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BRIEF COMMUNICATION

Mannitol, polyethylene glycol and NaCl induced polypeptide changes during *in vitro* culture of three tomato cultivars

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Abstract

Three cultivars of tomato Pusa Ruby, Arka Vikas and Pusa Early Dwarf were subjected to osmotic stresses induced by mannitol, polyethylene glycol and NaCl *in vitro*. Polypeptide patterns were analyzed during each stress treatment to differentiate between tolerant and sensitive cultivars. The stresses induced more stress proteins in cv. Pusa Ruby compared to other two cultivars indicating it to be relatively osmotic stress tolerant.

Additional key words: *Lycopersicon esculentum,* osmotic stress.

Abiotic stresses such as heat, drought, cold and salinity cause major yield constraints in all crops. All these environmental stresses bring about osmotic stress in plants which, in turn, alter gene expression and induce many new proteins (Altman 2003, Grover *et al.* 2003). Expressions of drought and salt induced polypeptides have been reported in several plants (Hurkman *et al.* 1989, Uma *et al.* 1995, Jyothsnakumari *et al.* 2009). It has been recorded that the stress induced proteins are better expressed in tolerant genotypes compared to sensitive ones (Pelah *et al.* 1997, Kawasaki *et al.* 2001, Jyothsnakumari *et al.* 2009, Roy *et al.* 2009, Alizadeh *et al.* 2010).

In vitro osmotic stress in plants can be induced by polyethylene glycol (PEG), mannitol and NaCl. The aim of the present study was to determine how drought and salt response could be used as a selection marker to differentiate between tolerant and sensitive cultivars of tomato so that the best cultivar could be selected for making drought tolerant transgenics. In fact, the most tolerant tomato cultivar identified in the *in vivo* drought stress investigations (Roy *et al.* 2009), were transformed *via Agrobacterium* using *bspA* gene encoding boiling stable protein of aspen for drought tolerance (Roy *et al.* 2006a,b).

The seedlings of the three tomato (*Lycopersicon esculentum* L.) cultivars, Pusa Ruby, Arka Vikas and Pusa Early Dwarf, were raised on Murashige and Skoog (1962; MS) basal medium. To induce osmotic stress *in vitro* by mannitol, leaf explants were excised from 6-week-old seedlings and cultured on regeneration medium (MS + 3 mg dm^{-3} BA + 0.3 mg dm^{-3} IAA) supplemented with mannitol in different concentrations (2, 4, 6, 8 and 10 %). 24 explants were grown and the experiment was repeated thrice. To induce osmotic stress by PEG, leaf explants were cultured on the regeneration media supplemented with 2, 4, 6, 8, and 10 % PEG-4000. To induce salt stress, leaf explants were grown on regeneration medium supplemented with 20, 40, 60, 80, 100, 200 and 300 mM NaCl. Control leaf explants were cultured on regeneration medium without mannitol, PEG or NaCl. Leaf explants with developing calli and shoots, cultured for 30 d, were frozen in liquid nitrogen and kept at -80 °C.

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Abbreviations: ABA - abscisic acid; BA - N^6-benzyladenine; BSA - bovine serum albumin; EDTA - ethylenediaminetetraacetic acid; IAA - indole-3-acetic acid; LEA - late embryogenesis abundant protein; MS - Murashige and Skoog; PAGE - polyacrylamide gel electrophoresis; PEG - polyethylene glycol; SDS - sodium dodecyl sulphate.
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Proteins of leaves were extracted following the protocol of Vu *et al.* (1982). Samples were homogenized with pestle and mortar in extraction buffer (50 mM Tris HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiotreitol and 5 mM isoascorbate). Crude protein extracts were centrifuged at 12 000 *g* for 20 min at 4 °C. Proteins in the supernatant were estimated following the protocol of Peterson (1977) at 750 nm, using bovine serum albumin as standard. For sodiumdodecyl sulphate (SDS)-PAGE, 100 μg total soluble proteins were precipitated by adding 4 volumes of cold acetone and then centrifuged at 10 000 *g* for 10 min at 4 °C. Pellet obtained was dried and dissolved in 0.02 cm^3 sample buffer (62.5 mM Tris HCl, pH 6.8, 2 % SDS, 10 % β-mercaptoethanol, 25 % glycerol and 0.01 % bromophenol blue).

Proteins were separated by 15 % SDS-PAGE (Laemmli 1970). Each lane was loaded with 100 μg of total proteins and run at 25 mA for 2 h in *Mini Protean II* apparatus (*Bio-Rad*, Hercules, USA). Gels were stained with 0.2 % (m/v) Coomassie blue in 40 % methanol and 10 % glacial acetic acid. They were destained in 40 % methanol containing 10 % glacial acetic acid, photographed and analyzed in *Gel Documentation 2000 System* (*Bio-Rad*).

Protein profile studies revealed that several new polypeptides developed in explants cultured on mannitol, PEG and NaCl supplemented media in all the three cultivars. Since the 29 kDa polypeptide was a major stress induced protein during *in vivo* water stress in the three cultivars investigated earlier (Roy *et al.* 2009), special attention was focused on its expression during *in vitro* stress treatments.

After one month of culture, leaf explants grown on mannitol supplemented medium showed stunted growth and reduction in the number of shoots (data not shown). For all the three cultivars, 10 % mannitol was lethal while 8 % was sub-lethal. Control leaf explants yielded good morphogenic response in terms of number and growth of shoots on regeneration medium. In SDS-PAGE analysis, the control sample of Pusa Ruby showed 12 polypeptides of molecular masses 21, 26, 27, 32, 33, 35, 38, 40, 46, 50, 55 and 72 kDa (Fig. 1*A*). The expression of higher Mr polypeptides, *i.e.*, 38, 40, 46, 50, 55 and 72 kDa were very low in control plants. Explants cultured on 4 to 10 % mannitol containing medium showed new polypeptides of Mr 22, 28 and 29 kDa (Fig. 1*A*). In Arka Vikas, 28 and 29 kDa polypeptides developed very feebly in 6, 8 and 10 % mannitol-treated plants compared to those in Pusa Ruby (Fig. 1*B*). Plants grown on 6 % mannitol, retained all the bands of control, in even higher intensities, compared to 2 and 4 % treated samples. They developed polypeptides of 38, 40, 46, 50 and 55 kDa in high intensities. The 29 and 40 kDa polypeptides were best expressed in leaves of plants grown on 8 % mannitol. Though plants grown on 10 % mannitol retained all the polypeptides as on 8 % mannitol but the expression of high Mr polypeptides (38, 40, 46, 50 and 55 kDa) was

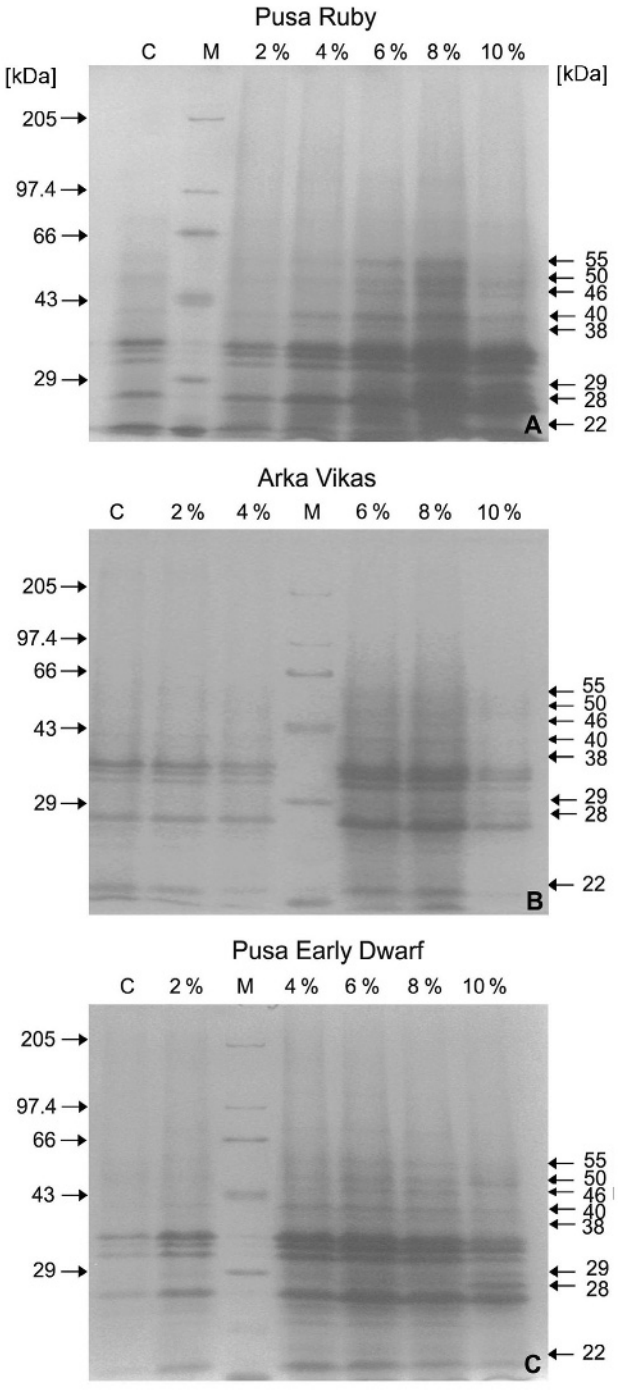

Fig. 1. Effect of mannitol (2 - 10 %) on total protein profile of leaves of the three tomato cultivars grown *in vitro* for 1 month. *A* - Protein profile of Pusa Ruby leaves; *lane* 1: control (C), *lane* 2: molecular mass markers (M), *lanes* 3 - 7: leaves cultured on 2, 4, 6, 8 and 10 % mannitol supplemented regeneration media, respectively. *B*, *C* - The same, of Arka Vikas and Pusa Early Dwarf leaves, respectively. In *B* and *C*, the M *lane* is shifted to the fourth and third positions, respectively.

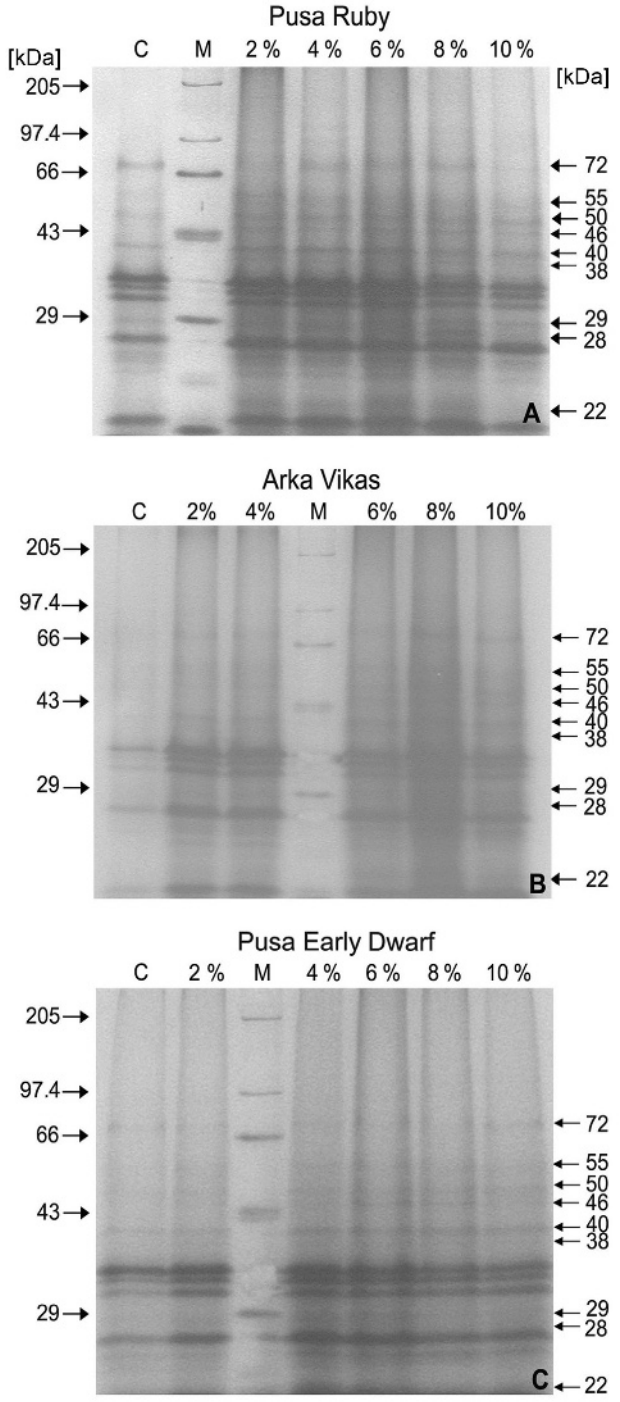

Fig. 2. Effect of 2 - 10 % PEG on polypeptide profiles of the three tomato cultivars. *A* - Protein profile of Pusa Ruby leaves, *lane* 1: control (C), *lane* 2: molecular mass markers (M), *lanes* 3 - 7: leaves cultured on 2, 4, 6, 8 and 10 % PEG supplemented regeneration media, respectively. *B*, *C* - The same, of Arka Vikas and Pusa Early Dwarf leaves, respectively. In *B* and *C*, the M *lane* is shifted to the fourth and third positions, respectively.

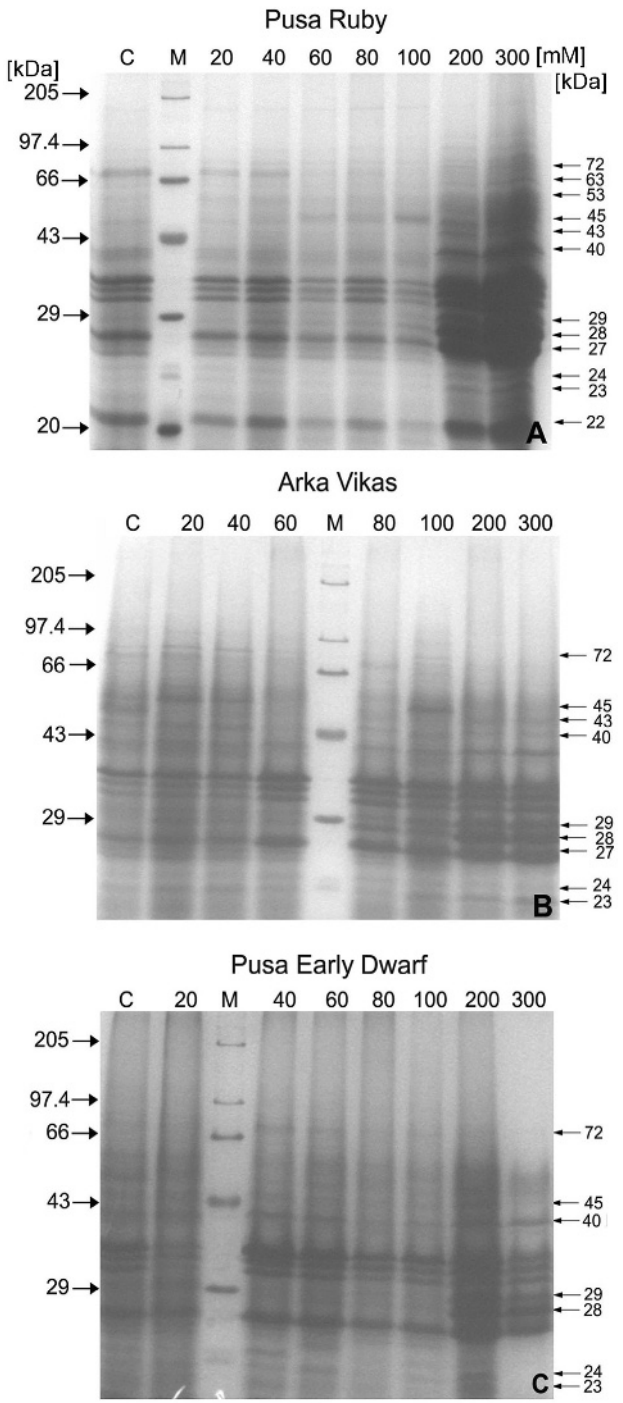

Fig. 3. Effect of 20 - 300 mM NaCl supplemented medium, on total protein profiles of the three tomato cultivars. *A* - Protein profile of Pusa Ruby leaves; *lane* 1: control (C), *lane* 2: molecular mass markers (M), *lanes* 3 - 9: leaves cultured on NaCl supplemented regeneration media. *B*, *C* - Total proteins of Arka Vikas and Pusa Early Dwarf leaves, respectively. In *B* and *C* the M *lane* is shifted to the fourth and third positions, respectively.

poorer. The protein profiles of Pusa Early Dwarf (Fig. 1*C*) also showed similar patterns of polypeptides but with lower intensities than those in Pusa Ruby. The protein profile pattern in Pusa Early Dwarf was better

than that of Arka Vikas and quite similar to Pusa Ruby but the intensities of 28 and 29 kDa polypeptides were less than in Pusa Ruby (Fig. 1*C*). The overall protein expression pattern was better in Pusa Ruby compared to the other two cultivars, in terms of their number as well as the intensity, indicating that Pusa Ruby is most tolerant to mannitol stress.

Explants grown on PEG supplemented media for one month, also showed stunted growth and reduction in the number of shoots. For all the three cultivars, 12 % concentration was lethal (data not shown). The SDS-PAGE of control explants of all the cultivars showed the same 12 polypeptides of 21, 26, 27, 32, 33, 35, 38, 40, 46, 50, 55 and 72 kDa (Fig. 2*A*-*C*) as mentioned above. In cv. Pusa Ruby (Fig. 2*A*), the intensity of these polypeptides progressively increased in explants grown on 2 to 10 % PEG. In addition, three new polypeptides (Mr 22, 28 and 29 kDa) were also induced. There was no remarkable difference between the protein profiles of explants cultured on mannitol (Fig. 1*A*) and PEG (Fig. 2*A*) supplemented media but the intensity of 29 kDa polypeptide was lower in PEG supplemented medium. Protein profile of Arka Vikas (Fig. 2*B*) was similar to that of Pusa Ruby but the protein bands were less intense. Protein profile of Pusa Early Dwarf (Fig. 2*C*) was also quite similar to Pusa Ruby, but the 29, 49, 50, 55 and 72 kDa polypeptides accumulated in very low intensity. Again, the protein profile results of explants grown under PEG stress showed better expression in Pusa Ruby than in the other two cultivars.

After one month of culture, 20 - 300 mM NaCl supplemented media severely affected caulogenic response of leaf explants, 200 mM being lethal for all cultivars. The control samples of all the three cultivars showed the same 12 polypeptides (Fig. 3*A*-*C*). Explants of Pusa Ruby cultured on NaCl supplemented medium showed higher intensity of these polypeptides and 9 new polypeptides of Mr 22, 23, 24, 28, 29, 43, 45, 53 and 63 kDa were induced (Fig. 3*A*). In contrast, the expression of 72 kDa polypeptide gradually declined with the rise in NaCl concentration. It was very interesting to note that though there was no morphogenesis on 200 and 300 mM NaCl, many polypeptides were highly expressed. Arka Vikas (Fig. 3*B*) and Pusa Early Dwarf (Fig. 3*C*) showed similar patterns of protein profiles but the intensity of 27, 28 and 29 kDa polypeptides were remarkably higher in Pusa Ruby compared to the other two cultivars. The results again indicated that, compared to other two cultivars, the proteins were better expressed in Pusa Ruby under salt stress.

Since PEG is a non-penetrating and non-ionic stress inducing compound, probably, it had a less detrimental effect on cells compared to mannitol (Gangopadhyay *et al.* 1997) or NaCl. Consequently, PEG brought less changes in cellular metabolism. NaCl is known to be the most detrimental osmotic agent (Gangopadhyay *et al.* 1997) and induced more new proteins compared to the other two osmotic agents used.

Several studies have shown upregulation of transcripts during different abiotic stresses in tolerant genotypes while they were absent in sensitive ones (Pelah *et al.* 1997, Jyothsnakumari *et al.* 2009, Roy *et al.* 2009). Similar results were obtained during the present study. The resistant cultivars can overcome the stress due to their ability to stimulate protein synthesis (Kawasaki *et al.* 2001). The present findings with resistant and sensitive cultivars are consistent with the published data. The results of the *in vitro* protein profile studies indicated that cultivar Pusa Ruby is the most tolerant cultivar among the three investigated. The results obtained *in vitro* coincide with the *in vivo* drought stress experiments carried out in these cultivars (Roy *et al.* 2009).

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