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Proline status of genetically stable salt-tolerant *Brassica juncea* L. somaclones and their parent cv. Prakash

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ABSTRACT

Three Brassica juncea L. somaclones (SR-1, -2 and -3) selected in vitro for NaCl-tolerance, non-selected somacone (CP-5) and parent cv. 'Prakash' were characterized for their free proline contents in the absence of stress and as a function of increasing salt stress. In the R_0 generation, 'SR-3' somaclone had ca. three times higher free proline as compared to parent 'Prakash' and other somaclones. Somaclone, SR-1, turned out to be sterile. The other somaclones were carried forward to the R_2 generation after making selections for yield and yield components in the R_1 generation. 'SR-3' bred true for its high proline accumulating characteristic. The somaclone 'SR-3' thus had a stable genetic variation for proline overproduction. Free proline content in 7-day-old whole seedlings and 6-week-old plant leaf tissue, increased with the increase in salt stress in all the lines but at differential rates. The magnitude of increase in free proline was much higher in 'SR-3' lines as compared to parent 'Prakash' and 'SR-2' salt-tolerant somaclones. Under salt stress, in leaf tissue, one of the 'SR-3' derived lines (SR3P6-2) accumulated as much as 269 µmoles of free proline as compared to ca. 20 µmoles per g dry weight in parent 'Prakash' and 'SR-2' line. It was interesting to note that there was a 'critical point' concentration of NaCl beyond which the endogenous level of free proline rose sharply. Somaclonal lines (SR3P6-2, SR2P1-2 and CP5-2) which were found to have higher salt-tolerance indices, also had higher 'critical points' as compared to the other relatively salt sensitive genotypes. The relationship between relative water content and osmotic potential of leaves under salt-stress also showed a relatively higher degree of osmotic adjustment in the selected somaclones, the maximum being in SR-3 derived lines.

INTRODUCTION

Proline status of plant organs and cell cultures continues to be an active area of research in stress physiology (Stewart and Larher 1980, Aspinall and Paleg 1981, Sheoran and Nainawatee 1990). Elevated levels of proline are believed to protect plant tissues against stress by acting as N-storage compound, osmo-solute and hydrophobic protectant for enzymes and cellular structures (Stewart and Lee 1974, Le Rudulier et al. 1984). Proline effected salt-tolerance has been reported in several crops and cell cultures (Mathur et al. 1980, Kueh and Bright 1982, Dix et al. 1984, Pandey and Ganapathy 1985, Chandler and Thorpe 1987, Hassan and Wilkins 1988, Kumar and Sharma 1989). On the contrary, there are reports where selected salt resistant and unselected lines did not differ in proline accumulation in response to salt-stress (Dix and Pearce 1981, Jain et al. 1987). Thus, enhanced proline levels could merely be a stress effect rather than the cause of stress tolerance. Widholm (1988) observed that proline over-producing (15-30 times) carrot suspension cultures were not tolerant to salt, mannitol or PEG stress or freezing, indicating the non-involvement of proline in salt-tolerance. Dreier (1983a,b) on the other hand, established that the level of NaCl treatment (critical point), beyond which the proline content of the tissue rises sharply, is higher for salt-tolerant plants than the salt-sensitive plants. The 'critical point' concept also holds true for an in vitro selected salt-tolerant cell line of eggplant (Jain et al. 1987). We have selected genetically stable salt-tolerant plants by screening highly morphogenic cotyledon explant cultures of Brassica juncea L. cv. Prakash at high NaCl concentrations (Jain et al. 1989, 1990) by the procedure outlined in Fig.1. Of the three variants, SR-1 was sterile. The other two (SR-2 and SR-3) could be carried forward to the R_2 generation where they showed higher salt-tolerance indices (ratio of plant performance with respect to a component in the presence and absence of salt-stress) for agronomic traits (plant biomass, seed weight, seed yield etc.). This report concerns the salt effected proline status of seedling and leaf tissue of parent and the variants selected in the presence (SR-1, -2 and -3) and absence (CP-5) of NaCl. The degree of osmotic adjustment was also assessed by estimating the relative water content and osmotic potential of leaves of different lines.

MATERIAL AND METHODS

Plant materials. The in vitro selected NaCl-tolerant somaclones (SR-1, -2 and -3) and non-selected somaclone (CP-5) of B. juncea and their parent progenitor cv. Prakash were used in the present study. The protocol, in brief, followed for the in vitro selection of NaCl-tolerant variants and their characterization is described in Fig.1. Somaclonal lines CP5-2, SR2P1-2 and SR3P2-1, P6-1 and P6-2 represent the R_2 generation raised from the selected agronomically superior R_1 plants of CP-5, SR-2, and SR-3 origin, respectively. The designations, e.g. P6 and -2 in SR3P6-2, refer to the plant number selected in the R_0 and R_1 generation, respectively. The salt-tolerance level of different genotypes was in the decreasing order of SR3P6-2, SR2P1-2, SR3P6-1, SR3P2-1, CP5-2 and Prakash (Jain et al. 1990).

Proline assay. Proline was extracted in 3% sulphosalicylic

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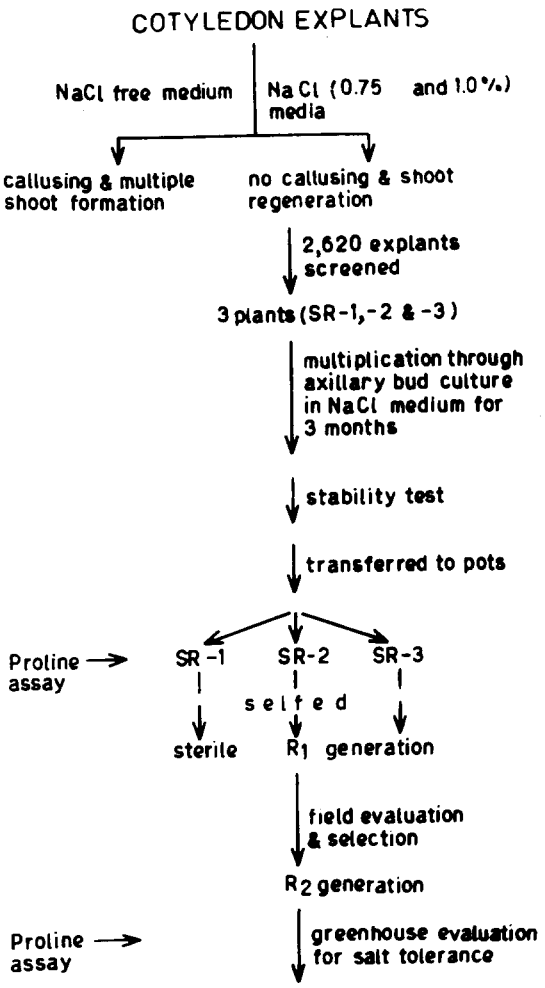

Fig.1 Scheme followed for the selection of salt-tolerant plants in B. juncea

acid and estimated by the colorimetric method of Bates et al. (1973). Proline estimations were carried out in the R_0 and R_2 generations of different genotypes (Fig.1). In the R_0 generation, leaves of 6-week-old SR-1, -2 and -3 salt-tolerant, non-selected somaclone CP-5, and seed raised plants were used for proline estimation. The electrical conductivity of solutions (EC) and of saturated soil extract (ECe) was determined by the method of Richards (1954). The ECe of potting soil varied between 1.9 to 2.3 mmhos/cm. In the R_2 generation, proline was estimated in 7-day-old seedlings and leaves of 6-week-old plants. Seedlings were raised by germinating seeds in sterilized Petri plates containing 0, 50, 100 and 150 mM NaCl solutions which respectively correspond to EC of 0, 3.9, 7.8 and 11.7. Seedlings were washed thoroughly with chilled distilled water before the extraction of proline. To determine the free proline content at leaf stage, plants were grown to maturity in polyethylene lined earthen pots having 5 kg sand of 27% field capacity as described earlier (Jain et al. 1990). The pots were irrigated weekly to field capacity with NaCl solutions of 0, 30, 60 and 90 meq/l prepared in the Hoagland (Arnon and Hoagland 1940) solution. EC of the solutions were 2.1, 4.2, 6.1 and 8.1 mmhos/cm, respectively, at 25°C. In between the irrigations, pots were thoroughly drained with water to bring the ECe down to 1.5. Mean ECe recorded after 6 weeks was 2.5, 5.0, 7.1 and 9.1 in pots irrigated with 0, 30, 60 and 90 meq/l NaCl solution, respectively. Each proline estimation included several seedlings or leaf tissue of several plants from experiments replicated thrice.

Water relations. Measurements of plant water relations were made on leaves of 6-week-old plants using the methods described by Kumar et al. (1984). Osmotic potential (π) was measured with a Model 5100-B Vapour Pressure Osmometer (Wescor, Ins. Logan, Utah, USA). Relative water content (ξ) was estimated by taking 10-12 discs of 5 mm diameter each from the middle portion of a leaf. The measurements were made on four fully expanded leaves of different plants between 12.00 to 14.00 hour. Since both ξ and π are interdependent variables, the linear regression was fitted with ξ as a dependent variable. Reciprocal of slope 'b' (1/b) is a measure of osmotic adjustment ($\ln \xi = a - b \ln \pi$).

RESULTS

Proline content in R_0 plants. In the R_0 generation, in vitro selected plants grown without salt stress differed in the leaf free proline content (Table 1). While the leaf tissue of CP-5, SR-1 and SR-2 had proline contents similar to the parent cv. Prakash, SR-3 leaves contained nearly three times higher proline.

Table 1. Free proline content in leaf tissue of parent cv. Prakash and R_0 plants of selected salt-tolerant and non-selected somaclones of B. juncea. Values = mean ± s.d.

Genotype	Free proline content (µmoles/g dry weight)
Prakash	4.6 ± 0.5
CP-5	5.5 ± 0.7
SR-1	5.2 ± 0.4
SR-2	4.4 ± 0.3
SR-3	17.0 ± 2.6*

*Significantly different from the parent 'Prakash' at 0.05 level

The somaclone SR-1 grew slowly and turned out to be sterile, while SR-2 and -3 could be selfed and carried forward to the R_2 generation. In the R_1 generation, all the variants were grown without salt-stress for the purpose of seed multiplication.

Proline content vs. salt-stress in the R_2 generation. All the genotypes, including SR-3, bred true for the proline accumulating characteristic (Table 2). Free proline content in whole seedlings as well as in leaf tissue increased, though differently, in different lines with the increase in salt stress (Table 2). Seedlings of SR-3 derived P6-1 and P6-2 somaclonal lines showed higher proline content, both in the absence of salt stress and at different levels of salt stress as compared to the parent cv. Prakash and CP5-2. Maximum accumulation of proline at the highest salt stress was also observed in these two lines. At 150 mM NaCl treatment seedlings of the other three somaclones had significantly ($P<0.05$) lower levels of free proline than the parent cv. Prakash.

In the leaf tissue also, SR-3 derived lines had higher proline content than the Prakash and unselected CP5-2 or even compared to the SR-2 salt tolerant line, both in the absence and presence of different levels of NaCl stress (Table 2). Increase in the proline content with increasing NaCl stress was relatively less and insignificant ($P < 0.05$) in the cv. Prakash and SR-2 line. The SR-3 lines, on the contrary, showed a sharp and significant increase in proline content at 60 and/or 90 meq/l salt stress. The increased level in SR-3 derived lines was in the range of 130-270 µmoles as against 20-45 µmoles per g dry tissue of the other lines. Strikingly, the leaves

Table 2. Effect of different salinity levels on free proline content in seedlings and leaves of parent cv. Prakash and R_2 progenies of some of the non-selected and selected salt-tolerant somaclones of B. juncea. Values = mean ± s.d.

NaCl treatment (mM)	Free proline content (μmoles/g dry weight)					
	Prakash	CP5-2	SR2P1-2	SR3P2-1	SR3P6-1	SR3P6-2
Seedlings[a]						
0	2.9 ± 0.4	3.0 ± 0.8	3.6 ± 0.3	6.0 ± 0.5	7.7 ± 0.6	5.3 ± 0.7
50	4.3 ± 0.4	4.2 ± 0.4	4.0 ± 1.1	4.2 ± 0.4	18.4 ± 1.9	6.1 ± 1.9
100[c]	36.6 ± 7.8*	15.3 ± 4.1	9.5 ± 1.0	38.2 ± 9.7*	68.8 ±14.9*	15.7 ± 1.9
150[c]	130.7 ± 9.7*	77.6 ± 6.6*	75.2 ± 3.2*	69.3 ±19.2*	183.3 ±22.4*	220.4 ±16.4*
Leaves[b]						
0	5.8 ± 0.3	6.5 ± 0.2	5.4 ± 0.7	7.7 ± 1.1	16.2 ± 5.1	16.8 ± 1.6
30	10.4 ± 2.0	11.5 ± 1.0	14.1 ± 1.3	24.3 ± 3.0	24.2 ± 3.5	23.0 ± 1.4
60[c]	20.7 ± 4.0	8.5 ± 1.0	20.6 ± 2.6	82.8 ± 7.0*	155.7 ±16.6*	37.3 ± 6.9
90[c]	23.6 ± 2.5	45.4 ± 6.3*	19.0 ± 1.3	129.5 ±50.6*	64.8 ± 1.4*	269.4 ±51.0*

* Significantly different from the respective control at 0.05 level.
a All the somaclones were significantly different from the parent cv. Prakash at 0.05 level.
b SR-3 derived lines were significantly different from the parent cv. Prakash at 0.05 level.
c Significant effect of NaCl treatment at 0.05 level.

of SR3P6-3 showed a 45-fold enhancement in proline level under stress, as against 4-fold in the parent cv. Prakash.

The pattern of increase in free proline as a function of salt-stress varied in different genotypes. In SR3P6-2 as well as CP5-2, a sharp increase in proline content was observed only at 150 mM NaCl in seedlings and at 90 meq/l in the leaf tissue. While the seedlings of SR-2 line showed a sharp increase at 150 mM NaCl, the leaves did not show any appreciable increase in proline content at any of the stress levels. In the other lines, either there was no sharp increase or the increase was at relatively lower (100 mM or 60 meq/l) NaCl concentrations.

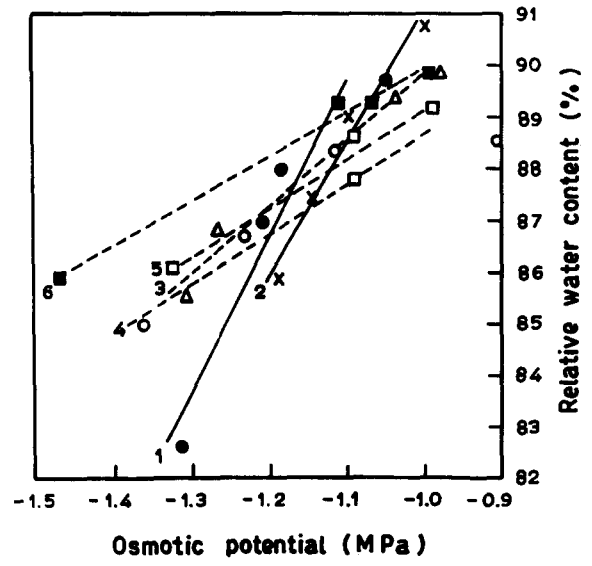

Fig.2. Relationship between osmotic potential and relative water content of leaves as a function of salt-stress in in vitro selected NaCl-tolerant (- -) and non-selected (—) plants of B. juncea. 1. Prakash (●), 2. CP5-2 (×), 3. SR2P1-2 (Δ), 4. SR3P2-1 (O), 5. SR3P6-1 (□) and 6. SR3P6-2 (■).

Osmoregulation in the R_2 generation. The relationship between the osmotic potential and relative water content of leaves of different genotypes drawn according to Morgan (1983) is shown in Fig.2. The lower degree of slope (b) is indicative of a higher level of osmoregulation. The six genotypes showed large variations in their b values. In SR3P6-1 and -2 the slope of the lines was of a lower degree (b = 0.12) as compared to the parent 'Prakash' (0.35) and the non-selected somaclone CP5-2 (0.32). Thus, the two SR-3 lines had a nearly three times higher level of osmoregulation (1/b) as compared to the controls. SR2P1-2 and SR3P2-1 had intermediate b values of 0.18 and 0.22 respectively.

DISCUSSION

Proline is a well known osmoprotectant against a variety of stresses in plants (Stewart and Lee 1974, Flowers et al. 1977, Aspinall and Paleg 1981, Sheoran and Nainawatee 1990). In this study also, an increase in free proline content has been observed in all the genotypes with increasing NaCl stress in the medium or irrigating solution. The mechanism of tolerance provided by higher proline levels was not studied, however, proline has been shown earlier by other workers to be involved in osmotic adjustments and protection against denaturation of enzymes and sub-cellular organelles (Stewart and Lee 1974, Le Rudulier et al. 1984).

Salt-tolerant somaclone (SR-3) had three times higher level of free proline compared to the parent cv. Prakash and also bred true for this characteristic over two generations. This indicates that the somaclone has a stable genetic change for proline overproduction. Such inherently higher levels of proline in SR-3 compares well with that reported in the leaves of hydroxyproline resistant plants of barley (Kueh and Bright 1982) and shoots of Nicotiana sylvestris (Dix et al. 1984) and sodium sulfate tolerant callus of B. napus (Chandler and Thorpe 1987). The higher proline level in the SR-3 somaclone possibly enabled it to survive the lethal salt level during screening, and thus made cell proliferation and shoot regeneration feasible. The relationship between relative water content and osmotic potential is used for the assessment of osmo-regulation in plants which in turn has been used as a selection criterion for stress tolerant genotypes (Morgan 1983, 1984, Turner and Begg 1981).

The SR-3 somaclonal lines showed higher levels of osmoregulation. The proline overproduction may result from a relaxed feed back inhibition of the regulatory step enzyme (Widholm 1988), increased activity of the enzymes involved in the synthesis and/or inhibition of enzymes involved in the degradation of proline (Kandpal et al. 1981) or gene amplification (Watad et al. 1983). Salt tolerance has been positively correlated earlier with the inherent ability for proline overproduction at the callus and/or plant level in many plant species (Kueh and Bright 1982, Dix et al. 1984, Pandey and Ganapathy 1985, Chandler and Thorpe 1987, Hassan and Wilkins 1988, Kumar and Sharma 1989).

The other salt-tolerant somaclone (SR-2) though did not show either an inherent high level of proline or a marked salt effected increase in the proline concentration both in the seedlings and leaves, yet it had a high salinity tolerance index (Jain et al. 1990). This somaclone also showed a relatively higher osmotic adjustment than the controls. The salt-tolerance mechanism of this line is currently under investigation to check for the presence of an osmoticum other than proline. Though algal cells are known to utilize a single osmoticum, tobacco cells were reported by Binzel et al. (1987) to accumulate several organic solutes in response to NaCl stress.

Dreier (1983a,b) and Jain et al. (1987) postulated a concept that the critical point (the level of NaCl concentration above which endogenous free proline content of the plant tissue increases sharply) of salt-tolerant genotypes is higher than the salt-sensitive ones. In comparison to the parent cv. Prakash, SR3P2-1 and SR3P6-1, the other salt selected lines SR3P6-2 and SR2P1-2 and unselected CP5-2 had higher 'critical points and accordingly were found to be more salt-tolerant as reported earlier (Jain et al. 1990). It is also seemingly clear that proline is accumulated at higher levels under acute stress conditions, perhaps, as a last resort to survive and to avoid osmotic death. Thus, the genotypes differing in the degree of salt-tolerance will have different critical points.

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