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Regeneration of fully nitrate reductase – deficient mutants from protoplast culture of *Nicotiana plumbaginifolia* (Viviani)

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Summary. Protoplast-derived colonies of haploid *N. plumbaginifolia* were selected for by chlorate resistance in media supplemented with casamino acids. Eighty resistant lines were confirmed by a second passage on a higher concentration of chlorate. Frequency of spontaneous mutation ranged from 10^{-5} to 10^{-6}. Fifty of the resistant lines could be regenerated into plants, and 30 were characterized biochemically. Ninety percent were fully deficient for nitrate reductase activity. The lines were further tested for xanthine dehydrogenase activity and subsequently classified as defective in the apoenzyme (*nia* type, 26 lines) or the cofactor (*cnx* type, 4 lines). Two groups had been identified up until now within the *cnx* type by growth tests on high concentrations of molybdate supplied to the medium. Nitrate reductase deficiency was stably and continously expressed in both variant cell cultures and regenerants. Genetic analysis demonstrated that nitrate reductase deficiency was inherited as a single recessive nuclear gene.

Key words: Genetic analysis – Inheritance – Nitrate reductase deficiency – *N. plumbaginifolia*

Introduction

Nitrate reductase deficient mutants (NR^-) have been reported in several plant species. In most cases they were selected for by chlorate resistance, one of the rare available positive screening procedures leading to an auxotroph phenotype. In this way the NR^- types were obtained using M2 seeds of *Arabidopsis thaliana* (Oostindier-Braaksma and Feenstra 1973), barley (Bright et al. 1982), and pea (Feenstra and Jacobsen 1980), cell suspensions of tobacco (Müller und Grafe 1978; Müller 1981), *Datura innoxia* (King and Khanna 1980), and *Rosa damascena* (Murphy and Imbrie 1981), or protoplast cultures of *N. plumbaginifolia* (Marton et al. 1982 a). Direct screening for in vivo NR activity (Kleinhofs et al. 1980) and a total isolation procedure for auxotroph selection (Strauss et al. 1981) also enable the selection for NR deficiency in barley and *Hyoscyamus muticus* respectively. Except for certain barley mutants reported by Bright et al. (1982), all those islated at plant level were shown to maintain residual NR activity. Fully NR deficient lines were described in cell suspension and protoplast culture systems, but plant regeneration followed by genetic analysis was reported only in the amphitetraploid species *N. tabacum*. The tobacco mutants were shown to be double recessive mutants for both *nia* and *cnx* types (Müller 1981, 1982). In the case of *N. plumbaginifolia,* plant regeneration was achieved only in cell lines with residual NR activity (Marton et al. 1982 a).

In this paper we report on isolation, regeneration, and biochemical and genetical characterization of several fully NR deficient mutants selected from haploid protoplasts of *N. plumbaginifolia,* a true diploid species (2n = 2x = 20).

Materials and methods

Protoplast and callus culture. Screening conditions

Haploid protoplast mother plants were cultured as previously described (Negrutiu 1981). Two haploid lines, P1 and Y1-AEC^R, were used as a protoplast source. The latter contains a mutation for resistance to the lysine analogue, S-aminoethyl-L-cysteine (AEC^R; monogenic recessive, unpublished). Media used during the experiments are listed in Table 1. They all were sterilized by autoclaving.

Table 1. Culture media and their use

Code	Use and composition of the medium	References
	Protoplast culture	
KA2	– 1/2 strength MS macroelements + 1 mM organic acids For a detailed description see ref.	Negrutiu (1981)
P7A	– 1/3 strength MS macroelements + 0.33 mM organic acids + 0.25 g/l casamino acids; the rest as KA2	
	Dilution and selection	
MD8cM	– as MAP (see ref.), 0.25 g/l casamino acids replacing the mineral nitrogen in MS macroelements	Negrutiu and Muller (1981)
MDs5	– as above, 5 mM $(NH_4)_2$ succinate replacing the casamino acids	
	Plant regeneration	
R4s	– as R4 (see ref.), except for sucrose (0.5 g/l) and mannitol (18 g/l); $(NH_4)_2$ succinate replaces the mineral nitrogen (15 mM)	Bourgin et al. (1979)
	Callus maintenance	
MSCls	– as R4s, except for hormones: 3 mg/l NAA + 0.1 mg/l BAP, and sucrose (30 g/l)	
MSCln	– as MSCls, with 10 mM KNO_3 as sole nitrogen source	
	Rooting, seed germination, and culture of PMP's	
R'SAs	– as R'SA (see ref.), 10 mM $(NH_4)_2$ succinate replacing the mineral nitrogen	Durand 1979 Negrutiu (1981)
Ms	– as R'SA, with 1/2 strength MS macro- and 1/10 microelements; 10 mM $(NH_4)_2$ succinate replacing the mineral nitrogen	
Mn	– as Ms, with 10 mM KNO_3 as sole nitrogen source	

Note: this table only details media components that are required to sustain growth of NR^- phenotypes
Abbreviations: *MS* salt solutions as described by Murashige and Skoog (1962); *NAA* naphthaleneacetic acid; *BAP* 6-benzylaminopurine; *PMP* protoplast mother plants

Non-mutagenized and UV irradiated (25 erg. s^{-1} mm^{-2}, 15W germicidal lamp) protoplasts were inoculated either in KA_2 or P7A medium. Ten to eleven day old cultures were washed by sedimentation and diluted to 5,000 colonies per ml in MD8cM medium. $KClO_3$ was added in the following concentrations: 50, 75, and 100 mM. Surviving colonies were transferred onto the same medium supplemented with 125 mM $KClO_3$.

Callus cultures were maintained on MSCls medium.

Protoplasts isolated from NR^- regenerants were cultured on KA_2 or P7A media for 10 days and subsequently diluted into MDs5.

Plant regeneration, growth, and seed-setting

Regeneration was induced on R4s medium. Two to three weeks later green spots or shoot primordia were transferred to a hormone free medium (R'SAs) for plantlet formation and rooting. For seed-setting the plantlets were either grafted on wild type plants or potted in perlite. A mineral solution containing ½ strength MS macroelements and 5–10 mM $CaCO_3$ was used to water the plants.

Nitrate reductase assay

The screening of lines for in vivo NR activity was carried out on young to fully expended leaves of in vitro propagated plantlets (Ms medium). To fully induce the NR enzyme, leaves were incubated overnight in the dark at 27 °C in R'SA with as N source either 40 mM KNO_3 or 10 mM NH_4 succinate + 40 mM KNO_3. Both media were filter sterilized at pH 5.6. The leaves were subsequently sliced and assayed according to Jaworski (1971) using an 0.1 M Na/K phosphate buffer pH 7.5, containing 40 mM KNO_3 and 4% propanol. NO_2 release was measured after 3 h. Values for wild type enzyme activity averaged 1169 (±427 s.d.) nmoles NO_2 produced per gram fresh weight in 3 h. In vivo NR activity of callus cultures was performed as described by Marton et al. (1982a).

Xanthine dehydrogenase activity

Xanthine dehydrogenase (XDH) activity was assayed as described by Mendel and Müller (1976).

Results

1 Isolation and regeneration of chlorate resistant phenotypes

Data presented in Table 2 show that 81 confirmed resistant lines were isolated from a total of $1.84 \cdot 10^7$

Table 2. Selection for chlorate resistance in haploid protoplast culture of *N. plumbaginifolia*

Experiment	Selection 1				Selection 2	
Haploid stock	P1		Y1-AEC^R		P1	
Chlorate conc.	50 mM	100 mM	50 mM	100 mM	75 mM	
No. of exposed protoplasts	6.7×10^6	7×10^6	1.5×10^6	1.5×10^6	1.7×10^6	6×10^6
Mutagen treatment	–	–	–	–	–	UV[a]
Survival rate (S%)	84	84	90	90	100	100 (80)[b]
Protoplast culture medium	P7A	P7A	P7A	P7A	KA2	KA2
No. of isolates	108	6	14	0	63	156 green 62 brown
Confirmed on 125 mM chlorate	66	5	9	–	1	65 green 0 brown
Mutation frequency per surviving cell	1.2×10^{-5}	8.5×10^{-7}	6.6×10^{-6}	7.4×10^{-7}	6×10^{-7}	1.3×10^{-5}

[a] One day old protoplasts were exposed to UV irradiation
[b] Survival rate in mutagenized plates as compared to controls is given in brackets

non-mutagenized haploid protoplasts; they were retained following a second passage on selection medium containing higher levels of chlorate (125 mM). The spontaneous mutation frequency of chlorate resistance varied with the strength of selection pressure (50 or 100 mM chlorate) from approximately 10^{-5} to 10^{-6} (also see Brock et al. 1973). A more than 20-fold increase in mutation frequency occurred at UV doses causing low lethality.

This paper describes spontaneously occurring resistant lines selected in a first series of experiments (Table 2). Fifty out of the 80 initial lines were regenerated into plants; only 30 were studied in more detail as losses (10 lines) or difficulties in maintaining the regenerants due to morphological abnormalities (11 lines) occurred.

2 *Expression of the resistance*

a) Cell cultures. Contrary to what was stated by Marton et al. (1982 a), wild type cultures grew actively on media containing nitrate (medium MSCln) as a sole nitrogen source provided exposure to light intensities above 2,000 lux was ensured. None of the chlorate-resistant lines was able to proliferate on the nitrate medium: the culture exhibited some increase in size due to cell enlargement but ceased growth within 5–7 days following subculture, and gradually turned brownish.

Proliferation of resistant lines was supported by addition of amino acids, NH_4 succinate, or by a combination of NH_4 salts with various Krebs' cycle intermediates or $CaCO_3$ (5 mM). All cell lines maintained their resistance to inhibitory concentrations of chlorate (150–200 mM in MD8cM medium) after prolonged subculture in the absence of selection pressure.

Similar results were obtained with protoplast cultures initiated from regenerated resistant plants. Such protoplasts failed to proliferate in nitrate media, and exhibited chlorate resistance when diluted to low densities into MDs5 medium (Fig. 1).

b) Regenerated plants. Variant regenerants were routinely maintained as cuttings on Ms (NH_4 succinate as nitrogen source) medium with agar or perlite as substrate. When transferred onto Mn (KNO_3 as sole nitrogen source) medium, their leaves turned gradually white and the roots stopped growing and became brownish with characteristic protuberances at the root

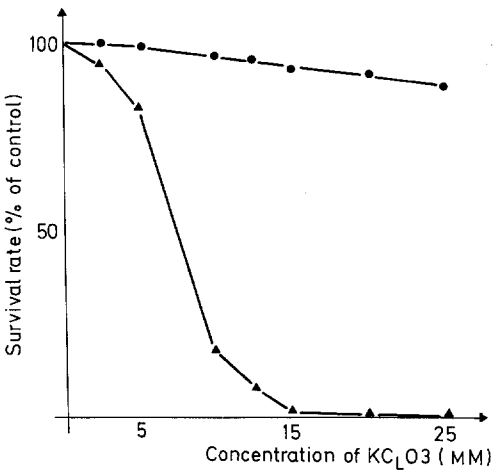

Fig. 1. Survival of wild type (▲) and NR$^-$ (●) protoplast-derived colonies (10 day old) on various concentrations of chlorate. Protoplast culture medium: KA2. Dilution to 200 protoplast-derived colonies per ml in MDs5 medium. Plating efficiency after dilution in controls was 42.2%. Each point represents the average of at least three replicates. SE values were less than 10%.

Table 3. Distribution of *nia* and *cnx* types of NR^- lines as identified in three distinct selection groups

Selection group (haploid stock and KC103 concentration)	Resistant lines		Mutant type	
	Regenerated	Tested for NR and XDH activity	*nia*	*cnx*
P1 – 50 mM	40	25	23	2
P1 – 100 mM	4	2	1	1
Y1 – AEC^R – 50 mM	6	3	2	1
Total	50	30	26	4

apex. The resistance to chlorate was more markedly expressed at plant level than in cell culture; 38 of the regenerated lines were tested on a Ms + chlorate medium and tolerated up to 100–125 mM chlorate. Wild type regenerants were completely inhibited by 10 mM chlorate. Sensitive plantlets were never observed among the many variant regenerants tested.

Potted and grafted resistant plants grew and developed normally. Occasionally some chlorosis appeared on fully expended leaves.

3 Biochemical basis of the resistance

Nitrate reductase activity. Screening of the resistant lines for NR activity was performed with regenerated resistant plants. Twenty-seven lines were fully deficient, while in three other lines residual NR activity (< 3%) could be occasionally measured. Similar results were obtained with grafts and callus cultures.

Xanthine dehydrogenase activity and molybdate response. All lines were assayed for XDH activity. Four out of 30 were identified as deficient in both NR and XDH activity and designated as *cnx* type, i.e. Mo cofactor defective; the remaining 26 were designated as *nia* types, i.e. defective in the apoenzyme (Mendel et al. 1981). *cnx* plants have a common distinguishable morphology as compared to *nia* types: flimsy shoots, narrow leaves of rather dark green colour. The *nia*/*cnx* ratio was 26 : 4. The four *cnx* lines (Np–*cnx* 20; *cnx* 27; *cnx* 82; *cnx* 103) were further tested for growth on a NO_3^-/NH_4^+ medium (R'SA) supplemented with unphysiologically high concentrations of Na_2MoO_4: 0.1–1.5 mM. Two of them (*cnx* 20 and *cnx* 82) were able to proliferate on this medium, and showed a phenotype similar to that of wild type plants at optimal molybdate concentration of 0.3 mM.

Table 3 gives the distribution pattern of *nia* and *cnx* lines isolated from two distinct haploid stocks and at two different selection concentrations of chlorate.

4 Genetical basis of the NR deficiency

Segregation tests have been performed with several lines on Mn and Ms + 15 mM $KClO_3$ media (Table 4). The lines could be crossed to wild type plants indicating that spontaneous doubling of the haploid genome occurred in the selected cells during the in vitro passage. All F1 progenies behaved as wild types (i.e. growth on nitrate and sensitivity to chlorate). All progenies from six selfed *nia* regenerants (*nia* 8, *nia* 26, *nia* 36, *nia* 39, *nia* 56, *nia* 84) failed to grow on nitrate as sole nitrogen source but grew on toxic concentrations of chlorate (Fig. 2). F2 progenies from two *nia* mutants tested (*nia* 7 and *nia* 26) showed a 3 : 1 growing/not growing segregation frequency on nitrate, and a 1 : 3 resistant/susceptible segregation frequency on chlorate. Another *nia* mutant (*nia* 18) and *cnx* 103 segregated as a 1 : 2 : 0 ratio, which could be explained by the presence of a lethal factor in the wild type

Table 4. Segregation of chlorate resistance and NR^- phenotypes among progenies of regenerated plants germinated on Mn (nitrate as sole N source) or chlorate supplemented Ms (NH4 succinate as N source) media

Genotype	Culture medium	Response		*P*
		Growing	Not growing	
*nia*26/*nia*26 selfed	Mn	0	150	–
	Ms + KC103	150	0	–
*nia*7/*nia*7 × + / + (F1)	Mn	150	0	–
	Ms + KC103	0	150	–
*nia*7/ + selfed (F2)	Mn	412	120	0.5 – 0.7
	Ms + KC103	87	302	0.3 – 0.5
*cnx*103/*cnx*103	Mn	150	0	–
× + / + (F1)	Ms + KC103	0	150	–
*cnx*103/ + selfed (F2)	Mn	133	65	0.3 – 0.5
*nia*18/ + selfed (F2)	Mn	209	104	0.3 – 0.5
*nia*26/ + selfed (F2)	Mn	482	164	0.3 – 0.5

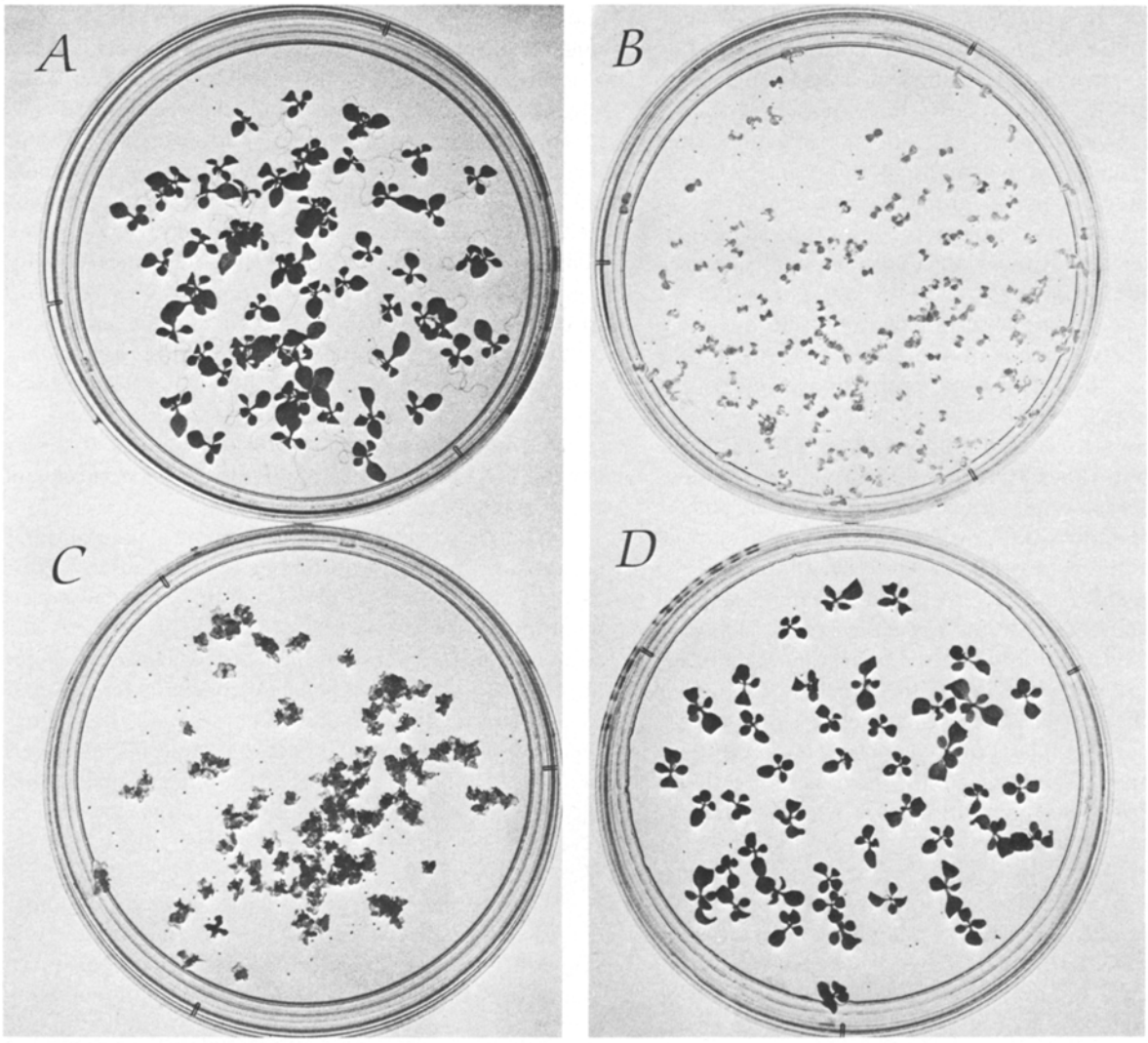

Fig. 2. Growth response of progeny seedlings from selfed wild type (**A** and **C**) and NR^- regenerated plant (Np-*nia* 26; **B** and **D**) on media with nitrate as sole N source (*upper line*) or NH_4 succinate + 15 mM KC103 (*lower line*). Note the curly appearance of wild type seedlings on chlorate

pollen. The results indicated that the loss of NR activity and resistance to chlorate as a consequence of *nia* and *cnx* type of mutations were inherited as single recessive nuclear genes. Homozygous NR^- seeds geminated normally on nitrate as the sole source of nitrogen; their cotyledons gradually turned white and the seedlings died without forming true leaves. The fully deficient phenotype was expressed accurately at various developmental stages of the mutant plants. On the contrary, progeny from selfed *nia* 13, a mutant with less than 3% residual NR activity, grew slowly on Mn medium, developing roots and several true leaves of pale green colour. Nevertheless, *nia* 13 seedlings showed resistance to chlorate when germinated on Ms + 15 mM $KClO_3$.

Diallelic crosses with 6 *nia* mutants produced progeny that failed to grow on nitrate media, suggesting that all *nia* mutants tested were allelic to each other. As expected, genetic complementation occurred in the cross between a *nia* (*nia* 26) and a *cnx* (*cnx* 103) mutant.

Discussion

A similar work on mutagenized and non-mutagenized haploid protoplasts of *N. plumbaginifolia* subjected to selection for resistance to chlorate has recently been published (Marton et al. 1982a). Both partially (67%) and fully (21%) NR^- lines were identified. NA (*nia*) and NX (*cnx*) types were present in a seven to four

ratio among the analysed strains. Plant regeneration could be obtained only in clones containing residual NR activity, which was sufficient to allow close to normal growth of the NR^- lines under restrictive conditions. Four types of NR^- lines were distinguished by complementation in somatic hybrids (three types in the *cnx* and one in the *nia* group; Marton et al. 1982 b).

In our case, non-mutagenized protoplasts undergoing rather similar selection conditions were subjected to a second and stronger selection pressure before the induction of regeneration under non-selective conditions. Ninety percent of the tested lines were fully deficient for NR activity, the proportion of *nia* to *cnx* clones being 26 to 4. This is much closer to what was reported in tobacco (36 : 4) by Müller (1982). Three types of NR^- lines have been identified up to now. Further enzymatic and genetic tests within the *nia* and the *cnx* groups should indicate the exact number of complementation types existing in our batch of mutants.

Several NR^- lines were isolated from a haploid plant stock already carrying a mutation for resistance to AEC that was previously selected in protoplast culture.

From a more general point of view, the above results indicate that selection for chlorate resistance with haploid protoplasts of *N. plumbaginifolia* yielded a large number of resistant lines, demonstrating that a highly efficient screening system is available for the isolation of auxotrophic mutants dependent on a reduced N source. Mutation frequencies of chlorate resistance are rather high suggesting that several genes could be implicated. This was shown to be the case in *Aspergillus* (Marzluf 1981) and *Neurospora* (Tomsett 1982) where the nitrate assimilation was shown to require the function of at least 10 and 8 genes repectively. In the case of *N. plumbaginifolia,* four types of NR^- clones have been reported until now (Marton et al. 1982 b), which is the highest number identified in a higher plant. In tobacco, both *nia* and *cnx* mutants were shown to be double mutants (Müller 1982 and personal communication). We present evidence that NR deficiency in *N. plumbaginifolia* is inherited as a single recessive gene.

The fact that fully NR^- mutants could be isolated by us in much higher proportion than previously reported in this species is most probably due to the "enrichment" step operated among the presumptive resistant isolates (the isolated colonies underwent a second passage on higher concentrations of chlorate). The mutagen treatment per se does not appear to play a part in this matter: the relative proportion of fully deficient versus partially deficient lines was shown to be similar ($\sim$ 30%) in spontaneously and mutagen induced resistant lines (Marton et al. 1982 a).

Our results, as those reported in tobacco, prove that it is possible to regenerate fully NR deficient plants, and that there is no need for complementation by somatic fusion (Marton et al. 1982 b) to "restore" the morphogenetic ability in such mutants. However, only 50 out of 80 initial isolates could be regenerated into plants with routine procedures, and even then 22% of them showed various degrees of morphological abnormalities. The fact that this occurred with non-mutagenized protoplast cultures passaged a relatively reduced number of times (approximately four times) before regenerating plants suggests that a true diploid species such as *N. plumbaginifolia* may be more susceptible to the action of factors generating somaclonal variation than polyploid ones such as tobacco[1]. Limiting to a minimum the in vitro passage stage of selected mutants, and using low killing rates at mutagenesis are two essential prerequisites for a successful regeneration of variant cultures.

It is interesting to note that neither uptake nor other classes of mutants (Oostindier and Feenstra 1973; Murphy and Imbrie 1981) could be identified by us with the described selection system in spite of the relatively large number of lines tested. However as not all selected lines were biochemically characterized, and as approximately 12% of the chlorate resistant lines reported by Marton et al. (1982 a) showed normal levels of NR activity, it can not be excluded that other mechanisms of resistance than NR deficiency can be revealed by screening for chlorate resistance in *N. plumbaginifolia.*

The NR deficiency was stably and continuously expressed in both variant cell culture and plants. Fully NR deficient mutants represent excellent biochemical markers as they respond to a double complementing screening: resistance to chlorate and inability to use nitrate as a sole source of nitrogen. In addition, selection and counter-selection conditions are rather stringent. All this is essential with respect to the potential use of NR deficient mutants as markers in genetic manipulation experiments. Furthermore, such nutritional deficient mutants isolated through positive selection schemes can be used in reconstruction experiments of auxotroph isolation aiming to improve the screening for auxotrophs by enrichment procedures. Last but not least, the high frequencies of mutation in this system makes it very suitable for studies on mutagenesis with plant cells. An obvious application would be the recovery of backmutants and other kind

1 Note that although poorly documented at present, available information suggests that the ability of cell cultures to regenerate plants may be altered to different extents, or even prevented, by the screening procedure employed or by the mutation per se (i.e. by affecting the morphogenetic process directly, by disrutping a regulative pathway which is involved in morphogenesis etc.)

of revertants and their use in more sophisticated studies on mutagenesis and molecular genetics.

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