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Identification and characterization of genes with unstable transcripts (*GUT*s) in tobacco

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Abstract

Plants and other higher eukaryotes have the ability to recognize and target specific transcripts for rapid decay from among the majority of relatively stable mRNAs present within cells. However, little is known about the nature of unstable transcripts in plants, or the mechanisms that facilitate their rapid degradation. As a first step toward understanding how plants distinguish between unstable and stable transcripts, a novel differential screen was used to identify cDNAs for genes with unstable transcripts (*GUT*s), solely on the basis of the instability of their mRNAs. cDNA probes were prepared from tobacco cells that had been depleted of highly unstable mRNAs by treatment for 90 min with a transcriptional inhibitor, and from control, untreated cells. *GUT* clones were selected on the basis of weak hybridization to the former probe relative to the latter probe. Half-life measurements performed on the mRNAs hybridizing to eight *GUT* clones indicated that each was unstable, with a half-life on the order of about an hour or less. All eight of the cDNAs corresponded to new tobacco genes, and four showed sequence similarity with genes from other species, including the eukaryotic family of DNAJ homologs, a tomato wound-inducible protein, and histone H3. In addition to providing information about the types of transcripts that are inherently unstable in plants, the GUT clones should provide excellent tools for the identification of *cis*- and *trans*-acting determinants of mRNA instability.

Introduction

Control at the level of mRNA stability is an important component in the regulation of many eukaryotic genes [11, 25, 28, 33]. This is because mRNA decay rates define the rapidity with which new steady-state transcript levels can be achieved after changes in the transcription rate of a gene. Most mRNAs in higher eukaryotes appear to be relatively stable, with half-lives on the order of several hours [4]. Unstable transcripts, with half-lives on the order of an hour or less, tend to

The nucleotide sequence data reported will appear in the dbEST datadase under accession numbers 44367–44379 and to the GenBank under accession numbers T18327–T18339.

encode proteins that are required only transiently in cells, such as those with critical roles in the rate of regulation of cellular growth and differentiation. Rapid alterations in the synthesis of these proteins is thought to be facilitated by the instability of the corresponding mRNAs [25]. A number of sequence elements have been identified within unstable transcripts that mediate the targeting of those mRNAs for rapid degradation, presumably via their interactions with specific binding factors [11, 28]. One well-characterized example is a sequence element termed the ARE, which is an AU-rich stretch in the 3′-untranslated region (UTR) containing one or more copies of an AUUUA motif [28]. AREs have been shown to play an important role in the rapid degradation of certain lymphokine, cytokine, and proto-oncogene transcripts (reviewed in [28]).

Control of gene expression at the level of mRNA stability is likely to be particularly important in plants [11, 33]. As sessile organisms, plants are unable to move away from adverse stimuli and are obliged to respond by altering endogenous gene expression, often within minutes. As discussed above, unstable transcripts are expected to figure prominently in rapid changes in gene expression that are manifest at the mRNA level. Rapid changes would be more difficult to achieve for the average stable mRNA because, even after an immediate cessation of transcription, such a transcript could remain functional for several hours. Thus in plants, in addition to genes with a role in growth and development, genes involved in other rapid (e.g., environmental) responses might also be apt to encode unstable transcripts. The functions of the few plant proteins that are known to be encoded by unstable transcripts generally support this premise. These proteins include PvPRP1, which participates in the response of bean cells to pathogen attack (the half-life of its transcript appears to decrease markedly following the treatment of bean cells with an elicitor [35]); phytochrome, which mediates many light-regulated responses [31]; and the Small Auxin Up RNAs (SAURs) of soybean [9, 17] and *Arabidopsis* [10; P. Gil and P.J. Green, unpublished], which encode proteins of unknown function, but whose expression properties are consistent with a role in auxin-induced cell elongation [9, 17]. However, the mechanisms that target these and other unstable transcripts for rapid degradation in plants remain to be elucidated.

Initial insights have been provided by recent studies examining the effect of specific sequence elements on reporter transcript stability in transformed tobacco cell lines [22]. In these experiments, transcription was halted by the addition of actinomycin D (ActD), and the subsequent decay of the reporter transcripts was monitored by northern blotting [22]. This approach has enabled us to identify two very different sequences that can confer instability on mRNAs in plants. One, termed DST, is highly conserved within the 3′-UTRs of all the SAURs reported to date [10, 18, 22]. When present in two copies in the 3′-UTRs of reporter genes, the DST element leads to a pronounced reduction in mRNA half-life [22]. The other sequence, consisting of 11 overlapping copies of the AUUUA motif, was also found to markedly decrease reporter transcript half-lives [23]. These observations demonstrate that sequence-specific recognition of unstable transcripts occurs in plants. However, it is unlikely that the DST and AUUUA elements are representative of the repertoire of instability determinants present in plant transcripts. We will need to identify and study a variety of unstable plant transcripts to obtain direct information about the complexity of different mechanisms by which specific transcripts are selected for rapid degradation.

As a first step toward this goal, we report here the application of a novel differential screening approach to identify genes with unstable transcripts (*GUT*s) in tobacco. The *GUT*s, which were identified solely on the basis of the instability of their transcripts, provide tools for the further characterization of mRNA decay pathways, and have yielded additional information concerning the kinds of proteins that are encoded by unstable transcripts in plants.

Materials and methods

Preparation of cDNA probes and RNA isolation

Culture conditions for the *Nicotiana tabacum* cv. Bright Yellow 2 (BY-2) cells [19] used in this work have been described elsewhere [22]. These cells have also been referred to as NT-1 cells [2, 22, 23]. To generate cDNA probes for the differential screen, duplicate BY-2 cultures at 3 days post sub-culture were left untreated (control cells), or treated for 90 min with the transcriptional inhibitor ActD at 100 μg/ml (this concentration of ActD was previously shown to inhibit incorporation of [^{3}H]-uridine into poly(A)$^+$ RNA by 94% [22]). To test the effect of the protein synthesis inhibitor cycloheximide (CHX) on *GUT* transcript accumulation, BY-2 cells were treated for 90 min with both ActD, at 100 μg/ml, and CHX, at 50 μg/ml [13]. Cells were harvested by centrifugation at 700 × *g* and frozen in liquid nitrogen. Total RNA was isolated essentially as described [20], except that the extraction solution was buffered with 80 mM Tris at pH 7.5 (in place of 25 mM sodium citrate, pH 7) and a second CsCl cushion, at 2.8 M, was included in the gradient. Poly(A)$^+$ RNA was prepared from these samples using oligo-dT columns (5 Prime – 3 Prime, Boulder, CO), and 1 μg of each poly(A)$^+$ RNA was used as template for the preparation of random-primed first-strand cDNA probes, as described [29].

Isolation of GUT *clones*

The BY-2 cell cDNA library used for screening for *GUT*s (the generous gift of Dr G. An) was prepared from RNA isolated from BY-2 cells 3 days after subculture, and cloned into λZAP II (Stratagene) with *Eco* RI-*Not* I adaptors [8]. The library was plated at low density (5000 plaques per 150 mm plate). Duplicate lifts were prepared, using standard protocols [29], and were prehybridized and hybridized as described [34]. Promising clones were plaque purified and converted into plasmid form [5]. For northern and Southern blot analyses, the *GUT* inserts were labelled with [^{32}P] by random priming [7].

Half-life determinations

Determinations of *GUT* mRNA half-lives were performed as described previously [22]. Briefly, BY-2 cell lines expressing a 35S-GUS-3C reference gene [22] were treated with ActD at 100 μg/ml, and RNA was isolated from aliquots of cells harvested every 30 min thereafter. Northern blots of total RNA were prepared and hybridized to *GUT* and GUS probes as described [22]. Quantitation of *GUT* and GUS mRNA half-lives was performed using a phosphorimager as described previously [22]. For each *GUT* probe, at least three northern blots were prepared with RNA isolated from time-course experiments performed using two independent cell lines.

Southern analysis

DNA was isolated from *Nicotiana tabacum* SR1 using a cetyltrimethylammonium bromide (CTAB) extraction procedure [26]. Then 10 μg of this DNA, digested with Eco RV or Hind III, were separated on 1% agarose gels and transferred to Hybond N membrane, as recommended by the manufacturer (Amersham). Prehybridization, hybridization, and washing of the Southern blots were performed using the same conditions as for the northern blots [34].

Sequence analysis

Sequencing was performed at the Plant Biochemistry Facility at the Plant Research Laboratory. Plasmid DNA from each *GUT* cDNA clone (or subclone) was prepared using Magic miniprep columns (Promega), and sequenced using *Taq* cycle sequencing and the Applied Biosystems 373A automatic sequencer, as recommended by the manufacturer. Nucleotide sequences of 250–300 bp from each end of the cloned *GUT* fragments were compared to sequences in the protein databases using the BLASTX algorithm under default parameter settings [1]. The nucleotide sequences of any *GUT* clones for which no data-

base matches were found at the amino acid level were compared to the nucleotide databases using the BLASTN algorithm [1].

Results and discussion

Identification of GUT *cDNA clones*

We have found previously that treatment of BY-2 cells with actinomycin D (ActD) effectively inhibits RNA polymerase II transcription [22], and have used this inhibitor as a tool to measure the half-lives of destabilized reporter transcripts in the absence of mRNA synthesis [22, 23]. ActD treatment should lead to a rapid decrease in the levels of endogenous unstable transcripts. For example, after 90 min of ActD treatment, a transcript with a half-life of 20 min would be present at only 6% of its level in control, untreated cells, whereas the levels of an average transcript, with a half-life of several hours [4, 25], would remain essentially unchanged. This disparity between levels of unstable transcripts in ActD-treated cells and their levels in untreated cells formed the basis for the differential screen described in this report. cDNA probes prepared using poly(A)$^+$ RNA from untreated cells should contain a full complement of BY-2 mRNA sequences, and should hybridize to each clone in the library. In contrast, the 90 min ActD probe will be essentially depleted of sequences corresponding to highly unstable transcripts, and should hybridize weakly or not at all to clones corresponding to unstable transcripts.

A total of ca. 300 000 plaques of a BY-2 cell cDNA library were screened with control and ActD cDNA probes, as described in Materials and methods. To minimize the selection of false-positive clones, the first filter lifted from each library plate was hybridized to the ActD first-strand cDNA probe, and the second to the control probe. Most plaques did not show significantly decreased hybridization to the ActD probe relative to that of the control probe, consistent with the premise that most plant mRNAs have half-lives greater than 90 min. However, about 150 plaques were identified that hybridized weakly to the ActD probe relative to the control probe, and these were isolated and rescreened. Of these, six (clones 3-2, 7-2, 8-1, 8-2, 8-3 and 15) were designated potential *GUT*s, as the plaques continued to show a diminished hybridization signal with the ActD probe. Purified plaques of potential *GUT* clones were converted into plasmid form, and the plasmid DNA was digested with the restriction endonuclease *Eco* RI to release the *GUT* cDNA fragment(s). Some individual λ clones had more than one *Eco* RI fragment. Southern and northern analyses indicated that these corresponded to separate cDNAs that had become fused during the preparation of the library. The *Eco* RI fragments were subcloned, and the clone names appended with an alphabetical modifier (e.g. λ clone 8-2 contained two separate *GUT* cDNAs, viz. *GUT* 8-2a and *GUT* 8-2b).

Further evaluation of the potential *GUT* clones was performed by examining transcript levels in the control and ActD-treated BY-2 cells. The *Eco* RI fragments from the potential *GUT* clones were labelled with [^{32}P]-dCTP to similar specific activities and hybridized to northern blots of total RNA from control and ActD-treated BY-2 cells. Quantitation of the northern blots shown in Fig. 1 demonstrated that from the original six λ clones, a total of 8 cDNA clones (3-2, 7-2a, 7-2c, 8-1, 8-2a, 8-2b, 8-3, 15) appeared to be *GUT*s, because they exhibited a more than twofold decrease in transcript abundance during 90 min of ActD treatment (see Table 1). One clone (7-2b) that showed little difference in transcript levels in the cells treated with ActD (Table 1) was designated a *NOTGUT*, and was used as a control for subsequent experiments.

Cycloheximide effect

It has been suggested that treatment with the protein synthesis inhibitor cycloheximide (CHX) tends to stabilize unstable transcripts in yeast and other systems [14]. These data have been interpreted as implying either that the unstable transcript in question is only rapidly degraded while it is being actively translated (i.e. an effect in *cis*),

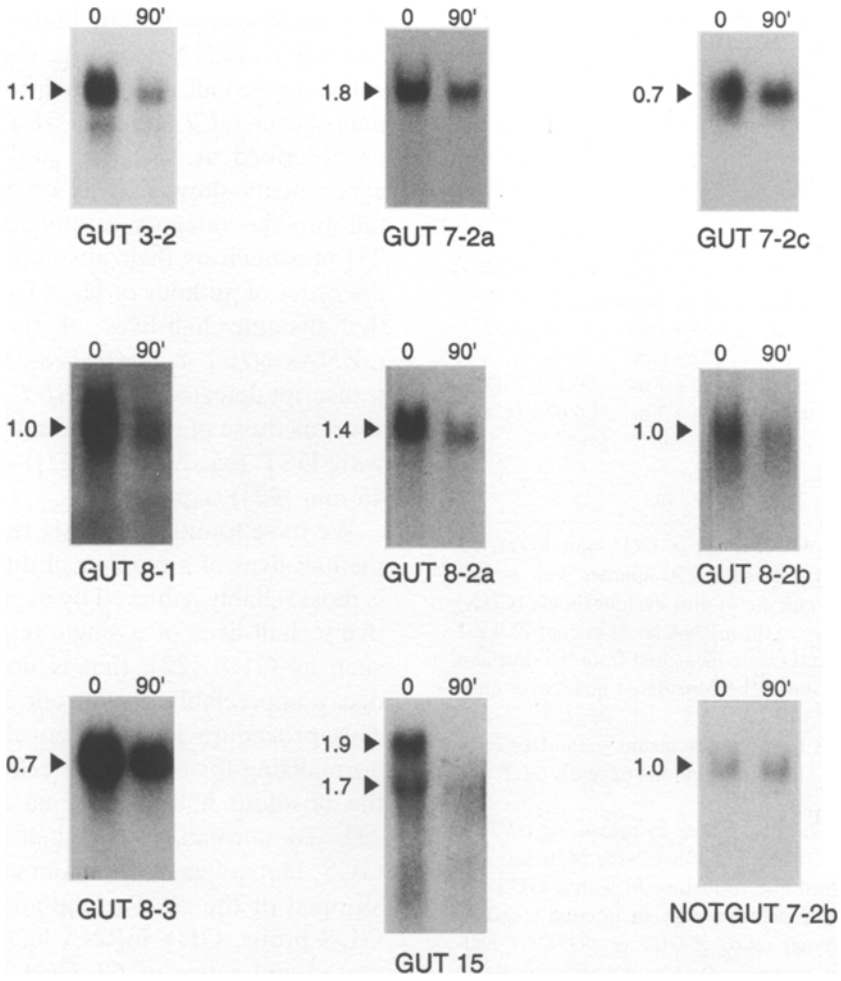

Fig. 1. Initial characterization of potential *GUT*s. Northern blots of 10 μg total RNA isolated from control, untreated BY-2 cells (0), and from cells treated for 90 min with ActD (90′) were hybridized to the indicated *GUT* and *NOTGUT* probes. Sizes of the major transcripts detected by each probe (arrowheads) were calculated relative to RNA molecular weight markers (Gibco/BRL).

or that a labile protein factor participates in the degradation of the mRNA (i.e. an effect in *trans*) [14, 28]. We were interested in examining the effect of CHX on the accumulation of *GUT* and *NOTGUT* transcripts in BY-2 cells. Therefore, we compared *GUT* transcript levels in RNA isolated from BY-2 cells that had been treated for 90 min with both ActD and CHX, and in RNA isolated from control, untreated cells. The data shown in Table 1 indicate that CHX treatment does not affect all unstable transcripts similarly in BY-2 cells (Table 1). In most cases, the *GUT* transcripts did not decline in the presence of ActD plus CHX, as would be expected if CHX inhibited their decay. However, CHX did not eliminate the decay of the larger of the two transcripts identified by the *GUT* 15 cDNA probe. In contrast, the accumulation of the smaller *GUT* 15 transcript in the ActD + CHX-treated cells increased markedly (Table 1). We have preliminary evidence that there are two different classes of *GUT* 15 cDNA (C.B. Taylor and P.J. Green, unpublished) and it is possible that the CHX effect on each class is different. Why the smaller *GUT15* transcript (and to a lesser extent some of the other transcripts) increased in abundance during CHX treatment is unknown. Under our conditions, ActD inhibits transcription by 94% within 30 min

Table 1. *GUT* and *NOTGUT* mRNA Levels and Half-Lives

GUT	ActD effect (fold)[1]	CHX effect (fold)[1]	Absolute half-life (*GUT*)[2]	Relative half-life (*GUT*/GUS)[3]
3-2	3.0↓	1.5↑	75 ± 30 m	1.26 ± 0.30
7-2a	2.6↓	ND	75 ± 16 m	0.99 ± 0.20
7-2c	2.2↓	1.5↑	52 ± 10 m	0.84 ± 0.20
8-1	4.0↓	2.0↑	42 ± 10 m	0.77 ± 0.20
8-2a	2.6↓	1.1↓	45 ± 10 m	0.77 ± 0.08
8-2b	2.8↓	2.0↑	56 ± 16 m	0.95 ± 0.33
8-3	3.0↓	1.0↕	52 ± 6 m	0.79 ± 0.16
15 1)	18.0↓	6.0↓	32 ± 7 m	0.37 ± 0.12
2)	5.5↓	6.5↑	54 ± 7 m	0.67 ± 0.29
NOTGUT				
7-2b	1.1↓	2.3↑	> 3 hr	> 3

[1] The increase (↑) or decrease (↓) in *GUT* and *NOTGUT* mRNA in NT-1 cells treated for 90 minutes with actinomycin D (ActD) or with ActD plus cycloheximide (CHX) was calculated relative to the mRNA levels in control BY-2 cells. The data for ActD were measured from the northern blots in Fig. 1, and should be considered qualitative measurements.

[2] The absolute mRNA half-lives are means ± standard error of at least three half-life determinations for each *GUT* and *NOTGUT*.

[3] Relative half-lives were determined by measuring *GUT* or *NOTGUT* and GUS mRNA half-lives (see Materials and methods) in BY-2 cell lines transformed with a GUS reference construct used previously as an internal standard [22]. Values are average ratios of *GUT* or *NOTGUT* half-life to GUS half-life, ± standard error, and were calculated from at least three different northern blots for each *GUT* and *NOTGUT*.

ND, not determined

[22] but it is conceivable that CHX could induce transcription before this inhibition is complete. A CHX effect on transcription has been suggested previously in plants (unpublished results in [3] and is known to occur in other systems [15, 16].

GUT *mRNA half-lives*

To confirm that the *GUT*s did indeed encode unstable transcripts, *GUT* mRNA decay rates were measured by northern blotting of RNAs isolated from BY-2 cells at 30 min intervals after the addition of ActD (see Materials and methods). Representative northern blots of *GUT* and the *NOTGUT* mRNA decay are shown in Fig. 2. To calculate the half-lives of their transcripts, the signals of each *GUT* and *NOTGUT* were quantitated as described in Materials and methods. These experiments show that all of the *GUT* mRNAs fall into the category of unstable transcripts [4, 25] inasmuch as their absolute half-lives are on the order of an hour or less (Table 1). Moreover, the absolute half-lives of three of the *GUT* mRNAs (*GUT* 8-1, *GUT* 8-2a, and the larger transcript detected by the *GUT* 15 probe) were as short as those of reporter transcripts destabilized with DST (ca. 33 min [22]) or AUUUA (ca. 45 min [23]) sequences.

We have found previously that comparison of the half-lives of a number of different transcripts is most reliably achieved by expressing them relative to half-lives of a single reference transcript, such as GUS [22], that is unstable enough to decay appreciably during our 2.5-h experiment. This procedure provides an effective means of normalizing for cell-line to cell-line variations in the absolute half-lives of each transcript [22, 23]. To normalize *GUT* half-lives to those of GUS, blots of each time-course experiment were stripped of the *GUT* probe and hybridized to a GUS probe. GUS mRNA half-lives were quantitated and ratios of *GUT*/GUS half-lives (relative half-lives) were calculated as described [22]. Table 1 shows that, as expected, the rank order of *GUT* mRNA stabilities is similar, whether they are expressed as absolute or relative half-lives. The relative half-life of the *NOTGUT* 7-2b transcript was estimated at > 3, because it did not degrade appreciably over the 150 min time course (Fig. 2 and Table 1). Thus, based on the relative half-life calculations, the *GUT* transcripts degrade at least 2.4- to 8-fold faster than the stable *NOTGUT* transcript (Table 1).

Structural characteristics of the GUT *s and* NOTGUT

To investigate the number of genes that hybridize to the individual *GUT* probes, Southern analyses

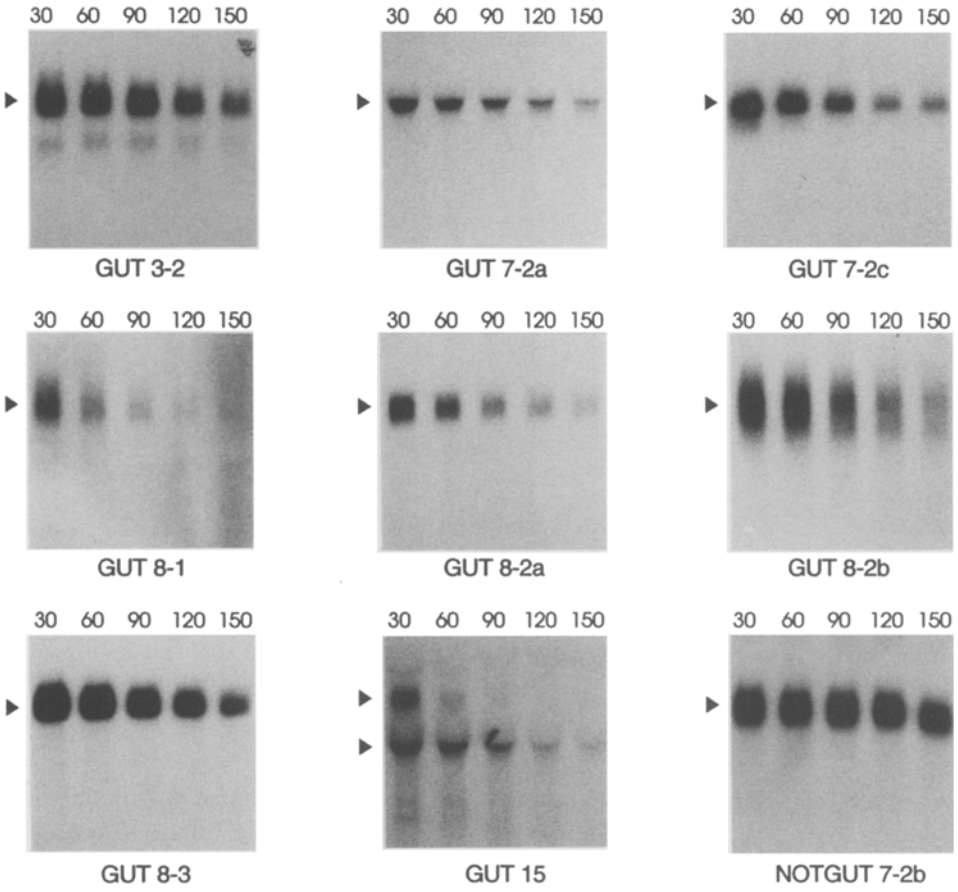

Fig. 2. Time-course experiments to determine *GUT* transcript half-lives.Northern blots of 10 μg total RNA, isolated from BY-2 cells at the indicated times (in minutes) after the addition of 100 μg/ml ActD to the cultures. Blots were hybridized to the indicated *GUT* and *NOTGUT* probes. Half-life measurements from these and additional experiments were determined following quantitation of the signals using a Phosphorimager, and are summarized in Table 1.

were performed as described in Materials and methods. The results are shown in Fig. 3, and the gene numbers estimated in Table 2. The most complex patterns were obtained with the *GUT* 8-1 and *GUT* 8-2b probes, which correspond to an unidentified protein and histone H3, respectively (see below). These probes hybridized to >10 fragments in each digest (Fig. 3). Conversely, the *GUT* 3-2, *GUT* 7-2c and *GUT* 15 cDNA probes hybridized to 1–5 bands per digest (Fig. 3), indicating that they are encoded by single or few copy genes in tobacco, and that the genomic DNA used in these experiments was digested to completion.

In general, the sizes of the *GUT* and *NOTGUT* cDNA clones correlate with the sizes of the corresponding transcripts. With the exception of *GUT* 3-2, all of the *GUT* and *NOTGUT* cDNA probes are of equal size or smaller than the major transcripts they detect on northern blots (see Table 2). Nonetheless, in some cases (e.g., *GUT*s 3-2, 7-2c and 15), it is clear that an individual *GUT* cDNA probe hybridizes to more than one transcript in BY-2 cells (see Figs. 1 and 2). The origins of these smaller transcripts are unknown at present. One intriguing possibility is that some may represent *GUT* mRNA degradation products. However, it is more likely that they are full-length mRNAs derived from genes closely related to the *GUT*s. The presence of two different cDNAs in a single *GUT* probe cannot be ruled out, especially for *GUT* 3-2, where the cloned

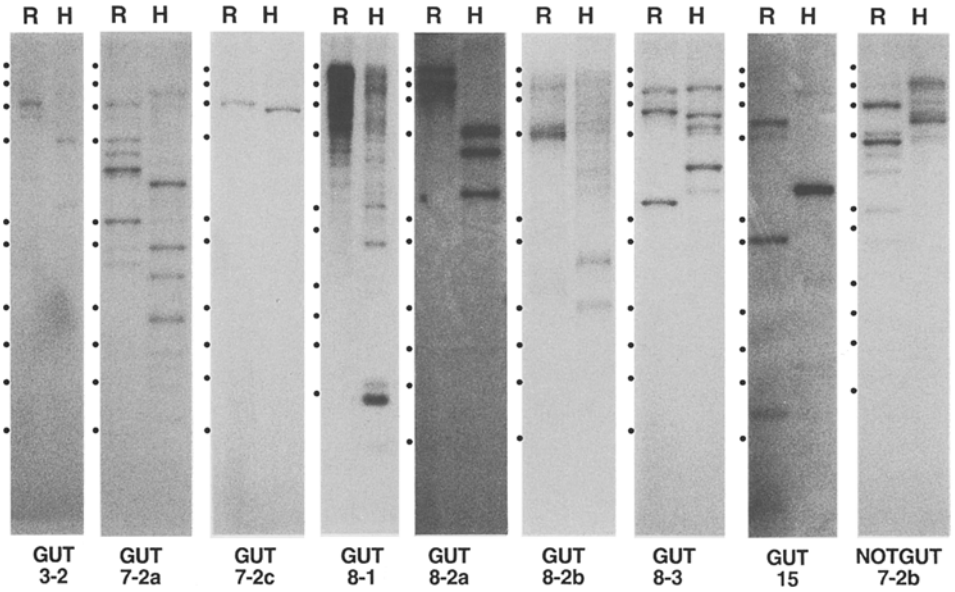

Fig. 3. Southern analysis of *GUT* and *NOTGUT* genes. Southern blots of tobacco genomic DNA digested with *Eco* RV (R) or *Hin*d III (H) were hybridized to the indicated *GUT* and *NOTGUT* probes. Dots to the left of each panel represent the positions of DNA molecular weight markers (λ/*Hin*d III and ϕX174/*Hae* III) in each gel. From top to bottom, these correspond to: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 1.4, 1.1, 0.9, and 0.6 kb.

insert is about three times larger than the major transcript it detects on northern blots (Table 2). The northern blot experiments in Figs. 1 and 2 also serve to demonstrate that the basal expression levels of the *GUT*s differ markedly from one another, despite the instability of all their transcripts. For example, *GUT*s 3-2 and 8-3 are expressed at very high levels in control BY-2 cells, but *GUT*s 7-2a and 15 are expressed at levels that are barely detectable on northern blots (Figs. 1 and 2). Moreover, the sizes of the *GUT* transcripts range from 1.9 kb for the larger *GUT* 15 mRNA, to 0.7 kb for *GUT*s 7-2c and 8-3, indicating that transcript size per se is unlikely to influence transcript half-life.

GUT *sequence similarities*

Information about the possible functions of protein products of the *GUT*s can be obtained by identifying similarities between the sequences of the GUTs and known protein sequences. This information may suggest reasons why the corresponding transcript is unstable. A similarity search of deduced amino acid sequences from the ends of each *GUT* and *NOTGUT* cDNA clone against protein sequences in the databases using the BLAST algorithm [1] reveals that four of the *GUT*s (7-2a, 7-2c, 8-2a, and 8-2b) and *NOTGUT* 7-2b are similar to previously characterized proteins. These similarities are discussed further below. For the remaining *GUT*s (3-2, 8-1, 8-3, and 15), there were no database matches at the amino acid level, suggesting that these *GUT*s represent novel cDNAs. A subsequent similarity search of the nucleotide sequences of these *GUT* cDNAs against the nucleotide databases confirmed this contention.

As shown in Fig. 4A, sequence from one end of the *GUT* 7-2a cDNA suggests that this GUT is a novel plant member of the eukaryotic family of *Escherichia coli* DnaJ homologues. These proteins, which have been identified in yeast, *Drosophila* and human cells, are thought to interact with Hsp70 proteins and to play roles in protein sorting [32]. GUT 7-2a possesses each of the residues that define the N-terminal 'J domain' of this

Table 2. Structural characteristics of *GUT*s and *NOTGUT*s

GUT	Transcript size (Kb)	cDNA size (Kb)	Estimated gene number[1]	Sequence similarity[2]	Accession numbers[3]
3-2	1.1	2.8	1–2	none	44367
7-2a	1.8	0.75	5–10	DNA J	44368
					44377
7-2c	0.7	0.35	1–2	W.I.P	44370
8-1	1.0	1.0	> 10	none	44371
					44378
8-2a	1.4	1.4	2–5	E2	44372
					44379
8-2b	1.0	0.6	> 10	Histone	44373
8-3	0.7	0.6	2–5	none	44374
15 1)	1.9				44375
2)	1.7	1.7	2–5	none	44376
NOTGUT					
7-2b	1.0	0.4	5–10	S5 protein	44369

[1] Gene numbers were estimated from the approximate number of bands on Southern blots of tobacco DNA (Fig. 3).
[2] See Fig. 4, and text.
[3] dbEST.

family of proteins [32]. The C-terminal domains of the eukaryotic DnaJ homologues are generally less conserved, and are thought to modulate the specificity of their interactions with Hsp70 proteins. It is not surprising, then, that sequence from the other end of the *GUT* 7-2a clone appears to have no similarity with previously characterized DnaJ proteins. Interestingly, the *GUT* 7-2a cDNA probe hybridizes with multiple bands on Southern blots (see Fig. 3), suggesting that tobacco may contain a family of DnaJ homologues.

Deduced amino acid sequence of GUT 7-2c (sequence from each end of the 350 bp *Eco* RI fragment overlaps in the middle) is similar to a fragment of a tomato wound-inducible protein (see Fig. 4B). Although the kinetics of induction of the homologous tomato transcript after wounding have not been described, in general wound-inducible proteins are rapidly induced at the mRNA level in response to wounding in plants [e.g. 27], and therefore conform to our predictions as to the type of genes that are likely to have unstable transcripts. GUT 8-2a has restricted similarity to the extreme N-terminus of the ubiquitin-conjugating enzyme E2 of *Drosophila melanogaster* (63% similarity over 44 amino acids; Fig. 4C), but its similarity to plant E2s is weaker. Whether or not the E2 enzymes have unstable transcripts has not been evaluated.

The strongest similarity between a GUT sequence and one on the databases is that of GUT 8-2b which, over the 600 bp cloned fragment, is 99% identical to maize histone H3 (Fig. 4D). Rapid changes in the levels of cell-cycle-regulated histone genes is thought to be facilitated by the instability of their transcripts [24]. Indeed, the histone H3 transcript has previously been characterized as unstable in mammalian cells [30], so it is not surprising that the corresponding tobacco gene was identified in our screen for *GUT*s. The sequences controlling the rapid degradation of animal cell-cycle-regulated histone transcripts involve a stem-loop structure in the 3′ end [12]. It is not clear whether this is also the case for plant histone mRNAs, which lack strong sequence conservation in their 3′-UTRs and, unlike animal histone transcripts, are polyadenylated [21].

NOTGUT 7-2b is also similar to a protein in the databases. The deduced amino acid sequence of the *NOTGUT* 7-2b cDNA is 97% similar to rat

A

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>SP:DNAJ_ECOLI DNAJ PROTEIN. Length = 376

Score = 248 (122.7 bits).  Identities = 44/88 (50%).  Positives = 69/88 (78%)

GUT 7-2a:   19 VPKGASDEQIKRAYRKLALKYHPDKNPGNEEANTKFAEINNAYEVLSDSEKKNIYDRYGEEGLKQHAASGGGRGAGMNIQDIFSQFFG 282
               V+K A + +I++AY++LA+KYHPD+N G+ EA++KF EI++AYEVL+DS+K+  YD+YG  +  Q + +GGG G+G +  DIF++ FG
DNA J:      12 VSKTAEEREIRKAYKRLAMKYHPDRNQGDKEAEAKFKEIKEAYEVLTDSQKRAAYDQYGHAAFEQGGMGGGGFGGGADFSDIFGDVFG 99
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B

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>PIR:S19773 Wound-induced protein - Tomato (fragment). Length = 76

Score = 77 (39.5 bits)                Score = 62 (31.8 bits)                Score = 53 (27.2 bits)
Identities = 15/16 (93%)              Identities = 11/20 (55%)              Identities = 13/25 (52%)
Positives = 16/16 (100%)              Positives = 15/20 (75%)               Positives = 17/25 (68%)

GUT 7-2c:   72 WIVAASIGAVEALKDQ 119    120 GFARWNYALRSLHHYAKTNL 179          225 ASAADISGEKFRKTEESLNKVMGLS 299
               WIVAAS+GAVEALKDQ                G  RWNY+LRSL + +K N+                +S+     +EK  K+EESL KVM LS
W.I.P.:      1 WIVAASVGAVEALKDQ 16      18 GLCRWNYPLRSLAQHTKNNV 37           47 SSSITTKSEKMEKSEESLRKVMYLS 71
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C

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>PIR:S19157 *Ubiquitin-conjugating enzyme - Fruit fly (Drosophila melanogaster). Length = 147

Score = 80 (39.7 bits). Identities = 15/44 (34%). Positives = 28/44 (63%).

GUT 8-2a:  298 AVKRILQEVKEMQSNPSDDFMSLPLEENIFEWQFGIRGPRDSEF 429
               A+KRI  E+ ++  +P+ +  + P+ +++F WQ  I GP DS +
Drome E2:    2 ALKRINKELQDLGRDPPAQCSAGPVGDDLFHWQATIMGPPDSPY 45
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D

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>sp|P05203|H3_MAIZE HISTONE H3. Length = 136

Score = 668 (315.4 bits). Identities = 135/136 (99%). Positives = 135/136 (99%)

GUT 8-2b:   92 MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRFRPGTVALREIRKYQKSTELLIRKLPF 161
               MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRFRPGTVALREIRKYQKSTELLIRKLPF
Histone H3:  1 MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRFRPGTVALREIRKYQKSTELLIRKLPF 69


GUT 8-2b:  162 QRLVREIAQDFKTDLRFQSSAVAALQEAAEAYLVGLFEDTNLCAIHAKRVTIMPKDIQLPRRIRGERA 499
               QRLVREIAQDFKTDLRFQSSAVAALQEAAEAYLVGLFEDTNLCAIHAKRVTIMPKDIQL RRIRGERA
Histone H3: 70 QRLVREIAQDFKTDLRFQSSAVAALQEAAEAYLVGLFEDTNLCAIHAKRVTIMPKDIQLARRIRGERA 136
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E

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>SP:RS5_RAT 40S RIBOSOMAL PROTEIN S5. Length = 204
Score = 217 (111.0 bits). Identities = 45/49 (91%). Positives = 48/49 (97%).

 NOTGUT 7-2b:  19 TGARESAFRNIKTIAECLADELINAAKGSSNSYAIKKKDEIERVAKANR 165
                  TGARE+AFRNIKTIAECLADELINA KGSSNSYAIKKKDE+ERVAK+NR
    Rat S5:   156 TGAREAAFRNIKTIAECLADELINARKGSSNSYAIKKKDELERVAKSNR 204
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Fig. 4. Sequence similarities of *GUT* and *NOTGUT* cDNAs. Nucleotide sequence data were obtained from both ends of each *GUT* and *NOTGUT* cDNA clone and compared to sequences on databases using the default parameters of the BLASTX algorithm as described in Materials and methods. Shown here are the alignments produced from the BLAST similarity searches. A. GUT 7-2a, B. GUT 7-2c, C. GUT 8-2a, D. GUT 8-2b, E. NOTGUT 7-2b. Score and (+) refer to alignment score and similar amino acids, as defined by the BLAST algorithm [1].

40s ribosomal protein S5 over a stretch of 49 amino acids. The stability of the *NOTGUT* 7-2b transcript is consistent with a 'housekeeping' role for the corresponding protein.

Beyond their similarities to sequences in the databases, a number of other interesting structural features of the *GUT* cDNAs have emerged. First, a comparison of identifiable *GUT* 3′-UTRs using the DNASIS program (Hitachi) indicated that there are no obvious conserved motifs. There are occasional AUUUA motifs, but these do not occur in highly AU-rich regions resembling the AREs, nor are they restricted to *GUT* 3′-UTRs, occurring also in the *NOTGUT* sequence. While a contribution of these AUUUA elements in the instability of the *GUT* transcripts cannot be ruled out, previous studies have shown that the mere presence of an AUUUA element is insufficient to cause mRNA instability (e.g. see [23] for a discussion). Secondly, *GUT* 8-1, which gives a dif-

fuse signal on northern blots, with consistently high background signals, has two curious repeats that are quite close together. An $(AC)_8$ motif (which, if translated, would correspond to a threonine-histidine repeat) is found some 50 bases upstream of a stretch of 13 C residues (potentially encoding four prolines). The significance of these sequences is not clear but, interestingly, a search of the nucleotide databases with the *GUT* 8-1 sequence showed that the former motif is related to a human genomic minisatellite sequence.

It is not surprising that no sequence conservation was found within the *GUT* 3′-UTRs. With the possible exception of the AREs in mammals, little such conservation has been found between different unstable transcripts in animals. Recently, an extensive search of the nucleotide databases for broadly conserved elements in the non-coding regions of a large number of eukaryotic genes was performed [6]. Interestingly, the only similarities identified in this search were between pairs of homologous genes from different species, not between groups of otherwise unrelated genes [6]. An alternative reason for the apparent lack of sequence conservation among the *GUT*s is that the relatively small number of different *GUT* clones that were isolated in this initial screen may preclude an exhaustive analysis. The fact that only a single cDNA for each different *GUT* gene was identified suggests that the current collection of *GUT*s is not complete, and that additional *GUT*s are likely to exist.

Conclusions and future prospects

The data presented in this paper demonstrate that the differential screening strategy is a promising approach for the isolation of *GUT*s in tobacco, which may be broadly applicable to the identification of genes with unstable transcripts in other species. Using this approach, we have identified nine previously uncharacterized tobacco cDNAs. These include eight *GUT*s, with mRNA half-lives in the range of about an hour or less, on the same order as those of transcripts characterized as unstable in mammalian cells [4, 25]. Four of the *GUT*s represent completely novel cDNAs, whereas the other four *GUT*s, and the *NOTGUT*, encode proteins with similarity to proteins in the databases. These similarities conform to our predictions concerning the kinds of proteins likely to be encoded by unstable or stable transcripts. The *GUT*s should constitute excellent tools for evaluating the mechanisms by which unstable transcripts are recognized and targeted for rapid degradation in plants. In particular, it will be most informative to delineate the sequences within the *GUT* transcripts that mediate their instability. Moreover, it should also be possible to investigate the roles of processes such as translation, deadenylation and protein binding in the mRNA decay pathways of a number of individual unstable transcripts. These efforts should substantially increase our understanding of the range of different mechanisms by which unstable transcripts are targeted for rapid degradation in plants.

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