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Lysine overproducer mutants with an altered dihydrodipicolinate synthase from protoplast culture of *Nicotiana sylvestris* (Spegazzini and Comes)

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Summary. Two S-(2-aminoethyl)L-cysteine (AEC) resistant lines were isolated by screening mutagenized protoplasts from diploid *N. sylvestris* plants. Both lines accumulated free lysine at levels 10 to 20-fold higher than in controls. Lysine overproduction and AEC-resistance were also expressed in plants regenerated from the variant cultures. A feedback insensitive form of dihydrodipicolinate synthase (DHPS), the pathway specific control enzyme for lysine synthesis, was detected in callus cultures and leaf extracts from the resistant lines. Aspartate kinase (AK), the other key enzyme in the regulation of lysine biosynthesis, was unaltered in the mutants. Crosses with wild type plants indicated that the mutation conferring insensitivity to feedback in DHPS, with as result overproduction of lysine and resistance to AEC, was inherited as a single dominant nuclear gene.

Key words: *Nicotiana sylvestris* – Protoplasts – AEC-resistance – Lysine overproduction – Feedback insensitive dihydrodipicolinate synthase – Expression

Introduction

Most crop plants are deficient in certain essential amino acids, lysine being the most limiting one in cereals. The understanding of regulatory loops in lysine biosynthesis in higher plants and isolation of mutants with increased lysine content are therefore matters of deep concern.

In both bacteria and Angiosperms lysine biosynthesis is controlled at the level of aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS) (Bryan et al. 1970; Cheshire and Miflin 1975; Aarnes 1977). Mutants possessing feedback insensitive forms of such enzymes should overproduce the corresponding amino acids. Selection for lysine + threonine – resistance resulted in overproduction of threonine, but not lysine, as reported in maize (Hibberd et al. 1980), tobacco (Bourgin et al. 1980), barley (Bright et al. 1982), and carrot (Catoir et al. 1983). Where analysed, the resistance was inherited as a monogenic dominant or semi-dominant trait, and associated with altered forms of AK. To obtain lysine overproduction, earlier data with microorganisms indicated that S-aminoethylcysteine, an analog of lysine, was rather efficient in isolating mutants that accumulate lysine (Nakayama et al. 1966; Sano and Shiio 1970; Brock et al. 1973; Demain 1975). Among the few AEC- resistant variants described in higher plants two were reported to overproduce free lysine (rice callus cultures, Chaleff and Carlson 1974; tobacco cell suspensions, Widholm 1976), but neither genetic nor biochemical evidence were made available in support of their results thus far. In orther cases strains characterized by altered uptake or incorporation mechanisms were isolated, e.g. in barley (Bright et al. 1979) and suspension cultures of *Arabidopsis thaliana* (Negrutiu et al. 1978), respectively. Furthermore, an AEC-resistant culture isolated in carrot cell suspensions (Matthews et al. 1980) was believed to represent a cell-variant; this could also be the case with an anther-derived AEC-resistant callus of rice (Shaeffer and Sharpe 1981).

This report describes the isolation from a protoplast culture system of diploid *N. sylvestris* (2n = 24) of AEC-resistant, lysine overproducing cell cultures and plants. The genetic and biochemical data are discussed in terms of designing appropriate selection strategies when breeding for improved nutritional quality in plants.

Abbreviations: AK = aspartate kinase (EC 2.7.2.4); DHPS = dihydrodipicolinate synthase (EC 4.2.1.52); AEC = S-(2-aminoethyl)L-cysteine

Material and methods

Isolation and culture of protoplasts

In vitro growth conditions of protoplast mother plants, protoplast isolation and culture have been described elsewhere (Negrutiu and Mousseau 1980, 1981). Diploid protoplasts were used throughout the experiments. Criteria for determination of plating efficiency were previously reported by Durand (1979). The plating efficiency in the control plates was 90%–100% as measured after two weeks in culture.

Mutagen treatment

Freshly isolated protoplasts were either UV irradiated ($25\ erg \cdot mm^{-2} \cdot s^{-1}$; 15 W, Sylvania germicidal lamp) or incubated for 45 min in wash medium containing 0.5% ethylmethane-sulphonate (EMS).

Selection conditions and plant regeneration

Fourteen–eighteen day old protoplast cultures were washed by sedimentation and incubated on a 10 ml layer of agar medium at a final density of 10^4 protoplast-derived colonies per ml as previously reported (Negrutiu and Muller 1981). AEC was added by sterile filtration in the dilution medium at a final concentration of 0.04–0.05 mM. Three weeks later resistant isolates were transferred to solid medium containing 0.05 mM AEC; after another three weeks regeneration was attemped as described by Bourgin et al. (1979) in the presence or absence of the analog. The regenerant were propagated on hormone free media.

Seed setting and seed germination

Resistant regenerants were transferred to the greenhouse and crossed with the wild type as male partner. Seeds were sterilized for 30 min in 5% $Ca(OCl)_2$ and seeded on agar medium containing half strength Murashige ans Skoog basal medium with 10 g/l sucrose and 0.1 mM filter-sterilized AEC.

Chromosome counts

Root tips from greenhouse plants or in vitro cuttings were pretreated for 3–4 h in a 0.5% colchicine solution at 14 °C, fixed in a 3 : 1 ethanol-acetic acid mixture, and coloured in an acetic-orcein solution as described by Sharma and Sharma (1972).

Amino acid analysis

Leaf tissue from greenhouse or in vitro cuttings (4–5 weeks old) was homogenized and extracted three times with a mixture of methanol/chloroform/water (12/5/1, v/v/v) (Bieleski and Turner 1966). Free amino acid pool from callus (10–14 days in culture) was extracted by boiling the material in 70% ethanol (Bright et al. 1979). Chlorophyll was removed by adding two parts of chloroform and three parts of water to the extract. The aqueous layer was evaporated and the residue was redissolved in 2 N HCl and hydrolysed at 100 °C under vacuum. The extracts were subsequently dissolved in loading buffer and analyzed in a LKB Biochrom 4101 amino acid analyzer on DC 6 A resin using a four buffer system.

AK and DHPS assay

Leaf tissue (1–5 g of mesophyll) from plants at various developmental stages was extracted in phosphate buffer (100 mM, pH 8) containing 1 mM EDTA, 10 mM mercaptoethanol, 10 mM diethylcarbamate, and 10% (v/v) glycerol. For AK assay 1 mM threonine was added to the extraction mixture. The extract was centrifuged (10 min, 12,000 rpm) and the supernatant was brought to 66% saturation with $(NH_4)_2SO_4$. The precipitated protein was redissolved in 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA, 1.4 mM mercaptoethanol and 20% (v/v) glycerol. Ammoniumsulphate was removed by passing the extract through a Sephadex G-25 column. For DHPS extraction, addition of mercaptoethanol in the dialysis buffer was omitted. All procedures were conducted at 4 °C. Protein concentrations was determined by the method of Lowry et al. (1952).

AK activity was determined by colorimetric dosage of aspartylhydroxamate with $FeCl_3$ reagent (Bryan et al. 1970). The assay mixture contained 10 mM Mg-ATP, 0.5 mM hydroxylamine and 50 mM K-aspartate. One hundred µl extract (1–3 mg protein) was added to obtain a final volume of 0.5 ml incubation mixture and was incubated at room temperature for 1 h. Controls lacking aspartate were included for all assays. A unit of AK activity is defined as the amount of enzyme producing 1 µmole of aspartylhydroxamate per minute in the mentioned incubation conditions.

DHPS activity was assayed using the O-ABA (aminobenzaldehyde) method of Yugari and Gilvarg (1965). The assay mixture contained 1.5 mmole DL-aspartylsemialdehyde neutralized just before use with KOH, 37 mM pyruvate, 0.05 M tris-HCl pH 8.2. Before assay 0.5 mg O-ABA solubilized in 35 µl ethanol was added to the reaction mixture. The reaction was initiated by adding 50 µl extract containing ca. 0.5 mg protein. The final volume of the mixture was 1 ml. After 30 min incubation at 37 °C the reaction was stopped by adding 200 µl 10% TCA. The colour was allowed to develop for 80 min and after centrifugation the A 540 was recorded. One unit of DHPS activity is defined as the amount of activity necessary to produce a change in A 540 of 0.001/min.

Results

1 Choice of the selection agent

Growth inhibition tests with three lysine analogs, namely AEC, δ-hydroxylysine (DHL) and α-aminocaprylic acid (ACA), relief of the inhibition by lysine, and in vitro assays for AK and DHPS activity and inhibition were performed.

Growth inhibition tests with seedlings of *N. sylvestris* showed that AEC exerted high toxicity at rather low concentrations (total growth inhibition at 0.05 mM, as determined by fresh and dry weight increase of the seedlings three weeks after transfer onto AEC medium; data not shown). DHL and ACA were required at approximately 10 times higher concentrations to produce similar inhibitory effects. Lysine in 4–10-times higher concentrations than AEC restored almost completely the growth inhibition produced by AEC. Lysine at concentrations used in restoration experiments was not inhibitory per se. Lysine was less efficient in countering the toxic effects of DHL and ACA. A similar response was observed with protoplast-derived colonies after dilution in media containing AEC (inhibitory concentration of 0.005 mM as calculated from survival curves after plat-

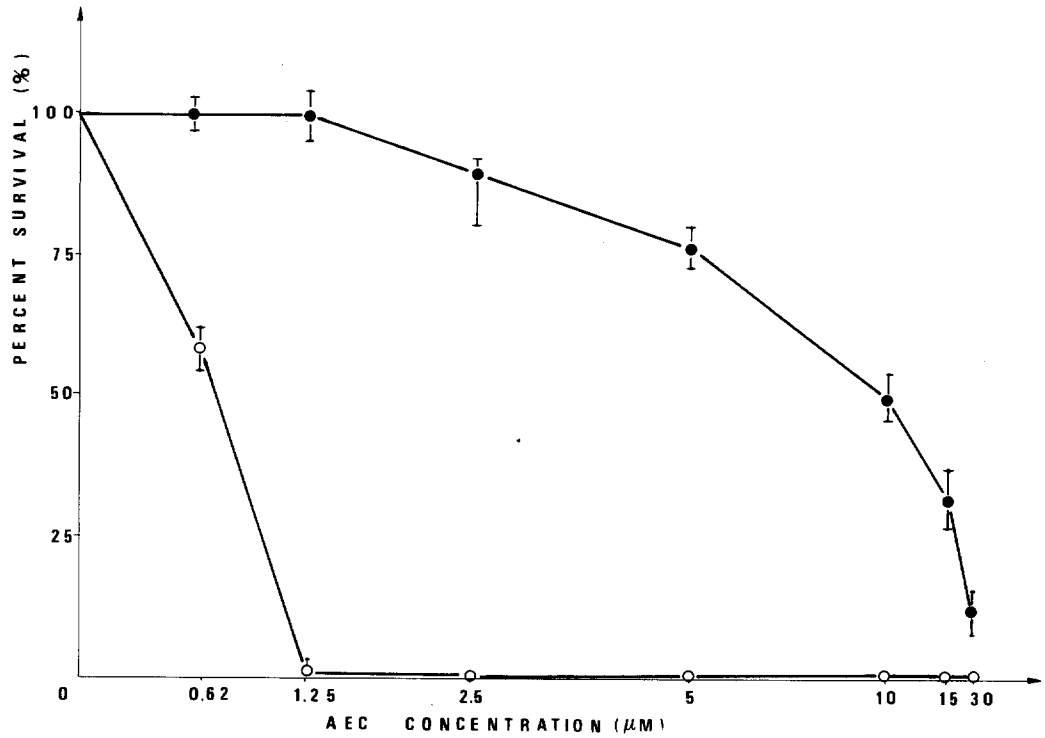


Fig. 1. Survival curves in 14 day old protoplast cultures from wild type (○) and RAEC-1 (●) plants following dilution (250 colonies/ml) in AEC containing media. Surviving colonies were scored three weeks after dilution. Plating efficiencies (PED %, Negrutiu and Muller 1981) were 70% (wild type) and 33% (RAEC-1), respectively. AEC concentrations are plotted on a semilogarithmic scale. Bars represent SE

ing 14 day old p-colonies at low densities; cf. Negrutiu and Muller 1981 and Fig. 1). AEC had a rapid toxic effect resulting in the killing of the cell population.

Enzyme assays. AK and DHPS were assayed in leaf tissues of *N. sylvestris* at different stages with respect to activity and allosteric inhibition (Table 1). Sixty to 80% of the AK activity was inhibited by lysine, the other part by threonine. DHPS was more strictly controlled by lysine than AK (50% inhibition at 22 μm lysine for DHPS and 400 μM for AK, respectively). In both cases lysine could be replaced by AEC as feedback inhibitor. However, 6–10 times higher concentrations were needed to reach equal inhibition levels. The structural requirements for DHPS inhibition were rather specific. No substitution of α, ε or caboxyl groups were tolerated. From different substituted lysines tested (α-acetyl, ε-acetyl-lysine, lysine hydroxamate, δ-hydroxylysine) only DHL showed a significant inhibitory effect at concentrations below 1 mM (75% inhibition at 1 mM).

2 Isolation of resistant lines

Non-mutagenized and mutagenized populations of diploid protoplasts were subjected to selection against AEC approximately two weeks after the initiation of the cultures. Two resistant colonies were isolated in two independent experiments from mutagenized cultures only. They were further confirmed as resistant by a second transfer on selection medium. The selection conditions appear to be very stringent as no false positives were observed. The results are presented in Table 2. Mutant frequency is expressed on a "per surviving colony" basis.

3 Expression of the resistance at cellular level

Callus cultures subcultured in the absence of the selection pressure for 3–5 passages preserved their resistance to AEC. Occasionally nonresistant sectors could be observed during culture. Both lines exhibited high levels of resistance to AEC (0.2–0.3 mM), i.e. 30–60 times higher as compared to wild type cultures which were completely inhibited by 0.005 mM AEC (Table 3). Protoplast-derived colonies (cloned cultures) from regenerated resistant plants were also resistant to AEC (see Negrutiu and Muller 1981): reconstruction experiments of mutant isolation with the resistant lines indicated than an expression time of $\geqq 10$ days (corresponding to colonies of $\geqq 10$ cells/aggregate) and a maximal density of 1 $(1.5) \cdot 10^4$ colonies per ml were necessary in order to recover the AEC-resistant phenotype under the described screening conditions. Callus

Table 1. Characteristics of aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS) in leaf extracts of wild type and RAEC plants. lys = lysine; thre = threonine

	Aspartate kinase[a]		Dihydrodipicolinate synthase[a]		
	Wild type	RAEC-1	Wild type	RAEC-1	RAEC-2
Specific activity					
mU/mg	0.8	0.8			
U/mg			12	15	11
Inhibition (% of total activity)					
Inhibitor					
none	0	0	0	0	0
lys (10 mM)	65	74			
thre (10 mM)	19	17			
lys + thre (10 mM)	89	88			
AEC (2 mM)	50	50			
lys (0.1 mM)			99	40	25
AEC (0.1 mM)			33	12	–
AEC (1 mM)			97	43	–

[a] AK activity gradually decreased with age of the plants; no significant changes in DHPS activity and pattern of allosteric inhibition occurred during plant development (from primary rosette to flowering stage)

Table 2. Isolation of AEC-resistant lines in protoplast culture of diploid *N. sylvestris*. Mutant frequencies are expressed on the basis of "per surviving colonies"

Total no. of exposed protopl.	Mutagen treatment	Survival rate (%)	No. of resistants		Mutant frequency
			Isolated	Confirmed	
10^7 (control)	–	100	0	–	–
10^7 (control)	–	90	0	–	–
$4.1 \cdot 10^7$	UV (700erg/mm²)	50	1	1 (RAEC-1)	$5 \cdot 10^{-8}$
$3.3 \cdot 10^7$	EMS (0.5%; 45 min)	65	1	1 (RAEC-2)	$5 \cdot 10^{-8}$

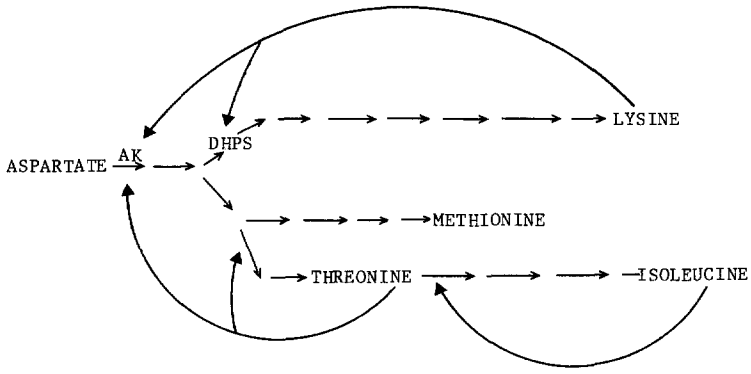
Table 3. Level of AEC toxicity in protoplast-derived cells (p-cells), callus culture, regenerants, and seedlings from wild type and AEC-resistant mutants

Type of tissue	AEC concentration (mM)	
	Wild type (M2)	RAEC-1/RAEC-2
Callus	0.005	0.2 /0.3
p-cells (14 day old)	0.0025	0.03–0.06
Regenerants	0.02	0.2 /0.2
Cuttings	0.02	0.2 /0.3
Seedlings	0.0375	0.15/–

cultures of the AEC-resistant lines became sensitive to the analog when grown on AEC (0.1 mM) in the presence of non-toxic concentrations of threonine and methionine (0.1 mM each) (Table 4). The results suggested that AK was unaltered in the AEC-resistant lines as feedback inhibition of the enzyme by endogenous high levels of lysine plus exogenously supplied threonine and methionine (and eventually AEC) would result in lysine shortage, with as consequence enhanced incorporation of AEC and inhibition of growth (see Scheme 1).

4. Expression of the resistance at plant level

Regeneration in the presence or absence of selection pressure produced AEC-resistant plantlets. Cuttings



Scheme 1. Inhibition of enzymes of the aspartate pathway of amino acid biosynthesis in plants. *Bold arrows* indicate inhibition of enzyme activity by the end product amino acids (from Bryan 1976). *AK* = aspartate kinase; *DHPS* = dihydrodipicolinate synthase

Table 4. Growth response of wild type (M2) and RAEC-2 callus on media supplemented with AEC and amino acids of the aspartate pathway. Concentrations are given in mM. Lys = lysine; Thre = threonine; Meth = methionine

Callus line	Control	Thre (0.1) Meth (0.1)	Thre (0.1) Meth (0.1) AEC (0.1)	Thre (0.1) AEC (0.1) Meth (0.1) Lys (0.05)	Lys (0.05) AEC (0.1)
M2	4+	3+	0	0/+	+
RAEC-2	4+	3+/4+	0	3+	3+

Table 5. Free amino content in callus and leaf tissue of wild type and AEC-resistant lines. Figures represent % of total free amino acids. Absolute amounts of free lysine are given in brackets (nmole/gram fresh wt). Each figure represents the mean of at least three replicates

Amino acid[a]	Callus			Leaves		
	Wild type	RAEC-1	RAEC-2	Wild type	RAEC-1	RAEC-2
Aspartate[b]	5.9±0.9	6.1±1.6	3.9±1.2	16.5±2.8	10.9±1.5	26.0±3.7
Threonine	2.1±0.4	1.6±0.5	1.0±0.3	1.8±0.1	1.2±0.1	1.2±0.1
Glutamate[b]	42.4±3.5	31.6±6.8	43.0±5.1	47.1±3.2	24.3±4.1	45.8±3.3
Methionine	traces	traces	traces	traces	traces	traces
Isoleucine	0.8±0.1	1.6±0.4	0.9±0.2	0.6±0.1	1.9±0.2	0.3±0.1
Lysine	1.2±0.4 (174)	15.4±3.3 (2580)	16.3±2.1 (2550)	0.6±0.1 (100)	17.7±3.5 (3220)	8.8±2.1 (1650)
Arginine	22.3±2.3	8.5±2.9	8.2±2.4	17.4±1.1	8.2±2.4	5.6±3.1
Total	14,500 (±2453)	16,750 (±3610)	15,650 (±3900)	16,100 (±1440)	18,100 (±2260)	18,650 (±3120)

[a] Other amino acids showed no significant changes and were therefore omitted
[b] The given figures represent (aspartate + asparagine) and (glutamate + glutamine)

from resistant plants initiated normal rosette growth and root formation in media containing 0.1–0.2 mM AEC, while wild type cuttings died within 7–10 days without inducing any roots at much lower concentrations of AEC (0.02 mM). Occasionally sensitive plants were observed among the tested regenerants (more frequently with RAEC-1), suggesting either a chimeral state of the original isolates or chromosomal instability in the lines.

The regenerants were tested for cross-resistance to inhibitory concentrations of lysine + threonine; both wild type and AEC-resistant regenerants were equally sensitive to 2 mM lysine + 1 mM threonine.

5 Molecular basis of the resistance

Amino acid analysis. Free amino acid composition was analysed in various tissues and organs of wild type and resistant lines. Table 5 details the content in glutamate,

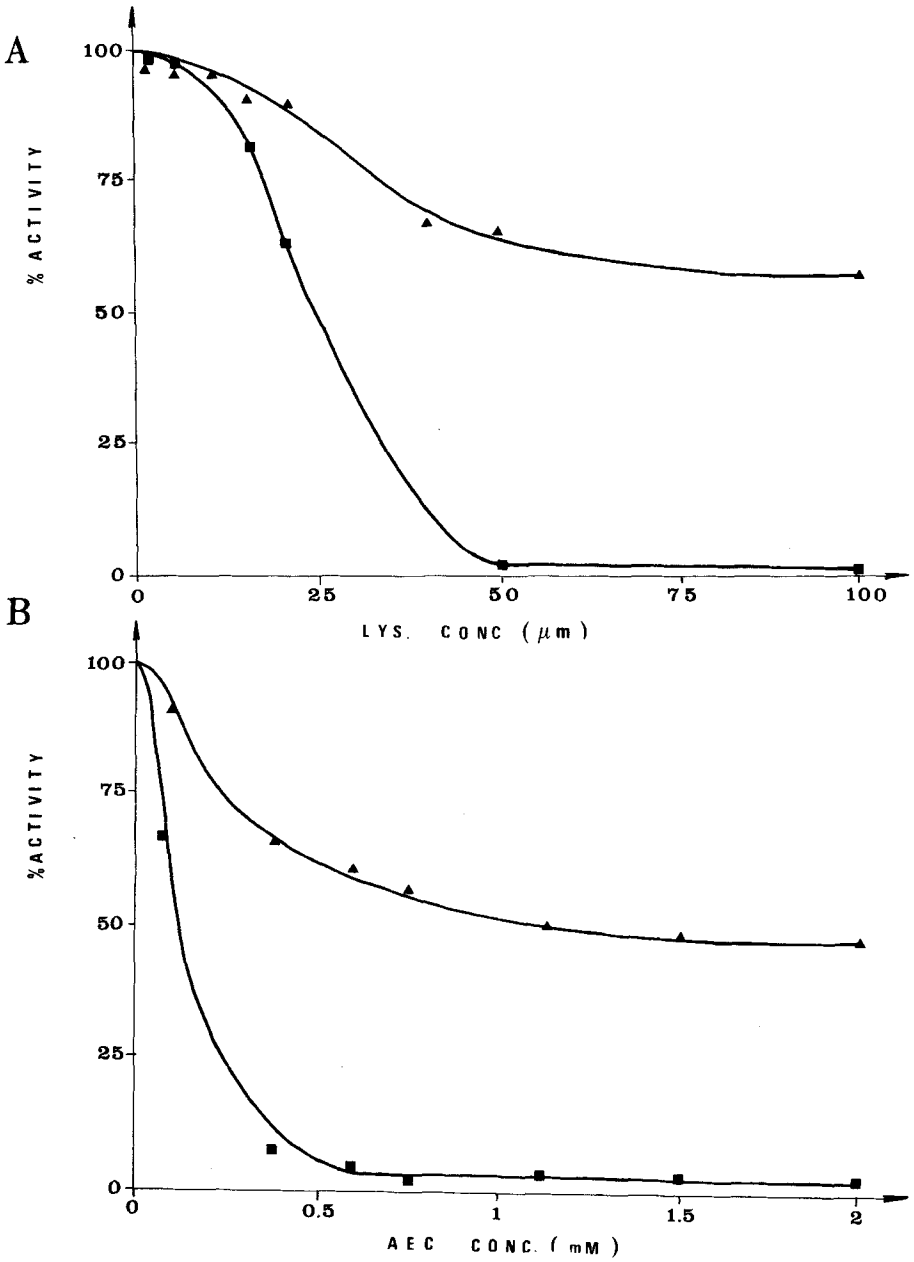


Fig. 2. Effect of lysine (*A*) and AEC (*B*) on the activity of dihydrodipicolinate synthase (DHPS) in leaf extracts of wild type (■ and RAEC-1 plants (▲)

arginine and aspartate, the three major amino acids in *N. sylvestris,* and amino acids of the aspartate pathway. The lysine pool was markedly increased in resistant calluses (15-fold), regenerated plants (up to 28-fold), and cloned cultures as compaired to controls. In absolute values the amount of lysine per g fresh weight increased from 100–170 nmole to 2,500 nmole (callus) and up to 3,200 nmole (regenerants). Beside slight modifications in threonine, glutamate, aspartate or isoleucine content, resistant calluses and regenerants exhibited an important drop in free arginine (2.5 fold; occasionally the reduction in arginine approached a 10-fold decrease).

Increase in lysine production was also recorded in root and leaf tissue from F_1 resistant progenies of RAEC-1. On the contrary, fruit tissue and immature seeds (assayed 12–14 days after pollination) from the same F_1 progenies showed no modification in free-lysine pool as compared to controls.

Enzyme properties. Assays for DHPS and AK activity and allosteric inhibition were performed with leaf tissue of resistant and control plants. There was no detectable change in the specific activity of both enzymes. No differences were observed in the inhibition of AK by lysine, threonine, or AEC in mutant and wild type plants

(Table 1). On the other hand, important differences were noticed in the inhibition pattern of DHPS (Fig. 2). Control tissues contained a DHPS that was completely inhibited at 100 μM lysine. The maximal inhibition of DHPS from resistant plants by lysine or AEC was estimated at 50% in RAEC-1 and 33% in RAEC-2. The presence of a feedback insensitive form of DHPS could be ascertained in callus cultures and at various stages of plant development, from young rosette to flowering plants.

5 Genetic basis of lysine overproduction

When transferred to greenhouse, RAEC-1 and RAEC-2 plantlets developed a phenotypically normal rosette stage. Subsequently, RAEC-2 formed abnormal chlorotic leaves producing no shoot at all. The chromosome complement was variable, ranging from 26 to 39.

RAEC-1 regenerants exhibited a range of phenotypes with respect to plant growth habit, leaf size, shape and chlorophyll content, flower morphology, earliness of flowering. Most often the plants developed an abnormal apical inflorescence, more or less normal flowers being formed on axilary branches. They were all male sterile; crossed with wild type pollen they produced seeds. Chromosome counts in root tips of progeny plants showed aneuploid chromosome numbers. Usually the somatic chromosome complement in these plant was 26. Certain root meristems were chimeric hyperdiploids, with chromosome numbers ranging from 26 to 32. When germinated on an AEC containing medium, such progenies segregated into a 1 sensitive: 1 resistant ratio (Table 6), indicating that RAEC-1 was isolated as heterozygote (Rr) and that the mutation was inherited as a monogenic dominant trait. The aneuploid condition of the material was associated in certain offsprings such as RAEC-1/5 : 9 with a larger proportion of aberrant seedling phenotypes which made a clear-cut distinction between resistant and sensitive segregants more difficult. However, generally speaking, resistant seedlings developed normally on inhibitiory concentrations of AEC (0.1–0.15 mM). Control plants and sensitive segregants exhibited symptoms of growth inhibition 7–10 days after germination: cotyledons turned gradually white, roots penetrated the agar only exceptionally and remained extremely short, no elongation of the hypocotyl occurred, and leaves when formed appeared chlorotic and abnormal (Fig. 3).

Table 6. Chromosome complement and segregation ratio in six backcrossed progenies (BC-2) from a AEC-resistant, lysine overproducer plant (RAEC-1/5)

Plant no.	Chromosome no.	Segregation on AEC medium		
		Resistant	Sensitive	*P*
RAEC-1/5: 9	26–30	97	145	0.01–0.001
RAEC-1/5:11	26–30	136	157	0.2 –0.5
RAEC-1/5:15	26–28	184	201	0.2 –0.5
RAEC-1/5:20	26	246	258	0.5 –0.8
RAEC-1/5:24	26–28	150	184	0.05–0.1
RAEC-1/5:28	26	91	102	0.2 –0.5

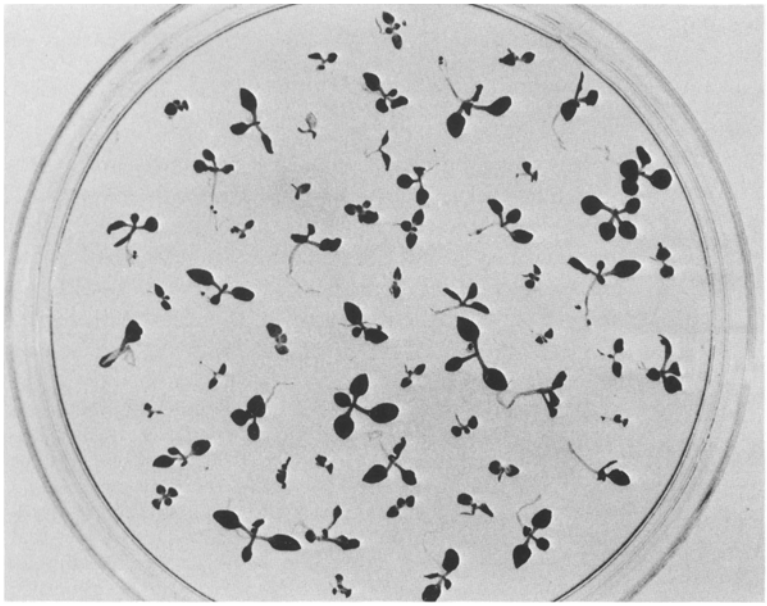


Fig. 3. Growth response to inhibitory concentrations of AEC (0.1 mM) of segregating, sensitive and resistant, progenies from a heterozygote (Rr) resistant plant

A backcross-programme was initiated with RAEC-1 with the aim of recovering diploid male fertile mutant plants. After 2 cycles of backcrossing there was a significant improvement of the material with respect to leaf morphology, apical dominance, vigour and seed-setting, but the aneuploid state and male sterility were maintained. It was estimated that further backcrossing was required to completely balance the genotype of the mutant. Among the many resistant progenies tested for an altered DHPS two plants exhibited only 33% inhibition to inhibitory concentrations of lysine. The presence in certain aneuploid plants of an extra-chromosome bearing the resistance gene could explain this pattern of inhibition.

Discussion

This work brings further evidence on the nature of genetic control of lysine biosynthesis in a higher plant. Biochemical and genetic data are presented about a mutation conferring insensitivity to feedback in DHPS, the pathway specific control enzyme for lysine biosynthesis, with, as result, overproduction of lysine and resistance to AEC. Genetic evidence suggested that the resistance was due to a dominant mutation in a single nuclear gene, the mutant cell line being isolated as a heterozygote. Biochemical data were in agreement with genetic evidence as a 50% decrease in sensitivity of DHPS to inhibitory concentrations of lysine of AEC were registered in resistant callus, regenerants and F_1 resistant progeny. AK, the other key enzyme in the regulation of lysine synthesis, was unaltered in the mutant in either activity or allosteric inhibition properties. The fact that the mutants exhibited no cross-resistance to lysine + threonine, and were sensitive to AEC in the presence of threonine and methionine corroborates these data.

Lysine overproduction was accompanied by an important drop in free arginine content. A similar drop in free arginine was observed in a lysine + threonine-resistant, threonine overproducer mutant of *N. sylvestris* (unpublished results). It is thus doubtful that the reduction in arginine pool could be directly correlated to a specific lysine effect on arginine biosynthesis (see Miflin 1971; Cattoir et al. 1980, 1981 a). Consequently, a more general effect of amino acids on nitrogen metabolism at the level of arginine pool (cf. Filner 1966; Heimer and Filner 1971; Behrend and Mateles 1975) has to be envisaged.

The reported results shed more light on several aspects related to the isolation of overproducer mutants in plant cell cultures, namely the experimental system, the selection agent, the expression of the mutation at cellular and plant level, and the regulatory mechanism.

The experimental system

Leaf protoplasts from diploid plants were used in these experiments. Previous reports have shown that variants resistant to AEC isolated from established cell cultures could not be regenerated into plants (Chaleff and Carlson 1974; Widholm 1976; Negrutiu et al. 1978; Matthews et al. 1980). Furthermore, in the case of carrot (Matthews et al. 1980; Cattoir, unpublished) selection for AEC-resistance resulted in isolation of cell-variants: their "resistance" was due to preexisting, tissue-specific variation. In our own experiments, response of *N. sylvestris* cell suspensions to selection conditions for AEC-resistance as well as for plant regeneration were rather unsatisfactory (data not shown). On the contrary, mesophyll protoplasts of *N. sylvestris* were shown to be genetically and physiologically homogenous (Magnien et al. 1980), exhibiting high morphogenic potential (Bourgin et al. 1979). We have shown here that the selection conditions were rather stringent with protoplast-derived colonies (no false positives) and that both AEC-resistant lines could be regenerated. As regeneration was induced shortly after initiating the cultures, their aneuploidy could be due to the joint effect of mutagen treatment, selection agent, and in vitro passage.

The selection agent

As with bacteria (Umbarger 1971), selection for amino acid analog-resistance within various plant species yielded somewhat different categories of mutants: in the case of AEC, uptake, incorporation, and lysine accumulating mutants could be isolated (Widholm 1976; Negrutiu et al. 1978; Bright et al. 1979; Cattoir et al. 1981 b). AEC acted in both cell culture and plant tissues of *N. sylvestris* as a potent analog of lysine as demonstrated by in vitro and in vivo experiments. It has been previously shown that the toxic effect(s) of AEC was caused by incorporation into proteins (Stern and Mehler 1965; Bright et al. 1979; also see Negrutiu et al. 1978). It is obvious that lysine overproduction is an efficient mechanism of resistance against AEC as it competes out the analog from actively being incorporated into proteins.

Expression of the selected trait in cell culture and regenerants

Lysine overproduction and AEC-resistance were permanently and similarly expressed in both mutant cell culture and plants. The induced mutation, designated *dhps-r1,* has been characterized biochemically and genetically and represents an excellent marker: AEC-resistance is expressed very early in protoplast culture and germinating seeds. DHPS, a stable enzyme, can be easily extracted and assayed.

The accumulation of free lysine in cultured cells and leaves was important (10-30-fold) considering the heterozygous condition of the isolates. Further analysis of homozygous plants should enable us to establish the full effect of the mutation on lysine pool and protein composition and content, and its eventual consequences on developmental processes. The fact that there was no apparent expression of lysine accumulation in immature seed tissues of resistant plants is meaningful on two

accounts. Important differences could exist amoung species and/or particular amino acids with respect to their translocation and interconversion (Schumacker 1967). The case of lysine is less doccumented thus far (Dierks-Ventling 1983). On the other hand, we have evidence that threonine can accumulate in immature seeds from a lysine + threonine-resistant mutant of *N. sylvestris* (manuscript in preparation). As a matter of fact, accumulation of free threonine in both seedlings and seeds of barley and maize overproducers has recently been reported (Bright et al. 1982; Hibberd and Green 1982). Before more general conclusions could be drawn on this matter and its eventual implications in crop breeding, it should be noticed that with the exception of a tryptophan overproducer line in tobacco (Widholm 1978) mutations to amino acid overproduction induced and selected in cell culture systems were expressed in vegetative organs of mutant plants. In more general terms, selection for feedback-insensitive mutants in amino acid synthesis could find a wide field of application in breeding of forage crops at least. Such mutations inherited as monogenic, dominant or semidominant traits, represent valuable tools in plant breeding.

The regulatory mechanisms

Succesful isolation of lysine overproducing mutants depends on the understanding of regulatory loops in the metabolism of lysine and other aspartate derived amino acids. Endproduct inhibition (at the level of enzyme activity) consists in a general control of the whole pathway by regulation of AK activity, and a specific control on the lysine pathway at the level of DHPS. In all plant species tested DHPS appeared very sensitive to low lysine concentrations. The major effectors of plant AK are lysine and threonine. They inhibit AK activity either in an additive or a cooperative way. The proportion of AK sensitive to lysine (AKlys) verus AK sensitive to threonine (AKthre) varies with species and type of tissue (Davies and Miflin 1977; Bryan 1976). Active growth of plant tissues is often accompanied by an important increase in AKlys, while AKthre is less subjected to fluctuations. In several species (e.g. barley, maize, sunflower) AKthre is always very low (Bryan et al. 1970; Aarnes 1974; Cheshire and Miflin 1975; Shewry and Miflin 1977). Consequently, under conditions of lysine accumulation (e.g. feedback insensitive DHPS) the carbon flow down the pathway that should satisfy the requirements for methionine and threonine could be insufficient. Our results show that in *N. sylvestris,* a species where AKthre is reasonably high, there is no need to fully desensitize the control of lysine biosynthesis: a unique mutation in DHPS is sufficient to obtain an important overproduction of lysine. Such results can also be expected in other plant species exhibiting a similar pattern of regulation in the AK, such as wheat, pea, or soybean (Wong and Dennis 1973; Aarnes and Rognes 1974; Matthews and Widholm 1978). A different selection strategy would appear necessary in the case of the first group of species mentioned. Brock et al. (1973) suggested that two mutations (a modified DHPS and AK) were required to obtain lysine overproduction in barley. As a matter fact, selection for AEC-resistance in barley resulted in isolation of uptake mutants only (Bright et al. 1979). Lysine overproducers could be isolated by selecting first for lysine + threonine-resistant, AK-feedback insensitive mutants, followed by a second round of selection against AEC. Mutants with a densitized AK are known in both maize (Hibberd et al. 1980) and barley (Bright et al. 1982; Cattoir, unpublished). We are using such barley mutants in a selection programme for resistance to AEC in order to verify the above hypothesis.

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Note added in proof

Male fertile plants were recovered after a third backcross cycle in the progeny of plants 20 and 24 (cf. Table 6). Crosses were performed with the wild type as female partner; segregation tests on AEC plates demonstrated than the resistance trait was transmitted through the male germline as well (1:1 ratio). Selfed progeny segregated in a ratio close to three resistant: one sensitive phenotypes ($P = 0.15–0.2$), confirming the monogenic dominant nature of the described mutation. In addition, in the same F2 progeny an albino seedling mutation was observed which segregated independently of the resistance to AEC.