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Changes in sugars, sucrose synthase activity and proteins in salinity tolerant callus and cell suspension cultures of *Brassica oleracea* L.

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Abstract

Salt tolerant callus and cell suspension cultures of *Brassica oleracea* L. var. *botrytis* were obtained by the selection of cells from cultures growing in medium supplemented with 85, 170, and 255 mM NaCl. Salt adapted calli and cell suspensions differed in their RNA and protein concentrations. These concentrations tend to diminish in calli and increase in cell suspensions, both at one or three weeks periods of growth in NaCl. Contents of sucrose and reducing sugars, however, accumulate similarly both in calli and cell suspensions after NaCl treatments. The activity of sucrose synthase was higher in salt adapted cells than in controls. Calli exposed to 255 mM NaCl for six months synthesized a 27 kDa polypeptide, while a 13 kDa polypeptide present in control conditions was absent under salinity. Several high molecular mass polypeptides (> 200 kDa) were visualized in control calli and at moderate salt concentrations, when conditions of the gel were modified.

Additional key words: adaptation, cauliflower, NaCl, RNA.

Introduction

In recent years there has been an increasing interest in the breeding of salt-resistant crop plants to be used in coastal areas that require heavy irrigation. Cell cultures seem to be more uniform in their response to stress conditions than whole plants, especially when cultured in liquid media. Exposure of cells to salt stress causes a set of metabolic and developmental changes (Cheesman 1988, Hasegawa *et al.* 2000). Some of these biochemical and structural changes allow plants to adjust to the new conditions.

Variation in salinity tolerance has been observed *Brassica* species (Chandler and Thorpe 1987, Paek *et al.* 1988, Chen and Plant 1999, Kawasaki *et al.* 2001, Miki *et al.* 2001a, Lutts *et al.* 2001) but the underlying metabolic changes occurring in salt tolerant *Brassica* cell lines have not been understood yet.

In this paper we analyze the total RNA and soluble protein concentrations in salt tolerant cell cultures, as well as the pattern of polypeptides synthesized by salt tolerant calli after *in vivo* labelling with ^{35}S-methionine. Sucrose, reducing sugar concentrations, and the activity of sucrose synthase in salt tolerant *Brassica oleracea* var. *botrytis* calli and cell suspension cultures have also been evaluated as biochemical indexes of salt tolerance.

Material and methods

Adaptation of callus and cell suspension cultures to NaCl: Callus cultures of cauliflower (*B. oleracea* L. var. *botrytis*) were induced from cotyledons of 10-d-old seedlings. Cell suspension cultures were obtained from

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Abbreviations: 2,4-D - 2,4-dichlophenoxyacetic acid; PMSF - phenyl methyl sulphonyl fluoride; PVPP - polyvinylpolypyrrolidone.

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the callus cells. These two cell lines were maintained by transferring an inoculum every three weeks (calli) or one week (cell suspensions) to fresh Murashige and Skoog (1962) medium supplemented with 0.2 mg cm^{-3} dichlorophenoxyacetic acid (2,4-D), 0.1 mg cm^{-3} kinetin and 3.5 % (m/v) sucrose as a carbon source. For the solid medium 0.8 % (m/v) *Difco Bacto* agar was added. The pH of the medium was adjusted to 5.8 before autoclaving. Salt adaptation and further selection was accomplished by transferring the six-month-old stock cultures (control) to the same medium with the addition of 85, 170, 255, and 342 mM NaCl. All cultures were incubated at 25 °C (16-h photoperiod; irradiance 4.05 W m^{-2} in calli, or 0.45 W m^{-2} in cell suspensions). Salt adaptation experiments were repeated three times in 50 plastic Petri dishes (calli) or 10 Erlenmeyer flasks (cell suspensions) for each tested condition.

Dry mass was determined by drying the biomass in an oven at 80 °C for 72 h. For the experimental analysis, 6-month-old control and salt tolerant cultures of calli and cell suspensions were ramdomly taken at the end of one and three weeks of the growth cycle from Petri dishes or Erlenmeyer flasks, respectively. For the experimental determination we considered the periods of time of one and three weeks both for calli and cell suspensions, although they were maintained in different conditions.

RNA and protein determinations: Extraction and quantification of total RNA was done according to Verwoerd-Ben *et al.* (1989). For each evaluation, 400 mg of fresh material was used. Total soluble protein content was measured according to Bradford (1976). Approximately 500 mg of fresh material was homogenized in a pre-cooled mortar with 1.5 cm^3 of 0.1 M Tris-HCl buffer (pH 8.0) containing 1 % β-mercaptoethanol, 0.005 % (m/v) phenyl methyl sulphonyl fluoride (PMSF) as protease inhibitor, and 10 % (m/v) polyvinylpolypyrrolidone (PVPP) to remove the phenolic compounds.

***In vivo* labelling and protein extraction:** Six-month-old control and salt tolerant calli in the 15th day of the growth cycle were incubated in liquid medium containing 3.7 GBq cm^{-3} of ^{35}S-methionine (specific activity > 40.07 GBq $mmol^{-1}$) on a rotary shaker for 44 h. After labelling, calli were washed and protein extraction was carried out as described above.

Electrophoresis: Electrophoresis of soluble proteins extracted from calli (control and NaCl tolerant) was performed according to Laemmli (1970) on 12.5 % and 7.5 % (m/v) SDS polyacrylamide slab gels. Samples with similar radiactivity (8.333 s^{-1}) were loaded on the gels, and size markers were added. Gels were fluorographed as described by Laskey and Mills (1975). After fluorography, gels were stained with Coomassie blue.

Determination of sucrose and reducing sugars: For these determinations 500 mg of fresh calli or cell suspensions were washed three times with mannitol solution in order to prevent sugar exit, and to remove remaining exogenous sugars. Then, cells were homogenized in 0.1 M Tris-HCl (pH 7.6), centrifuged at 1 224 *g* for 15 min at 0 °C and the supernatant was collected. Sugars were determined as Somogy (1952) and Nelson (1944) described using D-glucose as standard. Sugar contents were calculated according to Paek *et al.* (1988).

Sucrose synthase activity: Sucrose synthase (UDP-D-glucose : D-fructose 2-D glucosyl transferase, EC 2.4.1.13) extraction, purification and activity measurements were performed according to Hawker (1967). The substrates of the enzyme reaction were UDP-glucose and D-fructose labelled with 3.76 GBq $mmol^{-1}$ of ^{14}C (specific activity > 11.1 MBq $mmol^{-1}$), and 10 mM of oxidized glutathione was added to the reaction mixture to inhibit the reverse reaction of sucrose synthase as invertase and thereby sucrose cleavage, according to the following reaction:

UDPG + ^{14}C-fructose ↔ ^{14}C-sucrose + UDP

Statistical analysis: Data were compared by analysis of variance and Student's *t*-test.

Results

Brassica control calli transferred to 85, 170, and 255 mM NaCl turned brown and several sectors were necrotic by the end of the first subculture (3 weeks), with the appearance of small growing or surviving yellowish zones on the surface of the cell. The number of these zones was reduced when increasing salt concentration. These surviving zones were selected and subcultured on the respective media. Salt adaptation of calli was considered as 100 % (when cultures were free from necrotic areas) after three subcultures (9 weeks) for calli treated with 85 or 170 mM NaCl and five subcultures (15 weeks) when treated with 255 mM NaCl. In cell suspensions three subcultures (3 weeks) on 85 and 170 mM NaCl and five subcultures (5 weeks) on 255 mM NaCl were necessary for adaptation. Calli or cell suspensions transferred to 342 mM NaCl stopped growing and became completely necrotic after 3 weeks of culture.

Total RNA concentration decreased in calli and increased in cell suspensions during the subculture cycle with salt treatments. This behaviour was maintained

similar after 1 or 3 weeks of growth cycle in increasing concentrations of NaCl (Fig. 1*A,B*).

Total soluble protein concentration in salt tolerant calli was diminished at the end of the growth cycle, while it was increased with increasing salinity in cell suspensions (Fig. 1*C,D*).

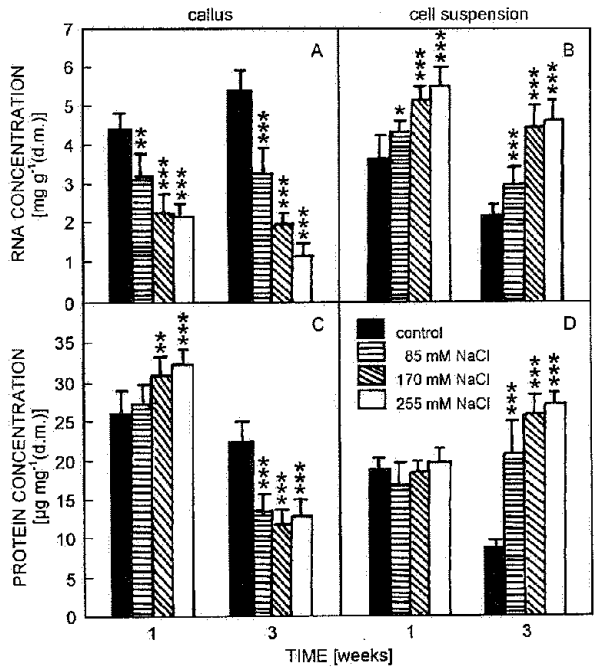

Fig. 1. Total RNA (*A,B*) and soluble protein (*C,D*) concentrations in 6-month-old and salt tolerant calli and cell suspension cultures of *B. oleracea* var. *botrytis* after 1 and 3 weeks of subculture. Means of three experiments ± SD shown as *vertical bars*; *,**,*** - differ from the control at 5, 1, and 0.1 % confidence levels, respectively.

SDS-polyacrylamide gel electrophoresis (12.5 %) showed a new band of 27 kDa protein in 255 mM adapted calli and a band of 13 kDa protein which was present in control calli, disappeared in salt treated cells (Fig. 2*A*). Another band which seems to be only present under conditions of salinity (33 kDa) has been further confirmed to be also present in control conditions. On the other hand, 7.5 % polyacrylamide gels (Fig. 2*B*) revealed two intense bands and two minor bands, all corresponding to proteins with molecular masses higher than 200 kDa. These bands were only present in controls and 85 mM NaCl treated calli and absent from those exposed to higher salt cancentrations (Fig. 2*B*). Coomassie blue stained gels showed no effect of NaCl on the pattern of polypeptides (data not shown).

Sugar (sucrose and reducing sugars) concentration increased with salt treatments similarly in calli and cell suspensions, although there were a general sugar decrease observed at the end of the third week of growth period compared to the first week (Fig. 3*A-D*).

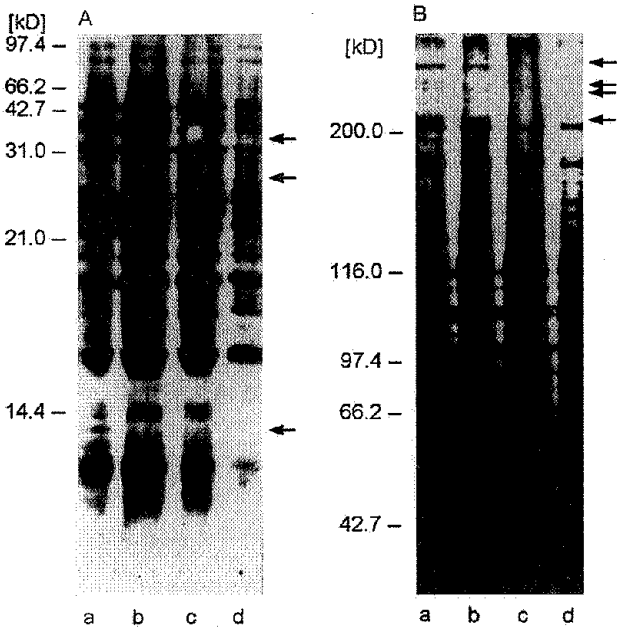

Fig. 2. Fluorographs of 12.5 % (*A*) and 7.5 % (*B*) SDS-polyacrylamide gel electrophoresis of polypeptides synthesized by 6-month-old control and salt treated calli at day 15 of subculture, incubated with ^{35}S-methionine for 44 h. *A* - control, *B* - 85 mM, *C* - 170 mM, *D* - 255 mM NaCl.

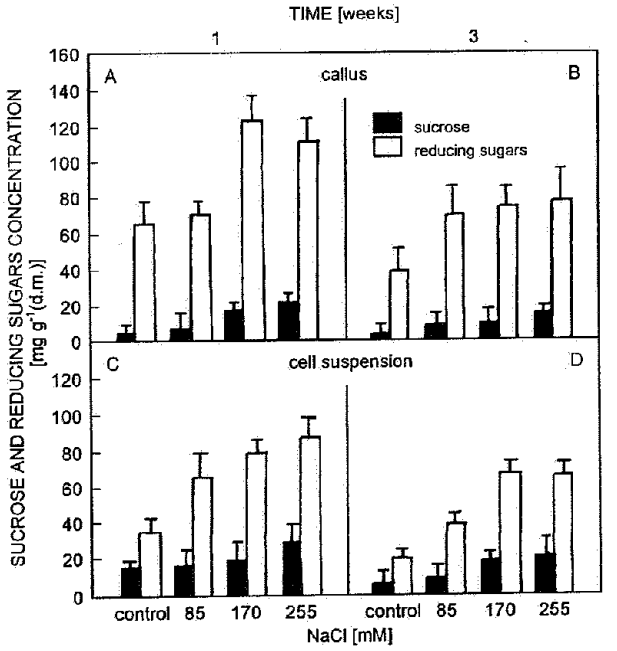

Fig. 3. Total sucrose and reducing sugars contents in 6-month-old control and salt tolerant callus (*A,B*) and cell suspension (*C,D*) cultures of *B. oleracea* var. *botrytis* after 1 and 3 weeks of subculture. Means ± SD shown as *vertical bars*. All values differ from their control at 0.1 % confidence level.

In vitro sucrose synthase activity in calli extracts increased with salt treatments after the first and third week of growth cycle, compared to controls (Fig. 4*A*). In cell suspensions, this activity was also enhanced with salt treatments (Fig. 4*B*).

Discussion

Brassica callus and cell suspension cultures may be adapted to NaCl by the stepwise selection of a few salt tolerant cells. This has also been observed in rice calli (Reddy and Vadyanath 1986, Lutts *et al.* 2001, Miki *et al.* 2001a,b) and tobacco cell suspensions (Wataad *et al.* 1991). This progressive selection of salt tolerant cells observed in *Brassica* cell cultures suggests that the population of *Brassica oleracea* var. *botrytis* may be heterogeneous to salt tolerance.

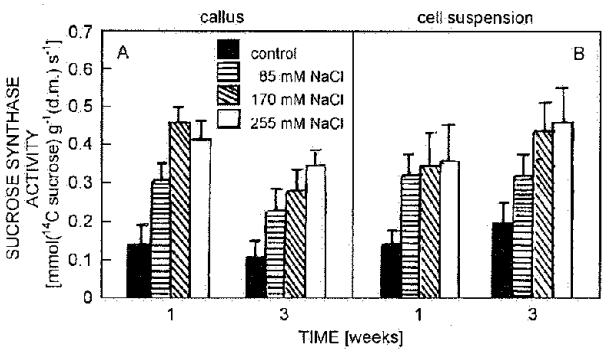

Fig. 4. *In vitro* sucrose synthase activity in 6 month-old control and salt tolerant callus and cell suspension (*A*,*B*) cultures of *B. oleracea* var. *botrytis* after 1 and 3 weeks of subculture. Means ± SD shown as *vertical bars*. All values of this figure differ from their controls at 0.1 % confidence levels.

Our data of RNA (mostly rRNA) and protein concentration give little evidence of adaptation process (Fig. 1). Moreover, there is not common response in the two cell culture systems analyzed, indicating a different metabolic and physiological state of calli and cell suspensions. The different culture conditions on which they have been maintained (time of subculture) could partially explain these results, suggesting some relationship between age of subculture (three weeks for calli and one week for cell suspensions) and RNA or protein accumulation. These results agree with previous observations in *Brassica napus* var. *oleifera* and wild *Brassica oleracea* callus cultures adapted to drought and salinity (Montoya 1989). There must be also taken into consideration that calli undergo some processes of differentiation (*i.e.* increase of vascular elements; Montoya 1989) which are not shared by cell suspensions under salinity and drought.

It has been reported that several compounds such as proline and polyamines increase with salt treatments (Le Dily *et al.* 1991, Nanjo *et al.* 1999, Shankhdhar *et al.* 2000). Also, an increase in the content of certain enzymes of the glucose pathway, *e.g.*, PEP-carboxylase has been reported in cell suspension cultures from plants which are able to make the transition to CAM photosynthetic pathway when growing under salinity conditions (Thomas *et al.* 1992), suggesting an important role of sugar metabolism under salinity (Hasegawa *et al.* 2000).

The greater sugar accumulation observed in salt tolerant *Brassica* cultures in parallel with the increase in salinity levels seems to be a common mechanism of either calli or cell suspension cultures for salt adaptation (Fig. 3). This sugar accumulation could contribute to balance the osmotic strength inside and outside the cells, thus preventing cellular dehydration, and providing a source of energy needed under saline conditions to fuel a more active cell metabolism (Das *et al.* 1990, Fieuw and Willebrink 1990, Koch 1996) as also occurs in calli adapted to grow under drought conditions (Montoya 1989).

Sugar accumulation could also be related to the high sucrose synthase activity that is observed *in vitro* in those cells (Fig. 4). This behaviour might be due to activation of the pre-existing enzymes or to *de novo* enzyme synthesis. Unfortunately, we cannot establish any relationship between sucrose synthase and polypeptides present in controls, which increase their synthesis under salinity conditions (Wang *et al.* 1999).

The greater reducing sugar content observed in *Brassica* salt tolerant cells under higher NaCl concentrations (Fig. 3) suggests that *in vivo* the sucrose synthase functions preferentially as invertase, by degrading the sucrose translocated from the medium to the cells. The need for hexoses may explain the higher hexose uptake of cultured cells under stress (Warren *et al.* 1986, Roberts-Oehlschlager *et al.* 1990, Huber and Huber 1996). The physiological role played by this enzyme might be related to the metabolic requirements of the tissue for hexoses which is associated with its more saline environment.

In our case, both calli and cell suspensions respond similarly to the salt treatments, though cell suspensions show a faster rate to achieve tolerance, if we establish the absence of necrotic areas in the cultures growing under salinity conditions as a criterion for tolerance.

Finally, the pattern of protein synthesis shows some changes in the expression of proteins from calli after salt treatments (Fig. 2). A unique polypeptide of 13 kDa is present only in control conditions and absent under salinity conditions. Moreover, another 27 kDa poly-

peptide is visible under NaCl treatments. Those results confirmed previous data (Martín *et al.* 1993). Some changes may also be required for sustained growth at these conditions. Similar changes on gene expression under salinity have been reported (Maslenkova *et al.* 1992, Reviron *et al.* 1992, Chen and Plant 2000).

In conclusion, our studies indicate that salt tolerance in *Brassica* calli and cell suspension cultures occurs at least partially, by specific changes in the metabolic and physiological state of the cell, which are evidenced by the accumulation of osmoregulators like sucrose and reducing sugars, mainly controlled by the sucrose synthase/ invertase activity, and a differential expression of some polypeptides.

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