Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 480-487

ISOLATION AND CHARACTERIZATION OF A 25 kDa ANTIFUNGAL PROTEIN FROM FLAX SEEDS

Jeffry R. Borgmeyer[1], Christine E. Smith and Q. Khai Huynh

Department of Protein Biochemistry, Monsanto Corporate Research,
The Monsanto Company, St. Louis, MO 63198

Received July 13, 1992

We have purified a 25 kDa protein from flax seeds to homogeneity by polyethyleneimine precipitation, ammonium sulfate precipitation, chitin extraction, Mono S cation exchange and C18 reversed phase column chromatographies. The purified protein strongly inhibited the growth of the agronomically important pathogen *Alternaria solani*, the causative agent of tomato early blight and in synergy with nikkomycin Z strongly inhibited the human pathogen *Candida albicans*. Amino terminal sequence analysis of the purified protein indicated that it has a high degree of homology to other reported pathogenesis-related antifungal proteins.

Pathogens which infect plants must overcome both passive and active plant defense systems (1,2). The passive defense system involves natural barriers present in the cell wall which inhibit microbial penetration, such as lignin, tannins, phenols and cellulose (1,2,5). In addition, although plants lack an immune system (3,4), they have evolved an active defense system in which proteins are synthesized in response to pathogen attack (1,2,5). These proteins are referred to as "pathogenesis related" (PR) proteins (1,5). PR proteins are secreted into the intercellular and intracellular spaces of leaves and are often found in relative abundance in the seeds of plants (5,6). Characteristically, these proteins are relatively protease and heat resistant and soluble at low pH (7,8). PR proteins have recently been categorized into five classes (1,5,9). PR protein classes two and three contain the chitinases (1,5,10,11) and β-1,3-glucanases (1,5,12,13) respectively. Classes one and four include proteins whose mechanism of action remain unknown (1,5). Class one contains serologically related proteins found in

[1]To whom correspondence should be addressed: Monsanto Company, Mail Zone AA4E, 700 Chesterfield Village Parkway, St. Louis, MO 63198. FAX 314-537-7264.

The abbreviations used are: PR, pathogenesis-related; TMV, tobacco mosaic virus; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis, TFA, trifluoroacetic acid.

0006-291X/92 $4.00

a wide variety of plants with molecular weights around 15 kDa which are induced upon infection by tobacco mosaic virus (TMV) (1,5,14,15). Class four includes acidic proteins with molecular weights of 13 to 14.5 kDa which are also induced by TMV infection and have sequence homology to the wound induced proteins of potato (1,5,16,17). Class five PR proteins, also known as thaumatin or osmotin like proteins, due to their extensive sequence homologies to these two proteins, have been reported to have membrane permeabilizing activities (1,5,18). While, little is known for certain concerning their role *in planta*, many PR proteins have been reported to be antifungal *in vitro* (11,18-24).

We are interested in studying the role of antifungal PR proteins in plants as well as in their application for the treatment of candidiasis and in the development of fungal resistant plants through gene transfer. With this in mind we have reported on the purification, characterization and cloning of chitinases with antifungal properties (22,24,25), and recently reported the purification, characterization and complete amino acid sequence of a 22 kDa antifungal protein from corn (23). Here we describe the isolation and characterization of a related 25 kDa protein from flax with antifungal properties against an agronomically important pathogen of tomato which causes early blight (*A. solani*) and which acts in synergism with nikkomycin Z to strongly inhibit the growth of the human pathogen *C. albicans*.

MATERIALS AND METHODS

Materials - Organically grown seeds of flax (*Linum usitatissimum*) were obtained from Arrowhead Mills, Inc. Colloidal chitin was prepared from acetylation of chitosan as described by Hirano *et al.*, (26). The Mono-S column (HR 5/5) was from Pharmacia LKB Biotechnology. The Vydac C18 reversed phase (4.6 x 250 mm, 5 micron particle size) column was obtained from Alltech Associates, Inc. The plant pathogens *A. solani* and *Phytopthera infestans* were provided by the Agricultural Group of Monsanto Company. *C. albicans* was provided by G.D. Searle. Silver stain SDS-PAGE molecular weight standards were obtained from Bio-Rad Laboratories. Nikkomycin Z was obtained from Calbiochem. Other reagents were of the highest grade commercially available.

Purification of a 25 kDa Antifungal Protein - All purification procedures were carried out at 4°C unless otherwise noted. 50 g of flax seed was extracted in 0.5 liter of 10 mM sodium acetate buffer, pH 5.0. The precipitate from the extract was discarded after centrifugation and 10 % polyethyleneimine was added dropwise to a final concentration of 0.25 %. The precipitate was removed by centrifugation and solid ammonium sulfate was added to the supernatant to 60 % of saturation. After stirring overnight the precipitate was collected by centrifugation and extensively dialyzed against 20 mM sodium bicarbonate buffer, pH 8.4. The dialyzed fraction was then incubated with 20 g of colloidal chitin in 50 ml of 20 mM sodium bicarbonate buffer, pH 8.4 for 1 hour at room temperature. The fraction which was not bound to chitin was collected by centrifugation, dialyzed against a 20 mM sodium acetate buffer, pH 5.0 and fractionated on a Mono-S column equilibrated with the above buffer. All fractions were collected and assayed for antifungal activity. A fraction which eluted at 130 mM NaCl showed strong antifungal activity. Final purification was achieved by reversed phase C18 chromatography. Materials were eluted from the column with an

increasing gradient of acetonitrile containing 0.1 % TFA. A fraction eluting with 48 % acetonitrile showed strong antifungal activity. SDS-PAGE and subsequent amino acid sequencing of this fraction showed that it contained a homogeneous protein which has a molecular weight of about 25 kDa.

Assay of Antifungal Activity - Antifungal activity against *A. solani* was carried out using a hyphal extension-inhibition assay as described by Roberts and Selitrennikoff (11). Fungal mycelium were harvested from actively growing fungal plates and placed into the center of Petri dishes containing the nutrient agar. After incubation of these dishes for 72 hr at room temperature to allow for mycelial growth, sterile filter paper discs were laid on the agar surface in front of the advancing fungal mycelium and then 35 µl of the protein solutions were applied to the discs. The plates were then further incubated at room temperature for 20 hrs. In this manner, if the protein being tested is an antifungal protein, a crescent shaped zone of inhibition is observed around the disc. Antifungal activity against *C. albicans* was carried out essentially as previously described by Roberts and Selitrennikoff (18). Saboroud dextrose agar was autoclaved and cooled to 45 ^{0} C. A suspension of *C. albicans* was added to a concentration of 2×10^4 organisms ml^{-1} , nikkomycin Z was added to a concentration of 0.2 µg ml^{-1} and 10 ml portions were added to Petri plates. Sterile paper discs were placed on the solidified agar, 30 ul samples of the protein solution were applied to the discs and the plates were incubated overnight at 37 ^{0}C. Clear zones of no fungal growth were seen around discs containing inhibitory concentrations of antifungal protein.

Other Methods - Protein concentration was determined by amino acid compositional analysis. Purity of the purified protein was confirmed by SDS-PAGE (27) with silver staining and by amino acid sequencing. Automatic Edman degradation of the purified protein was performed using an Applied Biosystems Model 470A sequenator (22).

RESULTS AND DISCUSSION

The presence of a protein in flax seeds which in synergism with nikkomycin Z inhibits *C. albicans* was first noted by Vigers *et al.* (28), however the protein was never purified or characterized. In the present study we have purified this antifungal protein from flax seeds to homogeneity by polyethyleneimine precipitation, chitin extraction, ammonium sulfate precipitation, Mono S cation exchange and C18 reversed phase chromatography. Flax seeds are extremely mucilaginous, making purification of proteins from this source very difficult. A key step in the purification was the separation of the mucilaginous material from the protein of interest. Initial attempts to achieve this by ammonium sulfate precipitation, dialysis and acetone precipitation, all failed. However, adding polyethyleneimine (29) to a final concentration of 0.25% resulted in not only removing DNA but also precipitated the mucilaginous material as well as numerous contaminating proteins, the protein of interest remained in the supernatant. The clarified supernatant could then be handled by conventional purification processes. The supernatant was concentrated by ammonium sulfate precipitation. Chitinases which can also be antifungal and therefore interfere with the bioassay were removed by affinity binding to colloidal chitin. Subsequent cation exchange chromatography on Mono S resulted in a major purification step, with the antifungal protein eluting with 130 mM NaCl as illustrated in Fig. 1A. Final purification by C18 reversed phase chromatography succeeded in removing several small molecular weight proteins, with the

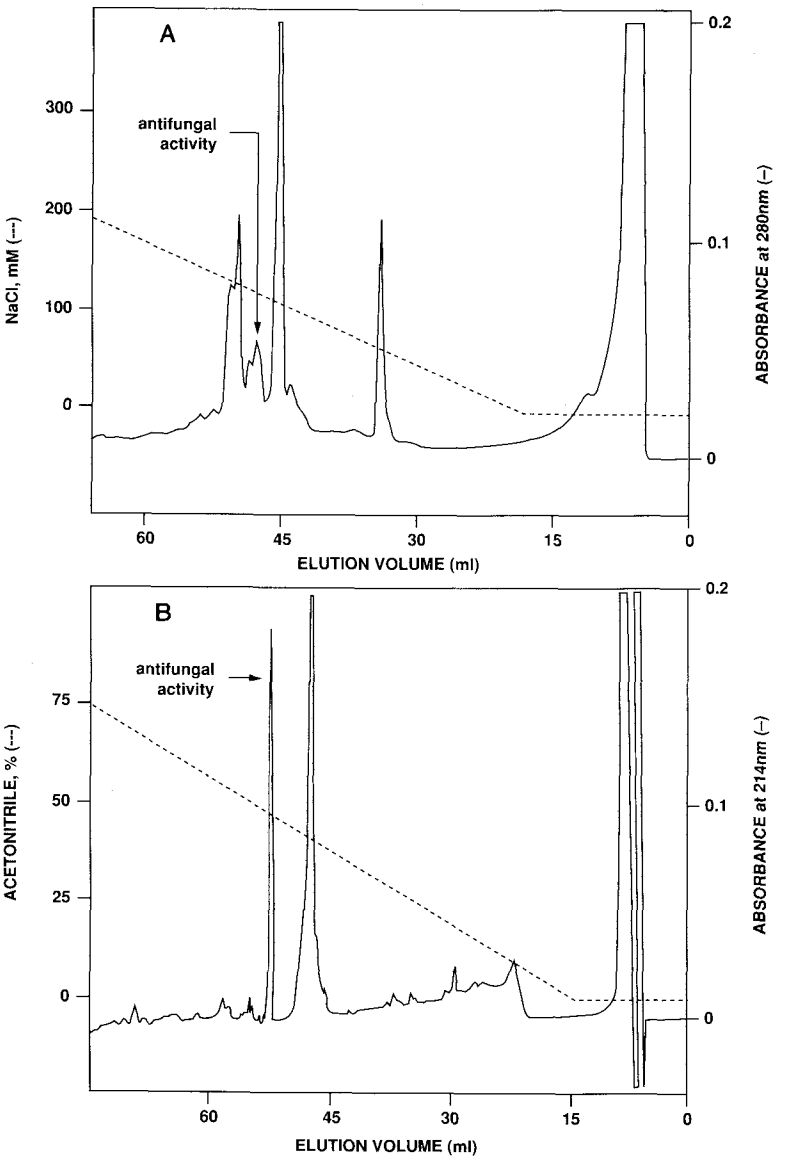

Fig. 1. A: Fractionation of flax components by Mono S chromatography following polyethyleneimine precipitation, ammonium sulfate precipitation and chitin extraction. B: Final purification by C18 reversed phase chromatography following Mono S chromatography. See "Materials and Methods" for details of the purification.

antifungal protein eluting with 48 % acetonitrile as illustrated in Fig. 1B. The purified protein has a molecular weight of about 25 kDa as determined by SDS-PAGE (Fig. 2). From 50 grams of flax seed about 24 μg of the protein was obtained. The purified protein strongly inhibited the growth of *A. solani* (tomato early blight) with as little as 1.0 μg per disc (Fig. 3A), but did not inhibit the fungal pathogen responsible for causing late blight in potato *Phytopthera infestans* (data not shown). As little as 1.0 μg of protein acted synergistically with nikkomycin Z to inhibit the growth of *C. albicans* as shown in Fig. 3B. Neither the 25 kDa

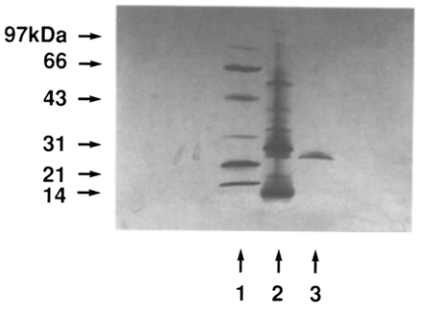

Fig. 2. SDS-PAGE of the purified antifungal protein. As indicated: lane 1, molecular weight standards; lane 2, flax seed extract; and lane 3, purified protein after C18 reversed phase chromatography.

protein or nikkomycin Z alone are capable of inhibiting the growth of *C. albicans*. These results suggest that the gene for this protein may be a promising candidate for the genetic engineering of fungal resistant crops and that the protein together with nikkomycin Z may be of use in treating candidiasis (22,23,28,31,37).

To gain an insight as to the structure-function relationship of this antifungal protein approximately 150 picomoles of purified protein was subjected to automatic Edman degradation and its NH_2-terminal amino acid sequence of 37 residues was established as shown in Fig. 4. Results from a computer search for homology with other published protein sequences showed that the NH_2-terminus has no homology with any β-1,3-glucanase or chitinase sequences published to date (22,24,30-32). However, the NH_2-terminus of the purified protein exhibited a high degree of sequence homology with a sweet protein thaumatin (33) and several other PR class five proteins as summarized in Fig. 4. The purified flax protein has a 68 % sequence identity among the first 37 amino acids with a 22 kDa antifungal protein isolated from corn (23), and an antifungal protein referred to

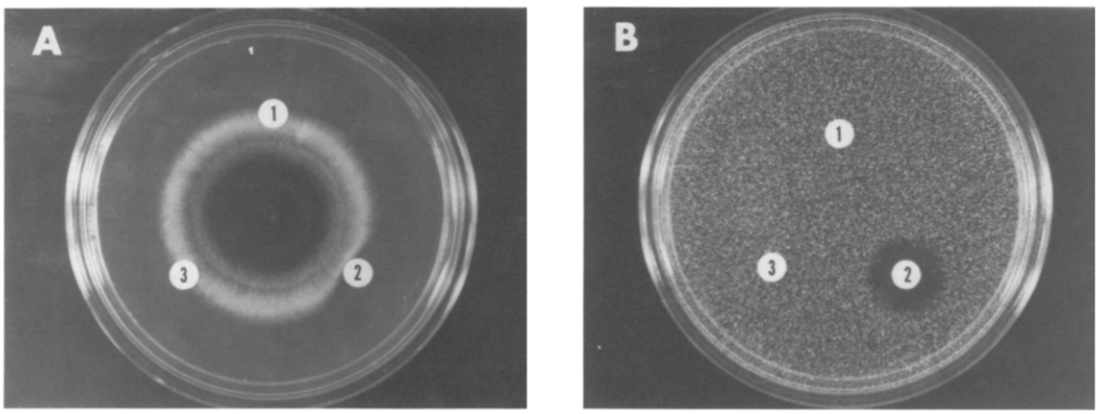

Fig. 3. In vitro antifungal activity of the purified flax protein against *Alternaria solani* (A) and *Candida albicans* (B). Discs 1, Buffer control (20 mM sodium acetate buffer, pH 5.0; discs 2, 1.0 µg of purified flax protein; discs 3, 1.0 µg of purified flax protein following boiling. See "Materials and Methods" for details of the *in vitro* antifungal assays.

FLAX	A R F D I Q N K C* P Y T V W A A S V P V . . . . . G G G R Q L N S G Q T W* X I D A P	
CORN 22 kDa (23)	A V F T V V N Q C P F T V W A A S V P V . . . . . G G G R Q L N R G E W W R I T A P	68 %
CORN Zeamatin (28)	A V F T V V N Q C P F T V W A A S V P V . . . . . G G G R Q L N R G E W W R I T A P	68 %
CORN A/TI (38)	A V F T V V N Q C P F T V W A A S V P V . . . . . G G G R Q L N R G E W W R I T A P	68 %
NP24 (34)	A T I E V R N N C P Y T V W A A S T P I . . . . . G G G R R L N R G Q T W V I N A P	65 %
TOBACCO PR-R (36)	A T F D I V N K C T Y T V W A A A S P . . . . . G G G R R L D S G Q S W S I N V N	65 %
OSMOTIN (35)	A T I E V R N N C P Y T V W A A S T P I . . . . . G G G R R L D R G Q T W V I N A P	62 %
AP24 (21)	A T I E V R N N C P Y T V W A A S T P I . . . . . G G G R R L D R G Q T W V I N A P	62 %
BARLEY-S (37)	A T F T V I N K C Q Y T V W A A A V P A . . . . . G G G Q K L D A G Q T W S I X X P	62 %
THAUMATIN (33)	A T F E I V N R C S Y T V W A A A S K G D A A L D A G G R Q L N S G E S W T I N V E	57 %
BARLEY-R (37)	A T I T V V N R C S Y T V W P G A L P . . . . . G G G V R L D P G Q R W A L N M P	43 %
TOMATO PR-23 (39)	A T F E V R N N C P Y T V W A A S T P I . . . . . G Q T W V I N A P R I W G R X H A	41 %
CONSENSUS	A . . . . . N . C . . T V W . . . . . W	

Fig. 4. NH_2-terminal amino acid sequence of the purified flax antifungal protein in comparison to those of the corn 22 kDa antifungal protein , zeamatin, the corn α–amylase/trypsin inhibitor, tomato NP24, tobacco PR-R, osmotin, tobacco AP24, barley protein S, thaumatin, barley protein R, and tomato PR-2. X denotes unidentified amino acids. * denotes a presumed cysteine or tryptophan residue. The dots denote spaces required for optimal alignment. Only proteins for which at least 37 amino acid residues are published are included in this comparison.

as zeamatin, also isolated from corn (28) (it is unclear whether these two antifungal proteins from corn are identical, as the complete sequence of zeamatin has not been published). The flax protein has a 68 % sequence identity to the α–amylase/trypsin inhibitor also isolated from corn seed (38). The flax antifungal protein sequence also has a high degree of homology with several salt induced proteins, NP24, a 24 kDa protein isolated from tomato (65 % sequence identity) (34), and 62% sequence identity with osmotin, a 24 kDa protein isolated from tobacco (34). AP24, a TMV induced antifungal protein isolated from tobacco (21) has a 62 % sequence identity among the first 37 amino acids to the antifungal flax protein. In addition the antifungal flax protein shows a high degree of sequence identity (65 %), to another PR protein isolated from tobacco, referred to as PR-R (36), as well as to two 23 kDa antifungal proteins from barley referred to as protein R (43 %) and protein S (62 %) (37). While we do not know the mode of action of this antifungal protein from flax, zeamatin has been shown to have membrane-permeabilizing activity (18,28) and considering the high degree of sequence identity to the flax protein (68 %) it is likely the mode of action of the flax antifungal protein is similar.

In conclusion, the results in this study together with our previous results (23) as well as results from other groups (18,21,28,34-39) provide further evidence for the existence of a novel class of homologous antifungal proteins distributed in a wide variety of plants that are classified as class five PR proteins which show a high degree of homology to the sweet protein thaumatin (33).

Acknowledgments - We would like to thank Dr. L.D. Bell for his strong encouragement and support of this project. In addition, we wish to thank J.F. Zobel for performing the automatic amino acid compositional analysis and S.A. Sammons for help in establishing sequence homology comparisons.

REFERENCES

1. Linthorst, H. (1991) *Critical Reviews in Plant Sciences* **10(2)**, 123-150.
2. Bell, A.A. (1981) *Ann. Rev. Plant Physiol.* **32**, 21-81.
3. Clarke, A.E., and Know, R.B. (1979) *Dev. Comp. Immunol.* **3**, 571-589.
4. Chester, K.S. (1933) *Q. Rev. Biol.* **8**, 129-154.
5. Bol, J., Linthorst, H., and Cornelissen, B. (1990) *Ann. Rev. Phytopathol.* **28**, 113-138.
6. Parent, J., and Asselin, A. (1984) *Can. J. Bot.* **62**, 564-569.
7. Van Loon, L. (1976) *J. Gen. Virol.* **309**, 375-379.
8. Van Loon, L. (1982) In *Active Defense Mechanisms in Plants, edited by R.K.S. Wood, New York, Plenum,* 247-273.
9. Van Loon, L., Gerritsen, Y., and Ritter, C. (1987) *Plant Mol. Biol.* **9**, 593-609.
10. Legrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6750-6754.
11. Roberts, W.K., and Selitrennikoff, C.P. (1986) *Biochim. Biophys. Acta* **880**, 161-170.
12. Kauffmann, S., Legrand, M., Geoffroy, P., and Fritig, B. (1987) *EMBO J.* **6**, 3209-3212.
13. Lamb, C.J., Lawton, M.A., Dron, M. and Dixon, R.A. (1989) *Cell* **56**, 215-224.
14. Antoniw, J., Ritter, C., Pierpoint, W., and Van Loon, L. (1980) *J. Gen. Virol.* **47**, 79-87.
15. Antoniw, J., White, R., Barbara, D., Jones, P., and Longley, A. (1985) *Plant Mol. Biol.* **4**, 55-60.
16. Kauffman, S., Legrand, M., and Fritig, B. (1990) *Plant Mol. Biol.* **14**, 381-390.
17. Linthorst, J., Danhash, N., Brederode, F., Van Kan, J., De Wit, P., and Bol, J. (1991) *Mol. Plant-Microbe Interact.* **4(6)**, 586-592.
18. Roberts, W.K., and Selitrennikoff, C.P. (1990) *J. of Gen. Microbiology*. **136**, 1771-1778.
19. Roberts, W.K., Laue, B. and Selitrennikoff, C.P. (1988) *Annal of the New York Academy of Sciences.* **544**, 141-150.
20. Mauch, F., Mauch-Mani, B., and Boller, T. (1988) *Plant Physiol.* **88**, 936-942.
21. Woloshuk, C., Meuilenhoff, J., Sela-Buurlage, M., van den Elszen, P., and Cornelissen, B. (1991) *The Plant Cell* **3**, 619-628.
22. Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R., and Shah, D. (1992) *J. Biol. Chem.* **267**, 6635-6640.
23. Huynh, Q.K., Borgmeyer, J.R., and Zobel, J.F. (1992) *Biochem. and Biophys. Res. Commun.* **182**, 1-5.
24. Verburg, J.G., and Huynh, Q.K. (1991) *Plant Physiol.* **95**, 450-455.
25. Verburg, J.G., Smith, C.E., Lisek, C.A., and Huynh, Q.K. (1992) *J. Biol. Chem.* **267**, 3886-3893.
26. Hirano, S., Ohe, Y., and Ono, H. (1987) *Carbohydr. Res.* **47**, 315-320.
27. Laemmli, U.K. (1970) *Nature* **227**, 680-685.
28. Vigers, A.J., Roberts, W.K. and Selitrennikoff, C.P. (1991) *Mol. Plant-Microbe Interact.* **4**, 315-323.
29. Gegenheimer, P. (1990) *Methods in Enzymology* **182**, 174-193.
30. De Loose, M., Alliotte, G., Gemetello, C., Gielen, J., Soetaert, P., Van Montagu, M., and Inze, D. (1988) *Gene* **70**, 13-23.
31. Leah,R., Tommerup, H., Svendesn, I., and Mundy, J. (1991) *J. Biol. Chem.* **266**, 1564-1573.
32. Fincher, G.B., Lock, P.A., Morgan, M.M., Lingelbach, K., Wettenhall, R. E., Mercer, J.F.B., Brandt, A., and Thomsen, K.K. (1986) *Proc. Natl. Sci. Acad. U.S.A.* **83**, 2081-2085.
33. Iyengar, R.B., Smits, P., van der Ouderaa, F., van der Wel, H., van Brou wershaven, J., Ravestein, P., Richters, G., and van Wassenaar, P.D. (1979) *Eur. J. Biochem.* **96**, 193-204.

34. King, G.J., Turner, V.A., Hussey, C.E., Wurtele, E.S., and Lee, S.M. (1988) *Plant Mol. Biol.* **10**, 401-412.
35. Singh, N.K., Nelson, D.E., Kuhn, D., Hasegawa P.M., and Bressan, R.A. (1989) *Plant Physiol.* **90**, 1096-1101.
36. Payne, G., Middlesteadt, W., Williams, S., Desai, N., Parks, T.D., Dincher, S., Carnes, M., and Ryals, J. (1988) *Plant Mol. Biol.* **11**, 223-224.
37. Hejgaard, J., Jacobsen, S., and Svendsen, I. (1991) *FEBS* **291**, 127-131.
38. Richardson, M., Valdes-Rodrigues, S., and Blanco-Labra, A. (1987) *Nature* **327**, 432-434.
39. Rodrigo, I., Vera, P., Frank, R., and Conjero, V. (1991) *Plant Mol. Biol.* **16**, 931-934.