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Mutational analysis of the DST element in tobacco cells and transgenic plants: Identification of residues critical for mRNA instability

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ABSTRACT

DST (downstream element), an approximately 40-base sequence derived from the 3′ untranslated region (UTR) of *SAUR* (small auxin up RNA) genes, represents one of only a few sequence elements that have been demonstrated directly to target transcripts for rapid decay in plant cells. Substitution mutations were made in conserved regions of the DST element containing the sequences ATAGAT and GTA, which are invariant among several *SAUR* genes. The mutant DST elements were inserted into the 3′ UTR of a β-globin reporter gene and then assessed for their ability to destabilize the reporter transcript in stably transformed BY-2 tobacco cells. Their effect on reporter mRNA accumulation in both intact transgenic tobacco plants and stably transformed BY-2 cells was also measured. Five- and six-base substitutions in the ATAGAT and GTA regions of DST, respectively, resulted in inactivation of the element as an instability determinant in all systems tested. Smaller, two-base substitution mutations within the ATAGAT and GTA regions had varying effects on DST function in BY-2 cells, ranging from little or no effect to significant increases in reporter mRNA half-life and accumulation. In contrast, all two-base substitution mutations tested resulted in inactivation of DST in intact tobacco leaves. Together, these results indicate that bases within both the ATAGAT and GTA regions of DST are required for its function as an mRNA instability determinant in both BY-2 cells and leaves of transgenic plants, and that the sequence requirements for DST to function in leaves are more stringent.

Keywords: mRNA stability; posttranscriptional; RNA decay; RNA degradation; *SAUR*

INTRODUCTION

All organisms require the carefully regulated expression of many genes for normal growth and development and to be able to adapt to ever changing environmental conditions. Although in the past much of the effort to understand gene regulation was devoted to transcriptional mechanisms, more recently it has become increasingly clear that posttranscriptional mechanisms also play important roles in the control of gene expression (Gallie, 1993; Sullivan & Green, 1993; Vierstra, 1993; Surdej et al., 1994). Posttranscriptional regulatory mechanisms provide organisms with added speed and flexibility of control that would not be available with transcription alone. Because plants must adapt rapidly to changing conditions from which they cannot flee, the added speed and flexibility provided by posttranscriptional control may be particularly important for them.

One important posttranscriptional regulatory mechanism is the control of mRNA stability. Clearly, the level to which a particular mRNA accumulates within a cell is dictated not only by its rate of synthesis, but also by its rate of decay. Although little is known about the mechanisms whereby mRNAs are degraded in plant cells, it is becoming clear that mRNA stability contributes to the regulation of many plant genes (Gallie, 1993; Green, 1993; Sullivan & Green, 1993). Because most plant and mammalian transcripts are relatively stable, with half-lives on the order of hours (Siflow & Key, 1979; Brawerman, 1993), it appears that most messages are inherently stable unless they are actively destabilized.

A first step toward understanding how unstable transcripts are recognized and degraded is to identify

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cis-acting sequences within unstable mRNAs that target them for rapid turnover. Chimeric reporter gene constructs have been particularly useful for the identification of such sequences. Perhaps the most extensively studied instability determinants are the AUUUA-containing motifs in the 3′ untranslated regions (UTRs) of unstable mammalian protooncogene and lymphokine transcripts such as those of c-*fos* and GM-CSF (Greenberg & Belasco, 1993; Chen & Shyu, 1995). It has been shown recently that repeats of the sequence AUUUA, when present in the 3′ UTR of reporter transcripts, can cause mRNA instability in plant cells (Ohme-Takagi et al., 1993).

The use of chimeric reporter constructs has also been used to identify an instability determinant in the 3′ UTR of a class of unstable plant transcripts encoded by the auxin-inducible *SAUR* genes (Newman et al., 1993). The *SAUR* (small auxin up RNA) genes were first identified in soybean (McClure et al., 1989) and subsequently in mungbean (Yamamoto et al., 1992) and *Arabidopsis thaliana* (Gil et al., 1994). Although the function of the *SAUR* gene products is not known, their expression is generally correlated with auxin-induced cell elongation (McClure & Guilfoyle, 1989; Franco et al., 1990). The half-lives of the soybean *SAUR* transcripts have been estimated to be between 10 and 50 min (McClure & Guilfoyle, 1989; Franco et al., 1990). The 3′ UTRs of the *SAUR* genes contain a 40-bp conserved sequence motif, the DST (downstream) sequence (Fig. 1), which has been suggested to control the stability of the soybean *SAUR* transcripts (McClure et al., 1989; Franco et al., 1990). Newman et al. (1993) directly tested whether DST could act as an instability determinant. They demonstrated that, in cultured tobacco cells (BY-2) when two copies of the DST element were present in the 3′ UTR of β-glucuronidase (GUS) or β-globin reporter transcripts, the half-life of the message was decreased markedly relative to that of reporter transcripts containing no insert or a spacer control insert. Transgenic tobacco plants expressing DST-containing reporter transcripts showed a corresponding decrease in mRNA accumulation. So far, DST, repeats of AUUUA (Ohme-Takagi et al., 1993), and premature nonsense codons (A. van Hoof & P.J. Green, unpubl. data) are the only *cis*-acting sequences that have been demonstrated directly to function as mRNA instability determinants in plants.

In the present study, through mutagenesis of the DST element, we have identified residues within DST that allow it to act as an instability determinant in plant

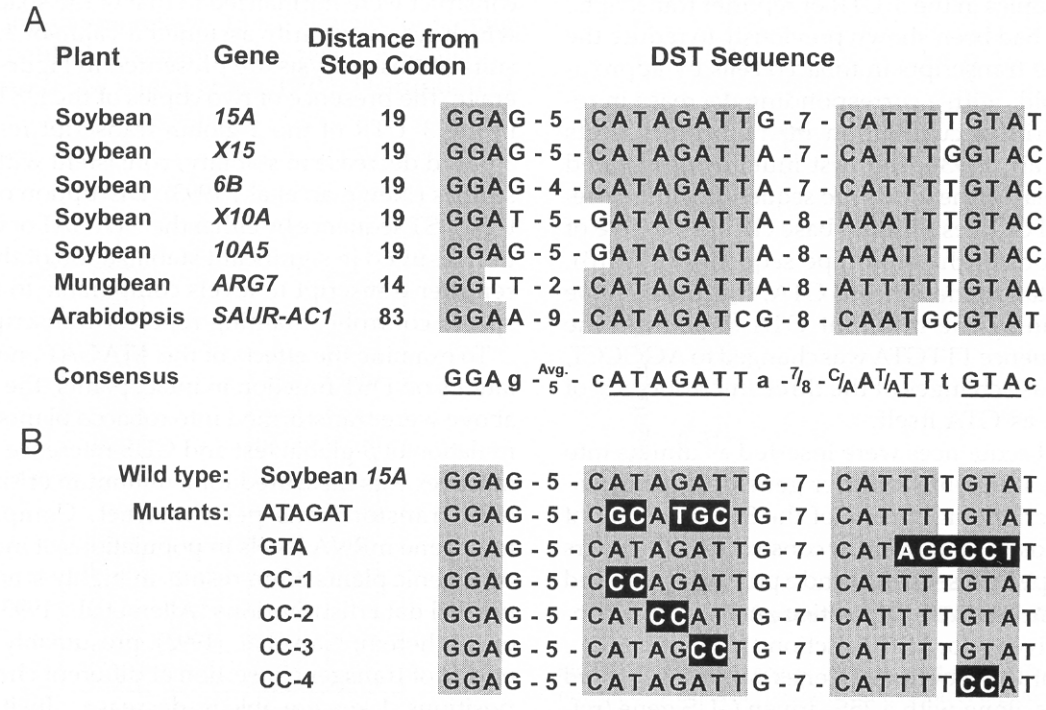

FIGURE 1. Sequence of wild-type and mutant DST elements. The elements consist of three conserved regions (shown in gray) separated by two variable regions. Numbers indicate the number of bases separating the conserved regions. **A:** DST sequences identified in seven *SAUR* genes are aligned (McClure et al., 1989; Yamamoto et al., 1992; Gil et al., 1994). All elements are located in the 3′ UTR at the indicated distances downstream of the stop codon. For the consensus, residues are shown as follows: underlined residues are invariant among all seven genes; capitalized residues are conserved in six of the seven genes; capitalized residues separated by a slash are conserved among all seven genes with each residue present in at least three of the seven genes; lower case residues are conserved in four or five of the seven genes. **B:** Sequence of the wild-type element derived from the soybean *SAUR 15A* gene and the mutant versions of that element used in this study. Mutant bases are shown as white on a black background.

cells. The identification of critical residues within DST should aid in the identification of functional DST elements in other plant genes and the *trans*-acting factors and mechanisms involved in the decay of DST-containing transcripts.

RESULTS

Regions containing the invariant sequences ATAGAT and GTA are required for DST-mediated instability

Comparison of DST sequences from the 3′ UTRs of several soybean *SAUR* genes as well as *SAUR* genes from mungbean and *Arabidopsis* reveals the presence of three highly conserved regions within the DST element (Fig. 1A). Within two of these conserved regions are several nucleotides that are identical among all reported *SAUR*-derived DST sequences. One region contains the invariant sequence ATAGAT and a second region contains the invariant sequence GTA. The absolute conservation of these regions suggests that they might be important for DST to function as an mRNA instability determinant in plant cells.

As a first step toward testing this hypothesis, mutations were made in a wild-type DST sequence derived from the soybean *SAUR 15A* gene. When present as two tandem copies in the 3′ UTR of reporter transcripts, this sequence had been shown previously to reduce the stability of the transcripts in tobacco cells by approximately fourfold, with a corresponding decrease in reporter transcript accumulation in transgenic plants (Newman et al., 1993). The first mutant, designated ATAGAT, replaced the wild-type sequence with the sequence GCATGC, resulting in base changes in five of the six positions of the wild-type sequence (Fig. 1B). A second mutation, designated GTA, was made in the region containing the invariant GTA sequence. The wild-type sequence TTTGTA was changed to AGGCCT, resulting in base changes of the three nucleotides 5′ of GTA, as well as GTA itself.

The mutant sequences were inserted as dimers into the 3′ UTR of a β-globin reporter gene (test gene) under the transcriptional control of the 35S promoter of cauliflower mosaic virus. Similar constructs with dimers of the wild-type DST sequence and a polylinker-derived spacer sequence served as positive and negative controls, respectively, for DST function (Newman et al., 1993). Each of the globin test genes was transformed into BY-2 cells along with a 35S-driven GUS gene (reference gene) on the same vector to serve as an internal standard. As previously reported (Newman et al., 1993; Ohme-Takagi et al., 1993), normalization of the test gene transcript half-life to that of a cotransformed reference gene (to give a relative half-life) results in less experimental variation, presumably because experimental variation (e.g., slight differences in growth rates among independently transformed cell lines) tends to affect the test and reference mRNAs coordinately. For each construct, independent, stably transformed cell lines expressing both the β-globin test gene and the GUS reference gene were identified and used for half-life analyses. Results of typical half-life experiments for DST, spacer, and ATAGAT mutant constructs are shown in Figure 2. Following inhibition of transcription using 100 μg/mL of actinomycin D (ActD) (Newman et al., 1993), the level of the DST-containing β-globin test transcript (see central autoradiogram and solid line in corresponding graph) declined more rapidly than that of the cotransformed GUS gene (dashed line in graph). In contrast, levels of both the spacer control-containing β-globin transcript and the ATAGAT mutant-containing β-globin transcript declined more slowly than those of the cotransformed GUS gene. For the experiments shown, the relative half-life (β-globin/GUS) of the DST-containing transcript was 0.34, whereas the relative half-lives for the spacer control- and ATAGAT mutant-containing transcripts were 1.26 and 1.12, respectively.

Relative half-lives of the β-globin test transcript were calculated for each cell line analyzed, and for each construct the relative half-lives of all cell lines analyzed were averaged. A minimum of four independent cell lines was analyzed for each construct. For greater ease of comparison, the average relative half-lives for each construct were normalized to that of the spacer control, which was arbitrarily assigned a value of 1.0. The results of this analysis are presented in Figure 3. On average, the presence of two copies of the DST sequence in the 3′ UTR of the β-globin transcript resulted in a 3.6-fold decrease in stability, consistent with previous studies (Newman et al., 1993). Disruption of the wild-type DST sequence by either the ATAGAT or GTA mutation resulted in significant stabilization of the β-globin reporter transcript to levels comparable to that of the spacer control-containing reporter transcript.

To examine the effects of the ATAGAT and GTA mutations on DST function in intact plants, the constructs above were transformed into tobacco plants and accumulation of β-globin test and GUS reference transcripts in leaves was measured for a minimum of six independent transformants per construct. Comparisons of transgene mRNA levels in populations of independent transgenic plants often results in highly scattered non-normal data distributions (Allen et al., 1993 and references therein; Nap et al., 1993), presumably due to the effects of transgene insertion at different chromosomal positions. We were able to decrease, albeit not eliminate, this variation by normalizing the β-globin mRNA levels to those of the cotransformed GUS gene, an approach used previously in similar studies (Kuhlemeier et al., 1987; Newman et al., 1993; Ohme-Takagi et al., 1993). Consistent with the BY-2 half-life results, Figure 4 shows that, in transgenic plants, two copies of the DST sequence in the 3′ UTR of the β-globin test gene re-

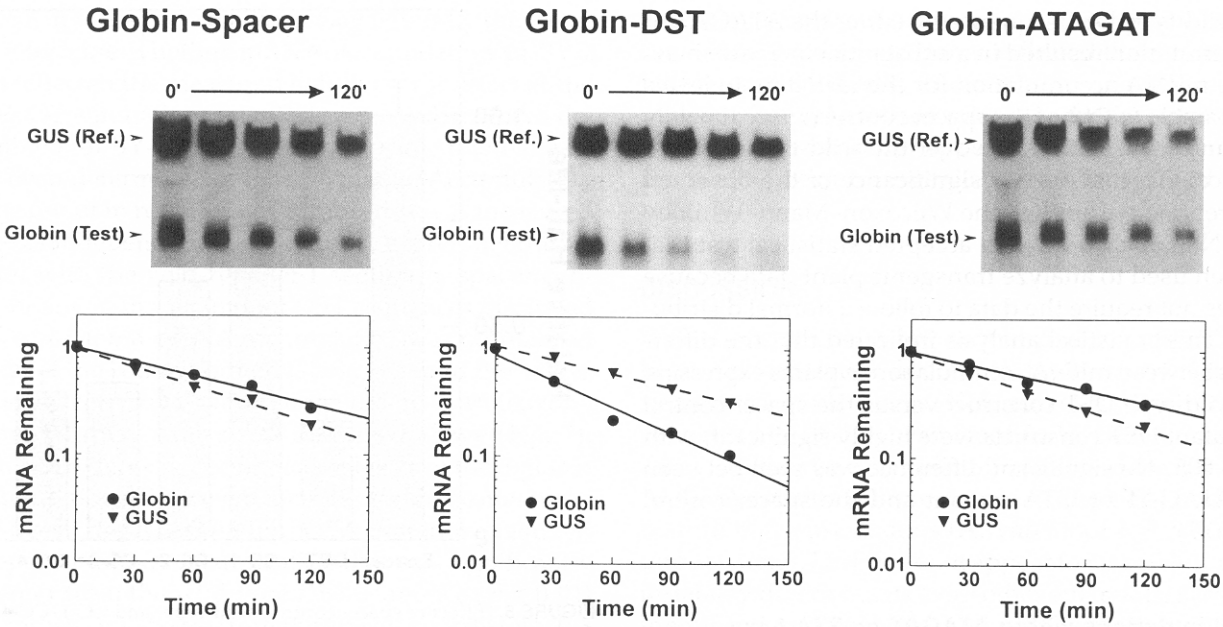

FIGURE 2. Decay of transcripts in tobacco (BY-2) cells stably transformed with various reporter constructs. Top: For the indicated transformed cell lines, RNA was isolated from samples harvested every 30 min after transcription was inhibited with 100 μg/mL ActD. RNA gel blots containing 20 μg of RNA per lane were probed for β-globin and GUS transcripts. Bottom: Radioactive signals from the upper panels were quantified using a PhosphorImager, normalized to the zero time point, and subjected to linear regression analysis. Data are from single experiments, but are representative of the experiments presented in Figure 3.

sulted in an average of about a fivefold decrease in mRNA accumulation relative to a test gene containing the spacer control. As before, this result is similar to that reported by Newman et al. (1993). Disruption of

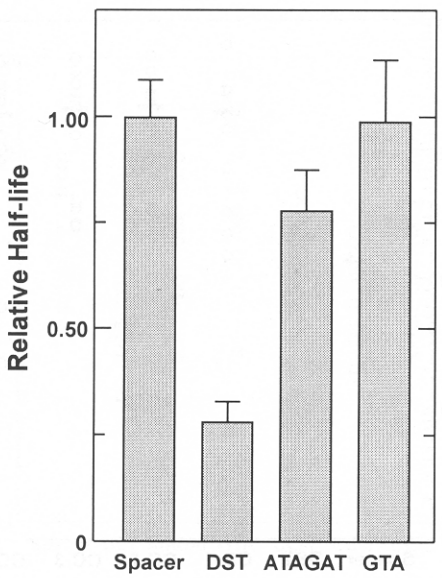

FIGURE 3. Effect of spacer control, wild-type, ATAGAT, and GTA mutant DST elements on the stability of β-globin test transcripts in BY-2 cells. For each cell line tested as in Figure 2, the half-life of the β-globin test transcript was normalized to the half-life of the transcript of the cotransformed GUS reference gene. For each construct, the relative half-lives for a minimum of four independent cell lines were averaged and normalized to the spacer control, which was arbitrarily assigned a value of 1.0. Error bars represent standard error.

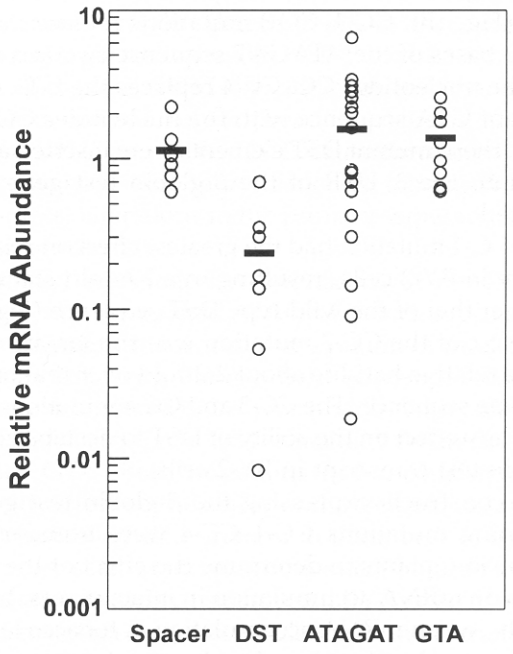

FIGURE 4. Effect of spacer control, wild-type, ATAGAT, and GTA mutant DST elements on the accumulation of β-globin test transcripts in tobacco plants. For each independent transformed tobacco plant, RNA was isolated from a leaf, subjected to RNA gel blot analysis, and hybridization with β-globin and GUS probes. Radioactive signals were quantified using a PhosphorImager. β-Globin mRNA levels for each individual plant were normalized to the mRNA levels of the cotransformed GUS reference gene (Newman et al., 1993). Relative β-globin mRNA accumulation for each independent transformant is shown (open circles). Average relative accumulation for each construct is shown as a solid bar. Number of individuals analyzed for each construct are: spacer, 6; DST, 7; ATAGAT, 20; GTA, 10.

the wild-type DST sequence by either the ATAGAT or GTA mutation resulted in a substantial increase in average mRNA accumulation for the test gene to levels comparable to that of the spacer control (approximately 6.6- and 6.0-fold increase over the wild-type DST sequence). To confirm the significance of the observed differences, we applied the Wilcoxon–Mann–Whitney test (Nap et al., 1993), an accepted statistical test that is often used to analyze transgenic plant data because it does not require the data to follow a normal distribution. This statistical analysis indicated that the differences between mRNA accumulation in plants expressing the wild-type DST construct versus the spacer control or mutant DST constructs were highly significant, with $p < 0.005$. No significant difference was seen between the ATAGAT or GTA mutant and the spacer control ($p > 0.7$).

Small mutations within ATAGAT or GTA have varying effects on the ability of DST to destabilize transcripts

To define to a higher degree of resolution which residues within the ATAGAT and GTA regions are important for DST to function as an mRNA instability determinant, a second set of mutations, designated CC-1–CC-4, was made (Fig. 1B). CC-1–CC-3 mutations replace the invariant bases of the ATAGAT sequence two at a time with the nucleotides CC. CC-4 replaces the GT of the invariant GTA sequence with the nucleotides CC. As before, these mutant DST elements were inserted as dimers into the 3′ UTR of the β-globin test gene and analyzed.

The CC-1 mutation had the greatest effect on relative half-life in BY-2 cells, resulting in a 2.6-fold stabilization over that of the wild-type DST sequence (Fig. 5). The effect of the CC-2 mutation was similar, increasing the relative half-life about 2.2-fold over that of the wild-type sequence. The CC-3 and CC-4 mutations had little if any effect on the ability of DST to destabilize the β-globin test transcript in BY-2 cells.

The constructs expressing the β-globin test genes containing mutations CC-1–CC-4 were transformed into tobacco plants to determine the effect of the mutations on mRNA accumulation in intact plants. Interestingly, when mRNA accumulation in tobacco leaves was measured and analyzed as described above for the ATAGAT and GTA mutations, increased accumulation of the β-globin test transcript was seen for all four of the 2-base substitution mutants, with an average increase in accumulation of 8.8- to 10.5-fold over test transcripts containing the wild-type DST sequence (Fig. 6). Statistical analysis using the Wilcoxon–Mann–Whitney test indicated that the increase in accumulation relative to the wild-type DST element was highly significant, with $p < 0.01$. No significant difference in mRNA accumulation was seen between CC-1–CC-4 mutants and the spacer control ($p > 0.27$).

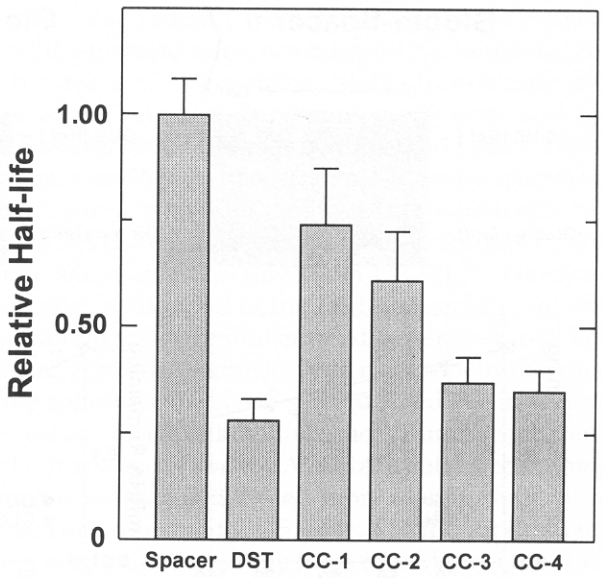

FIGURE 5. Effect of spacer control, wild-type, and CC-1–CC-4 mutant DST elements on the stability of β-globin test transcripts in BY-2 cells. A minimum of four independent cell lines for each of the indicated constructs was analyzed as in Figure 3. Error bars represent standard error.

All four CC mutations seem to affect mRNA accumulation in tobacco plants, but only CC-1 and CC-2 appear to affect mRNA half-life in BY-2 cells; thus, recognition

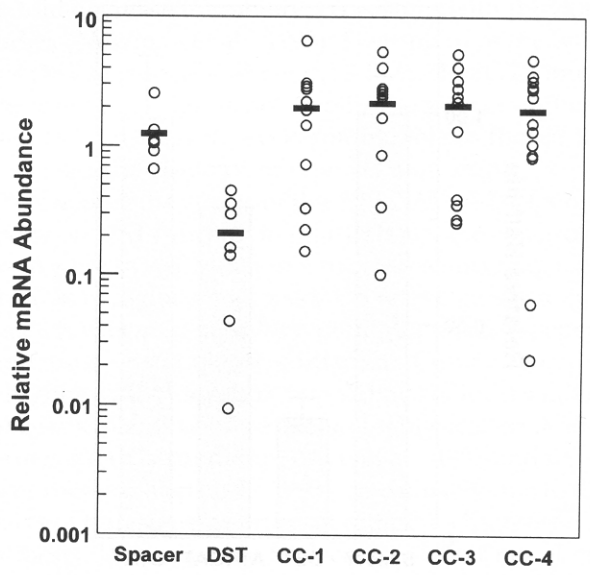

FIGURE 6. Effect of spacer control, wild-type, and CC-1–CC-4 mutant DST elements on the accumulation of β-globin test transcripts in tobacco plants. mRNA accumulation for each of the indicated constructs was analyzed as in Figure 4. Relative β-globin mRNA accumulation for each independent transformant is shown (open circles). Average relative accumulation for each construct is shown as a solid bar. Number of individuals analyzed for each construct are: spacer, 6; DST, 7; CC-1, 12; CC-2, 10; CC-3, 13; CC-4, 14.

of DST in these two cell types may be subtly different. To determine whether mRNA accumulation in BY-2 cells reflected the measured half-lives, the effect of the CC-1–CC-4 mutations on mRNA accumulation was determined for BY-2 cells in much the same manner as it had been determined in tobacco plants. Accumulation of the β-globin reporter gene transcript was measured for 8–24 independent transformants expressing the reporter with the wild-type DST sequence, spacer control, or any of the six mutant DST sequences discussed above. β-Globin mRNA accumulation was normalized to that of the cotransformed GUS gene, and the values for each construct were subjected to a Wilcoxon–Mann–Whitney analysis as described above (Fig. 7). With one exception, accumulation levels of the β-globin test transcript in BY-2 were consistent with the measured half-lives. The ATAGAT and GTA mutations resulted in reporter mRNA accumulation that was significantly different from the wild-type DST sequence ($p < 0.01$) and indistinguishable from the spacer control ($p > 0.3$ and $p > 0.5$, respectively). CC-1 and CC-2 mutations resulted in β-globin mRNA accumulation intermediate between DST and the spacer control (i.e., significantly different from either DST or spacer, with $p < 0.05$). Accumulation of the transcript containing the CC-4 mutation was not significantly different from the wild-type DST ($p > 0.3$). Only accumulation of the β-globin transcript with the CC-3 mutant DST element was inconsistent with the measured half-life, with transcript accumulation being significantly different from the wild-type DST element ($p < 0.01$) and indistinguishable from the spacer control ($p > 0.29$). Thus, with the exception of the CC-3 mutation, transcript accumulation of the β-globin test genes reflected their measured half-lives in BY-2 cells. The difference in mRNA accumulation between tobacco plants and BY-2 cells for the CC-4 mutant transcript appears to be an inherent difference between transgenic tobacco leaves and BY-2 cells.

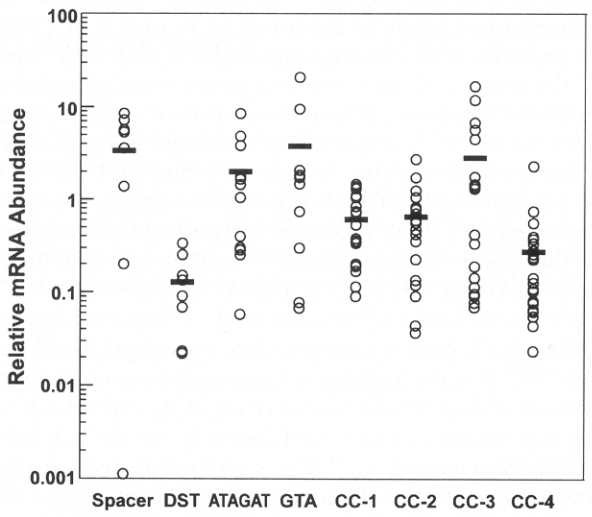

FIGURE 7. Effect of spacer control, wild-type, and mutant DST elements on the accumulation of β-globin test transcripts in BY-2 cells. RNA was isolated from independent transformed BY-2 cell lines and subjected to RNA gel blot analysis and hybridization as in Figure 4. Relative β-globin mRNA accumulation for each independent cell line is shown (open circles). Average relative accumulation for each construct is shown as a solid bar. Number of individuals analyzed for each construct are: spacer, 8; DST, 8; ATAGAT, 12; GTA, 10; CC-1, 18; CC-2, 19; CC-3, 19; CC-4, 26.

DISCUSSION

DST is one of only a few sequence elements that has been demonstrated directly to target transcripts for rapid decay in plant cells (Newman et al., 1993; Ohme-Takagi et al., 1993; A. van Hoof & P.J. Green, unpubl. data). Unlike the other plant instability determinants, AUUUA repeats (Ohme-Takagi et al., 1993) and premature nonsense codons (A. van Hoof & P.J. Green, unpubl. data), DST does not appear to resemble known instability determinants from other eukaryotic systems. Identification of sequences within the element that are required for DST activity should aid in the identification of functional DST elements in other plant genes. Further, mutant DST sequences could prove useful in understanding the mechanisms whereby DST-containing transcripts are recognized and degraded. For these reasons, we made a series of mutant DST elements and assessed the ability of these mutant DST sequences to function as instability determinants in the context of a β-globin reporter transcript. All the mutations were made in regions of DST that are invariant among those elements derived from soybean, mungbean, and *Arabidopsis SAUR* genes (Fig. 1). Because there is no apparent conserved secondary structure among the DST sequences, alterations in the primary sequence were of prime concern.

Initially, we made two mutations of the DST sequence, one designated ATAGAT, which replaced five of six invariant nucleotides in a region of the DST sequence containing the invariant sequence ATAGAT, and one designated GTA, which replaced six of six nucleotides in a region of DST containing the invariant sequence GTA. Both mutations had a consistent and dramatic effect on the ability of the element to function as an instability determinant in plant cells. These effects were seen in increased message half-life and accumulation in stably transformed BY-2 cells, as well as in increased message accumulation in intact transgenic tobacco plants. These data indicate that both the ATAGAT and GTA regions are required for DST to function as an instability determinant in plant cells.

To identify residues critical to DST's destabilizing effect to a higher degree of resolution, we made a second set of mutations within the DST sequence, replacing the invariant ATAGAT sequence two bases at a time with C residues (CC-1–CC-3) or by replacing the GT of the invariant GTA with C residues (CC-4). When tested for their effect on reporter transcript half-life in BY-2

cells, CC-1 and CC-2 had the greatest effect, substantially increasing mRNA half-life over that of transcripts containing the wild-type element. In the case of CC-1, the reporter half-life approached that of transcripts containing the spacer control element. The CC-2 mutation had somewhat less of an effect, resulting in a half-life intermediate between that caused by the wild-type element and the spacer control element. In contrast, CC-3 and CC-4 mutations had little if any effect on reporter mRNA half-life in BY-2 cells. The lack of a stabilizing effect by the CC-3 mutation may indicate that the 3′ portion of the ATAGAT region is less critical for DST function in BY-2 cells. That the CC-4 mutation had no effect on test transcript stability was unexpected because it alters two of the invariant GTA residues. However, the original GTA mutation disrupted a stretch of three T residues, in addition to the invariant GTA. This region is pyrimidine-rich in all known *SAUR* DST sequences, suggesting that the T- or pyrimidine-rich nature of the GTA region may be important for DST function. When reporter mRNA accumulation was measured in BY-2 cells for transcripts containing the CC-1, CC-2, and CC-4 mutations, mRNA accumulation mirrored the measured half-lives. For CC-3, we observed an apparent disparity between the effect of the mutation on reporter mRNA half-life and reporter mRNA accumulation in BY-2 cells, the cause of which is unclear. One possibility is that this mutation enhances mRNA accumulation without stabilizing the transcript (e.g., by enhancing transcriptional elongation).

Interestingly, CC-1–CC-4 mutant elements all led to increased accumulation of the β-globin reporter mRNA in intact transgenic tobacco plants to levels comparable to that of the spacer control element. This finding suggests that recognition of the DST element may be somewhat different in different types of cells. This is most evident for the CC-4 mutation, which had little effect on reporter mRNA half-life or accumulation in BY-2 cells, but resulted in a significant increase in β-globin mRNA accumulation in leaves of intact tobacco plants. That the GTA sequence is required for DST function in leaves is consistent with its high degree of conservation. Such differential effects could be the result of differences in the concentration or composition of the cellular factors that recognize and mediate the decay of DST-containing transcripts in the different types of cells used in this study. Different cell types in intact plants could use such a mechanism to posttranscriptionally regulate the abundance of DST-containing mRNAs in different tissues and organs. The ease with which intact transgenic plants can be generated could facilitate further studies along these lines.

In contrast to AUUUA-containing mRNA instability determinants, it is unclear whether DST might function as an instability determinant in non-plant systems. DST sequences in the 3′ UTR of two auxin-inducible tobacco transcripts have been reported (van der Zaal et al., 1991). Therefore, it seems likely that DST might act to limit the accumulation of *SAUR* and other DST-containing transcripts in a variety of plant species. Recently, we have found that DST can also limit transcript accumulation in *Arabidopsis* (M.L. Sullivan, D. Thompson, & P.J. Green, unpubl. data), which should allow further characterization of DST-mediated mRNA decay through genetic approaches. Such genetic approaches, along with biochemical methods, will no doubt aid in the elucidation of the mechanisms by which DST sequences accelerate mRNA decay in higher plants.

MATERIALS AND METHODS

Plant materials

Nicotiana tabacum cv Bright Yellow 2 [BY-2 (also called NT-1)] cells (An, 1985) and *N. tabacum* SR-1 plants were cultured as described previously (Newman et al., 1993). Stably transformed BY-2 cell lines and transgenic SR-1 plants were generated by *Agrobacterium tumefaciens*-mediated transformation as described previously (Newman et al., 1993) using the *A. tumefaciens* strain LBA4404 harboring the appropriate constructs. Transformed cell lines and plants were initially screened for β-glucuronidase (GUS) reporter gene expression by histochemical staining (Jefferson et al., 1986) and subsequently for expression of the β-globin gene by RNA gel blotting.

Gene constructions

p1069, a pBluescript derivative with a *Bgl* II–*Bam*H I insert comprising two tandem copies of the wild-type DST sequence derived from the soybean *SAUR 15A* gene served as the template for mutagenesis. Site-directed mutagenesis was performed essentially as described by Kunkel et al. (1987). The presence of the desired mutations in both DST copies and the absence of extraneous mutations was confirmed by automated sequencing (Plant Biochemistry Facility, Michigan State University). Test genes were constructed in a pUC vector intermediate with the cassette structure *Sac* I-35S-*Bgl* II-*Xba* I-globin-*Bam*H I-E9-*Cla* I to create β-globin test genes (Newman et al., 1993). Wild-type and mutant DST sequences and the spacer control sequence (a tandem repeat of the sequence GATCCGATCTAGAGTCGACCTGCAGGCATGCA AGCTATCTCTAGAA [Newman et al., 1993]) were inserted as 90-bp *Bgl* II–*Bam*H I fragments into the unique *Bam*H I site in the 3′ UTR of the β-globin test gene, approximately 250 bp upstream of the E9 poly(A) sites (Fang et al., 1989). Each test gene was moved as a *Sac* I–*Cla* I fragment into the polylinker of p851 (Newman et al., 1993), a pMON505-derived plant transformation vector (Rogers et al., 1987) containing a 35S-driven GUS-3C gene.

RNA methods

RNA was isolated from BY-2 cells and tobacco leaves by the GTC method and used for RNA gel blots and hybridization as described previously (Newman et al., 1993). Hybridization probes corresponding to β-globin or GUS-coding regions

were labeled using ^{32}P dCTP by the random primed method of Feinberg and Vogelstein (1983). Radioactive signals were detected and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, California). All blots were probed with β-globin and quantified before being probed with GUS to minimize background, as described previously (Newman et al., 1993). For mRNA accumulation experiments, all blots used for a given comparison were hybridized at the same time with the same hybridization probes.

Half-life determination and analysis of mRNA accumulation

Half-life measurements for BY-2 cell lines were made essentially as described by Newman et al. (1993). Briefly, three to four days following subculture, actinomycin D (ActD) was added to BY-2 cultures to a concentration of 100 μg/mL. Thirty minutes following ActD addition, 10-mL samples were harvested and frozen in liquid nitrogen every 30 min for 120 min. Following analysis of RNA on RNA gel blots as described above, values for each time course were subjected to linear regression using SigmaPlot (Jandel Scientific, Corte Madera, California) to calculate mRNA half-lives.

To measure mRNA accumulation in transgenic tobacco plants, primary transformed tobacco plants expressing the GUS reference gene were grown to the 10- to 14-leaf stage, and a fully expanded leaf from the third pair from the apex was harvested and frozen in liquid nitrogen prior to RNA isolation and RNA gel blot analysis as described above. To measure mRNA accumulation in BY-2 cells, RNA was isolated from independent stably transformed cell lines and was subjected to RNA gel blot analysis as described above. Messenger RNA accumulation data for tobacco plants and BY-2 cell lines were analyzed by the Wilcoxon–Mann–Whitney test (Nap et al., 1993).

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