Mendelian inheritance of streptomycin resistance in *Nicotiana plumbaginifolia*

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Received April 10, 1991/Revised version received July 30, 1991 – Communicated by J. M. Widholm

ABSTRACT

In a previous study two haploid streptomycin-resistant clones of *Nicotiana plumbaginifolia* were isolated. The chromosome number of one of these clones has now been doubled through leaf-midvein culture and the resultant diploids were characterized genetically. Our results show that streptomycin resistance in this clone is conditioned by a recessive nuclear gene. Haploid protoplasts of this streptomycin-resistant mutant were selected for chlorate resistance. All clones obtained from the selection were deficient in nitrate reductase activity in addition to resistance to streptomycin. Genetic analysis of progeny of one of these clones revealed that the genes for streptomycin resistance and for the apoenzyme of nitrate reductase are unlinked.

INTRODUCTION

Streptomycin is an aminoglycoside antibiotic which interferes with protein synthesis on bacterial-type ribosomes (Spotts and Stanier 1961, Davies et al. 1964). Mutants resistant to this antibiotic have been isolated from a wide variety of organisms including bacteria, algae, and higher plants. In *Escherichia coli* the resistance is caused by alterations in the 30S subunit of the ribosome (Ozaki et al. 1969; Montandon et al. 1986; Melancon et al. 1988). In the green alga *Chlamydomonas reinhardtii* both nuclear and cytoplasmic mutations have been shown to confer streptomycin resistance (Sager 1954; Lee and Jones 1973). The mechanism of the resistance caused by the nuclear mutations is not clear, while in the cytoplasmic mutants the resistance is due to alterations in the small subunit of the chloroplast ribosome (Harris et al. 1989; Liu et al. 1989). In higher plants there is only one case in which streptomycin resistance has been found to be conferred by a nuclear gene (Maliga 1981); in all other cases the resistance is conferred by a mutation in the chloroplast DNA (Etzold et al. 1987; Galili et al. 1989; Fromm et al. 1989) and is inherited maternally (Maliga et al. 1975; Fluhr et al. 1985; To et al. 1989).

In a previous study we (To et al. 1989) successfully isolated 20 streptomycin-resistant clones from protoplast cultures of haploid *Nicotiana plumbaginifolia*. Two of these clones were haploid and sterile, therefore, they could not be characterized genetically. In the present study, we doubled the chromosome number of one of these clones and studied the mode of inheritance of the resistance trait. Our results show that streptomycin resistance in this

clone is conditioned by a recessive nuclear gene, *sr*.

N. plumbaginifolia has been shown to be a model species in somatic cell genetics (Maliga 1984) but there is presently no genetic map. One area of our research with this species is the construction of genetic stocks with multiple markers for gene mapping. As a first step in achieving this goal, we selected chlorate-resistant clones from the streptomycin-resistant mutant and determined the linkage relationship between *sr* and the structural gene for the apoenzyme of nitrate reductase.

MATERIALS AND METHODS

Plant material. The haploid streptomycin-resistant clone, SR0003, used in this study was isolated from protoplast cultures of haploid *Nicotiana plumbaginifolia* in the presence of 1 mg/ml streptomycin sulfate (To et al. 1989) and was propagated by culturing lateral buds in MS (Murashige and Skoog 1962) basal medium containing 1% sucrose and 0.8% agar. The chromosome number of this clone was doubled by culturing leaf midveins on MS basal medium supplemented with 0.1 mg/l 1-naphthaleneacetic acid (NAA) and 0.5 mg/l 6-benzylaminopurine (BAP).

Isolation of nitrate reductase-deficient clones. Nitrate reductase-deficient (NR⁻) colonies were selected from protoplast cultures of haploid SR0003 according to the scheme of Grafe et al. (1986). The method for protoplast isolation and culture have been described (Huang and Chen 1988). Colonies that were resistant to 40 mM KClO₃ but were unable to utilize nitrate as the sole nitrogen source were transferred to AA medium (Müller and Grafe 1978) supplemented with 0.2 mg/l BAP for shoot induction. Shoot cuttings were embedded in hormone-free AA medium for root formation.

Chromosome number determination. Roots were collected from in vitro grown plants. They were treated with 2 mM 8-hydroxyquinoline at 18° to 20°C for 3 h, fixed in ethanol-acetic acid (3:1) overnight, stained by the Feulgen method, and treated with 1% pectinase for 1 h. In the preparation of slides for chromosome counts, root tips were squashed in a drop of 45% acetic acid.

Enzyme assays. In vivo nitrate reductase (NR) activity was measured by the method of Jaworski (1971) using 200 mg of leaf tissue. Xanthine dehydrogenase (XDH) activity was detected in crude extracts of leaf tissue after electrophoretic separation in polyacrylamide gels, as described by Mendel and

Müller (1976).

Genetic analysis. To study the inheritance of streptomycin resistance, diploid plants of SRO003 were crossed reciprocally with the wild type. The F_1 plants were then self-fertilized and backcrossed to SRO003. Seeds from the crosses were germinated on MS basal medium containing 1 mg/ml streptomycin sulfate. Green seedlings were considered to be resistant while white ones were sensitive.

One NR^- clone isolated from SRO003, SR-NR⁻8, was used to study the linkage relationship between the genes for streptomycin resistance and for the apoenzyme of NR. Plants of this clone could not grow on their own roots, therefore, they were grafted onto plants of *N. tabacum* cv. Wisconsin 38. Flowering grafts were crossed reciprocally to wild-type *N. plumbaginifolia*. The F_1 plants were then backcrossed to SR-NR⁻8. Seeds from the crosses were germinated on BSR medium (Gabard et al. 1987) containing 1 mg/ml streptomycin sulfate. Seedlings resistant to the antibiotic were subsequently transferred to BN_2 medium (Gabard et al. 1987) to test their ability to utilize nitrate as the sole nitrogen source, while those sensitive to the antibiotic were transferred first to streptomycin-free BSR medium for recovery, and then to BN_2 medium. Seedlings which could not grow on BN_2 medium were classified as NR^- .

RESULTS AND DISCUSSION

The diploid plants obtained from leaf-midvein culture of haploid SRO003 were normal in appearance and were fertile. All seedlings grown from seeds obtained by self-fertilization of haploid SRO003 were streptomycin resistant. F_1 hybrid plants between SRO003 and the wild type were all streptomycin sensitive regardless the direction of the crosses. In the backcross and F_2 generations, streptomycin-sensitive and resistant plants segregated in a 1:1 and 3:1 ratio, respectively (Table 1, Fig. 1). These data indicate that streptomycin resistance in SRO003 is governed by a recessive nuclear gene. We propose the symbol *sr* for this gene.

Table 1. Inheritance of streptomycin resistance in the mutant SRO003

Generation	No. seedlings		Test ratio	χ^2	P
	S ^a	R			
P:					
wt selfed	657	0			
SRO003 selfed	0	461			
F_1 :					
wt x SRO003	217	0			
SRO003 x wt	102	0			
BC ₁ :					
(wt x SRO003)					
x SRO003	242	256	1:1	0.39	>.5
(SRO003 x wt)					
x SRO003	71	63	1:1	0.48	>.2
F_2 :					
(wt x SRO003)					
selfed	449	155	3:1	0.14	>.5
(SRO003 x wt)					
selfed	185	65	3:1	0.13	>.5

^aS and R represent sensitive and resistant, respectively, when seeds germinated on medium containing 1 mg/ml of streptomycin sulfate.

Streptomycin resistance conferred by a recessive, nuclear mutation has also been reported in *N. sylvestris* (Maliga 1981) and *C. reinhardtii* (Sager 1954; Lee and Jones 1973). In the latter species, Harris

Fig. 1. Tests for streptomycin resistance where white seedlings show bleaching (susceptibility). Seeds from self-fertilization of wt *N. plumbaginifolia* (A), self-fertilization of mutant SRO003 (B), backcross (wt x SRO003) x SRO003 (C), and self-fertilization of (wt x SRO003) (D) were germinated on medium containing 1 mg/ml of streptomycin sulfate.

et al. (1977) have shown that all the mutants analyzed by them are allelic, and Spiess and Arnold (1975) have found that the electrophoretic mobility of the chloroplast ribosomal proteins of one mutant is different from that of the wild type. These results seem to suggest that the nuclear streptomycin resistance locus in *C. reinhardtii* may code for a chloroplast ribosomal protein. In the case of SRO003, *sr* could be a structural gene for a chloroplast ribosomal protein, or a gene affecting the uptake of the antibiotic or its inactivation.

Leaf protoplasts of haploid SRO003 were used for isolation of plants deficient in NR activity. From approximately 2×10^5 protoplast-derived cells plated in medium containing 40 mM $KClO_3$, 21 chlorate-resistant colonies were isolated. Subsequent tests showed that all of these were unable to grow on medium with nitrate as the sole nitrogen source. Chlorate-resistant colonies have also been isolated from protoplast cultures of haploid *N. plumbaginifolia* by other investigators. The frequency of chlorate-resistant colonies in this study is within the range of variation reported by Gabard et al. (1987) but is higher than those obtained by Márton et al. (1982) and Negrutiu et al. (1983). This difference is likely caused by the utilization of different selection procedures.

Nineteen out of 21 chlorate-resistant colonies produced plants upon transfer to the regeneration medium. The chromosome numbers of regenerated plants were present in a geometric series such as 10, 20, and 40 (Table 2), suggesting that endomitosis and/or endoreduplication are mainly responsible for the changes.

Diploid plants of the chlorate-resistant clones were tested for NR activity. The results revealed that 13 clones had no detectable NR activity and 3 clones had partial NR activity (Table 2). Tests for XDH activity showed that all clones except No. 10 had this enzyme activity (Table 2). Thus, among the 16 NR^- clones only one is presumably deficient in the molybdenum cofactor and was classified as the *cnx* type, while the remaining clones are likely defective

Table 2. Chromosome numbers, nitrate reductase (NR) and xanthine dehydrogenase (XDH) activities of plants regenerated from the chlorate-resistant clones isolated from protoplast cultures of haploid SRO003

Chlorate-resistant clone	No. plants with the following chromosome number			NR activity (% of wt) ^a	XDH activity
	10	20	40		
1	0	0	7	nd ^b	nd
3	0	0	5	nd	nd
4	0	3	0	0	+
5	0	4	0	0	+
6	0	4	0	0	+
7	5	2	0	0	+
8	0	3	0	0	+
9	4	2	0	0	+
10	0	5	0	1.4	-
11	0	3	0	6.0	+
12	7	0	0	nd	nd
13	0	5	0	0	+
14	0	3	1	0	+
15	0	7	0	0	+
16	0	7	0	0	+
17	1	2	1	0	+
18	0	4	1	0	+
20	0	7	0	9.7	+
21	0	6	6	0	+

^aNR activity of wt: 812 nmoles NO₂⁻/g fresh weight·3h

^bnd: not determined

in the apoenzyme and were classified as the *nia* type. In contrast to this result, Gabard et al. (1987) found that approximately 40% NR⁻ mutants isolated from protoplast cultures of haploid *N. plumbaginifolia* were the *cnx* type. The recovery of a high proportion of *cnx* mutants has been attributed to an improvement in culture conditions which favor the regeneration of *cnx* callus (Gabard et al. 1987).

Table 3. Inheritance of streptomycin resistance and nitrate reductase deficiency in double mutant SR-NR⁻8

Generation	Total	Streptomycin sensitive		Streptomycin resistant	
		NR ⁺	NR ⁻	NR ⁺	NR ⁻
P:					
wt selfed	530	530	0	0	0
SR-NR ⁻ 8 selfed	312	0	0	0	312
F ₁ :					
wt x SR-NR ⁻ 8	556	556	0	0	0
SR-NR ⁻ 8 x wt	408	408	0	0	0
BC ₁ :					
(wt x SR-NR ⁻ 8)					
x SR-NR ⁻ 8	190	56	44	47	43
(SR-NR ⁻ 8 x wt)					
x SR-NR ⁻ 8	187	51	43	56	37

The NR⁻ clones isolated from SRO003 were designated as the SR-NR⁻ series. The only *cnx* clone in this study had partial NR activity, could grow in soil without special care, and was able to set seeds upon self-fertilization. Further characterization of this clone is necessary. Previous studies show that in *N. plumbaginifolia* there is only one structural gene for the apoenzyme of NR (Márton et al. 1982; Negrutui et al. 1983; Gabard et al. 1987). Clone SR-NR⁻8 was chosen to study the linkage relationship between the genes for streptomycin resis-

tance and for the apoenzyme of NR. As shown in Table 3, all seedlings from self-fertilization of SR-NR⁻8 were streptomycin resistant and NR⁻. F₁ plants between SR-NR⁻8 and the wild type were all streptomycin sensitive and NR⁺ regardless the direction of the crosses. The ratio of streptomycin sensitive to resistant and of NR⁺ to NR⁻ fit the 1:1 ratio in backcrosses (wt x SR-NR⁻8) x SR-NR⁻8 ($\chi^2 = 0.526$ and 1.346 , respectively) and (SR-NR⁻8 x wt) x SR-NR⁻8 ($\chi^2 = 0.005$ and 3.898 , respectively). In both cases the four phenotypic classes fit the 1:1:1:1 ratio ($\chi^2 = 2.210$ and 4.551 , respectively). From these we conclude that *sr* and the structural gene for the apoenzyme of NR are unlinked.

The double mutant SR-NR⁻8 could be propagated in vitro in medium containing diammonium succinate and was fertile when grafted onto plants of *N. tabacum* cv. Wisconsin 38. Thus, leaf protoplasts from the double mutant or its anther-derived haploids can be used for selection of other mutations such as herbicide resistance (Chaleff and Ray 1984) and valine resistance (Marion-Poll et al. 1988) until the desired multiple-marker stocks are obtained. The multiple-marker stocks are useful for gene mapping.

ACKNOWLEDGEMENTS

We are grateful to S. Y. Tung for technical assistance. This study was supported by Grant No. NSC79-0211-B002-04 from the National Science Council, Republic of China.

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