Genetics of the DST-mediated mRNA decay pathway using a transgene-based selection

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Abstract

mRNA sequences that control abundance, localization and translation initiation have been identified, yet the factors that recognize these sequences are largely unknown. In this report, a transgene-based strategy designed to isolate mutants of *Arabidopsis thaliana* that fail to recognize these sequences is described. In this strategy, a selectable gene and a screenable marker gene are put under the control of the sequence element being analyzed and mutants are selected with altered abundance of the corresponding marker RNAs. The selection of mutants deficient in recognition of the DST (downstream) mRNA degradation signal is used as a test-case to illustrate some of the technical aspects that have facilitated success. Using this strategy, we report the isolation of a new mutant, *dst3*, deficient in the DST-mediated mRNA decay pathway. The targeted genetic strategy described circumvents certain technical limitations of biochemical approaches. Hence, it provides a means to investigate a variety of other mechanisms responsible for post-transcriptional regulation.

Introduction

Rates of mRNA degradation are highly variable in eukaryotic cells and these differences allow for precise control of gene expression [1]. Mechanisms facilitating rapid mRNA decay are of particular interest because they provide a means for organisms to adjust RNA levels quickly in response to changes in transcription. Several sequences that target transcripts for rapid turnover in plants have also been identified. These include the DST (downstream) element [2], the AUUUA repeat [3] and the premature stop-codons [4]. To gain insights into the molecular basis of sequence-specific recognition and degradation of unstable mRNAs, a genetic approach was devised to isolate *Arabidopsis* mutants defective in DST-mediated mRNA degradation (*dst* mutants; [5]). Such a strategy is advantageous because (i) genes corresponding to the mutant phenotypes, by definition, affect the process *in vivo*; (ii) basic information about the mechanisms of mRNA degradation may be obtained by studying the mutants; (iii) genetic analysis is not limited by preconceived mechanistic ideas (e.g. the cellular factor that recognizes an RNA degradation sequence may itself be an RNA molecule, not a protein); and (iv) experimental limitations of biochemical approaches, such as unstable or low abundance proteins or the presence of non-specific RNA-degrading activities in protein extracts, are eliminated.

In this report, we describe the development of the transgenic system that was used to select the *dst1* and *dst2* mutants described previously [5], as well as a new mutant *dst3*, which are defective in DST-mediated mRNA decay. Emphasis has been placed on the theoretical and practical aspects of the approach that may be relevant to its application to other sequence-specific post-transcriptional regulatory systems.

A genetic approach for isolation of mutants defective in DST-mediated mRNA decay

The strategy to isolate mutants defective in the DST-mediated decay pathway was based on the use of both a selectable and screenable marker transcript that could be destabilized by the insertion of DST sequences. After mutagenesis of transgenic plants harbouring these genes, the goal was to select isolates with increased levels of the selectable marker transcript. Mutations in *trans*-factors required for the function of the DST element were our major interest, and these would be expected to increase the mRNA abundance of both the selectable and the screenable marker genes. In contrast, mutations in *cis* would result in increased mRNA abundance only of the selectable marker mRNA. To identify the optimal marker genes two antibiotic resistance genes, *HPH* (hygromycin phosphotransferase) and *DHFR* (dihydrofolate reductase), and two screenable markers, *Globin* (β-globin) and *GUS* (β-glucuronidase), were examined. As shown in

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Abbreviations used: DHFR, dihydrofolate reductase; DST, downstream; GUS, β-glucuronidase; HPH, hygromycin phosphotransferase; UTR, untranslated region.

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A selection strategy for the isolation of mutants defective in the DST-mediated decay pathway

(A) A comparison of the effect of the DST sequences on the mRNA abundance of selectable and screenable reporter genes. The histogram depicts the normalized abundance of reporter transcripts in pooled transgenic Arabidopsis calli relative to the non-destabilized control message. Multiple experiments were performed for each reporter gene and each experiment represents at least 100 independent calli. Error bars represent S.D. (B) Relevant steps in this mutant isolation strategy using the HPH and GUS markers. Arrows represent 35S promoter sequences, boxes represent coding sequences and 3′-UTRs respectively. T-DNA border sequences are signified by vertical lines.

Figure 1(A), insertion of two copies of the DST element (DST × 2) into the 3′-UTR (untranslated region) of these genes reduced the abundance of the DHFR transcript to a much lesser extent (<2-fold) than it did for HPH, GUS or Globin transcripts (approx. 4-fold), indicating that DHFR would not be an appropriate selectable marker for the dst mutant selection strategy. Insertion of four copies of the DST element into the HPH, GUS and Globin transcripts had an even greater effect than two copies, as expected.

On the basis of these results a T-DNA plasmid (p1519) was then constructed using HPH as the selectable marker gene and GUS as the screenable marker gene, each containing a tetramer of the DST element inserted into the 3′-UTR. A single transgenic line, homozygous for a single insertion of the T-DNA, was chosen for mutagenesis. This line also reflected the average mRNA abundance in a population of transgenic plants.

The selection

The selection of the dst mutants from mutagenized p1519 plants is summarized in Figure 1(B). Ethylmethane sulphonate was chosen over insertional mutagenesis because of its high mutation frequency. Seeds from multiple p1519 T3 plants were pooled so that adequate T4 seeds would be available for ethylmethane sulphonate mutagenesis [6]. A total of 794 000 M2 seeds from 46 independent groups were plated on to 100 µg/ml hygromycin, the lowest concentration of hygromycin that killed the parental plants. After 3–4 weeks, 338 plants had developed at least two true leaves and all were carried forward. Not surprisingly, most of these plants were escapes because only approx. 20% exhibited heritable hygromycin resistance. Most did not increase HPH mRNA and could be transport mutants. No candidates for cis mutations were found presumably because four copies of the DST element were present and only two were required for activity [2]. In the end, the mutants isolated were very rare since only three hygromycin-resistant plants met our stringent criteria of heritable increases in HPH-DST and GUS-DST mRNA abundance. Of these, dst1 and dst2 were isolated relatively early in the selection process and were found to be in independent genes [5].
Figure 2 | *dst3* is a novel mutation affecting the DST-mediated mRNA decay pathway

(A) Representative Northern blot of HPH mRNA in the *dst* mutants. mRNA abundance was measured in the rosette leaves of *dst3*, the parental line p1519, the non-destabilized control p1493 and in previously isolated mutants *dst1* and *dst2*. The abundance of HPH-DST mRNA has been normalized to that of translation initiation factor 4A, *eIF4A*. (B) Histogram representing abundance of HPH-DST transcripts from (A) normalized to *eIF4A*.

*dst3* defines a new gene in the DST-mediated mRNA degradation pathway

A third mutant, *dst3*, isolated during the later phase of the selection is shown in Figure 2(A). The level of HPH-DST transcript in *dst3* is approx. 3.5-fold higher relative to p1519 and this increase in mRNA abundance is similar to that observed in *dst1* and *dst2* (Figure 2B). The half-life of HPH mRNA was calculated to be 1.5-fold greater in *dst3* when compared with that in p1519 (results not shown), which is as expected for a 3–4-fold RNA increase (for a discussion, see [5]). This indicates that the *dst3* mutation results in an increase in mRNA stability that explains the increased abundance of the HPH-DST transcript.

To evaluate whether *dst3* was allelic with *dst1* or *dst2*, a complementation test was performed. Similar to *dst1* and *dst2*, *dst3* is partially dominant, a characteristic of these mutations that complicates standard complementation tests. The F1 progeny (*dst3*/DST3) of a cross between *dst3* and p1519-31 have HPH-DST mRNA abundance levels that are intermediate between *dst3*/dst3 plants and p1519-31 (DST3/DST3). The results of crosses to *dst1*/dst1 or *dst2*/dst2 were similar to those of the cross to p1519-31, indicating that *dst1*, *dst2* and *dst3* are probably affecting three distinct genes. Assuming there are no unusual genetic interactions of these partially dominant mutations, if *dst1* or *dst2* had been allelic with *dst3*, these crosses would have probably resulted in F1 plants with high levels of HPH-DST mRNA abundance.

Future prospects

Targeted genetic approaches have been used previously to isolate mutations that affect the function of specific promoter sequences [7–9]. In this report, the utility of such a procedure has been extended to a post-transcriptional cis-regulatory element. The approach described has broad applicability and provides a means to study many post-transcriptional regulatory pathways (e.g. translational control or protein turnover) using genetic analysis. To this end, multiple selectable and screenable marker genes could be tested for the impact of the appropriate cis-acting element as in Figure 1. This could be followed by (i) either chemical or insertion mutagenesis of the corresponding transgenic plants (depending on the expected mutant frequency) and (ii) selection.

The genetic isolation of the *dst* mutants coupled with functional genomic approaches (e.g. microarray experiments with *dst1*) have provided insight about the biological significance of the DST-mediated decay pathway, i.e. its association with the circadian clock [10]. Analysis of the effect of the different *dst* mutants on circadian gene expression should further aid in unravelling the mechanisms that underlie post-transcriptional control of clock-controlled gene expression.

References


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