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Regulation of storage-protein synthesis in pea (*Pisum sativum* L.) cotyledons under conditions of sulphur deficiency

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The effects of sulphur deficiency on the expression of storage-protein genes in developing pea (*Pisum sativum*) cotyledons were studied. Legumin-gene transcription was decreased by S-deficiency, but not to the same extent as the decrease in the level of legumin mRNA. Vicilin-gene transcription was not significantly affected. Control of gene expression may thus occur during transcription and/or post-transcriptional events.

INTRODUCTION

When peas (*Pisum sativum*) are grown under conditions of sulphur deficiency, the levels of legumin, one of the two major seed storage proteins, and other relatively sulphur-rich proteins are reduced in the mature seeds (Randall *et al.*, 1979), whereas levels of vicilin, the other major storage protein, which contains no sulphur amino acids, are slightly increased. Pulse–chase labelling experiments carried out with immature cotyledons have demonstrated a greatly reduced level of legumin synthesis (80–88% reduction) under conditions of S-deficiency (Chandler *et al.*, 1983), and assays using cloned cDNA species as specific probes were used to demonstrate that this reduction was primarily due to reduced levels of legumin mRNA. Furthermore, levels of legumin mRNA and legumin synthesis were shown to increase in S-deficient plants shortly after the restoration of S-supply, suggesting that regulation of legumin-gene expression at the transcriptional level was occurring in response to change in sulphur availability. Similar studies have been carried out on a number of seed proteins, both of legume (Chandler *et al.*, 1984) and cereal species (Shewry *et al.*, 1983).

The aim of our investigation was to establish whether or not the decreased amounts of legumin synthesized in S-deficient plants were a result of control of gene expression at the level of transcription of the legumin genes.

MATERIALS AND METHODS

Plant material

Plants were grown from pea (*Pisum sativum* L., variety Feltham First) seeds under normal and S-deficient conditions as previously described (Evans *et al.*, 1979; Gatehouse *et al.*, 1982), except that chlorides were substituted for sulphates in the aqueous nutrient solution (at 8 mM concn.) to give sulphur deficiency. Under S-deficient conditions, the plants were smaller with fewer pods.

Isolation of total protein and total RNA

Total protein was isolated from developing cotyledons as described earlier (Gatehouse *et al.*, 1982). Identical volumes of extracts from equal weights of freeze-dried cotyledons were analysed by SDS/polyacrylamide-gel electrophoresis with 17% (w/v) acrylamide in the separating gel, and the gel was stained with Coomassie Blue. The protein levels were quantified by scanning the gel electrophoretogram with a Gilford 2000 spectrometer.

The isolation of total RNA from pea cotyledons was performed by the SDS/proteinase K method of Hall *et al.* (1978). RNA was assayed by absorbance, assuming $A_{260}^{1mg/ml} = 24$. The RNA was glyoxalated and analysed on 1.5%-(w/v)-agarose gel as described by Gatehouse *et al.* (1982).

Isolation of nuclei and transcription

Nuclei were isolated from fresh cotyledons by using the procedure described by Evans *et al.* (1984). Final preparations were stored at −80 °C in 50% (v/v) glycerol. The nuclei were examined by fluorescence microscopy and counted by means of a modified Fuchs–Rosenthal haemocytometer.

Transcription in isolated nuclei in 140 μl assay mixtures containing [α-^{32}P]UTP, at 26 °C for 3 min and 45 min, and the isolation of RNA from transcribed nuclei, were described by Evans *et al.* (1984).

cDNA plasmids

The preparation and isolation of cDNA clones specific for pea legumin (pDUB 3 and pDUB 6, previously referred to as 'pRC 2.11.7' and 'pAD 4.4'), pea vicilin M_r 47000 [pDUB 7 (pAD 3.4)], pea vicilin M_r 50000 [pDUB 2 (pRC 2.2.1)], and pBR 322 are described elsewhere (Croy *et al.*, 1982; Lycett *et al.*, 1983, 1984). Inserts were isolated from the cDNA plasmids and labelled with ^{32}P by nick-translation (Gatehouse *et al.*, 1982).

Assays of mRNA species

The detection and quantitative comparison of single mRNA species in total RNA preparations from developing cotyledons were carried out by the 'Northern Blot' technique. RNA species were denatured with glyoxal, subjected to electrophoresis on 1.1%-agarose gels, transferred to nitrocellulose sheets and hybridized to cDNA probes as previously described (Gatehouse *et al.*, 1982). After hybridization, filters were dried and

Abbreviations used: d.a.f., days after flowering; SDS, sodium dodecyl sulphate.

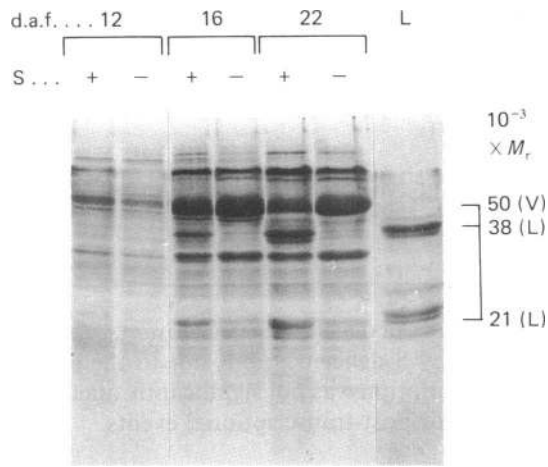

Fig. 1. SDS-/polyacrylamide-gel-electrophoretic pattern of protein extracts from developing pea cotyledons of plants grown under normal (S^+) and S-deficient conditions (S^-)

Abbreviations used: V, vicilin; L, legumin. Track L is the legumin standard.

autoradiographed at −70 °C with an intensifying screen. The levels of mRNA species were quantified by scanning the X-ray film with a Gilford 2000 spectrometer.

Assays of specific nuclear transcripts.

Plasmid cDNAs were restricted with endonuclease *Bam*H1 (pDUB 3, pDUB 6, pDUB 7 and pDUB 2) or *Eco*R1 (pBR 322) (according to the suppliers' instructions), and the digests were electrophoresed on 0.7% agarose gels, transferred to nitrocellulose sheets by the 'Southern' technique and hybridized to ^{32}P-labelled nuclear transcripts as described in detail by Evans *et al.* (1984). Similar numbers of nuclei from control and S-deficient cotyledons were taken for transcription, and specific cDNA species immobilized on to nitrocellulose filters were hybridized to the same amount of input [^{32}P]RNA radioactivity (c.p.m.). Recombinant plasmid pBR 322 was used as a control on each filter. Filters were autoradiographed by exposure to Fuji RX X-ray film by using a Du Pont Lightning Plus intensifying screen at −80 °C for 14 days. The levels of specific run-off transcripts in isolated nuclei were estimated by laser densitometry [2202 Ultrascan densitometer (LKB, Bromma, Sweden) equipped with 2220 recording integrator] of the X-ray films and comparison of peak areas.

RESULTS

The stages of seed development in pea var. Feltham First under the standard growth conditions employed have been described previously (Gatehouse *et al.*, 1982). Cotyledon development occurs during the period 8–21 d.a.f. under these conditions. Accumulation of the major storage proteins, legumin (M_r 38000 and M_r 21000) and vicilin (M_r 50000 and M_r 47000) in control and S-deficient plants was examined in two separate experiments by SDS/polyacrylamide-gel electrophoresis (Fig. 1) and quantitative densitometry of the stained gels. Vicilin and legumin polypeptides were at higher levels [1.4(±0.2)–1.8(±0.4)-fold] in control compared with S-deficient plants at early to mid-cotyledon development stages. As development proceeded, the ratio of accumulated legumin in control compared with S-deficient plants increased [to 2.6(±0.4)-fold by the end of cotyledon development], whereas the ratio of accumulated vicilin 50000-M_r polypeptide in cotyledons of control compared with S-deficient plants decreased [to 0.64(±0.07)-fold by the end of cotyledon development], so that S-deficient plants had higher levels of this polypeptide than had the control. Vicilin 33000-M_r polypeptide (the product of post-translation proteolysis of the 47000-M_r precursor polypeptide) followed a pattern of accumulation in control and S-deficient plants similar to that of the vicilin 50000-M_r polypeptide, suggesting that expression of all the vicilin genes was affected in essentially the same way.

The relative levels of storage-protein mRNA species in control and S-deficient plants midway (15 d.a.f.) through

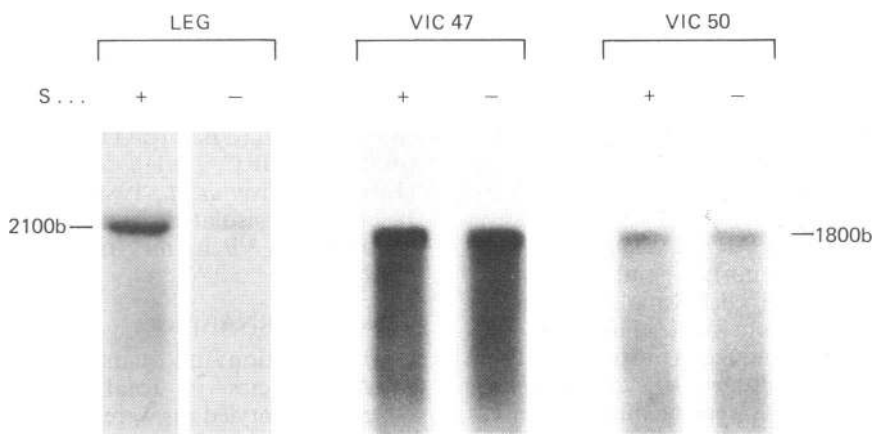

Fig. 2. 'Northern'-blot assays of specific mRNA species in total RNA from normal and S-deficient cotyledons

Total RNA (10 μg/track) from 15 d.a.f. pea cotyledons of plants grown under normal (S^+) and sulphur-deficient (S^-) conditions were glyoxalated, electrophoresed on 1.1% agarose gel, blotted on to nitrocellulose paper and hybridized to the specific ^{32}P-labelled cDNA inserts (4.6×10^6 c.p.m.): LEG, pDUB 6 for legumin, sp. radioactivity 3.6×10^7 c.p.m./μg; VIC 47, pDUB 7 for M_r-47000 vicilin, sp. radioactivity 7.5×10^7 c.p.m./μg; VIC 50, pDUB 2 for M_r-50000 vicilin, sp. radioactivity 3.4×10^7 c.p.m./μg. 2100b and 1800b indicate legumin and vicilin mRNA species respectively.

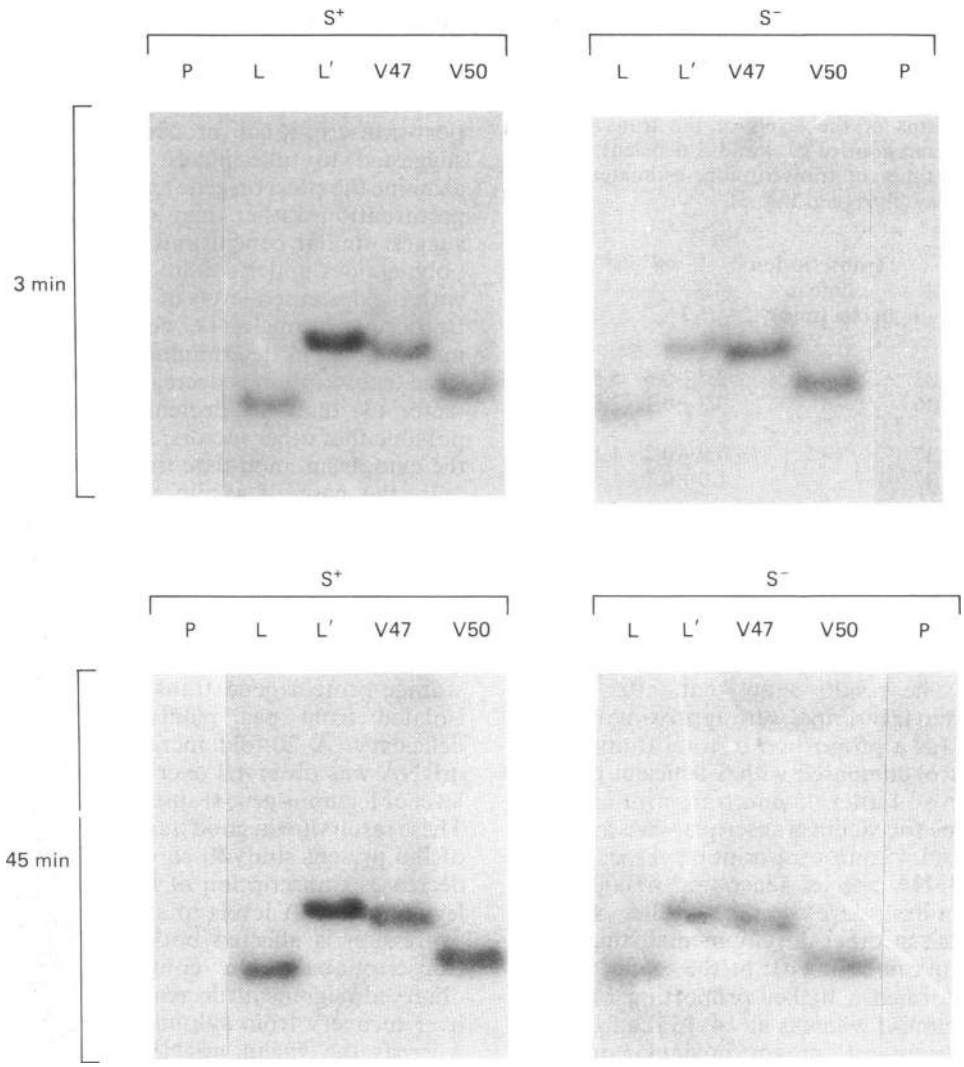

Fig. 3. Hybridization of ^{32}P-labelled transcripts synthesized during 3 min and 45 min transcription by nuclei (6.5×10^5) isolated from pea cotyledons (15 d.a.f.) of plants grown under normal (S^+) and S-deficient (S^-) conditions to specific cDNA plasmids immobilized on to nitrocellulose filters

Plasmid DNA species (5 μg/tract), containing inserts for pea legumin (lane L, pDUB 3; lane L′, pDUB 6), vicilin M_r 47000 (lane V47, pDUB 7), vicilin M_r 50000 (lane V50, pDUB 2) and vector pBR 322 (lane P), were digested with restriction enzymes, separated on 0.7% agarose gels, transferred to nitrocellulose and hybridized to ^{32}P-labelled transcripts (7.2×10^6 c.p.m.).

cotyledon development were estimated in duplicate by Northern-blot hybridizations of total cotyledon RNA to cDNA plasmids shown to encode the different polypeptides. Results (Fig. 2) showed that legumin mRNA levels were decreased in S-deficient plants, whereas levels of vicilin mRNA species (as detected by either of the vicilin cDNA probes) were little changed. Quantitative densitometry of two autoradiographs gave mean ratios of mRNA species in control and S-deficient plants of 6.0($\pm$2.0) for legumin, compared with 1.3($\pm$0.1) for vicilin. Control and S-deficient plants yielded similar amounts of total RNA per g fresh weight of cotyledons at this developmental stage.

The synthesis of mRNA species was investigated by assay of specific transcripts produced by nuclei isolated from control and S-deficient plants. Similar numbers of nuclei per g fresh weight of cotyledons were isolated at corresponding developmental stages under both treatments. Transcripts were labelled by incubating nuclei with labelled UTP, and specific transcripts were identified by hybridization of total transcripts to 'Southern' blots of specific cDNA plasmids. Relative levels of specific transcripts were estimated by densitometry of the resulting autoradiographs. We have previously established a linear relationship between the intensity of the band and the amount of ^{32}P-labelled RNA used in the hybridization for this type of DNA transfer (Evans *et al.*, 1984). Transcripts were isolated after 3 min (at which time transcription was occurring at a constant rate) and at 45 min (when transcription was essentially complete as measured by acid-precipitable radioactivity). Fig. 3 shows one of the results from two experiments of hybridizing transcripts produced by nuclei isolated from cotyledons of S-deficient and control plants at a mid-cotyledon development stage (15 d.a.f.) to the seed storage-protein cDNA species, and quantitative analysis

Table 1. Quantification of transcription in isolated nuclei of pea cotyledons (15 d.a.f.) from plants grown under control and S-deficient conditions

Given are mean ratios of the levels of the transcripts (duplicate assays) from control (S^+) and S-deficient (S^-) cotyledons, at two times of transcription, estimated by densitometry of X-ray films (e.g. Fig. 3).

Seed protein	cDNA plasmid	Transcription time in nuclei (min)...	S^+/S^- 3	S^+/S^- 45
Legumin	pDUB3		2.7±0.6	5.5±0.4
	pDUB6		2.3±0.1	2.8±0.4
Vicilin				
47000 M_r	pDUB7		0.9±0.2	1.1±0.1
50000 M_r	pDUB2		1.0±0.1	1.5±0.2

of the transcription results is given in Table 1. The ratios of the specific transcripts are given as mean values from duplicate assays with the corresponding deviations from the mean values. The results show that, after 3 min transcription, legumin transcripts were approximately 2.5 times as abundant (as a proportion of total transcripts) in nuclei from control compared with S-deficient plants; this ratio was approx. 4 after 45 min transcription. The corresponding ratios for vicilin transcripts were approx. 1.0 and 1.3 for 3 and 45 min transcription respectively; both the vicilin cDNA species (encoding 47000- and 50000-M_r polypeptides) gave similar results. Similar results were obtained at other developmental stages (12 and 14 d.a.f.) (results not shown); at the earlier stage, vicilin transcripts formed a higher proportion of total transcripts than legumin, whereas at 14–15 d.a.f. vicilin and legumin transcripts were present in similar proportions, in agreement with previous results (Evans *et al.*, 1984).

DISCUSSION

The present results for levels of accumulated storage-protein polypeptides in the cotyledons of developing seeds in S-deficient compared with control pea plants are consistent with those reported by other authors. However, we were unable to obtain as great a decrease in accumulated legumin as that reported by Chandler *et al.* (1983), who used a pea variety (Greenfast) with a low legumin content, whereas pea var. Feltham First has a high legumin content. As the two pea varieties also show differences in their developmental patterns of protein synthesis [compare results in Chandler *et al.* (1984) with those in Gatehouse *et al.* (1982)], the present results are not wholly comparable with previous reports. However, in agreement with those previous reports, a marked decrease in levels of legumin mRNA under conditions of S-deficiency was observed, whereas levels of vicilin mRNA species were not significantly altered.

Assays of specific transcripts produced by nuclei isolated from cotyledons of developing pea seeds have shown that a correlation exists between the accumulation of mRNA species and the relative activities of transcription of the appropriate genes, and it was concluded that control of gene expression was being exerted at the transcriptional level (Evans *et al.*, 1984). However, control of expression by other factors, such as post-transcriptional processing of mRNA, was also suggested to take place. The present results, which examine the effect on gene expression of an environmental perturbation, rather than a developmental progression, suggest similar conclusions. The lower level of legumin polypeptides under conditions of S-deficiency correlates with the decreased levels of legumin mRNA and reduced transcripts in nuclei, i.e. control of transcription is the major factor in determining gene expression. However, since transcription is decreased by a significantly smaller factor (3) than the decrease in mRNA level (6), it is possible that other factors, such as stability of mRNA in the cytoplasm, modulate the transcriptional control.

In the case of vicilin-gene expression, we do not observe a higher level of transcription in S-deficient plants compared with control, although the S-deficient plants accumulate more vicilin, and thus the observed difference seems to be due wholly to post-transcriptional factors.

While this paper was in preparation, a paper by Beach *et al.* (1985) was published examining the changes in storage-protein gene transcription in cotyledon nuclei isolated from pea plants recovering from sulphur deficiency. A 20-fold increase in the level of legumin mRNA was observed over the recovery period, but the level of legumin-gene transcription increased only 2-fold. These results are in good qualitative agreement with those of the present study in showing that sulphur deficiency decreases transcription of legumin genes, but decreases legumin mRNA levels to a greater extent, i.e. that gene expression is affected both at transcription and post-transcriptionally. In contrast, Beach *et al.* (1985) observed a significant decrease in vicilin-gene transcription over recovery from sulphur deprivation (up to 6-fold), whereas the vicilin mRNA level decreased only very slightly. Under our conditions, no corresponding effect of S-deficiency on vicilin-gene transcription was observed.

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