This is to certify that the

dissertation entitled

THE XRN FAMILY OF 5'-3' EXORIBONUCLEASES
IN ARABIDOPSIS THALIANA

presented by

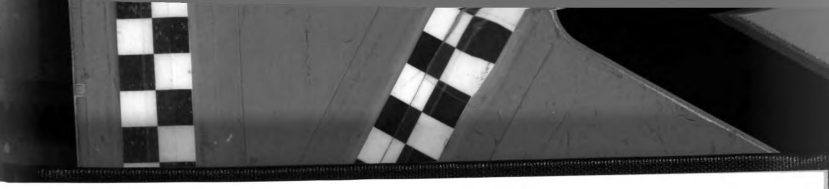
JAMES PAUL KASTENMAYER

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Pamela J. Green
Major professor

Date 5/2/01



THE XRN FAMILY OF 5-3' EXORIBONUCLEASES IN ARABIDOPSIS THALIANA

By

James Paul Kastenmayer

AN ABSTRACT OF A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biology

Department of Cell and Molecular Biology

2001

Professor Pamela J. Green

ABSTRACT

THE XRN FAMILY OF 5'-3' EXORIBONUCLEASES IN ARABIDOPSIS THALIANA

By

James Paul Kastenmayer

The regulated expression of many genes requires selective and active degradation of mRNAs in the cytoplasm. Insight into the manner by which plants identify targets for rapid degradation has been gained in recent years, but there are many unresolved questions about what occurs following this recognition event. In particular, the RNases which catalyze mRNA decay in plants, and other multicellular eukaryotes, have not been identified. The main goal of this dissertation was to investigate the possible role of members of the *Arabidopsis thaliana* XRN-family of exoribonucleases in cytoplasmic mRNA degradation, with a minor goal of examining the importance of deadenylation to the mechanism of mRNA decay in plants.

The XRN-family of 5'-3' exoribonucleases was first described in budding yeast, and is present in many eukaryotes, including plants. However, the XRN-family in Arabidopsis has features that differ from those of other eukaryotes. Arabidopsis lacks an ortholog of Xrn1p, the cytoplasmic enzyme in yeast responsible for the decay of the majority of mRNAs, but expresses three orthologs of Xrn2p/Rat1p, a nuclear rRNA and snoRNA processing enzyme in yeast. To characterize the basic features of the three Arabidopsis XRN-like enzymes (AtXRNs), assays which made use of heterologous expression in yeast *xrn* mutants were employed. The results of these experiments demonstrated that all three of the AtXRNs are active as 5'-3' exoribonucleases when expressed in yeast. In addition, the results of the studies of the AtXRNs in yeast

indicated that AtXRN4, in contrast to AtXRN2 or AtXRN3, might be a cytoplasmic enzyme, and could therefore have a role in mRNA degradation.

To investigate these possibilities, the intracellular location of AtXRN4 in plant cells was examined by localization of an AtXRN4-GFP fusion protein and mRNA decay analyzed in an *xrn4* mutant. AtXRN4-GFP accumulates in the cytoplasm, in contrast to AtXRN2-GFP and AtXRN3-GFP which are targeted to the nucleus. To directly examine AtXRN4's potential role in mRNA degradation in Arabidopsis, the decay of mRNAs in an *xrn4* mutant was examined using DNA microarrays and northern blots. The preliminary results of these experiments indicate that the decay of at least three mRNAs is apparently altered in the *xrn4* mutant. AtXRN4 could be a key enzyme in the decay pathways of these mRNAs.

The mechanism by which mRNAs are degraded in plant cells is unknown. Rapid deadenylation is often a first step in the decay of unstable mRNAs in yeast and mammalian systems, and could be a first step in the decay of some mRNAs in plants. To investigate the importance of deadenylation to rapid mRNA decay in plants, the rate of deadenylation of mRNAs in plants was examined using several methods.

ACKNOWLEDGEMENTS

First and foremost I would like to acknowledge my mentor Pam Green. Pam has made so many contributions to this dissertation and my development as a researcher that I couldn't begin to list all of them. Without her support and encouragement, not to mention her remarkable enthusiasm, the research described in this dissertation would never have been completed.

As with any Ph.D., there are many people who contributed to the work. I would like to specifically recognize the technical support of Linda Danhof. The lab has always been well maintained with almost any reagent you could want (look up!) and for anything that we didn't have it would be ordered and received quickly. Linda has also supervised a veritable army of undergraduates who have provided many helping hands. I would also like to specifically recognize the efforts of Chris Behrens. Chris joined the lab as a professorial assistant at a time when there were a lot of exiting experiments to be done, but before we could do the experiments, a lot of work was required. His assistance proved invaluable to several experiments in this thesis. I am also indebted to Ambro van Hoof who supervised me during my rotation, and provided help with the experiments using the yeast *xrn1Δ* mutant. I'd also like to thank Shawn Anderson for providing me with basic molecular biology skills while I was a technician, and for encouraging me to attend graduate school at MSU.

Perhaps equally important are those who contributed to the atmosphere of the lab. I've really enjoyed my experience as a graduate student in Pam's lab in large part because of the other students and post-docs in the lab. I'd like to thank Mark Johnson, Nikki

LeBrasseur, Rodrigo Gutiérrez, Preet Lidder and Gustavo MacIntosh for being great colleagues and friends.

Last, but certainly not least, I would like to thank my parents, my brother Tom, my sister Susi and my fiancé Robin for their support and encouragement. Graduate school has been a very exciting and rewarding experience and I am fortunate that I have been able to share this with them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

ARE-AU-rich element

DAPI-4',6'-diamidino-2-phenylindole

DST-down stream element

EST-expressed sequence tag

GFP-green fluorescent protein

NLS-nuclear localization sequence

NMD-nonsense mediated mRNA decay

PARN-poly(A) ribonuclease

PAT-PCR-poly(A) test PCR

PCR-polymerase chain reaction

PGRR-putative glucose regulated repressor protein

PTGS-post transcriptional gene silencing

RACE-rapid amplification of cDNA ends

RT-reverse transcription

SD-synthetic drop out medium

snoRNA-small nucleolar RNA

CHAPTER 1

DEGRADATION OF mRNAs: CIS-ACTING ELEMENTS, THE BASAL mRNA DECAY MACHINERY AND THE REGULATION OF mRNA TURNOVER

INTRODUCTION

Of particular importance to the expression of many genes are mechanisms which alter mRNA abundance. Clearly, changing the rate of transcription is an important way that mRNA abundance is altered. However, the rate at which transcripts are degraded also plays a critical role in establishing the abundance of transcripts, and is a key determinant of the rate at which new steady-state transcript levels can be achieved. The focus of early research on mRNA degradation in higher plants has been on identifying and characterizing RNA sequence elements that target individual transcripts for rapid mRNA degradation. While these studies have provided key insight into the regulation of mRNA stability, little is known about the RNases which are responsible for degrading mRNAs in plants, or in other multicellular eukaryotes. This chapter briefly describes well-characterized examples of sequence elements which mediate rapid mRNA degradation in plants. It is likely that these elements mediate interactions between the mRNAs and the RNases that degrade them. Thus, studies of rapid mRNA degradation mediated by specific sequence elements may shed light on the activities responsible for transcript degradation. The discussion of sequences which mediate rapid mRNA degradation in plants is followed by a description of the enzymes that function in mRNA decay in yeast, a well-characterized system with respect to the mechanism of mRNA decay. However, there are several differences between mRNA decay in higher plants and yeast which may have important consequences for the mechanism and regulation of mRNA degradation in plants.

INTRODUCTION

Of partic[illegible] alter mRNA stab[illegible] that mRNA stab[illegible] also plays a cru[illegible] the determinant of [illegible] to identifying focus of early [illegible] for rapid and characteriz[illegible] into the regulation of mRNA degrad[illegible] responsible for degrading mRNA stabili[illegible] briefly describes mRNAs in plants [illegible] of rapid mRNA well-characteriz[illegible] interactions between the degradation in [illegible] of rapid mRNA degradation mRNAs and the RNas[illegible] on the activities responsible for mediated by spec[illegible] which mediate rapid mRNA transcript degradation. [illegible] of the enzymes that function in mRNA degradation in plants [illegible] to the mechanism of mRNA decay in yeast, a well-characterized [illegible] decay. However, there are several differences between mRNA decay in higher plants and yeast which may have important consequences for the mechanism and regulation of mRNA degradation in plants.

INSTABILITY DETERMINANTS TARGET SPECIFIC mRNAs FOR RAPID DEGRADATION

Modulation of gene expression for many genes involves changing the steady-state abundance of mRNAs. The steady-state abundance is a dynamic condition that is achieved when mRNA synthesis balances mRNA decay. Altering either the mRNA synthesis rate or the rate of mRNA degradation can change the steady-state, but the combination of the rates of these two processes determines the speed at which new steady-state levels can be achieved. This effect is most clear in the case where the synthesis rate of the mRNA decreases. If an mRNA decays rapidly, it will have a short half-life and its abundance will decrease rapidly following a reduction in its synthesis rate. The unstable mRNA can quickly achieve a new steady-state level. In contrast, if the mRNA decays slowly, it will persist for a longer period of time following inhibition of transcription, and a greater amount of time is required to attain the same new steady-state level. Thus, following a decrease in transcription rate, mRNA stability limits the speed at which mRNA steady-state levels decrease, with unstable mRNAs able to reach lowered steady-state levels faster than stable mRNAs. A less intuitive consequence of mRNA stability is its affect on the rate at which mRNA steady-state abundance can increase. Following an increase in mRNA synthesis rate, a stable transcript will accumulate at a faster rate than an mRNA of lesser stability; however, the unstable mRNA will more quickly reach its new-steady state level than will the stable mRNA. Therefore, following a change in mRNA synthesis rate, unstable mRNAs reach new steady-state levels faster than stable mRNAs. By destabilizing mRNAs through selective degradation, cells can enhance regulation of gene expression at the level of transcription.

In order for cells to selectively target specific transcripts for degradation, the process of mRNA decay must be regulated. An indication of this regulation is that all mRNAs do not have the same stability. The half-lives of mRNAs from different genes, as well as the stability of particular mRNAs at different times in the life of an organism, can vary over a wide range. In plants, most mRNAs have half-lives on the order of several hours; however, within this population of relatively stable mRNAs there are transcripts which are highly unstable with half-lives on the order of minutes (Gutiérrez et al., 1999). To achieve this differential stability, cells must be capable of recognizing particular mRNAs and targeting them for rapid degradation among a population of stable mRNAs. Much of the work on mRNA stability in plants, as well as in mammalian cells, has focused on identifying nucleotide sequences within unstable mRNAs, referred to as "mRNA instability determinants", that serve as signals to target specific mRNAs for rapid degradation. The way in which instability determinants stimulate decay of specific mRNAs is unknown; however, they likely function to directly or indirectly affect the activity of RNases involved in mRNA degradation in plants. Rapid mRNA degradation mediated by these elements is both constitutive as well as regulated in response to stimuli. The following examples of instability determinants from plants highlight these types of regulation.

Constitutive mRNA decay mediated by the DST-element

A well characterized mRNA instability determinant from plants is the DST element. The DST (down-stream) element is a sequence that is conserved in the 3' untranslated regions (UTRs) of many *SAUR* (small auxin up RNA) genes. First described

in soybean, transcripts encoded by the *SAUR* genes are induced by auxin, and are some of the most unstable mRNAs known in plants, with half-lives of between 10 and 50 minutes (McClure and Guilfoyle, 1989; Franco et al., 1990). The presence of a conserved sequence in the 3'UTR of many of the *SAUR* genes, the DST sequence, indicated that this element might mediate mRNA instability (McClure et al., 1989). This function for the DST element was subsequently demonstrated. Insertion of two copies of a DST element into the 3' UTR of a reporter RNA is sufficient to trigger rapid decay of an otherwise stable RNA in transformed tobacco cells in culture, and to decrease mRNA abundance in leaves of reporter RNAs in transgenic tobacco plants (Newman et al., 1993). Studies of mRNA decay mediated by the 3' UTR of the *SAUR-AC1* gene of Arabidopsis, which contains one DST sequence and several partial DST sequences, indicate that the DST element also functions as an mRNA instability element in Arabidopsis, and that its instability function is not controlled by auxin (Gil and Green, 1996). In fact, the DST element may be an example of an instability sequence that functions to constitutively target mRNAs for rapid degradation. Constitutive mRNA degradation mediated by the DST element may function to continuously effect the rates of induction and repression of *SAUR* genes, thus allowing more rapid changes in the steady-state levels of transcripts containing the DST-element (e.g. in response to auxin).

Premature stop codons

A specialized class of mRNA instability sequence that could be considered to function as a constitutive instability elements are early nonsense codons. In plant, animal and fungal systems, the presence of a nonsense codon upstream of its normal position

will target transcripts for rapid decay (Hentze and Kulozik, 1999; van Hoof and Green, 1996). This nonsense-mediated mRNA decay (NMD) likely functions to target improperly processed mRNAs for decay to prevent the translation of truncated, possibly deleterious, proteins. Research on NMD in recent years has focused on elucidating how NMD targets are distinguished from normal mRNAs, sometimes referred to as 'RNA surveillance'. The position of the nonsense codon is important. In plants and yeast, if the early stop codon is within the first 60% of the open reading frame, the transcript is targeted by NMD (Peltz et al., 1993; van Hoof and Green, 1996). This similarity between yeast and plants may indicate that the machinery that recognizes NMD targets in plant resembles that of yeast cells. It is not clear how cells recognize transcripts with early stop codons; however the position dependence of a stop codon's ability to trigger NMD may point to the importance of processes occurring at the 3' end of the transcript during translation termination. One current model for NMD in yeast proposes that changes in the mRNP-domain structure of the 3' end of the mRNA is triggered by premature termination, and that these structural changes lead to rapid mRNA decay (Hilleren and Parker, 1999). However, in at least one case in yeast, a sequence element located in the 3' end of a NMD substrate has been implicated in triggering NMD, which may indicate that recognition of specific sequence elements in response to premature termination of translation is important (Peltz et al., 1993). Work in mammalian cells has also pointed to the potential importance of interactions of the translation machinery with proteins bound to the 3' end of mRNAs.

Recognition of early nonsense codons in mammalian cells involves an interesting link with the process of mRNA splicing. The ability of an early stop codon to trigger

NMD in mammals depends on the position of the stop codon relative to the final exon-exon junction. If the stop codon is greater than 50-55 nt upstream of the last exon-exon splice site, it will trigger NMD (Nagy and Maquat, 1998). This observation led to the idea that splicing in mammalian cells results in a 'mark' being deposited on the transcript indicating where it was spliced and that it may be the proximity of the early stop codon to this mark that is sensed by the NMD machinery. Interestingly, recent work in mammalian cells has identified several proteins that are bound to transcripts following their splicing, including several splicesome associated factors (Le Hir et al., 2000). It is not yet known whether these proteins function in the recognition of premature stop codons in NMD; however the position of these protein complexes near exon-exon junctions indicates they could have a function in the recognition of NMD substrates.

Regulated mRNA degradation mediated by the SRE

In contrast to mRNAs that are rapidly degraded in a constitutive manner, the decay of some mRNAs is regulated in response to stimuli. Such regulated decay allows for cells to quickly adjust the expression of specific genes in response to a stimulus. One such example is the Amy3 transcript of rice which encodes an α-amlyase. The Amy3 transcript exhibits rapid changes in abundance in response to carbohydrate availability. Addition of sucrose to rice cells in culture results in a rapid decrease in the Amy3 mRNA due to repression of its transcription and selective degradation of the Amy3 transcript (Chan and Yu, 1998). Sequences mediating the rapid decay of the Amy3 mRNA have been mapped to the 3'UTR and are called the sucrose response element (SRE). Interestingly, the SRE contains one copy of the motif AUUUA, a sequence known to

target mRNAs for rapid decay in animal and plant cells when present in multiple copies (Chen and Shyu, 1995). Regulation of *Amy3* is one example of the role that mRNA stability plays in modulating gene expression in response to stimuli.

Regulated mRNA decay is widespread in plants and has been described for the Fed1 transcript in pea in response to light in a mechanism that involves changes in translation (Petracek, et al., 1998), for the PvPRP1 transcript in bean in response to fungal elicitor (Sheng et al., 1991), the CIM1 transcript in soybean in response to cytokinin (Downes et al., 1998) and the cystathionine gamma-synthase mRNA in Arabidopsis in response to methionine (Chiba, et al., 1999).

5'-3' EXORIBONUCLEASES LIKELY FUNCTION IN mRNA DECAY IN PLANTS

While several examples of instability determinants have been identified in plants and other organisms, it is not known how they function to target mRNAs for rapid degradation. One possibility is that they may recruit a 'basal mRNA degradation machinery', a set of proteins required for the degradation of the majority of mRNAs. In this model, most mRNAs, both stable and unstable would be degraded by a common set of RNases. Sequence-specific RNA binding proteins may bind to instability determinants and recruit or activate components of this basal mRNA decay machinery, resulting in rapid degradation of specific mRNAs. This function of instability determinants would be analogous to sequence elements in promoters involved in recruiting or activating the basal transcription machinery at particular genes. It is also possible that some instability determinants are recognition sites for endoribonucleases that cleave the mRNA internally. A basal mRNA decay machinery might degrade the products of this initial

cleavage. Both of these models invoke the presence of RNases that are involved in the degradation of many transcripts and which may therefore be considered to be part of a basal mRNA decay machinery. While the components of a basal mRNA decay machinery in plants are as yet unknown, the decay of several transcripts in plants indicates that degradation by 5'-3' exoribonucleases may be an important feature of the decay of some mRNAs in plants.

Natural mRNA degradation intermediates

In plant cells, as in other eukaryotic cells, the process of mRNA degradation is quite rapid, and intermediates of mRNA degradation usually do not accumulate to detectable levels. However, in at least two cases in plants, the degradation of specific mRNAs is accompanied by the accumulation of mRNA decay intermediates. Degradation of the PhyA mRNA in oat seedlings, and the SRS4 mRNA in petunia and tobacco plants each results in the accumulation of specific mRNA decay intermediates (Higgs and Colbert, 1994; Tanzer and Meagher, 1995). However, as discussed below, these intermediates differ from each other, and indicate that both 5'-3' as well as 3'-5' exoribonuclease-mediated mRNA decay pathways likely function in plants.

PhyA mRNA degradation intermediates in oat seedlings accumulate as a series of increasingly shorter transcripts. This pattern of degradation intermediates is consistent with exoribonuclease-mediated degradation from both ends (Higgs and Colbert, 1994). Approximately 75% of these intermediates appear to be the result of degradation from the 5' end, with the remainder being due to degradation from both ends simultaneously. Exoribonuclease-mediated degradation has also been implicated in mRNA degradation of the SRS4 mRNA in petunia and tobacco. However, an important difference in the

proposed mechanism of SRS4 mRNA degradation from that of PhyA turnover is that an endoribonuclease(s) likely cleaves the SRS4 transcript at an early step. The SRS4 degradation intermediates consist of several discrete bands indicative of endoribonuclease-mediated cleavage, and are likely subsequently degraded by exoribonucleases (Tanzer and Meagher, 1995). It is not known if the mechanisms of PhyA and SRS4 mRNA degradation are particular to these transcripts, or if other mRNAs are degraded by mechanisms involving exoribonucleases. Interestingly, exoribonucleases have also been implicated in mRNA degradation during post transcriptional gene silencing (PTGS), a process which resembles 'normal' mRNA degradation in several respects.

mRNA degradation intermediates induced by PTGS

PTGS, first described in plants and subsequently discovered in several eukaryotes, is the simultaneous down-regulation of multiple genes that share high levels of sequence identity (reviewed in Sijen and Kooter, 2000). This down-regulation is accomplished at the post-transcriptional level by rapid degradation of PTGS substrates. Insight into the mechanism of mRNA degradation mediated by PTGS has been gained by the analysis of mRNA decay intermediates that accumulate during PTGS. The structures of these decay intermediates are consistent with PTGS-mediated mRNA degradation resembling the proposed mechanism for SRS4 degradation, with initial endoribonuclease cleavage followed by exoribonuclease degradation. In several cases, targets of PTGS accumulate as truncated RNAs that are believed to be mRNA degradation intermediates (Goodwin et al., 1996; Lee et al., 1997; Tanzer et al., 1997; van Eldik et al., 1998). PTGS-mediated degradation of β-1,3 glucanse transcripts, and an RNA encoding the coat protein of

tobacco etch virus (TEV-CP) in tobacco, results in the accumulation of several decay intermediates as discrete bands detected on northern blots (van Eldik et al., 1998; Goodwin, et al., 1996). It is likely that these bands represent decay intermediates generated by an endoribonuclease(s) (van Eldik et al., 1998). In addition to discrete bands, a continuous distribution of products can be detected in plants when either the β-1,3 glucanse or the TEV-CP genes are silenced, indicative of exoribonuclease activity. The products proposed to be generated by an endoribonuclease are likely degraded by exoribonucleases from both ends (van Eldik et al., 1998). While these studies point to a possible similarity between the mechanism of 'normal' mRNA decay and PTGS-mediated decay, the mechanism of PTGS involves more than cleavage by endo- and exoribonucleases.

An important feature of PTGS in multiple systems is the accumulation of small (21-23 nt) RNAs which are products of the degradation event (Hamilton and Baulcombe, 1999; Zamore et al., 2000). Studies using end-labeled substrates and a cell extract from Drosophila cells which carries out PTGS (referred to as RNAi in Drosophila) indicates that PTGS substrates are cleaved one or twice and can be cleaved anywhere throughout the transcript to generate the 21-23 nt RNAs (Zamore et al., 2000). This indicates that PTGS-induced degradation may result in the production of many transcripts that have been cleaved internally. These products could be substrates for the same exoribonucleases that might catalyze 'normal' mRNA degradation.

It is worth noting that the intracellular location of PTGS-mediated mRNA decay is not known. There is evidence indicating that the decay is cytoplasmic in plants, as some viruses that replicate in the cytoplasm are targets of PTGS (reviewed in Sijen and

Kooter, 2000). However, in at least one case, silencing of chalcone synthase in petunia, products of PTGS activity are enriched in the nucleus (Metzlaff et al., 1997). The RNases that function in the PTGS machinery may be located in the cytoplasm, similar to the expected location of the basal mRNA decay machinery, or in the nucleus, or in both intracellular compartments.

Degradation of RNAs in plant cell extracts

The 7 methyl-guanosine cap at the 5' end of eukaryotic mRNAs has functions in both mRNA stability and translation (reviewed in Jacobson and Peltz, 1996). The possible role of the cap in promoting mRNA stability was established over 20 years ago by the demonstration that capped RNAs are more stable than non-capped RNAs when incubated in wheat germ extracts (Shimotohno et al., 1977a). Analysis of the decay products of RNAs incubated in wheat germ extract indicated that the decay might be catalyzed by a 5'-3' exoribonuclease that produced 5'-mononucleotides, but which could not degrade capped RNAs. Subsequently, an exoribonuclease exhibiting these characteristics was partially purified from wheat germ extract in the late 1970s (Shimotohno and Miura, 1977b). Although nothing more has been reported about this, RNase from plants, the 5'-3' exoribonuclease Xrn1p has been shown to be a major enzyme in mRNA decay in yeast.

Xrn1p, A 5'-3' EXORIBONUCLEASE, IS A MAJOR COMPONENT OF THE BASAL mRNA DECAY MACHINERY OF YEAST

Perhaps the strongest evidence that 5'-3' exoribonucleases play a role in mRNA degradation in plants is that this type of activity is a key component of mRNA decay in several mRNA decay pathways in yeast and might be conserved in plants. Xrn1p is a

constituent of the basal mRNA decay machinery in yeast, a group of proteins that catalyze the decay of the majority of mRNAs. In yeast, the majority of mRNAs are degraded by the deadenylation-dependent-decapping pathway (Figure 1-1 , center; reviewed in McCarthy, 1998). The first step in this pathway is the removal of the poly(A) tail, most likely by a complex of proteins containing Ccr4p and Caf1p (Tucker et al., 2001). Interestingly, removal of the poly(A) tail is an early step in mRNA decay conserved in several systems, including mammalian cells (Shyu et al., 1991) and *Chlamydomonas rheinhardtii* (Baker, 1993); however, the enzyme(s) responsible have not been definitively identified. While it is not known if deadenylation plays an important role in mRNA degradation in higher plants; the Arabidopsis genome encodes an ortholog of mammalian PARN (poly(A) ribonuclease; Johnson, 2000). PARN has been suggested to be the nuclease that catalyzes deadenylation of mRNAs in mammalian cells (Körner, 1998, Gao, 2000).

Although deadenylation is a common first step in mRNA degradation in multiple systems, the steps of mRNA degradation following deadenylation have only been definitively identified in yeast. Following deadenylation in yeast, mRNAs are decapped by Dcp1p, and degraded to completion by the 5'-3' exoribonuclease Xrn1p. Surprisingly, while a potential Dcp1p ortholog can be found in the Arabidopsis genome (Kastenmayer et al., 1998; Gutiérrez et al., 1999) Xrn1p orthologs are absent, and have not been found in the available sequences from additional higher plants (Chapter 3).

Figure1-1. mRNA decay pathways in yeast. The major deadenylation-dependent-decapping decay pathway is shown in the middle. The majority of mRNAs in yeast are believed to be degraded through this pathway. On the left is shown a specialized version of the major pathway which functions on mRNAs containing early non-sense codons. Transcripts degraded through this pathway are not deadenylated prior to decapping. On the right is shown a 3'-5' decay pathway catalyzed by a complex of 3'-5' exoribonucleases known as the exosome. This pathway is believed to play a minor role in mRNA degradation.

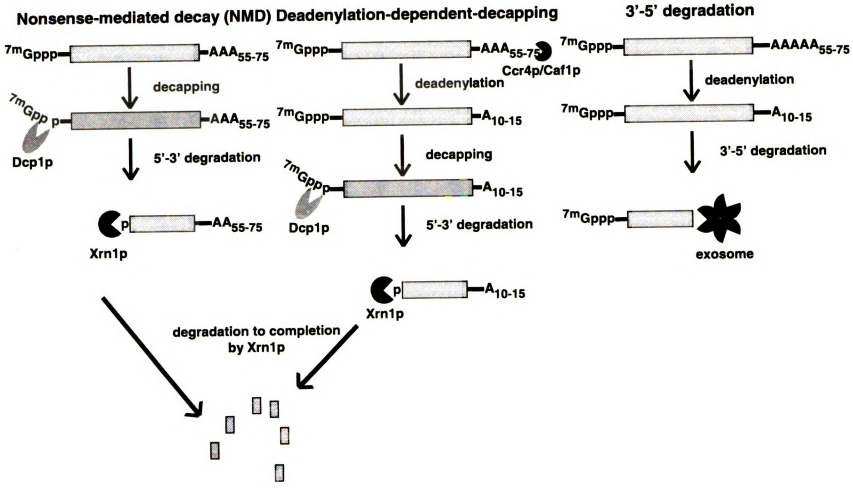


Figure 1-1. mRNA decay pathways in yeast

However, Xrn1p orthologs are present in a number of eukaryotes which may indicate that Xrn1p-mediated mRNA degradation is conserved in a variety of eukaryotes, but is lacking from higher plants.

In addition to this major mRNA decay pathway (Figure 1-1 center), additional pathways exist which catalyze degradation of mRNAs in yeast. The NMD pathway, responsible for degrading mRNAs with premature stop codons, does not require prior deadenylation, but is dependent on Xrn1p-mediated degradation (Figure 1-1, left). Transcripts in yeast are also degraded from the 3' end by a complex of exoribonucleases known as the exosome (Figure 1-1 right; Jacobs-Anderson 1998). Exosome-mediated degradation likely occurs in mammalian and plant cells, as orthologs of components of the exosome have been found in both of these systems (Mitchell et al., 1997; Gutiérrez et al., 1999, Chekanova et al., 2000).

Elucidation of these known mRNA degradation pathways in yeast depended on two complementary experimental approaches, the analyses of mRNA decay intermediates and analyses of mutants of the basal mRNA decay machinery. As mentioned previously, mRNA degradation in eukaryotic cells usually does not result in the accumulation of detectable mRNA decay intermediates. The absence of naturally occurring mRNA decay intermediates for the majority of transcripts has precluded the ability to elucidate mRNA degradation pathways based on the structures of these intermediates or the kinetics of their appearance or disappearance during the course of degradation. This obstacle has been in large part overcome in studies on mRNA decay in yeast.

Expression of poly(G)-containing genes in yeast results in the accumulation of poly(G)-stabilized mRNA degradation intermediates

It is possible to trap mRNA decay intermediates in yeast by the introduction of stable secondary structures, such as poly(G) tracts of 18 nts, into mRNAs. These stable secondary structures inhibit exoribonuclease-mediated mRNA decay in yeast, resulting in the accumulation of poly(G)-stabilized mRNA decay intermediates (Muhlrad et al., 1994; Jacobson-Anderson et al., 1998). Interestingly, similar experiments have failed to produce poly(G)-stabilized mRNA decay intermediates in plant and mammalian cells, an indication that mRNA decay may differ mechanistically between yeast and multicellular eukaryotes (Johnson, 2000). The majority of the poly(G)-stabilized mRNA decay intermediates which accumulate in yeast begin at the 5' end of the poly(G) tract and end at the poly(A) tail which has been shortened to 10-12 adenosines (Muhlrad et al., 1994). These intermediates are consistent with mRNA degradation occurring preferentially from the 5' end, most likely catalyzed by a 5'-3' exoribonuclease which is blocked by the poly(G) tract.

The demonstration that Xrn1p generates the poly(G)-stabilized mRNA decay intermediates was accomplished in two ways. The activity of Xrn1p on RNA substrates *in vitro* was examined, and mRNA turnover was analyzed in an *xrn1Δ* mutant. Xrn1p exhibits 5'-3' exoribonuclease activity *in vitro*, releases 5'-mononucleotides, and importantly, its progression through RNA substrates is blocked by poly(G) tracts (Stevens, 1979; Poole and Stevens, 1997). The most compelling evidence that Xrn1p functions in mRNA degradation *in vivo* is that many mRNAs are stabilized in *xrn1Δ* cells, including mRNAs containing early stop codons (Larimer et al., 1992; Hsu and

Stevens, 1993). In addition, formation of poly(G)-stabilized mRNA decay intermediates is greatly reduced in *xrn1Δ* cells, with a concomitant increase in the steady-state level of the full-length poly(G)-containing mRNA (Decker and Parker, 1993).

An additional discovery made through the analysis of poly(G)-containing genes in *xrn1Δ* cells was the exosome mediated mRNA degradation pathway (Figure 1-1 A, right). 5'-3' mediated mRNA degradation appears to predominate mRNA decay in yeast; poly(G)-stabilized mRNA decay intermediates consistent with this decay pathway accumulate to high levels (Jacobson-Anderson et al., 1998). However, when 5'-3' decay is inhibited by deletion of DCP1, poly(G)-stabilized mRNA decay intermediates accumulate which retain the full 5'-end of the mRNA and end at the 3' end of the poly(G) tract (Jacobson-Anderson et al., 1998). Further studies demonstrated that these intermediates are due to degradation from the 3' end by the exosome, and that these intermediates accumulate to low levels in wildtype yeast (Jacobson-Anderson et al., 1998). Thus the analysis of poly(G)-stabilized mRNA decay intermediates, in combination with studies of *xrn1* mutants, was critical not only to the elucidation of Xrn1p's function in mRNA degradation in yeast, but also to the discovery of additional mRNA decay pathways.

Xrn1p has roles in rRNA and snoRNA processing in addition to catalyzing cytoplasmic mRNA degradation. rRNAs are synthesized as large precursors which are first cleaved internally by endoribonucleases, and then trimmed to their mature forms by exoribonucleases (for current review see Kressler et al., 1999). Xrn1p trims the 5' ends of several pre-rRNAs, and the exosome trims the 3' ends of these pre-rRNAs. Xrn1p has a similar role in the maturation of the 5' ends of small nucleolar RNAs (snoRNAs).

snoRNAs can be divided into two classes based on conserved sequences present in the RNA, the C/D box and H/ACA box classes. These RNAs serve as guides for rRNA processing, including 2'OH methylation directed by members of the C/D class (Kiss-Laszlo et al., 1996) and pseudouridylation by members of the H/ACA class (Ganot et al, 1997). Many snoRNAs in yeast are encoded in introns of mRNAs and mature through two separate pathways. In the first pathway, the intron is spliced out as an intron lariat by the mRNA splicing machinery. Following debranching of the intron lariat, the ends of the intron are trimmed by exoribonucleases, including Xrn1p, to yield the mature snoRNA (Villa et al., 1998). A second pathway of snoRNA processing involves the direct cleavage of the mature snoRNA out of the mRNA by endoribonuclease cleavage, and does not depend on exoribonuclease trimming (Villa et al., 1998). The role of Xrn1p in trimming of the 5' ends of rRNAs and snoRNAs was revealed by analysis of *xrn1Δ* cells in which the 5' ends of these RNA species are not trimmed to their mature length (Henry et al., 1994; Villa et al., 1998). In addition to trimming rRNA and snoRNA 5' ends, Xrn1p degrades the rRNA processing intermediate ITS1 (Stevens et al., 1991).

THE FUNCTION OF Xrn2p/Rat1p, A SECOND 5'-3' EXORIBONUCLEASE OF YEAST, PARTIALLY OVERLAPS WITH THE FUNCTIONS OF Xrn1p

Xrn1p's function in both rRNA and snoRNA processing is shared with the second known 5'-3' exoribonuclease in yeast, Xrn2p/Rat1p. Xrn2p/Rat1p is highly similar in sequence and enzymatic activity to Xrn1p. Xrn2p/Rat1p is also a 5'-3' exoribonuclease that is blocked by poly(G) tracts (Poole and Stevens, 1997). The processing of rRNAs and snoRNAs exhibit similar defects in *xrn1* and *xrn2/rat1* mutants indicating overlap in function (Henry et al., 1994, Villa et al., 1998). Double mutants exhibit the most

dramatic defects. However, while both XRNs of yeast share these similarities, Xrn2p/Rat1p differs from Xrn1p in two important ways. First, Xrn2p/Rat1p is targeted to the nucleus while Xrn1p is found in the cytoplasm (Heyer et al., 1995, Johnson, 1997). Second, Xrn2p/Rat1p is encoded by an essential gene, while *xrn1Δ* cells are viable (Larimer et al., 1992; Amberg et al., 1992) indicating that Xrn2p/Ratp1 likely has a function(s) distinct from that of Xrn1p. Interestingly, while Xrn2p/Rat1p is targeted to the nucleus, it appears to have activity on mRNAs, an activity that can be detected when *XRN1* is deleted (Decker and Parker, 1993; He and Jacobson, 2001). This could be due to a residual amount of Xrn2p/Rat1p remaining in the cytoplasm that is active on mRNAs.

An additional activity of Xrn2p/Rat1 may be degradation of pre-mRNAs in the nucleus. In the *rat1-1* strain after a shift to the non-permissive temperature, several pre-mRNA species are elevated, including mRNAs which are improperly spliced (Bousquet-Antonelli, 2000). Similar results were observed with mutants of the exosome, indicating decay of pre-mRNAs in the nucleus may be catalyzed in part by the same enzymes involved in mRNA degradation in the cytoplasm. The essential function of Xrn2p/Rat1p is unknown, but impaired degradation of pre-mRNAs in the nucleus catalyzed by Xrn2p/Rat1p could be one reason that *rat1-1* cells arrest growth, while *xrn1Δ* cells are viable.

Further evidence for distinct functions of the XRN proteins of yeast comes from the analysis of the *xrn1Δ* and *rat1-1* mutants. Mutation of either of the yeast XRN genes results in distinct phenotypes. Loss of Xrn1p function results in hypersensitivity to the microtubule depolymerizing drug benomyl and defects in karyogamy, recombination and

sporulation (Kim et al, 1990). The conditional *rat1-1* allele of *XRN2/RAT1* was cloned in a screen for mutants which failed to properly transport mRNA from the nucleus (RNA trafficking RAT mutants; Amberg et al., 1992), and the conditional allele *tap1* in a screen for suppressors of a mutated RNA polymerase III promoter (transcriptional activator protein; Di Sengi et al., 1993). That the phenotypes of the *xrn1Δ* and *rat1-1* strains differ indicates that Xrn1p and Xrn2p/Rat1p likely have distinct functions in yeast.

LINKS BETWEEN INSTABILITY DETERMINANTS AND THE BASAL mRNA DECAY MACHINERY

As mentioned above, the manner in which instability determinants function to increase mRNA degradation rates is not known. However there is evidence that the basal mRNA decay machinery could be regulated by these sequence elements. In support of this model are experiments showing that each of the steps of the major deadenylation-dependent-decapping pathway of yeast occur at different rates when RNAs bearing instability determinants are compared to mRNAs lacking such elements.

The most well characterized function of instability determinants is to increase deadenylation rates. Several instability elements from yeast are known to increase the rate at which unstable mRNAs are deadenylated as do AREs from mammalian cells (Decker and Parker, 1993; Chen and Shyu, 1995). It is easy to imagine that instability determinants in yeast could function to recruit the Crr4p/Caf1p complex to unstable mRNA s, leading to rapid deadenylation followed by decapping. The rate of decapping is also stimulated by several instability determinants in yeast (Muhlrad and Parker, 1992; Muhlrad et al., 1994), and the activity of Dcp1p might also be a target of instability elements. AREs in mammalian cells may have a similar function as instability

determinants in yeast, although the components of the mRNA decay machinery, and the steps of mRNA degradation following deadenylation are unknown in mammalian cells. AREs might recruit PARN in animal cells (or AtPARN in plant cells?) to unstable mRNAs to facilitate their rapid deadenylation. Interestingly, a decapping activity has recently been characterized in mammalian cell extracts that is stimulated by the presence of an ARE in the 3' UTR of substrate RNAs (Gao et al., 2001). These data indicate that a conserved mechanism of some instability determinants might be to stimulate deadenylation and decapping, and also support a model in which decapping is a regulated step in the in the decay of some mammalian transcripts.

The least is known about regulation of the last step in the deadenylation-dependent-decay-pathway, degradation from the 5' end by Xrn1p. However, several experiments indicate that the activity of Xrn1p might be regulated. Yeast two-hybrid assays have shown that proteins related to the SM-proteins of splicing, LSMs, interact with the C-terminus of Xrn1p (Fromont-Racine, 2000). The significance of this interaction is unknown; however, it is worth noting that these Lsm proteins are members of a complex known to physically interact with the decapper Dcp1p and are required for mRNA decapping in yeast (Tharun et al., 2000). This could indicate that the Lsm proteins may have a function in regulating the access of Xrn1p to the 5' end of mRNAs following decapping, a process mediated by the interaction of the Lsms with the C-terminus of Xrn1p. The apparent absence of Xrn1p orthologs from higher plants (Chapter 3) may indicate that this type of regulation does not occur in plant cells. Additional evidence for regulation of 5'-3' decay following decapping comes from the observation that a point mutation in *eIF5A* results in the accumulation of mRNAs which

are decapped, but stable (Zuk and Jacobson, 1998). Xrn1p may be unable to gain access to the 5' ends of these transcripts. Finally, accumulation of decapped mRNAs in an *xrn1Δ* strain is enhanced when genes required for NMD, *UPF1*, *NMD2* and *UPF3*, are also deleted, indicating that proteins involved in NMD might regulate 5'-3' decay (He and Jacobson, 2001). Since this observation is only seen in the absence of Xrn1p function, its significance to general mRNA degradation is unclear, but may be a result of changes in the activity of Xrn2p/Rat1p on mRNAs in the cytoplasm (He and Jacobson, 2001).

SCOPE OF THIS THESIS

Rapid turnover of mRNAs can be broken down into two phases, recognition of substrates for rapid decay and active degradation. Several instability determinants have been identified and characterized providing important insight into how genes can be regulated in plants at the level of mRNA stability. Recently, Arabidopsis mutants which are unable to efficiently recognize and degrade mRNAs bearing the DST element have isolated (Johnson et al., 2000). These mutants will likely provide key insight into the manner in which the DST element is recognized. As the function of the DST element, and other instability determinants, likely involves modulation of the activities which ultimately degrade mRNAs, identification of components of the basal mRNA decay machinery in plants may yield insight into the function of instability determinants as well as into mRNA degradation in general. The main goal of this thesis was to identify RNases which might catalyze cytoplasmic mRNA degradation in plants. Chapter 2 describes how assays were developed to assess basic features of an XRN-family member

identified in *Arabidopsis thaliana* (AtXRN2). In Chapter 3, these assays are applied to investigate the potential role of all three of XRN-family members in mRNA degradation in Arabidopsis. Evidence is presented that although XRN1 orthologs are absent from Arabidopsis, and probably higher plants, the Xrn2p/Rat1p-like protein AtXRN4 may function in cytoplasmic mRNA decay in Arabidopsis. Chapter 4 describes the identification of T-DNA insertion alleles in each of the *AtXRN* genes. Preliminary analysis of mRNA decay in Arabidopsis seedlings mutant for *AtXRN4* using cDNA microarrays is presented. These experiments indicate that AtXRN4 may have a role in catalyzing mRNA degradation in Arabidopsis. Chapter 5 describes attempts to address the possibility that deadenylation is a key step in the mechanism of mRNA degradation in plants stimulated by the presence of a DST element or a synthetic ARE. The most important contribution of this thesis is likely to be the discovery that AtXRN4 may degrade some mRNAs in Arabidopsis. This would provide the first evidence that the XRN-family functions in mRNA decay in higher eukaryotes and would be the first example of an RNase with a general role in degrading mRNAs in a multicellular eukaryote.

REFERENCES

Amberg, D. C., Goldstein, A. L., and Cole, C. N. (1992). Isolation and characterization of *RAT1*: An essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. Genes Dev. **6**, 1173-1189.

Baker, E. J. (1993). Control of poly(A) length. Control of Messenger RNA Stability, J. Belasco and G. Brawerman eds (New York: Academic Press), pp.367-415.

Bousquet-Antonelli, C., Presutti, C., and Tollervey, D. (2000). Identification of a regulated pathway for nuclear pre-mRNA turnover. Cell **102**, 765-775.

Brown, C. E. and Sachs, A. B. (1998). Poly(A) tail length control in *Saccharomyces cerevisiae* occurs by message-specific deadenylation. Mol. Cell. Biol. **18**, 6548-6559.

Chan, M. T. and Yu, S. M. (1998). The 3' untranslated region of a rice α-amylase gene functions as a sugar-dependent mRNA stability determinant. Proc. Nat. Acad. Sci. USA **95**, 6543-6547.

Chen, C. Y. A. and Shyu, A. B. (1995). AU-rich elements: Characterization and importance in mRNA degradation. Trends Biochem. Sci. **20**, 465-470.

Chiba, Y., Ishikawa, M., Kijima, F., Tyson, R. H., Kim, J., Yamamoto, A., Nambara, E., Leustek, T., Wallsgrove, R. M., and Naito, S. (1999). Evidence for autoregulation of cystathionine gamma-synthase mRNA stability in arabidopsis. Science **286**, 1371-1374.

Decker, C. J. and Parker, R. (1993). A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev. **7**, 1632-1643.

Di Sengi, G., McConaughy, B. L., Shapiro, R. A., Aldrich, T. L., and Hall, B. D. (1993). TAP1, a yeast gene that activates the expression of a tRNA gene with a defective internal promoter. Mol. Cell. Biol. **13**, 3424-3433.

Downes, B. P. and Crowell, D. N. (1998). Cytokinin regulates the expression of a soybean β-expansin gene by a post-transcriptional mechanism. Plant Mol.Biol. **37**, 437-444.

Franco, A. R., Gee, M. A., and Guilfoyle, T. J. (1990). Induction and superinduction of auxin-responsive mRNAs with auxin and protein synthesis inhibitors. J. Biol. Chem. **265**, 15845-15849.

Fromont-Racine, M., Mayes, A. E., Brunet-Simon, A., Rain, J. C., Colley, A., Dix, I., Decourty, L., Joly, N., Ricard, F., Beggs, J. D., and Legrain, P. (2000). Genome-wide protein interaction screens reveal functional networks involving Sm-like proteins. Yeast **17**, 95-110.

Gao, M., Fritz, D. T., Ford, L. P., and Wilusz, J. (2000). Interaction between a poly(A)-specific ribonuclease and the 5' cap influences mRNA deadenylation rates in vitro. Mol. Cell **5**, 479-488.

Gao, M., Wilusz C.J., Peltz, S. W., and Wilusz, J. (2001). A novel mRNA-decapping activity in HeLa cytoplasmic extracts is regulated by AU-rich elements. EMBO J. **20**, 1134-1143.

Gil, P. and Green, P. J. (1996). Multiple regions of the *Arabidopsis SAUR-AC1* gene control trascript abundance: the 3' untranslated region functions as an mRNA instability determinant. EMBO J. Journal **15**, 1678-1686.

Goodwin, J., Chapman, K., Swaney, S., Parks, T. D., Wernsman, E. A., and Dougherty, W. G. (1996). Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. Plant Cell **8** , 95-105.

Gutiérrez, R. A., MacIntosh, G. C., and Green, P. J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci. **4**, 429-438.

Hamilton, A. J. and Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286**, 950-952.

He, F. and Jacobson, A. (2001). Upf1p, Nmd2p, and Upf3p regulate the decapping and exonucleolytic degradation of both nonsense-containing mRNAs and wild-type mRNAs. Mol. Cell. Biol. **5**, 1515-1530.

Henry, Y., Wood, H, Morrissey, J. P., Petfalski, E., Kearsey S, and Tollervey, D. (1994). The 5' end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. EMBO J. **13**, 2452-2463.

Hentze, M. W. and Kulozik, A. E. (1999). A perfect message: RNA surveillance and nonsense-mediated decay. Cell **96**, 307-310.

Heyer, W.-D., Johnson, A. W., Reinhart, U., and Kolodner, R. D. (1995). Regulation and intracellular localization of *Saccharomyces cerevisiae* strand exchange protein 1 (Sep1/Xrn1/Kem1), a multifunctional exonuclease. Mol.Cell.Biol. **15**, 2728-2736.

Higgs, D. C. and Colbert, J. T. (1994). Oat phytochrome A mRNA degradation appears to occur via two distinct pathways. Plant Cell **6**, 1007-1019.

Hilleren, P. and Parker, R. (1999). mRNA surveillance in eukaryotes: Kinetic proofreading of proper translation termination as assessed by mRNP domain organization? RNA **5**, 711-719.

Hsu, C. L. and Stevens, A. (1993). Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. Mol.Cell.Biol. **13**, 4826-4835.

Jacobs Anderson, J. S. and Parker, R. (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J. Journal **17**, 1497-1506.

Jacobson, A. and Peltz, S. W. (1996). Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. Annu. Rev. Biochem.**65**, 693-739.

Johnson, A. W. (1997). Rat1p and Xrn1p are functionally interchangeable exoribonucleases that are restricted to and required in the nucleus and cytoplasm, respectively. Mol.Cell.Biol. **17**, 6122-6130.

Johnson, M. A. (2000). Genetic Determinants of mRNA Stability in Plants. Ph.D. dissertation, Michigan State University

Johnson, M.A., Perez-Amador M.A., Lidder, P. and Green, P.J. (2000) Mutants of Arabidopsis defective in a sequence-specific mRNA degradation pathway. Proc. Natl. Acad. Sci. USA **97**, 13991-13996.

Kastenmayer, J. P., van Hoof, A., Johnson, M. & Green, P. J. (1998) in *Plant Molecular Biology*, eds. Raikhel, N., Last, R., Morelli, G., & LaShavo, F. (Springer-Verlag, Berlin), pp. 125-133.

Kim, J., Ljungdahl, P. O., and Fink, G. R. (1990). kem mutations affect nuclear fusion in Saccharomyces cerevisiae. Genetics **126**, 799-812.

Kiss-László, Z., Henry, Y., and Kiss, T. (1998). Sequence and structural elements of methylation guide snoRNAs essential for site-specific ribose methylation of pre-rRNA. EMBO J. **17**, 797-807.

Körner, C. G., Wormington, M., Muckenthaler, M., Schneider, S., Dehlin, E., and Wahle, E. (1998). The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of *Xenopus* oocytes. EMBO J. **17**, 5427-5437.

Kressler, D, Linder, P., and De la Cruz, j. (1999). Protein trans-acting factors involved in ribosome biogenesis in Saccharomyces cerevisiae. Mol. Cell. Biol. **12**, 7897-7912.

Larimer, F. W., Hsu, C. L., Maupin, M. K., and Stevens, A. (1992). Characterization of the XRN1 gene encoding a 5'-->3' exoribonuclease: sequence data and analysis of disparate protein and mRNA levels of gene-disrupted yeast cells. Gene **120**, 51-57.

Le Hir, H., Maquat, L. E., Maquat, L. E., and Moore, M. J. (2000). The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. EMBO J. **19**, 6860-6890.

McCarthy, J. E. G. (1998). Posttranscriptional control of gene expression in yeast. Micro. Mol. Biol. Rev.**62**, 1492-1553.

McClure, B. A. and Guilfoyle, T. J. (1989). Rapid redistribution of auxin-regulated RNAs during gravitropism. Science **243**, 91-93.

Metzlaff, M., O'Dell, M., Cluster, P. D., and Flavell, R. B. (1997). RNA-mediated RNA degradation and chalcone synthase A silencing in Petunia. Cell **88**, 845-854.

Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997). The exosome: A conserved eukaryotic RNA processing complex containing multiple 3'-->5' exoribonucleases. Cell **91**, 457-466.

Muhlrad, D., Decker, C. J., and Parker, R. (1994). Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev. **8**, 855-866.

Muhlrad, D. and Parker, R. (1992). Mutations affecting stability and deadenylation of the yeast MFA2 transcript. Genes and Dev. **6**, 2100-2111.

Nagy, E. and Maquat, L. E. (1998). A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. Trends Biochem.Sci. **6**, 198-199.

Newman, T. C., Ohme-Takagi, M., Taylor, C. B., and Green, P. J. (1993). DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell **5**, 701-714.

Peltz, S. W., Brown, A. H., and Jacobson, A. (1993). mRNA destabilization triggered by premature translational termination depends on at least three *cis*-acting sequence elements and one *trans*-acting factor. Genes Dev. **7**, 1737-1754.

Petracek, M. E., Dickey, L. F., Nguyen, T. T., Gatz, C., Sowinski, D. A., Allen, G. C., and Thompson, W. F. (1998). Ferredoxin-1 mRNA is destabilized by changes in photosynthetic electron transport. Proc. Natl. Acad. Sci. USA **95**, 9009-9013.

Poole, T. L. and Stevens, A. (1997). Structrual modifications of RNA influence the 5' exoribonucleolytic hydrolysis by XRN1 and HKE1 of *Saccharomyces cerevisiae*. Biochem.and Biophys. Res. Comm.**235**, 799-805.

Sachs, A. B. and Deardorff, J. A. (1992). Translation initiation requires the PAB-dependent poly(A) ribonuclease in yeast. Cell **70**, 961-973.

Sheng, J., D'Ovidio, R., and Mehdy, M. C. (1991). Negative and positive regulation of a novel proline-rich protein mRNA by fungal elicitor and wounding. Plant J. **1**, 345-354.

Shimotohno, K., Kodama Y., and Hashimoto, J. and Miura K. (1977a). Importance of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis. Proc. Natl. Acad. Sci. USA **74**, 2734-2738.

Shimotohno, K. and Miura, K. (1977b). A novel 5'-exonuclease which degrades uncapped mRNA. Proceedings of the 1977 Molecular Biology Meeting of Japan , 83-85.

Shyu, A.-B., Belasco, J. G., and Greenberg, M. E. (1991). Two distinct destabilizing elements in the c-*fos* message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev. **5**, 221-231.

Sijen, T. and Kooter, J. M. (2000). Post-transcriptional gene-silencing: RNAs on the attack or on the defense? BioEssays **22**, 520-531.

Stevens, A. (1979). Evidence for a 5'-3' direction of hydrolysis by a 5'-mononucleotide-producing exoribonuclease from saccharomyces cerevisiae. Biochem. Biophys.Res. Comm. **86**, 1126-1132.

Stevens, A., Hsu, C. L., Isham K.R., and Larimer, F. W. (1991). Fragments of the internal transcribed spacer 1 of pre-mRNA accumulate in *Saccharomyces cerevisiae* lacking 5'-3'exoribonuclease 1. J. Bacteriol. **173.**, 7024-7028.

Tanzer, M. M. and Meagher, R. B. (1995). Degradation of the soybean ribulose-1,5-bisphosphate carboxylase small-subunit mRNA, SRS4, initiates with endonucleolytic cleavage. Mol.Cell.Biol. **15** , 6641-6652.

Tharun, S., He, W. H., Mayes, A. E., Lennertz, P., Beggs, J. D., and Parker, R. (2000). Yeast Sm-like proteins function in mRNA decapping and decay. Nature **404**, 515-518.

Tucker, M., Valencia-Sanchez, M. A., Staples, R. R., Chen, J., Denis, C. L., and Parker, R. (2001). The Transcription Factor Associated Ccr4 and Caf1 Proteins are Components of the Major Cytoplasmic mRNA Deadenylase in *Saccharomyces cerevisiae*. Cell **104**, 377-386.

Van Eldik, G. J., Litiere, K., Jacobs, J. J. M. R., Van Montagu, M., and Cornelissen, M. (1998). Silencing of β-1,3-glucanase genes in tobacco correlates with an increased abundance of RNA degradation intermediates. Nucleic Acids Res. **26**, 5176-5181.

van Hoof, A. and Green, P. J. (1996). Premature nonsense codons decrease the stability of phytohemagglutinin mRNA in a position-dependent manner. Plant J. **10**, 415-424.

Villa, T., Ceradini F, Presutti, C., and Bozzoni, I. (1998). Processing of theintron-encoded U18 small nucleolar RNA in the yeast *Saccharomyces cervisiae* relies on both exo- and endonucleolytic activities. Mol. Cell. Biol. **18** , 3376-3383.

Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101**, 25-33.

Zuk, D. and Jacobson, A. (1998). A single amino acid substitution in yeast eIF-5A results in mRNA stabilization. EMBO J. **17**, 2914-2925.

CHAPTER 2

DEVELOPMENT OF ASSAYS TO STUDY THE XRN-FAMILY OF ARABIDOPSIS

The original form of this manuscript is in press in *Methods in Enzymology*. Reference: Kastenmayer J. P., Johnson M.J. and Green P.J. "Analysis of XRN-orthologs Through Complementation of Yeast Mutants and Localization of XRN-GFP Fusion Proteins". It has been modified to fit within the context of this thesis.

INTRODUCTION

A surprising result of our investigation of the XRN-family in Arabidopsis was the discovery that multiple orthologs of Xrn2p/Rat1p of yeast are encoded in the Arabidopsis genome (discussed in Chapter 3). However, analysis of the complete sequence of the Arabidopsis genome indicates that many segmental duplications have occurred in the evolution of the genome, and that many genes are members of multi-gene families in Arabidopsis (Vision et al., 2000). In the years to come, one of the challenges facing the plant community will be determining the individual functions of members of multi-gene families. This chapter describes the use of specific yeast mutants to study the enzymatic activities and intracellular locations of the three members of the XRN-family in Arabidopsis (AtXRNs). The use of these yeast strains was of great aid in rapidly characterizing the basic features of the AtXRN-family, and, as discussed in Chapter 3, allowed important questions regarding the activities and intracellular locations of the AtXRNs to be addressed. In addition, the results of these experiments directed the subsequent emphasis on genetic studies of *AtXRN4* described in Chapter 4. This chapter serves primarily as a guide for those wishing to study XRN-family members from other eukaryotes but also provides a theoretical framework for how one might begin to distinguish between members of a multi-gene family.

The XRN-family of 5'-3' exoribonucleases was first described in *Saccharomyces cerevisiae* in which it consists of two related proteins, Xrn1p and Xrn2p/Rat1p. These enzymes are similar in sequence and enzymatic activity but differ in their intracellular locations and cellular functions. Xrn1p is an abundant cytoplasmic enzyme and plays a major role in the degradation of mRNAs in the cytoplasm, as well as trimming the 5'

ends of rRNAs and degrading rRNA processing intermediates (Heyer et al., 1995; Hsu and Stevens, 1993; Henry et al., 1994; Stevens et al., 1991). In contrast to Xrn1p, Xrn2p/Rat1p is a nuclear protein that functions in the processing of rRNA and snoRNAs (Johnson, 1997; Petfalski et al., 1998, Villa et al., 2000). In other eukaryotes for which sequence is available, the XRN-family consists of a single member of the Xrn1p-like class and a single member of the Xrn2p/Rat1p-like class. In contrast, the Arabidopsis genome encodes three Xrn2p/Rat1p orthologs and no Xrn1p orthologs (Chapter 3). The function of these XRN-enzymes, or the XRN enzymes of other multicellular eukaryotes are unknown. Since certain aspects of mRNA degradation appear to differ between yeast and multicellular eukaryotes, differences that could be due to mechanisms specific to the XRN enzymes of multicellular eukaryotes, an investigation of these XRNs is warranted.

Insight into the possible cellular function of XRN orthologs can be gained by examining their exoribonuclease activities and intracellular locations. The exoribonuclease activity of recombinant XRN enzymes has been examined on RNA substrates *in vitro*, an approach used to study the mouse Xrn1p ortholog mXRN1p (Bashkirov et al., 1997). The intracellular location of Xrn1p and its ortholog from mouse, mXRN1, has been examined by immunocytochemistry using anti-XRN antibodies (Bashkirov et al., 1997; Heyer, 1995). The localization of Xrn2p/Rat1p and its orthologs from Arabidopsis has been studied with XRN-GFP fusion proteins (Johnson, 1997; Chapter 3). While these approaches can yield detailed information about the characteristics of XRN enzymes, a few simple experiments performed prior to such detailed analyses can give rapid insight into the basic features of XRN enzymes. These experiments have the advantage that they are easy to perform, and their results can

enhance subsequent studies of the XRN-enzymes both *in vitro* and in their native contexts.

Analysis of XRN-enzyme activity on poly(G)-containing mRNAs

Making use of RNA substrates with labeled 5' or 3' ends, Stevens and co-workers demonstrated that both Xrn1p and Xrn2p/Rat1p function as RNases that preferentially degrade RNAs from the 5' end *in vitro* (Poole and Stevens 1997). A modification of these studies was the use of RNA substrates which contained secondary structures known to block mRNA degradation *in vivo*, such as poly(G) tracts. Tracts of poly(G) are able to form stable structures in which four guanosines interact thorough non-Watson-Crick base-pairing (Kang et al., 1992). By introducing poly(G) tracts or stem-loops into substrate RNAs, it was shown that sequences 3' of the stable structure were not effectively degraded by Xrn1p or by Xrn2p/Rat1p, indicating that these structures inhibited the XRNs progression through the RNA (Poole and Stevens, 1997). Therefore, this approach confirms that the XRNs are active as exoribonucleases, demonstrates that they are blocked by poly(G) tracts, and that they degrade RNA from the 5' end.

The inability of the XRNs of yeast to progress through poly(G) tracts *in vitro* is consistent with studies of mRNA degradation in yeast. Experiments performed several years ago showed that the expression of genes in yeast which contain poly(G) tracts of 18 guanosines results in the accumulation of mRNA degradation intermediates that begin at the poly(G) tract and end at the poly(A) tail (Muhlrad et al., 1994). That the abundance of such poly(G)-stabilized mRNA decay intermediates is greatly reduced when the *XRN1* gene is deleted led to the hypothesis that Xrn1p degrades mRNAs from the 5' end and

that its progression through mRNAs is inhibited by poly(G) tracts (Muhlrad et al., 1994). The demonstration that Xrn1p's activity is indeed blocked by poly(G) tracts *in vitro* is strong support for this hypothesis. Therefore, the accumulation of poly(G)-stabilized mRNA decay intermediates, when poly(G)-containing genes are expressed in yeast, is a demonstration of Xrn1p's enzymatic function. As discussed below, the accumulation of poly(G)-stabilized mRNA decay intermediates in yeast in the absence of Xrn1p function can serve as a rapid method to analyze the activity of XRN orthologs from other organisms.

Given the utility of the poly(G) tract approach to understanding mRNA decay in yeast, the application of a similar approach to other eukaryotes may also lead to insight into mRNA degradation. The accumulation of poly(G)-stabilized mRNA decay intermediates similar to those observed in yeast could indicate that XRN-mediated degradation occurs. Such intermediates have been observed in *Chlamydomonas rheinhardtii* (Gera and Baker, 1998), an indication that degradation by an XRN-like enzyme may occur in this organism. However, in several plant and mammalian systems, expression of poly(G) tract-containing genes does not result in poly(G)-stabilized mRNA degradation intermediates (Johnson, 2000). The analysis of poly(G)-containing mRNAs in other eukaryotes may indicate if the absence of poly(G)-stabilized mRNA decay intermediates is the result of a mRNA degradation mechanism common to multicellular eukaryotes, or is particular to specific groups of eukaryotes. Such knowledge should aid in determining the extent to which the mechanism of mRNA decay in multicellular eukaryotes differs from the major mRNA decay pathway in yeast.

Use of a yeast xrn1Δ mutant and poly(G)-containing mRNAs to analyze the enzymatic activity of XRN enzymes

A possible explanation for the lack of poly(G)-stabilized mRNA decay intermediates in plants was that one of the AtXRNs might progress through the stable secondary structures formed by poly(G) tracts. However, as described in Chapter 3, each of the AtXRNs are unable to progress through poly(G) tracts. It is likely that blockage by poly(G) tracts is an inherent property of XRN enzymes. This property is experimentally advantageous, as it allows for the exoribonuclease activity of the XRN enzymes to be rapidly assessed.

A simple test of the potential exoribonuclease activity of an XRN enzyme is to express it in a *xrn1Δ* yeast strain and analyze the degradation of poly(G)-containing mRNAs. If an XRN protein is active as an exoribonuclease, and degrades transcripts from the 5' end, then it will likely generate poly(G)-stabilized mRNA decay intermediates similar to those produced by Xrn1p. Studies of mouse mXRN1 (Bashkirov et al., 1997) and *Drosophila* Pacman (Till et al., 2000) have made use of *xrn1* complementation, but have not studied poly(G)-containing genes. Expression of mXRN1 or Pacman in an *xrn1* mutant was shown to reduce the abundance of endogenous mRNAs which normally accumulate to high levels in the absence of Xrn1p. It was further shown that this reduction in mRNA abundance when Pacman was expressed in the *xrn1* mutant was due to increased mRNA degradation rates. Studies of *xrn1* complementation can be enhanced by the analysis of poly(G)-containing genes. This approach does not require mRNA half-life determinations, as the accumulation of an mRNA decay intermediate is monitored. This property could be advantageous if expression of the XRN does not completely restore mRNA turnover, since small differences in mRNA degradation rates

can be hard to detect. In addition, it enables the direction of catalysis to be determined as well as the effect of poly(G) tracts on the progression of the XRN enzymes to be addressed. A further advantage of this approach is that it can yield results for both nuclear-targeted as well as cytoplasmic exoribonucleases, and therefore can be used to study Xrn1p orthologs as well as Xrn2p/Rat1p orthologs (see below and Chapter 3).

Complementation of the *xrn1Δ* mutant was used to study AtXRN2 of *Arabidopsis thaliana*. AtXRN2 was expressed from a 2μ plasmid (p1954 in Figure 2-2 A) in an

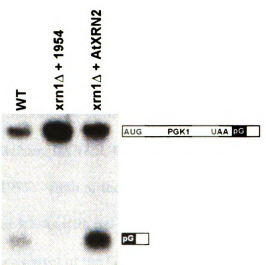


Figure 2-1. Analysis of the enzymatic activity of AtXRN2 on poly(G)-containing mRNAs when expressed in yeast lacking Xrn1p (*xrn1Δ*). A cDNA encoding AtXRN2 was inserted into p1954 (see Figure 2-2) and expressed in an *xrn1Δ* strain. The *xrn1Δ* strain expresses two poly(G)-containing genes, PGK1 and MFA2, each of which has a 18 guanosine tract in the 3' UTR. The accumulation of the poly(G) reporter mRNA PGK1, and its corresponding poly(G)-stabilized mRNA decay intermediate were analyzed by northern blot. 20μg of total RNA was separated on a 1.2% agarose formaldehyde gel, and transferred to a membrane which was hybridized with a radiolabeled oligonucleotide complementary to the poly(G)-containing PGK1 mRNA. The structure of the poly(G) reporter transcript and its poly(G)-stabilized decay intermediate generated by Xrn1p or AtXRN2 are shown at right

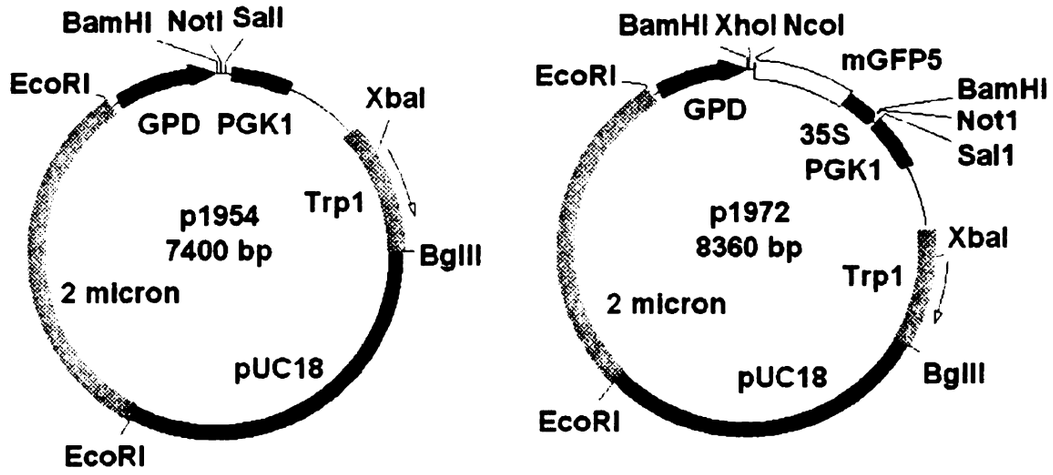
xrn1Δ strain which also expresses two poly(G)-containing reporter genes, PGK1 and MFA2. Expression of AtXRN2 in the *xrn1Δ* strain leads to a decrease in the accumulation of the full length PGK1 reporter RNA, and the formation of a poly(G)-stabilized PGK1 mRNA decay intermediate similar that formed by Xrn1p in wildtype (Figure 2-1). Similar results were obtained when the MFA2 poly(G) reporter was examined (data not shown). This indicates that AtXRN2 is active as a 5'-3' exoribonuclease, can complement the mRNA turnover defect of the *xrn1Δ* mutation, and is blocked by poly(G) tracts when expressed in yeast.

xrn1Δ complementation-Yeast strains (WT) yRP841 (MATα, *trp1-Δ1*, ura*3-52*, *leu2-3,112*, *lys2-201*, cup::LEU2pm), and (*xrn1Δ*) yRP884 (MATa, *trp1-Δ1*, *ura3-52*, *leu2-3,112*, *lys2-201*, cup::LEU2pm, *XRN1*::URA3) have been previously described (Caponigro and Parker, 1995). Both of these strains harbor chromosomal genes encoding PGK1 and MFA2 reporter RNAs with poly(G) tracts of 18 guanosines in their 3' UTRs. These genes are under the control of the GAL1 upstream activating sequence, requiring growth in galactose to induce expression. High levels of expression of heterologous proteins can be accomplished with the yeast expression vector pG1 (Schena et al., 1991). We have modified the polylinker of this vector to include an additional unique restriction site (*Not*I, p1954 in Figure 2-2) and generated a vector that can be used to generate GFP-fusions for protein localization studies in yeast (p1972 in Figure 2-2). The vector p1972 has a unique *Xho*I site (Figure 2-2 B).

The yRP841 and yRP884 strains are easily transformed with the vectors described in Figure 2-2 using standard methods. We recommend the following procedure, which

includes a small-volume sub-culturing step, to obtain good growth of the transformed strains and high expression levels of the poly(G) reporter genes. A single colony of each transformant is used to inoculate 1ml of SD medium containing 2% glucose, which is

A.



B.

```
Bam HI                    Not I                              Sal I
      |                     |                                  |
      GGATCCCCCGGGCTGCAGGAATTCGCGGCCGGAATTCGATATCAGCTTATCGATACCGTCGAC
p1954 ----------------------------------------------------------
      CCTAGGGGGCCCGACGTCCTTAAGCGCCGGCCTTAAGCTATAGTCGAATAGCTATGGCAGCTG

Bam HI                    Xho I                              Nco I
      |                     |                                  |
      GGATCCGATCCTTCAAGCTCGAGACCACTCGGATCTCTCTCTAATCGCCCGATCGGAATCCGCCATGGT
p1972 ----------------------------------------------------------
      CCTAGGCTAGGAAGTTCGAGCTCTCGGTGAGCCTAGAGAGAGATTAGCGGCTAGCCTTAGGCGGTACCA
```

Figure 2-2. Derivatives of pG1 vector for expression of proteins in yeast. (A) p1954 is a mulitcopy plasmid (2μ) with the GPD promoter and PGK1 terminator. For expression in yeast, sequences can be inserted between the promoter and terminator using the unique *Bam*HI, *Sal*I or *Not*1 sites. p1972 is similar to p1954 but contains the mGFP5 derivative of GFP(von Arnim et al., 1998) and also contains the 35S terminator for the cauliflower mosaic virus. Fusion of GFP to the C-terminus of a protein is accomplished by insertion of an open reading frame into the *Nco*I site. This vector could also be used to express proteins (not as a GFP fusion) using the *Xho*I site not available in p1954.(B) Polylinker sequence of p1954 and p1972. Unique enzyme sites are indicated.

grown for 2 days at 28°C in a shaker. A 20 µl aliquot of each two-day culture is used to inoculate 1ml of SD containing 2% galactose (to induce expression of poly(G) reporters) which is grown for an additional one to two days. The cells grown in SD+galactose are used to inoculate 30 mls of SD+galactose to an OD_{600} of 0.05. It is possible to inoculate the 30 ml SD+galactose culture directly with the initial SD+glucose culture; however, this can result in a significant lag in growth and poor growth. The 30 ml culture is grown to an OD_{600} of 0.3-0.4 (usually takes 2 days) and harvested by centrifugation in 50 ml conical tubes. Total RNA is isolated using the method described previously (Parker et al., 1991), and analyzed using standard northern blotting techniques. The probes used for analysis of the PGK1 and MFA2 reporter RNAs are oligonucleotides PGK1: 5'-AATTGATCTATCGAGGAATTCC-3', and MFA2: 5'-ATATTGATTAGATCAGGAATTCC-3' as previously described (Caponigro and Parker, 1995). The oligonucleotides are 5' end labeled as follows: 300 ng of oligonucleotide and 400µCi of [γ-P^{32}]-ATP (ICN) are incubated with 10 units of T4 polynucleotide kinase (Roche) and kinase buffer (supplied by the manufacturer) in a 20 µl total volume at 37°C for 1 hour. The labeled oligonucleotide is isolated from free nucleotide by gel filtration chromatography using a NucTrap column (Stratagene).

Determining the intracellular localization of XRN-family members

The cellular function of XRN-family members is dependent upon their intracellular locations. As mentioned above, yeast Xrn1p is cytoplasmic, while Xrn2p/Rat1p is nuclear. However, as described in Chapter 3, AtXRN4, an Xrn2p/Rat1p ortholog, accumulates in the cytoplasm. This indicates that the intracellular location of

an XRN-family member may not be reliably predicted based on its similarity to either Xrn1p or Xrn2p/Rat1p. In addition, while AtXRN2 is targeted to the nucleus (Chapter 3), expression of AtXRN2 in yeast led to the accumulation of a poly(G) intermediate similar to the one produced by cytoplasmic Xrn1p (Figure 2-1). Similarly, over-expression of Xrn2p/Rat1p in yeast can partially complement the mRNA turnover defect of *xrn1Δ* cells (Poole and Stevens). It is likely that nuclear-targeted XRNs accumulate at some level in the cytoplasm when expressed to high levels. The cytoplasmic accumulation of nuclear XRN-proteins is advantageous as it facilitates the analysis of their exoribonuclease activity on mRNAs. However, this indicates that *xrn1Δ* complementation apparently does not distinguish between cytoplasmic and nuclear enzymes, and that additional experiments are required to examine the intracellular location of an XRN-enzyme. Prior to in-depth localization studies, insight into intracellular localization can be gained by an additional yeast complementation experiment using the *xrn2/rat1* mutant *rat1-1*^{ts}.

If expression in the *xrn1Δ* strain indicates that an XRN enzyme is active as an exoribonuclease, complementation of an *xrn2/rat1* mutant can then be tested. Xrn2p/Rat1p is encoded by an essential gene, and cells harboring the *rat1-1*^{ts} mutation rapidly arrest their growth at the non-permissive temperature (Amberg et al., 1992). Rescuing the *rat1-1*^{ts} mutation appears to require an active exoribonuclease in the nucleus (Johnson, 1997). This indicates that complementation of *rat1-1*^{ts} by an XRN protein may serve as an additional assay for exoribonuclease activity as well as for nuclear targeting.

Complementation studies of *rat1-1*^{ts} were employed to gain insight into the intracellular locations of the AtXRNs. The results of these experiments, which will be discussed in Chapter 3, indicated that complementation of *rat1-1*^{ts} by heterologous

proteins is dependent on the same intracellular location requirements as for the endogenous yeast proteins. Furthermore, the localization of the AtXRNs in plant cells was consistent with complementation of *rat1-1*^{ts} serving as an indicator of intracellular location. Therefore, *rat1-1*^{ts} complementation may serve as an indication not only of the intracellular location of XRN-proteins when expressed in yeast, but perhaps also in their native contexts.

Complementation of rat1-1^{ts}- The yeast strains employed are FY86 (MATα, *ura3-52*, *his3Δ200*, *leu2Δ1*) and DAt1-1 (MATα, *ura3-52*, *leu2Δ1*, *trp1Δ63*, *rat1-1*) (Amberg et al., 1992). This particular *rat1-1*^{ts} strain allows for the use of the same expression vectors employed in *xrn1*Δ complementation as transformants can be selected for growth in the absence of tryptophan. After transformation, single colonies are used to inoculate 1 ml of SD medium. Following growth overnight, the cultures are diluted to equal OD_{600} with SD medium and the cultures are streaked on plates. The best results have been obtained by streaking out 3 µl of the overnight culture which had been diluted to an OD_{600} of approximately 1.0. This amount proved optimal since no growth of the *rat1-1*^{ts} cells was observed at the non-permissive temperature, while with greater volumes or with a lesser dilution, some growth can occur which can complicate the analysis. 3 µl of the diluted cells are pipeted onto two SD plates and spread with a sterile loop. One plate is incubated at the permissive temperature (26° C) and the other at the non-permissive temperature (37° C). Incubation of these plates for three days will result in abundant growth of the wildtype and complemented *rat1-1*^{ts} strains. The *rat1-1*^{ts} strain transformed with the yeast XRN2/RAT1 gene is used as a positive control. It should be noted that

complementation of the *rat1-1*^{ts} strain by heterologous proteins may not result in growth at 37° C equivalent to *rat1-1*^{ts} complemented with RAT1 (Chapter 3). Therefore, if it appears that growth does not occur in three days, the plates should be incubated for a longer period to determine if the cells are growing very slowly. The *rat1-1*^{ts} strain transformed with empty vector (p1954) will not grow even when incubated for seven days at the non-permissive temperature. In addition, the growth of the transformed *rat1-1*^{ts} strain at the permissive temperature could also be informative. Overexpression of Xrn1p in wildtype cells has been reported to adversely affect growth rate (Bashkirov et al., 1995; Page et al., 1998), and it is possible that high levels of expression of other XRN-proteins could affect growth of the *rat1-1*^{ts} strain. If so, this may be evident at the permissive temperature. Expression of the AtXRNs using the vectors in Figure 2-2 had no apparent effect on the growth of the *rat1-1*^{ts} strain at the non-permissive temperature indicating that the level of expression obtained with these vectors does not appear to inhibit growth. A final note with respect to *rat1-1*^{ts} complementation relates to the solid SD medium used for growing the transformed strains. While some protocols indicate that SD medium can be autoclaved, we have found that the complemented *rat1-1*^{ts} grows very poorly on autoclaved medium. The SD medium should be filter-sterilized.

Localization of XRN-GFP fusion proteins in yeast

A more direct approach to protein localization is the analysis of fusions of the XRNs to the green fluorescent protein (GFP), a technique successfully used to study Xrn2p/Rat1p of yeast and the AtXRNs (Johnson, 1997; Chapter 3). Analysis of XRN-GFP fusions expressed in yeast can be used to confirm the results of *rat1-1*^{ts}

complementation. As mentioned above, we have constructed a derivative of the pG1 vector that allows for the expression in yeast of proteins with mGFP5 fused to the C-terminus (p1972, Figure 2-2; von Arnim et al., 1998). Insertion of an open reading in the unique *Nco*I site is used to generate the XRN-GFP fusion plasmid. Prior to protein localization studies in any system, it should be demonstrated that the XRN-GFP fusions retain exoribonucleolytic activity and that *rat1-1*^{ts} complementation is not affected by the fusion. This is easily accomplished by testing if the XRN-GFP fusion retains the ability to generate a poly(G)-stabilized mRNA decay intermediate when expressed in the *xrn1*Δ strain and does not differ in ability to complement *rat1-1*^{ts} relative to the XRN protein without the GFP fusion. Fusion of GFP to the C-terminus of the yeast XRN proteins does not appear to adversely affect their function (Johnson, 1997) and as described in Chapter 3 had no apparent effect on the function of the AtXRNs. If the XRN-GFP protein retains exoribonucleolytic activity and is not altered in ability to complement *rat1-1*^{ts}, its localization can then be determined by fluorescence microscopy.

Localization of XRN-GFP proteins in yeast cells- The *rat1-1*^{ts} strain is transformed with the plasmid for expression of the XRN-GFP fusion using standard methods. GFP without a fusion, expressed from p1972 (Figure 2), can be used as a control as this protein is distributed uniformly across cells and is not localized preferentially to the nucleus or cytoplasm (von Arnim, et al., 1998). The growth of the transformants should be carried out in a manner similar to that used for the *rat1-1*^{ts} complementation studies described above. Following dilution with SD medium to an OD_{600} of approximately 1, the cells are grown at 26° for an additional 4 hours. To stain nuclear DNA, DAPI (Sigma) is added to

a final concentration of 0.5 μg/ml, for the final 30 minutes of the 4 hour growth. It is sometimes difficult to obtain adequate staining of yeast nuclear DNA with DAPI. Staining can be enhanced by the addition of 20% ethanol and incubation for approximately 5 minutes. This treatment does not appear to effect the intracellular localization or distribution of the AtXRN-GFP fusions. However, it leads to decreased intensity of GFP fluorescence, and a rapid quenching of GFP fluorescence for some AtXRN-GFP fusion proteins. For proteins whose expression is low in yeast, the decrease in GFP fluorescence due to ethanol treatment may make it difficult to obtain representative images of protein localization before GFP fluorescence decreases below detection. Optimization of ethanol concentration or incubation times may be required to obtain the best results for individual XRN-GFP fusions.

CONCLUSIONS

A complete understanding of the function of XRN proteins requires an examination of their enzymatic activity and their intracellular localization. These two aspects of XRN proteins can be rapidly investigated by complementation of *xrn1* and *xrn2/rat1* yeast mutants. The advantages of these experiments are that they are easy to perform, and in the case of *rat1-1*^{ts} complementation, give insight into the function of XRN-enzymes in their native contexts. Use of these assays allowed for the basic characteristics of the AtXRNs to be examined, and as described in Chapter 3, enabled the investigation of the possible role of an AtXRN in mRNA degradation.

REFERENCES

Amberg, D. C., Goldstein, A. L., and Cole, C. N. (1992). Isolation and characterization of *RAT1*: An essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. Genes Dev. **6**, 1173-1189.

Bashkirov, V. I., Scherthan, H., Solinger, J. A., Buerstedde, J-M., and Heyer, W.-D. (1997). A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. J. Cell Biol. **136**, 761-733.

Bashkirov, V. I., Solinger JA, and Heyer WD. (1995). Identification of functional domains in the Sep1 protein (=Kem1, Xrn1), which are required for transition through meiotic prophase in *Saccharomyces cerevisiae*. Chromosoma **104**, 215-222.

Caponigro, G. and Parker, R. (1995). Multiple functions for poly(A)-binding protein in mRNA decapping and deadenylation in yeast. Genes Dev. **9**, 2421-2432.

Gera, J. F. and Baker, E. J. (1998). Deadenylation-dependent and -independent decay pathways for beta1-tubulin mRNA in *Chlamydomonas reinhardtii*. Mol.Cell.Biol. **18**, 1498-1505.

Henry, Y., Wood, H, Morrissey, J. P., Petfalski, E., Kearsey S, and Tollervey, D. (1994). The 5' end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. EMBO J. **13**, 2452-2463.

Heyer, W.-D., Johnson, A. W., Reinhart, U., and Kolodner, R. D. (1995). Regulation and intracellular localization of *Saccharomyces cerevisiae* strand exchange protein 1 (Sep1/Xrn1/Kem1), a multifunctional exonuclease. Mol.Cell.Biol. **15**, 2728-2736.

Hsu, C. L. and Stevens, A. (1993). Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. Mol.Cell.Biol. **13**, 4826-4835.

Johnson, A. W. (1997). Rat1p and Xrn1p are functionally interchangeable exoribonucleases that are restricted to and required in the nucleus and cytoplasm, respectively. Mol.Cell.Biol. **17**, 6122-6130.

Johnson, M. A. (2000). Genetic Determinants of mRNA Stability in Plants. Ph.D. dissertation. Michigan State University

Kang,C., Zhang, X., Ratliff, R., Moyzis, R., and Rich, A. (1992) Crystal structure of four-stranded Oxytricha telomeric DNA. Nature **365**, 126-31.

Muhlrad, D., Decker, C. J., and Parker, R. (1994). Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev. **8**, 855-866.

Petfalski, E., Dandekar, T., Henry, Y., and Tollervey, D. (1998). Processing of the precursors to small nucleolar RNAs and rRNAs requires common components. Mol.Cell Biol. **18**, 1181-1189.

Poole, T. L. and Stevens, A. (1997). Structrual modifications of RNA influence the 5' exoribonucleolytic hydrolysis by XRN1 and HKE1 of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Comm. **235**, 799-805.

Schena, M., Picard, D, and Yamamoto, K. R. (1991). Vectors for constitutive and inducible Expression in Yeast. Methods Enzymol. **194**, 389-398.

Stevens, A., Hsu, C. L., Isham K.R., and Larimer, F. W. (1991). Fragments of the internal transcribed spacer 1 of pre-mRNA accumulate in *Saccharomyces cerevisiae* lacking 5'-3' exoribonuclease 1. J. Bacteriol. **173.**, 7024-7028.

Till, D. D., Linz, B., Seago, J. E., Elgar, S. J., Marujo, P. E., Elias, M. D., Arraiano, C. M., McClellan, J. A., McCarthy, J. E. G., and Newbury, S. F. (1998). Identification and developmental expression of a 5'-3' exoribonuclease from *Drosophila melanogaster* . Mech. Dev. **79**, 51-55.

Villa, T., Ceradini F, Presutti, C., and Bozzoni, I. (2000). Processing of the intron-encoded U18 Small nucleolar RNA in the yeast *Saccharomyces cervisiae* relies on both exo- and endonucleolytic activities. Mol. Cell. Biol. **18** , 3376-3383.

Vision, T.J., Brown, D.G., and Tanksley, S.D. (2000) The origins of duplications in Arabidopsis. Science **290**: 2114-2117.

von Arnim, A. G., Deng, X. W., and Stacey, M. G. (1998). Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. Gene **221**, 35-43.

Xue, Y., Bai, X. X., Lee, I., Kallstrom, G., Ho, J., Brown, J., Stevens, A., and Johnson, A. W. (2000). *Saccharomyces cerevisiae RAI1* (YGL246c) is homologous to human *DOM3Z* and encodes a protein that binds the nuclear exoribonuclease Rat1p. Mol. Cell. Biol. **20**, 4006-4015.

CHAPTER 3

NOVEL FEATURES OF THE XRN-FAMILY IN ARABIDOPSIS: EVIDENCE THAT AtXRN4, ONE OF SEVERAL ORTHOLOGS OF NUCLEAR Xrn2p/Rat1p, FUNCTIONS IN THE CYTOPLASM

In its original form, this manuscript was published in the *Proceedings of the National Academy of Sciences*, Reference: Kastenmayer and Green (2000) Novel Features of the XRN-Family of Arabidopsis: Multiple Orthologs of Nuclear Xrn2p/Rat1p and Evidence that AtXRN4 Functions in the Cytoplasm. PNAS 97: 13985-90. Control experiments which could not be included in the publication have been incorporated in this chapter as well as analysis of the complete Arabidopsis genome with respect to the XRN-family.

ABSTRACT

The 5'-3' exoribonucleases, Xrn1p and Xrn2p/Rat1p, function in the degradation and processing of several classes of RNA in *Saccharomyces cerevisiae*. Xrn1p is the main enzyme catalyzing cytoplasmic mRNA degradation in multiple decay pathways, while Xrn2p/Rat1p functions in the processing of rRNAs and snoRNAs in the nucleus. Much less is known about the XRN-like proteins of multicellular eukaryotes; however, differences in their activities could explain differences in mRNA degradation between multicellular and unicellular eukaryotes. One such difference is the lack in plants and animals of mRNA decay intermediates like those generated in yeast when Xrn1p is blocked by poly(G) tracts that are inserted within mRNAs. We investigated the XRN-family in *Arabidopsis thaliana* and found it to have several novel features. The Arabidopsis genome contains three XRN-like genes (*AtXRN*s) that are structurally similar to Xrn2p/Rat1p, a characteristic unique to plants. Furthermore, our experimental results and sequence database searches indicate that Xrn1p orthologs may be absent from higher plants. The lack of poly(G) mRNA decay intermediates in plants cannot be explained by the activity of the AtXRNs, as they are blocked by poly(G) tracts. Finally, complementation of yeast mutants and localization studies indicate that two of the AtXRNs likely function in the nucleus, while the third acts in the cytoplasm. Thus, the XRN-family in plants is more complex than in other eukaryotes, and if an XRN-like enzyme plays a role in mRNA decay in plants, the likely participant is a cytoplasmic Xrn2p/Rat1p ortholog, rather than an Xrn1p ortholog.

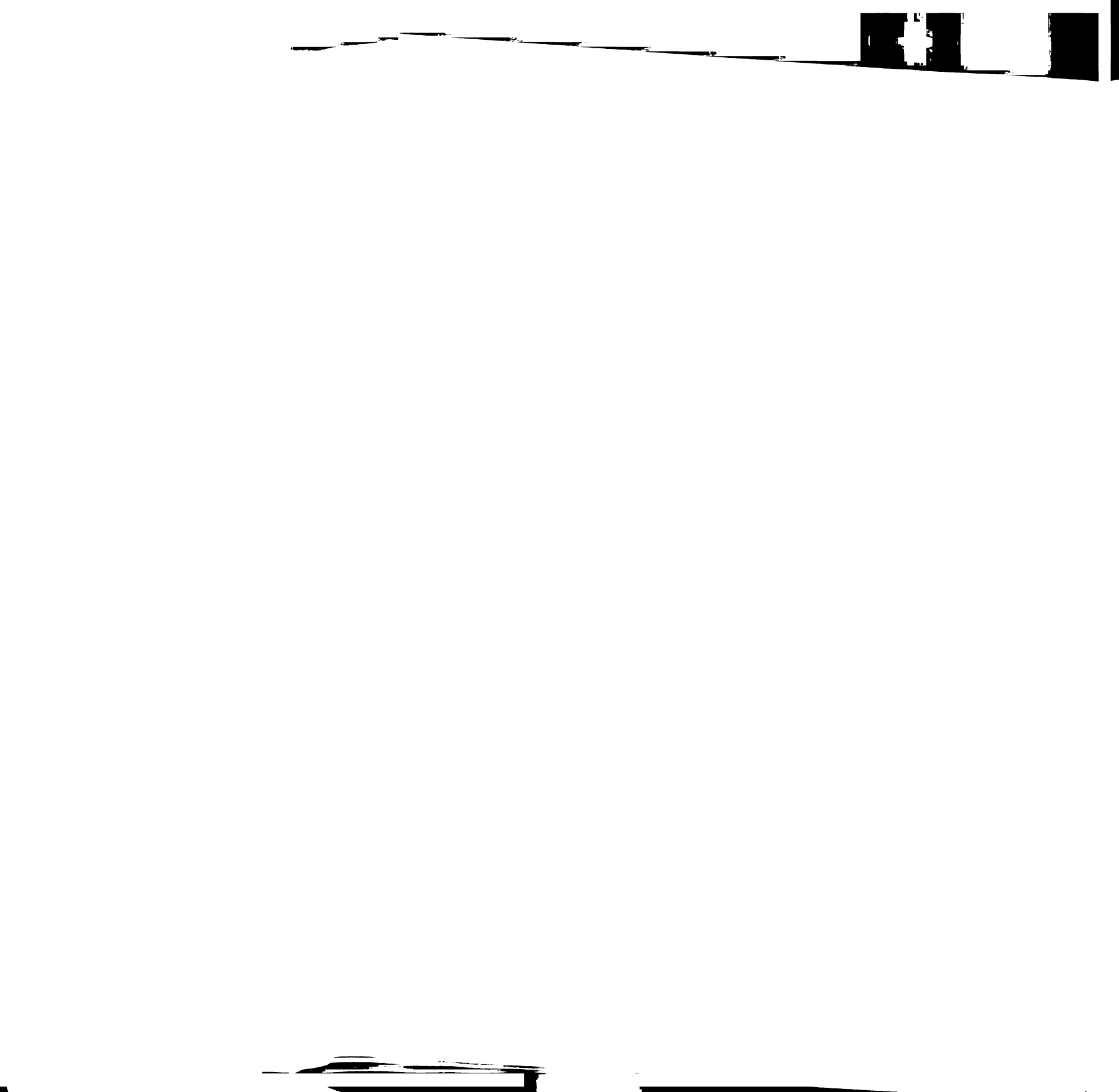
INTRODUCTION

5'-3' exoribonucleases play key roles in many RNA processing pathways, including mRNA degradation and the processing of rRNA and snoRNAs. In the yeast *Saccharomyces cerevisiae*, Xrn1p and Xrn2p/Rat1p are particularly prominent in these processes. These two exoribonucleases are highly related in sequence and enzymatic activity, but they differ with respect to their main *in vivo* substrates, intracellular locations, and relative abundance. Xrn1p catalyzes the degradation of the majority of mRNAs, is cytoplasmic, and is highly expressed (1,2). It is the main enzyme catalyzing mRNA degradation of decapped mRNAs in both the deadenylation-dependent-decapping as well as the nonsense-mediated decay (NMD) pathways (3). In the deadenylation-dependent-decapping pathway, transcripts are deadenylated, decapped, and then degraded 5'-3' by Xrn1p. The NMD pathway is similar, except that decapping and subsequent 5'-3' degradation by Xrn1p is not dependent on prior deadenylation. In addition to mRNA degradation, Xrn1p functions in the maturation of the 5' ends of rRNAs, and degrades rRNA processing intermediates (4, 5). Processing of rRNA 5' ends is a function shared with Xrn2p/Rat1p. In contrast to Xrn1p, Xrn2p/Rat1p is located primarily in the nucleus, is essential, and is expressed at lower levels than Xrn1p (6, 7). It is believed to be the major activity responsible for trimming the 5' ends of several rRNAs, and also trims the 5' ends of many snoRNAs during their maturation (8, 9).

Studies of Xrn1p's role in mRNA degradation in yeast were aided by the analyses of mRNA decay intermediates. While mRNA degradation intermediates usually do not accumulate to detectable levels in eukaryotic cells, it is possible to trap them in yeast by the insertion of a poly(G) tract into a mRNA. Expression of poly(G)-containing mRNAs

in yeast cells results in the accumulation of mRNA decay intermediates which begin at the 5' end of the poly(G) tract and end at the poly(A) tail, an indication that mRNA degradation is catalyzed from the 5' end in yeast (10-12). The demonstration that Xrn1p cannot progress through poly(G) tracts *in vitro* (13), and genetic studies which showed that the generation of the poly(G)-stabilized mRNA decay intermediates *in vivo* is almost exclusively dependent on Xrn1p (12), implicated Xrn1p as a major enzyme in mRNA degradation. Thus, the ability to generate mRNA decay intermediates by insertion of poly(G) tracts, in combination with studies of *xrn1* mutants, was crucial in determining Xrn1p's role in mRNA degradation in yeast cells. However, expression of poly(G) containing mRNAs in plant or animal cells does not result in the accumulation of poly(G)-stabilized mRNA decay intermediates, despite the presence of XRN-like enzymes in these organisms (14, 15, L. Maquat, A.-B. Shyu, G. Goodall, personal communications). This indicates that there is likely a difference in the mechanism by which mRNAs are degraded in multicellular eukaryotes compared to yeast.

To investigate this difference, we cloned three members of the Arabidopsis XRN-family and examined their enzymatic activities through heterologous expression in yeast. As the absence of poly(G)-stabilized mRNA decay intermediates in plant cells could most easily be explained by the AtXRNs progressing directly through poly(G) tracts, we investigated their activity on poly(G)-containing mRNAs. All three AtXRNs are blocked by poly(G) tracts when expressed in yeast, indicating that the absence of poly(G)-stabilized mRNA decay intermediates in plant cells is likely due to a novel mechanism. Beyond addressing the absence of poly(G)-stabilized mRNA decay intermediates in plant cells, our experiments provide evidence that the number, type and intracellular



distribution of these Xrn2p/Rat1p orthologs is unique in Arabidopsis, observations that may have important implications for the mechanism of mRNA turnover in higher plants.

MATERIALS AND METHODS

Cloning of AtXRN3 and AtXRN4 cDNAs and analyses of sequences.

The EST H4B9T7 (accession no. W43714), which contains the entire AtXRN2 open reading frame, was obtained from the Arabidopsis Biological Resource Center (http://aims.cps.msu.edu/aims/). The 3' end of a cDNA clone for AtXRN3 was isolated by using the internal *Sac*I to *Cla*I fragment of H4B9T7 as a probe to screen the PRL2 library (16). The 5' end of the AtXRN3 cDNA was obtained by rapid amplification of cDNA ends (RACE), using as a template, cDNAs generated from seven day old Arabidopsis seedlings grown on plates containing 1x MS medium and 3% sucrose. These template cDNAs were produced with a Marathon 5' 3' RACE kit (Clonetech). The primers used for amplification were the Marathon AP1 primer and an AtXRN3 cDNA specific primer PG469 5'-GCTCTGGAAGTGCATGCGAACTTGC-3'. The full-length AtXRN3 sequence was constructed by ligation of 5' RACE product and partial cDNA. The AtXRN4 cDNA was generated by RT-PCR and 3' RACE using the above described seedling cDNAs as template. The 5' end of the AtXRN4 cDNA was obtained with PG676 5'-CCTTCAAGCTCGAGACCAC-3', and PG704 5'-CCCGAAGCCGCACCAGTAGAGGA-3', the 3' end with PG734 5'-CCCATACCATTATGCTCC-3' and AP1. The full length AtXRN4 sequence was constructed by ligation of 5' and 3' RT-PCR products into the yeast expression vector

PG1 (described below) to generate p2038. The sequences of all PCR products were determined, and matched the corresponding genomic sequences.

Complementation of yeast mutants

All of the studies in yeast employed derivatives of the shuttle vector pG1 (17) with the AtXRN cDNAs inserted between the *Bam*HI and *Sal*I sites. They were p1846 (AtXRN2), p1958 (AtXRN3), and p2038 (AtXRN4). Yeast strains yRP841 (MATα, *trp1-Δ1*, *ura3-52*, *leu2-3,112*, *lys2-201*, cup::*LEU2*pm), and yRP884 (MATa, *trp1-Δ1*, *ura3-52*, *leu2-3,112*, *lys2-201*, cup::LEU2pm, *XRN1*::*URA3*), generously provided by Dr. Roy Parker, were used to study the activity of the AtXRNs on poly(G) mRNAs as described (18).

Yeast strains FY86 (MATα, *ura3-52*, *his3Δ200*, *leu2Δ1*) and DAt1-1 (MATα, *ura3-52*, *leu2Δ1*, *trp1Δ63*, *rat1-1*), generously provided by Dr. Charles Cole, were used to study *rat1-1*^{ts} complementation (19). Over-night liquid cultures of *rat1-1*^{ts} transformants were diluted to a similar OD_{600}, streaked on duplicate plates, and one plate incubated at 26° C and the other at 37 °C for three days.

Northern blot analyses of RNA from Arabidopsis plants

Total RNA from most tissues was isolated as described in (20) from *Arabidopsis thaliana* ecotype Columbia grown in soil for 30 days under standard conditions. The root tissue was harvested from seedlings grown on MS medium for 14 days. 20 μg of total RNA was separated with a 1% agarose formaldehyde gel, transferred to nylon membrane

(Nytran plus, Schleicher and Shuell), and hybridized with AtXRN gene specific probes. The gene specific probes used were the *Xho*I to *Not*I fragment of H4B9T7(AtXRN2), the *Xba*I to *Not*I fragment of p1958 (AtXRN3), and the *Xho*I to *Not*I fragment of p2038 (AtXRN4).

Southern Blot Analysis

15 μg of genomic DNA was incubated overnight with 100 units of the restriction enzymes indicated in the text. The restriction digests were separated with a 1% agarose gel, and blotted to nylon membrane. A separate blot was generated and hybridized with each of the gene specific probes.

Construction of GFP-fusions and localization studies

The open reading frames of AtXRN2 and AtXRN4 were amplified by PCR and inserted into the *Nco*I site of pAVA393 (21) for studies in onion epidermal cells. The correct sequence of all PCR products was verified. The primers used were: PG773 5'-CCATGGAACTGTTTTGGGAGG-3' and PG774 5'-CCATGGGTGTACCGTCGTTTT-3' for AtXRN2, and PG766 5'-GGAATCCGCCATGGGAGTACCGGC-3' and PG767 5'-CCATGGACAAGTTTGCACCTGC-3' for AtXRN4.

For localization studies in yeast, the AtXRN4-GFP fusion was expressed in *rat1-1*^{ts} from p2039, a pG1 derivative containing an AtXRN4-GFP fusion. Transformed cells were grown overnight at the permissive temperature, diluted to an OD_{600} similar to that used for *rat1-1*^{ts} complementation and photographed with a Kodak DC120 camera

(Kodak) and a Zeis Axiophot fluorescence microscope (Zeis) using appropriate filters. Treatment with 20% ethanol was used to facilitate DAPI staining and did not effect AtXRN4-GFP localization (data not shown).

Bombardment of onion epidermal cell layers was carried out as described (22), with the exception that 1.0 μm gold particles were used and the amounts of DNA were as indicated in Figure 3-6. The plasmids used were: pGFP-GUS and pAVA 367(GFP-NIa) (21), p2046 (AtXRN2-GFP), and p2042 (AtXRN4-GFP). Transformed onion epidermal layers were incubated on plates containing 1X MS medium and 3% sucrose for 20-24 hours in the dark and then photographed as described above.

RESULTS

AtXRN2, AtXRN3 and AtXRN4 are orthologs of the *Saccharomyces cerevisiae* protein Xrn2p/Rat1p

To identify XRN-like sequences of Arabidopsis, we searched the GenBank database for sequences similar to Xrn1p. Portions of three chromosomal sequences, TAC K16E1 (accession no. AB022210), BAC F10A5 (accession no. AC006434), BAC F20D21 (accession no. AC005287) and two ESTs, H4B9T7 (accession no. W43714) and H4B8T7 (accession no. W43713) contained sequences highly similar to Xrn1p. The two ESTs correspond to the XRN-like gene present on TAC K16E1. Analysis of the entire sequence of the EST H4B9T7 revealed an open reading frame highly similar to Xrn1p, as well as to Xrn2p/Rat1p. Due to its greater similarity to Xrn2p/Rat1p, the protein encoded by H4B9T7 was designated AtXRN2 (accession no. AF286720). cDNAs corresponding

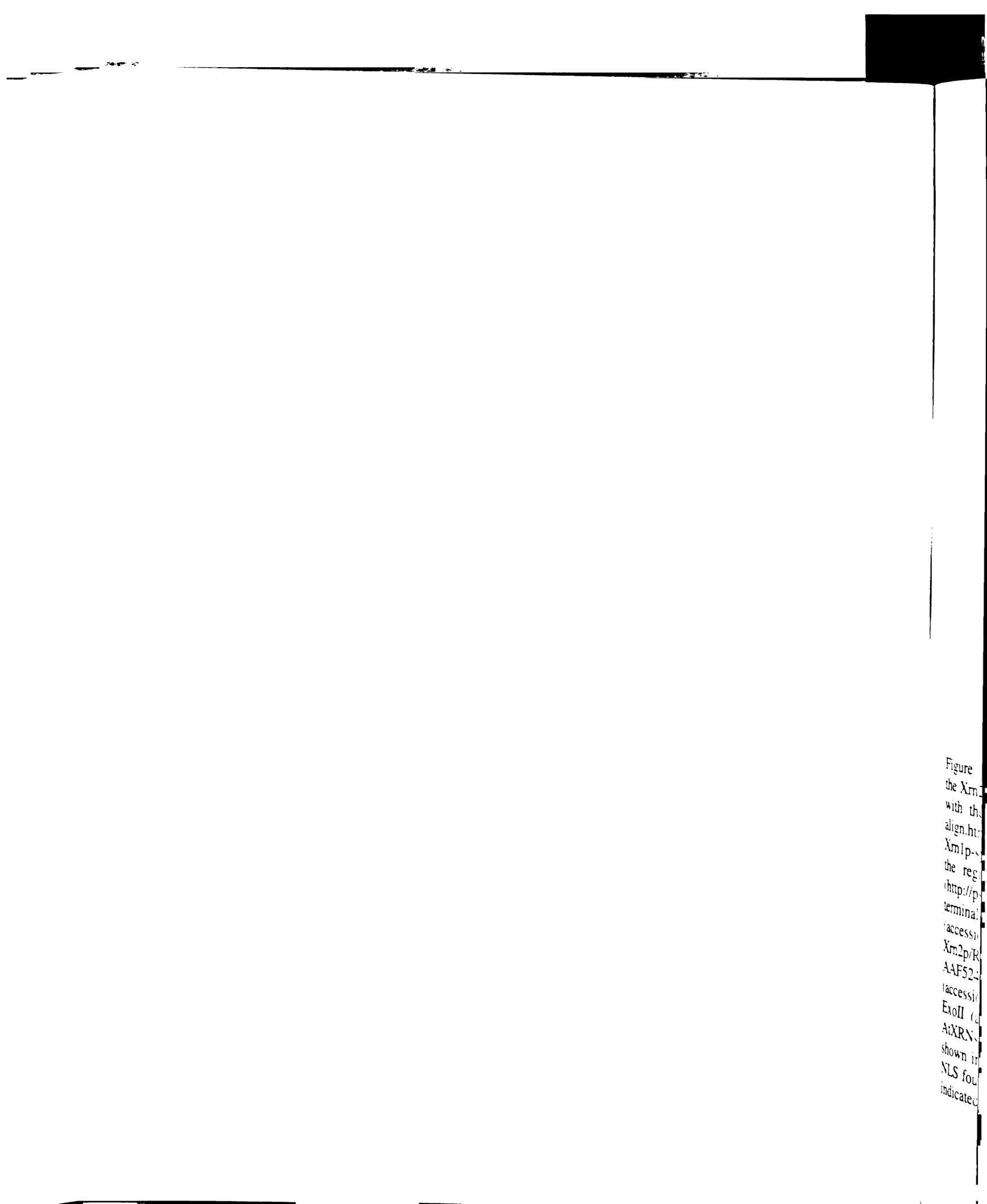
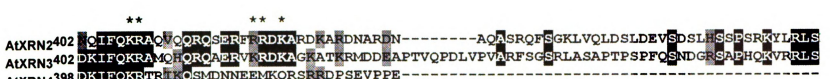


Figure 3-1. The Arabidopsis proteins AtXRN2, AtXRN3 and AtXRN4 are orthologs of the Xrn2p/Rat1p protein of *S. cerevisiae*. (A) Members of the XRN family were aligned with the program CLUSTALW (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) revealing conserved regions of the XRN-family (black regions) and an Xrn1p-specific domain (gray regions). Bipartite NLS consensus motifs (diamonds), in the regions indicated by the bracket, were identified with the program PSORT (http://psort.nibb.ac.jp). The half diamond in AtXRN4 indicates the lack of the C-terminal portion of a consensus bipartite NLS. The AtXRNs, *M. musculus* Dhm1 (accession no. I49635), *S. pombe* Dhp1 (accession no. S43891), *S. cerevisiae* Xrn2p/Rat1p (accession no. NP_014691), *D. melanogaster* gene product (accession AAF52452), *Mus musculus* mXrn1 (accession no. CAA62820), *D. melanogaster* Pacman (accession no. CAB43711), *S. cerevisiae* Xrn1p (accession no. P22147) and *S. pombe* ExoII (accession no. P40383) were aligned. (B) The amino acid sequences of the AtXRNs which correspond to the bracket in part A are shown. Identical residues are shown in black, similar residues are in gray. The basic residues constituting a bipartite NLS found in AtXRN2 and AtXRN3, only part of which is conserved in AtXRN4, are indicated by asterisks.

A.


AtXRN2
A. thaliana
AtXRN3
A. thaliana
AtXRN4
A. thaliana
Dhm1
M. musculus
D.m. Xrn2
D. melanogaster
Xrn2p/Rat1p
S. cervisiae
Dhp1
S. pombe
mXrn1
M. musculus
Pacman
D. melanogaster
Xrn1p
S. cerevisiae
Exoll
S. pombe
= 40-50% identity
=15-20% similarity

B.


AtXRN2 402
AtXRN3 402
AtXRN4 398

to the remaining XRN-like sequences were obtained by cDNA library screening and RT-PCR as described in Materials and Methods. The protein encoded by the cDNA corresponding to the XRN-like gene on BAC F10A5 was designated AtXRN3 (accession no. AF286719), and the protein encoded by the cDNA corresponding to the XRN-like gene on BAC F20D21 was designated AtXRN4 (accession no. AF286718).

By comparing amino acid sequences, it is possible to distinguish between Xrn1p-like and Xrn2p/Rat1p-like proteins because members of the Xrn1p-like class have a carboxy-terminal domain specific to this class (gray boxes Figure 3-1 A). The AtXRNs lack this carboxy-terminal domain. An additional characteristic of Xrn1p-like class is the closer spacing of the four N-terminal most conserved regions relative to that of the Xrn2p/Rat1p-like class (black boxes Figure 3-1A). The spacing of these N-terminal conserved regions in the AtXRNs is most like members of the Xrn2p/Rat1p-like class. Based on these sequence features, we classified the AtXRNs as Xrn2p/Rat1p orthologs.

Additional experiments were carried out to identify XRN-like sequences from Arabidopsis and other plant species that were more similar to Xrn1p than to Xrn2p/Rat1p; however, no evidence for XRN1-like sequences in plants was obtained. Extensive searches of sequence databases, including the complete Arabidopsis genome, have not yielded evidence for an XRN1-like gene in Arabidopsis or any other plant species. The absence of XRN1-like sequences from the complete Arabidopsis genome, and from the variety of sequences available from other plant species, makes it likely that Xrn1p orthologs are absent from higher plants.

AtXRN2, AtXRN3 and AtXRN4 are expressed in the major organs of Arabidopsis

The 3' ends of the AtXRN cDNAs, including a portion of the open reading frames and 3' untranslated regions, are not conserved, and were used to generate gene specific probes to study the expression of the AtXRNs using northern blots. These probes were able to specifically recognize the individual *AtXRN* genes on Southern blots (Figure 3-2 A). As seen in Figure 3-2 B, all three AtXRN transcripts were detected in roots, leaves, stems, and flowers. The levels of the AtXRN transcripts were similar to each other, and to themselves in different tissues, relative to the control eIF4A (quantitation not shown).

AtXRN2, AtXRN3 and AtXRN4 function as 5'-3' exoribonucleases and are blocked by poly(G) tracts when expressed in yeast

The enzymatic activity of the AtXRNs could explain the absence of poly(G)-stabilized mRNA decay intermediates in plants. The simplest explanation could be that the XRN-like enzymes of plants (and possibly other multicellular eukaryotes) can progress directly through poly(G) tracts degrading poly(G)-containing mRNAs to completion. To examine this possibility, and to determine if the AtXRNs were functional as exoribonucleases, the activity of each of the AtXRNs on poly(G) mRNAs was tested through heterologous expression in yeast. The AtXRN2, AtXRN3 and AtXRN4 proteins were expressed from a multicopy plasmid in wildtype and *xrn1*Δ yeast strains. These strains expressed two poly(G)-containing genes, PGK1 and MFA2, under the control of the GAL1 upstream activating sequence. For each gene, two transcripts, full-length and poly(G)-stabilized mRNA decay intermediate, readily were detected in northern blots of

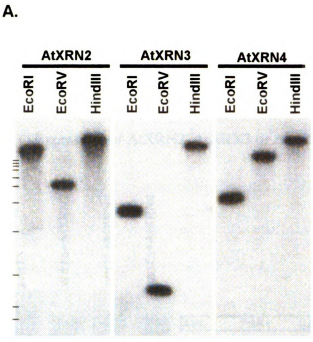


Figure 3-2. Analysis of *AtXRN* expression. (A) Gene specific probes generated from the 3' ends of the AtXRN cDNAs are specific for the individual *AtXRN* genes on Southern blots. 15μg of genomic DNA was digested with the restriction enzymes indicated, separated with a 1% agarose gel and blotted to nylon membrane. Each blot was hybridized with the AtXRN gene specific probe indicated at top of each blot. Tick marks indicate the size of molecular weight markers, 10 kb to 1.6 kb and 1 kb. (B) *AtXRN2*, *AtXRN3* and *AtXRN4* are expressed in the major Arabidopsis organs. Northern blot analysis was carried out on 20 μg total RNA isolated from the indicated organs. Gene specific probes for the AtXRNs, as well as a probe against the eIF4A transcript which served as a control, were used in the hybridization. AtXRN4 was analyzed separately and is shown relative to the eIF4A control for that experiment. R=roots, L=leaves, S=stems, F=flowers

RNA isolated from wildtype yeast (Figure 3-3). In contrast, in *xrn1*Δ cells, little or no poly(G) intermediate accumulated for either reporter transcript as previously observed (Figure 3-3, and ref. 11). Expression of AtXRN2, AtXRN3 or AtXRN4 in the *xrn1*Δ

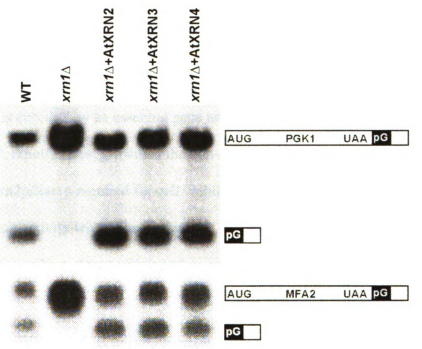


Figure 3-3. All three AtXRNs function as exoribonucleases which are blocked by poly(G) tracts when expressed in yeast. The AtXRNs were expressed in the *xrn1*Δ strain, and the accumulation of the poly(G) reporter mRNAs PGK1 (top) and MFA2 (bottom) was analyzed by northern blot. The structure of the poly(G) reporters is shown at right, and the AtXRN expressed in the *xrn*Δ strain is shown above.

cells resulted in a decrease in the abundance of the full-length reporter mRNAs, and in an increase in the accumulation of the poly(G) intermediates for both reporter transcripts (Figure 3-3). This result indicates that all three AtXRNs function as 5'-3' exoribonucleases which are able to degrade mRNAs, and that they are blocked by poly(G) tracts when expressed in yeast. Therefore, the absence of poly(G)-stabilized

mRNA decay intermediates in plant cells is unlikely due solely to an AtXRN progressing through poly(G) tracts and indicates that additional cellular proteins would likely be required for one of the AtXRNs to degrade poly(G)-containing mRNAs in plant cells.

AtXRN2 and AtXRN3 but not AtXRN4 complements the *rat1-1*^{ts} mutation

All three of the AtXRNs are structurally more similar to Xrn2p/Rat1p than to Xrn1p, indicating that they may have a nuclear function as does Xrn2p/Rat1p. Xrn2p/Rat1p is encoded by an essential gene and cells harboring a temperature sensitive allele, *rat1-1*^{ts}, rapidly arrest growth at the non-permissive temperature (19). The function of Xrn2p/Rat1p required for cell viability is unknown, but is thought to be exoribonuclease activity within the nucleus (7). Coupled with our observation that the AtXRNs have exoribonuclease activity when expressed in yeast (Figure 3-3), successful complementation of *rat1-1*^{ts} would imply nuclear localization in yeast cells.

The AtXRN yeast expression plasmids were introduced into the *rat1-1*^{ts} strain and the growth of the transformants on solid medium was monitored. Expression of the AtXRNs did not alter the growth of the *rat1-1*^{ts} strain at the permissive temperature, indicating that expression of the AtXRNs had no deleterious effects on the growth of the *rat1-1*^{ts} strain in this assay (Figure 3-4). At the non-permissive temperature, expression of AtXRN2 and AtXRN3 in the *rat1-1*^{ts} strain restored growth, albeit to a lesser extent than the XRN2/RAT1 control (Figure 3-4). This indicated that AtXRN2 and AtXRN3 likely entered the nucleus and replaced the essential function of Xrn2p/Rat1p. In contrast, expression of AtXRN4 did not rescue the growth arrest of the *rat1-1*^{ts} strain indicating that it likely did not enter the yeast nucleus.

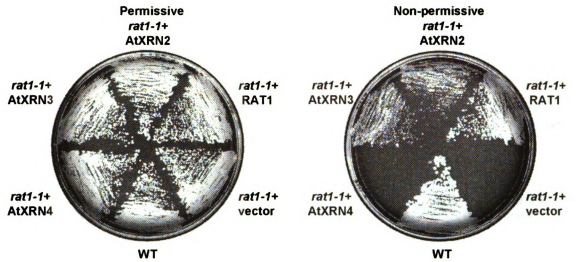


Figure 3-4. AtXRN2 and AtXRN3 but not AtXRN4 complement the *rat1-1* ^{ts} mutation. The *rat1-1* ^{ts} strain was transformed with the expression plasmids employed in Figure 3-3 and the growth of the transformants monitored at the permissive (left) and non-permissive (right) temperatures.

AtXRN2-GFP is targeted to the nucleus while AtXRN4-GFP is cytoplasmic

Complementation of *rat1-1*^{ts} indicated that the AtXRNs likely differ regarding nuclear targeting, and therefore might differ with respect to nuclear localization sequences (NLS). The AtXRNs are about 65% identical to each other in the regions encompassing the XRN-family conserved domains (black boxes Figure 3-1 A); however, AtXRN4 lacks about 90 amino acids present in AtXRN2 and AtXRN3 (indicated by bracket in Figure 3-1 A). These 90 amino acids of AtXRN2 and AtXRN3 contain an obvious bipartite NLS beginning at amino acid 407 (diamonds in Figure 3-1 A). The bipartite NLS is a well-characterized motif that targets proteins to the nucleus in plants and other eukaryotes, and consists of two basic regions separated by a variable spacer

(23). The N-terminal domain of the NLS in also conserved in AtXRN4, but as a result of the sequence deletion (relative to the other AtXRNs), the C-terminal region of this NLS

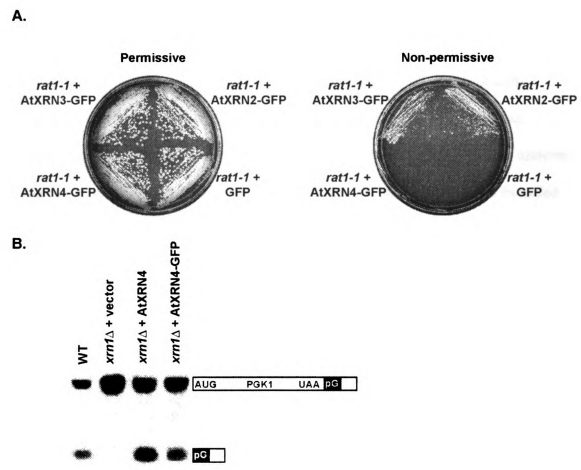


Figure 3-5. AtXRN-GFP fusion proteins are functional. (A) The *rat1-1* ^{ts} strain was transformed with AtXRN-GFP expression plasmids and the growth of the transformants monitored at the permissive (left) and non-permissive (right) temperatures as in Figure 3-4. (B) AtXRN4-GFP is functional as an exoribonuclease. AtXRN4-GFP was expressed in the *xrn1*Δ strain, and the accumulation of the poly(G) reporter mRNA PGK1 was analyzed by northern blot. The structure of the poly(G) reporter is shown at right, and the AtXRN expressed in the *xrn*Δ strain is shown above.

is absent (Figure 3-1 B). If AtXRN2 and AtXRN3 were targeted to the nucleus, but AtXRN4 was not, this would explain the AtXRNs differential ability to complement *rat1-1*^{ts} and could indicate that AtXRN4 has a cytoplasmic function.

To examine this possibility, an AtXRN4-GFP fusion was expressed and characterized first in yeast and subsequently in plant cells. The RNase activity of the AtXRN4-GFP fusion was confirmed by its ability to generate a poly(G)-stabilized mRNA decay intermediate when expressed in *xrn1*Δ cells (Figure 3-5 B), and, like AtXRN4, AtXRN4-GFP did not complement *rat1-1*^{ts} (Figure 3-5 A). Yeast cells expressing AtXRN4-GFP exhibited two expression patterns, uniform cytoplasmic fluorescence, and spots which varied in both size and number (Figure 3-6). The uniform fluorescence was distributed evenly across the yeast cells, but appeared to be excluded from the nucleus. Exclusion from the nucleus is illustrated by the cells within the box, where a region without fluorescence (indicated by the arrow) corresponds to DAPI stained nuclear DNA (Figure 3-6 A, Overlay). Similarly, the spots did not correspond to the nucleus (Figure 3-6 A, Overlay). Although we cannot rule out that some AtXRN4-GFP may enter the nucleus, these results indicate that the most likely reason for the inability of AtXRN4 to complement *rat1-1*^{ts} is due to its exclusion from the nucleus, and might indicate that AtXRN4 functions as a cytoplasmic protein in Arabidopsis

To examine the intracellular location of the AtXRNs in plant cells, AtXRN-GFP fusion proteins were transiently expressed in onion epidermal cells by particle bombardment (22). As expected, based on successful *rat1-1*^{ts} complementation, AtXRN2-GFP showed high fluorescence in the nucleus, an expression pattern similar to the nuclear GFP-NIa protein (21) (Figure 3-6 B). In addition to a general nuclear localization, AtXRN2-GFP accumulated in bright spots within the nucleus that may represent targeting of AtXRN2-GFP to the nucleoli. As an ortholog of Xrn2p/Rat1p of yeast, AtXRN2 may also function in rRNA processing and could be targeted to this

Figure 3-6. AtXRN2-GFP and AtXRN4-GFP localization. (A) AtXRN4-GFP was expressed in the *rat1-1*^{ts} strain, and GFP fluorescence compared with DAPI stained nuclear DNA. The GFP and DAPI images of the boxed cells are shown superimposed (Overlay). (B) Plasmids encoding AtXRN2-GFP and AtXRN4-GFP were used to transform onion epidermal cells by particle bombardment. GFP fluorescence of AtXRN2-GFP and AtXRN4-GFP was compared to that of GFP-GUS, a primarily cytoplasmic protein, and GFP-NIa which is targeted to the nucleus. The onion epidermal cell images were obtained with a 20X objective. A 40X image of AtXRN4-GFP 1μg is also shown. The arrows indicate the nuclei.

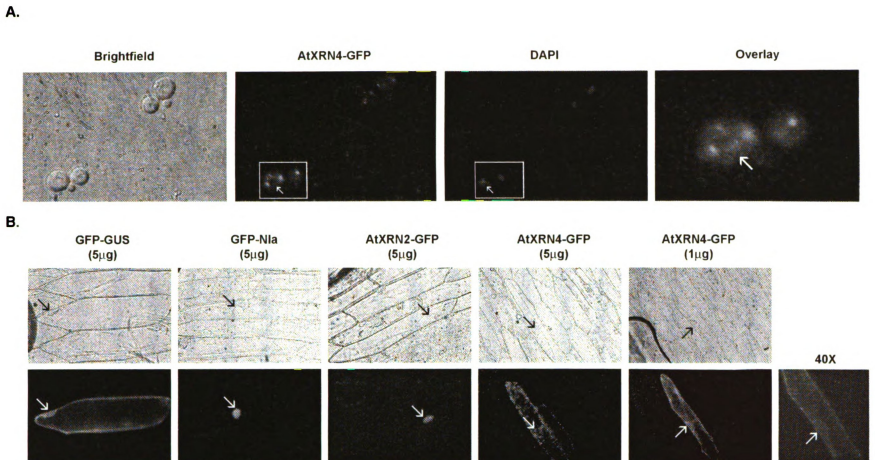


Figure 3-6. AtXRN2-GFP and AtXRN4-GFP localization.

sub-nuclear region. Similar to AtXRN2-GFP, transient expression of AtXRN3-GFP in onion epidermal cells revealed a nuclear localization (data not shown). In contrast to the intense nuclear fluorescence of AtXRN2-GFP, the intracellular distribution of AtXRN4-GFP appeared more similar to that of GFP-GUS, a protein known to accumulate in the cytoplasm (21). In addition, similar to its expression in yeast, AtXRN4-GFP accumulated as both uniform fluorescence and as spots that were not detected within the nucleus (Figure 3-6B). Since the AtXRN4-GFP spots may have been cytoplasmic inclusions (24), we tested if lowering AtXRN4-GFP expression would diminish the number of spots. As seen in Figure 3-6 B, (AtXRN4-GFP 1 μg), reducing the amount of DNA used in the bombardment resulted in a reduction in the number and size of the spots. A higher magnification shows uniform AtXRN4-GFP fluorescence and the reduction in the number of spots (AtXRN4-GFP 1μg, 40X). Thus, reducing the amount of DNA decreases the number and size of the spots, while not effecting the cytoplasmic localization of AtXRN4-GFP.

DISCUSSION

In this study, we investigated the XRN-family in Arabidopsis and discovered it has several features not found in other eukaryotes. The Arabidopsis genome encodes an unusual number and distribution of XRN enzymes. All three AtXRNs are Xrn2p/Rat1p orthologs, and Xrn1p orthologs are apparently absent from higher plants. The absence of a Xrn1p ortholog is surprising since they have been described from *S. pombe* (25), *M. musculus* (26), *D. melanogaster* (27), and are present in sequence databases from a variety of other eukaryotes (Kastenmayer and Green, unpublished). This indicates that

Xrn1p-like function may be conserved in eukaryotes; however, the lack an Xrn1p ortholog from Arabidopsis, and possibly higher plants, would require that the conserved function be catalyzed by different RNase. This study indicates that Xrn1p-like function may be carried out by AtXRN4-like enzymes in higher plants. AtXRN4 functions as an exoribonuclease, and is cytoplasmic where it could function in the degradation of mRNAs.

A potential difference in the mechanisms of mRNA decay between yeast and multicellular eukaryotes is indicated by the accumulation of poly(G)-stabilized mRNA decay intermediates in yeast, but not in higher eukaryotes (14, 15, L. Maquat, A.-B. Shyu, G. Goodall, personal communications). In plant cells, expression of mRNAs containing poly(G) tracts does not lead to the accumulation of poly(G)-stabilized mRNA decay intermediates like those observed when Xrn1p is blocked by the insertion of poly(G) tracts in mRNAs in yeast (14). One possible explanation for the absence of poly(G)-stabilized mRNA decay intermediates in higher eukaryotes may be that the XRNs from multicellular eukaryotes are more robust than their yeast counterparts and can progress directly through poly(G) tracts. Alternatively, these XRNs may be blocked by poly(G) tracts, but additional proteins, such as RNA helicases, resolve the structures formed by poly(G) tracts thus allowing XRN-like enzymes to complete degradation. It is also possible that a highly active 3'-5' mRNA degradation pathway in plants degrades through poly(G) tracts from the 3' end. However, while our experiments in plant cells were designed to detect poly(G)-stabilized mRNA decay intermediates due to the action of a 3'-5' exoribonuclease mediated pathway, or intermediates due to degradation from both ends of the mRNA simultaneously ["poly(G) stub" (28)], such intermediates were

not detected. A 3'-5' pathway in plants would therefore also likely require the activity of an RNA helicase to degrade poly(G)-containing mRNAs. RNA helicases are likely associated with mRNA degradation machinery in eukaryotes (29), and since is it is known that greater than 30 RNA helicase-like genes are present in the Arabidopsis genome (30), such activities could be quite prevalent in the cytoplasm. It is unlikely that blockage of XRN proteins by poly(G) tracts in yeast depends on cellular factors particular to yeast, as poly(G) tracts and other stable secondary structures inhibit both Xrn1p and Xrn2p/Rat1p *in vitro* with only purified components present (13). Although the XRNs from animals have not been directly tested for their ability to progress through poly(G) tracts, the five XRNs that have so far been tested do not progress through poly(G). This indicates that blockage by poly(G) tracts is likely an inherent property of XRN enzymes, and that the absence of poly(G)-stabilized mRNA decay intermediates in animal cells is likely due to a similar reason as in plants.

Several observations indicate that some mRNAs in plants may be degraded by 5'-3' exoribonuclease-mediated pathways that could involve AtXRN4 in Arabidopsis, or AtXRN4-like enzymes of other plants. The most compelling evidence comes from studies of degradation intermediates of PhyA mRNA in oat seedlings. The majority of these intermediates exist as a series of transcripts lacking increasing amounts of the 5' end, consistent with degradation mediated by a 5'-3' exoribonuclease (31). Degradation of at least some of mRNAs from the 5' end in plants may resemble the major mRNA decay pathway in yeast in which mRNAs are degraded from the 5' end by Xrn1p following their deadenylation and decapping. In addition to AtXRN4, orthologs of other components relevant to this pathway exist in the Arabidopsis genome (32), including

PAB2. PAB2 is a poly(A) binding protein which is able to function in the coupling of deadenylation to mRNA decay when expressed in yeast (33). Alternatively, endoribonuclease cleavage may initiate mRNA decay, with AtXRN4 catalyzing the degradation of the products of endoribonuclease cleavage. Such a mechanism has been proposed for the degradation of SRS4 mRNA in petunia (34), as well as for the degradation of mRNAs in a variety of plant species targeted by post-transcriptional gene silencing (35-37). Post-transcriptional gene silencing is a phenomenon which occurs in plants, animals and fungi in which mRNAs that share a high degree of similarity are selectively degraded (reviewed in 38). Because both cytoplasmic and nuclear mRNA decay may be involved in this process, any of the AtXRNs could participate.

The presence of three Xrn2p/Rat1p orthologs in Arabidopsis is a particularly interesting aspect of the XRN-family in Arabidopsis. Since AtXRN2 and AtXRN3 are both targeted to the nucleus, they may have functions similar to Xrn2p/Rat1p, such as rRNA or snoRNA processing (8,9). These functions may be redundant, substrate-specific or specific to particular cell types. Insight into these possibilities may be gained by a more detailed analysis of their expression patterns in different cells, intranuclear distributions and enzymatic activities. Another implication of multiple XRN2/RAT1 orthologs in Arabidopsis is the potential to gain insight into the evolution of gene function. The three AtXRN genes are likely to have arisen due to duplication of an XRN2/RAT1-like gene. The deletion of the region encoding the bipartite NLS during duplication may have given rise to an AtXRN4-like protein. Loss of the NLS would allow this protein to gain cytoplasmic function, and this protein may have eventually replaced a Xrn1p ortholog. That a Xrn2p/Rat1p protein can replace Xrn1p function is

supported by the observation that Xrn2p/Rat1p expressed in the cytoplasm of yeast can complement an *xrn1*Δ strain (7). Therefore, mRNA degradation in plants may resemble the major mRNA decay pathway of yeast; however, in contrast to yeast, the final degradation of mRNAs could be catalyzed by a cytoplasmic Xrn2p/Rat1p-like enzyme such as AtXRN4. This may be a feature of mRNA degradation unique to the plants, as this is the only eukaryotic kingdom from which XRN1-like genes are apparently absent.

Ultimately, it should be possible to determine the substrates and functions of the AtXRNs in Arabidopsis by the isolation and characterization of *xrn* mutants. Analyses of these mutants may aid in dissecting the potentially overlapping roles of AtXRN2 and AtXRN3 in the nucleus. It should be noted that analyses of *xrn1* and *xrn2/rat1* mutants has implicated Xrn1p as having a meiosis specific role independent from its exoribonuclease activity (39), and Xrn2p/Rat1p in nucleocytoplasmic mRNA trafficking (19). Thus, analysis of Arabidopsis *xrn* mutants may provide further insight not only into mRNA decay and RNA processing in plants, but also into functions of the XRN-family in general.

Acknowledgements: We thank Dr. Mark Johnson and Dr. Ambro van Hoof for helpful comments on this manuscript, Chris Behrens for contributing to the localization studies, and Linda Danhof for expert technical assistance. This work was supported by the National Science Foundation (grant no. IBN 9408052), U.S. Department of Energy (grant no. DE-FG02-91E920021) and the U.S. Department of Agriculture (grant no. 9801498)

REFERENCES

1-**Heyer, W.-D., Johnson, A. W., Reinhart, U., and Kolodner, R. D.** (1995). Regulation and intracellular localization of *Saccharomyces cerevisiae* strand exchange protein 1 (Sep1/Xrn1/Kem1), a multifunctional exonuclease. Mol. Cell. Biol. **15**, 2728-2736.

2-**Hsu, C. L. and Stevens, A.** (1993). Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. Mol. Cell. Biol. **13**, 4826-4835..

3-**Tharun, S. and Parker, R.** (1997) in *mRNA Metabolism & Post-Transcriptional Gene Regulation*, eds. Harford, J. B. & Morris, D. (Wiley-Liss Inc, pp. 181-200.

4-**Henry, Y., Wood, H, Morrissey, J. P., Petfalski, E., Kearsey S, and Tollervey, D.** (1994). The 5' end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. EMBO J. **13**, 2452-2463.

5-**Stevens, A., Hsu, C. L., Isham K.R., and Larimer, F. W.** (1991). Fragments of the internal transcribed spacer 1 of pre-mRNA accumulate in *Saccharomyces cerevisiae* lacking 5'-3'exoribonuclease 1. J. Bacteriol. **173.**, 7024-7028.

6-**Kenna, M., Stevens, A., McCannon, M. & Douglas, M. G.** (1993) An essential yeast gene with homology to the exonuclease-encoding XRN1/KEM1 gene also encodes a protein with exoribonuclease activity. *Mol. Cell. Biol.* **13**, 341-350.

7-**Johnson, A. W.** (1997). Rat1p and Xrn1p are functionally interchangeable exoribonucleases that are restricted to and required in the nucleus and cytoplasm, respectively. Mol. Cell. Biol. **17**, 6122-6130

8-**Petfalski, E., Dandekar, T., Henry, Y., and Tollervey, D.** (1998). Processing of the precursors to small nucleolar RNAs and rRNAs requires common components. Mol. Cell. Biol. **18**, 1181-1189.

9-**Villa, T., Ceradini F, Presutti, C., and Bozzoni, I.** (1998). Processing of the intron-encoded U18 small nucleolar RNA in the yeast *Saccharomyces cervisiae* relies on both exo- and endonucleolytic activities. Mol. Cell. Biol. **18** , 3376-3383.

10-**Vreken, P. & Raué, H. A.** (1992) The rate-limiting step in yeast PGK1 mRNA degradation is an endonucleolytic cleavage in the 3'-terminal part of the coding region*Mol. Cell. Biol.* **12**, 2986-2996.

11-**Decker, C. J. and Parker, R.** (1993). A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev. **7**, 1632-1643.

12-**Muhlrad, D., Decker, C. J., and Parker, R.** (1994). Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev. **8**, 855-866.

13-**Poole, T. L. and Stevens, A.** (1997). Structrual modifications of RNA influence the 5' exoribonucleolytic hydrolysis by XRN1 and HKE1 of *Saccharomyces cerevisiae.* Biochem.and Biophys. Res. Comm.**235**, 799-805.

14-**Johnson M.A.** (2000). Genetic Determinants of mRNA Stability in Plants. Doctoral Dissertation Michigan State University.

15-**Kastenmayer, J. P., van Hoof, A., Johnson, M. & Green, P. J.** (1998) in *Plant Molecular Biology*, eds. Raikhel, N., Last, R., Morelli, G., & LaShavo, F. (Springer-Verlag, Berlin), pp. 125-133.

16-**Newman, T. C., de Bruijn, F., Green, P. J., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N. V., Somerville, S. C., Tomashow, M., Retzel, E., and Somerville, C. R.** (1994). Genes Galore: A summary of methods for accessing results from partial sequencing of anonymous Arabidopsis cDNA clones. Plant Physiol. **106**, 1241-1255.

17-**Schena, M., Picard, D. & Yamamoto, K. R.** (1991). Vectors for constitutive and inducible gene expression in yeast.*Methods Enzymol.* **194**, 389-398.

18-**Caponigro, G. and Parker, R.** (1995). Multiple functions for poly(A)-binding protein in mRNA decapping and deadenylation in yeast. Genes Dev. **9**, 2421-2432.

19-**Amberg, D. C., Goldstein, A. L., and Cole, C. N.** (1992). Isolation and characterization of *RAT1*: An essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. Genes Dev. **6**, 1173-1189.

20-**Taylor, C. B., Bariola, P. A., DelCardayré, S. B., Raines, R. T. & Green, P. J.** (1993). RNS2: a senescence-associated RNase of Arabidopsis that diverged from the S-RNases before speciation (1993) *Proc. Natl. Acad. Sci. USA* **90**, 5118-5122.

21-**von Arnim, A. G., Deng, X. W., and Stacey, M. G.** (1998). Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. Gene **221**, 35-43.

22-**Vargona, M. J., Schmidt, R. J. & Raikhel, N.** (1992). Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. *Plant Cell* **4**, 1213-1227.

23-**Hicks, G. R. & Raikhel, N.** (1995). Protein import into the nucleus: an integrated view. *Annual Rev. of Cell Dev. Biology* **11**, 155-188.

24-**Stacey, M. G., Hicks, S. N. & von Arnim, A. G.** (1999). Discrete domains mediate the light-responsive nuclear and cytoplasmic localization of Arabidopsis COP1*Plant Cell* 11, 349-363.

25-**Szankasi, P. & Smith, G. R.** (1996). Requirement of S. pombe exonuclease II, a homologue of S. cerevisiae Sep1, for normal mitotic growth and viability. *Current Genetics* **30**, 284-293.

26-**Bashkirov, V. I., Scherthan, H., Solinger, J. A., Buerstedde, J-M., and Heyer, W.-D.** (1997). A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. J. Cell Biol. **136**, 761-733.

27-**Till, D. D., Linz, B., Seago, J. E., Elgar, S. J., Marujo, P. E., Elias, M. D., Arraiano, C. M., McClellan, J. A., McCarthy, J. E. G., and Newbury, S. F.** (1998). Identification and developmental expression of a 5'-3' exoribonuclease from *Drosophila melanogaster* . Mech. Dev. **79**, 51-55.

28-**Jacobs-Anderson, J. and Parker, R.** (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J. **17**, 1497-1506.

29-**Jacobs Anderson, J. S. & Parker, R.** (1996) RNA turnover: The helicase story unwinds. *Current Biology* **6**, 780-782.

30-**Aubourg S, Kreis M & Lecharny A** (1999) The DEAD box RNA helicase family in Arabidopsis thaliana. *Nucleic Acids Res* **27**, 628-636.

31-**Higgs, D. C. and Colbert, J. T.** (1994). Oat phytochrome A mRNA degradation appears to occur via two distinct pathways. Plant Cell **6**, 1007-1019.

32-**Gutiérrez, R. A., MacIntosh, G. C., and Green, P. J.** (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci. **4**, 429-438.33. Palanivelu, R., Belostotsky, D. A. & Meagher, R. B. (2000) *Plant Journal* 22, 187-198.

34-**Tanzer, M. M. and Meagher, R. B.** (1995). Degradation of the soybean ribulose-1,5-bisphosphate carboxylase small-subunit mRNA, SRS4, initiates with endonucleolytic cleavage. Mol.Cell.Biol. **15** , 6641-6652.

35-**Tanzer, M. M., Thompson, W. F., Law, M. D., Wernsman, E. A. & Uknes, S.** (1997). Characterization of post-transcriptionally suppressed transgene expression that confers resistance to tobacco etch virus infection in tobacco. *Plant Cell* **9**, 1411-1423.

36-**van Eldik G.J., Litiere K., Jacobs J.J., Van Montagu M & Cornelissen M** (1998). Silencing of beta-1,3-glucanase genes in tobacco correlates with an increased abundance of RNA degradation intermediates *Nucleic Acids Res.* **26**, 5176-5181.

37-**Metzlaff, M., O'Dell, M., Cluster, P. D. & Flavell, R. B.** (1997). RNA-mediated RNA degradation and chalcone synthase A silencing in Petunia. *Cell* **88**, 845-854.

38-**Sijen, T. and Kooter, J. M.** (2000). Post-transcriptional gene-silencing: RNAs on the attack or on the defense? BioEssays **22**, 520-531.

39-**Solinger J.A., Pascolini D & Heyer WD** (1999) Identification of functional domains in the Sep1Protein (=Kem1, Xrn1), which is required for transition through meiotic prophase in *Saccharomyces cerevisiae. Mol. Cell Biol.* **9**, 5930-5942.

CHAPTER 4

ANALYSIS OF mRNA DEGRADTION IN AN *xrn4* MUTANT INDICATES THAT AtXRN4 MAY FUNCTION IN mRNA TURNOVER

INTRODUCTION

As mentioned in Chapter 1, analyses of mutants of components of the basal mRNA decay machinery in yeast were critical in demonstrating the role that particular proteins play in the major mRNA decay pathway. Similarly, characterization of mutants of the *AtXRN* genes has the potential to shed light on their individual functions in Arabidopsis. In yeast, deletion of *XRN1*, which encodes the cytoplasmic 5'-3' exoribonuclease Xrn1p, decreases the rate of mRNA degradation and results in the accumulation of mRNAs to high levels (Larimer et al., 1992). In addition, the mRNAs which accumulate have short poly(A) tails, and are decapped (Hsu and Stevens, 1993). These observations not only demonstrated that Xrn1p likely degrades mRNAs, but also indicated that deadenylation and decapping most likely precede decay catalyzed by Xrn1p. Therefore, the analysis of the structures of mRNAs which accumulated in *xrn1* mutants facilitated the elucidation of the sequence of events in the major mRNA decay pathway in yeast. A second benefit of these analyses was the discovery of an additional mRNA decay pathway in yeast, the 3'-5' exosome-mediated decay pathway.

The exosome is a complex of multiple 3'-5' exoribonucleases involved in mRNA degradation and rRNA processing reactions (van Hoof and Parker, 1999). There are orthologs of exosome components in Arabidopsis and mammals, indicating that mRNA degradation catalyzed from the 3' end by the exosome may be conserved in eukaryotes (Gutiérrez et al., 1999; Mitchell et al., 1997). The role of the exosome in mRNA decay was discovered when the expression of poly(G)-containing genes was analyzed in yeast cells mutant for components of the deadenylation-dependent-decapping pathway. Expression of poly(G)-containing genes in wildtype yeast results in the accumulation of

an mRNA decay intermediate which begins at the poly(G) tract and ends at the poly(A) tail (Muhlrad et al., 1994). Deletion of the decapping enzyme gene *DCP1*, results in a decrease in the amount of this mRNA decay intermediate (Jacobs-Anderson et al., 1998). However, a new a poly(G)-stabilized mRNA decay intermediate, consistent with degradation from the 3' end, accumulates to high levels. It was subsequently found that a small amount of this intermediate can be detected in wildtype yeast (Jacobs-Anderson et al., 1998). This insight led to the discovery of the 3'-5' mRNA decay pathway of yeast which is less active than the 5'-3' pathway, but is easily detectable when degradation from the 5' end is reduced. Thus, analysis of mutants of the basal mRNA decay machinery facilitated the elucidation of the mechanism of the major mRNA decay pathway as well uncovered additional mRNA decay pathways.

AtXRN4, a cytoplasmic exoribonuclease, may be part of a basal mRNA decay machinery in Arabidopsis. Analysis of mutants of *AtXRN4* might allow for its role in mRNA decay to be examined, and could also facilitate the discovery of new mRNA degradation pathways. Recently, a collection of T-DNA insertion mutants of *Arabidopsis thaliana* has been generated (Krysan et al., 1999). This collection can be screened by PCR to identify T-DNA insertion mutants of genes of interest. Through PCR screening of this population, multiple insertions were found in each of the *AtXRN* genes. Due to AtXRN4's potential role as an mRNA degrading enzyme, RNA metabolism in an *xrn4* mutant was characterized to the greatest extent by a combination of primer extension, northern blot, and DNA microarray. The results of these analyses indicate that AtXRN4 is likely encoded by a non-essential gene, as homozygous *xrn4* T-DNA insertion mutants could be obtained. Preliminary analysis of mRNA decay in an *xrn4* mutant indicates that

the decay of three mRNAs may be impaired in the mutant, and that AtXRN4 could play a role in the degradation of these transcripts in Arabidopsis.

RESULTS

Identification of T-DNA insertion alleles of the *AtXRN* genes

The T-DNA insertion population which was screened for *xrn* mutants was generated by infiltration of thousands of wildtype Arabidopsis plants with a T-DNA vector that confers resistance to kanamycin (Krysan et al., 1999). Genomic DNA isolated from pools of the infiltrated plants was screened by PCR for T-DNA insertions in the *AtXRN* genes by the Arabidopsis Functional Genomics Consortium (AFGC; http://afgc.stanford.edu/). Two rounds of PCR were used to identify a sub-pool made up of 25 pools of 9 plants (referred to as "J pools"). These pools were obtained from the Arabidopsis Biological Resource Center (http://aims.cps.msu.edu/aims/) and single plants harboring the T-DNA inserts of interest were identified. Figure 4-1 A shows a schematic diagram of the T-DNA alleles found for each of the *AtXRN* genes. Table 1 summarizes the position of each of the T-DNAs relative to the start codon, the intron/exon borders, as well as whether single plants harboring each of the *xrn* alleles have been identified. In all cases, the T-DNA insertions are found within the *AtXRN* coding regions, a position which might allow for transcripts to be produced from the mutant locus. However, should the mutated *xrn* genes get expressed, truncated AtXRN proteins lacking the C-terminus would be generated due to the intervening T-DNA sequence. Based on deletion analysis of Xrn1p of yeast, it is likely that these truncated proteins would be inactive; small

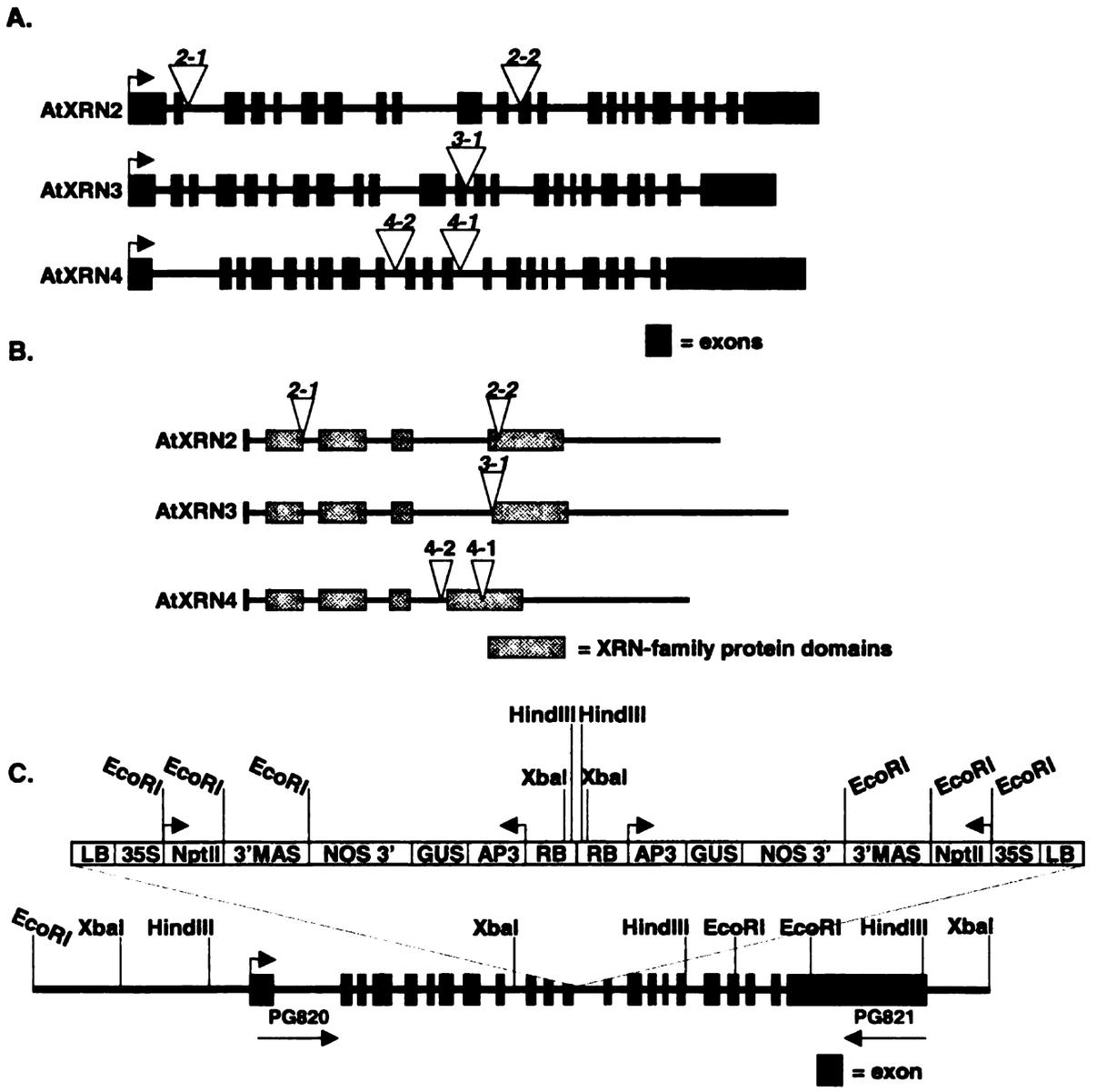


Figure 4-1. Schematic diagram of T-DNA insertions in the *AtXRN* genes and proteins. (A) The location of each of the T-DNAs in the *AtXRN* genes is indicated by a triangle above which the name of the allele is given. Exons are indicated by black boxes. (B) The position of the T-DNAs in the AtXRN proteins is indicated to show the amount of the protein which could be synthesized in each of the mutant backgrounds. Gray boxes indicate regions conserved in the XRN-family of proteins. (C) Detailed diagram of the *xrn4-1* allele showing the position of restriction enzyme cleavage sites. At least two copies of the T-DNA are present in this insertion. LB=left border, RB=right border, Mas 3'= mannopine synthase terminator, NptII=neomycin phosphotransferase, 35S=35 S promoter, Nos 3'=nopaline synthase terminator, GUS= β glucuronidase, AP3=portion of the APETELA3 promoter, PG820 and PG821=primers used to screen the insertion collection.

deletions of the C-terminus of Xrn1p abolish exoribonuclease activity (Page et al., 1998). Therefore, it is likely that plants homozygous for each T-DNA insertion would lack the exoribonuclease activity of the corresponding AtXRN protein.

AtXRN4's cytoplasmic localization is consistent with a role in mRNA degradation (Chapter 3), and plants with a mutation in the *AtXRN4* gene may be defective in mRNA decay. To examine this possibility, plants harboring the *xrn4* alleles were further characterized. The most progress has been made on the *xrn4-1* allele. Experiments on plants harboring the *xrn4-2* allele have been prevented due to the fact

Table 4-1
Position of T-DNA insertions in *AtXRN* genes

The position of each of the T-DNAs relative to the start codon, and whether the insertion is in an intron or an exon is shown. The identification of single plants from the J-pools is indicated

Allele	Distance from ATG	intron/exon	Single plant
xrn2-1	+553	intron	yes
xrn2-2	+4159	exon	yes
xrn3-1	+3493	intron	yes
xrn4-1	+3592	intron	yes
xrn4-2	+2915	intron	yes

that the generation of this insertion was likely accompanied by a chromosomal deletion. While uncommon, T-DNA insertion sometimes results in large scale genomic rearrangements and deletions of the chromosomal DNA flanking the insertion point; such deletions are sometimes lethal when homozygous (Krysan et al., 2000). The lethality of chromosomal deletions due to T-DNA insertion could result from loss of individual

essential genes, or the simultaneous deletion of a large numbers of genes may be lethal. Based on PCR, chromosomal DNA 5' of the *xrn4-2* insertion point was likely deleted. Although it was not possible to determine the extent of the deletion, plants homozygous for the *xrn4-2* mutation could not be recovered, indicating that the deletion is likely lethal when homozygous. However, as described below, it was possible to obtain homozygous *xrn4-1* plants, indicating that the lethality of the *xrn4-2* mutation is unlikely due to the mutation of the *AtXRN4* gene. Figure 4-1 C shows a schematic diagram of the characteristics of the *xrn4-1* locus determined by PCR (data not shown) and Southern blot analysis (Figure 4-2 A). At least two T-DNAs are present at this locus with the left border facing both the 5' and 3' ends of the *AtXRN4* gene.

Isolation of *xrn4-1* homozygotes

A plant containing the *xrn4-1* mutation was identified in J pool 2902 plant #44, by PCR screening with the internal AtXRN4 primer PG820 shown in Figure 1-1 C, and the T-DNA left border primer JL-202. PCR analysis indicated that this plant was heterozygous for the *xrn 4-1* mutation (data not shown). The pollen from this plant was used in a cross to wildtype, and the progeny of this cross (F1) were selected on kanamycin. The kanamycin resistant seedlings were transferred to soil, grown, and allowed to set seed (F2). F2 seeds were plated on kanamycin containing medium and scored for resistance. As expected for a single insert, the majority of F2 plants segregated 3:1 for kanamycin resistance. The progeny of one plant segregated in a ratio of approximately 5:1 indicating an additional T-DNA(s) was segregating in this plant. To identify homozygous *xrn4-1* plants, 50 kanamycin resistant F2 seedlings of a population

segregating approximately 3:1 for kanamycin resistance (269 resistant:88 sensitive) were screened by PCR using three primers PG820, PG821 (shown in Figure 4-1 C) and the T-DNA left border primer JL-202. PCR analysis of 30 of the kanamycin selected F2 plants revealed the presence of *xrn4-1* heterozygous and homozygous plants in a ratio of approximately 2:1 (data not shown).

A genomic Southern blot was carried out on DNA isolated from the progeny of an F2 plant (F3) determined to be an *xrn4-1* homozygote by PCR. Southern blot analysis confirmed that the F3 plants were homozygous for the *xrn4-1* mutation (Figure 4-2 A). The mobility of the bands detected with an AtXRN4-specic probe indicated that at least

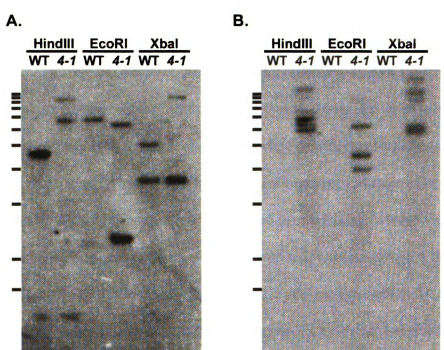


Figure 4-2. Southern blot analysis of wildtype (WT) and *xrn4-1* plants. 10μg of genomic DNA was digested with the restriction enzymes indicated above each lane. The digested DNA was separated with a 0.8% agarose gel and blotted to membrane. (A) The membrane was hybridized with probes complementary to the *AtXRN4* gene. (B) The same blot as in A was stripped and hybridized with probes complementary to the NOS 3' terminator. The lines to the left of the blots indicate the migration of molecular weight markers: 11kb, 10kb, 9kb, 8kb, 7kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1.6kb and 1.0kb.

two T-DNAs are present in the *xrn4-1* locus. For example, cleavage of DNA isolated from the *xrn4-1* plants with *Hind*III, with cleaves on both sides of the T-DNA insertion and in the right border sequence, (see Figure 4-1 for position of *Hind*III sites) resulted in bands of approximately 9500b, 6500 bp and 1300 bp (Figure 4-2 A). The total mass of these bands (approximately 17300 bp) is consistent with at least two of the approximately 5 kb T-DNAs inserted into the 7.0 kb *AtXRN4* gene. Multiple T-DNAs inserted at one position is quite common, and has been found in additional T-DNA mutants, including the *xrn2-1* allele (data not shown). Hybridizing the Southern blot with a probe specific for the NOS terminator present in the T-DNA revealed a more complex pattern than expected for two T-DNAs inserted at the *xrn4-1* locus. It is likely that additional T-DNA-derived sequence lies between the right borders shown in Figure 4-1 C. Consistent with this possibility is the fact that no PCR products were generated when primers complementary to the GUS coding region, which were designed to amplify across the right borders, were used. This region likely contains sequence too large to be amplified. A second possibility is that an additional T-DNA(s) is segregating in these *xrn4-1* seedlings. However, the parental plant (F2) was selected from a population that was segregating in a ratio of 3:1 for kanamycin resistance, the expected result for a single insertion. A final possibility is that there are fragments of the T-DNA which lack the kanamycin resistance cassette inserted elsewhere in the genome and these fragments were not detected when the kanamycin segregation ratios were calculated.

Less than full-length AtXRN4 transcripts are expressed in *xrn4-1* seedlings

Since the *xrn4-1* T-DNA insertion is distal to the *AtXRN4* promoter it was likely that transcription from the *xrn4-1* locus would produce transcripts containing the 5' end of the AtXRN4 transcript fused to T-DNA derived sequence. To examine this possibility, RNA isolated from wildtype seedlings and from the progeny of a *xrn4-1* heterozygote, which were selected on kanamycin, was analyzed by northern blot. Multiple smaller than full-length bands were detected in the RNA from the *xrn4-1* plants but not in RNA isolated from the wildtype plants (Figure 4-3). The less than full-length bands present in the *xrn4-1* plants are likely due to aberrant splicing of transcripts containing the 5' end of AtXRN4 and portions of T- DNA derived sequence. These bands may arise if sequences transcribed from the T-DNA contain cryptic splice sites. Differential use of these sites might result in splicing of the AtXRN4-T-DNA fusion transcripts in several different places. Consistent with this hypothesis, an RT-PCR product of expected size can be generated with a primer which anneals in the 5' end of the AtXRN4 transcript, PG821,and a primer which anneals in the left border sequence of the T-DNA, JL-202 (data not shown). Sequencing of the end of this product confirmed that it corresponds to an AtXRN4 transcript fused to sequence derived from the left border of the T-DNA and also revealed that a stop codon in-frame with the AtXRN4 open reading frame lies immediately downstream of the AtXRN4-T-DNA junction. The results of RT-PCR with the left border primer and a primer which anneals in the 3' end of the AtXRN4 transcript indicates that several products are likely produced which include AtXRN4 sequence lying downstream of the T-DNA insertion (data not shown).

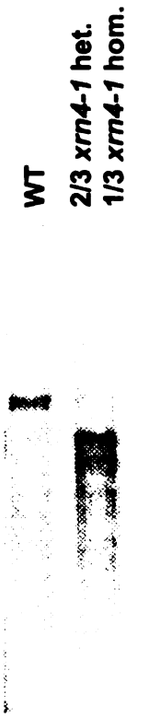


Figure 4-3. Less than full-length AtXRN4 transcripts are expressed in *xrn4-1* seedlings. 1 μg of poly(A) enriched RNA from wildtype and the progeny of an *xrn4-1* heterozygote was separated with a 1% agarose/formaldehyde gel, transferred to membrane, and hybridized with probes specific for the 3' end of AtXRN4.

Full-length AtXRN4 transcript is not detected in homozygous *xrn4-1* seedlings

Since the *xrn4-1* insertion resides in an intron, it was possible that the T-DNAs might be spliced out of transcripts generated from this locus, and that full-length AtXRN4 transcript might be expressed in this mutant. While northern blot analysis of *xrn4-1* heterozygotes indicated that full-length AtXRN4 transcript was likely greatly reduced due to the T-DNA insertion (Figure 4-3), it remained possible that a small amount of full-length transcript might be synthesized in the mutant. Since a small amount of full-length transcript might not be detected by northern analysis, RT-PCR, a more sensitive technique, was employed. Total RNA was isolated from the leaves of

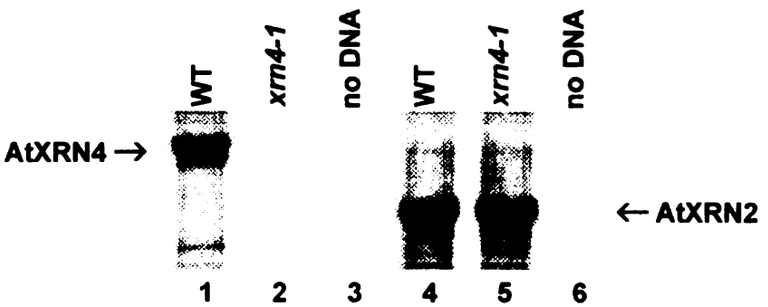


Figure 4-4. Full-length AtXRN4 transcripts are not detected in *xrn4-1* homozygotes. RT-PCR was carried out on total RNA from wildtype and *xrn4-1* homozygous plants. AtXRN4-specific primers PG820 and PG821 (shown in Figure 4-1 C) were used in lanes 1-3 and AtXRN2-specific primers PG840 and PG841 were used in lanes 4-6. The PCR products were separated with a 1% agarose gel and transferred to membrane. The membrane was hybridized with probes complementary to AtXRN2 and AtXRN4.

wildtype and *xrn4-1* plants and used to generated cDNAs. These cDNAs were used in a PCR reaction with primers PG820 and PG821. These primers anneal in the 5' and 3' ends of the *AtXRN4* gene on opposite sides of the T-DNA (shown in Figure 4-1 C). These primers gave a robust PCR product when used with cDNAs generated from wildtype plants (Figure 4-4 lane 1). In contrast, these primers did not yield PCR products when used with cDNAs generated from *xrn4-1* homozygotes (Figure 4-4 lane 2). As a control for the quality of the cDNAs, RT-PCR was carried out using primers specific for the AtXRN2 transcript. Similar amounts of this product were seen in samples from wildtype and *xrn4-1* homozygous mutants indicating that the inability to detect RT-PCR products corresponding to full-length AtXRN4 transcript in *xrn4-1* plants was not due to poor cDNA quality (Figure 4-4, lanes 4 and 5). The preceding analysis indicated that full-length AtXRN4 transcripts are likely absent in *xrn4-1* plants, and that these mutants would be suitable for further analyses.

Phenotypic Characterization of *xrn4-1* homozygotes

The vector used to generate the T-DNA insertion collection contains a few hundred bases of the *APETELA3* promoter (Krysan et al., 1999; shown as AP3 in Figure 4-1 C). Plants containing this T-DNA have been reported to undergo silencing of the endogenous *APETELA3* gene (Krysan et al., 1999) a homeotic gene involved in the development of sepals and stamens (Jack et al., 1992). The morphology of flowers on plants homozygous for the *xrn4-1* mutation is indicative of *APETELA3* silencing. Similar to petals from plants mutant for *APETELA3*, the petals of homozygous *xrn4-1* plants are similar in morphology to sepals, but in contrast to sepals, have white regions at their tops (data not shown and Jack et al., 1992). *APETELA3* is also involved in stamen development and strong *APETELA3* mutant alleles are male sterile (Jack et al., 1992). Consistent with silencing of *APETELA3*, the fertility *xrn4-1* homozygotes was greatly reduced, but not completely abolished (data not shown). Since the phenotypes apparently due to *APETALA3* silencing-dependent morphological phenotypes were only detected in mature plants, the search for molecular phenotypes was restricted to two-week old *xrn4-1* seedlings with the hope that any phenotypes detected would be as a result of mutation of *AtXRN4* and not *APETELA3* silencing.

Preliminary Analysis of mRNA decay in *xrn4-1* seedlings using cDNA microarrays

A likely phenotype for the *xrn4-1* mutation is impaired degradation of mRNAs. However, since *xrn4-1* plants appear to have no aberrant morphological phenotypes (other than the *APETELA3* silencing-associated phenotype mentioned above), it seemed unlikely that large numbers of transcripts would be mis-expressed in the mutant, and that microarray analysis might aid in identifying a small number of transcripts elevated in the

xrn4-1 mutant. Since the reduction in fertility due to *APETELA3* silencing limited the amount of homozygous mutants that could be analyzed, a more crude analysis was undertaken. mRNA degradation in wildtype plants was compared to degradation in the progeny of an *xrn4-1* heterozygote selected on kanamycin. In this experiment wildtype is compared to plants of which 2/3 are *xrn4-1* heterozygotes and 1/3 are homozygotes. Due to the crude nature of this experiment the hybridization was limited to a single slide. While four slides are normally used to account for technical variation, it was hoped that a few transcripts which were the most elevated in *xrn4-1* seedlings could be detected and that these might be substrates of AtXRN4.

To compare mRNA degradation in wildtype and *xrn4-1* seedlings, a cordycepin time course experiment was carried out as described (Johnson et al., 2000). Two week old seedlings were transferred to liquid medium, equilibrated for 30 minutes in buffer, followed by the addition of the transcription inhibitor cordycepin to a final concentration of 150 μg/ml. Seedlings were collected after 0 and 120 minutes of incubation with cordycepin. Total RNA was isolated from the 120 minute samples and used to generate probes that were hybridized to the AFGC 11 K microarray (Schaffer et al., 2000). The AFGC array contains approximately 11,000 clones from the PRL2 library (Newman et al., 1994). The 120 minute samples were compared because a greater difference in transcript abundance due to differences in degradation rates would be expected to exist between these samples rather than between samples at steady-state. For example, if an mRNA is stabilized two-fold in the *xrn4-1* mutant compared to wildtype, its abundance at steady state would be two-fold higher in the mutant (assuming that the transcription rate is unaffected by the *xrn4-1* mutation). While a two-fold difference can be detected on a

microarray, it is close to the limit of detection. However, after a 120 minute treatment with cordycepin, the difference in degradation rates of the two mRNAs would result in a greater than two-fold difference in abundance. Thus, it was predicted to be easier to detect subtle changes in mRNA stability by comparing differences in abundance of transcripts after the 120 minute of cordycepin treatment rather than at steady-state.

As expected, the abundance of the vast majority of transcripts was unchanged in the mutant relative to wildtype after the 120 minute cordycepin treatment. However, approximately 130 transcripts were detected as elevated greater than two-fold in the *xrn4-1* mutant relative to the wildtype, while a similar number were detected as reduced greater than two-fold. The 10 most elevated and the 10 most reduced transcripts in *xrn4-1* seedlings are shown in Tables 4-2 and 4-3, respectively. If representative of true differences, the change in abundance of these transcripts in *xrn4-1* seedlings could arise from either changes in transcription rate, or changes in stability. To distinguish between these possibilities, the decay kinetics of several of these transcripts was compared in mutant and wildtype using northern blots. As northern blots do not require as much RNA as microarray analysis, it was feasible to examine the expression of these transcripts in the small number of homozygous *xrn4-1* seedlings which could be obtained.

The cordycepin time course was repeated on wildtype and *xrn4-1* homozygous seedlings. Total RNA was isolated from the 0 and 120 minute time points, and the decay of several transcripts which were detected as elevated in *xrn4-1* seedlings on microarrays was examined by northern blot. Since transcript elevation is the most likely primary effect of mutation of *AtXRN4*, northern blot analysis was used to examine transcripts which were elevated in the mutant. A reduction in transcript abundance is most likely

Table 4-2. Transcripts elevated in *xrn4-1* seedlings detected by microarrays

The 10 most-elevated transcripts in *xrn4-1* seedlings are indicated. The normalized ratio of signal intensity is shown, as well as annotation from TIGR (http//:www.tigr.org)

clone name	*xrn4-1*/wt at t=120	annotation
214A6T7	5.4	2s storage protein-like
123N21T7	4.3	12s cruciferin seed storage protein (atcru3)
126C19XP	4.1	putative auxin-regulated protein
136E11T7	3.8	12s seed storage protein precursor
124N17T7	3.2	lipid transfer protein, putative
171N2T7	3.2	putative glucose regulated repressor protein
201A11T7	3.2	putative protein
176K14T7	3.0	not assigned
115F4T7	2.9	not assigned
107I3T7	2.8	isocitrate lyase

due to a secondary effect of the *xrn4-1* mutation, and transcripts whose abundance decreased have not yet been examined by northern blot. Two transcripts, 214A6T7, annotated as encoding a 2S seed storage protein and 124N17T7, annotated as encoding a putative lipid transfer protein (annotation from TIGR, http://www.tigr.org/), appear to be elevated in *xrn4-1* plants and to decay more slowly than in the wildtype (Figure 4-5). The apparent stabilization of these transcripts in *xrn4-1* seedlings is between 3-5 fold, a value similar to the degree of mRNA stabilization seen in yeast when 5'-3' decay is inhibited by deletion of the *XRN1* or *DCP1* genes (Larimer and Stevens, 1992; Beelman et al., 1996). The fact that the elevation of the 214A6T7 and124N17T7 transcripts in the *xrn4-1* mutant appears to be due to increased stability indicates that they may be direct targets of AtXRN4 and that their decay warrants further study.

Table 4-3. Transcripts reduced in *xrn4-1* seedlings detected by microarrays

The 10 most-reduced transcripts in *xrn4-1* seedlings are indicated. The normalized ratio of signal intensity is shown, as well as annotation from TIGR (http//:www.tigr.org)

clone name	*xrn4-1*/wt at t=120	annotation
196M20T7	0.29	glycine-rich protein, AtGRP-5
123M2T7	0.29	similar to protein kinase 1
206B23T7	0.28	unknown protein
40B8T7	0.28	putative ABC transporter
247A3T7	0.28	beta-N-acetylhexosaminidase-like protein
196J20T7	0.27	ATP dependent copper transporter
222A14T7	0.26	probable imbibition protein - wild cabbage
176A10T7	0.25	glucosyltransferase like protein.
G7H8T7	0.18	hypothetical 213.7 KD protein YCF1
H5D4T7	0.16	contains similarity to heat shock protein

One additional transcript appears to decay in the *xrn4-1* mutant in a manner that differs from wildtype. The EST 171N2T7 encodes a protein that is annotated as a putative glucose regulated repressor, and will be referred to as PGRR. Northern blot analysis of the cordycepin time course with probes generated from the 171N2T7 clone was carried out and is shown in Figure 4-6. While the PGRR transcript is elevated in the mutant, it appears to decay at a similar rate in both the *xrn4-1* mutant and in wildtype, with a half-life of approximately 85 minutes. This indicates that its elevation in *xrn4-1* seedlings may be due to an increase in its transcription rate. However, an additional band could be detected in the RNA isolated from the *xrn4-1* mutant (indicated by the arrow in

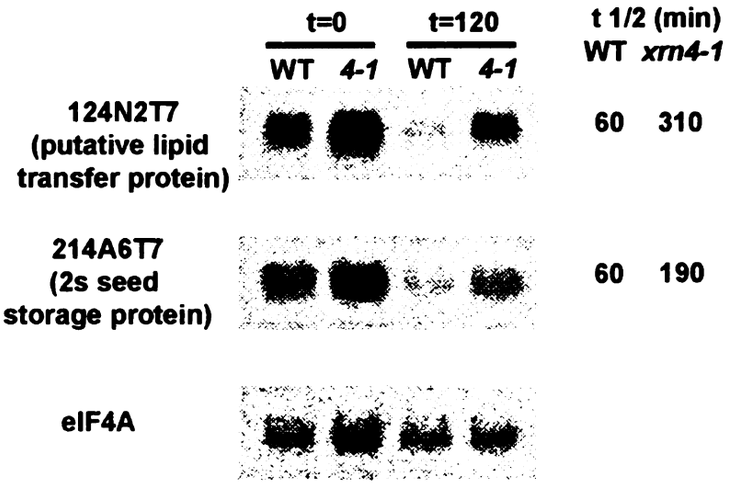


Figure 4-5. Two transcripts detected as elevated in *xrn4-1* seedlings by microarray appear to be stabilized 3-5 fold. Total RNA was isolated from wildtype and *xrn4-1* seedlings of 0 and 120 minute time points of a cordycepin time-course. 10 μg of each sample was separated with a 1% agarose/formaldehyde gel and blotted to membrane. The membrane was hybridized with the probes indicated on the left. eIF4A served as a loading control and the half-live values shown at the right were calculated normalized to eIF4A.

Figure 4-6). This band can also be detected in wildtype plants, but only at the zero time point of the cordycepin time course, indicating that it likely decays rapidly in wildtype seedlings (Figure 4-6, compare lanes 1 and 3). In contrast, the abundance of this band does not change appreciably in *xrn4-1* seedlings over the time course (Figure 4-6, compare lanes 2 and 4). While the source of this additional band is not yet clear, it might represent an mRNA decay intermediate which accumulates in the *xrn4-1* mutant. In addition to this putative mRNA decay intermediate, a 'smear' can be seen which extends from the full-length down to the mobility of the putative intermediate. This smear can be seen most clearly in the RNA from the mutant but can also be detected in RNA from

wildtype seedlings (Figure 4-6, compare lanes 1 and 2). Over the cordycepin time-course this smear decreases in intensity, which is likely due to its degradation.

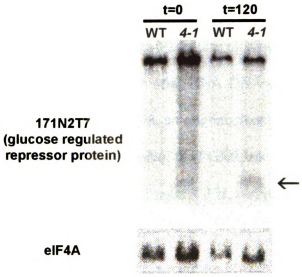


Figure 4-6. The decay of the PGRR transcript is accompanied by the accumulation of an additional band in *xrn4-1* seedlings. Total RNA was analyzed as described in Figure 4-5 and hybridized with probes generated from the EST 171N2T7, and eIF4A as a loading control. The arrow indicates the additional band detected in *xrn4-1* seedlings.

To characterize the putative mRNA decay intermediate that accumulates in *xrn4-1* seedlings it was further analyzed by northern blot. Based on an alignment of the sequence of the 171N2T7 clone with the *PGRR* gene, this clone corresponds to the 3' end of the *PGRR* gene. Since probes generated from 171N2T7 detected the putative mRNA decay intermediate, it seemed likely that the putative intermediate corresponded to the 3' end of the transcript. To examine this possibility, northern blot analysis was carried out with a probe that anneals towards the 5' end of the transcript. The EST 188L22T7 includes sequence complementary to the 5' end of the *PGRR* gene. A portion of this EST, which is predicted to anneal approximately 500 nt 5' of where probes generated

from 171N2T7 hybridize, was used for northern analysis. As seen in Figure 4-7 A, these probes (probe 1 in Figure 4-7 A) do not detect the additional band in the *xrn4-1* mutant. This result indicates that the additional band likely corresponds to the 3' end of the PGRR transcript. To further investigate this possibility the ability of the putative mRNA decay intermediate to be cleaved with a complementary oligonucleotide and RNase H was examined. RNase H degrades the RNA of an RNA-DNA duplex. Total RNA from the *xrn4-1* mutant was incubated with PG978, an oligonucleotide complementary to a region approximately 300 bp from the 3' end of the PGRR transcript, and RNase H. This resulted in cleavage of the additional band (Figure 4-1 B, compare lanes 3 and 4), indicating that it most likely corresponds to the 3' end of the PGRR transcript. A final observation about the structure of this putative mRNA decay intermediate is that it likely has no poly(A) tail, or at most a short poly(A) tail. Removal of the poly(A) tail *in vitro* using oligo dT and RNase H should result in an increase in the mobility of the putative intermediate if it were polyadenylated. However, treatment with RNase H and oligo dT had no apparent effect on the mobility of the putative decay intermediate (Figure 4-7 B compare lanes 3 and 5). Figure 4-7 B also shows more clearly than Figure 4-6 that a small amount of the putative mRNA decay intermediate can be detected in RNA from wildtype plants.

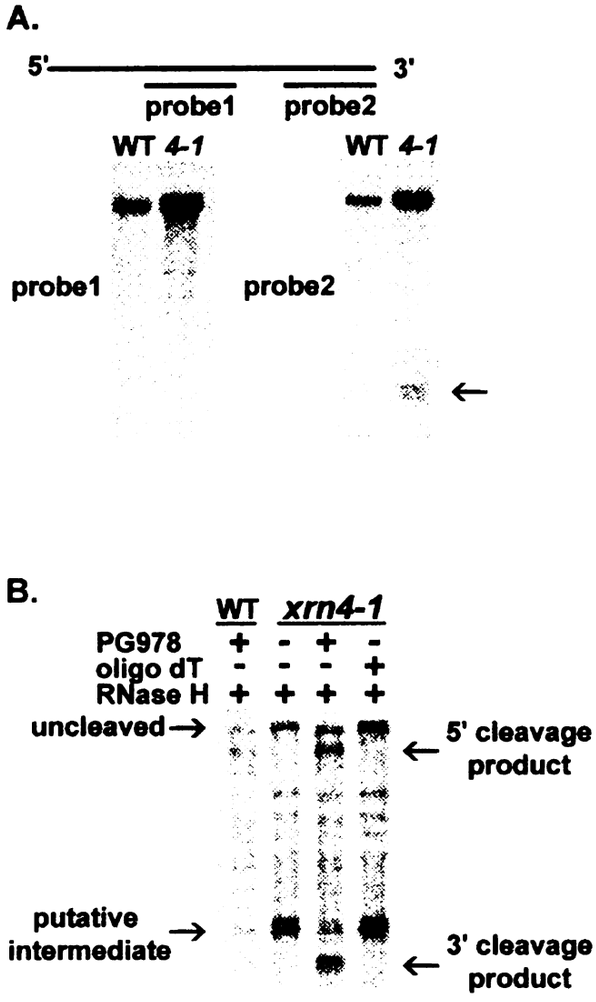


Figure 4-7. The additional band detected in *xrn4-1* seedlings corresponds to the 3' end of the PGRR transcript. (A) Northern blot analysis of RNA from wildtype and *xrn4-1* plants with probes that hybridize in the middle of the PGRR transcript (probe1) and probes complementary to the 3' end (Probe 2).Total RNA from the rosette leaves of mature wildtype and *xrn4-1* plants was analyzed as described in Figure 4-5. The arrow indicates the putative 3' mRNA decay intermediate. (B) RNase H cleavage of RNA from wildtype and *xrn4-1* rosette leaves. 20μg of total RNA was incubated with the oligonucleotides indicated at the top in the presence of RNaseH. The RNase H cleavage reactions were separated with a 2% agarose/formaldehyde gel and blotted to membrane. The membrane was hybridized with probes complementary to the entire PGRR transcript. PG987 is complementary to the 3' end of the PGRR transcript

MATERIALS AND METHODS

PCR screening for T-DNA insertion mutants

Primers for each of the AtXRNs were selected using the suggested parameters described by the AFGC knockout facility (http://www.biotech.wisc.edu/Arabidopsis/). The primers used to screen for insertions in the AtXRN genes are described in table 4-4.

Table 4-4. Primers used to screen for T-DNA insertions in the *AtXRN* genes

gene	primer name	primer sequence
AtXRN2	PG840:	5'-TTGATAGGCTTTTTGTTATGGTTCGACCT-3
	PG841:	5'-GTTTTATCATTTTCGCCTCTTCCATCATG-3
AtXRN3	PG842:	5'-CCAAGAATCATAATTTCTGCCGGAATAGA-3'
	PG843:	5'-TGAGCCAAAGCCTTTGACGTGTTTATAGT-3'
AtXRN4	PG820:	5'-ATACCCGAAGTCAATTAGTGACGTCGTTG-3'
	PG821:	5'-TGGACTACTGTTCATGACGAATTCCTTTG-3'.

The AFGC used these primers in combination with T-DNA border primers to screen superpools of DNA for insertions in the *AtXRN* genes by PCR. Following the identification of superpools containing insertions in the *AtXRN*s, the positions of the insertions were determined by sequencing of the PCR products. The insertions lying closest to the 5' end of each of the *AtXRN* genes were selected for further screens. Secondary PCR screens were carried out by the AFGC to identify the sub-pools containing DNA with the *xrn* insertions. Following this PCR, seeds were obtained corresponding to 25 pools of seeds from nine plants which had been used to generate the DNAs used in the second round (J-pools). These seeds were planted, and J-pools with each of the *xrn* alleles were identified. To identify single plants with the *xrn* alleles individual leaves were harvested from J-pool plants and analyzed by PCR. DNA was

isolated using an abbreviated CTAB method. Individual leaves were freeze-dried and ground to powder by shaking with 2.5 mm zirconia/silica beads (Biospec Products). The powered leaves were incubated in 500 µl of CTAB buffer (Saghai-Maroof, et al., 1984) for 30 minutes at 68 °C, chloroform extracted, and the DNA was ethanol precipitated. The PCR conditions were identical to those described by the AFGC website. Southern blots of 5 µl of the PCR products were used to confirm their identity.

Northern blot analysis

Total RNA was isolated from wildtype and *xrn4-1* Arabidopsis plants (WS) plants as described (Newman et al, 1993). Poly(A) enriched RNA was obtained with an Oligotex kit and used to compare *AtXRN4* expression in wildtype and *xrn4-1* homozygotes (Qiagen). 10 µg of total RNA was used for the comparison of mRNA decay in wildtype and *xrn4-1* homozygotes. The RNA was separated with 1% agarose gels, transferred to nylon membrane and hybridized with random primed probes.

RT-PCR

Total RNA isolated from single leaves of wildtype and *xrn4-1* plants was reverse transcribed with Superscript II (Gibco) and PCR amplified with primers PG820 and PG821 to detect AtXRN4, and PG840 and PG841 to detect AtXRN2 using the AFGC PCR protocol. The PCR products were analyzed by Southern blot.

Southern blot analysis

Genomic DNA was isolated from wildtype and *xrn4-1* homozygous mutants using the CTAB method (Saghai-Maroof et al., 1984). 10 μg of DNA was incubated with the restriction endonucleases indicated in Figure 4-2, separated with a 0.9% agarose gel and transferred to a Nytran membrane. To detect *AtXRN4*, the blot was hybridized with probes generated from the PCR product of PG820 and PG821. To detect the T-DNA, probes generated from the NOS 3' end were used.

Microarray analysis

Total RNA was isolated from wildtype and the progeny of an *xrn4-1* heterozygote selected on kanamycin after the 120 minute treatment with cordycepin. 100 μg of this RNA was reverse transcribed using an oligo dT primer in the presence of Cy dyes to generate the microarray probes as described (Schaffer et al., 2001). The cordycepin time course was carried out on two-week old seedlings using 150 μg/ml of cordycepin as previously described (Johnson et al., 2000). Microarray data was normalized as described (Schaffer et al., 2001).

DISCUSSION

Microarray analysis of *xrn4-1* seedlings resulted in the identification of two transcripts which were apparently stabilized in the *xrn4-1* mutant, and a third transcript whose decay may give rise to an mRNA decay intermediate in *xrn4-1* plants. These

results provide support for the hypothesis that the cytoplasmic exoribonuclease AtXRN4 could function in the decay of some mRNAs.

The apparent stabilization of the 214A6T7 and 124N17T7 transcripts in *xrn4-1* seedlings is consistent with AtXRN4 functioning in a mRNA decay pathway similar to the major deadenylation-dependent-decapping pathway of yeast. However, a more detailed analysis of the structures of these transcripts in *xrn4-1* seedlings would be required to address this possibility, and could shed light on the pathway by which these mRNAs are degraded. In yeast, deletion of the *XRN1* gene, which encodes the cytoplasmic 5'-3'exoribonuclease, results in the accumulation of mRNAs which have shortened poly(A) tails, and which are decapped (Hsu and Stevens, 1993). These changes in structure would likely not be observed with standard northern blots and require closer examination. If the two transcripts which are apparently stabilized in *xrn4-1* seedlings are also decapped and have short poly(A) tails, this would indicate that they might be degraded by a pathway that resembles the deadenylation-dependent-decapping pathway of yeast. If they retain the cap or a long poly(A) tail this would likely point to a different mechanism. With respect to the poly(A) tail, it seems likely that the elevated transcripts retain at least a short poly(A) tail. The probes used to hybridize the microarray were generated by reverse transcription of total RNAs using an oligo dT primer. In order for the transcripts which were elevated in the *xrn4-1* mutant to have been detected as elevated on microarrays, they would have had to have at least enough poly(A) tail to be efficiently primed with oligo dT. It is possible that additional transcripts which are degraded by AtXRN4 might not have been detected by the microarray if they accumulated with very short poly(A) tails and were inefficiently

reverse transcribed. It may be possible to hybridize the microarray with randomly primed probes generated from *xrn4-1* plants to detect these species.

The decay of the PGRR transcript, which is accompanied by the accumulation of the 3' end of the transcript in *xrn4-1* seedlings, may indicate that AtXRN4 participates in an mRNA decay pathway which differs from the major pathway of yeast. This putative intermediate appears to be present at low levels in wildtype plants but is more abundant in the *xrn4-1* mutant (Figure 4-6) and might represent a natural mRNA decay intermediate that is stabilized due to mutation of *AtXRN4*. The decay kinetics of the PGRR transcript indicates that the mechanism of its decay may differ from the 124N2T7 and 214A6T7 transcripts, the transcripts which are apparently stabilized by the *xrn4-1* mutation. Impaired degradation following decapping would be expected to result in an increase in the stability of the full-length transcript. However, the full-length PGRR transcript decays in the *xrn4-1* mutant with similar kinetics as in wildtype (Figure 4-6). This could indicate that an early step in the degradation of this transcript is internal cleavage by an endoribonuclease. Mutation of *AtXRN4* would likely not affect the activity of such an endoribonuclease, and the full-length would decrease at the same rate in wildtype and the *xrn4-1* mutant. If the 3' product of the initial endoribonuclease cleavage is a substrate for AtXRN4, this product might accumulate in the *xrn4-1* mutant. An activity like the exosome, a complex of 3'-5' exoribonucleases which functions in 3'-5' mRNA decay in yeast (van Hoof and Parker, 1999), might degrade the 5' cleavage product. The putative mRNA decay intermediate can be detected at low levels in wildtype plants (Figure 4-7) which may indicate that it is inefficiently degraded in the wildtype by AtXRN4. The presence of a stable secondary structure in the 3' end of the

PGRR transcript might inhibit AtXRN4's progression through the PGRR mRNA, similar to the blockage of AtXRN4 by poly(G) tracts (Chapter 3).

FUTURE EXPERIMENTS

Additional characterization of *xrn4-1*

The analysis of mRNA decay in homozygous *xrn4-1* seedlings by microarray should be repeated to confirm previous results, as well as to identify additional candidates for AtXRN4 substrates. It is likely that many targets of AtXRN4 may not have been identified in the relatively crude experiment described. An additional important experiment with respect to the *xrn4-1* mutation is to determine if expression of wildtype AtXRN4 in *xrn4-1* seedlings can complement any of the phenotypes of the *xrn4-1* plants. This should help to confirm if the phenotypes observed are due to mutation of *AtXRN4*. A final experiment with respect to the *xrn4-1* mutation is to try to determine if this mutation causes loss of AtXRN4's exoribonuclease activity. A transcript consisting of the 5' end of AtXRN4 fused to sequences derived from the T-DNA can be detected in *xrn4-1* seedlings and a portion of the N-terminus of the AtXRN4 protein may be produced in AtXRN4 plants. However, it is unlikely that this truncated protein would have exoribonuclease activity as Xrn1p mutants in yeast with smaller deletions in the C-terminus are inactive (Page et al, 1998). Nevertheless, it should be possible to determine if the truncated AtXRN4 protein which could be expressed in *xrn4-1* plants has activity. This would most easily be accomplished by expressing the truncated AtXRN4 protein in an *xrn1Δ* yeast strain and analyzing the accumulation of poly(G)-stabilized mRNA decay intermediates as described for the AtXRNs in Chapter 3.

While more analyses of *xrn4-1* seedlings should be carried out, characterization of mRNA turnover with additional *xrn4* alleles should also facilitate the investigation of AtXRN4's role in mRNA degradation. Isolation of additional T-DNA alleles (from a T-DNA insertion population generated with a T-DNA that does not have *APETELA3* promoter sequence) is currently underway. In addition to T-DNA insertion mutants, plants with reduced levels of AtXRN4 transcript might also be examined. The expression of RNAs which are self-complementary and which are also complementary to an endogenous gene has been used to decrease the expression of several genes in Arabidopsis (Chuang; and Meyerowitz, 2000). These self-complementary RNAs are able to form 'panhandle' structures that are believed to trigger post-transcriptional gene silencing (Waterhouse, 1998; Smith et al., 2000). Expression of AtXRN4-panhandle RNAs could be used to reduce the expression of *AtXRN4* and the decay of mRNAs could be examined in these plants.

Characterization of the PGRR transcript to gain insight into mRNA decay mechanisms

Expression of the PGRR transcript under the control of a regulated promoter in plants compromised for AtXRN4 function should allow for several aspects of its decay to be addressed. Such an experiment should allow for the determination if the full-length PGRR transcript and its putative intermediate share a precursor-product relationship. This is an important step in demonstrating that the putative intermediate is generated from decay of the full-length. In addition, cloning and characterizing the sequence of this putative intermediate will useful in determining the mechanism of its production. Analysis of this putative mRNA decay intermediate could also shed light on AtXRN4 independent mRNA degradation. It is likely that the putative intermediate decays slowly

in *xrn4-1* seedlings, indicating that additional activities which can catalyze the degradation of this transcript are present in the cytoplasm of Arabidopsis cells. It may be possible to use genetics to identify proteins which could degrade this intermediate.

A second direction of future research on mRNA decay in *xrn4* mutants may yield insight into mRNA decay in general. While it appears that AtXRN4 may catalyze the degradation of some mRNAs, the decay of the majority of transcripts appears unaffected in *xrn4-1* seedlings. It is likely that several mRNA decay pathways function in Arabidopsis; however, the manner in which specific mRNAs would be targeted to particular mRNA decay pathways is unclear. As mentioned in Chapter 1, instability determinants which target transcripts for rapid degradation likely modulate components of the basal mRNA decay machinery. A comparison of the sequences of all transcripts stabilized in *xrn4* mutants may reveal common sequence or structural motifs required for AtXRN4-specific degradation. It may be possible to identify sequence elements which regulate AtXRN4's activity, or are involved in directing AtXRN4 activity. This could provide important information about how AtXRN4 is regulated.

REFERENCES

Beelman, C. A., Stevens, A., Caponigro, G., LaGrandeur, T. E., Hatfield, L., Fortner, D. M., and Parker, R. (1996). An essential component of the decapping enzyme required for normal rates of mRNA turnover. Nature **382**, 642-646.

Chuang, C.F. and Meyerowitz, E.M. (2000) Specific and heritable genetic interference by double-stranded RNA in Arabidopsis thaliana. Proc. Natl. Acad. Sci.USA **97**, 4985-4990.

Gutiérrez, R. A., MacIntosh, G. C., and Green, P. J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci. **4**, 429-438.

Henry, Y., Wood, H, Morrissey, J. P., Petfalski, E., Kearsey S, and Tollervey, D. (1994). The 5' end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. EMBO J. **13**, 2452-2463.

Hsu, C. L. and Stevens, A. (1993). Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. Mol.Cell.Biol. **13**, 4826-4835.

Jack, T., Fox, G. L., and Meyerowitz, E. M. (1994). Arabidopsis homeotic gene APETALA3 ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. Cell **76**, 703-716.

Jacobs-Anderson, J. and Parker, R. (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J. **17**, 1497-1506.

Johnson, M. A. (2000). Genetic Determinants of mRNA Stability in Plants. Ph.D. dissertation. Michigan State University.

Kastenmayer, J. P., van Hoof, A., Johnson, M. & Green, P. J. (1998) in *Plant Molecular Biology*, eds. Raikhel, N., Last, R., Morelli, G., & LaShavo, F. (Springer-Verlag, Berlin), pp. 125-133.

Krysan, P. J., Young, J. C., and Sussman, M. R. (1999). T-DNA as an Insertional Mutagen in Arabidopsis. Plant Cell **11**, 2283-2290.

Larimer, F. W., Hsu, C. L., Maupin, M. K., and Stevens, A. (1992). Characterization of the XRN1 gene encoding a 5'-->3' exoribonuclease: sequence data and analysis of disparate protein and mRNA levels of gene-disrupted yeast cells. Gene **120**, 51-57.

Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997). The exosome: A conserved eukaryotic RNA processing complex containing multiple 3'-->5' exoribonucleases. Cell **91**, 457-466.

Muhlrad, D., Decker, C. J., and Parker, R. (1994). Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev. **8**, 855-866.

Newman, T. C., de Bruijn, F., Green, P. J., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N. V., Somerville, S. C., Tomashow, M., Retzel, E., and Somerville, C. R. (1994). Genes Galore: A summary of methods for accessing results from partial sequencing of anonymous Arabidopsis cDNA clones. Plant Physiol. **106**, 1241-1255.

Newman, T. C., Ohme-Takagi, M., Taylor, C. B., and Green, P. J. (1993). DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell **5**, 701-714.

Page, A. M., Davis, K., Molineux, C., Kolodner, R. D., and Johnson, A. W. (1998). Mutational analysis of exoribonuclease I from *Saccharomyces cerevisiae*. Nucleic Acids Res. **26**, 3707-3716.

Petfalski, E., Dandekar, T., Henry, Y., and Tollervey, D. (1998). Processing of the precursors to small nucleolar RNAs and rRNAs requires common components. Mol. Cell. Biol. **18**, 1181-1189.

Saghai Maroof, M. A., Biyashev, R. M., Yang, G. P., Zhang, Q., and Allard, R. W. (1994). Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations, and population dynamics. Natl. Acad. Sci. USA **91**, 5466-5470.

Schaffer, R., Landgraf, J., Accerbi, M., Simon, V., Larson, M., and Wisman, E. (2000). Microarray analysis of diurnal and circadian-regulated genes in Arabidopsis. Plant Cell **13**, 113-123.

Smith N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G., and Waterhouse, P.M. (2000). Total silencing by intron-spliced hairpin RNAs. Nature **407**, 319-320.

van Hoof, A. and Parker, R. (1999). The exosome: A proteasome for RNA? Cell **99**, 347-350.

Villa, T., Ceradini F, Presutti, C., and Bozzoni, I. (1998). Processing of the intron-encoded U18 small nucleolar RNA in the yeast *Saccharomyces cervisiae* relies on both exo- and endonucleolytic activities. Mol. Cell. Biol. **18** , 3376-3383.

Waterhouse, P. M., Graham, H. W., and Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA **95**, 13959-13964.

CHAPTER 5

INVESTIGATION OF THE ROLE OF DEADENYLATION IN mRNA DECAY IN HIGHER PLANTS

INTRODUCTION

A first step in the elucidation of how an mRNA instability determinant regulates mRNA decay is to determine the mechanism by which mRNAs bearing the instability determinant are degraded. Once these steps are known, it is then possible to investigate which steps might be modulated by the instability determinant to bring about rapid decay. A well characterized instability determinant in plants is the DST-element, a sequence found in the 3' UTRs of SAUR genes (Sullivan and Green, 1996). Insertion of two copies of the DST-element from the SAUR-15A gene into the 3' UTR of a reporter mRNA is sufficient to target an otherwise stable transcript for rapid decay in plant cells (Newman et al., 1993). Mutational analyses indicated that the DST element functions in a sequence dependent manner, and that mutation of the ATAGAT region of the DST element inhibits its destabilizing function (Sullivan and Green, 1995). An additional instability determinant which functions in plant cells is a sequence based on the AU-rich elements (AREs) of several mammalian instability determinants. A synthetic ARE containing 11 copies of the pentamer AUUUA targets transcripts for rapid decay in plant cells (Ohme-Takagi, 1993). While it has been demonstrated that both the DST-element and the synthetic ARE function in a sequence specific manner to target mRNAs for rapid degradation, their mode of action is unknown. There are several possible ways that they might function to stimulate mRNA turnover; however, a likely possibility is that they destabilize mRNAs by causing rapid deadenylation.

Many studies have pointed to the role of the poly(A) tail in stabilizing mRNAs when injected into plant or animal cells (reviewed in Baker, 1993). That transcripts with poly(A) tails are more stable than transcripts lacking poly(A) tails when injected into

cells indicated that the poly(A) tail might provide a protective role *in vivo*. Furthermore, as had been hypothesized for the mRNA stabilizing function of the 5' cap, these experiments indicated that the regulated removal of the poly(A) tail may have a function in the regulation of mRNA stability. This appears to be the case for several unstable mammalian and yeast transcripts in which poly(A) tail removal is the first step of mRNA degradation.

The link between deadenylation and mRNA degradation was first made in studies of the unstable mammalian c-fos mRNA. The c-fos transcript contains an ARE in its 3' UTR which contains multiple copies of the pentamer AUUUA. The degradation rate of the unstable c-fos mRNA was compared to that of a stable control transcript. For both populations, deadenylation occurred over time; however, the unstable mRNA was deadenylated more rapidly, and this rapid deadenylation depended on the presence of the ARE (Wilson and Treisman, 1988). A key observation of mRNA decay directed by the c-fos AREs was that deadenylation preceded decay of the mRNA. This indicated that deadenylation was likely required for the decay of ARE-containing mRNAs and that removal of the poly(A) tail might stimulate mRNA degradation. Since these initial studies, the function of several different classes of AREs in increasing deadenylation rates has become well established (Chen and Shyu, 1995). Increasing the rate of deadenylation also appears to be a common feature of instability determinants in yeast (Muhlrad and Parker, 1992; Caponigro and Parker, 1996) and deadenylation is a first step in the degradation of several yeast transcripts (Decker and Parker, 1993). Deadenylation preceding mRNA decay has also been observed in *Chlamydomonas rheinhardtii* (Baker, 1993).

A current model for how deadenylation triggers mRNA degradation involves the link between the 5' and 3' end of mRNAs. There are several models for how the 5' and 3' ends of mRNAs might interact mediated by the poly(A) binding protein (PAB) (reviewed in Jacobson, 1996). This interaction has been proposed to stabilize mRNAs in yeast that are degraded by the deadenylation-dependent-decapping pathway (Caponigro and Parker, 1996). According to this model, the interaction between the ends of the mRNA mediated through PAB stabilizes transcripts by preventing them from being decapped by Dcp1p. In support of this model, PAB deletions result in premature decapping and decay of mRNAs in yeast (Caponigro and Parker, 1995). In yeast, transcripts are deadenylated to 10-12 adenylates prior to their decapping and degradation (Decker and Parker, 1993). Since a poly(A) tail of 10-12 adenylates is unlikely to bind PAB with high efficiency(Sachs et al., 1987), one effect of deadenylation might be to disrupt the interactions of PABs with the poly(A) tail. This would in turn disturb the interaction of the ends of mRNA mediated by PAB leading to decapping and degradation of transcripts in yeast. This model is attractive because it explains in part how modulation of deadenylation could lead to differences in mRNA half-lives in yeast. However, it is not clear if such an explanation is applicable to the turnover of transcripts in mammalian cells, which are deadenylated prior to their degradation.

The poly(A) tails of several transcripts bearing AREs in mammalian cells are shortened to approximately 35 nts prior to degradation (Shyu et al., 1991), a length which could still support binding of at least one PAB (Sachs et al., 1987). Therefore, the effect of shortening the poly(A) tail in mammalian cells may be more complex than disrupting the interactions of the 5' and 3' ends of mRNAs by loss of PAB binding. Nevertheless,

there is indirect evidence that deadenylation may lead to decapping of some mRNAs in mammalian cells and mRNAs containing AREs are rapidly decapped in mammalian cell free extracts (Gao et al., 2001; Couttet et al., 1997).

To gain insight into the potential role of deadenylation in the mechanism of mRNA decay in plants, mRNA decay directed by the synthetic ARE and the DST element was examined in tobacco cells. The fact that AREs likely target some mammalian transcripts for decay by increasing the deadenylation rate indicates that the synthetic ARE may have a similar mechanistic function in plants cells. The DST-element could use a similar mechanism, or may have a function distinct from instability determinants such as AREs. To address these possibilities, attempts were made to measure and compare the deadenylation rates of stable mRNAs, and those destabilized by insertion of two copies of the DST element or the synthetic ARE, in tobacco cells. However, while it was possible to measure the deadenylation rate of a stable reporter mRNA, it was not possible to measure the deadenylation rate of unstable mRNAs due to their low steady-state abundance.

RESULTS AND DISCUSSION

If the decay of some transcripts in plants resembles the mechanism of decay of many mRNAs in yeast and mammalian cells, rapid deadenylation may precede, and be required for degradation. The DST element or the synthetic ARE may function as instability determinants in plants by increasing the deadenylation rate of transcripts bearing these sequences and thus lead to rapid mRNA degradation. If true, reporter transcripts containing two copies of the DST sequence or the synthetic ARE should be

deadenylated more rapidly than stable control transcripts lacking these elements. In addition, reporter RNAs bearing two copies of a DST element which has been mutated in one of the regions required for instability function, the ATAGAT region, should also be deadenylated more slowly. To examine these hypotheses, attempts were made to measure and compare the deadenylation rates of unstable reporter transcripts containing two copies of the DST element (DSTX2), stable transcripts containing two copies of the mutated DST element (ATAGATX2), unstable transcripts bearing the synthetic ARE consisting of 11 overlapping AUUUA pentamers (AUUUAX11) and a stable spacer control for the synthetic ARE which is AU-rich but lacks AUUUA pentamers.

Previous studies of the instability function of the DST element made use of a transformed suspension culture of tobacco cells, NT cells, which expressed reporter genes (Newman et al., 1993, Ohme-Takagi et al., 1993). The reporter genes consisted of the cauliflower mosaic virus 35S promoter, the murine β-globin coding region, and the pea rbcS-E9 polyadenylation sequence (Figure 4-1 A). Instability elements, and control sequences which do not alter mRNA stability, were inserted in the 3' UTR between globin and E9. These constructs were used to stably transform NT cells and the decay rates of transcripts expressed from these genes was measured in the absence of on-going mRNA synthesis. The transcriptional inhibitor actinomycin D was added to the transformed cells grown in liquid culture, aliquots of these cells were taken at intervals, and mRNA decay was followed by northern blot analysis of RNAs isolated from each time-point.

NT cells were stably transformed with each of the constructs described above and used for actinomycin D time courses. To measure the deadenylation rates of the reporter

RNAs two approaches were taken, the RNase H cleavage method which has been used to measure deadenylation rates of transcripts in yeast (e.g. Muhlrad et al., 1994) and a newly developed method called the poly(A) test (PAT), which makes use of RT-PCR (Sallés and Strickland, 1999).

Analysis of deadenylation of Globin-ATAGATX2-E9 by RNase H cleavage

The RNase H cleavage method relies on an increase in the electrophoretic mobility of mRNAs in polyacrylamide gels due to loss of the poly(A) tail over time. To more easily detect this change in mobility, the reporter transcripts are cleaved close to the poly(A) tail to produce small species which undergo more substantial changes in mobility due to differences in the length of the poly(A) tail. RNase H is used to cleave the reporter RNAs. RNase H cleaves the RNA of an RNA:DNA duplex, thus the reporter RNAs can be cleaved into two pieces if incubated with a complementary DNA oligonucleotide in the presence of RNase H. Cleavage with PG244, an oligonucleotide which is complementary to the 3' end of the globin coding region, yields a 5' cleavage product corresponding to the majority of the 5' end of the reporter mRNA, and a smaller 3' cleavage product corresponding to the E9 sequence and the poly(A) tail. Since the E9 polyadenylation sequence directs poly(A) tail addition at four sites, four cleavage products corresponding to the 3' end of the reporter RNA are produced. The mobility of these short 3' cleavage products undergoes substantial changes in migration due to loss of the poly(A) tail over time, and it is this change in migration that is used to derive the deadenylation rate.

The deadenylation rate of the Globin-ATAGATX2-E9 RNAs were examined

first. For transcripts at steady-state, the distribution of poly(A) tails spans from transcripts with tails which are newly synthesized and are the longest, to older transcripts with shorter tails due to deadenylation. This distribution results in a heterogeneous population of transcripts, but the reporter RNAs must be cleaved close to the 3' end for this population to be detected. The heterogeneity is not detected for the un-cleaved

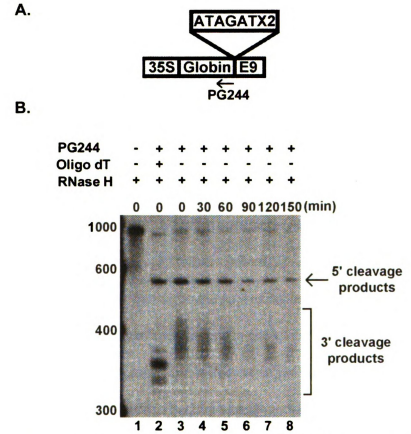


Figure 5-1. The poly(A) tail of Globin-ATAGATX2-E9 shortens over time. (A) Schematic diagram of reporter gene expressed in NT cells. 35S: promoter from cauliflower mosaic virus, Globin: murine globin coding region, E9: polyadenylation sequence from pea rbcS gene, PG244: globin-specific oligonucleotide. (B) RNase H cleavage analysis of Globin-ATAGATX2-E9 actinomycin D time course. 20 µg of total RNA of the indicated time points was cleaved with RNase H and PG244. The poly(A) tail of the RNA from the first time point was removed *in vitro* with oligo dT and RNase H in a separate reaction The cleaved RNAs were separated with a 6% polyacrylamide 8.0 M urea gel, and transferred to a membrane which was hybridized with probes specific for globin. The mobility of molecular weight markers is shown at the left and the time of acitnomycin D treatment at the top.

transcript (Figure 5-1 B, lane 1), but when the reporter RNA is cleaved with PG244 and RNase H, the 3' cleavage products are detected on northern blots as a diffuse band (Figure 5-1 B, lanes 3-8). To demonstrate that the diffuse band is due to differences in poly(A) tail length, the poly(A) tails of the 3' cleavage products from first time point (t=0) were removed *in vitro* prior to electrophoresis. This was accomplished by incubating the RNA with RNase H in the presence of oligo dT and the Globin-specific oligonucleotide PG244, resulting in cleavage of the reporter RNA and degradation of the poly(A) tails of the 3' cleavage products. If the diffuse band detected on northern blots is due to heterogeneity in length of the poly(A) tails, *in vitro* removal of the poly(A) tail should result in the disappearance of the diffuse band. The band should then migrate as a discrete band of the size expected for the 3' cleavage product without poly(A) tails. In this case, the four sizes of the E9 3' UTR are expected. As can be seen in Figure 5-1 B lane 2, *in vitro* removal of the poly(A) tail of Globin-ATAGATX2-E9 transcripts generated distinct bands corresponding to the approximate sizes expected for the E9 3' UTR. The variation in intensity of these bands is due to differential use of the poly(A) addition sites, with the most intense band reflective of polyadenylation at site two (Hunt, 1989). This indicates that the diffuse band detected on northern blots is due to heterogeneity in length of the poly(A) tails and can be used to monitor changes in deadenylation.

To measure the deadenylation rate over time, the initial length of the poly(A) tail was calculated and its shortening rate over the time-course determined. The mobility of the top of the diffuse band at time zero, which corresponds to transcripts with the longest poly(A) tails, was compared to molecular weight markers. The size of the 3' RNase H

cleavage product in which site two of the E9 tail has been used, the site at which most of the reporter RNAs were polyadenylated, is 340 nt (Figure 5-1 B, lane 2). The top of the diffuse band at time zero migrates to a position of approximately 450 nt (Figure 5-1 B, lane 3). This indicates that the poly(A) tail is approximately110 nt long at time zero. This length is intermediate between yeast and mammals which have poly(A) tails of approximately 75 nt and 300 nt, respectively (Baker, 1993). To calculate the degree to which the poly(A) tail shortens, the change in the mobility of the top of the diffuse band is monitored over time. As can be seen in lanes, the top of the diffuse band migrates further down the gel with increasing time due to deadenylation (Figure 5-1 B, lanes 3-8). The average change in mobility over the entire time-course reflects a deadenylation rate of approximately 0.5 nt/min, a value similar to stable mRNAs measured in mammalian and yeast systems (Shyu et al., 1991; Decker and Parker, 1993). A repeat of this analysis with an independently generated time course of the same stable ATAGATX2 RNA resulted in a similar measurement (data not shown).

The next step in the analysis was to measure the deadenylation rate of the unstable Globin-DSTX2-E9 and Globin-AUUUAX11-E9 transcripts. If these sequences function by increasing deadenylation rate, deadenylation of transcripts bearing these elements would be expected to occur faster than rate of deadenylation of the stable Globin-ATAGATX2-E9 transcripts determined above. Unfortunately, due to technical limitations, such a measurement was not obtained.

The steady-state abundance of Globin-DSTX2-E9 and Globin-AUUUAX11-E9 transcripts is quite low due to their high degradation rates. When the RNase H cleaved RNA was separated by electrophoresis, the intensity of the signal detected on a northern

blot was further reduced due to the diffuse nature of the 3' cleavage product. This made the low abundance transcripts even more difficult to detect. The diffuse band corresponding to the 3' end of the Globin-DSTX2-E9, or Globin-AUUUAX11-E9, was not detected in any of several experiments when polyacrylamide gels were used. The 3' cleavage product could be observed when agarose gels were used, indicating that these reporter RNAs could be cleaved. However, concentrations of up to 4% agarose failed to resolve the small differences in the migration of these cleavage products, or of the 3' cleavage products of a repeat of the Globin-ATAGATX2-E9 time course (data not shown).

Analysis of deadenylation of Globin-ATAGATX2-E9 by PAT-PCR

As an alternative to the RNase H cleavage method, an RT-PCR method was attempted. For RT-PCR to be applicable to measuring changes in poly(A) tail lengths, the reverse transcription reaction must generate cDNAs which contain as much as possible of the poly(A) tail-derived sequence. A method to generate cDNAs which include poly(A) derived sequence has been recently developed called PAT-PCR (Sallés and Strickland, 1999; diagrammed schematically in Figure 5-2 A). In this method, oligo dT_{12-18} is annealed to the RNA. The oligonucleotides anneal along the length of the poly(A) tails, but can leave some of the 3' end without poly(A) annealed. These oligonucleotides are phosphorylated on their 5' ends and are ligated together with DNA ligase. An excess of an 'anchor primer', which consists of poly(T) followed by 16 bases of a non T-rich sequence, is added to this mixture of total RNA and ligated oligo dT. The anchor primer can anneal to any un-paired poly(A) tail at the 3' end. Following

annealing of the anchor primer, the oligo dT and anchor primers are ligated to each other with DNA ligase. This oligo dT-anchor primer ligation product is used as the primer for reverse transcription, thus incorporating sequence from the poly(A) tail in the first strand cDNA. The cDNAs generated are used in a PCR reaction with a gene specific primer and a primer which anneals to the anchor sequence. Radiolabled dATP is included in the PCR reaction to label the PCR products which are separated by PAGE and detected with autoradiography.

The RNA from an actinomycin D time course of cells expressing Globin-ATAGATX2-E9 was analyzed by PAT-PCR. It should be noted that PAT-PCR was developed to monitor changes in poly(A) tail length of transcripts with a discrete poly(A) distribution at steady state and whose abundance doesn't change over time (Sallés et al., 1999). It was unclear if PAT-PCR would work on transcripts whose poly(A) tails vary over a wide range in length, and for transcripts which are decaying over time. The main concern was that PAT-PCR would not distinguish between disappearance of full-length transcript over time without loss of the poly(A) tail, and deadenylation occurring over time. To simulate a deadenylation-independent decay of mRNA, the cDNAs generated from one time point (time 15 for Globin-ATAGATX2-E9) were serially diluted, with cDNAs from non-transformed NT cells added to maintain the same total template concentration across the dilution series. The cDNAs were subjected to PCR analysis. The expected result was that a diffuse band of PAT-PCR products corresponding to the distribution of differing poly(A) tail lengths at steady-state would be detected. Across the dilution series the diffuse band should not decrease in length but should decrease in

overall radioactive signal due to the dilution of the template. However, this was not the case (Figure 5-2 B). The top of the PAT-PCR products appeared lower on the gel across

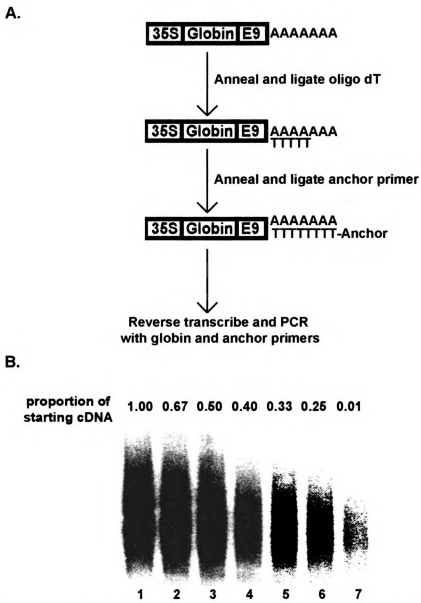


Figure 5-2. Use of the PAT-PCR method to measure deadenylation (A) Schematic diagram of the PAT-PCR strategy. (B). PAT-PCR of a dilution series of Globin-ATAGATX2-E9 cDNAs generated from the 15 minute time point of an actinomcyin D time course. PAT-PCR was carried out as described in the text and the reactions were separated on a non-denaturing 6% acrylamide gel. Above each lane is shown the amount of cDNA from the first time point used for each PCR reaction of the dilution series.

the dilution series similar to what would be expected for deadenylation over time. It appears that the longer products are not detected below a certain concentration. From this result it appears that PAT-PCR, at least in its present state, causes loss of transcript over time to mimic a decrease in the length of the poly(A) tail over time. While this effect is minimal with up to a two-fold dilution (Figure 5-2 B compare lanes 1-3) with dilutions greater than two-fold the effect is quite significant, giving the appearance that the poly(A) tail has shortened by greater than 25 nucleotides. As the expected change in abundance of unstable mRNAs would likely exceed two-fold in an actinomycin D time course, it would be difficult to distinguish between loss of the poly(A) tail and inefficient PCR of long poly(A)-tailed mRNAs. Despite these drawbacks to PAT-PCR, there are several ways that the technique might be optimized to measure deadenylation rates of decaying mRNAs in the future.

If the measurement of deadenylation were restricted to early time points such that the change in mRNA abundance is not very great, this may alleviate the effect of loss of transcripts over time. However, as it is known that deadenylation exhibits biphasic kinetics in yeast cells (Muhlrad and Parker, 1992), restricting measurement to early time points might result in a measurement not reflective of the over all deadenylation rate. Alternatively, it may be possible to use equivalent amounts of RNA for each time point. For example, if the reporter RNA is known to decay three-fold after a particular time interval, three-fold more RNA for this time point could be used in the PAT-PCR. This could have the effect of reducing the effects of decreasing amounts of reporter RNA over time and allow for a deadenylation rate to be calculated. Either of these approaches might be successful in determining if deadenylation rates correlate with mRNA instability

triggered by DST or AREs. However, prior to more optimization, it would be beneficial to modify the overall design of the experiment to gain more information about the relationship between deadenylation and mRNA degradation.

A significant limitation to the analysis of changes in poly(A) tail lengths of a steady-state RNA population generated from a constitutive promoter is that the relationship between deadenylation and decay cannot be fully determined. While it is possible to determine if mRNA instability correlates with a higher rate of deadenylation, it is not possible to determine if deadenylation precedes, and therefore might be required for, mRNA decay. To make such a determination requires the analysis of a synchronously synthesized population of transcripts to determine if removal of the poly(A) tail precedes disappearance of the mRNA. Such a population could be generated by a promoter that is rapidly induced and repressed.

Recently, a transcript that is rapidly and transiently induced has been discovered in Arabidopsis (R.A. Gutiérrez and P.J. Green, unpublished). If the promoter of the gene encoding this transcript mediates its expression kinetics, this promoter might be a good way to control the expression of reporter genes in Arabidopsis. It could allow for the generation of a synchronous population of reporter transcripts whose deadenylation could be measured and compared to the kinetics of decay in intact Arabidopsis seedlings. A further modification to the expression of the reporter gene would be to use a transcription terminator which directs polyadenylation at only one site. The reporter genes in this study made use of the pea rbcS-E9 polyadenylation sequence which directs polyadenylation at four sites (Hunt, 1988). Transcripts polyadenylated at each of these sites are produced which leads to overlapping of the 3' RNase H cleavage products

generated from these transcripts on northern blots. This makes the determination of deadenylation rates more challenging, since it is difficult to determine where the top of the diffuse band for each of these heterogeneous mRNAs migrates. The use of a regulated promoter and a "stricter" polyadenylation sequence should greatly enhance the ability to determine if deadenylation precedes the decay of some mRNAs in plants.

CONCLUSIONS AND FUTURE PROSPECTS

Although technical limitations prevented a comparison of deadenylation rates of a stable and unstable reporter RNAs in tobacco cells, the results presented here indicate that such measurements may be possible. The RNase H-cleavage method was successful in measuring the deadenylation rate of a stable mRNA and this method may be appropriate for future studies. As a limitation of this method is the difficulty of detecting RNase H cleavage products of mRNAs expressed at low levels, application of this method will require expression levels that surpass those achieved by the 35S promoter used in the experiments described above. The transiently induced promoter described above will likely give higher expression levels than the 35S promoter, and likely can be used to produce a synchronous population of transcripts. The use of the RNase H cleavage method in combination with this promoter has the greatest potential to address the role of deadenylation in the function of the DST element and the synthetic ARE in stimulating mRNA decay in plants.

MATERIALS AND METHODS

RNase H cleavage

A DNA oligonucleotide complementary to the 3' end of the globin coding region, PG244: 5'-CCCAATGCCATAATACTCG-3', was used to direct cleavage or the reporter transcripts. 20 μg of total RNA was incubated with 2 μg of oligonucleotide at 65°C for 10 minutes in a water bath. Over the course of about one hour, the water bath was slowly cooled to room temperature. The reactions were then placed on ice for 5 minutes. RNase H digestions are incubated at 37°C for 1 hour in 50 μl of RNase H reaction mix containing 4 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 mM KCl, 1 mM DTT and three units of RNase H (GIBCO-BRL). The reactions were then ethanol precipitated and resuspended in formamide loading buffer containing: 10 mM EDTA, 1 ug/ul xylene cyanol and 1ug/ul bromophenol blue in 100% formamide (Hilamond and Sproat, 1994).

Actinomycin D time courses and northern analysis

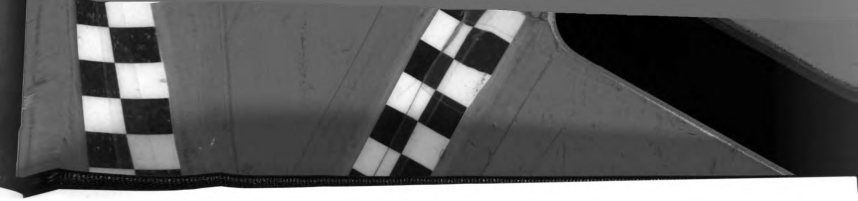
Time courses were performed with 50 ml cultures of stably transformed NT cells five days after sub-culture using 500 ug/ml of actinomycin D as previously described (Newman et al., 1993). Total RNA was isolated from 5 ml aliquots of the NT cells at 15 or 30 minute time intervals of treatment with actinomycin D. Northern blot analyses of RNase H cleaved RNAs were conducted as follows. RNase H cleavage reactions were separated with 6% polyacrylamide (30:1 acrylamide: bis-acrylamide) 8.0 M urea gels. The gels were 15 cm long and were run at 300V for 10 hours. Following electrophoresis, the RNA was transferred to Zetaprobe membrane (Biorad) using a Hoeffer TE42 transfer

apparatus (Hoeffer). The transfer was done at 300 mA for 12 hours in 0.5X TBE at 4 °C. Pre-hybridizations and hybridizations were done using standard techniques. The best results were obtained by washing once with 2XSSC 0.1% SDS for 30 minutes at 65 °C.

PAT-PCR

The RT-PCR reactions were carried out according to the method of Sallés et al., (1999) as follows: 20 µg of total RNA was incubated in a 7 µl volume with 20 ng of oligo dT [$pd(T)_{12-18}$ (Pharmacia)] at 65 °C for 10 minutes. The reactions were transferred to 42 °C for ligation of the oligo dT primers. This ligation was conducted in a 20 µl volume including 4 µl 5 X RT buffer (Gibco) 2µ 0.1 M DTT, 1µl mixture of all four dNTPs at 10 mM each, 1µl of 10 mM ATP and 1 µl of high concentration T4 DNA ligase (10 Weiss U/µl). This reaction mix was incubated for 30 minutes at 42 °C. Following ligation of the oligo dT primers, 200 ng of the anchor primer PG 461: 5'-GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT-3' was added and the reaction incubated at 16 °C for 2 hours to ligate the anchor primer to the poly dT. Following ligation of the anchor primer, the reaction was incubated at 42 °C for two minutes after which 200 U of Superscript reverse transcriptase (Gibco) was added. Reverse transcription was carried out at 42 °C for 1 hour. PCR was carried out with the following cycles: 2 minutes at 94 °C, 20 cycles of 1 minute at 94 °C, 2 minutes at 58 °C and 3 minutes at 68 °C. The best results were obtained with Advantage cDNA polymerase mix (Clontech). The PCR reactions included 5 µCi of α-^{32} P dATP (NEN). The primers used for the PCR were the Globin specific primer PG485, which is the reverse complement of

PG244, and a primer identical to the anchor primer PG461, but lacking the thymidylates at the 3' end (primer PG 484). Use of the anchor primer in the PCR as recommended in Sallés et al., (1999) resulted in the amplification of non-specific products. These products were greatly reduced by substituting the anchor primer with primer PG484. The PAT-PCR reactions were separated on a non-denaturing 6% polyacrylamide gel and exposed to film.



REFERENCES

Baker, E. J. (1993). Control of poly(A) length. Control of Messenger RNA Stability, J. Belasco and G. Brawerman eds (New York: Academic Press), pp.367-415.

Brown, C. E. and Sachs, A. B. (1998). Poly(A) tail length control in *Saccharomyces cerevisiae* occurs by message-specific deadenylation. Mol. Cell. Biol. **18**, 6548-6559.

Caponigro, G. and Parker, R. (1995). Multiple functions for poly(A)-binding protein in mRNA decapping and deadenylation in yeast. Genes Dev. **9**, 2421-2432.

Caponigro, G. and Parker, R. (1996). mRNA turnover in yeast promoted by the MATα1 instability element. Nucleic Acids Res. **24**, 4304-4312.

Chen, C. Y. A. and Shyu, A. B. (1995). AU-rich elements: Characterization and importance in mRNA degradation. Trends Biochem. Sci. **20**, 465-470.

Couttet, P., Fromont-Racine, M., Steel, D., Pictet, R., and Grange, T. (1997). Messenger RNA deadenylylation precedes decapping in mammalian cells. Proc. Natl. Acad. Sci. USA **94**, 5628-5633.

Decker, C. J. and Parker, R. (1993). A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev. **7**, 1632-1643.

Gao, M., Fritz, D. T., Ford, L. P., and Wilusz, J. (2000). Interaction between a poly(A)-specific ribonuclease and the 5' cap influences mRNA deadenylation rates in vitro. Mol. Cell **5**, 479-488.

Gao, M., Wilusz C.J., Peltz, S. W., and Wilusz, J. (2001). A Novel mRNA-decapping Activity in HeLa Cytoplasmic Extracts is Regulated by AU-rich Elements. EMBO J. **20**, 1134-1143.

Hunt, A. G. and MacDonald, M. H. (1989). Deletion analysis of the polyadenylation signal of a pea ribulose-1,5-bisphosphate carboxylase small-subunit gene. Plant Mol. Biol. **13**, 125-138.

Jacobson, A. (1996). Poly(A) metabolism and translation: The closed loop model. In Translational Control. M. Hershey, M. Mathews,. and N. Sonenberg eds (New York: Cold Spring Harbor Press) pp 451-480.

Johnson, M. A. (2000). Genetic Determinants of mRNA Stability in Plants. Ph.D. dissertation. Michigan State University

Körner, C. G., Wormington, M., Muckenthaler, M., Schneider, S., Dehlin, E., and Wahle, E. (1998). The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of *Xenopus* oocytes. EMBO J. **17**, 5427-5437.

Lamond, A. I. and Sproat, B. S. (1994). In RNA Processing, A Practical Approach. S. J. Higgins and B. D. Hames eds (New York: IRL Press) p. 127.

Muhlrad, D., Decker, C. J., and Parker, R. (1994). Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev. **8**, 855-866.

Muhlrad, D. and Parker, R. (1992). Mutations affecting stability and deadenylation of the yeast MFA2 transcript. Genes Dev. **6**, 2100-2111.

Newman, T. C., Ohme-Takagi, M., Taylor, C. B., and Green, P. J. (1993). DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell **5**, 701-714.

Ohme-Takagi, M., Taylor, C. B., Newman, T. C., and Green, P. J. (1993). The effect of sequences with high AU content on mRNA stability in tobacco. Proc. Natl. Acad. Sci. USA **90**, 11811-11815.

Sachs, A. B., Davis, R. W., and Kornberg, R. D. (1987). A Single Domain of Yeast Poly(A)-binding Protein is Necessary and Sufficient for RNA Binding and Yeast Viability. Mol.Cell.Biol. **7**, 3268-3276.

Sachs, A. B. and Deardorff, J. A. (1992). Translation initiation requires the PAB-dependent poly(A) ribonuclease in yeast. Cell **70**, 961-973.

Sallés, F. J., Richards, W. G., and Strickland, S. (1999). In Methods, a Companion to Methods in Enzymology. A. Jacobson and S.W. Peltz eds (New York: Academic Press) pp 38-45.

Shyu, A.-B., Belasco, J. G., and Greenberg, M. E. (1991). Two distinct destabilizing elements in the c-*fos* message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev. **5**, 221-231.

Tucker, M., Valencia-Sanchez, M. A., Staples, R. R., Chen, J., Denis, C. L., and Parker, R. (2001). The Transcription Factor Associated Ccr4 and Caf1 Proteins are Components of the Major Cytoplasmic mRNA Deadenylase in *Saccharomyces cerevisiae*. Cell **104**, 377-386.

Wilson, T. and Treisman, R. (1988). Removal of poly(A) and consequent degradation of c-fos mRNA facilitated by 3' AU-rich sequences. Nature **336**, 396-399.

SYNOPSIS

SYNOPSIS

Many of the experiments carried out on in this dissertation were based on the assumption that mRNA decay in plants may resemble mRNA decay in yeast, but also might differ in several important ways. The experimental results presented in this dissertation indicate that mRNA degradation of some mRNAs in plants likely occurs thorough a pathway which resembles the major deadenylation-dependent-decapping pathway of yeast; however, this degradation is most likely catalyzed by AtXRN4, an ortholog of the nuclear yeast enzyme Xrn2p/Rat1. Furthermore, experiments reported in this dissertation indicate that additional highly active mRNA degradation pathways exist in Arabidopsis.

The presence of orthologs in the Arabidopsis genome of components of the major 5'-3' , as well as the 3'-5' mRNA decay pathways of yeast (Kastenmayer et al., 1998; Gutiérrez, et al., 1999) indicates that mRNA degradation pathways in yeast may be conserved in higher plants. In addition, the presence of such mRNA decay pathways in plants is supported in several cases by the structures of natural mRNA decay intermediates (e.g. Tanzer and Meagher, 1995). However, the striking absence of an Xrn1p ortholog from Arabidopsis and other plants species clearly indicates that mRNA decay catalyzed from the 5' end in higher plants differs from mRNA degradation in yeast. In addition, the absence of poly(G)-stabilized mRNA decay intermediates in plant cells, like those observed in yeast due to blockage of Xrn1p, further indicates that mRNA decay differs between plants and yeast (Kastenmayer et al., 1998; Johnson, 2000). The discovery that the Xrn2p/Rat1p ortholog AtXRN4 is cytoplasmic, rather than nuclear (Chapter 3), indicates that 5'-3' mRNA decay in Arabidopsis may be catalyzed by

AtXRN4. However, as AtXRN4 is blocked by poly(G) tracts (Chapter 3), its activity cannot solely account for the absence of poly(G)-stabilized mRNA decay intermediates in Arabidopsis. AtXRN4 may catalyze the degradation of mRNAs, similar to Xrn1p in yeast, but might function in concert with additional cellular proteins which facilitate the degradation of highly structured mRNAs.

Although AtXRN4 may have an Xrn1p-like function with respect to mRNA degradation, it is likely that AtXRN4's contribution to mRNA degradation in Arabidopsis is not as great as Xrn1p's role in mRNA decay in yeast. In contrast to Xrn1p, *AtXRN4* is not highly expressed (Chapter 3), and the AtXRN4 protein is unlikely to be particularly abundant. This could indicate that mRNA degradation catalyzed from the 5' end is a less prominent mRNA decay pathway in plants than in yeast. The preliminary observation that the abundance of relatively few transcripts is altered in the *xrn4-1* mutant (Chapter 4) is consistent with this possibility.

Taken together, the experiments presented in this dissertation support the hypothesis that the degradation of some mRNAs in plants resembles 5'-3' mRNA decay in yeast, with the exception that the Xrn2p/Rat1p ortholog AtXRN4 may catalyze mRNA degradation in Arabidopsis. In addition, studies of mRNA degradation in the *xrn4-1* mutant indicate that additional mRNA decay pathways exist in Arabidopsis. In the future, it will be interesting to address the contribution that AtXRN4 makes to global mRNA degradation as well as the mRNA degradation pathway in which AtXRN4 functions.

REFERENCES

Gutiérrez, R. A., MacIntosh, G. C., and Green, P. J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci. **4**, 429-438.

Johnson, M. A. (2000). Genetic Determinants of mRNA Stability in Plants. Ph.D. dissertation. Michigan State University

Kastenmayer, J. P., van Hoof, A., Johnson, M. & Green, P. J. (1998) in *Plant Molecular Biology*, eds. Raikhel, N., Last, R., Morelli, G., & LaShavo, F. (Springer-Verlag, Berlin), pp. 125-133.

Tanzer, M. M. and Meagher, R. B. (1995). Degradation of the soybean ribulose-1,5-bisphosphate carboxylase small-subunit mRNA, SRS4, initiates with endonucleolytic cleavage. Mol.Cell.Biol. **15** , 6641-6652.