and roots of Arabidopsis seedlings have the capacity to synthesize IAA, thus potentially contributing to the auxin needed for growth and development (Ljung et al., 2001). In the case of cotyledons, IAA is produced in localized sites (e.g. hydathodes) (Aloni, 2004; see also Fig 5H and 6F in this paper). IAA from the aerial part is also known to be actively transported through the vascular parenchyma to the roots (Teale et al., 2006). Therefore, endogenous auxin accumulated in the upper part of the hypocotyl in TIBA-treated seedlings may be due to blockage of auxin basal transport.

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

All these results support the conclusion that auxin induces *SIGA20ox1* 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 \textit{SlGA20ox} expression is associated with an increase of GA content. Frigerio et al. (2005) suggested that auxin has a direct effect on \textit{AtGA20ox1} and \textit{AtGA2ox2} 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 \textit{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 \textit{SlGA20ox1} 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 \textit{SlGA20ox1} 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 \textit{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).
**SlGA20ox1** expression is regulated by light in etiolated seedlings

The expression of *SlGA20ox1: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 *SlGA20ox1*. Interestingly, *SlGA20ox1* 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 *SlGA20ox1* 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 *SlGA20ox1* 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 GA$_1$ content and elongation has been reported (Gil and García-Martínez, 2000; Reid et al., 2002).

In summary, our results show that the promoter of *SlGA20ox1* (a gene encoding an enzyme of GA biosynthesis from tomato) can be expressed in diverse vegetative and reproductive organs of Arabidopsis using the construct *SlGA20ox1: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 *SlGA20ox1* 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 SlGA20ox1: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 SlGA20ox1:GUS constructs with specific promoter-deleted regions may be a convenient system to identify DNA elements involved in SlGA20ox1 feed-back, auxin and light regulation.

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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 hydatode; 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 ± 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), *AtGA20os3* (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
numbers below the name of each gene indicate position of the base relative to the first coding ATG.
Fig. 1

A

Promoter

ATG

ATT

I1

I2

I3

E1

E2

E3

B

pCAMBIA 1381Z

-834 bp

GUS

C

TCTCTTTTGGTTATGTGAAAAGTATAGAAATAAATTATTTTTAAATAAAAGAAAATAATCG
TCTCAAATCCAAATAGTTAGATGACACCAGCACTGACTATTTAAAGAATAGTAGTGAAGAA
GAGTATGGTCAATGGGAAGGGTTGAGTTGTAATGGTTCAATCCACCAAGGAAAGAATC
CAAACACCATGGGGAACGTTTTCCCTGTCACAGTGAAACAAAACAAACATATG
TTTTATCTTATTTTAACCTTTACAAACTCTTCTATTTATTTGATTGTTAATTAAATTTT
TTAAAATATAGTTGAATATTTTTATGTTACAGTATATGTAATGTACTATTGTTTTTTAAAATAATACAT
GGATAAGGGGAAAAATGAGGAAATTAAAGGAAAGATGAAACTTTATCATGAAAAAT
GAAAGTTATATATATGTCAATCGATTTGAGTCATAAAAGATTTCACAATGAAATTTTCTGATATAT
TTTTGAAACTAAAATAATAGTCTCATAATCTTATCTCATACAAAAAACTATAATTATATTGAGAA
GCTCTCTAGTAATGAATAGAGAAGAATACAAAGCAAAACATAGAAAGGACATCTTCAAT
TTAAGGATTGCTAAGCCTTTTGGACAATTTATCTATTTTTTTGTTAATGAGAGACCCAT
AAAGATTATTTTTTTTCACCTATTTTCCTCAACTATTAATTATTAATTTAGTCTTCAAT
CTCTCTTTTATTTCTTTCAATTTTGGCCTTCAAAATTATAATCACTAGTCTTTGCAT
GCAATTATAatg
Fig. 2

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

Secondary root length (mm)

Roots with stained columella (%)

Secondary root length (mm)
Fig. 3
Fig. 4

A

[Bar graph showing data for CONTROL, GA3, PCB, PCB + GA3]

B

[Images showing seedling growth under different conditions: CONTROL, GA3, PCB, PCB + GA3]

C

[Images of gel electrophoresis results for 2SGA20ox1 and ACTINE genes under different conditions: CONTROL, GA3, PCB, PCB + GA3]
Fig. 5

**SIGA20ox1:GUS**

- A
- B
- C
- D
- E
- F
- G

**DR5:GUS**

- H
- I

**Legend**

- A: 2 mm
- B: 2 mm
- C: 1 mm
- D: 2 mm
- E: 1 mm
- F: 2 mm
- G: 0.5 mm

**Description**

- **A**: An image of a plant with a 2 mm scale bar.
- **B**: An image of a plant with a 2 mm scale bar.
- **C**: An image of a plant with a 1 mm scale bar.
- **D**: An image of a plant with a 2 mm scale bar.
- **E**: An image of a plant with a 1 mm scale bar.
- **F**: An image of a plant with a 2 mm scale bar.
- **G**: An image of a plant with a 0.5 mm scale bar.

**Genetic Constructs**

- **SIGA20ox1:GUS**
- **DR5:GUS**
Fig. 6

<table>
<thead>
<tr>
<th>Control</th>
<th>+TIBA</th>
<th>Excised root</th>
<th>+BA</th>
<th>Excised root + BA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
</tr>
<tr>
<td>SIGA20ox1:GUS</td>
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<tr>
<td>F</td>
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<tr>
<td>DR5:GUS</td>
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</table>
Fig. 7

SIGA20ox1::GUS

DR5::GUS