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PROLINE BIOSYNTHESIS IN A PROLINE-ACCUMULATING BARLEY MUTANT

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Abstract—A barley (*Hordeum vulgare* L) mutant, R5201, selected for resistance to 4 mM *trans*-4-hydroxyproline had a 3–6 fold increase in the soluble proline content of the leaf compared with the parent cultivar, Maris Mink The mutant converted more [U-^{14}C]glutamic acid to free proline in the leaves than Maris Mink but incorporation into protein proline was similar Incorporation of radioactivity into proline was inhibited by exogenous proline more in Maris Mink than R5201, suggesting that feedback inhibition of proline biosynthesis is relaxed, but not absent in the mutant When [1-^{14}C]ornithine was the precursor, both R5201 and Maris Mink incorporated similar small amounts of label into soluble and protein proline More protein proline was formed by both genotypes from labelled glutamic acid than from labelled ornithine There may exist two routes of proline formation, where the glutamate pathway is synthetic and the ornithine pathway is catabolic

INTRODUCTION

In plants, it has been suggested that proline is formed from glutamic acid by way of glutamic semialdehyde and pyrroline-5-carboxylic acid (P5C) [1] An alternative route is proposed from ornithine by α- or δ-deamination to glutamic semialdehyde or α-ketoaminovaleric acid [2, 3] However, these routes have not been unequivocally proven We have previously described the isolation and genetic characterisation of three barley (*Hordeum vulgare* L) mutants resistant to *trans*-4-hydroxyproline The mutants are allelic and in each case the single, partially dominant nuclear gene is associated with an increase in the soluble content of proline [4, 5] We report here a more detailed examination of the free amino acid content of one of the mutants, R5201, and an analysis of the products of the metabolism of radioactively labelled glutamic acid, ornithine and proline These studies suggest that proline synthesis from glutamic acid is regulated by proline and that this regulation is partially relaxed in the mutant

RESULTS

Amino acid content of the leaves of R5201 and Maris Mink

The soluble amino acid content was examined in young leaves of three different ages by extraction and automated amino acid analysis (Table 1) The total amount of free amino acids was similar in the three samples of each genotype, with the exception of asparagine which was abundant in 9-day old leaves but was considerably decreased in older leaf samples The only major difference between the two genotypes was in the content of proline This was increased from 0 23 to 1 47 μmol/g fr wt leaf in R5201 The proportion of proline in the leaf proteins was unchanged, being 5 91 mol% in the mutant and 5 98 mol% in Maris Mink

The effect of exogenous proline on the synthesis of proline from [U-^{14}C]*glutamic acid*

Turgid excised leaf segments of R5201 and Maris Mink were fed for 2 hr with [^{14}C]glutamic acid with or without cold proline in an illuminated leaf chamber The uptake of [^{14}C]glutamic acid was similar in both R5201 and Maris Mink and the inclusion of cold proline at 10 mM did not affect the uptake nor the proportion of the label in the various fractions (water-soluble, protein-containing residue, chloroform-soluble and CO_2)

In the water-soluble fraction, radioactivity was recovered mainly in aspartic acid, γ-aminobutyric acid and α-ketoglutaric acid The analytical system used did not separate asparagine and glutamine from glutamic acid and so any label in these compounds would be counted with unchanged glutamic acid Analysis of one water soluble fraction using 2-dimensional paper chromatography, however, showed that glutamine (15%) and asparagine (8%) were major products (data not shown) The proportion of recovered label in soluble proline was low but that in protein proline was higher (7–15% of the label in the protein hydrolysates) Little or no radioactive arginine was detected in the soluble fraction but in the protein hydrolysates up to 8% of the activity was recovered in arginine (Fig 1) There was a substantial reduction in radiolabelled proline both in the soluble and protein fractions in the presence of cold proline, but it did not affect the distribution of label into arginine or other amino acids analysed Total proline formation from [^{14}C]glutamic acid in Maris Mink was inhibited 97% by external proline In R5201 the total proline formation in the absence of proline was greater than in Maris Mink,

Table 1 Free amino acids in three different leaf samples of Maris Mink and mutant R5201

	Free amino acid content* (μmol/g fr wt)					
	First leaf 9 days		First leaf 13 days		Second leaf 13 days	
Amino acid	Maris Mink	R5201	Maris Mink	R5201	Maris Mink	R5201
Asp	8 75 ± 0 49	8 61 ± 0 82	8 41 ± 0 84	9 41 ± 0 76	6 56 ± 1 03	6 96 ± 0 38
Thr	2 55 ± 0 10	2 41 ± 0 06	2 21 ± 0 23	2 97 ± 0 06	1 68 ± 0 18	1 32 ± 0 42
Ser	7 01 ± 1 13	7 31 ± 1 00	6 21 ± 0 82	5 12 ± 0 58	3 39 ± 0 18	3 38 ± 0 49
Asn	12 27 ± 6 03	18 48 ± 7 50	0 86 ± 0 10	1 55 ± 0 98	1 29 ± 0 18	3 18 ± 1 96
Glu	12 98 ± 1 45	11 79 ± 0 71	12 46 ± 2 78	17 13 ± 1 48	13 31 ± 0 98	13 35 ± 1 01
Gln	10 48 ± 1 81	11 33 ± 3 01	2 90 ± 3 93	7 23 ± 1 60	5 14 ± 1 28	4 86 ± 2 18
Pro	0 26 ± 0 02	1 54 ± 0 14	0 18 ± 0 01	1 40 ± 0 30	0 26 ± 0 02	1 46 ± 0 09
Gly	4 39 ± 1 73	6 63 ± 1 08	2 37 ± 0 82	2 65 ± 1 08	2 11 ± 0 46	3 21 ± 0 57
Ala	3 86 ± 0 88	3 57 ± 0 63	5 95 ± 0 27	5 52 ± 1 32	3 51 ± 0 29	3 35 ± 0 75
Val	1 05 ± 0 23	1 25 ± 0 11	0 65 ± 0 09	0 79 ± 0 09	0 63 ± 0 09	0 69 ± 0 15
Ile	0 30 ± 0 12	0 45 ± 0 10	0 15 ± 0 06	0 18 ± 0 09	0 16 ± 0 04	0 23 ± 0 02
Leu	0 16 ± 0 07	0 24 ± 0 04	0 16 ± 0 06	0 24 ± 0 22	0 13 ± 0 03	0 20 ± 0 03
Tyr	0 19 ± 0 02	0 19 ± 0 02	0 13 ± 0 01	0 13 ± 0 06	0 16 ± 0 03	0 18 ± 0 01
Phe	0 28 ± 0 07	0 33 ± 0 05	0 21 ± 0 05	0 25 ± 0 08	0 19 ± 0 01	0 25 ± 0 02
γAB†	0 47 ± 0 04	0 38 ± 0 01	0 30 ± 0 14	0 25 ± 0 11	0 13 ± 0 05	0 21 ± 0 14
Lys	0 35 ± 0 01	0 41 ± 0 05	0 14 ± 0 05	0 17 ± 0 05	0 12 ± 0 01	0 17 ± 0 01
His	1 08 ± 0 15	1 43 ± 0 35	0 07 ± 0 01	0 09 ± 0 02	0 21 ± 0 07	0 39 ± 0 10
Trp	1 23 ± 0 14	1 32 ± 0 25	nd‡	nd	nd	nd
Arg	0 25 ± 0 14	0 34 ± 0 11	0 10 ± 0 06	0 06 ± 0 02	0 06 ± 0 01	0 15 ± 0 04
Total	68 26	78 00	43 45	55 05	39 02	43 54

*Each value is the mean ± standard deviation of triplicate samples
†γ-Aminobutyric acid
‡Not detected

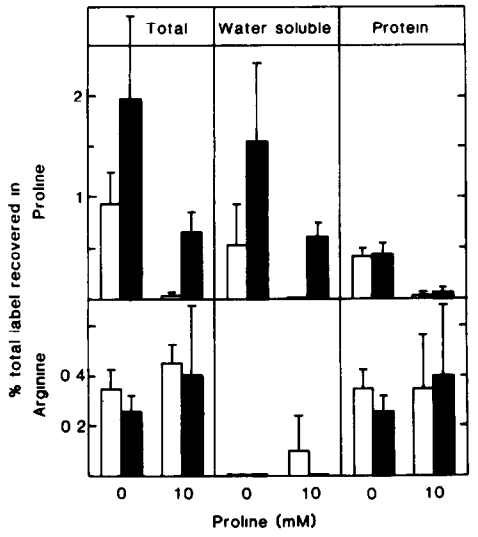

Fig 1 Incorporation of [U-^{14}C]glutamic acid into proline and arginine of Maris Mink (□) and R5201 (■) in the presence (10) and absence (0) of 10 mM cold proline into the total, water-soluble and protein fractions Each column is either the mean of triplicate treatments (in proline) or duplicate treatments (in arginine) (three leaves per treatment) Bars represent standard deviations Total dpm recovered (= 100%) for Maris Mink were 5 10 × 10^5 dpm and 4 95 × 10^5 dpm respectively and for R5201, 5 19 × 10^5 dpm and 5 55 × 10^5 dpm respectively in the absence (0) and presence (10) of 10 mM cold proline

and was inhibited only 65% by externally fed proline (Fig 1)

The incorporation of [1-^{14}C]*ornithine into proline and other amino acids*

[^{14}C]Ornithine was fed to excised 6-day old leaves of Maris Mink and R5201 The effect of prefeeding the leaves with 10 mM glutamic acid was also studied Ornithine was readily converted into citrulline and arginine in both genotypes and this was not affected by prefeeding the leaves with cold glutamic acid (Table 2) Most of the label in arginine was in the protein fraction The incorporation of label into the other amino acids, aspartate, glutamate, P5C, proline and organic acids such as α-ketoglutaric acid was much less Incorporation into proline was low and unlike the case with [^{14}C]glutamic acid feeding, the amount of labelled proline in the soluble and protein fractions was similar Incorporation of [^{14}C]ornithine into proline was not affected by cold glutamate Maris Mink and R5201 contained similar low levels of radioactively labelled proline

Metabolism of [U-^{14}C]*proline*

[^{14}C]Proline metabolism was examined in whole 4-day old plants fed for 4 hr In both mutant and control plants a substantial proportion of the label remained as proline with the remainder being metabolised to aspartic acid, glutamic acid and α-ketoglutaric acid (Table 3) The mutant incorporated a similar amount as the parent into

Table 2 Distribution of label from [1-^{14}C]ornithine in proline, arginine and citrulline in illuminated leaves of Maris Mink and mutant R5201

	Distribution of ^{14}C label (% recovered label in water soluble + protein fractions)			
	No prefeeding		Glu prefeeding	
Amino acid	Maris Mink	R5201	Maris Mink	R5201
Proline	0 2	0 4	0 6	0 4
	0 3	0 7	0 5	0 7
Arginine	25 8	36 2	11 6	17 4
	12 6	26 9	18 6	28 2
Citrulline	2 9	4 1	6 1	2 6
	6 6	6 1	2 4	4 8
Total dpm recovered (= 100%)	$6\,80 \times 10^5$	$7\,01 \times 10^5$	$8\,65 \times 10^5$	$5\,14 \times 10^5$

Results are values from duplicate feedings for each treatment Labelled proline and arginine were recovered from both soluble and protein fractions, citrulline only from the soluble fraction

Table 3 Distribution of label from [U-^{14}C]proline in illuminated young plants of Maris Mink and mutant R5201

	Distribution of [^{14}C]label (% recovered label in water soluble + protein fractions)			
	Soluble		Protein	
Compound	Maris Mink	R5201	Maris Mink	R5201
α-Ketoglutarate*	60±12	69±085	0	0
Aspartate	17±03	15±20	55±05	12±01
Glutamate	107±05	73±025	26±07	14±02
Proline	252±20	398±294	345±30	336±25

Results are means ± standard deviation of duplicate feedings for 4 hr to whole plants grown from embryos Total dpm recovered (= 100%) were $5\,29 \times 10^5$ dpm for Maris Mink and $6\,94 \times 10^5$ dpm for R5201

*Label comigrating with authentic α-ketoglutaric acid on the autoanalyser

protein bound proline but retained more proline in the soluble fraction

DISCUSSION

Analysis of the entire free amino acid pool in leaves showed only one consistent difference between the mutant R5201 and the parent, Maris Mink, which was an increased content of soluble proline in R5201 This confirms previous analysis by the acid ninhydrin method [4] and demonstrates the specific nature of the mutation in R5201 Similar increases in soluble proline were also observed in the roots and in leaves of mutant plants grown to maturity (data not shown) The level of asparagine in 13-day old leaves was lower than that of 9-day old leaves in both genotypes This may be due to the initial presence of asparagine transported from the seed [6] There were no other significant changes in the other amino acids at the different leaf ages Analysis of the protein amino acids of the leaves (data not shown) showed that the protein amino acid composition of the two genotypes was similar, including that of proline

Proline biosynthesis from glutamic acid was inhibited by proline Inhibition of proline formation by proline has been demonstrated in maize roots [7] and tissue culture cells [8] It suggests that an enzyme in the biosynthetic route to proline is subject to feedback regulation Both the increased labelling of proline in the mutant under normal conditions and the less severe inhibition by external proline suggest that feedback regulation of this enzyme in the mutant may be reduced, causing the observed increase in free proline Such a situation has been found in barley mutants resistant to lysine plus threonine which contain aspartate kinase isoenzymes in which feedback regulation by lysine is reduced or lacking [9, 10] The enzyme subject to feedback regulation by proline is not known P5C reductase, the last enzyme on the route from glutamic acid is not inhibited by proline [11, 12] and the two enzyme activities glutamate kinase and glutamic semialdehyde dehydrogenase, which are thought to produce P5C, have yet to be measured in plant systems However, proline-sensitive glutamate kinase preparations from bacteria

have been reported [13] so this enzyme is the most likely candidate for regulation in plants The mutant still retains the capacity for substantially greater proline accumulation under stress [4, 14] suggesting that further mutational increases in proline accumulation may be possible Arginine biosynthesis was not affected by exogenous proline indicating that the formation of arginine from glutamate occurs by a route different from that of proline This is probably through the acetylated pathway via ornithine [1]

Proline and arginine were, however, both formed from [^{14}C]ornithine Arginine and citrulline are the expected major products of ornithine metabolism [15, 16] Agreement between duplicates was poor but there was no evidence that incorporation of [^{14}C]ornithine between the two genotypes was different Proline formation was similar in the presence and absence of glutamic acid suggesting that glutamic acid is not an intermediate Such a pathway via ornithine transaminase has been shown in pea cotyledons [2] and *Cucurbito pepo* [17] Less of the proline formed from ornithine was recovered in protein when compared with the formation from glutamic acid and formation was similar in the mutant and parent These observations are consistent with this proline formation being degradative rather than synthetic as is found in *Neurospora crassa* [18]

[^{14}C]Proline fed to young plants was readily incorporated into protein Maize roots fed with [^{14}C]proline were also found to incorporate label into protein proline (44–51%) [7] R5201 retained more of the labelled proline than Maris Mink This was retained in the soluble pool with the amount of label in protein proline being similar in the two genotypes This may reflect trapping by the higher free proline pool(s) in the mutant

EXPERIMENTAL

Plant material Seeds of *Hordeum vulgare* L cv Maris Mink and the homozygous mutant line, R5201, were obtained from glasshouse grown plants

Amino acid analysis Plants of R5201 and Maris Mink were grown from seeds in Eff compost Leaf samples (three leaves of about 350 mg per sample) were taken at the 9th and 13th day, extracted with MeOH–$CHCl_3$–H_2O (12 5 3) [19] After separation of the aq and organic layers, the aq extract was dried under vacuum at 50° and redissolved in 1 ml 0 01 M HCl These samples were analysed for their soluble amino acid content with an LKB 4400 automated amino acid analyser Values were corrected for losses of 15% determined by recovery of added radioactive isoleucine

L[U-^{14}C]*Glutamic acid feeding* Maris Mink and R5201 plants were grown from dissected mature embryos on solidified Murashige and Skoog salt medium (basal medium) [4] for four days and then in soil for a further 7 days Leaves excised from these 11-day old plants were fed with [U-^{14}C]glutamic acid using leaf chambers [20] Leaf segments were placed in slots in a perspex block (six slots per block) and held upright by a wire framework The labelled soln (50 μl) was placed in each slot The leaves were placed in a glass vial (40 cm^3) stoppered with a rubber bung A steady supply of humidifed air was passed through the vial (*ca* 400 ml/min) Leaves were illuminated by a 150 W lamp, 30 cm from the chamber with the light first passing through a water tank The leaves were fed for 2 hr and samples of three leaves (*ca* 107 mg) were extracted with MeOH–$CHCl_3$–H_2O CO_2 was trapped by passing the outlet air through 25 ml 2 5 M KOH The aq fraction was dried and redissolved in 1 ml 0 01 M HCl for amino acid analysis The plant residue was hydrolysed under N_2 in a sealed tube using 3 ml 6 M HCl, for 20 hr at 110°, dried and redissolved in 0 01 M HCl for amino acid analysis Radioactive amino acids were separated on an NC2 automated amino acid analyser in which 80% of the effluent was diverted to a fraction collector and 20% passed through the ninhydrin reaction coil to give the recorded trace The collected fractions (1 4 ml) were counted in 3 5 ml tritosol scintillant [21] with 87 ± 5% counting efficiency About 20% of the label loaded was either lost in the column or in low counting fractions which were not considered

DL[1-^{14}C]*Ornithine feeding* Embryos of R5201 and Maris Mink were grown on basal medium for 6 days Excised leaves from these plants were fed with [^{14}C]ornithine with and without prefeeding with cold glutamic acid and for 1 hr in the same way as the glutamic acid feeding

[U-^{14}C]*Proline feeding* Four-day old plants, grown from embryos, were each fed with [^{14}C]proline in 0 2 ml (0 2 μCi) liquid basal medium for 4 hr The whole plant (leaf, root and scutellum) was extracted with MeOH–$CHCl_3$–H_2O soln and the aq sample (soluble amino acid) and residue sample after hydrolysis (protein hydrolysate) were analysed on an NC2 automatic amino acid analyser for incorporation of label Each sample consisted of five plants of *ca* 180 mg fr wt

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