2005

ISOLATION AND CHARACTERIZATION OF THE FOUR ARABIDOPSIS THALIANA POLY(A) POLYMERASE GENES

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ABSTRACT OF DISSERTATION

Lisa Renee Meeks

The Graduate School
University of Kentucky
2005
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ARABIDOPSIS THALIANA POLY(A) POLYMERASE GENES

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Lisa Renee Meeks
Lexington, Kentucky

Director: Dr. Arthur G. Hunt, Professor of Agronomy
Lexington, Kentucky

2005

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ABSTRACT OF DISSERTATION

ISOLATION AND CHARACTERIZATION OF THE FOUR *ARABIDOPSIS THALIANA* POLY(A) POLYMERASE GENES

Poly(A) tail addition to pre-mRNAs is a highly coordinated and essential step in mRNA maturation involving multiple cis- and trans-acting factors. The trans-acting factor, poly(A) polymerase (PAP) plays an essential role in the polyadenylation of mRNA precursors. The *Arabidopsis thaliana* genome contains four putative PAP genes. We have found, using in silico analysis and transgenic plants expressing GUS under the control of the four PAP promoters, that each of these genes is expressed in overlapping, yet unique patterns. This gives rise to the possibility that these genes are not redundant and may be essential for plant survival. To further test this, inducible RNAi and T-DNA mutagenized plants were obtained and analyzed. Plants lacking all, or most, of each PAP gene product, due to RNAi induction, were not viable at any of the stages of plant growth tested. Furthermore, T-DNA PCR analysis determined that no plants containing a homozygous mutation, were viable. This data reveals that lack of any of the four PAP gene products has a significant effect on the plants ability of survive, thus indicating that each PAP gene is essential. Finally, transient expression experiments with each of the full length PAP cDNAs fused to GFP showed that the PAP I, PAP II and PAP IV gene products are localized throughout the nucleus and within nuclear speckles. The cellular localization of PAP III could not be determined.

KEYWORDS: Poly(A) Polymerase, mRNA 3'-End Formation, RNA-Processing, Poly(A) Tail, *Arabidopsis thaliana*

Lisa Renee Meeks

11/21/2005
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DEDICATION

I would like to dedicate this dissertation to my father, Tom Meeks, who passed in 1992. His love for me knew no bounds and I miss him as much today as the day he died, if not more.
ACKNOWLEDGEMENTS

I would like to thank everyone at the University of Kentucky that formed my environment during this time and helped me to grow in so many ways. I would especially like to thank my advisor, Dr. Arthur G. Hunt, whose guidance, patience and understanding has helped me through this rather difficult PhD project as well as many personal matters. His technical and editorial advice was essential to the completion of this dissertation and has taught me innumerable lessons and insights on the workings of academic research in general. Thank you for not giving up on me through the years! My thanks also go to the members of my committee, Drs. Joseph Chappell, Robert Houtz, Martha Peterson and Michael Barrett for their advice and guidance. In addition, I would like to give a special thank you to Dr. John Snyder for his willingness to serve as may outside examiner. I would also like to thank Dr. Sharon Perry for her assistance in the microscopy room and Dr. Randy Dinkins for his friendship and support.

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CHAPTER ONE

Literature Review

In the nucleus of eukaryotic cells, RNA polymerase II (Pol II) transcribes large pre-messenger RNAs (pre-mRNA) that extend between 100 to 4000 nucleotides beyond the 3’-end of the mature mRNA. For these pre-mRNAs to be translated, they must first undergo post-transcriptional modifications including the addition of a 5’-7-methyl guanine cap, exon/intron splicing, addition of a poly(A) tail to the 3’-end in a non-template dependant manner and subsequent transport of the mature mRNAs from the nucleus to the cytoplasm where they can be used as a substrate for translation.

Almost all mRNAs in eukaryotes undergo polyadenylation. The only known exceptions are the replication-dependent histone mRNAs in metazoan organisms, which have a 3’ stem-loop structure instead of a poly(A) tail (Marzluff and Pandey, 1988). The addition of a poly(A) tail occurs in two steps that are tightly coupled by the basal polyadenylation machinery in vivo but can be experimentally uncoupled in vitro. The first step requires the removal of the 3’-terminal non-coding fragment through an ATP dependent, endonucleolytic cleavage of the nascent transcript at a specific site in the pre-mRNA. This step is closely followed by polymerization of adenosine residues to the upstream 3’-OH of the 5’ cleavage product (Keller and Minvielle-Sebastia, 1997; Wahle and Kuhn, 1997; Zhao et al., 1999a; Figure 1.1). Poly(A) tail lengths range from about 75 residues in the yeast Saccharomyces cerevisiae to nearly 300 residues in humans (Butler et al., 1990; Wickens, 1990; Brawerman, 1981). Efficient polyadenylation occurring at a specific location requires intricate interactions between cis signals on the nascent transcript and trans-acting factors contained in the multi-subunit polyadenylation complex (Minvielle-Sebastia et al., 1998; Wahle and Ruegsegger, 1999; Zhao et al., 1999a).

3’-end processing is not only essential for the addition of the poly(A) tail, it is also coupled to other events in the nucleus such as transcription termination (Whitelaw and Proudfoot, 1986; Logan et al., 1987; Connelly and Manley, 1988), mRNA splicing (Niwa et al., 1990a; Niwa et al., 1990b; Boelens et al., 1993; Lutz et al., 1996) and mRNA export from the nucleus (Whitelaw and Proudfoot, 1986; Connelly and Manley,
The link between transcription and polyadenylation has been well studied and many associations have been found between these two processes. The nucleotide sequences found on the nascent RNA signaling the site of polyadenylation are, in effect, also a transcription termination signal for Pol II. Defects in these sequences bring about loss of mRNA 3’-polyadenylation as well as defective transcription termination, which leads to transcription read-through into downstream genes (Whitelaw and Proudfoot, 1986; Connelly and Manley, 1988). Through yeast mutational studies, several components of the polyadenylation apparatus have been shown to be required for correct Pol II termination (Dichtl et al., 2002a). In mammalian cells, deletion of the C-terminal domain (CTD) of the largest subunit of Pol II devastated the cell’s ability to perform capping, splicing and polyadenylation, thus showing the critical interplay of these three major pre-mRNA processing events (McCracken et al., 1997). Furthermore, direct interactions of polyadenylation factors with the Pol II CTD, as well as transcriptional factors, have been established both genetically and biochemically and in yeast, it was found that separate domains on individual protein factors were devoted to either transcription termination or polyadenylation signaling (Barilla et al., 2001; Dichtl et al., 2002a; Kyburz et al., 2003; Aranda and Proudfoot, 2001; Sadowski et al., 2003; Licatalosi et al., 2002). There is a highly coordinated link between polyadenylation, splicing and exon definition, particularly definition of the last exon where protein-protein interactions between splicing and polyadenylation factors occur at the polyadenylation signal (Robberson et al., 1990; Berget, 1995; Niwa et al., 1990a; Niwa et al., 1990b; Niwa and Berget, 1991a; Niwa and Berget, 1991b; Scott and Imperial, 1996; Cooke et al., 1999; Vagner et al., 2000; Cooke and Alwine, 2002; Boelens et al., 1993; Lutz et al., 1996; McCracken et al., 2002). Finally, the link between poly(A) tail addition and mRNA export from the nucleus to the cytoplasm has been demonstrated through the generation of yeast mutants defective in 3’-end processing. These yeast strains not only show defects in polyadenylation but also reduced nuclear export of polyadenylated mRNAs and a decline in recruiting abilities for nuclear export factors (Hammell, 2002).

The poly(A) tail itself plays important roles in mRNA metabolism. Ever changing environments require that cells be able to adjust mRNA levels quickly and efficiently. One method to alter mRNA levels is through mRNA turnover and removal of
the polyadenylate tail. This step is frequently the first and rate-limiting step of this process (Bernstein et al., 1989; Beelman and Parker, 1995; Boeck et al., 1996; Carpousis et al., 1999; Curtis et al., 1995; Ford et al., 1997; Lewis et al., 1995; Caponigro and Parker, 1995). Regulated increases in the length of the tail have also been observed. For example, tail lengths of vasopressin mRNAs can vary in a circadian type rhythm and can rapidly increase in times of dehydration in rats (Robinson et al., 1988, Carrazana et al., 1988). Polyadenylate tails also influence the efficiency of translation through interactions with the 5’-cap to help load the RNA onto the 40S ribosomal subunit and subsequently stimulate translation (Gallie, 1991; Craig et al., 1998; Proweller and Butler, 1997; Preiss and Hentze, 1998; Tarun and Sachs, 1995; Sachs et al., 1997). Regulation of gene expression, through the alternative usage of polyadenylation sites, can occur thus changing the capacity of coding or non-coding sequences in the 3’ region of the mRNA (Foulkes et al., 1993; Takagaki et al., 1996; Proudfoot, 1986).

RNA Sequences Directing 3’-End Processing

Sequences controlling location and efficiency of mRNA cleavage and polyadenylation are contained within the nascent transcript. Surprisingly, such a seemingly simple process has been found to require intricate interactions between several cis-acting factors and a multitude of trans-acting proteins (Edmonds, 2002). These cis-acting signals are responsible for orchestrating the multi-subunit protein factors required for cleavage and polyadenylation. The processes involved in polyadenylation have been well studied and appear to be universal, however, diverse organisms use apparatus with unique features to accomplish pre-mRNA 3’-end maturation.

In mammals, at least 3 cis-elements on the nascent transcript are required to define the exact location of the cleavage site. These core elements include the A-rich element consisting of the hexanucleotide sequence AAUAAA, the GU- or U-rich downstream element (DSE) and the site of polyadenylation (Figure 1.2). There are also several auxiliary elements located upstream and/or downstream from these core elements. These signals are responsible for regulating the efficiency of 3’-processing through recruitment of regulatory factors and by assuring that the core is open and accessible to the polyadenylation machinery (Wahle and Keller, 1996; Zarudnaya et al., 2003). 70-
90% of polyadenylated animal mRNAs contain the hexanucleotide AAUAAA, or an AUUAAA variant, located 10-30 nucleotides upstream of the cleavage site that is absolutely required for both the cleavage and polyadenylation steps in mRNA 3’ processing of most RNA transcripts (Fitzgerald and Shenk, 1981; Higgs et al., 1983; Montell et al., 1983; Wickens and Stephenson, 1984; Wickens, 1990; Manley, 1988; Wahle and Keller, 1992). The variant AUUAAA directs cleavage, in vitro, with an efficiency of 66%, compared to the wild type AAUAAA level (Graber et al., 1999a; Graber et al., 1999b; Beaudoin et al., 2000; MacDonald and Redondo, 2002; Sheets et al., 1990; Proudfoot and Brownlee, 1976).

The second, less conserved element of the core polyadenylation signal is the U-rich or GU-rich DSE. This signal is located within 50 nucleotides downstream of the poly(A) site (Proudfoot, 1991; Chen et al., 1995; Takagaki and Manley, 1997). The distance between the hexanucleotide element and the DSE determines the effectiveness of a particular cleavage site on the RNA and the efficiency of the cleavage reaction (MacDonald et al., 1994, Mason et al., 1986; Cole and Stacy, 1985; Conway and Wickens, 1987; Sadofsky and Alwine, 1984; Simonsen and Levinson, 1983; Gil and Proudfoot, 1987; McDevitt et al., 1986). It is not unusual for a polyadenylation signal to have more than one DSE (Chou et al., 1994; McDevitt et al., 1986; Gil and Proudfoot, 1987).

The site of cleavage, which ultimately becomes the point of poly(A) addition, is referred to as the poly(A) site. While there is no strong consensus sequence in the area lying between the AAUAAA motif and the DSE, cleavage was found to occur on the 3’-side of an A residue in ~70% of vertebrate mRNAs and, in 59% of all genes analyzed the pentultimate residue was a C residue (Sheets et al., 1990). Thus, a CA dinucleotide usually characterizes the poly(A) site for most genes. A few mammalian genes have been found to contain a U-rich area surrounding the CA dinucleotide that may play a role in determining the strength of the polyadenylation signal (Moreira et al., 1995; Brackenridge and Proudfoot, 2000).

The core elements govern the site of cleavage and the efficiency of the polyadenylation reaction. However, other auxiliary sequences found on the RNA molecule, along with the secondary structure of the transcript itself, can play important
roles in poly(A) site choice and efficiency. For example, viral poly(A) sites commonly have a U-rich enhancer sequence located upstream of the AAUAAA element termed upstream sequence enhancers (USE) (Simonsen and Levinson, 1983; Bhat and Wold, 1987; DeZazzo and Imperiale, 1989; DeZazzo et al., 1991; DeZazzo et al., 1992; Prescott and Falck-Pedersen, 1992, 1994; Sittler et al., 1994; Silver Key and Pagano, 1997; Carswell and Alwine, 1989; Lutz and Alwine, 1994; Schek et al., 1992; Russnak and Ganem, 1990; Brown et al., 1991; Cherrington and Ganem, 1992; Valsamakis et al., 1991). A computer analysis found a large number of human genes with a high concentration of U-rich elements (USE) upstream of the AAUAAA signal (Legendre and Gautheret, 2003). A G-rich, auxiliary downstream enhancer (AUX DSE) sequence located 3’ of the DSE has been described to positively influence the polyadenylation process in the SV40 L pre-mRNA and to be present in ~34% of mammalian poly(A) signals (Bagga et al., 1995; Bagga et al., 1998; Chen and Wilusz, 1998; Lou et al., 1996; Lou et al. 1999; Arhin et al., 2002). These sequences are thought to act as recognition sites for factors stabilizing the polyadenylation complex. Negative regulatory elements have also been found upstream of the poly(A) sites for the U1A and the bovine papillomavirus late poly(A) sites and downstream of the poly(A) site in promoter-proximal HIV-1 (Furth et al., 1994; Gunderson et al., 1994; Gunderson et al., 1997; Ashe et al., 1997).

RNA secondary structure is also thought to play an important role in polyadenylation. However, this aspect of 3’-processing has not been extensively studied. Preliminary results have demonstrated that secondary structures, along with the cleavage and polyadenylation signals, can enhance or retard cleavage site selection. For example, chimaeric RNA containing the AAUAAA hexamer in a secondary structure had a much lower cleavage rate compared to pre-mRNAs in which this structure was absent (Chen and Wilusz, 1998). In addition, both a stem loop structure containing the core poly(A) signals in the HIV-1 transcript and base pairing in hepatitis delta virus transcripts reduce polyadenylation efficiency (Klasens et al., 1999; Hsieh et al., 1994). On the other hand, stem loop structures have been shown to enhance 3’-end processing. In the murine immunoglobulin M secretory transcript, a stem loop structure containing a DSE is critical for positively regulating the poly(A) site (Phillips et al., 1999). This up regulation is also
seen in human T-cell leukemia virus types 1 and 2 and bovine leukemia virus transcripts in which a stem loop structure brings together the AAUAAA signal and the cleavage site which are almost 300 nucleotides apart (Guntaka, 1993). Interestingly, ~27% of pre-mRNA sequences in a database containing poly(A) signals have the potential to form G-quadruplexes which are structures made up of DNA or RNA containing clusters of G-repeats that form four-stranded structures composed of stacked G-tetrads (Patel et al., 1999; Zarudnaya et al., 2002). This structure has not been well studied and what effect it has on polyadenylation is not well understood at this time.

Unlike mammalian cells, yeast polyadenylation signals have been more difficult to discern. Yeast RNAs contain no highly conserved sequence motif equivalent to the AAUAAA hexanucleotide domain found in mammalian cells and the yeast signals that have been identified demonstrate a degeneracy more analogous to the USEs and DSEs found at mammalian polyadenylation sites (Humphrey et al., 1994; Guo and Sherman, 1996a, 1996b). Like mammals, the yeast _Saccharomyces cerevisiae_ contains at least three different cis elements that contribute to the efficiency and accuracy of yeast 3’-end processing. The UA-rich efficiency element (EE) is located at a variable distance upstream of the cleavage site and functions to enhance the strength of a polyadenylation site (Russo et al., 1991, 1993; Egli et al., 1995; Guo et al., 1995a; Henikoff et al., 1983; Henikoff and Cohen, 1984; Irniger et al., 1991; Irniger et al., 1992; Irniger and Braus, 1994; Hou et al., 1994). A somewhat conserved, A-rich positioning element (PE) is the yeast element most similar to the AAUAAA mammalian signal and is responsible for defining the site of cleavage to approximately 20 nucleotides downstream from it (Guo et al, 1995b; Guo and Sherman 1996a, 1996b; Graber et al., 1999a; Graber et al., 1999b; van Helden et al., 2000). The third yeast 3’-end processing signal is the actual site of polyadenylation, which occurs most frequently at a PyA (Py = pyrimidine) sequence. As is the case in mammals, the area flanking the PyA tends to be U-rich (Graber et al., 1999a; Graber et al., 1999b; Bennetzen and Hall, 1982; Heidmann et al., 1994; Russo et al., 1993; Figure 1.2). Variations in the number of U’s present in this area give rise to poly(A) signals of differing efficiencies (Graber et al., 1999a; Graber et al., 1999b; Bennetzen and Hall, 1982; Heidmann et al., 1994; Russo et al., 1993; Dichtl and Keller, 2001). General requirements for the sequences located downstream of the cleavage site
are not well understood. While some genes require downstream signals for efficient cleavage, others seem not to be affected by deletions of nearly all downstream sequences (Hyman et al., 1991; Aranda et al., 1998; Egli et al., 1995; Hou et al., 1994; Peterson and Myers, 1993; Sadhale and Platt, 1992; Chen and Nordstrom, 1992). Finally, the secondary structures around signal sequences have not been intensely studied in yeast, but for some yeast genes they may be important in regulating 3’-end formation (Hyman et al., 1991; Sadhale and Platt, 1992; Doktycz et al., 1998; Stumpf et al., 1996).

In many yeast genes, a group of poly(A) sites can be located downstream of the efficiency and positioning elements. In contrast, most mammalian genes have only one poly(A) site located 3’ of the AAUAAA (Aranda et al., 1998; Hegemann and Fleig, 1993; Ryu and Mertz, 1989). Interestingly, some yeast polyadenylation signals can function in either orientation (Sadhale and Platt, 1992; Aranda et al., 1998; Irniger et al., 1991; Peterson and Mayers, 1993) and not only are efficiency and positioning elements degenerate, they are also redundant giving rise to extremely complex polyadenylation signals (Egli et al., 1995; Guo et al., 1995a; Henikoff et al., 1983).

3’-end processing in plants is not as well defined as in mammals and yeast. However, the cis-elements have been well characterized for several plant genes (Hunt, 1994; Li and Hunt, 1997; Wu et al., 1993; Rothnie, 1996; Graber et al., 1999a; Loke et al., 2005). Like other organisms, the process of mRNA 3’-end formation in plants also requires at least three cis-acting elements, the near-upstream element (NUE), the far-upstream element (FUE), both highly degenerate, and the cleavage site itself, which, like other organisms, consists of a PyA dinucleotide surrounded by a U-rich region (Rothnie, 1996; Hunt, 1994; Graber et al, 1999b; Fig 1.2). Plant genes often contain more than one NUE as well as multiple polyadenylation sites. In this situation, each poly(A) site is controlled by a separate NUE (Wu et al., 1993; Dean et al., 1986; MacDonald et al., 1991; Mogen et al., 1992). The NUE, positioned ~10 to 30 nucleotides upstream of the cleavage site, indicates where cleavage will take place. This signal is somewhat similar to the mammalian hexanucleotide sequence in that it can contain the AAUAAA motif but it can also be other related or unrelated sequences and may be as large as 10 nucleotides (Li and Hunt, 1995). The U rich FUE, which may contain upwards of 100 nucleotides, is found approximately 100 nucleotides upstream of the cleavage site and aides in
determining the efficiency of the polyadenylation site (Hunt, 1994; Rothnie, 1996; Li and Hunt, 1997). These elements can influence polyadenylation at more than one poly(A) site within a single gene. NUE and FUE elements are modular and proper spacing between them is very important to their function (Wu et al., 1994; Rothnie et al., 1994).

Comparison of the cis-acting signals in mammals, yeast and plants demonstrates that the yeast and plant poly(A) signals are most similar in that both lack a DSE to promote cleavage efficiency as seen in mammals. However, while the placements of each of the polyadenylation elements differ among these three organisms, there is some evolutionary conservation as each organism contains an A-rich sequence, a U-rich sequence and a site of polyadenylation surrounded by a U-rich region. Also, it appears that spacing between these elements determines the rate of cleavage and polyadenylation at a specific poly(A) site.

**Nuclear mRNA 3’-end Cleavage and Polyadenylation Machinery**

Proper and efficient polyadenylation is an intricate process involving several protein complexes as well as at least three cis-acting RNA sequence elements. In 1985, Moore and Sharp began to isolate and characterize components of the mammalian cleavage and polyadenylation machinery and were soon followed by Butler and Platt (1988) who purified and identified similar proteins in yeast. These organisms showed a high degree of conservation in the machinery needed for mRNA 3’-end maturation although some differences were also found (Figures 1.3 and 1.4).

Mammalian poly(A) tail addition occurs using a specific set of proteins for both cleavage and polyadenylation. The machinery involved in the cleavage step includes the cleavage and polyadenylation specificity factor (CPSF), cleavage-stimulatory factor (CstF), cleavage factors Im and IIIm (CF Im and CF IIIm), RNA polymerase II (Pol II) and poly(A) polymerase (PAP). CPSF and PAP are also key players, along with poly(A)-binding protein nuclear I (PABPN1), in polyadenylation (Minvielle-Sebastia and Keller, 1999; Wahle and Ruegsegger, 1999; Zhao et al, 1999a; Figure 1.3).

CPSF is a multimeric complex composed of 5 polypeptides. The first four are identified by their molecular weights of 160, 100, 73 and 30 kDa (CPSF-160, CPSF-100, CPSF-70, and CPSF-30) (Christofori and Keller, 1988; Gilmartin and Nevins, 1989;
Takagaki et al., 1988, 1989; Bienroth et al., 1991, Murthy and Manley, 1992). The fifth protein is called the factor interacting with PAP or FIP1 for short (Kaufmann et al., 2004; Figure 1.3). CPSF-160 contains sequences resembling the RNP1 and RNP2 type motifs found in many RNA-binding proteins and is responsible for binding the AAUAAA signal found in mammalian polyadenylation signals (Jenny and Keller, 1995; Murthy and Manley; 1995; Gilmartin et al., 1995). This interaction, however, is not stable and addition of PAP alone results in reduced enzymatic activity (Murthy and Manley, 1995). Only through interactions with the other four CPSF subunits, CstF and PAP, is a stable and processive complex formed on the RNA (Barabino et al., 1997; Bienroth et al., 1991; Jenny and Keller, 1995; Jenny et al., 1996; Murthy and Manley, 1992). CPSF-160 also interacts with the CTD of Pol II suggesting that CPSF-160 not only aids in coordination and regulation of cleavage and polyadenylation, but it may also play a role in transcription termination (McCracken et al., 1997). CPSF-100 and CPSF–73 show 23% identity and 49% similarity and appear to be closely related, but their functions are not well understood (Jenny et al., 1994; Jenny et al., 1996). CPSF-100 has been shown to interact with symplekin which is thought to aid in assembly of CstF as well as the entire polyadenylation complex (Takagaki and Manley, 2000) and CPSF-73 has been shown to interact with CF IIAm (described below) (Takagaki and Manley, 2000; de Vries et al., 2000). Both proteins contain a β-CASP domain, which is found in other enzymes involved in nucleic acid cleavage (Callebaut et al., 2002). This domain consists of a conserved zinc-binding motif that plays a central role in catalysis for these enzymes. The β-CASP domain found in CPSF-100 and CPSF-73 make these subunits prime candidates for the, as of yet, unidentified nuclease responsible for cleavage of pre-mRNA before subsequent addition of the poly(A) tail. CPSF-100, however, contains substitutions within this motif, which reduce the likelihood that this is the subunit responsible for cleavage. On the other hand, CPSF-73 does not contain substitutions in this domain and therefore may be the elusive enzyme responsible for the actual cleavage of the RNA molecule during 3’-end maturation (Aravind, 1999; Callebaut et al., 2002). Further evidence for this possibility has been revealed through UV-cross-linking experiments in which a protein with a similar molecular weight as CPSF-73 can be seen in the vicinity of the pre-mRNA cleavage site (Ryan et al., 2004). CPSF-30 is thought to aid CPSF-160 in
the recognition of RNA substrates, through its interaction with the USE cis-acting factor, and to stabilize the polyadenylation complex through an interaction with PABPN1 (described below; Chen et al., 1999). The last subunit of the CPSF complex is FIP1 (Kaufmann, et al., 2004). This 66-kDa protein interacts with CPSF-30, CPSF-160, CstF-77, PAP and with the poly(U) rich USEs in RNAs and is thought to help define the poly(A) site. Binding of FIP1 to this cis-acting factor stimulates the activity of PAP with or without the AAUAAA motif. However, the activity was not as high as that seen with the CPSF-AAUAAA interaction (Kaufmann et al., 2004).

Cleavage-stimulatory factor (CstF) is a multi-subunit complex that interacts with the DSE as well as CPSF and is required for cleavage but not for poly(A) addition (Gilmartin and Nevins, 1989; Takagaki et al. 1992). It is composed of three polypeptides of 77, 64 and 50 kDa (Gilmartin and Nevins, 1991; Takagaki et al., 1990; Takagaki and Manley, 1994; Figure 1.3). CstF-77 bridges the other two CstF subunits and interacts with CPSF-160 and PAP to help stabilize the polyadenylation complex (Takagaki and Manley, 1994; Takagaki and Manley, 2000, Murthy and Manley, 1995). CstF-64 is the subunit responsible for recognizing and binding the DSE of precursor RNA through an RNP-type RNA binding domain (Gilmartin and Nevins, 1991; Takagaki et al., 1992; Takagaki and Manley, 1997; MacDonald et al., 1994; Beyer et al., 1997; Wilusz and Shenk, 1988). It also interacts with symplekin and a transcription factor termed positive cofactor 4 (PC4) (Takagaki and Manley, 2000; Calvo and Manley, 2001). The final subunit, CstF-50, like CPSF-160, has been found to interact with the CTD of Pol II. These interactions further demonstrate the connections found between pre-mRNA transcription and polyadenylation (McCracken et al., 1997).

Like CstF, cleavage factors I and II (CF Im and CF IIm) are required only for pre-mRNA nucleolytic cleavage. CF Im is composed of three major subunits of 68, 59 and 25 kDa and a fourth minor subunit of 72 kDa (Figure 1.3). The three larger subunits appear to be related, sharing similar amino acid domains and the three smaller proteins can be UV cross-linked to RNA substrates (Ruegsegger et al., 1996; Ruegsegger et al., 1998). In vitro, functional CF Im can be reconstituted using only the 68- and 25-kDa subunits. However, due to the amino acid similarity between the three larger proteins, CF Im-72 and –59 may also be able to interact with CF Im 25 to constitute different
dimer isoforms of the CF Im complex (Takagaki et al., 1989; Ruegsegger et al., 1998). Gel retardation assays have shown that purified CF Im has a high affinity for RNAs containing polyadenylation signal sequences and may increase the stability of the 3’-end processing complex. Kinetic assays of the cleavage reaction indicate that interactions of CF Im with the RNA substrate may be an early step in the assembly of the 3’-end processing machinery. CF Im would then facilitate the recruitment of other processing factors (Ruegsegger et al., 1996; Ruegsegger et al., 1998). The domain organization of CF Im-68 shows a strong similarity to the superfamily of RS-rich splicing factors. Interestingly, CF Im-72, -68 and –25 co-migrate with snRNP splicing factors in sucrose gradient and co-immunoprecipitate with U1 snRNP due to an interaction between CF Im-25 and the U1 70K protein (Awasthi and Alwine, 2003). These interactions again demonstrate the cohesiveness of mRNA 3’-end processing with other processes involved in producing a mature and functional mRNA molecule (Ruegsegger et al., 1998). The second cleavage factor, CF IIm, remains somewhat elusive and thus far has not been purified to homogeneity. Therefore, it has not been characterized to the extent of other cleavage and polyadenylation factors. Fractionation and purification of HeLa cell nuclear extracts separated CF IIm into two components, CF IIAm, which is an essential part of RNA cleavage and CF IIBm, which is thought to play more of a stimulatory role in cleavage (de Vries et al., 2000; Figure 1.3). Proteins identified in the CF IIAm fraction included CF Im, some splicing and transcription factors and Pcf11p and Clp1p, which are homologs of yeast proteins necessary for pre-mRNA 3’-end maturation (see below). The role, if any, that each of these proteins plays in mammalian mRNA maturation is not yet clear, but research has shown that both are true subunits of CF IIAm and hClp1 bridges CF Im and CPSF (de Vries et al., 2000).

RNA polymerase II (Pol II) is well known for its role in transcribing protein-encoding genes. However, the interactions of Pol II with proteins involved in other mRNA processing events, such as capping, splicing, cleavage and polyadenylation, requires the inclusion of Pol II as a necessary factor in these processes. In general, these interactions take place through a unique domain protruding from the large subunit of the enzyme known as the carboxy-terminal domain (CTD). The CTD consists entirely of repeats, 52 in mouse and humans, of the 7-amino-acid consensus sequences YSPTSPH
The enzyme actually responsible for adding adenosine residues to cleaved RNAs was first observed in the early 1960s and became known as poly(A) polymerase (PAP) (Edmonds and Winters, 1976). In vertebrates, multiple forms of PAP mRNA are synthesized from a single gene by alternative splicing (Raabe et al., 1991; Wahle et al., 1991; Thuresson et al., 1994; Ballantyne et al., 1995; Gebauer and Richter, 1995; Zhao and Manley, 1996). The longest mRNA forms encode PAPs I (~82 kDa) and II (~78 kDa), which are distinguished from each other by their C-terminal sequences (Raabe et al., 1991). Other, shorter mRNAs (PAPs III, IV, V, and VI) arise from competition between alternative splicing and polyadenylation sites on the pre-mRNA precursor. However, the predicted protein products have not been found. Functions of these shorter forms are not known at this time but they may be components of an autoregulatory mechanism (Raabe et al., 1991; Wahle et al., 1991; Ballantyne et al., 1995; Gebauer and Richter, 1995; Zhao and Manley, 1996). Of these five PAPs, PAP II is the predominant form in most cells (Martin and Keller, 1996; Martin et al., 1999) and is required for both cleavage and polyadenylation (Raabe et al., 1991; Wahle et al., 1991). This high fidelity enzyme incorporates AMPs onto the mRNA in a non-templated dependent manner and when isolated from the rest of the polyadenylation complex is able to synthesize a tail through non-specific polyadenylation (Manley, 1983; Moore et al., 1986; Wahle, 1991; Zarkower et al., 1986). The poly(A) tail arising from PAP alone is of unregulated length and can be added to any RNA. In this case, the enzyme functions in a purely distributive manor, binding only one adenosine molecule per polymerization event (Edmonds, 1982). Upon the addition of PABPNI (described below) or CPSF, the processivity of the enzyme increases to <10 nucleotides polymerized for each binding
event. Only after the simultaneous addition of both PABPNI and CPSF can PAP synthesize a full-length poly(A) tail in a single processive event and at a specific polyadenylation signal (Bienroth et al., 1993).

To gain a better understanding of the structure of mammalian PAP, Martin et al. (2000) crystallized a truncated bovine PAP in complex with divalent cations and a substrate analog, 3’-dATP. At a 2.5-angstrom resolution, PAP was found to contain three domains including a highly conserved, N-terminal catalytic domain, a central domain and a C-terminal RNA-binding domain. The catalytic domain is structurally homologous to the catalytic “palm” domain of other nucleotidyl transferases such as DNA polymerase β and kanamycin phosphotransferase (Davies et al., 1994; Pelletier et al., 1994; Sawaya et al., 1997) and consists of five-stranded mixed β-sheets and two α-helices (Holm and Sander, 1995; Martin and Keller, 1996; Martin et al., 1999). This domain includes three aspartate residues that are conserved among the nucleotidyl transferase superfamily and is the site of binding for ATP and metal ions (Holm and Sander, 1995; Martin and Keller, 1996; Zhao and Manley, 1996). These aspartate residues, located at positions 113, 115, and 167, coordinate two Mn$^{2+}$ metal ions and position them where the 3’-dATP molecules interact with the active site and catalyze an in-line attack of the RNA’s 3’-OH group on the α-phosphate of the incoming ATP (Wittmann and Wahle, 1997). A central β-strand connects the catalytic domain to the central domain. This central domain does not resemble pol β but does show structural similarity to the allosteric activity domain of ribonucleotide reductase R1. This region is composed of a four-helix bundle and a three-stranded mixed β-sheet and is connected to the C-terminal domain through a hinge-like motif. The C-terminal, RNA-binding, domain is a compactly folded globular domain that resembles an RNA-recognition motif fold and consists of a four-stranded antiparallel β-sheet, flanked by two helices to one side as seen in ribosomal protein S6, phenylalanyl-tRNA synthetase and sex-lethal protein (Lindahl et al., 1994; Goldgur et al., 1997; Handa et al., 1997; Martin et al., 2000). The domain responsible for RNA binding is located between amino acids 488 and 508 (Martin and Keller, 1996). This domain overlaps with the region needed for AAUAAA dependent activity (Thuresson et al., 1994), CPSF binding (Murthy and Manley, 1995) and encompasses a nuclear localization signal (NLS-1; Martin and Keller,
In most DNA- and RNA-binding proteins, an overlap is found between the NLS and the polynucleotide-binding domains. NLS-1 together with a second NLS (NLS-2) about 140 residues downstream of the first are required for efficient localization of PAP to the nucleus (Dingwall and Laskey, 1991, Raabe et al., 1994). The sequence surrounding NLS-2 is dispensable for both the catalytic activity and for the RNA substrate binding activity of PAP (Martin and Keller, 1996; Raabe et al., 1994; Zhelkovsky et al., 1995). This C-terminal region also contains multiple phosphorylation sites for the cyclin-dependent kinase (cdk) p34<sup>cdc2</sup>-cyclin B. It is through this phosphorylation/dephosphorylation that PAP is regulated during the cell cycle and oocyte maturation with enzymatic activity decreasing upon phosphorylation (Abuodeh et al., 1998; Colgan et al., 1996; Colgan et al., 1998; Ballantyne et al., 1995). The C-terminal 69 residues of PAP hosts several protein-protein interactions including CF 1-25 which is known to aid in the assembly of the polyadenylation machinery at the mRNA 3' processing site and could play an important part in the coordination of this assembly and/or the regulation of the PAP (Kessler et al., 1995; Kim and Lee, 2001) and with CFI through the 25 kDa subunit (Kim et al., 2001). Amino acids 720 to 739 contain a PAP regulatory domain (PDR) that links polyadenylation to pre-mRNA splicing events via protein-protein interactions with splicing factors U1A and U2AF65. These splicing factors have been well documented as PAP regulatory proteins when bound to the PDR (Boelens et al., 1993; Gunderson et al., 1994; Gunderson et al., 1997; Vagner et al., 2000) and with CFI through the 25-kDa subunit (Kim et al., 2001).

Other active PAPs, arising from different genes, have been found and include an intronless, testis-specific, cytoplasmic PAP, named TPAP (PAP<sub>β</sub>). TPAP is expressed predominantly in round spermatids and thought to be involved in the additional extension of poly(A) tails of pre-existing mRNAs in haploid germ cells (Kashiwabara et al., 2000). Another PAP gene, neo-PAP, has been found in a human tumor cell cDNA library. And finally, PAPγ, which is encoded by a separate gene, termed PAPOLG (Lee et al., 2000; Topalian et al., 2001; Kyriakopoulou et al., 2001). Neo-PAP, an SRP RNA adenylating enzyme, is overexpressed in the nucleus of human neoplasms and unlike other mammalian PAPs, there is only one form of neo-PAP. It is very similar to the active forms of PAP mentioned above, however, the CTD of neo-PAPs differs from these other
previously identified PAPs. These differences give rise to alternatives in the phosphorylation states of neo-PAP and suggest that they are regulated by distinct mechanisms (Topalian et al., 2001). PAP\(\gamma\) is a 90 kDa, nuclear localized isoform with similar sequences and exon/intron patterns to that of PAP (77-kDa) and it is thought to have resulted from gene duplication (Thuresson et al., 1994). This enzyme was first purified as an SRP RNA adenylating enzyme, containing a PAP regulatory domain (PRD). This gene acts very similar to the previously described PAPs. However the CTD does differ to allow for different phosphorylation patterns (Perumal et al., 2001; Kyriakopoulou et al., 2001). This gives rise to the possibility that each of these PAP isoforms performs a specific task within the cell and these tasks may be further regulated within different steps of the cell cycle.

CPSF and PAP suffice for poly(A) addition to a pre-cleaved RNA substrate. However, rapid elongation and control of poly(A) tail length requires an additional factor, PABPN1 (Bienroth et al., 1993). The 33-kDa PABPN1 contains a glutamate-rich N-terminal domain, a single RNP domain in its middle region and an arginine-rich C-terminal domain. The protein tends to form oligomers and binds to the poly(A)tail with a minimum interaction site size of \~10-11nts (Nemeth et al., 1995; Wahle et al., 1993). Upon binding, a circular protein-RNA complex is formed which may control the length of the poly(A) tail (Wahle, 1995). PABPN1 binds to PAP to tether it to the RNA and stimulate polyadenylation (Bienroth et al., 1993; Chen et al., 1999; Kerwitz et al., 2003). Although there are substantial differences between yeast and mammals in terms of the nature of the polyadenylation signal, a striking degree of conservation among trans-acting protein subunits utilized during mRNA 3’-maturation is seen between these organisms (Wahle and Ruegsegger, 1999; Zhao et al., 1997). Fractionation of whole-cell yeast extracts has identified several distinct activities that contribute to the efficiency and accuracy of yeast 3’-end cleavage and polyadenylation (Chen and Moore 1992; Kessler et al., 1996). Factors responsible for recognition of the poly(A) signal, accurate endonucleolytic cleavage and subsequent polyadenylation of the RNA precursor are cleavage factors I and II (CF I and CF II), polyadenylation factor I (PF I), poly(A) polymerase (Pap1p) and poly(A) binding proteins (Pab1p and Nab2p) (Lingner, 1991a, 1991b; Hector et al., 2002). A factor containing both CF II, PF I and other
polyadenylation subunits has been identified by affinity chromatography. This factor can provide both CF II and PF I activities and has been designated the cleavage-polyadenylation factor (CPF) (Ohnacker et al., 2000; Figure 1.4).

Cleavage Factor I (CF I) is responsible for recruiting the polyadenylation complex near the polyadenylation site. The CF I complex is functionally analogous to mammalian CPSF in that it is necessary for both cleavage and polyadenylation. However, their subunit compositions are different. CF I can be separated into two activities, CF IA and CF IB (Kessler et al., 1996). CF IA consists of four polypeptides identified as Rna14p (76 kDa), Rna15p (38 kDa), Pcf11p (70 kDa), and Clp1p (50 kDa; cleavage/polyadenylation protein 1) (Amrani et al., 1997a; Kessler et al., 1996; Minvielle-Sebastia et al., 1994; 1997; Preker et al., 1997). The Rna14 gene gives rise to three unique transcripts generated through alternative polyadenylation sites within the same gene (Minvielle-Sebastia et al., 1991; Mandart and Parker, 1995) and the protein can be found in both the nucleus and the mitochondria (Bonneaud et al., 1994; Rouillard et al., 2000). Its role in the mitochondria is not well understood at this time and will not be addressed in this review. The Rna14p plays a similar role in yeast as that of CstF-77 in mammals in that it forms a bridge between CF IB and CF IA by connecting Rna15p and Hrp1p (see below; Gross and Moore, 2001a; Takagaki and Manley, 1994). Interactions between Rna14p and a subunit of PF I, Pfs2p (described below), have also been observed (Gross and Moore, 2001a). These interactions are thought to contribute to the binding strength and/or specificity of CF I. Rna14p has also been shown to associate with the phosphorylated CTD of Pol II (Barilla et al., 2001). Rna15p is the subunit responsible for the recognition of the A-rich positioning element (PE), which occurs through the RNP-type RNA-binding motifs found on Rna15p. This interaction is strengthened when Rna15p is in complex with Hrp1p (see below) and Rna14p (Gross and Moore, 2001b; Minvielle-Sebastia et al., 1991; Takagaki and Manley, 1994). The RNP-type RNA-binding motifs are similar to those found in the mammalian counterpart of Rna15p, CstF-64, however, they cannot recognize the same RNAs (Takagaki and Manley, 1997). Rna15p also interacts with a cell-cycle specific transcription factor Res2p, a factor required for transcription termination (Aranda and Proudfoot, 2001) and, like CstF-64, Rna15p also interacts with the transcriptional co-activators Mbp1 and Sub1
(PC4 in mammals; Calvo and Manley, 2001; Aranda and Proudfoot, 2001). Pcf11p and Clp1p show similarity to subunits found in the mammalian CF IIm complex (hPcf11 and hClp1; de Vries, 2000). Pcf11p interacts with both Rna15p and Clp1p (Amrani et al., 1997a; Zhao et al., 1999a; Gross and Moore, 2001a) and with the CTD of Pol II (Barilla et al., 2001; Sadowski et al., 2003).

CF IB contains only the 73-kDa polypeptide, Hrp1p (Kessler et al., 1997). The HRP1 gene was previously identified as a gene encoding a protein involved in mRNA export that can be shuttled between the nucleus and the cytoplasm (Kessler et al., 1997). Its role in mRNA 3’-end maturation is recognizing and binding the UA-rich enhancer element through RNP-type RNA-binding motifs of which there are two, RNP1 and RNP2 (Chen and Hyman, 1998; Kessler et al., 1997; Valentini et al., 1999; Henry et al., 1996; Minvielle-Sebastia and Keller, 1999). Structurally, Hrp1p is related to the mammalian heterogeneous nuclear ribonucleoproteins (hnRNPs), a family of nuclear RNA-binding proteins (Weighardt et al., 1996; Kessler et al., 1997). Hrp1p interacts with both Rna14p and Rna15p and is thought to play an important role in regulating cleavage site utilization (Kessler et al., 1997; Minvielle-Sebastia et al., 1998).

The second cleavage factor (CF II) has been implicated in poly(A) site recognition through two of its four subunits, Yhh1p (150 kDa) and Ysh1p (100 kDa) (Stumpf and Domdey, 1996; Zhao et al., 1997). The remaining subunits are Ydh1p (105 kDa) and Pta1p (90 kDa) (Zhao et al., 1997; Zhao et al., 1999b). Yhh1p, the yeast homolog of CPSF-160 (Stumpf and Domdey, 1996), is an RNA-binding protein hypothesized to be involved in U-rich cleavage/poly(A) site recognition, due to its preference to interact with U-rich sequences (Dichtl et al., 2002a; Dichtl et al., 2002b). This subunit was also found to interact with the CTD of Pol II, Ydh1p (see below), Rna14p, Pcf11p and weakly to Clp1p (Dichtl and Keller, 2001; Kyburz et al., 2003). Ydh1p and Ysh1p appear, like their mammalian homologs CPSF-100 and CPSF-73, respectfully, to be related (Zhao et al., 1997; Jenny et al., 1996). Ydh1p, like Yhh1p, have been shown to interact with the CTD of RNA polymerase II and to play a role in cleavage/poly(A) site recognition (Dichtl and Keller, 2001; Dichtl et al., 2002a; Kyburz et al., 2003). There are multiple interactions between the Ydh1p protein and other 3’-processing subunits including Yhh1p, Ysh1p, Pta1p, Pfs2p and Pcf11p as well as itself.
These interactions could indicate that this subunit plays an important role in the assembly and structural order of the CPF complex and in associating yeast 3’-end processing with Pol II mediated transcription (Kyburz et al., 2003). Ysh1p interacts with Cilp1p, Pcf1lp, and Yth1p (see below; Barabino et al., 2000; Kyburz et al., 2003). The smallest subunit of CF II is a protein encoded by PTA1, initially identified as an essential gene affecting pre-tRNA processing (O’Conner, 1992; Zhao et al., 1999b). Not much is known about the role Pta1p plays in mRNA 3’-end polyadenylation other than it interacts with Ydh1p (Kyburz et al., 2003). Finally, Pta1p has also been shown to interact with the CTD of Pol II (Rodriguez et al., 2000).

PF I, a multi-protein complex needed for poly(A) addition, but not for cleavage (Chen and Moore, 1992), contains Fip1p (factor interacting with poly(A) polymerase), Yth1p, Pfs1p or Mpe1p and Pfs2p (Preker et al., 1995, 1997; Ohnacker et al., 2000; Vo et al., 2001). Fip1p, a 55 kDa protein and yeast homolog of the human Fip1, directly interacts with Pap1p (see below), Yth1p (see below), and weakly with Rna14p (Barabino et al., 1997; Preker et al., 1995). Fip1p is the only protein known to directly interact with Pap1p and alone it can alter the processivity of this enzyme. By blocking the access of Pap1p to the RNA substrate, Fip1p inhibits poly(A) extension, shifting Pap1p from a processive to a distributive mode of poly(A) synthesis (Preker et al., 1995; Zhekovsky et al., 1998; Helmling et al., 2001). Fip1p is also thought to aid in polyadenylation by tethering Pap1p to the CPF complex and to RNA through its interactions with Pfs2p and Yth1p (see below; Barabino et al., 1997; Barabino et al., 2000; Ohnacker et al., 2000) as well as to CF I through its interaction with Rna14p (Preker et al., 1995). Yth1p (the yeast homolog of mammalian CPSF-30) interacts with Ysh1p, Pfs2p, Pfs1p and Fip1p (Preker et al., 1995; Barabino et al., 1997; Barabino et al., 2000; Ohnacker et al., 2000). Like the CF II subunits, Yhh1p and Ydh1p, Yth1p is also able to identify U-rich sequences on the RNA molecule and appears to aid in cleavage site selection as well as polyadenylation (Barabino et al., 2000). The 58 kDa, Pfs1p, short for polyadenylation factor I subunit, is thus far uncharacterized. However, a protein preliminarily characterized under the name Mpe1p, is thought to be the Pfs1 protein previously identified. This protein has been shown to interact with Pcf1lp, a subunit of CF IA, but does not appear to be directly involved in the CPF complex. However, it is required for specific cleavage and
polyadenylation of the pre-mRNA substrate (Vo et al., 2001). The last subunit of the PF I complex is the 53 kDa protein Pfs2p (most likely yeast homolog to mammalian CstF-50) and, like Pfs1p, it has not been well characterized. Pfs2p interacts with Rna14p, Ysh1p and Fip1p forming a physical link between CF II-PFI and CF IA (Ohnacker, 2000). Interestingly, in the fission yeast Schizosaccharomyces pombe, Pfs2p has also been shown to play a role in chromosome dynamics. Mutants of this gene product showed defects in chromosomal attachment to the mitotic spindle, giving rise to a chromosome missegregation, as well as defects in mRNA 3’-end processing (Wang et al., 2005). This new finding is a further demonstration of the multitude of interactions found between mRNA 3’-end processing factors and other cellular processes.

The yeast poly(A) polymerase, Pap1p, is the enzyme that synthesizes the poly(A) tail and was the first 3’-end processing factor to be purified and identified from yeast (Lingner et al., 1991b; Patel and Butler, 1992). Sequence comparisons show that the yeast and mammalian PAP proteins are very similar within the first 400 N-terminal amino acids, a region that contains the N-terminal catalytic domain, the central domain and the C-terminal RNA-binding domain (Martin and Keller, 1996; Ohnacker et al., 1996). Like its mammalian counterpart, the crystal structure of a truncated form of the enzyme (the N-terminal 537 amino acids), alone and in complex with 3’-dATP, has been generated (Bard et al., 2000). When isolated from other proteins involved in mRNA 3’-end maturation, Pap1p retains polymerase activity, however, unlike their mammalian counterpart that adds adenosine residues to the RNA primer in a distributive manner, the yeast Pap1p adds them in a processive manner giving rise to long, non-templated, stretches of adenosine nucleotides to an RNA primer at unspecified sites (Zhelkovsky et al., 1998). It is only after the addition of Fip1p that Pap1p can function in a regulated and controlled fashion (Helmling et al., 2001). Unlike the mammalian system, yeast Pap1p is not necessary for efficient cleavage of RNA (Mandart and Parker, 1995).

Yeast, like mammals, controls poly(A) tail growth through poly(A) binding proteins. In yeast, the major hnRNPs associated with the poly(A) tails of mRNA in both the nucleus and cytoplasm are Pab1p and Nab2p (70 kDa and 55 kDa respectfully) (Anderson et al., 1993; Adam et al., 1986; Setyono and Greenburg, 1981; Swanson and Dreyfuss, 1988; Hector et al., 2002). In the nucleus, Nab2p and/or Pab1p are thought to
bind the poly(A) tail as it is being manufactured then, at some length, Nab2p and/or Pab1p suppresses the activity of Pap1p by preventing its further access to the RNA substrate. Nab2p and/or Pab1p may also participate in mRNA export to the cytoplasm. In the cytoplasm, Pab1p binds the adenosine residues and helps to recruit a poly(A)-specific nuclease (PAN) which deadenylates the poly(A) tail until a tail length of 50 to 90 nucleotides is reached (Brown and Sachs, 1998; Deardorff and Sachs, 1997; Lowell et al., 1992; Zhelkovsky et al., 1998; Hector et al., 2002). PAN contains at least two subunits, Pan2p (127 kDa), which interacts with Pab1p, and Pan3p (76 kDa), which directly interacts with Pan2p (Boeck et al., 1996). Deletion of either PAN gene results in an increase in mRNA poly(A) tail length as well as the loss of Pab1p-stimulated PAN activity (Boeck et al., 1996; Brown et al., 1996). Pab1p remains associated with the tail and, with the translation initiation machinery, forms a ‘closed loop’ structure (Jacobson, 1996) that promotes translation initiation by recruiting 40S ribosomal subunits and interacting with the initiation factor eIF4G which, directly bind to the cap-binding protein eIF4E (Tarun and Sachs, 1996; Tarun et al., 1997; Wells et al., 1998). Pab1p can also be regulated by Pbp1p (Pab1p-binding protein). Pbp1p may either suppress the activity of Pab1p or prevent the association of PAN with Pab1p (Mangus et al., 1998). Once Pab1p is removed from the polyadenylate tail, deadenylation-dependent mRNA turnover begins (Tarun and Sachs, 1995, 1996; Tarun et al., 1997; Caponigro and Parker, 1995). Interactions of Pab1p and CF IA, specifically with Rna15p, have also been demonstrated (Minvielle-Sebastia et al., 1997; Amrani et al., 1997b).

The interplay of RNA polymerase II with yeast mRNA 3’-end processing factors has been noted. However, what influence each has on the other is not well understood. Pol II has not been identified specifically as a yeast mRNA 3’-end processing factor, as in mammals, and there have been no reports of the cleavage and polyadenylation steps being affected by mutations of Pol II. On the other hand, mutations involving the poly(A) signals and/or mRNA 3’-end maturation machinery can result in transcription defects (Russo and Sherman, 1989; Birse et al., 1998; Greger et al., 2000). Also, as mentioned above, there are several interactions involving the CTD of Pol II and yeast cleavage and polyadenylation subunits such as Pcf1lp a subunit of CF IA and three subunits from CF II (Pta1p, Yhh1p and Ydh1p; Barilla et al., 2001; Rodriguez et al.,
There is also a link between 3’-end processing machinery (Rna15p) and transcriptional co-activators (Sub1 and Mbp1) known to aid in initiation of transcription by Pol II (Aranda and Proudfoot, 2001; Calvo and Manley, 2001).

Our understanding of the plant polyadenylation apparatus, compared to the mammalian and yeast systems, is not as well developed. However, progress is being made and many plant genes have been found that encode proteins that share homology with mammalian and yeast polyadenylation factors (Arabidopsis Genome Initiative, 2000; Table 1.1). Recently, several protein-protein interactions have been found between some of these proteins.

Plant poly(A) polymerase was first described in 1968 (Sachar) and has since been observed in many plant species such as maize, tobacco, wheat, spinach, pea and Arabidopsis (Mans and Huff, 1975; D’Alessandro and Srivastava, 1985; Burkhard and Keller, 1974; Kapoor et al., 1993; Verma and Sachar, 1994; Lisitsky et al., 1996; Das Gupta et al., 1995; Li et al., 1996; Hunt, 2000). Some of the enzymes described represent chloroplast-localized PAPs involved in RNA degradation and will not be discussed further (Burkhard and Keller, 1974; Das Gupta et al., 1995, 1998). The other types of plant PAPs are responsible for the addition of a polyadenylate tract to the 3’-end of cleaved mRNAs. These PAPs differ in their apparent molecular weights and subunit structures but all have similar biochemical properties to each other and to the mammalian and yeast PAPs. Multiple forms of these PAPs have been found in wheat and Arabidopsis (Sharma et al., 2002; Addepalli et al., 2004). Protein column purification shows that germinated wheat embryos contain two forms of monomeric PAP (PAPI and PAPII) both ~65 kDa and both which are activated upon phosphorylation in nonspecific polyadenylation assays (Sharma et al., 2002). Four PAP-encoding genes (PAP I-IV, corresponding to chromosomes I-IV) have been identified in Arabidopsis, each giving rise to a functional PAP as determined in non-specific polyadenylation assays (Hunt, 2000; Addepalli et al., 2004). These four proteins show a high degree of conservation with the bovine PAPs at their N-terminus (Figure 1.5) but the C-termini have few similarities among themselves or with other eukaryotic PAPs (Figure 1.6). Three of the four enzymes (PAP I, PAP II and PAP IV) contain 700-800 amino acids (~83-95 kDa).
with a definable nuclear localization signal while the fourth (PAP III) is much smaller (482 amino acids, 57 kDa) and lacks the NLS. RT-PCR analysis has shown that the four PAP gene products give rise to alternatively spliced forms within specific plant tissue indicating that alternative splicing may be an important regulator for gene expression (Addepalli et al., 2004; Figure 1.7). Another type of regulation for these enzymes appears to be hormonal. Gibberellic acid, when applied to embryo-less wheat half-seeds was found to stimulate PAP activity while abscisic acid and auxin both inhibited activity (Berry and Sachar, 1981; 1982; 1983). cAMP, which has been reported to mimic hormones and to act as a second messenger in other plant systems, has also been shown to play a stimulatory role in PAP activity in *Cicer arietinum* (Chick pea) (Assmann, 1995; Bolwell, 1995; Praveen et al., 1997; Praveen et al., 2000). An interaction between at least one form of the chromosome II PAP and the recombinant Arabidopsis CPSF-100 protein has been seen. However, this interaction is unique to plants and has not been noted in other eukaryotic systems demonstrating the uniqueness of polyadenylation machinery among organisms (Elliott et al., 2003). Mutations in the CPSF-100 or CPSF-73 genes leads to embryo lethality and thus suggests that polyadenylation factors may also play roles in other plant processes (Meinke et al, 2003; Xu et al., 2004). The Arabidopsis FY protein (a homolog of the yeast Pfs2p protein) helps to regulate flowering-time through an interaction with FCA, a flowering-time regulatory protein. This interaction gives rise to alternative polyadenylation of FCA-encoding RNAs, thus helping to control FCA expression levels (Simpson et al., 2003). Another difference between the plant and mammalian system is observed in the Arabidopsis homologs to CstF-50 and CstF-77, in that the AtCstF-50 and –77 subunits do not interact as is seen in mammals. However, the interaction between AtCstF-77 and –64 proteins is conserved (Takagaki and Manley, 2000; Yao et al., 2002).

Poly (A) binding proteins have also been identified in Arabidopsis. PAB3 was found to rescue a yeast strain mutated at the Pab1p Delta loci by delaying the onset of mRNA deadenylation (Chekanova and Belostotsky, 2003). A second PABP, PAB5, which is restricted to pollen and ovule development and early embryogenesis, was also capable of rescuing a PABP deficient yeast strain (Belostotsky and Meagher, 1996).
Figure 1.1. Eukaryotic mRNA Processing.

Endonucleolytic cleavage between the AAUAAA and a U/GU rich region downstream produces an upstream fragment ending with a 3'-OH and a downstream fragment beginning with 5’-phosphate. The polyadenylate tail is added to the upstream fragment and the downstream fragment is degraded (Adapted from Wahle and Keller, 1996).
Figure 1.2. Depiction of a Simplistic Poly(A) Signal For Mammals, Yeasts and Plants.

USE, auxiliary upstream enhancer; DSE, downstream element; EE, efficiency element; PE, positioning element; FUE, far-upstream element; NUE, near-upstream element; nt, nucleotide; CA and PyA, dinucleotide sequence where cleavage actually occurs on the 3’-side (Adapted from Rothnie, 1996).
Figure 1.3. Schematic Representation of the Mammalian mRNA 3’-End Processing Complex.

The mammalian cleavage complex assembles through a combination of cis-elements and trans-acting factors (see text for details). Subunits involved in individual factors are represented by unique color patterns specific for that factor.
Figure 1.4. Schematic Representation of the Yeast mRNA 3’-End Processing Complex.

The yeast cleavage complex assembles through a combination of cis-elements and trans-acting factors (see text for details). Subunits involved in individual factors are represented by unique color patterns specific for that factor.
Table 1.1. Arabidopsis Genes Encoding Polyadenylation Apparatus Subunits.

This set of data is annotated according to the mammalian and yeast polyadenylation factor subunit that corresponds to the Arabidopsis gene, and organized according to the relevant mammalian factor (e.g., CPSF, CstF, etc. (http://www.uky.edu/~aghunt00/polyA2010.html).

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Figure 1.5. Alignment of the Conserved Portion of the Four Arabidopsis PAPs with the Bovine PAP.

The longest predicted amino acid sequences from the four Arabidopsis nPAP genes were aligned with each other and the bovine PAP sequence (Genbank accession CAA43782) using ClustalW and the alignments of the first 500 amino acids (approximately) displayed using MacBoxshade. Amino acids that are identical in all five polypeptides are shaded black, with white lettering. Positions that are similar and conserved are shaded gray, with black lettering. The N-terminal nuclear localization signal of the bovine enzyme is underlined (Addepalli et al., 2004).
Figure 1.6. Alignment of the C-Terminal Domains of the Four Arabidopsis PAPs with the Bovine PAP.

The longest predicted amino acid sequences from the Arabidopsis nPAP genes on chromosomes I, II, and IV were aligned with each other and the bovine PAP sequence (Genbank accession CAA43782) using ClustalW and the alignments of the C-terminal 200-300 amino acids (approximately) displayed using MacBoxshade. Amino acids that are identical in all five polypeptides are shaded black, with white lettering. The nuclear localization signal of the bovine enzyme is underlined with solid black lines, and the domain that interacts with U2AF is highlighted in bold with a thicker black line (Vagner et al., 2000; Addepalli et al., 2004).
Figure 1.7. Alternative Splicing of PAP-Encoding mRNAs In Different Plant Tissues.

Four hundred nanograms of total RNA isolated from the indicated tissue (denoted on the top: L – leaf, S – stem, R – root, F – flower) was analyzed by RT-PCR using primers that flank intron 6 for PAP I, PAP II and PAP IV and intron 5 for PAP III. RT-PCR products were separated on agarose gels and visualized. The direction of migration of the DNA is from top (negative) to bottom (positive). Results obtained with primers specific for an Arabidopsis tubulin gene (At5g62690) are shown for comparison (Addepalli et al., 2004).
CHAPTER TWO
Characterization of Promoter Expression Patterns Derived From Four Arabidopsis thaliana Poly(A) Polymerase Genes

INTRODUCTION

An essential step in mRNA maturation in eukaryotic cells is the accurate and efficient processing of the 3’-end of the primary RNA transcript, resulting in the addition of a poly(A) tail (Proudfoot, 1991). This process has been shown to affect other cellular events such as transcription termination (Whitelaw and Proudfoot, 1986; Logan et al., 1987; Connelly and Manley, 1988), mRNA splicing (Niwa et al., 1990a; Niwa et al., 1990b; Boelens et al., 1993; Lutz et al., 1996) and mRNA export from the nucleus (Whitelaw and Proudfoot, 1986; Connelly and Manley, 1988). The poly(A) tail itself appears to be involved in numerous aspects of RNA metabolism including mRNA nuclear export efficiency, message stability, and initiation of translation (Bernstein et al., 1989; Beelman and Parker, 1995; Boeck et al., 1996; Carpousis et al., 1999; Curtis et al., 1995; Ford et al., 1997; Lewis et al., 1995; Caponigro and Parker, 1995; Gallie, 1991; Craig et al., 1998; Proweller and Butler, 1997; Preiss and Hentze, 1998; Tarun and Sachs, 1995; Sachs et al., 1997).

Poly(A) polymerase (PAP) is the enzyme that catalytically adenylates pre-mRNAs in mammals and yeast. In vertebrates, multiple isoforms of PAP have been identified and, initially, two PAP forms were described, PAP I (70 kDa) and PAP II (83 kDa). These two isozymes differ in their C-terminus and are generated by alternative splicing (Raabe et al., 1991; Wahle et al., 1991; Zhao and Manley, 1996). In mammalian PAP I, the C-terminal region contains multiple phosphorylation sites and is important for cell cycle regulation and oocyte maturation (Abuodeh et al., 1998; Colgan et al., 1996; Colgan et al., 1998; Ballantyne et al., 1995). Mammals also contain truncated forms of PAP mRNAs corresponding to the 5’ half of the gene, but they are not translated in vivo (Wahle et al., 1991; Ballantyne et al., 1995; Gebauer and Richter, 1995; Zhao and Manley, 1996; Martin and Keller, 1996).
Our knowledge about mRNA 3'-end maturation in yeast and mammals greatly exceeds our understanding of this process in plants. Due to the extended involvement of eukaryotic 3'-end processing and processing factors in other cellular events, our lack of knowledge about plant polyadenylation may hamper our understanding of many of these cellular processes. Extensive investigation of the polyadenylation signals of several plant genes has been undertaken and we now have a firm grasp on the nature of these signals (Hunt, 1994; Wu et al., 1993; Rothnie, 1996). Many plant homologs to yeast and mammalian polyadenylation trans-acting factors have been identified in databases (Table 1.1) but little is known about their roles in 3'-end maturation or about their cellular and tissue localization or their biochemical natures. To date, the most well studied plant polyadenylation factor is the catalytic enzyme PAP (Mans and Huff, 1975; D’Alessandro and Srivastava, 1985; Burkhard and Keller, 1974; Kapoor et al., 1993; Verma and Sachar, 1994; Lisitsky et al., 1996; Das Gupta et al., 1995; Li et al., 1996; Hunt, 2000; Sharma et al., 2002; Addepalli et al., 2004; Berry and Sachar, 1981; 1982; 1983; Assmann, 1995; Bolwell, 1995; Praveen et al., 1997; Praveen et al., 2000; Elliott et al., 2003). In Arabidopsis, PAP is coded by a gene family consisting of four members located on chromosomes I, II, III and IV. The primary sequences of the proteins encoded by these genes (PAP I, PAP II, PAP III and PAP IV, respectively) show a high degree of conservation in the N-terminal 2/3 of the enzymes with each other as well as with the bovine PAP I but the C-termini show a large degree of variability among themselves and when compared to bovine PAP I (Addepalli, 2004; Figures 1.5 and 1.6). Moreover, PAP III lacks this portion altogether (Addepalli et al., 2004; Figure 1.6). This is the portion of the mammalian enzyme that is activated, upon phosphorylation, during the cell cycle (Abuodeh et al., 1998; Colgan et al., 1996; Colgan et al., 1998; Ballantyne et al., 1995). However, this does not rule out other mechanisms of cell-cycle regulation for the Arabidopsis PAPs. This is demonstrated in the yeast PAP enzyme that can undergo cell cycle regulated phosphorylation and like the previously mentioned PAPs, this enzyme contains a unique C-terminal domain (Mizrahi and Moore, 2000).

In Arabidopsis, previous Northern blot and RT-PCR analysis has shown that these genes are differentially expressed in various Arabidopsis tissues in unique and overlapping patterns (Figure 1.7). Northern blot analysis showed that each of the four
PAP genes were expressed to a significant extent in flowers and that PAPs I, II and III were expressed, to varying degrees, in stems. PAPs I, III and IV could also be found in the roots. PAP III was the predominantly expressed PAP mRNA in the leaves. Furthermore, RT-PCR analysis demonstrated that alternatively spliced variants of these genes had unique expression patterns within these tissues (Figure 1.7). This indicates that these splicing events may play a regulatory role in Arabidopsis PAP gene expression (Addepalli et al., 2004).

To extend our understanding of these PAP gene expression profiles, we used an in vivo approach in which we fused the 5’ UTR from each PAP gene to the β-glucuronidase (GUS) reporter gene then subsequently transformed these constructs into Arabidopsis plants and selected at least five transgenic T2 lines for each promoter construct for analysis. Here we report the characterization of these Arabidopsis transgenic plant lines expressing GUS under the control of the four Arabidopsis PAP gene promoters.
RESULTS

The PAP promoter::GUS Expression Construct and Transgenic Lines

Preliminary Northern blot and RT-PCR analysis of the four Arabidopsis PAP gene products indicates that these genes appear to be regulated in a tissue specific manner (Addepalli et al., 2004). Promoter regions, defined as the nucleotide sequence contained between the PAP start codon and the coding region of the proceeding gene, of the PAP genes were aligned using CLUSTALW and no significant homology was found (data not shown). To gain a better understanding of how these genes are expressed during plant growth and development, we produced at least five Arabidopsis lines expressing β-glucuronidase (GUS) under control of the promoter of each of the four PAP genes. The expression pattern of each promoter was monitored using GUS activity as a histochemical marker and evaluated in plants at various stages of growth (Figures 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 and 2.10; Summarized in Tables 2.1, 2.2 and 2.3). Results obtained from each of the five Arabidopsis lines, for a single PAP promoter, showed similar results (not shown).

PAP promoter::GUS Expression in One-Day-Old Embryos

Figure 2.3 shows GUS expression in cotyledons for the PAP I, PAP III and PAP IV promoters. The PAP I and PAP IV promoters powered GUS expression throughout the cotyledons with the PAP I promoter showing slightly more GUS expression than the PAP IV promoter. The PAP III promoter gave rise to GUS expression only in the cotyledon tips. The PAP II promoter produced no GUS activity in one-day-old embryos.

Expression of PAP promoter::GUS Constructs in Light-Grown Seedlings

The expression of the chimeric PAP promoter::GUS constructs in 6-day-old light-grown seedlings is shown in Figure 2.4. In these seedlings, the expression patterns of PAP I and PAP IV promoters were almost identical with GUS being expressed throughout the cotyledons and hypocotyls but confined to the vascular tissue in the radicle. The most notable difference between these two promoters could be seen in the radicle. Here the PAP I promoter was more active than the PAP IV promoter. The PAP
II promoter was also active in the hypocotyls, cotyledon and radicles but, in the two latter tissues, expression was limited to the vascular tissue. Compared to the PAP I and PAP IV promoters, the PAP II promoter was less active. The PAP III promoter showed the most unique pattern with GUS being expressed strongly at the cotyledon tips and vascular tissue of the radicle but only slightly in the hypocotyl. Interestingly, the PAP III promoter was the only one active in the radicle tip (Figure 2.4, PAP III inlay). In the primary leaves, PAP I, PAP III and PAP IV promoters were active but to different degrees. The PAP II promoter produced no significant GUS expression in this tissue while the PAP I and PAP IV promoters were active throughout the leaves. The PAP III promoter was active only in the basal marginal region of primary leaves (Figure 2.5).

PAP Expression in Mature Plants and Their Organs

To investigate the activities of the four PAP promoters in adult plants, PAP promoter::GUS transformed plants were grown in the greenhouse for 3 to 6 weeks and subsequently assayed for GUS activity. The various reporter gene constructs were expressed in various tissues of the mature plants (as shown in Figures 2.6, 2.7, 2.8, 2.9 and 2.10) and again displayed unique and overlapping gene expression patterns.

All four PAP promoters were active in the rosette leaves of 3 to 4-week-old plants (Figure 2.6). The PAP I and PAP IV promoters were active throughout the leaves, especially in the vascular tissue and leaf petioles. The PAP II promoter was also active in the leaf petioles and vascular systems but not to the extent seen with the PAP I and PAP IV promoters. The PAP III promoter was most active in the petioles of the young leaves and young leaf tips. Some light staining of the leaf vascular systems could also be seen.

Figure 2.7 shows that the PAP I, PAP III and PAP IV promoters are active in the primary and secondary root systems. The promoters from the PAP I and PAP IV genes showed very similar patterns, appearing to be confined to the vascular system with the PAP I promoter being the most active. The PAP I plants also showed very light staining in the root tips (Figure 2.7, PAP I inlay) that was not seen in the 6-day-old seedlings (Figure 2.4). The PAP III promoter was active throughout the root tissue excluding the elongation zone but was quite strong in the root tip itself (Figure 2.7, PAP III inlay). The PAP II promoter was not active to a noticeable degree in the roots or root tips.
A quite diverse expression pattern was observed among the various gene family members in flowers (Figure 2.8). The PAP I promoter::GUS plants contained only light staining in the filament and vascular tissue of the petal. The PAP II plants showed heavy staining in the style, receptacle and pedicel and only light staining of sepal vascular tissues. The PAP III promoter activity was seen in the stigma and the pollen of mature anthers, but not immature anthers (not shown). The PAP promoter most widely expressed in flowers was the PAP IV promoter, which was very active in pollen, sepals, style and stigma and lightly in the pedicel (not shown).

Figure 2.9 shows that PAP IV is the most heavily expressed PAP gene family member in siliques and is found throughout the silique structures and was the only PAP to show GUS activity in the seed, confined to the funiculus (Figures 2.9 and 2.10). PAP I, PAP II and PAP III promoters showed GUS activity in the abscission zone, in addition, PAP II had activity in the valve of the silique and PAP III in the stigma (Figure 2.9).

To obtain an estimate of the relative levels of each of the four Arabidopsis PAP gene products, locus Ids, corresponding to each PAP, were used to query the Genevestigator *Arabidopsis thaliana* Microarray Database and Analysis Toolbox (Zimmermann et al., 2004). Each of the PAP gene products was shown to accumulate in all developmental stages (Figure 2.11). However, the PAP IV product accumulation levels are consistently at least twofold higher than those of the remaining PAPs. The second highest accumulator was PAP I, followed by PAP II then PAP III. A second analysis of accumulation by tissue rather than developmental stage produced similar results.
In mammals and yeast, poly(A) polymerases (PAPs) have been shown to play a vital role in polyadenylation of pre-mRNAs but little is known about their activities in plant polyadenylation reactions (Minvielle-Sebastia and Keller, 1999; Wahle and Ruegsegger, 1999; Zhao et al, 1999a). Searches of public databases found four potential Arabidopsis PAP genes. The proteins encoded by these genes show a strong resemblance to each other, especially at their N-termini. Recombinant proteins for each were found to be active in non-specific polyadenylation assays indicating that they are each functional enzymes (Addepalli, 2004). Previous studies have shown that the gene products for these four genes are expressed in a tissue specific manner and that alternative splicing can affect their expression patterns (Addepalli et al., 2004). The physiological significance of having four PAP genes has yet to be determined. When gene duplication occurs, there is the potential that the enzymatic properties of the isozymes are redundant. On the other hand, these duplication events could lead to increased variation in the expression profiles of the individual enzymes in which each evolves differing enzymatic characteristics and roles within differing cells and or tissues throughout the life cycle of the plant. Microarray experiments (https://www.genevestigator.ethz.ch/; Figure 2.11) and previous RT-PCR analysis for these gene products have shown that these genes are expressed throughout the plant at specific lifecycle stages. To shed light on the developmentally regulated expression patterns of the four Arabidopsis PAP genes, a visual global comparative of the PAP promoters, fused to a reporter gene, could allow us to follow expression changes, for each PAP promoter, during the development of a single plant. In addition, this spatial-temporal analysis might offer clues that would help to determine if these genes share redundant or non-functional roles in the plant. To carryout this study, we introduced GUS fusions containing DNA sequences located 5’ of the PAP protein coding regions into the Arabidopsis genome.

We found that, while each PAP promoter::GUS construct shows a unique activity profile, there are several overlaps in their activity patterns. All four of the PAP promoters powered GUS activity in seedlings, mature leaves, flowers, siliques, and seeds to varying degrees and location within these tissues (Tables 2.1, 2.2 and 2.3; Figures 2.4, 37.
In embryos, seedlings and the rosette, the PAP I and IV promoters gave the highest GUS activity for all the tissues examined. In embryos, GUS expression caused by these PAP promoters was restricted to the cotyledons (Figure 2.3). In seedlings and mature plants, expression was located throughout the cotyledon, hypocotyl, primary leaves and rosette leaves, especially in the vascular tissues, but was restricted to the vascular tissue of the radicle and roots (Figures 2.4, 2.5, 2.6 2.7). These results paralleled expression results from digital northerns obtained from array/gene chip analysis (Figure 2.11). Of the remaining two PAP promoters, PAP II showed no GUS activity in 1-day-old embryos and PAP III had GUS staining only at the tips of the cotyledons (Figure 2.3). The PAP II and PAP III promoters also produced GUS activity throughout the seedling hypocotyl and radicle of about the same strength and in the vascular tissue of the cotyledon and rosette leaves with GUS staining being slightly stronger with PAP II than PAP III (Figures 2.4, and 2.6). However, the PAP III promoter showed very strong GUS activity in the cotyledon leaf tip and, interestingly, was the only PAP promoter to show GUS activity in the radicle tip (Figures 2.4 and 2.6). Comparison of these results with digital northerns paralleled findings for the PAP II promoter but not for the PAP III promoter. While we showed GUS activity for the PAP III promoter in these tissues approximately equal to the PAP II promoter in many cases, digital northerns showed little to no expression in any of these tissues for the PAP III gene (Figures 2.4, 2.6 and 2.11). In the primary leaves and mature roots, the PAP II promoter showed no GUS activity while the PAP III promoter displayed GUS expression only in the basal marginal region of primary leaves and throughout the root tissue. These results are exactly opposite to what was seen in digital northerns (Figures 2.5, 2.7 and 2.11). In mature root tips, both the PAP I, which did not show GUS activity in the radicle tip, and PAP III promoters displayed GUS activity (Figures 2.4 and 2.7). In flowers, GUS activity was seen in both male and female organs, as well as in basic floral structures such as petals, receptacle and pedicels (Figure 2.8). In the filament and the vascular tissue of the petals, the PAP I promoter was the only promoter that gave rise to GUS activity. For petals, digital northerns showed expression of PAP I, PAP II and PAP IV with PAP IV being the most highly expressed, followed by PAP II and then PAP I. It also showed PAP II being expressed in the stamen, to similar levels as PAP I and PAP
III, but filament and anthers, individually, were not analyzed. Both PAP II and PAP IV promoters showed GUS expression in the style, sepals, and pedicel but at differing locations and to differing degrees. In the these tissues, GUS expression from the PAP II promoter was very heavy in the pedicle but confined to the vascular tissue of the sepals and to the tip of the style whereas the PAP IV promoter showed GUS throughout the style and lightly in the pedicel. The PAP II promoter showed GUS activity lightly in the vascular tissues of sepals and continued down into the receptacle was heavily stained. On the other hand, GUS expression from the PAP IV promoter was heavy throughout the sepal, especially in the vascular tissue but not including the receptacle. Digital northerns showed that PAP IV had the highest expression in all these tissues (Figures 2.8 and 2.11). In the stigma and anther, specifically the pollen, GUS activity for PAP III and IV promoters was noted. However, GUS from the PAP III promoter was found only in immature pollen grains. This echoes what was found in digital northerns and, in fact, this was one of only two places that digital northerns showed elevated expression levels for PAP III, the other being in the seed (Figures 2.8 and 2.11). In siliques, the PAP IV showed the most GUS activity, which could be seen throughout the tissue. The remaining PAP promoters had GUS expression in the abscission zone and, in the case of the PAP II promoter, in the valve region. In digital northerns, PAP IV was expressed the highest followed by PAP I, PAP II and PAP III (Figures 2.9 and 2.11). The PAP IV promoter was the only promoter showing GUS activity in seeds and limited to the funiculus. This PAP was also seen in digital northerns, which also showed expression for the remaining PAPs (Figures 2.10 and 2.11).

Promoter expression analysis and digital northerns confirm that each of the four Arabidopsis PAP genes is expressed throughout the plant tissues investigated. However, comparisons between promoter activities of a specific gene to levels of products for that gene, using array/chip analysis, must be carefully considered. In many cases, transcriptional activation of an endogenous promoter does not always give rise to a “stable”, endogenous gene product, nor does it shed light on the possibility of alternative splicing events of the gene products which could alter results found within digital Northern experiments depending on where the probe hybridizes. Therefore, results from promoter activation studies may not always correlate with results obtained using
techniques that investigate gene products. This being said, all we can do is note the similarities and differences with interest but remember that differences may be the result of the previously mentioned reasons.

It was previously shown that each of the Arabidopsis PAP genes can give rise to products that have undergone alternative splicing (Figure 1.7) which, in the studies performed earliew, gave rise to mRNAs that would contain an early stop codon (Addepalli, 2004). Therefore, we must take into consideration the idea that the products arising from transcriptionally active promoters may not give rise to products that are translated into functional proteins, or translated at all for that matter. Some of these products may play more of a regulatory role within cells and deserves further investigation. Nevertheless, these studies show that the four Arabidopsis PAPs are potentially expressed throughout the plant in a temporal-spatial manner.

Interestingly, the PAP gene whose product is the most dissimilar of the four PAPs, PAP III, was shown to have very little accumulation of gene product, in digital northerns (Figure 2.11) throughout much of the life cycle, except in the stamen and slightly in seeds. However, PAP promoter::GUS analysis does not correlate with digital northerns. These analyses have shown that the promoter of the PAP III gene is able to power GUS activity in different tissues and at different times throughout plant development and therefore may play several roles in plant growth and development and indicates that the PAP III gene is a functional gene. Interestingly, this promoter was the only PAP promoter active in the radicle tips of 6-day-old seedlings (Figure 2.4) and could indicate that this PAP plays a role in regulating gravitropism and growth of the root. This portion of the root contains a quiescent center, located just behind the root cap consisting of four seemingly inactive cells. Quiescent and meristematic cells are different in their sensitivity to environmental stresses such as radiation. For example, meristematic cells stop dividing when exposed to x-rays while quiescent cells are unaffected by radiation and soon begin dividing to reform the meristem. Cells in the quiescent center function as a reservoir to replace damaged cells of the meristem. This is important because it organizes the patterns of primary growth in roots van den Berg et al., 1995; van den Berg et al., 1997). The PAP III promoter appeared to be active throughout all the stages of growth in a specific spatial pattern. Because the PAP III promoter was
the only one of the four Arabidopsis PAPs to show GUS activity in these young roots, it may play an important role in the quiescent center and/or meristamic region that the other PAPs cannot fulfill.

While the PAP III gene product is the least like the other Arabidopsis PAPs, it does have similarities with a cytoplasmic spermatocyte-specific PAP (TPAP) identified in mouse (Kashiwabara et al., 2000). Like PAP III, TPAP lacks the C-terminal extension that contains the C-terminal phosphorylation sites and the nuclear localization signals in other mammalian PAPs (Figure 1.6). We have shown, using digital northerns, that the PAP III gene product accumulates in the male organs of Arabidopsis and the PAP III promoter was able to induce GUS expression in mature pollen (Figures 2.8 and 2.11). While these findings do not directly link the PAP III gene to cytoplasmic gametogenic mRNA polyadenylation, it does allow for the possibility of a role for PAP III in this process and is an area deserving further investigation.

It is obvious from these findings that these four Arabidopsis PAP genes are not simply duplications whose products perform redundant functions. Each PAP gene promoter is expressed in a unique pattern that implies that each of the four Arabidopsis PAPs plays an important role within the plant. While this work gives insights into the transcriptional expression patterns for these four genes, it does not consider other likely levels of control beyond the level of transcription that may also play important roles in the ultimate control of PAP activities for these four genes. Assessment of function, i.e. confirmation of the specific reaction catalyzed by each of the genes, as well as the cellular location, poses an even greater challenge. Promoter analysis can tell one whether a gene is transcriptionally active but it cannot identify which reactions the gene product is catalyzing. To answer these questions, further studies are needed.
MATERIALS AND METHODS

Plant Material

*Arabidopsis thaliana* ecotype Columbia was obtained from Lehle Seeds (Round Rock, TX) and used throughout this study. Seeds were germinated and plants cultivated in the greenhouse until maturity with a 16-h light and 8-h dark regime at 22°C.

Preparation of PAP Promoter Constructs and Plant Transformation

To analyze promoter activity the nucleotide sequence between the ATG start codon and the coding regions of the upstream gene for each of the four Arabidopsis poly(A) polymerase genes (corresponding to the chromosome number each gene was located on), AtPAP I, AtPAP II, AtPAP III and AtPAP IV (At1G17980, At2G 25850, At3G06560, At4G32850, respectively) were amplified by PCR. The PAP I, PAP II, PAP III and PAP IV promoters (734 bp, 781 bp, 2111 bp and 1041 bp respectively), upstream of the ATG start codon were amplified with the PII5'1SalI (5'-GTCGACGAGGGCTTATT CAGATGCATTATTAG-3’) and PII3’1NcoI (5’-CCATGGCTATCCTACTTACTTTTGAGCATAG-3’) primer sets, respectively (Table 2.4). For PCR amplification, 25-50-ng total genomic DNA from wild type plants, 100-ng of primers, 0.8-mM dNTPs, 5.0-µl of Ultra HF PCR buffer (Stratagene) and 2.5-units of Pfu Turbo DNA polymerase (Stratagene) were used in 50-µl PCR reactions. PCR amplifications were run for 35 cycles of 92°C for 1 min, 55°C for 1 min and 72°C for 2 min. The amplification products were cloned into pGEM (Promega) and the inserts sequenced. For the latter, sequencing reactions were carried out with the BigDye terminator kit and analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems) following the recommendations of the supplier. Sequence data were analyzed using MacVector software (Kodak). The promoter fragments were excised from pGEM with SalI and Ncol.
and cloned into SalI and NcoI digested pCAMBIA1303 vector (Figures 2.1 and 2.2). pCAMBIA is a binary vector designed for plant transformation and includes the GUSA reporter gene along with hygromycin (Hpt) and kanamycin (Kan) selectable marker genes.

Plant Transformation and Growth

Expression constructs were transferred to Agrobacterium tumefaciens strain GV-3850 and the helper plasmid PRK-2013 by tri-parental mating described by Schardl et al. (1987). Arabidopsis thaliana plants, ecotype Columbia (COL), were transformed using the floral dip method as described by Clough and Bent (1998). These transformants were allowed to grow and seed in the greenhouse under long day conditions (22°C, 16-h light). Seeds were selected on germination medium containing 25-mg/L hygromycin (Sigma). At least five independent homozygous T2 lines for each construct were examined for GUS expression. Homozygous lines were identified by determining ratios of selective marker inheritance in T3 plants on hygromycin-containing media.

Histochemical Localization of GUS Expression

Histochemical analysis of GUS activity in transgenic plants was performed essentially as described by Stomp (1992). Plant tissues were incubated at 37°C for 24 h in a 100-mM sodium phosphate buffer (pH 7.2, 0.5-mM potassium ferrocyanide, 0.5-mM potassium ferricyanide, 0.1% Triton X-100) containing 0.1-mM 5-bromo-4-chloro-3-indolyl glucuronide. Subsequently, the samples were then transferred to 70% ethanol to remove the chlorophyll. Data was recorded by photography (see the following section).

Microscopy Techniques and Photography

Zeiss Stemi SV11 and Zeiss Axioplan 2 microscopes were used for visualizing embryos as well as adult structures. Photographs were taken using a Zeiss Axiocam MRc5 and visualized using AxioVision 4.1 software (Zeiss, Jena, Germany). Images were processed using Adobe ImageReady software (version 2.0; Adobe Systems, San Jose, CA).
In Silico Analysis

Locus identifiers were submitted to the Genevestigator *Arabidopsis thaliana*
microarray database and analysis toolbox (Zimmermann et al., 2004) at
https://www.genevestigator.ethz.ch, where they were assayed against 1434
developmental and tissue-specific Arabidopsis microarray experiment results (Edgar et
al., 2002; Rocca-Serra et al., 2003; Parkinson et al., 2005; Craigon et al., 2004).
Table 2.1. GUS Activity Analysis For Each Arabidopsis PAP Promoter In 1-Day-Old Embryos.

Results were obtained using GUS staining patterns observed in figure 2.3. X - heavy stain; / - light stain.

<table>
<thead>
<tr>
<th>1-DAY-OLD EMBRYOS</th>
<th>PAP I</th>
<th>PAP II</th>
<th>PAP III</th>
<th>PAP IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledons</td>
<td>X</td>
<td></td>
<td>/ (TIPS)</td>
<td>X</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radicle Tip</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2. GUS Activity Analysis For Each Arabidopsis PAP Promoter In 6-Day-Old Light Grown Seedlings.

Results were obtained using GUS staining patterns observed in figures 2.4 and 2.5. X - heavy stain; / - light stain; VT – vascular tissue; BMR - basal marginal region.

<table>
<thead>
<tr>
<th>6-DAY-OLD LIGHT-GROWN SEEDLINGS</th>
<th>PAP I</th>
<th>PAP II</th>
<th>PAP III</th>
<th>PAP IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledons</td>
<td>X</td>
<td>/ (VT)</td>
<td>/ (VT)</td>
<td>X</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>X</td>
<td>/</td>
<td>/</td>
<td>X</td>
</tr>
<tr>
<td>Radicle</td>
<td>X (VT)</td>
<td>/ (VT)</td>
<td>/ (VT)</td>
<td>/ (VT)</td>
</tr>
<tr>
<td>Radicle Tip</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Primary Leaves</td>
<td>X</td>
<td>X (BMR)</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. GUS Activity Analysis For Each Arabidopsis PAP Promoter In 3-6-Week-Old Plants.

Results were obtained using GUS staining patterns observed in figures 2.6, 2.7, 2.8, 2.9 and 2.10. X - heavy stain; / - light stain; VT – vascular tissue; MA – mature anthers.

<table>
<thead>
<tr>
<th>3-6-WEEK-OLD PLANT</th>
<th>PAP I</th>
<th>PAP II</th>
<th>PAP III</th>
<th>PAP IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosetta</td>
<td>X</td>
<td>/ (VT)</td>
<td>/ (MB/VT)</td>
<td>X</td>
</tr>
<tr>
<td>Roots</td>
<td>X (VT)</td>
<td></td>
<td>X</td>
<td>X (VT)</td>
</tr>
<tr>
<td>Root Tips</td>
<td>/</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>X</td>
<td>/ (VT)</td>
<td>/ (VT)</td>
<td>X</td>
</tr>
<tr>
<td>Filament</td>
<td>/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Petal</td>
<td>/ (VT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Style</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Receptacle</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pedicle</td>
<td></td>
<td>X</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Sepal</td>
<td></td>
<td>/ (VT)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Stigma</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Pollen</td>
<td></td>
<td></td>
<td>X (MA)</td>
<td>X</td>
</tr>
<tr>
<td>Siliques</td>
<td></td>
<td></td>
<td>/</td>
<td>X</td>
</tr>
<tr>
<td>Seed</td>
<td></td>
<td></td>
<td>/</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4. Oligonucleotides used for cloning and sequencing.

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER DESIGNATION</th>
<th>SEQUENCE (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAPI</td>
<td>PI 5’ SalI</td>
<td>GTCGACGGGTTATTCAGATGCATTATTAG</td>
</tr>
<tr>
<td></td>
<td>PI 3’ Neol</td>
<td>CCATGGCTATCTGATCTTTTC</td>
</tr>
<tr>
<td>PAPII</td>
<td>PI 1 5’ SalI</td>
<td>GTCGACTTTCACCGCAGAGCGAGACCA</td>
</tr>
<tr>
<td></td>
<td>PI 1 3’ Neol</td>
<td>CCATGGGACAAAGACCTTAACACTAGAAA</td>
</tr>
<tr>
<td>PAPIII</td>
<td>III 5’ SalI</td>
<td>GTCGACTTCCGATCAGAGAAAGCAAACGATCAA</td>
</tr>
<tr>
<td></td>
<td>III 3’ Neol</td>
<td>CCATGGCAGCTACGAAAATTTGAAAGAAAGT</td>
</tr>
<tr>
<td>PAPIV</td>
<td>IV 5’ SalI</td>
<td>GTCGACTGGATTCTAAATATCAAAGTTGCTCTCC</td>
</tr>
<tr>
<td></td>
<td>IV 3’ Neol</td>
<td>CCATGGCATAACAGATTGATAATCCTTAAG</td>
</tr>
</tbody>
</table>
Figure 2.1. Schematic Representation of the T-DNA of the Plasmid pCAMBIA 1303.

Numbers at the end of each PAP promoter::GUS construct represents, in base pairs, the length of the promoter sequence as determined by the length of the nucleotide sequence between the each PAP ATG start codon and the coding region of the upstream gene. The restriction sites SalI and NcoI were added to the promoter sequences during PCR amplification. These fragments were inserted into the SalI and NcoI digested pCAMBIA 1303 vector then transformed into Arabidopsis via Agrobacterium mediated transformation using the floral dip method. Seeds were selected on basic media containing hygromycin.
Figure 2.3. Analysis of GUS Activity In 1-Day-Old Seedlings of Arabidopsis Plants Transformed With PAP promoter::GUS Fusion Constructs.

GUS activity was detected after an overnight reaction period in 1-day-old Arabidopsis plants harboring one of the four Arabidopsis PAP promoter regions (736 bp, 783 bp, 2114 bp and 1834 bp, respectively) fused to GUS.
**Figure 2.4.** Analysis of GUS Activity In 6-Day-Old Seedlings of Arabidopsis Plants Transformed With PAP promoter::GUS Fusion Constructs.

GUS activity was detected after an overnight reaction period in 6-day-old T2 transgenic Arabidopsis seedlings harboring one of the four Arabidopsis PAP promoter regions (736 bp, 783 bp, 2114 bp and 1834 bp, respectively) fused to GUS.
**Figure 2.5.** Analysis of GUS Activity In Primary Leaves of 6-Day-Old Arabidopsis Plants Transformed With PAP promoter::GUS Fusion Constructs.

GUS activity was detected after an overnight reaction period in primary leaves of 6-day-old T2 transgenic Arabidopsis primary leaves harboring one of the four Arabidopsis PAP promoter regions (736 bp, 783 bp, 2114 bp and 834 bp, respectively) fused to GUS.
**Figure 2.6.** Analysis of GUS Activity In Rosette Leaves of 3 to 4-Week-Old Arabidopsis Plants Transformed With PAP promoter::GUS Fusion Constructs.

GUS activity was detected after an overnight reaction period in 3-4 week-old T2 transgenic Arabidopsis rosette leaves harboring one of the four Arabidopsis PAP promoter regions (736 bp, 783 bp, 2114 bp and 1834 bp, respectively) fused to GUS.
Figure 2.7. Analysis of GUS Activity In Roots From 4-Week-Old Arabidopsis Plants Transformed With PAP promoter::GUS Fusion Constructs.

GUS activity was detected after an overnight reaction period in 4 week-old T2 transgenic Arabidopsis roots harboring one of the four Arabidopsis PAP promoter regions (736 bp, 783 bp, 2114 bp and 1834 bp, respectively) fused to GUS.
**Figure 2.8.** Analysis of GUS Activity In Mature Flowers From 6-Week-Old Arabidopsis Plants Transformed With PAP promoter::GUS Fusion Constructs.

GUS activity was detected after an overnight reaction period in 6 week-old T2 transgenic Arabidopsis flowers harboring one of the four PAP promoter regions (736 bp, 783 bp, 2114 bp and 1834 bp, respectively) fused to GUS. Plant structures are denoted with arrows (F: filament; STY: style; R: receptacle; PED: pedicel; SP: sepal; STI: stigma).
Figure 2.9. Analysis of GUS Activity In Siliques From 6-Week-Old Arabidopsis Plants Transformed With PAP promoter::GUS Fusion Constructs.

GUS activity was detected after an overnight reaction period in 6 week-old T2 transgenic Arabidopsis siliques harboring one of the four Arabidopsis PAP promoter regions (736 bp, 783 bp, 2114 bp and 1834 bp, respectively) fused to GUS.
Figure 2.10. Analysis of GUS Activity In Seeds From 6-Week-Old Arabidopsis Plants Transformed With PAP promoter::GUS Fusion Constructs.

GUS activity was detected after an overnight reaction period in 6 week-old T2 transgenic Arabidopsis seeds harboring one of the four Arabidopsis PAP promoter regions (736 bp, 783 bp, 2114 bp and 1834 bp, respectively) fused to GUS.
Figure 2.11. Average Relative Intensity of the Four Arabidopsis PAP Gene Products.

*In silico* analysis of the four Arabidopsis PAP gene product accumulations from 1434 developmental gene chip experiments. Results are given by developmental stage (X-axis) and in terms of gene chip-normalized expression levels (Y-axis). Expression levels are shown to scale. Developmental stages are as follows: 1, 1.0-5.9 days; 2, 6.0-13.9 days; 3, 14.0-17.9 days; 4, 18.0-20.9 days; 5, 21.0-24.9 days; 6, 25.0-28.9 days; 7, 29.0-35.9 days; 8, 36.0-44.9 days; 9, 45.0-50.0 days. Analyses performed via the Genevestigator website https://www.genevestigator.ethz.ch.
CHAPTER THREE

Effects of Silencing the Four Poly(A) Polymerase Gene Family Members in *Arabidopsis thaliana*

INTRODUCTION

Most mRNAs originating within the nuclei undergo 3’-end polyadenylation during mRNA maturation. This polyadenylation is a two-step reaction that involves RNA cleavage, at a specific site, then subsequent addition of a polyadenylate tract. Poly(A) tails play many important roles in RNA metabolism including facilitating nuclear export, increasing the efficiency of translation initiation, and mRNA stabilization in the cytoplasm (Bernstein et al., 1989; Beelman and Parker, 1995; Boeck et al., 1996; Carpousis et al., 1999; Curtis et al., 1995; Ford et al., 1997; Lewis et al., 1995; Caponigro and Parker, 1995; Gallie, 1991; Craig et al., 1998; Proweller and Butler, 1997; Preiss and Hentze, 1998; Tarun and Sachs, 1995; Sachs et al., 1997). Accurate and efficient 3’-end polyadenylation, in both mammals and yeast, requires a multitude of trans-acting factors and can be coupled to transcription as well as other RNA-processing reactions (Wahle and Ruegsegger, 1999; Minvielle-Sebastia and Keller, 1999; Zhao et al., 1999a; Edmonds, 2002; Proudfoot, 2004; Zorio and Bentley, 2004). Poly(A) addition is catalyzed by the enzyme poly(A) polymerase (PAP) a member of the nucleotidyl transferases family (Aravind and Koonin, 1999; Holm and Sander, 1995; Martin and Keller, 1996). In *vitro*, this enzyme can add adenylate residues to RNAs in a non-specific reaction in a non-constitutive fashion. In biological systems, PAP associates with additional proteins to add the polyadenylate tract in a substrate specific, constitutive manner (Minvielle-Sebastia and Keller, 1999; Preker et al., 1997). Polyadenylation has been well studied in both mammals and yeast. However, in plants this process is not as well understood. To further our understanding of mRNA maturation in plants, we have employed several molecular genetic techniques to determine what role PAP plays in overall plant development.
Manipulation of plant metabolism through the use of molecular genetic techniques has been used since the isolation of the first cDNA encoding an enzyme in plants (Bedbrook et al., 1980), introduction of foreign DNA through the use of the Agrobacterium Ti plasmid (Hernalsteens et al., 1980) and the establishment of plant transformation techniques (Bevan, 1984; Horsch et al., 1985). With these methods, plant lines can be developed that partially or completely lack the expression of specific gene products. These plant mutants can be used to provide additional insights into functions of enzymes in metabolism and the essentiality of isozymes from multigene families.

Loss of function mutations in plants, mutated through the insertion of foreign DNA randomly within chromosomes, can be created using transposons (Sundaresen et al., 1995; Martienssen, 1998) or the T-DNA of Agrobacterium tumefaciens (Azpiroz-Leehan and Feldmann, 1997). Agrobacterium tumefaciens, the plant pathogenic bacterium, has been found to preferentially transfer part of its DNA, the T-DNA, into the transcribed regions, or in their vicinity, of a plant cells nuclear genome (Koncz et al., 1989; Mathur et al., 1998; Lindsey et al., 1993). T-DNA insertions, unlike those obtained from transposons, are physically stable through multiple generations and do not transpose subsequent to insertion (Radhamony et al., 2005). T-DNA, in nature, encodes proteins necessary for biosyntheses of plant growth factors and bacterial nutrients (the opines), which are not necessary for T-DNA transfer. In the laboratory, these genes can be replaced by any DNA, such as resistance markers and/or reporter genes, which can subsequently be introduced into the plant genome (Tinland, 1996) and used for gene identification based on reporter gene expression. This method, unlike classical mutagenesis methods, eliminates the need for mutant phenotypes, which can be easily overlooked or difficult to obtain for redundant or essential genes (Springer, 2000). Once plants containing the reporter genes have been isolated, various cloning or PCR-based strategies can be used to determine which gene contains the insertion.

T-DNA tagging has become a powerful tool in several ways; it allows large populations of genes to be mutagenized then subsequently allows for easier isolation of the tagged genes, through the use of reporter genes (Springer, 2000). It also allows mutations within essential genes, which would lead to plant death in the homozygous state, to be maintained in heterozygous plants. T-DNA tagging also has advantages over
other approaches for understanding gene function in that other methods may be
correlative and may not prove a casual relationship between gene sequence and function.
For example, microarray analysis can be used to understand gene expression regulation
through the determination of the presence of a gene product, but factors other than
mRNA levels may also determine the activity of a gene. However, these in situ
expression studies cannot prove a casual relationship between a gene sequence and its
function. On the other hand, null mutations for a gene of interest may allow insight into
the effect of a gene’s absence on an organism’s ability to function.

A variety of methods have been developed to generate and isolate mutants in
known genes of Arabidopsis by T-DNA insertional mutagenesis and, through these
methods, large populations of tagged mutants are generated and screened for insertions.
Furthermore, plant lines containing identified gene disruptions can now be obtained from
these large insertion-mutagenized collections. These plants can be tested for phenotypes
that are caused due to the loss of function of the gene. Surprisingly, many knockouts
show no visible, directly scorable phenotypes, which is presumably due to partial and
complete functional redundancy, as well as, the ability of higher plants to adapt to various
stresses and constraints without undergoing morphological changes or due to our inability
to detect slight physiological alterations in fitness (Bouche and Bouchez, 2001). While
there are limitations to what T-DNA insertional mutagenesis can provide alone, it can be
an important companion to investigations aimed at determining the necessicity of specific
genes and/or functions of individual genes in the whole organism

RNA interference (RNAi) has also become a powerful tool to knock out
expression of specific genes in a variety of organisms. RNAi is a form of homology-
dependent gene silencing common to fungi, animals, and plants (Bosher and Labouesse,
2000). Although some specifics of the silencing mechanism may differ between
kingdoms, double-stranded RNA seems to be a universal initiator of RNAi (Bosher and
Labouesse, 2000). The essence of RNA-induced gene silencing is the delivery of double-
stranded RNA (dsRNA) into an organism, or cell, to induce a sequence-specific RNA
degradation mechanism that effectively silences a targeted endogenous gene. In plants,
expression of self-complementary RNAs from introduced transgenic constructs has
proved to be a rapid and consistent initiator of RNAi for several genes in *Arabidopsis thaliana* (Chuang and Meyerowitz, 2000; Smith et al., 2000).

Because there are four PAP gene family members, each active in non-specific polyadenylation assays and expressed in unique and overlapping patterns (see chapter 2 and Addepalli et al, 2004), we wanted to explore which, if any, of these isozyzmes were essential to plant development and viability. Previous studies on promoter activity of each of the four PAP genes showed that each PAP is expressed in overlapping and unique patterns (see above). However, it has not been determined if the enzymatic activities of the four PAP isozymes play redundant roles or if all four gene products are necessary for plant viability. To address these questions and to further our understanding of the four PAP genes, we employed T-DNA insertion and RNAi techniques to eliminate or suppress each of these four gene products. In each case, we found that loss of each of the four individual PAP gene products was lethal. PCR genotyping of individual T2 T-DNA insertional mutants confirmed that homozygous mutant plants were not produced which strongly suggesting that each of these genes are necessary for plant growth. This conclusion was also drawn from plants whose endogenous PAP gene products were eliminated, or drastically reduced, by the induction of PAP::RNAi expression. Taken together, these results indicate that the four PAP gene products do not share redundant roles within Arabidopsis plants but that expression of each of these genes is essential for plant growth.
RESULTS

Molecular Characterization of PAP T-DNA Insertion Mutants

Analysis of the recently completed Arabidopsis genome has indicated that four PAP genes can be found in this model plant (Addepalli et al., 2004). To investigate the functions of these genes and to determine if any of the four Arabidopsis PAP gene products are essential, we searched for mutants in public T-DNA insertion line collections. At least one line for each PAP gene family member was identified in the SIGnAL T-DNA express database and obtained (Alonso et al, 2003; Table 3.1; Figure 3.1). T2 plants from these lines showed no obvious growth or developmental defects when compared to wild type plants grown under standard growth conditions suggesting that T-DNA insertions into the PAP genes did not affect normal plant growth. To determine if the individual PAP genes are essential, a PCR genotyping assay was used to analyze at least 35 individual T2 plants from each line. This analysis was carried out in two PCR reactions using gene-specific primers spanning the insertion site and a gene-specific primer with a primer specific for the T-DNA insert (Table 3.1). Each of these two primer combinations should give rise to one predictable PCR product from the single wild type allele in wild type plants or a single mutant allele in homozygous plants and two predictable PCR products, one from the wild type allele and one from the mutant allele, in heterozygous plants. Results from PCR analysis of genomic DNA showed no plants that were homozygous for the insertion (Table 3.2). The lack of any progeny homozygous for any of the T-DNA insertions suggests that the inactivation of each gene leads to plant death. The lines for three of the PAP genes, PAP I, PAP II, and PAP IV, showed ratios of heterozygous to wild type close to 2:1 indicating that the insertions led to seeds that were either embryo lethal or unable to germinate. On the other hand, lines with T-DNA insertions in the PAP III gene showed ratios closer to 1:1. This ratio has been shown to be more indicative of a gametophyte lethal mutation (Howden et al., 1998).
RNA Interference Analysis of PAP Genes

To further substantiate the results found with the T-DNA lines that strongly indicate that each of the four PAP genes products are necessary for plant viability, we employed a second method of reducing or eliminating each of the endogenous PAP gene products, through selective degradation, known as RNA interference (RNAi). Due to the possibility that each of the four PAP gene products are essential for basic cell function and development as suggested above, constitutive RNAi expression would prevent transformed plants from producing subsequent viable generations. To overcome this potential problem, the inducible RNAi pTA7001 vector, requiring the topical application of dexamethasone for induction was used (Figure 3.2). This system can allow for plants to grow “normally” until induction of the RNAi construct. This also allowed us to observe effects of RNAi induction at different stages of post-germination plant development. Because the PAP gene family members share substantial sequence identity within the first 2/3 of the N-terminal regions, PAP-specific nucleotide sequences arising from the 3’ part of the C-terminal protein coding region, in the case of PAP II, or the 3’UTRs, for PAPs I, III and IV, (Table 3.3), were cloned into the dexamethasone inducible RNAi pTA7001 vector (see Methods). These constructs, as well as the empty pTA7001 vector, were then individually introduced into wild type Arabidopsis plants, using the floral dip method of Agrobacterium mediated transformation. The resulting seeds were germinated on selective media containing the antibiotic hygromycin to isolate transformants (see Methods). At least three plant lines for each PAP gene, except PAP II in which only one line was produced, was used for RNAi induction experiments. Plants from each line were transferred to soil then treated with the dexamethasone inducer every two to three days starting at or close to the 6-leaf stage. New untreated plants were induced every week to determine what effects the loss of the endogenous gene would have on plants throughout various stages of development. Before induction, no phenotypic differences were observed between the PAP::RNAi lines and the wild type plants at any stage of plant development, indicating that insertion of the RNAi construct into the plant genome itself did not produce noticeable effect on “normal” plant development and that subsequent observed changes were due to effects arising from loss of the endogenous RNA (Figure 3.3 and 3.4). Within 2 weeks of continued induction,
PAP::RNAi lines stopped growing and began to show signs of decline throughout each developmental stage until plant death (Figures 3.3 and 3.4).

Previously it was noted that the pTA7001 vector alone, without the addition of cloned DNA, could show negative effects on plant growth following dexamethasone treatment. To address this concern, transgenic plant lines containing the empty pTA7001 vector were induced with dexamethasone, alongside plants containing the PAP::RNAi construct. Figures 3.3 and 3.4 show that alterations in plant growth did occur. However, differences could be seen in the rate of decline in plant health between plants containing the empty vector and those containing the PAP::RNAi constructs. Empty pTA7001 plants undergoing induction beginning at, or close to the 6-leaf-stage, continued to display normal growth patterns for several weeks after the plants containing the PAP::RNAi constructs began to show altered growth patterns, compared to induced and uninduced control plants. pTA7001 plants that were not induced until and after the bolting stage also showed signs of altered growth in rosette leaves but bolt growth, flowering, seed production and seed germination were not altered compared to control plants (Figures 3.3 and 3.4). This was not the case for plants containing the PAP::RNAi constructs in which dexamethasone treatment led to plant death.

These results, taken with those found upon analysis of the T-DNA lines (see above), strongly suggest that eliminating, or substantially reducing, each of the PAP gene products is detrimental to plant health and that each PAP gene product is essential for plant viability.

**Analysis of Endogenous PAP RNA Levels in PAP-RNAi Plants**

The steroid-inducible system used in these experiments relies on the constitutive expression of GVG, a chimeric transactivator, which activates the expression of the gene cloned in front of the recognition site in the DNA upon binding the steroid (Aoyama and Chua, 1997). Therefore, the possibility exists that overexpression of the GVG may cause non-target genes to be activated. To verify that the repression of specific PAP gene expression was due the overexpression of the RNAi portion of the constructs and not because of the expression of the GVG chimeric transactivator (Aoyama and Chua, 1997), the expression of the PAP genes was monitored in the transgenic PAP::RNAi lines and
compared with lines transformed with the same vector (pTA7001) containing no PAP coding sequence and to wild type plants. Also, to eliminate concerns that each of the PAP::RNAi constructs was affecting the levels of multiple PAP gene products, we performed RT-PCR analysis using total RNA prepared from each PAP::RNAi line to compare transcript levels of each PAP gene family member and wild type plants. This analysis would also eliminate the possibility that the dexamethasone was affecting PAP RNA levels in plants lacking the RNAi insert. Total RNA was isolated from wild type plants, as well as plants containing the PAP::RNAi or the empty pTA7001 vector, at the six-leaf-stage prior to induction. RNA was again purified from these plant lines seven days after induction. These RNAs were subjected to RT/PCR analysis using primers from the dissimilar 3’-regions of each PAP gene. These primers had previously been found to be specific for each of the four PAP gene products (Addepalli, 2004, Table 3.3). These results are shown in Figure 3.5. Amplification of the actin gene product was used as a constitutive control to show that equal amounts of RNA had been used. PAP-RNAi lines showed a substantial decrease, or complete loss, in transcript levels for the endogenous PAP targeted by each particular PAP::RNAi construct when compared to the remaining PAPs and to PAP RNA levels in pTA7001 and wild type, induced and uninduced controls. The three remaining PAP transcript levels remained consistent to PAPs found in wild type uninduced and dexamethasone treated control plants (Figure 3.5). These results eliminate the possibility that more than one PAP gene product was affected in plants containing the PAP::RNAi constructs from each of the four PAP genes and that dexamethasone was not responsible for loss of gene expression.
DISCUSSION

We attempted to address the function of the four PAP gene family members of Arabidopsis. However, because each of the four PAP gene products shows high sequence identity, it is unclear whether the PAP homologues have overlapping or unique functions. Prior PAP promoter expression studies have suggested that one or more of the PAPs could play redundant roles due to overlapping patterns of the four PAP promoter::GUS activities observed but no two PAP promoters shared identical expression profiles. The data presented here offer insights into the importance of the four Arabidopsis poly(A) polymerase genes.

We identified Arabidopsis plants containing independent T-DNA insertion alleles of the four Arabidopsis PAP genes. Analysis of the progeny resulting from selfing plants heterozygous for each mutant allele did not identify a plant homozygous for the insertion for any of the mutant alleles. However, PAP I, PAP II and PAP IV all showed a heterozygous to wild type ratio of 2:1, indicative of embryo lethal or seed viability mutations (Table 3.2 a). Statistical analysis of this result, using the chi-square ($X^2$) test for goodness of fit, demonstrated that the observed results are in good agreement (P >0.05) with the phenotypic ratio of 2:1 for a single dominant nuclear gene (Table 3.2 b and c). The Arabidopsis PAP III gene, mutated using T-DNA insertions, was unique in that it showed heterozygous to wild type ratios close to 1:1 (Table 3.2 a). Statistical analysis using the chi-square test showed that these results were in agreement (P >0.05) with the phenotypic ratio of 1:1 (Table 3.2 b and c). This ratio has been shown to result from gametophyte lethality, either male or female, due to the loss of the T-DNA containing homozygote plants, as well as half of the heterozygote plants. It is unlikely that the PAP III gene product plays an essential role in both male and female gametogeneis for, if this were the case, the ratios would most likely be less than 1:1 due to the loss of both gametes which would confer full penetrant gametophytic lethality. In this case, T-DNA would not be transmitted to the progeny at all and could not be detected in PCR screens. Expansion and differentiation of germ cells requires intricate processes involving the regulation of many cellular changes. These changes give rise to germ cells containing mRNA transcripts of varying lengths indicating that germ cell maturation
requires flexibility in gene expression and protein function. This flexibility can arise through alterations in transcriptional initiation utilizing alternative start sites, exon splicing resulting in altered proteins, polyadenylation to control mRNA stability and translational timing (Walker et al., 1999). This indicates that PAP(s) may play important roles in regulation of germ cell maturation. Previous GUS expression analysis using the PAP III promoter indicates that PAP III is expressed in the stigma as well as in mature pollen (Figure 2.8; Table 2.3). However, it is impossible to determine whether it is the male or female gametes lost in these lines without further studies involving reciprocal crosses between heterozygous and wild type plants.

Arabidopsis seed development has been shown to be an intricate process involving an estimated 500 to 1,000 essential genes (McElver et al., 2001). Also, a significant number of genes specifically expressed in anthers and/or pollen have been identified (Twell, 1994). Arabidopsis PAPs have all been shown to possess nonspecific poly(A) polymerase activity (Addepalli et al., 2004; Hunt et al., 2000). The loss of this enzyme, could lead to the loss of some, or all, of the predicted genes essential for gametogenesis and seed development. The observation that three of the four Arabidopsis PAPs showed embryo lethal ratios, could indicate that each of the three PAPs are responsible for polyadenylating the genes necessary for seed development in either an overlapping or unique fashion and more detailed analysis will be necessary to determine which gene products each PAP polyadenylates. While the PAP III protein appears to be involved in gametogenesis, it may also play a role in seed development as well and what role this protein plays in Arabidopsis vitality will also involve further experimentation.

The inviability of RNAi::PAP plants lacking functional PAP gene products suggests that each member of the gene family has specialized unique and essential functions that apparently play important roles in plant vitality (Figures 3.3 and 3.4). Unfortunately, because of the lethality of the PAP homozygous mutants and the lack of any noticeable phenotype in heterozygous plants, we are unable to precisely determine for which functions these proteins are essential. In mammal and yeast systems, PAPs have been identified as the enzyme responsible for adding polyadenylate tails to nascent-mRNAs (Raabe et al., 1991; Wahle et al., 1991; Lingner et al., 1991b; Patel and Butler, 1992). In these systems, pre-mRNA polyadenylation has been shown to play many roles.
in cellular and mRNA metabolism such as transcription termination (Whitelaw and Proudfoot, 1986; Logan et al., 1987; Connelly and Manley, 1988) mRNA splicing (Niwa et al., 1990a; Niwa et al., 1990b; Boelens et al., 1993; Lutz et al., 1996), mRNA export from the nucleus (Whitelaw and Proudfoot, 1986; Connelly and Manley, 1988), mRNA stability (Bernstein et al., 1989; Beelman and Parker, 1995; Boeck et al., 1996; Carpousis et al., 1999; Curtis et al., 1995; Ford et al., 1997; Lewis et al., 1995; Caponigro and Parker, 1995), efficiency of translation (Gallie, 1991; Craig et al., 1998; Proweller and Butler, 1997; Preiss and Hentze, 1998; Tarun and Sachs, 1995; Sachs et al., 1997), and regulation of gene expression (Foulkes et al., 1993; Takagaki et al., 1996; Proudfoot, 1986; reviewed in chapter one). The four Arabidopsis PAP enzymes are active and have been shown to add polyadenylate tracts to RNA in non-specific polyadenylation assays (Hunt, 2000; Addepalli, 2004) and may possibly play similar roles in Arabidopsis, as the enzymes of mammals and yeast. If this is the case, the lethality, induced by loss of the PAP gene products, may occur due to the loss of polyadenylation of nascent-mRNAs. If poly(A) tails play vital roles in other plant functions as, they do in yeast and mammals, these defects could interfere with many essential processes within the cell. This argument is supported by the reports that mutations in other plant polyadenylation homologs, CPSF-100 and CPSF-73, lead to embryo lethality (Meinke et al., 2003; Xu et al., 2004).

It is interesting that induction of the RNAi constructs for PAP I and PAP II showed a severe, down-regulation of these gene products but not a total loss of the transcripts after seven days of dexamethasone treatments (Figure 3.5). Despite a small amount of gene expression remaining, these mutants were unable to survive dexamethasone treatment. This indicates that these enzymes play critical roles in plant viability and that a total down-regulation of these genes is not necessary to lead to plant death.

We have demonstrated in these studies that the four Arabidopsis PAPs are essential genes. Each appears to play vital roles in seed development, as demonstrated by T-DNA mutational analysis and in cellular functions that are necessary for viability throughout the plant life cycle as demonstrated by RNAi analysis. What role each
individual PAP plays within Arabidopsis is however, undetermined and will require future investigation.
MATERIALS AND METHODS

Plant Material

*Arabidopsis thaliana* ecotype Columbia was obtained from Lehle Seeds (Round Rock, TX) and used throughout this study. Seeds were germinated and plants cultivated in the greenhouse, until maturity, with a 16-h light and 8-h dark regime at 22°C.

Seed Pools of T-DNA Mutants

Seed pools of T-DNA-mutagenized *Arabidopsis thaliana*, in the PAP I, PAP II and PAP III genes, were acquired from the Arabidopsis Biological Resource Center (Columbus, OH) or from the Sussman and Amasino laboratories at the University of Wisconsin-Madison, for PAP IV. The mutant lines were allowed to self-pollinate and T2 seeds were harvested and germinated in soil, in the greenhouse, under normal long-day growth conditions.

Determination of Genotypes by PCR

Genotyping for T-DNA mutants was performed on at least 35 T2 plants from each transgenic T-DNA line using a PCR based method. Gene-specific and T-DNA specific (Lba1 for Salk T-DNA lines and p745 for WiscDsLox T-DNA lines) oligonucleotide primer sets were designed to determine if plants were homozygous wild type, homozygous mutant or heterozygous (Table 3.1). DNA was extracted from leaves taken from 3-4-week old, soil grown plants using a rapid homogenization plant DNA extraction kit (Caragen) with the following modified protocol. 200-µl DNA lysis buffer (100-mM Tris-HCL, pH 8.0; 50-mM EDTA, pH 8.0; 500-mM NaCl) was added to 100-mg leaf tissue and homogenized in the provided homogenizer or with mortar and pestle then centrifuged 30 seconds at <10,000 RPM. An additional 280-µl DNA lysis buffer was added along with 37.5-µl 20% SDS. The sample was placed in a 65°C water bath for 10 minutes. 94-µl 5M KAc was added and the sample was placed on ice for 5 minutes. The samples were then centrifuged at >13,000 RPM for 5 minutes after which the supernatant was transferred to a clean 1.5-ml Eppendorf tube. 600-µl phenol/chloroform (1:1) was added and the samples were centrifuged 5 minutes at 12,000 RPM. The supernatant was
removed and 360-µl of isopropyl alcohol was added. The samples were centrifuged 10 minutes at >13,000 RPM and the pellet was washed with 70% EtOH and allowed to air dry. Finally, the pellet was resuspended in 30-µl of water. For PCR amplification, 25-50-ng of genomic DNA, 100-ng of each primer (Table 3.1), 2.5-µl of 50-mM MgCl₂, 5-µl of 2.5-mM dNTPs, 5-µl of 10X PCR buffer (Gibco/BRL) and 0.2-units of Taq DNA polymerase (Gibco/BRL) were used in 50-µl PCR reactions. PCR amplifications were run for 35 cycles of 92°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes.

**PAP::RNAi Plasmid Constructs**

For PAP I::RNAi and PAP IV::RNAi constructs, a portion of the 3’ UTRs (225 bp and 138 respectfully) were generated by PCR from wild type genomic DNA using the oligonucleotide primers PAPI 5’A RNAi (5’-CCCCTCGAGGCTAGCTGGCTTTTCCC TACATTGCCAAGAATCTC-3’) and PAPI 3’B RNAi (5’-AAAAAGCTTAGATCTGT GAAGTAAACTCAACCCAGACTTTTTATTTAT-3’) for PAPI and PAPIV 5’ RNAi (5’-CCCCTCGAGGCTAGCGAAGGTATAGGCAGAGAAAGCATTGGTGGTGGT-3’) and PAPIV 3’ RNAi (5’-AAAAGCTTAGATCTAAAGCATTCATCATCATTCCAGACA TTATATAATCAT-3’) for PAPIV (Table 3.3) and cloned into pGEM (Promega). The sense and anti-sense fragments were excised using XhoI-HindIII and BglII-Nhel (respectfully, restriction sites were included in oligonucleotide primer sequence). The sense fragment was cloned into Sall and HindIII sites of the pBluescript-RNAi vector. This construct was then digested with BamHI and SpeI and the anti-sense fragment was cloned into this site. After amplification, this construct was digested with XhoI and XbaI to excise the sense/intron/anti-sense portion. This fragment was then cloned in pTA7001 digested with XhoI and SpeI. PAP III::RNAi construct and the PAP II::RNAi plant lines were kindly donated by Dr. Arthur Hunt (University of Kentucky) and Dr. Quinn Li (Miami University).

**Plant transformation**

PAP::RNAi constructs, as well as the empty pTA7001 vector, were transferred to *Agrobacterium tumefaciens* strain GV-3850 and the helper plasmid PRK-2013 as described by Schardl et al. (1987). The floral dip method as described by Clough and
Bent (1998) was used to transform *Arabidopsis thaliana* plants, ecotype Columbia (COL). These transformants were allowed to grow and seed in the greenhouse under long day conditions (22°C, 16-h light). T1, and subsequently T2, plants were selected on germination medium containing 25-mg/L hygromycin (Sigma). T2 plants resistant to hygromycin were transferred to soil and grown in the greenhouse under normal growth conditions. At least 3 independent homozygous lines, except for CH II in which only 1 line was obtained, for each construct were treated with glucocorticoid. Homozygous lines were identified by determining ratios of selective marker inheritance in T3 plants on hygromycin-containing media.

*Glucocorticoid Treatments*

To induce the glucocorticoid-regulated transcriptional activator, dexamethasone (Sigma), an analog of glucocorticoid, was dissolved in 100% ethanol to a 25-µM solution. For systemic induction, the plants were sprayed with a solution containing 25-µM of dexamethasone onto the aerial portions of the plants. T2 PAP::RNAi transformants, as well as pTA7001 control and wild type plants, at the 6-leaf-stage, were treated with dexamethasone every two to three days for 21 days with a new set of plants beginning dexamethasone treatment every seven days. Control plants were sprayed with a solution containing 0.1% ethanol. Plants were photographed using a Nikon Coolpix 4300 digital camera and images processed using Adobe ImageReady software (version 2.0; Adobe Systems, San Jose, CA).

*RT-PCR Analysis*

Total RNA was isolated from plants at the 6-leaf-stage before and after 7 days of dexamethasone induction using Trizol (Invitrogen) per manufacture’s instructions. First strand cDNA was made with oligo-dT using the ProSTAR™ Ultra HF RT-PCR system (Stratagene) following the manufacturer’s specifications. For PCR amplification, 1.5-µl of the first strand reaction, 200-mg of primers, 0.8-mM dNTPs, and 5.0-µl of Ultra HF PCR buffer (Stratagene) were used in 50-µl PCR reactions. PCR amplifications were run for 20 cycles of 92°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. Oligonucleotide primer sets for each PAP gene product were; PAP I -5’B (5’-
GGGCCCGGGTATACCTGAGGACTTAGATTATTCACAGGACTCC-3’)/3’B (5’-GGGGAATTCCGTATACTCCACGTCGTTTGGCCAAAATCT-3’), PAP II – 5’421 (5’-TTGGCTGAAATGGAAAGAAGTGACTG-3’)/3’1020 (5’-AAACTGGAATTGCTC TGTCATAACAC-3’), PAP III – 5’2 (5’-GGATCCATTCCAAAACAATGTAGATGTGC TTATCCTTTC-3’)/3’2 (5’-GGGAATTCGTATAACCTCGCTCTTTGGCCCATAT TT-3’), PAP IV – 5’I (5’-CCCGGGTGTCGCCACAGGATCTGGATTCTCTC-3’)/3’B (5’-ACGACGGTCCCAGACAGGAAATCAGACGCTC-3’), Actin 5’ (5’-CTCATGAGA TTCTCACTGAGAGAGTTAC-3’)/3’ (5’-TTAGAAGCATTTCCTGTGAAACAGCC ATGG-3’) (Table 3.3). The PCR products were visualized on 1.5% agarose gels.
Table 3.1. Arabidopsis PAP T-DNA lines, location within the gene and oligonucleotides used for PCR analysis.

<table>
<thead>
<tr>
<th>Gene and T-DNA Lines</th>
<th>Genomic Position of Insertion Downstream of the ATG Initiation Codon (BP) (I): Intron (E) Exon</th>
<th>Primer Designation</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>AtPAP I - AT1G17980</td>
<td>WiseDsLox4-13-4 161.14, 1587 (I)</td>
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<td>3’9</td>
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Table 3.2. Segregation Analysis of Arabidopsis Plant Lines Containing T-DNA Inserts In the PAP Genes.

(a): Ratio of heterozygous to wild type; (b): value calculated for the segregation ratio 2:1* or 1:1**; (c): Calculated P value based on $X^2$. $P = 0.05$ was chosen as a critical limit, such that the predicted ratio was not rejected for $P$ values $>0.05$. * indicates a significant ratio of heterozygous to wild type plants in a 2:1 (*) or 1:1 (**) ratio.

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Table 3.3. List of oligonucleotides used for cloning and sequencing.

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Figure 3.1. Schematic Diagram of T-DNA Insertions In the Four Arabidopsis PAP Genes.

T-DNA insertions in Arabidopsis PAP genes were screened by PCR, using DNA from individual plants as templates with gene-specific primers and T-DNA primers. Small arrows indicate direction of T-DNA inserts, filled boxes indicate exons, and white spaces indicate introns.
Figure 3.2. Schematic Representation of the pTA7001 Vector.

Structure of the pTA7001 plasmid within the left and right T-DNA borders. A portion of the four Arabidopsis PAP cDNAs were cloned in both the sense and antisense orientations into the multiple cloning site of the binary plasmid pTA7001 vector (Aoyama and Chua, 1997; see Methods). RB: right T-DNA border; 35S: 35S promoter of *Cauliflower mosaic virus*; GVG: glucocorticoid-inducible chimeric transcription factor; E9: pea rbcS-E9 polyadenylation sequence; NOS: nopaline synthetase promoter; HPT: hygromycin phosphotransferase coding sequence; NOS₅: nopaline synthetase polyadenylation sequence; 6XUAS-46: GVG-regulated promoter; MCS: multiple cloning site; 3A₅: rbcS-3A polyadenylation sequence; LB: left T-DNA border (Adapted from Aoyama and Chua, 1997).
Figure 3.3. Comparison of Morphological Phenotypes of PAP::RNAi Mutants Treated With Dexamethasone Starting At the 6-Leaf-Stage.

Top panel shows wild type and transgenic plants containing PAP::RNAi constructs or empty pTA7001 vector after treatment with 25-μM dexamethasone and 0.1% ethanol, every two to three days, starting at the 6-leaf stage. Bottom panel shows wild type and transgenic plants containing PAP::RNAi constructs or empty pTA7001 vector after treatment with a solution containing 0.1% ethanol, every two to three days, starting at the 6-leaf-stage. Plants were photographed after 14 days of treatment.
Figure 3.4. Comparison of Morphological Phenotypes of PAP::RNAi Mutants Treated With Dexamethasone After Bolting.

Top panel shows wild type and transgenic plants containing PAP::RNAi constructs or empty pTA7001 vector after treatment with 25-µM dexamethasone and 0.1% ethanol, every two to three days, starting after plants had started to bolt. Bottom panel shows wild type and transgenic plants containing PAP::RNAi constructs or empty pTA7001 vector after treatment with a solution containing 0.1% ethanol, every two to three days, starting after the plants had started to bolt. Plants were photographed after 21 days of treatment.
Leaf tissue from the PAP::RNAi, pTA7001 empty vector transgenic lines and wild type plants was used to isolate RNA before and after dexamethasone treatment. The RNA was then analyzed by RT-PCR using primers specific for each PAP gene as well as actin control primers. Panels A, B, C and D contain RT-PCR results from each of the four PAP::RNAi lines before and after dexamethasone treatment. Panels E and F contains RT-PCR results for the empty pTA7001 vector transgenic line and wild type plants.
CHAPTER FOUR

Cellular Localization of Arabidopsis Genes Encoding PAP Homologs

INTRODUCTION

The synthesis of pre-mRNA from DNA requires a series of highly synchronized and regulated events that take place in the nucleus of most eukaryotic cells. These pre-mRNAs are then transported to the cytoplasm and translated into polypeptides that are subsequently processed to become a mature folded protein. The generation of a mature mRNA involves the addition of the 5'-methyl-GpppG cap structure, splicing, cleavage and synthesis of a poly(A) tail. The signals necessary for the precise addition of the poly(A) tail, as well as the tail itself, play important roles in many steps involved in mRNA maturation including transcription termination (Whitelaw and Proudfoot, 1986; Logan et al., 1987; Connelly and Manley, 1988), mRNA splicing (Niwa et al., 1990a; Niwa et al., 1990b; Boelens et al., 1993; Lutz et al., 1996), mRNA export from the nucleus (Whitelaw and Proudfoot, 1986; Connelly and Manley, 1988), mRNA turnover (Bernstein et al., 1989; Beelman and Parker, 1995; Boeck et al., 1996; Carpousis et al., 1999; Curtis et al., 1995; Ford et al., 1997; Lewis et al., 1995; Caponigro and Parker, 1995), efficiency of translation (Gallie, 1991; Craig et al., 1998; Proweller and Butler, 1997; Preiss and Hentze, 1998; Tarun and Sachs, 1995; Sachs et al., 1997), and regulation of gene expression (Foulkes et al., 1993; Takagaki et al., 1996; Proudfoot, 1986).

Polyadenylation takes place in both the nucleus and the cytoplasm (Zhao et al., 1999a; Ballantyne et al., 1995). In the nucleus, virtually all newly synthesized mRNAs are polyadenylated but, on the other hand, cytoplasmic polyadenylation occurs on a subset of mRNAs carrying cytoplasmic polyadenylation signals. In mammals and yeast, the processes involved in poly(A) tail addition have been extensively studied and many insights into the cis and trans acting factors necessary to produce a mature and accurate poly(A) tail have been identified. However, compared to other eukaryotic systems, mRNA 3'-end formation and polyadenylation in plants is poorly understood.
Polyadenylation signals for several plant genes have been characterized and have led to a better understanding of the RNA sequences required for mRNA 3’-end polyadenylation (Hunt, 1994; Li and Hunt, 1997; Wu et al., 1993; Rothnie, 1996; Graber et al., 1999a; Loke et al., 2005). However, many questions remain about factors needed to recognize these plant polyadenylation signals and about the overall 3’-end polyadenylation process. Recent completion of the Arabidopsis genome-sequencing project has allowed for the identification of potential plant homologs for most of the polyadenylation factors required for 3’-end processing in other eukaryotic systems (Table 1.1). As of yet, many of these homologs have yet to be analyzed for their possible roles in plant polyadenylation.

In mammals and yeast, poly(A) polymerase (PAP) is a key component of the polyadenylation apparatus. This enzyme is responsible for the catalytic addition of the polyadenylate tract to the 3’-end of the cleaved pre-mRNA and is required for both the cleavage and polyadenylation reaction (Raabe et al., 1991; Wahle et al., 1991; Zhao et al., 1999a; Edmonds, 2002; Wahle and Ruegsegger, 1999; Colgan and Manley, 1997; Keller and Minvielle-Sebastia, 1997; Wahle and Kuhn, 1997). Multiple PAP genes can be found in mammals as well as alternatively spliced transcripts arising from these genes (Lee et al., 2000; Kashiwabara et al., 2000; Kyriakopoulou et al., 2001; Perumal et al., 2001; Topalian et al., 2001). In Arabidopsis, four genes have been identified that encode possible poly(A) polymerases. These genes reside on chromosomes I, II, III, and IV and have been designated PAP I, PAP II, PAP III and PAP IV, respectively (Addepalli et al., 2004). The products arising from each of these genes have been cloned and sequenced and their polypeptides analyzed. Three of the four genes, PAP I, PAP II and PAP IV, give rise to proteins ranging in size from 700-800 amino acids and contain a putative nuclear localization signal (NLS; underlined in figures 1.5 and 1.6). The fourth PAP, PAP III, produces a much smaller gene product of 482 amino acids and lacks an identifiable NLS as well as 200-300 amino acids from the C-terminal portion of the protein, compared to the other three Arabidopsis PAP proteins. The PAP IV protein has previously been shown to localize to the nucleus of transformed tobacco leaf cells (Forbes, unpublished results). The fourth PAP, PAP III, produces a much smaller gene product of 482 amino acids and lacks an identifiable NLS as well as 200-300 amino acids...
from the C-terminal portion of the protein, compared to the other three Arabidopsis PAP proteins. These findings suggested that the PAP III gene might have resulted from a gene duplication event and gives rise to a non-functional protein. However, recombinant proteins for each PAP have been shown to possess non-specific PAP activity in vitro (Addepalli et al., 2004). In mammals, a cytoplasmic PAP isoform, specific to the spermatocytes, has been identified that, like the Arabidopsis PAP III gene, lacks both the NLS and C-terminal region found in other PAPs (Kashiwabara et al., 2000). In addition, previous studies (see above) have indicated that the PAP III promoter, as well as the remaining three PAP promoters, were able to power GUS expression in a unique spatial-temporal pattern. RNAi and T-DNA knockout plants also demonstrated that each of the four PAP genes is essential for plant viability.

In this study, we have utilized the highly visible, internal fluorophores, GFP (green fluorescent protein) and DsRed, as reporter genes in an attempt to determine the cellular localization of the four Arabidopsis PAP proteins in onion epidermal cells. Using particle bombardment, we co-transformed onion epidermal cells with empty DsRed vector and vectors containing GFP attached to each of the four Arabidopsis PAPs. We found that the PAP I, PAP II, and PAP IV proteins are localized to the nucleus as expected but, more specifically, can localize as nuclear speckles as well as diffusely throughout the nucleoplasm. Unfortunately, transformations containing PAP III::GFP constructs failed to give rise to viable cells and cellular localization could not be determined.
RESULTS

Plasmid Construction

In order to evaluate PAP cellular localization, the pGDG binary vector (Goodin et al., 2002, Figure 4.1), which allows for a high level of transient expression of an autofluorescent protein, was fused, at the 3’ end, to each of the PAP protein coding sequences and introduced into onion epidermal cells by particle bombardment (see Materials and Methods). The onion epidermis was used due to its large, living, transparent cells, ideal for visualizing autofluorescent proteins. In addition, unlike green plant tissues, these cells have low levels of autofluorescence. These constructs were co-transformed with the empty vector, pGDR, expressing a red fluorescent protein (DsRed), as a control.

PAP I, PAP II and PAP IV Proteins Are Localized to the Nucleus

Fluorescence microscopic studies of the transformed onion cells indicate that DsRed autofluorescence was found throughout the cells (Figures 4.2 A, 4.3 A and 4.4 A), whereas PAP I, PAP II and PAP IV proteins were localized to the nucleus as seen by the condensed spots of green fluorescence in panel B of figures 4.2, 4.3 and 4.4, respectively. Furthermore, while these PAPs were diffuse throughout the nucleoplasm, there were distinctive accumulations seen in structures reminiscent of those previously described as nuclear speckles (Figure 4.5; Nagatani, 2004; Tillemans et al., 2005; Docquier et al., 2004). The nuclear localization of the PAP proteins was identical with that of 4´,6-diamidino-2-phenylindole (DAPI), which stains double-stranded DNA in the nucleus (Figures 4.2 C, 4.3 C and 4.4 C). Over-laying the PAP::GFP images onto the DAPI images (Figures 4.2 D, 4.3 D and 4.4 D) demonstrate that the PAP I, PAP II and PAP IV proteins are exclusively localized in nuclei.

Cellular Localization of PAP III Could Not Be Determined

Co-transformation of the PAP III::GFP construct with the empty DsRed control vector did not generate viable cells, unlike cells transformed with DsRed alone. Five particle bombardment transformations of onion epidermal cells, with both the CH
III::GFP and pGDR vectors, produced no viable co-transformed cells. On two attempts, a total of three cells were found expressing DsRed alone. These results were compared to particle bombardment results using pGDR alone in which an average of 42 cells, per experiment, were found expressing DsRed. This result indicates that overexpression of the PAP III gene product was detrimental to the onion cells. Therefore, cellular localization of the PAP III gene product could not be determined using this method.
DISCUSSION

Processing of mRNA precursors is a critical step in gene expression. These steps are comprised of an integrated series of reactions mediated by a large and complex set of protein factors (Zhao et al., 1999a; Edmonds, 2002; Wahle and Ruegsegger, 1999; Colgan and Manley, 1997; Keller and Minvielle-Sebastia, 1997; Wahle and Kuhn, 1997; Rothnie, 1996; Hunt, 1994). Poly(A) polymerase, has been shown to play several critical roles in RNA processing. It not only participates in endonucleolytic cleavage of pre-mRNA and catalysis of poly(A) synthesis, it also interacts with other proteins that may help coordinate polyadenylation, splicing and exon definition (Robberson et al., 1990; Berget, 1995; Niwa et al., 1990a; Niwa et al., 1990b; Niwa and Berget, 1991a; Niwa and Berget, 1991b; Scott and Imperial, 1996; Cooke et al., 1999; Vagner et al., 2000; Cooke and Alwine, 2002; Boelens et al., 1993; Lutz et al., 1996; McCracken et al., 2002). The polyadenylation reaction is intertwined with other steps involved in mRNA synthesis such as transcription termination (Whitelaw and Proudfoot, 1986; Logan et al., 1987; Connelly and Manley, 1988), mRNA splicing (Niwa et al., 1990a; Niwa et al., 1990b; Boelens et al., 1993; Lutz et al., 1996), mRNA export from the nucleus (Whitelaw and Proudfoot, 1986; Connelly and Manley, 1988), mRNA stability (Bernstein et al., 1989; Beelman and Parker, 1995; Boeck et al., 1996; Carpousis et al., 1999; Curtis et al., 1995; Ford et al., 1997; Lewis et al., 1995; Caponigro and Parker, 1995), efficiency of translation through interactions with the 5’-cap (Gallie, 1991; Craig et al., 1998; Proweller and Butler, 1997; Preiss and Hentze, 1998; Tarun and Sachs, 1995; Sachs et al., 1997), and regulation of gene expression, through the alternative usage of polyadenylation sites (Foulkes et al., 1993; Takagaki et al., 1996; Proudfoot, 1986). These examples demonstrate the significant role of polyadenylation in regulation and control of gene expression.

In *Arabidopsis thaliana*, *in silico* analysis has identified four potential PAP genes. Three of the four genes, PAP I, PAP II and PAP IV, contain ~750 amino acids and possess a putative nuclear localization signal (NLS; Underlined in Figure 1.5). PAP III, on the other hand, appears to be much smaller, 482 amino acids, and lacks any recognizable NLS (Addepalli et al., 2004, Figure 1.6). To determine if each of the PAP I,
PAP II, and PAP IV proteins were directed into the nucleus, we generated constructs containing each of the full-length proteins attached to GFP. As expected, each of these proteins was localized throughout the nucleoplasm of onion epidermal cells (Figures 4.2, 4.3 and 4.4). Moreover, each of these PAPs was concentrated in speckles within the nucleus (Figure 4.5). Several types of nuclear speckling structures have been identified and include cleavage bodies, Cajal/coiled bodies, splicing speckles and paraspeckles. The role of cleavage bodies is not yet fully understood but several factors involved in the mammalian cleavage and polyadenylation of pre-mRNAs have been localized to cleavage bodies and include symplekin, CstF and CPSF (Spector, 2001; Schul et al., 1996; Hofmann et al., 2002). Cleavage bodies can overlap with, or are adjacent to, Cajal/coiled bodies (Schul et al., 1999). Cajal/coiled bodies are found, most abundantly, in very transcriptionally active and proliferating cells in the vicinity of nucleoli (Gall, 2000). It is within the Cajal/coiled bodies that the biogenesis of snRNPs takes place with the snRNPs subsequently being moved to the splicing speckles (Sleeman and Lamond, 1999).

Splicing speckles are thought to be storage sites for mRNA splicing factors which can either be recruited from the speckles to sites of transcription or genes can be recruited to the vicinity of the speckles where mRNAs are then transcribed within the speckles (Lamond and Spector, 2003; Misteli et al., 1997; Shopland et al., 2003; Moen et al., 2004). In mammals, almost 200 proteins have been purified from splicing speckles and contain proteins involved in splicing including various snRNAs (small nuclear ribonucleic acids), snRNPs (small nuclear ribonucleoproteins), serine/arginine-rich proteins (SRps), and RNA polymerase II (Spector, 1993; Fu and Maniatis, 1990; Thiry, 1993; de Jong et al., 1996; Misteli and Spector, 1997; Singer and Green, 1997; Saitoh et al., 2004; Rappsilber et al., 2002; Zhou et al., 2002; Neubauer et al., 1998; Hartmuth et al., 2002; Will et al., 2002; Jurica et al., 2002). In addition to these proteins, the mammalian polyadenylation factors PABPN1 and PAP have been found in these domains (Calado and Carmo-Fonseca, 2000; Schul et al., 1998; Krause et al., 1994). Paraspeckles are generally irregularly shaped bodies that are usually clustered together and are often closely adjacent to splicing speckles (Fox et al., 2002). These domains are thought to play roles in transcription and RNA splicing as well as pre-mRNA processing as indicated by the localization of mammalian CF I to these areas (Dettwiler et al., 2004).
While it is interesting that polyadenylation factors are found in many of these bodies, it is not understood how they come together to accomplish poly(A) tail addition. In our studies, we have shown that the Arabidopsis PAP I, PAP II and PAP IV proteins localize, not only diffusely throughout the nucleoplasm, but to also as speckles within onion cell nuclei. However, which body or bodies each of these PAPs localize to is not known and further experimentation will be required to answer this question.

In contrast to the three larger PAP proteins, which contain an identifiable NLS and have been shown to enter the nucleus, PAP III lacks a predictable NLS. However, not all proteins that are targeted to the nucleus contain a recognizable NLS. In these cases, the NLS is not necessarily evident within the primary amino acid sequence, rather protein transport to the nucleus appears to occur at the level of the biochemical properties of the protein and to some degree on the proteins secondary structure (Endo et al., 1989; Hammen et al., 1994). To determine if PAP III was also nuclear localized, we transformed onion epidermal cells with a construct containing the full-length PAP III protein attached to GFP. However, unlike PAP I, PAP II and PAP IV, PAP III subcellular localization could not be determined by these studies. Presumably, the overexpression of PAP III was detrimental to cellular functions and did not allow for viable onion cells. While this cannot exclude PAP III from being a nuclear localized protein, a similar result was also observed in Drosophila in which in vivo overexpression of PAP during embryogenesis causes a dramatic elongation of poly(A) tails and a loss of specificity during cytoplasmic polyadenylation, which in turn, resulted in embryonic lethality. In other words, regulation of the PAP level is essential for controlled cytoplasmic polyadenylation and cell viability (Juge et al., 2002). The lack of the nuclear localization signal for the Arabidopsis PAP III gene and the similar result found upon overexpression of this protein, leading to lethality, as is seen in overexpression of the Drosophila cytoplasmic PAP suggests that PAP III may play a role in cytoplasmic polyadenylation within Arabidopsis cells.

Subcellular localization is related to protein function in that, proteins have evolved to function optimally in a specific subcellular localization. Therefore, the final destination of a protein, within cells, is crucial to its function. Identifying the subcellular localization of the four Arabidopsis PAP proteins will help to specify where they are
targeted within cells and could aide in determining interactions with other proteins and small metabolites in their local environment. Future studies will be necessary to fully understand the undoubtedly complex behaviors of each of these PAPs genes.
MATERIALS AND METHODS

GFP and DsRed Fusion Constructs

For transient GFP assays, the pGDG (GFP) fusion plasmid was used (Goodin et al., 2002). Briefly, the coding region of the four Arabidopsis PAPs were amplified, using PCR, with the oligonucleotide primers 5′ Sal I (5′-GTCGACATGGCTAGTGTCCAGCA AAATGGGCAACGG-3′) and 3′ APA I (5′-GGGCCCTTACTTGCCATTGGTTTTGCC TAGAGACGT-3′) for PAP I, 5′16 Bgl II (5′-AGATCTATGGTGAGTACTCAACAAC GCACGGACG-3′) and 3′11 Bgl II (5′-AGATCTTCTTGTATTTGCATAAAACCCATAAT GGGTT-3′) for PAP II, and 5′7 Sal I (5′-GTCGACATGGCTAGTGCCAGCAAAATGGGCAACGG-3′) and 3′6 Bam HI (5′-GGATTCACATTTAACCTCCATACCATCAG CTTCTCT-3′) for PAP III, 5′ (Table 4.1) in the 5′ to 3′ direction using a full-length cDNA as template. The PAP IV GFP clone was kindly donated by Kevin Forbes. PCR products were subcloned into the EcoRV site of pGEM-T Easy (Promega), per the manufacturer’s instructions, and resulting clones were sequenced with T7 and SP6 oligonucleotide primers. PGEM-PAP clones were then digested with Sal I and APA I for PAP I, Bgl II for PAP II and Sal I and Bam HI for PAP III and the resulting fragments were ligated, into Sal I and APA I digested pGDG plasmid for PAP I, Bam HI digested pGDG plasmid for PAP II and Sal I and Bam HI digested pGDG plasmid for PAP III, by T4 DNA ligase (Invitrogen). Recombinants were sequenced with oligonucleotide primers 3′120 (5′-AACATCTTGCAAGTGCTTCTCGAGCTCTCTT-3′) for PAP I, 3′1 (5′-GACAGCATTTGCATCCTCCACCATCTGA-3′) for PAP II and 3′3 (5′-GTTAGACTAAAACACCTTCTGCCACAACGGTGCCAGTT-3′) for PAP III, to determine if the GFP fusions were in frame (Table 4.1). For controls, pGDR (DsRed) (Gooding et al., 2002) empty vectors were co-localized with PAP::GFP constructs.

Particle Bombardment

The fusion plasmids were introduced into onion epidermal skin cells by particle bombardment using a PDS1000 DuPont Bio-Rad Microprojectile delivery system (Bio-Rad Laboratories) per the manufacturer’s instructions. Briefly, 0.5-mg of gold microcarriers (1µm) per shot, were vortexed vigorously in 1-ml 70% ethanol (V/V) for 3-
5 minutes and then allowed to soak for 15 minutes. Microparticles were pelleted, ethanol removed, washed three times in 1-ml sterile water, and then resuspended in 15-µl sterile water. To this, 2-µg of each DNA, 50-µl 2.5-M CaCl₂ and 20-µl 0.1-M spermidine were added with constant vortexing. Vortexing was continued for 3 minutes. Microparticles were pelleted in microfuge for 2 seconds, supernatant removed, and the pellet washed with 140-µl of 70% ethanol, then 140-µl of 100% ethanol and finally resuspended in 12-µl of 100% ethanol.

For macrocarrier preparation, suspended microcarriers were spread in the center of macrocarrier (Biorad Labs, USA) and installed in the particle gun assembly per the manufacturer’s instructions. For all experiments, a helium pressure of 1100 psi was selected. The distance between rupture disk and macrocarrier was adjusted to 8-10 cm from the onion tissue. Following bombardment, the tissue was transferred to T- agar media, incubated at 25°C and then analyzed 24-48 hours after bombardment.

Detection and Photography of GFP/DdRed Expression and DAPI staining of Onion Epidermal Cells

Localization of GFP and DsRed expression in onion cells was determined using a Zeiss Axioplan 2 microscope with a Zeiss AttoArc 2 light source. Excitation and emission wavelengths for GFP, were 470-nm and 500-nm, respectively, and for DsRed these values were 550-nm and 590-nm, respectively, and for DAPI, 358-nm and 461-nm, respectively. Transformed cells were then stained with 2.5-µg/ml 4’,6-diamidino-2-phenylindole (DAPI) and 0.5% Triton X-100 in phosphate buffer saline (PBS) for 30 min at room temperature. Photographs were taken using a Zeiss Axiocam Mrc5 and visualized using AxioVision 4.1 software (Zeiss, Jena, Germany). Images were processed using Adobe ImageReady software (version 2.0; Adobe Systems, San Jose, CA).
### Table 4.1. List of oligonucleotides used for cloning and sequencing.

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<th>GENE</th>
<th>PRIMER DESIGNATION</th>
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<td>3’APA I</td>
<td>GGGCCCTTACCTTGCCATTGGTTTTGGCTAGAGACGTAACATCTTGCAAGTGCTTCCTT</td>
</tr>
<tr>
<td></td>
<td>3’120</td>
<td></td>
</tr>
<tr>
<td>PAPII</td>
<td>5’16 Bgl II</td>
<td>AGATCTATGGGTGATGACTCAACAGACGACG</td>
</tr>
<tr>
<td></td>
<td>3’11 Bgl II</td>
<td>AGATCTTTCATGGATTGGCATTAACCAATAATGGGTT</td>
</tr>
<tr>
<td></td>
<td>3’1</td>
<td>GACAGCATTTTGCATCCTCCACCCTCTG</td>
</tr>
<tr>
<td>PAPIII</td>
<td>5’7 Sal I</td>
<td>GTCGACATGGCTAGTGTCACCAGCAAAATGGGCAACGG</td>
</tr>
<tr>
<td></td>
<td>3’6 Bam HI</td>
<td>GGATTCACATTAAATCCCATACCATCAAGCTTCTCCT</td>
</tr>
<tr>
<td></td>
<td>3’3</td>
<td>GTTGAAGCTAAAAACACCTTCCTGCGCAACGTCGGTT</td>
</tr>
</tbody>
</table>
Figure 4.1. Depiction of the pGDG and pGDR Vectors.

A. Schematic representations of pGDG and pGDR. LB (left border) and RB (right border) denote the borders of the T-DNA; Nopaline synthase (Nos polyA) are used as polyadenylation signals. B. Sequence of the multiple cloning site of pGDR. Note that the PstI site can be used only in pGDG because the DsRed2 coding sequence contains a PstI site.
Figure 4.2. Cellular Localization of PAP I::GFP.

Fluorescence of onion epidermal cells co-expressing DsRed and PAP I::GFP constructs. (A) and (B) depict the autofluorescent protein DsRed and the nuclear localized fluorescence of PAP I fused with GFP, respectively. (C) 4,6-diamidino-2-phenylindol (DAPI)-staining of the nucleus shown in (A) and (B). (D) Merged image of panels (B) and (C). The onion cell was transformed using particle bombardment.
Figure 4.3. Cellular Localization of PAP II::GFP.

Fluorescence of onion epidermal cells co-expressing DsRed and PAP II::GFP constructs. (A) and (B) depict the autofluorescent protein DsRed and the nuclear localized fluorescence of PAP I fused with GFP, respectively. (C) 4,6-diamidino-2-phenylindol (DAPI)-staining of the nucleus shown in (A) and (B). (D) Merged image of panels (B) and (C). The onion cell was transformed using particle bombardment.
**Figure 4.4.** Cellular Localization of PAP IV::GFP.

Fluorescence of onion epidermal cells co-expressing DsRed and PAP IV::GFP constructs. (A) and (B) depict the autofluorescent protein DsRed and the nuclear localized fluorescence of PAP I fused with GFP, respectively. (C) 4,6-diamidino-2-phenylindol (DAPI)-staining of the nucleus shown in (A) and (B). (D) Merged image of panels (B) and (C). The onion cell was transformed using particle bombardment.
Figure 4.5. Distribution of Fluorescence Within the Nucleus of PAP I::GFP, PAP II::GFP and PAP IV::GFP.

Images of onion cell nuclei showing fluorescence of PAP I::GFP (A), PAP II::GFP (B) and PAP IV::GFP (C) constructs. The onion cell was transformed using particle bombardment.
CHAPTER FIVE

Summary and Future Directions

The regulated expression of genes is critical for all forms of life to effectively survive and thrive in their environments. Gene expression may change in response to physical signals from the environment, interactions between species, and signals within an organism. Altering DNA structure, transcriptional modifications, stability or translation of mRNA, and post-translational modification of proteins are all examples of the many levels of regulatory mechanisms employed by organisms.

After transcription, pre-messenger RNA (pre-mRNA) must undergo substantial processing before it can be exported from the nucleus to the translation machinery. This processing, as well as transcription itself, must be coordinated for efficient and regulated gene expression to occur. Pre-mRNA processing events include capping, splicing and polyadenylation. In addition, mRNA and protein diversity can arise through variations in post-transcriptional processing leading to multiple isoforms generated from single genes. Regulation of transcription, as well as mRNA stability and export, are important factors in regulating expression levels of a gene.

Polyadenylation has been shown to require a complex assembly of protein factors at the 3’-end of the pre-mRNA (Wahle and Ruegsegger, 1999; Zhao et al., 1999a). The enzyme responsible for catalyzing the poly(A) tail is poly(A) polymerase (PAP). This enzyme belongs to a family of related nucleotidyltransferases and is a single enzymatically active polypeptide (Wahle and Ruegsegger, 1999; Minvielle-Sebastia and Keller, 1999; Zhao et al., 1999a; Edmonds, 2002). Poly(A) polymerases can be found in both the nucleus and cytoplasm of eukaryotic cells. Cytoplasmic PAPs can add additional adenosine residues to mRNAs that had previously undergone polyadenylation in the nucleus. This additional regulation of poly(A) tails has been found to be critical for diversity of developmental decisions (Wickens et al., 2000; Richter, 2000).

Unlike poly(A) polymerases from plants, which are just beginning to be described (Hunt, 2000; Addepalli et al., 2004), poly(A) polymerases from yeast and mammals have been well characterized (Zhao, 1999a; Wahle and Ruegsegger, 1999;). It has long been
known that PAPs exist in many plant species (Sachar, 1968; Mans and Huff, 1975; D’Alessandro and Srivastava, 1985; Burkhard and Keller, 1974; Kapoor et al., 1993; Verma and Sachar, 1994; Lisitsky et al., 1996; Das Gupta et al., 1995; Li et al., 1996; Hunt, 2000). We have recently identified four PAP genes (PAP I-IV, corresponding to chromosomes I-IV) in *Arabidopsis thaliana* by *in silico* analysis of the Arabidopsis genome using bovine PAP data. There is a high degree of amino acid conservation between the Arabidopsis PAPs and the bovine PAP especially in the N-terminal portions of the proteins but the C-termini show great variability when compared to each other and to the bovine PAP (Figures 1.5 and 1.6). Three of the four PAPs (PAP I, PAP II and PAP IV) are 700-800 amino acids (~83-95 kDa) in size and contain a definable nuclear localization signal. The fourth PAP (PAP III) appears much smaller (482 amino acids, 57 kDa) and is missing the nuclear localization signal (Hunt, 2000; Addepalli et al., 2004; Figures 1.5 and 1.6). Furthermore, each of the Arabidopsis PAP genes produce functional enzymes as demonstrated in non-specific polyadenylation assays and each is found in potentially alternatively spliced forms (Addepalli et al., 2004; Figure 1.7). Northern blot and RT-PCR analysis also indicate that these forms are differentially expressed in various plant tissues in unique and overlapping patterns (Figure 1.7). These findings indicate that each of the PAPs may play an important and distinct role in the regulation of gene expression (Hunt, 2000; Addepalli et al., 2004).

Results presented here add to our understanding of the four Arabidopsis PAP genes. In summary, gene expression analysis was done using *in silico* analysis and the fusion of the 5’ UTR from each PAP gene to the β-glucuronidase (GUS) reporter gene, which was subsequently transformed into Arabidopsis plants. These results showed unique and overlapping gene expression profiles which imply that these genes are not redundant and may play unique roles within the plant (Chapter 2; Figures 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.10 and 2.11; summarized in Tables 2.1, 2.2 and 2.3). These results were further substantiated in chapter three where analysis of gene knockout (T-DNA and RNAi) plant lines showed that each of the PAP gene products is necessary for plant viability (Table 3.3; Figures 3.3 and 3.4). Finally, determination of cellular localization was attempted for the four PAPs. PAP I, PAP II and PAP IV were all localized to the nucleus of onion epidermal cells (Chapter 4; Figures 4.2, 4.3, 4.4) and more specifically,
were localized within speckles of the nucleus (Figure 4.5). Transformation of PAP III::GFP constructs lead to onion cell death and cellular localization of PAP III could not be determined.

These results have given insights to the gene expression and essentiality for each Arabidopsis PAP gene but many questions remain to be answered. The results from chapter two begin to address the expression profiles for the PAP genes but they do not allow for unique expression patterns of alternatively spliced variants. To gain a full understanding of how each of these genes participates in polyadenylation and gene regulation, it is important to understand how each alternatively spliced transcript is expressed within the plant. Two methods of determining these expression patterns that could be employed are in situ hybridization and further utilization of the gene chip technology. In both cases, the first step would involve identifying and cloning each of the variants to be used as probes in these studies. In situ hybridization can be used to detect individual nucleic acids using specific RNA probes in both individual cells as well as in tissue sections. In the second strategy, alternatively spliced variants could be used as probes in microarray analysis. This technology can distinguish transcripts that are up to 90% identical. Therefore, using portions of the variants that are significantly unique between family members would allow for the expression pattern of each variant to be monitored.

While both of these techniques could provide important clues to the expression patterns of each PAP gene, limitations do exist. For example, isolating and cloning each of the alternatively spliced variants could prove to be very difficult and time consuming in that some forms of the gene products may be in low abundance, unstable or may only be produced at specific times during the life of the plant or at a specific location within the plant. Also, particular environmental cues may lead to alternative forms that would not be present in plants unexposed to these specific environments.

Another interesting avenue of study and one that would provide insight into the regulatory mechanisms governing PAP gene and protein expression and function, is an in depth analysis of each of the PAP promoter regions. Promoter regions are the DNA sequences, usually directly 5’ to the coding sequence, which are required for basal and/or regulated transcription of a gene and are generally known as cis-acting elements. These
elements function as binding sites for effector proteins, called transcription factors (TFs). It is the interaction of suitable sets of TFs with the promoter sequence that allows for the transcriptional initiation complex to be formed (Zawel and Reinberg, 1995). Many promoters are composed of modular units that convey specific functionality to the promoter in specific signaling pathways and in tissue specific manners (Firulli and Olson, 1997). Also, orientation and distances between the TF binding sites may affect transcription initiation (Klingenhoff et al., 1999; Kel et al., 1999).

While there are many techniques available for this type of analysis, I will only discuss a few. In silico analysis can be used to find known transcription factor binding sites and cis-acting elements but, in many cases, cannot take into account the flexibility of promoter modules. Deletion analysis and saturation mutagenesis, in which a library of mutations within the promoter sequence is generated, could be used to identify and evaluate cis-acting elements within the promoter. Finally, gel retardation assays could be used to determine whether a specific nuclear protein binds to a promoter fragment that contains different combinations of cis elements and, if so, determine if they bind to each element, one or none. This type of analysis could aid in determining promoter modules. Unfortunately, these studies will not allow for a full understanding of the PAP promoter regions because regulation of transcription does not depend on cis elements alone. The structure of the DNA itself can affect transcription by either allowing or preventing accessibility of the binding sites. While the DNA structure can be predicted in silico, these predictions are still in their infancy.

The exact role of each of the PAPs in Arabidopsis remains unclear. Results from plants containing gene knockouts showed that each of the PAPs is essential for plant viability (Chapter 3; Table 3.3; Figures 3.3 and 3.4) but further investigations are needed to determine the functions of each gene product. T-DNA mutagenized plants with insertions in the PAP I, PAP II and PAP IV genes all showed segregation ratios of 2:1 and for PAP III a 1:1 ratio was found (Table 3.3). For PAP I, PAP II and PAP IV, the 2:1 ratio suggests that these genes may be involved in embryo development or seed viability. As no homozygous plants were identified for these mutagenized genes, determining where the initial effect of the missing PAP gene product has on these homozygous plants will provide further insights on functions of these genes’ products. The first step in
determining if homozygosity leads to embryo lethality or a mutation in seed viability is to observe T-DNA mutant plants to determine if abnormal embryos can be identified. If abnormal embryos exist, then the mutation is probably embryo lethal. To substantiate this hypothesis, single-embryo RT-PCR could be carried out to determine if abnormal embryos contain only the disrupted allele. If this is the case, then these embryos are homozygous for the mutant PAP gene. Abnormalities in these seeds can be investigated using microscopy to compare normally developing seeds to those that appear abnormal. These observations may provide insights into what stage of seed development was affected by lack of the PAP gene product. If embryos appear normal, germination of the seeds can be carried out and ratios of germinated and un-germinated seeds can be obtained. Homozygous seeds, lacking the functional PAP gene product, should constitute one fourth of the seed population. These un-germinated seeds can also be tested using RT-PCR to confirm this result. Further insights into when PAP genes are expressed during seed development can be done with RT-PCR using RNA extracted from wild type Arabidopsis seeds during different stages of development.

In PAP III mutants, a segregation ratio of 1:1 (Table 3.3) was observed indicating that this may be a gametogenic mutation. However, it is not known if this mutation is associated with male or female gametogenesis. Promoter expression analysis of the PAP III promoter showed GUS expression in both the stigma and mature pollen (Figure 2.8; Table 2.3). Therefore, we are unable to predict whether it is the male or female gametogenesis that is affected by the loss of the PAP III gene product. To answer this question, reciprocal backcrosses between wild type plants and the T-DNA mutant lines will be required. If transmission of the mutation is through the male, the backcross between wild type pollen and a heterozygous plant should give an F2 ratio of 1:1. If the mutation were through the female, then this cross would only yield wild type plants.

Insights into gene regulation and function can also be found by determining the subcellular localization of the genes products. Subcellular localization of proteins determines their ability to interact with other proteins in their local environments. Results from chapter four suggest that PAP I, PAP II and PAP IV localize to the nucleus of onion epidermal cells as was expected due to the presence of the nuclear localization signal found within the predicted gene products. More specifically, these proteins could
be found throughout the nucleoplasm as well as in speckled bodies within the nucleus (Figures 1.6, 4.2, 4.3, 4.4 and 4.5). Subcellular localization for PAP III, however could not be determined as transformation of PAP III::GFP (green fluorescent protein) constructs into onion epidermal cells presumably led to cell death due to over expression of the PAP III gene product (Chapter 4 results). These results were obtained by fusing the full-length cDNA from each PAP gene to GFP. Therefore, there exists the possibility that alternatively spliced variants from each gene may be localized to different areas within cells. To determine if this is the case, these experiments would have to be repeated using these variants. This may also allow for the determination of the PAP III gene product.

Further experiments to determine exactly where PAP I, PAP II and PAP IV are localized within the nucleus could also aide in determining where, and perhaps how, polyadenylation takes place within the nucleus. Several structures have been identified within the nucleus that give rise to this type of speckling and include cleavage bodies, Cajal/coiled bodies, splicing speckles and paraspeckles (Chapter 4 discussion). To determine were the PAP I, PAP II and PAP IV gene products localize within the nucleus, co-transformations with the PAP::GFP constructs and constructs containing proteins, specific for each nuclear structure, fused with a differing florescent protein, would have to be done.

What role each of the four PAPs plays within the Arabidopsis plant and throughout its life cycle has yet to be fully understood. However, due to the alternate splicing variants and the unique and overlapping gene expression profiles, their participation in polyadenylation and gene regulation will, more than likely, be complex due to the necessity for adaptation and survival in a multitude of environments. This complexity allows the organism to quickly alter and finely control its responses to the environment and it is evident that continuing research on these four PAPs is needed in order to understand what role each plays in gene control.

What might be the reasons for the existence of multiple PAP genes in Arabidopsis thaliana? Perhaps each of the proteins contain distinctive properties that allow cells to fine tune the efficiency of 3’-end polyadenylation or to exercise greater control upon the poly(A) tail length of specific genes and thus provide additional regulations of RNA
stability. On the other hand, each of the PAPs may be functionally equivalent to one another but permit specific quantitative controls of PAP levels in individual tissues and/or cell growth states. PAP levels were shown to be tightly regulated (Zhao and Manley, 1998). Therefore, using more than one PAP may allow greater control under many unique conditions. In this study, we found that each of the Arabidopsis PAP genes shows unique and overlapping expression patterns, each is essential for plant viability and the PAP I, PAP II and PAP IV gene products are localized within the nucleus.
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