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Nucleotide Metabolism in Germinating Seeds

THE RIBONUCLEIC ACID OF *PISUM ARVENSE*

BY G. R. BARKER AND JOSÉ A. HOLLINSHEAD
Department of Chemistry, The University, Manchester 13

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Preliminary studies have been reported (Barker & Douglas, 1960) of the variations of RNA, ribonuclease and protein in the cotyledons and in the root and shoot of the developing pea seedling, and a possible mechanism was suggested for the translocation of RNA from the cotyledon to the growing parts of the plant. This possibility is now being investigated. Earlier experiments were carried out with *Pisum sativum* (var. English Wonder), but in subsequent batches of seed, germination and growth under the cultural conditions used showed considerable variation between individual plants, and it was not possible to obtain reliable values for the RNA content of the plants in the early stages of growth. Much more uniform results have been obtained with seed of *Pisum arvense*. The present paper records experiments designed to establish conditions for studying translocation of nucleotide material from cotyledon to the growing seedling. The RNA content of the cotyledons and the course of the incorporation of [³²P]phosphate during the early stages of growth of *P. arvense* have been measured. A satisfactory method is described for the determination of RNA in cotyledon tissue.

METHODS AND MATERIALS

Total phosphate was determined by the method of Fiske & Subbarow (1925). Difficulty was experienced in decolorizing the solution after incineration, but this was achieved by the addition of 1 drop of 60% (w/v) perchloric acid followed by 1 drop of 100-volume hydrogen peroxide. Pentose was determined by the method of Drury (1948). All analyses were carried out in duplicate.

Ribonucleic acid of Pisum arvense. Peas were soaked for 36 hr. in water at 22°, removed from the water and cooled to 0°. A sample (200 g.) was ground with solid CO₂, and crude RNA was prepared by the method of Markham (1955). The crude material was dissolved in dilute NaOH at pH 8 (50 ml.) and brought to pH 7. A small residue was removed by centrifugation and pure RNA was extracted with 90% (w/v) phenol by the method of Kirby (1956). The purified material had a value for $\epsilon(P)$, as defined by Chargaff & Zamenhof (1948), of 8100. The $E_{280\text{ m}\mu}/E_{260\text{ m}\mu}$ ratio was 0.49. Warburg & Christian (1941–42) give a value of 0.49 for the $E_{280\text{ m}\mu}/E_{260\text{ m}\mu}$ ratio for yeast RNA. This value for the ratio indicates the presence of 0.1% of protein by Layne's (1957) method of calculation; according to Kalckar's (1947) equation the value corresponds to a complete absence of protein.

Determination of ribonucleic acid. The method of Smillie & Krotkov (1960) was modified to make it suitable for cotyle-

don tissue. Material for analysis (approx. 30 mg.) was homogenized in a glass homogenizer. Acid-soluble and lipid material was removed from the homogenate by the method of Smillie & Krotkov (1960), except that 95% (v/v) ethanol saturated with sodium acetate was used for the initial extraction of lipid (Marko & Butler, 1951) to avoid degradation of nucleic acids. The dried residue was extracted for 1 hr. at room temperature with 1.5 ml. of *N*-NaOH and centrifuged. The process was repeated with 1 ml. of *N*-NaOH. The combined supernatants were treated with 4 ml. of absolute ethanol and 3.25 ml. of acetic acid, and the precipitated DNA-protein complex was centrifuged and washed twice by centrifugation with aq. 70% (v/v) ethanol brought to pH 4 with acetic acid. The combined supernatants that constitute the RNA extracts gave no test for deoxypentose by the Ceriotti (1952) reaction; the DNA-protein complex contained no pentose.

Nucleotide material in the RNA extracts was measured by: (a) determination of pentose, (b) determination of total phosphorus, and (c) measurement of the extinction at 260 μ . In each case calibration curves were used based on RNA prepared as described above. The results are recorded in Table 1 (columns a, b and c). Examination of the ultra-violet-absorption spectrum of the RNA extracts indicated gross contamination with protein. A correction for the contribution to the extinction at 260 μ from protein in the RNA extracts was therefore made as described below. A sample (0.2 ml.) of the RNA extract was brought to pH 7 (bromothymol blue) with *N*-NaOH, the required volume of alkali being determined on a separate sample. Protein was determined in the resulting solution by the method of Lowry, Rosebrough, Farr & Randall (1951). Similar analyses were carried out with crystalline bovine plasma albumin and a graph was constructed relating extinction at 750 μ obtained in the above analyses and the extinction at 260 μ of solutions of the protein at the same concentration. A linear relationship was found in the range used. The extinction at 260 μ of the RNA extract from the cotyledons was then corrected for its content in protein by using this graph. The validity of the method of correction was tested as follows.

Samples of cotyledon were extracted as described by Smillie & Krotkov (1960). Protein was determined in one sample of the RNA fraction and the concentration of RNA was calculated from the extinction at 260 μ after correction as described above. A further sample of the RNA fraction was purified by ion-exchange chromatography as described by Smillie & Krotkov (1960), and the RNA concentration in the original extract was calculated from the extinction of the purified material at 260 μ . In view of the results obtained in five series of experiments (Table 2) the method of analysis and correction used to obtain the results in Table 1 (column d) was adopted for the present work.

Growth of seed. The seed used was *P. arvense* (var. Minerva Maple). Germination and growth of seedlings were carried out in a ventilated growth box maintained at $22 \pm 1^\circ$ and illuminated by fluorescent tubes (Mazda 'warm white') giving an illumination of between 1500 and 1800 ft. candles at a distance of 30 in. below the tubes. *P. arvense* resembles *P. sativum* in being a long-day plant (Moore & Bond, 1962), and continuous illumination was found to be satisfactory.

Effect of phosphate on initial growth. Batches of peas were soaked for 17 hr. in 100 ml. of KH_2PO_4 solutions of various

concentrations. The seed was then planted in vermiculite and watered with distilled water daily. After 7 days the lengths of the radicles were measured. The results are shown in Table 3.

Method of culture. To ensure that conditions used for the incorporation of phosphate into the germinating seed allowed normal growth, plants were grown to maturity as described below. The complete nutrient solution was similar to that of Hewitt (1952), ferric citrate being replaced by ferric ethylenediaminetetra-acetate (Jacobson, 1951). The solution contained, in 2.5 ml.: $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (2 g.), KNO_3 (0.85 g.), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.95 g.), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.5 g.), ferric ethylenediaminetetra-acetate solution (25 ml.) and micro-nutrient solution (2.5 ml.). The ferric ethylenediaminetetra-acetate solution contained, in 180 ml.: 0.4 g. of tetrasodium ethylenediaminetetra-acetate and 1.5 ml. of 10% (w/v) FeCl_3 . The micro-nutrient solution contained, in 250 ml.: $\text{MnCl}_2 \cdot \text{H}_2\text{O}$ (0.5 g.), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05 g.), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.16 g.), H_3BO_3 (0.5 g.) and Na_2MoO_4 (0.0125 g.). The phosphate-free nutrient solution had the same composition

Table 1. *Comparison of methods for determining ribonucleic acid in cotyledons of Pisum arvense*

Tissue was homogenized, and, after the removal of methanol-soluble material, acid-soluble material and lipids, nucleotides derived from RNA were extracted as described in the Methods and Materials section. The RNA content of the cotyledons was calculated from determinations of (a) pentose, (b) total phosphorus and (c) extinction at 260 μ . In column (d) are given values obtained after correction of the value of the extinction at 260 μ for the presence of protein as described in the text.

Sample	RNA content (mg./sample) calculated by method			
	(a)	(b)	(c)	(d)
A	0.36	0.24	0.29	0.25
B	0.17	0.14	0.14	0.13
C	0.15	0.36	0.26	0.14
D	0.12	0.43	0.13	0.06
E	0.14	0.39	0.17	0.07

Table 2. *Assessment of reliability of method of correcting analyses for ribonucleic acid*

The RNA fraction was prepared from samples of cotyledon tissue as described in the Methods and Materials section. The RNA concentration in the RNA extract was calculated (i) from measurements of extinction at 260 μ , (ii) from measurements of extinction at 260 μ after correction for the presence of protein and (iii) from measurements of the extinction at 260 μ after purification of the RNA extract by treatment with ion-exchange resin as described in the text.

Sample	RNA content (mg./sample) calculated by method		
	(i)	(ii)	(iii)
F	0.44	0.33	0.36
G	0.31	0.20	0.22
H	0.53	0.39	0.38
I	0.34	0.19	0.18
J	0.41	0.25	0.26

as the complete nutrient solution except for the replacement of the NaH_2PO_4 by Na_2SO_4 (0.25 g.). Batches of ten peas were treated under the following conditions:

(a) Group A. Peas were soaked in distilled water for 17 hr., sown in vermiculite in crystallizing dishes and watered daily with distilled water.

(b) Group B. Peas were soaked for 17 hr. in 0.01M- KH_2PO_4 , sown as above, supplied after 4 days with 50 ml. of phosphate-free nutrient solution supplemented at weekly intervals with a further 10 ml. of the same solution and watered daily with distilled water.

(c) Group C. Peas were soaked for 17 hr. in distilled water, sown as above, supplied after 4 days with 50 ml. of complete nutrient solution, supplemented at weekly intervals with 10 ml. of the same solution and watered daily with distilled water.

After 2 weeks, plants in group A showed stunted growth relative to those in groups B and C, but later resumed normal growth. No distinction could be observed between plants in groups B and C. Flowers appeared at 8 weeks in group B, 9 weeks in group C, and 10 weeks in group A.

Peas were grown under conditions B above, with 0.01M- $\text{KH}_2^{32}\text{PO}_4$ of various specific radioactivities up to 250 $\mu\text{Ci/l.}$ No difference was detected over a period of 15 days in the germination and in the growth of shoot and root between peas grown under these conditions and those grown under conditions B with non-radioactive phosphate.

Radioactivity experiments. Radioactivity was measured in a liquid counter type 6M (20th Century Electronics Ltd., Croydon, Surrey) having an efficiency of 8.1%. All measurements were carried out in triplicate, counting being continued until a statistical error of not more than 5% was obtained. Radioactivities were corrected for decay to the beginning of the experiment.

To ascertain the optimum conditions for the uptake of orthophosphate, batches of ten peas were soaked for various times in 10 ml. of 0.01M- $\text{KH}_2^{32}\text{PO}_4$ having a specific radioactivity of approx. 250 $\mu\text{Ci/l.}$ (obtained from The Radiochemical Centre, Amersham, Bucks., and diluted with non-radioactive 0.01M- KH_2PO_4 before use to give the required specific radioactivity). The seed was rinsed with water, blotted with filter paper and homogenized in 75 ml. of water. The homogenate was diluted with water to 250 ml. Total phosphate was determined in a sample (0.4 ml.) of the resulting suspension, the solution being diluted to 10 ml. before measurement of the intensity of the blue colour. Measurements of radioactivity were made on the solutions used in the colorimetric determination of phosphorus and corrections made for the absorption by molybdate ions. Samples (0.1 ml.) of the original solution and of the solution remaining after removal of the peas were diluted to 100 ml. and the radioactivities of the diluted solutions were measured. The results are shown in Table 4.

RESULTS

Ribonucleic acid content of cotyledons. Peas were soaked in water and grown under condition C described above. At intervals samples of cotyledon tissue were analysed for RNA as described in the Methods and Materials section. The results from two experiments are shown in Fig. 1 (curve A).

Peas were grown as above, and 3 days after sowing the roots were removed 1 cm. from the cotyledons and the shoots removed at the plumule arch. The cut surfaces were covered with lanolin,

Table 3. *Effect of phosphate concentration on initial growth of Pisum arvense*

Batches of peas were soaked for 17 hr. in KH_2PO_4 of various concentrations, planted in vermiculite, and after 7 days the lengths of the radicles were measured. The results are given as means $\pm$ S.E.M. with the numbers of peas indicated in parentheses. The effect of low and high ionic strength is indicated by the difference between the mean length ($\pm$ S.E.M.) of radicle at the concentration indicated and the mean length of radicle at the optimum concentration.

Concn. of KH_2PO_4 (M) ...	0.005	0.01	0.02	0.04	0.06	0.08
Mean lengths of radicle (mm.)	63.7 $\pm$ 3.6 (10)	89.3 $\pm$ 9.3 (10)	78.4 $\pm$ 5.3 (10)	66.5 $\pm$ 11.3 (9)	65.7 $\pm$ 11.5 (9)	41.2 $\pm$ 12.3 (10)
Difference of means	25.6 $\pm$ 9.9	—	10.9 $\pm$ 10.7	22.8 $\pm$ 14.6	23.6 $\pm$ 14.8	48.1 $\pm$ 15.1

Table 4. *Uptake of [^{32}P]phosphate by seed of Pisum arvense*

Batches of ten peas were soaked in 10 ml. of 0.01M- $\text{KH}_2^{32}\text{PO}_4$ (0.1 ml. diluted with water to 100 ml. gave 434.7 $\pm$ 9.4 counts/min.), removed and homogenized. Radioactivity and total phosphorus were determined in samples (1/625) of the homogenate as described in the Methods and Materials section. After removal of the peas, radioactivity was determined in samples (0.1 ml.) of the remaining solution (approx. 10 ml.) after dilution to 100 ml. with water. The radioactivities are given as means $\pm$ S.E.M.

Time of soaking (hr.) ...	4	8	10.5	13	17	20	24
Radioactivity in sample of homogenate (counts/min.)	45.0 $\pm$ 1.9	93.2 $\pm$ 2.6	116.8 $\pm$ 2.1	113.8 $\pm$ 2.5	281.0 $\pm$ 5.2	214.1 $\pm$ 4.0	228.5 $\pm$ 4.7
Total phosphorus in sample (mg.)	0.021	0.020	0.019	0.017	0.019	0.019	0.021
Relative specific radioactivity	1.53	3.32	4.38	4.78	10.54	8.04	7.76
Radioactivity in remaining liquid (counts/min.)	455.4 $\pm$ 6.0	482.6 $\pm$ 6.0	488.1 $\pm$ 4.6	497.4 $\pm$ 8.6	380.6 $\pm$ 5.9	416.9 $\pm$ 5.9	402.0 $\pm$ 6.0

and the peas were replanted in vermiculite and grown as described above. Samples of cotyledon tissue were analysed for RNA and the results are shown in Fig. 1 (curve B).

Incorporation of [32 P]phosphate into cotyledons. Peas were soaked for 17 hr. in 0.01M-potassium dihydrogen [32 P]phosphate (specific radioactivity approx. 45 μ Ci/l.) (100 ml. for ten peas), and grown in vermiculite under condition B as described in the Methods and Materials section. At intervals samples of cotyledon tissue (30–50 mg.) from about five seeds were weighed, homogenized in 5 ml. of methanol and fractionated by the method of Smillie & Krotkov (1960, p. 43). Radioactivity was determined in the methanol-soluble fraction (10 ml.) and the acid-soluble fraction (10 ml.). The crude dried nucleic acid fraction (residue C; Smillie & Krotkov, 1960, p. 43) was extracted twice with 5 ml. of 5% (w/v) perchloric acid at 90° for 15 min. The combined extracts were diluted to

10 ml. and the radioactivity was determined. The results are shown in Fig. 2. In view of the results obtained with the crude nucleic acid fraction, this was not separated into the RNA and DNA components. The lipid and protein fractions were devoid of radioactivity. In one experiment the acid-soluble extract from cotyledons taken 5 days after planting (10 ml.) was neutralized, 10N-sulphuric acid and 5% (w/v) ammonium molybdate were added and the resulting solution (20 ml.) was extracted twice with 10 ml. of 2-methylpropan-1-ol to remove inorganic phosphate (cf. Ochoa, Krakow & Basilio, 1963). The radioactivities of the combined 2-methylpropan-1-ol extracts and of the residual aqueous phase were respectively 174.1 ± 4.3 and 717.5 ± 8.5 counts/min.

DISCUSSION

Growth of seed. The results shown in Table 3 indicate that treatment of seed with 0.01M-potassium dihydrogen phosphate stimulates the growth of the radicle over the first 7 days compared with seeds treated with phosphate at lower concentration. This stimulation is statistically significant. At higher concentrations, growth is not significantly depressed until the molarity reaches 0.08. Mulvani & Pollard (1939) found that germination of various seeds takes place most readily in solutions of low ionic strength, and the results suggest that the same applies in the present case. In the earlier stages of the treatment with 0.01M-potassium dihydrogen phosphate, water is taken up preferentially as shown by the increase in the specific radioactivity of the ambient solution during the first 13 hr. (Table 4); during the subsequent period phosphate ions are taken up preferentially. In view of these results and the fact that uptake of water may be a limiting factor in germination, it was decided to supply orthophosphate to peas before germination in 0.01M-solution. Growth of plants to maturity indicated that the administration of phosphate before germination as in method B (see the Methods and Materials section) had no deleterious effect. Under these conditions [32 P]phosphate was satisfactorily taken up within 17 hr. (Table 4) and no radiation effect was observed with the amounts of radioactivity used.

Determination of ribonucleic acid. Various modifications of the method of Schmidt & Thannhauser (1945) for the separation of the RNA fraction from DNA for analytical purposes have been described (for review of methods see Fleck & Munro, 1961). Smillie & Krotkov (1960), working with plant material, used a single extraction at 37° for 16 hr. with 0.3N-sodium hydroxide. On the other hand, one extraction with N-alkali at room temperature has been recommended for this type of tissue

Fig. 1. Changes in RNA content of cotyledons during germination of *P. arvense*: Δ , $\bullet$, two experiments with normal plants; $\blacksquare$, cotyledons from which root and shoot were removed.

Fig. 2. Incorporation of [32 P]orthophosphate into the methanol-soluble (Δ), acid-soluble ($\bullet$) and RNA + DNA ($\blacksquare$) fractions of the cotyledons of *P. arvense*.

(Cooper & Loring, 1957; Heyes, 1960). In the present experiments with pea cotyledons it was found that a short extraction with *N*-sodium hydroxide was preferable in that less protein was extracted under these conditions. On the other hand between 25 and 37 % of the nucleotides of RNA remained unextracted by one treatment with *N*-sodium hydroxide for 1 hr. at room temperature; two extractions removed not less than 98 % of the ribonucleotides from the crude nucleic acid fraction. By this method of treatment contamination of the RNA fraction was reduced to a minimum.

The results recorded in Table 1 (columns *a*, *b* and *c*) show no correlation between the three values obtained for RNA cotyledon tissue based respectively on determinations of pentose, phosphorus and extinction at 260 $m\mu$. The first two of these methods may give errors due to contamination of the RNA extracts with variable amounts of non-nucleotide pentose-containing and phosphorus-containing materials. Spectroscopic determination of nucleotides in RNA extracts is subject to error due to absorption at 260 $m\mu$ by protein material, and no entirely satisfactory method is known for eliminating this. A method of estimating the error due to protein has been put forward by Fleck & Munro (1962), but in the present series of experiments negative values for RNA were sometimes obtained by their method. Difficulty in preparing a specimen of cotyledon protein free from nucleotide material prevented a more reliable correction being made by this method. Similar difficulties arise in the use of Layne's (1957) and Kalckar's (1947) methods for estimating gross contamination by protein. The weakness of all these methods stems from the fact that, not only does protein material contribute to the extinction at the wavelength of maximal absorption by the nucleotides, but, conversely, nucleotides contribute to extinction at the wavelength used for estimating the extent of contamination by protein. In the method of correction now put forward, the contribution to the absorption at 260 $m\mu$ by protein is calculated from measurements of extinction at 750 $m\mu$ which are not affected by the presence of nucleotide material. The choice of protein used for estimating the correction is arbitrary, since, even if it had been practicable to obtain a nucleotide-free protein from the cotyledon tissue, the possibility would have remained that such a preparation might differ in composition from the protein present in the RNA extracts. However, the method adopted is justified by the agreement between results obtained by its use and those given by the ion-exchange technique of Smillie & Krotkov (1960) (columns ii and iii in Table 2). Treatment of RNA fraction with ion-exchange resin was introduced by Smillie & Krotkov (1960) for the removal of contaminants other

than protein, but this treatment has been found to be satisfactory for removal of peptide material from RNA (De Deken-Grenson & De Deken, 1959). The method described in the present paper is more convenient for general purposes than the use of ion-exchange resins, and is considered to be satisfactory for the study of changes in RNA content of the cotyledons during growth. It is not claimed that the procedure is applicable directly to other tissue and, as with other methods, its suitability for a given material would need to be checked by comparison with other analytical procedures.

Ribonucleic acid content of cotyledons. The results expressed in Fig. 1 (curve *A*), though showing some variation between results from the two experiments, indicate that a decrease of the RNA content of the cotyledons occurs throughout the first 2 weeks of growth and that the rate of decrease is greatest at about the third and fourth days. This tendency is in agreement with results recorded for cotyledons of *Vigna sesquipedalis* (Osawa & Oota, 1953), although in this case depletion of cotyledon RNA was complete in about 1 week. In other examples strict comparison is difficult. Thus Cherry & Hageman (1961) record a general decrease in the RNA of the scutellum of germinating corn seed, although an initial rise in the RNA of the mitochondrial fraction was observed. On the other hand, an increase has been observed in the RNA content of germinating peanuts (Marcus & Feeley, 1962), but this case, in which germination is epigeal, is probably not comparable. The peanut seedling shows other differences from those of *P. arvense* that are discussed below.

Incorporation of [32 P]phosphate into the cotyledon. The results discussed above differ from those recorded for peanut cotyledons (Marcus & Feeley, 1962) and those previously obtained with *P. sativum* (Barker & Douglas, 1960) in exhibiting no initial rise in the RNA of the cotyledons. In an attempt to study the transport of RNA from the cotyledons to the growing parts of the plant, seeds were germinated in the presence of inorganic [32 P]phosphate. The results obtained (curve *A* in Fig. 2) indicate that the RNA of the cotyledons is not appreciably labelled by [32 P]orthophosphate, although it is shown that seeds germinate and grow normally when supplied exogenously with orthophosphate under the conditions used. Further, there is active phosphorus metabolism in the tissue since less than 20 % of the radioactivity of the acid-soluble fraction is due to inorganic phosphate. These results are in essential agreement with the situation in germinating seed of *V. sesquipedalis* (Oota & Osawa, 1954), although incorporation in this case was measured over only a short period of time. Thus it appears that in the cotyledons of *P. arvense* and *V. sesquipedalis* neither net synthesis of RNA nor exchange between RNA

phosphorus and inorganic phosphate occurs. By contrast, in the peanut cotyledon, where an initial increase of RNA occurs, labelling of the RNA by [^{32}P]orthophosphate is observed (Marcus & Feeley, 1962). Thus, in the various cases quoted, the results of analyses of RNA and of labelling experiments are in agreement, and it is suggested that the situation in the peanut on the one hand and in *P. arvense* and *V. sesquipedalis* on the other reflect a metabolic distinction between seeds exhibiting epigeal and hypogeal germination respectively. Owing to the difficulties encountered in germinating *P. sativum* satisfactorily for analytical purposes, we are unable at present to assess the situation in that organism.

Transport of ribonucleic acid. From the results expressed in Fig. 1 (curve A) and Fig. 2, it appears that cotyledon RNA does not undergo active metabolism. However, the results obtained with plants from which root and shoot had been removed (curve B in Fig. 1) suggest, in agreement with Oota & Osawa (1954), that the normal decrease in cotyledon RNA is not due to autolysis, but is due to a translocation brought into play by the growing plant. This conclusion is also in accordance with general considerations discussed by Varner, Balce & Huang (1963). It is not known whether or not the labelling of the acid-soluble fraction observed in the present experiments involves ribose-bound phosphate residues. This is at present under investigation, and, as yet, the occurrence of degradation and resynthesis of cotyledon RNA cannot be completely excluded. It cannot therefore be decided whether translocation occurs at the macromolecular level as envisaged by Ledoux & Huart (1962), or whether it occurs at a lower level of molecular complexity as suggested by Barker & Douglas (1960).

SUMMARY

1. A method is described for the determination of RNA which is suitable for cotyledon tissue.

2. The RNA content of the cotyledons of germinating seed of *Pisum arvense* falls during the first 2 weeks of growth. This decrease is delayed by the removal of the root and shoot.

3. The acid-soluble fraction of the cotyledons is labelled by exogenously supplied [^{32}P]orthophosphate, but no significant labelling of the cotyledon RNA is observed.

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