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# Salt tolerance of *Solanum tuberosum* L. overexpressing an heterologous osmotin-like protein

D. EVERS*, S. OVERNEY**, P. SIMON**, H. GREPPIN** and J.F. HAUSMAN*

*CRP-Centre Universitaire, CREBS,*
*162A, Avenue de la Faïencerie, L-1511 Luxembourg, Grand Duchy of Luxembourg**
*Laboratoire de Biochimie et de Physiologie Végétales,*
*3, place de l'Université, Université de Genève, CH-1211 Genève 4, Switzerland***

**Abstract**

Potato (*Solanum tuberosum* L. cv. Bintje) was transformed with a cDNA clone encoding an osmotin-like protein. Transgenic and non-transgenic *in vitro* plants were subjected to NaCl for 3 weeks. The shoot and root development was slightly affected by salinity indicating that the salt condition used was a mild stress. The endogenous proline content of the osmotin-like transformed clone only raised slightly as compared to the non-transformed genotype, where a marked increase in proline content could be observed as a result to salt stress. These data provide evidence for the involvement of osmotin-like proteins in the mechanisms of salt tolerance in potato plants.

*Additional key words*: adaptation, osmoprotectant, osmotin, potato, proline, salinity.

## Introduction

The progressive salinization of irrigated lands limits the future of agriculture in some of the most productive areas of the world. Therefore salinity is one of the major abiotic stresses affecting plants worldwide. The understanding of the adaptive mechanism by which the plants cope with the presence of salt in their environment still remains incomplete, partly because of the complexity of salt stress which presents an ionic component on the one hand and an osmotic component on the other hand. Plant cells respond to cellular dehydration caused by salinity by synthesis of low molecular mass organic compounds and/or accumulation of ions in the vacuole. To some extent, plant cells are able to restore the pressure potential of the cell. This

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Fax: (+352) 46 6644413, e-mail: evers@crpcu.lu

phenomenon has been called osmoregulation (*e.g.* Leshem and Kuiper 1996).

It is commonly accepted that the mechanisms of salt tolerance or salt adaptation are regulated by a number of genes. Among them, osmotin and osmotin-like proteins are encoded by a multigene family that is highly conserved in the *Solanaceae* (Zhu *et al.* 1995). Expression of these proteins is under the control of different environmental and hormonal stimuli (Singh *et al.* 1989, LaRosa *et al.* 1992, Zhu *et al.* 1993, 1996). It appears that osmotin-like proteins may play an important role in plant tolerance to different kinds of stresses, *e.g.*, osmotic stress (LaRosa *et al.* 1992), chilling (Zhu *et al.* 1996), and fungal infection (Liu *et al.* 1996, Abad *et al.* 1996). Osmotin was originally isolated from tobacco cell lines adapted to salts and indeed, numerous studies have shown a putative role for osmotin in osmotic stress (Kononowicz *et al.* 1992, LaRosa *et al.* 1992). Overexpression of an osmotin-like gene in plants is thus an interesting tool to study the effect of salt stress in higher plants.

Another well known phenomenon in response to salt stress is the accumulation of proline (*e.g.* Rhodes *et al.* 1986, Rhodes 1987, Hervieu *et al.* 1995, Lutts *et al.* 1996). Although the precise role of this accumulation is not yet well established, the study of proline as a marker of salt tolerance is generally accepted (Lin and Kao 1996, Lutts *et al.* 1996). Proline accumulates in the cytoplasm without having any detrimental effect on cytosolic enzyme activities (Lutts *et al.* 1996). Some authors suggest that it plays a role in the intracellular osmotic adjustment between the cytoplasm and the vacuole (Delauney and Verma 1993). Venekamp (1989) suggests that proline overproduction in stressed conditions is an attempt to regulate cytosolic pH.

In the present work, we investigated whether salt tolerance can be altered by genetically manipulating the expression of an osmotin-like protein from *Arabidopsis thaliana* in transgenic potato (Capelli *et al.* 1997). Transgenic plants showing overexpression of the gene were assayed for salt stress. In this first study, morphological attributes and the evolution of proline content were followed in order to assess their implication in the adaptation of non-transgenic and of transgenic potato plants to salt.

## Materials and methods

**Plants and culture conditions:** *In vitro* potato (*Solanum tuberosum* L. cv. Bintje) plantlets (S0) were axenically cultured, in tubes or polyethylene jars, on a multiplication medium (Murashige and Skoog 1962; MS) supplemented with 0.8 % bactoagar (*Difco*) and 3 % sucrose. Plant culture maintenance and/or multiplication were realized by monthly cutting potato shoots into 3 to 4 node pieces with subsequent sub-culturing on new multiplication medium.

***Agrobacterium* strain and plant transformation:** *Agrobacterium tumefaciens* strain LBA 4404 carrying the plasmid pBinosm was used for plant inoculations. In this plasmid, the osmotin-like cDNA lacking a fragment encoding a short C-terminal extension, was put under the control of the CaMV35S + 1/2 promoter and the poly-adenylation signal of the nopaline synthase gene, and was inserted into the polylinker

site of the vector pBin19 (Bevan 1984). The neomycine phosphotransferase II (NPTII) gene in T-DNA providing the resistance to kanamycin was used as the marker. Potato stem sections were transformed as described by Barker *et al.* (1992, 1994). Amplification by the polymerase chain reaction (PCR) of NPTII gene was performed to confirm the presence of the T-DNA from the binary vector pBinosm in the kanamycin-resistant plants. Genomic DNA of potato was extracted from leaf tissues of transgenic and control (non transgenic) plants as described by Edwards *et al.* (1991). Overexpression of the transgene was controlled both at the messenger (Northern analysis) and protein level (SDS-PAGE). The transgenic potato clone (S3) was used for the salt stress analyses.

**Salt stress experiments:** Freshly cut apical shoots (15 mm long) were kept on MS0 medium (multiplication medium without NaCl). For salt stress treatments, isolated new plantlets (7 d after cutting, 15 shoots per jar) were transferred on MS medium supplemented with NaCl to a final concentration of 100 mM or on fresh MS0 medium (control) for 21 d.

Morphological features were measured after 21 d of cultivation on salt-containing and control media. The shoot and the root lengths corresponded to the longest shoot and root per explant. The number of roots per explant was established by taking into account only rooted shoots. Dry mass of the explants was determined by drying until constant mass was achieved.

For proline determination, whole shoots were sampled after 0, 1, 3, 7, 15, and 21 d of culture on control and on NaCl-containing media. Free proline content was determined by colorimetry after extraction of fresh matter with salicylic acid under agitation for 1 h (Bates *et al.* 1973).

For data analyses, *ANOVA* analysis of variance and Student-Newman-Keuls tests were used for multiple comparison of treatment means.

## Results and discussion

The two genotypes used in this work showed different behaviour already in non-stressing conditions. Indeed, the S3 shoots were significantly smaller than the S0 shoots after 21 d of culture on MS0 medium (Fig. 1*A*). The number of nodes per shoot was significantly higher for S3 plants as compared to the S0 plants (Fig. 1*C*). This higher number of buds indicates a reduction of cell elongation (Bandurski *et al.* 1995). Cell elongation is modulated by two interrelated processes, osmotic uptake of water and extension of the existing cell wall (Cleland 1986). The transformation of the plant with a continuously-expressed gene coding for an osmotin-like protein could interfer with the uptake of the osmotic solutes needed to maintain a continuous growth. It can be hypothesized that this effect on cell elongation interacts with auxin production in the shoots. This could explain the evolution observed for the rooting system of the two genotypes, *i.e.* the transformed plants have longer and more roots as compared to the S0 (Fig. 1*E,F*). Indeed, expression phase of the rooting has been correlated with reduced endogenous content of auxin (Gaspar *et al.* 1992, 1994).

In a previous work, we studied the effect of different salts like NaCl, $Na_2SO_4$, $MgCl_2$ and $MgSO_4$ supplied at concentrations up to 300 mM on potato. We have shown that S0 potato plants grown *in vitro* on 100 mM salt-containing media were or were not able to reverse the stress depending on the concentration and the toxicity of the ions (Evers *et al.* 1998). Shoot and root development were affected first, followed at higher salt concentrations by shoot and root necrosis (Evers *et al.* 1997, 1998). The results of the present work show that explant viability was not affected by 100 mM NaCl since no shoot necrosis and no significant reduction of fresh and dry masses were observed for the S0 and S3 genotypes after 21 d of culture on salt-containing medium (Table 1).

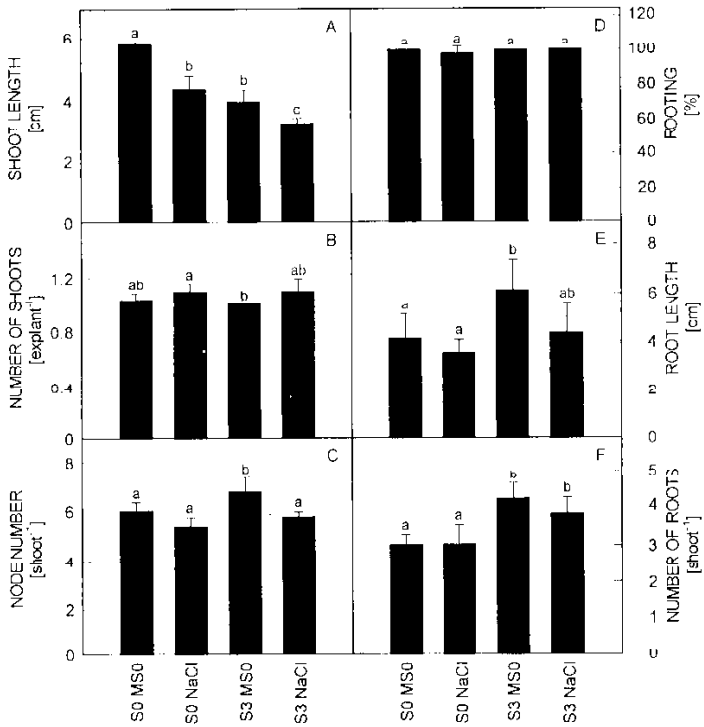


Fig. 1. Effect of salinity (100 mM NaCl-supplemented MS0-medium) on shoot length (*A*), number of shoots per explant (*B*), node number per shoot (*C*), rooting percentage (*D*), root length (*E*), and number of roots per rooted explant (*F*) of non-transgenic (S0) and osmotin-like transgenic (S3) potato plants. Means ± SD of 50 explants. Different letters indicate significant differences at $P = 0.05$ and $P = 0.01$ (for node number).

Clear reduction of the shoot length and of the root length was found under salt stress (Fig. 1*A*,*E*). The reduction of the shoot length was more important in the case of the S0 plants (-25 %) as compared to the S3 plants (-18 %). The shoot length was 3.9 cm in average for the S3 plants after 21 d on MS0 medium as compared to 5.8 cm

for the S0 plants. Similar results have been observed in *Carthamus* by Gadallah (1996), in rice by Lutts *et al.* (1996) and in potato by Evers *et al.* (1998).

Shoot production per explant was not affected by 100 mM NaCl (Fig. 1*B*); the number of nodes per shoot remained unchanged in the case of the S0 genotype, whereas a reduction could be observed in the case of the S3 genotype (Fig. 1*C*).

Table 1. Fresh and dry masses of non-transgenic (S0) and osmotin-like transgenic (S3) potato plants cultivated on MS0 and 100 mM NaCl-supplemented media. Means ± SD of 50 explants. Different letters indicate significant differences at $P = 0.05$.

| Conditions | Fresh mass [mg shoot$^{-1}$] | Dry mass [mg shoot$^{-1}$] |
|---|---|---|
| S0 MS0 | 63.4 ± 7.0 a | 5.4 ± 0.6 x |
| S0 NaCl | 62.1 ± 12.9 ab | 5.6 ± 1.2 xy |
| S3 MS0 | 82.8 ± 20.3 ab | 7.6 ± 1.6 y |
| S3 NaCl | 74.9 ± 6.8 b | 6.9 ± 0.7 y |

All the plants rooted at 100 % in both control and salt conditions after 21 d of culture. Thus the rooting percentage was not affected by salinity (Fig. 1*D*). The S3 plants had longer roots than the S0 plants, *i.e.* 6.2 cm in average for the S3 plants as compared to 4.1 cm in average for the S0 plants after 21 d on MS0 medium. Salinity induced a significant reduction of the root length in the S3 plants (-29 %) as compared to the control plants on MS0 medium but not in the S0 plants (Fig. 1*E*).

In both S0 and S3 genotypes, NaCl did not affect the number of roots per shoot (Fig. 1*F*). A higher number of roots per shoot was observed in the S3 plants than in the S0 plants in both control and salt-stressing condition.

According to Lichtenthaler's (1996) concept of stress, the condition used in the present work should be considered as a mild stress, as it does not induce any damaging effects to the plants, *i.e.* only slightly different growth of roots and shoots was observed.

Proline is commonly considered to act as a compatible osmotic solute. It is believed to protect plant tissues against stressing agents, however its role continues to be controversial (Lin and Kao 1996). A drastic accumulation of proline in plants under salt stress has been commonly observed (Hervieu *et al.* 1995, Lin and Kao 1996, Lutts *et al.* 1996). In the present work, the proline content of the S0 genotype cultivated on the MS0 medium was slightly higher than the proline content of the transformed genotype which remained at a very low level all over the cultivation time (Fig. 2). Under NaCl stress, a drastic and continuous increase beginning on day 1 of cultivation was observed in the case of the S0 clone, whereas the proline content of the transformed clone raised only moderately. Measuring the ratio of proline content for stressed/non-stressed plants, a drastic increase is observed for both genotypes during the first 3 d of culture followed by a stabilization until 21 d (Fig. 2, inset). According to the literature, the results concerning the proline content indicate a better tolerance to salt of the osmotin-like transformed potato plants. Indeed, Lutts *et al.* (1996) observed higher proline concentrations in salt-sensitive vs. salt-tolerant rice

cultivars. Since growth is generally considered to be an energy-requiring process, elevated proline levels caused by NaCl are most likely acting as a way to save energy by inhibiting growth and as a readily utilizable source of energy and amino groups once NaCl is relieved. Lin and Kao (1996) suggested that proline accumulation in NaCl-treated roots and shoots is associated with the ionic rather than the osmotic component of NaCl stress.

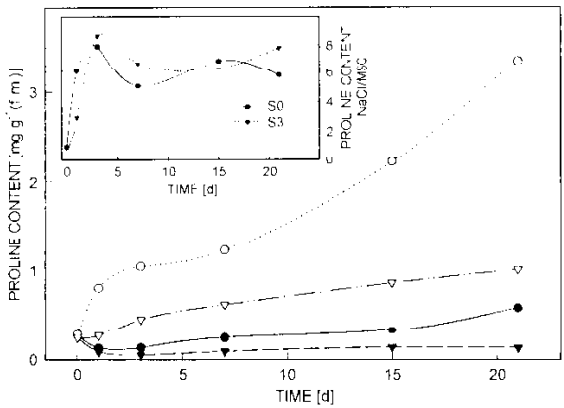


Fig. 2. Effect of salinity (100 mM NaCl-supplemented MS0-medium) on proline content of non-transgenic (S0) (*closed circles* - MS0 medium, *open circles* - NaCl-supplemented medium) and osmotin-like transgenic (S3) (*closed triangles* - MS0 medium, *open triangles* - NaCl-supplemented medium) potato plants. Data from a representative experiment. The ratio of the proline content of the explants on salt-supplemented medium divided by the proline content of the explants on MS0 medium is shown in the inset.

In the present study, the pattern of a natural salt tolerance mechanism was modified by overexpressing an osmotin-like protein in transgenic potato plants in order to enhance salt tolerance. Osmotin is known to be the product of an osmotically induced gene (Vartanian 1996). Osmotin is a basic isoform of pathogenesis-related protein group 5 that has been shown to possess antifungal activity (Liu *et al.* 1996). It accumulates abundantly in NaCl- and desiccation-adapted cells of tobacco (Singh *et al.* 1985, 1987b). The osmotin gene is regulated by a variety of environmental and hormonal signals including wounding, ethylene, salt, abscisic acid, desiccation, UV light, fungal infections (Chang *et al.* 1995, Liu *et al.* 1994, Nelson *et al.* 1992, Singh *et al.* 1987a, 1989). In this work, we attempted to study the role of osmotin-like protein genes in salt tolerance. Our results revealed no significant morphological differences between the non-transformed and the transformed genotypes exposed to salt stress. However, considering proline, a well-known marker of salt stress, significant differences between the two genotypes were found, indicating different levels in salt tolerance.

A next step consists in studying ion accumulation rates and distribution in different organs of the plants. In fact, salinity stress frequently induces an increase in Na and Cl content as well as a decrease in K, Ca, $NO_3$ and P concentrations (Lutts *et al.*

1996). The present plant system seems to be particularly interesting to study ion distribution as the pattern of ion distribution differs among genotypes differing in salt tolerance (Lutts *et al.* 1996). A correlation could be drawn between the differential accumulation of proline in genotypes differing in their resistance to salt stress and the accumulation of toxic ions. In parallel, we also intend to investigate the distribution and the evolution of soluble carbohydrates especially those known to be osmoprotectants.

## References


Abad, L.R., Paino d'Urzo, M., Liu, D., Narasimham, M.L., Reuveni, M., Zhu, J.K., Niu, X., Singh N.K., Hasegawa, P.M., Bressan, R.A.: Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. - Plant Sci. **118**: 11-23, 1996.

Bandurski, R.S., Cohen, J.D., Slovin, J.P., Reinecke, D.M.: Auxin biosynthesis and metabolism. - In: Davies, P.J. (ed.): Plant Hormones. Pp. 39-65. Kluwer Academic Publishers. Dordrecht - Boston - London 1995.

Barker, H., Reavy, B., Kumar, A., Webster, K.D., Mayo, M.A.: Restricted virus multiplication in potatoes transformed with the coat protein gene of potato leafroll luteovirus: similarities with a type of host gene-mediated resistance. - Ann. appl. Biol. **120**: 55-64, 1992.

Barker, H., Webster, K.D., Jolly, C.A., Reavy, B., Kumar, A., Mayo, M.A.: Enhancement of resistance to potato leafroll virus multiplication in potato by combining the effects of host genes and transgenes. - Mol. Plant-Microbe Interact. 7: 528-530, 1994.

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water stress studies. - Plant Soil **39**: 205-207, 1973.

Bevan, M.W.: Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res. **12**: 8711-8721, 1984.

Capelli, N., Diogon, T., Greppin, H., Simon, P.: Isolation and characterization of a cDNA clone encoding an osmotin-like protein from *Arabidopsis thaliana*. - Gene **191**: 51-56, 1997.

Chang, P.F.L., Cheah, K.T., Narasimhan, M.L., Hasegawa, P.M., Bressan, R.A.: Osmotin gene expression is controlled by elicitor synergism. - Physiol. Plant. **95**: 620-626, 1995.

Cleland, R.: The role of hormones in wall loosening and plant growth. - Aust. J. Plant Physiol. **13**: 93-103, 1986.

Delauney, A.J., Verma, D.P.S.: Proline biosynthesis and osmoregulation in plants. - Plant J. **4**: 215-223, 1993.

Edwards, K., Johnstone, C., Thompson, C.: A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. - Nucl. Acids Res. **19**: 1349, 1991.

Evers, D., Schmit, C., Mailliet, Y., Hausman, J.F.: Growth characteristics and biochemical changes of poplar shoots *in vitro* under sodium chloride stress. - J. Plant Physiol. **151**: 748-753, 1997.

Evers, D., Hemmer, K., Hausman, J.F.: Salt stress induced biometric and physiological changes in *Solanum tuberosum* L. cv Bintje grown *in vitro*. - Acta Physiol. Plant. **20**: 3-7, 1998.

Gadallah, M.A.A.: Abscisic acid, temperature and salinity interactions on growth and some mineral elements in *Carthamus* plants. - Plant Growth Regul. **20**: 225-236, 1996.

Gaspar, T., Kevers, C., Hausman, J.F., Berthon, J.Y., Ripetti, V.: Practical uses of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots. - Agronomie **12**: 757-765, 1992.

Gaspar, T., Kevers, C., Hausman, J.F., Ripetti, V.: Peroxidase activity and endogenous free auxin during adventitious root formation. - In: Lumsden, P.J., Nicholas, J.R., Davies, W.J. (ed.): Physiology, Growth and Development of Plants in Culture. Pp. 289-298. Kluwer Academic Publishers. Dordrecht - Boston - London 1994.

Hervieu, F., LeDily, F., Huault, C., Billard, J.P.: Contribution of ornithine aminotransferase to proline accumulation in NaCl-treated radish cotyledons. - Plant Cell Environ. **18**: 205-210, 1995.

Kononowicz, A.K., Nelson, D.E., Singh, N.K., Hasegawa, P.M., Bressan, R.A.: Regulation of the osmotin gene promoter. - Plant Cell **4**: 513-524, 1992.

LaRosa, P.C., Chen, Z., Nelson, D.E., Singh, N.K., Hasegawa, P.M., Bressan, R.A.: Osmotin gene expression is posttranscriptionally regulated. - Plant Physiol. **100**: 409-415, 1992.

Leshem, Y.Y., Kuiper, P.J.C.: Is there a GAS (general adaptation syndrome) response to various types of environmental stress? - Biol. Plant. **38**: 1-18, 1996.

Lichtenthaler, H.K.: Vegetation stress: an introduction to the stress concept in plants. - J. Plant Physiol. **148**: 4-14, 1996.

Lin, C.C., Kao, C.H.: Proline accumulation is associated with inhibition of rice seedling root growth caused by NaCl. - Plant Sci. **114**: 121-128, 1996.

Liu, D., Raghothama, K.G., Hasegawa, P.M., Bressan, R.A.: Osmotin overexpression in potato delays development of disease symptoms. - Proc. nat. Acad. Sci. USA **91**: 1888-1892, 1994.

Liu, D., Rhodes, D., Paino D'Urzo, M., Xu, Y., Narasimhan, M. L., Hasegawa, P.M., Bressan, R.A., Abad, L.: *In vivo* and *in vitro* activity of truncated osmotin that is secreted into the extracellular matrix. - Plant Sci. **121**: 123-131, 1996.

Lutts, S., Kinet, J.M., Bouharmont, J.: Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. - Plant Growth Regul. **19**: 207-218, 1996.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 473-497, 1962.

Nelson, D.E., Raghothama, K.G., Singh, N.K., Hasegawa, P.M., Bressan, R.A.: Analysis of structure and transcriptional activation of an osmotin gene. - Plant mol. Biol. **19**: 577-588, 1992.

Rhodes, D.: Metabolic responses to stress. - In: Stumpf, P.K., Conn, E.E. (ed.): The Biochemistry of Plants: A Comprehensive Treatise. Vol. 12. Physiology of Metabolism. Pp. 201-241. Academic Press, San Diego 1987.

Rhodes, D., Handa, S., Bressan, R.A.: Metabolic changes associated with adaptation of plant cells to water stress. - Plant Physiol. **82**: 890-903, 1986.

Singh, N.K., Handa, A.K., Hasegawa, P.M., Bressan, R.A.: Proteins associated with adaptation of cultured tobacco cells to NaCl. - Plant Physiol. **79**: 126-137, 1985.

Singh, N.K., Bracker, C.A., Hasegawa, P.M., Handa, A.K., Buckel, S., Hermodson, M.A., Pfankoch, E., Regnier, F.E., Bressan, R.A.: Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. - Plant Physiol. 85: 529-536, 1987a.

Singh, N.K., LaRosa, P.C., Handa, A.K., Hasegawa, P.M., Bressan, R.A.: Hormonal regulation of protein synthesis associated with salt tolerance in plant cells. - Proc. nat. Acad. Sci. USA **84**: 739-743, 1987b.

Singh, N.K., Nelson, D.E., Kuhn, D., Hasegawa, P.M., Bressan, R.A.: Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. - Plant Physiol. **90**: 1096-1101, 1989.

Vartanian, N.: Mutants as tools to understand cellular and molecular drought tolerance mechanisms. - Plant Growth Regul. **20**: 125-134, 1996.

Venekamp, J.H.: Regulation of cytosol acidity in plants under conditions of drought. - Physiol. Plant. **76**: 112-117, 1989.

Zhu, B., Chen, T.H.H., Li, P.H.: Expression of an ABA responsive osmotin-like gene during the induction of freezing tolerance in *Solanum commersonii*. - Plant mol. Biol. **21**: 729-735, 1993.

Zhu, B., Chen, T.H.H., Li, P.H.: Activation of two osmotin-like protein genes by abiotic stimuli and fungal pathogen in transgenic potato plants - Plant Physiol. **108**: 929-937, 1995.

Zhu, B., Chen, T.H.H., Li, P.H.: Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. - Planta **198**: 70-77, 1996.