# Two genes for threonine accumulation in barley seeds

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cholesterol. The time constant of exchange then varies in proportion to $(1+F)$, and approaches the limiting value of $k_1$ as $F$ approaches zero. This prediction is again, at least qualitatively, in agreement with reported observations[9,10,12].

Thus the phase partition theory[8] accounts for the exchange kinetics of homologous artificial systems as well as for the natural heterologous system in blood. The idea of transfer by collision is therefore redundant. Since the specific rate constants $k_1$ and $k_3$ can be estimated quite accurately (Table 1) the effect of temperature will provide information on the energy by which cholesterol and probably other apolar compounds are bound in different dispersed phases.

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# Two genes for threonine accumulation in barley seeds

**Simon W. J. Bright, Joseph S. H. Kueh, Julian Franklin, Sven Erik Rognes* & Benjamin J. Miflin**

Biochemistry Department, Rothamsted Experimental Station, Harpenden AL5 2JQ, UK
* Botanical Institute, University of Oslo, Blindern, Oslo 3, Norway

**Cereal seed protein is the major direct source of dietary protein for man and also serves as an indirect source through the feeding of livestock. For non-ruminant animals, such as man, swine and poultry, most cereal seed proteins are deficient in lysine and threonine and much effort has been devoted to their improvement[1]. Supplementation of barley diets for pigs with lysine and threonine is sufficient to produce a feed with protein of high biological value[2]. Mutant lines that accumulate these amino acids in the seed could therefore be of agricultural value. The synthesis of lysine, threonine and methionine, which are all derived from aspartic acid, is regulated in plants by a series of feedback loops[3,4]. This regulation is such that when barley embryos are germinated on a medium containing lysine plus threonine (LT medium) the plants are starved of methionine and fail to grow[5]. Plants which do grow in these conditions may contain enzymes that are no longer feedback-regulated and that may allow enhanced amounts of end-product amino acids to accumulate. We report here the identification of two genes in barley, mutations in which lead to decreased feedback sensitivity of the enzyme aspartate kinase, resistance to lysine plus threonine and, in some cases, to accumulation of soluble threonine in the seed.**

From an $M_2$ population of $10^4$ mature embryos ($M_1$ treated with sodium azide) three mutants resistant to lysine plus threonine (2–3 mM) were selected for further study. These were R(Rothamsted) 2501 (ref. 6), R3004 and R3202 which produced sensitive and resistant progeny in the $M_3$ generation. Pure-breeding resistant lines were obtained from R3004 and R3202 and, with difficulty, from R2501. The designation R3004 or R3202 refers henceforth to the $M_3$ and subsequent generations of such lines.

Both R3202 and R3004 plants grew better than controls in a range of concentrations of lysine and threonine (Fig. 1). R3202 plants were generally larger and had better root growth than R3004. Both mutant lines had lost the synergistic inhibition of growth by lysine plus threonine (Fig. 2) but were still slightly inhibited by lysine alone. This was reduced by addition of arginine. Neither R3202 nor R3004 were resistant to the lysine analogue *S*-(2-aminoethyl)cysteine.

To characterize the genetic basis of resistance, mutant plants were crossed among themselves and with normal barley cv. Golden Promise. Crosses with Golden Promise (Table 1) showed a segregation of three resistant to one sensitive in the $F_2$ generation, consistent with resistance in each case being due to the action of a single dominant nuclear gene. When R3202 and R3004 were reciprocally crossed, in the $F_2$ generation a ratio of 15 resistant plants to 1 sensitive plant was observed (Table 1), suggesting that the two genes *Lt1b* and *Lt2* are unlinked. Progeny of single $F_1$ plants of reciprocal crosses of heterozygous R2501-derived plants (*Lt1a*/+) with R3202 (*Lt1b*/*Lt1b*) were scored in the same way for resistance (Table 1). Three plants (C, D and E) gave no sensitive progeny in the $F_2$ generation, suggesting that they had received a resistance gene from each parent and that these two genes are allelic or closely linked. However, two plants (A and B) produced sensitive progeny, suggesting that they had received the wild-type sensitive gene from R2501; this hypothesis is supported for A by the segregation ratio of 33 : 8 (not significantly different from 3 : 1) and for B by the finding that, when 13 of the 15 resistant $F_2$ plants were self-pollinated and their $F_3$ progeny tested, 9 of them gave rise to some sensitive plants (pooled data 108 : 44 not significantly different from 3 : 1). No segregation data are available for the cross R2501 × R3004.

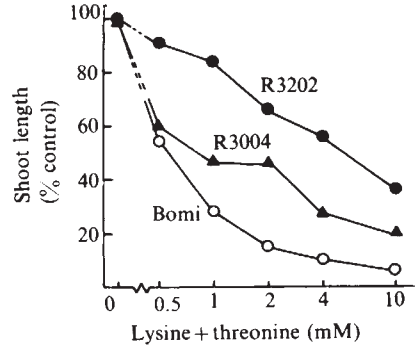


**Fig. 1** Growth of two mutants R3004 (▲) and R3202 (●) and their parent cultivar Bomi (○) in a range of equimolar concentrations of lysine and threonine. Mature embryos were hand dissected and grown on a basal medium containing agar, salts, sucrose and vitamins in Petri dishes under lights at 25 °C as in ref. 5. Amino acids were filter sterilized before addition to autoclaved medium. After 7 days, 7–16 plants were removed and the length of the shoot from the tip of the first leaf to the base was measured. Results are expressed as percentages of the mean value of each genotype in the absence of lysine and threonine. 100% values ranged from 46 to 61 mm with standard error values less than 5 mm.

**Table 1** Genetics of resistance to lysine plus threonine in three mutants crossed amongst themselves and with cv. Golden Promise

| $F_1$ parents female × male | | $F_1$ genotype at two loci *lt1* | *lt2* | No. of $F_2$ plants resistant | sensitive |
|---|---|---|---|---|---|
| R2501 × Golden Promise | | *Lt1a/+* | *+/+* | 92 | 28 |
| Golden Promise × R2501 | | *+/Lt1a* | *+/+* | 50 | 18 |
| R3004 × Golden Promise | | *+/+* | *Lt2/+* | 131 | 44 |
| Golden Promise × R3004 | | *+/+* | *+/Lt2* | 113 | 36 |
| R3202 × Golden Promise | | *Lt1b/+* | *+/+* | 37 | 15 |
| Golden Promise × R3202 | | *+/Lt1b* | *+/+* | 74 | 27 |
| R3004 × R3202 | | *+/Lt1b* | *Lt2/+* | 183 | 13 |
| R3202 × R3004 | | *Lt1b/+* | *+/Lt2* | 151 | 10 |
| R2501 × R3202 | A | *+/Lt1b* | *+/+* | 33 | 8 |
| | B | *+/Lt1b* | *+/+* | 15 | 1 |
| | C | *Lt1a/Lt1b* | *+/+* | 110 | 0 |
| | D | *Lt1a/Lt1b* | *+/+* | 61 | 0 |
| R3202 × R2501 | E | *Lt1b/Lt1a* | *+/+* | 58 | 0 |

Mature embryos were germinated for 1 or 2 days on basal medium before transfer to LT medium. Resistance was scored after 7 days' total growth. Resistant plants, when grown in the presence of lysine plus threonine, had long green shoots and pale scutella, whereas sensitive plants, derived either from the original selections or from control plants of several cultivars, had short yellow shoots and dark pigmentation on the scutellum and at the base of the leaf. The absence of darkening of the scutellum was used as an absolute indicator of resistance. Ratios were analysed by $\chi^2$ test. In crosses with cv. Golden Promise the R2501-derived plant used was homozygous (*Lt1a/Lt1a*), in crosses with other mutants the R2501-derived plants were heterozygous (*Lt1a/+*). R3202 (*Lt1b/Lt1b*) and R3004 (*Lt2/Lt2*) plants were homozygous for resistance at the *Lt1* and *Lt2* loci, respectively. Crosses were performed with glasshouse grown plants. Flowers on plants of the female parents were emasculated and enclosed in cellophane bags for 2–4 days before pollination with anthers from selected male parents. Bags were kept over the ears until collected.

Aspartate kinase (AK) in barley can be separated into the three isoenzymatic forms AKI, AKII and AKIII (ref. 6); the latter two are sensitive to feedback inhibition by lysine. Analysis of the properties of these isoenzymes[7] has shown that R2501 and R3202 both have an AKII with greatly decreased sensitivity to inhibition by lysine and a normal AKIII, whilst R3004 has an unchanged AKII but an AKIII that is relatively unaffected by lysine. These results, taken with the genetic data, suggest that resistance to lysine plus threonine in R2501 and R3202 is due to mutations in a gene *lt1* causing altered feedback sensitivity of AKII and in R3004 is due to a mutation in a gene *lt2* which decreases lysine inhibition of AKIII. The genes *lt1* and *lt2* are, therefore, likely to be structural loci for the two aspartate kinase isoenzymes. The absence of tight regulatory control of aspartate kinase would allow sufficient flux down the pathway to satisfy the methionine requirement in LT medium.

It has been suggested that mutants having such altered regulatory feedback properties might produce cereal plants with grains of improved nutritional quality[8,9]. Analyses of the soluble amino acids of seeds (Table 2) showed that the mutation in R3004 caused a greater than 12-fold increase in threonine. Similar increases have been previously observed in R2501 (ref. 6). The absence of such accumulation in R3202 may be because there was less of the mutant enzyme form[7]. An increase in threonine of 100 μmol per g nitrogen would be sufficient to increase the total threonine content of the seed (~1,400 μmol per g nitrogen) by about 7%. Increases of this order have been measured. This would decrease the amount of extra threonine required for optimum nutrition of monogastric animals fed on barley meal plus vitamins[10]. Seed of R3004 has been multiplied and will be used to test this in rat feeding trials. The agronomic performance of the R3004 line, even in the absence of outcrossing and reselection, is not markedly affected when compared with the parent Bomi.

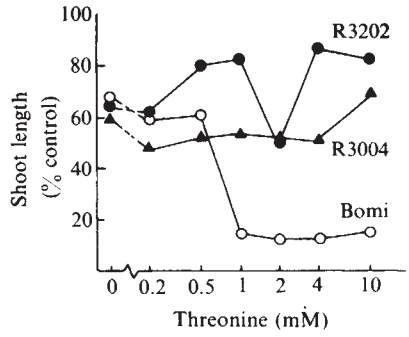


**Fig. 2** Growth of two mutants R3004 (▲) and R3202 (●) and Bomi (○) in a range of threonine concentrations with lysine held constant at 3 mM. Mature embryos were grown and 12–19 plants measured as in Fig. 1. 100% values ranged from 49 to 67 mm with standard error values less than 5 mm.

None of the mutants has enhanced levels of soluble lysine. This is consistent with the lack of resistance to the lysine analogue aminoethylcysteine and is probably because the activity of the first enzyme unique to lysine biosynthesis—dihydrodipicolinic acid synthase—is also tightly regulated by lysine[11].

Variant plant cells accumulating tryptophan[12] and threonine[13] as a consequence of altered enzymatic regulation have been selected. However, it is only possible to examine the effects of such changes on seed composition by using fertile plants. Our results confirm the view that it is possible to select mutants with relaxed feedback control which can accumulate nutritionally essential amino acids in the seed. A maize mutant which accumulates threonine in the seed[14] has also been identified. We view these mutants as a significant step towards the development of a barley grain of balanced amino acid content.

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**Table 2** Soluble amino acids in grains of normal barley and three mutants

| | Soluble content (nmol per mg nitrogen) | | | | |
|---|---|---|---|---|---|
| | Pooled controls | | | | |
| Amino acid | I | II | R3004 | R3202 | R2501 |
| Thr | 9±3 | 12±5 | 116±36 | 16±5 | 147±21 |
| Lys | 12±5 | 12±5 | 15±3 | 18±5 | 19±8 |
| Ala | 49±13 | 47±10 | 41±6 | 68±14 | 29±5 |
| Val | 18±10 | 28±12 | 17±5 | 38±10 | 19±4 |
| Glx | 143±25 | 140±36 | 122±20 | 199±39 | 112±22 |
| Asx | 167±27 | 181±28 | 122±18 | 237±45 | 131±24 |
| Total (17/20) | 580 | 740 | 745 | 905 | 743 |
| Seed nitrogen (mg N per g dry wt) | 14–18 | 22–27 | 22–27 | 16–19 | 24–29 |

Milled grain samples were extracted with 5% cold trichloroacetic acid plus 10 mM 2-mercaptoethanol as in ref. 6 and analysed on a Technicon TSM autoanalyser. Values are expressed as mean±s.d. for at least eight analyser runs and four grain samples. Tryptophan and cysteine were not determined; methionine peaks were always too small to quantify as they were overlapping with a small unidentified peak. Glutamine and asparagine were hydrolysed to the free acid before analysis. Data for R2501 are taken from ref. 6 for comparison and are for pooled seeds from heterozygous *Lt1a/+* individuals. Nitrogen was determined by Kjeldahl analysis. The two control groups were: I, Bomi and sensitive lines segregating from the original R3202 selected plant; II, sensitive lines segregated from the original R2501 selected plant.

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