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EXPRESSION OF THE *SAUR-AC1* GENE OF *ARABIDOPSIS THALIANA*

By

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ABSTRACT

EXPRESSION OF THE *SAUR-AC1* GENE OF *ARABIDOPSIS THALIANA*

By

Pedro Gil

The Small Auxin Up RNA (*SAUR*) genes were originally characterized in soybean where they encode a set of unstable transcripts that are rapidly induced by auxin. In this study, the expression of a *SAUR* gene, designated *SAUR-AC1*, from *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia is characterized. The promoter of *SAUR-AC1* gene contains putative regulatory motifs conserved among soybean *SAUR* promoters, as well as sequences implicated in the regulation of other genes in response to auxin. Accumulation of *SAUR-AC1* mRNA is readily induced by natural and synthetic auxins and by the translational inhibitor cycloheximide. Moreover, several auxin- and gravity-response mutants of *Arabidopsis* exhibit decreased accumulation of the *SAUR-AC1* mRNA in elongating etiolated seedlings. These studies indicate that *SAUR-AC1* will be a useful probe of auxin-induced gene expression in *Arabidopsis*. To monitor the contribution of *SAUR* promoter and downstream sequences on mRNA accumulation, chimeric genes containing different regions of the *SAUR-AC1* gene have been introduced into *Arabidopsis* and tobacco plants. These experiments demonstrate that the *SAUR-AC1* promoter region

is responsible for auxin- and cycloheximide-induced mRNA accumulation in transgenic *Arabidopsis* and tobacco plants. In both types of plants, the promoter region is most active in aerial organs, including flowers, and also displays preferential expression in tissues that are potential targets for auxin action. In contrast, *SAUR-AC1* downstream sequences are partially responsible for cycloheximide-induced mRNA accumulation, but do not participate in auxin induction in transgenic tobacco. *SAUR* downstream sequences mediate an effect on mRNA accumulation in tobacco plants and stably transformed cells, with both the coding region and the 3' untranslated region (UTR) independently limiting mRNA accumulation levels. In order to measure the mRNA half-lives of chimeric transcripts including different regions of the *SAUR-AC1* transcript, we have used the tetracycline-regulated Top10 promoter system. Upon treatment with tetracycline, this promoter selectively stops transcription of transgenes in stably transformed tobacco cell lines. We have found that the coding region of *SAUR-AC1*, although sufficient to limit mRNA accumulation, does not decrease stability of the mRNA. The *SAUR-AC1* 3'UTR, which contains the putative mRNA instability determinant DST element conserved in plant *SAUR* genes, was shown to function as a mRNA instability determinant in plant cells. Consistent with the idea that multiple unstability determinants are present in 3'UTR of *SAUR-AC1*, disruption of a conserved motif of the DST does not create a stable mRNA.

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CHAPTER 1

INTRODUCTION

A number of plant mRNAs have been identified that accumulate rapidly following induction with natural or synthetic auxins (Theologis, 1986). In excised elongating soybean hypocotyls, one family of transcripts, known as the Small Auxin Up RNAs (*SAUR*s), begins to accumulate within 2.5 min after auxin application (McClure and Guilfoyle, 1987). Although the function of the *SAUR* gene products is unknown, the transcripts have been localized in tissues that are targets for auxin-induced cell elongation (Gee *et al.*, 1991). The observation that *SAUR*s appear before auxin-induced cell elongation is observed (McClure and Guilfoyle, 1987; Vanderhoef and Stahl, 1975), suggests that they may contribute to the process (McClure *et al.*, 1989). A correlation between *SAUR* gene expression and cell elongation has also been observed during the gravitropic response. In gravity-stimulated seedlings, an asymmetric accumulation of the *SAUR* transcripts is evident before visible bending of the plants occurs (McClure and Guilfoyle, 1989). The *SAUR* transcripts accumulate in the cells that are destined to elongate, presumably due to a rapid redistribution of endogenous auxin (McClure and Guilfoyle, 1989; Li *et al.*, 1991).

In an effort to elucidate mechanisms of auxin signal transduction, a number of mutants have been isolated that have altered responses to auxin (Estelle, 1992) and/or defects in gravitropism (e.g. Bullen *et al.*, 1990), a process considered to be controlled by auxin. Many of these mutants have been isolated in *Arabidopsis* because of the widely recognized advantages of this system for molecular genetics (Somerville, 1989). The analysis of auxin-responsive gene expression could provide a particular advantage in the characterization of these mutants because many of their physiological traits are tedious to score quantitatively, or develop too slowly to provide insight into the early events in auxin action. The main reason that auxin-responsive gene expression, as a means to characterize mutants, has received a minimal amount of attention to date is the paucity of well-characterized auxin-regulated genes of *Arabidopsis* that are suitable for these studies.

The accumulation of the soybean *SAUR* transcripts in response to auxin is due, at least in part, to transcription (Franco *et al.*, 1990), and is likely to involve regulatory sequences in the promoter region (Li *et al.*, 1994). The soybean *SAUR10A* promoter is mostly active in epidermal and cortical cells of transgenic tobacco stems (Li *et al.* 1991). Since *SAUR* transcripts were also localized in similar tissues of soybean hypocotyls (Gee *et al.*, 1991), it is likely that, as in the case of other plant genes, tissue-specificity of *SAUR* genes is determined by promoter sequences. However, it is unknown whether the localized expression of the soybean *SAURs* is common to all *SAUR* genes.

During gravitropism, *SAUR* transcripts disappear very rapidly from cells that are not targeted for enhanced elongation (McClure and Guilfoyle, 1989). This result, together with mRNA half-life measurements made using the transcriptional inhibitor Actinomycin D (Franco *et al.*, 1990) indicated that *SAUR* transcripts are very short-lived. In plants, as in other eukaryotic organisms, unstable transcripts seem to be the exception and not the norm. Experiments done in soybean cells estimated the half-lives of most plant mRNAs on the order of several hours (Silflow and Key, 1979). In general, the low stability of eukaryotic transcripts is an active process that depends on the presence of RNA sequences within the transcripts. Examples of RNA sequences that promote mRNA decay have been found both in coding regions and 3' untranslated regions (UTRs) of unstable transcripts of yeast and mamalian cells (Sachs, 1993). In some cases, multiple mRNA instability elements are present in a transcript. For example, multiple instability determinants were detected in the human *c-fos* mRNA, which remained unstable after one of its mRNA instability determinants had been inactivated (Shyu *et al.* 1989). This message encodes a transcriptional factor involved in the regulation of cell growth and development.

It has been observed that two synthetic elements cause mRNA instability in plants. One of this sequences consists of 11 copies of the AUUUA sequence motif. The AUUUA motif is present in multiple copies in the 3'UTR of certain unstable mammalian mRNAs, including *c-fos* and granulocyte-macrophage colony stimulating factor (GM-CSF) (Peltz *et al.* 1991). The AUUUA sequences are necessary for the 3'UTR of *c-fos* to function as an mRNA stability determinant (Shyu *et al.* 1989). In stably transformed tobacco cells, insertion of the AUUUA repeats into the 3'UTR of reporter transcripts was found to induce rapid transcript decay (Ohme-Takagi *et al.*, 1993). In transgenic tobacco plants insertion of the AUUUA repeats into the 3'UTR of a globin reporter gene was also shown to decrease mRNA accumulation (Ohme-Takagi *et al.*, 1993). Although AUUUA motifs have been observed in endogenous plant transcripts, it is not known whether they function as mRNA instability determinants.

A second sequence that functions as an mRNA instability determinant is a dimer of the DST element. The DST element consists of about 40 bases and is conserved downstream the coding region of the *SAUR* genes (McClure and Guilfoyle, 1989). A synthetic dimer of the DST element has been shown to destabilize the mRNA of reporter genes when inserted in their 3' UTRs (Newman *et al.*, 1993). In addition, the accumulation of reporter transcripts containing DST dimers was also limited in transgenic tobacco, suggesting that DSTs can also target transcripts for rapid decay in plants. However, only isolated dimers of a DST element have been shown to be sufficient to cause rapid decay of reporter transcripts, therefore it is still necessary to test if DST sequences contribute to the instability of native *SAUR* transcripts. At present, there is no evidence supporting the hypothesis that auxin affects the stability of the *SAUR* transcripts or the function of DST sequences (Li *et al.*, 1991; Newman *et al.*, 1993), suggesting that DST sequences act constitutively to destabilize the *SAUR* transcripts (Newman *et al.*, 1993).

To provide a *SAUR* gene that could be used as a molecular probe for the analysis of gravity-response and auxin-response mutants, as well as to identify potentially important regulatory features of *SAUR* genes, we sought to isolate a *SAUR* gene from *Arabidopsis*. In this study, we describe the isolation and characterization of the auxin-inducible *SAUR-AC1* gene of *Arabidopsis*. This gene shares many structural features with its soybean counterparts, including the presence of a DST sequence in the 3' UTR. The identification of auxin- and gravity-response mutants with defects in *SAUR-AC1* expression indicated that this gene should be an effective tool for studying auxin signal transduction in *Arabidopsis*. The role of the different regions of the *SAUR-AC1* gene in the control of its expression has also been investigated. By using chimeric genes we have observed that the *SAUR-AC1* promoter is responsible for auxin induction and preferential expression in certain tissues of *Arabidopsis* and tobacco. Another goal was to determine which sequences were responsible for *SAUR* mRNA instability. We used a regulated promoter to measure directly mRNA accumulation and mRNA half-lives of chimeric messages containing transcribed *SAUR* sequences. These studies demonstrate that the 3'UTR of *SAUR-AC1* limits accumulation of a transcript by targeting it for rapid degradation.

CHAPTER 2

MATERIALS AND METHODS

Isolation and Sequencing of *SAUR-AC1* Genomic and cDNA Clones

Primers corresponding to two highly conserved regions of the soybean *SAUR* open reading frames (ORFS) were synthesized as follows: 5' GCAGTCTATGT(T/C)GGAGA 3' and 5' CA(T/A)GG(T/A)ATTGTGAG(G/A)CC 3'. Amplification of DNA sequences flanked by these primers was accomplished by two sequential polymerase chain reaction (PCR) experiments using genomic DNA of *Arabidopsis thaliana* (L.) ecotype Columbia as template. The initial amplification was carried out in a volume of 100 μL and contained 100 ng of heat-denatured *Arabidopsis* genomic DNA, 163 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM of each dNTP, and 2.5 units of Taq DNA polymerase (Perkin Elmer). Reactions were subjected to 25 cycles of 94°C for 1 min, 37°C for 2 min, and 72°C for 3 min. After a final 7 min incubation at 72°C, the PCR products were separated on a low melting temperature agarose gel and the major product (130-150 bp) was excised and used for a second round of PCR under the same conditions as above. The major PCR product was gel purified, blunt-end ligated into a plasmid vector, and the clones were sequenced using the dideoxy method of Sanger *et al.*, (1977). The deduced amino acid sequence of one of the PCR clones was found to be 78% identical to the soybean consensus and was used as a probe to isolate the corresponding gene designated *SAUR-AC1*. The gene resides on an EcoRI fragment of genomic DNA approximately 6 kb in length isolated from an *A. thaliana* (L.) ecotype Columbia genomic library that was kindly provided by Drs. Carrie Schneider and

Chris Somerville. A cDNA clone identical to positions +45 to +485 was isolated from an *Arabidopsis* cDNA library described previously (Taylor and Green, 1991) that was kindly provided by Drs. Alex Gasch and Nam-Hai Chua. Both the cDNA clone and the genomic clone were sequenced on both strands to generate the sequence shown in Figure 3-1.

Transgenic Arabidopsis Plants

A. thaliana ecotype RLD plants used for transformation were grown in square Petri dishes (80 X 25 mm) containing AGM medium (4.3 g/L Murashige-Skoog salts [Sigma], 3% [w/v] sucrose, 1X Murashige-Skoog vitamins [Sigma], 2.5 mM MES, pH 5.7, 8 g/L phytagar [Gibco]) placed vertically to facilitate collecting roots from the agar surface under sterile conditions. Root transformation was performed as described by Valvekens *et al.* (1988). Etiolated seedlings of transgenic *Arabidopsis* were grown in complete darkness for 7 to 10 days in Petri dishes (80 X 25 mm) containing AGMK medium (AGM medium with 100 μg/mL Kanamycin) under sterile conditions. Transgenic *Arabidopsis* plants for histochemical studies were grown in a 1:1:1 mixture of fine vermiculite, perlite and sphagnum pea under a 16-h light/8-h dark cycle at 20°C.

Transgenic Tobacco Plants

N. tabacum SR-1 plants were grown and transformed as previously described by Newman *et al.* (1993). Plantlets testing positive for expression of the *GUS* reference gene were transferred to soil, under conditions of 16-h light/8-h dark at 27°C and 75% humidity. For analysis of RNA levels (Figure 3-7), plants were grown to the 10- to 14-leaf stage, and a fully expanded leaf of the third pair from the apex was harvested and frozen in liquid nitrogen prior to extraction of RNA.

BY-2 Cells

Untransformed *Nicotiana tabacum* cv Bright Yellow 2 (BY-2) cultured cells, were grown and subcultured as described previously by Newman *et al.* (1993). To facilitate transformation, constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation under the conditions recommended by the manufacturer (Bio-Rad: 25 μF, 2.5 kV, 200 Ω). *Agrobacterium* strains containing the construct of interest were used to transform BY-2 cells as described previously (An, 1985) with minor modifications described by Newman *et al.* (1993). In the case of Top10 chimeric genes, 2 rounds of transformation of the same cells were necessary. The first transformation was performed to introduce the *35S-TETVp16* construct. This construct contains the hygromycin resistance (*HPT*) gene, making possible for transformed cells to be selected on NTCH medium (NT medium with 500 μg/mL carbenicilin and 50 μg/mL hygromycin). Individual transformed calli were tested for expression of the TETVp16 gene by Northern blot analysis as described below. Transformed lines with highest TETVp16 mRNA levels were selected and named BY-2(TetVp16). They were grown in liquid cultures and retransformed with Top10 constructs. These constructs included a kanamycin resistance (*NPTII*) gene, making possible a selection for retransformed cells using NTKCH medium (NT medium with 100 μg/mL kanamycin, 500 μg/mL carbenicilin and 50 μg/mL hygromycin). After 3 to 4 weeks individual calli were transferred to fresh plates and cultured at 28°C. Transformed lines were screened for expression of reporter *GUS* genes by enzymatic assay (β-Glucuronidase) and subsequently by Northern blot analysis. Liquid cultures were generated by resuspending transformed calli in 10 mL of NTKCH liquid medium (without phytagar), and subsequently were subcultured weekly.

Gene Constructions and Site-directed Mutagenesis

Test genes were constructed in pBluescript SKII- (Stratagene) intermediate vectors that have the following cassette structure: SacI-Promoter-BglII-XbaI-Coding region-BamHI-3'UTR-ClaI. The *SAUR-GUS-E9* test gene contains the -2300 to +30 promoter region of *SAUR-AC1* fused to the *GUS-E9* gene, constructed by exchanging the 3'UTR of the *GUS-3C* and the *CAT-E9* genes described by Fang *et al.* (1989). The *SAUR-GUS-E9* gene was inserted between the SacI and ClaI sites of the polylinker of the plasmid p847 described by Newman *et al.* (1993). The *35S-SAUR-E9* gene includes the (-940 to +9) 35S promoter (Fang *et al.* 1989), the +61 to +352 fragment of the *SAUR-AC1* gene which contains 26 nucleotides of the 5' untranslated leader and the complete coding region followed by the *E9* 3'UTR of the *CAT-E9* gene described by Fang *et al.* (1989). The *35S-Globin-SAUR* gene includes the same 35S promoter fused to the human β-globin coding region described by Newman *et al.* (1993), followed by the +353 to +1347 *SAUR-AC1* 3'UTR. The *35S-SAUR-SAUR* gene includes the same 35S promoter fused to the +61 to +1347 fragment of the *SAUR-AC1* gene. These three genes and the 35S-*Globin-E9* gene, which contains the same 35S promoter fused to the globin coding region and the *E9*

3'UTR fragments described above, were inserted between the SacI and ClaI sites of the polylinker of the plasmid p851 described by Newman *et al.* (1993). Top10 constructs were derivatives of pMON505 (Rogers *et al,*, 1987) that contain a *Top10-GUS-3C* reference gene cassette inserted in the HpaI site and test genes *Top10-Globin-E9*, *Top10-SAUR-E9* and *Top10-Globin-SAUR* inserted between the SacI and ClaI sites of the polylinker. The Top10 promoter consists of the EcoRI-BglII fragment of pTop10 (Weinnann *et al.*, 1994). Coding regions and 3'UTRs are identical to the corresponding constructs driven by the 35S promoter. To generate the 35S-TetVp16 construct, the EcoRI-HindIII fragment containing sequences encoding TETVP16 from the plasmid pTet1-Vp16 described by Weinnann *et al.* (1994) was inserted between the EcoRI and HindIII sites of the binary vector pBIG-HYG (Becker, 1990). Site-directed mutagenesis of the ATAGAT sequence of the DST element was performed as described by Kunkel *et al.* (1987). We used as a template a *Globin-SAUR* pBluescript SKII(-) intermediate vector and the primer: 5' GGAATATACAATACGCATGCCGTAATTGATC 3'. ATAGAT mutant clones were screened for the presence of the SphI restriction site present in the mutagenic oligo and their identities were confirmed by DNA sequencing.

Auxin and cycloheximide treatments

Transgenic etiolated seedlings from *Arabidopsis* and tobacco were sectioned at the base of the hypocotyls and the roots were discarded. The hypocotyls were cut into 2- to 3- mm sections and incubated in KPSC (10 mM potassium phosphate, pH 6; 2% [w/v] sucrose, 50 μM chloranphenicol) medium for 4 hours to deplete endogenous auxins (McClure and

Guilfoyle, 1987). Samples were transferred to fresh buffer with or without 50 μM 2,4-D or 70 μM cycloheximide for 1h at 28°C and then frozen in liquid nitrogen.

Histochemical Analyses

Intact seedlings and hand-cut sections were placed directly into a solution containing 1 mM X-Gluc, 0.025 mM KFeCN, 0.025 mM K_2FeCN, 0.01% Triton X-100, 50 mM NaH_2PO_4 pH 7, 1 mM EDTA and 10 mM β-mercaptoethanol and vacuum infiltrated for 30 minutes. Subsequently, they were incubated at 37°C for 12 hours or until sufficient staining developed. Destaining and mounting of the samples were performed as described by Benfey *et al.*, (1989). Photographs were taken using a Wild M420 microscope (Heerburg).

S1 Nuclease Protection Analysis

The 5' end of the *SAUR-AC1* mRNA was analyzed by S1 nuclease protection as described previously (Newman *et al.*, 1993), with minor modifications. The probe was a single-stranded DNA fragment covering the sequence from -414 to +141 in Figure 3-1 that was labeled according to Nagy *et al.* (1987). A primer complementary to the sequence from +119 to +141 was used to prime synthesis of the probe and generate a sequencing ladder to size the protection products. 20 μg of total RNA from *Arabidopsis* seedlings treated with 2,4-D as described above were used for the S1 nuclease protection reaction.

RNA isolation and Northern Blot Analysis

Total RNA was isolated from 7 to 10-day old etiolated seedlings, fully developed leaves from mature tobacco plants or BY-2 cells, essentially as described by Newman *et al.* (1993), except that a second phenol extraction was performed after solubilizatium of the lithium chloride precipitate. Total RNA samples were denatured and separated on formaldehyde/agarose gels. Following transfer to Biotrace HP (Gelman), the blots were treated as described by Taylor and Green (1991). Radioactive DNA probes were synthesized using a random primed DNA kit (Boehringer) as recommended by the supplier. Coding region probes were made using as templates XbaI-BamHI fragments isolated from pBluescript intermediates. The probes were *GUS, Globin, SAUR* and *CAT*. 3'UTR probes were made using as templates BamHI-ClaI fragments from pBluescript intermediates. The probes were *E9* and *SAUR*.

Half-Life Determination

Half-life determinations were performed on stably transformed BY-2 cell lines three to four days after subculture. Tetracycline was added to the culture to a final concentration of 10 μg/mL, and 10 mL samples were removed from the culture every 15 min for 75 min. Each sample was immediately sedimented at 700 *g* for 1 min and frozen in liquid nitrogen. Following analysis of the RNA on RNA gel blots as described above, signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The values for each time course were subjected to linear regression analysis using SigmaPlot (Jandel Scientific, Corte Madera, CA) to calculate mRNA half-lives.

CHAPTER 3

CHARACTERIZATION OF THE *SAUR-AC1* GENE FOR USE AS A MOLECULAR GENETIC TOOL IN *ARABIDOPSIS*

RESULTS

Structure of the *SAUR-AC1* Gene

The soybean *SAUR* genes contain several stretches of highly conserved amino acids and no introns (McClure *et al.*, 1989). Based on these observations, we devised a strategy to generate an *Arabidopsis SAUR* probe from genomic DNA using the polymerase chain reaction (PCR). Oligonucleotide primers corresponding to the most highly conserved regions of the soybean *SAUR* genes were synthesized as described in Materials and Methods, and used to amplify *Arabidopsis* genomic DNA. A major amplification product of the expected size (about 140 bp) was isolated, reamplified and cloned into a plasmid vector. The inserts from twenty-five clones were partially or completely sequenced, but only one was found to contain sequences homologous to the soybean *SAUR* genes. This insert was used as a probe to isolate genomic and cDNA clones of the corresponding gene, which was designated *SAUR-AC1* (for *Arabidopsis* Columbia *SAUR* gene 1).

Figure 3-1 shows the nucleotide and deduced amino acid sequence of *SAUR-AC1*. Similar to soybean *SAUR* genes (McClure *et al.*, 1989), *SAUR-AC1* potentially encodes a small (89 amino acid) polypeptide and contains no introns. Approximately 400 bp of the promoter region and 500 bp 3' of the gene have been sequenced, allowing for the identification of a TATA box at -39, and two putative poly(A) signals (underlined

Legend to Figure 3-1. The nucleotide and deduced amino acid sequences of the *SAUR-AC1* gene. Capitalized nucleotides indicate transcribed sequences. Numbers on the left refer to the nucleotide sequence. The first nucleotide of the *SAUR-AC1* mRNA is numbered +1. The sequence for the putative TATA box (-39) and promoter sequences (Z element, -51; DUE element, -84; NDE element, -105; A box, -255) found in other auxin-regulated promoters, and in the *SAUR-AC1* promoter, are boxed. A DST element in the 3' UTR is indicated by shading. Two possible poly (A) signals are underlined. All nucleotide positions designate the 5'-proximal nucleotide of the corresponding sequence.

This figure represents the data derived from sequencing SAUR-AC1 cDNA and genomic clones.
Cloning and sequencing of the genomic clones were performed by Yang Liu and Elizabeth Verkamp

```
-416  tag tca aat ttt cct ttc ttg cca cag aat ttt ttt gaa ggc ttt tgt
-368  gac ttt gtc agt ata taa tca aat cat gtg gtc ctg tct ttt gaa tat
-320  cat tga gaa gaa ggt atg act taa tca gtg ata gag act tgt ggt tca
-272  tta ggt aca caa ctt cat gtc cct aca taa tct ata gag aga ttt ggc
-224  atg tga tgt ctt tga gac aaa taa gcc cca atg aag tta tca tgt agg
-176  aca tca tta gac act aac ttc tca gac acc att tat tga ttt gtt ctt
-128  ggt tta gta tct aca acc caa agc cat gtg ctc tgt aat taa aaa gaa
 -80  aaa cct caa aaa tac tta atc att tct ttc ata cgt tcg ttt ata taa
 -32  caa gta act aaa ccc ctt tag att cac aaa ctA AAG AAG ATC CAA TTT

  17  TAA AAT CTC AAA GCT TTC TCC AAG ACT AAG AAA CAT TTA AGC TTC AGG

                                  Met Ala Phe Leu Arg Ser Phe Leu Gly Ala
  65  AAA ACA TAA GGG AAA ATA ATG GCT TTT TTG AGG AGT TTC TTG GGT GCT

      Lys Gln Ile Ile Arg Arg Glu Ser Ser Ser Thr Pro Arg Gly Phe Met
 113  AAG CAA ATT ATT CGA AGG GAA TCA TCG TCG ACA CCA AGA GGA TTC ATG

      Ala Val Tyr Val Gly Glu Asn Asp Gln Lys Lys Lys Arg Tyr Val Val
 161  GCG GTC TAT GTA GGA GAG AAT GAT CAG AAG AAG AAG AGA TAT GTG GTG

      Pro Val Ser Tyr Leu Asn Gln Pro Leu Phe Gln Gln Leu Leu Ser Lys
 209  CCG GTT TCA TAC TTA AAC CAG CCT TTG TTT CAA CAA CTG TTG AGT AAA

      Ser Glu Glu Glu Phe Gly Tyr Asp His Pro Met Gly Gly Leu Thr Ile
 257  TCT GAG GAA GAG TTT GGT TAT GAT CAT CCA ATG GGC GGC TTA ACA ATA

      Pro Cys His Glu Ser Leu Phe Phe Thr Val Thr Ser Gln Ile Gln  *
 305  CCA TGT CAT GAA TCT TTG TTC TTC ACA GTC ACA TCT CAG ATA CAA TGA

 353  AGT ACT ATA CTA CAA CAT TTC CAT ATT TTT TTT AGA TTG TTA GCT AAT

 401  TTC CCC TGG AGA TAA TTG TAA ATT GTT TCA ATG AGA AGA ATA TAC AAT

 449  ACA TAG ATC GTA ATT GAT CAA TGC GTA TTT GCA TGT Taa tac att tgt

 497  gtc ttg tac caa aaa aag gaa tta tac att tgt gtc att taa ctc tgg
 545  aca cca tac att tcg tca tta cag tga aac ggc aga att tga aca ctc
 593  att att ctg gtt ggt agt tat ttc cat att tct caa aga aca ttt atg
 641  tga cta tta tca ttc ctt gcg aca act gta ata atg aga aaa ctt ggt
 689  att ttt ttt ggc tct tca tat aag ttg ttt aaa ata ggt ttc gaa gcc
 737  caa agc cca taa att aaa cgc cta aca ttc acg cgc tct ttg act atg
 785  gtt gct tag gaa cag atg cgc gtg ggg aag ttg gca ccg ttt ttt ctc
 833  ttg ctt aca tct att ttt ttt tct taa acg tct att tat ttg ctt tac
 881  gtc att gta acg ttt gtt tgt ttt ctc tgt atc gtt agt tgt tgt aca
 929  ctt gta cta tgg acg ttg aac tgc ggt tag tcc ggt tca gct agc att
 977  ttt taa atg tac atc tat ttt ctt att gat tat gtg tat aat gtt att
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Figure 3-1. The nucleotide and deduced amino acid sequences of the *SAUR-AC1* gene.

in Figure 3-1) located at +439 and +446 (37 and 44 bp, respectively, upstream of poly(A) addition site of the cDNA clone).

The calculated molecular mass and isoelectric point of the *SAUR-AC1* polypeptide are 10 kD and 9.2, respectively. The amino acid sequence does not contain a typical signal sequence, ER retention signal, or N-glycosylation signal, suggesting that the SAUR-AC1 polypeptide does not enter the secretory pathway. However, it is possible that SAUR-AC1 is a nuclear protein because it contains two short regions of basic amino acids (amino acids 18 to 23 and 47 to 50, as numbered in Figure 3-2) that may form a bipartite nuclear localization signal (Raikhel, 1993).

The deduced amino acid sequence of SAUR-AC1 is aligned in Figure 3-2 with that of the soybean *SAUR* consensus (McClure *et al.*, 1989) and a mung bean *SAUR* cDNA, designated ARG7 (Yamamoto *et al.*, 1992), that was recently isolated. The soybean *SAUR* genes are least similar upstream of amino acid 31 (McClure *et al.*, 1989), and the *SAUR-AC1* gene shows little similarity to the soybean or *ARG7* sequences in this region. In contrast, the sequences are highly similar within the region amplified initially with PCR from amino acid 38 to 87. Between these residues, *SAUR-AC1* is 78% identical to the soybean SAUR consensus and 80% identical to the *ARG7* amino acid sequence. Over the same region, the nucleotide sequence of *SAUR-AC1* is 75% identical to that of *ARG7* and 74% identical to the soybean prototype, *SAUR-15A* (McClure *et al.*, 1989). There were two mismatches between the *SAUR-AC1* nucleotide sequence and each of the two primers used for PCR, which could explain the low frequency of SAUR clones isolated in that

```
                            1                      25                       50                       75                       100
                            .                       .                        .                        .                        .
SAUR CONSENSUS (Soybean)    MGFRlpGIRk-aSf--anq----asskavdvp-KGYlaVYVGe---KmrRFvIPVSYlNqPSFQdLLsQAEEEFGYdHPmGGLTIPCsEdvFq-iTscL--N
                            * *    :*   **  * *     **     * :*::******   * :*:*:******** ** *** :***************** * :*  :** :  :
SAUR-AC1                    MAF----LR---SFLGAKQIIRRESSS---TP-RGFMAVYVGENDQKKKRYVVPVSYLNQPLFQQLLSKSEEEFGYDHPMGGLTIPCHESLFFTVTSQI--Q
                            * *    :*   *   *::     **    :* :*::*******  *  *:***** ******* *** :********** ****** ****  :** :  *
ARG7 (Mung bean)            MGFRLPGIRKTLS---ARNE---ASSKVLDAPPKGYLAVYVGEN-MK--RFVIPVSHLNQPLFQDLLSQAEEEFGYDHPM-GLTIPCSEDLFQHITSCLSAQ
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Figure 3-2. Alignment of the deduced amino acid sequences of *SAUR-AC1*, the consensus sequence for the soybean *SAUR* genes (McC lure et al., 1989) and the *ARG7* cDNA from mung bean (Yamamoto et al., 1992). Identical amino acids are marked with asterisks and similar amino acids with colons. Similar amino acids are grouped as: A, S, T; N, Q; D, E; I, L, M, V; H, K, R; and F, W, Y.

experiment (<5%). Beyond amino acid 87, three of the five amino residues most highly conserved among the soybean *SAUR* genes are also found in *SAUR-AC1*. There are conservative changes at the other two positions. The overall size of the coding region is similar for all *SAUR* genes.

The promoter region of *SAUR-AC1* contains several sequence motifs that are homologous to those found in the soybean *SAUR* genes and other auxin-inducible genes (see boxes in Figure 3-1). These include the first 6 bp of the 8-bp A box and the last 7 bp of the 10-bp Z element, implicated in auxin induction of the pea PS-IAA4/5 and PS-IAA6 promoters (Ballas *et al.*, 1993), and nos promoter (An *et al.*, 1990), respectively. *SAUR-AC1* also contains a 12/14 bp match with the DUE element, and a 12/16 bp match with the NDE element, which are the most highly conserved sequences in soybean *SAUR* promoters (McClure *et al.*, 1989). Thus, there are many candidates for auxin-responsive elements upstream of the *SAUR-AC1* TATA box.

In Southern analyses shown in Figure 3-3, a probe covering the *SAUR-AC1* coding region hybridizes to a single band of genomic DNA unless the coding region contains a site for the restriction enzyme used (e.g., Hinc II and AccI). The lack of detection of other *SAUR* genes in these hybridizations, which were performed at high stringency, indicates that this probe is specific for the *SAUR-AC1* gene under these conditions. Several faint bands can be detected in most digests if low stringency conditions are used (data not shown). This is consistent with the observation that other *SAUR* genes are

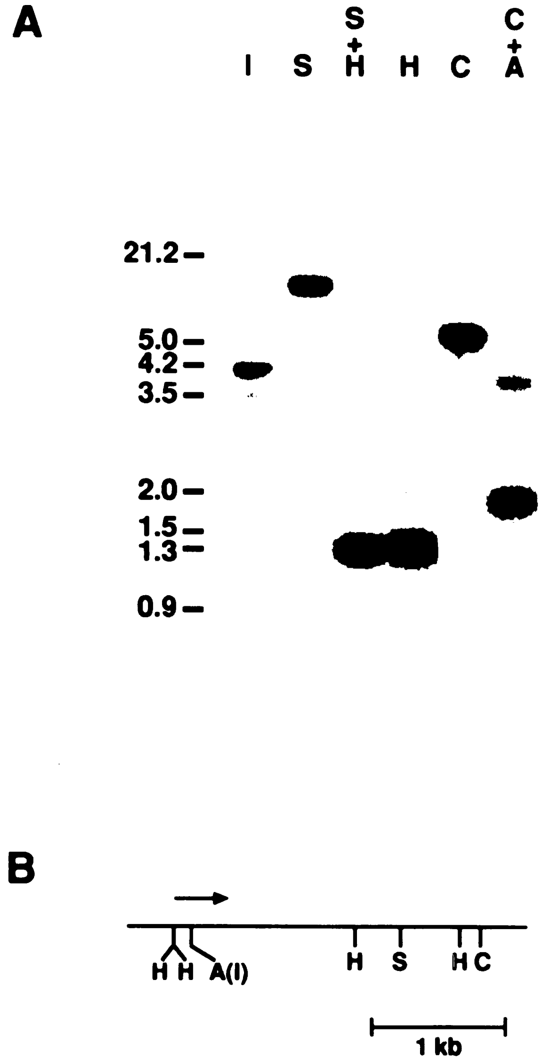

Figure 3-3. Southern blot hybridization analysis of the *SAUR-AC1* gene. (A) Samples of 10 μg of *Arabidopsis* genomic DNA were cut with HincII (I), SacI (S), SacI and HindIII (S+H), HindIII (H), ClaI (C) or ClaI and AccI (C+A) as indicated. The blot was hybridized to a ^{32}P-labeled probe covering the *SAUR-AC1* coding region as described in Materials and Methods. Size markers in kb are indicated on the left. (B) Partial restriction map of the *SAUR-AC1* gene derived from the genomic clone and its nucleotide sequence. Restriction enzymes abbreviations are as in (A). The arrow indicates the position of the *SAUR-AC1* coding region.

present in *Arabidopsis* (Guilfoyle *et al.*, 1992), but the sequences of these genes and their regulatory properties have not been described.

Structure of the *SAUR-AC1* mRNA

To map the 5' end of the *SAUR-AC1* mRNA, a single-stranded probe covering the region between -414 and +141 was annealed to total RNA from 2,4-D treated seedlings and digested with S1 nuclease, as described in Materials and Methods. Figure 3-4 shows the S1 protection products electrophoresed adjacent to a sequencing ladder of the probe. The 5' ends are clustered within a 7-bp region, with start sites at two A residues (see asterisks in Figure 3-4) being favored. The A residue designated as +1 in Figure 3-1 corresponds to the most 5' of the two As.

The 3' end of the *SAUR-AC1* mRNA shown in Figure 3-1 (at +485) was deduced from the position of poly(A) addition within the cDNA clone. Since only one cDNA clone was isolated, the presence of additional 3' ends cannot be ruled out; however, if they exist they may be located nearby, because the two putative poly(A) signals are adjacent to each other at about the expected distance (Mogen *et al.*, 1992) from the poly(A) site of the cDNA. Perhaps the most interesting sequence motif conserved among the 3' ends of *SAUR* genes is the DST element implicated in mRNA instability. One feature of DST sequences is that they consist of three highly conserved sequences separated by two more variable sequences (McClure *et al.*, 1989; Newman *et al.*, 1993). As shown in Figure 3-5, *SAUR-AC1* contains a DST element with these characteristics that is located 10 bases upstream of the poly(A) addition site of the transcript (see Figure 3-1).

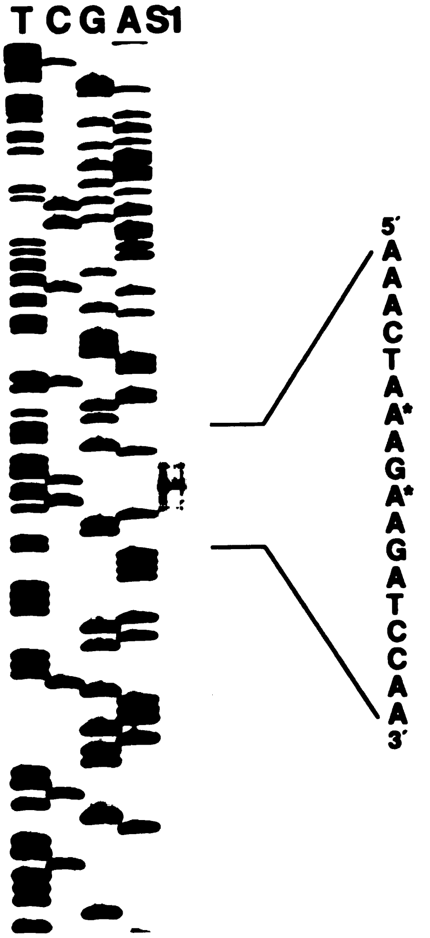

Figure 3-4. S1 protection analysis of the 5' end of the *SAUR-AC1* transcript. Transcriptional start sites were detected by alignment of the protected DNA fragment with a sequencing ladder of the sense strand of the *SAUR-AC1* DNA. The major protection products in the S1 lane are marked with asterisks on the nonsense strand sequence to the right.

This experiment was performed by Yang Liu

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Induction of SAUR-AC1 Expression in Wild-type and Mutants of Arabidopsis

Most studies of *SAUR* gene expression in soybean have been conducted using etiolated elongating soybean hypocotyl sections that respond rapidly to auxin treatment (McClure and Guilfoyle, 1987; McClure *et al.*, 1989). In an effort to use an analogous system to investigate the effect of auxin on *SAUR-AC1*, we performed a series of induction experiments using segments of etiolated *Arabidopsis* seedlings as described in Materials and Methods. Seedlings were incubated for 4 h in buffer in order to deplete endogenous auxin (McClure *et al.*, 1989) and then treated with either the synthetic auxin 2,4-D or the natural auxin IAA for 1 h. The level of *SAUR-AC1* is below the level of detection following the depletion step as shown in Figure 3-6, and the same was true in untreated seedlings (data not shown). Treatment with either 2,4-D or IAA led to a significant induction of the *SAUR-AC1* mRNA. The effect of the protein synthesis inhibitor cycloheximide (CHX) was also investigated because of the inductive effect of CHX on the soybean *SAUR* genes (Franco *et al.*, 1990). A very large increase in *SAUR-AC1* expression was observed following a 1-h treatment with CHX as shown in Figure 3-6. Treatment with 2,4-D plus CHX induced only slightly more *SAUR-AC1* mRNA than CHX alone. This is in contrast to the superinduction of *SAUR* mRNA that occurs in soybean seedlings subjected to treatment with 2,4-D and CHX (Franco *et al.*, 1990).

One of the main reasons for isolating an *Arabidopsis SAUR* gene is its potential

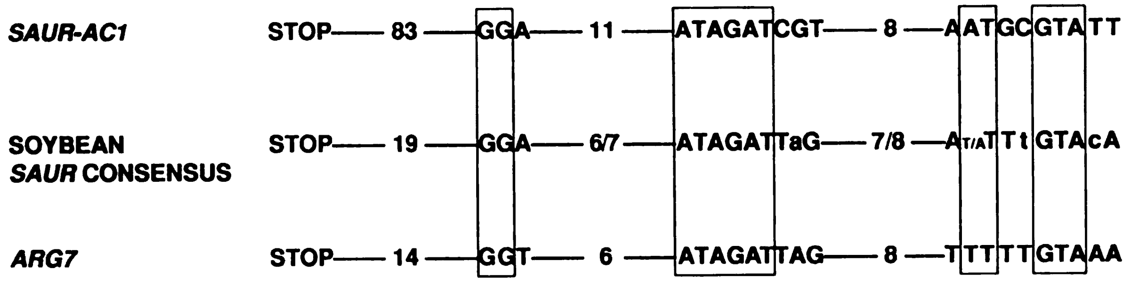

Figure 3-5. Alignment of DST sequences found in *SAUR* genes. Nucleotides that are identical in *SAUR-AC1*, *ARG7*, and the soybean consensus described by McClure *et al.* (1989) are boxed. The number of nucleotides in the variable regions separating each box or separating the DST elements from their respective STOP codons are indicated.

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use as a molecular probe to study signal transduction in a system amenable to molecular genetics. Therefore, it was of interest to investigate whether known mutants of *Arabidopsis* exhibit deficiencies in the expression of *SAUR-AC1*. We chose to focus on mutants with altered auxin and gravity responses because the influence of both of these stimuli on *SAUR* expression in soybean has been well documented.

Figure 3-7A shows a comparison of *SAUR-AC1* expression in wild-type and two auxin-resistant mutants of *Arabidopsis*, following 2,4-D treatment for 1 h. The most severe effect on transcript accumulation was exhibited by seedlings of the *axr2-1* mutant, where little or no expression was routinely observed. As shown in Figure 3-7C, when expression is observed in *axr2-1*, the level is less than 5% of the wild-type level. The *aux1-7* mutant induces about 45% less *SAUR-AC1* mRNA than the wild type.

Defects were also observed when gravity-response mutants (Bullen *et al.*, 1990; Bullen, 1992) were assayed for *SAUR-AC1* expression. Seedlings of two of these mutants, *mg20* and *mg421*, exhibit reduced induction of *SAUR-AC1* mRNA in response to 2,4-D, whereas seedlings of the third (*mg65*) induced wild-type levels of the mRNA (Figures 3-7B and 3-7C). None of the gravity-response or auxin-response mutants in this study express detectible *SAUR-AC1* mRNA in etiolated seedlings without 2,4-D treatment (data not shown).

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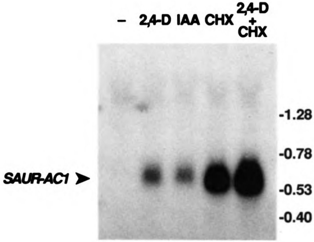

Figure 3-6. Northern analysis of *SAUR-AC1* mRNA levels in etiolated seedlings of *Arabidopsis*. Seedlings were depleted for endogenous auxin, and then subjected to one of the following 1-h treatments: none (-); 50 μM 2,4-D (2,4-D); 10 μM IAA (IAA); 70 μM cycloheximide (CHX); or 50 μM 2,4-D, 70 μM cycloheximide (2,4-D + CHX). Samples of 20 μg of total RNA were separated on a formaldehyde gel and blotted to nylon membrane. The blot was hybridized with a ^{32}P-labeled probe covering the *SAUR-AC1* coding region as described in Materials and Methods.

DISCUSSION

The isolation and characterization of the *SAUR-AC1* gene of *Arabidopsis* described in this report serves two important purposes. First, because *Arabidopsis* is rather distantly related to the legumes from which the other reported *SAUR* genes derive, the structural features conserved in *SAUR-AC1* should suggest which characteristics are of importance to *SAUR* genes in general. Second, the identification of an auxin-regulated *SAUR* gene of *Arabidopsis* provides a means to exploit the unique genetic resources of this model system so that the nature and mechanisms of auxin responses can be investigated at the molecular level.

Within the promoter regions of the soybean *SAUR* genes, the most prominent conserved elements are the NDE and DUE elements, both of which are also found in the *SAUR-AC1* promoter, albeit in the opposite order. Several other sequence motifs that have been implicated in auxin-responsive expression, either experimentally or on the basis of sequence conservation, are also present upstream of the *SAUR-AC1* transcription start site. In addition to the A box and the Z element shown in Figure 1, the *SAUR-AC1* promoter contains multiple copies of both the R and CCATT elements conserved among other auxin-inducible genes (Conner *et al.*, 1990; Gielen *et al.*, 1984, Slighton *et al.*, 1986; Hagen *et al.*,1991). In the future, it should be possible to employ deletion and gain of function

Legend to Figure 3-7. Expression of *SAUR-AC1* in auxin-resistant and gravity-response mutants of *Arabidopsis*. *(A) SAUR-AC1* mRNA accumulation in wild-type and auxin-resistant mutants of *Arabidopsis* ecotype Columbia. (B) *SAUR-AC1* mRNA accumulation in wild-type and gravity-response mutants of *Arabidopsis* ecotype Estland. Northern blots contained 20 μg total RNA per lane from wild-type or mutant seedlings following 2,4-D treatment as described for Figure 6. (C) Histogram of the relative accumulation of *SAUR-AC1* mRNA in 2,4-D treated, etiolated seedlings of *Arabidopsis* mutants expressed as a percentage of the expression in the appropriate wild-type parent. The results represent the data average ± the standard deviation from three independent experiments, each of which has been normalized to an endogenous internal standard as described in Materials and Methods.

The gravity-response mutants were analyzed in collaboration with Vladimir Orbovic and Ken Poff

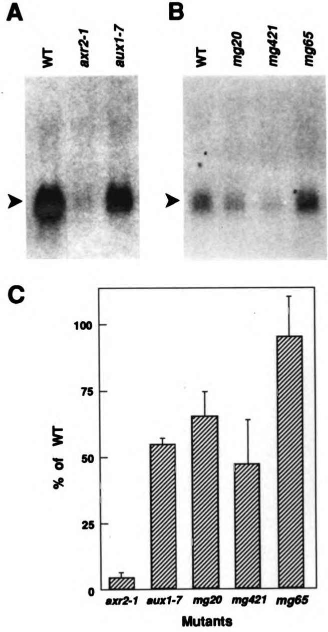

Figure 3-7. Expression of *SAUR-AC1* in auxin-resistant and gravity-response mutants of *Arabidopsis*.

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experiments to delineate the contribution of each of these sequence motifs to the transcriptional control of *SAUR-AC1*.

Another potentially important sequence, the DST element, was identified in the 3' UTR of the SAUR-AC1 transcript. DST sequences have also been identified downstream of the other SAUR coding regions. As in the case of SAUR-AC1, the cDNA sequence of ARG7 (Yamamoto *et al.*, 1992), demonstrates that the DST sequence falls within the transcribed region. The other *SAUR* mRNAs have not been mapped, but based on the proximity of the DST sequences to the coding regions in the soybean *SAUR* genes, it is likely that the DST elements are transcribed as part of the mRNAs in these cases as well. DST sequences have been proposed to contribute to the instability of the soybean *SAUR* transcripts (McClure *et al.*, 1989., Li *et al.*, 1991) and recently it was shown that DST sequences can destabilize both GUS and globin reporter transcripts in tobacco (Newman *et al.*, 1993). These observations indicate that the DST element within the *SAUR-AC1* mRNA may function as a determinant of mRNA instability. The alignment of DST sequences among SAUR genes (see Figure 3-5) demonstrates that the ATAGAT and GTA sequences in the second and third blocks are invariant and thus may be important functional determinants.

The time required for a transcript to reach a new steady state (higher or lower) after a change in transcription is dependent on the stability of the mRNA. A common characteristic of unstable transcripts is that they can be induced or repressed rapidly. The marked induction of the *SAUR-AC1* mRNA within 1 h in response to natural and synthetic auxins is therefore consistent with the transcript having a short half-life. In addition, many

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unstable transcripts can be induced by CHX, as is *SAUR-AC1*. At present it is unknown whether the CHX effect occurs because 1) a labile *trans*-acting factor required for rapid mRNA degradation or transcriptional repression is depleted, or 2) rapid degradation of the *SAUR* mRNA requires translation in *cis*. The soybean *SAUR* genes are also induced by CHX but this induction differs from that of *SAUR-AC1* in two respects. First, for the soybean *SAUR* genes, the mRNA accumulation following CHX treatment is only sightly greater than that following 2,4-D treatment. However, in the case of *SAUR-AC1*, CHX is a much stronger inducer of mRNA accumulation than is 2,4-D. The second difference, which is particularly striking, relates to the lack of superinduction of *SAUR-AC1* when CHX and 2,4-D treatments are combined. With the soybean *SAUR* genes, a prominent superinduction effect was observed under the same conditions (Franco *et al.*, 1990). This distinction indicates that the mechanisms by which CHX, and/or 2,4-D, act to induce *SAUR-AC1* and the soybean *SAUR* genes may differ.

One of the most promising approaches to the study of plant regulatory mechanisms is the isolation and analysis of mutants. In *Arabidopsis*, a number of mutants have been isolated that exhibit altered responses to hormones and to external stimuli that are presumed to be mediated by hormones. Although most of these mutants have been thoroughly characterized physiologically, in many cases the molecular analysis has been limited by the lack of availability of suitable molecular probes. The work described in this report demonstrates that the *SAUR-AC1* gene should provide a useful molecular marker for the study of both gravity and auxin-response mutants because seedlings of several of

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these mutants have observable defects in *SAUR-AC1* expression. The auxin-responsive mutants investigated in this study, *aux1-7* and *axr2-1*, were both isolated as auxin resistant, but they differ from each other with respect to many other characteristics such as their inheritance pattern, resistance to other hormones, response to gravity, and patterns of growth (Pickett *et al.*, 1990; Wilson *et al.*, 1990). Therefore, it is not surprising that the *aux1-7* and *axr2-1* mutations have different effects on *SAUR-AC1* expression. The accumulation of the *SAUR-AC1* transcript in response to 2,4-D is almost completely blocked in etiolated seedlings of *axr2-1* whereas only a modest reduction is evident in *aux1-7* seedlings. At present it is unknown whether the residual effect of the *aux1-7* mutation on SAUR-AC1 expression is due to incomplete inactivation of the *AUX1* gene in this mutant or because the mutation alters a pathway that is not totally responsible for induction of *SAUR-AC1* by auxin.

A modest reduction in expression of *SAUR-AC1* is also seen with two of the three gravity-response mutants analyzed. Although all of the mutants studied (including the auxin-resistant mutants) have some defect in gravitropism, this defect does not appear to correlate directly with *SAUR-AC1* mRNA levels in the mutant seedlings. In *mg20*, gravitropism is normal in root but altered in stem (Bullen, 1992; Wilson *et al.*, 1990), whereas in *aux1-7* the opposite is true. *mg421* exhibits defects in both root and stem gravitropism (Bullen, 1992); yet *mg20*, *aux1-7*, and *mg421* have similar effects on *SAUR-AC1* expression in seedlings (Fig. 3-7C). This lack of correlation also extends to *mg65* and *axr2-1*, both of which affect the gravity response in roots and shoots. Little or no *SAUR-*

AC1 expression was detected in *axr2-1* seedlings, while mRNA accumulation was induced normally in *mg65*. However, it should be noted that defects in gravitropism may be more likely to affect the distribution of the *SAUR-AC1* mRNA rather than its overall accumulation in the seedling. Work done with soybean *SAUR* genes has demonstrated that the redistribution of *SAUR* expression can be visualized using tissue printing (McClure and Guilfoyle, 1989) or promoter fusion studies (Li *et al.*, 1991). Similar studies with the *SAUR-AC1* gene may now be designed to reveal more subtle defects among the mutants affecting the gravity response.

The most obvious difference among the mutants studied in this report has to do with growth. With the exception of *axr2-1*, all of the mutants exhibit a normal stature. In contrast, *axr2-1,* which is the most deficient in *SAUR-AC1* expression, displays an extreme dwarf phenotype and very short internodes (Wilson *et al.*, 1990). Examination of the cellular anatomy of *axr2-1* has indicated that the primary reason for the shortened internodes is a reduction in cell elongation rather than cell number (Timpte *et al.*, 1992). Although the rapid induction of cell elongation by auxin is well documented, it has been difficult to attribute the elongation deficiency in *axr2-1* to a defect in auxin action because this mutant is highly pleiotropic (Wilson *et al.*, 1990). However, SAUR expression is highly correlated with the rapid effects of auxin on cell elongation. Therefore, the strong effect of *axr2-1* on *SAUR-AC1* expression and on elongation lends support to the possibility that the growth defect in *axr2-1* is the direct result of a defect in auxin action.

SAUR-AC1 may be useful not only in the evaluation of known phenotypes of auxin and gravity-response mutants, but may also detect phenotypes that are not morphologically evident. Moreover, the *SAUR-AC1* gene will likely be an expedient tool in evaluating double mutants for epistatic relationships and thus aid in efforts to elucidate how the components that comprise auxin signal transduction chains interact. Conversely, it is also likely that the aforementioned mutants can be used to enhance future expression studies of the *SAUR-AC1* gene as well as studies of SAUR function.

CHAPTER 4

EFFECT OF PROMOTER SEQUENCES ON THE EXPRESSION OF *SAUR-AC1*

RESULTS

Analysis of the activity of the *SAUR-AC1* promoter region in Arabidopsis plants

One of our goals was to test if the promoter region of *SAUR-AC1* controls transcript accumulation in *Arabidopsis*. With this aim in mind, we constructed a chimeric gene with the promoter region of *SAUR-AC1* fused to a GUS reporter transcript. Etiolated seedlings of transgenic *Arabidopsis* plants from two independent lines containing the *SAUR-GUS-E9* test gene and the *35S-CAT-3C* reference gene (Figure 4-1) were subjected to auxin or cycloheximide treatments. These treatments were identical to those found to induce accumulation of the endogenous *SAUR-AC1* transcript in *Arabidopsis* (Chapter 3). The accumulation of the test and reference transcripts was monitored by Northern blot analysis as shown in Figure 4-2. The *SAUR-AC1* promoter region (-2300 to +30) directed low GUS transcript accumulation in etiolated seedlings depleted of endogenous auxins. When auxin was supplied exogenously, the GUS-E9 transcript accumulated to significantly higher levels. A similar effect was observed in seedlings treated with cycloheximide. Such treatments had little effect on the accumulation of the reference transcript. This indicated that changes in GUS-E9 mRNA levels were specifically mediated by the *SAUR-AC1* promoter region and not due to a general increase on transcriptional activity of auxin- or cycloheximide treated cells.

Legend to the Figure 4-1. Vector with test and reference genes. The *SAUR-GUS-E9* gene was engineered into the binary vector p841 as described in Materials and Methods. The *SAUR-GUS-E9* test gene contains a 2Kb region of *SAUR-AC1* followed by the coding region of *GUS* gene and the E9 3'UTR, derived from the pea *RBCS-E9* gene. The construct containing the *SAUR-GUS-E9* test gene also includes a *35S-CAT-3C* reference gene. This gene consists of the 35S promoter followed by sequences encoding a bacterial chloranphenicol acetyl-transferase (*CAT*) and the 3' UTR of the pea *RBCS-3C* gene. Arrows indicate the direction of the coding regions.

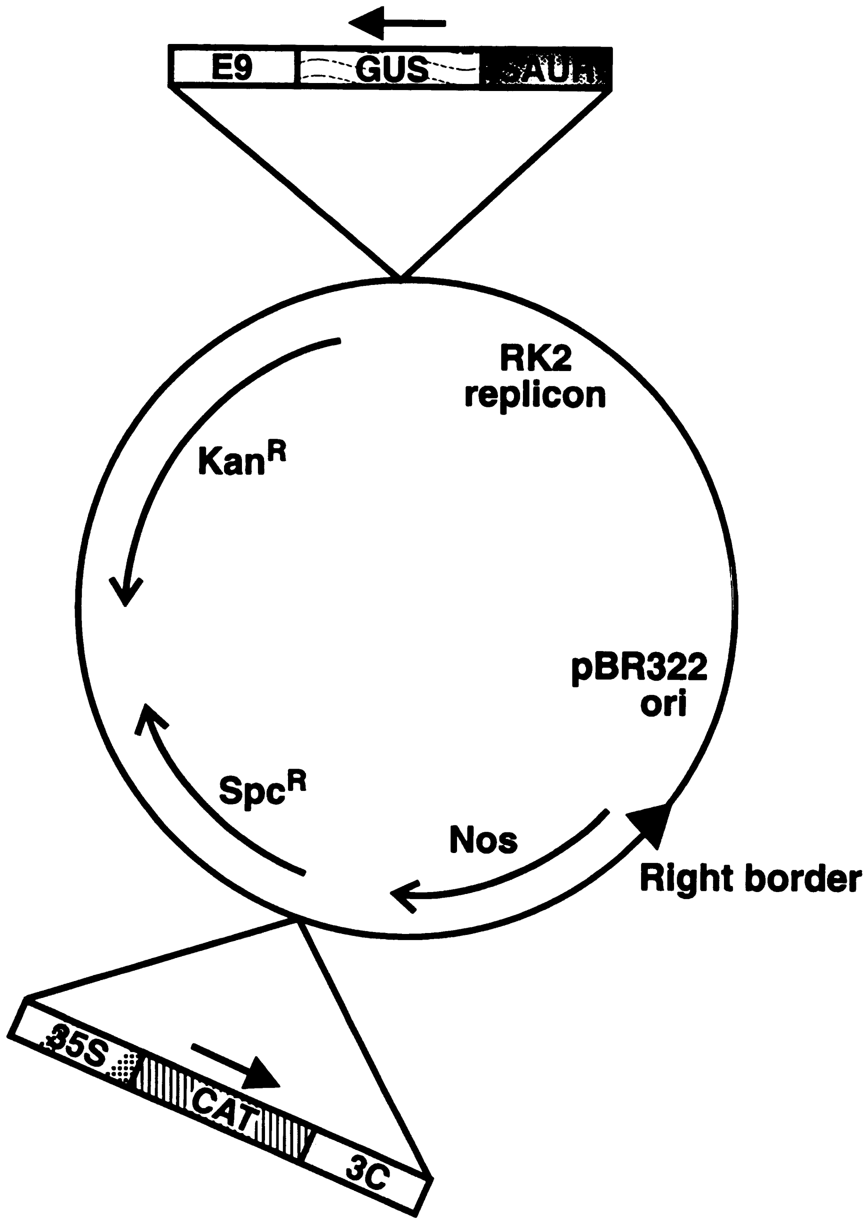

Figure 4-1. Vector with test and reference genes.

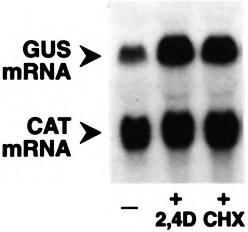

Figure 4-2. Induction of the *SAUR-AC1* promoter in transgenic *Arabidopsis*. Northern blot of 20 μg of RNA isolated from transgenic *Arabidopsis* containing the *SAUR-GUS-E9* test gene and the *35S-CAT-3C* reference gene. Sections of etiolated seedlings (progeny of regenerated transgenic plants) were depleted of endogenous auxin (-) or additionally treated for 1 hour with 50 μM 2,4-D (2,4-D) or 70 μM cycloheximide (CHX). The blot was hybridized with a GUS probe and a CAT probe.

We also sought to identify the *Arabidopsis* tissues where the *SAUR-AC1* promoter is active. Such tissues are potential targets for *SAUR-AC1* mRNA accumulation, thus indicating where the regulatory machinery is most likely to be active. A histochemical analysis of transgenic *Arabidopsis* plants containing the *SAUR-GUS-E9* gene described above was therefore performed. Promoter activity can be monitored in transgenic plants indirectly by observing where the activity of the *GUS* gene product, β-glucuronidase, is located. This method has been used extensively to localize the activity of a variety of plant promoters. Figure 4-3 shows the tissues where the *SAUR-AC1* promoter is active in *Arabidopsis*. This pattern of expression was confirmed by analyzing three transgenic lines. The *SAUR-AC1* promoter was active in plants grown both in darkness and light. GUS activity was present in all aerial tissues of etiolated seedlings (Figure 4-3A, upper panel). However, it was notably predominant in the elongating region of the hypocotyl (Figure 4-3A lower panel). Moderate GUS activity was occasionally detected in root tips (Figure 4-3A lower panel). Floral stems of light grown plants displayed highest GUS activity in cortical cells and vascular tissues (Figure 4-3C), although low GUS activity was also present in other tissues. Transverse sections of rosette leaves indicated high GUS activity in the vascular tissue, epidermis and adjacent cortex in the midrib, and in mesophyll cells of the lamina (Figure 4-3D). In addition high GUS activity was also detected in mature flowers (Figure 4-3E).

Legend to the Figure 4-3 Histochemical analysis of the expression of the *SAUR-GUS-E9* gene in transgenic *Arabidopsis*. (A) 7-day old etiolated seedling. (B) 7-day old light grown seedling. (C) Transverse section of floral stem. (D) Transverse section of midrib of a rossette leaf. (E) Mature flower

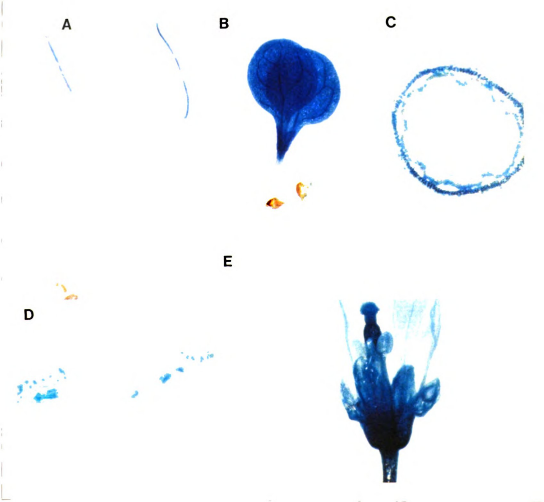

Figure 4-3. Histochemical analysis of the expression of the *SAUR-GUS-E9* gene in transgenic *Arabidopsis*.

Analysis of the activity of the *SAUR-AC1* promoter region in tobacco plants

The next question to address was whether the *SAUR-AC1* promoter was regulated in a similar fashion in tobacco. This was important because we wanted to use tobacco, a plant easily transformed, as a model system to understand the role of *SAUR* transcribed sequences in regulating transcript accumulation. The use of tobacco would allow the transcripts of the transgenes to be monitored by Northern blot analysis, without interference from the endogenous *SAUR-AC1* transcript. In addition, the soybean *SAUR10A* promoter was previously characterized in tobacco using a *SAUR-GUS* promoter fusion (Li *et al.* 1991). Thus, the study of the activity of the *SAUR-AC1* promoter in tobacco allows the activity of both *SAUR* promoters to be compared directly.

The strategy used to study the activity of the *SAUR-AC1* promoter region in *Arabidopsis* was also used to investigate the behavior of this promoter region in transgenic tobacco. Etiolated seedlings of transgenic tobacco containing the *SAUR-GUS-E9* gene were depleted of endogenous auxins or treated with either 2,4-D or cycloheximide. The co-transformed *35S-CAT-3C* reference gene was also present in the transgenic seedlings to serve as an internal standard. Northern blot analysis of mRNA accumulation revealed that the *SAUR-AC1* promoter region was sufficient to direct auxin-inducible expression in transgenic tobacco and accumulation of the *GUS-E9* transcript upon treatment with cycloheximide (Figure 4-4). The induction of the *SAUR-AC1* promoter under these

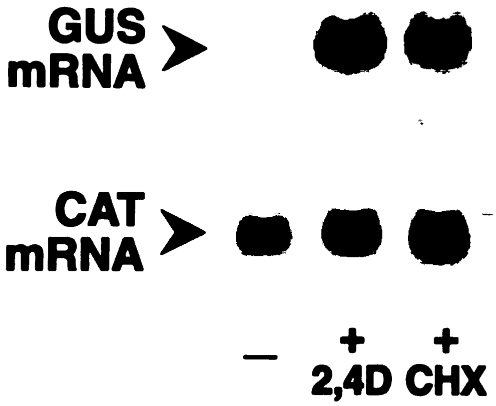

Figure 4-4. Induction of the *SAUR-AC1* promoter in transgenic tobacco. Northern blot of 20 μg of RNA isolated from transgenic tobacco containing the *SAUR-GUS-E9* test gene and the *35S-CAT-3C* reference gene. Etiolated seedlings were depleted yof endogenous auxin (-) or additionally treated for 1 hour with 50 μM 2,4-D (2,4-D) or 70 μM cycloheximide (CHX). The blot was hybridized with a GUS probe, stripped and hybridized with a CAT probe.

conditions was nearly identical in tobacco and *Arabidopsis*, the only difference being that the activity of the promoter appeared weaker in transgenic tobacco.

By using tobacco plants containing the *SAUR-GUS-E9* reporter gene as a visible marker, we have also performed a histochemical analysis of the transcriptional activity of the *SAUR-AC1* promoter region in tobacco (Figure 4-5). The *SAUR-AC1* promoter is mostly active in tobacco aerial tissues (Figure 4-5A). On occasion, we could detect low activity in the root tips (data not shown). Etiolated seedlings showed a similar pattern in tobacco and in *Arabidopsis*, but with lower GUS activity in tobacco (data not shown). Stems of mature tobacco plants displayed highest GUS activity in epidermal cells, cortical cells and vascular tissues (Figure 4-5B). Leaves also display high GUS activity with expression in the vascular tissue, the epidermis and its adjacent cortex (Figure 4-5C). Interestingly, an asymmetrical distribution of GUS activity was observed in the leaf laminae. Strong GUS activity was consistently present in the palisade parenchyma of the mesophyll (Figure 4-5D). GUS activity could also be seen in transgenic tobacco flowers (Figure 4-5E).

DISCUSSION

The expression of the *SAUR-AC1* gene in plants, is regulated at at least two different levels. The first level of regulation consists of the auxin-induction of *SAUR-AC1* mRNA accumulation that is mediated by the promoter region presumably by transcriptional activation. The second level consists of the differential activity of the promoter

Legend to the Figure 4-5 Histochemical analysis of the expression of the *SAUR-GUS-E9* gene in transgenic tobacco. (A) 7-day old tobacco seedling grown under white light seedling. (B) Transverse section of stem from mature plant. (C) Transverse section of midrib of a mature leaf. (D) Transverse section of the lamina of a mature leaf. (E) Petal from a mature flower

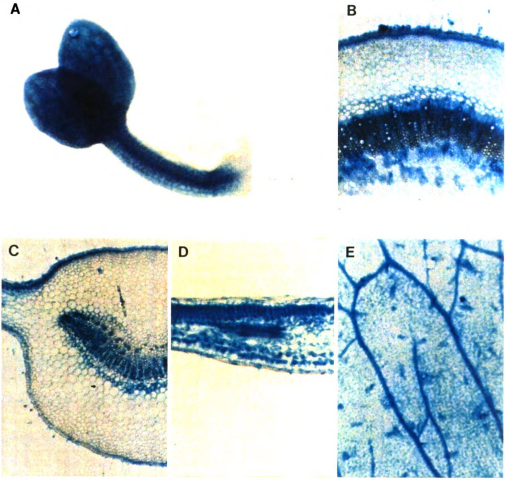

Figure 4-5. Histochemical analysis of the expression of the *SAUR-GUS-E9* gene in transgenic tobacco

region of *SAUR-AC1* in certain tissues of the plant.

With respect to transcriptional control of the accumulation of *SAUR-AC1* mRNA, we have found that the *SAUR-AC1* promoter region is responsible for auxin induction of the *SAUR-AC1* transcript. This is consistent with the presence of multiple sequences in the *SAUR-AC1* promoter which are conserved in other auxin-inducible promoters (Chapter 3). Less probable, but still possible is that other promoter sequences or a 30bp sequence from the 5'UTR may participate in auxin induction. We have also found that cycloheximide-induced accumulation of the *SAUR-AC1* transcript is, at least in part, mediated by promoter sequences. This indicates that this protein synthesis inhibitor is likely to promote transcriptional activation of the *SAUR-AC1* promoter, and suggests that a repressor may be a component of the auxin signal transduction pathway. Alternatively, cycloheximide could function to stimulate phosphorylation and thus stimulate transcriptional activation. This latter effect has been detected studying the activity of promoters from mammalian proto-oncogenes like *c-fos* and *c-jun* in cells treated with cycloheximide (Edwards and Mahadevan, 1992).

By generating transgenic *Arabidopsis* plants containing the *SAUR-GUS-E9* gene, we also generated a potential tool for the study of auxin-signal transduction. First, because the *SAUR-AC1* promoter is auxin inducible, these plants could allow mutants with diminished auxin-induction of the *SAUR-AC1* promoter to be isolated. Limited auxin-induction of *SAUR-AC1* mRNA accumulation occurs in several auxin-resistant and gravity-responsive mutants (Chapter 3). Second, plants containing the *SAUR-GUS-E9* gene can be crossed with mutants suspected to be affected in processes controlled by auxin. Accordingly, the effect of the mutations on the activity of the promoter could be examined.

The second level of regulation of *SAUR-AC1*, namely the spatial pattern of mRNA accumulation, is also most likely to be transcriptional, since the promoter region of *SAUR-AC1* is preferentially active in certain plant tissues. In etiolated seedlings of *Arabidopsis*, maximum activity of the promoter is localized to the elongating region of the hypocotyls, a fact consistent with the proposed role of *SAUR* gene products in cell elongation (McClure and Guilfoyle, 1989). In addition, transgenic tobacco stems display high promoter activity in the epidermis, a tissue suggested to direct auxin-induced cell elongation in stems (Nick *et al.*, 1990). GUS activity in epidermal cells was also observed in *Arabidopsis* albeit at a lower level. In leaves, it is interesting that an asymmetry of the distribution of GUS activity in mesophyll cells was observed, with stronger GUS activity in the upper section of the leaves. Again this effect was more evident in tobacco, but exhibited by *Arabidopdsis* as well. The high promoter activity in vascular tissues where auxin has been implicated in the differentiation process (Clutter, 1960), indicates that *SAUR-AC1* may play a role in cell differentiation in addition to auxin-induced cell-elongation. To reveal possible roles of auxin in specific tissues, mutants with altered localization of the expression of the *SAUR-AC1* gene can be identified and they could be affected in a subset of auxin responses. Interestingly, the *axr3* mutant is the first example of a mutant which displays ectopic activity of the *SAUR-AC1* promoter in roots (H.M.Ottoline Leyser, F. Bryan Pickett, Sunnethra Dharmaseri and Mark Estelle, unpublished results).

In transgenic tobacco, the activity of the *SAUR-AC1* promoter shows a similar pattern of tissue-specific expression in tobacco compared to the soybean *SAUR10A* promoter. However, certain differences could indicate that there are divergences between species or that different members of *SAUR* gene families are not expressed in identical fashion. For instance, the presence of *SAUR* expression in mature floral organs seems to be unique to *SAUR-AC1*. In contrast, the soybean *SAURs* are expressed in elongating filaments of the stamen (Li *et al.*, 1991). The different patterns of tissue-specific localization of *SAUR* expression among divergent *SAUR* promoters should make it easier to identify conserved promoter sequences mediating preferential expression in different tissues.

CHAPTER 5

EFFECT OF TRANSCRIBED SEQUENCES OF *SAUR-AC1* ON mRNA STABILITY

RESULTS

Effect of downstream sequences on SAUR-AC1 transcript accumulation

One of the goals of the experiments in this chapter was to test whether *SAUR* transcribed sequences contributed to auxin- and cycloheximide-induced mRNA accumulation. To this end, we used seedlings of transgenic tobacco plants containing the *35S-SAUR-SAUR* test gene and the *35S-GUS-3C* reference gene (Figure 5-1). These seedlings were subjected to treatments with 2,4-D and cycloheximide identical to the ones used to test the promoter region (Chapter 4). The effects were monitored by Northern blot analyses. As shown in Figure 5-2, application of the synthetic auxin 2,4-D had little effect on *SAUR-AC1* mRNA levels compared to the untreated control. The low induction of the *35S-SAUR-3C* gene after treatment with 2,4-D can be accounted for by a small inductive effect on the 35S promoter (1.4-fold increase in transcript accumulation for both *GUS* reference and *SAUR* test transcripts). In contrast, there is a specific increase in the amount of the *SAUR* message detected upon treatment with cycloheximide, compared to the GUS reference transcript. Interestingly, when downstream sequences of the *SAUR-AC1* were expressed under the control of the strong 35S promoter, very little *SAUR* mRNA accumulated. This indicated that downstream sequences constitutively limited the accumulation of the *SAUR*

Legend to the Figure 5-1. Structure of test and reference genes. A series of test and reference chimeric genes made for this study are shown. First, second and third boxes represent promoter region, coding region and 3'UTR respectively. The *35S-SAUR-SAUR* gene consists of the CaMV 35S promoter followed by the *SAUR-AC1* gene coding region and 3' UTR. The *35S-Globin-E9* and *Top10-Globin-E9* genes contain the 35S and Top10 promoters respectively, followed by the coding region of the human β-*globin* gene and the *E9* 3' UTR, derived from the pea *RBCS-E9* gene. The *35S-SAUR-E9* and *Top10-SAUR-E9* genes contain the 35S promoter and the Top10 promoter respectively, followed by the *SAUR-AC1* coding region and the *E9* 3' UTR. The *35S-Globin-SAUR* and *Top10-Globin-SAUR* contain the 35S and Top10 promoters followed by the β-*globin* coding region and the 3' UTR of *SAUR-AC1*. The *Top10-Globin-MSAUR* is identical to the *Top10-Globin-SAUR* except the sequence ATAGAT in the 3'UTR of *SAUR-AC1* was replaced by a SphI restriction site. All constructs in which test genes are under control of the 35S promoter also contain a *35S-GUS-3C* reference gene in which the *GUS* coding region is under the control of the 35S promoter and polyadenylation signals are provided from the 3' UTR of the pea *RBCS-3C* gene. Similarly, constructs in which test genes are under the control of the Top10 promoter also include a *Top10-GUS-3C* reference gene. Test genes along with suitable reference genes were engineered into the binary vector p951 (described in Chapter 2).

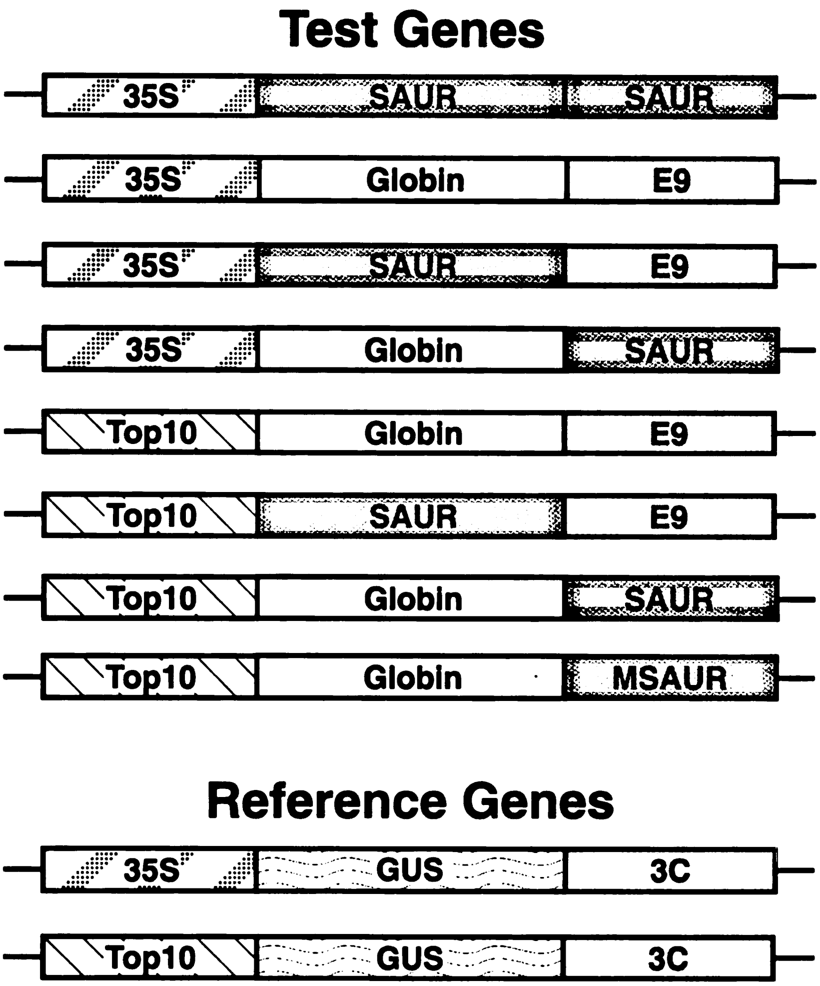

Figure 5- 1. Structure of test and reference genes.

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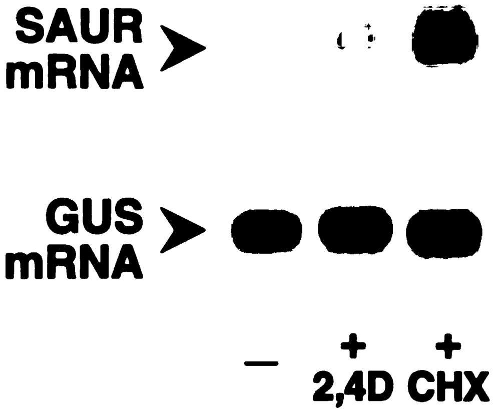

Figure 5-2. Effect of transcribed sequences on mRNA accumulation. Etiolated seedlings (the progeny of a primary transformant expressing the *35S-SAUR-SAUR* gene) were treated as described in Figure 4-2. RNA was isolated from the treated seedlings and 20 μg per lane was used for Northern blot analysis. The blot was hybridized first with a *SAUR-AC1* probe, stripped and reprobed with a GUS probe to generate the upper and lower panels, respectively.

transcript, an observation consistent with the idea that *SAUR-AC1* mRNA is rapidly degraded. To localize downstream sequences that limit *SAUR-AC1* mRNA accumulation, we have compared the mRNA levels of transgenes driven by an identical promoter containing different regions of *SAUR-AC1*. Our strategy was to replace the coding region or the 3'UTR of a control *35S-Globin-E9* test gene by equivalent *SAUR-AC1* sequences, introduce the constructs into tobacco plants, and compare transcript accumulation. The *35S-Globin-E9* gene consisted of the 35S promoter followed by the human β-*globin* coding region and the 3' UTR of the *RBCS-E9* gene (Figure 5-1). By replacing the β-*Globin* coding region by the *SAUR-AC1* coding region, we generated the *35S-SAUR-E9* test gene (Figure 5-1). The *35S-Globin-SAUR* test gene was generated by replacement of the *E9* 3'UTR of the *35S-Globin-E9* by the *SAUR-AC1* 3'UTR (Figure 5-1). In all constructs, as well as in the *35S-SAUR-SAUR* construct described above, there was also a *35S-GUS-3C* reference gene present on the same plasmid (Figure 5-1). We quantified test and reference mRNA levels by Northern blot analysis from at least seven independent transgenic tobacco plants for each construct. We compared pairs of constructs using the common part of the chimeric transcripts as a probe in simultaneous hybridization. For each plant, mRNA levels of test genes were normalized to the reference GUS-3C transcript to help to minimize position effects. To examine differences in transcript accumulation due to the coding region of *SAUR-AC1*, the *35S-GLOBIN-E9* and the *35S-SAUR-E9* test genes were compared (Figure 5-3A, first panel). For the same purpose, and also to test whether the *SAUR-AC1* coding region and its 3' UTR might act synergistically, mRNA

Legend to Figure 5-3. The effect of *SAUR-AC1* transcribed sequences on mRNA accumulation in transgenic tobacco plants. Dots represent test mRNA accumulation normalized to the internal standard GUS mRNA accumulation (Test mRNA/GUS mRNA) for each independent transgenic tobacco plant as determined by Northern Blot analysis. Bars represent the average value for each construct. To monitor the effect of *SAUR* transcribed sequences, test mRNA accumulation was compared for pairs of test genes that differ only in the region to be tested. Blots to be compared were simultaneously hybridized to the test probe which corresponded to the common part of the test transcripts being compared as a probe and the GUS reference probe. (A) To monitor the effect of the coding region, we compared mRNA levels from plants expressing the *35S-Globin-E9* gene to those of plants expressing the *35S-SAUR-E9* gene, and mRNA levels of plants expressing the *35S-GLOBIN-SAUR* gene to those expressing the *35S-SAUR-SAUR* gene. (B) To monitor the effect of the 3' UTR, we compared mRNA levels of plants expressing the *35S-GLOBIN-E9* gene to those of plants expressing the *35S-GLOBIN-SAUR* gene, and mRNA levels of plants expressing the *35S-SAUR-E9* gene to those of plants containing the *35S-SAUR-SAUR* gene.

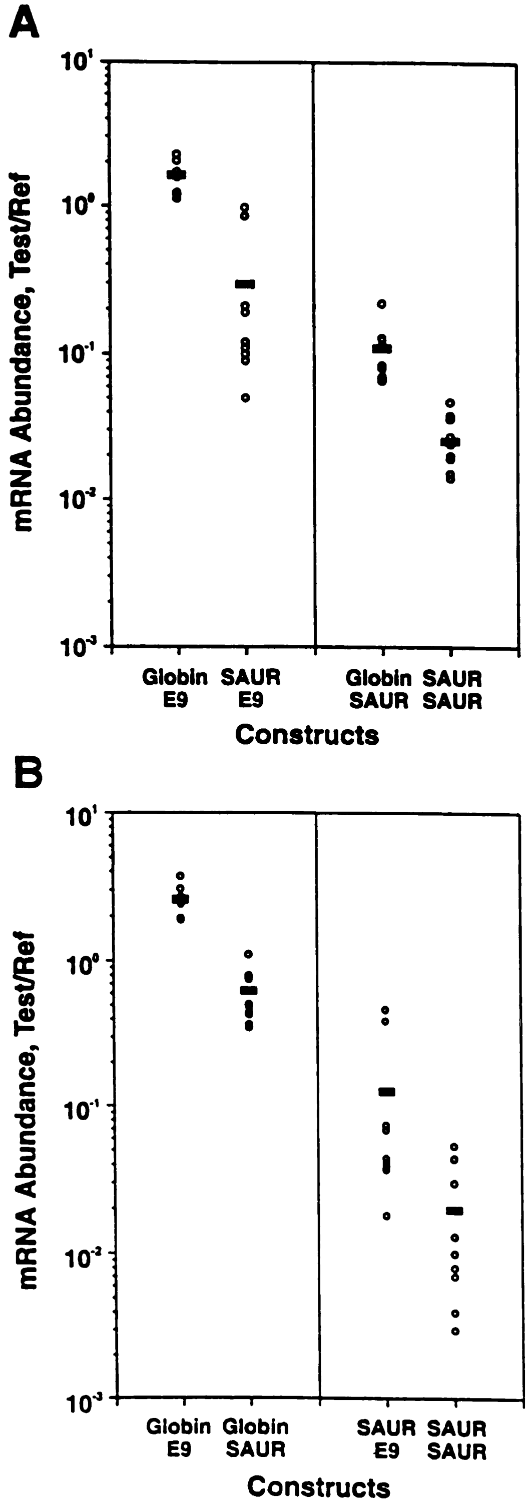

Figure 5-3. Effect of *SAUR-AC1* transcribed sequences on mRNA accumulation in transgenic tobacco.

accumulation of the *35S-GLOBIN-SAUR* and the *35S-SAUR-SAUR* test genes were also compared (Figure 5-3A, second panel). The data showed that replacement of the *Globin* coding region by the *SAUR* coding region resulted in an average decrease in transcript accumulation of about five-fold whether the coding region was followed by the E9 3' UTR or the *SAUR* 3' UTR.

The *SAUR-AC1* 3'UTR was examined in a similar fashion for its effect on transcript accumulation in transgenic plants. The data in the first panel of Figure 5-3B shows a comparison of the abundance of the *GLOBIN-E9* test transcript to the *GLOBIN-SAUR* transcript. Expression levels of the *35S-SAUR-E9* gene were also compared to the *35S-SAUR-SAUR* gene in the second panel of Figure 5-3B. Again the results were quite similar with both sets of constructs. Presence of the *SAUR-AC1* 3'UTR caused a four-fold and a six-fold decrease in mRNA levels when fused to the *Globin* or *SAUR* coding regions, respectively.

To further test the limited transcript accumulation due to *SAUR-AC1* transcribed sequences, we compared the levels of similar transcripts driven by the Top10 promoter, instead of the 35S promoter. Obtaining the same results with both promoters would exclude the possibility that the effects on mRNA accumulation were mediated by the specific interaction between the 35S promoter and *SAUR-AC1* dowstream sequences. In addition, to confirm that the effect on transcript accumulation caused by *SAUR-AC1* sequences observed in transgenic tobacco plants occurs also in tobacco cells, transcript accumulation was compared in stably transformed tobacco BY-2 cells. This was important because the BY-2 system, previously shown to be an effective model system for mRNA half-life measurements (Newman *et al.*, 1993; Ohme-Takagi *et al.* 1993), could then be applied to the study of *SAUR-AC1* sequences. To test the effect caused by coding region sequences, a large number (>50) of transformed calli containing the *Top10-Globin-E9* or the *Top10-SAUR-E9* test genes, together with a *Top10-GUS-3C* reference gene (Figure 5-1), were pooled and RNA was isolated and subjected to Northern blot analysis (Figure 5-4A). A *GUS-3C* reference gene under control of the Top10 promoter was present with each test gene to allow comparison of ratios of test to reference gene expression levels. Similarly, to monitor the effect of the *SAUR-AC1* 3'UTR on transcript accumulation, pools of transformed calli containing the *Top10-Globin-E9* or the *Top10-Globin-SAUR* constructs (Figure 5-1) were compared as shown in Figure 5-4B. From these experiments, we determined that both the coding region or the 3'UTR of *SAUR-AC1* are individually sufficient to decrease mRNA accumulation four-fold in comparison to the *GLOBIN-E9* transcript in stably transformed BY-2 calli, mimicking not only qualitatively but also quantitatively the effect seen in transgenic tobacco plants.

Effect of SAUR-AC1 transcribed sequences on mRNA stability

To determine if the low accumulation of the chimeric messages containing the *SAUR-AC1* coding region or 3'UTR was due to their rapid turnover, we directly measured mRNA half-lives of test genes by shutting off transcription in stably transformed tobacco celllines. The decay of test and reference transcripts could therefore be followed in the absence of on-going mRNA synthesis. Our first approach was to treat liquid cultures of stably

Legend to Figure 5-4. Effect of the *SAUR-AC1* coding region and 3' untranslated region on transcript accumulation in tobacco cell lines. BY-2(TetVp16) tobacco cell lines were stably retransformed with a *Top10-GLOBIN-E9*, a *Top10-GLOBIN-SAUR* or a *Top10-SAUR-E9* test genes. A *Top10-GUS-3C* reference gene was also cotransformed in each case, and RNA was isolated from pools of transformed calli. Northern blots containing 30 μg total RNA per lane were analyzed to compare mRNA levels of the *Top10-Globin-E9* gene and the *Top10-SAUR-E9* gene (A) or the *Top10-Globin-E9* gene and the *Top10-Globin-SAUR* gene (B).

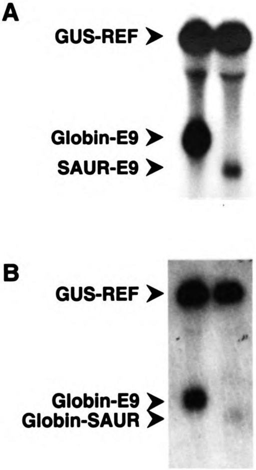

Figure 5-4. Effect of the *SAUR-AC1* coding region and 3' untranslated region on transcript accumulation in tobacco cell lines.

transformed BY-2 cell lines containing the *35S-Globin-E9*, *35S-SAUR-E9* and *35S-Globin-SAUR* test genes with the transcriptional inhibitor Actinomycin D. Subsequently mRNA decay rates were monitored as previously described (Newman *et al.* 1993). No significant effects on mRNA stability due to *SAUR-AC1* sequences were observed in those experiments (data not shown). We suspected that the general inhibitory effect on transcription by Actinomycin D may have interfered with the function of the possible *SAUR-AC1* mRNA instability determinants. To circumvent this potential problem, we measured mRNA half-lives of test and reference genes using the Top10 promoter (Weinnann *et al.*, 1994). This regulated promoter allowed us to specifically shut off transcription of test and reference genes without affecting transcription of endogenous genes.

Top10 consists of a series of Tet operators upstream of a TATA box and is dependent on the presence of a transcriptional activator, TETVP16 which binds to the Tet operators. TETVP16 is a protein fusion between the DNA-binding domain of the bacterial Tet repressor and the activation domain of the transcription factor VP16 of Herpes Simplex Virus (Weinnann *et al.*, 1994). Treatment with tetracycline rapidly inactivates TETVP16, and this in turn shuts off transcription from Top10. In order to measure the decay rates of our test and reference transcripts driven by the Top10 promoter, we first transformed BY-2 tobacco cell lines with a construct which contains the sequences encoding the fusion protein TETVP16 under the control of the 35S promoter. Subsequently, we selected stably transformed cell lines with the highest level of expression for the TETVP16 mRNA (data not shown). These cell lines were then retransformed with constructs containing test and reference genes under the control of the Top10 promoter. To measure mRNA half-lives, we treated stably double-transformed liquid cultures of BY-2 cells with tetracycline and RNA was isolated from samples withdrawn at 15 min intervals thereafter. Time course experiments were performed for transformed cell lines containing the *Top10-GLOBIN-E9* gene (Figure 5-5A), the *Top10-SAUR-E9* gene (Figure 5-5B), or the *Top10-GLOBIN-SAUR* gene (Figure 5-5C). All transformed cell lines also contained the *Top10-GUS-3C* reference gene. Northern blots were prepared, quantified, and mRNA half-lives were plotted (Figure 5-5D). These experiments showed that the half-lives of the GLOBIN-E9 transcript and the SAUR-E9 transcript were not significantly different. In contrast, the GLOBIN-SAUR transcript displayed a very short half-life of less than 20 minutes in the three lines tested. Because GUS is the reference transcript in all constructs, we could also determine that its half-life was less than 30 minutes in tobacco cells. We also calculated the relative mRNA half-life for each test gene, i.e. the ratio of the test mRNA half-life divided by the reference GUS mRNA half-life. Relative mRNA half-lives are better indicators of direct differences between mRNA decay rates than absolute mRNA half-lives as demonstrated previously (Newman *et al.*, 1993). The histogram in Figure 5-5E displays the relative half-lives of the test transcripts determined for at least three independent cell lines. When we compared the mRNA half-lives of the *Top10-Globin-E9* and the *Top10-SAUR-E9* test transcripts in at least three independent transformed cell lines we do not observe any significant difference in their relative decay rates. This indicated that the *SAUR-AC1* coding region sequences did not contain an mRNA instability determinant. We also compared the mRNA

Legend to Figure 5-5 Determination of mRNA half-lives. Stably retransformed BY-2 (TetVp16) cell lines expressing the indicated test genes and a co-transformed *Top10-GUS-3C* reference gene were treated with 10 μg/ml tetracycline, and RNA was isolated from samples withdrawn at 15 min intervals thereafter. Northern blots containing 30 μg total RNA per lane were hybridized to test and reference probes. Representative blots for cell lines transformed with the *Top10-GLOBIN-E9* test gene (A), the *Top10-GLOBIN-SAUR* test gene (B) or the *Top10-SAUR-E9* (C) test gene are shown. (D) Radioactive signals from the blots were quantified, plotted, and subjected to linear regression analysis. Solid squares represent Globin-E9 mRNA levels, solid triangles represent SAUR-E9 mRNA levels, solid circles represent Globin-SAUR mRNA levels. Reference mRNA levels for these test transcripts, are represented by open squares, open triangles and open circles, respectively. (E) Histogram representing the data from at least three independent cell lines per construct analyzed as in D above. Relative half-lives were calculated by normalizing the half-lives of the Globin test transcripts to that of the GUS reference transcripts in each experiment. The relative half-life of the Globin-E9 transcript was arbitrarily assigned a value of one and other values were adjusted accordingly. Error bars represent the standard deviation.

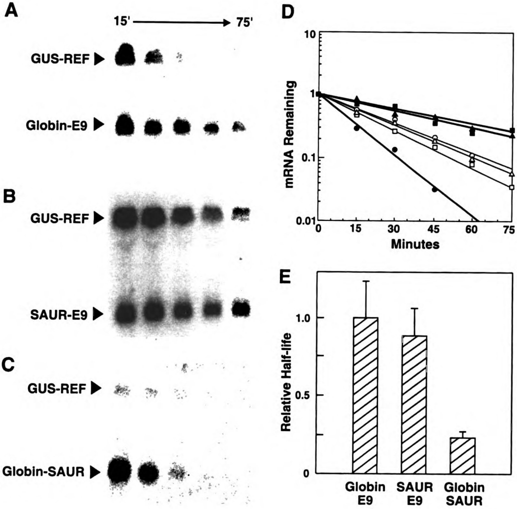

Figure 5-5. Determination of mRNA half-lives.

half-life of the *Top10-Globin-E9* and the *Top10-Globin-SAUR* test transcripts in a similar manner. In this case, we found that the presence of the *SAUR* 3'UTR led to a decrease of the mRNA half-life of the test transcript of about 5-fold. By comparing this resultto the effect on mRNA steady-state levels, we observed that the reduction in mRNA stability caused by the *SAUR* 3'UTR was of similar magnitude to the reduction that it causes in mRNA accumulation (as shown in Figures 5-4B and 5-5B). This indicates that an mRNA instability determinant can completely explain the decrease on mRNA accumulation caused by the *SAUR-AC1* 3'UTR.

Effect of a mutation in a conserved region of the DST element on mRNA accumulation and mRNA stability

The DST element present in the 3' UTR of several plant *SAUR* genes, consists of three blocks of conserved motifs separated by two variable regions of conserved length (Figure 3-5). There is one well conserved DST element in the 3'UTR of the *SAUR-AC1* gene. Tamdem repeats of a synthetic DST derived from the soybean *SAUR15A* have previously been shown to target transcripts for rapid decay when inserted in the 3'UTR of *GUS* or *Globin* reporter genes (Newman *et al.*, 1993). Because the 3'UTR of *SAUR-AC1* functions as an mRNA instability determinant, we wanted to test if a mutation in the DST element could stabilize the mRNA. To this end, we have modified the 3'UTR of the *SAUR-AC1* gene by site-directed mutagenesis of the conserved sequence present in the middle of the DST element, replacing the invariant ATAGAT with the sequence GCATGC (Figure 5-6A). This mutation is known to inactivate a synthetic dimer of the DST sequence (M.Sullivan and P.Green, unpublished results). A test gene incorporating the mutant *SAUR* 3' UTR, designated *Top10-Globin-MSAUR*, was transformed along with a *Top10-GUS-3C* reference gene into BY-2(TetVp16) cells. We first monitored the accumulation of the Globin-MSAUR transcript compared to that of the Globin-E9 and Globin-SAUR transcripts. As described above for other test genes, we pooled more than 50 stably transformed calli for each construct, isolated total RNA from the pools, and analyzed the accumulation of test transcripts by Northern blot analysis. As shown in Figure 5-6B, the levels of the *Globin-MSAUR* transcript were similar to those of the *Globin-SAUR* transcript, and the levels of the *GUS-3C* reference transcript were also similar in pools of calli transformed with either construct.

Since a mutation disrupting the ATAGAT sequence conserved in DST elements did not affect mRNA accumulation, such a mutation should not affect mRNA decay rates, which are in part responsible for mRNA levels. To confirm this, we decided to test whether the sequence ATAGAT in the DST sequence was required for the instability of the Globin-SAUR transcript. We measured directly mRNA half-lives of test transcripts using the tetracycline-regulated Top10 promoter as detailed above. The histogram in Figure 5-7B displays the relative half-lives of *Globin-E9*, *Globin-SAUR* and *Globin-MSAUR* test transcripts for at least two independent stably transformed BY-2 cell lines for each construct. When we compared the relative mRNA half-lives of the *Top10-Globin-SAUR* and the *Top10-Globin-MSAUR* test genes we did not observe any significant difference in mRNA decay rate. This indicated that the ATAGAT sequence of the 3'UTR

Legend to Figure 5-6 Effect on transcript accumulation and mRNA stability of a mutation in the *SAUR-AC1* 3'UTR that alters the ATAGAT sequence conserved in DST elements. (A) Comparison of the wild-type DST element in the 3'UTR of the Globin-SAUR transcript to the mutant DST element in the Globin-MSAUR transcript. (B) A tobacco cell line stably transformed with the 35S-TETVP16, was retransformed with a construct containing the *Top10-GLOBIN-MSAUR* gene and the *Top10-GUS-3C* reference gene. RNA was isolated from pools of transformed calli and Northern blots containing 30 μg total RNA per lane were performed to compare mRNA levels of the *Top10-Globin-E9* gene, the *Top10-Globin-SAUR* gene and the *Top10-Globin-MSAUR* gene. (C) Histogram that represents the effect on mRNA stability of the wild-type *SAUR-AC1* 3'UTR compared to the effect of the mutant MDST1 *SAUR-AC1* 3'UTR. Time courses, Northern blot analyses and calculations of the relative mRNA half-life of the Globin-MSAUR transcript were performed as described in Figure 5-5. The histogram represents the data from at least two independent cell lines per construct. Error bars represents the standard deviation, except in the case of the *Globin-MSAUR* transcript in which it represents the range of separation of two values.

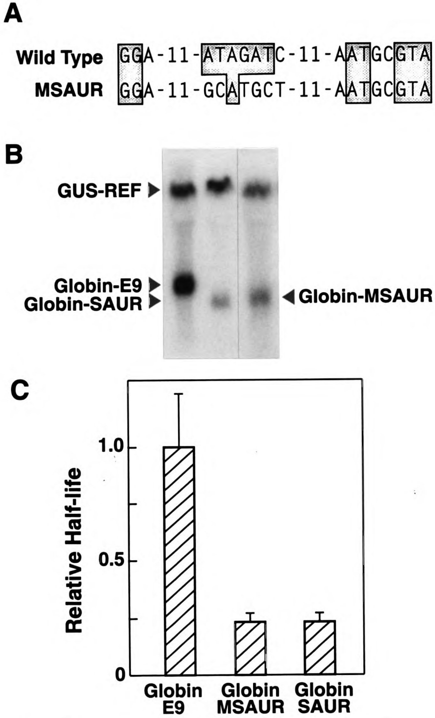

Figure 5-6 Effect on transcript accumulation and mRNA stability of a mutation in the *SAUR-AC1* 3'UTR that alters the ATAGAT sequence conserved in DST elements.

SAUR-AC1, absolutely conserved in all known DST elements, was not required for rapid mRNA decay.

DISCUSSION

A second level of regulation of the expression of the *SAUR* genes, in addition to transcriptional regulation, exists because they encode unstable transcripts. We first showed that the *SAUR-AC1* 3'UTR limits transcript accumulation in transgenic tobacco and stably transformed cultured cells. It could be argued that the presence of sequences downstream the *SAUR-AC1* 3'UTR may regulate the accumulation of the Globin-SAUR transcript. However, in this study, we also present evidence showing that the 3'UTR from *SAUR-AC1* functions as a potent mRNA instability determinant. This indicates that both effects are mediated by transcribed sequences in the 3'UTR, because the 3'UTR causes an effect on mRNA stability of similar magnitude to its effect on mRNA accumulation and an mRNA instability determinant must be in the transcript.

The 3'UTR of *SAUR-AC1* is of particular interest. Not only is it the first example of a native plant sequence element shown to function as an mRNA instability determinant, but because it also contains one copy of the DST element, which is conserved in the 3'UTR of plant *SAUR* genes. Two copies of this element has previously been shown to target reporter transcripts for rapid decay (Newman *et al.*, 1993), suggesting it could also have a role in the mRNA instability of SAUR transcripts. DST sequences are interesting because unlike many other eukaryotic mRNA unstability determinants, they are not AUrich sequences and they are well conserved in a family of genes across plant species. It is possible that they mediate a mechanism to control mRNA stability that is novel and unique for plants. However, we have determined that a five base pair substitution in the invariant ATAGAT motif of the *SAUR-AC1* DST element does not result in increased transcript stability in the context of the *SAUR-AC1* 3'UTR. This can be interpreted in several ways. One possibility is that there are multiple mRNA instability determinants in the 3'UTR of *SAUR-AC1*. Several unstable transcripts contain multiple instability determinants and the DST covers only the last third of the 3'UTR. In addition, two copies of a synthetic DST element were required to destabilize GUS and Globin reporter transcripts in BY-2 cells (Newman *et al.*, 1993). Perhaps in the native context another sequence is required for DST to destabilize *SAUR* transcripts. Alternatively, the disruption of the ATAGAT motif may not be sufficient to inactivate the DST element. We do not favor this hypothesis, because a similar mutation inactivates a synthetic dimer of DST elements inserted in the 3'UTR of reporter genes (M.Sullivan and P.J. Green, unpublished results). However, it could be the case that *SAUR* sequences flanking the DST element and absent in the synthetic dimer, stabilize the interaction with a DST-binding factor. A third possibility is that the 3'end of *SAUR-AC1* was changed by the ATAGAT mutation such that sequences controlling polyadenylation were affected. This could result in a negative indirect effect on mRNA stability that would compensate the lack of function of the DST element. The ATAGAT mutation had no detectable effect on the size of the transcript on Northern blots (data not shown), suggesting that if polyadenylation were affected by the mutation, it resulted in the inclusion of only a small number of bases.

Further mutagenesis studies will be necessary to investigate the contribution of the DST to the instability of the *SAUR-AC1* mRNA.

Concerning the kinetics of redistribution of *SAURs* during gravitropism, the presence of the 3'UTR of *SAUR-AC1* can account for the instability of *SAURs* deduced from RNA tissue-printing. Those experiments showed that *SAURs* are undetectable in the upper side of etiolated seedlings 20 minutes after they are placed horizontally (McClure and Guilfoyle, 1989). This is consistent with the fact that when the 3'UTR of *SAUR-AC1* is present in a test transcript, it causes a decrease in mRNA stability enough to render an absolute mRNA half-life of less than 20 minutes. We have also observed that *SAUR-AC1* mRNA accumulation is unaffected by auxin, indicating that the instability of the *SAUR-AC1* transcript is probably constitutive. The function of the DST elements inserted in reporter genes was also found not to be affected by auxin (Newman *et al.*, 1993). Therefore, it appears that the *SAUR-AC1* mRNA is inherently unstable in order to respond rapidlyto changes in transcription in response to auxin.

The general transcriptional inhibitor Actinomycin D eliminates the rapid mRNA decay caused by the 3'UTR of *SAUR-AC1*. This may explain the half-life of soybean *SAURs* in pea seedlings, after treatment with Actinomycin D, that was more than 40 minutes (Franco *et al.* 1990). This effect also suggests the presence of labile components in the mRNA degradation machinery, although it might just be an unspecific effect caused by the drug.

The coding region of *SAUR-AC1* has a negative effect on the accumulation of the messages in which it is present. In this respect, a recent report indicates that the coding region of soybean *SAURs* is responsible for cycloheximide induction (Li *et al.*, 1994). The induction by cycloheximide of *SAUR-AC1* mRNA accumulation, when this transcript is driven by a promoter that shows little or no induction, indicates that transcribed sequences also mediate cycloheximide induction of mRNA accumulation. However, we have not detected any effect on mRNA stability due to this region. It is possible that the cycloheximide effect on mRNA accumulation caused by a soybean *SAUR* coding region is related to the limited transcript accumulation caused by the *SAUR-AC1* coding region. If that is the case, both effects could be due to the presence of a cycloheximide-inducible transcriptional elongation factor.

The coding region effect on mRNA abundance is independent of the 3'UTR effect. When the accumulation of test transcripts was monitored in transgenic tobacco plants, we observed that the contributions of coding region and 3'UTR in limiting mRNA accumulation occurred similarly when both regions were present or when they acted separately. This indicates that two separate pathways limit mRNA accumulation of *SAUR* transcripts, and is consistent with only one them involving mRNA instability. In addition, both processes occurred in transgenic tobacco plants and in stably transformed tobacco cell lines, indicating that the mechanisms that regulate *SAUR* mRNA accumulation are likely to be of general importance in plant cells.

CHAPTER 6

CONCLUSIONS

The main goal of this study was to identify sequences within unstable plant mRNAs that mediate rapid mRNA decay in plants. At the onset of this project, the plant genes that were best candidates to achieve this goal were a family of auxin inducible soybean genes, named *SAURs*. The *SAUR* transcripts displayed short mRNA half-life and contained a particular sequence motif, the DST element, conserved in the dowstream region of five members of the soybean SAUR gene family. We decided to clone and characterize the *SAUR-AC1* gene from *Arabidopsis*, instead of analyzing one of the soybean *SAURs*, because of the advantages of *Arabidopsis* as a genetic model system. With these observations as a starting point, the *SAUR-AC1* gene of *Arabidopsis* was shown to be auxin inducible (Chapter 3). Sequences in the promoter region were found to be responsible for auxin-induction and for preferential expression in aerial tissues (Chapter 4). Transcribed regions of *SAUR-AC1* constitutively limited transcript accumulation, both coding region and 3'UTR contributing to the process (Chapter 5).

The 3'UTR of *SAUR-AC1* promotes rapid transcript decay in tobacco cells, as well as low transcript accumulation in tobacco plants (Chapter 5). This suggests that it also performs a similar function in *Arabidopsis* destabilizing the *SAUR-AC1* transcript. During the time when these experiments were performed, Newman *et al.* (1993) demonstrated that a synthetic dimer of a soybean DST element, was sufficient to destabilize reporter transcripts in tobacco cells. Surprisingly, we have found that a conserved region of the DST element of *SAUR-AC1* was not required for rapid transcript decay (Chapter 5). This could indicate that multiple instability elements are present in the 3'UTR of *SAUR-AC1*. This redundancy may be required to assure the low accumulation of the *SAUR-AC1* transcript. Consistent with this idea, we have also observed that the *SAUR-AC1* coding region also limited transcript accumulation, although in this case an mRNA unstability determinant was not responsible for this effect (Chapter 5).

A second goal of this project was to generate a tool to study auxin signal transduction pathways. When *SAUR-AC1* was cloned we first confirmed that its transcript was auxin inducible (Chapter 3). This indicated that the *SAUR-AC1* gene could be used as a molecular marker to study *Arabidopsis* mutants suspected to be affected in auxin metabolism. As an example, we have shown that several auxin-resistant and gravity responsive mutants display altered expression of *SAUR-AC1* in response to exogenous auxin (Chapter 3). In addition, the sequences in the *SAUR-AC1* promoter region are responsible for auxin-induced activity of a *SAUR-GUS* promoter fusion and for preferential promoter activity in certain tissues of transgenic *Arabidopsis* plants (Chapter 4). Therefore, it should also be possible to cross these transgenic plants to mutants with defects in processes involving auxins, to test for differences in promoter activity in different tissues in the mutants.

One of the long-term goals of this project was to understand the molecular mechanisms that plants use for the rapid degradation of unstable mRNAs. The first step was to localize cis-acting elements responsible to target messages for rapid decay which we have achieved identifying the 3'UTR of *SAUR-AC1* as an mRNA instability determinant. The second step will be to characterize the factors that are likely to interact with such elements. This can be achieved by cross-linking of proteins that interact with the 3' UTR of *SAUR-AC1*. A different approach, possible in *Arabidopsis*, can be to identify mutations in trans that stabilize transcripts containing the *SAUR-AC1* 3' UTR. This should make it possible to clone and characterize molecular components involved in mRNA decay.

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