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Auxin-Induced Expression of the Soybean SAUR Locus in Transgenic Petunia

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Abstract. Three auxin-inducible genes of the soybean SAUR (small auxin up RNAs) locus, along with two flanking genes, have been transferred into petunia using *Agrobacterium tumefaciens* T-DNA transformation. Genomic analysis showed that two transgenic petunia plants contained one copy of the SAUR locus, and one plant contained approximately 10 copies of the transferred locus. Gene-specific probes were used to probe RNA blots, and our results indicated that the three genes were transcriptionally induced by the addition of the auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) to excised young leaves of the transgenic plants. This suggests that elements involved in auxin-regulated gene expression are strongly conserved in soybean and petunia. The two plants containing one copy of the SAUR locus showed nearly equal expression of all three SAUR genes after 2,4-D induction, while the plant with 10 copies showed a dramatic under-expression of two of the three genes following auxin administration. RNase protection analysis of the transcript for SAUR locus gene 6B showed that the transcription start site for this gene in the three transformed petunia plants was identical to that in soybean.

The SAUR (small auxin up RNAs) locus (McClure 1987; McClure et al. 1989) of soybean contains at least three different genes that are transcriptionally induced by a variety of auxins (McClure 1987; McClure and Guilfoyle 1987). The three genes, 6B, 10A5, and 15A, code for poly (A^+) RNAs of 530–560 nucleo-

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tides (McClure and Guilfoyle 1987). These three genes along with two additional highly homologous genes are clustered on an 8 kilobase (kb) genomic fragment of soybean DNA (McClure et al. 1989). It is not known at this time whether the two additional genes which border the 6B, 10A5, and 15A genes are also expressed in response to auxin application. Copy number reconstruction experiments suggest that there are one or two copies of the SAUR locus per soybean genome (McClure 1987; McClure et al. 1989). Sequence analysis of the SAUR cDNAs (McClure 1987; McClure et al. 1989) and the SAUR genomic locus (McClure et al. 1989) has revealed that all three of the auxin-induced genes are related, and each contains a TATA box at 25–30 base pairs (bp) upstream of the transcription start site as determined by S_1 mapping (M. Gee, unpublished results). In addition to the TATA box element, other conserved sequence elements are found in the 5′-flanking regions of all five genes; however, the significance of the upstream sequence is not known.

The functions of the products encoded by the SAUR locus genes are not known at this time. No significant homology has been found with other plant or animal genes of known function. It is clear, however, from a variety of growth and developmental studies that the genes are only induced by physiologically active auxins and that expression of the genes shows a strong correlation with cell extension (McClure 1987; McClure and Guilfoyle 1987). In addition, we have recently demonstrated that SAUR gene expression is regulated within intact seedlings which are gravistimulated (McClure and Guilfoyle 1989). The accumulation of these RNAs is largely restricted to the cortex and epidermis of the hypocotyl regions active in cell elongation. Therefore, even in the absence of external auxin application, the SAUR RNAs are most abundant in cells that are about to undergo or are in the process of rapid cell extension.

To gain more information of the possible functions of the SAUR locus gene products and the putative auxin-regulatory elements associated with these genes, it will be necessary to mutate these genes in vitro and reintroduce them into plants. For these studies, it is necessary to identify a suitable host plant that was easily transformable and regenerable. Because of the low efficiency of transformation and subsequent regeneration of soybean plants, mutation studies could not be conducted with soybean. At the same time, the recipient plant had to be similar enough to soybean so that the transferred soybean genes maintained their auxin-inducibility in the transgenic plant. To determine whether analysis of these genes might be carried out in transgenic petunia plants, we transferred 7 kb of the SAUR locus DNA into petunia via *Agrobacterium*-mediated T-DNA transformation. Petunia provided an easily transformable system that was also easily regenerated (Rogers et al. 1986). We report herein on three transgenic petunia plants that expressed the SAUR locus soybean genes in response to auxin application.

Materials and Methods

Transformation of Petunia

A 7-kb XhoI-HindIII SAUR DNA fragment of the soybean genome was subcloned from pXB21 (McClure et al. 1989) into the XhoI-HindIII sites in the

polylinker of the plant transformation vector, pMON505 (provided by Monsanto Corp., St. Louis, MO, USA). Bacterial matings and petunia (*Petunia hybrida* var. Mitchell diploid) leaf disc transformations were conducted using procedures described by Horsch et al. (1985) and Rogers et al. (1986). Transgenic plants were selected and verified by testing them for production of nopaline (Rogers et al. 1986) or by removing a leaf from a putative transgenic plant and allowing it to recallus on Murashige and Skoog medium (MS) (Murashige and Skoog 1962) plates containing 300 μg/ml kanamycin.

RNA and DNA Isolation and Blotting

Genomic DNA was isolated from 1- to 2-cm petunia leaves as described by Delaporta et al. (1983). The genomic DNA was incubated at 37°C for 12 h in 100 mM Tris-HCl (pH 7.5) containing 0.2% SDS and 200 μg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN, USA), phenol extracted twice, and precipitated with ethanol. Twenty micrograms of digested genomic DNA was electrophoresed on a 1% agarose gel (Maniatis et al. 1982) and blotted onto Zetaprobe (BioRad, Richmond, CA, USA). For RNA isolations, 1- to 2-cm leaves were removed from transgenic petunias and shaken in incubation media (2% sucrose, 10 mM potassium phosphate, pH 6) without auxin or incubation media containing 1 mM 2,4-dichlorophenoxyacetic acid (2,4-D) for 1 h. The high concentration of auxin was utilized because the SAUR genes in soybean show an approximately linear increase in expression as the 2,4-D concentration is increased from 1 μM to 1 mM (McClure and Guilfoyle 1987). The tissue was frozen in liquid N_2, and the RNA was extracted as described by Rochester et al. (1986). Soybean (*Glycine Max* var. Wayne) RNA was extracted from 3-day-old elongating hypocotyl sections after a 1 h incubation in 50 μM 2,4-D (McClure and Guilfoyle 1987). Ten micrograms of poly (A^+) petunia RNA prepared by passing total RNA through oligo (dT) cellulose one time (McClure 1987; McClure and Guilfoyle 1987), or 10 μg of total soybean RNA were electrophoresed on glyoxal gels (McMaster and Carmichael 1977) and blotted onto Zetaprobe. Gene-specific probes for RNA blots were prepared by synthesizing ^{32}P-labeled antisense RNA from cDNAs in pGEM vectors (Promega, Madison, WI, USA) which contained the 3′-untranslated regions for the gene 6B, 10A5, or 15A. Probes for genomic blots were prepared by synthesizing ^{32}P-labeled antisense RNA from full length cDNAs of genes 6B, 10A5, and 15A in Bluescript vectors (Stratagene, La Jolla, CA, USA). Blots were hybridized in 50% formamide, 5× Denhardt's, 5× SSC, 1 mg/ml denatured, sonicated salmon sperm DNA, 0.1 mg/ml polyadenylic acid, 0.2% SDS, 10 mM sodium phosphate (pH 7.0) at 42°C (Maniatis et al. 1982). All blots were washed in 0.2 × SSC and 1% SDS at 65°C. Autoradiograph signals were quantitated using an EC910 densitometer (E-C Apparatus Corp., St. Petersburg, FL, USA).

RNAse Protection Analysis

A 900 bp fragment containing 175 bp of the gene 6B coding region and 625 bp

of 5′-flanking sequences was subcloned into pGEM3 (Promega). A^{32}P-labeled RNA strand complementary to the gene 6B mRNA was synthesized and used with 10 μg petunia poly (A^+) RNA or 10 μg total soybean RNA in a protection assay described by Falvalora et al. (1980) with one modification. Instead of using S_1 nuclease, 300 μl digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 300 mM NaCl) containing RNAses A (40 μg/ml) and T_1 (2 μg/ml) were added to degrade the nonhybridized RNA. The RNAse digestion was stopped by adding 20 μl 10% SDS and 50 μg proteinase K and incubating at 37°C for 15 min. The reaction was phenol extracted and precipitated by adding 20 μg tRNA and 1 ml ethanol (for additional details see Promega Riboprobe Technical Bulletin).

Results

Genomic Blot Analysis

A 7-kb fragment containing auxin-regulated SAUR genes 6B, 10A5, and 15A and the two flanking genes X15 and X10A (i.e., it is not known whether the two flanking genes are responsive to auxin) (Fig. 1) was inserted into the *Agrobacterium tumefaciens* Ti vector pMON505 and transferred into petunia (Rogers et al. 1986). Three of the regenerated petunia plants, referred to as plants 2, 3, and 23, were confirmed as being transgenic based on kanamycin resistance (Rogers et al. 1986) and were used for further characterization of the SAUR locus genes.

Genomic blot analysis of the three transgenic plants was carried out to ensure that the entire SAUR locus had been transferred and had not been rearranged during transfer to petunias. Genomic DNA isolated from plants 2, 3, and 23 was digested with XbaI and HindIII to produce a 4.5-kb fragment containing genes 6B and 10A5 along with a 2.5 kb fragment containing gene 15A (Fig. 2B). The resulting Southern blot was probed with ^{32}P-labeled probes generated from nearly full-length cDNA clones for all three genes. Figure 2A shows that transgenic plants 3 and 23 contain approximately one copy of the entire SAUR locus per genome, while plant 2 contains approximately 10 copies of the SAUR locus per genome. Copy numbers were quantitated by densitom-

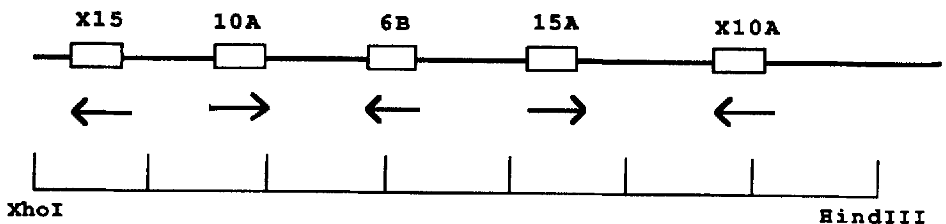

Fig. 1. Map of the SAUR locus in soybean. Genes 10A5, 6B, and 15A are used as hybridization probes in this study, and each of these three genes has been shown to be induced by auxin treatment (McClure 1987; McClure and Guilfoyle 1987). Genes X15 and X10A are highly homologous to the three internal genes, but the two flanking genes have not been shown to be expressed. In transgenic experiments described here, all five genes were transferred to petunia.

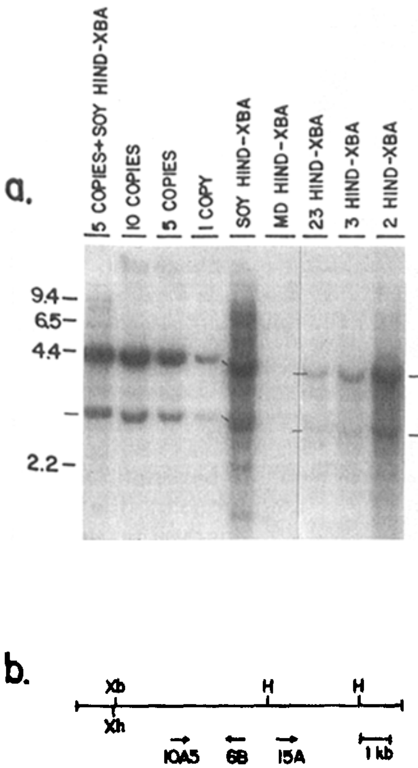

Fig. 2. (A) Genomic blot of petunia and soybean DNA. The blots were probed as described in Material and Methods. A reconstruction experiment is shown to the left where 1, 5, and 10 gene equivalents were blotted to estimate the copy number in petunia. The petunia or soybean nuclear DNA was cut with restriction endonucleases XbaI and HindIII. The untransformed petunia plant is labeled MD. Transgenic petunia plants are labeled 23, 3, and 2. (B) Map of the SAUR locus showing the XbaI, XhoI, and HindIII restriction sites. The XbaI to HindIII fragment is 4.5 kb and contains the auxin-responsive genes 10A5 and 6B, as well as the flanking gene X15, and the HindIII to HindIII fragment is 2.5 kb and contains the auxin-responsive gene 15A and the flanking gene X10A.

etry. The SAUR locus fragments were also apparent in soybean genomic DNA, but were absent from the nontransformed petunia genomic DNA.

RNA Blot Analysis

RNA blots were used to determine if the SAUR genes were induced in the three transgenic petunias when exogenous auxin was applied to excised young leaves. Poly (A^+) RNA was isolated from excised transgenic petunia leaves after a 1-h incubation in either incubation media minus auxin or incubation media containing 1 mM 2,4-D. The RNAs were blotted and hybridized with gene-specific ^{32}P-labeled probes generated from cDNAs that corresponded to the 3′-untranslated region of genes 6B, 10A5, and 15A. Under the conditions used, these probes did not cross-hybridize with one another (based on Southern blotting). Figure 3A shows that all three SAUR genes were induced by 2,4-D in plants 3 and 23 that contained a single copy of the soybean SAUR locus. Surprisingly, plant 2, which contains approximately 10 copies of the soybean SAUR locus, produced a transcript for gene 6B when auxin was administered, but produced only very weak signals corresponding to transcripts from genes 10A5 and 15A. The auxin-induced transcription responses were

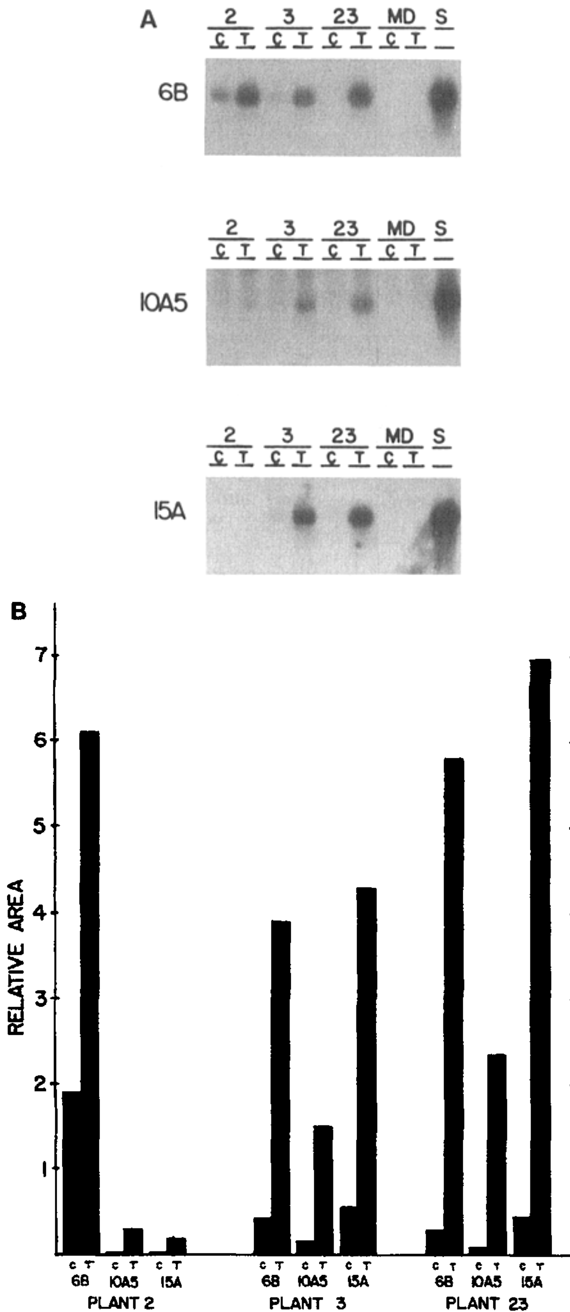

Fig. 3. (**A**) RNA blots of petunia and soybean RNA. RNA was isolated, blotted, and probed as described in Materials and Methods. MD is untransformed petunia, 2, 3, and 23 are transgenic petunia, and S is auxin-treated (50 μM 2,4-D for 1 h) soybean elongating hypocotyl sections. C is control or untreated petunia; T is 2,4-D-treated petunia. The size of the RNA detected in each case is 530–560 nucleotides. (**B**) Densitometer quantitation of the RNA blot results as shown in (**A**).

quantitated by densitometry, and these results are shown in Figure 3B. SAUR-like transcripts were not detected in nontransformed petunia plants.

RNAse Protection Analysis

To ensure that the 2,4-D-induced transcripts in the transgenic petunia plants had been initiated accurately, at least in the case of gene 6B, RNAse protection assays of transcript 6B from soybean and the transgenic plants were compared. Figure 4 shows that the initiation site of transcript 6B in soybean and in all three transgenic petunia plants is identical.

Discussion

We are interested in identifying *cis*-acting DNA elements which confer auxin-inducibility to certain plant genes, and ultimately in identifying the *trans*-acting factors that interact with the *cis*-acting elements. To identify the auxin-responsive *cis*-acting DNA elements, it is necessary to manipulate these elements (e.g., in vitro mutagenesis) and reintroduce them into a suitable host plant for transgenic analysis or into protoplasts for transient expression analysis. Because of the tissue-specific expression observed with the auxin-inducible SAUR locus genes (McClure and Guilfoyle 1989), it is unlikely that we will be able to thoroughly examine expression in transient assays with electroporated protoplasts; therefore, we have initiated efforts at identifying a suitable host plant for carrying out transgenic analysis. Soybean itself is not a suitable host for transgenic studies because of its currently low efficiencies for transformation and regeneration. On the other hand, petunia is a good host because it is easily and efficiently transformed and regenerated (Rogers et al. 1986). If we are going to employ petunia for transgenic studies, it is first necessary to demonstrate that soybean auxin-inducible elements are functional in petunia. In this report, we have demonstrated the utility of employing transgenic petunia for analysis of the auxin-inducible soybean *cis*-acting elements.

Sequence analysis of the SAUR locus in soybean has revealed highly conserved sequence elements within 250 bp of the 5′-flanking regions of five clustered genes (McClure et al. 1989; see Fig. 1). It is unclear which of these con-

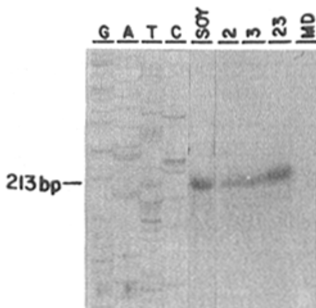

Fig. 4. RNAse protection analysis to determine the start site of transcription for gene 6B in soybean (soy) and transgenic petunia plants (2, 3, and 23). MD is untransformed petunia. A sequencing ladder is shown to the left (G, A, T, C).

served *cis*-acting sequences are responsible for the auxin-inducibility. The 7 kb SAUR fragment used for the transformation studies described here contains about 1.2 kb of DNA sequence flanking each of the three genes analyzed, and these flanking regions are likely to contain the *cis*-acting elements that confer auxin-inducibility as well as tissue- and organ-specificity (McClure and Guilfoyle 1989; McClure et al. 1989). It will now be necessary to examine the upstream regions of individual soybean SAUR genes in transgenic petunia in order to identify the specific elements involved in the regulation of their expression.

In the studies described here, we found that transgenic petunias containing one copy of the soybean SAUR locus expressed SAUR genes 6B, 10A5, and 15A in response to applied auxin. On the other hand, when 10 copies of the gene cluster were transferred to petunia, two of the soybean genes, 10A5 and 15A, were dramatically under-expressed in auxin-stimulated plants. Only gene 6B appeared to respond to auxin administration in the petunia plant with 10 copies of the SAUR locus. The 10-copy transgenic petunia plant appears to have the same SAUR gene organization as the single copy petunia plants and soybean plants (Fig. 2), so scrambling of the DNA during transformation does not appear to explain the poor auxin-inducibility of genes 10A5 and 15A in petunia carrying multiple copies of the SAUR locus. It is possible that two of the genes are methylated and inactive in the 10-copy plant, although it is unclear why this would happen in the 10-copy plant, but not in singly-copy plants. Another possibility is that a transcription factor is present in limited amounts in petunia, and that gene 6B has a higher affinity for this factor than genes 10A5 and 15A. This might then result in the failure of genes 10A5 and 15A to be expressed in response to applied auxin when multiple copies of gene 6B are present. We are currently attempting to test this latter possibility in transgenic petunias.

It is worth pointing out that while the soybean SAUR genes are expressed under auxin control in transgenic petunia plants, young petunia leaves produce 10- to 50-fold less SAUR transcripts than plumules of 7-day-old etiolated soybean seedlings. However, transcription was generally induced 10- to 20-fold in the transgenic petunia leaves while being induced only three- to four-fold in soybean plumules treated with 1 mM 2,4-D. This contrasts with the 50- to 100-fold induction observed with excised, auxin-depleted soybean elongating hypocotyl sections. It should also be noted that transgenic petunia plants, like soybean, produced SAUR transcripts in young leaves and petioles (i.e., soybean has also been shown to produce SAUR transcripts in elongating hypocotyl regions in the absence of applied auxin) without being induced with exogenous auxin. Because of the organ and tissue-specific expression of the SAUR genes observed in soybean seedlings (McClure and Guilfoyle, 1987, 1989), it will be important to carefully evaluate the auxin induction of the soybean SAUR genes in different tissues and organs at different maturity stages of regenerated petunia plants. To facilitate such studies in transgenic petunias, we are in the process of obtaining petunias transformed with the SAUR 5′-flanking regions fused to the reported gene, glucuronidase (GUS).

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