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 a 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 other 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 (Shaeffler 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.
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·mm⁻²·s⁻¹; 15 W, Sylvania germicidal lamp) or incubated for 45 min in wash medium containing 0.5% ethyl-methane-sulphonate (EMS).

Selection conditions and plant regeneration

Fourteen-eighty eight day old protoplast cultures were washed by sedimentation and incubated on a 10 ml layer of agar medium at a final density of 10⁹ 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 attempted as described by Bourgin et al. (1979) in the presence or absence of the analog. The regenerants 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 and 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 acids were determined by colorimetric dosage of aspartylhydroxamate with FeCl₃ 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

I 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-