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Isolation and characterization of valine-resistant mutants of *Nicotiana plumbaginifolia*

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Summary. Haploid mesophyll protoplasts of *Nicotiana plumbaginifolia* were mutagenized by UV-irradiation. Protoplast-derived colonies were then selected for valine resistance on a medium containing 5 or 10 mM valine. From the resistant calli, plants were regenerated. Resistance was inherited as a recessive Mendelian character in seven clones. Mutations conferring valine resistance were shown to be allelic. Protoplast-derived cells of L-valine-resistant plants were also resistant to L-threonine. Resistance to valine was based on a reduced valine uptake rate.

Key words: Valine resistance – *Nicotiana plumbaginifolia* – Protoplast – Amino acid uptake

Introduction

Valine resistance is a convenient marker for somatic plant cell genetics (Bourgin 1976, 1978) and has recently been used to optimize mutagenesis procedures on protoplasts of *Nicotiana tabacum* (Grandbastien et al. 1985) and of other *Nicotiana* species (Vunsh et al. 1982; Marion-Poll et al. 1985, 1986; Nielsen et al. 1985).

N. tabacum valine-resistant mutants belonged to two groups. Mutants of the first group were affected in acetolacetate synthase, the first enzyme of the biosynthetic pathways of valine, leucine and isoleucine. This character was transmitted as a single Mendelian dominant character (Wallsgrove et al. 1984; Bourgin et al. 1985). Mutants of the second group were deficient for uptake of valine and various other amino acids, threonine for example, and were shown to be affected in neutral and acidic amino acid transport but not in basic amino acid transport (Bourgin et al. 1985; Borstlap et al. 1985). Valine uptake deficiency was transmitted to the progeny as a digenic recessive character (Bourgin et al. 1985). In haploid *N. tabacum* plants two independent loci were shown to be involved in the valine resistance due to amino acid uptake deficiency because of the amphidiploid structure of the *N. tabacum* genome (Grandbastien et al. 1985).

Nicotiana plumbaginifolia, a true diploid *Nicotiana* species from which haploid plants are available (Bourgin et al. 1979), has recently become a model species in plant cell genetics (Maliga 1984). Various types of mutants have been obtained: deficient in nitrate assimilation (Márton et al. 1982; Negrutiu et al. 1983; Gabard et al. 1986), antibiotic-resistant (Cséplö and Maliga 1982) or amino acid-auxotrophs (Sidorov et al. 1981; Negrutiu et al. 1985).

Isolation of valine-resistant calli from *N. plumbaginifolia* protoplasts has been described by Nielsen et al. (1985), to test the efficiency of mutagenic treatments. No plants were regenerated and the nature of the mutation was not investigated. We report here the isolation and characterization of valine-resistant plants obtained from mutagenized mesophyll protoplasts of *N. plumbaginifolia*. The aim of this work was the optimization of the conditions of selection of *N. plumbaginifolia*-resistant mutants and obtaining monogenic mutants useful for plant cell genetics.

Materials and methods

Plant material, protoplast isolation and mutagenesis

A haploid clone of *Nicotiana plumbaginifolia* (cv Viviani) was used as protoplast source. The plants were cultured in a greenhouse, as previously described (Chupeau et al. 1974).

Mesophyll protoplasts were isolated and cultured at a density of 2×10^4 cells/ml in medium T_0 (Chupeau et al. 1974). Then 1 day after isolation, protoplasts were irradiated with a germicidal lamp (G30T8, Mazda) at an incident dose rate of 12.5 erg/mm²/s for 40 s and kept in the dark.

Conditions of selection and regeneration

After 5 days of culture in the dark in medium T_0, p-cells (protoplast-derived cells) were subcultured in medium C containing 0.05 μM naphthalene acetic acid at a density of 5×10^3 divided cells/ml (Muller et al. 1983). Then 2 weeks later derived colonies were plated at a density of 10^3 colonies/ml on the same medium C but containing 0 or 0.22 M mannitol, 0.5% agarose and 5 or 10 mM L-valine. After 1 month of culture on selective medium, surviving colonies were subcultured onto modified medium B (20 mM KNO_3 as sole nitrogen source, 14 mM sucrose and no mannitol) (Bourgin et al. 1979). Regenerated plantlets were propagated in vitro on the same medium and were transferred to the greenhouse.

Characterization of the selected clones

Protoplasts were isolated from the regenerated plants and 5 days later, the p-cells were subcultured at a density of 10^2 divided cells/ml in medium C containing different concentrations of L-valine or L-threonine. Seed progeny of the fertile plants was tested for valine resistance on the modified medium B containing 0.5 mM L-valine.

Amino acid uptake experiments were performed on p-cells after 6 days of culture in medium T_0 at a density of 2×10^4 cells/ml. Labelled (U-^{14}C) L-valine (275 mCi/mmol) or L-lysine (300 mCi/mmol), obtained from Amersham, were added to 12 ml of cell suspension (0.3 μCi/ml) and incubated in Erlenmeyer flasks in an orbital shaker (50 rpm) at 25 °C. Aliquots of 0.5 ml were taken at 5, 10, 15, 30 min and suspended in 5 ml of a solution of 3% (w/v) KCl and 0.2% (w/v) $CaCl_2$, filtered on glass fiber filters (Whatman GFA) and washed 3 times with the same solution. The radioactivities of the filters were then counted and referred to the cell number.

Results

Selection of valine-resistant colonies

Various UV doses were compared for their killing effect and a dose of 500 erg/mm² inducing a 50% decrease in plating efficiency was used in further experiments. After 5 days of culture, 60% of the control and 30% of the irradiated protoplasts were divided. Conditions for the efficient recovery of valine-resistant colonies have been determined after using various selection schemes (Table 1). The frequency of valine-resistant clone recovery was highest (4×10^{-4}) when selection was initiated 20 days after protoplast isolation and mutagenesis, and the selective medium contained 5 or 10 mM valine but no mannitol (Table 1).

Test of valine resistance in regenerated plants

Plant regeneration was obtained in 42 valine-resistant clones (Table 1). Out of these, plants representing 37 clones were tested for valine resistance. P-cells (protoplast-derived cells) of regenerated plants of 22 clones were resistant to toxic concentrations of valine when grown at low cell density (Table 1, Fig. 1). Out of the 22 clones, 13 were also tested and found to be threonine resistant (Fig. 2). P-cells of plants sensitive to valine were also sensitive to threonine.

Inheritance of valine resistance

Mutants were selected from haploid protoplasts, but most of the regenerated plants were diploid, as already described for nitrate reductase-deficient mutants (Gabard et al. 1986). To study the transmission of valine resistance to the progeny, seeds were germinated on medium B containing 0 or 0.5 mM valine. Wild type (WT) seedlings were killed by 0.5 mM valine (the

Table 1. Recovery of valine-resistant mutants under different selective conditions

Mutagenic treatment	No	UV	UV	UV	UV
Selective medium contained:					
Valine concentration (mM)	5	5	5	10	10
Mannitol concentration (M)	0	0	0.22	0	0.22
No. of colonies selected	7×10^4	1.1×10^5	1.5×10^5	1.5×10^5	1.5×10^5
No. of resistant clones	1	48	9	50	1
Frequency of valine resistance[a]	1.4×10^{-5}	4.4×10^{-4}	6×10^{-5}	3.3×10^{-4}	7×10^{-6}
No. of clones in which plants were regenerated	1	20	2	19	0
Resistance in regenerated plants[b]	1/1	9/16	1/1	11/19	–
Seed transmission	1/1	6/7	1/1	5/8	–

[a] The average frequency is the number of valine-resistant clones/number of colonies (divided p-cells) subcultured 5 days after protoplast isolation

[b] Resistance (no. of resistant clones/no. of tested clones) has been confirmed by testing p-cells or seed progeny resistance of regenerated plants. If regenerated plants were male sterile, resistance of F_2 progeny was determined. In this case, only a determined proportion of seedlings were resistant to valine (see Table 2)

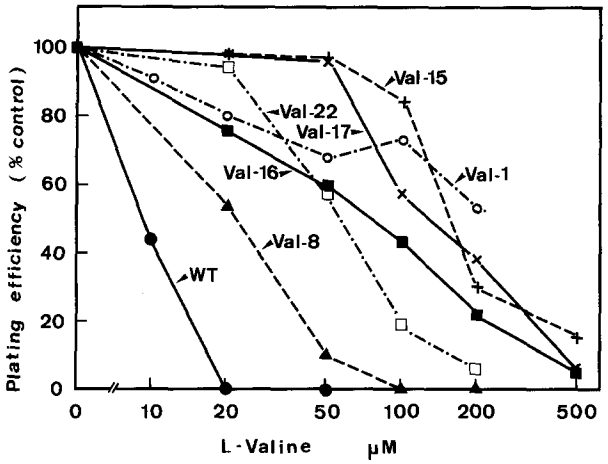

Fig. 1. Test of valine resistance of regenerated plants by p-cell culture

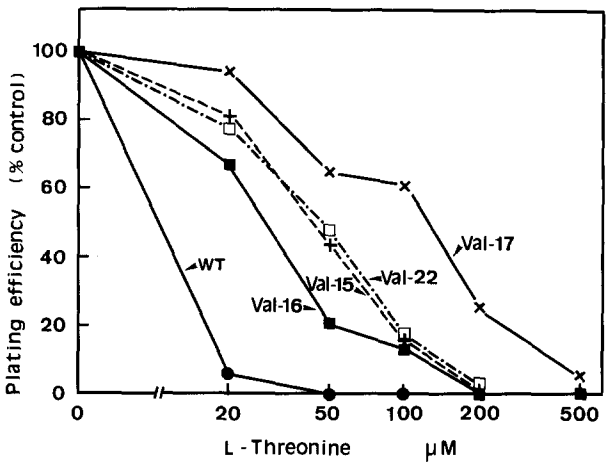

Fig. 2. Test of threonine resistance of regenerated plants by p-cell culture

cotyledons bleached). Seedlings were classified as resistant to valine if they could grow on a medium containing 0.5 mM valine (Table 1).

Plants representing 7 clones, Val-12, Val-15, Val-16, Val-17, Val-22, Val-30 and Val-46, set seed upon selfing. Out of these, Val-16 was the only regenerated clone after selection on medium B containing 0.22 mM mannitol and only Val-22 was obtained without mutagenic treatment (Table 1). P-cells of all these plants, except for the clone Val-30, were resistant to valine and threonine. About 90% of the seedlings obtained after selfing were resistant to 0.5 mM valine for all clones except Val-30 (Table 2). Two plantlets sensitive to valine were rescued from the progeny of clones Val-15 and one from clone Val-22, transferred to the greenhouse and selfed. P-cells of these plants were resistant to valine. Their progeny was again tested for resistance to valine. More than 90% of seeds were resistant to valine for the three clones tested, suggesting that although these clones were phenotypically sensitive in the seed test, they were genetically identical to seedlings initially scored as resistant.

Plants representing 33 clones were crossed with the wild type. Plants of 28 clones gave seeds after crossing. Among the 5 other clones, sterile plants of two were completely abnormal and developed poorly; plants of another grew normally but had a modified leaf morphology, whereas plants regenerated from the two last clones showed normal vegetative development. After crossing, seeds of 19 clones could be tested for valine resistance and all were sensitive to valine (Table 2). P-cells of the F_1 plants tested (Val-15 × WT, Val-17 × WT and Val-22 × WT) were also sensitive to valine when grown at low cell density (Val-15 result in Fig. 3).

F_2 seed progeny was obtained by self-fertilization of F_1 plants representing 14 clones. In 10 clones that were resistant in the p-cell assay of regenerated plants, about

Table 2. Inheritance of valine resistance of seed progeny of some of the mutants

Valine (mM)	0	0.5				
		No. of resistant (R) or sensitive (S) seedlings				
		Observed		Expected		P^f
		R	S	R	S	
Selfed progeny						
WT	76/76[a]	0	79	0	79	
Val-15	78/78	76	7	83	0	
Val-22	89/92	61	8	69	0	
Crosses with WT[b]						
Val-15	36/36	0	28	0	28	
Val-22	35/35	0	38	0	38	
F_2 progeny[c]						
Val-1	85/85	20	70	22	68	0.7
Val-8	33/36	30	110	35	105	0.4
Val-15	48/48	27	85	28	84	0.9
Val-16[d]	139/174	20	50	18	52	0.6
Val-17[d]	30/40	16	46	16	46	1
Val-22	61/61	10	22	8	24	0.5
F_3 progeny[e]						
Val-1	31/31	82	3	85	0	
Val-8	36/36	76	11	87	0	
Val-22	84/84	61	8	69	0	

[a] No. of growing seedlings/no. of germinated seedlings
[b] Crosses with wild type (F_1) were obtained using wild type plants as pollinisator
[c] F_2 progeny obtained by selfing F_1 plants
[d] Here, 25% of the F_2 (and selfed) progeny are albino plants for Val-16 and abnormal plants Val-17. They could be distinguished from sensitive plants, but could not be classified in sensitive and resistant plants. These genetic traits were segregating independently from valine resistance. They are not included in the number of resistant or sensitive plants
[e] F_3 progeny obtained by selfing valine-resistant F_2 plants
[f] P value of χ^2 test

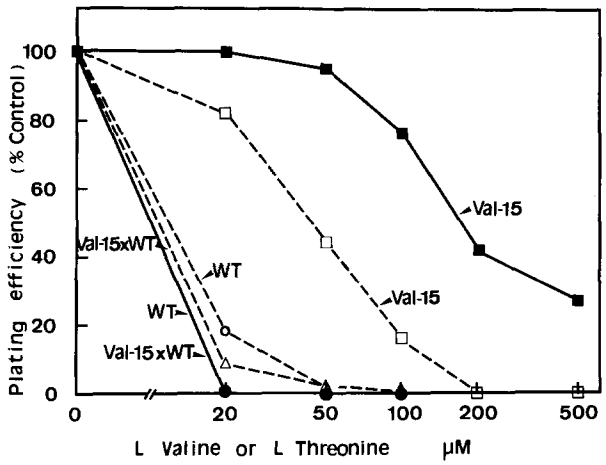

Fig. 3. Test of valine (——) and threonine (– – –) resistance of p-cells derived from a Val-15 plant and its F_1 progeny (Val-15 × WT)

Table 3. Complementation test to determine allelism of valine resistance mutations[a]

♀	♂				
	Val-12	Val-15	Val-16	Val-17	Val-22
Val-12	+	nt[b]	nt	+	+
Val-15	nt	+	nt	+	+
Val-16	nt	nt	+	+	+
Val-17	nt	nt	+	+	+
Val-22	nt	nt	nt	nt	+

[a] +: seedlings derived from cross were resistant to 0.5 mM valine
[b] nt: not tested

Table 4. Relative uptake rate of ^{14}C-L-valine and ^{14}C-L-lysine of valine-resistant p-cells (protoplast-derived cells)

Clone tested	^{14}C-valine	^{14}C-lysine
WT	100[a]	100[a]
Val-15	5	nt[b]
Val-16	2	75
Val-17	5	74
Val-22	3	74
Val-15 × WT	100	nt
Val-16 × WT	81	nt
Val-17 × WT	112	nt

[a] 100% = 5 nmol L-valine absorbed/h per 10^6 cells; 0.4 nmol L-lysine absorbed/h per 10^6 cells
[b] nt: not tested

25% of the F_2 seedlings were resistant to 0.5 mM valine. F_2 progeny of 4 clones sensitive at the cellular level could not grow on a medium containing 0.5 mM valine. Plants representing these 4 clones, therefore, were most probably regenerated from wild type cells escaping the selection process. More than 90% of the F_3 seed progeny, obtained by selfing F_2 valine-resistant plants, were resistant to 0.5 mM valine (Table 2).

Complementation groups

Different genes could be mutated in the *N. plumbaginifolia* genome leading to the same recessive valine-resistant phenotype. A cross between such recessive mutants affected at different loci should lead to a valine sensitive progeny. Crosses between plants representing clones Val-12, Val-15, Val-16, Val-17 and Val-22 indicate that they belong to the same complementation group (some data in Table 3).

Amino acid uptake of p-cells

The p-cells of the valine-resistant mutants exhibited a reduced valine uptake rate: 2% to 5% that of wild type cells. Lysine uptake was less affected (75% that of wild type). The F_1 plants were shown to have a valine uptake rate close to normal (Table 4).

Discussion

We describe in this paper the isolation and characterization of valine-resistant plants of *N. plumbaginifolia* obtained after mutagenesis of mesophyll haploid protoplasts. Mutation frequencies obtained in the experiments (Table 1) are comparable to those published for valine resistance (Nielsen et al. 1985) or nitrate reductase deficiency (Gabard et al. 1986) in *N. plumbaginifolia*. A high percentage, 60%, of regenerated plants from the selected clones were shown to be resistant (Table 1). These results show that valine resistance is a convenient marker to study the efficiency of mutagenic treatments on *N. plumbaginifolia* protoplasts, as shown previously with other *Nicotiana* species (Vunsh et al. 1982; Grandbastien et al. 1985).

The study of the valine resistance of the seed progeny (Table 2) shows that only 90% of the population (selfed progeny and F_3 progeny) instead of 100% are resistant to valine. This suggests that regenerated plants may not be homozygote, although it is clear that they carry recessive mutations. (Crosses with wild type give rise only to sensitive progeny.) The results obtained in F_2 progeny analysis are in agreement with the transmission to progeny of a monogenic recessive marker and therefore suggest that the regenerated plants were homozygote for this marker. This is confirmed by finding that valine-sensitive plants rescued from the selfed progeny of regenerated resistant plants were valine-resistant upon retesting and by the occurrence of a similar proportion of seedlings with a sensitive phenotype in the progeny of resistant selfed F_2

plants. Appearance of phenotypically sensitive plants after selfing resistant regenerates was probably due to the heterogeneity of the seed population collected on homozygote plants (size, germination delay ...). This may reveal the partial inability of these homozygote plants to mature their seeds appropriately, due to a defect in amino acid transport. The distortion increases when the valine concentration is higher. (For example only 60% of the selfed progeny of Val-15 plants is resistant to 1 mM valine). We would probably have better ratios by using a slightly lower valine concentration than 0.5 mM, but we found this concentration convenient for distinguishing between sensitive and resistant plants.

All the valine-resistant plants of *N. plumbaginifolia* obtained are deficient in valine uptake. This character has been shown to be recessive and transmitted to the progeny as monogenic Mendelian trait. Valine uptake deficiency is a digenic character in *N. tabacum,* in accordance with the polyploid origin of the species (Bourgin et al. 1985). A parallel situation is found for another genetic trait, nitrate assimilation deficiency (mutation of the nitrate reductase structural gene: "nia" mutants), which is digenic in *N. tabacum* (Müller et al. 1983) and monogenic in *N. plumbaginifolia* (Negrutiu et al. 1985).

The second type of mutant affected in valine biosynthesis (Wallsgrove et al. 1984), described by Bourgin et al. (1985), has not been isolated in our experiments using *N. plumbaginifolia* protoplasts. It is probably more difficult to select because the level of valine resistance of p-cells derived from this *N. tabacum* mutant is very low, compared to mutants deficient for valine uptake.

P-cells of mutants resistant to valine were also found to be resistant to threonine (Fig. 2). They are affected in valine uptake but only slightly in lysine uptake (Table 4). Although limited, these observations confirm the results obtained with valine-resistant *N. tabacum* mutants which show that they are deficient in neutral and acidic amino acid uptake and not in basic amino acid uptake (Borstlap et al. (1985). They can be compared to mutants obtained in barley, which are resistant to a lysine analog and are impaired in basic amino acid transport but not in neutral and acidic amino acid transport (Bright et al. 1983). Nevertheless, we have observed a slightly reduced lysine uptake in our valine-resistant mutants (Table 4). A similar observation has been described for the *N. tabacum* valine-resistant mutants (Borstlap et al. 1985), indicating that there is probably an interaction between the two transport mechanisms.

Uptake mutants such as described in this paper should facilitate the understanding of amino acid transport in flowering plants.

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