Natural Inhibitors of Germination and Growth, VII
Synthesis of Ribulosebisphosphate Carboxylase in Darkness and Its Inhibition by Coumarin

Helena Burghardt, Harald Brunner, Ralf Oelmüller, Friedrich Lottspeich*, Ulrike Oster and Wolfhart Rüdiger

Botanisches Institut der Universität München, Menzinger Straße 67, D-80638 München, Bundesrepublik Deutschland
* Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-82152 Martinsried, Bundesrepublik Deutschland

Z. Naturforsch. **49c,** 321–326 (1994); received March 10, 1994

Dedicated to Prof. Aloysius Wild on the occasion of his 65th birthday

Lepidium sativum, Cruciferin, Ribulosebisphosphate Carboxylase, Inhibition of Transcription

Cress *(Lepidium sativum)* seeds were germinated in darkness. Seedlings were investigated for soluble proteins by SDS-PAGE. Two proteins were identified by microsequencing: the small subunit of ribulosebisphosphate carboxylase (SSU) and the alpha subunit of the storage protein cruciferin. Net synthesis of small and large subunits of ribulosebisphosphate carboxylase (SSU and LSU) was investigated by Western blot. Net synthesis of both subunits was inhibited by coumarin. To the contrary, net synthesis of cruciferin was increased by coumarin. With specific cDNA probes, we determined steady state levels of the corresponding mRNAs (rbcS mRNA for SSU, rbcL mRNA for LSU). Both mRNAs can be detected in dry seeds; their amount increases during germination in the dark. Incubation with coumarin inhibits this increase. Inhibition of development by coumarin on the level of transcription is discussed.

Introduction

Coumarin (2H-1-benzopyran-2-one) is known since a long time as regulator of plant growth and development (review: Brown, 1981). Being produced by some plants, coumarin is listed as an allelophatic chemical (Putnam, 1983; Valio, 1973). In this connection, mainly its action as germination inhibitor has been considered (Williams and Hoagland, 1982; Reynolds, 1989). It inhibits root growth similar as several phenolic acids do (Glass, 1976). Tolerant species are believed to metabolize coumarin and detoxify it in this way (Sivan *et al.*, 1965). However, there are effects different from this inhibitory action: Svensson (1972) reported *e.g.* an increase of net DNA synthesis, decrease of net RNA synthesis and increase of the protein content per cell by coumarin in roots of maize and wheat. The lack of interaction of coumarin with several metabolic inhibitors led Svensson (1972) to the conclusion that coumarin effects already existing structures or enzymes which were not defined however.

In the course of our studies on protein patterns during germination of cress seeds, we characterized a seed protein as carboxy terminal fragment of the heat shock protein HSP70 (Oster *et al.*, 1992). This fragment occurs naturally in dry seeds but disappears normally during germination. Coumarin inhibits the degradation of this fragment. The opposite behaviour was found for a 17 kDa protein. We describe here its identification as the small subunit of ribulosebisphosphate carboxylase. This observation prompted a study on the accumulation of both subunits (SSU and LSU) of this enzyme on protein and mRNA level during germination of cress seeds in the dark and the influence of coumarin on these processes.

Materials and Methods

Treatment of plant material

Seeds of garden cress (*Lepidium sativum* L.) were either germinated with water ("water control") or treated with coumarin under otherwise identical conditions. Coumarin (final conc. 9×10^{-3} M) was applied to the filter paper as described before (Oster *et al.*, 1992). Cress seeds

Reprint requests to Prof. Dr. W. Rüdiger.
Telefax: (089) 17861-185.

0939–5075/94/0500–0321 $ 03.00

(15 per Petri dish, diameter 4.5 cm) were evenly distributed on the filter paper which was moistured with 1.5 ml of either water or coumarin solution. The Petri dishes were then incubated at 24 °C and 80% relative humidity in the dark for the indicated time. Sowing and harvesting was performed under dim-green safelight.

Extraction of soluble proteins

Seeds or seedlings of 4 Petri dishes were collected, frozen with liquid nitrogen and homogenized with 8 ml 50 mM Tris/HCl, pH 8.3, with mortar and pistil. The homogenate was centrifuged at 12,000×*g* for 15 min. Precooled acetone (final conc. 70%, v/v) was then added to the supernatant and the mixture incubated at −80 °C for 15 h. The protein precipitate was then separated by centrifugation (8000×*g* for 15 min) and dissolved in 100 µl 9.5 M urea. Protein was determined according to Lowry *et al.* (1951). The solution was diluted with sample buffer (Laemmli, 1970) to a final conc. of 4 µg protein/µl.

Electrophoresis and blotting procedure

Electrophoresis was performed according to Laemmli (1970) on gels (1 mm) with 12% acrylamide. The stacking gel was modified according to Makowsky and Ramsby (1993). 200 µg protein were applied per lane. The proteins were then transfered by the "semi-dry" method (Kyhse-Andersen, 1984) to either nitrocellulose (for immunodetection) or to glass fiber sheets (for sequence analysis). Immunodetection was performed according to Beisiegl (1986); the antisera against LSU from *Sinapis alba* and SSU from *Pisum sativum.* respectively, were applied in a 1:500 dilution. Microsequencing was performed according to Eckerskorn *et al.* (1988) after collection of the respective band from 10 lanes.

RNA isolation and hybridization

Total RNA was prepared according to Paulsen and Bogorad (1988) with the modifications of Kittsteiner *et al.* (1991). Labelling of DNA probes were performed with random primers (Feinberg and Vogelstein, 1983).

Results and Discussion

In a previous paper (Oster *et al.*, 1992), we compared protein patterns of dry seeds, dark-grown seedlings and coumarin-treated seeds of *Lepidium sativum* L. In the course of these experiments, we noticed a protein band sized about 17 kDa in the fraction of soluble proteins which was detectable after Coomassie staining only in the seedlings but not in the dry seeds or coumarin-treated seeds (Fig. 1). In order to identify the protein, we separated soluble proteins by SDS-PAGE, blotted the proteins of 10 lanes (200 µg total protein each) to siliconized glass fiber and applied the collected spots to gas phase sequencing. The resulting N-terminal sequence I K V W P P I G K K K F identified the protein as the small subunit of ribulosebisphosphate carboxylase/oxygenase (SSU, Fig. 2). The homology with known sequences of SSU from higher plants is high (between 58 and 83% identity).

In order to investigate the formation of SSU in dark-grown seedlings in more detail, we investigated extracts from seeds, seedlings and coumarin-treated seeds by immunostaining. Besides an antiserum against SSU, another antiserum against the large subunit of ribulosebisphosphate carboxylase/

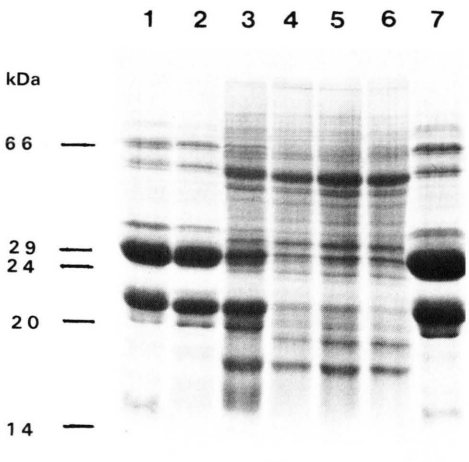

Fig. 1. Separation of soluble proteins extracted from etiolated cress seedlings grown for the indicated time. 1 = dry seeds; 2–6 = germinated in water for 24 h (2), 48 h (3), 72 h (4), 96 h (5), 120 h (6); imbibed with 9 mM coumarin for 48 h (7). Extracted proteins (see Materials and Methods) were applied to the gel (200 µg protein per lane). The gel was stained with Coomassie Brilliant Blue. Arrows indicate marker proteins.

A

```
                5         10        15        20        25        30
  1  N T D I T S N G E R V K C↑M Q V W P P I G K K K F E T L S Y
 31  L P P L T R D Q L L K E V E Y L L R K G W V P C L E F E L L
 61  K G F V Y G E H N K S P R Y Y D G R Y W T M W K L P M F G T
 91  T D P A Q V V K E L D E V V A A Y P E A F V R V I G F N N V
121  R Q V Q C I S F I A H T P E S Y
```

B

I K V W P P I G K K K F	*Lepidium sativum* (this paper)
M Q : : : : : : : : :	pea, *cv.* Feltham Fist
M Q : : : : : : : : :	soybean
M Q : : : : L : L : : :	spinach
M Q : : : : Y : : : : Y	tobacco
M Q : : : I E : I : : :	wheat
M Q : : : : E : L : : :	*Lemna gibba*
M S M K T L P K E R R :	*Synechococcus* PCC 6301
M S : : T L P K E R R Y	*Anabaena* PCC 7120

Fig. 2. Alignment of the N-terminal sequence of the 17 kDa protein from cress seedlings with sequences of SSU. A: Comparison with the SSU precursor from *Pisum sativum* (Bedbrook *et al.*, 1980). The cleavage site between signal peptide and mature SSU is indicated by arrow. B: Alignment with N-terminal partial sequences of SSU from different species. Data from Andrews and Lorimer (1987).

oxygenase (LSU) was used in these studies. Both subunits are present already in dry seeds (Fig. 3). During the first 24 h of imbibition, the amount of both subunits remains as low as in the dry seeds. We observe an increase of the amount of both subunits during germination in the dark, *i.e.* 48 to 72 h after imbibition (lanes 3 and 4). No further increase is found upon prolonged incubation in the dark, *i.e.* 96 and 120 h after imbibition (lanes 5 and 6). In the presence of coumarin, no increase in the content of SSU or LSU can be observed 48 h after imbibition (lane 7). The amount of both subunits remains at the level of that in dry seeds under these conditions. This means that neither net synthesis nor net degradation is observed in the presence of coumarin. This differs from the situation with cruciferin (see below): we observed an increase in the amount of cruciferin during incubation with coumarin (Fig. 1). The increase demonstrates that translation in general is not inhibited by coumarin.

The anti-SSU antiserum gives a positive cross-reaction also with LSU, with the 32 kDa fragment of HSP70 (Oster *et al.*, 1992) and a major protein of apparent size 30 kDa. We determined also the identity of this protein by microsequencing: the sequence R Q S L G V P P Q L G N E identified it as the α-subunit of cruciferin, the storage protein of Brassicaceae (Fig. 4). The cross-reaction is significantly weaker than the proper reaction with SSU. We assume that a few epitopes in the cross-reacting proteins are similar to the authentic epitopes in SSU. The protein bands which react – besides LSU – with the anti-LSU antiserum are probably proteolysis products of LSU, indicating degradation in spite of constant accumulation, *i.e.* a steady turnover of LSU in the dark.

Synthesis of a protein in germinating seeds can either be derived from mRNA pre-existing in seeds or from mRNA newly formed during germination. In order to test this situation in cress, we determined the steady state levels of mRNA for

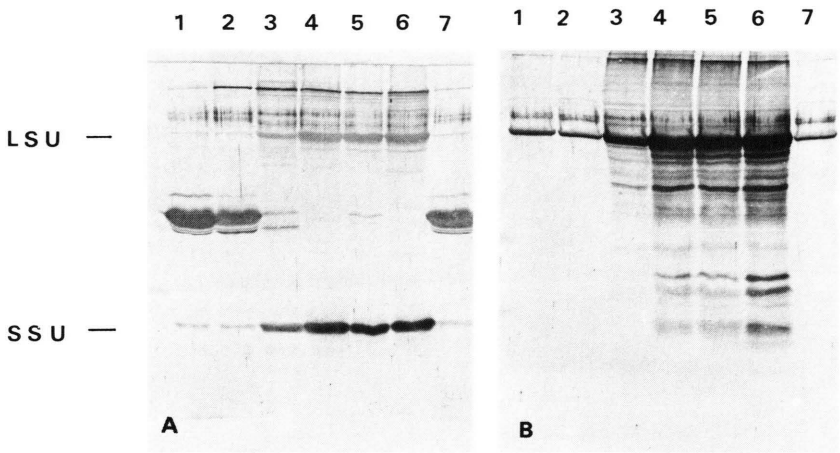

Fig. 3. Immunostaining of subunits of ribulosebisphosphate carboxylase/oxygenase in cress plants (*Lepidium sativum* L.). Immunostaining A: with anti-SSU antiserum, B: with anti-LSU antiserum. Lanes 1–7 as in Fig. 1. Position of SSU and LSU are indicated by arrows.

1	R	Q	S	L	G	V	P	P	Q	L	G	N	E	13	*Lepidium sativum* (this paper)
61	:	:	:	:	:	:	:	:	:	:	:	:	A	96	*Rhaphanus sativus* (Depigny-This *et al.*, 1992)
61	:	:	:	:	:	:	:	:	:	:	:	:	A	96	*Brassica napus* (Rödin *et al.*, 1990, 1992)
88	:	:	:	:	:	:	:	:	:	:	Q	:	:	126	*Arabidopsis thaliana* (Raynal *et al.*, 1990)

Fig. 4. Comparison of the N-terminal sequence of the 30 kDa protein from cress seedlings with sequences of the alpha subunit of cruciferin. Identical amino acids are indicated by colon.

SSU (rbcS mRNA) and LSU (rbcL mRNA) under the same physiological conditions as for protein analysis (Fig. 5). We find low but detectable levels of both rbcS and rbcL mRNA in dry seeds. This level does not change significantly until 24 h after imbibition. The levels are definitely higher than the levels in roots of 96 h old seedlings. The level of both mRNAs increases stepwise during further incubation with water in the dark; in 96 h old seedlings, it is about 4- to 5-fold for rbcL mRNA and about 10-fold for rbcS mRNA compared to the level in seeds. A further increase is observed in light-grown seedlings. The light-induced increase is more pronounced for rbcL mRNA than for rbcS mRNA. Coumarin-treated seeds contain the same amounts of both mRNAs as dry seeds even 48 h after imbibition.

These results suggest that new synthesis of both mRNAs occurs during germination in the dark and that this synthesis is inhibited by coumarin. Increased steady state levels can principally also be achieved by a decreased rate of degradation even with a constant raté of synthesis. We consider this alternative unlikely in our plant material; it would imply a high rate of degradation in dry seeds that are generally known for slow metabolism. During germination, the high rate of degradation should be maintained only in the presence of the inhibitor coumarin but should decline without the inhibitor. The likely explanation of the observed effects is the inhibition of transcription of rbcS and rbcL genes by coumarin. It cannot be decided at this moment whether coumarin is an inhibitor of transcription or whether inhibits specifically the activation of a gene (our group of genes) responsible for development.

Many papers deal with rbcS and rbcL gene expression and its regulation (reviews: Lehmann and Parthier, 1985; Manzara and Gruissem, 1988; Dean *et al.*, 1989; Spreitzer, 1993). Developmental control can be considered as the basic regulation which operates in the dark and in the light in both dicotyledonous (Berry *et al.*, 1985; Degenhardt *et al.*, 1991; Harn *et al.*, 1993) and monocotyledonous plants (Rapp and Mullet, 1991). Developmental regulation of rbcS transcripts in connection with source-sink transcriptions has recently been discussed in correlation with metabolic factors related to the carbohydrate content (Krapp *et al.*, 1993); it remains to be shown whether such a regulation is of general importance. Developmental regulation is superimposed by light regu-

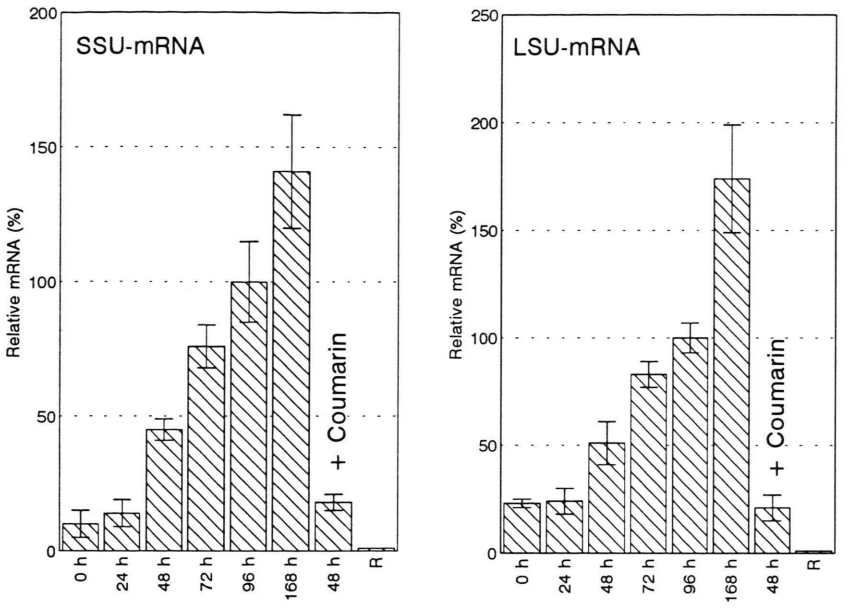

Fig. 5. Steady state levels of rcbS and rbcL mRNA in cress seedlings grown in darkness for the indicated time. Equal amounts of total RNA were dot-blotted and hybridized with labelled probes for rbcS and rbcL. Values are means of 3 experiments. 0 h = dry seeds; 168 h = plants grown under white light; R = roots of 96 h etiolated plants.

lation. Light regulated synthesis of SSU is primarily under transcriptional control: regulation of rbcS gene expression *via* phytochrome is well documented. Light regulation of the synthesis of LSU is possibly quite different: evidence for translational control has been accumulated in this case (Sivan *et al.*, 1965; Inamine *et al.*, 1985). This complex regulation leads finally to a stoichiometric accumulation of both subunits in the mature enzyme. However, the accumulation of the subunits and their mRNAs is not strictly coordinated under all experimental conditions (Berry *et al.*, 1985; Harn *et al.*, 1993).

Very early stages of development do not contain measurable amounts of LSU or SSU peptides and rbcL mRNA or rbcS mRNA in *Amaranthus* cotyledons (Berry *et al.*, 1985) or in barley primary leaves (Rapp and Mullet, 1991). The developmental state of dry cress seeds and 24 h-old seedlings (this paper) corresponds to about 3 day-old *Amaranthus* seedlings (Berry *et al.*, 1985) or leaf sections 3 to 5 (1–4 cm from the leaf base) of dark-grown 4 day-old barley plants (Rapp and Mullet, 1991): we find both LSU and SSU mRNAs and peptides in the dark. We find furthermore cab mRNA 48 h-old dark-grown seedlings (Kittsteiner *et al.*, 1991); this corresponds also the section 5 of the barley leaf (Rapp and Mullet, 1991). Later developmental stages in the dark contain smaller amounts of cab mRNA in both plants (Kittsteiner *et al.*, 1991; Rapp and Mullet, 1991). In cress seedlings, a steady increase of both rbcS mRNA and rbcL mRNA is observed during development in the dark (this paper). In *Amaranthus* and barley, the amount of rbcL mRNA increases for a longer time of development than the amount of rbcS mRNA (Berry *et al.*, 1985; Rapp and Mullet, 1991). These observations indicate that the particular developmental program is – within a certain frame – species-specific.

Acknowledgements

Wolfhart Rüdiger thanks the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt, for financial support.

Andrews T. J. and Lorimer G. H. (1987), Rubisco: Structure, Mechanisms, and Prospects for Improvement. In: The Biochemistry of Plants (P. K. Stumpf and E. E. Conn, eds.), **Vol. 10,** Academic Press, San Diego, pp. 131–218.

Beisiegl U. (1986), Protein blotting. Electrophoresis **7,** 1–18.

Berry J. O., Nikolau B. J., Carr J. P. and Klessig D. F. (1985), Transcriptional and post-transcriptional regulation of ribulose-1,5-bisphosphate carboxylase gene expression in light- and dark-grown *Amaranthus* cotyledons. Mol. Cell Biol. **5,** 2238–2246.

Brown S. A. (1981), Coumarins. In: The Biochemistry of Plants (P. K. Stumpf and E. E. Conn, eds.), **Vol. 7,** Academic Press, New York, pp. 269–300.

Dean C., Pichersky E. and Dunsmuir P. (1989), Structure, evolution, and regulation of rbcS genes in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40,** 415–439.

Degenhardt J., Fiebig C. and Link G. (1991), Chloroplast and nuclear transcripts for plastid proteins in *Arabidopsis thaliana:* tissue distribution in mature plants and during seedling development and embryogenesis. Bot. Acta **104,** 455–463.

Depigny-This D., Raynal M., Aspart L., Delseny M. and Grellet F. (1992), The cruciferin gene family in radish. Plant Mol. Biol. **20,** 467–479.

Eckerskorn C., Mewes W., Goretzki H. and Lottspeich F. (1988), A new siliconized glass fiber as support for protein-chemical analysis of electroblotted proteins. Eur. J. Biochem. **176,** 509–519.

Feinberg A. P. and Vogelstein B. (1983), A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13.

Glass A. D. M. (1976), The allelopathic potential of phenolic acids associated with the rhizosphere of *Pteridium aquilinum.* Can. J. Bot. **54,** 2440–2444.

Harn C., Khayat E. and Daie J. (1993), Expression dynamics of genes encoding key carbon metabolism enzymes during sink to source transition of developing leaves. Plant Cell Physiol. **34,** 1045–1053.

Inamine G., Nash B., Weissbach H. and Brot N. (1985), Light regulation of the synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase in peas: evidence for translational control. Proc. Natl. Acad. Sci. U.S.A. **82,** 5690–5694.

Kittsteiner U., Brunner H. and Rüdiger W. (1991), The greening process in cress seedlings. II. Complexing agents and 5-aminolevulinate inhibit accumulation of cab-mRNA coding for the light-harvesting chlorophyll *a/b* protein. Physiol. Plant. **81,** 190–196.

Krapp A., Hofmann B., Schäfer C. and Stitt M. (1993), Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the "sink regulation" of photosynthesis? The Plant Journal **3,** 817–828.

Kyhse-Andersen J. (1984), Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Meth. **10,** 203–209.

Laemmli U. K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Lehmann J. and Parthier B. (1985), Ribulose-1,5-bisphosphat-Carboxylase/Oxygenase – Schlüsselenzym der Kohlenstoffassimilation. Biol. Rdsch. **23,** 207–224.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951), Protein measurement with the folin phenol reagent. J. Biol. Chem. **193,** 265–275.

Makowsky G. S. and Ramsby M. L. (1993), pH Modification to enhance the molecular sieving properties of sodium dodecyl sulfate – 10% polyacrylamide gels. Anal. Biochem. **212,** 283–285.

Manzara T. and Gruissem W. (1988), Organization and expression of the genes encoding ribulose-1,5-bisphosphate carboxylase in higher plants. Photosynth. Res. **16,** 117–139.

Oster U., Kardinal C., Burghardt H., Werner B., Lottspeich F. and Rüdiger W. (1992), Natural inhibitors of germination and growth VI. Detection of a carboxyterminal fragment of the heat shock protein HSP70 after coumarin treatment. J. Plant Physiol. **40,** 110–115.

Paulsen H. and Bogorad L. (1988), Diurnal and circadian rhythms in the accumulation and synthesis of mRNA for the light-harvesting chlorophyll *a/b*-binding protein in tobacco. Plant Physiol. **88,** 1104–1109.

Putnam A. R. (1983), Allelopathic chemicals. C&EN, 34–45.

Rapp J. C. and Mullet J. E. (1991), Chloroplast transcription is required to express the nuclear genes rbcS and cab. Plastid DNA copy number is regulated independently. Plant Mol. Biol. **17,** 813–823.

Raynal M., Grellet F., Laudie M., Meyer Y., Cooke R. and Delseney H. (1990), Accession number Z17659, Code ATTS0233, EMBL Data Heidelberg.

Reynolds T. (1989), Comparative effects of heterocyclic compounds on inhibition of lettuce fruit germination. J. Exp. Bot. **40,** 391–404.

Rödin J., Ericson M. L., Josefsson L. G. and Rask L. (1990), Characterization of a cDNA clone encoding a *Brassica napus* 12S protein (cruciferin) subunit. J. Biol. Chem. **265,** 2720–2723.

Rödin J., Sjödahl S., Josefsson L. G. and Rask L. (1992), Characterization of a *Brassica napus* gene encoding a cruciferin subunit: estimation of sizes of cruciferin gene families. Plant Mol. Biol. **20,** 559–563.

Sivan A., Mayer A. M. and Poljakoff-Mayber A. (1965), The metabolism of exogenous coumarin by germinating lettuce seeds and seedlings. Israel. J. Bot. **14,** 69–73.

Spreitzer R. J. (1993), Genetic dissection of rubisco structure and function. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44,** 411–434.

Svensson S.-B. (1972), The effect of coumarin on growth, production of dry matter, protein and nucleic acids in roots of maize and wheat and the interaction of coumarin with metabolic inhibitors. Physiol. Plant. **27,** 13–24.

Valio I. F. M. (1973), Effect of endogenous coumarin on the germination of seeds of *Coumarouna odorata* Aublet. J. Exp. Bot. **24,** 442–449.

Williams R. D., Hoagland R. E. (1982), The effects of naturally occurring phenolic compounds on the seed germination. Weed Sci. **30,** 206–212.