PERGAMON

Phytochemistry 52 (1999) 225–231

PHYTOCHEMISTRY

Methionine-induced stabilization of Bowman–Birk protease inhibitor mRNA

Yuchang Cheng[a,1], James T. Madison[b,2], John F. Thompson[b,*]

[a]*Section of Plant Biology, Division of Biological Sciences, Cornell University, Ithaca, NY 14853, USA*
[b]*US Plant, Soil and Nutrition Laboratory, Agricultural Research Service, United States Department of Agriculture, Tower Road, Ithaca, NY 14853, USA*

Received 21 July 1998; received in revised form 10 December 1998; accepted 10 February 1999

Abstract

Supplemental methionine in a complete culture medium increases the Bowman-Birk-type protease inhibitor (BBPI) level in cultured developing soybean (*Glycine max*, L. Merrill) cotyledons. The increase of BBPI level was paralleled by an increase in the mRNA level. Nuclear run-on analysis showed that transcriptional activities of the BBPI genes were not different between cotyledons cultured in media with or without added methionine.

Kinetics of changes in BBPI mRNA levels suggested a change in BBPI mRNA stability upon adding or omitting methionine from the medium. Direct measurement of BBPI mRNA by the use of cordycepin revealed that BBPI mRNAs were much more stable in cotyledons cultured in medium supplemented with methionine than in cotyledons cultured in medium without methionine. No major difference or heterogeneity in the length of BBPI mRNA poly(A) tails in cotyledons cultured in medium with or without met was seen. Published by Elsevier Science Ltd.

Keywords: *Glycine max*; Leguminosae; Soya bean; Transcriptional activity; Bowman–Birk-type protease inhibitor; mRNA stability; Poly(A)

1. Introduction

Two types of protease inhibitors are found in soybean seeds: the Kunitz inhibitor and the Bowman–Birk-type protease inhibitors (BBPI) (Tan-Wilson & Wilson, 1986). The Kunitz inhibitor has a molecular weight (*M*r) of 21 kDa, containing 3% sulfur amino acids (Tan-Wilson & Wilson, 1986). BBPIs are considerably smaller, (approximately 7–8 kDa) in a single polypeptide chain of 70–80 amino acid residues, about 20% being sulfur amino acids (Tan-Wilson & Wilson, 1986).

At least three different BBPIs have been described in soybean seeds, including the classical Bowman–Birk protease inhibitor, (the one originally identified by Bowman and Birk), the C II inhibitor and the inhibitor PI IV (Tan-Wilson et al., 1987). Genes encoding these proteins contain extensive regions of homology (Tan-Wilson et al., 1987; Joudrier, Foard, Floener, & Larkins, 1987). Both the Kunitz inhibitor and BBPIs are packaged into protein bodies in developing seeds and degraded during germination (Horisberger & Tacchini-Vonlanthen, 1983a, 1983b; Wilson, 1988). The function of these inhibitors is not clear, although roles as a minor storage protein, a regulator of endogenous proteases, or a defense agent against insects have been suggested (Ryan, 1981).

Studies of sulfur nutrition using intact plants have shown that plants grown under sulfur-deficient conditions produce seeds containing decreased amounts of sulfur-rich proteins, along with an increase of seed proteins containing low levels of sulfur-containing

* Corresponding author. Tel.: +1-607-255-0665; fax: +1-607-255-1132.
E-mail address: jft3@cornell.edu (J.F. Thompson)
[1] Present address: Institute of Botany, Academia Sinica, Nankang, Taipei 11529, Taiwan, Peoples Republic of China.
[2] Present address: 1010 Islamorada Blvd., Punta Gorda, FL 33955, USA.

0031-9422/99/$ - see front matter Published by Elsevier Science Ltd.
PII: S0031-9422(99)00107-7

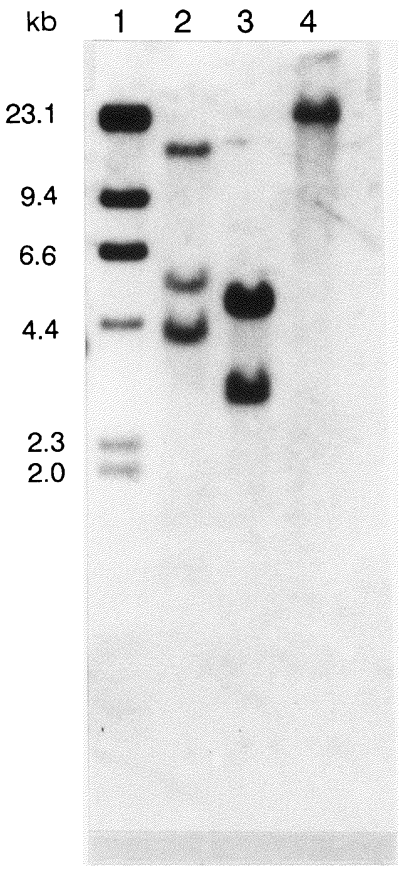

Fig. 1. Southern blot analysis of soybean genome DNA. Total soybean DNA (5 μg) was digested with EcoR I (lane 2), Nde I (lane 3), Xba I (lane 4), and separated on a 0.8% agarose gel. cDNA (clone 6–7) was used as a probe. Lane 1 contains the *Hind* III-digested λ DNA. Numbers on the left side indicate sizes of the *Hind* III fragments.

amino acids (Thompson & Madison, 1992). We have also shown a similar effect when in vitro cultured soybean cotyledons are grown in a complete medium supplemented with methionine (met medium) or a medium without added met (basal medium). Furthermore, by using a differential screening method to screen a cDNA library, we were able to identify a cDNA clone (designated 6–7), that contains the 3′-portion of a mRNA encoding a BBPI, which is sulfur-rich and preferentially expressed in cotyledons cultured in methionine (met) medium (Biermann, de Banzie, Handelsman, Thompson, & Madison, 1998).

In this study, we used the isolated cDNA clone (6–7) as a probe to show that the regulation of BBPI gene expression in cultured soybean cotyledons by met occurs at the post-transcriptional level, mostly due to a met-induced stabilization of BBPI mRNA.

2. Results

2.1. BBPI is encoded by a multigene family

To determine the number of genes that hybridize to clone 6–7 in the soybean genome, the 6–7 cDNA plasmid (Biermann et al., 1998) was used to probe total soybean DNA digested with several restriction enzymes that do not cut within the probe cDNA. Fig. 1 shows that under high stringency conditions (washed with 0.2 × SSC at 65°C), clone 6–7 hybridized with three EcoR I DNA fragments (lane 2), two Nde I DNA fragments (lane 3) and one Xba I DNA fragment (lane 4). No other bands were detected even at low stringency (washed with 2 × SSC at 65°C), (data not shown). These results indicated the presence of a small number of BBPI DNA sequences in the soybean genome.

2.2. BBPI protein level, BBPI mRNA level and transcriptional activity of the BBPI genes in the presence and absence of methionine

The mechanism that results in the increased expression of BBPI genes in cotyledons cultured in met medium was investigated by comparing BBPI protein levels, BBPI mRNA levels and transcriptional activities of BBPI genes between cotyledons cultured in basal medium and cotyledons cultured in met medium. Table 1 row 3 shows that met does not increase the translational efficiency nor does it act at the posttranslational level. The fact that met increases BBPI and its mRNA (rows 1 and 2) to the same extent shows that met does not increase translation efficiency in cotyledons cultured in met medium or decrease BBPI

Table 1
Ratios of BBPI protein level, mRNA level and transcriptional activity of the BBPI genes in soybean cotyledons cultured with or without methionine. Relative levels of Bowman–Birk Protease Inhibitor mRNA and transcriptional activity were measured on an X-ray film with a laser densitometer as described in the experimental section; arbitrary units were used. The average BBPI level was 147 ± 17.3 μg equivalent of lima bean BBPI per gram fresh weight ($n = 5$) for cotyledons cultured in basal medium for 6 d; (Mean ± standard error)

Parameter	Methionine/basal	Number of independent measurements
BBPI protein level	6.8 ± 1.1	6
BBPI mRNA level	6.4 ± 1.3	5
Transcriptional activity of BBPI genes	0.9 ± 0.1	2

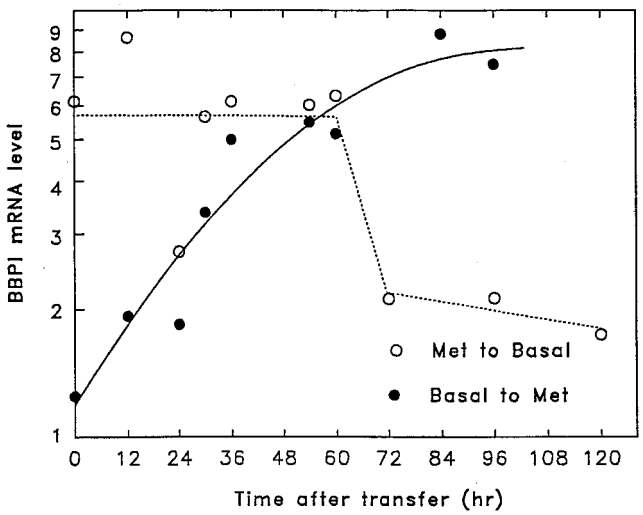

Fig. 2. Kinetic changes of BBPI mRNA in cultured soybean cotyledons after being switched from basal medium to methionine medium or from methionine medium to basal medium. Arbitrary units were used to express the BBPI mRNA level.

protein degradation. These results suggested that the regulation of the BBPI gene expression by met was primarily confined to post-transcriptional changes in the level of BBPI mRNA.

2.3. Kinetics of changes in BBPI mRNA levels

To further understand the control mechanism that elevated the BBPI mRNA level in cotyledons cultured in met medium, the kinetics of changes of BBPI mRNA levels in cultured cotyledons upon addition of met to the basal-medium or removal of met from met medium were characterized.

We cultured cotyledons in basal or met medium for 6 days, and then transferred cotyledons either from basal to met medium or vice versa. Cotyledons were harvested at different times after being transferred and the level of BBPI mRNA at each time point after transfer was determined.

As shown in Fig. 2, the BBPI mRNA level in cultured cotyledons was increased after being transferred from basal to met medium. No significant delay was observed. The BBPI mRNA level continued to increase for about 3 days before a new steady state was reached. On the other hand, a rapid drop of the BBPI mRNA level was observed when cotyledons were transferred from met to basal medium after a lag period of 2–3 days. This lag period correlates well with the decrease of the free met pool in soybean cotyledons when they are transferred from met to basal medium, since Holowach, Madison, & Thompson (1986) showed that a dramatic decrease of the free met pool only occurs 2–4 days after the transfer. Thus, the results suggest changes of the BBPI mRNA level in

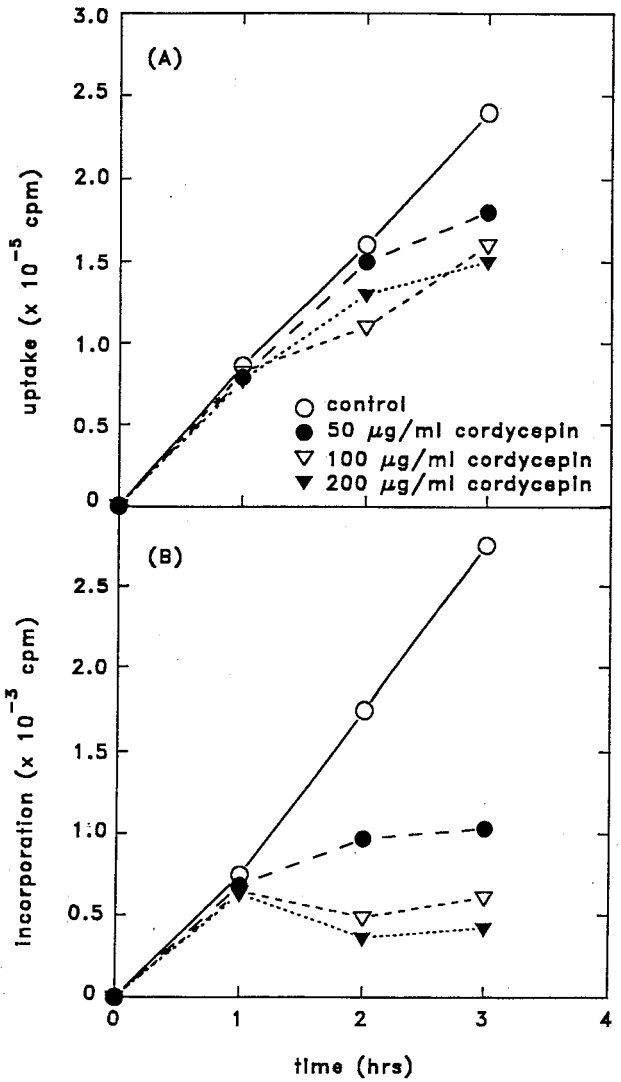

Fig. 3. The effect of cordycepin on uridine uptake and RNA synthesis in soybean cotyledons. (A) Uptake of [^{3}H]uridine (10 μCi/ml) in the presence of various amounts of cordycepin. (B) RNA synthesis in the presence of various concentrations of cordycepin.

cultured cotyledons was dependent on the concentration of the uncombined met. Furthermore, since the time required for change of mRNA levels from one steady state to another steady state is dependent on the mRNA stability (Hargrove & Schmidt, 1989), the difference between kinetics of BBPI mRNA accumulation upon addition of met and the kinetics of BBPI mRNA decrease upon removal of met also indicated an alteration of BBPI mRNA stability by met.

2.4. Measurement of BBPI mRNA stability by using cordycepin

The half-life of BBPI mRNA was directly measured by using the transcription inhibitor, cordycepin (3′-deoxyadenosine), which terminates transcription once

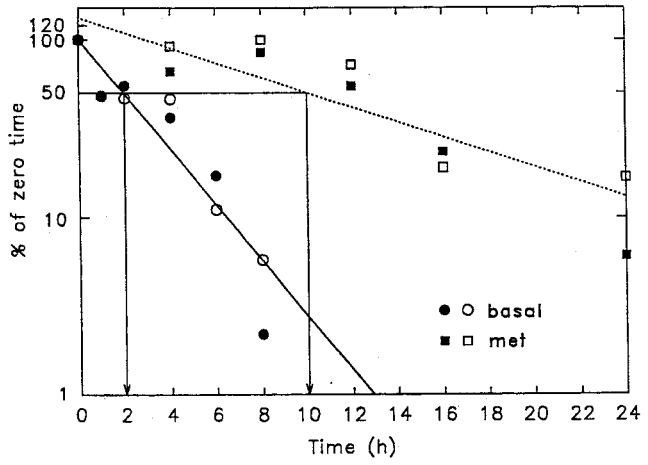

Fig. 4. Decay kinetics of BBPI mRNA in soybean cotyledons cultured in basal or methionine medium. Soybean cotyledons were cultured in basal or met medium for 6 days, and then transferred to the same medium containing 100 μg/ml cordycepin. First samples (time zero) were taken after one hour of cordycepin treatment. The level of BBPI mRNA at each time point was expressed as a percentage of the amount at time zero. The different symbols represent two separate experiments. The lines were determined by the least squares method (sigma plot).

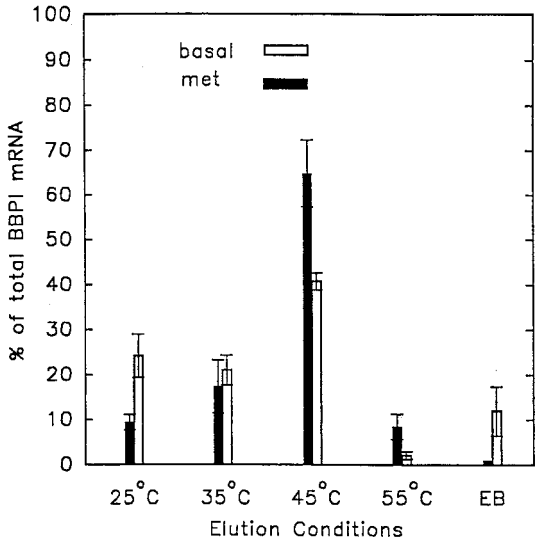

Fig. 5. Poly(U)-Sepharose thermal elution profile of BBPI mRNA in soybean cotyledons cultured in basal or methionine medium. Results presented represent means (± standard error) of three separate experiments and are expressed as percentage of total poly(U)-binding BBPI mRNA.

it is incorporated into RNA (Spiegel & Marcus, 1975). We cultured cotyledons in basal or met medium for 6 days and then transferred them to the same medium (basal to basal, met to met) containing 100 μg/ml cordycepin for 1 h before starting to harvest samples. While the uptake of [^{3}H]-uridine was only slightly inhibited (Fig. 3A), incorporation into RNA (Fig. 3B) was totally inhibited by 100 μg/ml cordycepin after one hour (which was used as zero time in Fig. 4). At different time points, cotyledons were harvested and the level of BBPI mRNA was determined. The decay kinetics of BBPI mRNA are shown in Fig. 4. We estimated that BBPI mRNA had a half-life of about 2 h in cotyledons cultured in basal medium and a half-life of about 10 h in cotyledons cultured in met medium. Thus, we conclude, from the results presented, that an increase in BBPI mRNA stability was the most important factor in the higher level of the BBPI mRNA in cotyledons cultured in met medium as compared to basal medium.

2.5. Poly(A) tail length and BBPI mRNA stability

Shortening of the poly(A) tail of mRNA has been suggested to be the initial and rate-limiting step for the degradation of some eukaryotic mRNAs (Bernstein & Ross, 1989). If the stabilization effect of met on BBPI mRNA was the result of inhibition of shortening of the poly(A) tail of BBPI mRNA, the size distribution of poly(A) tails of BBPI mRNA in cotyledons cultured in basal medium would be different from that in cotyledons cultured in met medium. To characterize the size distribution of the poly(A) tails of BBPI mRNA, thermal elution of total RNA isolated from cotyledons cultured in different media were performed by using a poly(U)-Sepharose column (Jacobson, 1987). As shown in Fig. 5, most BBPI mRNAs, either from cotyledons cultured in met medium or cotyledons cultured in basal medium for 6 days, were eluted at 45°C, which is consistent with the evidence that most cytoplasmic mRNAs have a steady-state distribution of a poly(A) tail about 40–60 nucleotides in length (Jacobson, 1987). Thus, the results showed that there was no major difference in the size distribution between poly(A) tails of BBPI mRNA in cotyledons cultured in basal medium and those in cotyledons cultured in met medium, which suggests that shortening of the poly(A) tail was not the rate-limiting step in BBPI mRNA degradation and was not involved in the met stabilization of BBPI mRNA.

3. Discussion

By using the cDNA plasmid 6–7 as the probe, we have shown that BBPIs in soybean seeds are encoded by a small multigene family. The data are consistent with previous results showing that there are at least three different, but highly homologous, Bowman–Birk type protease inhibitors present in soybean seeds (Tan-Wilson et al., 1987). However, our result is different from that described by Hammond, Foard, & Larkins (1984), who concluded that there is only one single copy gene that encodes the BBPI in the soybean gen-

ome although they used a cDNA clone which is very similar to ours with only 11 bases different out of 125 bases (cf 1 vs 15). Moreover, in our DNA blot (Fig. 1), we did not observe any bands, under high or low hybridization stringencies, that corresponded to those of Hammond et al. (1984), despite the fact that we both used DNA isolated from the same cultivar, Provar. The reason for this discrepancy is not clear and deserves further investigation.

We have also shown that met enhances the level of the BBPIs, which are rich in sulfur amino acids, by stabilizing BBPI mRNA in cultured soybean cotyledons. In a study of the effect of sulfur nutrition on pea seed protein gene expression, it was shown that levels of legumin and albumin 1 mRNAs, both of which encode sulfur-rich proteins, were low in pea seeds of plants grown under sulfur deficiency (Spencer, Rerie, Randall, & Higgins, 1990). When plants are recovering from sulfur deficiency, there is a rapid increase in the level of legumin and albumin 1 mRNAs within 1–2 days, while the transcriptional activities of these genes are not changed during that period. These results suggest that expression of pea legumin and albumin 1 genes in response to sulfur availability are regulated by a post-transcriptional mechanism, which is consistent with the results reported here. Comparable examples of regulation of synthesis of proteins in seeds by the availability of nutrient sulfur have also been obtained in other species (e.g., lupin, barley, cowpea, rape, sunflower and wheat; reviewed in Spencer et al. (1990)). In all cases, plants grown under sulfur-deficient conditions produce seeds containing decreased amounts of sulfur-rich proteins, along with an increase of seed proteins containing a low level of sulfur amino acids. Furthermore, the met effect on soybean seed protein synthesis has also been demonstrated in transgenic petunia seeds by transferring a gene encoding the β subunit of β-conglycinin into petunia (Fujiwara, Hirai, Chino, Komeda & Naito, 1992). It appears that plants of different species use a common mechanism to regulate seed protein synthesis, depending upon the availability of sulfur. By changing the stability of mRNA in response to environmental factors, plants could adapt to their environment quickly and use their nutritional resources more efficiently (Hargrove & Schmidt, 1989).

Messenger RNA turnover is now recognized as a major control point in the regulation of gene expression (Alber & Green, 1996). It has been shown that some mRNAs can be degraded from their 3′ end by first removal of the poly(A) tail (e.g., c-*fos* and c-*myc* mRNAs in mammalian cells (Bernstein & Ross, 1989)). Nevertheless, some mRNAs are probably degraded initially through an endonucleolytic cleavage (e.g., 9E3 mRNA in chicken cells (Stoeckle & Hanafusa, 1999)), and subsequently the intermediates are degraded by exonucleases. Results of thermal elution of BBPI mRNA from a poly(U)-Sepharose column did not support the idea that removal of poly(A) tails is the rate-limiting step in degradation of BBPI mRNA (Fig. 5). In spite of the fact that the proportion of BBPI mRNA of cotyledons cultured in basal medium that eluted at 25°C was higher than those cultured in met medium (Fig. 5), we suggest that this elevated level could represent partially degraded BBPI mRNA (possibly generated by endonucleolytic cleavage), which would be expected to be higher in cotyledons cultured in basal medium than in cotyledons cultured in met medium. The increase in the proportion of BBPI mRNA of cotyledons cultured in basal medium eluted in the elution buffer (EB) fraction reflects the fact that BBPI mRNA is less stable in cotyledons cultured in basal medium since destabilization of a mRNA species increases the percentage of the newly synthesized mRNA, which would contain longer poly(A) tails, in the whole mRNA population.

In any event, the mechanism by which BBPI mRNAs are stabilized in cotyledons cultured in met medium remains to be established. We anticipate that its elucidation will facilitate our long-term effort to improve soybean seed quality by increasing the sulfur content in seed proteins.

4. Experimental

4.1. Plant material and culture

Soybean plants (*Glycine max* L. Merr. cv Provar) were grown in a glasshouse in a mixture of sand and soil on a modified Hoagland's nutrient solution (Thompson, Madison, & Muenster, 1977). Immature soybean cotyledons (20–50 mg) were cultured in a complete medium (Thompson et al., 1977) which included 1.5 mM sulfate (basal medium). For met medium, L-met was added to the basal medium at a final concentration of 8.4 mM.

4.2. Measurement of Bowman–Birk type protease inhibitor

Bowman–Birk type protease inhibitor activity was assayed as described by Biermann et al. (1998) where benzoyl-DL-arginine p-nitroanilide was used as the substrate with purified lima bean inhibitor as a reference.

4.3. Isolation of DNA and RNA

DNA was isolated from soybean leaf tissue as described (Saghai-Maroof, Soliman, Jorgensen, & Allard, 1984). Total RNA was isolated from soybean

cotyledons by the method of Wadsworth, Redinbaugh, & Scandalios (1988).

4.4. Southern blots

Isolated soybean genomic DNA (5 μg) was digested with different restriction enzymes, electrophoresed in 0.8% agarose, and transferred to a GeneScreen[3] membrane (DuPont) (Maniatis, Fritsch, & Sambrook, 1982). Hybridization was performed in 5 × SSC, 5 × Denhardt's solution, 5% dextran sulfate, 0.5% SDS, 100 μg/mL of salmon sperm DNA, 50 mM sodium phosphate, pH 6.5, at 65°C overnight (Maniatis et al., 1982). The hybridization probes were prepared by random hexamer primer labeling of linearized cDNA clone 6–7 and phage λ DNA with α-[^{32}P]dATP using the Multiprime DNA labeling system (Amersham). After hybridization, the membrane was washed in 0.2 × SSC at 65°C, (or 2 × SSC at 65°C for low stringency), and autoradiographed at −70°C with Kodak X-Omat AR film.

4.5. Quantification of BBPI mRNA levels

Since, using cDNA 6–7 as a probe, only a single band was detected on Northern blots (Biermann et al., 1998), we used slot blots to measure BBPI mRNA levels in cultured cotyledons. Total RNA, 5 or 10 μg, was blotted onto a GeneScreen membrane by using a Bio-Dot SF apparatus (Bio-Rad), and then hybridized with the labeled cDNA 6–7 probe as described above. After hybridization, the membrane was washed in 0.3 × SSC at 65°C. For quantification of BBPI mRNA levels, hybridization signals were estimated using a laser densitometer (LKB). In each case, a duplicate membrane was hybridized to a labeled clone, pGmr-1 (a gift from Dr E. Zimmer), which contains the soybean 18 S and 25 S ribosomal gene sequences, and these hybridization signals were used to normalize the BBPI signals.

4.6. Nuclear run-on analysis

Nuclei were isolated from soybean cotyledons as described by Cox and Goldberg (1988). Nuclei containing about 20 μg of DNA were used for each nuclear run-on assay. The assay conditions were the same as described by Cox and Goldberg (1988). Reactions were terminated after 15 min, and labeled RNA was isolated as described (Marzluff & Huang, 1985). Hybridization was carried out in DNA excess by blotting 2 μg of linearized BBPI cDNA 6–7 and rDNA (pGmr-1) onto a GeneScreen membrane under the conditions described above. After hybridization, the membrane was washed in 0.3 × SSC at 65°C, and autoradiographed. The autoradiograph was quantified by laser densitometry. The transcriptional activity of BBPI genes was estimated after the signal hybridized to cDNA 6–7 was normalized to the signals hybridized to rDNA.

4.7. Measurement of cordycepin effects on uridine uptake and RNA synthesis

Soybean cotyledons (100–130 mg) were labeled with [5,6-^{3}H]uridine (40 Ci/mmol)(10 μCi/ml) in the presence of various concentrations of cordycepin. At different times, cotyledons were harvested, washed and ground in 2 volumes (w/v) of the extraction buffer (5% SDS, 0.15 M LiCl, 0.05 M EDTA and 0.05 M Tris-HCl, pH 9). Debris was removed by centrifugation at 10,000 *g* for 30 min, aliquots of the supernatant were taken, and the RNA in the remaining supernatant was precipitated by 5% TCA. Radioactivities in the supernatant and the precipitate were measured by liquid scintillation as the total uptake of uridine and the synthesis of RNA, respectively.

4.8. Elution of RNAs from poly(U)-Sepharose column

Thermal elution of RNAs from poly(U)-Sepharose columns was performed as described in Jacobson (1987). RNA eluted in each fraction was blotted onto a GeneScreen membrane and hybridized with the labeled cDNA 6–7 probe as described.

[3] Mention of company names or commercial products does not imply recommendation or endorsement by the United States Department of Agriculture over others not mentioned.

Acknowledgements

We thank Dr E. Zimmer for providing us the pGmr-1 clone. We also thank Drs J. B. Nasrallah and D. B. Stern for their critical reviews of the manuscript. The Martin Scholarship fund provided crucial support.

References

Alber, M. L., & Green, P. J. (1996). Control of mRNA stability in higher plants. *Plant Molecular Biology*, *32*, 63.

Bernstein, P., & Ross, J. (1989). Poly(A), poly(A) binding protein and the regulation of mRNA stability. *Trends in Biochemical Science*, *14*, 373.

Biermann, B. J., de Banzie, J. S., Handelsman, J., Thompson, J. F., & Madison, J. T. (1998). Methionine and sulfate increase a Bowman-Birk-type protease inhibitor and its messenger RNA in soybeans. *Journal of Agricultural and Food Chemistry*, *46*, 2858.

Cox, K. H., & Goldberg, R. B. (1988). Analysis of plant gene ex-

pression. In C. H. Shaw, *Plant molecular biology: a practical approach* (p. 1). Oxford: IRL Press.

Fujiwara, T., Hirai, M. Y., Chino, M., Komeda, Y., & Naito, S. (1992). Effects of sulfur nutrition on expression of the soybean seed strorage protein genes in transgenic petunia. *Plant Physiology*, *99*, 263.

Hammond, R. W., Foard, D. E., & Larkins, B. A. (1984). Molecular cloning and analysis of a gene coding for the Bowman-Birk protease inhibitor in soybean. *Journal of Biological Chemistry*, *259*, 9883.

Hargrove, J. L., & Schmidt, F. H. (1989). The role of mRNA and protein stability in gene expression. *Federation of American Societies of Experimental Biology Journal*, *3*, 2360.

Holowach, L. P., Madison, J. T., & Thompson, J. F. (1986). Studies on the mechanism of regulation of the mRNA level for a soybean storage protein subunit by exogenous L-methionine. *Plant Physiology*, *80*, 561.

Horisberger, M., & Tacchini-Vonlanthen, M. (1983a). Ultrastructural localization of Bowman-Birk inhibitor on thin sections of *Glycine max* (soybean) cv. Maple Arrow by the gold method. *Histochemistry*, *77*, 37.

Horisberger, M., & Tacchini-Vonlanthen, M. (1983b). Ultrastructural localization of Bowman-Birk inhibitor on thin sections of *Glycine max* (soybean) cv. Maple Arrow by the gold method. *Histochemistry*, *77*, 313.

Jacobson, A. (1987). Purification and fractionation of poly(A)$^+$ RNA. *Methods in Enzymolology*, *152*, 254.

Joudrier, P. E., Foard, D. E., Floener, L. A., & Larkins, B. A. (1987). Isolation and sequence of cDNA encoding the soybean protease inhibitors PI IV and C-II. *Plant Molecular Biology*, *10*, 35.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982). *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Marzluff, W. F., & Huang, R. C. C. (1985). Transcription of RNA in isolated nuclei. In B. D. Hames, & S. J. Higgins, *Transcription and translation: A practical approach* (p. 89). Oxford, UK: IRL Press.

Ryan, C. A. (1981). Proteinase inhibitors. In A. Marcus, *Biochemistry of plants*, *vol. 6* (p. 351). New York: Academic Press.

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., & Allard, R. W. (1984). Ribosomal DNA spacer-length polymorphism in barey: Mendalian inheritance, chromosomal location and population dynamics. *Proceedings of the National Academy of Science USA*, *81*, 8014.

Spencer, D., Rerie, W. G., Randall, P. J., & Higgins, T. J. V. (1990). The regualtion of pea seed storage protein genes by sulfur stress. *Australian Journal of Plant Physiology*, *17*, 355.

Spiegel, S., & Marcus, A. (1975). Polyribosome formation in early wheat embryo germination independent of either transcription or polyadenylation. *Nature*, *256*, 228.

Stoeckle, M. Y., & Hanafusa, H. (1999). Processing of 9E3 mRNA and regulation of its stability in normal and Rous sarcoma virus-transformed cells. *Molecular and Cellular Biology*, *9*, 4738.

Tan-Wilson, A. L., & Wilson, K. A. (1986). Relevance of multiple soybean trysin inhibitor forms to nutritional quality. In M. Friedman, *Nutritional and toxicological significance of enzyme inhibitors in foods* (p. 391). New York: Plenum Press.

Tan-Wilson, A. L., Chen, J. C., Duggan, M. C., Chapman, C., Obach, R. S., & Wilson, K. A. (1987). Soybean Bowman-Birk trypsin isoinhibitor: classification and report of a glycine-rich trypsin inhibitor class. *Journal of Agricultural and Food Chemistry*, *35*, 974.

Thompson, J. F., & Madison, J. T. (1992). The effect of sulfate and methionine on legume proteins. In H. Rennenberg, C. H. Brunold, L. J. De Kok, & I. Stulen, *Sulfur nutrition and sulfur assimilation in higher plants* (p. 145). The Hague, Netherlands: SBP Academic Publishing BV.

Thompson, J. F., Madison, J. T., & Muenster, A. E. (1977). In vitro culture of immature cotyledons of soya bean (*Clycine max* L. Merr.). *Annals of Botany*, *41*, 29.

Wadsworth, G. J., Redinbaugh, M. G., & Scandalios, J. G. (1988). A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. *Analytical Biochemistry*, *172*, 279.

Wilson, K. A. (1988). The proteolysis of trysin inhibitors in legume seeds. *Critical Review of Biotechnology*, *8*, 197.