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Production of sodium-chloride-tolerant *Brassica juncea* plants by in vitro selection at the somatic embryo level

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Summary. Somatic embryos, developed from hypocotyl segments of light-grown seedlings of *Brassica juncea* cv RLM198, were subjected to selection at varying concentrations of sodium chloride (NaCl). Plants were developed from proliferated somatic embryos selected on NaCl-containing medium. The selections were characterized for salt tolerance, esterase isozyme pattern, and proline accumulation. It has been found that: (i) selected tolerant lines showed better root growth, shoot growth, and fresh weight accumulation on salt-containing medium when compared to the control; (ii) salt tolerance was transmitted to the next generation in seed progeny of tolerant plants grown in the absence of exposure to salt; (iii) both the starting material and the tolerant selections accumulated proline, even when grown in salt-free medium. On salt-containing medium, however, the differences in accumulated proline between the control and tolerant lines became more pronounced, and (iv) the patterns of esterase isozymes of two tolerant selections were similar but distinctly different from that of the parental control.

Key words: *Brassica juncea* in vitro selection – Stress tolerance – Correlated somatic embryo-whole plant response to salt stress

Introduction

In vitro selection for stress tolerance, when relevant restrictive conditions can be created, is an attractive choice, because tolerance to most stresses is governed by complex genetic control, and the prevailing lack of knowledge about the mechanism through which tolerance operates limits success of selection in conventional breeding procedures. The in vitro selection, in turn, also has limitations, such as: (i) the difficulty of regenerating plants from the selected tolerant cell lines, and (ii) the occasional lack of correlation between in vitro tolerance and whole plant response. Overcoming the regeneration problem will remove a major hurdle and allow valid evaluation of the effectiveness of genetic manipulation through in vitro selection. We have earlier reported (Kirti and Chopra 1989) that efficient, single-step, somatic embryogenesis can be induced in cultured hypocotyl explants of *Brassica juncea*. Such somatic embryos, produced under non-selective conditions, have been screened for tolerance to NaCl and genetically stable, salt-tolerant mustard has been obtained. The results of this work are reported in this paper.

Materials and methods

Brassica juncea (L) Czern cv RLM198 was used to produce somatic embryos to select for salt tolerance. Hypocotyl explants from 5- to 7-day-old, light-grown seedlings were induced to produce callus and somatic embryos. The hypocotyl region of somatic embryos was proliferated into shoots on MS medium (Murashige and Skoog 1962), supplemented with 2% sucrose and 0.25 mg/l 6-benzylaminopurine (Kirti and Chopra 1989). Plantlets were raised from such shoots.

In initial experiments, attempts were made to produce somatic embryos on induction medium containing 1% NaCl. The frequency of embryogenesis in this procedure was low and the small number of embryos that were produced failed to germinate on NaCl-containing proliferation medium. In subsequent experiments, somatic embryos were produced on normal induction medium, but were screened on proliferation medium containing various concentrations of NaCl. On the proliferation medium, secondary embroyid production is suppressed. Shoots from embryos proliferating on NaCl-containing media were subjected to two further cycles of selection on medium contain-

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ing 1.25% NaCl to produce well-formed shoots. These shoots were transferred to NaCl-free, half-strength MS medium with 1% sucrose for rooting. Plantlets raised from axillary bud culture of shoots obtained from in-vitro-germinated seed of the parental cultivar served as control.

To assess the growth potential of selections under stress, plantlets were grown on liquid medium containing one-fourth strength MS salts and 1.29% NaCl (EC: 15 dS/m). Growth was computed as percent gain compared to the control.

Plantlets selected for NaCl tolerance in vitro were hardened in a growth chamber at 25±1 °C, 16 h photoperiod, 200 μE $m^{-2}s^{-1}$, and 80% relative humidity. These plants were transferred to pots and grown under natural conditions. Selfed seed of the selections was used to assess NaCl tolerance. Data on root length, shoot length, and fresh weight were recorded on 1-week-old seedlings. Germination and growth potential of seedlings raised from seed of tolerant selections were assessed on half-strength MS solidified medium containing 1.05% NaCl (EC: 12 dS/m).

Free proline accumulated in the leaves was estimated in salt-tolerant selections grown for 1 week on NaCl-free or NaCl-supplemented, one-fourth strength MS liquid medium (1.05%, EC: 12 dS/m). Fully expanded young leaves were used to estimate free proline, according to Bates et al. (1973).

For esterase isozyme analysis, 500 mg of 5-day-old seedlings, grown under 16 h (25 °C) light/8 h (20 °C) dark in a growth chamber, was macerated in 0.5 ml of 50 m*M* TRIS-Cl buffer (pH 7.5) containing 0.1% insoluble polyvinyl pyrrolidone. The mixture was centrifuged and the supernatant was used to study the isozyme pattern as per Eduardo-Vallejos (1983).

Results

On induction medium containing 1% NaCl, the frequency of somatic embryogenesis was very low: out of 510 hypocotyl explants, only 29 produced one embryo each. These embryos failed to develop on proliferation medium containing 1% NaCl. Survival of somatic embryos produced on normal induction medium also suffered on salt-containing medium (Table 1). At 1% NaCl concentration, the survival rate was only 1.6%.

Seven somatic embryos that survived on NaCl-containing proliferations medium for about 12 weeks produced well-formed shoots. These were rooted to raise 65 plantlets. Selfed seed was obtained from these plants, and its germinability was assessed on NaCl-containing, half-strength MS medium. Seeds from three of the selections (S-10, S-51, and S-63) had significantly better germination on NaCl-containing medium (Table 2) and these were subjected to a more detailed assessment. Their early growth, measured from 7-day-old seedlings maintained on half-strength, solidified MS medium containing 1.05% NaCl (EC: 12 dS/m), was significantly superior to that of the control (Table 3).

After germination and the initial growth on NaCl-containing medium, seedlings were transferred to the liquid NaCl-free MS medium and maintained in the growth chamber for 1 month. These plantlets were transferred for 10 days into NaCl-containing liquid medium again and growth was assessed as the percent gain in root length, shoot length, and fresh weight. Salt tolerance of selections was evident from better growth, as compared to the control (Table 4).

Proline accumulation was measured in salt-tolerant and control plantlets. In the control, there was no substantial difference in free proline levels between plantlets growing on NaCl-free and NaCl-containing medium. In contrast, the tolerant selections had lower proline levels

Table 1. Recovery of salt-tolerant somatic embryos on media with different NaCl concentrations

NaCl in proliferation medium (%)	EC of the medium (dS/m)	Somatic embryos		
		Plated (no.)	Surviving	
			(no.)	(%)
0.0 (Control)	2.6	100	91	91.0
1.0	12	1528	24	1.6
1.25	15	207	6	2.9
1.50	18	1238	9	0.7
1.60	20	582	0	0.0
1.80	22	601	0	0.0
2.00	24	2111	0	0.0

Table 2. Germinability of seeds of salt-tolerant selections on NaCl-containing medium (NaCl: 1.05%; EC: 12 dS/m)

Genotype	Germination	
	Actual (%)	Compared to control (%)
Control	51.25	–
S-10	61.95**	+20.88
S-51	66.26**	+29.29
S-63	66.56**	+29.87

** Significant at $t_{1\%}$

Table 3. Growth parameters of 1-week-old seedlings of salt-tolerant selections on NaCl-containing medium (NaCl: 1.05%; EC: 12 dS/m)

Genotype	Root length		Shoot length		Fresh weight	
	Actual (cm)	Gain over control (%)	Actual (cm)	Gain over control (%)	Actual (g)	Gain over control (%)
Control	3.36	–	2.78	–	0.032	–
S-10	4.67**	+39.0	3.94**	+41.7	0.059**	+ 84.4
S-51	4.47**	+33.0	3.86**	+38.8	0.045**	+ 40.6
S-63	6.03**	+79.5	4.31**	+55.0	0.064**	+100.0

** Significant at $t_{1\%}$

Table 4. Growth of salt-tolerant plantlets in NaCl-containing liquid medium (NaCl: 1.29%; EC: 15 dS/m)

Genotype	Gain in root length		Gain in shoot length		Gain in fresh weight	
	(%)	Compared to control (%)	(%)	Compared to control (%)	(%)	Compared to control (%)
Control	11.9	–	10.3	–	15.2	–
S-10	12.1	+ 1.7	14.7	+ 38.8	29.2*	+ 90.8
S-51	24.8**	+108.4	26.2**	+154.4	34.2**	+125.0
S-63	15.3	+ 25.2	18.1*	+ 56.3	34.3**	+125.0

* Significant at $t_{5\%}$
** Significant at $t_{1\%}$
Measured after 10 days' growth

Table 5. Proline accumulation in salt-tolerant selections on normal and NaCl-containing liquid medium (NaCl: 1.05%; EC: 12 dS/m)

Genotype	Proline (μg/g fresh weight)	
	NaCl-free medium	NaCl-containing medium
Control	386.0	498.4
109-3-6[a]	912.0	2704.0
7-1[a]	1064.0	2868.0
6-2[a]	293.0	2848.0
S-10[b]	1511.4	4765.0
S-51[b]	673.2	7524.0
S-63[b]	752.4	886.0

[a] Plantlets derived from selected somatic embryos
[b] Plantlets derived from selfed seed of salt-tolerant selections

Table 6. Survival of somatic embryos from second cycle selection on NaCl-containing medium (NaCl: 1.00%; EC: 12 dS/m)

Genotype	Somatic embryos		
	Plated (no.)	Surviving	
		(no.)	(%)
Control (1st selection cycle)	1528	24	1.6
S-51	45	23	51.1
S-63	221	125	56.6

in the NaCl-free medium, but showed increased free proline when grown on NaCl-containing medium (Table 5). The experiment was repeated three times with similar results. Esterase isozyme patterns were studied for selections 10 and 63, along with the control, in 5-day-old seedlings obtained from seed grown on NaCl-containing

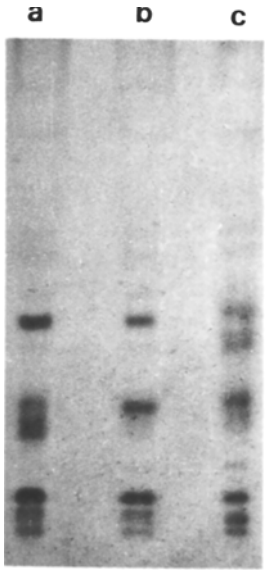

Fig. 1. Esterase isozyme patterns in two salt-tolerant selections: selection 63 (*a*), selection 10 (*b*), and control (*c*)

medium in the growth chamber. Major isozyme differences were observed between the selections and the control. It was interesting to note that both selections had similar band patterns (Fig. 1). Selection 51, which was subsequently studied, also had a band pattern similar to that observed for selections 10 and 63.

Somatic embryos were raised from S-51 and S-63 and tested on proliferation medium containing 1.0% NaCl. It was observed that the survival and proliferation rates of these somatic embryos were more than 50%, whereas the survival rate of embryos from the initial selection was only 1.6% (Table 6). S-10, however, did not produce somatic embryos, even after prolonged culture on embryo induction medium.

Discussion

In-vitro-selected cell lines, even when verified for salt tolerance, have often been found to lose their regeneration potential (Meredith 1984; McCoy 1987a). Perhaps for this reason, embryogenesis was drastically inhibited and the somatic embryos failed to germinate when NaCl was added to the induction medium. In our effort, this difficulty was overcome by inducing somatic embryos on NaCl-free medium and subsequently challenging them to restrictive conditions. This procedure has proved effective.

A positive correlation between cellular tolerance and plant level tolerance is essential for utilizing in vitro procedures of selection for NaCl tolerance. Whole plant response correlates positively with cell response in some

cases (Orton 1980; McCoy 1987b) but not in others (Flowers et al. 1985). In our work, selection for tolerance was based on the survival of somatic embryos on selection medium. The selected tolerance was retained by the rooted plantlets and transmitted to the next generation. Superior seed germinability and growth of plantlets in the generation following selection clearly point to the stability of tolerance of the selections. Thus, the selection for NaCl tolerance at the somatic embryo level is genetic in nature and is transmitted through seed. Adopting a different approach, Jain et al. (1990) were also able to obtain stable salt tolerance in *B. juncea*. Transmission of tolerance through a seed generation is also unambiguously indicated by high frequency proliferation on NaCl-containing medium of somatic embryos arising from selections S-51 and S-63.

Doubts have been expressed about the practical use of in vitro selection for salt tolerance, because the resistance has, in some cases, been diluted over generations. Two causes can lead to such instability. (i) The tolerance may be related to gross chromosome structural alterations, such as translocations, whose progressively reduced transmission can cause loss of phenotype. It is expected that such chromosomal rearrangements will adversely affect fertility of the plants. (ii) The observed tolerance may be a consequence of epigenetic variation. The tolerance selected in our experiments is, however, genetic and is transmitted throug a seed generation. We infer that instability does not operate in our selections because: (i) the pollen fertility and seed set in majority of the tolerant plants was high; (ii) the tolerant somatic embryos were of spontaneous origin and were produced on normal medium without imposed salt stress.

For practical application, the selections need to be observed for tolerance during reproductive phase and for yield. This, however, has not yet been done. It is also important to mention that tolerance of different selections, currently being characterized on the basis of growth parameters, varies and it should be possible to select a level of tolerance at which penalty on grain yield is not high.

Many plants accumulate proline in response to water and salt stress (Chandler et al. 1986; Chandler and Thorpe 1987). It is believed that the accumulated proline mediates tolerance by serving as a source of cytoplasmic osmoticum (Handa et al. 1983; Watad et al. 1983; Daines and Gould 1985) and protecting cytoplasmic enzymes and cellular structures (Le Rudulier et al. 1984). It has also been argued that proline counteracts the inhibitory effect of NaCl (Pandey and Ganapathy 1985). A relationship has been observed between the concentration of NaCl to which the plants are exposed and the extent of proline accumulation in sensitive and tolerant plants. Salt tolerance has been well correlated with proline accumulation in NaCl-tolerant callus cultures of chickpea. There also appears to be a critical NaCl concentration in culture medium above which the endogenous free proline content of tissue rises sharply. This critical concentration is higher for salt-tolerant plants and lower for salt-sensitive ones (Dreier 1983). In our work it has been observed that both the parental cultivar RLM 198 and the tolerant selections accumulate proline under salt-free condition. The difference of accumulated proline, however, becomes more pronounced in response to NaCl exposure.

The Brassicas show interspecific variability for salt tolerance (Chandler et al. 1986) indicating that the characteristic is genetically controlled. It should, therefore, be possible to identify introgressed tolerance by subjecting material from wide crosses to in vitro selection.

Our findings suggest that in vitro selection can yield stable, salt-tolerant plants.

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