Dual promoter of *Agrobacterium tumefaciens* mannopine synthase genes is regulated by plant growth hormones (auxin/cytokinin-regulated promoters of opine synthase genes/bacterial luciferase/video imaging)

W. H. R. Langridge*, K. J. Fitzgerald*, C. Koncz†, J. Schell‡, and A. A. Szalay**

*University of Alberta, Plant Molecular Genetics and Plant Biotechnology Centre and Department of Cell Biology, Medical Sciences Building, Edmonton, AB T6G 2P5, Canada; and †Max Planck Institut für Züchtungsforschung, D-5000 Köln 30, Federal Republic of Germany

Contributed by J. Schell, December 22, 1988

ABSTRACT Temporal and spatial distribution of mannopine synthase (*mas*) promoter activity was determined throughout the development of transgenic tobacco plants using bacterial luciferase *luxA* and *luxB* as reporter genes. Luciferase activity was determined by luminometry in *vitro* and visualized by computer-enhanced single-photon video imaging in *vivo*. The activity of the *mas* dual promoters increased basipetally in developing plants and was wound-inducible in leaf and stem tissue. Hormone biosynthetic with isolated plant tissues and tumors deficient in the transferred DNA (T-DNA)-encoded genes *iaaM*, *iaaH*, and *ipt* indicated that activity of the *mas* dual promoters is regulated by auxin and enhanced by cytokinin in both differentiated and tumorous plant cells.

Transfer and integration of a well-defined region of Ti plasmids (transferred DNA, T-DNA) from soil agrobacteria into the nuclear genome of plants mediates the morphogenetic transformation of plant cells to tumors (1–3). Expression of the T-DNA genes *iaaM*, *iaaH*, and *ipt* plays a key role in the maintenance of cell division and the suppression of transformed plant cell differentiation (4–7). Other T-DNA genes, such as the 1' and 2' genes of the right T-DNA (T*R*-DNA), specify the synthesis of metabolites (opines) that are secreted from transformed plant tissues and that serve as nutrients for free-living pathogenic agrobacteria (8–12). T-DNA genes carry transcriptional regulatory elements recognized in plants and demonstrate various levels of expression in plant tumors (13–15). Analysis of the promoters of the left T-DNA (T*L*-DNA)-encoded nopaline and octopine synthase genes indicates that they contain defined transcriptional enhancers (16, 17). The expression of T*L*-DNA gene 5 promoter–octopine synthase gene fusion was found to be regulated in a tissue-specific fashion in transgenic plants (18). Similarly, the mannopine synthase (mas) 1',2' dual promoters are from the T*R*-DNA were found to be functional in transformed plant tissues (19).

In this manuscript, we report that expression of the *mas* 1',2' dual promoters of the T*R*-DNA is regulated throughout development of transformed tobacco plants using the bacterial luciferase *luxA* and *luxB* as reporter genes (20). Tumor formation in *Agrobacterium tumefaciens*-infected plants occurs in response to elevated levels of auxin- and cytokinin-synthesized from T*L*-DNA encoded genes (7). To examine the involvement of T*L*-DNA encoded genes in the localized activation of the *mas* 1',2' dual promoters, transformed tobacco plants were infected with *A. tumefaciens* octopine strains containing functional or inactivated *iaaH*, *iaaM*, or *ipt* genes. Further, we provide evidence for regulation of the *mas* 1',2' promoters by auxin and cytokinin.

The light-emitting luciferase reporter enzyme has enabled us to quantitatively measure activity of the *mas* promoter fusions in cell-free extracts in vitro as well as to visualize changes in promoter activity in response to various physiological and hormonal stimuli in isolated tissues and organs, by computer enhanced low-light video imaging in vivo.

Our results indicate that the expression of *mas* genes is induced by wounding and is regulated by auxin and cytokinin in normal and tumors plant tissues.

MATERIALS AND METHODS

Transgenic Plants. *Nicotiana tabacum* SR1 (21) plants carrying a transcriptional fusion of *Vibrio harveyi* luciferase *luxA* and *luxB* genes to the promoters of T*R*-DNA-encoded *mas* 1' and 2' genes were obtained by protoplast cocultivation (22, 23) and leaf-disc infection (24) using *A. tumefaciens* strain *GV3101* (pMP90RK) (18) containing plasmid pPCV701-luxA&B, as described (20). Plants were maintained in sterile tissue cultures on MS hormone-free agar medium (25). Calli were initiated from leaf sections and maintained in an MS medium containing 5 μM naphthaleneacetic acid (NAA) and 1 μM benzylaminopurine (BAP). Tumors were incubated and maintained as described (18). For Northern RNA hybridization analysis poly(A)* RNA was prepared (26), separated on formaldehyde gels, and blotted onto nitrocellulose filters (27).

Luminescence Assay. Plant tissues [40–60 mg (fresh weight)] were homogenized in 1.0 ml of luciferase reaction buffer (50 mM sodium phosphate, pH 7.0/50 mM 2-mercaptoethanol/0.4 M sucrose) and cleared by centrifugation in an Eppendorf centrifuge for 5 min at 4°C. After determination of protein concentration (28), the extracts were supplemented with 0.1% bovine serum albumin and aliquots were assayed for luciferase activity in a luminometer (20). The luminometric measurements were standardized with defined amounts of purified luciferase. Light emission standard, 1 light unit (L.U.) is equivalent to 1.6 × 10⁶ photons per sec.

Imaging of Light Emission in Plant Organs and Tissues. Bioluminescence was detected in transgenic tobacco plants as described (29). Tissues and organs of transformed plants or whole plantlets were placed in plastic culture dishes adjacent to a filter paper strip saturated with an aqueous emulsion of the volatile luciferase substrate decanal. Samples were transferred to the chamber of a photon-counting video camera–photomultiplier system. The chamber was darkened, and the number and distribution of photons emitted from the plant tissues were recorded. Routinely, an adequate number of photons were collected in 30 min to reconstruct a well-defined image of bioluminescent tissues.

Aminoglycoside Phosphotransferase (APH(3')II) Assay. The expression of *mas* promoter luciferase gene fusions was compared with the nopaline synthase promoter–APH(3')II

Abbreviations: T-DNA, transferred DNA; T*R*- and T*L*-DNA, right and left transferred DNA, respectively; NAA, naphthaleneacetic acid; BAP, benzylaminopurine; L.U. light unit(s); ABA, abscisic acid; APH(3')II, aminoglycoside phosphotransferase.

*To whom reprint requests should be addressed.
gene fusion contained in pPCV701uxA&B T-DNA, as internal standard. Tissue extracts were prepared as described (18) and assayed for APH(3')II activity using kanamycin sulfate and (γ-32P)ATP substrates (19). In samples containing identical amounts of protein, the relative activity of APH(3')III enzyme was determined by densitometric scanning of kanamycin phosphate spots on autoradiograms.

RESULTS

Tissue Specificity of mas Promoters. The mas genes are transcribed from closely linked mas 1' and 2' dual promoters of the T5-DNA (19). To study regulation of the mas promoters, luciferase was used as a reporter system. The luxA and luxB genes encoding a heterodimeric luciferase in Vibrio harveyi were converted to structural gene cassettes, linked to the mas 1',2' dual promoters in vector pPCV701uxA&B (20), and transformed into tobacco plants. We have reported (20) that the expression of mas promoter-fused luciferase genes results in synthesis and assembly of a functional luciferase enzyme, conferring light emission in plants. Quantitative transcript analysis demonstrated that similar amounts of luxA and luxB transcripts were synthesized from the mas 1' and 2' promoters in transformed plant tissues (Fig. 1), indicating that sequences located in a 200-base-pair region between the 1' and 2' promoters, regulate bidirectional transcription. Thus, luciferase activity may reflect changes in transcriptional activity of both mas promoters.

The luciferase reporter gene system has provided a sensitive tool to identify temporal and spatial activity of the mas promoters in cell cultures and differentiated plants. Comparable quantitative data were obtained by in vitro luminometric determination of light emission in cell-free extracts prepared from calli, plantlets, and the tissues of vegetative and flowering plants (Table 1). Activity of the mas promoters was also monitored throughout the ontogeny of transgenic plants. Callus were induced from leaves of transformed plants and regenerated to flowering plants. Light emission from tissues was measured during each stage of development by luminometry and computer-enhanced video imaging.

Calli maintained at a high auxin to cytokinin ratio displayed ~200-fold higher activities than differentiated plant tissues (Table 1). At low auxin to cytokinin ratios, calli formed shoots and the activity of the mas promoters decreased. In seed-derived plantlets, luciferase was expressed in roots at much higher levels than in other organs. Shoot tips of soil-grown plants displayed the lowest activity when compared with other tissues. In stems, leaves, and petioles of nonflowering plants, a gradual increase in luciferase activity was observed from the shoot apex toward the base. In the stem, maximum luciferase activities were located in the cambium and vascular tissues. This result may reflect the high density of cells in vascular tissues. Leaves displayed a gradient of bioluminescence, resulting in a 30-fold increase in luciferase expression from the leaf base to the tip (Fig. 2B). During flowering, the basipetal expression gradient disappeared resulting in an increased level of luciferase expression throughout all stem and leaf tissues examined. In flowers, 2 days prior to opening a dramatic increase of luciferase activity was detected in nonfused portions of the corolla (Fig. 2C). A basipetal expression gradient was also found in all flower tissues examined (Table 1). The mas promoters were silent in pollen, but became highly active within the first hour of pollen germination (results not shown).

A comparable distribution of luciferase activity was found in plants transformed with the fused luxAB genes linked to the mas 1' or 2' promoter. Fusion of the luxA and -B genes resulted in the expression of a 78-kDa single bacterial luciferase polypeptide in transformed plants (A. Escher and A. A. S., unpublished work). The spacial distribution of nopaline synthase promoter-driven APH(3')II gene activity, although displaying some variability in plant tissues, differed in pattern and level of expression from the described activity of the mas promoter (ref. 18, data not shown).

The basipetal luciferase activity gradient in plant organs, the change of reporter gene expression in calli by modification of auxin to cytokinin ratios, and alteration in the

![Fig. 1. Hybridization of poly(A)+ RNA (10 µg) prepared from leaves of transformed tobacco plants containing the mas promoter luciferase fusion to the Sal I luxA DNA fragment (A) and to the BamHI luxB DNA fragment (B) of pPCV701uxA&B DNA, used as probes. Hybridization of APH (3')III DNA probe, isolated as a Bcl-I-BamHI fragment from plasmid pPCV02 DNA (18), to the plant poly(A)+ RNA sample is shown (C). Identical amounts of DNA fragments were labeled and probed with similar specific activities were used for hybridization (18). The numbers at the left of each lane indicate the size of the hybridizing RNA in bases.](image-url)
Fig. 2. Low-light video-image analysis of mas promoter activity in plant organs using the bacterial luciferase reporter enzyme. All photographs were prepared by superimposing the bioluminescent image upon the video image of the plant organ. Color calibration bar from bottom to top indicates increasing numbers of photons. (A) Expression of mas promoter luciferase A and B gene fusion in stem internode sections of flowering (Bottom) and nonflowering (Middle) transgenic tobacco plants. Stem sections from an untransformed tobacco plant are also shown (Top). Stem sections from each plant are arranged horizontally from left to right from the base to the shoot apex. (B) Activity of the mas promoters in leaf tissues. Lower left and right leaves correspond to the 10th and 4th leaves, respectively, below the vegetative shoot apex of a 30-cm-tall transgenic plant. The top leaf (horizontal) is from an untransformed tobacco plant. [Note: low expression in young leaf (Right) and higher luciferase activity in the tip and margin of the older leaf (Left).] (C) Activity of the mas promoters in sepal, stigma, and petals of the corolla of transgenic tobacco plant flower (Left), longitudinal section through flower from transformed plant (Middle), and a flower from an untransformed plant (Right) are shown. (D) Activation of axillary buds after apical meristem removal. One of two 40-cm-tall identical transgenic tobacco plants was decapitated. Both plants were incubated at room temperature for 12 hr. Stem segments (8 cm) from the top of the decapitated plant (Right) and the intact plant (Left) were sliced along the longitudinal axis and placed side by side in a culture dish. Bioluminescence was measured for 1 hr. (E) Inhibition of the activity of the mas promoters. (Inset left) Stem section excised from the ninth internode below the shoot apex of a 60-cm-tall nonflowering plant incubated for 12 hr on filter paper saturated with 5 μM NAA. (Inset right) Internodal segment of apical stem (3 cm) excised from the same region placed on the upper surface of a stem section. Inhibition of luciferase activity in serial stem sections was measured by video-image analysis. (Upper and lower left) Sections were treated with auxin only. (Upper and lower right) Stem sections were covered for 12 hr with stem segments prior to low-light video analysis. Curve (in red) at base of the panel indicates the distribution of photons detected in the area delineated by the horizontal blue lines. (F) Activity of the mas 1', 2' dual promoters in wild-type A. tumefaciens stem tumors induced on transgenic tobacco plants. (Upper) From left to right: stem section from an untransformed plant; section excised 1 cm above stem tumor on a transgenic plant; section through the center of stem tumor; and section excised 1 cm below stem tumor. All tissue sections (Upper) were measured by low-light video-image analysis immediately after excision from the stem; only the tumor tissue emits light. (Lower) Stem sections are identical to the upper row with respect to their position in the tumorous transgenic plant. As a positive control, luciferase activity was measured 12 hr after incubation of the sections in 5 μM NAA.

Luciferase pattern of expression during flowering indicate that auxin plays a significant role in mas promoter regulation.

**Activity of the mas 1',2' Dual Promoters Is Stimulated by Auxin.** The shoot apex and leaf primordia are known centers of auxin synthesis and their removal temporarily arrests polar auxin transport in vegetative seed plants (30). To test the correlation between an auxin gradient in the plant and the relative activity of the 1'2' dual mas promoters, the shoot
apex of several transgenic plants was removed and the cut stem was treated with 10 μM NAA. This treatment resulted in a 130-fold increase in reporter enzyme activity when compared to stem samples taken immediately after removing the shoot apex (Table 2). Activity of the luciferase reporter enzyme also increased about 50-fold in untreated stem sections, indicating a wound-induced activation of the mas promoters. Since the activity of the nopaline synthase promoter-driven APH(3')II gene increased only 3-fold, we concluded that extracellular addition of auxin enhances expression of the mas 1',2' dual promoter–luciferase gene fusion.

In stem sections obtained from the ninth internode below the apex, treatments with cytokinin (1 μM BAP) increased the wound-induced level of luciferase gene expression to ~50% of that observed when auxin (10 μM NAA) was added (Fig. 3A). In stem sections incubated with auxin, a 50-fold increase in luciferase activity was detected. Addition of cytokinin to auxin-treated sections did not significantly increase the level of luciferase expression (Fig. 3A).

To follow auxin-dependent activation of the mas promoters, leaf discs were incubated with increasing amounts of auxin in the presence of 0.3 μM BAP. Over 25 hr, a continuous increase of light production was detected that correlated with auxin concentration and that reached maximum activity 4–5 days after incubation of the leaf discs in MS medium (Fig. 3B and data not shown).

Induction of the mas promoters in stem sections and leaf discs may be due to wound-induced ethylene production. However, treatment of stem sections with the ethylene-generating compound chloroethyl phosphonic acid (10 μg/ml) or with ethylene inhibitors—e.g., cobalt chloride (0.1 mM) and aminovinylglycine (0.1 mM) did not enhance or inhibit luciferase expression, when added alone or with 10 μM NAA. In contrast, stem sections incubated in the auxin transport inhibitor 1,3,5-triodobenzoic acid applied at concentrations of 1 μM to 1 mM resulted in a 10–99% inhibition of mas promoter activity.

The Apical Meristem Contains a Factor That Inhibits Auxin Stimulation of mas Promoter Activity. Bioassay results indicated that the mas promoters are activated by auxin. However, low reporter-enzyme activity detected in shoot tips and in young leaves known to actively synthesize auxin, contradicted the bioassay results. Observations described below provide explanations for this apparent contradiction.

Application of shoot segments (3 cm long), derived from the stem apex, to the upper surface of stem sections (2 mm) on filter paper saturated with 5 μM NAA, resulted in almost complete inhibition of luciferase expression in the stem discs measured 12 hr after application of the stem segment (Fig. 2D). This result indicates that the shoot apex produces an inhibitory substance that is probably transported basipetally in the stem and that down-regulates the dual mas promoters by counteracting auxin stimulation.

Conversion of leaf tissues to protoplasts resulted in a 500-fold increase in mas promoter activity, independent of hormone concentration, during protoplast isolation. When protoplast cultures were allowed to form calli, luciferase activity was similar to that detected in calli derived from organ explants. The extent of mas promoter activation due to protoplast formation clearly exceeded the wound-induced response, indicating that an inhibitor was removed from leaf tissues by the protoplast isolation procedure.

Within 12 hr after removal of the shoot apex in nonflowering plants, the light emission of axillary buds increased dramatically (Fig. 2E). This result indicates that either auxin concentration is increased in axillary buds or that an inhibitor is removed in the absence of apical dominance.

Comparison of these results with models that explain the mechanism of apical dominance (31) and inhibition of auxin action (30), we have found a correlation between the observed physiological properties of the putative inhibitor and abscisic acid (ABA). Treatments of auxin-activated stem sections with 10 μM to 1 mM ABA resulted in a 22–67% inhibition of mas promoter activity, respectively. Whether the inhibitor is identical to ABA or to other auxin-inhibited ABA-like compounds proposed to balance auxin action in stems and leaves remains to be determined.

T-DNA Genes Influence the Activity of the mas 1',2' Dual Promoters. The above observations indicate that a positive regulatory role of exogenously provided auxin led us to study regulation of the dual mas promoters in tumor cells containing the T-DNA genes iaaM, iaaH, and ipt, which specify the intracellular synthesis of auxin and cytokinin.

**Table 2. Wound and auxin-mediated activation of mas promoters in stem sections from decapitated transgenic plants**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Auxin</th>
<th>Luciferase activity, L.U./μg of protein</th>
<th>Fold increase in luciferase activity, units/μg of protein</th>
<th>Fold increase in APH(3')II, APH activity, units/μg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2A</td>
<td>0.5</td>
<td>1</td>
<td>3.0</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>24.0</td>
<td>48</td>
<td>8.5</td>
</tr>
<tr>
<td>72</td>
<td>0</td>
<td>33.0</td>
<td>66</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>24</td>
<td>0.3</td>
<td>187.0</td>
<td>134</td>
<td>16.4</td>
</tr>
<tr>
<td>72</td>
<td>0.3</td>
<td>294.0</td>
<td>146</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Nonflowering 1.0-m-tall tobacco plants were decapitated in the middle of the 10th internode below the shoot apex and the stem was encased in a Tygon tubing sleeve to form a well. The cut stem surface was treated with water or 10 μM NAA. Enzyme activities were assayed in homogenates of 4-mm-thick stem sections excised at selected time intervals.

**Fig. 3.** (A) Influence of auxin and cytokinin on mas promoter activity in stem sections. Sections excised 10–12 internodes below the shoot apex of nonflowering plants were incubated on filter paper discs saturated with water (△); 1 μM BAP (●); 10 μM NAA (○); 1 μM BAP and 10 μM NAA (△); or a mixture of 1 μM BAP, 10 μM NAA, and cycloheximide at 5.0 μg/ml (○). At selected time intervals, stem slices were homogenized and assayed for luciferase activity. (B) Auxin activation of mas promoters in leaf discs. Discs (7 mm) were excised from a young fully expanded leaf and incubated on filter paper saturated with a solution of 0.3 μM BAP (△) or BAP supplemented with: 0.5 μM NAA (○), 5 μM NAA (●), or 40 μμM NAA (○). Luciferase activity was measured by luminometric assay.
Table 3. *iaaM* and *iaaH* genes regulate mas promoters in tumors

<table>
<thead>
<tr>
<th>Agrobacterium strain</th>
<th>Mutation in T-DNA</th>
<th>Luciferase activity, L.U./µg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD-1</td>
<td></td>
<td>149</td>
</tr>
<tr>
<td>A6#328</td>
<td><em>iaaM</em></td>
<td>13</td>
</tr>
<tr>
<td>A6#393</td>
<td><em>iaaH</em></td>
<td>2</td>
</tr>
<tr>
<td>A6#338</td>
<td><em>ipt</em></td>
<td>69</td>
</tr>
</tbody>
</table>

Transgenic tobacco plants were inoculated with *A. tumefaciens* strain LD1 containing wild-type Ti plasmid B6S3 and with strains A6#328, A6#393, and A6#338 carrying deletions of *iaaM*, *iaaH*, and *ipt* genes, respectively (32). Luciferase activity was measured in combined tissue extract of four tumor slices. In experiments in which the stem was inoculated with mutant strains, luciferase activities were determined in stem sections containing the inoculation site.

Tumors were induced on stems of transgenic tobacco plants with wild-type and mutant *A. tumefaciens* strains carrying deletions of either *iaaM*, *iaaH*, or *ipt* genes in the T-DNA of the Ti plasmid B6S3 (32). Wild-type tumors, 1-2 fold, expressed the luciferase-reporter enzyme at ≈150-fold higher levels than those detected in stem sections above or below the tumor (Fig. 2F). In the absence of *iaaM* or *iaaH* genes, luciferase levels at infection sites were identical to those found in uninfected stem sections (Table 3). Deletion of the *ipt* gene resulted in a decrease in luciferase activity to ≈50% of that found in wild-type tumors. These results indicate that the activity of the *mas* promoters is regulated by the ratio of auxin to cytokinin in tumor tissues.

**DISCUSSION**

The results described above indicate that the promoter activity of the *mas* gene derived from the T-DNA of an *A. tumefaciens* Ti plasmid is regulated in both tumorous and differentiated plant cells by phytohormones. This observation is intriguing since bacterial Ti and Ri plasmids carry in their T-DNA sets of genes that encode functions involved in the synthesis of auxin and cytokinin or in the determination of auxin sensitivity of plant cells. Auxin induction of the *mas* promoters and the modulation of their activity by the ratio of auxin to cytokinin suggests that a regulatory circuit involving T-DNA genes may exist that permits the fine tuning of T-DNA gene expression in response to physiological changes. The fact that in contrast to other T-DNA genes, the *mas* genes and the auxin biosynthesis genes *iaaM* and *iaaH* have been conserved during evolution in different T-DNAs of Ti and Ri plasmids further supports this hypothesis.

Hormonal induction of luciferase in stem sections and leaf discs and the influence of deleting the T-DNA tumor genes *iaa* and *ipt* indicate that cytokinin enhances and auxin mediates activation of the dual *mas* promoters. Induction of the *mas* promoters by wounding suggests a possible involvement of ethylene in their activation. However, we could not find evidence supporting this assumption. At this time, it cannot be ruled out that ethylene produced in vivo may modify the activity of the dual *mas* promoters in certain plant organs by reducing the uptake and polar transport of auxin (33).

Analogous to wound induction in leaf and stem tissues, protoplast isolation resulted in a rapid increase in activity of the *mas* promoters. The low levels of luciferase expression found in young stem and leaf tissues and the inhibition of wound-induced promoter activation by stem-derived substances indicate that the *mas* promoters can be repressed through inhibition of auxin action. Such a compound could be ABA since it is known that auxins maintain the synthesis of a high level of ABA-like substances that accumulate in leaves, reduce auxin levels in decapitated stem, and inhibit the growth of axillary buds (30, 31).

The observations described here contribute to the general scientific interest in crown-gall and hairy-root systems. Genes, such as the *mas* genes, carried by prokaryotic plasmids have acquired cis elements that permit their function to be fine tuned by auxin and cytokinin levels after their transfer and integration into plant cells.

We thank D. O’Kane for supplying light emission standards and luminometer standardization methods, O. Olsson for helpful discussions, and W. Su, K. Benschop, and A. Szenthe for technical assistance. This research was supported by Grant PCM-8410753 from the National Science Foundation and by a Government of Alberta Establishment Grant to A.A.S. We are indebted to L. White for typing this manuscript.