Volume 316, number 2, 119–122 FEBS 12022 January 1993

Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens*

Antonio Molina, Ana Segura and Francisco García-Olmedo

Laboratorio de Bioquímica y Biología Molecular, ETS Ingenieros Agrónomos-UPM, E-28040 Madrid, Spain

Received 22 October 1992

Four homogeneous proteins (Cw_{18}, Cw_{20}, Cw_{21}, Cw_{22}) were isolated from etiolated barley leaves by extraction of the insoluble pellet from a Tris-HCl (pH 7.5) homogenate with 1.5 M LiCl and fractionation by reverse-phase high-performance liquid chromatography. All 4 proteins inhibited growth of the pathogen *Clavibacter michiganensis* subsp. *sepedonicus* ($EC_{50}s = 1–3 \times 10^{-7}$ M) and had closely related N-terminal amino acid sequences. The complete amino acid sequences of proteins Cw_{18} and Cw_{21} were determined and found to be homologous to previously described, non-specific lipid transfer proteins from plants (32–62% identical positions). The proteins also inhibited growth of the bacterial pathogen *Pseudomonas solanacearum* ($EC_{50}s = 3–6 \times 10^{-7}$ M) and the fungus *Fusarium solani* ($EC_{50}s = 3–20 \times 10^{-6}$ M). A homologous protein from maize leaves (Cw_{41}) was purified in a similar manner and also found to have inhibitory properties. A synergistic effect against the fungus was observed when protein Cw_{21} was combined with thionins. A defense role for non-specific lipid transfer proteins from plants is proposed.

Lipid transfer protein; Plant pathogen; Thionin

1. INTRODUCTION

Non-specific lipid transfer proteins (nsLTPs) from plants are to some extent 'actresses in search of a role' [1,2]. Members of this protein family have been reported in a variety of tissues from both mono- and dicotyledon species, including barley aleurone [3], spinach leaves [4,5], maize seeds [6,7], castor beans [8,9], carrot embryos [10,11], wheat seeds [12] and tomato stems [13]. Although it was initially thought that they were probably involved in cytoplasmic lipid shuttling between organelles, more recent findings, which showed that they were synthezised as precursor proteins with N-terminal sequences [7,14] and that they were secreted [11,15], seem to contradict the above possibility. In the course of an investigation of possible defense proteins in barley, we have identified a group of closely related nsLTPs in young etiolated leaves that are potent growth inhibitors of bacterial and fungal pathogens, and that show a clear synergistic effect in combination with thionins. A nsLTP from maize leaves was similarly isolated and was also found to be inhibitory.

Correspondence address: F. García-Olmedo, Laboratorio de Bioquímica y Biología Molecular, ETS Ingenieros Agrónomos-UPM, E-28040 Madrid, Spain. Fax: (34) (1) 543 4879.

Abbreviations: nsLTP, non-specific lipid transfer protein; EDTA, ethylene-diamine-tetracetic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SGE, starch-gel electrophoresis; cfu, colony forming units.

*Patent application P 9101258 (24.05.91) PCT/EP92/01130.

2. MATERIALS AND METHODS

2.1. *Materials*

Barley cvs. Betzes and Bomi were sown in vermiculite and grown in the dark for 7 days. Leaf material was collected and immediately frozen in liquid nitrogen. Maize cv INIA 1986 was similarly processed. Bacterial pathogens *Clavibacter michiganensis* subsp. *sepedonicus*, strain C5, and *Pseudomonas solanacearum* strain P2, and the fungal pathogen *Fusarium solani* strain 1, were from the ETSIA collection (Madrid). Basic protein BP1 from barley and a mixture of the purified α1- and β-thionins from wheat were the gift of C. Hernández-Lucas.

2.2. *Purification procedure*

Frozen leaf material (20 g) was ground to powder in liquid nitrogen, using a mortar and pestle, and extracted once with 80 ml of buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and twice with 80 ml of distilled H_2O. The resulting pellet was then extracted with 50 ml of 1.5 M LiCl at 4°C for 1 h, dialyzed against 5 l of H_2O, using a Spectra/Por 7 (MWCO:2000) membrane (Spectrum Medical Ind. Inc.), and freeze-dried. The extract was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on an ultrapore C3 column (1 × 25 cm; 5 μm particle; 300 Å pore) from Beckman, using a H_2O/2-propanol gradient, 0.1% trifluoroacetic acid, at 0.5 ml/min, as indicated in the legend of Fig. 1A. Fractions were collected by hand and freeze-dried.

2.3. *Characterization*

The purified proteins were subjected to 3 types of electrophoretic separations: SDS-PAGE in preformed gradient gels (4–20% acrylamide; Bio-Rad) according to the manufacturers instructions; starch-gel electrophoresis (SGE) at pH 3.2, in aluminum lactate buffer; and SGE at pH 8.3 in Tris-HCl [16]. Electrospray ionization mass spectrometry was performed using a VG Biotech BIO-Q instrument by M-Scan Inc. (PA, USA). Amino acid sequencing was carried out by automated Edman degradation (Applied Biosystems) of the intact proteins or of the appropriate peptides generated with the specific proteases listed in Fig. 2A, following the manufacturers instructions.

2.4. *Inhibition tests*

Proteins were dissolved in water and added to microtiter wells prior

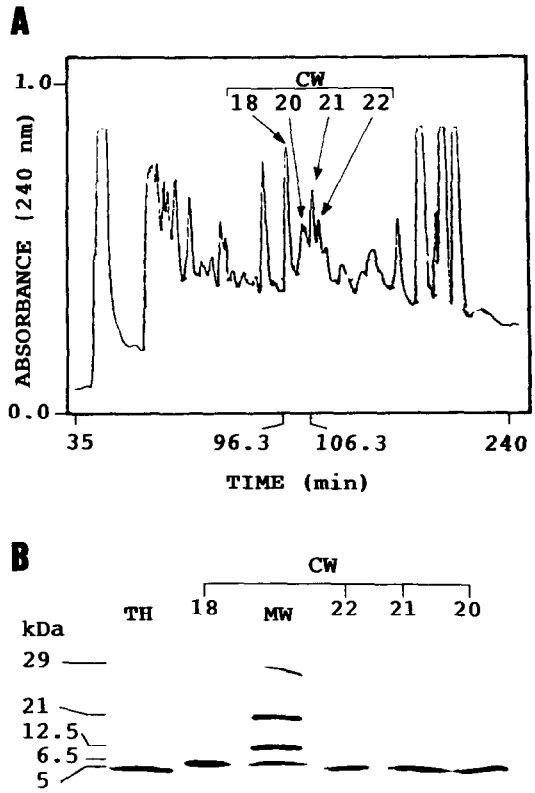


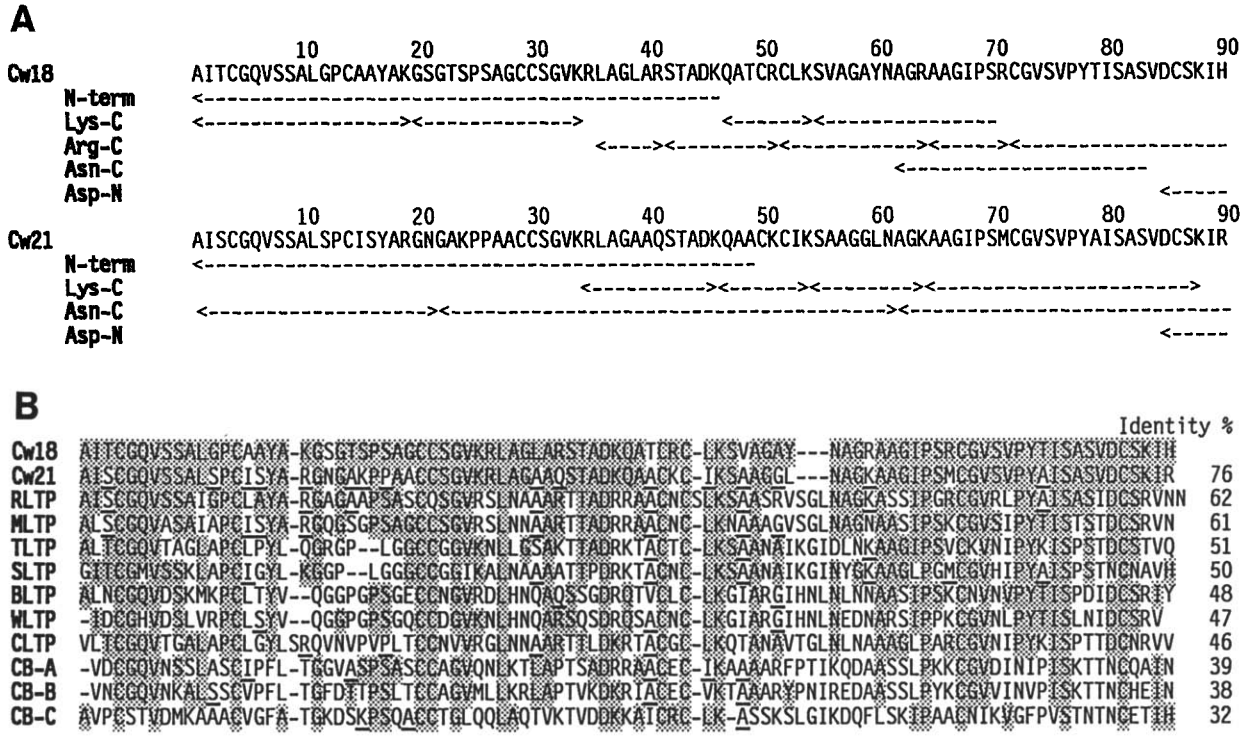
Fig. 1. Purification of proteins Cw_{18}, Cw_{20}, Cw_{21} and Cw_{22}. (A) RP-HPLC fractionation of the 1.5 M LiCl extract from the insoluble pellet from a Tris-HCl (pH 7.5) homogenate from etiolated barley leaves. The gradient used was H_2O (0.1% trifluoroacetic acid)–2-propanol linear 0–30% for 180 min, followed by linear 30–50% for 15 min. (B) Separation by SDS-PAGE of the purified proteins. Molecular mass markers (MW) were carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), cytochrome *c* (12.5 kDa), bovine lung trypsin inhibitor (6.5 kDa) and thionin (TH) from barley endosperm (5 kDa).

to innoculation. Bacteria were inoculated at a final concentration of 10^4 cfu/ml in sterile microtiter plates at final volumes of 150 μl (100 μl protein + 50 μl nutrient broth, Oxoid). After 16–24 h of incubation at 28°C, growth was recorded by measuring absorbance at 490 nm in an ELISA plate reader. In the case of the fungus, spores were collected from 8-day-old cultures grown at 25°C on potato dextrose agar plates (Difco) and stored at −80°C in 20% glycerol. Spore suspensions (10^4/ml) were incubated in potato dextrose broth with the indicated amounts of added proteins (final volume 75 μl) in microtiter plates at 25°C for 26–44 h, and growth was determined as described above [17].

3. RESULTS

The insoluble pellet obtained from a Tris-HCl (pH 7.5) homogenate of etiolated barley leaves (cv. Betzes) was extracted with 1.5 M LiCl and the extract was fractionated by RP-HPLC as shown in Fig. 1A. The fractions designated Cw_{18}, Cw_{20}, Cw_{21}, and Cw_{22} were active against the bacterial pathogen, *C. michiganensis* subsp. *sepedonicus* in a preliminary screening and gave single bands with apparent molecular masses of about 6.5 kDa when subjected to gradient SDS-PAGE (Fig. 1B). They also appeared as homogeneous when fractionated by SGE/pH 3.2 and SGE/pH 8.3 (not shown).

Determination of the N-terminal amino acid sequences of the 4 proteins showed that they were closely related to each other and homologous to previously described plant nsLTPs (Fig. 2). The first 16 N-terminal residues from proteins Cw_{20}, Cw_{21}, and Cw_{22} were identical and differed at 4 positions from those of protein Cw_{18}. Yield of the 4 proteins taken together was 20–60 μg per g fresh weight ($2–6 \times 10^{-6}$ M). Proteins Cw_{18} and Cw_{21} were purified on a larger scale from cv. Bomi and completely sequenced, following the strategy indicated in Fig. 2A. Homology was fully corroborated, as shown



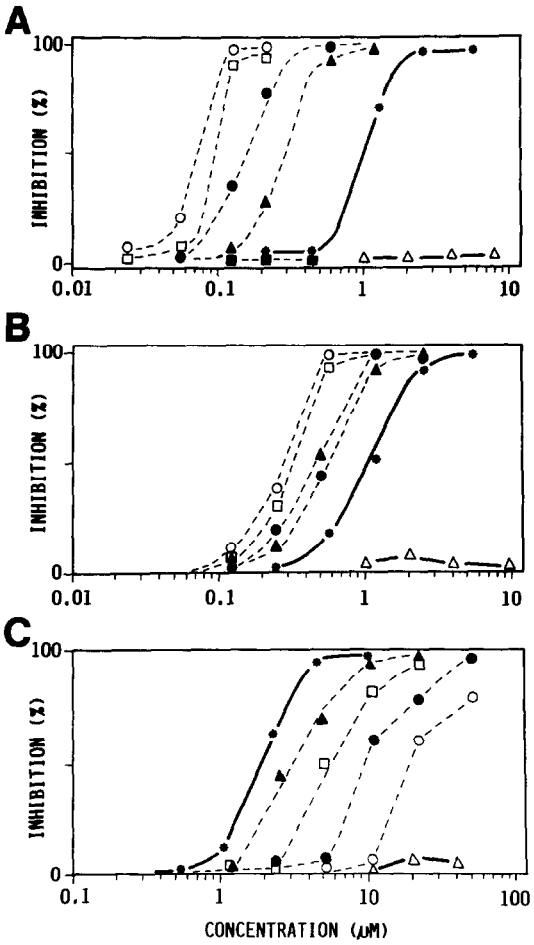


Fig. 3. In vitro inhibition tests with the following plant pathogens: (A) *C. michiganensis* subsp. *sepedonicus*. (B) *P. solanacearum*. (C) *F. solani*. The following symbols are used: Cw_{18} (□), Cw_{20} (●), Cw_{21} (▲), Cw_{22} (gn), Cw_{18} incubated with trypsin (Boehringer, Mannheim) at 37°C for 18 h in 0.1 M NH_4HCO_3 (pH 8.1) buffer (■), $\alpha 1 + \beta$ thionins from wheat (*), and BP1 (△). Cw_{18} incubated in above buffer at 37°C for 18 h retained full inhibitory activity (not shown).

in Fig. 2B, where an alignment of the new sequences with previously described nsLTPs has been carried out. The molecular mass calculated from the amino acid sequence for Cw_{18} differed from that determined from the mass spectrum (8774.04 Da) by less than 1 Da, while that for Cw_{21} (8671.34 Da) showed a difference of 16.49

←

Fig. 2. Amino acid sequences of proteins Cw_{18} and Cw_{21} from Bomi barley. (A) Sequencing strategy. Besides the N-terminal sequence of the intact proteins (N-term), those of the indicated peptides, obtained with different proteases, were determined. Endoproteases used were the following: Lys-C (Boehringer-Mannheim); Arg-C, syn. clostrapain (Promega); Asn-C (Pierce); Asp-C (Boehringer-Mannheim). (B) Alignment of the amino acid sequences from Cw_{18} and Cw_{21} proteins with those from previously described nsLTPs. Amino acid residues that are identical to those in Cw_{18} are shaded and those identical with Cw_{21} are underlined. Sequences aligned were the following: RLTP from ragi [20], MLTP from maize [7], TLTP from tomato [13], SLTP from spinach [5], BLTP from barley [3], WLTP from wheat [12], CLTP from carrots [11] and CB_A, CB_B and CB_C from castor bean [8,9].

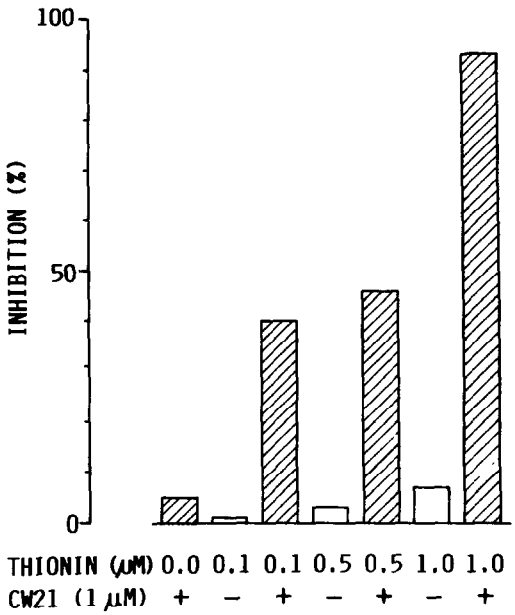


Fig. 4. Synergism between thionins ($\alpha 1 + \beta$ from wheat) and Cw_{21} against the fungus, *F. solani*. Inhibition was greater than additive ($P < 0.05$) at the 3 concentrations tested.

Da, which was probably due to the oxygen from the methoxy group in the single methionine at position 71 (Fig. 2A).

The Cw proteins were about 10-fold more active towards *C. michiganensis* subsp. *sepedonicus* than the thionins included in the experiment as the positive control (Fig. 3A). A basic protein (BP1) with an apparent M_r of about 6 kDa, which had undergone the same purification steps as the Cw proteins, was used as the negative control. The inhibitory activity was destroyed by trypsin (Fig. 3A) and by papain (not shown). The Cw proteins were also more active than thionins towards the bacterial pathogen *P. solanacearum* (Fig. 3B), while they were less active than thionins against the fungus *F. solani* (Fig. 3C). A synergistic effect of nsLTPs with thionins was observed against the fungus (Fig. 4). The combined effect of thionins and nsLTPs against bacteria was merely additive (not shown).

The same purification procedure used for the barley Cw proteins was applied to obtain proteins with the same apparent molecular mass and activity from maize etiolated leaves (Fig. 5). The N-terminal amino acid sequence of the most abundant of these proteins (Cw_{41}), which had an EC_{50} of 2 μM towards *C. michiganensis* subsp. *sepedonicus*, was determined (Fig. 5C) and found to be closely related (93% similarity; 79% identity) to a previously described nsLTP from a different maize cultivar [7].

4. DISCUSSION

Proteins Cw_{18} and Cw_{21} are closer to each other (76% identity; 85% similarity) than to the other known members of the nsLTP family in plants (32–62% identity).

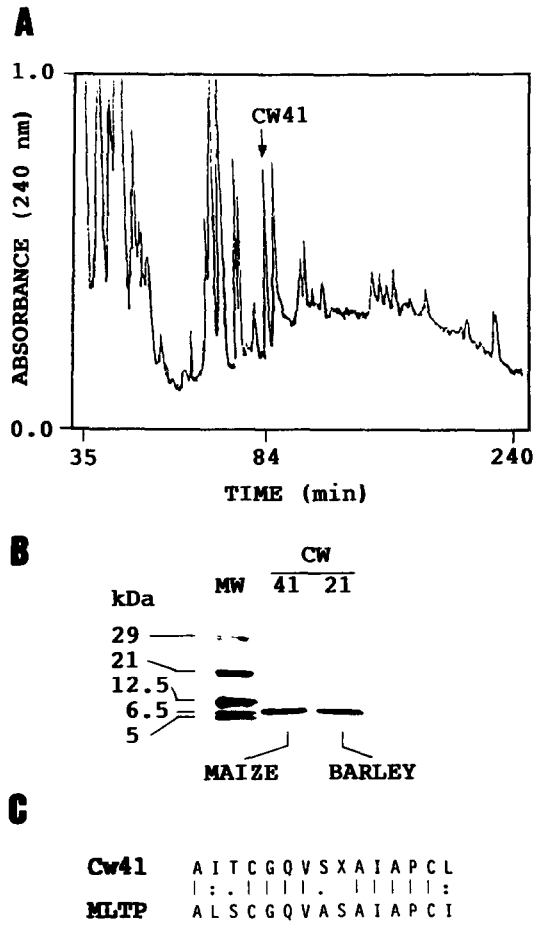


Fig. 5. Purification of protein Cw_{41} from etiolated leaves of maize cv. INIA 1986. (A) RP-HPLC fractionation of the 1.5 M LiCl pellet as in Fig. 1. (B) Separation of purified protein by SDS-PAGE. (C) N-Terminal amino acid sequence of Cw_{41} aligned with that of a previously described nsLTP from maize [7]. Other details as in Fig. 1.

They are also more similar to nsLTPs previously isolated from maize and ragi (61–62% identity) than to the nsLTP identified in barley aleurone (48% identity). These results are in accord with previous suggestions that different types of nsLTPs with different tissue specificity exist in a given species [8,9]. All amino acid residues that were highly conserved in previously described members of this family, including the 8 cysteines, are also conserved in the sequences reported here.

The supposition that the in vitro antibiotic activity of the barley proteins is a common feature of nsLTPs is supported by the observation that a member of the family chosen at random, i.e. Cw_{41} from maize, has been found to be active. In this context, three aspects of the Cw proteins are to be noted: (i) they are active at concentrations below those reported for other putative defense proteins, such as thionins, thaumatin-like proteins [18], chitinase C and ribosome inactivating protein K [19]; (ii) they are present in plant tissues at concentrations which are well above those required for complete inhibition in vitro of the pathogens tested; (iii) the activity ranking of the genetic variants differs markedly for the different pathogens, indicating some degree of specificity. These properties, together with their ability to act synergistically against pathogens in combination with the thionins, which are present in the same tissue, strongly suggest that one of the principal roles of endogenous nsLTPs in plants is that of defense against pathogens. This role would be congruent with observations in maize and carrots, where nsLTPs are expressed largely in the epidermal cells of developing aerial parts of the plants and are secreted [1,11,15], and in tomato, where it has been shown that a nsLTP gene can be induced by abiotic stress [13].

Acknowledgements. We are indebted to M. Moyer (CIBA-GEIGY Corp., USA), C. Buesa (Servei Sequenciacio, Universitat Barcelona), and G. Jimenez (CSIC, Madrid), for their contribution to the amino acid sequences, and to D. Lamoneda, J. García and C. Rojas for technical assistance. This work was supported by the Comisión Interministerial de Ciencia y Tecnolgía (Grants BIO90–0084 and PETRI 90–0065).

REFERENCES

[1] Chasan, R. (1991) Plant Cell 3, 842–843.
[2] Yamada, M. (1992) Plant Cell Physiol. 3, 1–6.
[3] Mundy, J. and Rogers, J.C. (1986) Planta 169, 51–63.
[4] Kader, J.C., Julienne M. and Vergnolle, C. (1984) Eur. J. Biochem. 1, 411–416.
[5] Bouillon, P., Drischel, C., Vergnolle, C., Duranton, H., and Kader, J.C. (1987) Eur. J. Biochem. 166, 387–391.
[6] Douady, D., Grosbois, M., Guerbette, F. and Kader, J.C. (1986) Plant Sci. 45, 151–156.
[7] Tchang, F., This, P., Stiefel, V., Arondel, V., Morch, M.-D, Pagés, M., Puigdomenech, P., Grellet, F., Delseny, M., Bouillon, P., Huet, J.-C., Guerbette, F., Beauvais-Cante, F., Duranton, H., Pernollet, J.-C. and Kader, J.-C. (1988) J. Biol. Chem. 263, 16849–16855.
[8] Takishima, k., Watanabe, J., Yamada, M. and Mamiya, G. (1986) Biochim. Biophys. Acta 986, 301–309.
[9] Takishima, K., Watanabe, J., Yamada, M., Suga, T. and Mamiya, G. (1988) Eur. J. Biochem. 190, 107–112.
[10] De Vries, S.C., Booij, H., Janssen, R., Vogels, R., Saris, L., LoSchiavo, F., Terzi, M. and van Kammen, A. (1988) Genes Dev. 2, 462–476.
[11] Sterk, P., Booij, H., Schellekens, G.A., van Kammen, A., and De Vries, S.C. (1991) Plant Cell 3, 907–921.
[12] Simorre, J.P., Caille, A., Marion, D., Marion, D. and Ptak, M. (1991) Biochemistry 30, 11600–11608.
[13] Torres-Schumann, S., Godoy, J.A and Pintor-Toro, J.A. (1992) Plant Mol. Biol. 18, 749–757.
[14] Bernhard, W.R., Thoma, S., Botella, J. and Somerville, C.R. (1991) Plant Physiol. 18, 164–170.
[15] Sossountzov, L., Ruiz-Avila, L., Vignols, F., Joliot, A., Arondel, V., Tchang, F., Grosbois, M., Guerbette, F., Miginiac, E., Delseny, M., Puigdomench, P. and Kader, J.C. (1991) Plant Cell 3, 923–933.
[16] García-Olmedo, F., Sotelo, I. and García-Faure, R. (1968) Anal. Inst. Nac. Invest. Agr. 17, 433–443.
[17] Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A. and Vanderleyden, J. (1990) FEMS Microbiol. Lett. 69, 65–60.
[18] Hejgaard, J., Jacobsen, S. and Svendsen, I. (1991) FEBS Lett. 291, 127–131.
[19] Leah, R., Tommerup, H., Svendsen, I and Mundy, J. (1991) J. Biol. Chem. 266, 1564–1573.
[20] Campos, F.A.P. and Richardson, M. (1984) FEBS Lett. 167, 221–225.