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High threonine producer mutant of *Nicotiana sylvestris* (Spegg. and Comes)

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Summary. Mutagenesis and the subsequent selection of mesophyll diploid protoplasts of *Nicotiana sylvestris* on growth inhibitory concentrations of lysine plus threonine has led to the isolation of an LT-resistant mutant. Regeneration of this line (RLT 70) and analysis of its descendants demonstrated the dominant monogenic nuclear character of the resistance gene, further named *ak-LT1*. When the inhibition properties of aspartate kinase were examined in the homozygous mutant, lysine-sensitive activity could no longer be detected. In comparison, 70%–80% of the wild-type enzyme activity was usually inhibited by lysine, and the rest by threonine. Evidence for the existence of at least two AK isoenzymes was obtained by ion-exchange chromatography, where two peaks of activity could be detected: the first one to be eluted is lysine sensitive, and the second one threonine sensitive. One consequence of the altered regulation of AK in the mutant was the enhanced production of soluble threonine. Threonine accumulation was observed to occur throughout the life cycle of the mutant plant as well as in its different organs. In particular, leaves exhibited a 45-fold increment of soluble threonine, which corresponds to a 13-fold increase in total threonine: almost one-third of the total amino acids was free and protein-bound threonine. In RLT 70 seeds, 20% of the free amino acid pool was in the form of threonine (70-fold accumulation compared to the wild type), and total threonine content was increased five fold. As a general rule, the other amino acids were also more abundant in RLT 70 seeds, such that the total of amino acids present was between two to four times higher, but in contrast with the situation encountered in leaves, this was also due to a higher protein-bound amino acid content.

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Key words: Amino acid biosynthesis – Aspartate kinase – Feedback inhibition – Threonine overproduction – *Nicotiana sylvestris*

Abbreviations: *AK*, aspartate kinase; *DHDPS*, dihydrodipicolinate synthase; *AEC*, S-(2-aminoethyl) L-cysteine; *LT*, lysine plus threonine; *AdoMet*, S-adenosyl-methionine

Introduction

Amino acid imbalance is an important factor in determining the nutritional value of various food and feed grains. Cereal crops are mostly deficient in lysine and threonine, and legume crops generally lack methionine. Increasing the amount of the desired essential amino acid in the soluble amino acid fractions of the plant thus represents a possible approach for improving crop quality. Such an increase could be obtained if the regulatory mechanisms controlling the biosynthetic pathway of that particular amino acid were abolished, or at least strongly diminished. The main controls exerted during the biosynthesis of the aspartate-derived amino acids lysine and threonine are the feedback inhibition of aspartate kinase (AK) by lysine and/or threonine, and the feedback inhibition of dihydrodipicolinate synthase (DHDPS) by lysine (Bryan 1980). These two enzymes are therefore potential targets of mutagenesis-selection procedures in the aim of isolating mutants that overproduce one or possibly both amino acids (Jacobs et al. 1987).

Selection, after mutagenesis, on medium containing toxic concentrations of lysine plus threonine has led to the isolation of a certain number of such LT-resistant mutants, of which the following have been analysed as plants: carrot (*Daucus carota* L.) (Cattoir-Reynaerts et al. 1983), maize (*Zea mays* L.) (Hibberd et al. 1980,

Hibberd and Green 1982; Miao et al. 1988; Diedrick et al. 1990) barley (*Hordeum vulgare* L.) (Bright et al. 1982) and tobacco (*Nicotiana tabacum* L.) (Bourgin et al. 1982). The simultaneous presence of these two amino acids at high levels causes the complete feedback inhibition of aspartate kinase activity, therefore not only hindering further synthesis of lysine and threonine but, more importantly preventing the synthesis of methionine, which is not supplemented in the medium (Green and Phillips 1974). Thus, growth inhibition is caused by methionine starvation and can be relieved if methionine or its precursors are added to the medium. To bypass this situation, an altered regulation, i.e. an aspartate kinase less sensitive to feedback inhibition, could appear and consequently lead to the accumulation of one or more end-product amino acids. In fact, in all of the LT-resistant mutants analysed so far, an overproduction of soluble threonine has been observed that is due to the presence of an aspartate kinase less sensitive to a feedback by lysine (while the threonine control, when existing, is unaffected).

Our interest in isolating a LT-resistant in *Nicotiana sylvestris* resides in the existence of a second mutant in this species (RAEC-1) (Negrutiu et al. 1984) that is unique in being resistant to a lysine analogue S-2-(aminoethyl)-L-cysteine (AEC). An altered DHDPS, which is totally desensitized to the normal stringent lysine feedback inhibition, leads to this amino acid's overproduction in the pool of free amino acids: levels reaching 30% of the total are found in leaves against 1.5% in the wild type. This enzyme has already been purified and characterized in the aim of developing a cloning approach with which to transform crops and consequently enhance their nutritional value (Ghislain et al. 1990). On the other hand, a classical cross between both *N. sylvestris* mutants – LT-resistant and threonine overproducer on one side, and AEC-resistant and lysine overproducer on the other - makes it possible to obtain a double mutant. Characterization of this exceptional double mutant at the level of its free amino acid content could provide further information on the internal regulation of the aspartate pathway, such as the priorities existing among the different branches leading to the biosynthesis of the four essential amino acids: lysine, threonine, isoleucine and methionine.

This report describes the isolation and the characterization of a highly threonine-overproducing *Nicotiana sylvestris* LT-mutant. The accumulation of soluble threonine has been studied throughout the plant's life cycle and in different organs. Furthermore, aspartate kinase, which is affected in the mutant, has been partially purified using anion-exchange chromatography and its inhibitory properties analysed. Thus, the direct relationship between the effect of the mutation on the enzymatic regulation of the aspartate pathway and modifications in the content of specific free amino acids could be established.

Materials and methods

Isolation of the mutant

Protoplast isolation and culture were conducted as described earlier (Negrutiu et al. 1984). Fresh diploid protoplasts (3.8×107) from leaves of *Nicotiana sylvestris* (Speggazini and Comes) were UV irradiated with 700 erg/mm^2 (15 W Sylvania lamp). Post-mutagenized protoplast culture was done following Negrutiu et al. (1984). The selection concentration used was of 0.75 m*M* lysine plus 0.25 m*M* threonine, both added to the dilution medium by sterile filtration. After 3 weeks resistant colonies were transferred to solid medium containing the same amino acid combination. Regeneration was attempted 3 weeks later as established by Bourgin et al. (1979). Confirmation of the resistance was done using 2 m*M* lysine plus 1.5 m*M* ($L_2T_{1.5}$) threonine for calli, and 3 m*M* lysine plus 2 m*M* threonine (L_3T_2) for plants and seeds. Resistant regenerants were transferred to the greenhouse and crossed with the wild type as male partner.

Progeny test for resistance

Seeds were sterilized in a commercial solution of sodium hypochloride for 10 min, washed 3×10 min in sterile water and seeded on agar medium containing half-strength Murashige and Skoog (1962) basal medium with 10 g/l sucrose plus lysine (2 m*M*) + threonine (1 m*M*) (L_2T_1), both filter-sterilized.

Amino acid analysis

Free amino acids. Between 100 to 500 mg of plant material was homogenized with a mortar and pestle in the presence of methanol:chloroform:water (12:5:1) (Bieleski and Turner 1966). Calli free amino acid determination was done with an additional step: the material was boiled in 70% ethanol for 10 min (Bright et al. 1979). At each of the three successive extractions, the homogenate was centrifuged, and the supernatants pooled. Chlorophyll (and the organic fraction) was removed by adding two parts chloroform plus one part water to the extract. The aqueous amino acid-containing (upper) layer was isolated and completely evaporated. The residue was resuspended in water plus 2*N* HCl, and placed under vacuum at 110 °C to hydrolyse the amides. Again left to evaporate at 85 °C, the residue was resuspended in Loading Buffer (Na-citrate, pH 2.2) and automatically analysed in a Biotronik LC 5001 amino acid analyser by ion-exchange chromatography using a five buffer system and ninhydrin detection.

Protein-bound amino acids. The pellet recovered after the different free amino acid extractions was resuspended in 1 ml 6.25 *N* HCl and placed under vacuum at 110 °C for 24 h. The hydrolysate was further left to evaporate at 85 °C, resuspended in 1 ml Loading Buffer and analysed as described above.

Total amino acids (free+protein-bound). Plant material was ground with a mortar and pestle. One millilitre 6.25 *N* HCl was added, and the extract was further treated according to the protein-bound protocol.
All samples were kept at −20 °C while waiting to be analysed.

Partial purification of aspartate kinase

Leaf tissue from plantlets between 2 and 10 weeks of age was used as starting material. All procedures were carried out at 4 °C unless otherwise indicated.

Leaf tissue (5–10 g) was extracted in 2 vol. 50 m*M* potassium phosphate (pH 7.5), 1 m*M* EDTA, 2 m*M* lysine, 2 m*M* threonine, 10 m*M* β-mercaptoethanol, 10 m*M* diethyldithiocarbamate (DIECA), 50 m*M* KCl and 20% (v/v) glycerol using a Waring Blendor. The homogenate was then filtered through a few layers of Miracloth. After centrifugation at 20,000 *g* for 20 min to remove cell debris, the supernatant was brought to 60% saturation with ammonium sulfate. The precipitate obtained after centrifugation at 20,000 *g* for 20 min was redissolved in 0.2 ml per original g leaves, of dialysis buffer (25 m*M* TRIS-HCl (pH 7.5), 1 m*M* EDTA, 50 m*M* KCl and 10% (v/v glycerol). Ammonium sulfate and other low-molecular-weight compounds were removed by passing the extract through a Sephadex G-25 column equilibrated with the dialysis buffer. The green fractions which contained the AK activity, were pooled, and 500 µl was loaded onto a Pharmacia Fast Protein Liquid Chromatography (FPLC) Mono Q HR 5/5 anion-exchange column previously equilibrated in dialysis buffer. AK activity was eluted with a 30-ml KCl gradient, 0.5 ml/min flow rate, into 0.5-ml fractions.

Assay for aspartate kinase activity

Radioactive assay. This assay was preferentially used when only small quantities of plant material were available, or when activity was very low (Rognes et al. 1983). L-(U-^{14}C)aspartate (Amersham) was first purified using a Dowex 1-X-8 anion-exchange resin (Biorad) to eliminate any contaminating neutral substances. The final concentration of the differents reagents in a assay volume of 100 µl were the following: 12 m*M* L-(U-^{14}C) aspartate (0.83 mCi/mmole), 10 m*M* ATP, 12 m*M* $MgCl_2$ and 400 m*M* hydroxylamine adjusted to pH 7.5 with KOH prior to use. The reaction was initiated by adding 5–500 µg enzymatic extract. After a 60-min incubation at 30 °C, 28 µl 12% (w/v) TCA containing 50 m*M* β-aspartylhydroxamate as carrier were added to stop the reaction. (^{14}C)Aspartylhydroxamate was separated from the excess (^{14}C)aspartate by paper chromatography using either high voltage (3 kV for 50 min) or normal (50 mA for 90 min) electrophoresis in sodium acetate pH 5.0. After a quick ninhydrin colouring, the product closest to the origin was counted in a liquid scintillation counter (LKB 1211 Rackbeta). The activity of the enzyme is expressed in pkat/ml (pkat or pmole per sec).

Hydroxamate assay. A modified form of the aspartylhydroxamate assay initially described by Bryan et al. (1970) has been developed in which the assay components add up to 1 ml. These include 100 µl 100 m*M* TRIS-HCl (pH 7.5), 400 µl 250 m*M* aspartic acid (pH 7.5), 25 µl 400 m*M* ATP, 25 µl 400 m*M* $MgCl_2$, 150 µl 3.33 *M* hydroxylamine (pH 7.5) and 100 µl H_2O, or 100 m*M* lysine, or 100 m*M* threonine. The reaction was started by adding 200 µl of enzyme to the test solution: this was subsequently incubated at 30 °C for 60 min. The assay was stopped by the addition of 200 µl 25% (w/v) $FeCl_3$ and 8% (w/v) TCA dissolved in 2 *N* HCl; colour was allowed to develop for 40 min. Blanks were included, in which aspartate was added just before the colour reagent. Absorbance was measured at 505 nm, after a 5 min centrifugation at 12,000 rpm to remove the precipitated proteins. A unit of AK activity is defined as the amount of enzyme producing 1 mmole of aspartylhydroxamate per minute.

Protein determination was done following the method of Bradford (1976), using a solution of bovine serum albumin (BSA) as the standard.

Assay for dihydrodipicolinate synthase activity

This assay was performed as described by Ghislain et al. (1990).

Table 1. Segregation of lysine + threonine resistance in seedling progeny of the RLT 70 original line

	Resistant	Sensitive	χ^2	*P*
Test cross				
RLT 70 × wild type	137	145	0.23 (1:1)	0.58–0.65
wild type × RLT 70	126	118	0.26 (1:1)	0.58–0.65
RLT 70 (selfed)	137	44	0.05 (3:1)	0.82

Results

Origin and characterization of LT-resistant RLT 70

Non-mutagenized and mutagenized populations of diploid protoplasts were submitted to selection using growth inhibitory concentrations of lysine (0.75 m*M*) plus threonine (0.25 m*M*) ($L_{0.75}T_{0.25}$) Resistant lines (12) were isolated from mutagenized cultures only, and their resistance as regenerated plants was tested on 3 m*M* lysine plus 2 m*M* threonine (L_3T_2). One line in particular (RLT 70) was confirmed to be resistant, and subsequently produced seeds. Analysis of its descendents revealed that the resistance trait was heterozygous in the parental plant and inherited as a monogenic dominant trait (Table 1). Sensitive seedlings were identified 8 days after germination: cotyledons gradually turned white and the short roots barely penetrated the agar, further development of the seedlings was blocked. Selfing of the resistant offsprings of RLT 70 did not result in homozygote types due to an altogether poor growth and low fertility. Back-crosses with the wild type followed by subsequent selections of the resistant types on L_2T_1 medium were necessary to obtain phenotypically normal plants and to finally recover the resistance property in the homozygous state. Biochemical and enzymatic analysis were primarily performed on this homozygous RLT 70 line.

Enzymatic properties

The basis of the LT resistance was investigated by determining the properties of the lysine- and/or threonine-inhibited enzymes of the aspartate pathway. AK and DHDPS activities were therefore measured and their inhibition pattern established in leaf tissue for both resistant RLT 70 and wild-type (WT) plants. No significant change in the specific activity of either enzyme could be detected. In both plants, DHDPS remained strongly inhibited by lysine (I_{50} at 15 µ*M*), and, to a lesser extent, by its analogue S-2-(aminoethyl)-L-cysteine (AEC) (I_{50} at 0.1 m*M*). This result was consistent with a test for cross resistance to AEC, where both WT and RLT 70 plantlets were equally sensitive to 0.1 m*M* of the analogue.

The inhibition pattern of AK determined in the wild type however differed greatly from the one measured in

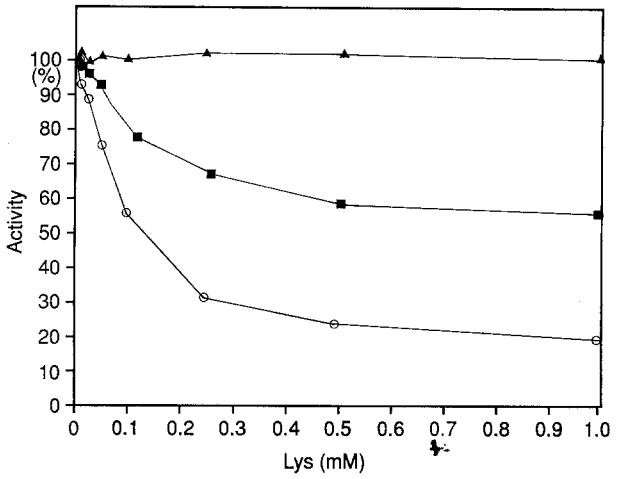

Fig. 1. Effect of lysine on the activity of aspartate kinase in leaf extracts of wild type (○), heterozygote (■) and homozygote (▲) RLT 70 (activity expressed as a percentage of the total)

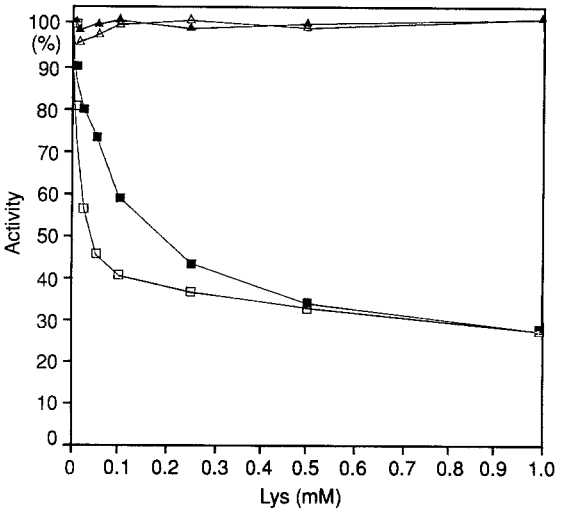

Fig. 2. Effect of lysine, and of lysine + AdoMet (0.2 m*M*) on the activity of aspartate kinase in leaf extracts of wild type (■, □) and homozygote RLT 70 (▲, △) (activity expressed as a percentage of the total)

Table 2. Inhibition of aspartate kinase in wild type and RLT 70 mutant in percentage of total enzymatic activity, in the presence of lysine and/or threonine at a final concentration of 10 m*M*

	+Lysine	+Threonine	+Lysine +Threonine
Wild type	70–80	20–30	90–100
RLT 70			
Heterozygote	30–40	20–30	50– 60
Homozygote	0–10	20–30	20– 30

the RLT 70 mutant. Up to 80% of the total AK activity of the wild-type enzyme is sensitive to feedback inhibition by lysine (I_{50} of 90 μ*M*) while the remaining 20% is feedback controlled by threonine (I_{50} of 110 μ*M*). When both amino acids are added to the assay, a total additive inhibition of the AK activity is observed, suggesting the presence of at least two isoenzymes, one lysine-sensitive, the other threonine-sensitive. In contrast, the homozygote RLT 70 displayed an AK totally desensitized to feedback inhibition by lysine (tested up to a final concentration of 10 m*M*). The heterozygote presented an intermediary situation in which 35%–40% of the AK activity was still inhibited by lysine (Fig. 1; Table 2). In both homozygote and heterozygote plants the fraction of AK activity sensitive to threonine remained unchanged. The ratio between AK lysine-sensitive and AK threonine-sensitive was also investigated during plant development and in "in vitro" culture conditions (cell suspensions and calli) in both wild type and mutant plants. No modification in this proportion could be observed (data not shown).

Since AdoMet rather than methionine itself appears to regulate methionine synthesis (Rognes et al. 1980) it appeared interesting to test its potential effect on AK activity. On its own AdoMet has actually little effect up to concentrations of 0.5 m*M*, but can inhibit 25% of the AK activity at 1 m*M*. Although the enzyme activity in wild-type *N. sylvestris* is predominantly inhibited by lysine, the presence of AdoMet greatly enhances the enzyme sensitivity towards this inhibitor, primarily in the low concentration range. The enzyme from the homozygote RLT 70, however, did not show any decrease of activity in the presence of both lysine and AdoMet (Fig. 2). Thus, no synergistic inhibition of the mutant enzyme activity due to lysine + AdoMet could be established. The addition of AdoMet to threonine in the assay did not affect the normal threonine feedback inhibition of aspartate kinase in the wild type as well as in the mutant.

A second potential inhibitor of AK activity was tested: AEC, a lysine analogue. AEC exerted a feedback inhibition of the wild-type enzyme that was between 6 to 8 times weaker than the control exerted by lysine, reaching 47% of the activity at 2 m*M* against 80% at the same concentration of lysine. No inhibition of the enzyme from the homozygote RLT 70 was observed up to a final concentration of 10 m*M* of AEC (Fig. 3).

Separation of the isoenzymes was attempted by ion-exchange chromatography using a Mono Q column with a FPLC system. The best results were obtained with a 30-ml gradient of 50–350 m*M* KCl: all of the AK activity eluted as one peak, preceding the major protein elution peak. Lysine-sensitive and threonine-sensitive activities, although not fully separated, could be measured using the radioactive assay. The lysine-sensitive AK was the first one to be eluted (Fig. 4). This elution profile

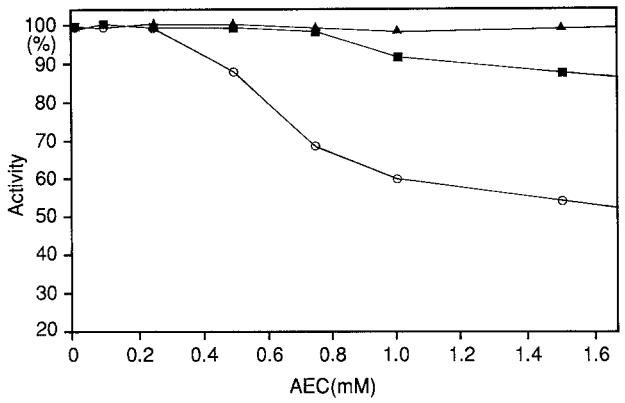

Fig. 3. Effect of AEC on the activity of aspartate kinase in leaf extracts of wild type (o), heterozygote (■) and homozygote (▲) RLT 70 (activity expressed as a percentage of the total)

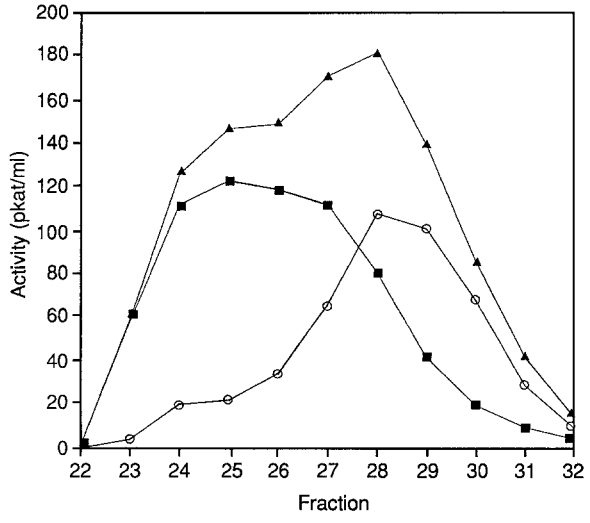

Fig. 4. Elution of wild-type aspartate kinase activity (▲) from an FPLC Mono Q anion exchange column (50–350 m*M* KCl gradient in 30 ml). The lysine-sensitive activity (■) is eluted before the threonine-sensitive activity (o) (expressed in pkat/ml)

strongly suggests the presence of at least two isoenzymes of AK in *Nicotiana sylvestris*.

Amino acid analysis

The free amino acid content of both the LT-resistant RLT 70 and the wild type was analysed in various tissues and organs during the life cycle of the plant. The evolution of free threonine overproduction in leaf tissue during RLT 70 development is shown in Fig. 5. Although the early stages of growth of RLT 70 exhibit a threonine content barely superior to that of the wild type, a peak which could reach 70% of the total (against 6% in the wild type) was observed just before flowering occurred. This actually corresponds to a +45-fold increase:

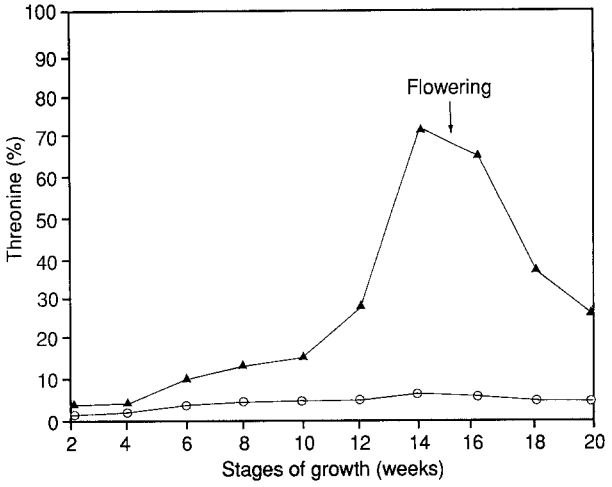

Fig. 5. Evolution of the free threonine production in wild type (o) and mutant RLT 70 (▲) in leaf extracts during the plant life cycle (expressed as a percentage of the total pool of free amino acids)

389 nmoles/g fresh weight in the wild type to be compared to 18,239 nmoles/g fresh weight in RLT 70.

Since the considerable increase in the pool of free amino acids is due to the large amounts of unbound threonine, its substraction from the total should provide a better approximation of the real values of the other amino acids. From Table 3 it is quite clear that with respect to the aspartate-derived amino acids a considerable increase in free isoleucine content (10 times) occurs, as well as to a lesser extent, an increase in methionine (5 times) and lysine (5 times). The pool of free aspartate is equivalent in both plants, demonstrating that this high overproduction of threonine, and to a lesser extent of the other aspartate-derived amino acids, did not lead to aspartate starvation. Finally, the total pool of free amino acids in RLT 70 is a factor of four-to-five greater. This is mainly due to the soluble threonine increment, as well as to the isoleucine and lysine contribution, but also to a slight global increase in the concentration of the other amino acids.

When protein-bound amino acids in the leaves of the RLT 70 mutant were analysed, no modification of the spectrum occurred. Furthermore, the total amount of protein present in the mutant was found to be identical to that found in the wild type (data not shown). The consequences of an increased soluble threonine accumulation in the leaves on total (protein-bound + free) amino acid concentration has been evaluated in RLT 70. Total threonine content was 119 nmole/g fresh weight in the mutant against the 9 nmole/g observed in the wild type, which corresponds, respectively to 28% and 5% of the total (free + protein-bound) amino acids. Thus, almost one-third of all the amino acids in the mutant RLT 70 leaves was in the form of threonine, mostly unbound.

Table 3. Free amino acid analysis of wild-type and RTL 70 leaves: absolute values (in nmoles g^{-1} of fresh weight) and percentages with threonine included and excluded

Amino acid	Absolute values (nmoles g^{-1} fresh weight)		Threonine included (%)		Threonine excluded (%)	
	W.T.	RLT 70	W.T.	RLT 70	W.T.	RLT 70
Aspartate	**910**	**1,053**	**15.5**	**3.9**	**16.6**	**12.2**
Threonine	**389**	**18,239**	**6.6**	**67.8**	–	–
Serine	601	813	10.2	3.0	10.9	9.4
Glutamate	1,197	1,391	20.3	5.2	21.8	16.1
Proline	524	583	8.9	2.2	9.5	6.7
Glycine	482	450	8.2	1.7	8.8	5.2
Alanine	681	437	11.6	1.6	12.4	5.0
Cysteine	63	199	1.1	0.7	1.2	2.3
Valine	234	243	4.0	0.9	4.3	2.8
Methionine	**28**	**151**	**0.5**	**0.6**	**0.5**	**1.7**
Isoleucine	**70**	**844**	**1.2**	**3.1**	**1.3**	**9.7**
Leucine	101	349	1.7	1.3	1.8	4.0
Tyrosine	83	200	1.4	0.7	1.5	2.3
Phenylalanine	79	245	1.3	0.9	1.4	2.8
Histidine	288	923	4.9	3.4	5.2	10.7
Lysine	**109**	**503**	**1.9**	**1.9**	**2.0**	**5.8**
Arginine	49	284	0.8	1.1	0.9	3.3
Total	5,888	26,907			5,499	8,668

Table 4. Total (free + protein-bound) amino acid analysis of wild-type and RLT 70 leaves: absolute values (in nmoles g^{-1} fresh weight) and percentages

Amino acid	Wild type (nmoles g^{-1} fresh weight)	RLT 70	Wild type (% of total)	RLT 70
Aspartate	18,006	29,345	10.3	7.3
Threonine	**9,134**	**118,960**	**5.2**	**29.6**
Serine	9,697	15,962	5.5	4.0
Glutamate	18,009	28,796	10.3	7.2
Proline	9,505	14,355	5.4	3.6
Glycine	17,163	28,024	9.8	7.0
Alanine	18,028	27,646	10.3	6.9
Cysteine	150	510	0.1	0.1
Valine	12,929	20,068	7.4	5.0
Methionine	1,892	4,536	1.1	1.1
Isoleucine	9,430	17,139	5.4	4.3
Leucine	15,580	24,234	8.9	6.0
Tyrosine	4,216	7,790	2.4	1.9
Phenylalanine	7,918	12,600	4.5	3.1
Histidine	3,114	7,856	1.8	2.0
Lysine	13,323	29,556	7,6	7.4
Arginine	6,998	14,722	4.0	3.7
Total	175,092	402,099		

Moreover, a global increase of 60% of all the other amino acids (free + protien-bound) was observed that was exclusively due to a free amino acid contribution (Table 4).

Analysis of the content of free amino acids in immature seeds, flowers, and calli revealed large amounts of soluble threonine in all cases (respectively 42%, 30%, and 25% of the total against 11%, 2% and 2% in the wild type) (Table 5). A general increase in the total of free amino acids was also observed in these organs, mainly a result of free threonine increment, but also from an enhanced content of the other amino acids.

Special attention was paid to the mature seeds of RLT 70 with respect to the extent of threonine accumulation. Free threonine content reached one-fifth of the total pool, which corresponds to a 70-fold increase (Table 6). However, all the other amino acids were also more abundant (mean of 17 times), such that the pool of free amino acids is approximately 20 times greater. Thus the consequences of the mutation on total (free + protein-bound) amino acid content in seeds are that threonine content is multiplied by a factor of six and that there is a ± four-fold increase in all the other amino acids, which differs from the situation encountered in leaves. As a matter of fact, although in both cases the protein-bound amino acid spectrum was unchanged in the mutant (seeds or leaves), the seeds were observed to have a significantly higher protein content, thereby increasing considerably the global contribution of amino acids in the composition of the seed (Table 7).

Discussion

The isolation of a homozygote RLT 70 threonine overproducer

The selection procedure based on the use of mesophyll protoplasts of *N. sylvestris* led to the isolation of a highly

Table 5. Content of some free amino acids in leaves, flowers, calli and immature seeds of wild type (W.T.) and RLT 70 mutant (expressed as percentage of the total free amino acid pool, in nmoles g^{-1} fresh weight)

Amino acid	Leaves		Flowers		Calli		Immature seeds	
	W.T.	RLT 70	W.T.	RLT 70	W.T.	RLT 70	W.T.	RLT 70
Aspartate	15.1	6.4	25.0	9.6	13.0	18.1	17.8	15.0
Threonine	6.3	75.2	2.3	29.6	1.4	24.6	11.3	41.5
Glutamate	19.4	5.8	30.3	30.2	29.2	23.7	25.3	21.6
Methionine	0.5	0.6	0.2	0.4	0.4	0.4	0.1	0.1
Isoleucine	1.7	2.7	1.6	2.0	2.0	3.0	1.2	0.8
Lysine	1.9	2.6	0.6	0.8	0.6	1.3	1.0	1.5
Arginine	1.2	1.6	0.3	0.6	2.3	3.1	1.1	4.3
Total	7,228	25,971	55,998	68,460	1,060	9,629	30,019	63,783

Table 6. Free amino acid analysis of wild-type and RLT 70 mutant mature seeds: absolute values (in nmoles g^{-1} fresh weight) and percentages

Amino acid	Wild type (nmoles g^{-1} fresh weight)	RLT 70	Wild type (% of total)	RLT 70
Aspartate	276	7,534	12.4	19.8
Threonine	**130**	**9,393**	**5.8**	**24.6**
Serine	134	1,945	6.0	5.1
Glutamate	493	4,651	22.1	12.2
Proline	388	2,409	17.4	6.3
Glycine	125	3,695	5.6	9.7
Alanine	175	3,012	7.8	7.9
Cysteine	15	245	0.7	0.6
Valine	94	829	4.2	2.2
Methionine	ND	46	–	0.1
Isoleucine	34	365	1.5	1.0
Leucine	38	480	1.7	1.3
Tyrosine	109	263	4.9	0.7
Phenylalanine	30	503	1.4	1.3
Histidine	108	877	4.8	2.3
Lysine	26	608	1.2	1.6
Arginine	55	1,301	2.5	3.4
Total	2,230	38,156		

ND: Not detected

Table 7. Total (free + protein-bound) amino acid analysis of wild-type and RLT 70 mutant mature seeds: absolute values (in nmoles g^{-1} fresh weight) and percentages

Amino acid	Wild type (nmoles g^{-1} fresh weight)	RLT 70	Wild type (% of total)	RLT 70
Aspartate	60,356	246,571	10.1	11.9
Threonine	**20,189**	**112,329**	**3.4**	**5.4**
Serine	26,812	106,312	4.5	5.1
Glutamate	98,489	301,513	16.5	14.5
Proline	51,235	65,573	8.6	3.2
Glycine	60,643	174,330	10.1	8.4
Alanine	40,858	212,709	6.8	10.2
Cysteine	2,378	12,987	0.4	0.6
Valine	33,254	126,346	5.6	6.1
Methionine	7,400	28,000	1.2	1.4
Isoleucine	30,650	99,134	5.1	4.8
Leucine	37,458	142,770	6.3	6.9
Tyrosine	11,375	40,436	1.9	2.0
Phenylalanine	31,296	95,741	5.2	4.6
Histidine	17,243	57,234	2.9	2.8
Lysine	17,600	61,098	2.9	2.9
Arginine	51,385	194,837	8.6	9.4
Total	598,621	2,077,919		

threonine-overproducing LT-resistant line: RLT 70. Progeny tests performed on plants regenerated from RLT 70 demonstrated the dominant monogenic nuclear character of the mutation, which shall further be referred to as *ak-LT1* (Table 1). This dominant Mendelian transmission of the LT resistance has also been observed in other species such as carrot (Cattoir-Reynaerts et al. 1983), barley (two distinct lysine-sensitive AK isoenzymes corresponding to two unlinked loci, *Lt1* and *Lt2*) (Bright et al. 1982; Rognes et al. 1983) or maize (only the lysine-sensitive AK enzyme detected up to now, but probably controlled by two distinct independant genes, *Ask-LT19* and *Ask-LT20* (Diedrick et al. 1990). The usefulness of such mutations that are inherited as dominant or codominant nuclear traits in plant breeding programmes, can never be overemphasized.

The availability of a lysine-overproducing *Nicotiana sylvestris* mutant (RAEC-1) with an altered DHDPS (Negrutiu et al. 1984) allowed us to cross this mutant with the RLT 70 threonine-overproducing mutant. Free amino acid analysis showed only lysine overproduction in the F_1 double heterozygote offsprings. Further studies should provide more information concerning regulation in the aspartate family in general, and the consequences of the presence of two mutated enzymes in the same biosynthetic pathway on the pool of free amino acids.

The study of aspartate kinase

Aspartate kinase, the first enzyme of the aspartate pathway, is known to be regulated in plants by two major effectors: lysine and threonine. However, no general feedback inhibition pattern can be ascribed: some species are inhibited by lysine alone (maize) (Dotson et al. 1990); most are inhibited lysine and threonine, such as barley (Rognes et al. 1983) spinach (Kochar et al. 1986), *sorghum* (Piryns et al. 1988) *Arabidopsis* (Vernaillen et al. 1985), *Lemna* (Giovanelli et al. 1989); even threonine alone can inhibit, such as in *Pisum* (Aarnes and Rognes 1974). Furthermore, in rapidly growing tissues there is usually a greater proportion of the lysine-sensitive enzyme present. *Nicotiana sylvestris* AK activity can be inhibited up to 80% by lysine, and most of the last 20% threonine. The additive inhibition of its activity when both effectors are simultaneously present suggests the existence of isoenzymes. Previous studies in a number of plants have clearly demonstrated the presence of AK isoforms. Three independant AK isoenzymes were resolved in barley using ion-exchange chromatography: AKI (65% sensitive to threonine only), AKII (lysine sensitive only) and AKIII (sensitive to lysine + AdoMet only) (Rognes et al. 1983). In spinach, two isofunctional forms of AK were separated on the basis of their different molecular weights using gel filtration: one was preferentially sensitive to lysine control (AKI); the second exclusively to threonine (AKII) (Kochar et al. 1986). Two lysine-sensitive AK isoforms seem to coexist in carrot, as shown by the biphasic heat inactivation curve (Relton et al. 1988). The presence of AK isoenzymes in *Nicotiana sylvestris* is confirmed in the present study by ion-exchange chromatography, in which two peaks of activity could be distinguished, although not fully separated. As in spinach, the lysine-sensitive activity was the first to be eluted. The next step will be to pool the fractions corresponding to each peak, and individually rechromatography them in order to confirm their different feedback regulation sensitivities.

The half-inhibition value of 90 μM that was determined for the lysine-sensitive AK isoform in *Nicotiana* is intermediate to those reported for other plants: 10 μM for the *Sorghum* enzyme (Piryns et al. 1988) 175 μM for the carrot enzyme(s) (Relton et al. 1988) and 360; 400 μM for the two barley isoenzymes (Arruda et al. 1984). The concentration of 110 μM threonine to reach the half-inhibition of the other *Nicotiana* isoenzyme is comparable to 60 μM for the *Lemna* enzyme (Giovanelli et al. 1989) and 120 μM for the *Sorghum* enzyme (I. Piryns, personal communication).

The comparison of the inhibition patterns of aspartate kinase of *N. sylvestris* wild type and homozygote RLT 70 revealed the complete insensitivity of the mutant enzyme to feedback inhibition by lysine (up to 10 mM), without affecting the threonine control. In addition, the observation that only half of the AK lysine-sensitive fraction in the heterozygote RLT 70 is insensitive to feedback control can be associated with the diploid nature of *N. sylvestris* and to a monogenic control of the inhibition property of this isoenzyme.

Of the LT-resistant mutants analysed to date, all have exhibited decreased sensitivity to lysine feedback inhibition (in carrot, barley and maize) (Cattoir-Reynaerts et al. 1983; Arruda et al. 1984; Diedrick et al. 1990). In maize the I_{50} values for lysine were approximately 2- to 4-fold and 76-fold higher for aspartate kinase from heterozygote (Ask-LT19/+) and homozygote (Ask2-LT20/Ask2-LT20) plants, respectively, than the wild-type enzyme.

Once we had clearly established that enzyme regulation was affected by the mutation, it appeared interesting to test two other potential effectors of the activity of the AK-lys enzyme: AdoMet and AEC. The cooperative inhibition of lysine + AdoMet has been reported for a number of plant aspartate kinases (Rognes et al. 1980), and the *Nicotiana sylvestris* wild-type enyzme is no exception to this general property. In contrast, but as expected, the enzyme of the homozygote RLT 70 was not inhibited by the simultaneous presence of lysine and AdoMet. AEC, a lysine analogue is expected to feedback inhibit the AK-lysine sensitive isoenzyme just as its model, although perhaps to a lesser extent. This was effectively the case for the wild-type *N. sylvestris,* which was observed to be six-to eight-fold less sensitive to the analogue than to its normal lysine control. In contrast, the homozygous mutated enzyme was not sensitive to 10 mM AEC, suggesting that both inhibitors regulate the wild-type enzyme in a similar manner.

Amino acid content

This study demonstrates the considerable impact that an altered regulation of one key enzyme belonging to a biosynthetic pathway can have on the concerned end products. Among those of the aspartate family, free threonine comprises up to 70% of the total pool of free amino acids in RLT 70 *N. sylvestris* leaves (18,239 mmoles/g) against 6% in the wild type (389 mmoles/g, or 45 times more). The maximal accumulation of soluble threonine occurs just before flowering, suggesting a possible translocation of this amino acid from the leaves to the seeds. By substracting the amount of threonine from the total amount of free amino acids in the mutant as well as in the wild type, a clear increase in the other end products of the aspartate pathway was revealed. A substantial increase in free isoleucine (10 times) was observed, indicating that further enzymatic regulation of that branch of the aspartate pathway (such as at the level

of threonine dehydratase) is not stringent. On the other hand, DHDPS controls the lysine biosynthesis more tightly, so that soluble lysine content was multiplied by a factor of five only. The last end product, methionine, displayed a similar increment. It should also be noted that the free aspartate pool is comparable in both wild type and RLT 70 mutant, and is thus not a limiting factor to the overproduction of one or more end products. RLT 70 seeds displayed a 70-fold increase in soluble threonine as well as a ± 17 times increment in the rest of the amino acids. In comparison, carrot LT-resistant embryos presented an 8-fold free threonine and a 2.5-fold free isoleucine increment (Cattoir-Reynaerts et al. 1983). Two barley mutants had 12 times more soluble threonine in their seeds than the wild type ones (Arruda et al. 1984). Seeds from maize AK mutants presented 14.5 nmoles/mg dry weight of free threonine against 0.50 nmoles/mg in the wild-type grain (Diedrick et al. 1990). The total free amino acids in *N. sylvestris* RLT 70 leaves and seeds increased 4–5 times and 20 times, respectively, essentially a result of free threonine overproduction. In seeds, however, a global increase in the other amino acids also contributed to the greater pool size observed. In comparison, maize mutant kernels showed only a 90% increase in their total free amino acids, mainly due to a higher free threonine content.

When the total amino acids of the leaves were examined, it appeared that up to 30% of the total consisted of threonine, mostly soluble, against only 5% in the wild type. Moreover, a global increase of 60% in all the other amino acids was observed, exclusively due to free amino acid augmentation. Effectively, no modification in the protein-bound amino acid spectrum could be determined in the RLT 70 mutant leaves, and total protein content was similar in both types of leaves. In RLT 70 seeds, total threonine was multiplied by a factor of six and total pool by a factor of four, but not only due to the free amino acid contribution. In fact, although the protein-bound amino acid spectrum was unchanged in the mutant (as for the leaves), total protein content was significantly higher in the mutant seeds. In maize mutants it appeared that the soluble amino acid contribution was insufficient to justify the global threonine levels observed (from 30.0 nmole/g dry weight in the wild type to 50.5 nmole/g in the mutant) and that a shift in the protein-bound amino acid spectrum was essentially responsible for this.

The high threonine contents encountered when analysing this LT-resistant mutant of *Nicotiana sylvestris* confirms the validity of the developed approach for improving crop quality, as well as the potential value of the mutated gene.

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