The regulation of phosphatidylcholine biosynthesis in rye (*Secale cereale*) roots

Stimulation of the nucleotide pathway by low temperature

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The incorporation of [^{14}C]choline chloride and [^{14}C]glycerol into segments taken from rye (*Secale cereale* L., cv. Rheidal) roots was greater in segments from roots grown at 5 °C than in segments taken from roots growing at 20 °C. The incorporation was measured at the temperature at which the root had been growing. Measurements *in vitro* of the enzymes of the nucleotide pathway showed activity of choline kinase (EC 2.7.1.32), choline-phosphate cytidylyltransferase (EC 2.7.7.15) and cholinephosphotransferase (EC 2.7.8.2) to be higher in homogenates from the cooler roots when assayed at 5 °C than the activities assayed at 20 °C in the 20 °C-root homogenates. Changes *in vivo* in the pool sizes of the CDP-base intermediates with temperature, relative differences in nucleotide-pathway-enzyme activities and a pulse-chase experiment with [^{14}C]choline indicated that the rate-limiting step for phosphatidylcholine biosynthesis in this tissue, at both temperatures, was the reaction catalysed by cytidylyltransferase.

INTRODUCTION

PtdCho and PtdEtn are the major acyl lipids found in the roots of the chill-resistant cereal winter rye (*Secale cereale*) (Clarkson *et al.*, 1980). In rye roots, precursor incorporation studies and measurements of relative enzyme activities have shown that PtdCho is synthesized primarily by the nucleotide pathway rather than by transmethylation of PtdEtn (Kinney, 1983). We have previously shown that the concentration of PtdCho and PtdEtn in roots grown at 5 °C was over twice that of roots grown at 20 °C (Kinney *et al.*, 1983). Incorporation of [^{32}P]P_i into the phospholipids of intact roots suggested that the stimulation of PtdCho and PtdEtn synthesis at low growth temperature was compatible with the increased concentrations found in the root tissues (Kinney, 1983). The catabolism of PtdCho and PtdEtn was unaffected by the root growth temperature (Kinney *et al.*, 1983). Horvath & van Hasselt (1985) have shown that chill-sensitive cucumber (*Cucumis sativus*) plants lose PtdCho and PtdEtn when they are cooled. The PtdCho or PtdEtn content of these plants could be increased by treatment of the plants with either choline chloride or ethanolamine, and this treatment increased the chill-resistance of the plants. It is possible, therefore, that the increases in the PtdCho and PtdEtn content of rye roots induced by low temperature may be related to the chill-resistance of this species. The present paper investigates the mechanism by which germination and growth at low, non-freezing, temperatures stimulates PtdCho biosynthesis in rye roots. We show that low growth temperature has its effect on PtdCho synthesis by increasing the activity of the enzymes of the nucleotide pathway, and that the rate-limiting step is the reaction

catalysed by choline-phosphate cytidylyltransferase (EC 2.7.7.15).

MATERIALS AND METHODS

Materials

[methyl- ^{14}C]Choline chloride, [methyl- ^{14}C]choline phosphate, cytidine 5-diphospho[methyl- ^{14}C]choline, [^{14}C]glycerol and [2- ^{14}C]ethan-1-ol-2-amine hydrochloride were obtained from Amersham International and ICN. Scintillant was purchased from L.I.P. International. Enzymes were from Sigma. All other chemicals were from either Sigma or BDH and were of the highest available grade.

Growth of plants

Seeds of rye (*Secale cereale* L. cv. Rheidal) were washed for 4 min in 10% (w/v) NaOCl and then allowed to imbibe for 6 h at room temperature in continuously aerated distilled water. They were then placed on damp filter paper in a glass Petri dish and germinated, without light, at either 5 or 20 °C, for 24 h. The seedlings were then transferred to black 5-litre beakers containing nutrient solution (Clarkson, 1976). The nutrient solution was replaced every 5 days and the pots topped up with deionized water every 24 h.

The beakers were placed in water baths maintained at either 5 ± 0.5 °C (cool roots) or 20 ± 0.5 °C (warm roots). The baths were located in a temperature-controlled growth cabinet which maintained the air temperature at a constant 20 ± 2 °C with a 16 h/8 h light/dark cycle. The light intensity during the light period was $200 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at the top of the pots.

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

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Plants grown for 9 days at 20 °C were at a similar stage of overall development as plants grown at 5 °C for 18 days. This estimate was based on shoot/root length, weight and leaf surface area (Kinney, 1983). These two ages were used throughout the study for comparison of temperature effects.

Precursor incorporation and choline pulse-chase

Roots grown at 5 or 20 °C were cut into a series of 10 mm segments. Segments were weighed and then placed in small vials with 100 mM-Pipes buffer, pH 7.0 (5 ml/g fresh wt. of tissue), which contained 8 μ M- (1.87 GBq/mmol)-[methyl-¹⁴C]choline chloride, -[2-¹⁴C]ethanolamine hydrochloride or -[¹⁴C]glycerol and infiltrated in a vacuum desiccator for 5 min at room temperature. The vials were then incubated at 5 or 20 °C for 0–120 min in a shaking water bath. In the pulse-chase experiments, 10 mm segments were incubated in buffer containing 1 mM-[methyl-¹⁴C]choline chloride (37 kBq/mmol) for 15 min and then transferred to buffer containing 1 mM unlabelled choline chloride for 0–3 h.

After incubation for the required time, the segments were washed in buffer, dried, frozen under liquid N₂ and ground to a fine powder in a pestle and mortar. The powder was extracted for 1 h with cold chloroform/methanol (2:1, v/v) and then filtered through glass fibre. The aqueous and organic phases were separated with 0.5 ml of distilled water, followed by centrifugation at 1000 g for 10 min. The organic phase was washed three times with ice-cold methanol/water (5:4, v/v) and the washings combined with the aqueous phase. Labelled compounds were separated on silica-gel G thin-layer plates using chloroform/propan-2-ol/triethylamine/methanol/0.25% KCl (30:25:18:9:6, by vol.) as a solvent system for the lipids in the organic phase (Touchstone *et al.*, 1980) and methanol/0.6% NaCl/NH₃ (10:10:1, by vol.) for the choline-containing compounds in the aqueous phase (Choy *et al.*, 1977). After elution from the plates with methanol, the radioactivity of each compound was measured by liquid-scintillation counting.

Pool size measurements

Roots (2 g fresh wt.) grown at 5 or 20 °C were frozen with liquid N₂, ground to a fine powder in a pestle and mortar and extracted for 1 h with 8 ml of cold 35% (w/v) trichloroacetic acid to which known amounts of radiolabelled choline compounds had been added to facilitate detection and estimate recovery. The extracted powder was removed by centrifugation and then re-extracted with 15% (w/v) trichloroacetic acid. The trichloroacetic acid extracts were combined and the trichloroacetic acid removed with 3 × 10 ml of diethyl ether. Choline-containing compounds in the aqueous layer were separated by chromatography as described above.

Choline and choline phosphate were estimated by the method of Barak & Tuma (1981), and CDP-choline was estimated as choline after incubation with 4 units of potato (*Solanum tuberosum*) acid phosphatase (Sigma type II) and 140 units of 5'-nucleotidase (Sigma grade III) for 10 h at 30 °C in 0.1 M-citrate buffer, pH 4.8.

Betaine was measured in the trichloroacetic acid/diethyl ether extracts by the method of Barak & Tuma (1981).

Tissue homogenization and enzyme assays

Roots were cut into 10 mm segments and homogenized on ice for 10 min, using a pre-cooled pestle and mortar, in 100 mM-Tricine buffer, pH 7.5, which contained 10 mM-dithiothreitol, 1 mM-EDTA, 10 mM-KCl and 20% (w/v) sucrose. The homogenate was filtered through two layers of cheesecloth and then centrifuged at 250 g for 10 min. The supernatant was centrifuged at 100000 g_{av} for 60 min at 4 °C in a MSE PrepSpin ultracentrifuge with a fixed-angle MSE aluminium rotor. The pellet was suspended in 1 ml of homogenization medium.

Choline kinase (EC 2.7.1.32) was assayed by the method of Burt & Brody (1975) and activity was found exclusively in the 100000 g/60 min supernatant. Optimal conditions for the assay were found to be 2 mM-ATP, 1 mM-MgCl₂, 0.8 mM-[methyl-¹⁴C]choline chloride (1.87 GBq/mmol) and 100 mM-aminopropanediol buffer, pH 9.0. Addition of an ATP-regenerating system, as described by Burt & Brody (1975), did not affect the activity of the enzyme in this assay. It was assumed, therefore, that the ADP produced during the course of this reaction did not inhibit the kinase activity.

Choline-phosphate cytidyltransferase (EC 2.7.7.15) was measured by the method of Choy *et al.* (1977). Most activity (70–80%) was found in the 100000 g/60 min pellet, the remaining activity being found in the supernatant. Optimal conditions for both the pellet and the supernatant activity were found to be 10 mM-MgCl₂, 5 mM-CTP, 4 mM-[methyl-¹⁴C]choline phosphate (2.05 MBq/mmol) and 100 mM-Pipes buffer, pH 7.5.

Cholinephosphotransferase (EC 2.7.8.2) was measured by the method of Lord *et al.* (1972). Most activity (> 95%) was recovered in the 100000 g/60 min pellet, and optimal conditions for the assay were found to be 2 mM-MgCl₂ and 2 mM-CDP-[methyl-¹⁴C]choline (2.15 MBq/mmol) in 100 mM-Tricine buffer, pH 7.5. The addition of various concentrations of mixed-species 1,2-diacylglycerol, either as an emulsion in 0.3% Tween 80 or as a sonicated dispersion in Tricine buffer, did not have any effect on the enzyme activity. It was assumed that a saturating amount of this co-substrate was available in the 100000 g/60 min pellet.

Protein was measured by the method of Bradford (1976).

RESULTS AND DISCUSSION

Choline, ethanolamine and glycerol incorporation into the lipid fraction of rye roots was linear with time (results not shown), the label from both choline and glycerol appearing predominantly in PtdCho (Table 1). Label from externally added glycerol was preferentially incorporated into PtdCho rather than into PtdEtn; it was also found that label from ethanolamine was incorporated into PtdEtn at little more than half the rate of choline incorporation into PtdCho (Table 1), even though the uptake rate of these two precursors was similar (approx. 4.0 kBq/h per g fresh wt. at 20 °C). The difference in glycerol incorporation rates between PtdCho and PtdEtn was much greater than the 2-fold difference in headgroup-precursor incorporation (close to 100-fold), and this may reflect a difference in the rates of synthesis *de novo* of these two lipids. Differential rates of synthesis can contribute to the different contents of

Table 1. Incorporation of ^{14}C -labelled precursors into the PtdCho and PtdEtn of root segments from roots grown at 5 °C (cool roots) and 20 °C (warm roots)

The incorporations were measured at the temperature at which the plants had been growing. Experimental details are given in the text. Results are means of duplicates from three assays; the S.E.M. was 5% of the mean or less.

Precursor . . . Incorporation into . . . Roots	Incorporation (pmol/h per g fresh wt. of tissue)					
	Choline		Ethanolamine		Glycerol	
	PtdCho	PtdEtn	PtdCho	PtdEtn	PtdCho	PtdEtn
Warm	18.0	0.09	0.40	10.8	50.0	0.8
Cool	38.4	0.15	0.65	24.9	80.0	1.1

different phospholipids in a tissue, although in rye roots the PtdCho and PtdEtn contents are similar (Kinney *et al.*, 1983). Incorporation of ethanolamine into PtdCho was very low (Table 1), which confirms our previous observation that the methylation of PtdEtn does not contribute significantly to PtdCho synthesis in this tissue (Kinney, 1983).

The incorporation of both choline and glycerol into PtdCho was over twice as great in the cool root segments as in segments from warm roots (Table 1). We emphasize that the incorporations were measured at the temperature at which the roots had been growing. The uptake of choline by the segments was similar at both temperatures (4.0 kBq/h per g fresh wt. at 20 °C and 3.3 kBq/h per g fresh wt at 5 °C), uptake being calculated as the total amount of label in the tissue after incubation and washing. Incorporation of ethanolamine into PtdEtn was also 2-fold higher at 5 °C, although incorporation of ethanolamine into PtdCho was only marginally higher at the cooler temperature (Table 1). These data indicate an increased PtdCho (and PtdEtn) synthesis via the nucleotide pathway in cooled roots as compared with warm roots.

The results of the choline pulse-chase experiment suggest that the rate-limiting step of the nucleotide pathway for PtdCho biosynthesis in this tissue was the reaction catalysed by choline-phosphate cytidyltransferase. At the end of the 15 min labelling period, at both temperatures, the bulk of the label was associated with choline phosphate. In the cool roots there was no detectable label remaining in the choline pool at the end of the pulse. In the warm roots the label still associated with the choline pool (90 Bq/g fresh wt.) remained constant throughout the course of the experiment. During the following incubation period label was lost from the choline phosphate at a rate similar to the appearance of label in PtdCho, approx. 18.3 Bq/h per g fresh wt. at 20 °C and 41.7 Bq/h per g fresh wt. at 5 °C (Fig. 1). A comparison of the contents of choline-containing metabolites in warm and cool roots (Table 2) also supports the hypothesis that there was an increased PtdCho synthesis via the nucleotide pathway in the cool roots. The contents of choline and CDP-choline were less in the cool roots, suggesting higher choline kinase and cholinephosphotransferase activities. The content of choline phosphate, however, was greater in the cool roots than in the warm roots. This higher choline phosphate pool could represent a decreased or unchanged

cytidyltransferase activity. It could also mean that there was an increase in cytidyltransferase activity that was substantially smaller than the increase in choline kinase activity, thus leading to greater amounts of choline phosphate. This latter suggestion is strongly supported by measurements of the enzyme activities *in vitro* (Table 3). The activities of all three enzymes of the nucleotide pathway were greater in extracts from cool roots, assayed at 5 °C, than in extracts from warm roots assayed at 20 °C. The increase in the activity of the cytidyltransferase (2.6-fold) corresponded closely to the increased PtdCho content of cooled roots, which was

Fig. 1. Flux of ^{14}C choline through the nucleotide pathway for PtdCho biosynthesis in segments taken from rye roots grown at 5 °C (cool roots) and 20 °C (warm roots)

Segments were incubated with 1 mM labelled choline for 15 min and then transferred to 1 mM unlabelled choline for 0–3 h. Experimental details are given in the text. Open symbols represent data from warm roots and closed symbols from cool roots. ○, ●, Choline phosphate; △, ▲, PtdCho. No label was observed in choline and CDP-choline extracted from cool-root segments during the course of the experiment. A constant amount of label was observed in the choline (90 Bq/g fresh wt. of tissue) and CDP-choline (60 Bq/g fresh wt. of tissue) extracted from warm-root segments throughout the sources of the experiment. Points represent the means for two determinations; the S.E.M. was 10% of the mean or less.

Table 2. Concentrations of choline-containing compounds in rye roots grown at 5 °C (cool roots) and 20 °C (warm roots)

Experimental details are given in the text. Results are means of three determinations; the S.E.M. was 12% of the mean or less.

Choline-containing compound	Concn. (nmol/g fresh wt. of tissue)	
	Roots . . .	
	Cool	Warm
Choline	420	760
Choline phosphate	1625	367
CDP-choline	55	171
PtdCho	443	148
Betaine	300	300

2.2-fold on a dry-weight basis (Kinney *et al.*, 1983) and 3.0-fold on a fresh-weight basis (Kinney *et al.*, 1983, and Table 2). The increase in the activity of the other two enzymes (3.6-fold for the choline kinase and 5.3-fold for the cholinephosphotransferase) was much greater. These data further support the hypothesis that the cytidylyltransferase activity was controlling the rate of PtdCho synthesis in this tissue and are in agreement with the published data for pea (*Pisum sativum*) stems treated with indol-3-ylacetic acid (Price-Jones & Harwood, 1983) and many animal tissues (Vance & Pelech, 1984). We did not observe any change in activity of the soluble cytidylyltransferase (Table 3). The levels of choline kinase activity were considerably higher than the levels of cytidylyltransferase and cholinephosphotransferase activities (Table 2). This observation is probably related to the high levels of choline phosphate measured in the xylem exudates of a number of plant species (Maizel *et al.*, 1956) and supports the hypothesis that plant roots may be the site of choline phosphate synthesis for PtdCho synthesis in leaves (Martin & Tolbert, 1983).

We do not know the mechanism by which the activities of the enzymes of the nucleotide pathway for PtdCho biosynthesis were increased by growth at low temperatures. The ratio of cool-root cholinephosphotransferase activity measured at 5 °C to that measured at 20 °C (0.7) and the corresponding ratio of activities of warm-root phosphotransferase (0.5) were similar (Table 3), as were the apparent Michaelis constants of this enzyme for CDP-choline (2.7 μ M at 5 °C and 3.1 μ M

at 20 °C; Kinney, 1983). These data suggest that the temperature kinetics and substrate affinities of the cholinephosphotransferase are similar in warm and cool roots. The percentage diminution in activities of the warm- and cool-root choline kinase and cytidylyltransferase with decreased assay temperature are also similar (Table 3). Thus it is possible that there may have been a greater concentration of cholinephosphotransferase enzyme, and possibly of the other enzymes of the nucleotide pathway, in roots grown at 5 °C when compared with 20 °C-grown roots. This may have arisen from an increased protein synthesis or decreased protein degradation in the cool roots. Laroche & Hopkins (1986) have reported rates of protein synthesis *in vitro* of polysomes isolated from cold-grown (10 °C) and warm-grown (25 °C) rye leaves. They found that amino acid incorporation in polysomes from cold-grown leaves, measured at 10 °C, was over twice that of warm-grown leaf polysomes measured at 25 °C. A similar situation may exist in rye roots, resulting in increased concentrations of phospholipid-synthesizing proteins. However, we cannot rule out at present the existence of isoenzymic forms of these proteins. Investigation of protein-synthesis patterns in warm and cool roots would be of value.

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REFERENCES

- Barak, A. J. & Tuma, D. J. (1981) *Methods Enzymol.* **72**, 287–292
 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
 Burt, A. M. & Brody, S. A. (1975) *Anal. Biochem.* **65**, 215–224
 Choy, P. C., Lim, P. H. & Vance, D. E. (1977) *J. Biol. Chem.* **252**, 7673–7677
 Clarkson, D. T. (1976) *Planta* **132**, 297–304
 Clarkson, D. T., Hall, K. C. & Roberts, J. K. M. (1980) *Planta* **149**, 464–471
 Horvath, I. & van Hasselt, P. R. (1985) *Planta* **164**, 83–88
 Kinney, A. J. (1983) D.Phil Thesis, University of Oxford
 Kinney, A. J., Clarkson, D. T. & Loughman, B. C. (1983) *Biochem. Soc. Trans.* **11**, 390–391
 Laroche, A. & Hopkins, W. G. (1986) *Plant Physiol.* **80**, S43
 Lord, J. M., Kagawa, T. & Beevers, H. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2429–2432
 Maizel, J. V., Benson, A. A. & Tolbert, N. E. (1956) *Plant Physiol.* **31**, 407–408

Table 3. Activities *in vitro* of the enzymes of the nucleotide pathway for PtdCho biosynthesis in extracts of roots grown at 5 °C (cool roots) and 20 °C (warm roots)

Results are means $\pm$ S.E.M. for triplicates of five to ten assays. Experimental details are given in the text.

Roots	Assay temperature (°C)	Activity (nmol/h per mg of protein)			
		Choline kinase	Soluble cytidylyltransferase	Membrane cytidylyltransferase	Choline-phosphotransferase
Warm	5	34 $\pm$ 5	0.0032 $\pm$ 0.0003	0.0047 $\pm$ 0.0007	0.017 $\pm$ 0.003
	20	43 $\pm$ 6	0.0037 $\pm$ 0.0003	0.0088 $\pm$ 0.0016	0.034 $\pm$ 0.004
Cool	5	156 $\pm$ 8	0.0031 $\pm$ 0.0004	0.0210 $\pm$ 0.0011	0.180 $\pm$ 0.020
	20	284 $\pm$ 28	0.0035 $\pm$ 0.0003	0.0539 $\pm$ 0.0052	0.257 $\pm$ 0.018

Martin, B. A. & Tolbert, N. E. (1983) *Plant Physiol.* **73**, 464–470
Price-Jones, M. J. & Harwood, J. L. (1983) *Biochem. J.* **216**, 627–631

Touchstone, J. C., Chen, J. C. & Beaver, K. M. (1980) *Lipids* **15**, 61–62
Vance, D. E. & Pelech, S. L. (1984) *Trends Biochem. Sci.* **9**, 17–20

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