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Salt-tolerant mutants in glycophytic salinity response (GSR) genes in *Catharanthus roseus*

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Abstract The periwinkle *Catharanthus roseus* shares glycophytic properties of crop plants. To contribute towards an understanding of the glycophytic response to salinity, large populations of M_2 seeds having an origin in nitroso-methyl urea and ethyl methane sulphonate treatments were screened for germination with 250 mM of NaCl. Out of the nine mutant lines so recovered, which tolerated salt stress due to loss of the normal glycophytic salinity response (*GSR*), the characteristics of six *gsr* mutants are reported here. All six, *gsr-1* to *gsr-6*, differed from the wild-type in both seedling and adult-plant morphological characters beside being salt tolerant. The mutations in them were inherited as monogenic recessive alleles at the corresponding wild-type loci. The trans-complementation tests revealed that the *gsr-1* to *gsr-6* mutants specified six complementation groups. The mutant seedlings generally accumulated more proline and glycine betaine, constitutively, than the wild-type. The mutant plants transpired lower amounts of water and accumulated higher amounts of proline under drought stress. It was inferred that the products of the six *GSR* genes defined here are involved in the regulation of salt stress, as well as cell division, developmental and/or morphogenetic pathway(s), in *C. roseus*.

Keywords *Catharanthus roseus* · Salt-tolerant mutants · Glycophytic salinity response · Proline · Glycine-betaine · Recessive Mendelian inheritance

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Introduction

Plants demonstrate wide variation in their tolerance to salt, while the halophytes inhabiting the coastal areas can complete their life cycles under salinity, whereas the inland glycophytes, including the crop plants, are relatively sensitive to salt stress (Greenway and Munns 1980). The properties of the inordinately salt-sensitive and salt-tolerant mutants of glycophytic plants such as soybean, barley, tobacco, tomato and *Arabidopsis thaliana* (Kueh and Bright 1982; Saleki et al. 1993; Vazquez-Flota and Loyola-Vargas 1994; Fellner and Sawhney 2001) show that the deleterious effect of salt stress on seed germination and plant survival, growth and development results mainly from repression and induction of salt-responsive genes, especially those concerned with the protection of cells by the accumulation of proline, other osmolytes or of specific ions (Alia et al. 1988; Bray 1993; Werner and Finkelstein 1995; Liu and Zhu 1997; Yancey et al. 1982). Since the different glycophytic species would have evolved differentially, the salt tolerance mechanism must be studied in diverse plants to determine the minimum genetic complement necessary for improving salt tolerance in food and industrial crops. Here, we report on the pleiotropy of recessive salt-tolerant mutants affected in six genes in the tropical glycophyte periwinkle, *Catharanthus roseus*.

Materials and methods

Seeds of the variety Nirmal of *C. roseus* pre-soaked in water for 8 h were mutagenised with ethyl methane sulfonate (EMS, 0.002–0.2%, v/v) and N-nitroso-N-methyl urea (NMU, 0.0002–0.2%, w/v), and sown to obtain 5,850 M_1 plants to produce M_2 seeds. Approximately 1.08×10^6 M_2 seeds (about 1.04×10^6 and 0.44×10^5 from EMS and NMU treatment, respectively) and 3.25×10^3 control (wild-type) seeds were screened for germination in the presence of 250 mM of NaCl, the salt concentration that completely inhibited the germination of wild-type seeds (Pandey-Rai et al. 2001). Six of the nine mutants whose seedlings survived on 250-mM NaCl were included in this study.

The morphological and biochemical properties of the wild-type and mutants were compared at seedling and adult-plant stages as follows, in experiments replicated three times. The sample size per treatment per replication was ten seedlings or five adult plants. The design of experiments for the statistical analysis was after Cochran and Cox 1957 (Mishra et al. 2001). One week-old seedlings, germinated on filter-paper circles moistened with double-distilled water, were irrigated with 0.150 and 200- or 250-mM of NaCl for 7 days. The proline and betaine contents in the control and treated seedlings were assayed according to Bates et al. (1973) and Grieve and Gratton (1983), respectively. The proline contents of leaves were similarly estimated in 8-week-old plants irrigated with water or NaCl solution (150, 200 or 250 mM), every 24 h for 7 days. The sodium and potassium contents in the seedlings were determined using the method of Jackson (1973), and the digital flame photometer model CC22D Elico. of Pvt. Ltd. The seedlings and adult plants of the above experiments were grown at 25 ± 2 °C under about 2,200 lux of white fluorescent light using a 18-h light: 6-h dark cycle. To compare the effects of drought on wild-type and mutants, 9-month-old plants were irrigated to 80% soil moisture content. The pot and soil surfaces were tightly covered with a polythene sheet to avoid direct evaporation of soil water. The pots were placed in a greenhouse under natural-light conditions. The soil moisture and proline content of leaves were assayed at a 1-month interval until 3 months.

The vegetative and reproductive descriptors were recorded periodically on 36-week-old treated and untreated plants. To carry out genetic complementation tests, the M_3 and M_4 generation mutant plants and wild-type plants were reciprocally crossed in all possible combinations. The salt sensitivity test was conducted on 30 seeds. The F_1 seeds that germinated on 250 mM of NaCl and/or on water were transplanted to observe the descriptors. For inheritance studies a separate set of F_1 seeds was used to raise the F_2-generation selfed seeds. One set of F_2 seeds was used to ascertain germination over 250 mM of NaCl, and another set to obtain adult plants for morphological description. Seeds formed on F_2 plants were employed to study the behavior of F_3 progenies.

Results

Heritability of distinguishing features of the salt-tolerant mutants

The six mutants possessing reduced sensitivity to NaCl used in this study demonstrated pleiotropic phenotypes distinguishable from each other and that of the wild-type (Table 1). The mutants have been called *gsr-1* to *gsr-6*, where *gsr* refers to absence of the normal glycophytic salinity response. The mutants have now been maintained by selfing for six generations. The phenotypes, the ability of seeds to produce seedlings after sowing on 250-mM NaCl medium, and characteristic seedling and adult plant morphologies, were displayed by the progenies of the six *gsr* mutants over all the generations with nearly complete penetrance.

The seedlings of the *gsr-1* to *gsr-4* mutants were smaller in size than those of *GSR*, due to the shorter hypocotyls. The *gsr-2* mutant bore only one cotyledon, in contrast to dicotyledony in the seedlings of GSR and other *gsr* mutants. The *gsr-3* and *gsr-4* seedlings bore long cotyledons. The roots of *gsr-1* and *gsr-2* seedlings were of small size. Branched roots were formed on *gsr-5* seedlings. The cotyledons had a dark green colour in *gsr-1*, *gsr-3* and *gsr-6* seedlings, and a yellowish-green colour in *gsr-2* and *gsr-4* seedlings. The adult plants of *gsr-1*, *gsr-3* and *gsr-4* were short statured. Branching was profuse in *gsr-1*. The petioles of leaves were of small size in *gsr-1*, *gsr-2* and *gsr-4*. The plants bore leaves with a smaller lamina in *gsr-2*, *gsr-3*, *gsr4* and *gsr-5*. The leaf lamina was oblong in *GSR*, obovate in *gsr-1*, elliptic in *gsr-2*, *gsr-5* and *gsr-6*, and lanceolate in *gsr-3* and *gsr-4*. The leaves of *gsr-3*, *gsr-4* and *gsr-5* were yellowish. The flower corolla tube was of small size in *gsr-1*, *gsr-3*, *gsr-4* and very long in *gsr-6*. The pods were of small size and fewer seeded in *gsr-2*, *gsr-3* and *gsr-4*, and was of larger size in *gsr-6* as compared to *GSR*.

Complementation and inheritance-pattern analyses

The seedling and adult plant phenotypes of F_1 progeny from crosses between wild-type and *gsr* mutants in all combinations are given in Table 2. The observations showed that the seedling salt tolerance and plant morphology were inherited as recessive traits. The F_1 seeds obtained from the reciprocal crosses between the six mutants failed to germinate on 250 mM of NaCl medium. The adult F_1 plants from these crosses had normal morphology of the parent cv Nirmal. Contrastingly, the inbred seeds and plants of mutants demonstrated their typical characteristics. These results of the complementation analysis confirmed that different genes had mutated in the *gsr-1* to *gsr-6* mutants.

The observations on the F_2 progenies of the crosses summarized in Table 3 showed that the segregation behavior of salt tolerance and the altered morphology of the *gsr-1* to *gsr-6* mutants fitted the Mendelian monogenic recessive transmission pattern. The behaviour of F_3 progenies from *gsr* × *GSR* crosses confirmed these observations (Table 4).

Relative sensitivity of *gsr* mutants to salt stress

The relative differences in the sensitivity of wild-type and mutants to salt stress were determined by sowing their seeds on media containing 0-, 150- and 200-mM NaCl. While the mutant seeds germinated on all the media, the wild-type seeds did not germinate on 200-mM NaCl. The sensitivities of the wild-type and mutants were compared in terms of six seedling parameters (Table 5). On the whole, the exposure of mutant seeds to NaCl increased the time period taken for germination by about 20%, and decreased hypocotyl and root sizes by about 40 and 50%, respectively, and fresh and dry weight of seedlings by about 45 and 30%, respectively. Mutants differed in their germination response to different concentrations of NaCl; *gsr-4* had the slowest germination. As compared to others, the hypocotyls were of larger size in *gsr-4* and *gsr-6*; the roots were of small size in *gsr-1*, *gsr-2*, *gsr-4* and *gsr-5*, of intermediate size in *gsr-6* and of large size in *gsr-3*. The mutants fell into the following order in terms of the dry weight of their seedlings: *gsr-1*<*gsr-2* and *gsr-4*<*gsr-3*, *gsr-5* and *gsr-6*.

Table 1 Morphological features of wild-type and salt-tolerant mutants of *C. roseus*

Relevent strain/ genotype[a]	Important features of the plant at the		Origin/ reference
	Seedling stage	Adult flowering stage	
GSR	Large thin hypocotyl; cotyledons two, green in colour (137B)[b], obovate in shape; root long, unbranched	Tall; stem branched, colour green (137B), texture pubescent, late branching; leaf petiole long, lamina-large, oblong in shape, colour dark green (137A), texture pubescent, number more, apex mucronate, base acute; flower corolla tube long; pods slightly curved, large, many seeds per pod	Kulkarni et al. 1999
gsr-1	Small thick hypocotyl; cotyledons two, very dark green (139B), oval in shape; root very small, unbranched	Dwarf; stem hyper-branched, colour very dark green (139B), texture pubescent, early branching; leaf petiole size very small, lamina large, obovate in shape, very dark green (139A), texture pubescent, number less, apex cuspidate, base obtuse; flower corolla tube small; pods highly curved, large, many seeds per pod	Ethyl methane sulfonate (EMS) mutagenesis
gsr-2	Small thin hypocotyl; cotyledon one, yellowish green (147B), obovate in shape, slightly curved; root small, unbranched	Tall; stem unbranched, colour green (137B), texture pubescent, late branching; leaf petiole size small, lamina small-sized, elliptic in shape, yellowish green (147A), texture pubescent, number less, apex acute, base acute; flower corolla tube long; pods slightly curved, small; several seeds per pod	EMS mutagenesis
gsr-3	Small thick hypocotyl; cotyledons two, dark green (137A), ob-lanceolate in shape; root long, unbranched	Semidwarf; stem unbranched, colour yellowish green (147B), texture pubescent, late branching; leaf petiole long, lamina small-sized, lanceolate in shape, yellowish green (147A), texture pubescent, number more, apex acute, base attenuate; flower corolla tube small; pods very small, unequal; several seeds per pod	EMS mutagenesis
gsr-4	Small thin hypocotyl; cotyledons two, yellowish green (147A), lanceolate in shape; root long, unbranched	Semidwarf; stem branched, colour yellowish green (147B), texture glaucous, late branching; leaf petiole small, lamina small-sized, lanceolate in shape, yellowish green (147B), texture glaucous, number more, apex acute, base attenuate; flower corolla tube small; pods slightly curved, very small; several seeds per pod	EMS mutagenesis
gsr-5	Large thin hypocotyl; cotyledons two, green (137B), obovate in shape; root very long, multibranched	Tall; stem multibranched, colour yellowish green (147B) texture pubescent, late branching; leaf petiole long, lamina small-sized, elliptic in shape, yellowish green (146A), texture pubescent, number more, apex mucronate, base acute; flower corolla tube long; pods slightly curved, large; many seeds per pod	EMS mutagenesis
gsr-6	Large thin hypocotyl; cotyledons two, dark green (137A), elliptic in shape; root long, unbranched	Tall; stem branched, colour very dark green (139A), texture glaucous, late branching; leaf petiole long, lamina large, elliptic in shape, colour dark green (139B), texture glaucous, number more, apex mucronate, base acute; flower corolla tube very long; pods slightly curved, very large, many seeds per pod	NMU

[a] = Each of the genotypes is homozygous for the concerned allele
[b] = Given in parenthesis are the colour codes of the Royal Horticulture Society: 137A, 137B, 139A, 139B = green group; 146A,147A,147B = yellow-green group

Table 2 Phenotypes of F_1 seedlings and adult plants obtained from reciprocal crosses between wild-type (*GSR*) and salt-resistant mutants (*gsr*) in the periwinkle *C. roseus*[a]

Genetic stock	*GSR*	*gsr-1*	*gsr-2*	*gsr-3*	*gsr-4*	*gsr-5*	*gsr-6*
GSR	+S[b] +M[c]	+S +M	+S +M	+S +M	+S +M	+S +M	+S +M
gsr-1	+S +M	–S[d] –M[e]	+S +M	+S +M	+S +M	+S +M	+S +M
gsr-2	+S +M	+S +M	–S –M	+S +M	+S +M	+S +M	+S +M
gsr-3	+S +M	+S +M	+S +M	–S –M	+S +M	+S +M	+S +M
gsr-4	+S +M	+S +M	+S +M	+S +M	–S –M	+S +M	+S +M
gsr-5	+S +M	+S +M	+S +M	+S +M	+S +M	–S –M	+S +M
gsr-6	+S +M	+S +M	+S +M	+S +M	+S +M	+S +M	–S –M

[a] ≥100 seedlings and ≥25 adult plants were studied for each test
[b] +S = salt sensitivity in F_1 seedlings
[c] +M = wild-type adult F_1 plant morphology
[d] –S = salt tolerance in F_1 seedlings
[e] –M = mutant morphology in adult F_1 plants; +M and –M morphologies were typical

Table 3 Morphology and/or response to salt stress (250 mM of NaCl) in the parent and F_2 generation progeny seedlings and adult plants. The plants obtained by their transplanting had a respective mutant morphology

Parents (P) and filial generations of crosses (♀×♂)	No. of seeds tested	Number of seeds that demonstrated		χ^2 test of plants (3:1)	Number studied at 9-months of age	Morphologically		χ^2 test (3:1)
		Wild-type Salt sensitive phenotype	Mutant type Salt resistant phenotype[a]			Wild-type phenotype	Mutant phenotype	
gsr^+ gsr^+ (Gsr^+)	72	72	0					
gsr-1$^-$gsr-1$^-$ (Gsr-1$^-$)	69	0	69					
gsr-2$^-$gsr-2$^-$ (Gsr-2$^-$)	68	0	68					
gsr-3$^-$gsr-3$^-$ (Gsr-3$^-$)	70	0	70					
gsr-4$^-$gsr-4$^-$ (Gsr-4$^-$)	61	0	61					
gsr-5$^-$gsr-5$^-$ (Gsr-5$^-$)	62	0	62					
gsr-6$^-$gsr-6$^-$ (Gsr-6$^-$)	66	0	66					
Gsr-1$^-$ × Gsr^+ (F_2)	565	425	140	0.01	66	49	17	0.02
Gsr^+ × Gsr-1$^-$ (F_2)	100	79	21	0.85	108	78	30	0.4
Gsr-2$^-$ × Gsr^+ (F_2)	793	586	207	0.52	206	154	52	0.01
Gsr^+ × Gsr-2$^-$ (F_2)	665	425	140	0.02	106	82	24	0.32
Gsr-3$^-$ × Gsr^+ (F_2)	267	212	55	2.7	52	41	11	0.4
Gsr^+ × Gsr-3$^-$ (F_2)	79	60	19	0.03	222	169	53	0.2
Gsr-4$^-$ × Gsr^+ (F_2)	220	168	52	0.2	100	75	25	0
Gsr^+ × Gsr-4$^-$ (F_2)	99	70	29	0.97	98	75	23	0.12
Gsr-5$^-$ × Gsr^+ (F_2)	90	69	21	0.13	50	39	11	0.24
Gsr^+ × Gsr-5$^-$ (F_2)	92	70	22	0.05	105	78	27	0.03
Gsr-6$^-$ × Gsr^+ (F_2)	402	301	101	0.003	108	83	25	0.2
Gsr^+ × Gsr-6$^-$ (F_2)	480	359	121	0.01	67	49	18	0.12

Table 4 Stability of *gsr* mutant phenotypes in F_3 progenies arising from *gsr* × *GSR* crosses

Cross	Behaviour of F_3 progeny seedlings raised from F_2 plants processing:						
	Wild-type morphology				Mutant morphology		
	Number of progenies screened	Number of progenies that gave:			Number of progenies screened	Number of progenies that gave:	
		Only salt-sensitive seedlings of wild-type morphology[a]	Both salt-sensitive wild-type morphology bearing[a] + salt-tolerant typical mutant morphology bearing seedlings[bc]	Seedlings bearing any new morphologies		Salt-tolerant seedlings bearing typical mutant morphology[bc]	Seedlings bearing any new morphologies
gsr-1 × *GSR*	100	21	56+23	0	47	47	0
gsr-2 × *GSR*	230	49	130+51	0	76	76	0
gsr-3 × *GSR*	210	56	102+52	0	63	63	0
gsr-4 × *GSR*	130	29	71+30	0	40	40	0
gsr-5 × *GSR*	115	23	67+25	0	35	35	0
gsr-6 × *GSR*	130	33	68+29	0	40	40	0

[a] Germinated on 150-mM NaCl and died on transfer to 250-mM NaCl medium

[b] Germinated on 150-mM NaCl and survived on transfer to 250-mM NaCl medium

[c] Seedlings were transplanted and resulting plants were observed. At the flowering stage no new morphologies were apparent

Among the six mutants, the time taken for the seeds to germinate into seedlings possessing fully open cotyledons was about 1.2 to 1.8-fold higher on 200 mM NaCl medium than on distilled water, the maximum difference being in *gsr-1*. There was about a 1.1 to 1.6-fold reduction in the hypocotyl length and dry weight of seedlings, a 1.3 to 3.3-fold reduction in root length and a 1.3 to 2.1-fold reduction in the fresh weight of seedlings by exposure to 200-mM NaCl stress in the seedlings of the different mutants. The most affected were gsr-6 in hypocotyl length and fresh seedling weight, gsr-5 in root length and gsr-1 in the dry weight of seedlings. Taking all the parameters into consideration, the mutants could be arranged in the following decreasing order of sensitivity

Table 5 Effect of the NaCl stress on the seedling growth parameters in salt-resistant mutants and the salt-sensitive wild-type in the periwinkle *C. roseus*

Relevent genotype[a]/statistic	Concentration of NaCl (mM)	Germination span time (days)[b]	Hypocotyl length (mm)	Root length (mm)	Fresh weight of ten seedlings (mg)	Dry weight of ten seedlings (mg)
GSR	0	3.3 ± 0.2	21.2 ± 0.3	18.0 ± 0.1	127.7 ± 1.5	6.7 ± 1.2
	150	4.3 ± 0.4	16.1 ± 0.1	10.2 ± 0.2	75.3 ± 1.2	5.6 ± 1.0
	200	–[c]	–	–	–	–
gsr-1	0	3.0 ± 0.1[d]	13.0 ± 0.3	12.1 ± 0.1	87.7 ± 1.2	6.3 ± 0.5
	150	3.5 ± 0.2	12.1 ± 0.1	11.3 ± 0.2	69.7 ± 1.1	5.7 ± 0.3
	200	5.3 ± 0.1	11.2 ± 0.2	9.20 ± 0.3	45.0 ± 0.9	4.0 ± 0.4
gsr-2	0	3.5 ± 0.3	16.3 ± 0.1	13.0 ± 0.1	77.0 ± 1.0	7.0 ± 1.0
	150	4.3 ± 0.4	14.2 ± 0.2	11.2 ± 0.3	61.0 ± 0.8	7.0 ± 1.1
	200	4.8 ± 0.1	14.1 ± 0.1	6.10 ± 0.1	46.0 ± 1.5	6.3 ± 1.5
gsr-3	0	3.3 ± 0.1	16.2 ± 0.1	29.0 ± 0.2	115.7 ± 1.5	8.7 ± 1.0
	150	3.8 ± 0.1	13.3 ± 0.2	21.1 ± 0.3	97.0 ± 1.0	7.7 ± 0.9
	200	4.0 ± 0.2	13.4 ± 0.1	21.2 ± 0.4	75.1 ± 0.5	6.7 ± 0.5
gsr-4	0	4.8 ± 0.1	15.1 ± 0.1	12.1 ± 0.1	120.0 ± 1.5	7.0 ± 1.5
	150	6.3 ± 0.1	13.2 ± 0.2	11.0 ± 0.3	95.0 ± 2.0	6.0 ± 1.2
	200	6.3 ± 0.2	13.4 ± 0.1	8.20 ± 0.1	94.0 ± 1.1	5.7 ± 1.0
gsr-5	0	4.3 ± 0.1	14.0 ± 0.1	13.0 ± 0.3	121.0 ± 1.0	8.0 ± 1.1
	150	5.5 ± 0.1	13.3 ± 0.3	11.2 ± 0.1	54.0 ± 1.5	6.7 ± 1.2
	200	5.8 ± 0.1	12.2 ± 0.4	4.40 ± 0.3	93.0 ± 1.9	6.6 ± 1.1
gsr-6	0	4.0 ± 0.1	26.0 ± 0.1	18.0 ± 0.1	144.0 ± 1.0	9.0 ± 1.0
	150	4.3 ± 0.2	18.1 ± 0.2	11.5 ± 0.2	102.0 ± 0.8	7.7 ± 1.5
	200	4.8 ± 0.1	16.2 ± 0.3	11.6 ± 0.2	69.0 ± 0.9	6.3 ± 1.4
Mean	0	3.8 ± 0.3	17.8 ± 0.2	16.4 ± 0.2	113.2 ± 8.8	7.6 ± 0.4
	150	4.6 ± 0.4	14.1 ± 0.1	12.5 ± 0.1	87.7 ± 5.9	6.7 ± 0.3
	200	4.5 ± 0.8	11.1 ± 0.2	8.40 ± 0.3	60.3 ± 10.9	5.1 ± 0.9
Grand mean		4.3 ± 0.3	14.3 ± 0.2	12.3 ± 0.2	86.9 ± 15.1	6.5 ± 0.8
Critical difference (CD) at 99% level of confidence to compare:						
(1) Genotypic effects		1.1	3	2	1.9	0.8
(2) NaCl concentration effects		0.5	1	1	1.3	0.6
(3) Genotypic effects at a NaCl concentration		1.4	3	2	3.5	1.7
(4) NaCl concentration effects in a genotype		1.6	4	2	3.9	1.6

[a] Genotypes are homozygous for the respective allele
[b] Opening of cotyledons was taken as a sign of germination
[c] Nil germination in 14 days
[d] Each observation in the tables gives the average measurement in a treatment ±SE

towards 200-mM NaCl stress: *gsr-4* > *gsr-5* > *gsr-3* > *gsr-2* > *gsr-1* > *gsr-6*.

Differential sodium uptake and potassium retention in seedlings under salt stress

It will be seen from the Table 6 that seedlings of the wild-type and mutants grown on distilled water for about 2 weeks had a comparable Na^+ content of about 0.2% on a dry weight basis. In the similarly treated seedlings the K^+ content of the wild-type and *gsr-1*, *gsr-3*, *gsr-4* and gsr-6 was about 1.0%. The K^+ content of the seedlings of *gsr-2* and *gsr-5* was however about 25% higher. Exposure to NaCl increased the Na^+ content and decreased the K^+ content of seedlings. The average increase of Na^+ over all the genotypes was 2- and 3.7-fold in seedlings grown over 150- and 250-mM NaCl, respectively, as compared to those grown in distilled water. The presence of 150- and 250-mM NaCl decreased the K^+ content of seedlings by 1.3- and 1.7-fold respectively. The Na^+ content of the wild-type seedlings increased about 2.7-fold and that of mutant seedlings 3.6 to 4.5-fold upon exposure to 250 mM of NaCl. The NaCl stress resulted in the accumulation of 1.3 to 1.7-fold higher amounts of Na^+ in the mutant seedlings as compared to the wild-type seedlings. Na^+ accumulation was highest in the seedlings of the *gsr-2*, *gsr-4* and *gsr-5* mutants. Interestingly the K^+ content of the wild-type and mutants was similarly lower by about 1.7-fold under the NaCl rich incubation conditions. Again, K^+ reduction was more pronounced in the seedlings of *gsr-2*, *gsr-4* and *gsr-5* mutants.

Proline and glycine–betaine accumulation

The observations summarized in Tables 7 and 8 showed that the mutant seedlings and adult plants accumulated more proline, on average about 1.7-times more proline, than the wild-type when grown without exogenous NaCl. The proline accumulation in *gsr-2* was twice that in the wild-type, under these conditions. The seedlings of all

Table 6 Sodium (Na^+) and potassium (K^+) contents in the seedlings of wild-type (GSR) and glycophytic-response minus salt-tolerant mutants (*gsr*) of the periwinkle *C. roseus* germinated under normal and NaCl stress conditions

Relevent genotype/ statistic	% Na^+ in dried germinated seedlings that had been grown on medium containing:				% K^+ in dried germinated seedlings that had been grown on medium containing:			
	No NaCl	150 mM NaCl	250 mM NaCl	Mean	No NaCl	150 mM NaCl	250 mM NaCl	Mean
GSR	0.19 ± 0.01	0.36 ± 0.05	0.53 ± 0.04	0.36	1.04 ± 0.10	0.82 ± 0.20	0.61 ± 0.10	0.82
gsr-1	0.20 ± 0.02	0.43 ± 0.01	0.75 ± 0.01	0.46	0.99 ± 0.20	0.84 ± 0.10	0.69 ± 0.09	0.84
gsr-2	0.18 ± 0.01	0.48 ± 0.02	0.81 ± 0.02	0.49	1.25 ± 0.10	0.96 ± 0.20	0.59 ± 0.08	0.93
gsr-3	0.19 ± 0.03	0.39 ± 0.01	0.68 ± 0.10	0.42	0.99 ± 0.40	0.85 ± 0.04	0.61 ± 0.02	0.81
gsr-4	0.21 ± 0.01	0.39 ± 0.04	0.88 ± 0.11	0.49	0.99 ± 0.30	0.91 ± 0.01	0.67 ± 0.04	0.85
gsr-5	0.20 ± 0.02	0.44 ± 0.08	0.87 ± 0.01	0.51	1.31 ± 0.11	0.88 ± 0.02	0.74 ± 0.05	0.97
gsr-6	0.19 ± 0.04	0.43 ± 0.09	0.72 ± 0.12	0.45	1.04 ± 0.40	0.75 ± 0.04	0.59 ± 0.04	0.79
Mean	0.20 ± 0.03	0.42 ± 0.02	0.75 ± 0.05	0.45	1.09 ± 0.05	0.86 ± 0.10	0.64 ± 0.02	0.86
Critical difference at the 99% level of confidence to compare:								
(1) genotypes: 0.10					0.14			
(2) NaCl treatments: 0.05					0.11			
(3) Genotypes at a NaCl concentration: 0.09					0.19			
(4) Effect of NaCl concentrations in a genotype: 0. 13					0.21			

Table 7 Content of free proline in 2-week-old seedlings of the wild-type (*GSR*) and salt-tolerant mutants (*gsr*) of the periwinkle *C. roseus* grown in the presence and absence of NaCl. CD at 99% level of confidence to compare: genotypes ≤0.1, NaCl treatments ≤0.1, genotype at a NaCl concentration ≤0.2, NaCl treatments in a genotype ≤0.2

Genotype	Proline content (μM/g fresh weight) in seedlings grown in the presence or absence of NaCl			
	0 mM NaCl	150 mM NaCl	200 mM NaCl	Mean
GSR	1.1 ± 0.1	2.0 ± 0.3	2.4 ± 0.5	1.8 ± 0.4
gsr-1	1.9 ± 0.4	2.2 ± 0.1	2.3 ± 0.9	2.1 ± 0.1
gsr-2	2.2 ± 0.1	3.2 ± 0.2	3.8 ± 0.4	3.1 ± 0.5
gsr-3	1.5 ± 0.3	1.8 ± 0.2	3.1 ± 0.2	2.1 ± 0.5
gsr-4	1.4 ± 0.2	1.8 ± 0.2	3.0 ± 0.3	2.1 ± 0.4
gsr-5	1.3 ± 0.9	1.8 ± 0.5	3.0 ± 0.1	2.0 ± 0.8
gsr-6	1.6 ± 0.2	2.6 ± 0.1	3.5 ± 0.3	2.6 ± 0.5
Mean	1.6 ± 0.1	2.2 ± 0.2	3.0 ± 0.2	2.3 ± 0.4

Table 8 Content of free proline in 8-week-old plants of the wild-type (*GSR*) and salt-tolerant mutants (*gsr*) of the periwinkle *C. roseus* grown in the presence and absence of NaCl. CD at 95% and 99% levels of confidence to compare: genotypes = 1.4, 2.3, NaCl effects = 2.7, 3.6, genotype at a NaCl concentration = 4.7, 6.2, NaCl treatments in a genotype = 4.6, 6.2

Genotype	Proline content (μM/g fresh weight) in plants grown with and without NaCl			
	0 mM NaCl	150 mM NaCl	200 mM NaCl	Mean
GSR	1.5 ± 0.5	2.7 ± 0.4	3.8 ± 0.2	2.7 ± 0.7
gsr-1	2.6 ± 0.7	2.8 ± 0.5	4.8 ± 0.4	3.4 ± 0.7
gsr-2	3.1 ± 0.1	3.7 ± 0.3	5.7 ± 0.3	4.2 ± 0.8
gsr-3	2.0 ± 0.8	4.0 ± 0.1	5.1 ± 0.2	3.7 ± 0.9
gsr-4	2.6 ± 0.1	4.1 ± 0.1	5.2 ± 0.1	4.0 ± 0.4
gsr-5	2.1 ± 0.1	3.2 ± 0.2	4.5 ± 0.3	3.3 ± 0.6
gsr-6	2.3 ± 0.1	4.8 ± 0.3	5.4 ± 0.4	4.2 ± 0.8
Mean	2.3 ± 0.2	2.2 ± 0.3	5.0 ± 0.2	2.3 ± 0.4

Table 9 Glycine-betaine content in 2-week-old seedlings of the wild-type (*GSR*) and salt-tolerant (*gsr*) mutants of the periwinkle *C. roseus* grown in the presence and absence of NaCl. CD at 95 and 99% levels of confidence to compare: genotypes = 1.4, 2.3, NaCl effects = 2.7, 3.6, genotype at a NaCl concentration = 4.7, 6.2, NaCl treatments in a genotype = 4.6, 6.2

Genotype	Glycine-betaine content (μM/g dry weight) in seedlings grown with and without NaCl			
	0 mM NaCl	150 mM NaCl	200 mM NaCl	Mean
GSR	17.8 ± 1.1	23.6 ± 1.5	35.5 ± 0.2	25.6 ± 5.2
gsr-1	23.0 ± 0.9	33.7 ± 0.7	38.3 ± 0.4	31.7 ± 4.5
gsr-2	29.8 ± 0.5	40.3 ± 0.4	51.2 ± 0.3	40.4 ± 6.2
gsr-3	25.0 ± 0.3	34.2 ± 0.4	40.8 ± 0.1	33.3 ± 4.6
gsr-4	27.8 ± 0.1	31.0 ± 0.3	44.4 ± 0.2	34.4 ± 5.1
gsr-5	25.9 ± 0.3	35.0 ± 0.9	40.3 ± 1.1	33.8 ± 4.2
gsr-6	23.7 ± 0.4	40.8 ± 0.5	51.2 ± 0.3	38.6 ± 8.0
Mean	24.7 ± 1.5	34.1 ± 2.2	43.1 ± 2.3	34.0 ± 0.4

Table 10 Amount of water taken up after last irrigation by wild-type (*GSR*) and salt-tolerant (*gsr*) mutants of the periwinkle *C. roseus*

Genotype	Percent water taken up by adult plant from soil irrigated with a measured amount of water		
	4-weeks	8-weeks	12-weeks
GSR	31.3 ± 4.1	73.0 ± 4.2	80.0 ± 4.2
gsr-1	11.0 ± 0.3	31.1 ± 0.4	60.5 ± 1.2
gsr-2	18.7 ± 0.4	48.1 ± 3.1	74.5 ± 1.0
gsr-3	23.0 ± 1.5	63.2 ± 2.1	78.0 ± 1.6
gsr-4	14.9 ± 0.8	58.1 ± 0.2	75.2 ± 1.2
gsr-5	14.1 ± 1.2	60.5 ± 0.3	76.3 ± 1.1
gsr-6	26.2 ± 1.1	60.9 ± 1.6	75.4 ± 1.2
Mean	19.9 ± 2.8	56.4 ± 3.4	74.3 ± 3.9
CD[a]	2.3	5.6	10.8

[a] = CD, critical difference to compare genotype with a 99% level of confidence

Table 11 Free proline content in water stressed plants of wild-type (*GSR*) and salt-tolerant mutants (*gsr*) of the periwinkle *C. roseus*

Genotype	Proline content in adult plant (μM/g fresh weight) at different times after withdrawal of irrigation			
	4-weeks	8-weeks	12-weeks	Mean
GSR	21.6 ± 3.1	35.2 ± 1.0	32.4 ± 0.5	29.7 ± 10.3
gsr-1	28.4 ± 0.5	38.3 ± 1.2	40.5 ± 0.1	35.7 ± 3.7
gsr-2	37.0 ± 1.0	44.0 ± 1.6	44.5 ± 1.2	41.8 ± 2.4
gsr-3	22.7 ± 1.3	36.1 ± 2.0	42.1 ± 0.2	33.6 ± 7.6
gsr-4	26.9 ± 0.9	35.1 ± 1.8	38.3 ± 0.3	33.4 ± 3.4
gsr-5	24.9 ± 1.4	35.3 ± 1.2	38.3 ± 0.3	32.8 ± 4.1
gsr-6	31.2 ± 0.2	37.1 ± 1.0	38.9 ± 0.2	35.7 ± 2.3
Mean	27.5 ± 2.0	37.3 ± 1.2	39.1 ± 5.9	34.7 ± 0.5
CD[a]	2.5	6.8	2.6	

[a] = CD, critical difference to compare genotypes with a 99% level of confidence

the genotypes accumulated more proline when exposed to NaCl stress. In the wild-type and all the mutants except *gsr-1*, the proline concentration in seedlings germinated in the presence of 200-mM NaCl was 1.7 to 2.3-fold higher than that in the absence of NaCl supplementation. Exposure to NaCl increased proline concentration only by a factor of 1.2 in the *gsr-1* seedlings. The levels of proline in the adult plants of the wild-type and individual mutants, tested with and without NaCl exposure, were similar. Irrespective of NaCl treatments, *gsr-2* and *gsr-6* plants accumulated a relatively higher level of proline.

It will be further seen from Table 9 that the mutant seedlings accumulated about 1.4-fold more glycine-betaine than the wild-type seedlings, without NaCl stress. All genotypes accumulated more glycine-betaine under NaCl stress than without NaCl stress. Under 200 mM NaCl, the *gsr-2*, *gsr-4* and *gsr-6* seedlings accumulated more glycine-betaine than the seedlings of the other genotypes. Whereas the level of proline accumulation under 200 mM NaCl versus 0 mM NaCl was similarly increased in *Gsr* and *gsr-6*, the inducible accumulation of proline was lower in other genotypes.

Improved toleration of drought stress by *gsr* mutants

It will be seen from the results summarized in Table 10 that the mutant plants evaporated 1.3 to 2.4-fold less water than the wild-type by the end of 2 months from the withdrawal of irrigation. By the end of 12 weeks of such imposed drought conditions, the plants of *gsr-1* had evaporated about 25% less water than plants of other genotypes. The mutants could be arranged in the following order in terms of their drought tolerance: *gsr-1* > *gsr-2* > *gsr-3* to *gsr-6* > wild-type (*GSR*). The drought-affected plants of the *gsr-2* mutant accumulated much more proline than the similarly affected plants of the other genotypes (Table 11).

Discussion

The above described properties of six monogenic recessive mutants of *C. roseus* in this angiospermic species have revealed the identity of corresponding complementation groups of genes whose products/functions are involved in the suppression of innate tolerance to saline conditions and in morphogenetic organ development. Some of the concerned characteristics of the mutants isolated and examined here are discussed below in terms of the information on response to NaCl stress gathered from studies on other plant species.

Available evidence suggests that the degree to which NaCl stress is tolerated in glycophytic plant species is determined by the coordinated expression of the genes that are responsible for the various protective homeostatic mechanisms, and of those that lower the efficiency

and effectiveness of the salt tolerance-giving mechanisms. This idea that salt tolerance is a quantitative trait is based on the heritable ecotypic differences in salt tolerance between land races of several weeds and crops (Zhu 2000), and on the recovery of both salt-sensitive and salt-tolerant mutants in certain plants where salt tolerance has been genetically analyzed (Werner and Finkelstein 1995; Zhu et al. 1998; Tsugane et al. 1999).

Mutants in five genes whose wild-type alleles are necessary for salt tolerance have been identified in *A. thaliana* (Wu et al. 1996; Liu and Zhu 1997; Liu et al. 2000). Additionally, single gene mutants that confer enhanced salt tolerance are known in a fern (Warne and Hickok 1987), *A. thaliana* (Saleki et al. 1993; Werner and Finkelstein 1995; Tsugane et al. 1999), soybean (Abel 1969) and barley (Kueh and Bright 1982). Nine genes based on salt-tolerant properties in their recessive mutants have been defined in *A. thaliana* (Werner and Finkelstein 1995; Zhu et al. 1998; Quesada et al. 2000). Based on the properties of these mutants, it is possible to suggest that the products of such *A. thaliana* genes and of those defined in *C. roseus* in the present work must somehow be involved in the down-regulation of mechanisms concerned with salt tolerance in respective species.

A seedling's/plant's response to NaCl stress is expected to be the coordinated outcome of the responses of various kinds of cells present in all its tissues and organs to the primary hyper-osmotic and -ionic stresses, and any secondary stress such as oxidative stress. Intracellular accumulation of high concentrations of Na^+ and Cl^- may cause cell death by such effects as disruption of the organization of nuclear, cytoplasmic and/or organelle membranes, metabolic toxicity, de-regulation of gene expression, attenuation of nutrient uptake and genetic damage (Yeo 1998; Hasegawa et al. 2000). Halophytes are able to protect their vital functions by compartmentalization of toxic ions into vacuoles (Flowers et al. 1977, 1986). Glycophytic cells also possess vacuoles and therefore may escape from hyper-ionic damage by vacuolization of the toxic ions (Apse et al. 1999; Shi et al. 2000). Both halo- and glyco-phytes are known to achieve osmotic adjustment in their cells by local accumulation of organic solutes (Flowers et al. 1986; Greenway and Munns 1980). A cell's internal osmotic potential is known to be lowered by the presence of charged compatible osmolytes such as proline and glycine-betaine (Yoshiba et al. 1995; Bohnert and Shen 1999). The compatible osmolytes are also known to lower the intracellular concentrations of reactive oxygen species produced under conditions of NaCl stress (Hayashi et al. 1997). The differences in salt tolerance between different plant types and different tissues/organs in a plant, and temporal differences in a tissue/organ, are likely to arise from differential regulation of the fluxes of Na^+ and expression of pathways for the synthesis and catabolism of osmolytes, and of proteins and enzymes that may protect cells against damage that may occur (Rhodes and Samaras 1994; Kasuga et al. 1999; Hasegawa et al. 2000). Some of these mechanisms have been ascribed protective/remedial roles against NaCl stress in certain available salt-tolerant mutants. The salt tolerance in a soybean mutant has been related to its ability to exclude Cl^- (Abel 1969). The barley salt-tolerant mutants have been shown to accumulate more than a normal level of proline (Kueh and Bright 1982). Salt tolerance in certain *A. thaliana* mutants has been found to be correlated with the overproduction of proline and/or glycine-betaine (Saleki et al. 1993; Werner and Finkelstein 1995; Zhu et al. 1998; Quesada et al. 2000).

The NaCl tolerance mutants of *C. roseus* in this study appears to be related in some measure to the hyper-accumulation of proline and glycine-betaine. The seedlings of all the six mutants accumulated significantly more proline and glycine-betaine without being exposed to exogenous NaCl (constitutive accumulation). The amount of constitutively accumulated proline was nearly double in the mutants of the *GSR-1* and *GSR-2* genes, as compared to that in the wild-type *GSR* seedlings. The exposure to NaCl vastly increased the proline and glycine-betaine contents of the mutants of all the *GSR* genes, except in the mutants of the *GSR-1* and *GSR-2* genes where the inducible accumulation of proline was quite low. The mutants of the *GSR-2* and *GSR-6* genes accumulated more proline than the wild-type in seedlings and adult plants under conditions of NaCl stress.

The amino-acid proline is synthesized from the amino-acid glutamic acid (Delauney and Verma 1993; Kishore et al. 1995). The quaternary ammonium compound glycine-betaine is synthesized from choline (Rathinasabapathi et al. 1997). The constitutive and NaCl inducible expression levels of the pathways to the synthesis of glutamic acid and choline, and subsequent extensions of these pathways to the syntheis of proline and glycine-betaine and the catabolism rates of the two osmolytes, are expected to determine the concentrations of proline and glycine-betaine in the NaCl-stressed wild-type and *gsr* mutants of *C roseus*. The observed differences in the constitutive and inducible accumulation levels between the mutants indicate that the pathways to proline and glycine-betaine must be differentially suppressed by the activities of the *GSR-1* to *GSR-6* genes. Since the NaCl – inducible regulatory mechanism for proline accumulation has been largely negated by the *gsr-1* mutation, the product of the *GSR-1* gene must be an element, perhaps a transcription factor, in the promotion of proline synthesis under NaCl stress.

High concentrations of salt in the rhizosphere create drought-like conditions and cause a water deficit in plants. All the *C. roseus* mutants – *gsr-1* to *gsr-6* – withstood water deficiency better than the wild-type and accumulated more proline than the wild-type under these conditions. Among the mutants, *gsr-2* accumulated more proline than the others. It is possible to suggest that the product of the *GSR-2* gene may have a genetically negative role on the mechanisms concerned with NaCl stress remediation.

The seedling and adult-plant morphologies of the *gsr-1* to *gsr-6* mutants of *C. roseus* are pleiotropically different

from those of the wild-type and there are large differences between the morphologies of the mutants. The responses of the mutants to NaCl stress were observed to be quantitatively different in terms of the various parameters used. These properties mean that the mutants reveal a novel stress tolerance mechanism(s) operating in plants. The products of the *GSR-1* to *GSR-6* genes must be involved in NaCl-stress control and in the determination of cell-division, differentiation and morphogenesis processes, both at seedling and adult-plant stages. These genes encode proteins which perhaps have regulatory effects on the expression of other genes affecting diverse functions. The *GSR* genes may specify transcription factors, proteases or other kinds of macromolecules that allow control over the quantities of the products of genes regulated by them. Since *gsr-1* to *gsr-6* mutants are able to integrate cellular and whole-body protective responses to NaCl stress, and are morphologically altered, the *GSR-1* to *GSR-6* genes may control a signalling cascade involving the interplay of different growth regulators such as brassinosteroids, gibberellins, auxins, cytokinins, abscisic acid and ethylene (Wilkinson et al. 1995; Ishitani et al. 1997; Morgan and Drew 1997; Shinozaki and Yamaguchi-Shinozaki 1997; Leung and Giraudat 1998; Pandey-Rai et al. 2001).

The properties of the mutants in the six genes identified in *C. roseus* in this study indicate that there are several pathways for controlling the damage caused by salt stress in plants. It can also be suggested that these pathways, which must be independent up to the points affected by the mutations described here, may subsequently remain independent or interact with each other for the final effect. The mutations described provide base material to uncover the hierarchical relationships between the genes defined by them. The mutants have indeed provided morphological and genetical markers to pyramid salt tolerance in *C. roseus*. It should be possible to test the hypothesis that halophytes have originated from glycophytes by loss-of-sensitivity functions (Hasegawa et al. 2000). Further study of these mutants will help in the understanding of salt-perception, – accumulation and – toleration mechanisms. Prospective work on the *GSR*-genes is expected to provide material for improving the salt tolerance of food and industrial crop plants.

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