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Characterization and regeneration of salt- and water-stress mutants from protoplast culture of *Nicotiana plumbaginifolia* (Viviani)

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Summary. Protoplast-derived colonies of haploid *N. plumbaginifolia* leaves were used to select for resistance to NaCl, KCl and polyethylene glycol 6000 (PEG). Salt- and PEG-tolerant cell lines were isolated on the basis of growth in a culture medium containing inhibitory concentrations of either NaCl or KCl (200 m*M*) or PEG (25%). The frequency of resistant lines ranged from 10^{-5} to 10^{-6}. One resistant line from each treatment was regenerated into plants. All resistant lines produced 10–25 times more proline than the wild type when grown on a non-selective medium. Similar values were also observed in the leaves of resistant progeny plants. In each mutant line, salt or PEG resistance was transmitted as a single dominant nuclear gene as shown by segregation ratios in progenies of crosses between resistant and wild-type plants. The latter observation demonstrates clearly the existence of a genetic basis for increased salt tolerance.

Key words: Protoplast selection – Salt- and water stress-resistant mutants – Proline overproduction – *Nicotiana plumbaginifolia* – Dominant gene

Introduction

There is considerable potential in the application of cell culture techniques in genetic engineering for future plant improvement (Rains et al. 1980). Cellular tolerance to salt and water stress is a fertile area for basic research, with possible applications in agriculture (Handa et al. 1983). The specific mechanisms conferring tolerance are not clearly understood, and traditional breeding approaches to salt tolerance have generally failed to identify a clear genetic basis for the trait (Tal 1984; Epstein et al. 1980). Several reports on salt-tolerant cell lines obtained in vitro selection methods are available, and NaCl-tolerant plants of tobacco (Nabors et al. 1980; Dix and Street 1975), rice (Vajrabhaya et al. 1989), flax (McHughen and Swartz 1984) and alfalfa have been regenerated. In a few cases, relatively stable variants have been obtained and tolerance has been retained in the progeny of regenerated plants (Nabors et al. 1980; Bhaskaran et al. 1986). However, the genetic basis for salt tolerance was not clearly established in any of these investigations.

The development of sophisticated cellular in vitro techniques based on protoplast culture combined with mutagenesis and appropriate positive selection methods has resulted in a wide spectrum of biochemical variants and mutants in *N. plumbaginifolia* (Negrutiu 1990). Such mutants have proved to be fruitful for studying metabolic pathways and gene regulation in plants (Negrutiu et al. 1984; Jacobs et al. 1987). An important point is that any such new trait that has been selected at the cellular level can be expressed at the plant level and then transferred to the next generation through meiosis. In that context the identification of single gene mutations that contribute to salt and water stress tolerance would provide a way to identify tolerance mechanisms at the cellular and molecular levels. Resistance to growth inhibition caused by salt has been obtained by incorporating NaCl or KCl into the culture medium. Water stress was created by the addition of PEG to the culture medium, which reduces free water concentrations extracellularly (Caplan et al. 1990).

In the investigation described in this paper, we used haploid protoplasts of *N. plumbaginifolia* to select single gene mutants tolerant to salt (NaCl or KCl) and water stress (PEG) that are characterized by proline overproduction.

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Materials and methods

Selection of salt- and PEG-tolerant cell lines through protoplast culture

Protoplasts from haploid *N. plumbaginifolia* plants were isolated and cultured as already described (Negrutiu et al. 1981). The mutagenic treatment was performed 1 day after protoplast isolation. They were exposed to UV light at a dose of 25 erg/mm^2 per second for 20 s and then placed in the dark for 2 days. One week later the surviving colonies were washed by sedimentation and transferred at the final density in a MDS medium containing the selection agents at a concentration of 200 m*M* for NaCl and KCl and 25% for PEG 6000. These concentrations were chosen on the basis of lethality curve established for wild-type cells growing on media containing such compounds (Sumaryati et al. in preparation). The culture medium was renewed every 2 weeks. After 6 weeks, the resistant colonies were transferred on MAP_1 solid medium containing the same selection agents for propagation. Four weeks later actively growing callus lines were used for free amino acid analysis. The stability of the resistance to the selection agent was tested by transferring part of the calli onto non-selective medium and after three successive subcultures over 4 weeks, by putting them back on salt- or PEG-containing medium. All the media used in the experiments were prepared according to Installé et al. (1985).

Regeneration and growth of salt- and PEG-tolerant plants

Regeneration was induced by transferring the calli from MAP_1 to RP medium. Two to three weeks later calli with shoot primordia were transferred onto hormone-free medium (R'SA) to assure plantlet formation and rooting; selection agents were present at the same concentrations. Regenerated plants growing in vitro were then transferred to the greenhouse.

Analysis of resistance in progenies: genetics and growth

To determine the genetic basis of the resistance in the selected lines, the salt- and PEG-resistant mutants were crossed with the wild type and selfed or back-crossed. In the crosses mentioned here, the salt- and drought-resistant mutants were used as female parents. Progeny seeds were sterilized with sodium hypochloride for about 15 min, then washed with sterile water. To favour germination, seeds were kept on a wet filter paper under light for 2 days at 26 °C and then transferred onto a solid MS medium containing 175 m*M* salt (NaCl or KCl) or 20% PEG 6000. The resistance or sensitivity of the plantlets was estimated 8 weeks after sowing. Confirmation of resistance was also established by inducing callus from leaves of resistant plants (R_0) and transferring 200 mg of callus onto a MAP_1 medium containing the selection agents. Final callus fresh weight after 4 weeks was determined for five replicates per cell line.

Amino acid analysis

Free amino acids in callus and 1- to 2-month-old plantlets were analysed by homogenizing 1 g of callus with a mixture of methanol, chloroform and water (12 : 5 : 1) (Bieleski and Turner 1966). Amino acid determination in callus required an additional step: the material was boiled in 70% ethanol for 10 min (Bright et al. 1978). Chlorophyll was recovered by adding two parts of chloroform plus one part of water to the extract. The aqueous layer was taken and completely evaporated. The residue was redissolved in concentrated HCl and hydrolysed under vacuum at 110 °C. After evaporation at 85 °C, the extracts were subsequently resuspended in the loading buffer (Na-citrate, pH 2.2) and analysed in an amino acid analyser (Biotronik LC 5001).

Results

Isolation and regeneration of salt- and PEG-resistant cell lines

Proline and two analogues of proline (azetidine-2-carboxylic acid and *trans*-4-hydroxy-L-proline) were tested together with salt (NaCl and KCl) and polyethylene glycol for inhibition of callus growth of the wild type. Inhibition curves allowed us to determine the concentrations of salt and PEG used in the selection experiments on the basis of a lethality effect for most of the wild-type cells (LD > 90%). Selection conditions and the isolation of resistant colonies and regenerants are briefly reported in Table 1. The frequency of resistant cell lines was about 10^{-5}; however only a few resistant clones were able to regenerate into fertile plants. The tolerant cell lines and plants which were further characterized were designated RNaCl-1, RKCl-1 and RPEG-1.

Characterization of selected salt-resistant lines

Growth rate in the presence of the selection agents. The salt-resistant lines RNACl-1 and RKCl-1 grew clearly better than the wild type. While a 100 m*M* concentration reduced callus fresh weight by 50% of the control value in the wild type, both tolerant lines were only slightly affected. Doubling the salt concentration to 200 m*M* completely blocked growth in wild-type cells, while the fresh weight increase was only reduced by 50% in both salt-resistant lines. The growth patterns of NaCl- and KCl-resistant lines were very similar (Fig. 1). The addition of 5% PEG in the culture medium resulted in a fresh weight increase for both the wild type and the resistant line (Fig. 2). The inhibitory effects of PEG were only noticeable at concentrations above 10%; under these conditions the RPEG-1 line grew significantly faster than the control line and maintained a 30% growth rate on 25% PEG while the wild type was completely inhibited.

Proline content. Wild-type callus cultures grown in the presence of a range of salt concentrations showed a par-

Table 1. Selection conditions applied for salt and PEG resistance in haploid protoplast cultures of *N. plumbaginifolia*

Selective agent	NaCl	KCl	PEG
Concentration used	200 m*M*	200 m*M*	25%
No. of protoplasts	10^6	10^6	10^6
No. of resistant clones	12	10	10
No. of confirmed resistant lines	1	1	3
Frequency of resistant clones	10^{-6}	10^{-6}	$3.3 \cdot 10^{-5}$

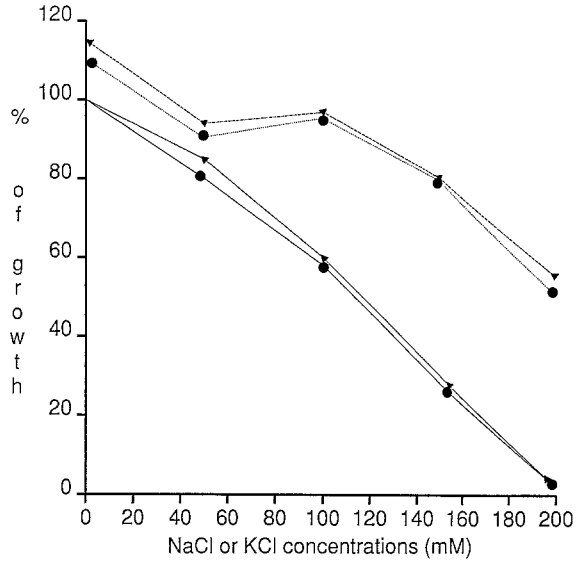

Fig. 1. Growth of salt-resistant lines and wild-type callus in the presence of increasing concentrations of NaCl or KCl. Mean fresh weights were determined after 4 weeks of culture on control and supplemented media. The values are means of five replicates (25 calli per plate). Wild type (—), RNaCl-1 (· · ·) RKCl-1 (– –) on NaCl (●) or KCl (▼)

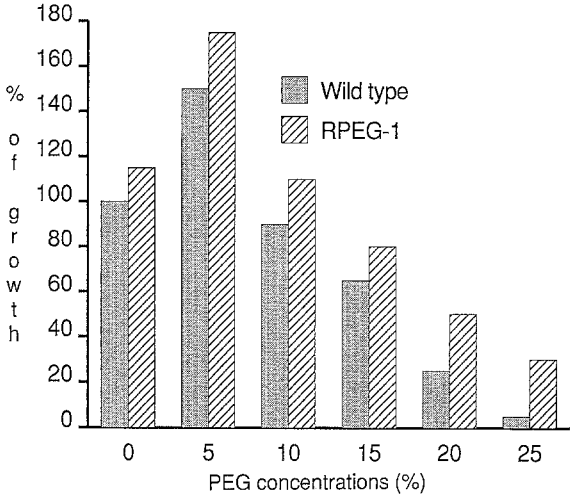

Fig. 2. Growth of PEG-resistant and wild-type callus in the presence of increasing concentrations of PEG. Mean fresh weights were determined after 4 weeks of culture on control and PEG-supplemented media. The values are means of five replicates (25 calli per plate)

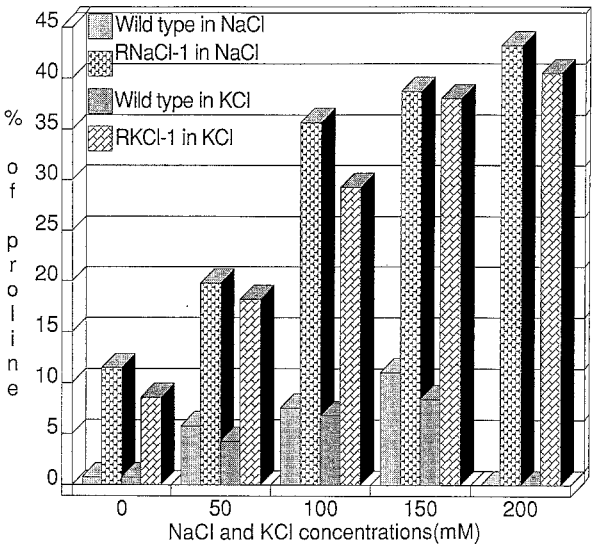

Fig. 3. Proline content as percentage of total free amino acids in callus from wild type, RNaCl-1 and RKCl-1. Callus were subcultured for 4 weeks in the presence of increasing salt concentration

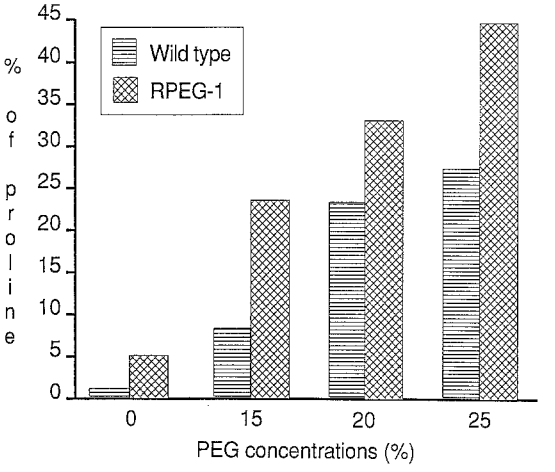

Fig. 4. Proline content as percentage of total free amino acids in callus from wild type and RPEG-1. Callus cultures were subcultured for 4 weeks in the presence of increasing PEG concentration

allel increase in their free proline content, which on 150 m*M* NaCl or KCl reached six- to eightfold the values determined on the non-saline medium (Fig. 3). The same response could be observed for the tolerant lines, RNaCl-1 and RKCl-1; however they accumulated about 10–15 times more free proline than the wild-type callus on the non-saline medium (Table 2). A further increase in proline accumulation was observed in response to salinized media: very high values (30–40% of the total free amino acids as proline) were reached in the presence of 150–200 m*M* salt. At 200 m*M* the inhibition of the wild-type cells was such that no overproduction of proline could be detected. Proline accumulation also increased in response to water stress due to the incorporation of increasing PEG concentrations in the culture medium (Fig. 4). This is again true for both the wild type and the RPEG-1 line. The resistant line is characterized by a marked increase in free proline content on a 0% PEG culture medium (5% instead of 0.8% for the wild type) (Table 2). Very high proline content values were obtained for the resistant line growing on 20–25% PEG (eightfold

Table 2. Free amino acid analysis of wild-type and resistant callus from the lines RNaCl-1, RKCl-1 and RPEG-1 grown on non-selective medium. Absolute values (in nmol g^{-1} fresh weight) and percentages

Amino acids	Wild type		RNaCl-1		RKCl-1		RPEG-1	
	nmol/g	(%)	nmol/g	(%)	nmol/g	(%)	nmol/g	(%)
Aspartate	956	10.5	676	7.2	709	7.7	1,236	13.0
Threonine	630	6.9	660	7.1	630	6.8	121	1.2
Serine	529	5.8	343	3.7	330	3.6	292	3.0
Glutamate	3,699	40.9	3,844	41.4	3,800	41.5	3,742	39.3
Proline	69	0.8	950	10.2	784	8.6	460	5.0
Glycine	158	1.8	70	0.7	72	0.7	197	2.0
Alanine	140	1.5	147	1.5	195	2.1	480	5.0
Cysteine	ND	ND	10	0.1	10	0.1	94	0.9
Valine	679	7.5	590	6.3	580	6.3	179	1.8
Methionine	ND	ND	10	0.1	9	0.1	9	0.1
Isoleucine	187	2.0	21	0.2	20	0.2	71	0.7
Leucine	316	3.5	94	1.0	140	1.0	58	0.6
Tyrosine	211	2.3	147	1.5	140	1.5	117	1.2
Phenylalanine	134	1.5	199	2.1	230	2.5	356	3.7
Histidine	1,057	11.7	1,094	11.8	1,050	11.5	978	10.2
Lysine	57	0.6	39	0.4	35	0.3	502	6.1
Arginine	196	2.1	375	4.0	410	4.4	489	5.1
Total	9,018		9,269		9,144		9,512	

ND, Not detected

Table 3. Free amino acid analysis of wild-type and F_2 progeny plants from the resistant lines RNaCl-1, RKCl-1 and RPEG-1. Leaves from greenhouse plants were used. Absolute values (in nmol g^{-1} fresh weight) and percentages

Amino acids	Wild type		RNaCl-1		RKCl-1		RPEG-1	
	nmol/g	(%)	nmol/g	(%)	nmol/g	(%)	nmol/g	(%)
Aspartate	1,157	14.7	1,198	9.8	923	6.7	819	5.6
Threonine	501	6.4	652	5.3	679	4.9	783	5.4
Serine	287	3.6	254	2.8	431	3.1	588	4.1
Glutamate	2,684	34.3	1,636	13.4	1,941	14.2	1,785	12.4
Proline	400	5.1	5,179	42.7	6,044	44.3	5,926	41.2
Glycine	560	7.1	652	5.3	725	5.3	860	5.9
Alanine	796	10.1	817	6.7	855	6.2	814	5.6
Cysteine	ND	ND	76	0.6	87	0.6	177	1.2
Valine	349	4.5	448	3.7	562	4.1	585	4.0
Methionine	ND	ND	28	0.2	13	0.1	14	0.1
Isoleucine	118	1.5	143	1.2	130	1.0	150	1.0
Leucine	143	1.8	35	0.3	60	0.4	185	1.2
Tyrosine	218	2.8	147	1.2	217	1.6	221	1.5
Phenylalanine	167	2.1	200	1.6	254	1.8	342	2.3
Histidine	215	2.7	277	2.2	314	2.3	614	4.2
Lysine	174	2.2	211	1.7	217	1.5	252	1.7
Arginine	32	0.4	135	1.1	155	1.1	151	1.0
Ornithine	16	0.2	28	0.2	30	0.2	110	0.8
Total	7,817		12,116		13,640		14,376	

ND, Not detected

the initial value on a 0% PEG medium). In contrast to the situation observed with salinized media, wild-type cells exposed to such PEG concentrations still contained high level of proline (more than 25% free proline in the total pool of amino acids) although they were severely affected in their growth rate.

Stability in the properties of the resistant lines

When the selected callus lines were transferred from the selective medium to a non-selective one for three successive subcultures of 4 weeks each and then put back on a medium with the original concentrations of NaCl, KCl,

Table 4. Fresh weight values of wild-type and resistant callus lines grown for 4 weeks on media containing 1.2% NaCl, 1.2% KCl and 25% PEG (6,000), respectively. Initial weights vary between 200 and 250 mg

Callus line	Final fresh weight (g)			
	Control medium	+1.2% NaCl	+1.2% KCl	+25% PEG
Wild type	2.75	0.22 (8.0)	0.21 (7.6)	0.22 (8.0)
RNaCl-1	3.82	2.25 (59.9)	2.15 (56.2)	2.16 (56.5)
RKCl-1	3.86	2.12 (54.9)	2.16 (55.9)	2.18 (56.4)
RPEG-1	3.95	2.29 (57.9)	2.19 (55.4)	2.21 (55.9)

Percentages for the corresponding control are given in brackets. The standard deviation for five replicates was less than 5%

Table 5. Segregation for salt and PEG resistance (R) and sensitivity (S) in progenies from back-crosses between salt-resistant mutants and the wild type and from F_2 in the case of PEG-resistant mutants

Mutants	Salt or PEG concentration	Number of plantlets		*P* values
		R	S	
RNaCl-1	NaCl 175 m*M*	95	100	1:1<0.05
RKCl-1	KCl 175 m*M*	100	93	1:1<0.05
RPEG-1	PEG 20%	150	45	3:1<0.05

R, resistance; S, sensitive

or PEG, they maintained an active growth comparable to the one observed originally (Table 4). Cross-resistance tests were also performed and the fresh weight values mentioned in Table 4 show that a resistant line originally selected against one stress factor is also resistant to the others. These tests were performed on callus induced on MAP_3 medium from leaves of resistant progeny plants obtained for each of the selected lines. The level of proline overproduction was also determined after three subcultures on a non-selective medium. Again, a high free proline content was observed in callus of RNaCl-1 (10% of the total amino acid content), RKCl-1 (8%) and RPEG-1 (4%) (Fig. 5). Similar values were also obtained in the leaves of resistant plants from progenies of crosses involving the three selected lines. Fully developed leaves from resistant plants growing in non-selective conditions in the greenhouse contained up to 8 times the free proline content of the wild type leaves (Table 3). Proline in leaves of RNaCl-1, RKCl-1 and RPEG-1 represent more than 40% of the total soluble amino acid pool. As shown in Table 3, the increase in the total free amino acids for the three resistant lines was due to this marked increase of proline production.

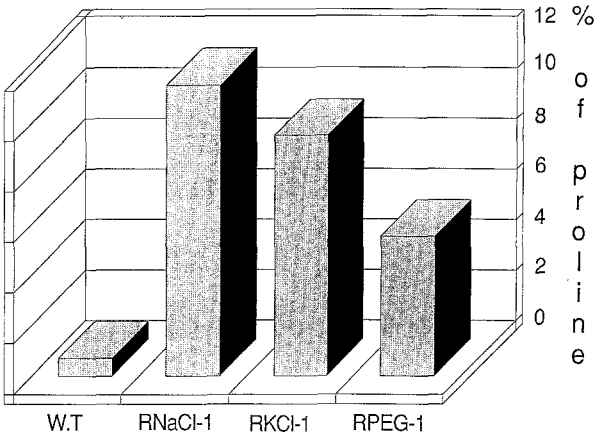

Fig. 5. Proline content as percentage of total free amino acids in callus tissue obtained from progeny plants of RNaCl-1, RKCl-1 and RPEG-1 and wild type, respectively. Callus was subcultured for 4 weeks on a non-selective medium

Fig. 6. Regenerated salt-resistant plant RNaCl-1 (*a*) compared to the wild type (*b*)

Genetic basis of salt- and water-stress resistance

The demonstration of a true mutation requires the crossing of plants regenerated from the respective selected cell lines and the subsequent analysis of their progenies. A few plants were regenerated from the selected calli, one from each type of salt-resistant line and three from PEG-tolerant lines. Spontaneous doubling of the haploid genome most probably occurred during the in vitro phase.

Fig. 7a–c. Segregation of tolerant versus sensitive plantlets in the progeny (BC2) of two successive back-crosses for RNaCl-1 (*a*) and RKCl-1 (*b*) and from F_2 for RPEG-1 (*c*). Seeds were sown on selective medium: RNaCl-1 or RKCl-1 on 175 m*M* NaCl or KCl, and RPEG-1 on 20% PEG (6000)

While self-pollination followed by seed set was successful for RPEG-1, seeds from the salt-resistant plants were only obtained in back-crosses due to the male sterility of both RNaCl-1 and RKCl-1. F_1 progenies were taller than both the original regenerants and the wild type (Fig. 6). The resistance to the corresponding selection agent was uniformly expressed in such F_1 plants growing in petri plates on Mn medium. For RNaCl-1 and RKCl-1, a second back-cross had to be performed to obtain BC_2 progeny seeds. For RPEG-1, F_2 seeds were produced by selfing of the hybrid F_1 plants. In order to determine a possible segregation for resistance, progeny seeds were sown on a Mn medium containing either salt (NaCl or KCl, 175 m*M*) or 20% PEG 6000. RNaCl-1 and RKCl-1 are both characterized by a 1 : 1 segregation of tolerant versus sensitive seedlings (Table 5, Fig. 7). Non-tolerant seedlings were identified 2 weeks after germination by the bleaching of the cotyledons. The observed ratio revealed that the dominantly expressed resistance segregates as a single Mendelian gene. Upon selfing of the regenerated PEG-resistant line all of the R_1 seeds produced were resistant to 20% PEG. F_2 seeds segregated in a 3 : 1 ratio, which indicates that the PEG-resistance trait is also controlled by a single dominant gene and that the RPEG-1 line behaves as a homozygote.

Discussion

In this paper we have characterized three types of mutants resistant to distinct selection agents, namely NaCl, KCl and PEG. The use of haploid protoplasts in combination with a well-defined mutagenic selection procedure appeared to be an appropriate protocol for obtaining stress-tolerant plants. Sublethal doses of the selection agents (LD > 90%) probably ruled out possible interference with any physiological adaptation by all cells or a fraction of them during the culture. Plants were also regenerated in the presence of the stressing agents in the medium. The few investigations where stable mutants have been obtained through in vitro selection made use of high levels of selection pressure (Nabors et al. 1980; Bressan et al. 1982). Though the biochemical mechanisms of stress tolerance in our mutants remain unknown, we found evidence that a mutation for increased cellular tolerance can also be expressed at the plant level and that at least part of the salt- and water-stress tolerance can be ascribed to changes occurring at the cellular level. While some plant species display a tolerance relying on anatomical and physiological features of the whole plant, others show a cellular mechanism of tolerance that also operates at the plant level (Smith and McComb 1981). The production of an osmoprotectant compound such as proline at high levels, is one of the adaptation responses of plants to salinity and water stress (Stewart and Lanker 1980; Le Rudulier et al. 1984). We observed an important proline overproduction in the three mutants, as well in the R_0 plant progenies and at callus level. The accumulation of free proline may reach 40% of the total free amino acids. The basic question remaining is whether the free proline could physiologically function as an osmoprotectant preventing damage to the cell (Dix and Pearce 1981). It is interesting to note that the mutants which accumulate 10- to 15-fold more proline than the wild type on saline medium are still capable of a further increase in proline concentration in response to salinized media (see Figs. 3 and 4). This can be explained by the existence of two mechanisms for producing proline, one being deregulated by the mutation and the other still responsive to stress conditions.

Cross-resistance of the three mutants to the selection agents may indicate similar mechanisms of tolerance to NaCl, KCl and PEG (Ben-Hayyim 1985). In each case it was possible to obtain fully developed regenerants, somewhat taller than the wild-type plants. However, the salt-resistant plants were male sterile, even after two successive back-crosses with the wild type. Though somaclonal variation may be involved, the high salt concentration of the medium may have an effect on the fertility of the plants. Salt and drought tolerance have been often as-

cribed to a complex, multigenic phenomenon (Nabors 1990). In our case, the genetic basis for salt and drought tolerance depends on a single dominant gene. The tolerance trait was analysed in F_2 and back-cross progenies by growth tests at the seedling level. Screening conditions were similar to those used in the cell selection system (200 m*M* NaCl or KCl and 25% PEG), and clear Mendelian segregation ratios between resistant and sensitive plants were recorded. In *Nicotiana tabacum* regenerated salt-tolerant plants were recovered, but the genetic basis of the tolerance remains unclear (Nabors et al. 1980). In the fern *Ceratopteris*, Warne and Hickok (1987) have described single gene mutants tolerant to NaCl by means of a selection protocol using the haploid sexual phase. Until now, stable, inheritable tolerance to PEG has not been reported.

The successful selection, characterization and genetic analysis of these salt- and water-stress mutants provide opportunities for further elucidation of salt and drought stress at the cellular and molecular levels. Studies are being performed to establish if the mutation alters the regulation of proline biosynthesis by decreasing the feedback inhibition exerted by the amino acid on γ-glutamyl kinase, the first enzyme of the pathway.

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