MOLECULAR ANALYSIS OF SULFUR-RESPONSIVE EXPRESSION OF PEA ALBUMIN 1 IN TRANSGENIC PLANTS

Amanda Jane Ellery

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DECLARATION

The work described in this thesis was carried out between February, 1993 and August, 1996 under the supervision on Dr T.J. Higgins. The work is my own except where otherwise stated in the text, and has not been previously submitted for any other degree.

Amanda Ellery.
ABSTRACT

Seeds of the pea (Pisum sativum) contain several storage proteins with high proportions of sulfur amino acids. Pea albumin 1 (PA1) contains 11% cysteine and methionine. PA1 expression is regulated by sulfur nutrition, via a post-transcriptional process, possibly by preferential destabilization of the PA1 transcript during sulfur-deficiency. Expression of a modified PA1 gene in tobacco leaves has previously been shown to respond to sulfur nutrition. In this study, a novel method for growing tobacco plants in vitro under controlled sulfur conditions was developed. Leaves from sulfur-adequate and sulfur-deficient transgenic plants grown in this system were used to measure decay of PA1 transcripts in the presence of a transcription inhibitor.

Plants transformed with genetic constructs containing fragments of the PA1 gene fused to a reporter gene whose expression was not regulated by sulfur, were used to locate a sulfur-regulatory region in the 3' flanking region of the PA1 transcript. A 323 nucleotide fragment, spanning nucleotides 10-333 of the PA1 3' flanking region, conferred a small response to sulfur deficiency. This response was enhanced when a further 173 nucleotides of PA1 3' flanking region were included in the reporter gene construct. Future experiments to determine more precisely, which PA1 RNA sequences are necessary for sulfur-responsive gene expression are described.

Post-transcriptional regulation of gene expression often involves physical interactions between specific RNA sequence or structural elements and cellular proteins. Protein extracts from pea cotyledons were probed with a transcript containing the 323 nucleotide region conferring sulfur-responsive gene expression to identify proteins capable of associating with the sulfur-regulatory region of the PA1 transcript. Proteins in pea cotyledons from plants grown under sulfur-adequate and sulfur-deficient conditions formed different complexes with transcripts from the PA1 3' flanking region. UV-crosslinking analyses confirmed that different proteins were associated with the PA1 3' flanking region in sulfur-adequate and sulfur-deficient extracts. Four proteins which bound in a sulfur-dependent manner to a transcript containing the 323 nucleotide sulfur regulatory region were identified. These proteins may have a role in regulating PA1 mRNA abundance in response to sulfur nutrition.

Further experiments which could further examine the nature of the mechanism regulating sulfur-responsive gene expression, and characterize the molecular components of the response are discussed, as is the significance of this mechanism for future modification of agriculturally useful species.
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CHAPTER 1

REVIEW OF LITERATURE
Chapter 1. Review of Literature

1.1 INTRODUCTION

Australian pastures commonly do not provide sufficient sulfur-containing amino acids to allow grazing sheep to fulfil their genetic potential for wool growth. Wool growth rate responses to high-sulfur amino acid diets and subcutaneous or abomasally injected cysteine and methionine have been observed. Some seed storage proteins contain moderate to high levels of sulfur amino acids. These proteins may be useful for improving the nutritional status of pastures if introduced into pasture species by genetic engineering. Some sulfur-rich proteins however, are sensitive to soil sulfur levels and are poorly expressed in plants grown on sulfur-deficient soils. Investigation of the molecular events resulting from poor sulfur nutrition will provide insight into both the basic question of how plants respond to changes of environment, and also into how this response might be controlled for successful accumulation of high-sulfur proteins under field conditions where sulfur status varies.

This review will examine the need for improving pastures with respect to sulfur amino acid composition and potential sources of sulfur-rich proteins available for transformation into pasture species. The responses of these proteins and especially that of the pea seed storage protein, pea albumin 1 (PA1), to sulfur nutrition, and the post-transcriptional mechanism by which these responses occur will be discussed. A discussion of general features controlling messenger RNA stability will be followed by specific examples from both plant and mammalian systems, in which gene expression is regulated by post-transcriptional processes.

The involvement of RNA-binding proteins (RNA-BPs) in the modulation of mRNA stability in some examples will be discussed, illustrating the frequency of occurrence of regulatory RNA/protein interactions. These examples, along with a summary of the current knowledge of stability determining elements in the mRNA of PA1, will form the basis of a discussion of a possible mechanism for the response of this sulfur-rich protein to soil sulfur nutrition.

1.1.1 Nutritional requirements for wool production

Keratin is the major protein of wool and contains 9-13% sulfur amino acids while the plant and microbial protein which is available for absorption and utilization by sheep, after digestion of food in the rumen contains only 2-3% sulfur amino acids (Cottle, 1988). Improvements in wool growth of penned sheep have been observed in response
to cysteine-rich blood meal feed supplements (Marston, 1932) and infusions of cysteine or methionine into the abomasum (Reis and Schinckel, 1963). Wool growth responses to methionine administered to the abomasum have also been reported in grazing sheep (Langlands, 1970), indicating that certain pastures may lack sufficient sulfur amino acids to support maximal wool growth. These improvements in wool growth cannot be attributed to an increase in metabolic energy from the amino acid supplements, as infusion of equivalent amounts of amino nitrogen did not affect wool growth (Reis and Schinckel, 1963).

Larger responses occurred when casein was supplied than when an equivalent amount of cysteine alone was supplied. This indicates that cysteine may be more effectively converted to wool protein if it is consumed in the presence of other amino acids. Dove and Robards (1974) also found that wool growth was greatest in response to a protein-rich diet and suggested that sulfur amino acids were utilized more efficiently for wool growth if consumed with an adequate supply of the other amino acids required for protein synthesis.

Other factors can also influence the magnitude of the response of wool growth to sulfur-supplementation, including the age and reproductive status of the animal (Stewart et al., 1993), initial wool growth rate (Pickering and Reis, 1993) and genetic potential for wool growth (Williams, 1995). Diet quality however, is important and may be more readily manipulated than other factors.

Two important conclusions can be drawn from these studies of the effects of sulfur amino acids on wool growth. Firstly, any sulfur amino acid supplement must either be administered post-ruminally (i.e. to the abomasum), or be resistant to degradation during passage through the rumen. Cysteine and methionine are rapidly degraded in the rumen, with up to 90% of intra-ruminally injected methionine deaminated in one hour (Maramatsu et al., 1994), so they are of little benefit for raising sulfur availability to sheep if supplied orally (Doyle and Bird, 1975; Doyle and Moir, 1979). Furthermore, the efficiency of utilization of sulfur amino acids for wool production may decline if the supplement is not consumed daily (Langlands, 1970), hence it seems important to provide a rumen-stable sulfur amino acid source which can be consumed daily as a part of the animal's diet if wool production is to be improved. A whole protein supplement included in the diet would most effectively fulfil these requirements.
Secondly, greatest utilization efficiencies result from provision of sulfur amino acids in the presence of other amino acids. Again, a protein rich in sulfur amino acids would be useful. In light of these conclusions it is instructive to assess potential sources of sulfur amino acid-rich, rumen-stable proteins which could be supplied to grazing sheep as a feed supplement. Given that the technology is now available to genetically transform important pasture and grain legumes such as subclover (Trifolium subterraneum; Khan et al., 1993), field peas (Pisum sativum; Schroeder et al., 1993) and lupins (Lupinus angustifolius; L. Molvig, personal communication), such a protein could be incorporated into standing pasture or grain supplements.

1.2 PLANT-DERIVED 'HIGH-SULFUR' PROTEIN

1.2.1 Sources of sulfur amino acid-containing proteins

Many crop plants, and probably many other plant species, produce seed storage proteins which include high levels of sulfur amino acids. These proteins have been investigated both in terms of their potential to supply increased levels of sulfur amino acids to grazing sheep and also in order to understand the responses of such proteins to environmental stresses, particularly nutrient deficiency.

Seed storage proteins, which provide nutrition for the developing embryo, can be broadly categorized on the basis of their sulfur amino acid compositions into 'sulfur-rich' and 'sulfur-poor'. Many crop species produce both types in their grains. This distinction has been observed in the seeds of lupins (Blagrove et al., 1976), soybeans (Glycine max, Gayler and Sykes, 1985), barley (Hordeum vulgare, Rahman et al., 1983), wheat (Triticum aestivum) and rye (Secale cereale; Shewry et al., 1983), rapeseed (Brassica napus) and sunflower (Helianthus annuus; Spencer et al., 1990) and peas (Schroeder, 1984; Higgins, 1984). Thus there are many potential sources of plant-derived, sulfur amino acid-containing proteins which could be incorporated into the diets of grazing sheep via genetic manipulation of pasture species.

The sulfur-rich albumin fractions of sunflower (McNabb et al., 1994) and pea (Spencer et al., 1988) have been shown to be relatively resistant to degradation by rumen microbes in in vitro experiments. It is likely that these proteins could pass through the rumen without being converted to low quality microbial protein, thereby providing a supply of intact, sulfur amino acid-rich protein for utilization in wool production.
Relative levels of these storage proteins are regulated by soil sulfur nutrition in all of the above-mentioned species. In general, soil sulfur deficiency causes a reduction in the levels of sulfur-rich proteins and a compensating increase in the levels of low-sulfur proteins, so that the total seed protein content is maintained. There are however, some exceptions and the responses of some of the most important crop species are summarised in Table 1.1.

Peas are probably the most extensively studied of the plant species known to contain high-sulfur seed storage proteins, and the metabolism of pea seed storage proteins in response to sulfur deficiency has been particularly well characterised. The major storage proteins of pea seeds are vicilin (40% of seed protein) and legumin (approximately 25% of seed protein; Schroeder, 1984). Vicilin does not contain any sulfur amino acids (Spencer et al., 1983) while legumin contains 1.7% sulfur amino acids (Lycett et al., 1984).

In addition to legumin, pea seeds produce three major albumins which also contain sulfur amino acids: pea albumin 1 (PA1 - 11% sulfur amino acid, Higgins et al., 1986), pea albumin 2 (PA2 - 3.5% sulfur amino acid, Higgins et al., 1987) and pea albumin 3 (PA3 - 2.6% sulfur amino acid; Schroeder, 1984). PA1 is the major contributor to pea seed sulfur amino acid content, providing approximately 50% of total sulfur amino acid in pea seeds (Higgins et al., 1986) but the abundance of PA1, as well as those of legumin and PA3, are reduced during sulfur deficiency (Randall et al., 1979; Chandler et al., 1983). These declines in protein levels are accompanied by compensatory increases in the levels of low-sulfur proteins, particularly vicilin, to maintain total seed protein content (Spencer et al., 1990). Under conditions of severe sulfur deficiency, total seed protein levels are reduced (Beach et al., 1985; Chandler et al., 1984) but the levels of the sulfur-rich proteins undergo a greater decline than those of the low-sulfur proteins, indicating that they are still preferentially suppressed by poor sulfur nutrition.

The responses of sulfur-rich seed storage proteins to soil sulfur nutrition may form a barrier to consistently expressing these proteins in pasture species, at the levels required to improve wool growth rates, under field conditions. If a gene for a sulfur-rich protein is poorly expressed in transgenic pasture plants grown in sulfur-deficient soil, the altered variety may be of no benefit for wool growth in some environments. Extensive analysis of the mechanisms by which regulation of seed protein composition by soil sulfur level occurs will be required if this problem is to be overcome.
Table 1.1 Responses of high- and low-sulfur seed storage proteins to sulfur deficiency

<table>
<thead>
<tr>
<th>Species</th>
<th>High-S proteins</th>
<th>Response to S deficiency</th>
<th>Low-S proteins</th>
<th>Response to S deficiency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pea</td>
<td>legumin</td>
<td>down</td>
<td>vicilin</td>
<td>up</td>
<td>1,2</td>
</tr>
<tr>
<td></td>
<td>PA1</td>
<td>down</td>
<td>lectin</td>
<td>up</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PA2</td>
<td>none</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PA3</td>
<td>down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lupins</td>
<td>conglutin γ</td>
<td>down</td>
<td>conglutin β</td>
<td>up</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>conglutin α</td>
<td>down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>barley</td>
<td>B hordein</td>
<td>down</td>
<td>C hordein</td>
<td>none</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>D hordein</td>
<td>down</td>
<td>NPN</td>
<td>up</td>
<td></td>
</tr>
<tr>
<td>wheat</td>
<td>glutenins (low MW)</td>
<td>down</td>
<td>glutenins (high MW)</td>
<td>up</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>albumins</td>
<td>down</td>
<td>gliadins (α,β,γ,ω)</td>
<td>up</td>
<td></td>
</tr>
<tr>
<td></td>
<td>triticins</td>
<td>down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>soybean</td>
<td>glycinin</td>
<td>down</td>
<td>β-conglycinin</td>
<td>up</td>
<td>6</td>
</tr>
<tr>
<td>rapeseed</td>
<td>napin</td>
<td>down</td>
<td>cruciferin</td>
<td>up</td>
<td>7</td>
</tr>
<tr>
<td>sunflower</td>
<td>2-4S albumins</td>
<td>down</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>helianthinins</td>
<td>down</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


PA: pea albumin; MW: molecular weight.

High-sulfur and low-sulfur proteins were distinguished by the relative amounts of sulfur-amino acids contained in each class of proteins, in each species. Low-sulfur proteins contain only trace amounts of cysteine or methionine, or lack these amino acids entirely. High-sulfur proteins contain up to 23% sulfur-amino acids, in the case of sunflower seed albumin (Körtt et al., 1991).
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The differential responses in expression of pea seed proteins to sulfur deficiency also provide a unique opportunity to study molecular aspects of a plant response to a specific environmental stress. The work described in this dissertation aims to identify some of the molecular events leading to the reduction of PA1 accumulation in response to sulfur deficiency.

1.2.2 Regulation of sulfur-rich proteins by sulfur nutrition

Expression of the PA1 and legumin genes in pea has been extensively studied and some general conclusions about the mechanisms by which expression of genes encoding sulfur-rich proteins is regulated can be drawn. Gene expression can be regulated at four major points during the progress of protein production, each of which could potentially be targeted during sulfur deficiency. Control may be exerted over transcription, post-transcriptional events including mRNA processing, export of transcripts to the cytoplasm and mRNA stability, translation and post-translational events such as protein processing and protein turnover rates.

Transcription of pea seed storage protein genes is temporally regulated during seed development (Gatehouse et al., 1982; Chandler et al., 1984). Inadequate sulfur nutrition during seed growth superimposes a second level of regulation of seed protein gene expression over this developmental control.

Given that sulfur-rich proteins are most affected by sulfur deficiency, a mechanism involving post-translational amino acid recycling was proposed (Chandler et al., 1983; Spencer et al., 1990). Under this model, translation of all proteins would continue at the same rate during sulfur deficiency but sulfur-rich storage proteins would be selectively degraded to support continued synthesis of more essential housekeeping proteins while supply of sulfur amino acids was limiting. This model was not supported by $^{14}$C labelled amino acid experiments. Legumin synthesized in sulfur-deficient cotyledons was as stable as that synthesized in sulfur-adequate cotyledons over a 20 hour chase period. This indicated that the reduction in legumin level in sulfur-deficient peas was the result of a reduction in the rate of synthesis of this protein rather than an increased rate of protein turnover (Chandler et al., 1983).

Protein synthesis rates may be regulated either by reduced availability of the specific mRNA encoding the protein in question, or by reduced translation of the mRNA, due to a shortage of amino-acyl-tRNAs. Because the sulfur-rich proteins are preferentially
affected by sulfur stress, a shortage of sulfur amino-acyl-tRNAs (cys-tRNA and met-tRNA) might restrict translation of proteins containing high levels of these amino acids. However despite a general reduction in amino-acyl-tRNA availability in sulfur-deficient plants, there is no evidence for a specific loss of sulfur amino-acyl-tRNAs (Macnicol, 1983), indicating that the supply of sulfur amino-acyl-tRNAs does not specifically limit translation of mRNAs encoding proteins containing high levels of sulfur amino acids.

Other observations also indicate that expression of sulfur-rich proteins is not limited by the availability of sulfur amino-acyl-tRNAs during sulfur deficiency. Firstly, while PA2 contains 2.6% sulfur amino acids (compared with 1.6% in legumin), its accumulation in the developing pea seed is not affected by sulfur nutrition (Spencer et al., 1990). Secondly, expression of vicilin is preferentially up-regulated during sulfur deficiency yet the primary vicilin polypeptide includes a sulfur-rich signal peptide which is cleaved off as the protein is translocated into the endoplasmic reticulum (Higgins et al., 1988). This peptide contains 5.4% cysteine and methionine, presenting a sulfur-rich sequence to the translation machinery. Taken together, these observations indicate that the availability of sulfur amino-acyl-tRNAs does not inhibit translation of sulfur-rich peptides during sulfur deficiency.

The remaining possibility for control of sulfur-rich protein expression in response to sulfur deficiency is a reduction in specific mRNA abundance either due to a reduction in transcription rate, or to an increased rate of mRNA turnover. By measuring steady-state mRNA levels and transcription rates in pea cotyledons recovering from sulfur-stress it was determined that turnover of mRNA for legumin and PA1 increased during sulfur stress. During a 48 hour period of recovery from sulfur deficiency, rates of both legumin and PA1 transcription varied less than two fold. In the same period the steady-state level of legumin mRNA increased 20-fold (Beach et al., 1985) while that of PA1 increased 9-fold (Higgins et al., 1986). These discrepancies between transcription rate, measured by \textit{in vitro} nuclear run-on transcription experiments, and steady-state mRNA levels imply that there is a change in stabilities of PA1 and legumin mRNAs in response to changes in sulfur nutrition, as well as a small change in transcription rate.

Rates of mRNA turnover for PA1 or legumin in sulfur-deficient peas have not been measured \textit{in vivo} for technical reasons but it appears that regulation of the expression of genes for PA1 and other high-sulfur proteins, at least in peas, occurs via a post-transcriptional pathway. This pathway may involve either selective destabilization of
mRNAs for sulfur-containing proteins during sulfur deficiency, or specific stabilization of inherently unstable transcripts when sulfur nutrition is adequate.

Stability of mRNA can be modulated in a variety of ways. General controls of mRNA stability as well as key examples from plant and animal systems will be discussed in the following sections.

1.3 GENERAL CONTROLS OF mRNA STABILITY

Messenger RNA turnover can be controlled by both general stability determinants which occur almost universally in eukaryote mRNAs, and by specific sequence or structural stability determinants which allow the transcripts in which they occur to be recognized and differentially regulated.

Poly(A) tails added to the 3' end of mRNA molecules before export to the cytoplasm are present in almost all eukaryotic messages. These tails can range in length from approximately 90 nucleotides in yeast (Groner and Phillips, 1975) to about 300 nucleotides in vertebrates (Brawerman, 1981). Only histone mRNAs lack this poly(A) tail, and the 3' ends of these transcripts are characterized by a conserved hairpin loop structure (Marzluff and Pandey, 1988). Poly(A) tails may have functions in nuclear processing and transport of mRNA, and initiation of translation (Brawerman, 1981; Peltz et al., 1991). Poly(A) tails have also been widely implicated in control of mRNA stability (reviewed by Beelman and Parker, 1995; Ross, 1995). Generally, poly(A) is complexed with multiple copies of poly(A)-binding protein (PABP) which can both protect the poly(A) from rapid degradation, and can also act to facilitate poly(A) removal during mRNA metabolism (Baker, 1993).

When complexed with PABP, poly(A) has been proposed to protect the body of the mRNA from degradation by non-specific exoribonucleases. This proposition is supported by several observations. Firstly, most polyadenylated messages are degraded more slowly than deadenylated messages (Peltz et al., 1991), indicating a protective role for poly(A) tracts. Secondly, depletion of PABP from protein extracts results in rapid degradation of polyadenylated mRNAs in vitro, but addition of excess PABP to PABP-depleted extracts restores the stability of poly(A) mRNAs (Bernstein et al., 1989), indicating that both poly(A) and PABP are necessary to protect mRNA from rapid decay. Finally, deadenylation has been shown to precede mRNA degradation in many instances; for example, mRNA for the mammalian proto-oncogene, c-fos, remains stable until the
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poly(A) tail has been removed (Shyu et al., 1991). Similarly, poly(A) shortening of c-myc mRNA was observed after 5 to 15 minutes of incubation of polysome-based, cell-free mRNA decay reactions. This corresponded closely with the time at which c-myc mRNA degradation products began to accumulate, indicating that poly(A) tail removal was required before c-myc mRNA degradation could proceed (Brewer and Ross, 1988).

Deadenylation can expose transcripts to degradation by 3' to 5' exoribonucleases, and intermediates consistent with this pathway have been observed for yeast PGK1 mRNA (Muhlrad and Parker, 1994; Muhlrad et al., 1995) and oat phytochrome mRNA (Higgs and Colbert, 1994). Nevertheless, some mRNAs can be stable in the absence of a poly(A) tail (Shyu et al., 1991; Chen et al., 1994).

The other structure which appears to protect eukaryotic mRNA from indiscriminate decay is the 5' 7-methylguanosine cap structure (Stevens, 1993). It is an important component in translation initiation (Shatkin, 1985) and has been implicated in control of mRNA stability. Uncapped mRNAs are less stable than capped transcripts in both oocytes (Furuichi et al., 1977; Drummond et al., 1985) and in in vitro experiments (Peltz et al., 1987). This structure has been proposed to protect mRNAs from 5' to 3' exoribonuclease activity (Furuichi et al., 1977), although to date, decapping activities and 5' to 3' exoribonucleases have mainly been isolated from and characterized in yeast (Saccharomyces cerevisiae) (Stevens, 1988; 1993). A putative decapping enzyme with 5' to 3' exoribonuclease activity however, has been described in mouse sarcoma cells (Coutts and Brawerman, 1993).

In addition to these separate roles there is some evidence to indicate that there may be interactions between these 5' and 3' structures which regulate the rate at which mRNA is degraded (Decker and Parker, 1994). Studies in yeast in which 5' to 3' degradation is blocked have shown that decapped mRNAs accumulate after deadenylation (Larimer and Stevens, 1990; Muhlrad et al., 1994; 1995). This indicates that deadenylated mRNA may become a substrate for decapping and subsequently, for decay in a 5' to 3' direction.

Some examples of plant and mammalian genes whose expression is at least partially regulated at the level of mRNA stability by specific stability-determining sequence elements will be discussed below (summarised in figure 1.1)
Figure 1.1 Summary of mRNA instability determinants discussed in chapter 1. PhyA: phytochrome A; SAUR: small auxin-up RNA; rbcS: rubisco small subunit; PvPRP1: bean proline-rich protein 1; GM-CSF: granulocyte macrophage-colony stimulating factor; PA1: pea albumin 1; ORF: open reading frame; UTR: untranslated region; CR: coding region; ARE: AU-rich element; IRE: iron-responsive element; SRE: sulfur-responsive element; DST: downstream element.
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<table>
<thead>
<tr>
<th>mRNA</th>
<th>Determinant</th>
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<tr>
<td>phyA</td>
<td>poly(A)+ 5' exonuclease</td>
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<td>poly(A)- 5'/3' exonuclease</td>
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<td>SAUR</td>
<td>ORF and 3' UTR DST</td>
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<td>ferredoxin</td>
<td>5' UTR &amp; CR determinant</td>
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<td>rbcS</td>
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<td>PvPRP1</td>
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<td>GM-CSF</td>
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<td>c-myc</td>
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<td>3'-terminal stem-loop</td>
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<td>PA1</td>
<td>CR &amp; 3' UTR SREs</td>
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1.4 POST-TRANSCRIPTIONAL REGULATION IN PLANT NUCLEAR GENES

The development-specific pattern of seed storage protein occurs by temporal regulation of transcription initiation (section 1.2.2), although accumulation of non-seed proteins in seeds appears to be prevented by a post-transcriptional mechanism (Walling et al., 1986; Sorensen et al., 1989). While transcriptional regulation provides a level of control appropriate to relatively slow developmental changes, plants also require a means to effect rapid changes in gene expression since, being sessile organisms, they must respond rapidly to environmental stimuli.

Post-transcriptional control of gene expression at the level of mRNA stability has two main advantages for plants which are unable to escape from unfavourable environmental conditions. Firstly, the stability of a given mRNA affects both its steady-state concentration and the rate at which a new steady-state level can be achieved after a change in transcription rate.

Using the equations of Hargrove and Schmidt (1989), both Morton (1993) and Ross (1995) have shown that a more stable mRNA will have a higher concentration at steady-state than an unstable mRNA being transcribed at the same rate (since steady-state concentration is a function of both synthesis rate and degradation rate), but the more stable mRNA will also take longer to reach a new steady-state level following a change in transcription rate. For example, if transcription of a particular gene ceases, the half life of the existing transcripts determines the time taken for those transcripts to be cleared from the cytoplasm. Transcripts with short half lives will be cleared from the cell more rapidly than those that are stable for longer. The advantage of this for plants is that genes which will be subject to rapid changes in expression, such as those regulated by light or temperature can be expressed as short-lived mRNAs, and those whose expression will need to be buffered against sudden changes in environment can be expressed as more stable mRNAs (Green, 1993).

The second feature of post-transcriptional regulation is that the half life of a given mRNA need not necessarily be fixed, but can be modulated in response to changes in environmental conditions. This flexibility allows for a high degree of control over rapid changes in gene expression. Thus for a given gene being transcribed at a given rate, the concentration of its mRNA will be a function of both the 'inherent' stability of the mRNA and any modulation of that stability by environmental or cellular factors.
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It is important to distinguish between inherently unstable mRNAs and mRNAs which are specifically destabilized in response to certain stimuli. Plant nuclear-encoded genes can be subject to both levels of regulation. Most eukaryotic mRNA species decay with half lives of up to 30 hours (Silflow and Key, 1979; Brawerman, 1993) therefore they may be considered stable unless specifically destabilized (Sullivan and Green, 1993). Some mRNA species in contrast, are inherently unstable and both types of mRNAs will be discussed below.

1.4.1 Plant genes with inherently unstable transcripts

1.4.1.1 Phytochrome A

Phytochrome plays an important role in the light-dependent development of plants and expression of phytochrome A is itself regulated in response to light. Transcription of phytochrome genes has been shown to decline by 65% (Quail et al., 1986) to 90% (Lissemore and Quail, 1988) on exposure of dark-grown plants to white or red light. Colbert et al. (1985) observed a 90% decline in phytochrome mRNA within three to five hours of red light irradiation of oat (Avena sativa) seedlings. This indicated either that phytochrome A mRNA is specifically destabilized by exposure to light, or that it is inherently unstable, since transcripts of most plant genes persist for many hours after cessation of transcription (Silflow and Key, 1979).

Seeley et al. (1992) established that in the absence of transcription, phytochrome A mRNA in dark-grown (etiolated) oat seedling decayed with a half life of approximately 60 minutes, compared with approximately 90 minutes in plants exposed to a pulse of red light, indicating that phytochrome A mRNA is inherently unstable compared with other eukaryotic mRNAs. This instability allows rapid clearance of phytochrome A mRNA following the decline in transcription induced by irradiation.

1.4.1.2 Auxin responsive mRNAs

Auxins can induce changes in cell extension within 20 minutes, requiring rapid changes in gene expression (Edelmann and Schopfer, 1989). A number of small mRNAs, called small auxin-up RNAs (SAURs) are induced in soybean hypocotyls within 2.5 minutes of auxin treatment (McClure and Guilfoyle, 1987), and have been estimated to have half lives of between 10 and 50 minutes (McClure and Guilfoyle, 1989). The induction of
SAURs by auxin is due to a six-fold increase in the rate of transcription initiation (McClure et al., 1989) however the short half lives of the mRNAs allow them to attain the new, higher steady-state level rapidly (McClure and Guilfoyle, 1987).

SAURs contain a 40 base pair element in their 3' untranslated regions (UTRs) referred to as the downstream (DST) element (McClure and Guilfoyle, 1989). This element consists of three highly conserved sequences separated by two variable sequences, and has been observed in auxin-responsive genes of soybean, mung bean, Arabidopsis and tobacco (Newman et al., 1993). DST elements have been proposed to control SAUR transcript stability (McClure et al., 1989; Franco et al., 1990). Rates of decay of chimeric mRNAs containing either β-glucuronidase (GUS) or β-globin coding regions, as well as two copies of the DST sequence in the 3' UTR, were measured in stably transformed tobacco cells in culture and in transgenic tobacco plants. The presence of DST elements in the 3' UTR caused 3.6- to 4.8-fold decreases in stabilities of GUS+2xDST and β-globin+2xDST transcripts compared with GUS and β-globin transcripts which did not contain DST elements (Newman et al., 1993).

These results indicate that the DST elements were recognized as mRNA instability determinants in plant cells. It should be noted however, that two copies of the DST element were required for destabilization in this study, yet in its normal context in the 3' UTR of SAURs, only a single DST element is present (Li et al., 1994), indicating that the DST element alone may not be sufficient to direct rapid mRNA turnover.

1.4.2 Plant genes with transcripts of modulated stability

While mRNA instability permits rapid adjustment to changing environmental conditions, modulation of mRNA stability allows for an extra level of control of gene expression in response to exogenous signals. There are several examples of regulated mRNA stability in plants and the exogenous signals eliciting such regulation range from light and temperature to pathogen attack and ripening signals (reviewed by Sullivan and Green, 1993). Specific examples will be discussed here.

1.4.2.1 Ferredoxin

Ferredoxin is a major component of the photosynthetic electron transport system and in peas, is encoded by the nuclear gene Fed-1 (Elliott et al., 1989b). Expression of Fed-1 is regulated by light at both transcriptional (Gallo-Meagher et al., 1992) and post-
transcriptional (Elliott et al., 1989a) levels, with Fed-1 mRNA accumulating rapidly after exposure to light.

A region spanning 38 nucleotides of the 5' leader sequence and one third of the ferredoxin coding region can confer a two- to five-fold increase in reporter mRNA abundance in response to light, in the absence of any change in transcription rate (Dickey et al., 1992). This discrepancy between transcription rate and mRNA accumulation rate may indicate that Fed-1 mRNA is specifically stabilized in light grown tissue.

Vorst et al. (1993) showed that expression of the Arabidopsis gene ferredoxin A (FedA) in response to light has both transcriptional and post-transcriptional components. Ferredoxin promoter-GUS fusions showed a two- to four-fold increase in transcriptional activity after exposure to light, while expression of the intact FedA gene in Arabidopsis increased 20-fold. Run-on transcription experiments with isolated Arabidopsis nuclei showed that transcription increased only 2-fold in response to light, indicating that there may be up to a 10-fold stabilization of FedA mRNA in response to light.

1.4.2.2 Rubisco small subunit

Expression of another photosynthetic protein, ribulose-1,5-bisphosphate carboxylase (rubisco) is also regulated by light. Transcription of the nuclear gene rbcS, which encodes the small subunit of this multimeric protein has been shown to be regulated by phytochrome (Silverthorne and Tobin, 1984). However, there may also be a post-transcriptional component to the light responses of various members of the potato rbcS gene family (Fritz et al., 1991). When light-grown plants were transferred to darkness, rbcS mRNA disappeared after 24 hours, whereas in light-grown plants in which transcription was inhibited by cordycepin, rbcS mRNA was still detectable after 36 hours. Thus the loss of rbcS mRNA on transfer to darkness could not be accounted for solely by cessation of transcription.

This data should be interpreted with caution, as cordycepin treatment appeared to stabilize rbcS messages in the dark. The discrepancy between rbcS levels in plants transferred to the dark and those in which transcription was inhibited but which remained in the light may have been due to cordycepin-induced stabilization of the rbcS message, rather than to stabilizing effects of light.
Nevertheless, various members of the \( rbcS \) gene family responded differentially to dark treatment. Since transcription of \( rbcS \) genes is known to be inhibited in the dark via a phytochrome-mediated pathway, this differential loss of \( rbcS \) transcripts in the dark has been assumed to result from differential rates of mRNA decay for individual members of the \( rbcS \) gene family. This result was supported by those of Wanner and Gruissem (1991) who found that although transcription rate was the 'primary determinant' of \( rbcS \) mRNA accumulation, subtle changes in levels of individual \( rbcS \) genes were regulated at the level of mRNA stability. In a comparison between etiolated and light-grown seedlings it was found that transcription rates of the genes \( rbcS \) 1 and \( rbcS \) 2 were 2.5-fold higher in light-grown than in etiolated seedlings, but that steady-state mRNA levels were approximately five-fold greater, indicating that light stabilized these mRNAs.

The five tomato \( rbcS \) genes show a high degree of DNA sequence conservation in the protein coding region (Sugita and Gruissem, 1987), indicating that modulation of \( rbcS \) mRNA stability is likely to be mediated via divergent sequences in the 5' and/or 3' untranslated regions. Involvement of 3' UTR sequences in \( rbcS \) mRNA decay has been implicated by a study of \( rbcS \) mRNA degradation products in soybean and transgenic petunia (Thompson et al., 1992). Degradation products of the soybean \( rbcS \) SRS4 mRNA were generally intact at the 5' end but were deadenylated and variously degraded at the 3' end, indicating that some feature towards the 3' ends of these transcripts may be targeted by the decay mechanism.

Similarly in \textit{Lemna gibba}, six \( rbcS \) genes were highly conserved in the coding region, but divergent in 5' and 3' UTRs (Silverthorne et al., 1990). Expression studies revealed differential patterns of mRNA accumulation between \( rbcS \) genes in roots and fronds. These accumulation patterns did not correlate with the differences in transcription rates of the individual genes (Silverthorne and Tobin, 1990). Taken together with the sequence divergence only in untranslated regions of the \( rbcS \) genes, these data indicate that the tissue-specific expression of \( rbcS \) genes may be post-transcriptionally regulated via sequences in 5' or 3' UTRs in \textit{L. gibba}.

1.4.2.3 Pathogen Response

Fungal elicitors can cause a rapid increase in levels of mRNAs encoding a variety of defence-related proteins, for example; cell wall proteins, chitinases and glucanases (Dixon and Harrison, 1990). They can also cause rapid reductions in the levels of other mRNAs. The mRNA for bean proline-rich protein (PvPRP1) is one example. Treatment of
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*Phaseolus vulgaris* cells with *Colletotrichum* elicitors resulted in reduction of PvPRP1 message level to approximately 6% of its original level within 3 hours (Zhang *et al.*, 1993). Nuclear run-off experiments showed that transcription was not affected by the elicitor, indicating that the decline in mRNA level may have been due to destabilization of PvPRP1 message. Actinomycin D (ActD) treatment stabilized PvPRP1 mRNA in elicitor-treated cells (half life: 18 hours compared with 45 minutes in elicited cells not treated with ActD). However in cells treated with ActD but not elicitor, PvPRP1 mRNA decayed with a half life of approximately 60 hours. Clearly therefore, the fungal elicitor destabilized PvPRP1 mRNA.

Further information about the mechanism of this destabilization is available from studies with translation inhibitors (Zhang *et al.*, 1993). Various translation inhibitors inhibited elicitor-dependent PvPRP1 mRNA decay by between 10 and 75% depending on the efficiency of translation inhibition. Taken together with the data indicating that PvPRP1 mRNA was stabilized by transcription inhibitors, these data indicate that *de novo* protein synthesis was required for elicitor induced degradation of PvPRP1 mRNA. This may indicate that a new protein involved in PvPRP1 mRNA decay is synthesized upon treatment with fungal elicitor, or that a very labile protein which must be constantly re-synthesized is involved.

A 50 kilo dalton (kD) RNA-binding protein specific for the PvPRP1 message was identified by UV-crosslinking experiments (Zhang and Mehdy, 1994). This protein, referred to as PRP-BP, recognized a 27 nucleotide U-rich element in the 3' UTR of PvPRP1 mRNA. PRP-binding activity was up-regulated in elicitor-treated cells and reached a level five fold higher than in unelicited cells immediately prior to the rapid decline in PvPRP1 mRNA level induced by elicitor treatment.

Experiments with agents capable of reducing (dithiothreitol (DTT), β-mercaptoethanol), oxidizing (diamine) or alkylating (N-ethylmaleimide) sulfhydryl groups showed that reduced -SH groups were required for binding, and that up-regulation of PRP-BP binding activity by fungal elicitor was likely to be the result of a post-translational modification involving SH groups. This was in contrast to previous results which indicated that on-going transcription and translation were required for PvPRP1 mRNA destabilization (Zhang *et al.*, 1993). It is likely therefore, that PRP-BP may form part of a multi-component complex involved in the destabilization of PvPRP1 mRNA in elicitor-treated cells. Nevertheless, this was the first reported identification of a cytoplasmic mRNA-binding protein interacting specifically with a nuclear-encoded mRNA in plants.
1.5 POST-TRANSCRIPTIONAL REGULATION IN CHLOROPLAST GENES

The organization and expression of plastid genes differs substantially from that of nuclear-encoded genes. This area has been reviewed in depth by Gruissem and Schuster (1993) and it is clear that post-transcriptional processes play a major role in regulating gene expression during differentiation and development of the chloroplast. Although there are general changes in plastid transcriptional activity during development of chloroplasts, these changes appear to affect all plastid genes similarly and do not account for differential mRNA accumulation during chloroplast development (Deng and Gruissem, 1987; Deng et al., 1987).

Patterns of mRNA accumulation and stability during chloroplast development have been studied for several chloroplast genes (Deng and Gruissem, 1987; Kim et al., 1991). The discrepancy between transcription rate and transcript accumulation is best exemplified by comparisons between expression of the genes for rubisco large subunit (rbcL) and photosystem II D1 protein (psbA) genes (Gruissem and Schuster, 1993). In etioplasts, psbA was transcribed at a higher rate than rbcL, yet rbcL transcript accumulated to a 7-10 fold higher level, indicating that psbA mRNA was unstable in the dark. On illumination the transcriptional activities of both genes increased in response to chloroplast maturation but their relative rates remained similar. However in mature chloroplasts, the level of psbA mRNA actually exceeded that of rbcL mRNA, indicating that psbA mRNA was selectively stabilized by light (Deng and Gruissem, 1987).

These results were supported by those of Kim et al., (1991) who found that the half life of psbA mRNA increased from 8 hours to greater than 40 hours during barley chloroplast development, while that of rbcL mRNA increased from 12 to 15 hours over the same period. Similarly Klaff and Gruissem (1991) reported an increase in psbA mRNA half life from 4.75 hours to 10.25 hours during leaf maturation, while rbcL mRNA half life increased from 4.5 to 5 hours over the same interval in spinach leaves.

Modulation of mRNA stability therefore, plays an important role in the regulation of gene expression in the chloroplast, since there does not appear to be any gene specific regulation of transcription initiation yet the levels of various mRNAs vary considerably throughout the course of plastid development and leaf maturation.
1.6 POST-TRANSCRIPTIONAL REGULATION IN MAMMALIAN CELLS

The importance of mRNA instability for effecting rapid changes in steady-state mRNA levels has been discussed in relation to plant cells, however it is also of great significance for mammalian cells. Transcripts from genes encoding the lymphokines and cell growth factors involved in regulation of cell growth and differentiation have unstable transcripts, as do histone mRNAs which are required only at a specific point in the cell cycle. This allows these mRNAs to accumulate rapidly after transcription of the genes is initiated, and also allows for rapid reduction in mRNA and protein levels when the gene product is no longer required. Failure to regulate the stability of certain proto-oncogene transcripts can result in tumour formation (Chen and Shyu, 1995). Hence post-transcriptional regulation of mRNA stability is clearly important in mammalian cells.

Several decay pathways have been elucidated for mammalian mRNAs. These pathways can be broadly subdivided into deadenylation-dependent and deadenylation-independent (Decker and Parker, 1994; Beelman and Parker, 1995). Loss of the poly(A) tail plays a major role in initiating the decay of many eukaryotic mRNAs, as discussed previously. However almost all eukaryotic mRNAs carry a poly(A) tail, yet they decay with half lives ranging from a few minutes to many hours (Brawerman, 1993). This diversity of mRNA decay rates suggests that there may be other, message-specific determinants of mRNA stability. Some examples of different types of stability determinants and their actions are discussed below.

1.6.1 Proto-oncogene and cytokine mRNAs

Genes that are rapidly induced upon exposure to stimuli such as growth factors, cytokines and neurotransmitters are called 'early response genes' and their transcripts from these genes decay rapidly upon transport into the cytoplasm (Greenberg and Belasco, 1993). Two distinct classes of early response genes can be identified on the basis of their modes of regulation. In the first class, expression is transiently elevated in response to extracellular stimuli by stabilization of their mRNA, which is otherwise unstable. Expression of the second class in contrast, is induced by a rapid, transient increase in transcription in response to extracellular stimuli.
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1.6.1.1 AU-rich elements

A well characterised example of the first class of early response genes is granulocyte macrophage-colony stimulating factor (GM-CSF). GM-CSF controls differentiation, proliferation and functional activation of granulocytes and macrophages in blood (reviewed by Metcalf, 1985). Shaw and Kamen (1986) observed that when T-cells were activated with lectin, GM-CSF mRNA disappeared within 30 minutes of transcription inhibition by actinomycin D. A highly conserved AU-rich sequence in the 3' UTR of GM-CSF mRNA containing several copies of the pentanucleotide motif AUUUA was found to destabilize β-globin mRNA. β-globin mRNA with a GC-rich sequence inserted into its 3’ UTR was stable for at least 2 hours but when the GM-CSF AU-rich region was cloned into the β-globin 3' UTR, the resulting mRNA decayed with a half life of less than 30 minutes indicating that this region was a potent mRNA destabilizing element.

The destabilizing activity of the GM-CSF AU-rich element (ARE) appeared to be modulated by the nature of the activating agent. For example, GM-CSF mRNA in T-cells treated with lectin was destabilized whereas in T-cells treated with a phorbol-diester the message was stabilized, indicating that the GM-CSF ARE may have a function in regulating GM-CSF expression in response to various stimuli.

The AUUUA motif is also present in the 3’ UTRs of a number of other cytokine and proto-oncogene 3’ UTRs (Greenberg and Belasco, 1993). The proto-oncogene c-fos, a member of the second class of early response genes, also contains a destabilizing ARE in the 3’ UTR of its mRNA. Deletion of this region results in transformation of this gene to an oncogene (Meijlink et al., 1985). In contrast to the GM-CSF ARE, the destabilizing activity of the ARE in the c-fos 3’ UTR is not affected by exposure to growth factor or cytokine but rather is constitutively active (Shyu et al., 1989; Lindsten et al., 1989). The different responses mediated by these two AREs indicates that there may be independent pathways by which mRNAs containing these sequences are degraded.

Some plant transcripts also contain AU-rich motifs (Ainley et al., 1988; Takahashi et al., 1989) and a synthetic sequence containing 11 AUUUA repeats has been shown to destabilize reporter transcripts in cultured tobacco cells (Ohme-Takagi et al., 1993). The mechanism by which this sequence targets mRNA for rapid decay is unknown.
1.6.1.2 How does the ARE initiate decay of mammalian mRNA?

Both c-fos-type and GM-CSF-type AREs have been shown to direct rapid deadenylation prior to degradation of the body of the mRNA (Shyu et al., 1991; Chen et al., 1994; Chen et al., 1995). The c-fos ARE directs biphasic decay of mRNA, with deadenylation as a first step. This deadenylation step, which does not appear to require intact AUUUA pentanucleotides in the AU-rich region, occurs in a synchronous manner leaving 30 to 60 adenosines on all molecules (Chen et al., 1995). Subsequent decay of the mRNA apparently depends on the presence of intact AUUUA motifs.

In contrast the GM-CSF ARE appears to direct a processive loss of the poly(A) tail, resulting in complete deadenylation of transcripts (Chen et al., 1995). These differences indicate either that different RNases participate in poly(A) removal from the two types of transcripts, or that a single RNase is involved and its processivity is modulated by the nature of the ARE.

Attempts have been made to identify a common, minimal functional sequence amongst various types of AU-rich elements (Chen and Shyu, 1994; Lagnado et al., 1994; Stoecklin et al., 1994) but the diversity of responses among different ARE-containing transcripts in different cell types and under different growth conditions (Ross, 1995) suggests that more complex mechanisms may be involved in targeting the various transcripts for degradation. It is possible that AREs exert their influence on mRNA stability via interactions with various trans-acting factors (Schuler and Cole, 1988; Ross, 1995), expression of which is regulated by cell type and growth conditions.

1.6.1.3 ARE-binding proteins

Several proteins with the capacity to bind to AU-rich elements (AUBPs) have been described recently. A protein in Jurkat cells capable of binding to a synthetic RNA sequence containing four iterations of the AUUUA motif was named the AU-binding factor (AUBF; Malter, 1989). AUBF appears to stabilize GM-CSF mRNA by masking AUUUA instability determinants, since addition of AUUUA-containing competitor mRNAs caused five-fold destabilization of GM-CSF mRNA (Rajagopalan and Malter, 1994). A protein binding to the ARE of c-myc mRNA has also been described in erythroleukaemia cells (Brewer, 1991). Another ARE-binding protein, called AUF, was identified on the basis of both its affinity for the c-myc ARE and its ability to degrade polysome-bound c-myc mRNA.
Other factors binding specifically to AREs have been described. A group of three AUBPs designated AU-A, AU-B and AU-C have been isolated from human T-lymphocytes (Bohjanen et al., 1991; 1992). AU-A associated with both GM-CSF and c-myc type AREs. It was constitutively expressed and appeared to be located primarily in the nucleus. It has since been shown to shuttle between nucleus and cytoplasm (Katz et al., 1994), indicating that it may be involved in transport of ARE-containing transcripts to the cytoplasm. AU-B and AU-C in contrast, bind only to AREs containing three or more overlapping iterations of the AUUUA motif and do not recognize the dispersed AUUUA motifs found in c-myc and c-fos type AREs. These AUBPs are only expressed in stimulated T-cells (Bohjanen et al., 1991), in which GM-CSF mRNA was shown to be unstable (Shaw and Kamen, 1986). Under conditions which stabilize GM-CSF mRNA binding activity of AU-B is decreased. Taken together these data may indicate that these mRNA binding proteins are directly involved in regulating lymphokine mRNA stability in stimulated T-cells.

In summary, the AUBF appears to protect GM-CSF mRNA from degradation by masking the AUUUA instability determinant, while AU-B and AU-C may actively destabilize the same mRNA by binding to the same sequence. mRNA stability will be determined by the balance between stabilizing and destabilizing mRNA-associated proteins in any given cell type.

1.6.1.4 Coding region determinants

In addition to the AREs in their 3' UTRs, c-myc and c-fos contain instability elements in their coding regions. The c-fos coding region contains a 320 nucleotide sequence which can induce a four-fold destabilization of mRNA when introduced, in-frame, into the β-globin coding region (Shyu et al., 1989; 1991). This instability-determining region is bound by two proteins which are necessary for destabilization of c-fos mRNA (Chen et al., 1992).

c-myc mRNA contains a 180 nucleotide segment at the 3' end of the coding region (coding region determinant - CRD) which, when introduced in-frame into β-globin mRNA, resulted in 2-3 fold destabilization of the β-globin mRNA (Herrick and Ross, 1994). This region was found to be recognized by a 70 kD polysomal protein (Bernstein et al., 1992). Addition of this 180 nucleotide fragment to in vitro mRNA decay assays resulted in 8-fold destabilization of polysomal c-myc mRNA and also destabilized CRD-
containing β-globin mRNA. It was proposed that the 70 kD protein normally binds to the c-myc CRD and protects it from endoribonucleolytic attack. Addition of exogenous CRD mRNA may have titrated the CRD-BP away from c-myc mRNA, exposing endoribonuclease-sensitive sites. Consistent with this argument, Bernstein et al. (1992) observed endonucleolytic c-myc cleavage products in the presence but not in the absence of competitor mRNA. These products were not observed with mRNA containing the c-myc 3' UTR instability determinant, indicating that the two regions mediated mRNA decay via different pathways.

1.6.2 Iron-regulated gene expression

One of the best characterized examples of post-transcriptional regulation of gene expression in mammalian systems is the iron responsive element found in the mRNAs encoding ferritin, transferrin receptor (TfR) and erythroid 5-aminolevulinate synthase (ALAS). Products of these mRNAs are involved in cellular iron metabolism and their expression is regulated by intracellular iron concentration. Ferritin is a major intracellular iron storage protein while TfR transports iron into the cell. The actions of ferritin and TfR together, control intracellular iron homeostasis; TfR imports iron into the cell when it is lacking and ferritin sequesters excess iron to prevent toxicity. ALAS in contrast, is the rate limiting enzyme regulating heme biosynthesis and is thus involved in iron utilization.

Expression of ferritin and TfR is coordinately regulated in response to cellular iron availability (Harford and Klausner, 1990). Abundant intracellular iron results in a reduction in TfR production, as iron import is not required. In contrast, ferritin synthesis increases under these conditions to sequester the excess iron. Conversely when iron is scarce ferritin synthesis declines, preventing iron sequestration and TfR synthesis increases, resulting in enhanced import of iron into the cell.

1.6.2.1 Iron Responsive Element

Iron dependent expression of ferritin is regulated at the level of translation and appears to be mediated by a 28 nucleotide stem-loop structure located in the 5' UTR of the mRNA (Aziz and Munro, 1987; Hentze et al., 1987). This structure is highly conserved between many vertebrate ferritins (Leibold and Munro, 1988), and has been designated the iron responsive element (IRE). The IRE is the sole determinant of repression of ferritin translation during iron starvation (Caughman et al., 1988). A similar element is present in the 5' UTR of ALAS mRNA (Cox et al., 1991), translation of which has been shown
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to be similarly repressed during iron starvation (Bhasker et al., 1993). This mechanism allows heme synthesis to be regulated in response to iron availability.

TfR mRNA contains five stem-loop structures, similar to the IRE of ferritin, in its 3' UTR (Casey et al., 1988a; Harford, 1993). Furthermore, the region of the TfR 3' UTR containing these structures coincides with the region conferring iron responsiveness (Casey et al., 1988b). Treatment of cells with the iron chelator desferrioxamine resulted in a 1.6 fold increase in TfR transcription rate but this was not sufficient to account for the 15-30 fold increase in levels of chimeric mRNAs containing regulatory regions of the TfR 3' UTR (Müllner and Kühn, 1988). TfR mRNA stability in the presence and absence of iron has been measured, and during iron starvation TfR mRNA decayed with a half life of approximately 30 hours. When iron was abundant however, TfR mRNA decayed with a half life of approximately 1.5 hours. Site-directed mutagenesis confirmed that the 3' UTR stem-loop structures were required for mRNA stabilization during iron starvation (Horowitz and Harford, 1992; Casey et al., 1989).

Degradation of TfR mRNA has been shown to be initiated by an endonucleolytic cleavage between the third and fourth stem-loop elements of the 3' regulatory region. In contrast to decay of the early response gene mRNAs discussed earlier, this cleavage was not preceded by poly(A) shortening (Binder et al., 1994).

1.6.2.2 Iron Regulatory Protein

The capacity of the IRE to regulate expression of two different genes via different mechanisms in response to the same stimulus indicated the possible involvement of one or more trans-acting protein factors in the process. A cytoplasmic protein with affinity for the ferritin IRE was isolated from rat cells and its affinity for the IRE was shown to decrease in the presence of iron (Leibold and Munro, 1988). A second protein similarly regulated in its affinity for IRE by iron, was identified in human cells (Rouault et al., 1988). Identification of these proteins, known as iron regulatory proteins (IRPs), supported the proposition of Caughman et al. (1988) that the ferritin IRE functioned as a binding site for a translational repressor (Walden et al., 1988). Regulation of IRE-binding activity of IRPs by iron provides an attractive model to explain the iron regulated translation of ferritin (Klausner et al., 1993). Under conditions of low cellular iron, the IRP has a high affinity for ferritin IRE and the resulting IRE/IRP complex would inhibit translation initiation. When iron is abundant, the IRP has low affinity for ferritin mRNA, allowing translation to proceed.
The IRP has also been shown to bind to the IRE-containing region of the TfR 3' UTR (Koeller et al., 1989), protecting up to four of the stem-loop structures (Müllner et al., 1989). The TfR mRNA-binding activity of this protein was induced by the addition of desferrioxamine and declined rapidly after addition of iron. IRP inactivation kinetics correlated with loss of TfR mRNA (Müllner and Kühn, 1988), suggesting that loss of IRP binding activity was directly related to degradation of TfR mRNA in response to iron. A model could be envisaged in which binding of the IRP to the 3' IRE would protect it from the endonucleolytic cleavage identified by Binder et al. (1994), preventing further decay of the body of the TfR mRNA.

1.6.2.3 Structure and function of the IRP

The IRP has been investigated in detail with respect to structural features which might contribute to its function. It has been shown that conversion between the low affinity IRP present in cells with adequate iron and the high affinity form which binds IREs in cells deprived of iron does not require protein synthesis (Hentze et al., 1989), and that low affinity IRP can be activated in vitro by reducing agents (Yu et al., 1992). Therefore it is likely that a post-translational mechanism switches the IRP between low and high affinity forms depending on the iron status of the cell.

Strikingly, the IRP contains all 23 active site residues of the mitochondrial enzyme, aconitase (Rouault et al., 1990; Philpott et al., 1991). Aconitase contains a [4Fe-4S] cluster at the active site. The fourth iron molecule is highly labile and loss of this molecule abolishes aconitase activity. The IRP contains an identical [4Fe-4S] cluster and has been shown to act as a functional cytoplasmic aconitase in cells with adequate iron (Haile et al., 1992a). Loss of the labile iron molecule from the iron-sulfur cluster, while abolishing aconitase activity, is not sufficient to induce IRE-binding activity. However when the [Fe-S] cluster was more extensively disrupted, RNA binding activity was observed (Haile et al., 1992b). If iron depletion of cells were to result in disassembly of the [Fe-S] cluster, this mechanism could be envisaged as a means of sensing cellular iron concentration and regulating expression of the relevant genes accordingly.

Klausner et al. (1993) proposed a model, based on the structure of mitochondrial aconitase, in which the integrity of the [Fe-S] cluster defined the conformation of the IRP. In either the [4Fe-4S] or [3Fe-4S] state the [Fe-S] cluster would hold the four domains of the protein together, permitting access of the aconitase substrate, citrate, but
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not mRNA. Disruption of the [Fe-S] cluster would allow flexibility in the structure, exposing mRNA binding sites, previously inaccessible due to the conformation maintained by the [Fe-S] cluster.

The interaction between IRE and IRP provides an attractive example of a mechanism by which the abundance of a vital nutrient can control gene expression. By regulating the affinity of a common RNA-binding protein for these mRNAs via a specific structural interaction, iron simultaneously regulates expression of several proteins involved in its metabolism. The IRP has been shown to tolerate only very small deviations in structure of the IRE (Kikinis et al., 1995), indicating that iron-dependent regulation of gene expression is limited to only those transcripts containing IREs.

1.6.3 Other examples of post-transcriptional regulation

The ARE and IRE discussed above represent the most highly characterized examples of post-transcriptional regulation of mammalian gene expression. However there are several other instances in which mRNA stability plays a role in regulating gene expression. Tubulin and histone mRNAs are degraded by unique pathways and will be discussed here to illustrate the diversity of mechanisms by which mRNA stability may be controlled.

1.6.3.1 Tubulin

The microtubules which form part of the cytoskeleton are composed of heterodimeric subunits containing α- and β-tubulin. When microtubule polymerization is blocked by colchicine, synthesis of α- and β-tubulin is specifically inhibited by the increase in concentration of tubulin monomers (Cleveland et al., 1981). The decrease in tubulin synthesis in response to elevated concentration of tubulin monomers appears to be due to destabilization of mRNA in the cytoplasm (Caron et al., 1985; Pittenger and Cleveland, 1985). Auto-regulation of β-tubulin mRNA stability by tubulin concentration is conferred by the first 13 translated nucleotides of the β-tubulin coding region (Yen et al., 1988a). These nucleotides encode the first four amino acids, Met-Arg-Glu-Ile, which must be translated in order for tubulin synthesis to be auto-regulated (Pachter et al., 1987; Gay et al., 1989). Mutations which change this peptide sequence abolish auto-regulation (Yen et al., 1988b). The factors which interact with this nascent peptide to effect tubulin mRNA degradation are as yet unknown.
1.6.3.2 Histone

Like tubulin, production of histone proteins throughout the cell cycle is autoregulated. An excess of histone protein appears to specifically destabilize histone mRNA (McLaren and Ross, 1993). Histone expression is highly regulated during the cell cycle, with histones only being produced during DNA synthesis (Marzluff and Pandey, 1988). Following cessation of DNA synthesis, histone mRNAs are rapidly degraded and it appears from in vitro studies (Peltz and Ross, 1987) that the presence of histone protein can accelerate the process 3 to 6 fold. It has been proposed that translation of histone continues after DNA synthesis has stopped, generating excess histone proteins which have no DNA with which to associate. The accumulating histone proteins act in some way to trigger histone mRNA decay (Ross, 1995).

Histone mRNAs are not polyadenylated, rather they contain a highly conserved stem-loop structure at the 3' end (Birnstiel et al., 1985; Marzluff, 1992). This structure appears to be involved in transport of histone mRNAs to the cytoplasm (Williams et al., 1994), translation (Sun et al., 1992) and regulation of histone mRNA stability (Pandey and Marzluff, 1987) by association with a stem-loop-binding protein (Pandey et al. 1991).

1.7 RNA-BINDING PROTEINS AND mRNA STABILITY

The preceding discussion of constitutive and modulated mRNA instability in mammalian cells illustrated the importance of mRNA/protein interactions for controlling mRNA stability. Trans-acting factors appear to be involved in mRNA decay processes for the AU-rich cytokine and oncogene sequences, c-myc and c-fos coding region stability determinants, transferrin receptor response to iron starvation and histone auto regulation during the cell cycle. This array of protein/mRNA interactions allows for a high level of regulation of mRNA stability in response to various stimuli, since the RNA-binding activities of these proteins can themselves be subject to modulation by exogenous or intracellular conditions; for example, affinity of the IRP for TfR mRNA changes in response to intracellular iron concentration.

It is indicative of the complexity of mRNA metabolism that none of the mRNA-binding proteins described to date has proved sufficient to solely direct mRNA decay. It appears more likely that these proteins form protein/mRNA complexes which, through further protein/protein interactions, are targeted for rapid decay.
Apart from the general association between poly(A) and poly(A)-binding protein, less is known about specific interactions between proteins and mRNAs from nuclear-encoded genes in plants. The only example to date is the PRP reported by Zhang and Mehdy (1994), which binds to bean PvPRP1 mRNA in response to fungal elicitors. PvPRP1 mRNA is destabilized under these conditions and it has been speculated that the PRP plays some role in this destabilization (section 1.4.2.3).

In chloroplasts however, mRNA/protein associations are an integral part of normal 3' end-processing and have been shown to play an important role in determining chloroplast mRNA stability.

1.7.1 Protein/RNA interactions in chloroplasts

Chloroplast-encoded genes produce mRNAs containing characteristic inverted repeat (IR) sequences in their 3' UTRs. These IR sequences are capable of forming stem-loop structures (Stern et al., 1989) and appear to be involved in the stabilization of chloroplast transcripts (Stern and Gruissem, 1987). Regulation of mRNA stability is the primary means by which expression of chloroplast genes is controlled (section 1.4).

Despite their resemblance to prokaryotic transcription termination structures (Hayes et al., 1996; Gruissem and Schuster, 1993), the stem-loops in chloroplast transcripts do not effectively direct transcription termination but act as RNA processing and stabilization signals (Stern and Gruissem, 1987). Mature chloroplast 3' ends are formed by exonucleolytic processing in a 3' to 5' direction, following an endonucleolytic cleavage downstream of the stem-loop structure (Stern and Kindle, 1993), suggesting that the stem-loop may protect upstream sequences by impeding progression of the 3'-5' exoribonuclease. The stem-loop therefore, defines the point at which the processed transcript ends.

The stem-loop structures alone however, are not sufficient to control mRNA stability and these structures have been shown to interact with proteins in chloroplast extracts (Stern et al., 1989). Schuster and Gruissem (1991) cloned the nuclear gene encoding a 28 kD mRNA-binding protein which recognized the inverted repeats of four chloroplast mRNAs, including that for the rubisco large subunit (rbcL). Abundance of this mRNA-binding protein was developmentally regulated in cotyledons, leaves and stems, correlating closely with the development-dependent accumulation of rbcL mRNA (See section 1.4). This protein was proposed to be involved in 3' end-processing and mRNA
stability, since depletion of it from chloroplast extracts inhibited in vitro 3' end-processing and resulted in mRNA decay (Schuster and Gruissem, 1991).

Subsequently a set of mRNA-binding proteins recognizing various 3' inverted repeat sequences has been identified (reviewed by Gruissem and Schuster, 1993). Most recently, Hayes et al. (1996) have purified and characterized a high molecular weight complex consisting of at least six protein components which interact with the petD mRNA 3' stem-loop and sequences flanking it, to regulate 3' end formation and mRNA stability.

Processing and stability of chloroplast mRNAs is extremely complex and requires several nuclear-encoded proteins to be either bound to, or in close proximity to, the structural element at the 3' end of the transcript. Taken together with the examples from mammalian systems, it appears that mRNA-associated proteins frequently influence gene expression via control of mRNA stability.

1.8 REGULATION OF PA1 mRNA ABUNDANCE

Although post-transcriptional regulation of gene expression often involves modulation of mRNA stability, rates of decay of the PA1 transcript in sulfur-adequate and sulfur-deficient plant tissue have not been measured directly. Expression of the PA1 gene has been shown to respond to sulfur nutrition and regulatory regions in the PA1 message have been sought by Morton (1993). In that study, both the coding region and 3' UTR of the PA1 mRNA, when expressed separately in transgenic tobacco, were shown to respond independently to sulfur deficiency, although the greatest response was observed in constructs containing both coding region and 3' UTR together. This situation seems similar to the c-myc stability determinants located in both the coding region and the 3' UTR. The results of Morton's experiments however, showed only that there were two regions of RNA which participated in regulation of PA1 expression, they did not establish that this regulation was the result of a change in stability of the PA1 transcript.

In the case of PA1, subsequent attention has been focused on the 3' regulatory region. A series of 3' deletions from the 3' UTR appeared to show that the sulfur responsive element (SRE) was located in the first 199 nucleotides of the 3' UTR, ie. within the mature mRNA. Examination of possible secondary structures showed that the whole 3' UTR of PA1 was likely to be rich in secondary structure but that nucleotides 109-199 of the 3' UTR were capable of forming a stem-loop structure.
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Morton (1993) proposed a mechanism by which sulfur deficiency might act to alter the stability of PAI mRNA. The model supposed that sulfur-regulated mRNAs would contain a specific sequence or structural element which is recognized by an mRNA-binding protein. This protein would take an 'active' conformation, in which it would bind to PAI mRNA and function as an endoribonuclease, during sulfur deficiency. This activated endoribonuclease would cleave the mRNA in the sulfur responsive element (SRE), upstream of the poly(A) tail, exposing the body of the mRNA to the actions of abundant, non-specific exoribonucleases. In this way the SRE-containing mRNA would be rapidly and specifically degraded under sulfur-deficient conditions.

When sulfur supply was adequate, the mRNA-binding protein would remain 'inactive' and unable to associate with the SRE. The transcript would remain intact and protected from rapid degradation by its 5' cap and poly(A) tail. Given that none of the mRNA-binding proteins identified to date have been able to direct mRNA decay in isolation, a single SRE-binding protein would be unlikely to bind and cleave SRE-containing mRNAs. Nevertheless if some additional factors are built into the model, differential recognition of SRE-containing mRNAs by proteins seems a feasible mechanism by which the response to sulfur might occur. For example, if a complex of several proteins such as that identified in chloroplast extracts by Hayes et al. (1996) assembled in response to sulfur deficiency and associated with PAI mRNA, a component of this complex might initiate decay. Alternatively, an mRNA/protein complex may form a substrate for an RNA degrading pathway which mRNA alone does not, so although the RNA-binding protein does not itself, degrade the mRNA, its association with the transcript would target the transcript for decay.

1.9 AIMS OF THE CURRENT WORK

The primary aim of the work described in this dissertation was to test the model of control of PAI mRNA stability by sulfur availability described in section 1.8 with respect to its three main assumptions. Although there is evidence to suggest that PAI mRNA is specifically destabilized in response to sulfur deficiency, this has never been directly demonstrated and the possibility that regulation may occur at alternative steps during processing of PAI pre-mRNA has not been formally excluded. Since the primary supposition of the proposed mechanism for regulation of PAI expression is that PAI mRNA is specifically destabilized during sulfur deficiency, it was important to examine whether there was a difference between the half-lives of PAI mRNA in sulfur-adequate and sulfur-deficient tissues.
Knowledge of the kinetics with which the PA1 transcript decays might provide information about the mechanism by which degradation occurs, therefore studies of *in vitro* mRNA stabilities in sulfur-adequate and sulfur-deficient tissue were carried out. In fact, it was not possible to accurately measure *in vitro* decay of PA1 mRNA in the leaves of transgenic tobacco, therefore attention was focused on clarifying the RNA factors involved in regulation of PA1 mRNA abundance *in vivo* and identifying possible regulatory protein factors which may also participate in regulation of PA1 expression in response to sulfur nutrition.

It was necessary to confirm the location of the 3' SRE. Morton hypothesized that since the region between nucleotides 109 and 199 of the 3' UTR were capable of forming a stem-loop structure, and since the region spanning nucleotides 10-199 of the 3' UTR appeared to confer sulfur-responsiveness on an unresponsive reporter gene in transgenic tobacco, it was likely that this region contained the SRE. Therefore it was necessary to confirm this result and further delineate the SRE.

More importantly, the model invokes participation of some form of mRNA-binding protein, specifically recognizing PA1 RNA sequence or structural elements, in modulating the stability of the PA1 transcript. As mentioned previously, such a protein has been described only in association with the response of *PvPR1* mRNA to pathogen attack. The final aim of this work was to identify and characterize PA1 RNA-associated proteins from sulfur-adequate and sulfur-deficient pea cotyledons. Any proteins displaying differential affinities for PA1 mRNA sequences under sulfur-adequate and sulfur-deficient conditions would be considered candidates for involvement in the response to sulfur.

This dissertation describes work dissecting the RNA, protein and mechanistic components of the response of PA1 expression to sulfur deficiency, with a view to understanding the molecular events taking place during this response. An understanding of these events will provide insight into how plants react, at the molecular level, to the problem of varying nutrient supply encountered constantly in the field.

In addition to such fundamental insights, information arising from studies of molecular aspects of the sulfur response may provide a basis for strategies to overcome sulfur-responsiveness in transgenic plants. Sulfur-rich seed storage proteins have shown promise as a source of rumen-stable sulfur amino acids (section 1.2.1). It is envisaged
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that these proteins will be provided to sheep in transgenic pasture plants in future, and these transgenic plants will be subject to a range of levels of sulfur nutrition in the field. PA1 has been shown to be regulated by sulfur deficiency in the leaves of transgenic tobacco (Morton, 1993) so it is likely that expression of genes for sulfur-rich proteins will also be regulated in leaves of pasture species. To obtain a consistent level of transgene expression in pasture plants it will be desirable to overcome sulfur-responsive expression of sulfur-rich proteins. It will be important to have a basic understanding of the events involved in this response before it can be altered.

Here I will show that a region of PA1 pre-mRNA located in the 3' UTR, downstream from the polyadenylation site, can regulate accumulation of reporter gene mRNA in transgenic plants in a sulfur-dependent fashion. Proteins from sulfur-adequate and sulfur-deficient pea cotyledons, which associate with this region of the PA1 3' UTR, have been identified. These proteins are candidates for further investigation, to determine their potential roles in regulation of sulfur-dependent PA1 expression.
CHAPTER 2

MATERIALS AND METHODS
Chapter 2. Materials and Methods

2.1 LOCATION OF THE 3' SRE

2.1.1 Plasmid construction and plant transformation

Five of the six chimeric gene constructs used in this series of experiments had already been made and transferred to tobacco. Their construction has been described in detail by Morton (1993) and their structures are summarized in Table 2.1. Briefly, a PA1 genomic clone from pea seed (pTJ6, EMBL database accession No. M81864) was modified for expression in leaves by replacing the PA1 promoter and 5' untranslated region with that from a ribulose-1,5-bisphosphate carboxylase small subunit gene from petunia (SSU301, Dean et al., 1985). This construct (SSU-PA1-PA11700) contained 481 base pairs of PA1 coding region and 1.7 kb of PA1 3' flanking region (Fig. 4.1). It was used as a positive control in sulfur deficiency experiments (section 2.1.2).

Similarly, a 1.28 kb cDNA from an avian ovalbumin gene (McReynolds et al., 1978) was modified for expression in leaves by inserting it between the SSU301 promoter and the cauliflower mosaic virus 35S (CaMV 35S) 3' region (Fig. 3.1). This construct was used as a negative control in sulfur deficiency experiments (section 2.1.2) and it was also used as an expression cassette for fragments of the PA1 3' flanking region (Fig. 3.1). Fragments beginning at nucleotide 10 of the PA1 3' flanking region, and extending to nucleotide 199 (SSU-ovalb-PA1189), 333 (SSU-ovalb-PA1323) or 506 (SSU-ovalb-PA1496) were inserted between the 3' end of the ovalbumin cDNA and the CaMV 35S transcription termination signal (Fig. 4.1). The positions of these deletion fragments within the PA1 3' flanking region are illustrated in figure 2.1. Transgenic tobacco plants containing these constructs, as well as plants transformed with the SSU-PA1-PA11700 construct were made available by Roger Morton.

![Diagram of the PA1 3' flanking region showing the relative positions of the EcoRV site 10 nucleotides beyond the stop codon, the polyadenylation signal beginning 162 nucleotides downstream of the stop codon and the ends of the deletion fragments tested in in vivo sulfur deficiency experiments.](image-url)
Table 2.1 Chimeric gene constructs previously made and used in experiments to locate the 3' sulfur responsive element of PA1. With the exceptions of pJO02 and pSJ21, these constructs were made by Roger Morton. pJO02 was made by Pamela Dunsmuir and pSJ21 was made by Megan Griffiths. All binary plasmids were constructed in pGA492 (An, 1986).

<table>
<thead>
<tr>
<th>Base plasmid</th>
<th>Binary plasmid</th>
<th>Promoter/5' UTR</th>
<th>Coding region</th>
<th>3' Flanking region</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJO02</td>
<td>pSJ21</td>
<td>petunia SSU</td>
<td>PA1</td>
<td>PA1 (1.7 kb)</td>
<td>SSU-PA1-PA1_{1700}</td>
</tr>
<tr>
<td>pRM23</td>
<td>pBRM23</td>
<td>petunia SSU</td>
<td>ovalbumin</td>
<td>CaMV 35S</td>
<td>SSU-ovalb-35S</td>
</tr>
<tr>
<td>pRM14</td>
<td>pBRM14</td>
<td>petunia SSU</td>
<td>ovalbumin</td>
<td>PA1 (0.496 kb)</td>
<td>SSU-ovalb-PA1_{496}</td>
</tr>
<tr>
<td>pRM33</td>
<td>pBRM33</td>
<td>petunia SSU</td>
<td>ovalbumin</td>
<td>PA1 (0.323 kb)</td>
<td>SSU-ovalb-PA1_{323}</td>
</tr>
<tr>
<td>pRM32</td>
<td>pBRM32</td>
<td>petunia SSU</td>
<td>ovalbumin</td>
<td>PA1 (0.189 kb)</td>
<td>SSU-ovalb-PA1_{189}</td>
</tr>
</tbody>
</table>

A sequence spanning nucleotides 10 - 109 of the PA1 3' UTR was amplified from the genomic clone pTJ6 using the polymerase chain reaction (PCR). The PCR product was inserted into the XbaI site of pGEM3zf(+) (Promega). This 99 nucleotide fragment was then excised from pGEM3zf(+) with EcoRV and HincII, and ligated into the Smal site of pRM23, between the 3' end of the ovalbumin cDNA and the CaMV 35S transcription terminator to produce pRM42.

pRM42 was linearized 5' to the CaMV 35S 3' region with EcoRI and was ligated into the EcoRI site of the binary vector pGA492 (An, 1986) to produce pBRM42. pGA492 contained a tetracycline resistance gene and a kanamycin resistance gene (NPTII) flanked by NOS 5' and 3' regions. The kanamycin resistance gene was used as a selectable marker for transformed plants while the tetracycline resistance gene was used to select transformed bacteria. Orientation of the insert with respect to the kanamycin resistance gene was determined by restriction enzyme digestion (data not shown). The ovalbumin expression cassette was convergent with the kanamycin resistance gene (Fig. 2.2).
pBRM42 was transferred from *Eschericia coli* to *Agrobacterium tumefaciens* (strain AGL 1) by triparental mating (Lazo *et al.*, 1991). The T-DNA between the border regions, containing both the kanamycin resistance gene and the ovalbumin expression cassette, was transformed into tobacco (*Nicotiana tobaccum* cv. Wisconsin 38) by *Agrobacterium*-mediated transfer, using the method of Horsch *et al.* (1985), as modified by Higgins *et al.* (1988). Plants were regenerated from transformed cells on media containing kanamycin (100 mg/L), for selection of transformed regenerants.

Putative transformants were screened for expression of ovalbumin mRNA by northern analysis (data not shown) and the plant expressing the highest level of ovalbumin mRNA was selected for further analysis. The selected plant was vegetatively propagated to obtain several genetically identical plants for use in sulfur deficiency assays, so as to minimise variation in transgene expression between individual plants.
2.1.2 Plant material and growth

Tobacco plants were grown in washed river sand in 15 cm pots, in the glasshouse under natural light. Plants were watered daily with 400 ml of nutrient solution, containing a range of sulfate concentrations (Castle and Randall, 1987, modified by Morton, 1993).

The basal nutrient solution contained: 5 mM potassium nitrate (KNO₃), 4 mM calcium nitrate (Ca(NO₃)₂), 1 mM ammonium nitrate (NH₄NO₃), 1 mM potassium dihydrogen phosphate (KH₂PO₄), 0.1 mM ferric citrate, 23 µM boric acid (H₃BO₃), 4.5 µM manganese chloride (MnCl₂·4H₂O), 0.7 µM zinc chloride (ZnCl₂), 0.4 µM cupric chloride (CuCl₂·2H₂O) and 0.22 µM molybdic acid (H₂MoO₄). Sulfate was supplied as magnesium sulfate (MgSO₄) and concentrations ranged from 10 µM to 1 mM (0.32 - 32 ppm). Magnesium concentration was maintained by varying magnesium chloride (MgCl₂) between 1 mM and 1.99 mM.

After being transferred to the glasshouse, plants were watered for two weeks with a nutrient solution containing 0.2 mM sulfate. Plants were then divided into four groups, two of the groups were watered with a solution containing 1 mM sulfate until harvest. The remaining two groups were watered with a solution containing 0.1 mM sulfate for a further one week, then with a 10 µM sulfate solution until harvest. When the sulfur-deficient plants displayed visible symptoms of sulfur deficiency (yellowing of the younger leaves, Fig. 2.3), usually after five to eight days on 10 µM sulfate, the first leaf greater than 7 cm in length was harvested from each plant and frozen in liquid nitrogen for RNA extraction. A second harvest was usually taken about five days after the first, to provide back up material.

2.1.3 Experimental design and analysis

Plants were grown in four groups on a single glasshouse bench. Each group contained: one plant carrying the positive control construct (SSU-PA1-PA1700); two plants carrying the negative control construct (SSU-ovalb-35S); and two plants carrying each construct to be tested (SSU-ovalb-PA1ₓ). Within each group of plants the position of each pot was randomly assigned. Two of the four groups of plants were watered with full nutrient solution (+S) and the remaining two groups were watered with reduced sulfur nutrient solution (-S), as outlined below.
Figure 2.3 Sulfur deficiency symptoms in tobacco. The plant on the left was grown under sulfur deficiency (described above) while the plant on the right was supplied with adequate sulfur.
This was a blocked design with a block consisting of one +S and one -S group, but since +S and -S groups were separated for ease of watering the design was not fully randomized. Therefore blocks were disregarded and data was analysed for the effect of sulfur treatment only.

To determine whether the response to sulfur deficiency of the test construct differed from that of the negative control construct, analysis of covariance was performed on ovalbumin expression levels using the level of actin expression as the covariate. The main assumption underlying analysis of variance is that experimental errors have a common variance. Because actin and ovalbumin expression levels were measured with different probes, the measured values were of different scales and the variances were not equal. The variances were however, proportional to the means of the measured values therefore logarithmic transformation was performed on the primary data to equalize variances (Steel and Torrie, 1980). Analysis of covariance was then performed on the transformed data.

### 2.1.4 RNA extraction

Frozen leaves were weighed and ground to a powder in liquid nitrogen. Two volumes (2 ml/g tissue) of extraction buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 8, 1 mM ethylenediaminetetra-acetate (EDTA), 0.1% SDS) were added and the slurry was allowed to partially thaw. To the slurry was added 1.5 volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and the solution was mixed thoroughly. After centrifuging at 16000 x g for 5 minutes, nucleic acids were precipitated from the aqueous phase by 0.1 volume of 2 M sodium acetate (CH₃COONa.3H₂O) and two volumes of cold ethanol. Nucleic acids were pelleted by centrifugation at 16000 x g for 10 minutes. The pellet was dissolved in distilled water (1 ml per gram of original tissue) and the RNA precipitated by 0.25 volumes of 10 M LiCl. The solution was maintained on ice for at least 1 hour then RNA was pelleted by centrifugation at 16000 x g for 10 minutes. RNA was suspended in distilled water (500 µl/g original tissue) and reprecipitated in ethanol. Finally RNA was washed with 70% ethanol and resuspended in distilled water (200 µl/g tissue). RNA concentrations were determined by absorbance at 260 nm, assuming a molar extinction coefficient of 0.025 for RNA, and assuming that all absorbance at 260 nm was due to RNA.
2.1.5 RNA analysis

Total RNA was size-fractionated by electrophoresis through agarose containing 5% formaldehyde. RNA (5 µg) was denatured at 65°C for 5 minutes in the presence of 50% formamide, 17.5% formaldehyde, 20 mM MOPS (3-[N-morpholino]propane-sulfonic acid), 5 mM NaOAc, 1 mM EDTA) and 10 µg ethidium bromide. RNA was electrophoresed on a 1.4% agarose gel containing 20 mM MOPS and 5% formaldehyde, at 100 volts for 2-2.5 hours. Gels were photographed and the positions of major ribosomal bands marked. RNA was transferred to nylon membrane (Hybond-N, Amersham) by overnight blotting and was cross-linked to the membrane by exposure to UV (254 nm, 250 µW/cm²) for 2 minutes (Khandjian, 1987). Membranes were dried at 37°C.

2.1.6 Probe synthesis and hybridization

RNA probes to detect PA1, ovalbumin and actin messages were synthesized in vitro by run-off transcription (Melton et al., 1984). Plasmids containing PA1 (RM7), ovalbumin (RM25) and actin (pAct24, Cotton et al., 1990) coding regions were linearized at the 5' end of the coding region inserts, to generate transcripts in antisense orientation (complementary to the relevant mRNA). Template DNA was transcribed with T7 (RM7 and RM25) or T3 (pAct24) RNA polymerase (Promega) in the presence of 32P UTP (Amersham). Reactions also contained 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol (DTT), 40 units RNasin (Promega), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP and 1 µM UTP in a total volume of 20 µl. The reactions were incubated at 37°C for 1 h and treated with RNase-free DNase (Promega) at 37°C for 10 minutes. Unincorporated nucleotides were removed by gel filtration in a 1 ml Sephadex G-50 spin column.

mRNA on nylon membranes was detected by Northern blot analyses using radioactive RNA probes. Membranes were pre-hybridized with hybridization solution [50% formamide, 10% SDS, 2 x SSPE (3 M NaCl, 0.2 M Na₂H₂PO₄, 20 mM EDTA, pH7.4, Sambrook et al., 1989), 0.5% Herring sperm DNA (Sigma), 10% dextran sulfate; 1 ml/10 cm² membrane] for one hour at 65°C to block any non-specific probe binding sites on the membrane. Radioactive RNA probes (1 x 10⁶ cpm/cm² of membrane) were then added and hybridized with membranes overnight at 65°C in the same hybridization solution. Non-specifically bound probe was removed by washing twice in 2 x SSC at room temperature for 10 minutes, then twice in 2 x SSC [20 x SSC (standard citrate
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saline) 3 M NaCl, 0.3 M sodium citrate], 0.1% SDS. 0.1% sodium pyrophosphate at 65°C for 20 minutes. Membranes were exposed to phosphor screens (Amemiya and Miyahara, 1988) for 18 - 96 hours. Images were detected using a Molecular Dynamics Phosphorimager (Johnston et al., 1990). The intensity of radioactive bands was determined by volume integration, using Molecular Dynamics ImageQuant™ software. If non-specific hybridization was detected after autoradiography, an additional ten minute wash at 65°C in 0.1 x SSC, 0.1% SDS, 0.1% sodium pyrophosphate was performed.

Differences in gel loading and transfer were corrected by removing primary probes from the membranes using 0.1% SDS at 100°C, and probing with a soybean actin probe (Cotton et al., 1990). Counts obtained for PA1 and ovalbumin probes were divided by counts obtained for the actin probe from the relevant sample, to provide a value for PA1 or ovalbumin expression, normalized for the effects of gel loading and transfer.

2.2 RNA STABILITY EXPERIMENTS

2.2.1 Plant growth and sulfur deficiency in vitro

Sorbarods (Sigma) are inert, paper-based cylindrical supports in which plants may be grown in solution culture. These supports were surrounded by cotton wool and placed in 15 cm deep tissue culture pots before autoclaving. The sorbarods and cotton wool were saturated with 50 ml of a nutrient solution containing: 5 mM KNO3, 4 mM Ca(NO3)2, 1 mM NH4NO3, 1 mM KH2PO4, 0.1 mM iron citrate, 23 µM H3BO3, 4.5 µM MnCl2.4H2O, 0.7 µM ZnCl2, 0.4 µM CuCl2.2H2O and 0.22 µM H2MoO4 and either: 1 mM MgSO4 and 1 mM MgCl2; or 2 mM MgCl2 without MgSO4. Excess solution was then poured off, before tobacco plants growing in MSO agar (Murashige and Skoog, 1962) were subcultured into the sorbarods. Plants were allowed to grow for two weeks then subcultured again, into a second sorbarod, saturated with the same nutrient solution. After a further one week, transcription inhibitor experiments were performed on excised leaves. Only leaves from nodes one to three were harvested.

2.2.2 Transcription inhibitor experiments

Excised leaves were cut into approximately 1 cm squares. Five pieces were immediately transferred to liquid nitrogen for RNA isolation and determination of initial message levels and five pieces were placed into 5 cm petri dishes. Each dish contained 1 mM PIPES (pH 7), 1 mM sodium citrate, 1 mM KCl, 15 mM sucrose (Jiang et al., 1994) and
either 200 µg/ml cordycepin (Sigma) or no cordycepin. Petri dishes containing leaf pieces, buffer and cordycepin were placed in a bell jar and leaf pieces were vacuum infiltrated for five minutes then swirled gently on an orbital shaker. At intervals, leaf pieces were removed from dishes, drained briefly and frozen in liquid nitrogen.

2.2.2.1 RNA extraction and analysis

The frozen tissue (approximately 0.3 g) was ground in liquid nitrogen in a mortar. Extraction buffer (section 2.1.4) was added and ground until a slurry formed. The slurry was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and spun in an eppendorf centrifuge for 5 minutes. Nucleic acid was precipitated from the aqueous phase by addition of an equal volume of isopropanol and incubation on ice for 30 minutes, and eppendorf centrifugation for 5 minutes. The pellet was washed with 70% ethanol the resuspended in 20 µl of distilled water. Nucleic acid concentration was determined by absorbance at 260 nm. Northern analysis was performed as described previously (section 2.1.5).

2.2.2.2 Probes

The following probes were used in Northern analyses of RNA from transcription inhibitor experiments, in addition to the PA1 probe described previously (section 2.1.6). Cab - random primed from a 0.5 kb fragment of pCab2211 corresponding to the 3' end of the coding region from a petunia chlorophyll a/b binding protein gene (Dunsmuir et al., 1983).

rbcS - random primed from a 0.469 kb fragment of pNTSS23 corresponding to exon 3 of a tobacco rubisco small subunit gene (Mazur and Chui, 1985).

18S ribosomal RNA gene - random primed from pTA250-10, containing the 18S region of a wheat ribosomal cistron (Gerlach and Bedbrook, 1979).

These probes were prepared using an Amersham random-priming kit and were hybridized to the membranes at 42ºC, using the same hybridization solution as was used for riboprobes (section 2.1.6). The cab probe was used to measure the half life of the chlorophyll a/b-binding protein mRNA, which has been shown to have a relatively short half life, while the SSU probe measured decay of the rubisco small subunit mRNA which has a longer half life (Jiang et al., 1994). The rate of decay of PA1 mRNA was compared with the decay rates of the cab and SSU messages to determine whether PA1
In all cases the sequences of the inserted fragments were determined by Taq DyeDeoxy™ Terminator cycle sequencing (Applied Biosystems) and checked against the sequence of pTJ6.
mRNA decayed with kinetics similar to a short-lived mRNA species or a long-lived mRNA species.

The 18S ribosomal RNA probe was used to measure the abundance of the 18S ribosomal RNA. The intensity of this band was not influenced by transcription inhibitor treatment and was indicative of gel loading, therefore it was used to correct loading differences.

2.3 IDENTIFICATION OF RNA-BINDING PROTEINS

2.3.1 In vitro transcription

Fragments of the PA1 3' flanking region, corresponding to the fragments used for in vivo sulfur deficiency experiments (section 2.1.1) were subcloned into vectors containing promoters for SP6 and T7 RNA polymerases. A similar strategy was used to insert each fragment into the vectors. Briefly, fragments spanning nucleotides 0-333, 0-199, 0-99, 99-199 and 199-333 of the PA1 3' flanking region were amplified from the genomic clone, pTJ6, by PCR. The 0-333, 0-99, 99-199 and 199-333 fragments were ligated into XbaI sites in the multiple cloning site of pGEM3zf(+) (Promega) to create pRM27, pRM39, pAE1 and pAE5 respectively (Fig. 2.4). The 199 nucleotide fragment spanning nucleotides 0-199 of the PA1 3' flanking region was inserted into the XbaI site in the multiple cloning site of pSP64polyA (Promega) to create pRM30. Plasmids pRM27, pRM30 and pRM39 were made by Roger Morton.

Prior to in vitro transcription, template plasmids were linearized at HindIII (RM27, RM39, AE1, AE5) or BamHI (RM30) sites. These sites arose from multiple cloning sites of the vectors and were 3' to the ends of the inserts. Full-length, run off transcripts were generated by in vitro transcription of template DNA by either T7 (RM27, RM39, AE1 and AE5) or SP6 (RM30) RNA polymerase (Promega) in the presence of 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 40 units RNasin, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP and 35 µM UTP. ³²P UTP was also included in the 20 µl reactions, which were incubated at 37°C for 1 hour. DNA template was removed by addition of 1 unit of DNase (Promega) for 10 minutes at 37°C. Probes were treated with phenol:chloroform and twice precipitated with ethanol at -80°C. Incorporation of radioactive UTP was measured by precipitation with trichloroacetic acid (TCA). The lengths of these in vitro transcribed RNA fragments were verified by comparing the unlabelled transcripts with leaf ribosomal RNA from tobacco (data not shown).
Unlabelled transcripts were generated in the same way except that radioactive UTP was omitted and equal concentrations (0.5 mM) of all nucleotides were used. These transcripts were not ethanol precipitated after the phenol:chloroform step but were directly quantified by absorbance at 260 nm.

Figure 2.4 Diagram of the PA1 3' flanking region and its sub-fragments which were used for in vitro transcription reactions, to generate probes for RNA-binding protein assays. The fragments were cloned into the transcription vectors pGEM3zf(+) or pSP64polyA, which were linearized at HindIII or BamH1 sites respectively prior to in vitro transcription from T7 or SP6 promoters.
2.3.2 Plant material

Pea seeds used for crude and nuclear extracts were kindly donated by T.J. Higgins and Don Spencer. They were grown under conditions described by Chandler et al. (1983). Briefly, peas (Pisum sativum cv. Greenfeast) were grown at 20°C with 16 hour photoperiod. Plants were grown in sand and watered with nutrient solutions containing 1 mM MgSO₄ for control plants and 0.05 mM MgSO₄ for sulfur-deficient plants until the first flower. Sulfur was omitted from sulfur-deficient solution thereafter. Pea cotyledons harvested at 19-20 days after flowering (DAF) were used for total and nuclear protein extract preparation.

2.3.3 Total protein extracts

Peeled and frozen pea cotyledons (1 g) were ground in a mortar with 1 ml of binding buffer (10 mM HEPES, pH 7.5, 3.5 mM MgCl₂, 40 mM KCl, 1 mM DTT and 5% glycerol). The slurry was spun at 10000 x g for 5 minutes and supernatant removed. Protein concentration was determined by Bradford assay (Bradford, 1976). The supernatant was aliquotted, frozen in liquid nitrogen and stored at -80°C.

2.3.4 Nuclear protein extracts

Peeled, frozen pea cotyledons (5 g) were homogenized using a Nibblex homogenizer in 5 ml extraction buffer [1 M hexylene glycol, 10 mM PIPES, pH 7, 10 mM MgCl₂, 5 mM β-mercaptoethanol (Watson and Thompson, 1986)]. The slurry was squeezed with a coarse wire mesh and the resulting liquid filtered through steel mesh with a pore width of 200 µm. The remaining pea tissue was re-homogenized in a further 5 ml extraction buffer, the resulting liquid was filtered through the 200 µm mesh and added to the previous filtrate. The residue in the mesh was then washed with 2.5 ml extraction buffer, which was also added to the previous filtrate.

After centrifugation of the homogenate at 1000 x g for 10 minutes, the pellet containing nuclei was resuspended in 2.5 ml nuclei wash buffer (0.1 mM mannitol, 30 mM HEPES, pH 7.5, 10 mM MgCl₂, 5 mM β-mercaptoethanol). The suspension was again centrifuged at 1000 x g for 5 minutes and the nuclei resuspended in 300 µl storage and sonication buffer (20 mM HEPES, pH 7.5, 420 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol (v/v), 5 mM β-mercaptoethanol). Nuclei were lysed on ice for 1
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hour and disrupted either by sonication with two 5 second bursts at 50 W or by repeated passage through 18, 21 and 25 gauge needles, using a 1 ml syringe.

The nuclear extract was spun at 10000 x g for 10 minutes and the supernatant dialysed for a total of 3 hours against 6 x 500 ml of dialysis buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 0.2 mM EDTA, 20% glycerol (v/v), 5 mM β-mercaptoethanol). The supernatant was then centrifuged at 10000 x g for 8 minutes. Protein concentration was determined by Bradford assay before the supernatant was aliquotted and frozen in liquid nitrogen.

2.3.5 Gel mobility shift assay

RNA transcribed and labelled in vitro (Specific activity ~ 2 x 10⁷ cpm/µg) was denatured at 85°C for five minutes then chilled on ice for two minutes. The probe (20 000 cpm/µl) was incubated with protein extract (10 µg) in the presence of 5 µg E. coli MRS600 tRNA (Boehringer Mannheim) in binding buffer (section 2.3.3) in a total volume of 15 µl at room temperature for 10 minutes. Loading buffer (8% ficoll (Sigma), 0.02% bromophenol blue, 0.04% xylene cyanol) was added and samples loaded on a 4% native polyacrylamide minigel containing 0.25 x TBE (22 mM Trizma Base, pH 8; 22 mM boric acid, 1.56 mM EDTA). The gel was pre-electrophoresed at 100 V for 10 minutes prior to loading and samples were run at 100 V, at 4°C with recirculating buffer (0.25 x TBE) for 2 - 3 hours. After electrophoresis gels were dried under vacuum at 80°C for 2 hours and exposed to X-ray film (Fuji) at -80°C, or phosphor screen overnight.

2.3.6 RNase protection assay

Reactions were carried out as described for the gel mobility shift assay and after incubation of the RNA transcripts with protein extracts, 1 unit of RNase T1 (Boehringer Mannheim) were added to each reaction. Reactions were incubated for a further 10 minutes at room temperature and then mixed with loading buffer as described above, before loading on 4% non-denaturing polyacrylamide gels containing 0.25 x TBE. Gels were electrophoresed, dried and fluorographed as described above (section 2.3.5).
2.3.7 UV-crosslinking assay

Reactions were carried out as described for the gel mobility shift assay except that \(1 \times 10^5\) cpm of probe were used instead of \(2 \times 10^4\) cpm and in some cases, probes were not heat denatured prior to addition to binding reactions. After incubation of the RNA transcripts with protein extracts, the reactions were irradiated for 15 minutes with UV light (254 nm, 1 mW/cm\(^2\)) in a Stratalinker (Stratagene). RNA was then digested with 4 µg RNase A (Sigma) for 10 minutes at 37°C. Reactions were boiled for 5 minutes in SDS loading buffer (125 mM Tris-HCl, pH 7.6, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 4% β-mercaptoethanol) then cooled to room temperature before being separated on 15% SDS-PAGE. Gels were electrophoresed at 100 V for approximately 3 hours, then dried and fluorographed as described for gel mobility shift assays (section 2.3.5).
CHAPTER 3

EVALUATION OF A METHOD FOR MEASURING PA1 mRNA STABILITY
Chapter 3. A Method For Measuring PA1 mRNA Stability

3.1 INTRODUCTION

Pea plants grown in the absence of sulfur exhibit a 9-fold increase in abundance of PA1 message in the seeds in the 48 hours following transfer to a sulfur-containing medium. Over the same interval there is no appreciable change in the rate of transcription of the PA1 gene, as measured by nuclear run-on transcription assay (Higgins et al., 1986). This discrepancy between transcription rate and accumulation of PA1 mRNA indicates that the PA1 transcript was stabilized upon introduction of an adequate level of sulfur to the pea cotyledons. However the accuracy with which measures of transcription made in vitro reflect in vivo RNA synthesis rates is uncertain (Thompson and White, 1991) and decay of PA1 mRNA in sulfur-adequate and sulfur-deficient plants has not been measured directly. Nevertheless, a change in stability of the PA1 transcript has been proposed as the mechanism by which the steady-state level of PA1 mRNA is regulated in response to a change in sulfur nutrition.

In order to demonstrate a connection between the change in steady-state mRNA level and a possible change in PA1 mRNA stability in response to changes in sulfur nutrition, it was necessary to devise a method for measuring the rates of decay of mRNA in sulfur-deficient and sulfur-adequate plant material. Both intact cells and cell-free decay systems have previously been used to demonstrate the effects of various mRNA elements on mRNA stability, particularly in mammalian cell culture systems (Belasco and Brawerman, 1993; Ross, 1995).

In plants, cell-free decay systems have been described for measuring decay of chloroplast mRNAs (Gruissem et al., 1986; Klaff, 1995) and the decay of oat phytochrome A mRNA has been measured in a polysome-based, in vitro method (Byrne et al., 1993). In that study a transcription inhibitor, cordycepin, was used to determine in vivo mRNA decay rates for actin, β-tubulin and phytochrome A mRNAs then decay rates for the same, polysome-associated mRNAs were measured in vitro. The in vitro system appeared to accurately reproduce in vivo decay processes as the rank order of mRNA half-lives measured in vitro was the same as that observed during in vivo in the presence of cordycepin.

Most studies of mRNA degradation in plants however, have made use of transcription inhibitors in intact cells. Many studies in which plant mRNA stability has been directly measured have also made use of cell suspension cultures, in which transcription inhibitors can be readily and uniformly taken up by all cells. Destabilization of the bean
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PvPRPl mRNA after exposure of cultured cells to fungal elicitor was measured this way (Zhang et al., 1993), as were the effects of DST (Newman et al., 1993) and AU-rich sequences (Ohme-Takagi et al., 1993) on mRNA stability. There are some examples where intact plant tissue has been exposed to transcription inhibitors and decay of endogenous mRNA species measured. The half-life of phytochrome A mRNA was measured by immersing abraded oat coleoptiles in a buffer containing cordycepin (Seeley et al., 1992). The results obtained by this method were subsequently confirmed by in vitro decay experiments (Byrne et al., 1993). Decay rates of the rubisco small subunit mRNA in light- and dark-grown plants were measured in intact potato plants grown in medium containing cordycepin (Fritz et al., 1991) and the effect of antisense RNA on stability of rubisco small subunit mRNA was investigated by incubating leaf discs from tobacco plants transformed with antisense rbcS in cordycepin (Jiang et al., 1994).

Suspension cultures transformed with the PA1 gene were not available to study stability of PA1 mRNA, therefore intact leaf material from tobacco plants transformed with a PA1 gene which was modified to be expressed in leaves (SSU-PA1-PA11700, Fig. 4.1) was used in mRNA decay experiments to determine the half-lives of PA1 mRNA in sulfur-adequate and sulfur-deficient plants. An approach similar to that employed by Jiang et al. (1994) was taken and the results of these experiments are presented here. Leaves were excised from sulfur-adequate and sulfur-deficient tobacco plants grown in tissue culture (described below) and were incubated in the transcription inhibitor, cordycepin. Leaf pieces were collected at intervals after addition of cordycepin, and total nucleic acids were extracted. The mRNA levels for PA1, chlorophyll a/b-binding protein (cab) and rubisco small subunit (rbcS) were measured (section 2.2.3).

A novel method for inducing sulfur deficiency in tissue culture, rather than glasshouse-grown, plants was devised. Plants grown in this system showed the same response of PA1 expression to sulfur nutrition as observed in glasshouse-grown plants. However, a method using excised tobacco leaf pieces for measuring mRNA stability did not produce reliable estimates of mRNA decay rates. All mRNAs measured underwent rapid, unregulated decay in this experimental system. This chapter describes the development of both the tissue culture system for inducing sulfur deficiency, and the mRNA decay reactions, as well as outlining problems in both systems and alternative methods by which the half-life of PA1 mRNA might be measured.
3.2 RESULTS

3.2.1 A response to sulfur occurred in tissue culture plants

In order to provide a plentiful supply of sulfur-adequate and sulfur-deficient leaf material of uniform developmental stage, a method for inducing sulfur deficiency in tissue culture plants was developed. Tobacco plants were grown in paper-based cylinders (Sorbarods; Sigma) surrounded by cotton wool in 10 cm high tissue culture pots (Fig. 3.1). The Sorbarods and cotton wool were saturated with 50 ml of sterile nutrient solution containing sulfate concentrations of either 1 mM (+S) or zero (-S). Tobacco plantlets were subcultured from stocks growing on nutrient agar, leaving only the top three leaves attached to the stem. The plants were grown in sorbarods for two weeks, after which each plant was subcultured into a fresh sorbarod, saturated with the same medium as the plant had been growing in previously. Again, only the top three leaves were left on the plant. After one week in the second pot, plants grown in the absence of sulfur exhibited visual symptoms of sulfur deficiency (yellowing leaves, see Fig. 3.1). At this time the top two or three fully expanded leaves were harvested for mRNA analysis.

Figure 3.2 shows that sulfur deficiency induced in these cultured plants caused a 9-fold reduction in the level of PA1 mRNA. This reduction in PA1 mRNA level in response to sulfur deficiency was comparable to responses reported in glasshouse grown tobacco (chapter 4; Morton, 1993) and in sulfur-deficient pea cotyledons (Higgins et al., 1986). This method therefore, is a suitable means of inducing the same response in PA1 expression as has been observed in transgenic tobacco grown under sulfur deficiency in the glasshouse. It also provides a simple means of obtaining moderate quantities of developmentally similar leaf material for mRNA stability studies.

3.2.2 Determination of the minimum effective concentration of transcription inhibitor

Cordycepin (3' deoxyadenosine) is a structural analogue of adenosine (Kaczka et al., 1964). Incorporation of this molecule into an RNA chain prevents further elongation due to the absence of the 3' oxygen, hence cordycepin acts as a transcription inhibitor. Cordycepin at a concentration of 0.1 mM has been reported to cause aberrations in nucleoli of onion root-tip cells (Stockert et al., 1970). In order to minimize the possible secondary effects of transcription inhibitors on cell metabolism it is desirable to use them at the minimum effective concentration (Seeley et al., 1992; Jiang et al., 1994).
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Figure 3.1 Tobacco plants growing in liquid culture on sorbarods and cotton wool. Plants were grown in -S (left) or +S (right) medium for three weeks, by which time sulfur deficiency symptoms were clearly visible. At this stage leaves were harvested for mRNA stability studies.

Figure 3.2. PAI mRNA levels in sulfur-adequate and sulfur-deficient tobacco plants grown in sorbarod culture. Values are the averages of mRNA levels in three individual plants, error bars represent standard deviations. PAI mRNA levels were determined by northern analysis and loading errors were corrected by normalization to actin mRNA levels. Error bars represent standard deviations.
To determine this minimum concentration for tobacco leaf pieces, leaf pieces from untransformed tissue culture-grown tobacco plants were incubated with concentrations of cordycepin from 0 to 500 µg/ml for six hours. Total nucleic acids were isolated from leaf pieces and the levels of cab mRNA were analysed by northern blot. Cab mRNA was selected as an indicator of transcriptional activity as it has a short half life (less than one hour; Jiang et al., 1994). The steady-state level of this short-lived mRNA would be expected to decline in response to inhibition of transcription more rapidly than that of a more long-lived mRNA, therefore interruptions to transcription may be detected more rapidly if the abundance of a short-lived mRNA species is monitored. Cab mRNA has previously been used for this purpose by Seeley et al. (1992). The membrane on which cab mRNA level was measured was then stripped and probed for the 18S ribosomal RNA. The intensity of this band was not affected by cordycepin at any concentration therefore the 18S ribosomal signal was used as an internal standard, against which to normalize other signals to correct errors in gel loading or transfer of RNA to membranes.

Figure 3.3 shows that over six hours, cab mRNA level declined as the concentration of cordycepin increased. However no further reduction was achieved by using concentrations in excess of 200 µg/ml, therefore subsequent experiments were performed in the presence of 200 µg/ml cordycepin. Similar profiles for inhibition of mRNA accumulation by cordycepin have been reported for phytochrome A mRNA in oat coleoptiles (Seeley et al., 1992) and rbcS mRNA in tobacco leaf pieces (Jiang et al., 1994). In these studies cordycepin was used at concentrations of 100 µg/ml and 150 µg/ml respectively, to inhibit mRNA synthesis while 50 µg/ml was used in a study of cab mRNA stability in soybean cell suspension (Romero and Lam, 1993). A concentration of 500 µg/ml was used to inhibit cab mRNA accumulation in primary oat leaves (Byrne et al., 1993). The cordycepin concentration used to inhibit mRNA synthesis in this experiment therefore, was within the range reported by other workers using similar methods. It was approximately 12-fold less than the concentration used in whole potato plants by Fritz et al. (1991).
Figure 3.3 Determination of the minimum concentration of cordycepin capable of inhibiting cab mRNA accumulation. (a) cab mRNA levels in leaf pieces incubated with increasing concentrations of cordycepin for six hours. Levels of cab mRNA were analysed by northern blot, quantified by volume integration and normalized to the levels of 18S ribosomal RNA (b).
3.2.3 Decay of PAI mRNA in sulfur-adequate and sulfur-deficient tobacco leaf

Having demonstrated that 200 µg/ml of cordycepin was effective in preventing cab mRNA synthesis in excised tobacco leaf pieces, the same conditions were used to estimate the half-lives of PAI, cab and rbcS mRNAs in leaf pieces taken from sulfur-adequate and sulfur-deficient tobacco plants grown on sorbarods in time course experiments.

Leaf material from plants grown on sorbarods was cut into 1 cm squares and maintained on ice, in a dish of water to minimize mRNA degradation while sufficient material was prepared for sampling at all time points. This took about 30 minutes. Material from several plants was pooled to obtain enough for each sampling, but +S and -S leaf pieces were kept separate. Four petri dishes were prepared for each time point; one dish containing 200 µg/ml cordycepin and another with no cordycepin were prepared for sulfur-adequate leaf pieces and duplicate dishes were prepared for sulfur-deficient leaf pieces. Five leaf pieces were randomly allocated to each dish. The dishes for the time zero sampling did not contain cordycepin, but were otherwise treated the same as dishes for subsequent samplings. Leaf pieces were then vacuum infiltrated in a bell jar with cordycepin for five minutes. The dishes containing the leaf pieces were then placed on an orbital shaker and swirled gently at room temperature.

RNA levels were measured at 15 minute intervals over a 90 minute time course which began at the end of the vacuum infiltration period, once all dishes were placed on the shaker. Leaf pieces were removed from the cordycepin solution, drained briefly and frozen in liquid nitrogen. After the last sample had been frozen, RNA was extracted from all samples and mRNA levels analysed by northern blot.

Each set of samples (+S/+cordycepin, +S/-cordycepin, -S/+cordycepin, -S/-cordycepin) from the seven time points was run on a separate gel and transferred to a separate nylon membrane. All membranes were probed at the same time, using the same probe. In the case of sulfur-deficient material incubated without cordycepin, the sample from the 90 minute time point was not analysed as the yield of RNA was not sufficient to run on a gel. Membranes were probed for signals from PAI, cab and rbcS mRNA. Errors in loading or transfer of RNA to the membrane were corrected by normalizing to the 18S ribosomal RNA level, which was not substantially affected by cordycepin.
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Cab and rbcS were chosen as examples of unstable and stable mRNA species respectively, because the decay rates of these transcripts have previously been measured in tobacco leaf discs under experimental conditions similar to those used in this current experiment. The tobacco cab mRNA has been shown to decay with a half-life of approximately 45 minutes while the tobacco rbcS mRNA decayed with a half-life of at least 5 hours (Jiang et al., 1994). Because the decay kinetics of PAI mRNA were unknown, the rates of decay of cab and rbcS mRNAs were measured as well as that of PAI mRNA, to determine whether PAI mRNA decayed with kinetics similar to an unstable (cab) or a stable (rbcS) mRNA. These two mRNA species of known stability also acted as controls, to determine whether or not the experimental system developed for measuring decay of PAI mRNA reproduced results obtained previously with a similar system (Jiang et al., 1994).

Figure 3.4(a) shows that in sulfur-adequate leaves, the levels of mRNA for PAI, cab and rbcS declined to 50% of their original levels within 10 to 15 minutes. The level of PAI mRNA continued to fall but after 45 minutes the level of this mRNA began to rise again. The initial decay of the cab message was faster than anticipated [half-life of 10 minutes vs 45 minutes (Jiang et al., 1994)] however the cab mRNA level fluctuated between 30% and 50% of the original level between 15 and 60 minutes before returning to its original level by 90 minutes. The reason for this fluctuation is unknown. Contrary to expectations, the rbcS mRNA also decayed with a half-life of approximately 15 minutes. Its level then began to rise again and by 90 minutes, the level of rbcS mRNA was 188% of the initial level. Again, the reason for the accumulation of rbcS mRNA after 15 minutes of incubation in cordycepin is unknown.

The rapid initial rates of decay of cab and rbcS mRNAs were not consistent with previously reported values. To test whether the results observed in the presence of cordycepin were indicative of the true rates of decay of these mRNA species in vivo, or artifacts of the experimental procedure, a parallel experiment was carried out in the absence of cordycepin. Results of this experiment are shown in Fig. 3.4(b). Levels of cab and rbcS mRNAs fluctuated over the 90 minute duration of the experiment. Although the magnitude of the fluctuations in levels of the cab and rbcS mRNAs were large, they were centred around 100% of the initial level, indicating that there was probably no net change in level of these messages over the duration of the experiment, as would be expected in the presence of ongoing transcription. The magnitude of these fluctuations however, may indicate that the procedure was subject to errors.
Figure 3.4 Decay of PA1 (○), cab (●) and rbcS (△) mRNA species in sulfur-adequate tobacco leaf pieces during incubation in the presence (a) and absence (b) of 200 µg/ml cordycepin. mRNA levels were measured over a 90 minute time course and error in loading were corrected by normalization of PA1, cab or rbcS mRNA level to the level of 18S ribosomal RNA for the corresponding sample.
PAI mRNA in contrast, decayed with a half-life of less than 15 minutes in the absence of cordycepin. By 45 minutes the level of this mRNA species had declined to 7.9% of its original level and fluctuated slightly thereafter. PAI mRNA was undetectable by 90 minutes. In contrast to the situation with cab and rbcS mRNA, the fluctuations in PAI mRNA level were not centred around 100%. The half-life of approximately 10 minutes estimated for PAI mRNA from this data represents an upper limit to the true half-life, since transcription was not inhibited and degraded mRNA molecules were being replaced by freshly synthesized molecules.

The reason for the rapid decline in PAI mRNA level in the absence of cordycepin is unknown but it may indicate that the rapid decay observed for PAI mRNA in the presence of cordycepin may have been caused by the handling of the leaf tissue required for preparation of this experiment (ie. cutting, transfer to petri dishes, infiltration). If this was the case, decay of all three mRNA species may have been due to the actions of non-specific ribonucleases triggered by wounding of the tissue during preparation, and in that case, would not reflect the rate at which natural decay processes occur in the intact plant.

This hypothesis was supported by the results of an experiment in which leaf material was harvested and placed directly into liquid nitrogen, before material for the rest of the experiment was harvested. In this experiment, leaf tissue was harvested from sulfur-adequate plants transformed with the PAI gene (SSU-PAI-PAI1700), as described previously. The leaf pieces were infiltrated with buffer but not cordycepin and samples were taken at 15 minute intervals up to one hour after vacuum infiltration. There was an interval of approximately 30 minutes between the initial harvest of material directly into liquid nitrogen and the harvest immediately after vacuum infiltration. There was an interval of approximately 30 minutes between the initial harvest of material directly into liquid nitrogen and the harvest immediately after vacuum infiltration.

During this interval the levels of both PAI and rbcS mRNAs declined by approximately 70% (Fig. 3.5(a) and (b)). Thereafter, both mRNAs decayed with half-lives of approximately 35 to 40 minutes. Again, these figures represent upper limits to the true half-lives of PAI and rbcS mRNAs, as transcription was not inhibited. Nevertheless, this result showed that large proportions of both PAI and the normally stable rbcS mRNA were lost during the period of preparation for the experiment. Therefore reliable estimates of mRNA degradation rates in tobacco leaves could not be obtained because rapid decay of PAI and rbcS transcripts was initiated before measurement of mRNA levels began. The discrepancy between the very short half-life observed for cab mRNA and the longer half-lives reported previously (Jiang et al., 1994; Romero and Lam, 1993).
suggest that cab mRNA was also degraded in an uncontrolled fashion by non-specific ribonucleases.

This problem was exacerbated in leaves from sulfur-deficient plants as they appeared to contain an even higher level of non-specific ribonuclease activity than sulfur-adequate leaf tissue. In the presence of cordycepin, PA1, cab and rbcS mRNAs all decayed extremely rapidly. Levels of these messages declined to 2.7% to 5% of their original levels within 15 minutes, resulting in half-lives of less than 10 minutes. Subsequently, the levels of all three mRNAs fluctuated between 0.7% and 16.7% of their initial levels (Fig. 3.6(a)).

In the absence of cordycepin, the pattern of mRNA abundance was different from that in the presence of cordycepin but again, there was no distinction between the behaviour of the PA1, cab and rbcS mRNAs over the first 30 minutes of the experiment. The mRNA levels for all three genes increased in the first 15 minutes, before declining to between 21% and 33% of initial levels by 30 minutes. The resulting half-lives were between 25 and 30 minutes, with rbcS mRNA marginally more stable than PA1 or cab. After 30 minutes the levels of all three mRNA species fluctuated widely (Fig. 3.6(b)).

The reasons for the erratic fluctuations in the mRNA levels are unknown, but the lack of variation in degradation rates between the three mRNA species both in the presence and absence of transcription inhibitor, indicates that they were not degraded in a controlled fashion by the normal decay pathways for those mRNA species. Rather, they may have been degraded by abundant, non-specific ribonuclease activities triggered during preparation of the leaf material for the experiments. It appears that the use of transcription inhibitors with excised leaf pieces was not an appropriate method for measuring mRNA decay rates in sulfur-adequate and sulfur-deficient tobacco leaves.
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Figure 3.5 Loss of PA1 (●) and rbcS (○) mRNA in tobacco leaf pieces during preparation of material for time course experiments, and for one hour thereafter (a). Leaves were excised from cultured tobacco plants and five 1 cm² pieces were immediately frozen in liquid nitrogen. This sample was called time zero. Remaining leaves were then cut into 1 cm squares and kept in ice cold water until enough pieces for all time points were cut. This process took about 30 minutes, after which these pieces were vacuum infiltrated with buffer and samples harvested at 15 minute intervals, beginning immediately after vacuum infiltration. (b) Quantification of mRNA levels measured in (a).
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Figure 3.6 Decay of PA1 (○), cab (●) and rbcS (△) mRNA species in sulfur-deficient tobacco leaf pieces during incubation in the presence (a) and absence (b) of 200 µg/ml cordycepin. mRNA levels were measured over a 90 minute time course and error in loading were corrected by normalization of PA1, cab or rbcS mRNA level to the level of 18S ribosomal RNA for the corresponding sample. Data from the 90 minute time point from the experiment conducted without cordycepin is not presented because insufficient RNA was obtained from this sample to prepare for electrophoresis.
3.3 DISCUSSION

Post-transcriptional regulation of gene expression has been reported for many plant genes (chapter 1). In many cases, discrepancies between rates of transcription measured in vitro and levels of mRNA accumulation measured in vivo have been presented as evidence for changes in rates of mRNA degradation (Green, 1993; Sullivan and Green, 1993) however rates of mRNA degradation are rarely measured directly, largely due to the technical difficulties of doing so in plants tissue. Studies in which mRNA stability has been directly measured, to confirm that discrepancies between transcription rates and steady-state mRNA levels did, in fact, result from changes in mRNA stability will be discussed in relation to the method developed to assess stability of PA1 mRNA in this present study.

Experiments using excised shoots, roots or leaf discs are more suitable for measuring mRNA decay rates than methods using intact plants, since rapid penetration of the cells by transcription inhibitors can be facilitated by vacuum infiltration or mechanical abrasion of the tissue surface and lower concentrations of inhibitor are required. Stability of phytochrome A mRNA has been measured in excised oat coleoptiles (Seeley et al., 1992) with the cuticles abraded, to facilitate uptake of cordycepin (Schopfer, 1989). A second example of an experimental system using excised tissue was the use of leaf discs from tobacco to measure the effect of antisense rbcS RNA on the stability of endogenous rbcS mRNA (Jiang et al., 1994). In this experiment a single leaf disc was taken from the first fully expanded leaf of glasshouse-grown tobacco plants. Discs from six individual plants were used for each point in the time course over which rbcS mRNA decay was measured, thus large numbers of plants would have been required for these experiments. The leaf discs were incubated in 150 µg/ml cordycepin for up to 2 hours and RNA from the six pooled leaf discs for each time point was analysed.

The leaf material used in this experiment most closely approximated the type of material available for analysis of PA1 mRNA stability in sulfur-adequate and sulfur-deficient transgenic tobacco, so the method developed to measure decay of PA1 mRNA was based on the method of Jiang et al. (1994), although several modifications were made.
Chapter 3. A Method For Measuring PA1 mRNA Stability

3.3.1 *In vitro* production of sulfur-deficient tobacco

Producing sulfur-deficient tobacco in the glasshouse is both time- and space-consuming, and to reproduce the experimental setup of Jiang *et al.* (1994) for a seven-point time course for both +S and -S plants, tested in the presence and absence of cordycepin, using only one leaf disc from each plant would have required 168 plants. This was impossible given the constraint of space in the glasshouse therefore an alternative method for generating sulfur-deficient tobacco leaf tissue was devised. This method involved growing plants essentially in liquid culture, supported by inert, paper-based cylinders in tissue culture pots, as described in chapter 2. Using this method it was possible to control sulfur availability and to induce sulfur deficiency in three weeks rather than four to five weeks in the glasshouse. Many more plants could be produced in a given space using this method and the leaves of these *in vitro* cultured plants were more developmentally homogeneous than those of glasshouse-grown plants.

The PA1 mRNA levels in the first three expanded leaves of each plant were found to be similar (data not shown) therefore more leaves per plant could be used for experiments. To further reduce the number of plants required, the three leaves taken from each plant were cut into four or six 1 cm² pieces and all pieces were mixed, in order to distribute between-plant variation among all samples. From this pool of leaf pieces, five were randomly selected for each petri dish and one petri dish was harvested at each time point. This novel method for producing sulfur-deficient leaf tissue was successful in that it produced tissue in which accumulation of PA1 mRNA was up to nine fold less than in corresponding sulfur-adequate tissue, a response comparable to that achieved in glasshouse-grown plants. There were however, some significant drawbacks to this method. The major problem was that being grown *in vitro*, with high humidity at 27°C, these plants were susceptible to bacterial contamination of the medium. Contamination rates varied between experiments, and in most cases the contaminating bacteria did not appear to be pathogenic, however in some cases plant growth appeared to be retarded and the effects of contamination on gene expression were unknown. Severely affected plants were not used in mRNA stability experiments but low levels of contamination may have contributed to the erratic results observed in some experiments.

A second disadvantage was that there appeared to be a very fine distinction between plants which were sufficiently deficient in sulfur to trigger the response in PA1 expression, and those which were not. Not all plants reached this stage at the same time,
making it difficult to select plants of uniform sulfur-status for use in experiments. To minimize the effects of this problem and that of bacterial contamination it was necessary to ensure that all plantlets were completely uniform in size and development at the outset, and to grow more plants than were actually necessary for the experiment, so that a uniform set could be selected. This reduced the advantage of this method in terms of the number of plants required, as extra plants were needed to over come the shortcomings of the method. It was not always possible to obtain a uniform set of plants to use in mRNA decay experiments and this too, may have contributed to the fluctuations observed in the results of those experiments.

Finally, the plants appeared able to effectively recycle sulfur from lower leaves, to maintain growth of the youngest leaves. It was difficult to chase sulfur from the plants effectively, to achieve a uniform level of sulfur deficiency. The ability of plants to recycle sulfur from lower leaves was unexpected since export of sulfur from expanded leaves can be inhibited during sulfur deficiency (Adiputra and Anderson, 1995), however sulfur supply for newly expanded leaves may be preferentially mobilized from existing leaves under some conditions (Bell et al., 1995). This factor also meant that extreme care was needed to ensure that starting plantlets were uniform in size, to minimize variation in initial sulfur pool size.

In summary, this novel method for growing sulfur-deficient tobacco plants in vitro was able to reproduce the sulfur-responsive expression of PA1 observed in glasshouse-grown plants, whilst allowing plants to be grown with reduced requirements for space and time. It did not however, overcome the problem of variation in levels of PA1 expression and responsiveness to sulfur between individual plants which was observed in the glasshouse-grown plants and is described in chapter 4. In addition, the method had new problems with respect to bacterial contamination and sulfur recycling, not previously encountered with glasshouse-grown plants. Therefore, while it is potentially a useful method for producing sulfur-deficient plants for a range of purposes, this in vitro method will require refinement if it is to produce reliable, reproducible results. Variation in development and sulfur status of the leaf tissue probably contributed to the inconsistent results obtained in mRNA decay experiments, but other factors relating to the experimental procedure may also have contributed.
3.3.2 Inhibition of transcription and mRNA decay

The second component of this method for measuring mRNA decay rates involved inhibiting transcription in the leaf pieces with cordycepin and measuring the rate of loss of existing mRNA populations. Cordycepin was chosen for this study in preference to the other transcription inhibitor commonly used in plants, actinomycin D, primarily in order to be able to compare the half-lives observed for cab and rbcS mRNAs with those obtained by Jiang et al. (1994) in the presence of cordycepin. Both cordycepin and actinomycin D have been shown to prolong the half-lives of unstable mRNAs in both plant and mammalian cells (Fritz et al., 1991; Seeley et al., 1992; Zhang et al., 1993; Shyu et al., 1991). The stabilizing effect of cordycepin in the studies by Fritz et al. and Seeley et al. was small compared with the 24-fold stabilization of PvPRP1 mRNA by actinomycin D (Zhang et al., 1993), so cordycepin was considered less likely to cause artifactual stabilization of mRNAs in the present study.

Since it is an analogue of adenosine, cordycepin may interfere with ATP synthesis in mitochondria. However the concentration used by Seeley et al. (100 µg/ml) has been shown to have no effect on respiration or ADP/O ratios in mung bean mitochondria (Cline et al., 1974). Therefore the risk of secondary metabolic effects should be minimized if the concentration of cordycepin used for transcription inhibition is in the order of 100 µg/ml. In these present experiments, cordycepin was used at a concentration of 200 µg/ml, therefore it was possible that metabolic aberrations may have affected mRNA decay, but it seems unlikely that this was the cause of unregulated mRNA decay, as Fritz et al. (1991) used a concentration of cordycepin 12 times higher than was used in these experiments and still observed regulated mRNA decay.

While the experimental procedure used to measure rates of decay of cab, rbcS and PA1 mRNAs was similar to the conditions used by Jiang et al. (1994) with the previously noted difference in the source of leaf material, the decay rates observed for cab and rbcS mRNAs in sulfur-adequate leaves were not in agreement with those observed by Jiang et al. (1994). Cab mRNA in light-grown, cordycepin-treated tissue has previously been reported to decay with a half-life of between 45 minutes (Jiang et al., 1994) and two hours (Romero and Lam, 1993), while in the absence of cordycepin Romero and Lam (1993) observed a half-life of approximately ten hours. In the experiments described here, in sulfur-adequate leaf material treated with cordycepin cab mRNA decayed with a half-life of approximately ten minutes. This was 4.5-fold faster than reported by Jiang et al. and 12-fold faster than in the experiments of Romero and Lam (1993). In the absence...
Chapter 3. A Method For Measuring PAI mRNA Stability

of cordycepin the level of cab mRNA fluctuated around 100% of the original level and did not fall below 50%, as expected in the presence of ongoing transcription. The reduction in the steady-state level of cab mRNA in the presence, but not in the absence of cordycepin indicated that synthesis of cab mRNA was inhibited by cordycepin but the reason for the rapid decay of this message following inhibition of transcription is not known.

Rubisco small subunit mRNA was chosen as an example of a more stable mRNA species, as its half-life was estimated to be approximately five hours by Jiang et al. (1994). This mRNA was not expected to decay appreciably during the 90 minutes over which these mRNA decay experiments were conducted. However, after 15 minutes the level of rbcS mRNA had fallen to approximately 50% of its original level. This large deviation from decay kinetics previously reported for rbcS mRNA indicated that the method used to measure the rates of decay of cab and rbcS mRNA in healthy tobacco leaf pieces did not reproduce the results of Jiang et al. (1994). Furthermore, the similarities in the initial mRNA decay rates observed for cab, rbcS and PAI, which decayed with a half-life of approximately 12 minutes, suggested that these mRNA species were being degraded indiscriminately by non-specific ribonucleases rather than via the controlled, message-specific pathways by which mRNA is normally degraded. The reason for the increase in level of all mRNAs after 30-45 minutes of incubation in cordycepin is not known.

Since the major difference between the method described here and that described by Jiang et al. (1994) was in the preparation of the leaf pieces, it was likely that the discrepancies in results arose during this procedure. In contrast to the method of Jiang et al., in which a single leaf disc was taken from each plant, several leaves were first removed from the plant and then cut into smaller pieces. This involved considerable handling and wounding of the tissue, and about 30 minutes elapsed between harvesting the first leaf and the beginning of the experiment. Therefore it is possible that the handling and wounding of the tissue during preparation induced activity of non-specific ribonucleases which began to degrade the mRNA before material for the first time point was frozen in liquid nitrogen. It was confirmed that 70% of PAI mRNA was lost between the initial harvest and time zero, by harvesting some leaf pieces directly into liquid nitrogen, to assess the level of PAI mRNA actually present in the plant. Thus, it appears that a very rapid mRNA decay process was initiated during preparation of the leaf material, and this may have caused the rapid decay of PAI, cab and rbcS mRNAs.
Alternatively, the faster rate of loss of transcripts in sulfur-deficient leaves may be attributed to fewer transcripts for each species of mRNA being present initially. Quantification of bands from the Northern blots used to assess RNA decay indicates that there were fewer transcripts in sulfur-deficient leaves than in sulfur-adequate leaves, in all cases. However, the increased rate of decay of all transcripts in sulfur-deficient leaves could not be attributed solely to reduced transcript levels. Further experimentation is required to determine the nature of ribonuclease activity in sulfur-deficient tissue.
Decay rates for cab and rbcS mRNAs were measured both as controls for the experimental technique and as examples of short- and long-lived mRNAs respectively. The decay of PAI mRNA was to have been compared with the decay of cab and rbcS mRNAs, to determine whether it was similar to that of a short-lived or a long-lived transcript. It was anticipated that PAI mRNA would decay in a manner similar to the long-lived message in sulfur-adequate tissue, and would decay rapidly in sulfur-deficient tissue. All three transcripts however, behaved similarly, revealing a shortcoming in the method. It was not therefore, possible to determine whether PAI mRNA behaved as a stable or an unstable message, even in sulfur-adequate tissue.

In the presence of 200 µg/ml cordycepin the levels of cab, rbcS and PAI mRNAs in sulfur-deficient leaves fell to 5%, or less, of their original levels within 15 minutes and fluctuated between zero and 17% thereafter. This initial rate of decay was even faster than that observed in sulfur-adequate tissue and indicates that if indeed, these mRNAs were attacked indiscriminately by non-specific ribonucleases during preparation of the experimental material, then these ribonucleases may be more prevalent, or more active in sulfur-deficient tissue. This is not unlikely, given that it was more difficult to isolate undegraded total RNA from sulfur-deficient leaves than from sulfur-adequate leaves in both experiments described here and in chapter 4. In the absence of cordycepin all three mRNA species were stabilized somewhat, reaching 50% of their original levels after approximately 25 minutes of incubation.

Although PAI mRNA was degraded faster in sulfur-deficient than in sulfur-adequate tissue as predicted by the model discussed in chapter 1, this was not due to specific destabilization of PAI mRNA by factors present in sulfur-deficient leaf tissue. The destabilization affected cab, rbcS and PAI mRNAs equally and thus, was likely to have been caused by non-specific factors during preparation.

In summary, the method used to measure rates of decay of PAI mRNA in sulfur-adequate and sulfur-deficient tobacco leaf tissue was based on that of Jiang et al. (1994) however due to shortcomings in both the growth conditions of the plants and the experimental protocol, this method failed to reproduce the results of Jiang et al. for the two mRNA species adopted as standards, cab and rbcS. The system did not differentiate between stable and unstable mRNAs and in the absence of ongoing transcription, all mRNAs examined were degraded at similar rates. This experimental system is therefore, inappropriate for detecting differences in the stability of PAI mRNA in response to sulfur availability. The hypothesis that the reduction in abundance of PAI mRNA in sulfur-
deficient plants is caused by specific destabilization of PA1 mRNA remains an attractive one, however at this stage it remains unproven. If this question is to be resolved an alternative means of measuring mRNA stability must be devised.

3.3.3 Alternative methods for measuring mRNA stability

The methods for measuring mRNA discussed so far in this chapter have used excised, but intact plant pieces (Jiang et al., 1994; Seeley et al., 1992) as the source of mRNA for study. These studies have also measured decay of existing mRNA populations by Northern blot analyses rather than by measuring decay of radioactively labelled mRNA in pulse-chase experiments. Pulse-chase experiments are particularly troublesome in plant tissues as it is difficult to label mRNA precursors to a sufficient specific activity (Parker et al., 1990) and it is also difficult to achieve an adequate chase of cold nucleotide (Cox et al., 1973). Other methods for measuring mRNA decay which use protoplasts or cell suspension cultures as a source of mRNA, but which do not employ pulse-chase techniques, have been described and mRNA decay has also been extensively studied in cell-free decay systems using a variety of mRNA sources.

3.3.3.1 Cell-free in vitro mRNA decay

A variety of cell-free methods for measuring mRNA degradation in mammalian systems have been reported (reviewed by Ross, 1993). In these types of experiments, mRNA or messenger ribonucleoprotein (mRNP) is incubated with cell extracts likely to contain ribonucleases. Decay is measured by removing aliquots from the reaction at intervals after incubation was started, and measuring the amount of a particular mRNA species remaining by northern analysis.

In plants, fewer cell-free methods have been described although the methods used have been based on the same principles as those used to characterize decay of mammalian mRNA. Degradation of transcripts for chloroplast-encoded genes has been observed in extracts from lysed chloroplasts (Stern et al., 1989; Klaflf, 1995) but only one method has been described for studying decay of nuclear-encoded mRNAs from plant cells in vitro. Byrne et al. (1993) described a method based on those used to measure decay of polysome-associated mammalian mRNAs (Ross and Kobs, 1986; Ross et al., 1987). In this method, polysomes isolated from etiolated oat shoots were used as the source of mRNA and degradation enzymes. The in vitro decay system exhibited selective decay.
and degraded actin, β-tubulin and phytochrome A mRNAs with half-lives in the same rank order as was observed when decay was measured in vivo, by inhibition of transcription with cordycepin. Moreover, the half-life of β-tubulin mRNA was decreased in polysomes isolated from leaves exposed to a pulse of red light prior to polysome isolation. This destabilization of β-tubulin mRNA in response to red light also occurs in vivo. Therefore the method appears to accurately reproduce changes in mRNA stability induced by changes in physiological state.

Although this method is straightforward, it may not be appropriate for measuring changes in stability of PA1 mRNA in response to sulfur deficiency. Results from chapter 4 indicated that the process by which PA1 expression responds to sulfur deficiency may occur in the nucleus rather than on polysomes. Nevertheless, a system could be envisaged in which nuclei or nuclear extracts substitute for polysomes in this type of experiment. Nuclei could be extracted from the leaves of transgenic plants containing PA1, or from pea cotyledons where PA1 is naturally expressed. After incubation of the nuclei, nuclear RNA would be extracted and degradation of PA1 mRNA measured.

3.3.3.2 Protoplasts

Gallie and Young (1994) used protoplasts isolated from maize endosperm to study the effects of promoters, introns and untranslated regions on transgene expression in that tissue. In that study, in vitro transcribed mRNA for a reporter gene, luciferase, was introduced into endosperm protoplasts by electroporation. The mRNA was translated in the protoplasts and luciferase activity assayed over time.

A similar approach could be used to measure mRNA decay kinetics by introducing in vitro transcribed mRNA into protoplasts then collecting aliquots of protoplasts at intervals and analysing the decay of the introduced mRNA. The advantage of this method is that the population of introduced mRNA molecules is homogeneous and its time of introduction to the cell mRNA degradation machinery is accurately known. Transcription inhibitors are not required so the risk of artifactual stabilization of the mRNA is minimized.

This approach was evaluated as a method for measuring decay of PA1 mRNA in protoplasts from sulfur-adequate and sulfur-deficient tobacco leaves. Mesophyll protoplasts were isolated from the leaves of sulfur-adequate and sulfur-deficient, glasshouse-grown tobacco but protoplasts derived from sulfur-adequate leaves did not
survive electroporation even at low voltages. Protoplasts from sulfur-deficient leaves were even more fragile and were extremely difficult to manipulate without damage. This approach was not pursued further, as it was not possible to introduce \textit{in vitro}-transcribed RNA without lysing the protoplasts.

### 3.3.3.3 Cell suspension culture

Transcription inhibitors, primarily actinomycin D, have been used with tobacco cell suspensions both to identify genes with unstable transcripts (Taylor and Green, 1995), and to examine the effects of sequence elements on mRNA stability (Newman \textit{et al.}, 1993; Ohme-Takagi \textit{et al.}, 1993).

The effects of both DST elements (Newman \textit{et al.}, 1993) and sequences with high AU content (Ohme-Takagi \textit{et al.}, 1993) on reporter gene mRNA stability have been studied in cell suspension culture. In these experiments, actinomycin D was added to the suspension and aliquots were removed at intervals, to monitor the decay of mRNA after inhibition of transcription. In this way, both DST and AU-rich elements were shown to destabilize reporter gene transcripts. In both cases, accumulation of these reporter gene mRNAs was reduced in transgenic plants, relative to accumulation of reporter gene transcripts lacking DST of AU-rich inserts. These correlations suggest that this method for measuring mRNA decay reflects the mRNA degradation processes occurring \textit{in vivo}.

There are two main advantages to using cell suspension cultures as a source of mRNA, rather than intact plant tissue. Firstly, the transcription inhibitor can be introduced into all cells at a rapid rate, without the need for vacuum infiltration, reducing the physical disruption to the cell. This may reduce the possibility of uncontrolled mRNA degradation in response to wounding. Secondly, samples for all time points can be harvested from the same population of cells (Byrne \textit{et al.}, 1993), reducing the likelihood of variation between samples which may arise from differences in tissue pieces taken from different plants when whole leaves or leaf pieces are used.

The disadvantage of experiments using suspension cultures in conjunction with transcription inhibitors is exemplified by the results of Zhang \textit{et al.} (1993) who found that \textit{PvPRP}1 mRNA was rapidly degraded in cells exposed to elicitor from \textit{Colletotrichum}. When cells were treated with elicitor alone, \textit{PvPRP}1 mRNA decayed with a half-life of approximately 45 minutes but when elicitor and actinomycin D were added together, the half-life of this mRNA species increased to 18 hours. Actinomycin D artificially
stabilized PvPRPl mRNA, presumably by inhibiting the synthesis of a labile factor required for the degradation process (Zhang et al., 1993).

In plants, it has recently become possible to inhibit synthesis of a single species of mRNA by expressing it from the tetracycline-repressible Top10 promoter (Weinmann et al., 1994). Transcription from this promoter is rapidly inhibited by the addition of low concentrations of tetracycline to cell cultures. Expression of the gene of interest and a reference gene can be driven from this promoter and decay of their mRNAs studied in the presence of tetracycline, while transcription of other mRNAs continues. This system was recently used to investigate the effects of the coding region and 3' UTR of an Arabidopsis SAUR on reporter gene mRNA stability (Gil and Green, 1996). This system showed that the SAUR 3' UTR was an effective mRNA destabilizing element, while similar experiments using actinomycin D to inhibit transcription did not distinguish between reporter genes carrying SAUR 3' UTRs and those carrying E9 3' UTRs.

This selectively repressible promoter therefore, allows measurement of mRNA decay in plant cell cultures without the use of metabolic inhibitors which can interfere with metabolic and degradation processes. A method for culturing plant cells in sulfur-deficient medium has been described (Rennenberg and Kaiser, 1989) but it is not known whether the response of PA1 to sulfur can be reproduced in cell culture. If expression of PA1 is sulfur-responsive in cell culture, the Top10 promoter might offer a reliable means of measuring PA1 mRNA stability in sulfur-adequate and sulfur-deficient cells, making it possible to determine whether the decreased accumulation of PA1 mRNA in sulfur-deficient transgenic plants is entirely caused by destabilization of the message, as proposed in the model outlined in chapter 1.

Although it has not yet been possible to prove that sulfur-responsive expression of PA1 is regulated by a change in stability of PA1 mRNA, other aspects of the mechanism by which sulfur regulates expression of PA1 were examined, assuming that the PA1 transcript is destabilized during sulfur deficiency. Experiments in which regions of PA1 RNA were tested for their ability to confer sulfur responsive gene expression in vivo are described in the next chapter. Identification of putative regulatory PA1 RNA-binding proteins is described in chapter 5.
CHAPTER 4

LOCATION OF THE 3' SULFUR RESPONSIVE ELEMENT
4.1 INTRODUCTION

Post-transcriptional regulation of gene expression in response to specific external stimuli is often mediated by specific sequences. These sequences are frequently located in the 3' UTR but also occur elsewhere in RNA transcripts (chapter 1; see Sullivan and Green, 1993; Ross, 1995 for reviews). An extensive study was carried out to identify and locate sequences in PAI mRNA which controlled the response to sulfur (Morton, 1993).

Using chimeric gene constructs expressed in the leaves of transgenic tobacco, it was found that the PAI coding region and a 1700 nucleotide sequence flanking the 3' end of the PAI coding region were each able to confer sulfur responsiveness on otherwise unresponsive reporter gene constructs. The responses conferred by these individual regions were not as great as the response observed when both regions were expressed together in tobacco leaves, indicating that both regions were required for a 'complete' response to sulfur.

The 1700 nucleotide 3' flanking region was further dissected to precisely locate, and define the limits of the SRE in the 3' flanking region. Fragments of PAI 3' UTR and flanking region 189, 323 and 496 nucleotides in length and beginning 10 nucleotides downstream of the translation stop codon were inserted into an unresponsive reporter gene construct which contained the coding region of the avian ovalbumin gene (Fig. 4.1). The ovalbumin gene was chosen for this study as the protein it encodes is sulfur-rich, but its expression in plants was not regulated by sulfur nutrition.

All three fragments conferred 2.5- to 4.5-fold reductions in ovalbumin mRNA levels in response to sulfur deficiency, indicating that the sulfur responsive element was contained within the first 199 nucleotides of the 3' flanking region and that additional sequences beyond this point did not substantially enhance magnitude of the response to sulfur. A polyadenylation site is located 167 nucleotides downstream of the translation stop codon in PAI mRNA and the sequenced cDNA ends a further 16 nucleotides downstream of the AAUAAA sequence thought to form part of some plant polyadenylation signals (Hunt, 1994; Messing et al., 1983). Therefore it is likely that the mature PAI mRNA ends approximately 183 nucleotides into the 3' flanking region. Since the first 199 nucleotides of the 3' flanking region were sufficient to confer sulfur-responsiveness, it appeared likely that sequences not contained in the mature transcript did not affect PAI mRNA abundance in response to sulfur deficiency.
Chapter 4. Location of the 3' Sulfur Responsive Element

Further evidence supporting a role for sequences contained within the mature PA1 message, specifically nucleotides 109-199, came from a construct in which the rubisco small subunit 5' UTR was replaced with that of ovalbumin. This change resulted in loss of sulfur-responsiveness and structural prediction analysis showed that the ovalbumin 5' UTR could pair with nucleotides 117-198 of the PA1 3' UTR while the small subunit 5' UTR could not. It was speculated that this predicted structural interaction between the 5' and 3' ends might interfere with a structural element required for sulfur-responsiveness, further implicating involvement of the 189 nucleotide segment in the response to sulfur deficiency.

The 189 nucleotide fragment of PA1 3' UTR was used to search the Genbank and EMBL databases for similar sequences in other known sulfur-responsive genes. Such similarities between sulfur responsive genes would provide evidence of a common mechanism by which genes that are regulated by sulfur deficiency might be recognized, but no similarities were found. Analysis of predicted secondary structures formed by mRNAs for various sulfur-rich seed proteins was also carried out but unlike the cases of the IRE (Koeller et al., 1989) and insulin-like growth factor II (Scheper et al., 1996), in which characteristic secondary structures are conserved between species, no striking similarities were observed (Morton, 1993).

Peng et al. (1996) observed that although not sharing any significant sequence similarity, instability domains in the 3' UTRs of c-jun and c-fos mRNAs were recognized by the same set of cytoplasmic factors. The instability domains of these two mRNAs were also found to be functionally interchangeable to a certain degree, indicating that they may have been regulated by the same set of factors. In light of this observation, the lack of sequence or structural similarity between mRNAs for different sulfur-responsive seed genes does not necessarily mean that they are not regulated by a common mechanism in different species. It may be however, that the recognition site for a regulating factor is more complex than a single conserved sequence or structure.

The results of the deletion study indicated that the 189 nucleotide region, spanning nucleotides 10-199 of the PA1 3' UTR, should be characterized in more detail in order to find the smallest possible section of PA1 mRNA capable of conferring sulfur-responsiveness. This would facilitate studies aimed at identifying protein/mRNA interactions involved in the response to sulfur (discussed in chapter 5).
Chapter 4. Location of the 3’ Sulfur Responsive Element

**CONSTRUCT**

**SJ21**

(SSU-PAl-PAl1700)

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**RM23**

(SSU-ovalb-35S)

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<td>1.26kb</td>
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**EXPRESSION CASSETTE**

**RM14**

(SSU-ovalb-PAl496)

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**RM33**

(SSU-ovalb-PAl323)

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**RM32**

(SSU-ovalb-PAl189)

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**RM42**

(SSU-ovalb-PAl99)

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Figure 4.1 Gene constructs used for in vivo sulfur-stress experiments:

**SJ21**: positive control; rubisco small subunit promoter and 5’ UTR - PAl coding region - PAl 3’ flanking region.

**RM23**: negative control; rubisco small subunit promoter and 5’ UTR - ovalbumin coding region - CaMV 35S 3’ UTR and termination sequence.

**RM23** also acted as the expression cassette. Fragments of the PAl 3’ UTR and flanking region were inserted between the ovalbumin coding region and the CaMV 35S 3’ UTR of the expression cassette. The sizes of the PAl fragments inserted into the expression cassette were 0.496 kb, 0.323 kb, 0.189 kb and 0.099 kb to make RM14, RM33, RM32 and RM42 respectively.
Chapter 4. Location of the 3' Sulfur Responsive Element

The experiments described in this chapter were designed to locate the 3' SRE within the 189 nucleotide fragment, and to test the hypothesis that the region between nucleotides 117 and 199 of the PAI 3' UTR contains the minimal sulfur-responsive element. The approach taken was similar to that described by Morton (1993). Initially a 99 base pair fragment spanning nucleotides 10-109 of the PAI 3' UTR was inserted into the ovalbumin expression cassette (SSU-ovalb-PA199; section 2.1.1) and tested for its ability to confer sulfur-responsiveness in transgenic tobacco. The 189 nucleotide fragment (SSU-ovalb-PA1189) was tested simultaneously but in contrast to results obtained by Morton (1993), neither it nor the 99 nucleotide fragment (SSU-ovalb-PA199) conferred a response to sulfur. Therefore the remaining fragments in the deletion series were also tested and both the 323 and 496 nucleotide fragments conferred sulfur-responsiveness on ovalbumin expression.

4.2 RESULTS

4.2.1 Generation of transgenic tobacco containing SSU-ovalb-PA199

A fragment of PAI mRNA spanning nucleotides 10-109 of the 3' UTR was subcloned into the ovalbumin expression cassette (pRM23). The gene construct (SSU-ovalb-PA199-CaMV35S) was then subcloned into the binary vector pGA492 (cloning described in section 2.1.1). This plasmid (pBRM42) was transferred to Agrobacterium tumefaciens by triparental mating. The T-DNA was transferred to tobacco by Agrobacterium-mediated transformation and plants were regenerated in the presence of 100 mg/L kanamycin (section 2.1.1). Putative transformants were screened for expression of ovalbumin mRNA by Northern blot analysis. Of the 17 plants screened, 10 expressed transcripts which hybridized with the ovalbumin probe (Fig. 4.2(a)). All of these plants expressed two bands which hybridized to the ovalbumin probe and in each plant the two bands were of approximately equal intensity. The transformant expressing the highest level of ovalbumin mRNA was selected for further analysis (transformant RM42-13).

The faster migrating band in RM42-13 was the same size as the ovalbumin mRNA produced by a plant containing the control plasmid pRM23 (Fig. 4.2(b)). It has previously been confirmed that the ovalbumin transcript in that control plant was of the correct size (Morton, 1993). The slower migrating band in RM42-13 was not the result of non-specific hybridization, as it was not present in mRNA isolated from plants transformed with the pGA492 vector only (AGA492, Fig. 4.2(b)). It is also unlikely that the upper band is the result of the insertion of the 99 nucleotides of PAI 3' UTR into the
expression cassette because the size difference between these two bands was equivalent to the difference between the 1.7 kb and 1.4 kb ribosomal RNA bands (data not shown). Insertion of only 99 nucleotides would not be expected to cause a mobility shift of this magnitude, therefore the lower band in RM42-13 encoded the ovalbumin mRNA and 99 nucleotides of the PA1 3' UTR.

This larger band in RM42 transformants is unlikely to have resulted from a random gene rearrangement during transformation, as it was present in every plant expressing ovalbumin coding region mRNA, but may result from utilization of an alternative polyadenylation site downstream of the CaMV 35S transcription termination sequence. This explanation for the presence of this band is supported by the fact that it was not present in one of the plants used in the sulfur deficiency experiment (Fig. 4.7(a)). All of these plants were genetically identical to the original RM42-13 plant which expressed two bands containing ovalbumin mRNA, therefore the additional band must have arisen during processing of the transcript rather than from a gene rearrangement during transformation. Since the two bands were of equal intensity in RM42 transformants, they were analysed together to determine the total amount of SSU-ovalb-PA199 present in these plants.

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Figure 4.2 (a) Northern blot showing ovalbumin mRNA levels in 10 of the plants transformed with the RM42 gene construct. Total RNA from these plants was probed with a riboprobe containing the first 417 nucleotides of the ovalbumin coding region. (b) Northern blot showing ovalbumin transcripts produced by plants transformed with pGA492, pBRM23 and pBRM42.
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Transgenic plants containing 99 and 189 nucleotide fragments of PA1 3' UTR in the ovalbumin expression cassette (SSU-ovalb-PA199 and SSU-ovalb-PA1189 respectively, Fig. 4.1) were grown under sulfur-adequate and sulfur-deficient conditions (section 2.1.2), and levels of ovalbumin mRNA were measured. Eight SSU-ovalb-PA199 and eight SSU-ovalb-PA1189 plants were grown, of which four of each were grown under sulfur deficiency. In addition, eight plants containing the negative control construct (SSU-ovalb-35S) and four carrying the positive control construct (SSU-PA1-PA11700) were included in the experiment. Four SSU-ovalb-35S and two SSU-PA1-PA11700 plants were grown under sulfur deficiency. SSU-PA1-PA11700 plants were not included in statistical analyses but were used to assess the severity of the sulfur deficiency induced in each experiment. This construct contains both PA1 coding region and 3' UTR SREs, and consistently responded to sulfur deficiency.

The level of ovalbumin mRNA in each plant was measured by northern blot analysis and was normalized to the level of actin mRNA in the same plant, to correct for any differences between samples in the amount of RNA loaded on the gel (section 2.1.5). Ovalbumin mRNA levels from plants containing fragments of PA1 3' UTR (SSU-ovalb-PA199, SSU-ovalb-PA1189, SSU-ovalb-PA1323 and SSU-ovalb-PA1496) were compared with levels from plants not containing PA1 sequence (SSU-ovalb-35S) by analysis of covariance. Actin mRNA levels were used as the covariate. This analysis measured the effects of sulfur treatment and construct across all samples, and also the interaction between treatment and construct. This interaction described the difference in response to sulfur treatment between two constructs. A statistically significant interaction between sulfur treatment and construct indicated that the response of the 'test' construct to sulfur deficiency was different from that of the negative control construct. This analysis was necessary because the level of ovalbumin mRNA in RM23 plants was reduced by sulfur deficiency in some experiments. This response has been observed previously (Morton, 1993) and is thought to be due to effects of sulfur deficiency on the small subunit promoter, possibly caused by reduced photosynthetic capacity of sulfur-deficient plants whose leaves became yellow.
4.2.2 Neither 99 nor 189 nucleotides of PA1 3' UTR confer sulfur-responsiveness

The results of a sulfur deficiency experiment in which SSU-ovalb-PA1_{99} and SSU-ovalb-PA1_{189} were tested are shown in figures 4.3 - 4.6. PA1 and ovalbumin mRNA levels from individual plants are shown. For each construct, the average value for PA1 or ovalbumin mRNA level in sulfur-adequate plants was divided by the average value in sulfur-deficient plants to obtain the magnitude of the response to sulfur deficiency. The level of PA1 mRNA from the positive control construct was reduced 4.7-fold in response to sulfur deficiency (Fig. 4.3) while the level of ovalbumin mRNA from the negative control construct declined by 1.15-fold (Fig. 4.4). Northern blots of PA1 and ovalbumin mRNA levels in SSU-PA1-PA1_{1700} and SSU-ovalb-35S controls respectively are shown here as examples. The average values for normalized ovalbumin mRNA levels from sulfur-adequate and sulfur-deficient SSU-ovalb-35S plants were compared using the student t-test and the difference was not significant at the 95% level, therefore SSU-ovalb-35S was not considered to respond to sulfur deficiency.

Normalized ovalbumin mRNA levels from SSU-ovalb-PA1_{99} plants declined 1.1-fold in response to sulfur deficiency (Fig. 4.5). This response was not significantly different from that of SSU-ovalb-35S plants ($P_{\text{interaction}} = 0.8$) indicating that the 99 nucleotide region of PA1 3' UTR contained in SSU-ovalb-PA1_{99} did not contain sequence(s) capable of conferring sulfur-responsiveness.

The plant from replicate 2.2 of the sulfur deficiency treatment did not express the larger ovalbumin transcript present in all other SSU-ovalb-PA1_{99} plants. It was not possible to analyse the bands separately so they were analysed together. This meant that the value obtained for ovalbumin mRNA level for plant -S 2.2 was approximately half of what it would have been if the second band had been present at the same intensity. The lack of this upper band did not influence the conclusion that the 99 nucleotide fragment of PA1 3' UTR did not confer sulfur-responsiveness, as the average values for ovalbumin expression in sulfur-deficient SSU-ovalb-PA1_{99} plants were only slightly lower than those for sulfur-adequate plants. If the value from the sulfur-deficient plant 2.2 had been doubled, the difference between average ovalbumin mRNA levels for sulfur-adequate and sulfur-deficient plants would have been even smaller.
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Figure 4.3 Effect of sulfur deficiency on PA1 expression in SSU-PA1-PA1700 (SJ21) plants

(a) Northern analysis of PA1 mRNA levels. Total mRNA was hybridized to an RNA probe complementary to the first 257 nucleotides of the PA1 coding region.

(b) Northern analysis of actin mRNA levels. Total mRNA was hybridized to an RNA probe derived from soybean actin coding region (Cotton et al., 1990)

(c) Quantification of PA1 mRNA levels. PA1 and actin mRNA levels were quantified using a phosphorimager and PA1 message levels were normalized to actin message levels to correct for differences in loading and transfer between samples.

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Figure 4.4 Effect of sulfur deficiency on ovalbumin expression in SSU-ovalb-35S (RM23) plants

a) Northern analysis of ovalbumin mRNA levels. Total mRNA was hybridized to an RNA probe complementary to the first 417 nucleotides of the ovalbumin coding region.

b) Northern analysis of actin mRNA levels. Total mRNA was hybridized to an RNA probe derived from soybean actin coding region (Cotton et al., 1990)

c) Quantification of ovalbumin mRNA levels. Ovalbumin and actin mRNA levels were quantified using a phosphorimager and ovalbumin message levels were normalized to actin message levels to correct for differences in loading and transfer between samples.
Chapter 4. Location of the 3' Sulfur Responsive Element

a) Northern analysis of ovalbumin mRNA levels.

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b) Northern analysis of actin mRNA levels.

c) Quantification of ovalbumin mRNA levels.

Figure 4.5 Effect of sulfur deficiency on ovalbumin expression in SSU-ovalb-PA199 (RM42) plants.

- a) Northern analysis of ovalbumin mRNA levels.
- b) Northern analysis of actin mRNA levels.
- c) Quantification of ovalbumin mRNA levels.
Chapter 4. Location of the 3' Sulfur Responsive Element

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ovalbumin

b) actin

c) 

Figure 4.6 Effect of sulfur deficiency on ovalbumin expression in SSU-ovalb-PA1 189 (RM32) plants

- a) Northern analysis of ovalbumin mRNA levels.
- b) Northern analysis of actin mRNA levels.
- c) Quantification of ovalbumin mRNA levels.
Chapter 4. Location of the 3' Sulfur Responsive Element

Ovalbumin mRNA levels increased slightly in sulfur-deficient SSU-ovalb-PA1189 plants (Fig. 4.6). The magnitude of this increase was 1.32-fold and was not significantly different from the response of SSU-ovalb-35S plants ($P = 0.85$). This indicated that this region did not, in fact, contain a sufficient length of PA1 sequence to confer sulfur-responsiveness. Possible reasons for the discrepancy between this result and that of Morton are discussed in section 4.3.

The sulfur-deficient SSU-ovalb-PA1189 plant from replicate 2.2 expressed two ovalbumin transcripts, similar to those expressed in SSU-ovalb-PA199 plants. This band may have arisen from an alternative polyadenylation signal derived from the vector. This band was not included in analysis of ovalbumin mRNA levels in these plants.

4.2.3 323 nucleotides of PA1 3' UTR confers sulfur-responsiveness

Since the 189 nucleotide region in the SSU-ovalb-PA1189 construct did not confer sulfur-responsiveness on expression of ovalbumin mRNA, the remaining constructs in the PA1 3' deletion series were re-tested under my conditions. In a separate sulfur deficiency experiment plants transformed with pRM33, a construct containing 323 nucleotides of PA1 3' UTR (SSU-ovalb-PA1323; Fig. 4.1) were tested. The results of this experiment are shown in figures 4.7 - 4.9. In this experiment, there was a six-fold reduction in PA1 mRNA levels in sulfur-deficient positive control (SSU-PA1-PA11700) plants compared with sulfur adequate plants (Fig. 4.7). Levels of ovalbumin message from SSU-ovalb-35S plants increased 1.4-fold in response to sulfur deficiency (Fig. 4.8). The reason for this is unknown but this difference was not significant and SSU-ovalb-35S was not considered sulfur-responsive.

Normalized ovalbumin mRNA levels in SSU-ovalb-PA1323 plants declined 1.5-fold in response to sulfur deficiency (Fig. 4.9). The difference between the small negative effect of sulfur deficiency on ovalbumin mRNA level in SSU-ovalb-PA1323 plants and the small positive effect on ovalbumin mRNA level in SSU-ovalb-35S plants was significant at the 95% level of confidence ($P_{interaction} = 0.018$). Because the effect of sulfur deficiency on ovalbumin mRNA level in SSU-ovalb-PA1323 plants was small, a t-test was applied to the mean values for ovalbumin mRNA levels in sulfur-adequate and sulfur-deficient plants. The difference was found to be significant at the 90% level but not at the 95% level.
It is likely that the 323 nucleotide region of PA1 3' UTR contained in pRM33 does contain sequence(s) conferring sulfur-responsiveness. This indicated that sequences not contained within the mature mRNA were required to effect the response to sulfur.

4.2.4 496 nucleotides of PA1 3' UTR confers a large response to sulfur

The remaining construct in the deletion series, SSU-ovalb-PA1_{496} which contained a 496 nucleotide region of PA1 3' UTR, was tested in a separate experiment. PA1 expression in positive control (SSU-PA1-PA1_{1700}) plants showed a 6.5-fold response to sulfur deficiency (Fig. 4.10), comparable to that observed in the experiment described above (section 4.2.3). Ovalbumin mRNA levels from SSU-ovalb-35S also declined in response to sulfur deficiency. A 2.4-fold difference in level of ovalbumin mRNA between sulfur-adequate and sulfur-deficient plants was observed (Fig. 4.11) indicating that transcription from the rubisco small subunit promoter was substantially inhibited by sulfur deficiency in this experiment. The reason for this is unknown, as the sulfur deficiency induced in this experiment was only slightly more severe than that observed in the previous experiment.

Nevertheless, the normalized ovalbumin mRNA levels from SSU-ovalb-PA1_{496} plants showed a 7.2-fold response to sulfur deficiency (Fig. 4.12). The magnitude of this response was significantly greater than that of SSU-ovalb-35S (P_{interaction} < 0.001) indicating that the 496 nucleotide fragment of PA1 3' UTR conferred a response to sulfur, over and above the effect of inhibition of transcription from the SSU promoter. This result indicated that not only were sequences beyond the polyadenylation signal required for sulfur-responsiveness, but that this response was enhanced by sequences downstream from nucleotide 333, which marks the end of the 323 nucleotide fragment in SSU-ovalb-PA1_{323}.

The results described above indicated that the first 333 nucleotides of the PA1 3' UTR conferred a small degree of sulfur-responsiveness on the ovalbumin reporter gene, and that the minimal element conferring this response was likely to be located between nucleotides 199 and 333. Sequences located downstream of this region appeared to enhance the magnitude of the response to sulfur, conferred by the minimal SRE.
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Figure 4.7 Effect of sulfur deficiency on PA1 expression in SSU-PA1-PA1₁₇₀₀ plants. Northern blots were probed for PA1 mRNA then reprobed for actin mRNA. Actin mRNA levels acted as internal standards for total RNA loading. Levels of PA1 mRNA were quantified by Phosphorimager and normalized to actin mRNA levels.

Figure 4.8 Effect of sulfur deficiency on ovalbumin expression in SSU-ovalb-35S plants. Ovalbumin and actin mRNA levels were determined by Northern blot analysis and quantified as described above.
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Figure 4.9 Effect of sulfur deficiency on ovalbumin expression in SSU-ovalb-PA1323 (RM33) plants
a) Northern analysis of ovalbumin mRNA levels.
b) Northern analysis of actin mRNA levels.
c) Quantification of ovalbumin mRNA levels.
Figure 4.10 Effect of sulfur deficiency on PAI expression in SSU-PA1-PA1_{1700} plants. PAI and actin mRNA levels were determined by Northern blot analysis and quantified as described for Figure 4.7.

Figure 4.11 Effect of sulfur deficiency on ovalbumin expression in SSU-ovalb-35S plants. Ovalbumin and actin mRNA levels were determined by Northern blot analysis and quantified as described for Figure 4.8.
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a) Northern analysis of ovalbumin mRNA levels.

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b) Northern analysis of actin mRNA levels.

c) Quantification of ovalbumin mRNA levels.

Figure 4.12 Effect of sulfur deficiency on ovalbumin expression in SSU-oval-PAl496 (RM14) plants

a) Northern analysis of ovalbumin mRNA levels.
b) Northern analysis of actin mRNA levels.
c) Quantification of ovalbumin mRNA levels.
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4.3 DISCUSSION

Studies of PAI gene expression have indicated that the decline in accumulation of this protein during sulfur deficiency was caused by a post-transcriptional reduction in mRNA level, specific for the PAI gene (Higgins et al., 1986). This was confirmed by Morton (1993) who showed that when PAI was expressed from the rubisco small subunit promoter in leaves of transgenic tobacco, the concentration of PAI mRNA was reduced at least 7-fold in response to sulfur deficiency, while the concentration of ovalbumin mRNA expressed from the same promoter declined approximately 1.2-fold. This indicated that, while sulfur deficiency may have slightly inhibited transcriptional activity from the small subunit promoter, a post-transcriptional process was the major determinant of PA1 mRNA concentration in response to sulfur deficiency.

PAI mRNA was found to contain at least two regions capable of conferring a response to sulfur deficiency: the coding region and a 1700 nucleotide 3' flanking region which included the 3' UTR. The sulfur-responsive element in the 3' flanking region remained functional when placed downstream of an ovalbumin cDNA reporter gene and its location was analysed by a series of deletions from the 3' end of the flanking region. The resulting fragments began at nucleotide 11 of the 3' UTR and were 496, 323 and 189 nucleotides in length.

The results from that analysis (Morton, 1993) suggested that only sequences contained within the 189 nucleotide fragment were required to confer sulfur-responsiveness on the reporter gene which, although encoding a sulfur-rich protein, was not regulated by sulfur-status in plants. Computer analysis of possible mRNA secondary structures suggested that the sulfur-responsive element would be located in the distal (3'-most) end of the 189 nucleotide fragment (section 1.9; Morton, 1993).

This prediction was tested in the experiments described here. Two gene constructs were planned. The first (SSU-ovalb-PAI99) contained the first 99 nucleotides of the 189 nucleotide fragment, inserted 3' to the ovalbumin reporter gene while the second construct would contain the remaining 90 nucleotides of the 189 nucleotide fragment. The construct containing the first 99 nucleotides of the PAI 3' UTR was not expected to respond to sulfur deficiency but expression of the second construct would have been expected to be sulfur-responsive. This expectation was based on a computer analysis of secondary structure, which predicted that this 90 nucleotide region of PAI 3' UTR would be able to interact physically with the 5' UTR of ovalbumin but not with that of the
rubisco small subunit. When constructs containing these regions were tested in sulfur deficiency experiments, ovalbumin mRNA levels from the construct containing the rubisco small subunit 5' UTR, but not the construct containing the ovalbumin 5' UTR, responded to sulfur status. The structural interaction between the ovalbumin 5' UTR and PA1 3' UTR may have disrupted a structure in the PA1 3' UTR, involved in the sulfur response (Morton, 1993).

When pRM42 was transferred to tobacco plants there was no significant response in ovalbumin mRNA level to sulfur deficiency (Fig. 4.5). This observation supported the prediction that this region of the PA1 3' UTR did not contain sequence or structural elements involved in the response of PA1 expression to sulfur deficiency.

The second construct which was to contain the putative sulfur-responsive element, was not tested for two reasons. Firstly, the 90 nucleotide region of PA1 3' UTR could not be subcloned into the ovalbumin expression cassette in the desired orientation. Clones containing the fragment in the reverse orientation were readily obtained but no colony containing the fragment in the desired orientation was ever isolated.

Secondly, the results of an experiment performed concurrently with the SSU-ovalb-PA199 sulfur deficiency experiment made this fragment redundant. To confirm that the 189 nucleotide fragment in SSU-ovalb-PA1189 conferred sulfur responsiveness as reported by Morton (1993), eight plants containing the 189 nucleotide fragment (SSU-ovalb-PA1189) were included in the experiment, four of which were grown under sulfur deficiency. Contrary to expectations, there was no significant difference between the response of ovalbumin mRNA level in the SSU-ovalb-PA1189 plants and that observed in SSU-ovalb-35S plants, which did not contain any PA1 sequence (figures 4.6 and 4.4). This result indicated that the 189 nucleotide fragment of PA1 3' UTR did not contain sequences which alone, were capable of conferring sulfur-responsiveness on ovalbumin mRNA expression. Given this result, and the difficulties in subcloning the 90 nucleotide fragment, the construct containing that region was not pursued since in isolation, those 90 nucleotides would be unlikely to confer sulfur-responsiveness. This region may however, contain sequences which are necessary but not sufficient for sulfur-responsive gene expression.

There are two possible reasons for the discrepancy between present and previous results with the 189 nucleotide fragment. The first is variation in gene expression level within the population of transgenic plants. In the present experiments, twice the number of
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Plants were tested for each construct compared with previous experiments. Four SSU-PA1-PA1_{1700} plants were grown and two of these were grown under sulfur-deficient conditions. Eight plants were grown for each of SSU-ovalb-35S, SSU-ovalb-PA1_{199} and SSU-ovalb-PA1_{189} and four of each were deprived of sulfur. In contrast, Morton grew only two SSU-PA1-PA1_{1700} plants, four SSU-ovalb-35S plants and four of each test construct, subjecting half of these to sulfur deficiency. The larger experiment appeared to demonstrate greater variation in ovalbumin mRNA levels between individual plants containing the same construct, even within the sulfur-adequate plants. This variation was unexpected, since all plants containing a given construct were vegetatively propagated from a single parent plant and were genetically identical, however variation in transgene expression level was observed between individual plants derived from a single parent even in tissue culture plants. This variation may have been overcome by using a much larger number of plants in each experiment.

Some of the variation observed in this and subsequent sulfur deficiency experiments may reflect the fact that it was not possible to harvest leaves of exactly the same age from all plants. While plants were initially selected for uniformity of size and development, it was not possible to ensure that all plants grew at identical rates once transferred to the glasshouse. Although the leaves harvested were between 7 cm and 11 cm in length, substantial differences in the maturity of these leaves were observed. There may have been a smaller range of plant growth and development rates in the smaller experiments, allowing for greater uniformity in leaf size and stage of development at harvest. Variation in leaf age at harvest may have contributed to variation in gene expression.

The second possible cause of the differences in results between this present experiment and previous experiments in which SSU-ovalb-PA1_{189} plants have been tested is that the severity of sulfur deficiency induced in this experiment may have been less than in previous experiments. In the present experiment, the level of PA1 mRNA in positive control plants was reduced 4.7-fold in response to sulfur deficiency while in Morton's experiment, the response was 7-fold, indicating that a greater degree of sulfur deficiency was induced in earlier experiments. However, the results presented here are representative of several independent experiments, in which varying degrees of sulfur deficiency were observed. In one experiment, a six-fold reduction in PA1 mRNA level was observed in sulfur-deficient SSU-PA1-PA1_{1700} plants but no difference in ovalbumin mRNA levels between sulfur-adequate and sulfur-deficient SSU-ovalb-PA1_{189} plants was detected. This data was not presented because of extreme variation in ovalbumin expression levels, but it demonstrates that the variation in ovalbumin

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expression, and inability of the 189 fragment of PA1 3' UTR persists in the presence of 
more severe sulfur deficiency. Therefore it is likely that failure to observe a consistent 
response in ovalbumin mRNA expression to sulfur deficiency in SSU-ovalb-PA1_{189} 
plants was due to the lack of an independent sulfur-responsive element in this region 
rather than failure to induce a sufficiently severe sulfur deficiency.

Given the contrast between the present results from SSU-ovalb-PA1_{189} plants and those 
observed previously, the remaining members of the PA1 3' deletion series were re-tested 
in separate experiments to determine the location of the SRE. In SSU-ovalb-PA1_{323} 
plants, which contained the 323 nucleotide fragment of PA1 3' UTR, sulfur deficiency 
induced a 1.5-fold reduction in ovalbumin mRNA level (Fig. 4.9). This response is 
smaller than the 4.6-fold response observed for this construct previously (Morton, 1993) 
but again, the experiment size was doubled and considerable variation in ovalbumin 
mRNA levels was observed, even among sulfur-adequate plants. This result indicated 
that sequence capable of conferring a small response to sulfur deficiency was present in 
the 323 nucleotide fragment of PA1 3' UTR.

Finally plants transformed with a construct containing the 496 nucleotide fragment of 
PA1 3' UTR and flanking region (SSU-ovalb-PA1_{496}) were tested. This 496 nucleotide 
region conferred a 7.2 fold response to sulfur deficiency on ovalbumin mRNA level (Fig. 
4.12). Morton (1993) also observed a response to sulfur with SSU-ovalb-PA1_{496}, 
however that response was considerably smaller than that observed here (2.6-fold vs 7.2- 
fold). The reason for this discrepancy in the magnitude of sulfur-responsiveness of 
SSU-ovalb-PA1_{496} is not apparent, since the degree of sulfur deficiency induced in the 
present experiment (a 6.5-fold reduction in PA1 mRNA level) was comparable to that 
reported by Morton (7-fold; 1993).

The response conferred by the 496 nucleotide fragment of PA1 3' flanking region in 
SSU-ovalb-PA1_{496} exceeded that observed for the SSU-PA1-PA1_{1700}. This contrasts 
with a previous observation that the 1700 nucleotide 3' flanking region alone, did not 
confer as great a response as the PA1 coding region and flanking region together. This 
present observation indicates that the coding region and 3' flanking region SREs may act 
in a redundant rather than additive fashion, since the 496 nucleotide fragment of 3' 
flanking region alone conferred a response of a similar magnitude to that observed when 
both the PA1 coding region and 3' UTR were present together. Taken together, the 
results of the sulfur deficiency experiments with SSU-ovalb-PA1_{496} and SSU-ovalb- 
PA1_{323} plants also indicate firstly, that sequences downstream of the polyadenylation
signal participate in sulfur-responsiveness and secondly, that additional sequence beyond the 'minimal' SRE can enhance the response to sulfur deficiency.

Results of all three sulfur deficiency experiments described in this chapter are summarized in figure 4.13. From these graphs it can be seen that increasing lengths of PA1 3' flanking region inserted downstream of the ovalbumin coding region confer increasing levels of sulfur-responsiveness on the abundance of ovalbumin mRNA. At least 323 nucleotides of PA1 3' UTR and flanking region were required to confer sulfur-responsiveness and the magnitude of the response was substantially enhanced when the next 173 nucleotides of PA1 3' UTR were added. These observations contrast with previous observations which indicated that only 189 nucleotides of PA1 3' UTR were required to confer sulfur-responsiveness, and that the presence of additional sequence downstream from this point did not increase the magnitude of the response markedly.

Not only do these new conclusions indicate that the SRE (or SREs) may be located further downstream of the translation stop site than previously thought, but they also provide some insight into the cellular location of the proposed post-transcriptional event which regulates the level of PA1 mRNA in response to sulfur deficiency.

It is common for gene expression to be regulated by sequence elements in the 3' UTR of the mRNA (chapter 1; Ross, 1995) but in many eukaryotic genes whose expression is regulated in this way, the regulatory element is located 5' to the polyadenylation signal. Hence these regulatory sequences are retained within the 3' UTR of the mature transcript when it is transported to the cytoplasm. Furthermore, these sequences often interact with cytoplasmic proteins. These protein/mRNA interactions appear to mediate cytoplasmic mRNA decay events and can determine the half-life of an mRNA molecule once it reaches the cytoplasm.

Unprocessed PA1 precursor mRNA contains three copies of the AAUAAA motif associated with polyadenylation (Wahle, 1995; Hunt, 1994) located 42, 103 and 162 nucleotides into the 3' UTR. In mammalian mRNAs the AAUAAA sequence is located 10-30 nucleotides upstream of the cleavage and polyadenylation site (Wahle, 1995). Polyadenylation signals in plants are more complex than those in mammalian mRNAs, but often contain AAUAAA or a similar sequence between 10 and 40 nucleotides upstream of the cleavage site (Li and Hunt, 1995). Since the PA1 cDNA ends at nucleotide 183 of the 3' UTR (Higgins et al., 1986), it is likely that this AAUAAA
Figure 4.13  Average responses of ovalbumin mRNA levels to sulfur deficiency. Error bars represent standard deviation.

a) Average levels of ovalbumin mRNA in sulfur-adequate and sulfur-deficient SSU-ovalb-PA199 plants.
b) Average levels of ovalbumin mRNA in sulfur-adequate and sulfur-deficient SSU-ovalb-PA1189 plants.
c) Average levels of ovalbumin mRNA in sulfur-adequate and sulfur-deficient SSU-ovalb-PA1323 plants.
d) Average levels of ovalbumin mRNA in sulfur-adequate and sulfur-deficient SSU-ovalb-PA1496 plants.
sequence beginning at nucleotide 162 of the PA1 3' UTR is the signal for polyadenylation of the PA1 mRNA.

All sequences downstream from the 3' end of the PA1 cDNA can be considered not to be included in the mature mRNA. Since the fragment spanning nucleotides 10-199 did not confer sulfur-responsiveness on expression of ovalbumin mRNA (Fig. 4.6) but the fragment spanning nucleotides 10-333 did, it appears that sequence between nucleotides 199 and 333 contained a sulfur-responsive element. This sequence is removed prior to transport of the mRNA to the cytoplasm and is not contained within the mature PA1 message, therefore the 3' SRE must act within the nucleus to reduce PA1 mRNA abundance.

The observation that sequence even further downstream of the polyadenylation signal (nucleotides 333-506) enhanced sulfur-responsiveness supported the conclusion that reduction in PA1 mRNA concentration in response to sulfur deficiency is the result of a nuclear post-transcriptional process. This conclusion was also supported by unpublished observations (T.J. Higgins) that the abundance of PA1 mRNA is reduced in the nuclear fraction of RNA from sulfur-deficient peas. It would be necessary to isolate nuclear RNA from sulfur-adequate and sulfur-deficient plants carrying the PA1 3' flanking sequences, to determine whether similar responses to those observed in total RNA could be observed in reporter gene mRNA levels in the nuclear RNA fraction. It will also be important to establish precisely, how far downstream of the polyadenylation site the PA1 primary transcript ends, to determine the functional significance of this putative downstream enhancer element.

The observation that the 496 nucleotide fragment conferred a substantially greater response to sulfur deficiency on ovalbumin mRNA expression than did the 323 nucleotide fragment (7.2-fold vs 1.5-fold, figure 3.13) contrasted with a previous observation that the two fragments conferred responses of similar magnitudes (2.6-fold vs 4.6-fold; Morton, 1993), and indicated that downstream sequences may participate in PA1 sulfur-responsiveness. At this stage it is unknown whether the region spanning nucleotides 333 to 506 contained a second, independent SRE, or whether it acted as an enhancer for the SRE contained within the 323 nucleotide fragment.

These two possibilities could be resolved by making a gene construct in which the 173 nucleotides of PA1 3' flanking region from the end of the 323 nucleotide fragment to the end of the 496 nucleotide fragment were subcloned into the ovalbumin expression
Chapter 4. Location of the 3' Sulfur Responsive Element

cassette. If this 173 nucleotide region conferred sulfur-responsiveness, it would indicate the presence of an independently functioning SRE in that part of the transcript. If no response was observed, that would indicate that the previous 323 nucleotides, or some part thereof, were required for an enhancer element in the 333-506 nucleotide region to function.

A similar approach was taken to determine whether the region spanning nucleotides 199-333 could confer a sulfur response independent of the preceding 199 nucleotides. This construct was not pursued due to cloning difficulties similar to those described earlier. Nevertheless, if these two experiments could be performed it would be possible to define more precisely, the location, number and maximum size of sequence elements involved in the response of PA1 expression to sulfur nutrition. Morton (1993) used this approach to eliminate the possibility that sequence between nucleotides 500 and 1700 of the PA1 3' flanking region may have contained an additional SRE. This region failed to confer sulfur-responsiveness, indicating that it did not contain any independently functioning sulfur-responsive elements.

The results obtained for this current series of experiments do not support the mechanism by which PA1 expression has been proposed to respond to sulfur nutrition (Morton, 1993). The earlier proposal was based on a generic model for mRNA decay in which instability determinants located in the 3' UTR of an mRNA act as substrates for endonucleolytic cleavage internal to the protective structures of the 5' cap and poly(A) tail. Rapid decay would then proceed via the activities of abundant, non-specific exonucleases. Although endonucleolytic cleavage is the first step in mRNA degradation in some cases (Binder et al., 1989; Brown et al., 1993), many mRNA decay pathways described to date are more complex, involving shortening of the poly(A) tail before digestion of the body of the mRNA begins (Wilson and Treisman, 1988; Peppel and Baglioni, 1991; Stoeckle and Guan, 1993). For example, degradation of the body of c-myc mRNA does not begin until the poly(A) tail has been reduced to about 40 adenylate residues. Subsequent, rapid decay is initiated by an endonucleolytic cleavage in the 3' UTR (Brewer and Ross, 1988).

An example of decay initiated by endonucleolytic cleavage internal to protective structures is the decay of the mRNA encoded by the chloroplast gene, psbA. Differential mRNA decay is the primary means by which chloroplast gene expression is regulated in response to chloroplast development (chapter 1). In vitro mRNA decay experiments using lysed chloroplasts have been used to elucidate the cleavage sites and subsequent decay.
mechanisms for psbA mRNA (Klaff, 1995). Decay of psbA mRNA was found to be initiated by endonucleolytic cleavages in the 5' part of the coding region. These cleavages generated large intermediate products which were subsequently cleaved into smaller fragments. These small fragments were then rapidly degraded by exonucleases. Although chloroplast-encoded mRNAs lack poly(A) tails, they terminate in a 3' stem-loop structure which appears to regulate the rate of mRNA decay by protecting the body of the mRNA from exonucleolytic digestion (Stern and Gruissem, 1987).

In contrast to these cytoplasmic mRNA degradation pathways, the mechanism by which PA1 mRNA is degraded in response to sulfur deficiency appears to occur in the nucleus, prior to the addition of the poly(A) tail. Therefore, cleavage of the precursor mRNA and subsequent non-specific exonucleolytic digestion of the mRNA cannot completely explain the specific reduction in PA1 mRNA level during sulfur deficiency. Unprocessed mRNAs do not possess 3' terminal poly(A) tracts and all messages would be expected to be equally susceptible to exonucleolytic decay in the nucleus, therefore some feature specific to the PA1 precursor mRNA must target it for rapid decay during sulfur deficiency.

It seems likely that rather than being attacked by non-specific exoribonucleases following the loss of 3' terminal protective structures, PA1 precursor mRNA may be targeted for degradation by specific factors within the nucleus. Since reduction in PA1 message level occurs only during sulfur deficiency, it is possible that some physical feature of the PA1 pre-mRNA is specifically altered during sulfur deficiency. For example association of a protein with PA1 pre-mRNA may induce a change in RNA conformation. A structural alteration might allow the transcript to be specifically recognized by nuclear RNA degradation equipment. The existence of proteins binding to transcripts derived from the 3' end of the PA1 gene is investigated in chapter 5.

Regulation of gene expression by differential processing of precursor transcript to mature mRNA in the nucleus has been described in mammalian systems. Most recently, a cycloheximide-sensitive block in the flow of precursor transcript to mature mRNA has been shown to regulate expression of the cytokines interleukin-1α and 1β (IL-1α, IL-1β; Jarrous and Kaempfer, 1994) and interleukin-2 (IL-2; Gerez et al., 1995), in response to T-cell activation during immune responses.

In the case of IL-2, which plays an important role in mediating the strength of immune responses by controlling proliferation of activated T-cells (Efrat and Kaempfer, 1984;
Efrat et al., 1982), a transient elevation in IL-2 mRNA level is induced when lymphocytes are stimulated with a mitogen such as phytohaemagglutinin (PHA). Following PHA stimulation, transcription of IL-2 mRNA is induced and sustained for at least 24 hours, whereas the level of IL-2 mRNA reaches a peak 8 hours after stimulation and declines rapidly thereafter, returning to basal levels within 24 hours (Gerez et al., 1995). The steady state level of IL-2 mRNA at 8 hours post-stimulation does not correspond with the elevated rate of transcription.

In contrast to other examples in which discrepancies between transcription rate and steady state mRNA levels have occurred, the decline in IL-2 mRNA levels is not caused by destabilization of the mRNA in the cytoplasm. Instead, while transcription of IL-2 continues for at least 24 hours after it is induced, a block develops early after induction of IL-2 expression which prevents processing of newly synthesized precursor mRNA into mature mRNA in the nucleus. The decline in cytoplasmic IL-2 mRNA level after 8 hours is caused by the natural decay of mature IL-2 transcript in the absence of a continued influx of newly processed message.

RNase protection analysis showed that IL-2 mRNA precursors were expressed for up to 25 hours, while mature transcripts were undetectable after 10 hours indicating that processing of precursors became inhibited within 10 hours of induction of IL-2 expression. Although these precursors remained detectable until 25 hours after induction, degradation products appeared after 9 hours, indicating that unprocessed precursors were degraded in the nucleus.

The nature of the processing inhibitor is unknown but may be a labile protein, as inhibition of protein synthesis results in superinduction of IL-2 mRNA expression. In the presence of cycloheximide, IL-2 mRNA levels increase approximately 5-fold but no increase in IL-2 mRNA stability was observed, suggesting that superinduction was the result of increased processing of precursor mRNA. Since inhibition of processing only occurred in the presence of ongoing protein synthesis, it is likely that the inhibitor was a labile protein factor (Gerez et al., 1995).

A similar mechanism has been outlined for regulation of IL-1α and IL-1β expression in response to activation by retinoic acid. Ongoing transcription of IL-1α and IL-1β genes is induced by retinoic acid, yet only a transient wave of IL-1α and IL-1β mRNA is observed. Again, cycloheximide-sensitive inhibition of precursor mRNA processing
develops after induction of these genes and unprocessed precursors decay in the nucleus (Jarrous and Kaempfer, 1994).

Changes in efficiency of nuclear precursor mRNA processing have also been observed during the cell-cycle dependent expression of the DNA biosynthetic enzyme, thymidine kinase (Gudas et al., 1988), while nuclear degradation during precursor mRNA processing has been shown to account for the decrease in class 1 major histocompatibility complex mRNA in cells transformed with oncogenic human adenovirus 12 (Vaessen et al., 1987).

Although a mechanism for gene regulation involving nuclear processing has not been described in plants, a model of this type could explain the regulation of PA1 expression in response to sulfur deficiency, since this response has now been shown to involve sequences which would be expected to be present only in unprocessed PA1 precursor mRNA. It is not known whether PA1 mRNA precursors accumulate in the nucleus during sulfur deficiency, and if so, whether there is any difference in the levels to which such precursors accumulate between sulfur-adequate and sulfur-deficient plants. Therefore it is premature to invoke a model involving inhibition of precursor processing to explain the decline in PA1 mRNA levels in sulfur deficient plants. However if PA1 precursor mRNA was directed, via the sequences in the 3' flanking region, to an RNA degradation pathway within the nucleus rather than in the cytoplasm during sulfur deficiency, this could explain the involvement of sequences downstream of the polyadenylation site in reducing PA1 mRNA levels.

To test this hypothesis it would be necessary to measure levels of PA1 mRNA in nuclear RNA. Preliminary experiments of this type have indicated that PA1 mRNA levels are indeed depressed in nuclear RNA of sulfur-deficient plants (T.J. Higgins, unpublished observations) but the status of these transcripts with respect to processing and polyadenylation is unknown. To determine whether the 323 nucleotide or 500 nucleotide fragments of PA1 3' UTR are capable of directing a decline in the level of nuclear RNA for transcripts containing these regions it would be necessary to extract nuclear RNA from SSU-ovalb-PA1323 or SSU-ovalb-PA1496 plants following sulfur deficiency and examine levels of nuclear ovalbumin mRNA.

Nuclear RNA degradation may not be the only mechanism by which PA1 expression is regulated. The presence of a SRE in the coding region (Morton, 1993) indicates that there may be a separate, possibly co-translational, mechanism by which PA1 mRNA is
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degraded. Co-translational mRNA degradation has been described for several mammalian genes including c-myc (Dani et al. 1984). A degradation mechanism involving sequences downstream of the polyadenylation site has also been reported for c-myc (Guilhot et al., 1988), therefore it is possible that expression of some genes can be regulated in a post-transcriptional manner in both nuclear and cytoplasmic compartments.

The results described in this chapter indicate that sequences downstream of the polyadenylation site may target PA1 mRNA for rapid degradation during sulfur deficiency. The location of these sequences indicate that the process may occur in the nucleus rather than the cytoplasm, but precise details of the mechanism are unknown. These results do not however, rule out the possibility that there may be more than one mechanism by which PA1 mRNA is degraded during sulfur deficiency. It is possible that the sulfur-responsiveness conferred by the first 189 nucleotides of the 3' UTR observed by Morton (1993) may have been mediated by a separate cytoplasmic mechanism. If that is the case, it is not known why a similar result was not observed in the current experiments.

Regardless of the mechanism by which PA1 mRNA abundance is regulated by sulfur deficiency, a region located in the 3' flanking region of the PA1 transcript which mediates this response has been identified. Experiments were performed to determine whether there are proteins in pea cotyledons which associate with this region of PA1 RNA to regulate sulfur-responsive expression of PA1. These experiments are described in the next chapter.
CHAPTER 5

PA1 RNA-BINDING PROTEINS
5.1 INTRODUCTION

In pea seeds, levels of mRNAs encoding the sulfur-rich proteins PA1, PA3 and legumin are specifically reduced during sulfur deficiency. Expression of most other seed genes is not affected by changes in sulfur nutrition (Randall et al., 1979; Chandler et al., 1983). Thus the response of mRNA abundance in pea seeds and tobacco leaves to changes in sulfur nutrition is transcript-specific. Although there may be an increase in general ribonuclease activity in leaves of sulfur-deficient tobacco plants (chapter 3), the abundance of PA1 mRNA is specifically down-regulated relative to that of other transgenes (chapter 4) by sulfur deficiency. This specificity for PA1 transcripts in transgenic tobacco, and for some mRNAs encoding sulfur-rich proteins in pea cotyledons implies that there is a certain feature of these transcripts which allows them to be recognized by the enzymes involved in RNA degradation.

If an inherent feature of the transcript alone was the target for rapid degradation, these transcripts would be constitutively unstable. It has not been proven that the PA1 transcript is not inherently unstable (chapter 3), but it seems unlikely given that this mRNA species normally accumulates to high levels in developing pea cotyledons. Since the PA1 transcript probably becomes unstable as a consequence of a decline in sulfur nutrition, it is more likely that some cellular factor, probably a protein, is transiently associated with it during sulfur deficiency, and that this association causes the transcript to be rapidly degraded.

Interactions between proteins and RNA are involved in many processes during gene expression, but of particular interest in this instance, is the prevalence of interactions between proteins and RNA elements in the 3' UTR regulating mRNA stability (McCarthy and Kollmus, 1995). Examples of mRNAs being either targeted for rapid degradation by association with a cellular protein, or protected from degradation by a similar interaction have been reported in both plant and mammalian cells, as discussed in chapter 1.

In the case of mammalian cells, there are two well documented examples of mRNA stability being regulated by RNA-associated proteins. Iron-dependent regulation of genes involved in cellular iron metabolism is mediated by a regulatory mRNA-binding protein (section 1.6.2.2) and stability of mRNAs for early response genes during cell growth and differentiation is regulated by association of several mRNA-binding proteins with specific AU-rich sequences in the 3' UTRs of these transcripts (section 1.6.1.3).
Chapter 5. PAI RNA-Binding Proteins

To date there has been only one report of expression of a plant nuclear-encoded gene being regulated by association of a protein with mRNA. RNA-binding activity of the protein PRP-BP is highly correlated with the destabilization of its target mRNA, PvPRP1 from *Phaseolus vulgaris*, in the presence of fungal elicitor compounds (Zhang and Mehdy, 1994; Zhang *et al.*, 1993). Association of PRP-BP with an element in the 3' UTR of PvPRP1 mRNA is not sufficient to destabilize the transcript but it has been suggested that further interactions between bound PRP-BP and other proteins may facilitate rapid decay of PvPRP1 mRNA (Zhang and Mehdy, 1994).

Associations between mRNA and protein have been predicted to participate in decay of other short-lived plant mRNAs such as phyA, SAURs, ferredoxin and rbcS (chapter 1). RNA-binding proteins have also been shown to protect mature 3' ends of mRNAs for chloroplast-encoded genes from rapid exonucleolytic degradation, thereby controlling the rate at which these mRNAs are degraded in the chloroplast (section 1.7.1).

Interactions between specific RNA structures or sequences, and various proteins have been shown to regulate gene expression via effects on transcript stability, and discrete regions of the PAI transcript have been shown to confer diminished mRNA accumulation in response to sulfur deficiency. The major prediction of a proposed mechanism for sulfur-responsive gene expression was that these sequences in the coding region and 3' UTR of the PAI transcript would be recognized by specific proteins within the cell, only during periods of sulfur deficiency. The complex between PAI RNA and protein formed as a result of this association would target the PAI transcript for rapid degradation, possibly within the nucleus, as regions downstream from the 3' end of the mature mRNA appear to be involved (chapter 4).

Examples of regulation of gene expression by rapid turnover of precursor mRNAs in the nucleus have been reported (Leys *et al.*, 1984; Chen, 1988; Jarrous and Kaempfer, 1994; Gerez *et al.*, 1995) however to date, none of these examples has been directly linked to activity of pre-mRNA-binding proteins. For this reason, experiments described in this chapter, to identify proteins specifically associated with the PAI 3' UTR, were modelled on those examples described above, in which cytoplasmic half-life was modulated by interactions between mRNA elements and mRNA-binding proteins.

This chapter describes the identification, in total and nuclear protein extracts from sulfur-adequate and sulfur-deficient pea cotyledons, of protein complexes binding to fragments of the PAI 3' UTR. The RNA fragments spanned the first 333 nucleotides of the PAI 3'
A fragment spanning nucleotides 0-506 was excluded from this study because it was likely that a large number of non-specifically bound proteins would associate with this length of RNA, possibly obscuring true candidate regulatory RNA-binding proteins. More precise delineation of the elements within the PA1 3' UTR, which control sulfur-regulated gene expression will facilitate further investigation of regulatory proteins associated with PA1 RNA.
flanking region, as the responses to sulfur deficiency conferred by these fragments were known (chapter 4). There was no clear correlation between patterns of RNA-binding activity among the fragments and responses to sulfur conferred by these fragments, however different complexes were formed by extracts from sulfur-adequate and sulfur-deficient peas on any given fragment, and for a given extract, different protein binding patterns were observed with the various PA1 3' RNA fragments used as probes. UV-crosslinking experiments showed that a large number of polypeptides associated with most of the fragments. Some of these proteins bound to PA1 transcripts in a sulfur-dependent manner and may have a function in regulating accumulation of PA1 mRNA in response to sulfur nutrition.

5.2 RESULTS

Radioactive RNA fragments transcribed from sections of the PA1 3' untranslated and flanking regions were used as probes to identify proteins in pea cotyledons which interacted with sequences in the PA1 3' region. The RNA fragments used are described in table 5.1 (see also Fig. 2.4).

Table 5.1 Nucleotides included in in vitro transcribed RNA fragments used as probes for RNA-binding proteins in sulfur-adequate and sulfur-deficient pea cotyledons.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Nucleotides spanned</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM27</td>
<td>PA1 3' 0-333</td>
<td>PA10.333</td>
</tr>
<tr>
<td>RM30</td>
<td>PA1 3' 0-199</td>
<td>PA10.199</td>
</tr>
<tr>
<td>RM39</td>
<td>PA1 3' 0-109</td>
<td>PA10.109</td>
</tr>
<tr>
<td>AE1</td>
<td>PA1 3' 109-199</td>
<td>PA1109.199</td>
</tr>
<tr>
<td>AE5</td>
<td>PA1 3' 199-333</td>
<td>PA1199.333</td>
</tr>
<tr>
<td>negative control</td>
<td>Ovalbumin coding region 0-417 (antisense)</td>
<td>antisense ovalbumin</td>
</tr>
</tbody>
</table>

These fragments were used because, apart from the additional 10 nucleotides at the 5' end of each transcript containing PA1 sequence, these regions were identical to those tested for ability to confer a response to sulfur deficiency in transgenic plants (chapter 4). It was anticipated that the smallest fragment conferring a response to sulfur (PA10.333) might bind a protein which was not associated with other transcripts which do not confer any response to sulfur in vivo.
Pea cotyledons grown under sulfur-adequate and sulfur-deficient conditions (Chandler et al., 1983) were used as a source of proteins with potential to bind to PA1. Extracts of total protein and nuclear protein were prepared from sulfur-adequate and sulfur-deficient pea cotyledons (sections 2.3.3 - 2.3.4), and interactions between proteins in these extracts and PA1 RNA fragments were detected by gel mobility shift assay (section 2.3.5). The gel mobility shift assay detected the decrease in electrophoretic mobility of a radioactive RNA transcript which occurs when a protein or other factor is bound to the transcript. This decrease in mobility of the RNA probe is due to the additional molecular weight contributed by the bound factor.

5.2.1 Pea cotyledons contain PA1 RNA-binding proteins

Since PA1 mRNA is normally produced in pea seeds between 17 and 25 days after fertilization [DAF (Chandler et al., 1983)], sulfur-adequate and sulfur-deficient peas harvested between 17 and 20 DAF were used as a source of protein. Regulation of mRNA stability via mRNA-binding proteins normally occurs in the cytoplasm (McCarthy and Kollmus, 1995), but results of earlier experiments with nuclear PA1 pre-mRNA indicated that regulation of PA1 mRNA abundance might occur in the nucleus (T.J. Higgins, personal communication), as did the results of experiments described in chapter 4. Because the sub-cellular location of the PA1 regulatory mechanism and, hence, the likely location of regulatory proteins was not accurately known, initially an unfractionated, crude extract of pea seed protein was probed for proteins binding to RNA sequences from the PA1 3' region.

5.2.1.1 PA10-333

Mobility shifts were observed when the largest PA1 RNA fragment, PA10-333, was incubated with extracts from sulfur-adequate (+S) and sulfur-deficient (-S) pea seeds. The shift induced by incubation with the +S extract was larger than that caused by incubation with the -S extract (Fig. 5.1(a)). Pre-treatment of extracts with SDS and proteinase K inhibited complex formation (data not shown), confirming that the mobility shifts were caused by association of proteins with the RNA.

Both +S and -S extracts resulted in RNA/protein complexes spanning a large range of apparent sizes, compared with the relatively homogeneous size of the probe alone (compare lane 1 with lanes 2 and 3, Fig. 5.1(a)). Radioactivity was observed at sizes smaller than the probe itself, however this was likely to be the result of degradation of the
probe during preparation and manipulation, or possibly the result of prematurely terminated transcripts. The large complexes between PA10-333 and protein may indicate that several proteins were associated with this probe. The difference in electrophoretic mobility between the complexes formed by proteins from +S and -S extracts may indicate that different combinations of proteins bind the PA10-333 transcript. This difference in mobility of RNA/protein complex may form the basis of a sulfur-status-specific difference in proteins associating with a regulatory region of PA1 RNA, and may be relevant to an RNA-binding protein-mediated mechanism for control of PA1 mRNA abundance.

The pattern of protein binding to PA10-333 probe was consistently observed over many experiments, so PA10-333 was adopted as a standard. Protein interactions with other sections of the PA1 3' region were then classified as like, or unlike the pattern for PA10-333.

5.2.1.2 PA10-199

A fragment spanning nucleotides 0-199 of the PA1 3' UTR (PA10-199) formed complexes similar to those observed with PA10-333 (Fig. 5.1(b)). Again, the complex formed in the presence of extract from sulfur-adequate pea seeds was of lower electrophoretic mobility than that formed in the presence of extract from sulfur-deficient pea seeds and again, each complex spanned a large range of apparent molecular weights. The actual mobilities of both +S and -S complexes with PA10-199 were slightly greater than those observed for PA10-333 however this may reflect the diminished contribution to complex mobility from the RNA component, as PA10-199 is 134 nucleotides smaller than PA10-333.

5.2.1.3 PA10-109

The fragment spanning nucleotides 0-109 of PA1 3' UTR (PA10-109) also formed complexes similar to PA10-333 when incubated with both +S and -S pea extracts (Fig. 5.4(c)), however the difference in apparent size between the +S and -S complexes was less marked than in the two previous examples. This may indicate that a component(s) of the +S complex has been lost from the interaction with this small RNA fragment, or that an extra component(s) has been added to the -S complex, reducing the difference in electrophoretic mobility between the two complexes.
**Figure 5.1** Continued on next page.
Figure 5.1 Gel mobility shift assays detecting interactions between proteins from pea cotyledons grown under sulfur-adequate (+S) and sulfur-deficient (-S) conditions, and in vitro transcribed RNA fragments of PAI 3' untranslated and flanking regions. (a) Interactions between pea seed extracts and PAI\textsubscript{109-333}, (b) Interactions between pea seed extracts and nucleotides PAI\textsubscript{10-199}, (c) Interactions between pea seed extracts and PAI\textsubscript{199-333}, (d) Interactions between pea seed extracts and PAI\textsubscript{0-109}, (e) Interactions between pea seed extracts and PAI\textsubscript{109-199}. 
5.2.1.4 PAI199-333

A fragment containing the 134 nucleotides included in PAI0-333 but not in PAI0-199 (i.e. nucleotides 199-333 of the PAI 3' flanking region) also formed complexes with proteins in +S and -S extracts similar to those observed with PAI0-333, although the shifted bands appeared more defined than those occurring with PAI0-333 (Fig. 5.1(d)). In addition, a greater proportion of PAI199-333 RNA degradation products were observed in the presence of both extracts with this probe. In the case of PAI0-333, unbound RNA degradation products were virtually absent when protein extracts were present. Therefore it is possible that the heterogeneity of electrophoretic mobility observed for PAI0-333/protein complexes may be partly due to the contribution of partially degraded, but still bound, RNA probe. In the case of PAI199-333, it appears that these degradation products were not bound, reducing the range of mobilities exhibited by the complexes.

5.2.1.5 PAI109-199

In all cases described above, there was no radioactivity migrating at the position of the undegraded, free probe when RNA was incubated with either extract. The complexes formed between proteins in +S and -S extracts, and PAI0-199, PAI0-109 and PAI199-333 probes were considered essentially similar to those complexes formed with PAI0-333. In contrast, a fragment containing nucleotides included in PAI0-199 but not PAI0-109 (i.e., nucleotides 109-199 of the PAI 3' UTR) exhibited a different pattern of protein binding when incubated with both +S and -S extracts (Fig. 5.1(e)).

Incubation of PAI109-199 with protein extract from sulfur-adequate peas resulted in a small proportion of the labelled RNA migrating at a position approximately equal to that of the complex formed by PAI0-333 with proteins in the -S extract. This band, although faint, was more defined than those observed with other fragments, hence fewer protein components may be involved. The majority of the PAI109-199 RNA migrated at the same position as the free probe, when incubated with extract from +S peas. When PAI109-199 RNA was incubated with the -S extract, no mobility shift was observed, indicating that high-affinity binding sites for PAI RNA-binding proteins in sulfur-deficient peas were not located between nucleotides 109 and 199 of the PAI 3' UTR.

These experiments showed that at 17-20 DAF, pea cotyledons contained proteins capable of binding to sections of the PAI 3' region. There appeared to be many such proteins...
and with the exception of PA1\textsubscript{109-199}, there were no probe-specific patterns of protein binding.

5.2.2 Nuclear extracts of pea cotyledons contain PA1 RNA-binding proteins

Total protein extracts from both sulfur-adequate and sulfur-deficient pea cotyledons formed large complexes, possibly containing several proteins. Since results of the experiments described in chapter 4 indicated that levels of PA1 mRNA might be regulated by processes occurring within the nucleus, nuclear extracts from sulfur-adequate and sulfur-deficient pea cotyledons were probed to determine whether any of the PA1 RNA-binding proteins were present in the nucleus. It was considered possible that removal of the cytoplasmic fraction from the pea seed extracts might reduce the amount of PA1 RNA-associated protein, allowing better resolution of the relevant RNA/protein interactions. The same fragments of PA1 3' flanking region were used to probe nuclear extracts. All probes formed one or more complexes with proteins in nuclear extracts of +S peas, and in most cases the resulting bands were much more clearly defined than those observed in the presence of total pea seed protein extracts. Nuclear extracts from sulfur-deficient peas however, formed complexes with PA1 transcripts similar to those formed by total protein extracts.

5.2.2.1 PA1\textsubscript{0-333}

When PA1\textsubscript{0-333} was incubated with 10 µg of nuclear protein from sulfur-adequate peas, two distinct complexes were observed (Fig. 5.2(a) and 5.2(b) - lane 2). A sharply defined, low mobility complex (complex A) was formed. Almost immediately below this was a broader band (complex B) whose mobility extended to the position at which unbound PA1\textsubscript{0-333} transcript migrated. Several other bands of higher mobilities were observed, however they were also present in the lane containing transcript only, and probably represented RNA degradation products.

Complex B resembled the complex formed by proteins in the total protein extract from +S peas, although relative to the free probe, complex B appeared to be of higher electrophoretic mobility than the complex formed between PA1\textsubscript{0-333} and total +S extract. It is possible therefore, that complex B still contains a number of individual protein components. Complex A however, was very distinct and could contain as few as one protein component.
In contrast to the resolution of interactions between +S nuclear proteins and PA1\textsubscript{0-333} into two complexes, incubation of PA1\textsubscript{0-333} transcript with nuclear extracts from sulfur-deficient peas produced only one shifted band (complex C; Fig. 5.2(a) and (b), lane 3). In terms of both its average size and range of electrophoretic mobility, this complex resembled the one observed for this probe when incubated with total protein extract. Complex C occupied a region of the gel beginning at a position intermediate between the top of complex B and the bottom of complex A, but it did not extend as far as the position of the unbound probe. Little free probe was observed in this lane (lane 3, Fig 5.2(a) and (b)) but unbound degradation products were present.

5.2.2.2 PA1\textsubscript{0-199}

The use of an extract of nuclear protein rather than total protein extract in the gel mobility shift assay facilitated resolution of the large complex formed between proteins in sulfur-adequate pea seeds and nucleotides 0-199 of PA1 3' UTR, into three separate complexes (Fig. 5.2(a) lanes 4-6). The predominant shifted band was highly defined and designated complex D. The distinct nature of this band indicates that it probably contains fewer protein components than complex B. Two other bands were also observed, although these bands were less intense than complex D. One was complex A'. This complex was the same size as complex A, observed with PA1\textsubscript{0-333} but it does not necessarily contain the same protein components, as the difference in size between PA1\textsubscript{0-333} and PA1\textsubscript{0-199} may contribute to complex mobility. Hereafter, dashes ('') will be used to denote complexes which appear to be similar but which occur in profiles of different probes. The other complex observed for PA1\textsubscript{0-199}, complex E, was a well defined band which migrated to a position intermediate between complexes A and D (Fig. 5.2(a), lane 5).

Only one complex (complex F) was formed as a result of the interaction between nuclear proteins from sulfur-deficient pea seeds and PA1\textsubscript{0-199} (Fig. 5.2(a), lane 6). The band representing complex F was more highly defined than either complex C, or the complex formed between the PA1\textsubscript{0-199} transcript and the -S total protein extract. Complex F migrated faster than complex C, although the contribution of PA1\textsubscript{0-199} to the size of complex F would have been less than that of PA1\textsubscript{0-333} to complex C. This difference in probe size makes it difficult to predict whether there were protein components common to complexes C and F. Complexes D and F however, may share common proteins, as the sizes of these complexes overlap and in this case, the same probe was used.
**Chapter 5. PA1 RNA-Binding Proteins**

### Figure 5.2

Gel mobility shift assays showing RNA/protein complexes from nuclei of sulfur adequate (+S) and sulfur-deficient (-S) pea cotyledons associated with fragments of PA1 3' flanking region. (a) PA1_{0-333}, PA1_{0-199}, PA1_{0-109}. (b) PA1_{0-333}, PA1_{199-333} and PA1_{109-199}. Radioactive *in vitro* transcribed RNA fragments were incubated with 10 µg of nuclear protein from +S and -S pea cotyledons, and the resulting complexes were separated by electrophoresis on 4% non-denaturing polyacrylamide.

<table>
<thead>
<tr>
<th>a) Probe</th>
<th>PA1_{0-333}</th>
<th>PA1_{0-199}</th>
<th>PA1_{0-109}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>0 +S -S</td>
<td>0 +S -S</td>
<td>0 +S -S</td>
</tr>
</tbody>
</table>

<table>
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<th>b) Probe</th>
<th>PA1_{0-333}</th>
<th>PA1_{199-333}</th>
<th>PA1_{109-199}</th>
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<tr>
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<td>0 +S -S</td>
<td>0 +S -S</td>
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108
Small amounts of free PA10-199 probe were observed in the presence of both +S and -S nuclear extracts, as were degradation products migrating faster than unbound transcript.

5.2.2.3  **PA10-109**

The PA10-109 probe exhibited a pattern of protein binding very similar to that of PA10-199 when incubated with +S nuclear extracts (Fig. 5.2(a), lanes 7-9). Complex D* was the most predominant shifted band but complexes A" and E" were slightly more abundant than they were in the case of PA10-199. In addition, a large proportion of the PA10-109 transcript was unbound. Nuclear extracts of sulfur-deficient pea seeds interacted with PA10-109 transcript to produce a complex (F') resembling complex F, described for PA10-199 however this band was slightly less defined (compare lanes 6 and 9, Fig. 5.2(a)). Complexes F and F' may share common protein components but again, the difference in size between PA10-199 and PA10-109 means that this is difficult to determine.

5.2.2.4  **PA199-333**

The interactions between the PA199-333 transcript and nuclear extract from sulfur-adequate cotyledons closely resembled those formed with PA10-109. Complex D" was again, the most predominant shifted band but in the case of PA199-333, this band as well as those representing complexes A"" and E"", was a highly defined single band (Fig. 5.2(b), lanes 4-6), indicating that a small number of protein components from +S pea seeds interacted with nucleotides 199-333 of the PA1 3' flanking region. The majority of the probe was unbound (Fig. 5.2(b), lane 5).

Most of the PA199-333 probe was also unbound after incubation with nuclear extract from sulfur-deficient cotyledons but a small amount migrated as a poorly defined band (complex C') of electrophoretic mobility similar to complex C, observed in figure 5.2(b), lane 3. This indicates that some of the proteins from -S nuclear extracts which bound nucleotides 0-333 may also bind nucleotides 199-333 of the PA1 3' flanking region.

5.2.2.5  **PA109-199**

When PA109-199 was incubated with nuclear extracts from sulfur-adequate pea seeds, the bulk of this transcript was unbound, however small quantities of complexes A''' and D''' were clearly observed (Fig. 5.2(b), lane 8). There was no band corresponding to
complex E, indicating that the binding site for protein component(s) of complex E was not located in this region of the PAI 3' UTR. PAI\textsubscript{109-199} transcript was also largely unbound after incubation with the -S nuclear extract but a very faint band (complex D\textsuperscript{*}) corresponding to complex D\textsuperscript{**} was observed (Fig. 5.2(b), lane 9). Although radioactivity was observed at a position similar to that of complex D for other transcripts, in those cases it formed part of a larger complex (compare Fig 5.2(b), lane 9 with lanes 3 and 6, and Fig. 5.2(a), lanes 3,6 and 9). In the case of PAI\textsubscript{109-199}, the band at this position was a clearly defined, single band. This may indicate that PAI\textsubscript{109-199} transcript binds proteins from -S nuclear extract with low affinity relative to the other transcripts. This is in agreement with the results from mobility shift assays with total protein extracts from sulfur-deficient pea cotyledons, although no protein/RNA interactions were detected at all in those experiments.

In contrast to gel mobility shift assays with total pea seed extracts, in which PAI\textsubscript{0-199}, PAI\textsubscript{0-109} and PAI\textsubscript{199-333} exhibited binding patterns similar to those of PAI\textsubscript{0-333}, the complexes formed when PAI\textsubscript{0-199}, PAI\textsubscript{0-109} and PAI\textsubscript{199-333} were incubated with nuclear extracts from pea seeds appeared to be more defined and, in the case of extracts from sulfur-adequate peas, more numerous than those formed by PAI\textsubscript{0-333}. The binding patterns for PAI\textsubscript{109-199} generated by both total and nuclear extracts indicated that few proteins bound to this region of the PAI 3' UTR.

These experiments with nuclear protein extracts show that: a) there are proteins which associate with RNA sequences from the PAI 3' region in nuclei of sulfur-adequate pea cotyledons which are not present in the nuclei of sulfur-deficient pea cotyledons (for example, complex A); b) there are proteins in nuclear extract from +S peas which bind to the longest PAI transcript (PAI\textsubscript{0-333}), but not to other transcripts (ie. the components of complex B which are intermediate between complexes D and E); c) protein in complex E does not interact with nucleotides 109-199 of the PAI 3' UTR; d) there may be proteins in the -S nuclear extract which bind PAI\textsubscript{0-333} but not other transcripts (for example, the slower migrating components of complex C); and e) there do not appear to be any strong binding sites for proteins in -S nuclear extracts between nucleotides 109 and 199 of the PAI 3' UTR.

Comparing results obtained using nuclear and total protein extracts from pea seeds shows that the complexes formed by nuclear extracts were more highly defined than those formed by total protein extracts and may have contained subsets of the proteins contained in complexes formed by total protein extracts. The use of nuclear extracts reduced the
amount of non-specific binding to PA1 transcripts. There were differences in the patterns of RNA/protein interactions both between transcripts and between sulfur-adequate and sulfur-deficient nuclear extracts. These differences may be indicative of a nuclear mechanism, mediated by RNA-binding proteins, which could regulate abundance of PA1 mRNA in a sulfur-dependent manner.

5.2.3 Some RNA/protein complexes are RNase T1-resistant

The ability of the gel mobility shift assay to resolve the protein/RNA complexes formed between PA1 3' fragments and pea seed proteins was considerably improved when nuclear extracts were used rather than total protein extracts, indicating that a subset of the PA1 RNA-binding proteins in pea seeds were located in the nucleus. Nevertheless, some poorly defined RNA/protein complexes were still observed. In order to further improve resolution of these complexes, the gel mobility shift procedure was modified to include a ribonuclease protection step. Assays of this type have previously been used to resolve RNA-binding proteins associated with the c-myc coding region stability determinant (Prokipcak et al., 1993) and with chloroplast RNA 3' ends (Danon and Mayfield, 1991).

After incubation of radioactive transcripts with nuclear protein extracts, but before binding reactions were loaded on polyacrylamide gels, unbound RNA was digested with RNase T1, which cleaves single stranded RNA at guanosine residues. RNase treatment may dissociate weakly bound proteins from RNA transcripts. Depending on the size of the bound protein, RNase T1 may also degrade any RNA not protected by protein, reducing the contribution of the probe to the electrophoretic mobility of the complex. It was anticipated that digestion of unbound or weakly bound probe might facilitate both improved resolution of the large complexes observed in the presence of nuclear extracts (for example, complexes B and C) and comparison of similar complexes between different probes.

5.2.3.1 PA10.333

RNase treatment did not markedly affect the protein-binding pattern of the PA10.333 probe. Complexes A and B were still formed by proteins in +S nuclear extracts, although complex B appeared to be slightly more clearly defined under these conditions. This was probably due to the removal of the band containing unbound probe, which migrated to a position just below complex B when the reaction mixture was not treated with RNase T1. Formation of complex C, between PA10.333 and nuclear protein from sulfur-deficient pea
seeds was not markedly affected by treatment of the binding reaction with RNase T1 (Fig. 5.3(a), lane 3). Like complex B, removal of unbound PA1₀⁻₃₃₃ probe from the lowest extremity of complex C aided resolution of this complex and confirmed that the average mobility of complex C was slightly less than that of complex B.

5.2.3.2 PA1₀⁻₁₉₉

When PA1₀⁻₁₉₉ probe was incubated with nuclear extract from sulfur-adequate peas then treated with RNase T1, only complex D was observed (Fig. 5.3(a), lane 5). Complex D was the fastest migrating of the three complexes observed in this reaction when unbound probe was not removed, therefore complexes A' and E' (observed in the absence of RNase T1; Fig. 5.2(a)) may have represented multiple copies of complex D, binding along the length of the 199 nucleotide fragment. RNase T1 treatment may have separated this series of complexes. Alternatively, the interactions of proteins in complexes A' and E' with the PA1₀⁻₁₉₉ transcript may have been of low affinity and may have been disrupted by the action of RNase T1. A third possible reason that these two complexes were not observed in RNase protection assays is that the RNA fragments protected by the protein components of complexes A' and E' did not contain any radioactively labelled uridine residues, and hence were not detectable by fluorography. If complexes A and A' bind the same site, this explanation seems unlikely for complex A', as complex A was readily detected in association with PA1₀⁻₃₃₃ in the RNase protection assay.

Resolution of the complex formed between nuclear proteins from sulfur-deficient pea seeds and PA1₀⁻₁₉₉ was improved after digestion of unbound probe. In the absence of RNase T1 digestion a band more distinct than that representing complex C, but less distinct than that representing complex D was observed (complex F). When the RNase protection step was included, a band similar to the one representing complex D, although of slightly lower mobility, was observed [complex G; (Fig. 5.3(a), lane 6)]. This may indicate that some of the protein components of complex F did not bind to PA1₀⁻₁₉₉ with sufficient affinity to protect the transcript from degradation by RNase T1. After RNase digestion of unbound transcript, it was possible to identify distinct interactions between nuclear proteins from both sulfur-adequate and sulfur-deficient pea cotyledons and the first 199 nucleotides of the PA1 3' UTR.
Figure 5.3 RNase protection assays showing RNA/protein complexes from nuclei of sulfur adequate (+S) and sulfur-deficient (-S) pea cotyledons associated with fragments of PA1 3' flanking region. (a) PA10-333, PA10-199, PA10-109. (b) PA10-333, PA199-333 and PA109-199. Radioactive in vitro transcribed RNA fragments were incubated with 10 µg of nuclear protein from +S and -S pea cotyledons, then unbound transcript was digested with RNase T1 (1u) before complexes were separated by electrophoresis on 4% non-denaturing polyacrylamide.
Similarly, for PA10-109 single bands were observed representing interactions of the transcript with nuclear proteins from both sulfur-adequate and sulfur-deficient pea seeds (complexes G* and G'; Fig 5.3(a), lanes 8 and 9). In this case however, the mobilities of both complexes were similar to that of complex G, and no band corresponding to complex D was detected after incubation of the PA10-109 with nuclear proteins from sulfur-adequate peas (compare lanes 5 and 8). This may indicate either that an additional protein component was added to complex D, or that complex D was RNase-sensitive and was replaced with a less sensitive complex of slightly larger size. Both of the bands observed for PA10-109 were of much lower intensity than those observed for PA10-199 and PA10-333, indicating that protein binding sites within the first 109 nucleotides of the PA1 3' UTR may not be bound with high affinity by nuclear proteins in pea cotyledons.

While three well defined bands were observed following incubation of PA1199-333 with nuclear extract from sulfur-adequate pea seeds, only two bands were observed after treatment with RNase T1 (Fig. 5.3(b), lane 5). Complexes D'' and E'' were present but complex A'', which was present prior to RNase T1 digestion (Fig. 5.2(b)), was absent.

The band resulting from interactions between PA1199-333 and nuclear proteins from sulfur-deficient pea cotyledons (F'') was slightly more clearly defined in the RNase protection assay than in the gel mobility shift assay, but was not as well defined as those bands observed with PA10-199 and PA10-109. The band observed for PA1199-333 resembled complex F, which was observed after incubation of PA10-199 with sulfur-deficient extracts (but before RNase T1 digestion; Fig. 5.2(a)) and it covered a smaller range of mobilities than the RNase protected fragment of PA10-333. This smaller complex may be composed of a subset of the proteins in the RNase protected complex of PA10-333.

The PA1109-199 probe was resistant to degradation by RNase T1, so the mobilities of complexes observed here may be influenced by the presence of undegraded probe. A band of low mobility was observed in the lane containing PA1109-199 probe only (Fig.
This band was also observed in the presence of both extracts but its origin is unknown. It is unlikely to represent an interaction between pea nuclear protein and RNA therefore it was not considered further. Complex D"" was observed when PA1109-199 was incubated with nuclear extract from sulfur-adequate peas (Fig. 5.3(b), lane 8), consistent with the result of the gel mobility shift assay (Fig. 5.2(b), lane 8).

In contrast, the binding pattern observed with nuclear extract from sulfur-deficient peas was not consistent with previous results. A complex resembling complex C (C') was formed when PA1109-199 was incubated with this extract, in contrast with previous gel mobility shift experiments in which little or no binding was observed. This inconsistency may have been due to the fact that PA1109-199 was not substantially degraded by RNase T1. This meant that the intact transcript was effectively incubated with protein extract for the initial 10 minutes as well as the 10 minutes in which RNase was present, therefore the extra incubation time may have allowed for association with the probe of proteins which did not associate in the first 10 minutes.

Although treatment of PA1 RNA binding reactions with RNase T1 did not result in formation of simple complexes in all cases, it did improve the resolution of some complexes by removing unbound transcript which co-migrated with the complex (for example, complexes B and C). This treatment did result in defined, RNase-protected bands when PA0-199 and PA0-109 were incubated with nuclear extracts from both sulfur-adequate and sulfur-deficient pea cotyledons. Sulfur-related differences in electrophoretic mobilities of these bands were observed in the case of PA1199. In the case of PA1199-333, extra complexes or components which were not observed in PA10-199 or PA0-109, but which were present in PA0-333 were observed. Together with data from experiments using transgenic plants (chapter 4) which showed that nucleotides 199 to 333 of the PA 3' flanking region were involved in sulfur regulation, this may indicate that nucleotides 199 to 333 of the PA 3' flanking region contain binding sites for proteins relevant to sulfur-regulated gene expression.
5.2.4 Specificity of protein binding

RNase protection assays indicated that a number of RNA-binding proteins from pea seeds were associated with the first 333 nucleotides of the PA1 3' UTR and it was possible that some of these complexes resulted from interactions between the transcript and non-specific RNA-binding proteins. In order to examine the specificity of protein/RNA interactions, heparin was added to binding reactions. Heparin is a polyanion which can be used to eliminate non-specific RNA/protein interactions (Tanguay and Gallie, 1996).

Heparin was added to RNase protection assays at final concentrations of up to 5 µg/ml. After incubation with RNase T1, heparin was added and reactions were incubated for a further 10 minutes. Figure 5.4(a) shows that complexes A and B were formed when PA1-333 was incubated with nuclear extract from sulfur-adequate peas in the absence of heparin (lane 4). As the concentration of heparin was increased the intensity of complex A declined, while the resolution of complex B increased (lanes 5-8). In the presence of 5 µg/ml heparin, complex B formed a well defined band. This indicates that heparin reduced non-specific binding activity in complex B and therefore, facilitated resolution of two distinct complexes.

Higher concentrations of heparin may enhance resolution of the two complexes or alternatively, the pattern of RNA-binding proteins might be completely changed in the presence of higher concentrations of heparin. Over a range of heparin concentrations (0.07 µg/ml to 1000 µg/ml), Tanguay and Gallie (1996) observed that the profile of complexes formed by a purified wheat RNA-binding protein with the transcript of tobacco mosaic virus changed markedly. At heparin concentrations of greater than 200 µg/ml only one complex was formed. It may be possible to use higher concentrations of heparin to further reduce non-specific binding of proteins from sulfur-adequate pea seeds to PA1-333.

When heparin was added to binding reactions containing PA1-333 and extracts from sulfur-deficient peas however, there did not appear to be any effect of increasing heparin concentration on formation of complex C (Fig. 5.4(b)). It is possible that concentrations of heparin greater than 5 µg/ml may have reduced the amount of non-specific binding in this complex.

Unlabelled specific and non-specific competitor RNA transcripts were also used to gain an indication of the specificity of binding between pea seed nuclear proteins and PA1.
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RNA transcribed in antisense orientation from the ovalbumin coding region was used as a non-specific competitor, while unlabelled PA10-333 was used as a specific competitor. Competitor transcripts were pre-incubated with protein extracts for approximately five minutes before addition of labelled PA10-333. Binding patterns in the presence of up to 200-fold molar excess of these competitors were examined by gel mobility shift assay.

When non-specific competitor was added to binding reactions containing PA10-333 and +S extract in increasing molar excess, activity appeared to be displaced from complex A. In contrast, the intensity of complex B increased slightly (Fig. 5.5(a)). These results suggest that the specificity of complex A for PA10-333 may be less than that of complex B. This result was consistent with the results of experiments in which heparin was used as a non-specific competitor.

When specific competitor was added to the same reactions, the activity in complex B decreased slightly as the excess of unlabelled PA10-333 increased (Fig. 5.4(b)). This ability of PA10-333 to compete more efficiently than antisense ovalbumin may indicate that complex B contained protein components which bound specifically to PA10-333.

In contrast, neither specific nor non-specific competitors had a marked effect on formation of complex C by -S extract with PA10-333 (Fig. 5.5(c)). This may indicate that complex C consisted of proteins with lower specificity than complex B for PA10-333. The lack of specificity of binding however, does not necessarily imply a lack of function, as many nuclear proteins with functions in RNA-processing appear to bind proteins with lower specificity than cytoplasmic RNA-binding proteins (McCarthy and Kollmus, 1995). It may be important however, to repeat this experiment with a smaller fragment of RNA known to be important for sulfur-regulation of PA1 expression, as this may reduce non-specific binding and may facilitate identification of specifically bound proteins from sulfur-deficient pea nuclei.
Figure 5.4 RNase protection assays containing 0, 5, 2.5, 1.25 and 0.625 µg/ml heparin. Heparin was added to RNase protection assays containing PA10-333 and nuclear extract from (a) sulfur-adequate or (b) sulfur-deficient pea seeds after digestion of unbound probe. Reactions were then incubated at room temperature for 10 minutes.
### Chapter 5. PAI RNA-Binding Proteins

#### a) Probe PAlo-333

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- complex A
- complex B

#### b) Probe PAlo-333

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- complex C
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Figure 5.5 Continued next page.
Chapter 5. PA1 RNA-Binding Proteins

5.3.2 Functional characterization of polyadenylation factor PA1

5.3.2.1 Characterization of PA1 binding to RNA

Figure 5.5 Relative intensities of complexes A, B and C in the presence of 0, 50, 100 and 200-fold molar excesses of unlabelled competitor transcripts. (a) Antisense ovalbumin transcript was incubated with nuclear proteins from sulfur-adequate pea seeds before $^{32}$P-labelled PA10.333 probe was added. Complexes formed with the radioactive probe were separated on 4% polyacrylamide and the intensities of complexes A (○) and B (■) were quantified using phosphorimager technology. (b) Unlabelled PA10.333 transcript was added to sulfur-adequate nuclear extract before $^{32}$P-labelled PA10.333 probe was added. Intensities of complexes A (○) and B (■) were measured as described above. (c) Antisense ovalbumin (▲) and unlabelled PA10.333 (△) transcripts were incubated with nuclear extracts from sulfur-deficient pea seeds before addition of $^{32}$P-labelled PA10.333 transcript. Intensities of complex C in the presence of molar excesses of both competitor transcripts were measured as described for complexes A and B, above.
5.2.5 Preliminary characterization of polypeptides binding to PA1 3’ flanking region

Pea nuclei contain protein factors capable of binding to fragments of the PA1 3’ region. In some cases there were apparent differences in the protein/RNA complexes, related to the sulfur status of the pea seeds from which the extracts were made. UV-crosslinking experiments, which detect proteins in close physical proximity to RNA, were performed to determine the number and approximate sizes of the polypeptide components of each of these complexes.

UV-crosslinking analyses have been used to identify RNA-binding proteins associated with the PvPRP1 mRNA in *P. vulgaris* (Zhang and Mehdy, 1994), the *c-myc* coding region instability determinant (Bernstein *et al.*, 1992) and instability determinants in transcripts of ribonucleotide reductase R1 (Chen *et al.*, 1993) and R2 (Amara *et al.*, 1994). UV-crosslinking has also been used to identify multiple RNA-binding proteins associated with tumour necrosis factor receptor mRNA (Winzen *et al.*, 1996) and protein components binding to transcripts containing AREs (Malter, 1989; Bohjanen *et al.*, 1991; 1992). In these examples, protein/RNA complexes detected by gel mobility shift assay or RNase protection assay were shown to be composed of between one and three protein components, even though some of the complexes initially identified in non-denaturing polyacrylamide gels were poorly defined. Therefore it was anticipated that in the case of the PA1, transcripts which have been shown to associate with proteins from pea nuclei, the well defined complexes (for example, those formed by PA10-199 and PA10-109) might contain a single protein while the less distinct complexes might contain several protein components.

*In vitro*-transcribed, 32P-labelled fragments of PA1 3’ UTR and flanking region identical to those used in gel mobility shift and RNase protection assays were used in UV-crosslinking experiments. In addition, an antisense transcript containing the first 417 nucleotides of the ovalbumin coding region was included as a negative control, to identify any non-specific RNA-binding proteins present in the pea seed nuclear extracts. These radioactive transcripts were incubated with 10 µg of nuclear protein from sulfur-adequate or sulfur-deficient pea cotyledons for 10 minutes at room temperature. The reactions were then placed on ice and exposed to ultraviolet radiation for 15 minutes. Unbound RNA in each reaction was digested with 4 µg RNase A at 37°C for 10 minutes, with the exception of reactions containing PA1109-199 probe. PA1109-199 was highly resistant to digestion by both RNase A and RNase T1, therefore 100 µg RNase A were added to
these reactions. After RNase treatment, samples were boiled with 5 µl loading buffer containing SDS then loaded on to 15% polyacrylamide gels containing SDS (section 2.3.7).

The profile of RNA-binding proteins for each probe was quite complex. Nevertheless, it was possible to determine qualitative differences between the profiles generated by nuclear proteins from sulfur-adequate and sulfur-deficient pea seeds (Table 5.2). Figure 5.4(a) shows the profiles of RNA-binding polypeptides in nuclear extracts of sulfur-adequate pea cotyledons after incubation with all probes. Antisense ovalbumin transcript was used as a negative control for PA1-specific RNA-binding activity and when it was incubated with nuclear extract from sulfur-adequate peas, three major polypeptides were labelled due to crosslinking with the radioactive probe. Bands were observed with relative molecular masses (Mr) of 75 k, 50 k and 35 k (Fig. 5.6(a), lane 1). Less prominent bands were also observed at Mr = 18.5 k to 19 k. When nuclear extracts from sulfur-deficient peas were incubated with the negative control, the predominant band was at Mr = 50 k but there were other minor polypeptides in the profile, including one at Mr = 75 k (Fig. 5.6(b), lane 1). Hence pea nuclei contain polypeptides which associate closely with RNA sequences other than those contained in the PA1 3' region.

5.2.5.1 PA10.333

The three prominent bands were also observed after incubation of PA10.333 with extracts from sulfur-adequate peas. In addition, four proteins which did not bind to the negative control transcript were observed. Polypeptides of Mr = 120 k, 80 k and a doublet at Mr = 50 k were present. There was also a doublet at Mr = 35 k. There were indistinct bands at approximately Mr = 15 k and 20 k which were not present in the profile of the negative control (Fig. 5.6(a), lane 2).

A complex profile of polypeptides from nuclei of sulfur-deficient pea cotyledons was crosslinked to the PA10.333 probe (Fig. 5.6(b), lane 2). The Mr = 205 k polypeptide was present and it was more intense than the corresponding band in the profile of the negative control transcript. In addition there was a series of four bands at Mr = 115 k, 110 k, 80 k and 70 k, none of which appeared in the negative control profile. The prominent Mr = 50 k band was present and appeared to be flanked by polypeptides migrating to positions just above and just below it, forming a triplet centred about Mr = 50 k. These two additional polypeptides were not associated with the negative control. Two distinct bands were
observed at \( M_r = 35 \text{ k} \) and \( 32 \text{ k} \) and although they were present in the profile of the negative control, they were more intense in the \( \text{PAI}_{0.333} \) profile.

Radioactivity was also observed between approximately \( M_r = 18 \text{ k} \) and \( 22 \text{ k} \), but was not resolved into distinct bands. Therefore, excluding the proteins common to the antisense ovalbumin transcript, sulfur-deficient pea nuclei contained a different set of polypeptides associated with the first 333 nucleotides of the PAI 3' flanking region than nuclei from sulfur-adequate pea seeds. Predominant amongst these were polypeptides of \( M_r = 115 \text{ k} \), \( 110 \text{ k} \), \( 70 \text{ k} \), the upper band of the \( M_r = 50 \text{ k} \) triplet and a polypeptide of \( M_r 32 \text{ k} \).

5.2.5.2 \( \text{PAI}_{0.199} \)

The bands at \( M_r = 120 \text{ k} \) and \( M_r = 80 \text{ k} \) were more pronounced in the sulfur-adequate RNA-binding profile of \( \text{PAI}_{0.199} \) than in that of \( \text{PAI}_{0.333} \), while the \( M_r = 75 \text{ k} \) band was less prominent. The \( M_r = 50 \text{ k} \) doublet observed in the \( \text{PAI}_{0.333} \) was also less prominent. The \( M_r = 35 \text{ k} \) protein present in the profiles of the negative control and \( \text{PAI}_{0.333} \) was absent from the \( \text{PAI}_{0.199} \) profile but the upper band of the \( M_r = 35 \text{ k} \) doublet observed in the profile of \( \text{PAI}_{0.333} \) was present. Some activity was observed at \( M_r = 20 \text{ k} \) but no clearly resolved bands were present (Fig. 5.6(a), lane 3).

Fewer nuclear polypeptides from sulfur-deficient peas were crosslinked to the \( \text{PAI}_{0.199} \) probe than were crosslinked to \( \text{PAI}_{0.333} \) (Fig. 5.6(b), lane 3). The bands at \( M_r = 205 \text{ k} \) and \( M_r = 110 \text{ k} \) which were observed in the \( \text{PAI}_{0.333} \) profile were absent from that of \( \text{PAI}_{0.199} \). Radioactive bands were observed at \( M_r = 115 \text{ k} \), \( 80 \text{ k} \) and \( 70 \text{ k} \), and a triplet centred around \( M_r = 50 \text{ k} \) was observed. A \( M_r = 35 \text{ k} \) band was also present. The \( M_r = 32 \text{ k} \) band observed in the profile of \( \text{PAI}_{0.333} \) was absent, as were the low molecular weight bands. At least two bands, those at \( M_r = 70 \text{ k} \) and the upper band of the \( M_r = 50 \text{ k} \) triplet, were present in this profile but were not present in the profile generated by this probe when it was crosslinked to nuclear proteins from sulfur-adequate pea seeds.

5.2.5.3 \( \text{PAI}_{0.109} \)

The RNA-binding profile generated by sulfur-deficient extract for the \( \text{PAI}_{0.109} \) probe was similar to that of \( \text{PAI}_{0.199} \) except that no polypeptides less than \( M_r = 35 \text{ k} \) were observed (Fig. 5.6(a), lane 4). This indicates that the higher molecular weight RNA-binding polypeptides bound at sites within the first 109 nucleotides of the PAI 3' UTR,
although they also appear to bind at sites further downstream in the 3' region of PA1 RNA.

The RNA-binding polypeptide profile generated when the PA10.109 probe was crosslinked to nuclear protein from sulfur-deficient pea seeds was similar to that of PA10.199 (Fig. 5.6(b), lane 4). This track was more heavily loaded than PA10.199, resulting in the appearance of lower molecular weight bands between Mr = 18 k and 22 k. The Mr = 32 k and 110 k polypeptides which bound to the PA10.333 probe did not bind to either PA10.199 or PA10.109, indicating that the binding sites for these polypeptides were not located in the first 198 nucleotides of the PA1 3' UTR.

5.2.5.4 PA1199-333

It appears that binding sites for the Mr = 120 k and 80 k polypeptides in extracts from sulfur-adequate peas may be located between nucleotides zero and 109 of the PA1 3' UTR, as these bands were not observed in the RNA-binding protein profiles of either PA1199-333 or PA1109-199. The PA1199-333 probe interacted only with the Mr = 75 k, 50 k and 35 k polypeptides which were present in the profiles of all other transcripts, although the Mr = 35 k band observed in association with PA1199-333 was broader than that associated with the ovalbumin antisense transcript (Fig. 5.6(a), lane 5). This may indicate that both bands of the Mr = 35 k doublet observed in association with the PA10.333 probe were also bound to the PA1199-333 probe.

The profile of polypeptides from sulfur-deficient extracts crosslinked to the PA1199-333 probe resembled that of PA10.333 more closely than those of PA10.199 and PA10.109 (Fig. 5.6(b), lane 5). The Mr = 205 k polypeptide was present, as were the Mr = 110 k and Mr = 80 k polypeptides but the Mr = 115 k polypeptide observed in the profiles of PA10.199 and PA10.109 was absent. However, the Mr = 205 k polypeptide was also associated with the negative control and should be discounted. In contrast, the Mr = 110 k polypeptide was not associated with either the negative control or PA10.199 but appeared to bind to a site downstream of nucleotide 199. This band was not present in the profile of PA1199-333 generated by sulfur-adequate nuclear extract. The PA1199-333 sulfur-deficient profile also contained the Mr = 70 k polypeptide observed in the PA10.333 profile, as well as the triplet at Mr = 50 k. Bands of Mr = 35 k and 32 k were observed, as were lower molecular weight bands of between Mr = 18 k and 22 k. None of these bands appeared in the sulfur-adequate profile of PA1199-333.
The profile of polypeptides in sulfur-adequate extracts which bound to PAI$_{109-199}$ was similar to that of PAI$_{199-333}$ except that a prominent band was present at approximately $M_r = 30$ k (Fig. 5.6(a), lane 6). This band was unexpected since gel mobility shift and RNase protection assays showed that this probe was not strongly bound by nuclear proteins from pea seeds. The proteins which did bind to this region were within the size range of those binding to other probes, therefore the appearance of a unique band smaller than the predominant bands associated with other probes was unexpected. This band may have been caused by an undegraded section of probe, since the PAI$_{109-199}$ transcript was highly resistant to digestion by RNase A. The intense activity (arrowed, Fig. 5.6(a)) present at the lower end of the gel in this lane may also have been caused by incomplete digestion of unbound probe.

The PAI$_{109-199}$ probe appeared to be crosslinked to the $M_r = 205$ k and 70 k polypeptides from sulfur-deficient extracts, but binding in the range of $M_r = 80$ k to 115 k was indistinct (Fig. 5.6(b), lane 6). It was difficult to determine whether the $M_r = 110$ k polypeptide which appeared to bind exclusively to a site downstream from nucleotide 199 of the PAI 3' flanking region was present in this profile. Even if this polypeptide did bind to a site between nucleotides 109 and 199 of the PAI 3' UTR, the $M_r = 110$ k band was more intense in the profile of PAI$_{199-333}$ than in that of PAI$_{109-199}$. This may indicate that the $M_r = 110$ k polypeptide bound with greater affinity to sites between nucleotides 199 and 333 than to sites between nucleotides 109 and 199.

In contrast to the profiles of all other PAI 3' transcripts when crosslinked to RNA-binding proteins from sulfur-deficient pea cotyledons, the profile of PAI$_{109-199}$ lacked the top band of the triplet at $M_r = 50$ k and contained only a doublet at this position. The unique band of approximately $M_r = 30$ k which was observed when PAI$_{109-199}$ was crosslinked to proteins from sulfur-adequate pea cotyledons, was also observed in the profile of proteins from sulfur-deficient peas binding to this probe. Lower molecular weight bands of between $M_r = 18$ k and 22 k were also observed.

The patterns of RNA-binding activity in these UV-crosslinking experiments were complex and it was not possible to correlate individual polypeptides with complexes observed in native RNase protection assays. However, it was shown firstly, that there were polypeptides in nuclear extracts from pea cotyledons which show a degree of specificity for transcripts derived from the PAI 3' untranslated and flanking regions (ie
Figure 5.6 Polypeptides labelled by UV-crosslinking with radioactive transcripts derived from the 3' end of PA1. The proteins were from nuclei of (a) sulfur-adequate and (b) sulfur-deficient pea cotyledons. Nuclear extracts were incubated with an antisense ovalbumin transcript (lane 1) and PA10-333 (lane 2), PA10-199 (lane 3), PA10-109 (lane 4), PA1199-333 (lane 5) and PA1109-199 (lane 6). Bound proteins were first crosslinked to the radioactive transcripts and unbound RNA was digested with RNase A. The resulting protein/RNA complexes were resolved by SDS-PAGE and detected by autoradiography using a phosphorimager. Sizes of protein markers are given in approximate \( M_r \) (x 10^3).
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Table 5.2 Summary of the peptides from nuclear extracts of sulfur-adequate (+S) and sulfur-deficient (-S) pea seeds binding to each of the probes used in UV-crosslinking experiments. Ticks represent the presence of a peptide of a given size in the profile of each probe. Question marks represent bands which are not distinct in the profiles.

Letters refer to individual bands of doublets and triplets, for example 50a, 50b and 50c refer to the upper, middle and lower bands respectively of the triplet centred around Mr = 50 k.

<table>
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<tr>
<th>Polypeptide Size Mr (k)</th>
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<th>PAI₀-233</th>
<th>PAI₀-109</th>
<th>PAI₁₀-109</th>
<th>PAI₁₀₀-233</th>
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Chapter 5. **PA1 RNA-Binding Proteins**

those polypeptides which did not bind to the negative control). Secondly, some polypeptides bound preferentially to a subset of the PA1 probes, indicating that those polypeptides recognized defined sites within the PA1 3' UTR and flanking region. This finding was in agreement with results from native RNase protection assays, which indicated that there were differences between probes in the RNA/protein formed by both +S and -S extracts. Both UV-crosslinking experiments and RNase protection assays indicated that for +S and -S extracts, the largest number of polypeptide components bound to PA10.333.

Finally PA1 RNA-binding polypeptides were provisionally identified in sulfur-deficient pea cotyledons which were not present in nuclei of peas grown under sulfur-adequate conditions. Interactions between polypeptides of Mr = 110 k, 70 k, 50 k (the upper band of the Mr.= 50 k triplet) and 32 k and PA10.333 indicate that sulfur deficiency induces changes in the population of nuclear polypeptides binding to the 3' region of PA1 RNA which has been shown to control sulfur-responsive PA1 expression (Table 5.2). These polypeptides, which only associate with PA1 RNA during sulfur stress, may play a role in regulating PA1 expression.

### 5.3 DISCUSSION

Since post-transcriptional regulation of mRNA abundance often involves interactions between RNA-binding proteins and RNA elements, it was predicted that a protein(s) might associate with the regulatory regions of PA1 RNA in a sulfur-dependent manner. This proposed protein/RNA interaction might mediate the post-transcriptional process(es) by which PA1 expression is regulated. A 323 nucleotide fragment of the PA1 3' flanking region has been shown to confer sulfur-responsive gene expression, therefore this region was considered a possible target for a regulatory PA1 RNA-binding protein. Since the first 189 nucleotides of this 323 nucleotide region were not sufficient to confer sulfur-responsiveness, it was possible that the remaining 134 nucleotides would be a more specific target for protein binding.

Involvement of the first 189 nucleotides in regulation of sulfur-responsive gene expression has not been formally excluded however, since this region may be necessary but not sufficient for sulfur-responsive gene expression, therefore a region containing the first 333 nucleotides of the PA1 3' flanking region was considered. In addition to transcripts containing the 134 nucleotide region and the 333 nucleotide region, fragments of PA1 3' UTR and flanking region known not to confer sulfur-responsive gene
expression (see Fig. 2.4) were used as probes to identify proteins in pea cotyledons which bound to the putative regulatory regions of the PA1 3' region in a sulfur-dependent manner.

### 5.3.1 PA1 RNA-binding proteins

Four experimental approaches were taken to study interactions between pea seed proteins and transcripts derived from the 3' end of PA1 RNA. Initial gel mobility shift analyses of PA1 RNA-binding activity in total protein extracts from sulfur-adequate and sulfur-deficient pea cotyledons harvested between 17 and 20 DAF revealed that both sulfur-adequate and sulfur-deficient cotyledons contained proteins capable of binding to all five transcripts from the PA1 3' UTR and flanking region. The protein/RNA complexes formed appeared large (based on electrophoretic mobility), suggesting that several protein components were involved in each case. The average sizes of complexes formed by proteins from sulfur-adequate cotyledons were greater than those formed by proteins from sulfur-deficient cotyledons, indicating that different populations of PA1 RNA-binding proteins were present in each extract. Further investigation showed that PA1 RNA-binding proteins were present in nuclear extracts from sulfur-adequate and sulfur-deficient pea seeds and again, there were sulfur-dependent differences in the mobilities of the RNA/protein complexes.

In general, only one major complex was formed between nuclear proteins from sulfur-deficient pea seeds and PA1 transcripts but proteins from sulfur-adequate peas formed between one and three complexes, depending on the probe used. In RNase protection assays however, a maximum of two complexes was observed for sulfur-adequate extracts, indicating that some complexes were unstable. The differences in size and number of protein/RNA complexes between extracts from sulfur-adequate and sulfur-deficient pea cotyledons are indicative of sulfur-dependent differences in the PA1 RNA-binding protein population, and may be relevant to sulfur-responsive regulation of PA1 mRNA abundance.

Sulfur-adequate nuclear extract formed a large complex (complex B) with both the 333 nucleotide and 134 nucleotide transcripts in RNase protection assays, but components of this complex also recognized transcripts from other regions of the PA1 3' UTR. Transcript-specific differences in complexes formed by nuclear extracts from sulfur-deficient cotyledons were difficult to detect, but it appeared that a larger group of proteins were associated with the 333 nucleotide transcript than with the smaller transcripts. Thus
it appeared that there were proteins in both sulfur-adequate and sulfur-deficient nuclear extracts which associated with transcripts for the two putative regulatory regions but not with other transcripts, however from the results of these gel mobility shift and RNase protection assays with nuclear extracts, it was not possible to identify a specific protein which was associated with either of these fragments in a sulfur-dependent manner.

5.3.2 Characterization of PA1 RNA-binding polypeptides

UV-crosslinking experiments were performed in order to determine more precisely, the number and size of PA1 RNA-binding polypeptides present in sulfur-adequate and sulfur-deficient pea seed extracts. These crosslinking experiments confirmed that a complex profile of polypeptides from both sulfur-adequate and sulfur-deficient pea seeds were associated with all five sections of the PA1 3' untranslated and flanking regions. Not all of these were specific to PA1 RNA sequences however, and the polypeptides binding to the negative control were not considered further as they are unlikely to be specifically involved in sulfur-responsive gene expression.

In each extract there was a group of polypeptides which bound to the PA1 3' transcripts, but not to the negative control transcript. In the nuclear extract from sulfur-adequate peas there was a total of five polypeptides of Mr greater than 20 k which bound to PA1 3' transcripts but not to the negative control. Within this group, some polypeptides bound in a probe specific manner but there were no polypeptides which bound exclusively to either the 333 nucleotide transcript or the 134 nucleotide transcript. Protein/RNA complexes of Mr less than 20 k were also present in some cases but were poorly resolved in the 15% polyacrylamide used for these experiments and therefore could not be considered further in this analysis. These small polypeptides may however, be important for regulation of PA1 expression and should be studied separately in future experiments.

5.3.2.1 Putative sulfur regulatory proteins

In extracts from sulfur-deficient pea cotyledons there were at least eight polypeptides which bound transcripts from the PA1 3' region with higher affinity than the negative control transcript. These polypeptides were considered to be candidates for sulfur-dependent regulatory proteins. A comparison of the 'PA1-specific' polypeptides in the profiles of the 333 nucleotide transcript shows that of these eight polypeptides, there were four which were present in sulfur-deficient extracts but not in sulfur-adequate extracts (Table 5.2). Polypeptides of Mr = 110 k, 70 k, approximately 51 k and approximately 32
k bound to this region in a sulfur-dependent manner. Of these four polypeptides, the Mr = 110 k and 32 k proteins also bound to the transcript spanning nucleotides 199-333 of the PA1 3' flanking region, but not to the transcripts containing the first 199 nucleotides of the PA1 3' UTR.

The Mr = 32 k polypeptide did appear to be present in the profile of the transcript containing only nucleotides 109-199 of the PA1 3' UTR, however this region (PA1_{109-199}) exhibited conflicting results in the four separate analyses. Gel mobility shift assays with total and nuclear protein extracts indicated that few proteins from sulfur-deficient pea seeds bound to this region, while RNase protection and UV-crosslinking experiments indicated that it was recognized by a large complex of several polypeptides from sulfur-deficient peas. The reason for this discrepancy is unknown, however the presence of the Mr = 32 k and 30 k polypeptides in this profile might be attributed to poor degradation of this probe by RNase A in UV-crosslinking experiments and therefore these bands may not represent polypeptides which play a role in vivo during sulfur deficiency.

The Mr = 110 k polypeptide in contrast, appeared to bind with higher affinity to the 134 nucleotide transcript containing nucleotides 199-333 of the PA1 3' flanking region than to any other transcript. Therefore at least one, and possibly two polypeptides were specifically associated with this 134 nucleotide sequence under sulfur-deficient conditions. This RNA sequence is likely to be involved in sulfur-responsive expression of PA1 because nucleotides 10-333 of the PA1 3' flanking region confer sulfur-responsive gene expression but nucleotides 10-199 do not, therefore while polypeptides associated with other regions of the PA1 primary transcript may be involved in sulfur-responsive gene expression, the polypeptides associated with this 134 nucleotide regulatory region may play a role in controlling expression of PA1 in response to sulfur nutrition.

In summary, UV-crosslinking experiments confirmed that there were protein components in nuclear extracts from sulfur-deficient pea cotyledons which associated with the both the 333 nucleotide and the 134 nucleotide PA1 transcripts, and which were not present in nuclear extracts from sulfur-adequate cotyledons. A group of four polypeptides which bound to the first 333 nucleotides of the PA1 3' flanking region in a sulfur-dependent manner was identified. Two of these polypeptides from sulfur-deficient extracts may be uniquely associated with the 134 nucleotide region of the PA1 3' flanking region which confers sulfur-responsive gene expression. These four polypeptides are candidates for
further characterization to determine their likely roles in regulation of PA1 expression in response to sulfur nutrition.

The complexity of the profiles of RNA-binding polypeptides associated with PA1 3' transcripts in both sulfur-adequate and sulfur-deficient nuclear extracts means that, at this stage, I can only speculate on the possible roles of these proteins in regulating expression of PA1. Several common themes uniting well characterized examples in which post-transcriptional regulation of gene expression is mediated by RNA-binding proteins can be identified however, and it is possible to assess sulfur-dependent regulation of PA1 expression with respect to these characteristics. These characteristic features of examples in which gene expression is regulated by RNA-binding proteins are: a high level of complexity of interactions between proteins and RNA elements; a high level of specificity of these interactions; location of regulatory RNA elements in the 3' untranslated regions of transcripts; and a predominantly cytoplasmic location of these regulatory protein/RNA interaction. Comparison of sulfur-regulated expression of PA1 with previously characterized systems, with respect to these characteristics highlights some similarities with other examples, but also some significant differences. These are discussed below.

**Complexity**

A characteristic feature emerging from studies of mammalian systems in which gene expression is regulated by interactions between regulatory proteins and RNA elements, is the complexity of these interactions. Interactions between RNA and proteins which regulate mRNA stability can be highly complex in terms of the numbers of protein components involved, the multiple functions of some regulatory proteins and the variety of effectors which can regulate the RNA-binding activity of these proteins. One of the most complex RNA-binding protein profiles reported to date is that generated in chick liver extracts after estrogen treatment. Estrogen induces a group of eight RNA-binding proteins with high affinity for elements in the 3' UTRs of estrogen-regulated transcripts such as apolipoprotein II (apoII) and vitellogenin. A minimal RNA element of 150 nucleotides in the 3' UTR of apoII mRNA is required for binding of all eight estrogen-induced proteins (Margot and Williams, 1996).

The RNA-binding protein profiles generated after crosslinking of pea nuclear proteins to the 333 nucleotide transcript of PA1 3' flanking region resembled that of estrogen-treated chicken liver extracts after crosslinking to apoII mRNA. In both cases an array of protein complexes was generated, indicating that many proteins may have been assembled into
multiprotein ribonucleoprotein complexes. In the case of estrogen induction, assembly of this complex is associated with a decrease in cytoplasmic stability of apoII mRNA and at least one of the proteins is thought to participate directly in mRNA turnover (Margot and Williams, 1996). Therefore, multi-component ribonucleoproteins can be associated with accelerated RNA turnover in response to exogenous stimuli.

Similarly, at least five proteins which bind to the AU-rich elements (AREs) commonly found in 3' UTRs of short-lived mammalian mRNAs have been identified by UV-crosslinking experiments (Curatola et al., 1995 and references therein; chapter 1). While the roles of these ARE-binding proteins remain unclear, some are associated with active destabilization of mRNA (DeMaria and Brewer, 1996; Bohjanen et al., 1991; Brewer and Ross, 1989) and others with stabilization of mRNA molecules (Rajagopalan and Malter, 1994; Gillis and Malter, 1991). Still others resemble components of heteronuclear ribonucleoprotein (hnRNP) complexes and may be involved in transport of ARE-containing transcripts to the cytoplasm (Katz et al., 1994; Hamilton et al., 1993). Thus, regulatory protein/RNA interactions can involve several different proteins and may participate in multiple processes which ultimately determine mRNA abundance.

Other examples of multiple proteins interacting with RNA elements to regulate RNA abundance include the iron regulatory proteins and proteins involved in 3' end formation of chloroplast mRNAs. Two iron regulatory proteins are regulated by a variety of stimuli and control expression of iron responsive genes in response to changes in iron availability (Samaneigo et al., 1994; Müllner et al., 1989), oxidative stress (Pantopolous and Hentze, 1995) and inflammation (Cairo and Pietrangelo, 1995). A multiprotein complex has been reported to control the formation and stability of 3' ends of chloroplast mRNAs (Hayes et al., 1996). Participation of multiple proteins in rapid turnover of PvPRPl during fungal infection has also been implied by the observation that interaction of a single RNA-binding protein with PvPRPl mRNA was not sufficient to initiate rapid turnover (Zhang and Mehdy, 1994). This may indicate that other proteins are also involved in the process.

Therefore, there are many examples of RNA/protein interactions which are associated with regulation of mRNA abundance and which involve several protein components. The identification of multiple proteins binding to PA1 3' regions therefore, is not unprecedented but the very large number of RNA/protein complexes observed was unexpected. It may be explained by the fact that RNA-binding proteins were sought in nuclear, rather than cytoplasmic extracts. In general, there are many proteins associated with various degrees of specificity, with pre-mRNA in nuclei (Dreyfuss et al., 1993;
Konarska and Sharp, 1986). The hnRNP proteins are involved in many aspects of RNA processing and transport, therefore it is possible that some of the proteins which bound to PA1 RNA transcripts may be involved in processing of primary RNA transcripts. The proteins which bound to PA1 transcripts in a sulfur-dependent manner however, are likely to be specifically involved in regulating the response of PA1 expression to sulfur deficiency.

Specificity

Many regulatory protein/RNA interactions appear to be highly specific (for example, Chen et al., 1993; Amara et al., 1994; Winzen et al., 1996). Gel mobility shift and RNase protection experiments using both heparin and unlabelled in vitro-transcribed RNA as competitors indicated that although there may be proteins in sulfur-adequate pea seeds which have specificity for the PA1.333 transcript (for example, complex B; Fig. 5.4(a)), the interactions between this transcript and most nuclear RNA-binding proteins from sulfur-deficient pea seeds (complex C) may not be highly specific (Fig. 5.5(c)). It is possible however, that some of the polypeptides from sulfur-deficient extracts identified by UV-cross linking analysis may bind PA1.333 with greater specificity than complex C. This could be examined by performing competition experiments using UV-crosslinking rather than RNase protection to identify polypeptides which bound specifically to the regulatory region of the PA1 transcript.

Location of RNA elements and binding proteins

Regulatory RNA-binding proteins can bind to RNA elements located in the 5' UTR, coding region or 3' UTR, depending on the mRNA species (Table 5.3). In most examples reported to date however, the RNA element with which a protein interacts to regulate expression of the particular RNA, is located in the 3' UTR. There does not appear to be any consensus in the size or location of these elements within the 3' UTR and they range in size from 22 nucleotides in the procα(I) collagen mRNA (Määttä and Penttinen, 1993) to an element in insulin-like growth factor II (IGFII) mRNA which contains two individual elements of 103 nucleotides and 323 nucleotides separated by 2 kB. These two elements and the intervening RNA fold into a secondary structure recognized by a 50 kD protein (Scheper et al., 1996).

The 333 nucleotide transcript from the PA1 3' flanking region therefore, is within the range of reported sizes for RNA-binding protein target sites, although it is larger than
many. The 134 nucleotide transcript spanning nucleotides 199-333 is more similar to the sizes of other reported elements but it is not known whether this fragment can function in isolation from the preceding 199 nucleotides to confer sulfur-responsiveness. If this 134 nucleotide fragment is the minimal sulfur-responsive element the proteins binding to this region of the PA1 primary transcript represent the complete set of putative sulfur-regulatory proteins.

The putative sulfur regulatory interaction between PA1 RNA and pea seed proteins occurs in the region 3' to the translation stop codon, but in contrast to most of the previously reported protein/RNA interactions, the regulatory region of PA1 RNA has been shown to include sequences which lie beyond the polyadenylation site. All of the examples listed in table 5.3, in which RNA abundance was regulated by proteins binding to elements in 3' UTRs involve elements located in the mature mRNA, therefore the proposed mechanism by which PA1 RNA abundance is regulated differs from most other examples with respect to the location of the RNA regulatory element within the 3' flanking region. Most of the regulatory proteins in these examples summarized in table 5.3 were localized in the cytoplasm. The location of the PA1 sulfur regulatory element outside, rather than within, the mature mRNA also reflects a likely contrast in the sub-cellular localization of putative regulatory proteins determining the level of PA1 mRNA in response to sulfur nutrition, since the regulatory process is likely to occur in the nucleus rather than in the cytoplasm.

The location of the PA1 sulfur regulatory element in the primary, rather than the mature, transcript is consistent with the identification in nuclei of sulfur-deficient pea seeds, of polypeptides which bind to the minimal sulfur responsive element in the PA1 primary transcript. While apparently differing from previously reported examples of post-transcriptional control of gene expression, locations of PA1 RNA regulatory elements and putative regulatory RNA-binding polypeptides are consistent with each other and suggest that sulfur-responsive expression of PA1 may be regulated by a novel, nuclear mechanism.
Table 5.3 Summary of recent examples in which gene expression is regulated by binding of proteins to RNA sequence or structural elements.

Abbreviations: TNFr - tumour necrosis factor receptor; IGF II - insulin-like growth factor II; CYP - cytochrome P-450; SLBP - stem-loop binding protein; apoII - apolipoprotein II; CRD-BP - coding region determinant-binding protein; ARE - AU-rich element; AUBF - ARE-binding factor; IRE - iron responsive element; IRP - iron regulatory protein; TIR - transferrin receptor.

<table>
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<tr>
<th>ELEMENT</th>
<th>SIZE OF ELEMENT (nucleotides)</th>
<th>LOCATION OF ELEMENT</th>
<th>SIZE OF PROTEINS</th>
<th>REGULATION OF BINDING ACTIVITY</th>
<th>ACTION</th>
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<td>81</td>
<td>coding region</td>
<td>75 kD, 80 kD</td>
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<td>IGF II</td>
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<td>Scheper et al., 1996</td>
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<td>SIZE OF ELEMENT (nucleotides)</td>
<td>LOCATION OF ELEMENT</td>
<td>SIZE OF PROTEINS</td>
<td>REGULATION OF BINDING ACTIVITY</td>
<td>ACTION</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>c-myc</td>
<td>182</td>
<td>coding region</td>
<td>70-75 kD (CRD-BP)</td>
<td>-</td>
<td>mRNA stabilization</td>
<td>Bernstein et al., 1992 Prokipcak et al., 1993</td>
</tr>
<tr>
<td>c-myc ARE</td>
<td>unknown</td>
<td>3' UTR</td>
<td>37 kD, 40kD (AUF)</td>
<td>-</td>
<td>mRNA destabilization</td>
<td>Brewer, 1991</td>
</tr>
<tr>
<td>ARE</td>
<td>synthetic (AUUUA) repeat</td>
<td>3' UTR</td>
<td>37 kD (AUF)</td>
<td>-</td>
<td>mRNA destabilization</td>
<td>DeMaria and Brewer, 1996</td>
</tr>
<tr>
<td>GM-CSF ARE</td>
<td>unknown</td>
<td>3' UTR</td>
<td>42 kD (AUBF)</td>
<td>-</td>
<td>mRNA stabilization</td>
<td>Rajagopalan et al., 1994</td>
</tr>
<tr>
<td>ARE</td>
<td>synthetic (AUUUA) repeat</td>
<td>3' UTR</td>
<td>34 kD (AU-A)</td>
<td>-</td>
<td>mRNA transport from nucleus</td>
<td>Katz et al., 1994</td>
</tr>
<tr>
<td>ARE</td>
<td>synthetic (AUUUA) repeat</td>
<td>-</td>
<td>AU-A 34 kD</td>
<td>-</td>
<td>transport destabilization</td>
<td>Bohjanen et al., 1992; 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AU-B 30 kD</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AU-C 43 kD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARE</td>
<td>synthetic (AUUUA) repeat</td>
<td>-</td>
<td>45 kD (AUBF)</td>
<td>-</td>
<td>mRNA stabilization</td>
<td>Malter and Hong, 1991</td>
</tr>
<tr>
<td>ARE</td>
<td>synthetic (AUUUA) repeat</td>
<td>-</td>
<td>45 kD (AUBF)</td>
<td>-</td>
<td>mRNA stabilization</td>
<td>Malter, 1991</td>
</tr>
<tr>
<td>IRE</td>
<td>678 (TIR)</td>
<td>3' UTR</td>
<td>98 kD (IRP1)</td>
<td>iron</td>
<td>mRNA stabilization/translation inhibition</td>
<td>Koeller et al., 1989 Mullner et al., 1989</td>
</tr>
<tr>
<td>IRE</td>
<td>51 (Ferritin)</td>
<td>5' UTR</td>
<td>105 kD (IRP2)</td>
<td>iron</td>
<td>mRNA stabilization/translation inhibition</td>
<td>Samaneigo et al., 1994 Henderson et al., 1993 Iwai et al., 1995 Guo et al., 1995</td>
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In summary, expression of many genes is regulated at the post-transcriptional level by interaction of mRNA-binding proteins with specific mRNA elements, often located within the 3' UTR of the transcript. These mRNA-binding proteins frequently control mRNA levels by modulating the cytoplasmic stability of the transcripts with which they associate, although the mechanisms by which they do so are currently poorly understood. While PAI mRNA abundance appears to be regulated by a post-transcriptional mechanism which may involve effects on RNA stability, and in which RNA-associated proteins may participate, there are two features of this mechanism which distinguish it from other examples described previously. The first is the fact that the 134 nucleotide region of PAI pre-mRNA controlling sulfur-dependent expression of PAI is outside the mature message, and secondly the protein factors which associate with the regulatory region of PAI pre-mRNA in a sulfur-dependent manner occur in pea nuclei. These two factors indicate that the process which regulates the abundance of PAI mRNA in response to sulfur nutrition occurs in the nucleus, although the mechanism by which the PAI pre-mRNA/protein complexes result in reduced PAI mRNA levels during sulfur deficiency is yet to be elucidated.

PAI expression may be regulated by sulfur nutrition via a novel, post-transcriptional mechanism mediated by nuclear proteins associated with pre-mRNA. This mechanism may involve effects on nuclear stability of the PAI pre-mRNA, however other pre-mRNA processing reactions may also be affected. Further characterization will be required to resolve this mechanism and approaches which could be taken will be discussed in the final chapter.
CHAPTER 6

GENERAL DISCUSSION
6.1 INTRODUCTION

Expression of a pea albumin 1 gene in developing pea seeds and leaves of transgenic tobacco is regulated by plant sulfur nutrition. During periods of sulfur deficiency, expression of this gene is down-regulated by a post-transcriptional mechanism and at least two independent elements within the PA1 transcript have been shown to mediate this response. Results from previous experiments have indicated that this regulation involves specific destabilization of the PA1 transcript, but this has not yet been directly demonstrated. The experiments described here are based on the assumption that the PA1 transcript is destabilized during sulfur deficiency. I conducted experiments to characterize the molecular components involved in down-regulation of PA1 expression during sulfur deficiency. Experiments investigating stability of PA1 mRNA were inconclusive and have been discussed fully in chapter 3.

6.1.1 RNA elements

It was of interest to identify RNA and protein components which might participate in sulfur-regulated PA1 expression. Previous experiments showed that large scale regions in both the PA1 coding region and the 3' flanking region independently conferred sulfur-responsive expression (Morton, 1993). I have shown that sequences in a 323 nucleotide fragment spanning nucleotides 10 to 333 of the PA1 3' flanking region conferred sulfur-responsive expression on a reporter gene, while a 189 nucleotide fragment spanning nucleotides 10 to 199 did not. This indicated that a sulfur-responsive element was located within a 134 nucleotide region of the PA1 transcript, spanning nucleotides 199 to 333 of the 3' flanking region. This region was downstream of the polyadenylation site, which is located at nucleotide 162 of the 3' untranslated region. While sequences required for sulfur-responsive gene expression are contained within this 134 nucleotide region, it is possible that sequences in the previous 189 nucleotides which do not themselves, confer sulfur-responsiveness, may be required for function of elements located between nucleotides 199 and 333.

A 496 nucleotide fragment containing nucleotides 10 to 506 of the PA1 3' flanking region conferred a larger response to sulfur deficiency than nucleotides 10 to 333, indicating that elements enhancing the sulfur response conferred by the 333 nucleotide region may be located downstream of nucleotide 333. The region spanning nucleotides 199 to 333 of the PA1 3' flanking region therefore, contains an element(s) capable of conferring sulfur-responsive gene expression, however there may be quantitative elements influencing the sulfur response located downstream in the 3' region.
The location of the putative regulatory elements, downstream from the polyadenylation site in the PA1 3' flanking region, provides information about the cellular compartment in which PA1 expression is likely to be regulated. Sequences downstream from the polyadenylation site are not included in the mature mRNA which is exported to the cytoplasm therefore, for these sequences to influence PA1 mRNA levels, regulation must occur in the nucleus. Regulation of gene expression involving changes in stability of nuclear pre-mRNA has been reported (Leys et al., 1984; Chen, 1988; Jarrous and Kaempfer, 1994; Gerez et al., 1995) but these examples are not as well characterized as the numerous examples in which cytoplasmic stability of mRNA is regulated.

Measurement of the levels of both nuclear and cytoplasmic PA1 RNA in sulfur-adequate and sulfur-deficient cells will indicate whether the distribution of PA1 transcripts between these two compartments changes during changes in sulfur nutrition. If the mechanism regulating sulfur-responsive gene expression occurs in the nucleus, concentration of both nuclear and cytoplasmic PA1 RNA would be expected to decline during sulfur deficiency. In contrast, if the response occurs in the cytoplasm, the level of PA1 RNA in the nucleus should be unaffected by sulfur deficiency while the level in the cytoplasm would be expected to decline.

6.1.2 PA1 RNA-binding proteins

Post-transcriptional regulation of gene expression frequently involves interactions between regulatory RNA elements and cellular proteins which recognize those elements. Protein/RNA interactions are involved in many post-transcriptional processes, including intron-processing, polyadenylation and transport of mRNA to the cytoplasm. Interactions between protein and RNA can also initiate rapid RNA decay, protect RNA from decay, or otherwise influence the accumulation of mRNA by as yet unknown pathways. It is possible that sulfur responsive elements in PA1 RNA associate with RNA-binding proteins specifically during sulfur deficiency, and that these protein/RNA interactions mediate sulfur-responsive expression of PA1. PA1 RNA-binding proteins might initiate rapid decay of the PA1 transcript either directly, by acting as endoribonucleases and exposing internal sites in PA1 RNA to non-specific exoribonucleases, or indirectly, by otherwise targeting PA1 RNA for rapid decay.

Gel mobility shift and RNase protection experiments showed that pea seeds contained proteins which formed ribonucleoprotein complexes with transcripts derived from the 3'
end of the PA1 primary transcript. Complexes formed with these transcripts by nuclear protein extracts from pea seeds grown under sulfur-adequate conditions differed from those formed by extracts from sulfur deficient pea seeds, indicating that they contained different protein components.

UV-crosslinking of RNA/protein complexes showed numerous polypeptides from pea seed nuclei in association with the 333 nucleotide sequence containing the sulfur regulatory element from the PA1 3' flanking region. Four of these polypeptides were unique to sulfur-deficient pea cotyledons and of these four, polypeptides of Mr 110 k and 32 k appeared to bind preferentially to the 134 nucleotide region between nucleotides 199 and 333. The presence of proteins which bound to the regulatory region of the PA1 primary transcript and which were unique to sulfur-deficient cotyledons indicates that these proteins may have a role in regulating accumulation of PA1 mRNA when peas are grown in sulfur-deficient soils.

### 6.2 FUTURE WORK

In order to further dissect the mechanism by which sulfur regulates expression of PA1, I have suggested the following approaches for future work.

#### 6.2.1 RNA elements

Elements in the 3' flanking region of the PA1 transcript are involved in regulating abundance of PA1 mRNA in response to sulfur nutrition, but the number, size and structure of these elements remains unclear. If the nature of RNA elements regulating abundance of PA1 mRNA is to be understood, it will be necessary to map the regions in greater detail. First, it will be necessary to assess the regions spanning nucleotides 199-333 and 333-506 to determine their ability to confer sulfur responsiveness. This will allow estimation of a maximum size for the regulatory RNA elements. It will then be necessary to establish a minimum size for the functional sulfur responsive element. In some examples of post-transcriptional regulation of gene expression, regulatory regions as small as 20 nucleotides have been identified (Amara et al., 1994).

Once a minimal regulatory element has been identified, it should be possible to identify important sequence and structural features of it by mutational analysis. This detailed analysis of the sulfur responsive element in the PA1 3' flanking region will quickly generate a large number of constructs and it would be impractical to test them all in sulfur-
If this technique was adopted, it would be desirable to ensure developmental and nutritional uniformity of the leaves, since variation in tissue type and sulfur-status may have contributed to the variation in gene expression between plants and between experiments observed in chapter 4. X-ray fluorography could be used to obtain a correlation between leaf sulfur-status and PAI expression, so that variation between experiments could be examined in terms of the degree of sulfur deficiency achieved in each experiment.
stress experiments using transgenic plants. It will be desirable to devise a less cumbersome method for assessing sulfur-responsive gene expression. It may be possible to use alternative promoters and coding regions to increase the level of expression of the reporter gene. This construct could be introduced into sulfur-adequate and sulfur-deficient leaves using a biolistic technique, which may provide a simpler method for studying the sulfur response.

6.2.2 RNA-binding proteins

Four polypeptides which bound to a 333 nucleotide putative sulfur-regulatory region of the PA1 3' flanking region during sulfur deficiency were identified by UV-crosslinking experiments. Many other polypeptides also bound this region but may not be involved in the sulfur response. It will be desirable to eliminate binding of some of these other polypeptides before attempting to determine the roles of the putative sulfur-regulatory proteins (SRPs) in sulfur-regulated expression of PA1. This could be achieved in several ways. It may be possible to remove non-specifically bound components by including heparin in binding reactions, as low concentrations of this compound were shown to reduce non-specific binding in some complexes in RNase protection assays. Alternatively, a non-specific, unlabelled RNA transcript, such as antisense ovalbumin, could be used as a competitor for non-specific RNA-binding polypeptides in UV-crosslinking experiments.

More detailed analysis of the region of PA1 pre-mRNA conferring sulfur-responsiveness may more precisely define the sulfur-responsive element and may reduce the size of the region under consideration. Using a smaller transcript in the UV-crosslinking assay may reduce the number of non-specific polypeptides associated with it and more stringent crosslinking conditions (for example, time of exposure to UV, UV intensity) should also be investigated.

Alternative methods for identifying proteins specifically associated with the regulatory region of PA1 RNA include excising the bound complex from the non-denaturing gel mobility shift assays and separating the protein components by SDS-PAGE (for example, Scheper et al., 1996), or using a 'north-western' analysis. This technique would avoid crosslinking in solution of proteins in close physical proximity to the probe but of no functional significance. The disadvantage of this method is that the proteins are first denatured by SDS-PAGE and then immobilized on a membrane. Under these conditions they may not be in a conformation which is able to recognize the RNA probe.
It is desirable to purify these putative SRPs from sulfur-deficient nuclear extracts, so that they can be investigated individually. Protocols for affinity purification of RNA-binding proteins have been used to purify the iron regulatory protein IRP1 (Rouault et al., 1989; Neupert et al., 1990). A small SRE immobilized on a solid support could provide a useful affinity matrix for the isolation of the SRPs from nuclear extracts of sulfur-deficient peas.

Once the putative SRPs have been purified it should be possible to establish a link between binding activity and regulation of PA1 expression, for instance by mutational analysis of the SRE. Mutations which abolished SRP binding and which also abolished sulfur-responsive expression of PA1 would provide evidence for the direct participation of RNA-binding proteins in sulfur-regulation of PA1 expression. If the same mutation also affected the stability of the PA1 transcript in a sulfur-dependent manner, a link would be established between protein binding and a mechanism regulating PA1 expression.

Mutational analysis of this type demonstrated the requirement for binding of the IRE by IRP for iron-regulated expression of ferritin by showing that mutations disrupting the stem of the IRE abolished both IRP binding and iron regulation (Kikinis et al., 1995). It may be possible to elucidate the binding site(s) of putative sulfur regulatory proteins by mutational studies, or by using short, antisense oligonucleotides to interfere with binding in specific regions of the PA1 regulatory region (Winzen et al., 1996).

Having demonstrated the involvement of putative SRPs in sulfur-regulated expression of PA1, it would be useful to clone the genes for these proteins. Knowledge of the nucleotide and amino acid sequences of sulfur regulatory proteins may be useful in attributing functions to the proteins, by comparison with other known RNA-binding proteins.

6.3 SIGNAL TRANSDUCTION OF SULFUR DEFICIENCY

Cloning the genes for these proteins will also make it possible to examine the expression of these genes during the development of sulfur deficiency and it may be possible to determine how expression of these regulatory proteins is itself, regulated. For example, it may be possible to determine the nature of the effector molecule which regulates expression and/or activity of SRPs. In vivo, provision of almost any sulfur source including sulfate, methionine, cysteine, glutathione or mercaptoethanol to sulfur-deficient
cultured peas pods stimulates expression of legumin (Chandler et al., 1984). It is unknown whether each of these compounds acts independently to affect gene expression or whether a common metabolite is the sole effector. It should be possible to determine in vitro which compounds influence either expression or activity of the regulatory proteins and hence, determine how the level of sulfur is sensed and transduced to an effect on gene expression.

This type of characterization has been carried out for iron-regulated gene expression in mammalian cells. Two IRPs have been cloned and characterized. Each IRP binds IREs in an iron-dependent manner (Henderson et al., 1993) but the abundance and activities of these RNA-binding proteins are regulated by two distinct mechanisms (Samaneigo et al., 1994; Iwai et al., 1995). They are expressed in different tissues (Samaneigo et al., 1994) and respond to different stimuli [in addition to iron (Schalinske and Eisenstein, 1996)], allowing a high degree of flexibility and sensitivity in regulation of iron responsive gene expression.

Although currently, not enough is known about sulfur-regulated expression of genes for high-sulfur proteins in plants to draw strong parallels between this system and iron-regulated gene expression in mammalian cells, it is possible that a system of similar complexity involving RNA-binding proteins mediates sulfur-regulated gene expression. Detailed characterization of all components of the system will be required for a comprehensive understanding of how nutritional signals originating in the soil are able to mediate expression of multiple genes in seeds of peas and other plants. At this stage it appears that a set of proteins associate with a region of PA1 pre-mRNA in the nucleus during sulfur deficiency, and that this RNA/protein association in some way inhibits accumulation of mature PA1 mRNA in the cytoplasm, possibly by interfering with transcript processing or by otherwise targeting PA1 pre-mRNA for rapid decay in the nucleus.

Sulfur-regulation is not confined to expression of PA1, but is a widespread feature of regulation of seed protein composition in many species (Spencer et al., 1990; chapter 1). Once the mechanism of sulfur regulation of PA1 expression is elucidated, it will be interesting to examine how applicable the mechanism is to other sulfur-responsive genes. Within the pea seed for instance, do the regulatory proteins associated with the PA1 transcript also associate with legumin and PA3 transcripts to down-regulate expression of these genes during sulfur deficiency? Conversely, do these regulatory proteins play any part in up-regulation of vicilin during sulfur deficiency? IRPs can induce transferrin
expression and repress ferritin expression in response to iron-starvation by binding to different regulatory regions of those transcripts (Klausner et al., 1993). One way to gain a preliminary indication of whether or not the putative SRPs identified in association with PA1 are also associated with other sulfur-regulated transcripts, would be to measure the ability of transcripts such as legumin and vicilin to compete with the PA1 transcript for these RNA-binding factors.

If these proteins are involved in a general mechanism by which gene expression is regulated by sulfur nutrition, there may be similar regulatory proteins in other plant species. If the genes for SRPs from pea could be cloned, they could be used to identify sulfur regulatory proteins in plants such as soybean and lupin. This may have implications for attempts to raise the value of these seeds as feed supplements by expressing high-sulfur proteins in them.

Since expression of PA1 in leaves of transgenic tobacco appears to be regulated by sulfur in an identical manner to its expression in pea seeds (Morton, 1993), it is possible that the regulatory mechanism (and putative regulatory proteins) is conserved between organs as diverse as pea seeds and tobacco leaves. Therefore it would be interesting to examine whether SRPs are also present in the leaves of pasture species such as subterranean clover and lucerne, which are targets for improved nutritional value via expression of high-sulfur proteins in leaves. An understanding of how these proteins interact with their RNA binding sites may provide insight into the most efficient ways to desensitize expression of high-sulfur proteins to fluctuating levels of soil sulfur in the field.

It is also interesting to note that accumulation of β-conglycinin subunits from soybean was regulated by sulfur when these subunits were expressed in petunia (Petunia hybrida) seeds, even though expression of petunia seed storage protein genes is not regulated by sulfur nutrition (Fujiwara et al., 1992). Together with the observation that genes for high-sulfur storage proteins from pea seeds were regulated in tobacco leaves, this observation indicates that the mechanism by which sulfur regulates gene expression may be highly conserved. The apparent conservation of a mechanism for sulfur-regulated gene expression, even in organs and plant species where it does not appear to be utilized, may indicate that the mechanism serves some other, more essential, purpose and has been adopted to regulate accumulation of seed storage proteins.

It is intriguing that expression of seed genes to be regulated by a rapid, post-transcriptional mechanism in response to changes in sulfur nutrition because while plant
roots might be expected to be exposed to moderately rapid changes in sulfur availability as a consequence of weather conditions (Rennenberg et al., 1990), the supply of sulfur to the seed could be expected to be buffered against sudden changes in sulfur nutrition, at least to a certain extent, by the ability of plants to recycle sulfur from older leaves (Adiputra and Anderson, 1995). This may indicate that the post-transcriptional mechanism already served another purpose (for example, regulating uptake of sulfate in roots, where fluctuating sulfur conditions might be expected) and during the process of evolution this pre-existing mechanism has been adapted for regulating expression of genes for seed storage proteins.

The abundance of mRNAs encoding proteins involved in uptake and transport (Smith et al., 1995) and assimilation of sulfate (Logan et al., 1996) have recently been shown to be regulated by sulfur nutrition. It is tempting to speculate that genes involved in transport, assimilation and utilization of sulfate might be regulated by a common mechanism in plants, in the same way as genes involved in transport, storage and utilization of iron are coordinately regulated by iron availability in mammalian cells. At this stage however, there is insufficient evidence to support this proposition. It will be important to know whether or not there is a common mechanism controlling these different activities, as future manipulation of the sulfate regulatory mechanism to enhance expression of high-sulfur proteins in transgenic plants may have consequences for uptake and assimilation of sulfate by these plants.

### 6.6 CONCLUDING REMARKS

An understanding of how soil sulfur status is able to regulate expression of genes involved in uptake, assimilation and utilization of sulfur is needed, so that sulfur metabolism of plants can be exploited to improve agricultural production and nutritional qualities of pasture species. The aim of this project was to characterize the molecular components of the mechanism by which sulfur regulates expression of a gene encoding a sulfur-rich storage protein from pea seeds. A region of the PAI pre-mRNA which mediated sulfur-responsive gene expression was located and preliminary evidence for proteins from nuclei of sulfur-deficient pea seeds which bound to this regulatory region of the PA1 transcript was obtained.

It has previously been proposed that PA1 RNA-binding proteins might specifically destabilize PA1 mRNA during sulfur deficiency. It was not possible to accurately assess the relative stabilities of PA1 transcripts in sulfur-adequate and sulfur-deficient leaves.
from transgenic tobacco plants and at this stage, it is not possible to predict what roles the putative sulfur regulatory proteins play in the regulation of gene expression by sulfur. It does appear likely however, that a novel, nuclear mechanism possibly involving protein-mediated effects on primary transcript stability regulates accumulation of PA1 mRNA in response to sulfur availability.

These results provide a basis for future research into sulfur-regulated expression of plant genes. Outcomes of future research may indicate how the expression of genes for high-sulfur proteins may be best manipulated for various agricultural applications, including improved nutritional quality of pasture legumes and grain supplements. These findings will need to be integrated with results from field studies of behaviour of transgenes to determine the consequences for the plant, of high level expression of foreign, sulfur-rich proteins under sulfur-limited conditions to determine the most effective strategies for achieving reliable, high-level expression of sulfur-rich proteins under field conditions.
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