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Two Feedback-Insensitive Enzymes of the Aspartate Pathway in *Nicotiana sylvestris*[1]

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ABSTRACT

Lysine and threonine overproducer mutants in *Nicotiana sylvestris*, characterized by an altered regulation of, respectively, dihydrodipicolinate synthase and aspartate kinase activities, were crossed to assess the effects of the simultaneous presence of these genes on the biosynthesis of aspartate-derived amino acids. The monogenic dominant behavior of both resistance traits was confirmed, and their loci were found to be unlinked. Study of the inhibition properties of dihydrodipicolinate synthase and aspartate kinase activities in RAEC-1 × RLT 70 confirmed the heterozygote state of both mutations, because only half of their lysine-sensitive activity could still be inhibited by this negative effector. Analysis of the free amino acid pool during the growth of the double mutant revealed a major free lysine overproduction reaching up to 50% of the total pool, whereas the other aspartate-derived amino acids remained equally or even less abundant than in the wild type. An abnormal phenotype was clearly associated with such high levels of lysine accumulation, which points out the possible role of this amino acid in the developmental features of the plant. Comparison of the amino acid content, free and total (free + protein-bound), between the wild type, the two mutants, and the double mutant obtained by crossing them brings new insights on the regulation of the aspartate pathway, and on its implications in relationship to plant nutritional value improvement.

The essential amino acids lysine, threonine, isoleucine, and methionine are biosynthesized in higher plants following a branched pathway derived from a common precursor: aspartate (6). Understanding the inner regulation effective during this process is essential with respect to basic plant metabolism, and to possible modification of the nutritional value of crops. Nonruminant feed or food based on cereals only, which are well known to be poor in lysine and threonine, are indeed growth-limiting in diets unless supplemented (12). As a consequence, numerous attempts to improve the content of these two amino acids have been conceived, among which is the modification of the regulatory processes involved in their biosynthesis (24).

A close examination of the lysine and threonine biosynthesis has put forth the existence of several enzymic steps feedback-controlled by these two end products (Fig. 1) (24). Essentially, two enzymes play a predominant regulatory role: AK[3] (EC 2.7.2.4), the first enzyme of the overall pathway, feedback-regulated by lysine and/or threonine, and DHDPS (EC 4.2.1.52), the first enzyme of the lysine-specific branch, feedback-controlled by lysine only. Mutants possessing feedback-insensitive forms of these enzymes should thus overproduce lysine or threonine, or possibly both.

Mutagenesis selection methods based on the growth inhibition caused by the presence of a lysine analog, AEC, or of LT (leading to methionine starvation) (17) in the culture medium, have led to the isolation of resistant mutants in various plant species. Resistant cell lines to AEC have been isolated in maize and potato, but without modification of the lysine content (13, 20). AEC-tolerant *Pennisetum* accumulated free lysine 5 to 7 times that of the control in vegetative tissues, but no genetic or biochemical evidence was available (3). In barley, wheat, maize, carrot, and *Arabidopsis*, recessive AEC-resistant mutants were characterized by a decrease of AEC uptake (5, 21–23, 29). Rice plants resistant to AEC, regenerated from anther-derived calli, resulted in seeds of higher protein-bound lysine and storage protein content (27). One *Nicotiana sylvestris* AEC-resistant plant (RAEC-1) regenerated from protoplast culture exhibited in leaves up to 28 times more free lysine than the wild type (26). The parent plant was heterozygotic for the AEC resistance trait, which was inherited as monogenic dominant nuclear gene. Only 50% of the DHDPS activity of this mutant could be inhibited by lysine (or AEC). This modification of the regulatory control of the lysine branch was associated with the overproduction of lysine in the free amino acid pool of the plant. To date, RAEC-1 is a unique example of a mutation in the regulatory properties of the DHDPS enzyme and of its characterization.

In contrast with the AEC selection in which different categories of mutants were yielded (uptake, overproduction), LT selection mainly produced mutants that accumulate free threonine due to an AK less sensitive to feedback inhibition by lysine (2, 4, 8, 9, 18, 19, 23). No overproduction of this amino acid was observed, presumably due to the further stringent DHDPS control. In *N. sylvestris*, one LT-resistant plant (RLT 70) exhibited in leaves and in seeds a 45-fold and a 70-fold increase of soluble threonine, respectively (11). In both cases, total (free + protein-bound) threonine content

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[3] Abbreviations: AK, aspartate kinase; DHDPS, dihydrodipicolinate synthase; AEC, *S*-(2-aminoethyl)L-cysteine; LT, lysine plus threonine.

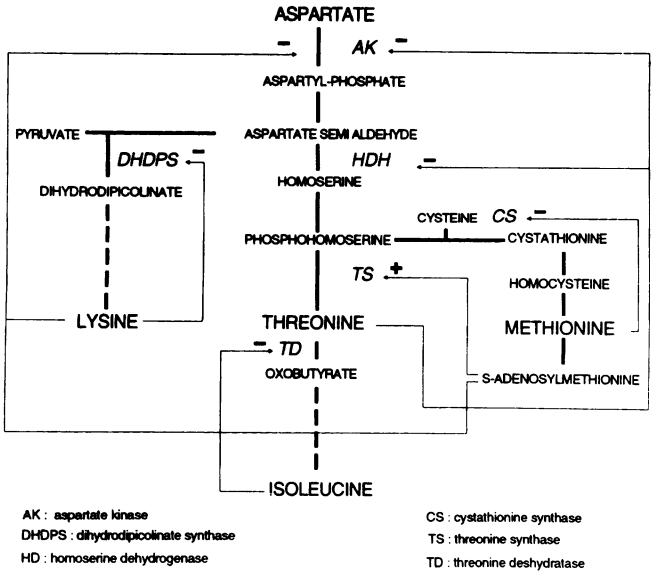

Figure 1. Main regulatory controls (determined *in vitro*) occurring in the biosynthetic pathway of the aspartate-derived amino acids in plants. Plus and minus signs indicate, respectively, stimulation and inhibition of the enzyme activity next to which they stand.

was greater in the mutant. The dominant monogenic nuclear transmission of the resistance trait was established, associated with an AK less sensitive to feedback inhibition by lysine.

Earlier attempts to isolate plants carrying both mutations by consecutive selection procedures were not conclusive. As such, the novelty of this report resides in the genetic and biochemical characterization of a unique double mutant, RAEC-1 × RLT 70, obtained by crossing the two *N. sylvestris* mutants: RAEC-1 (accumulating free lysine as the result of an altered regulation of DHDPS activity), and RLT 70 (overproducing free threonine consequently to a modified control of AK activity). The comparison of its properties with those of the two mutant parents brings valuable information concerning the internal regulation of the branched aspartate-derived biosynthetic pathway.

MATERIALS AND METHODS

Isolation of the Mutant

Isolation and characterization of both RAEC-1 and RLT 70 *Nicotiana sylvestris* mutants have been reported earlier (11, 26). Both were grown simultaneously in the greenhouse, and the female partner was emasculated before pollen maturation. Seeds issued from the cross were harvested and stored at 4°C.

Progeny Test for Resistance

Seeds were sterilized in ordinary bleaching water for 10 min, washed three times for 10 min in sterile water, and seeded on agar medium containing half-strength Murashige and Skoog (25) basal medium with 10 g/L sucrose containing either selection agent: lysine (2 mM) + threonine (1 mM) (L_2T_1) or AEC (0.1 mM), both filter-sterilized.

Amino Acid Analysis

Free Amino Acids

Between 100 and 500 mg of fresh plant material were homogenized using a mortar and pestle in the presence of methanol:chloroform:water (12:5:1) (1). Calli free amino acid extraction required an additional step: the material was boiled in 70% ethanol for 10 min (5). At each of the three successive extractions, the homogenate was centrifuged and the supernatants pooled. Chl (and the organic fraction) was removed by adding to the extract two parts chloroform plus one part water. 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 hydrolyze the amides. After evaporation at 85°C, the residue was resuspended in loading buffer (Na-citrate, pH 2.2) and automatically analyzed in a Biotronik LC 5001 amino acid analyzer 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 of 6.25 N HCl placed under vacuum at 110°C for 24 h. The hydrolysate was left to evaporate at 80°C, resuspended in 1 mL of loading buffer, and analyzed as described above.

Total Amino Acids (Free + Protein-Bound)

Plant material was ground with mortar and pestle. One milliliter of 6.25 N HCl was added, and the extract was further treated following the protein-bound protocol.

All samples were kept at −20°C while waiting to be analyzed.

Partial Purification of AK

Leaf tissue from 2- to 10-week-old plantlets was used as starting material. All procedures were carried out at 4°C unless otherwise indicated.

Leaf tissue, between 5 and 10 g, was extracted in 2 volumes of 50 mM potassium phosphate (pH 7.5), 1 mM EDTA, 2 mM lysine, 2 mM threonine, 10 mM β-mercaptoethanol, 10 mM diethyldithiocarbamate, 50 mM KCl, and 20% (v/v) glycerol using a Waring Blendor. The homogenate was 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/original g leaves of dialysis buffer, 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM KCl, and 10% (v/v) glycerol. Ammonium sulfate and other low mol wt compounds were removed by passing the extract through a Sephadex G-25 column equilibrated with the dialysis buffer. The green fractions containing the AK activity were pooled and used as such for the inhibition studies.

Assay for AK Activity

The hydroxamate assay initially described by Bryan *et al.* (7) has been partially modified as follows: the assay compo-

nents add up to 1 mL and include 100 μL of 100 mM Tris-HCl (pH 7.5), 400 μL of 250 mM aspartic acid (pH 7.5), 25 μL of 400 mM ATP, 25 μL of 400 mM $MgCl_2$, 150 μL of 3.33 M hydroxylamine (pH 7.5), and 100 μL of H_2O, 100 mM lysine, or 100 mM threonine. The reaction was started by the addition 200 μL of enzyme incubated at 30°C for 60 min. The assay was stopped by the addition of 200 μL of 25% (w/v) $FeCl_3$ and 8% (w/v) TCA dissolved in 2 N HCl, and color was allowed to develop for 40 min. Blanks were included in which aspartate was added just before the color reagent. Absorbance was measured at 505 nm after a 5-min centrifugation to remove the precipitated proteins at 12,000 rpm. One unit of AK activity is defined as the amount of enzyme producing 1 nmol aspartylhydroxamate/min (with an absorption coefficient of 750 M^{-1} cm^{-1} for aspartylhydroxamate).

Assay for DHDPS Activity

This assay was performed as described by Ghislain *et al.* (14). One unit of DHDPS activity is defined as the amount of enzyme causing an increase in absorbance of 0.001 min^{-1} at 550 nm.

RESULTS

Isolation and Developmental Features of the Double Mutant

Because both parents, RAEC-1 and RLT 70, were originally isolated as heterozygotes for their respective resistance property, selfing of the plants and subsequent selection of their progeny were necessary to obtain the mutation in the homozygous state. The two homozygous mutants, RAEC-1 and RLT 70, were then crossed using each mutant as a female as well as a male partner, and the F_1 offsprings were sown on AEC- or LT-containing media. No sensitive phenotypes are observed on either selection plate, as expected from the dominant nature of both mutations (Table I). Tests for absence of cross-resistance of RLT 70 to 0.1 mM AEC and of RAEC-1 to L_2T_1 were performed: wild-type and mutant parents are equally sensitive to the selection procedure, their growth being totally inhibited approximately 8 d after germination.

Development of the F_1 plantlets transferred to the greenhouse is perfectly normal until young rosettes of between 5 and 10 leaves are formed. At that stage, the plant phenotype starts to change dramatically, showing a lack of apical dominance and of internodes, modification of leaf size, reduction of leaves almost to the ribs, and decreased Chl content (Fig. 2). No stem elongation or flower formation has ever been observed, although all the crosses were performed with phenotypically normal mutant parents. The F_1 population can be maintained at this growth level for many months without further development.

Among a homozygous population of the parent RAEC-1 grown in the greenhouse, a modified phenotype very similar to the one developed by the double mutant appears with a frequency of about 1 plant out of 15. This plant is also sterile, as no flowers are formed at all. The possibility of a relation between the abnormal phenotype and an aneuploid state of the plant genome was rejected after chromosome counting using root tips of the mutant showed the normal 2n=24 of *N. sylvestris* (data not shown).

Table I. *Segregation Analysis of the Progenies Obtained from Crosses Performed with RLT 70 and RAEC-1*

The two homozygous mutants RLT 70 and RAEC-1 were crossed, using each mutant as a female as well as a male partner, and the F_1 offsprings sown on L_2T_1- and AEC-containing media.

	L_2T_1 (Lys 2 mM + Thr 1 mM)		AEC (0.1 mM)	
	Resistant	Sensitive	Resistant	Sensitive
Selfing				
RAEC-1 × RAEC-1	0	231	243	0
RLT 70 × RLT 70	219	0	0	227
Crosses				
RAEC-1 × RLT 70	215	0	224	0
RLT 70 × RAEC-1	232	0	221	0

Enzymic Properties

DHDPS

Maximal inhibition of DHDPS activity in RAEC-1 × RLT 70 by lysine is 50%, as for the heterozygote RAEC-1 enzyme, and presents a similar I_{50} of approximately 25 μM (Table II; Fig. 3). The homozygous RAEC-1 parent, however, presents a DHDPS totally desensitized to feedback inhibition by lysine (tested up to a final concentration of 10 mM), as well as by its analog, AEC. The wild-type enzyme was characterized earlier with an I_{50} value of 15 μM (14).

AK

The study of the AK inhibition pattern in RAEC-1 × RLT 70 has shown that lysine can inhibit only 35 to 40% of the total enzyme activity, which is comparable to the situation encountered for the heterozygote RLT 70 AK enzyme instead of the usual 70 to 80% in the wild type (Table II; Fig. 4). On the other hand, an AK totally insensitive to the feedback inhibition by lysine characterizes the homozygote parent RLT 70. In all cases, the inhibition values for threonine remain unaffected, varying between 20 and 30% of the AK activity.

No detectable change in the specific activity of DHDPS or AK between the wild type, the two parental mutants, and RAEC-1 × RLT 70 at a given developmental stage can be found (typical specific activity values encountered in *N. sylvestris* after the gel filtration step were of 15 units/mg for DHDPS and of 1.4 units/mg for AK).

Amino Acid Content

Free Amino Acids in Leaves

Leaf free amino acid content of the double heterozygote RAEC-1 × RLT 70 was analyzed throughout the plant growth and compared to the values obtained for the wild type and for each parental mutant, RAEC-1 and RLT 70 (Tables III and IV). A few major observations can be noted.

First, lysine is the only amino acid of the aspartate pathway

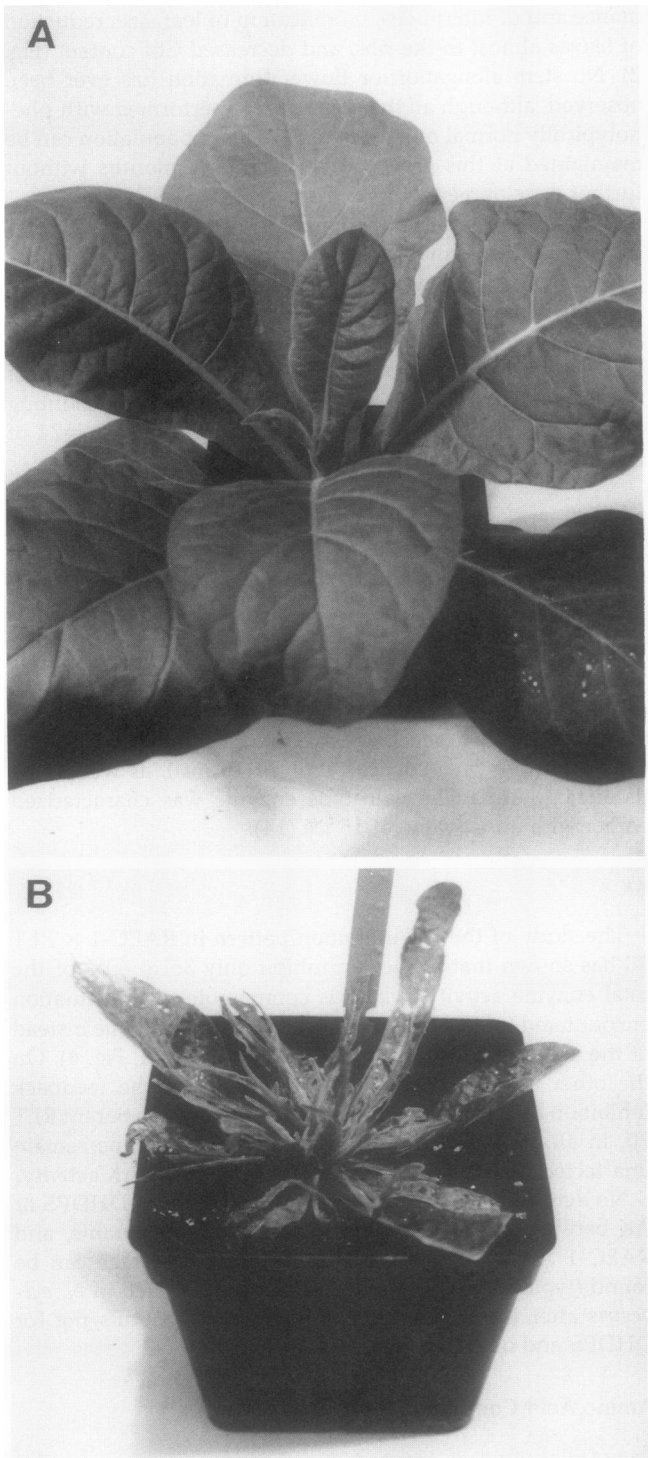

Figure 2. Wild-type *N. sylvestris* phenotype (A) compared to the aberrant morphology developed by double mutant RAEC-1 × RLT 70 (B).

Table II. *Comparison of Maximal Inhibition by Lysine of AK and DHDPS Activities*

DHDPS and AK activities from the wild type, the parental mutants, and RAEC-1 × RLT 70 were measured, as well as their maximal inhibition by lysine (final concentration of 10 mM), expressed as a percentage of the total activity.

Genotype	AK	DHDPS
Wild type	80	100
RAEC-1 × RLT 70		
Double heterozygote	40	50
RAEC-1		
Heterozygote	80	50
Homozygote	80	0
RLT 70		
Heterozygote	40	100
Homozygote	0	100

overproduced by RAEC-1 × RLT 70 (1785 nmol/g compared to 92 nmol/g, *i.e.* a 20-fold multiplication of the wild-type content, but up to 50% free lysine has sometimes been observed). In contrast, threonine, isoleucine, and methionine contents are similar or even reduced, especially in the older plants. When compared to the parental lysine overproducer RAEC-1, the levels of lysine accumulated in RAEC-1 × RLT 70 appear noticeably superior (30% compared to 20%). Concerning RAEC-1, however, complementary data must be provided. When considering a homozygous population of RAEC-1 grown in the same conditions, lysine overproduction ranges from 2 (no accumulation) to 30% (amount comparable to the RAEC-1 × RLT 70 lysine content). Normal phenotypes may accumulate up to approximately 25% of free lysine, beyond which an aberrant morphology tends to develop. Such an array of variation is not observed among a RAEC-1 × RLT 70 population in which all plants without exception contain 30% or more free lysine, together with the abnormal

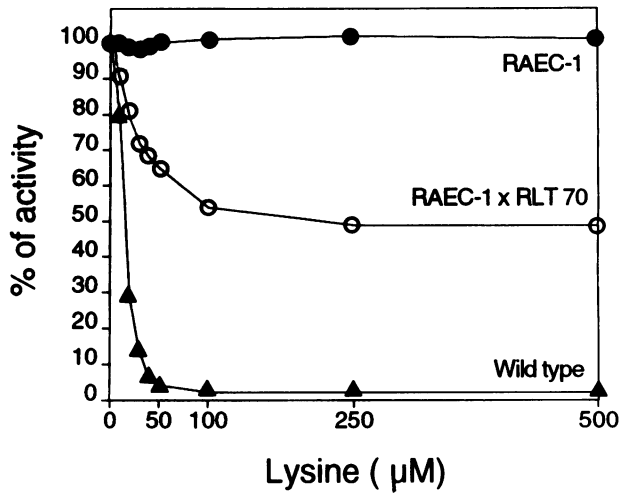

Figure 3. Inhibition of the DHDPS activity (expressed as a percentage of the total activity) in the presence of lysine, from wild type (▲), parental mutant RAEC-1 (●), and RAEC-1 × RLT 70 (○) plants.

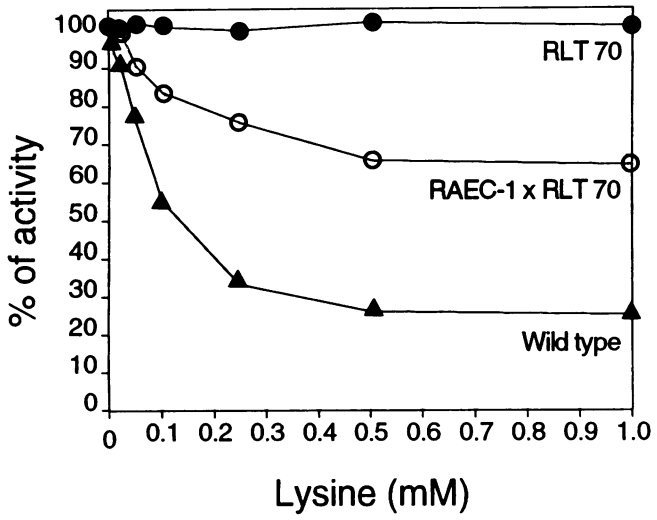

Figure 4. Inhibition of the AK activity (expressed as a percentage of the total activity) in presence of lysine, from wild type (▲), parental mutant RLT 70 (●), and RAEC-1 × RLT 70 (○) plants.

phenotype. It should also be noted that free proline content, often related as a response to stress, is higher in RAEC-1 × RLT 70 than in both parental mutants.

Second, when the entire pool of free amino acids is considered, RAEC-1 × RLT 70 presents a slightly higher total compared to that of the wild type and equivalent with that of RAEC-1 (but much smaller than that of RLT 70). This difference is essentially due to the high lysine content of RAEC-1 × RLT 70. The free aspartate pool does not seem limiting with regard to the production of either amino acid derived from it. Finally, a distinct increase of the lysine overproduction occurs during plant development, with a maximum after approximately 12 weeks of growth (Fig. 5).

Total (Free + Bound) Amino Acids in Leaves

The impact of the lysine overproduction on total (free + bound) amino acids has been evaluated in leaves and compared to the wild type and to both parents' situations (Table V). Total lysine content in RAEC-1 × RLT 70 is increased by 2.3-fold, whereas threonine, isoleucine, and methionine are diminished by approximately 20%. It should be stated that none of the mutants displays a shift in the protein-bound amino acid spectrum (data not shown). Global content of amino acids in leaves of RAEC-1 × RLT 70 resembles (or may be slightly inferior to) that encountered in the wild type and RAEC-1, as opposed to what is observed in RLT 70, where the protein content is remarkably increased.

Table III. *Comparison of the Free Amino Acid Pool at an Early Stage (Plantlets of Approximately 10 Leaves, Grown in Vitro)*

Analysis of the free amino acid pool was performed in young leaves from RAEC-1 × RLT 70, RAEC-1 (normal phenotype), RLT 70, and wild type (expressed in absolute values as nmol/g fresh weight, and in relative values as percentage of the total).

Amino acid	Wild Type		RAEC-1 × RLT 70		RAEC-1		RLT 70	
	nmol/g	%	*nmol/g*	%	*nmol/g*	%	*nmol/g*	%
Aspartate	1814	17.6	1982	16.0	2341	16.8	2483	16.2
Threonine	79	0.8	112	0.9	141	1.0	1084	7.1
Serine	151	1.5	178	1.4	168	1.2	157	1.0
Glutamate	6219	60.3	7468	60.4	8988	64.5	9451	61.8
Proline	236	2.3	352	2.9	139	1.0	235	1.5
Glycine	387	3.8	485	3.9	399	2.9	532	3.5
Alanine	380	3.7	436	3.5	484	3.5	398	2.6
Cysteine	ND[a]		ND		ND		ND	
Valine	91	0.9	110	0.9	102	0.7	102	0.7
Methionine	ND		ND		ND		ND	
Isoleucine	59	0.6	62	0.5	65	0.5	67	0.4
Leucine	78	0.8	77	0.6	83	0.6	92	0.6
Tyrosine	143	1.4	143	1.2	169	1.2	131	0.9
Phenylalanine	132	1.3	158	1.3	137	1.0	95	0.6
Histidine	440	4.3	454	3.7	573	4.1	362	2.4
Lysine	61	0.6	311	2.5	115	0.8	61	0.4
Arginine	41	0.4	32	0.3	40	0.3	42	0.3
Total	10311		12360		13944		15292	

[a] ND, Not detected.

Free Amino Acids in Calli

Comparison of the amino acid analysis of seed-induced calli obtained after several subcultures (resulting in a stabilization of the amino acid spectrum) of the wild type and of the three mutants underlines the characteristic lysine overproduction occurring in RAEC-1 × RLT 70 (20 times more than in the wild type) (Table VI). Interestingly, the same levels are observed in RAEC-1 calli. The other aspartate-derived amino acids in RAEC-1 × RLT 70 are certainly not accumulated and generally are even less abundant than in the wild-type calli. Total content of free amino acids is comparable in all four lines.

DISCUSSION

Isolation and Developmental Features of RAEC-1 × RLT 70

The simultaneous presence of two mutations coding for a desensitization of DHDPS and of AK to inhibition by lysine was obtained by crossing homozygote mutants RAEC-1 (26) and RLT 70 (11). Genetic analysis of this unique double mutant confirmed the monogenic dominant behavior of both mutations and indicated that their loci were unlinked. This was corroborated by then using heterozygous (instead of homozygous) parents in the cross: a 1:1 resistant-sensitive ratio was then observed when progeny were sown on either AEC or L_2T_1 selection medium. Resistant plants on one medium transferred to the other resegregated in a 1:1 resistant-sensitive ratio (data not shown). No differences in the transmission of either mutation was noted after reciprocal crosses, which attests to the nuclear status of both genes. Although both parents presented a perfectly normal phenotype throughout their growth, offsprings of the cross invariably developed a very abnormal phenotype and were completely sterile. The RAEC-1 parent, however, does sporadically produce a comparable phenotype, systematically linked to a very high free lysine content (above 25% of the free pool).

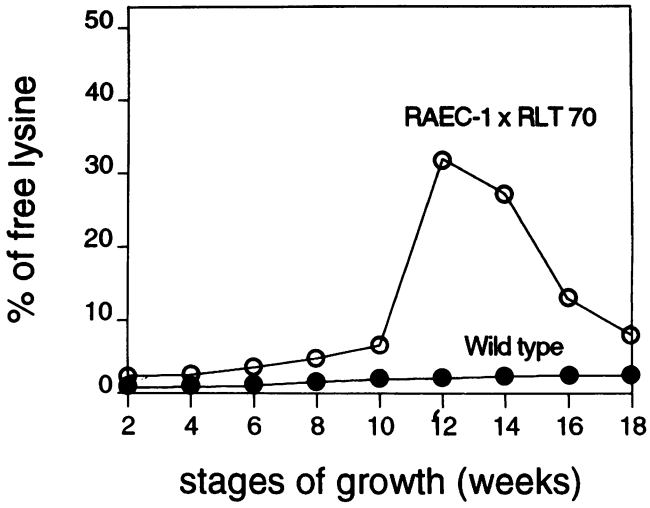

Figure 5. Evolution of the free lysine content in leaves (expressed as a percentage of the total pool of free amino acids) during the growth cycle (expressed in weeks) of wild type (●) and RAEC-1 × RLT 70 (○) plants.

In transgenic *N. tabacum* plants containing the dapA gene from *Escherichia coli* (which codes for an enzyme 200-fold less sensitive to inhibition by lysine *in vitro*), up to 200-fold elevation of lysine was observed in plants presenting an aberrant morphology similar to that of RAEC-1 × RLT 70 (16). An earlier study has demonstrated that a limited number

Table IV. *Comparison of the Free Amino Acid Pool in Adult Plants*

Analysis of the free amino acid pool was performed in leaves (adult plants, grown in the greenhouse) from RAEC-1 × RLT 70, RAEC-1, RLT 70, and wild type (expressed in absolute values in nmol/g fresh weight and in relative values as percentage of the total).

Amino Acid	Wild Type		RAEC-1 × RLT 70		RAEC-1		RLT 70	
	nmol/g	%	*nmol/g*	%	*nmol/g*	%	*nmol/g*	%
Aspartate	694	14.6	1286	21.7	1042	16.3	1977	6.6
Threonine	335	7.1	51	0.9	159	2.5	18779	62.9
Serine	462	9.7	80	1.4	422	6.6	932	3.1
Glutamate	1037	21.9	852	14.4	1087	17.0	1927	6.5
Proline	376	7.9	952	16.1	303	4.7	624	2.1
Glycine	448	9.5	69	1.2	496	7.8	573	1.9
Alanine	453	9.6	207	3.5	489	7.7	494	1.7
Cysteine	24	0.5	132	2.2	60	0.9	278	0.9
Valine	181	3.8	51	0.9	180	2.8	281	0.9
Methionine	22	0.5	5	0.1	30	0.5	184	0.6
Isoleucine	83	1.8	17	0.7	78	1.2	752	2.5
Leucine	101	2.1	118	1.9	377	1.3	64	1.1
Tyrosine	61	1.3	17	0.3	75	1.2	261	0.9
Phenylalanine	67	1.4	30	0.5	101	1.6	275	0.9
Histidine	262	5.5	302	5.1	368	5.8	1099	3.7
Lysine	92	1.9	1785	30.1	1380	21.6	697	2.3
Arginine	43	0.9	32	0.5	79	1.2	362	1.2
Total	4741		5932		6395		29872	

Table V. *Comparison of Total (Free + Bound) Amino Acid Content in Adult Plants*

Analysis of the total (free + bound) amino acid content was performed in leaves (adult plants, grown in the greenhouse) from RAEC-1 × RLT 70, RAEC-1, RLT 70, and wild type (expressed in absolute values in nmol/g fresh weight and in relative values as percentage of the total).

Amino Acid	Wild Type		RAEC-1 × RLT 70		RAEC-1		RLT 70	
	nmol/g	%	*nmol/g*	%	*nmol/g*	%	*nmol/g*	%
Aspartate	38122	13.6	40649	13.5	39002	13.2	46218	7.4
Threonine	14137	5.0	10579	3.5	11110	3.7	181650	29.2
Serine	14598	5.2	12252	4.1	14141	4.8	26702	4.3
Glutamate	36418	13.0	40460	13.4	39078	13.2	46224	7.4
Proline	11509	4.1	13621	4.5	12363	4.2	21645	3.5
Glycine	25222	9.0	27804	9.2	27015	9.1	43225	6.9
Alanine	24928	8.9	26760	8.9	26804	9.0	41769	6.7
Cysteine	359	0.1	831	0.3	307	0.1	607	0.1
Valine	18923	6.7	18138	6.0	19123	6.5	33114	5.3
Methionine	2765	1.0	2107	0.7	2378	0.8	7501	1.2
Isoleucine	13347	4.8	11721	3.9	12760	4.3	28111	4.5
Leucine	22005	7.8	22224	7.4	22586	7.6	36220	5.8
Tyrosine	6780	2.4	3452	1.1	6098	2.1	11959	1.9
Phenylalanine	13680	4.9	11727	3.9	12592	4.2	19117	3.1
Histidine	8173	2.9	7861	2.6	6832	2.3	13215	2.1
Lysine	17807	6.3	40261	13.3	32751	11.0	43723	7.0
Arginine	11982	4.3	11516	3.8	11774	4.0	22212	3.6
Total	280755		301963		296714		623212	

Table VI. *Comparison of the Free Amino Acid Pool in Calli*

Analysis of the free amino acid pool was performed in calli (after several subcultures) of RAEC-1 × RLT 70, RAEC-1, RLT 70, and wild type (expressed in absolute values in nmol/g fresh weight and in relative values as percentage of the total).

Amino Acid	Wild Type		RAEC-1 × RLT 70		RAEC-1		RLT 70	
	nmol/g	%	*nmol/g*	%	*nmol/g*	%	*nmol/g*	%
Aspartate	1646	5.3	872	2.5	2239	6.3	699	2.1
Threonine	119	0.4	122	0.4	114	0.3	1769	5.2
Serine	179	0.6	90	0.3	169	0.5	177	0.5
Glutamate	18499	59.6	21280	62.1	22911	64.2	22310	66.1
Proline	3819	12.3	3384	9.9	3333	9.3	1995	5.9
Glycine	1111	3.6	579	1.7	487	1.4	998	3.0
Alanine	2229	7.2	2863	8.4	1097	3.1	2088	6.2
Cysteine	23	0.1	40	0.1	32	0.1	35	0.1
Valine	239	0.8	328	1.0	201	0.6	469	1.4
Methionine	140	0.5	125	0.4	49	0.1	507	1.5
Isoleucine	246	0.8	210	0.6	149	0.4	729	2.2
Leucine	235	0.8	249	0.7	157	0.4	333	1.0
Tyrosine	452	1.5	358	1.0	360	1.0	196	0.6
Phenylalanine	309	1.0	175	0.5	397	1.1	258	0.8
Histidine	1614	5.2	1627	4.8	1771	5.0	1023	3.0
Lysine	99	0.3	1958	5.7	2001	5.6	124	0.4
Arginine	93	0.3	33	0.1	212	0.6	21	0.1
Total	31052		34288		35679		33731	

of amino acids were capable of altering very specifically the developmental pattern of *Marchantia polymorpha* gemmalings (10). The presence of lysine in the media led to the formation of a rosette pattern of growth (no more apical dominance), derived from a thickened, irregular thallus. Persistance of the abnormalities required the presence of the amino acid, at a minimal concentration, a situation possibly related in *N. sylvestris* mutants to the critical value of approximately 25% of free lysine in the pool, below which normal plant phenotypes are observed, but beyond which morphological irregularities develop. One proposed mechanism consisted of the modification of structure or composition of histones composed essentially of basic amino acids (such as lysine), which would result in abnormal ontogeny through differential transcription of the genome.

This putative correlation between a high free lysine content and a modified phenotype in *N. sylvestris* RAEC-1 × RLT 70 deserves more investigation, and among other studies, the polyamine spectrum should be analyzed regarding the direct intervention of several members of the aspartate family, *i.e.* lysine, *s*-adenosylmethionine, and aspartate, in polyamine biosynthesis (28).

Enzymic Properties

The heterozygote state of both mutations in RAEC-1 × RLT 70 was further confirmed by the inhibition properties of DHDPS and AK activities in the presence of lysine: only half of their lysine-sensitive activity could still be inhibited by this negative effector.

Free Amino Acid Content

Considering the amino acid analysis of the double mutant RAEC-1 × RLT 70, the most striking feature is undoubtedly the exclusive overproduction of lysine, and even to a certain extent, at the expense of the other aspartate-derived amino acids. As deduced from previous studies, control of the lysine biosynthesis seems to occur mainly at two levels: AK (I_{50} of 90 μM lysine) and DHDPS (I_{50} of 15 μM lysine). However, DHDPS is the most stringently regulated enzyme of the pathway and as such, should allow a lysine overproduction if desensitized. This is effectively the case in RAEC-1 plants and calli, in which high lysine contents can be reached.

Although systematically observed in the latter, RAEC-1 leaf tissues present a great variation in free lysine amounts, which remains difficult to explain. If RAEC-1 × RLT 70 calli present very similar levels of lysine to those observed in RAEC-1 calli, RAEC-1 × RLT 70 leaves exhibit, in contrast, a high free lysine content throughout the F_1 plants. From this comparison, it appears that in calli issued from RAEC-1 and RAEC-1 × RLT 70, lysine overproduction has reached a maximum. Thus, the fact that AK is mutated does not increase the free lysine content of RAEC-1 × RLT 70. In addition, the heterozygote status of DHDPS in the double mutant does not affect the level reached by free lysine. This has also been observed in parents RAEC-1 and RLT 70, which presented the same overproduction levels, respectively, of lysine and of threonine in heterozygotes or homozygotes. In leaf tissue, the variation in the production of lysine could be linked to a certain developmental and tissue specificity, which is absent in calli.

Considering the decrease in the other aspartate-derived free amino acids, threonine, isoleucine, and methionine in RAEC-1 × RLT 70 older leaves, DHDPS regulation is probably the most stringent control in the entire aspartate family, such that when desensitized, the lysine branch constitutes a sink draining most of the aspartate semialdehyde synthesized. In fact, although both mutations separately lead to the overproduction of different amino acids, *i.e.* lysine and threonine, their simultaneous presence clearly shows the predominance of the lysine biosynthesis over that of threonine. Aspartate, however, does not seem to be a limiting factor to the overproduction, and even is more abundant in the mutants than in the wild type, in contrast with its limiting role proposed by Giovanelli *et al.* (15). Free proline content in RAEC-1 × RLT 70 is higher, which attests to the stressed situation of growth of this mutant.

The role of AK as an important regulatory step for the entry of 4-carbon units into the aspartate family of amino acids has been previously investigated *in vivo* in *Lemna paucicostata* (15). These findings indicate that lysine and threonine *in vivo* do not normally limit AK activity severely enough to limit the carbon flux through the aspartate kinase step. In fact, the relative stringency of AK as a control should be considered in relation to the pathway branch followed. In the case of RLT 70, an AK desensitized to feedback control overproduces (in high amounts) as a direct consequence the only amino acid whose biosynthesis is not further strictly controlled, *i.e.* threonine. The role of AK is predominant, if not unique. When RAEC-1 × RLT 70 is examined and compared to its parent RAEC-1, free lysine levels reached in leaves are always only slightly superior to those of RAEC-1, and equivalent in calli. Apparently, AK does have a minor influence, but is overruled by DHDPS, whose sole desensitization is sufficient to lead to a lysine overproduction.

Overproduction in the double and in the two parental mutants always presents a maximum, which in phenotypically normal plants coincides with the stem elongation preceding the flower initiation. Considering the altered development of RAEC-1 × RLT 70, overproduction can only be related to time (in weeks of growth), but presents, nevertheless, a peak of lysine accumulation.

Total Amino Acid Analysis

Repercussion of the free lysine accumulation in RAEC-1 × RLT 70 (and RAEC-1) on the total (free + bound) amino acid content was very clear: total lysine was doubled in the mutant when compared to the wild type. Because no shift in the protein-bound amino acid spectrum of RAEC-1 × RLT 70 could be observed (just as for both parental mutants; data not shown) and global protein quantity was similar to the wild-type content (like RAEC-1, but as opposed to RLT 70), the increase of total lysine present in the double mutant was attributed to the free lysine overproduction only.

Free Amino Acid Overproduction and Plant Nutritional Value Improvement

This report presents a unique double mutant, RAEC-1 × RLT 70, overproducing free lysine in such amounts that total

lysine content in leaves is doubled, whereas the amount of threonine present is only slightly affected. This high accumulation of free lysine in leaves has been associated in this study of RAEC-1 × RLT 70 and RAEC-1 mutants to the abnormal phenotype, mainly characterized by reduced leaf blade surface, absence of stem elongation, and sterility.

The need for a modulation of the amount of free lysine present in the pool of free amino acids will arise if this hypothesis is effectively confirmed. One approach to solve this problem consists of acting on the rate of synthesis of lysine, involving the cloning of the mutated *dhdps* gene and its integration into an expression cassette designed either to maintain a certain level of lysine accumulation throughout growth or to target a specific tissue (for example, the endosperm of seeds) for lysine accumulation. Transformed plants with normal phenotypes should then be analyzed to determine if from a breeder's point of view, the levels of lysine reached are still significant.

Another way to avoid these morphological changes would be to trap the overproduced lysine in a modified storage polypeptide exceptionally rich in lysine codons. Such a lysine sink should counterbalance the negative effects associated with a high free lysine content.

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