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Analysis of mRNA stabilities during pollen development and in BY2 cells

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Summary

RNA stability is important in post-transcriptional gene expression. However, its role during plant male gametophyte development has not been studied in detail, probably because of technical difficulties and because earlier work suggested that, in general, mRNAs expressed during late pollen development would be stable. We determined mRNA stabilities of 12 pollen-expressed genes. We measured pollen mRNA decay during *in planta* development and *in vitro* maturation after transcriptional inhibition with ActinomycinD (ActD). Because five of these genes were also expressed in somatic cells we also measured RNA decay in BY2 cells after transcriptional inhibition with ActD. Ten of the mRNAs were highly stable during pollen development ($t_{1/2}$ > 30 h), including all seven pollen-specific mRNAs. However, two mRNAs showed a logarithmic decrease as pollen development proceeded, both *in planta* and *in vitro*. In fact, for one of these two, *GUT8-2b*, the mRNA level decreased significantly within 3 h. Our results show that many pollen-expressed mRNAs are indeed long-lived but that at least some mRNAs undergo decay during pollen maturation. Thus, there is no overall cytoplasmic condition in pollen that stabilizes mRNAs and specific mRNA turnover mechanisms must exist. We detected reciprocal mRNA stabilities with two genes that are expressed in both BY2 cells and pollen. *GUT15* mRNA, known to be unstable ($t_{1/2}$ < 3 h) in BY2 cells, was highly stable in pollen. In contrast, *GRP2* mRNA decayed in pollen but was highly stable in BY2 cells. Therefore, we conclude that mRNA stability can be tissue-specific.

Introduction

Detailed information on the stability of specific mRNAs and on other mechanisms of post-transcriptional regulation is available in many eukaryotes, such as yeast, *C. elegans*, drosophila, frog, mouse and human (Ross, 1995; Sachs, 1993; Stebbins-Boaz and Richter, 1997). There are usually considered to be three groupings of mRNA stability. Unstable mRNAs have half-lives of less than about 3 h. They frequently encode regulatory proteins or proteins that are induced in response to environmental signals; examples include *c-fos* and *c-myc* (Stebbins-Boaz & Richter, 1997). Most mRNAs are moderately stable and have half-lives of several hours. Highly stable mRNAs have half-lives of more than 1 day and frequently occur in terminally differentiated cells; examples include the globin mRNAs (Weiss and Liebhaber, 1994). The half-lives of different messages within the same cell can range from less than 30 min to more than 1 day (Sachs, 1993), implying that features of the mRNA contribute to stability. In animal reproductive cells, long-lived mRNAs are common. For example, in mammalian sperm and *Xenopus* oocytes, transcription stops before development is completed. Thus, many mRNAs must be pre-synthesized, but they are protected from degradation and premature translation because they are masked by proteins containing Y-box motifs (Stebbins-Boaz and Richter, 1997; Tafuri *et al.*, 1993; Wolffe, 1994). Developmental cues or fertilization subsequently leads to dramatic increases in protein synthesis due to recruitment of these masked mRNAs onto polysomes.

In plants, information on the stability of specific mRNAs is limited, as most studies have focused on short-lived mRNAs that are expressed in somatic cells (Abler and Green, 1996). If pollen is considered terminally differentiated then it might not contain mRNAs that undergo rapid turnover. The genes expressed in pollen have been grouped into two classes based on their expression patterns during development: 'early' genes, first expressed after the release of the microspores from the tetrad and 'late' genes, first expressed after the first microspore mitosis. From their work with transcriptional and translational inhibitors, Mascarenhas and co-workers proposed (about 25 years ago) that mRNAs of genes expressed during late pollen development might be stable and stored for later translation during pollen germination, in analogy with mRNA storage in animal reproductive cells (reviewed in Mascarenhas, 1990). However, the stability of individual mRNAs were not measured at that time. Many groups have since shown that the steady state levels of many pollen-expressed mRNAs increase during maturation (reviewed in Mascarenhas, 1990; Mascarenhas, 1993; McCormick, 1993). Such increases during late pollen development could reflect an inherent stability of the

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Abbreviations: ActD, Actinomycin D; $t_{1/2}$, RNA half-life; UTR, untranslated region.

mRNA but could also reflect a higher transcription rate relative to the decay of the mRNA. Thus, despite the fact that there is still little direct evidence for highly stable mRNAs in pollen, this concept was widely accepted.

Whether all or most mRNAs are highly stable in a specific cell type has major implications for the mechanisms that underlie their stability. In a scenario where all mRNAs are highly stable a cytoplasmic environment might exist that prevents mRNA decay, as was observed in oocytes and sperm cells (Wolffe, 1994). This contrasts with a scenario where some mRNAs decay, suggesting that specific cis- and trans-acting factors determine the stability of individual mRNAs, as observed in somatic cells (Abler and Green, 1996; Ross, 1995; Sachs, 1993).

In order to eventually determine the mechanisms that control mRNA stability during pollen development, we needed to determine the stability of mRNAs expressed during pollen development. Therefore, we exploited a tobacco *in vitro* pollen maturation system (Touraev *et al*., 1995) in order to effectively use the transcriptional inhibitor ActD and measure relative RNA stabilities. We also measured steady state mRNA levels at different time points during normal maturation in the anther, since a decline in the total amount of mRNA during *in planta* development also implicates mRNA decay. Ten of the mRNAs tested indeed turned out to be highly stable during late pollen development. Two less stable pollen mRNAs were identified, but no decay shorter than 12 h was found. In addition, we found that the stability of mRNAs in pollen is not necessarily correlated with their stability in somatic cells.

Results

In planta *mRNA expression patterns during late pollen development*

We selected 12 pollen-expressed mRNAs for analysis, including mRNAs for (putative) regulatory proteins as well as mRNAs for housekeeping proteins. Some of the mRNAs are pollen-specific or encode pollen-specific isoforms of somatically expressed proteins, while others are also expressed in somatic tissues. In addition, we selected two mRNAs because they have a short half-life in BY2 cells (Taylor and Green, 1995). Seven of the selected mRNAs are pollen-specific *(NT59*, *TP10*, *C27X, TAC112*, *NTP303*, *PDC2, NeIF4a8)* and are expressed at high levels in mature tobacco pollen, as judged by RNA gel blot analysis. *NT59* (Kulikauskas and McCormick, 1997) and *TP10* (Rogers and Lonsdale, 1992) are tobacco homologues of the well studied *LAT59* and *LAT56* genes; they encode proteins that are similar to pectate lyases and are presumed to be involved in the synthesis of the growing pollen tube wall (Kulikauskas and McCormick, 1997; references therein). *C27X* (Tebutt *et al*., 1994) encodes a polygalacturonase, also thought to be involved in pollen tube wall synthesis. *NTP303* encodes a pollen-specific protein that has homology to ascorbate oxidase (Weterings *et al*., 1992). *TAC112* encodes a pollen-specific actin isoform (Thangavelu *et al*., 1993). *PDC2* encodes a pollen-specific isoform of pyruvate decarboxylase (Bucher *et al*., 1995) and is involved in anaerobic fermentation in pollen. *NeIF4a8* encodes a pollen-specific translation initiation factor and was of special interest in this study because its steady state mRNA levels do not increase during late pollen development (Brander and Kuhlemeier, 1995). *ADH1* encodes alcohol dehydrogenase and, like *PDC2*, is involved in anaerobic fermentation in pollen, but is also expressed in leaf (Bucher *et al*., 1995). *NTK1* is a shaggy-like serine-threonine protein kinase homologue that is also expressed in all sporophytic tissues tested (Einzenberger *et al*., 1995).

GRP2 encodes a protein with a Y-box motif (Obokata *et al*., 1991). In mammals, Y-box genes are expressed in most cell types and can function both in transcriptional regulation in somatic cells and in RNA stabilization in reproductive cells. We were intrigued by this observation and therefore wanted to determine the expression of *GRP2*. RNA gel blot analysis showed equivalent expression in BY2 cells, root, leaf, sepals, petals, ovary, pistils, seed pods, uni-cellular microspores and early bi-cellular pollen, but surprisingly *GRP2* mRNA was not detected in mature pollen (data not shown). To precisely determine the *in planta* decay rate of *GRP2* mRNA, we isolated mRNA from bi-cellular pollen of anthers collected at different times during development and performed RNA gel blot analysis. The intensity of the hybridization signal at each time point was quantified and is represented in Figure 1. Intriguingly, *GRP2* mRNA levels steadily declined, unlike most previously characterized pollen-expressed mRNAs. The decrease of *GRP2* mRNA was logarithmic over an extended period of time. Since the steady state mRNA level is the sum of transcription and mRNA decay, the logarithmic decrease during *in planta* development can be used to calculate the maximum possible half-life for *GRP2* mRNA; it was 15.8 ± 4.2 h. The expression of other genes whose steady state mRNAs levels are known to increase during pollen development was determined using the same filters and these increases were confirmed (Figure 1, Table 1). Lastly, we confirmed (Figure 1) that the level of *NeIF4a8* mRNA is constant throughout pollen development, as reported previously (Brander and Kuhlemeier, 1995).

Because *GRP2* mRNA levels decreased during pollen development, we were interested to identify other mRNAs that might decay during pollen development. We therefore tested the *GUT* genes (genes with unstable transcripts) because they were known to be unstable in BY2 cells (Taylor and Green, 1995). First we needed to determine

which of the GUT genes might be expressed in pollen. By RNA gel blot analysis we measured the levels of *GUT7-2a*, *GUT8-2a*, *GUT8-2b* and *GUT15* mRNA in BY2 cells, early bi-cellular pollen and mature pollen. Similar levels of *GUT7-2a*, *GUT8-2a* and *GUT15* mRNA were observed in BY2 cells and mature pollen, but their mRNAs were low or undetectable in early bi-cellular pollen (data not shown). In contrast, *GUT8-2b* was expressed in early bi-cellular pollen, but not in mature pollen (data not shown). *GUT15* and *GUT8-2b* were hybridized to RNA isolated from bi-cellular pollen at different times during development. The mRNA level of *GUT15* increased, while the mRNA level of *GUT8-2b* decreased (Figure 1). The calculated maximum half-life for *GUT8-2b* mRNA is 12.3 h ± 3.6 h.

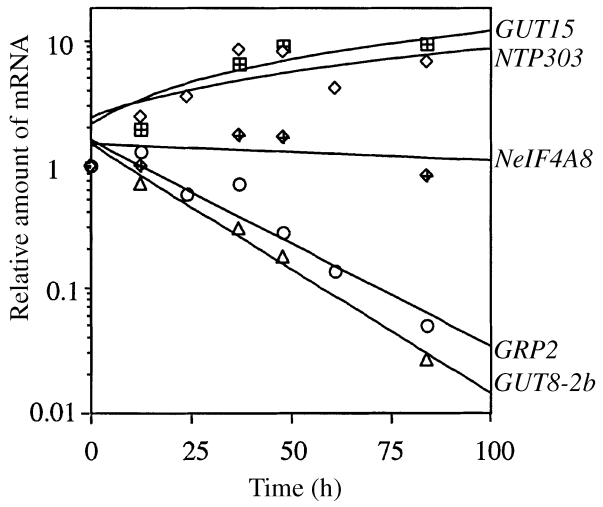

Figure 1. *In planta* steady state levels of five pollen-expressed mRNAs. RNA was extracted from pollen at different timepoints and analyzed by RNA blotting. Pollen is early bi-cellular at t = 0 h; at t = 100 h the anthers are dehisced and pollen is fully mature.

We can thus distinguish three classes of gene expression during late pollen development. Most of the tested genes have expression profiles expected for late pollen genes, with increases in steady state mRNA levels during late pollen development. However, the *NeIF4a8* mRNA expression level remains constant throughout pollen development, and the *GRP2* and *GUT8-2b* mRNA levels decline during late pollen development. With these results alone we cannot distinguish between the relative contributions of transcription and mRNA stability to these mRNA levels. Therefore we set up a system to determine the stability of each mRNA during pollen maturation.

Table 1. Developmental profiles and half-lives of mRNAs in pollen and BY2 cells

mRNA	References	Function/Homology	*In planta* pollen[a]	*In vitro* pollen[b]	BY2 cells[b]
Pollen specific genes					
NTP303	(Weterings *et al.*, 1992)	ascorbate oxidase	up[3]	stable[3]	
C27X	(Tebutt *et al.*, 1994; Rogers and Lonsdale, 1992)	polygalacturonase	up[2]	stable[2]	
TAC112	(Thangavelu *et al.*, 1993)	actin	up[2]	stable[6]	
TP10	(Rogers and Lonsdale, 1992)	pextate lyase, LAT56	up*	stable[2]	
NT59	(Kulikauskas and McCormick, 1997)	pectate lyase, LAT59	up*	stable[2]	
NeIF4A8	(Brander and Kuhlmeier, 1995)	elongation initiation factor 4A	steady[6]	stable[4]	
PDC2	(Bucher *et al.*, 1995)	pyruvate decarboxylase	steady*	stable[4]	
Pollen and BY2 expressed genes					
ADH1	(Bucher *et al.*, 1995)	alcohol dehydrogenase	up[1]	stable[3]	stable[3]
NTK1	(Einzenberger *et al.*, 1995)	shaggy-like serine/ threonine protein kinase	up[2]	stable[1]	stable[3]
GUT15	(Taylor and Green, 1995; Van Hoof *et al.*, 1997)	not known	up[3]	stable[2]	1.1 h ± 0.3 h[5]
GUT3-2	(Taylor and Green, 1995)	not known	nt	nt	1.5 h ± 0.2 h[2]
GUT8-2b	(Taylor and Green, 1995)	not known	12.3 h ± 3.6 h[5]	16.7 h ± 7.7 h[5]	2.9 h ± 0.8 h[3]
GUT8-2b -			28.9 h ± 8.6 h[4]		
GRP2	(Obokata *et al.*, 1991)	glycine-rich protein	15.8 h ± 4.2 h[5]	stable[6]	stable[3]
GRP2 -				14.2 h ± 4.5 h[5]	

[a]mRNA levels during normal *in planta* development; up, mRNA levels increase; steady, mRNA levels are constant throughout development; numbers, time it takes *in planta* (in hours with standard deviation) for the mRNA to decrease to 50%.; *results taken from the literature. nt, not tested.

[b]*In vitro* ActD experiments; stable, $t_{1/2}$ is >30 h; numbers, $t_{1/2}$ (in hours with standard deviation); values in parentheses represent times (in hours with standard deviation) for *GUT8-2b* and *GRP2* mRNA levels to decrease to 50% *in vitro* in the absence of ActD. Superscript numbers indicate the number of separate experiments performed.

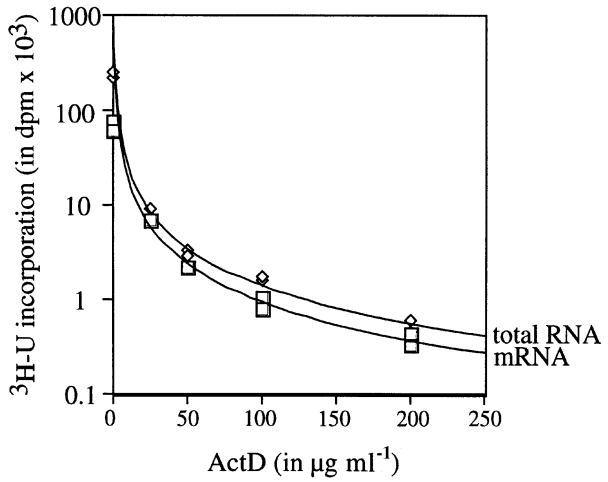

Figure 2. Transcriptional inhibition by ActD during *in vitro* pollen maturation.

ActD transcriptional inhibition during in vitro *pollen maturation*

To measure mRNA half-lives we used ActD, a potent inhibitor of transcription. Treatment with 100 μg ml^{-1} ActD resulted in 94% inhibition of *de novo* RNA synthesis in tobacco BY2 cells (Newman *et al.*, 1993). We tested whether ActD can block *de novo* RNA synthesis in developing tobacco pollen. ActD was added in different concentrations to *in vitro* maturing pollen and ^{3}H-Uridine was added to label *de novo* transcribed mRNAs. Figure 2 shows that 100 μg ml^{-1} ActD inhibited 98.6% of the ^{3}H-Uridine incorporation into mRNA. This concentration of ActD also blocked transcription of ribosomal mRNA because less than 1% of the ^{3}H-Uridine was incorporated in total RNA.

In vitro tobacco pollen maturation is equivalent to normal development (Touraev *et al.*, 1995), as demonstrated by a pollen germination test performed 2 days after the onset of maturation. Touraev *et al.* (1995) further demonstrated that *in vitro* matured pollen can achieve fertilization when transferred to a stigma. It was important to test the viability of the pollen during longer periods of ActD treatment because we might find mRNAs with long half-lives. Fluorescein diacetate staining revealed no decrease in viability when *in vitro* maturing pollen was treated with 100 μg ml^{-1} ActD for 30 h (data not shown), although germination was inhibited.

Most but not all mRNAs are highly stable during pollen development

We determined the stability of all 12 pollen-expressed mRNAs using ActD and *in vitro* maturation of early bicellular pollen. Total RNA was isolated at different timepoints after transcriptional inhibition with ActD (100 μg ml^{-1}) and analyzed by RNA gel blots. RNA was also isolated at the same timepoints from untreated cultures to determine and compare the profiles of the mRNAs in *in vitro* cultured pollen with those in the *in planta* situation. Figure 3 shows typical results from one of these experiments. Without ActD treatment the pollen-specific mRNAs *C27X, TAC112* and *NTP303* increase during *in vitro* maturation, as they do *in planta* (Figure 3, right). After ActD treatment the mRNA levels remained constant (Figure 3, left). Thus, these mRNAs are highly stable throughout pollen maturation as was predicted for most late pollen mRNAs (Mascarenhas, 1990). In similar experiments we assayed the mRNA levels for the other genes. *PDC2*, *ADH1*, *NTK1*, *NeIF4a8*, *NT59*, *TP10* and *GUT15* also qualified as highly stable since no decay was observed after transcriptional inhibition with ActD (Table 1). Of all these messages, only one (*NeIF4a8*) ever displayed a half-life less than 30 h ($^{ActD}t_{1/2}$ of 23.6 h; in only one out of four experiments). *GUT8-2b* and *GRP2* were hybridized to the same membranes used for the highly stable mRNAs. Within the first 3 h a clear decay of *GUT8-2b* was observed in each separate experiment. Its relative mRNA levels continued to follow a logarithmic decrease so that the $^{ActD}t_{1/2} = 16.7 \pm 7.7$ h. This confirmed that RNA decay can be monitored in pollen after a transcriptional block with ActD. Because the *in vitro* $^{ActD}t_{1/2}$ is very similar to the *in planta* $^{max}t_{1/2}$ we conclude that little or no *GUT8-2b* transcription takes place during late pollen development. The mRNA of *GRP2* was stabilized by ActD so that only in two out of six experiments was its half-life less than 30 h ($^{ActD}t_{1/2}$ of 20 h and 25 h; see also Figure 3b). ActD sometimes appears to stabilize mRNAs, perhaps because it inhibits transcription of genes whose products are needed for mRNA degradation (Abler and Green, 1996; Ross, 1995). It is also possible that *GRP2* is seemingly stabilized because ActD slows down the pollen maturation process. However, we think this is less likely because in the same experiment *GUT8-2b* mRNA decayed. Because of the stabilization of *GRP2* mRNA, we cannot exclude that ActD somewhat stabilized other messages and thereby might have slightly affected the half-lives of the mRNAs classified as highly stable. Nevertheless, we can conclude that *GRP2* mRNA decays both *in planta* and during *in vitro* pollen development in the absence of ActD. Because *GUT8-2b* and *GRP2* mRNA levels decline during late pollen development, it is possible that they correspond to early genes (Mascarenhas, 1990). Nonetheless, the finding that *GUT8-2b* and *GRP2* mRNAs are less stable during late pollen development indicates that differential RNA decay mechanisms are present in the cytoplasm of the developing pollen grain.

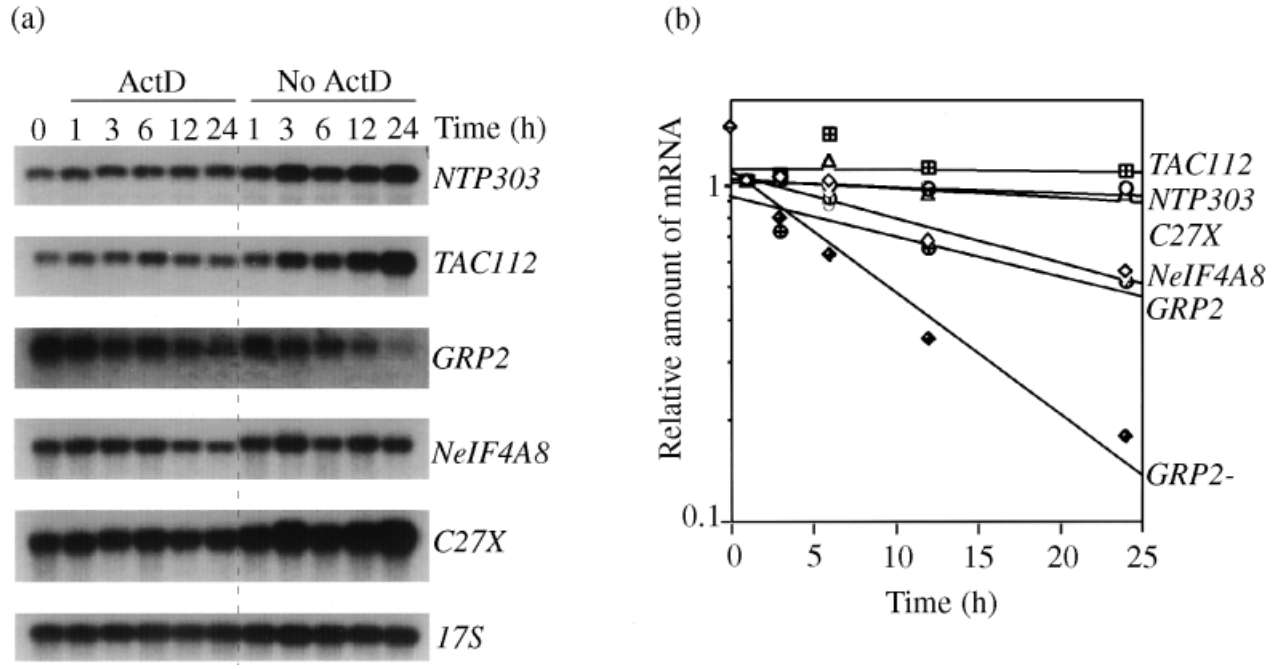

Figure 3. mRNA stability of five pollen-expressed genes.
(a) RNA blot analysis showing mRNA levels at different timepoints during *in vitro* pollen maturation with or without transcriptional inhibition by ActD.
(b) Graphical representation of the data in (a), showing relative mRNA levels after transcriptional inhibition by ActD. *GRP2-*, levels of *GRP2* mRNA in the absence of ActD.

Differential mRNA stability between pollen and somatic cells

A comparison of mRNA stability in pollen versus somatic cells might help identify any elements that are specific for RNA turnover in pollen. This comparison was possible because five of the 12 genes we studied are also expressed in BY2 cells (Table 1). In the *in vitro* pollen maturation experiments using ActD as a transcriptional inhibitor, we found that *GUT15* mRNA was highly stable and that *GUT8-2b* mRNA was moderately stable. Both mRNAs were previously identified as unstable in BY2 cells (Taylor and Green, 1995). We measured the mRNA stability of *GRP2*, *NTK1* and *ADH1* in BY2 cells. Figure 4 shows that none of these mRNAs decayed in BY2 cells after transcriptional inhibition with ActD. As controls, three unstable messages (*GUT3-2*, *GUT15* and *GUT8-2b*) were hybridized to the same blots (Figure 4). The half-lives of these *GUT* mRNAs were similar (Table 1) to those reported earlier (Taylor and Green, 1995).

Thus, we identified all possible classes of mRNA stability between the two cell types (Table 1). *ADH1* and *NTK1* mRNAs were highly stable in both cell types. *GRP2* mRNA was moderately stable during pollen development, but was highly stable in BY2 cells. *GUT15* was unstable in BY2 cells but highly stable in pollen, while *GUT8-2b* mRNA was unstable in BY2 cells (Taylor and Green, 1995; Table 1) but was moderately stable in pollen.

Discussion

Despite the detailed information on mRNA stability in animal reproductive cells, the information on mRNA stability during pollen development was incomplete. We confirmed the long-held but untested assumption that, as in animal gametes, most mRNAs are extremely stable in pollen. Precise measurements and many repetitions allowed us to identify less stable mRNAs in pollen, indicating the existence of differential RNA decay mechanisms in pollen. We must therefore conclude that the situation in pollen is different from that in animal reproductive cells, where a transcriptional arrest coincides with overall mRNA stabilization (Ross, 1995; Stebbins-Boaz and Richter, 1997; Wolffe, 1994). Both transcription (Dircks *et al.*, 1996; Mascarenhas, 1993; McCormick, 1993; Muschietti *et al.*, 1994) and RNA degradation are ongoing during late pollen development. PAB5, a poly(A) binding protein that is highly abundant in pollen, may play a role in regulating the balance between mRNA degradation and stability (Belostotsky and Meagher, 1996). We presume that c*is*-determinants, probably located in the 3′-UTRs, will influence mRNA stability in pollen. Although the features mediating the stability of long-lived mRNAs in plants have not been examined, in mammals several highly stable mRNAs have pyrimidine-rich tracts in their 3′UTRs. Because related protein complexes bind these 3′UTRs (Holcik and Liebhaber, 1997), it is believed that a general

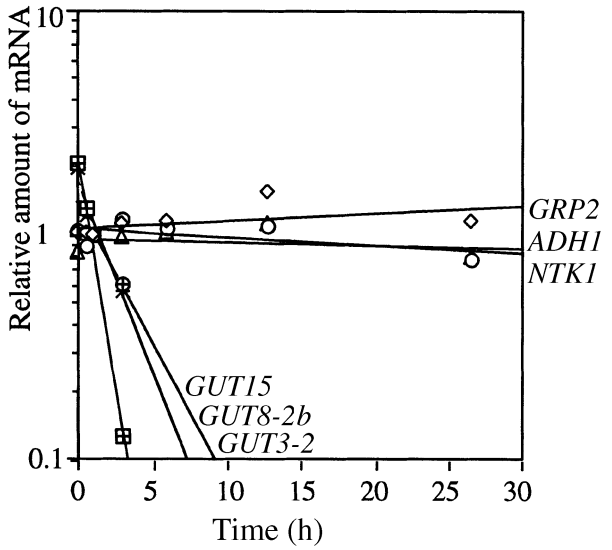

Figure 4. mRNA stability of six genes expressed in BY2 cells.
Graphical representation of the relative mRNA levels measured after transcriptional inhibition with ActD.

mechanism exists to stabilize mRNAs. Unfortunately, it is premature to analyze sequences in the 3′UTRs of pollen mRNAs because the published 3′UTR sequences of these mRNAs are often incomplete (B. Ylstra and S. McCormick, unpublished results).

In mammalian cells it has been shown that half-lives of mRNAs can differ in different cell types (Ross, 1995). For example, the α2-globin mRNA in the CS mutant is relatively unstable in erythroid cells and stable in non-erythroid cells (Weiss and Liebhaber, 1994). In erythroid cells wild-type α2-globin is stabilized by an erythroid-specific mRNA–protein complex that recognizes a pyrimidine-rich motif in the 3′-UTR (Morales *et al.*, 1997). The mRNA of the CS mutant is less stable because the formation of this stabilizing complex is blocked. Here we showed that an individual mRNA species can have different half-lives in different plant cell-types. Interestingly, mRNA half-lives cannot only differ between the different cell-types (*GUT8-2b*), but we also found examples of mRNAs that are unstable in one cell-type and completely stable in the other (see Table 1, *GRP2* and *GUT15*). The differential stabilities of the *GUT15*, *GRP2* and *GUT8-2b* mRNAs in pollen and BY2 cells suggest that tissue-specific factors regulate these differences. Although not yet studied for the *GUT* mRNAs, in BY2 cells the instability of other short-lived messages is mediated by AU-rich elements in the 3′ UTR (Abler and Green, 1996). If the 3′UTR elements or other features of the *GUT* mRNAs mediate their instability in BY2 cells, our results suggest that pollen does not express trans-acting factors that recognize such elements.

Experimental procedures

In vitro *culture conditions and plant materials*

Nicotiana tabacum Samsun NN plants were grown in the greenhouse or in a growth chamber under a 16 h day/8 h night regimen at 25°C with 15 000 lux light at pot level. For each timepoint or ActD concentration, early bi-cellular pollen was isolated from five flowers and cultured in AMGlu medium (Touraev *et al.*, 1995). To test for the quality of the *in vitro* culture, pollen was transferred after 2 nights in AMGlu medium to GQ germination medium or was stained with 0.1% fluorescein diacetate (FDA) (Muschietti *et al.*, 1994; Touraev *et al.*, 1995). *N. tabacum* Bright Yellow 2 (BY2) cells were grown in liquid medium at 28°C on a shaker in the dark. Three- to 4-day-old cultures were used and transcriptionally inhibited with 100 μg ml^{-1} ActD as described previously (Newman *et al.*, 1993). Viability of the BY2 cells was not reduced after 30 h incubation in ActD, as judged by FDA staining.

In vivo *^{3}H-Uridine labeling during* in vitro *pollen maturation*

To determine the concentration of ActD required to inhibit *de novo* RNA transcription *in vivo,* ActD (from a 5 mg ml^{-1} stock solution in 100% ethanol) was added in concentrations ranging from 0 to 200 μg ml^{-1} to 5 ml overnight cultures containing early bi-cellular pollen of five flower buds in AMGlu medium. Experiments were performed in duplicate. After 1.5 h, 100 μCi ^{3}H-[5,6] Uridine (Amersham, Arlington Heights, IL, USA) was added to each culture and incubated for 4 h. Total RNA was isolated and 10 μg of each sample was separated on a 1.2% agarose gel. The mRNA fraction, defined as that which migrates between the 18S and 5S ribosomal bands, was cut out from each lane with a razor blade. The radioactivity of the mRNA fractions or 6 μg total RNA was measured with a scintillation counter.

Quantitative RNA gel blot analysis

Tissues were homogenized for 15 min on a vortex in the presence of 4 M guanidine, 25 mM Na Citrate, 0.5% sarkosyl, 50 μM β-mercapto ethanol, 20 μM EDTA and approximately 0.5 g of 425–600 μm glass beads (Sigma, St. Louis, MO, USA) and RNA was isolated as described previously (Logemann *et al.*, 1986). RNA concentrations were measured at a wavelength of 260 nm, diluted as necessary and measured again to obtain equal concentrations. Within a specific experiment equal amounts of between 5 and 8 μg of total RNA were loaded on 1.2% agarose and 5.2% formaldehyde gels, electrophoresed and blotted onto S & S Nytran membranes (Schleicher and Schuell, Keene, NH, USA) and UV cross-linked. Hybridizations were performed in 7% SDS, with 1 mM EDTA, 1% BSA and 0.5 M Na_2HPO_4, pH 7.2 at 65°C overnight, followed by three washes in either 0.3× SSPE or 0.1× SSPE and 0.1% SDS. Quantitation of the signal was performed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). Data were transferred to CA-Cricketgraph III version 1.01 (Computer Associates Int. Inc., Islandia, NY, USA) that plots the data in a logarithmic graph and gives an equation from which half-lives were calculated. For the short-lived mRNAs ($t_{1/2}$ < 3 h, i.e. the *GUT*s in the BY2 cells) only timepoints < 5 h were included in the half-life calculation. During *in planta* development, mRNA half-life was defined as the time at which the amount of steady-state mRNA declines to 50% of the initial value.

Synthesis and analysis of the probes

A *GRP2* fragment (Obokata *et al*., 1991) was isolated by PCR (elongation at 60°C) on *N. sylvestris* DNA with the primers 5′-CTAAGGAACCGTCAA(A/G)TGGTT-3′ and 5′-CTCCAGACTCA-ACTTCGAA(C/T)TC-3′, generating a 162 bp fragment comprising the putative Y-box motif (Wolffe, 1994) and excluding the glycine-rich region that could cause cross-hybridization to other genes encoding glycine-rich sequences. A *NTK1* fragment was isolated by PCR (elongation at 55°C) on *N. tabacum* DNA with the primers 5′-TCATAAACCAAGGGACGGGCACTG-3′ and 5′-ATGACTTCAGTAGGCTTAGCACC-3′, generating a 1230 bp fragment of the coding region (Einzenberger *et al*., 1995). *PDC2* was isolated from pollen mRNA by RT–PCR (elongation at 55°C) using the primers 5′-AGAAATATAGTACTCGAGGTGTGGG-3′ and 5′-CTTAAGTTACCAGAAGGATGTGGG-3′, generating a 700 bp cDNA fragment (Bucher *et al*., 1995). *ADH1* was similarly isolated using the primers 5′-GCGGCCGCCTGCTGGGCAGGTCAT-3′ and 5′-AGCAAGGCCACAGCTCCCAAGCC-3′, generating a 628 bp cDNA fragment (Bucher *et al*., 1995). Fragments were ligated into the pMOS*Blue* T-vector (Amersham) and verified by sizing on agarose gels and by sequence analysis using dye-labeled terminators (Perkin-Elmer, Foster City, CA, USA). For *NT59* the probe was a 300 bp fragment corresponding to amino acids 21–120 of the tobacco *NT59* protein (Kulikauskas and McCormick, 1997). A 400 bp 17S probe was generated with the primers 5′-GCCGAATTCAGTAATTCTAGAGCTAATAC-3′ and 5′-GCCAAGCTTCAGACACTAAAGCGCCCGGTA-3′ (Takaiwa *et al*., 1985) on rice genomic DNA, and was used to confirm equal loading in Figure 3. *GUT* plasmids (Taylor and Green, 1995) were gifts from P.J. Green (Michigan State University, East-Lansing, MI, USA); inserts were obtained by *Eco*RI digests. *GUT15* detects two messages (Van Hoof *et al*., 1997), but only the lower band was measured and discussed in this paper. All other inserts were derived from plasmids containing the entire or almost entire cDNA sequences and were gifts: *NTP303* (Weterings *et al*., 1992) from J.A.M. Schrauwen (Katholieke Universiteit Nijmegen, the Netherlands), *TP10* (Rogers and Lonsdale, 1992), *C27X* (Rogers and Lonsdale, 1992; Tebutt *et al*., 1994) and *TAC112* (Thangavelu *et al*., 1993) from D. Lonsdale (John Innes Institute, Norwich, UK) and *NeIF4a8* (Brander and Kuhlemeier, 1995) from C. Kuhlemeier (Universität Bern, Bern, Switzerland). The probes were labeled (Feinberg and Vogelstein, 1984) with [α-^{32}P]dATP (Amersham).

All probes were tested by Southern blot analysis using *Eco*RI and *Bam*HI (data not shown). *GRP2* and *NeIF4a8* detected a single locus in diploid tobacco (*N. sylvestris*) and two loci in tetraploid tobacco (*N. tabacum*). Using the same stringent washing conditions (0.1× SSPE and 0.1% SDS at 65°C) the *NTP303*, *ADH1*, *TAC*25, *NT59* and *TP10* probes also detected a single locus in diploid tobacco, in accordance with the literature (Bucher *et al*., 1995; Rogers and Lonsdale, 1992; Thangavelu *et al*., 1993; Weterings *et al*., 1992). The *GUT15* and *GUT8-2b* probes each hybridized to two bands in diploid tobacco, and the *C27X*, *NTK1* and *PDC2* probes hybridized to two to four bands in diploid tobacco.

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