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Short communication

Regulation of *Glyine max* Ornithine Decarboxylase by Salt and Spermine

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We examined the effect of CsCl and spermine on the induction of ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis form *Glycine max* axes. Transcription of the ODC gene was induced by 0.1 and 1 mM of CsCl, and the amount of putrescine was increased 3.5-fold by 1 mM CsCl treatment. Spermine also induced the expression of the ODC gene in a dose dependent manner. However, CsCl provoked an increase in the active phosphorylated ERK (pERK), a central element of the mitogen-activated protein kinase (MAPK) cascade. Our data demonstrates an interaction between the ODC induction and the MAPK signaling pathway, and suggests that the latter may be involved in cell signaling in salt-stressed plants.

Keywords: Ornithine decarboxylase, Cesium chloride, Spermine, pERK

Introduction

Polyamines are low molecular weight cations at physiological pH. They are widely distributed in all living organisms. Polyamine levels and biosynthesis may be an integral part of the response mechanism in plants to various environmental stresses, such as mineral deficiency, low pH, osmotic stress, and salts (Tiburcio *et al.*, 1990). In animals, plants and fungi, ornithine decarboxylase (ODC) catalyzes the first and rate-limiting step in polyamine biosynthesis (Tabor and Tabor, 1984; Pegg, 1986; Heby and Persson, 1990), the decarboxylation of ornithine to produce putrescine. ODC is known to be one of the most highly regulated enzymes (Seiler and Heby, 1988). It also has a very short half-life.

Regulation of ODC can be found at the transcriptional and translational levels, as well as the levels of mRNA and protein stability (Murakami *et al.*, 1985; Katz and Kahana, 1987; Persson *et al.*, 1988; Grens and Scheffler, 1990; Chen and Chen, 1992). Unlike bacteria and mammals, plants have an additional pathway to form putrescine from arginine by arginine decarboxylase (ADC) (Tiburcio *et al.*, 1993, 1994). ADC activity levels have been correlated with many abiotic stresses in plants. Plants stimulate putrescine accumulation by the activity of the stress-induced ADC, when subjected to stresses, such as potassium deficiency, pH (Young and Galston, 1983), osmotic stress (Flores and Galston, 1982), nutrient (Basso and Smith, 1974), UV light (Kramer *et al.*, 1991), and pollutants (Priebe *et al.*, 1978). The role of the ADC pathway in the putrescine accumulation under stress conditions is relatively well established. However, the importance of the relative ODC pathway in putrescine accumulation during stress conditions is still poorly understood.

In mammalian cells, the activation of the three mitogen-activated protein kinase (MAPK) pathways, JNK (also known as stress-activated protein kinase (SAPK)), extracellular signal-regulated kinase (ERK) (Itoh *et al.*, 1994; Matsuda *et al.*, 1995), and p38 by osmotic stress has been shown so far. In recent studies, the signaling pathway that leads to the induction of ornithine decarboxylase by ERK in human ECV40 cells was also reported (Flamigni *et al.*, 2001). With the aim of understanding the molecular mechanism that regulates the ODC gene expression by CsCl and spermine, we determined how the ODC activity and expression level changed in salt stressed soybean axes. In addition, we studied whether the ERK pathway was essential for ODC induction that is stimulated by CsCl.

Materials and Methods

Overproduction of *N. glutinosa* ODC in *E. coli* Cloning of the *Nicotian glutinosa* cDNA library was performed as previously described (Lee and Cho, 2001). *E. coli* BL 21 (DE3) was transformed with the pGEX2T-ODC plasmid. The transformants were tested for the ODC expression upon induction with IPTG. *E. coli* BL 21 (DE3) cells, carrying the expression plasmid containing *N. glutinosa* ODC, were grown overnight at 37°C in a LB medium (containing 50 μg/ml ampicillin) (Sambrook *et al.*, 1989). The cells were diluted 100-fold into the same medium and allowed to grow until A_{600} reached 0.5. To induce the expression of the gene, IPTG

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was added to the culture to produce a final concentration of 1 mM. The cells were incubated for 4 h, harvested by centrifugation (5,000 × *g*; 10 min) and sonicated in phosphate-buffered saline (PBS). The cell lysate was centrifuged at 13,000 × *g* for 20 min. The resulting supernatant was used for purification on Glutathione-S-transferase Sepharose 4B resin in a batch procedure, according to the manufacturer's recommendations. Recombinant *N. glutinosa* was recovered from the fusion protein by thrombin cleavage, then purified according to the manufacturer's recommendations.

Antibody preparation The purified *N. glutinosa* ODC was used to immunize the rat. Two hundred μg of the enzyme that was dissolved in PBS, containing 1 mM DTT, was emulsified with Freund's complete adjuvant. It was used for the first injection. One month later the rat was injected twice at intervals with 200 μg each of the enzyme in Freund's incomplete adjuvant. The rat was bled 10 days after the last injection. Two ml of antiserum was centrifuged and stored at −20°C.

Plant material Soybean seeds (*Glycine max*) were sterilized with 1% sodium hydrochloride, then washed extensively with distilled water. Sterilized seeds were germinated at 25°C in the dark. Soybean seeds were grown for 3 days in the dark in 0.1, 1, and 10 mM of CsCl, NaCl, and spermine. After germination, the axes were collected and stored at −70°C for future use.

Polyamine extraction and analysis An equal volume of 20% (w/v) trichloroacetic acid was added to the salt treated *Glycine max* axes. The mixture was vortex-mixed vigorously and centrifuged at 13,000 rpm for 10 min. The dansylated polyamines were separated on silica gel 60 plates with a chloroform/triethylamine (25 : 2, v/v) solvent system.

Enzyme activity assay The *Glycine max* ODC activity was assayed at 37°C for 60 min by the liberation of $^{14}CO_2$ from L-[carboxy^{14}C]ornithine as a substrate (Kim and Cho, 1993). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol $^{14}CO_2$/h. The protein concentration was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

Gel electrophoresis and immunoblot analysis SDS-polyacrylamide gel electrophoresis was performed by the Laemmli method (Laemmli, 1976) using a Tris-Glycine buffer [25 mM Tris, 200 mM Glycine, 0.1% SDS (w/v), pH 8.3] and a 12.5% separating gel. For immunoblot analysis, after SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes (Millipore. Bedford, MA). The immunocomplex with antibodies prepared against the recombinant *Nicotiana glutinosa* ODC was detected with the alkaline phosphatase Western-blot analysis system (Boehringer Mannheim). Bands for the immunoblots were scanned with a UVP Easy Digital Image analyzer to comparatively quantify activities and protein values, which were expressed as a percentage of the reference (that of the 3-day old *Glycine max* axes that were only grown in water).

Western blot analysis for ERK protein Immunoblot analyses were performed in order to determine the expression level of the phospho-ERK protein in *G. max* axes. Aliquots of the samples were mixed with a sample buffer and heated at 95°C for 15 min. The respective protein samples were applied to a 5% polyacrylamide gel and subjected to electrophoresis. The prestained SDS-PAGE standard (broad range, Bio-Rad, Hercules, USA) was used as a molecular weight marker. The proteins were then transferred onto a nitrocellulose membrane using a transblot chamber with a Tris buffer. The membranes were incubated with the rat anti-rabbit pERK (HRP conjugated, DAKO, Glostrup, Denmark) for 2 h at room temperature, then subjected to ECL Western blot (Amersham

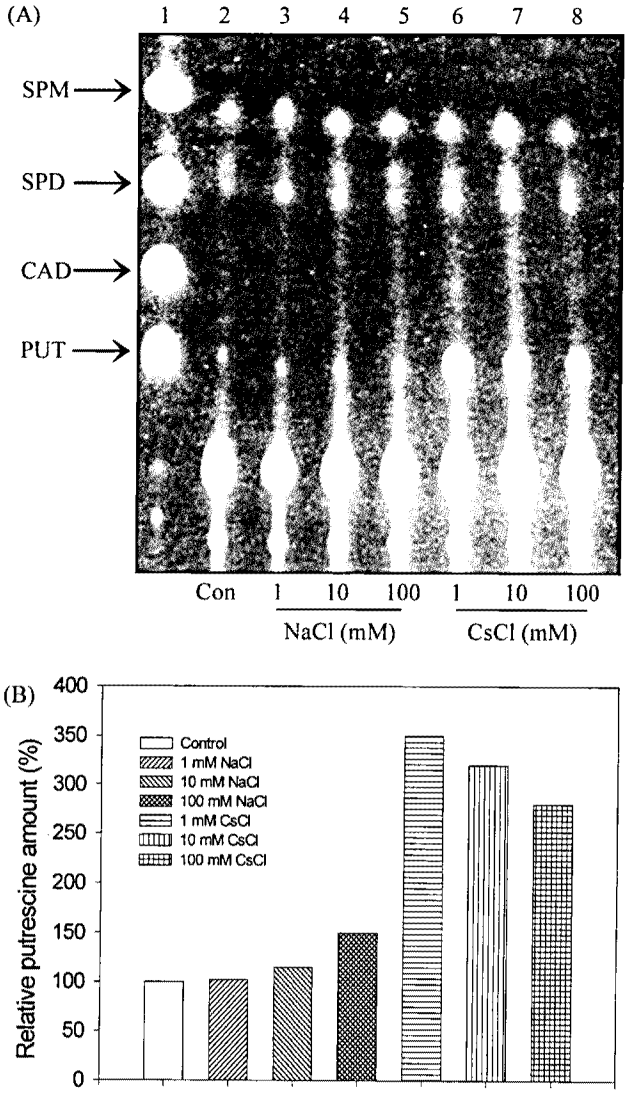

Fig. 1. Detection of putrescine from NaCl and CsCl treated *Glycine max* axes. (A) Polyamines were derivatized with dansyl chloride and subjected to a TLC analysis. Lane 1, Polyamine standard, putrescine (PUT), cadaverine (CAD), spermidine (SPD), spermine (SPM); Lane 2, control axes; Lane 3, axes grown at 1 mM NaCl; Lane 4, axes grown at 10 mM NaCl; Lane 5, axes grown at 100 mM NaCl; Lane 6, axes grown at 1 mM CsCl; Lane 7, axes grown at 10 mM CsCl; Lane 8, axes grown at 100 mM CsCl. (B) The relative putrescine amounts are shown as bar diagrams. The data are presented as a percentage of the control amount of putrescine grown with no salts.

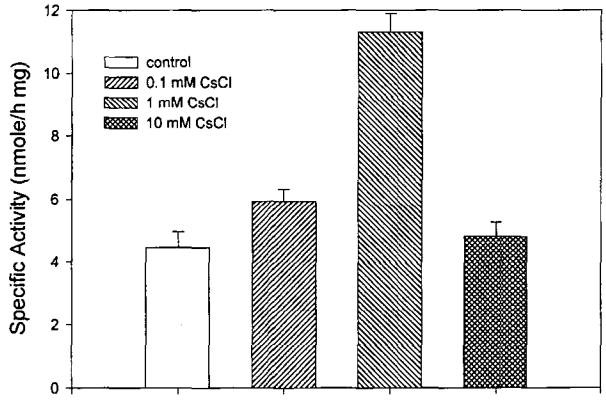

Fig. 2. Effect of CsCl on ODC activity of *Glycine max* axes. Enzyme activity was measured in a 40 mM Tris buffer (pH 8.0) that contained 2 mM PLP, 2 mM DTT and 0.1 mM EDTA at 37°C for 1 h using L-[carboxy^{14}C]ornithine as a substrate. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 nmol of $^{14}CO_2$ per hour. Values are mean±SE obtained from the three independent experiments.

International Inc., Buckinghamshire, England). Positive immunoreactive bands were quantified densitometrically and compared to the controls.

Results and Discussion

Effect of salts and spermine on ODC and putrescine levels

Apart from the well-documented physiological regulation of ODC activities in animal cells, little is known about the metabolic or molecular mechanisms that regulate the synthesis of ODC in plants. When *Glycine max* axes were grown in water that contained 0.1 to 10 mM of CsCl, NaCl, or spermine, the growth of the axes decreased as the salt concentrations increased. However, all of the axes that were grown for three days had the same amount of protein. To study the effects of salt on putrescine levels of *G. max* axes, samples were derivatized with dansylchloride and separated on silica gel TLC plates. There was a marked increase (3.5-fold) in the putrescine levels with the 1 mM CsCl treatment, but NaCl did not significantly change the putrescine level. The ODC activity was measured in order to study the possible relationship between CsCl induced putrescine levels and the activation of the putrescine synthesizing enzyme. Figure 2 shows the changes in the specific ODC activity by the CsCl treatment. The ODC activity increased by more than 2-fold at 1 mM of the CsCl treatment, and slowly decreased at 10 mM of CsCl. We also analyzed the activity of ADC (the alternative pathway for putrescine biosynthesis) in both NaCl and CsCl treated *G. max* axes, but the ADC activity did not change significantly (data not given). In an attempt to explain the increase in the putrescine level by the CsCl treatment that is associated with enhancement of the ODC enzyme activity, an anti-*N. glutinosa* antibody was used to detect the induction of ODC expression levels (Fig. 3). In plants, ODC and ADC

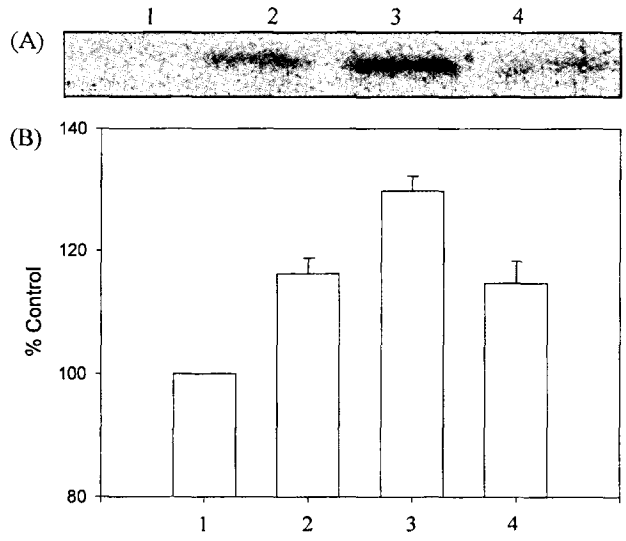

Fig. 3. Effects of CsCl on the induction of *Glycine max* ODC. (A) Total protein form axes grown at different concentrations of CsCl were loaded in each lane for Western blot analysis using an anti-*N. glutinosa* ODC antibody. Lane 1, control ODC; Lane 2, ODC induction from 10 mM CsCl treated axes; Lane 3, ODC induction from 1 mM CsCl treated axes; Lane 4, ODC induction from 0.1 mM CsCl treated axes. (B) Protein immunoblots from *Glycine max* axes treated as in (A) ($n = 3$ for each group) were analyzed by densitometry and expressed as a percentage of the control.

appear to have specific roles in plant growth and physiology (Tabor and Tabor, 1984; Evans and Malmberg, 1989). In osmotically stressed oat leaves, putrescine accumulates dramatically and activates the pathway catalyzed by ADC (Borell *et al.*, 1996). This stress response occurred very rapidly during the first few hours of treatments, however, it then decreased rapidly (Flores and Galston, 1984). Since 3 day-old axes were used to detect the enzyme activities, the ADC activity may have reached negligible values at 72 h. Thus, the low activity of ADC and ODC prevented the accumulation of putrescine in NaCl treated axes. Sheahan *et al.* (1993) demonstrated that Cs^+ acts as a potassium transport analog. Watson *et al.* (1996) reported an increase of ADC and putrescine levels by adding 3 mM of CsCl to *Arabidopsis thaliana* seedlings. Differing from the reports on *A. thaliana*, *G. max* exhibited a peak of 1.5-fold increase in ODC protein levels at 10 mM of CsCl treatment. The decrease in putrescine and ODC induction levels above 10 mM of CsCl may due to the toxic effects on *G. max* axes (Santos *et al.*, 1999). These results suggest that plant species can respond differently in regulating putrescine levels under CsCl stress.

During our previous work on recombinant *N. glutinosa* ODC (Lee and Cho, 2001), spermine activated the ODC activity, which differs from the previous report on *Saccharomyces cerevisiae* ODC (Tyagi *et al.*, 1981). For a detailed study of the induction of ODC protein by spermine treatment, immunochemical studies were performed (Fig. 5).

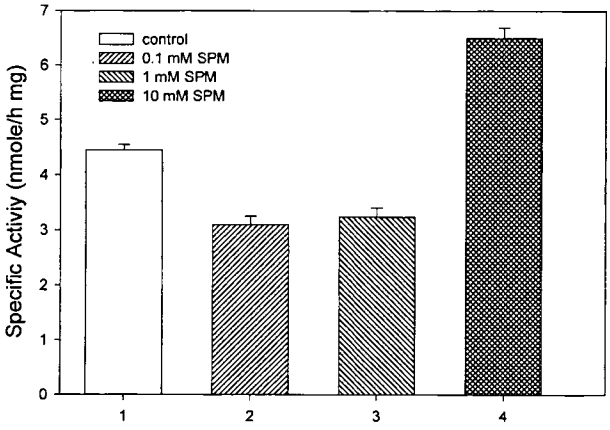

Fig. 4. Effects of spermine (SPM) on ODC activity of *Glycine max* axes. Enzyme activity was measured in a 40 mM Tris buffer (pH 8.0) that contained 2 mM PLP, 2 mM DTT and 0.1 mM EDTA at 37°C for 1 h using L-[carboxy^{14}C]ornithine as a substrate. Values are mean±SE obtained from the three independent experiments.

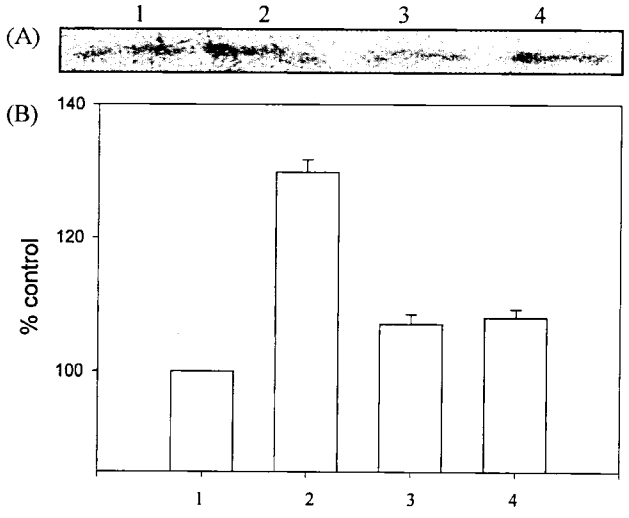

Fig. 5. Effects of spermine (SPM) on the induction of *Glycine max* ODC. (A) Total proteins from axes grown at different concentrations of spermine were loaded in each lane for Western blot analysis using an anti-*N. glutinosa* ODC antibody. Lane 1, control ODC; Lane 2, ODC induction from 10 mM spermine treated axes; Lane 3, ODC induction from 1 mM spermine treated axes; Lane 4, ODC induction from 0.1 mM spermine treated axes. (B) Protein immunoblots from *Glycine max* axes that were treated as in (A) ($n = 3$ for each group) were analyzed by densitometry and expressed as a percentage of the control.

The 35% increase in ODC protein levels at 10 mM with the spermine treatment indicates an induction of protein expression, rather than protein modification by spermine. This accounts for the increase in ODC activity (Fig. 4).

Activation of pERK by CsCl Recently, over 60 hormones, mitogens, and recognized signal transducers were reported to

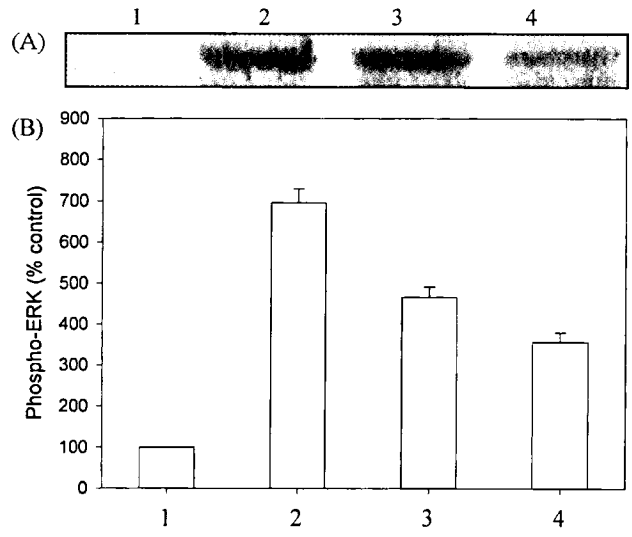

Fig. 6. phospho-ERK is activated by CsCl in *Glycine max* axes. (A) Total protein from axes grown at different concentrations of CsCl were loaded in each lane for Western blot analysis using an anti-phospho-ERK antibody, described in "Materials and Methods". Corresponding bands are as follows: Lane 1, control pERK; Lane 2, pERK induction from 10 mM CsCl treated axes; Lane 3, pERK induction from 1 mM CsCl treated axes; Lane 4, pERK induction from 0.1 mM CsCl treated axes. (B) Protein immunoblots from *Glycine max* axes that were treated as in (A) ($n = 3$ for each group) were analyzed by densitometry and expressed as a percentage of the control.

increase ODC activity in various target tissues and cells (Scalabrino *et al.*, 1991; Scalabrino and Lorenzini, 1991). Although a large variety of stimulus is able to induce ODC in the target cell, the involvement of MAPK subfamilies in ODC induction has scarcely been investigated. Quite recently, there were reports on the requirement of ERK on the expression of ODC in leukemia L1210 cells (Flamigni *et al.*, 1999) as well as the induction of ODC by histamine and ATP being mediated by ERK and p38 MAPK (Flamigni *et al.*, 2001). The present study shows that the CsCl induced ODC expression is mediated by ERK, the subtype of MAPK, in plants. Using antibodies that are specific for phosphorylated ERK (pERK), we performed an immunoblot analysis on samples that were grown in CsCl solutions (Fig. 6.). The increased rate of pERK was 3.5, 4.6, and 7 fold under 0.1, 1, and 10 mM of CsCl treatment, respectively. The correlation between the high expression of pERK and the increase in ODC expression levels are consistent with the results that have been reported in animal cells (Flamigni *et al.*, 1999, 2001). The intermediate steps from the extracellular signaling molecules to ERK, and to the down stream induction of ODC, have not been completely defined. In mammalian cells, a number of agonists that act on G-protein receptors stimulated ERK cascade through the involvement of either Gα or Gβr subunits. In some cases, the ERK activation was mediated by PKC, as well as some other routes independent of changes in

the PKC activation (Lopez-Ilsaca, 1998; Kim *et al.*, 1999). The signal cascade that leads to the ODC overexpression also remains largely unknown in plants, but this research shows that the ERK pathway is essential for ODC induction under CsCl stimulation. Additional studies on extracellular stress and signal transduction pathways will help define how the ODC gene is regulated in plants.

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References

Basso, L. C. and Smith, T. A. (1974) Effect of mineral deficiency on amine formation in higher plants. *Phytochemistry*, **3**, 875-883.

Borrell, A, Besfort, R. T., Masgrau, C. and Tiburcio, A. F. (1996) Regulation of arginine decarboxylase by spermine in osmotically stressed oat leaves. *Physiol. Plant* **98**, 105-110.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Chen, Z. P. and Chen, K. Y. (1992) Mechanism of regulation of ornithine decarboxylase gene expression by asparagine in a variant mouse neuroblastoma cell line. *J. Biol. Chem.* **267**, 6946-6951.

Evans, P. T. and Malmberg, R. L. (1989) Do polyamines have roles in plant development? *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 235-269.

Flamigni, F., Facchini, A., Capanni, C., Stefanelli, C., Tantini, B. and Caldarera, C. M. (1999) p44/42 mitogen-activated protein kinase is involved in the expression of ornithine decarboxylase in leukemia L1210 cells. *Biochem. J.* **341**, 363-369.

Flamigni, F., Facchii, A., Giordano, E., Tantini, B. and Stefaneli, C. (2001) Signaling pathway leading to the induction of ornithine decarboxylase: Opposite effect of p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK inhibitors. *Biochem. Pharmaol.* **61**, 25-32.

Flores, H. E. and Galston, A. W. (1982) Polyamines and plant stress: activation of putrescine by osmotic shock. *Science* **217**, 1259-1261.

Flores, H. E. and Galston, A. W. (1984) Osmotic stress-induced polyamine accumulation in cereal leaves. I. Physiological parameters of the response. *Plant Physiol.* **75**, 102-109.

Grens, A. and Scheffler, I. E. (1990) The 5'- and 3'-untranslated regions of ornithine decarboxylase mRNA affect the translational efficiency. *J. Biol. Chem.* **265**, 11810-11816.

Heby, O. and Persson, L. (1990) Molecular genetics of polyamine synthesis in eukaryotic cells. *Trends Biochem. Sci.* **15**, 153-158.

Itoh, T., Yamauchi, A., Miyai, A., Yokoyama, K., Kamada, T., Ueda, N. and Fujiwara, Y. (1994) Mitogen-activated protein kinase and its activator are regulated by hypertonic stress in Madin-Darby canine kidney cells. *J. Clin. Invest.* **93**, 2387-2392.

Katz, A. and Kahana, C. (1987) Transcriptional activation of mammalian ornithine decarboxylase during stimulated growth. *Mol. Cell. Biol.* **7**, 2641-2643.

Kim, D. C. and Cho, Y. D. (1993) Purification and properties of ornithine decarboxylase from *Glycine max* axes. *J. Biochem. Mol. Biol.* **26**, 192-197.

Kim, J. Y., Yang, M. S., Oh, C. D., Kim, K. T., Ha, M. J., Kang, S. S. and Cheu, J. S. (1999) Signaling pathway leading to an activation of mitogen-activated protein kinase by stimulating M3 muscarinic receptor. *Biochem. J.* **337**, 275-280.

Kramer, G. F., Norman, H. A., Krizek, D. T. and Mirecki, R. M. (1991) Influence of UV-B radiation on polyamines, lipid peroxidation and membrane lipids in cucumber. *Phytochemistry* **30**, 2101-2108.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, Y. S. and Cho, Y. D. (2001) in Supplement to Plant and Cell Physiology, Fukuoka, Japan, March 23-26, Abstract 654, pp s198.

Lopez-Ilsaca, M. (1998) Signaling from G-proteincoupled receptors to mitogen-activated protein (MAP)-kinase cascades. *Biochem. Pharmacol.* **56**, 269-277.

Matsuda, S., Kawasaki, H., Moriguchi, T., Gotoh, Y. and Nishida, E. (1995) Activation of protein kinase cascades by osmotic shock. *J. Biol. Chem.* **270**, 12781-12786.

Murakami, Y., Fujita, K., Kameji, T., Otsonomia, K., Kanamoto, R. and Hyashi, S. (1985) in *Recent Progress in Polyamine Research*, Selmeci, L., Brosnasl, M. E. and Eiler, N. (eds.), pp. 75-85, Akademai, Kiado, Budapest.

Pegg, A. E. (1986) Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* **234**, 249-62.

Persson, L., Holm, I. and Heby, O. (1988) Regulation of ornithine decarboxylase mRNA translation by polyamines, Studies using a cell-free system and a cell line with an amplified ornithine decarboxylase gene. *J. Biol. Chem.* **263**, 3528-3533.

Priebe, A., Klein, H. and Jager, H. J. (1978) Role of polyamines in SO_2^- polluted pea plants. *J. Exp. Bot.* **29**, 1045-1050.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Santos-Mello, R., Schmidt, T., Neuhauss, E. and Almeida, A. (1999) Induction of micronuclei by CsCl *in vivo* and *in vitro*. *Mutat. Res.* **446**, 239-244.

Scalabrino, G. and Lorenzini, E. C. (1991) Polyamines and mammalian hormones. Part II: Paracrine signals and intracellular regulators. *Mol. Cell. Endocrinol.* **77**, 37-56.

Scalabrino, G., Lorenzini, E. C. and Ferioli, M. E. (1991) Polyamines and mammalian hormones. Part I: Biosynthesis, interconversion and hormone effects. *Mol. Cell. Endocrinol.* **77**, 1-35.

Seiler, N. and Heby, O. (1988) Regulation of cellular polyamines in mammals. *Acta. Biochem. Biopy. Hung.* **23**. 1-35.

Sheahan, J. J., Ribeironeto, L. and Sussman, M. R. (1993) Cesium-insensitive mutants of *Arabidopsis thaliana*. *Plant J.* **3**, 647-656.

Tabor, C. W. and Tabor, H. (1984) Polyamines. *Annu. Rev. Biochem.* **53**, 749-790.

Tiburcio, A. F., Kaur-Swahney, R. and Galston, A. W. (1990) *Polyamine Metabolism:* in *The Biochemistry of Plants*. 16, pp 283-325, Academic Press.

Tiburcio, A. F., Besford, R. T., Capell, T., Borrell, A., Testillano, P. S. and Risueòo, M. C. (1994) Mechanisms of polyamine action during senescence responses induced by osmotic stress.

J. Exp. Bot. **45**, 1789-1800.

Tiburcio, A. F., Campos, J. L., Figueras, X. and Besford, R. T. (1993) Recent advances in the understanding of polyamine functions during plant development. *Plant Growth Regul.* **12**, 331-340.

Tyagi, A. K., Tabor, C. W. and Tabor, H. (1981) Ornithine decarboxylase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **236**, 12156-12163.

Watson, M. B. and Malmberg, R. L. (1996) Regulation of *Arabidopsis thaliana* Heynh arginine decarboxylase by potassium deficiency stress. *Plant Physiol.* **111**, 1077-1083.

Young, N. D. and Galston, A. W. (1984) Physiological control of arginine decarboxylase activity in potassium deficient oat shoots. *Plant Physiol.* **76**, 331-335.