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Rapid Report

Chlorsulfuron blocks 2,4-D-induced cell enlargement and NADH oxidase in excised sections of soybean hypocotyls

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Abstract

Elongation of soybean hypocotyl sections induced by the auxin herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), was blocked by micromolar concentrations of the sulfonylurea herbicide chlorsulfuron (2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-trizin-2-yl)amino]carbonyl]benzenesulfonamide). The inhibition at equimolar concentrations of 2,4-D and chlorsulfuron was overcome by a 10- or 100-fold excess of 2,4-D. Stimulation by 2,4-D of an 2,4-D-responsive NADH-oxidase activity of the soybean plasma membrane also was blocked by the presence of concentrations of chlorsulfuron equimolar to the 2,4-D. Chlorsulfuron alone was largely without effect on either cell elongation or the auxin-stimulated NADH-oxidase activity over a range of chlorsulfuron concentrations. The results show a clear correlation between inhibition of auxin-stimulated cell elongation and the inhibition of an auxin-stimulated component of a plasma membrane NADH-oxidase activity.

Keywords: Chlorsulfuron; Herbicide; Auxin; NADH oxidase; Plasma membrane; Plant growth; (Soybean)

Previous studies have identified an NADH-oxidase activity associated with plasma membranes of soybean hypocotyls and other plant sources [1,2]. The activity was stimulated by the plant growth hormones of the auxin type such as indole-3-acetic acid (IAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) [3,4] suggesting some relationship to the process of cell enlargement or its control [2]. However, only a few opportunities have been afforded in which it was possible to attempt to correlate the activity of the plasma membrane-associated NADH oxidase and auxin-stimulated growth [5–7]. Specifically, an inhibitor specific for both the auxin-stimulated component of cell enlargement and the auxin-stimulated component of the plasma membrane NADH oxidase having little or no effect on constitutive levels of the NADH oxidase and of cell enlargement was sought. In this report, we provide evidence that the sulfonylurea herbicide chlorsulfuron (2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-trizin-2-yl)amino]-carbonyl]benzenesulfonamide) represents such an inhibitor. While the herbicidal effects of chlorsulfuron are known to be mediated through an interaction with a vestigial quinone site of acetolactate synthetase [8], the effect of chlorsulfuron on the NADH oxidase also may be exerted through interaction with a quinone site. As such, chlorsulfuron inhibition emerges as a valuable experimental tool for further investigation of plasma membrane NADH-oxidase function in plant cells.

Seeds of soybean (*Glycine max* L. Merr., cv. Williams) were soaked 4 to 6 h in deionized water, planted in moist vermiculite, and grown 4 to 5 days in darkness at 25°C in foil-covered 18 cm × 23 cm × 10 cm plastic boxes normally without supplemental additions of water. One or two cm long segments, cut 5 mm below the cotyledons, were harvested under low laboratory lighting and used for elongation measurements (1 cm segments) and isolation of membranes (2 cm segments).

For isolation of plasma membrane segments (40 g) were chopped with razor blades in 40 ml of homogenization medium (0.3 M sucrose, 50 mM Tris-Mes [pH 7.5], 10 mM KCl, 1 mM $MgCl_2$, 1 mM PMSF). The homogenates were filtered through one layer of Miracloth (Chicopee Mills, NY) and centrifuged for 10 min at 6000 × *g* (HB-4 rotor). The supernatant was recentrifuged at 60 000 × *g* (Beckman SW 28 rotor) for 30 min and the pellets were

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resuspended in 0.25 M sucrose with 5 mM potassium phosphate (pH 6.8). Plasma membrane vesicles were prepared using a 16 g aqueous two-phase partitioning system [9]. Resuspended 60 000 × *g* pellets were mixed with 6.4% (w/w) Polyethylene Glycol 3350 (Fisher), 6.4% (w/w) Dextran T500 (Pharmacia), 0.25 M sucrose, and 5 mM potassium phosphate (pH 6.8). After mixing the tubes by 40 inversions, the phases were separated by centrifugation at 750 × *g* for 5 min. The lower phase was repartitioned twice with fresh lower phases. The upper phases were diluted approximately four-fold with buffer and collected by centrifugation at 100 000 × *g* for 30 min. The membranes were stored frozen at −70°C prior to assay. The yield was 1 to 2 mg of plasma membrane protein.

The assay for the plasma membrane NADH oxidase was in 50 mM Tris-Mes buffer (pH 7.0), 150 μM NADH in the presence of 1 mM potassium cyanide, the latter to inhibit any mitochondrial NADH oxidases contaminating the plasma membranes. The assay was started by the addition of 0.1 mg of plasma membrane protein. The reaction was monitored by the decrease in the absorbance at 340 nm with 430 nm as reference, using an SLM DW-2000 spectrophotometer in the dual wavelength mode of operation or a Hitachi Model U3210. The change of absorbance was recorded as a function of time by a chart recorder. The specific activity of the plasma membrane was calculated using an absorption coefficient of 6.21 mM^{-1} cm^{-1} and expressed a nmol min^{-1} per mg protein.

Assays were initiated by addition of NADH and measured over 5 min after an equilibration period of 10 min. After each addition, the assay was continued for 10 min with the steady state rate between 5 and 10 min being reported. Control rates were linear for 50 min or longer. Unless indicated otherwise, results from duplicate determinations were averaged.

For growth studies, hypocotyl segments, 1 cm in length, were harvested from the zone of cell elongation under dim laboratory light (1.5 μE s^{-1} m^{-1}) by cutting 5 mm below the cotyledons. The segments were floated on aqueous solutions, 2 ml/10 sections, containing the test substances. 2,4-D solutions were aqueous and prepared without aid of co-solvent. Chlorsulfuron was dissolved in DMSO and assayed in 0.1% DMSO. Growth was estimated to the nearest 0.5 mm. Values for initial lengths were subtracted and appropriate controls without inhibitor and with and without 2,4-D were included in each determination. Experiments were repeated 3-times with similar results.

Protein content was determined by the bichinchoninic acid (BCA) procedure [10]. Standards were prepared with bovine serum albumin.

Elongation of 1 cm segments of hypocotyl segments from etiolated seedlings of soybean was stimulated by 2,4-D (Fig. 1). The stimulation was proportional to the logarithm of the concentration of 2,4-D over the range 10^{-8} to 10^{-4} M (Figs. 1 and 2A). At the near optimum concentration of 10^{-5} M, the rate of elongation was approximately twice that in the absence of 2,4-D.

When 2,4-D-stimulated elongation was measured in the presence of 1 μM chlorsulfuron, the 2,4-D response was reduced or eliminated at 10^{-7} and 10^{-6} 2,4 − D and reduced at 10^{-5} M 2,4-D (Fig. 1). At higher concentrations of 2,4-D, e.g. 10^{-4} M, growth rates in the presence or absence of chlorsulfuron were similar (Fig. 2A). However, growth inhibition at 10^{-3} M 2,4-D relative to 10^{-4} M 2,4-D observed in the absence of chlorsulfuron was not observed in its presence (Fig. 2A). In the absence of 2,4-D, the chlorsulfuron by itself was without significant effect on elongation of the 1 cm hypocotyl segments (Fig. 2A and 2B).

The antagonism between chlorsulfuron and 2,4-D was observed when the concentration of chlorsulfuron exceeded or was approximately equal to the concentration of 2,4-D (Figs. 1 and 2A). If the chlorsulfuron concentration was 1 μM, antagonism was observed at 2,4-D concentrations of 1 μM or less (Figs. 1 and 2A). However, 10 μM chlorsulfuron was required to inhibit if the 2,4-D concentration was 10 μM (Fig. 2B).

In the absence of chlorsulfuron, 2,4-D at 10^{-3} M was supraoptimal (Fig. 2A) and growth was reduced compared to 10^{-4} M. In the presence of chlorsulfuron, the response curve was clearly shifted to higher 2,4-D concentrations in that growth was not reduced even at 10^{-3} M.

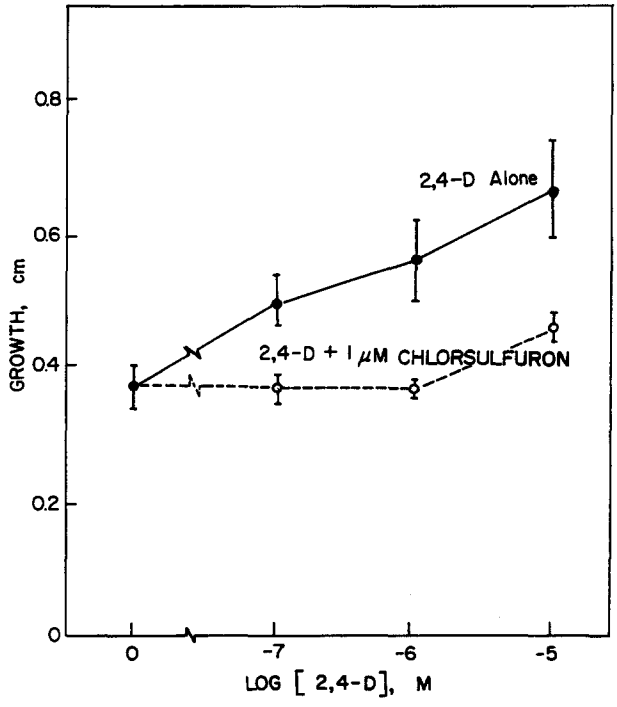

Fig. 1. Stimulation of growth of 1 cm segments cut from dark grown soybean seedlings in response to increasing concentrations of the auxin herbicide, 2,4-D, and its inhibition by the presence of the sulfonylurea herbicide, chlorsulfuron. Note that only the 2,4-D-stimulated growth was inhibited.

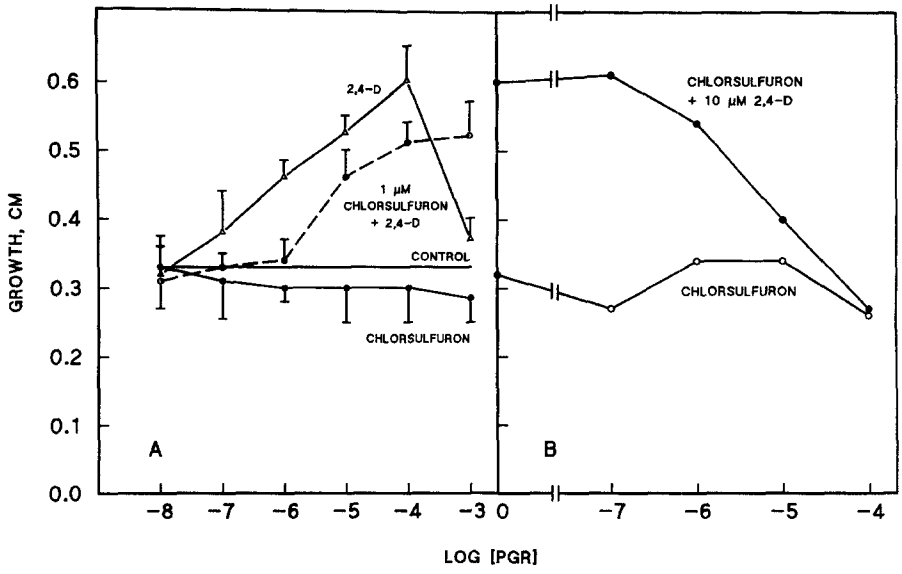

Fig. 2. As in Fig. 1 from another series of experiments in which a wider range of 2,4-D concentrations was examined and compared to chlorsulfuron alone. (A) Open symbols are the growth response to varying concentrations of 2,4-D in the presence of chlorsulfuron. Triangles are growth as a function of 2,4-D concentration in the absence of chlorsulfuron. Solid symbols are the growth response to varying concentrations of chlorsulfuron alone. (B) Growth response to varying concentrations of chlorsulfuron in the presence or absence of 10 μM 2,4-D. PGR = plant growth regulator.

Plasma membrane vesicles isolated from the etiolated hypocotyl segments of soybean exhibited an NADH-oxidase activity stimulated by 2,4-D (Fig. 3). In membrane preparations where a basal rate had been established following the addition of NADH, the addition of 1 μM 2,4-D approximately doubled the rate of NADH oxidation in parallel to effects on steady-state growth. Chlorsulfuron (1 μM) had only a small effect on the rate of NADH

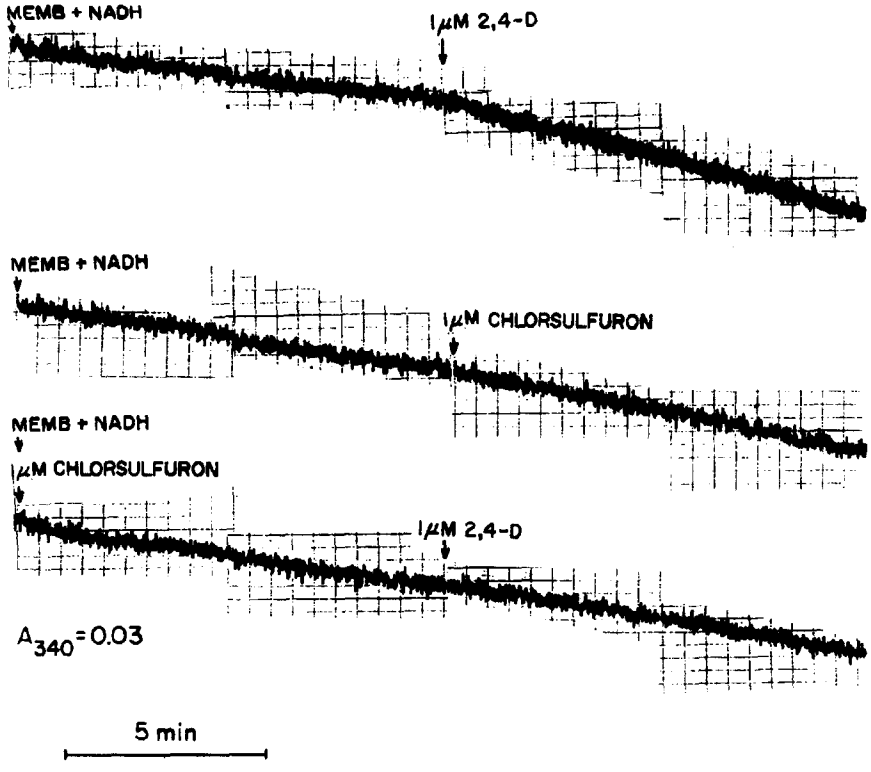

Fig. 3. Spectrophotometer traces measuring NADH oxidation by isolated vesicles of plasma membrane prepared from dark-grown seedlings of soybean and stimulation by 2,4-D (top trace). Chlorsulfuron, by itself, was without effect or slightly stimulatory to NADH oxidation (middle trace). Pretreatment of the membranes with chlorsulfuron (bottom trace) prevented the stimulation of NADH oxidation by 2,4-D.

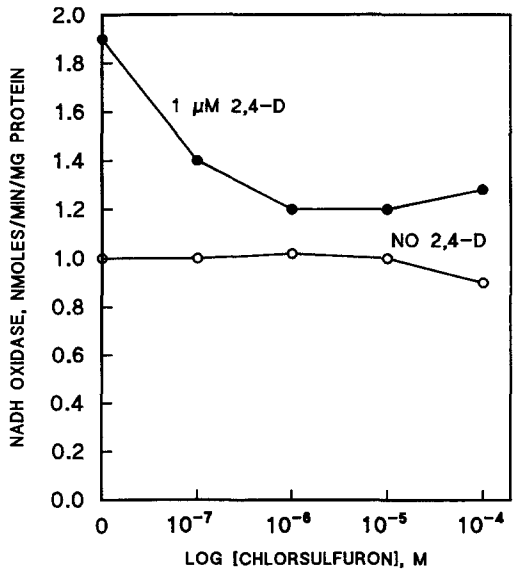

Fig. 4. NADH oxidase activity as a function of chlorsulfuron concentration in the presence or absence of 1 μM 2,4-D.

oxidation. If the membranes were first treated with 1 μM chlorsulfuron followed by 1 μM 2,4-D, the NADH-oxidase activity was no longer responsive to the 2,4-D addition (Fig. 3).

The response of the 2,4-D-stimulated oxidation of NADH by the isolated plasma membrane vesicles to chlorsulfuron exhibited a maximum response at 1 μM chlorsulfuron when followed by 1 μM 2,4-D. In the absence of 2,4-D, the chlorsulfuron had little or no effect on the rate of NADH oxidation (Fig. 4). The chlorsulfuron was added as a DMSO solution with the final concentration of DMSO being 0.1%. This concentration of DMSO was constant throughout the experiment including the no chlorsulfuron controls.

Extraction of lyophylized soybean plasma membranes for 6 h at 23°C reduced the activity of the NADH oxidase by 80 to 90% (Table 1). The heptane extract, when added back and the heptane evaporated, restored 60 to 70% of both the basal activity and the 2,4-D-stimulated activity. When CoQ_{10} (ubiquinone) was added to the extracted membranes in heptane and the heptane was removed by evaporation, activity also was restored. There was less restoration, however, of the 2,4-D-stimulated activity than that obtained with the heptane extract added back.

Chlorsulfuron is a representative of the class of herbicides known collectively as sulfonylureas [11]. Their mechanism of herbicidal action is through the inhibition of acetolactate synthase, a key enzyme in the pathway of branched chain amino-acid synthesis [12–15]. The enzyme catalyzes two reactions. One reaction is the condensation of two pyruvate molecules to produce CO_2 and α-acetolactate, a precursor of valine and leucine. The other reaction is the condensation of pyruvate and α-ketobutyrate to form CO_2 and 2-acetohydroxybutyrate, a precursor of isoleucine. Inhibition is complex and time-dependent [12,16,17]. Tightest binding is under conditions of enzyme turnover [12,17]. The herbicide binding site has been suggested to represent a quinone site based on the observation that the sequence of pyruvate oxidase [18] is very similar to that of acetolactate synthase [13,19–23] and competition of ubiquinone-Q (Q_o) with radiolabeled sulfometuron methyl for the binding site of acetolactate synthase [8]. An unusual feature of acetolactate synthase has been its absolute requirement for FAD [24,25] which is typically more characteristic of oxidoreductases.

While the molecular target for 2,4-D remains unknown, Morré et al. [3] observed that the oxidation of NADH by isolated plasma membrane vesicles of soybean was stimulated by 2,4-D. This stimulation did not require addition of endogenous electron acceptors such as ascorbate radical or hexacyanoferrate III and electron transfer was presumably either to oxygen or endogenous electron acceptors associated with the plasma membrane [1,2]. The activity was not a peroxidase [26] and was distinguished from other NADH oxidases by being resistant to cyanide [4]. The response of the activity to auxin was specific for growth active analogs of both natural (indole-3-acetic acid = IAA) and synthetic auxins and was exhibited even by purified or partially purified enzyme preparations [4]. Designated NADH oxidase I [5], the auxin-stimulated activity correlated with the stimulation of growth by auxins in excised stem sections [6,7].

It was therefore of interest in the present study to examine the effect of chlorsulfuron on the 2,4-D-stimulated NADH-oxidase activity of isolated plasma membrane vesicles of soybean. In the absence of 2,4-D, chlorsulfuron had little or no effect on the activity. However, in the presence of chlorsulfuron, the ability of 2,4-D to stimulate the NADH-oxidase activity was reduced or eliminated depending on the chlorsulfuron/2,4-D ratio. The plasma membrane-located NADH oxidase stimulated by auxin has not yet been characterized completely. However, it is clearly distinct from either pyruvate oxidase or acetolactate

Table 1
Response of NADH oxidase activity of soybean plasma membranes to heptane extraction and readdition of coenzyme Q_{10}

Membrane treatment	Specific activity, nmol/min per mg protein ± S.D. ($n = 8$)	
	basal	2,4-D stimulated
Control	1.2 ±0.25	1.7 ±0.4
Extracted	0.15 ± 0.08	0.2 ±0.02
+ heptane extract	0.75 ± 0.1	1.25 ± 0.05
+ 10 μM CoQ_{10}	0.75 ± 0.1	1.0 ±0.1

Lyophylized soybean plasma membranes (250 mg of protein) were extracted with 5 ml heptane for 6 h at 23°C in the dark. The heptane was decanted and evaporated. The heptane extracts or ubiquinone (CoQ_{10}) were added back to the membrane in 5 ml heptane. The heptane was removed by evaporation and the membranes were taken up in 50 mM Tris-chloride (pH 7.4) for assay. The 2,4-D concentration was 1 μM.

synthase [2]. The activity of the NADH oxidase of liver plasma membranes appears to be influenced by quinones [27]. Certain quinone analogs inhibit the activity. The activity is reduced by extraction of plasma membranes by lipid solvents efficient in quinone extraction. Addition back of the lipid extracts restores activity. The activity can be restored as well by adding back quinones in place of the lipid extracts.

When studies similar to those described by Sun et al. [27] were carried out with soybean plasma membranes, the NADH-oxidase activity was markedly reduced by heptane extraction. The activity could be partially restored by adding back the heptane-extracted materials or by the addition of 10 μM ubiquinone to the extracted membranes. The observation that herbicidal sulfonylureas appear to occupy vestigial quinone sites [8], would be consistent with the observed inhibition by chlorsulfuron of both acetolactate synthase and the 2,4-D-stimulated component of the plasma membrane NADH oxidase. The latter, however, may be more related to the specific inhibition of auxin-stimulated growth, whereas the herbicidal effects are more clearly the result of inhibition of acetolactate synthase.

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References

[1] Morré, D.J. and Brightman, A.O. (1991) J. Bioenerg. Biomembr. 23, 469–-489.
[2] Morré, D.J. (1994) J. Bioenerg. Biomembr. 26, 421–433.
[3] Morré, D.J., Navas, P., Penel, C. and Castillo, F.J. (1986) Protoplasma 133, 195–197.
[4] Brightman, A.O., Barr, R., Crane, F.L. and Morré, D.J. (1988) Plant Physiol. 86, 1264–1269.
[5] Brightman, A.O. and Morré, D.J. (1991) in Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport. II. Plants (Crane, F.L., Morré, D.J. and Löw, H., eds.), pp 85–110, CRC Press, Boca Raton, FL.
[6] Morré, D.J., Brightman, A.O., Wu, L.-Y., Barr, R., Leak, B. and Crane, F.L. (1988) Physiol. Plant. 73, 187–193.
[7] Morré, D.J., Crane, F.L., Barr, R., Penel, C. and Wu, L.-Y. (1988) Physiol. Plant. 72, 236–240.
[8] Schloss, J.V., Lawrence, M.C. and Van Dyk, D.E. (1988) Nature 331, 360–362.
[9] Sandelius, A.S., Barr, R., Crane, F.L. and Morré, D.J. (1987) Plant Sci. 48, 1–10.
[10] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F. H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[11] Duke, S.O. (1990) Environ. Health Perspect. 87, 263–271.
[12] LaRossa, R.A. and Schloss, J.V. (1984) J. Biol. Chem. 259, 8753–8757.
[13] Falco, S.C. and Dumas, K.S. (1985) Genetics 109, 21–35.
[14] Chaleff, R.S. and Mauvais, C.J. (1984) Science 224, 1443–1445.
[15] Ray, T.B. (1984) Plant. Physiol. 75, 827–832.
[16] Muhitch, J. M., Shaner, D. L. and Stidham, M. A. (1987) Plant. Physiol. 83, 451–456.
[17] Schloss, J.V. (1984) in Flavins and Flavoproteins (Bray, R.C., Engel, P.C. and Mayhew, S.G., eds.), pp. 737–740, De Gruyter, Berlin.
[18] Grabau, C. and Cronan, J.E., Jr. (1986) Nucleic Acids Res. 14, 5449–5460.
[19] Wek, R.C., Hauser, C.A. and Hatfield, G.W. (1985) J. Nucleic Acids Res. 13, 3995–4010.
[20] Friden, P., Donegan, J., Mullen, J., Tsui, P., Freundlich, M., Eoyang, L., Weber, R. and Silverman, P. M. (1985) J. Nucleic Acids Res. 13, 4011–4027.
[21] Lawther, R.P., Calhoun, D.H., Adams, C.W., Hauser, C.A., Gray, J. and Hatfield, G.W. (1981) Proc. Natl. Acad. Sci. USA 78, 922–925.
[22] Squires, C.H., De Felice, M., Devereux, J. and Calvo, J.M. (1983) J. Nucleic Acids Res. 11, 5299–5313.
[23] Mazur, B.J., Chui, C.-F. and Smith, J.K. (1987) Pl. Physiol. 85, 1110–1117.
[24] Stømer, F.C. and Umbarger, H.E. (1964) Biochem. Biophys. Res. Commun. 17, 587–592.
[25] Schloss, J.V., Van Dyk, D.E., Vasta, J.F. and Kutny, R.M. (1985) Biochemistry 24, 4952–4959.
[26] Crane, F.L., Barr, R., Brightman, A.O. and Morré, D.J. (1989) Plant Physiol. 89 (Suppl.), 146.
[27] Sun, I.L., Sun, E.E., Crane, F.L., Morré, D.J., Lindgren, A. and Löw, H. (1992) Proc. Natl. Acad. Sci. USA 89, 11126–11130.