Volume 291, number 1, 117–121 FEBS 10230 October 1991

ADONIS 001457939100940L

Phytochrome control and anoxia effect on the activity and expression of soybean seedling lipoxygenases 1 and 2

Mauro Maccarrone, Gerrit A. Veldink and Johannes F.G. Vliegenthart

Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands

Received 3 August 1991

The effects of different light irradiations and anoxia treatment on the activity and expression of the lipoxygenases 1 (LOX-1) and 2 (LOX-2) of soybean seedlings have been investigated. A phytochrome-mediated decrease of the specific activities of both isoenzymes was found which was due to the regulation of the gene expression at the transcriptional level. The anoxia treatment of the seedlings led to a decrease of both lipoxygenase specific activities as well, but this effect was attributable to the reduction of the amount of the two isoenzymes, without changes in the steady-state levels of specific mRNAs. Therefore, light and anoxia are both regulators of soybean lipoxygenases, but their mechanism of action at the molecular level is different.

Lipoxygenase-1; Lipoxygenase-2; Phytochrome; Anoxia; Soybean

1. INTRODUCTION

Lipoxygenases (E.C. 1.13.11.12) comprise a class of non-heme iron-containing dioxygenases, which oxidize unsaturated fatty acids containing a 1,4-*Z*,*Z*-pentadiene system to conjugated hydroperoxy acids. These enzymes are widely distributed in nature among plants and animals (for a review on plant lipoxygenases see [1]. The first lipoxygenase was isolated from soybeans (*Glycine max*) and was later designated lipoxygenase-1 (LOX-1), being one of the four distinct isoenzymes (LOX-1, -2, -3a and -3b) present in these plants [2]. Soybean seeds contain considerable amounts of LOX-1 and LOX-2, which differ in their kinetic features [2,3] and are encoded by two different genes [4,5].

Lipoxygenase activity in mustard (*Sinapis alba*) seedlings has been reported to be inhibited by light with an all-or-nothing phytochrome-mediated mechanism [6]. A decrease in lipoxygenase activity upon light irradiation has been shown also in squash (*Cucurbita moscata*) seedlings [7]. Many aspects of soybean physiology are light-dependent [8], thus a possible effect of light irradiation on soybean lipoxygenase was investigated.

Anaerobic conditions are known to cause dramatic changes in a variety of biochemical features of plants [9,10]. In particular, drastic modifications of the patterns of proteins synthesized under anaerobiosis have been described [11]. In soybean seedlings subjected to anoxia, rapid changes in the protein synthesis occur which are due to a fast dissociation of polyribosomes [12]. Therefore, the effect of anaerobiosis on LOX-1 and -2 was studied, taking into account that these enzymes were proposed to induce and maintain natural anaerobiosis during soybean germination [13].

The aim of this paper was to investigate the possible role of light and anoxia as regulating factors of soybean lipoxygenase activity and expression. We present evidence that light regulation is at the transcriptional level, while anoxia plays a role at the translational level of gene expression.

Abbreviations: LOX-1, lipoxygenase-1; LOX-2, lipoxygenase-2; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; NC, nitrocellulose.

Correspondence address: J.F.G. Vliegenthart, Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands

2. MATERIALS AND METHODS

Chemicals were of the purest analytical grade. Nitrocellulose filters (0.45 μm) were purchased from Bio-Rad; [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci=37 GBq) and Hybond-N membranes (0.45 μm) were from Amersham; Polynucleotide kinase (50 000 U/mg) was from Boehringer.

2.1. *Plant material*

Soybean (*Glycine max* (L.) Merrill) seeds were soaked in tap water overnight at room temperature, then they were grown at 22°C in a greenhouse, in the dark, and were watered daily with tap water. Seedlings were homogenized at 4°C in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM PMSF. Homogenates were centrifuged for 15 min at 10 000 × *g*, the supernatants were collected and centrifuged twice for 5 min at 12 000 × *g*. The final supernatants were used without further purification for protein assay, enzyme activity and immunochemical determinations.

2.2. *Assay of lipoxygenase activity*

Enzyme activity was measured polarographically, at 25°C, in a solution of 1.8 mM linoleic acid in air-saturated 0.1 M sodium borate

buffer, pH 9.0 (LOX-1), or 0.1 M sodium phosphate buffer, pH 6.6 (LOX-2).

Protein concentration was determined according to Bradford [14], using BSA as a standard.

2.3. *Enzyme-linked immunosorbent assay (ELISA)*

The procedure described by Leaper and Newbury [15] was adopted, coating immunoplates with 50 μg/well of each sample. Rabbit anti-LOX-1 polyclonal antibodies, purified according to Vernooy-Gerritsen [16], were used as first antibody (diluted 500-fold). Goat-anti-rabbit IgGs conjugated with alkaline phosphatase (GAR-AP, Bio-Rad) were used as second antibody (diluted 3000-fold). The substrate for the alkaline phosphatase reaction was *p*-nitrophenyl-phosphate, 0.5 mg/ml in 0.5 M diethanolamine buffer pH 9.8, and color development was recorded at 405 nm. LOX-1 and -2, purified as already reported [17], were reacted with the anti-LOX-1 antibodies. The A_{405} values of the crude preparations of soybean seedlings were within the range that yielded a linear relationship between the amount of LOX-1 and -2 and the extinction. Controls were carried out by using rabbit non-immune serum instead of anti-LOX-1 antibodies; controls also included wells coated with BSA (100 μg/well).

2.4. *Immune dot-blotting*

The assay was performed according to Hawkes [18], dotting onto NC filters 10 μg/well of each sample with a microfiltration Bio-Dot apparatus (Bio-Rad). Anti-LOX-1 antibodies (diluted 1:1500) and GAR-AP (Bio-Rad), diluted 1:3000, were used as first and second antibody, respectively. BSA was included as a control, at different concentrations.

2.5. *RNA isolation and Northern blot analysis*

Total RNA was isolated from soybean seedlings according to Verwoerd [19] and its purity and intactness were checked according to Logemann [20]. RNA (40 μg/lane) was electrophoresed on a 1% agarose gel containing 3.3% formaldehyde, transferred to Hybond-N membranes and subjected to Northern blot following the procedure of Meinkoth and Wahl [21]. Two oligonucleotides were prepared with a Biosearch 8600 DNA Synthesizer: 5′-CTGCTGAGCCATCAGGGTTAAC-3′, specific for the LOX-1 mRNA [4]; 5′-CTCCTCTGTTCAGGATTCCCGA-3′, specific for the LOX-2 mRNA [5]. The oligonucleotide probes were labeled at their 5′ ends with polynucleotide kinase in the presence of [γ-^{32}P]ATP (3000 Ci/mmol). The ^{32}P-probes (sp. act. 10^9 cpm/mg) were separately used in the Northern blot analyses. Pre-hybridizations and hybridizations were performed at 45°C as previously described [22], using homochromatography mix I [21] as a carrier at a final concentration of 100 μg/ml. The hybridization buffer activity was 2.10^6 cpm/ml.

2.6. *Illumination conditions and anaerobic treatment*

White, blue and red light irradiations were all delivered by a white fluorescent tube (TLD 36W/33, Philips; irradiance=3 mW/cm^2). Blue light was obtained by filtering the light through a blue (ICI, 727) perspex filter, red light by filtering the light through a red (ICI, 4400) perspex filter; far-red light was delivered by an incandescent lamp (GLS 100W, Philips; irradiance=42 mW/cm^2), filtered through red (ICI, 4400) and green (ICI, 6600) perspex filters [15]. During irradiation of plant material the temperature of the plant cabinet was kept at 22°C. After illumination the seedlings were frozen at −80°C until use.

Soybean seedlings were subjected to anoxia according to Reggiani [9]. The experiments were performed in jars containing water, which had been previously flushed with helium (5 min) and pure nitrogen (1 h), in order to achieve complete anaerobiosis [23]. The anaerobic conditions were kept throughout the experiments by continuously flushing the jars with pure N_2. At fixed times seedlings were harvested and frozen at −80°C until use.

Each data point reported in this paper is the mean of three independents experiments (SD<5%).

3. RESULTS

3.1. *Effect of light and anaerobic conditions on lipoxygenase activity*

Dark-grown, 3-day-old soybean seedlings were exposed to different combinations of light (Table I), in order to investigate a possible phytochrome regulation. Both lipoxygenase activities decreased to 50% of the dark control when the seedlings were exposed to white light or to brief pulses of red light. Brief pulses of far-red light, delivered soon after the red ones, reversed the red radiation effect and repeated photoreversibility was observed. Similarly, a blue light pulse led to a 50% decrease of lipoxygenase activity, an effect fully reversed by subsequent far-red light irradiation.

LOX-1 and -2 specific activities of 3-day-old, dark-grown soybean seedlings subjected to anaerobic stress for periods ranging from 12 to 36 h are shown in Fig. 1 (top). Both activities decreased to approximately 30% of the untreated control after 24 h of anoxia, then remained unaltered for the following 12 h of treatment.

3.2. *Lipoxygenase content in light- and anoxia-treated seedlings*

An ELISA test was performed on different amounts of pure LOX-1 and -2 by using anti-LOX-1 polyclonal antibodies. These antibodies were able to cross-react with both the isoenzymes, though LOX-2 reacted less than LOX-1 (Figure 3, inset). Therefore, the anti-LOX-1 antibodies were used to estimate the overall amount of LOX-1 and -2 in the seedlings. The lipoxygenase content of the samples was initially evaluated with an immune dot-blot assay. It is shown in Fig. 2 that seedlings exposed to pulses of red light or blue light displayed a decrease in the amount of LOX, when compared to the dark control. A decrease in the lipoxygenase content was also observed in the seedlings subjected to the anaerobic treatment and was proportional to the duration of the treatment (Fig. 2). Because the dot-blot

Table I

Effect of light treatment on lipoxygenase activity

Treatment	Specific Activity (μmol $O_2 \cdot min^{-1} \cdot$ mg P^{-1})	
	LOX-1	LOX-2
Control (2 h dark)	2.21	6.58
2 h white-light	1.10	3.24
5 min red + 115 min dark	1.18	3.49
5 min far-red +115 min dark	2.30	6.24
5 min red + 5 min far-red +110 min dark	2.30	6.43
5 min red + 5 min far-red + 5 min red + 105 min dark	1.24	3.20
15 min blue + 105 min dark	1.18	3.36
15 min blue + 5 min far-red +100 min dark	2.05	6.25

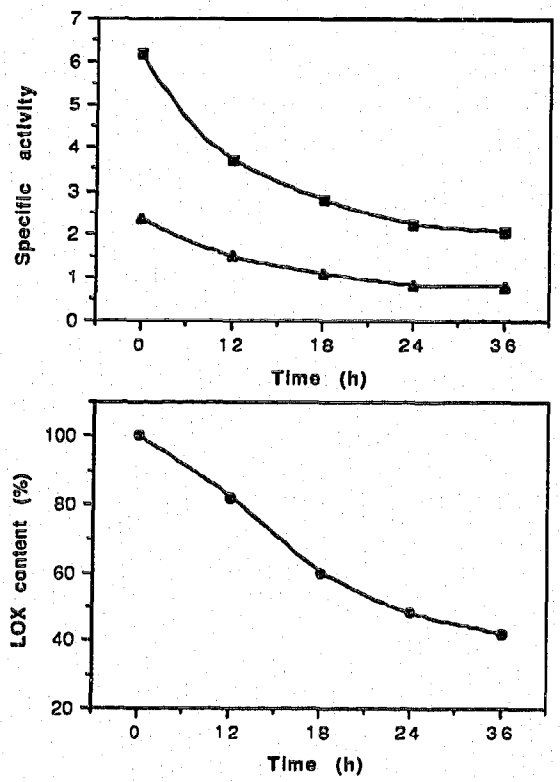

Fig. 1. Top. Decrease of LOX-1 (full triangles) and LOX-2 (full squares) specific activities of dark-grown, 3-day-old soybean seedlings, subjected to anaerobic conditions for the indicated periods. Specific activities are expressed as $\mu mol\ O_2 \cdot min^{-1} \cdot mg\ P^{-1}$. Bottom. LOX content of the same samples as in the top, estimated with the ELISA test. The LOX content is expressed as percentage of the control value (100 ng·mg P^{-1}).

analysis allowed only a rough estimate of the amount of lipoxygenases in the samples, an ELISA test was set up. In Fig. 3 it is shown that in seedlings exposed to white, red or blue light the LOX content was less than 50% of the dark control, a finding which was well correlated to the decrease of LOX-1 and -2 specific activities in the same samples. When the ELISA was performed on the anoxia-treated samples, the results shown in Fig. 1 (bottom) were obtained, indicating that the LOX content of the seedlings decreased in a way parallel to the decrease of the lipoxygenase activities (Fig. 1, top).

3.3. *Northern blot analysis*

The steady-state level of mRNAs coding for LOX-1 and LOX-2 was analysed in seedlings subjected to the same light and anoxia treatment as above. The pattern obtained by hybridizing with the LOX-1 probe is shown in Fig. 4 and was superimposable with the one obtained by hybridizing with the LOX-2 probe. Upon irradiation with white, red or blue light the level of the specific mRNAs decreased as compared to the level of the dark control (lanes A–D). On the other hand, the mRNAs remained the same as the control in the seedlings subjected to anaerobic conditions, whatever the duration of the treatment (lanes E–H).

4. DISCUSSION

The profiles of LOX-1 and -2 specific activities in dark-grown soybean seedlings were determined in preliminary experiments, which indicated high activity of both isoenzymes in the early stages of germination, with a maximum on day 3 and a slow decrease between days 3 and 5 of growth (data not shown). Thus 3-day-old, dark-grown seedlings were chosen as a reference to ascertain the possible effect of light and anoxia on the lipoxygenases.

Soybean seedlings showed a decrease in LOX-1 and -2 specific activities after irradiation with white light (Table I). This decrease is regulated by the morphogenic pigment phytochrome, since the activities were decreased by brief pulses of red light, the effects of which were reversed by brief pulses of far-red [24]. When the effect of the type of light was investigated by irradiating the seedlings with blue light, the same lipoxygenase inhibition as the one due to red illumination was observed (Table I). Since the blue light effect was erased by far-red, the blue photoresponse is attributable to the activation of phytochrome [25] and not to the activation of a blue light photoreceptor [26]. The LOX content of the light- treated seedlings decreased in the same way as the specific activity of the samples (Fig. 2 and 3). Therefore, the phytochrome system regulates the lipoxygenase synthesis and/or degradation. In order to ascertain whether this control was at least in part transcriptional, total RNA was isolated and specific probes for LOX-1 and LOX-2 were used to measure the steady-state levels of mRNAs. As indicated in Fig. 4 (lanes A–D) a decrease in the mRNAs level of the irradiated samples was found, compared to the dark control. Thus it can be concluded that light controls the LOX-1 and -2 expression acting at the transcriptional level.

Many examples exist of phytochrome-mediated transcriptional regulation of enzymes [27], also in soybeans [28]. Our finding of a phytochrome-mediated regulation of the activity and expression of LOX-1 and -2 may have a physiological meaning, since lipid metabolism is implicated in the photoresponses of seeds [29]. Moreover, our results support the hypothesis for a role of

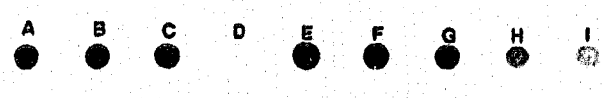

Fig. 2. Detection of LOX by dot-blotting. A, dark control; B, red light; C, blue light; D, BSA (10 μg); E, anoxia control (untreated); F, +12 h anoxia; G, +18 h anoxia; H, +24 h anoxia; I, +36 h anoxia; L, BSA (20 μg). Light treatments were as reported in Table I.

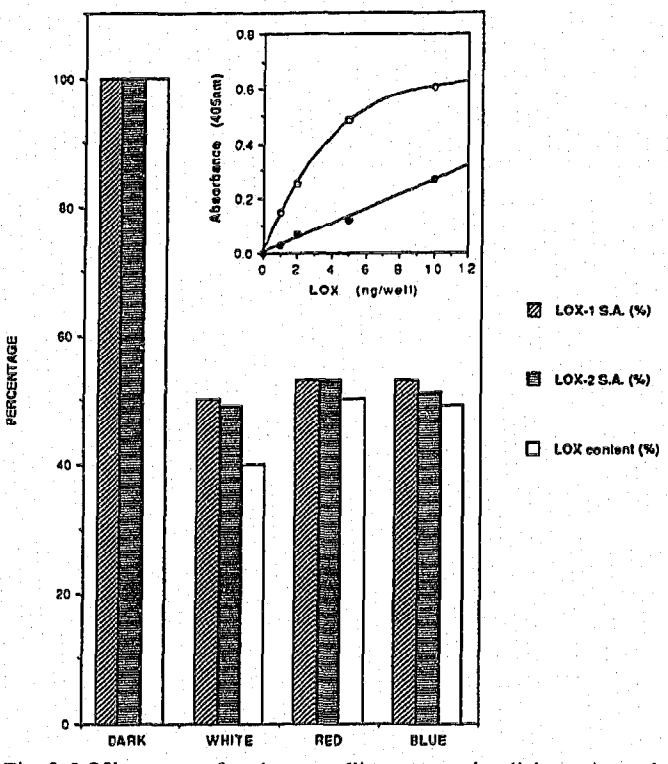

Fig. 3. LOX content of soybean seedlings exposed to light, estimated by the ELISA test and expressed as percentage of the LOX content of the dark control (100 ng·mp P^{-1}). The decrease in the amount of enzyme is paralleled by the decrease in LOX-1 and -2 specific activities (S.A.) in the same samples (S.A. values from Table I). Inset. Reactivity of pure LOX-1 (empty circles) and LOX-2 (full circles) to the anti-LOX-1 antibodies, evaluated with the enzyme-linked immunosorbent assay.

these enzymes in the biosynthesis of the photosynthetic organelles in germinating soybeans [30].

Like light irradiation, anoxia led to a decrease in LOX activities of soybean seedlings (Fig. 1, top). The decrease of activity was paralleled by the decrease in the amount of LOX in the samples (Fig. 1, bottom, and Fig. 2), whereas the steady-state level of the LOX-1 and -2 mRNAs was the same as in the untreated control, independent of the duration of the treatment (Fig. 4, lanes E–H). On the other hand, dark-grown, 3-day-old seedlings kept for 36 h under aerobic conditions did not display any decrease in the LOX content, compared to the untreated control (data not shown). These results indicate that the anaerobic conditions played a role on the lipoxygenase synthesis and/or degradation, acting at the translational level of the LOX genes expression. This seems to be a general mechanism operating in the seedlings during anaerobic stress, since it has been reported that a large reduction of protein synthesis occurs in soybeans subjected to anoxia, without significant changes in the mRNA stability [12].

Different hypotheses may be proposed on the physiological significance of the lipoxygenase sensitivity to anaerobiosis. The decrease of LOX activities may be

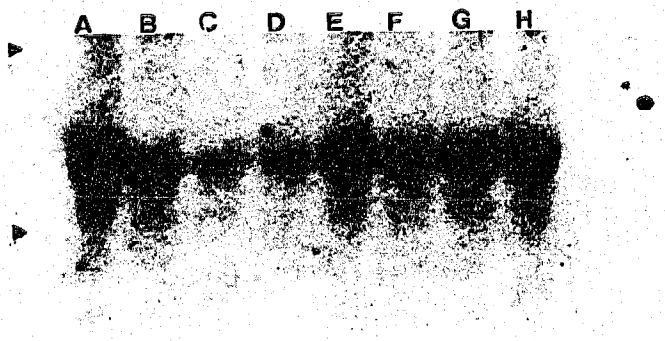

Fig. 4. Northern blot of total RNA (40 μg/lane) probed with the oligonucleotide specific for the LOX-1 mRNA. A, dark control; B, white light; C, red light; D, blue light; E, anoxia control (untreated); F, +12 h anoxia; G, +24 h anoxia; H, +36 h anoxia. Light treatments were as reported in Table I. Arrows indicate the position of the ribosomal RNAs (25S and 18S).

related to both the high sensitivity of plant cell membranes to anoxia [10] and the reduced need for the seedlings to create and maintain the natural anaerobiosis in the early stage of germination [13,31]. On the other hand, the decrease of LOX content upon anaerobic treatment could have a more general explanation. In fact, the synthesis of LOX-1 and -2 could be halted, in order to allow the synthesis of different, specific 'anaerobic proteins' [11], necessary for the seedlings during the anoxia stress.

Finally, it is noteworthy that both LOX-1 and -2 respond in the same way to the regulating factors under study, so that a differential regulation cannot be proposed to explain the presence of the two isoenzymes in the same cell. Since LOX-1 and -2 have the same intracellular localization in germinating soybean seeds [32], the differences in their enzymatic features might give a reason for their co-existence in the cell.

Acknowledgements: The authors thank Dr J. Verhagen for helpful discussions. The friendly assistance of Drs P. van Aarle and Drs A. Bovy is also gratefully acknowledged. This investigation was supported by EC Grant SC1-0197.

REFERENCES

[1] Vliegenthart, J.F.G. and Veldink, G.A. (1982) in: Free Radicals in Biology (Pryor, W.A., ed.) Vol. V, pp. 29–64, Academic Press, New York.
[2] Axelrod, B., Cheesbrough, T.M. and Laakso, S. (1981) Methods Enzymol. 71, 441–451.
[3] Bild, G.S., Bhat, S.G., Ramadoss, C.S. and Axelrod, B. (1978) J. Biol. Chem. 253, 21–23.
[4] Shibata, D., Steczko, J., Dixon, J.E., Hermodson, M., Yazdanparast, R. and Axelrod, B. (1987) J. Biol. Chem. 262, 10080–10085.
[5] Shibata, D., Steczko, J., Dixon, J.E., Andrews, P.C., Hermodson, M. and Axelrod, B. (1988) J. Biol. Chem. 263, 6816–6821.
[6] Mohr, H. and Oelze-Karow, H. (1976) in: Light and Plant Development (Smith, H., ed.) pp. 257–284. Butterworths, London.
[7] Surrey, K. and Barr, E.M. (1966) Plant Physiol. 41, 780–786.

[8] Britz, S.J. (1990) Photochem. Photobiol. 52, 151–159.
[9] Reggiani, R., Hochkoeppler, A. and Bertani, A. (1989) Plant Physiol. 91, 1197–1201.
[10] Vartapetian, B.B. and Zakhmilova, N.A. (1990) Protoplasma 156, 39–44.
[11] Sachs, M.M., Freeling, M. and Okimoto, R. (1980) Cell 20, 761–767.
[12] Lin, C.Y. and Key, J.L. (1976) J. Mol. Biol. 26, 237–247.
[13] Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1977) Prog. Chem. Fats Other Lipids 15, 131–166.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Leaper, L. and Newbury, H.J. (1989) Plant Sci. 64, 79–90.
[16] Vernooy-Gerritsen, M., Veldink, G.A. and Vliegenthart, J.F.G. (1982) Biochim. Biophys. Acta 708, 330–334.
[17] Finazzi-Agro', A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 326, 462–470.
[18] Hawkes, R., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147.
[19] Verwoerd, T.C., Dekker, B.M.M. and Hoekema, A. (1989) Nucleic Acids Res. 17, 2362.
[20] Logemann, J., Schell, J. and Willmitzer, L. (1987) Anal. Biochem. 163, 16–20.
[21] Meinkoth, J. and Wahl, G. (1984) Anal. Biochem. 138, 267–284.
[22] Maccarrone, M., Rossi, A., D'Andrea, G., Amico sante, G. and Avigliano, L. (1990) Anal. Biochem. 188, 101–104.
[23] Reggiani, R., Brambilla, I. and Bertani, A. (1985) J. Exp. Bot. 36, 1193–1199.
[24] Morohashi, Y. (1989) Plant Cell Physiol. 30, 811–815.
[25] Butler, W.L., Hendricks, S.B. and Siegelman, H.W. (1964) Photochem. Photobiol. 3, 521–528.
[26] Fluhr, R. and Chua, N.-H. (1986) Proc. Natl. Acad. Sci. USA 83, 2358–2362.
[27] Tobin, E.M. and Silverthorne, J. (1985) Annu. Rev. Plant Physiol. 36, 569–593.
[28] Berry-Lowe, S.L. and Meagher, R.B. (1985) Mol. Cell. Biol. 5, 1910–1917.
[29] Hendricks, S.B. and Borthwick, H.A. (1965) in: Chemistry and Biochemistry of Plant Pigments (Goodwin, T.W., ed.) pp. 405–436. Academic Press, New York.
[30] Vernooy-Gerritsen, M., Bos, A.L.M., Veldink, G.A. and Vliegenthart, J.F.G. (1983) Plant Physiol. 73, 262–267.
[31] Puntarulo, S. Beconi, M.T., Sanchez, R.A. and Boveris, A. (1987) Plant Sci. 52, 33–39.
[32] Vernooy-Gerritsen, M., Leunissen, J.L.M., Veldink, G.A. and Vliegenthart, J.F.G. (1984) Plant Physiol. 76, 1070–1079.